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Lifestyle of beer spoiling lactic acid bacteria

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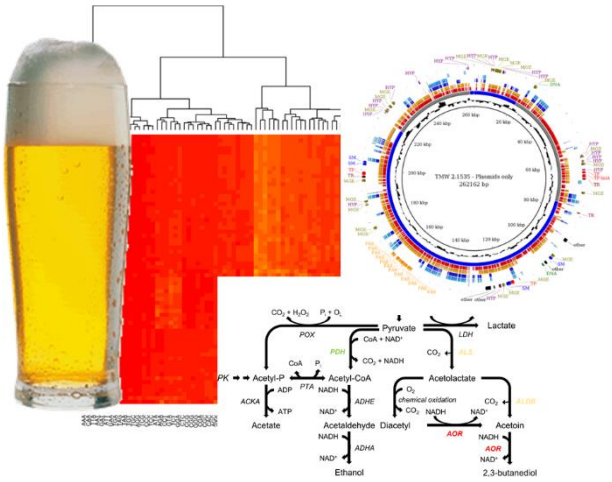

Bayerisches Reinheitsgebot, 23.4.1516

„Wir wollen auch sonderlichen, das füran allenthalben in unnsern Steten, Märckten und auf dem Lannde, zu kainem Pier merer Stückh, dann allain Gersten, Hopffen unnd Wasser, genommen und geprauchte sollen werden.“ Das Buech der gemeinen Landpot, Landsordnung, Satzung vnnd Gebreuch des Fürstenthumbs in Obern vnnd Nidern Bairn. Jm Fünftzehenhundert vnnd Sechtzehendem Jar aufgericht.

Doctoral thesis

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Vorwort

Die vorliegende Arbeit entstand im Rahmen eines durch Haushaltsmittel des BMWi über die AiF-Forschungsvereinigung Wissenschaftsförderung der Deutschen Brauwirtschaft e.V. geförderten Projektes (AiF 17576 N).

Teilergebnisse der vorliegenden Arbeit wurden vorab in Fachzeitschriften publiziert. Siehe Kapitel 15: „List of Publications and Student Theses“.

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Abbreviations

AA(s)	amino acid(s)
ABC	ATP-binding cassette
ADI	arginine deiminase
AGDI	agmatine deiminase
BSA	beer spoilage ability
BSP	beer spoilage potential
CV	coefficient of variation
DMG(s)	diagnostic marker gene(s)
DNA	deoxyribonucleic acid
FA(s)	fatty acid(s)
FAS	fatty acid biosynthesis
GABA	γ-aminobutyric acid
GAD	glutamate decarboxylase
HGT	horizontal gene transfer
HP	hazard potential
hmw	higher-molecular-weight
MALDI-TOF MS	Matrix-assisted-Laser-Desorption-Ionization-Time-Of-Flight Mass Spectrometry
MDR	multidrug transporter
MFS	major facilitator superfamily
MIC	minimum inhibitory concentration
LAB	lactic acid bacteria
L.	*Lactobacillus*
LTA(s)	lipoteichoic acid(s)
OA(s)	organic acids(s)
P.	*Pediococcus*
RI	refractive index
pmf	proton motive force
TCA	tricarboxylic acid

Contents

1 Introduction

Beer, as we know it, is a fermented alcoholic beverage with the basic ingredients water, malted barley, hops and yeast, as fixed by the German purity law from 1918 (Preedy, 2009). The story of beer, however, starts about 7,000 years before in the old cities of Mesopotamia. It slowly became the beverage we are used to, when the Bavarian duke Wilhelm IV decided in 1516 that Bavarian brewers have to exclusively use water, malted barley and hops. Especially the usage of hops, which do not only contribute to a better taste, was an important milestone in the history of beer (Meußdoerffer and Zarnkow, 2014; Preedy, 2009). So what is so special about (hopped) beer, explaining its propagation all over the world?

Beer, “the liquid bread,” was a safe source for water, nutrients and vitamins during the Dark Age and later on, while the access to clean water was not self-evident. Further, beer contains alcohol, while the urge to get drunk is as old as humanity. Drunkenness does and did go along with social gathering, as beer is and was mostly drunk in company. The mutually drinking of beer often defined the affiliation to a group and the social situation in the past. Finally, beer was and is considered as a healthy and nutritious food, while in past days it was even used as source material for pharmaceuticals or for sedation during the treatment of sick persons (Meußdoerffer and Zarnkow, 2014). Nowadays consumers expect a safe and long-lasting product with a consistent tasty flavor. Beer spoilage is a phenomenon hardly known by the majority of consumers, while its occurrence changes the product towards an unpleasant sensory appearance, including acidification, haze, off-flavors and slimy consistency (Suzuki, 2011). A spoiled or as unpleasant deemed beer might cause a consumer to discard the beer or even change the beer brand long-term, while the latter is no problem for the consumer, considering the 1,352 breweries, which are only found in Germany (www.brauer-bund.de; Statistik der Brauwirtschaft in Zahlen 2006-2015).

In contrast, beer spoilage is a problem for brewers. German breweries produced 9.56 billion liters of beer in 2014, employing more than 25,000 workers resulting in at least 7.9 billion Euro volume of sales (www.brauer-bund.de; Statistik der Brauwirtschaft in Zahlen 2006-2015). In principle, brewers also want to produce a safe and microbiological stable product with a consistent tasty flavor, but breweries also make up a distinct economic sector, while beer is the product. Brewers want to sell a steady and tasty product, which ties consumers to their brand. Thus, any change in quality or sensory appearance of the product is harmful for the brand and consequently for business. Beer-spoiling bacteria cause such changes, while the potential economic damage by beer spoilage is not only caused by a loss of consumer’s confidence, but also because a spoiled batch of beer has to be recalled and discarded (Sakamoto and Konings, 2003). Consequently, brewers rely on a reliable quality control for the

detection of harmful contaminants in order to prevent the abovementioned economic damage or, simplified, they need to find those bacteria, which spoil their product before they actually do it.

Finally, from the bacteria´s perspective, beer represents a rather hostile environment, characterized by the presence of several antibacterial hurdles. These hurdles, especially the presence of hops, result in a very stable beverage, which so far has been found to prevent the growth of the vast majority of bacteria, including foodborne pathogens such as *Salmonella* and *Staphylococcus* (Vriesekoop et al., 2012). Beer-spoiling bacteria have to come up with strategies to survive and even grow in this harsh environment. Thus, beer is a pleasure for the consumer and a valuable product for the brewer, but it is also an ecological niche for bacteria, whose occupation is only possible for a small number of specialists.

The following chapters provide background information about beer as an ecological niche, about beer-spoiling lactic acid bacteria and their adaption to beer. In any case, it is strongly recommended to read the chapter “Terminology and definitions” (4.1, p. 51) before proceeding to the results and discussion chapters.

1.1 Beer and hops

1.1.1 The environment beer and its composition

In general, beer can be classified into two types, depending on the yeast used for fermentation, while several other ingredients and the fermentation conditions affect the taste, but also the microbial stability. Besides the type of yeast, malt (germinated and dried grain) and water, especially the variety and amount of hops influence the sensory appearance and the susceptibility of beer towards spoilage. Top fermented beers represent only a small percentage of the total beer production/consumption worldwide and include amongst others the beer types Ale, Porter, Stout, Kölsch, Alt and Wheat beer (Weissbier). Bottom fermented beers include the most common beer types Pilsner and Lager beer. There is no general correlation of these two types to spoilage susceptibility, which is mainly defined by pH and hop content. These parameters, especially the hop content, vary dramatically within the subordinate beer types, taking wheat beer with approximately 8 to 15 International Bitterness Units (IBU) or a Stout with 30 to 45 IBU. Despite or maybe even because of the fermentation of the raw materials by yeast, beer is a very complex beverage that contains about 800 organic compounds (Preedy, 2009). Some general compounds and there concentrations are outlined in Table 1.

Table 1: The composition of beer - a rough overview for some selected compounds and compound groups. Typical concentrations are given as well as the number of compounds related to a compound group and the source or agent of the respective compounds. Adapted and modified (Hardwick, 1995; Preedy, 2009). Note that the composition of those beers used within this study is outlined in Appendix 1 (p. 328).

Compound(s)	Concentration	Number of compounds	Source or agent
Water	90 - 94 %	1	Water
Ethanol	3 - 5 % v/v	1	Yeast, malt
Carbohydrates	1 - 6 % w/v	~100	Malt
Carbon dioxide	3.5 - 4.5 g/l	1	Yeast, malt
Inorganic salts	500 - 4,000 mg/l	~25	Water, malt
Total nitrogen content	300 - 1,000 mg/l	~100	Yeast, malt
Organic acids	50 - 250 mg/l	~200	Yeast, malt
Vitamin B compounds	5 - 10 mg/l	13	Yeast, malt

The primary sources of carbohydrates in beer are three polysaccharides, which are degraded during malting and mashing (heating of malt and water) in order to provide the necessary sugar for yeast fermentation. These polysaccharides, starch, pentosan and β-glucan, are degraded mainly by plant enzymes applying different temperatures resulting in a liquid extract called wort, which contains remaining polysaccharides, but also oligo-, di- and monosaccharides. About 64 to 77 % of these carbohydrates are fermentable by yeast, while maltose accounts for 50 to 60 % of them. As yeast mainly ferments saccharose, glucose, fructose, maltose and maltotriose, and because wort additionally contains various other sugars, although most of them only in traces compared to fermentable sugars, beer ends up with a total carbohydrate content of 20 to 30 g/l. Besides the quantitatively most abundant starch derived sugars, the final product contains up to 100 different carbohydrates, while the majority is made up by “non-fermentable” dextrins and α-glucans (~90 %) (Preedy, 2009). The fermentation of sugars by yeast results in the production of ethanol, carbon dioxide (0.35 to 0.45 %) and organic acids (OA), while the last two compounds are responsible for the in general acidic pH (3.8 - 4.7) of the final product, which contributes to microbial stability of beer (Preedy, 2009).

Beer contains malt and yeast derived “proteins”, which are found in concentrations of around 0.3 to 1 g/l (up to 5 g/l, personal communication) (Preedy, 2009). Brewers usually measure their protein applying the Kjeldahl method, which does not distinguish amino acids (AA), peptides and proteins. However, during malting, mashing and wort boiling, most proteins are degraded and therefore the majority of “protein” is available as peptides and amino acids. After fermentation, about 30 % of the total nitrogen compounds remain in the final product, including proteins (~25 %, > 3,500 kD) as well as peptides and free amino acids, the latter two making up 75 % (Abernathy et al., 2009).

Lipids and fatty acids (FA) also come from malt, while starting with a lipid content of 3 % in case of barley, only very little remains in the final product (0.1 %). In addition, yeast metabolism modifies the composition of the present fatty acids, increasing the proportion of short-chain fatty acids (C8 to C10) and decreasing the share of long-chain fatty acids (C12 to C18). Thus, finished beers contain about 15 to 30 mg/l fatty acids, most of them with a short-chain length of C4 to C10 (Preedy, 2009).

Another very important ingredient of beer is hop (*Humulus lupulus*), where the pine-like cones, from the virgin female plant, are used to flavor and stabilize beer. Hops are added to wort, followed by heating, while especially three hop components are essential for the brewing process and the final product. The essential oils and the phenolic compounds have an impact on flavor and aroma of the product. The so-called resins, separated into soft resins and hard resins, confer beer its bitterness. During wort boiling important soft resin compounds, known

as humulones (α-acids), are oxidatively isomerized to iso-α-acids, which represent the major bitter compounds in beer, but also exhibit a strong antibacterial activity (Preedy, 2009).

Malt is also a rich source of vitamins of the B-group, while some of them are crucial as growth factors for yeast and consequently utilized. Thus, beer contains considerably less of these vitamins compared to wort, which is especially distinct in case of biotin and pantothenic acid. Further, beer contains purines and pyrimidines, which result from nucleic acid degradation, while their concentration, based on literature apparently, varies dramatically from 0.2 to 139 µg/ml. Beer is further considered as “rich” with respect to inorganic compounds. Their concentration and composition strongly depends on the water used for brewing, while in total about 25 different inorganic compounds are found. Other beer compounds are higher alcohols, aldehydes, esters, ketones and sulphur compounds, while some of them have a high impact on the flavor of the product (Preedy, 2009). For further information on the composition of beer, the reader is referred to the comprehensive work of Victor R. Preedy (2009), which was the basis for this chapter.

1.1.2 The environment beer, its hurdles and bacterial tolerance

Beer is considered as a safe and stable beverage, which is characterized by various hurdles for bacterial growth. These hurdles directly result from the brewing process, while the corresponding selective properties contribute to beer´s microbial stability and shelf live. Bacteria have to cope with the metabolic products of yeast and the resulting environment as well as with processing hurdles, like the almost oxygen free bottling technology (Vriesekoop et al., 2012). Thus, they find an environment with a high concentration of ethanol, a low pH, reduced oxygen content (~ less than 0.3 ppm), high carbon dioxide concentration and the presence of antibacterial hop bitter compounds. Further, as the consequence of fermentation by yeast, beer is considered a low nutrient environment (Suzuki, 2011).

1.1.2.1 Ethanol

A hurdle for bacterial growth is the presence of ethanol, which is a metabolic product of the brewer’s yeast and mostly present in concentrations between 3.5 to 5 % (v/v) (Vriesekoop et al., 2012). The general antibacterial properties of ethanol have been described to affect cell membrane functions, which might even lead to cell membrane leakage and consequent inactivation (Ingram, 1990). A synergistic inhibitory effect with acid stress was observed for *Listeria monocytogenes* (Barker and Park, 2001) and *Escherichia coli* O157:H7 (Jordan et al., 1999). Haft et al. (2014) proposed an inhibition of *E. coli´s* transcription and translation by ethanol, while they conclude that this is caused by a direct effect on ribosomes and RNA

polymerase, leading to an uncoupled transcription and translation. Further, ethanol may affect a range of other cell functions, including enzyme activities and cell morphology (Vriesekoop et al., 2012). Bacterial tolerance towards ethanol has been suggested to be conferred by changes of the membrane lipid composition and adaptions/mutations of ribosomal genes and the RNA polymerase (Haft et al., 2014).

1.1.2.2 Low pH and acid stress

Beer has a low pH (3.4 to 4.8) because of the brewing process, especially influenced by yeast fermentation (Preedy, 2009; Vriesekoop et al., 2012). The causing agents, mainly small organic acids, are considered to diffuse passively through the cell membrane, followed by dissociation because of the comparatively higher intracellular pH (dependent on pKa). Thus, protons are released leading to intracellular acidification, affecting the transmembrane proton gradient (ΔpH) and consequently the proton motive force (pmf) (van de Guchte et al., 2002). This is considered to reduce the pmf-dependent uptake of nutrients and may finally result in metabolic exhaustion, also because of a negative effect on the activity of enzymes as well as a damaging effect on DNA (van de Guchte et al., 2002). There is also a synergistic effect of a low pH with ethanol and the antibacterial effect of hops (Barker and Park, 2001; Simpson, 1993a).

LAB are able to use amino acids to generate energy at a low pH and to counteract acid stress. Decarboxylation of amino acids, coupled with electrogenic transport, results in the consumption of protons and the generation of ATP via the pmf (van de Guchte et al., 2002). Tyrosine, glutamate and histidine are decarboxylated to their corresponding amines, tyramine, γ-aminobutyric acid (GABA) and histamine, exchanging substrate and product by antiport systems (Higuchi et al., 1997; Molenaar et al., 1993; Wolken et al., 2006). The arginine deiminase (ADI) pathway results in the production of ornithine, ATP, NH_3 and CO_2 (Mozzi et al., 2010; Zuniga et al., 2002). The agmatine deiminase (AGDI) pathway represents a similar system, using agmatine as substrate while producing putrescine and NH_3 (Lucas et al., 2007). Mixed-acid fermentation by homofermentative LAB or a general shift of pyruvate metabolism by LAB can also result in non-acidic end products (Mozzi et al., 2010) and therefore represent a potential metabolic strategy of pH homeostasis. Further, responses to acid stress can be the utilization of the malolactic fermentation, citrate fermentation, the urease system and the electrogenic transport of end products. Additionally, ATP dependent expulsion of protons, changes in cell envelope structures and repair mechanisms to fix acid induced damage of proteins and DNA are described as stress responses, all of them requiring energy (van de Guchte et al., 2002).

1.1.2.3 Oxygen

The low concentration of oxygen, which is getting less because of modern bottling techniques, prevents the growth of most aerobic bacteria (Suzuki, 2011). Concentrations of oxygen are found to range from 0.4 to 4 mg/l (Preedy, 2009). For LAB the absence of oxygen or the presence of only low amounts of oxygen does not represent a real stress, as these bacteria are in general facultative anaerobic or microaerophilic (Holzapfel and Wood, 2014). This property/hurdle has more significance for other bacteria, as it prevents growth of strictly aerobic contaminants such as *Acetobacter pasteurianus* (Back, 1994) and enables the growth of the strictly anaerobic beer-spoiling bacteria of the genus *Pectinatus* and *Megasphaera* (Suzuki, 2011). In case of heterofermentative LAB, low levels of oxygen, depending on metabolic capabilities, might be advantageous as oxygen can be used as an alternative electron acceptor allowing a higher energy yield from hexoses (Arskold et al., 2008; Zaunmuller et al., 2006). However, the presence of oxygen is in general associated with negative effects for LAB because of oxidative stress (van de Guchte et al., 2002).

1.1.2.4 Carbon dioxide

Carbon dioxide is produced during primary and secondary fermentation, while it can also be added additionally through the brewer by carbonation (Preedy, 2009). Carbon dioxide inhibits the growth of bacteria in several ways. Carbonisation creates an anaerobic environment, with the already described consequences (see 1.1.2.3). In addition, it causes a decrease of the pH, affects the cell membrane and has an influence on carboxylation and decarboxylation reactions, resulting in an inhibitory effect on growth (Vriesekoop et al., 2012). A general inhibitory effect of CO_2 on the growth of foodborne pathogens such as *Bacillus cereus*, *Enterococcus faecalis* or *Listeria monocytogenes* has been demonstrated in various studies as summarized by Vriesekoop et al. (2012). While CO_2 might even be stimulatory for LAB at low concentrations (< 0.3 g/l), those amounts typically found in beer (3.5 - 4.5 g/l) have an inhibitory effect (Suzuki, 2015).

1.1.2.5 Nutrient (limitation) utilization

Beer is considered as “a poor medium because nutrients are almost depleted by the fermentative activates of brewing yeast (Suzuki, 2011)”, while its composition was already detailed in chapter 1.1.1 (p. 3). Hop tolerance mechanisms are supposed to have a high energy demand (Vogel et al., 2010). Further, the low pH of beer, in combination with the other antibacterial hurdles, is likely to affect the metabolism of LAB in beer. In consequence, the

metabolic strategies of beer-spoiling LAB may be of importance for adaption to and growth in beer.

So far, only little is known about the metabolism of LAB growing in beer and its significance. Quite early a positive relationship between the beer spoilage ability of lactobacilli and their metabolic versatility was observed, while no particular biochemical traits could be connected to beer spoilage potential (BSP) (Dolezil and Kirsop, 1980). Fernandez and Simpson (1993) did reject this finding and did not find a correlation of hop resistance, being the most important determinant in beer, to metabolic products or sugar profile of various strains of lactobacilli and pediococci. In another study, they investigated the relation of beer spoilage by *Lactobacillus* (*L.*) *brevis* and *Pediococcus* (*P.*) *damnosus* to 56 parameters of 17 different lager beers. A statistical significant relationship of beer spoilage susceptibility to eight beer parameters could be found, including the content of free amino nitrogen, total soluble nitrogen content as well as the concentrations of various individual amino acids and maltotriose. Low concentrations of the respective parameters correlated with higher beer resistance to spoilage (Fernandez and Simpson, 1995). Suzuki et al. (2005b) investigated the metabolism of important heterofermentative beer-spoiling LAB species *L. brevis*, *L. lindneri* and *L. paracollinoides*. For each species, they determined the content of 34 metabolites for a single strain with the ability to grow in pilsner-type beer. Major findings were a high relevance of citrate, pyruvate, malate and arginine for growth in beer, but also for ATP production under hop stress. Malolactic fermentation and citrate utilization, as well as the ADI system were suggested to be important for beer-spoiling LAB (Suzuki et al., 2005b).

1.1.2.6 Antibacterial hops

Hops are added to wort and are considered as the major antibacterial hurdle for gram positive bacteria in the final product (Vriesekoop et al., 2012). Hops, namely the iso-α-acids, which form upon heat induced oxidation during wort boiling (Preedy, 2009), have two modes of action. They act as proton ionophores, thereby dissipating the transmembrane gradient, which leads to a decrease of intracellular pH (Behr and Vogel, 2009; Simpson, 1993b). As a result, the pmf-dependent uptake of nutrients is hampered and essential enzyme reactions are inhibited (Sakamoto and Konings, 2003; Simpson, 1993a; Yansanjav et al., 2004). A second mode of action was described by Behr and Vogel (2010), which showed that iso-α-acids participate in transmembrane redox reactions, thus inducing oxidative (redox) stress. Schurr, Hahne, et al. (2015) found that the manganese-binding induced oxidative stress has a superior significance for the antibacterial effect of iso-α-acids regarding *L. brevis*. Other harmful effects of hops have been described, among them the loss of intracellular Mn^{2+}, while Mn^{2+} is important

for LAB energy generation and redox homeostasis, and an induction of membrane leakage in case of *Bacillus subtilis* (Behr et al., 2007b; Suzuki, 2011; Vriesekoop et al., 2012).

Hops are considered as the most important antibacterial hurdle in beer, representing the major determinant of microbial beer stability. Beer spoilage ability is regarded to be species-associated, resulting in a comparatively low number of species with relevance for beer spoilage. Nevertheless, these species partially contain strains with and without beer spoilage ability. Hop resistance was shown to be a major distinguishing property for intraspecies differentiation (Suzuki et al., 2006). The origin of current beer-spoiling LAB seems to be tightly connected to the usage of hops, while it is suggested that hop resistance is mainly conferred by horizontal gene transfer (HGT). This is supported by the fact that a comparative analysis of hop tolerance genes and their respective genetic clusters revealed a high nucleotide sequence similarity (~ 99 %) between phylogenetically non-related beer-spoiling species (Suzuki, 2015). In addition, the first available genome sequences of brewery isolated LAB confirmed that these genes and their clusters are found on plasmids (Behr et al., 2015; Bergsveinson, Pittet, et al., 2015; Pittet et al., 2012).

Cell membrane associated hop tolerance mechanisms

Most tolerance mechanisms have been described to be associated with the cell membrane. They base on the assumption that hop bitter acids confer their protonophore property via intrusion into the cell, where they lose a proton and cause acidification (Sakamoto et al., 2001; Simpson, 1993b). Two multidrug transporters (MDR) have been described, HorA as an ABC type MDR and HorC as a pmf-dependent MDR. Both are suggested to act as hop efflux transporters. In consequence, a reduced net influx of undissociated and membrane-permeable hop bitter acids is proposed, limiting the antibacterial protonophore effect on the cell (Sakamoto et al., 2001; Suzuki et al., 2005a). The corresponding genes, *horA* and *horC*, are prominent diagnostic marker genes (DMGs) for the species independent identification of beer-spoiling LAB, as the presence of these plasmid-encoded genes has shown a good correlation to beer spoilage ability (Haakensen et al., 2008; Suzuki et al., 2006). HitA was proposed as another mediator of hop resistance in *L. brevis*, while this potential divalent cation transporter is suggested to counteract the intracellular Mn^{2+} leakage caused by hops, thus reducing the harmful effect of iso-α-acids (Hayashi et al., 2001). Further, the intracellular acidification by hop bitter acids was found to induce pmf-generating activities and the membrane located proton-translocating ATPase in order to extrude protons (Suzuki, 2015). These active hop tolerance mechanisms have been proposed to provide the cells enough time to develop long-term and passive hop tolerance (Vogel et al., 2010). Behr et al. (2006) found a change in *L. brevis* membrane fatty acid composition towards the incorporation of more saturated fatty

acids upon hop adaption. This reduces the membrane fluidity, protects the cell from acid stress and potentially from hop stress (Behr et al., 2006). The significance of membrane biosynthesis and modification was further supported by the results of Schurr, Behr, et al. (2015) and Preissler (2011).

Cell wall associated hop tolerance mechanisms

While expression of HitA provides a mechanism of Mn^{2+} homeostasis, Mn^{2+} binding by lipoteichoic acids (LTAs) is considered as a long-term passive hop tolerance mechanism. Behr et al. (2006) found an increase of higher-molecular-weight (hmw) LTAs in *L. brevis* upon acid and hop stress. This is considered to improve the cell wall barrier function, but also hypothesized to be a reservoir for divalent cations, such as Mn^{2+}. These hmw LTAs have a higher potential to bind Mn^{2+} and thus compete for them with hop bitter acids. As hops require Mn^{2+} to exert their full antibacterial properties, the LTA mediated Mn^{2+} homeostasis can be considered as important hop tolerance mechanism (Behr et al., 2006; Schurr, Hahne, et al., 2015).

Other hop tolerance mechanisms and cellular responses of LAB to hop stress

The expression of Mn^{2+} dependent enzymes has been reported to be upregulated upon hop stress in *L. brevis*. Affected are also enzymes that are considered to play a role for energy generation and redox homeostasis. The upregulation of Mn^{2+} dependent enzymes might result from the hop-dependent reduction of available Mn^{2+}, thus compensating the decreased enzyme activity by an increase of enzyme amount. Further, these enzymes might compete for Mn^{2+} with hop compounds, thus reducing their antibacterial effect (Behr et al., 2007a). The observed upregulation of hop-inducible proteins, the enhanced LTA production and HitA are all suggested to control the intracellular Mn^{2+} content, consequently counteracting hop induced oxidative stress (Vogel et al., 2010). Morphological shifts into smaller rods and a consequently reduced surface area have been observed for *L. brevis* and *L. lindneri* adapted to beer. It is hypothesized that these changes lead to a reduction of defense perimeters and help to deploy active and membrane-bound resistance mechanisms more efficiently (Asano et al., 2007). Finally, acid tolerance mechanisms, such as the ADI system and the glutamate decarboxylase (GAD) system, have been shown to improve *L. brevis* growth under hop stress by supporting the maintenance of the pmf (Behr et al., 2006; Schurr et al., 2013).

1.2 Beer-spoiling lactic acid bacteria

While there are other important beer-spoiling microorganism, such as the Gram-negative and strictly anaerobic members of *Pectinatus* and *Megasphaera* as well as some wild yeasts such as *Dekkera* spp., the vast majority of spoilage incidents is caused by LAB. From 1980 to 2002 up to 90 % of all spoilage incidents in Germany, were caused by LAB from the genera *Lactobacillus* and *Pediococcus* (Back, 1994; Suzuki, 2011). Beer-spoiling LAB cause acidification, sedimentation, haze, turbidity, off-flavors, biogenic amine production and ropiness (slime formation), depending on species and strains (Kalač et al., 2002; Suzuki, 2011).

There are about 20 LAB species with a general significance for beer spoilage, which can be classified according to their general hazard potential (HP) for the product, as done by Hutzler et al. (2013). Those LAB species with a very high hazard potential include *L. backii*, *L.* (*para*-)*collinoides*, *L. paucivorans, L. brevis*, *L. lindneri* and *P. damnosus*, while the latter three are considered as the major beer spoilers (Hutzler et al., 2013; Suzuki, 2015). High hazard potential LAB include *L. acetotolerans*, *L.* (*para*-)*buchneri*, *L.* (*para*-)*casei*, *L. coryniformis*, *L. perolens*, *L. plantarum*, *L. rossiae*, *P. claussenii* and *P. inopinatus*. Further, Hutzler et al. (2013) classifies *Lactococcus lactis* and *Leuconostoc* (*para*-)*mesenteroides* as species with a positive tendency for beer spoilage, while an adaption is necessary for these species to grow in beer. This general classification applies to species, while within the single species the actual ability to grow in and spoil beer (beer spoilage ability) may vary significantly. Beer spoilage ability of LAB strains is additionally affected by their physiological conditions (adaption to beer/hop bitter acids) and dependent on isolation source and the beer type, while the significance of the encountered antibacterial hurdles was already detailed in chapter 1.1.2 (p. 5). Species and strains with a short lag-phase in beer and thus a quick adaption have been further designated as obligate beer-spoiling species by Back (1994). The contamination of beer occurs either via primary contamination, during beer production (e.g. from raw materials), or via secondary contamination, which happens during filling in the packaging hall (Suzuki, 2015).

1.2.1 Pediococcus claussenii

P. claussenii is considered to have a high hazard potential and was initially described in 2002 by Dobson et al. (2002). It is characterized by non-motile, non-spore-forming coccoid cells, occurring in tetrads and pairs. *P. claussenii* is homofermentative and microaerophilic, grows up to 40° C with an optimum at 28° C and was found to show growth from pH 4.5 to 8.0. Acid production was observed from glucose, fructose, mannose, *N*-acetylglucosamine, amygdalin,

arbutin, cellobiose, aesculin, gentiobiose, trehalose, salicin, ribose and mannitol as well as from maltose in a strain-dependent manner (Dobson et al., 2002). Upon growth in beer, citrate, malate as well as cellobiose were found to be utilized (Pittet et al., 2013). EPS production was observed for some isolates, while this property was shown to be unstable upon repeated broth cultivation (Dobson et al., 2002). All strains of this species have been isolated from brewery environments so far (Suzuki, 2015).

There are no statistics about spoilage incidents for 1980 to 2002, while *P. claussenii* seems to be of subordinate significance for Germany, considering a percentage share of 0 to 1 % for the period from 2010 and 2013 (Suzuki, 2015). *P. claussenii* is more often found as a primary contamination and additionally characterized by a strain specific beer spoilage potential, while the majority of all isolates is characterized by beer spoilage ability, causing acidification, turbidity and in some cases ropiness (Haakensen et al., 2008).

P. claussenii ATCC BAA-344 (TMW 2.340/DSM 14800^T) was the first beer-spoiling strain, for which a complete genome sequencing and a comparative transcriptomic project were performed (Pittet et al., 2012; Pittet et al., 2013). Especially the transcriptome comparison of this strain, growing in beer and in MRS medium, revealed interesting insights into the multifactorial adaption of *P. claussenii* to beer. Regarding the chromosome, the most significant upregulation, referring to growth in beer in comparison with MRS, was found for genes of the AGDI pathway, the citrate and malate fermentation operons, a cellulase and a cellobiose (maltose)-specific phosphotransferase system (PTS). The first three mentioned traits are suggested to play a role in acid tolerance and hop tolerance, counteracting acidification. Several other operons have been upregulated, most of them found to revolve around the transport and utilization of various substrates (e.g. mannitol, trehalose and thiamine) (Pittet et al., 2013). Additional upregulations were found to be associated with the GO terms fatty acid biosynthesis (FAS), proton transport, oxidative stress, ion transport, cell wall processing and DNA repair. ATCC BAA-344 is characterized by six to eight plasmids (unstable). It was found that most (up to 97 %, pPECL-8) of the plasmid-encoded genes were significantly upregulated upon growth in beer, while only three genes in total have been upregulated in MRS. Those genes upregulated in beer are mostly hypothetical proteins, while those upregulated genes with a given annotation comprise the *horA* cluster, including the already mentioned hop transporter, but also genes predicted to be involved in phospholipid and cell wall biosynthesis. Further upregulated plasmid-encoded genes are associated with conjugation, DNA protection, oxidative stress and restriction modification. Pittet et al. (2013) finally found that the upregulation of these genes was not solely driven by transcriptional regulation, but also by the copy number of these plasmids (Pittet et al., 2013).

1.2.2 *Pediococcus damnosus*

P. damnosus, initially described by Claussen in 1903 (Holzapfel and Wood, 2014), has also been considered as LAB species with very high hazard potential (Hutzler et al., 2013). It is described as microaerophilic, homofermentative, non-motile, non-spore-forming and characterized by spheroid cells, which form tetrads or diplococci and short chains. Growth was observed from 8 to 30° C, with an optimum at rather low temperatures such as 22 to 25° C. The optimal pH is 5.5, while *P. damnosus* also grows at lower pH values (e.g. pH 4.5). Glucose, fructose, mannose, cellobiose and galactose are fermented, while in other cases the production of acid was also shown for maltose, melizitose, sucrose, arbutin and salicin (Dobson et al., 2002; Holzapfel and Wood, 2014). This is rather in contrast to the statement that *P. damnosus* has a comparatively narrow sugar consumption spectrum (Suzuki, Asano, Iijima and Kitamoto, 2008). *P. damnosus* is further known to produce acetoin and diacetyl, and for the strain-specific ability to produce exopolysaccharides (EPS), resulting in a buttery off-flavor and ropiness in beer, respectively. Although *P. damnosus* is considered to be closely associated with the brewery environment (Suzuki, 2015), it was repeatedly found within wineries, where it takes part in the malolactic fermentation, a second fermentation of wines in order to reduce wine acidity by the conversion of malic acid to lactic acid. It also can cause wine spoilage due to EPS production (Delaherche et al., 2004; Juega et al., 2014).

Between 1980 and 2013, *P. damnosus* caused 1.2 to 31 % of all beer spoilage incidents recorded in Germany. *P. damnosus* is mainly a primary contaminant; beer spoilage ability is strain-dependent and leads to sediment formation, acidification as well as off-flavors such as diacetyl (Back, 2005; Haakensen et al., 2008; Suzuki, 2011, 2015). *P. damnosus* is commonly found associated with yeast and beer. Further, it is also known to be one of the most frequent contaminants in fermentation and maturation processes, which may cause an unexpected rise in the diacetyl level during the brewing processes. As in case of *L. lindneri*, *P. damnosus* was found to adhere to the brewing yeast, which is considered to induce premature sedimentation of yeast cells. Both species tend to be latent in fermentation and maturation during the brewing process, while both seem to need beer-specific components for optimal growth (Back, 2005; Storgards et al., 1997; Suzuki, 2015). A principal correlation of beer spoilage ability to the presence of *horC* and *horA* was found for *P. damnosus* (Haakensen et al., 2008).

1.2.3 Lactobacillus backii

L. backii is another species with very high hazard potential and is again considered as obligate beer-spoiling species (Bohak, 2006; Hutzler et al., 2013). "*Lactobacillus backi*" has been described by Bohak (2006), while it has been redesignated to *L. backii*, as a consequence of

initial misnomer, by Tohno et al. (2013). *L. backii* is part of the *L. coryniformis* group, whose members are in general considered as facultatively heterofermentative (Holzapfel and Wood, 2014), while *L. backii* is characterized as a homofermentative, facultatively anaerobic, non-motile and non-spore-forming species within the initial description and the redesignation. Acid is produced from glucose, fructose, mannose, mannitol, sorbitol, *N*-acetylglucosamine, arbutin and salicin. Further, *L. backii* DSM 18080^T was found to exhibit β-glucosidase, β-galactosidase and N-acetyl-β-glucosaminidase activity. Growth was observed between 10 to 37° C, with an optimum at 28° C, and a pH from 3.5 to 8.0, with an optimum of 4.5 to 6.5. Rod-shaped cells are unregularly shaped, characterized by rounded ends, while they occur singly, in pairs and in short chains (Bohak, 2006; Tohno et al., 2013). So far, *L. backii* has been only described to be isolated from brewery environments and is therefore considered unique to the brewing environment (Bohak, 2006; Iijima et al., 2007; Suzuki, 2015).

Because of the comparatively recent description of *L. backii*, spoilage-incident statistics are only available for 2010 to 2013, while the proportion of spoilage by this organism made up 4.8 to 10.9 % in Germany. However, because of the high similarity to *L. coryniformis*, it is possible that *L. backii* was identified as *L. coryniformis* in the past, which is indicated by the fact that the latter species is even missing in recent statistics, while a prevalence of 1 to 10 % was found from 1980 to 2002 (Back, 1994, 2005; Suzuki, 2011, 2015). Primary contamination is dominant over secondary contamination, while beer spoilage by *L. backii* is causing turbidity, sedimentation and slight acidification (0.1 to 0.2 pH units). All described strains have been shown to possess beer spoilage ability and a comparatively high hop tolerance, while *horA* and *horC* were found in some and in all strains, respectively (Bohak, 2006; Iijima et al., 2007).

1.2.4 Lactobacillus lindneri

L. lindneri is considered to have a very high hazard potential and according to Back (2005), it is an obligately beer-spoiling species. It was initially mentioned by Henneberg in 1901, further specified by Lindner (1909) and validly published by Back et al. (1996). *L. lindneri* belongs to the *L. fructivorans* group, is obligately heterofermentative, microaerophilic, non-spore-forming, non-motile and grows best at pH values around 4.6 to 5.2 and not above temperatures of 30° C (Back, 2005; Back et al., 1996; Holzapfel and Wood, 2014). Cells are non-motile and rod-shaped, occurring as single cells, in pairs or in chains. As in case of *L. brevis*, cells show a strong tendency to alter their morphology upon incubation in beer. *L. lindneri* is considered as a brewery-specific microorganism (Suzuki, 2015), although it has been reported that some strains have been isolated from winery environments, where they are considered as spoilage organism, potentially responsible for the formation of indole from tryptophan (Arevalo-Villena et al., 2010). It has a very narrow sugar utilization profile, while so far only glucose, maltose

and in some cases fructose have been shown to be fermented. This narrow sugar profile has been suggested to be the consequence of *L. lindneri´s* deep association with the environmental niche beer (Suzuki, 2015). Pyruvate, citrate, malate and glutamate were consumed by *L. lindneri* upon growth in beer (Suzuki et al., 2005b). The cell wall of this bacterium lacks teichoic acids. Further, *L. lindneri* was found to tolerate ethanol concentrations up to 7 % as well as very high hop contents (45 IBU). Growth of *L. lindneri* seems to be significantly enhanced by the presence of an unidentified or at least unpublished growth factor, suggested to come from yeast metabolism (Back, 2005; Back et al., 1996).

Beer spoilage by *L. lindneri* caused up to 25 % of all documented spoilage incidents in Germany during 1980 to 2013, while primary contamination seems to dominate over secondary contamination (Back, 1994, 2005; Suzuki, 2011, 2015). With the exception of an artificial plasmid-cured variant, lacking *horC*, there are no reports about strains lacking beer spoilage ability. Thus, some brewing microbiologists argue that in contrast to *L. brevis*, *L. lindneri* is an innate beer spoiler (Suzuki et al., 2005a). Beer spoilage by *L. lindneri* causes turbidity, sedimentation and a slight acidification. Strains of this species have been shown to exhibit a hard-to-cultivate characteristic upon primary isolation on regular laboratory media, while this hard-to-cultivate state can be gradually reduced and induced by repeated passages in MRS and beer, respectively. This hard-to-cultivate state is suggested to be caused by a profound adaption to the beer environment, amongst others characterized by a low pH and a completely different availability of nutrients compared to MRS media (Suzuki, 2011). Strains of *L. lindneri* have been shown to adhere to brewing yeast cells, which suggests that this species is latent during fermentation and maturation of beer (Storgards et al., 1997).

1.2.5 Lactobacillus paracollinoides

L. paracollinoides is characterized by a very high hazard potential and as an obligate beer-spoiling LAB species (Back, 2005; Hutzler et al., 2013). It has been validly described by Suzuki, Funahashi, et al. (2004), while Ehrmann and Vogel (2005) found that this species has been referred to previously using the not validly described (by Van Laer in 1892) name "*L. pastorianus*". *L. paracollinoides* belongs to the *L. collinoides* group with six heterofermentative species, three of them isolated from fermented beverages (Holzapfel and Wood, 2014). It is described as a facultatively anaerobic, heterofermentative, rod shaped, non-motile and non-spore-forming organism, while cells occur singly or in short chains. Growth was observed at 15° C but not at 45° C (Suzuki, Funahashi, et al., 2004). Acid production was observed from ribose, xylose, glucose, maltose and melibiose, while it has been shown that *L. paracollinoides* is using pyruvate, arginine and maltotriose upon growth in beer (Suzuki, Funahashi, et al., 2004; Suzuki et al., 2005b). *L. paracollinoides* has been repeatedly isolated

from the brewery environment and is suggested to be unique to the brewing industry (Suzuki, 2015). In contrast, *L. paracollinoides* has been isolated from cider, where it is considered to be responsible for the production of biogenic amines (Ladero et al., 2011), and in green-olive fermentations (Lucena-Padros et al., 2014).

As in case of *L. backii*, there are no long-term statistics available, while only a low prevalence (0 to 3.6 %) was found for the period from 2010 to 2013. Analogous to *L. backii*, the high similarity of *L. paracollinoides* to *L. collinoides* might have caused misidentifications in the past (Suzuki, 2015). Secondary contamination is found at a higher frequency compared to primary contamination, while spoilage is characterized by turbidity and acidification (Hutzler et al., 2013; Suzuki, Funahashi, et al., 2004). As in case of *L. lindneri*, *L. paracollinoides* comprises strains with hard-to-cultivate status and the only described non-spoiling strains were obtained artificially. The lack of ORF5, which is now known to be located within the *horC* cluster, was found to correlate to the inability of the generated variants to spoil beer (Suzuki et al., 2006; Suzuki, Ozaki, et al., 2004).

1.2.6 Lactobacillus brevis

L. brevis is a species with very high hazard potential and the most frequent beer-spoiling organism (Back, 1994; Suzuki, 2011, 2015). *L. brevis* was first mentioned in 1919 as *Betabacterium breve* and finally described as *L. brevis* in 1934 in Bergey´s (Holzapfel and Wood, 2014), while some strains have been designated (miss-assigned) as *L. frigidus* or *L. diastaticus* in the past (Back, 1994, 2005; Suzuki, 2015). *L. brevis* belongs to the *L. brevis* group, containing ten species with facultatively or obligately heterofermentative fermentation. *L. brevis* is a physiologically versatile species, occupying multiple niches, while it has been isolated from various sources such as milk, cheese, sauerkraut, sourdough, silage, cow manure, faeces, honey, the intestinal tracts of humans and rats, and of course from beer and the brewery environment (Back, 2005; Holzapfel and Wood, 2014; Preissler, 2011). It is characterized by non-motile, rod-shaped cells, occurring singly and in short chains (Holzapfel and Wood, 2014; Kandler, 1983), while a morphological shift was observed upon growth in beer, resulting in a reduced cell size compared to other conditions (Asano et al., 2007). *L. brevis* is obligate heterofermentative, facultative anaerobic and grows from 15 to 45° C (Holzapfel and Wood, 2014). It is metabolically versatile, illustrated by a broad sugar spectrum with up to 12 (strain dependent) fermentable sugars (Holzapfel and Wood, 2014) and the ability to use the GAD system and the ADI pathway (Behr et al., 2006; Behr et al., 2007b; Schurr et al., 2013). *L. brevis* has been reported to have a relaxed sugar utilization, fermenting even more than one sugar at once (Kim et al., 2009). Upon growth in beer, *L. brevis* was shown to utilize pyruvate, citrate, malate, arginine, glutamate and tyrosine (Suzuki et al., 2005b).

For the period from 1980 to 2013, *L. brevis* is considered to be responsible for 39 to 53 % of all spoilage incidents (Back, 1994, 2005; Suzuki, 2011, 2015), causing turbidity, sedimentation, acidification and slimy beer, but no diacetyl off-flavor (Back, 2005). Secondary contamination seems to be more frequent, while beer spoilage ability is a strain-specific property, which also correlates to the source of isolation, as it has been shown that non-brewery isolates in general lack beer spoilage ability (Nakagawa, 1978; Preissler, 2011; Suzuki, 2015). Based on sequence analysis, recent MALDI-TOF MS results, proteomic research and sugar fermentation profiles, it is suggested that beer-spoiling *L. brevis* has adapted in a stepwise manner and consequently forms a phylogenetically distinct group within the species (Suzuki, 2015). It is important to consider that the antibacterial properties of hops, as well as potential hop tolerance mechanisms (e.g. *HorA*, *HorC* (Suzuki, 2015)) have almost exclusively been studied with *L. brevis* as model organism. *L. brevis* is not only the best investigated beer-spoiling LAB species, but also in general a well-studied organism, which is reflected by about 140 publications with reference to this species within title or abstract.

Recently, Bergsveinson, Baecker, et al. (2015) presented the genome of the rapid beer-spoiling *L. brevis* BSO 464, while they found the strain to possess eight different plasmids. They performed plasmid curing experiments as well as a consequent characterization of the resulting variants with respect to hop tolerance and beer spoilage ability. They conclude that besides the already described plasmid-encoded gene clusters, containing the accepted hop tolerance genes *horA* and *horC*, beer spoilage ability and hop resistance are also affected by other plasmids without any well-characterized genes. They could confirm that successful growth of *L. brevis* in beer is a multifactorial process and that it requires complex, plasmid-encoded genetics beyond the bacterial chromosome. This is strikingly indicated by the fact that BSO 464, lacking specific plasmids, even lost its ability to grow in beer. HorC was found to have the most significant impact on the beer spoilage ability of *L. brevis* BSO 464 (Bergsveinson, Baecker, et al., 2015; Bergsveinson et al., 2012).

1.3 The detection and identification of beer-spoiling lactic acid bacteria

Usually the first step in quality control includes sampling and cultivation of contaminants using detection media (e.g. MRS, NBB, ABD, UBA and BMB). These detection media should specifically detect relevant contaminations within a preferably short period, also those strains with a hard-to-cultivate state (Suzuki, Asano, Iijima, Kuriyama, et al., 2008). Specific detection media, such as ABD and NBB, are generally used as a first step for routine quality control and have been shown to improve the detection of beer-spoiling LAB substantially with respect to sensitivity and detection time (Back, 2005; Suzuki, Asano, Iijima, Kuriyama, et al., 2008). Thus, one ends up with the detection of brewery LAB, while an identification or an evaluation of the actual beer spoilage ability is not yet achieved. As beer spoilage ability is strain-dependent for some species and because these detection media also enrich LAB species, which are not necessarily capable of growing in the final product, a consequent identification and a potential evaluation of the actual beer spoilage ability is essential for efficient quality control (Suzuki, Asano, Iijima and Kitamoto, 2008).

A species identification, in order to find out if the detected contamination includes LAB species with very high or high hazard potential can be carried out using various methods, while some approaches were also implemented into actual quality control products. Using species or even group specific PCR approaches (e.g. VIT® Bier plus *L. brevis*, Vermicon, Munich, Germany), relevant species can be identified within a few hours after detection (Suzuki, Asano, Iijima and Kitamoto, 2008). Another method for a fast and reliable identification of brewery relevant LAB employs the identification via MALDI-TOF MS (Kern et al., 2013; Kern et al., 2014; Wieme et al., 2014). Other species identification methods have been proposed over the last decades, for example based on ribotyping, fluorescence *in situ* hybridization (FISH), loop-mediated isothermal amplification (LAMP) and immunoassays (Suzuki, Asano, Iijima and Kitamoto, 2008), while not all of these methods are applicable for brewery quality control.

In order to determine the actual beer spoilage ability, a forcing test, including the inoculation of the product with the detected microorganism and the consequent assessment of visible growth or other spoilage related effects, represents the most reliable method. However, despite high accuracy, these tests take up to several weeks, while at the time results are available, the product is already irretrievably distributed in the market. Thus, rapid and reliable methods are needed to predict the ability of LAB strains to grow in and spoil beer (Suzuki, Asano, Iijima and Kitamoto, 2008). Besides some approaches to predict the beer spoilage ability by rapid growth tests (e.g. resazurin assay, (Preissler et al., 2010)), most effort in

research focused on the identification of molecular DMGs, which will be detailed in the next chapter.

1.3.1 Molecular detection of lifestyle genes

DMGs with a good correlation to beer spoilage ability and a specifity for brewery and beer-spoiling LAB, at best linked to a biological function that is connected to LAB growth under hop stress (major determinant) and in beer, can be referred to as lifestyle genes of beer-spoiling LAB. In the past two decades, several mostly plasmid-encoded DMGs have been identified, some of them with validity within a single species and others within several. The most prominent lifestyle genes comprise *horA*, *horC* and *hitA*, while the presence of the former two DMGs has been shown for various LAB species, including all with a very high hazard potential (Suzuki et al., 2006). Suzuki et al. (2006) tested 130 strains of various species and frequent brewery isolates, finding that all beer-spoiling strains were either positive for *horA* or *horC*. The usage of both DMGs resulted in no false negative results, while false positive signals were also obtained. It is very important to note that DMGs as *horA* and *horC* allow a very precise targeting (primers), as these genes and their surrounding clusters are highly conserved on DNA level (~ 99 %) and do not show high DNA similarities to other homologous genes, which might result in false positive identifications. As these genes are spread by HGT, also considering their significance within various LAB species, they can also detect potentially yet uncharacterized species, which would not be targeted by a species-specific system (Suzuki, Asano, Iijima and Kitamoto, 2008; Suzuki et al., 2006).

Other potential DMGs have been proposed, while these genes, including *bsrA* and *bsrB* (both predicted to be ABC MDRs, chromosomally encoded) together with *hitA,* so far have been shown to be of minor relevance (Bergsveinson et al., 2012; Haakensen, Schubert, et al., 2009; Suzuki, 2015). Chromosomally encoded DMGs have been proposed for *L. brevis*, while several genes were identified, amongst others, associated with transcriptional regulation and the cell envelope, which were present in beer-spoiling strains but not in non-spoiling strains (e.g. *cinA*, *arsR*) (Behr et al., 2015). Despite the doubtless general significance of *horA* and *horC*, these genes have also been found to be missing in some beer-spoiling strains or to be present in non-spoiling LAB (Haakensen, Pittet, et al., 2009; Haakensen et al., 2008; Suzuki et al., 2006).

The ability to spoil beer was found for several species and strains to be unstable, especially when isolates were propagated in laboratory media, while the loss of beer spoilage ability was observed to correlate with the loss of lifestyle genes such as *horA* and *horC*. This strengthens the hypothesis that specific lifestyle genes are necessary for the growth in beer and that these

represent consequently appropriate targets for quality control (Suzuki, 2015). On the other side, recent plasmid curing experiments have also shown that especially the relevance of *horA* for *L. brevis* might be overestimated (Bergsveinson, Baecker, et al., 2015). Consequently, the research for novel DMGs and true lifestyle genes for beer-spoiling LAB is still ongoing.

2 Hypotheses and Objectives

If we look for novel target genes for a fast and reliable DNA-based quality control in breweries, in order to provide consumers with an impeccable product, it is important to understand the mechanisms underlying the growth of beer-spoiling lactic acid bacteria (LAB) in their ecological niche beer. In this study, we want to provide the reader with a comprehensive genome analysis of LAB species with high and very high hazard potential. Along this genome analysis, supported by physiological, metabolic, proteomic and genetic data, we aim to describe shared and species-specific strategies, which will finally provide new insights into the lifestyle of beer-spoiling LAB in beer. Based on these insights and supported by comparative genomics, we further aim to identify novel diagnostic marker genes (DMGs) and lifestyle genes for the identification of beer-spoiling LAB in quality control.

Hypotheses: Beer-spoiling LAB can be characterized by a common lifestyle, which allows them to overcome the beer-specific antibacterial hurdles. This adaption to the harsh environment beer is the consequence of brewery- and beer-spoilage-specific genes and genome properties. Beer-spoiling LAB genomes are characterized by a number of specific genes, which are not present in non-spoiling strains. These genes are connected to their ability to grow in beer, because they support the organism to counteract and handle beer-specific hurdles. Consequently, these genes represent DMGs or even lifestyle genes, which can be used for the targeted discrimination of beer-spoiling and non-spoiling strains of LAB.

This hypotheses result in the following objectives.

General objectives:

Describe shared (metabolic) strategies and species-specific strategies and evaluate their role and the role of genomic plasticity for the lifestyle of beer-spoiling LAB in beer. Consequently, identify traits (genes), which enable and support the adaptation to the niche beer and evaluate their potential as targets for quality control.

Further objectives:

Establish a reliable reference method for the determination of beer spoilage ability and determine the stability and transferability of these results into other beer systems

Characterize the spoilage characteristics of LAB with very high and high hazard potential, brewery and non-brewery isolates in order to assess the requirement for intraspecies

discrimination of LAB and to provide consistent reference data for the evaluation of DMGs and other rapid detection systems

Obtain complete genome sequences of LAB with very high hazard potential and varying beer spoilage ability to perform a comprehensive comparative genome analysis

Identify the LAB lifestyle in beer: metabolic capabilities and strategies of beer-spoiling LAB in beer and under hop stress, stress tolerance with respect to beer specific hurdles, as well as genomic traits, which are connected to beer spoilage ability and the adaptation to beer and the brewery environment

Delineate the roles of genetic setting *versus* genomic plasticity and phenotypic plasticity in the expression of a lifestyle enabling growth in beer

Identify those traits (DMGs), which enable (or support) growth in beer and which are consequently candidate genes for the identification of beer-spoiling LAB

Evaluate and validate the predicted DMGs in order to provide novel targets for brewing-microbiological quality control and establish potential links between DMGs and their significance for the LAB lifestyle in beer (lifestyle genes)

Propose a distinct detection and differentiation system for beer-spoiling LAB

3 Material and Methods

In this chapter, in most cases, only the general methodology is described. The experimental design will be addressed within the corresponding result chapters, if appropriate.

3.1 Microorganisms, media and culture conditions

3.1.1 Microorganisms

Table 2 lists all strains, alternative identifiers and isolation sources. Strains, used for single experiments, are mentioned in the corresponding result sections and/or in the corresponding material and methods section.

Table 2: List of used strains. All strains are listed together with their species, strain number (TMW), alternative identifiers and the isolation source. *L.* = *Lactobacillus*, *P.* = *Pediococcus*. TMW = Technische Mikrobiologie Weihenstephan, Alternative ID (DSM, ATCC), NB = non-spoiling variant, T = type strain.

Species	TMW	Alternative ID	Isolation source
P. claussenii	2.340	DSM 14800^T	spoiled beer
P. claussenii	2.1531		unpasteurised beer
P. claussenii	2.1545		unpasteurised beer
P. claussenii	2.50		brewery environment
P. claussenii	2.51		brewery environment
P. claussenii	2.53		brewery environment
P. claussenii	2.54		brewery environment
P. claussenii	2.56		brewery environment
P. claussenii	2.59		brewery environment
P. claussenii	2.60		brewery environment
P. claussenii	2.61		brewery environment
P. claussenii	2.62		brewery environment
P. claussenii	2.64		brewery environment
P. claussenii	2.65		brewery environment
P. claussenii	2.67		brewery environment
P. damnosus	2.4	DSM 20331^T	lager beer yeast
P. damnosus	2.125		unknown
P. damnosus	2.1532		bottled beer
P. damnosus	2.1533		brewery environment
P. damnosus	2.1534		brewing yeast sample
P. damnosus	2.1535		pilsner beer
P. damnosus	2.1536		winery environment
P. damnosus	2.1546		brewery environment
P. damnosus	2.1547		pilsner beer - unfiltrated

P. damnosus	2.1548		brewery environment
P. damnosus	2.1549		winery environment
P. damnosus	2.1635		Kellerbier
P. damnosus	2.1636		Kellerbier
P. damnosus	2.1637		brewing environment
P. damnosus	2.1638		brewery fermentation tank
P. damnosus	2.1639		brewery environment
P. damnosus	2.1640		wheat beer
P. damnosus	2.1641		brewery environment
P. damnosus	2.1642		pilsner beer - unfiltrated
P. damnosus	2.1643		winery environment
L. backii	1.1299	DSM 18080^{T}	spoiled lager beer
L. backii	1.1430		spoiled beer
L. backii	1.1432		spoiled beer
L. backii	1.1883		spoiled beer
L. backii	1.1988		light wheat beer
L. backii	1.1989		beer
L. backii	1.1990		brewery environment
L. backii	1.1991		brewery environment
L. backii	1.1992		brewery environment
L. backii	1.2002		brewery environment
L. backii	1.2003		brewery environment
L. backii	1.2004		wheat beer
L. backii	1.2005		pilsner beer
L. backii	1.2070		brewery environment
L. backii	1.2071		brewery environment
L. backii	1.2072		dark lager beer
L. backii	1.2073		brewery environment
L. backii	1.2077		brewery environment
L. backii	1.2078		brewery environment
L. backii	1.2079		unpasteurised beer
L. lindneri	1.88	DSM 20690^{T}	spoiled beer
L. lindneri	1.1285	DSM 20692	spoiled beer
L. lindneri	1.1286	DSM 20692^{NB}	spoiled beer
L. lindneri	1.1433		spoiled beer
L. lindneri	1.1993		beer
L. lindneri	1.2006		brewery environment
L. lindneri	1.2007		brewery environment
L. lindneri	1.2008		brewery environment
L. lindneri	1.2009		brewery environment
L. lindneri	1.2080		brewery environment
L. lindneri	1.2081		brewery environment
L. lindneri	1.2082		brewery environment
L. lindneri	1.456		brewery environment
L. lindneri	1.481		brewery environment
L. paracollinoides	1.696	DSM 15502$^{T\text{-}NB}$	brewery environment
L. paracollinoides	1.1979	DSM 20197^{NB}	beer
L. paracollinoides	1.1994		brewery environment

L. paracollinoides	1.1995		pilsner beer
L. paracollinoides	1.2010		pilsner beer
L. brevis	1.6	DSM 20054^T	faeces
L. brevis	1.1326	ATCC 367	silage
L. brevis	1.100		sourdough
L. brevis	1.1205		sourdough
L. brevis	1.1282		brewery environment
L. brevis	1.1369		honey fermentation
L. brevis	1.1370		honey fermentation
L. brevis	1.2048		sausage swell
L. brevis	1.230		spoiled beer
L. brevis	1.240		spoiled beer
L. brevis	1.302		brewery environment
L. brevis	1.313		brewery environment
L. brevis	1.315		brewery environment
L. brevis	1.317		brewery environment
L. brevis	1.465		brewery environment
L. brevis	1.473		brewery environment
L. brevis	1.474		brewery environment
L. brevis	1.476		brewery environment
L. brevis	1.485		brewery environment
L. brevis	1.507		brewery environment
L. plantarum	1.9	DSM 20174^T	pickled cabbage
L. plantarum	1.1308		brewery environment
L. plantarum	1.277		palm wine
L. plantarum	1.1789		faeces
L. plantarum	1.321		brewery environment
L. paracasei	1.1434		human colon
L. paracasei	1.304		brewery environment
L. paracasei	1.427		sourdough
L. paracasei	1.1982		water kefir
L. paracasei	1.1882		brewery environment
L. parabuchneri	1.429	DSM 5707^T	human saliva
L. parabuchneri	1.345		brewery environment
L. parabuchneri	1.1306		sourdough
L. parabuchneri	1.2083		wheat beer
L. parabuchneri	1.2084		brewery environment
L. harbinensis	1.1453		parmesan cheese
L. harbinensis	1.1455		parmesan cheese
L. harbinensis	1.2085		dark lager beer
L. harbinensis	1.2086		brewing yeast sample
L. harbinensis	1.2087		brewery environment
L. paucivorans	1.1424	DSM 22467^T	yeast storage tank
L. paucivorans	1.2063		sausage swell
L. sanfranciscensis	1.1304		sourdough
L. helveticus	1.695	DSM 20075^T	emmental cheese

3.1.2 Media

3.1.2.1 Lactic acid bacteria growth media

LAB were cultivated using NBB-Agar, NBB-Bouillon (Döhler, Darmstadt, Germany) and MRS media. The term $mMRS_1$ will always refer to the medium described in Table 3 with a pH of 6.2, if not indicated otherwise. Modifications of $mMRS_1$ are mentioned in the particular sections.

Table 3: Composition of $mMRS_1$. Compounds (chemicals) are listed together with the corresponding supplier and, if available, the purity grade. Concentrations are given in g/l.

Compound	Supplier & Grade	Concentration (g/l)
Peptone from casein	Merck Millipore, Billerica, USA, for microbiology	10
Meat extract	Merck Millipore, Billerica, USA, for microbiology	5
Yeast extract	Roth, Karlsruhe, Germany, for bacteriology	5
K_2HPO_4 * 3*H_2O	Merck Millipore, Billerica, USA, p.a.	4
KH_2PO_4	Merck Millipore, Billerica, USA, ≥ 99 %	2.6
NH_4Cl	Roth, Karlsruhe, Germany, ≥99.5 %	3
L-Cysteine-HCl monohydrate	Roth, Karlsruhe, Germany, ≥98.5 %	0.5
Tween 80	Gerbu, Heidelberg, Germany, purified	1
$MgSO_4$ * 7H_2O	Merck Millipore, Billerica, USA, p.a.	0.2
$MnSO_4$ * H_2O	Merck Millipore, Billerica, USA, ≥98 %	0.038
D-Maltose	Merck Millipore, Billerica, USA, ≥98 %	10
D-Glucose	Merck Millipore, Billerica, USA, for microbiology	5
D-Fructose	Merck Millipore, Billerica, USA, ≥99 %	5

In some cases, the acidic environment and the low content of manganese in beer should be simulated in order to adapt cultures to these conditions or for other experimental reasons. In these cases, the same medium (Table 3) was used with the following alterations: No L-cysteine hydrochloride, pH 4.3, 0.16 mg/l $MnSO_4$ * H_2O $MgSO_4$ * 7H_2O 98 mg/l (as found in beer, personal communication). This medium will be designated as $mMRS_2$ or blb (beer like broth). All media were autoclaved at 121° C for 20 min, in order to obtain sterility. Temperature sensitive components were sterile filtered (pore size 0.2 µm) and added after autoclaving. Sugars were autoclaved separately to avoid Maillard reactions. In case of plates, 15 g/l agar was added. The pH of media was adjusted with 2 to 6 M HCl or NaOH.

3.1.2.2 Medium additives

Table 4 lists relevant additives used for different experiments.

Table 4: Relevant medium additives. Additives are listed with purity grade and supplier of the respective chemical. TLC = thin-layer chromatography, HPLC = high-performance liquid chromatography, NA = not available.

Additive	Grade	Supplier
Carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP)	≥ 97% (TLC)	Sigma-Aldrich, St. Louis, USA
Calcimycin (A23187)	≥ 97% (TLC)	Sigma-Aldrich, St. Louis, USA
2,4-dinitrophenol (DNP)	moistened with water, ≥ 97.0%; ≥ 15% water	Sigma-Aldrich, St. Louis, USA
Hydrogen peroxide	≥ 30%	Merck Millipore, Billerica, USA
Iso-α-acids	30 % (+/-1 %) w/w (HPLC - CO2 extract)	Hopsteiner, Mainburg, Germany
Nigericin	≥ 97 % (TLC)	Sigma-Aldrich, St. Louis, USA
Valinomycin	≥ 97 % (TLC), ≥ 90 % (HPLC)	Sigma-Aldrich, St. Louis, USA
Tween 20	purified	Gerbu, Heidelberg, Germany
Tween 60	NA	Sigma-Aldrich, St. Louis, USA
Tween 80	purified	Gerbu, Heidelberg, Germany
D-Arabinose	≥ 99 %	Roth, Karlsruhe, Germany
D-Fructose	≥ 99 %	Sigma-Aldrich, St. Louis, USA
D-Glucose	≥ 99.5 %	Sigma-Aldrich, St. Louis, USA
D-Gentiobiose	≥ 98 %	Sigma-Aldrich, St. Louis, USA
D-Galactose	NA	Merck Millipore, Billerica, USA
D-Isomaltose	≥ 98 %	Sigma-Aldrich, St. Louis, USA
D-Lactose	≥ 98 %	Sigma-Aldrich, St. Louis, USA
D-Mannitol	≥ 98 %	Roth, Karlsruhe, Germany
D-Maltose	≥ 98 %	Merck Millipore, Billerica, USA
D-Ribose	≥ 99 %	Sigma-Aldrich, St. Louis, USA
D-Sucrose	≥ 99 %	Sigma-Aldrich, St. Louis, USA
D-Sorbitol	≥ 99.5 %	Sigma-Aldrich, St. Louis, USA
D-Xylose	≥ 99 %	Sigma-Aldrich, St. Louis, USA
L-Arginine	≥ 99 %	Merck Millipore, Billerica, USA
L-Glutamic acid	≥ 99 %	Sigma-Aldrich, St. Louis, USA
L-Histidine	≥ 99 %	Roth, Karlsruhe, Germany
L-Tyrosine	≥ 99 %	Merck Millipore, Billerica, USA
Sodium acetate trihydrate	≥ 99.5 %	Roth, Karlsruhe, Germany
Tri sodium citrate dihydrate	≥ 99 %	Roth, Karlsruhe, Germany
DL-Malic acid	≥ 97 %	Roth, Karlsruhe, Germany
Succinic acid	≥ 99.5 %	Roth, Karlsruhe, Germany

3.1.2.3 Beers

Thirteen different beers (Table 5), varying in source, brewing style, pH and hop content, were used throughout this work. All beers were degassed and sterile filtered (0.2 µM, Rapid-Flow Filters, Thermo Scientific, Waltham, USA). Appendix 1 (p. 328) includes additional analytical data about the beers from brewery 1.

Table 5: Beers used for growth experiments. ID-Numbers (1 to 4) refer to different breweries, e.g. wheat beer 1 and pilsner beer 1 were produced by brewery 1. Some basic parameters are given, including the fermentation type, special properties (e.g. organic production), the gravity of the wort used for the respective beer (measure of fermentable sugar content), the alcohol (ethanol) content, the pH and the international bitterness units (IBUs) as a measure for the hop content (1 IBU ~ 1 ppm Iso-α-acids).

ID	Fermentation type	Special	Gravity (wt. %)	Alcohol (v/v %)	pH	IBU
Wheat beer 1	top fermented	Kristall	12.5	5.5	4.4	14
Wheat beer 2	top fermented		12.7	5.3	4.2	13
Wheat beer 3	top fermented	organic	12.4	5.4	4.4	11
Wheat beer 4	top fermented		12.4	5.4	4.3	12
Lager beer 1	bottom fermented		11.5	5.1	4.3	18
Lager beer 2	bottom fermented		11.7	4.9	4.3	22
Lager beer 3	bottom fermented	organic	12.0	4.8	4.5	21
Lager beer 4	bottom fermented		11.6	4.9	4.6	17
Pilsner beer 1	bottom fermented		11.5	5.1	4.4	29
Pilsner beer 2	bottom fermented		11.9	4.9	4.5	33
Pilsner beer 3	bottom fermented	organic	11.2	4.7	4.5	27
Pilsner beer 4	bottom fermented		11.3	4.9	4.4	28
Pilsner beer 5	bottom fermented		11.2	4.9	4.3	40

3.1.3 Culture conditions and storage

If not stated otherwise, cultivation/incubation was carried out in $mMRS_1$ pH 6.2 at 25° C. Reaction tubes containing liquid media were filled to the maximum in order to obtain an oxygen-reduced environment. For the same purpose, the wells of microtiter plates were overlaid with paraffin oil and agar plates were incubated using the Anaerocult® C system (both Merck Millipore, Billerica, USA).

All Strains, upon receipt from third parties, were streaked out on $mMRS_1$ agar and incubated until single colonies were visible. Single colonies were analysed using Matrix-assisted-Laser-Desorption-Ionization-Time-Of-Flight Mass Spectrometry (MALDI-TOF MS, see 3.3.1.4, p. 38) in order to test whether the indicated species of the isolates was correct. After identification/confirmation, each strain was sub-cultured three times on $mMRS_1$ plates to get

them into a comparably physiological state. Afterwards, 50 ml $mMRS_1$ were inoculated with a single colony, followed by incubation at 25° C. A sufficient amount of cells was harvested by centrifugation, suspended in $mMRS_1$ with a final proportion of 40 % glycerol and stored at -80° C. From glycerol cultures, strains were reconstituted for usage by streaking them on $mMRS_1$ or NBB (Döhler, Darmstadt, Germany) agar.

Standard preculture:

Unless noted otherwise, $mMRS_1$ liquid precultures were used in order to obtain cell suspensions for all kind of experiments. 1.8 ml $mMRS_1$ pH 6.2 was inoculated with a single colony (= biological replicate) and incubated for 4 days at 25° C before use. These precultures will be named standard precultures consequently.

3.2 Growth dynamics and bacterial physiology

3.2.1 Methods to determine bacterial cell count and growth

Starting approximately with a concentration of 10^7 cells/ml, cell suspensions start to get turbid. This turbidity (optical density = OD) results from the absorbance, reflection and scattering of light by cells and can be measured using a photometer. By approximation and in a specific measurement range (usually OD < 0.3), the cell density of vegetative growing cells is proportional to the measured absorbance (Bast, 2014b). In this study growth was mainly detected by the determination of the OD at λ = 590 nm. Measurements were performed with a spectrophotometer Novaspec II (Pharmacia Biotech, Uppsala, Sweden) or a Tecan Sunrise Plate Reader (Männedorf, Swiss).

For the determination of the total cell-count, bacteria were counted using a hemocytometer. These microscopically chambers, with a defined volume (V = 0.25 nl) and marks for orientation and better counting, are filled with a cell suspension while cells are enumerated using a microscope (Bast, 2014a).

As described by Twigg (1945), bacterial metabolism leads to a reduction of resazurin (blue) to resorufin (pink - irreversible) and finally from resorufin to dihydroresorufin (colorless - reversible). In some cases, resazurin was used as a measure for growth. Table 6 shows the composition of the used resazurin solutions.

Table 6: Composition of the resazurin stock solution and resazurin indication buffer.

Resazurin stock solution	6.75 mg in 2.5 % (v/v %) ethanol
Resazurin indication buffer	200 µl resazurin stock solution + 1 ml 500 mM Tris buffer at pH 8.8

3.2.2 Gradient microtiter plates – RoboSeq® 4204S

A pipette robot, RoboSeq® 4204S (MWG Biotech AG, Ebersberg, Germany), was used in order to create concentration gradients of given compounds in microtiter plates. Each well was filled with a final volume of 230 µl. The system was covered by a self-made microbiological cabinet for sterile working. The robot was programed to create a gradient from 0 % of the maximum concentration of additive X to 100 %, in 10 % steps. The layout created by the robot is illustrated by Figure 1.

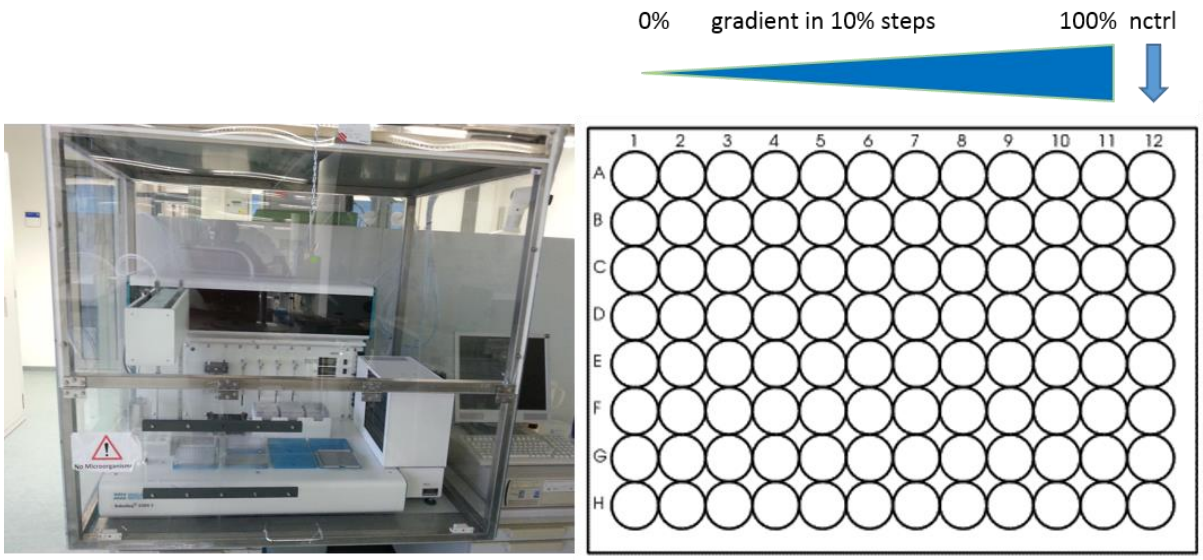


Figure 1: RoboSeq® 4204S with laminar flow and layout of gradient plates - created by RoboSeq® 4204S. nctrl = negative control containing 0% of additive X, not inoculated.

3.2.3 Growth under stress conditions

Growth challenge experiments, with exceptions, were carried out using microtiter plates with 10 % step gradients of additives as described above (3.2.2, p. 30). Table 7 lists the compounds or parameters that were tested (for purity and supplier see Table 4, p. 27), the maximal and minimal concentration applied and the intended stress quality induced thereby. In addition, the basic medium used for each stress quality is indicated.

14 ml $mMRS_1$ pH 6.2 were inoculated with 2 % of a standard preculture and incubated at 25° C until stationary phase. Afterwards, cells were harvested by centrifugation and suspended in $mMRS_1$ pH4.3 to an OD_{590} of 2. Wells were inoculated with 5 µl of this cell suspension and overlaid with 75 µl of paraffin oil. Growth at 25° C was tracked at λ = 590 nm for 7 days, using the Tecan Sunrise Plate Reader (Männedorf, Swiss). Raw data were analysed and visualized using MS Excel (Microsoft, Redmond. USA) and the R package grofit (Kahm et al., 2010). For details regarding the evaluation of the obtained growth data, see chapter 3.6.1 (p. 44). The test was performed with three biological replicates as well as controls, containing the test media without inoculation.

Table 7: Stress qualities applied in growth challenge experiments. The intended stress quality, the used basic medium and the stress compound/parameter are listed together with the range tested for the respective stress compounds/parameters Phosphate buffer[1] = same concentrations of phosphate compounds and Mn^{2+}/Mg^{2+} in buffer, as found in $mMRS_1$, CCCP = carbonyl cyanide m-chlorophenyl hydrazone.

Stress quality	Basic medium	Compound/Parameter	Range
Alcohol stress	$mMRS_1$ pH 4.3	ethanol absolute	0 - 20 % v/v
Acid stress	$mMRS_1$	pH adjusted with HCl	2.75 - 6.2
Hop stress	$mMRS_2$ pH 4.3	iso-α-acids	0 - 50 ppm
Oxidative stress	$mMRS_1$/$mMRS_2$ pH 4.3	H_2O_2	0 - 5 mM
Ionophore stress	$mMRS_1$ pH 4.3	CCCP	0 - 0.2 mM
Ionophore stress	$mMRS_1$ pH 4.3	calcymicin	0 - 20 µM
Ionophore stress	$mMRS_1$ pH 4.3	DNP	0 - 1 mM
Ionophore stress	$mMRS_1$ pH 4.3	nigericin & valinomycin	0 - 10 µM
Lack of nutrients	$mMRS_1$ pH 4.3	phosphate buffer[1]	0 - 100 % of $mMRS_1$
Lack of Mn^{2+} and Mg^{2+}	$mMRS_1$ pH 4.3/6.2	$mMRS_1$ without $MnSO_4$ and $MgSO_4$	0 - 100 % of $mMRS_1$

3.2.4 Growth in the presence of iso-α-acids for HPLC analysis

14 ml of $mMRS_1$ were inoculated with 2 % of a standard preculture and incubated at 25° C until stationary phase. 50 ml of $mMRS_2$, containing 50 % of the minimal inhibitory concentration of iso-α-acids for a given strain, as well as $mMRS_1$ pH 6.2 and $mMRS_2$ pH 4.3 without iso-α-acids, were inoculated with cells from the working culture to an OD_{590} of 0.001 - 0.010 and incubated at 25° C. Samples were taken regularly in order to trace pH and OD_{590}. After incubation (time depending on species and strain), residual samples were frozen at -20° C for HPLC analysis (see 3.5, p. 42). The test was performed with three biological replicates as well as controls, containing the test media without inoculation.

3.2.5 Growth in beer with additives

The effect of different additives to lager beer 1 on the growth of LAB was investigated. All additives and their concentration can be found in Table 8 (p. 33), their purity as well as the respective supplier in Table 4 (p. 27).

Table 8: Beer-variants (supplemented beers) used for growth experiments in lager beer 1. All variants base on lager beer 1. In addition, the respective additives are given as well as their concentration.

Beer-variant	Additive	Concentration
Lager beer 1	no additives	
Lager$_{pH5.0}$	6 M NaOH for pH adjustment	
Lager + sugars	arabinose, fructose, glucose, gentiobiose, galactose, isomaltose, lactose, mannitol, maltose, ribose, sucrose, sorbitol, xylose	1 mM each
Lager + amino acids	tyrosine, arginine, histidine, glutamic acid	2 mM Tyr, 10 mM others
Lager + organic acids	malate, citrate, pyruvate, succinate	10 mM each
Lager + minerals	$MgSO_4$ * 7*H_2O/$MnSO_4$ * H_2O	0.2 g/l/0.038 g/l
Lager + fatty acids	Tween 20/60/80	1 g/L each
Lager + peptone	peptone from casein	10 g/L

The concentration of carbohydrates, amino acids and organic acids in lager beer 1 was either based on the available HPLC data or adapted from Suzuki et al. (2005b). Approximately the threefold amount of the particular substance or substance class was added to lager beer 1 for the growth experiments. The added amount of minerals, Tween and peptone matched to concentrations found in $mMRS_1$. The growth experiment was also conducted using lager beer 1 without additive (lager$_{pH4.3}$) and lager beer 1 with pH 5.0 (lager$_{pH5.0}$). 10 ml of lager$_{pH5.0}$ were inoculated with 2 % of a standard preculture and incubated at 25° C until visible growth was observed or maximally 14 days, respectively. Afterwards, cells were harvested by centrifugation and suspended in lager$_{pH5.0}$ to an OD_{590} of 2. 200 µl of the eight additive beer-variants were inoculated with 5 µl of cell suspension and wells were overlaid with 75 µl paraffin oil. Microtiter plates were incubated at 25° C and the OD_{590} was tracked for 30 days using the Tecan Sunrise Plate Reader (Männedorf, Swiss). Raw data were analysed and visualized using MS Excel (Microsoft, Redmond. USA). The maximum OD_{590} within 30 days was used in order to compare the impact of a given additive on LAB strains. The test was performed with three biological replicates as well as controls, containing the test media without inoculation.

3.2.6 Evaluation of beer spoilage potential and hop resistance

3.2.6.1 Resazurin test - Determination of the metabolic activity in beer

The metabolic activity of LAB was determined in four different beers (wheat, lager, pilsner beer 1 and pilsner beer 5), using resazurin as redox indicator (see 3.2.1, p. 30). Therefore, the method described by Preissler et al. (2010) was used with modifications. 14 ml working cultures, either containing $mMRS_1$ pH 6.2 or $mMRS_2$ pH 4.3 (or both), were inoculated with 2 % of a standard preculture and incubated at 25° C until stationary phase. Afterwards, cells were harvested by centrifugation and suspended in wheat beer 1 to an OD_{590} of 2.

Subsequently, 200 µl of beer, provided in microtiter plates, were inoculated with 5 µl of the cell suspension and overlaid with 75 µl paraffin oil. Duplicates of each microtiter plate were incubated at 25° C for 6 and 30 days, respectively, before 5 µl of resazurin indication buffer (Table 6, p. 30) was added. Microtiter plates were further incubated for 3 h at 25° C, before absorbance at λ = 570 and 600 nm was measured using a Tecan Sunrise Plate Reader (Männedorf, Swiss). Wells were also evaluated visually for color change. The test was performed with three biological replicates and two technical replicates per strain, as well as control wells containing the test beers without inoculation.

3.2.6.2 MIC test variant 1 - Determination of the minimal inhibitory concentration (MIC) for iso-α-acids using resazurin

Working cultures (14 ml) and cell suspensions in wheat beer with an OD_{590} = 2 were prepared exactly as stated under 3.2.6.1 (p. 33). MIC-tests were performed in microtiter plates containing 230 µl/well $mMRS_2$ pH 4.3 and an increasing amount of iso-α-acids (maximum concentration depending on species, from 35 to 80 ppm). Microtiter plates were prepared using the RoboSeq® 4204S as described in chapter 3.2.2 (p. 30). 10 µl of resazurin stock solution (Table 6, p. 30) was added to each well before inoculation with 10 µl of cell suspension. Microtiter plates were incubated for 6 days at 25° C using the Anaerocult® C system (Merck Millipore, Billerica, USA). Subsequently, 50 µl of a 500 mM tris buffer (pH 8.8) was added to each well, followed by visual assessment of color changes. The test was performed with three biological replicates and two technical replicates per strain, as well as control wells containing the test beers without inoculation.

3.2.6.3 MIC test variant 2 - Determination of the MIC for iso-α-acids using resazurin

Working cultures (14 ml) containing $mMRS_2$ pH 4.3 were inoculated with 5 % of a NBB-Bouillon (Döhler, Darmstadt, Germany) preculture (1.8 ml), which was inoculated with a single colony and incubated for 4 days at 25° C, followed by incubation of working cultures until stationary phase. Afterwards, cells were harvested by centrifugation and suspended in $mMRS_2$ pH 4.3 to an OD_{590} of 2. MIC-tests were performed in microtiter plates containing 230 µl/well $mMRS_2$ pH 4.3 and an increasing amount of iso-α-acids.

Eight $mMRS_2$ media with different concentrations of iso-α-acids, in each case prepared from the same iso-α-acid stock solution (20 mg/ml of ISO30 extract in $Ethanol_{abs}$, see Table 4, p. 27), were tested for all strains:

- 0 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm

As in case of MIC test variant 1, 10 µl of resazurin stock solution (Table 6, p. 30) was added to each well before inoculation with 10 µl of cell suspension. Further implementation and evaluation was carried out as described for MIC test variant 1 (p. 34). The test was performed with three biological replicates per strain, as well as control wells containing the test beers without inoculation.

3.2.6.4 MIC test variant 3 - Determination of the MIC for iso-α-acids by optical density

MIC test variant 3 was conducted as variant 2 with the following modifications:

- 240 µl/well filling volume ($mMRS_2$) for microtiter plates
- no addition of resazurin
- growth was determined by measurement of OD_{590}, using the Tecan Sunrise Plate Reader (Männedorf, Swiss)

3.2.6.5 Beer spoilage test

Strains were tested using a beer spoilage test as described by Suzuki et al. (2005b), with modifications. 10 ml of lager beer 1 with elevated pH (= $lager_{pH5.0}$, pH 5.0 adjusted with 6 M NaOH) were inoculated with 2 % of a standard preculture and incubated at 25° C until visible growth was observed or maximally 14 days, respectively. After visible growth in $lager_{pH5.0}$, the total cell count (hemocytometer, see 3.2.1, p. 30) was determined and test beers (wheat, lager, pilsner, 10 ml) were inoculated with approximately 5 x 10^3 cells/ml (final concentration). The inoculated test beers were incubated at 25° C and examined every second day for visible growth. After 60 days, the OD_{590} and the pH were determined, while the rest of selected samples was frozen at -20° C for HPLC analysis (see 3.5, p. 42). The test was performed with three biological replicates and control triplicates containing the test beers without inoculation.

In order to probe the effect of additional fatty acids in beer on the beer spoilage potential of *P. damnosus*, lager beer 1 was supplemented with a Tween mixture (Tween 80, 60, 20, 1 g/l each, see Table 4, p. 27) as an additional test beer. Implementation and evaluation was performed as for other test beers.

3.2.7 Kinetics of genetic instability - *P. claussenii* TMW 2.53

TMW 2.53 cell smear from $mMRS_1$ agar plates was suspended in 500 µl $mMRS_1$ pH 6.2. 10 ml $lager_{pH5.0}$ as well as 10 ml $mMRS_1$ pH 6.2 were inoculated with 150 µl of the abovementioned cell suspension. The cell suspension used for inoculation was tested for *horC* and M13 with PCR (3.4.2, p. 39). After incubation of 8 days at 25° C, cells grown in $lager_{pH5.0}$ were plated on NBB-Agar and cells from $mMRS_1$ on $mMRS_1$ agar. Plates were incubated for 8 days at 25° C, before nine colonies (1. Generation = 1GEs) from each setup were tested with colony PCR for *horC* and M13 (in total 18). In addition, 1GEs were stored on NBB and $mMRS_1$ plates, respectively, followed by determination of beer spoilage potential, using the beer spoilage test, (3.2.6.5, p. 35) and acquisition of MALDI-TOF MS high quality spectra, applying the plain cell extraction method (3.3.1.1,p. 37). Two 1GEs from the MRS-MRS and the lager-NBB setup, respectively, were chosen for further propagation. 10 ml $lager_{pH5.0}$ as well as 10 ml $mMRS_1$ pH 6.2 were inoculated with the selected 1GEs from the storage plate, followed by incubation of 8 days at 25° C and plating as described above. 20 colonies (2. Generation = 2GEs) descending from the selected 1GEs, ten from the MRS-MRS and ten from the lager-NBB setup, were tested for *horC* and M13 with PCR (in total 120). Four selected 2GEs were tested for their beer spoilage potential and used for the generation of 3GEs, analogous to what was described above. As above, ten 3GEs, from each setup, were tested for *horC* and M13 with PCR (in total 40).

The whole procedure is also illustrated by Figure 34 (p. 122) in the corresponding results part. In order to check, if a *regain* of beer spoilage potential is possible, a 2GE, without *horC* and M13, lacking the ability to grow in pilsner beer 1, was subjected to a beer spoilage test where the preculture in $lager_{pH5.0}$ was supplemented with pasteurized cells of a variant having *horC* and M13. In another approach 30 µg DNA (*horC*, M13 positive) of TMW 2.54-SB was added to the preculture. As control, the same 2GE was incubated without any supplementation. In addition, the pasteurized cells were subjected to the same beer spoilage test, as a control for proper pasteurization.

3.3 Proteomics - Matrix-assisted-Laser-Desorption-Ionization-Time-Of-Flight Mass Spectrometry

3.3.1.1 Sample preparation

Three different sample preparation methods, described by Kern et al. (2013), were applied and are specified below. Mostly direct transfer methods, such as cell smears (CS) and an extension of CS by an “on-target extraction” using formic acid (CS/FA), were sufficient for species identification. For CS single colonies or cell smear were picked from agar plates and the cell material was smeared directly onto a stainless steel target (Bruker Daltonics, Bremen, Germany) and overlaid with HCCA matrix solution (Bruker Daltonics, Bremen, Germany). In case of CS/FA 1 µl of 70 % formic acid was added to the cell smear on the target and, after air-drying of the cell material, HCCA matrix was added. In both cases, samples had to be air-dried after HCCA matrix addition and before measurement. If CS or CS/FA preparation were insufficient for identification or high quality mass spectra were desired, a plain cell extraction (CE) was carried out. Therefore, a 1 µl loop of cell material was picked from plates or ~1 ml of a liquid culture was harvested by centrifugation. Biomass, collected from broth, was washed with H_2O and resuspended in 300 µl H_2O, while cell material from plates was resuspended directly in 300 µl H_2O. Inactivation of bacteria was achieved by addition of absolute ethanol to the sample (end-concentration of 70 v/v %), followed by protein extraction using formic acid, water and acetonitrile (HPLC grade, 35:15:50, v/v %). 1 µl of the extract was applied onto the target and, after air drying, spots were overlaid with HCCA matrix, followed by measurement (Kern et al., 2013).

3.3.1.2 MALDI-TOF MS analysis

Mass spectra were generated with a Microflex LT MALDI-TOF MS from Bruker Daltonics (Bremen, Germany), provided with a nitrogen laser (λ = 337 nm). The device is operating in a linear positive ion detection mode under the control of Biotyper Automation Control 3.0 (Bruker Daltonics, Bremen, Germany). If not stated otherwise, the mass spectra (in a range from 2,000 – 20,000 Da) of 240 laser shots were collected to create sum spectra. A bacterial standard was obtained from Bruker Daltonics (Bremen, Germany) and used for external mass calibration (Kern et al., 2013).

3.3.1.3 Data export and processing

MALDI-TOF MS spectra were exported and processed as described by Kern et al. (2014). Raw data were exported with FlexAnalyisis 3.3 (Bruker Daltonics, Bremen, Germany) and processed using a MASCAP (Mantini et al., 2010) based software using GNU Octave (www.octave.org). Peak processing, detection and alignment was performed according to Mantini et al. (2010). For alignment and clustering of peaks, the limit of distance tolerance was set to 600 ppm.

3.3.1.4 Routine species identification

Each isolate was checked for correct species assignment, using the CS or the CS/FA method (3.3.1.1, p. 37), before cryo conservation and after reconstitution from cryo cultures. Identification was obtained from the MALDI-Biotyper 3 (Bruker Daltonics, Bremen, Germany).

3.3.1.5 Species, strain and spoilage potential identification using MASCAP

Single colonies from $mMRS_1$ agar were streaked extensively on NBB-Agar (Döhler, Darmstadt, Germany) and incubated at 25° C, until a well-grown lawn was observed. Incubation time differed from 3 to 10 days, depending on species and strain. CE was carried out as described in chapter 3.3.1.1 (p. 37) and three technical replicates were measured for four biological replicates (per strain). In total, five biological replicates were measured on five different days and in one case by a third person (24 technical replicates), in order to cover biological and technical variance as good as possible. Spectra were exported and processed as described in chapter 3.3.1.3 (p. 38). A database entry was created for each strain from the 24 technical replicates, measured by the third person. All other spectra were aligned and assigned to the database created. In addition, spectra were used for cluster analysis, while *Euclidian* based distances were calculated using octave and plotted using R (3.6.1, p. 44).

3.4 Molecular biology

3.4.1 Isolation of total bacterial DNA

Total DNA for molecular biology was extracted using the E.Z.N.A. Bacterial DNA Kit (Omega Bio-Tek, Norcross, USA) according to the manufacturer. Cells were washed with 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), before applying the lysozyme treatment. DNA was resolved from the columns with elution buffer or H_2O and stored at -20° C.

High molecular weight DNA for genome sequencing was isolated using the Genomic-tip 100/G (Qiagen, Venlo, Netherlands) kit according to the manufacturer, with modifications. Cell lysis was adapted with respect to lysis time and the concentration of lysing enzymes, depending on strain and species. 16 h incubation (maximum) and the double amount of lysozyme/proteinase K (as recommended) were sufficient in order to obtain clear lysates for hard to lyse strains.

3.4.2 Polymerase Chain Reaction

Taq Core Kit 10 (MP Biomedicals, Santa Ana, USA) was used according to the manufacturer. Primers were obtained from MWG Biotech AG (Ebersberg, Germany) and applied with an end concentration of 0.5 µM per reaction. All relevant primers are listed in Appendix 2 (p. 330), together with their sequence. At least 1.25 U of *Taq* DNA polymerase was used per reaction. If not stated otherwise, PCR buffer with $MgCl_2$ was used. A Mastercycler gradient (Eppendorf AG, Hamburg, Germany) was utilized for thermo-profiles, which were designed according to the “Guidelines for PCR programs” of the *Taq* Core Kit 10. Products were analysed with agarose gel electrophoresis (see 3.4.3, p. 40). PCR was mostly conducted without prior DNA isolation. Therefore, either a single colony was suspended in 200 µl H_2O or, depending on cell density, 0.5 to 2 ml liquid cultures were harvested by centrifugation and resuspended in 200 µl H_2O. Finally, 2 µl of these opaque cells suspensions were used as template for PCR. In every case, where PCR was performed without prior DNA isolation, the used cell suspensions were tested for suitability with RAPD-PCR and/or 16S rDNA PCRs, as controls. Only if these were positive, PCRs with other targets (e.g. *horC*, M05) were accounted as valid. All primers for the amplification of potential DMGs were used at an annealing temperature of 50° C. In addition, they were designed to result in products ranging from 100 to 200 bp.

3.4.2.1 RAPD-PCR

For the differentiation of LAB on strain level, a random amplified polymorphic DNA analysis - PCR (RAPD-PCR) was performed, again using the *Taq* Core Kit 10 (MP Biomedicals, Santa Ana, USA). Table 9 and Table 10 describe the composition of a single reaction (50 µl) and the temperature profile used.

Table 9: RAPD-PCR reaction. The respective components are listed together with their used quantity.

Component	Quantity
H_2O	32.25 µl
10x PCR Taq-Buffer (without. $MgCl_2$)	5 µ
$MgCl_2$ (25mM)	7 µl
dNTPs (each 10 mM)	2 µl
M13V primer	0.50 µl
Taq polymerase (5 U/µl)	0.25 µl
DNA template	10 - 200 ng
Total Volume	50 µl

Separation of products was performed using agarose gel electrophoresis (see 3.4.3, p. 40). Data processing and cluster analysis was done with Bionumerics (3.6.2.9 p. 49).

Table 10: RAPD-PCR - Thermo-profile. Applied temperatures, the duration of each step and the repeats are shown.

Temperature (° C)	Duration (min)	Repeats
94	3	
40	5	x 3
72	5	
94	1	
60	2	x 32
72	3	

3.4.3 Agarose gel electrophoresis

Nucleic acids were separated, corresponding to their size, using 1 - 2 % gels (agarose in 0.5 x TBE-buffer). Separation was accomplished by applying an electric tension of 80 - 250 V in 0.5 x TBE (using an Electrophoresis Power Supply EPS 300, Pharmacia Biotech, Uppsala, Sweden). 6x Loading Dye and different GeneRuler DNA Ladders (Thermo Fisher Scientific, Waltham, USA) were used according to requirements. For visualization dimidium bromide and

an UVT-28 M transilluminator (Herolab, Wiesloch, Germany) were used. Pictures were taken with a CCD camera.

RAPD products (3.4.2.1, p. 40) were separated on 1.3 % TBE gels at 90 V and 70 mA. 6 µl of loading dye were mixed with 26 µl of the reaction and 10 µl were applied to the gels. Gels were stopped and stained when the bromophenol band reached 75 % of a 14 cm gel.

3.5 High-performance liquid chromatography

3.5.1 Sample preparation

Precipitation of proteins in biological samples, necessary for the persistence of HPLC systems and proper separation, was carried out using two different methods as described by Capuani et al. (2012)

For amino acid and organic acid analysis, this was accomplished by mixing 50 µl of perchloric acid (70 v/v %) with 1 ml of sample. After incubation at 4° C for 24 h, precipitation was achieved by centrifugation (15,000x g, 10 min). Subsequently the supernatant was filtrated (0.2 µM pore size) and ready for HPLC analysis. If necessary, the samples were further diluted with H_2O or 0.1 M HCl (Capuani et al., 2012). For carbohydrate analysis, 750 µl of the sample were mixed with 450 µl of a 10 % (w/v) $ZnSO_4*7H_2O$ solution and afterwards with 450 µl 0.5 M NaOH. After incubation for 20 min at 25° C, the supernatant was obtained by centrifugation and filtered as described above. Dilution, if necessary, was carried out using H_2O (Capuani et al., 2012).

3.5.2 Carbohydrate analysis

Carbohydrates were analysed using a Carbopac PA20column (Dionex, Sunnyvale, USA), combined with an electrochemical detector (ICS-5000, Dionex, Sunnyvale, USA) as previously described by Capuani et al. (2012)

HPLC grade eluents:

- A & B: Water (Macron, Avantor, Center Valley, USA)
- C: 100 mM NaOH (J.T. Baker, Avantor, Center Valley, USA)
- D: 1 M Na-acetate (Sigma-Aldrich, St. Louis, USA)

A gradient as described by Capuani et al. (2012), with a flow rate of 0.5 ml/min and a column temperature of 25° C, was used for carbohydrate separation.

- 0 min: 37.5 % B and 25.5 % C → 24 min: 100 % C → 34 min: 100 % D → 44 min: 37.5 % B and 25.5 % C

3.5.3 Amino acid analysis

Amino (amino acids, biogenic amines) quantification was done as described by Schurr et al. (2013) with a Gemini C18 column (Phenomenex, Aschaffenburg, Germany), a Dionex UltiMate 3000 HPLC system (Dionex, Idstein, Germany) utilizing an UV-detector measuring at 338 or 269 nm, in combination with the following HPLC grade eluents:

- A: 20 mM Na_2HPO_4, 20 mM NaH_2PO_4 (both Merck Millipore, Billerica, USA), 0.8 % tetrahydrofuran (Roth, Karlsruhe, Germany), pH 7.8
- B: 30 % acetonitrile, 50 % methanol (both Roth, Karlsruhe, Germany), 20 % Water (Macron, Avantor, Center Valley, USA)

A gradient as described by Schurr et al. (2013), with a flow rate of 0.8 ml/min and a column temperature of 40° C, was used for separation:

- 0 min: 0 % B → 16 min: 64 % B → 19 min: 100 % B → 22 min: 100 % B → 22.25 min 0 % B

Before separation, samples were subjected to pre-column amino group derivatisation using o-phthalaldehyde-3-mer-captopropionic (OPA) acid and 9-fluorenylmethyl chloroformate (FMOC), as described by Bartok et al. (1994).

3.5.4 Organic acid and sugar analysis

Organic acids and sugars from MRS were quantified as previously described (Geissler et al., 2016) employing a Dionex UltiMate 3000 HPLC system (Dionex, Idstein, Germany) with a Rezex ROA-Organic Acid H+ (8%, Phenomenex, Aschaffenburg, Germany) and a RI-101 detector (Shodex, München, Germany). Analytes were separated at a constant flow rate of 0.7 ml/min with a column temperature of 85° C for 30 min. Sulfuric acid (Rotipuran, Roth, Karlsruhe, Germany) with a concentration of 5 mM served as mobile phase.

3.5.5 Quantification

Quantification was achieved using external HPLC grade standards and the Chromeleon evaluation software version 6.80 (Dionex, Idstein, Germany). Calibration was performed within the range of interest employing at least three different concentrations and five measurements for each standard.

3.6 Genomics, bioinformatics and statistical analysis

3.6.1 Statistics and data visualization

Most data were evaluated and visualized using Microsoft Excel (Redmond, USA) and R Software (3.1.0, http://www.rproject.org/). Table 11 lists the R packages used and their purpose.

Table 11: R packages used. Package and functions used are given together with the purpose.

Package	Functions used	Purpose/Usage
adegenet	find.clusters, dapc, scatter.dapc	Discriminant analysis of principle components (Jombart and Ahmed, 2011)
grofit	grofit, grofit.control	Fitting biological growth curves with R (Kahm et al., 2010)
hmisc & corrplot	corrplot, rcorr	Calculation and visualization of Spearman´s rank correlation (Harrell, 2014; Wei, 2013)
graphics	boxplot, stripchart	Visualization of distributions using descriptive statistics (R Core Team)
VennDiagram	venn.diagram	Visualization of venn diagrams (Chen and Boutros, 2011)
gplots	heatmap.2	Cluster analysis - creation of heatmaps including dendrogram based on hierarchical clustering (Warnes, 2015)
wordcloud	wordcloud	Plot a wordcloud for the visualization of word frequencies within a text (Fellows, 2014)

Correlation coefficients were interpreted according to Mukaka (2012), only if significance was given (CI 0.95). Correlation coefficient (ρ) interpretation:

- 0.9 to 1.0: very high correlation
- 0.7 to 0.9: high correlation
- 0.5 to 0.7: moderate correlation
- 0.3 to 0.5: low correlation
- 0.0 to 0.3: negligible correlation

DMG-statistics: The quality of DMGs was tested with Spearman´s rank correlation and Fisher´s exact test, corrected applying Bonferroni-Holm (Fisher, 1925; Holm, 1979). Precision, sensitivity (recall), specifity, accuracy f-measure, false positive rate and false negative rate as well as the total correct assignments (accuracy) by a specific DMG, were calculated using a confusion matrix (with Excel) and adduced to assess the quality of a given DMG.

In general, differences (similarities) between samples/groups were calculated using the student´s T-test, the Kruskal-Wallis test and the Mann-Whitney U-test (Kruskal and Wallis, 1952; STUDENT, 1908; Wilcoxon, 1945).

3.6.2 Genome analyses

The complete genomes of 17 bacterial strains, of five different species, were sequenced with single molecule real time (SMRT) sequencing (Eid et al., 2009; McCarthy, 2010), assembled, annotated and submitted to GenBank (Burks et al., 1985; Clark et al., 2016). Strains were selected based on a detailed characterization and are listed in Table 12 (p. 56).

3.6.2.1 Genome sequencing

High molecular weight DNA was isolated (3.4.1, p. 39) and sequenced at GATC Biotech (Konstanz, Germany) by SMRT sequencing (Eid et al., 2009; McCarthy, 2010). Employing P4-C2 chemistry, more than 200 Mb of raw data were generated from one to two SMRT cells (1x120 min movies), loaded with libraries having an insert size of 8 - 12 kb.

3.6.2.2 Genome assembly

Raw data were assembled using SMRT-Analysis (v 2.2.0 p2, Pacific Biosciences, Menlo Park, USA), applying the hierarchical genome assembly process (HGAP2/3) developed by Chin et al. (2013). Assemblies were checked with respect to several quality criteria: subread N50, mean read score, pre-assembly yield, number of contigs, contig N50, coverage and average consensus concordance.

Manual curation of assemblies, in order to end up with complete genomes, was mainly done as suggested by PacBio and is described online (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/Finishing-Bacterial-Genomes). Polished assemblies (fasta), obtained from assemblies using the RS_HGAP_Assembly_3 protocol were split into contigs using BioPerl (http://www.bioperl.org), employing the Bio::SeqIO system. Contigs were tested for redundancy using NCBI BLAST (Altschul et al., 1990; Camacho et al., 2009) and checked for overlapping ends (see Figure 2, p. 47) with the dotplot tool Gepard (Krumsiek et al., 2007). In addition, each contig was examined for conspicuous coverage behaviour and mapping quality (polishing) with SMRT-View 2.30 (Pacific Biosciences, Menlo Park, USA), while a decrease in mapping quality, at both ends of a contig, also indicated overlapping ends. A correct genome assembly was also evaluated with BridgeMapper (RS_BridgeMapper), which is part of the SMRT-Analysis software.

Redundant or non-sense contigs (e.g. completely covered by another contig) were discarded, while all other contigs were circularized in case overlapping ends were present. Therefore, an *in silico* break was introduced into the contig, followed by reassembly/cirularization using minimus2 (AMOS, http://amos.sourceforge.net). The resulting contigs were checked for proper circularization using Gepard. Further, all circularized contigs were examined using Gepard and NCBI BLAST versus the original contigs, in order to confirm that 100 % of the initial sequence information was retained. All chromosomes of a specific species were aligned and reordered with respect to each other employing Mauve (Darling et al., 2004; Darling et al., 2007; Rissman et al., 2009).

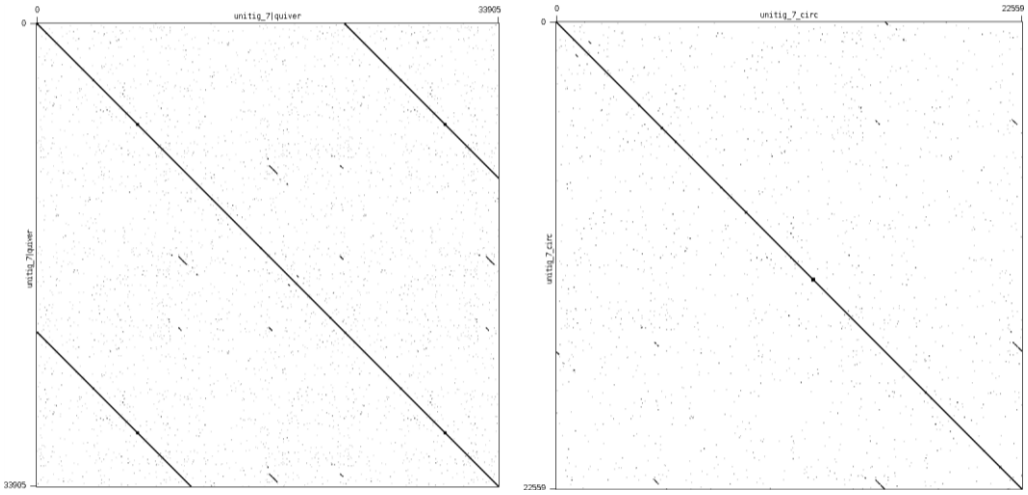


Figure 2: Dotplot of circular DNA sequence against itself (directly derived from SMRT analysis) - created with Gepard. Left side: before circularization with minimus2, right side: after circularization. Overlapping ends are indicated by parallel lines spanning from the y-axis to the x-axis of the Dotplot.

All circularized contigs, as well as those where a circularization was not possible, were merged together and deposited as a reference for a resequencing job by SMRT-Analysis, using the RS-Resequencing_1 protocol. Resequencing was repeated until an average reference consensus accordance of 100 % was accomplished. Each resequencing job was examined with respect to quality criteria as mentioned above, using SMRT-Analysis statistics and SMRT-View. The consensus sequence of each genome was obtained from the last resequencing job and stored as fasta-file. This genome fasta file served as input for all consequent genome analysis applications, including annotation.

3.6.2.3 Genome annotation

Genomes were submitted to RAST (Rapid Annotations using Subsystems Technology) for annotation, using default settings: classic RAST, RAST as gene caller, automatically fix errors, backfill gaps (Aziz et al., 2008; Overbeek et al., 2014). Annotation was completed using RAST2BADGE (Behr et al., 2016). No manual curation was performed. Single annotations were probed, using NCBI BLASTp (Altschul et al., 1990; Camacho et al., 2009), in cases of relevance for specific analyses. Further, genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (Angiuoli et al., 2008).

3.6.2.4 Genome submission - NCBI

A bioproject (PRJNA290141) was created, followed by the addition of biosamples for all strains sequenced. Locus tags and protein IDs were requested from the NCBI or assigned automatically. Submission was done as described online (http://www.ncbi.nlm.nih.gov/genbank/genomesubmit). While the genomes of *P. damnosus* were submitted together with RAST annotations, all other genomes were submitted without and were consequently annotated by the NCBI Prokaryotic Genome Annotation Pipeline (Angiuoli et al., 2008).

3.6.2.5 Acquisition of published genomes and processing for comparative genomics

Additional genomes were obtained from NCBI GenBank as genbank files (Burks et al., 1985; Clark et al., 2016). Files were downloaded from UNIX command line (wget) from ftp (ftp://ftp.ncbi.nlm.nih.gov/) and consequently converted to fasta files using genbank_to_fasta.py (Lee Bergstrand). Fasta files were annotated with RAST (3.6.2.3, p. 47) in order to obtain consistently annotated genomes for comparison.

3.6.2.6 Extraction of genomic properties

General genomic properties (GC content, coding density etc.) were extracted using in-house Bash tools, CMG biotools (Vesth et al., 2013) and Psortb for subcellular localization (Yu et al., 2010). The number and diversity of insertion elements/transposons was determined with ISfinder (www.is.biotoul.fr) (Siguier et al., 2006).

3.6.2.7 The identification of diagnostic marker genes and shared gene contents

DMGs were identified using BlAst Diagnostic Gene finder (BADGE). The program was designed and written within this project, in order to identify potential DMGs by comparative genomics. Program design, implementation and other information can be found within the corresponding publication and the program manual (Behr et al., 2016). If not stated otherwise, BADGE was used with default settings, only changing the minimum target occurrence depending on question and genomes to be compared.

3.6.2.8 Sequence alignment and primer design

After selection of DMGs for further evaluation (e.g. PCR), the corresponding align.fasta files (containing all identified sequences of a specific DMG) created by BADGE for every DMG, were aligned using Clone Manager 9 (Scientific & Educational Software). After *multi-way* alignment (exhaustive pairwise alignments), applying the *standard linear* scoring matrix, a *merged* consensus sequence was created. Again using Clone Manager 9, DMG specific primer pairs were designed, producing products in a range of 100 to 200 bp. Primers were used for PCR, as described in chapter 3.4.2 (p. 39).

3.6.2.9 Phylogenetics and phylogenomics

Phylogenetic trees were constructed based on RAPD-PCR patterns, sequence comparison of the 16S rDNA and other genetic markers, fragmented all-against-all comparison (phylogenomics), codon usage, amino acid usage, proteome comparison and *Pancore*-analysis.

RAPD-PCR patterns (3.4.2.1, p. 40) were clustered with Bionumerics (Applied Maths, Sint-Martens-Latem, Belgium). Hierarchical clustering was done by calculating similarities applying *Pearson*, followed by cluster analysis using the *Ward* method. Genetic marker sequences were extracted from uniform RAST annotated genomes, using an in house bash tool. Sequences were aligned with ClustalW and clustered using Splits Tree, applying the UPGMA method (Dress et al., 1996; Huson, 1998; Larkin et al., 2007; Thompson et al., 1994). Phylogenomics for genomes, chromosomes and plasmidomes was done, applying a fragmented alignment using Gegenees (Agren et al., 2012) with standard settings. Data were exported as nexus files and clustered with Splits Tree, applying the UPGMA method (Dress et al., 1996; Huson, 1998). Marker alignment and phylogenomic based trees were completed with TreeGraph2 (Müller and Müller, 2004; Stöver and Müller, 2010). Codon usage and amino acid usage were calculated with CMG-Biotools, followed by heatmap construction and hierarchical clustering (UPGMA) using the R gplots package (Vesth et al., 2013; Warnes, 2015). Pangenome trees were also calculated with CMG-Biotools, applying standard settings (Snipen and Ussery, 2010; Vesth et al., 2013).

3.6.2.10 Pan, core and accessory genomes

Pan, core and accessory genomes on protein level were calculated using CMG-Biotools and BADGE, in each case applying a 50/50 cutoff. Proteins were considered to be in the same family, if 50 % of the alignment was identical and the length of the alignment was more than 50 % of the longest family member sequence (Behr et al., 2016; Vesth et al., 2013). Accessory, strain and group specific genes were also calculated on DNA level using BADGE (Behr et al., 2016).

3.6.2.11 Metabolic reconstruction and functional analysis

Functional categorization was performed using the SEED subsystems (Aziz et al., 2008; Overbeek et al., 2014) as well as the cluster of orthologous groups (COG) categories (Galperin et al., 2015; Tatusov et al., 1997), applying the approach of Andreas Leimbach and the corresponding Perl tool cdd2cog. The SEED subsystem analysis allows an assignment of predicted genes to a hierarchical three-level categorization system, ranging from category, subcategory to subsystem. The COG enrichment ends with a classification into 23 categories, with no subdivision. Note that in case of the Seed subsystems a given gene can be assigned to several subsystems. The proportion (coverage) of genes assigned to SEED subsystems and COG categories will be mentioned in the corresponding sections. Settings for COG assignment are less stringent, consequently resulting in more assignments, but also higher error rates. Metabolic capabilities were analysed using the KEGG mapper (Kanehisa and Goto, 2000; Kanehisa et al., 2014; Okuda et al., 2008), supported by NCBI BLASTp analysis (Altschul et al., 1990; Camacho et al., 2009). The function/annotation of individual genes of interest was evaluated in detail on protein level using the STRING database (Snel et al., 2000; Szklarczyk et al., 2015).

3.6.2.12 Visualization of genome comparison

Genomic architecture is illustrated by BLAST ring images and whole genome alignments using BRIG and Mauve, respectively (Alikhan et al., 2011; Darling et al., 2004; Darling et al., 2007; Rissman et al., 2009). Mauve was used applying standard settings. BLAST output of BRIG was modified before image creation as follows: Coding density: 100 % identity, 100 % query/subject coverage; BLAST hits of other genomes were filtered accordingly, applying 95 % identity, 95 % query/subject coverage.

4 Results

Since 26 to 118 strains were characterized within the single experiments, the majority of data is summarized in tables and/or included into the appendix (p. 328). Most result chapters start with a short summary of the most important findings, followed by detailed subsections, showing and describing the actual data. Further, the experimental design will be described, if appropriate, as the material and methods chapter contains general methods and protocols only.

4.1 Terminology and definitions

The following terms and definitions will be used throughout the results and discussion chapters and are necessary to follow:

Cell density ≈ OD_{590}: The optical density at a wavelength of 590 nm was used a measure of cell density throughout this study. Note that an increase or decrease of the OD potentially could also result from any other change of light absorption, reflection and scattering by broth or cells. Thus, it is only a measure and does not necessarily correlate in a linear way with the actual cell number.

Hazard potential: LAB species are classified according to their general hazard potential for beer. This classification applies to species and was adopted from Hutzler et al. (2013). Species can have a very high or high hazard potential, a positive tendency for beer spoilage and no hazard potential. Species with very high hazard potential also include strains without beer spoilage ability. Definition of a hazard potential serves as a rough measure for the significance of a species for brewing microbiology and is based on spoilage statistics, literature and experience of Hutzler et al. (2013). It is not a measured and clearly defined trait as beer spoilage potential and ability for those strains actually characterized within this study.

Beer spoilage potential: Refers to the ability to grow in beers with increasing antibacterial properties. This property was determined experimentally within this study for all single strains. Thus, it is not a property assigned to a whole species. There are four beer spoilage potential groups ranging from no beer spoilage potential to strong beer spoilage potential.

Beer spoilage ability: Refers to the ability to grow in (at least) lager beer, representing the average beer regarding antibacterial properties. Includes all strains with middle and strong

beer spoilage potential. As beer spoilage potential, this property was determined experimentally within this study for all single strains.

Hop tolerance: If not stated otherwise, this refers to the minimum inhibitory concentration (MIC) of iso-α-acids, which was determined for each strain experimentally. The MIC serves as a measure for hop tolerance.

Core-species: *L. brevis*, *L. backii*, *L. paracollinoides*, *L. lindneri*, *P. claussenii* and *P. damnosus*. All species regarded to have a very high hazard potential and *P. claussenii*. Focused within this study.

Plasmidome: Totality of plasmids derived from a single strain/genome.

Brewery strain/genome/plasmid/plasmidome: Refers to the isolation source of the corresponding strain, genome etc. Will be used to avoid inconvenient phrases. Consequently, all other will be referred as “other” or “non-brewery” entities.

Diagnostic marker gene (DMG): A gene, capable of differentiating two strains or groups.

Lifestyle: The lifestyle composes from properties, strategies and capabilities of LAB, a species or a strain, which are related to the successful growth in beer and/or can be associated with an enhanced tolerance to beer specific hurdles, thus conferring a potential advantage.

Lifestyle gene: A DMG, where a connection to the lifestyle of a particular bacterium within a given environment is established. E.g. based on physiology or metabolism.

4.2 Physiological characterization of lactic acid bacteria according to their beer spoilage potential

4.2.1 Acquisition, species-control and storage

118 isolates (Table 2, p. 23) out of various sources were checked for correct species assignment with MALDI-TOF MS (3.3.1.4, p. 38) and propagated 4 times in $mMRS_1$ pH 6.2 in order to obtain strains in a comparably physiological state. For all isolates, species membership and purity were confirmed and strains were stored at -80° C.

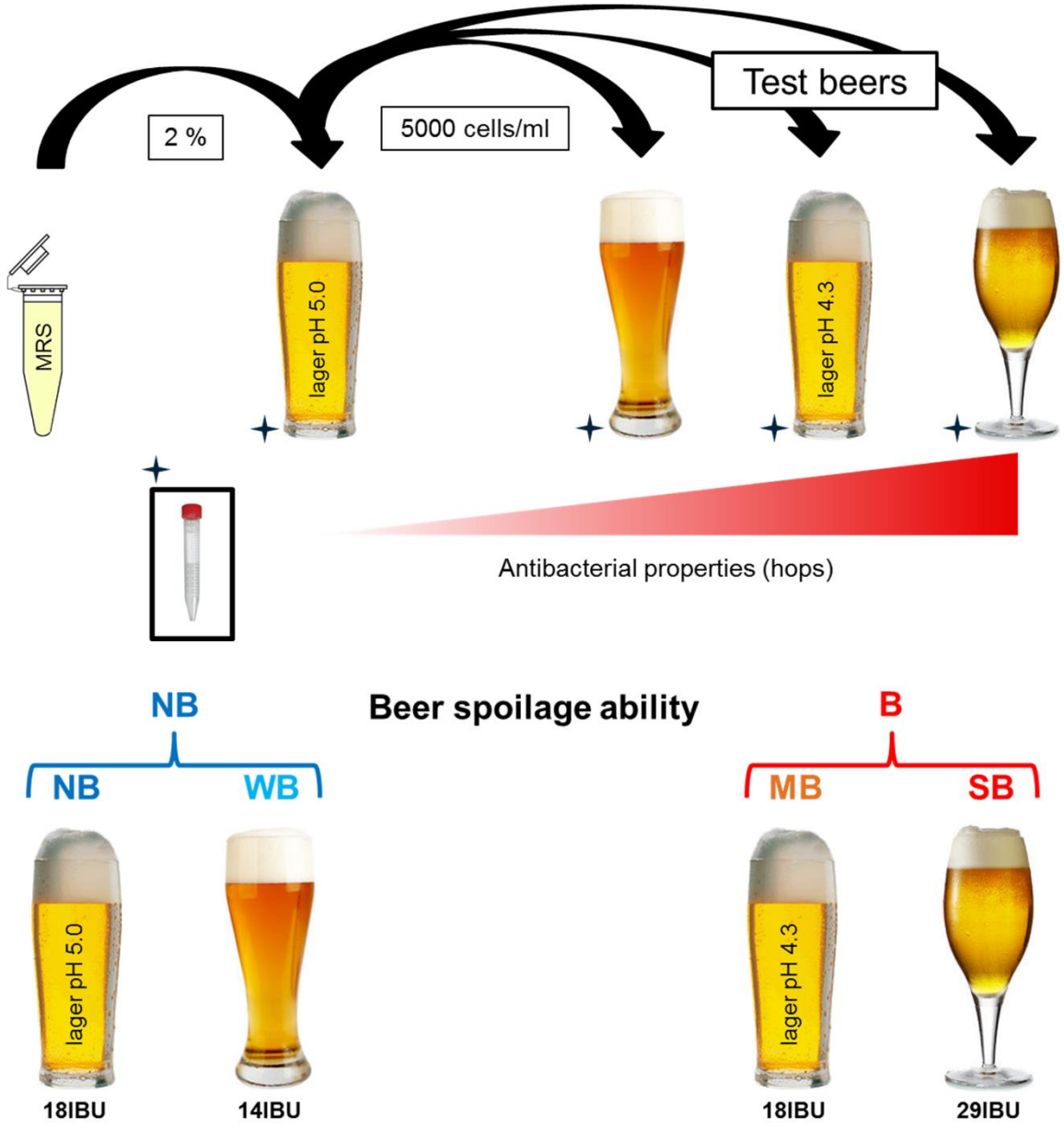
4.2.2 Initial characterization and strain selection for detailed characterization

Within an initial screening 55 strains comprising the core six species were tested employing the comparatively rapid resazurin test and characterized with respect to their adaptive beer spoilage potential in beer (Preissler et al., 2010). This was done in order to reduce the number of strains for a detailed characterization, resulting in a small set of strains with maximum diversity for method optimization, the evaluation of other methods to determine the beer spoilage ability/potential and the consequent selection of a reference method, which would be applied to an extended set of strains. 28 strains were selected for detailed characterization; covering a broad range with respect to beer spoilage potential, isolation source and considering special properties (e.g. slime production, *P. claussenii* TMW 2.340). Data are summarized in Appendix 3 (p. 332).

4.2.3 Detailed characterization: beer spoilage ability and potential, selection of a reference method and strain selection for genome sequencing

A selection of 28 strains was characterized in detail regarding their adaptive and constitutive beer spoilage potential in order to establish a reliable reference method for the determination of beer spoilage ability/potential and to select strains for genome sequencing. The classification of strains, employing the resazurin test (Preissler et al., 2010) and the beer spoilage test according to Suzuki et al. (2005b), were tested for accordance and validity using explorative data analysis, indicating that the classification of strains based on the beer spoilage test has a better correlation to the totality of data. Therefore, the beer spoilage test was chosen as reference method for the determination of beer spoilage potential and beer spoilage ability. Figure 3 (p. 55) illustrates the beer spoilage test as well as the consequent classification of strains into beer spoilage potential and beer spoilage ability groups. 17 strains were chosen for genome sequencing. For each species, if available, strains with preferably different beer spoilage potential, RAPD pattern and source were selected, also considering the presence of published hop tolerance genes (*horA*, *horC* and *hitA*). Further, at least two strains of each species and at least one strain of each beer spoilage potential group were chosen for sequencing in order to cover a maximum diversity with respect to species, beer spoilage potential and genetic background.

Table 12 (p. 56) summarizes the results from the detailed characterization for all strains selected for genome sequencing. Note that the beer spoilage potential of some strains was tested repeatedly, either because of variable DMG profiles or due to variable growth behaviour. For some strains listed in Table 12, a variable beer spoilage potential and ability was observed, which is marked. The table shows those data, which were the foundation of the selection process for genome sequencing. The concluding characterization and classification of these strains can be found in the next chapter (4.2.4, p. 57). A detailed description of the method optimization, of the statistical analysis for reference method selection and all other results of the detailed characterization are found within the Supplementary section 1 (9, p. 229), including figures, tables and additional comments.



Beer spoilage potential	Meaning	Range of validity
NB	no potential	no growth in all test beers
WB	weak potential	growth in wheat beer
MB	medium potential	growth in lager
SB	strong potential	growth in pilsner

Figure 3: Illustration of beer spoilage test - workflow and classification-rules. After cell transfer from $mMRS_1$ to beer with an elevated pH of 5.0, cells can adapt to the beer environment at reduced antibacterial properties. Test beers with increasing antibacterial properties, mainly determined by hop content, are inoculated with a low number of cells in order to mimic realistic conditions. Based on their growth behaviour, strains are classified with respect to beer spoilage ability and beer spoilage potential, as shown at the bottom of the figure. IBU = International bitter units (measure for hop content).

Table 12: Selection of strains for genome sequencing. All strains, which were chosen for the genome sequencing, are listed together with beer spoilage potential (BSP) related characteristic. Beer spoilage ability (BSA) and potential as defined by beer spoilage test: Strong BSP (SB), middle BSP (MB), weak BSP (WB), no BSP (NB); BSA (B/NB). [V] = variable BSP/BSA, here results of first three biological replicates tested with resulting categories, selection criteria for genome sequencing. Categorization (BSP) according to resazurin test: abbreviations with same meaning but defined by resazurin test (see Supplementary section 1). Hop tolerance: Minimum inhibitory concentration (MIC) of iso-α-acids, adaptive (-a) and constitutive (-c). RAPD-PCR: Cluster within species each strain belongs to, labeled using their initials (e.g. Lb = *L. backii*). Lifestyle genes: present = +, absent = -, NA = not available, t2 = tested separately, not included in detailed characterization.

Strain information			**Spoilage characteristics**			**Hop tolerance**		**Lifestyle genes & genetic diversity**			
Species	**TMW**	**Source**	**BSA**	**BSP**	**BSP Resazurin**	***MIC-c (ppm)***	***MIC-a (ppm)***	***RAPD***	***horA***	***horC***	***hitA***
P. claussenii	2.53	brewery	NB[V]	WB[V]	WB	10 +/- 0	15 +/- 0	Pc1	-	-/+	-
P. claussenii	2.54	brewery	B	SB	MB	15 +/- 0	22 +/- 3	Pc2	+	+	-
P. damnosus	2.1532	beer	NB	NB	NB	NA	NA	Pd2	+	+	-
P. damnosus	2.1533	brewery	B	SB	MB	10 +/- 1	21 +/- 1	Pd2	+	+	+
P. damnosus	2.1534	brewing yeast	NB	NB	NB	NA	6 +/- 3	Pd2	+	+	-
P. damnosus	2.1535	pilsner beer	B	SB	WB	4 +/- 2	22 +/- 1	Pd1	+	+	+
P. damnosus	2.1536	winery	NB	NB	NB	NA	12 +/- 5	Pd1	-	-	-
L. backii	1.1988	wheat beer	B	SB	SB	16 +/- 4	33 +/- 8	Lb2	+	+	-
L. backii	1.1989	beer	B	SB	SB	28 +/- 0	58 +/- 4	Lb1	-	+	-
L. backii	1.1991	brewery	B	SB	SB	19 +/- 2	28 +/- 0	Lb1	+	+	-
L. backii	1.1992	brewery	B	MB[V]	WB	9 +/- 4	14 +/- 0	Lb1	-	+	-
L. backii	1.2002[t2]	brewery	NB[V]	WB[V]	MB	19 +/- 0	25 +/- 11	NA	-/+	+	-/+
L. lindneri	1.1993	beer	B[V]	MB[V]	SB	NA	23 +/- 0	Ll2	-/+	-/+	-
L. lindneri	1.481	brewery	B	MB	SB	15 +/- 0	NA	Ll2	+	-	-
L. paracoll.	1.1979	beer	NB	NB	NB	10 +/- 0	15 +/- 0	Lp1	-	-	-
L. paracoll.	1.1994	brewery	B	SB	WB	18 +/- 3	30 +/- 0	Lp2	+	+	-
L. paracoll.	1.1995	pilsner beer	B	SB	WB	12 +/- 3	27 +/- 3	Lp1	+	+	+

4.2.4 Comprehensive and concluding characterization of beer spoilage potential and beer spoilage ability

Alternative methods (e.g. PCR) for the discrimination of beer-spoiling and non-spoiling strains need a reliable reference to validate their predictive accuracy. Therefore, 118 strains, comprising 13 species, were classified with respect to beer spoilage potential and beer spoilage ability based on the results of the beer spoilage test in order to provide a consistent reference. For each species, at least one strain was found lacking beer spoilage ability (in test beers of brewery 1). About 70 % of all brewery isolates were characterized by beer spoilage ability, while about 10 % of the non-brewery isolates were classified into this group. Independent of the origin, beer spoilage potential was variable for biological replicates of 25 % of all strains. Therefore, for all strains a beer spoilage potential range and an average beer spoilage potential were defined. Turbidity and acidification of test beers, caused by LAB, did correlate more to species than to beer spoilage potential and beer spoilage ability. The adaption time showed high to very high correlation to beer spoilage potential. All three parameters were affected by the antibacterial properties of the corresponding test beers. Strains were also tested for hop tolerance, showing that there was only a significant correlation of hop tolerance to beer spoilage potential of two of 13 species, *L. brevis* and *L. paracollinoides*. Note that the classification into groups within this section is final and will be used throughout the rest of the thesis.

All strains were characterized with the beer spoilage test (3.2.6.5, p. 35) and assigned to the respective beer spoilage potential and beer spoilage ability groups. According to Suzuki et al. (2005b) strains were additionally assigned to two groups based on the ability to grow in lager beer 1, which will be referred to as beer-spoiling strains. In addition, strains were tested for adaptive hop tolerance (MIC tests variant 2/3, 3.2.6.3 (p. 34)). In case of variable strains, tests were performed repeatedly in order to determine the range of beer spoilage potential (e.g. WB-MB) and an average beer spoilage potential. The classification was done as described in chapter 4.2.3 (p. 54). Raw data are partially originating from student projects under my supervision. Table 13 (p. 58) lists all strains with their beer spoilage ability, beer spoilage potential and MIC of iso-α-acids.

Table 13: Conclusive classification of strains into beer spoilage ability (BSA) and beer spoilage potential (BSP) groups. BSA and BSP were determined applying the beer spoilage test (3.2.6.5, p. 35). This classification is final and does not coincide in a few cases with the classification done within the detailed characterization (4.2.3, p. 54). Classification based on beer spoilage test: strong BSP (SB) - growth in pilsner 1, middle BSP (MB) - growth in lager beer 1, weak BSP (WB) - growth in wheat beer 1, no BSP (NB) - no growth in test beers; BSA (B/NB) defined by growth in lager beer 1. BSP and BSA are once listed as range to illustrate the variability found for some strains. Additionally, the average BSP/BSA is shown and the MIC of iso-α-acids, as a measure for hop tolerance, as average +/- standard deviation. The average BSP/BSA were defined (calculated) applying the following rules: Simple median point: e.g. 5 x SB, 1 x WB → **SB**, average category: 3 x WB, 3 x SB → **MB**, "safe side": 3 x MB, 3 x SB → **SB**.

	TMW	BSA range	Average BSA	BSP range	Average BSP	MIC (ppm)
P. claussenii	2.340	NB-B	B	WB-SB	MB	20 +/- 0
	2.1531	NB	NB	NB	NB	20 +/- 0
	2.1545	NB-B	B	WB-SB	MB	10 +/- 0
	2.50	NB-**B**	B	WB-**SB**	SB	30 +/- 0
	2.51	B	B	SB	SB	20 +/- 0
	2.53	NB-**B**	B	NB-**SB**	SB	20 +/- 0
	2.54	B	B	SB	SB	30 +/- 0
	2.56	B	B	SB	SB	30 +/- 0
	2.59	B	B	SB	SB	30 +/- 0
	2.60	B	B	MB	MB	30 +/- 0
	2.61	B	B	MB	MB	20 +/- 0
	2.62	B	B	SB	SB	37 +/- 5
	2.64	B	B	SB	SB	30 +/- 0
	2.65	B	B	MB	MB	37 +/- 5
	2.67	B	B	SB	SB	30 +/- 0
P. damnosus	2.4	B	B	MB	MB	5 +/- 0
	2.125	NB	NB	NB	NB	5 +/- 0
	2.1532	NB	NB	NB	NB	7 +/- 3
	2.1533	B	B	SB	SB	15 +/- 0
	2.1534	NB	NB	NB	NB	7 +/- 3
	2.1535	B	B	SB	SB	10 +/- 0
	2.1536	NB	NB	NB	NB	5 +/- 0
	2.1546	NB	NB	NB	NB	5 +/- 0
	2.1547	NB	NB	NB	NB	5 +/- 0
	2.1548	B	B	MB	MB	5 +/- 0
	2.1549	NB	NB	**NB**-WB	NB	5 +/- 0
	2.1635	NB	NB	NB	NB	NA
	2.1636	B	B	SB	SB	5 +/- 0
	2.1637	B	B	SB	SB	5 +/- 0
	2.1638	NB	NB	NB	NB	5 +/- 0
	2.1639	NB	NB	NB	NB	5 +/- 0
	2.1640	NB	NB	NB	NB	5 +/- 0
	2.1641	B	NB	NB	NB	5 +/- 0
	2.1642	NB	B	SB	SB	8 +/- 5
	2.1643	NB	NB	NB	NB	5 +/- 0

	TMW	BSA	Average BSA	BSP	Average BSP	MIC (ppm)
L. backii	1.1299	B	B	SB	SB	30 +/- 0
	1.1430	B	B	SB	SB	30 +/- 0
	1.1432	B	B	SB	SB	30 +/- 0
	1.1883	B	B	SB	SB	50 +/- 0
	1.1988	B	B	SB	SB	43 +/- 5
	1.1989	B	B	SB	SB	50 +/- 0
	1.1990	B	B	SB	SB	33 +/- 5
	1.1991	B	B	SB	SB	37 +/- 5
	1.1992	B	B	MB-**SB**	SB	15 +/- 0
	1.2002	NB-B	B	WB-SB	MB	17 +/- 2
	1.2003	B	B	SB	SB	30 +/- 0
	1.2004	NB	NB	**NB**-WB	NB	40 +/- 0
	1.2005	NB-B	B	WB-SB	MB	30 +/- 0
	1.2070	B	B	SB	SB	37 +/- 5
	1.2071	B	B	**MB**-SB	MB	50 +/- 0
	1.2072	B	B	SB	SB	50 +/- 0
	1.2073	B	B	SB	SB	40 +/- 0
	1.2077	B	B	MB	MB	47 +/- 5
	1.2078	B	B	SB	SB	37 +/- 5
	1.2079	NB	NB	NB-**WB**	WB	50 +/- 0
L. lindneri	**1.88**	NB-**B**	**NB**	NB-**MB**	**NB**	**10 +/- 0**
	1.1285	B	B	MB	MB	5 +/- 0
	1.1286	B	B	MB	MB	8 +/- 3
	1.1433	B	B	MB	MB	10 +/- 0
	1.1993	NB-B	B	NB-SB	MB	40 +/- 0
	1.2006	**NB**-B	NB	**NB**-SB	NB	15 +/- 0
	1.2007	NB-**B**	B	NB-**SB**	SB	10 +/- 0
	1.2008	B	B	MB-**SB**	SB	5 +/- 0
	1.2009	NB-**B**	B	WB-**SB**	SB	5 +/- 0
	1.2080	B	B	SB	SB	10 +/- 0
	1.2081	B	B	SB	SB	20 +/- 0
	1.2082	B	B	SB	SB	15 +/- 0
	1.456	B	B	SB	SB	15 +/- 0
	1.481	B	B	MB	MB	15 +/- 0
L. paracoll.	1.696	NB	NB	WB	WB	20 +/- 0
	1.1979	NB	NB	NB	NB	10 +/- 0
	1.1994	B	B	SB	SB	30 +/- 0
	1.1995	B	B	SB	SB	20 +/- 0
	1.2010	B	B	SB	SB	30 +/- 0

	TMW	BSA	Average BSA	BSP	Average BSP	MIC (ppm)
L. brevis	1.6	NB	NB	WB	WB	10 +/- 0
	1.1326	NB	NB	NB	NB	12 +/- 3
	1.100	NB	NB	WB	WB	20 +/- 0
	1.1205	**NB**-B	NB	**WB**-SB	WB	15 +/- 0
	1.1282	B	B	SB	SB	47 +/- 6
	1.1369	NB	NB	WB	WB	15 +/- 0
	1.1370	B	B	MB-SB	SB	40 +/- 0
	1.2048	**NB**-B	NB	**WB**-MB	WB	10 +/- 0
	1.230	B	B	SB	SB	37 +/- 6
	1.240	B	B	SB	SB	50 +/- 0
	1.302	NB	NB	WB	WB	17 +/- 12
	1.313	B	B	SB	SB	50 +/- 0
	1.315	B	B	SB	SB	47 +/- 6
	1.317	B	B	SB	SB	37 +/- 5
	1.465	B	B	SB	SB	50 +/- 0
	1.473	B	B	SB	SB	18 +/- 3
	1.474	B	B	SB	SB	30 +/- 10
	1.476	B	B	SB	SB	47 +/- 6
	1.485	B	B	SB	SB	33 +/- 6
	1.507	B	B	MB-**SB**	SB	30 +/- 10
L. plant.	**1.9**	**B**	**B**	**SB**	**SB**	**15 +/- 0**
	1.1308	NB	NB	WB	WB	15 +/- 0
	1.277	NB	NB	WB	WB	30 +/- 0
	1.1789	NB	NB	NB	NB	10 +/- 0
	1.321	NB	NB	NB-WB	NB	18 +/- 2
L. paracas.	1.1434	**NB**-B	NB	**NB**-MB	NB	15 +/- 0
	1.304	NB	NB	NB-**WB**	WB	27 +/- 5
	1.427	NB	NB	NB	NB	27 +/- 5
	1.1982	NB	NB	**NB**-WB	NB	10 +/- 0
	1.1882	NB	NB	NB-WB	WB	50 +/- 0
L. parabu.	1.429	NB	NB	WB	WB	30 +/- 0
	1.345	NB	NB	NB-**WB**	WB	27 +/- 5
	1.1306	NB	NB	WB	WB	15 +/- 0
	1.2083	NB	NB	WB	WB	30 +/- 0
	1.2084	NB-**B**	B	WB-**MB**	MB	15 +/- 0
L. harbin.	1.1453	NB	NB	NB	NB	50 +/- 0
	1.1455	NB	NB	NB	NB	50 +/- 0
	1.2085	NB	NB	WB	WB	15 +/- 0
	1.2086	NB	NB	WB	WB	20 +/- 0
	1.2087	**NB**-B	NB	**WB**-MB	WB	50 +/- 0
L[1]	1.1424	NB	NB	WB	WB	30 +/- 0
	1.2063	NB	NB	NB	NB	30 +/- 0
L[2]	1.1304	NB	NB	NB	NB	NA
L[3]	1.695	NB	NB	NB	NB	NA

L. paracoll.(*inoides*); *L. plant.*(*arum*); *L. paracas.*(*ei*); *L. parabu.*(*chneri*); *L. harbin.*(*ensis*); *L*[1] = *L. paucivorans*; *L*[2] = *L. sanfranciscensis*; *L*[3] = *L. helveticus*

4.2.4.1 Beer spoilage potential, source and distribution

The distribution of beer spoilage potential and beer spoilage ability within the single species was determined, in order to evaluate if a species specific or a brewery environment specific detection system would be sufficient for quality control.

69 % of all brewery isolates were characterized by beer spoilage ability, while 91 % of the non-brewery isolates had no beer spoilage ability. Of 81 investigated strains belonging to very high hazard potential species, 67 % were found to possess beer spoilage ability, with more than 50 % being able to grow in pilsner beer. High hazard potential species were characterized by 46 % beer-spoiling strains, while only 29 % were classified to have a strong beer spoilage potential. Without *P. claussenii*, so far considered as high hazard potential LAB (Hutzler et al., 2013), only 10 % were characterized by beer spoilage ability. Figure 4 (p. 62) illustrates that all investigated species comprised strains with no or weak beer spoilage potential. The proportion of strains with no beer spoilage ability was most prominent for those species, where strains out of different sources were investigated (e.g. *P. damnosus*, *L. brevis*). In case of *L. backii*, *L. lindneri* and *P. claussenii*, only brewery isolates were characterized (available). All three species showed a high percentage share (86 - 93 %) of strains with beer spoilage ability. In case of the high hazard potential species *L. plantarum, L. paracasei, L. parabuchneri* and *L. harbinensis*, only two of 20 strains possessed beer spoilage ability. All species comprised strains with unstable beer spoilage potential (in total 25 %), also illustrated by Figure 4. The highest proportion of unstable strains was found for *L. lindneri*, while all unstable *L. lindneri* strains were characterized by individual biological replicates with and without beer spoilage ability.

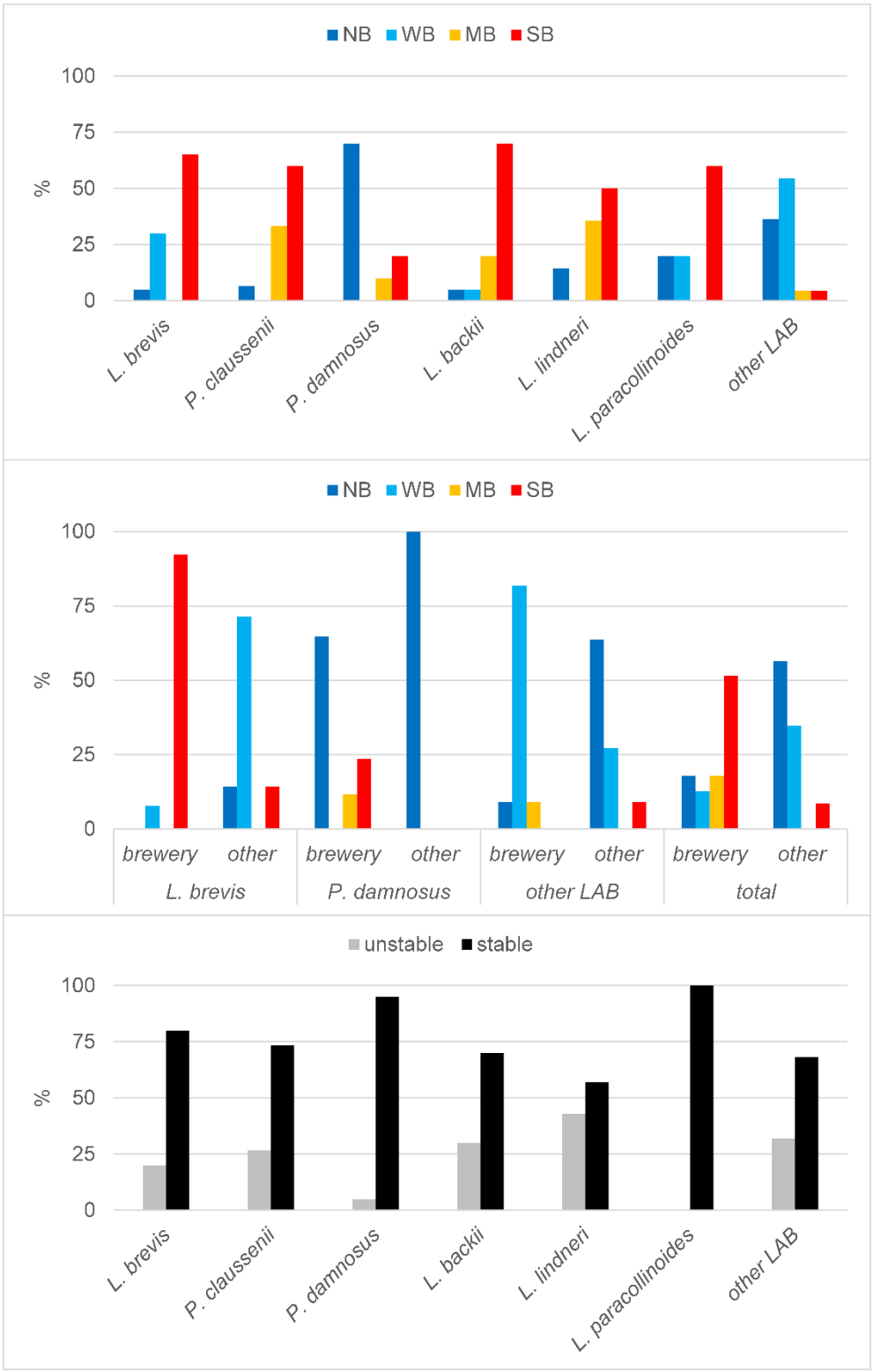


Figure 4: Beer spoilage potential – descriptive statistics: Percentage share of beer spoilage potential (BSP) groups within each species (top panel), dissected in case of those species where isolates were obtained from brewery and other sources (middle panel). In addition, the percentage share of strains with unstable BSP is shown in the bottom panel for all strains. Other LAB = *L. plantarum*, *L. paracasei*, *L. parabuchneri*, *L. harbinensis* and *L. paucivorans*. SB = strong BSP, MB = middle BSP, WB = weak BSP, NB = no BSP.

4.2.4.2 Acidification, turbidity and adaption

Appendix 6 and Appendix 7 (p. 336) contain detailed data about turbidity, acidification and adaption (time) for single strains and species, respectively. Turbidity (OD_{590}) and acidification (pH) were also used to validate visual determinations (growth). These parameters can also be used as measures for the intensity of spoilage, days until visible growth as a measure for adaption. Spearman´s rank correlation (3.6.1, p. 44) was used to test for significant correlations of these parameters to beer spoilage potential.

A significant high to very high negative correlation to beer spoilage potential was found for the adaption time (ρ = -0.80 to -95) in wheat, lager and pilsner beer, but not in $lager_{pH5.0}$. Figure 5 (p. 64) illustrates that strains with higher beer spoilage potential adapted faster to beer, in this case wheat beer 1. With exceptions, the adaption time increased with raising antibacterial properties of beer (wheat → pilsner), as also shown in Figure 5 (p. 64). Only low to moderate correlations to beer spoilage potential were found for pH and the OD_{590} after 60 days (wheat, lager, pilsner beer). This is also illustrated in the upper panels of Figure 6 (p. 65) and Figure 7 (p. 66). Analogous to the adaption time, the turbidity decreased from wheat to pilsner beer and increasing beverage stability. Acidification was mostly species dependent as illustrated by Figure 7 (p. 66). While *L. backii* and *L. lindneri* strains kept the pH near or even above the initial pH of wheat beer (pH 4.4 +/- 0.1), *L. paracollinoides* and *P. damnosus* strains lowered the pH down to 3.8 or lower. *L. lindneri* strains with middle to strong beer spoilage potential did either not grow in wheat beer, but in lager/pilsner, or were characterized by poor growth performance in this beer (adaption, turbidity). All core-species adapted faster to beer as the high hazard potential species *L. plantarum, L. paracasei, L. parabuchneri, L. harbinensis* and the very high hazard potential species *L. paucivorans* (other LAB). While core-species adapted to wheat beer in 4 to 13 days (in average, see Appendix 7, p. 341), the abovementioned other LAB had an adaption time of 21 +/- 7 days.

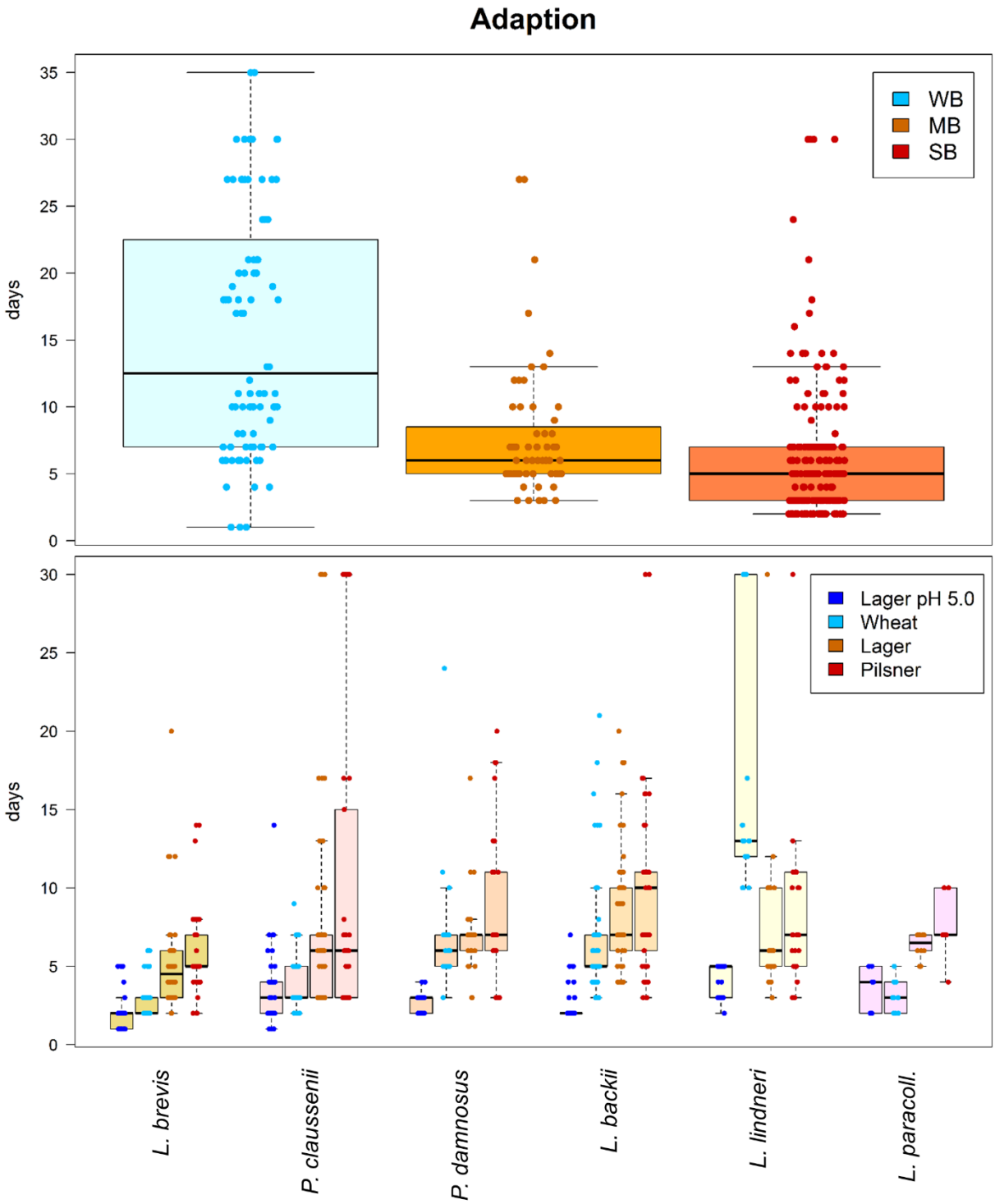


Figure 5: Adaption of lactic acid bacteria to beer - days until visible growth in beer spoilage test. The distribution of data is shown, illustrated by boxplots, combined with stripplots, including all biological replicates instead of average values. The top panel shows the days until visible growth in wheat beer for all strains assigned to the groups WB, MB and SB. The bottom panel shows the adaption time for all SB strains of the core-species in four different beers with increasing antibacterial properties (wheat → pilsner). SB = strong beer spoilage potential (BSP), MB = middle BSP, WB = weak BSP, NB = no BSP.

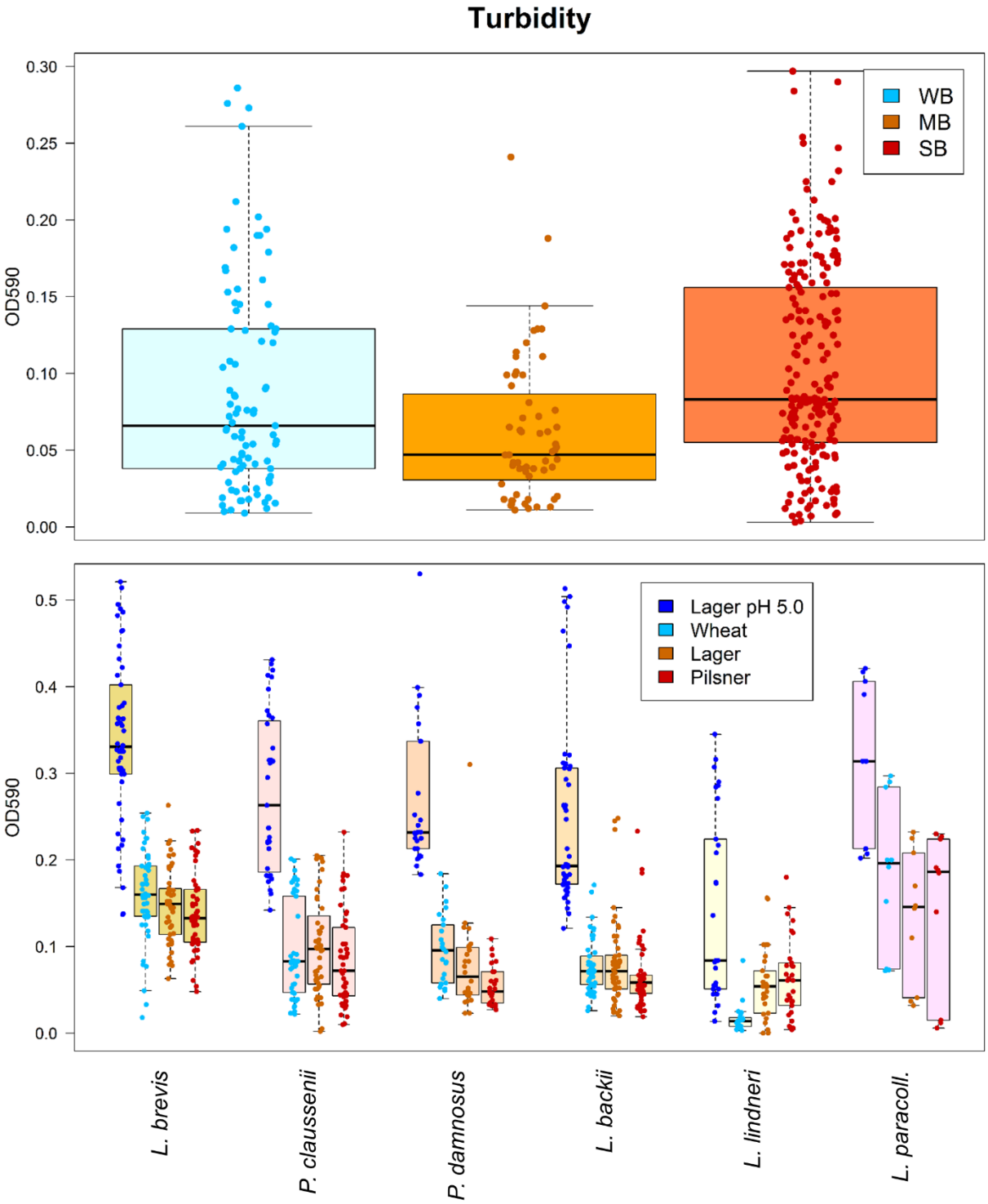


Figure 6: Turbidity of beer caused by lactic acid bacteria - OD_{590} after 60 days, determined in beer spoilage test. The distribution of data is shown, illustrated by boxplots, combined with stripplots including all biological replicates instead of average values. The top panel shows the OD_{590} for all strains in wheat beer assigned to the groups WB, MB and SB. The bottom panel shows the OD_{590} for all SB strains of the core-species in four different beers with increasing antibacterial properties (wheat →pilsner). SB = strong beer spoilage potential (BSP), MB = middle BSP, WB = weak BSP, NB = no BSP.

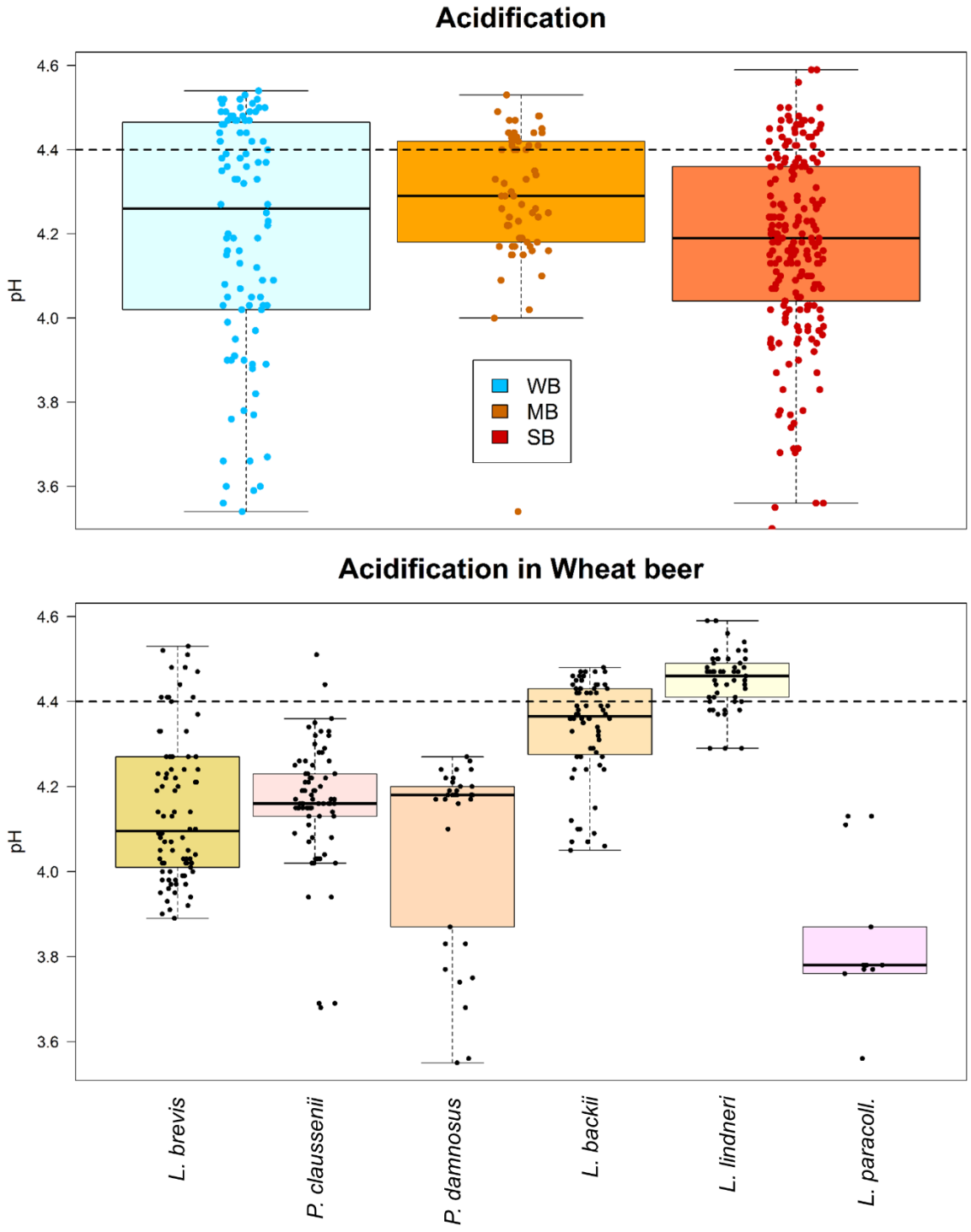


Figure 7: Acidification caused by lactic acid bacteria - pH after 60 days, determined in beer spoilage test. The distribution of data is shown, illustrated by boxplots, combined with stripplots including all biological replicates instead of average values. The top panel shows the pH in wheat beer after 60 days for all strains assigned to the groups WB, MB and SB. The bottom panel shows the pH after 60 days for all WB, MB and SB strains in wheat beer, summarized to the species level. The dashed black bar indicates the pH determined for wheat beer (4.4 +/- 0.1). SB = strong beer spoilage potential (BSP), MB = middle BSP, WB = weak BSP, NB = no BSP.

4.2.4.3 Hop tolerance

113 strains were tested for hop tolerance in $mMRS_2$ at a pH of 4.3 and eight different concentrations of iso-α-acids, applying the MIC test variants 2 and 3 (3.2.6.3, p. 34). All MICs, determined for three biological replicates, are listed in Table 13 (p. 58). Hop tolerance was tested for preadapted cells and correlation (3.6.1, p. 44) to beer spoilage potential was calculated.

Figure 8 and Figure 9 (p. 68-69) show the hop tolerance of all strains, sorted from high to low tolerance and color coded according to average beer spoilage potential. A significant high correlation to beer spoilage potential was found for *L. brevis* and *L. paracollinoides*, a tendency for increased hop tolerance in case of strong beer-spoiling strains for *P. claussenii*. Of all 13 species, *L. backii* (MIC = 37 +/- 10 ppm) showed the highest average tolerance for iso-α-acids, while *P. damnosus* was characterized by the lowest hop tolerance (MIC = 6 +/- 3 ppm). A prediction of beer spoilage ability from hop tolerance (MIC), as determined here, was not possible.

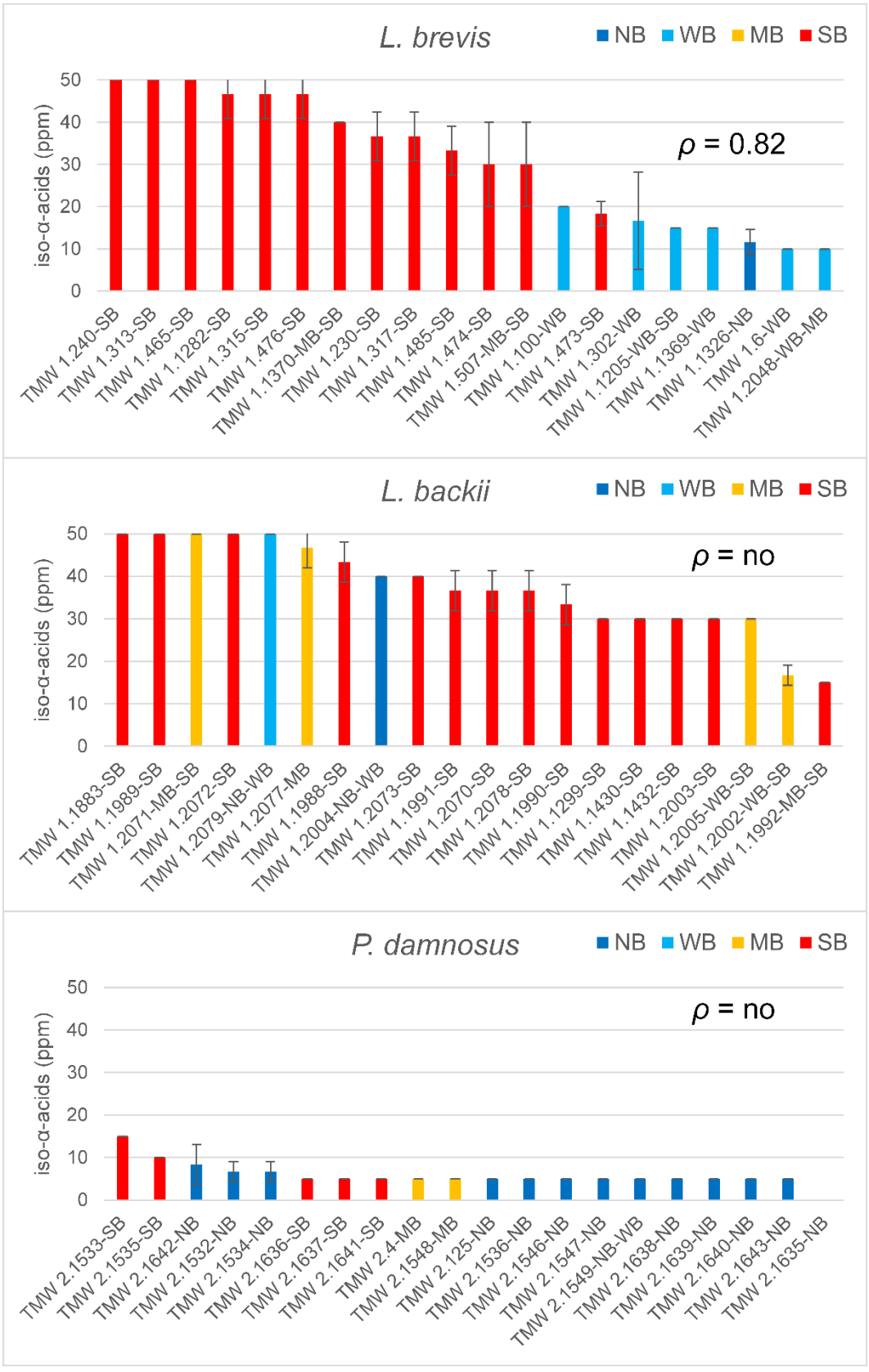


Figure 8: Minimum inhibitory concentrations of iso-α-acids, determined for *L. brevis*, *L. backii* and *P. damnosus*. Illustrated as average +/- standard deviation. Spearman´s rank correlation (ρ) to average spoilage potential (BSP) is shown. SB = strong BSP, MB = middle BSP, WB = weak BSP, NB = no BSP.

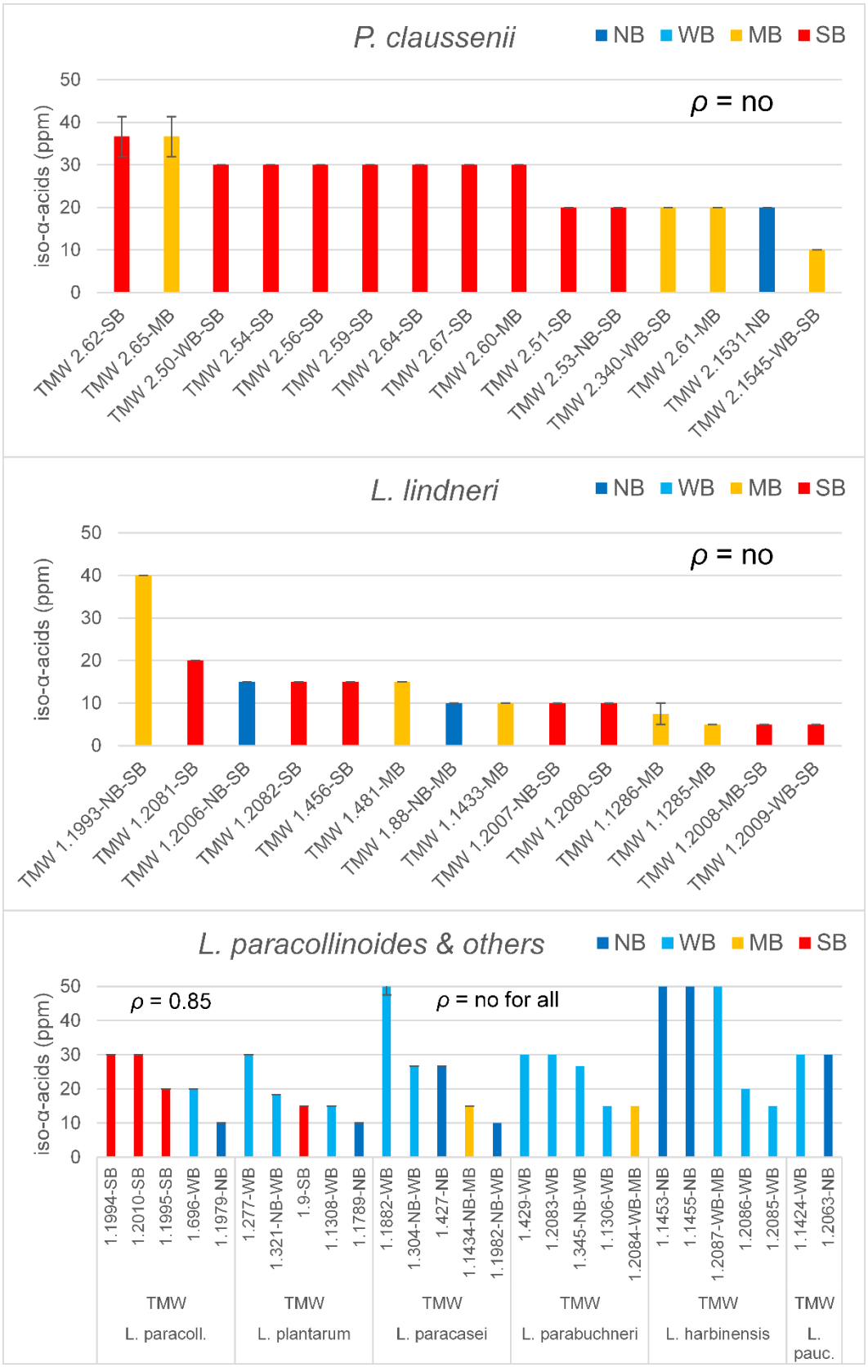


Figure 9: Minimum inhibitory concentrations of iso-α-acids, determined for *P. claussenii*, *L. lindneri* and other species. Illustrated as average +/- standard deviation. Spearman´s rank correlation (ρ) to average spoilage potential (BSP) is shown. SB = strong BSP, MB = middle BSP, WB = weak BSP, NB = no BSP.

4.2.5 Transferability of beer spoilage test results

It was tested, whether the results of one beer spoilage test using beers from a brewery *X* are transferable and valid for other beers with similar properties. Therefore, the beer spoilage test was conducted for a selection of 23 strains using three other beer-sets from three other breweries. Each set included a wheat, a lager and a pilsner beer from the respective brewery. Beers of the same brewing type differed with respect to hop content (3 to 5 IBU), pH (0.2 to 0.3 units) and ethanol content (0.1 to 0.3 v/v %). The resulting strain classifications were compared to each other and to the classification based on the beer spoilage test done with beers from brewery 1.

57 % of all strains were assigned to the same beer spoilage potential group in all beer-sets, while 70 % were classified consistently regarding beer spoilage ability. In four cases, strains with no beer spoilage ability in beer-set 1 were classified as beer spoilers in one or more other beer-set (cf. Table 14, p. 71). The same was true for four beer-spoiling strains (according to beer-set 1), but vice versa. In case of *P. damnosus* and *L. paracollinoides*, the accordance between the different beer-sets was 100 %, while none of the investigated *L. lindneri* strains showed a consistent beer spoilage potential or ability. For all four beer-sets, it could be observed that *L. lindneri* strains, even if they showed good growth in lager or pilsner beer (MB - SB), often completely lacked growth in wheat beers. Appendix 8 (p. 342) contains beer spoilage data (turbidity, acidification and adaption) obtained for the alternative beer-sets. Raw data are partially originating from student projects under my supervision.

Table 14: Transferability test - classification of lactic acid bacteria strains into beer spoilage potential (BSP) and beer spoilage ability (BSA) groups, based on beer spoilage tests using different beer-sets. The average BSP and BSA are shown. Classification based on beer spoilage test: SB = strong BSP, MB = middle BSP, WB = weak BSP, NB = no BSP; BSA (B/NB) defined by growth in lager beer. B1 to B4: beers used from brewery 1 to 4. Consistent classification is indicated by green color.

Species	Strain	B1	B2	B3	B4	B1	B2	B3	B4
		Average BSP				Average BSA			
L. brevis	TMW 1.313	SB	SB	SB	SB	B	B	B	B
	TMW 1.465	SB	SB	SB	SB	B	B	B	B
	TMW 1.302	WB	SB	MB	MB	NB	B	B	B
	TMW 1.1326	WB	NB	WB	NB	NB	NB	NB	NB
P. claussenii	TMW 2.50	SB	SB	SB	SB	B	B	B	B
	TMW 2.54	SB	SB	SB	SB	B	B	B	B
	TMW 2.1545	MB	MB	WB	MB	B	B	NB	B
	TMW 2.1531	NB	NB	WB	MB	NB	NB	NB	B
P. damnosus	TMW 2.1532	NB	NB	NB	NB	NB	NB	NB	NB
	TMW 2.1533	SB	SB	SB	SB	B	B	B	B
	TMW 2.1535	SB	SB	SB	SB	B	B	B	B
	TMW 2.1536	NB	NB	NB	NB	NB	NB	NB	NB
L. backii	TMW 1.2002	MB	SB	SB	SB	B	B	B	B
	TMW 1.2004	NB	WB	NB	WB	NB	NB	NB	NB
	TMW 1.1989	SB	SB	SB	SB	B	B	B	B
	TMW 1.1991	SB	SB	SB	SB	B	B	B	B
L. lindneri	TMW 1.88	NB	SB	SB	SB	NB	B	B	B
	TMW 1.2006	NB	SB	SB	NB	NB	B	B	NB
	TMW 1.2008	SB	SB	SB	NB	B	B	B	NB
	TMW 1.2009	SB	SB	SB	NB	B	B	B	NB
L. paracoll.	TMW 1.1979	NB	NB	NB	NB	NB	NB	NB	NB
	TMW 1.1994	SB	SB	SB	SB	B	B	B	B
	TMW 1.1995	SB	SB	SB	SB	B	B	B	B

4.2.6 Growth dynamics of lactic acid bacteria in beer with additives and under beer specific stress conditions

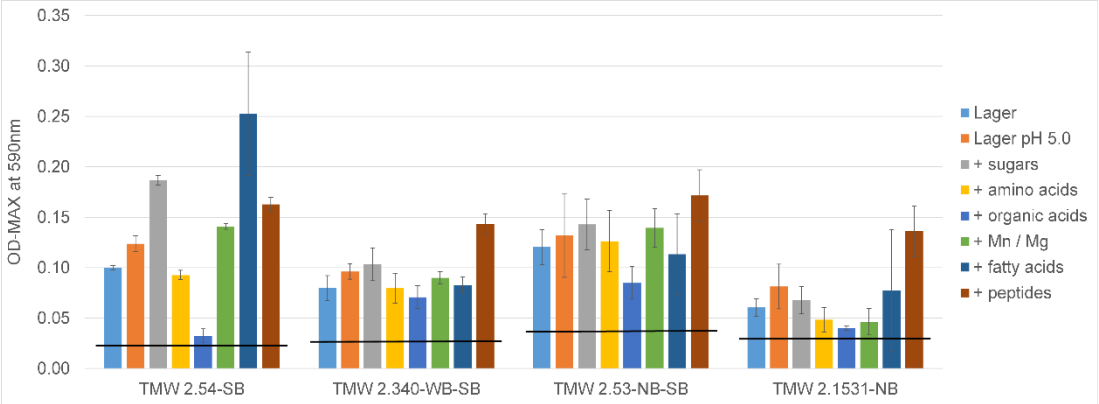
4.2.6.1 Growth in beer with additives

The effect of different additives on the growth behaviour of 26 core-species strains in lager beer 1 was investigated. This was done in order to evaluate the significance of beer specific parameters for single species and strains and in order to evaluate the significance of these parameters for the susceptibility of beer towards spoilage. The impact of an increased carbohydrate, amino acid, organic acid, fatty acid, mineral and peptide content as well as of an elevated pH was tested with respect to growth promoting and inhibitory effects. Distinct and species-common growth enhancing effects could be observed for peptide addition in case of *P. claussenii*, carbohydrates for *L. lindneri*, and for *P. damnosus* in lager beer 1 supplemented with fatty acids. Strains of *L. brevis*, *L. paracollinoides* and *P. claussenii* with weak and no beer spoilage potential, showed an extended log phase and a shortened lag phase in lager beer 1 (better/faster adaption), supplemented with peptides. The addition of organic acids had a negative effect on growth of 16 strains of five species. Only *L. lindneri* was not significantly affected.

26 strains of six different species were characterized, as described in chapter 3.2.5 (p. 32). Cells were preadapted in $lager_{pH5.0}$ and lager beer 1 variants, supplemented with the abovementioned additives, were inoculated with a high amount of cells (approximately to OD_{590} = 0.01 to 0.04), allowing even strains with no beer spoilage potential to grow to a certain extent in lager beer 1 without modifications (for comparison). Growth was tracked for 30 days at 25° C. The maximum OD_{590} was used as a measure for growth, in order to compare the different beer variants. All differences with more than two standard deviations were considered as significant. The composition and concentration of additives can be found in Table 8 (p. 33). Single growth curves are not shown but will be referred to within the text. Raw data are partially originating from student projects under my supervision.

4.2.6.1.1 Pediococcus claussenii

The addition of peptone (peptides and amino acids) led to a significantly enhanced growth of all strains (Figure 10). Except for TMW 2.54-SB, an extended log phase and a comparatively shorter lag phase were observed in this beer variant. In contrast, the addition of amino acids did not affect *P. claussenii´s* growth. The addition of organic acids resulted in an inhibition of growth of all strains except TMW 2.340-MB, which is characterized by slime production.



Strain	Spoilage potential	pH 5.0	Sugars	AA	OA	MN	FA	PE
TMW 2.54	SB	↑	↑	→	↓	↑	↑	↑
TMW 2.340	WB-SB	→	→	→	→	→	→	↑
TMW 2.53	NB-SB	→	→	→	↓	→	→	↑
TMW 2.1531	NB	→	→	→	↓	→	→	↑

Figure 10: Growth of *P. claussenii* strains in lager beer 1, with and without additives. **Bar chart:** The maximum measured OD_{590} within 30 days at 25° C is shown. Average +/- standard deviation. The black bar corresponds to the OD_{590} at day 0. **Table:** Significant differences with respect to lager beer 1 without additives are indicated by arrows: ↑ = enhanced growth, ↓ = reduced growth, → = no significant impact. AA = amino acids, OA = organic acids, MN = Mn^{2+}/Mg^{2+}, FA = fatty acids, PE = peptides and AA, Classification based on beer spoilage test: SB = strong beer spoilage potential (BSP), MB = middle BSP, WB = weak BSP, NB = no BSP.

4.2.6.1.2 Pediococcus damnosus

The addition of fatty acids to lager beer 1 caused an improved growth of four of five strains (Figure 12, p. 75). In case of TMW 2.1534-NB, a tendency for improved growth could be seen when single replicates (growth curves), grown in lager beer 1, with and without the addition of fatty acids, were compared to each other. All NB strains showed an extended log phase in beer with fatty acids. SB strains were characterized by a distinct reduction of the OD_{590} after 12 days, when supplemented with fatty acids. This is shown exemplary for TMW 2.1535-SB. This effect was not observed in any other beer variant. Supplementary organic acids had a negative effect on the growth of *P. damnosus* strains, resulting in reduced maximum turbidity.

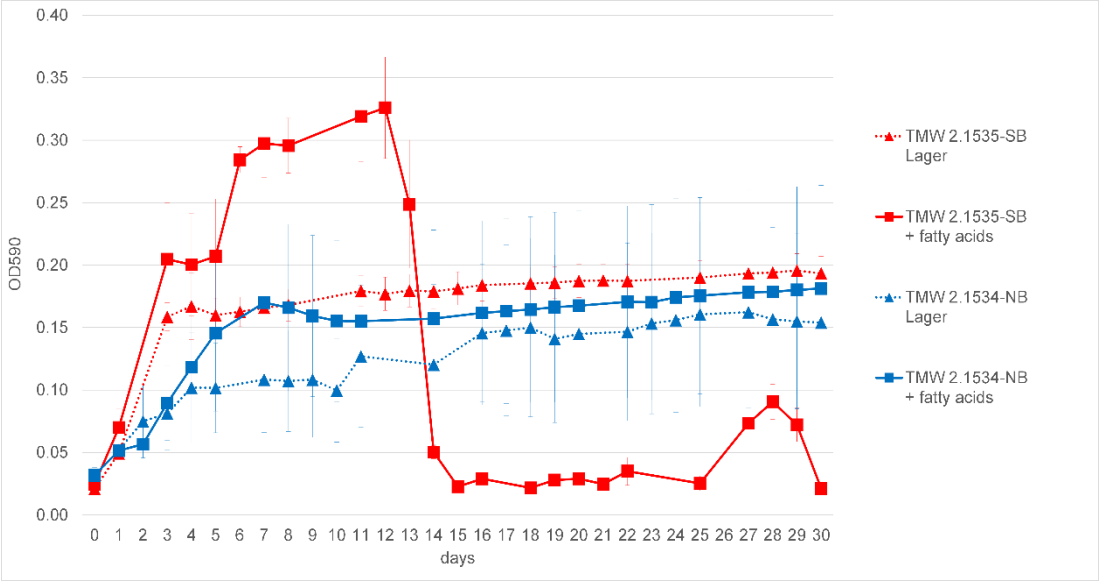
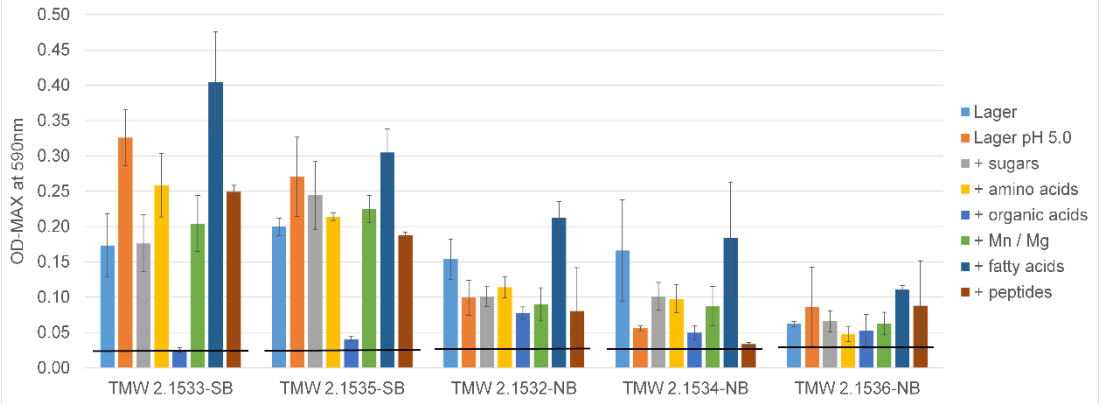


Figure 11: Growth of *P. damnosus* in lager beer 1, with and without additional fatty acids. Growth is shown for the first 20 days at 25° C, for one strong spoiling and one non-spoiling strain, exemplary. TMW 2.1534-NB was characterized by an extended log phase and enhanced turbidity, when supplemented with fatty acids. This is also the case for TMW 2.1535-SB, while here growth is also characterized by a distinct decrease of OD_{590} starting after 12 days. The fatty acid beer variant was the only variant tested resulting in such a growth behaviour for TMW 2.1535-SB and TMW 2.1533-SB (not shown).

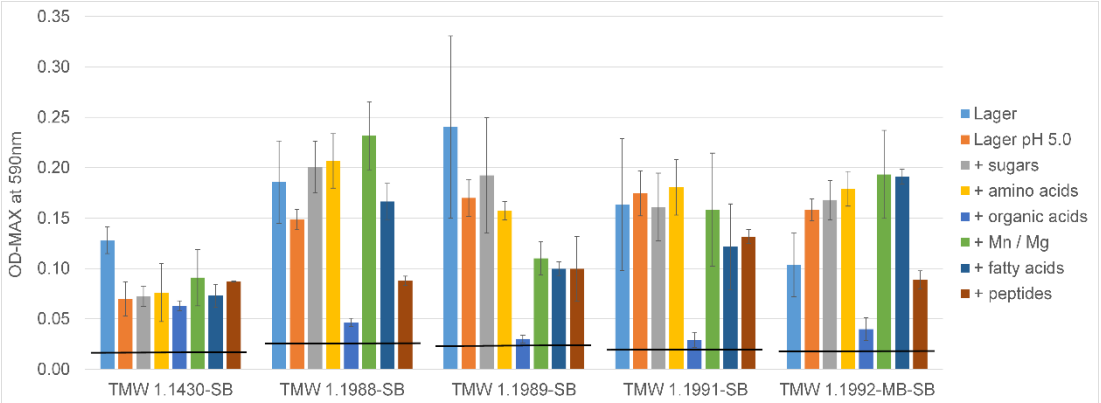


Strain	Spoilage potential	pH 5.0	Sugars	AA	OA	MN	FA	PE
TMW 2.1533	SB	↑	→	→	↓	→	↑	→
TMW 2.1535	SB	→	→	→	↓	→	↑	→
TMW 2.1532	NB	→	→	→	↓	↓	↑	→
TMW 2.1534	NB	→	→	→	→	→	→	→
TMW 2.1536	NB	→	→	→	→	→	↑	→

Figure 12: Growth of *P. damnosus* strains in lager beer 1, with and without additives. **Bar chart:** The maximum measured OD_{590} within 30 days at 25° C is shown. Average +/- standard deviation. The black bar corresponds to the OD_{590} at day 0. **Table:** Significant differences with respect to lager beer 1 without additives are indicated by arrows: ↑ = enhanced growth, ↓ = reduced growth, → = no significant impact. AA = amino acids, OA = organic acids, MN = Mn^{2+}/Mg^{2+}, FA = fatty acids, PE = peptides and AA, Classification based on beer spoilage test: SB = strong beer spoilage potential (BSP), MB = middle BSP, WB = weak BSP, NB = no BSP.

4.2.6.1.3 Lactobacillus backii

Strains with stable, strong beer spoilage potential, were not positive affected by any additives tested, while TMW 1.1992 (MB-SB) with the comparatively lowest beer spoilage potential within the tested set, was positive affected by the addition of sugars, amino acids, minerals and fatty acids (Figure 13, p. 76).

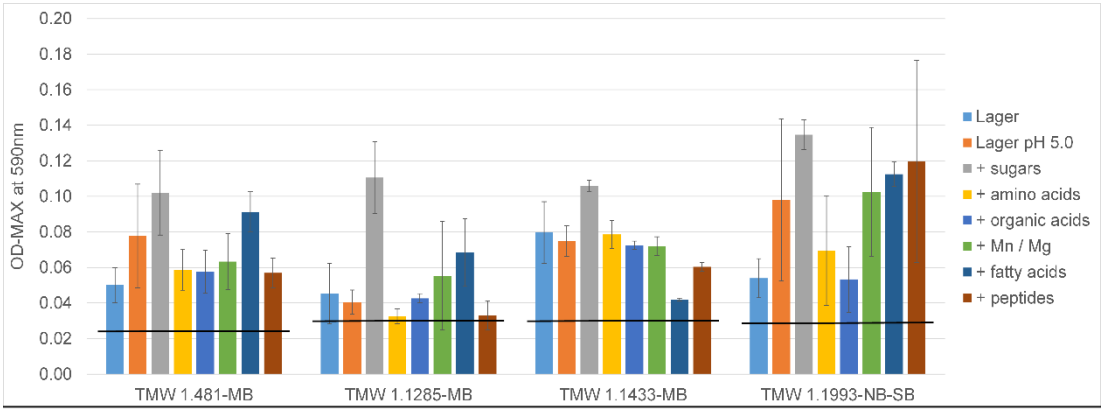


Strain	Spoilage potential	pH 5.0	Sugars	AA	OA	MN	FA	PE
TMW 1.1430	SB	↓	↓	→	↓	→	↓	↓
TMW 1.1988	SB	→	→	→	↓	→	→	↓
TMW 1.1989	SB	→	→	→	↓	→	→	→
TMW 1.1991	SB	→	→	→	↓	→	→	→
TMW 1.1992	MB-SB	→	↑	↑	↓	↑	↑	→

Figure 13: Growth of *L. backii* strains in lager beer 1, with and without additives. **Bar chart:** The maximum measured OD_{590} within 30 days at 25° C is shown. Average +/- standard deviation. The black bar corresponds to the OD_{590} at day 0. **Table:** Significant differences with respect to lager beer 1 without additives are indicated by arrows: ↑ = enhanced growth, ↓ = reduced growth, → = no significant impact. AA = amino acids, OA = organic acids, MN = Mn^{2+}/Mg^{2+}, FA = fatty acids, PE = peptides and AA, Classification based on beer spoilage test: SB = strong beer spoilage potential (BSP), MB = middle BSP, WB = weak BSP, NB = no BSP.

4.2.6.1.4 Lactobacillus lindneri

In case of *L. lindneri,* a growth enhancing effect was observed in lager with sugar addition for three of four strains (Figure 14, p. 77), while the same tendency can also be seen for TMW 1.1285-MB looking at the bar chart. Growth curves in the same beer variant were different with respect to slope and duration of log phase, while both parameters were increased. Two strains were positively affected by the addition of fatty acids, while TMW 1.1433-MB showed reduced growth in that specific beer variant.

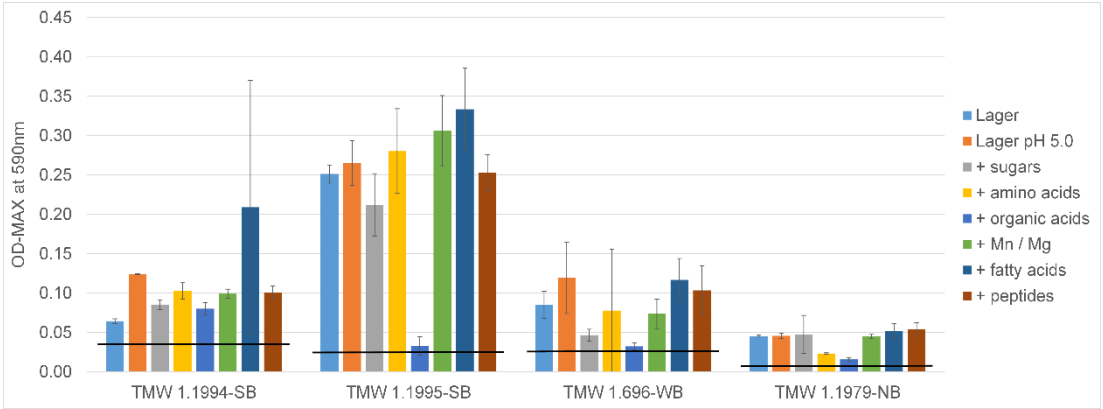


Strain	Spoilage potential	pH 5.0	Sugars	AA	OA	MN	FA	PE
TMW 1.481	MB	⇨	⇧	⇨	⇨	⇨	⇧	⇨
TMW 1.1285	MB	⇨	⇧	⇨	⇨	⇨	⇨	⇨
TMW 1.1433	MB	⇨	⇨	⇨	⇨	⇨	⇩	⇨
TMW 1.1993	NB-SB	⇨	⇧	⇨	⇨	⇨	⇧	⇨

Figure 14: Growth of *L. lindneri* strains in lager beer 1, with and without additives. **Bar chart:** The maximum measured OD590 within 30 days at 25° C is shown. Average +/- standard deviation. The black bar corresponds to the OD590 at day 0. **Table:** Significant differences with respect to lager beer 1 without additives are indicated by arrows: ⇧ = enhanced growth, ⇩ = reduced growth, ⇨ = no significant impact. AA = amino acids, OA = organic acids, MN = Mn^{2+}/Mg^{2+}, FA = fatty acids, PE = peptides and AA, Classification based on beer spoilage test: SB = strong beer spoilage potential (BSP), MB = middle BSP, WB = weak BSP, NB = no BSP.

4.2.6.1.5 Lactobacillus paracollinoides

Three of four strains were characterized by reduced growth in the beer variant containing supplementary organic acids, while TMW 1.1994-SB was positively affected. The same strain showed enhanced turbidity in all beer variants in comparison to lager beer 1. Figure 15 (p. 78) illustrates the heterogeneous situation found for this species. The adaption to beer, illustrated by a reduced lag phase and extended log phase compared to beer without additives, was enhanced in the peptide variant in case of TMW 1.1979-NB and TMW 1.696-WB (single growth curves not shown).

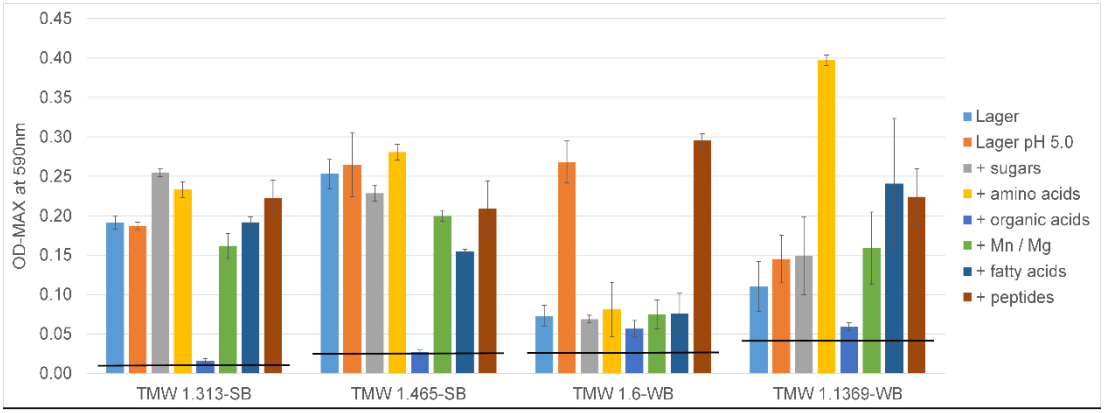


Strain	Spoilage potential	pH 5.0	Sugars	AA	OA	MN	FA	PE
TMW 1.1994	SB	↑	↑	↑	↑	↑	→	↑
TMW 1.1995	SB	→	→	→	↓	→	→	→
TMW 1.696	WB	→	↓	→	↓	→	→	→
TMW 1.1979	NB	→	→	↓	↓	→	→	→

Figure 15: Growth of *L. paracollinoides* strains in lager beer 1, with and without additives. **Bar chart:** The maximum measured OD590 within 30 days at 25 ° C is shown. Average +/- standard deviation. The black bar corresponds to the OD590 at day 0. **Table:** Significant differences with respect to lager beer 1 without additives are indicated by arrows: ↑ = enhanced growth, ↓ = reduced growth, → = no significant impact. AA = amino acids, OA = organic acids, MN = Mn^{2+}/Mg^{2+}, FA = fatty acids, PE = peptides and AA, Classification based on beer spoilage test: SB = strong beer spoilage potential (BSP), MB = middle BSP, WB = weak BSP, NB = no BSP.

4.2.6.1.6 Lactobacillus brevis

Additional amino acids had a positive effect on growth of two strains, while a similar tendency for the other strains is indicated by the bar chart of Figure 16 (p. 79). Both non-brewery isolates with weak beer spoilage potential showed a strong response to the addition of peptides and a tendency for better growth in lager$_{pH5.0}$. By supplying these strains with peptides (TMW 1.6) and amino acids (TMW 1.1369), both reached a higher turbidity as the strong spoiling strains. For both WB strains, the addition of peptides altered the growth curves not only with respect to the maximum OD_{590} but also with respect to lag phase (adaption) and the duration of log phase. The addition of organic acids to lager beer 1 resulted in total inhibition of both strains with strong beer spoilage potential. Strong beer-spoiling strains were also characterized by a negatively affected growth in beer containing elevated amounts of Mn^{2+}/Mg^{2+}.



Strain	Spoilage potential	pH 5.0	Sugars	AA	OA	MN	FA	PE
TMW 1.313	SB	⇨	⇧	⇧	⇩	⇨	⇨	⇨
TMW 1.465	SB	⇨	⇨	⇨	⇩	⇩	⇩	⇨
TMW 1.6	WB	⇧	⇨	⇨	⇨	⇨	⇨	⇧
TMW 1.1369	WB	⇨	⇨	⇧	⇨	⇨	⇨	⇧

Figure 16: Growth of *L. brevis* strains in lager beer 1, with and without additives. **Bar chart:** The maximum measured OD590 within 30 days at 25 ° C is shown. Average +/- standard deviation. The black bar corresponds to the OD590 at day 0. **Table:** Significant differences with respect to lager beer 1 without additives are indicated by arrows: ⇧ = enhanced growth, ⇩ = reduced growth, ⇨ = no significant impact. AA = amino acids, OA = organic acids, MN = Mn^{2+}/Mg^{2+}, FA = fatty acids, PE = peptides and AA, Classification based on beer spoilage test: SB = strong beer spoilage potential (BSP), MB = middle BSP, WB = weak BSP, NB = no BSP.

4.2.6.2 Growth under stress conditions – adaption and tolerance

Beer is often referred to as a beverage with several antibacterial hurdles for bacterial growth (Vriesekoop et al., 2012). In order to check the tolerance and adaption of core-species LAB strains to these beer specific hurdles or stress conditions, growth experiments, testing different stress qualities (hops, ethanol, pH) and intensities, were conducted. Hops can be considered as main hurdle for bacterial growth, while the antibacterial properties of hops are composed of their ionophore character and their potential to induce oxidative stress (Schurr, Hahne, et al., 2015). Therefore, strains were checked for potential cross-resistances to other ionophores (uncouplers) and oxidative stress induced by H_2O_2. The best species independent correlation to beer spoilage potential was observed for ethanol and iso-α-acids tolerance, while the latter was distinct for *L. brevis* and *L. paracollinoides*. A positive correlation of oxidative stress tolerance to hop tolerance was found, while the former, at low Mn^{2+} content, had a high to very high correlation to beer spoilage potential of *P. claussenii* and *L. brevis*. The tolerance to various ionophores did not correlate to hop tolerance. Based on the obtained growth data, *L. backii* shows the highest tolerance and adaption to beer specific stress conditions, while *P. damnosus* is ranked as last in both cases. *P. claussenii* is ranked as number one with respect to overall stress tolerance compared to the other species. Again, *P. damnosus* is ranked last.

26 strains were tested for their growth behaviour in $mMRS_1$ and $mMRS_2$ variants containing different additives or lacking specific components, respectively. Up to 12 different stress intensities were checked for the following stress qualities: acid stress, ethanol stress, hop stress, nutrient limitation, manganese limitation, oxidative stress induced by H_2O_2 at low/high manganese content and ionophore stress induced by 2,4 dinitrophenol (DNP), carbonyl cyanide m-chlorophenyl hydrazone (CCCP), calcimycin and a combination of valinomycin and nigericin. Growth was tracked for 7 days at 25° C. Table 4 (p. 27) lists all additives used, while chapter 3.2.3 (p. 31) describes the applied stress qualities and intensities tested. Raw data were generated within a student´s project under my supervision. More than 8200 growth curves were generated and evaluated using groFit (cf. 3.6.1, p. 44), extracting three parameters, the maximum OD_{590}, the duration of the lag phase (lag) and the maximum specific growth rate (μ_{max}), for comparison and statistics. Figure 17 illustrates the results for a single strain and a single stress quality, graphically. Depending on species and strain, stress qualities affected either all parameters or only single ones.

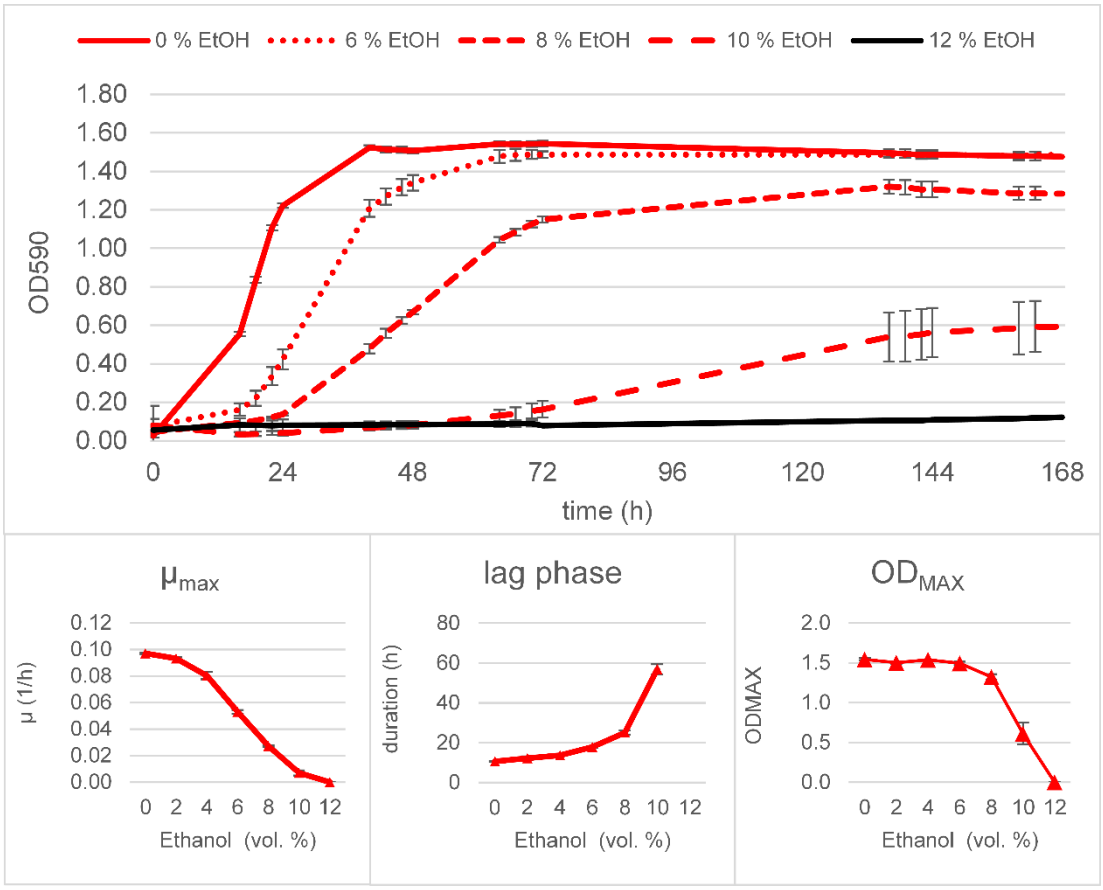


Figure 17: Growth parameter illustration. The maximum turbidity (OD_{MAX}), the maximum specific growth rate (μ_{max}) and the duration of the lag phase were extracted for all growth data obtained. This example illustrates, graphically, the growth data and the resulting parameters for *L. brevis* TMW 1.465-SB, grown in $mMRS_1$ pH 4.3 and an increasing ethanol content. Growth curves (upper panel) are shown for some selected concentrations. The lower panels show the values of the extracted parameters (e.g. μ_{max}) plotted versus the ethanol concentration. In this example, all three parameters were affected by an increase of the ethanol content. EtOH = Ethanol.

The correlation of growth parameters under different stress qualities and intensities to beer spoilage potential and each other was investigated applying spearman´s rank correlation (see. 3.6.1, p. 44), including the abovementioned growth parameters and the minimum inhibitory concentrations (MIC) determined. For each species, the average MIC values were calculated for all stress qualities (Table 15, p. 83). Species were ranked according to their average MIC values and assigned a tolerance rank (Table 17, p. 84). In addition, species were ranked according to their adaption to beer specific stress qualities at (near at) beer-typical intensities (Table 16, p. 83). Therefore, the most affected growth parameter (μ_{max}, OD_{MAX}, lag) was used for each particular stress quality. For the ranking, the selected parameter was correlated to the same parameter without stress. Rankings were summarized, resulting in total tolerance, ionophore tolerance, oxidative stress tolerance and the tolerance as well as the adaption to beer specific stress qualities. The following stress qualities and intensities were

included to determine the adaption to beer specific stress: acid stress (pH 4.25), ethanol stress (6 v/v %), hop stress (15 ppm iso-α-acids), nutrient limitation (20 % of $mMRS_1$, arbitrary) and manganese (magnesium) limitation (0.16 mg/l, concentration found as in beer).

Appendix 9 (p. 341) lists all strains included as well as the MIC values, determined by the lack of growth, for all stress qualities, with the exception of nutrient and manganese limitation. In case of both last-named stress qualities, growth was observed in all intensities for all strains with single replicate exceptions.

A species independent low positive correlation of MIC values to beer spoilage potential was found for ethanol ($\rho = 0.49$) and iso-α-acids ($\rho = 0.45$). In addition, a low to high positive correlation of hop tolerance (MIC iso-α-acids) to MIC values for H_2O_2 in $mMRS_2$ ($\rho = 0.44$) and $mMRS_1$ ($\rho = 0.70$) was found. With the exception of DNP (low positive, $\rho = 0.44$), no significant correlation of hop tolerance to the tolerance towards other ionophores was found. Dissected to the species level, the significance of iso-α-acids for beer spoilage potential became distinct for two of six species. This is illustrated by a very high positive correlation for *L. brevis* ($\rho = 0.94$) and *L. paracollinoides* ($\rho = 0.94$). For *P. claussenii* ($\rho = 0.82$) and *L. brevis* ($\rho = 0.94$), a high to very high positive correlation of oxidative stress tolerance at low manganese content (H_2O_2 in $mMRS_2$) to beer spoilage potential was observed. *L. brevis* in addition was characterized by perfect correlation ($\rho = 1$) of ethanol tolerance to beer spoilage potential.

Tolerance and adaption rankings can be found in the following Tables.

Table 15: Average minimum inhibitory concentrations (MICs) of tested stress qualities, determined for core-species. Stress qualities and units are given. MICs are listed as mean +/- standard deviation.

Stress (unit)	*P. damnosus*	*P. claussenii*	*L. backii*	*L. brevis*	*L. lindneri*	*L. paracoll.*
Acid (pH)	3.60 +/- 0.22	2.75 +/- 0.00	3.13 +/- 0.14	3.00 +/- 0.00	3.38 +/- 0.14	3.25 +/- 0.00
Ethanol (v/v %)	10 +/- 3	11 +/- 1	16 +/- 1	11 +/- 1	9 +/- 1	13 +/- 2
Iso-α-acids (ppm)	10 +/- 4	24 +/- 5	25 +/- 14	19 +/- 13	33 +/- 16	8 +/- 3
Oxidative, high Mn2+ (mM)	1.8 +/- 0.3	3.9 +/- 0.3	2.9 +/- 0.5	3.6 +/- 0.3	3.5 +/- 0.4	1.8 +/- 0.7
Oxidative, low Mn2+ (mM)	1.9 +/- 0.2	3.1 +/- 0.3	3.3 +/- 1.2	3.4 +/- 0.5	4.5 +/- 0.6	1.3 +/- 0.3
K+ Ionophores - Valinomycin & Nigericin (µM)	7 +/- 4	> 10	8 +/- 3	> 10	3 +/- 3	5 +/- 4
H+ ionophore - DNP (mM)	0.1 +/- 0.0	0.3 +/- 0.1	0.1 +/- 0.1	0.4 +/- 0.1	0.2 +/- 0.1	0.1 +/- 0.1
Divalent cations ionophore - Calcimycin (µM)	16 +/- 9	> 100	23 +/- 13	50 +/- 18	28 +/- 35	67 +/- 40
H+ ionophore - CCCP (mM)	0.08 +/- 0.05	> 0.2	0.13 +/- 0.07	0.14 +/- 0.04	0.04 +/- 0.03	0.05 +/- 0.01

Table 16: Adaption ranking based on growth characteristics under beer specific stress conditions. First place and last place are marked. Stress qualities chosen include acid, ethanol and hop stress as well as nutrient limitation and manganese limitation. Intensities were chosen as close as possible to typical concentrations found in beer. In case of nutrient limitation, 20 % mMRS$_1$ was chosen, as the growth at lower concentrations resulted in hard-to-fit growth curves (hard to fit for groFit, resulting in less reliable growth parameters). It does not reflect the nutrient situation found in typical beers.

Stress – adaption to	*P. damnosus*	*P. claussenii*	*L. backii*	*L. brevis*	*L. lindneri*	*L. paracoll.*
Acid - pH 4.25	6	2	5	4	3	1
Ethanol - 6 v/v %	6	4	2	3	1	5
Hop - 15 ppm iso-α-acids	5	2	1	4	3	5
Nutrient limitation - 20 % of mMRS$_1$	6	3	2	4	1	5
Manganese limitation - Mn^{2+} 0.16 mg/l	2	3	4	1	5	6
Total (beer) adaption	6	2	1	4	3	5

Table 17: Tolerance ranking based on average minimum inhibitory concentrations (MICs) of each species. First place and last place are marked. Summarized ranks are based on medians of single ranks. Beer specific: includes acid, ethanol and iso-α-acids; oxidative total: includes H_2O_2 (mMRS$_1$ and mMRS$_2$) and iso-α-acids; ionophores: includes valinomycin & nigericin, 2,4 dinitrophenol (DNP), carbonyl cyanide m-chlorophenyl hydrazine (CCCP), calcimycin and iso-α-acids.

Stress	***P. damnosus***	***P. claussenii***	***L. backii***	***L. brevis***	***L. lindneri***	***L. paracoll.***
Acid (pH)	6	1	3	2	5	4
Ethanol	5	3	1	3	6	2
Hop (iso-α-acids)	5	3	2	4	1	6
Oxidative, high Mn^{2+}	5	1	4	2	3	6
Oxidative, low Mn^{2+}	5	4	3	2	1	6
K^+ Ionophores - Valinomycin & Nigericin	4	1	3	1	6	5
H^+ ionophore - DNP	6	2	4	1	3	4
Divalent cations ionophore - Calcimycin	6	1	5	3	4	2
H^+ ionophore - CCCP	4	1	3	2	6	5
Overall tolerance	6	1	3	2	4	4
Beer specific	5	2	1	2	5	4
Oxidative total	5	3	3	2	1	6
Ionophores	5	1	3	2	4	5

4.3 Genomics

4.3.1 Genome sequences

17 strains were chosen for whole genome sequencing. Genomes were sequenced using SMRT sequencing (McCarthy, 2010), assembled using SMRT-Analysis (Pacific Biosciences, Menlo Park, USA) and annotated with RAST (Aziz et al., 2008; Overbeek et al., 2014), as described in chapter 3.6.2 (p. 45). All 17 genomes could be assembled to *complete* status, resulting in a chromosome and zero to ten plasmids. Annotation was not curated manually, while in case where a specific gene or locus was of interest, additional analysis was carried out. This is stated in the corresponding sections. Table 18 (p. 86) lists important metrics of all sequenced genomes. For comparative analysis, additional genomes were downloaded from GenBank (Burks et al., 1985; Clark et al., 2016) and adduced, in a variable manner, for comparative analysis. They are listed together with assembly level, accession number and source in Appendix 10 (p. 347). The selection was primarily based on the availability of complete genomes in August 2015 and the following criteria:

- Genus *Lactobacillus*: all genomes with assembly level *complete* and *chromosome*
    - Exceptions: *L. brevis* TMW 1.6, TMW 1.313, TMW 1.465 - level *scaffold* → characterized in various experiments
    - *L. sanfranciscensis* TMW 1.1304 - level *scaffold* → next relative species (ANI/16S) to *L. lindneri*
    - *L. rossiae* DSM 15814, *L. coryniformis* DSM 20001 - level *scaffold* → because of their relevance for brewing microbiology (Hutzler, et al., 2013)
- Order *Lactobacillales*: Reference genomes of *Leuconostoc mesenteroides* ATCC8293 and *Lactococcus lactis* IL 1403, because of their potential relevance for brewing microbiology (Hutzler, et al., 2013)
- Reference genomes of *Bacillus subtilis* 168 and *Escherichia coli* K-12 substr. MG1655 → accepted model organism

Table 18: Genome metrics for sequenced strains. All biosamples are part of the bioproject PRJNA290141. Accession numbers are given for all contigs. Coverage = average coverage of assemblies, Contigs = Chromosome plus plasmids, PEG = Protein encoding genes based on RAST annotation, Beer spoilage potential (BSP) based on growth behaviour: SB = strong BSP, MB = middle BSP, WB = weak BSP, NB = no BSP. Average is given.

Species	Strain	Biosample	NCBI accession	Coverage	Size (Mbp)	Contigs	GC content (%)	PEG (RAST)
P. claus.	TMW 2.53-SB	SAMN04505731	CP014933 - CP014935	192	1.95	3	37.1	1895
	TMW 2.54-SB	SAMN04505732	CP014936 - CP014939	146	1.99	4	37.1	1940
P. damnosus	TMW 2.1532-NB	SAMN03876481	CP012269 - CP012274	144	2.20	6	38.3	2060
	TMW 2.1533-SB	SAMN03876482	CP012275 - CP012282	92	2.40	8	38.4	2259
	TMW 2.1534-NB	SAMN03876483	CP012283 - CP012287	138	2.28	5	38.3	2162
	TMW 2.1535-SB	SAMN03876484	CP012288 - CP012293	142	2.51	6	38.6	2330
	TMW 2.1536-NB	SAMN03876485	CP012294 - CP012296	137	2.17	3	38.3	2017
L. backii	TMW 1.1988-SB	SAMN04505726	CP014623 - CP014633	121	2.82	11	40.8	2671
	TMW 1.1989-SB	SAMN04505727	CP014873 - CP014880	89	2.85	8	40.8	2646
	TMW 1.1991-SB	SAMN04505728	CP014881 - CP014889	99	2.82	9	40.7	2590
	TMW 1.1992-SB	SAMN04505729	CP014890 - CP014898	109	2.78	9	40.8	2621
	TMW 1.2002-MB	SAMN04505730	CP014899 - CP014906	168	2.84	8	40.7	2653

Species	Strain	Biosample	NCBI accession	Coverage	Size (Mbp)	Contigs	GC content (%)	PEG (RAST)
L. lindneri	TMW 1.481-MB	SAMN04505733	CP014907 - CP014911	143	1.39	1	34.3	1347
	TMW 1.1993-SB	SAMN04505734	CP014872	262	1.45	5	34.4	1429
L. paracoll.	TMW 1.1979-NB	SAMN04505735	CP014912 - CP014914	107	3.32	3	47.0	2953
	TMW 1.1994-SB	SAMN04505736	CP014915 - CP014923	102	3.66	9	46.8	3363
	TMW 1.1995-SB	SAMN04505737	CP014924 - CP014932	88	3.75	9	46.6	3378

4.3.2 Genome analysis

The genomes of all six core-species were analysed in detail with respect to general genomic properties and genome structure, the distribution of functional categories (functional analysis) and predicted metabolic capabilities. The whole genome analysis section orients itself on the previous detailed genome analysis about *L. sanfranciscensis* by Vogel et al. (2011). In a first subsection, the general genomic properties are described, including, amongst others, parameters such as genome size, GC content or the number of rRNA operons. The second part contains the functional analysis. The SEED subsystem analysis allows an assignment of predicted genes to a hierarchical three-level categorization system, ranging from category (Nucleosides and Nucleotides), subcategory (Pyrimidines) to subsystem (De Novo Pyrimidine Synthesis) (Aziz et al., 2008; Overbeek et al., 2014). The COG enrichment ends with a classification into 23 categories with no subdivision (Galperin et al., 2015; Tatusov et al., 1997). Both classification systems allow a more global and functional view on the genome of an organism, as all genes are assigned to comprehensible, functional categories. In a third part, the metabolic capabilities were predicted, based on KEGG EC numbers and verified/checked by BLASTp/STRING (Altschul et al., 1990; Camacho et al., 2009; Kanehisa and Goto, 2000; Kanehisa et al., 2014; Okuda et al., 2008; Snel et al., 2000; Szklarczyk et al., 2015). Metabolic capabilities were predicted for more than 20 pathways, including, amongst others, carbohydrate, amino acid, purine and pyrimidine, fatty acid and vitamin metabolism.

The comprehensible genome analysis of a single genome of *L. sanfranciscensis* (Vogel et al., 2011) took nine pages in a typical journal. As this analysis includes 24 genomes, which were not only analysed but also compared to each other, the results are offered in a supplementary section (67 pages). All details, depicted in individual chapters for each species, can be found in Supplementary section 2 (10, p. 239). All relevant data and results, which are necessary to comprehend any thoughts and theories in the discussion, will be recapped explicitly in the corresponding discussion sections.

4.3.3 Comparative genomics of beer-spoiling lactic acid bacteria

As stated in the introduction, several LAB species are known to be of general significance for the brewery environment, while they are further classified into categories, according to their general hazard potential (species level) for the product. In order to evaluate if these species share specific properties and if some of these properties correlate to their significance for the brewery environment and their hazard potential, as classified by Hutzler et al. (2013), a comprehensive genomic comparison was conducted. Potential (chromosomal) requirements for the general ability of species to grow in beer should be worked out. Further, a genomic analysis was done concentrating on beer spoilage ability and potential (strain level) for the actually physiological characterized strains and species.

4.3.3.1 General genomic properties

It was investigated, if several genomic properties correlate to the general hazard potential and isolation source (brewery/non-brewery). Therefore, we analysed the respective properties for 114 genomes using cluster and correlation analysis. Significant correlations are only mentioned when they are at least moderate ($\rho = 0.5$ to 0.7). Based on these comparisons, we could not find a species independent and distinct relation of hazard potential or isolation source to genome size, chromosome size, number of proteins, GC content, coding density, codon usage, amino acid usage and subcellular localization of proteins. A moderate correlation was found for the quantity of plasmids, while the correlation to source ($\rho = 0.63$) was higher as to hazard potential ($\rho = 0.58$). Figure 18 (p. 90) shows the number of plasmids found for the included genomes in descending order, labeled according to the respective species hazard potential and the strain´s isolation source.

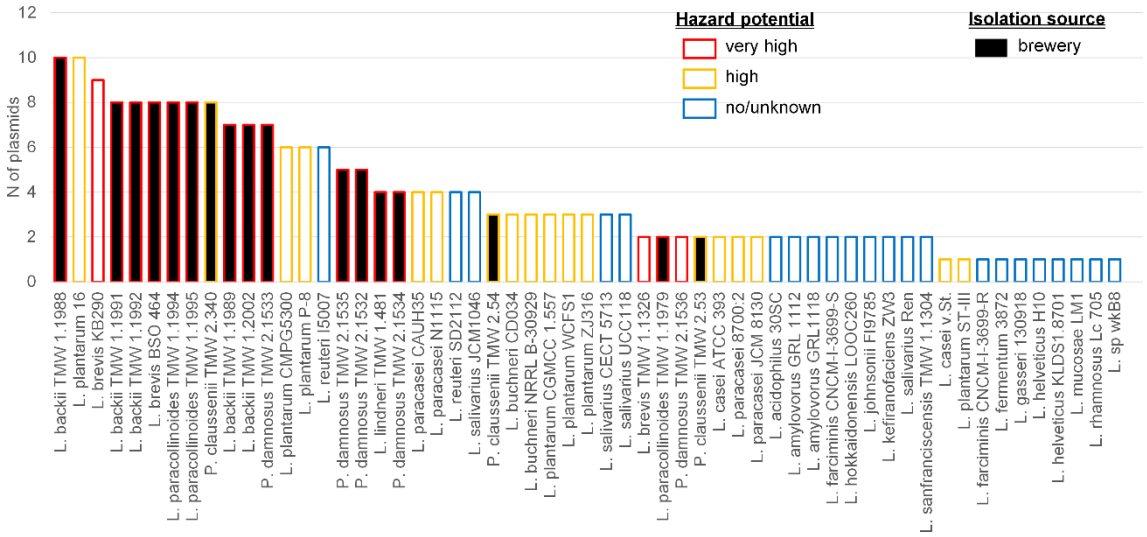


Figure 18: Number of plasmids in context of hazard potential and isolation source. The number of plasmids is shown for all complete genomes. Most brewery isolates carry four or more plasmids. *L. casei* v.St. = various strains (7) of *L. casei* containing one plasmid.

4.3.3.2 Phylogenetic and phylogenomic analysis, and environmental association

Based on various phylogenetic measures and marker genes we investigated the relation of the general hazard potential to phylogeny. Phylogenomic and phylogenetic analysis were done as described in chapter 3.6.2.9 (p. 49). LAB species with very high and/or high hazard potential did not cluster together based on 16S rDNA, 23S rDNA, *rpoA*, *rpoB* and *recA*. Further, they did not group together based on a phylogenomic analysis (fragmented all-vs-all alignment, Gegenees) of their chromosomes. Neither a proteome based comparison, followed by clustering, nor a pan-genome tree analysis resulted in distinct clusters, correlating to general hazard potential or isolation source.

In order to evaluate possible unknown habitats of the investigated core-species, we performed a BLAST (NCBI) analysis of their 16 rDNA versus the NCBI environmental database. In case of *L. backii*, *L. lindneri* and *P. claussenii* we did not find matches with an identity of 99 % or higher. For *P. damnosus,* we found a high identity match within a full-scale municipal waste compost sample, for *L. paracollinoides* a match to a sample taken from human skin and for *L. brevis* several matches to varying environmental samples. These include fermented grain from Chinese liquor fermentations and again the abovementioned compost sample.

4.3.3.3 Mobile genetic elements: insertion sequences and transposons

We investigated the relation of hazard potential and isolation source to the number of insertion sequences (IS), while IS-elements were predicted using ISfinder (Siguier et al., 2006). There was no species independent significant (spearman´s rank) correlation to source (brewery/non-brewery) or to hazard potential. Resolving the source to four distinct groups, a relation to source became apparent as illustrated by Figure 19. Brewery genomes are characterized by a significantly (CI 0.95) higher number of IS-elements compared to genomes isolated from the gastrointestinal tract, faeces and from plant sources. Figure 19 also illustrates that genomes of isolates from milk and dairy products are characterized by the comparatively highest number of IS-elements, although the difference to genomes of brewery isolates is not significant ($p = 0.07$). Significance was tested using the Kruskal-Wallis test and the Mann-Whitnery U-test.

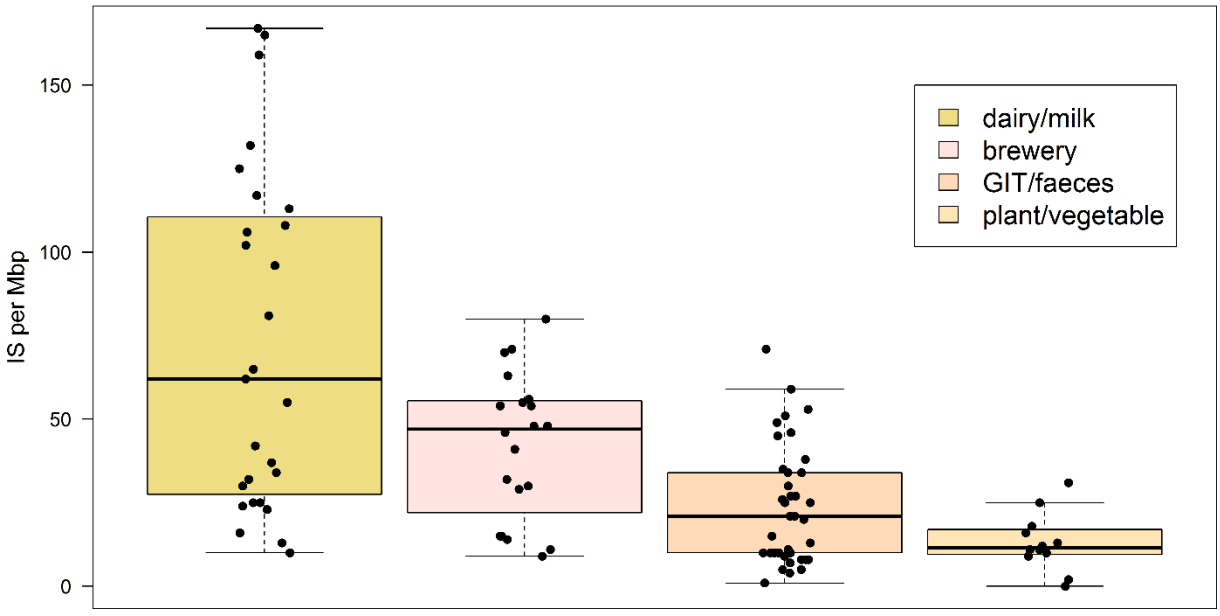


Figure 19: Number of insertion sequences (IS) per Mbp within lactic acid bacteria genomes. IS elements were predicted using ISfinder (Siguier et al., 2006). Only those genomes were accounted which were part of a group with more than five members. Thus, 96 of 114 genomes were assigned to one of the four defined groups. Dairy/milk: contains all isolates from human and animal milk as well as from all dairy products. Brewery: all brewery isolates. GIT/faeces: all isolates from human and animal gastrointestinal tracts and faeces as well as two samples of a human vagina. Plant/vegetable: all isolates from plant and vegetable fermentations.

A relation of isolation source to the number of IS elements was also observed within single species. Brewery genomes of *L. brevis* were found to carry 53 +/- 17 IS/Mbp, while the other isolates were characterized by 16 +/- 12 IS/Mpb. In case of *P. damnosus,* we found the number of IS-elements to correlate to beer spoilage potential and isolation source. Strong beer-spoiling brewery *P. damnosus* strains were characterized by 76 +/- 5 IS/Mbp, brewery non-spoiling strains by 51 +/- 3 IS/Mbp and the non-spoiling winery isolate by 27 IS/Mbp. These intraspecies relations to isolation source were not restricted to LAB species with relevance for brewing microbiology.

IS elements were found on chromosomes and on plasmids. In addition, brewery-specific and plasmid-encoded genetic clusters (for details see 4.3.3.7, p. 99), amongst others encoding for established and novel DMGs such as *horA*, *horC* and *fabZ*, were found to contain or to be flanked by one or two transposases in 19 of 20 cases. While the presence-of/flanking-by the transposase was found to be conserved, the encountered transposase sequences were not identical in all cases (with respect to the same cluster).

4.3.3.4 Functional analysis - SEED & COG

A relation of hazard potential and isolation source to functional pattern, the distribution of genes assigned to SEED/COG based categories, was investigated. Functional pattern were obtained as described in chapter 3.6.2.11 (p. 50) and evaluated using cluster analysis.

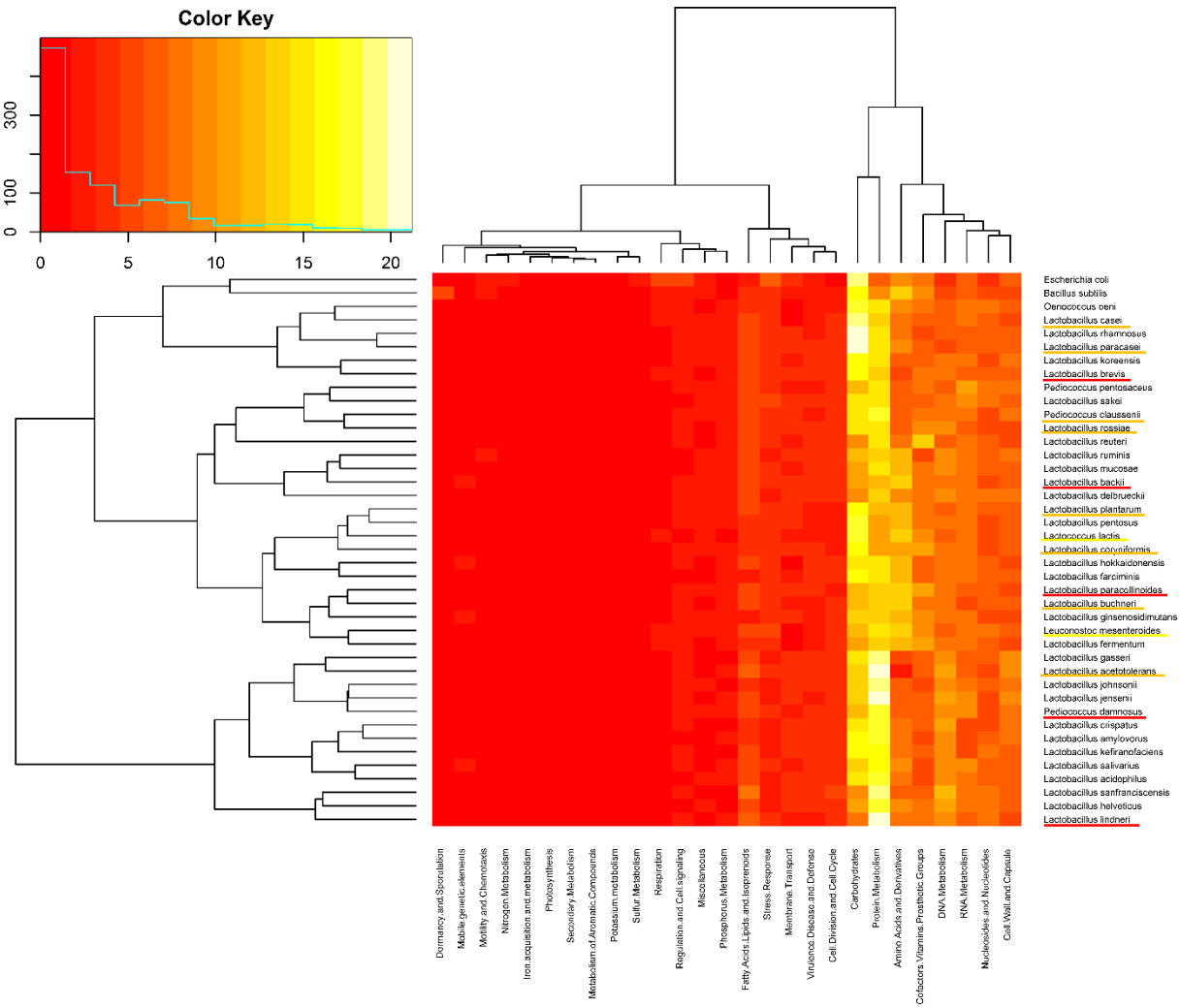


Figure 20: Cluster analysis of lactic acid bacteria genomes, based on SEED functional pattern. Functional pattern result from chromosomal SEED subsystem analysis, based on proportion of assignments to the respective SEED categories. The “color key” window shows the occurrence (y-axis) of all found proportions (%, x-axis). All strains of each species clustered together. Therefore, the respective strains have been “summarized” to species level, in order to obtain a better overview. Species are underlined in colors corresponding to their hazard potential: red = very high, orange = high, yellow = positive tendency.

Cluster analysis was performed based on total counts and based on proportions, which were calculated relative to the sum of functionally assigned genes. LAB species with very high and/or high hazard potential did not cluster together based on genome and chromosomal functional pattern obtained for SEED/COG categories, SEED subcategories and SEED subsystems. An exemplary result is shown in Figure 17 (p. 93). Plasmidomes partially clustered brewery and hazard potential strains together, which will be detailed in chapter 4.3.3.7 (p. 99).

Spearman's rank correlation was adduced to investigate potential correlations of functional groups, counts and proportions, to hazard potential and isolation source. Significant correlations are only mentioned when they are at least moderate (ρ = 0.5 to 0.7). On chromosomal level, we found a moderate correlation (ρ = 0.60) of hazard potential to the COG category secondary metabolites (biosynthesis and transport). While no correlation was found for SEED categories, several moderate correlations (ρ = 0.53 +/- 0.03) to hazard potential were found for SEED subcategories and subsystems. These correlations were further investigated and were the consequence of species effects or simply correlated to genome size.

Species effects: Most complete genomes, adduced for this comparative study, were available for *L. casei*, *L. paracasei* and *L. plantarum*. These bacteria are considered as LAB species with high hazard potential (Hutzler et al., 2013). In most cases, correlation resulted from these strains/species, whereas those species with very high hazard potential were rather average with respect to the count/proportion of a given category, subcategory etc. Similar effects were observed for other species. Correlation analysis was also conducted for average values for each species, removing these effects. Consequently, the observed correlations of certain functional groups to hazard potential were no longer observed.

Functional analysis of plasmidomes revealed no correlations to hazard potential. Isolation source "brewery environment" showed a moderate correlation to the COG categories lipid transport and metabolism (ρ = 0.56), as well as to defense mechanisms (ρ = 0.54). A detailed analysis of brewery plasmidomes can be found in chapter 4.3.3.7 (p. 99).

4.3.3.5 Pan and core genome analysis

Another approach to derive shared (chromosomal) properties for brewery-relevant LAB included the calculation of core genomes and the subsequent analysis of them. Core genomes, unions and intersections were calculated for all genomes using CMG-biotools (Vesth et al., 2013), as described in chapter 3.6.2.10 (p. 50). The core genome of all LAB species with very high or high hazard potential contains 295 gene families. LAB with very high hazard potential and *P. claussenii*, which should be reconsidered as species with very high hazard potential based on our results (see rationale in 5.1.2, p. 143), share a core of 399 protein families. All core gene families are chromosomally encoded. The stepwise de- and increase of core and pan genome is illustrated below by Figure 21.

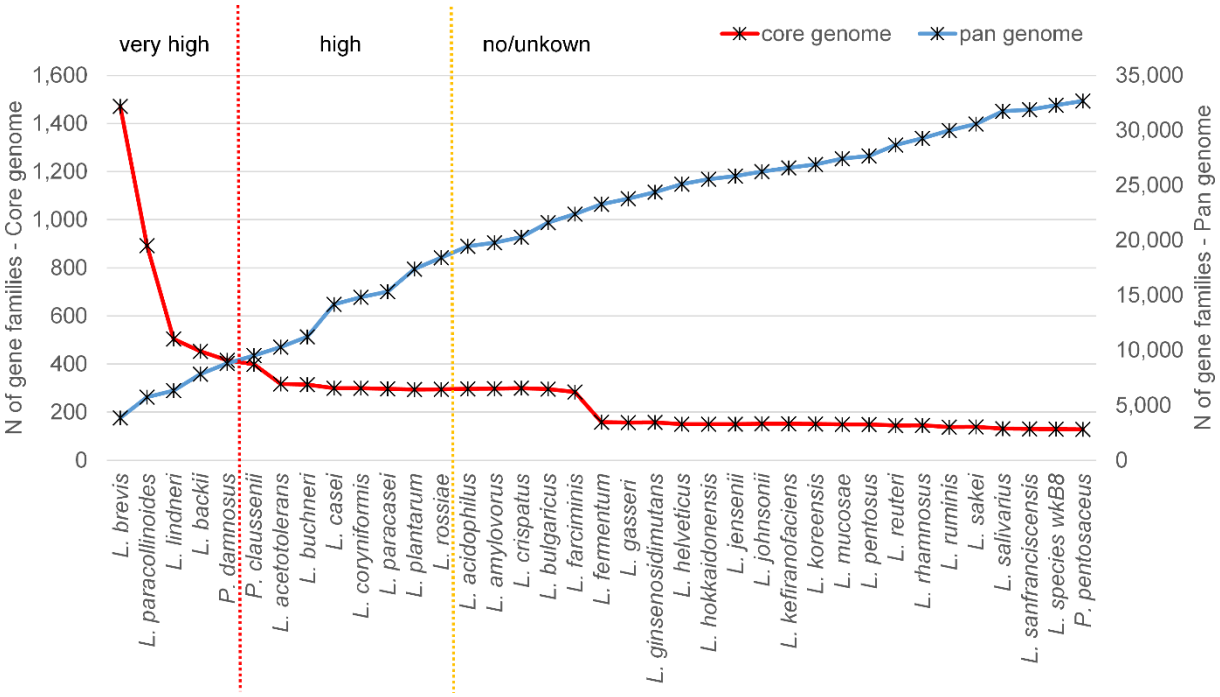


Figure 21: PanCore-Plot of 37 lactic acid bacteria species. Pan and core gene families were calculated with CMG-biotools, applying standard settings (BLASTp, 50 % identity and 50 % query/subject coverage). The hazard potential groups are indicated by dotted lines. Note that the graph starts with the core and pan genome of *L. brevis*. The pan genome increases with every genome added to the calculation, while the core genome decreases. Those values at the end of the graph do not illustrate the size of the core and pan genome of *P. pentosaceus,* but of all LAB genomes included. Distinct steps between two (expected) groups can generally be used to illustrate that everything before the step is characterized by a distinct group specific number of genes.

Both abovementioned core genomes do not contain any gene, which is specific or unique for the corresponding group, as any included gene was also found within other genomes. Further, we investigated if these core genomes are also encoded in total in any other LAB species, in order to evaluate if the whole sets of genes are specific for the corresponding hazard potential or not. The core genome of LAB with high to very high hazard potential was found to be covered by 90 to 100 % by strains/genomes without a documented hazard potential. It is completely encoded by *L. hokkaidonensis*, *L. koreensis*, *L. pentosus* and several *L. rhamnosus* strains. The same was found for the core genome of LAB with very high hazard potential and *P. claussenii*. Examples are *L. koreensis* and *L. pentosus*, while the latter species was once reported to be isolated from a home-brewed beer (Todorov and Dicks, 2004). LAB with high hazard potential were found to encode for 84 to 100 % of the very high hazard potential core genome. The corresponding value was 81 to 100 % in case of those species, which are considered to have no relevance. This observation did not change, when *P. claussenii* was excluded from the very high hazard potential group. The overall core genome of 37 LAB species contained 129 gene families. The shared gene content of LAB species with relevance for the brewery environment and a known hazard potential is not specific or unique for this group.

Figure 22 (p. 97) shows the SEED subsystem analysis of the abovementioned calculated core genomes. Most genes were assigned to the SEED categories protein metabolism, RNA metabolism, DNA metabolism and carbohydrate metabolism. Within these categories, we found mainly genes related to general metabolic and regulatory functions, e.g. protein biosynthesis, DNA repair or central carbon metabolism.

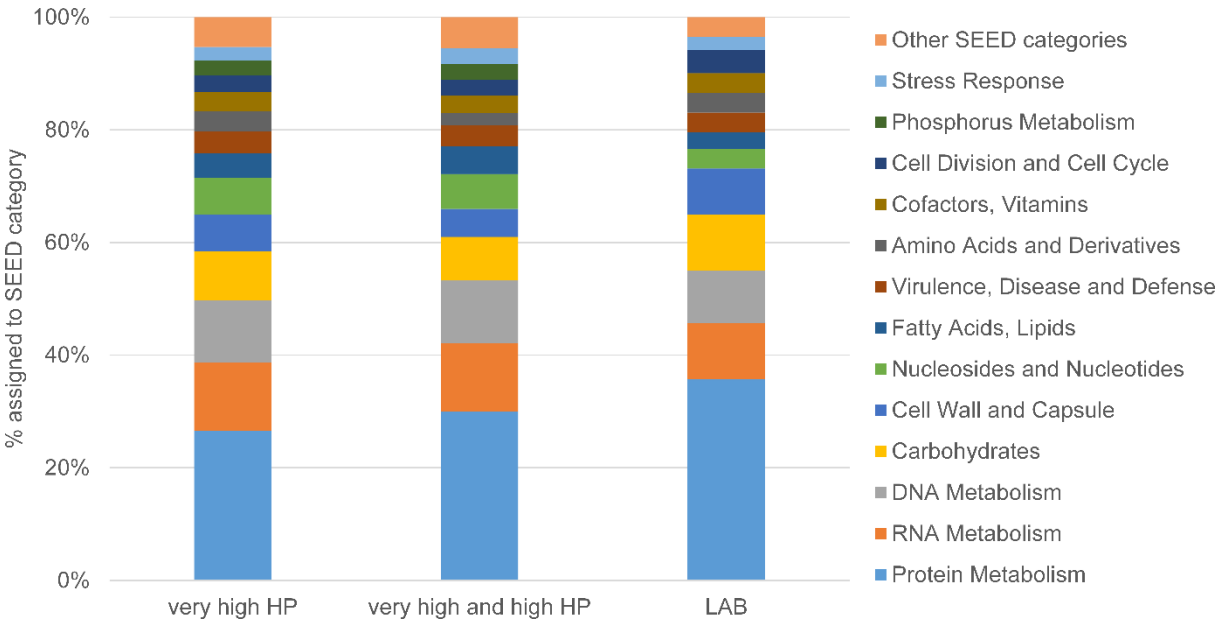


Figure 22: SEED subsystem analysis of core genomes of lactic acid bacteria with very high hazard potential, very high and high hazard potential and all included LAB genomes. Core genomes were calculated for all strains with very high hazard potential (HP), very high and high hazard potential and for all LAB genomes included in this study. The proportion of core genes assigned to SEED categories is shown. All categories with a proportion less than 2 % are summarized as “Other SEED categories.”

4.3.3.6 Sequence-based genome and proteome comparison

As no properties/traits/features were found to be exclusively present in all species/strains of interest, e.g. to those with very high hazard potential, sequence based comparison were conducted looking for any similarities defining the groups of interest. Comparisons were done with Gegenees (Agren et al., 2012) and BADGE (Behr et al., 2016) on DNA and protein level. As already stated, species with very high and/or high hazard potential did not cluster together based on chromosomal and whole genome comparison. This was found on DNA and protein level, while no chromosomal genes were found to be distinctly enriched within any of the predefined target groups. In contrast, a fragmented all-against-all comparison of all plasmidomes resulted in a distinct cluster of all brewery isolates with very high and high hazard potential (see Figure 23, p. 98). It is important to note that this cluster contains only brewery plasmidomes and not all plasmidomes of a particular hazard potential species. The cluster is the consequence of a shared pool of genes, which is not only based on shared functions or protein sequences, but is also based on highly similar DNA sequences, as outlined in the following chapters.

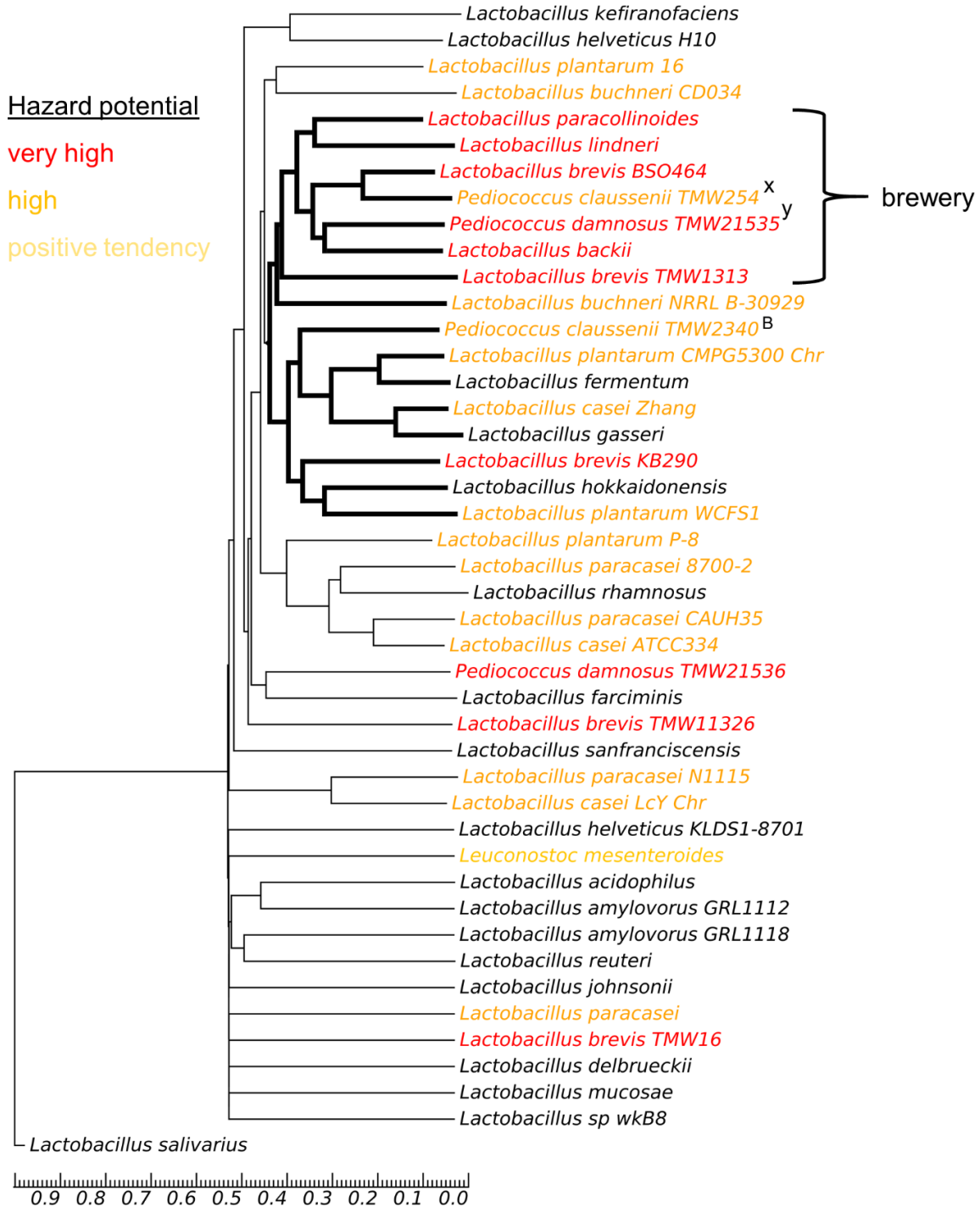


Figure 23: Phylogenomic tree of lactic acid bacteria plasmidomes, based on a fragmented all-against-all comparison (Agren et al., 2012). If all strains of a species clustered together, they were condensed to the respective species. Elsewhere strain designations were kept for all or for representative strains (see x and y). The bold subtree contains all brewery isolates, while the included upper subordinate subtree contains all brewery isolates, with the exception of *P. claussenii* TMW 2.340-MB. *L. buchneri* NRRL B30929 was isolated from an ethanol production plant. Note that all non-brewery strains of the high hazard potential species (*L. brevis* KB 290, TMW 1.1326-NB and TMW 1.6-WB, *P. damnosus* TMW 2.1536-NB) do cluster elsewhere based on their plasmid sequences. x = represents TMW 2.54-SB and TMW 2.53-SB, y = all brewery isolates of *P. damnosus*.

4.3.3.7 Comparative analysis of brewery plasmids

General properties

Brewery isolates where found to have up to ten plasmids, ranging in size from less than 5 kbp to 144 kbp. They comprise GC contents from 34.6 to 46.6 % and consequently do not show a consistent codon or amino acid usage. Nevertheless, based on the codon usage of their plasmidomes, most brewery isolates cluster together as illustrated by Figure 25 (p. 100). In total, we found 105 plasmids, while 19 pairs of nearly identical plasmids (95 % identity, 90 % coverage) were found, ending up with 86 individual plasmids. With three exceptions, all plasmid pairs were found within a single species. Within these species, plasmids were shared by a single pair of strains:

- *L. backii* TMW 1.1989-SB and TMW 1.2002-MB share all (seven) plasmids
- *P. damnosus* TMW 2.1532-NB and TMW 2.1534-NB share four plasmids
- *P. damnosus* TMW 2.1533-SB and TMW 2.1535-SB share two plasmids
- *P. claussenii* TMW 2.53-SB and TMW 2.54-SB share two plasmids
- *L. paracollinoides* TMW 1.1979-NB and TMW 1.1995-SB share one plasmid
- *L. paracollinoides* TMW 1.1995-SB and *P. claussenii* TMW 2.340-MB share two plasmids
- *L. brevis* BSO 464 and *P. claussenii* TMW 2.340-MB share one plasmid

The subcellular localization of the predicted proteins does not differ from the average of all other investigated plasmids, as illustrated by Figure 24 (p. 100).

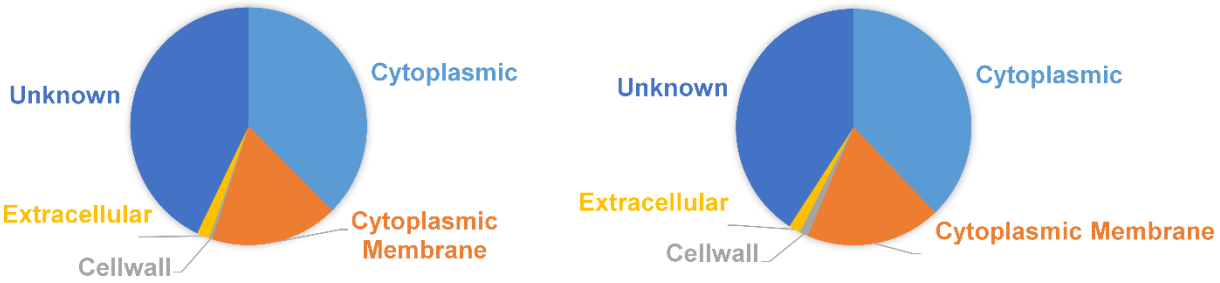


Figure 24: Subcellular localization of plasmidome proteins in percentage. All brewery isolate derived plasmids have been “pooled,” as well as all remaining plasmids, which do not represent a real entity. Distribution is shown for the brewery plasmidome and the “other” plasmidome. **Left** - brewery plasmidome, in detail → Cytoplasmic: 37.5 %; Cytoplasmic membrane: 17.5 %; Cell wall: 0.4 %; Extracellular: 1.8 %; Unknown: 42.8 %. **Right** - “other” plasmidome, in detail → Cytoplasmic: 37.8 %; Cytoplasmic membrane: 18.6 %; Cell wall: 1.2 %; Extracellular: 1.6 %; Unknown: 40.8 %.

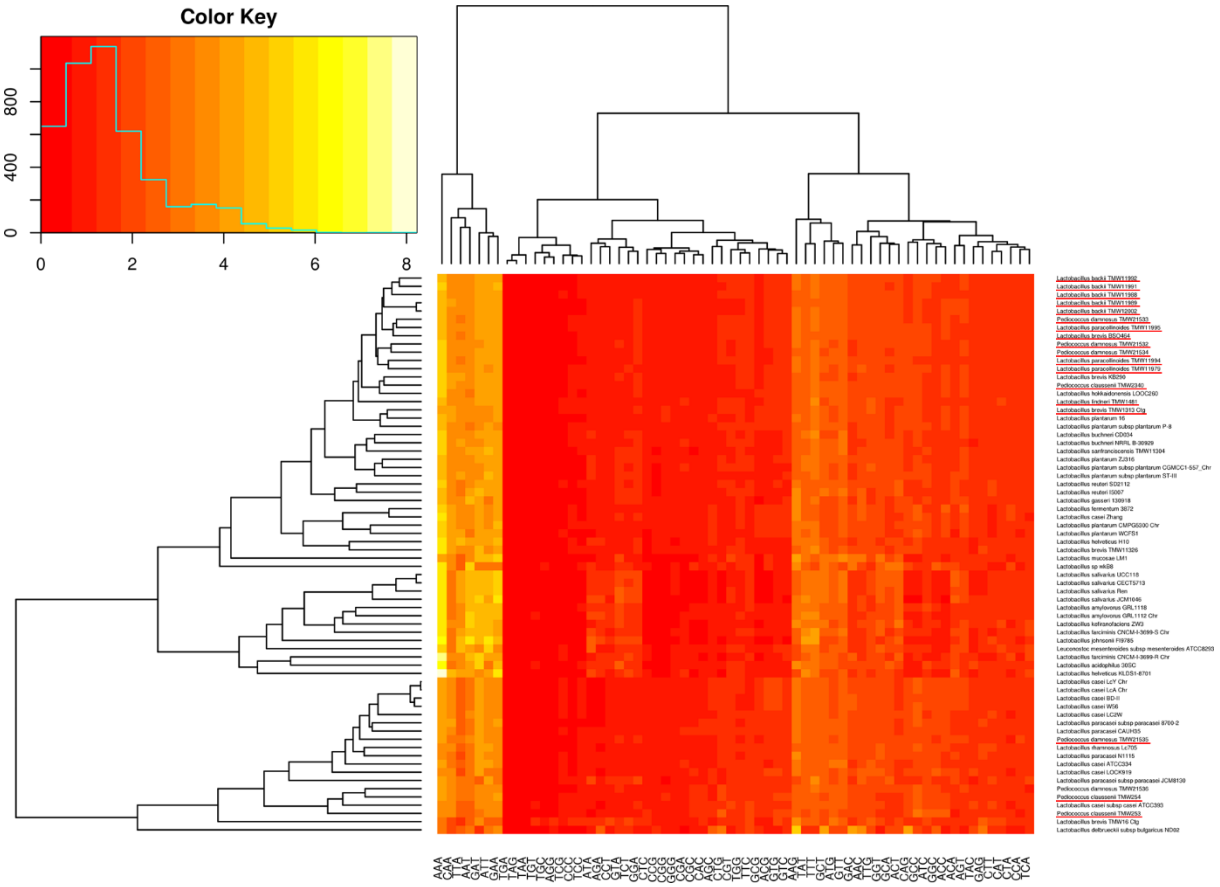


Figure 25: Codon usage of lactic acid bacteria plasmidomes - dendrogram and heatmap. The “color key” window shows the occurrence (y-axis) of all found proportions (%, x-axis). Brewery isolate derived plasmidomes are underlined in red.

Functional analysis - SEED subsystem analysis

The protein encoding genes of all brewery plasmidomes, as well as all plasmid-derived scaffolds of TMW 1.313-SB were “pooled” and analysed as a whole. The same was done for all other plasmids, although they are not derived from the same source. The SEED subsystem analysis, manually reworked, gave the following global distributions:

- Brewery plasmids: 23 % subsystem coverage, 35 % hypothetical proteins, 15 % mobile element proteins, which were not assigned to the corresponding SEED category, and 27 % other not classified genes
- Other plasmids: 21 % subsystem coverage, 42 % hypothetical proteins, 12 % mobile element proteins, which were not assigned to the corresponding SEED category, and 25 % other not classified genes

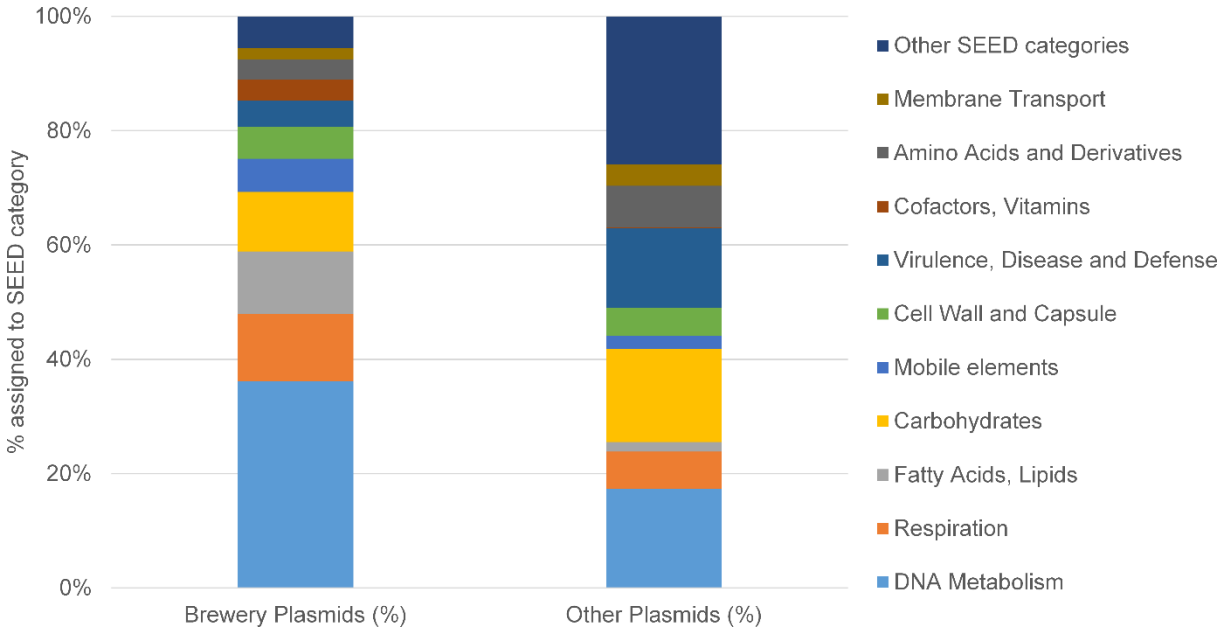


Figure 26: SEED subsystem analysis of brewery and non-brewery plasmidomes. The proportion of genes assigned to SEED categories is shown. All categories with a proportion less than 2 % within the brewery plasmid group are summarized as “Other SEED categories.” Note that the group “Other plasmids” contains sequences of various sources and does not represent a uniform entity. Further, it is important to note that the high proportion of genes assigned to the category “Respiration” is mainly the consequence of a systematic SEED annotation/subsystem-analysis error. Within the brewery plasmids, 76 of 87 assignments to this category are based on a consistent misclassification of a plasmid replication associated gene as an anaerobic respiratory reductase by RAST.

Figure 26 (p. 101) shows the distribution of SEED categories within the brewery plasmids, as well as the corresponding values for the other plasmids for comparison. Detailed data and comparison of the particular plasmids within the single species can be found within Supplementary section 2 (10, p. 239), in the corresponding species chapters.

Enriched categories were broken down with respect to those subsystems making up the majority within these categories. Their proportion of the respective category and their distribution within the core-species` plasmidomes are illustrated by Table 19 (p. 103). The relevance of single subsystems differs, while some subsystems were found to be present in several species. Besides the high abundance of subsystems belonging to the category DNA metabolism, the subsystem teichoic and lipoteichoic acids biosynthesis were found to be most prevalent.

Table 19: Distribution of SEED subsystems within brewery plasmidomes. SEED categories are shown in descending order, according to their overall proportion within brewery plasmidomes. Subsystems making up the majority within these categories are listed, together with their proportion within the respective category. Brewery plasmidomes are colored yellow. For comparison, non-brewery plasmidomes of the core-species are shown as well. Note that this hit-map shows the presence (+) and absence (-) of genes assigned to particular SEED subsystem. The corresponding genes are not necessarily identical sequences. v = presence indicated *in vitro* (PCR). Beer spoilage potential (BSP) is shown, where available: SB = strong BSP, MB = middle BSP, WB = weak BSP, NB = no BSP.

Category	Subsystem	% of category	*L. backii* TMW 1.1988-SB	*L. backii* TMW 1.1991-SB	*L. backii* TMW 1.1992-SB	*L. backii* TMW 1.1989-SB	*L. backii* TMW 1.2002-MB	*P. damnosus* TMW 2.1533-SB	*P. damnosus* TMW 2.1535-SB	*P. damnosus* TMW 2.1532-NB	*P. damnosus* TMW 2.1534-NB	*P. claussenii* TMW 2.53-SB	*P. claussenii* TMW 2.54-SB	*P. claussenii* TMW 2.340-MB	*L. brevis* BSO464	*L. brevis* TMW 1.313-SB	*L. paracollinoides* TMW 1.1994-SB	*L. paracollinoides* TMW 1.1995-SB	*L. paracollinoides* TMW 1.1997-NB	*L. lindneri* TMW 1.481-MB	*P. damnosus* TMW 2.1536-NB	*L. brevis* KB290	*L. brevis* TMW 1.1326-NB	*L. brevis* TMW 1.6-WB
DNA metabolism	Type I restriction-modification	84	+	+	+	+	+	+	+	-	-	v	+	+	+	-	+	+	+	-	-	+	-	-
	DNA topoisomerases, Type I, ATP-independent	16	+	+	+	+	+	+	+	+	+	v	+	+	+	-	+	+	+	-	-	+	-	-
Respiration	Anaerobic reductases - ferredoxin reductase	13	+	+	+	-	-	-	-	-	-	+	+	-	+	-	-	+	-	-	+	+	+	-
Fatty acids, Lipids	Fatty acid biosynthesis FASII - complete cluster	100	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrates	Maltose uptake and utilization	23	+	-	-	-	-	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
	Citrate/Malate metabolism, transport, and regulation	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-
	Alpha-acetolactate operon - acetoin / butanediol	13	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-
	Dihydroxyacetone kinases	12	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	D-ribose utilization	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Mobile elements	Phage proteins	100	+	-	+	-	-	+	+	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-
Cell wall and capsule	Teichoic and lipoteichoic acids biosynthesis	46	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	-	-	-	-
	Murein hydrolases / Peptidoglycan recycling	19	-	-	-	-	-	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-
	Sortase	17	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Virulence, disease and defense	Copper homeostasis	59	+	-	+	+	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-
	Cobalt-zinc-cadmium resistance	21	+	-	+	+	+	-	+	-	-	v	+	-	-	-	-	-	-	-	-	+	-	-
	Arsenic resistance	24	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Cofactors, Vitamins	Biotin biosynthesis / utilization	100	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-
Amino acids and derivatives	Glu, Gln, Asp and Asn biosynthesis	50	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-
	Agmatine deiminase pathway	32	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
	Creatine and creatinine degradation	13	-	-	-	-	-	-	-	-	-	v	+	+	-	-	-	-	-	-	-	-	-	-
	Serine biosynthesis	5	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
Membrane transport[1] / Potassium metabolism[2]	Copper transport system[1]	73	+	-	+	+	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-
	Magnesium transport system[1]	27	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-
	Potassium homeostasis/transport system[2]	100	+	-	+	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-

BADGE analysis - homologous DNA

As the SEED subsystem analysis only covers 23 % of all plasmid genes, we investigated the distribution of brewery plasmid genes, their occurrence in non-brewery isolates and if they are part of genetic clusters, based on sequence similarity. Therefore, we used BADGE, looking specifically for highly similar, homologous, shared DNA. Threshold values for homologous genes were set to 90 % sequence identity and 90 % subject-to-query/query-to-subject coverage.

Of 3,381 brewery plasmid-encoded genes, 589 were found to be present in at least two brewery plasmidomes. 543 of them are shared by those 19 (nearly) identical plasmid pairs mentioned above, while only 25 % of these genes were found exclusively within these plasmids. The other 75 % are encoded in at least one more brewery plasmidome, showing that the shared gene content of brewery plasmidomes is not only based on the presence of identical plasmids. In order to compare the degree of shared information within a species and between species, a plasmid gene pool was defined for each species and each pair of species. A gene was considered part of the genetic pool, if it occurred twice within a species, or at least twice within a pair of species (at least one strain of each species). Table 20 (p. 105) illustrates that the gene pool within a species is always the largest. Further, it shows that especially *L. backii* and *P. damnosus* share a high amount of plasmid-encoded genetic information. While 41 % of all shared genes are only present within a single species, the rest was found in at least two species, including 17 genes with occurrence in all six core-species-brewery-plasmidomes (see Figure 27, p. 105). 27 % (160 genes) of all shared genes were also found within the analysed non-brewery plasmids, thus being not brewery-specific. Note that these other plasmids include those of non-brewery *L. brevis* and *P. damnosus* strains, as well as 164 additional plasmids comprising 19 other LAB species. These encode in total for more than 5,400 genes.

Figure 28 (p. 106) illustrates that the shared genetic pool consists of highly similar genes, while those genes, which were also found within the other plasmidomes, have a higher similarity within the brewery plasmidomes compared to their equivalents within the other plasmidomes.

Table 20: Brewery plasmidomes - the number of shared plasmid-encoded genes between species. Species were summarized, omitting non-brewery strains, which were added to all other non-brewery plasmidomes. Only those 589 genes are considered, which were found to be encoded by at least two different brewery strains/plasmidomes. A gene was further considered part of the gene pool, if it occurred at least twice within a species, or at least twice within a pair of species. Note that a gene being part of a gene pool does not have to be absent within another species or the other plasmids. This figure is not about exclusively shared genes. Threshold values for homologous genes were set to 90 % sequence identity and 90 % subject-to-query/query-to-subject coverage.

	L. backii	*P. damnosus*	*L. paracollinoides*	*P. claussenii*	*L. brevis*	*L. lindneri*	Other plasmids
L. backii	379	177	116	103	97	24	54
P. damnosus	177	300	87	83	72	25	110
L. paracollinoides	116	87	249	91	88	27	120
P. claussenii	103	83	91	183	70	21	98
L. brevis	97	72	88	70	165	23	97
L. lindneri	24	25	27	21	23	36	23

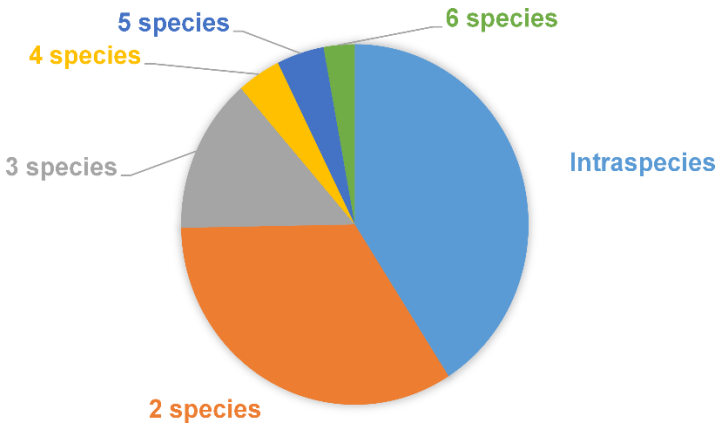


Figure 27: Brewery plasmidomes - the proportion of shared plasmid-encoded genes between species. The proportion of genes shared within and between two to six core-species. The same genes were considered as stated in Table 20. In detail → Intraspecies: 41 %; two species: 34 %; three species: 14 %; four species: 4 %; five species: 4 %; six species: 3 %. Threshold values for homologous genes were set to 90 % sequence identity and 90 % subject-to-query/query-to-subject coverage.

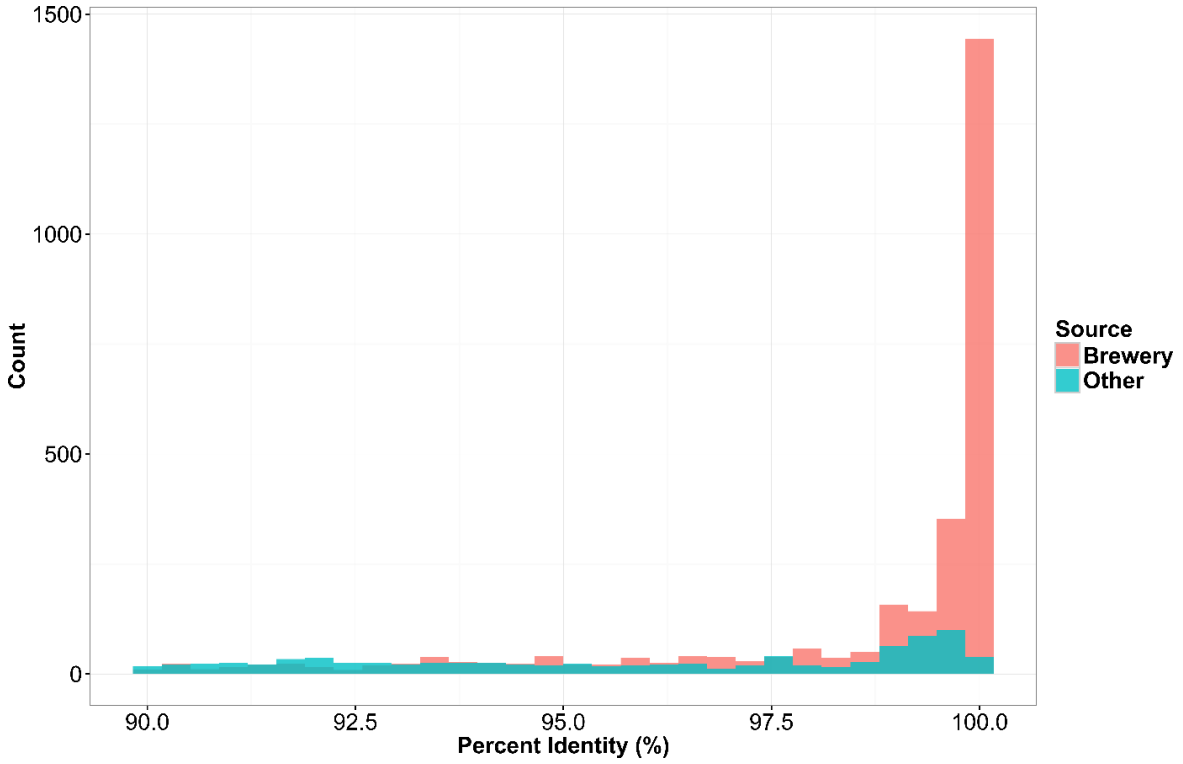


Figure 28: Brewery plasmidomes - sequence similarity within brewery plasmid gene pool. The histogram shows the total count of BLAST hits versus sequence similarity (percent identity) of these matches. We found 589 genes shared by at least two brewery plasmidomes. The BADGE settings for the identification of these 589 genes were set to 90 % sequence identity and 90 % subject-to-query/query-to-subject coverage. All 589 genes were blasted against all brewery plasmids and against all non-brewery plasmids. The red histogram shows the similarity of these 589 shared genes within the brewery plasmidomes, from minimum 90 % (Identity threshold) to 100 %. The blue histogram shows the sequence similarities of those 160 genes, which were also identified in non-brewery plasmids, but those similarities to non-brewery plasmid genes.

We also found that the amount of shared genes between beer-spoiling strains of two different species partially exceeds the shared gene content within a species, compared to strains lacking beer spoilage ability within the species. For example, both beer-spoiling strains of *P. damnosus* share 84 +/- 10 genes with the beer-spoiling *L. backii* strains, 61 +/- 5 with the non-spoiling brewer isolates and only 18 +/-2 with winery isolate *P. damnosus* TMW 2.1536-NB. The shared gene content on strain level is illustrated by Figure 29 and Table 21 (p. 108). Most genes with high prevalence within the brewery plasmidomes were found to be partially or exclusively encoded within contiguous pieces of DNA, while Table 22 (p. 109) shows the distribution of these genetic clusters. *HorA* and *horC*, as well as their corresponding genetic clusters, have the highest prevalence within the investigated brewery plasmidomes. In case of *horC*, we found two general cluster variants, while the truncated variant consists of *horC* and *horB* only. Further, we identified 16 additional clusters, some of them found within various species and others restricted to single species or groups.

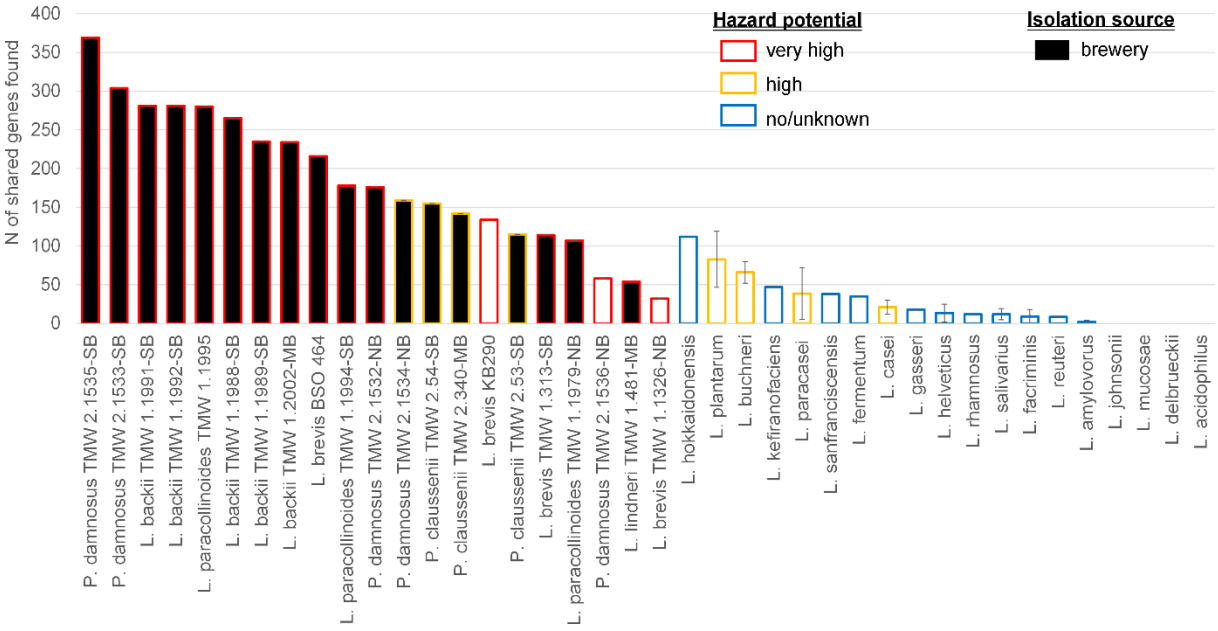


Figure 29: Brewery plasmidomes - the number of genes shared with the shared gene pool. The number of shared genes is shown for all single strains of the core-species. Only those 589 genes are considered, which are encoded by at least two different brewery plasmidomes. Other species were summarized, represented by an average value and standard deviation. The hazard potential and the isolation source are indicated. Beer spoilage potential (BSP) is shown, where available: strong BSP (SB), middle BSP (MB), weak BSP (WB), no BSP (NB). Threshold values for homologous genes were set to 90 % sequence identity and 90 % subject-to-query/query-to-subject coverage.

	L. backii TMW 1.1988-SB	*L. backii* TMW 1.1991-SB	*L. backii* TMW 1.1992-SB	*L. backii* TMW 1.1989-SB	*L. backii* TMW 1.12002-MB	*P. damnosus* TMW 2.1533-SB	*P. damnosus* TMW 2.1535-SB	*P. damnosus* TMW 2.1532-NB	*P. damnosus* TMW 2.1534-NB	*P. damnosus* TMW 2.1536-NB	*P. claussenii* TMW 2.53-SB	*P. claussenii* TMW 2.54-SB	*P. claussenii* TMW 2.340-MB	*L. paracollinoides* TMW 1.1994-SB	*L. paracollinoides* TMW 1.1995-SB	*L. paracollinoides* TMW 1.1997-NB	*L. brevis* BSO464	*L. brevis* TMW 1.313-SB	*L. brevis* KB290	*L. brevis* TMW 1.1326-NB	*L. lindneri* TMW 1.481-MB
L. backii TMW 1.1988-SB		121	118	77	78	88	91	35	36	27	27	33	29	42	48	22	53	13	37	11	17
L. backii TMW 1.1991-SB	121		130	70	73	104	85	42	41	27	34	45	44	55	61	34	66	16	51	13	21
L. backii TMW 1.1992-SB	118	130		77	79	85	86	36	36	25	33	40	49	49	61	26	54	15	48	13	16
L. backii TMW 1.1989-SB	77	70	77		159	67	82	34	35	15	26	29	29	30	46	19	55	16	36	11	14
L. backii TMW 1.2002-MB	78	73	79	159		68	82	34	35	15	25	29	29	31	47	20	58	16	37	11	16
P. damnosus TMW 2.1533-SB	88	104	85	67	68		152	66	56	20	29	37	43	48	54	23	55	13	49	12	23
P. damnosus TMW 2.1535-SB	91	85	86	82	82	152		65	56	16	29	37	24	43	49	21	53	14	35	10	19
P. damnosus TMW 2.1532-NB	35	42	36	34	34	66	65		104	10	12	21	24	25	30	13	28	7	19	8	15
P. damnosus TMW 2.1534-NB	36	41	36	35	35	56	56	104		10	14	23	24	25	32	13	30	6	18	8	15
P. damnosus TMW 2.1536-NB	27	27	25	15	15	20	16	10	10		8	10	11	14	16	12	17	5	35	5	9
P. claussenii TMW 2.53-SB	27	34	33	26	25	29	29	12	14	8		74	4	10	36	9	28	6	10	9	5
P. claussenii TMW 2.54-SB	33	45	40	29	29	37	37	21	23	10	74		36	19	42	16	47	12	16	10	10
P. claussenii TMW 2.340-MB	29	44	49	29	29	43	24	24	24	11	4	36		35	45	24	41	14	37	9	18
L. paracollinoides TMW 1.1994-SB	42	55	49	30	31	48	43	25	25	14	10	19	35		73	45	44	11	35	10	24
L. paracollinoides TMW 1.1995-SB	48	61	61	46	47	54	49	30	32	16	36	42	45	73		65	64	19	39	14	19
L. paracollinoides TMW 1.1997-NB	22	34	26	19	20	23	21	13	13	12	9	16	24	45	65		33	10	27	8	12
L. brevis BSO464	53	66	54	55	58	55	53	28	30	17	28	47	41	44	64	33		28	49	15	22
L. brevis TMW 1.313-SB	13	16	15	16	16	13	14	7	6	5	6	12	14	11	19	10	28		15	3	4
L. brevis KB290	37	51	48	36	37	49	35	19	18	35	10	16	37	35	39	27	49	15		15	13
L. brevis TMW 1.1326-NB	11	13	13	11	11	12	10	8	8	5	9	10	9	10	14	8	15	3	15		6
L. lindneri TMW 1.481-MB	17	21	16	14	16	23	19	15	15	9	5	10	18	24	19	12	22	4	13	6	

Table 21: Brewery plasmidomes - the number of shared plasmid-encoded genes between strains. Note that only strains of core-species are shown. Further, note that in case of TMW 1.313-SB a draft genome was used. Brewery plasmidomes are colored yellow. The red box indicates the fact that both beer-spoiling strains of *P. damnosus* share more genes with the beer-spoiling *L. backii* strains, as with the non-spoiling strains of the same species. Threshold values for homologous genes were set to 90 % sequence identity and 90 % subject-to-query/query-to-subject coverage. Beer spoilage potential (BSP) is shown, where available: SB = strong BSP, MB = middle BSP, WB = weak BSP, NB = no BSP.

Table 22: Brewery plasmidomes - distribution of genetic clusters within brewery plasmidomes. Brewery plasmidomes are colored yellow. For comparison, non-brewery plasmidomes of core-species are shown. Brewery-specific DNA : + = no high identity hits (50 % query/subject coverage, 75 % identity) in nr/nt BLAST database with exception of brewery isolates. Beer spoilage potential (BSP) is shown, where available: SB = strong BSP, MB = middle BSP, WB = weak BSP, NB = no BSP. + = present & - = absent → with respect to genes assigned to these subsystems. v = presence indicated *in vitro* (PCR), p = partially present.

Cluster	DMG	Function	brewery specific DNA	*L. backii* TMW 1.1988-SB	*L. backii* TMW 1.1991-SB	*L. backii* TMW 1.1992-SB	*L. backii* TMW 1.1989-SB	*L. backii* TMW 1.2002-MB	*P. damnosus* TMW 2.1533-SB	*P. damnosus* TMW 2.1535-SB	*P. damnosus* TMW 2.1532-NB	*P. damnosus* TMW 2.1534-NB	*P. claussenii* TMW 2.53-SB	*P. claussenii* TMW 2.54-SB	*P. claussenii* TMW 2.340-MB	*L. brevis* BSO464	*L. brevis* TMW 1.313-SB	*L. paracollinoides* TMW 1.1994-SB	*L. paracollinoides* TMW 1.1995-SB	*L. paracollinoides* TMW 1.1997-NB	*L. lindneri* TMW 1.481-MB	*P. damnosus* TMW 2.1536-NB	*L. brevis* KB290	*L. brevis* TMW 1.1326-NB	*L. brevis* TMW 1.6-WB
FAS-Cluster	M05 (*fabZ*)	Type II fatty acid biosynthesis	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
horA-Cluster	*horA* / ORF5	Hop export / cell envelope modification	-	+	+	-	-	-	+	+	+	+	v	+	+	+	-	+	-	-	+	-	-	-	-
horC-Cluster-1	*horB* / *horC*	Hop export / cell envelope modification	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	-	+	-	-	-	-	-	-
horC-Cluster-2	*horB* / *horC*	Truncated version - *horB* / *horC* only	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
hitA-Cluster	*hitA*	Mn^{2+} / Mg^{2+} transport	+	-	-	-	-	v	+	+	-	-	-	-	-	+	+	-	v	-	-	-	-	-	-
Cluster 1		Acetoin / butanediol metabolism	+	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-
Cluster 2		Peptidoglycan recycling / metal ABC transport	+	-	-	-	-	-	+	+	+	+	-	-	-	p	-	-	-	-	-	-	-	-	-
Cluster 3		Sugar utilization / DNA modification	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Cluster 4	M09	Maltose utilization	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cluster 5	M28	Unknown	+	+	+	+	+	+	+	+	-	-	+	+	-	+	-	p	p	-	-	-	-	-	-
Cluster 6	M03	Metal ABC transport	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cluster 7		Unknown	+	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cluster 8		Biotin utilization / cyclopropane FA synthesis	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Cluster 9		Conjugation	-	-	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-
Cluster 10	M42	Various (Lipase / NADH peroxidase)	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-
Cluster 11		Unknown (Enolase)	+	+	+	+	-	-	+	+	-	-	+	+	-	+	-	-	+	-	-	-	-	-	-
Cluster 12		Agmatine deiminase pathway	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
Cluster 13		Sugar transporter	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Cluster 14	M22 / M23	Maltose utilization	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
Cluster 15		Biotin utilization	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4.3.4 The type II fatty acid biosynthesis cluster

Partial results of this chapter have been published (Behr et al., 2016).

A plasmid-encoded and highly conserved fatty acid biosynthesis (FAS) cluster was identified using BADGE, while one gene of this cluster was also tested as DMG (M05 - *fabZ*, see 4.4, p. 115). The plasmid-encoded cluster was found to be present in all five genomes of *L. backii*, as well as in both beer-spoiling strains of *P. damnosus*. In addition, all sequenced strains of both species were found to have an incomplete chromosomally encoded type II FAS, which consists of a small cluster coding for *accABCD* and spread genes for *acpP*, *fabG*, *fabH* as well as *fabD* in case of *P. damnosus*. In contrast, the other core-species genomes included a chromosomally-encoded complete FAS.

Figure 30 (p. 111) illustrates the localization of the FAS cluster on *P. damnosus* plasmids and the lack of it in case of the three NB strains. Based on this *in silico* prediction, all three genome sequenced NB strains of *P. damnosus* are not able to produce long-chain fatty acids, as they lack the enzymes for chain elongation FabF, FabZ and FabI. While there are some structural differences within the seven evaluated FAS clusters, the gene content and the respective DNA sequences are highly conserved. Figure 31 (p. 112) shows the organization of the FAS cluster for *P. damnosus* TMW 2.1535-SB and TMW 2.1533-SB, as well as *L. backii* TMW 1.2002-MB and TMW 1.1988-SB. The FAS cluster is flanked by two putative transposases, while the IS2 sequence is identical in all strains and the ISL3 sequence is different in TMW 1.2002-MB. There are two genes within the cluster encoding for FabZ, while they are only homologous and have different DNA sequences.

Figure 32 (p. 113) shows a dendrogram of selected brewery LAB plasmids based on a phylogenomic analysis. As in case of the *horC* and the *horA* clusters, the FAS cluster is found on different plasmids, which are not necessarily identical.

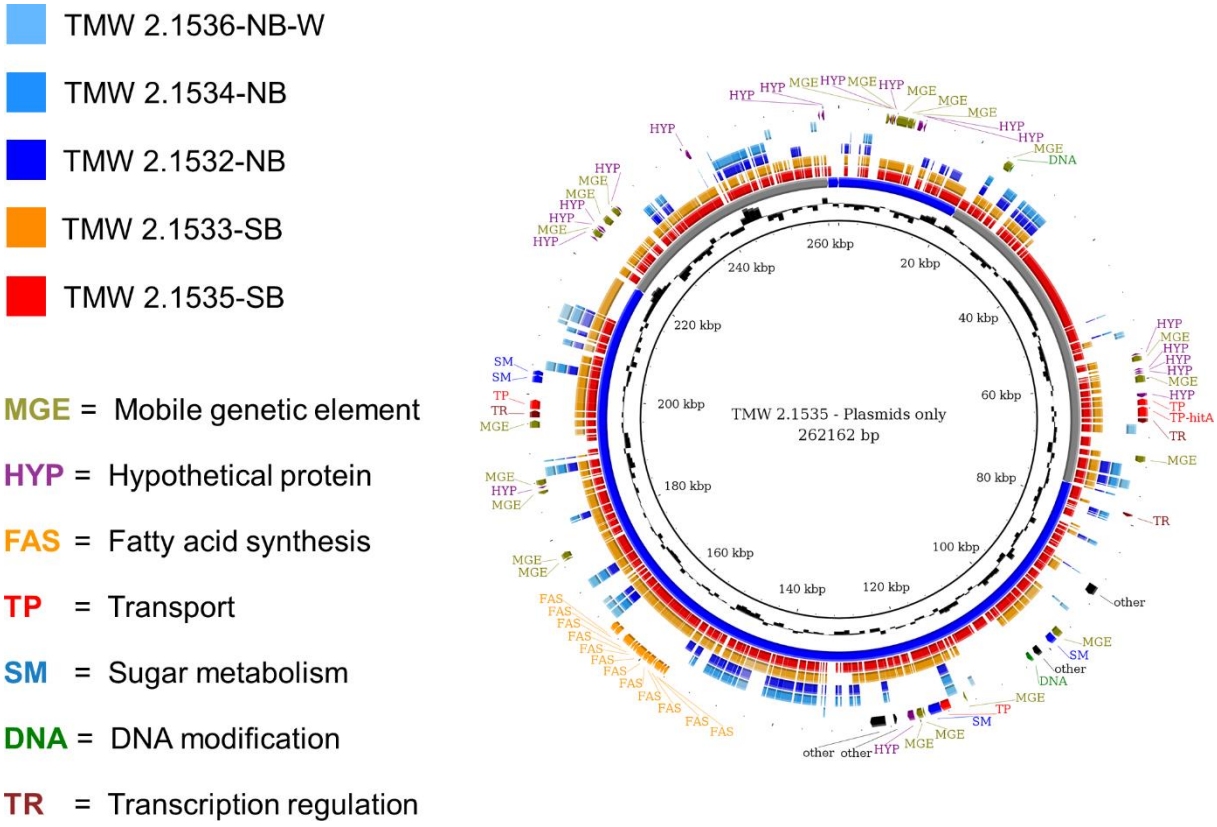


Figure 30: BLAST ring image of all *P. damnosus* plasmids. All rings are described from the inside to the outside: ring 1 (black) represents the total plasmid sequence of TMW 2.1535-SB as reference with bp coordinates; ring 2 (black) shows the GC content of TMW 2.1535-SB; ring 3 consists of arcs with different lengths and alternating coloration (grey/blue) representing the different plasmids of TMW 2.1535-SB; ring 4 (red) shows all BLAST hits of TMW 2.1536-SB ORFs versus its own plasmids illustrating the coding density; ring 5 (orange) shows all BLAST hits of TMW 2.1533-SB ORFs versus the reference; rings 6-8 (blue shades) show all BLAST hits of the NB strains ORFs versus the reference, ring 9 contains all DMGs identified by BADGE mapped to the reference, located in the gaps of the rings 6-8. The identified DMGs include a type II FAS cluster. SB = strong beer spoilage potential (BSP), NB = no BSP.

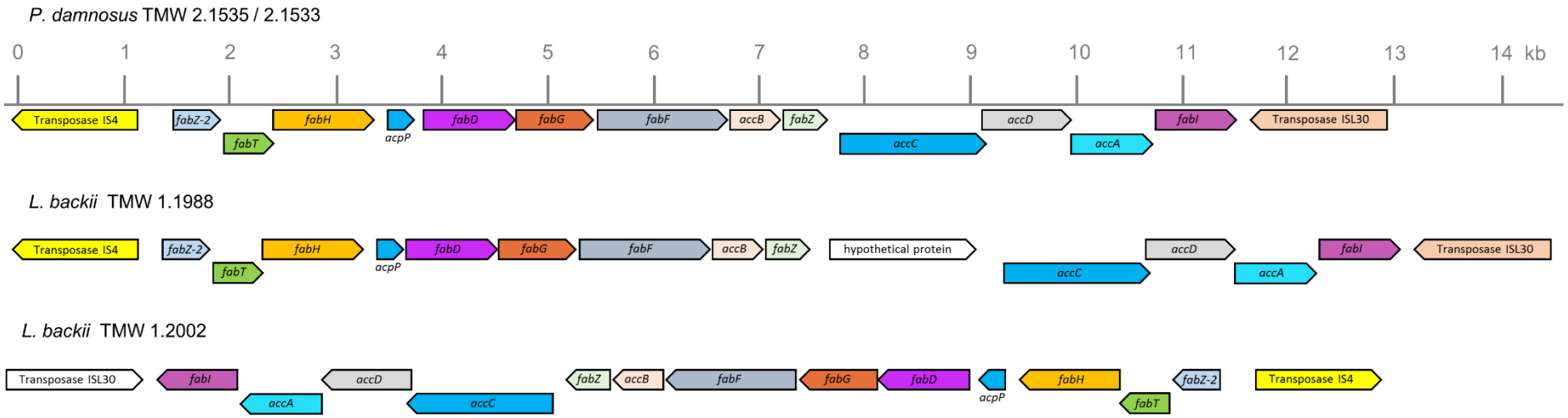


Figure 31: Organization of the identified fatty acid biosynthesis (FAS) cluster. The plasmid-encoded FAS cluster is illustrated for four strains. While the organization is slightly different, the gene content and the sequence is highly conserved. All genes labeled in the same colors are at least 99 % identical with 99 % coverage (to each other). White color indicate that there is no corresponding sequence in the other clusters shown. The IS4 family transposase is identical in all cases, while the ISL30 transposases are different in some cases, with respect to DNA sequence. Note that there are two *fabZ* genes, while the one targeted as diagnostic marker gene is labeled as *fabZ*. The acyl carrier protein (ACP), encoded by *acpP*, caries all intermediates of the FAS. Initiation of FAS is catalysed by the acetyl-CoA carboxylase, consisting of four proteins encoded by *accABCD*. The products of *fabD* and *fabH* are necessary to transfer the resulting malonyl group to ACP and to form the first intermediate of the FAS pathway. The elongation is catalysed by FabF, FabG, FabZ and FabI.

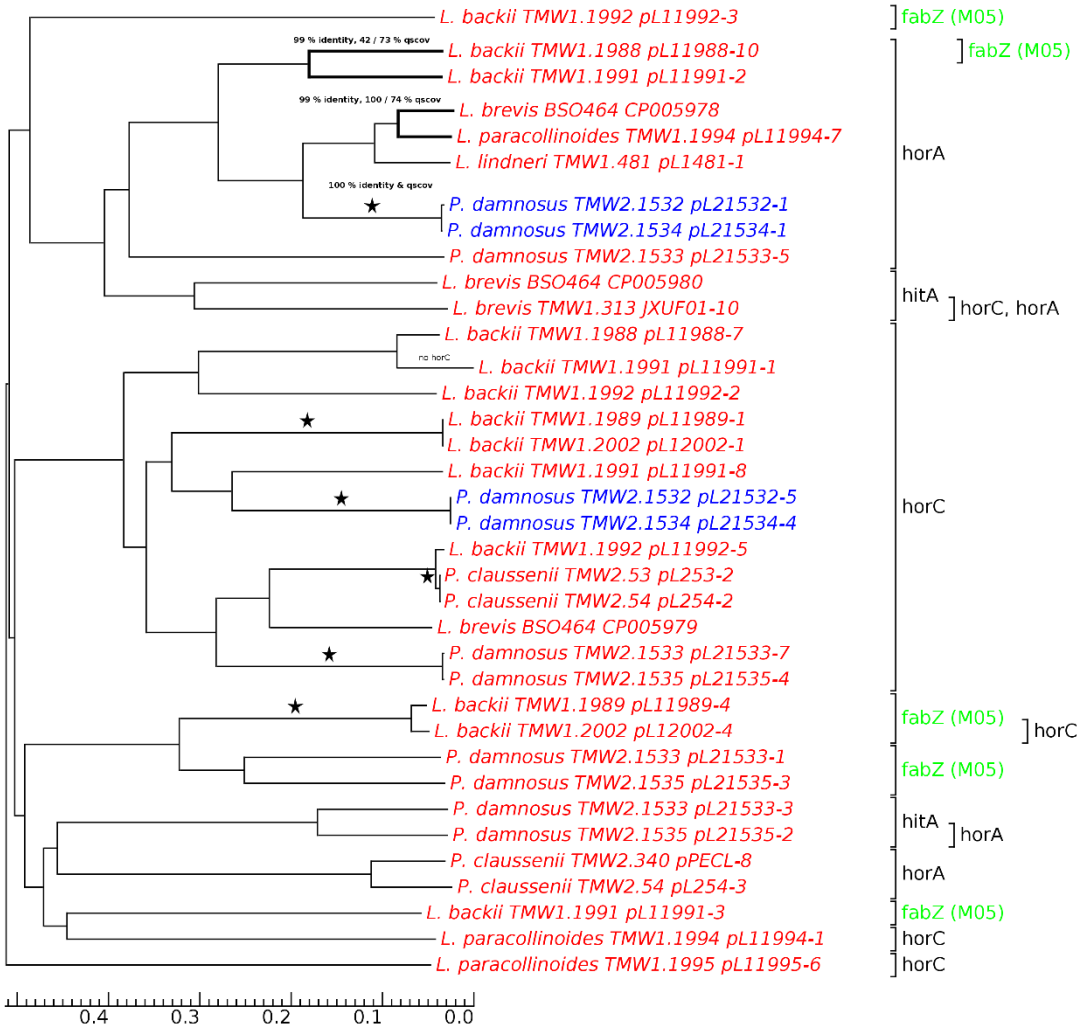


Figure 32: Dendrogram based on fragmented all-against-all comparison (phylogenomics) of a selection of brewery plasmids. The distribution of the FAS, *horA* and *horC* clusters in plasmid sequences is indicated. Only those plasmids are included, which carry either of the abovementioned clusters. The presence of the respective clusters is indicated by *fabZ*, *horA* and *horC*. A scale bar indicates the distance. (Almost) Identical plasmids (95 %) are labeled with a star. Plasmid labels of strains with beer spoilage ability are written in red, non-spoiler plasmids in blue.

4.3.5 The identification of diagnostic marker genes

In order to identify potential lifestyle genes by comparative genomics, a bioinformatics tool (BADGE) was designed and written for the fast and reliable identification of DMGs (Behr et al., 2016).

In case of *L. brevis,* the identified DMGs mainly corresponded to those identified in a previous study (Behr et al., 2015). 58 DMGs were identified present only in both SB strains and not in TMW 1.1326-NB and TMW 1.6-WB. The identification of species specific DMGs for *L. brevis* was not focused here, as promising DMGs for this species were already present from the abovementioned study (Behr et al., 2015). For *P. claussenii* the strain TMW 2.53-NB-SB, initially selected as weak spoiler, turned out to be genetically and physiological (beer spoilage potential) unstable, while the *variant* sequenced proved to be a strong spoiler. The same was true for *L. backii*, where all sequenced strains finally turned out to have beer spoilage ability. In both cases, no reliable extraction of potential discriminatory species-specific lifestyle genes was possible. In case of *L. lindneri,* two MB strains were sequenced, allowing no differentiation. For *L. paracollinoides,* 183 DMGs were identified, present in both SB strains and absent in TMW 1.1979-NB. BADGE comparison of three NB and two SB strains resulted in 66 DMGs specific for the strong spoiling strains of *P. damnosus* (Behr et al., 2016). DMGs were, with one exception, plasmid-encoded and mainly coding for hypothetical proteins, mobile genetic elements or part of a FAS cluster.

Species independent BADGE runs were conducted, while no novel DMGs with validity in all species were found. Those DMGs with relevance in two or more species are almost exclusively encoded on plasmids and often arranged in clusters. The results of these species independent comparisons were adduced for the selection of promising DMGs for PCR evaluation and validation. By comparing the identified DMGs in case of *P. damnosus* or *L. paracollinoides* to the genomes of obligate beer spoilers, such as *L. lindneri* or *L. backii*, the significance of the available DMGs could be estimated, supporting the selection process.

4.4 Evaluation and validation of diagnostic marker genes

Partial results of this chapter have been published (Behr et al., 2016).

In silico predicted DMGs for the differentiation of beer spoilage potential and beer spoilage ability groups of *L. brevis*, *L. lindneri*, *L. backii*, *L. paracollinoides*, *P. claussenii* and *P. damnosus* were evaluated and validated using PCR. Candidate genes, predicted by BADGE (see 4.3.5, p. 114), were selected based on function as well as on functional and spatial clustering. Within clusters, DMGs were selected by means of quality, while those genes were preferred with a maximum distinction from related DNA sequences. If possible, DMGs with relevance in more than one species were chosen. In addition, published and relevant information/data were adduced for the selection process. After DMG selection, sequence alignment and primer design, additional non-sequenced strains were tested for the presence of up to 45 DMGs using PCR (3.4.2, p. 39). In addition, strains were tested for published lifestyle genes *horA*, *horC*, *hitA*, *cinA* and *arsR,* for comparison. The potential value of the tested DMGs was assessed using various statistical measures, including Fisher´s p and several confusion-matrix-related parameters.

Nine novel, species independent DMGs were found to be of potential value. A combination of *horC,* M19 and M34 (BSA-MIX) was found to be able to detect 100 % of all beer spoilers within the six core-species (0 % false negative rate) in a species independent way, with a false positive rate of 77 %. For comparison, a combination of *horA* and *horC* resulted in the detection of 94 % of all beer spoilers, with 58 % false positive identifications of non-spoiling strains. Species-specific DMGs/DMG-combinations showed a higher discriminatory power with respect to beer spoilage ability. M05 (*fabZ*), encoding for an enzyme of a type II FAS cluster, allowed a 100 % correct discrimination of beer-spoiling and non-spoiling strains of *P. damnosus*. A more exact differentiation of beer spoilage potential groups was only possible for *L. paracollinoides* (cf. Table 38, p. 308). Table 23 shows quality metrics for nine novel DMGs with a potential value for the species independent identification of core-species LAB strains with beer spoilage ability. Published DMGs are shown for comparison.

Table 23: Species independent diagnostic marker genes (DMGs) - evaluation and validation. The relation of DMGs to beer spoilage ability was tested with Fisher´s exact test for significance (indicated in red, p-value < 0.05). True positive (TP), false positive (FP), true negative (TN) and false negative (FN) results were counted and used for the calculation of accuracy, precision, sensitivity, specifity and F-measure. Accuracy = TP+TN/(TP+FP+TN+FN) → total correct identifications, measure of correctness; precision = TP/(TP+FP) → a measure for the probability that the identification is correct; Sensitivity = TP/(TP+FN) → a measure for the proportion of positives that are correctly identified as such; Specifity = TN/(TN+FP) → a measure for the proportion of negatives that are correctly identified as such; F-measure = 2TP/(2TP+FP+FN) → harmonic mean of precision and sensitivity.

DMG	Accuracy	Precision	Sensitivity	Specifity	F-meas.	TP	FP	TN	FN	P-value
horC[a]	0.73	0.83	0.79	0.59	0.81	53	11	16	14	5.40E-04
horA[a]	0.69	0.80	0.76	0.52	0.78	51	13	14	16	1.37E-02
hitA[a]	0.42	0.84	0.24	0.88	0.37	16	3	23	51	2.56E-01
arsR[a]	0.35	1.00	0.10	1.00	0.19	7	0	26	60	1.84E-01
cinA[a]	0.36	1.00	0.10	1.00	0.19	7	0	27	60	1.87E-01
M05	0.54	0.96	0.37	0.96	0.54	25	1	25	42	7.32E-04
M03	0.67	0.88	0.63	0.77	0.73	42	6	20	25	9.92E-04
M15	0.57	0.94	0.43	0.92	0.59	29	2	24	38	1.12E-03
M37	0.65	0.84	0.63	0.69	0.72	42	8	18	25	1.00E-02
M28	0.57	0.86	0.48	0.81	0.62	32	5	21	35	1.74E-02
M43	0.67	0.80	0.72	0.54	0.76	48	12	14	19	2.97E-02
M42	0.58	0.83	0.52	0.73	0.64	35	7	19	32	3.68E-02
M19[b]	0.68	0.75	0.82	0.31	0.79	55	18	8	12	2.59E-01
M34[b]	0.60	0.75	0.67	0.42	0.71	45	15	11	22	4.71E-01

[a] = for comparison to novel DMGs [b] = not statistical significant discriminatory potential (no DMG), but useful as marker gene for the species independent identification of beer-spoiling LAB (e.g. *L. backii, L. lindneri, L. paracollinoides* and *P. claussenii*)

We could not identify and validate species independent DMGs with a significant correlation to beer spoilage potential. As we suggest treating contaminations with *L. backii, L. lindneri, L. paracollinoides* and *P. claussenii* as a general threat for the product (see rationale in discussion, 5.1.2, p. 143), the transposases M19 and M34 can be used to detect all strains of these species in a single.

PCR results for all relevant DMGs, as well as the corresponding DMG statistics (e.g. correlation to spoilage potential, etc.) for all six core-species, can be found in Supplementary section 3 (11, p. 301). Species-specific DMGs and DMG systems for the discrimination of beer-spoiling and non-spoiling strains of *L. brevis* and *P. damnosus* are detailed below and in Table 24 to Table 25 (p. 118). We also identified four novel DMGs, M21, M22, M37 and M38, for the differentiation of beer-spoiling and non-spoiling strains of *L. paracollinoides*, as illustrated by Table 38 (p. 308).

Promising species-specific DMGs and DMG combinations, for the discrimination of strains with and without beer spoilage ability, with high accuracy based on PCR evaluation and validation:

- *P. damnosus*: M05 **(and** *horA* **or** *horC***)** → 100 % accuracy (and sensitivity, etc.)
- *L. brevis*: *horC* **and/or** M37 → 90 % accuracy, 92 % precision, sensitivity and F-measure, 86 % specifity; TMW 1.302 displayed beer spoilage ability in beer-sets of three other breweries (see 4.2.5, p. 70) → considering TMW 1.302 as beer spoiler: the system improves to 95 % accuracy, 100 % precision and specifity, 93 % sensitivity, 96 % F-measure

Thirteen selected DMGs were also tested with seven additional high hazard potential species, while no or only limited accuracy and relevance were found. M05 (*fabZ*) was found in two strains of *L. paucivorans*, one isolated from the brewery environment (TMW 1.1424-WB) and one from a sausage swell (TMW 1.2063-NB). The strain from the brewery environment was additionally tested positive for *horA* and *horC*. Only TMW 1.1424-WB was able to grow in wheat beer.

Table 24: *Lactobacillus brevis* diagnostic marker genes (DMGs) - evaluation and validation. The relation of DMGs to beer spoilage ability was tested with Fisher´s exact test for significance (indicated in red, p-value < 0.05). True positive (TP), false positive (FP), true negative (TN) and false negative (FN) results were counted and used for the calculation of accuracy, precision, sensitivity, specifity and F-measure, which is the harmonic mean of precision and sensitivity (confusion matrix). For calculation/description of parameters, see Table 23 (p. 116).

DMG	Accuracy	Precision	Sensitivity	Specifity	F-meas.	TP	FP	TN	FN	P-value
***horC*[a]**	0.85	1.00	0.77	1.00	0.87	10	0	7	3	3.10E-03
***horA*[a]**	0.70	0.82	0.69	0.71	0.75	9	2	5	4	1.60E-01
***hitA*[a]**	0.74	1.00	0.62	1.00	0.76	8	0	6	5	1.81E-02
***arsR*[a]**	0.68	1.00	0.54	1.00	0.70	7	0	6	6	4.36E-02
***cinA*[a]**	0.70	1.00	0.54	1.00	0.70	7	0	7	6	4.45E-02
M02	0.68	1.00	0.54	1.00	0.70	7	0	6	6	4.36E-02
M15	0.68	1.00	0.54	1.00	0.70	7	0	6	6	4.36E-02
M15	0.74	0.90	0.69	0.83	0.78	9	1	5	4	5.73E-02
M37[b]	0.74	0.90	0.69	0.83	0.78	9	1	5	4	5.73E-02

[a] = for comparison to novel DMGs [b] = not statistical significant discriminatory potential, but useful in combination with *horC*

Table 25: *Pediococcus damnosus* diagnostic marker genes (DMGs) - evaluation and validation. The relation of DMGs to beer spoilage ability was tested with Fisher´s exact test for significance (indicated in red, p-value < 0.05). True positive (TP), false positive (FP), true negative (TN) and false negative (FN) results were counted and used for the calculation of accuracy, precision, sensitivity, specifity and F-measure, which is the harmonic mean of precision and sensitivity (confusion matrix). For calculation/description of parameters, see Table 23 (p. 116).

DMG	Accuracy	Precision	Sensitivity	Specifity	F-meas.	TP	FP	TN	FN	P-value
***horC*[a]**	0.6	0.4	0.9	0.4	0.6	6	8	5	1	3.54E-01
***horA*[a]**	0.6	0.5	1.0	0.4	0.6	7	8	5	0	1.14E-01
***hitA*[a]**	0.7	0.6	0.4	0.8	0.5	3	2	11	4	2.90E-01
M05	1.0	1.0	1.0	1.0	1.0	7	0	13	0	1.29E-05
M03	0.9	0.8	1.0	0.8	0.9	7	2	11	0	4.64E-04
M42	0.9	0.8	1.0	0.8	0.9	7	2	11	0	4.64E-04
M18	0.9	1.0	0.6	1.0	0.7	4	0	13	3	7.22E-03
M01	0.8	0.8	0.6	0.9	0.7	4	1	12	3	3.07E-02
M02	0.8	0.8	0.6	0.9	0.7	4	1	12	3	3.07E-02

[a] = for comparison to novel DMGs [b] = not statistical significant discriminatory potential, but useful in combination with *horC*

4.4.1 FabZ, fatty acids and spoilage potential of *P. damnosus*

Results of this chapter were previously published (Behr et al., 2016).

The effect of additional fatty acids on the growth behaviour of five *P. damnosus* strains in lager beer 1 was investigated (Table 26). Only TMW 2.1535, *fabZ*-positive and characterized by a strong beer spoilage potential, was able to grow without an additional fatty acid source. *FabZ*-negative brewery isolates, positive for at least one published hop tolerance gene, were found to grow only in lager beer 1 with Tween.

Table 26: Growth of *P. damnosus* in lager beer 1 with and without Tween addition. Five strains were checked for growth in lager beer 1 with and without the addition of Tween, applying a modified beer spoilage test (3.2.6.5, p. 35). Only the *fabZ* positive TMW 2.1535-SB was able to grow in lager beer 1 without Tween. W = winery environment. Strong beer spoilage potential (BSP) = SB, no BSP = NB. *fabZ* (M05) PCR positive = +, PCR negative = -; growth in lager beer 1 and lager beer 1 + Tween = +, no growth = -.

Strain/TMW	BSP	*horA*	*horC*	*hitA*	*fabZ*	Lager beer 1	+ Tween
2.1535	SB	+	+	+	+	+	+
2.1532	NB	+	+	-	-	-	+
2.125	NB	+	-	+	-	-	+
2.1639	NB	-	+	-	-	-	+
2.1643-W	NB	-	-	-	-	-	-

The fatty acid composition of TMW 2.1532-NB and TMW 2.1535-SB was investigated upon growth in lager$_{pH5.0,}$ as all strains of *P. damnosus* were able to grow in this beer variant. In addition, the fatty acid composition of TMW 2.1535-SB after growth in lager$_{pH4.3}$ was determined. Samples were taken from beers after visible growth occurred, followed by freeze-drying of samples and analysis of cellular fatty acids by the DSMZ (Braunschweig, Germany). A comparison of the fatty acid composition of TMW 2.1532-NB and TMW 2.1535-SB in lager$_{pH5.0}$ revealed a higher ratio (0.6 > 0.5) of unsaturated fatty acids to saturated fatty acids in case of the strong spoiling strain (Figure 33, p. 120). In addition, the average chain length of TMW 2.1535-SB was higher, resulting from more C17 to C20 fatty acids. Further, the percentage share of cyclopropyl fatty acids was higher in case of TMW 2.1535-SB. In lager pH 4.3, the proportion of UFAs increased in case of TMW 2.1535-SB (from 0.6 to 1.0), mainly conditioned by a quadruplication of oleic acid (C18:1). TMW 2.1535-SB carries a plasmid-encoded complete type II FAS cluster, while TMW 2.1532-NB has an incomplete FAS. Appendix 11 (p. 351) shows the detailed proportion of fatty acids identified for all three setups.

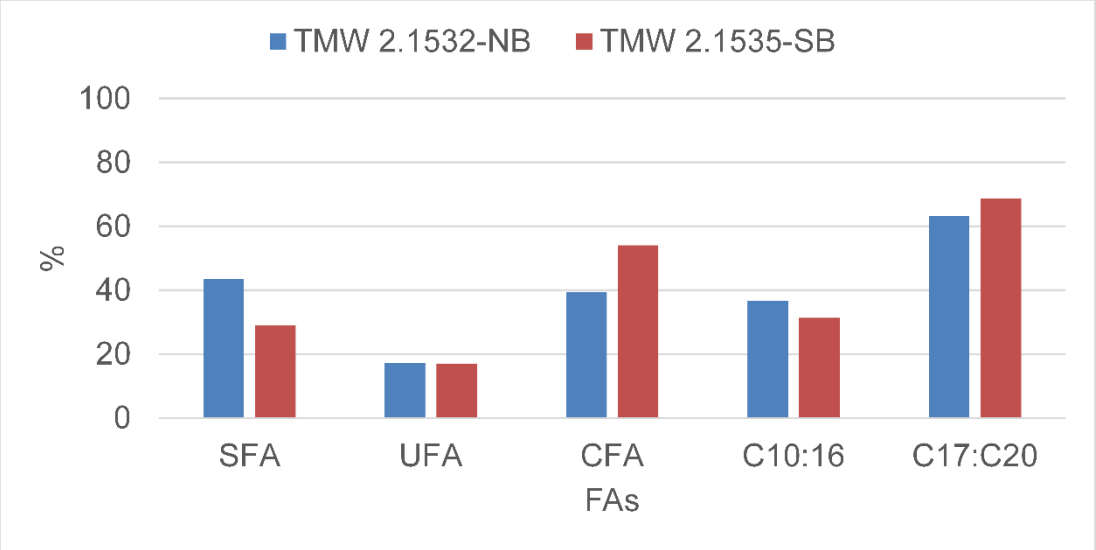


Figure 33: Summarized cellular fatty acid composition of *P. damnosus* strains after growth in beer. Two strains, with (TMW 2.1535-SB) and without (TMW 2.1532-NB) a plasmid-encoded type II FAS cluster, were analysed for their total fatty acid composition after growth in lager$_{pH5.0}$. Results are summarized. SFA = saturated fatty acids, UFA = unsaturated fatty acids, CFA = cyclopropyl fatty acids, C10 to C20 = chain length of fatty acids. Strong beer spoilage potential (BSP) = SB, no BSP = NB.

We attempted to investigate the fatty acid composition of more SB and NB strains. The first comparison also showed the presence of long-chain fatty acids in the non-spoiling strain TMW 2.1532. This was unexpected, as this strain lacks the FAS cluster. Thus, it was hypothesized that the carry-over of fatty acids from mMRS$_1$ to lager$_{pH5.0}$ was responsible for this effect. Consequently, we changed the protocol for the inoculation of lager$_{pH5.0}$, washing cells in a 50 mM potassium-phosphate buffer at pH 5.0, followed by a resting period in the same buffer for 24 h at 25° C and inoculation of lager$_{pH5.0}$ with 2 % of this washed cell suspension. This did not affect the growth behaviour of the strong spoiling strains in lager$_{pH5.0}$, while the non-spoiling strains did either not grow at all or only to very small amounts and with very long adaption times (> 30 days). Consequently, even at a bigger scale, we were not able to obtain a sufficient amount of NB-cells, grown in beer, for fatty acid analysis. Note that NB strains were capable of growing in lager$_{pH5.0}$ when inoculated with an unwashed mMRS$_1$ preculture, or washed and supplied with Tween, or washed and supplied with supernatant of the fermented mMRS$_1$ preculture.

To further demonstrate the relevance of the FAS cluster for *P. damnosus* growth, we checked the growth behaviour of washed cells of NB and SB strains in lager$_{pH5.0}$ and mMRS$_1$, with and without Tween. Growth was only affected in case of the NB strains. This illustrated by an example: The non-spoiling strain TMW 2.1536 showed an OD$_{590}$ of 3.89 after 7 days in mMRS$_1$ with Tween and an OD$_{590}$ of 0.067 without Tween, initially inoculated to an OD$_{590}$ of 0.050.

For comparison, TMW 2.1535-SB was characterized by OD values of 4.33 with and 4.61 without Tween. The same effect was observed in the case of $lager_{pH5.0}$ and the other investigated strains.

4.4.2 Genetic instability of beer-spoiling lactic acid bacteria and its connection to an unstable spoilage potential

It is known that hop resistance and the ability to spoil beer can get lost, which is connected to the loss of know lifestyle genes such as *horA* and *horC* (Suzuki et al., 2006). Some strains tested showed a variable and unstable DMG profile as well as an unstable beer spoilage potential. In addition, there were cases, where a discrepancy of *in silico* (genome sequences) and *in vitro* (DMG PCR) was observed for investigated DMGs. Both was the case for TMW 2.53 (NB to SB), which was tested for kinetics of instability and the significance of this phenomenon for beer spoilage potential. This was achieved by repeated subcultivation in $mMRS_1$ and $lager_{pH5.0}$, accompanied by an evaluation of offspring (plating) with respect to DMGs (PCR) and beer spoilage potential (beer spoilage test). The workflow of this experiment is illustrated by Figure 34 (p. 122).

HorC was characterized by higher stability, especially in $lager_{pH5.0}$, while repeated subcultivation in $lager_{pH5.0}$ resulted in higher stability of both DMGs tested. M13 stability was lesser in both setups, while the proportion of variants loosing M13 was higher when grown in $mMRS_1$. Only variants containing both or at least one DMG were able to grow in pilsner beer 1. By mixing a pasteurized offspring variant with both DMGs with a vital variant lacking *horC* and M13, the ability to grow in pilsner beer 1 could be restored. In another example, it was shown that the loss of *fabZ* in case of *P. damnosus* TMW 2.1636 correlates with the loss of beer spoilage ability, illustrating the importance of *fabZ* and the instability of DMGs.

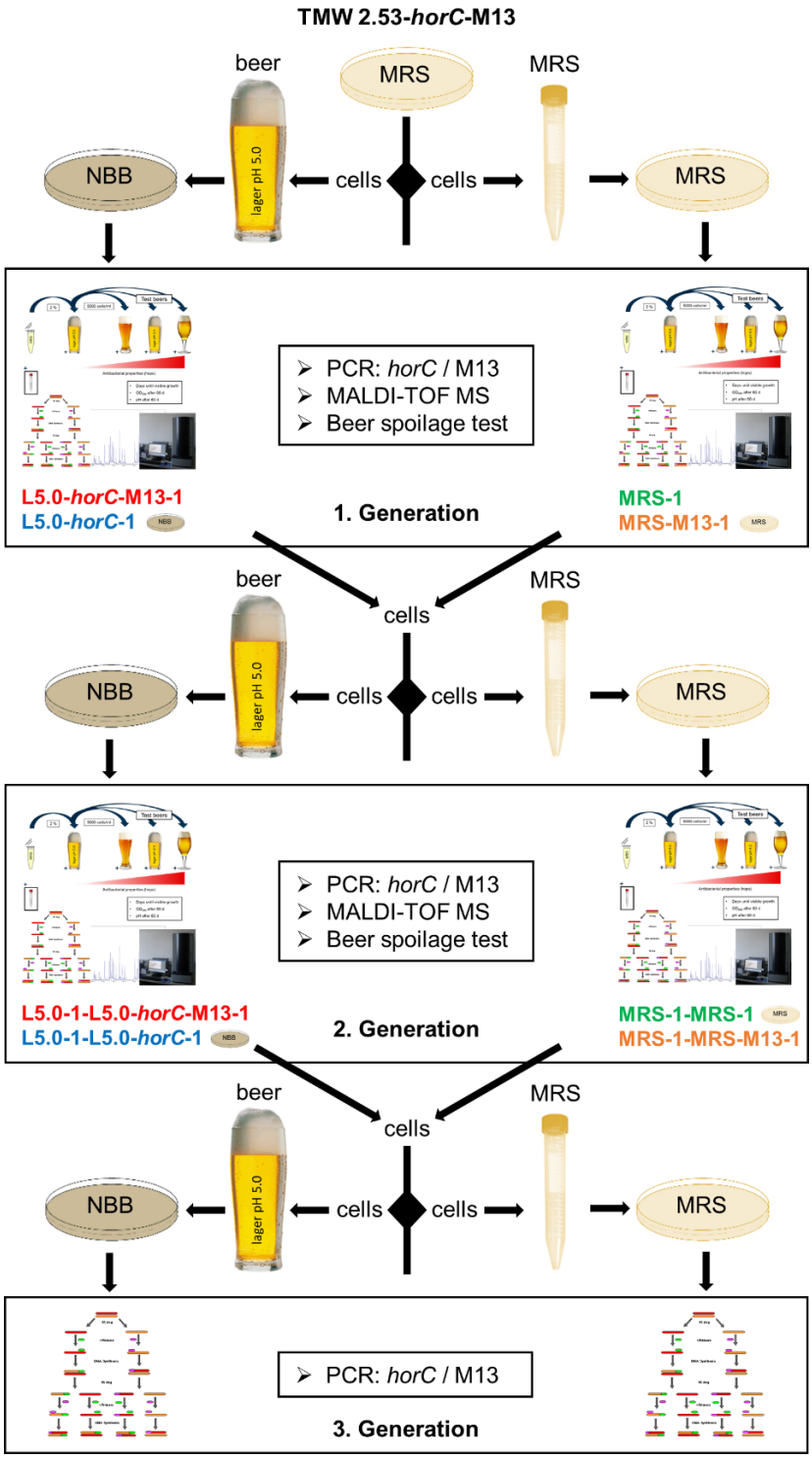


Figure 34: Genetic instability of *P. claussenii* TMW 2.53 - experimental design. Cells of TMW 2.53, tested positive for *horC* and M13, were used to inoculate lager$_{pH5.0}$ and mMRS$_1$ pH 6.2, followed by plating on NBB-Agar and mMRS$_1$ plates. Resulting colonies (offspring) were tested for presence of *horC* and M13 by PCR and for beer spoilage potential. MALDI-TOF MS spectra were recorded. A selection of offspring (see color code/naming for lineage) of the first generation, including all four possible genetic variants, was subjected to the same treatment as described above, resulting in a second generation. A third generation was generated accordingly, while offspring was only checked for genetic variants using PCR. Cells of given variants were not mixed. Arrows just indicate the general workflow.

Genetic instability was tested using PCR (3.4.2, p. 39), targeting *horC* and M13, found on pL254-2/pL253-2 and pL254-3, respectively. TMW 2.54 and TMW 2.53, isolated from the same brewery, have a chromosomal average nucleotide identity (ANI) of 100 % and share two plasmids (pL254-1/pL253-1; pL254-2/pL253-2). Plasmid 2 contains the *horC* cluster, while pL254-3 (no homolog in sequenced genome of TMW 2.53) contains M13 and the *horA* cluster. TMW 2.53 was tested positive for M13/*horA* in various, but not all cases before this experiment. The beer spoilage potential of a selection of offspring, comprising all genetic variants generated, was investigated applying the beer spoilage test (3.2.6.5, p. 35). In addition, it was checked if potential genetic variants could be differentiated using MALDI-TOF MS (3.3 p. 37) in order to enable a larger screening to improve statistics. The presence/absence neither of *horC* nor of M13 were trackable using MALDI-TOF MS.

TMW 2.53 cells, used for the initial inoculation of this test, were positive for *horC, horA* and M13 (*in vitro ≠ in silico*). By repeated subcultivation, although in different proportions depending on origin and propagation medium, genetic variants were generated lacking both, one or none of the tested DMGs. In total M13 was more unstable than *horC* and both DMGs were more stable when TMW 2.53 was grown in $lager_{pH5.0}$. In average, when both DMGs were present before propagation, *horC* was present in 100 % of the $lager_{pH5.0}$ offspring and 83 % of the MRS offspring, while M13 got lost in 16 and 41 % of all cases investigated, respectively. Repeated propagation in $lager_{pH5.0}$ resulted in higher genetic stability in case of both genes tested. TMW 2.53-MRS-1, lacking both DMGs, did not regain one or both DMGs nor the ability to grow in pilsner beer 1 spontaneously (in generation 2 or 3). Figure 35 (p. 124) illustrates the genetic (in)stability observed within the propagation experiment. Note that no statistics are shown for the lineage of TMW 2.53-MRS-1, as all 60 offspring tested were negative for both DMGs.

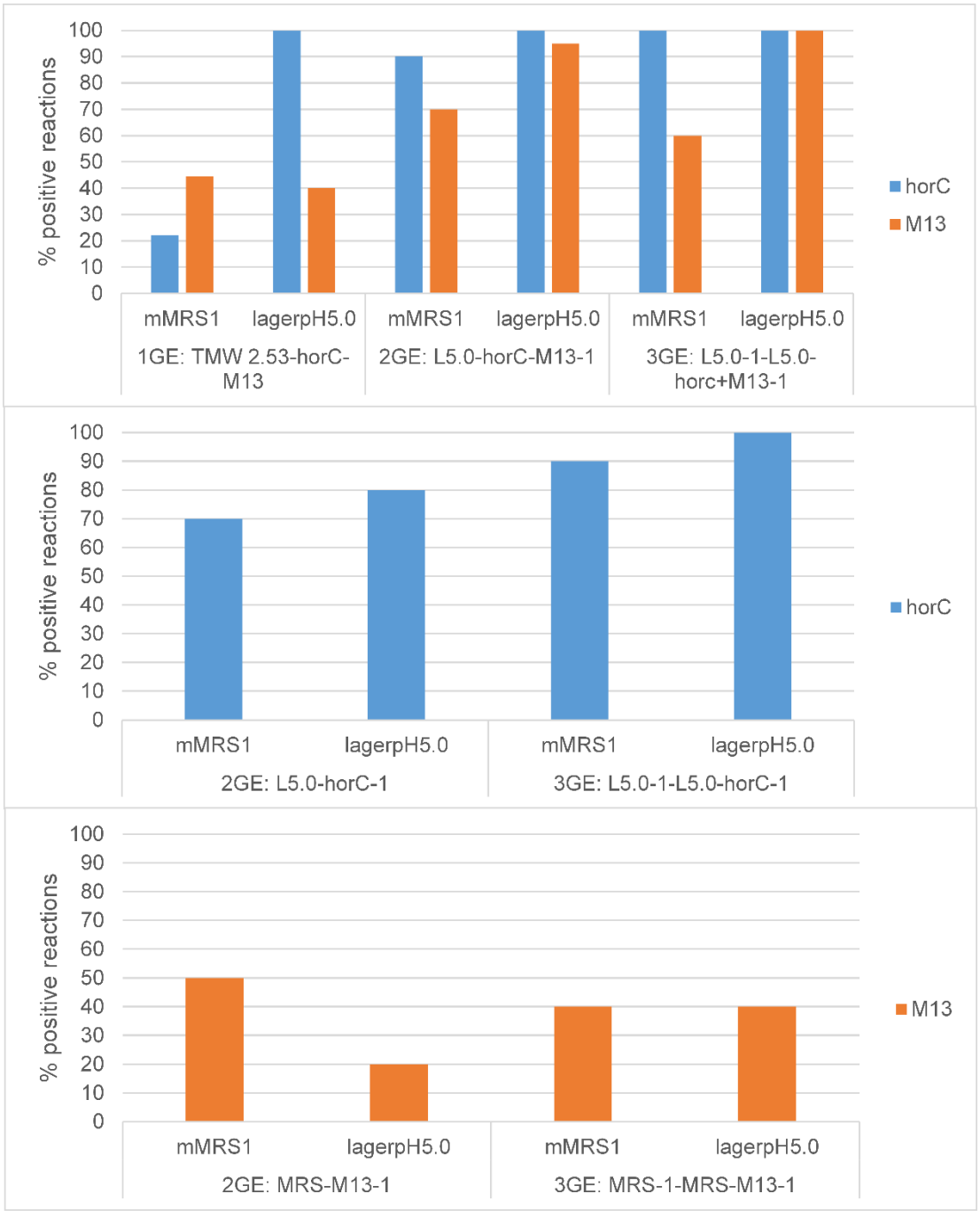


Figure 35: Genetic instability of *P. claussenii* TMW 2.53 - stability statistics. Colonies (offspring) of different generations (1GE - 3GE), descending from defined genetic variants (e.g. (TMW 2.53) L5.0-horC-M13-1, positive for both), were tested for the presence of *horC* and/or M13 by PCR. The proportion of positive reactions is shown. In general 10 colonies of each setup ($lager_{pH5.0}$/$mMRS_1$) were tested, with exception of the offspring of L5.0-horC-M13-1 and MRS-1 (not shown), where 40 colonies were checked with PCR. Note that the designations of the GE-variants used for propagation also describes their lineage.

The beer spoilage potential of the genetic variants is illustrated by Figure 36. The presence of *horC* correlated with faster adaption to lager beer 1 and a higher turbidity. Only variants with both or at least one DMG were able to grow in pilsner beer. Results are also summarized by Table 27 (p. 126).

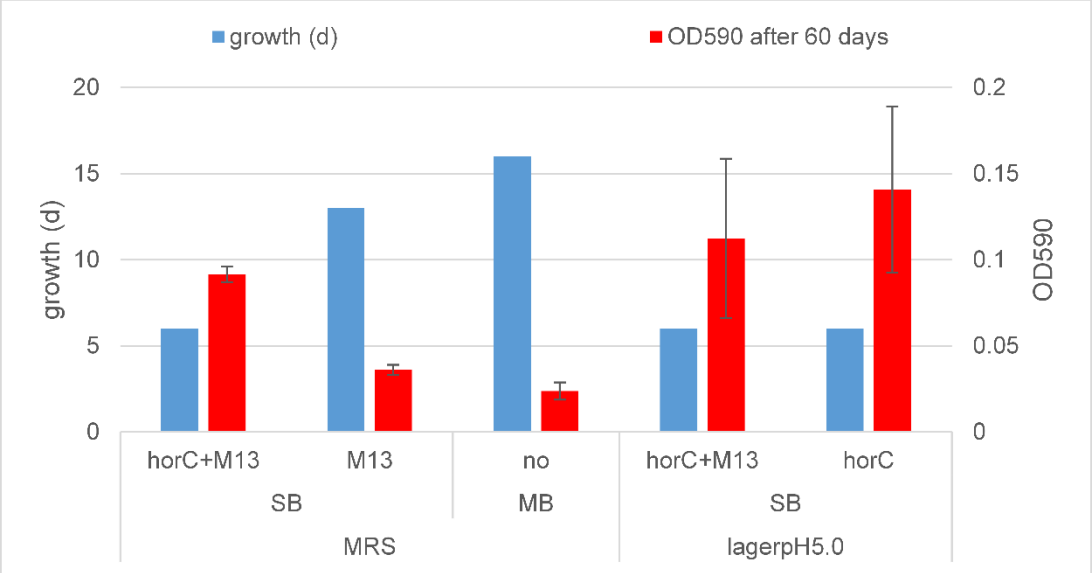


Figure 36: Growth of *P. claussenii* TMW 2.53 genetic variants in lager beer 1. Results are summarized for genetic variants, originating from the same setup ($lager_{pH5.0}$/$mMRS_1$). In addition to beer spoilage potential (BSP) group, the genetic variant is indicated as well as the media where the variants originated. Adaption (growth (days)) and turbidity (OD_{590}) are shown. The lack of *horC* has a distinct effect on both parameters. Strong BSP = SB, middle BSP = MB.

It was also tested, if the addition of *horC*/M13 containing DNA of TMW 2.54-SB, or of pasteurized cells of a *horC*/M13 variant of TMW 2.53, could restore the ability to grow in pilsner beer 1. TMW 2.53-MRS-1, lacking *horC* and M13, was only able to grow in lager beer 1 (MB), while the addition of pasteurized cells of TMW 2.53-L5.0-*horC*-M13 enabled TMW 2.53-MRS-1 to grow in pilsner beer. The addition of DNA had no such effect. Sterility controls of pasteurized cells of TMW 2.53-L5.0-horC-M13 showed no growth in any of the test beers, in $lager_{pH5.0}$ and $mMRS_1$ pH 6.2.

Table 27: Beer spoilage potential and genetic variants of *P. claussenii* TMW 2.53. The generation, the medium (setup) where the corresponding origin was incubated and the names of the resulting variants (offspring) are given. The color code, applied to the variants, illustrates their lineage. The offspring of the second generation is colored as the corresponding origin. In case of TMW 2.53-MRS-1-MRS-1, the test was conducted with three technical replicates (1-3), with and without the addition of pasteurized TMW 2.53-L5.0-*horC*-M13-1 cells. Replicate 1 was also incubated with 30 µg of genomic DNA of TMW 2.54 (*horC*, M13 positive). Strong beer spoilage potential (BSP) = SB, middle BSP = MB, weak BSP = WB. NA = not available.

Generation / setup / variant			Growth i. lager beer 1 (d)	OD_{590} i. lager beer 1	horC	M13
1. Generation	MRS	horC-M13-1-SB	6	0.087	+	+
		horC-M13-2-SB	6	0.096	+	+
		M13-1-SB	13	0.039	-	+
		M13-2-SB	13	0.033	-	+
		1-MB	16	0.028	-	-
		2-MB	16	0.017	-	-
		3-MB	16	0.029	-	-
		4-MB	16	0.021	-	-
		5-MB	16	NA	-	-
	L5.0	horC-M13-1-SB	6	0.101	+	+
		horC-M13-2-SB	6	0.065	+	+
		horC-M13-3-SB	6	0.095	+	+
		horC-M13-4-SB	6	0.189	+	+
		horC-1-SB	6	0.098	+	-
		horC-2-SB	6	0.098	+	-
		horC-3-SB	6	0.198	+	-
		horC-4-SB	6	0.108	+	-
		horC-5-SB	6	0.201	+	-
2. Generation	MRS	L5.0-1-horC-M13-1-SB	5	0.203	+	+
		MRS-1-M13-1-MB	11	0.115	-	+
		MRS-1-1-1-WB	no	0	-	-
		MRS-1-1-2-MB	>30	0.036	-	-
		MRS-1-1-3-MB	>30	0.042	-	-
		MRS-1-1-1 + DNA-MB	>30	0.036	NA	NA
		MRS-1-1-1 + L5.0-horC-M13-1-past.-SB	>30	0.063	NA	NA
		MRS-1-1-2 + L5.0-horC-M13-1-past.-SB	>30	0.062	NA	NA
		MRS-1-1-3 + L5.0-horC-M13-1-past.-SB	>30	0.051	NA	NA
	L5.0	L5.0-horC-M13-1 pasteurized-NB	no	0	+	+
		L5.0-1-horC-M13-1-SB	3	0.205	+	+
		L5.0-1-horC-1-SB	5	0.202	+	-

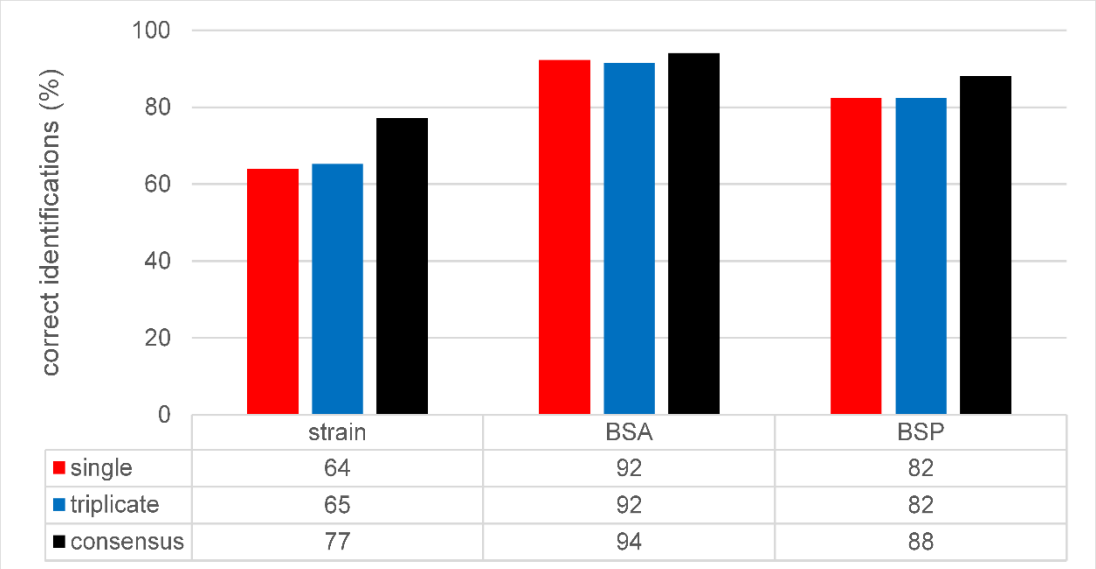
In another experiment, TMW 2.1636-SB was tested 10 times (biological replicates) for the ability to grow in lager beer 1 within a “truncated” beer spoilage test. Only one test beer was inoculated with cells from $lager_{pH5.0}$, the remaining procedure was the same as described in chapter 3.2.6.5 (p. 35). One biological replicate was not able to grow in lager beer 1. All replicates were tested for the presence of *horA*, *horC* and *fabZ*. All nine replicates with beer spoilage ability were tested positive for the abovementioned DMGs, while the NB-replicate was positive for all DMGs with the exception of *fabZ*.

4.5 Species, strain and group identification using MALDI-TOF MS

MALDI-TOF MS was tested for its ability to identify relevant LAB on species, strain and group level, regarding beer spoilage ability and potential. Species identification was 100 % correct for all 1770 spectra recorded. Overall strain level identification was 65 % for biological replicates and 77 % for consensus spectra made from five biological replicates. The proportion of correct strain-level identifications varied between species. Besides those species where only five strains were investigated in total, the best results for strain identification were obtained for *L. brevis* (88 %) and *P. damnosus* (73 %). In case of *L. lindneri,* only 29 % of all biological replicates were identified correctly on strain level, while the proportion of correct identifications increased to 71 % when consensus spectra were used. Beer spoilage ability prediction was correct for 92 % of all biological replicates. In case of beer spoilage potential, this proportion decreased to 82 %. The assignment to beer spoilage ability and potential groups is mainly driven by strain identification when the best (first) database entry is adduced. Based on the second database entry, in total 76 % and 52 % of all triplicates were assigned correctly with respect to beer spoilage ability and beer spoilage potential. At all levels, the best overall results were obtained with consensus spectra.

15 high quality spectra of 118 strains, characterized with respect to beer spoilage ability and beer spoilage potential (4.2.4, p. 57), were generated and used for a database search (3.3.1.5, p. 38). The database entry was generated based on 24 high quality spectra generated by a third person in order to consider the preparative variation. Spectra processing, alignment and consequent identification were done with a MASCAP (Mantini et al., 2010) and octave (www.octave.org) based bioinformatics pipeline. Database search was conducted for single spectra, triplicate spectra representing a biological replicate and consensus spectra, made from five biological replicates and therefore from 15 spectra in total. In addition, consensus spectra were clustered and visualized as dendrogram using R Software (3.6.1, p. 44). Raw data partially originate from student projects under my supervision.

Species identification was 100 % correct for single (technical replicates), triplicate (biological replicates) and consensus spectra (five biological x three technical replicates). Strain level identification improved from single to triplicate and finally consensus spectra, as illustrated by Figure 37.



	strain	BSA	BSP
single	64	92	82
triplicate	65	92	82
consensus	77	94	88

Figure 37: MALDI-TOF MS - total correct strain, beer spoilage ability and spoilage potential group identifications (%) using single, triplicate and consensus spectra. Three single spectra (technical replicates) were made for each biological replicate (triplicate), while five biological replicates (consensus) were measured in total. BSA = Beer spoilage ability, BSP = Beer spoilage potential.

In case of *L. paracollinoides*, *L. plantarum*, *L. paracasei*, *L. parabuchneri*, *L. harbinensis* (five strains investigated, each) and *L. paucivorans* (two strains) only small sets were investigated, characterized by high proportions of correct strain level identifications, ranging from 76 % to 92 %. Using consensus spectra, 100 % correct identifications on strain level could be obtained for the abovementioned species. In case of the other species, 14 to 20 strains were included, while the proportions of correct identifications using triplicates, adducing the first database entry or the second one, are shown in Figure 38 (p. 130). Correct identification (%) on all levels was best for *L. brevis* and worst for *L. lindneri*.

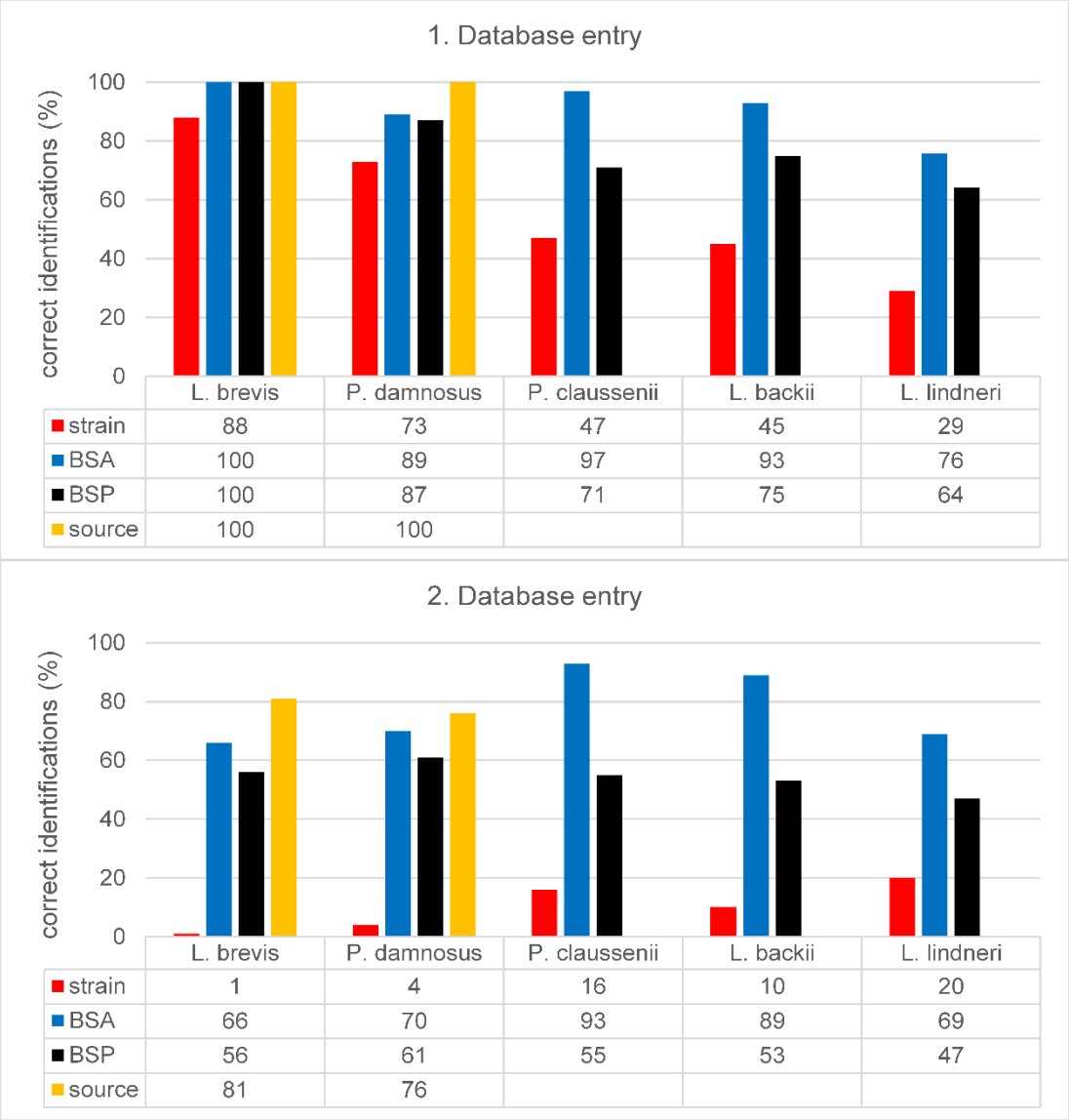


Figure 38: MALDI-TOF MS - correct strain, isolation source, beer spoilage ability and spoilage potential group identifications (%) on different levels using triplicate spectra of those species were more than ten strains were investigated. Each triplicate corresponds to a biological replicate and is based on three single spectra (technical replicates). The top panel shows the results based on the first (best) database entry matching the spectra, the bottom panel is based on the second database entry. BSA = beer spoilage ability, BSP = beer spoilage potential, source = isolation source brewery/non-brewery.

Cluster analysis of consensus spectra (Figure 39, p. 131) shows that species cluster together, while no distinct branches or clusters were found which separate beer spoilage ability and beer spoilage potential groups within single species. The high proportion of correct identifications regarding beer spoilage potential and ability is not reflected by a distinct clustering of beer-spoiling and non-spoiling strains based on their low molecular weight proteome.

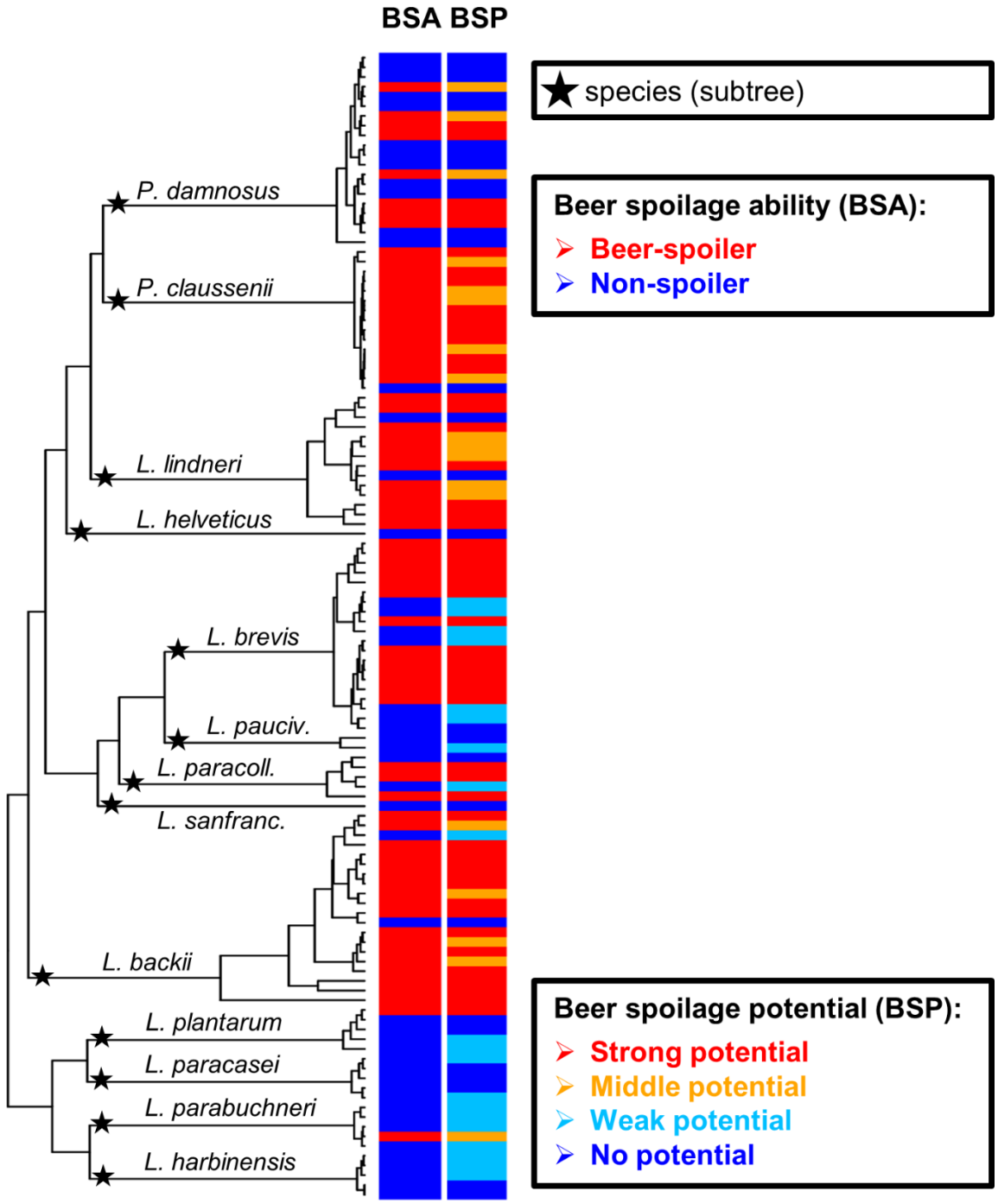


Figure 39: MALDI-TOF MS - cluster analysis of consensus spectra. All consensus spectra were clustered based on euclidian distance. Results are shown as dendrogram. Color-codes correspond to beer spoilage ability and potential groups and are detailed in the corresponding boxes.

4.6 Cluster analysis based on M13-V RAPD-PCR

55 strains, comprising all six core-species, were analysed with M13-V RAPD-PCR (3.4.2.1, p. 40). This was done to test this method for its predictive ability and in order to illustrate the genetic diversity of isolates. Based on the band patterns, similarity coefficients were calculated applying *Pearson's* correlation. Cluster analysis was done with the *Ward* method and plotted as dendrogram.

All six species form distinct clusters, while they differ with respect to intraspecies RAPD pattern diversity (see Figure 40, p. 133). The highest diversity was found for *L. backii*, the lowest for *L. lindneri*. There are no subtrees, within the single species, which correlate to beer spoilage ability or potential. A prediction of beer spoilage potential or beer spoilage ability based on a M13-V RAPD-PCR pattern was therefore not possible.

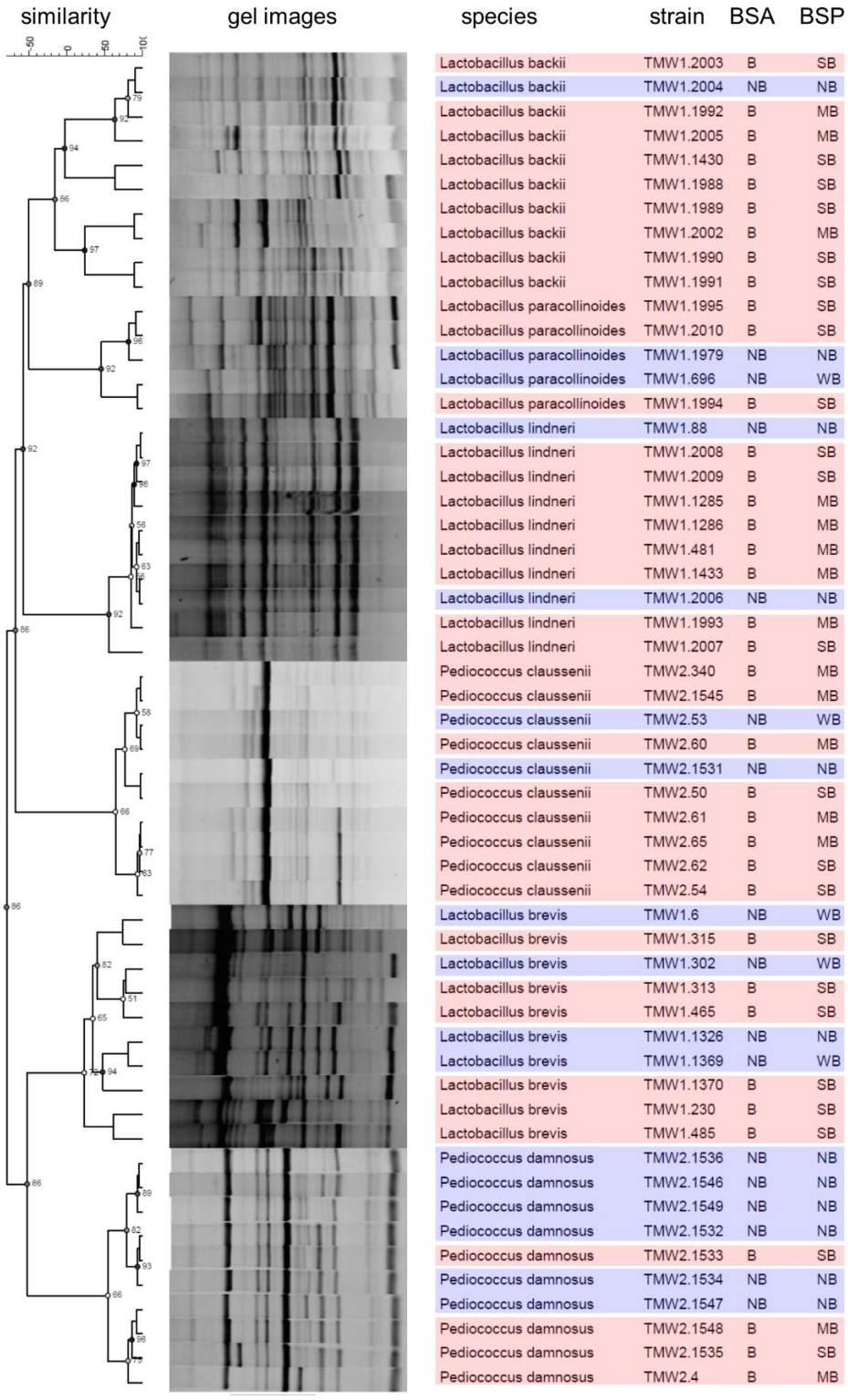


Figure 40: M13-V RAPD-PCR based dendrogram of 55 lactic acid bacteria strains of the core species. Similarity was calculated according to *Pearson*, followed by cluster analysis using the *Ward* method. Strains with beer spoilage ability (BSA) are labeled red, non-spoilers blue. Species form separate clusters, while no distinct subtrees are differentiating the beer spoilers from non-spoilers within the single species. Average beer spoilage potential (BSP) based on beer spoilage test: strong beer spoilage potential (BSP) (SB), middle BSP (MB), weak BSP (WB), no BSP (NB); BSA (B/NB).

4.7 Metabolism of beer-spoiling lactic acid bacteria

The whole chapter 4.7, also tables and figures, partially correspond to a recent publication about the metabolism of LAB in beer (Geissler et al., 2016).

4.7.1 Metabolism in beer

We investigated the metabolic capabilities and strategies of 26 strains, comprising all core-species, with varying beer spoilage potential. Metabolic capabilities of all strains, from no to strong beer spoilage potential, were investigated in lager beer with elevated pH ($lager_{pH5.0}$) and consequently reduced antibacterial properties. Metabolic data were also collected from the same lager beer with a pH of 4.3 ($lager_{pH4.3}$), which prevents the growth of strains with no beer spoilage ability and reveals the actual metabolism and the metabolic strategies of true beer-spoiling strains in a typical, regarding the pH, lager beer. We found no correlation of metabolic capabilities regarding carbohydrate, organic acid and amino acid metabolism to beer spoilage potential and ability. Pediococci strains utilized mainly glucose, fructose as well as the disaccharides gentiobiose and trehalose. *L. backii* consumed mannitol and sorbitol in addition to glucose and fructose. No pentoses were used by homofermentative strains investigated. *P. claussenii* strains were all characterized by agmatine utilization and concomitant putrescine production. The homofermentative species *P. claussenii* and *L. backii* produced acetic acid in addition to lactic acid in beer. Heterofermentative LAB were characterized by a more diverse sugar metabolism including different disaccharides as well as pentoses. Further, heterofermentative LAB exhibited distinct amino acid metabolism resulting in the production of biogenic amines. Citrate was utilized by *P. claussenii* and *L. lindneri*, as well as a single strain of *L. brevis*, while malate was utilized by all species except *L. paracollinoides*.

A detailed description of the experimental design, the metabolism of the investigated strains in $lager_{pH5.0}$ and $lager_{pH4.3}$, as well as an exact description of the concomitant statistical analyses can be found in Supplementary section 4 (11, p. 301). All relevant data and results, which are necessary to comprehend any thoughts and theories in the discussion, will be recapped in the discussion explicitly.

4.7.1.1 Metabolism in lager$_{pH5.0}$

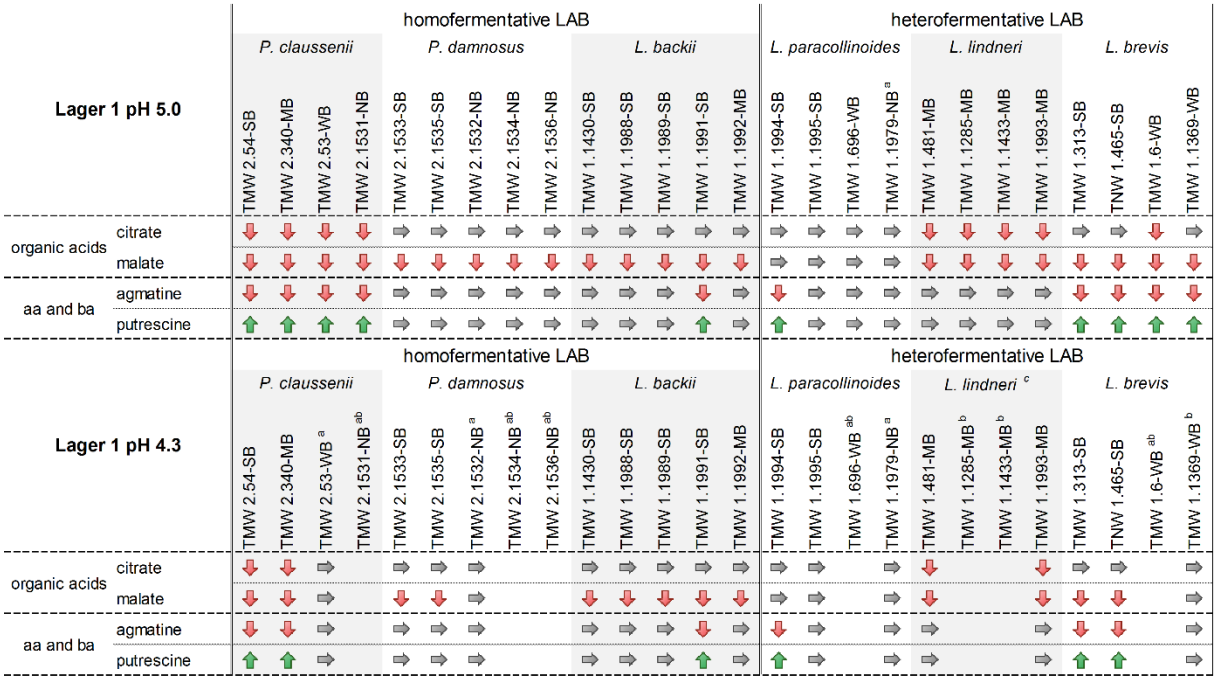
In order to determine the metabolic capabilities of all investigated strains, regardless of their beer spoilage potential, the metabolism in lager$_{pH5.0}$ (less antibacterial) was examined (see Table 28). Figure 41 (p. 137) compares the metabolism in lager$_{pH5.0}$ and lager$_{pH4.3}$ for selected strains, comprising all core-species.

Table 28: Metabolism of lactic acid bacteria in lager$_{pH5.0}$: arrows indicate % production (vertically upwards > 25 %, 45° upwards > 15 %), consumption (vertically downwards > 25 %, 45° downwards > 15 %) or no change (horizontal = 0 ± 15%) relative to the amounts found in non-inoculated beer (column on the right side), GABA = γ-aminobutyric acid. Note that the beer spoilage potential groups are based on the specific beer spoilage test, where the samples for HPLC analysis were taken. Data are described in more detail within Supplementary section 4 (12.2, p. 313).

		homofermentative LAB														heterofermentative LAB												
		P. claussenii				*P. damnosus*					*L. backii*					*L. paracollinoides*				*L. lindneri*				*L. brevis*				
		TMW 2.54-SB	TMW 2.340-MB	TMW 2.53-WB	TMW 2.1531-NB	TMW 2.1533-SB	TMW 2.1535-SB	TMW 2.1532-NB	TMW 2.1534-NB	TMW 2.1536-NB	TMW 1.1430-SB	TMW 1.1988-SB	TMW 1.1989-SB	TMW 1.1991-SB	TMW 1.1992-MB	TMW 1.1994-SB	TMW 1.1995-SB	TMW 1.696-WB	TMW 1.1979-NB[a]	TMW 1.481-MB	TMW 1.1285-MB	TMW 1.1433-MB	TMW 1.1993-MB	TMW 1.313-SB	TNW 1.465-SB	TMW 1.6-WB	TMW 1.1369-WB	non-inoculated control (µM)
hexoses	glucose	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↘	↑	↓	↓	↓	↓	↘	↓	↓	↓	↓	254 ± 7
	fructose	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	→	↓	↓	↓	↓	↓	↓	↓	↓	350 ± 12
	galactose	↗	→	→	→	↘	↓	→	→	→	→	→	→	→	→	↓	↓	↓	→	↘	→	→	→	↓	↓	↓	↓	57 ± 1
pentoses	arabinose	→	→	→	↗	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	↓	↓	↓	↓	230 ± 7
	xylose	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	↓	→	→	↓	→	↘	↓	→	↓	↓	↓	326 ± 9
disaccharides	isomaltose	↓	→	→	→	↓	↓	→	→	→	→	→	→	→	→	→	↓	↓	→	→	→	→	→	↓	↓	↓	↓	1028 ± 21
	lactose	→	→	→	→	↘	↘	→	→	→	→	→	→	↓	→	→	↓	→	→	→	→	→	→	↓	↓	→	→	231 ± 7
	gentiobiose	↓	↓	↓	↓	↓	↓	↓	↓	↓	→	→	→	→	→	→	→	→	→	→	→	→	→	↘	↘	↘	→	387 ± 13
	trehalose	↓	↓	↓	↓	↓	↓	↓	↓	↓	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	218 ± 8
sugar alcohols	sorbitol	↗	→	→	↗	↘	→	→	→	→	↓	↓	↓	↓	↓	→	→	→	→	→	↗	→	→	→	→	→	→	133 ± 3
	mannitol	→	↓	↓	↓	→	↗	→	→	→	↓	↓	↓	↓	↓	→	→	→	→	→	→	→	→	↑	↑	↑	↑	111 ± 4
organic acids	acetate[b]	↑	↑	↑	↑	→	→	→	→	→	↑	↑	↑	↑	↑	↑	↑	↑	→	↑	↑	↑	↑	↑	↑	↑	↑	424 ± 54
	lactate	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	→	↑	↑	↑	↑	↑	↑	↑	↑	851 ± 68
	pyruvate	↓	↘	↓	↘	→	↓	↘	↓	↘	↘	↓	↓	↓	↓	↓	↓	↓	→	↓	↓	↓	↓	↓	↓	↓	↘	568 ± 44
	succinate	↘	↓	↘	↘	→	↘	↘	→	→	→	→	↗	→	→	↓	↓	↓	→	↓	↓	↓	↓	↓	↓	↓	↓	3165 ± 168
proteinogenic aminoacids (aa)	arginine	→	→	→	→	→	→	→	→	→	→	→	→	→	→	↘	↓	↓	↓	→	→	→	→	↓	↓	↓	↓	212 ± 3
	asparagine	→	→	→	→	→	→	→	→	→	→	→	→	→	→	↓	↓	↘	→	↓	↓	↓	↓	→	→	→	→	93 ± 2
	aspartic acid	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	↓	→	→	↗	→	→	↓	↓	↓	↓	↓	152 ± 4
	glutamic acid	→	→	→	→	→	→	↘	→	↘	→	→	↗	→	→	↘	→	→	↘	→	→	↑	↑	↓	↓	↓	↓	121 ± 9
	histidine	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	↓	↓	↓	↓	→	→	→	→	147 ± 2
	lysine	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	↘	→	→	→	→	→	→	↘	→	→	→	123 ± 1
	methionine	→	→	→	→	→	→	↘	→	↘	↓	↓	↓	↘	→	↘	↘	→	→	↘	↘	↘	→	→	↓	↓	↓	38 ± 1
	threonine	↗	→	→	↗	↗	↗	→	→	→	→	→	→	→	→	↘	↘	→	→	→	→	→	→	→	→	→	→	88 ± 1
	tryptophan	↓	↓	↘	↘	↓	↓	↘	→	→	→	→	→	↓	→	↘	↘	→	→	→	↘	→	→	↘	→	→	→	143 ± 2
	tyrosine	→	→	→	→	→	→	→	→	→	↓	→	→	→	→	→	→	→	→	→	→	→	→	↓	↓	↓	↓	296 ± 4
amines and other aa	GABA	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	↑	↗	→	→	↗	↗	↗	↑	↑	↑	↑	316 ± 3
	tyramine	→	→	→	→	→	→	→	→	→	↑	→	→	→	→	→	→	→	→	→	→	→	→	↑	↑	↑	↑	66 ± 1
	ornithine	→	→	→	→	→	→	→	→	→	→	→	↗	→	→	↑	↑	↑	↑	→	→	→	→	↑	↑	↑	↑	31 ± 0
	histamine	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	↑	↑	↑	↑	→	↗	→	→	132 ± 3

[a]: no visible growth in lager$_{pH5.0}$. [b] instead of 15 and 25 %, a 50/100 % rule for arrow indication was applied in case of acetate

Table 29: Semiquantitative analysis of metabolism of lactic acid bacteria in lager$_{pH5.0}$ and lager$_{pH4.3}$. Citrate, malate, agmatine and putrescine utilization (vertically downwards), and production (vertically upwards), are indicated by arrows. No change is indicated by a horizontal arrow. Note that the beer spoilage potential groups are based on the specific beer spoilage test, where the samples for HPLC analysis were taken. An explanation why these analytes were analysed semiquantitatively, as well as how this was done, can be found in Supplementary section 4 (12.1, p. 310).



[a]: no growth in beer, [b]: no data obtained, [c]: low cell density compared to other species.

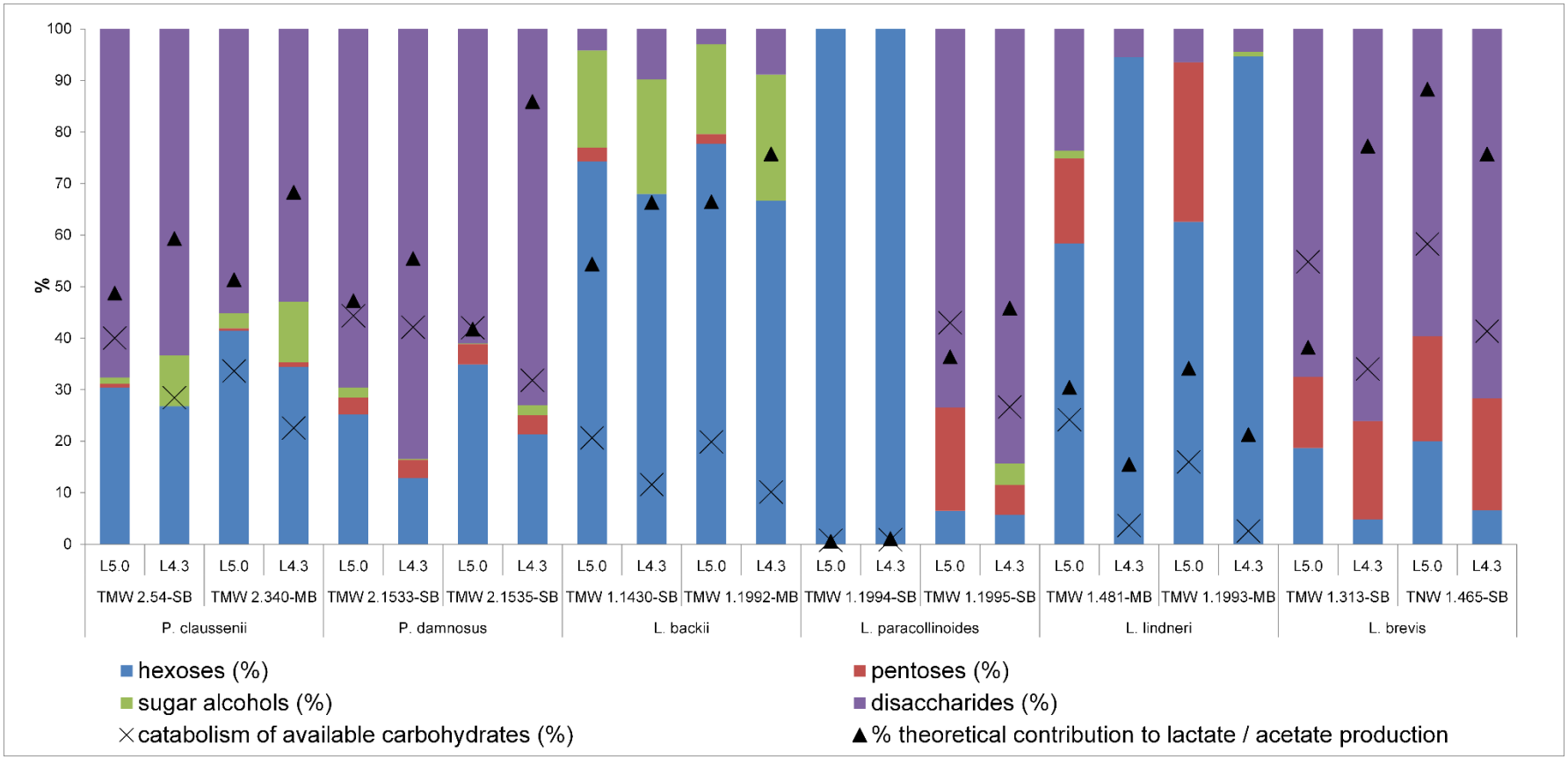


Figure 41: Conversion of carbohydrates in lager$_{pH5.0}$ and lager$_{pH4.3}$. The proportion (%) of hexoses, pentoses, sugar alcohols and disaccharides of the total consumed carbohydrates is shown for a selection of strains with growth at both pH values. Thus, the bar chart illustrates the sugar profile of the respective strain. The catabolism of available carbohydrates (% of total available) is shown (crosses) as well as their theoretical contribution to the lactic and acetic acid (total acid) concentrations measured (triangles). The latter parameter was calculated by dividing the stoichiometric possible total acid by the measured total acid and serves as a rough measure for the significance of analytes for energy supply. Note that the beer spoilage potential groups are based on the specific beer spoilage test where the samples for HPLC analysis were taken. L5.0 = Lager$_{pH5.0}$, L4.3 = Lager$_{pH4.3}$.

4.7.1.2 *Metabolism in lager$_{pH4.3}$*

In lager$_{pH4.3}$, the metabolism of MB and SB strains was investigated at a beer-typical pH of 4.3.

Table 30: Metabolism of lactic acid bacteria in lager$_{pH4.3}$: arrows indicate % production (vertically upwards > 25 %, 45° upwards > 15 %), consumption (vertically downwards > 25 %, 45° downwards > 15 %) or no change (horizontal = 0 ± 1 5%) relative to the amounts found in non-inoculated beer (column on the right side), GABA = γ-aminobutyric acid. Note that the beer spoilage potential groups are based on the specific beer spoilage test, where the samples for HPLC analysis were taken. Data are described in more detail within Supplementary section 4 (12.3, p. 317).

		homofermentative LAB													heterofermentative LAB											
		P. claussenii				*P. damnosus*					*L. backii*					*L. paracollinoides*				*L. lindneri* [c]				*L. brevis*				
		TMW 2.54-SB	TMW 2.340-MB	TMW 2.53-WB[a]	TMW 2.1531-NB[ab]	TMW 2.1533-SB	TMW 2.1535-SB	TMW 2.1532-NB[a]	TMW 2.1534-NB[ab]	TMW 2.1536-NB[ab]	TMW 1.1430-SB	TMW 1.1988-SB	TMW 1.1989-SB	TMW 1.1991-SB	TMW 1.1992-MB	TMW 1.1994-SB	TMW 1.1995-SB	TMW 1.696-WB[ab]	TMW 1.1979-NB[a]	TMW 1.481-MB	TMW 1.1285-MB[b]	TMW 1.1433-MB[b]	TMW 1.1993-MB	TMW 1.313-SB	TNW 1.465-SB	TMW 1.6-WB[ab]	TMW 1.1369-WB[b]	non-inoculated control (µM)
hexoses	glucose	↓	↓	↑		↓	↓	→			↓	↓	↓	↓	↓	↑	→		→	↘			↓	→	→		→	95 ± 1
	fructose	↓	↓	→		↘	↓	→			↓	↓	↓	↓	↓	↑	↑		→	↓			↘	→	→		→	372 ± 24
	galactose	→	→	→		↘	↓	→			→	→	→	→	→	↓	↓		→	→			→	↓	↓		→	69 ± 0
pentoses	arabinose	→	→	→		→	→	→			→	→	→	→	→	→	→		→	→			→	↓	↓		→	242 ± 2
	xylose	→	→	→		→	→	→			→	→	→	→	→	→	→		→	→			→	→	↓		→	341 ± 2
disaccharides	isomaltose	↘	→	→		↓	↘	→			→	→	→	→	→	→	↓		→	→			→	↓	↓		→	1120 ± 10
	lactose	→	→	→		↘	→	→			→	→	→	↓	→	→	↓		→	→			→	↘	↓		→	239 ± 4
	gentiobiose	↓	→	→		↓	↓	→			→	→	→	→	→	→	→		→	→			→	→	→		→	581 ± 39
	trehalose	↓	↓	→		↓	↓	→			→	→	→	→	→	→	→		→	→			→	→	→		→	248 ± 5
sugar alcohols	sorbitol	→	→	→		→	→	→			→	→	↓	→	→	→	→		→	→			→	→	→		→	154 ± 1
	mannitol	↓	↓	→		→	→	→			↓	↓	↓	↓	↓	→	↘		→	→			→	↗	→		→	111 ± 1
organic acids	acetate[d]	↑	↑	→		→	→	→			↑	↑	↑	↑	↗	↑	↑		→	↑			↗	↑	↑		→	197 ± 4
	lactate	↑	↑	→		↑	↑	→			↑	↑	↑	↑	↑	↑	↑		→	↑			↑	↑	↑		→	732 ± 27
	pyruvate	↓	↓	→		↓	↓	→			↓	↓	↓	↓	↓	↓	↓		→	↓			→	↓	↓		→	612 ± 61
	succinate	→	↓	→		→	→	→			→	→	→	→	→	↓	↓		→	→			→	↓	↓		→	3136 ± 1
proteinogenic aminoacids (aa)	arginine	→	→	→		→	→	→			→	→	→	→	→	→	↓		→	→			→	↓	↓		→	219 ± 12
	asparagine	→	→	→		→	→	→			→	→	→	→	→	↘	↘		→	↓			↘	→	→		→	74 ± 6
	aspartic acid	→	→	→		→	→	→			→	→	→	→	→	→	↓		→	→			→	→	→		→	116 ± 7
	glutamic acid	→	→	→		→	→	→			→	→	→	→	→	→	→		→	→			→	↓	↓		→	158 ± 8
	histidine	→	→	→		→	→	→			→	→	→	→	→	→	→		→	↓			→	→	→		→	165 ± 16
	lysine	→	→	→		→	→	→			→	→	→	→	→	→	↓		→	→			→	→	↘		→	92 ± 6
	methionine	→	→	→		→	→	→			→	→	→	→	→	→	→		→	→			→	↗	→		→	30 ± 1
	threonine	→	→	→		→	→	→			→	↗	↗	↑	→	→	→		→	→			→	→	→		→	50 ± 5
	tryptophan	→	→	→		→	→	→			→	→	→	→	→	→	→		→	→			→	↗	→		→	150 ± 2
	tyrosine	→	→	→		→	→	→			↓	→	→	→	→	→	→		→	→			→	↓	↓		→	322 ± 26
amines and other aa	GABA	→	→	→		→	→	→			→	→	→	→	→	→	→		→	→			→	↑	↑		→	345 ± 10
	tyramine	→	→	→		→	→	→			↑	→	→	→	→	→	→		→	→			→	↑	↑		→	43 ± 3
	ornithine	→	→	→		→	→	→			→	→	→	→	→	↑	↑		→	→			→	↑	↑		→	25 ± 1
	histamine	↓	↓	→		→	→	→			→	↗	→	↓	→	↓	→		→	↑			→	↓	↓		→	119 ± 11

[a]: no growth in lager$_{pH4.3}$, [b]: no data obtained, [c]: low cell density compared to other species. [d] instead of 15 and 25 %, a 50/100 % rule for arrow indication was applied in case of acetate

4.7.2 Metabolism in MRS variants

For comparison, the metabolism of all strains was also investigated in $mMRS_1$ pH 6.2, $mMRS_2$ pH 4.3 and $mMRS_2$ pH 4.3 with the addition of iso-α-acids (see 3.2.4, p. 32). Growth and pH were tracked, while the maximum OD_{590} was adduced as measure for growth. Samples for HPLC analysis were taken at the end of fermentation, which took different periods depending on species. As only glucose, fructose and maltose were present in all MRS variants, no direct comparison to beer with respect to sugar metabolism was possible. The metabolism was investigated with respect to distinct effects caused by a low pH (acid stress, $mMRS_2$ pH 4.3) and low pH with additional hop stress ($mMRS_2$ pH 4.3 with the addition of iso-α-acids, 50 % MIC), in comparison to reference conditions ($mMRS_1$ pH 6.2) or each other. Note that refreactive index (RI) detection, used for the simultaneous quantification of carbohydrates and organic acids, is not suited for proper ethanol quantification. Thus, ethanol production could only be determined in terms of present or absent, while this task was especially difficult in case of the hop stress media, as the added iso-α-acids were dissolved in ethanol as solvent. Although a calibration with standard solutions was conducted, we could not obtain a reliable relation of signal to concentration. Thus, in case of the hop stress media, we were not able to distinguish reliably between added ethanol and fermentation-based ethanol. In case of the other media, where non-inoculated controls were found to have no ethanol signal at all, a principle statement about ethanol production was possible. Results referring to reference conditions and acid stress conditions apply to all tested strains, if not stated differently. Findings regarding the metabolism under hop stress only refer to those strains with evident growth under hop stress ($OD_{590} > 0.01$).

We could neither detect production of acetate by *P. claussenii* and *L. backii* strains growing in MRS at two different pH values, nor under hop stress, while a production of acetate was found in beer for both species growing in beer. Nevertheless, a distinct metabolic shift was observed for all six core-species when exposed to hop stress. All strains with evident growth in the MRS variant with supplemented iso-α-acids were characterized by a similar metabolic response (see Figure 42, p. 140). The following findings refer to a comparison of the metabolism found in $mMRS_2$ pH 4.3 with and without hop addition, while an increase or decrease refers to a ratio of the respective parameters (“hop stress divided by acid stress”): Firstly, the amount of sugar substrates consumed increased or the ratio of carbohydrate utilization to cell density raised, compared to acid stress only. Secondly, the amount of lactate production decreased unproportional to sugar utilization, but proportional ($R^2 = 0.6$) to the % reduction of cell density. Thus, under hop stress, there is an enhanced sugar utilization at a concurrent lower cell density and a reduced lactic acid production. This results in less acidification as compared to acid stress only, which is supported by pH data. The pH of hop stress media after fermentation was

higher compared to acid stress in all cases where evident growth occurred in both media, despite the fact that most strains consumed more carbohydrates (see Figure 42). Note that in case of *L. brevis* the total amount of organic acids, lactic and acetic acid, did not decrease under hop stress, as observed for the other core-species. Instead, the ratio of lactate to acetate decreased, reflecting more acetate, and again, less lactate production.

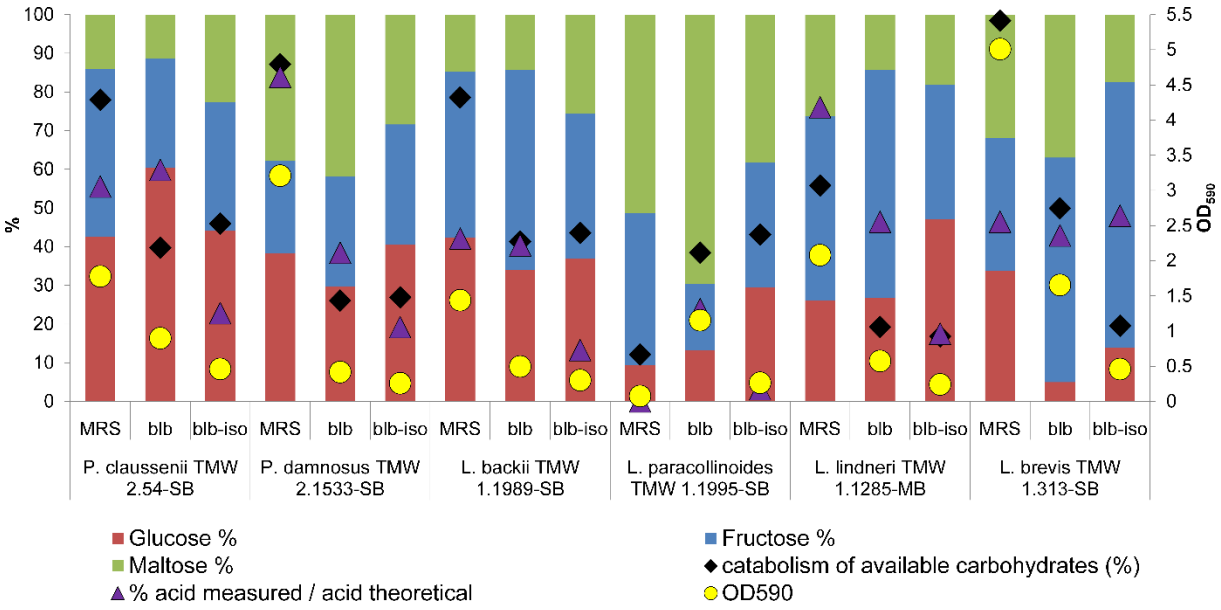


Figure 42: Carbohydrate catabolism of core-species in different MRS variants - exemplary for six beer-spoiling strains. The proportion (%) of glucose, fructose and maltose of total consumed sugars is shown by the bar chart, illustrating the sugar profile of the respective strain. The catabolism of available carbohydrates (% of total available) is shown, as well as the ratio of measured lactic and acetic (if produced) acid to theoretical possible organic acid concentrations, calculated under the assumption that each C6 compound could be degraded to two molecules of acid. The OD_{590} is shown as a measure of cell density. Acetate was only produced by *L. brevis* TMW 1.313-SB, while the ratio of lactate to acetate decreased from MRS (7.0) to blb (2.2) to blb-iso (1.9), demonstrating that also *L. brevis* produced less lactate under hop stress, compared to acid stress only. Ethanol was produced under acid stress by *L. paracollinoides* TMW 1.1995-SB and *L. brevis* TMW 1.313-SB. MRS = $mMRS_1$ pH 6.2, blb = $mMRS_2$ pH 4.3, blb-iso = $mMRS_2$ pH 4.3 + 50 % MIC iso-α-acids. Average values for all parameters are shown for three biological replicates.

Amino acid metabolism was most prominent in case of *L. brevis*, as also found in beer. Glutamate utilization and the concomitant GABA production was found to be stimulated by hop stress in case of the beer-spoiling *L. brevis* strains. For details regarding single species and all investigated strains, the reader is referred to Supplementary section 5 (11, p. 301), dedicated to the metabolism of single core-species in MRS variants. All relevant data and results, which are necessary to comprehend any thoughts and theories in the discussion, will be recapped explicitly in the discussion.

5 Discussion

Hop tolerance is known to be an important shared trait of beer-spoiling LAB, while beer is an environment characterized by multiple hurdles, thus making beer spoilage ability a multifactorial trait. Apart from the static (chromosomal) genetic setting, phenotypic plasticity, reflected by the regulated adaptation to changing environmental conditions, is important for the lifestyle of beer-spoiling LAB in beer (Behr et al., 2006; Behr et al., 2007b; Bergsveinson et al., 2012; Haakensen et al., 2008; Pittet et al., 2013; Suzuki et al., 2006). We found that, on one hand, beer-spoiling LAB are characterized by mostly species-specific, chromosomally encoded metabolic settings conferring adaptation to the niche beer. On the other hand, strains of different species share a dynamic mobile genetic pool, encoding additional traits beyond hop tolerance, which finally enable and facilitate LAB growth in beer. Thus, genomic plasticity is an important part of LAB´s lifestyle in the brewery environment. The respective additional genes contribute, amongst others, to cation homeostasis, oxidative stress tolerance and cell envelope modification and metabolism. These were suggested to be relevant for beer spoilage potential, while their significance so far has only been indicated in terms of phenotypic plasticity (Suzuki, 2015). Further, the ability to produce long-chain fatty acids was found to be essential for LAB growth in the low-fat environment beer. This and other shared traits comprise lifestyle genes for the classification of LAB according to their beer spoilage ability in quality control.

5.1 The beer spoilage potential and ability of lactic acid bacteria with very high and high hazard potential

5.1.1 Establishment of a reference method - Classification and transferability

Each quick-reference method for the identification of beer-spoiling LAB, e.g. DMG based, needs to be tested by means of a reliable reference method, classifying strains into beer-spoiling and non-spoiling strains. A two-step, forcing-like beer spoilage test, using three test beers from the same brewery with increasing antibacterial properties, was compared to the resazurin test developed by Preissler et al. (2010), while in both cases strains were subsequently classified into beer spoilage potential groups. The latter method allows a higher throughput, the usage of more test beers, is less tedious and material-intensive, while providing faster results. However, a comparison of the classifications showed that the resazurin test is underestimating the beer spoilage potential of various strains and is therefore not suitable as a reliable reference method. Further, explorative data analysis of all spoilage related data showed a better accordance with the classification of LAB based on the beer spoilage test. Besides statistical reasons, the beer spoilage test offers several other advantages. Growth is detected as is, without changing the system by adding a beer-alien substance such as resazurin or paraffin oil. The temporal resolution is higher and more spoilage related data can be obtained, e.g. pH and turbidity. In contrast to the resazurin test, the inoculum size (~5000 cells/ml) reflects realistic conditions encountered in the brewery. Finally, this kind of beer spoilage test is very similar to a forcing test (exception: test beer is not source beer) and is also the standard procedure to evaluate the beer spoilage ability by the community (Asano et al., 2007; Haakensen et al., 2008; Iijima et al., 2007; Suzuki et al., 2005b). This allows a better transfer of data and makes it easier to estimate the validity of third party results for comparison. Nevertheless, we extended the beer spoilage test using three test beers with increasing antibacterial properties, instead of a single lager beer, which allows a more exact classification. The beer spoilage test was used for the characterization and classification of strains and will be referred as reference method.

In 25 % of all cases, we could not stably classify strains into a distinct beer spoilage potential group, while this is apparently not the result of an unstable or non-reliable method. Instead this results from the unstable beer spoilage potential of LAB strains, as a consequence of genetic instability, which will be dealt with in subsequent sections (e.g. 5.2.2, p. 166), but is also a known issue (Suzuki, 2015).

The transferability of classifications was tested with beers from other breweries. Classification into beer spoilage ability groups was consistent in 70 %, in case of beer spoilage potential only in 57 % of all strains, while transferability strongly depended on species. The stability of classifications was dramatically different within the tested species. Thus, it is not surprising that the classification in case of *L. lindneri*, characterized by a very large number of unstable (regarding beer spoilage potential) strains, showed the lowest transferability. The opposite was true for *P. damnosus*, which was characterized by stable beer spoilage potential and a consistent classification within alternative beer systems, indicating high transferability.

In conclusion, we could establish a reference method for the determination of beer spoilage potential and beer spoilage ability for LAB with very high and high hazard potential, while the reliability, stability and transferability of the applied classification system varies between species. A lack of stability and transferability most likely results from the unstable beer spoilage potential of LAB strains, which is suggested to be the consequence of genetic instability.

5.1.2 Beer spoilage potential and beer spoilage ability - The need for intraspecies differentiation

118 strains, comprising 13 species with very high and high hazard potential, were characterized using the beer spoilage test and subsequently classified into beer spoilage ability and beer spoilage potential groups, according to their ability to grow in beers with increasing antibacterial properties. All other methods, which were tested for their ability to predict beer spoilage potential and ability, were consequently tested for their accordance with this reference method.

As expected, LAB with very high hazard potential showed a higher proportion of beer-spoiling strains (67 %) compared to high hazard potential LAB (46 %). This becomes even more evident if we reclassify *P. claussenii* as a species with very high hazard potential, which is suggested here. Only 10 % of the remaining 20 high hazard potential strains were able to grow in lager beer, thus characterized as beer-spoiling strains. With one exception, all *P. claussenii* strains were able to grow in lager beer 1, while even 60 % have a strong beer spoilage potential. The only strain with no beer spoilage potential was found to be able to grow in lager beer of another brewery within the transferability test. Thus, all 15 strains have to be considered as beer-spoiling strains. Since *P. claussenii* has been shown to have comparable spoilage characteristics (adaption, acidification, turbidity) like the other very high hazard potential species, and because we could show that *P. claussenii* strains lacking *horA* and *horC* are also able to grow in lager beer, this species has to be reconsidered as a species with very high hazard potential. This will be immediately applied within this thesis.

Beer spoilage ability clearly correlated to isolation source, while only 9 % of all non-brewery isolates were characterized by beer spoilage ability. This is in tune with other literature (Suzuki, 2015) and makes sense considering the fact that those capabilities, which are necessary to grow in beer, are not chromosomally (and therefore not universally/stable within a species) encoded, but conferred by brewery-specific mobile genetic elements. Further, most non-brewery isolates (57 %) were not able to grow in wheat beer, characterized by comparatively low antibacterial properties. With two exceptions, all remaining non-brewery isolates (35 %) were able to grow in wheat beer only, in addition characterized by comparatively long adaption times and low cell densities. This is in tune with the assumption that brewery-derived LAB have adapted to the environment beer and that beer spoilage ability is not necessarily an innate species-trait (Suzuki, 2015).

Despite these general tendencies, regarding hazard potential and isolation source, beer spoilage ability and potential distributions vary between species. In case of *P. claussenii*, *L. backii* and *L. lindneri* we found more than 25 % strains with an unstable beer spoilage potential, while 86 to 93 % of all strains exhibit beer spoilage ability. There was no or only poor transferability of the applied classification systems regarding *L. lindneri* and *P. claussenii*. In case of *L. backii,* only one strain of 20 was characterized by the lack of beer spoilage ability. Further, we could only obtain brewery isolates of these species, while it was already suggested that these bacteria might be brewery-specific microorganism (Suzuki, 2015). Altogether, the low proportion of non-spoiling strains, the high share of unstable strains and the fact that the predictive accuracy of any rapid-reference method referring to the beer spoilage test is subsequently limited (transferability/stability), suggest that a species identification is sufficient or even more suitable in case of these species. This is also suggested for *L. paracollinoides*, although we observed no unstable beer spoilage potential nor any issues with transferability. Nevertheless, both non-spoiling strains were previously obtained from the DSMZ and it was shown that strains of this species lose hop resistance genes upon cryo-conservation (which is confirmed in this study), consequently lacking beer spoilage ability (Suzuki et al., 2006). As both non-spoiling strains, used in this study, are likely to be artificial, and because *L. paracollinoides* is characterized by a generally low prevalence (0 to 3.6 %) in breweries, an intraspecies differentiation might be unrewarding and is consequently not recommended.

In case of *L. brevis* and *P. damnosus,* we have a different situation, characterized by comparatively low instability and good to perfect transferability. These two species were characterized by 35 to 70 % non-spoiling strains. In case of *L. brevis*, we found a strong relation of beer spoilage ability to isolation source, which confirms that *L. brevis* strains from other sources mostly lack beer spoilage ability (Nakagawa, 1978). Nevertheless, almost 75 % of all non-brewery isolates were able to spoil wheat beer, while we also found one honey-isolate

with strong beer spoilage potential. Therefore, and because *L. brevis* is a rather ubiquitous species, which can be transferred into the brewery environment from various sources, an intraspecies differentiation is necessary, especially in order to decrease the number of false positive results, which might result from a species identification. Although none of the *P. damnosus* winery isolates was able to spoil beer, an intraspecies differentiation is necessary, as only 35 % of all brewery isolates were found to possess beer spoilage ability. Regarding other species with high hazard potential (e.g. *L. plantarum*, *L. paracasei*), although they were not focused within this study, an intraspecies differentiation is suggested because of a distinct strain-specific beer spoilage potential.

In conclusion, we were able to classify all included strains and species with respect to beer spoilage potential and beer spoilage ability. Our results suggest, also considering the available spoilage statistics (Back, 1994, 2005; Suzuki, 2011, 2015) that an intraspecies differentiation in brewing quality control, with respect to beer spoilage ability/potential, might be unrewarding in case of *P. claussenii*, *L. paracollinoides*, *L. backii* and especially in case of *L. lindneri*. With regard to *L. brevis* and *P. damnosus* we provide consistent reference data for the evaluation and validation of DMGs and other rapid detection system, since an intraspecies differentiation is necessary in order to avoid a high number of false positive results based on species detection.

5.1.3 The significance of spoilage associated characteristics

There was a significant high to very high correlation of the adaption time in test beers to beer spoilage potential. This shows that strains with a strong beer spoilage potential are not only able to grow in beers with higher antibacterial properties (e.g. pilsner), but also show a faster adaption to the unfavourable conditions in beer. However, this correlation was not found in case of $lager_{pH5.0}$, where 116 of 118 strains were able to grow. This confirms the outstanding importance of a low pH for the stability of beer with respect to spoilage, also contingent upon the pH-dependent antibacterial properties of hops (Fernandez and Simpson, 1995; Simpson, 1993b). It also shows that all kind of approaches, where the beer spoilage ability of a strain is supposed to be predicted based on its growth in diluted or even buffered beer-broth mixtures, have no predictive ability. In routine quality control, beer samples are often diluted with NBB and/or water in order to reduce the selectivity of beer (pH > 5.0). This is intended to detect obligate beer-spoiling LAB, but also bacteria with only potential relevance or indirect significance for beer spoilage (Back, 1994, 2005). However, we found even strains from completely different sources (e.g. sausages, honey, faeces), lacking any beer spoilage potential, to grow in $lager_{pH5.0}$ or in NBB. In quality control, it is important to understand that the ability to grow in beer/water or beer/NBB mixtures (with an elevated pH) does not give a reliable

information about the actual product hazard by a contamination. If quality control relies solely on the usage of detection media and reinoculation tests, e.g. forcing test, it should be considered that only the inoculation of the test beer without modification gives a reliable result regarding beer spoilage ability. We further found that species with a very high hazard potential show a faster adaption to beer, compared to high hazard potential species, emphasizing their close association with the environment beer. We could neither detect a species independent correlation of acidification nor cell density to beer spoilage potential, while all three spoilage characteristics are species dependent and dependent on the used beer (decrease/increase of parameter with increasing antibacterial properties, wheat → pilsner).

In conclusion, we found that strains with high beer spoilage potential cannot only grow in beers with higher stability, but also show a faster adaption to beer. An increase of the pH (to 5.0) makes beer susceptible to LAB without beer spoilage potential and from all kind of sources, emphasizing the role of the low pH hurdle.

5.1.4 The role of hop tolerance for beer spoilage potential and ability

Hop resistance is considered the major distinguishing determinant regarding beer spoilage ability (Suzuki, 2011). In contrast to what was expected, we could not find a species independent correlation of beer spoilage ability or potential to hop tolerance in $mMRS_2$ pH 4.3. A significant high correlation was only found for *L. brevis* and *L. paracollinoides*. This could mean that the applied method to determine hop tolerance is not suited for this task, as suggested by Haakensen, Schubert, et al. (2009). They could show that the correlation of beer spoilage ability to a hop tolerance test in MRS is affected by the “strength” of MRS (dilution of nutrients), the ethanol content and the physical state of MRS (agar/broth). It was concluded that hop tolerance is affected by other beer specific properties, such as nutrient limitation and ethanol presence. However, all 118 strains showed an improved growth in $mMRS_2$ pH 4.3 without hops compared to any test beer or $lager_{pH5.0}$, while in some cases the addition of even low amounts of iso-α-acids to $mMRS_2$ completely inhibited growth. This was especially prominent for *P. damnosus* and *L. lindneri*. In both cases, we found strains, which were not even able to grow in $mMRS_2$ pH 4.3 with 5 ppm iso-α-acids within 6 days, while they were characterized by visible growth in beers with 8 to 26 ppm iso-α-acids within 5 to 7 days. In addition, one has to consider that in case of the beer spoilage test, 10 ml of beer were inoculated with around 1 to 2 µl of a low cell density preculture (OD_{590} ~ 0.05 to 0.2, ~ 5000 cells/ml), while the MIC tests (200 µl) were inoculated with 5 µl of a concentrated cell suspension (OD_{590} = 1 to 2, ~ 10^9 cells/ml). Altogether, this indicates that in case of these species hop tolerance is even enhanced in beer, or the other way around that MRS lacks specific components which are necessary to establish hop tolerance. This is in tune with the

finding that certain unidentified/unpublished components of beer even promote the growth of these species (Back, 2005; Suzuki, 2015). On the other side, we identified several strains, especially within the high hazard potential species (e.g. *L. paracasei*, *L. harbinensis*), which lack any beer spoilage potential, while they are able to grow in MRS with iso-α-acids concentrations of up to 50 ppm (compare pilsner beer 1: 26 ppm). This indicates that hop tolerance, as a standalone trait, is not sufficient for growth in beer and that beer spoilage ability cannot be predicted solely based on this parameter.

In conclusion, not only the ability to grow in beer, but also hop tolerance is a multifactorial trait, which is apparently affected by other parameters, as already suggested (Haakensen, Schubert, et al., 2009). However, the idea that beer even enhances/activates hop tolerance of some LAB species has to be further investigated. The predictive ability of hop resistance tests, regarding beer spoilage ability and potential, is not sufficient.

5.2 The lifestyle of beer-spoiling lactic acid bacteria in beer

5.2.1 Comparative genomics - The genomic preconditions

We were able to obtain the complete genome sequences of 17 LAB strains with varying beer spoilage ability. A comprehensive reference text (see Supplementary Section 2 - Genome analyses, p. 239) about the genome analysis of these relevant organisms is provided including a detailed description of their predicted metabolic capabilities. These data were further used for a comprehensive comparative genome analysis. Note that the following sections are almost exclusively based on *in silico* prediction, which will not be stressed repeatedly by using terminology such as "*in silico*" or "predicted" etc. Where available, *in silico* predicted traits will be discussed along and supported by physiological data.

5.2.1.1 Chromosomal preconditions and "the core genome of spoilage"

Beer-spoiling LAB are known to be not closely related to each other based on 16 rDNA sequences and do not cluster together in distinct branches within LAB (Sun et al., 2015; Suzuki, 2015), which can be confirmed here. We wanted to investigate, which properties actually make them a distinct group (e.g. very high hazard potential) and which properties are actually shared by these organism, allowing them to grow in and spoil beer. Traits, e.g. genes, which have a distinct association with the ability of LAB to grow in beer, could be used for the specific detection of beer-spoiling LAB.

The analysis of further phylogenetic markers confirmed that these bacteria are not a monophyletic group within the LAB, indicating that their adaption to the niche beer was a convergent process. Further, LAB with very high and/or high hazard potential could not be related to each other based on genome size, chromosome size, number of proteins, GC content, coding density, codon usage, amino acid usage, proteome similarity, chromosome similarity, functional pattern and subcellular localization of proteins. To the contrary, beer-spoiling LAB include species with completely different genomic preconditions. Exemplarily, they comprise genomes, which belong to the smallest (*L. lindneri*) and to the largest (*L. paracollinoides*) within the lactobacilli, characterized by completely different codon usage and GC contents from 34.3 % (*L. lindneri*) to 47.0 % (*L. paracollinoides*). Regarding chromosomal properties, beer-spoiling LAB species cover the whole diversity within the genus *Lactobacillus*.

Although LAB with very high and/or high hazard potential share a common chromosomal core genome, we found these sequence based core genomes to be not specific for these groups, as they were covered even to 100 % by other LAB species without any documented significance for brewing microbiology. These core genomes are characterized by genes almost exclusively related to basal functions, e.g. translation, transcription and central carbon metabolism, making it unlikely that these genes represent those specific traits, which are mandatory for growth in beer. Besides the fact that these core genomes are not specific for the groups of interest, we found that combinations of genomes (the calculation of their core genomes), derived from a similar number of strains and species, results in comparable numbers of core genes. To a certain extent, core genomes within the genus *Lactobacillus* seem to be a simple product of genome number and threshold values for homologous genes, while these shared genes not necessarily reflect a similar lifestyle or adaption to a specific niche. This is also illustrated by the chromosomally encoded metabolic capabilities of these bacteria. Very high hazard potential LAB include homofermentative and heterofermentative species, some of which are characterized by completely different carbohydrate utilization systems, including metabolically very versatile bacteria (*L. brevis*) and metabolically restricted species (*L. lindneri*), while these differences were also confirmed by HPLC data. *L. paracollinoides* and *L. backii* seem to be prototroph for 19 to 20 amino acids, while *L. brevis* or *L. lindneri* are auxotroph for most amino acids. *L. brevis* encodes for amino acid decarboxylation (glutamate, tyrosine) systems, the ADI and the AGDI system and consequently produces various biogenic amines, which has been confirmed by HPLC analysis. These systems very likely contribute to *L. brevis* energy generation and pH homeostasis. In contrast, *P. damnosus* does neither encode for any of these systems nor did we observe any biogenic amine production. There are various other examples, regarding vitamin biosynthesis, nucleotide metabolism and other metabolic pathways, which emphasize that there is apparently no distinct, shared and chromosomally encoded metabolic foundation, which is obligatory for LAB to grow in beer. This is in accordance with a previous study, which also came to the conclusion that the “(chromosomal) core genome” of *L. brevis* BSO 464 plays at best a minimal role for hop tolerance and beer spoilage ability (Bergsveinson, Baecker, et al., 2015). Instead, these bacteria have species-specific metabolic strategies to grow in beer, accompanied by shared plasmid-encoded traits, which are necessary to grow in and spoil beer.

5.2.1.2 The role of plasmids for the adaption to beer and the brewery environment

We wanted to investigate the role of mobile DNA for the adaption of brewery isolates to beer and the brewery environment and consequently answer the question if this adaption is mainly a plasmid-encoded, dynamic and unstable trait. Brewery isolated genomes are characterized by up to ten plasmids and a high amount of plasmid-encoded information, representing up to 10.6 % of their total DNA, while we found a positive correlation of the number of plasmids to hazard potential, isolation source and spoilage potential. Within the genus *Lactobacillus*, we could only find a higher ratio of plasmidome to genome size for strains (complete genomes) of *Lactobacillus salivarius*. *L. salivarius* plasmids are considered to contribute to niche adaption, in this case the mammalian gastrointestinal tract (Behr et al., 2016; Raftis et al., 2014). In order to evaluate the role of brewery-plasmids for niche adaption and the lifestyle of beer-spoiling LAB, we conducted a comprehensive plasmidome analysis. The whole chapter partially corresponds to a previous publication by Behr et al. (2016).

Within individual species, we found that those strains with a higher beer spoilage potential were mostly characterized by a higher number of plasmids. Thus, we focused on their analysis, revealing that there is a shared pool of highly homologous, plasmid-encoded genes, in various cases organized as genetic clusters. 59 % of all homologous genes were found to be shared by two or more species, supporting the postulated hypothesis that hop resistance, beer spoilage ability and thus niche adaption are mediated and transferred via HGT (Suzuki et al., 2006). We further found that various genes and clusters are encoded on otherwise different plasmids and flanked by transposases, indicating that a distinct proportion of brewery DNA is transferred transposon-mediated, as suggested by Suzuki (2015). Beer-spoiling strains were found to share more genes with each other as with non-spoiling strains, while several beer-spoiling strains of different species share even more genes with each other as with non-spoiling strains of the same species. Thus, the number of shared homologous genes is apparently related to niche adaption.

Previous studies indicated that some plasmids or plasmid-encoded genes are obligatory for beer spoilage ability (Bergsveinson, Baecker, et al., 2015; Suzuki et al., 2006), while others have an auxiliary function (Bergsveinson, Baecker, et al., 2015; Pittet et al., 2013). The active hop tolerance mechanisms, shared by all six core-species, are mediated by *horA* and *horC* and apparently have a significance within all species, which makes sense if we consider them as a first defense line against hops (Vogel et al., 2010). On the other side, only *L. backii* and *P. damnosus* were found to encode for a complete FAS cluster, while this piece of homologous DNA was not found within any of the other plasmidomes. As the other species encode a complete chromosomal FAS, there is no need for an additional plasmid-encoded FAS cluster.

This indicates that brewery LAB do not just randomly take up mobile DNA or plasmids, but specifically take what they need from a brewery-specific pool of DNA.

Functional analysis of brewery plasmidomes revealed an enrichment of certain functional categories, while the vast majority of all plasmid-encoded/shared genes encodes for either hypothetical proteins, or products with a non-clearly defined or non-informative biological function. Figure 43 (p. 152) highlights the most abundant genes and functions found on these brewery plasmids, in a wordcloud. Not all of these genes and functions are actually brewery-specific, while many of them are still interesting, as they are noticeable abundant. However, while most of the functions, for example FAS, are not specific for the brewery environment, many genes and clusters are specific for the brewery environment on DNA sequence level, such as the FAS cluster.

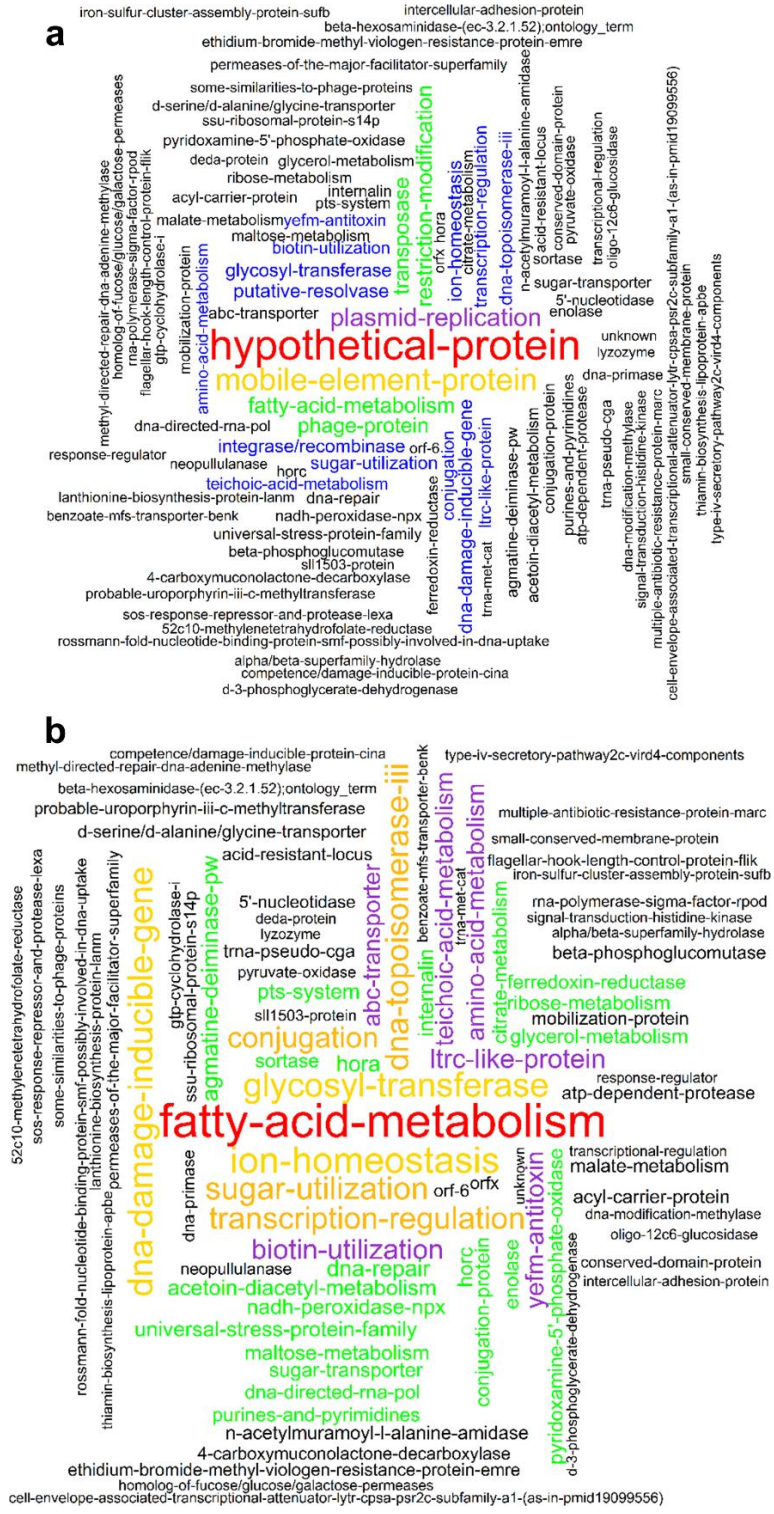


Figure 43: Wordcloud of brewery plasmidome-encoded genes and global functions - illustration of gene and function/trait frequencies. Genes with a distinct association to a global function, e.g. conjugation or fatty acid metabolism, were summarized. In contrast to the SEED subsystem analysis or a comparable analysis, all genes/functions are depicted. The upper panel (a) shows all brewery genes/functions, which occur more than two times in the total brewery plasmidome. The lower panel (b) shows the same but without genes/functions lacking an informative biological function (e.g. hypothetical protein, mobile element protein). Thus, the lower panel has a different scaling (color/font size).

In conclusion, the comparative analysis of brewery plasmidomes emphasizes the known relevance of cation homeostasis, oxidative stress tolerance and cell envelope metabolism for hop tolerance and beer spoilage potential. Further, we suggest that plasmid-encoded metabolic traits, which allow the reduction of the acid load, e.g. by producing non-acidic end products such as diacetyl or butanediol, contribute to the acid tolerance of beer-spoiling LAB. These metabolic strategies are suggested to be part of their lifestyle in the low pH environment beer. The abundance of plasmid-encoded, partially brewery-specific genes, encoding for various functions, which might confer an advantage in the brewery environment or can be linked (in theory) to an improved growth in beer, suggests that these plasmids are the foundation for LAB beer spoilage potential and consequently important for their lifestyle in beer. This is supported by the fact that plasmid-encoded DMGs are and were found to have high to perfect correlations to beer spoilage ability (Behr et al., 2016; Haakensen et al., 2008; Suzuki et al., 2006), while our results support the idea that the presence of distinct DMGs/clusters/plasmids is mandatory for beer spoilage ability (obligatory) or affects beer spoilage potential (auxiliary). As the vast majority of all plasmidome genes is uncharacterized or even lacks any informative annotation, further research is necessary to deduce the significance of single plasmids, clusters and genes for beer spoilage potential and niche adaption. The established plasmidome sequences of beer-spoiling strains of six very high hazard potential LAB species are the foundation for plasmid curing experiments and transcriptomics, while both approaches will help to identify and differentiate obligatory and auxiliary genetic entities. This will further improve the selection process of DMGs for diagnostic purposes, as true, obligatory lifestyle genes (e.g. *horC*, *fabZ*) represent perfect targets for quality control.

The potential role of some interspecies shared genes and clusters, part of the flexible brewery gene pool, will be discussed in detail in the following sections, while species-specific properties will be dealt with in separate sections.

5.2.1.2.1 Carbohydrate metabolism - Sugars and organic acids

This chapter partially corresponds to previous publications by Behr et al. (2016) and Geissler et al. (2016).

Metabolic traits, which allow exploiting additional energy sources, a more efficient utilization of substrates, or to reduce the stress-load encountered in beer, might be advantageous and thus contribute to an improved growth in beer. Brewery plasmids were found to encode several proteins associated with carbohydrate metabolism, including citrate/malate utilization, pyruvate metabolism and maltose utilization.

Citrate and malate have been identified as energy sources under hop exposure and as important substrates for growth in beer in case of *L. brevis* and *L. lindneri* (Suzuki et al., 2005b). All *L. lindneri* strains have been found to utilize malate upon growth in beer. Thus, an additional plasmid-encoded utilization and transport system for malate, as encoded by *L. lindneri* TMW 1.481-MB (also chromosomally encoded), might be advantageous for growth in beer. Malolactic fermentation is suggested to result in pmf generation, thus providing energy, e.g. for transport processes without the creation of reducing equivalents (Juega et al., 2014; Olsen et al., 1991). In addition, the acidity of the environment is reduced by the conversion of the dicarboxylic malic acid to the monocarboxylic lactic acid, thus potentially reducing the acid load (Juega et al., 2014). So far, *L. paracollinoides* was not shown to utilize citrate in beer (Suzuki et al., 2005b). We found a complete citrate fermentation cluster on pL11995-2 of *L. paracollinoides* TMW 1.1995-SB. However, citrate was not consumed by this strain upon growth in beer.

The ability to produce the unwanted off-flavor diacetyl (indirectly) from pyruvate is a plasmid-encoded trait in case of *P. damnosus*, while the corresponding alpha-acetolactate operon (Cluster 1, see Table 22, p. 109) was only found for the brewery genomes. *L. paracollinoides* chromosomes were all found to encode the respective enzymes, while the strong spoiling strain TMW 1.1995 is also characterized by an additional, plasmid-encoded alpha-acetolactate operon.

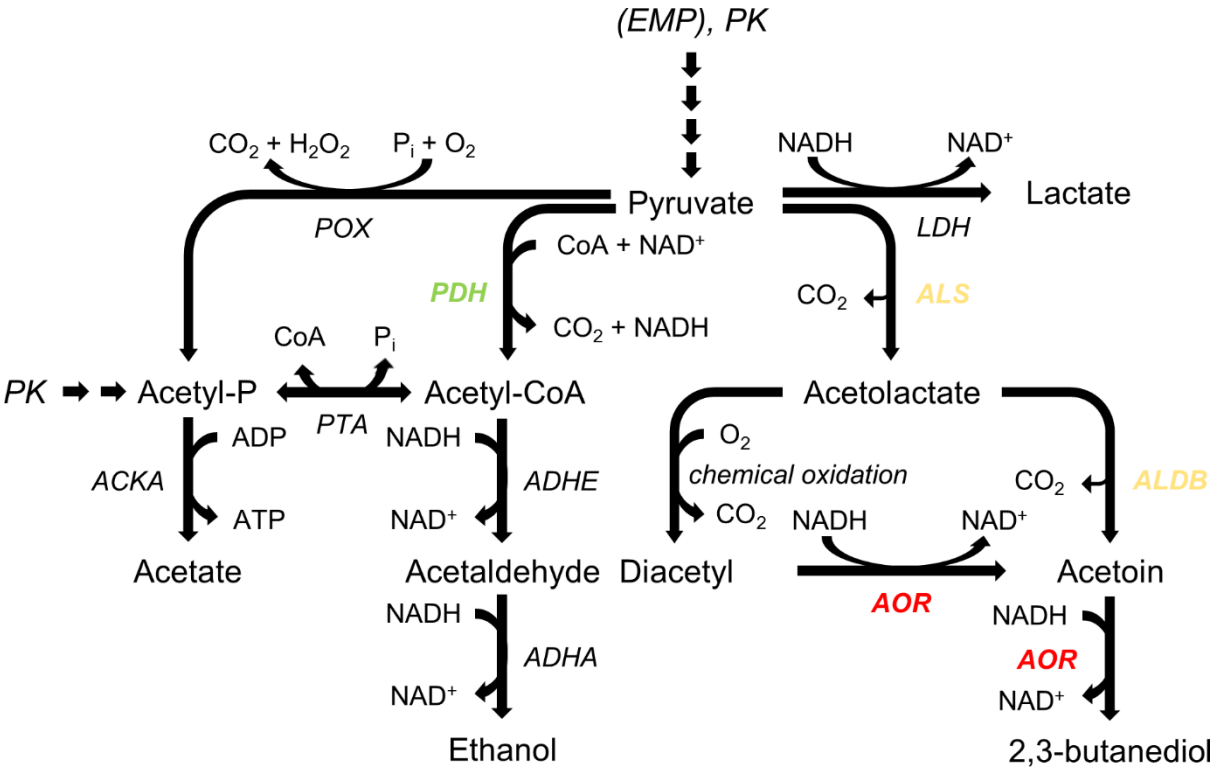


Figure 44: Pyruvate metabolism of *P. damnosus* and *L. paracollinoides*. EMP = Embden–Meyerhof–Parnas, PK = Phosphoketolase (EC 4.1.2.9), LDH = lactate dehydrogenase (EC 1.1.1.27/28), **ALS** = acetolactate synthase (EC 2.2.1.6) and **ALDB** = alpha-acetolactate decarboxylase (EC 4.1.1.5) - both plasmid-encoded and only found within brewery plasmidomes in case of *P. damnosus*, chromosomally encoded by all *L. paracollinoides* genomes and additionally plasmid-encoded by *L. paracollinoides* TMW 1.1995-SB, **AOR** = Acetoin reductase (EC 1.1.1.4) - chromosomally encoded by TMW 2.1533-SB, TMW 2.1535-SB, TMW 2.1536-NB (winery) and all *L. paracollinoides*, POX = pyruvate oxidase (EC 1.2.3.3); **PDH** = Pyruvate dehydrogenase (*L. paracollinoides* only); ACKA = acetate kinase (EC 2.7.2.1), PTA = phosphate acetyltransferase (EC 2.3.1.8), ADHE = acetaldehyde dehydrogenase (EC 1.2.1.10), ADHA = alcohol dehydrogenase (EC 1.1.1.1).

The alpha-acetolactate operon encodes for acetolactate synthase (EC 2.2.1.6) and alpha-acetolactate decarboxylase (EC 4.1.1.5). The former enzyme is responsible for the decarboxylation of pyruvate to acetolactate, which is spontaneously oxidized to the off-flavor diacetyl. Alternatively, acetolactate is decarboxylated to acetoin, while both potential products, acetoin and diacetyl, are non-acidic (see Figure 44).

Altogether, we suggest that this metabolic capability is not only troubling brewers, but also represents a metabolic advantage for growth in beer. The production of non-acidic end products in an intrinsically acidic environment might be an advantage. Nevertheless, *P. damnosus*, lacking a respiratory chain, has to recycle its NAD using alternative electron acceptors, as this would normally happen during lactate production. Therefore, it is interesting that both sequenced beer-spoiling *P. damnosus* and *L. paracollinoides* TMW 1.1995-SB

encode a chromosomal acetoin reductase (EC 1.1.1.4), allowing the subsequent reduction of acetoin/diacetyl to 2,3-butanediol, recycling NAD. Thus, these strains are potentially able to channel pyruvate to butanediol, instead of lactate, recycling two molecules of NAD per pyruvate while producing non-acidic end products. The production of diacetyl by *P. damnosus* in beer is a known issue (Back, 1994, 2005; Suzuki, 2015), as well as the general production of secondary metabolites as acetoin and butanediol by LAB contaminants in beer (Saxby, 1996). However, as we did not quantify these metabolites, we cannot confirm their production upon growth in beer for those strains investigated. The production of diacetyl by *P. damnosus* TMW 2.1535-SB in lager beer 1 was once confirmed with direct infusion mass spectrometry (data not shown).

Functional and sequence analysis revealed the presence of plasmid-encoded maltose phosphorylase(s) (clusters 4/14, see Table 22, p. 109) in case of individual brewery-strains of *L. backii*, *L. brevis*, *L. paracollinoides* and *P. damnosus*. In addition, all investigated core-species genomes were found to encode a chromosomal maltose phosphorylase. Maltose is considered to be a major carbohydrate in beer (Preedy, 2009) and all metabolically investigated strains of the core-species, with one exception, were shown to be generally capable of maltose degradation. However, we could not find a pronounced significance of maltose utilization for LAB metabolism in beer, as only three out of 26 strains consumed maltose in lager beer 1 pH 4.3 with a concomitant low degree of consumption. This is in accordance with a previous finding of Suzuki et al. (2005b), which could not observe a utilization of maltose by *L. brevis*, *L. lindneri* and *L. paracollinoides* in beer. We also tested all core-species strains for the presence of the DMGs M9 and M23, part of clusters 4/14, by PCR. Our results confirmed that this trait, or the predicted genes for maltose phosphorylase, have no significant correlation to beer spoilage potential or ability, as illustrated by Fisher`s p values of 0.713 and 0.216. As we further found that the availability of maltose can vary dramatically, even within a specific type of beer, it might make sense that beer-spoiling LAB do not focus on the utilization of this unstable substrate in beer. If we take one step back and consider the brewery itself as the LAB environment, the (improved) ability to utilize maltose could be of use in other brewery areas. LAB are not only found in the final product, but spread over the production area, including malt, wort and the fermentation tanks (Bamforth, 2001; Bokulich et al., 2015). Wort is a nutrient-rich, “high”-pH (~ 5.5) broth (Bamforth, 2001; Preedy, 2009), while for example *P. damnosus* is known to be a frequent contaminant of the fermentation process (wort fermentation by yeast). The maltose content in beer is comparatively low (50 +/- 100 mg/L (Preedy, 2009), 2.8 mg/L lager beer 1) and *P. damnosus* does not encode for a PTS system for maltose, most probably representing the most efficient active substrate transport system from an energetic point of view (Neves et al., 2005). On the other hand, *P. damnosus* does encode PTS systems for those sugars, which were actually found to be consumed in

lager beer 1. In order to grow in the harsh environment beer, LAB need to invest high amounts of energy (making efficient metabolic systems an important trait), especially for active hop tolerance mechanisms (Vogel et al., 2010), while the high energy demand of LAB due to hop stress could be clearly demonstrated within this study. Consequently, maltose is a rather unattractive substrate in beer, while the high amount of maltose in wort (56 to 59 g/l) and the considerable reduced antibacterial properties of wort, concomitant with the potentially reduced energy costs, might make maltose an attractive substrate in that specific area. *L. lindneri*, another species that is suggested to be latent during wort fermentation, was screened with PCR for the presence of cluster 14/M23, finding all tested strains to be positive for this maltose utilization cluster. The potential relevance of brewery plasmids for the growth within other areas of the beer production site might also explain the observation that some of these plasmids do not significantly affect beer spoilage ability and potential, as observed for *L. brevis* BSO 464 (Bergsveinson, Baecker, et al., 2015).

In conclusion, we identified interspecies, plasmid-encoded traits, which allow the utilization of alternative substrates, improved NAD recycling and a shift towards less acid metabolic products. Any metabolic process reducing the acidity of the environment can be considered as an important part of the lifestyle of beer-spoiling LAB in beer. In addition, these traits offer new possibilities for a more efficient energy generation, which is necessary considering the high energy demand by active hop tolerance mechanisms (Vogel et al., 2010). We also suggest that the (improved) ability to consume maltose is of minor relevance for LAB growth in beer, while it might be an important trait for the growth in other brewery areas such as wort.

5.2.1.2.2 Amino acid metabolism

A conserved cluster for a complete agmatine deiminase pathway (AGDI, Cluster 12 see Table 22, p. 109) was found, encoded by the plasmidomes of two *L. paracollinoides* strains and *L. backii* TMW 1.1991-SB. The AGDI pathway allows the production of 2 ATP and 2 NH_3 from agmatine, resulting in the biogenic amine putrescine, while agmatine is often found in beer also as a potential metabolic product of yeast metabolism (Galgano et al., 2012; Kalač et al., 2002). Agmatine consumption as well as putrescine production were confirmed by HPLC analysis for *L. backii* TMW 1.1991-SB and *L. paracollinoides* TMW 1.1994-SB, while this was not detected for the other strains of both species. *L. paracollinoides* TMW 1.1979-NB, also positive for cluster 12, did not show growth in lager beer 1 with pH 5.0/4.3. This indicates that the AGDI pathway has rather an auxiliary function, and, all alone, is not sufficient for successful growth in beer. Nevertheless, ATP generation, alkalization of the intracellular space and a no-cost substrate/product antiport make it a very attractive system for LAB at a low pH (Mozzi et al., 2010; Zuniga et al., 2002). Acidification of test beers by *L. backii* TMW 1.1991-SB (and

L. backii TMW 1.1990-SB, same source brewery, RAPD-pattern, DMG profile) was highest within this species, accompanied by about twice as much lactic acid production compared to the other investigated *L. backii* strains. It is conceivable that the alkalization of the cell/environment due to the production of NH_3 supported the strain to withstand this higher degree of acidification.

5.2.1.2.3 Cell wall and capsule

Brewery plasmidomes were found to be enriched in functions associated with the SEED category cell wall and capsule. Several shared genes and enriched functions, especially associated with the biosynthesis and modification of teichoic/lipoteichoic acids and peptidoglycan metabolism were identified, while most of these genes were found on distinct genetic clusters such as cluster 2, the *horA* and the *horC* clusters. Suzuki et al. (2006) already stated that the highly conserved regions flanking *horA* and *horC* might potentially be involved in cell envelope metabolism, while a general relevance of the cell wall and LTAs for hop resistance was already detailed in the introduction. Two genes of the *horA* and the *horC* clusters, respectively, are considered to potentially participate in cell envelope biogenesis and the glycosylation of teichoic acids, while the significance of teichoic acids for hop tolerance was already shown by Behr et al. (2006). Interestingly, the DMG PCR-screening revealed that M43 (*horC* cluster), encoding for a glycosyltransferase associated with cell wall modification, does also occur independently from the *horC* cluster. We further found some potential enzymes involved in teichoic acid biosynthesis, which supports the idea that (L)TAs have a significance for brewery LAB. However, while a modification of LTAs has been identified as a specific response of *L. brevis* to acid and hop stress (Behr et al., 2006), this is hardly a part of *L. lindneri´s* lifestyle in beer, as this species was found to lack cell wall teichoic acids (Back, 2005; Back et al., 1996). This lack of teichoic acids was found to be reflected by an apparent lack of genes associated with this trait.

Genes potentially involved in peptidoglycan recycling and peptidoglycan biosynthesis were identified. Two different N-acetylmuramyl-L-alanine amidases (AMIs, EC 3.5.1.28) were found, one variant on cluster 2 (see Table 22, p. 109), which is encoded by all brewery *P. damnosus* and *L. brevis* BSO 464, and another variant encoded by *L. brevis* TMW 1.313-SB and *L. paracollinoides* TMW 1.1995-SB. These autolysins separate the glycan strand from the peptide, allowing the further degradation/recycling of the stem peptides and the remaining glucans (Vollmer et al., 2008). Cluster 2 additionally encodes for a potential high-affinity zinc-uptake-system protein (ZnuA), which makes sense considering the fact that several AMIs need Zn^{2+} for their enzymatic activity (Vollmer et al., 2008). Recent comparative proteomic research with *L. brevis* TMW 1.465-SB indicated an upregulation of proteins involved in peptidoglycan

metabolism under acid and hop stress (Behr et al., 2007b). Anyway, the actual role of AMIs for growth in beer or hop tolerance remains elusive. This is also due to the fact that the physiological functions of AMIs in general are not known very well, while it is suggested that these enzymes are mainly involved in cell separation and peptidoglycan recycling (Vollmer et al., 2008). In general however, peptidoglycan hydrolases are necessary for cell wall growth and thus also for bacterial growth, while they are considered as pacemaker enzymes regarding growth (Ghuysen and Hackenbeck, 1994). It has been observed that some lactobacilli, e.g. *L. fructivorans* and *L. homohiochi*, increase their cell wall thickness upon increasing ethanol stress (Higashi, Aoki, et al., 1988; Higashi, Yamamoto, et al., 1988). It could be possible that the here found genes have a potential role for cell wall growth and consequently for the stress tolerance of brewery LAB, e.g. against ethanol. However, this hypothesis has to be tested by comparing the cell walls of strains with and without the respective genes.

5.2.1.2.4 Ion transport and homeostasis

Within the brewery enriched SEED categories virulence, disease and defense as well as membrane transport, we found several genes associated with ion transport and homeostasis, encoded within various plasmidomes of five core-species.

In detail, most of these genes were found to be linked to copper homeostasis, namely to the ATP-dependent export of copper ions (*copB*). Copper content in beer is found to range from 0.01 to 1.55 mg/ml, mainly derived from raw materials (Suzuki, 2015). For bacteria, copper is an essential trace nutrient but also, depending on the concentration, a toxic element. The antibacterial properties of copper are suggested to be induced by Fenton-type reactions and other redox reactions (thiol oxidation, hydroxylation). Mrvcic et al. (2013) found that the tolerance to copper is species dependent, while they observed a significant, concentration-dependent, inhibitory effect on three different LAB species including *L. brevis*. However, those concentrations, which were actually shown to exhibit an inhibitory effect (> 50 mg/ml), were in a different range than those contents typical for beer. As ten of 17 brewery plasmidomes were found to encode for copper homeostasis related genes, this biological function might still be of relevance. It is known that hops, e.g. iso-α-acids, are able to chelate various cations, including Cu^{+}, with a high affinity (Parsons et al., 2002; Ting, 2015). Further, the antibacterial activity of trans-isohumulone was shown to be increased by the presence of monovalent cations (K^{+}, Rb^{+}, NH_4^{+}, Na^{+}, Li^{+}), although Cu^{+} itself was not tested (Simpson and Smith, 1992). One possible explanation for the predicted emphasis of copper homeostasis might thus be that hop-bound Cu^{+}, analogous to Mn^{2+}, participates in a redox-active system potentially causing oxidative stress. This would be possible as Cu^{+} can act as oxidant and reductant. In consequence, the export of copper by CopB from the cell might either represent a potential

mechanism to prevent the cell from copper induced oxidative damage (Gupta and Lutsenko, 2012; Mrvcic et al., 2013; Solioz et al., 2010), or might help to reduce the antibacterial action of hops. However, there are no concrete data about the antibacterial properties of hops in combination with copper, nor about the relevance of copper itself for beer-spoiling LAB. Thus, a systematic investigation of copper tolerance as well as hop tolerance as a function of copper content is necessary to understand the potential role of copper homeostasis for beer spoilage potential. Alternatively, copper homeostasis is of relevance in other areas of the brewery environment, as it is known that the concentration of copper decreases during the production process from wort to the final product (Zufall and Tyrell, 2008). Finally, copper homeostasis related genes were also found in 11 other included non-brewery plasmidomes, while almost all of them (10 of 11) were found to belong to LAB associated with (isolated from) milk and the GIT (e.g. *L. salivarius*, *L. paracasei* and *L. reuteri*). Interestingly, these copper-homeostasis-related genes, such as CopB, were found to cluster in two plasmid-encoded groups based on multiple sequence alignment. One group consists of those genes found in *L. salivarius*, while the other cluster contains the remaining sequences, brewery and milk/GIT-derived, with sequence similarities of 93 to 100 %. This indicates that these homologous genes might have been introduced into the breweries by human or animals.

We further identified a brewery-specific DNA sequence encoding a magnesium transporter (CorA). It is located on a distinct cluster together with the already published lifestyle gene *hitA*, which is a potential hop inducible Mn^{2+}/H^{+} symporter, and a transcriptional regulator of the *TetR* family (M02). We found the *hitA* cluster encoded by brewery *L. brevis*, *P. damnosus*, and, for the first time, encoded by *L. backii* and *L. paracollinoides* strains. PCR screening of 65 strains of these four species showed a significant relation (Fisher´s $p = 0.027$) of *hitA* (and probably the whole cluster) to beer spoilage ability. Further, Preissler (2011) found that *hitA*-positive *L. brevis* strains had a comparatively higher tolerance to hops than those without that gene, while this can only partially be confirmed based on our results, indicating a moderate but not significant correlation to hop tolerance. Our results (e.g. low sensitivity (39 %) of *hitA* as DMG) indicate that *hitA* is not essential for beer spoilage ability and hop tolerance but has an auxiliary function for growth in beer. Behr et al. (2006) could show that *hitA* is overexpressed in *L. brevis* under acid and hop stress. As both genes are predicted to be under the control of the same transcriptional regulator, it is conceivable that *corA* is also overexpressed under these beer-typical conditions. CorA is considered to be a major ubiquitous Mg^{2+} uptake system, which is involved in Mg^{2+} homeostasis in all kind of bacteria (Kehres et al., 1998). Interestingly, Preissler (2011) observed Mg^{2+} uptake and a concomitant release of Mn^{2+} by *hitA*-positive *L. brevis* TMW 1.313-SB grown in pilsner beer. As Mn^{2+} and Mg^{2+} are suggested to be interchangeable with respect to some physiological functions (Jakubovics and Jenkinson, 2001), the observed ion homeostasis could be a specific strategy to reduce the antibacterial

effects of hops, as additionally the antibacterial properties of hops were found to be less pronounced (compared to Mn^{2+}) (Behr and Vogel, 2009) or even reduced in the presence of Mg^{2+} (Preissler, 2011; Simpson and Smith, 1992). The association of *hitA* and *corA* encoded on a shared cluster, potentially simultaneously regulated, was previously unknown, but indicates that not only manganese but also magnesium homeostasis could be important for the growth of beer-spoiling LAB in beer.

In conclusion, plasmid-encoded and shared genes were found associated with ion homeostasis and the transport of metals (additionally for cobalt/zinc/cadmium and potassium). Given the fact that these metals partially have antibacterial properties themselves (Tsakalidou and Papadimitriou, 2011) and considering the knowledge about the evident linkage of hop`s antibacterial properties to the content of various cations (Behr and Vogel, 2009; Simpson and Smith, 1992), the presence of such a high number of ion transport related genes indicates that active ion homeostasis, not only in case of manganese, is an important biological process for LAB growing in beer and/or the brewery environment and thus part of their lifestyle in beer.

5.2.1.2.5 Oxidative stress

A NADH peroxidase (M42, also part of Cluster 10, see Table 22, p. 109) was encoded by genes in 11 brewery beer-spoiling plasmidomes, while the DNA-sequence itself is not specific for the brewery environment and was found in other LAB genomes. PCR screening revealed a species independent enrichment of M42 in beer-spoiling strains (Fisher´s $p = 0.037$ for core-species), which is especially prominent for *P. damnosus*. NADH-peroxidases (*npx*, EC 1.11.1.1) catalyse the reduction of H_2O_2 to H_2O using NADH as electron donor, thus counteracting oxidative stress but also offering the possibility to recycle NAD (Kang et al., 2013). Further, we found a ferredoxin reductase (Fdx) and a ferritin-like antioxidant protein (Dps), again encoded by several brewery plasmidomes, but also by non-brewery isolates. Both are also considered part of the cellular defense against oxidative stress (Imlay, 2013; Kang et al., 2013). An auxiliary role of these genes for growth in beer is conceivable considering the fact that only small amounts of oxygen in beer, together with Fe^{2+} or Cu^{+}, can cause the formation of H_2O_2 and other reactive oxygens species (Preedy, 2009). Further, oxidative (redox) stress was previously identified as a major antibacterial property of hops (Behr and Vogel, 2010; Schurr, Hahne, et al., 2015), although it remains elusive how this could participate in the generation of H_2O_2 or any other ROS. A potential significance of Npx and Fdx for growth in beer is also supported by the findings of Bergsveinson, Baecker, et al. (2015). They demonstrated that the loss of pLb464-8, which carries both abovementioned genes, had a significant negative effect on *L. brevis* BSO 464 growth behaviour in beer, while its relevance for hop tolerance was not clear. They suggest that pLb464-8 might be important to cope with

other stresses found in beer (Bergsveinson, Baecker, et al., 2015). This could be oxidative stress, although pLb464-8 encodes for 45 other potential genes with different functions/annotations. The same research group could also demonstrate that several oxidative-stress-related genes of *P. claussenii* ATCC BAA-344 (TMW 2.340-MB), including the plasmid-encoded *dps* gene, were significantly upregulated upon growth in beer (Pittet et al., 2013), while a general significance of oxidative stress tolerance for *L. brevis* hop tolerance was indicated by various other studies (Behr et al., 2006; Behr et al., 2007b; Behr and Vogel, 2010; Vogel et al., 2010).

We found a positive, species independent correlation of hop tolerance to oxidative stress tolerance. In case of *P. claussenii* and *L. brevis*, the tolerance to oxidative stress at low Mn^{2+} concentrations was even found to correlate to a high degree to beer spoilage potential. It is also interesting that especially *L. lindneri*, considered to be an obligate (innate) beer-spoiling species, is characterized by the highest tolerance to oxidative stress within all core-species. Anyway, it has to be noted that beer is filled virtually oxygen free these days (Suzuki, 2011). In consequence, it might be as in case of the maltose utilization clusters described above. Oxidative stress related genes might be more relevant in other brewery areas. Wort is aerated before yeast fermentation and contains about 8 to 12 mg/l O_2. This makes the emergence of ROS and oxidative stress essentially more likely compared to beer (0.4 to 4 mg/l) (Preedy, 2009).

In conclusion, the comparative plasmidome analysis emphasizes the importance of oxidative stress tolerance and redox homeostasis for beer-spoiling LAB in beer and/or the brewery environment, while the occurrence of the respective genes in non-brewery isolates and non-spoiling strains indicates that they have a rather auxiliary function and that they are not suited as DMGs for the differentiation of beer-spoiling and non-spoiling strains.

5.2.1.2.6 Others

Cluster 5 (DMG 28, see Table 22, p. 109), shared by beer-spoiling brewery plasmidomes of all core-species, except *L. lindneri*, was predicted to encode for seven genes. Only one gene could be assigned to a different annotation as hypothetical or unknown, leaving the potential function of the whole cluster unknown. However, that specific annotated gene is predicted to encode for a member of the universal stress protein family (UspA). The UspA family is an orthologous group of proteins, which is considered to be involved in the molecular response to various stress qualities including ethanol, oxidants, uncouplers and others. Nevertheless, despite the ubiquitous distribution of UspA proteins within bacteria, archaea and plants, the exact biochemical functions of these proteins are still unknown (Kvint et al., 2003). DMG M28

was found to have a significant (Fisher´s p = 0.017, core-species) species independent relation to beer spoilage ability with a precision of 84 %. This indicates that this brewery-specific cluster has a general relevance for beer spoilage ability, while its actual function remains elusive. *UspA* and M28, a hypothetical protein, are also encoded on pLb464-4 (CP005981), while pLb464-4 was found to be one of two plasmids (pLb464-8, see section about oxidative stress), which had an auxiliary positive effect on the growth behaviour of *L. brevis* BSO 464 in beer.

Cluster 7 is shared by the beer-spoiling *P. damnosus* TMW 2.1535-SB and four out of five *L. backii* plasmidomes, while it encodes five potential proteins loosely related to benzoate degradation and acid tolerance. The role of acid tolerance was discussed repeatedly.

Cluster 11 (see Table 22, p. 109) is shared by nine beer spoilers' plasmidomes and encodes for eight genes. Again almost all (7) of them are either hypothetical or without a useful annotation, thus leaving the actual function of this brewery-specific cluster unclear. One gene encodes for a brewery-specific enolase (EC 4.2.1.11) sequence, while it is unclear what kind of advantage an additional copy of this gene might have. Nevertheless, it was observed that different enolase isoforms are used by yeast, depending on the carbon source used (Avilan et al., 2011). As a plasmid-encoded potential enolase was not found in any other *Lactobacillus* plasmidome, its presence might contribute to a metabolic advantage in beer or the brewery environment. The chromosomal enolase gene of *P. claussenii* ATCC BAA-344 (TMW 2.340-MB) was found to be strongly upregulated (22 fold) in beer (Pittet et al., 2013), supporting a potential relevance of this trait.

Further, shared genes were found related to the uptake of unknown substrates, growth at cold temperatures (*ltrA*), DNA restriction/modification, DNA repair and others. However, unfortunately the vast majority of all genes encodes for hypothetical proteins and other without a defined biological function. As we now know the plasmid sequences and the potential gene sequences for six very high hazard LAB species including more than 20 strains, it is important to get a deeper knowledge of their relevance in order to "discard" those that have no relevance for beer spoilage. We think that the approach performed by Bergsveinson, Baecker, et al. (2015), including the generation of plasmid-cured variants and the consequent characterization of their beer spoilage potential with respect to some key parameters, will help to better understand the role of these plasmids and clusters. Finally, we suggest that not all of these genes, described within chapter 5.2.1.2, are actually relevant for LAB growth in beer, but might play a role for the growth within other areas of the brewery environment, eventually even in environments outside the brewery.

5.2.1.3 The role of the fatty acid biosynthesis for beer spoilage ability

This chapter partially corresponds to a previous publication by Behr et al. (2016).

We found a highly conserved fatty acid biosynthesis (FAS) cluster encoded in all *L. backii* as well as in both beer-spoiling *P. damnosus* plasmidomes. Interestingly, the FAS cluster was found on quite different plasmids, while the presence of two cluster-flanking transposases indicates that this piece of DNA may also be transferred in a transposon-mediated way. Both species lack a complete chromosomal FAS, while we could show that only those strains have beer spoilage ability, which encode for this cluster or which were positively tested for the DMG *fabZ*, which is part of the cluster. *P. damnosus* non-spoiling strains, lacking the FAS cluster, were characterized by very poor growth in $mMRS_1$ (OD_{590} ~0.01) without the addition of a fatty acid source. In contrast, *fabZ*-positive strains were not affected by the presence or absence of Tween in MRS. On the other hand, non-spoiling *P. damnosus* strains, lacking the FAS cluster but encoding *horC*, were capable to grow in beer when supplemented with a fatty acid source. Further, it was demonstrated that the loss of *fabZ* comes along with the loss of beer spoilage ability in case of *P. damnosus* TMW 2.1636-SB. Altogether, these data indicate that the availability (either biosynthesis or supplemented) of fatty acids is essential for *P. damnosus* growth in beer. As long-chain fatty acids are almost depleted in beer as a consequence of yeast metabolism (Preedy, 2009), it makes sense that beer-spoiling LAB are capable to produce long-chain fatty acids by *de novo* biosynthesis. Interestingly, we found all three *P. damnosus* winery isolates to be *fabZ* negative, suggesting that the ability to produce fatty acids is no part of *P. damnosus´* lifestyle in wine. While beer contains about 15 to 30 mg/l fatty acids with a low proportion of long-chain fatty acids (C12 to C18), (red) wine is characterized by fatty acid concentrations of 270 to 960 mg/l, while long-chain fatty acids with chain lengths from C12 to C18 make up the majority (Yunoki et al., 2004). As there is no shortage of long-chain fatty acids in wine, winery *P. damnosus* do not rely on the ability to produce fatty acids *de novo*.

We do not conclude that *horC*, *horA*, *hitA*, or any other not yet characterized gene found on these brewery plasmids, have no significance for *P. damnosus* or *L. backii* beer spoilage ability. Nevertheless, our results indicate that these genes are of subordinate significance, in a hierarchical way. It is even likely that both, the FAS cluster and hop resistance genes like *horC*, are needed for successful growth in beer. This is supported by the inability of the winery isolate *P. damnosus* TMW 2.1643-NB, lacking *fabZ*, *horC*, *horA* and *hitA*, to grow in beer with an additional fatty acid source. Further, we characterized two strains of the very high hazard potential species *L. paucivorans*, being the only species besides *L. backii* and *P. damnosus* that was tested positive for *fabZ*. Only the brewery isolate TMW 1.1424-WB was able to grow

in wheat beer, additionally characterized by *horA* and *horC*. In contrast, the non-spoiling strain TMW 1.2063, which was isolated from a sausage, was negative for both hop resistance genes. On the one hand, this indicates that active hop tolerance mechanisms associated with the cell membrane have no value if the cell is not capable to build an intact membrane. On the other hand, it indicated that the ability to produce fatty acids *de novo* alone is also not sufficient for beer spoilage ability.

Interestingly, all brewery plasmidomes of *P. damnosus* as well as four out of five plasmidomes of *L. backii* carry one of two identified clusters (clusters 8/15, see Table 22, p. 109) containing genes for the uptake and utilization of biotin. Biotin is required as a covalently attached cofactor of the FAS-initiating enzyme acetyl-CoA carboxylase (Zhang and Rock, 2008). Cluster 8 in addition encodes for a cyclopropane-fatty-acyl-phospholipid synthase and a fatty acid binding protein of the DegV family. Both clusters were not found within the other core-species plasmidomes. The analysis of *P. damnosus* fatty acid composition, grown in beer at two pH values (5.0/4.3), showed in general a high proportion of (C19:0) cyclopropane fatty acids, which was also found to be enriched in case of the acid-stressed *L. brevis* TMW 1.465-SB (Behr et al., 2006). An incorporation of cyclopropane fatty acids is considered to render membranes less permeable, thus protecting the cell from unwanted penetration by small acids or hops (Beales, 2004; Schurr, Behr, et al., 2015), while cyclopropane fatty acids are generally considered to be important for bacterial stress tolerance (Montanari et al., 2010). Although some of these abovementioned functions (genes) are additionally chromosomally encoded (depending on strain/species, differently encoded on DNA level), their occurrence emphasizes the importance of fatty acid and membrane metabolism for *L. backii* and *P. damnosus* beer spoilage ability.

Previous findings based on analytical, proteomic and transcriptomic data support the hypothesis that *de novo* FAS and in general membrane lipid related metabolism are important processes for LAB beer spoilage ability (Behr et al., 2006; Behr et al., 2007b; Pittet et al., 2013; Schurr, Behr, et al., 2015). Pittet et al. (2013) found the chromosomal FAS cluster of *P. claussenii* TMW 2.340-MB to be upregulated in beer, while they also found other plasmid-encoded genes (part of *horA* cluster), which are associated with phospholipid biosynthesis, to be upregulated. The relevance of FAS is further supported by the fact that all other very high hazard and high hazard potential species encode for a complete FAS cluster. We checked all included LAB genomes for the presence of a complete FAS and found that 89 % of all very high and high hazard potential genomes (including *L. backii* and *P. damnosus*) encode for it, including all species of interest (with single strain exceptions). Interestingly, only 61 % of all other genomes were characterized by a complete FAS. Thus, hazard potential might generally be triggered by the ability to produce long-chain fatty acids.

We conclude that the availability of long-chain fatty acids is essential for successful growth of beer-spoiling LAB in the low fat environment beer. Consequently, the ability to produce fatty acids *de novo* is an important part of the lifestyle of *L. backii* and *P. damnosus* in beer, while this ability alone is apparently not sufficient for growth in beer, requiring additional, hop-tolerance-related and plasmid-encoded genetics.

5.2.2 A dynamic environment, a dynamic genome and the origin of beer-spoiling lactic acid bacteria

Breweries are sometimes extreme and very dynamic environments, characterized by complex, ever-changing, abiotic and biotic circumstances. There are temperatures from 4 to 100° C, pH values from 3.4 to 6.6, aerobic and anaerobic areas and a drastically changing nutrient situation, while all respective compartments are connected to each other making cross contamination a very likely process (Bamforth, 2001; Bokulich et al., 2015; Preedy, 2009). Bacteria are able to respond to changing environmental conditions by phenotypic plasticity or by adaptive evolution (genomic plasticity) (Casacuberta and Gonzalez, 2013). Mobile genetic elements including plasmids and transposons, which are referred to as “the flexible gene pool,” can be shared within an environment and contribute to adaptive evolution by HGT, thus allowing bacterial “evolution in quantum leaps” (Casacuberta and Gonzalez, 2013; Frost et al., 2005; Hacker and Carniel, 2001; Top and Springael, 2003; Wiedenbeck and Cohan, 2011). The flexible gene pool consists of mobile genetic elements, which might confer a selective advantage. It also contains so called selfish DNA-molecules e.g. IS elements, which are considered to simply promote their own spread (Hacker and Carniel, 2001). However, even those IS elements, which do not transfer functional DNA, e.g. a metabolic operon within a transposon, can have a positive effect on genome plasticity and thus on environmental adaption (Casacuberta and Gonzalez, 2013).

Beer-spoiling LAB can also be found within other areas of the brewery environment and are consequently exposed to the abovementioned varying environmental conditions. Phenotypic plasticity as a response to beer, hop or acid exposure was demonstrated here, but also in various other studies before, including metabolic, physiological and morphological adaptions (Asano et al., 2007; Behr et al., 2006; Behr et al., 2016; Geissler et al., 2016; Schurr et al., 2013; Suzuki et al., 2005b). Genomic plasticity, more precisely the uptake of hop resistance genes such as *horA* and *horC* by HGT, was already suggested to be responsible for the emergence/origin of beer-spoiling LAB, transforming originally non-spoiling strains into harmful contaminants (Suzuki, 2015). However, we suggest that the comparatively high number of mobile genetic elements, plasmids and IS elements (see 4.3.3.1, p. 89/4.3.3.3, p. 91) contributes to multiple niche adaption within the dynamic brewery environment. In accordance

with a previous study (Bergsveinson, Baecker, et al., 2015), we further suggest that only a defined amount of the (flexible) shared gene (plasmid) pool is actually obligatory or auxiliary for growth in beer, while other traits might confer distinct advantages within other brewery areas (see 5.2.1.2, p. 150), supporting the multiple niche hypothesis.

Brewery plasmids, important lifestyle genes and beer spoilage potential/ability have been further shown to be unstable, while we could confirm and extend the relation of mobile genetic elements (plasmids/transposon flanked cluster → composite transposons) to beer spoilage potential and ability. Further, we could show that the (in)stability of lifestyle genes (plasmids) relies on the selective pressure applied (see 4.4.2, p. 121), while the instability of spoilage potential and its relation to genetic instability was already known (Suzuki, 2015). We could show that even hop resistance genes, and presumably the corresponding plasmids, underlie a different selective pressure, while the lack of pressure apparently results in the increased loss of now unnecessary/adverse genetic material. On the other side, we obtained results which indicate that beer spoilage potential, and thus the potential for a better environmental adaption, can be regained by a potential uptake of brewery DNA upon selective pressure. Initial transformation/co-incubation experiments indicate that *P. claussenii* is able to improve its beer spoilage potential in the presence of beer-spoiling DNA (in the form of a pasteurized *horC* and *horA* positive beer-spoiling strain) in beer, although this has to be rechecked carefully (see 4.4.2, p. 121). However, Haakensen et al. (2008) found that several LAB strains changed their DMG profile (e.g. *horA* gain) after repeated passages in beer. This supports the idea of short-term adaption by genomic plasticity and DNA uptake in beer.

The significance of genomic plasticity for brewery LAB within the dynamic brewery environment is further indicated by the finding that 95 % of all published and novel identified shared genetic clusters were found to be either flanked by one or two transposases, or to contain an IS element within the cluster, thus indicating a potential distribution via a transposon-mediated way. This is supported by the finding that identical clusters were repeatedly found on quite different plasmids (see 4.3.4, p. 110). IS elements and transposons may act as vectors for HGT, while it is known that these mobile genetic elements can confer new metabolic capabilities or defense mechanisms, thus representing an advantage for bacterial adaption (Casacuberta and Gonzalez, 2013). The comparatively high number of IS elements within brewery genomes, also correlative to spoilage potential in case of some species (*L. brevis*, *P. damnosus*), might further contribute to niche adaption and adaption to changing conditions by increasing the mutation rate within a single cell or population independently from a potential transfer of genetic material. As environmental stress can induce transposition events, they are finally considered to play a key role in translating environmental

changes (stress sensing) into changes at the genomic level, thus playing a very important role for environmental adaption (Casacuberta and Gonzalez, 2013).

P. damnosus, *L. lindneri* and *L. paracollinoides* are suggested to be brewery-specific microorganism. Similarly, *L. backii* is considered to be unique to the brewery environment (Suzuki, 2015). Despite the fact that the three first named species were also isolated from other sources (Bae et al., 2006; Delaherche et al., 2004; Juega et al., 2014; Ladero et al., 2011; Lucena-Padros et al., 2014), we think this statement has to be questioned. Beer virtually lacks any long-chain fatty acids (Preedy, 2009), thus making their biosynthesis obligatory. Still we found two important beer-spoiling species, *P. damnosus* and *L. backii*, to be characterized by a plasmid-encoded FAS cluster, concurrently lacking a stable chromosomal FAS. Neither *horA* nor *horC* have been ever found to be chromosomally and thus stably encoded by beer-spoiling LAB. Beer contains all 20 amino acids as well as various peptides; still *L. paracollinoides* and *L. backii* seem to be prototroph for all or almost all amino acids, respectively (10, p. 239). Further, *L. paracollinoides*, in general characterized by an extensive predicted metabolic capacity, is predicted to be capable of producing various vitamins of the B group (10.5.3, p. 284), while these nutrients are apparently present in sufficient amounts for LAB growth in beer. This is supported by the fact that those species without the predicted ability to produce B-vitamins, such as *P. damnosus*, grow successfully in beer. The same is true for the amino acid example, where *L. brevis* demonstrates that intensive amino acid biosynthesis is not necessary for growth in beer (10.6.2, p. 292). Why keep these traits, in case these organisms are indeed brewery-specific and closely associated to breweries over time? *L. lindneri* successfully demonstrates that only little metabolic capabilities and a small genome (10.4, p. 269) are necessary for growth in beer. Altogether, this argues against a long-term adaption or an actual “speciation” of these species within beer. Further, we conclude that these species are not brewery-specific but brewery-typical, probably introduced into the brewery from other (plant) sources. Bokulich et al. (2015) could show that hop pellets as well as grain samples are contaminated by important very high hazard LAB. They also found that the contact with beer predicts the distribution of hop resistance genes such as *horA*, *horC* and *hitA*, on brewery surfaces. While they found LAB such as *L. brevis*, *L. lindneri* or *Pediococcus* on hop pellets, they could not detect the abovementioned hop resistance genes on this raw material. This indicates that in the moment these bacteria meet the respective DNA (flexible shared gene pool), they acquire the ability to grow in beer. Thus, we coincide with the hypothesis of Suzuki (2015): hop resistance genes and brewery DNA chose LAB as their hosts for their own survival (selfish genes) and conversely LAB “allow” (selective pressure) the state of “symbiosis” in order to gain decisive advantages within the brewery environment.

HGT and mobile genetic elements allow bacteria to build on their unique, pre-existing adaptions to either invade new niches, adapt to changing environments or in order to improve their performance in the current niche (Casacuberta and Gonzalez, 2013; Cohan and Koeppel, 2008; Frost et al., 2005; Hacker and Carniel, 2001). Mobile genetic elements are just like open source software: try, improve and share with others (Frost et al., 2005). In conclusion, we suggest that beer-spoiling LAB are not necessarily (solely) persistent residents of the brewery environment, long-term and statically adapted to a single niche like beer, waiting for their chance. Instead, we suggest that these bacteria are introduced into the brewery from raw materials, while they adapt to the dynamic brewery environment by an uptake and a release of niche-specific genes from a flexible (shared) gene pool. This genomic plasticity is further increased due to transposable elements. The coding of capabilities which are necessary in one part of the brewery but not in another (e.g. hop resistance, raw materials ↔ wort/beer) within unstable entities, makes sense in an ever-changing environment. Thus, we conclude that a high degree of genomic plasticity is part of the lifestyle of brewery and beer-spoiling LAB within the brewery environment.

5.2.3 Shared metabolic strategies and features

The whole chapter 5.2.3 partially corresponds to a recent publication about the metabolism of LAB in beer (Geissler et al., 2016).

As a consequence of fermentation by yeast, beer contains only limited amounts of readily fermentable substrates, which is considered as another antibacterial hurdle bacteria have to cope with (Suzuki et al., 2005b; Vriesekoop et al., 2012). This accepted statement is found all over literature and will be questioned in subsequent sections. Further, it is suggested that active hop tolerance mechanisms act as a first line defense, while these defense mechanisms are considered very energy consuming (Sakamoto et al., 2001; Suzuki, 2015; Suzuki et al., 2005a; Vogel et al., 2010). Altogether, it is conceivable that a multi-hurdle, harsh environment such as beer requires a sufficient amount of energy generation by beer-spoiling LAB in order to survive and grow. Therefore, we investigated the metabolic capabilities and metabolic strategies of LAB with very high hazard potential, growing in beer and under hop stress, in order to answer the question if there are distinct and obligatory metabolic capabilities for beer spoilage ability. Shared metabolic strategies, which outline a distinct and common feature of LAB lifestyle in beer, will be detailed in this chapter, as well as generally valid statements about LAB metabolism in beer and under hop stress. Species-specific traits will be dealt with in the corresponding species chapters (5.2.4, p. 178).

5.2.3.1 The role of metabolic capabilities for beer spoilage potential

Based on the available data about the composition of beer regarding carbon, energy and nitrogen sources (Preedy, 2009) and supplemented by the analytical data obtained within this study, we suggest that there is no actual “lack” of these compounds in beer. In contrast, beer is a rather substrate-diverse and nutrient rich environment, offering bacteria various carbohydrates, all kind of amino acids and other nitrogen sources. This substrate-diversity makes it possible that LAB with very different metabolic capabilities and strategies (e.g. *P. damnosus* ↔ *L. brevis*) are able to grow successfully in beer. Consequently, we found that the (measured) metabolic capabilities (HPLC, lager$_{pH5.0}$) of LAB species with very high hazard potential, regarding carbohydrate, amino acids and organic acid metabolism, did not correlate to beer spoilage potential or beer spoilage ability, but to fermentation type (homofermentative/ heterofermentative) and species. To be exact, this means that we did not find a specific metabolic trait or pathway, related to the abovementioned substance classes, which is obligatory for beer-spoiling LAB or which differentiates beer-spoiling from non-spoiling strains. The corresponding metabolic capabilities were almost exclusively found to be conserved (HPLC data/chromosomally encoded) within a species, thus showing that they are not decisive with respect to beer spoilage potential.

In contrast to the other substance classes, long-chain fatty acids are indeed depleted in beer, most probably by yeast fermentation (Preedy, 2009). We consequently suggest that the metabolic capability to produce fatty acids is in general mandatory for beer-spoiling LAB in order to grow in beer.

5.2.3.2 Shared strategies and features

We found a relation of metabolism in beer to fermentation type (homo-/heterofermentative), as well as distinct species-specific strategies, showing that there is no exclusive metabolic strategy which has to be used for energy generation and growth in beer. Shared strategies and features will be discussed below.

5.2.3.2.1 Global strategies

Altogether, we found that beer-spoiling LAB have a more diverse metabolism in beer as previously expected, while the spectrum of relevant substrates for heterofermentative LAB, including organic acids and arginine, was confirmed and extended by a diverse sugar metabolism and the utilization of various amino acids. Homofermentative LAB are characterized by a distinct, mostly species specific carbohydrate metabolism and a

consequent focus on the uptake of these substrates via energy-efficient PTS systems, a less pronounced or even lacking amino acid metabolism and a shift to mixed acid fermentation in case of *L. backii* and *P. claussenii*.

Interestingly, we found beer-spoiling LAB not generally focused on those carbohydrates, which are considered by brewers to be the major beer saccharides. These include sugars such as glucose, fructose and maltose, while all of them are so-called fermentable sugars, indicating their usage/usability by yeast. Their content in beer varies dramatically, depending on the performance of yeast fermentation and the initial wort composition, while a summary of sugar contents in various beers all over Europe reveals extreme variations (e.g. glucose 0 to 8 g/l) (Preedy, 2009). Thus, it might be a specific strategy of beer-spoiling LAB to focus on those substrates, which are not fermented by yeast and probably are not subjected to these strong fluctuations. Further, we suggest that beer-spoiling LAB can be globally characterized by metabolic capabilities and strategies to avoid the formation of acidic end products, especially lactic acid, in order to reduce the acid stress and the concomitant increase in hop´s antibacterial properties. Especially heterofermentative species are characterized by the production of biogenic amines, while the underlying metabolic pathways, in accordance with previous literature (Behr et al., 2006; Schurr et al., 2013; Suzuki et al., 2005b; Suzuki et al., 2006), are suggested to play an important role for their pH homeostasis and hop tolerance in beer.

There are many conceivable metabolic adaptions of LAB to unfavored conditions, including alternative pyruvate metabolism, mixed acid fermentation, the utilization of alternative electron acceptors (organic acids, fructose) as well as energy and pH-homeostasis related amino acid metabolism (Holzapfel and Wood, 2014; Mozzi et al., 2010; van de Guchte et al., 2002). Based on genome analysis and the comprehensive evaluation of LAB metabolism in beer, we conclude that beer-spoiling LAB, although by a different extent, apply all of these strategies for a successful growth in the harsh environment beer.

5.2.3.2.2 Homofermentative LAB

Homofermentative beer-spoiling LAB can be characterized by a more or less species-specific and distinct carbohydrate fermentation profile in beer, with a focus on hexoses, sugar alcohols and disaccharides, especially trehalose in case of pediococci and mannitol instead of disaccharides for *L. backii* strains. Interestingly, carbohydrates were preferred by all three species, which were predicted to be utilized via PTS systems. Exemplary, trehalose was found to be the preferred carbohydrate in beer in case of *P. damnosus* and *P. claussenii*, while both species encode a utilization of trehalose via PTS uptake and trehalose-6-phosphate hydrolase

(EC 3.2.1.93). PTS systems most probably represent the most efficient active transport systems from an energetic point of view, as only one ATP is needed for transport and activation of a sugar (Neves et al., 2005). The hydrolysis of trehalose-6-phosphate consequently results in glucose-6-phosphate and glucose, which can be further metabolized via glycolysis. Pittet et al. (2013) could show that genes involved in the PTS-uptake and utilization of trehalose and mannitol, by *P. claussenii* TMW 2.340 (MB, ATCC BAA-344T), were up-regulated during growth in beer.

We further found that homofermentative LAB are characterized by metabolic capabilities and strategies to reduce the production of acidic end products, especially lactic acid. Lactic acid is considered to have a very inhibitory effect on LAB at a low pH, which is due to its function as a weak acid, while also other mechanisms of lactate toxicity have been suggested (Siezen et al., 2002). *P. claussenii* and *L. backii* are characterized by a shift to mixed acid fermentation upon growth in beer. We could neither detect production of acetate by *P. claussenii* and *L. backii* strains growing in MRS at two different pH values (acid stress) nor under hop stress, while acetate was produced in beer at both pH values. Thus, we conclude that the shift to mixed acid fermentation is not solely triggered by a low pH or hops, but represents a specific response of these LAB to the complex conditions found in beer. Our results further show that the production of acetate is preferred over the production of lactate in beer. In case of *P. claussenii*, the acetate apparently results from citrate fermentation, which is supported by analytical data, genome analysis and a transcriptomic study with TMW 2.340-MB performed by Pittet et al. (2013). Based on *in silico* prediction, citrate is finally decarboxylated to acetate and pyruvate without the generation of NADH. As no NAD has to be recycled, *P. claussenii* is free to generate extra ATP via the acetate kinase (EC 1.2.3.3), resulting in even more acetate (see Figure 45, p. 173). Alternatively, pyruvate can be decarboxylated to the non-acidic end products diacetyl and acetoin, reducing the acid load due to the own metabolism. Additional energy, the fact that no reducing equivalents are formed (Hugenholtz, 1993) and the potential contribution of citrate fermentation to pH homeostasis (Siezen et al., 2002) make citrate fermentation an attractive pathway in beer. *L. backii* strains were not found to utilize citrate nor to encode for the metabolic capability. Thus, the acetate is apparently the product of acetate kinase (EC 1.2.3.3), which indicates that *L. backii* might use an alternative electron acceptor to recycle its NAD in order to produce more ATP via acetate production. *P. damnosus* was not found to produce acetate, while based on genome analysis, a production of acetate or ethanol is possible in the presence of oxygen. Nevertheless, we suggest that also *P. damnosus* switches his pyruvate metabolism, from lactate production only, to diacetyl, acetoin and butanediol production in order to restrict excessive lactate production and acidification of the environment (for details see 5.2.1.2, p. 150).

Besides citrate utilization by *P. claussenii*, we found all three homofermentative species to utilize the organic acids pyruvate and malate, while the advantage of pyruvate for a strictly fermentative organism is self-explanatory. The benefits by malolactic fermentation regarding pH homeostasis and energy generation were already described in a previous chapter (see 5.2.1.2, p. 150). Genes related to malate and citrate utilization were found to be upregulated in *P. claussenii* ATCC BAA-344 (TMW 2.340-MB) upon growth in beer (Pittet et al., 2013).

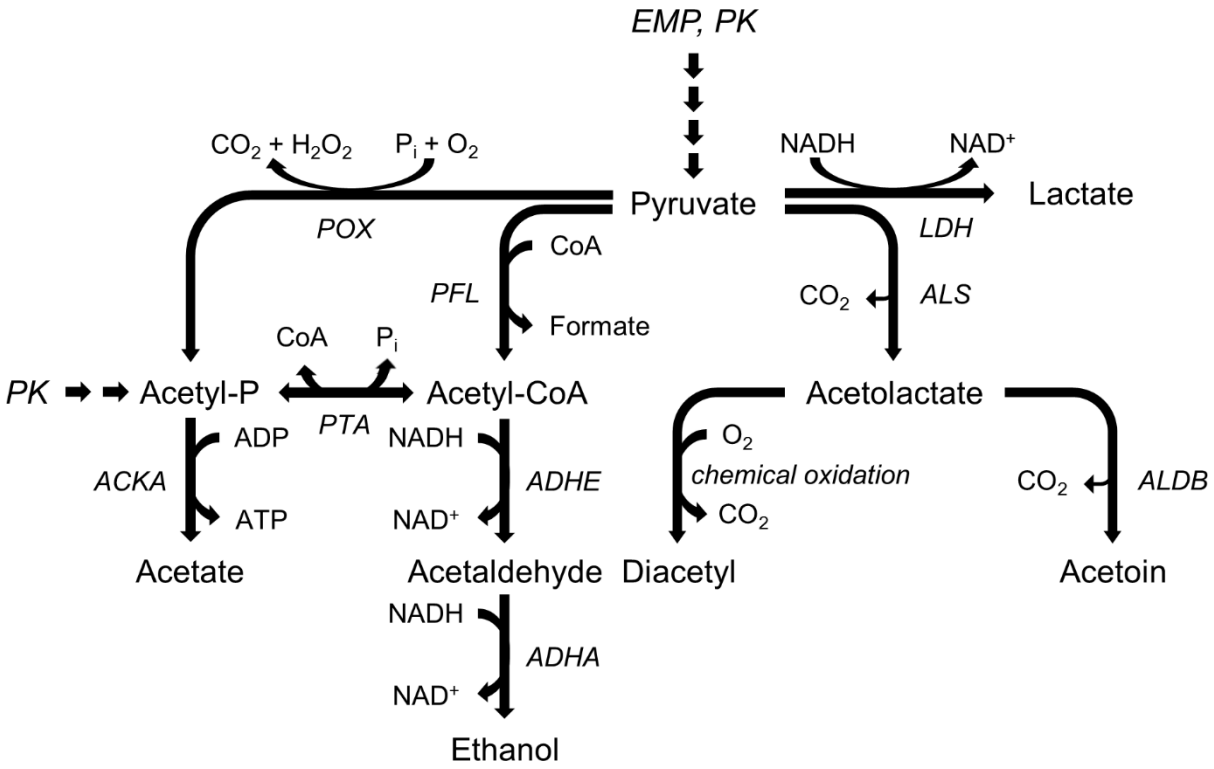


Figure 45: Pyruvate metabolism of *P. claussenii* and *L. backii* based on *in silico* prediction. EMP = Embden–Meyerhof–Parnas, PK = Phosphoketolase (EC 4.1.2.9), LDH = lactate dehydrogenase (EC 1.1.1.27/28), ALS = acetolactate synthase (EC 2.2.1.6), ALDB = alpha-acetolactate decarboxylase (EC 4.1.1.5), POX = pyruvate oxidase (EC 1.2.3.3); ACKA = acetate kinase (EC 2.7.2.1), PFL = pyruvate formate lyase (EC 2.3.1.54), PTA = phosphate acetyltransferase (EC 2.3.1.8), ADHE = acetaldehyde dehydrogenase (EC 1.2.1.10), ADHA = alcohol dehydrogenase (EC 1.1.1.1).

The relevance of pH homeostasis related amino acid metabolism for growth in beer and acid/hop tolerance was shown for heterofermentative LAB in previous studies (Behr et al., 2006; Schurr et al., 2013; Suzuki et al., 2005b). Transcriptomic analysis revealed the chromosomal AGDI pathway of *P. claussenii* ATCC BAA-344 (TMW 2.340-MB) to be the most affected gene cluster within its genome, comparing growth in MRS and beer. The transcript levels of the respective mRNAs were increased 64 to 269 fold compared to MRS (Pittet et al., 2013). We found all sequenced *P. claussenii* strains to encode for the AGDI pathway, while all

four physiological characterized strains consumed agmatine and produced putrescine. Whereas the AGDI pathway is stably encoded within the *P. claussenii* chromosome and thus most likely a species-specific trait, putrescine production from agmatine was found to be a strain-specific and plasmid-encoded trait in case of *L. backii* TMW 1.1991-SB. This was already discussed in chapter 5.2.1.2 (p. 150), together with the beneficial aspects of the AGDI pathway regarding pH homeostasis and ATP generation. All *L. backii* strains were found to be capable to decarboxylate tyrosine to tyramine in MRS. While free tyrosine is also present in beer, that specific metabolic trait does not seem to be too important for *L. backii* growth in beer, as only one strain, TMW 1.1430-SB, actually used this system in beer. It is further interesting to note that we were not able to identify the corresponding enzymes and transport system, neither on DNA, protein or functional level. *P. damnosus* is characterized by the lack of any amino acid deiminase or decarboxylase ability/activity. Thus, *P. damnosus* is the only very high hazard potential species investigated, which is apparently not producing any biogenic amines, making this species from a sanitary point of view to the best suited for biological acidification of wort or the production of sour beers. Compared to heterofermentative LAB, the investigated homofermentative species, especially *P. damnosus*, are characterized by a less pronounced amino acid metabolism, indicating alternative strategies of pH homeostasis, which were already suggested above (avoidance of acidic end products/alternative pyruvate metabolism/mixed acid fermentation). Alternatively, the respective bacteria do not rely on a transmembrane gradient, allowing intracellular acidification.

In average 61 % of the total acid produced in $lager_{pH5.0}$ or $lager_{pH4.3}$ could be explained by the sugar catabolism investigated, while the % theoretical contribution of all carbohydrates was calculated under the assumption that all consumed sugars are finally degraded to lactic and acetic acid. As it is very likely that a distinct proportion of carbohydrates is either used for anabolism or finally degraded to an alternative end product, which was not quantified here, the observed carbohydrate metabolism represents even less of the actual fermentative metabolism of these LAB. Other energy sources such as citrate, malate and pyruvate have to be considered as well as not investigated carbohydrates, e.g. cellobiose, kojibiose or maltotriose and amino acids.

5.2.3.2.3 Heterofermentative LAB

Metabolism of heterofermentative LAB was more heterogeneous between and within the investigated species, while especially strains of *L. paracollinoides* showed a high intraspecies variance. As in case of homofermentative pediococci, the metabolism of disaccharides seems to play an important role for growth in beer of *L. paracollinoides* and *L. brevis*. In contrast to homofermentative strains, all three heterofermentative species are capable to ferment the

available pentoses, while these are interestingly not the preferred substrates (compared to hexoses/disaccharides) for *L. lindneri* and *L. paracollinoides.* Nevertheless, this should not be over interpreted, as the theoretical contribution of the investigated carbohydrates to total acid production was quite low for both species, indicating that other substrates play a major role. However, while Suzuki et al. (2005b) identified organic acids and arginine as major substrates of heterofermentative LAB in beer, we found all three species to utilize at least two different sugars, in case of *L. brevis* up to eight different. Thus, we conclude that the utilization of carbohydrates is also of relevance for heterofermentative LAB growth in beer.

As in case of homofermentative LAB, we observed a tendency to produce acetate rather than lactate, illustrated by lactate to acetate ratios near 1 or less. A switch to alternative pyruvate metabolism by beer-spoiling *L. brevis*, because of acid and hop stress, was already suggested (Behr et al., 2006; Behr et al., 2007b). Based on genomic predictions, *L. brevis* and *L. paracollinoides* have the ability to produce diacetyl, acetoin and butanediol, besides lactate, acetate and ethanol. These alternative fates of pyruvate allow the recycling of NAD and the production of non-acidic end products. *L. lindneri* apparently only encodes the formation of lactate, acetate and ethanol from pyruvate and phosphoketolase pathway, lacking an alpha-acetolactate operon and thus lacking the ability to produce acetoin and diacetyl. Interestingly, both genomes were found to encode for acetoin reductase (EC 1.1.1.4), allowing the utilization of diacetyl and acetoin as electron acceptors. Diacetyl is not only a metabolic product of beer-spoiling LAB, but also a metabolic product of yeast fermentation (Preedy, 2009), while it was already mentioned that *L. lindneri* is also considered to be latent during wort fermentation (Back, 2005; Storgards et al., 1997; Suzuki, 2015). Thus, the metabolic capability to use diacetyl as extern electron acceptor could be an advantage for *L. lindneri*, resulting in more energy generation and less lactate production.

Organic acids were used as substrates by all heterofermentative species investigated, while only pyruvate was utilized by all three species. *L. paracollinoides* did show neither citrate fermentation nor malolactic fermentation, which is in accordance with a previous study (Suzuki et al., 2005b). However, we found all three sequenced strains to encode for malolactic fermentation and TMW 1.1995-SB for citrate fermentation. As these pathways were apparently not used in beer, we conclude that the metabolic versatile species *L. paracollinoides* has alternative strategies for pH homeostasis. As in case of *P. claussenii*, *L. lindneri* seems to focus on organic acid metabolism, indicated by malate and citrate fermentation. Malolactic fermentation was found to be a stable trait of *L. brevis*, whereas citrate fermentation is strain specific, which is in accordance with the genome analysis and previous observations (Fryer, 1970). Altogether, we could confirm the relevance of organic acid metabolism for heterofermentative LAB (Suzuki et al., 2005b), although we conclude that citrate fermentation

is not necessary for *L. brevis*, as none of the analyzed beer-spoiling strains was characterized by citrate utilization nor by the metabolic capability.

Amino acid metabolism, decarboxylation and deiminase reactions were found to be very prominent within heterofermentative LAB. All species included strains producing at least one biogenic amine, while especially *L. brevis* uses most known pH and energy-generation related systems, including the ADI, AGDI as well as the GAD system, additionally characterized by tyrosine decarboxylase. *L. brevis* converted 97 +/- 3 % of all available precursors to biogenic amines, emphasizing the importance of amino acid metabolism for this species, while a relevance of amino acid metabolism for acid and hop tolerance was indicated repeatedly (Behr et al., 2006; Schurr et al., 2013; Suzuki et al., 2005b; Suzuki et al., 2006). An addition of tyrosine, arginine and glutamate to beer improved the growth of *L. brevis*, especially remarkable in case of non-spoiling strains. This is also in tune with the findings of Fernandez and Simpson (1995) that the content of some free amino acids correlated negatively to the susceptibility of beer towards spoilage, as *L. brevis* TMW 1.1369-WB, normally only able to grow in wheat beer, showed the highest turbidity (compared to strong spoilers) in lager beer with the addition of the abovementioned amino acids. However, the marked biogenic amine production of *L. brevis* makes this organism also less suitable for technological applications such as sour beer production, at least from a sanitary point of view. Tyramine and histamine are considered to be potential health risks for some consumers, e.g. using monoamine oxidase inhibitors, while histamine was found to be produced by *L. lindneri*, which is confirmed here (Kalač et al., 2002; Preedy, 2009). Kalač et al. (2002) suggested that the formation of biogenic amines in beer is mainly due to contaminations with LAB, while it was also concluded that lactobacilli are more important biogenic amine producers compared to pediococci. This can be partially confirmed, while we would rather suggest that the production of biogenic amines in beer is mainly done by heterofermentative very high hazard LAB. An active pH homeostasis, presumably triggered by the amino acid metabolism of heterofermentative LAB is illustrated exemplary by a simple experiment, where we added the amount of in beer produced acids (by *L. lindneri* TMW 1.1433-MB) to a non-fermented beer. The pH of the chemically acidified control was 4.3, while the pH of beer acidified by *L. lindneri* TMW 1.1433-MB was 4.7.

As in case of the homofermentative LAB, our data show that we did not discover the entirety of heterofermentative LAB energy and carbon metabolism in beer. Especially for *L. paracollinoides* and *L. lindneri*, a further analysis is necessary to reveal additional energy and carbon sources by these bacteria, while in case of *L. paracollinoides* maltotriose and maltodextrin are likely substrates (Suzuki et al., 2005b). The observed increase of glucose and fructose concentrations upon growth in beer suggests the degradation of oligosaccharides.

5.2.3.2.4 The energy demand of hop tolerance and the avoidance of lactic acid production under hop stress

Hops are considered to increase the energy demand by LAB. This is firstly because hops act as pH dependent proton ionophores (Behr and Vogel, 2009; Simpson, 1993b) thus reducing the pmf and consequently the pmf dependent uptake of substrates (Suzuki, 2011). Secondly, hop tolerance itself is considered energy-intensive, especially as the active hop transporters HorC and HorA rely on energy in terms of ATP and pmf (Sakamoto et al., 2001; Suzuki, 2015; Suzuki et al., 2005a; Vogel et al., 2010). In order to illustrate the observed metabolic responses to hop exposure, the reader is supplied with a distinct example, on the basis of *P. claussenii* TMW 2.54-SB (*horA* and *horC* positive), while all proportions adduced refer to a reduction or increase of a parameter, comparing growth in a MRS variant with pH 4.3 without (acid stress) and with additional hops (hop stress):

- The OD_{590} as a measure for cell density decreased by 49 % under hop stress
- The production of lactic acid decreased by 49 % under hop stress
- Despite a decrease of cell density and lactic acid production, the amount of consumed carbohydrates increased by 22 % under hop stress
- Despite an increased carbohydrate utilization, the pH decreased to a lesser extent under hop stress: acid stress → pH 3.4; hop stress → pH 3.9

These findings apply to the other very high hazard LAB species, while we found this effect to be especially prominent in case of pediococci and *L. paracollinoides* and least distinct for *L. brevis*. Based on the findings there are two major conclusions:

1. Hop exposure, a potential metabolic/anabolic (e.g. cell wall modification) response to it and/or hop tolerance cause an increased substrate and energy demand in beer-spoiling LAB. We were able to demonstrate an increased substrate utilization in relation to cell density, as a distinct response to hop exposure.

2. Upon hop exposure, beer-spoiling LAB reduce the broth-acidification (to a minimum), which makes sense considering that the antibacterial properties of hops increase by ~50 % with a decrease of the pH of 0.2 units (Simpson, 1993b; Simpson and Smith, 1992). Despite a relative, in several cases even an absolute increase of carbohydrate utilization, we observed a reduced production of lactic acid and comparatively little acidification under hop stress.

The distinct discrepancy of carbohydrate utilization and lactate production under hop stress either results from anabolic processes or a shift in pyruvate/acetyl-phosphate, metabolism. Only *L. brevis* was shown to produce acetate under acid and hop stress. This indicates that the other core-species shift their metabolism towards non-acidic products, such as ethanol, acetoin, diacetyl and butanediol, which is in accordance with their predicted metabolic capabilities. A similar metabolic response as described above, although less prominent, was observed comparing the metabolism of core-species in $lager_{pH5.0}$ and $lager_{pH4.3}$ (decrease in pH → increase in hops antibacterial properties). Thus, we conclude that beer-spoiling LAB show a specific metabolic reaction to the exposure with hops, resulting in an increased substrate utilization and energy demand and the avoidance of acidic products.

5.2.4 Species-specific strategies and characteristics - Lifestyle and detection

The vast majority of all information and publications about beer-spoiling LAB is based on research with *L. brevis*, while many principles and concepts were simply extrapolated to other species. Based on a comprehensive genome analysis and supported by metabolic, physiological and genetic data, we shortly describe six important very high hazard potential LAB beer species with respect to relevant characteristics, as well as their individual metabolic and physiological capabilities and strategies. Based on these characteristics we aim to give insights into the lifestyle of these important brewery contaminants in beer as well as ideas how to handle these organisms in quality control. If not stated otherwise, comparison of parameters refers to a comparison of these parameters within the core-species.

5.2.4.1 Pediococcus claussenii

Strains of *P. claussenii* spoil a typical lager beer within 3 to 30 days, causing turbidity and acidification similar to *L. brevis*, as well as the production of the biogenic amine putrescine. Based on *in silico* prediction *P. claussenii* is also capable of diacetyl “production,” which is in accordance with previous observations (Hutzler et al., 2013; Suzuki, 2015). All tested isolates originate from the brewery environment and are characterized by beer spoilage ability, while beer spoilage potential was unstable in 25 % of all cases. *P. claussenii* is well adapted to beer, which is indicated by a high tolerance and adaption to beer specific hurdles, while cell density was hardly affected by a beer typical pH of 4.25, an ethanol content of 6 % (vol/vol) and a hop content of 15 ppm (~ lager beer). Within the core-species, we found *P. claussenii* to have the highest tolerance to a low pH (MIC 2.75) and ionophores, altogether suggesting that *P. claussenii* either does counteract intracellular acidification very effectively or does not rely on pmf, potentially using alternative ion gradients. The utilization of the AGDI pathway, malate and citrate fermentation and the concomitant switch to mixed acid fermentation might be good

candidates for an active pH homeostasis and thus be important for its lifestyle in beer, while the significance of these metabolic traits is in accordance with previous findings (Pittet et al., 2013). The high tolerance towards ionophores (including K^+ and divalent cations) might be explained by a comparatively (to all other included genomes) high number of ion transport and homeostasis related genes, as well as a high number of LTA biosynthesis related genes, while LTAs are considered to be important for ion homeostasis and hop tolerance (Behr et al., 2006).

A generally high basal hop tolerance (MIC 26 +/- 7 ppm) did not correlate to beer spoilage ability, while even strains lacking known hop transport genes, were characterized by a basal hop tolerance and beer spoilage ability. Thus, *horA* and *horC* are not obligatory for beer spoilage ability and basal hop tolerance of *P. claussenii*. Kind of opposed to this, we found that growth in beer with a higher hop content, pilsner beer 1 with 26 ppm, apparently relies on the presence of at least one "*hor"*-gene. The presence of both lifestyle genes had a significant impact on beer spoilage potential, also illustrated by cell density and adaption time, while *horC* is the preferred/dominant hop tolerance gene/mechanism, as previously found for *L. brevis* BSO 464 (Bergsveinson, Baecker, et al., 2015). This suggests *horC* to be the best-suited beer-spoilage-lifestyle-gene for *P. claussenii*, which can be confirmed based on our DMG screening.

Oxidative stress tolerance at a low, beer typical Mn^{2+}/Mg^{2+} content correlated to beer spoilage potential, emphasizing the role of oxidative stress in beer. We could identify several oxidative-stress-related, plasmid-encoded genes in case of the sequenced strains, including a ferredoxin reductase and a potential NADH peroxidase (DMG M42), while the latter gene was found to be encoded by ten of 15 characterized strains. Thus, we suggest that oxidative stress tolerance is an important property of beer-spoiling *P. claussenii*.

Our physiological and analytical data, together with the available genome analysis, fit to previous data about the transcriptome of *P. claussenii* in beer (Pittet et al., 2013), allowing us comparatively deep insights into the metabolism of this species growing in beer. Energy is generated from glucose, fructose, gentiobiose, trehalose and mannitol, whereas these substrates, with exception of glucose, are apparently taken up via energy-efficient PTS systems and consequently channelled into glycolysis. Cellobiose utilization (also via PTS) in beer was indicated in a previous study (Pittet et al., 2013). Further energy is generated from pyruvate, malolactic fermentation and citrate fermentation, while the latter trait is presumably mainly responsible for the acetate production by this homofermentative LAB species. The metabolism of these organic acids might contribute to pH and redox homeostasis by NAD recycling. There is no distinct metabolism of any free amino acids, while the observed conversion of the aminoguanidine agmatine to putrescine, via the AGDI pathway, results in ATP production and alkalization. The addition of peptone to beer had a significant growth

enhancing effect on *P. claussenii*, suggesting that the utilization of peptides might also be part of its metabolic strategy. Based on genome prediction, *P. claussenii* encodes for the respective transport and degradation systems, while beer is known to provide a part of its nitrogen in terms of peptides (Preedy, 2009). As *P. claussenii* is further auxotroph for the majority of all amino acids, it is likely that the utilization of peptides is important for its successful growth in beer. The beneficial effect of peptone is not due to a potential increase in agmatine content, which was tested.

Finally, it is suggested to (re)classify *P. claussenii* as a very high hazard potential LAB species, while an intraspecies differentiation in quality control, e.g. using *horC*, is not recommended and rewarding because of the high proportion of beer spoilers with partially unstable beer spoilage ability, the low abundance of this species in breweries (cost-benefit ratio) and the ability of *horA*/*horC* negative strains to spoil beer.

5.2.4.2 Pediococcus damnosus

P. damnosus is quite contrary compared to *P. claussenii*. Spoilage of a typical lager beer was characterized by sedimentation and acidification, while visible spoilage occurred after 6 to 30 days. Only brewery isolates are predicted to be capable to produce the unwanted off-flavor diacetyl, which was confirmed exemplary (in lager beer 1), while *P. damnosus* is not characterized by the production of biogenic amines. *P. damnosus* is characterized by a distinct, stable and transferable strain specific beer spoilage potential, while the ability to grow in beer was found to definitely rely on a plasmid-encoded trait. The role of *fabZ*, the availability of fatty acids and their biosynthesis was already discussed in detail in previous chapters (see 5.2.1.3, p. 164). However, the fact that an essential metabolic capability for growth in beer is plasmid-encoded instead of stably integrated into the chromosome (together with various other adaptions to that specific environment), is in sharp contrast to the idea that *P. damnosus* is a brewery-specific microorganism. However, the ability to produce fatty acids in the low-fat environment beer is a distinct feature of *P. damnosus* lifestyle in beer, but apparently not in wine, which is a beverage characterized by a comparatively high content of long-chain fatty acids (Preedy, 2009; Yunoki et al., 2004).

In contrast to *P. claussenii*, *P. damnosus* is characterized by the least tolerance and adaption to beer specific hurdles, which is interesting considering the success of this species as the second abundant beer-spoiling LAB species. There was no shift to mixed acid fermentation, no citrate utilization and no detectible or any potential (predicted) amino compound metabolism related to energy generation and pH homeostasis. All other core-species were found to encode for at least one of the abovementioned metabolic capabilities. Consequently, it is logical that

P. damnosus is characterized by the lowest tolerance and adaption to acid stress, while this does not mean that *P. damnosus* employs no metabolic strategies in order to reduce the acid stress in beer. Malolactic fermentation and especially a shift of pyruvate metabolism towards non-acidic end products are likely to contribute to pH and redox homeostasis (see 5.2.1.2, p. 150).

Hop tolerance in MRS did not correlate to beer spoilage ability and potential, while it was already suggested in a previous section that hop tolerance of *P. damnosus* might be triggered by other beer specific properties (5.1.4, p. 146). Nevertheless, our results indicate that one of the "*hor*"-genes, together with the FAS cluster, is necessary for successful growth in beer, while we could not establish a correlation of hop resistance to the presence of these genes. Besides the indicated significance of both hop tolerance related clusters for growth in beer, our results further suggest an importance of oxidative stress tolerance (NADH peroxidase, M42), ion homeostasis and cell wall (LTA) metabolism for *P. damnosus* in beer.

Carbohydrate metabolism is more diverse as expected, with a focus on disaccharides. Glucose, fructose, galactose, isomaltose and preferably trehalose and gentiobiose are used as carbon and energy sources in beer. With the exception of glucose and isomaltose, substrates are taken up by PTS systems and utilized via glycolysis. Galactose-6-phosphate is metabolized using the tagatose-6-phosphate pathway. Isomaltose is transported into the cell using a potential ABC transporter and further hydrolyzed by a oligo-1,6-glucosidase (EC 3.2.1.10), resulting in glucose for further utilization. The other disaccharides are preferred over isomaltose, emphasizing the focus on energy efficient uptake of substrates via PTS systems. Pyruvate and malate are used as substrates, while malate is presumably utilized via malolactic fermentation. Carbohydrates are further degraded to lactic acid and presumably to diacetyl, acetoin and butanediol, in order to recycle NAD and for pH homeostasis. Amino acids for protein biosynthesis apparently derive from peptide degradation, as *P. damnosus* does neither consume reasonable amounts of amino acids nor possess the ability to produce all of them *de novo*. Fatty acids are produced *de novo* and modified to cyclopropyl fatty acids in order to obtain a membrane with a reduced permeability to small acids and hops (see 5.2.1.3, p. 164).

P. damnosus comprises strains with and without a stable ability to grow in beer, while this ability, even in case of beers with low antibacterial properties such as wheat beer, is correlative to the metabolic capability to produce long-chain fatty acids. We suggest an intraspecies identification of beer-spoiling strains in quality control using *fabZ* in combination with *horA* and *horC*.

5.2.4.3 Lactobacillus backii

L. backii is considered to be a brewery-specific and obligate beer-spoiling species with very high hazard potential (Bohak, 2006; Hutzler et al., 2013; Suzuki, 2015). Beer spoilage potential was unstable in about 25 % of all cases, whereas beer spoilage ability was constant for four strains in four beer systems. While we do suggest to treat any contamination with *L. backii* as a serious threat for the product (90 % beer-spoiling strains), we were also able for the first time to identify a strain (TMW 1.2004) of this species lacking beer spoilage ability. As in case of *P. damnosus,* we suggest the plasmid-encoded ability to produce fatty acids *de novo* to be essential for beer spoilage ability, also indicating an origin of this species from outside the brewery.

A typical lager beer is spoiled within 4 to 19 days, while spoilage is characterized by turbidity and little to no acidification (ΔpH ~ 0.1), as observed by Bohak (2006). The low degree of acidification is typical for *L. backii*, which can also be considered as a distinct strategy of stress prevention (acid stress → concomitant hop stress). Metabolic adaptions, which are potentially involved in *L. backii* pH homeostasis, include mixed acid fermentation, malolactic fermentation as well as a general shift in pyruvate metabolism to non-acidic products (e.g. acetoin). Single strains were found to conduct acid tolerance related amino acid metabolism, producing biogenic amines, such as tyramine and putrescine.

L. backii seems to be generally well adapted for growth in beer, characterized by the highest overall adaption and tolerance to the major beer specific hurdles. Consequently, we found the cell density to be hardly affected by a beer typical pH of 4.25, an ethanol content of 6 % (vol/vol) and a hop content of 15 ppm (~ lager beer). This high degree of adaption is further supported by the fact that we could not improve the growth of strong beer-spoiling *L. backii* in beer by the addition of any nutrients, nor by any other modification. As in case of *P. damnosus* we found various plasmid-encoded, partially brewery-specific genes and clusters related to ion homeostasis, oxidative stress (NADH peroxidase) tolerance and and cell wall (LTA) metabolism, which potentially contribute to the adaption of this species to the multi-hurdle environment beer.

Hop tolerance in MRS did not correlate to beer spoilage potential and was found to be generally high (37 +/- 10) within all strains, which is in accordance with previous findings (Bohak, 2006). We can further confirm the presence of *horC* in 100 % of all strains, while *horA* was only found in some strains (Iijima et al., 2007). The prevalence of *horC* suggests that it is the preferred/dominant hop tolerance gene/mechanism, as in case of *L. brevis* and *P. claussenii*.

Glucose, fructose and mannitol are the major carbon and energy sources. As in case of the other homofermentative LAB, those substrates are preferred which are transported via PTS systems. Malolactic fermentation and pyruvate utilization contribute to energy generation, potentially supplemented by strain specific metabolic traits, including tyrosine decarboxylation (TMW 1.1430-SB), the utilization of the AGDI-pathway and lactose utilization (TMW 1.1991-SB). TMW 1.1991-SB was characterized by a considerable improved growth in lager beer, compared to the other *L. backii* strains, indicating a potential advantage conferred by the abovementioned traits. A shift to mixed acid fermentation in beer was observed, while an almost equimolar production of acetic and lactic acid, without citrate utilization, suggest the usage of an unknown alternative electron acceptor for NAD recycling and the consequent improved ATP generation by acetate production. *L. backii* is characterized by a proteolytic system for the uptake and utilization of peptides and further predicted to be prototroph for all amino acids except lysine.

Beer-spoiling strains of *L. backii* can be identified by a simultaneous detection of *horC* and *fabZ*. Nevertheless, a high proportion of unstable strains and the low proportion of non-spoiling strains make an intraspecies differentiation unrewarding. *L. backii* should be further treated as a general threat for the product.

5.2.4.4 Lactobacillus lindneri

Spoilage of a typical lager beer occurred within 9 +/- 4 days, characterized by turbidity, sedimentation, slight acidification (partially alkalization in wheat beers) and the production of histamine. *L. lindneri* is characterized by an unstable (~50 %) and unpredictable beer spoilage potential, accompanied by a distinct genetic instability, while our results indicate that each strain is apparently capable of spoiling lager beer, although not each strain is able to spoil each lager beer. However, any attempt of an intraspecies differentiation is consequently not reliable. Thus, we suggest to treat every contamination with *L. lindneri* as threat for the product, although we identified some species independent DMGs, which were capable to detect all *L. lindneri* strains (M22, M23 and M34). Note that we do not suggest to handle *L. lindneri* on the species level because we think it is an innate beer-spoiling species (Suzuki et al., 2005a), but because of the unstable and unpredictable behaviour of this species, which was not only found within the beer spoilage tests, but was a constant phenomenon observed in other physiological, analytical, proteomic and genetic experiments. While all five other core-species were found to show reduced growth performance (adaption, turbidity and acidification) with an increase of the antibacterial properties of beer (wheat → lager → pilsner beer), *L. lindneri* was characterized by a comparatively improved growth in lager and pilsner beer. Several strains were not even able to grow in wheat beer, but characterized by strong growth in pilsner beer.

Replicates of an individual strain were characterized by quite different growth curves in various laboratory media, a different metabolism or different DMG profiles. The identical sample preparation for mass spectra generation with MALDI-TOF MS, with a single strain, resulted in high quality spectra once and in impractical low quality spectra the other time. Although it does sound unscientifically, one can say that *L. lindneri* is a “hard-to-characterize” species, also because of the additional poor culturability.

Nevertheless, we were able to obtain some information about this interesting species, although the reader should keep in mind the potential restricted validity because of the abovementioned circumstances. *L. lindneri* is well-adapted (third rank within core-species) to beer specific hurdles, while an ethanol content of 6 % (vol/vol) did even improve its growth with respect to the maximum cell density. The evaluation of *L. lindneri* growth at different pH values, revealed an optimal growth pH of 4.38 +/- 0.5, thus even lower as previously assumed (4.6 to 5.2, (Back, 2005; Back et al., 1996)). Considering a pH of 3.4 to 4.8 as typical for beer, *L. lindneri* seems to be well adapted concerning this hurdle. Acid tolerance is potentially conferred/improved by histidine decarboxylation with the concomitant production of histamine, citrate fermentation and malolactic fermentation, while all of these metabolic traits may also contribute to energy generation.

Hop tolerance was again found to show no correlation to beer spoilage potential, while we cannot confirm that this species is in general characterized by a high basal hop tolerance (Back, 2005; Back et al., 1996), based on our hop tolerance tests in MRS media. However, we found most strains to show good growth in pilsner beer with up to 33 IBUs, but no or only little growth in wheat beers with about 12.5 IBUs, indicating that the hop content is not necessarily the decisive hurdle in case of *L. lindneri*. Both “*hor*”-genes are used by this species, while previous results indicate *horC* to be the preferred/dominant hop tolerance gene/mechanism (Suzuki et al., 2005a).

L. lindneri was further found to have the highest tolerance to oxidative stress at a beer typical Mn^{2+}/Mg^{2+} content, compared to the other core-species. Within the core-species, *L. lindneri* is the only species, which is predicted to encode for a manganese catalase, a non-heme enzyme which is considered to be advantageous under microaerophilic oxidative stress by H_2O_2 (Whittaker, 2012). Anyway, as the activity of these enzymes relies on the presence of Mn^{2+}, it is surprising that the oxidative stress tolerance of *L. lindneri* decreases at increasing Mn^{2+} concentration.

Our metabolic analysis further indicates that we still might have missed a significant proportion of *L. lindneri´s* energy metabolism in beer, as the amount of lactic and acetic acid produced,

could not nearly be explained by the actually detected metabolism of sugars. However, we can confirm the proposed high relevance of citrate, malate and pyruvate as important substrates of beer-spoiling *L. lindneri* (Suzuki et al., 2005b), while *L. lindneri* is also the only species which was not negatively affected by the addition of high amounts of organic acids to beer (see 4.2.6, p.72). The catabolism of organic acids is likely to contribute to lactate and acetate production in beer, while the semiquantitative assessment of citrate and malate does not allow a calculation of this contribution. The supply with energy by organic acid metabolism is further complemented by a metabolism of glucose, fructose and histidine. An addition of sugars to beer, including glucose, fructose and maltose resulted in a significantly increased cell density in beer, indicating that the sugar content in beer might be limiting for this species. *L. lindneri* has a proteolytic system for the uptake and utilization of peptides as amino acid source, while being auxotroph for at least seven amino acids.

L. lindneri is further characterized by seven complete rRNA operons, as it was also found in case of the next relative, *L. sanfranciscensis* (Vogel et al., 2011). Both species are considered to be strongly adapted to their environment (sourdough, beer), characterized by a comparatively small genome (*L. sanfranciscensis* 1.3 Mbp, *L. lindneri* 1.4 Mbp) and thus by a very high rRNA operon density, which is in general considered to be important to achieve high growth rates and beneficial for the adaption to rapidly changing environments (Klappenbach et al., 2000). The dynamics of the brewery environment were already discussed in detail (5.2.2, p. 166). We also tested if the genome-sequenced strain of *L. sanfranciscensis*, TMW 1.1304, is able to spoil beer, as both species were found to cluster together based on various parameters, emphasizing their close association. While we could observe growth in lager$_{pH5.0}$, TMW 1.1304-NB was not able grow in any of the actual test beers. This indicates that the inability of *L. sanfranciscensis* to grow in beer at a typical pH of 4.3 is connected either to acid tolerance or to hop tolerance, while *L. sanfranciscensis* TMW 1.1304-NB lacks any known hop tolerance gene. We further identified 363 genes (BADGE, protein level, default settings), which were specific for *L. lindneri*, including those genes necessary for histidine uptake and decarboxylation to histamine. However, altogether it is very likely that the lack of hop tolerance genes is the major determinant.

L. lindneri is not known to produce the off-flavor diacetyl (Suzuki, 2015), while interestingly we found both strains to encode for an acetoin reductase (EC 1.1.1.304) which catalyses the reduction of diacetyl to acetoin and butanediol. If we reconsider that *L. lindneri* is also found to be present during fermentation, where diacetyl is a by-product of yeast or a contamination with *P. damnosus*, it might make sense to use diacetyl as an alternative electron acceptor.

5.2.4.5 Lactobacillus paracollinoides

Spoilage by *L. paracollinoides* occurs within 6 to 7 days in a typical lager beer, accompanied by turbidity, cell flocculation, strong acidification and a strain and pH dependent production of the biogenic amines GABA and putrescine, while a production of histamine has been reported for strains isolated from cider samples (Ladero et al., 2011). “Diacetyl production” is encoded, although neither tested here nor reported so far. Beer spoilage potential was found to be stable, transferable and significantly correlated to hop tolerance in MRS and the presence of hop resistance genes. Because of a small sample size, five strains, we ended up with various DMGs with potential perfect correlation to beer spoilage ability, some of them even allowing the differentiation of beer spoilage potential groups. As only *horC* positive strains were shown to possess beer spoilage ability, which is in accordance with previous findings (Suzuki, Ozaki, et al., 2004), we suggest that *horC* is again the preferred/dominant hop tolerance gene/mechanism. Nevertheless, all non-spoiling variants of this species were either obtained artificially or lost their beer spoilage ability due to laboratory mishandling (Suzuki et al., 2006), while the general abundance of this species seems to be negligible (0 to 3.6 %, (Suzuki, 2015)). An intraspecies differentiation is thus unrewarding and not recommended.

Like *L. lindneri*, *L. paracollinoides* is characterized by poor growth on laboratory media such as MRS and NBB, while the genomic preconditions and the actual and predicted metabolic capabilities are as different as they could be within the genus *Lactobacillus*. In contrast to *L. lindneri*, *L. paracollinoides* is characterized by a predicted prototrophy for all proteinogenic amino acids (two of three strains), able to produce various vitamins of the B group, a complete pentose phosphate pathway and of course various other metabolic capabilities as the genome is 2.5 times bigger as the genome of *L. lindneri*. However, both species comprise hard-to-cultivate strains, while we conclude that this state is not related to metabolic versatility or narrowness and obviously not to genome size or complexity.

Despite the comparatively best adaption to a beer typical pH of 4.25, *L. paracollinoides* was ranked as penultimate species regarding the adaption and tolerance to beer specific hurdles, but also regarding a general stress tolerance to ionophores, oxidative stress and others. However, these tolerance tests have been conducted in MRS media and do not necessarily reflect the actual adaption of this species to the respective hurdles in beer. Strong beer-spoiling *L. paracollinoides* caused the strongest turbidity in beer, comparing all beer-spoiling strains tested, which illustrates their profound adaption to beer and its hurdles. This adaption is also reflected by the high amount of plasmid-encoded genes, which can be associated with a potential advantage for their growth in beer. Amongst others, genes with relation to ion

homeostasis, cell wall metabolism or pH homeostasis are likely to improve the survival and growth of *L. paracollinoides* in beer.

We could not define a distinct and shared metabolic strategy for *L. paracollinoides* in beer, as both strains with evident growth in $lager_{pH4.3}$ were characterized by quite different metabolism. However, there were some shared traits, which will be detailed below. In contrast to the other core-species, we found *L. paracollinoides* neither using malolactic fermentation nor citrate fermentation, while the former trait was found to be encoded by all investigated *L. paracollinoides* genomes and the latter trait on a plasmid of TMW 1.1995-SB. The lack of citrate and malate utilization as well as the usage of pyruvate are thus confirmed (Suzuki et al., 2005b) and further extended by a metabolization of succinate. Another conserved trait was the increase of the fructose content in beer, which was accompanied by an increase of glucose in case of TMW 1.1994-SB, thus indicating the utilization of non-investigated di-, oligo- or polysaccharides. All genomes were found to encode for a maltodextrin glucosidase (EC 3.2.1.20), which is potentially responsible for the increasing glucose concentrations, derived from maltotriose or maltodextrins, in case of TMW 1.1994-SB. Beer contains little amounts of fructan, stachyose and sucrose as potential fructose sources (Krahl et al., 2009; Preedy, 2009), while we could not identify the respective enzymes and pathways. Further, we found both strains to use galactose, which is taken up via a permease and further catabolized using the Leloir pathway. Additional ATP is generated using the ADI pathway and the plasmid-encoded and strain-specific AGDI pathway, while both pathways presumably contribute to an improved acid tolerance. Although not confirmed by analytical data or reported so far, we suggest redox homeostasis and pH homeostasis to be supported by an avoidance of lactate production, resulting in diacetyl, acetoin and butanediol. However, especially in case of TMW 1.1994-SB we observed a big discrepancy between the detected metabolic products (lactate and acetate) and the observed carbohydrate catabolism. This shows that we are far from comprehending the metabolism of this very high hazard potential LAB species.

Comparing both strong spoiling strains, which were actually investigated, regarding their metabolism in beer, we found TMW 1.1995-SB to be more successful. Interestingly, TMW 1.1995-SB showed a dramatically higher cell density (4 to 20 times) upon growth in all test beers compared to TMW 1.1994-SB. As both genomes encode for the hop tolerance genes *horA* and *horC*, the observed difference might be related to their metabolism in beer. TMW 1.1995-SB was found to use isomaltose and lactose, indicating that the utilization of these disaccharides as additional energy sources is an advantage in beer. However, the metabolic capabilities for the utilization of lactose via beta-galactosidase and Leloir pathway, as well as isomaltose via isomaltase are encoded by both of them. Thus, we conclude that the utilization of these substrates is not the reason for the comparatively greater success of

TMW 1.1995-SB, but the consequence of it. An improved adaption or tolerance of TMW 1.1995-SB to beer and its hurdles could allow more growth and results in a higher substrate demand.

5.2.4.6 Lactobacillus brevis

L. brevis is the most important beer-spoiling organism (Suzuki, 2015), while spoilage of a typical lager beer occurs within 5 +/- 3 days. No other species was found to adapt so fast, while spoilage was characterized by strong turbidity, acidification and the production of biogenic amines such as tyramine, GABA and putrescine. The formation of diacetyl is possible based on *in silico* prediction, while it was neither observed here nor in previous studies (Suzuki, 2015). The correlation of beer spoilage ability to isolation source, as well as a distinct strain-dependent beer spoilage potential were confirmed (Suzuki, 2015). Four of 20 strains were found to have an unstable beer spoilage potential, while only two of them were characterized by biological replicates with and without beer spoilage ability, both of them non-brewery isolates. We could further confirm hop tolerance to be the decisive property in case of this species, while all beer-spoiling strains, with one exception, were found to be positive for either *horA* or *horC.* Despite the fact that we identified novel DMGs, which will support the necessary and recommended intraspecies differentiation of this species, *horC* seems to be the preferred/dominant hop tolerance gene/mechanism (Bergsveinson, Baecker, et al., 2015; Bergsveinson et al., 2012). In case a brewery is producing wheat beer only, we do not recommend an intraspecies differentiation. With a single exception, all *L. brevis* strains, independently from their source or any hop resistance gene, were found to spoil wheat beer.

As stated within the introduction, *L. brevis* is a physiologically and metabolically versatile species, which is isolated from diverse, regarding the nutrient composition different, sources (Holzapfel and Wood, 2014). This diversity is also reflected by their genomes, while we found all six strains to have an accessory genome of in average 58 %, meaning that less than 50 % of all genes were found to be conserved within all six genomes. Beer-spoiling or brewery *L. brevis* strains, respectively, are suggested to be a distinct (phylogenetical) group within the species (Suzuki, 2015). Our genomic analysis further supports this hypothesis, since all three brewery genomes show more similarities to each other as compared to the non-brewery genomes, despite an overall high chromosomal variation within this species. Differences/similarities (44 genes shared by all three brewery genomes and not present in non-brewery genomes) were not restricted to the respective plasmidomes, but also reflected by chromosomal fragments and genes. Interestingly, two of these fragments encode for 11 genes related to the uptake and utilization of pectin or arabinoxylan derived hexuronic acids (galacturonate, glucuronate and fructuronate), as well as for the uptake and degradation of

polygalacturonates. This indicates that these strains may be introduced into the breweries from plant sources, or that the utilization of these plant derived carbon sources represent an advantage in beer, while beer is known to contain arabinoxylan and pectin derived sugars (Preedy, 2009). However, the abovementioned and other chromosomal genes were successfully tested as DMGs for beer-spoiling *L. brevis* in a previous study (Behr et al., 2015). We tested two of these chromosomal DMGs, *cinA* and *arsR*, with an extended set of strains, confirming that these DMGs occur only within the group of brewery isolates. However, not all brewery isolates were found to possess them (low sensitivity of DMGs), reducing their suitability for quality control. Nevertheless, if we think back to BSO 464, losing the ability to spoil beer with the loss of the *horC* harbouring plasmid (Bergsveinson, Baecker, et al., 2015), it becomes clear that these chromosomal genes are not always sufficient for beer spoilage ability. On the other side, we characterized an isolate from honey (TMW 1.1370-SB), lacking any know hop tolerance genes, but characterized by strong beer spoilage potential. We conclude that the lifestyle of *L. brevis* is also defined by the chromosomal setting of niche-specific genes, while the actual ability to spoil beer (lager beer) is a mostly plasmid-encoded trait.

Still, what makes *L. brevis* the most successful beer-spoiling organism? We inoculated a lager beer with strong beer-spoiling strains of all six core-species, while we found *L. brevis* to be dominant over the other species (plating and species differentiation with MALDI-TOF MS, data not shown), even after a short period. This dominance is also illustrated by the high abundance of this species in breweries and beer spoilage statistics. *L. brevis* beer spoilage ability, with exceptions (TMW 1.1370-SB), relies on the presence of plasmid-encoded genes. *L. brevis* seems to accumulate all kind of brewery-specific DNA, while especially strong beer-spoiling strains are characterized by up to 21 potential DMGs. As in case of the other species we found various plasmid-encoded genes, related to global functions with a potential relevance for growth in beer, including ion homeostasis, oxidative stress or cell wall metabolism (LTAs). However, these and the in general relevant hop tolerance clusters containing *horA* and *horC*, were also found in case of the other species and do consequently not explain the dominance/success of this species. Considering all those shared properties mentioned above, the answer to our question has to reside within the species (chromosome), its adaption and tolerance to beer specific hurdles, or its metabolic strategies.

L. brevis was found to have a high intrinsic tolerance to various stress qualities, ranked on the second place in comparison to the other core-species regarding beer specific stress, oxidative stress, ionophores and overall stress. In case of oxidative stress, we found *L. brevis* to encode for a comparatively high number of tolerance related genes, while we found both beer-spoiling strains to be characterized by an improved growth rate and an increased cell density in the

presence of about 2 mM H_2O_2. However, in comparison to *P. claussenii*, *L. backii* and *L. lindneri* we found a rather limited adaption of *L. brevis* to beer specific hurdles, illustrated by considerable strong growth inhibition by beer typical stress intensities of the respective hurdles (e.g. pH 4.25).

The metabolic diversity of *L. brevis* might be a reason for its success in beer and its comparatively fast adaption to beer. *L. brevis* was found to have the highest number of monosaccharide metabolism-related genes within all investigated LAB genomes. All investigated carbohydrates, except trehalose and sorbitol, were found to be converted in $lager_{pH5.0}$ or $lager_{pH4.3}$, including hexoses, pentoses and disaccharides. At a beer typical pH of 4.3, however, galactose, isomaltose and arabinose are the preferred sugars, thus showing that *L. brevis* does not solely focus on hexose, pentose or disaccharide metabolism, respectively. *L. brevis* was shown to have a relaxed sugar utilization control, resulting in the parallel usage of more than on sugar (Kim et al., 2009). This flexibility might be one reason for the fast adaption of *L. brevis* to beer. The already diverse sugar metabolism is supplemented by malolactic fermentation and in some cases, not correlative to source and beer spoilage ability, by citrate fermentation. Finally, we found *L. brevis* to use amino acid decarboxylation systems for tyrosine and glutamic acid as well as the ADI and AGDI pathway, releasing at least three different biogenic amines into the beer. ADI pathway and GAD pathway were already shown to contribute to acid and hop tolerance of *L. brevis* (Behr et al., 2006; Schurr et al., 2013). Thus, it is conceivable that the other systems also play a role for the successful growth in beer, either because of ATP production and alkalization or by pmf generation and electrogenic transport. The relevance of amino acid metabolism for successful growth in beer was tested by the addition of amino acids and peptone to lager beer, while especially those strains without beer spoilage ability were strongly affected, characterized by even higher cell densities as the strong beer-spoiling strains. This indicates that the lack of plasmid-encoded hop tolerance can be compensated by an increased amino acid and peptide metabolism (in case these substrates are available in comparatively higher concentrations), presumably because of the concomitant alkalization and energy generation. In conclusion, metabolic versatility with a focus on amino acid metabolism is suggested to be the key of *L. brevis´* lifestyle and success in beer.

5.3 The importance of beer specific hurdles for the growth of lactic acid bacteria and for the susceptibility of beer

Publications about beer-spoiling bacteria usually start with the same kind of statements about the environmental conditions in beer. An example from the last extensive review about beer-spoiling bacteria:

“Beer has been recognized as a microbiologically stable beverage. This is due to the presence of ethanol (0.5–10%w/w), hop bitter compounds (ca. 17–55 ppm of iso-α-ac-ids), high carbon dioxide content (approximately 0.5% w/v), low pH (3.8–4.7) and reduced concentration of oxygen (generally less than 0.3 ppm). Beer is also a poor medium because nutrients are almost depleted by the fermentative activities of brewing yeast (Suzuki, 2011). “

It is known that these hurdles, in the above-mentioned intensities, inhibit the growth of foodborne pathogens and the vast majority of all other brewery contaminants (Suzuki, 2011), but what is their significance (treated as single individual hurdles) regarding the stability of beer towards spoilage by LAB with very high hazard potential and their beer spoilage potential?

A grading of single hurdles, based on the state of knowledge, follows.

5.3.1 Ethanol - 0.6 to 12.7 % (v/v)

A beer typical ethanol content of about 4 to 6 % (v/v) could not prevent but only slow down the growth of LAB species with very high hazard potential. It was further shown that beer typical ethanol concentrations exert only limited negative effects on cellular enzyme activities (Vriesekoop et al., 2012). Fernandez and Simpson (1995) tested the susceptibility of 17 lager beers to spoilage by the important beer-spoiling organism *Lactobacillus* (*L.*) *brevis* and *Pediococcus* (*P.*) *damnosus*, while they did not find a significant correlation of spoilage susceptibility to ethanol content in a range from 3.47 to 6.05 % (v/v)). Pittet et al. (2011) could show that there is no significant correlation of beer spoilage ability of 61 LAB strains to ethanol tolerance, further finding that this trait is essentially conserved within single species. However, beers with a considerable higher ethanol content have been shown to possess a general higher stability towards microbial spoilage (Shimwell, 1935) and an improved resistance towards a range of foodborne pathogenic bacteria as *Escherichia coli* O157:H7 and *Salmonella typhimurium* (Menz et al., 2011). Further, we found a significant low positive correlation of ethanol tolerance to beer spoilage potential for 24 strains of the core-species, which was mainly driven by *L. brevis*. In conclusion, the ethanol content may be only relevant at the edges

of what is typically found. Within a 7-day MIC test, we found 13 of 30 tested strains, including all *L. lindneri* strains, to be completely inhibited by 10 % (v/v) ethanol, while this range is only of relevance for extremely strong beers, such as German Doppelbock and Eisbock. Finally, alcohol free beers showed a higher spoilage susceptibility in case of *L. brevis* (Preissler, 2011).

5.3.2 pH value - 3.8–4.7

The tolerance to low pH values alone does not determine the beer spoilage ability of LAB with very high hazard potential. The average minimum inhibitory pH value for 30 strains of all six core-species was found to be 3.19 +/- 0.28. Consequently, only some sour beers, e.g. Berliner Lambic with a pH of 3.1, could be considered stable based on acidity only. However, the elevation of the pH of lager beer 1 from 4.3 to 5.0 resulted in successful growth of 116 of 118 tested strains, also by almost all non-brewery isolates without any beer spoilage potential. In contrast, only 69 strains were able to grow in lager beer 1 with pH 4.3. This dramatic effect is very likely the consequence of a synergism of pH and hops, considering that the antibacterial properties of hops increase by ~50 % with a decrease of the pH of 0.2 units (Simpson and Smith, 1992) (Simpson, 1993a). The significance of the pH hurdle is further emphasized by the expended effort by beer-spoiling LAB to counteract acidification in beer, as described in various chapters within this thesis. In conclusion, the pH is one of the most important hurdles for LAB growth in beer, showing the highest correlation to product susceptibility as determined by Fernandez and Simpson (1995).

5.3.3 Oxygen/carbon dioxide - ~0.3 ppm/0.5 % (w/v)

Neither the distinct effect of oxygen nor of carbon dioxide were investigated within this study, while our genome analysis emphasizes a relevance of oxidative stress tolerance within the brewery environment and potentially in beer. Recently, Bergsveinson, Redekop, et al. (2015) demonstrated the significance of carbon dioxide as a strong selective hurdle for LAB beer spoilage ability, regarding a finished and packaged beer. Especially the latter hurdle might be a promising target for future research.

5.3.4 Nutrient limitation - “poor medium”

Regarding carbon, nitrogen and energy sources, we conclude that their amount in beer is not a true limit for LAB growth in beer, although we could find that the spoilage susceptibility of lager beer could be increased by the addition of amino acids, peptides or sugars, depending on species and strain. Further, the amount of these compounds had an impact on the extent

of spoilage (degree of turbidity). However, growth or no growth, and thus beer spoilage ability, is not in general determined by the available content of the abovementioned nutrients. The comparison of metabolism in $lager_{pH5.0}$ to $lager_{pH4.3}$ revealed that the growth of most LAB was not limited by the amount of available carbohydrates, indicated by the partially high amount of in principle fermentable but non-utilized sugars, organic acids and amino acids. The diversity of observed metabolic strategies in beer also argues against nutrient limitation as kind of a selective pressure. Finally, the composition of beer, as detailed in other literature about beer outside the research area "beer spoilage," does not coincide with the accepted statement about beer as a poor medium for bacteria, at least considering carbohydrates or proteins. In contrast, there is a sugar concentration of about 20 to 30 g/l in beer, which is as much as some laboratory media have (e.g. MRS Broth: 20 g/l), although most of the sugar does not exist as monosaccharides. The "protein" concentration goes up to 5 g/l (MRS Broth: 20 g protein extracts), while 75 % are considered to be available as free amino acids and peptides smaller as 3,500 kD (Abernathy et al., 2009). However, beer contains only small amounts of long-chain fatty acids and some vitamin B members are found in low concentrations (Preedy, 2009). Based on our results, we conclude that the availability of long-chain fatty acids does determine if LAB are able or not to grow in beer. This is supported by a correlation of the metabolic capability to produce fatty acids *de novo* to beer spoilage ability of the important very high hazard species *L. backii* and *P. damnosus*.

We suggest to avoid or modify the often-found general statements about the "lack of nutritive substances," the "almost depleted" nutrient content or the "low nutrient availability" (Behr et al., 2015; Suzuki, 2011; Vriesekoop et al., 2012). These statements are not differentiated enough to reflect the actual situation found in beer. Still they established within the specific research area "beer spoilage", while they are even in contrast to basic knowledge about beer´s nutrient content as described in other "beer literature (Preedy, 2009)".

5.3.5 Hops

We could not find a species independent correlation of hop tolerance in MRS to spoilage ability and potential. In case of *P. damnosus* and *L. lindneri* we found almost all strains to be completely inhibited by a typical/average hop content, while the collected data do not coincide with beer spoilage ability and potential of both species (5.2.4.4, p. 183), indicating that hop tolerance depends on other beer specific properties for these two species. The other core-species, with single strain exceptions, are not inhibited by beer typical hop contents in MRS, reflected by an average MIC of 31 +/- 12 ppm iso-α-acids. Nevertheless, the confirmed abundance and significance of hop resistance genes, such as *horA* and *horC* (Bergsveinson, Baecker, et al., 2015; Bokulich et al., 2015; Haakensen et al., 2008; Pittet et al., 2013; Suzuki

et al., 2006), strongly indicates that hops are the major determinant of strain-specific beer spoilage potential and ability. This is also reflected by the general observation that very high hazard LAB, except *L. lindneri*, grew best in wheat beer, followed by lager and pilsner beer, while the used test beers were characterized by almost identical pH values but increasing hop content. Only in case of *L. lindneri,* we conclude that the hop content is not the major decisive hurdle, as detailed in chapter 5.2.4.4 (p. 183). Thus, we agree with the opinion of hops being a major hurdle of beer, determining the susceptibility of beer to spoilage (Simpson, 1993a). On the other side, we observed a high hop tolerance of several strains lacking any beer spoilage potential in MRS, as well as a lack of hop tolerance by several strong beer-spoiling strains. Thus, hop tolerance as a standalone trait is not sufficient for growth in beer, is apparently affected by other parameters in beer (Haakensen, Schubert, et al., 2009), and beer spoilage ability cannot be predicted solely based on hop tolerance.

Behr and Vogel (2009) found no correlation (cross-resistance) of hop tolerance to the tolerance to single proton ionophores (CCCP, DNP in *L. brevis*), while proton uncoupling is considered a major mode of action of hops (Behr and Vogel, 2009; Simpson, 1993b). Despite a low positive correlation to DNP ($\rho = 0.44$), we did also not find a correlation for five other ionophores and five other species (see 4.2.6.2, p. 80), confirming that hop tolerance is not an arbitrary tolerance to all kind of ionophores (Behr and Vogel, 2009). In contrast, the moderate positive correlation ($\rho = 0.57$) of hop tolerance to oxidative stress tolerance supports the findings of Schurr, Hahne, et al. (2015), emphasizing the relevance of the second mode of action of hops, oxidative stress.

Altogether, we conclude that single hurdles, at beer typical intensities are mostly not sufficient for total inhibition of very high hazard LAB. Likewise, spoilage potential and ability cannot be deduced of their tolerance to a single hurdle. Beer is a multihurdle environment, while the total inhibitory effect is very likely a product of an additive or even a synergistic effect of the present hurdles, as indicated by previous results (Barker and Park, 2001; Haakensen, Schubert, et al., 2009; Jordan et al., 1999; Simpson and Smith, 1992). A prediction of spoilage ability based on a single tolerance hurdle is apparently not reliable, while the susceptibility of beer seems to be related to all of them, but especially to the combination of these stress qualities.

5.4 The identification of beer-spoiling lactic acid bacteria

As initially stated, brewers need to perform quality control in order to provide consumers with a stable and safe product. Thus, detection and identification of harmful contaminations with beer-spoiling LAB has to be reliable and decisive, fast and highly selective, as well as uncomplicated and cheap. While the usage of detection media such as NBB allows the detection and enrichment of contaminations, they do not provide an identification, do not allow the tracking of contamination routes within the brewery by strain identification (source tracking), or give any information about the actual product hazard. Forcing tests are still considered the gold standard for the prediction of beer spoilage ability and thus for the product hazard due to a given contaminant, while these tests are quite tedious and simply take too long. Consequently, rapid and reliable alternatives are desired.

With reference to the beer spoilage test, we probed the suitability of the resazurin test, hop tolerance tests, RAPD-PCR, MALDI-TOF MS and a range of established and new DMGs for the prediction of beer spoilage potential and ability of LAB with very high and high hazard potential. The resazurin test (Preissler et al., 2010) seems to have a certain predictive ability in case of *L. brevis,* while we observed a massive underestimation of spoilage potential and ability in case of the other very high hazard LAB species. Further, a prediction of beer spoilage ability based on a rapid hop tolerance test was also shown to be not suitable (5.1.4, p. 146 and 5.3, p. 191). Both finger printing techniques, DNA (RAPD-PCR) and protein-based (MALDI-TOF MS) could not reliably differentiate beer-spoiling and non-spoiling strains. Consequently, these methods are also not suitable for the prediction of beer spoilage potential and ability, while both techniques are useful for species and strain differentiation/identification (Kern et al., 2013; Kern et al., 2014; Tompkins et al., 1996; Wieme et al., 2014).

In conclusion, we were able to predict novel DMGs by comparative genomics, evaluated their significance using PCR and validated the value of several novel target genes. Especially the identified lifestyle genes, where a link of the DMG to the LAB lifestyle in beer is either indicated or even proven, will be interesting targets for the brewing-microbiological quality control. Based on a hybrid approach, we provide a distinct detection and differentiation system for the identification of harmful contaminations with very high hazard potential LAB. This system includes a species and strain identification with MALDI-TOF MS and a consequent differentiation of beer-spoiling and non-spoiling strains using lifestyle genes. The following chapters describe such a system as well as some alternative approaches and their potential implementation.

5.4.1 Species and strain identification using MALDI-TOF MS

MALDI-TOF MS is a powerful tool for species identification of bacteria, also for those encountered in the brewery environment (Kern et al., 2013; Wieme et al., 2014), while Kern et al. (2014) also successfully demonstrated the method´s suitability for a strain-level differentiation of *L. brevis*. We obtained 15 high quality spectra each for 118 strains, comprising 13 species with high and very high hazard potential, confirming the reliability of MALDI-TOF MS for brewery LAB species identification with 100 % correct assignments. Strain-level differentiation was successful in 65 % of all cases. A high proportion of correct strain identifications was found for *L. brevis* (88 %) and *P. damnosus* (73 %) and therefore for those species, which were characterized by a distinct strain-specific beer spoilage ability. We also tried to differentiate LAB strains with respect to beer spoilage potential and ability, while we conclude that the high number of positive group assignments regarding beer spoilage ability and potential is driven by correct strain identifications and does not arise from distinct low molecular weight proteome pattern (no biomarker/cluster see Figure 39, p. 131), which are specific for these groups. Species identification is an important step in microbial quality control in breweries (Wieme et al., 2014), as the species identity could help to reveal flaws of a brewing system and to improve the hygienic design (primary ↔ secondary contamination, differences in resistance to disinfectants, heat etc. (Back, 1994, 2005; Back et al., 1996)).

Altogether, MALDI-TOF MS represents a fast and reliable method for the species identification of brewery contaminants. Depending on species, it also offers the possibility of a strain identification, which allows the tracking of contamination routes as well as the evaluation of cleaning procedures.

5.4.2 Lifestyle genes for the identification of beer-spoiling lactic acid bacteria

In order to complement the available set of lifestyle genes for a more reliable and improved identification of beer-spoiling LAB, we generated 17 genomes of very high hazard LAB with varying beer spoilage potential and performed a comparative genome analysis. For the prediction of novel DMGs, we designed and programmed the BlAst Diagnostic Gene findEr (BADGE), a simple and fast bioinformatics tool which is addressed in detail within a previous publication (Behr et al., 2016). We successfully used BADGE to predict DMGs, which were present in beer-spoiling genomes but not in non-spoiling genomes. Employing a PCR assay, we evaluated 45 predicted DMGs and consequently validated the significance of some novel and promising DMGs. PCR was performed without prior DNA extraction using single colonies from NBB plates. This allows the prediction of beer spoilage ability within a few hours after detection. It is also possible to further reduce the detection time by analysing (PCR) beer without a previous cultivation-based preenrichment (e.g. QuickGEN, Gen-ial, Troisdorf, Germany).

We could not identify novel DMGs with a high discriminatory power within all focused very high hazard core-species, while our analysis confirmed that *horC* is the most abundant and dominant species independent lifestyle gene. Nevertheless, we provide a new set of partially species independent DMGs and potential lifestyle genes for an optimization of already existing marker systems. The provided DMG and primer sequences, in combination with the gathered quality data, regarding their accuracy, precision etc., allow the deriving of requirements-oriented barcode systems for the classification of LAB with very high hazard potential. Depending on product line, resources, the desired precision, the intended effort and the actual ambition (risk-taking), different solutions can be drafted, which will be outlined in the following subsections and Table 31 (p. 201). Note that we found established and novel DMGs to be of minor or no significance for the investigated high hazard potential (excluding *P. claussenii*, reclassified) species. As we did not sequence and compare the genomes of these high hazard potential species, we suggest that an independent approach is necessary to deduce valid DMGs for these species. The following detection systems only affect very high hazard potential core-species.

5.4.2.1 A species independent approach for the identification of beer-spoiling lactic acid bacteria

Based on the lifestyle gene *horC* and the novel DMGs M19 and M34 (BSA-MIX), we identified all beer-spoiling strains of LAB species with very high hazard potential, using only three target genes, whereby also 77 % of all non-spoilers were identified as beer-spoiling strains (false positive rate). Note that the detection of only one of these DMGs was assessed to be sufficient for a positive finding (see Figure 46, p. 199). Both novel DMGs are not specific for the brewery environment but abundant and typical. As both genes are associated with genome plasticity, plasmid replication and transposition, they are consequently typical for the lifestyle of beer-spoiling LAB and thus can be considered as lifestyle genes. They are neither obligatory nor decisive for beer spoilage ability, but indicative. As a primary endeavour of brewery quality control is to avoid any false negative identifications (high sensitivity), the abovementioned combination represents a distinct enhancement to the often suggested combination of *horA* and *horC* (Haakensen et al., 2008; Suzuki et al., 2006), which resulted in the identification of 95 % of all beer-spoiling strains with a false positive rate of 58 %. Note that such a system can be arbitrarily extended using further brewery typical or specific DMGs (e.g. *horA*, M37, M43, see 4.4, p. 115) with good sensitivity and precision, resulting in less risk to miss a potential harmful contamination, but also less specifity (more false positive).

Such a DMG-based, species independent quality control is compact, fast and results in high sensitivity, while the chance for a release of a threatened but undetected batch of beer is consequently low(ered). However, such an approach does not provide a species or strain identification and thus no source tracking. Further, it results, because of a quite undifferentiated system, in a very low specifity (22 %), which might lead to an unnecessary waste of product. Considering the high sensitivity and the fact that such a system is apparently interesting for a quality control approach with a focus on risk minimisation, a consideration of each contamination (detection media only) as a potential product hazard might be a better solution. This way there is no need for additional technology (Thermocycler etc.) and knowledge, which is especially interesting for small breweries with little resources.

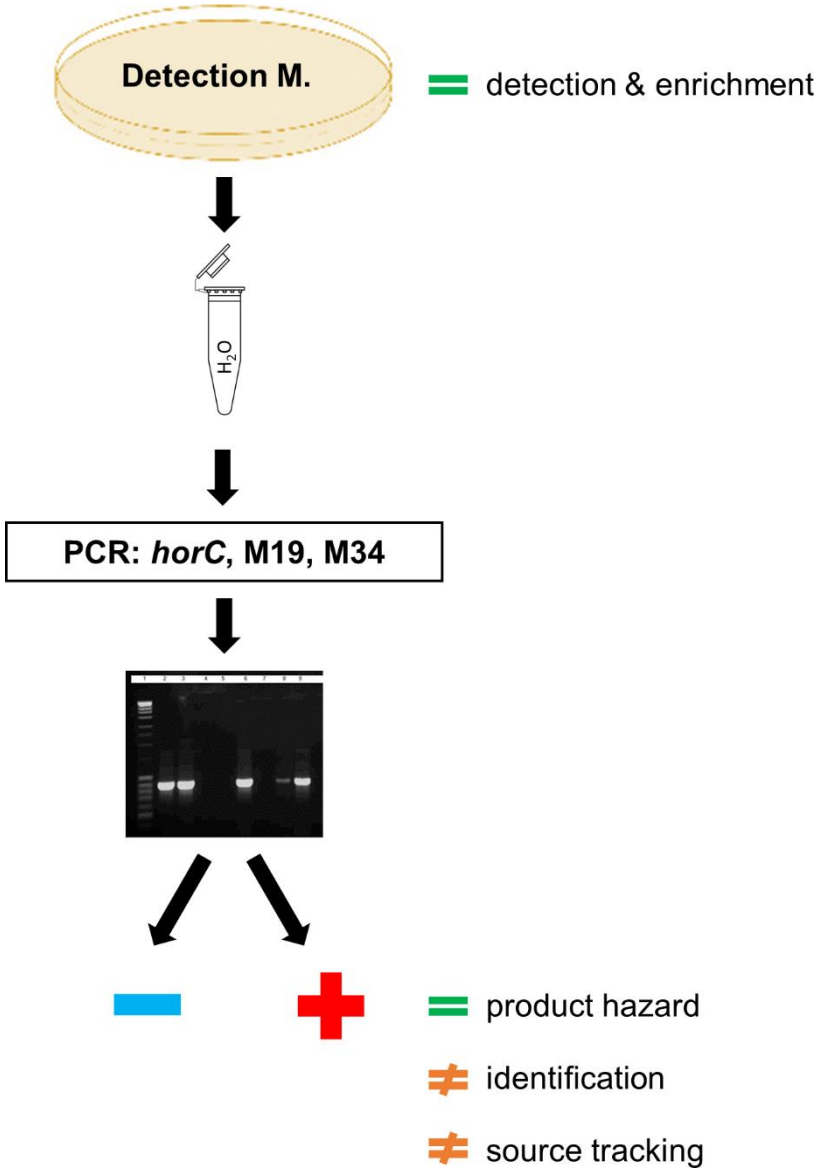


Figure 46: A species independent PCR approach for the identification of beer-spoiling strains of very high hazard potential LAB species using the BSA-MIX. Contaminations/cells are detected and enriched using media (in this case NBB). Consequently, cells (a colony) are suspended in 200 µl water. 2 µl of such a (opaque) suspension are subjected to PCR as template. Alternatively, PCR is made without prior enrichment (e.g. QuickGEN, Gen-ial, Troisdorf, Germany). The presence of any of the DMGs *horC*, M19 and M34 counts as a positive result. Control reactions to test the cell suspension for the suitability for PCR are strongly recommended (e.g. 16S rDNA, M13-V).

5.4.2.2 A species dependent approach for the identification of very high hazard potential lactic acid bacteria

Because of instability, low transferability, a low number of non-spoiling strains and/or a lack of significance (see 5.1.2, p. 143) we suggest that a species identification is sufficient for *L. backii, L. lindneri, L. paracollinoides* and *P. claussenii*. Preliminary species identification can be achieved using species-specific PCR based systems (e.g. First-Beer Differentiation PCR Kit, Gen-ial, Troisdorf, Germany) or other non-DNA-based approaches, such as MALDI-TOF MS. A detection of *L. backii, L. lindneri, L. paracollinoides* and *P. claussenii* is counted as product hazard, without consequent intraspecies differentiation. A detection of *L. brevis* and *P. damnosus* is followed by an intraspecies differentiation, which is necessary and recommended, among other things, because of a distinct strain-specific beer spoilage ability and a high relevance of both species for beer spoilage (see 5.1.2, p. 143). The lifestyle genes *fabZ*, *horA* and *horC* are used in case of *P. damnosus*, while the presence of *fabZ* and one of the “hor”-genes together, is counted as positive result. Applying this system, we were able to differentiate 20 strains into beer-spoiling and non-spoiling strains 100 % correctly. *L. brevis* is differentiated using *horC* and M37, while the presence of either is counted as positive results. 18 (19, see 4.4, p. 115) of 20 strains were correctly identified as beer-spoiling or non-spoiling strains using *horC* and M37. Altogether, despite the fact that we did no differentiate on a strain-level within four of six core-species, this approach led to a detection of 99 % of all beer-spoiling strains with a false positive rate of 30 %. This way we end up with a specifity (70 %) of the identification system, where a strain differentiation actually pays off for quality control, as it is possible to differentiate the majority of harmful and harmless contaminations with very high hazard potential LAB.

This approach allows the detection, identification and classification of contaminants with respect to the actual product hazard, while providing improved accuracy, precision and specifity, compared to the species independent approach introduced in the previous chapter (5.4.2.1, p. 198). On the other side this approach is costlier, more tedious, slightly riskier (sensitivity 99 % compared to 100 % of BSA-MIX) and time consuming, while it still does not provide a possibility for source and strain tracking.

Table 31: Comparison of two proposed quality control systems for the identification of harmful contaminations with beer-spoiling lactic acid bacteria with very high hazard potential. The relation of two distinct detection systems to beer spoilage ability of core-species was tested with Fisher´s exact test for significance (p-value < 0.05). **Species independent approach:** The presence of any of the DMGs *horC*, M19 or M34 (BSA-MIX) counts as a positive result. **Species independent approach:** A detection of *L. backii, L. lindneri, L. paracollinoides* and *P. claussenii* is counted as positive result, without consequent intraspecies differentiation. A detection of *L. brevis* and *P. damnosus* is followed by an intraspecies differentiation using *fabZ* and *horA* or *horC* in case of *P. damnosus,* and *horC* or M37 in case of *L. brevis*. True positive (TP), false positive (FP), true negative (TN) and false negative (FN) results were counted and used for the calculation of accuracy, precision, sensitivity, specifity, false positive rate, false negative rate and F-measure, which is the harmonic mean of precision and sensitivity (confusion matrix). The comparatively better quality value is highlighted in bold red letters. BSA-MX = detection of *horC* and/or M19 and/or M34. Accuracy = TP+TN/(TP+FP+TN+FN) → total correct identifications, measure of correctness; precision = TP/(TP+FP) → a measure for the probability that the identification is correct; Sensitivity = TP/(TP+FN) → a measure for the proportion of positives that are correctly identified as such; Specifity = TN/(TN+FP) → a measure for the proportion of negatives that are correctly identified as such; False positive rate = FP/(FP+TN); False negative rate = FN/(FN+TP); F-measure = 2TP/(2TP+FP+FN).

	Species independent approach: BSA-MIX	**Species dependent approach**
Accuracy	0.78	**0.90**
Precision	0.76	**0.89**
Sensitivity	**1.00**	0.99
Specifity	0.22	**0.70**
F-measure	0.86	**0.94**
True positive	**67**	66
False positive	21	**8**
False positive rate	0.78	**0.30**
True negative	6	**19**
False negative	**0**	1
False negative rate	**0.00**	0.01
Fisher´s p-value	3.64E-04	**1.11E-12**

5.4.3 A hybrid approach for brewery quality control

After a detection of contaminants, using detection and enrichment media such as NBB, we suggest to perform a preliminary species (and strain) identification with MALDI-TOF MS. We suggest (see 5.1.2, p. 143) to treat any contamination with *L. backii, L. lindneri, L. paracollinoides* and *P. claussenii* as a threat for the product, while MALDI-TOF MS allows a fast and reliable identification of these species, but only limited source (strain-level: 29 to 47 % correct) tracking. In case of *L. brevis* and *P. damnosus*, together causing the majority of spoilage incidents (Suzuki, 2015), MALDI-TOF MS mostly allows the additional differentiation of strains and thus the potential identification of contamination routes. For *L. brevis* and *P. damnosus*, we further suggest to perform an intraspecies differentiation with respect to beer spoilage ability using lifestyle genes and PCR, using the same DMG system as detailed in the previous chapter (5.4.2.2, p. 200). Figure 47 (p. 203) illustrates the suggested hybrid approach.

In addition to detection, identification and a classification with respect to beer spoilage ability (see 5.4.2.2, p. 200), this hybrid approach allows a strain-level identification and thus the tracking of contamination routes. Brewery-specific databases can be created, which would not only allow the tracking of contamination routes, but also facilitate a long-term mapping of the microbial contamination of the brewery, which could help to improve quality control and quality management by improving the hygienic design. Strain-level differentiation further verifies if cleaning is/was efficient and sufficient (reoccurrence of a distinct strain). MALDI-TOF MS has low consumable costs, allows high-throughput and a reliable and fast identification of LAB with relevance for brewing quality control (Kern et al., 2013; Kern et al., 2014; Wieme et al., 2014). However, MALDI-TOF MS has also high initial investment costs, which makes these systems less attractive for small to medium breweries (Wieme et al., 2014). We suggest that the hybrid approach is especially interesting for big breweries with appropriate resources or diagnostic laboratories, which offer quality control related services for the brewing industry.

At the end, a modern quality control might be a tailored quality control for each brewery. The own product line can be tested for the validity of the suggested test systems. We provide additional DMGs and primer sequences, which could improve the quality control for other product lines. We further provide an easy-to-use bioinformatics tool, BADGE (Behr et al., 2016), for the extraction of novel DMGs from the generated genome sequences, in case the own product line is characterized by a different spoilage susceptibility. A differentiated approach like this can be gradually adapted to the respective product line, consequently allowing the efficient and tailored differentiation of harmful and harmless contaminations.

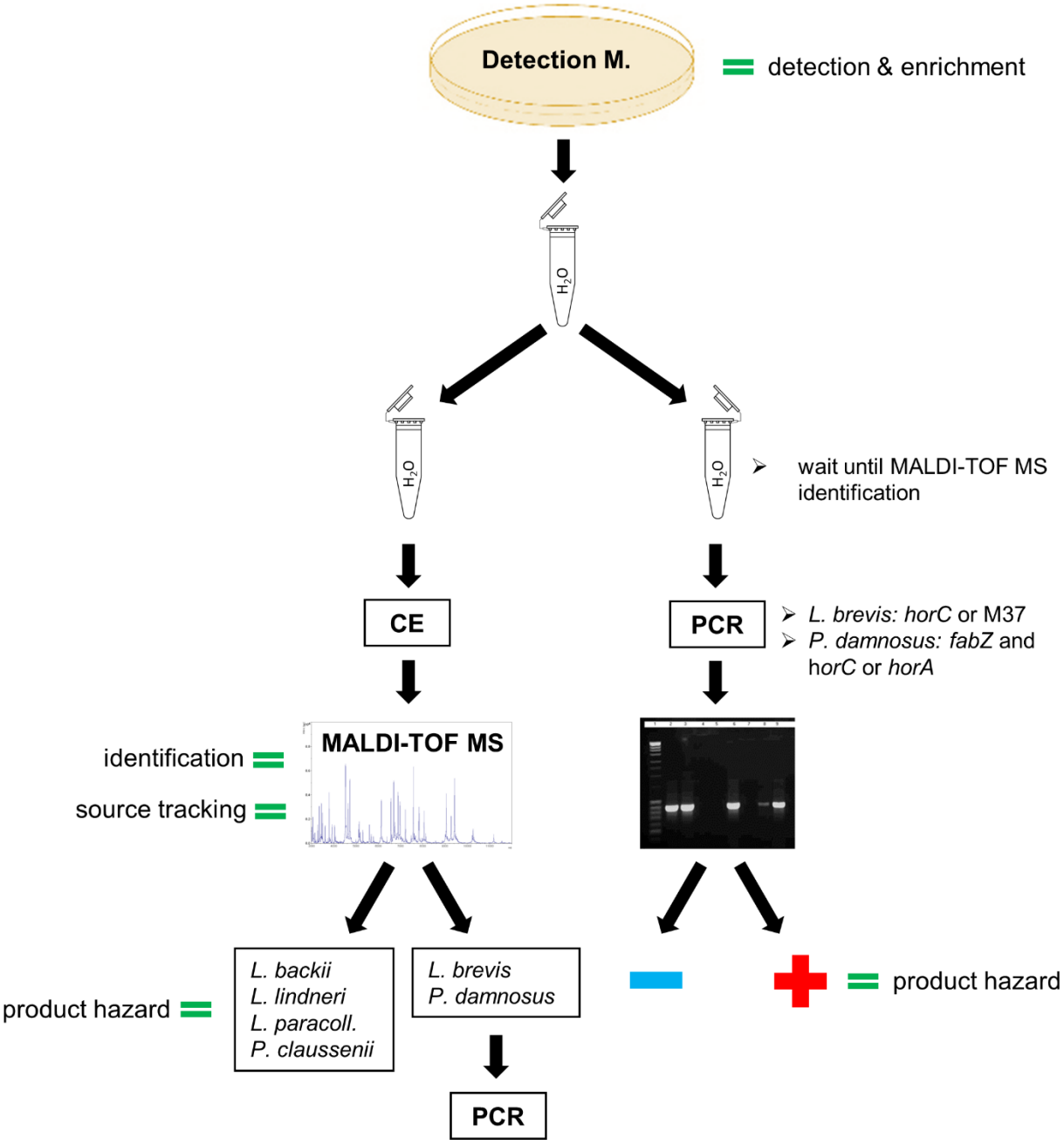


Figure 47: A species dependent hybrid approach for the identification of beer-spoiling strains of very high hazard potential LAB species. Contaminations/cells are detected and enriched using media (in this case NBB) and consequently cells (a colony) are suspended in 200 µl water. 10 µl are stored for colony PCR, while 190 µl are used for a short cell extraction (CE) and MALDI-TOF MS analysis, which allows a preliminary species and a potential strain identification (source tracking). In case the finding is positive for *L. brevis* or *P. damnosus*, a subsequent intraspecies differentiation with respect to beer spoilage ability is performed, in order to evaluate the actual product hazard due to the contamination. Therefore, 2 µl of the (opaque) cell suspension are subjected to PCR as template. Control reactions to test the cell suspension for the suitability for PCR are strongly recommended (e.g. 16S rDNA, M13-V).

6 Summary

Beer is a pleasure for the consumer and a valuable product for the brewer, but it is also a harsh ecological niche for bacteria. Its occupation is only possible for a small number of species, while beer spoilage ability is mostly a strain-specific trait. This is because beer is characterized by various antibacterial hurdles including a high concentration of ethanol, a low pH, reduced oxygen content, high carbon dioxide concentration, a low nutrient content and the presence of antibacterial hops (Suzuki, 2011; Vriesekoop et al., 2012). For quality control, it is important to understand the lifestyle of beer-spoiling lactic acid bacteria (LAB) in their ecological niche beer and consequently to understand which strategies and lifestyle genes allow them to grow in the multihurdle environment beer.

118 strains, comprising 13 LAB species, were characterized and classified with respect to beer spoilage potential and beer spoilage ability, while this study focused on the (very) high hazard potential species *P. claussenii*, *P. damnosus*, *L. backii*, *L. lindneri*, *L. paracollinoides* and *L. brevis* (core-species) (Hutzler et al., 2013). 26 strains of these core-species, varying in beer spoilage potential and isolation source, were investigated for their growth dynamics in beer-variants with various additives, their tolerance to beer-specific and related stress qualities (hurdles) and their metabolism in beer, under acid and under hop stress. 17 of these strains were further chosen for single molecule real time sequencing (McCarthy, 2010), raw data were assembled to complete genomes and annotated. Using the tool BlAst Diagnostic Gene finder, written within this project (Behr et al., 2015), we predicted diagnostic marker genes (DMGs) for the identification of beer-spoiling LAB. 45 DMGs were evaluated and validated with 118 strains, using PCR. Along a comprehensive genome analysis, comprising 24 core-species genomes and up to 90 additional LAB genomes, supported by the abovementioned physiological and metabolic data, shared and species-specific strategies were described, providing new insights into the lifestyle of beer-spoiling LAB in beer. This was further done to identify potential lifestyle genes, which are genes where a link of the DMG to the LAB lifestyle in beer is either indicated or even proven. We finally probed the suitability of a range of established and new DMGs/lifestyle genes for the prediction of beer ability of LAB with very high and high hazard potential.

Beer-spoiling LAB are known to be not closely related to each other based on 16 rDNA sequences (Suzuki, 2015). The analysis of further phylogenetic markers and different phylogenomic analyses confirmed that these bacteria are not a monophyletic group within the LAB, emphasizing that their adaptation to the niche beer was a convergent process. However, which properties actually make them a distinct group? We found no distinct interspecies

relation to various chromosomal properties, nor did we find a specific “core genome of beer spoilage.” We did not find a specific metabolic trait or pathway, related to sugars, organic acids and amino acids, which is obligatory for beer-spoiling LAB. The corresponding metabolic capabilities were almost exclusively conserved within a species, showing that they are not decisive with respect to beer spoilage ability. However, there are many conceivable metabolic adaptions of LAB to unfavored conditions including alternative pyruvate metabolism, mixed acid fermentation, the utilization of alternative electron acceptors as well as energy and pH-homeostasis related amino acid metabolism (Holzapfel and Wood, 2014; Mozzi et al., 2010; van de Guchte et al., 2002). Our results indicate that beer-spoiling LAB, although by a different extent, apply all of these strategies for a successful growth in the harsh environment beer. We finally verified the proposed enhanced energy-demand of LAB exposed to hops (Vogel et al., 2010), indicating that all metabolic strategies which improve energy economy, are important for their lifestyle in beer.

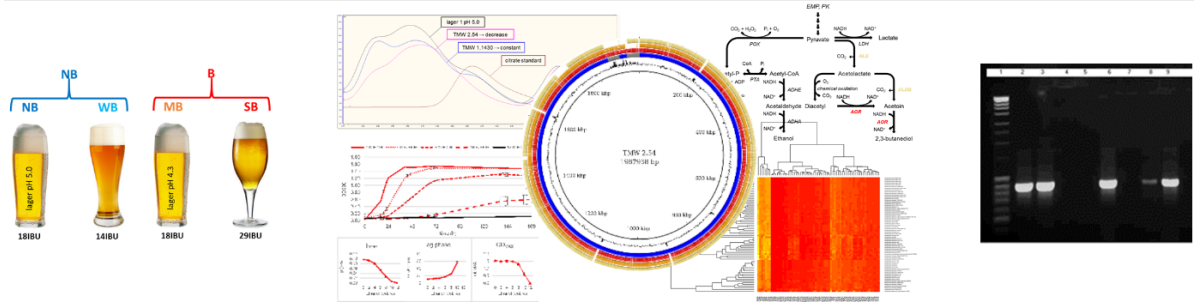
We further found a distinct relation of the isolation source brewery, the hazard potential and the beer spoilage potential to the number of plasmid-encoded, brewery-specific and brewery-typical (enriched) genes and, in general, to the amount of mobile genetic elements. Breweries are sometimes extreme and very dynamic environments characterized by complex, ever-changing, abiotic and biotic circumstances (Preedy, 2009). Bacteria are able to respond to changing environmental conditions by phenotypic and/or by genomic plasticity (Casacuberta and Gonzalez, 2013). Genomic plasticity, more precisely the uptake of hop resistance genes by horizontal gene transfer, was already suggested to be responsible for the origin of beer-spoiling LAB, transforming originally non-spoiling strains into harmful contaminants (Suzuki, 2015). However, we suggest that genomic plasticity contributes to multiple niche adaption within the dynamic brewery environment. Beer-spoiling LAB are not necessarily (solely) persistent residents of the brewery environment, long-term and statically adapted to a single niche like beer, waiting for their chance. Instead, we suggest that these bacteria are introduced into the brewery from raw materials (Bokulich et al., 2015), while they adapt to the dynamic brewery environment by an uptake and a release of niche-specific genes from/to a shared gene pool. This pool contains genes and genetic clusters, most of them associated with transposases, which are shared by all beer-spoiling strains of all core-species and others, which are only relevant for a selection of species. We identified traits, which allow the utilization of alternative substrates, improved NAD recycling and a shift towards less acid metabolic products or alkalization, while any metabolic process reducing the acidity of the environment can be considered as an important part of the lifestyle of beer-spoiling LAB in beer. Further, the comparative analysis of brewery plasmidomes emphasizes and extends the known relevance of cation homeostasis, oxidative stress tolerance and cell envelope metabolism for beer spoilage potential (Suzuki, 2015). In case of *P. damnosus* and *L. backii*, we found the

plasmid-encoded ability to produce long-chain fatty acids to be essential for beer spoilage ability in the low-fat environment beer (Preedy, 2009), while *fabZ*, part of the brewery-specific fatty acid biosynthesis-cluster, proved to be an outstanding lifestyle gene for the identification of beer-spoiling strains of these species. We were able to predict further novel DMGs by comparative genomics, evaluated their significance using PCR and validated the value of several novel lifestyle genes for beer-spoiling LAB. We provide three distinct detection and differentiation systems for the identification of harmful contaminations with very high hazard potential LAB core-species, while all of them outcompete approaches relying on already published DMGs. A hybrid approach, including a species and strain identification with MALDI-TOF MS and a consequent differentiation of beer-spoiling and non-spoiling strains using lifestyle genes, was further found to be the best solution with respect to accuracy, precision and specifity.

In conclusion, beer spoilage ability is a plasmid-encoded, dynamic trait. The combination of mostly species-specific chromosomal and shared plasmid-encoded traits and strategies enables LAB to overcome the beer-specific antibacterial hurdles. Beer-spoiling LAB are different with respect to their chromosomal prerequisites, while they share a number of plasmid-encoded lifestyle genes, which can be used for the targeted discrimination of beer-spoiling and non-spoiling LAB.

Graphical summary: A global overview of the most important lifestyle-features and metabolic strategies is shown in terms of categories. The occurrence/relevance of a category within the single core-species is indicated by yellow and red color, while the latter indicates that the corresponding traits are (rare exceptions) essential for beer spoilage ability. If available, the respective lifestyle genes are shown. The counteracted stress qualities and the purpose of all categories, with respect to their lifestyle in beer, are shown and color-coded. The *evidence-level* is indicated: GG = genomics and genetics; PM = physiology and metabolism; L = literature (see p. 212 for literature adduced). The applied workflow/study-design is sketched below the table.

Lifestyle and metabolic strategies	**Lifestyle genes**	**Stress, Hurdle and Purpose**	**homofermentative**			**heterofermentative**		
			P. claussenii	***P. damnosus***	***L. backii***	***L. paracollinoides***	***L. brevis***	***L. lindneri***
Hop transport	*horA, horC*	hop	GG,L	GG,L	GG,L	GG,L	GG,L	GG,L
Avoidance of lactic acid production		acid, hop	PM	PM	PM	PM	PM ,L	PM
Genomic plasticity	M03, M19, M34	adaptive evolution	GG,PM	GG	GG	GG	GG	GG
Teichoic and lipoteichoic acid metabolism	M43	acid, hop	GG,L	GG,L	GG,L	GG,L	GG,L	
Cation homeostasis	*hitA*	oxidative, hop	GG	GG	GG	GG	GG,L	
Oxidative stress tolerance	M42, M18	oxidative, hop	GG,L	GG	GG	GG	GG,L	
Alternative pyruvate metabolism		acid, hop	GG,L	GG,PM ,L	GG	GG	GG,L	
Malolactic fermentation		acid, hop, energy	GG,PM	GG,PM	GG,PM		GG,PM ,L	GG,PM ,L
Arginine/agmatine deiminase		acid, hop, energy	GG,PM ,L		GG,PM	GG,PM ,L	GG,PM ,L	
Amino acid decarboxylation		acid, hop, energy			PM	PM	GG,PM ,L	GG,PM ,L
Alternative electron acceptors		acid, hop, energy	GG,PM ,L				GG,PM ,L	GG,PM ,L
Mixed-acid fermentation		acid, hop, energy	GG,PM		GG,PM			
Fatty acid biosynthesis	*fabZ*	acid, hop		GG,PM	GG			



7 Zusammenfassung

Bier ist ein beliebtestes Genussmittel sowie ein profitables Produkt der Brauwirtschaft. Gleichzeitig handelt es sich um eine anspruchsvolle ökologische Nische für eine kleine Anzahl von Spezialisten, darunter bierverderbende Milchsäurebakterien (MSB). Deren Fähigkeit zum Wachstum in Bier ist eine teilweise stammspezifische Eigenschaft. Dabei müssen MSB einige Hürden nehmen: ein hoher CO_2 sowie ein niedriger O_2 Gehalt, Ethanol, ein niedriger pH Wert und antibakterielle Hopfensäuren (Suzuki, 2011; Vriesekoop et al., 2012). Für die mikrobielle Qualitätskontrolle in Brauereien ist es daher wichtig die Lebensweise (Lifestyle) bierverderbender MSB in der ökologischen Nische Bier sowie die angewandten Überlebensstrategien zu verstehen. Daraus ergeben sich mögliche Lifestyle-Gene, welche den MSB erlauben in Bier zu wachsen und der Qualitätskontrolle als mögliche Zielgene zur Verfügung stehen.

118 Stämme 13 verschiedener Spezies wurden physiologisch in Bierverderbspotential- und Bierverderbsfähigkeit-Gruppen eingeteilt, wobei der Fokus dieser Arbeit auf *P. claussenii*, *P. damnosus*, *L. backii*, *L. lindneri*, *L. paracollinoides* und *L. brevis* (Kernspezies) lag. Diese Kernspezies zeichnen sich durch ein (sehr) hohes Bierschädlichkeitspotential aus (Hutzler et al., 2013). 26 Stämme, variierend in Verderbspotential und Isolationsquelle, wurden auf ihr Wachstum in verschiedenen substituierten (z.B. Zugabe von Aminosäuren) Biervarianten, sowie ihren Metabolismus in Bier, unter Säure- und Hopfenstress untersucht. Des weiteren wurde das Wachstum dieser 26 Stämme bei bierspezifschen Stressqualitäten (Hürden) evaluiert. Von diesen Stämmen wurden mittels *single molecule real time sequencing* (McCarthy, 2010) 17 vollständige Genome generiert und annotiert. Innerhalb einer ausführlichen Genomanalyse wurden 24 (Kernspezies-Genome) bis zu 114 MSB-Genome analysiert bzw. miteinander verglichen. Anschließend wurden mit Hilfe des BlAst Diagnostic Gene finder (Behr et al., 2015) diagnostische Markergene (DMGs) für die Identifizierung bierverderbender MSB bestimmt, wobei 45 DMGs anhand der 118 Stämme mittels PCR evaluiert und validiert wurden. Unter Einbezug aller ermittelten Daten wurden gemeinsame und spezies-spezifische Strategien abgeleitet, um daraus Informationen über die Lebensweise bierverderbender MSB zu gewinnen. Die daraus resultierenden Erkenntnisse wurden verwendet um Lifestyle-Gene zu identifizieren, also DMGs mit einer naheliegenden oder nachgewiesenen Verbindung zur Lebensweise der MSB in Bier. Die potentiellen DMGs und Lifestyle-Gene wurden abschließend auf ihre Anwendbarkeit zur Vorhersage der Bierverderbsfähigkeit von MSB mit hohem und sehr hohem Bierschädlichkeitspotential untersucht.

Basierend auf der 16S rDNA stellen bierverderbende MSB keine monophylogenetische Gruppe dar (Suzuki, 2015), wobei dies durch die Analyse weiterer phylogenetischer Marker sowie phylogenomische Analysen bestätigt wurde. Dies deutet auf eine konvergente Anpassung hin, wobei sich die Frage stellt, welche Eigenschaften die Gruppe der Bierverderber definieren? Weder besitzen Bierverderber ein für diese Gruppe spezifisches *Core*-Genom, noch zeichnen sie sich durch andere distinkte, chromosomale Gemeinsamkeiten aus. Bezüglich organischer Säuren, Aminosäuren und Kohlenhydraten konnten keine spezifischen, oder für den Bierverderb obligatorischen, speziesunabhängigen metabolischen Eigenschaften definiert werden. Dass die jeweiligen metabolischen Fähigkeiten innerhalb der einzelnen Spezies fast ausschließlich konserviert vorliegen, zeigt dass diese nicht entscheidend sind für die jeweilige (stamm-spezifische) Bierverderbsfähigkeit. Dennoch ist davon auszugehen, dass die jeweiligen metabolischen Strategien bierverderbender MSB zu einem erfolgreichen Wachstum in Bier beitragen. Dabei gibt es verschiedene mögliche Anpassungen von MSB an ungünstige Bedingungen, u.a.: alternativer Pyruvatstoffwechsel, Verwendung alternativer Elektronenakzeptoren und pH-Homöostase/energiegenerierender Aminosäurestoffwechsel (Holzapfel and Wood, 2014; Mozzi et al., 2010; van de Guchte et al., 2002). Die hier erbrachten Ergebnisse deuten darauf hin, dass bierverderbende MSB, wenn auch in unterschiedlichem Ausmaß, alle oben genannten Strategien anwenden. Der bereits postulierte erhöhte Energiebedarf von bierverderbende MSB unter Hopfenstress (Vogel et al., 2010) konnte in dieser Arbeit verifiziert werden, woraus sich schließt, dass alle metabolischen Strategien, welche zu einer effizienteren Energiegewinnung beitragen, einen wichtigen Anteil an deren Lebensweise innehaben.

Es wurde ein distinkter Zusammenhang zwischen der Isolationsquelle Brauerei, dem Bierschädlichkeitspotential und dem Bierverderbspotential auf der einen Seite und der Anzahl plasmid-kodierter, brauerei-spezifischer und -typischer (angereicherter) Gene auf der anderen Seite gefunden, sowie ein genereller Zusammenhang zur Anzahl an mobilen genetischen Elementen. Brauereien können als teilweise extreme Umwelt betrachtet werden, charakterisiert durch sich ständig ändernde biotische und abiotische Bedingungen (Preedy, 2009), wobei Bakterien diesen Veränderungen mit phänotypischer und/oder genomischer Plastizität begegnen können (Casacuberta and Gonzalez, 2013). Genomische Plastizität, genauer gesagt die Aufnahme von Hopfenresistenz-Genen durch horizontalen Gentransfer, wurde bereits als eine Ursache für das Auftauchen/die Entstehung von bierverderbenden MSB vorgeschlagen, wobei durch die Aufnahme der Gene ursprünglich harmlose Kontaminanten zu bierverderbende MSB transformiert werden (Suzuki, 2015). In dieser Arbeit wird nahegelegt, dass genomische Plastizität zusätzlich zur Anpassung an multiple Nischen innerhalb des dynamischen Brauerei-*Environments* beiträgt. Bierverderbende MSB sind nicht notwendigerweise (ausschließlich) persistente Kontaminanten der Brauerei - langfristig und

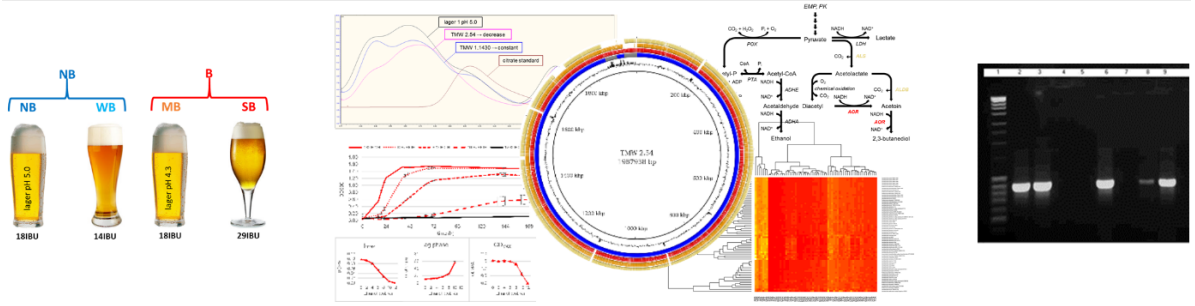
statisch angepasst an eine einzige Nische wie Bier. Da MSB (ohne Hopfentoleranz-Gene) durch Rohstoffe in die Brauerei eingetragen werden können (Bokulich et al., 2015), wird angenommen, dass sich diese durch Aufnahme und Abgabe von nischen-spezifischen Genen eines geteilten genetischen Pools an ihr dynamisches Habitat anpassen. Dieser genetische Pool enthält spezies-spezifische und spezies-unabhängige Gene und genetische Cluster, ein Großteil assoziiert mit Transposons. Dabei wurden genetische Elemente identifiziert, welche unter anderem die Verwendung alternativer Substrate, ein verbessertes NAD-Recycling und erhöhte Säuretoleranz ermöglichen, wobei jeglicher metabolischer Prozess, welcher die Ansäuerung in Bier verringert, als wichtiger Bestandteil des Lifestyles bierverderbender MSB gilt. Des weiteren untermauert die vergleichende Analyse der Brauerei-Plasmidome die bereits indizierte Relevanz der Toleranz gegenüber oxidativem Stress, sowie von Kationen-Homöostase und zellhüll-assoziiertem Metabolismus für das Bierverderbspotential (Suzuki, 2015). Die plasmid-codierte Fähigkeit zur Biosynthese von langkettigen Fettsäuren ist im Fall von *P. damnosus* und *L. backii* eine essentielle Eigenschaft für das Wachstum in der fettsäurearmen Nische Bier. Hierbei zeigte sich *fabZ*, Teil des entsprechenden Clusters, als herausragendes Lifestyle-Gen für die Identifizierung bierverderbender Stämme dieser Spezies.

Die vergleichende Genomik ermöglichte die Ableitung weiterer DMGs, welche mittels PCR evaluiert und validiert wurden. Anhand neuer und etablierter Lifestyle-Gene wurden drei unterschiedliche Nachweissysteme zur Identifikation schädlicher Kontaminationen mit bierverderbenden MSB (Kernspezies) erarbeitet, wobei alle drei Systeme eine Verbesserung bisheriger Ansätze, welche ausschließlich auf der Verwendung etablierter DMGs basieren, darstellen. Ein Hybrid-Ansatz wird vorgeschlagen, welcher sich durch ein hohes Maß an Richtigkeit, Präzision und Spezifität auszeichnet. Dieser besteht aus einer Spezies und Stammidentifizierung mittels MALDI-TOF MS und anschließender Differenzierung bierverderbender und nichtverderbender Stämme mittels Lifestyle-Genen (PCR).

Abschließend kann gesagt werden, dass Bierverderbsfähigkeit eine plasmid-kodierte, dynamische Eigenschaft ist. Die Kombination aus meist spezies-spezifischen chromosomalen und spezies-unabhängigen plasmid-kodierten Eigenschaften und Strategien ermöglicht es MSB die bierspezifischen Hürden zu nehmen und in Bier zu wachsen. Bezüglich chromosomaler Voraussetzungen sind bierverderbende MSB unterschiedlich, während sie einen Pool spezies-unabhängiger Lifestyle-Gene teilen. Diese Gene ermöglichen die zielgerichtete Differenzierung bierverderbender und nichtverderbender MSB.

Graphische Zusammenfassung: Die Tabelle gibt einen Überblick über globale Kategorien Lebensweise-assoziierter Eigenschaften und metabolischer Strategien. Die Relevanz bzw. das Vorkommen einer Kategorie innerhalb der Kernspezies ist in Gelb hervorgehoben, für den Bierverderb essentielle Eigenschaften in Rot. Dazugehörige Lifestyle-Gene, soweit verfügbar, sind aufgeführt. Für jede Kategorie ist die jeweilige Stress-qualität gezeigt, also jene bierspezifische Hürde, welcher durch den genannten Mechanismus entgegengewirkt wird. *Evidenzlevel*: GG = Genomik und Genetik; PM = Physiologie und Metabolismus; L = Literatur (siehe S. 204 für herangezogene Literatur). Das Design der Studie ist in groben Zügen unterhalb der Tabelle skizziert.

Lebensweise und metabolische Strategien	**Lifestyle-Gene**	**Stress, Hürde und Aufgabe**	**homofermentativ**			**heterofermentativ**		
			P. claussenii	***P. damnosus***	***L. backii***	***L. paracollinoides***	***L. brevis***	***L. lindneri***
Hopfentransport	*horA, horC*	Hopfen	GG,L	GG,L	GG,L	GG,L	GG,L	GG,L
Vermeidung von Milchsäureproduktion		Säure, Hopfen	PM	PM	PM	PM	PM ,L	PM
Genomische Plastizität	M03, M19, M34	adaptive Evolution	GG,PM	GG	GG	GG	GG	GG
Teichon/Lipoteichonsäure-metabolismus	M43	Säure, Hopfen	GG,L	GG,L	GG,L	GG,L	GG,L	
Kationen-Homöostase	*hitA*	Oxidativ, Hopfen	GG	GG	GG	GG	GG,L	
Oxidativer Stress	M42, M18	Oxidativ, Hopfen	GG,L	GG	GG	GG	GG,L	
Alternativer Pyruvatmetabolismus		Säure, Hopfen	GG,L	GG,PM ,L	GG	GG	GG,L	
Malolaktische Fermentation		Säure, Hopfen, Energie	GG,PM	GG,PM	GG,PM		GG,PM ,L	GG,PM ,L
Arginine/Agmatine Deiminase		Säure, Hopfen, Energie	GG,PM ,L		GG,PM	GG,PM ,L	GG,PM ,L	
Aminosäure Decarboxylierung		Säure, Hopfen, Energie			PM	PM	GG,PM ,L	GG,PM ,L
Alternative Elektronenakzeptoren		Säure, Hopfen, Energie	GG,PM ,L				GG,PM ,L	GG,PM ,L
Gemischte Säuregärung		Säure, Hopfen, Energie	GG,PM		GG,PM			
Fettsäurebiosynthese	*fabZ*	Säure, Hopfen		GG,PM	GG			



Literatur zur graphischen Zusammenfassung: (Back, 1994, 2005; Behr et al., 2006; Behr et al., 2007b; Behr and Vogel, 2009, 2010; Bergsveinson, Baecker, et al., 2015; Bergsveinson, Pittet, et al., 2015; Bergsveinson et al., 2012; Dobson et al., 2002; Haakensen et al., 2008; Hutzler et al., 2013; Iijima et al., 2007; Kalač et al., 2002; Pittet et al., 2012; Pittet et al., 2013; Sakamoto et al., 2001; Schurr et al., 2013; Schurr, Behr, et al., 2015; Schurr, Hahne, et al., 2015; Suzuki, 2015; Suzuki, Funahashi, et al., 2004; Suzuki et al., 2005a, b; Suzuki et al., 2006; Vogel et al., 2010).

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9 Supplementary Section 1 - Detailed characterization

9.1 Detailed characterization for strain selection

28 strains, comprising six different species, were selected based on the results of the initial characterization (4.2.2, p. 53) and characterized in detail. Methods for the determination of hop resistance and metabolic activity in beer were optimized with respect to incubation time before characterization. Resazurin tests (3.2.6.1, p. 33) and MIC-tests (3.2.6.2, p. 34) were done for preadapted (adaptive potential) and non-adapted (constitutive potential) cells. In addition, strains were tested with a beer spoilage test (3.2.6.5, p. 35) according to Suzuki et al. (2005b). The presence of already published lifestyle genes, suggested to correlate with beer spoilage ability, was tested for three biological replicates using PCR (3.4.2, p. 39). M13-V RAPD-PCR was done as a measure for genetic diversity in order to support the selection process of strains for genome sequencing. Based on RAPD-PCR band patterns, similarity coefficients were calculated applying *Pearson's* correlation. Cluster analysis was done with the *Ward* method and plotted as dendrogram (Figure 51, p. 234). In case of the resazurin test, strains were assigned to beer spoilage potential groups applying the following rules, based on a visual assessment of color change (cf. Figure 48, p. 230):

- No beer spoilage potential (NB): + ≤ wheat 1 beer adaptive
- Weak potential (WB): + ≥ wheat beer 1 constitutive or lager beer 1 adaptive
- Middle potential (MB): + ≥ lager beer 1 constitutive or pilsner beer 1 adaptive
- Strong potential (SB): + ≥ pilsner beer 1 constitutive or pilsner beer 5 adaptive

Results are summarized in Appendix 4 (p. 334) and show the data of the resazurin test and the classification into the resazurin based beer spoilage potential groups, the corresponding MIC values as a measure for hop tolerance and the classification of strains into beer spoilage potential groups based on the resazurin test. The classification is also listed in Table 32 (p. 233).

Figure 48 (p. 230), Figure 49 (p. 230) show example results for both methods.

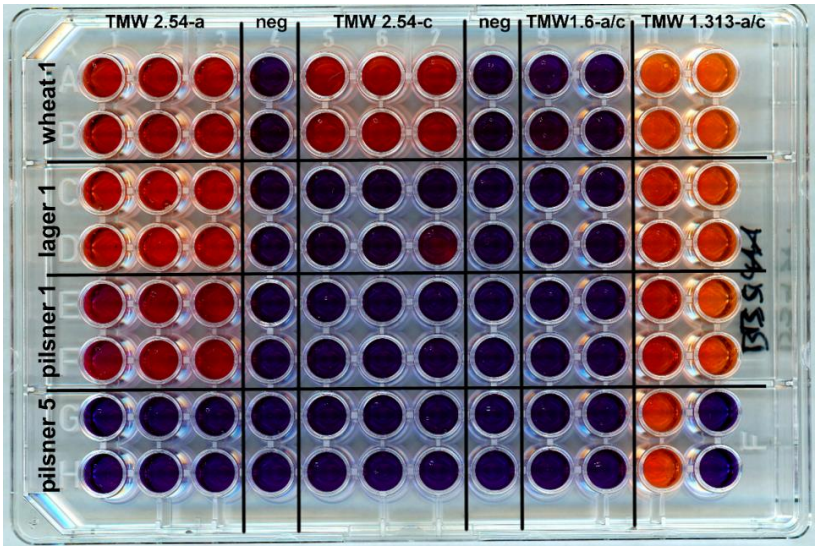


Figure 48: Illustration of the rapid resazurin test. After incubation of 6 days at 25° C, resazurin is added to all wells of the microtiter plate. Metabolically active cells cause a reduction of blue resazurin to orange/pink resorufin, indicating growth in beer. In this example, the adaptive (-a) and constitutive (-c) beer spoilage potential (BSP) were tested for *P. claussenii* TMW 2.54. Based on the abovementioned rules, the strain was assigned to the MB group. Preadapted cells of TMW 2.54 are capable of growing in wheat, lager and pilsner beer 1. Without pre-adaption, growth was detected in wheat beer 1 only. Neg = non-inoculated negative control/sterile control; TMW 1.6/TMW 1.313: biological controls, TMW 1.6 with low BSP/TMW 1.313 with strong BSP.

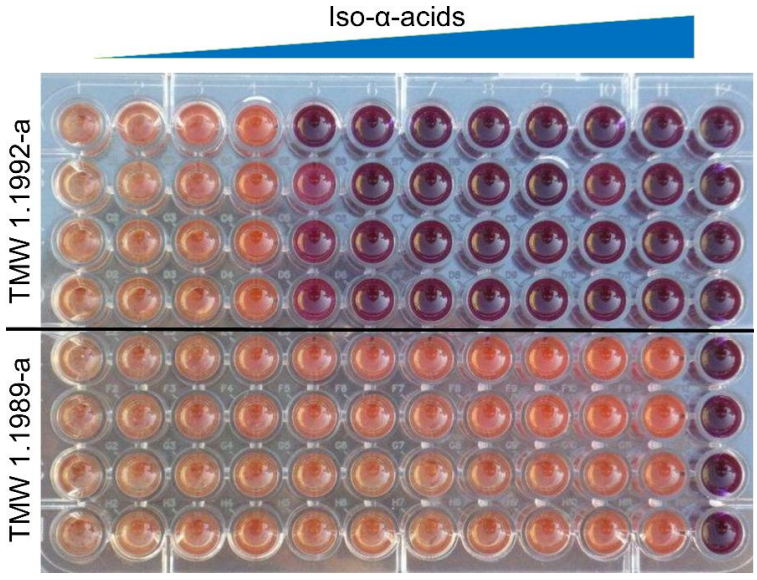


Figure 49: Illustration of a minimum inhibitory concentration (MIC) test using resazurin. After incubation of 6 days at 25° C, tris-buffer (pH 8.0) was added to all wells containing the cells, resazurin and an increasing amount of iso-α-acids. Metabolically active cells cause a reduction of blue resazurin to orange/pink resorufin indicating growth in $mMRS_2$ with increasing antibacterial properties. In this example the adaptive (-a) tolerance to iso-α-acids was tested for *L. backii* TMW 1.1989 and TMW 1.1992. TMW 1.1989 was able to grow in all inoculated wells, indicating high hop tolerance (> 50 ppm). The last row contains $mMRS_2$ only, serving as sterile control.

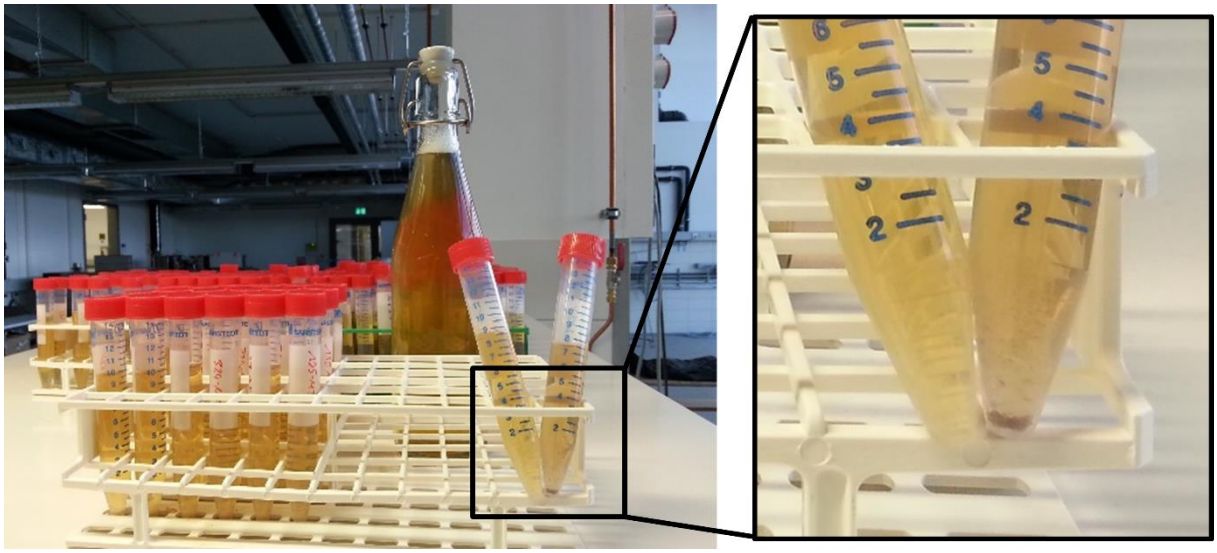


Figure 50: Photograph of beer spoilage test tubes. Tubes are checked periodically for visible growth. The tube on the right side shows strong sedimentation caused by *P. damnosus* TMW 2.1535. The left tube is the sterile control.

Figure 50 illustrates a beer spoilage test according to Suzuki et al. (2005b). The following rules were used for categorization.

- No beer spoilage potential (NB): no growth in test beers
- Weak potential (WB): growth in wheat beer 1
- Middle potential (MB): growth in lager beer 1
- Strong potential (SB): growth in pilsner beer 1

According to Suzuki et al. (2005b), strains were additionally assigned to two groups based on the ability to grow in lager beer 1 (lager beer as *average* beer with respect to pH, hop content), which will be referred to as beer-spoiling strains (beer spoilage ability):

- No beer spoilage ability (NB): no growth in lager beer 1
- beer spoilage ability (B): growth in (at least) lager beer 1

Table 32 (p. 233) shows the classification of strains into the corresponding groups, based on this specific beer spoilage test. Note that the final categories each strain belongs to and more detailed data (turbidity, acidification and adaption) regarding the growth behaviour obtained from the beer spoilage test can be found in chapter 4.2.4 (p. 57).

The presence of known DMGs within the characterized set of strains is summarized in Appendix 5 (p. 335).

The significance of *horA*, *horC* and *hitA* differed and there was a species dependent relation to beer spoilage potential and ability. A detailed evaluation of marker profiles of all tested strains as well as the correlation of marker genes to beer spoilage ability, potential and isolation source can be found in chapter 4.4 (p.115).

Figure 51 (p. 234) shows a dendrogram based on the RAPD-PCR patterns of the characterized strains. Preferably, different strains (within one species) were chosen for genome sequencing, attempting to cover a high diversity within one species. Therefore, two clusters were assigned for each species, defined by the first branching within each species.

Table 32: Detailed characterization of beer spoilage potential - categorization based on the beer spoilage test (3.2.6.5, p. 35). Group assignment based on beer spoilage test: strong beer spoilage potential (BSP) (SB) - growth in pilsner beer 1, middle BSP (MB) - growth in lager beer 1, weak BSP (WB) - growth in wheat beer 1, no BSP (NB) - no growth in test beers; beer spoilage ability (BSA - B/NB) defined by growth in lager beer 1. Categories (Cat.) based on the resazurin test are also listed for comparison. All strains chosen for genome sequencing are marked in grey.

Species	TMW	BSA	BSP	Cat. Resazurin
L. brevis	1.6	NB	WB	NB
L. brevis	1.1369	NB	WB	WB
L. brevis	1.313	B	SB	SB
L. brevis	1.465	B	SB	SB
P. claussenii	2.340	B	MB	WB
P. claussenii	2.1531	NB	NB	WB
P. claussenii	2.53	NB	WB	WB
P. claussenii	2.54	B	SB	MB
P. damnosus	2.1532	NB	NB	NB
P. damnosus	2.1533	B	SB	MB
P. damnosus	2.1534	NB	NB	NB
P. damnosus	2.1535	B	SB	WB
P. damnosus	2.1536	NB	NB	NB
L. backii	1.1430	B	SB	SB
L. backii	1.1988	B	SB	SB
L. backii	1.1989	B	SB	SB
L. backii	1.1990	B	SB	SB
L. backii	1.1991	B	SB	SB
L. backii	1.1992	B	MB	WB
L. lindneri	1.1285	B	MB	SB
L. lindneri	1.1286	B	MB	SB
L. lindneri	1.1433	B	MB	WB
L. lindneri	1.1993	B	MB	SB
L. lindneri	1.481	B	MB	SB
L. paracoll.	1.696	NB	WB	NB
L. paracoll.	1.1979	NB	NB	NB
L. paracoll.	1.1994	B	SB	WB
L. paracoll.	1.1995	B	SB	WB

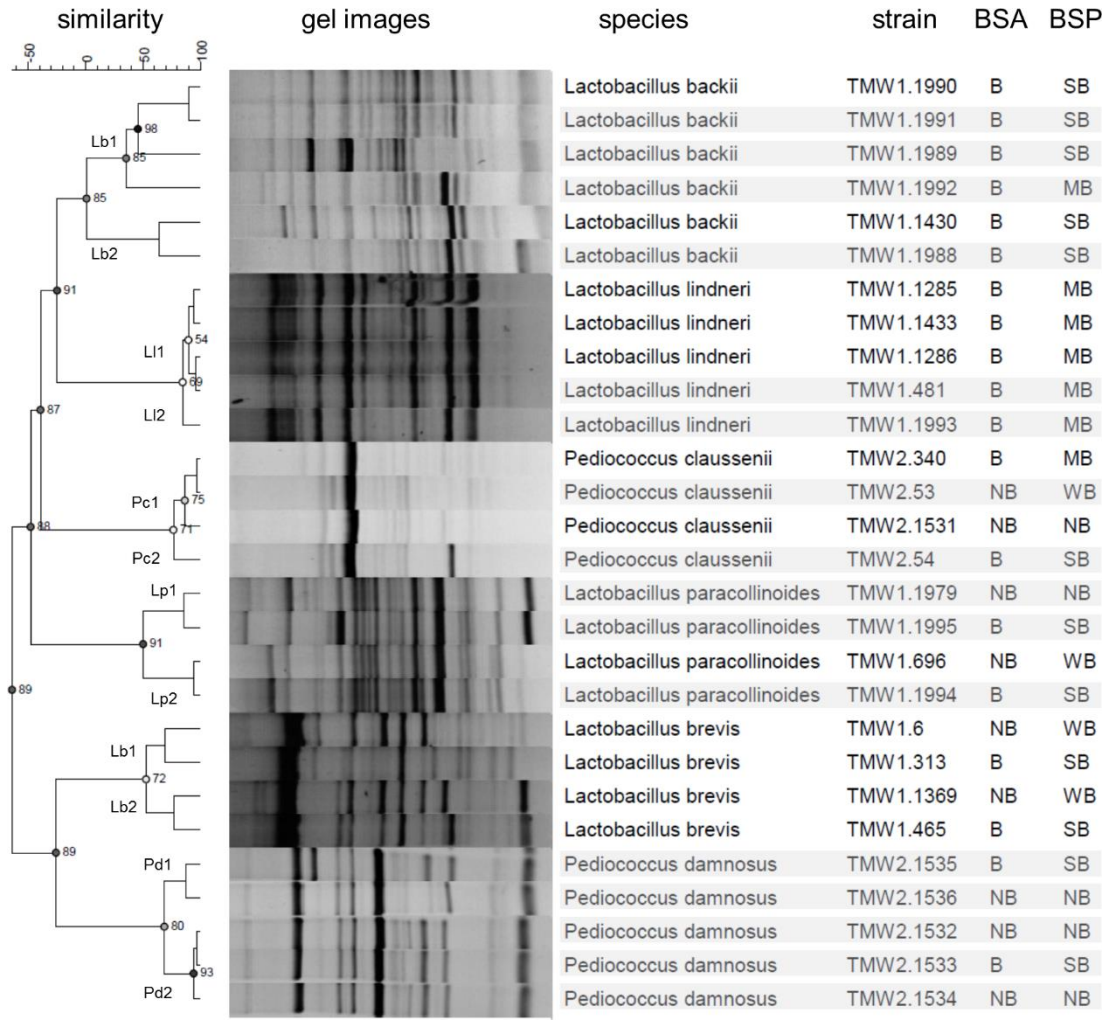


Figure 51: M13 RAPD-PCR dendrogram of strains characterized within the detailed characterization - illustrating the genetic diversity of characterized strains. Similarity was calculated according to *Pearson*, followed by cluster analysis using the *Ward* method. Within each species strains are assigned to two clusters, labeled using their initials (e.g. Lb = *L. backii*) followed by the numbers 1 or 2. All strains chosen for genome sequencing are marked in grey. Beer spoilage potential (BSP) based on this specific beer spoilage test (Table 32): strong BSP (SB), middle BSP (MB), weak BSP (WB), no BSP (NB); beer spoilage ability (BSA - B/NB).

9.2 Selection of a reference method for beer spoilage potential determination

In order to establish a reference method for the determination of beer spoilage ability and beer spoilage potential, all available data from the detailed characterization were analysed, using explorative data analysis. Categorization systems following simple man-made and logical rules, based on either the resazurin test or the beer spoilage test, were tested with respect to accordance and significance. Results showed that the included data match better to a classification of strains based on the beer spoilage test.

Physiological and genetic data obtained within the detailed characterization were tested for their accordance with the classification systems based on the resazurin test (3.2.6.1, p. 33) and the beer spoilage test (3.2.6.5, p. 35).

The following data were included into explorative data analysis and the construction of a heatmap:

- Resazurin test - metabolic activity in beer (constitutive/adaptive):
    - visual assessment of metabolic activity after 6 and 30 days
    - technical evaluation: the difference of OD_{570} to OD_{600}
- Beer spoilage test:
    - visual assessment of growth
    - days until visible growth as a measure for adaption time
    - OD_{590} after 60 days as a measure for turbidity
    - pH after 60 days as a measure for acidification potential
- Absence/presence of published lifestyle genes with known correlation to beer spoilage potential
- Minimum inhibitory concentration (MIC) for iso-α-acids (constitutive/adaptive)

Discriminant analysis of principle components (DAPC) was performed, (3.6.1, p. 44) while all data were scaled and centered before calculation. Using the 'find.clusters' function, 4 optimal (defined by data) clusters were defined, retaining ten principal components, explaining more than 90 % of the cumulative variance, followed by DAPC based on these 4 clusters. By plotting the first two discriminant functions of a DAPC, the distance or proximity of two groups is illustrated (Figure 52 (a), p. 237). All other classifications (Figure 52 (b, c, d)) are only indicated by alternative labelling, according to the different beer spoilage potential classification systems. The totality of data tested, matched best to the classification systems based on the beer spoilage test, illustrated by a clearer separation of groups as in case of the resazurin test based classification. This observation was confirmed using cluster analysis (not shown).

The quality of the tested classification systems is also illustrated by a heatmap (Figure 53), where all strains were sorted according to their overall beer spoilage potential (related data). All data were normalized in a range of 0 (blue) to 1 (red), while values in between are illustrated by a floating color code. A value of 1 corresponds to a characteristic of a given parameter indicating (correlating to) high beer spoilage potential, while a value of 0 means the opposite. For example the presence of lifestyle genes as *horA*, *horC* and *hitA* in general correlates to a higher beer spoilage potential and is therefore illustrated by a value of +1 (red color in heatmap), while the lack of these genes is represented by a value of 0 (blue). Next to the heatmap, the strains are listed and colored depending on the group they belong to. This is shown for all three classification systems. A distinct separation of same-colored blocks indicates a good quality for a given classification system, which is perfect for the categorization into two beer spoilage ability groups, employing the beer spoilage test. In addition, the classification into beer spoilage potential groups based on the beer spoilage test has a better quality (accordance) than the classification based on the resazurin test. Consequently, the beer spoilage test was chosen as reference method for the characterization of the beer spoilage ability and potential for all other strains.

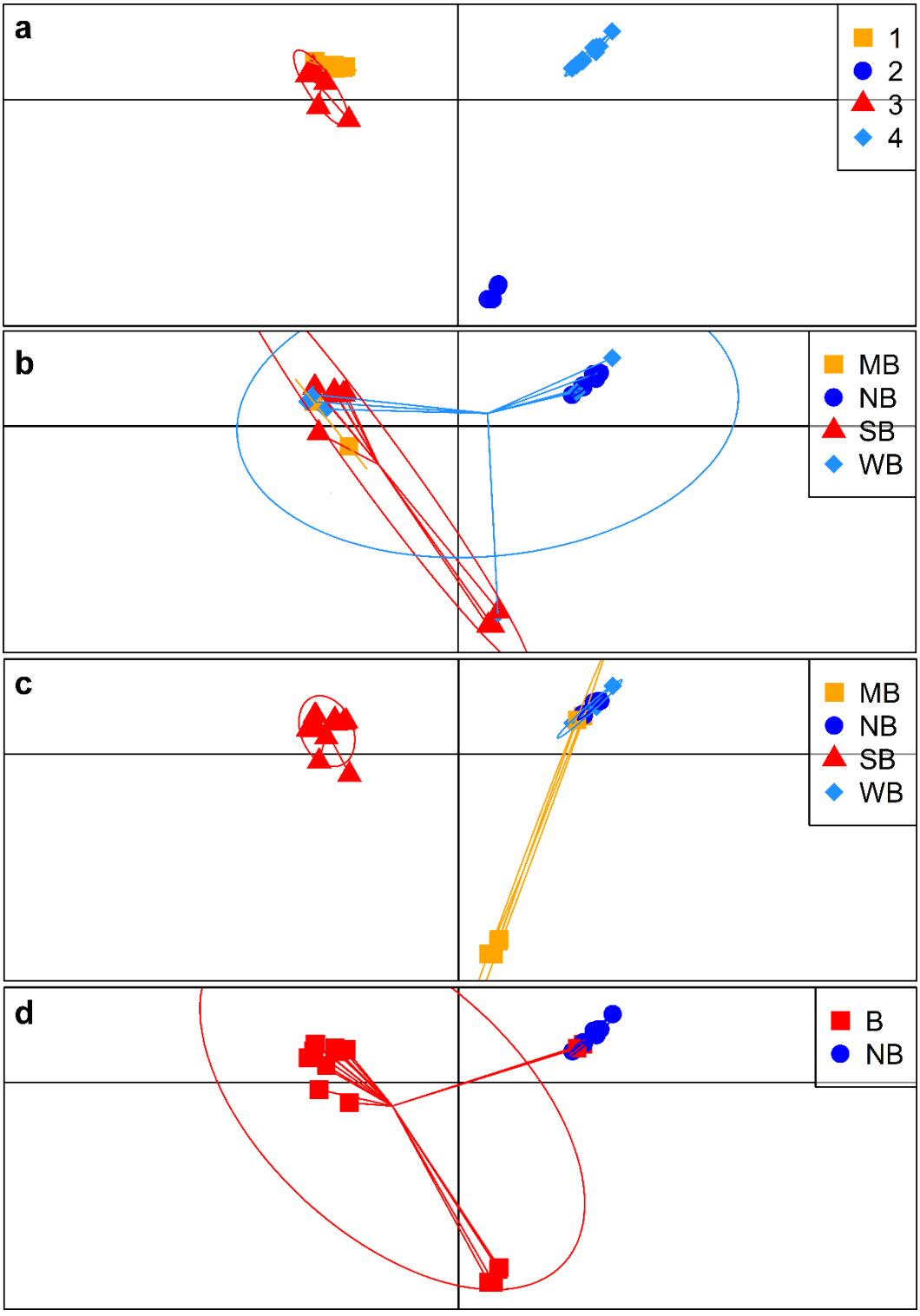


Figure 52: Explorative data analysis and testing of beer spoilage classification systems - scatterplots of first 2 principal components of the DAPC. A discriminant analysis of discriminant functions (DAPC) was done including all data from the detailed characterization, with the exception of RAPD-PCR. Each symbol corresponds to a single strain. **(a)** Four optimal clusters defined by 'find.clusters'; **(b,c,d)** data labeled according to: **(b)** beer spoilage potential (BSP) groups based on resazurin test; **(c)** BSP groups based on beer spoilage test; **(d)** beer spoilage ability groups based on beer spoilage test.

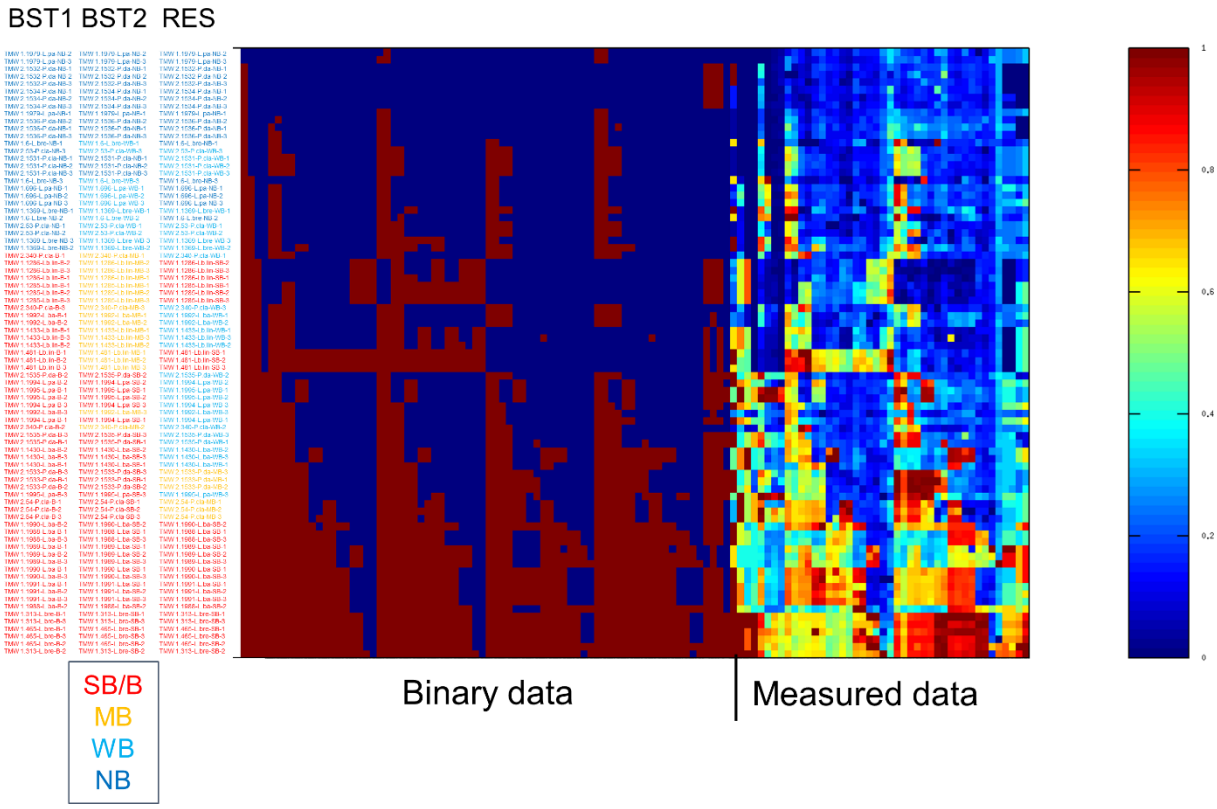


Figure 53: Heatmap, illustrating the overall beer spoilage potential (BSP) for 28 strains and the accordance with the beer spoilage classification systems used. On the left side, the y-axis labels (strains - three biological replicates) are listed in triplicate and color-labeled according to the three classification systems used. A distinct separation of same-colored blocks indicates a good classification system. Binary data include all parameters, which could only attain a value of 1 and 0. Measured data were normalized within a range of 0 to 1. A value of 1 (red) corresponds to a characteristic indicating high BSP, while values towards 0 (blue) indicate no or low BSP. BST1 = beer spoilage ability (BSA) according to beer spoilage test; BST2 = BSP according to beer spoilage test; RES = BSP according to resazurin test. SB = strong BSP; MB = middle BSP; WB = weak BSP; NB = no BSP; BSA (B/NB).

10 Supplementary Section 2 - Genome analyses

This supplementary section contains the genome analysis of 24 genomes of the core-species. General genomic properties, functional analysis and metabolic prediction were performed as described in chapters 3.6.2 (p. 45) and 4.3.2 (p. 88). All genomes sequenced within this project were analysed, including some previously published genomes. Included genomes are listed in each species chapter.

Functional analysis: In order to find and highlight exceptional and interesting features, the functional pattern of 114 strains, comprising 37 species of the genus *Lactobacillus*, were compared to each other with respect to total count of a given category and the respective proportion in relation to the total assignments/genome size. Note that in case of the Seed subsystems a given gene can be assigned to several subsystems. Also, consider that in general only about 40 to 50 % of all genes can be assigned to a SEED subsystem. The proportion (coverage) of genes assigned to SEED subsystems and COG categories will be mentioned in the corresponding chapters.

Note that the following chapters are based on *in silico* prediction and that this fact will not stressed repeatedly. If not stated differently, metabolic predictions apply to all genomes of a given species.

10.1 *Pediococcus claussenii*

10.1.1 General genomic properties

Two complete genomes of the beer-spoiling strains TMW 2.53-SB and TMW 2.54-SB were obtained in this study and supplemented with the genome of the slime producing, beer-spoiling strain TMW 2.340-MB (alias ATCC BAA-344) (Pittet et al., 2012). Both newly sequenced genomes could be assembled to one chromosome and two or three plasmids, respectively. *In vitro* results strongly indicate that the genetic unstable strain TMW 2.53 (NB-SB) also harbours three plasmids (same three as TMW 2.54-SB, pL254-3), while the sequenced *variant* only contained two plasmids. The chromosome size of *P. claussenii* ranges from 1.83 Mbp to 1.89 Mbp with a consistent GC content of 36.8 %. The chromosomes show a high degree of synteny with respect to general structure, resulting in one consistent block, as illustrated by Figure 55 (p. 241). TMW 2.340-MB harbours eight plasmids with 1,815 bp to 36,388 bp and GC contents from 34.9 to 41.2 %. The genomes of TMW 2.54-SB and TMW 2.53-SB contain three (two) plasmids ranging from 23,149 bp to 40,099 bp with a GC content between 40.2 and 44.5 %. The found plasmids differ not only in GC content from the chromosomes, but also with respect to codon usage (Figure 55). In total, 1,882 to 1,940 open reading frames (ORFs) were found, resulting in a coding density of about 87 %, while the core genome contained 1,687 gene families and the pan genome 1,993. Four complete rRNA operons, 55 tRNAs and two pseudo-tRNAs were found to be encoded. Figure 55 shows the predicted subcellular localization of *P. claussenii* proteins.

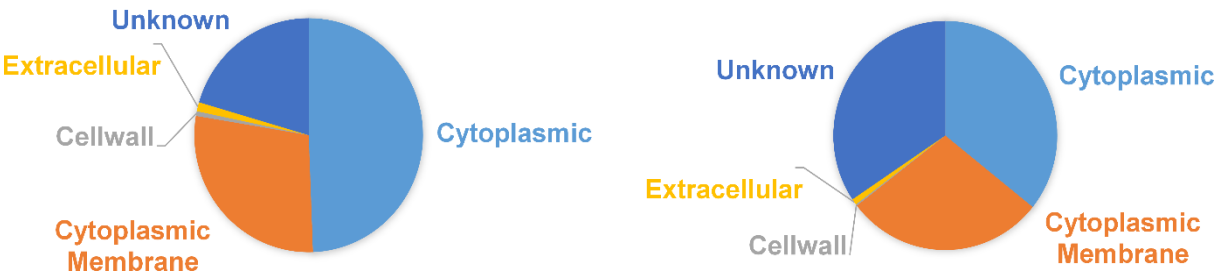


Figure 54: Subcellular localization of *P. claussenii* proteins in %. Distribution is shown for the average values for all three strains. **Left** – chromosomally encoded proteins, in detail → Cytoplasmic: 49.4 +/- 0.3 %; Cytoplasmic membrane: 28.2 +/- 0.1 %; Cell wall: 0.6 +/- 0.1 %; Extracellular: 1.3 +/- 0.1 %; Unknown: 20.5 +/- 0.3. **Right** - plasmid encoded proteins, in detail → Cytoplasmic: 35.7 +/- 2.7 %; Cytoplasmic membrane: 28.8 +/- 7.3 %; Cell wall: 0.2 +/- 0.3 %; Extracellular: 0.9 +/- 0.6 %; Unknown: 34.4 +/- 4.6.

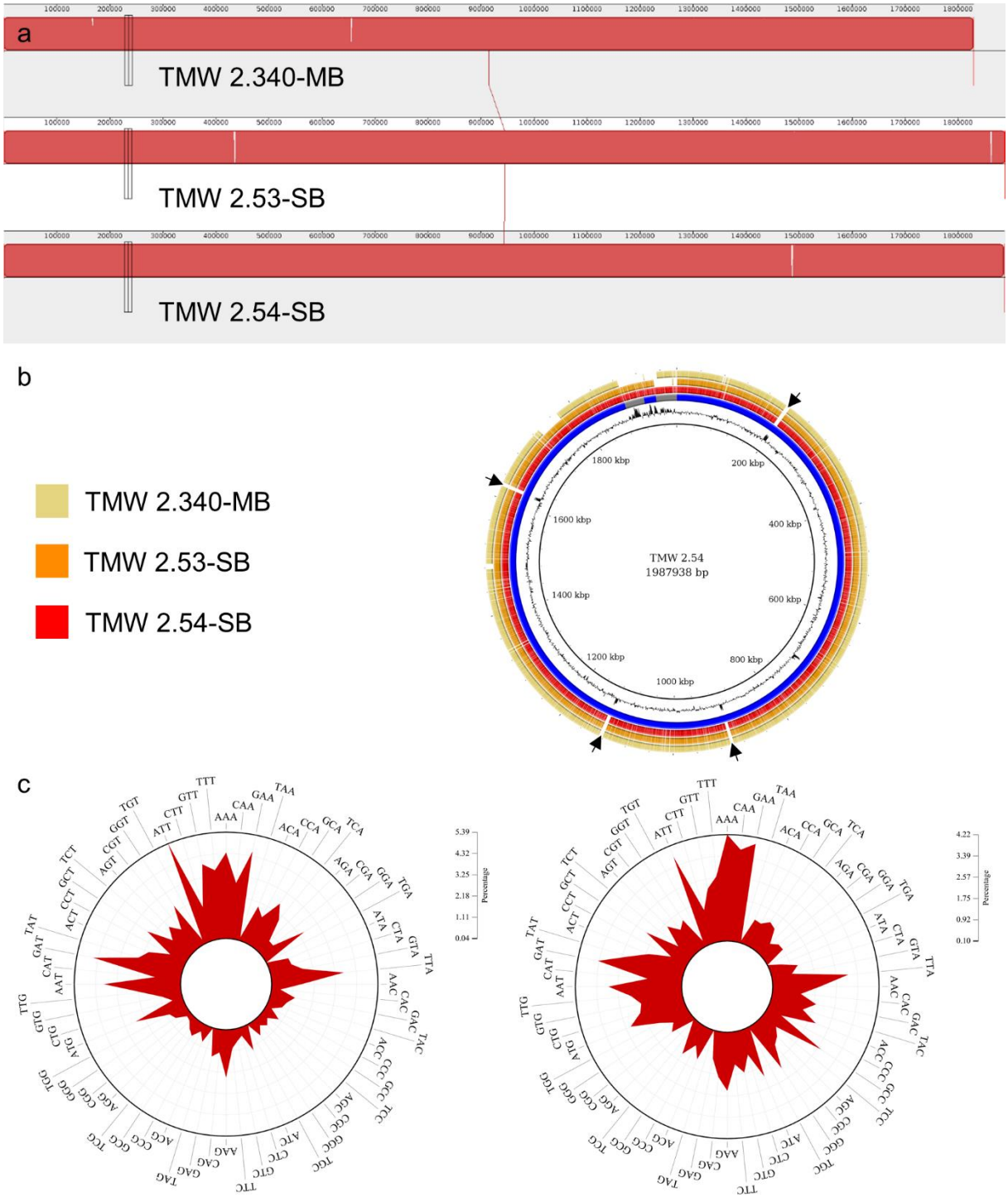


Figure 55: Illustration of the general genomic properties of *P. claussenii*. **(a)** Chromosome alignment of *P. claussenii* genomes, illustrating the high degree of chromosomal synteny **(b)** BLAST ring image of all *P. claussenii* genomes. All rings are described from the inside to the outside: ring 1 (black) represents the total genome sequence of TMW 2.54-SB as reference with bp coordinates; ring 2 (black) shows the GC content; ring 3 consists of arcs with different lengths and alternating coloration (grey/blue), representing the different contigs of TMW 2.54-SB; ring 4 (red) shows all BLAST hits of TMW 2.54-SB ORFs versus its own genome, illustrating the coding density; rings 5/6 (orange) show all hits of TMW 2.53-SB/TMW 2.340-MB ORFs versus the reference; black arrows indicate rRNA operon localization **(c)** Codon usage of TMW 2.54-SB chromosome (left) and plasmidome (right), exemplary.

10.1.2 Functional analysis - SEED and COG

46 to 47 % of the found ORFs could be assigned to SEED subsystems and the corresponding classification system. Of the remaining unassigned ORFs, 336 to 347 were annotated as hypothetical proteins. In case of the COG enrichment, 90 to 93 % could be assigned to a functional category, while 21.5 % were assigned to the global category “poorly characterized.” In both cases, the functional analysis showed a highly conserved pattern in case of the chromosomes (Figure 56, p. 243). In total, most genes were assigned to categories related to translation, transcription and carbohydrate metabolism, followed by genes associated with amino acid and cell envelope metabolism. In comparison, *P. claussenii* was not ranked within the top 10 (in comparison to the 114 investigated lactobacilli genomes) for any investigated SEED or COG category. In contrast, this species was found to be ranked within those 10 strains, having the lowest number and proportion of genes associated with nucleotide transport and metabolism. On the SEED subcategory and subsystem level, *P. claussenii* is found within the top 10 regarding teichoic and LTA biosynthesis, cobalamin biosynthesis and cation transport.

The SEED subsystem coverage for the plasmidomes is only 15.5 %, while about 75 % of all genes could be assigned to COG categories. Besides general plasmid features, at least 5 % or more could be related to cell envelope biogenesis, ion transport and metabolism, as well as defense mechanisms and stress response. In addition, several genes were associated with carbohydrate and lipid metabolism.

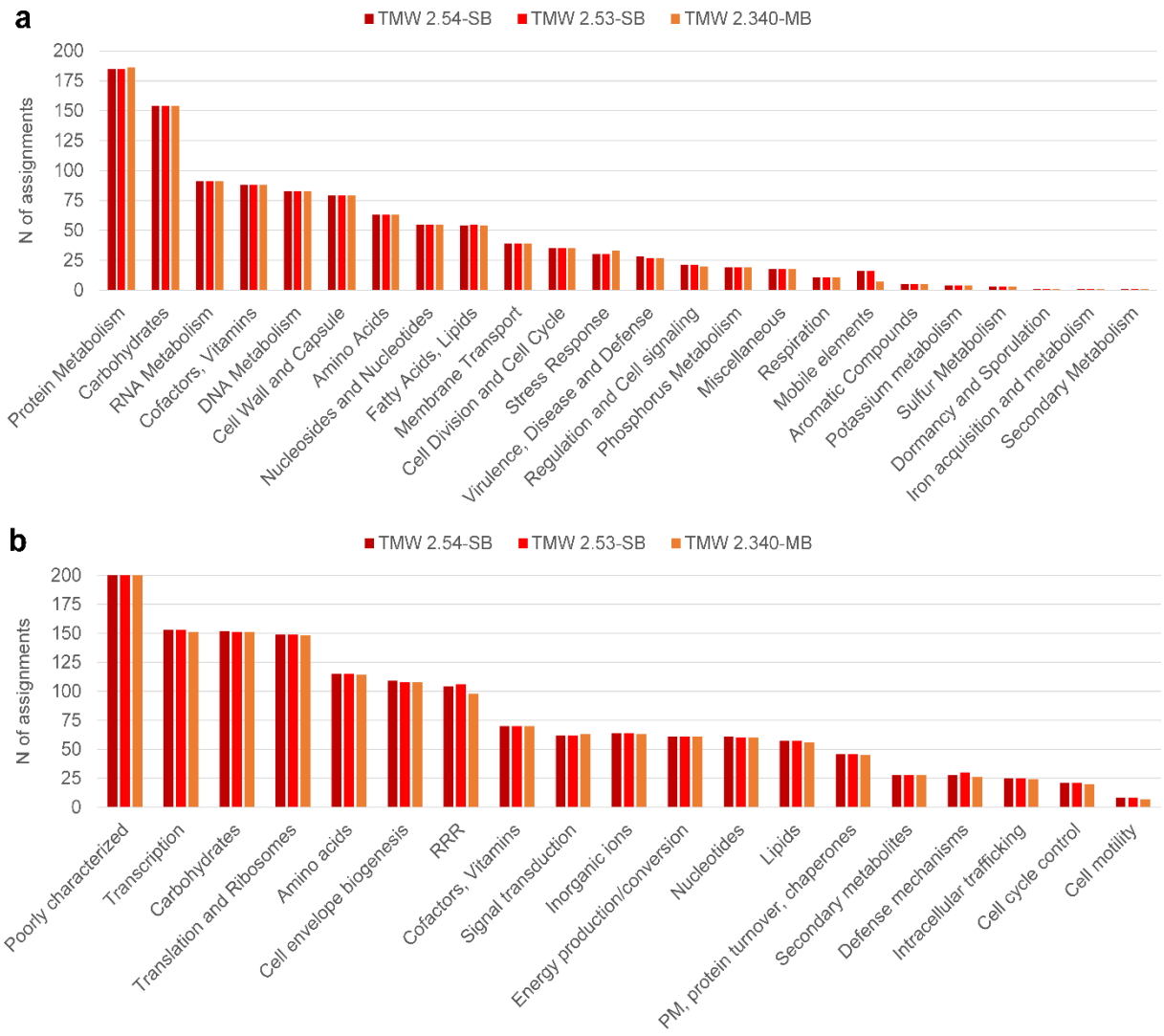


Figure 56: Functional analysis of *P. claussenii* chromosomes. The total number of proteins assigned to the single categories is shown for SEED categories **(a)** and COG categories **(b)** in descending order. In case of the COG category “Poorly characterized,” the actual values range from 357 to 369. SEED subsystem coverage: 47.9 +/- 0.4; COG coverage: 92.7 +/- 0.6. RRR = Replication, recombination and repair, PM = posttranslational modification.

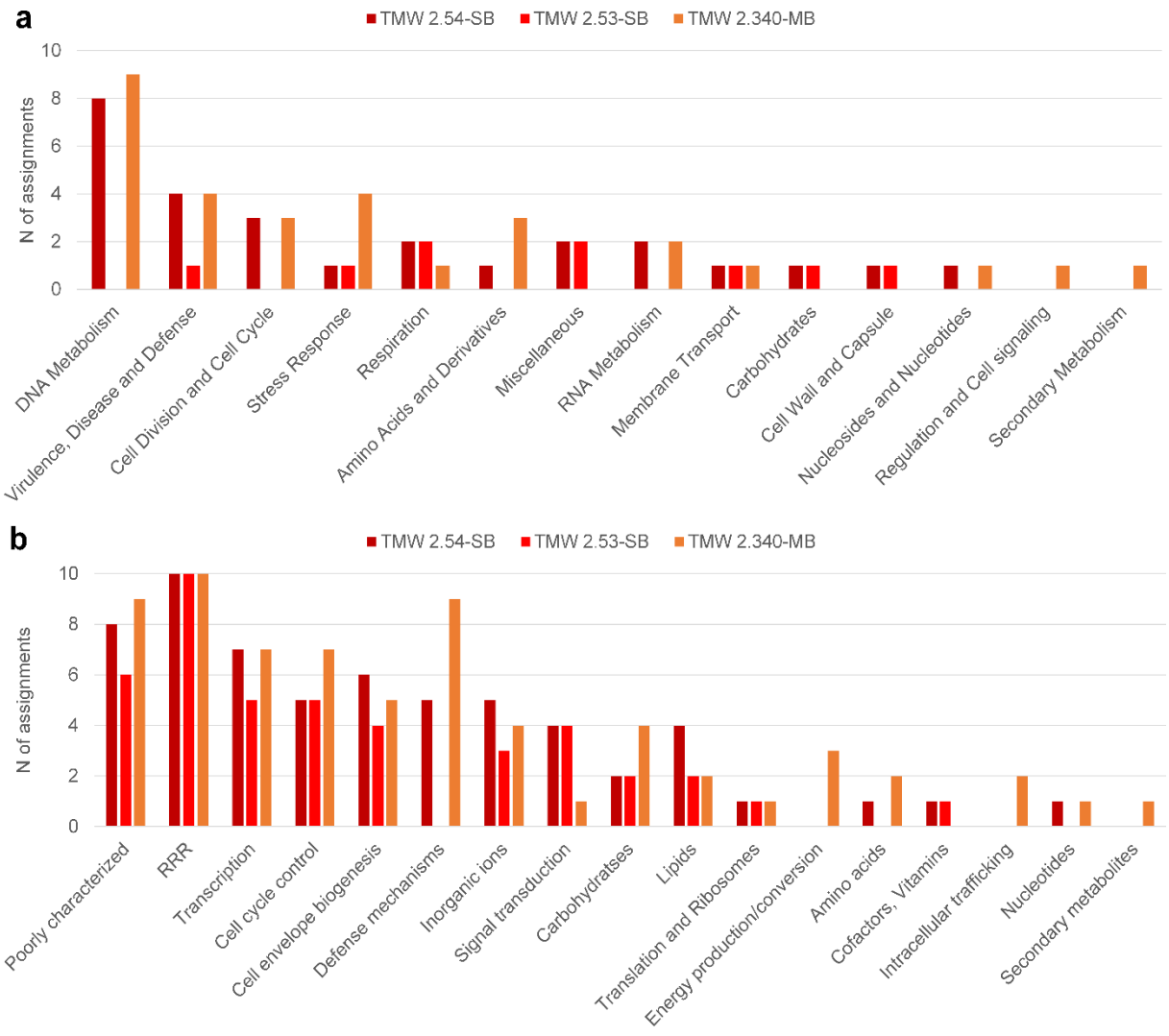


Figure 57: Functional analysis of *P. claussenii* plasmidomes. The total number of proteins assigned to the single categories is shown for SEED categories **(a)** and COG categories **(b)** in descending order. In case of the COG category RRR, 39 assignments were found for all three strains. SEED subsystem coverage: 15.5 +/- 5.9; COG coverage: 74.5 +/- 4.7. RRR = Replication, recombination and repair.

10.1.3 Metabolic capabilities

10.1.3.1 Carbohydrates and central metabolism

All genes for glycolysis and phosphoketolase pathway are present. With exception of transaldolase (EC 2.2.1.2), we found all genes of the pentose phosphate pathway, including those needed for the synthesis of 5-phospho-alpha-D-ribose 1-diphosphate (PRPP), a central intermediate of bacterial metabolism. Phosphotransferase systems (PTS), as well as the necessary enzymes to channel the respective substrates into glycolysis, were found for fructose, mannose, trehalose, cellobiose and mannitol. In addition, not clearly defined transport systems were found for maltose and isomaltose, spatially located next to the enzymes for their utilization, a maltose phosphorylase (EC 2.4.1.8) and an isomaltase (EC 3.2.1.10). No maltodextrin glucosidase (EC 3.2.1.20) was found. Further, all enzymes for the Leloir pathway of galactose utilization are present. Glycerol and gluconate permeases were found as well as the necessary enzymes for their utilization. The way of glucose uptake remains unclear, while genes potentially encoding for a permease and associated with a PTS system were found. In case of ribose, a potential transport system was found as well as a ribokinase (EC 2.7.1.15), allowing the utilization within the pentose phosphate pathway. Several 6-phospho-beta-glucosidases (EC 3.2.1.86) were found, located next to not clearly defined transport systems.

10.1.3.2 Pyruvate metabolism, organic acids and TCA cycle

All enzymes are present to produce ethanol, acetate, lactate (L/D-lactate) and acetoin from pyruvate as illustrated by Figure 45 (p. 173). In addition, the anaplerotic enzyme pyruvate carboxylase (EC 6.4.1.1) was found. All genes were found which are necessary for the uptake and utilization of malate and citrate. No enzymes of the TCA cycle were found.

10.1.3.3 Proteolytic system and amino acids

A cell wall-bound, extracellular protease was not found, while transport systems for oligo, di- and tripeptides are present. Several different endopeptidases, aminopeptidases and oligo-/tri-/dipeptidases are encoded. In addition, potential transport systems were found for alanine, glutamine/glutamic acid, glycine, lysine, methionine, threonine and branched amino acids (valine, leucine, isoleucine), as well as unspecified amino acid transporter. The amino acid biosynthesis of *P. claussenii* is incomplete and the remaining, predicted capabilities are mostly isolated from the central carbon metabolism (Figure 58, p. 246). A direct connection to the central carbon metabolism is only evident for asparagine/aspartic acid, glutamine/glutamic acid and proline. A complete auxotrophy is indicated for histidine, tryptophan, tyrosine,

phenylalanine, valine, leucine, isoleucine and threonine. All other amino acids can potentially be synthesized from other amino acids (e.g. serine, glycine, cysteine) or from other compounds (homocysteine → methionine). All three genomes were found to encode a complete AGDI pathway.

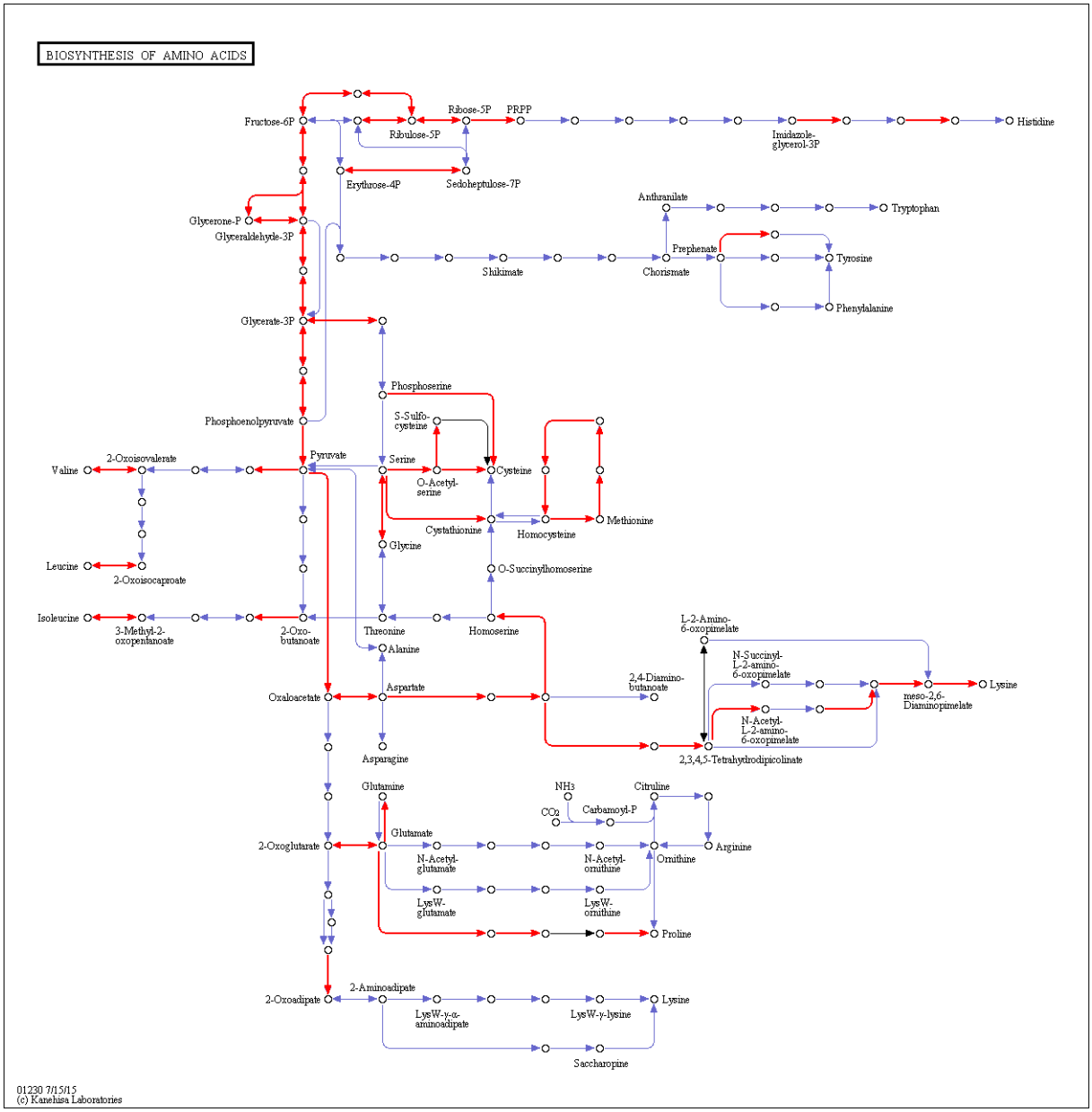


Figure 58: Predicted pathway of amino acid biosynthesis - *P. claussenii* TMW 2.54-SB. Red arrows indicate a reaction, which is present, based on EC numbers. The figure was obtained from the KEGG PATHWAY mapping tool. The metabolic capabilities of *P. claussenii*, with respect to amino acid biosynthesis, were identical for all three strains. Alanine can be synthesized from cysteine by cysteine desulfurase (EC 2.8.1.7), which is not indicated in the map. Glutamate and aspartate can be interconverted by aspartate transaminase (EC 2.6.1.1). Asparagine can be synthesized from aspartate using the asparagine synthetase (EC 6.3.5.4), which is also not part of this map.

10.1.3.4 Purines and Pyrimidines

Inosinmonophosphate (IMP) cannot be synthesized from PRPP and therefore the purine metabolism cannot be fed from the pentose phosphate pathway. From IMP, both nucleobases and all kind of nucleotides can be produced, as well as the nucleoside adenosine. Enzymes for the synthesis of guanosine from GMP seem to be missing. A potential xanthine permease and a xanthine phosphoribosyltransferase (EC 2.4.2.22) were found, allowing the padding of purine metabolism via xanthosine 5'-phosphate (XMP).

The biosynthesis of uridine monophosphate (UMP), from PRPP and glutamine, was found to be complete, while the enzymes for the biosynthesis of cytidine, cytosine, thymidine and thymine were not found. Nucleotides for RNA and DNA can be made from UMP. Uracil phosphoribosyltransferase (EC 2.4.2.9), necessary for the interconversion of UMP and uracil, is present. A potential uracil permease is encoded.

10.1.3.5 Fatty acid biosynthesis

A complete chromosomally encoded type II FAS was found, as well as the necessary peripheral enzymes for membrane integration. A cyclopropane-fatty-acyl-phospholipid synthase was found.

10.1.3.6 Vitamins and Co-Factors

A complete biosynthesis cluster for vitamin B12 (cobalamin), as well as an ECF (energy-coupling factor) transport system were found as previously reported by Pittet et al. (2012). The biosynthetic enzymes for the direct thiamine precursors, the condensation of the pyrimidine and the thiazole component to thiamine-phosphate and the interconversion of thiamine to the active thiamine-phosphate are present. A direct connection to the central metabolism remains unclear. A potential ECF transporter for the uptake of thiamine is present. Folate can be synthesized from GTP and chorismate, while both precursors are not synthesized via the known and common pathways (PRPP to GTP, PEP/fructose-6-P to chorismate). In addition, a potential ECF transporter for folate was found.

Genes for the biosynthesis of riboflavin are missing, while enzymes for the synthesis of FMN from riboflavin and the interconversion to FAD are present. Again, a potential ECF transporter was found. Members of the vitamin B6 group cannot be synthesized *de novo*, while enzymes for interconversions as well as a potential ECF transporter are present. In case of the vitamin B3 complex, no genes were found for the biosynthesis of nicotinate and nicotinamide from

tryptophan and aspartate, while NAD can be made from nicotinate and nicotinamide. No associated transport system was found. For biotin, we could not identify any genes involved in the biosynthesis, but a potential ECF transporter.

10.1.3.7 Stress response and tolerance

Acid stress: The complete AGDI pathway was found, which results in the formation of 2 NH_3. NH_3 can also be produced by the found asparaginase (EC 3.5.1.1). As described above, all genes necessary for malate and citrate fermentation are encoded.

Oxidative stress: Of 13 in detail investigated genes, associated with oxidative stress, six to seven were found to be present in *P. claussenii:* thioredoxin and thioredoxin reductase (EC 1.8.1.9), NADH peroxidase, RecA, a manganese transport system (MntH) and ferroxidase (EC 1.11.1.1); in case of TMW 2.340-MB, a protein similar to glutathione reductase was also detected.

Hop stress: TMW 2.53-SB and TMW 2.54-SB were found to have the complete *horC* cluster. TMW 2.54-SB and TMW 2.340-MB have the complete *horA* cluster, while its presence in some variants of TMW 2.53-SB is indicated by *in vitro* results (4.4.2, p. 121). The genes encoding for the BsrA/BsrB ABC multidrug transporter are present in all three genomes.

10.1.3.8 Others

19 to 24 mobile element proteins, two to five transposase and 14 to 38 phage related genes were found. In each case, the number was higher for the strong spoiling strains TMW 2.54 and TMW 2.53. 18 competence related genes are present. 16 to 17 and 14 to 15 genes related to peptidoglycan and (lipo-) teichoic acid biosynthesis were found, respectively.

10.2 *Pediococcus damnosus*

10.2.1 General genomic properties

Five complete genomes were generated as published previously (Behr et al., 2016). The chromosome size ranges from 2.07 Mbp to 2.25 Mbp and therefore shows a way higher variation as in case of *P. claussenii*. The GC content is 38.2 to 38.9 %. The chromosome alignment shows a high degree of synteny between the brewery non-spoiling strains TMW 2.1532 and TMW 2.1534, while the other three strains show extensive rearrangements of in general conserved blocks (Figure 59, p. 250). Based on phylogenomic analysis, the chromosomes of both beer-spoiling strains have a higher similarity to the winery isolate as to the non-spoiling brewery isolates. Starting with two plasmids in case of the winery isolate, four to seven plasmids were found for the brewery isolates, with GC contents from 37.3 to 43.3 %. Plasmid size ranges from 7,678 bp (TMW 2.1536-NB-WB) up to 143,870 bp (TMW 2.1535-SB), which can be considered as a megaplasmid. In case of the beer-spoiling strains, more than 10 % of the genome is plasmids based, resulting in 2.40 to 2.51 Mbp genome sizes. Higher ratios of plasmid to chromosome length were only found for *Lactobacillus salivarius*, which is also known to possess megaplasmids. In comparison, the other *P. damnosus* strains have whole genome sizes ranging from 2.17 to 2.28 Mbp. Figure 59 b (p. 250) illustrates that more than 95 % of the information shared by the beer-spoiling strains and not present in the non-spoiling strains, is found on plasmids. Besides GC content, some plasmids differ also in terms of codon and amino acid usage from the respective chromosome. In total, 2,017 to 2,330 open reading frames (ORFs) were found, resulting in a coding density of about 84 %, while the core genome contains 1,580 gene families and the pan genome 2,374. Consequently, the accessory genome makes up 19 to 32 %, while this value is 12 % in case of *P. claussenii*. Four complete rRNA operons, 60 tRNAs and two pseudo-tRNAs were found. Figure 60 (p. 251) shows the predicted subcellular localization of *P. damnosus* proteins. In case of plasmid proteins, the proportion of proteins with unknown subcellular localization increases.

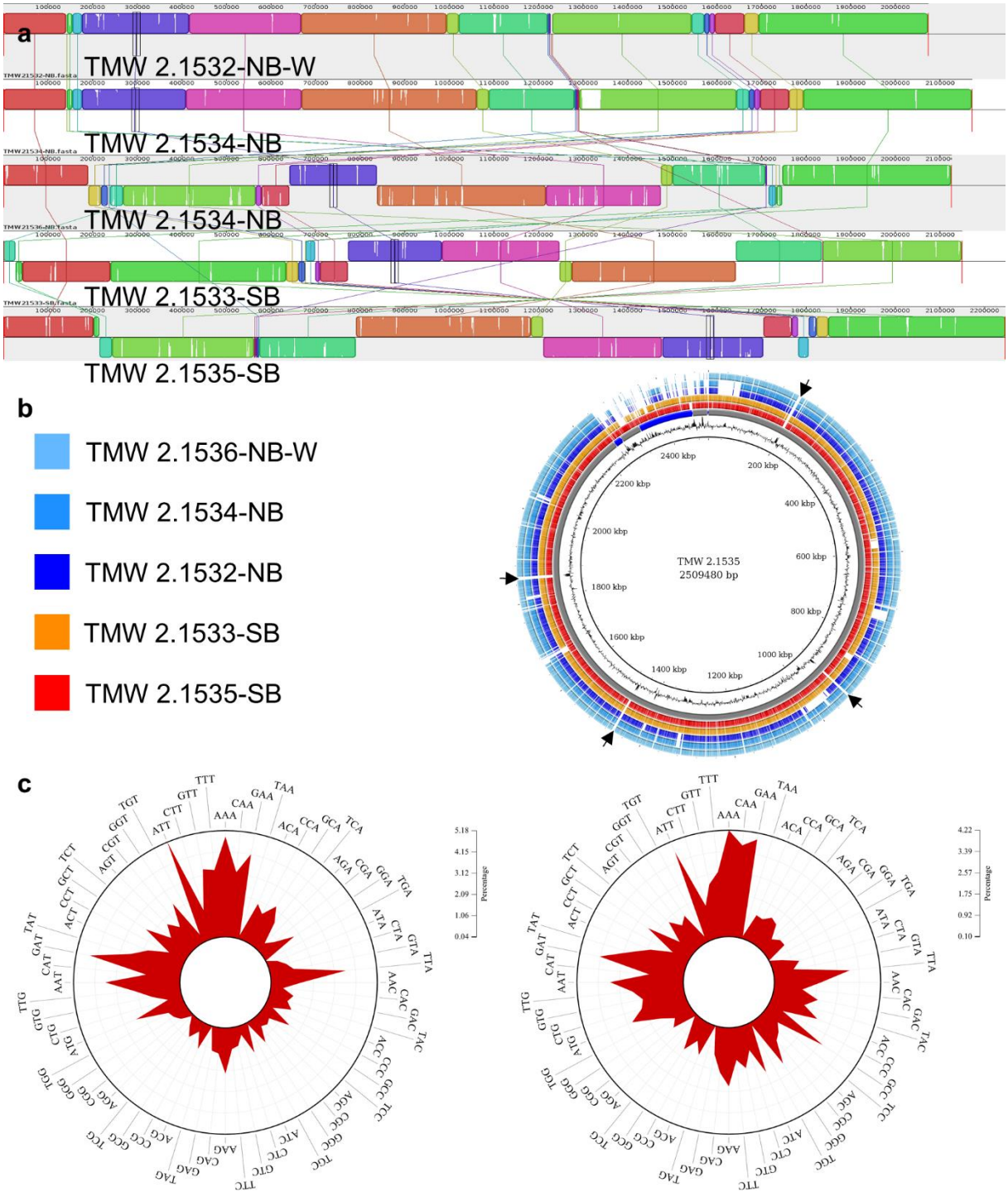


Figure 59: Illustration of the general genomic properties of *P. damnosus*. **(a)** Chromosome alignment **(b)** BLAST ring image of all *P. damnosus* genomes. All rings are described from the inside to outside: ring 1 (black) represents the total genome sequence of TMW 2.1535-SB as reference with bp coordinates; ring 2 (black) shows the GC content; ring 3 consists of arcs with different lengths and alternating coloration (grey/blue), representing the different contigs of TMW 2.1535-SB; ring 4 (red) shows all BLAST hits of TMW 2.1535-SB ORFs versus its own genome, illustrating the coding density; ring 5 shows the BLAST hits of TMW 2.1533-SB ORFs versus the reference; rings 6-8 (blue) show hits of the NB strains ORFS versus the reference; black arrows indicate rRNA operon localization **(c)** Codon usage of TMW 2.1535 chromosome (left) and plasmidome (right), exemplary.

Figure 60: Subcellular localization of *P. damnosus* proteins in %. Distribution is shown for the average values for all five strains. **Left** - chromosomal proteins, in detail → Cytoplasmic: 49.9 +/- 0.9 %; Cytoplasmic membrane: 25.9 +/- 0.6 %; Cell wall: 1.3 +/- 0.1 %; Extracellular: 1.4 +/- 0.1 %; Unknown: 21.5 +/- 0.6. **Right** - plasmid proteins, in detail → Cytoplasmic: 38.2 +/- 2.1 %; Cytoplasmic membrane: 16.5 +/- 2.5 %; Cell wall: 0.3 +/- 0.4 %; Extracellular: 2.1 +/- 1.5 %; Unknown: 21.5 +/- 0.6.

10.2.2 Functional analysis - SEED and COG

41 +/- 2 % of the found ORFs could be assigned to SEED categories. Of the remaining unassigned ORFs, 395 to 480 were annotated as hypothetical proteins. In case of the COG enrichment, 85 to 87 % could be assigned to a functional category, with about 22 % poorly characterized proteins. With an average coefficient of variation (CV) of 8 (SEED) to 9 % (COG) for all investigated categories, *P. damnosus* shows a much higher chromosomal functional diversity as *P. claussenii* (CV 1.4 %), fitting to the results of pan/core genome analysis and the chromosomal alignment. The COG enrichment shows a significant difference between the brewery isolates and the winery isolate TMW 2.1536-NB, with respect to the amount of genes associated with replication, recombination and repair (RRR) for the chromosomal analysis. Otherwise, no correlation of any feature to a grouping based on source or beer spoilage potential could be found on chromosome level, as illustrated by Figure 61 (p. 253). As in case of the phylogenomic analysis, the functional analysis of the chromosome groups both beer-spoiling strains closer to the winery isolate and not with the other brewery isolates. Compared to other lactobacilli genomes, a quite high proportion (in relation to assigned functions) of genes is associated to DNA and RNA metabolism based on SEED categorization, while this trend was not found for the total count of those features. In case of TMW 2.1533-SB, more than 10 % of the assigned genes are related to DNA metabolism, which is only exceeded by *L. sanfranciscensis* and *L. acetotolerans*. COG enrichment resulted in a comparatively low amount of genes related to energy production and conversion.

Functional analysis of plasmids resulted in a SEED subsystem coverage of 22 +/- 2% and a COG enrichment coverage of 74 +/- 2 %. Based on functional analysis, the following categories

and subordinate levels are noticeable with respect to isolation source and/or beer spoilage potential of the investigated genomes/strains (see Figure 62, p. 254):

The number of carbohydrate metabolism related genes is enriched in case of the strong spoiling strains (SEED/COG). All four brewery isolates contain a small alpha-acetolactate operon, allowing the formation of acetoin from pyruvate, as well as genes for maltose utilization. Both beer spoilers additionally encode for a glycoside hydrolase, associated with chitin degradation. TMW 2.1536-NB (winery isolate) plasmids were found to contain genes for glycerol and dihydroxyacetone metabolism. A lack of genes associated with DNA metabolism (SEED category) in case of the winery isolate and a higher number in case of the beer-spoiling strains within the brewery isolates was found. The difference results from the presence of DNA topoisomerases and a type I restriction modification system. This finding is supported by the difference in RRR proteins found in COG analysis. The number of genes related to fatty acid and lipid metabolism (SEED/COG) is strongly enriched in case of the beer-spoiling strains. This is due to the presence of a complete FAS cluster. In case of the winery isolate, no genes with relation to cell wall biogenesis (SEED/COG) are present. In contrast, all brewery isolates carry plasmid-encoded genes for peptidoglycan recycling and, in two cases, for teichoic acid glycosylation. With respect to vitamins and cofactors, all brewery isolates have been found to encode for a potential biotin ECF transporter. Behind the SEED categories membrane transport, virulence, disease and defense (VDD) and potassium metabolism, we found transport systems for potassium, copper and magnesium, while the latter is associated with *hitA*, a putative and published manganese transporter correlative to beer spoilage ability. All these features were found only in the beer-spoiling plasmidomes, while this difference in inorganic ion transport is also indicated by the COG enrichment. Further, the categories protein metabolism and “nucleosides and nucleotides” were only found for the brewery plasmids, encoding a S14p ribosomal protein with high similarity to the one of *L. plantarum* and a 5'-nucleotidase (EC 3.1.3.5), amongst others involved in purine salvage.

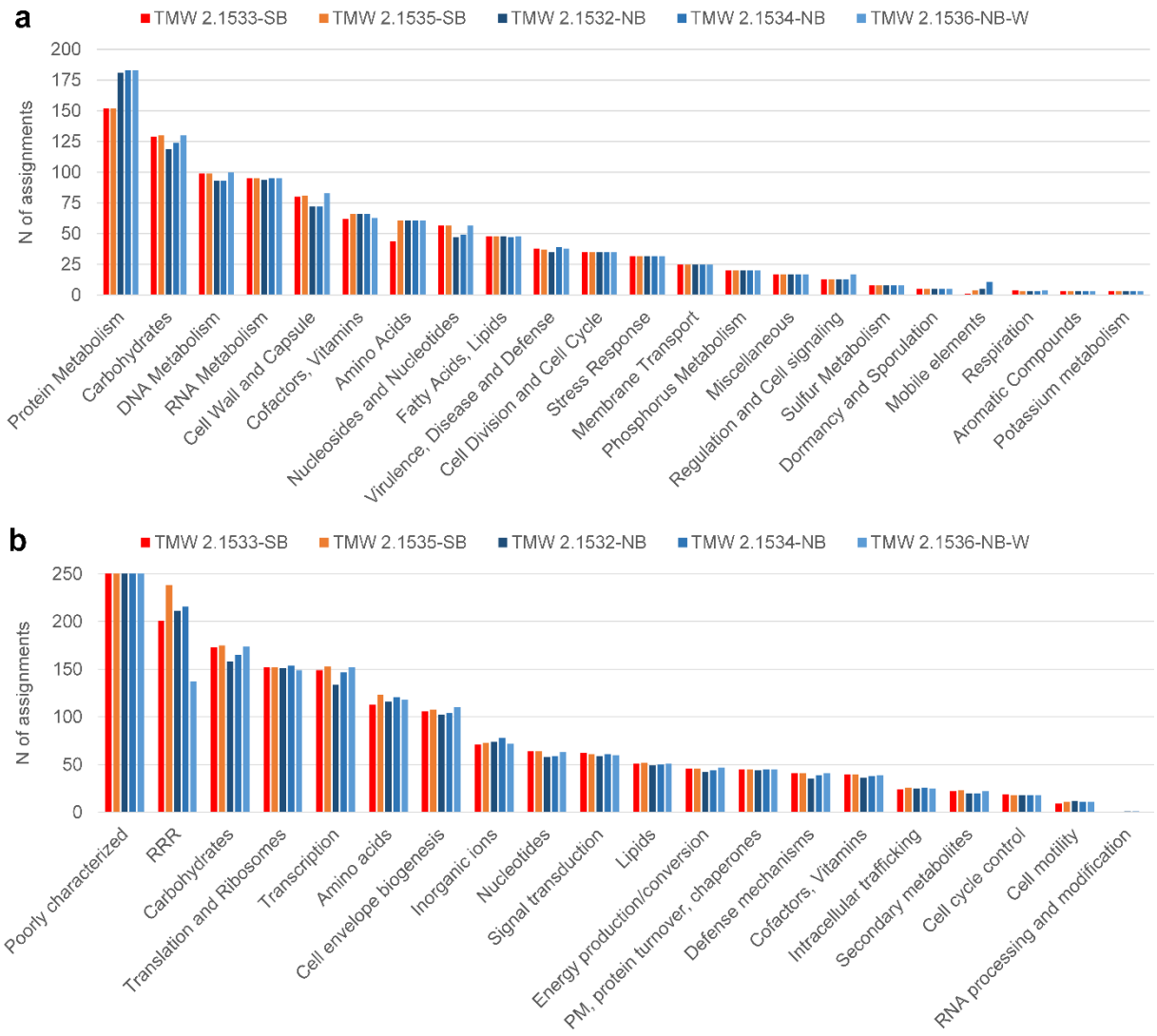


Figure 61: Functional analysis of *P. damnosus* chromosomes. The total number of proteins assigned to the single categories is shown for SEED categories **(a)** and COG categories **(b)** in descending order. The difference in protein metabolism (SEED) results from a RAST subsystem assignment error. All proteins missing, in comparison to the other strains, are found on sequence and annotation (text) level, but were not assigned to the corresponding category/subcategory/subsystem. In case of the COG enrichment, the four brewery isolates were found to have more genes associated with replication, recombination and repair (RRR), compared to the winery isolate. In case of the COG category “Poorly characterized,” the actual values range from 278 to 403. SEED subsystem coverage: 42.1 +/- 1.2; COG coverage: 89.4 +/- 0.9. RRR = Replication, recombination and repair, PM = posttranslational modification.

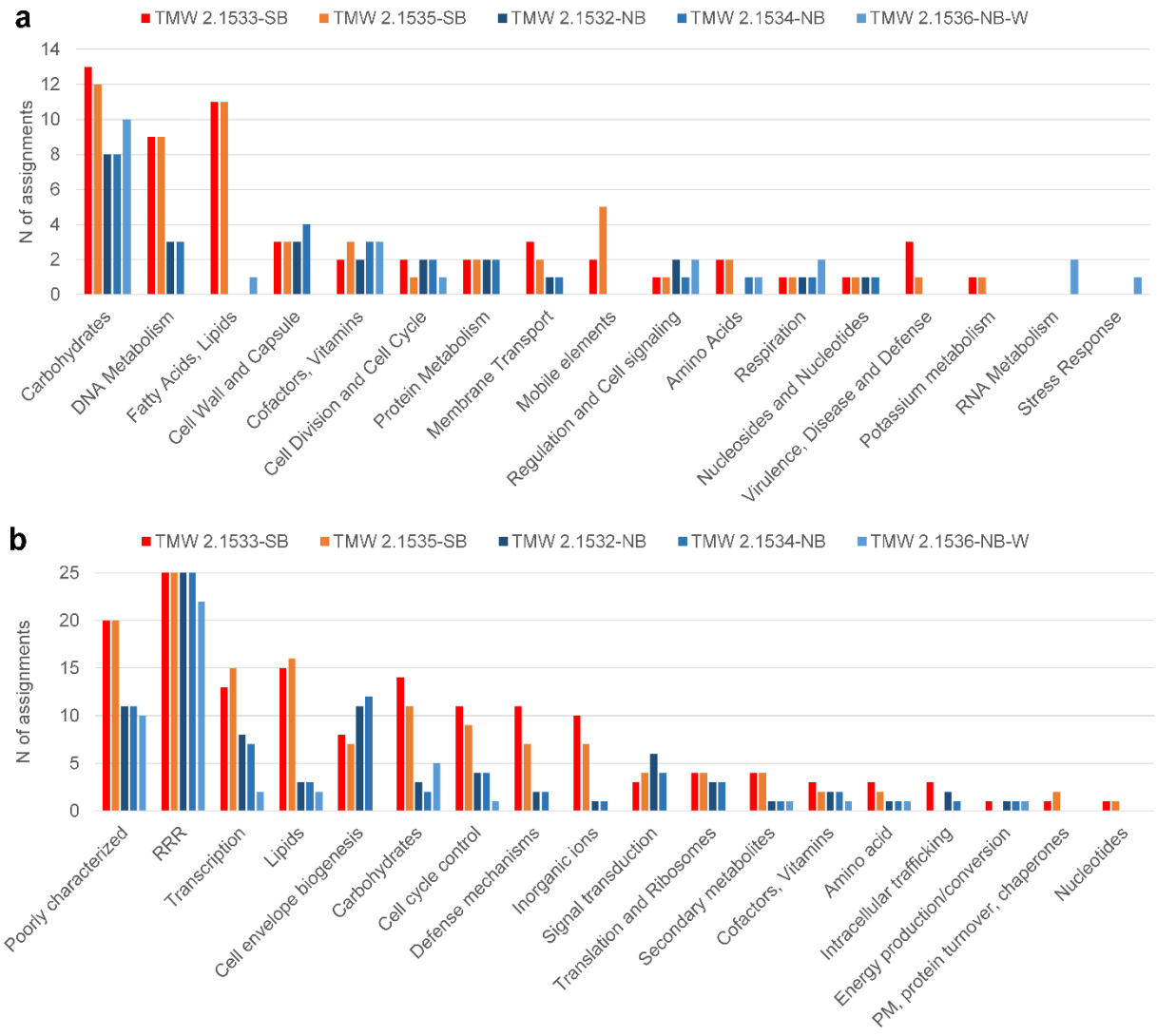


Figure 62: Functional analysis of *P. damnosus* plasmidomes. The total number of proteins assigned to the single categories is shown for SEED categories **(a)** and COG categories **(b)** in descending order. In case of the COG category RRR, the actual values are 79 to 90 for beer-spoiling strains, 36 to 42 for brewery isolate non-spoilers, 22 for the winery isolate TMW 2.1536-NB. SEED subsystem coverage: 21.8 +/- 2.2; COG coverage: 74.2 +/- 2.0. RRR = Replication, recombination and repair, PM = posttranslational modification.

10.2.3 Metabolic capabilities

10.2.3.1 Carbohydrates and central metabolism

A complete glycolysis and phosphoketolase pathway were found. The pentose phosphate pathway is complete with the exception of transaldolase (EC 2.2.1.2). Enzymes and PTS systems for the utilization of fructose, mannose, sucrose, trehalose and cellobiose were found. In addition, not clearly defined transport systems (MFS/ABC) were found for maltose and isomaltose, spatially located next to the enzymes for their utilization, a maltose phosphorylase (EC 2.4.1.8) and an isomaltase (EC 3.2.1.10). Further, a maltodextrin glucosidase (EC 3.2.1.20) was identified. Galactose is fed into glycolysis using the tagatose-6-phosphate pathway, while an unspecified PTS system is found in front of the respective genes. Glycerol ABC transporter were found as well as the necessary enzymes for utilization. TMW 2.1536-NB, isolated from wine, additionally has a plasmid-encoded glycerol transport system. The way of glucose uptake remains unclear, while genes potentially encoding for a permease and associated with a PTS system were found. Several 6-phospho-beta-glucosidases (EC 3.2.1.86) were found, located next to not clearly defined transport systems. In one case, a beta-glucoside PTS system is found located next to it.

10.2.3.2 Pyruvate metabolism, organic acids and TCA cycle

All strains are capable to produce ethanol, acetate and lactate (L/D-lactate) from pyruvate, as illustrated by Figure 63 (p.256). Both enzymes responsible for the formation of acetolactate and acetoin were only found for the 4 brewery isolates, encoded on plasmids. Acetoin reductase (EC 1.1.1.4) was found chromosomally in both beer-spoiling strains and the non-spoiling strain from the winery environment. Thus, only the beer-spoiling strains are capable to produce 2,3-butanediol from pyruvate. In addition, the anaplerotic enzyme pyruvate carboxylase (EC 6.4.1.1) was found. Uptake and utilization of malate is encoded. The TCA cycle is incomplete, while only one enzyme, fumarate hydratase (EC 4.2.1.2), was found to be present.

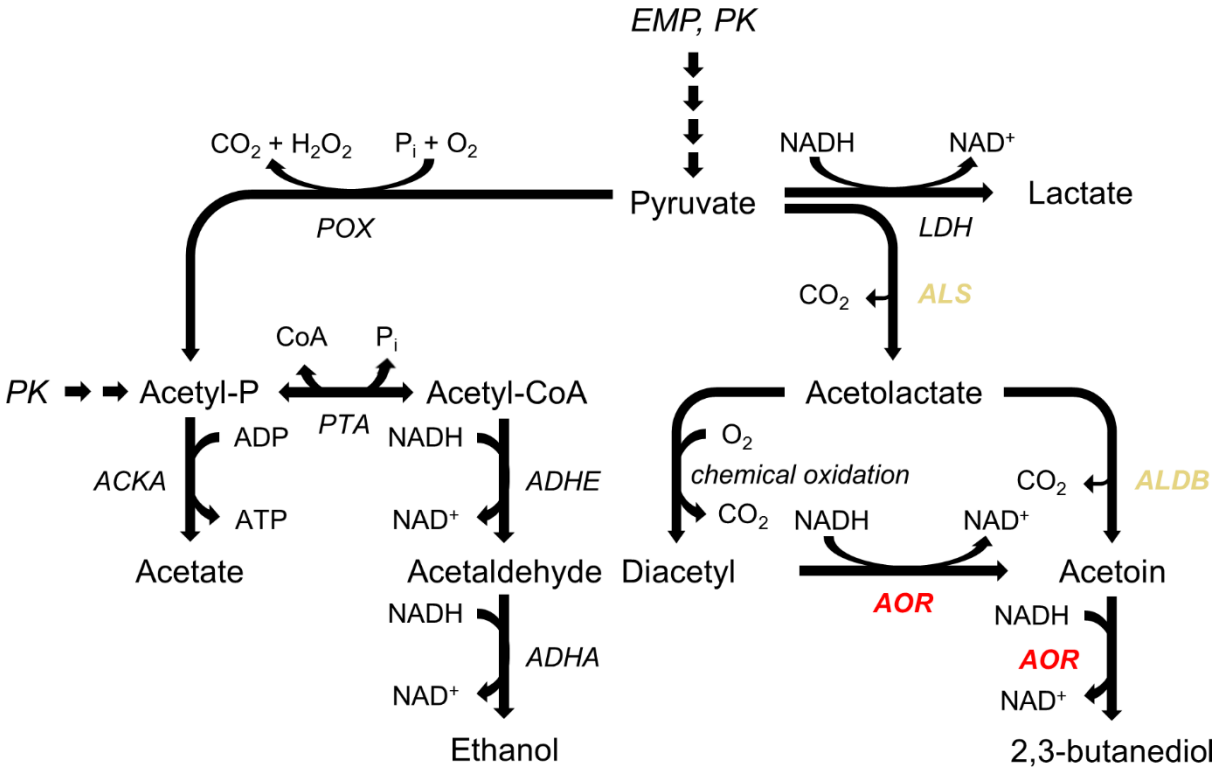


Figure 63: Pyruvate metabolism of *P. damnosus*. EMP = Embden–Meyerhof–Parnas, PK = Phosphoketolase (EC 4.1.2.9), LDH = lactate dehydrogenase (EC 1.1.1.27/28), **ALS** = acetolactate synthase (EC 2.2.1.6) and **ALDB** = alpha-acetolactate decarboxylase (EC 4.1.1.5) - both plasmid-encoded and only found within brewery genomes, **AOR** = Acetoin reductase (EC 1.1.1.4) - chromosomally encoded by TMW 2.1533-SB, TMW 2.1535-SB, TMW 2.1536-NB-W, POX = pyruvate oxidase (EC 1.2.3.3); ACKA = acetate kinase (EC 2.7.2.1), PTA = phosphate acetyltransferase (EC 2.3.1.8), ADHE = acetaldehyde dehydrogenase (EC 1.2.1.10), ADHA = alcohol dehydrogenase (EC 1.1.1.1).

10.2.3.3 Proteolytic system and amino acids

A cell wall-bound, extracellular protease was not found, while transport systems for oligo, di- and tripeptides are present. Several different endopeptidases, aminopeptidases and oligo-/tri-/dipeptidases are encoded. In addition, potential transport systems were found for alanine, glutamine/glutamic acid, glycine, methionine and branched amino acids, as well as unspecified amino acid transporter. An incomplete amino acid biosynthesis was found, indicating auxotrophy for histidine, tryptophan, tyrosine, phenylalanine, threonine and all branched amino acids. With exception of TMW 2.1533-SB, we found all enzymes to produce aspartate, asparagine, cysteine and methionine from pyruvate. Glutamate, glutamine and proline are potentially made from 2-oxoglutarate. Lysine, arginine, alanine, glycine and serine can be synthesized from precursors or interconverted, but the respective reaction sequences are not connected to the central carbon metabolism (see Figure 64).

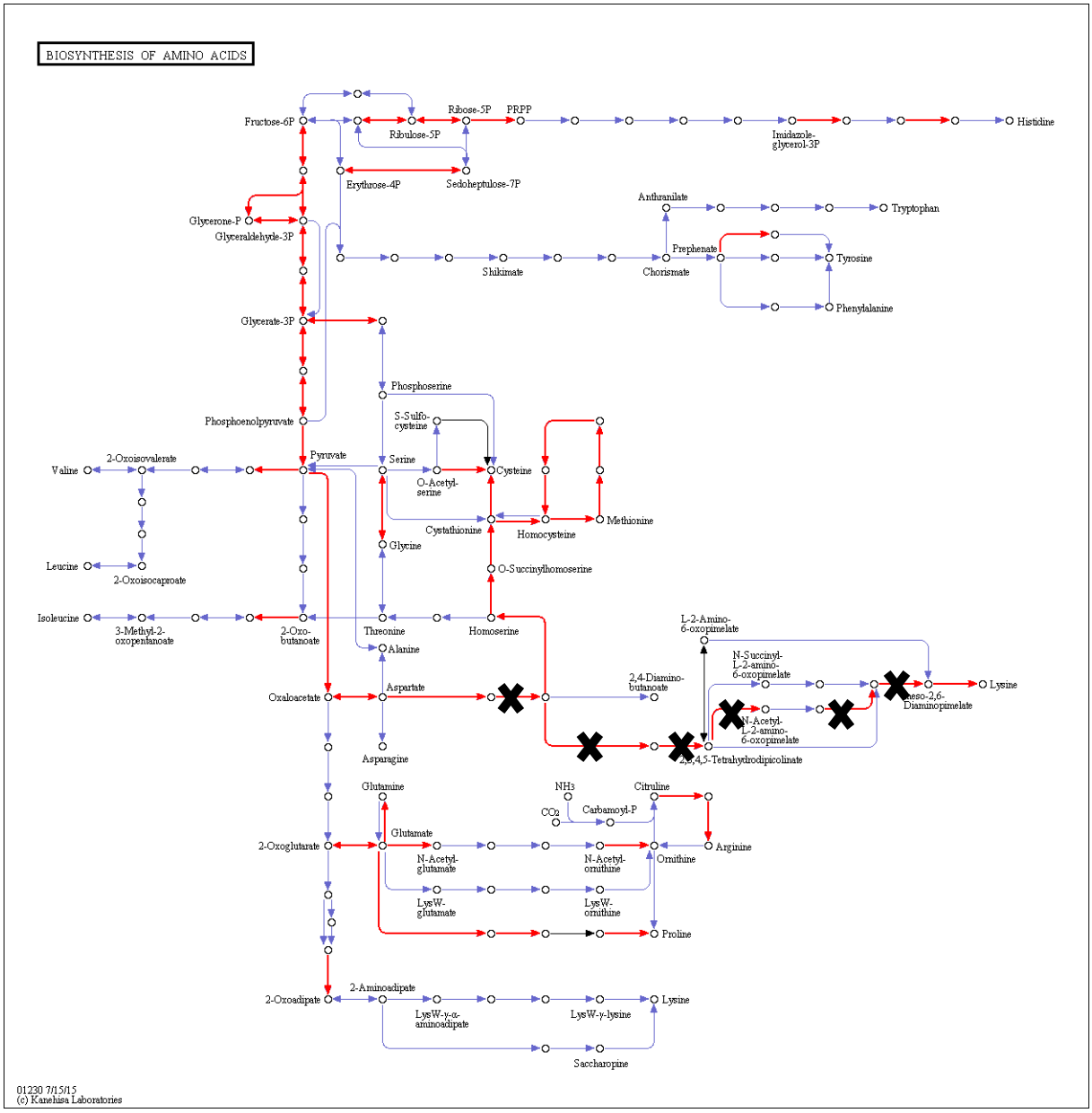


Figure 64: Predicted pathway of amino acid biosynthesis - *P. damnosus*. Red arrows indicate a reaction, which is present, based on EC numbers. The figure was obtained from the KEGG PATHWAY mapping tool. This figure is based on TMW 2.1535-SB. Alanine can be synthesized from cysteine by cysteine desulfurase (EC 2.8.1.7), which is not indicated in the map. Glutamate and aspartate can be interconverted by aspartate transaminase (EC 2.6.1.1). Asparagine can be synthesized from aspartate using the Asparagine synthetase (EC 6.3.5.4), which is also not part of this map. All strains have the same reaction map as TMW 2.1535-SB, with the exception of TMW 2.1533-SB. Genes encoding for the respective enzymes, which were not found in case of TMW 2.1533-SB are marked with a black cross.

10.2.3.4 Purines and Pyrimidines

IMP cannot be synthesized from the pentose phosphate pathway. From IMP all nucleobases, nucleosides and nucleotides can be produced. A potential xanthine permease and a xanthine phosphoribosyltransferase (EC 2.4.2.22) were found, allowing the padding of purine metabolism via xanthosine 5'-phosphate (XMP).

The biosynthesis of UMP from PRPP and glutamine was found to be complete. All enzymes were found for the formation of all nucleotides, all nucleosides, uracil, uridine and thymine. Uracil phosphoribosyltransferase (EC 2.4.2.9), necessary for the interconversion of UMP and uracil, is present. A potential uracil permease is encoded.

10.2.3.5 Fatty acid biosynthesis

The chromosomal type II FAS is incomplete. Both beer-spoiling strains carry a complete plasmid-encoded FAS cluster. For details, see 4.3.4 (p. 110). Genes for peripheral enzymes and for the modification to cyclopropane fatty acids were found for all strains.

10.2.3.6 Vitamins and Co-Factors

No vitamin biosynthetic pathway was found to be complete. Most genes were found in case of folate biosynthesis and thiamine metabolism. In both cases, enzymes for the synthesis from precursors are partly available, while no connection to the central metabolism was found. Folate can be synthesized from dihydroneopterin and 4-aminobenzoate, while the latter cannot be synthesized from chorismate. Thiamine-phosphate pyrophosphorylase (EC 2.5.13), catalysing the penultimate reaction of thiamine biosynthesis, was found, while the hydroxyethylthiazole kinase (EC 2.7.1.50), necessary for the formation of the finale thiazole component, was not found.

In case of the vitamin B3 and B6 complexes, as well as riboflavin, all enzymes for an interconversion of the different active forms were found, but not for biosynthesis. Biosynthetic genes for biotin and vitamin B12 (Cobalamin) are missing as well. Potential ECF transporter were found for riboflavin, pyridoxine (vitamin B6) and biotin as well as other transport systems for thiamine.

10.2.3.7 Stress response and tolerance

Acid stress: malolactic fermentation and asparaginase (EC 3.5.1.1) were found to be the only acid tolerance related systems investigated.

Oxidative stress: Of 13 in detail investigated genes, associated with oxidative stress, seven to eight were found to be present in *P. damnosus:* thioredoxin and thioredoxin reductase (EC 1.8.1.9), NADH peroxidase, RecA, a manganese transport system (MntH) and ferroxidase (EC 1.11.1.1). A protein similar to glutathione reductase was also detected. TMW 2.1532-NB, TMW 2.1534-NB and TMW 2.1535-SB were found to have a potential NADH oxidase.

Hop stress: All brewery genomes are characterized by complete *horA* and *horC* clusters. Both beer-spoiling strains additionally encode for *hitA*.

10.2.3.8 Others

Two (winery isolate) to seven plasmids were found. The number of mobile element proteins correlates with the number of plasmids and ranges from 49 (winery isolate) to 181. The same is true for the count of transposase related genes. 15 to 48 phage related genes were found. In average, 13 genes connected to competence were found. 17 and 14 genes related to peptidoglycan and (lipo-) teichoic acid biosynthesis are present, constantly within all strains.

10.3 *Lactobacillus backii*

10.3.1 General genomic properties

SMRT-sequencing and assembly resulted in five complete genomes of beer-spoiling *L. backii* strains. The chromosome size ranges from 2.55 Mbp to 2.67 Mbp with a CV of 2.1 %. The GC content is 40.8 to 40.9 %. The chromosome alignment shows ten conserved blocks with partly different arrangements. Based on chromosome size, phylogenomic analysis, ANI and the amount of shared genes, *L. backii* genomes can be subdivided into two groups. TMW 1.1989-SB and TMW 1.2002-MB have a bigger chromosome (~ 0.1 Mbp), a higher ANI to each other and share almost 100 % of the annotated information. Figure 66 (p. 261) illustrates these differences. Seven to ten plasmids were found with GC contents from 34.7 to 43.9 %. Plasmid sizes range from 7,030 bp to 70,980 bp, while a partial plasmid sequence with only 2,095 bp was found for TMW 1.1992-SB. The proportion of plasmid sequences represents 6.3 to 9.4 % of the genomes, ending with genome sizes of 2.78 to 2.85 Mbp. In total 2,590 to 2,671 open reading frames (ORFs) were found, resulting in a coding density of about 81.5 %, while the core genome contains 1,924 gene families and the pan genome 2,889. As in case of *P. damnosus*, the accessory genome makes up a high proportion, about 27 +/- 1 %. Five complete rRNA operons were found, which are also indicated in Figure 66 b. TMW 1.1988-SB encodes for 69 tRNAs, TMW 1.1992-SB for 64 tRNAs and the other strains for 65 tRNAs. All strains were found to have two pseudo-tRNAs. Figure 66 shows the predicted subcellular localization of *L. backii* proteins. In case of plasmid proteins, the proportion of proteins with unknown subcellular localization increases. Besides GC content, some plasmids differ also in terms of codon and amino acid usage from the respective chromosome.

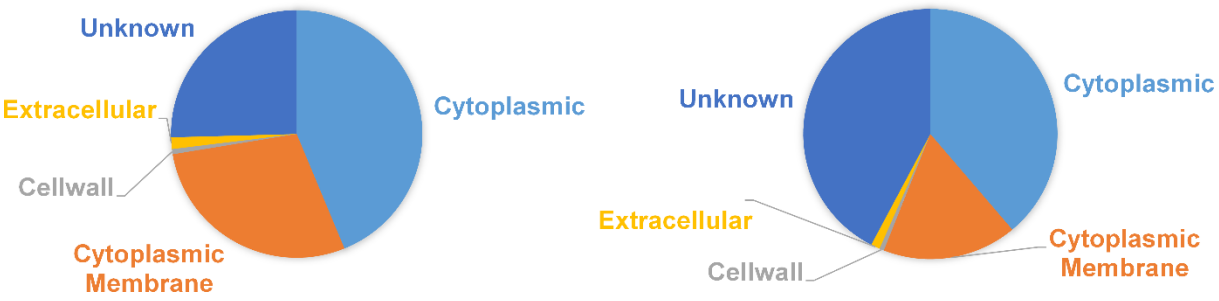


Figure 65: Subcellular localization of *L. backii* proteins in %. Distribution is shown for the average values for all five strains. **Left** - chromosomal proteins, in detail → Cytoplasmic: 43.8 +/- 0.5 %; Cytoplasmic membrane: 28.6 +/- 0.5 %; Cell wall: 0.7 +/- 0.0 %; Extracellular: 1.5 +/- 0.1 %; Unknown: 25.4 +/- 0.9. **Right** - plasmid proteins, in detail → Cytoplasmic: 38.8 +/- 2.7 %; Cytoplasmic membrane: 17.1 +/- 1.9 %; Cell wall: 1.0/1.5 % for TMW 1.1989/TMW 1.2002, 0.0 to 0.4 for others; Extracellular: 1.2 +/- 0.1 %; Unknown: 42.3 +/- 2.0.

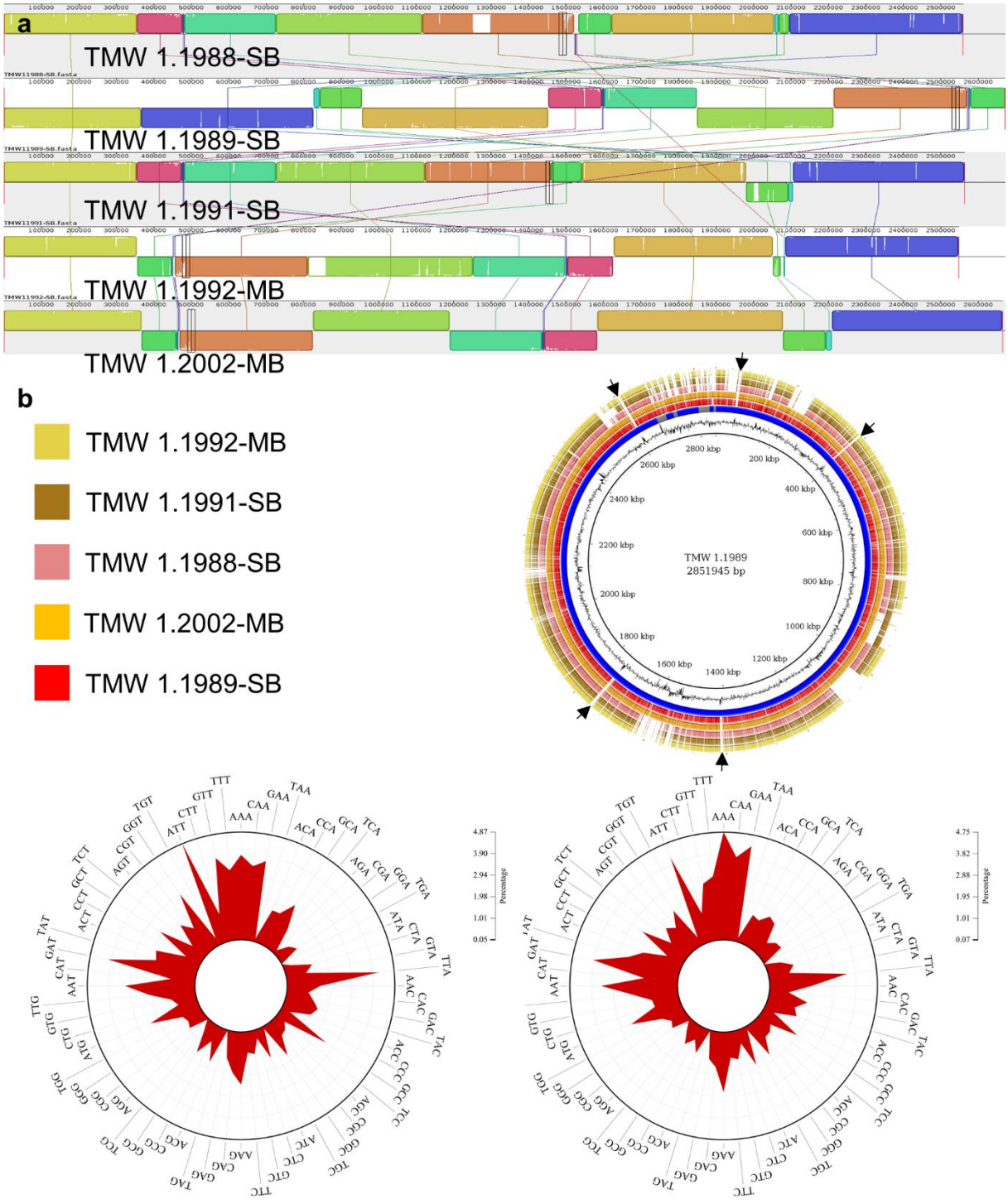


Figure 66: Illustration of the general genomic properties of *L. backii*. **(a)** Chromosome alignment **(b)** BLAST ring image of all *L. backii* genomes. All rings are described from the inside to the outside: ring 1 (black) represents the total genome sequence of TMW 1.1989-SB as reference with bp coordinates; ring 2 (black) shows the GC content; ring 3 consists of arcs with different lengths and alternating coloration (grey/blue), representing the different contigs of TMW 1.1989-SB; ring 4 shows all BLAST hits of TMW 1.1989-SB ORFs versus its own genome illustrating, the coding density; rings 5 to 8 show the BLAST hits of the other genomes ORFs versus the reference; black arrows indicate the localization of rRNA operons (no ORF hits) **(c)** Codon usage of 1.1989-SB chromosome (left) and plasmidome (right), exemplary.

10.3.2 Functional analysis - SEED and COG

41 +/- 1 % of the found ORFs could be assigned to SEED categories. Of the remaining unassigned ORFs, 573 to 647 were annotated as hypothetical proteins. In case of the COG enrichment, 83 to 86 % could be assigned to a functional category, with about 23 % poorly characterized proteins. In total, most genes were assigned to categories related to amino acid metabolism, protein metabolism, DNA and RNA metabolism and carbohydrate metabolism, followed by genes associated with vitamins/cofactors and the cell envelope. In comparison, *L. backii* is characterized by a high number and proportion (13.6 to 15.1 % in relation to total SEED assignments) of genes related to amino acid metabolism, while only one species, *L. paracollinoides*, was found to have more genes classified into this SEED category. This is mainly due to the presence of biosynthetic genes, as described in the next section about the metabolic capabilities. Further top 10 rankings could be observed within the SEED categories DNA and RNA metabolism, stress response, virulence disease and defense, as well as iron acquisition. In case of the COG enrichment, we could not identify special features in comparison to the other genomes investigated. We could not identify functional groups correlative to the beer spoilage potential of this species.

In a similar range as *P. damnosus*, *L. backii* shows a higher (chromosomal) functional variation (CV 3 to 8 %) as *P. claussenii*. Chromosomal functional analysis confirms the subdivision into two groups, while TMW 1.1989-SB and TMW 1.2002-MB group together. One main difference can be seen for the category cofactors & vitamins (SEED & COG), resulting from the presence of a complete molybdenum cofactor (moco) biosynthesis cluster in case of the above named strains. A second major difference was found for nitrogen metabolism. This results from the presence of a nitrate reductase (EC 1.7.99.4) for the same strains, directly located next to the moco-cluster. Next to these genes, several ORFs were found potentially involved in vitamin K2 biosynthesis. Both mentioned vitamins are related to nitrate reduction. Further differences are associated with choline metabolism (SEED - cell wall and capsule), one-carbon Metabolism and DNA metabolism (RRR in case of COG).

The SEED subsystem coverage for the plasmidomes is 23 +/- 1 %, while about 73 % of all genes could be assigned to COG categories. Most genes are related to DNA metabolism (SEED) and RRR (COG), as well as fatty acids and lipids. The latter results from a plasmid-encoded FAS cluster, which was found to be nearly identical to the one found for *P. damnosus.* As in case of *P. damnosus*, all brewery isolates have been found to encode for a potential biotin ECF transporter. In three strains, several genes were assigned to the category carbohydrates, for example related to pyruvate metabolism and glycerol utilization. With exception of TMW 1.1991-SB, all strains are characterized by plasmid-encoded features

connected to the subsystem copper homeostasis (SEED category - Virulence, Disease and Defense). The same, but with exception of TMW 1.1992-SB, is true for the subsystem teichoic and LTA biosynthesis. The comparatively higher number of amino acid utilization (SEED/COG) features in case of TMW 1.1991-SB is due to the presence of a complete cluster for the AGDI pathway. For further details, see Figure 68 (p. 264).

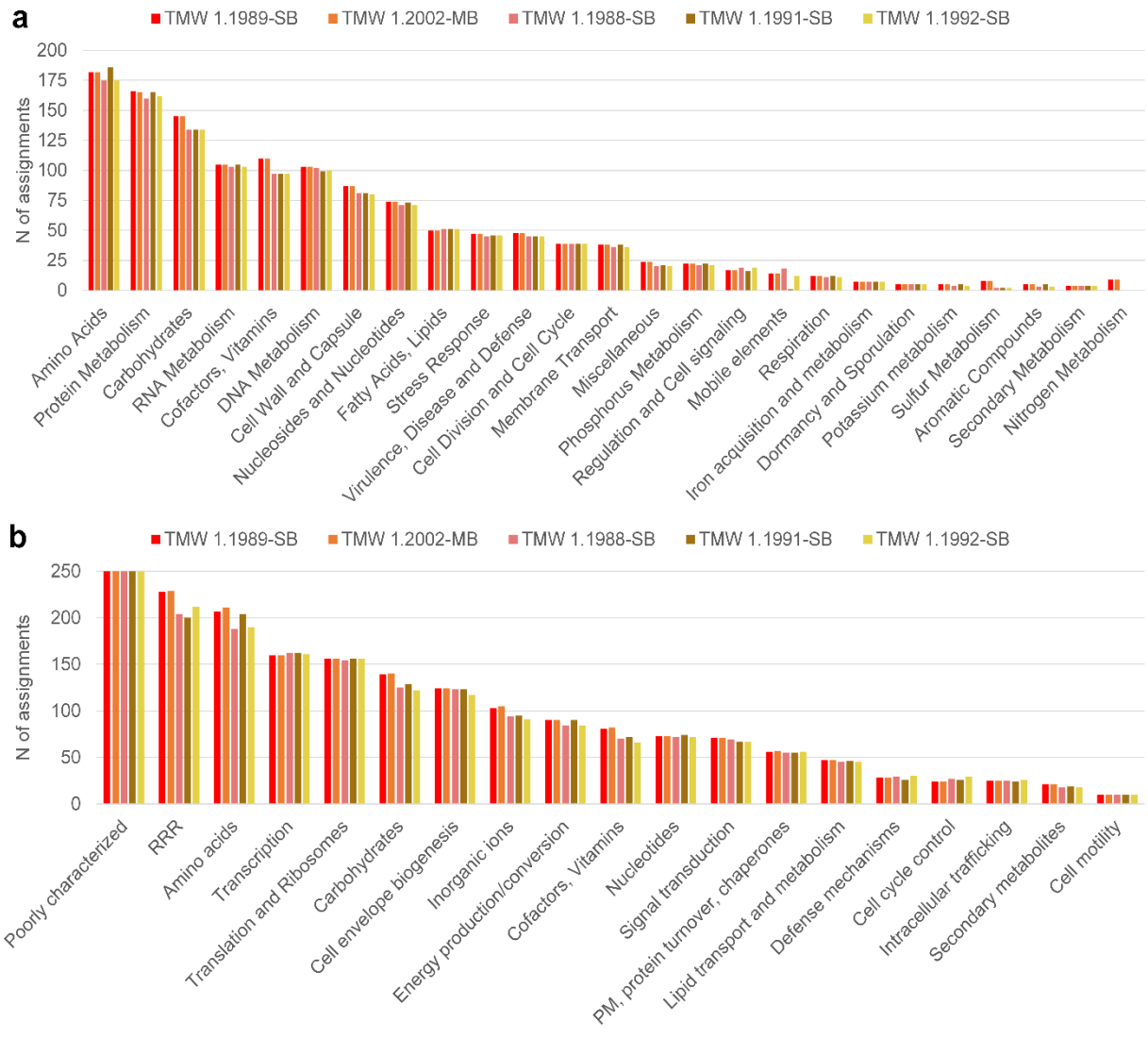


Figure 67: Functional analysis of *L. backii* chromosomes. The total number of proteins assigned to the single categories is shown for SEED categories **(a)** and COG categories **(b)** in descending order. In case of the COG category “Poorly characterized,” the actual values range from 469 to 493. SEED subsystem coverage: 42.6 +/- 0.7; COG coverage: 86.2 +/- 1.0. RRR = Replication, recombination and repair, PM = posttranslational modification.

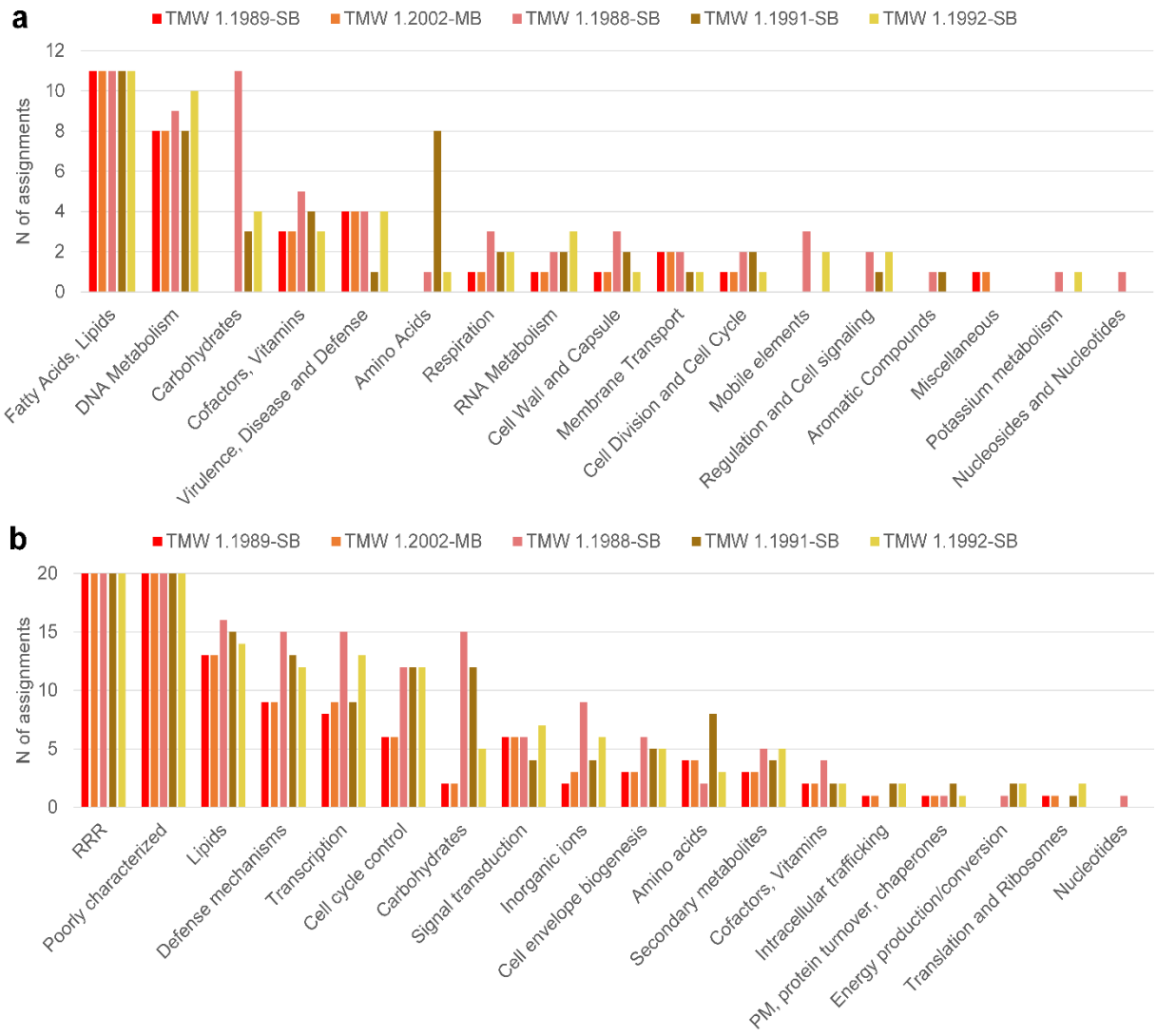


Figure 68: Functional analysis of *L. backii* plasmidomes. The total number of proteins assigned to the single categories is shown for SEED categories **(a)** and COG categories **(b)** in descending order. In case of the COG category RRR, the actual values are 40 to 85, poorly characterized: 21 to 36. SEED subsystem coverage: 22.8 +/- 1.0; COG coverage: 73.4 +/- 2.9. RRR = Replication, recombination and repair, PM = posttranslational modification.

10.3.3 Metabolic capabilities

10.3.3.1 Carbohydrates and central metabolism

A complete glycolysis and phosphoketolase pathway were found. The pentose phosphate pathway is complete, with the exception of transaldolase (EC 2.2.1.2), while in case of TMW 1.1988-SB, TMW 1.1991-SB and TMW 1.1992-MB an ambiguous gene encoding for this protein was found. Enzymes and PTS systems for the utilization of fructose, mannose, cellobiose, mannitol and sorbitol were found. In addition, not clearly defined transport systems were found for maltose, spatially located next to a maltose phosphorylase (EC 2.4.1.8). Further, a maltodextrin glucosidase (EC 3.2.1.20) was identified. A potential plasmid-encoded isomaltase (EC 3.2.1.10) was found next to an undefined transport system in case of TMW 1.1991-SB. Neither Leloir pathway nor tagatose-6-phosphate pathway, for the utilization of galactose, are present. A Glycerol transporter was found as well as the necessary enzymes for utilization. Several enzymes related to trehalose utilization were found, while the sequence analysis was ambiguous. RAST annotation resulted in trehalose utilization; BLASTp suggests kojibiose as the used substrate. Glucose is presumably taken up by the glucose permease GlcU. A 6-phospho-beta-glucosidases (EC 3.2.1.86) was found with a beta-glucoside PTS system next to it.

10.3.3.2 Pyruvate metabolism, organic acids and TCA cycle

All strains are capable to produce ethanol, acetate, lactate (L/D-lactate) and acetoin from pyruvate. Based on prediction, the pyruvate metabolism is identical to the one found for *P. claussenii* and can be obtained from the respective figure (Figure 45, p. 173). In addition, the anaplerotic enzymes pyruvate carboxylase (EC 6.4.1.1) and phosphoenolpyruvate carboxykinase (EC 4.1.1.49) were found. Uptake and utilization of malate is encoded. The TCA cycle is incomplete, while all enzymes for the reaction sequence from oxaloacetate via citrate, isocitrate and oxalosuccinate to 2-oxoglutarate are present.

10.3.3.3 Proteolytic system and amino acids

A cell wall-bound, extracellular protease was not found, while transport systems for di- and tripeptides are present. Several different endopeptidases, aminopeptidases and oligo-/tri-/dipeptidases are encoded. In addition, potential transport systems were found for alanine, arginine, asparagine/aspartate glutamine/glutamate, glycine, lysine, methionine, serine, tyrosine and branched amino acids, as well as unspecified amino acid transporter.

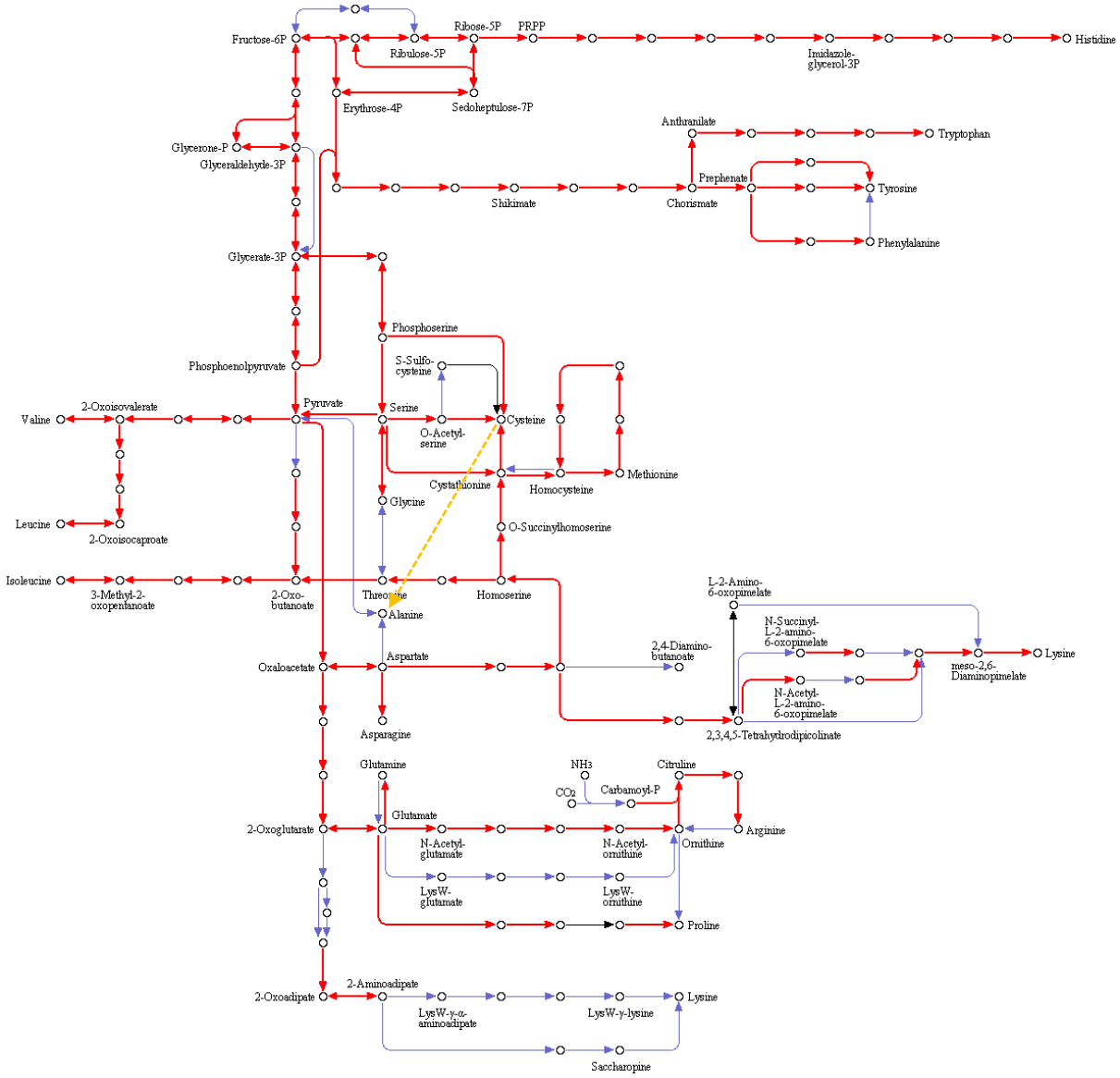


Figure 69: Amino acid biosynthesis - *L. backii*. Red arrows indicate a reaction, which is present, based on EC numbers. The figure was obtained from the KEGG PATHWAY mapping tool. The metabolic capabilities of *L. backii* with respect to amino acid biosynthesis were identical for all five strains. This figure is based on TMW 1.1989-SB. Alanine can be synthesized from cysteine by cysteine desulfurase (EC 2.8.1.7), which is not part of the original KEGG map. It is now indicated by a dotted orange arrow. Asparagine is synthesized from aspartate, using the Asparagine synthetase (EC 6.3.5.4), which is normally not part of this KEGG map. The reaction from aspartate to aspartic acid was mapped anyway, for clarity. The apparent gap within the lysine biosynthesis from aspartate may be closed by the enzyme that is mapped above the gap, or a non-annotated aminotransferase.

Based on prediction, *L. backii* is prototroph for all amino acids except lysine (see Figure 69, p. 266), while alanine and asparagine are synthesized via “alternative enzymes.” Alanine is made from cysteine, using the cysteine desulfurase (EC 2.8.1.7), and asparagine from aspartate via asparagine synthase (EC 6.3.5.4). In case of lysine, the presence of a non-

specified aminotransferase would close the gap. TMW 1.1991-SB is characterized by a plasmid-encoded AGDI-pathway.

10.3.3.4 Purines and Pyrimidines

IMP can be made from PRPP. All nucleobases, nucleosides and nucleotides can be produced from IMP. A potential xanthine permease and a xanthine phosphoribosyltransferase (EC 2.4.2.22) were found, allowing the padding of purine metabolism via xanthosine 5'-phosphate (XMP).

The biosynthesis of UMP from PRPP and glutamine is complete. All enzymes were found for the formation of all nucleotides, all nucleosides, uracil, uridine and thymine. Uracil phosphoribosyltransferase (EC 2.4.2.9), necessary for the interconversion of UMP and uracil, is present. A potential uracil permease is encoded.

10.3.3.5 Fatty acid biosynthesis

The chromosomal type II FAS is incomplete. All strains carry a complete plasmid-encoded FAS cluster. For details, see 4.3.4 (p. 110). Genes for peripheral enzymes and for the modification to cyclopropane fatty acids were found for all strains.

10.3.3.6 Vitamins and Co-Factors

Folate can be synthesized via GTP and chorismate, while both precursors can be made from the central carbon metabolism. Based on prediction, the same is true for riboflavin, which is produced from GTP and ribulose-5-phosphate. In both cases, all enzymes for interconversions of the different variants are present, e.g. from FMN to FAD and enzymes for the *glutamatization* of folate. The biosynthetic enzymes for the direct thiamine precursors, the condensation of the pyrimidine and the thiazole component to thiamine-phosphate and the interconversion of thiamine to the active thiamine-phosphate are present. A direct connection to the central metabolism remains unclear.

Potential ECF transport systems for folate, riboflavin, pyridoxine (vitamin B6), thiamine, niacin and biotin were found. Enzymes for interconversions of vitamin B6 variants and nicotinate and nicotinamide (further to NAD/NADP) are present. Cobalamin cannot be synthesized de novo, while a potential permease was found.

10.3.3.7 Stress response and tolerance

Acid stress: malolactic fermentation and asparaginase (EC 3.5.1.1) were found to be the only acid tolerance related systems investigated. TMW 1.1991-SB has a plasmid-encoded cluster for the AGDI pathway.

Oxidative stress: Of 13 in detail investigated genes, associated with oxidative stress, seven were found to be present in *L. backii:* thioredoxin and thioredoxin reductase (EC 1.8.1.9), NADH peroxidase, RecA, a manganese transport system (MntH) and ferroxidase (EC 1.11.1.1). A protein similar to glutathione reductase was also detected.

Hop stress: All isolates are characterized by a complete *horC* cluster. In addition TMW 1.1989-SB and TMW 1.2002-MB possess a second, truncated *horC* cluster, consisting of a mobile element protein as well as *horB* and *horC* only. A complete *horA* cluster is encoded in case of TMW 1.1988-SB and TMW 1.1992-SB. Based on *in vitro* results 1.2002-MB is also positive for *horA* and *hitA*, which was not found in the respective genome sequences nor within the unmapped reads.

10.3.3.8 Others

Seven to ten plasmids were found. The number of mobile element proteins correlates with the number of plasmids and ranges from 98 to 141. 14 to 38 genes related to transposases, 16 to 52 related to phages were found. The latter number correlates with the number of plasmids. 13 genes connected to competence were found.16 and five genes related to peptidoglycan and (lipo-) teichoic acid biosynthesis, respectively, are present constantly within all genomes.

10.4 *Lactobacillus lindneri*

10.4.1 General genomic properties

Two genomes were sequenced resulting in complete chromosomes, one plasmid in case of TMW 1.481-MB and three partial plasmid sequences for the same strain. The chromosome size ranges from 1.39 Mbp to 1.42 Mbp in case of TMW 1.481-MB. Similar chromosome (genome) sizes are found for all sequenced genomes of the *L. fructivorans* group (~ 1.3 to 1.4 Mbp), e.g. for *L. sanfranciscensis* (1.30 Mbp). The chromosomal GC content is 34.3 %. The chromosome alignment shows three conserved blocks and two strain specific pieces (see Figure 71, p. 270). Only one complete plasmid was found with a size of 14,963 bp and a GC content of 41.5 %. Further, three partial plasmid sequences, with sizes from 3,920 to 11,167 bp and GC contents from 37.3 to 42.9 %, were found for TMW 1.481-MB. *In vitro* results indicate that TMW 1.1993-SB originally/normally also harbours plasmids, as the strain was tested positive for *horA*, *horC* and the novel marker-sequence M12. All of them are found on plasmids in case of the other species investigated and are not encoded on TMW 1.1993-MB´s chromosome or found within the unmapped reads. Both strains share a core genome of 1,305 gene families, with a pan genome of 1,405, resulting in coding densities of 87.8 +/- 0.1 %. Seven complete rRNA operons, 60 tRNA genes and one pseudo-tRNA were found. Figure 70 shows the predicted subcellular localization of *L. lindneri* proteins. Besides GC content, TMW 1.481-MB plasmids differ also in terms of codon and amino acid usage from the respective chromosome.

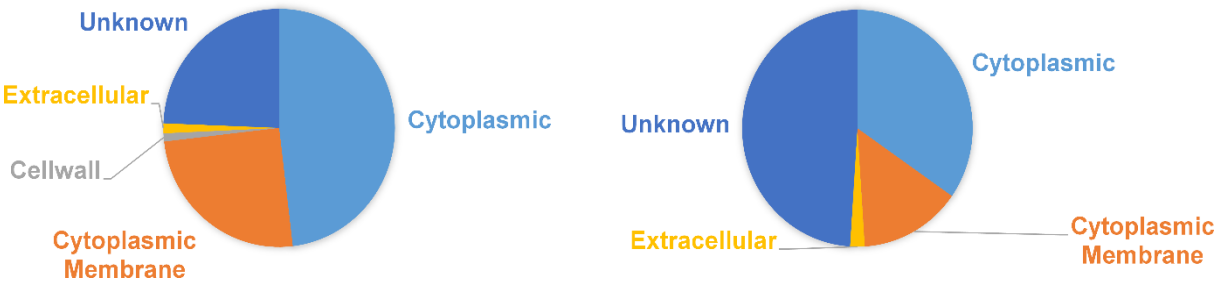


Figure 70: Subcellular localization of *L. lindneri* proteins in %. Distribution is shown for the average values for both strains. **Left** - chromosomal proteins, in detail → Cytoplasmic: 48.1 +/- 0.3 %; Cytoplasmic membrane: 25.0 +/- 0.2 %; Cell wall: 1.1 +/- 0.0 %; Extracellular: 1.4 +/- 0.0 %; Unknown: 24.4 +/- 0.5. **Right** - plasmid proteins (TMW 1.481-MB only), in detail → Cytoplasmic: 34.7 %; Cytoplasmic membrane: 14.3 %; Extracellular: 2.0 %; Unknown: 49 %.

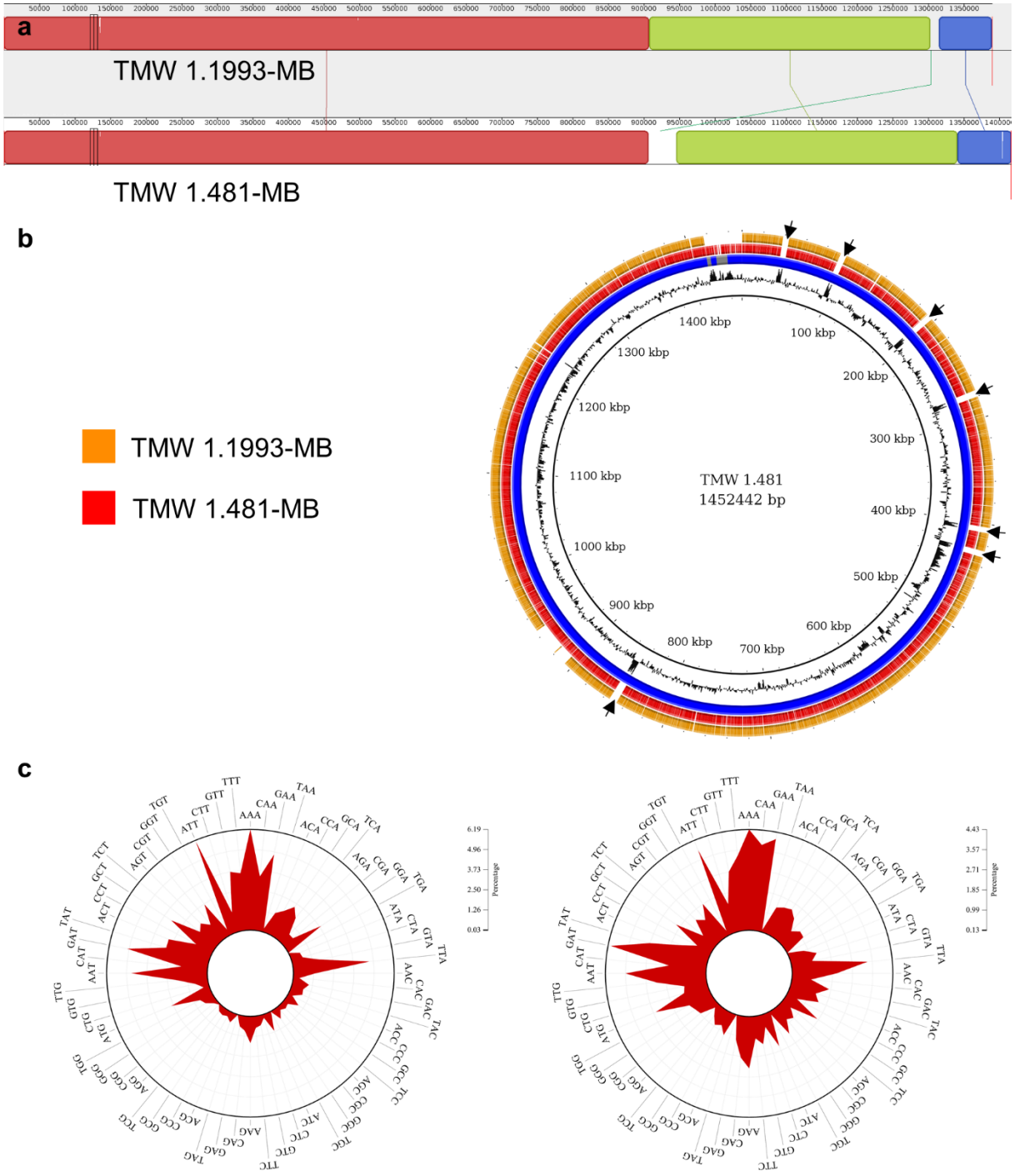


Figure 71: Illustration of the general genomic properties of *L. lindneri*. **(a)** Chromosome alignment **(b)** BLAST ring image of all *L. lindneri* genomes. All rings are described from the inside to the outside: ring 1 (black) represents the total genome sequence of TMW 1.481-MB as reference with bp coordinates; ring 2 (black) shows the GC content; ring 3 consists of arcs with different lengths and alternating coloration (grey/blue), representing the different contigs of TMW 1.481-MB; ring 4 shows all BLAST hits of TMW 1.481-MB ORFs versus its own genome, illustrating the coding density; ring 5 shows the BLAST hits of TMW 1.1993-MB ORFs versus the reference; black arrows indicate the localization of rRNA operons **(c)** Codon usage of TMW 1.481-MB, chromosome (left) and plasmidome (right), exemplary.

10.4.2 Functional analysis - SEED and COG

52 +/- 1 % of the found ORFs could be assigned to SEED categories, while the remaining ORFs contain 204 to 234 hypothetical proteins. In case of the COG enrichment, 93 +/- 1 % could be assigned to a functional category, with about 22 % poorly characterized proteins. As in case of the other species, most genes are associated with translation, transcription and other basic metabolism. Based on total feature count, *L. lindneri* is not characterized by any comparatively prominent SEED category, while in relation to its genome size it was found to be ranked within the top 10 for protein metabolism, nucleosides and nucleotides and lipid metabolism. This is the consequence of the small genome size of *L. lindneri*. A similar trend, with respect to the above named categories, can be observed for other small genomes (*L. sanfranciscensis*, *L. acetotolerans*), as well as a general correlation of these categories to genome size. This is confirmed by COG enrichments. On the other side, *L. lindneri* is characterized by the smallest (COG) or second smallest (SEED) number of carbohydrate metabolism related genes, which results in a low number of fermentable carbohydrates (see section about metabolic capabilities). In addition, *L. lindneri* is ranked on the last place in case of cell wall and capsule related genes, resulting from the complete lack of genes for the biosynthesis of (lipo)teichoic acids. Further, *L. lindneri* has only 40 genes related to RNA processing and modification (SEED subcategory), while the average within the genus *Lactobacillus* was found to be 70.

The chromosomal functional pattern are highly conserved between both strains, as illustrated by Figure 72 (p. 272).Only seven genes of the TMW 1.481 plasmidome could be assigned to the SEED classification system, while 85 % of all genes could be assigned to COG categories. Most genes are related to DNA metabolism (SEED) and RRR (replication, recombination and repair, COG). One partial plasmid sequence was found to encode for a complete cluster for citrate utilization (SEED carbohydrates). For details, see Figure 72 c.

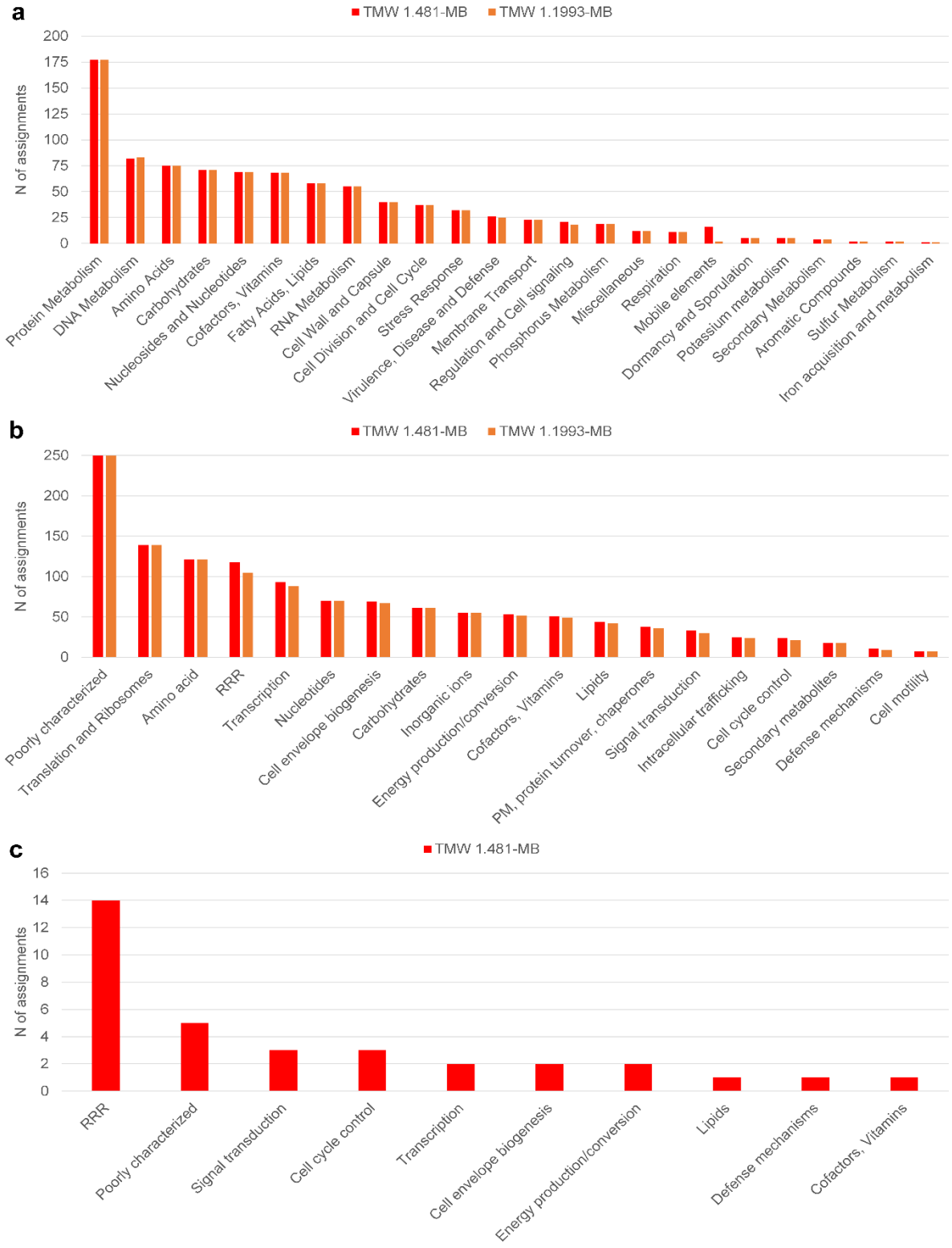


Figure 72: Functional analysis of *L. lindneri* genomes. The total number of chromosomal proteins assigned to the single categories is shown for SEED categories **(a)** and COG categories **(b)** in descending order. In case of the COG category “Poorly characterized”, the actual values range from 278 to 289. SEED subsystem coverage: 52.5 +/- 0.3; COG coverage: 94.7 +/- 0.3. **(c)** The lower panel shows the COG enrichment for TMW 1.481-MB plasmidome, COG coverage: 85 %. RRR = Replication, recombination and repair, PM = posttranslational modification.

10.4.3 Metabolic capabilities

10.4.3.1 Carbohydrates and central metabolism

Glycolysis is incomplete, lacking the key enzyme, while as expected a complete phosphoketolase pathway (PKP) was found. The pentose phosphate pathway is complete, with the exception of transaldolase (EC 2.2.1.2). No PTS transporters are present. Enzymes for the phosphorylation and activation of glucose and fructose for PKP exist. Maltose phosphorylase (EC 2.4.1.8) and beta-phosphoglucomutase (EC 5.4.2.6) are found next to a MFS type sugar transporter, allowing the utilization of maltose. No maltodextrin glucosidase (EC 3.2.1.20) was found. Two potential ribose transport proteins and ribokinase (EC 2.7.1.15) potentially allow ribose utilization. Glycerol kinase (EC 2.7.1.30) and glycerol-3-phosphate dehydrogenase (EC 1.1.1.94) are available for glycerol utilization, while no associated transport system was found. A gluconate permease and gluconokinase (EC 2.7.1.12) indicate the potential for gluconate utilization via the pentose phosphate pathway.

10.4.3.2 Pyruvate metabolism, organic acids and TCA cycle

All strains are capable to produce ethanol, acetate and lactate (L/D-lactate) from pyruvate and/or acetyl-phosphate (from PKP). Diacetyl and acetoin reducing enzymes are present, while the connection to pyruvate is missing (see Figure 73, p. 274). No additional anaplerotic enzymes were found. Uptake and utilization of malate and citrate is encoded. The TCA cycle is incomplete, while only fumarate hydratase was found (EC 4.2.1.2).

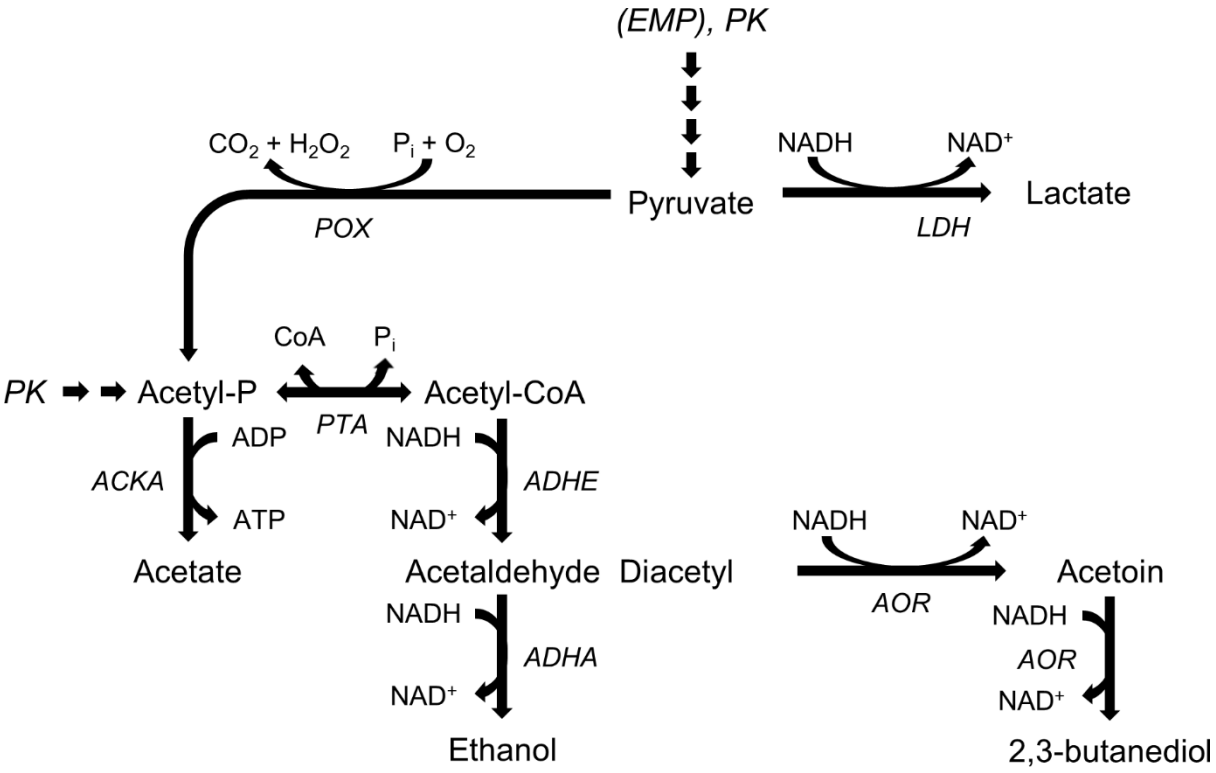


Figure 73: Pyruvate metabolism of *L. lindneri*. , EMP = Embden–Meyerhof–Parnas, PK = phosphoketolase (EC 4.1.2.9), LDH = lactate dehydrogenase (EC 1.1.1.27/28), POX = pyruvate oxidase (EC 1.2.3.3); ACKA = acetate kinase (EC 2.7.2.1), PTA = phosphate acetyltransferase (EC 2.3.1.8), ADHE = acetaldehyde dehydrogenase (EC 1.2.1.10), ADHA = alcohol dehydrogenase (EC 1.1.1.1), AOR = acetoin reductase (EC 1.1.1.4). EMP is not complete, while the enzyme phosphoketolase results in acetyl-phosphate and glyceraldehyde-3-phosphate, which is metabolized to pyruvate as known for EMP.

10.4.3.3 Proteolytic system and amino acids

A cell wall-bound, extracellular protease was not found, while transport systems for di- and tripeptides and potentially for oligopeptides are present. Endopeptidases, aminopeptidases and oligo-/tri-/dipeptidases are encoded. In addition, potential transport systems were found for alanine, arginine, glutamine/glutamate, glycine, lysine, methionine, serine and branched amino acids, as well as unspecified amino acid transporter systems. A direct biosynthesis from central carbon metabolism precursors is potentially possible for tryptophan and tyrosine. Other biosynthetic reaction sequences were found to be isolated, or the respective amino acids can only be made from other amino acids. An interconversion of alanine and cysteine, serine and glycine, glutamate and aspartate, as well as their derivatives glutamine and asparagine is possible. Arginine can be produced from citruline. Lysine biosynthesis remains ambiguous. Based on prediction *L. lindneri* is auxotroph for all branched amino acids, histidine, phenylalanine, methionine, threonine and most probably for proline. For details, see Figure 74.

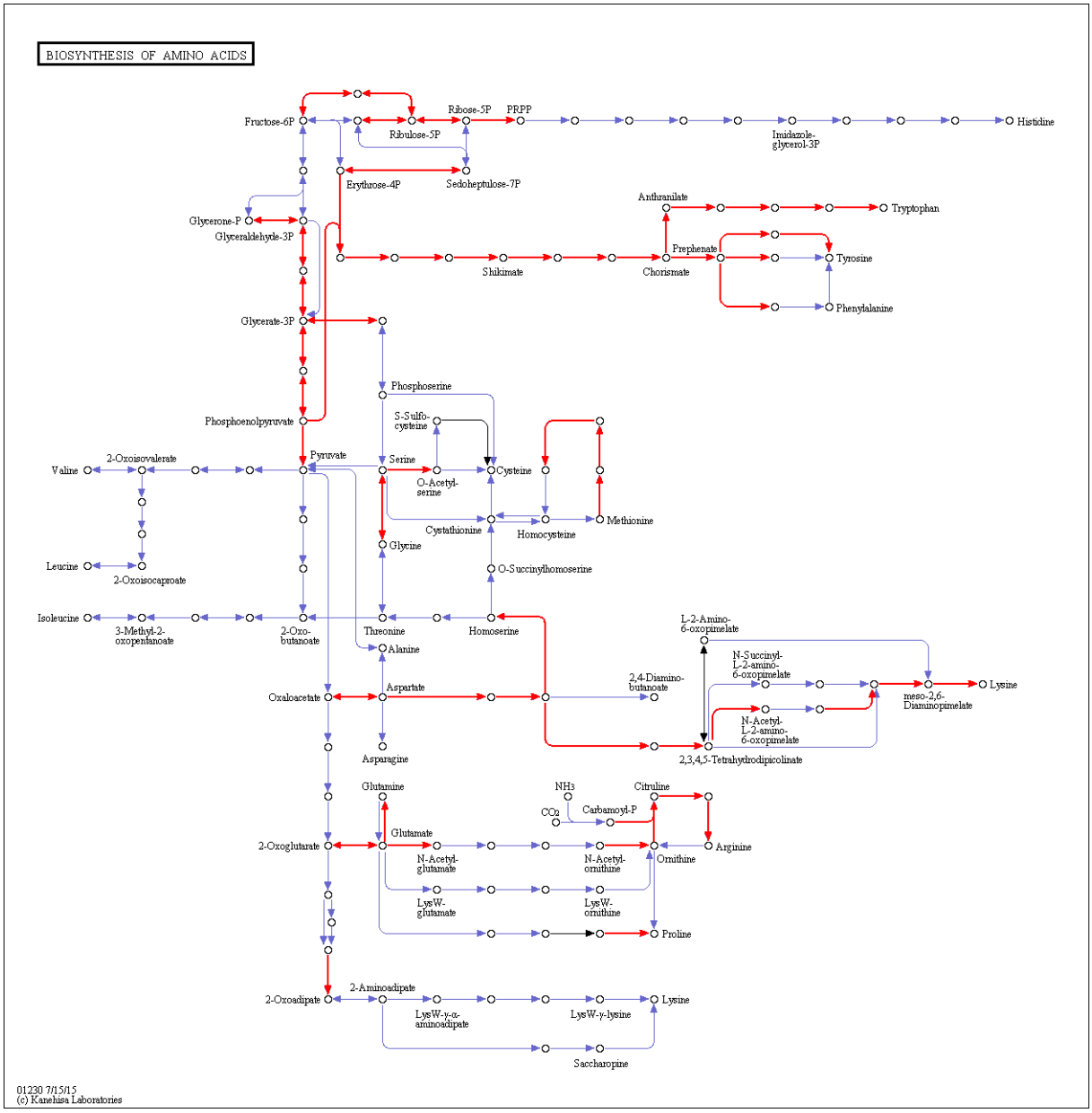


Figure 74: Predicted pathway of amino acid biosynthesis - *L. lindneri*. Red arrows indicate a reaction, which is present, based on EC numbers. The figure was obtained from the KEGG PATHWAY mapping tool. This figure is based on TMW 1.1993-MB. Alanine can be synthesized from cysteine by cysteine desulfurase (EC 2.8.1.7), which is not indicated in the map. Glutamate and aspartate can be interconverted by aspartate transaminase (EC 2.6.1.1). Asparagine can be synthesized from aspartate, using the Asparagine synthetase (EC 6.3.5.4), which is also not part of this map.

10.4.3.4 Purines and Pyrimidines

IMP can be made from PRPP. From IMP all nucleobases, nucleosides and nucleotides can be produced. A potential Cytosine/purine/uracil/thiamine/allantoin permease was found.

The biosynthetic pathway of UMP from PRPP and glutamine is complete. All enzymes were found for the formation of all nucleotides, all nucleosides, uracil, uridine and thymine. Cytosine production from CMP or cytidine is not encoded. Uracil phosphoribosyltransferase (EC 2.4.2.9), necessary for the interconversion of UMP and uracil, is present. A potential uracil permease is encoded.

10.4.3.5 Fatty acid biosynthesis

The chromosomal type II FAS is complete. Genes for peripheral enzymes and for the modification to cyclopropane fatty acids were found for all strains.

10.4.3.6 Vitamins and Co-Factors

The biosynthetic enzymes for the direct thiamine precursors, the condensation of the pyrimidine and the thiazole component to thiamine-phosphate and the interconversion of thiamine to thiamine-phosphate are present. As for all six in detail-investigated species, the necessary enzyme for the phosphorylation of thiamine-phosphate to thiamine-pyrophosphate (cofactor for phosphoketolase) is missing, while thiamine-pyrophosphate can be made directly from thiamine. FMN/FAD and NAD/NADP can be synthesized from riboflavin and nicotinate/nicotinamide, respectively, while no *de novo* biosynthesis is encoded. Dihydrofolate synthase (EC 6.3.2.12), necessary for the synthesis of folate derivatives is present, while a biosynthesis from chorismate is not encoded. ECF transporter were found for riboflavin, thiamine, folate and biotin. *L. lindneri* lacks any enzymes related to vitamin B6 and vitamin B12 metabolism.

10.4.3.7 Stress response and tolerance

Acid stress: A histidine decarboxylase (EC 4.1.1.22) and a potential antiporter for histidine/histamine was found in both genomes. Additionally, malolactic fermentation, citrate fermentation and asparaginase (EC 3.5.1.1) were found to be encoded.

Oxidative stress: Of 13 in detail investigated genes, associated with oxidative stress, eight were found to be present in *L. lindneri:* thioredoxin and thioredoxin reductase (EC 1.8.1.9),

catalase (EC 1.11.1.6), manganese catalase, NADH peroxidase, RecA and a manganese transport system (MntH). A protein similar to glutathione reductase was also detected.

Hop stress: TMW 1.481-MB possess a complete *horA* cluster. Based on *in vitro* results TMW 1.1993-MB is also positive for *horA* and *horC*, which was not found in the respective genome sequences nor within the unmapped reads.

10.4.3.8 Others

In case of TMW 1.481-MB, we found one plasmid and three partial plasmid sequences. The number of mobile element proteins ranges from seven to 15 (TMW 1.481-MB). Six genes related to transposases and four to 19 (TMW 1.481-MB) related to phages were found. 15 genes connected to competence were found. 16 genes related to peptidoglycan biosynthesis and no genes related to (lipo-) teichoic acid biosynthesis, respectively, are present constantly within all strains.

10.5 *Lactobacillus paracollinoides*

10.5.1 General genomic properties

Genome sequencing of two beer-spoiling and one non-spoiling strain resulted in three complete genomes. Chromosome size ranges from 3.24 Mbp to 3.48 Mbp with a consistent GC content of 47.2 %, while the smaller chromosome belongs to the non-spoiling strain TMW 1.1979. The same strain is characterized by only two plasmids. In contrast, both strong spoiling strains possess eight extrachromosomal elements. The 18 plasmids have a size between 13,353 and 57,063 bp, resulting in total genome sizes from 3.32 to 3.75 Mbp. The latter (TMW 1.1995-SB) represents the second largest *Lactobacillus* genome known, after *L. parakefiri*. The plasmid GC content ranges from 34.7 to 42.3 % and is therefore much lower than in case of the chromosomes, while they also differ with respect to codon usage from the chromosome. Mauve alignment illustrates a more similar chromosomal structure of TMW 1.1979-NB and TMW 1.1995-SB, while the beer-spoiling strains share more information on sequence level. This and the fact that most of the beer-spoiling specific DNA, but not everything, is located on plasmids, is illustrated by Figure 76 b (p. 279). All three strains share a core genome of 2,503 gene families, with a pan genome of 3,343 genes, resulting in accessory genomes proportions of 15 to 26 %. The coding density is about 79 %. Six complete rRNA operons, 65 to 68 tRNA genes and two pseudo-tRNA were found. Figure 75 shows the predicted subcellular localization of *L. paracollinoides* proteins.

Figure 75: Subcellular localization of *L. paracollinoides* proteins in %. Distribution is shown for the average values for all three strains. **Left** - chromosomal proteins, in detail → Cytoplasmic: 44.7 +/- 0.7 %; Cytoplasmic membrane: 27.3 +/- 0.3 %; Cell wall: 1.5 +/- 0.1 %; Extracellular: 1.5 +/- 0.1 %; Unknown: 24.9 +/- 0.4. **Right** - plasmid proteins (TMW 1.481-MB only), in detail → Cytoplasmic: 37.5 +/- 2.3 %; Cytoplasmic membrane: 14.6 +/- 0.8 %; Cell wall: 0.5 +/- 0.4 %; Extracellular: 1.9 +/- 0.5 %; Unknown: 45.5 +/- 3.6 %.

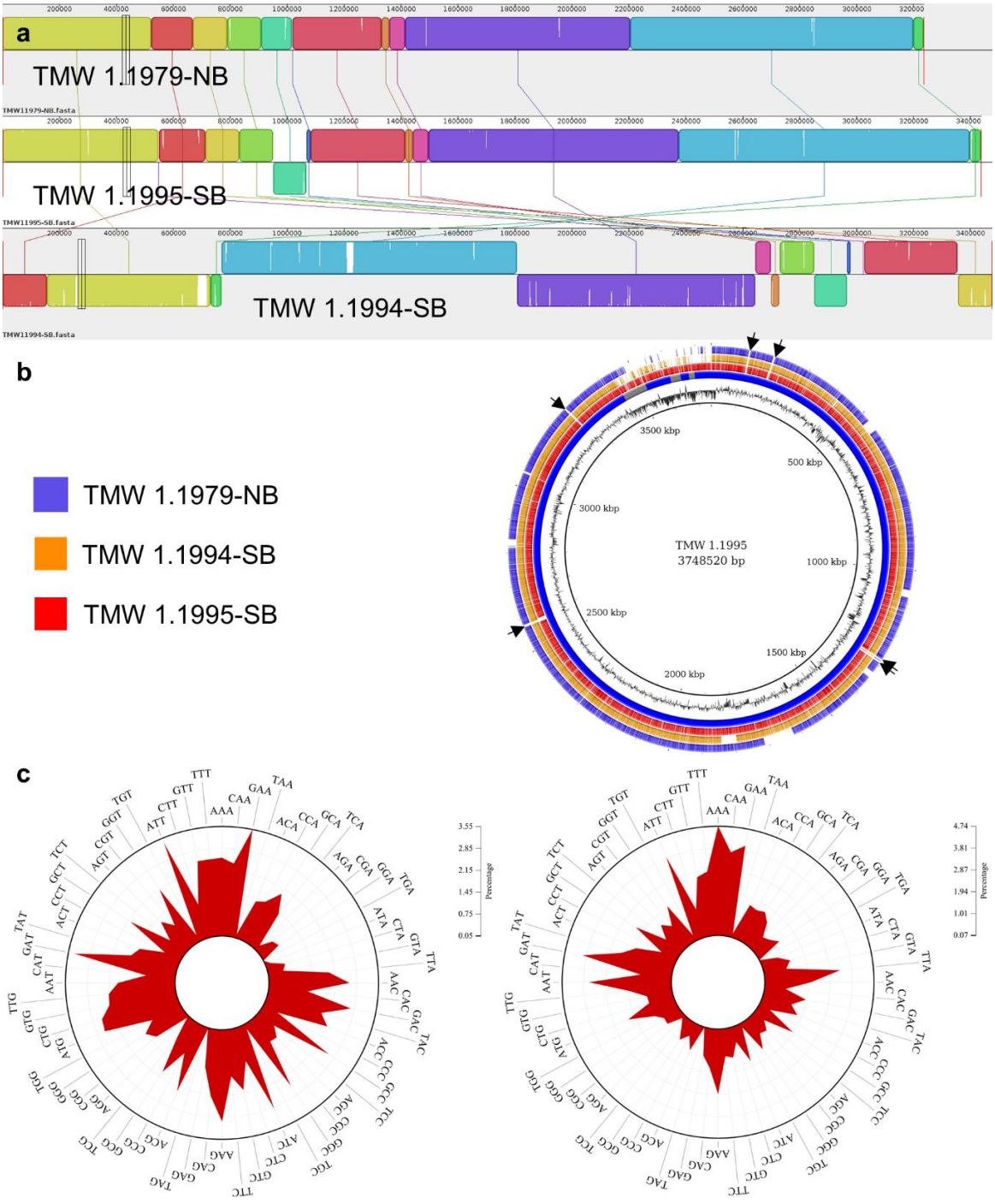


Figure 76: Illustration of the general genomic properties of *L. paracollinoides*. **(a)** Chromosome alignment **(b)** BLAST ring image of all *L. paracollinoides* genomes. All rings are described from the inside to the outside: ring 1 (black) represents the total genome sequence of TMW 1.1995-SB as reference with bp coordinates; ring 2 (black) shows the GC content; ring 3 consists of arcs with different lengths and alternating coloration (grey/blue), representing the different contigs of TMW 1.1995-SB; ring 4 shows all BLAST hits of TMW 1.1995-SB ORFs versus its own genome, illustrating the coding density; ring 5 (orange) shows the BLAST hits of TMW 1.1994-SB ORFs versus the reference; ring 6 (blue) shows the BLAST hits of TMW 1.1979-NB versus the reference; black arrows indicate the localization of rRNA operons (no ORF hits) **(c)** Codon usage of TMW 1.1995-SB, chromosome (left) and plasmidome (right), exemplary for *L. paracollinoides*.

10.5.2 Functional analysis - SEED and COG

41 +/- 1 % of the found ORFs could be assigned to SEED categories. Of the remaining unassigned ORFs, 587 to 734 were annotated as hypothetical proteins. In case of the COG enrichment, 87 to 90 % could be assigned to a functional category, with about 20 % poorly characterized proteins.

Compared to the other lactobacilli genomes, *L. paracollinoides* is characterized by the highest number of genes associated with amino acid transport and metabolism (SEED & COG), resulting in a high degree of predicted amino acid prototrophy, as described in the corresponding section about the metabolic capabilities. There is a high number of features related to vitamins and cofactors, while *L. paracollinoides* is not only characterized by a high number of genes assigned to this category, but also by a high proportion relative to the total number of assigned genes. Because of the comparatively large genome, further top 10 rankings were observed, e.g. for phosphorous metabolism, nitrogen metabolism, sulfur metabolism (all SEED) or energy production and conversion (COG).

Compared to *P. claussenii*, the chromosomal functional pattern is more diverse (CV SEED 5.4 %/COG 3.1 %), while this variation mainly results from differences between the two beer-spoiling strains and TMW 1.1979-NB. As illustrated in Figure 77(p. 282), this reposes primarily from differences in amino acid, carbohydrate and vitamin metabolism, which is apparent from SEED and COG classification. In case of the category amino acids, it results from the absence of several biosynthetic genes (TMW 1.1979-NB), as detailed in the corresponding section about the predicted metabolic capabilities (e.g. branched amino acids). The difference in genes associated with carbohydrate metabolism is based, amongst others, on the reduced number of genes related to xylose and xyloside utilization, as well as glycerol utilization in case of TMW 1.1979-NB. The difference, which can be seen for the category cofactors & vitamins (SEED & COG) and nitrogen metabolism (SEED), results from the presence of a complete moco biosynthesis cluster in case of the beer-spoiling strains, which is accompanied by the presence of a nitrate reductase (EC 1.7.99.4) located next to it.

Functional analysis of plasmids resulted in a SEED subsystem coverage of 29 +/- 4 % and a COG enrichment coverage of 71 +/- 3 %. Based on functional analysis, the following categories and subordinate levels are noticeable with respect to isolation source (all three from brewery environment) and beer spoilage potential of the investigated genomes/strains (see Figure 78, p.283):

The number of carbohydrate metabolism related genes is enriched in case of the beer-spoiling strains (SEED/COG). Functions associated with ribose and trehalose/maltose/kojibiose utilization are found in both beer-spoiling strains. TMW 1.1995-SB additionally was found to possess plasmid-encoded clusters related to citrate metabolism, maltose utilization and acetolactate metabolism (acetoin, butanediol). Further, both beer-spoiling strains have a slightly higher number of genes related to cell envelope biogenesis and membrane transport (both SEED), while the latter is based on a transport system for copper. TMW 1.1994-SB and TMW 1.1979-NB encode a complete cluster for the AGDI pathway, assigned to the category amino acids. The large number of genes related to DNA metabolism (SEED, RRR for COG), in case of all three strains, results of type I restriction modification systems.

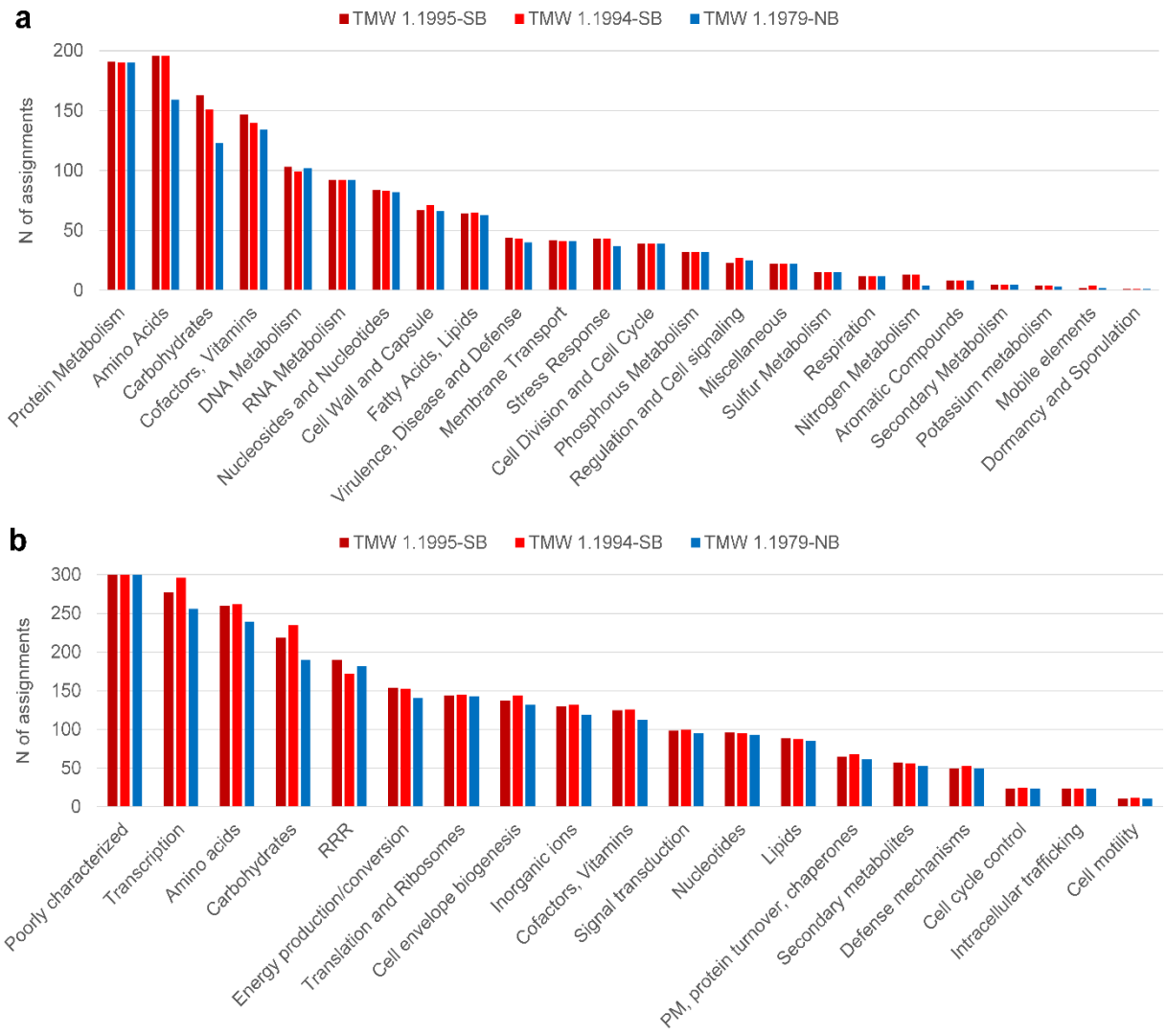


Figure 77: Functional analysis of *L. paracollinoides* chromosomes. The total number of proteins assigned to the single categories is shown for SEED categories (a) and COG categories (b) in descending (average) order. In case of the COG category "Poorly characterized," the actual values are 602, 656 and 636. SEED subsystem coverage: 42.3 +/- 0.7; COG coverage: 90.6 +/- 0.7. RRR = Replication, recombination and repair, PM = posttranslational modification.

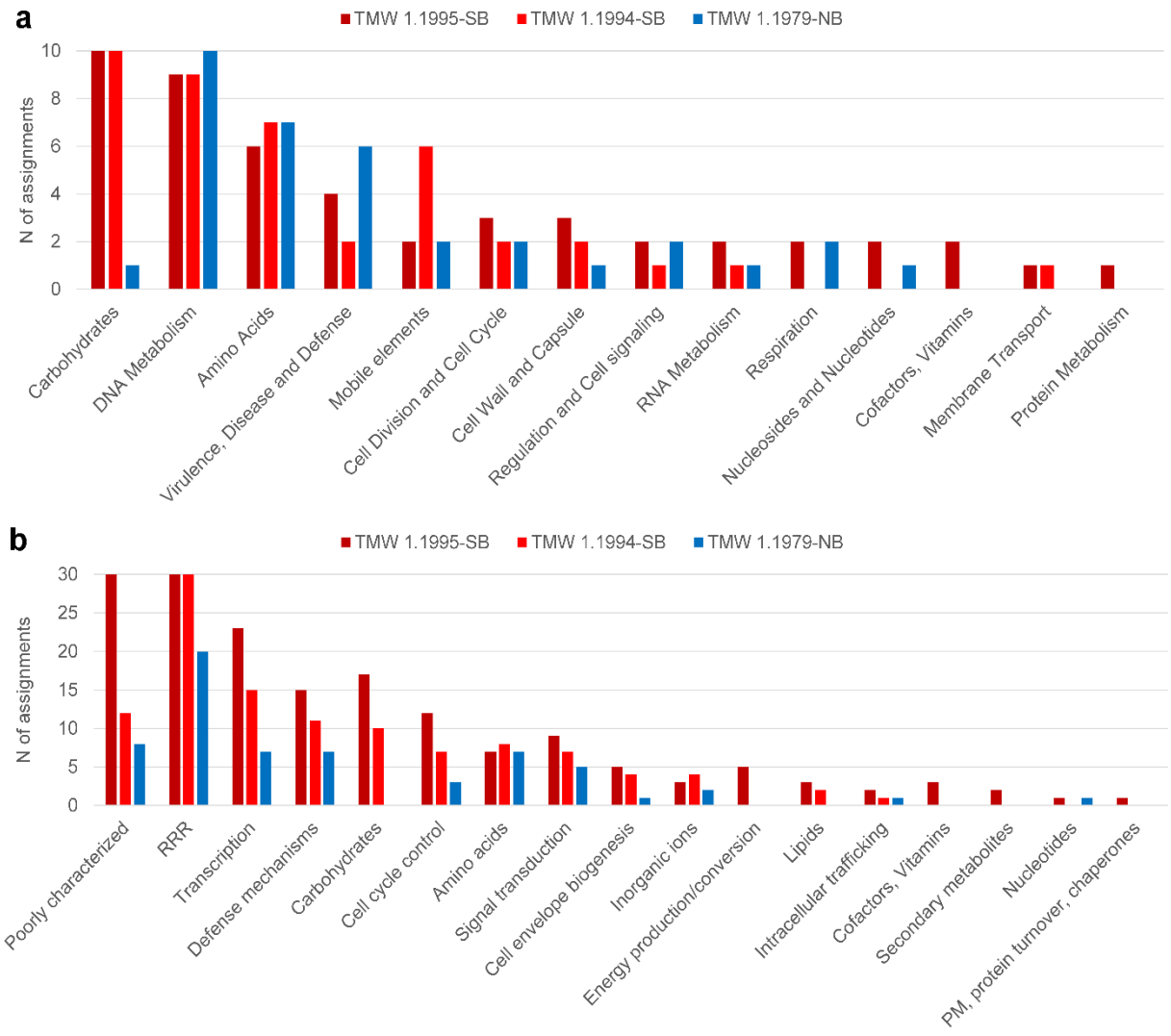


Figure 78: Functional analysis of *L. paracollinoides* plasmidomes. The total number of proteins assigned to the single categories is shown for SEED categories (a) and COG categories (b) in descending (average) order. In case of the SEED category carbohydrates, the actual value for TMW 1.1995-SB is 38. In case of the COG category “Poorly characterized,” the actual value for TMW 1.1995-SB is 32. RRR: actual values are 51 (TMW 1.1994-SB) and 91 (TMW 1.1995-SB). SEED subsystem coverage: 29.4 +/- 3.6; COG coverage: 70.6 +/- 2.6. RRR = Replication, recombination and repair, PM = posttranslational modification.

10.5.3 Metabolic capabilities

10.5.3.1 Carbohydrates and central metabolism

Phosphoketolase and pentose phosphate pathway are complete. Glycolysis is incomplete, lacking fructose-bisphosphate aldolase (EC 4.1.2.13). Only one complete PTS system was found, which, based on database search, is related to either fructose or mannose uptake. Mannose-6-phosphate isomerase (EC 5.3.1.8), which is necessary for isomerization of mannose-6-phosphate to fructose-6-phosphate, is missing. Two permease-like transport systems for galactose were found, as well as all enzymes for the Leloir pathway for galactose utilization. A potential lactose permease, as well as a beta-galactosidase (EC 3.2.1.23) for the consequent hydrolysis to glucose and galactose are encoded. Potential MFS transport systems were found for maltose and isomaltose, spatially located next to the enzymes for their utilization, a maltose phosphorylase (EC 2.4.1.8) and an isomaltase (EC 3.2.1.10). A maltodextrin glucosidase (EC 3.2.1.20) was found. Further, we found genes with ambiguous functions either associated with trehalose or kojibiose utilization. Both beer-spoiling strains encode for a beta-glucosidase (EC 3.2.1.21) and a 6-phospho-beta-glucosidase (EC 3.2.1.86). Enzymes for arabinose utilization are encoded, while no related transport system was found. In contrast, ribose (ABC type) and xylose transporter (proton symport) were found. Ribokinase (EC 2.7.1.15), necessary for ribose utilization, was found in all three genomes. The respective enzymes for xylose utilization were only found in case of the beer-spoiling strains. All genomes encode for a potential xyloside transport system and a beta-xylosidase (EC 3.2.1.37). Only the beer-spoiling strains carry genes for an alpha-xylosidase (EC 3.2.1.-). The same was found for a glycerol-3-phosphate ABC transporter, while all three strains have a glycerol uptake facilitator protein. Enzymes for the degradation of glycerol and glycerol-phosphate are present in all three genomes. Gluconate can be taken up presumably by a permease and utilized via pentose phosphate pathway.

10.5.3.2 Pyruvate metabolism, organic acids and TCA cycle

All enzymes are present to produce ethanol, acetate, lactate (L/D-lactate), acetoin and 2,3 butanediol from pyruvate, as illustrated by Figure 79 (p. 285). Ethanol or acetate can also be made from acetyl-phosphate, resulting from phosphoketolase reaction. In addition, the anaplerotic enzyme phosphoenolpyruvate carboxykinase (EC 4.1.1.49) was found. All genes were found which are necessary for the uptake and utilization of malate. Citrate utilization is only encoded by TMW 1.1995-SB (plasmid-encoded). As in case of *L. backii*, the TCA cycle is encoded from citrate to 2-oxoglutarate. Further, with exception of fumarate hydratase (EC 4.2.1.2), the remaining genes are missing.

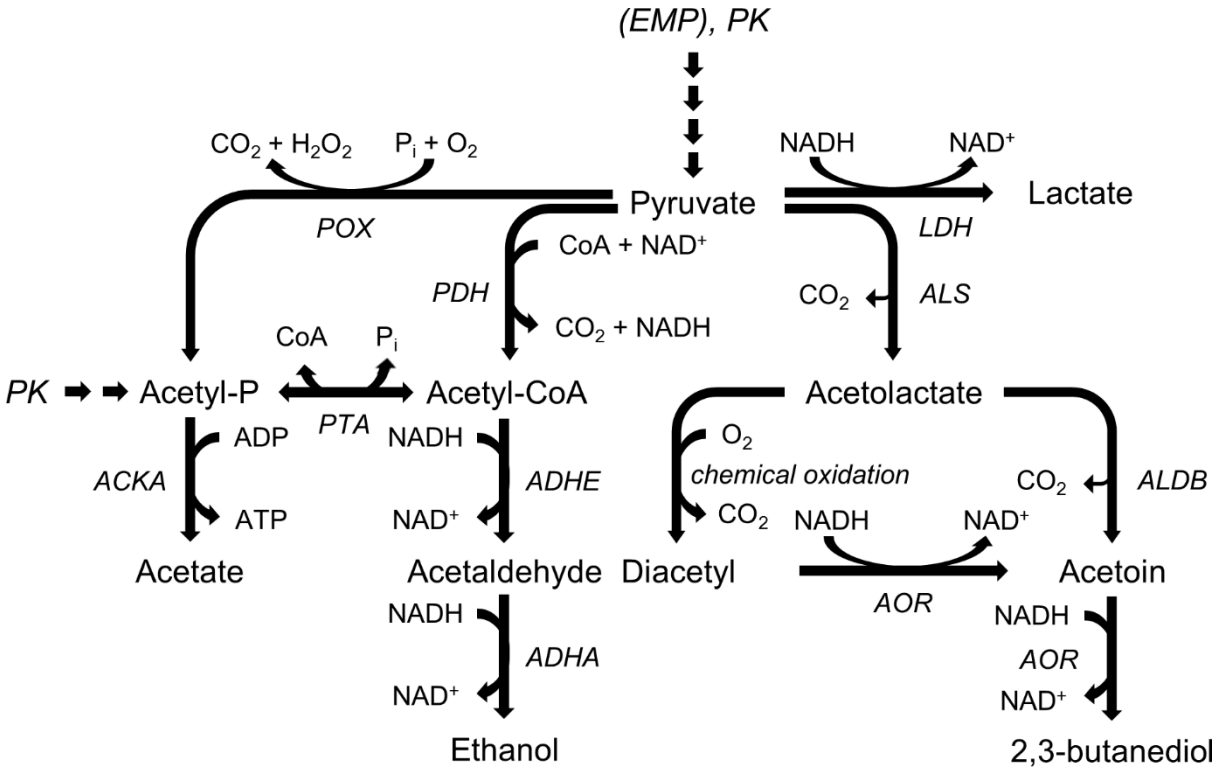


Figure 79: Pyruvate metabolism of *L. paracollinoides* and *L. brevis*. EMP = Embden–Meyerhof–Parnas, PK = Phosphoketolase (EC 4.1.2.9), LDH = lactate dehydrogenase (EC 1.1.1.27/28), ALS = acetolactate synthase (EC 2.2.1.6), ALDB = alpha-acetolactate decarboxylase (EC 4.1.1.5), AOR = acetoin reductase (EC 1.1.1.4), POX = pyruvate oxidase (EC 1.2.3.3), PDH = pyruvate dehydrogenase (EC 1.2.4.1), ACKA = acetate kinase (EC 2.7.2.1), PTA = phosphate acetyltransferase (EC 2.3.1.8), ADHE = acetaldehyde dehydrogenase (EC 1.2.1.10), ADHA = alcohol dehydrogenase (EC 1.1.1.1), EMP is not complete, while the enzyme phosphoketolase results in acetyl-phosphate and glyceraldehyde-3-phosphate, which is metabolized to pyruvate as known for EMP.

10.5.3.3 Proteolytic system and amino acids

A cell wall-bound, extracellular protease was not found, while transport systems for oligo, di- and tripeptides are present. Several different endopeptidases, aminopeptidases and oligo-/tri-/dipeptidases are encoded. In addition, potential transport systems were found for alanine, arginine, cysteine, glutamine/glutamic acid, glycine, lysine, methionine, threonine, proline, serine and branched amino acids as well as unspecified amino acid transporter. Both beer-spoiling strains are, based on prediction, capable to synthesize all 20 amino acids, which is illustrated and explained in detail in Figure 80 (p. 286). TMW 1.1979-NB lacks several enzymes for the connection of leucine, isoleucine and arginine biosynthesis to central carbon metabolism. In addition, the ultimate enzyme, necessary for methionine biosynthesis is missing. All three strains encode a complete ADI pathway, TMW 1.1979-NB and TMW 1.1994-SB additionally a plasmid-encoded AGDI pathway. In addition, glutamate

decarboxylase (EC 4.1.1.15) and asparaginase (EC 3.5.1.1) were found. No GABA/glutamate antiporter was found.

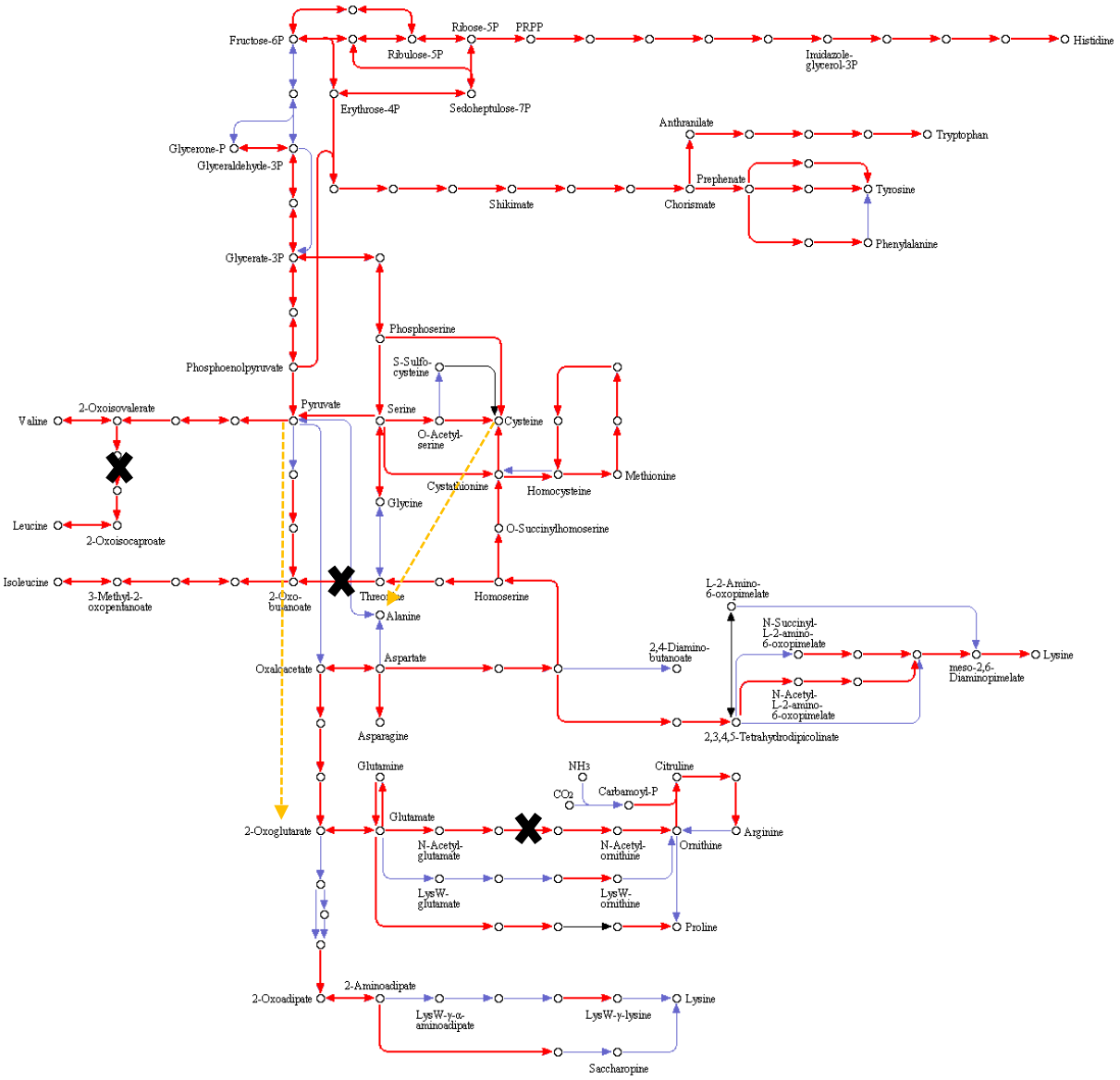


Figure 80: Predicted pathway of amino acid biosynthesis - *L. paracollinoides*. Red arrows indicate a reaction, which is present, based on EC numbers. The figure was obtained from the KEGG PATHWAY mapping tool. The metabolic capabilities of *L. paracollinoides* with respect to amino acid biosynthesis were identical for both beer-spoiling strains. This figure is based on the genome of TMW 1.1995-SB. Alanine can be synthesized from cysteine by cysteine desulfurase (EC 2.8.1.7), which is not part of the original KEGG map. It is now indicated by a dotted orange arrow. The part of the TCA cycle, necessary for 2-oxoglutarate production was found and is indicated by a dotted orange arrow. Therefore, 2-oxoglutarate can be produced from citrate, while oxaloacetate can be made from aspartate and 2-oxoglutarate by aspartate transaminase (EC 2.6.1.1.). The missing reactions (sequences) in case of TMW 1.1979-NB are marked with crosses.

10.5.3.4 Purines and Pyrimidines

IMP can be synthesized from PRPP. From IMP both nucleobases and all kind of nucleotides and nucleosides can be made. Xanthine permease, guanine-hypoxanthine permease and xanthine phosphoribosyltransferase (EC 2.4.2.22) were found, allowing the additional padding of purine metabolism via xanthosine 5'-phosphate (XMP).

UMP can be made from PRPP and glutamine. All enzymes for the biosynthesis of nucleotides, nucleosides and for the nucleobase thymine are encoded. Uracil phosphoribosyltransferase (EC 2.4.2.9), necessary for the interconversion of UMP and uracil, is present. A potential uracil permease was found.

10.5.3.5 Fatty acid biosynthesis

A complete chromosomally encoded type II FAS was found, as well as the necessary peripheral enzymes for membrane integration. A cyclopropane-fatty-acyl-phospholipid synthase was found as well.

10.5.3.6 Vitamins and Co-Factors

A complete biosynthesis cluster for vitamin B12 (cobalamin) as well as an ECF transport system were found. The biosynthetic enzymes for the direct thiamine precursors, the condensation of the pyrimidine and the thiazole component to thiamine-phosphate and the interconversion of thiamine to the active thiamine-phosphate are present. A direct connection to the central metabolism remains unclear, as well as a potential phosphorylation of thiamine-phosphate to thiamine-pyrophosphate, while the latter can be made from thiamine. A potential ECF transporter for the uptake of thiamine is present. Folate can be synthesized from GTP and chorismate, whereas both precursors can be synthesized from the central metabolism. In addition, a potential ECF transporter for folate was found. The anabolic enzymes for riboflavin biosynthesis from GTP and ribulose-5-phosphate are encoded, as well as the necessary enzymes for FMN and FAD production. As in case of folate, riboflavin biosynthesis is connected to the central metabolism and a potential ECF transporter was found. Pyridoxal 5'-phosphate synthase (EC 4.3.3.6) was found to be encoded, being the key enzyme for the biosynthesis of pyridoxal-5-phosphate from ribulose-5-phosphate and glyceraldehyde-3-phosphate. The enzymes for the interconversion of vitamin B6 variants and an ECF transporter were also found. NAD and $NADP^+$ can be derived from nicotinate and nicotinamide, while a biosynthesis from quinolinate is also encoded. A connection to the central metabolism remains elusive. The non-spoiling strain´s genome does not encode for nicotinamidase (EC 3.5.1.19),

an enzyme for the interconversion of nicotinate and nicotinamide. All three strains carry genes for a potential niacin transporter and a biotin ECF transporter.

10.5.3.7 Stress response and tolerance

Acid stress: The complete AGDI pathway was found in case of TMW 1.1979-NB and TMW 1.1994-SB, as well as the ADI pathway in all three genomes as mentioned above. NH_3 can also be produced by the found asparaginase (EC 3.5.1.1). All genes for malolactic fermentation are encoded. TMW 1.1195-SB encodes (plasmid) for citrate fermentation.

Oxidative stress: Of 13 in detail investigated genes, associated with oxidative stress, seven were found to be present in *L. paracollinoides:* thioredoxin and thioredoxin reductase (EC 1.8.1.9), NADH peroxidase, RecA, a manganese transport system (MntH) and ferroxidase (EC 1.11.1.1). A protein similar to glutathione reductase was also detected.

Hop stress: TMW 1.1979-NB does not carry any of the known hop stress associated genes. TMW 1.1995-SB has a complete *horC* cluster, while TMW 1.1994-SB was found to have the truncated version, with *horC* and *horB* only. TMW 1.1994-SB also carries the complete *horA* cluster. TMW 1.1995-SB was repeatedly tested positive for *hitA,* while neither the respective sequence nor the complete *hitA* cluster could be found within the genome. The respective sequence was found within the unmapped reads, while the corresponding read could not be integrated into the assembly.

10.5.3.8 Others

Two (TMW 1.1979-NB) to eight plasmids were found, 74 to 120 mobile element proteins, 23 to 32 transposase related genes, 28 to 42 phage related genes and 11 to 14 competence related genes. In each case, the number was higher for the beer-spoiling strains. 16 and 13 genes related to peptidoglycan and (lipo-) teichoic acid biosynthesis were found, respectively.

10.6 *Lactobacillus brevis*

10.6.1 General genomic properties

No *L. brevis* genomes were sequenced within this study. Six publicly available genomes were analysed, while four of them were also characterized with respect to beer spoilage ability of the respective strain, beer spoilage potential and other physiological properties. The corresponding strains are marked by a TMW number. Note that three genomes included, are draft genomes, which are consequently omitted in some analyses, where appropriate. The following genomes were included:

- TMW 1.313-SB, TMW 1.465-SB (both brewery isolates) and TMW 1.6-WB (faeces) → draft genomes (scaffold) with 12 to 46 contigs (Behr et al., 2015)
- TMW 1.1326-NB (ATCC 367, silage) → complete genome (Makarova et al., 2006)
- BSO464 (brewery) → complete genome/chromosome level (Bergsveinson, Pittet, et al., 2015), based on description, BSO 464 is considered as beer-spoiling
- KB290 (probiotic, fermented vegetable) → complete genome (Fukao et al., 2013)

The chromosome size of the complete (assembly level) *L. brevis* genomes ranges from 2.29 to 2.50 Mbp, while the largest scaffolds in case of the draft genomes were found to have a similar or even larger size (TMW 1.313-SB, 2.57 Mbp). The corresponding GC content is 45.6 +/- 0.8 %. Chromosome alignment (Figure 82 a, p. 291) and phylogenomic analysis show a comparatively high chromosomal variation, compared to the other species focused here. Two to nine plasmids were found in case of the complete genomes, ranging in size from 2,353 to 84,941 bp, with variable GC contents from 34.4 to 42.5 %. Altogether, the genome size of *L. brevis* was found to be 2.57 +/- 0.13 Mbp, while the brewery genomes have comparatively larger genomes. Besides GC content, some plasmids differ also in terms of codon and amino acid usage from the respective chromosome. Figure 82 b illustrates that the draft genomes of TMW 1.313-SB and TMW 1.465-SB are more similar to the complete genome of the brewery isolate BSO 464 as the other genomes analysed. Further, it reveals that in contrast to *P. damnosus*, brewery and most probably beer-spoiler specific DNA is not only found on plasmids, but also chromosomally located. In total, 2,219 to 2,606 open reading frames (ORFs) were found, resulting in a coding density of about 81 %, while the respective value was lower for the draft and higher for the complete genomes. The core genome contains 1,471 gene families and the pan genome 3,848. This results in an accessory genome of more than 58 % in average, indicating high genomic diversity. The number of RNA genes is variable, most likely also because of the draft nature of three of the six investigated genomes. In case of the

complete genomes, we found five to six rRNA operons, 47 to 62 tRNAs and one to three pseudo-tRNAs. Figure 81 shows the average predicted subcellular localization of *L. brevis* proteins and the codon usage.

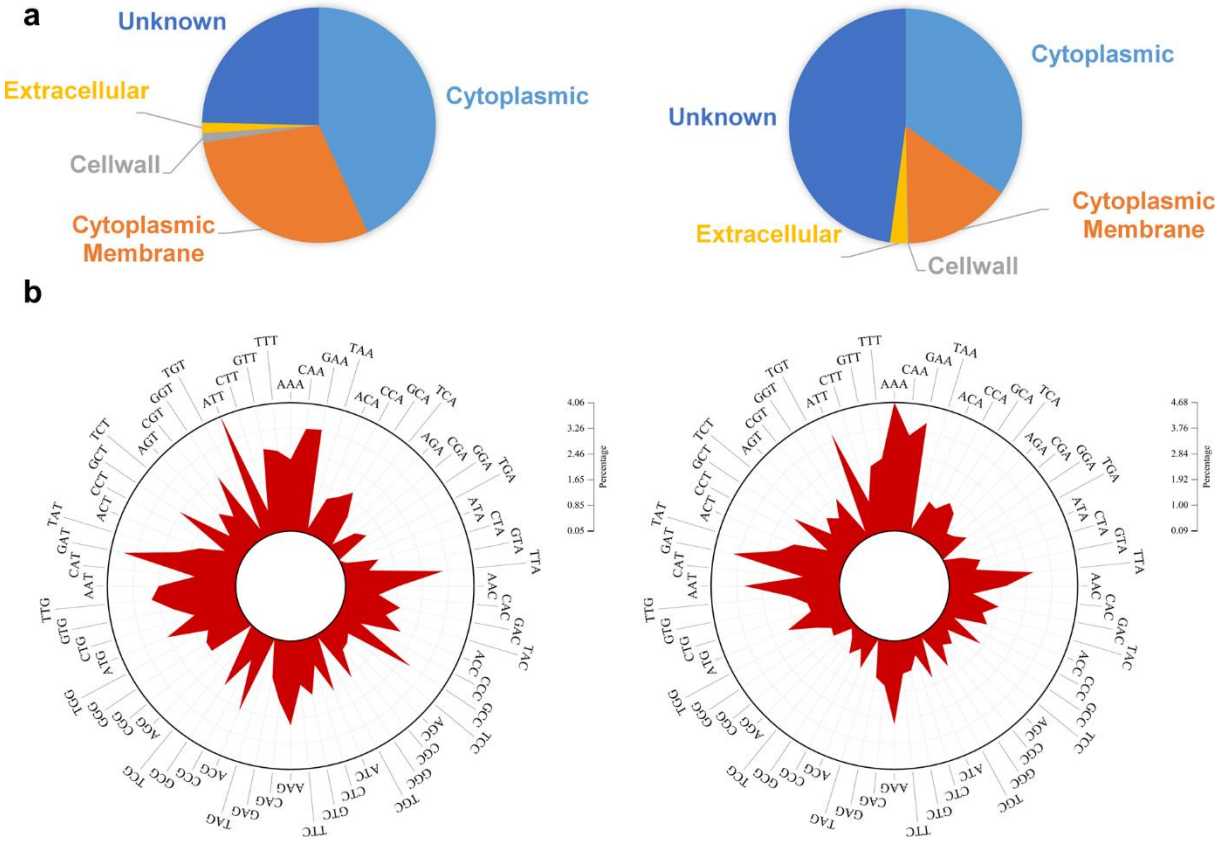


Figure 81: Subcellular localization of *L. brevis* proteins and codon usage. **(a)** Subcellular localization in %. **Left** - chromosomal proteins for all genomes except TMW 1.465-SB, in detail → Cytoplasmic: 43.2 +/- 2.7 %; Cytoplasmic membrane: 29.5 +/- 1.1 %; Cell wall: 1.3 +/- 0.1 %; Extracellular: 1.5 +/- 0.3 %; Unknown: 24.6 +/- 3.4. **Right** - plasmid proteins (BSO 464, KB290, TMW 1.1326-NB), in detail → Cytoplasmic: 34.7 +/- 4.0 %; Cytoplasmic membrane: 14.9 +/- 5.9 %; Cell wall: 0.1 +/- 0.2 %; Extracellular: 2.4 +/- 0.7 %; Unknown: 47.8 +/- 2.7 %. **(b)** Codon usage of TMW BSO 464, chromosome (left) and plasmidome (right), exemplary for *L. brevis*.

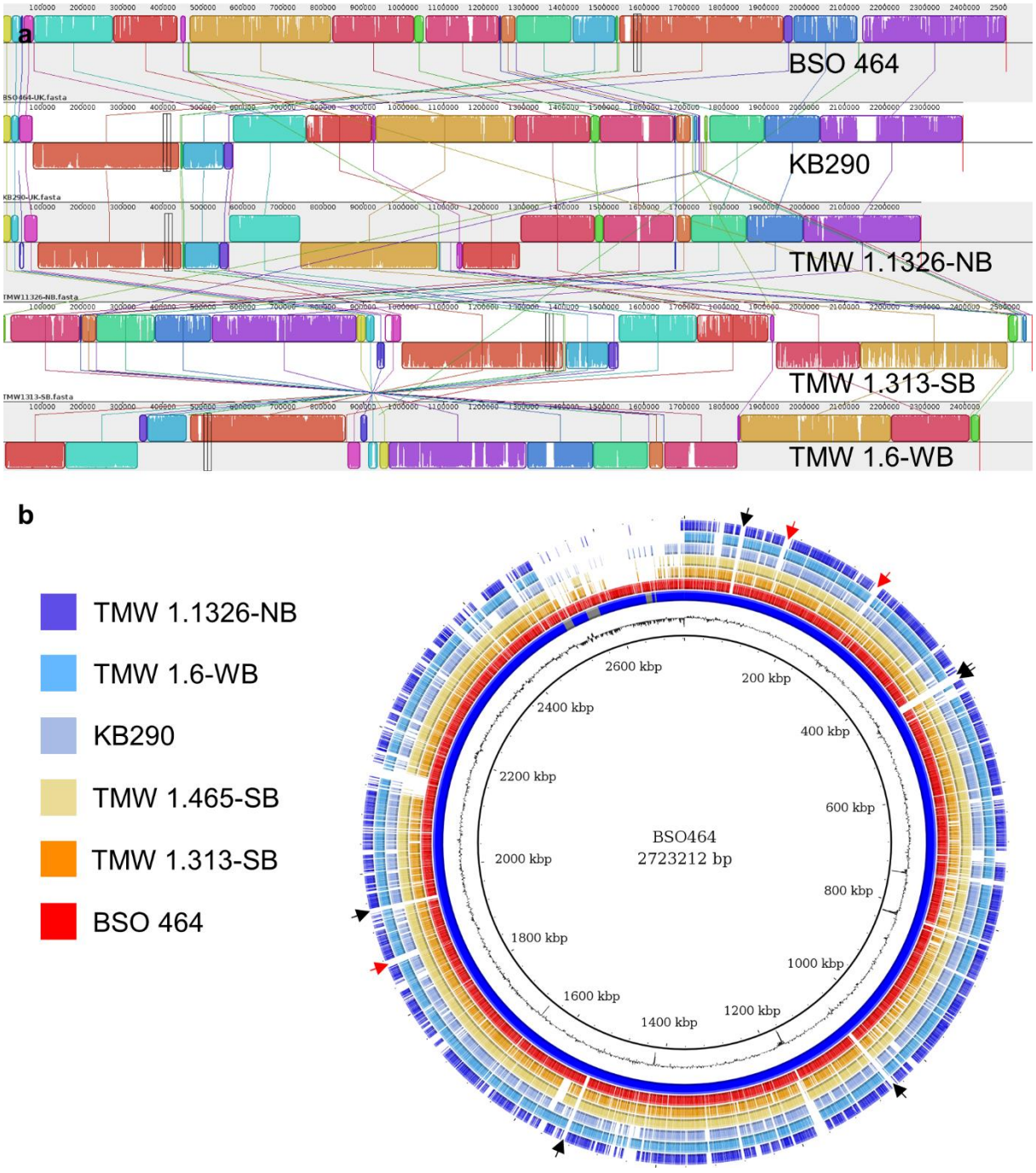


Figure 82: Illustration of general genomic properties of *L. brevis*. **(a)** Chromosome alignment. Note that TMW 1.313-SB and TMW 1.6-WB are draft genomes. Consequently, the largest scaffold was used. TMW 1.465-SB is missing (lacks a continuous chromosomal scaffold) **(b)** BLAST ring image. All rings are described from the inside to the outside: ring 1 (black) represents the total genome sequence of BSO 464 (considered as beer-spoiling) as reference with bp coordinates; ring 2 (black) shows the GC content; ring 3 consists of arcs with different lengths and alternating coloration (grey/blue), representing the different contigs of BSO 464; ring 4 shows all BLAST hits of BSO 464 ORFs versus its own genome, illustrating the coding density; rings 5 to 6 show the BLAST hits of TMW 1.313-SB and TMW 1.465-SB ORFs versus the reference; rings 6 to 9 shows the BLAST hits of KB290, TMW 1.6-WB and TMW 1.1326-NB versus the reference; **black** arrows indicate the localization of rRNA operons (no ORF hits); **red** arrows give examples of chromosomal loci which are only found within brewery isolates.

10.6.2 Functional analysis - SEED and COG

41 +/- 2 % of the found ORFs could be assigned to SEED categories. Of the remaining unassigned ORFs, 419 to 588 were annotated as hypothetical proteins. In case of the COG enrichment, 84 to 90 % could be assigned to a functional category, with about 22 % poorly characterized proteins.

As in case of the other species, most genes are associated with translation, transcription and other basic metabolism. Compared to the other lactobacilli genomes, *L. brevis* is characterized by the highest number of genes assigned to the SEED subcategory monosaccharide metabolism, which reflects the substrate diversity of this species, as detailed in the corresponding section about the metabolic capabilities. Further enriched SEED categories are fatty acids, stress response and iron acquisition (ranked first). The high count within the category “stress response” results mainly from a high number of genes related to the subcategory oxidative stress, where *L. brevis* is ranked first together with *L. plantarum*. The comparatively high number of genes found related to iron acquisition, results from several genes associated with heme uptake.

Chromosomal similarities between the beer-spoiling strains, including those which are specific for the brewery environment (see Figure 82, p. 291), are not reflected by a distinct functional pattern (e.g. a higher count of SEED/COG category compared to non-brewery isolates), with the exception of genes assigned to the COG category replication, recombination and repair. Note that this does not mean that there are no functional differences on subordinate levels or based on genes, which are not classified into the applied systems. For a detailed illustration, see Figure 83 (p. 294).

The functional analysis of *L. brevis* plasmids included only those, which are clearly identified as plasmids (found within the complete genomes, cf. Figure 84, p. 295). In case of a detailed description of a specific feature of interest, the particular plasmid like scaffolds and contigs were analysed downstream. The SEED subsystem coverage was found to be 26 +/- 5 %, the COG enrichment coverage 66 +/- 12 %. Based on functional analysis, the following categories and subordinate levels are noticeable with respect to isolation source and beer spoilage potential of the investigated genomes/strains:

Based on the total number of assigned genes, BSO 464 is characterized by a higher number of proteins related to the SEED categories mobile elements, which is due to the presence of several phage genes. Further, the SEED categories carbohydrates (enolase, xyloside transporter), cell wall and capsule, and protein metabolism are slightly enriched compared to

the non-brewery isolates. BSO 464 as well as the plasmid-like contigs of TMW 1.313-SB encode for genes related to the SEED subsystems peptidoglycan recycling and teichoic acid glycosylation (SEED category cell wall and capsule). Further, both were found to carry transport systems for manganese (*hitA*) and magnesium (SEED VDD), and genes assigned to the subsystem for vitamin B6 biosynthesis. In case of BSO 464, we also found the same S14p ribosomal protein (SEED protein metabolism) as in case of *P. damnosus*. In case of the SEED categories DNA metabolism and stress response, KB290, BSO 464 and TMW 1.313-SB were found to carry the same genes for a type I restriction modification system and some oxidative stress related functions.

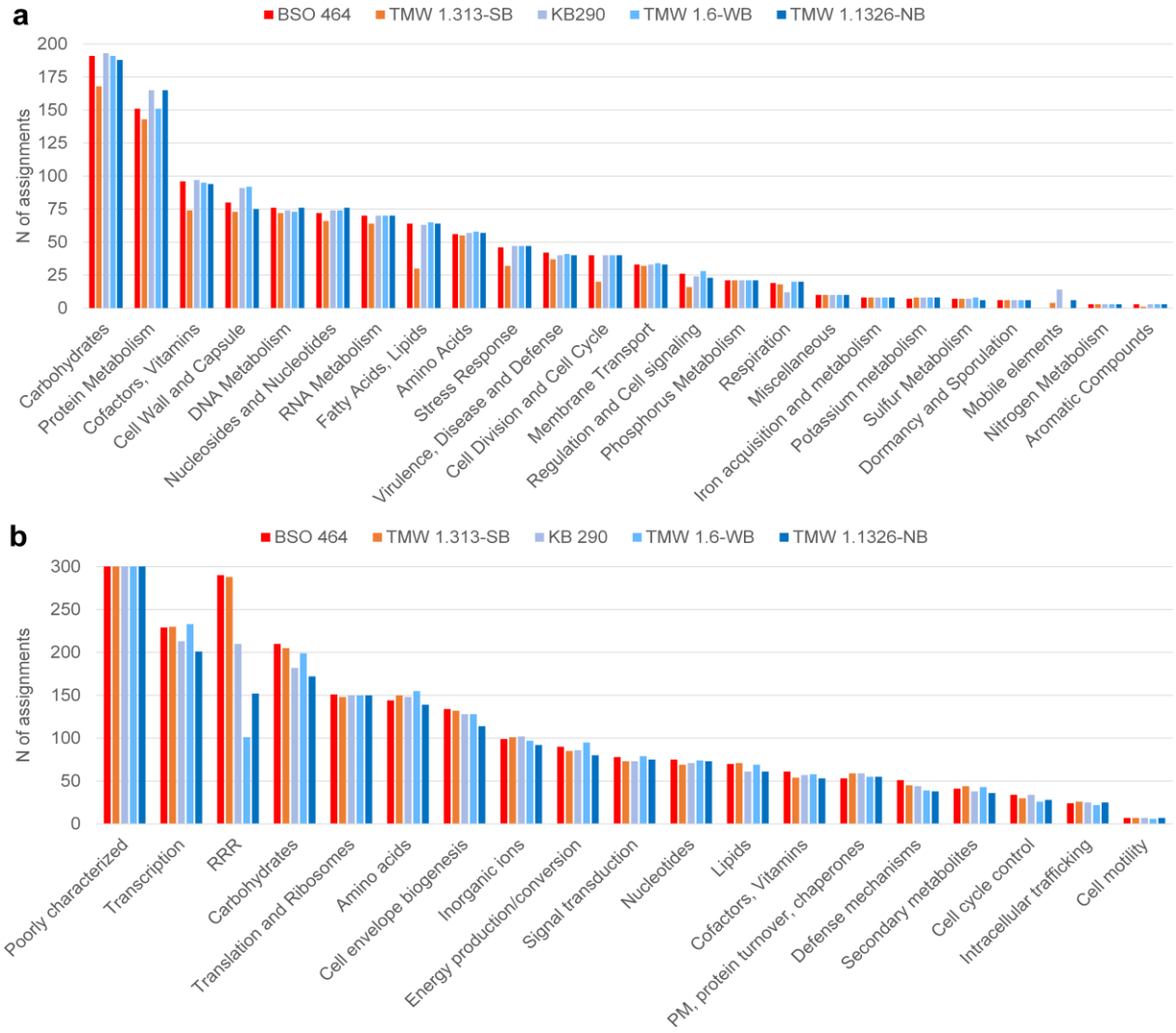


Figure 83: Functional analysis of *L. brevis* chromosomes. The total number of proteins assigned to the single categories is shown for SEED categories (a) and COG categories (b) in descending (average) order. Note that TMW 1.313-SB and TMW 1.6-WB are draft genomes. Consequently, the largest scaffold was used. TMW 1.465-SB is missing (lacks a continuous chromosomal scaffold). In case of the COG category “Poorly characterized,” the actual values range from 453 to 501. SEED subsystem coverage: 41.2 +/- 1.8; COG coverage: 91.4 +/- 2.8. RRR = Replication, recombination and repair, PM = posttranslational modification.

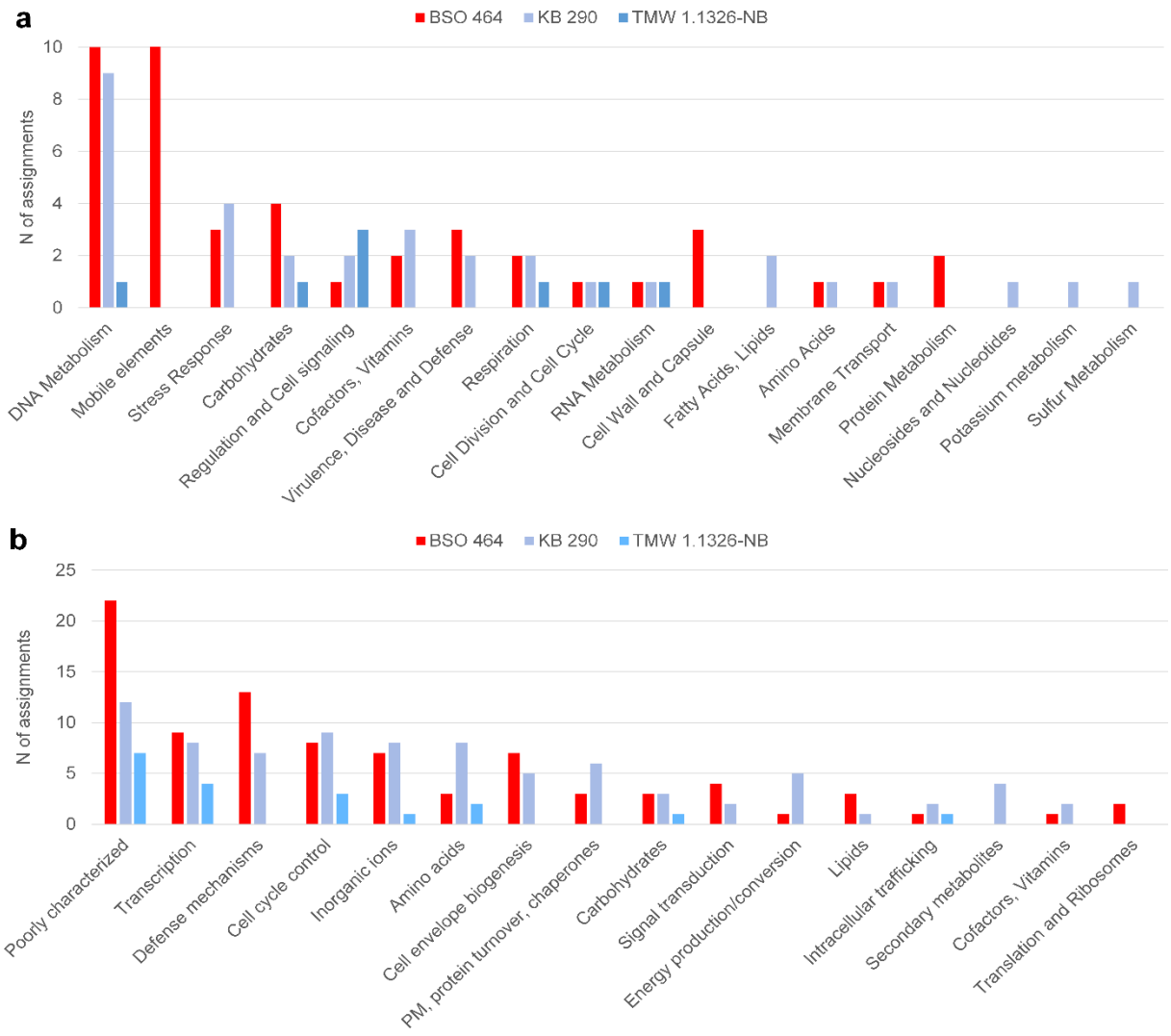


Figure 84: Functional analysis of *L. brevis* plasmidomes. The total number of proteins assigned to the single categories is shown for SEED categories (a) and COG categories (b) in descending (average) order. Data are only shown for complete genomes. SEED subsystem coverage: 29.4 +/- 3.6; COG coverage: 70.6 +/- 2.6. RRR = Replication, recombination and repair, PM = posttranslational modification.

10.6.3 Metabolic capabilities

10.6.3.1 Carbohydrates and central metabolism

Phosphoketolase (PKP) and pentose phosphate pathway are complete. Glycolysis is incomplete. The glucose transporter GlcU and a potential permease were found. As in case of *L. paracollinoides*, only one complete PTS system was found, related to either fructose or mannose uptake. Mannose-6-phosphate isomerase (EC 5.3.1.8) is encoded, potentially allowing the utilization of mannose-6-phosphate. Two permease-like transport systems for galactose were found, as well as all enzymes for the Leloir pathway for galactose utilization. A potential lactose permease, as well as a beta-galactosidase (EC 3.2.1.23) for the consequent hydrolysis to glucose and galactose are encoded. A potential MFS transport system was found for maltose, spatially located next to the enzyme for maltose utilization, a maltose phosphorylase (EC 2.4.1.8). The respective transport and utilization system for isomaltose was found for all strains except BSO 464. No maltodextrin glucosidase (EC 3.2.1.20) was found. Several genes have been annotated as enzymes for the utilization of trehalose, while none of those predicted functions could be confirmed by BLASTp analysis, which resulted in unspecified hydrolases and phosphorylases (kojibiose/maltose/trehalose). All genomes encode for proton symport transporter for xylose and arabinose, as well as for the necessary enzymes to channel them into the PKP. Xyloside transporter (XynT), alpha-xylosidase and beta-xylosidase (EC 3.2.1.37) are encoded in all cases. An ABC-like ribose transport system and ribokinase (EC 2.7.1.15) were found, allowing the utilization of ribose. In addition, all genomes encode for transport and utilization systems for glycerol, glycerate and gluconate. Further, we found enzymes for the utilization of fructuronate and glucoronate, as well as a glucuronide transporter. Only the three beer-spoiling strains encode for an oligogalacturonide transporter and a potential polygalacturonase (EC 3.2.1.15). Tagaturonate reductase (EC 1.1.1.58) and altronate hydrolase (EC 4.2.1.7), necessary for a further utilization of galacturonate via the pentose phosphate pathway were not found. A 6-phospho-beta-glucosidase (EC 3.2.1.86) was encoded in all genomes. Mannitol 2-dehydrogenase (EC 1.1.1.67), allowing the utilization of fructose as alternative electron acceptor, was found within all genomes.

10.6.3.2 Pyruvate metabolism, organic acids and TCA cycle

The pyruvate metabolism of *L. brevis* is, based on the enzymatic setting, identical to the one found for *L. paracollinoides*. Potential products include acetate, lactate, ethanol, acetoin, diacetyl and 2,3-butanediol. For a graphical illustration, see Figure 79 (p. 285). The anaplerotic enzyme phosphoenolpyruvate carboxykinase (EC 4.1.1.49) was found. All genes were found

which are necessary for the uptake and utilization of malate. Citrate utilization is strain specific and only encoded by BSO 464 and TMW 1.6-WB (chromosomally encoded). With exception of fumarate hydratase (EC 4.2.1.2), no enzymes of the TCA cycle were found.

10.6.3.3 Proteolytic system and amino acids

A cell wall-bound, extracellular protease was not found, while transport systems for oligo, di- and tripeptides are present. Endopeptidases, aminopeptidases and oligo-/tri-/dipeptidases are encoded. In addition, potential transport systems were found for alanine, arginine, cysteine, glutamine/glutamic acid, glycine, methionine, serine and branched amino acids, as well as unspecified amino acid transporter. The biosynthetic capabilities of *L. brevis* are limited, while in no case a direct connection of the corresponding reaction sequences to the central metabolism was found. *L. brevis* is auxotroph for at least histidine, tryptophan, valine, leucine, isoleucine, methionine, threonine, lysine, arginine and proline. All other amino acids can either be synthesized from direct precursor (tyrosine, phenylalanine), from each other (serine/glycine, asparagine/aspartate, glutamine/glutamate, alanine/cysteine) or from central carbon metabolites, such as 2-oxoglutarate and oxaloacetate. Neither of these central metabolites nor any other found reaction sequences are connected to the central metabolism (e.g. EMP, pentose phosphate pathway).

All investigated genomes encode for complete ADI and AGDI pathway. Further, we found tyrosine decarboxylase (EC 4.1.1.25) and glutamate decarboxylase (EC 4.1.1.15), together with their associated substrate/product antiporter systems, as well as asparaginase (EC 3.5.1.1) to be encoded in all cases.

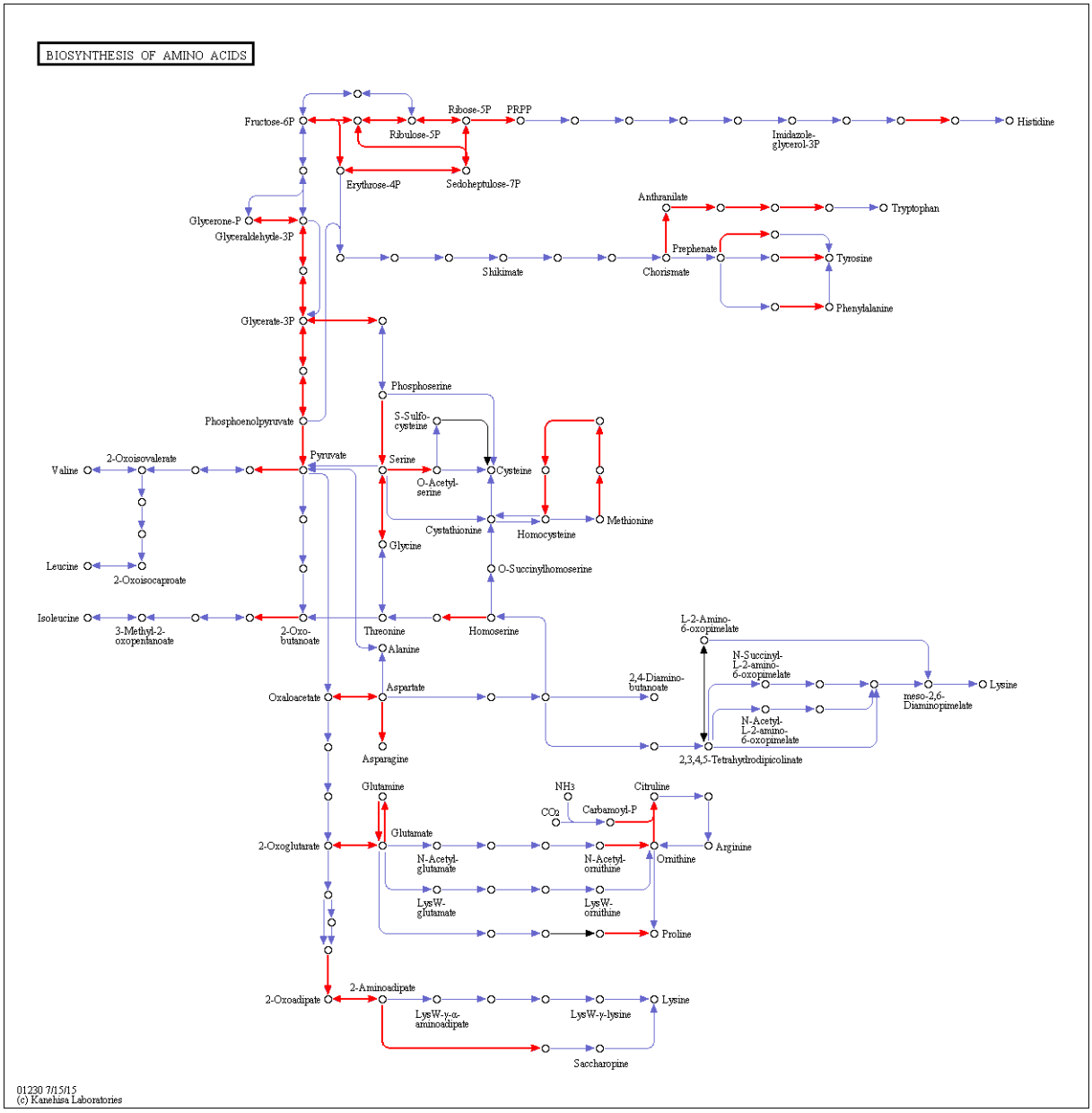


Figure 85: Predicted pathway of amino acid biosynthesis - *L. brevis*. Red arrows indicate a reaction, which is present, based on EC numbers. The figure was obtained from the KEGG PATHWAY mapping tool. This figure is based on BSO 464. Alanine can be synthesized from cysteine by cysteine desulfurase (EC 2.8.1.7), which is not indicated in the map. Glutamate and aspartate can be interconverted by aspartate transaminase (EC 2.6.1.1). Asparagine can be synthesized from aspartate, using the Asparagine synthetase (EC 6.3.5.4), which is also not part of this map.

10.6.3.4 Purines and Pyrimidines

IMP biosynthesis from PRPP or any other precursor is not encoded. From IMP both nucleobases and all kind of nucleotides and nucleosides can be made. Xanthine permease, guanine-hypoxanthine permease and xanthine phosphoribosyltransferase (EC 2.4.2.22) were found, allowing the padding of purine metabolism via xanthosine 5'-phosphate (XMP). Additional purine transport systems were found.

We did not find dihydroorotate dehydrogenase (EC 1.3.3.1), which is necessary for the biosynthesis of orotate from dihydroorotate. All enzymes for the biosynthesis of UMP from orotate and PRPP are encoded. All nucleotides, nucleosides and the nucleobase thymine can be produced from UMP. Uracil phosphoribosyltransferase (EC 2.4.2.9), necessary for the interconversion of UMP and uracil, is present. A potential uracil permease was found.

10.6.3.5 Fatty acid biosynthesis

A complete chromosomally encoded type II FAS was found, as well as the necessary peripheral enzymes for membrane integration. A cyclopropane-fatty-acyl-phospholipid synthase was found as well.

10.6.3.6 Vitamins and Co-Factors

Riboflavin biosynthesis from GTP and ribulose-5-phosphate as well as the consequent reactions to FMN and FAD are encoded, while GTP cannot be made from the PRPP, as described above. The biosynthetic enzymes for the direct thiamine precursors, the condensation of the pyrimidine and the thiazole component to thiamine-phosphate and the interconversion of thiamine to the active thiamine-phosphate are present. A direct connection to the central metabolism remains unclear, as well as a potential phosphorylation of thiamine-phosphate to thiamine-pyrophosphate, while the latter can be made from thiamine.

Folate, nicotinate/nicotinamide, vitamin B6 and vitamin B12 cannot be synthesized *de novo*. In case of folate, nicotinate/nicotinamide and vitamin B6 the respective enzymes for activation and interconversion of different derivatives were found. Potential ECF transporter for the uptake of thiamine, folate, riboflavin, pyridoxine and biotin are present.

10.6.3.7 Stress response and tolerance

Acid stress: The complete AGDI pathway as well as the ADI pathway was found in all genomes. NH_3 can also be produced by the asparaginase (EC 3.5.1.1). All genes for malolactic fermentation are encoded. Further, we found decarboxylases, as well as the corresponding antiport systems to exchange the respective products GABA and tyramine with the substrates glutamate and tyrosine. Citrate fermentation is encoded (plasmid) by TMW 1.6-WB and BSO 464.

Oxidative stress: Of 13 in detail investigated genes, associated with oxidative stress, eight were found to be present in *L. brevis:* thioredoxin and thioredoxin reductase (EC 1.8.1.9), NADH peroxidase (EC 1.11.1.1), catalase (EC 1.11.1.6), RecA, a manganese transport system (MntH) and ferroxidase (EC 1.11.1.1). A protein similar to glutathione reductase was also detected.

Hop stress: All three non-brewery isolates lack any known hop stress related genes. TMW 1.313-SB and BSO 464 encode the complete *horA*, *horC* and *hitA* clusters. TMW 1.465-SB was tested positive (PCR) for *horA*, *horC* and *hitA* (see 4.4, p. 115).

10.6.3.8 Others

Besides the three draft genomes, two to nine plasmids were found. The number of mobile element proteins ranges from two (TMW 1.6-WB, isolated from faeces) to 146 (TMW 1.313-SB, brewery isolate) and correlates to the isolation source. The same is true for transposases. For comparison, BSO464 (brewery isolate) and KB290 (probiotic strain) carry both plasmids with a total size of about 0.2 Mbp, while BSO464 was found to have 38 transposase related genes and KB290 only four. In addition, we found two to 62 phage associated genes, correlating with the number of plasmids. Further, the investigated genomes encode for 13 to 16 competence related genes, 16 genes for peptidoglycan and 14 for teichoic and LTA biosynthesis.

11 Supplementary Section 3 - Evaluation and validation of diagnostic marker genes

45 DMGs were tested with up to 118 strains, comprising 13 species employing PCR (3.4.2, p. 39) and gel electrophoresis (3.4.3, p. 40). Results are only shown for relevant DMGs (those with promising results) and the six core-species (see Table 33 to Table 38, p. 303). Table 39 (p. 309) describes all DMGs with relevance, together with supplementary information (e.g. annotation). Raw data were partially generated within a student's project under my supervision. All tables base on the same scheme. In order to remain clear (space problems), the basic table description is provided here in advance:

DMG parameters (rows/sections) and table-design are described top down:

Brewery environment specific: + = no megablast hits (high identity) in nr/nt BLAST database with exception of brewery isolates

Cluster: DMG was found within a cluster, [1] = also found outside of clusters

Function category: manually assigned functional category, for used abbreviations see Table 39 (p. 309).

DMG: ID/name of DMG

The **center** of the table indicates positive PCR reactions with **+** and negative reactions with **-**, the strains tested, their isolation source and their beer spoilage ability and potential (BSP): SB = strong BSP, MB = middle BSP, WB = weak BSP, NB = no BSP.

BSA (beer spoilage ability): The relation of DMGs to beer spoilage ability was tested with Fisher´s exact test for significance (indicated in red, p-value < 0.05). True positive (TP), false positive (FP), true negative (TN) and false negative (FN) results were counted and used for the calculation of accuracy, precision, sensitivity, specifity and F-measure, which is the harmonic mean of precision and sensitivity (confusion matrix). Accuracy = TP+TN/(TP+FP+TN+FN) → total correct identifications, measure of correctness; precision = TP/(TP+FP) → a measure for the probability that the identification is correct; Sensitivity = TP/(TP+FN) → a measure for the proportion of positives that are correctly identified as such; Specifity = TN/(TN+FP) → a measure for the proportion of negatives that

are correctly identified as such; F-measure = 2TP/(2TP+FP+FN); False positive rate = FP/(FP+TN); False negative rate = FN/(FN+TP); F-measure = 2TP/(2TP+FP+FN).

BSP (beer spoilage potential): spearman´s rank correlation (ρ) to BSP, x = no significant (p-value = 0.05) correlation.

BRE (brewery): Accuracy = TP+TN/(TP+FP+TN+FN) → total correct identifications of brewery isolates

Table 33: Diagnostic marker gene evaluation and validation for *P. damnosus*. For a detailed description about table features, calculated parameters and the meaning of all rows, see p. 301. Results for M05, M42, M03, M02, M01, *hitA*, *horA* and *horC* were previously published (Behr et al., 2016).

Brewery env. specific			-	+	+	+	+	-	+	+	-	-	-	+	-	-	-
Cluster			horA	horC	hitA		hitA	CI-6[1]	FAS	CI-4			CI-14[1]	CI-5	FAS[1]	CI-10[1]	horC[1]
Function category			HET	HET	CTP	MGE	TR	MGE	FAS	SMU	DNA	MGE	MGE	HYP	MGE	SR	GLT
DMG			***horA***	***horC***	***hitA***	**M01**	**M02**	**M03**	**M05**	**M09**	**M18**	**M19**	**M22**	**M28**	**M34**	**M42**	**M43**
brewery isolates	beer spoilage strains	TMW 2.1533-SB	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
		TMW 2.1535-SB	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
		TMW 2.1641-SB	+	+	-	+	+	+	+	-	+	+	-	-	+	+	+
		TMW 2.1636-SB	+	+	-	-	-	+	+	-	+	+	-	-	+	+	+
		TMW 2.1637-SB	+	+	-	-	-	+	+	-	+	+	-	-	+	+	+
		TMW 2.1548-MB	+	+	-	-	-	+	+	-	+	+	-	-	+	+	+
		TMW 2.4-MB	+	-	+	+	+	+	+	-	-	-	+	-	+	+	-
	non spoilage strains	TMW 2.125-NB	+	-	+	+	+	+	-	-	-	-	+	+	+	-	-
		TMW 2.1640-NB	+	+	+	-	-	+	-	+	-	+	+	+	+	+	+
		TMW 2.1639-NB	-	+	-	-	-	-	-	+	-	+	-	+	+	+	+
		TMW 2.1532-NB	+	+	-	-	-	-	-	-	-	+	-	-	+	-	+
		TMW 2.1534-NB	+	+	-	-	-	-	-	-	-	+	-	-	-	-	+
		TMW 2.1547-NB	+	+	-	-	-	-	-	-	-	+	-	-	+	-	+
		TMW 2.1635-NB	+	+	-	-	-	-	-	-	-	+	-	-	-	-	+
		TMW 2.1638-NB	+	+	-	-	-	-	-	-	-	+	-	-	+	-	+
		TMW 2.1642-NB	+	+	-	-	-	-	-	-	-	+	-	-	+	-	+
		TMW 2.1546-NB	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
winery i.		TMW 2.1536-NB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		TMW 2.1549-NB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		TMW 2.1643-NB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BSA		accuracy	0.60	0.55	0.70	0.80	0.80	0.90	1.00	0.65	0.85	0.50	0.70	0.60	0.60	0.90	0.55
		precision	0.47	0.43	0.60	0.80	0.80	0.78	1.00	0.50	1.00	0.40	0.60	0.40	0.47	0.78	0.43
		sensitivity	1.00	0.86	0.43	0.57	0.57	1.00	1.00	0.29	0.57	0.86	0.43	0.29	1.00	1.00	0.86
		specifity	0.38	0.38	0.85	0.92	0.92	0.85	1.00	0.85	1.00	0.31	0.85	0.77	0.38	0.85	0.38
		f-measure	0.64	0.57	0.50	0.67	0.67	0.88	1.00	0.36	0.73	0.55	0.50	0.33	0.64	0.88	0.57
		false positive rate	0.62	0.62	0.15	0.08	0.08	0.15	0.00	0.15	0.00	0.69	0.15	0.23	0.62	0.15	0.62
		false negative rate	0.00	0.14	0.57	0.43	0.43	0.00	0.00	0.71	0.43	0.14	0.57	0.71	0.00	0.00	0.14
		Fisher´s p-value	0.11	0.35	0.29	0.03	0.03	0.00	0.00	0.59	0.01	0.61	0.29	1.00	0.11	0.00	0.35
BSP		correlation (ρ)	x	x	x	0.54	0.54	0.79	0.98	x	0.68	x	x	x	x	0.79	x
BRE		accuracy	0.88	0.82	0.29	0.29	0.29	0.53	0.41	0.76	0.24	0.88	0.71	0.71	0.88	0.53	0.82

Table 34: Diagnostic marker gene evaluation and validation for *L. brevis*. For a detailed description about table features, calculated parameters and the meaning of all rows, see p. 301. Partial results for *arsR* and *cinA* were previously published (Behr et al., 2015).

Brewery env. specific			-	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-
Cluster			horA	horC	hitA			hitA	CI-6[1]			CI-14[1]	CI-14[1]	CI-5	FAS[1]		CI-10[1]	horC[1]
Function category			HET	HET	CTP	TR	CIN	TR	MGE	HYP	MGE	MGE	SMU	HYP	MGE	DNA	SR	GLT
DMG			***horA***	***horC***	***hitA***	***arsR***	***cinA***	**M02**	**M03**	**M15**	**M19**	**M22**	**M23**	**M28**	**M34**	**M37**	**M42**	**M43**
brewery isolates	beer spoilage strains	TMW 1.313-SB	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
		TMW 1.476-SB	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+
		TMW 1.465-SB	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	+
		TMW 1.315-SB	-	+	+	+	+	-	+	-	-	+	-	-	-	+	+	+
		TMW 1.317-SB	-	+	+	+	+	+	-	-	+	+	-	-	-	+	-	+
		TMW 1.485-SB	+	+	-	+	+	-	+	+	+	+	+	-	+	+	-	+
		TMW 1.230-SB	+	+	-	+	+	-	+	+	+	+	-	-	+	+	-	-
		TMW 1.474-SB	+	+	-	-	-	-	+	+	+	+	-	+	-	+	-	+
		TMW 1.473-SB	+	-	+	-	-	+	+	+	+	+	-	-	+	+	+	-
		TMW 1.240-SB	+	+	+	-	-	+	+	-	-	-	-	+	-	-	-	+
		TMW 1.507-SB	+	-	+	-	-	+	-	-	+	-	-	-	+	+	-	-
		TMW 1.1282-SB	-	+	-	-	-	-	+	-	+	-	-	-	+	-	-	+
oi.		TMW 1.1370-SB	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
bi.		TMW 1.302-WB	+	-	-	-	-	-	+	-	+	+	+	+	-	+	+	-
other isolates	non spoilage strains	TMW 1.2048-WB	+	-	-	-	-	-	+	-	-	+	-	-	+	-	+	+
		TMW 1.1369-WB	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
		TMW 1.6-WB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		TMW 1.100-WB	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
		TMW 1.1205-WB	-	-	NA	-	-	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		TMW 1.1326-NB	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
BSA		accuracy	0.70	0.85	0.74	0.68	0.70	0.68	0.74	0.68	0.68	0.63	0.37	0.53	0.53	0.74	0.37	0.74
		precision	0.82	1.00	1.00	1.00	1.00	1.00	0.83	1.00	0.73	0.75	0.67	0.83	0.83	0.90	0.60	0.90
		sensitivity	0.69	0.77	0.62	0.54	0.54	0.54	0.77	0.54	0.85	0.69	0.15	0.38	0.38	0.69	0.23	0.69
		specifity	0.71	1.00	1.00	1.00	1.00	1.00	0.67	1.00	0.33	0.50	0.83	0.83	0.83	0.83	0.67	0.83
		f-measure	0.75	0.87	0.76	0.70	0.70	0.70	0.80	0.70	0.79	0.72	0.25	0.53	0.53	0.78	0.33	0.78
		false positive rate	0.29	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.67	0.50	0.17	0.17	0.17	0.17	0.33	0.17
		false negative rate	0.31	0.23	0.38	0.46	0.46	0.46	0.23	0.46	0.15	0.31	0.85	0.62	0.62	0.31	0.77	0.31
		Fisher´s p-value	0.16	0.00	0.02	0.04	0.04	0.04	0.13	0.04	0.56	0.62	1.00	0.60	0.60	0.06	1.00	0.06
BSP		correlation (ρ)	0.36	0.70	0.57	0.51	0.51	0.51	0.45	0.51	x	x	x	x	x	0.50	x	0.50
BRE		accuracy	0.77	0.77	0.62	0.54	0.54	0.54	0.85	0.54	0.85	0.77	0.23	0.46	0.38	0.77	0.31	0.69

Table 35: Diagnostic marker gene evaluation and validation for *L. backii*. For a detailed description about table features, calculated parameters and the meaning of all rows, see p. 301.

Brewery env. specific			-	+	+	-	+	+	-	-	+	-	-	-
Cluster			horA	horC	hitA	CI-6[1]	FAS	CI-4		CI-14[1]	CI-5	FAS[1]	CI-10[1]	horC[1]
Function category			HET	HET	CTP	MGE	FAS	SMU	MGE	MGE	HYP	MGE	SR	GLT
DMG			***horA***	***horC***	***hitA***	**M03**	**M05**	**M09**	**M19**	**M22**	**M28**	**M34**	**M42**	**M43**
brewery isolates	beer spoilage strains	TMW 1.1299-SB	-	+	-	+	+	-	+	-	-	+	+	+
		TMW 1.1430-SB	+	+	-	+	+	+	+	-	+	+	+	+
		TMW 1.1988-SB	+	+	-	+	+	+	+	-	+	+	-	+
		TMW 1.1990-SB	+	+	-	+	+	-	+	-	+	+	+	+
		TMW 1.1991-SB	+	+	-	+	+	-	+	-	+	+	+	+
		TMW 1.1992-SB	-	+	-	+	+	-	+	-	+	+	+	+
		TMW 1.2003-SB	-	+	-	+	+	-	+	-	+	+	+	+
		TMW 1.2072-SB	+	+	-	+	+	+	+	-	+	+	-	+
		TMW 1.2073-SB	+	+	-	+	+	-	+	-	+	+	+	+
		TMW 1.2078-SB	+	+	-	+	+	-	+	-	-	+	+	+
		TMW 1.1432-SB	-	+	-	+	+	-	-	-	-	+	-	+
		TMW 1.2070-SB	+	+	+	+	+	-	-	-	+	+	+	+
		TMW 1.1883-SB	+	+	+	+	+	+	+	-	+	+	+	+
		TMW 1.1989-SB	-	+	-	+	+	-	+	-	+	+	+	+
		TMW 1.2002-MB	+/-	+	+/-	+	+	-	+	-	+	+	+	+
		TMW 1.2005-MB	+	+	-	+	+	-	+	-	-	+	+	+
		TMW 1.2071-MB	+	+	-	+	+	-	+	+	+	+	-	+
		TMW 1.2077-MB	+	+	-	+	+	-	+	-	-	+	+	+
	NB	TMW 1.2079-WB	+	+	-	+	+	-	+	-	-	+	+	+
		TMW 1.2004-NB	+	+	+	+	-	+	+	+	+	+	+	+
BSA		accuracy	0.65	0.90	0.20	0.90	0.95	0.25	0.80	0.10	0.70	0.90	0.70	0.90
		precision	0.87	0.90	0.75	0.90	0.95	0.80	0.89	0.50	0.93	0.90	0.88	0.90
		sensitivity	0.72	1.00	0.17	1.00	1.00	0.22	0.89	0.06	0.72	1.00	0.78	1.00
		specifity	0.00	0.00	0.50	0.00	0.50	0.50	0.00	0.50	0.50	0.00	0.00	0.00
		f-measure	0.79	0.95	0.27	0.95	0.97	0.35	0.89	0.10	0.81	0.95	0.82	0.95
		false positive rate	1.00	1.00	0.50	1.00	0.50	0.50	1.00	0.50	0.50	1.00	1.00	1.00
		false negative rate	0.28	0.00	0.83	0.00	0.00	0.78	0.11	0.94	0.28	0.00	0.22	0.00
		Fisher´s p-value	1.00	1.00	0.37	1.00	0.10	0.45	1.00	0.19	0.52	1.00	1.00	1.00
BSP		correlation (ρ)	x	x	x	x	0.73	x	x	-0.64	x	x	x	x
BRE		accuracy	0.75	1.00	0.20	1.00	0.95	0.25	0.90	0.10	0.70	1.00	0.80	1.00

Table 36: Diagnostic marker gene evaluation and validation for *P. claussenii*. For a detailed description about table features, calculated parameters and the meaning of all rows, see p. 301.

Brewery env. specific			-	+	-	-	+	-	-
Cluster			horA	horC		CI-14[1]	CI-5	CI-10[1]	horC[1]
Function category			HET	HET	MGE	MGE	HYP	SR	GLT
DMG			***horA***	***horC***	**M19**	**M22**	**M28**	**M42**	**M43**
brewery isolates	**beer spoilage strains**	TMW 2.51-SB	+	+	+	-	+	-	+
		TMW 2.54-SB	+	+	+/-	-	+	+	+
		TMW 2.56-SB	+	+	+	-	+	+	+
		TMW 2.59-SB	+	+	+	-	+	+	+
		TMW 2.62-SB	+	+	+	-	+	-	+
		TMW 2.64-SB	-	+	+	-	+	+	+
		TMW 2.53-SB	+/-	+/-	+/-	-	+/-	+	+
		TMW 2.67-SB	-	+	+	-	+	-	+
		TMW 2.50-SB	-	+/-	+	-	-	+	+
		TMW 2.61-MB	-	+	+	-	+	+	+
		TMW 2.65-MB	+	+	+	-	+	-	+
		TMW 2.1545-MB	+	-	+	-	-	+	+
		TMW 2.60-MB	-	-	+	-	-	+	+
		TMW 2.340-MB	-	-	+	+	-	-	-
	NB	TMW 2.1531-NB	-	-	+	-	-	+	+
BSA		accuracy	0.60	0.80	0.93	0.13	0.73	0.60	0.87
		precision	1.00	1.00	0.93	1.00	1.00	1.00	0.93
		sensitivity	0.57	0.79	1.00	0.07	0.71	0.57	0.93
		specifity	1.00	1.00	0.00	1.00	1.00	1.00	0.00
		f-measure	0.73	0.88	0.97	0.13	0.83	0.73	0.93
		false positive rate	0.00	0.00	1.00	0.00	0.00	0.00	1.00
		false negative rate	0.43	0.21	0.00	0.93	0.29	0.43	0.07
		Fisher´s p-value	0.47	0.27	1.00	1.00	0.33	0.47	1.00
BSP		correlation (ρ)	x	0.72	x	x	0.59	x	x
BRE		accuracy	0.47	0.73	1.00	0.07	0.67	0.67	0.93

Table 37: Diagnostic marker gene evaluation and validation for *L. lindneri*. For a detailed description about table features, calculated parameters and the meaning of all rows, see p. 301.

Brewery env. specific			-	+	-	-	-	-	-
Cluster			horA	horC	CI-6[1]		CI-14[1]	CI-14[1]	FAS[1]
Function category			HET	HET	MGE	MGE	MGE	SMU	MGE
DMG			***horA***	***horC***	**M03**	**M19**	**M22**	**M23**	**M34**
brewery isolates	**beer spoilage strains**	TMW 1.2007-SB	+	+	+	-	+	+	+
		TMW 1.2082-SB	+	+	+	+	+	+	+
		TMW 1.2008-SB	+	-	-	+	+	+	+
		TMW 1.2080-SB	+	-	-	+	+	+	+
		TMW 1.2081-SB	+	-	-	+	+	+	+
		TMW 1.456-SB	+	-	+	-	+	+	+
		TMW 1.2009-SB	-	-	-	-	+	+	+
		TMW 1.1285-MB	+	+	-	+	+	+	+
		TMW 1.1286-MB	+	+	-	+	+	+	+
		TMW 1.1993-MB	+/-	+/-	-	-	+	+	+
		TMW 1.1433-MB	+	-	+	+	+	+	+
		TMW 1.481-MB	+	-	+	+	+	+	+
	NB	TMW 1.2006-NB	+	-	-	+	+	+	+
		TMW 1.88-NB	-	-	-	+	+	+	+
BSA		accuracy	0.86	0.50	0.50	0.57	0.86	0.86	0.86
		precision	0.92	1.00	1.00	0.80	0.86	0.86	0.86
		sensitivity	0.92	0.42	0.42	0.67	1.00	1.00	1.00
		specifity	0.50	1.00	1.00	0.00	0.00	0.00	0.00
		f-measure	0.92	0.59	0.59	0.73	0.92	0.92	0.92
		false positive rate	0.50	0.00	0.00	1.00	1.00	1.00	1.00
		false negative rate	0.08	0.58	0.58	0.33	0.00	0.00	0.00
		Fisher´s p-value	0.27	0.51	0.51	1.00	1.00	1.00	1.00
BSP		correlation (ρ)	x	x	x	x	x	x	x
BRE		accuracy	0.86	0.36	0.36	0.71	1.00	1.00	1.00

Table 38: Diagnostic marker gene evaluation and validation for *L. paracollinoides*. For a detailed description about table features, calculated parameters and the meaning of all rows, see p. 301.

Brewery env. specific			-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-	+	-	-
Cluster			horA	horC	hitA		hitA	CI-6[1]			CI-14[1]	CI-14[1]			CI-5	FAS[1]				CI-10[1]	horC[1]
Function category			HET	HET	CTP	MGE	TR	MGE	MGE	HYP	MGE	SMU	PTP	MDR	HYP	MGE	DNA	DNA	HYD	SR	GLT
DMG			***horA***	***horC***	***hitA***	**M01**	**M02**	**M03**	**M19**	**M21**	**M22**	**M23**	**M24**	**M25**	**M28**	**M34**	**M37**	**M38**	**M40**	**M42**	**M43**
brewery i.	**B**	TMW 1.1995-SB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		TMW 1.2010-SB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		TMW 1.1994-SB	+	+	-	-	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-
	NB	TMW 1.696-WB	-	+/-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-
		TMW 1.1979-NB	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-
BSA		accuracy	1.00	0.80	0.80	0.80	0.80	0.80	0.80	1.00	1.00	0.80	0.80	0.80	0.80	0.60	1.00	1.00	0.80	0.80	0.80
		precision	1.00	0.75	1.00	1.00	1.00	1.00	0.75	1.00	1.00	0.75	0.75	0.75	1.00	0.60	1.00	1.00	1.00	1.00	1.00
		sensitivity	1.00	1.00	0.67	0.67	0.67	0.67	1.00	1.00	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	0.67	0.67	0.67
		specifity	1.00	0.50	1.00	1.00	1.00	1.00	0.50	1.00	1.00	0.50	0.50	0.50	1.00	0.00	1.00	1.00	1.00	1.00	1.00
		f-measure	1.00	0.86	0.80	0.80	0.80	0.80	0.86	1.00	1.00	0.86	0.86	0.86	0.80	0.75	1.00	1.00	0.80	0.80	0.80
		false positive rate	0.00	0.50	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.50	0.50	0.50	0.00	1.00	0.00	0.00	0.00	0.00	0.00
		false negative rate	0.00	0.00	0.33	0.33	0.33	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.33	0.33	0.33
		Fisher´s p-value	0.10	0.40	0.40	0.40	0.40	0.40	0.40	0.10	0.10	0.40	0.40	0.40	0.40	1.00	0.10	0.10	0.40	0.40	0.40
BSP		correlation (ρ)	0.97	x	x	x	x	x	x	0.97	0.97	x	x	x	x	x	0.97	0.97	x	x	x
BRE		accuracy	0.60	0.80	0.40	0.40	0.40	0.40	0.80	0.60	0.60	0.80	0.80	0.80	0.40	1.00	0.60	0.60	0.40	0.40	0.40

Table 39: Relevant diagnostic marker genes (DMGs). IDs and locus-tags of the respective genes within the genome of a representative.

DMG-ID	Representative/locus-tag	Annotation (RAST/BLASTp)	Functional category
M01	TMW 2.1533/ADU70_0027	plasmid partitioning protein	Mobile genetic element (MGE)
M02	TMW 2.1533/ADU70_0142	transcription regulator (TetR family)	Transcription regulation (TR)
M03	TMW 2.1533/ADU70_0196	transposases (IS30 family)	Mobile genetic element (MGE)
M05 (*fabZ*)	TMW 2.1533/ADU70_0261	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	Fatty acid biosynthesis (FAS)
M09	TMW 2.1533/ADU70_0186	aldose 1-epimerase	Sugar and maltose utilization (SMU)
M15	TMW 2.54/AYR58_09720	hypothetical protein	Hypothetical protein (HYP)
M18	TMW 2.340/PECL_1974	Non-specific DNA-binding protein Dps/Iron-binding ferritin-like antioxidant protein/Ferroxidase (EC 1.16.3.1) // DNA starvation/stationary phase protection protein	DNA modification (DNA)
M19	TMW 1.1995/AYR63_16445	YefM protein (antitoxin to YoeB)/prevent host death	Mobile genetic element (MGE)
M21	TMW 1.1995/AYR63_16435	hypothetical protein	Hypothetical protein (HYP)
M22	TMW 2.1533/ADU70_0197	transposases (IS30 family)	Mobile genetic element (MGE)
M23	TMW 1.1995/AYR63_15595	Aldose 1-epimerase (EC 5.1.3.3) Ontology_term KEGG_ENZYME:5.1.3.3	Sugar and maltose utilization (SMU)
M24	TMW 1.1995/AYR63_12365	Na(+)/H(+) antiporter	Proton transport protein (PTP)
M25	TMW 1.1995/AYR63_12450	drug resistance transporter2C EmrB/QacA family	Multidrug resistance (MDR)
M28	TMW 2.54/AYR58_09470	hypothetical protein	Hypothetical protein (HYP)
M34	TMW 1.1995/AYR63_00270	transposase (IS4 family)	Mobile genetic element (MGE)
M37	TMW 2.54/AYR58_09710	Type I restriction-modification system2C DNA-methyltransferase subunit M (EC 2.1.1.72)	DNA modification (DNA)
M38	TMW 1.1995/AYR63_16165	Type I restriction-modification system2C DNA-methyltransferase subunit M (EC 2.1.1.72)	DNA modification (DNA)
M40	TMW 1.1995/AYR63_15660	Alpha/beta superfamily hydrolase	Hydrolase (HYD)
M42	TMW 2.1533/ADU70_0144	NADH peroxidase/NAD(FAD)-dependent dehydrogenase	Stress response (SR)
ORF4 (M43)	TMW 2.54/AYR58_09530	glycosyltransferase/cell wall modification	Glycosyltransferase (GLT)
hitA	published control	Manganese transporter	Cation transport protein (CTP)
horA	published control	Multidrug ABC transporter	Hop efflux transporter (HET)
horC	published control	pmf dependent multidrug transporter	Hop efflux transporter (HET)
cinA	published control	Competence/damage inducible protein	Competence inducible protein (CIN)
arsR	published control	transcription regulator (ArsR family)	Transcriptional regulation (TR)

12 Supplementary Section 4 - Metabolism in beer

The whole chapter partially corresponds (also tables/figures) to a recent publication about the metabolism of LAB in beer (Geissler et al., 2016).

12.1 Experimental design and data evaluation

Samples were taken from the beer spoilage test (3.2.6.5, p. 35), performed within the detailed characterization (4.2.3, p. 54). In total, 39 substances were quantified using three different HPLC systems (3.5, p. 42). Consumption and production (as a concentration) were calculated by subtracting the concentrations (per analyte) of the particular sample (beer inoculated with strain) from the control (non-inoculated beer). To get % consumption and production, these values were divided by the control concentration and multiplied with 100. Metabolic data, obtained from $lager_{pH5.0}$, were normalized for discriminant analysis of principal components (DAPC, 3.6.1, p. 44), in order to look at the metabolic pattern regardless of high concentration differences among the investigated analytes, as follows: More than 25 % consumption of analyte ≙ value -1, consumption in a range from 15 to 25 % ≙ -0.5, more than 25 % production of analyte ≙ 1, a production in a range from 15 to 25 % ≙ -0.5. If deviation from control was less than 15 % in both directions, the corresponding value for DAPC analysis was set to 0. In case of acetate (because of low initial concentration and low sensitivity of the HPLC system for this compound) thresholds were 50 and 100 %, respectively. The optimal number of clusters was selected using *find.clusters*. *DAPC* was carried out based on groups defined by *find.clusters*. Other groupings, like species, beer spoilage potential or fermentation type were only indicated by alternative labeling and not by new calculation.

Using spearman´s rank correlation, the relation of metabolic parameters to beer spoilage potential was tested. The following metabolites were included into correlation analysis: for lactate, acetate, hexoses, pentoses, disaccharides, sugar alcohols, amines and ornithine the difference to the control (non-inoculated $lager_{pH5.0}$), the total acid production was set equal to the lactate and acetate production, the total carbohydrate conversion was set equal to all carbohydrates consumed or produced, the amino acid production was set to the conversion of glutamic acid, histidine, tyrosine and arginine.

Five different batches of lager beer 1 were investigated in order to define the stable sugar profile. Maltose content of untreated lager beer underlay strong variations in a range from 68 to 2215 µM (100.8% fluctuation). The behavior towards maltose, growing in beer, was checked in $lager_{pH5.0}$ for all strains once. All growing heterofermentative LAB strains consumed maltose.

In case of *P. claussenii*, maltose content was either not affected, or released in case of TMW 2.54-SB. Both SB strains of *P. damnosus* consumed maltose, while the growth of all three NB strains resulted in increased maltose contents. Two strains of *L. backii* used maltose; in the other three cases (strains) no changes were observed. However, maltose was not considered as a stable part of the sugar profile of lager beer 1 because of the high fluctuation.Table 28 (p. 135) and Table 30 (p. 138) show the % production and consumption of 29 analytes in $lager_{pH5.0}$ and $lager_{pH4.3}$. For a distinct illustration, only conversions higher than 15% are indicated. Ten additional investigated analytes are not shown because of no or only little conversion (relevance). The proportion (%) of hexoses, pentoses, sugar alcohols and disaccharides, relative to the total amount of carbohydrates consumed, is displayed (Figure 41, p. 137) for a selection of strains (two strains with growth at both pH values for each species), thereby resembling the sugar profile of each strain in the respective beer. In addition, the percentage catabolism of the available (measured) carbohydrates and the theoretical contribution of this carbohydrate catabolism to the amount of acetic and lactic acid measured (total acids produced) are shown. The latter is calculated dividing the maximum theoretical, stoichiometric (e.g. one hexose results in two acids) amount of total acids produced by the measured amount of total acids. It is used as a rough measure for the significance of a compound and gives an idea of the remaining unknown catabolism (resulting in total acid). The maximum theoretical amount of total acid from carbohydrates was calculated under the assumption that all hexoses and pentoses are finally (and mainly) degraded to lactate and acetate as end products.

Citrate, malate, agmatine and putrescine were analyzed semiquantitatively. Therefore, we analyzed the available HPLC chromatograms, while we identified the corresponding peaks of the components of interest (citrate, malate → Rezex ROA-Organic Acid H+, see 3.5.4, p. 43; agmatine, putrescine → Gemini C18, see 3.5.3, p. 43). A quantification was not conducted because of the following reasons: Citrate and malate as well as agmatine were found to be distinct shoulder peaks, which hamper any attempt for a reliable quantification. Putrescine was found to result in three peaks, while a calibration did result in very poor quality values. Thus, these metabolites were analysed semiquantitatively, resulting in three different states: decreasing, constant, increasing. Figure 86 shows an example for how these three different states were assessed, in this case citrate. Table 29 (p. 136) summarizes the results of the semiquantitative analysis for lager beer at pH 4.3 and pH 5.0.

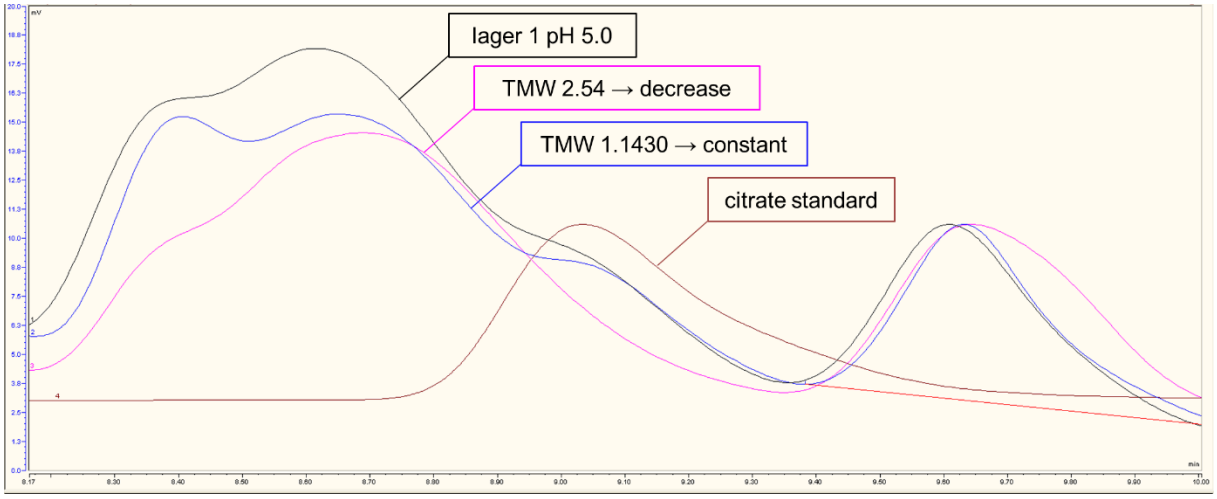


Figure 86: Semiquantitative citrate analysis - illustration of semiquantitative assessment. This figure shows how we assessed production and release in case of those analytes, for which an absolute quantification was not possible because of shoulder or split peaks. X-axis = retention time in min, y-axis = signal intensity in mv. *P. claussenii* TMW 2.54-SB shows a decreased signal intensity compared to the negative control ($lager_{pH5.0}$) at the retention time of citrate (see citrate standard). *L. backii* TMW 1.1430-SB does not show a decrease. Both results are in accordance with the *in silico* predicted ability to utilize citrate for these strains. A Dionex UltiMate 3000 HPLC with a Rezex ROA-Organic Acid H+ and a RI-101 detector was used. Separated at a constant flow rate of 0.7 ml/min with a column temperature of 85° C for 30 min. Sulfuric acid with a concentration of 5 mM served as mobile phase. For details, see 3.5.4 (p. 43).

In general, metabolic data are mostly illustrated by simplified figures and tables (see main part of this thesis, 4.7.1, p. 134), while complex illustrations are based on average values, lacking error bars, to enable a clear figure. The respective HPLC data with the corresponding standard or average deviations are available as “Supplementary Table 1” from the respective publication (Geissler et al., 2016).

12.2 Metabolism in lager$_{pH5.0}$

12.2.1 Homofermentative lactic acid bacteria

All homofermentative strains consumed glucose, fructose and no pentoses. Additionally, all pediococci strains used the disaccharides gentiobiose and trehalose, while *L. backii* strains utilized mannitol and sorbitol. Three of four strains of *P. claussenii* also metabolized mannitol. In case of *Pediococcus* strains, isomaltose was only consumed in noticeable amounts by strains with strong beer spoilage potential. In case of *L. backii*, only TMW 1.1991-SB was able to ferment lactose, being the strain with the highest cell density after 60 days of incubation. Acetate production by homofermentative LAB is indicated by lactate to acetate ratios from 3.7 to 9.3 for *P. claussenii* and from 0.8 to 3.1 for *L. backii. P. damnosus* produced lactate only. A part of the *Pediococcus* strains metabolized tryptophan and methionine. *L. backii* strains partially consumed methionine and tyrosine in case of TMW 1.1430-SB. The latter also produced tyramine. Agmatine consumption and putrescine production was observed for all *P. claussenii* strains and for *L. backii* TMW 1.1991-SB. Malate and pyruvate were used by all homofermentative species, while citrate was only utilized by *P. claussenii*.

12.2.2 Heterofermentative lactic acid bacteria

Glucose and fructose were consumed by *L. brevis* and *L. lindneri*, and released by three of four strains of *L. paracollinoides*. A production of mannitol could be observed for all *L. brevis* strains. In addition, *L. brevis* strains catabolized pentoses and disaccharides, in higher amounts especially arabinose and isomaltose, respectively. *L. brevis* strains with strong beer spoilage potential were degrading lactose. For *L. paracollinoides* there was no uniform carbohydrate utilization pattern, ranging from overall carbohydrate release (TMW 1.1994-SB) to a diverse carbohydrate pattern, using galactose, xylose, isomaltose and lactose (TMW 1.1995-SB). Although TMW 1.1979-NB did not show visible growth, glucose consumption was detected. Heterofermentative strains showed lactate to acetate ratios from 0.2 to 1.8, whereas TMW 1.1979-NB did not produce detectable acid. In contrast to homofermentative LAB, pyruvate and succinate were consumed by all heterofermentative strains, except TMW 1.1979-NB. All heterofermentative species included strains producing at least one biogenic amine. Strains of *L. paracollinoides* displayed arginine and asparagine consumption as well as ornithine production. *L. lindneri* strains were characterized mainly by consumption of histidine and production of histamine, as well as catabolization of asparagine. *L. brevis* strains consumed arginine, aspartic acid, glutamic acid, tyrosine and methionine (3 out of 4 strains), while producing GABA, tyramine and ornithine. Semiquantitative analysis

further revealed agmatine utilization and putrescine production in case of all *L. brevis* strains. All strains of *L. lindneri* and *L. brevis* TMW 1.6-WB showed consumption of citrate and malate, while the latter substrate was used by all *L. brevis* strains.

12.2.3 Multivariate statistics and comparative metabolic analysis

The pH is a major antibacterial hurdle of beer and strongly affects the antibacterial properties of hops (Fernandez and Simpson, 1995; Simpson and Smith, 1992). A general tendential metabolic shift was observed, comparing the metabolism of beer-spoiling strains in $lager_{pH5.0}$ and $lager_{pH4.3}$. While the optical density, as a measure for cell density, decreased by 70 +/- 13 %, the catabolism of available carbohydrates decreased only by 33 +/- 26 %. Thus, there was more carbohydrate utilization in relation to cell density, while this effect was especially prominent in case of pediococci and less distinct in case of *L. lindneri*. Exemplary, *P. damnosus* TMW 2.1533-SB reached a cell density (OD_{590}) of 0.061 in $lager_{pH4.3}$ and 0.280 in $lager_{pH5.0}$, while the amount of utilized sugars even increased by 2 % in $lager_{pH4.3}$. In case of *P. claussenii* and *L. backii*, the lactate to acetate ratio decreased, resulting from less lactate production (~50 % of concentration found in $lager_{pH5.0}$) compared to acetate production (~ 80 %). Similar effects were observed in case of the comparison of acid stress to hop stress in MRS, but more prominent. The distinct effect of hop stress on sugar and organic acid metabolism of beer-spoiling LAB was investigated in MRS in more detail (see 4.7.2, p. 139).

For $lager_{pH5.0}$ a DAPC analysis was carried out in order to highlight potential relations of species, beer spoilage potential and fermentation type to the investigated metabolism. Based on the ‘find.clusters’ function, four intrinsic (explained by the data) and optimal clusters (Figure 87, p. 315) were defined upon using five principal components, explaining more than 80% of the cumulative variance. Top right of Figure 87, the same data are species labeled. Homofermentative LAB cluster together closely, while heterofermentative LAB show more diversity (Figure 87, bottom right), with the highest intraspecies variability for *L. paracollinoides*. Only quantitative data were included within this analysis.

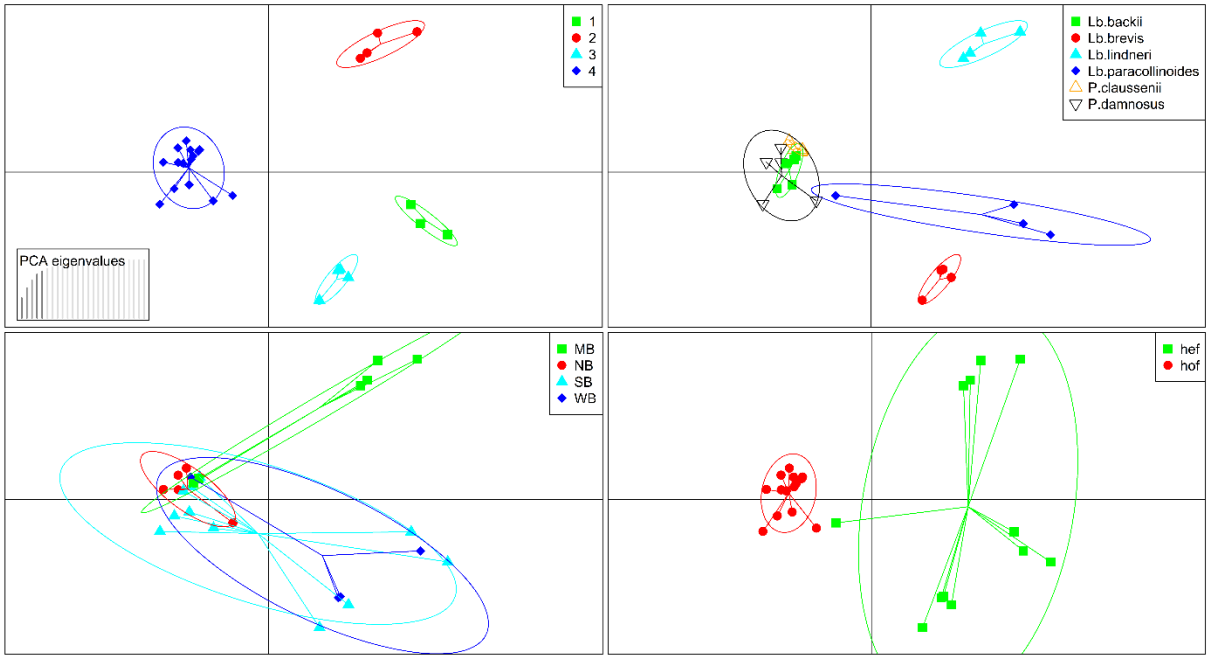


Figure 87: Discriminant analysis of principal components (DAPC) of metabolic capabilities - illustrated as scatterplot of first two principal components of the DAPC. All metabolic data, obtained for lager$_{pH5.0}$, were considered. Each point represents a single strain, while the coloration varies between the different panels. The figure illustrates that there are four metabolic clusters, while they roughly correspond to homofermentative LAB and three clusters for the investigated heterofermentative LAB. **Top left:** optimal clusters defined by *find.clusters*; also shown a scheme of the contribution of PCA *eigenvalues* to cumulative variance. **Top right:** data labeled according to species (outlier of *L. paracollinoides* is TMW 1.1979-NB). **Bottom left:** data labeled according to beer spoilage potential. **Bottom right:** data labeled according to fermentation type.

Comparing the optimal clusters and the species labeled data, one can see that cluster 2 corresponds to *L. lindneri*, cluster 3 to *L. brevis* and cluster 1 covers three of four strains of *L. paracollinoides*. TMW 1.1979-NB, lacking visible growth does cluster together with all homofermentative strains (outlier). Looking at the data labeled according to beer spoilage potential (Figure 87, bottom left), no distinctive separation can be observed, as the four detached data points of the MB group all belong to *L. lindneri*.

Correlation was tested using spearman´s rank correlation. Figure 88 shows that there is almost no correlation of the beer spoilage potential to the metabolic parameters included. The only significant correlation to beer spoilage potential was observed for lactate (r_S = 0.42) and total acid production (r_S = 0.46).

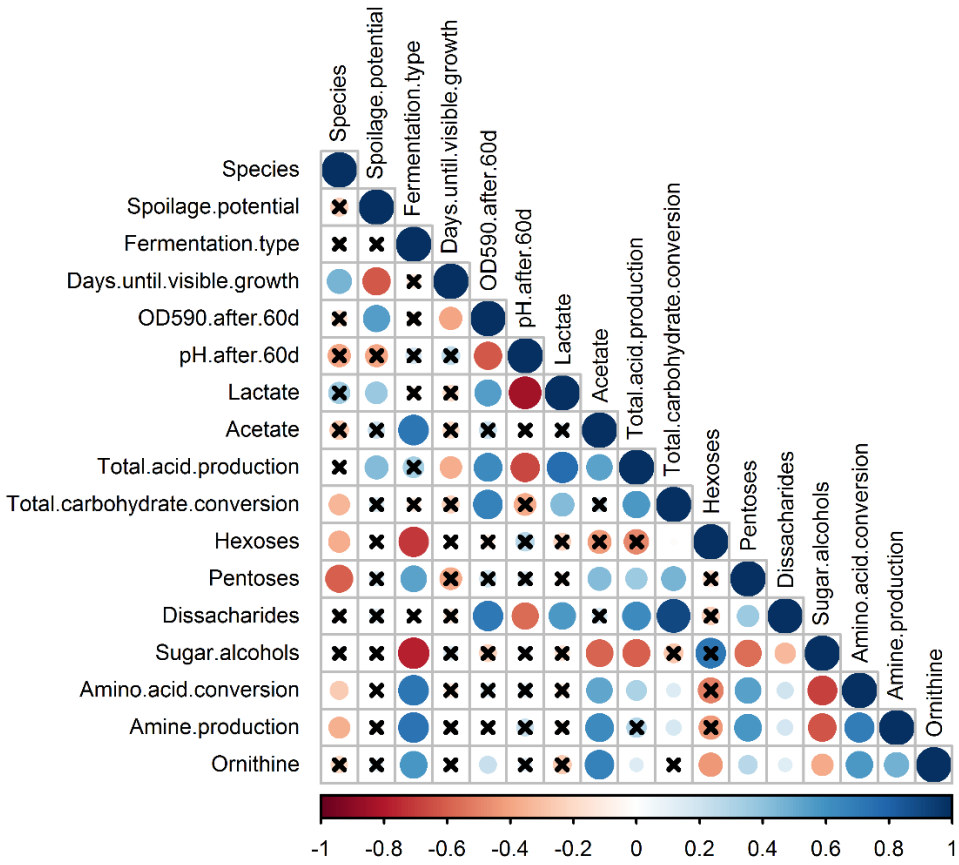


Figure 88: Spearman´s rank correlation of metabolic traits to each other and to beer spoilage potential - illustrated as corrplot. Red circles indicate negative correlation. Blue circles indicate positive correlation. The colors are linked to values between -1 and +1, using the scale at the bottom. If marked with a cross, the calculated correlation coefficient is not significant (p-value > 0.05). If not mentioned explicitly (e.g. lactate, hexoses), the difference in concentration to non-inoculated control beer was adduced as a measure for the respective metabolism. In case of amino acid conversion, only the most prominent were considered: glutamic acid, histidine, arginine and tyrosine.

Considering all metabolic data and both statistical tests, there is no pronounced correlation or relation of beer spoilage potential to metabolic capability, regarding carbohydrate, organic acid and amino acid metabolism, in lager$_{pH5.0}$. The metabolic patterns mostly correlate to species and fermentation type (heterofermentative/homofermentative).

12.3 Metabolism in lager$_{pH4.3}$

The focus of this chapter lies on differences of growth in lager$_{pH4.3}$ to lager$_{pH5.0.}$ If not stated otherwise, results refer to those strains with visible growth in both beers (lager$_{pH5.0}$/lager$_{pH4.3}$). If available, for every species one strain lacking visible growth was investigated for potential metabolism (control). Proportions (%) were calculated (as average values for all strains of one species) in relation to the total amount of consumed carbohydrates.

12.3.1 Homofermentative lactic acid bacteria

P. claussenii TMW 2.53-NB-SB, lacking visible growth in lager$_{pH4.3}$, showed a release of glucose. *P. claussenii* strains with evident growth in lager$_{pH4.3}$ showed a shift to more mannitol (from 2 % to 11 %, average of consumed carbohydrates) consumption. Trehalose utilization increased by 12 % compared to lager$_{pH5.0}$, while the utilization of glucose and gentiobiose was reduced by 12 % and 10 %, respectively. *P. damnosus* strains showed a tendency to use less glucose (decreased by 11 %), while trehalose consumption increased to 100% of the available content for both SB strains. In case of *L. backii*, the proportion of sugar alcohols used, although now restricted to mannitol, increased to 11 % of their carbohydrate consumption. In addition, glucose consumption was reduced by 40 % of the available content. Strains of *P. damnosus* were producing no acetate, while in case of *P. claussenii* and *L. backii*, the lactate to acetate ratio decreased. In lager$_{pH4.3,}$ strains of *L. backii* exhibited little until no acidification (see Appendix 6, p. 336). Succinate consumption became less and pyruvate utilization more for pediococci strains. Amino acid metabolism by homofermentative strains in lager$_{pH4.3}$ was even less pronounced compared to lager$_{pH5.0}$.

In average, 61 % of the total acid produced (in lager$_{pH5.0}$/lager$_{pH4.3}$) could be explained by carbohydrate catabolism investigated here.

12.3.2 Heterofermentative lactic acid bacteria

TMW 1.1995-SB did not show xylose and glucose consumption in lager$_{pH4.3}$, instead consuming mannitol. *L. lindneri´s* xylose utilization was not detectable anymore in lager$_{pH4.3}$, switching to hexose utilization only. Compared to lager$_{pH5.0}$, *L. brevis* strains showed a different sugar utilization. Hexose consumption was reduced by 14 %, as well as mannitol production (58%). In contrast, galactose, arabinose and isomaltose conversion remained nearly unaltered. Heterofermentative strains showed slightly higher lactate to acetate ratios compared to lager$_{pH5.0}$, ranging from 0.9 to 1.6. No acidification, rather a slight increase in pH of beer was observed for strains of *L. lindneri*. Strains of *L. lindneri* also showed a reduced amino acid metabolism in lager$_{pH4.3}$, compared to lager$_{pH5.0}$, retaining asparagine (both strains) and histidine utilization (TMW 1.481-MB) in addition to histamine release. It has to be mentioned that both strains showed a very low optical density after 60 days in lager$_{pH4.3}$. *L. brevis* strains retained most of their amino acid metabolism, while strains of *L. paracollinoides* showed a smaller diversity in amino acid metabolism, reduced mainly to the production of ornithine and the utilization of asparagine as a shared property.

For strains of *L. paracollinoides* (21 %) and *L. lindneri* (25 %), only a quarter of the total acid, produced in lager$_{pH5.0}$ or lager$_{pH4.3}$, could be explained by the carbohydrates analyzed. For *L. brevis,* 70 % could theoretically result from sugars and sugar alcohols investigated.

13 Supplementary Section 5 - Metabolism in MRS

13.1 *Pediococcus claussenii*

In general, the preferred substrate under each condition was found to be glucose, followed by fructose and maltose, while no acetate was produced under all conditions. There was a distinct shift in metabolism, comparing acid to hop stress, as illustrated by Figure 89 (p. 320). Under hop stress the total amount of consumed sugars increased, as well as the proportion of maltose, while the amount of produced lactate decreased dramatically. This is also illustrated by the ratio of measured lactate to theoretical possible lactate (stoichiometric (e.g. 1 hexose results in 2 acids (ethanol)) maximal amount of lactate produced). The lower the ratio, the higher the amount of carbon source, which is not degraded to lactate. This could either result from the presence of alternative electron acceptors or be the consequence of carbon sources used for anabolic processes. Note that the maximum cell density measured (OD_{590}) was found to be two to four times higher under acid stress without additional hops. In conclusion, TMW 2.54-SB and TMW 2.340-WB-MB consumed more sugar, more maltose and produced less lactate under hop stress, while they were characterized by an at least halved cell density compared to acid stress only. This results in less acidification, despite a higher amount of sugars used, which is illustrated by the pH course determined for each strain and MRS variant. Both strains with evident growth under hop stress condition showed a pH of 3.9 at the end of fermentation and a pH of 3.4 growing in $mMRS_2$ pH 4.3 without the addition of hops. There was not acetate or ethanol production.

Asparagine was consumed by all four strains in $mMRS_1$ pH 6.2 (~33 %) and two a lesser extent in $mMRS_2$ pH 4.3 (~ 15 %). This was not observed in $mMRS_2$ pH 4.3 with the addition of iso-α-acids nor in beer.

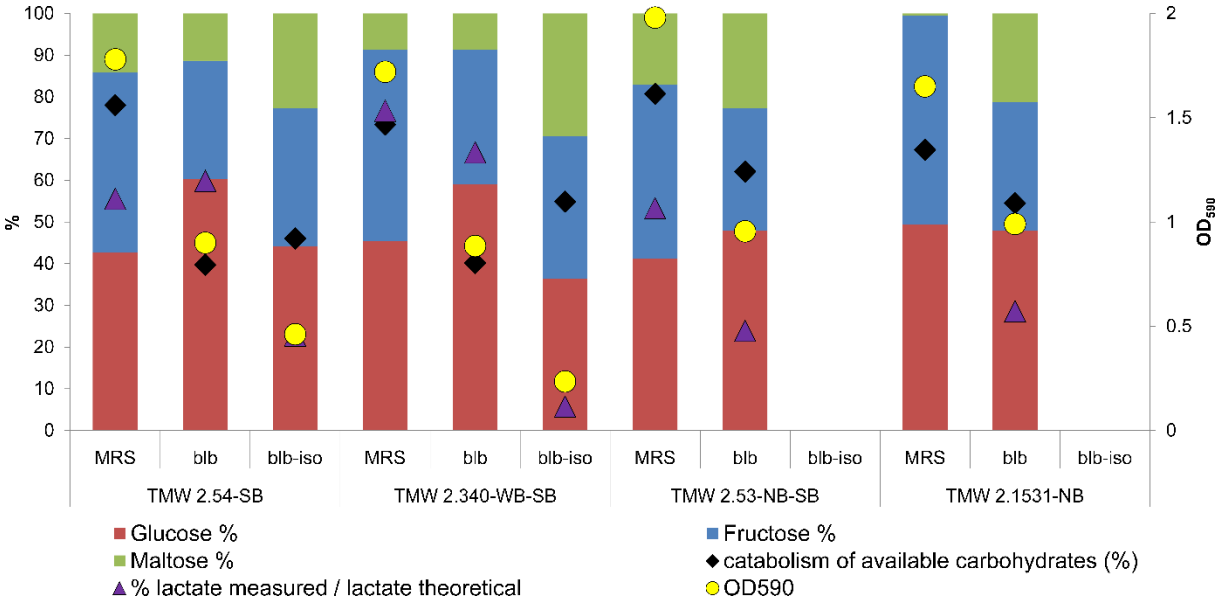


Figure 89: Carbohydrate catabolism of *P. claussenii* in different MRS variants. Data are only shown for those conditions, where evident growth occurred. The proportion (%) of glucose, fructose and maltose of total consumed sugars is shown by the bar chart, illustrating the sugar profile of the respective strain. The catabolism of available carbohydrates (% of total available) is shown, as well as the ratio of measured lactic acid to theoretical possible lactic acid concentrations, calculated under the assumption that each C6 compound could be degraded to two molecules of lactic acid. The OD_{590} is shown as a measure of cell density. Average values for all parameters are shown for three biological replicates. No acetate and no ethanol were produced. MRS = $mMRS_1$ pH 6.2, blb = $mMRS_2$ pH 4.3, blb-iso = $mMRS_2$ pH 4.3 + 50 % MIC iso-α-acids. Iso-α-concentrations applied: TMW 2.54: 7.5 ppm; others: 5 ppm.

13.2 *Pediococcus damnosus*

As in case of *P. claussenii*, glucose is the preferred substrate, while the proportion of consumed maltose and fructose (with respect to totally used sugars) differed between strains. TMW 2.1532-NB did not utilize any maltose under both conditions with evident growth, while TMW 2.1533-SB degraded the total available maltose under reference conditions. Two of three strains with evident growth under hop stress conditions showed an increased proportion of maltose consumption under this condition, as observed for *P. claussenii*. Analogously, we also observed a metabolic shift from acid stress to hop stress towards more carbohydrate utilization relative to cell density. In case of both beer-spoiling strains, the OD_{590} decreased by ~ 40 %, while the amount of carbohydrates utilized increased in case of TMW 2.1533-SB and only slightly decreased in case of TMW 2.1535-SB (~ 10 %). Further, the amount of lactic acid produced decreased by 49 and 62 %, respectively. This is also illustrated by the ratio of measured lactate to theoretical possible lactate, shown in Figure 90 (p. 322). The described metabolic shift is even more obvious for TMW 2.1534-NB, where the carbohydrate consumption reduced by 15 % and the cell density by 75 %. In conclusion, we found the same kind of metabolic shift as in case of *P. claussenii*: an increased sugar consumption relative to cell density and a decreased lactate production. For all three strains where evident growth occurred under all conditions, we found the least acidification (pH course) in case of hop stress. Exemplary, TMW 2.1533-SB broth, after 13 days of fermentation, had a pH of 3.4 in case of the reference condition, 3.7 under acid stress and 4.0 under hop stress. This is important considering the metabolic shift mentioned above, as the amount of sugars used, increased or remained nearly unaltered compared to acid stress alone. There was no acetate or ethanol production and no distinct metabolism of free amino acids under all conditions.

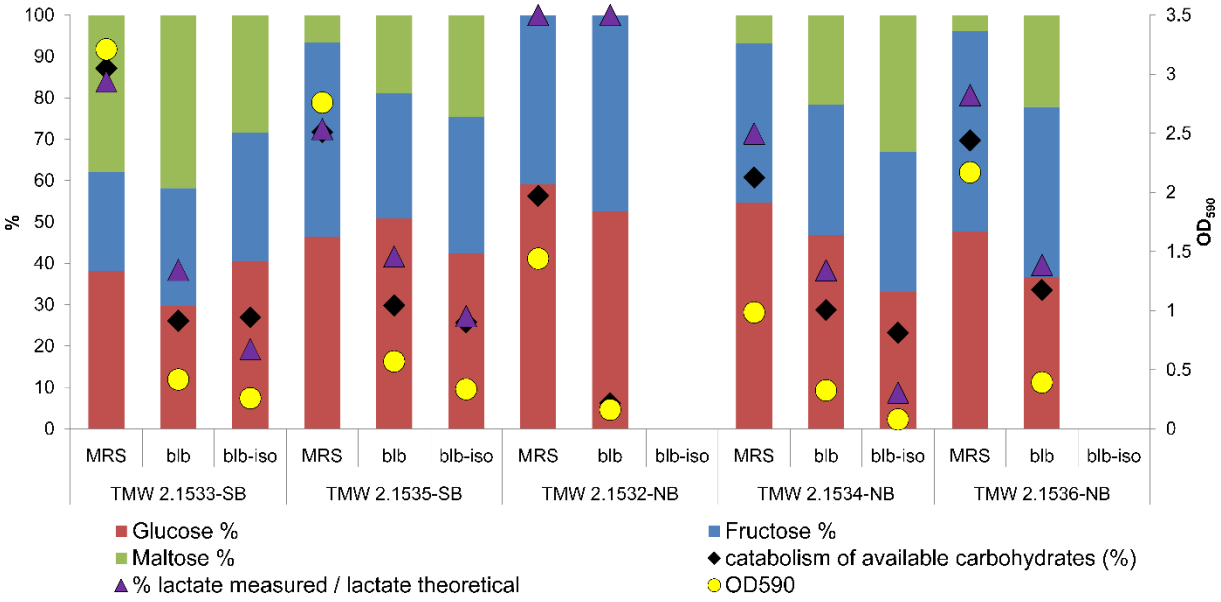


Figure 90: Carbohydrate catabolism of *P. damnosus* in different MRS variants. Data are only shown for those conditions, where evident growth occurred. The proportion (%) of glucose, fructose and maltose of total consumed sugars is shown by the bar chart, illustrating the sugar profile of the respective strain. The catabolism of available carbohydrates (% of total available) is shown, as well as the ratio of measured lactic acid to theoretical possible lactic acid concentrations, calculated under the assumption that each C6 compound could be degraded to two molecules of lactic acid. The OD_{590} is shown as a measure of cell density. Average values for all parameters are shown for three biological replicates. Note: In case of TMW 2.1532-NB the amount of measured lactic acid even exceeded the amount of theoretical calculated lactate, thus indicating the degradation of additional MRS components to lactate. No acetate and no ethanol were produced. MRS = $mMRS_1$ pH 6.2, blb = $mMRS_2$ pH 4.3, blb-iso = $mMRS_2$ pH 4.3 + 50 % MIC iso-α-acids. Iso-α-concentrations applied: TMW 2.1533: 5 ppm; others: 2 ppm.

13.3 *Lactobacillus backii*

The proportion of maltose consumption increased from acid stress condition to hop stress in all cases, while there was a general tendency (4 of 5 strains) to use more fructose instead of glucose at pH 4.3. Again, we observed more carbohydrate consumption and less lactate production relative to the observed cell density, illustrated by Figure 91. Analogously, the pH decreased to a lesser amount under hop stress conditions. While the cell density was reduced by 60 +/- 15 %, comparing acid stress and hop stress conditions, sugar utilization reduced by 21 +/- 20 %. At the same time, lactate production decreased by 63 +/- 9 %. In case of TMW 1.1989-SB, this shift is particularly prominent, as distinctly shown by Figure 91. There was not acetate or ethanol production under all conditions. All strains showed tyrosine degradation and tyramine release, either under all conditions (TMW 1.1430-SB, TMW 1.1989-SB, TMW 1.1991-SB, TMW 1.1992-SB) or only under hop stress (TMW 1.1988-SB). Only TMW 1.1430-SB was also characterized by tyramine production growing in beer.

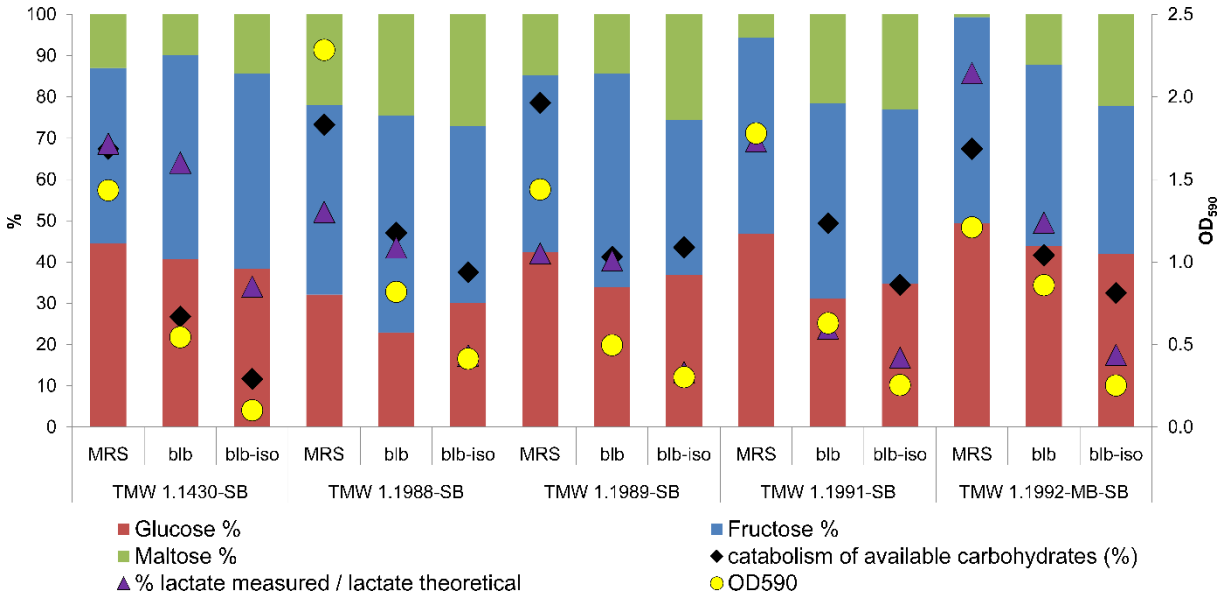


Figure 91: Carbohydrate catabolism of *L. backii* in different MRS variants. The proportion (%) of glucose, fructose and maltose of total consumed sugars is shown by the bar chart, illustrating the sugar profile of the respective strain. The catabolism of available carbohydrates (% of total available) is shown, as well as the ratio of measured lactic acid to theoretical possible lactic acid concentrations, calculated under the assumption that each C6 compound could be degraded to two molecules of lactic acid. The OD_{590} is shown as a measure of cell density. Average values for all parameters are shown for three biological replicates. No acetate was produced. MRS = $mMRS_1$ pH 6.2, blb = $mMRS_2$ pH 4.3, blb-iso = $mMRS_2$ pH 4.3 + 50 % MIC iso-α-acids. Iso-α-concentrations applied: TMW 1.1430 & TMW 1.1992: 9.5 ppm; TMW 1.1988: 8 ppm; TMW 1.1989: 14 ppm.

13.4 *Lactobacillus lindneri*

L. lindneri strains, with one exception, are characterized by enhanced growth at pH 4.3. There is no distinct sugar preference applicable for all strains. Those strains with evident growth under hop stress were found to change their metabolism in comparison to acid stress (which might not be considered as “stress,” if we compare to reference conditions): an increase of sugar utilization, a decrease of lactate production relative to cell density and less acidification after fermentation (pH). No acetate production was observed, while three of four strains released ethanol after growth at pH 4.3. The same three strains showed a utilization of histidine and a production of histamine at pH 4.3, while only TMW 1.1993-SB, characterized by the comparatively best growth of all four strains, was found to maintain this metabolism under hop stress. TMW 1.1285-MB was found to be different compared to the other strains, characterized by the comparatively best growth at reference conditions, a different sugar profile and the lack of histidine/histamine metabolism. Results are illustrated by Figure 92.

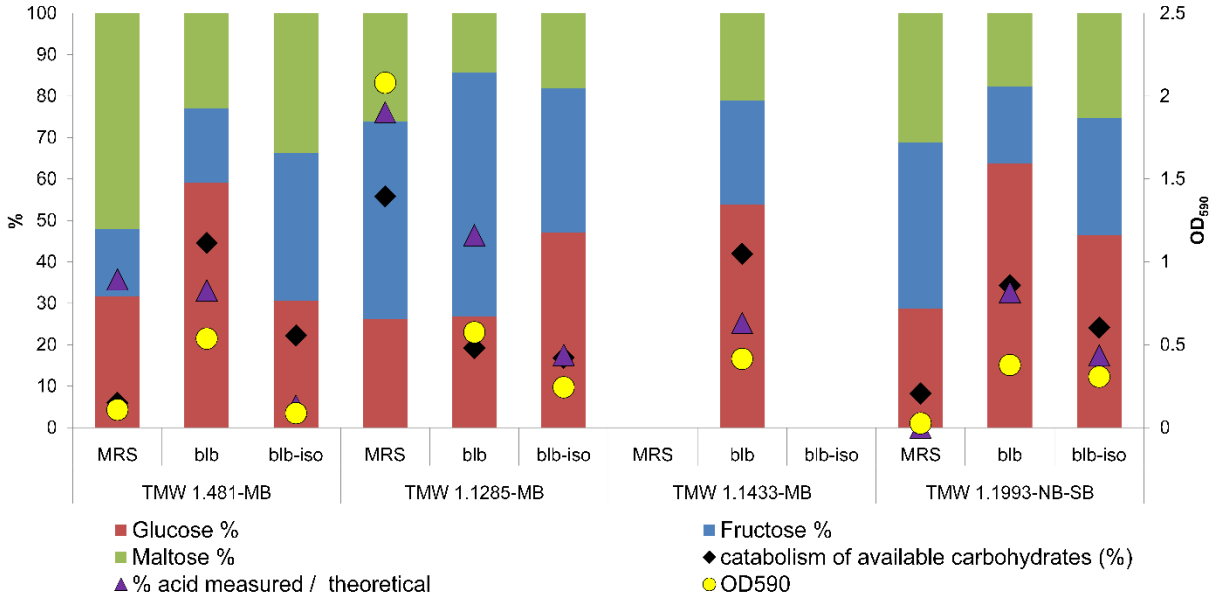


Figure 92: Carbohydrate catabolism of *L. lindneri* in different MRS variants. Data are only shown for those conditions, where evident growth occurred. The proportion (%) of glucose, fructose and maltose of total consumed sugars is shown by the bar chart, illustrating the sugar profile of the respective strain. The catabolism of available carbohydrates (% of total available) is shown, as well as the ratio of measured lactic acid to theoretical possible lactic acid concentrations, calculated under the assumption that each C6 compound could be degraded to two molecules of lactic acid. The OD_{590} is shown as a measure of cell density. Average values for all parameters are shown for three biological replicates. No acetate was produced. MRS = $mMRS_1$ pH 6.2, blb = $mMRS_2$ pH 4.3, blb-iso = $mMRS_2$ pH 4.3 + 50 % MIC iso-α-acids. Iso-α-concentrations applied: TMW 1.481, TMW 1.1993 & TMW 1.1285: 7.5 ppm; TMW 1.1433: 10 ppm.

13.5 *Lactobacillus paracollinoides*

Best growth occurred at pH 4.3 without hop addition, while all strains showed the lowest cell density at reference conditions. Only one strain showed evident growth ($OD_{590} > 0.01$) under hop stress conditions, exhibiting the same metabolic shift as the other core-species, characterized by an increase of sugar utilization and a decrease of lactate production relative to cell density (see Figure 93, p. 325). This resulted in less acidification compared to acid stress, reflected by a final pH value (after fermentation) of 4.1 with additional hop stress and a pH of 3.6 without it. Sugar profiles are different between strains, while both strong spoiling strains prefer maltose over fructose and glucose. Both SB strains produced ethanol at pH 4.3 and no acetate, while the non-spoiling strains produced both metabolites. Arginine utilization was observed for all four strains at reference conditions, while the consumption reduced dramatically at pH 4.3 in case of three strains, despite higher cell densities. Only TMW 1.1979-NB was characterized by more arginine utilization and ornithine production at pH 4.3.

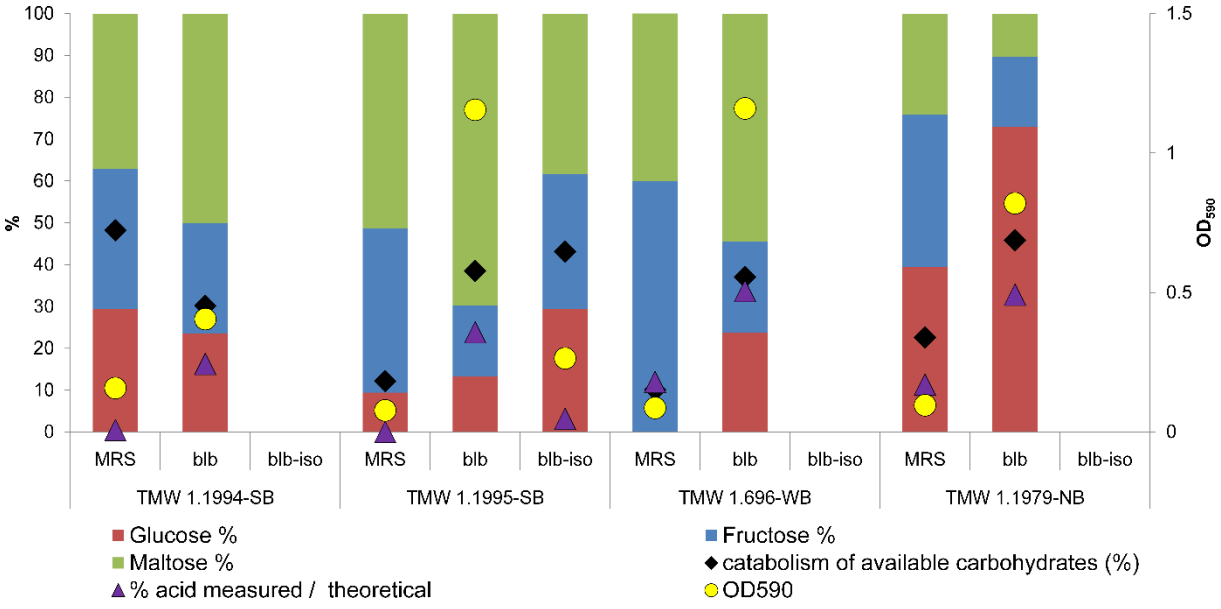


Figure 93: Carbohydrate catabolism of *L. paracollinoides* in different MRS variants. Data are only shown for those conditions, where evident growth occurred. The proportion (%) of glucose, fructose and maltose of total consumed sugars is shown by the bar chart, illustrating the sugar profile of the respective strain. The catabolism of available carbohydrates (% of total available) is shown as well as the ratio of measured lactic/acetic acid to theoretical possible lactic/acetic acid concentrations, calculated under the assumption that each C6 compound could be degraded to two molecules of lactic/acetic acid. The OD_{590} is shown as a measure of cell density. Average values for all parameters are shown for three biological replicates. MRS = $mMRS_1$ pH 6.2, blb = $mMRS_2$ pH 4.3, blb-iso = $mMRS_2$ pH 4.3 + 50 % MIC iso-α-acids. Iso-α-concentrations applied: TMW 1.696 & TMW 1.1979: 5 ppm; TMW 1.1994: 9 ppm; TMW 1.1995: 6 ppm.

13.6 *Lactobacillus brevis*

L. brevis is characterized by a different metabolic behavior as the other core-species. Fructose is preferred in most cases, accompanied by mannitol production. There is no distinct tendency of less organic acid (acetate/lactate) production in relation to cell density, comparing acid and hop stress conditions (see Figure 94, p. 327). The reduction (%) of lactate production is only slightly higher as the reduction of carbohydrate utilization, thus characterized by a less pronounced imbalance of these two parameters, compared to the other core-species. Ethanol and acetate were produced in all MRS variants, while the ratio of lactate to acetate decreased from reference conditions to acid stress and hop stress (where data are available), indicating a shift towards more acetate production. As in case of the other species, acidification was lowest under hop stress. Arginine/ornithine metabolism was observed in all cases with evident growth. Glutamic acid was consumed by all strains under reference conditions, releasing GABA. At pH 4.3, the glutamate/GABA metabolism was different between strains. Both strong spoiling strains showed a reduced conversion of glutamate to GABA, while it increased again (comparison acid/hop stress) in case of hop stress. TMW 1.6-WB showed the same glutamate/GABA metabolism under acid stress, compared to reference conditions, while TMW 1.1369-WB was characterized by GABA production without detectable glutamate utilization. With the exception of TMW 1.313-SB under acid stress, all strains showed tyrosine/tyramine conversion under all conditions with evident growth. Lysine was utilized by both SB strains under all conditions.

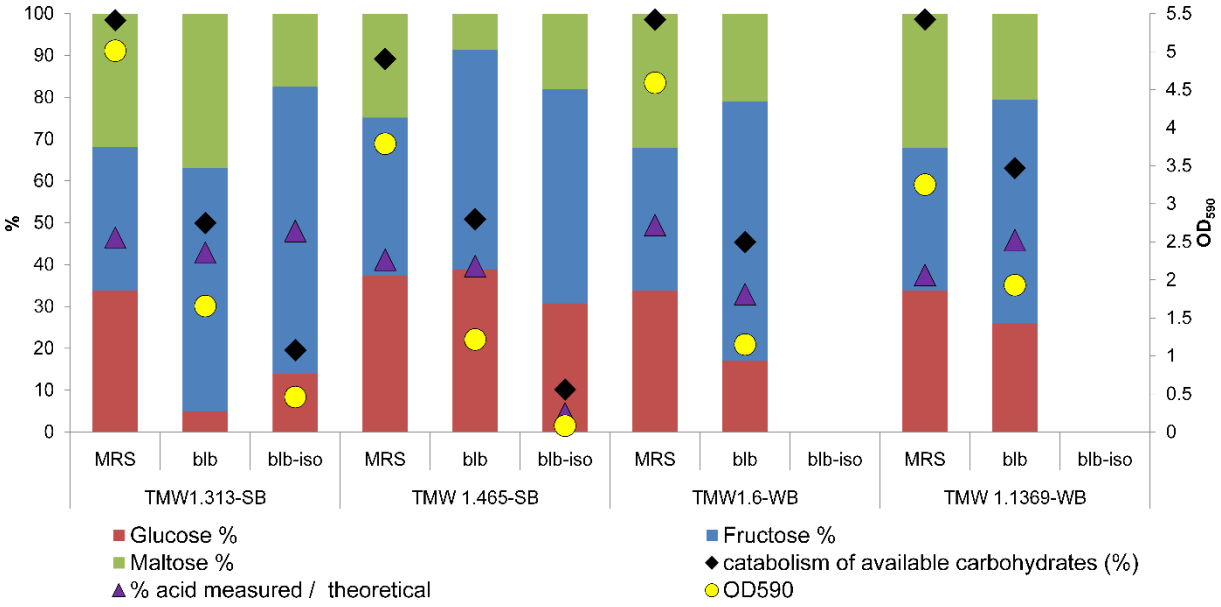


Figure 94: Carbohydrate catabolism of *L. brevis* in different MRS variants. Data are only shown for those conditions, where evident growth occurred. The proportion (%) of glucose, fructose and maltose of total consumed sugars is shown by the bar chart, illustrating the sugar profile of the respective strain. The catabolism of available carbohydrates (% of total available) is shown, as well as the ratio of measured lactic/acetic acid to theoretical possible lactic/acetic acid concentrations, calculated under the assumption that each C6 compound could be degraded to two molecules of lactic/acetic acid. The OD_{590} is shown as a measure of cell density. Average values for all parameters are shown for 3 biological replicates. MRS = $mMRS_1$ pH 6.2, blb = $mMRS_2$ pH 4.3, blb-iso = $mMRS_2$ pH 4.3 + 50 % MIC iso-α-acids. Iso-α-concentrations applied: TMW 1.313 & TMW 1.465: 15.5 ppm; TMW 1.1369: 3.5 ppm; TMW 1.6: 3 ppm.

14 Appendix - Tables and Figures

Appendix 1: Additional analytical data for beers of brewery 1. Total trace elements and long-chain fatty acids were determined by the BLQ (http://www.blq-weihenstephan.de/). Carbohydrate and amino acid/biogenic amine concentrations were determined within this study and are listed with average deviation.

Analyte	Wheat beer 1	Lager beer 1	Pilsner beer 1
Chloride (mg/l)	203	164	166
Nitrate (mg/l)	7.2	8.1	11.3
Hydrogen phosphate (mg/l)	649	517	543
Sulphate (mg/l)	222	151	170
Calcium (mg/l)	34	21	24
Magnesium (mg/l)	97.8	77.2	78.6
Potassium (mg/l)	529	460	483
Sodium (mg/l)	11	10.8	11.9
Iron (mg/l)	0.1	0.05	< 0.02
Copper	0.01	< 0.01	< 0.01
Manganese (mg/l)	0.26	0.06	0.07
Zinc (mg/l)	< 0.01	< 0.01	< 0.01
Aluminium (mg/l)	0.14	0.13	0.13
Silicon (mg/l)	22	30.7	31.6
Phosphor (mg/l)	290	240	250
Boron (mg/l)	0.11	0.09	0.09
Barium (mg/l)	0.03	0.02	0.02
Cobalt (mg/l)	< 0.05	< 0.05	< 0.05
Molybdenum (mg/l)	< 0.05	< 0.05	< 0.05
Strontium (mg/l)	0.119	0.067	0.070
Caproic acid/C6:0 (mg/l)	8	9	4
Caprylic acid/C8:0 (mg/l)	31	26	30
Decanoic acid/C10:0 (mg/l)	7	4	7
C12 to C18 fatty acids (mg/l)	< 2	< 2	< 2

Analyte	Wheat beer 1	Lager beer 1	Pilsner beer 1
Sorbitol (mg/l)	2.54 +/- 0.11	2.81 +/- 0.02	2.71 +/- 0.12
Mannitol (mg/l)	1.67 +/- 0.02	2.03 +/- 0.01	4.44 +/- 0.14
Arabinose (mg/l)	3.42 +/- 0.11	3.63 +/- 0.03	5.02 +/- 0.01
Galactose (mg/l)	0.85 +/- 0.04	1.24 +/- 0.00	2.07 +/- 0.05
Glucose (mg/l)	1.38 +/- 0.01	1.71 +/- 0.02	2.48 +/- 0.10
Xylose (mg/l)	4.31 +/- 0.11	5.11 +/- 0.04	4.35 +/- 0.12
Fructose (mg/l)	5.61 +/- 0.39	6.70 +/- 0.46	6.74 +/- 0.13
Isomaltose (mg/l)	34.52 +/- 0.47	38.33 +/- 0.42	38.27 +/- 0.64
Lactose (mg/l)	4.20 +/- 0.31	8.19 +/- 0.16	4.74 +/- 0.14
Gentiobiose (mg/l)	13.38 +/- 0.61	19.89 +/- 1.44	11.3 +/- 0.13
Maltose (mg/l)	3.47 +/- 1.01	2.84 +/- 0.02	3.21 +/- 0.03
Trehalose (mg/l)	1.97 +/- 0.03	8.48 +/- 0.15	4.6 +/- 0.06
Total[1] Sugars (mg/l)	~101	~77	~90
Pyruvate (mg/l)	54 +/- 5	54 +/- 5	40 +/- 8
Succinate (mg/l)	370 +/- 2	370 +/- 2	354 +/- 19
Lactate (mg/l)	66 +/- 2	66 +/- 2	84 +/- 3
Acetate (mg/l)	12 +/- 0	12 +/- 0	18 +/- 2
Total Organic acids (mg/l)	~524	~502	~496
Alanine (mg/l)	77.01 +/- 0.87	82.66 +/- 4.2	84.65 +/- 0.53
Arginine (mg/l)	36.90 +/- 0.59	38.22 +/- 2.45	32.92 +/- 0.33
Asparagine (mg/l)	12.34 +/- 0.27	9.73 +/- 0.75	29.71 +/- 0.11
Aspartate (mg/l)	20.27 +/- 0.56	15.48 +/- 0.93	24.86 +/- 0.13
Cysteine (mg/l)	26.48 +/- 0.12	22.40 +/- 1.31	21.86 +/- 0.04
Glutamate (mg/l)	17.81 +/- 1.36	23.32 +/- 1.22	21.60 +/- 0.50
Glycine (mg/l)	22.07 +/- 0.18	27.22 +/- 1.49	33.22 +/- 0.11
Histamine (mg/l)	14.66 +/- 0.36	13.20 +/- 1.20	6.31 +/- 0.15
Histidine (mg/l)	22.76 +/- 0.40	25.63 +/- 2.48	31.00 +/- 0.17
Isoleucine (mg/l)	25.66 +/- 0.24	20.56 +/- 1.25	30.64 +/- 0.16

Analyte	Wheat beer 1	Lager beer 1	Pilsner beer 1
Leucine (mg/l)	40.58 +/- 0.48	37.64 +/- 2.34	76.74 +/- 0.25
Lysine (mg/l)	17.91 +/- 0.12	13.40 +/- 0.91	13.93 +/- 0.08
Methionine (mg/l)	5.73 +/- 0.19	4.52 +/- 0.39	9.97 +/- 0.06
Ornithine (mg/l)	4.05 +/- 0.03	3.28 +/- 0.10	8.49 +/- 0.02
Phenylalanine (mg/l)	60.15 +/- 0.79	58.47 +/- 4.38	74.35 +/- 0.97
Serine (mg/l)	7.96 +/- 0.08	4.77 +/- 0.23	21.62 +/- 0.07
Threonine (mg/l)	10.43 +/- 0.16	5.96 +/- 0.77	18.98 +/- 0.07
Tryptophan (mg/l)	29.1 +/- 0.45	30.61 +/- 0.53	52.04 +/- 0.04
Tyramine (mg/l)	9.09 +/- 0.11	5.96 +/- 0.47	9.59 +/- 0.11
Tyrosine (mg/l)	53.58 +/- 0.74	58.41 +/- 4.98	66.75 +/- 0.24
Valine (mg/l)	53.06 +/- 0.83	58.40 +/- 3.90	81.10 +/- 0.46
γ-Aminobutyric acid (mg/l)	32.62 +/- 0.32	35.59 +/- 0.99	60.16 +/- 0.18
Total[1] Amino acids (mg/l)	~726	~540	~433

[1] Total refers to sum of all measured substances of a class. E.g. in case of sugars we could not include ribose or sucrose in our analysis, still they are in beer. Thus, these sums are not the total sum, but the sum of investigated analytes.

Appendix 2: Primer sequences. All sequences are given 5’ to 3’. Only relevant primer pairs, mentioned within the results part, are included.

Primer pair ID(S)	Forward Primer	Reverse primer
616V/609R (16S rDNA)	AGACTTTGATYMTGGCTCAG	ACTACYVGGGTATCTAAKCC
M13-V (RAPD)	GTTTTCCCAGTCACG	NA
M01	ATCGTGCTATGTTCGCACTC	CAAGGCCATCAGCACTTATCTC
M02	TACACCGCGGCAATTGAAG	GGGTTCGATAATCGCGTTCAG
M03	GGCAAACGGGTTGAATCTG	AGTGCTCGGTTCCATAGTC
M05	ATTGAGGCAATGGCTCAGAC	CGATCCGTGACCTAATCCAATG
M13	AGCGTTGGGCACTTGTTG	GAACGGCCACCACAGAAATC
M15	CAATGAGCGTCTTCCAGACAC	TGAGTCACCCTGTAACCTAGC
M18	GCTTGATGGCAGTCCTTACTC	TGCCGTGTTGGTAAAGGTC
M19	ATTGCTCGTTCAAATAGTC	CCCAATAATCATCATCTGATTC
M21	GCTGGTAAAGGATRCAGTGC	CAATACCGTTGCTYAACCGG
M22	TAAGGCATCACGGCAAGTC	CCTAACACGGTATCACCTTCC
M23	GGAGGGAGTAGGACTAACTTC	CACTGCTATCGTCAGGATTGG
M24	CGGTCTTGTCAGCAATAGTGAG	CCCAATTCTGCCCAGACTAATG
M25	TGCTCGGGCAGTCATTATTC	GATTTGGCGTGTTAGCAAGG
M28	AATAGTATGAGCAGCAAACC	CACGTCTAGTTAGTTCATTAGC
M34	AGCAAGCCCTCGCAAATG	TGCCCACGCCGTTTAATC
M37	GGCTTRTTTGCCGATRTG	ACGTCGCCTTGATGRTGG
M38	GGCCATTTGGTGATTCAGG	GTACAAAGGCGAAGTCTGC
M40	CGTCACGCGACCGATTTAG	CGTCAATCACGGCCTGAAC
M42	TCCAGCAGGTAAGCCAATG	CGCAGTAGCGAAGTGATAGTC
ORF4 (M43)	CATCACCAGCCTAACGATTGC	TCATTTGGATGCCGCCAAC
hitA	TTGCAATCAATGGCTGCTCG	TGCGGTCCCGCTAAGAATAC
horA	AATCTTAACCCTGCCGGTGG	TGGATTCGAGTGGTTGAGCC
horC	TACACAGAAACCCGTTCACC	CTGTGCGCTAATTCGTGATG
cinA	AGTGCAGCCGAAAGTTTAACTGGGG	ACAGCCACGAGCCATTGAGCG
arsR	TTTGTCCCAAGCTACTTCATCTGGC	TGGGCCATCCCCTGAGTCGT

Appendix 3: Initial characterization of the growth behaviour in beer. Strains were tested applying the resazurin test (3.2.6.1, p. 33) to determine their ability to stay metabolically active (growth) in four different beers after incubation of 6 and 30 days at 25° C: + growth/- no growth. In addition, the minimum inhibitory concentration (MIC) of iso-α-acids was determined (3.2.6.2, p. 34) for each strain: mean +/- standard deviation (ppm), NG = no growth in test setup and no data available (NA). As controls, four previously well-characterized strains of *L. brevis* were used. Beers and MIC tests were inoculated with preadapted cells ($mMRS_2$: beer like pH and Mg^{2+}/Mg^{2+} content). All strains chosen for the detailed characterization are marked in grey.

		Wheat 1		Lager 1		Pilsner 1		Pilsner 5		MIC hop
Species	**TMW**	**6 days**	**30 days**	**6 days**	**30 days**	**6 days**	**30 days**	**6 days**	**30 days**	**ppm**
L. brevis	1.6	+	+	-	-	-	-	-	-	8 +/- 2
L. brevis	1.1369	+	+	+	-	-	-	-	-	14 +/- 4
L. brevis	1.313	+	+	+	+	+	+	+	+	47 +/- 9
L. brevis	1.465	+	+	+	+	+	+	+	+	44 +/- 5
P. claussenii	2.340	+	+	-	-	-	-	-	-	15 +/- 0
P. claussenii	2.1531	+	+	-	-	-	-	-	-	15 +/- 0
P. claussenii	2.50	+	+	-	-	-	-	-	-	20 +/- 0
P. claussenii	2.53	+	+	-	-	-	-	-	-	15 +/- 0
P. claussenii	2.54	+	+	+	+	-	+	-	-	29 +/- 3
P. claussenii	2.60	+	+	+	+	-	-	-	-	30 +/- 11
P. claussenii	2.61	+	+	-	+	-	-	-	-	32 +/- 9
P. claussenii	2.62	+	+	-	+	-	-	-	-	25 +/- 0
P. claussenii	2.65	+	+	+	-	-	-	-	-	23 +/- 5
P. damnosus	2.4	+	+	-	-	-	-	-	-	8 +/- 2
P. damnosus	2.125	+	-	-	-	-	-	-	-	10 +/- 0
P. damnosus	2.1532	-	-	-	-	-	-	-	-	NG
P. damnosus	2.1533	+	+	-	+	-	-	-	-	10 +/- 0
P. damnosus	2.1534	-	-	-	-	-	-	-	-	NG
P. damnosus	2.1535	+	+	+	-	-	-	-	-	13 +/- 2
P. damnosus	2.1536	+	-	-	-	-	-	-	-	12 +/- 4
P. damnosus	2.1546	+	-	-	-	-	-	-	-	7 +/- 2
P. damnosus	2.1547	-	-	-	-	-	-	-	-	NG
P. damnosus	2.1547	-	-	-	-	-	-	-	-	NG
P. damnosus	2.1635	-	-	-	-	-	-	-	-	NG
P. damnosus	2.1636	-	-	-	-	-	-	-	-	NG
P. damnosus	2.1637	-	-	-	-	-	-	-	-	NG

		Wheat 1		Lager 1		Pilsner 1		Pilsner 5		MIC hop
Species	**TMW**	**6 days**	**30 days**	**6 days**	**30 days**	**6 days**	**30 days**	**6 days**	**30 days**	**ppm**
P. damnosus	2.1547	-	-	-	-	-	-	-	-	NG
P. damnosus	2.1635	-	-	-	-	-	-	-	-	NG
P. damnosus	2.1636	-	-	-	-	-	-	-	-	NG
P. damnosus	2.1637	-	-	-	-	-	-	-	-	NG
L. backii	1.1299	+	+	+	+	+	+	-	-	54 +/- 0
L. backii	1.1430	+	+	+	-	-	-	-	-	16 +/- 0
L. backii	1.1432	+	+	+	-	-	-	-	-	39 +/- 0
L. backii	1.1883	+	+	+	-	+	-	-	+	54 +/- 0
L. backii	1.1988	+	+	+	+	-	+	-	+	39 +/- 11
L. backii	1.1989	+	+	+	-	+	-	+	+	60 +/- 4
L. backii	1.1990	+	+	+	+	-	+	-	-	31 +/- 0
L. backii	1.1991	+	+	+	+	+	+	-	+	39 +/- 0
L. backii	1.1992	-	+	-	+	-	-	-	-	16 +/- 0
L. backii	1.2002	+	+	-	+	-	-	-	-	16 +/- 0
L. backii	1.2003	+	-	+	-	+	-	-	+	47 +/- 0
L. backii	1.2004	+	+	+	+	+	+	-	-	54 +/- 0
L. backii	1.2070	+	+	+	-	+	-	-	-	39 +/- 0
L. backii	1.2071	+	+	+	-	+	-	-	-	57 +/- 13
L. backii	1.2072	+	+	+	+	+	+	-	+	47 +/- 8
L. lindneri	1.88	-	-	-	-	+	+	-	+	NG
L. lindneri	1.1285	-	-	-	-	+	+	+	-	NG
L. lindneri	1.1286	-	-	-	-	+	+	+	+	NG
L. lindneri	1.1433	+	-	-	-	+	+	-	-	16 +/- 0
L. lindneri	1.1993	-	-	-	-	+	+	+	+	NG
L. lindneri	1.2006	-	-	-	-	+	+	-	+	NG
L. lindneri	1.2008	-	-	-	-	+	+	-	+	NG
L. lindneri	1.2080	+	-	-	-	+	+	-	-	16 +/- 0
L. lindneri	1.2081	-	-	-	-	+	+	-	+	NG
L. lindneri	1.2082	+	-	+	-	+	+	-	+	8 +/- 0
L. lindneri	1.456	+	-	+	-	+	+	+	-	16 +/- 0
L. lindneri	1.481	+	-	-	-	+	+	+	-	8 +/- 0
L. paracoll.	1.696	+	-	+	-	+	-	-	+	35 +/- 0
L. paracoll.	1.1979	-	-	-	-	-	-	-	-	5 +/- 0
L. paracoll.	1.1994	+	+	+	-	-	-	-	-	10 +/- 0
L. paracoll.	1.1995	+	+	+	+	-	-	-	-	25 +/- 0

Appendix 4: Detailed characterization of the growth behaviour in beer - resazurin test and MIC test. 28 strains were tested applying the resazurin test (3.2.6.1, p. 33) to determine their ability to stay metabolically active (growth) in four different beers after incubation of 6 and 30 days (6 d/30 d) at 25° C: + growth/- no growth. In addition, the minimum inhibitory concentration (MIC) of iso-α-acids (hop) was determined (3.2.6.2, p. 34) for each strain: mean +/- standard deviation (ppm), NG = No growth in test setup/no data available (NA). All strains chosen for genome sequencing are marked in grey. Cat. = Category. No BSP (NB): + ≤ wheat beer 1 adaptive, weak BSP (WB): + ≥ wheat beer 1 constitutive or lager beer 1 adaptive, middle BSP (MB): + ≥ lager beer 1 constitutive or pilsner beer 1 adaptive, strong BSP (SB): + ≥ pilsner beer 1 constitutive or pilsner beer 5 adaptive.

			Constitutive BSP – $mMRS_1$ pH 6.2									Adaptive BSP – $mMRS_2$ pH 4.3								
	Strain	**Cat.**	**Wheat 1**		**Lager 1**		**Pilsner 1**		**Pilsner 5**		**Mic hop**	**Wheat 1**		**Lager 1**		**Pilsner 1**		**Pilsner 5**		**Mic hop**
Species	**TMW**		**6d**	**30d**	**6d**	**30d**	**6d**	**30d**	**6d**	**30d**	**ppm**	**6d**	**30d**	**6d**	**30d**	**6d**	**30d**	**6d**	**30d**	**ppm**
L. brevis	1.6	NB	-	-	-	-	-	-	-	-	6 +/- 1	+	+	-	-	-	-	-	-	9 +/- 2
L. brevis	1.1369	WB	-	+	-	-	-	-	-	-	7 +/- 2	+	+	-	-	-	-	-	-	16 +/- 2
L. brevis	1.313	SB	+	+	+	+	+	+	-	-	31 +/- 3	+	+	+	+	+	+	+	+	45 +/- 3
L. brevis	1.465	SB	+	+	+	+	+	+	-	-	31 +/- 3	+	+	+	+	+	+	+	+	44 +/- 4
P. claussenii	2.340	WB	+	+	-	-	-	-	-	-	10 +/- 0	+	+	-	-	-	-	-	-	15 +/- 0
P. claussenii	2.1531	WB	+	+	-	-	-	-	-	-	10 +/- 0	+	+	-	-	-	-	-	-	15 +/- 0
P. claussenii	2.53	WB	-	+	-	-	-	-	-	-	10 +/- 0	+	+	-	-	-	-	-	-	15 +/- 0
P. claussenii	2.54	MB	+	+	-	-	-	-	-	-	15 +/- 0	+	+	+	+	+	+	-	-	22 +/- 3
P. damnosus	2.1532	NB	-	-	-	-	-	-	-	-	NG	-	-	-	-	-	-	-	-	NG
P. damnosus	2.1533	MB	+	+	-	+	-	-	-	-	10 +/- 1	+	+	+	+	-	-	-	-	21 +/- 1
P. damnosus	2.1534	NB	-	-	-	-	-	-	-	-	NG	-	-	-	-	-	-	-	-	6 +/- 3
P. damnosus	2.1535	WB	+	+	-	-	-	-	-	-	4 +/- 2	+	+	-	-	-	-	-	-	22 +/- 1
P. damnosus	2.1536	NB	-	-	-	-	-	-	-	-	NG	+	-	-	-	-	-	-	-	12 +/- 5
L. backii	1.1430	SB	+	+	+	-	+	-	-	-	19 +/- 4	+	+	-	-	-	+	-	-	21 +/- 4
L. backii	1.1988	SB	+	+	+	-	-	+	-	-	16 +/- 4	+	+	+	-	+	+	-	-	33 +/- 8
L. backii	1.1989	SB	+	+	+	-	-	+	-	+	28 +/- 0	+	+	+	-	+	+	+	+	58 +/- 4
L. backii	1.1990	SB	+	+	+	+	+	+	-	-	28 +/- 0	+	+	+	+	+	+	-	-	21 +/- 0
L. backii	1.1991	SB	+	+	+	+	+	+	-	-	19 +/- 2	+	+	+	+	+	+	-	-	28 +/- 0
L. backii	1.1992	WB	+	+	-	-	-	-	-	-	9 +/- 4	+	+	-	-	-	-	-	-	14 +/- 0
L. lindneri	1.1285	SB	+	-	-	-	-	-	+	-	15 +/- 0	-	-	-	-	-	-	+	-	NG
L. lindneri	1.1286	SB	-	-	-	-	-	-	+	-	5 +/- 0	-	-	-	-	-	-	+	-	NG
L. lindneri	1.1433	WB	+	+	-	-	-	-	-	-	15 +/- 0	+	-	-	-	-	-	-	-	NG
L. lindneri	1.1993	SB	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	-	-	-	-	-	+	+	23 +/- 0
L. lindneri	1.481	SB	+	-	+	-	+	-	+	-	15 +/- 0	+	-	+	-	+	-	+	-	NG
L. paracoll.	1.696	NB	-	-	-	-	-	-	-	-	11 +/- 4	+	+	-	-	-	-	-	-	20 +/- 5
L. paracoll.	1.1979	NB	-	-	-	-	-	-	-	-	10 +/- 0	-	-	-	-	-	-	-	-	15 +/- 0
L. paracoll.	1.1994	WB	+	-	-	-	-	-	-	-	18 +/- 3	+	+	-	-	-	-	-	-	30 +/- 0
L. paracoll.	1.1995	WB	-	-	-	-	-	-	-	-	12 +/- 3	+	+	+	+	-	-	-	-	27 +/- 3

Appendix 5: PCR on published lifestyle genes *horA*, *horC* and *hitA* within the detailed characterization (4.2.3, p. 54). Positive reactions with the expected product size are indicated by +. Negative reactions are indicated by -; V = variable marker profile; D = marker profile differs from published marker profile/genome data. All strains chosen for genome sequencing are marked in grey. The beer spoilage potential (BSP), determined by beer spoilage test, is listed as well. Strong BSP (SB), middle BSP (MB), weak BSP (WB), no BSP (NB).

Species	TMW	BSP	*horA*	*horC*	*hitA*
L. brevis	1.6	WB	-	-	-
L. brevis	1.1369	WB	-	-	-
L. brevis	1.313	SB	+	+	+
L. brevis	1.465	SB	+	+	+
P. claussenii	2.340^{D}	MB	-	-	-
P. claussenii	2.1531	NB	-	-	-
P. claussenii	2.53^{V}	WB	-	-/+	-
P. claussenii	2.54	SB	+	+	-
P. damnosus	2.1532	NB	+	+	-
P. damnosus	2.1533	SB	+	+	+
P. damnosus	2.1534	NB	+	+	-
P. damnosus	2.1535	SB	+	+	+
P. damnosus	2.1536	NB	-	-	-
L. backii	1.1430	SB	+	+	-
L. backii	1.1988	SB	+	+	-
L. backii	1.1989	SB	-	+	-
L. backii	1.1990	SB	+	+	-
L. backii	1.1991	SB	+	+	-
L. backii	1.1992	MB	-	+	-
L. lindneri	1.1285	MB	+	+	-
L. lindneri	1.1286^{D}	MB	+	+	-
L. lindneri	1.1433	MB	+	-	-
L. lindneri	1.1993^{V}	MB	-/+	-/+	-
L. lindneri	1.481	MB	+	-	-
L. paracoll.	1.696^{V}	WB	-	-/+	-
L. paracoll.	1.1979	NB	-	-	-
L. paracoll.	1.1994	SB	+	+	-
L. paracoll.	1.1995	SB	+	+	+

Appendix 6: Conclusive characterization (4.2.4, p. 57) of 118 strains regarding their beer spoilage potential (BSP), using the beer spoilage test (3.2.6.5, p. 35). The time until visible growth (days) is listed as a measure for adaption. In addition, turbidity (OD_{590}) and pH were determined after 60 days of incubation at 25° C. All values are given as average +/- standard deviation. no - 60 = no growth observed within 60 days. Average values were calculated in accordance with average BSP groups. This table continues for the next five pages.

		lager beer 1 pH5.0			wheat beer 1 - pH 4.4 +/- 0.1			lager beer 1 - pH 4.3 +/- 0.1			pilsner beer 1 - pH 4.4 +/- 0.1		
	TMW	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**
Lactobacillus brevis	TMW 1.6	4 +/- 1	0.20 +/- 0.11	4.51 +/- 0.06	11 +/- 4	0.12 +/- 0.08	4.37 +/- 0.08	no - 60	0.00 +/- 0.00	4.38 +/- 0.08	no - 60	0.00 +/- 0.00	4.34 +/- 0.04
	TMW 1.1326	3 +/- 0	0.10 +/- 0.02	4.81 +/- 0.02	no - 60	0.01 +/- 0.00	4.51 +/- 0.00	no - 60	0.00 +/- 0.00	4.47 +/- 0.01	no - 60	0.00 +/- 0.00	4.42 +/- 0.01
	TMW 1.100	1 +/- 0	0.36 +/- 0.05	3.97 +/- 0.03	14 +/- 4	0.07 +/- 0.02	4.01 +/- 0.02	no - 60	0.00 +/- 0.00	4.3 +/- 0	no - 60	0.00 +/- 0.00	4.33 +/- 0.00
	TMW 1.1205	1 +/- 0	0.40 +/- 0.06	3.97 +/- 0.09	14 +/- 7	0.08 +/- 0.04	4.00 +/- 0.05	no - 60	0.00 +/- 0.00	4.27 +/- 0.05	no - 60	0.00 +/- 0.00	4.35 +/- 0.02
	TMW 1.1282	1 +/- 0	0.25 +/- 0.04	3.99 +/- 0.01	5 +/- 0	0.09 +/- 0.07	4.27 +/- 0.21	7 +/- 0	0.11 +/- 0.04	3.77 +/- 0	5 +/- 0	0.14 +/- 0.04	3.85 +/- 0.00
	TMW 1.1369	2 +/- 0	0.34 +/- 0.01	4.26 +/- 0.01	20 +/- 1	0.15 +/- 0.01	4.09 +/- 0.00	no - 60	0.00 +/- 0.00	4.28 +/- 0	no - 60	0.00 +/- 0.00	4.36 +/- 0.01
	TMW 1.1370	3 +/- 1	0.40 +/- 0.20	4.12 +/- 0.08	6 +/- 1	0.13 +/- 0.02	4.16 +/- 0.08	10 +/- 3	0.12 +/- 0.05	4.16 +/- 0.12	14 +/- 0	0.10 +/- 0.00	4.09 +/- 0.02
	TMW 1.2048	1 +/- 0	0.29 +/- 0.04	3.99 +/- 0.11	11 +/- 1	0.09 +/- 0.01	4.19 +/- 0.00	no - 60	0.00 +/- 0.00	4.37 +/- 0	no - 60	0.00 +/- 0.00	4.40 +/- 0.00
	TMW 1.230	2 +/- 0	0.32 +/- 0.01	4.01 +/- 0.02	2 +/- 0	0.20 +/- 0.04	3.97 +/- 0.01	6 +/- 0	0.11 +/- 0.01	3.86 +/- 0.04	7 +/- 0	0.11 +/- 0.02	3.93 +/- 0.02
	TMW 1.240	1 +/- 0	0.34 +/- 0.07	3.88 +/- 0.00	3 +/- 0	0.17 +/- 0.02	4.00 +/- 0.01	3 +/- 0	0.08 +/- 0	3.75 +/- 0	5 +/- 0	0.06 +/- 0.01	3.83 +/- 0.00
	TMW 1.302	2 +/- 0	0.26 +/- 0.02	4.65 +/- 0.02	6 +/- 0	0.10 +/- 0.02	4.52 +/- 0.01	no - 60	0.00 +/- 0.00	4.49 +/- 0	no - 60	0.00 +/- 0.00	4.40 +/- 0.00
	TMW 1.313	2 +/- 1	0.37 +/- 0.11	4.08 +/- 0.12	2 +/- 0	0.14 +/- 0.05	4.12 +/- 0.10	4 +/- 1	0.16 +/- 0.04	3.91 +/- 0.12	5 +/- 1	0.17 +/- 0.04	3.94 +/- 0.11
	TMW 1.315	2 +/- 0	0.46 +/- 0.03	4.08 +/- 0.03	2 +/- 0	0.24 +/- 0.01	3.90 +/- 0.01	6 +/- 0	0.16 +/- 0.01	3.79 +/- 0.02	10 +/- 2	0.14 +/- 0.00	3.77 +/- 0.00
	TMW 1.317	2 +/- 0	0.38 +/- 0.05	3.88 +/- 0.01	2 +/- 0	0.20 +/- 0.03	4.24 +/- 0.00	4 +/- 0	0.22 +/- 0.03	3.87 +/- 0.04	4 +/- 0	0.15 +/- 0.03	3.83 +/- 0.02
	TMW 1.465	2 +/- 0	0.31 +/- 0.01	4.27 +/- 0.01	3 +/- 0	0.20 +/- 0.02	4.02 +/- 0.02	5 +/- 0	0.16 +/- 0.01	4.02 +/- 0	5 +/- 0	0.13 +/- 0.00	4.15 +/- 0.02
	TMW 1.473	1 +/- 0	0.34 +/- 0.04	3.94 +/- 0.00	3 +/- 2	0.20 +/- 0.01	4.14 +/- 0.06	10 +/- 7	0.17 +/- 0.02	3.9 +/- 0.03	3 +/- 2	0.13 +/- 0.02	3.99 +/- 0.04
	TMW 1.474	1 +/- 0	0.27 +/- 0.07	4.00 +/- 0.01	2 +/- 0	0.09 +/- 0.02	4.41 +/- 0.00	5 +/- 0	0.17 +/- 0.04	3.76 +/- 0.01	5 +/- 0	0.18 +/- 0.04	3.83 +/- 0.00
	TMW 1.476	1 +/- 0	0.38 +/- 0.02	3.95 +/- 0.01	2 +/- 0	0.14 +/- 0.01	4.14 +/- 0.00	5 +/- 0	0.14 +/- 0.02	3.79 +/- 0.01	5 +/- 0	0.14 +/- 0.04	3.98 +/- 0.05
	TMW 1.485	2 +/- 0	0.33 +/- 0.01	3.97 +/- 0.00	2 +/- 0	0.17 +/- 0.01	3.94 +/- 0.01	4 +/- 1	0.12 +/- 0.01	3.78 +/- 0.02	8 +/- 0	0.11 +/- 0.00	3.82 +/- 0.00
	TMW 1.507	1 +/- 0	0.23 +/- 0.07	4.13 +/- 0.04	6 +/- 0	0.12 +/- 0.02	4.00 +/- 0.02	2 +/- 0	0.07 +/- 0.03	4 +/- 0.02	2 +/- 0	0.06 +/- 0.00	4.14 +/- 0.00

		lager beer 1 pH5.0			wheat beer 1 - pH 4.4 +/- 0.1			lager beer 1 - pH 4.3 +/- 0.1			pilsner beer 1 - pH 4.4 +/- 0.1		
	TMW	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**
Pediococcus claussenii	TMW 2.340	14 +/- 0	0.21 +/- 0.01	4.23 +/- 0.01	9 +/- 0	0.04 +/- 0.00	4.18 +/- 0.03	30 +/- 0	0.04 +/- 0.01	4.2 +/- 0.04	no - 60	0.01 +/- 0.00	4.39 +/- 0.00
	TMW 2.1531	14 +/- 0	0.15 +/- 0.00	4.35 +/- 0.01	no - 60	0.00 +/- 0.00	4.42 +/- 0.01	no - 60	0.00 +/- 0.00	4.3 +/- 0	no - 60	0.01 +/- 0.00	4.40 +/- 0.00
	TMW 2.1545	6 +/- 4	0.19 +/- 0.03	4.12 +/- 0.08	4 +/- 1	0.06 +/- 0.03	4.36 +/- 0.09	7 +/- 0	0.00 +/- 0.00	4.24 +/- 0.01	no - 60	0.00 +/- 0.00	4.41 +/- 0.01
	TMW 2.50	5 +/- 3	0.28 +/- 0.09	4.12 +/- 0.13	7 +/- 4	0.06 +/- 0.02	4.21 +/- 0.09	14 +/- 6	0.06 +/- 0.03	4.14 +/- 0.1	26 +/- 6	0.03 +/- 0.04	4.12 +/- 0.05
	TMW 2.51	4 +/- 0	0.28 +/- 0.08	3.96 +/- 0.15	3 +/- 0	0.05 +/- 0.02	4.18 +/- 0.07	3 +/- 0	0.06 +/- 0.03	4.04 +/- 0.08	3 +/- 0	0.06 +/- 0.03	4.09 +/- 0.06
	TMW 2.53	3 +/- 4	0.24 +/- 0.10	4.31 +/- 0.26	5 +/- 4	0.08 +/- 0.06	4.17 +/- 0.10	12 +/- 9	0.10 +/- 0.06	4.05 +/- 0.12	12 +/- 11	0.10 +/- 0.07	4.07 +/- 0.17
	TMW 2.54	5 +/- 2	0.39 +/- 0.04	4.05 +/- 0.10	3 +/- 0	0.15 +/- 0.04	3.98 +/- 0.04	6 +/- 2	0.14 +/- 0.04	3.96 +/- 0.03	7 +/- 0	0.11 +/- 0.03	4.11 +/- 0.09
	TMW 2.56	3 +/- 0	0.31 +/- 0.00	4.12 +/- 0.03	3 +/- 0	0.12 +/- 0.04	4.19 +/- 0.06	3 +/- 0	0.14 +/- 0.04	3.83 +/- 0.33	3 +/- 0	0.14 +/- 0.06	4.11 +/- 0.07
	TMW 2.59	4 +/- 0	0.26 +/- 0.07	4.22 +/- 0.18	5 +/- 0	0.07 +/- 0.02	4.16 +/- 0.02	4 +/- 1	0.10 +/- 0.03	3.96 +/- 0.08	3 +/- 0	0.08 +/- 0.02	4.05 +/- 0.05
	TMW 2.60	5 +/- 2	0.18 +/- 0.01	4.25 +/- 0.09	5 +/- 0	0.07 +/- 0.02	4.15 +/- 0.04	7 +/- 0	0.06 +/- 0.01	4.07 +/- 0.08	no - 60	0.00 +/- 0.00	4.40 +/- 0.01
	TMW 2.61	6 +/- 0	0.15 +/- 0.02	4.17 +/- 0.01	6 +/- 0	0.15 +/- 0.03	4.15 +/- 0.00	6 +/- 0	0.12 +/- 0.01	4 +/- 0.02	no - 60	0.00 +/- 0.00	4.40 +/- 0.02
	TMW 2.62	5 +/- 0	0.24 +/- 0.02	4.18 +/- 0.10	5 +/- 0	0.08 +/- 0.04	4.18 +/- 0.02	10 +/- 0	0.08 +/- 0.02	4.09 +/- 0.06	17 +/- 0	0.05 +/- 0.01	4.04 +/- 0.03
	TMW 2.64	4 +/- 0	0.17 +/- 0.02	4.39 +/- 0.01	6 +/- 1	0.08 +/- 0.00	4.21 +/- 0.01	3 +/- 0	0.06 +/- 0.01	4.09 +/- 0.03	7 +/- 0	0.05 +/- 0.01	4.25 +/- 0.02
	TMW 2.65	6 +/- 0	0.19 +/- 0.03	4.32 +/- 0.02	5 +/- 0	0.06 +/- 0.03	4.22 +/- 0.04	7 +/- 0	0.05 +/- 0.01	4.06 +/- 0.04	no - 60	0.00 +/- 0.00	4.40 +/- 0.00
	TMW 2.67	4 +/- 0	0.36 +/- 0.01	3.91 +/- 0.02	3 +/- 0	0.17 +/- 0.01	3.69 +/- 0.00	7 +/- 0	0.14 +/- 0.02	3.64 +/- 0.01	3 +/- 0	0.14 +/- 0.00	3.71 +/- 0.00
Pediococcus damnosus	TMW 2.4	3 +/- 0	0.15 +/- 0.03	4.28 +/- 0.01	8 +/- 0	0.06 +/- 0.00	4.18 +/- 0.00	22 +/- 0	0.07 +/- 0.02	3.97 +/- 0	no - 60	0.00 +/- 0.00	4.39 +/- 0.00
	TMW 2.125	3 +/- 0	0.14 +/- 0.02	4.49 +/- 0.10	no - 60	0.00 +/- 0.00	4.47 +/- 0.01	no - 60	0.00 +/- 0.00	4.32 +/- 0.01	no - 60	0.00 +/- 0.00	4.36 +/- 0.00
	TMW 2.1532	3 +/- 1	0.17 +/- 0.04	4.36 +/- 0.06	no - 60	0.00 +/- 0.00	4.44 +/- 0.03	no - 60	0.01 +/- 0.00	4.32 +/- 0.01	no - 60	0.01 +/- 0.01	4.38 +/- 0.02
	TMW 2.1533	2 +/- 0	0.28 +/- 0.05	3.69 +/- 0.17	7 +/- 0	0.16 +/- 0.03	3.60 +/- 0.06	7 +/- 0	0.06 +/- 0.03	3.71 +/- 0.16	19 +/- 1	0.06 +/- 0.02	3.87 +/- 0.12
	TMW 2.1534	11 +/- 0	0.14 +/- 0.00	4.34 +/- 0.04	no - 60	0.00 +/- 0.00	4.43 +/- 0.00	no - 60	0.00 +/- 0.00	4.31 +/- 0	no - 60	0.01 +/- 0.00	4.40 +/- 0.00
	TMW 2.1535	3 +/- 1	0.40 +/- 0.06	3.64 +/- 0.09	6 +/- 1	0.12 +/- 0.03	3.8 +/- 0.05	7 +/- 0	0.11 +/- 0.01	3.77 +/- 0.11	7 +/- 0	0.09 +/- 0.01	3.89 +/- 0.07
	TMW 2.1536	14 +/- 0	0.10 +/- 0.01	4.37 +/- 0.08	no - 60	0.00 +/- 0.00	4.43 +/- 0.00	no - 60	0.00 +/- 0.00	4.31 +/- 0	no - 60	0.01 +/- 0.00	4.40 +/- 0.00
	TMW 2.1546	17 +/- 0	0.07 +/- 0.01	4.98 +/- 0.00	no - 60	0.00 +/- 0.00	4.50 +/- 0.00	no - 60	0.00 +/- 0.00	4.49 +/- 0.01	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
	TMW 2.1547	13 +/- 1	0.08 +/- 0.01	4.94 +/- 0.03	no - 60	0.01 +/- 0.00	4.50 +/- 0.00	no - 60	0.00 +/- 0.00	4.48 +/- 0.01	no - 60	0.00 +/- 0.00	4.40 +/- 0.00
	TMW 2.1548	7 +/- 0	0.17 +/- 0.04	4.29 +/- 0.05	7 +/- 0	0.07 +/- 0.02	4.20 +/- 0.07	30 +/- 0	0.04 +/- 0.00	4.21 +/- 0.01	no - 60	0.00 +/- 0.00	4.40 +/- 0.02
	TMW 2.1549	7 +/- 0	0.05 +/- 0.01	4.96 +/- 0.01	no - 60	0.02 +/- 0.03	4.40 +/- 0.15	no - 60	0.00 +/- 0.00	4.49 +/- 0.01	no - 60	0.00 +/- 0.00	4.41 +/- 0.01
	TMW 2.1635	2 +/- 0	0.10 +/- 0.01	4.66 +/- 0.14	no - 60	0.00 +/- 0.00	4.46 +/- 0.01	no - 60	0.00 +/- 0.00	4.31 +/- 0.01	no - 60	0.00 +/- 0.00	4.36 +/- 0.00
	TMW 2.1636	3 +/- 1	0.22 +/- 0.02	4.34 +/- 0.07	6 +/- 1	0.08 +/- 0.02	4.18 +/- 0.01	6 +/- 2	0.06 +/- 0.02	4.07 +/- 0.02	7 +/- 4	0.04 +/- 0.01	4.13 +/- 0.01

		lager beer 1 pH5.0			wheat beer 1 - pH 4.4 +/- 0.1			lager beer 1 - pH 4.3 +/- 0.1			pilsner beer 1 - pH 4.4 +/- 0.1		
	TMW	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**
P. damnosus	TMW 2.1637	3 +/- 0	0.23 +/- 0.02	4.35 +/- 0.07	6 +/- 1	0.08 +/- 0.03	4.22 +/- 0.03	6 +/- 1	0.06 +/- 0.02	4.11 +/- 0.03	5 +/- 2	0.04 +/- 0.01	4.16 +/- 0.04
	TMW 2.1638	3 +/- 0	0.19 +/- 0.01	4.27 +/- 0.12	no - 60	0.01 +/- 0.00	4.47 +/- 0.01	no - 60	0.00 +/- 0.00	4.32 +/- 0.01	no - 60	0.00 +/- 0.00	4.34 +/- 0.04
	TMW 2.1639	3 +/- 1	0.31 +/- 0.06	3.64 +/- 0.02	no - 60	0.00 +/- 0.00	4.50 +/- 0.03	no - 60	0.00 +/- 0.00	4.37 +/- 0.04	no - 60	0.00 +/- 0.00	4.40 +/- 0.04
	TMW 2.1640	3 +/- 1	0.36 +/- 0.06	3.57 +/- 0.10	no - 60	0.00 +/- 0.00	4.47 +/- 0.01	no - 60	0.00 +/- 0.00	4.32 +/- 0.01	no - 60	0.00 +/- 0.00	4.36 +/- 0.01
	TMW 2.1641	3 +/- 0	0.21 +/- 0.01	4.35 +/- 0.09	12 +/- 6	0.06 +/- 0.02	4.20 +/- 0.03	11 +/- 4	0.09 +/- 0.11	4.10 +/- 0.03	11 +/- 4	0.04 +/- 0.01	4.16 +/- 0.03
	TMW 2.1642	4 +/- 0	0.20 +/- 0.01	4.22 +/- 0.10	no - 60	0.00 +/- 0.00	4.45 +/- 0.03	no - 60	0.00 +/- 0.00	4.33 +/- 0.01	no - 60	0.00 +/- 0.00	4.35 +/- 0.00
	TMW 2.1643	4 +/- 0	0.13 +/- 0.08	4.40 +/- 0.36	no - 60	0.00 +/- 0.00	4.46 +/- 0.01	no - 60	0.00 +/- 0.00	4.31 +/- 0.01	no - 60	0.00 +/- 0.00	4.37 +/- 0.03
Lactobacillus backii	TMW 1.1299	2 +/- 0	0.16 +/- 0.01	4.86 +/- 0.00	5 +/- 0	0.08 +/- 0.00	4.37 +/- 0.01	6 +/- 1	0.08 +/- 0.01	4.25 +/- 0.01	5 +/- 0	0.06 +/- 0.00	4.30 +/- 0.01
	TMW 1.1430	4 +/- 0	0.20 +/- 0.00	4.56 +/- 0.01	4 +/- 0	0.06 +/- 0.01	4.30 +/- 0.01	4 +/- 0	0.06 +/- 0.00	4.29 +/- 0.00	11 +/- 0	0.05 +/- 0.00	4.33 +/- 0.00
	TMW 1.1432	2 +/- 0	0.18 +/- 0.00	4.84 +/- 0.00	5 +/- 0	0.27 +/- 0.29	4.33 +/- 0.00	5 +/- 0	0.08 +/- 0.02	4.23 +/- 0.01	6 +/- 1	0.07 +/- 0.00	4.28 +/- 0.01
	TMW 1.1883	2 +/- 0	0.50 +/- 0.00	3.87 +/- 0.00	7 +/- 0	0.08 +/- 0.00	4.46 +/- 0.00	7 +/- 0	0.24 +/- 0.01	3.80 +/- 0.01	7 +/- 0	0.20 +/- 0.02	3.75 +/- 0.02
	TMW 1.1988	2 +/- 0	0.19 +/- 0.00	4.50 +/- 0.01	5 +/- 0	0.07 +/- 0.01	4.27 +/- 0.00	7 +/- 0	0.06 +/- 0.00	4.24 +/- 0.01	11 +/- 0	0.06 +/- 0.00	4.32 +/- 0.00
	TMW 1.1989	3 +/- 0	0.17 +/- 0.01	4.67 +/- 0.00	7 +/- 0	0.08 +/- 0.00	4.36 +/- 0.00	9 +/- 0	0.07 +/- 0.00	4.27 +/- 0.00	11 +/- 0	0.06 +/- 0.00	4.32 +/- 0.01
	TMW 1.1990	2 +/- 0	0.30 +/- 0.01	4.31 +/- 0.01	5 +/- 0	0.12 +/- 0.01	4.10 +/- 0.00	11 +/- 0	0.08 +/- 0.00	4.08 +/- 0.00	11 +/- 0	0.06 +/- 0.00	4.28 +/- 0.02
	TMW 1.1991	2 +/- 0	0.31 +/- 0.00	4.30 +/- 0.00	4 +/- 0	0.09 +/- 0.00	4.07 +/- 0.00	10 +/- 0	0.08 +/- 0.00	4.06 +/- 0.02	10 +/- 0	0.05 +/- 0.01	4.29 +/- 0.02
	TMW 1.1992	5 +/- 3	0.22 +/- 0.04	4.58 +/- 0.05	14 +/- 4	0.05 +/- 0.01	4.34 +/- 0.09	19 +/- 2	0.04 +/- 0.01	4.28 +/- 0.05	18 +/- 7	0.04 +/- 0.00	4.36 +/- 0.03
	TMW 1.2002	4 +/- 2	0.21 +/- 0.04	4.14 +/- 0.19	11 +/- 4	0.11 +/- 0.06	4.28 +/- 0.17	9 +/- 0	0.07 +/- 0.01	4.34 +/- 0.01	16 +/- 0	0.06 +/- 0.01	4.38 +/- 0.00
	TMW 1.2003	5 +/- 0	0.17 +/- 0.02	4.21 +/- 0.19	6 +/- 0	0.16 +/- 0.02	4.40 +/- 0.04	14 +/- 0	0.12 +/- 0.02	4.30 +/- 0.02	17 +/- 0	0.07 +/- 0.02	4.22 +/- 0.01
	TMW 1.2004	4 +/- 1	0.32 +/- 0.20	3.86 +/- 0.29	no - 60	0.00 +/- 0.00	4.54 +/- 0.02	no - 60	0.00 +/- 0.00	4.45 +/- 0.03	no - 60	0.00 +/- 0.00	4.38 +/- 0.06
	TMW 1.2005	4 +/- 2	0.17 +/- 0.07	4.37 +/- 0.24	4 +/- 3	0.05 +/- 0.01	4.40 +/- 0.02	11 +/- 1	0.02 +/- 0.00	4.30 +/- 0.01	3 +/- 0	0.02 +/- 0.00	4.33 +/- 0.01
	TMW 1.2070	2 +/- 0	0.16 +/- 0.01	4.87 +/- 0.00	9 +/- 1	0.05 +/- 0.02	4.36 +/- 0.00	7 +/- 0	0.04 +/- 0.01	4.26 +/- 0.01	7 +/- 0	0.04 +/- 0.01	4.31 +/- 0.00
	TMW 1.2071	2 +/- 0	0.12 +/- 0.00	5.02 +/- 0.01	5 +/- 0	0.04 +/- 0.00	4.46 +/- 0.03	8 +/- 2	0.03 +/- 0.01	4.42 +/- 0.00	no - 60	0.00 +/- 0.00	4.48 +/- 0.01
	TMW 1.2072	2 +/- 0	0.47 +/- 0.03	3.87 +/- 0.01	3 +/- 0	0.07 +/- 0.00	4.42 +/- 0.00	4 +/- 0	0.11 +/- 0.00	4.13 +/- 0.00	4 +/- 0	0.10 +/- 0.00	4.23 +/- 0.00
	TMW 1.2073	2 +/- 0	0.30 +/- 0.01	4.44 +/- 0.00	6 +/- 0	0.12 +/- 0.00	4.23 +/- 0.01	6 +/- 0	0.13 +/- 0.01	4.11 +/- 0.00	6 +/- 0	0.11 +/- 0.01	4.17 +/- 0.00
	TMW 1.2077	2 +/- 0	0.14 +/- 0.01	4.54 +/- 0.45	10 +/- 0	0.06 +/- 0.01	4.44 +/- 0.00	8 +/- 3	0.06 +/- 0.02	4.30 +/- 0.00	no - 60	0.00 +/- 0.00	4.36 +/- 0.00
	TMW 1.2078	2 +/- 0	0.17 +/- 0.00	4.85 +/- 0.00	5 +/- 0	0.05 +/- 0.00	4.35 +/- 0.00	7 +/- 0	0.03 +/- 0.01	4.26 +/- 0.00	7 +/- 0	0.04 +/- 0.02	4.32 +/- 0.00
	TMW 1.2079	3 +/- 0	0.08 +/- 0.01	4.84 +/- 0.00	10 +/- 0	0.03 +/- 0.01	4.41 +/- 0.04	no - 60	0.00 +/- 0.00	4.33 +/- 0.00	no - 60	0.00 +/- 0.00	4.34 +/- 0.01

		lager beer 1 pH5.0			wheat beer 1 - pH 4.4 +/- 0.1			lager beer 1 - pH 4.3 +/- 0.1			pilsner beer 1 - pH 4.4 +/- 0.1		
	TMW	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**
Lactobacillus lindneri	TMW 1.88	5 +/- 0	0.06 +/- 0.03	4.64 +/- 0.03	no - 60	0.00 +/- 0.00	4.48 +/- 0.01	no - 60	0.00 +/- 0.00	4.47 +/- 0.03	no - 60	0.00 +/- 0.00	4.33 +/- 0.06
	TMW 1.1285	8 +/- 0	0.06 +/- 0.01	4.72 +/- 0.01	5 +/- 0	0.01 +/- 0.00	4.42 +/- 0.01	5 +/- 0	0.01 +/- 0.00	4.31 +/- 0.00	no - 60	0.00 +/- 0.00	4.38 +/- 0.00
	TMW 1.1286	7 +/- 1	0.06 +/- 0.01	4.71 +/- 0.02	5 +/- 0	0.01 +/- 0.00	4.41 +/- 0.00	5 +/- 0	0.01 +/- 0.00	4.31 +/- 0.00	no - 60	0.00 +/- 0.00	4.39 +/- 0.00
	TMW 1.1433	3 +/- 0	0.07 +/- 0.00	4.69 +/- 0.00	4 +/- 0	0.02 +/- 0.00	4.40 +/- 0.00	7 +/- 0	0.01 +/- 0.00	4.30 +/- 0.00	no - 60	0.00 +/- 0.00	4.38 +/- 0.00
	TMW 1.1993	3 +/- 0	0.09 +/- 0.02	4.69 +/- 0.08	14 +/- 0	0.02 +/- 0.00	4.46 +/- 0.01	30 +/- 0	0.01 +/- 0.00	4.34 +/- 0.00	no - 60	0.01 +/- 0.00	4.4 +/- 0.00
	TMW 1.2006	5 +/- 0	0.03 +/- 0.01	4.66 +/- 0.04	no - 60	0.00 +/- 0.00	4.47 +/- 0.00	no - 60	0.00 +/- 0.00	4.46 +/- 0.02	no - 60	0.00 +/- 0.00	4.34 +/- 0.05
	TMW 1.2007	5 +/- 0	0.04 +/- 0.03	4.66 +/- 0.05	16 +/- 7	0.04 +/- 0.03	4.42 +/- 0.04	11 +/- 1	0.03 +/- 0.01	4.20 +/- 0.05	12 +/- 1	0.04 +/- 0.02	4.16 +/- 0.00
	TMW 1.2008	5 +/- 0	0.05 +/- 0.02	4.61 +/- 0.03	10 +/- 3	0.03 +/- 0.02	4.39 +/- 0.09	7 +/- 1	0.06 +/- 0.03	4.14 +/- 0.08	7 +/- 1	0.06 +/- 0.02	4.05 +/- 0.00
	TMW 1.2009	5 +/- 0	0.06 +/- 0.04	4.66 +/- 0.01	12 +/- 2	0.03 +/- 0.02	4.47 +/- 0.02	7 +/- 1	0.02 +/- 0.02	4.21 +/- 0.05	8 +/- 1	0.03 +/- 0.03	4.08 +/- 0.00
	TMW 1.2080	3 +/- 0	0.25 +/- 0.05	3.98 +/- 0.00	no - 60	0.01 +/- 0.00	4.46 +/- 0.00	4 +/- 1	0.08 +/- 0.02	3.75 +/- 0.01	5 +/- 0	0.07 +/- 0.02	3.82 +/- 0.00
	TMW 1.2081	3 +/- 0	0.26 +/- 0.03	3.99 +/- 0.00	no - 60	0.00 +/- 0.00	4.45 +/- 0.00	5 +/- 0	0.06 +/- 0.01	3.75 +/- 0.01	3 +/- 0	0.06 +/- 0.02	3.80 +/- 0.00
	TMW 1.2082	4 +/- 0	0.31 +/- 0.02	4.03 +/- 0.01	30 +/- 0	0.01 +/- 0.00	4.58 +/- 0.01	4 +/- 0	0.16 +/- 0.00	3.88 +/- 0.00	4 +/- 0	0.15 +/- 0.03	3.94 +/- 0.00
	TMW 1.456	3 +/- 0	0.21 +/- 0.05	3.94 +/- 0.02	no - 60	0.00 +/- 0.00	4.44 +/- 0.01	5 +/- 0	0.06 +/- 0.01	3.71 +/- 0.01	11 +/- 0	0.13 +/- 0.01	3.77 +/- 0.00
	TMW 1.481	2 +/- 0	0.04 +/- 0.00	4.77 +/- 0.01	6 +/- 0	0.02 +/- 0.00	4.44 +/- 0.00	8 +/- 0	0.01 +/- 0.00	4.35 +/- 0.00	no - 60	0.00 +/- 0.00	4.44 +/- 0.00
L paracoll.	TMW 1.696	12 +/- 2	0.20 +/- 0.02	4.16 +/- 0.11	7 +/- 0	0.19 +/- 0.01	3.77 +/- 0.01	no - 60	0.00 +/- 0.00	4.29 +/- 0.01	no - 60	0.00 +/- 0.00	4.38 +/- 0.02
	TMW 1.1979	no - 60	0.02 +/- 0.00	4.88 +/- 0.00	no - 60	0.00 +/- 0.00	4.41 +/- 0.02	no - 60	0.01 +/- 0.00	4.30 +/- 0.00	no - 60	0.01 +/- 0.00	4.40 +/- 0.00
	TMW 1.1994	2 +/- 0	0.21 +/- 0.00	4.06 +/- 0.01	4 +/- 0	0.07 +/- 0.00	4.12 +/- 0.01	6 +/- 0	0.04 +/- 0.00	3.94 +/- 0.01	10 +/- 0	0.01 +/- 0.00	4.24 +/- 0.01
	TMW 1.1995	4 +/- 0	0.41 +/- 0.01	3.89 +/- 0.02	3 +/- 0	0.29 +/- 0.01	3.78 +/- 0.00	7 +/- 0	0.22 +/- 0.01	3.82 +/- 0.02	7 +/- 0	0.20 +/- 0.02	3.83 +/- 0.00
	TMW 1.2010	4 +/- 1	0.34 +/- 0.04	3.64 +/- 0.12	3 +/- 1	0.19 +/- 0.02	3.60 +/- 0.16	6 +/- 1	0.14 +/- 0.02	3.67 +/- 0.05	6 +/- 1	0.20 +/- 0.04	3.75 +/- 0.10
L. plant	TMW 1.9	1 +/- 0	0.26 +/- 0.00	4.11 +/- 0.01	11 +/- 0	0.15 +/- 0.01	3.95 +/- 0.01	12 +/- 11	0.08 +/- 0.03	3.94 +/- 0.11	4 +/- 0	0.08 +/- 0.01	3.97 +/- 0.01
	TMW 1.1308	1 +/- 0	0.33 +/- 0.06	3.98 +/- 0.05	16 +/- 2	0.09 +/- 0.05	3.94 +/- 0.06	no - 60	0.00 +/- 0.00	4.30 +/- 0.00	no - 60	0.00 +/- 0.00	4.33 +/- 0.00
	TMW 1.277	1 +/- 0	0.31 +/- 0.00	4.14 +/- 0.01	15 +/- 0	0.16 +/- 0.02	3.92 +/- 0.02	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
	TMW 1.1789	1 +/- 0	0.27 +/- 0.02	4.04 +/- 0.08	no - 60	0.01 +/- 0.00	4.45 +/- 0.00	no - 60	0.00 +/- 0.00	4.30 +/- 0.00	no - 60	0.00 +/- 0.00	4.33 +/- 0.00
	TMW 1.1789	1 +/- 0	0.21 +/- 0.05	4.32 +/- 0.17	no - 60	0.01 +/- 0.00	4.52 +/- 0.00	no - 60	0.00 +/- 0.00	4.38 +/- 0.00	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
L. paracas.	TMW 1.1434	1 +/- 0	0.20 +/- 0.04	4.15 +/- 0.03	no - 60	0.01 +/- 0.00	4.50 +/- 0.02	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
	TMW 1.304	1 +/- 0	0.15 +/- 0.00	4.11 +/- 0.00	27 +/- 0	0.09 +/- 0.00	3.85 +/- 0.03	no - 60	0.00 +/- 0.00	4.36 +/- 0.00	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
	TMW 1.427	1 +/- 0	0.16 +/- 0.00	4.13 +/- 0.00	no - 60	0.00 +/- 0.00	4.51 +/- 0.00	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.40 +/- 0.00
	TMW 1.1982	1 +/- 0	0.14 +/- 0.02	4.27 +/- 0.02	no - 60	0.01 +/- 0.00	4.50 +/- 0.01	no - 60	0.00 +/- 0.00	4.38 +/- 0.01	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
	TMW 1.1882	1 +/- 0	0.10 +/- 0.00	4.26 +/- 0.00	20 +/- 0	0.07 +/- 0.01	3.92 +/- 0.03	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.41 +/- 0.00

L. paracoll.(*inoides*); *L. plant*.(*arum*); *L. paracas*.(*ei*);

		lager beer 1 pH5.0			wheat beer 1 - pH 4.4 +/- 0.1			lager beer 1 - pH 4.3 +/- 0.1			pilsner beer 1 - pH 4.4 +/- 0.1		
	TMW	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**
L. parabu.	TMW 1.429	1 +/- 0	0.21 +/- 0.05	3.96 +/- 0.01	32 +/- 4	0.04 +/- 0.01	4.45 +/- 0.03	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.40 +/- 0.00
	TMW 1.345	1 +/- 0	0.29 +/- 0.03	3.98 +/- 0.01	24 +/- 0	0.03 +/- 0.01	4.10 +/- 0.03	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.40 +/- 0.00
	TMW 1.1306	1 +/- 0	0.25 +/- 0.01	4.01 +/- 0.00	27 +/- 0	0.13 +/- 0.01	4.36 +/- 0.00	no - 60	0.00 +/- 0.00	4.37 +/- 0.01	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
	TMW 1.2083	1 +/- 0	0.20 +/- 0.05	3.98 +/- 0.00	30 +/- 0	0.04 +/- 0.00	4.48 +/- 0.01	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
	TMW 1.2084	1 +/- 0	0.12 +/- 0.04	4.01 +/- 0.00	26 +/- 1	0.08 +/- 0.04	4.44 +/- 0.07	15 +/- 13	0.08 +/- 0.01	3.83 +/- 0.01	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
L. harbin.	TMW 1.1453	1 +/- 0	0.14 +/- 0.01	4.43 +/- 0.05	no - 60	0.01 +/- 0.00	4.53 +/- 0.01	no - 60	0.00 +/- 0.00	4.37 +/- 0.01	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
	TMW 1.1455	1 +/- 0	0.13 +/- 0.02	4.45 +/- 0.02	no - 60	0.01 +/- 0.00	4.52 +/- 0.00	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
	TMW 1.2085	1 +/- 0	0.16 +/- 0.03	4.07 +/- 0.05	27 +/- 0	0.21 +/- 0.04	3.66 +/- 0.00	no - 60	0.00 +/- 0.00	4.38 +/- 0.00	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
	TMW 1.2086	1 +/- 0	0.23 +/- 0.04	3.90 +/- 0.01	18 +/- 0	0.17 +/- 0.02	3.60 +/- 0.00	no - 60	0.00 +/- 0.00	4.36 +/- 0.01	no - 60	0.00 +/- 0.00	4.40 +/- 0.00
	TMW 1.2087	1 +/- 0	0.21 +/- 0.03	4.05 +/- 0.02	17 +/- 0	0.26 +/- 0.01	3.55 +/- 0.01	no - 60	0.04 +/- 0.06	4.27 +/- 0.13	no - 60	0.00 +/- 0.00	4.41 +/- 0.00
L[1]	TMW 1.695	no - 60	0.00 +/- 0.00	4.96 +/- 0.01	no - 60	0.01 +/- 0.00	4.52 +/- 0.01	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.37 +/- 0.00
L[2]	TMW 1.1424	7 +/- 4	0.18 +/- 0.15	4.33 +/- 0.40	8 +/- 3	0.01 +/- 0.00	4.48 +/- 0.02	no - 60	0.00 +/- 0.00	4.39 +/- 0.02	no - 60	0.00 +/- 0.00	4.39 +/- 0.02
	TMW 1.2063	12 +/- 0	0.10 +/- 0.03	4.67 +/- 0.04	no - 60	0.00 +/- 0.00	4.52 +/- 0.00	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.39 +/- 0.02
L[3]	TMW 1.1304	1 +/- 0	0.10 +/- 0.02	4.78 +/- 0.01	no - 60	0.01 +/- 0.00	4.52 +/- 0.00	no - 60	0.00 +/- 0.00	4.37 +/- 0.00	no - 60	0.00 +/- 0.00	4.40 +/- 0.00

L. parabu.(*chneri*); *L. harbin*.(*ensis*); *L[1] = L. helveticus*; *L[2] = L. paucivorans; L[3] = L. sanfranciscensis*

Appendix 7: Adaption, turbidity and acidification by lactic acid bacteria, determined within beer spoilage test and summarized for species. This table summarizes the results of Appendix 6 (p. 336). Average values were calculated for all strains with growth in the respective beers. Initial pH values of beers: wheat beer 1 pH 4.4 +/- 0.1; lager beer 1 pH 4.3 +/- 0.1; pilsner beer 1 pH 4.4 +/- 0.1.

Beer	Parameter	*L. brevis*	*P. claussenii*	*P. damnosus*	*L. backii*	*L. lindneri*	*L. paracoll.*
Lager beer 1 pH5.0	growth (days)	2 +/- 1	5 +/- 4	5 +/- 4	3 +/- 2	5 +/- 1	14 +/- 19
	OD_{590} (60 days)	0.32 +/- 0.12	0.24 +/- 0.09	0.20 +/- 0.10	0.23 +/- 0.13	0.09 +/- 0.08	0.24 +/- 0.13
	pH (60 days)	4.14 +/- 0.25	4.19 +/- 0.19	4.25 +/- 0.42	4.42 +/- 0.39	4.55 +/- 0.26	4.08 +/- 0.40
	ΔpH (60 days)	0.86 +/- 0.25	0.80 +/- 0.19	0.75 +/- 0.42	0.58 +/- 0.39	0.45 +/- 0.26	0.92 +/- 0.40
Wheat beer 1	growth (days)	6 +/- 6	5 +/- 3	7 +/- 4	8 +/- 6	13 +/- 12	4 +/- 2
	OD_{590} (60 days)	0.14 +/- 0.06	0.09 +/- 0.05	0.09 +/- 0.04	0.08 +/- 0.08	0.02 +/- 0.02	0.19 +/- 0.07
	pH (60 days)	4.15 +/- 0.17	4.16 +/- 0.14	4.07 +/- 0.22	4.34 +/- 0.12	4.45 +/- 0.07	3.80 +/- 0.21
	ΔpH (60 days)	0.25 +/- 0.17	0.24 +/- 0.14	0.33 +/- 0.22	0.06 +/- 0.12	-0.05 +/- 0.07	0.60 +/- 0.21
Lager beer 1	growth (days)	5 +/- 3	10 +/- 8	11 +/- 8	9 +/- 4	8 +/- 5	6 +/- 1
	OD_{590} (60 days)	0.14 +/- 0.05	0.09 +/- 0.05	0.07 +/- 0.05	0.08 +/- 0.05	0.04 +/- 0.04	0.13 +/- 0.07
	pH (60 days)	3.90 +/- 0.15	4.03 +/- 0.16	4.00 +/- 0.18	4.22 +/- 0.13	4.12 +/- 0.21	3.79 +/- 0.12
	ΔpH (60 days)	0.40 +/- 0.15	0.27 +/- 0.16	0.30 +/- 0.18	0.08 +/- 0.13	0.18 +/- 0.21	0.51 +/- 0.12
Pilsner beer 1	growth (days)	6 +/- 3	10 +/- 10	9 +/- 5	10 +/- 6	8 +/- 5	8 +/- 2
	OD_{590} (60 days)	0.14 +/- 0.05	0.09 +/- 0.05	0.05 +/- 0.02	0.07 +/- 0.04	0.06 +/- 0.04	0.14 +/- 0.09
	pH (60 days)	3.90 +/- 0.12	4.08 +/- 0.14	4.06 +/- 0.14	4.27 +/- 0.14	4.00 +/- 0.16	3.92 +/- 0.22
	ΔpH (60 days)	0.47 +/- 0.12	0.32 +/- 0.14	0.34 +/- 0.14	0.13 +/- 0.14	0.40 +/- 0.16	0.48 +/- 0.22

Appendix 8: Beer spoilage test (3.2.6.5, p. 35) with 23 strains using three beers each, from brewery 2, 3 and 4. The time until visible growth (days) is listed as a measure for adaption. In addition, turbidity (OD_{590}) and pH were determined after 60 days of incubation at 25° C. All values are given as average +/- standard deviation. no - 60 = no growth observed within 60 days. Average values were calculated in accordance with average beer spoilage potential groups. This table continues for the next two pages.

		wheat beer 2 - pH 4.2 +/- 0.1			lager beer 2 - pH 4.3 +/- 0.1			pilsner beer 2 - pH 4.5 +/- 0.1		
	TMW	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**
L. brevis	TMW 1.313	5 +/- 0	0.09 +/- 0.02	4.02 +/- 0.01	5 +/- 0	0.08 +/- 0.01	3.75 +/- 0.00	5 +/- 0	0.10 +/- 0.01	3.92 +/- 0.03
	TMW 1.465	5 +/- 0	0.07 +/- 0.01	4.05 +/- 0.00	5 +/- 0	0.15 +/- 0.03	3.76 +/- 0.01	7 +/- 0	0.10 +/- 0.01	3.98 +/- 0.01
	TMW 1.302	9 +/- 0	0.02 +/- 0.01	4.16 +/- 0.00	18 +/- 0	0.04 +/- 0.02	3.90 +/- 0.05	39 +/- 0	0.01 +/- 0.01	4.27 +/- 0.01
	TMW 1.1326	no - 60	0.01 +/- 0.01	4.19 +/- 0.00	no - 60	0.00 +/- 0.00	4.34 +/- 0.00	no - 60	0.00 +/- 0.00	4.45 +/- 0.00
P. claussenii	TMW 2.50	5 +/- 0	0.06 +/- 0.02	4.03 +/- 0.06	6 +/- 1	0.07 +/- 0.05	4.01 +/- 0.12	19 +/- 0	0.10 +/- 0.01	4.08 +/- 0.02
	TMW 2.54	3 +/- 0	0.10 +/- 0.02	4.01 +/- 0.08	5 +/- 0	0.15 +/- 0.04	3.99 +/- 0.06	15 +/- 4	0.09 +/- 0.03	4.05 +/- 0.06
	TMW 2.1545	no - 60	0.01 +/- 0.01	4.24 +/- 0.00	13 +/- 0	0.00 +/- 0.00	4.25 +/- 0.03	no - 60	0.00 +/- 0.00	4.44 +/- 0.00
	TMW 2.1531	no - 60	0.00 +/- 0.00	4.20 +/- 0.00	no - 60	0.00 +/- 0.00	4.34 +/- 0.00	no - 60	0.00 +/- 0.00	4.45 +/- 0.00
P. damnosus	TMW 2.1532	no - 60	0.01 +/- 0.00	4.20 +/- 0.00	no - 60	0.00 +/- 0.00	4.34 +/- 0.00	no - 60	0.00 +/- 0.00	4.44 +/- 0.00
	TMW 2.1533	7 +/- 0	0.12 +/- 0.02	3.47 +/- 0.01	6 +/- 1	0.02 +/- 0.01	3.54 +/- 0.07	9 +/- 0	0.02 +/- 0.01	3.74 +/- 0.02
	TMW 2.1535	11 +/- 1	0.08 +/- 0.01	3.83 +/- 0.01	6 +/- 1	0.07 +/- 0.00	3.45 +/- 0.01	11 +/- 2	0.09 +/- 0.01	3.69 +/- 0.04
	TMW 2.1536	no - 60	0.01 +/- 0.01	4.21 +/- 0.00	no - 60	0.00 +/- 0.00	4.34 +/- 0.00	no - 60	0.00 +/- 0.00	4.45 +/- 0.00
L. backii	TMW 1.2002	8 +/- 2	0.09 +/- 0.01	4.17 +/- 0.01	13 +/- 0	0.07 +/- 0.01	4.20 +/- 0.01	23 +/- 1	0.07 +/- 0.01	4.07 +/- 0.05
	TMW 1.2004	9 +/- 0	0.03 +/- 0.01	4.16 +/- 0.00	no - 60	0.00 +/- 0.00	4.31 +/- 0.01	no - 60	0.00 +/- 0.00	4.42 +/- 0.01
	TMW 1.1989	7 +/- 1	0.08 +/- 0.01	4.21 +/- 0.00	10 +/- 1	0.07 +/- 0.00	4.24 +/- 0.01	15 +/- 0	0.11 +/- 0.01	4.02 +/- 0.00
	TMW 1.1991	5 +/- 0	0.09 +/- 0.01	4.03 +/- 0.09	9 +/- 2	0.12 +/- 0.00	4.12 +/- 0.02	14 +/- 0	0.13 +/- 0.02	3.98 +/- 0.03
L. lindneri	TMW 1.88	no - 60	0.00 +/- 0.00	4.24 +/- 0.01	5 +/- 0	0.10 +/- 0.03	3.79 +/- 0.00	5 +/- 0	0.09 +/- 0.01	4.00 +/- 0.03
	TMW 1.2006	no - 60	0.00 +/- 0.00	4.26 +/- 0.00	5 +/- 0	0.02 +/- 0.02	3.82 +/- 0.02	5 +/- 0	0.05 +/- 0.02	4.06 +/- 0.11
	TMW 1.2008	no - 60	0.00 +/- 0.00	4.26 +/- 0.00	4 +/- 0	0.09 +/- 0.01	3.79 +/- 0.00	4 +/- 0	0.06 +/- 0.00	4.01 +/- 0.03
	TMW 1.2009	no - 60	0.00 +/- 0.00	4.25 +/- 0.00	4 +/- 0	0.09 +/- 0.03	3.81 +/- 0.00	4 +/- 0	0.06 +/- 0.00	4.09 +/- 0.01
L. para.	TMW 1.1979	no - 60	0.00 +/- 0.00	4.20 +/- 0.00	no - 60	0.00 +/- 0.00	4.34 +/- 0.00	no - 60	0.00 +/- 0.00	4.45 +/- 0.00
	TMW 1.1994	9 +/- 3	0.00 +/- 0.00	4.01 +/- 0.00	7 +/- 0	0.08 +/- 0.02	3.64 +/- 0.05	11 +/- 0	0.05 +/- 0.02	4.04 +/- 0.04
	TMW 1.1995	5 +/- 0	0.05 +/- 0.02	3.84 +/- 0.01	7 +/- 0	0.13 +/- 0.06	3.66 +/- 0.02	7 +/- 0	0.12 +/- 0.01	3.84 +/- 0.05

		wheat beer 3 - pH 4.4 +/- 0.1			lager beer 3 - pH 4.5 +/- 0.1			pilsner beer 3 - pH 4.5 +/- 0.1		
	TMW	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**
L. brevis	TMW 1.313	5 +/- 0	0.12 +/- 0.02	4.22 +/- 0.00	5 +/- 0	0.12 +/- 0.02	4.00 +/- 0.00	5 +/- 0	0.19 +/- 0.02	3.87 +/- 0.00
	TMW 1.465	2 +/- 0	0.08 +/- 0.03	4.22 +/- 0.00	5 +/- 0	0.10 +/- 0.01	4.00 +/- 0.00	7 +/- 0	0.16 +/- 0.04	4.02 +/- 0.03
	TMW 1.302	4 +/- 0	0.07 +/- 0.03	4.33 +/- 0.00	8 +/- 2	0.01 +/- 0.01	4.08 +/- 0.03	no-60	0.00 +/- 0.00	4.51 +/- 0.00
	TMW 1.1326	no-60	0.00 +/- 0.00	4.39 +/- 0.00	no-60	0.00 +/- 0.00	4.47 +/- 0.00	no-60	0.00 +/- 0.00	4.52 +/- 0.00
P. claussenii	TMW 2.50	4 +/- 0	0.06 +/- 0.02	4.08 +/- 0.06	4 +/- 0	0.06 +/- 0.01	4.23 +/- 0.01	8 +/- 0	0.00 +/- 0.00	4.43 +/- 0.00
	TMW 2.54	3 +/- 0	0.12 +/- 0.08	4.04 +/- 0.06	3 +/- 0	0.16 +/- 0.01	4.17 +/- 0.09	5 +/- 0	0.06 +/- 0.04	4.36 +/- 0.11
	TMW 2.1545	4 +/- 0	0.03 +/- 0.00	4.12 +/- 0.00	no-60	0.00 +/- 0.00	4.44 +/- 0.03	no-60	0.00 +/- 0.00	4.52 +/- 0.00
	TMW 2.1531	26 +/- 1	0.02 +/- 0.00	4.19 +/- 0.02	no-60	0.00 +/- 0.00	4.47 +/- 0.00	no-60	0.00 +/- 0.00	4.52 +/- 0.00
P. damnosus	TMW 2.1532	no-60	0.00 +/- 0.00	4.39 +/- 0.00	no-60	0.00 +/- 0.00	4.47 +/- 0.00	no-60	0.00 +/- 0.00	4.52 +/- 0.00
	TMW 2.1533	5 +/- 0	0.22 +/- 0.06	3.48 +/- 0.00	5 +/- 0	0.07 +/- 0.06	3.56 +/- 0.00	7 +/- 0	0.08 +/- 0.03	3.67 +/- 0.00
	TMW 2.1535	7 +/- 0	0.19 +/- 0.05	3.70 +/- 0.00	7 +/- 0	0.15 +/- 0.01	3.60 +/- 0.03	7 +/- 0	0.14 +/- 0.01	3.78 +/- 0.01
	TMW 2.1536	no-60	0.01 +/- 0.00	4.38 +/- 0.00	no-60	0.00 +/- 0.00	4.47 +/- 0.00	no-60	0.00 +/- 0.00	4.52 +/- 0.00
L. backii	TMW 1.2002	6 +/- 0	0.07 +/- 0.00	4.32 +/- 0.09	11 +/- 0	0.04 +/- 0.00	4.40 +/- 0.01	11 +/- 0	0.08 +/- 0.02	4.43 +/- 0.00
	TMW 1.2004	no-60	0.00 +/- 0.00	4.38 +/- 0.00	no-60	0.00 +/- 0.00	4.45 +/- 0.01	no-60	0.00 +/- 0.00	4.50 +/- 0.01
	TMW 1.1989	7 +/- 1	0.06 +/- 0.01	4.37 +/- 0.00	10 +/- 3	0.06 +/- 0.01	4.40 +/- 0.00	9 +/- 5	0.00 +/- 0.00	4.00 +/- 0.00
	TMW 1.1991	5 +/- 0	0.09 +/- 0.02	4.20 +/- 0.00	10 +/- 3	0.13 +/- 0.01	4.23 +/- 0.00	7 +/- 2	0.19 +/- 0.07	4.01 +/- 0.23
L. lindneri	TMW 1.88	no-60	0.00 +/- 0.00	4.39 +/- 0.01	5 +/- 0	0.08 +/- 0.06	4.04 +/- 0.03	5 +/- 0	0.10 +/- 0.02	3.79 +/- 0.00
	TMW 1.2006	5 +/- 0	0.05 +/- 0.06	4.32 +/- 0.04	5 +/- 0	0.07 +/- 0.02	4.08 +/- 0.06	5 +/- 0	0.11 +/- 0.06	4.01 +/- 0.20
	TMW 1.2008	6 +/- 0	0.00 +/- 0.00	4.00 +/- 0.00	6 +/- 0	0.08 +/- 0.02	4.05 +/- 0.00	6 +/- 0	0.11 +/- 0.01	3.80 +/- 0.00
	TMW 1.2009	6 +/- 0	0.00 +/- 0.00	4.33 +/- 0.00	6 +/- 0	0.09 +/- 0.01	4.09 +/- 0.01	7 +/- 1	0.03 +/- 0.01	4.38 +/- 0.08
L. para.	TMW 1.1979	no-60	0.00 +/- 0.00	4.38 +/- 0.00	no-60	0.00 +/- 0.00	4.47 +/- 0.00	no-60	0.00 +/- 0.00	4.52 +/- 0.00
	TMW 1.1994	4 +/- 0	0.08 +/- 0.03	3.96 +/- 0.00	7 +/- 0	0.00 +/- 0.00	4.11 +/- 0.02	27 +/- 7	0.03 +/- 0.01	4.24 +/- 0.01
	TMW 1.1995	3 +/- 0	0.17 +/- 0.05	3.93 +/- 0.01	3 +/- 0	0.11 +/- 0.02	3.91 +/- 0.00	3 +/- 0	0.10 +/- 0.03	3.94 +/- 0.03

		wheat beer 4 - pH 4.3 +/- 0.1			lager beer 4 - pH 4.5 +/- 0.1			pilsner beer 4 - pH 4.4 +/- 0.1		
	TMW	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**	**growth (days)**	**OD590 (60 days)**	**pH (60 days)**
L. brevis	TMW 1.313	5 +/- 0	0.12 +/- 0.01	4.16 +/- 0.03	5 +/- 0	0.10 +/- 0.01	4.26 +/- 0.00	5 +/- 0	0.11 +/- 0.02	4.14 +/- 0.02
	TMW 1.465	4 +/- 1	0.09 +/- 0.01	4.21 +/- 0	5 +/- 0	0.16 +/- 0.00	4.27 +/- 0.01	5 +/- 0	0.14 +/- 0.06	4.16 +/- 0.01
	TMW 1.302	7 +/- 0	0.04 +/- 0.01	4.27 +/- 0.01	7 +/- 0	0.06 +/- 0.00	4.56 +/- 0.00	no-60	0.04 +/- 0.01	4.46 +/- 0.02
	TMW 1.1326	no - 60	0.00 +/- 0.00	4.31 +/- 0.00	no - 60	0.00 +/- 0.00	4.57 +/- 0.01	no-60	0.00 +/- 0.00	4.43 +/- 0.00
P. claussenii	TMW 2.50	5 +/- 0	0.07 +/- 0.03	4.12 +/- 0.04	5 +/- 0	0.09 +/- 0.02	4.24 +/- 0.08	6 +/- 1	0.08 +/- 0.02	4.09 +/- 0.05
	TMW 2.54	3 +/- 0	0.10 +/- 0.03	4.07 +/- 0.08	3 +/- 0	0.13 +/- 0.03	4.19 +/- 0.10	3 +/- 0	0.11 +/- 0.01	4.09 +/- 0.07
	TMW 2.1545	4 +/- 0	0.03 +/- 0.01	4.26 +/- 0.01	4 +/- 0	0.04 +/- 0.00	4.22 +/- 0.00	no-60	0.00 +/- 0.00	4.44 +/- 0.01
	TMW 2.1531	no-60	0.00 +/- 0.00	4.31 +/- 0	yes-60	0.08 +/- 0.01	4.27 +/- 0.01	no-60	0.00 +/- 0.00	4.44 +/- 0.00
P. damnosus	TMW 2.1532	no-60	0.00 +/- 0.00	4.31 +/- 0	no-60	0.00 +/- 0.00	4.57 +/- 0.00	no-60	0.00 +/- 0.00	4.44 +/- 0.00
	TMW 2.1533	5 +/- 0	0.13 +/- 0.01	3.58 +/- 0	5 +/- 0	0.14 +/- 0.01	3.54 +/- 0.01	7 +/- 0	0.11 +/- 0.02	3.52 +/- 0.01
	TMW 2.1535	9 +/- 2	0.11 +/- 0.02	3.92 +/- 0	7 +/- 0	0.22 +/- 0.03	3.60 +/- 0.01	7 +/- 0	0.13 +/- 0.04	3.47 +/- 0.02
	TMW 2.1536	no-60	0.01 +/- 0.00	4.31 +/- 0	no-60	0.00 +/- 0.00	4.57 +/- 0.00	no-60	0.00 +/- 0.00	4.44 +/- 0.00
L. backii	TMW 1.2002	8 +/- 0	0.06 +/- 0.01	4.27 +/- 0.02	14 +/- 0	0.06 +/- 0.00	4.51 +/- 0.01	14 +/- 0	0.05 +/- 0.01	4.39 +/- 0.01
	TMW 1.2004	8 +/- 1	0.03 +/- 0.00	4.24 +/- 0	28 +/- 0	0.01 +/- 0.00	4.55 +/- 0.00	no-60	0.00 +/- 0.00	4.42 +/- 0.01
	TMW 1.1989	7 +/- 1	0.11 +/- 0.01	4.36 +/- 0.01	6 +/- 0	0.06 +/- 0.01	4.55 +/- 0.00	10 +/- 1	0.05 +/- 0.01	4.42 +/- 0.01
	TMW 1.1991	5 +/- 0	0.06 +/- 0.01	4.24 +/- 0.01	5 +/- 0	0.10 +/- 0.00	4.46 +/- 0.01	9 +/- 0	0.14 +/- 0.03	4.17 +/- 0.09
L. lindneri	TMW 1.88	no-60	0.00 +/- 0.00	4.34 +/- 0	no-60	0.00 +/- 0.00	4.64 +/- 0.00	9 +/- 0	0.00 +/- 0.00	4.14 +/- 0.10
	TMW 1.2006	7 +/- 0	0.04 +/- 0.04	4.3 +/- 0.08	no-60	0.00 +/- 0.00	4.63 +/- 0.01	no-60	0.00 +/- 0.00	4.50 +/- 0.00
	TMW 1.2008	no-60	0.00 +/- 0.00	4.34 +/- 0	no-60	0.00 +/- 0.00	4.64 +/- 0.00	no-60	0.00 +/- 0.00	4.50 +/- 0.00
	TMW 1.2009	8 +/- 0	0.01 +/- 0.00	4.34 +/- 0	no-60	0.01 +/- 0.00	4.61 +/- 0.00	no-60	0.00 +/- 0.00	4.48 +/- 0.00
L. para.	TMW 1.1979	no-60	0.01 +/- 0.00	4.31 +/- 0	no-60	0.00 +/- 0.00	4.57 +/- 0.00	no-60	0.00 +/- 0.00	4.44 +/- 0.00
	TMW 1.1994	5 +/- 1	0.06 +/- 0.01	3.92 +/- 0.01	4 +/- 0	0.08 +/- 0.01	3.92 +/- 0.01	7 +/- 0	0.11 +/- 0.01	3.74 +/- 0.02
	TMW 1.1995	5 +/- 0	0.14 +/- 0.04	3.9 +/- 0.02	4 +/- 1	0.19 +/- 0.02	3.90 +/- 0.01	6 +/- 2	0.20 +/- 0.06	3.82 +/- 0.03

Appendix 9: Minimum inhibitory concentrations (MICs) for various stress qualities - strain level. MICs were defined by the first concentration (intensity), where no growth was determined by turbidity measurement (OD_{590}). The respective units and concentrations are given. Strong beer spoilage potential (BSP) (SB), middle BSP (MB), weak BSP (WB), no BSP (NB). EtOH = ethanol, H_2O_2 ↑Mn^{2+} = oxidative stress in $mMRS_1$ pH 4.3, H_2O_2 ↓Mn^{2+} = oxidative stress in $mMRS_2$ pH 4.3, DNP = 2,4 dinitrophenol, CCCP = carbonyl cyanide m-chlorophenyl hydrazone, Cal. = Calcimycin, Val.&Nig. = Valinomycin and Nigericin. > = bigger than, as growth was detected in all intensities tested. NA = not available.

	Strain	Category	pH	EtOH	Iso-α-acids	H_2O_2 ↑Mn^{2+}	H_2O_2 ↓ Mn^{2+}	DNP	CCCP	Cal.	Val.&Nig.
			pH	v/v %	ppm	mM	mM	mM	mM	µM	µM
L. brevis	TMW 1.6	WB	3.00	10	5	3.5	3	0.2	0.12	30	> 10
	TMW 1.1369	WB	3.00	10	10	3.5	3	0.4	0.12	60	> 10
	TMW 1.313	SB	3.00	12	30	3.5	3.5	0.4	0.12	70	> 10
	TMW 1.465	SB	3.00	12	30	4.0	4.0	0.4	> 0.2	40	> 10
P. claussenii	TMW 2.340	WB-SB	2.75	10	25	3.5	3.0	0.4	> 0.20	> 100	> 10
	TMW 2.1531	NB	2.75	10	20	4.0	3.0	0.3	> 0.20	> 100	> 10
	TMW 2.53	NB-SB	2.75	12	20	4.0	3.0	0.3	> 0.20	> 100	> 10
	TMW 2.54	SB	2.75	12	30	4.0	3.5	0.3	> 0.20	> 100	> 10
P. damnosus	TMW 2.1532	NB	3.75	14	10	2.0	2.0	0.1	0.08	30	> 10
	TMW 2.1533	SB	3.25	12	10	1.5	2.0	0.1	0.16	20	> 10
	TMW 2.1534	NB	3.75	8	10	2.0	2.0	0.1	0.04	10	> 10
	TMW 2.1535	SB	3.75	10	15	1.5	1.5	0.1	0.04	10	3
	TMW 2.1536	NB	3.50	8	5	2.0	2.0	0.1	0.08	10	3

	Strain	Category	pH	EtOH	Iso-α-acids	H_2O_2 ↑Mn^{2+}	H_2O_2 ↓ Mn^{2+}	DNP	CCCP	Cal.	Val.&Nig.
			pH	v/v %	ppm	mM	mM	mM	mM	µM	µM
L. backii	TMW 1.1430	SB	3.00	14	15	3.5	2.5	0.1	0.02	10	7
	TMW 1.1988	SB	3.25	16	30	2.5	2.5	0.2	0.16	40	> 10
	TMW 1.1989	SB	3.25	16	30	3.0	3.0	0.1	0.16	20	> 10
	TMW 1.1991	SB	3.00	16	NA	2.5	5.0	0.1	0.16	20	3
	TMW 1.1992	MB-SB	3.25	18	NA	2.5	5.0	0.2	0.08	20	2
L. lindneri	TMW 1.1285	MB	3.50	8	40	3.0	5.0	0.1	0.02	10	1
	TMW 1.1433	MB	3.25	10	25	4.0	4.0	0.2	0.04	10	1
	TMW 1.1993	NB-SB	3.50	10	> 50	3.5	4.0	0.2	0.08	80	7
	TMW 1.481	MB	3.25	8	15	3.5	5.0	0.2	0.02	10	3
L. paracoll.	TMW 1.1979	NB	3.25	14	5	2.0	1.5	0.2	0.06	50	10
	TMW 1.696	WB	3.25	10	5	1.5	1.0	0.1	0.04	> 100	3
	TMW 1.1994	SB	3.25	14	10	2.5	1.5	0.1	0.04	> 100	3
	TMW 1.1995	SB	3.25	14	10	1.0	1.0	0.1	0.04	20	3

Appendix 10: Genomes, obtained from NCBI GenBank (Burks et al., 1985; Clark et al., 2016), adduced for various comparative genome analyses. Strain designation, assembly accession number and assembly level according to NCBI.

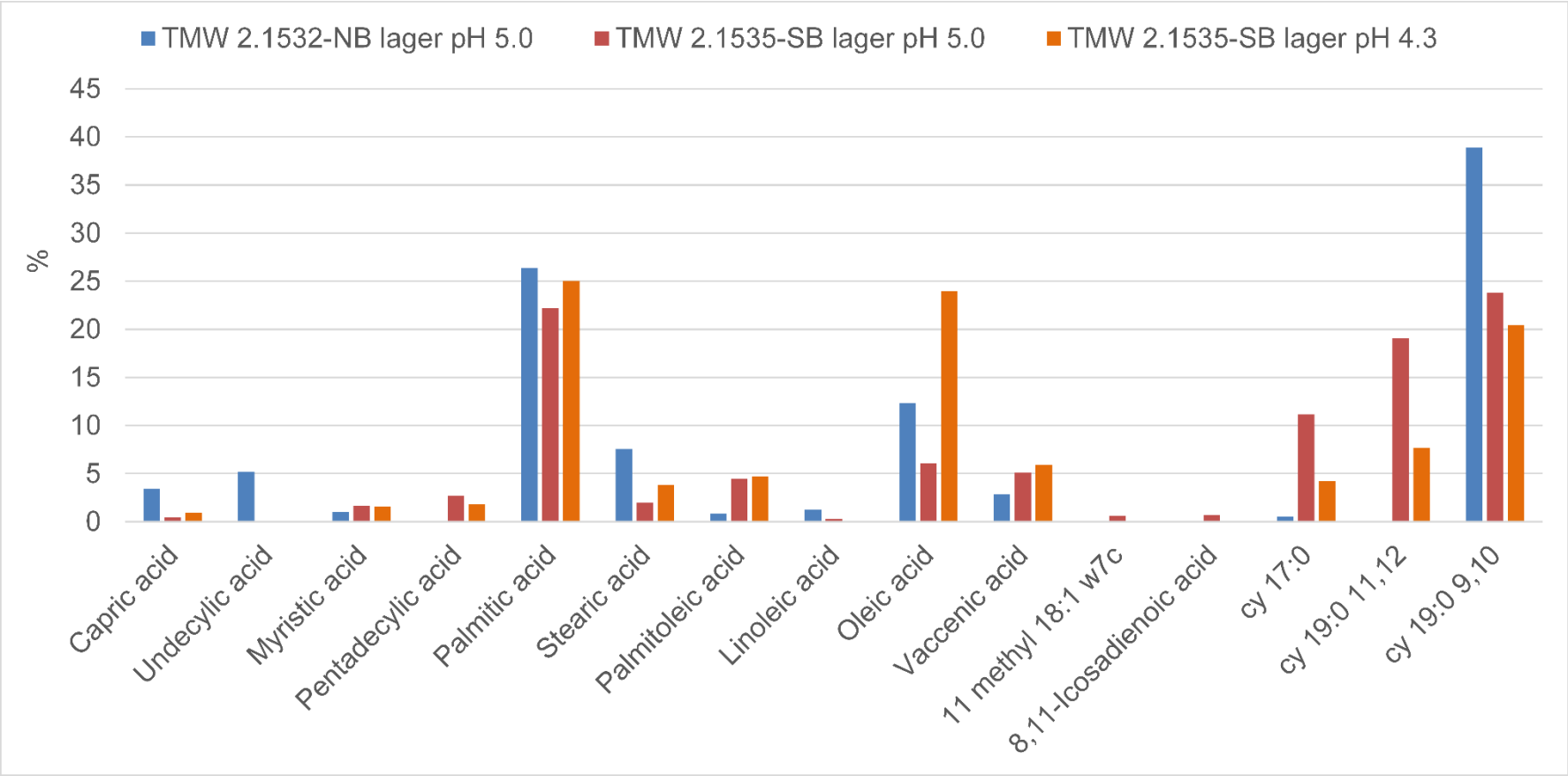
Species	Strain	Assembly accession	Assembly level
Lactobacillus acetotolerans	NBRC 13120	GCA_001042405.1	Complete Genome
Lactobacillus acidophilus	30SC	GCA_000191545.1	Complete Genome
Lactobacillus acidophilus	FSI4	GCA_000934625.1	Complete Genome
Lactobacillus acidophilus	La-14	GCA_000389675.2	Complete Genome
Lactobacillus acidophilus	NCFM	GCA_000011985.1	Complete Genome
Lactobacillus amylovorus	GRL 1112	GCA_000182855.2	Chromosome
Lactobacillus amylovorus	GRL1118	GCA_000194115.1	Complete Genome
Lactobacillus brevis	TMW 1.465	GCA_000833395.1	Contig
Lactobacillus brevis	TMW 1.313	GCA_000833405.1	Contig
Lactobacillus brevis	TMW 1.6	GCA_000833415.1	Contig
Lactobacillus brevis	ATCC 367	GCA_000014465.1	Complete Genome
Lactobacillus brevis	BSO 464	GCA_000807975.1	Chromosome
Lactobacillus brevis	KB290	GCA_000359625.1	Complete Genome
Lactobacillus buchneri	CD034	GCA_000298115.2	Complete Genome
Lactobacillus buchneri	NRRL B-30929	GCA_000211375.1	Complete Genome
Lactobacillus casei	12A	GCA_000309565.2	Complete Genome
Lactobacillus casei	ATCC 334	GCA_000014525.1	Complete Genome
Lactobacillus casei	BD-II	GCA_000194765.1	Complete Genome
Lactobacillus casei	BL23	GCA_000026485.1	Complete Genome
Lactobacillus casei	LC2W	GCA_000194785.1	Complete Genome
Lactobacillus casei	LcA	GCA_000400585.1	Chromosome
Lactobacillus casei	LcY	GCA_000388095.2	Chromosome
Lactobacillus casei	LOCK919	GCA_000418515.1	Complete Genome
Lactobacillus casei	Zhang	GCA_000019245.3	Complete Genome
Lactobacillus casei	ATCC 393	GCA_000829055.1	Complete Genome
Lactobacillus casei	W56	GCA_000318035.1	Complete Genome
Lactobacillus coryniformis	KCTC 3167	GCA_000166795.1	Scaffold

Species	Strain	Assembly accession	Assembly level
Lactobacillus crispatus	ST1	GCA_000091765.1	Chromosome
Lactobacillus bulgaricus	2038	GCA_000191165.1	Complete Genome
Lactobacillus bulgaricus	ATCC 11842	GCA_000056065.1	Complete Genome
Lactobacillus bulgaricus	ATCC BAA-365	GCA_000014405.1	Complete Genome
Lactobacillus bulgaricus	ND02	GCA_000182835.1	Complete Genome
Lactobacillus farciminis	CNCM-I-3699-S	GCA_001046795.1	Chromosome
Lactobacillus farciminis	CNCM-I-3699-R	GCA_001188635.1	Chromosome
Lactobacillus fermentum	3872	GCA_000466785.3	Complete Genome
Lactobacillus fermentum	CECT 5716	GCA_000210515.1	Complete Genome
Lactobacillus fermentum	F-6	GCA_000397165.1	Complete Genome
Lactobacillus fermentum	IFO 3956	GCA_000010145.1	Complete Genome
Lactobacillus gasseri	130918	GCA_000814885.1	Complete Genome
Lactobacillus gasseri	ATCC 33323	GCA_000014425.1	Complete Genome
Lactobacillus ginsenosidimutans	EMML 3141	GCA_001050475.1	Complete Genome
Lactobacillus helveticus	CNRZ32	GCA_000422165.1	Complete Genome
Lactobacillus helveticus	DPC 4571	GCA_000015385.1	Complete Genome
Lactobacillus helveticus	H10	GCA_000189515.1	Complete Genome
Lactobacillus helveticus	H9	GCA_000525715.1	Complete Genome
Lactobacillus helveticus	KLDS1.8701	GCA_000961015.1	Complete Genome
Lactobacillus helveticus	MB2-1	GCA_001006025.1	Complete Genome
Lactobacillus helveticus	R0052	GCA_000165775.3	Complete Genome
Lactobacillus hokkaidonensis	LOOC260	GCA_000829395.1	Complete Genome
Lactobacillus jensenii	JV-V16	GCA_000159335.1	Chromosome
Lactobacillus johnsonii	DPC 6026	GCA_000204985.1	Complete Genome
Lactobacillus johnsonii	FI9785	GCA_000091405.1	Complete Genome
Lactobacillus johnsonii	N6.2	GCA_000498675.1	Complete Genome
Lactobacillus johnsonii	NCC 533	GCA_000008065.1	Complete Genome
Lactobacillus kefiranofaciens	ZW3	GCA_000214785.1	Complete Genome
Lactobacillus koreensis	26-25	GCA_001050435.1	Complete Genome

Species	Strain	Assembly accession	Assembly level
Lactobacillus mucosae	LM1	GCA_000248095.3	Complete Genome
Lactobacillus paracasei	CAUH35	GCA_001191565.1	Complete Genome
Lactobacillus paracasei	L9	GCA_001244395.1	Complete Genome
Lactobacillus paracasei	N1115	GCA_000582665.1	Complete Genome
Lactobacillus paracasei	8700:2	GCA_000155515.2	Complete Genome
Lactobacillus paracasei	JCM 8130	GCA_000829035.1	Complete Genome
Lactobacillus pentosus	KCA1	GCA_000271445.1	Chromosome
Lactobacillus plantarum	16	GCA_000412205.1	Complete Genome
Lactobacillus plantarum	B21	GCA_000931425.1	Complete Genome
Lactobacillus plantarum	CMPG5300	GCA_000762955.1	Chromosome
Lactobacillus plantarum	JDM1	GCA_000023085.1	Complete Genome
Lactobacillus plantarum	CGMCC 1.557	GCA_001272315.1	Chromosome
Lactobacillus plantarum	P8	GCA_000392485.2	Complete Genome
Lactobacillus plantarum	ST-III	GCA_000148815.2	Complete Genome
Lactobacillus plantarum	WCFS1	GCA_000203855.3	Complete Genome
Lactobacillus plantarum	ZJ316	GCA_000338115.2	Complete Genome
Lactobacillus reuteri	IRT	GCA_001046835.1	Complete Genome
Lactobacillus reuteri	DSM 20016	GCA_000016825.1	Complete Genome
Lactobacillus reuteri	I5007	GCA_000410995.1	Complete Genome
Lactobacillus reuteri	JCM 1112	GCA_000010005.1	Complete Genome
Lactobacillus reuteri	SD2112	GCA_000159455.2	Complete Genome
Lactobacillus reuteri	TD1	GCA_000439275.1	Complete Genome
Lactobacillus rhamnosus	ATCC 8530	GCA_000233755.1	Complete Genome
Lactobacillus rhamnosus	ATCC 53103	GCA_000011045.1	Complete Genome
Lactobacillus rhamnosus	Lc 705	GCA_000026525.1	Complete Genome
Lactobacillus rhamnosus	LOCK900	GCA_000418475.1	Complete Genome
Lactobacillus rhamnosus	LOCK908	GCA_000418495.1	Complete Genome
Lactobacillus rossiae	DSM 15814	GCA_000428925.1	Scaffold
Lactobacillus ruminis	ATCC 27782	GCA_000224985.1	Complete Genome

Species	Strain	Assembly accession	Assembly level
Lactobacillus sakei	23K	GCA_000026065.1	Complete Genome
Lactobacillus salivarius	JCM1046	GCA_000758365.1	Complete Genome
Lactobacillus salivarius	CECT 5713	GCA_000143435.1	Complete Genome
Lactobacillus salivarius	Ren	GCA_001011095.1	Complete Genome
Lactobacillus salivarius	UCC118	GCA_000008925.1	Complete Genome
Lactobacillus sanfranciscensis	TMW 1.1304	GCA_000225325.1	Complete Genome
Lactobacillus sp.	wkB8	GCA_000761135.1	Complete Genome
Lactococcus lactis	IL1403	GCA_000006865.1	Complete Genome
Leuconostoc mesenteroides	ATCC 8293	GCA_000014445.1	Complete Genome
Pediococcus claussenii	ATCC BAA-344	GCA_000237995.2	Complete Genome
Pediococcus pentosaceus	ATCC 25745	GCA_000014505.1	Complete Genome
Pediococcus pentosaceus	SL4	GCA_000496265.1	Complete Genome

Appendix 11: Fatty acid composition of two *P. damnosus* strains after growth in beer. Two strains, with (TMW 2.1535-SB) and without (TMW 2.1532-NB) a plasmid-encoded type II fatty acid biosynthesis cluster, were analysed for their total fatty acid composition after growth in $lager_{pH5.0}$. TMW 2.1535-SB was also grown in $lager_{pH4.3}$ and analysed for cellular fatty acids. The proportion (%) of identified fatty acids is shown.



15 List of Publications and Student Theses

Peer-reviewed Journals

Jürgen Behr, Andreas J. Geissler, Patrick Preissler, Armin Ehrenreich, Angel Angelov, Rudi F. Vogel. Identification of ecotype-specific marker genes for categorization of beer-spoiling *Lactobacillus brevis*. Food Microbiol. 2015;51:130-138.

Andreas J. Geissler, Jürgen Behr, Kristina von Kamp, Rudi F. Vogel. Metabolic strategies of beer spoilage lactic acid bacteria in beer. Int J Food Microbiol 2016;216:60-68.

Jürgen Behr[x], Andreas J. Geissler[x], Jonas Schmid, Anja Zehe, Rudi F. Vogel. The Identification of Novel Diagnostic Marker Genes for the Detection of Beer Spoiling *Pediococcus damnosus* Strains Using the BlAst Diagnostic Gene findEr. Plos One 2016;11(3):e0152747.

[x] : joint first authorship

Oral presentations

Andreas J. Geissler, Jürgen Behr, Rudi F. Vogel. Lifestyle-Gene bierverderbender Milchsäurebakterien. Oral presentation at the Seminar Hefe und Mikrobiologie (Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität), Freising, Germany, 25.03.2015 to 26.03.2016.

Poster presentations

Andreas J. Geissler, Jürgen Behr, Rudi F. Vogel. Metabolic strategies of beer spoilage lactic acid bacteria. Poster presented at Young Scientist Symposium on Malting, Brewing and Distilling, Ghent, Belgium, 28.10.2014 to 30.10.2014.

Student theses

The following student theses were supervised. The resulting raw data were partially incorporated into this thesis with written permission by the respective students.

Bachelor theses:

Kristina von Kamp. Untersuchung von Wachstum und Metabolismus bierverderbender Milchsäurebakterien. 2014

Ferdinand Speer. Physiologie und Stresstoleranzen von bierverderbenden Milchsäurebakterien. 2014

Master theses:

Jonas Schmid. Untersuchungen von bierverderbenden Milchsäurebakterien mit Hilfe von MALDI-TOF-MS. 2014

Anja Zehe. Differenzierung bierverderbender Milchsäurebakterien mittels MALDI-TOF MS, PCR und Physiologie. 2015