TECHNISCHE UNIVERSITÄT MÜNCHEN

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Production of volatile metabolites in model fermentations and in raw sausages by *Lactobacillus sakei*

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Doctoral Thesis

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Contents

Abbrevations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
a_W	activity of water
c	concentration
cfu	colony forming unit
CoA	coenzym A
CO_2	carbon dioxide
c.rel.	relative concentration (related to the internal standard)
°C	degree Celsius
DNA	desoxyribonucleic acid
dNTPs	desoxyribonukleosidtriphosphates
et al.	et alii
eV	electrone volts
g	gram
GC	gas chromatography
h	hours
HCl	hydrocloric acid
HPLC	high performance liquid chromatography
H_2O bidest.	twice distilled water
H_2O dest.	distilled water
H_2SO_4	sulfuric acid
kb	kilo base
kg	kilogram
L	liter
μg	microgram
mg	milligram
min	minutes
ml	milliliter
μl	microliter
mM	nilli Mol

mmol	millimol
μmol	micromol
μm	micrometer
mMRS-medium	modified de Man Rogosa Sharpe medium
MS	mass spectrometry
NaCl	sodium chloride
NAD	nicotinamid adenine dinucleotide
NADH	reduced nicotinamid adenine dinucleotide
ng	nanogram
nm	nanometer
nmol	nanomol
OD	optical density
PCR	polymerase chain reaction
pH	pondus Hydrogenii
pK_a	acid dissociation constant
%	percent
PTS	phosphotransferase system
RAPD	random amplification of polymorphic DNA
rpm	rounds per minute
sec	seconds
SPME	solid phase micro extraction
subsp.	subspecies
TBE	tris boric acid ethylenediaminetetraacetic acid
TE	tris ethylenediaminetetraacetic acid
TMW	Technische Mikrobiologie Weihenstephan
TRIS	tris(hydroxymethyl)aminomethane
UV	ultraviolet
V	volts

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1 Introduction

1.1 Raw fermented sausages

1.1.1 Definition and history

Raw fermented or dry sausages are uncooked meat products and consist mostly of a mixture of lean meats and fat combined with salts, nitrate and/or nitrite, sugars and spices. Today meat starter cultures with defined properties are frequently used in the production processes. Different meats, calibers, degrees of chopping and production processes lead to a large variety of raw fermented sausage products (figure 1).

Figure 1: Different types of raw fermented sausages. (The figure was taken from the website: www.bleybestewurst.de).

Raw fermented sausages have been developed and produced for centuries in regions with moderate climates, especially in Europe. The most famous examples for dry sausages are the various types of sausages which were of Hungarian, German, Italian, French and Spanish origin. They are called salami, salchichón, saucisson, chorizo or pepperoni (Toldrá & Reig, 2007). The word salami comes from the Italian word "salame" which means salted meats (Hummerjohan, 2004).

During the ripening the a_W-values are reduced to 0.8 - 0.9 due to the water loss (Ziegler *et al.*, 1987; Astiasaran *et al.*, 1990; Fernández-Salguero *et al.*, 1994). The pH-values are usually below 5.0, while in south European countries a moderate

acidity with values of 5.0 - 5.5 are preferred (Acton & Dick, 1976; Astiasaran *et al.*, 1990).

Because cold temperatures (max. 20°C) are required for the fermentation and storage, the production was traditionally performed in winter months. The matured end-product is shelf-stable even under higher temperatures and can be stored over several weeks. This process was therefore used for long time preservation. Today the fermentations take place in air-conditioned rooms and therefore dry sausages are fabricated during the whole year and in all climates (Heinz & Hautzinger, 2007).

1.1.2 Raw materials, ingredients, production and fermentation

A variety of meat kinds can be used for raw fermented sausages and depends on regional traditions. The used meat sources are generally beef, pork, poultry, horse, donkey, camel, lamb or goat (Heinz & Hautzinger, 2007). A good hygienic quality is necessary to obtain a microbial safe end product. Raw fresh meat has usually a pH of 5.4 - 5.8 (Faustmann & Cassens, 1990). In most products pork back fat is used because it shows the lowest rancidity during long fermentation and storage processes (Heinz & Hautzinger, 2007). Raw fermented sausages are fabricated with 20 - 35 % fatty tissue and 65 - 80 % lean meat, from one or two meat types, e.g. beef and pork (Astiasaran *et al.*, 1990; Heinz & Hautzinger, 2007).

The addition of salt lowers the a_W-value of 0.96 - 0.97 in the sausage batter by absorbing water which presents an initial hurdle for spoilage and pathogenic bacteria (Astiasaran *et al.*, 1990; Heinz & Hautzinger, 2007). Also salt soluble proteins are extracted from the small lean meat particles. These solubilized or gelatinous proteins act like an adhesive between the interfaces of lean meat and fat particles in the sausage (Heinz & Hautzinger, 2007). Usually 26 - 30 g salt/kg sausage batter respectively 2.6 - 3.0 % are used (Astiasaran *et al.*, 1990; Heinz & Hautzinger, 2007; Toldrá & Reig, 2007). Due to the water loss the salt contents in ripened salamis are in the range of 3.03 - 6.14 % (Ziegler *et al.*, 1987; Casiraghi *et al.*, 1996; Samelis *et al.*, 1998). The salt is also used as a carrier for the curing agent sodium nitrite (Toldrá & Reig, 2007; Honikel, 2008). This additive is responsible for the red cured meat color and acts inhibitory against pathogenic bacteria. Nitrate can also be used, but it first has to be reduced to nitrite through bacterial activity. The nitrite is then reduced to nitric oxide which leads to the chemical curing reaction (Hutkins, 2006).

The addition of 0.2 - 0.7 % sugar leads to an accelerated growth of lactic acid bacteria (Lücke, 1994). Their sugar metabolism results in the accumulation of lactic

acid resulting in a low pH and the development of the typical flavor (Hutkins, 2006; Heinz & Hautzinger, 2007; Toldrá & Reig, 2007). Usually glucose and/or lactose are used. The addition of spices like pepper, garlic and paprika is used to improve the taste (Toldrá & Reig, 2007).

Figure 2: Typical ingredients for the production of salami (lean pork meat, minced beef, pork back fat, curing salt, starter culture, sugar mixed with pepper).

The production of traditional salami starts with the chopping of frozen pork meat followed by the pork back fat. Then the starter cultures together with sugar and spices are added. The chopping is done until the required particle size or granulation of the meat is reached. As next step minced beef is added and finally the curing salt. The sausage batter is then stuffed into casings. The stuffed sausages are then optionally immersed to potassium-sorbate to avoid fungal growth. Alternatively the sausages can be immersed in a mould bath for the growth of a desired coating (Hummerjohann, 2004; Heinz & Hautzinger, 2007; Toldrá & Reig, 2007). In the next few days the reddening of the meat takes place at temperatures of 20 - 25°C and a relative humidity of 90 % (Hummerjohann, 2004; Heinz & Hautzinger, 2007). Sausages without a mould coating can then optionally be treated with cold smoke. The non-smoked products are called "air dried" (Heinz & Hautzinger, 2007). The drying period takes several weeks at temperatures below 20°C and a relative humidity of 75 - 85 % (Hummerjohann, 2004; Heinz & Hautzinger, 2007). The fer-

mentation and drying times are determined by the sausages formulation and casing diameters (Heinz & Hautzinger, 2007; Toldrá & Reig, 2007). In small traditionally manufacturing processes the sausages mature up to several months, while the industrially fermented sausages are finished within three to four weeks (Heinz & Hautzinger, 2007). Figure 3 shows the manufacturing process.

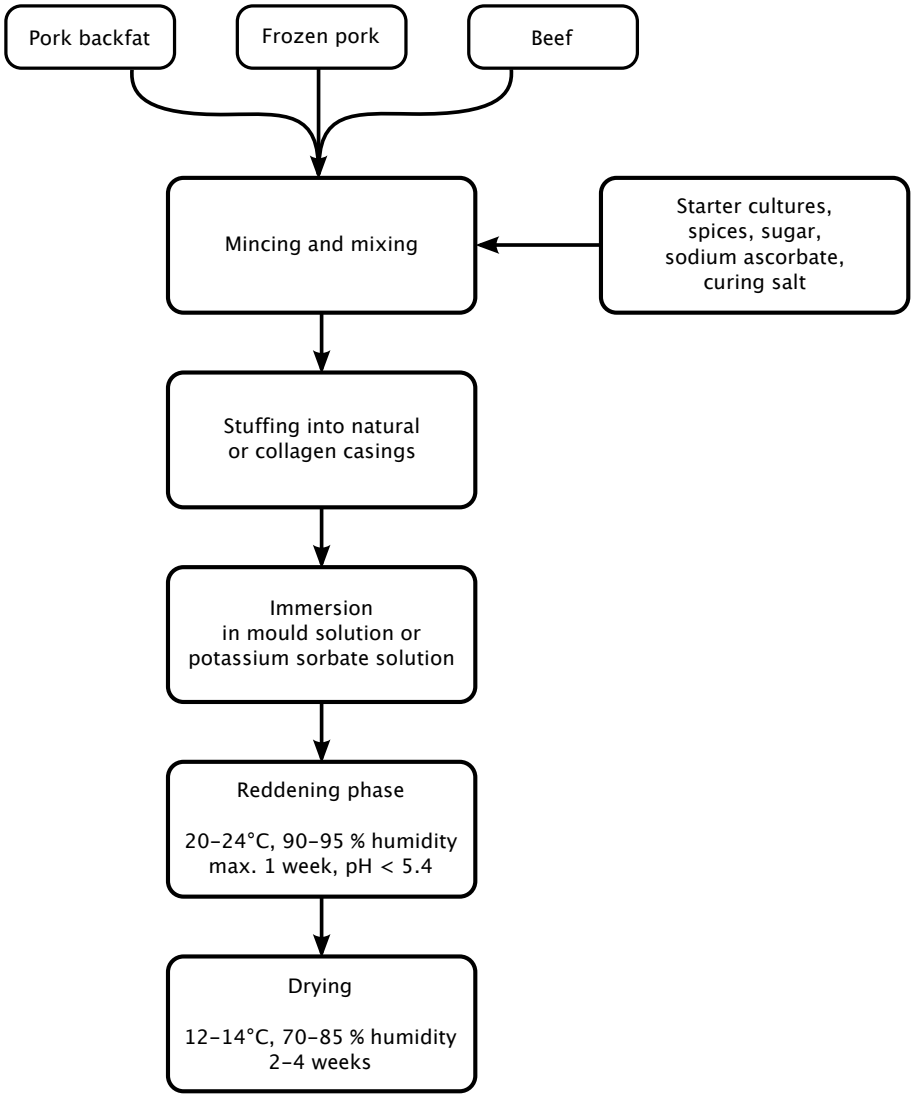


Figure 3: Manufacturing process of raw fermented sausages. (The figure was adapted from Hummerjohann, 2004).

1.2 Starter cultures for meat fermentations

In former times or in traditional processings raw sausages were fermented by the spontaneous house flora or inoculated by back slopping. However, today mainly starter cultures are used for the fermentation process. Bacteria species belonging to the genera *Lactobacillus*, *Pediococcus*, *Kocuria* and *Staphylococcus* are the dominant commercial starter cultures (Hammes *et al.*, 1990; Lücke, 1994; Hammes & Hertel,

1998). Most of these bacteria produce lactic acid and volatile flavor compounds. They are generally harmless in terms of the consumers' health and product spoilage and have therefore the QPS-status (Qualified Presumption of Safety) of the EFSA (European Food Safety Authority). Depending on the taste, texture and appearance of the desired product, specific cultures are selected. In most cases mixtures of different strains are used. Lactobacilli are used for a fast acidification while sausages made with pediococci showed a less acidic taste. Staphylococci are used for the fast reduction of nitrate and nitrite which leads to the stable red curing color and reduces the risk of fat rancidity. They also contribute to the development of flavor and texture by degradation of proteins and amino acids. The properties of bacteria used as meat starter cultures are listed in table 1.

Table 1: Properties of bacteria used as meat starter cultures. (Adapted from Hutkins, 2006).

Organism	Minimal temperature	Optimum temperature	Acid from glucose[1]	Nitrate reductase	Primary function
Lactobacillus sakei	4°C	32 - 35°C	+	-	acid production
Lactobacillus curvatus	4°C	32 - 35°C	+	-	acid production
Lactobacillus plantarum	10°C	42°C	+	-	acid production
Pediococcus pentosaceus	15°C	28 - 32°C	+	-	acid production
Pediococcus acidilacti	15°C	40°C	+	-	acid production
Kocuria varians	10°C	25 - 37°C	-	+	flavor
Staphylococcus carnosus	10°C	30 - 40°C	-	+	flavor
Staphylococcus xylosus	10°C	25 - 35°C	-	+	flavor

[1] Under anaerobic conditions

Additionally to the bacteria some strains of yeasts, mainly the genus *Debaromyces* and *Candida* and moulds, mainly the genus *Penicillium* are used in some manufacturing processes.

1.2.1 Lactobacilli

The genus *Lactobacillus* is the most abundant bacterial group in sausages. These are rod shaped gram-positive bacteria with a maximal size of 2 μm. The species *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum* and *Lactobacillus pentosus* are usually the dominant species on meat and selected strains are used as starter cultures.

L. sakei was first found and described as *L. sake* from the rice wine sake (Katagiri *et al*, 1934). Later it was renamed as *L. sakei* (Trüper & De' Clari, 1997). *L. sakei* and *L. curvatus* have been isolated from several raw fermented products of plant and animal origin, e.g. fermented vegetables, sourdough and smoked fish (Leroi *et al.*, 1998; Lyhs *et al.*, 1999; Lee *et al.*, 2005; Robert *et al.*, 2009). Nevertheless, the main habitats are meat products (Hammes *et al.*, 1990; Hugas *et al.*, 1993). In the year 1996 both species were splitted into two subspecies: *L. sakei* subsp. *sakei* and *L. sakei* subsp. *carnosus*; *L. curvatus* subsp. *curvatus* and *L. curvatus* subsp. *melibiosus* (Klein *et al.*, 1996).

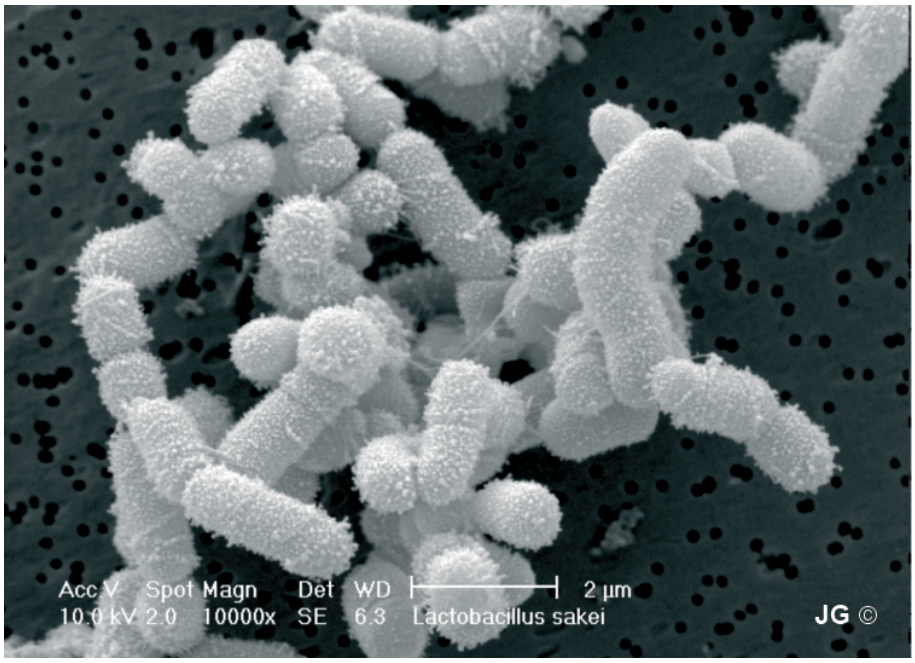


Figure 4: Scanning electron microscopic picture of *L. sakei* cells. (The picture was kindly provided by Jörg Groth, Stuttgart).

L. sakei and *L. curvatus* are well adapted to cured meat. They are able to grow up to 8 - 10 % salt, a pH of 4 - 5 and at temperatures of 10 - 15°C (Hugas *et al*, 1993; Coppola *et al.*, 1998; Samelis *et al.*, 1998; Papamanoli *et al.* 2003; Ammor *et al.*, 2005). Their metabolism is anaerobe, but they are aerotolerant due to a heme dependent catalase (Hertel *et al.*, 1998).

L. sakei and *L. curvatus* require glucose, maltose, mannose, ribose or sucrose as carbohydrate source for growth (Klein *et al.*, 1996; Lauret *et al.*, 1996; Veyrat *et al.*, 1996; Stentz *et al.*, 2001). The glucose is transported via the phosphoenolpyruvate-carbohydrate-phosphotransferase system into the cell and additionally by a PTS-independent permease (Lauret *et al.*, 1996). *L. sakei* and *L. curvatus* are facultative heterofermentative which leads to the production of lactic acid, ethanol, acetate and CO_2. This metabolic way gains 1 mol ATP/mol glucose. Both species are also able to use the homofermentative lactic acid fermentation which leads to the production of lactic acid as single product. This way provides 2 mol ATP/mol glucose. The chemical schemata of both pathways are shown in figure 5.

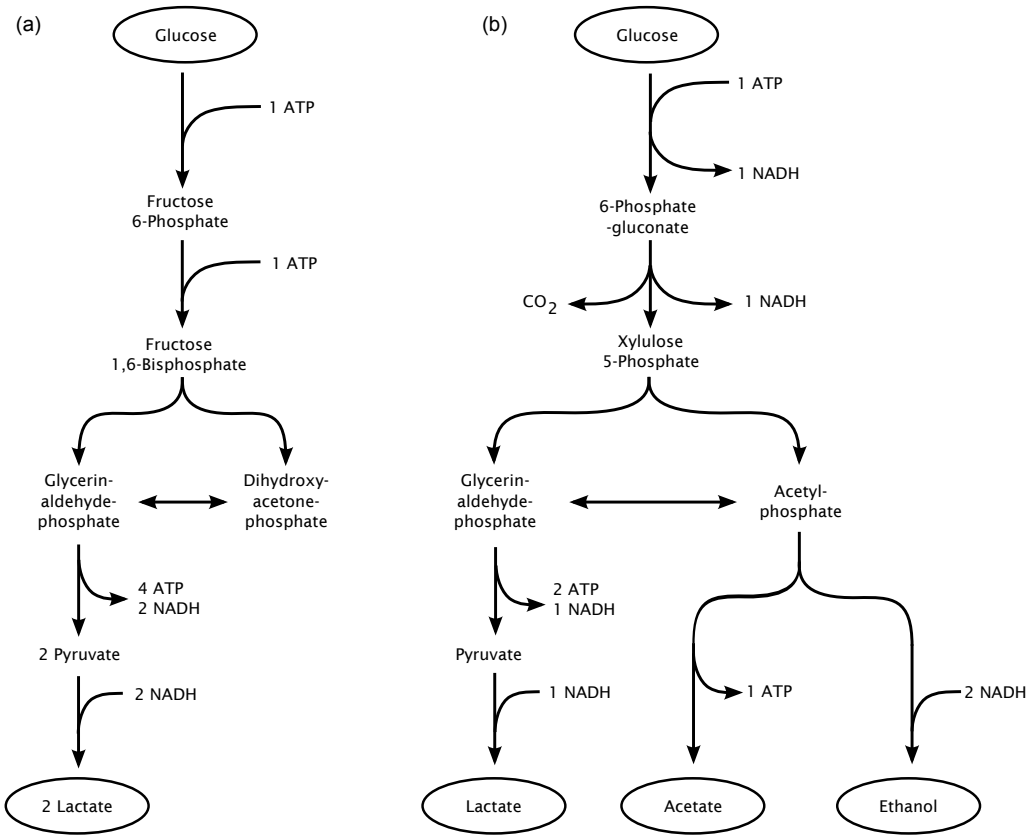


Figure 5: Lactic acid fermentation. (a) Homofermentative lactic acid fermentation. (b) Heterofermentative lactic acid fermentation. (Adapted from Hofvendahl & Hahn-Hägerdal, 2000).

In the early phase of sausage fermentation lactic acid bacteria contribute to the food safety and preservation due to the production of acids and the concomitant decrease of the pH. Additionally many gram-positive bacteria, such as lactic acid bacteria, produce bacteriocins (Tagg *et al.*, 1976; Klaenhammer, 1988). These are small, ribosomal synthesized, antimicrobial peptides or proteins. The majority of these bacteriocins cause membrane permeabilization, leading to the dissipation of membrane potential and the leakage of ions, ATP and other vital molecules from the target bacteria (Tagg *et al.*, 1976; Klaenhammer, 1988). *L. sakei* produces up to five different bacteriocins. These are sakacin A, G, K, P, and Q. *L. curvatus* produces curvacin A. These bacteriocins show high antimicrobial activity toward the food borne pathogen *Listeria monocytogenes* (Eijsink *et al.*, 1998; Katla *et al.*, 2001). Therefore bacteriocins or bacetriocin producing strains were often used as preservatives in the food industry (de Vyst & Leroy, 2007).

A large variety of strains are identified and collected. Some screening studies were performed concerning e.g. at acidification rates, growth under different conditions and/or utilization of different substrates (Ammor *et al.*, 2005; McLeod *et al.*, 2008). Further studies focussed at the genetic heterogeneity and/or the ability to produce biogenic amines (Berthier & Ehrlich, 1999; Bover-Cid *et al.*, 2001; Aymerich *et al.*, 2005; Chaillou *et al.*, 2009). These studies proofed that *Lactobacillus sakei* species are heterogenic, especially on genomic level. However, (based on these results) no studies investigated the capacity of the strains to produce flavor relevant volatile compounds and only little is known about the differences, which determine their applicability as meat starter.

1.2.2 Staphylococci

In sausages the most abundant species of staphylococci are *Staphylococcus carnosus* and *Staphylococcus xylosus*. *S. carnosus* was first isolated from dry fermented sausages and described by Schleifer & Fischer (1982). There are two subspecies known of *S. carnosus*: *S. carnosus* subsp. *carnosus* and *S. carnosus* subsp. *utilis*.

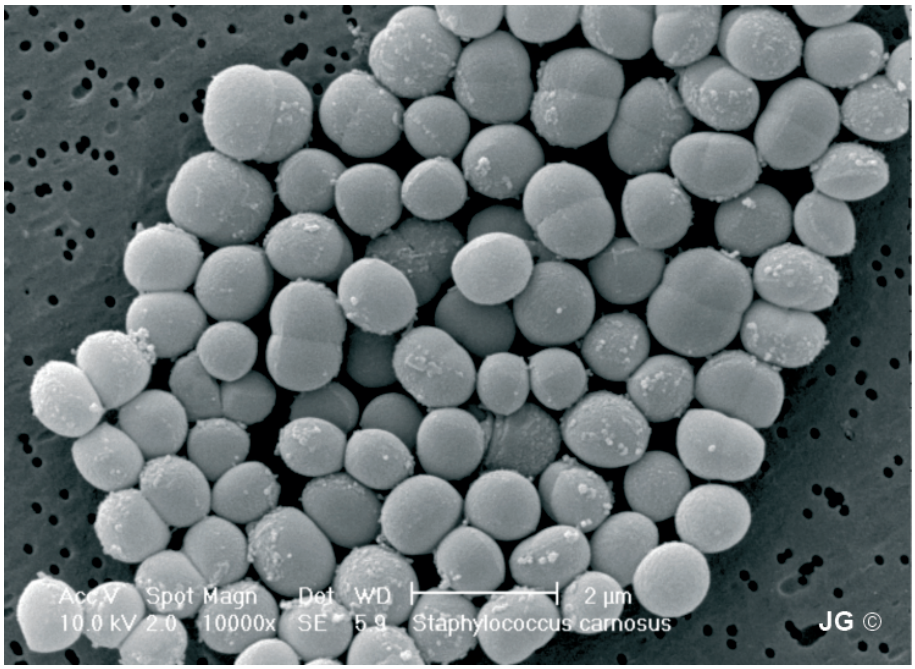


Figure 6: Scanning electron microscopic picture of *S. carnosus* cells. (Kindly provided by Jörg Groth, Stuttgart).

Staphylococci are coccoid shaped. They are facultatively anaerobic and are therefore metabolically active inside of sausages. Under anaerobic conditions they use nitrate as electron acceptor instead of oxygen. After depletion of nitrate the produced nitrite is further reduced to ammonium (Neubauer & Götz, 1996). The molecular and genetic characterization of the nitrate and nitrite reductase system of *S. xylosus* was done by Pantel *et al.* (1998) and Neubauer *et al.* (1999). The nitrite produced by the nitrate reductase of staphylococci is in the sausages further reduced to nitric oxide. This reacts in an acidic environment with myoglobin to nitrosyl-myoglobin. The nitrosyl-myoglobin is stable at room temperature and provides the desired bright red color of the meat product (Hutkins, 2006). The chemical schema of this reaction is shown in figure 7.

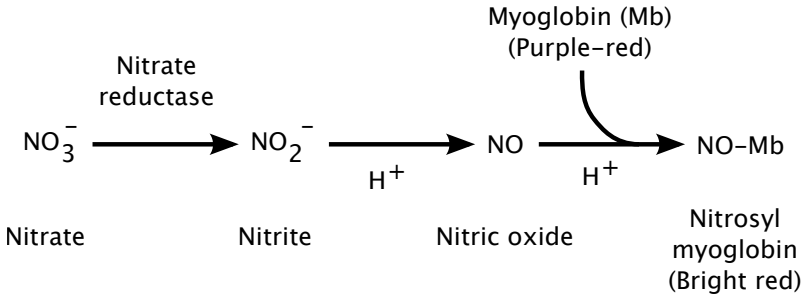


Figure 7: Nitrate and nitrite reaction in meat and formation of the red curing color. (Adapted from Hutkins, 2006).

Staphylococci contribute strongly to the aroma formation by their amino acid metabolism and lipolytic activities (Montel *et al.*, 1998).

1.2.3 Other meat starter microorganisms

The genus *Pediococcus* belongs together with the genera *Lactobacillus* and *Paralactobacillus* to the family of *Lactobacillaceae*. The most abundant species in the meat context are *P. acidilacti* and *P. pentosaceus*. They usually occur in form of diplococci or tetrads. They are anaerobic and have a homofermentative metabolism. Like other lactic acid bacteria some strains of pediococci produce a bacteriocin named pediocin which has its main inhibitory effect against *L. monocytogenes* (Foegeding *et al.*, 1992; Henderson *et al.*, 1992). In contrast to lactobacilli pediococci prefer higher temperatures and were therefore mainly used for fast and high temperature fermentations (Hammes & Knauf, 1994). In the United States pediococci are preferably used instead of staphylococci (Everson *et al.*, 1970; Smith & Palumbo, 1983; Hammes *et al.*, 1990).

Kocuria varians belongs to the family of *Micrococcaceae* and occurs in short chains or clusters. Like staphylococci it also produces the nitrate reductase and contributes to the reddening process of the meat product (Martín *et al.*, 2006).

The yeasts *Debaryomyces hansenii* and *Candida utilis* are also used as starter cultures for raw fermented sausages, mainly for Mediterranean style sausages with a moderate acidity. They can also contribute to the flavor generation by their amino acid metabolism (Olesen & Stahnke, 2000; Durá *et al.*, 2004).

Moulds of the genus *Penicillium* are often used as a surface mycoflora to prevent the growth of undesired moulds. Due to their lipolytic and proteolytic activity they also contribute to the flavor formation (Toledo *et al.*, 1997; Bruna *et al.*, 2001).

1.3 Metabolism of amino acids and its impact of fermented food and sausages

The bacterial metabolism of amino acids concerning to the production of volatile compounds is shown in figure 8. The details are described in the following sections.

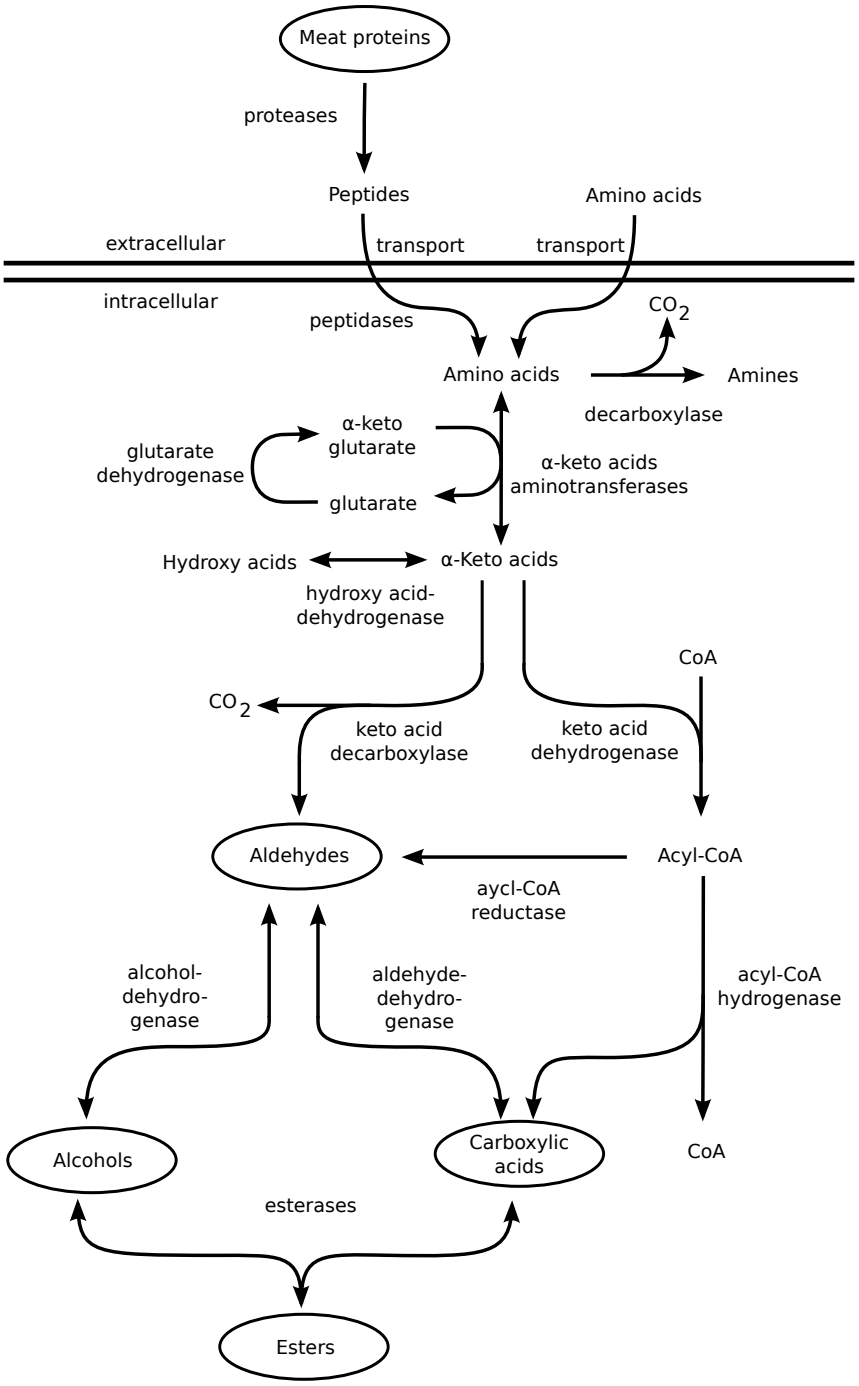


Figure 8: Amino acid metabolism leading to volatile flavor compounds in cheese and sausages. (Adapted from Helinck *et al.*, 2004; Smit *et al.*, 2005b; Liu *et al.*, 2008).

1.3.1 Transport of peptides and amino acids

Most lactic acid bacteria are deficient in the biosynthesis of several amino acids and therefore need a source in their habitat. *L. sakei* and *L. curvatus* require diverse amino acids for growth, e.g. lysine, methionine, leucine, isoleucine, tyrosine and valine (Lauret *et al.*, 1996; Møretrø *et al.*, 1998). The major part of amino acids in raw food material is bound in proteins. They are cleaved into peptides and amino acids by the peptidases of the raw material and/or the extracellular peptidases of the microorganisms and can then be transported into the cells. Peptidolytic activities are shown for *L. sakei* and *L. curvatus* (Fadda *et al.*, 1999). An overview of the known peptidases of lactic acid bacteria is given by Savijoki *et al.* (2006). Lactic acid bacteria have transporters for peptides as well as for single amino acids (Champomier-Vergès *et al.*, 2002; Kleerebezem *et al.*, 2003; Chaillou *et al.*, 2005).

1.3.2 Transaminase and decarboxylase reactions

The first intracellular step of the amino acid metabolism are either a decarboxylation producing biogenic amines or transamination producing α-keto acids. For the conversion of several types of amino acids like the branched-chain amino acids and aromatic amino acids specific transaminases are identified and characterized, mainly in lactococci (Gao & Steele, 1997; Yvon *et al*, 1997; Rijnen *et al*, 1999; Engels *et al.*, 2000; Yvon *et al.*, 2000). Screenings with different lactic acid bacteria showed that the transaminase activity is very low in most of the tested bacteria (Larrouture *et al.*, 2000; Smit *et al.*, 2004). The ability to produce biogenic amines is rather low in *L. sakei* and *L. curvatus* (Santos, 1998).

1.3.3 Degradation of α-keto acids to volatile compounds

The α-keto acids are the central intermediates in the catabolism of amino acids. They can be hydrogenated to the corresponding hydroxy acid, decarboxylated to aldehydes or converted to acyl-CoA by the keto acid dehydrogenase (Helinck *et al.*, 2004; Smit *et al.*, 2005b). The hydroxy acids are not part of the major flavor compounds and are not known precursors of flavor compounds (Yvon & Rijnen, 2001). Nevertheless several 2-hydroxy-acid dehydrogenases have been identified and characterised from lactic acid bacteria (Schütte *et al.*, 1984; Hummel *et al.*, 1985; Yamazaki & Maeda, 1986; Hummel *et al.*, 1988) and hydroxy acids were also found in some dairy fermentations (Gao *et al.*, 1998; Gummalla & Broadbent, 1999). Nevertheless,

this metabolic way is undesired in fermentation processes, because this hydrogenation leads to low α-keto acid concentrations, which are therefore less available for pathways leading to flavor compounds (Smit *et al.*, 2005b). No literature regarding the formation of hydroxy acids in fermented sausages can be found.

The α-keto acids can also be converted to aldehydes by a decarboxylase. In fermentation processes this pathway is more desired than the production of hydroxy acids, because aldehydes and the corresponding alcohols, carboxylic acids and esters belong to the main flavor compounds of fermented foods (Montel *et al.*, 1998; Smit *et al.*, 2005b; Longo & Sanromán, 2006). However the production of aldehydes is only described for some strains of lactic acid bacteria (Tucker & Morgan, 1967; Ayad *et al.*, 1999; Smit *et al.*, 2004). It seems that the aldehydes are used as electron acceptors to enable NAD recycling needed for the glycolysis and are immediately converted to the corresponding acids, or the α-keto acids are converted directly into the carboxylic acids by a keto acid dehydrogenase via acyl-CoAs. This seems to be the preferred pathway (Yvon & Rijnen, 2001; Thierry *et al.*, 2002; Helinck *et al.*, 2004; Smit *et al.*, 2005b).

An alcohol dehydrogenase is identified in many lactic acid bacteria (Zourari *et al.*, 1992; Nosova *et al.*, 2000; Jensen *et al.*, 2001). Although the conversation rates are rather low, alcohols and carboxylic acids can be connected to esters via esterases. An esterification activity was found in various lactic acid bacteria isolated from cheese including lactococci, lactobacilli, *Streptococcus thermophilus*, leuconostocs and pediococci (Liu *et al.*, 1998). Also staphylocci used in meat fermentations showed a strong esterification activity (Talon *et al.*, 1998; Casaburi *et al.*, 2006).

1.3.4 Aroma compounds of raw fermented sausages

The first studies concerning volatile compounds in food and raw fermented sausages were done in the 60s. Today more than 400 volatile compounds are described in raw fermented sausages (Schmidt & Berger, 1998; Edwards *et al.*, 1999; Meynier *et al.*, 1999; Ansorena *et al.*, 2001; Marco *et al.*, 2004). Since the 90s the method of gas-chromatography-olfactometry was established and the aroma relevant compounds could be identified from the large amount of volatile compounds and their olfactory thresholds could be identified (van Ruth, 2001; Marco *et al.*, 2007; d'Acampora *et al.*, 2008; Söllner & Schieberle, 2009; Gianelli *et al.*, 2011). The main flavor compounds of raw fermented sausages are listed in table 2.

Table 2: Main flavor compounds of raw fermented sausages as described by Montel *et al.*, 1998; Marco *et al.*, 2007; Söllner & Schieberle, 2009; Gianelli *et al.*, 2011 and Mair & Schieberle (personal communication).

Volatile compound	Flavor description	Olfactory threshold (μg/kg oil)	Probable origin of the compound
3-methylbutanal	malty	5.9	leucine
2-methylbutanal	malty	152	isoleucine
ethyl-2-methyl-propanoate	fruity	1.2 - 1.35	valine
ethyl-butanoate	fruity, strawberry	28 - 31	
ethyl-2-methyl-butanoate	fruity	0.26 - 0.28	isoleucine
ethyl-3-methyl-butanoate	fruity	0.68	leucine
hexanal	green, grassy, rancid	32 - 326	lipids and/or fatty acids
2-acetyl-1-pyrroline	roasty, popcorn	0.1	
nonanal	citrus, soapy, plastic	1000	lipids and/or fatty acids
acetic acid	vinegar, pungent	124 - 750	heterofermentative lactic acid fermentation
methional	cooked potato	0.2	methionine
(Z)-2-nonenal	fatty	4.9	lipids and/or fatty acids
(E)-2-nonenal	fatty, cucumber	900 - 978	lipids and/or fatty acids
propanoic acid	sweaty	3840	lipids and/or fatty acids
butanoic acid	rancid, cheesy	109 - 205	lipids and/or fatty acids
phenylacetaldehyde	floral	24 - 37	phenylalanine

Volatile compound	Flavor description	Olfactory threshold (μg/kg oil)	Probable origin of the compound
3-methylbutanoic acid	sweaty, dirty socks, cheesy	22 - 66	leucine
2-methylbutanoic acid	sweaty, dirty socks, cheesy	203	isoleucine
2-phenylethanol	honey, floral	367	phenylalanine
4-methylphenol	horse, manure	68	
2-phenylacetic acid	honey	360	phenylalanine
diacetyl	buttery	4.5 - 10	pyruvate metabolism

Table 2 shows that the flavor of raw fermented sausages is not determined by a single or only a few volatile compounds which provide the aroma impression of the sausage. It is rather a composition of many volatile compounds. Söllner & Schieberle (2009) showed that at least 33 different volatile compounds are necessary to imitate the flavor of salami. Table 2 also shows that some key aroma compounds seem to be derived from the involved microorganisms predominant from the amino acid metabolism. But in comparison to cheese products the formation of flavor compounds in sausages by microorganisms is less understood. In general the desired flavor of sausages can be archived by the selection of different kind of meats, fat contents, spices like pepper, garlic and paprika, and by the process, e.g. smoking, the use of moulds or the fermentation parameters. The other part of the flavor development is performed by the indigenous meat enzymes and the added microorganisms.

1.4 Aim of this study

The aim of this work was to screen various *L. sakei* strains for their ability to produce volatile compounds contributing to the aroma of fermented sausages. A main focus was on the metabolism of branched chain amino acids, their conversion to the corresponding volatile compounds and the identification of metabolic key reactions. Another item was to analyze the behavior of *L. sakei* during the fermentation of raw sausages focusing on bacterial growth, acidification and the production of volatile compounds. The third focus was the growth and the production of volatile compounds by *L. sakei* under stress conditions.

2 Materials and Methods

2.1 Materials

2.1.1 Devices, chemicals, bacterial strains etc.

Devices

Table 3: Devices

Devices	Model and Manufacturer
Gas chromatograph	Agilent Technologies 7890 A GC Systems, Santa Clara, CA, USA
Mass spectrometer	Agilent Technologies 5975 C VL MSD with triple axisdetector, Santa Clara, CA, USA
GC autosampler	Combi-Pal System, CTC Analytics AG, Zwingen, Switzerland
Capillary columm	Zebron/ ZB Wax; (length 60 m; diameter 0,25 mm; coat thickness 0,25 μm), Phenomenex, Torrance, CA, USA
SPME fiber	Assembly (23 Gaugen, 85 μm) carboxen/polydimethylsiloxane (CAR/PDMS), Supelco, Bellefonte, Pa, USA
GC-software	Agilent MSD ChemStation Software Rev. E. 02.00, Santa Clara, CA, USA
Database	NIST MS Search 2.0. 2002, Gaithersburg, MD, USA
HPLC autosampler	Gina 50, Gynkotek AG, Olten, Switzerland
HPLC columm	Rezex ROA-Organic Acid, Phenomenex, Torrance, CA, USA
HPLC columm heater	Gynkotek AG, Olten, Switzerland
HPLC UV-detector	Gynkotek AG, Olten, Switzerland
HPLC pump	680 HPLC Pump, Dionex GmbH, Idstein, Germany
Plate reader	Tecan Sunrise, TECAN Deutschland GmbH, Crailsheim, Germany
Plate reader-software	X Fluor 4, TECAN Deutschland GmbH, Crailsheim, Germany

Devices	Model and Manufacturer
Mincer	Le Hachoir, Tefal, Groupe SEB Deutschland GmbH, Offenbach/Main, Germany
Cutter	MTK 661, MADO Garant, Maschinenfabrik Dornhan GmbH, Dornhan, Germany
Sausage filler	HTW 6, Friedr. Dick GmbH & Co. KG, Deizisau, Germany
Conditioning cabinet	KBF 720, Binder GmbH, Tuttlingen, Germany
Ultraturrax	Art Miccra D-8, ART Prozess- & Labortechnik GmbH & Co. KG, Müllheim, Germany
pH-Meter	Knick pH 761 Calimatic, Knick elektronische Messgeräte GmbH & Co. KG, Berlin, Germany
Electrode	Mettler Toledo AG, Greifensee, Switzerland
Laminar flow sterile work bench	HERA safe, Heraeus Instruments GmbH, Hanau, Germany
Pipettes	Pipetman, Gilson S.A.S., Villiers-le-Bel, France
Vortexer	Vortex 2 Genie, Scientific Industries Inc., Bohemia, NY, USA
Incubator	Memmert INB series, Memmert GmbH & Co. KG, Schwabach, Germany
Autovlaves	2540 ELV, Systec GmbH, Wettenberg, Germany Varioklav, H + P Labortechnik, Oberschleißheim, Germany
Stirrer	RCT-Basic, IKA Labortechnik, IKA Werke GmbH & Co. KG, Staufen, Germany
Scales	SBA 52, Scaltec Instruments GmbH, Göttingen, Germany SI-234, Denver Instrument GmbH, Göttingen, Germany

Devices	Model and Manufacturer
Microscopes	Axiolab, Carl Zeiss MicroImaging GmbH, Göttingen, Germany Axiostar plus,Carl Zeiss MicroImaging GmbH, Göttingen, Germany
Camera	AxioCam ICc1, Carl Zeiss MicroImaging GmbH, Göttingen, Germany
Camera-software	AxioVision Rel. 4.7.2, Carl Zeiss MicroImaging GmbH, Göttingen, Germany
Water bath	MD 12 Lauda, LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, Germany
Centrifuges	Z 216 K, Hermle Labortechnik GmbH, Wehingen, Germany Z 383 K Hermle Labortechnik GmbH, Wehingen, Germany
PCR-cycler	Eppendorf Mastercycler gradient, Eppendorf AG, Hamburg, Germany
Microwave	LG, Intellowave, Willich, Germany
Gel electrophoresis	25 x 20 cm, Easy Cast electrophoresis system, Owl Separation Systems, Portsmouth, NH, USA
Power supply	LKB Bromma 2197
UV table	Herolab UVT 28M, Herlab GmbH Laborgeräte, Wiesloch, Germany
Gel documentation system	Intas GDS, Göttingen, Germany

Expendable materials

Table 4: Expendable materials

Materials	Type and Manufacturer
GC-vials	20 ml, VWR International GmbH, Darmstadt, Germany
Magnetic GC-caps with septum	Butyl/PTFE, VWR International GmbH, Darmstadt, Germany
HPLC-vials	1.5 ml, TECHLAB GmbH, Erkerode, Germany
HPLC-crimp-caps with septum	PTFE, TECHLAB GmbH, Erkerode, Germany
Microtiter plates	multi well plate 96-well flat bottom with lid, Sarstedt AG & Co., Nümbrecht, Germany
Reaction tubes	200 μl, 1.5 ml, 2 ml, Eppendorf AG, Hamburg, Germany
Sterile tubes	5 ml, 15 ml, 50 ml, Sarstedt AG & Co., Nümbrecht, Germany
Syringes	20 ml, BD Discardit, Becton Dickinson GmbH, Heidelberg, Germany
Sterile filter	Filtropur S 0.2 (0.2 μm), Sarstedt AG & Co., Nümbrecht, Germany
Microscope slides and cover glasses	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
Fibrous casings	caliber 55, Wiberg GmbH, Salzburg, Austria caliber 55, Walsroder FR natur, Case Tech GmbH, Walsrode, Germany

Chemicals

All used chemicals are listed in the table 5 in alphabetical order.

Table 5: Chemicals

Chemical	Purity and Manufacturer
Acetic acid	p.a., 100 %, Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acetoin	purum, $\geq$ 97 %, Fluka, Sigma-Aldrich GmbH, Steinheim, Germany
Agar	Difco, BD Sciences, Becton Dickinson GmbH, Heidelberg, Germany
Agarose	for gel electrophoresis, Biozym Scientific GmbH, Hessisch Oldendorf, Germany
α-Ketoisocaproic acid	Sigma-Aldrich GmbH, Steinheim, Germany
α-Ketoisovaleric acid	purum, $\geq$ 97 %, Fluka, Sigma-Aldrich GmbH, Steinheim, Germany
α-Keto-3-methyl pentanoic acid	$\geq$ 98 %, Sigma-Aldrich GmbH, Steinheim, Germany
Ammonium chloride	p.a., $\geq$ 99,5 %, Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Baird-Parker-Agar	Merck KGaA, Darmstadt, Germany
Boric acid	$\geq$ 99.5 %, Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bromphenol blue	sodium salt, AppliChem GmbH, Darmstadt, Germany
Butanoic acid	$\geq$ 99 %, Merck KGaA, Darmstadt, Germany
Chloramphenicol	$\geq$ 98 %, Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Curing salt	Metzgerei Häuslmair, Kirchdorf, Germany
Cyano-cobalamin	99 %, Sigma-Aldrich GmbH, Steinheim, Germany
D-(+)-Gluconic acid δ-lactone	purum, $\geq$ 99 %, Sigma-Aldrich GmbH, Steinheim, Germany
Diacetyl	puriss, p.a., 99 %, Fluka, Sigma-Aldrich GmbH, Steinheim, Germany
1,2-Dimethoxy-ethane	puriss, p.a., Sigma-Aldrich GmbH, Steinheim, Germany

Chemical	Purity and Manufacturer
Dimethyldisulfide	≥ 99 %, Sigma-Aldrich GmbH, Steinheim, Germany
Dimidiumbromide	≥ 98 %, Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dipotassiumhydrogen-phosphate-Trihydrate	p.a., Merck KGaA, Darmstadt, Germany
Egg yolk telluride	Sterile, 20 %, Merck KGaA, Darmstadt, Germany
Ethanol	VWR International GmbH, Darmstadt, Germany
Ethylenediaminetetra-acetic acid	≥ 98 %, Riedel-de Haën GmbH, Seelze, Germany
Folic acid	p. a., Sigma-Aldrich GmbH, Steinheim, Germany
Glucose	D(+)-Glucose-Anhydrate, Merck KGaA, Darmstadt, Germany
Glycerol	99.5 %, Gerbu Biotechnik, GmbH, Gaiberg, Germany
2,3-Heptanedione	≥ 97 %, SAFC, Sigma-Aldrich GmbH, Steinheim, Germany
2,3-Hexanedione	≥ 90 %, SAFC, Sigma-Aldrich GmbH, Steinheim, Germany
Hydrochloric acid	Merck KGaA, Darmstadt, Germany
Isoleucine	98 %, Merck KGaA, Darmstadt, Germany
L(+)-Ascorbic acid	sodium salt, ≥ 99 %, Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L-Cysteinhydro-chloride-Monohydrate	≥ 98,5 %, Carl Roth Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L-Leucine	≥ 99 %, Merck KGaA, Darmstadt, Germany
L-Valine	≥ 99 %, Fluka, Sigma-Aldrich GmbH, Steinheim, Germany
Magnesium sulfate-Heptahydrate	p.a., Merck KGaA, Darmstadt, Germany
Maltose	Maltose (Monohydrate), Merck KGaA, Darmstadt, Germany
Manganese(II)-Sulfate-Monohydrate	p.a., Merck KGaA, Darmstadt, Germany

Chemical	Purity and Manufacturer
Meat extract	dry granulated, Merck KGaA, Darmstadt, Germany
Methanol	Carl Roth Carl Roth GmbH & Co. KG, Karlsruhe, Germany
2-Methylbutanal	$\geq$ 90 %, Fluka, Sigma-Aldrich GmbH, Steinheim, Germany
3-Methylbutanal	$\geq$ 97 %, Sigma-Aldrich GmbH, Steinheim, Germany
2-Methylbutanoic acid	$\geq$ 98 %, Sigma-Aldrich GmbH, Steinheim, Germany
3-Methylbutanoic acid	$\geq$ 99 %, Sigma-Aldrich GmbH, Steinheim, Germany
2-Methyl-1-butanol	Sigma-Aldrich GmbH, Steinheim, Germany
3-Methyl-1-butanol	Sigma-Aldrich GmbH, Steinheim, Germany
2-Methylbutylacetate	$\geq$ 99 %, SAFC, Sigma-Aldrich GmbH, Steinheim, Germany
3-Methylbutylacetate	puriss, p.a., $\geq$ 99 %, Fluka, Sigma-Aldrich GmbH, Steinheim, Germany
2-Methylbutyl-2-methylbutyrate	$\geq$ 90 %, SAFC, Sigma-Aldrich GmbH, Steinheim, Germany
3-Methylbutyl-3-methylbutyrate	$\geq$ 98 %, SAFC, Sigma-Aldrich GmbH, Steinheim, Germany
2-Methylpropanal	$\geq$ 99 %, Sigma-Aldrich GmbH, Steinheim, Germany
2-Methylpropanoic acid	$\geq$ 97 %, Sigma-Aldrich GmbH, Steinheim, Germany
2-Methyl-1-propanol	Sigma-Aldrich GmbH, Steinheim, Germany
2-Methylpropyl-2-methylpropanoate	$\geq$ 99 %, SAFC, Sigma-Aldrich GmbH, Steinheim, Germany
Nicotinic acid	p.a., Sigma-Aldrich GmbH, Steinheim, Germany
Panthotenic acid	p.a., Sigma-Aldrich GmbH, Steinheim, Germany
Pepper	white, Ostmann Gewürze GmbH, Dissen a.T.W., Germany
Peptone	from caseine, pancreatically digested, granulated, Merck KGaA, Darmstadt, Germany

Chemical	Purity and Manufacturer
Perchloric acid	p.a., 70 %, Merck KGaA, Darmstadt, Germany
Potassium-dihydrogenphosphate	p.a., ≥ 99 % Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Potassium sorbate	purum, p.a., Sigma-Aldrich GmbH, Steinheim, Germany
Pyridoxin-HCl	Fluka, Sigma-Aldrich GmbH, Steinheim, Germany
Sodium chloride	p.a., ≥ 99 %, Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium hydroxide	p.a., Merck KGaA, Darmstadt, Germany
Sodium nitrate	p.a., Merck KGaA, Darmstadt, Germany
Sodium nitrite	p.a., ≥ 99 %, Merck KGaA, Darmstadt, Germany
Sucrose	Sigma-Aldrich GmbH, Steinheim, Germany
Sulfuric acid	p.a., 95 - 97 %, Merck KGaA, Darmstadt, Germany
Thiamin	Fluka, Sigma-Aldrich GmbH, Steinheim, Germany
Tris	ultra pure, MP Biomedicals, Solon, Ohio, USA
Tris-HCl	≥ 99.75 %, Gerbu Biotechnik, GmbH, Gaiberg, Germany
Tween 80	Tween 80 Polyethylene sorbitan monooleate, Gerbu Biotechnik GmbH, Gaiberg, Germany
Yeast extract	granulated, Merck KGaA, Darmstadt, Germany

Meat

Table 6: Meat

Meat	Origin
Lean pork	Metzgerei Häuslmair, Kirchdorf, Germany
Pork back fat	Metzgerei Häuslmair, Kirchdorf, Germany
Beef	Metzgerei Häuslmair, Kirchdorf, Germany

Kits for DNA-extraktion and -purification and PCR

Table 7: Kits for DNA-extraction and -purification and PCR

Purpose	Kit and Manufacturer
DNA-extraction	*E.Z.N.A*^{TM} Bacterial DNA Kit Prouct No. D3350-02, Omega Bio-Tek Inc., Norcross, GA, USA
DNA-purification	QIAquick QIAGEN PCR Purification Kit (250), Qiagen, Hilden, Germany
DNA-polymerase	Taq Core Kit, MP Biomedicals, Solon, Ohio, USA

Primer

Table 8: Primer (purchased from MWG-Biotech AG, Ebersberg, Germany)

Primer	Sequence
616v	5'-AGA GTT TGA T(CT)(AC) TGG CTC AG-3'
609r	5'-CA(GT) AAA GGA GGT GAT CC-3'
M13V	5'-GTT TTC CCA GTC ACG AC-3'

Bacterial Strains

Table 9: Bacterial Strains

Species	Strain number	Origin
Lactobacillus sakei	TMW 1.2	CTC 372, isolate from raw sausage (Spain)
Lactobacillus sakei	TMW 1.3	CTC 460, isolate from raw sausage (Spain)
Lactobacillus sakei	TMW 1.4	CTC 494 (new), CTC A4 (old), isolate from raw sausage (Spain)

Species	Strain number	Origin
Lactobacillus sakei	TMW 1.13	starter culture for cured meats
Lactobacillus sakei	TMW 1.22	LTH 677
Lactobacillus sakei	TMW 1.23	LTH 673, isolate from raw sausage
Lactobacillus sakei	TMW 1.30	
Lactobacillus sakei	TMW 1.46	LTH 678
Lactobacillus sakei	TMW 1.114	LTH 1641
Lactobacillus sakei	TMW 1.147	CTC 335
Lactobacillus sakei	TMW 1.148	CTC 341
Lactobacillus sakei	TMW 1.149	CTC 415
Lactobacillus sakei	TMW 1.150	CTC 430
Lactobacillus sakei	TMW 1.151	CTC 431
Lactobacillus sakei	TMW 1.152	CTC 439
Lactobacillus sakei	TMW 1.153	CTC 442
Lactobacillus sakei	TMW 1.154	CTC 445
Lactobacillus sakei	TMW 1.155	CTC 461
Lactobacillus sakei	TMW 1.161	LTH 1651
Lactobacillus sakei	TMW 1.162	
Lactobacillus sakei	TMW 1.163	
Lactobacillus sakei	TMW 1.165	
Lactobacillus sakei	TMW 1.402	LTH 2068, isolate from sauerkraut
Lactobacillus sakei	TMW 1.411	LTH 1756, isolate from sauerkraut
Lactobacillus sakei	TMW 1.412	LTH 1768, isolate from sauerkraut
Lactobacillus sakei	TMW 1.417	LTH 312, from sausage starter preparation
Lactobacillus sakei	TMW 1.454	LTH 673
Lactobacillus sakei	TMW 1.578	
Lactobacillus sakei	TMW 1.579	
Lactobacillus sakei	TMW 1.587	
Lactobacillus sakei	TMW 1.588	

Species	Strain number	Origin
Lactobacillus sakei	TMW 1.589	LTH 1182
Lactobacillus sakei	TMW 1.1189	DSM 20017T, LMG 9468, ATCC 15521
Lactobacillus sakei	TMW 1.1239	isolate from sourdough
Lactobacillus sakei	TMW 1.1240	isolate from sourdough
Lactobacillus sakei	TMW 1.1290	isolate from raw sausage
Lactobacillus sakei	TMW 1.1322	INRA 23 K, Institut National de la Recherche Agronomique, France
Lactobacillus sakei	TMW 1.1366	starter culture for Salami
Lactobacillus sakei	TMW 1.1383	LTH 681, starter culture for Salami
Lactobacillus sakei	TMW 1.1285	starter culture for Salami
Lactobacillus sakei	TMW 1.1386	LTH 5754, starter culture for Salami
Lactobacillus sakei	TMW 1.1388	LTH 3816, starter culture for Salami
Lactobacillus sakei	TMW 1.1392	LTH 1195, starter culture for Salami
Lactobacillus sakei	TMW 1.1393	starter culture for Salami
Lactobacillus sakei	TMW 1.1394 *Lactobacillus pentosus/plantarum*	starter culture for Salami
Lactobacillus sakei	TMW 1.1388	LTH 1189, starter culture for Salami
Lactobacillus sakei	TMW 1.1396	LTH 2389, starter culture for Salami
Lactobacillus sakei	TMW 1.1397	LTH 680, starter culture for Salami
Lactobacillus sakei	TMW 1.1398	LTH 1176, starter culture for Salami
Lactobacillus sakei	TMW 1.1399	LTH 1183, starter culture for Salami

Species	Strain number	Origin
Lactobacillus sakei	TMW 1.1474	LTH 2076
Lactobacillus sakei	TMW 1.1407	isolate from fermented fresh water fish (Norway)
Lactobacillus curvatus	TMW 1.7	DSM 20019, LTH 469
Lactobacillus curvatus	TMW 1.17	LTH 1174, isolate from raw sausage
Lactobacillus curvatus	TMW 1.27	
Lactobacillus curvatus	TMW 1.48	LTH 1635
Lactobacillus curvatus	TMW 1.49	LTH 1636
Lactobacillus curvatus	TMW 1.50	LTH 1637
Lactobacillus curvatus	TMW 1.51	LTH 1638
Lactobacillus curvatus	TMW 1.158	LTH 1649
Lactobacillus curvatus	TMW 1.166	
Lactobacillus curvatus	TMW 1.167	LTH 3265
Lactobacillus curvatus	TMW 1.401	LTH 2053, isolate from sauerkraut
Lactobacillus curvatus	TMW 1.407	LTH 1852, isolate from sauerkraut
Lactobacillus curvatus	TMW 1.408	LTH 1853, isolate from sauerkraut
Lactobacillus curvatus	TMW 1.421	LTH 1157, isolate from sausage material
Lactobacillus curvatus	TMW 1.439	LTH 683, isolate from Mettwurst
Lactobacillus curvatus	TMW 1.440	LTH 684, isolate from Hungarian Salami
Lactobacillus curvatus	TMW 1.591	
Lactobacillus curvatus	TMW 1.593	
Lactobacillus curvatus	TMW 1.594	
Lactobacillus curvatus	TMW 1.595	
Lactobacillus curvatus	TMW 1.596	
Lactobacillus curvatus	TMW 1.624	isolate from Italian raw sausage
Lactobacillus curvatus	TMW 1.1291	isolate from raw sausage

Species	Strain number	Origin
Lactobacillus curvatus	TMW 1.1365	
Lactobacillus curvatus	TMW 1.1381	LTH 2028, starter culture for Salami
Lactobacillus curvatus	TMW 1.1382	LTH 2029, starter culture for Salami
Lactobacillus curvatus	TMW 1.1384	LTH 1173, starter culture for Salami
Lactobacillus curvatus	TMW 1.1389	LTH 2001, starter culture for Salami
Lactobacillus curvatus	TMW 1.1390	LTH 1899, starter culture for Salami
Lactobacillus curvatus	TMW 1.1391	LTH 4261, starter culture for Salami
Lactobacillus farciminis	TMW 1.68	DSM 20184
Lactobacills paracasei	TMW 1.1387	LTH 2579
Pediococcus pentosaceus	TMW 2.800	
Kocuria varians	TMW 2.802	LTH 1529
Staphylococcus carnosus	TMW 2.801	LTH 3727
Lactobacillus spec.	TMW 1.191	

2.1.2 Media and solutions

If not explicitly noted the media and solutions were autoclaved and then stored at room temperature.

Modified mMRS-medium

Table 10: Modified mMRS-medium

Ingredients	Amount
Peptone	10 g
Yeast extract	5 g
Meat extract	5 g
Dipotassiumhydrogenphosphate-Trihydrate	4 g
Potassiumdihydrogenphosphate	2.6 g
Ammoniumchloride	3 g
L-Cysteinhydrochloride-Monohydrate	0.5 g
Tween 80	1 g
Agar (only for plates)	15 g
Maltose	10 g
Glucose	5 g
H_2O dest.	1 L

The sugar compounds were autoclaved separately and mixed after cooling. 1 ml Mg/Mn-standard-solution and 1 ml vitamin-mix were added. For the experiments analysing the volatile compounds only 3 g/L glucose and no maltose were used. For the bacteriocin assay, the covering agar was prepared with 7,5 g/L agar. The agar plates were stored at 4°C.

Magnesium-Manganese-standard-solution

Table 11: Magnesium-Manganese-standard-solution

Ingredients	Amount
Magnesium Sulfate-Heptahydrate	100 g
Manganese(II)-Sulfate-Monohydrate	50 g
H_2O dest.	1 L

The solution was sterilised by filtration through a filter with a pore size of 0.2 μm.

Vitamin-Mix

Table 12: Vitamin-Mix

Ingredients	Amount
Cobalamine	0.2 g
Folic acid	0.2 g
Nicotinic acid	0.2 g
Pantothenic acid	0.2 g
Pyridoxine-HCl	0.2 g
Thiamine	0.2 g
H_2O dest.	1 L

The solution was sterilised by filtration through a filter with a pore size of 0.2 μm. The storage was at -20°C.

Modified mMRS-medium containing amino acids and α-ketoacids

The modified mMRS-medium containing 0.3 % glucose was supplemented either with 25 mM leucine, isoleucine, valine as well as the corresponding α-ketoacids α-ketoisocaproic acid, α-keto-3-methylpentanoic acid and α-ketoisovaleric acid used. These media were sterilised by filtration through a filter with a pore size of 0.2 μm.

Modified mMRS-medium for growth curves and stress responses

The mMRS-medium containing 0.3 % glucose was used. As factors for stress salt and curing salt (concentrations 0 - 5 %), sodium nitrate (0 - 5 %), sodium nitrite (0 - 0.5 %) and different pH-value (pH 4 - 6.75) were used. The media containing sodium nitrite were sterilised by filtration through a filter with a pore size of 0.2 μm.

Internal Standard

1,2-Dimethoxy-ethane was mixed with H_2O dest. and diluted to a concentration of 1.924 μg/ml. This stock solution was stored at -20°C.

Baird-Parker-Agar

This agar was prepared in accordance to the manufacturer's instructions. Plates were stored at 4°C.

Glycerol stock solution

Table 13: Gycerol stock solution

Ingredients	Amount
Glycerol	80 % vol
H_2O dest.	20 % vol

TE-Buffer

Table 14: TE-Buffer

Ingredients	Amount
Tris-HCl	10 mM
Ethylenediaminetetraacetic acid	1 mM

10 x TBE Buffer

Table 15: 10 x TBE-Buffer

Ingredients	Amount
Tris	108 g
Boric acid	55 g
Ethylenediaminetetraacetic acid (0.5 M; pH 8)	40 ml
H_2O bidest.	ad 1 L

The working solution was diluted to 0.5 x with H_2O bidest.

Agarose gel

Table 16: Agarose gel

Ingredients	Amount
Agarose	3.5 g
0.5 x TBE-Buffer	270 ml

The agarose solution was heated in the microwave until the agarose was solved and transferred to the gel chamber.

Loading Dye

Table 17: Loading Dye

Ingredients	Amount
Sucrose	400 g
Bromphenol blue	2,5 g
H_2O bidest.	1 L

The loading dye was used without any kind of sterilisation.

16S-PCR

Table 18: 16S-PCR

Ingredients	Amount
H_2O bidest.	43.25 μl
10 x Buffer with magnesium chloride	5 μl
dNTPs	1 μl
Primer 616v	0.25 μl
Taq-polymerase	0.25 μl
DNA sample	1 μl

The PCR-solution was mixed directly before use.

RAPD-PCR

Table 19: RAPD-PCR

Ingredients	Amount
H_2O bidest.	32.2 μl
10 x Buffer	5 μl
Magnesium chloride	10 μl
dNTPs	2 μl
Primer M13V	0.5 μl
Taq-polymerase	0.3 μl
DNA sample	0.5 μl

The PCR-solution was mixed directly before use.

2.2 Methods

2.2.1 Growth conditions and storage

Bacteria were routinely grown at 30°C in modified mMRS medium and on agar plates. Bacterial stock solution were prepared with 1 ml glycerol stock solution, mixed with 0.8 ml grown bacteria and stored at -80°C.

2.2.2 Model fermentations

Model fermentations for the analysis of the volatile compounds were performed in modified mMRS-medium containing 0.3 % glucose to imitate conditions prevailing in fermented sausages. Over night cultures were washed twice with the modified medium. GC-vials were filled with 10 ml medium and inoculated with washed cells to a density of 10^6 cells/ml. 100 μl of the internal standard were added to all samples. Fermentations were performed for 5 days at 30°C. Samples were analyzed in minimum in duplicate at days 0 and 5.

2.2.3 Analysis of volatile compounds by SPME-GC-MS

Volatile compounds in the headspace of GC vials were determined by GC-MS analysis with SPME for sample collection. Before the analysis, the SPME fibres were preconditioned in the injection port of the GC as indicated by the manufacturer. The fibre was exposed to the samples for 30 min at 30°C. The GC oven temperature program started when the fiber was inserted and was held at 30°C for 15 min, ramped to 50°C at 3°C/min, then to 110°C at 4°C/min, to 150°C at 5°C/min, and to 250°C at 10°C/min and finally, held at 250°C for 10 min. The carrier gas was helium with a flow rate of 1.03 ml/min. The GC column was connected to the ion source of the mass spectrometer operating in the scan mode within a mass range of m/z 29 - 150. Ionisation was performed by electronic impact at 70 eV. The periodically calibration was performed by autotuning. Compounds were identified by comparison of the mass spectral data with those of the library Nist 2002 Mass Spectral Database. Additionally, selected compounds were identified by comparison of mass spectra and retentions times with those of authentic standards.

2.2.4 Quantification of volatile compounds

22 important volatile compounds were selected and quantified. 100 μg of each compound were diluted in 100 ml methanol. 1 ml of these solutions where then

further diluted in 100 ml H_2O dest. From these stock solutions appropriate dilutions were made and measured minimum in triplicate. 2-Methylpropanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, butanoic acid, acetic acid and acetoine were diluted directly in H_2O dest. Calibration curves of 6 different concentrations and 1,2-dimethoxy-ethane as internal standard were determined by GC-MS. The internal standard was used in a concentration of 19.24 nmol/ml respectively 19.24 nmol/g in all fermentations as well as in the quantifications.

2.2.5 Determination of the colony forming units

Overnight cultures were grown in modified mMRS-medium to the stationary phase. Appropiate dilutions were plated in triplicate on modified mMRS-agar plates or Baird Parker agar plates and incubated 24 - 48 h at 30°C. The colonies were counted and the numbers of colony forming units were calculated.

2.2.6 Determination of pH-values to analyse the acidification rate

Modified mMRS-medium with 0.3 % glucose was inoculated with 10^6 cells/ml. The pH in 3 ml was measured every two hours. This was performed for 28 hours. Final measurements were done after 38 hours and 5 days.

2.2.7 Analysis and quantification of lactic acid

Cultures were grown for five days in modified mMRS-medium with 0.3 % glucose. 50 μl perchloric acid was then added to 1 ml culture. Proteins were precipated over night by 4°C. The samples were centrifuged at 14000 rpm for 15 minutes. The supernatant was membrane filtrated into an HPLC vial. Until measurement the samples were stored at -20°C. The conditions for the HPLC analysis were: mobile phase was 5 mN H_2SO_4, flow rate at 0.6 ml/min, columm temperature at 85°C. The injection volume was 10 μl and and lactic acid was detected by UV-detection with a first wavelength of 225 nm and a second wavelength of 250 nm. The quantification of the lactic acid was done by comparison of the peak areas with those of standard solutions.

2.2.8 Analysis of volatile compounds with addition of amino acids and α-keto acids

This analysis was done in the same way as described in in sections 2.2.2 and 2.2.3. Additionally the modified mMRS-medium was supplemented either with 25 mM leucine, isoleucine, valine, α-ketoisocaproic acid, α-keto-3-methylpentanoic acid or α-ketoisovaleric acid.

2.2.9 Production of raw sausages

Dry fermented sausages were manufactured in batches of 3 kg. Each batch was made of: 1350 g (45 %) lean pork (goulash size, frozen), 750 g (25 %) pork back fat (frozen), 900 g (30 %) beef (mineced), 84 g (2.8 %) nitrite curing salt, 9 g (0.3 %) glucose, 3 g (0.1 %) pepper and 1.5 g (0.05 %) sodium ascorbate. The cutting procedure was done by the following scheme: lean pork 30 sec slow mode; pork back fat 20 sec slow mode; glucose, pepper, sodium ascorbate and bacteria 10 sec slow mode; 2 x 40 sec fast mode; beef 20 sec slow mode; nitrite curing mode 60 sec slow mode. For inoculation, 10^6 cells/g *L. sakei* and 10^7 cells/g for *S. carnosus* were used. The sterile control was made without the inoculation of a starter culture. Instead, glucono-δ-lactone was used for chemical acidification and chloramphenicol as antibiotic against spontaneous flora. The concentrations were 27 g glucono-δ-lactone/ 3 kg sausage meat and 300 mg chloramphenicol solved in ethanol / 3 kg sausage meat.

The mixtures were then stuffed into wet collagen casings, the final mass of each sausage was about 300 g. The sausages were then immersed in a 10 % potassium sorbate solution to prevent mould growth. The ripening was done in the conditioning cabinet with following gradient of temperature and humidity: day 0 24°C and 92 % humidity, reduction of weight 1 %; 1 - 2 day 24°C and 90 - 91 % humidity, reduction of weight 1.5 %; 2 - 3 day 20°C and 88 % humidity, reduction of weight 2 - 2.5 %; 3 - 5 day 20°C and 86 % humidity, reduction of weight 2 - 2.5 % per day; 5 - 6 day 18°C and 84 % humidity, reduction of weight 2 - 2.5 %; 6 - 7 day 18°C and 80 - 82 % humidity, reduction of weight 2 - 2.5 %; from 7th day 16°C and 76 - 78 % humidity, reduction of weight 1 - 2 % per day. The range for the humidity was varied due to the measured reduction of weight. In the first week the weight reduction of sausages was analysed every day, in the second week every second day and from the third week on twice a week. At the days 0, 1, 3, 5, 8, 14, 21, 28, 35 samples were taken for the analysis of pH, cfu/g and GC-MS analysis.

2.2.10 Chemical and mircrobial analysis of the sausages

The sausages were sliced and samples of 2 g were taken for the analysis of the pH-value and for microbial analysis. For the pH analysis the samples were minced with 20 ml H_2O dest. using an ultraturrax and the pH was measured with a pH-electrode. For GC-MS analysis sausage samples of 3 g were weightend in a GC-vial, 30 μl of the inertnal standard were added and the samples were measured as described in section 2.2.3.

For microbial analysis the samples were minced with 20 ml mMRS-medium and further diluted. Appropiate dilutions were plated in triplicate on modified mMRS-agar plates or Baird Parker plates and incubated 24 - 48 h at 30°C. At day 35 colonies from the plates were taken and the DNA was extracted. This was also done with the bacteria strains used for the inoculation. With the DNA 16S-PCRs and RAPD-PCRs were performed. The PCR cycles are given in tables 20 and 21.

Table 20: Cycles for the 16S-PCR

Step	Temperature	Time
1	94°C	5 min
2	94°C	45 sec
3	52°C	45 sec
4	72°C	45 sec
5	(Cycles 2 - 4 were repeated for 35 times)	
6	72°C	5 min

Table 21: Cycles for the RAPD-PCR

Step	Temperature	Time
1	94°C	3 min
2	40°C	5 min
3	72°C	5 min
4	(Cycles 1 - 3 were repeated for 3 times)	
5	94°C	1 min
6	60°C	2 min
5	72°C	3 min
6	(Cycles 5 - 7 were repeated for 32 times)	

For gelelectrophoresis samples were mixed with 10 μl loading dye and 7.5 μl were transfered to the gel. The DNA samples were then separated at 200 V for one hour. The gel was stained for 30 min in a dimidiumbromide solution, washed with H_2O dest. and visualised under UV-light. The band patterns of the RAPD samples from day 35 were compared to those of the pure cultures. The 16S-products were purified and the sequences were analysed by the company GATC Biotech AG in Konstanz (Germany). The sequences were then compared with the database NCBI/Blast.

2.2.11 Bacteriocin assay

The strains tested were grown overnight in modified mMRS-medium. Drops of overnight cultures were then put on agar plates and grown for 24 hours at 30°C. The sakacin P producing strain *L. sakei* TMW 1.454 was used as positive control. The indicator strains were also grown overnight in modified mMRS-medium. 10 ml melted covering agar was inoculated with 100 μl of the indicator cultures and then spaced on the whole agar plates. The plates were then again incubated at 30°C for 24 hours.

2.2.12 Analysis of volatile compounds in a sausage model system

The meat was prepared the same way as described in section 2.2.9. 3 g meat was inoculated with 10^6 cells/ml of *L. sakei* and/or 10^7 cells/ml of *S. carnosus* and stuffed into GC-vials. 30 μl of the internal standard was added and the samples were measured as described in section 2.2.3 at day 0 and 5. The fermentations were done at room temperature.

2.2.13 Growth curves

Microtiter plates were prepared with 200 μl in each well with medium presenting different stress factors and concentrations. The wells were then inoculated with 10^6 cells/ml from a washed overnight culture. The temperature in the plate reader was set to 30°C with the range between 28°C and 32°C. The measurement was done every two hours during a period of 24 hours. The absorption was measured at a wavelength of 590 nm after a short shaking of 10 sec.

2.2.14 Determination of the colony forming units and the cell morphology under stress conditions

The colony forming units were performed like described in section 2.2.5. The morphology of the cells was observed by light microscopy.

2.2.15 Analysis of volatile compounds under stress conditions

These analysis were done the same way as described in in section 2.2.3. The modified mMRS-media with 0,3 % glucose were prepared with 2.5 % salt, 0.125 % sodium nitrite or pH 5.5.

3 Results

3.1 Quantification of volatile compounds by SPME-GC-MS

The 22 volatile compounds 2-methylpropanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, butanoic acid, acetic acid, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, diacetyl, acetoin, dimethyldisulfide, 1-butanol, 2,3-hexanedione, 2,3-heptanedione, 2-methylbutyl-acetate, 3-methylbutyl-acetate, 2-methylpropyl-2-methylpropanoate, 2-methylbutyl-2-methylbutanoate and 3-methylbutyl-3-methylbutanoate were quantified as described in section 2.2.4. The relation of the peak area and the quantity of the compounds diacetyl and 2-methylpropanal was linear within the measured range. In all other cases the relation of the peak area and the quantity of the compounds was a polynomial equation. To give an example, the calibration curve of acetic acid is shown in figure 9 as an example.

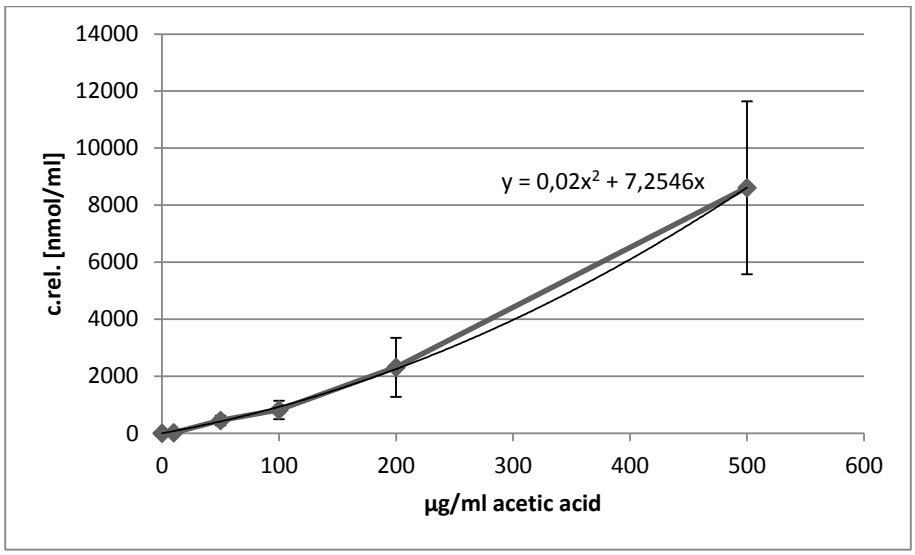


Figure 9: Calibration curve of acetic acid.

In table 22 the calculations of the quantifications for all 22 compounds are shown.

Table 22: Calculations of the quantifications.

Compound	Calibration range	Calibration equation
2-methyl-propanoic acid	$0 - 50\ \mu g/ml$	$c[\mu g/ml] = -4.3855 + \sqrt{19.2326 + c.rel./0.6999}$
2-methyl-butanoic acid	$0 - 50\ \mu g/ml$	$c[\mu g/ml] = -0.6045 + \sqrt{0.3654 + c.rel./4.2162}$

Compound	Calibration range	Calibration equation
3-methyl-butanoic acid	$0 - 50\ \mu g/ml$	$c[\mu g/ml] = -5.5025 + \sqrt{0.2525 + c.rel./4.2409}$
butanoic acid	$0 - 20\ \mu g/ml$	$c[\mu g/ml] = -0.655 + \sqrt{0.4290 + c.rel./2.9379}$
acetic acid	$0 - 500\ \mu g/ml$	$c[\mu g/ml] = -181.365 + \sqrt{32893 + c.rel./0.02}$
2-methylpropanal	$0 - 1000\ ng/ml$	$c[ng/ml] = c.rel./11.235$
2-methylbutanal	$0 - 1000\ ng/ml$	$c[ng/ml] = -241.3135 + \sqrt{58232 + c.rel./0.0204}$
3-methylbutanal	$0 - 500\ ng/ml$	$c[ng/ml] = -630.185 + \sqrt{397133 + c.rel./0.0073}$
2-methyl-1-propanol	$0 - 200\ ng/ml$	$c[ng/ml] = -18.2705 + \sqrt{333.812 + c.rel./0.0037}$
2-methyl-1-butanol	$0 - 5000\ ng/ml$	$c[ng/ml] = -1499.5 + \sqrt{2248500 + c.rel./0.0003}$
3-methyl-1-butanol	$0 - 10000\ ng/ml$	$c[ng/ml] = -3646 + \sqrt{13293316 + c.rel./0.0001}$
diacetyl	$0 - 5000\ ng/ml$	$c[ng/ml] = c.rel/0.6083$
acetoin	$0 - 100\ \mu g/ml$	$c[\mu g/ml] = -18.389 + \sqrt{338.1553 + c.rel./0.0144}$
dimethyldisulfide	$0 - 10\ ng/ml$	$c[ng/ml] = -12.6715 + \sqrt{160.5669 + c.rel./6.8559}$
1-butanol	$0 - 2000\ ng/ml$	$c[ng/ml] = -81.5 + \sqrt{6642.25 + c.rel./0.0015}$
2,3-hexanedione	$0 - 500\ ng/ml$	$c[ng/ml] = -60.9475 + \sqrt{3714.6 + c.rel./0.0019}$
2,3-heptanedione	$0 - 500\ ng/ml$	$c[ng/ml] = -45.2975 + \sqrt{2051.86 + c.rel./0.0131}$
2-methylbutyl-acetate	$0 - 20\ ng/ml$	$c[ng/ml] = -4.0955 + \sqrt{16.7731 + c.rel./0.176}$
3-methylbutyl-acetate	$0 - 500\ ng/ml$	$c[ng/ml] = -132.749 + \sqrt{17622 + c.rel./0.0904}$

Compound	Calibration range	Calibration equation
2-methylpropyl 2-methylpropanoate	$0 - 50\ ng/ml$	$c[ng/ml] = -6.9585 + \sqrt{48.4207 + c.rel./0.6485}$
2-methylbutyl- 2-methylbutanoate	$0 - 100\ ng/ml$	$c[ng/ml] = -39,375 + \sqrt{39,375 + c.rel./0,0358}$
3-methylbutyl- 3-methylbutanoate	$0 - 100\ ng/ml$	$c[ng/ml] = -9.565 + \sqrt{91.4892 + c.rel./0.0971}$

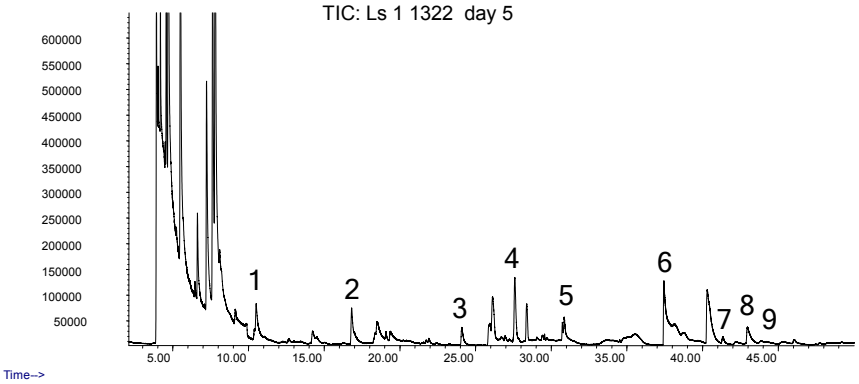
3.2 Screening of 51 *Lactobacillus sakei* and 30 *Lactobacillus curvatus* strains

The model fermentations were performed as described in section 2.2.3 with modified mMRS-medium. As comparison to the *Lactobacillus sakei* and *curvatus*, strains of *Lactobacillus paracasei*, *Lactobacillus farciminis*, *Staphylococcus carnosus*, *Pediococcus pentosaceus* and *Kocuria varians* were used. These strains were included in this study for a better overview concerning the metabolic spectrum of meat associated bacteria and to validate the analyzing method.

In the model fermentations nearly 30 volatile compounds were identified by comparison of their fragmentation patterns with thouse of the NIST-database. The identified compounds were carbondioxide, hexane, carbondisulfide, octane, butanal, 2-butanone, 2-methylbutanal, 3-methylbutanal, 2-ethylfuran, 2-pentanone, diacetyl, thiophene, toluene, dimethyldisulfide, 2-methylthiophene, 2-methyl-2-butenal, 3-methylbutyl-acetate, 2n-butylfuran, 1-butanol, 2-heptanone, limonene, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-pentylfuran, acetoin, 1-hexanol, acetic acid, 1-heptanol, benzaldehyde, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid. The compounds dimethyldisulfide, 1-butanol, 2-methyl-butanol, 3-methyl-butanol, acetoin, acetic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid were produced by the *L. sakei* and *L. curvatus* strains within five days (figure 10). All other volatile compounds were already found at day 0 in the medium and did not increase noticeable during the fermentation. All *L. sakei* and *L. curvatus* strains showed a similar volatile profile. Only the produced quantities varied. Dimethyldisulfide was produced in quantities of 0.5 - 1.8 ng/ml, 1-butanol was produced in quantities of 66 - 932 ng/ml,

2-methyl-1-butanol was produced in quantities of 34 - 160 ng/ml, 3-methyl-1-butanol was produced in quantities of 291 - 534 ng/ml, acetoin was produced in quantities of 4.6 - 10 μg/ml, acetic acid was produced in quantities of 33 - 118 μg/ml, 2-methylpropanoic acid was produced in quantities of 1.9 - 3.4 μg/ml, butanoic acid was produced in quantities of 5.7 - 8.4 μg/ml, 2-methylbutanoic acid was produced in quantities up to 0.1 μg/ml and 3-methylbutanoic acid was produced in quantities up to 0.8 μg/ml.

In contrast to the *L. sakei* and *L. curvatus* strains *L. paracasei*, *L. farciminis*, *S. carnosus*, *P. pentosaceus* and *K. varians* showed different profiles of volatile compounds (figure 10). *L. farciminis* and *L. paracasei* showed a high production of diacetyl with 6.9 μg/ml and 5.3 μg/ml. The produced amounts of the *L. sakei* and *L. curvatus* strains were only maximal 0.3 μg/ml. *L. paracasei* produced also the highest amount of dimethyldisulfide with 7.5 ng/ml. The production of 2-methyl-1-butanol and 3-methyl-1-butanol were the highest by *L. farciminis* and *P. pentosaceus* with amounts of 300 ng/ml and 230 ng/ml 2-methyl-1-butanol and 772 ng/ml and 1171 ng/ml 3-methyl-1-butanol. *P. pentosaceus* also produced the highest amount of acetoin (42 μg/ml). *S. carnosus* produced the largest quantities of 2-methylbutanoic acid and 3-methylbutanoic acid with values of 1,3 μg/ml and 4,4 μg/ml, respectively. Furthermore, the amounts of 2-methylpropanoic acid, butanoic acid and acetic acid were the highest in the culture with *S. carnosus*. *K. varians* was the only strain with no production of any acids. *S. carnosus* additionally degraded all aldehydes contained in the media. Chromatograms derived from various strains on day five of the fermentation period by SPME-GC/MS are shown in figure 10.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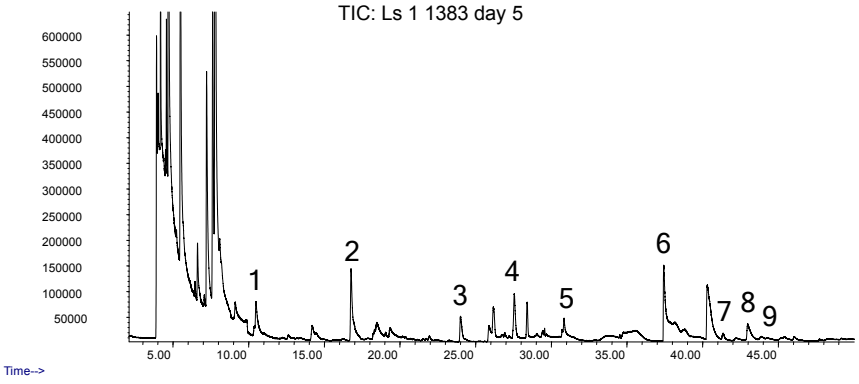
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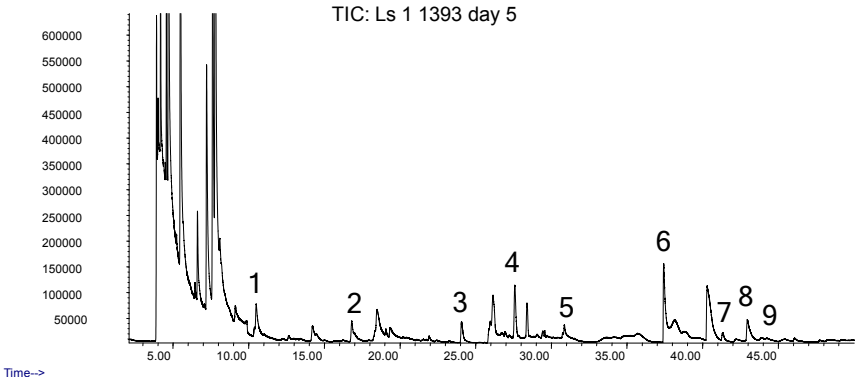
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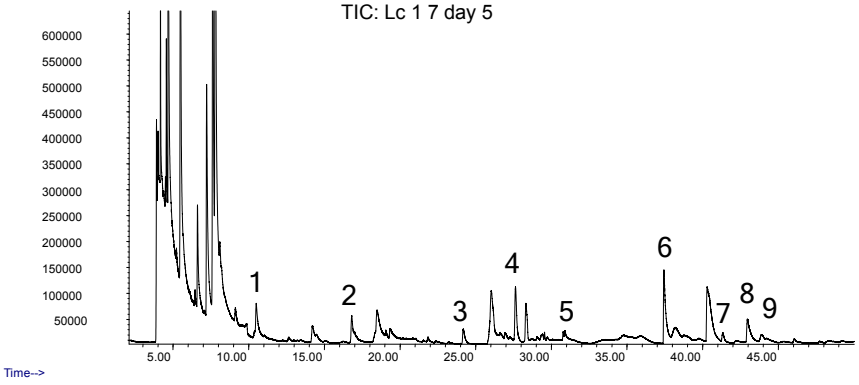
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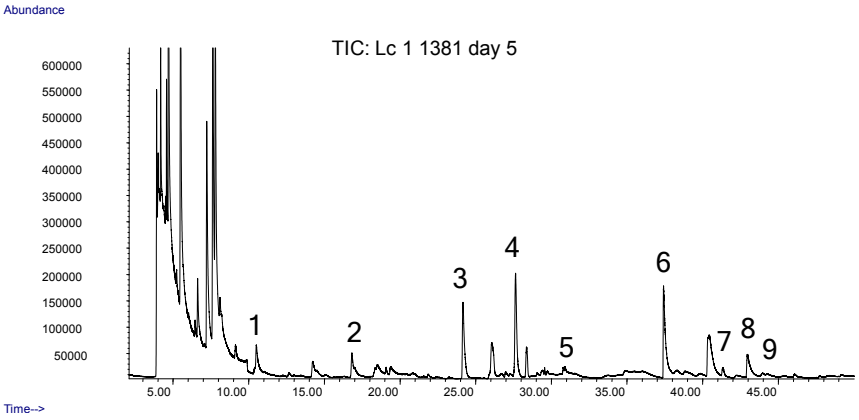
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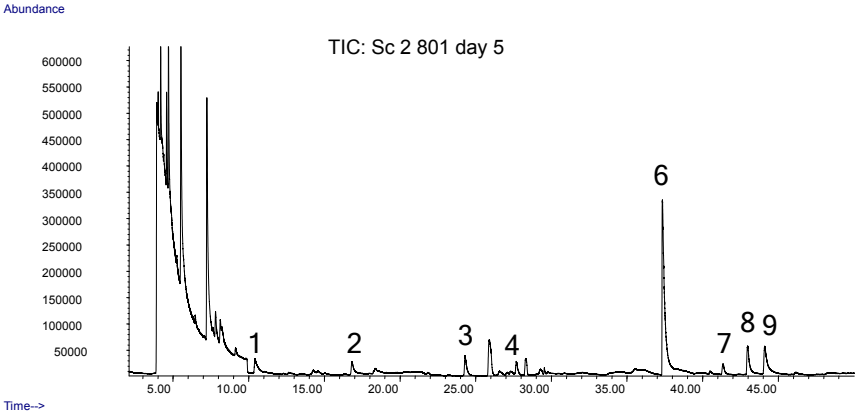
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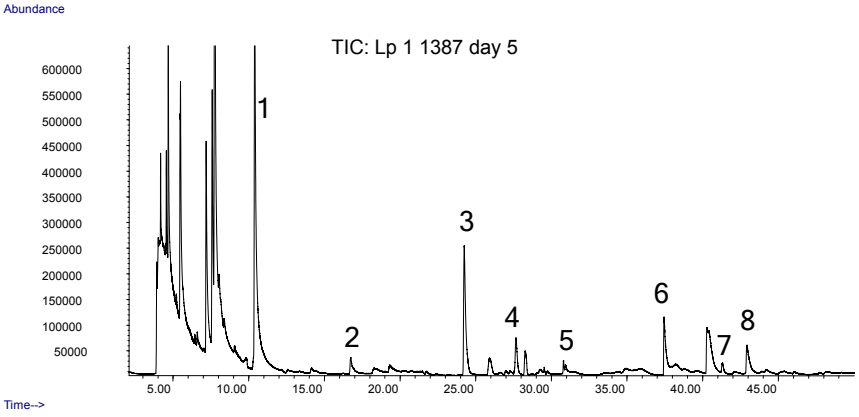
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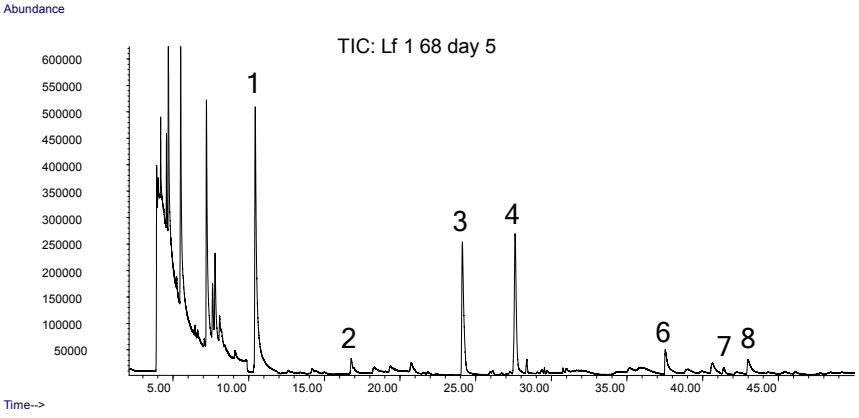
(g)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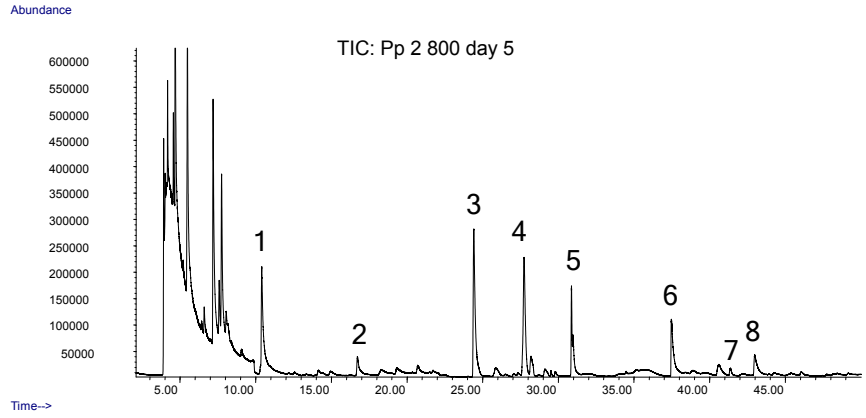

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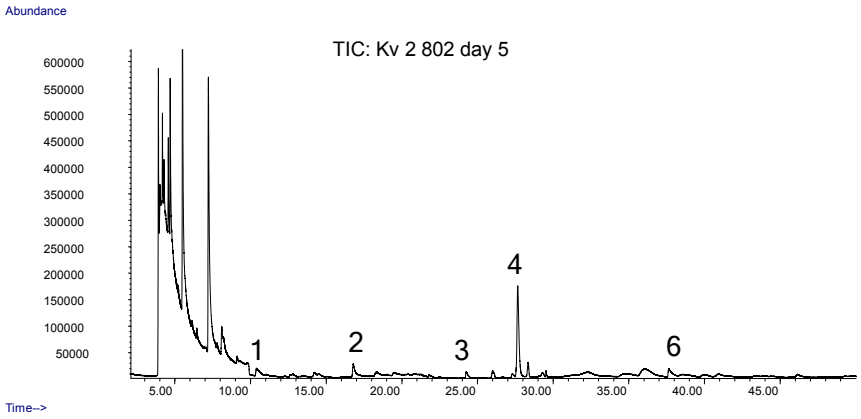


(j)

Figure 10: SPME-GC/MS analysis of the volatilomes of different bacterial species and strains after five days in modified mMRS-medium. Numbers of volatile compounds: 1 = diacetyl, 2 = dimethyldisulfide, 3 = 1-butanol, 4 = 2/3-methyl-1-butanol, 5 = acetoin, 6 = acetic acid, 7 = 2-methylpropanoic acid, 8 = butanoic acid, 9 = 2/3-methylbutanoic acid. (a) *L. sakei* TMW 1.1322. (b) *L. sakei* TMW 1.1383. (c) *L. sakei* TMW 1.1393. (d) *L. curvatus* TMW 1.7. (e) *L. curvatus* TMW 1.1381. (f) *S. carnosus* TMW 2.801. (g) *L. paracasei* TMW 1.1387. (h) *L. farciminis* TMW 1.68. (i) *P. pentosaceus* TMW 2.800. (j) *K. varians* TMW 2.802.

These results show that the used SPME-GC-MS method is appropriate to show differences in the metabolism of different bacterial species. Due to the fact that the *L. sakei* and *curvatus* strains showed similar volatile profiles, only the strains *L. sakei* TMW 1.1383 and 1.1393 were selected for further experiments. For comparison the strain *S. carnosus* TMW 2.801 was used.

3.3 Colony forming units, acidification rate and production of lactic acid by *L. sakei* TMW 1.1383, 1.1393 and *S. carnosus* TMW 2.801

Cultures were grown overnight in modified mMRS-medium with glucose and maltose for five days and plated like described in section 2.2.5. The strains reached colony forming units of $6*10^8$/ml. After five days in the culture of *S. carnosus* TMW 2.801 still $2 * 10^8$ colonies/ml were growing, while the cfu/ml of the *L. sakei* strains was only maximal $7 * 10^6$ (figure 11).

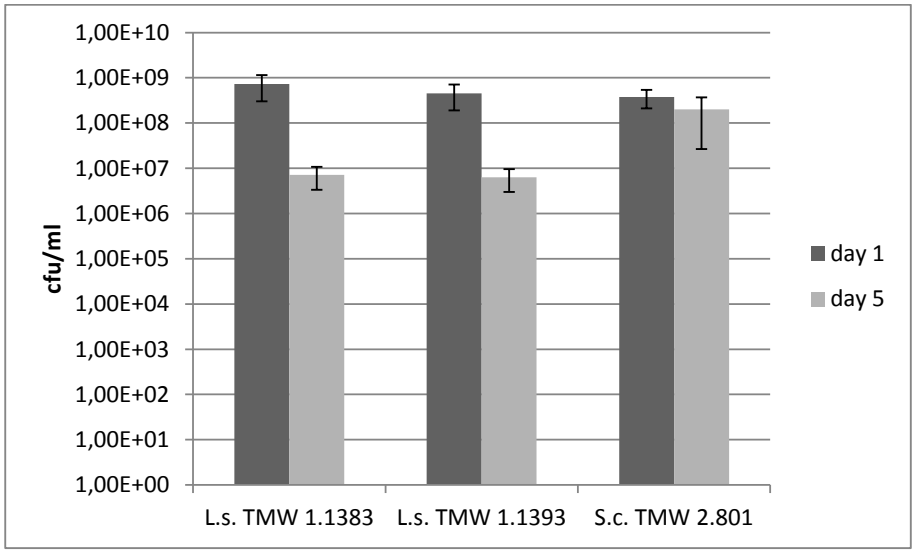


Figure 11: Colony forming units per ml in mMRS medium after one and five days of growth by the strains *L. sakei* TMW1.1383, 1.1393 and *S. carnosus* TMW 2.801.

The modified mMRS-medium with 0.3 % glucose was inoculated with 10^6 cells/ml as described in section 2.2.6. The medium showed a pH of 6.5 at the beginning of the experiment. After 8 hours the pH started to decrease in the cultures with *L. sakei* strains. After 14 hours the pH reached a value of 4.5 (figure 12). The acidification of the culture with the *S. carnosus* starts later and is slower. The decrease of the pH started after 10 hours and reached a pH of 4,8 after 28 hours (figure 12). After five days a pH of 4,7 was reached in all cultures (data not shown).

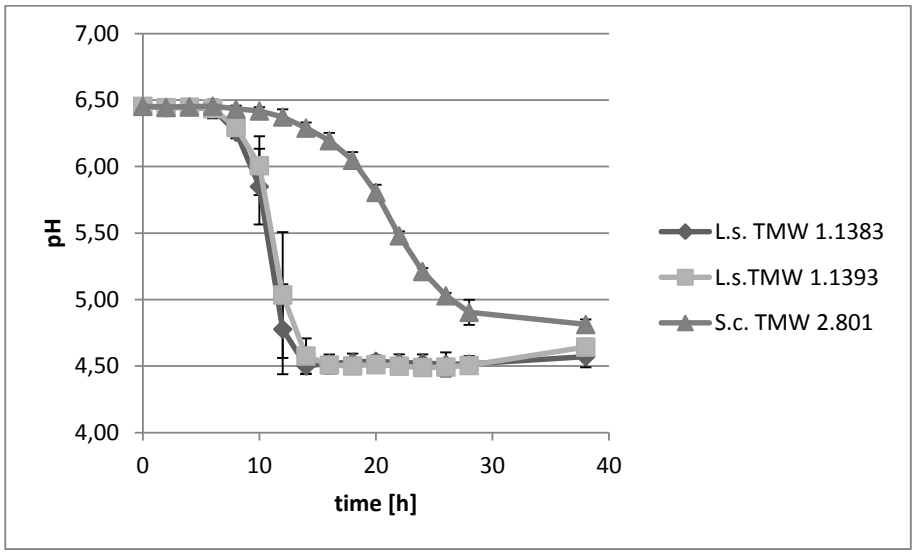


Figure 12: Acidification of the modified mMRS medium with 0.3 % glucose inoculated with 10^6 cells/ml of the strains *L. sakei* TMW 1.1383, 1.1393 and *S. carnosus* TMW 2.801.

The production of lactic acid in the modified mMRS-medium with 0.3 % glucose (16 mmol/L) was determined as described in section 2.2.7 after five days of cultivation. The amount of 0.3 % glucose was given in this and all other experiments, because this is the average concentration in the manufacturing of dry fermented sausages. All three strains produced 30 mmol/L lactic acid within five days of cultivation as shown in figure 13.

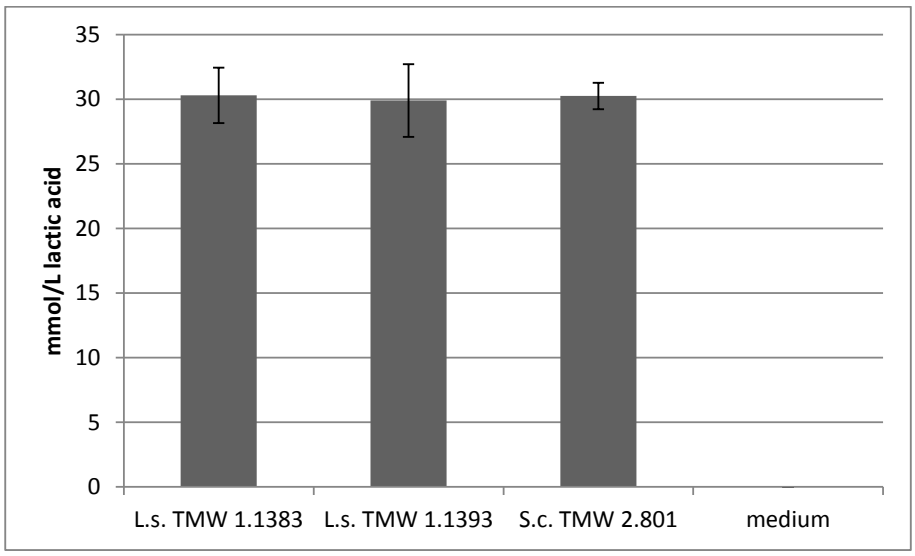
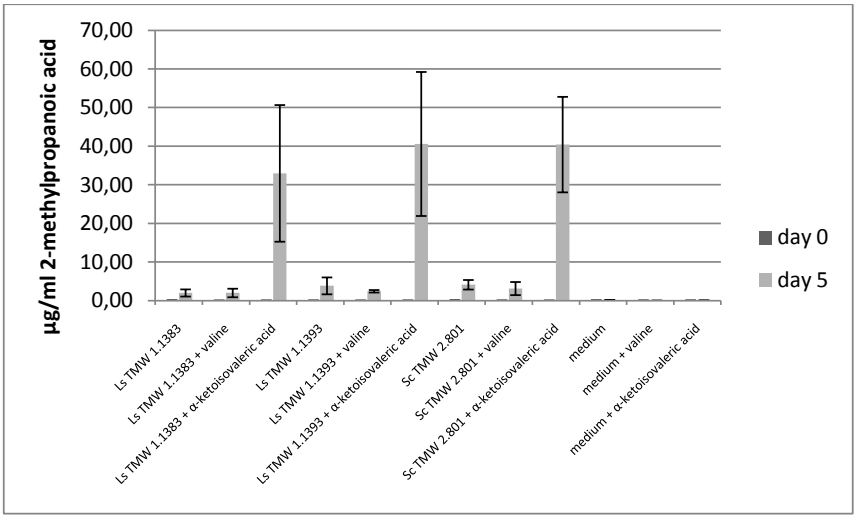


Figure 13: Production of lactic acid by the strains *L. sakei* TMW1.1383, 1.1393 and *S. carnosus* TMW 2.801 within five days in modified mMRS-medium with 0.3 % glucose.

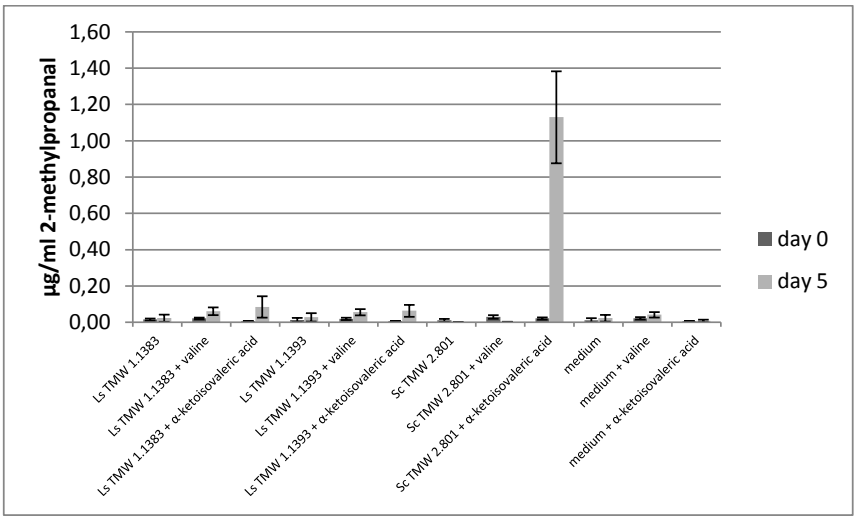
3.4 Production of volatile compounds by *L. sakei* TMW 1.1383, 1.1393, and *S. carnosus* TMW 2.801 cultivated with branched chain amino acids and branched chain α-keto acids

The strains were cultivated in the modified mMRS-medium with 0.3 % glucose and 25 mM branched chain amino acids, branched chain α-keto acids, or without supplement as described in section 2.2.8 The amount of volatile compounds were analysed at day 0 and 5.

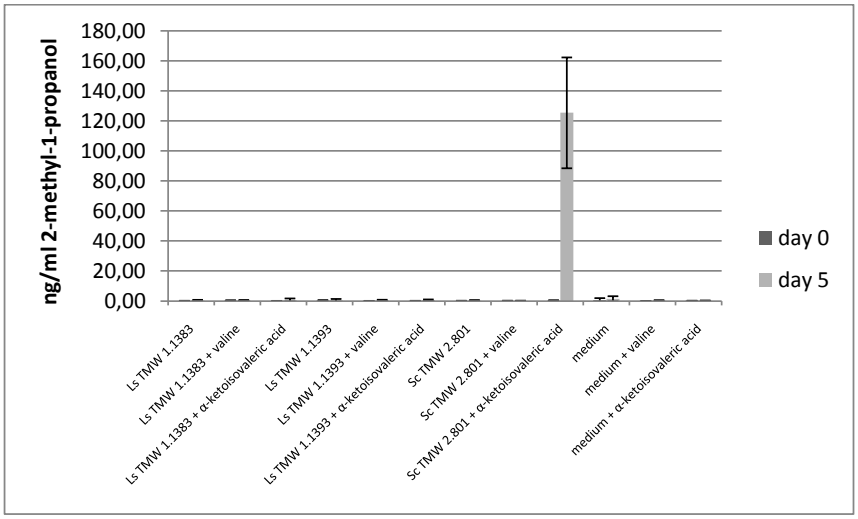
The supplementation of the amino acid valine showed nearly no effect to the production of volatile compounds (figure 14). In contrast the addition of α-ketoisovaleric acid increased the amount of 2-methylpropanoic acid 50 fold by the *L. sakei* and the *S. carnosus* strain (figure 14 a). The compounds 2-methylpropanal and 2-methyl-1-butanol were not produced by the *L. sakei* strains in higher amounts. Both compounds were only produced by the *S. carnosus* strain in higher amounts. The amount of 2-methylpropanal was increased 50 fold (figure 14 b). Without addition of α-ketoisovaleric acid the compound 2-methylpropanal was metabolized by the *S. carnosus* strain during the fermentation. The compound 2-methyl-1-propanol was only produced with the addition of α-ketoisovaleric acid by the *S. carnosus* strain (figure 14 c).



(a)



(b)

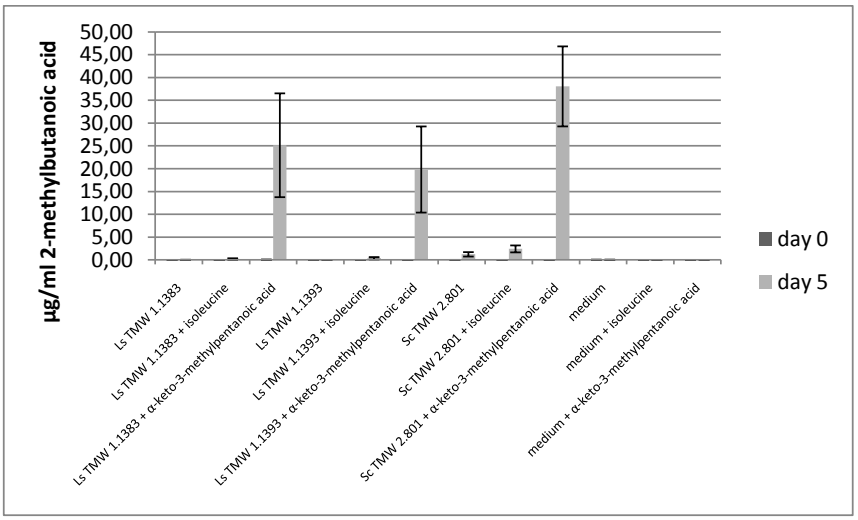


(c)

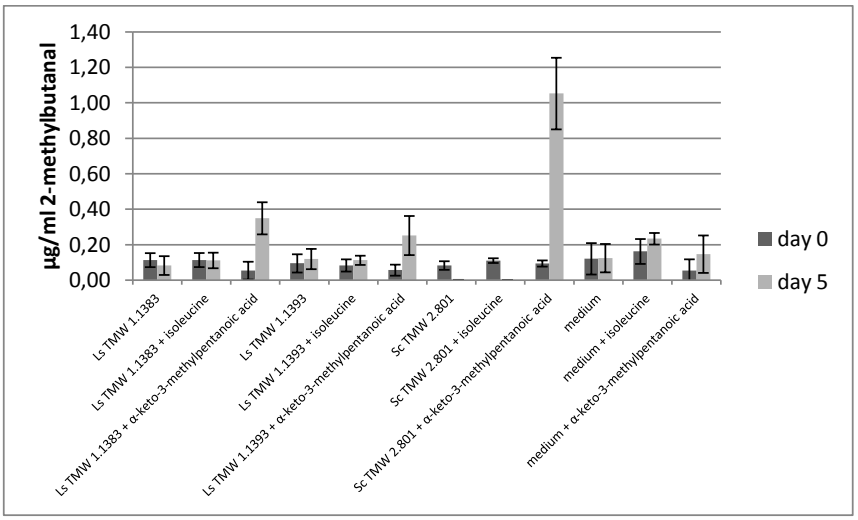
Figure 14: Production of volatile compounds by the strains *L. sakei* TMW 1.1383, 1.1393 and *S. carnosus* TMW 2.801 in medium without supplementation and with addition of 25 mM valine or α-ketoisovaleric acid. (a) Production of 2-methylpropanoic acid. (b) Production of 2-methylpropanal. (c) Production of 2-methyl-1-propanol.

The supplementation of the amino acid isoleucine showed nearly no effects to the production of volatile compounds similar to the supplementation of valine (figure 15). Only the production of 2-methylbutanoic acid was enhanced in the culture inoculated with *S. carnosus*. The addition of the α-keto acid showed, as in the previous experiment, increased amounts of the carboxylic acid. The amount of 2-methylbutanoic acid was even 300 fold, because it is only produced in very small quantities without addition of α-keto-3-methylpentanoic acid (figure 15 a). The production of the corresponding aldehyde and alcohol was increased only little by the

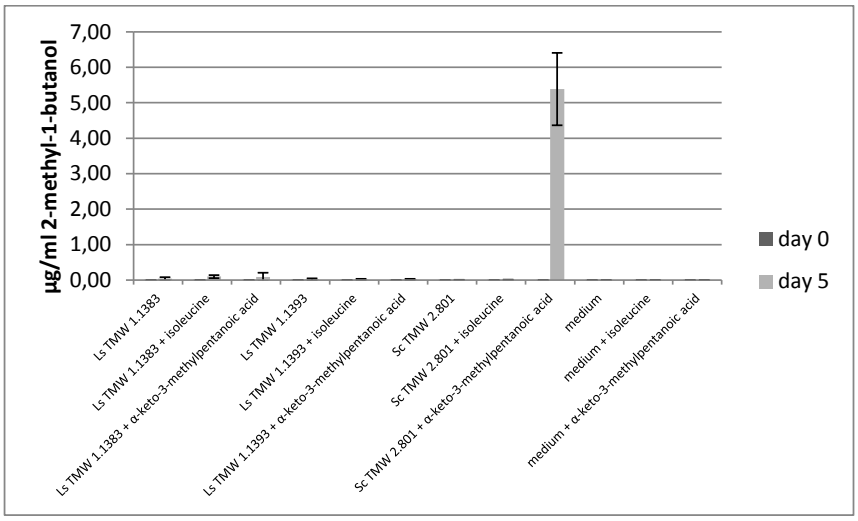
L. sakei strains. Again, both compounds were only produced by the *S. carnosus* strain in higher amounts. The compound 2-methylbutanal was increased 30 fold (figure 15 b). Without addition of α-keto-3-methylpentanoic acid the compound 2-methylbutanal was also degraded by the *S. carnosus* strain during the fermentation. The compound 2-methyl-1-butanol was increased 500 fold with the addition of α-keto-3-methylpentanoic acid (figure 15 c).



(a)



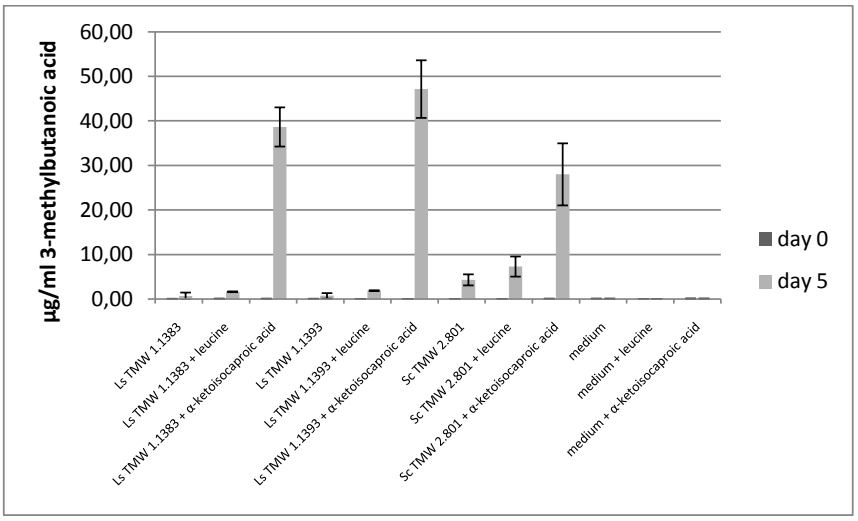
(b)



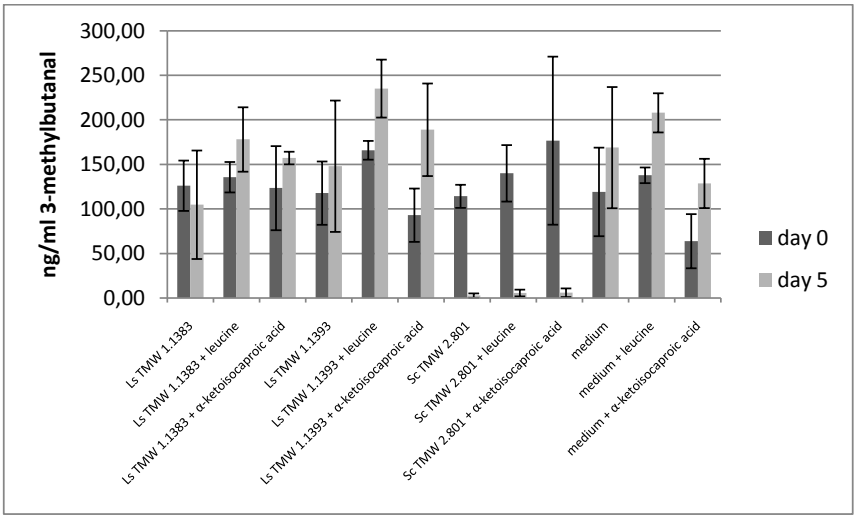
(c)

Figure 15: Production of volatile compounds by the strains *L. sakei* TMW 1.1383, 1.1393 and *S. carnosus* TMW 2.801 in medium without supplementation and with addition of 25 mM isoleucine or α-keto-3-methylpentanoic acid. (a) Production of 2-methylbutanoic acid. (b) Production of 2-methylbutanal. (c) Production of 2-methyl-1-butanol.

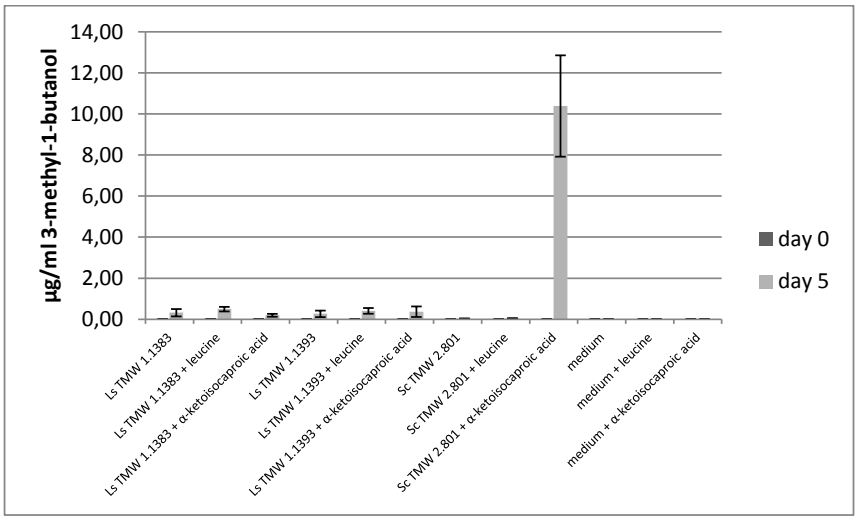
The addition of the amino acid leucine showed also little effects to the production of volatile compounds (figure 16). Only the production of 3-methylbutanoic acid was enhanced in the culture inoculated with *S. carnosus*. Like the other two α-keto acids the amount of the corresponding carboxylic acid was increased in this experiment. The amount of the 3-methyl-butanoic acid was increased 250 fold in the cultures with *L. sakei* and 40 fold in the cultures with *S. carnosus*. Without α-ketoisocaproic acid the production of 3-methylbutanoic acid was 6 times higher in the cultures with *S. carnosus* than in the cultures with *L. sakei* (16 a). In contrast to the α-ketoisovaleric acid and the α-keto-3-methylpentanoic acid the *S. carnosus* strain did not produce the corresponding aldehyde 3-methylbutanal. It was degraded like in the cultures without addition of α-keto acids (16 b). The compound 3-methyl-1-butanol was produced by the *L. sakei* strains independently from the supplementation of the medium (16 c and d). The *S. carnosus* produced only small amounts of 3-methyl-1-butanol without addition of α-ketoisocaproic acid. On the contrary, with addition of the α-ketoisocaproic acid the produced amount was increased 800 fold (16 c and d).


µg/ml 3-methylbutanoic acid
60,00
50,00
40,00
30,00
20,00
10,00
0,00
day 0
day 5
Ls TMW 1.1383
Ls TMW 1.1383 + leucine
Ls TMW 1.1383 + α-ketoisocaproic acid
Ls TMW 1.1393
Ls TMW 1.1393 + leucine
Ls TMW 1.1393 + α-ketoisocaproic acid
Sc TMW 2.801
Sc TMW 2.801 + leucine
Sc TMW 2.801 + α-ketoisocaproic acid
medium
medium + leucine
medium + α-ketoisocaproic acid

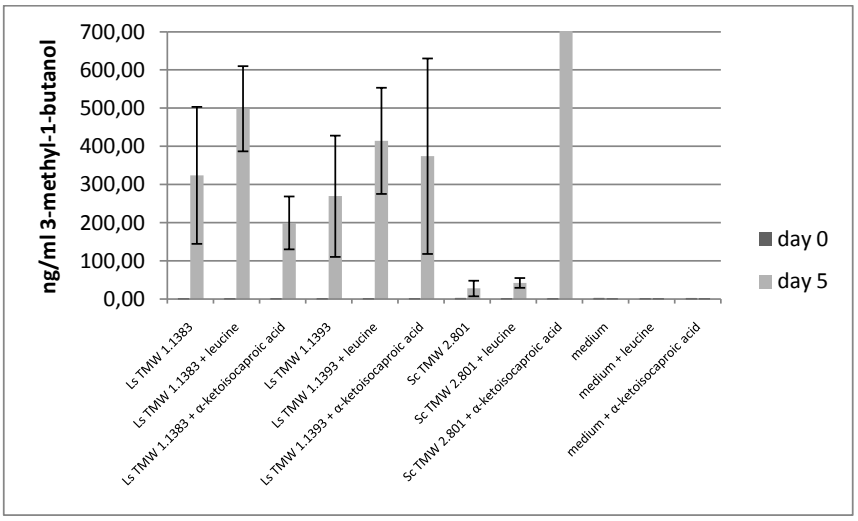
(a)


ng/ml 3-methylbutanal
300,00
250,00
200,00
150,00
100,00
50,00
0,00
day 0
day 5
Ls TMW 1.1383
Ls TMW 1.1383 + leucine
Ls TMW 1.1383 + α-ketoisocaproic acid
Ls TMW 1.1393
Ls TMW 1.1393 + leucine
Ls TMW 1.1393 + α-ketoisocaproic acid
Sc TMW 2.801
Sc TMW 2.801 + leucine
Sc TMW 2.801 + α-ketoisocaproic acid
medium
medium + leucine
medium + α-ketoisocaproic acid

(b)


µg/ml 3-methyl-1-butanol
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
day 0
day 5
Ls TMW 1.1383
Ls TMW 1.1383 + leucine
Ls TMW 1.1383 + α-ketoisocaproic acid
Ls TMW 1.1393
Ls TMW 1.1393 + leucine
Ls TMW 1.1393 + α-ketoisocaproic acid
Sc TMW 2.801
Sc TMW 2.801 + leucine
Sc TMW 2.801 + α-ketoisocaproic acid
medium
medium + leucine
medium + α-ketoisocaproic acid

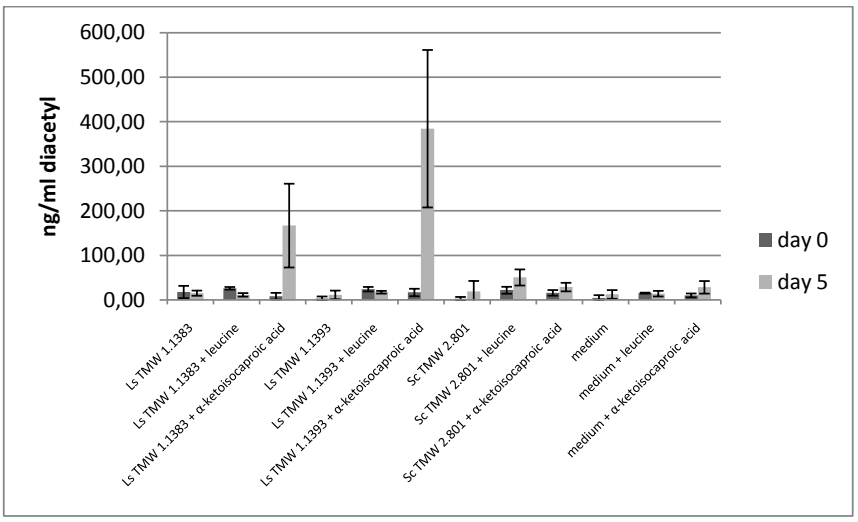
(c)



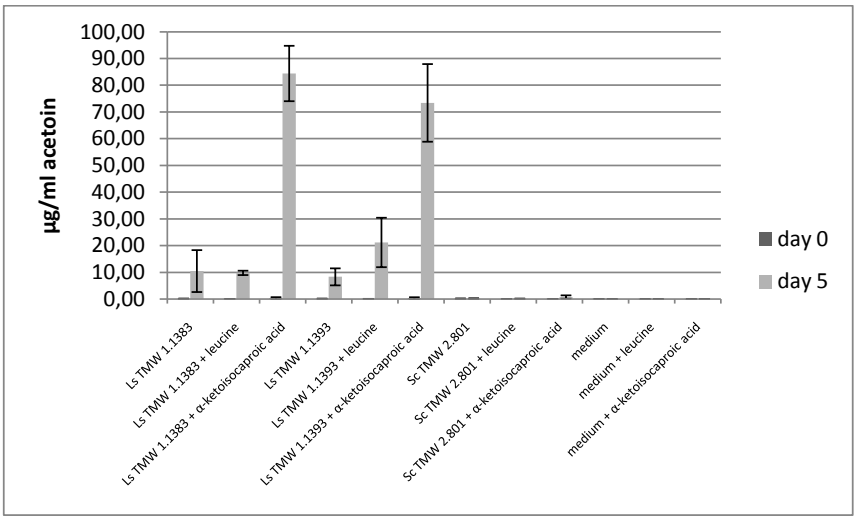
(d)

Figure 16: Production of volatile compounds by the strains *L. sakei* TMW 1.1383, 1.1393 and *S. carnosus* TMW 2.801 in medium without supplementation and with addition of 25 mM leucine or α-ketoisocaproic acid. (a) Production of 3-methylbutanoic acid. (b) Production of 3-methylbutanal. (c) and (d) Production of 3-methyl-1-butanol.

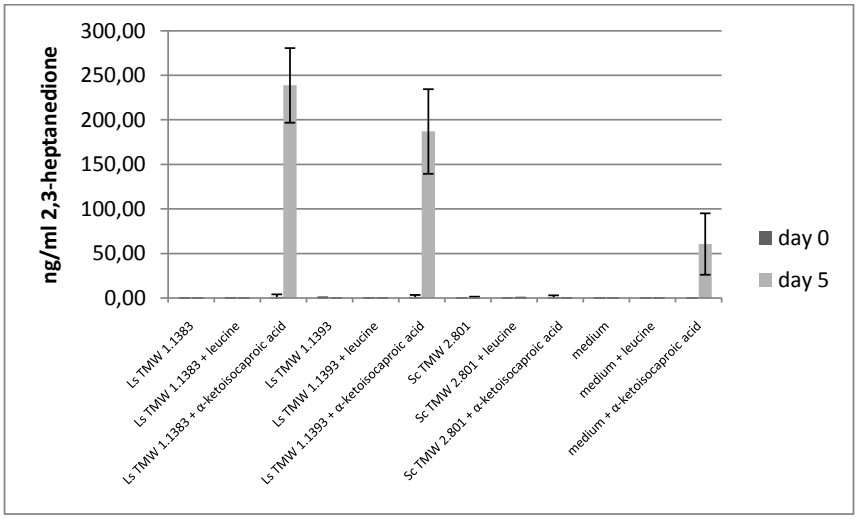
In addition to the volatile compounds derived directly from the α-keto acids the *L. sakei* strains produced 170 and 380 ng/ml diacetyl, 85 and 75 μg/ml acetoin and 240 and 190 ng/ml 2,3-heptanedione from α-ketoisocaproic acid (figure 17 a - c). With α-ketoisovaleric acid the strains produced 350 and 250 ng/ml 2,3-hexanedione (17 d).



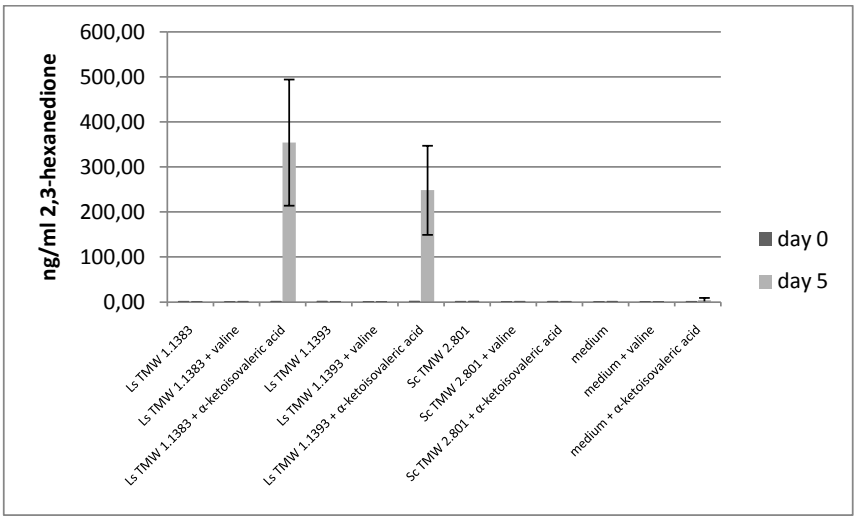
(a)


µg/ml acetoin
100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
day 0
day 5
Ls TMW 1.1383
Ls TMW 1.1383 + leucine
Ls TMW 1.1383 + α-ketoisocaproic acid
Ls TMW 1.1393
Ls TMW 1.1393 + leucine
Ls TMW 1.1393 + α-ketoisocaproic acid
Sc TMW 2.801
Sc TMW 2.801 + leucine
Sc TMW 2.801 + α-ketoisocaproic acid
medium
medium + leucine
medium + α-ketoisocaproic acid

(b)


ng/ml 2,3-heptanedione
300,00
250,00
200,00
150,00
100,00
50,00
0,00
day 0
day 5
Ls TMW 1.1383
Ls TMW 1.1383 + leucine
Ls TMW 1.1383 + α-ketoisocaproic acid
Ls TMW 1.1393
Ls TMW 1.1393 + leucine
Ls TMW 1.1393 + α-ketoisocaproic acid
Sc TMW 2.801
Sc TMW 2.801 + leucine
Sc TMW 2.801 + α-ketoisocaproic acid
medium
medium + leucine
medium + α-ketoisocaproic acid

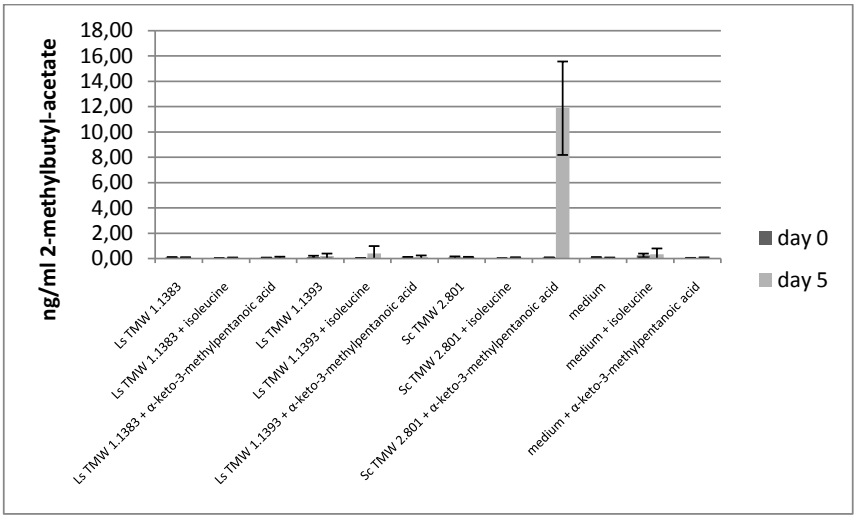
(c)



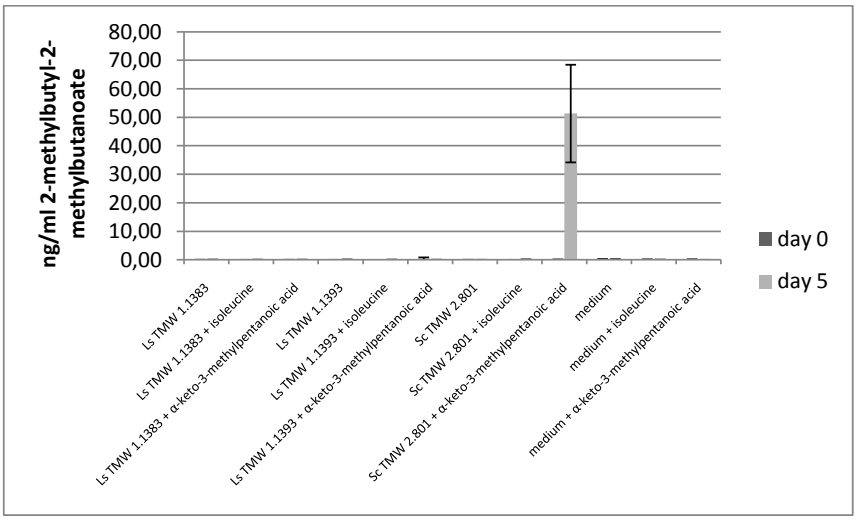
(d)

Figure 17: Production of additional volatile compounds by the strains *L. sakei* TMW 1.1383 and 1.1393 in medium without supplementation and with addition of 25 mM leucine, α-ketoisocaproic acid, valine or α-ketoisovaleric acid. (a) Production of diacetyl. (b) Production of acetoin. (c) Production of 2,3-heptanedione. (d) Production of 2,3-hexanedione.

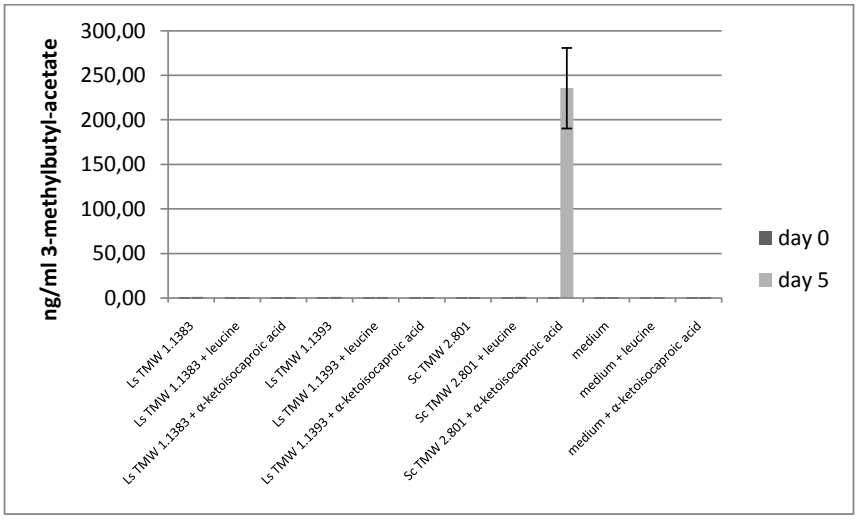
In addition to the volatile compounds derived directly from the α-keto acids the *S. carnosus* strain produced ester compounds (figure 18) With α-keto-3-methylpentanoic acid the strain produced 12 ng/ml 2-methylbutyl-acetate and 50 ng/ml 2-methylbutyl-2-methylbutanoate (figure 18 a and b). With α-ketoisocaproic acid the strain produced 240 ng/ml 3-methylbutyl-acetate and 80 ng/ml 3-methylbutyl-3-methylbutanoate (figure 18 c and d). With α-ketoisovaleric acid the strain produced 14 ng/ml 2-methylpropyl-2-methylpropanoate (figure 18 e).



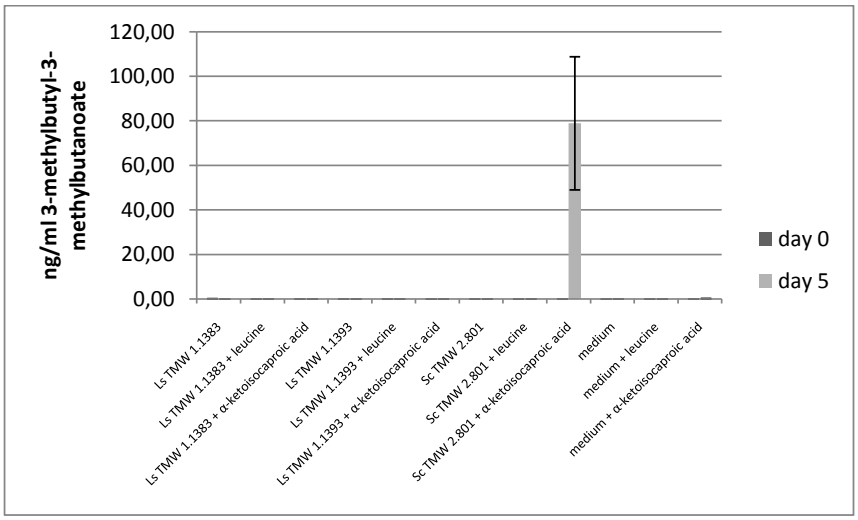
(a)


ng/ml 2-methylbutyl-2-methylbutanoate
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
day 0
day 5
Ls TMW 1.1383
Ls TMW 1.1383 + isoleucine
Ls TMW 1.1383 + α-keto-3-methylpentanoic acid
Ls TMW 1.1393
Ls TMW 1.1393 + isoleucine
Ls TMW 1.1393 + α-keto-3-methylpentanoic acid
Sc TMW 2.801
Sc TMW 2.801 + isoleucine
Sc TMW 2.801 + α-keto-3-methylpentanoic acid
medium
medium + isoleucine
medium + α-keto-3-methylpentanoic acid

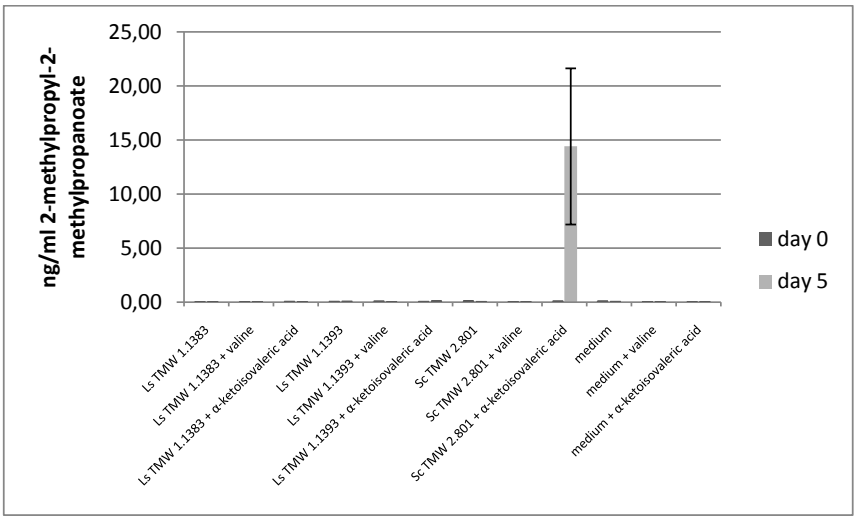
(b)


ng/ml 3-methylbutyl-acetate
300,00
250,00
200,00
150,00
100,00
50,00
0,00
day 0
day 5
Ls TMW 1.1383
Ls TMW 1.1383 + leucine
Ls TMW 1.1383 + α-ketoisocaproic acid
Ls TMW 1.1393
Ls TMW 1.1393 + leucine
Ls TMW 1.1393 + α-ketoisocaproic acid
Sc TMW 2.801
Sc TMW 2.801 + leucine
Sc TMW 2.801 + α-ketoisocaproic acid
medium
medium + leucine
medium + α-ketoisocaproic acid

(c)


ng/ml 3-methylbutyl-3-methylbutanoate
120,00
100,00
80,00
60,00
40,00
20,00
0,00
day 0
day 5
Ls TMW 1.1383
Ls TMW 1.1383 + leucine
Ls TMW 1.1383 + α-ketoisocaproic acid
Ls TMW 1.1393
Ls TMW 1.1393 + leucine
Ls TMW 1.1393 + α-ketoisocaproic acid
Sc TMW 2.801
Sc TMW 2.801 + leucine
Sc TMW 2.801 + α-ketoisocaproic acid
medium
medium + leucine
medium + α-ketoisocaproic acid

(d)



(e)

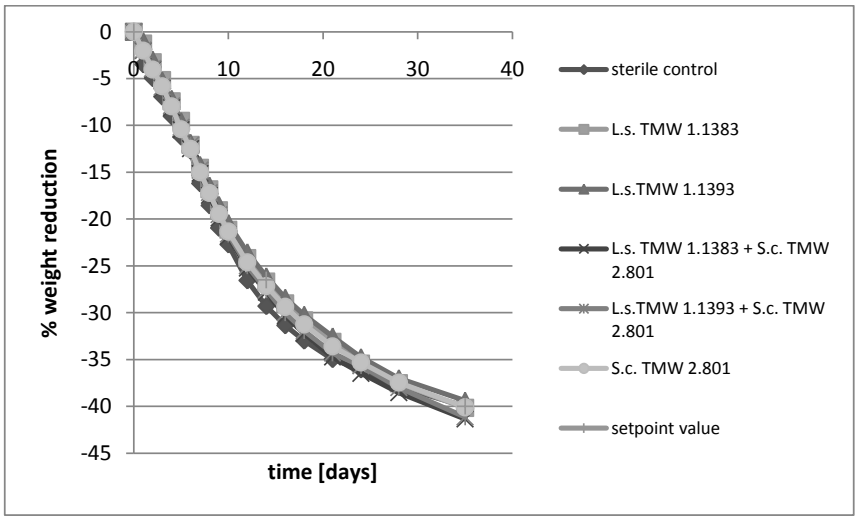
Figure 18: Production of additional volatile compounds by the strain *S. carnosus* TMW 2.801 in medium without supplementation and with addition of 25 mM isoleucine, α-keto-3-methylpentanoic acid, leucine, α-ketoisocaproic acid, valine, α-ketoisovaleric acid. (a) Production of 2-methylbutyl-acetate. (b) Production of 2-methylbutyl-2-methylbutanoate. (c) Production of 3-methylbutyl-acetate. (d) Production of 3-methylbutyl-3-methylbutanoate. (e) Production of 2-methylpropyl-2-methylpropanoate.

3.5 Production of raw sausages with *L. sakei* TMW 1.1383, 1.1393, and *S. carnosus* TMW 2.801

3.5.1 Reduction of weight during ripening

The sausages were manufactured and matured as described in section 2.2.9. The reduction of weight was similar in all experiments and there were nearly no differences between the different batches (figure 19 a). This showed that the conditions of the ripening process were identical and the sausages from different experiments are comparable to each other.

There were some differences in the appearance of the sausages between the different batches. The sausages which were inoculated with *S. carnosus* TMW 2.801 showed earlier the desired red color (figure 19 b). Also at the end of the fermentation these sausages had an attractive red color, while the sausages inoculated with *L. sakei* had more a brownish color. The sterile control showed a pink color at the beginning of the fermentation.



(a)

(b)

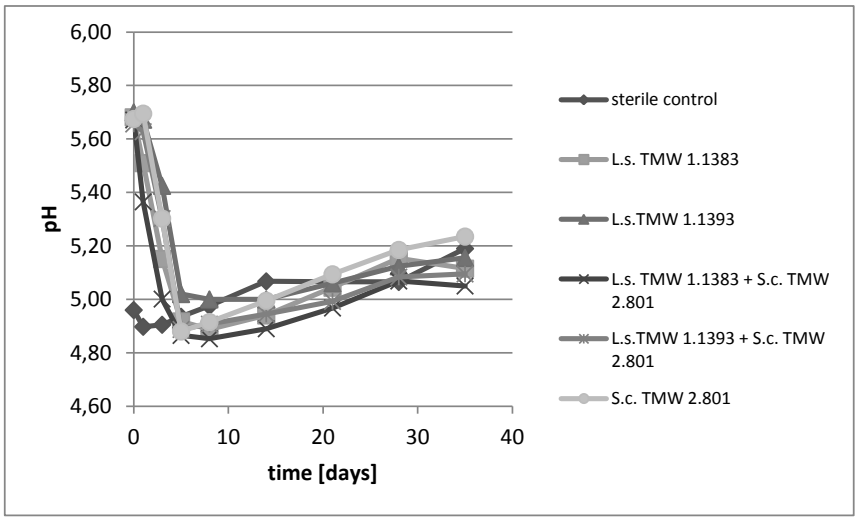
Figure 19: Ripening of the manufactured dry sausages. (a) Average reduction of weight during the fermentation. (b) Appearance of the sausages at day 2 of the fermentation. (In the front are some brownish sausages inoculated only with *L. sakei*.)

3.5.2 Developing of the pH-values during the ripening

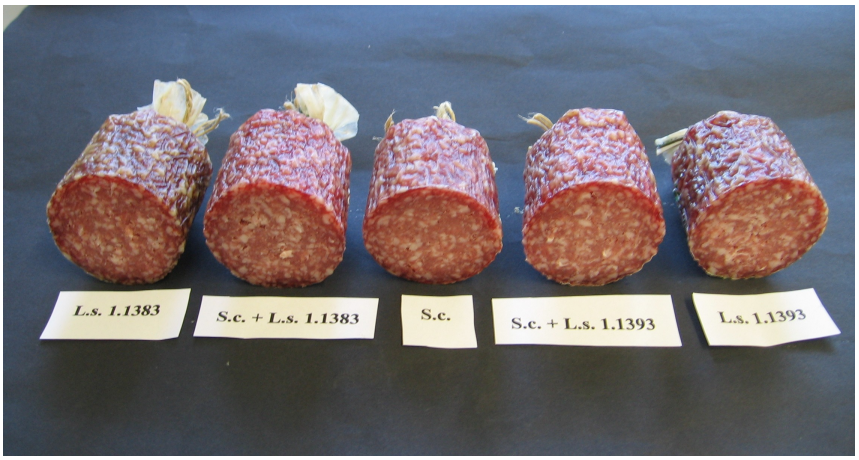
At the beginning of the fermentation the pH value was between 5.6 and 5.8. All batches showed the lowest pH-value (4.8 - 5.0) at day 5. Afterwards the pH values increased slightly and finished at day 35 with a pH in the range of 5.1 - 5.2 (figure 20 a). Except for the first three days the inoculation of different strains and

strain combinations did not lead to significantly different pH values during the fermentation. The combination of *L. sakei* TMW 1.1383 and *S. carnosus* showed the fastest acidification while the batch inoculated with *L. sakei* TMW 1.1393 showed the slowest acidification at the beginning of the fermentation (figure 20 a).

The sterile control already showed a pH value below 5 at day 0 (figure 20 a). During the fermentation the value increased to pH 5.2.



(a)



(b)

Figure 20: Ripening and appearance of the manufactured dry sausages. (a) Average development of the pH-values during the fermentation. (b) Appearance of the sausages at day 35.

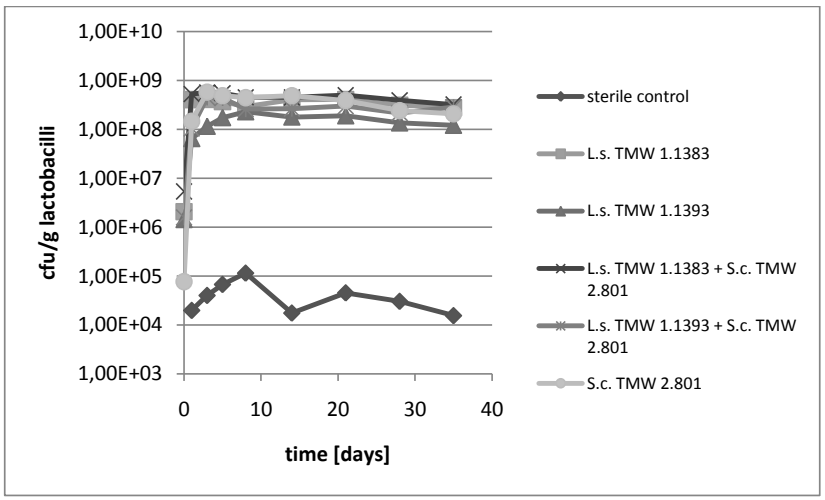
3.5.3 Development of colony forming units during the ripening

In all batches and experiments the colony forming units of lactobacilli reached a concentration of 10^8 and 10^9 cells/g sausage within 3 days. This value remained

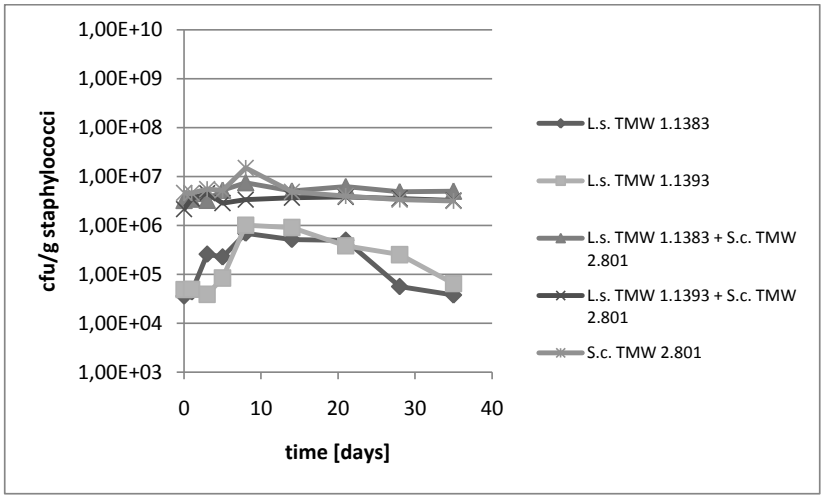
almost stable during the rest of the fermentation (figure 21 a). At the end of the ripening period the dominance of the starter culture was verified. In each case *L. sakei* TMW 1.1383 was the only *Lactobacillus* strain. In three of four experiments a second *Lactobacillus* strain grew in sausages inoculated with the strain *L. sakei* TMW 1.1393. This was observable in sausages inoculated with *L. sakei* TMW 1.1393 alone and together with *S. carnosus* TMW 2.801. In one of the experiments the additional strain was isolated and identified. The 16S analysis showed the sequence of another *L. sakein* strain. Figure 21 e shows the RAPD profile of the inoculated strains at day 0 and 35 and of the contaminant *Lactobacillus* strain. *L. sakei* TMW 1.1383 and *L. sakei* TMW 1.1393 did not show antagonistic effect towards the contaminant *Lactobacillus* strain and another sensitive *Lactobacillus* strain (figure 21 f). As positive control *L. sakei* TMW 1.454, which produces sakacin P, was used.

The colony forming units in the sterile control were between 10^4 and 10^5/g sausage (figure 21 a). Interesting was that the bacterial flora was relatively heterogenic. Two different *Staphylococcus*-species (*S. saprophyticus* and *S. spec.*, probably *S. carnosus*), two different *Kocuria*-species (*K. kristinae* and *K. rhizophila*), two different *Lactobacillus*-species, *Bacillus cereus*, *Macrococcus caseolyticus*, *Microbacterium oxydans* and *Serratia spec* could be isolated.

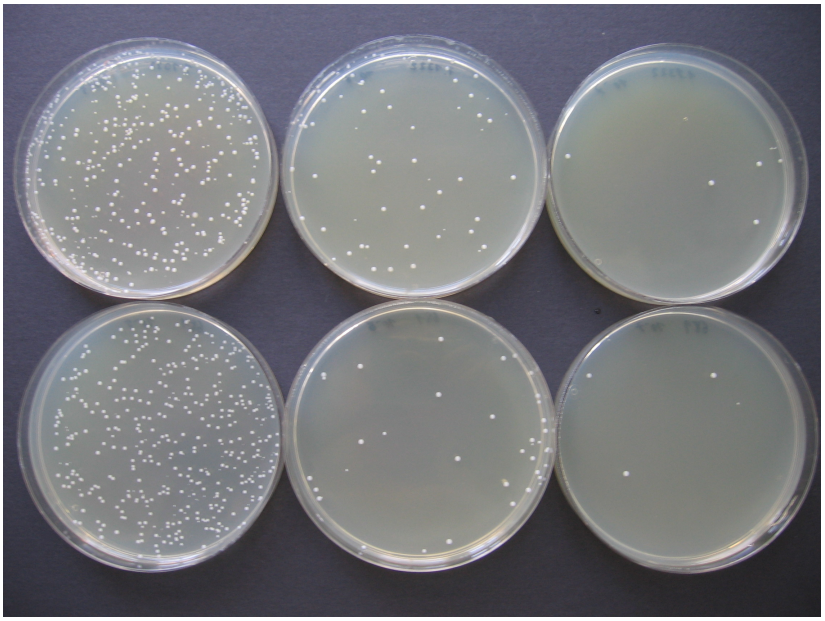
The colony forming units of staphylococci showed differences between the batches. If *S. carnosus* was inoculated as starter culture the colony forming units reached 10^6 to 10^7 cells/g sausage. In sausages inoculated only with lactobacilli the colony forming units of staphylococci were one to two orders of magnitude lower (figure 21 b).



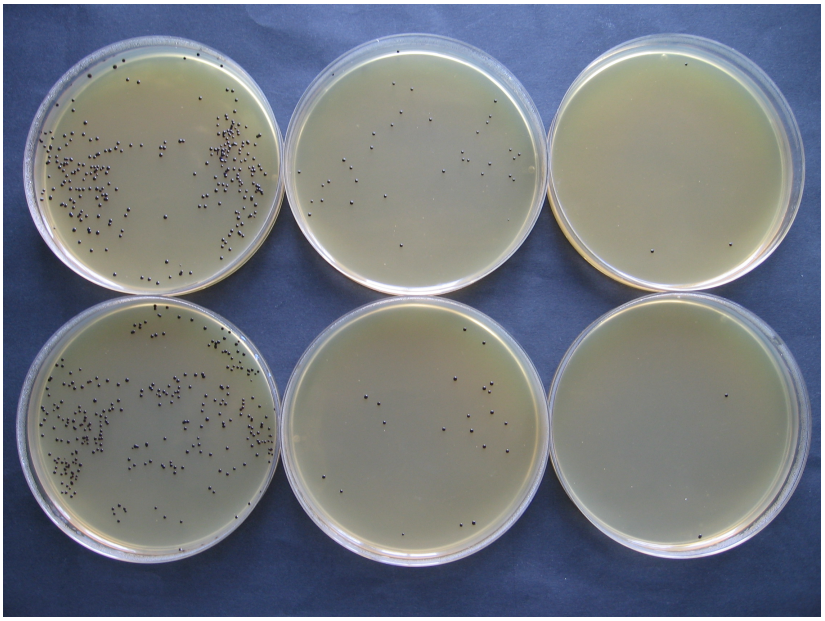
(a)


1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
cfu/g staphylococci
0
10
20
30
40
time [days]
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 + S.c. TMW 2.801
L.s. TMW 1.1393 + S.c. TMW 2.801
S.c. TMW 2.801

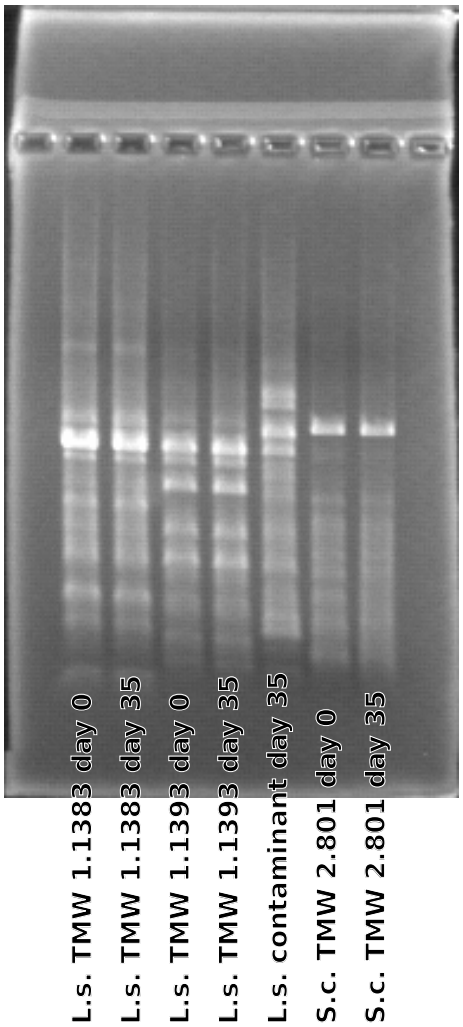
(b)



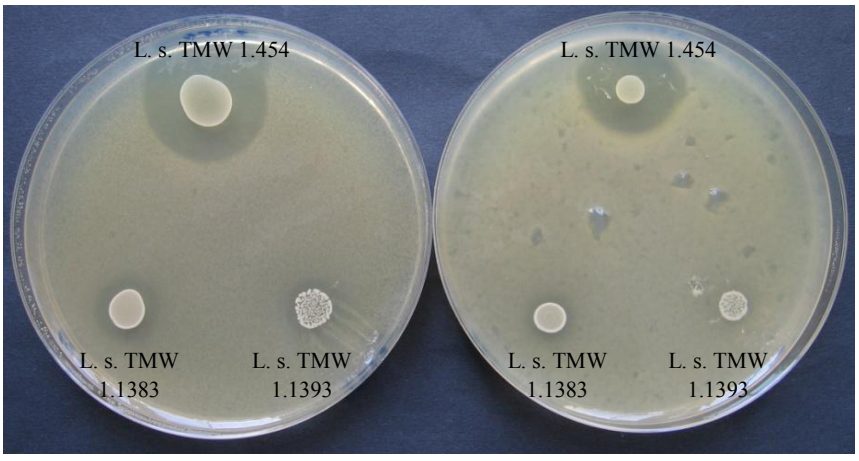
(c)



(d)



(e)



(f)

Figure 21: Development of the colony forming units during the fermentation. (a) Lactobacilli. (b) Staphylococci. (c) Colonies on mMRS-plates. (d) Colonies on Baird-Parker-plates. (e) RAPD profile of the strains at day 0 and 35. (f) Agar diffusion assay to detect acivity of bacteriocins toward the strain isolated from the sausage and *L. spec.* TMW 1.191 which is sensitive toward most bacteriocins.

3.5.4 Volatile compounds

In the produced sausages more than 100 volatile compounds were detected by comparison of their fragmentation patterns with thouse of the NIST database. Among them 2 hydrocarbons, 2 hydrocarbons, 11 aldehydes, 10 ketones, 22 alcohols, 11 carboxylic acids, 11 ester compounds, 8 sulfurcontaing compounds, 14 aromatic compounds, 2 oxygen containing heterocyclic compounds, 13 terpenes and 7 nitrogene containing compounds could be identified. The table 23 shows all identified compounds.

Table 23: Volatile compounds detected in the sausages.

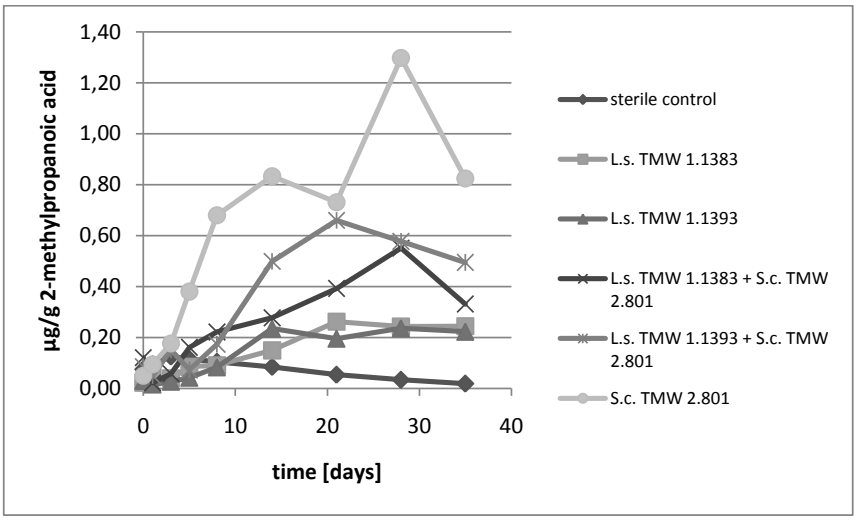
chemical class	compound
hydrocarbons	1,3-pentanediene
	1,4-pentanediene
aldehydes	acetaldehyde
	2-methylpropanal
	2-methylbutanal
	3-methylbutanal
	pentanal
	hexanal
	heptanal
	3-methyl-2-butenal
	3-methylhexanal
	2-heptenal
	2-octenal
ketones	acetone
	2-butanone
	2-pentanone
	2-heptanone
	2-nonanone
	3-octanone
	2,3-butanedione (diacetyl)
	3-hydroxy-2-butanone (acetoin)
	1-octen-3-on
	methylisobutylketone
alcohols	ethanol

chemical class	compound
	isopropylalcohol
	1-propanol
	1-butanol
	1-pentanol
	1-hexanol
	1-heptanol
	1-octanol
	2-butanol
	2-hexanol
	2-methyl-1-propanol
	2-methyl-1-butanol
	3-methyl-1-butanol
	3-methyl-2-butanol
	4-methyl-2-pentanol
	1-pentene-3-ol
	1-octen-3-ol
	2-propene-1-ol
	4-hexen-1-ol
	3-methyl-2-buten-1-ol
	3-methyl-3-buten-1-ol
	cyclohexanol
carboxylic acids	acetic acid
	propanoic acid
	butanoic acid
	pentanoic acid
	hexanoic acid
	sorbic acid
	2-methylpropanoic acid
	2-methylbutanoic acid
	3-methylbutanoic acid
	3-hexenoic acid
	4-hexenoic acid

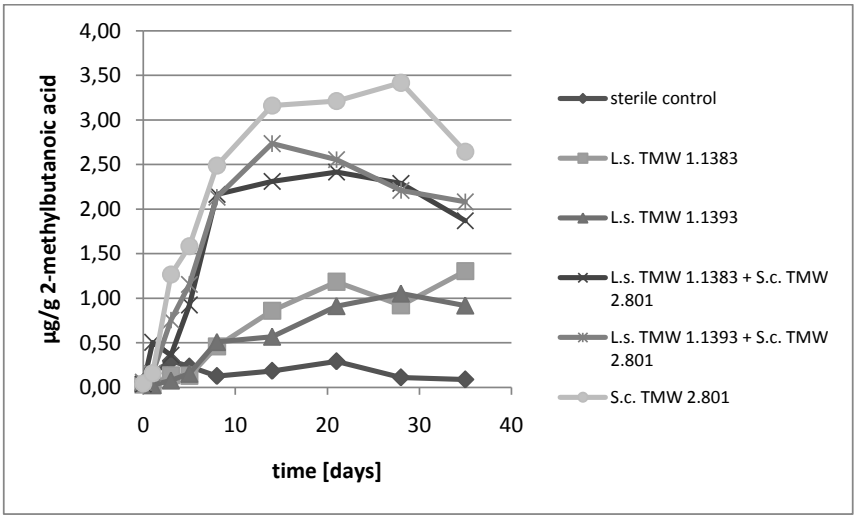
chemical class	compound
esters compounds	ethylacetate
	ethylbutanoate
	ethylhexanoate
	ethylsorbate
	ethyl-2-methylbutanoate
	ethyl-3-methylbutanoate
	ethyl-2-hydroxypropanoate
	ethyl-3-hexanoate
	ethyl-3-hexenoate
	ethyl-2,4-hexadienoate
	linalylpropionate
sulfurcontaing compounds	carbondisulfide
	dimethyldisulfide
	dimethyltrisulfide
	dimethylsulfoxide
	thiazole
	dimethylsulfon
	2-methylthiophene
	3-thiophencarboxyaldehyde
aromatic compounds	benzaldehyde
	3-methylbenzaldehyde
	phenylacetaldehyde
	phenyethylalcohol
	phenol
	4-methylphenol
	2-methoxyphenol
	toluol
	styrene
	3,4-dimethylstyrene
	acetophenone
	1-methyl-2-(1-methylethyl)-benzene
	1-methyl-3-(1-methylethyl)-benzene
	1-methyl-4-(1-methylethyl)-benzene

chemical class	compound
oxygen containing heterocyclic compounds	2-pentylfuran
	butyrolactone
terpenes	α-pinene
	β-pinene
	α-phellandrene
	β-phellandrene
	β-myrcene
	3-carene
	D-limonene
	m-cymene
	o-cymene
	1,3,8-p-menthatriene
	caryophyllene
	isocaryophyllene
	linalool
nitrogen containing compounds	pyridine
	dimethylpyrazine
	benzonitrile
	aniline
	N-ethylbenzeneamine
	4-(methylamino)-3-pentene-2-one
	4-methyl-2-pentanone oxime

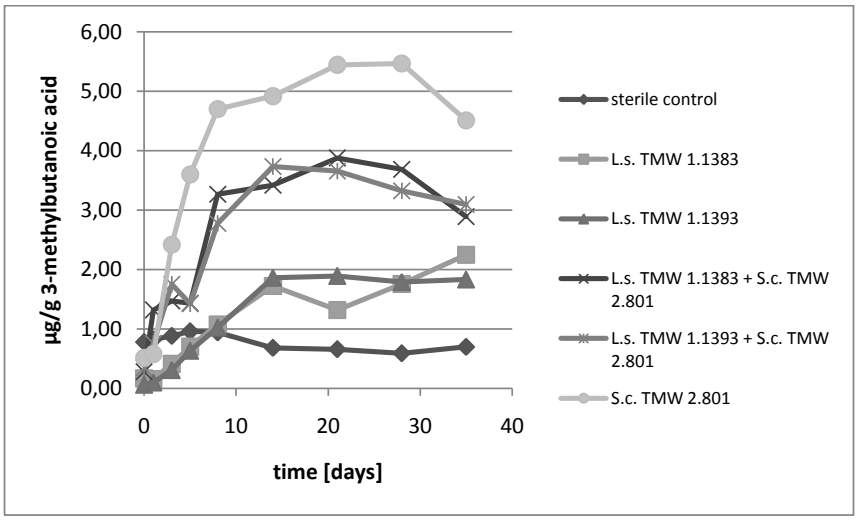
The amounts of the carboxylic acids deriving from branched chain amino acids were very low at the beginning of the sausage fermentation. They were produced during the fermentation of the inoculated sausages and reached the highest amounts at day 28 in the sausages inoculated with *S. carnosus*. The maximal amounts were 1.3 μg/g of 2-methylpropanoic acid, 3.4 μg/g of 2-methylbutanoic acid and 5.5 μg/g of 3-methylbutanoic acid (figure 22). The amounts of 2- and 3-methylbutanoic acid were clearly higher in the sausages inoculated with *S. carnosus* (figure 22 b and c). The non-inoculated control showed less production of these compounds (figure 22).



(a)



(b)

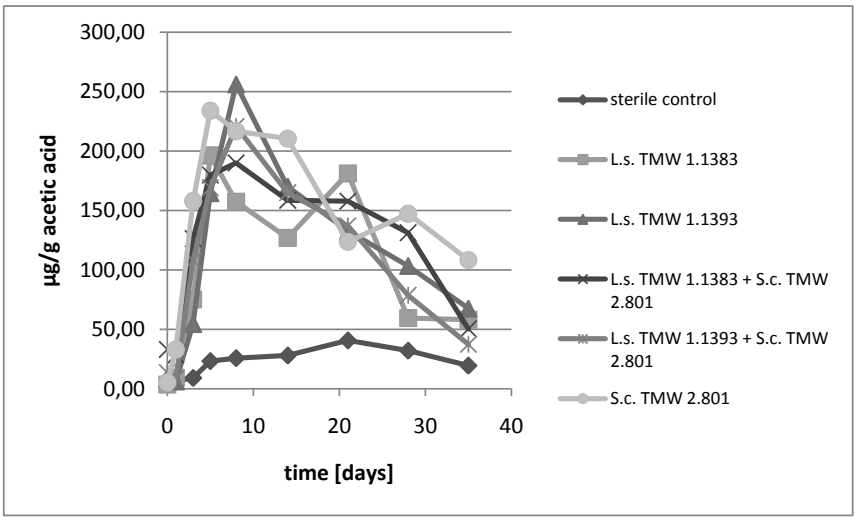


(c)

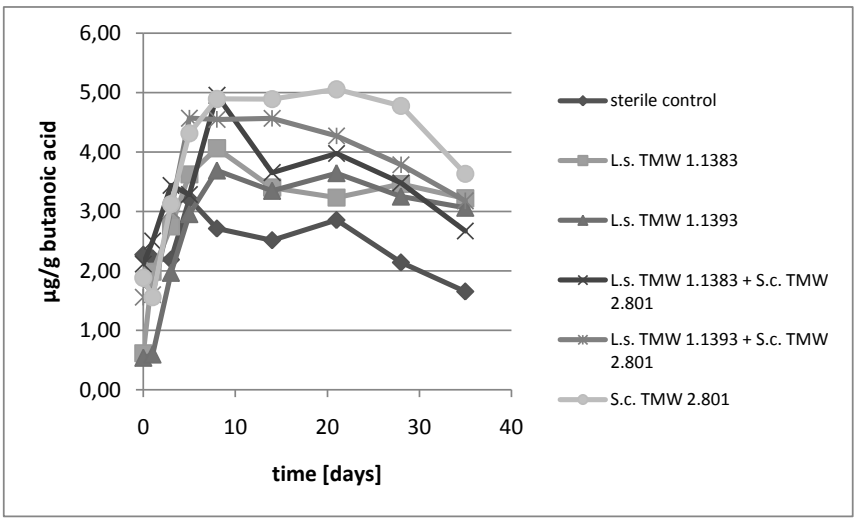
Figure 22: Quantities of the carboxylic acids derived from branched chain amino acids during the fermentation of the sausages. (a) 2-Methylpropanoic acid. (b) 2-Methylbutanoic acid. (c) 3-Methylbutanoic acid.

The amount of acetic acid was very low at the beginning of the fermentation, just like the previous carboxylic acids. The development of the acetic acid was similar in all batches except the sterile control, where only little amounts were produced. Noticeable is the rapid increase at the beginning with values up to 250 μg/g and the decrease of the amount of acetic acid to the end of the fermentation (figure 23 a).

In contrast to the previous carboxylic acids there were already higher amounts of butanoic acid with 0.5 - 2 μg/g at the beginning of the fermentation. The maximal amount could be detected at day 21 in the batch inoculated only with *S. carnosus* with a concentration of 5 μg/g. The differences in the amounts of butanoic acid between the batches were rather low (figure 23 b).



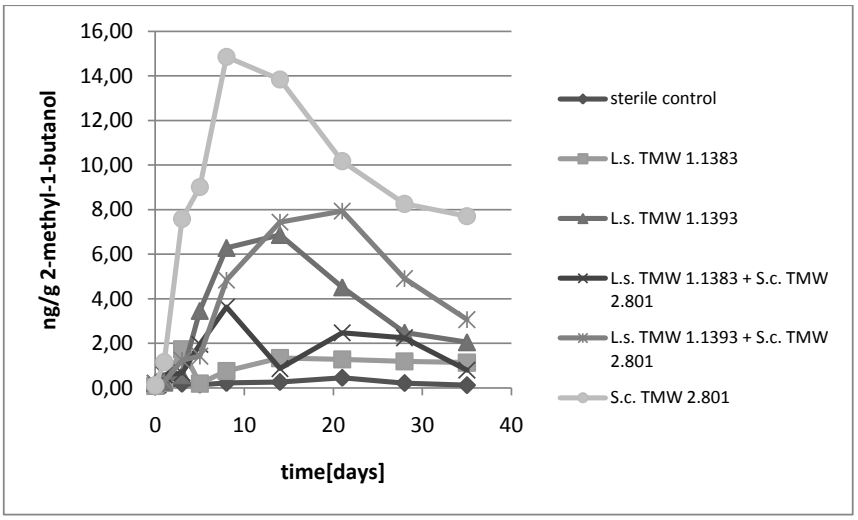
(a)



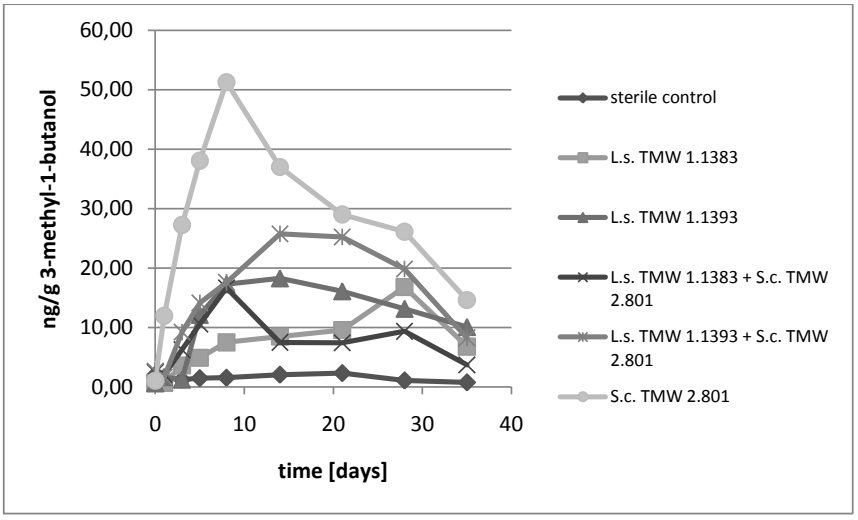
(b)

Figure 23: Quantities of acetic acid and butanoic acid during the fermentation of the sausages. (a) Acetic acid. (b) Butanoic acid.

At the beginning of the fermentation no 2-methyl-1-butanol and 3-methyl-1-butanol could be found. Therefore, these compounds were produced during the fermentation. The amounts of 2-methyl-1-butanol with up to 15 ng/g and 3-methyl-1-butanol with up to 51 ng/g at day 8 were the highest in the batches inoculated only with *S. carnosus*. In the sterile control there was almost no production of these both compounds (figure 24).



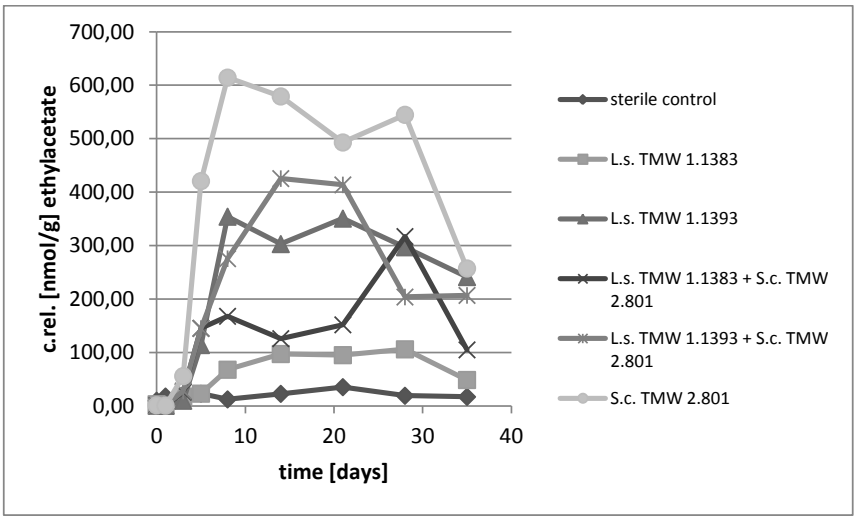
(a)



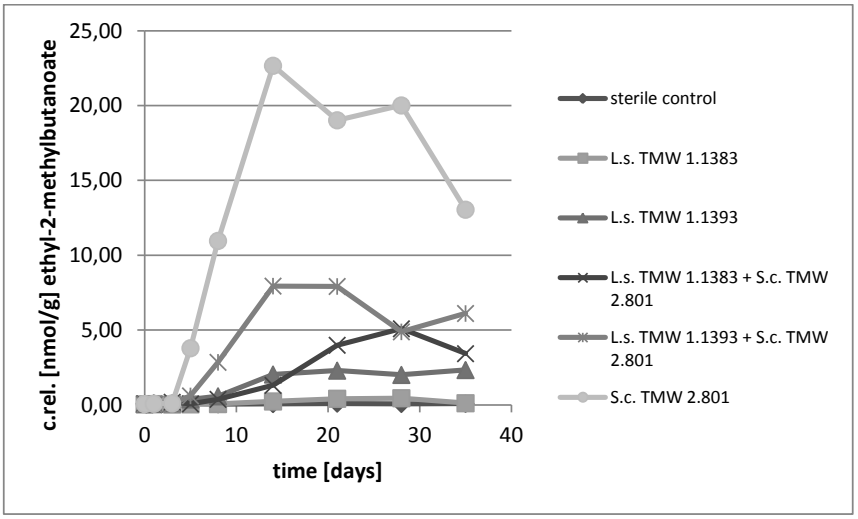
(b)

Figure 24: Quantities of 2-methyl-1-butanol and 3-methy-1-butanol during the fermentation of the sausages. (a) 2-Methyl-1-butanol. (b) 3-Methyl-1-butanol.

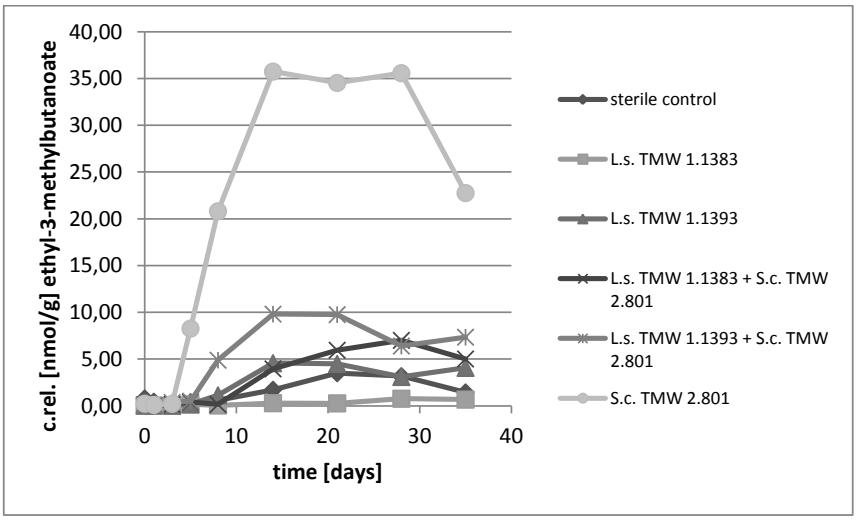
Ethylacetate was produced in every batch during the fermentation except in the sterile control (figure 25 a). The quantities of ethyl-2-methylbutanoate and ethyl-3-methylbutanoate showed large differences between the sausages inoculated with *S. carnosus* and the other batches (figure 25 b and c).



(a)



(b)



(c)

Figure 25: Quantities of ester compounds during the fermentation of the sausages. (a) Ethylacetate. (b) Ethyl-2-methylbutanoate. (c) Ethyl-3-methylbutanoate.

Like the ester compounds the production of phenylacetaldehyde in the sausages depends on the presence of *S. carnosus* (figure 26).

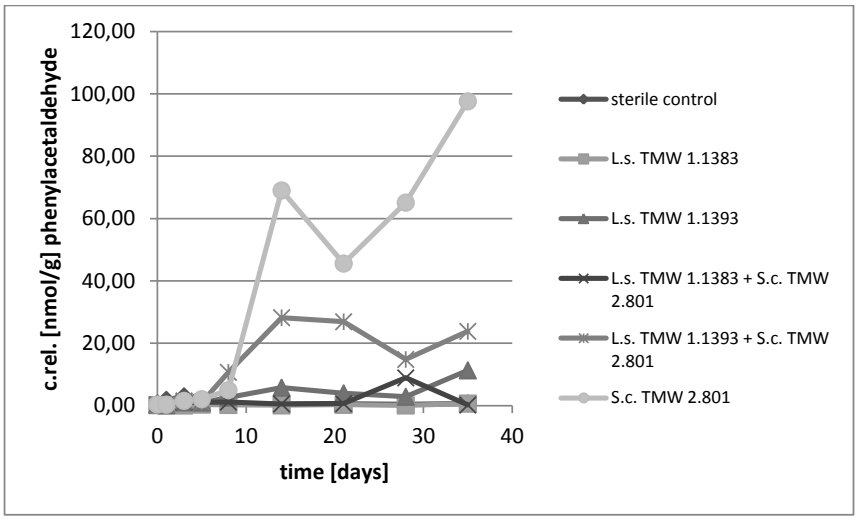
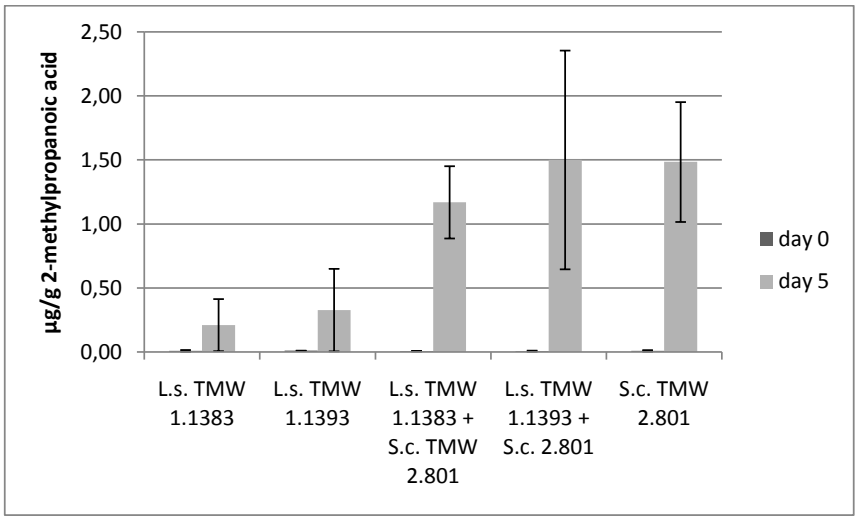


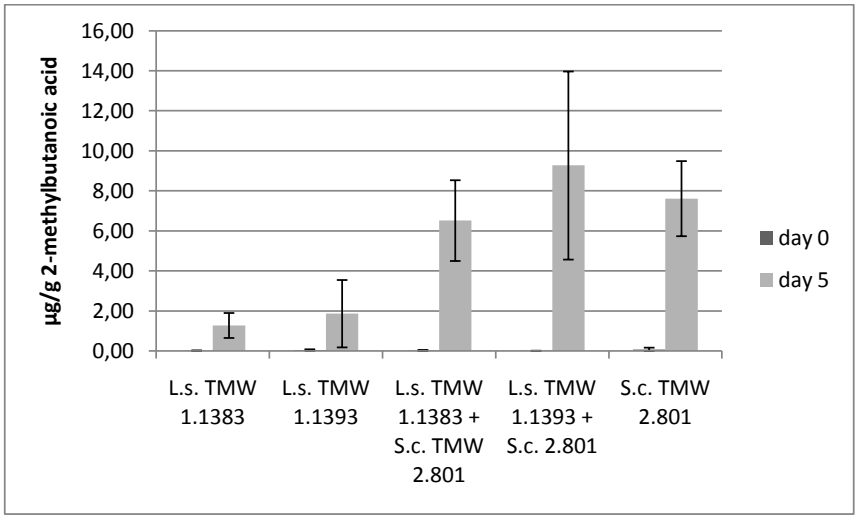
Figure 26: Quantities of phenylacetaldehyde during the fermentation of the sausages.

3.6 Formation of volatile compounds in a sausage model system with *L. sakei* TMW 1.1383, 1.1393, and *S. carnosus* TMW 2.801

The fermentations in the sausage model system were done as described in section 2.2.13. Within five days 2-methylpropanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid were produced in the sausage model system (figure 27). Like in the model fermentations with modified mMRS-medium and in the sausage fermentations the produced amounts were higher if *S. carnosus* was inoculated. The produced amount of 2-methylpropanoic acid is in the same range as in the sausages, while the produced amounts of 2-methylbutanoic acid and 3-methylbutanoic acid were 2 - 3 times higher in the sausage model system.



(a)



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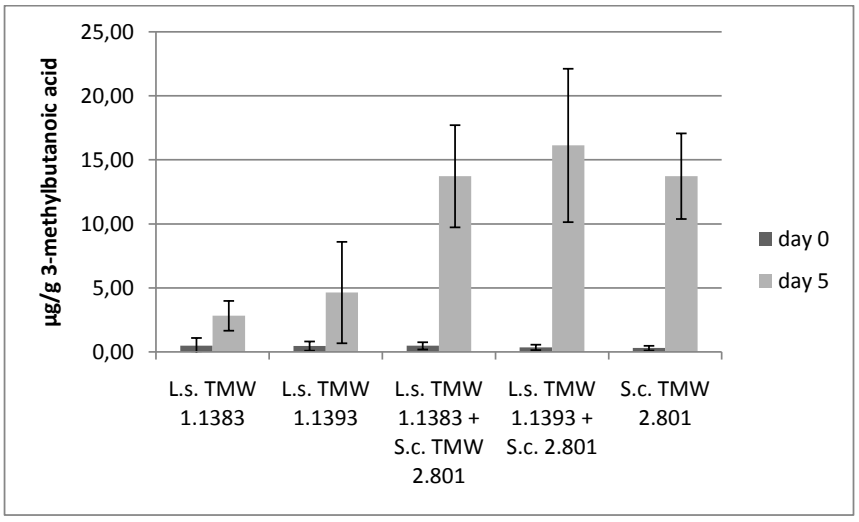
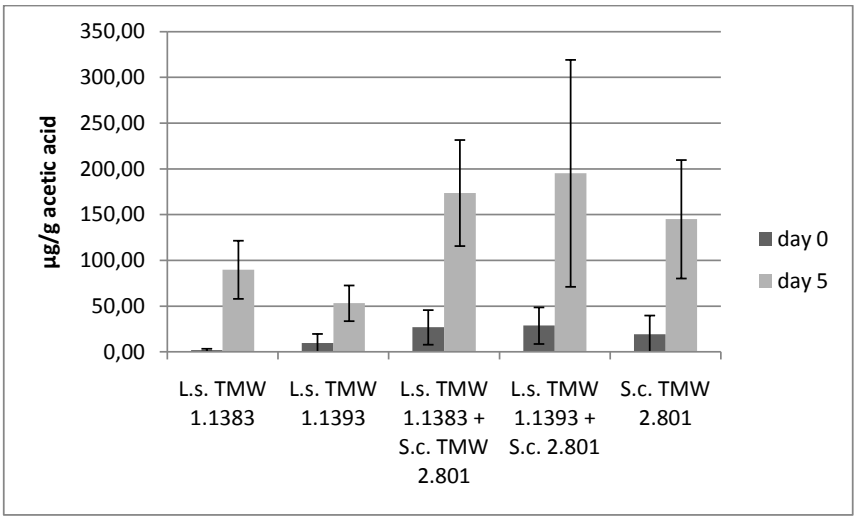


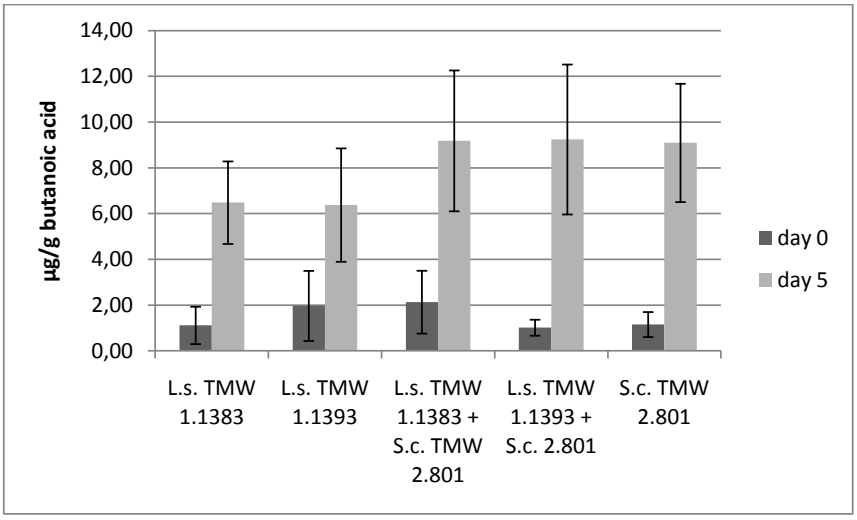
(c)

Figure 27: Production of the carboxylic acids derived from branched chain amino acids in the sausage model system. (a) 2-Methylpropanoic acid. (b) 2-Methylbutanoic acid. (c) 3-Methylbutanoic acid.

Acetic acid and butanoic acid were also produced in the sausage model system (figure 28). The produced amounts of acetic acid were in the same range as in the sausage fermentations. The produced amounts of butanoic acid in the sausage model system were a little higher than in the sausage fermentations.



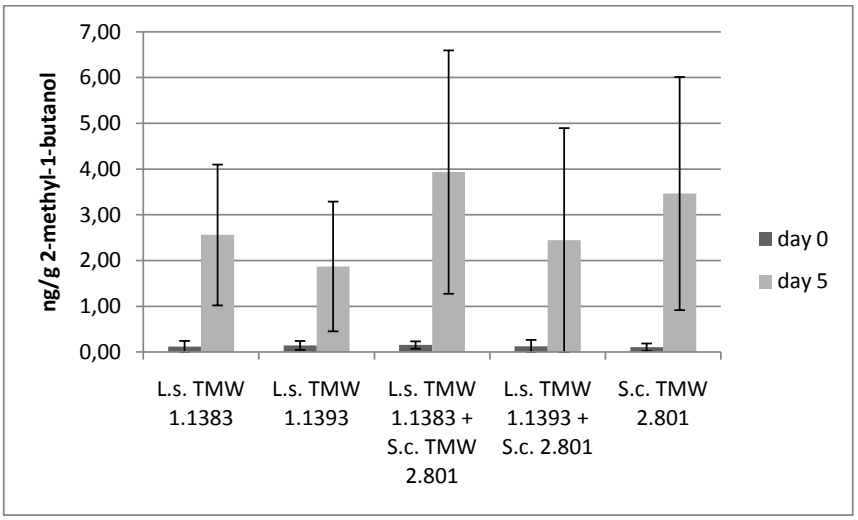
(a)



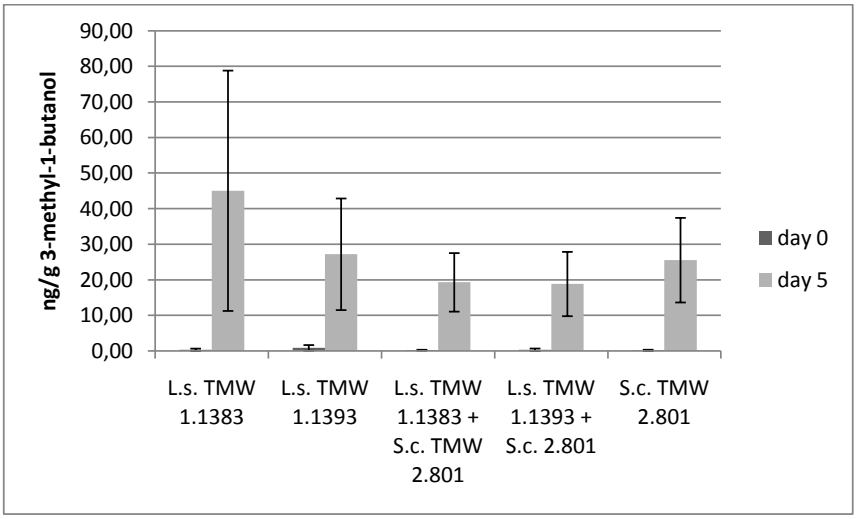
(b)

Figure 28: Production of acetic acid and butanoic acid in the sausage model system. (a) Acetic acid. (b) Butanoic acid.

2-methyl-1-butanol and 3-methyl-1-butanol were produced within five days in the sausage model system, unfortunately with a high range of variation (figure 29). The produced amounts of 2-methyl-1-butanol were lower than in the sausage fermentations, while the produced amounts of 3-methyl-1-butanol were in the same range.



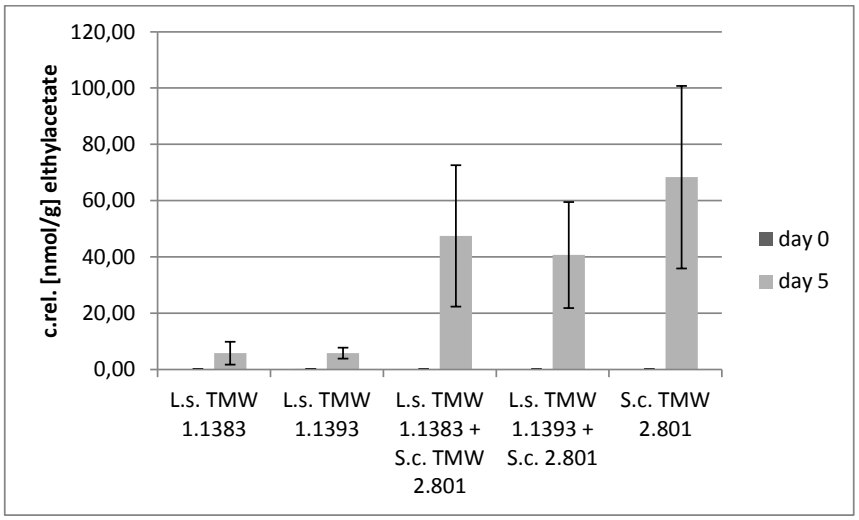
(a)



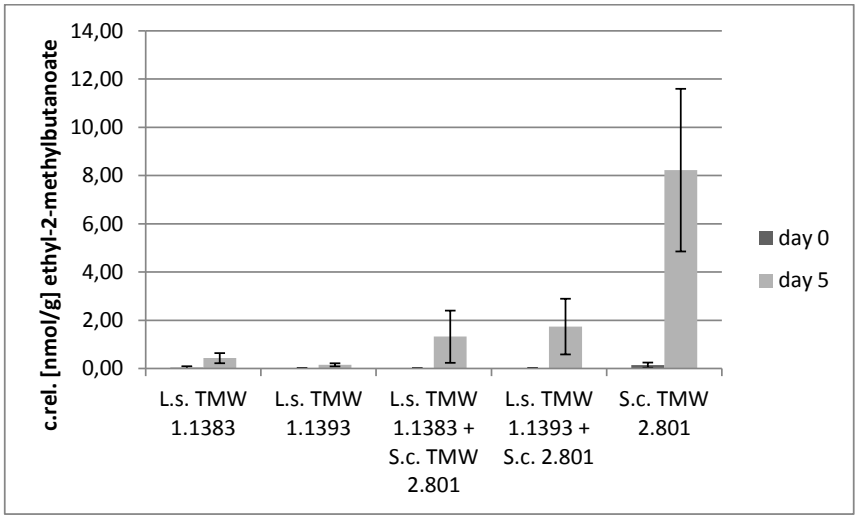
(b)

Figure 29: Production of 2-methyl-1-butanol and 3-methyl-1-butanol in the sausage model system. (a) 2-Methyl-1-butanol. (b) 3-Methyl-1-butanol.

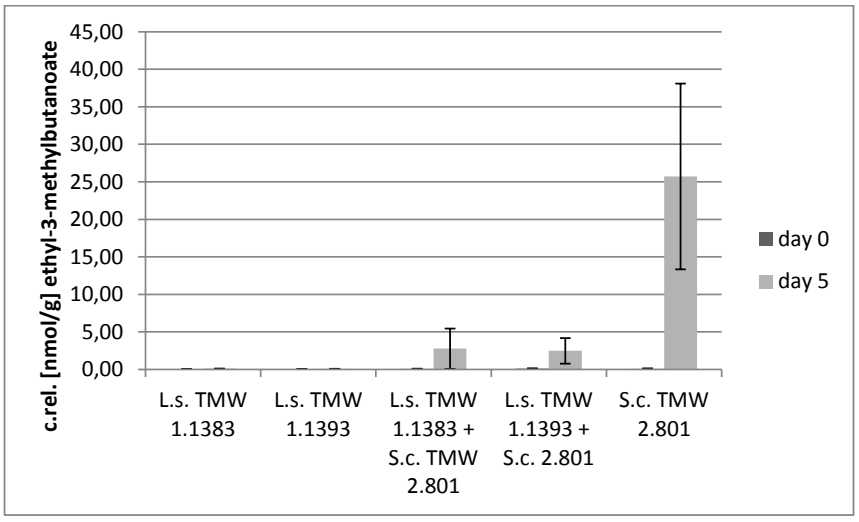
Like in the sausage fermentations a high production of ester compounds was observable in samples inoculated with *S. carnosus* (figure 30). The produced amounts of ethylacetate were much lower in the sausage model system than in the sausage fermentations, while the produced amounts of ethyl-2-methylbutanoate and ethyl-3-methylbutanoate were only slightly lower in the sausage model system.



(a)



(b)



(c)

Figure 30: Production of ester compounds in the sausage model system. (a) Ethylacetate. (b) Ethyl-2-methylbutanoate. (c) Ethyl-3-methylbutanoate.

A production of phenylacetaldehyde could be seen in the sausage model system, but the produced quantities were very low with a high range of variation and no differences were detectable between the batches (figure 31).

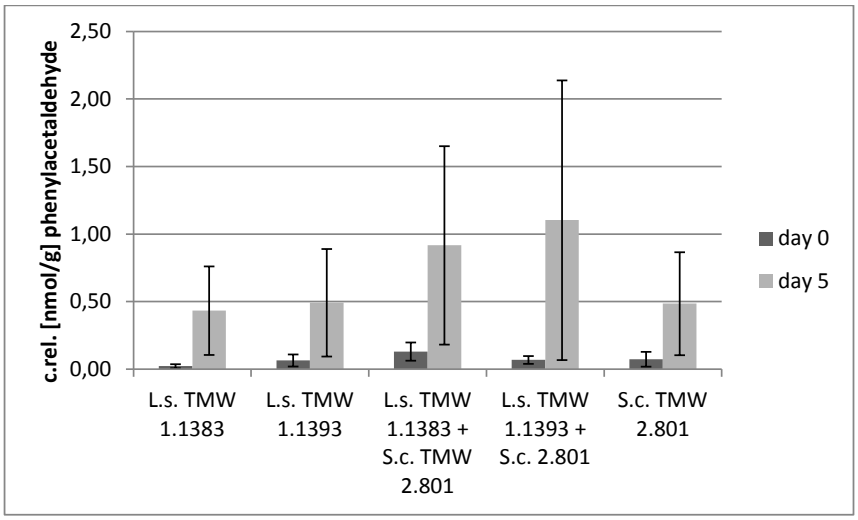


Figure 31: Production of phenylacetaldehyde in the sausage model system.

Within five days a production of 1-butanol was observable in the sausage model system (figure 32). This is in accordance to the model fermentation in modified mMRS-medium, where also 1-butanol was produced in the cultures with *L. sakei* as well as *S. carnosus*.

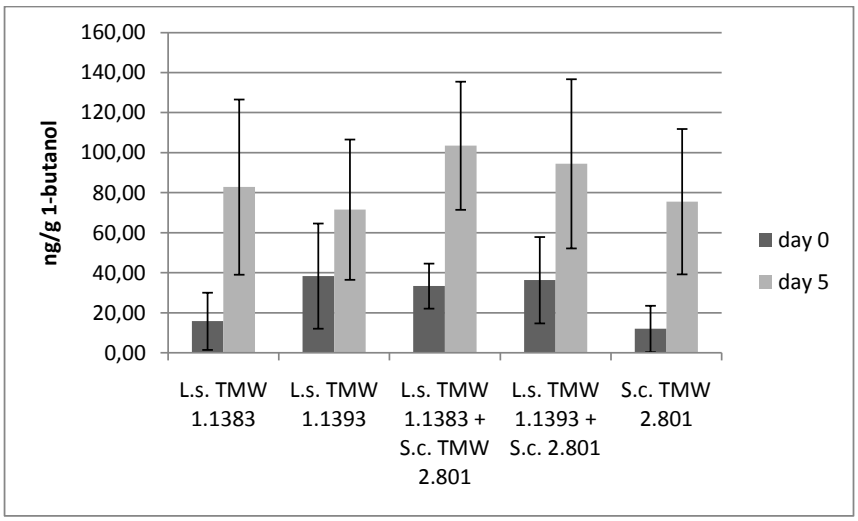


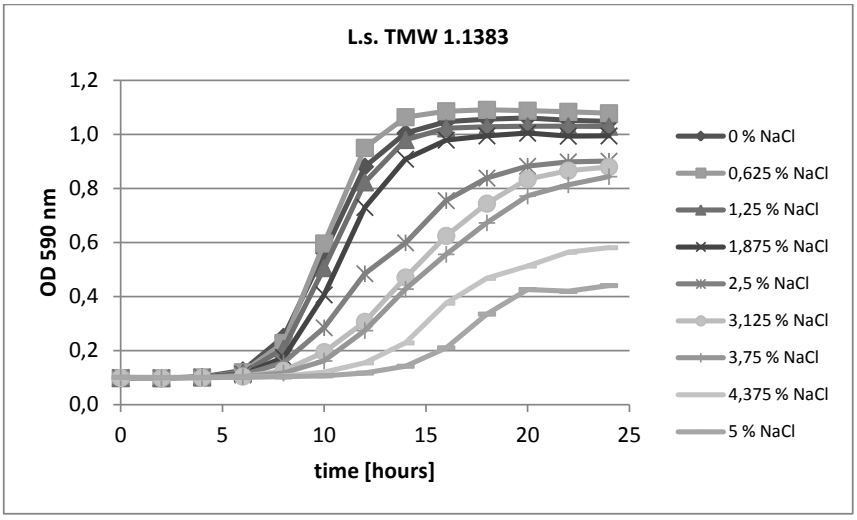
Figure 32: Production of 1-butanol in the sausage model system.

3.7 Growth and production of volatile metabolites by *L. sakei* TMW 1.1383 and 1.1393 under stress conditions

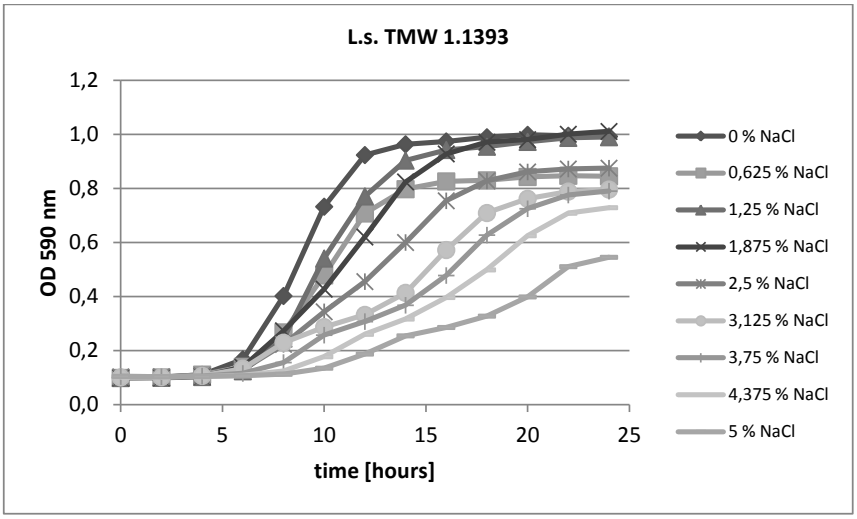
3.7.1 Growth curves under stress conditions

The bacterial growth was measured under different conditions of stress in microtiter plates as described in section 2.2.13. As stress factors salt, curing salt, nitrate, nitrite, and low pH values were used.

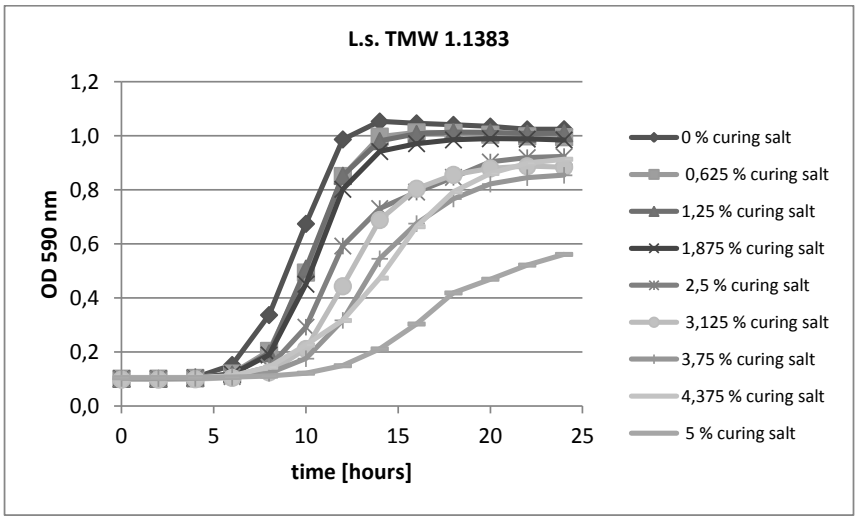
The growth curves of both *L. sakei* strains showed nearly no differences upon addition of salt or curing salt (figure 33). A concentration of 2.5 % salt or curing salt led to a growth impairment of the strains. It is noticeable that generally the strain *L. sakei* TMW 1.1383 showed a slightly better growth than the strain TMW 1.1393. The strain *L. sakei* TMW 1.1383 reached OD-values of nearly 1.1 at 590 nm, while the strain TMW 1.1393 reached only an OD of 1 at 590 nm.



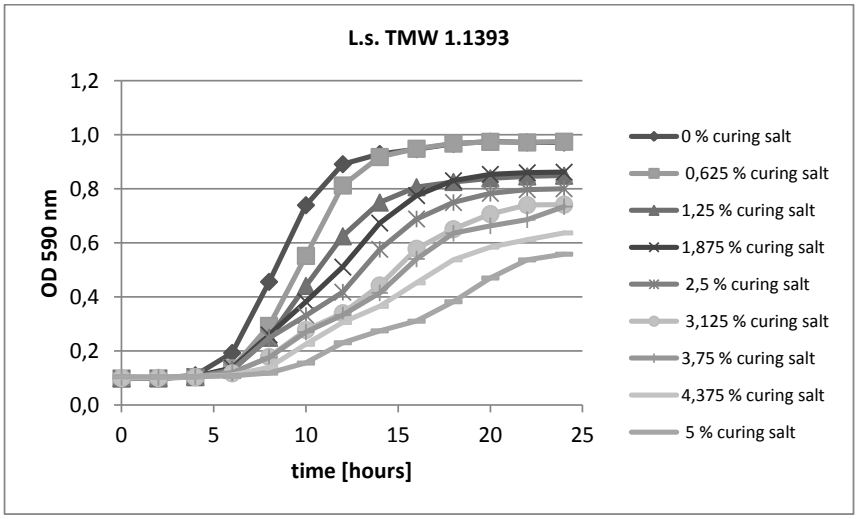
(a)



(b)



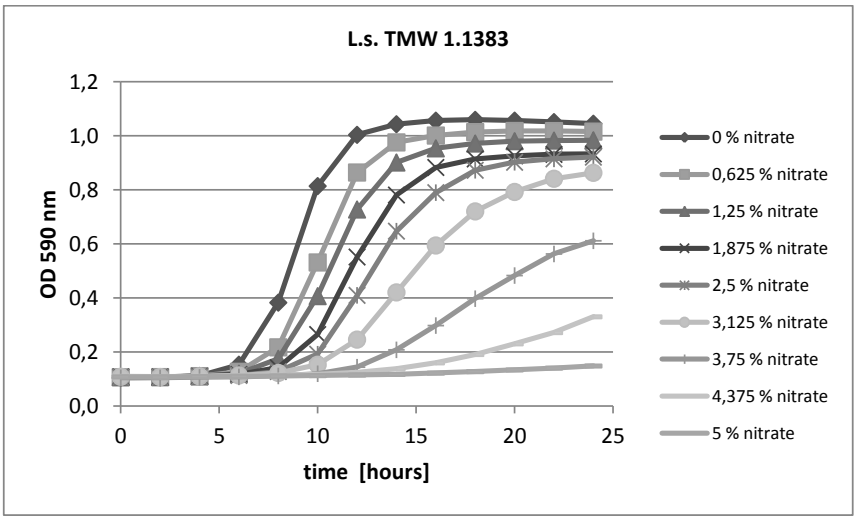
(c)



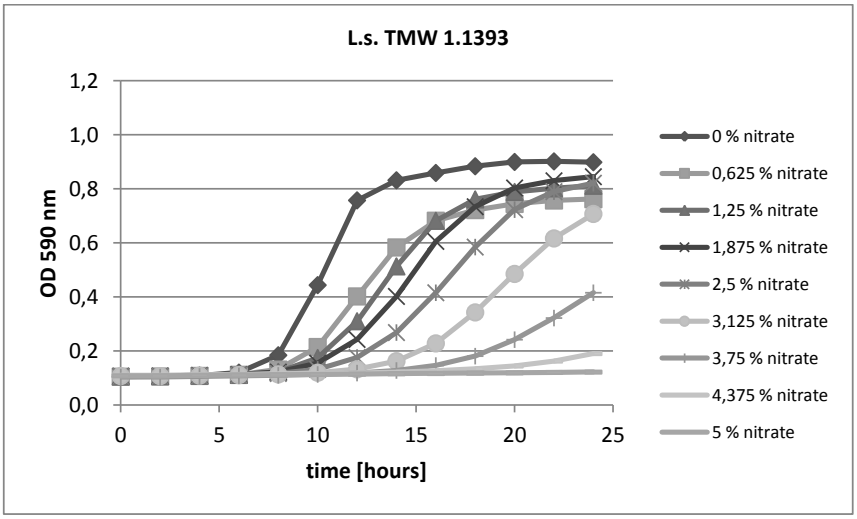
(d)

Figure 33: Growth of the strains *L. sakei* TMW 1.1383 and 1.1393 with different concentrations of salt and curing salt. (a) *L. sakei* TMW 1.1383 with 0 - 5 % NaCl. (b) *L. sakei* TMW 1.1393 with 0 - 5 % NaCl. (c) *L. sakei* TMW 1.1383 with 0 - 5 % curing salt. (d) *L. sakei* TMW 1.1393 with 0 - 5 % curing salt.

The growth curves with addition of nitrate showed that both strains were impaired from a concentration of 2.5 % nitrate (figure 34).



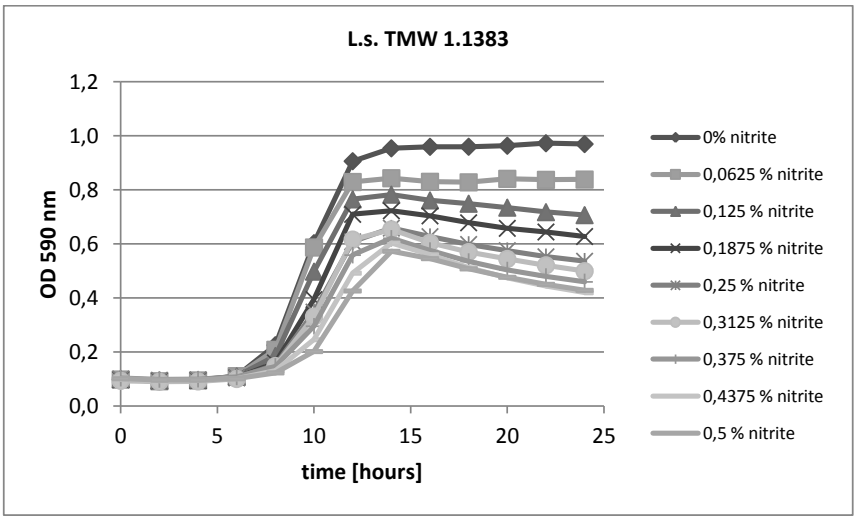
(a)



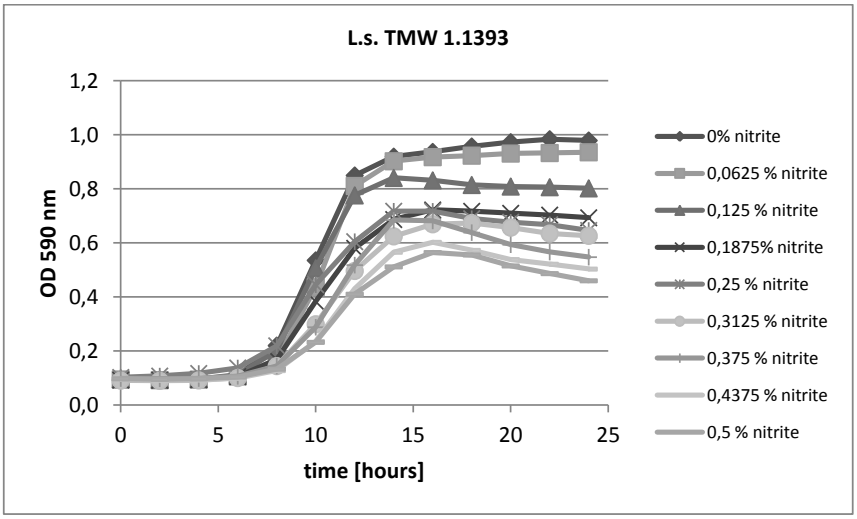
(b)

Figure 34: Growth of the strains *L. sakei* TMW 1.1383 and 1.1393 with different concentrations of nitrate. (a) *L. sakei* TMW 1.1383 with 0 - 5 % nitrate. (b) *L. sakei* TMW 1.1393 with 0 - 5 % nitrate.

To addition of nitrite both strains reacted more sensitively compared to nitrate. In this case a concentration of 0.125 % was sufficient to impair the bacterial growth (figure 35). The strain *L. sakei* TMW 1.1393 showed a slightly better resistance to nitrite than the strain TMW 1.1383 (figure 35).



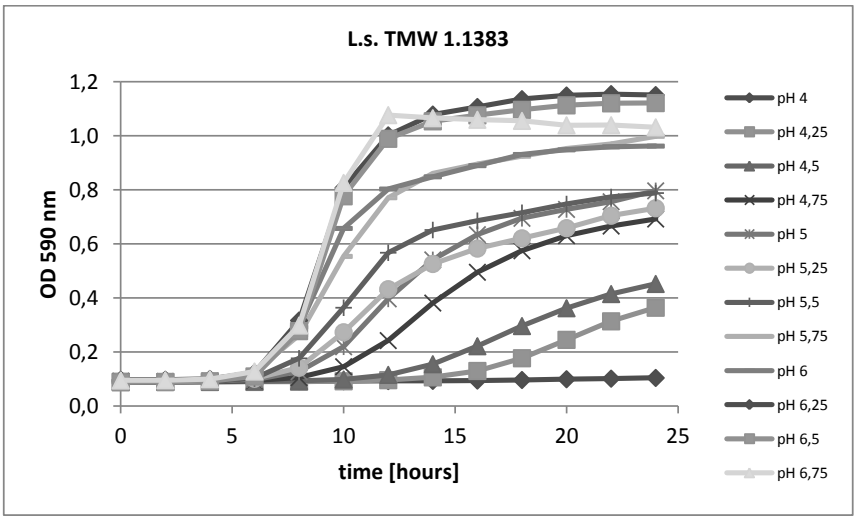
(a)



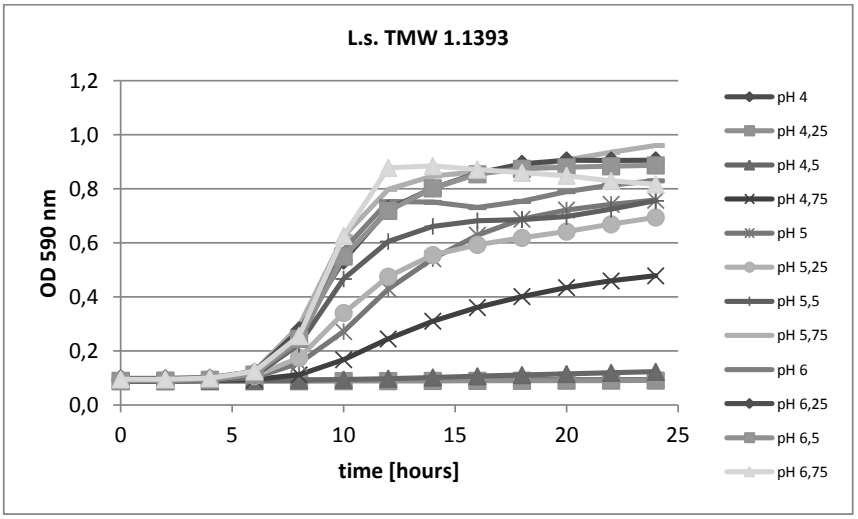
(b)

Figure 35: Growth of the strains *L. sakei* TMW 1.1383 and 1.1393 with different concentrations of nitrite. (a) *L. sakei* TMW 1.1383 with 0 - 0.5 % nitrite. (b) *L. sakei* TMW 1.1393 with 0 - 0.5 % nitrite.

The growth with different pH-values showed that the strain *L. sakei* TMW 1.1393 is much more sensitive to acid stress than the strain TMW 1.1393. The strain *L. sakei* TMW 1.1383 grew at pH 4.25 and 4.5, while the strain TMW 1.1393 did not grow below a pH of 4.75 (figure 36). In summary the strain *L. sakei* TMW 1.1383 showed a better growth at OD 590 nm with up to 1.2 as in previous experiments. The strain *L. sakei* TMW 1.1393 only reached values of OD 1.0 at 590 nm.



(a)



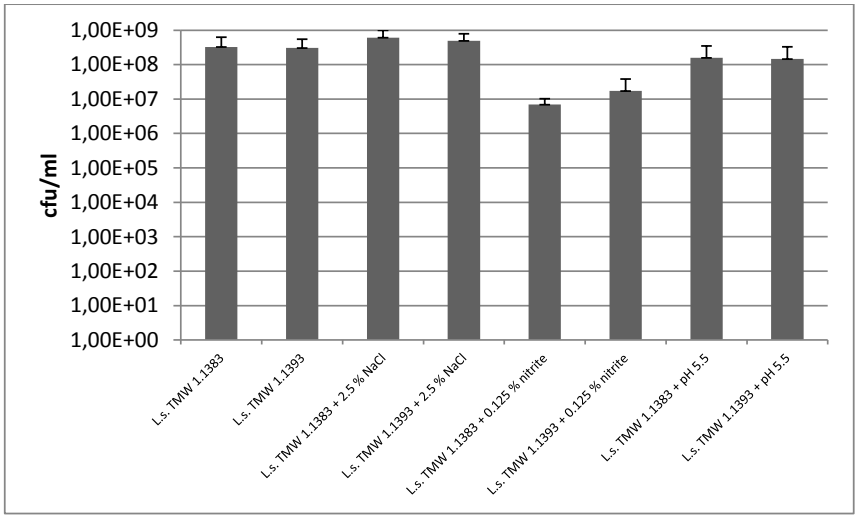
(b)

Figure 36: Growth of the strains *L. sakei* TMW 1.1383 and 1.1393 with different pH-values. (a) *L. sakei* TMW 1.1383 with pH 4 - 6.75. (b) *L. sakei* TMW 1.1393 with pH 4 - 6.75.

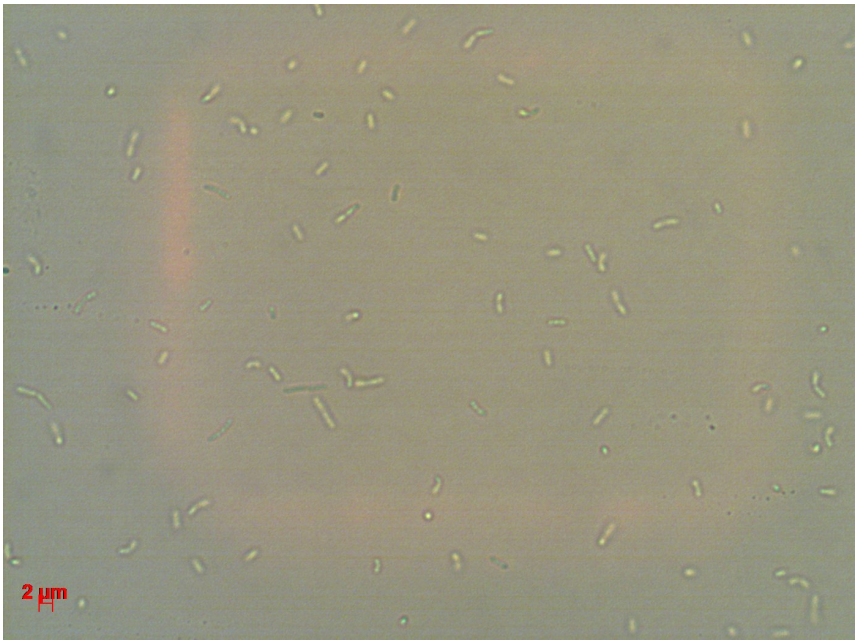
For both strains, the stress conditions, which limited the end OD at 590 nm to a minimum value of 10 % below maximum (in direct, undiluted measurements in the titer plates) were 2.5 % salt, 2.5 % curing salt, 2.5 % nitrate, 0.125 % nitrite and pH 5.5. For further experiments 2.5 % salt, 0.125 % nitrite and pH 5.5 were used. The experiments using curing salt and nitrate were not followed any further, because no other results were expected compared to those obtained with salt. Furthermore, 2,5 % nitrate is nearly the 200 fold amount of nitrate occurring in sausages.

The colony forming units were determined as described in section 2.2.5 under the mentioned stress conditions and without stress. In modified mMRS-medium with-

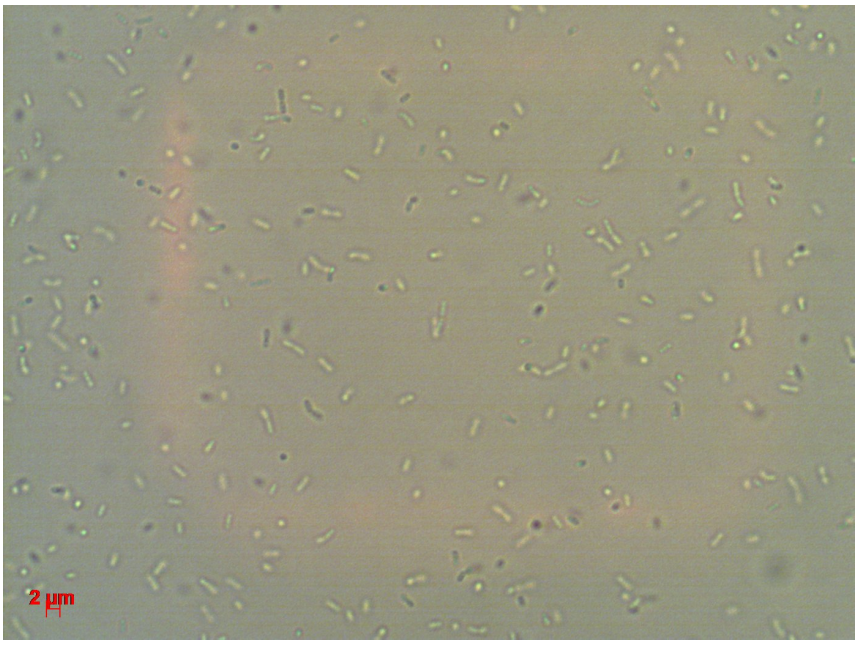
out stress conditions the *L. sakei* strains reached $3 * 10^8$ cfu/ml (figure 37 a). With 2.5 % NaCl the the strains reached $5.5 * 10^8$ cells/ml, while the OD of 590 nm was reduced under this condition. This was concomitant with a morphological change of the cells leading to a reduced cell size (figure 37 c) probably as a result of osmotic stress. With 0.125 % nitrite the colony forming units were drastically reduced to $6.9 * 10^6$ for *L. sakei* TMW 1.1383 and $1.7 * 10^7$ for *L. sakei* TMW 1.1393 (figure 37 a). At pH 5.5 the colony forming units were only reduced to $1.5 * 10^8$ (figure 37 a).



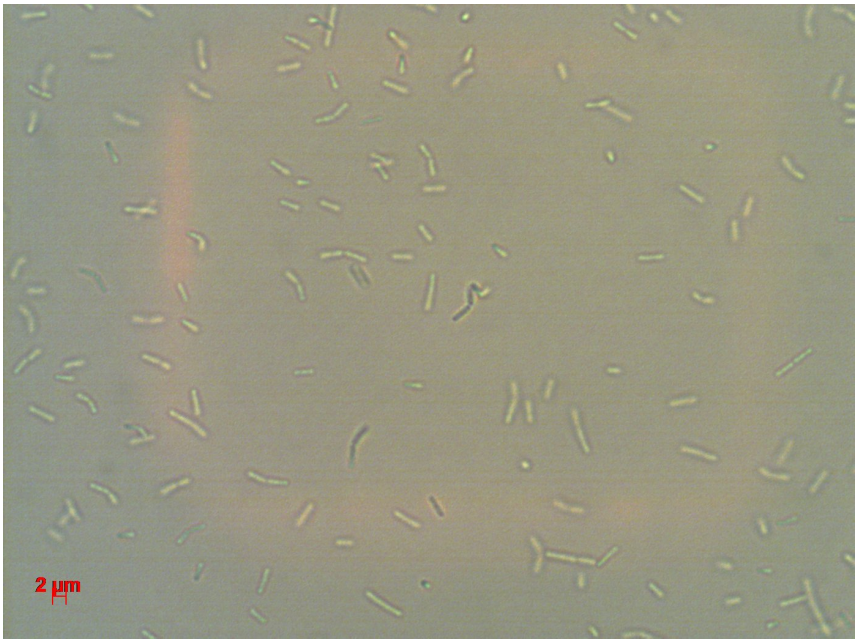
(a)



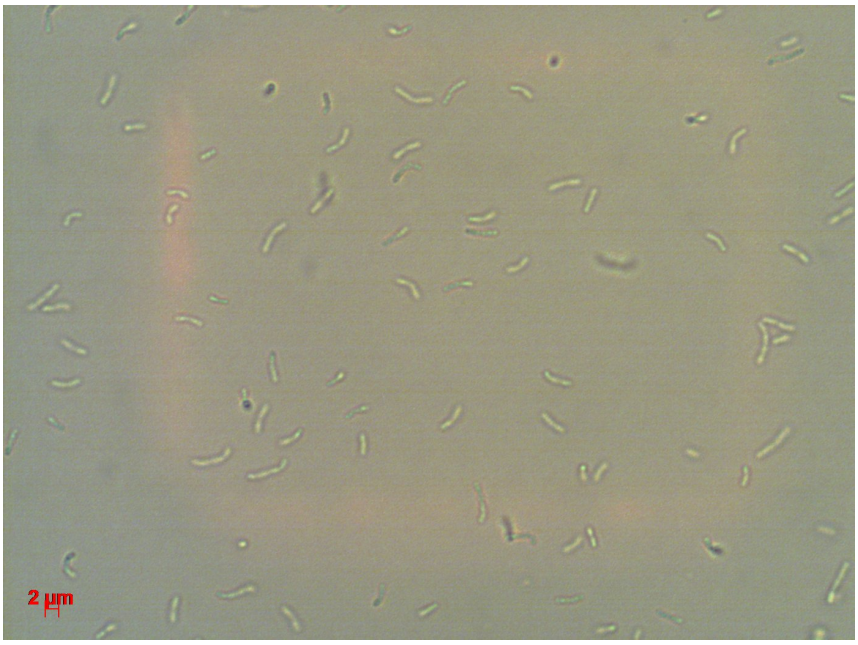
(b)



(c)



(d)



(e)

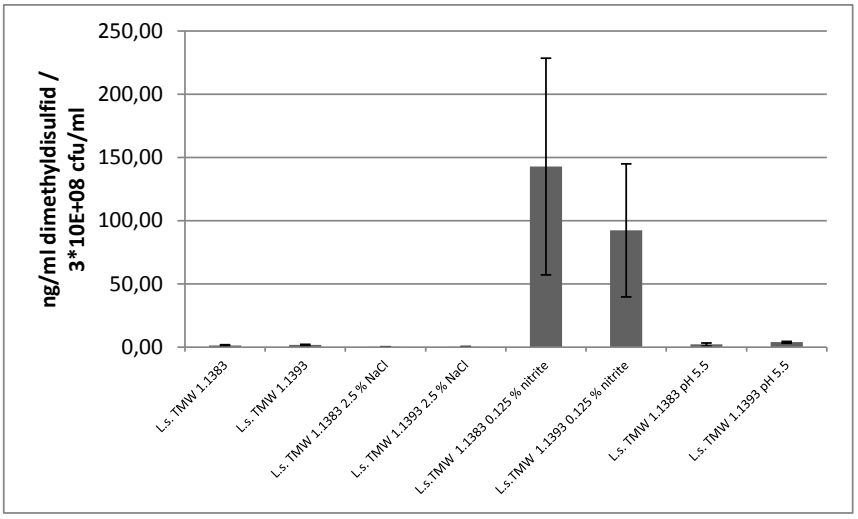
Figure 37: Colony forming units under the different stress conditions and cell morphologies. (a) Colony forming units under the different stress conditions. (b) Morphology of cells of *L. sakei* TMW 1.1383 after growth in modified mMRS without stress parameters. (c) Morphology of cells of *L. sakei* TMW 1.1383 after growth in modified mMRS with 2.5 % NaCl. (d) Morphology of cells of *L. sakei* TMW 1.1383 after growth in modified mMRS with 0.125 % nitrite. (e) Morphology of cells of *L. sakei* TMW 1.1383 after growth in modified mMRS with pH 5.5.

3.7.2 Production of volatile metabolites under stress conditions

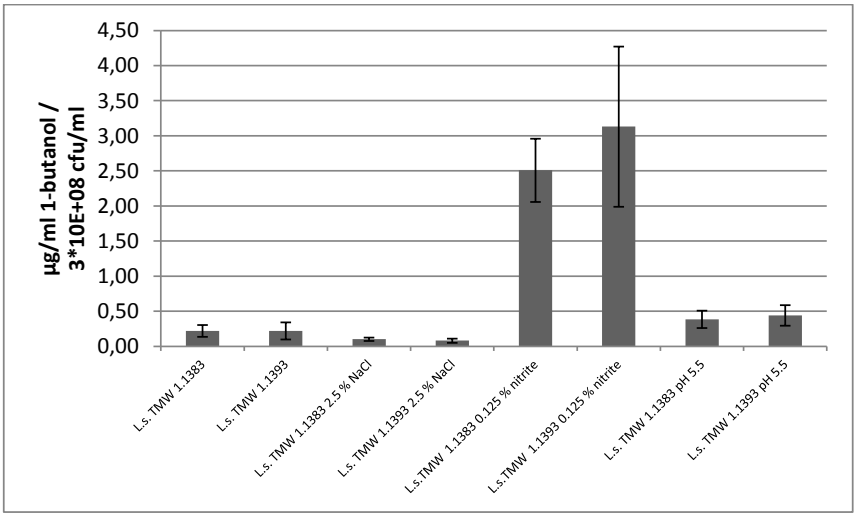
The analysis of volatile metabolites under stress conditions was performed as described in section 2.2.15. As stress factors 2.5 % salt, 0.125 % nitrite and pH 5.5 were used.

The cultivation under stress conditions changed the quantities of the produced amounts of dimethyldisulfide, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, acetoin, acetic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid (figure 38). The production of these compounds was reduced by cultivation with salt stress and massively increased with nitrite stress in consideration of the cell counts. The strain *L. sakei* TMW 1.1383 produced higher amounts of acetoin under nitrite stress than the strain *L. sakei* TMW 1.1393 (figure 38 e). The production of acetic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid was increased under pH stress

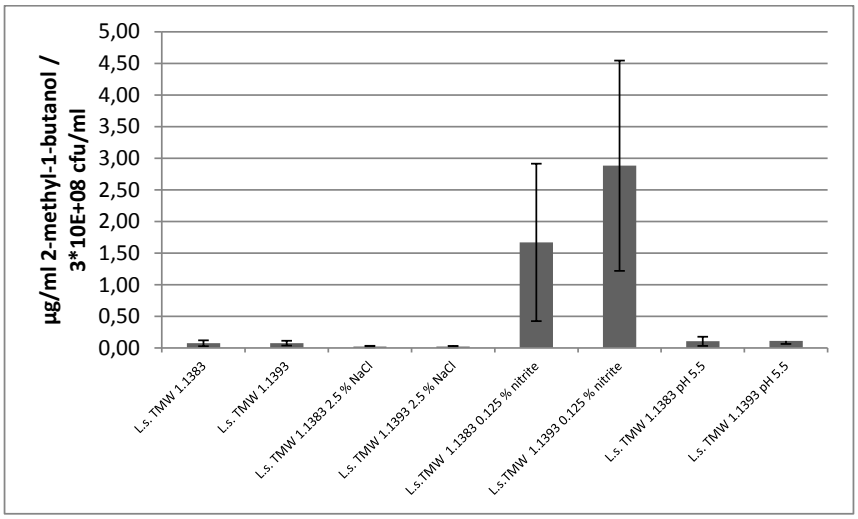
(figure 38 f - j). In the figures, the amounts of volatiles formed were normalized to a cfu of $3 * 10^8$ to enable quantitative comparison. As these numbers were already achieved after 24 h metabolites formed by significantly lower numbers present during the initial phase of fermentation can be neglected.



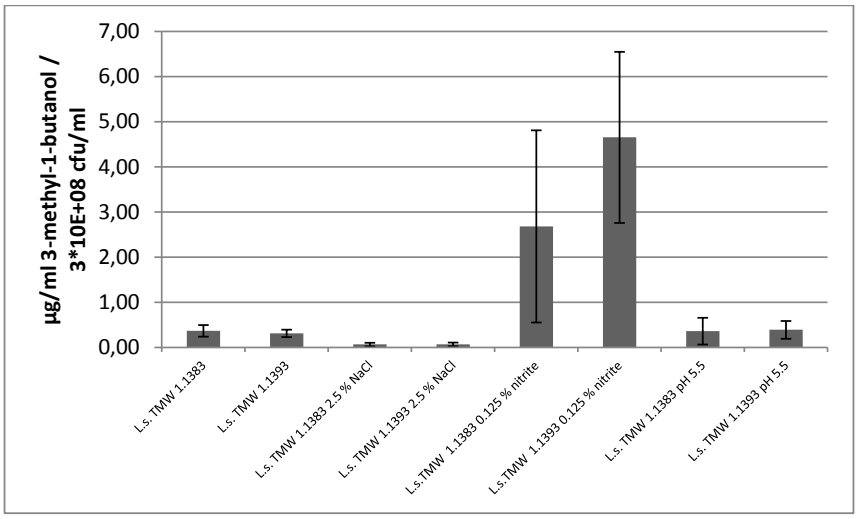
(a)



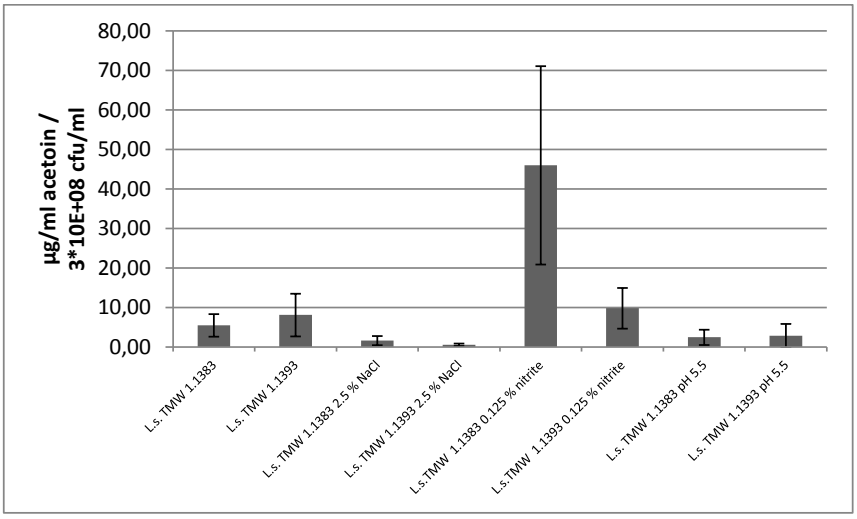
(b)


µg/ml 2-methyl-1-butanol / 3*10E+08 cfu/ml
5,00
4,50
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

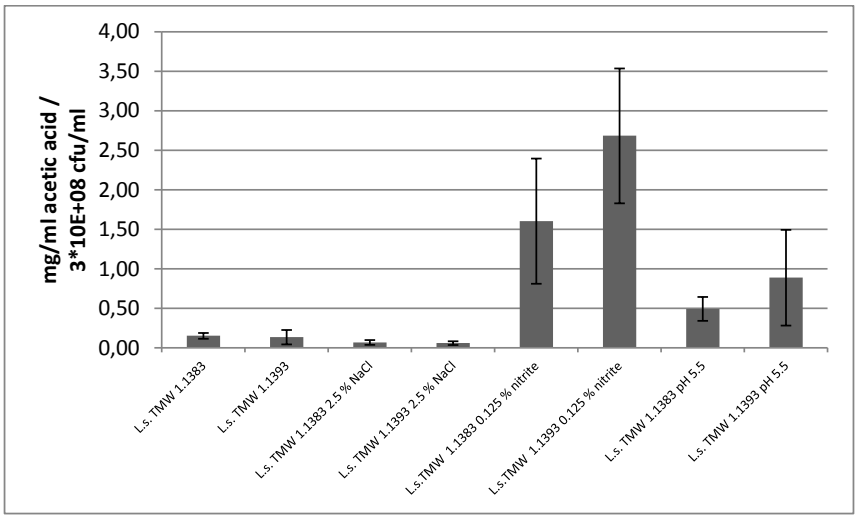
(c)


µg/ml 3-methyl-1-butanol / 3*10E+08 cfu/ml
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

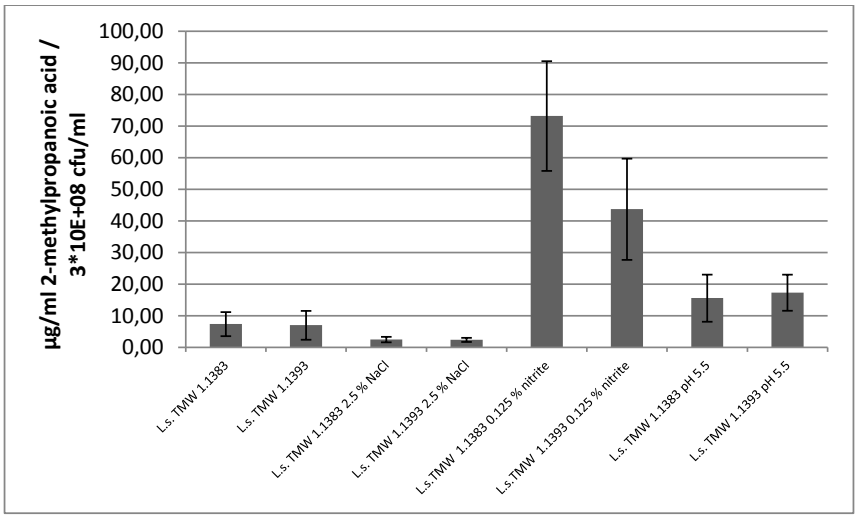
(d)


µg/ml acetoin / 3*10E+08 cfu/ml
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

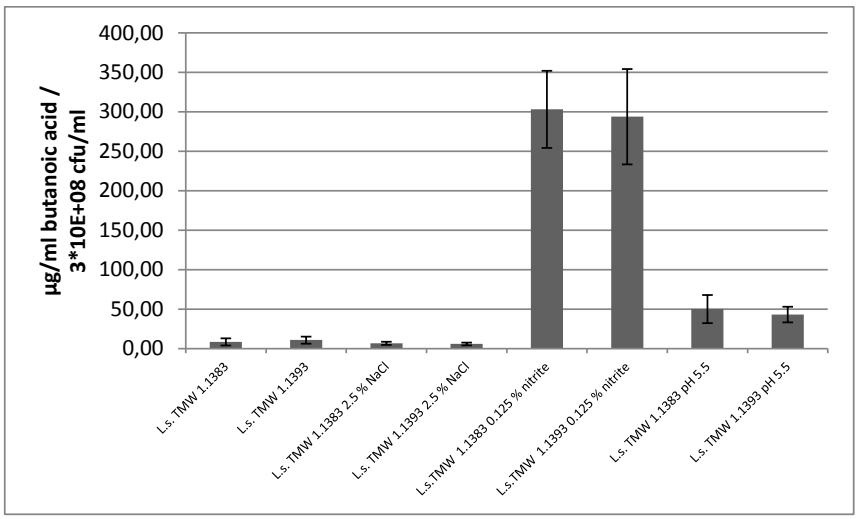
(e)


mg/ml acetic acid / 3*10E+08 cfu/ml
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

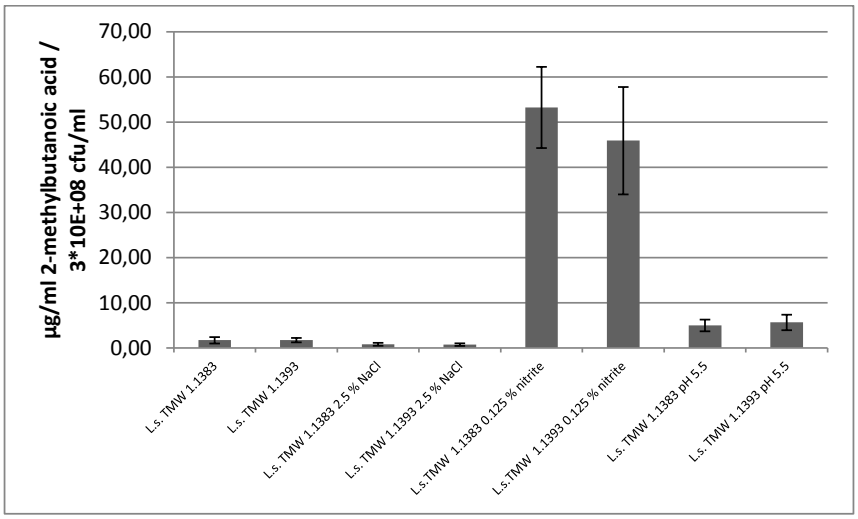
(f)


µg/ml 2-methylpropanoic acid / 3*10E+08 cfu/ml
100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

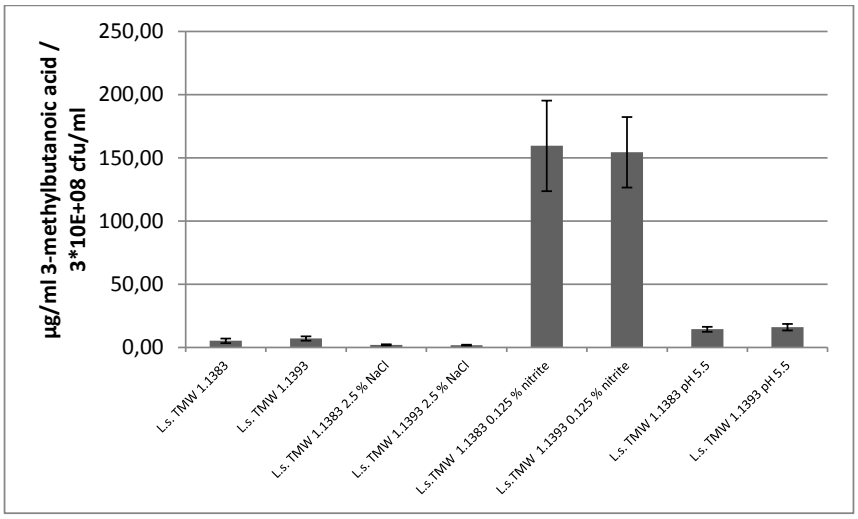
(g)


µg/ml butanoic acid / 3*10E+08 cfu/ml
400,00
350,00
300,00
250,00
200,00
150,00
100,00
50,00
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

(h)



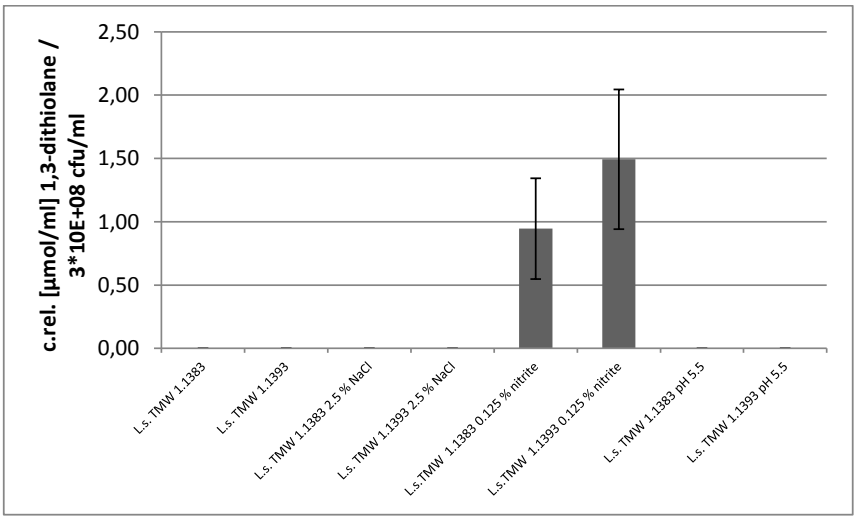
(i)



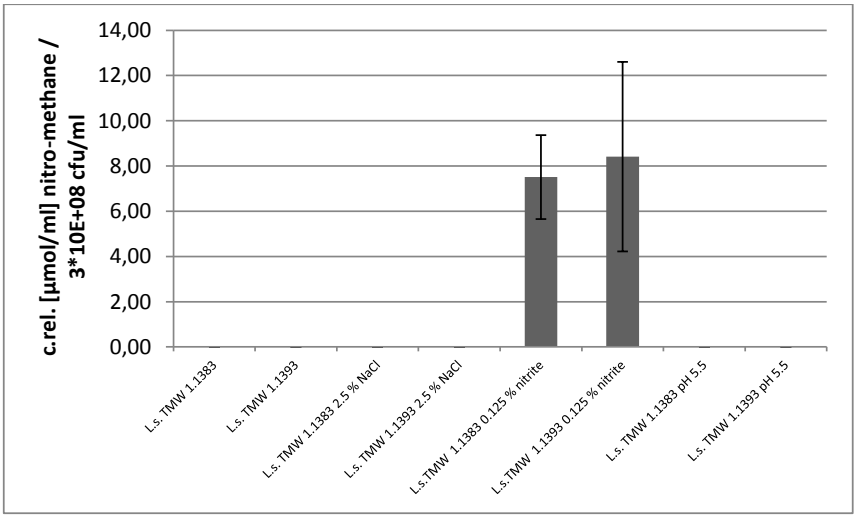
(j)

Figure 38: Production of volatile metabolites under stress conditions. (a) Production of dimethyldisulfide. (b) Production of 1-butanol. (c) Production of 2-methyl-1-butanol. (d) production of 3-methyl-1-butanol. (e) Production of acetoin. (f) Production of acetic acid. (g) Production of 2-methylpropanoic acid. (h) Production of butanoic acid. (i) Production of 2-methylbutanoic acid. (j) Production of 3-methylbutanoic acid.

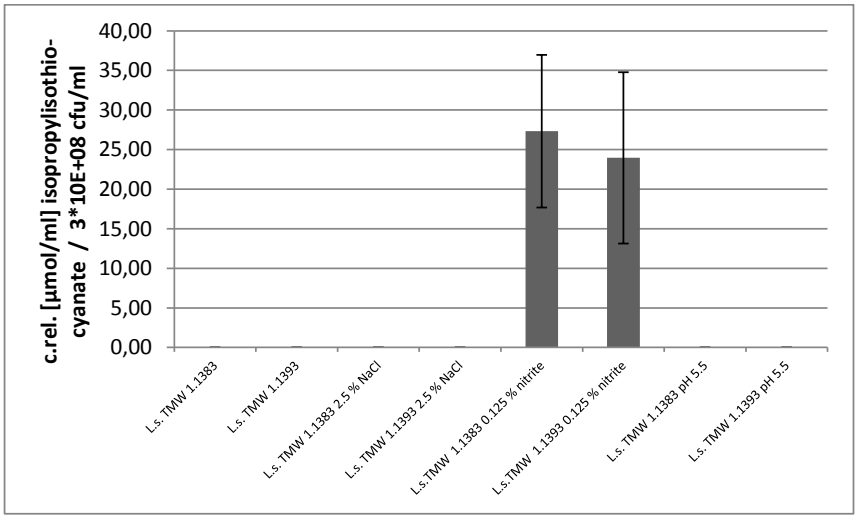
The cultivation under nitrite stress stress also led to the production of 1,3-dithiolane, nitro-methane, isopropylisothiocyanate, isothiocyanato-methane, 1-isothiocyanato-butane, 1,3-dithiane and isobutylisothiocyanate. These compounds were not observed when cultivated under standard conditions. (figure 39).


c.rel. [µmol/ml] 1,3-dithiolane / 3*10E+08 cfu/ml
2,50
2,00
1,50
1,00
0,50
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

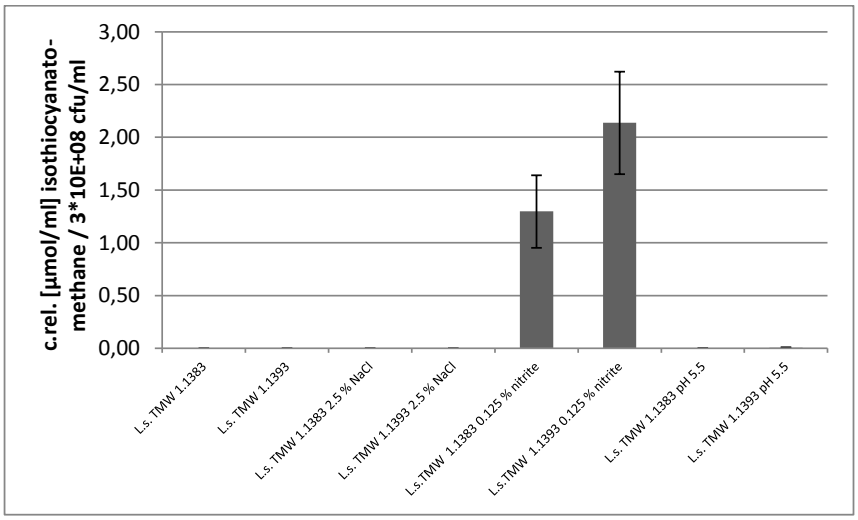
(a)


c.rel. [µmol/ml] nitro-methane / 3*10E+08 cfu/ml
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

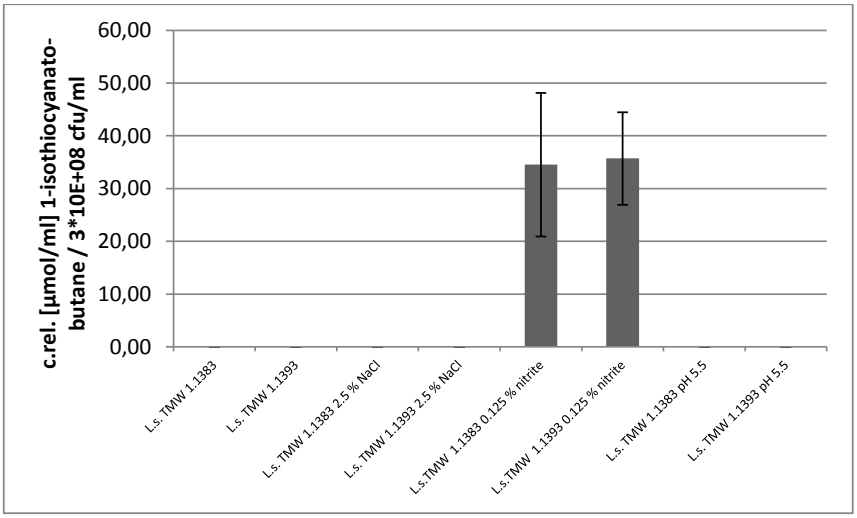
(b)


c.rel. [µmol/ml] isopropylisothio-cyanate / 3*10E+08 cfu/ml
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

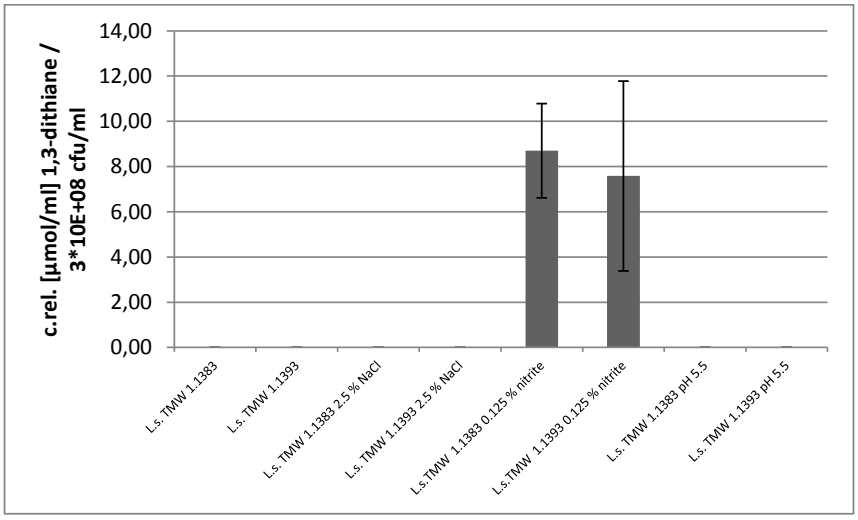
(c)


c.rel. [µmol/ml] isothiocyanato-methane / 3*10E+08 cfu/ml
3,00
2,50
2,00
1,50
1,00
0,50
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

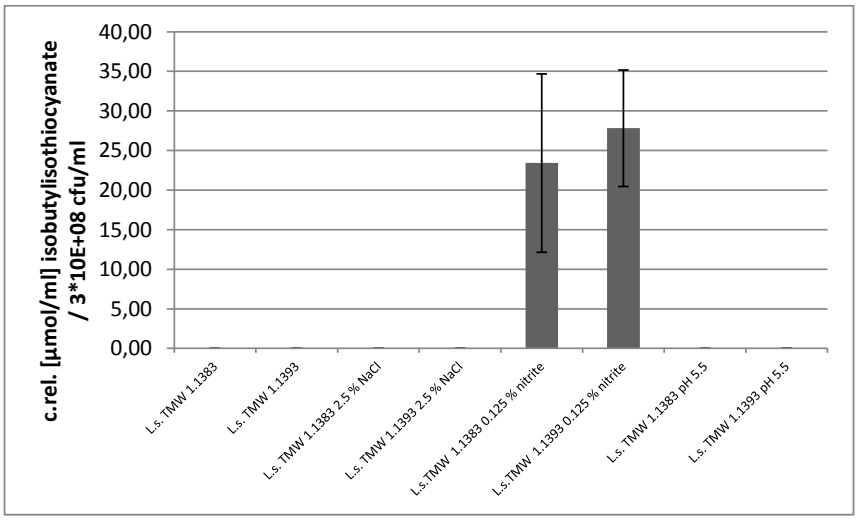
(d)


c.rel. [µmol/ml] 1-isothiocyanato-butane / 3*10E+08 cfu/ml
60,00
50,00
40,00
30,00
20,00
10,00
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

(e)


c.rel. [µmol/ml] 1,3-dithiane / 3*10E+08 cfu/ml
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
L.s. TMW 1.1383
L.s. TMW 1.1393
L.s. TMW 1.1383 2.5 % NaCl
L.s. TMW 1.1393 2.5 % NaCl
L.s.TMW 1.1383 0.125 % nitrite
L.s.TMW 1.1393 0.125 % nitrite
L.s. TMW 1.1383 pH 5.5
L.s. TMW 1.1393 pH 5.5

(f)



(g)

Figure 39: Production of the volatile metabolites under nitrite stress. (a) Production of 1,3-dithiolane. (b) Production of nitro-methane. (c) Production of isopropylisothiocyanate. (d) Production of isothiocyanato-methane. (e) Production of 1-isothiocyanato-butane. (f) Production of 1,3-dithiane. (g) Production of isobutylisothiocyanate.

4 Discussion

This work showed that the metabolism and the production of volatile compounds are similar in various *L. sakei* strains under growth in complex medium. In modified mMRS-medium with 0.3 % glucose (resembling meat conditions) they produced dimethyldisulfide, 1-butanol, 2-methyl-butanol, 3-methyl-butanol, acetoin, acetic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid. The profiles of volatile compounds from meat-associated bacterial species like *Lactobacillus sakei*, *L. paracasei*, *L. farciminis*, *Pediococcus pentosaceus*, *Kocuria varians* and *Staphylococcus carnosus* were different and distinguishable from each other. The conversion of branched-chain amino acids into volatile compounds is very limited in *L. sakei*, while the addition of the corresponding α-keto acid led to a multiplied production of carboxylic acids and ketones. Under these conditions and in contrast to *L. sakei S. carnosus* produced alcohols, carboxylic acid, ester compounds and aldehydes except 3-methylbutanal. The *L. sakei* strains are similar in their production of volatile compounds, but they show differences in their assertiveness against other strains during sausage fermentations. Used as starter culture the strain *L. sakei* TMW 1.1383 is virtually the only *Lactobacillus* strain throughout the whole fermentation, while the strain *L. sakei* TMW 1.1393 is much less assertive against other lactobacilli and often grows together with other *Lactobacillus* strains in the sausages. This observation seemed not to be caused by the formation of bacteriocins, which were negative for both strains. The highest concentrations of volatile compounds could be measured in sausages inoculated with *S. carnosus*. The fermentations in a meat model system led to the production of the same volatile compounds, rendering it a useful approach for the screening of starter strains. The growth under stress conditions present in fermented sausages showed that growth and metabolism of *L. sakei* are affected by these factors. For nitrate and nitrite higher amounts than those present in sausages were necessary to affect growth. Still, the production of volatile compounds is massively increased upon nitrite stress.

4.1 Quantification of volatile compounds by SPME-GC-MS

In this work all volatile compounds were determined by GC-MS analysis with SPME for sample collection. This technique was first established by Pawliszyn and coworkers in the early 90s. It was then introduced to analyze the volatile profile of

food samples. Summaries are given by Kataoka *et al.* (2000) and Plutowska *et al.* (2007). Marco *et al.* (2004) introduced this technique for the analysis of raw fermented sausages.

From 22 volatile compounds the calibration equations were calculated. The relation of the peak areas and the quantities of the compounds diacetyl and 2-methylpropanal were linear in the measured range. In all other cases the relation of the peak area to the quantity of the compounds was a polynomial equation. This was also observed by Siebert *et al.* (2005) for carboxylic acids. Flores & Hernández (2007) and Pino & Queris (2010) also observed that most volatile compounds show a linear range only in a defined calibration range.

The advantage of the SPME-GC-MS technique is that the volatiles compounds have not to be extracted by solvents and the measurements are done relatively fast, so that screenings with many samples can be performed. The disadvantages are that the different fiber types show a different selectivity towards various compounds and the volatile compounds have different binding rates to the fibers (Vergnais *et al.*, 1998; Roberts *et al.*, 2000; Marco *et al.*, 2004; Yu *et al.*, 2008). Therefore the chromatograms do not show the real composition of a sample. This has to be kept in mind especially when there are no direct quantifications done with all detected compounds. The adsorption rates of the compounds to the fibers are saturation curves which partly need many hours for saturation (Marco *et al.*, 2004). Therefore it is important that always the same adsorption times are used if measurements need to be compared. Another general disadvantage of the whole SPME-GC-MS system is that the device related fluctuations were multiplied with the fluctuations of the samples. This often leads to high standard deviations. Yang & Peppard (1994) also observed such effects.

4.2 Screening of 51 *Lactobacillus sakei* and 30 *Lactobacillus curvatus* strains

In model fermentations 51 *L. sakei* and 30 *L. curvatus* strains were screened for their potential to produce volatile compounds. Strains of *Lactobacillus paracasei*, *Lactobacillus farciminis*, *Staphylococcus carnosus*, *Pediococcus pentosaceus* and *Kocuria varians* were used as comparison to the *Lactobacillus sakei* and *curvatus* strains. These strains were included in this study to get a better overview about the metabolic spectrum of meat associated bacteria and to validate the analyzing

method. Arnold & Senter already (1998) established this method for the identification and differentiation of bacteria isolated from processed poultry.

Nearly 30 volatile compounds were identified in the fermentation broth. This shows that the method used here is appropriate to detect and analyse the bacterial production of these compounds. The *L. sakei* and *L. curvatus* strains produced dimethyldisulfide, 1-butanol, 2-methylbutanol, 3-methylbutanol, acetoin, acetic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid within five days. The produced amounts, the olfactory thresholds, and the flavor descriptions of these compounds are given in the table 24.

Table 24: Production of volatile compounds by *L. sakei* and *L. curvatus*, the olfactory thresholds of the compounds in liquid media and the flavor descriptions.

Volatile compound	Production by *L. sakei* and *L. curvatus*	Olfactory threshold in liquid media	References	Flavor description	References
dimethyl-disulfide	0.5 - 1.8 ng/ml	1.1 ng/ml	Giri *et al.*, 2010	cauliflower	Montel *et al.*, 1998
1-butanol	66 - 932 ng/ml	400 - 459 ng/ml	Pino & Queris, 2010; Giri *et al.*, 2010	fusel oil-like, solvent	Sabatini *et al.*, 2008; Giri *et al.*, 2010
2-methyl-1-butanol	34 - 160 ng/ml	16 - 300 ng/ml	Giri *et al.*, 2010; Pino & Queris, 2010	socks, fusel oil-like	Montel *et al.*, 1998; Giri *et al.*, 2010
3-methyl-1-butanol	291 - 534 ng/ml	4 - 280 ng/ml	Giri *et al.*, 2010; Pino & Queris, 2010	fruity, whiskey, balsamic	Montel *et al.*, 1998; Olivares *et al.*, 2009; Giri *et al.*, 2010

Volatile compound	Production by *L. sakei* and *L. curvatus*	Olfactory threshold in liquid media	References	Flavor description	References
acetoin	4.6 - 10 μg/ml	150 - 600 μg/ml	Bartowsky & Henschke, 2004	buttery	Montel *et al.*, 1998
acetic acid	33 - 118 μg/ml	2 μg/ml	Pino & Queris, 2010	vinegar, pungent, sour	Montel *et al.*, 1998; Kalua *et al.*, 2007
2-methyl-propanoic acid	1.9 - 3.4 μg/ml	6.6 μg/ml	Giri *et al.*, 2010	cheesy, fatty, savoury snacks	Montel *et al.*, 1998; Giri *et al.*, 2010; Olivares *et al.*, 2009
butanoic acid	5.7 - 8.4 μg/ml	0.11 - 0.65 μg/g oil	Marco *et al.*, 2007; Kalua *et al.*, 2007	rancid, cheesy	Montel *et al.*, 1998; Marco *et al.*, 2007; Kalua *et al.*, 2007
2-methyl-butanoic acid	0 - 0.1 μg/ml	0.2 μg/g	Söllner & Schieberle, 2009	sweaty, cheesy	Montel *et al.*, 1998; Söllner & Schieberle, 2009
3-methyl-butanoic acid	0 - 0.8 μg/ml	0.02 - 0.07 μg/g oil	Kalua *et al.*, 2007; Olivares *et al.*, 2009	sweaty, chees	Montel *et al.*, 1998; Kalua *et al.*, 2007; Olivares *et al.*, 2009

The formation of acetic acid results either from the heterofermentative metabolism or from a side reaction of the homofermentative carbohydrate metabolism, e.g. alternative degradation of pyruvate (Montel *et al.*, 1998). This is found in lactic acid bacteria as well as in staphylococci. Butanoic acid originates from the lipid metabolism (Montel *et al.*, 1998). Acetic and butanoic acid were produced in concentrations above the olfactory threshold by *L. sakei* and *L. curvatus* (table 24). This suggests that these species contribute to the flavor profile of fermented sausages by the production of acetic and butanoic acid, which belong next to lactic acid to the main aroma compounds (Montel et al., 1998; Söllner & Schieberle, 2009; cf. table 2).

2-Methyl-1-butanol, 3-methyl-1-butanol, 2-methylpropanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid can be derived from the branched chain amino acids isoleucine, leucine and valine, respectively (Montel *et al.*, 1998). All five compounds also belong to the main volatile compounds of dry fermented sausages (Montel *et al*, 1998; Meynier *et al.*, 1999; Marco *et al.*, 2004; Olivares *et al.*, 2009). The produced amounts of the alcohols are hardly above the olfactory threshold ranges (table 24). Therefore it is unlikely that there is a strong flavor impact by *L. sakei* and *L. curvatus* concerning these compounds in fermented sausages. In contrast to them the produced amounts of the carboxylic acids were above the olfactory threshold ranges and therfore an effect to the flavor of fermented sausages is expected. These compounds contribute strongly with a sweaty and cheesy flavor note to fermented sausages (Montel *et al.*, 1998; cf. table 2).

The production of dimethyldisulfide shows that the *L. sakei* and *L. curvatus* strains are able to metabolize the amino acid methionine, which sometimes leads to them being spoilage organisms in vacuum packed meats. Dimethyldisulfide imparts a flavor of cauliflower and can lead to an off-flavor in high concentrations (Montel *et al.*, 1998). It is often found in raw fermented sausages (Stahnke, 1994; Montel *et al.*, 1998; Meynier *et al.*, 1999). The produced amounts were mostly above the olfactory threshold range (table 24). Therefore it is possible that the production of dimethyldisulfide by *L. sakei* and *L. curvatus* affects the flavor of fermented sausages. 1-Butanol is a product of the alcoholic and heterolactic fermentation (Sabatini *et al.*, 2008). It is produced in olives fermented with lactobacilli (Sabatini *et al.*, 2008) and is another volatile compound found in dry sausages (Stahnke, 1994).

Acetoin is a part of the pyruvate catabolism (Montel *et al.*, 1998). It is produced in olives fermented with lactobacilli (Sabatini *et al.*, 2008) and is found in some sausages (Stahnke, 1994). But the produced amounts by *L. sakei* and *L. curvatus*

are far below the olfactory threshold range and therefore there is no impact to the flavor of fermented sausages expected by these species.

All *L. sakei* and *L. curvatus* strains showed a similar profile in the production of volatile compounds. Only the produced quantities varied. The largest differences were found in the production of 1-butanol and 2-methylbutanol. This shows that there are some differences in the conversion rates between the strains, but the whole metabolic capabilities to produces volatile compounds seems to be the same in all tested *L. sakei* and *L. curvatus* strains. This was unexpected, because genomic investigation showed that the diversity within the *L. sakei* species is quite high. Chaillou *et al.* (2009) showed that there is a variation of 25 % in the genomic size from 1515 kb to 2320 kb. Champomier *et al.* (1987) showed with DNA-DNA reassociation that the levels of relatedness are below 72 %, indicating that the species *L. sakei* exhibits important elements of genetic heterogeneity. Based on these findings it was expected that the capabilities to produce volatile compounds are probable different within this species. To date some other screening studies are done with *L. sakei* focusing e.g. at acidification rates, growth under different conditions and/or utilization of different substrates (Ammor *et al.*, 2005; McLeod *et al.*, 2008). Both studies showed among others that there are some differences between the strains in the growth and acidification rates.

The *S. carnosus* strain produced the largest quantities of 2-methylbutanoic acid and 3-methylbutanoic acid with 1.3 μg/ml and 4.4 μg/ml. Larrouture *et al.* (2000) also showed that *S. carnosus* produces preferred 3-methylbutanoic acid from leucine and no 3-methylbutanal and 3-methyl-1-butanol. The amounts of 2-methylpropanoic acid, butanoic acid and acetic acid were also the highest in the cultures with the *S. carnosus* strain. *S. carnosus* and *S. xylosus* are next to lactobacilli the main starter bacteria of fermented sausages. Therefore staphylococci strongly impact on the flavor due to their production of acids (Stahnke, 1995; Montel *et al.*, 1998). It is interesting that the *S. carnosus* strain TMW 2.801 oxidized the aldehydes present in the medium. Beck *et al.* (2002) showed for *S. xylosus* that the conversion rates of aldehydes into their corresponding acids are quite high. This also seems to be the case with the *S. carnosus* strains used. The aldehydes in the medium were partly (max. 6 %) used as precursors for the carboxylic acids.

In summary, the SPME-GC-MS method proved to be appropriate to analyze volatile compounds of bacterial origin and to visualize the differences in the metabolism of different bacterial species. The production of the compounds acetic acid,

butanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid is of special interest, because of their low olfactory threshold values and their strong impact of the aroma of raw fermented sausages (Montel *et al.*, 1998; Söllner & Schieberle, 2009; cf. table 2).

4.3 Colony forming units, acidification rate and production of lactic acid by *L. sakei* TMW 1.1383, 1.1393 and *S. carnosus* TMW 2.801

The *L. sakei* and the *S. carnosus* strain reached a density of nearly 10^9 cells/ml modified mMRS-medium within a day. This is the density which *L. sakei* also reached in the experimental sausages fermentations and in other sausages fermentations (Eerola *et al.*, 1996; Roig-Sagués & Eerola, 1997; Gardini *et al.*, 2002; Zdolec *et al.*, 2008). That shows that *L. sakei* is able to grow the same way in liquid media as well as in sausages. *S. carnosus* shows a better growth in liquid medium. This is mainly due to the fact that the growth of the *S. carnosus* is limited in dry fermented sausages through the fast acidification rates of the lactobacilli, which is shown by some authors (Sørensen & Jakobsen 1996; Papamanoli *et al.*, 2002; Søndergaard & Stahnke, 2002). After five days in culture the *S. carnosus* strain showed a better survival rate than the *L. sakei* strains (10^8 cfu/ml and 10^7 cfu/ml, respectively). Champomier-Vergès *et al.* (1999) observed that cultivated with glucose *L. sakei* reaches a density of 10^8 cfu/ml and after 20 hours of growth the cfu/ml decreases then rapidly.

The modified mMRS-medium with an initial pH of 6.5 was inoculated with 10^6 cells/ml. The *L. sakei* strains showed a fast acidification which started after 8 hours. After 14 hours a pH of 4.5 was reached. The strain *L. sakei* TMW 1.1383 was a little faster than the strain TMW 1.1393, but the differences were only marginal. The *S. carnosus* strain started after 10 hours with the acidification and reached a pH of 4.8 only after 48 hours. Ammor *et al.* (2005) showed in a screening with 36 different *L. sakei* strains that the acidification rates varied slightly. McLeod *et al.* (2008) also showed differences in the acidification rates of some *L. sakei* strains in a meat model system. In contrast to the *L. sakei* strains the *S. carnosus* showed a very slow acidification property. Coventry & Hickey (1991) showed that the growth and the acid production of *S. carnosus* is already impaired at pH 5.5 and at a pH of 4.7 the growth is inhibited, while *L. plantarum* and *P. pentosaceus* were not in-

fluenced by these conditions. They also showed that the *S. carnosus* reached a pH of 4.7 after 32 hours by growth with an initial pH of 6.0 (Coventry & Hickey, 1991). This is in accordance with the findings of this study, where the pH was in a similar range in the culture of *S. carnosus* after 32 h.

Within five days of cultivation the *L. sakei* strains and the *S. carnosus* strain produced 30 mmol/L lactic acid in the modified mMRS-medium with 0.3 % glucose. This shows that almost 100 % of the offered glucose is transformed into lactic acid. Not only lactic acid bacteria but also staphylococci produce lactic acid from glucose (Schleifer & Fischer, 1982; Montel *et al.*, 1993).

4.4 Production of volatile compounds by *L. sakei* TMW 1.1383, 1.1393, and *S. carnosus* TMW 2.801 cultivated with branched chain amino acids and branched chain α-keto acids

The addition of 0.25 mM of branched chain amino acids to the modified mMRS-medium showed in contrast to the α-keto acids no or only little effects towards the production of volatile compounds by *L. sakei*. This can be referred to limited transport of free amino acids and a low transaminase activity. Poolman (1993) summarized that branched chain amino acids are generally transported by cation-linked amino acid symporters. However, amino acid transport is a strain specific property, as shown along the example of *L. helveticus* (Nakajima *et al.*, 1998). Tavaria *et al.* (2002) screened various lactic acid bacteria strains for their uptake of amino acids, which was highest at pH 6. The uptake of amino acids correlated only poorly with the production of related volatile compounds (Tavaria *et al.*, 2002). Several genes for amino acid transporters could be found within the genome of *L. sakei* 23 K but there is no annotation of genes coding for transporters specific for branched chain amino acids whereas many other lactobacilli carry one or even more genes for these kinds of transporters. In the lack of a branched chain amino acid transporter these amino acids are probably not efficiently transported into the cell. Absence of this gene was demonstrated for most *L. sakei* strains used in a recent study including the strains TMW1.1383 and 1.1393 (Freiding *et al.*, 2011). A branched-chain amino acid transporter however is described for staphylococci (Vijaranakul *et al.*, 1998).

On the other hand, the added amino acids may not be used in a complex medium like mMRS, which alone provides enough amino acids for bacterial growth. Sinz &

Schwab (2011) showed with resting cells of the same *L. sakei* strains in a buffer system that the production of volatile compounds is enhanced by the addition of free amino acids.

A low transaminase activity of *L. sakei* is in line with the observation of Smit *et al.* (2004) who showed that only some strains of lactic acid bacteria have an effective transaminase reaction, and Freiding *et al.* (2011) who identified the transaminase as a major bottleneck in amino acid conversion by *L. sakei*. Furthermore, it has been demonstrated that different lactic acid bacteria require the addition of α-ketoglutarate and pyridoxal-5-phosphate to metabolize leucine (Larrouture *et al.*, 2000). The addition of α-ketoglutarate to cheese enhances the conversion of amino acids to aroma compounds (Yvon *et al.*, 1999). Staphylococci however have a branched-chain amino acid aminotransferase (Madsen *et al.*, 2002; Dordet-Frisoni *et al.*, 2007). In contrast to the *L. sakei* strains the *S. carnosus* showed a higher production of carboxylic acids in modified mMRS-medium. By the addition of branched-chain amino acids the production of 2- and 3-methylbutanoic acid was further enhanced by *S. carnosus*. But compared to approaches with the addition of α-keto-acids the addition of branched chain amino acids to *S. carnosus* cultures showed little effect. Limited enhanced conversation rates of added amino acids were found in another study with a *Staphylococcus* strain (Møller *et al.*, 1998). Masson *et al.* (1999) and Sinz & Schwab (2011) however showed with resting cells of *S. carnosus* that the production of 3-methylbutanal is elevated by incubation in buffer with leucine. Larrouture *et al.* (2000) showed that the production of 3-methylbutanoic acid from leucin can be increased by the addition of α-ketoglutarate and pyridoxal-5-phosphate to the *S. carnosus* culture.

Upon the addition of α-ketoisocaproic acid, α-keto-3-methyl-pentanoic acid and α-ketoisovaleric acid the *L. sakei* strains produced in the fermentations large amounts of 3-methylbutanoic acid, 2-methylbutanoic acid and 2-methylpropanoic acid. Since it is the only direct metabolite from the α-keto acids produced in large amounts by the *L. sakei* strains and no or only a little enhanced production of the corresponding aldehydes and alcohols is seen, it seems that these strains rather use the way of the ketoacid dehydrogenase via acyl-CoAs to produce the carboxylic acids than through the production of aldehydes as intermediate. An α-keto acid decarboxylase activity producing aldehydes is shown for *Lactococcus lactis* and *Lactobacillus delbrueckii* subsp. *lactis* (Helinck *et al.*, 2004; de la Plaza *et al.*, 2004; Smit *et al.*, 2005a; de Palencia *et al.*, 2006). But this reaction could neither be shown for other lactobacilli

(Helinck *et al.*, 2004; Smit *et al.*, 2004; de Palencia *et al.*, 2006) nor could the corresponding gene be described (Liu *et al.*, 2008).

In contrast the *S. carnosus* strain produced with addition of α-ketoisocaproic acid, α-keto-3-methyl-pentanoic acid and α-ketoisovaleric acid like *L. sakei* the corresponding carboxylic acids, but also high amounts of 3-methylbutanol, 2-methylbutanol, 2-methylpropanol, 2-methylbutanal and 2-methylpropanal. This suggests that the *S. carnosus* strain metabolizes the α-keto acids using the α-keto acid decarboxylase and the aldehyde dehydrogenase leading to the production of the corresponding aldehyde, alcohol and carboxylic acid. It was unexpected that the *S. carnosus* strain did not produce 3-methylbutanal from the α-ketoisocaproic acid. Beck *et al.* (2002) showed that the conversion rates of branched-chain aldehydes into the corresponding acids are very high in a strain of *S. xylosus*. So it is possible that 3-methylbutanal is not accumulated due to the fast conversion.

In the fermentation broth and in natural environments as meat α-ketoglutarate and other α-keto-acids are barely available and transporters for these compounds have consequently not been described. Still, in an acidic environment these α-keto-acids are (partly) protonated and should easily enter the cell serving as precursors upon their addition to the medium. The pK_a-values of the used α-keto acids are between 4.2 and 5.1, which is in the range of the mMRS-medium and meat upon bacterial growth. Therefore it is assumed that the α-keto acids passes the cell wall passively.

The possible pathways of the α-keto-acids conversions for *L. sakei* and *S. carnosus* are shown in figure 40.

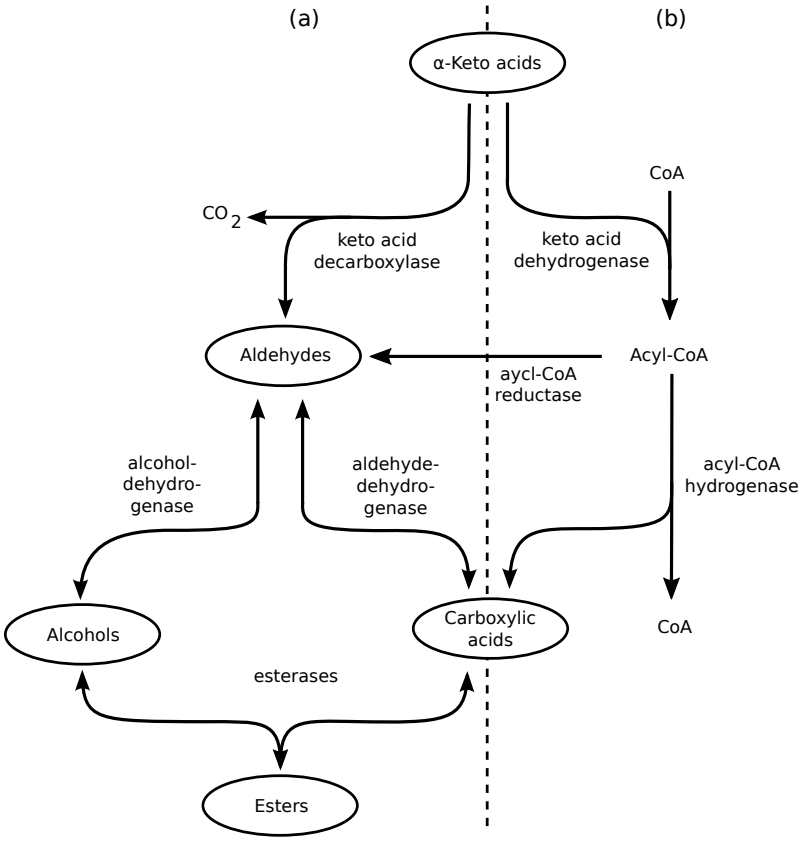


Figure 40: Probable pathway of the production of volatile compounds by *S. carnosus* (a) and *L. sakei* (b). (Adapted from Helinck *et al.* (2004)).

The production of esters by *S. carnosus* is not unexpected, but it is the first time shown that the production is induced by α-keto acids. Søndergaard & Stahnke (2002) reported that *Staphylococcus xylosus*, *carnosus* and *equorum* produced the esters 3-methyl-1-butylacetate, 2-methyl-1-butylacetate and 3-methylbutyl-3-methylbutanoate in sausages minces. Lipases from staphylococci were even used for the industrial production of flavor esters (Talon *et al.*, 1996; Ghamgui *et al.*, 2006; Karra-Châabouni *et al.*, 2006).

The ketones diacetyl and acetoin are produced via pyruvate metabolism, which is connected to the metabolism of sugars and citric acid (Bartowsky & Henschke, 2004) or they are derived from the amino acid asparagine via oxalacetate (Ardö, 2006). Upon increased formation of pyruvate (e.g. in the presence of citrate) α-acetolactate is formed, which is a precursor for diacetyl and acetoine and also for leucine. This pathway is regulated by high concentrations of leucine, which stimulates α-acetolactate decarboxylase producing acetoine (Goupil *et al.*,1996) and thus reducing leucin biosynthesis. Whenever in silico data show that leucin biosynthesis is not functional in *L. sakei* the regulatory mechanism of the α-acetolactate pool appears to be preserved. Still, the production of ketones by the *L. sakei* strains cul-

tivated with branched chain α-keto acids is not clearly understood and no literature is found describing the production of ketones by lactic acid bacteria under these conditions. Kieronczyk *et al.* (2001) showed that *L. paracasei* strains produced higher amounts of acteoine by cultivation with amino acids and α-ketoglutarate. In cheese fermented with lactic acid bacteria also ketones were found, e.g. diacetyl, acetoin and 2,3-hexanedione (Ayad *et al.*, 2004; Di Cagno *et al.*, 2007). Diacetyl also can be detected in fermented sausages (Olivares *et al.*, 2009). The production of 2,3-heptanedione can partly be chemical, because this compound was build to a certain amount even in the non-inoculated medium within 5 days. Figure 41 shows the general pyruvate metabolism and the formation of diacetyl and acetoin in lactobacilli.

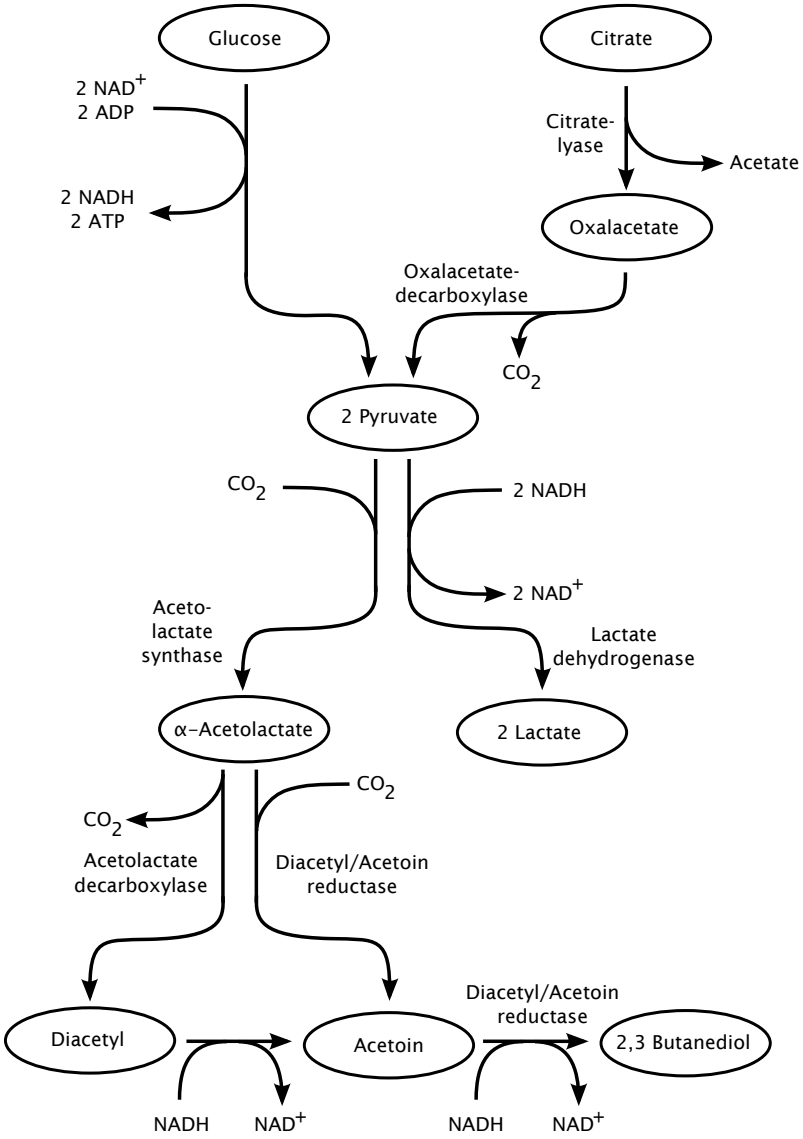


Figure 41: Schematic pathway showing the pyruvate metabolism. (Adapted from the website: www.nmpdr.org).

4.5 Production of raw sausages with *L. sakei* TMW 1.1383, 1.1393, and *S. carnosus* TMW 2.801

4.5.1 Reduction of weight during ripening

The reduction of weight was similar in all experiments and there were nearly no differences between the different batches. The set points were 25 % weight loss after 14 days and 40 % at the end of the fermentation. These set points were reached in each experiment. This showed that the conditions of the ripening process were identical and the sausages from different experiments are comparable to each other. The chosen set points were taken from industrial sausage fermentations and were in the range of other experimental sausage fermentations (Næs *et al.*, 1995; Blom *et al.*, 1996; Muguerza *et al.*, 2002).

The sausages which were inoculated with *S. carnosus* TMW 2.801 showed earlier the desired red color. Also at the end of the fermentation these sausages had a pleasant red color, while the sausages only inoculated with *L. sakei* had a more brownish color. This s in accordance with the observations of Stahnke *et al.* (2002), who showed that sausages inoculated with *L. curvatus* and *S. carnosus* show a more intensive red color than sausages inoculated with *L. curvatus* and *K. varians*.

4.5.2 Developing of the pH-values during the ripening

The fermentations started with a pH between 5.6 and 5.8. The lowest pH values were reached at day 5 with pH 4.8 to 5.0. At the end of the fermentation the pH values were between 5.1 and 5.2. The fastest acidification occurred in batches inoculated with *L. sakei* TMW 1.1383 and *S. carnosus* TMW 2.801. The slowest acidification was monitored in batches only inoculated with *L. sakei* TMW 1.1393. The sterile control showed already at the beginning of the fermentation a pH value below 5 and finished with a pH of 5.2 at day 35.

In the model system it already could be seen that the strain *L. sakei* TMW 1.1383 has a little more rapid acidification rate than the strain *L. sakei* TMW 1.1393. In this experiment these differences were even more distinct. Again this observation fits the data of Ammor *et al.* (2005) and McLeod *et al.* (2008) who showed also differences in the acidification rates of different *L. sakei* strains. The development of the pH during the ripening of the sausages is typical and described for many other sausage fermentation processes (Holley *et al.*, 1988; Johansson *et al.*, 1994; Pérez-Alvarez *et al.*, 1999; Bozkurt & Erkmen, 2002; Gardini *et al.*, 2002; Työppönen *et al.*,

2003). The decrease of the pH in sausages is mainly performed by the production of lactic acid (Demeyer *et al.*, 1979).

4.5.3 Development of the colony forming units during the ripening

independently from inoculation, in all batches and experiments with *L. sakei* the colony forming units of lactobacilli reached 10^8 to 10^9 cells/g sausage. This is also observed and described for other sausage fermentation processes (Eerola *et al.*, 1996; Roig-Sagués & Eerola, 1997; Gardini *et al.*, 2002; Zdolec *et al.*, 2008).

At the end of the ripening period the dominance of the starter culture was verified. In sausages inoculated with *L. sakei* TMW 1.1383 only this *Lactobacillus*-strain could be identified at the end of the fermentation. In the sausages which were inoculated with the *L. sakei* strain TMW 1.1393 a second strain was isolated in three of four experiments. The strain *L. sakei* TMW 1.1383 seems to be more assertive against other lactobacilli than the strain *L. sakei* TMW 1.1393. The production of bacteriocins does not seem to be the inhibitory substance of *L. sakei* TMW 1.1383, because both strains showed no inhibitory activity towards the contaminant strain isolated from the sausage and an indicator strain. The strain *L. sakei* TMW 1.1383 showed a slightly faster and better growth rate and a faster acidification rate than the strain *L. sakei* TMW 1.1939. It is unclear if this is sufficient to suppress the growth of other lactobacilli and needs further investigation. Garriga *et al.* (1996) already showed that there are differences in the competiveness of various *Lactobacillus* strains in fermented sausages. In general it seems that the competiveness of *L. sakei* strain is the main factor if a strain is appropriate as starter culture for the sausage fermentation or not.

The colony forming units in the sterile control were between 10^4 and 10^5/g sausage with a relatively heterogenic bacterial flora. But the growth of 10^4 to 10^5 cells/g sausage is metabolically insignificant and therefore these batches can be used as control without bacterial influence on the formation of volatile compounds. That through the use of antibiotics in sausages the bacterial growth can not completely be suppressed was also observed by Stahnke (1994).

The colony forming units of staphylococci showed differences between the batches. If *S. carnosus* was inoculated as starter culture the colony forming units were 10^6 to 10^7 cells/g sausage. In sausages inoculated only with *L. sakei* the colony forming units of staphylococci were one to two orders of magnitude lower. Similar results were shown by Montel *et al.* (1993) and Stahnke (1994) that the cfu of 10^7 staphy-

lococci/g sausage can only be reached if these bacteria were inoculated. Montel *et al.* (1993) and Bover-Cid *et al.* (1999) reported that sausages made with different staphylococci as starter strains also contained 10^6 to 10^7 staphylococci/g. In contrast to lactobacilli staphylococci are only limitedly able to grow in sausages due to the low pH (Sørensen & Jakobsen 1996; Papamanoli *et al.*, 2002; Søndergaard & Stahnke, 2002; Ravyts *et al.*, 2010).

4.5.4 Volatile compounds

In the produced sausages more than 100 volatile compounds were detected. Among them 2 hydrocarbons, 11 aldehydes, 10 ketones, 22 alcohols, 11 carboxylic acids, 11 ester compounds, 8 sulfur containg compounds, 14 aromatic compounds, 2 oxygen containing heterocyclic compounds, 13 terpenes and 7 nitrogen containing compounds were detected. These compounds were also found by other researches in dry fermented sausages (Schmidt & Berger, 1998; Edwards *et al.*, 1999; Meynier *et al*, 1999; Ansorena *et al.*, 2001; Marco *et al.*, 2004).

The formation of the volatile compounds generated by bacterial activities started mainly at day 3 of ripening which correlates with the end of the growth of the inoculated bacteria. This was also observed in a study of Johansson *et al.* (1994). This indicates that the metabolism of amino acids starts in sausages after growth and depletion of sugar.

Aldehydes

The aldehydes 2-methylpropanal, 2-methylbutanal and 3-methylbutanal are derived from the amino acids valine, isoleucine and leucine, respectively. Their formation can be of microbial origin and/or by the chemical Strecker reaction as suggested and discussed by several authors (Garcia *et al.*, 1991; Stahnke, 1995; Edwards *et al.*, 1999; Bruna *et al.*, 2001). Stahnke (1994) observed that these compounds were found in sausages inoculated with starter cultures as well as in noninoculated sausages treated with antibiotics. This fits the observation of this work where these compounds were also found in the sterile controls. The aldehydes pentanal, hexanal and heptanal are derived from the lipid autoxidation (Drumm & Spanier, 1991; Shahidi & Pegg, 1994). This process is enhanced by higher temperatures, the presence of nitrite and in an acidic environment (Stahnke, 1995).

Ketones

Many ketones are products of the lipid oxidation (Berdagué *et al.*, 1993). Methyl ketones can also be produced through microbial β-oxidation of saturated fatty acids with a following β-keto acid decarboxylation which is mainly associated with moulds (Okumura & Kinsella, 1985; Sunesen & Stahnke, 2003). Fadda *et al.* (2002a) showed that this pathway can also be found in staphylococci.

Alcohols

Alcohols are the largest chemical group found in the sausages. The alcohols 2-methyl-1-butanol, 3-methyl-1-butanol and 2-methyl-1-propanol are derived from the amino acids isoleucine, leucine and valine, respectively. 2-Methyl-1-propanol was found only in limited amounts while the production of 2-methyl-1-butanol and 3-methyl-1-butanol was strongly affected by the inoculated starter cultures. The highest amounts were build in the batches inoculated only with *S. carnosus* and no production was found in the sterile control. In the model fermentations the production of 2-methyl-1-butanol and 3-methyl-1-butanol were higher in the cultures with *L. sakei* than in the culture with *S. carnosus*. In this case the model system was less appropriate to predict the behavior of the bacterial strains in the sausages. Interestingly the *S. carnosus* produced high amounts of 2-methyl-1-butanol and 3-methyl-1-butanol if the media were supplemented with the corresponding α-keto acids of isoleucine and leucine. It is possible that there is a certain source of α-keto acids in the sausages or the metabolism of *S. carnosus* differs in model and in sausage fermentations. However Stahnke (1994) also reported, that sausages made with *S. xylosus* have shown a production of 3-methyl-1-butanol. In contrast in sausages without *S. xylosus* this compound was not detected. Montel *et al.* (1996) reported that *S. carnosus* showed the highest production of 3-methyl-1-butanol in a screening with different *Staphylococcus* species.

Carboxylic acids

During the ripening the branched chain carboxylic acids 2-methylpropanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid were produced. In the sterile controls these compounds were not produced. This shows that these compounds were of metabolic origin from the inoculated organisms, and derived from the amino acids valine, isoleucine and leucine. In the batches inoculated with *S. carnosus* the produced amounts were the highest. This correlates with the observations of the screening where the *S. carnosus* also produced higher amounts of these acids. Concerning the

production of carboxylic acids the screening in model system in modified mMRS is appropriate to get hints for the behavior of bacteria in sausages. The production of 2-methylpropanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid is of great importance for the aroma of dry fermented sausages due to the strong cheesy and sweaty flavor impression and their low olfactory threshold values (Montel *et al.*, 1998; Söllner & Schieberle, 2009).

The development of the acetic acid was similar in all batches except for the sterile control in which only little amounts were produced. This shows that the acetic acid is also of bacterial origin as already shown in the screening. But the produced amounts are rather less correlated with the inoculated cultures. The formation of acetic acid results either from the heterofermentative metabolism or by a side reaction of the homofermentative carbohydrate metabolism which is found in lactic acid bacteria as well as in staphylococci (Montel *et al.*, 1998). The production only takes place at the beginning of the fermentation together with the bacterial growth. This was also reported by Johansson *et al.* (1994). The amount of butanoic acid was also less strongly associated with the batch; even in the sterile control a certain amount of production was seen. It seems that butanoic acid can partly be built by chemical reactions next to bacterial activity which could be observed in the screening in model system. Mostly it originates from the lipid metabolism (Montel *et al.*, 1998). Nevertheless both compounds have a strong aroma and affect therefore strongly the flavor of dry fermented sausages (Söllner & Schieberle, 2009; cf. table 2).

Ester Compounds

The production of ethyl acetate, ethyl-2-and ethyl-3-methylbutanoate in the sausages is strongly affected by the inoculated starter cultures. The highest amounts were formed in the batches inoculated only with *S. carnosus* and no production could be found in the sterile control. This fits the findings of Stahnke (1994) who found these and other ester compounds in sausages fermented with *S. xylosus*, but not in a sterile control treated with antibiotics. The compounds ethyl-2-methylbutanoate and ethyl-3-methylbutanoate are derived from the amino acids isoleucine and leucine. Interestingly is that the *S. carnosus* produced high amounts of ester compounds if the media was supplemented with α-keto acids. It could be that there is a certain source of α-keto acids in the sausages or the metabolism of *S. carnosus* differs in model and in sausage fermentations. Montel *et al.* (1996) reported that *S. carnosus* showed the highest production of ethyl-2-methylbutanoate and ethyl-3-

methylbutanoate in a screening with different Staphylococcus species. Due to their fruity flavor and the low olfactory threshold values these compounds have an effect towards the aroma of dry fermented sausages (Montel *et al.*, 1998; Marco *et al.*, 2007; Söllner & Schieberle, 2009; cf. table 2).

Sulfur Containing Compounds

One of the most important sulfur containing compound in raw sausages is dimethyldisulfide (Montel *et al.*, 1998; Meynier *et al.*, 1999). It originates from the amino acid methionine and has a flavor of cauliflower with a low olfactory threshold (Montel *et al.*, 1998). But the production of dimethyldisulfide and carbondisulfide has to be avoided due to their negative aroma contribution (Montel *et al.*, 1998).

Aromatic compounds

Berdagué *et al.* (1993) suggested that most aromatic compounds are derived from the feedstuff, because their occurrence is not correlated to certain starter cultures and they are found in plant material used as animal food. Phenylacetaldehyde can be derived from the amino acid phenylalanine and has a floral flavor (Montel *et al.*, 1998; Marco *et al.*, 2007). It is mostly found in higher quantities in raw fermented sausages manufactured with staphylococci or micrococci (Ansorena *et al.*, 2000; Sunesen *et al.*, 2001; Hierro et al., 2005).

Nitrogen containing compounds

The nitrogen containing compounds seem not to be of interest or aroma active, because they are found in nearly all published sausages fermentations, but were not further mentioned in the discussions of the publications.

Terpenes

The terpenes originate from pepper (Ekundayo *et al.*, 1988; Luning *et al.*, 1994; Stahnke, 1994; Jiang & Kubota, 2004). They are not of microbiological or fermentative origin and are therefore not further discussed in this work.

4.6 Production of volatile compounds in a sausage model system

In the sausage model system the volatile compounds ethylacetate, ethyl-2-methylbutanoate, ethyl-3-methylbutanoate, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-bu-

tanol, acetic acid, 2-methylpropanoci acid, butanoic acid, phenylacetaldehyde, 2-methylbutanoic acid and 3-methylbutanoic acid were produced within five days. The production of ethylacetate, ethyl-2-methylbutanoate, ethyl-3-methylbutanoate, 2-methylpropanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid was highest in the samples inoculated with *S. carnosus*. This fits the data from the sausage fermentations, where these compounds were also mostly produced in the sausages inoculated with *S. carnosus*. The compound phenylacetaldehyde was produced only in very low quantities in the sausage model system. This seems to be due to the short fermentation time. The model fermentations were stopped after 5 days, while the production of phenylacetaldehyde started only after 8 - 14 days in the sausage fermentation. But longer fermentations are not practicable due to the inhibition of the drying in the GC-vial. Nevertheless the sausage model system is appropriate to study the production of ester compounds by staphylococci. This was not feasible in the model fermentation in modified mMRS-medium. To observe the production of acetic acid, 2-methylpropanoic acid, buanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid both model systems are equally well suited.

A similar sausage model system was developed and used by Fadda *et al.* (2002b) for the study of the protein degradation of lactobacilli.

4.7 Growth behavior and production of volatile metabolites by *L. sakei* TMW 1.1383 and 1.1393 under stress conditions

Modified mMRS-medium containing 0.3 % glucose was inoculated with 10^6 cells/ml of the *L. sakei* strains. As stress factors different concentrations of salt, curing salt, nitrate, nitrite and different pH were used. The respective growth rates were visualized along an increase of OD at 590 nm. The results show that both strains were impaired by 2.5 % salt and curing salt, 2.5 % nitrate, 0.125 % nitrite and pH 5.5 leading to 10 % lower OD, which were the chosen stress factors for the analysis of volatile compounds. Generally, the *L. sakei* strain TMW 1.1383 showed a better growth than the strain TMW 1.1393. The strain *L. sakei* TMW 1.1393 was also more sensitive to acidic stress. It did not grow at pH 4.5, while the strain *L. sakei* TMW 1.1383 still grew at pH 4.25. Only to nitrite stress the strain *L. sakei* TMW 1.1393 showed a better resistance than the strain TMW 1.1383. The observation of different growth rates fits the data of Ammor et al. (2005) and McLeod at al.

(2008) who showed differences in the growth rates of diverse *L. sakei* strains.

The production of volatile compounds was monitored under the following conditions: 2.5 % salt, 0.125 % nitrite and pH 5.5 after five days and calculated to the cell counts. The productions of the volatile compounds dimethyldisulfide, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, acetoin, acetic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid, and 3-methylbutanoic acid are affected differently by the stress factors. The production of these compounds was decreased with salt stress and increased with nitrite stress. The pH stress led also to higher amounts of the acids acetic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid, and 3-methylbutanoic acid.

4.7.1 Salt stress

L. sakei in general shows a high salt tolerance and most strains are able to grow up to 8 % NaCl (Hugas *et al.*, 1993; Coppola *et al.*, 1998; Samelis *et al.*, 1998; Papamanoli *et al.*, 2003; Ammor *et al.*, 2005). But this study and Korkeala *et al.* (1992) and Marsilio & Lanza (1998) show that their growth is already reduced at 2 - 4 % NaCl, what is in the range of the initial concentration in sausages. This shows that there is a stress potential by salt from the beginning of the manufacturing process. By growth with 2.5 % NaCl the OD of 590 nm is reduced although the colony forming units are nearly doubled. While the increase in cell numbers upon salt stress remains unexplained, the reduced cell size can be referred to osmotic stress and subsequent desiccation of the cells. This effect is already shown by Poirier *et al.* (1998) and Lemay *et al.* (2000). Korkeala *et al.* (1992) showed that the growth of *L. sakei* and *L. curvatus* is enhanced by 1 - 2 % NaCl and reduced from 3 % NaCl.

When growth with 2.5 % NaCl the production of all volatile compounds is decreased. It seems that the cells use their energy rather for the division than for the production of volatile compounds. Tjener *et al.* (2004) already showed that salt has a negative effect towards lactic acid bacteria leading to less acidification and less volatile compounds in a meat model system. This effect was also observed by Rozés & Peres (1996) in a study with *Lactobacillus plantarum* where the production of acetic acid was inhibited by salt concentrations from 4 %. Other studies showed that the bacteriocin production of lactobacilli is reduced through higher salt concentrations (De Vuyst, 1996; Leroy & de Vyst *et al.*, 1999; Delgado *et al.*, 2007). Marceau *et al.* (2004) showed that the protein expression is changed significantly in *L. sakei* by growth in mMRS-medium with 4 % NaCl.

4.7.2 Nitrate and nitrite stress

Nitrate and nitrite are usually used in concentrations of 0.014 % in the form of curing salt in fermented sausages and were degraded during the fermentation by the metabolic activity of staphylococci. The *L. sakei* are very durable towards nitrate. An amount of 2.5 %, which is nearly the 200 fold of the maximal concentration occurring in sausages, is necessary to influence the growth. Nitrite impairs the growth of *L. sakei* at 0.125 % nitrite, which is the 10 fold of concentrations occurring in sausages. Although the OD at 590 nm shows a moderate reduction of the cell density, the colony forming unit is massively decreased to 10^7 cells/ml suggesting that a large cell fraction of about $1.4 * 10^8$ is lethally damaged. By growth with 0.125 % nitrite the production of all produced volatile compounds is multiplied. This indicates that the production of volatile compounds could already be increased at nitrite values, which show no reduction of growth or the cell density. An increase of 2-methylbutanoic acid and 3-methylbutanoic acid by higher nitrite values in a meat model system inoculated with *S. xylosus* was also observed by Stahnke (1996). A difference between both *L. sakei* strains can be seen in the production of acetoin which is more increased with nitrite stress by the strain *L. sakei* TMW 1.1383. Remarkable is that with 0.125 % nitrite the compounds 1,3-dithiolane, nitro-methane, isopropylisothiocyanate, isothiocyanato-methane, 1-isothiocyanato-butane, 1,3-dithiane and isobutylisothiocyanate were formed. The formation of these compounds may result from chemical cross-reactions with the nitrite and the bacterial metabolites in the medium, because these compounds could hardly be detected in sausages and in fermentation cultures of lactic acid bacteria. Only Nitro-methane was produced in a sausage minces inoculated with *S. xylosus* or *S. carnosus* and suplementd with nitrate (Stahnke 1995; Stahnke, 1999). Like higher salt concentrations nitrate and nitrite can also show an inhibitory effect towards the production of bacteriocins in *L. sakei* (Leroy & de Vyst, 1999; Hugas *et al.*, 2002).

4.7.3 pH stress

The pH of fermented sausages usually starts at 5.7 and decreases below 5.0. The strain *L. sakei* TMW 1.1383 still grew at a pH of 4.25 and the strain *L. sakei* TMW 1.1393 at a pH of 4.75. This fits the findings of Papamanoli *et al.* (2003) and Ammor *et al.* (2005) who showed growth of *L. sakei* at pH 5 and below. The growth of the strains *L. sakei* TMW 1.1383 and TMW 1.1393 is already reduced at pH 5.5. At this pH the amounts of acetic acid, 2-methylropanoic acid, butanoic acid, 2-

methylbutanoic acid and 3-methylbutanoic acid are increased in the cultures. These acids belong to the main flavor compounds of dry fermented sausages (cf. table 2) and a pH of at least 5.5 is reached in all sausage fermentations. It seems that the release of these acids is enhanced by the acidic environment therefore contributes strongly to the aroma impression. Serazanetti *et al.* (2011) showed for a strain of *Lactobacillus sanfransiscensis* that the leucine catabolism and the production of the corresponding carboxylic acid are increased under acidic stress. Like high salt and nitrite concentrations a low pH also leads to a inhibation of the production of bacteriocins by *L. sakei* (Mørtvedt-Abildgaard *et al.*, 1995; Aasen *et al.*, 2000; Leroy & de Vyust, 2005).

The growth of the lactobacilli and the production of volatile compounds under influence of all these stress factors needs to be further investigated.

4.8 Conclusions

L. sakei and *L. curvatus* show similar patterns on the production of volatile compounds in liquid media and in raw fermented sausages. This is in contrast to their genetic heterogeneity reported by Berthier & Ehrlich (1999) and Chaillou *et al.* (2009) and was therefore not expected. Their ability to produce volatile compounds from added branched-chain amino acids is rather low in contrast to the addition of the corresponding α-keto acids in the acidified medium. In comparison to *S. carnosus* the *L. sakei* produce less flavor compounds in fermented sausages. In this environment the *L. sakei* are different assertive against other *Lactobacillus* strains. The *L. sakei* strains are also different resistant to pH- and nitrite stress. The production of volatile compounds is reduced by salt stress and massively increased by cultivation with nitrite stress. The cultivation with pH-stress also led to a higher amount of acids.

Despite the similarities of the *L. sakei* strains it is still possible through high cell numbers and long fermentation durations that little differences between strains can have an effect towards the quality of raw fermented sausages.

5 Summary / Zusammenfassung

In this study 51 *L. sakei* strains and 30 *L. curvatus* strains were screened for their ability to produce volatile compounds in model fermentations. All strains produced the compounds dimethyldisulfide, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, acetoin, acetic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acids. The quantities varied but the general metabolism concerning the production of these compounds seems to be the same in all strains. The chromatographic profiles of the *L. sakei* and *L. curvatus* strains clearly differed from those of other species like *S. carnosus*. The *L. sakei* strains TMW 1.1383 and 1.1393 were selected for further physiological analytics and compared with the behavior of the *S. carnosus* strain TMW 2.801. All three strains grew up to a density of 10^9 cells/ml in modified mMRS-medium with maltose and glucose and produced 30 mmol/L lactic acid from 0.3 % glucose. The *L. sakei* strains showed a faster acidification rate of the medium than the *S. carnosus* strain.

The cultivation with branched chain amino acids showed that the *L. sakei* strains did use them only scarcely to produce volatile compounds in a complex medium. With the supplementation of the α-keto acids α-ketoisocaproic acid, α-keto-3-methylpentanoic acid and α-ketoisovaleric the *L. sakei* strains however produced the corresponding carboxylic acids 3-methylbutanoic acid, 2-methylbutanoic acid and 2-methylpropanoic acid in large amounts. The *S. carnosus* strain produced these carboxylic acids and also the corresponding alcohols, esters and aldehydes except 3-methylbutanal. These findings led to the suggestion that the *S. carnosus* strain decarboxylates the α-keto acids to the corresponding aldehydes which were then further converted to alcohols, carboxylic acids and ester compounds. The *L. sakei* strains seem to produce the carboxylic acids by the keto acid dehydrogenase via acyl-CoAs. Additionally the *L. sakei* strains produced larger amounts of ketones like diacetyl and acetoin under these conditions.

In raw fermented sausages the *L. sakei* strain TMW 1.1383 showed a little better growth and a faster acidification than the strain TMW 1.1393. The *L. sakei* strain TMW 1.1393 was not assertive enough to suppress the growth of another *Lactobacillus* strain in the sausages. This difference between the two *L. sakei* strains concerning their assertiveness seemed not to be caused by the production of bacteriocins. The analysis of the volatile compounds of the sausages showed that the *S. carnosus* strain produced higher quantities of the aroma relevant compounds than

the *L. sakei* strains.

The cell yield as determined by final optical OD of the *L. sakei* strains was decreased by 10 % upon addition of 2.5 % salt, 0.125 % nitrite, or at a pH of 5.5. Except of nitrite stress the strain *L. sakei* TMW 1.1383 showed a better growth and a higher pH tolerance than the strain TMW 1.1393. By growth with salt stress the cell size was reduced, so that the colony forming unit was higher although the optical density was reduced. The production of volatile compounds was reduced by salt stress and massively increased by cultivation with nitrite stress. The cultivation with pH-stress also led to a higher amount of the acids acetic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid.

In dieser Arbeit wurden 51 *L. sakei* Stämme and 30 *L. curvatus* Stämme in Modellfermentationen untersucht hinsichtlich ihrer Fähigkeit, flüchtige Substanzen zu bilden. Alle getesteten Stämme produzierten die flüchtigen Substanzen Dimethyldisulfid, 1-Butanol, 2-Methyl-1-Butanol, 3-Methyl-1-Butanol, Acetoin, Essigsäure, 2-Methylpropansäure, Buttersäure, 2-Methylbuttersäure und 3-Methylbuttersäure. Die Mengen der gebildeten Substanzen variierten, aber der generelle Metabolismus hinsichtlich der Bildung dieser Substanzen scheint bei allen Stämmen gleich zu sein. Die chromatographischen Diagramme der *L. sakei* und *L. curvatus* Stämme zeigten klare Unterschiede zu denen von anderen Spezies wie z.B. *Staphylococcus carnosus*. Die *L. sakei* Stämme TMW 1.1383 und 1.1393 wurden daher ausgewählt für weitere physiologischen Untersuchungen und verglichen mit dem Verhalten des *S. carnosus* Stammes TMW 2.801. Alle drei Stämme wuchsen bis zu einer Zelldichte von 10^9 Zellen/ml in modifizierten mMRS-Medium mit Maltose und Glucose auf und bildeten 30 mmol/L Milchsäure bei Wachstum mit 0.3 % Glucose. Die *L. sakei* Stämme zeigte eine schnellere Ansäuerung des Mediums als der *S. carnosus* Stamm.

Die Kultivierung mit verzweigtkettigen Aminosäuren zeigte, dass die *L. sakei* Stämme in Vollmedium diese kaum nutzen, um flüchtige Substanzen zu produzieren. Der Zusatz der α-Ketosäuren α-Ketoisocapronsäure, α-Keto-3-Methylpentansäure and α-Ketoisovaleriansäure bewirkte, das die *L. sakei* Stämme die entsprechenden Carboxylsäuren 3-Methylbuttersäure, 2-Methylbuttersäure und 2-Methylpropansäure in hohen Mengen bildeten. Der *S. carnosus* Stamm hingegen bildete neben den Carboxylsäuren auch die entsprechenden Alkohole, Ester und Aldehyde außer 3-Methylbutanal. Diese Beobachtungen lassen darauf schließen, dass der *S. carnosus*

Stamm die α-Ketosäuren zu den entsprechenden Aldehyden decarboxyliert, die dann in die entsprechenden Alkoholen, Carboxylsäuren und Ester weiter verstoffwechselt wurden. Die *L. sakei* Stämme scheinen die Carboxylsäuren mittels der Ketosäuredehydrogenase über Acyl-CoAs zu bilden. Zusätzlich bildeten die *L. sakei* Stämme unter diesen Bedingungen größere Mengen an Ketonen wie Diacetyl und Acetoin.

Während der Rohwurstreifung zeigte der *L. sakei* Stammm TMW 1.1383 ein etwas besseres Wachstum und Säuerung als der Stamm TMW 1.1393. Der *L. sakei* Stamm TMW 1.1393 zeigte sich nicht durchsetzungsfähig genug, um das Wachstum eines anderen *Lactobacillus* Stammes in der Rohwurst zu unterdrücken. Diese Unterschiede in der Durchsetzungsfähigkeit der *L. sakei* Stämme schien nicht auf die Bildung von Bakteriozinen zurückzuführen zu sein. Die Analysen der flüchtigen Substanzen der Rohwürste ergab, dass der *S. carnosus* Stamm höhere Mengen der aromarelevanten Substanzen bildete als die *L. sakei* Stämme.

Die Stressbedingungen, welche die Endzelldichte der *L. sakei* um 10 % der OD reduzierten, waren 2.5 % Salz, 0.125 % Nitrit bzw. ein pH-Wert von 5.5. Der Stamm *L. sakei* TMW 1.1383 zeigte insgesamt ein leicht besseres Wachstumsverhalten und eine höhere Säuretoleranz als der Stamm TMW 1.1393 mit der Ausnahme von Nitritstress. Bei Wachstum unter Salzstress waren die Zellgrößen reduziert, so das die koloniebildenden Einheiten erhöht waren, obwohl die optische Dichte reduziert war. Die Bildung von flüchtigen Substanzen war reduziert mit Salzstress und stark gesteigert bei Kultivierung unter Nitritstress. Die Kultivierung unter pH-Stress führte ebenso zu gesteigerten Mengen von Essigsäure, 2-Methylpropansäure, Buttersäure, 2-Methylbuttersäure und 3-Methylbuttersäure.

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