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Effect of fat contact and changes in the aqueous phase of emulsions on high pressure inactivation of *Lactobacillus plantarum*

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Abbreviations and Symbols

°C	Degree Celsius
ATCC	American Type Culture Collection, Manassas, Virginia, USA
AU	Arbitrary units
a_w	Water activity
B.	*Bacillus*
CFA	Cyclopropane fatty acid
cfu	Colony forming units
CPB	Citrate-phosphate buffer
CSH	Cell surface hydrophobicity
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany
E.	*Escherichia*
em	Emission wavelength
ex	Excitation wavelength
FA	Fatty acid
FI	Fluorescence intensity
FTC	Fluorescein thiocarbamoyl
g	Standard acceleration of gravity (9.81 m/s^2)
GR	Guaranteed reagent
HCCA	α-cyano-4-hydroxycinnamic acid
HHP	High hydrostatic pressure
HPT	High pressure-temperature
IPB	Imidazole-phosphate buffer
L.	*Lactobacillus*
LAB	Lactic acid bacteria
LB	Luria-Bertani
M	Molar (mol L^{-1})

MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MATH	Microbial adhesion to hydrocarbons
min	Minute(s)
MRS	de Man, Rogosa, Sharpe
MTP	Microtiter plate
N	Viable cell count after treatment
N_0	Initial viable cell count
NaCl	Sodium chloride
n_D^{25}	Refractive index matched to the sodium D-line, i.e. measured at a wavelength of 589 nm, at 25 °C for a certain material
o/n	Overnight
O/W	Oil-in-water
OD_x	Optical density at wavelength x
Ph Eur	Pharmacopoea Europaea (European Pharmacopoea)
PI	Propidium iodide
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SFA	Saturated fatty acid
TMW	Technische Mikrobiologie Weihenstephan
TPCK	Tosyl phenylalanyl chloromethyl ketone
TUM	Technische Universität München
UFA	Unsaturated fatty acid
v/v	Volume/volume
WPI	Whey protein isolate
w/v	Weight/volume

1 Introduction

This study was performed with the aim of systematically investigating the influence of fat in combination with other food components on the high-hydrostatic pressure (HHP) inactivation of lactobacilli. Moreover, the effect of previous contact of *Lactobacillus* cells to fat and fat-associated compounds on their HHP sensitivity was examined. This chapter introduces HHP technology and gives an overview of the genus *Lactobacillus* and the model system, emulsions, used in this study.

1.1 High Hydrostatic Pressure (HHP) technology

HHP technology, also referred to as High Pressure Processing (HPP), describes the application of high pressure to a product or item via a pressure-transmitting fluid. There are a variety of applications for HHP technology, and due to its capability of inactivating a wide range of microorganisms it has great potential for the food industry. This section deals with the role of HHP in food preservation, the basic physico-chemical principles underlying HHP and its effect on the pressurized material with special focus on microbiological target structures, and gives a short overview of its practical implementation.

1.1.1 The role of HHP technology in food preservation

Today's consumers, especially in industrialized countries, have an increasing demand for healthy, fresh-tasting, minimally-processed food without additives (Torres Bello *et al.*, 2014). At the same time, convenient ready-to-eat meals, requiring little time and effort for preparation, are becoming more and more popular. To meet these demands and produce microbiologically safe and shelf-stable food at the same time, novel food processing technologies are needed in addition to conventional heat pasteurization and sterilization, traditional preservation methods such as fermentation, smoking and acidification, or the use of artificial or natural preservatives like salt or sugar. As a result, various innovative, non-thermal food preservation technologies have been developed over the last decades, including the application of irradiation, ultrasound, pulsed electric fields and oscillatory magnetic fields (Torres Bello *et al.*, 2014, Cebrián *et al.*, 2016). One of the most promising of these technologies is HHP, as it effectively inactivates most spoilage-associated and pathogenic vegetative bacteria, yeasts and molds as well as some quality-deteriorating enzymes, and therefore can extend shelf life, reduce spoilage and increase product safety (Torres Bello *et al.*, 2014). At the same time, HHP has a very low impact on valuable food characteristics, leaving organoleptic properties like color, texture and flavor, as

well as nutritional aspects such as vitamin content largely unaffected (Smelt, 1998, Balasubramaniam & Farkas, 2008, Considine *et al.*, 2008, Torres Bello *et al.*, 2014).
Furthermore, it minimizes the need for traditional preservatives like salt or acid on the one hand, and food additives on the other hand, thus allowing for increased sensorial quality as well as healthier and additive-free, so-called “green-label” products. Another advantage is that HHP can be applied after packaging, thus preventing recontamination after treatment, and allows for inactivation of spoilage microorganisms introduced during preparation (e.g. slicing, mixing of ingredients etc.) (Yamamoto, 2017). Since pressure acts simultaneously and quasi-instantaneously over the whole product, the process does not depend on product shape and size, and the problem of over-processing of the external and under-processing of the inner parts of the product, occurring with thermal preservation techniques, does not exist (Smelt, 1998, Considine *et al.*, 2008). Its mild effect on product properties makes HHP particularly suitable for the preservation of complex products like ready-to-eat meals and delicatessen composed of a variety of different ingredients that would be adversely affected by methods like heat pasteurization (van de Ven *et al.*, 2007). Altogether, HHP technology has the potential to produce high-quality, minimally-processed foodstuffs with extended shelf life and reduced preservative content, and is therefore increasingly used by the food industry.

1.1.1.1 History of HHP technology in food preservation

The first time HHP was applied on food products to prevent spoilage dates back to 1899 when pressurization at ~700 MPa was reported as an alternative preservation method for milk, being able to extend shelf life with a concomitantly lower effect on taste than heat (Hite, 1899). In the early 20th century, the American physicist Percy W. Bridgman explored the physical effects of HHP on various materials, established the phase transition diagram of water under pressure and discovered the denaturation of pressurized egg albumen (Bridgman, 1912, Bridgman, 1914, Balasubramaniam *et al.*, 2015). Whereas other industries such as the chemical, material and process engineering fields successfully exploited HHP technology throughout the 20th century, the food industry made use of elevated pressures only from the 1970s on, when coffee was decaffeinated by using the supercritical fluid technology with pressures of 30-50 MPa (King, 2014, Balasubramaniam *et al.*, 2015). The use of HHP for food preservation purposes began even later when increasing research effort in the 1980s enabled the introduction of the first high-pressure-treated products, namely fruit jams and jellies, on the Japanese market in 1990 (Balasubramaniam *et al.*, 2015, Yamamoto, 2017). Other countries like the USA and Spain followed with the commercialization of HHP treated guacamole and cured ham, respectively, and

since then both the number of commercialized products and the research activity on industrial as well as academic level has strongly increased (Balasubramaniam *et al.*, 2015, Yamamoto, 2017).

1.1.1.2 Current situation and future perspectives

In 2009 the production of HHP processed food reached an amount of more than 200 000 t (Heinz & Buckow, 2009), and raised to 350 000 t in 2012 (Torres Bello *et al.*, 2014) and more than 500 000 t in 2015 (Georget *et al.*, 2015), representing a worldwide annual market of about 2.5 billion US dollars, which makes HHP the most commercially developed non-thermal preservation technology (Torres Bello *et al.*, 2014, Balasubramaniam *et al.*, 2015). Since the introduction of the first products in the 1990s the range of HHP treated foodstuffs has dramatically broadened. Products available on the market include fruit juices and smoothies, vegetable products, sauces, dips and salad dressings, meat and seafood as well as plenty of ready-to-eat products (Balasubramaniam & Farkas, 2008, Torres Bello *et al.*, 2014, Balasubramaniam *et al.*, 2015).

The fact that HHP is applied after packaging makes it suitable for the preservation of ready-to-eat products. These products comprise convenience food as well as delicatessen, such as ready-made salads on the basis of meat, fish or other seafood, or dips and sauces. The first successful attempts of producing shelf-stable ranch dressing using HHP were undertaken in the USA (Waite *et al.*, 2009).

Despite this generally positive development, where HHP technology is becoming increasingly important to the food industry, several drawbacks hamper a more widespread use. First of all, high acquisition costs and an expensive and laborious maintenance of the equipment together with the relatively low throughput due to the limitation to batch or, at most, semi-continuous operation constrain the application of HHP to the high-value/high-price segment (Balasubramaniam *et al.*, 2015, Yamamoto, 2017). Moreover, the fact that the efficacy of HHP inactivation varies widely among bacterial species and even on the strain level has impeded the identification of appropriate surrogate organisms, which makes the acquisition of systematic data and process validation difficult (Balasubramaniam & Farkas, 2008, Considine *et al.*, 2008, Gänzle & Liu, 2015). Unlike thermal inactivation, where parameters like D- and z-values derived from log-linear models predict the survival of microorganisms very well, pressure-induced microbial inactivation over time is more variable, typically characterized by a strong tailing effect (i.e. non-linear inactivation) and therefore very difficult to model (Balasubramaniam & Farkas, 2008, Gänzle & Liu, 2015). In addition, inactivation efficiency strongly depends on the chemical

composition of the food matrix, with the pH and the concentration of salt, sugars and other solutes playing a major role. Other food components, such as fat, are sparsely characterized in terms of their effect on HHP inactivation. Furthermore, food matrices represent complex, and often inhomogeneous mixtures of various components that can act synergistically or antagonistically with each other. Additionally, pressure-induced microbial inactivation in food products not only depends on the direct lethal effect of pressure, but also on the post-pressure survival of the microorganisms in the food matrix (Balasubramaniam & Farkas, 2008, Gänzle & Liu, 2015). Depending on the composition, the food matrix can either hinder or promote the recovery of sublethally injured cells and thus increase or reduce the efficacy of the pressure treatment, respectively. For all these reasons, the acquisition of systematic data and process validation is very difficult such that currently the inactivation efficiency has to be determined case by case for each product in order to assure proper product safety for the consumer and compliance with the requirements of laws and directives. In general, eukaryotes like yeasts and molds are the most pressure-sensitive microorganisms followed by Gram-negative and then Gram-positive vegetative bacteria. Some fungal ascospores and most bacterial endospores, including dangerous pathogens such as *Clostridium botulinum*, are highly pressure-resistant, surviving the highest technically feasible pressures at ambient temperatures (Smelt, 1998, Torres Bello *et al.*, 2014, Gänzle & Liu, 2015). Hence, HHP alone cannot be used for sterilization, and treated products have to be stored under refrigerated conditions or exhibit features that prevent the growth of these organisms, such as a low pH value (Balasubramaniam *et al.*, 2015). However, a synergistic effect of the combination of HHP and heat > 70 °C has been reported by numerous studies (Balasubramaniam *et al.*, 2015). For intense treatment parameters above 400-600 MPa and 90-120 °C this method has been described as pressure assisted thermal sterilization (PATS) and is a promising approach for sterilization of foodstuffs with less detrimental effects on their nutritional value and the organoleptic properties compared to conventional heat preservation (Balasubramaniam & Farkas, 2008). Besides HHP pasteurization and PATS, there are a variety of possibilities where the use of elevated pressure may assist or replace other currently used food processing methods in the near future. These include pressure-assisted rapid freezing and thawing, the combined application of pressure and an electric field (also referred to as pressure-ohmic-thermal sterilization), the combination of pressure and electric field, high pressure homogenization and the pasteurization of delicate liquid foods using a combination of CO_2 and pressure (Balasubramaniam *et al.*, 2015). HPP treatment may also be assisted by the use of antimicrobial compounds such as bacteriocins or endolysins to reduce the required pressure levels and to eliminate tailing effects

(Balasubramaniam & Farkas, 2008, van Nassau *et al.*, 2017). Altogether, further dissemination of HHP technology in food preservation is hampered mostly by a still tremendous lack of knowledge. Therefore, more research also on a basic level is needed to identify the impact of different ingredients, food matrix components and characteristics on the inactivation behavior of spoilage microorganisms.

In addition to food preservation and shelf life extension, there are various alternative applications of HHP in food processing, such as to increase the activity of enzymes for fermentation processes, shucking of crustaceans, removal of allergens from rice and wheat by extrusion or the acceleration of water impregnation (soaking), e.g. of rice or soy beans (Yamamoto, 2017).

1.1.1.3 Practical implementation of HHP processing in food preservation

In industrial HHP pasteurization, pressures between 100 to 800 MPa (in most cases between 400 and 600 MPa) are applied for time periods of a few seconds to several minutes, depending on the organisms to be inactivated and the pressure sensitivity of the product, and can be combined with mild heat (20-50 °C) to enhance the lethal effect on microorganisms and additionally achieve inactivation of spoilage-associated enzymes (Heinz & Buckow, 2009, Torres Bello *et al.*, 2014, Yamamoto, 2017). With the exception of some semi-continuous systems used for liquid products, high-pressure plants are typically batch systems consisting of one or more thick-wall cylinders made of high strength steel alloy showing high fracture toughness and corrosion resistance (Balasubramaniam *et al.*, 2015). The product is immersed in a pressure-transmitting fluid inside the vessels, which, due to the negligible compressibility of liquids, uniformly and instantaneously transfers the pressure from the intensifier to the product. The pressure vessels are covered with appropriate closures and connected via an intensifier to a high pressure pump regulated by a computer-assisted control unit (Balasubramaniam *et al.*, 2015). Semi-continuous systems for liquid products consist of at least two connected vessels, with the first being charged, the second being pressurized and an optional third one being discharged at the same time (Balasubramaniam *et al.*, 2015). For industrial applications of batch systems, water is usually used as pressure-transmitting fluid, whereas glycol, mixtures of glycol and water or different oils are used for many laboratory applications, where extreme conditions are reached and pressure-induced temperature changes of the fluid play a greater role. The addition of glycol, for example prevents water from freezing during rapid pressure release, thus protecting the piping and vessel system from damage due to volume changes. HHP treatment is typically performed with the product in its final packaging. Therefore, flexible and pressure-resistant materials, especially in terms of permeability, release of compounds or separation of

layers, are used. These include polyethylene (PE), polyethylene terephthalate (PET) and polypropylene (PP), ethylene-vinyl alcohol (EVOH), polyamide (PA), and nylon films (Torres Bello *et al.*, 2014).

Since the inclusion of headspace air causes higher efforts to build up the target pressure due to its high compressibility, and solubilized oxygen shows increasing reactivity under pressure, thus affecting food quality, products are either vacuum-packed, or air inclusion is reduced as far as possible before HHP treatment (Torres Bello *et al.*, 2014, Balasubramaniam *et al.*, 2015, Yamamoto, 2017).

1.1.2 Physical principles underlying HHP

Laws of Thermodynamics:

The zeroth, first and second law of thermodynamics represent the basis for understanding the physicochemical phenomena observed during HHP treatments. The zeroth law of thermodynamics, the law of thermal equilibrium, states that two systems that are both in equilibrium with a third system are also in equilibrium with each other.

According to the first law of thermodynamics, the law of conservation of energy, the change in internal energy (*U*) of a closed system equals the sum of energy added to the system in the forms of work (W), heat (Q) and the sum of chemical potential (μ), where μ is the potential energy that can be absorbed or released during chemical reactions by *N* particles of *i* different types in the system (Job & Herrmann, 2006, Lenz, 2017):

$$\boldsymbol{dU} = \boldsymbol{dW} + \boldsymbol{dQ} + \sum_{\boldsymbol{i=1}}^{\boldsymbol{j}} \mu_{\boldsymbol{i}} \boldsymbol{dN_i} \qquad \text{(Eq. 1)}$$

During pressurization, *dW* represents the volumetric work and can thus be expressed as:

$$\boldsymbol{dW} = -\boldsymbol{pdV} \qquad \text{(Eq. 2)}$$

dQ represents the amount of heat energy added to the system and is generally positive (Lenz, 2017). The sum of the chemical potential *μ* is given by the number of reactive particles of a certain type with a certain chemical potential present in the system. At a constant pressure, the chemical potential ideally equals the partial molar Gibbs free energy. During phase transition under pressure the chemical potential can change. In case of chemical equilibrium (i.e. only one

type of particle is present) or in case of phase equilibrium, the sum chemical potential can be zero (Lenz, 2017).

The second law of thermodynamics states that all processes strive to achieve thermodynamic equilibrium over time, i.e. homogeneity of energy and matter. This means that the system tends to maximize the degree of disorder, also referred to as entropy (S). As a consequence, the total entropy in a closed system increases or remains equal, but never decreases over time.

During HHP processing, the amount of heat (Q) transferred to the system can be expressed as the temperature (T) of the system and the space where the heat comes from and goes to, multiplied with the increase in entropy (dS) (Lenz, 2017):

$$\boldsymbol{dQ = TdS} \qquad \text{(Eq. 3)}$$

In cases in which the sum of the chemical potential is negligible (see above), the inner energy U of a system can consequently be expressed in relation to pressure, volume, temperature and entropy as follows (Lenz, 2017):

$$\boldsymbol{dU = -pdV + TdS} \qquad \text{(Eq. 4)}$$

In addition to the thermodynamic fundamentals, there are three general principles that underlie the physicochemical processes during HHP treatment and help understand the accompanying observable phenomena:

Le Chatelier's principle:

According to the principle established by both Henry Le Chatelier and Ferdinand Braun independently, any physicochemical process, such as a chemical reaction, phase transition, change in molecular configuration or macromolecular arrangement, that is accompanied by a decrease in volume, is favored by pressure and vice versa (Cheftel, 1995, Balasubramaniam *et al.*, 2015). Hence, a pressure increase shifts the equilibrium towards the state with the lowest volume whereas pressure release favors an equilibrium shift towards the state with a higher volume (Cheftel, 1995).

Principle of microscopic ordering:

The principle of microscopic ordering states that, provided that the temperature is constant, an increase in pressure leads to a higher degree of molecular order. The fact that the same effect

(higher molecular order) is observed with a decrease in temperature, means that pressure and temperature act antagonistically on molecular structures (Balasubramaniam *et al.*, 2015).

Isostatic principle:

The isostatic principle states that pressure acts equally in all directions and is transmitted uniformly and (quasi-)instantaneously throughout the whole product, regardless of its size and geometry (Cheftel, 1995, Balasubramaniam *et al.*, 2015). This means that if a fluid is used to transmit pressure, all parts of a pressurized product experience the same pressure for the same amount of time so that over-processing of the outer parts and under-processing of the inner parts is not observed. It further explains why food products, as long as they contain no pores or air pockets, macroscopically retain their shape during HHP treatment (Balasubramaniam & Farkas, 2008).

1.1.3 Effects of HHP on the physicochemical properties of pressurized matter

On the basis of the physical principles described in the previous section and depending on their individual physicochemical characteristics, different materials can undergo various reversible and irreversible alterations under pressure. This section gives an overview of pressure-induced structural changes in food-relevant materials and biological structures, providing the molecular basis for the processes that are currently believed to be responsible for pressure-induced microbial inactivation.

1.1.3.1 Adiabatic heating

During pressure increase, the temperature of the pressurized material rises as a result of compressive work against intermolecular forces. Likewise, pressure release causes a decrease in temperature (Cheftel, 1995, Balasubramanian & Balasubramaniam, 2003). This phenomenon is called adiabatic compression heating, adiabatic heat of compression or shorter, adiabatic heating. The effect of adiabatic heating can be explained by the first law of thermodynamics as described previously by Lenz (2017) and Knoerzer *et al.* (2010): Assuming that there is no thermal exchange with the environment, i.e. ideal adiabatic conditions prevail, the temperature reached during compression can be calculated based on the assumption that the entropy change *dS* is a function temperature *T* and pressure *p*, i.e. *S = f(T,p)*:

$$dS = \left(\frac{\partial S}{\partial T}\right)_p dT + \left(\frac{\partial S}{\partial p}\right)_T dp \qquad \text{(Eq. 5)}$$

Assuming that the process is completely reversible, the overall change in entropy is zero and thus the compression heating rate can be expressed by rearranging the formula:

$$\frac{dT}{dp} = -\frac{\left(\frac{\partial S}{\partial p}\right)_T}{\left(\frac{\partial S}{\partial T}\right)_P} \quad \text{(Eq. 6)}$$

Using the appropriate Maxwell's relation that expresses the specific Volume V as the inverse of the density ρ:

$$\left(\frac{\partial S}{\partial p}\right)_T = -\left(\frac{\partial V}{\partial T}\right)_P \quad \text{(Eq. 7)}$$

the volume can be expressed as the inverse of the density:

$$v = f(p,T) = \frac{1}{\rho} \quad \text{(Eq. 8)}$$

Defining the isobaric heat capacity C_p as:

$$C_p(p,T) = T\left(\frac{\partial S}{\partial T}\right)_p \quad \text{(Eq. 9)}$$

the thermal expansion coefficient α_p, according to Bridgman (1912), as:

$$\alpha_p(p,T = \frac{1}{V}\left(\frac{\partial V}{\partial T}\right)_p \quad \text{(Eq. 10)}$$

and the compression heating coefficient k_C, according to Knoerzer *et al.* (2010), as:

$$k_C = f(p,T) = \frac{\alpha_p}{\rho C_p} \quad \text{(Eq. 11)}$$

the compression heating rate can be expressed as:

$$\frac{dT}{dP} = -\frac{\left(\frac{\partial S}{\partial P}\right)_T}{\left(\frac{\partial S}{\partial T}\right)_P} = \frac{V\left(\frac{1}{V}\left(\frac{\partial V}{\partial T}\right)_P\right)}{\frac{1}{T}\left(T\left(\frac{\partial S}{\partial T}\right)_P\right)} = \frac{T\alpha_p}{\rho C_p} = k_C T \quad \text{(Eq. 12)}$$

According to Equation 12, the temperature change experienced by pressurized material under adiabatic (i.e. no heat exchange with the environment), isentropic (i.e. constant entropy) conditions depends on its compressibility (i.e. thermal expansion), its density and its specific heat capacity. All three in turn depend on pressure and temperature (Lenz, 2017). Moreover, these parameters also depend on the material's thermophysical properties, meaning that different materials experience different temperature changes due to adiabatic heating (Knoerzer *et al.*, 2010). Therefore, the compression heating rate as a function of pressure and starting temperature has to be determined empirically for each material. At the moment, water is the only material for which holistic data exist, including mixture rules for water-soluble components (Knoerzer *et al.*, 2010). Due to the complex composition and variability of food products, the determination of pressure-temperature-dependent compression heating rates is very laborious and investigations concerning single food components were mostly carried out only for specific pressure-starting temperature combinations, so far (Knoerzer *et al.*, 2010). However, maximum adiabatic heating effects expected during pressurization can be calculated from empirical data obtained under nearly adiabatic conditions (Knoerzer *et al.*, 2010). The actual effect of adiabatic heating is generally less pronounced than theoretically calculated, since it is impossible in practice to completely eliminate heat transfer between a pressurized sample and the environment, and ideal adiabatic conditions are thus never reached. Nevertheless, compression and decompression can lead to significant temperature shifts that influence the effect of pressure on the sample and can support the lethal effect of HHP on microorganisms whereby the uniform and instantaneous adiabatic heating of the entire product increases process efficiency. The effect can also be used to reach the desired sterilization temperature during PATS treatments more quickly and, reversely, achieve a uniform and instantaneous cooling effect during pressure release. On the other hand, the effect of adiabatic heating has to be kept under control when treating temperature-sensitive products in order to avoid temperature-induced deterioration of the product, e.g. due to protein denaturation. The adiabatic heating of water is 2-3°C per 100 MPa (Cheftel, 1995).

1.1.3.2 Phase transition

Depending on temperature and pressure, materials adopt different states of aggregation, i.e. solid, liquid and gaseous, which differ in density and thus in the volume occupied by a certain number of molecules. For most materials, pressure and temperature act antagonistically, with higher temperatures being associated with larger volumes and lower densities due to increased molecular motion, whereas a pressure increase favors states with smaller volumes and higher

densities, according to Le Chatelier's principle. Therefore, the melting and boiling temperatures of a certain substance depend on the prevailing pressure and, vice versa, the phase transition pressures depend on the temperature. Unlike most chemical substances, water shows a decrease in melting temperature with higher pressures, remaining liquid down to -22 °C when pressurized with 210 MPa (Cheftel, 1995). This is due to the negative thermal expansion at temperatures below 4 °C, which means that a further reduction in temperature and the formation of ice crystals lead to an increase in volume at ambient pressure (~0.1 MPa). Furthermore, a pressure increase does not lead to normal ice crystals but diverse solid structures of crystalline and amorphous nature some of which are only metastable, as discovered by Bridgman and other researchers (Bridgman, 1912, Zheligovskaya & Malenkov, 2005, Zheligovskaya & Malenkov, 2006). In contrast, the pressure-induced phase-transition of oil, or more generally spoken lipids, follows the general rule, as a pressure increase of 100 MPa raises the melting temperature of triglycerides by > 10 °C (Cheftel, 1995). Thus, many food-relevant lipids undergo reversible crystallization, i.e. a phase change from liquid to solid during HHP treatment at ambient temperatures (Ferstl *et al.*, 2010). In terms of biological systems in general, the phase transition behavior of lipid membranes is of utmost importance for the survival or inactivation of microorganisms under HHP conditions. Aspects of changes in the phase state of biological membranes are introduced in detail in 1.1.4.1.4.

1.1.3.3 Dissociation equilibrium and pH value

The pH value of an aqueous system depends on the prevailing temperature and pressure, since both have an influence on the dissociation equilibrium of water itself as well as aqueous solutions. This section provides an insight into the physicochemical background underlying the pressure- (and temperature-)dependence of the pH value (Georget *et al.*, 2015). Some solutes dissolved in water, but also water molecules themselves, act as proton donors (acids) or acceptors (base), i.e. they can give or receive protons (H^+) to or from water molecules, respectively. This process achieves an equilibrium state that depends on intrinsic properties of the solute and extrinsic factors. This so-called dissociation equilibrium is based on the dissociation reaction shown in Equation 13 for acids:

$$\boldsymbol{HA + H_2O \leftrightarrow A^- + H_3O^+} \qquad \text{(Eq. 13)}$$

where HA is the acid (proton donor), H_2O is water, A^- the conjugated base and H_3O^+ an oxonium ion.

According to the law of mass action, the dissociation equilibrium is described by the equilibrium constant K, which depends on the concentration [ ] and activity coefficient (γ_i) of each component (Equation 14):

$$\boldsymbol{K = \frac{\gamma_{A^-} \cdot \gamma_{H_3O^+}}{\gamma_{HA} \cdot \gamma_{H_2O}} \cdot \frac{[A^-] \cdot [H_3O^+]}{[HA] \cdot [H_2O]}} \qquad \text{(Eq. 14)}$$

Since the concentration of water [H_2O] is very high (~55.5 M) compared to those of the other components, it can be assumed as constant and therefore be omitted from the equation. This results in the acid dissociation constant K_a, which describes the extent of dissociation of an acid into the conjugated base and oxonium and is defined as:

$$\boldsymbol{K_a = \frac{\gamma_{A^-} \cdot \gamma_{H_3O^+}}{\gamma_{HA}} \cdot \frac{[A^-] \cdot [H_3O^+]}{[HA]}} \qquad \text{(Eq. 15)}$$

Since the value of K_a differs among acids and can vary over several orders of magnitude, the additive inverse of its common logarithm, pK_a, is usually used to describe the dissociation constant (Equation 16):

$$\boldsymbol{pK_a = -log_{10}(K_a)} \qquad \text{(Eq. 16)}$$

Likewise, the additive inverse of the common logarithm of the activity of oxonium ions (divided by 1 mol L^{-1} to make the parameter dimensionless) is defined as the pH value, which is the most widely used way to describe acidity in an aqueous system (Equation 17).

$$\boldsymbol{pH = -log_{10}\left(\gamma_{H_3O^+} \cdot \frac{[H_3O^+]}{1\ mol \cdot L^{-1}}\right)} \qquad \text{(Eq. 17)}$$

The temperature and pressure dependence of the equilibrium constant was described already in 1887 by Planck who established the following equation (Planck, 1887):

$$\boldsymbol{\left(\frac{d \ln K}{dp}\right)_T = \left(\frac{\Delta V(p)}{RT}\right)} \qquad \text{(Eq. 18)}$$

with *p* being the pressure (MPa), *T* the absolute temperature (K), *R* the gas constant (8.3145 cm^3 MPa K^{-1} mol^{-1}) and *V* the reaction volume (cm^3 mol^{-1}), which is the difference between the partial volumes of the products and reactants, and itself depends on the pressure (Mathys *et al.*, 2008).

The transformation and integration of Planck's equation leads to an expression (Equation 19) that describes the pressure and temperature dependence of the acid equilibrium constant pK_a (Lenz, 2017):

$$pK_a = pK_a{}^0 + \frac{\log_{10} e}{RT} \int_{p^0}^{p} \Delta V(p)\,dp \qquad \text{(Eq. 19)}$$

Although the pH value is usually used to describe the acidity of aqueous systems, it only considers the concentration of oxonium ions and thus cannot accurately describe the pressure- or temperature-induced shifts of the dissociation equilibrium. In pure water with low and nearly equal concentrations in hydroxide (OH^-) and oxonium (H_3O^+) ions, for example, a pressure increase causes the dissociation of water molecules leading to higher, but still equal hydroxide and oxonium concentrations. In this case, the pH shift would be observed although neutral conditions are still prevailing. The pK_a value, in contrast, takes all reaction partners of water into consideration and thus describes equilibrium changes in the system more accurately (Mathys *et al.*, 2008).

The equilibrium shift towards the dissociated state observed under pressure results from the accompanying formation of electrical charges, which causes a reduction in volume (Drude & Nernst, 1894). In a process called hydration, electrostatic forces between a charged ion and the partially charged water molecules (dipoles) keep water in a specific orientation around the ion. Water molecules occupy a smaller volume when bound in hydration shells as compared to the unbound state. Thus, according to Le Chatelier's principle, pressure causes the dissociation of weak acids into negatively charged acid ions and free oxonium ions, leading to a shift in many food-relevant and biochemical buffer systems (Gross & Jaenicke, 1994).

1.1.3.4 Behavior of molecular interactions under pressure

The macroscopic appearance of organic materials depends on the intra- and intermolecular interactions within them, and changes in these interactions are the basis for the reversible and irreversible material changes observed during pressurization. Owing to the pressure-induced equilibrium shift stated in the principle of Le Chatelier, the effect of HHP on macromolecules depends on the susceptibility of their inter- and intramolecular interactions to volume changes. Being almost incompressible, covalent bonds are highly pressure-stable (Williamson, 2015). Accordingly, the primary structures of macromolecules like proteins, polysaccharides and nucleic acids show a HHP resistance up to 1-2 GPa (Mozhaev *et al.*, 1994). In contrast, non-covalent

interactions are generally more pressure-sensitive, since their bonding energy is distance-dependent (Balasubramaniam *et al.*, 2015). Hydrogen bonds, which are crucial for secondary and tertiary structures of many biological macromolecules, including proteins, are generally strengthened under pressure, since a pressure-induced volume decrease causes the approximation of the interaction partners (Cheftel, 1995). Electrostatic interactions (salt bonds) and, in part, hydrophobic interactions are impaired under pressure due to electrostriction during the formation of additional charges and the alignment of water molecules close to hydrophobic groups, respectively (Cheftel, 1995).

1.1.3.5 The effect of pressure on the physical properties of food

Although having a mild effect on food material in general, HHP can transiently or permanently alter the chemical state and appearance of foodstuffs depending on their chemical composition. The behavior of the main food constituents under HHP is explained in the following under consideration of their (macro-)molecular structures.

Proteins

Due to the large variety of different structures and functions, proteins are probably the most intensively studied molecules under pressure. Several types of molecular interactions are involved in the formation of a protein's 3-D structure. The primary structure, i.e. the polypeptide backbone and the amino acid residues, and the secondary structure (α-helix and β-sheet) are largely unaffected by HHP, since they are based on covalent and hydrogen bonds (see 1.1.3.4), respectively (Knorr *et al.*, 2006, Huang *et al.*, 2014). The tertiary (three-dimensional orientation of the polypeptide chain) and quaternary (composition of a protein complex of different subunits) structures, by contrast, are considerably pressure-sensitive (Knorr *et al.*, 2006). Thiol groups within a protein undergo oxidation and form disulfide bonds under pressure (Ma & Ledward, 2013). The native state of a protein contains cavities that can be empty or filled with water molecules, and thus has a larger partial molar volume than the unfolded, fully hydrated state (Williamson, 2015). Favoring volume reduction, HHP forces water molecules into void spaces and hydrophobic areas inside the protein, thus disrupting hydrophobic interactions. Furthermore, it breaks up electrostatic interactions (salt bonds) due to the volume reduction that accompanies hydration caused by the separation of electric charges (electrostriction) (Cheftel, 1995). This leads to partial or complete unfolding and denaturation as well as the dissociation of oligomers into their subunits. Depending on the pressure level and the individual protein, HHP-induced denaturation can be reversible or irreversible, leading to misfolded proteins, gelation or aggregation with other proteins after pressure release, especially in case of high protein

concentrations and at appropriate pH and ionic strength (Cheftel, 1995). Although for many proteins unfolding leads to a decrease in the total volume (Cheftel, 1995), the susceptibility to HHP-mediated denaturation highly depends on the three-dimensional structure of the actual protein. If denaturation is accompanied by a volume increase, pressure can also stabilize the native state and enhance its heat-stability (Knorr *et al.*, 2006). Unlike heat, HHP can leave parts of a protein unaffected, whereas others are reversibly or irreversibly changed (Knorr *et al.*, 2006). Partial protein denaturation starts at 100 MPa, and pressures higher than 200 MPa can cause severe damage to a protein's tertiary structure (Huang *et al.*, 2014). Although the threshold pressure for irreversible denaturation depends on the structure of the protein, pressure levels below 300 MPa are generally considered to induce reversible denaturation, whereas irreversible impairment is observed at higher pressures (Huang *et al.*, 2014). Moderate pressures between 100 and 300 MPa can thus be used to dissolve protein aggregates and refold misfolded proteins into their native state (Follonier *et al.*, 2012). HHP-induced denaturation leads to the inactivation of most enzymes (Mozhaev *et al.*, 1994). Furthermore, enzymatic reactions can be either favored or inhibited by HHP, depending on the accompanying volume changes (Eisenmenger & Reyes-De-Corcuera, 2009).

Protein denaturation plays a major role in HHP treatment of meat, which undergoes changes in both color and texture. Meat color depends on pressure-sensitive proteins like myosin and water soluble heme proteins like myoglobin (Ma & Ledward, 2013). Myosin denatures at 180–300 MPa resulting in opaqueness similar to that observed with cooked meat whereas the denaturation of myoglobin causes a color shift from red over pink at > 150 MPa to grayish-brown above 350 MPa due to oxidation of the heme pigment (oxymyoglobin Fe^{2+} into metmyoglobin Fe^{3+}) (Cheftel, 1995, Ma & Ledward, 2013).

For many protein isolates from vegetables, HHP has been shown to induce gelatinization (Torres Bello *et al.*, 2014). Regarding pressure sensitivity, milk proteins are the most extensively studied food proteins (Cebrián *et al.*, 2016). Pressure leads to unfolding of whey proteins and the exposure of chemical groups (sulfhydryl groups) that interact with other proteins (whey, casein), resulting in the formation of new interactions with other whey proteins and casein proteins (in milk). Upon pressure release, whey proteins that have not interacted with other proteins under pressure, return to the pre-pressure state (Huppertz 2006). The denaturation of whey proteins α-lactalbumin (α-la) and β-lactoglobulin (β-lg) in bovine milk occurs at pressures above 400 and 100 MPa, respectively, and casein micelles are disrupted and rearranged at pressures > 300 MPa (Huppertz *et al.*, 2006).

Fat

Being rather small and of low molecular weight, fat molecules *per se* do not undergo structural changes under food-relevant pressures. However, fat can reversibly undergo crystallization, i.e. phase transition from the liquid to the solid state during pressure build-up (Ferstl *et al.*, 2010). Since the fat fraction in foodstuffs is a rather inhomogeneous mixture of a variety of different molecular lipid structures, phase transition usually occurs over a large pressure/temperature range. HHP treatment at about 400 MPa and higher increases the susceptibility of polyunsaturated fatty acids to oxidation, as observed in fresh meat and fish products (Ma & Ledward, 2013). One reason for this is the HHP-induced formation of free radicals (Bragagnolo *et al.*, 2006).

Carbohydrates

Carbohydrates are either present as low-molecular sugar molecules or in the form of starch, where many sugar entities are linked to long linear and cross-linked macromolecules. Sugars are highly pressure-stable, whereas the intermolecular interactions between starch molecules are affected by pressure, leading to their rearrangement and the absorption of water molecules. As a consequence, starch, which usually occurs in the form of granules with low water solubility, swells and irreversibly adopts a gel-like structure (Stute *et al.*, 1996, Stolt *et al.*, 2000). The extent of gelatinization depends on the type of starch, pressure height, holding time and temperature (Stolt *et al.*, 2000, Bauer & Knorr, 2005).

Other food constituents

Due to the high pressure stability of covalent bonds, low-molecular compounds like lipids, saccharides and peptides are largely unaffected by HHP up to 1-2 GPa (Mozhaev *et al.*, 1994), and many health-promoting and value-adding compounds in food, such as vitamins, antioxidants color pigments etc. are relatively stable under pressure levels relevant for food processing (Oey *et al.*, 2008, Balasubramaniam *et al.*, 2015).

1.1.4 Inactivation of microorganisms by HHP

Microbial inactivation by HHP is a complex process that varies widely depending on pressure intensity, treatment time and other environmental factors as well the microorganism itself. The profile of HHP inactivation curves often follows a second-order development, with a shoulder at short holding times followed by a linear slope on semi-logarithmic application and a tail resulting from a pressure-resistant fraction of cells that is viable even after prolonged holding times (Cebrián *et al.*, 2016). The tailing effect is believed to result from heterogeneity of inherent

resistance within the population rather than genetic variation, since isolation and re-cultivation of the tail population does not lead to increased pressure resistance (Patterson, 2005). The phenomenon of tailing makes it difficult to extrapolate inactivation rates and calculate parameters, such as D-values and is one reason why HHP inactivation efficiency has to be assessed case-by-case for every new product (Patterson, 2005). Shoulders at the beginning of treatment, in contrast, are probably due to sublethal injury and the concomitant action of HHP on several cellular target structures (Mañas & Pagán, 2005). The following sections describe the cellular structures that are impaired by HHP and the effect of the factors influencing the extent of microbial HHP inactivation.

1.1.4.1 Cellular target structures for HHP

High pressure acts on vegetative bacterial cells by impairing the function of different cellular target structures and mechanisms (Mañas & Pagán, 2005). These damages occur, in part, simultaneously and their complex interplay is believed to cause first sublethal injury and finally inactivation (Georget *et al.*, 2015). Depending on the pressure intensity, different cellular components undergo changes in structure and/or functionality.

1.1.4.1.1 Enzymes and other proteins

The three-dimensional configuration of proteins, in particular of enzymes, is of utmost importance for their functionality, and small changes at the substrate recognition site or the catalytic center are sufficient to strongly alter an enzyme's activity (Torres Bello *et al.*, 2014). As described in 1.1.3.5, proteins can undergo unfolding and denaturation during pressure treatment. Pressure-induced dissociation of multimeric complexes (quaternary structure) and break-up of the three-dimensional configuration (tertiary structure) leads to a loss of functionality of many enzymes. In general, protein unfolding due to pressures below 200 MPa is considered reversible, whereas pressures of 300 MPa and higher cause irreversible denaturation (Yamamoto, 2017). However, the pressure intensity required to induce enzyme inactivation and the reversibility of unfolding largely depends on the pressure sensitivity of an individual protein and the surrounding matrix, with low-molecular, non-ionic solutes and kosmotropic ions like phosphate exerting a stabilizing effect (Zhang & Cremer, 2006). Furthermore, depending on its structure, an enzyme may also be activated by HHP (Huang *et al.*, 2014).

HHP-induced loss of proper enzyme functionality causes a variety of cellular malfunctions that eventually lead to inactivation, such as the impairment of the transmembrane pH gradient due to ATPase inactivation (Wouters *et al.*, 1998, Molina-Gutierrez *et al.*, 2002) or the accumulation of

reactive oxygen species (ROS) as a result of metabolic imbalance (Bloomfield *et al.*, 1998, Wuytack *et al.*, 2003).

Moreover, HHP induces stress responses in bacterial cells similar to those observed with heat and other stresses, including the expression of stress proteins like heat and cold shock proteins to keep HHP-induced damage in check (Huang *et al.*, 2014). Thus, the disruption of repair proteins required to restore functionality of other proteins is a central step during HHP-induced inactivation.

1.1.4.1.2 Nucleic acids

As the structure of nucleic acids is mostly based on covalent and hydrogen bonds, DNA and RNA per se are largely unaffected by HHP (Patterson, 2005). However, HHP induces the condensation of nucleic acids and causes degradation as a result of facilitated contact with endonucleases (Patterson, 2005, Huang *et al.*, 2014). Furthermore, binding of enzymes involved in processing of nucleic acids is hampered, and thus DNA replication, transcription and translation are inhibited under HHP, resulting in an impairment of cell division and gene expression.

1.1.4.1.3 Ribosomes

Ribosomes are complex structures consisting of nucleic acids, i.e. ribosomal RNA, and proteins. Their sophisticated three-dimensional structure represents a sensitive target for HHP. Ribosome formation, and consequently, protein synthesis is impaired by pressure levels starting from 50 MPa (Huang *et al.*, 2014), and HHP has been reported to cause misconfiguration of ribosomes and disintegration into subunits (Georget *et al.*, 2015).

1.1.4.1.4 Cytoplasmic membrane

Role and composition of the cytoplasmic membrane

The cytoplasmic membrane separates the intracellular space from the environment. Its integrity is essential for many physiological processes, such as energy generation, uptake of nutrients, disposal of metabolites, signaling and the maintenance of intracellular conditions like pH and osmotic pressure, which can be significantly different from those found outside the cell (Winter, 2015). The bacterial cell membrane mainly consists of amphiphilic phospholipids with a hydrophilic head group and a hydrophobic tail made up by two fatty acid chains linked via ester bonds. Based on the hydrophobic effect, phospholipids tend to keep their hydrophobic tail region away from bulk water, whereas the polar, hydrophilic head group seeks water contact. Thus, a bilayer with a hydrophobic core and hydrophilic surfaces is formed. In addition, large numbers of

proteins can be embedded in the bilayer, either spanning the whole membrane or being anchored and protruding from the inner or outer membrane surface.

Behavior of the membrane under pressure

Under physiological conditions, most biological membranes are in the liquid-crystalline (L_α) phase with their acyl chains in a melted, disordered conformation (Winter, 2015). However, a decrease in temperature or an increase in pressure cause tighter packing, restricting the motion of the phospholipid acyl chains and consequently leading to transitions from the L_α to the more rigid gel-like phases like the lamellar gel phase ($L_{\beta'}$ and $P_{\beta'}$) and lamellar-crystalline or subgel (L_c) phase. The reason for this is a an exothermic enthalpy change and a decrease in the partial molar volume during the liquid crystalline-to-gel transition (Matsuki, 2015, Winter, 2015). At pressure-induced phase transition, the bilayer shrinks laterally, and its thickness is increased due to a straightening of the acyl chains, i.e. a decrease of kinks and gauche conformers (Winter, 2015). Pressure reduces the lateral diffusion of membrane lipids in the liquid-crystalline phase and, even more strongly, through the transition to the gel phase, whereas rotational dynamics are largely unaffected (Winter, 2015).

Due to the antagonistic effect of temperature and pressure, the melting temperature is shifted towards higher temperatures by increasing pressure. This shift amounts to about 22°C/100 MPa in model membranes (Winter & Dzwolak, 2005). Membrane fluidity and phase transition pressure or temperature strongly depend on the molecular constitution of the membrane. Longer acyl chains and a higher degree of saturation decreases fluidity and shifts the phase transition towards higher temperatures and lower pressures, whereas chain shortening and unsaturation causes higher fluidity and a downshift in transition temperature and an upshift in transition pressure (Matsuki, 2015). Unsaturation of fatty acids leads to kinks in the acyl chain, resulting in a bulkier conformation with higher conformational freedom and thus looser packing of the membrane (Abe, 2015). Besides acyl chain constitution, phase transition behavior depends on the polar phospholipid head group, with smaller, tighter-packing head groups causing lower phase transition pressures than bulky or charged head groups (Matsuki, 2015, Winter, 2015).

By modifying the fatty acid composition, i.e. the hydrocarbon chain length, and the ratio of unsaturated to saturated, cis to trans unsaturated and branched to unbranched acyl chains as well as the degree of cyclopropanation, microorganisms adjust the fluidity of their membranes according to changes in environmental conditions, such as pH, ion strength, harmful compounds and temperature (Ulmer *et al.*, 2002, Denich *et al.*, 2003). The phase transition temperature of biological membranes is typically about 10 °C below the growth temperature (Winter, 2015).

Likewise, organisms living in the deep sea, where extreme temperatures of 2-4°C and pressures of up to 100 MPa prevail, adapt their membrane composition in a process called homeoviscous adaptation by incorporating increased amounts of cis-unsaturated fatty acids, in order to ensure proper fluidity (Allen *et al.*, 1999, Bartlett, 2002).

Effect of pressure on membrane functionality

Microbial cell membranes become porous and lose their barrier function under pressure. This permeabilization causes a loss of cytoplasmic matter such as proteins and allows external substances to enter the cytoplasm, resulting in an impairment of osmotic pressure and transmembrane pH gradient (Pagan & Mackey, 2000, Mañas & Mackey, 2004, Klotz *et al.*, 2010, Abe, 2015). Especially in Gram-negative bacteria, HHP-induced loss of membrane integrity has been associated with cell death (Klotz *et al.*, 2010, Charoenwong *et al.*, 2011).

Moreover, HHP leads to denaturation and inactivation of membrane-bound enzymes (Kato *et al.*, 2002) such as ion channels and ATP-driven transporters involved in different physiological processes like signaling, ion flux and nutrient uptake (Abe, 2013, Winter, 2015). Especially the pressure-induced transition from liquid-crystalline to gel phase has been associated with a loss of function of membrane-bound enzymes such as a multidrug resistance ABC transporter of *L. plantarum* (Wouters *et al.*, 1998, Ulmer *et al.*, 2000, Ganzle *et al.*, 2001, Ulmer *et al.*, 2002, Molina-Höppner *et al.*, 2004, Winter, 2015). Pressure has further been shown to weaken the interactions between lipids and proteins and to cause the detachment of integral and peripheral membrane-bound proteins (Winter, 2015). It has been shown that membrane-bound proteins and membrane lipids mutually influence their behavior under pressure. The structure and phase behavior of the lipid bilayer under different temperature-pressure conditions depend on the concentration of embedded proteins, but also changes of protein conformation and function can be influenced by the lipids (Winter, 2015).

Role of the membrane in bacterial high-pressure sensitivity

Owing to the above-mentioned HHP-induced damages, the cytoplasmic membrane is considered one of the main cellular target structures during bacterial HHP inactivation. In fact, the fatty acid composition and thus the fluidity and phase transition temperature of the cell membrane have been shown to strongly influence HHP sensitivity of bacteria (Casadei et al., 2002, Ulmer et al., 2002). Piezophilic deep-sea bacteria adjust their membrane fatty acid composition according to the pressure prevailing in their habitat in order to maintain proper fluidity and functionality (Yano et al., 1998). Their membranes are generally characterized by high amounts of unsaturated fatty acids (UFA), ensuring proper fluidity at high-pressure and low-

temperature conditions (Yano *et al.*, 1997). For *E. coli* cells in exponential phase, loss of membrane integrity has been found to directly correlate with cell death (Pagan & Mackey, 2000, Mañas & Mackey, 2004). Stationary phase cells and especially Gram-positive bacteria, in contrast, tend to maintain membrane integrity even when inactivated (Pagan & Mackey, 2000, Mañas & Mackey, 2004).

1.1.4.1.5 Oxidative stress (oxidation/redox equilibrium)

HHP induces a high degree of sublethal damage to cellular structures, resulting in a metabolic imbalance and, consequently, the accumulation of large quantities of reactive oxygen species (ROS) (Bloomfield *et al.*, 1998, Wuytack *et al.*, 2003). According to several studies, this kind of oxidative stress is believed to contribute to microbial inactivation. For example, HHP-induced ROS formation causes intracellular damage that finally leads to cell death in *E. coli* (Aertsen *et al.*, 2005) and leads to the induction of an oxidative stress response in yeast, where an overexpression of genes conferring resistance to oxidative stress enhances HHP tolerance (Bravim *et al.*, 2016). In addition, the incubation under anaerobic conditions increases the survival rate of HHP treated *Staphylococcus aureus* (Cebrian *et al.*, 2010) and *E. coli* cells (Aertsen *et al.*, 2005, Kimura *et al.*, 2017).

1.1.4.1.6 Intracellular pH

HHP generally causes the dissociation of weak acids, due to electrostriction (1.1.3.3). Therefore, many cytoplasmic acids undergo dissociation and lower the intracellular pH. HHP treatment of *L. plantarum* and *Lactococcus lactis* leads to a decrease in intracellular pH (Wouters *et al.*, 1998, Molina-Gutierrez *et al.*, 2002), whereas the loss of viability seems to be related to the inactivation of enzymes (ATPases) for the restoration of a proper pH gradient after pressurization (Molina-Gutierrez *et al.*, 2002, Molina-Höppner *et al.*, 2004).

1.1.4.2 Factors influencing the HHP susceptibility of microorganisms

Microbial inactivation by HHP depends on a variety of both intrinsic properties of the microorganisms and extrinsic parameters. The latter include general environmental factors and properties of the matrix, the microorganisms are embedded in during HHP treatment. Moreover, conditions experienced by the microorganisms prior to HHP treatment can alter their physiological state in such a way that HHP sensitivity is affected. Last but not least, the survival rate of pressure-treated microorganisms strongly depends on the post-pressure treatment, as the recovery of sublethally injured cells can be facilitated or inhibited by favorable or adverse environmental conditions, respectively.

1.1.4.2.1 The surrounding matrix during HHP treatment

Microbial inactivation by HHP strongly depends on the chemical composition of the surrounding matrix (Cebrián *et al.*, 2016). This is especially important when it comes to HHP processing of food, as foodstuffs represent complex matrices composed of many different biochemically active substances and differ widely among each other. The fact that HHP inactivation efficiency differs greatly among food products and is lower in many food matrices compared to standardized buffer systems (Cheftel, 1995, Patterson *et al.*, 1995, Gervilla *et al.*, 2000, Patterson, 2005, Smiddy *et al.*, 2005, Black *et al.*, 2007) has been one of the major impediments of HHP technology on its way towards a more widespread application. Especially milk and dairy products have frequently been reported to exert a protective effect (Patterson *et al.*, 1995, Garcia-Graells *et al.*, 1999, Gervilla *et al.*, 2000, O'Reilly *et al.*, 2000, Narisawa *et al.*, 2008), whereas also the opposite has been reported sometimes (O'Reilly *et al.*, 2000). A summary of the effects of the major individual food constituents on HHP inactivation efficiency is given in the following sections.

Organic and inorganic low-molecular solutes

When ions or other low-molecular compounds are dissolved in water, they tightly bind water molecules in hydration shells due to electrostatic interactions, thus reducing the water activity (a_w) of the solution. a_w is defined as the ratio of vapor pressure over a sample to the vapor pressure over pure water at the same temperature and assumes values between 0 (total dryness) and 1 (humidity over pure water). With the exception of some halophiles, microorganisms generally require a_w values of 0.9 or higher for proliferation. Therefore, many traditional food preservation methods like drying or the addition of salt or sugar are based on a_w reduction. Despite its general growth-inhibitory effect, low a_w has been associated with a reduced efficacy of temperature- or pressure-induced microbial inactivation (Van Opstal *et al.*, 2003, Georget *et al.*, 2015). For example, mono- and disaccharides have been reported to protect various microorganisms from high-pressure inactivation (Simpson & Gilmour, 1997, Van Opstal *et al.*, 2003, Molina-Höppner *et al.*, 2004) and a baroprotective effect of NaCl was shown on *Lactococcus lactis* (Molina-Höppner *et al.*, 2004), *E. coli* and *Saccharomyces cerevisiae* (Oxen & Knorr, 1993).

Different mechanisms have been proposed to underlie the baroprotective effect observed with high osmolarity, but so far no generalization could be made, as the extent of baroprotection is highly dependent on solute type and concentration rather than the a_w value in general (Oxen & Knorr, 1993, Molina-Gutierrez *et al.*, 2002, Molina-Höppner *et al.*, 2004, Koseki & Yamamoto,

2007, Georget *et al.*, 2015). The baroprotective effect of different solutes relies on the response of the microorganism to elevated concentrations of the solute and interactions of the solute with biomolecules, and ionic and non-ionic solutes seem to have different mechanisms of protection (Molina-Höppner *et al.*, 2004).

Sugar molecules, for example, are readily accumulated in the cell when present in high extracellular concentrations, since they have no detrimental effects but rather stabilize the cell membrane and proteins during physical and chemical stresses (Leslie *et al.*, 1995, Crowe *et al.*, 2001, Molina-Höppner *et al.*, 2004). Sugars are accumulated by microorganisms as a response to physical stress, such as heat (Singer & Lindquist, 1998), and piezophilic deep-sea bacteria accumulate compatible solutes like glutamate, betaine, alanine and beta-hydroxybutyrate to withstand elevated pressures during growth (Martin *et al.*, 2002). Compatible solutes are compounds that can be accumulated to high concentrations as they do not interfere with the enzyme functionality and metabolic processes and, in addition, stabilize proteins (Roessler & Muller, 2001, Georget *et al.*, 2015).

In contrast, most ionic solutes, except kosmotropic ones like sulfate or phosphate, which exert protein- and membrane stabilizing effects (Zhang & Cremer, 2006, Georget *et al.*, 2015), cannot be accumulated to high concentrations in the cell due to detrimental effects on protein and membrane structure and function. At high extracellular salt concentrations, microorganisms therefore accumulate compatible solutes from the surroundings to cope with the osmotic stress (Glaasker *et al.*, 1996, Glaasker *et al.*, 1996, Glaasker *et al.*, 1998, Molina-Höppner *et al.*, 2004). The protective effect of NaCl and other salts on cell viability and cell membrane is thus believed to result from accumulated compatible solutes rather than directly from Na^+ or Cl^- ions.

In real food, the protective effect of high solute concentrations can be compensated by reduced post-pressure recovery from sublethal damage (Van Opstal *et al.*, 2003, Duranton *et al.*, 2012). This is probably the reason why the preservation of dry-cured ham works as efficiently as to allow the introduction of this product on the European market, despite the low a_w (Georget *et al.*, 2015).

pH value

Most vegetative bacteria including several foodborne pathogens, like salmonella, staphylococci, Shiga toxin-producing *E. coli*, Listeria and *Cronobacter sakazakii* show increased pressure sensitivity in acidic environments (Stewart *et al.*, 1997, Garcia-Graells *et al.*, 1998, Alpas *et al.*, 2000, Ritz *et al.*, 2000, Koseki & Yamamoto, 2006, Ritz *et al.*, 2008, Arroyo *et al.*, 2011, Li *et al.*, 2016). Although lactobacilli exhibit high acid tolerance in general, their inactivation is enhanced

at low pH as well (Molina-Gutierrez *et al.*, 2002). Hence, the pressure-induced dissociation equilibrium shift (1.1.3.3) can further enhance the lethal effect of HHP per se (Georget *et al.*, 2015).

In addition to direct inactivation, the recovery of sublethally injured cells is hampered in low-pH environment (Garcia-Graells *et al.*, 1998, Linton *et al.*, 1999, Jordan *et al.*, 2001, Koseki & Yamamoto, 2006). The reason for the increased pressure sensitivity in low-pH environments is currently unknown, but the HHP induced loss of membrane integrity and of functionality of membrane-bound enzymes involved in pH homeostasis is believed to cause an intracellular pH shift that impairs intracellular molecular structures (Cebrián *et al.*, 2016).

Proteins

Data describing the extrinsic effect of proteins alone on the survival of microorganisms under pressure are rare, but they are generally associated with a baroprotective effect on microorganisms. For example, bovine serum albumin (BSA), whey protein as well as casein, although only when bound in micelles in the combination with minerals, inhibited bacterial inactivation by HHP (Simpson & Gilmour, 1997, Black *et al.*, 2007, Narisawa *et al.*, 2008). The mechanism underlying is not yet clear, but there is evidence for a physical basis of this effect, since cells incorporated in protein aggregates showed reduced injury than cells in planktonic suspension (Narisawa *et al.*, 2008). However, due to the great heterogeneity in terms of size, mass, shape, flexibility, charge and surface characteristics, such as cell surface hydrophobicity, a general effect of proteins on microbial inactivation is difficult to identify.

Fat

Compared to other food matrix parameters, only little data exists concerning the effect of fat on HHP inactivation of vegetative bacteria in general with results being inconsistent and partially contradictory. Some studies reported a baroprotective effect of fat, with inactivation of *Listeria monocytogenes* being reduced by a mixture of 30 % (v/v) olive oil in phosphate-buffered saline (PBS) compared to pure PBS (Simpson & Gilmour, 1997) and by increased fat contents in dry-cured ham at pressures above 700 MPa (Bover-Cid *et al.*, 2015). However, the same fat contents enhanced HHP inactivation of *Listeria monocytogenes* at lower pressure levels (Bover-Cid *et al.*, 2015) and an effect of fat on inactivation of *Salmonella enterica* in the same model system could not be observed (Bover-Cid *et al.*, 2017). Moreover, no clear effect of fat was observed during HHP inactivation of *Salmonella thyphimurium* and *Listeria innocua* in a minced chicken model system (Escriu & Mor-Mur, 2009), of different bacterial species in milk (Gervilla *et al.*, 2000, Ramaswamy *et al.*, 2009) and of different pathogens in meat to which olive oil was

added before pressurization (Kruk *et al.*, 2014). Moreover, the fatty acid composition in the surrounding matrix has been shown to promote or inhibit recovery of injured cells during storage after HHP treatment, as meat products with high linoleic acid contents showed lower bacterial growth than those with high oleic acid contents and control samples (Rubio *et al.*, 2007).

As a consequence, the mode of action of fat during HHP inactivation is still unclear and there is controversy about the existence of a general effect of the matrix constituent fat on HHP inactivation. Some authors hypothesized that local hotspots of reduced a_w that emerge due to the low miscibility of fat with aqueous systems, for example at the fat-water-interface or the surface of oil droplets in oil-in-water emulsions may cause baroprotection (Georget *et al.*, 2015). It has also been hypothesized that fat protects vegetative cells due to physical pressure absorption (Gervilla *et al.*, 2000). Enhancement of inactivation by fat could be based on higher adiabatic heating during compression compared to aqueous systems leading to higher temperatures (Georget *et al.*, 2015), an increase in the concentration of fat-soluble, antimicrobial compounds, a transfer of triglycerides from the food matrix to the cell membrane that alters its permeability, and the formation of fat crystals (Gervilla *et al.*, 2000). Since the melting temperature of triglycerides increases by more than 10°C per 100 MPa, lipids of oils, which are in a liquid state at ambient pressure, may crystallize under high pressure (Cheftel, 1995). The combination of baroprotective and sensitizing effects might be the reason for the contradictory observations made in the past (Gervilla *et al.*, 2000) and emphasize the need for more systematic investigations in this context.

1.1.4.2.2 Temperature

The temperature prevailing during pressurization has a great impact on bacterial HHP resistance. In general, high temperatures representing a stress factor by themselves enhance HHP inactivation (Alpas *et al.*, 2000, Patterson, 2005). This synergistic effect of heat and pressure is exploited during inactivation of highly pressure-resistant bacterial endospores by PATS. As shown by many studies, also very low temperatures support microbial HHP inactivation (Arroyo *et al.*, 1997, Arroyo *et al.*, 1999, Casadei *et al.*, 2002, Ritz *et al.*, 2008). In general, bacteria show the highest resistance when pressure-treated close to or a few degrees below the growth temperature (Sonoike *et al.*, 1992, Cheftel, 1995). The increased HHP sensitivity of bacterial cells when treated at elevated or low temperatures may at least partly be based on the interplay of pressure and temperature concerning the membrane phase state (Cebrián *et al.*, 2016).

1.1.4.2.3 Intrinsic factors of microorganisms

Species- and strain-specific variation

There is great variation among different species of microorganisms in terms of HHP sensitivity, and even strains of the same species can differ widely, e.g. by more than 6 log cycles (Patterson *et al.*, 1995, Simpson & Gilmour, 1997, Benito *et al.*, 1999, Cebrián *et al.*, 2016). Generally, eukaryotes like yeasts and molds are the most pressure-sensitive microorganisms, with pressures of 100-200 MPa being generally sufficient for inactivation. Vegetative bacteria are more pressure-resistant, requiring pressures between 200 and 600 MPa at moderate temperatures for inactivation. In general, Gram-positive bacteria are more resistant than Gram-negative bacteria, possibly because they possess a more rigid cell envelope (Mañas & Pagán, 2005). In addition, smaller, round-shaped cocci generally show higher resistance compared to larger, rod-shaped bacteria, but many exceptions to these rules have been observed (Gervilla *et al.*, 2000, Yuste *et al.*, 2004, Balasubramaniam & Farkas, 2008, Huang *et al.*, 2014). The highest pressure-resistance has been observed with bacterial endospores, some of which are able to survive pressures above 1 GPa (Cheftel, 1995, Smelt, 1998, Huang *et al.*, 2014). Their inactivation often requires elevated temperatures in addition to pressure (Balasubramaniam & Farkas, 2008).

Growth phase

Cells in exponential phase are generally more sensitive than those in stationary phase (McClements *et al.*, 2001, Mañas & Mackey, 2004, Considine *et al.*, 2008). This may at least in part be due to differences in the composition and fluidity of the cytoplasmic membrane, as shown for *E. coli* and the conversion of unsaturated into cyclopropane fatty acids, which increases membrane stability while retaining proper fluidity (Casadei *et al.*, 2002).

Growth conditions

A microorganism's history highly influences its physiological state and thus its sensitivity to various stresses, including HHP (Li *et al.*, 2009, Zotta *et al.*, 2013, Cebrián *et al.*, 2016). Especially the cultivation temperature can significantly affect the survival rates during subsequent HHP treatments (Cebrián *et al.*, 2016). There is evidence that this is done at least partly through changes in membrane composition and fluidity (Ulmer *et al.*, 2002). As mentioned in 1.1.4.2.2, bacteria show the highest resistance when pressure-treated a few degrees below the growth temperature (Sonoike *et al.*, 1992). Likewise, the pH of the growth medium has an impact on HHP sensitivity. For example, *L. plantarum* cells grown at pH 5.0 are more pressure resistant than cells grown at pH 7.0 (Wouters *et al.*, 1998). However, data on the effect of growth

medium composition in general, and especially with respect to food-relevant substances, on HHP sensitivity are very rare (Cebrián *et al.*, 2016). In particular, the uptake of fat or fat-associated substances, such as fatty acids, before HHP treatment, has not been investigated regarding HHP sensitivity, so far.

Previous stress conditions

When exposed to adverse environmental conditions, such as osmotic or oxidative stress, acidity or nutrient deficiency, as for example during transition to the stationary phase, bacterial cells induce a stress response based on gene expression. This is done by sigma factors, σ^S (encoded by the *rpoS* gene) in Gram-negative and SigB (*sigB*) in Gram-positive bacteria, which are intracellular proteins that bind to RNA polymerase and confer specificity for promoter of stress response genes (Mañas & Pagán, 2005, Cebrián *et al.*, 2014). The presence of these sigma factors and the associated expression of stress response genes cause morphological and physiological changes and enable the adaptation to the adverse conditions. There is evidence that this stress response also increases resistance to HHP and accounts for the enhanced HHP resistance generally observed in stationary phase (Mañas & Pagán, 2005). The depletion of the *rpoS* and *sigB* genes significantly reduces HHP resistance (Cebrián *et al.*, 2016), further substantiating this hypothesis. Bacteria possess a specific response to heat stress modulated by specific sigma factors regulating the expression of proteases and chaperons. Sublethal HHP treatments have been shown to induce the expression of heat shock proteins, and a previously applied heat shock with the expression of specific heat shock proteins protected *E. coli* cells against HHP inactivation (Pagan & Mackey, 2000, Aertsen *et al.*, 2004).

Cell surface characteristics

The bacterial cell surface can assume a rather hydrophilic or hydrophobic character, which decides upon a cell's affinity towards different surfaces and thus its localization in heterogeneous matrices (An & Friedman, 1998, Ly *et al.*, 2006, Giaouris *et al.*, 2009, Krasowska & Sigler, 2014). Cell surface hydrophobicity (CSH) is determined by structures on the surface of the cell envelope such as the surface (S)-layer consisting of proteins (van der Mei *et al.*, 2003), and depends on the growth phase and state as well as environmental factors (Beck *et al.*, 1988, Liao *et al.*, 2015). During HHP treatment in complex matrices, CSH-mediated localization, e.g. at the surface of oil droplets dispersed in an otherwise aqueous matrix may have an influence on microbial inactivation. Possible results of enhanced fat contact may be either protection on a physical basis or due to the uptake of certain compounds from the fat matrix or increased

inactivation due to stronger adiabatic heating of fat during pressure-build-up as compared to water.

1.2 Model organisms lactobacilli

This section provides a general overview of the genus *Lactobacillus* and information about the characteristics of the *Lactobacillus* species chosen for this study as well as the role of lactobacilli in food spoilage, in general.

1.2.1 The genus *Lactobacillus*

The genus *Lactobacillus* belongs to the order of *Lactobacillales* or lactic acid bacteria (LAB), which are part of the class *Bacillus*, and represents a large, taxonomically heterogeneous group of more than 170 species and 17 subspecies that, in part, differ widely in their genomic and metabolic properties (Goldstein *et al.*, 2015). Lactobacilli are Gram-positive, catalase-negative, rod-shaped bacteria that do not form spores (Goldstein *et al.*, 2015, Papadimitriou *et al.*, 2016). Being facultatively anaerobic, they do not rely on, but tolerate oxygen, although most lactobacilli grow better under microaerophilic conditions (Goldstein *et al.*, 2015). In general, lactobacilli are aciduric or even acidophilic, growing at pH values between 3 and 8 with pH optima between 5.5 and 6.2, whereas growth temperatures ranging from 2 to 53 °C are reported, depending on species and strain (Salvetti *et al.*, 2012). Lactobacilli grow on many growth media, including MRS (deMan, Rogosa, Sharpe) (De Man *et al.*, 1960). Their metabolism is characterized by the fermentation of sugars to lactic acid as the main product and they are currently categorized into three groups based on carbohydrate utilization and the resulting fermentation products (Hammes & Vogel, 1995, Hammes & Hertel, 2009), although the current taxonomic classification based on 16S rRNA analysis differs from this (Salvetti *et al.*, 2012): Obligate homofermentative lactobacilli mainly produce lactic acid from hexoses via the Embden-Meyerhof-Parnas pathway (EMP) or glycolysis, whereas facultative heterofermentatives use hexoses to produce lactic acid via the EMP and in case of low hexose levels can ferment pentoses and gluconate via an inducible phosphoketolase of the pentose phosphate (PP) pathway, resulting in acetic acid, ethanol and formic acid as end products (Salvetti *et al.*, 2012). Obligate heterofermentatives, in turn, produce lactic acid, ethanol or acetic acid and CO_2 from pentoses and hexoses solely via the so-called phosphogluconate pathway, which is the first part of the PP pathway (Hammes & Hertel, 2009, Salvetti *et al.*, 2012). Many *Lactobacillus* species are part of the human mouth, gastrointestinal or vaginal microbiota and generally exert beneficial effects on human health: First, they assist nutrition by metabolizing compounds of the human diet to low-molecular-weight metabolites, amino acids, and vitamins, which can be easily absorbed by the gut epithelial cells

(Liévin-Le Moal & Servin, 2014). Some *Lactobacillus* strains produce antioxidants, and the fatty acid metabolism of lactobacilli has been associated with beneficial effects on human health (Goldstein *et al.*, 2015). Second, they inhibit the growth of harmful microorganisms by competing for space and nutrients or by secreting bacteriocins or other antimicrobial compounds (Ljungh & Wadstrom, 2006, Liévin-Le Moal & Servin, 2014). Furthermore, they are able to modulate the human immune system by enhancing immune responses against pathogenic microorganisms or exerting anti-inflammatory effects (Ljungh & Wadstrom, 2006, Liévin-Le Moal & Servin, 2014). Due to their health-promoting properties, lactobacilli are advertised and sold as probiotics in food, such as yogurt, or in the form of special preparations (Turroni *et al.*, 2014). Lactobacilli are so-called *generally regarded as safe* (GRAS) microorganisms and only few cases of serious human infections, often associated with comorbidity, have been reported so far (Goldstein *et al.*, 2015).

Due to their ability of lactic acid fermentation, lactobacilli have been intensely used for food processing and preservation since ancient times – albeit unintentionally – and are now attracting more and more attention by the food industry, as advances in microbiological and genomic analyses allow for their targeted use to obtain desired organoleptic and probiotic product characteristics. *Lactobacillus* strains are especially used for the fermentation of milk to cheese, yogurt and other dairy products, and as starter cultures for raw meat fermentations (Buckenhuskes, 1993). Furthermore, they are part of sourdough communities and play a major role in the fermentation of vegetable food, such as olives or cabbage, e.g. to make sauerkraut or kimchi, and animal feed such as silage. Their role in food processing and preservation is mostly based on the production of lactic acid and the ability to grow and survive in acidic environment, where other, food-spoiling and pathogenic, microorganisms are unable to proliferate. Their acid tolerance enables considerable survival in low-pH gastric environments and the secretion of bacteriocins or related substances inhibiting the growth of competing spoilage-associated microorganisms (Liévin-Le Moal & Servin, 2014).

1.2.2 The role of lactobacilli in food spoilage

Apart from their beneficial effects on human health and their utilization for food processing, lactobacilli are associated with spoilage of various food products (Fernández Ramírez *et al.*, 2015). Although they pose no major risk for consumers' health and food safety, they constitute a challenge for the food industry as especially meat and fish products as well as sauces like ketchup, mayonnaise and salad dressings are prone to *Lactobacillus* spoilage (Smittle, 1977). Spoilage by lactobacilli is often characterized by acidification, the formation of sour off-flavors

and off-odors, discoloration and in some cases gas and slime production (Borch *et al.*, 1996, Vermeulen *et al.*, 2007). In this study, three spoilage-relevant *Lactobacillus* spp. are considered, namely facultatively heterofermentative *L. plantarum* and *L. sakei* and obligate heterofermentative *L. fructivorans* (Dicks & Endo, 2016). Their role in food spoilage is illustrated hereinafter.

1.2.2.1 *Lactobacillus fructivorans*

L. fructivorans is a generally slow-growing, obligate heterofermentative *Lactobacillus* species (Splittstoesser, 1982). The species was described for the first time in 1934 after isolation from spoiled salad dressing (Charlton) and was found repeatedly in spoiled mayonnaise and dressings (Kurtzman *et al.*, 1971, Smittle & Flowers, 1982) and tomato ketchup with a low pH of 3.8, causing further acidification, gas formation and off-odor (Bjorkroth & Korkeala, 1997). *L. fructivorans* is characterized by a relatively high acid and ethanol tolerance, with some strains isolated from sake growing in the presence of up to 20 – 21 % (v/v) ethanol and a considerably high heat resistance (Splittstoesser *et al.*, 1975). Growth of *L. fructivorans* has also been observed in salad dressing at pH values as low as 3.5 (Meyer *et al.*, 1989). This explains why *L. fructivorans* was found in spoiled vinegar preserves (Dakin & Radwell, 1971) and alcoholic beverages such as wine (Splittstoesser, 1982), premixed cocktails (Splittstoesser *et al.*, 1975) and sake (Suzuki *et al.*, 2008). *L. fructivorans* is comparably fastidious, growing only under certain conditions (Sanders *et al.*, 2015).

1.2.2.2 *Lactobacillus sakei*

Being a psychrotolerant species *L. sakei* grows well at low temperatures and is therefore primarily associated with the spoilage of meat and seafood products, which are usually stored at these conditions. Strains of *L. sakei* were found on various marinated (Schirmer *et al.*, 2009) and cooked pork products packed under modified atmosphere (Vasilopoulos *et al.*, 2008) or vacuum, such as ham (Kalschne *et al.*, 2015), bacon, emulsion-based sausages (Samelis *et al.*, 2000) and other specialties (Chenoll *et al.*, 2007, Laursen *et al.*, 2009). Furthermore, *L. sakei* was isolated from fresh beef vacuum and modified atmosphere-packed beef (Jääskeläinen *et al.*, 2016), cooked (Samelis *et al.*, 2000, Chenoll *et al.*, 2007, Geeraerts *et al.*, 2018) and marinated poultry products (Björkroth *et al.*, 2000, Björkroth *et al.*, 2005) as well as specialties like pasteurized French-style goose liver called *foie gras* (Matamoros *et al.*, 2010). It has also been associated with the spoilage of seafood including shrimps (Mejlholm *et al.*, 2008) and was shown to grow well on a variety of processed meat and seafood products at low temperatures (Mejlholm & Dalgaard, 2013).

1.2.2.3 *Lactobacillus plantarum*

Strains of the species *L. plantarum* show great genetic diversity and thus populate a variety of habitats, leading to a broad spectrum of food products prone to *L. plantarum* spoilage. In general, *L. plantarum* is less cold tolerant than *L. sakei*, but some strains were associated with spoilage of cold-stored products, such as *foie gras* (Matamoros *et al.*, 2010), vacuum-packed cooked pork products (Chenoll *et al.*, 2007) and marinated herring (Lyhs *et al.*, 2001). Like *L. fructivorans*, *L. plantarum* was isolated from salad dressing, mayonnaise, ketchup and related products (Kurtzman *et al.*, 1971, Smittle, 1977, Fernández Ramírez *et al.*, 2015, Sanders *et al.*, 2015), but also from marinades intended for the seasoning of meat (Lundstrom & Bjorkroth, 2007). Naturally occurring on plants, *L. plantarum* is frequently part of the microbiota during fermentation of beverages, but can also lead to the spoilage of fruit juices (Tajchakavit *et al.*, 1998, Bevilacqua *et al.*, 2010, Trinetta *et al.*, 2017) and of fermented apple (cider) and pear (perry) juice (Splittstoesser, 1982), must and (fortified) wine (Stratiotis & Dicks, 2002) due to malolactic fermentation and the resulting production of biogenic amines (Coton *et al.*, 2010, Sanders *et al.*, 2015).

1.3 Examination system: Emulsions

On the one hand, many foodstuffs potentially suitable for HHP processing are based on emulsions. Milk and many dairy products for example are natural emulsion systems, whereas mayonnaise and related sauces, dips and dressings are prepared by mixing oil with water and adding an emulsifying agent such as egg yolk for stabilization. On the other hand, during the preparation of emulsions individual parameters like fat content, oil droplet size and oil-water interface as well as the concentration of additional components, such as solutes in the aqueous phase, can be varied systematically. This makes emulsions a suitable system for the investigation of the interaction of fat and other food components and their combined effect on HHP inactivation of spoiling microorganisms. The following sections illustrate the properties of emulsion-based foods with special focus on microbial spoilage, the physical basics underlying emulsions, their stability and preparation methods.

1.3.1 Emulsion-based food

Emulsions are the basis for a variety of foodstuffs in which large proportions of fat are stably mixed with an aqueous component. These products include especially sauces like mayonnaise and similar bases as well as dips, dressings, spreads and ready-made salads prepared thereof. During the preparation of such fresh, un-cooked ready-to-eat products, the risk of introduction of exogenous spoilage microorganisms by the addition of e.g. spices or from containers, cutlery or

staff is very high (Georget *et al.*, 2015). The shelf-life of such products is usually extended by lowering the pH through the addition of organic acids, such as citric or acetic acid or salts of sorbic or benzoic acid (Vermeulen *et al.*, 2007, Panagou *et al.*, 2013). The concentrations of undissociated acids, NaCl, hexoses and disaccharides required to render emulsion-based products shelf-stable at ambient temperature can be calculated using the so-called CIMSCEE code, a formula established by the Committee of the Industries of Mayonnaises and Table Sauces of the European Economic Community (fr.: *Comité des Industries des Mayonnaises et Sauces Condimentaires de Communauté Economique Européenne*), which is widely used by food producers (Vermeulen *et al.*, 2007, Manios *et al.*, 2014). Although these conditions inhibit the growth of most spoilage and almost all pathogenic microorganisms (Smittle, 1977, Smittle, 2000), some lactobacilli and acid-tolerant molds and yeasts are able to grow even in such harsh environments (see 1.2.2). In addition, a tart and acidic taste is considered undesirable by many consumers, and the consumer acceptance of additional preservatives like non-acid antimicrobial substances such as nisin or gluconic acid is constantly decreasing. HHP has the potential to achieve adequate post-package reduction of the microbial load in complex, non-acidic food products without the need for artificial additives, while preserving quality attributes.

1.3.2 Physical and chemical principles of emulsions

Emulsions are mixtures of two naturally immiscible liquids, usually a hydrophilic and a lipophilic one, as for example water and oil. One of the two liquids forms the dispersed phase and is distributed as small droplets within the other liquid, the continuous phase. Emulsions in which oil is dispersed in water are called oil-in-water (O/W) emulsion, whereas in water-in-oil (W/O) emulsions, the aqueous phase is dispersed in a continuous lipid phase. While molecular interactions in aqueous systems are mainly based on hydrogen bonds and electrostatic interactions (in case of dissolved ions), lipophilic (organic) fluids mainly exhibit van der Waals forces. The lack of common molecular interaction forces means thermodynamic instability and causes high interfacial tensions between aqueous and organic liquids. Thus, the two liquids tend to separate in order to reduce their common contact area. The resulting phase separation manifests in coagulation of the droplets of the dispersed phase and finally results in the phase with lower density, in most cases the organic one, floating on top of the denser, mostly aqueous, phase.

1.3.3 Emulsifiers

Phase separation in emulsions is prevented by the addition of stabilizing agents known as emulsifiers. Many emulsifiers are surface-active agents, also called surfactants which act

through the reduction of the interfacial tension between the two phases. Alternative mechanisms to stabilize emulsions include the thickening of the continuous phase by the addition of hydrocolloids such as starches or gums, thus reducing the motion of dispersed droplets and the probability of their coagulation (Hasenhuettl & Hartel, 2008). Surfactants are amphiphilic compounds with both hydrophilic and hydrophobic parts. Hence, they are able to interact with the aqueous as well as the lipid phase and accumulate at the oil-water interface, reducing the interfacial tension and increasing the thermodynamic stability of the system. Therefore, they both facilitate the break-up of dispersed-phase droplets into smaller ones during emulsion preparation and impede their coagulation afterwards, also due to the surfactant layer formed on the droplet surface (Walstra, 1993, Degner *et al.*, 2014). Due to the wide range in hydro- and lipophilicity of amino acid side chains, proteins can serve as surfactants by folding and positioning at the oil-water interface in a way that lipophilic amino acid residues protrude into the lipid phase, whereas other, more hydrophilic parts of the molecule are situated in the aqueous phase (Hasenhuettl & Hartel, 2008). In addition to lowering the interfacial tension, adsorbed proteins increase the steric and electrostatic repulsion between dispersed fat droplets (Walstra, 1993, Degner *et al.*, 2014). Another widely used type of emulsifiers are small-molecule surfactants characterized by a hydrophilic head group and a lipophilic tail region, which is often larger in size. They are classified according to their hydrophilic region with non-ionic surfactants possessing uncharged hydrophilic parts, often comprising many hydroxyl (OH-) residues, and ionic surfactants being positively (cationic) or negatively (anionic) charged. Zwitterionic, also called amphoteric surfactants, in contrast, have both positive and negative charges. The electric charge of the hydrophilic part of ionic emulsifiers strongly enhances their hydrophilic character and their solubility in aqueous environments. Charged emulsifier molecules sitting on the surface of oil droplets further stabilize emulsions through electrostatic repulsion of the oil droplets from each other. The hydrophobic tail region is usually composed of one or two fatty acid residues that can vary in chain length and the degree of saturation (Degner *et al.*, 2014). Many foods naturally contain surfactants that are responsible for the products' physical characteristics and can be exploited as emulsifiers by the food industry. Examples are proteins originating from milk and dairy products like casein and whey proteins as well as lipoproteins and phospholipids (e.g. lecithin) from egg yolk or soy beans (Hasenhuettl & Hartel, 2008, Degner *et al.*, 2014). Interestingly, also microorganisms were demonstrated recently to function as emulsifiers for the formation of so-called Pickering O/W emulsions, which are characterized by solid particles as emulsifiers, due to proteins, polypeptides and polysaccharides on their surface ensuring adhesion to and coverage of the oil droplet surface (Firoozmand & Rousseau, 2016). In addition,

there exist a variety of synthetic surfactants including mono- and diglycerides as well as derivatives thereof, glycol and glycerol esters of fatty acids, and sorbitan and polyoxyethylene derivatives like polysorbates. The latter comprise the emulsifier used in this study, Tween® 80. Polysorbates are non-ionic surfactants consisting of a large polyoxyethylated sorbitan group as the polar head coupled to one fatty acid moiety (Hasenhuettl & Hartel, 2008). The final structure of polysorbates is achieved by a polymerization chain reaction in which ethylene oxide (oxirane) molecules are repeatedly added to the sorbitan group of a sorbitan fatty acid ester. The degree of ethoxylation, i.e. the number of coupled ethoxylate moieties, is highly variable due to the synthesis process, making polysorbates a heterogeneous mixture of molecules with different molecular weight, but similar emulsifying properties (Hasenhuettl & Hartel, 2008). Depending on the fatty acid species, which can vary between lauric acid, palmitic acid, stearic acid or oleic acid, different polysorbates exist, referred to as polysorbate 20, 40, 60 (or 65) and 80, or more commonly, as their trade names Tween 20, Tween 40, Tween 60 (65) and Tween 80, respectively. Tween 80 (EU food additive number E 433), with oleic acid as the lipophilic part, is widely used by the food, cosmetics and pharmaceutical industries, for example for the stabilization of ice cream and whipped toppings, chewing gum and bakery goods (Hasenhuettl & Hartel, 2008).

Besides its function as an emulsifier in food products, Tween 80 is widely used in microbiology, where it serves as an additive for microbial growth media (De Man *et al.*, 1960) that provides microbial cells with exogenous oleic acid, enhancing growth of many types of microorganisms (Johnsson *et al.*, 1995, Partanen *et al.*, 2001, Leccese Terraf *et al.*, 2012, Tan *et al.*, 2012) and protecting them against adverse environmental conditions, such as low pH (Corcoran *et al.*, 2007, Broadbent *et al.*, 2014), bile salts (Li *et al.*, 2011), freeze drying (Hansen *et al.*, 2015) and nutrient depletion (Al-Naseri *et al.*, 2013).

1.3.4 Stability of emulsions

Despite the presence of emulsifying agents, which, in many cases only delay their destabilization, emulsions undergo destruction over time. There are two principle mechanisms that lead to the irreversible destruction of emulsions (Bibette *et al.*, 2002): The first one, called Ostwald ripening, describes the transformation of the dispersed phase into bigger entities that are smaller in number due to the diffusion of molecules of the dispersed phase through the continuous phase. Since the surface area-to-volume ratio decreases with droplet size, the diffusion from small droplets to larger ones is preferred to the reverse process. Thus, bigger droplets become bigger, whereas smaller droplets are reduced in size over time until they

disappear completely. Through coalescence, in contrast, droplets of the dispersed phase come into direct contact tight enough to rupture the film of continuous-phase molecules between them, thus allowing their direct fusion. The bigger the dispersed droplets are, the weaker is the curvature of their surface and the larger the surface area that can get into contact with other droplets. Therefore, coalescence is favored by large droplet sizes and eventually leads to complete phase separation. There are other, reversible mechanisms that change the properties of an emulsion over time. Creaming or sedimentation are driven by gravitation and the differences in density between water and fat. If fat droplets dispersed in an aqueous continuous phase are not tightly packed but have enough space to move freely around each other, their lower density compared to water pushes them upwards and leads to their accumulation beneath the surface of the aqueous phase. Vice versa, sedimentation describes the accumulation of the denser dispersed phase particles at the bottom of the continuous phase, for example water droplets dispersed in oil. Creaming and sedimentation can be reversed by agitation of the emulsion (McClements, 2004). Depending on the molecular interaction forces on their surfaces, dispersed phase particles undergo aggregation to bigger particles, thus increasing the rate of creaming or sedimentation. Unlike coalescence, aggregation, also referred to as flocculation, does not lead to fusion of the particles and, therefore, is reversible (McClements, 2004). The extent and type of destabilization of emulsions depends on intrinsic factors like the ratio of dispersed to continuous phase, droplet size distribution and the type and concentration of the emulsifier, as well as extrinsic factors, such as temperature and mechanical stress.

1.3.5 Preparation of emulsions

The preparation of stable emulsions requires a fine distribution of the dispersed phase within the continuous phase. In principle, emulsions can be prepared in two ways. The first one is based on the generation of turbulent flows with a combination of elongational and shearing forces and is used e.g. by techniques using high-pressure homogenizers or colloid mills (Bibette *et al.*, 2002). The second way is the generation of laminar flows and gentle stirring, where, for example, temperature changes are used to induce phase transitions that result in the formation of small droplets (Bibette *et al.*, 2002). A two-step process using high-pressure homogenization is a method widely used in the food industry. In the first step, the mixture of lipid phase, aqueous phase and emulsifier is pre-homogenized by high-shear mixing to form a coarse, polydisperse pre-emulsion (Bibette *et al.*, 2002, Degner *et al.*, 2014). During the following high-pressure homogenization, the pre-emulsion is set under pressure and forced to pass through a small nozzle. The pressure drop and high speed create large shear forces and cavitation that rupture

the fat droplets into smaller ones (Bibette *et al.*, 2002). Homogenization parameters like stirring speed, pressure and temperature of the preparation are adjusted according to the desired emulsion characteristics, i.e. small or large droplets, the nature of the emulsifier and the composition of the lipid and the aqueous phase, as aqueous phase parameters like salt and sugar content, pH and the presence and concentration of biopolymers like proteins and starches can significantly influence emulsification behavior and stability (Hasenhuettl & Hartel, 2008, Degner *et al.*, 2014).

1.3.6 Characterization of emulsions

Besides several chemical characteristics like the nature and concentration of the emulsifier and the composition of the dispersed and the continuous phase, emulsions can be characterized by a variety of physical parameters. One of these is the droplet concentration usually described as the dispersed-phase volume fraction and defined as the volume of the dispersed-phase droplets divided by the total volume of the emulsion. In case of considerably thick interfacial layers, e.g. with protein-based emulsifiers, the effective volume fraction of the coated droplets can be significantly larger than that of the actual volume fraction of the dispersed phase. The dispersed-phase mass fraction is the equivalent to the volume fraction and based on the mass ratio between the dispersed and the continuous phase and equals the volume fraction if the two phases possess equal density. One of the most important emulsion characteristics is the size of the droplets. An emulsion is called monodisperse if all droplets have the same size. Real food emulsions, however, are typically polydisperse, possessing a range of particle sizes, also referred to as particle size distribution, rather than a single one. Among the most widely used methods to determine an emulsion's particle size (distribution) is based on light scattering/laser diffraction.

1.4 Motivation, project aim and working hypotheses

1.4.1 Motivation

Since its introduction in the 1990s, HHP processing has witnessed increasing utilization by the food industry due to several advantages over conventional food preservation techniques (see 1.1). Especially complex, heat-sensitive products, such as ready-to-eat salads seasoned with mayonnaise or related sauces, offer great potential for HHP technology. However, microbial inactivation by HHP is often less efficient in foodstuffs than standardized buffer solutions, and the complexity of food matrices makes it difficult to predict the outcome of HHP processing.

Therefore, the efficiency of HHP inactivation has to be assessed case-by-case for every single product.

To allow for a more widespread use of HHP technology more systematic data are required, facilitating the elaboration of product-specific treatment conditions that ensure product safety and adequate shelf life on the one hand, and prevent detrimental effects on product quality and excessive operating costs due to over-processing on the other hand. Moreover, generally accepted values regarding the effect of individual food matrix constituents on the efficiency of HHP inactivation written down in national and international standards could provide producers and legal authorities with reliable criteria and facilitate the market launch of novel HHP-processed products. Besides the acquisition of systematic data on the effect of the individual food components on HHP inactivation efficiency, it is necessary to evaluate the potential interplay between different matrix constituents, since food matrices always represent mixtures of different ingredients with potentially synergistic or antagonistic effects. Most food matrices are aqueous environments in which fat is more or less homogeneously distributed, but on the microscopic and molecular level always remains separate from the aqueous phase, e.g. as oil droplets dispersed in the continuous aqueous phase of an O/W emulsion. Thus, possible effects of fat and aqueous phase parameters of the food matrix on HHP inactivation should be independent of each other. However, systematic data in terms of the impact of fat on the action of other food matrix parameters like salt, sugar and protein content as well as the pH in the form of systematic data is still missing.

The cell envelope appears to play a role in HHP inactivation and bacterial localization in O/W emulsions has been hypothesized to influence inactivation. Cells with hydrophobic surfaces are ascribed with higher affinity for the lipid phase than hydrophilic ones, which might enhance their susceptibility to fat-mediated effects on HHP inactivation. Due to coating of the oil droplets in emulsifier-stabilized O/W emulsions with emulsifier molecules resulting in a loss of the net hydrophobic character, differences in HHP inactivation based on CSH-dependent localization can be excluded. Therefore, changes in HHP inactivation due to variations of the matrix should be independent of the CSH, unless other intrinsic features related with CSH contribute to HHP sensitivity.

In addition to the surrounding matrix, the efficiency of microbial inactivation depends on the intrinsic pressure resistance of the treated microorganisms, which in turn is the result of an interplay of the genetic background and the physiological state. The latter depends on the growth phase and environmental conditions like growth temperature, nutrient availability and possible stress conditions experienced previously. Spoilage microorganisms come into contact

with the food matrix during preparation at the latest, which may be long enough before HHP treatment to allow for an adaptation of their physiological state. So far, it is not known, whether the presence of fat exerts such a preconditioning effect, nor whether a possible cellular response to fat contact can change HHP resistance. A better understanding of the cellular response to fat contact can be helpful for the assessment of potential risks, the establishment of avoidance strategies during food preparation, processing and storage and the identification of appropriate processing conditions. Last but not least the investigation of a cellular response to fat facilitate the understanding of the mechanisms underlying both microbial HHP inactivation and the development of HHP resistance.

1.4.2 Aim of the project

The main objective of this study was to expand the knowledge about the effect of fat and fat-associated substances on the HHP inactivation efficiency of vegetative spoilage bacteria. The study consists of two major parts. In the first part, the impact of fat on the action of different food matrix parameters related to the aqueous phase was evaluated. To test for a potential interrelation defined emulsifier-stabilized O/W emulsions with rapeseed oil as the lipid phase were used as a model system. The NaCl and sucrose contents, the pH value and the type and concentration of proteins were varied in a food-relevant range at a constant droplet size and a fat content of 50 % (v/v) rapeseed oil corresponding to the amount of fat frequently found in emulsion-based food products. In the second part, the effect of contact to fat and fat-associated compounds on the physiological state and HHP sensitivity was investigated, and a potential connection between both features was assessed.

1.4.3 Working hypotheses

On the basis of the knowledge background and objectives outlined above, the following working hypotheses were established, providing the framework for the experimental work of the two parts of this study.

1.4.3.1 Mutual influence of fat and aqueous-phase parameters during HHP inactivation of bacteria in O/W emulsions

- The presence of fat does not change the effect of the aqueous phase parameters (I) NaCl content, (II) sucrose content, (III) pH value and (IV) protein type and content on HHP inactivation, as long as the water content in the aqueous phase remains constant.
- The response of treated bacteria to changes in the surrounding matrix in O/W emulsions regarding HHP sensitivity is independent of CSH.

1.4.3.2 Effect of preconditioning fat and fat-associated compounds on HHP sensitivity

- Prolonged residence of bacteria in O/W emulsion prior to HHP treatment changes HHP sensitivity, and these changes are independent of CSH, but their intensity depends on the temperature during the contact time.
- The presence of rapeseed oil (without emulsifier) during cultivation changes HHP sensitivity of hydrophobic but not hydrophilic bacteria due to favored contact to fat.
- Oleic acid is the most abundant fatty acid in rapeseed oil and constitutes the hydrophobic part of emulsifier Tween 80. Therefore, preconditioning by cultivation in the presence of Tween 80 or free oleic acid causes the same changes in HHP sensitivity as observed with rapeseed oil.
- Preconditioning by cultivation in the presence of rapeseed oil or associated compounds like Tween 80 provokes a cellular response that accounts for changes in HHP sensitivity. The cellular response to Tween 80 comprises an adaptation of the fatty acid composition of the cell membrane according to exogenous fatty acids and manifests in changes in the transcriptomic profile. The transcriptomic response to the presence of Tween 80, which is widely used in cultivation of lactobacilli, provides general insight in its growth promoting effect beyond preconditioning towards HHP tolerance.

2 Materials and Methods

2.1 Materials

2.1.1 Bacterial strains

Table 1: Bacterial strains used in this study. All strains used are part of the Technische Mikrobiologie Weihenstephan (TMW) strain collection.

Species	TMW No.	Alternative denotation	Origin
B. subtilis	2.742	DSM 10^T	Unknown
L. fructivorans	1.59	DSM 20203^T	Sourdough
L. fructivorans	1.452	LTH 669, Gent LAB 681	Unknown
L. fructivorans	1.1856	L1	Whey
L. sakei	1.13	-	Starter culture for cured meat
L. sakei	1.23	LTH 673	Raw sausage
L. sakei	1.151	CTC 431	Unknown
L. sakei	1.161	LTH 1651	Unknown
L. sakei	1.165	7L0201.35/ II-2	Sauerkraut
L. sakei	1.412	LTH 1768, Gent LAB 162	Unknown
L. sakei	1.704	Sg2	Sourdough
L. sakei	1.1189	DSM 20017^T, ATCC 15521	Moto (starter of sake)
L. sakei	1.1239	Lp46	Sourdough, ENITIAA Laboratoire de Microbiologie Alimentaire et Industrielle, Nantes, France
L. sakei	1.1322	INRA 23K	Unknown, Institut National de la Recherche Agronomique, France
L. sakei	1.1393	BB 3059	Starter organism for salami
L. sakei	1.1396	LTH 2389	Starter organism for salami
L. sakei	1.1399	LTH 1183	Starter organism for salami
L. sakei	1.1407	HEIDI1	Fermented freshwater fish (Norway)
L. sakei	1.1452	#11	Brenta cheese (Cavalese, Italy)
L. sakei	1.1474	LTH 2076	Unknown
L. sakei	1.1954	Nr. 51	Spoiled sausage
L. plantarum	1.1	CTC 305	Raw sausage (Hugas *et al.*, 1993)
L. plantarum	1.9	DSM 20174, LTH 478	Pickled cabbage
L. plantarum	1.25	LTH 2354	Portuguese raw sausage,
L. plantarum	1.38	LTH 232	Starter culture
L. plantarum	1.64	DSM 20205	Sourdough
L. plantarum	1.246	-	Blackcurrant drink
L. plantarum	1.277	-	Palm wine
L. plantarum	1.284	-	Unknown

Species	TMW No.	Alternative denotation	Origin
L. plantarum	1.409	LTH 1870, Gent LAB 159	Sauerkraut
L. plantarum	1.708	CTC 51	Raw sausage, Instituto de Investigación y Tecnología Agroalimentarias (IRTA), Monells, Spain)
L. plantarum	1.834	ULICE 24-4-147	Unknown, Unité de Laboratoire sur l'Innovation des Céréales (ULICE), Riom, France
L. plantarum	1.1204	-	Unknown
L. plantarum	1.1308	-	Unknown
L. plantarum	1.1478	WALA 01030879	Belladonna honey
L. plantarum	1.1594	-	Breast milk
L. plantarum	1.1623	-	Breast milk
L. plantarum	1.1728	-	Rice sourdough
L. plantarum	1.1732	-	Fermented Food (Syria)
L. plantarum	1.1789	-	Human feces (Syria)
L. plantarum	1.1792	-	Human feces (Syria)
L. plantarum	1.1808	-	Unknown
L. plantarum	1.1810	-	Unknown
L. plantarum	1.2089	S16	Unknown

2.1.2 Instruments

Table 2: Instruments used in this study.

Instrument	Model	Manufacturer
100x Microscopy objective lens	N-Achroplan 100x/1.25 Oil Iris	Carl Zeiss Microscopy GmbH, Munich, Germany
Absorbance microplate photometer	Sunrise™	Tecan Group Ltd., Männedorf, Switzerland
Absorbance microplate photometer	SpectraFluor	Tecan Group Ltd., Männedorf, Switzerland
Analytical balance	SI-234	Denver Instrument, Bohemia, NY, USA
Analytical centrifuge	LUMiFuge®	LUM GmbH, Berlin, Germany
Cascade Console Freeze Dry System	FreeZone Plus 2.5 Liter	Labonco Corporation, Kansas City, MO, USA
Colony counter	BZG 30	WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany
Colony counting imaging station	ColonyDoc-It™	Ultra-Violet Products Ltd, Upland, California, USA
Disperser-homogenizer	Miccra® D15	Miccra GmbH, Müllheim, Germany
Disperser-homogenizer	T25 digital ULTRA TURRAX®	IKA®-Werke GmbH & Co. KG, Staufen, Germany
Dual vessel high-pressure unit	TMW-RB	Knam Schneidetechnik GmbH, Langenargen, Germany
Electronically controlled manual dispenser	Multipette® stream	Eppendorf AG, Hamburg, Germany
Fluorescence microplate photometer	FLUOStar Omega	BMG Labtech GmbH, Ortenberg, Germany
MALDI-TOF mass spectrometry unit	Microflex LT	Bruker Daltonics, Bremen, Germany
Particle size analyzer	Mastersizer 2000	Malvern Instruments GmbH, Herrenberg, Germany
Phase contrast microscope	Axiostar plus	Carl Zeiss Microimaging GmbH, Jena, Germany
Refractometer	-	Carl Zeiss Microscopy GmbH, Munich, Germany
RGB camera for microscopy	AxioCam ICc1	Carl Zeiss Microimaging GmbH, Jena, Germany
Spectrophotometer	Novaspec II	Pharmacia Biotech, Uppsala, Sweden
Spectrophotometer	Novaspec Plus	GE Healthcare, Buckinghamshire, United Kingdom
Thermostating circulator for high pressure unit	FC 600	JULABO Labortechnik GmbH, Seelbach, Germany
Two-stage pressure homogeniser	APV Model 1000	APV Systems, SPX Flow Technology Rosista GmbH, Unna, Germany
Vacuum pump for freeze dry system	Type 302101	Ilmvac GmbH, Ilmenau, Germany

2.1.3 Software

Table 3: Software used in this study.

Program	Producer
AxioVS40 V 4.8.2.0	Carl Zeiss Microscopy GmbH, Munich, Germany
flexAnalysis 3.4	Bruker Daltonik GmbH, Bremen, Germany
flexControl 3.4	Bruker Daltonik GmbH, Bremen, Germany
ImageJ	National Institute of Health, Bethesda, MD, USA
MALDI Biotyper 3.0	Bruker Daltonik GmbH, Bremen, Germany
Maldi Biotyper Automation Control 2.2	Bruker Daltonik GmbH, Bremen, Germany
MARS Data Analysis Software	BMG LABTECH GmbH, Ortenberg, Germany
Mastersizer 2000 v5.60	Malvern Instruments GmbH, Herrenberg, Germany
Rockhopper	Brian Tjaden, Wesseley Colloge, MA, USA (McClure *et al.*, 2013, Tjaden, 2015)
SEPView 6.0	LUM GmbH, Berlin, Germany
SigmaPlot 12.5	Systat Software Inc., San Jose, CA, USA

2.1.4 Chemicals

Table 4: Chemicals used in this study.

Compound	Specification	Supplier
Acetonitrile	≥ 99.9 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Agar-agar	Bioscience-grade, granulated	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Antifoam B Emulsion	-	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
L-Aspartic acid	Ph Eur	Merck KGaA, Darmstadt, Germany
Casein	≥ 95 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Citric acid	≥ 99.5 %, p.a., ACS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L-Cysteine-HCl monohydrate	≥ 98.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol absolute	≥ 99.8 %	VWR Chemicals, Darmstadt, Germany
D(-)-Fructose	GR grade	GERBU Biotechnik GmbH, Heidelberg, Germany
D(+)-Glucose (monohydrate)	for microbiology	Merck KGaA, Darmstadt, Germany
HCCA/α-cyano-4-hydroxy-cinnamic acid	-	Bruker Daltonik, Bremen, Germany
Imidazole	puriss. p.a., ≥ 99.5 % (GC)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
KH_2PO_4	≥99 %, p.a., ACS	Merck KGaA, Darmstadt, Germany
K_2HPO_4 * 3 H_2O	for analysis	Merck KGaA, Darmstadt, Germany
Lauric acid	for synthesis	Merck KGaA, Darmstadt, Germany
Linoleic acid	≥99 %	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

Compound	Specification	Supplier
Linolenic acid	≥99 %	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Meat extract	for microbiology	Merck KGaA, Darmstadt, Germany
$MgSO_4 * 7H_2O$	ACS, Reag. Ph Eur	Merck KGaA, Darmstadt, Germany
$MnSO_4 * H_2O$	≥99 %, p.a., ACS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Myristic acid	for synthesis	Merck Schuchard OHG, Hohenbrunn, Germany
NaCl	≥99.5 %, p.a., ACS, ISO	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$Na_2HPO_4 * 2H_2O$	for analysis	Merck KGaA, Darmstadt, Germany
NH_4Cl	≥99.7 %, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Oleic acid	extra pure	Merck KGaA, Darmstadt, Germany
Palmitic acid	for synthesis	Merck Schuchard OHG, Hohenbrunn, Germany
Palmitoleic acid	≥98.5 %, analytical standard	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Peptone from casein	for microbiology	Merck KGaA, Darmstadt, Germany
Peptone from meat	for microbiology	Merck KGaA, Darmstadt, Germany
Polyethylene glycol (PEG 400)	Ph. Eur.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Rapeseed oil (Tip brand)	-	real,- SB-Warenhaus GmbH, Mönchengladbach, Germany
Ringer's solution (tablets)	-	Merck KGaA, Darmstadt, Germany
Stearic acid	for synthesis	Merck Schuchard OHG, Hohenbrunn, Germany
Sucrose	Standard Grade	GERBU Biotechnik GmbH, Heidelberg, Germany
Tributyrin	97 %, food grade	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Tween 20	for synthesis	Merck KGaA, Darmstadt, Germany
Tween 40	for synthesis	Merck KGaA, Darmstadt, Germany
Tween 60		Sigma Aldrich Chemie GmbH, Steinheim, Germany
Tween 80 (for standard mMRS)	pharmaceutical grade	GERBU Biotechnik GmbH, Heidelberg, Germany
Tween 80 (emulsions and specific experiments)	for synthesis	Merck KGaA, Darmstadt, Germany
Whey protein isolate	-	kindly provided by the Chair of Chair of Food and Bioprocess Engineering of the TU Munich, Freising, Germany
Yeast extract	for bacteriology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

2.1.5 Biochemical reagents and kits

Table 5: Biochemical reagents and kits used in this study.

Reagent/kit	Specification	Supplier
Bacterial Viability Kit L7012	-	Thermo Fisher Scientific, Waltham, MA, USA
Lysozyme 100.000 units/mg	RNA isolation	SERVA Electrophoresis GmbH, Heidelberg, Germany
Proteinase K 20 mg/mL	RNA isolation	GERBU Biotechnik GmbH, Heidelberg, Germany
RNAprotect Bacteria Reagent	RNA isolation	QIAGEN GmbH, Hilden, Germany
RNeasy mini kit	RNA isolation	QIAGEN GmbH, Hilden, Germany

2.1.6 Growth and Recovery Media

2.1.6.1 Modified MRS medium (mMRS)

Table 6: Composition of mMRS medium .

Compound	Concentration (g L^{-1})
Peptone from casein	10 g L^{-1}
Meat extract	5 g L^{-1}
Yeast extract	5 g L^{-1}
KH_2PO_4	4 g L^{-1}
K_2HPO_4 * 3 H_2O	2.6 g L^{-1}
NH_4Cl	3 g L^{-1}
Cystein-HCl	0.5 g L^{-1}
Tween 80	1 g L^{-1}
D-Glucose	7.5 g L^{-1}
D-Fructose	7.5 g L^{-1}
$MgSO_4$ * $7H_2O$	0.1 g L^{-1}
$MnSO_4$ * H_2O	0.036 g L^{-1}
Agar-Agar[1]	15 g L^{-1}

[1]added for solidification of the medium when agar plates were prepared

All components except the sugars, $MgSO_4$ and $MnSO_4$ were solubilized in 0.8 L deionized water and autoclaved at 121°C for 15 min. A 5x concentrated solution of glucose and fructose was autoclaved separately at the same conditions. A 1000x stock solution of $MgSO_4$ * 7 H_2O and $MnSO_4$ * 4 H_2O was filter-sterilized (0.2 µm pore size, Sarstedt, Nürnbrecht, Germany). Both solutions were added to the medium after autoclaving.

To prepare solid mMRS medium (mMRS agar), agar-agar was added in the concentration stated in Table 6 followed by vigorous mixing prior to autoclaving. The final medium was poured into 10 cm petri dishes.

2.1.6.1.1 mMRS medium buffered with citric acid and aspartic acid

mMRS medium with buffer capacities at different acidic pH values was prepared by adding citric acid and aspartic acid to basic mMRS medium to a final concentration of 20 mM each, resulting in a pH of 3.88, and adjusting the pH to the desired value using 6 M HCl or 4 M NaOH, respectively.

2.1.6.1.2 mMRS medium supplemented with NaCl

Prior to autoclaving, NaCl was added to the prepared mMRS medium to give concentrations of 5, 6, 7.5 and 10 % (w/v) in the final medium. After autoclaving (see 2.1.6.1), the a_w value of the final medium was measured.

2.1.6.1.3 mMRS without Tween 80 (mMRS-)

To prepare mMRS-, standard mMRS was prepared as described above, using the ingredients listed in Table 6 with the sole exception that Tween 80 was not included.

2.1.6.1.4 mMRS medium supplemented with different Tween types

The different Tween types (Tween 20, Tween 40, Tween 60, Tween 80) were solubilized in mMRS- at a concentration of 10 g L^{-1}, filter-sterilized (0.2 µm pore size, Sarstedt, Nürnbrecht, Germany) to exclude the possibility of deteriorating effects of autoclaving, and finally added to sterile mMRS- to give a concentration of 1 g L^{-1}. The resulting media are referred to as mMRST20, mMRST40, mMRST60 and mMRST80 throughout this manuscript, respectively. Although mMRST80 and standard mMRS medium have the same composition, mMRST80 was used in experiments where the effects of Tween supplementation were investigated to ensure comparability with mMRS media supplemented with other Tween types.

2.1.6.1.5 mMRS medium supplemented with free fatty acids

Free fatty acids, i.e. stearic acid, oleic acid, linoleic acid, linolenic acid, palmitic acid, palmitoleic acid, myristic acid, and lauric acid were solubilized in 95 % ethanol to a concentration of 50 mM. These stock solutions were added in a ratio of 1:1000 to sterile mMRS- to obtain a final fatty acid concentration of 50 µM. The FA stock solutions and the supplemented medium were stored at RT in the dark.

2.1.6.1.6 Selective mMRS agar containing NaCl

Prior to autoclaving, NaCl was added to the prepared mMRS medium (with agar-agar) to give concentrations ranging from 1 % (w/v) to 10 % (w/v) in 1 % increments in the final medium.

2.1.6.1.7 mMRS agar supplemented with tributyrin (mMRS-TB)

To prepare mMRS-TB, standard mMRS agar was prepared as described in above. After autoclaving, 10 mL L^{-1} tributyrin were added and homogenously distributed using a disperser-homogenizer (Miccra® D15, Miccra GmbH, Müllheim, Germany) equipped with a previously sterilized stirring rod. The medium was poured in 10 cm petri dishes and allowed to solidify.

2.1.6.2 *Bacillus subtilis* medium (DSMZ Medium 1)

Growth medium for *Bacillus subtilis* was prepared according to Nutrient agar No. 1 of the DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen*).

Table 7: Composition of growth medium for *Bacillus subtilis*.

Compound	Concentration
Peptone from casein	5 g L^{-1}
Meat extract	3 g L^{-1}

The ingredients were dissolved in water and autoclaved at 121 °C and 0.2 MPa for 20 min.

2.1.6.3 LB Agar

Table 8: Composition of Luria-Bertani (LB) agar.

Compound	Concentration
Peptone from casein	10 g L^{-1}
Yeast extract	5 g L^{-1}
NaCl	10 g L^{-1}
Agar-agar	20 g L^{-1}

The ingredients were dissolved in water and the pH was adjusted to 7.0 using 4 M NaOH and 6 M HCl. The prepared medium was autoclaved at 121 °C and 0.2 MPa for 20 min.

2.1.6.4 TS+ recovery diluent

All components were dissolved in water and autoclaved at 121 °C, 0.2 MPa for 15 min.

Table 9: Composition of TS+ recovery diluent.

Compound	Concentration
Peptone from casein	14 g L^{-1}
NaCl	8.5 g L^{-1}
Antifoam B emulsion	0.1 mL L^{-1}

All components were dissolved in water and autoclaved at 121 °C, 0.2 MPa for 15 min.

2.1.7 Buffers

2.1.7.1 Imidazole-phosphate buffer (IPB), pH 7

Table 10: Composition of IPB.

Compound	Concentration
KH_2PO_4	0.1 g L^{-1}
Na_2HPO_4 * 2 H_2O	4.45 g L^{-1}
Imidazole	1.7 g L^{-1}

Chemicals were solubilized in deionized water and the pH was adjusted to 7 using 6 M HCl followed by autoclaving at 121 °C, 0.2 MPa for 15 min.

2.1.7.1.1 IPB with different NaCl concentrations

NaCl was dissolved in freshly prepared, non-sterile IPB (pH 7) to concentrations of 0.5, 1.25, 2.5, 5, 12.5 and 25 % (w/v). The obtained IPB-NaCl solutions were autoclaved at 121 °C, 0.2 MPa for 15 min.

2.1.7.1.2 IPB with different sucrose concentrations

Sucrose was dissolved in freshly prepared, non-sterile IPB (pH 7) to concentrations of 5, 12.5, 25 and 50 % (w/v). The obtained IPB-sucrose solutions were autoclaved at 121 °C, 0.2 MPa for 15 min.

2.1.7.1.3 IPB containing protein components

Since autoclaving leads to denaturation of proteins due to high temperatures, whey protein isolate (WPI) and casein were introduced into sterile IPB. For the purpose of comparison, the same procedure was applied for peptone, although this additive is stable under autoclaving conditions. The three additives were incorporated into IPB in the concentrations stated in Table 11 and as described as follows:

Table 11: Concentrations of protein components incorporated in IPB.

Protein component	Concentration [% (w/v)]
Peptone from casein	2.5
	10
Whey protein isolate	2.5
	10
Casein	2.5

Peptone from casein, whey protein isolate and casein were provided in sterile measuring flasks and part of the final volume of sterile IPB was added. The mixtures were stirred under sterile conditions until the protein components were fully dissolved (peptone, WPI) or a homogenous mixture was obtained (casein). Then IPB was added until the final volume was reached, the mixtures were finally transferred into sterile glass bottles and stored at 4 °C to prevent spoilage.

2.1.7.2 Citrate-Phosphate Buffer (CPB)

Stock solutions of 50 mM citric acid and 100 mM Na_2HPO_4 in water were prepared and mixed as shown in Table 12 to obtain citrate-phosphate buffer (CPB) with the appropriate pH value (Table 12). The prepared buffer solutions were autoclaved at 121 °C, 0.2 MPa for 15 min.

Table 12: Mixing ratios of buffer components for McIlvaine citrate/phosphate buffer with different pH values.

pH	v(50 mM citric acid) (mL)	v(100 mM Na_2HPO_4) (mL)
7	176	824
6	368	632
5	485	515
4	614	386
3	794	206

2.1.7.3 Quarter-strength Ringer's solution

Sterile quarter-strength Ringer's was prepared from ready-to use tablets by dissolving 1 tablet in 500 mL deionized water and autoclaving at 121 °C, 0.2 MPa for 15 min.

2.2 Preparation and analysis of O/W emulsions

Besides chemical characteristics like oil-to-water ratio, the nature of the emulsifier and the composition of the aqueous phase emulsions are characterized by physical parameters like the droplet size and the overall droplet surface area. The droplets of the dispersed phase can be assumed as particles and therefore be analyzed by particle size measurement.

2.2.1 Preparation of O/W emulsions

Usually, surfactants are added to the continuous phase before emulsification but polysorbates represent an exception (Hasenhuettl & Hartel, 2008). Thus, Tween 80 was added to the lipid phase. Oil-in-water (O/W) emulsions containing 50 % rapeseed oil were prepared by addition of 5 g Tween 80 to 0.25 L rapeseed oil followed by stirring at RT overnight. On the next day, 0.25 L IPB containing the appropriate type and concentration of solute or CPB with the appropriate pH

value were added, respectively. The mixture was homogenized with an Ultra Turrax disperser at 23.000 rpm for 60 s to give a pre-emulsion. The final emulsion was obtained by two-stage pressure homogenization in an APV Model 1000 Homogenizer. The processing pressures (see Table 13) were determined empirically for each individual emulsion in order to obtain the highest possible conformity in specific surface area (SSA) and median droplet size (D(v,0.5)).

Table 13: Preparation of O/W emulsions with defined oil-water interface containing rapeseed oil and Imidazole/phosphate buffer (IPB) or Citric acid/phosphate buffer (CPB).

#	Buffer system	Aqueous phase parameter	Homogenization pressure	
			Step 1 (bar)	Step 2 (bar)
1	IPB	-	100	20
2	IPB	0.5 % NaCl	100	20
3	IPB	1.25 % NaCl	100	20
4	IPB	2.5 % NaCl	100	20
5	IPB	5 % NaCl	100	20
6	IPB	12.5 % NaCl	100	20
7	IPB	25 % NaCl	100	20
8	IPB	5 % sucrose	100	20
9	IPB	12.5 % sucrose	100	20
10	IPB	25 % sucrose	100	20
11	IPB	50 % sucrose	100	20
12	CPB	pH 7	100	20
13	CPB	pH 6	100	20
14	CPB	pH 5	100	20
15	CPB	pH 4	100	20
16	CPB	pH 3	100	20
17	IPB	-	300	60
18	IPB	2.5 % peptone	200	40
19	IPB	10 % peptone	100	20
20	IPB	2.5 % whey protein isolate	150	30
21	IPB	10 % whey protein isolate	100	20
22	IPB	2.5 % casein	150	30

2.2.2 Determination of droplet size and oil-water interface

The oil droplet size and the oil-water contact surface in the emulsions were determined using a Malvern Mastersizer 2000 particle size analyzer. This device determines the size of dispersed particles based on their ability to scatter a focused laser beam and under consideration of the refractive index of the dispersed material. The refractive index of rapeseed oil was determined to

1.472 (n_D^{25}) by means of a refractometer and used for calculation of emulsion characteristics, such as SSA, surface- and volume-weighted mean D(3,2) and D(4,3), respectively, and parameters describing the particle size distribution (D(v,0.1), D(v,0.5) and D(v,0.9)). To obtain reliable measuring values, the sample volume was adjusted to obtain an obscuration between 10 and 12 %.

2.2.3 Determination of creaming rate

Creaming rates of the prepared O/W emulsions were determined using a LUMiFUGE® analytical centrifuge. This device accelerates creaming by centrifugation and tracks the position of the oil/water phase boundary during the centrifugation process by repeatedly recording intensity profiles of transmitted light over the sample. The shift of the phase boundary over time is then used to calculate the creaming rate expressed as µm s^{-1}. In this way, the creaming behavior of the emulsions expected during standing at gravity for longer time periods can be simulated and evaluated in a very short time.

For conduction of measurements, special cuvettes provided by the device manufacturer were loaded with 400 mg O/W emulsion and centrifuged at 2474 rpm, 25 °C for 700 s. Transmission profiles were recorded every 10 s. Recorded intensity profiles were evaluated using the software SEPView® 6.0 provided by the device manufacturer under consideration of a phase separation threshold of 15 %, a measuring range between ~190 and ~290 mm (position on the sample) and a time interval of the entire 700 s of measurement.

2.2.4 Determination of emulsion shelf life

To determine the shelf life of the prepared O/W emulsions with respect to coalescence and creaming, emulsions were stored at 4 °C for a period of 9 days and inspected visually by the unaided eye and photo documentation every 24 h. Additionally, droplet size and SSA as well as creaming rates were determined as described in 2.2.2 and 2.2.3, respectively, at the end of the incubation period and compared to values determined immediately after preparation.

2.2.5 Stability of the prepared emulsions under high pressure

Prepared O/W emulsions were filled in 0.5 mL cryovials. The vials were closed, while care was taken to avoid the inclusion of air bubbles, and sealed with parafilm. The loaded vials were pressure-treated with 500 MPa, 5 min as described in 2.6.2. After HHP treatment, SSA and droplet size as well as creaming rate were determined as described in 2.2.2 and 2.2.3, respectively, and compared to values determined before HHP treatment.

2.3 General microbiological methods

2.3.1 Preparation of stock cultures

To prepare stock cultures of the *Lactobacillus* strains used in this study, mMRS agar plates were inoculated with a loopful of cryo culture from the *Technische Mikrobiologie Weihenstephan* (TMW) strain collection using the streaking technique and incubated aerobically at 30 °C overnight. For each strain one single colony was picked and used to inoculate 12 mL mMRS medium. The liquid culture was allowed to grow at 30 °C overnight and then centrifuged (5,000 × g, 5 min, RT) to harvest the cells. The supernatant was discarded. The pellet was resuspended in 0.8 mL of fresh mMRS medium, mixed with 0.8 mL of sterile 80 % (v/v) glycerol solution in a prepared cryo vials and stored at -80 °C. The identity on species level was confirmed by proteomic analysis using MALDI-TOF MS (see 2.4.1).

2.3.2 Preparation of pre-cultures

In order to have cultures in a standardized state for each experiment, per-cultures of the used strains were prepared prior to start of the actual experiments. Culture conditions varied among experiments and are thus stated specifically in the following sections.

Lactobacillus strains

Pre-cultures were prepared by inoculating sterile mMRS medium with a loopful of the stock culture and aerobic incubation at 30 °C overnight. This overnight culture (pre-culture) was further used to inoculate fresh medium (1 % (v/v)) for the individual experiments.

Bacillus subtilis

Pre-cultures of *Bacillus subtilis* TMW 2.742 were prepared by inoculating LB agar plates with a loopful of cryo culture from the TMW strain collection using the streaking technique and aerobic incubation at 37 °C overnight. A single colony was picked, transferred into fresh DSMZ medium 1 and incubated aerobically overnight with agitation to obtain a *B. subtilis* pre-culture.

2.3.3 Assessment of growth characteristics

Growth curves of bacterial strains in different conditions were recorded by measurement of the optical density at 600 nm (OD_{600}) in liquid cultures. For this, liquid growth medium was inoculated with pre-cultures of the respective *Lactobacillus* strains. The inoculum volume used is stated specifically for each experiment. 150 µL of the prepared culture were immediately loaded onto a sterile, transparent 96-well microtiter plate (F-bottom) (Sarstedt, Nürnbrecht, Germany) and overlaid with 50 µL of sterile paraffin oil to avoid evaporation during incubation. The plate

was incubated in microplate reader at controlled temperature and OD_{600} was repeatedly recorded in defined time intervals. Temperature, incubation time and measuring frequency varied according to the growth conditions examined and are stated specifically for each experiment. The plate was agitated with moderate intensity prior to each measurement for at least 15 s to resuspend cells that may have sedimented. Recorded growth curves were used to determine the maximum OD_{600} ($maxOD_{600}$), the maximum growth rate (μ_{max}) and, where applicable, the time to reach an OD_{600} of 0.1 of bacterial strains under given conditions.

To calculate μ_{max}, the natural logarithm of the measured OD_{600} values, $ln(OD_{600})$, was plotted against time and the growth rate μ was determined for each measuring interval using Equation 20.

$$\mu = \frac{\boldsymbol{ln(X_t) - ln(X_0)}}{\boldsymbol{t - t_0}} \qquad \text{(Eq. 20)}$$

with t = a certain time of measurement, t_0 = the previous measuring time; X_t = OD_{600} at time t; X_0 = OD at time t_0.

The regression line was calculated using the time intervals during exponential phase showing the highest and constant growth rates to finally determine μ_{max}, which is defined as the slope of the regression line.

2.3.4 Viable cell count and inactivation

500 µL of treated and control samples were mixed in a ratio of 1:1 with TS+ to ensure separation of potentially formed cell aggregates and then serially diluted (100 µL in 900 µL) in TS+. 100 µL of an appropriate sample dilution were plated on mMRS agar in duplicate using sterile glass beads. The agar plates were incubated for three days at 30 °C and colonies were counted. From the colony counts the concentration of viable cells (viable cell count) in the sample was calculated. Inactivation resulting from HHP treatment is expressed as the log reduction (LR) defined as the common logarithm of the viable cell count of the untreated control sample divided by the viable cell count of the treated sample:

$$\boldsymbol{LR = log_{10}(\frac{N_0}{N})} \qquad \text{(Eq. 21)}$$

with

N_0 = Concentration of viable cells in the untreated (control) sample [cfu mL^{-1}]

and

N = Number of viable cells in the (HHP) treated sample [cfu mL^{-1}]

2.3.5 Determination of maximum non-inhibitory NaCl concentration

The maximum non-inhibitory NaCl concentration was determined as described by Cebrián (Cebrián *et al.*, 2014). For this, mMRS medium was inoculated 1 % (v/v) with a pre-culture of the investigated strain and incubated at 30 °C for 24 h. Cells were harvested by centrifugation (5,000 × g, 5 min, RT), washed once, resuspended and serially diluted in IPB to an appropriate concentration. 100 µL of diluted cell suspension was plated on selective mMRS agar supplemented with 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 % (w/v) NaCl and standard mMRS agar as positive control. The plates were incubated at 30 °C for a period of 9 days and colonies were counted every day. The highest NaCl concentration that led to less than 20 % reduction in colony count after the full incubation period (9 days) was considered the maximum non-inhibitory concentration and used for examination of sublethal injury after HHP treatment.

2.3.6 Assessment of sublethal injury

For the determination of the amount of sublethally injured cells, pressure-treated cell suspensions were diluted in TS+ and plated as described in 2.3.4 with the sole difference that besides normal mMRS agar the suspensions were plated on mMRS supplemented with the maximum non-inhibitory NaCl concentration in parallel. The plates were incubated for 3 to 9 days at 30 °C to allow for cell recovery and colony formation. The extent of sublethal injury was calculated as the difference between colony count on mMRS agar and mMRS agar supplemented with the NaCl MNIC.

2.4 Characterization of bacterial strains

2.4.1 Proteomic analysis

2.4.1.1 Sample preparation

Samples were prepared for MALDI-TOF MS analysis according to the plain cell extraction procedure described by Kern *et al.* (Kern *et al.*, 2013): Fresh mMRS medium was inoculated with a pre-culture of the respective *Lactobacillus* strain and incubated at 30 °C overnight. 1 mL of the overnight culture was transferred into a 1.5 mL reaction tube and centrifuged (5,000 × g, 5 min, RT). The supernatant was discarded and the pellet resuspended in 300 µL deionized water by pipetting up and down followed by vortexing for 2 min. 900 µL ethanol abs. were added to inactivate the cells and the suspension was vortexed for another 2 min. The mixture was centrifuged (13,000 rpm, 2 min, RT), the supernatant was discarded and the pellet was allowed to dry in the opened reaction tube placed on a paper towel. The remaining ethanolic solution was finally removed by centrifugation (13,000 rpm, 2 min) followed by removal of residual liquid

using an appropriate pipette and drying for another 30 min with opened tube cap at RT. The dried pellet was resuspended in 50 µL 70 % aqueous formic acid (FA) solution by pipetting up and down and vortexing for 2 min. The suspension was mixed with 50 µL acetonitrile (ACN) by pipetting followed by vortexing for 2 min. In case of extraordinarily small pellets due to low yield of cell material, e.g. with *L. fructivorans* strains, the volume of added FA solution and ACN was adjusted, maintaining the FA solution:ACN ratio at 1:1. The preparation was centrifuged (13,000 rpm, 2 min) to spin down cell debris. 1 µL of the supernatant/preparation was placed on a clean stainless-steel target plate (Bruker Daltonics, Bremen, Germany) and allowed to dry at RT. The sample position was overlaid with 1 µL alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix solution in ACN. Care was taken to avoid touching the dried sample with the pipette tip. The prepared sample was allowed to dry at room temperature in the dark and inserted into the MALDI-TOF mass spectrometer.

2.4.1.2 MALDI-TOF MS measurement

MALDI-TOF MS analysis was performed using a Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) as described by Kern (Kern *et al.*, 2013). The device is equipped with a nitrogen laser (λ = 337 nm) and operates in linear positive ion detection mode under the control of the Biotyper Automation Control 2.0 software (Bruker Daltonics, Bremen, Germany). Per sample the mass spectra in a range from 2,000 to 20,000 Da of 240 laser shots were accumulated to create sum spectra. A bacterial test standard obtained from the device manufacturer was used for external mass calibration.

2.4.1.3 Data analysis

Species identification

Identification of the tested strains on species level was done by comparison of the recorded spectra to a reference database using the Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany). According to the reliability score obtained from the correlation of intensity of matching peaks and the similarity of spectra of the tested sample and data base entries, identification on species level or genus level or no identification was possible.

Comparative analysis

Recorded raw MALDI-TOF mass spectra were exported using the FlexAnalysis 3.4 software (Bruker Daltonics, Bermen, Germany) and processed using an inhouse software based on octave (https://www.gnu.org/software/octave/) run on an open sharedroot computer cluster (ATIX; http://opensharedroot.org). After pre-processing by smoothing, baseline subtraction and

normalization of signal intensities as described by Usbeck *et al.* (2013), all spectra of one species were summarized to give one consensus spectrum using mass spectrometry comparative analysis package (MASCAP) (Mantini *et al.*, 2010). Similarities between the consensus spectra of each strain were calculated using a software based on high-throughput multidimensional scaling (HiT-MDS) and finally mapped in a two-dimensional plane based on the Euclidean distance (Lauterbach *et al.*, 2017).

2.4.2 Cell surface hydrophobicity

CSH was determined using the Microbial Adhesion to Hydrocarbons (MATH) test described by (Rosenberg *et al.*, 1980) with some modifications: Cells were grown in mMRS medium to late exponential growth phase (OD_{600} ~0.8), harvested by centrifugation (5,000 × g, 5 min, 25 °C) and washed with and resuspended in IPB to an OD_{600} of 0.35-0.4. 4 mL of this cell suspension were transferred to round-bottom glass test tubes (10 mm diameter) containing 0.4 mL n-hexadecane or rapeseed oil, respectively, and incubated at 30 °C for 10 min. Afterwards, the test tubes were vortexed for 2 min at maximum speed followed by 15 min resting at RT to allow for complete separation of the aqueous and organic phases. The aqueous phase was carefully transferred into measuring cuvettes using a glass pipette and the OD_{600} was determined using a UV-VIS spectrophotometer. The degree of hydrophobicity was determined by calculating the ratio of the OD_{600} of the aqueous phase after agitation to the OD_{600} of the cell suspension before agitation and expressed in percent.

2.4.3 Spoilage potential

Acidity tolerance

To determine the acidity tolerance of *Lactobacillus* strains, growth characteristics were determined in mMRS-CA medium with pH values of 6.2, 4.5, 4.0 and 3.5 as described in 2.3.3. The medium was inoculated 2 % (v/v) with pre-cultures of the strains and incubated at 30 °C in a Sunrise microplate reader (Tecan, Männedorf, Switzerland) over a period of 96 h with OD_{600} measurements every 30 min.

Salinity tolerance

To determine the salinity tolerance of *Lactobacillus* strains, growth characteristics were determined in mMRS medium supplemented with 5, 6, 7.5 or 10 % (w/v) NaCl as described in 2.3.3. The medium was inoculated 2 % (v/v) with pre-cultures of the strains and incubated at 30 °C in a Sunrise microplate reader (Tecan, Männedorf, Switzerland) over a period of 96 h with OD_{600} measurements every 30 min.

Cold tolerance

To determine the cold tolerance of *Lactobacillus* strains, growth characteristics were determined in mMRS medium as described in 2.3.3 with the sole difference that the microtiter plates were incubated in refrigerating incubators and inserted into the microplate reader only for OD-measurement. The medium was inoculated 2 % (v/v) with pre-cultures of the strains and cultures were incubated at 4 °C and 10 °C over a period of 9 days. OD_{600} was measured in a Sunrise microplate reader (Tecan, Männedorf, Switzerland) every 24 h.

2.4.4 Potential to destabilize emulsions

2.4.4.1 Lipolytic activity

5 mL of fresh mMRS and DSMZ medium 1 were inoculated 1 % (v/v) with pre-cultures of *Lactobacillus* strains in mMRS medium and of *Bacillus subtilis* strain TMW 2.742 in DSMZ Medium 1, respectively. The prepared cultures were aerobically grown at 30 °C (*Lactobacillus*) and 37 °C with agitation (*B. subtilis*) to stationary growth phase. The overnight cultures were serially diluted in TS+ and an appropriate dilution was plated on mMRS-TB agar plates so that the formation of single colonies was ensured. Agar plates were incubated at 30 °C (*Lactobacillus*) and 37 °C (*B. subtilis*) and inspected visually every day for transparent halos around the colonies within the otherwise turbid agar. After formation of sufficiently big colonies that allowed to distinguish between lipolytically active and inactive strains the halo formation was documented by photography using a Colony Doc-It Imaging Station (Upland, CA, USA).

2.4.4.2 Proteolytic activity

Proteolytic activity of the selected strains was assessed by cleavage of fluorescein isothiocyanate-labeled Fluorescein thiocarbamoyl (FTC) casein. Every molecule of FTC casein is coupled to several residues of green-fluorescing fluorescein. Due to Förster Resonance Energy Transfer (FRET), fluorescein absorbs the fluorescence energy emitted by other fluorescein molecules in close proximity so that no fluorescence signal is emitted upon excitation with light of the appropriate wavelength. This quenching effect occurs between the fluorophores bound to one casein molecule. Cleavage of the casein polypeptide increases the distance between single fluorescein molecules and thus leads to an increase in fluorescence emission, which, in turn, is a measure for the proteolytic activity in a sample. For performing the assay, 5 mL of fresh mMRS and 5 mL of fresh DSMZ medium 1 were inoculated 1 % (v/v) with pre-cultures of *Lactobacillus* strains in mMRS medium and of *Bacillus subtilis* strain TMW 2.742 in DSMZ Medium 1, respectively. The prepared cultures were aerobically grown at 30 °C

(*Lactobacillus*) and 37 °C with agitation (*B. subtilis*) to stationary growth phase. The overnight cultures were centrifuged (5,000 × g, 5 min, RT), washed once and resuspended in IPB to an OD_{600} of ~0.5. A stock solution of fluorescein isothiocyanate-labelled (FTC-)Casein was diluted in IPB to a concentration of 10 µg mL^{-1}. Porcine trypsin was dissolved in IPP to final concentrations of 5 and 10 µg mL^{-1}. 100 µL of the prepared bacterial suspensions, trypsin solution (positive control) or plain IPB (negative control) were mixed with 100 µL FTC-Casein solution in the wells of a white 96-well microtiter plate (F-bottom). The microtiter plate was incubated in a microplate fluorescence reader (FLUOStar Omega, BMG Labtech GmbH, Ortenberg, Germany) at 30° C for 10 h. Fluorescence intensity was measured using a filter set with excitation/emission wavelength of 485/520 nm (gain: 300) every 10 min. The plate was agitated 10 s before each measuring cycle with 300 rpm (shaking mode: double orbital). Each sample was measured in triplicate. The fluorescence signal resulting from measurement of 200 µL IPB (blank) was subtracted from the mean value of the three wells to obtain the blank-corrected fluorescence intensity.

2.4.4.3 Degradation of emulsion constituents

Some constituents of the O/W emulsions used in this study, such as emulsifier Tween 80 as well as sucrose and peptone could potentially function as carbon sources. The ability of *Lactobacillus* strains to degrade these substances was assessed by examination of their growth behavior in IPB containing each of the substances and combinations of them in the concentrations present in the emulsions (see 2.2.1). Therefore, the following conditions were investigated:

1. Tween 80 (1 % (w/v))
2. Sucrose (5 / 12.5 / 25 / 50 % (w/v))
3. Peptone (2.5 / 10 % (w/v))
4. Sucrose (5 / 12.5 / 25 / 50 % (w/v)) + Tween 80 (1 % (w/v))
5. Peptone (2.5 / 10 % (w/v)) + Tween 80 (1 % (w/v))

For this, sterile IPB containing 5, 12.5, 25 and 50 % (w/v) sucrose or 2.5 and 10 % peptone from casein was prepared as described in 2.1.7.1. Tween 80 was added to standard IPB, IPB containing sucrose and IPB containing peptone to a final concentration of 1 % (w/v) followed by sterile filtration using 0.2 µm pore size filters.

5 mL of fresh mMRS medium was inoculated 1 % (v/v) with pre-cultures of *Lactobacillus* strains and grown at 30 °C for 24 h. Cultures were centrifuged (5000 × g, 5 min, RT), washed and resuspended in 5 mL IPB and finally used to inoculate the prepared IPB containing the emulsion

constituents (1 % (v/v)). Standard IPB without additives and mMRS medium served as negative and positive growth controls, respectively.

Growth curves were recorded as described in 2.3.3 using a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany) with incubation at 30 °C for 6 h in case of sucrose, peptone and combinations of sucrose or peptone with Tween 80, and for 15 h when only Tween 80 was present. The OD_{600} was recorded every 5 min. Intensity of growth was assessed by comparison of the maxOD_{600} value with the initial OD_{600} value at each condition.

2.5 Localization of cells in oil-water mixtures and O/W emulsions

2.5.1 Sample preparation

Localization in oil-water mixtures

Mixtures of IPB and rapeseed oil in a ratio of 1:1 were prepared by homogenization using a disperser-homogenizer (Miccra® D15, Miccra GmbH, Müllheim, Germany) at maximum speed for 1 min. Cells were added to the mixture to a final concentration of ~10^8 cfu mL^{-1}.

Localization in O/W emulsions

An O/W emulsion of 50 % rapeseed oil in IPB (1 % Tween 80) without additives in the aqueous phase was prepared as described in 2.2.1. Cells were added to the emulsion to a final concentration of ~10^8 cfu mL^{-1}. To visualize cells, fluorescence staining was performed by adding 3 µL SYTO® 9 (3.34 mM; Bacterial Viability Kit L7012, Thermo Fisher Scientific Inc., USA) to 1 mL of sample and incubating for 15 min at RT in the absence of light.

2.5.2 Microscopic evaluation

3 µL of the preparations were placed on a glass slide, covered with a glass coverslip and examined by bright field and fluorescence microscopy (only O/W emulsions) using an Axiostar plus microscope (Carl Zeiss Microscopy GmbH, Munich, Germany) equipped with 40x and 100x objectives, leading 400- and 1000-fold overall magnification, respectively. Immersion oil was added when the 100x objective was used. Images were captured with a 1.388- by 1.038-pixel RGB camera (AxioCam ICc1, Carl Zeiss Microscopy GmbH, Munich, Germany) and processed using the AxioVS40 V 4.8.2.0 software (Carl Zeiss Microscopy GmbH, Munich, Germany) and ImageJ.

2.6 HHP inactivation experiments

2.6.1 Sample preparation

2.6.1.1 HHP inactivation in emulsions

10 mL mMRS medium were inoculated 1 % (v/v) with pre-cultures of the strains of interest in mMRS medium and incubated at 30 °C for 24 h. The cultures were centrifuged (5,000 × g, 5 min, RT), washed once, and finally resuspended in 10 mL IPB, giving a concentration of ~10^9 cfu mL^{-1}. This stock suspension was diluted 1:50 in the respective O/W emulsion and in the corresponding pure aqueous phase in parallel to a concentration of ~10^7 cfu mL^{-1}. For this, 10 mL of the emulsion or buffer were prepared in sterile 50 mL Erlenmeyer flasks, each containing a sterile magnetic stirrer. 200 µL of the stock suspension was aseptically added to the flask followed by stirring at moderate speed and RT for 120 s under aseptic conditions.

2.6.1.2 HHP inactivation after growth in mMRS supplemented with Tween or free fatty acids

Pre-cultures of test strains in mMRS- were used to inoculate 10 mL of the respective growth medium. Cultures were incubated at 30 °C for 24 h, centrifuged (5,000 × g, 5 min, RT), washed once, and finally resuspended in 10 mL IPB, giving a concentration of ~10^9 cfu mL^{-1}. For determination of inactivation, the stock suspension was diluted 1:50 in IPB to give a concentration of ~10^7 cfu mL^{-1}. For assessment of the effect of HHP on metabolic activity, protein release and membrane permeability, the stock solution was used undiluted.

2.6.1.3 HHP inactivation in IPB after growth in mMRS-rapeseed oil mixture

150 mL sterile mMRS- were prepared in a suitable beaker under aseptic conditions and stirred using a disperser-homogenizer (Miccra® D15, Miccra GmbH, Müllheim, Germany) equipped with a previously sterilized stirring rod at moderate speed. During stirring, 150 mL rapeseed oil were slowly added over a period of 1 min to achieve maximum possible dispersion of oil droplets. After addition was completed, the mixture was stirred for 1 min at maximum speed. Afterwards, speed was reduced to the initial level and 10 mL aliquots of the mixture transferred to 15 mL tubes while stirring to avoid immediate phase separation and to ensure proper medium-oil ratio in the tube. The prepared 10 mL aliquots of the mixture were inoculated 1 % (v/v) with a pre-culture of test strains in mMRS-, where the inoculum volume referred to the volume of mMRS-medium, i.e. 5 mL. Control samples without oil were prepared consisting of 5 mL, the volume of mMRS- present in the cultures containing oil. Cultures were incubated at 30 °C for 24 h with agitation (200 rpm) to counteract phase separation and ensure maximum possible contact of the

lipid and aqueous phases. Cultures were centrifuged (5000 × g, 5 min, RT), washed three times, where care was taken to remove especially residual oil from the samples, and finally resuspended in 10 mL IPB, giving a concentration of ~10^9 cfu mL^{-1}. This stock suspension was diluted 1:50 in IPB to give a concentration of ~10^7 cfu mL^{-1}.

2.6.2 HHP treatment

An aliquot of 600 µL of the sample (cell suspension / emulsion) was transferred to 0.5 mL cryovials (Nunc CryoTube™ Vials, internal thread; Thermo Fisher Scientific, Waltham, MA, USA). Care was taken to avoid the inclusion of air bubbles. The cryovials were sealed with parafilm to prevent intrusion of pressure-transmitting fluid and leakage of sample material during pressure build-up and release, respectively.

Samples were placed in two parallel linked 7 mL pressure vessels equipped with thermostating jackets (high pressure unit TMW-RB, described earlier (Lenz & Vogel, 2014, Lenz *et al.*, 2015)). A mixture of 70% polyethylene glycol 400 (Carl Roth, Karlsruhe, Germany) and 30 % deionized water was used as pressure-transmitting fluid. Vessel temperature was held constant at 25 °C (FC 600; JULABO, Seelbach, Germany). Samples were incubated in the pressure vessel for 5 min prior to starting the pressure ramp to reach the desired starting temperature. Compression and decompression rates were kept constant at 200 MPa min^{-1}. Target pressure/holding time combinations were chosen according their capability of inactivating a significant portion of cells within a population.

2.6.3 Treatment of control samples

Untreated control samples

Reference samples for the determination of the initial viable cell count were stored at RT during HHP treatment and were evaluated simultaneously to the HHP-treated samples.

Heat-inactivated control samples

For experiments regarding HHP-induced membrane permeabilization, samples with completely inactivated cell populations, showing the maximum achievable membrane permeability, were required. For complete inactivation, suspensions were incubated at 100 °C for 15 min.

2.7 Effect of growth medium supplementation with fat-associated compounds

2.7.1 Transcriptomic analysis

Sample preparation

Sterile mMRS- was inoculated 1 % (v/v) with a pre-culture of TMW 1.708 in mMRS- and incubated at 30 °C for 24 h. 9 mL of fresh mMRS- were inoculated 1 % (v/v) with the 24 h culture and grown at 30 °C for 4 h to reach mid-exponential growth phase. 1 mL of either mMRS- or mMRS containing 10 g L^{-1} Tween 80, leading to a final Tween 80 concentration of 1 g L^{-1}, was added followed by further incubation at 30 °C for 0.5 h. Transcription was stopped by the addition of RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) to the culture. Thereafter, mRNA was isolated and purified using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Final RNA concentration was determined via light absorption spectrometry using a NanoDrop™ device (Wilmington, DE, USA).

RNA sequencing and data analysis

Samples were then sent to GATC Biotech (Konstanz, Germany) for RNA sequencing. Sequencing data were analyzed using the Rockhopper software (McClure *et al.*, 2013, Tjaden, 2015) on the basis of the genome of *L. plantarum* TMW 1.708 (Biosample: SAMN05805046 (Kafka *et al.*, 2017)). Genes with corrected p-values lower than 0.05 were considered to show significantly different expression levels. The 2.5 % quantiles with the greatest increase and decrease in expression level were considered for further investigation.

2.7.2 Growth characteristics

To determine the effect of growth medium supplementation with different Tween types, growth characteristics were determined in mMRS-, mMRST20, mMRST40, mMRST60 and mMRST80 as described in 2.3.3. The media were inoculated 1 % (v/v) with pre-cultures of the strains and incubated at 30 °C in a FLUOStar Omega microplate reader (BMG Labtech, Ortenberg, Germany) over a period of 30 h with OD_{600} measurements every 30 min.

2.7.3 Determination of fatty acid composition

For determination of cellular fatty acid profiles, sterile mMRS- was inoculated 1 % (v/v) with a pre-culture of TMW 1.25 or 1.708 and incubated at 30 °C for 24 h. Fresh mMRS-, mMRST20, mMRST40, mMRST60 or mMRST80 was inoculated 1 % (v/v) with this 24 h culture and incubated at 30 °C for another 24 h to give a cell density of ~10^9 cfu mL^{-1}. Cells were harvested by centrifugation (5,000 × g, 5 min, RT), washed three times in quarter-strength Ringer's solution

and finally freeze-dried using a FreeZone Plus 2.5 L freeze dry system (Labonco, Kansas City, MO, USA). The lyophilisate was stored under N_2 atmosphere and sent to the Identification Service of DSMZ (Braunschweig, Germany).

2.7.4 Determination of metabolic activity after HHP treatment

A stock solution of resazurin-Na salt (Serva, Heidelberg, Germany) in IPB was prepared with a concentration of 70 mM. Glucose and fructose were dissolved in IPB, each to a concentration of 15 g L^{-1}. The resazurin-Na salt stock solution was diluted in the sugar-IPB solution to a final concentration of 1 µM and filter-sterilized (0.2 µm pore size, Sarstedt, Nürnbrecht, Germany), giving the resazurin working solution. To measure metabolic activity, 100 µL resazurin working solution were mixed with 100 µL pressure-, heat- or untreated cell suspension (initial viable cell count 10^8 - 10^9 cfu mL^{-1}) and loaded onto white 96-well microtiter plates (F-bottom) (Nunc, Thermo Fisher Scientific, Waltham, MA, USA). Fluorescence intensity (ex/em: 544/590 nm) was measured during incubation at 30 °C in an Omega FLUOStar microplate reader (BMG Labtech, Ortenberg, Germany) every 120 s for a period of 30 min.

2.7.5 Measurement of protein release from cells after HHP treatment

An aliquot of 500 µL of a pressure-, heat- or untreated sample (initial viable cell count 10^8 - 10^9 cfu mL^{-1}) was transferred to a sterile reaction tube and centrifuged (2,800 × g, 15 min, 5° C). The supernatant was filter-sterilized (0,2 µM pore size, Sarstedt, Nürnbrecht, Germany) and protein concentration was measured on black 96-well microtiter plates (F-bottom, Greiner bio-one, Frickenhausen, Germany) using the Pierce™ Coomassie Plus (Bradford) assay kit (ThermoFisher, Waltham, MA, USA) according to the micro MTP protocol provided by the manufacturer. The samples were incubated at 25 °C in the absence of light for 10 min, followed by measuring absorbance at 595 nm in a FLUOStar Omega plate reader (BMG Labtech GmbH, Ortenberg, Germany). Bovine serum albumin provided with the kit was used to establish a standard curve for protein concentrations between 0 and 25 µg mL^{-1}.

2.7.6 Measurement of propidium iodide uptake under HHP

Uptake of propidium iodide (PI) and resulting fluorescence was used to measure HHP-induced membrane permeability, as described by Klotz *et al.* (2010) with some modifications. Suspensions of untreated and heat-inactivated (100 °C, 15 min) cells (see 2.6.3) were used as negative and positive controls, respectively, and were stained with PI simultaneously to the HHP-treated samples.

2.7.6.1 Propidium iodide staining

Total membrane permeability

For the determination of total (transient and permanent) membrane permeability, cell suspensions in IPB containing 10^8-10^9 cfu mL^{-1} were prepared as described in 2.6.1.2 and mixed with Propidium iodide (PI, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) to a final concentration of 3 µM before HHP treatment. Samples were HHP-treated as described in 2.6.2. After pressure release, 500 µL aliquots of HHP treated and control samples were washed twice in IPB (10,000 × g, 5 min, 25 °C) and finally resuspended in 1 mL IPB.

Permanent membrane permeability

For the determination of permanent membrane permeability, 500 µL aliquots of HHP treated (see 2.6.2) and control samples were mixed with PI to a final PI concentration of 3 µM immediately after pressure release, incubated for at least 15 min in the absence of light and subsequently purified as described above by washing twice in IPB (10,000 × g, 5 min, 25 °C) followed by resuspension in 1 mL IPB.

2.7.6.2 Fluorescence measurement

Fluorescence intensity was measured using a filter set with excitation/emission wavelengths of 485/620 nm on black 96-well microtiter plates (F-bottom, Greiner bio-one, Frickenhausen, Germany) using a FLUOStar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Data were normalized by dividing the measured fluorescence intensity values by the OD_{600} measured in parallel (U-bottom, non-sterile, Sarstedt, Nürnbrecht, Germany), in order to exclude effects of variations in cell concentration.

2.7.6.3 Microscopic analysis

For microscopic analysis, samples were treated as explained above with the exception that staining was performed using the LIVE/DEAD BacLight Bacterial Viability kit (Thermo Fisher Scientific, Waltham, MA, USA) containing Syto® 9 and PI. The final concentration of each dye in the sample was 3 µM. 5 µl stained sample spread on a glass microscope slide (Carl Roth, Karlsruhe, Germany) and covered with a coverslip, were examined under an Axiostar plus microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with an epifluorescence unit. Stained cells were visualized using epifluorescence light and appropriate filters. In all cases, a 100x objective was used, giving a total magnification of 1,000-fold. Images were captured with a 1.388- by 1.038-pixel RGB camera (AxioCam ICc1) and were processed with the AxioVS40 V 4.8.2.0 software (Carl Zeiss MicroImaging, Jena, Germany) and ImageJ.

2.8 Data analysis

Where indicated in the figures, differences between measured data were tested for statistical significance using the software SigmaPlot. Initially, the data groups were tested for normal distribution by means of the Shapiro-Wilk test and equality of variance. Criteria were met if p-values > 0.05 were obtained. Comparison of two data groups was performed using the Student's t-test if normal distribution and equal variance were confirmed, and using the non-parametric Mann-Whitney rank sum test otherwise. For comparison of more than two data groups, One-Way Analysis of Variance (ANOVA) followed by a post-hoc Student-Newman-Keuls test or Kruskal-Wallis One-Way ANOVA on ranks were performed for normally distributed data sets with equal variance or otherwise, respectively. Data sets were considered significantly different when p-values were < 0.05.

3 Results

This study was performed to assess the impact of fat on the aqueous-phase-dependent HHP inactivation of vegetative bacterial cells in model O/W emulsion using spoilage-associated *Lactobacillus* strains as model organisms. Furthermore, the cellular response to preconditioning by fat and fat-associated substances and its effect on HHP sensitivity was examined. Parts of the results presented in this this chapter were presented in recent publications (Reitermayer *et al.*, 2018), and "Interaction of fat and aqueous phase parameters during high-hydrostatic pressure inactivation of *Lactobacillus plantarum* in oil-in-water emulsions" (Reitermayer *et al*., currently under review). The selection of strains and determination of basic parameters of emulsions are in part consistent with the results presented in the PhD thesis of Thomas Kafka (2018), Lehrstuhl für Technische Mikrobiologie, Technische Universität München.

3.1 Model system O/W emulsions

O/W emulsions were used as a model system to investigate the interaction between fat and different aqueous phase parameters with respect to the HHP sensitivity of *L. plantarum* strains. Starting from an O/W emulsion containing 50 % (v/v) rapeseed oil dispersed in pressure-stable IPB at pH 7 and stabilized by 1 % (v/v) Tween 80 as emulsifier, aqueous phase parameters NaCl and sucrose concentration as well as the type and concentration of protein were varied in a food-relevant range. For the variation of the pH, citrate-phosphate buffer (CPB) was used as aqueous phase. In order to exclude possible effects of lipid phase parameters and allow for sound comparison of emulsions with varying aqueous phase parameters, differences in the area of the oil-water interface and droplet size were minimized as far as possible by adapting the process parameters of the two-step homogenization process for the individual emulsions. The storage stability of the prepared emulsions was assessed by visual inspection and reevaluation of the droplet size and oil-water interface after a storage period and by determination of the creaming rate. To ensure stability under the pressure conditions used in this study, emulsions were treated with the most intense pressure conditions used in this study and examined again with respect to droplet size oil-water interface and creaming rate after pressure treatment.

3.1.1 Emulsion characteristics

O/W emulsions with varying aqueous phase parameters were prepared as described in 2.2.1 and subsequently measured by laser diffraction to determine (I) the area of the oil-water interface expressed as Specific Surface Area (SSA), which is given by the surface area of all droplets in a given unit volume of emulsion and (II) the volume median diameter D(v,0.5), which

describes the droplet diameter at which 50 % of the population is smaller and 50 % is larger. The SSA values of all emulsions used in this study are shown in Figure 1, and the corresponding D(v,0.5) values are displayed in Figure 2. A complete overview of all emulsions and the corresponding manufacturing parameters are given in Table 17 (Appendix). The standard emulsion without additional solutes in the aqueous phase manufactured with homogenization pressures of 100 (step 1) and 20 (step 2) bar showed an average SSA of 3.74 m^2 g^{-1} and a D(v,0.5) of 2.58 µm. The addition of low amounts of NaCl to the aqueous phase slightly reduced the SSA and increased the droplet size, whereas with 25 % (w/v) NaCl the opposite was observed. With increasing sucrose concentrations SSA gradually increased while D(v,0.5) decreased to values of 4.63 m^2 g^{-1} and 2.13 µm, respectively. A change of the buffer system to CPB and variation of the pH value in the aqueous phase from 7 down to 3 had no impact on the droplet size and fat surface, as all emulsions prepared with CPB with the standard process parameters (100/20 bar) showed SSA values between 3.67 and 3.84 m^2 g^{-1} and D(v,0.5) between 2.44 and 2.57 µm (Figure 1C and Figure 2C). In contrast, the addition of protein to the aqueous phase resulted in an increase in SSA and a decrease in D(v,0.5). By adapting the process parameters (see Appendix Table 17) SSA values between 4.48 and 5.44 m^2 g^{-1} and D(v,0.5) values between 1.57 and 2.10 µm could be obtained (Figure 1D and Figure 2D). To obtain a comparable reference emulsion without additives, an additional standard emulsion containing plain IPB was prepared using process pressures of 300/60 bar, showing an SSA of 5.22 m^2 g^{-1} and a D(v,0.5) of 1.60 µm. The preparation of emulsions with casein concentrations higher than 2.5 % (w/v) was not possible since casein is insoluble in aqueous environments, making processing of the emulsion impossible.

Since only small differences in droplet size and overall fat surface were observed among emulsions with the same aqueous phase parameter, possible secondary effects resulting from the lipid phase could be excluded.

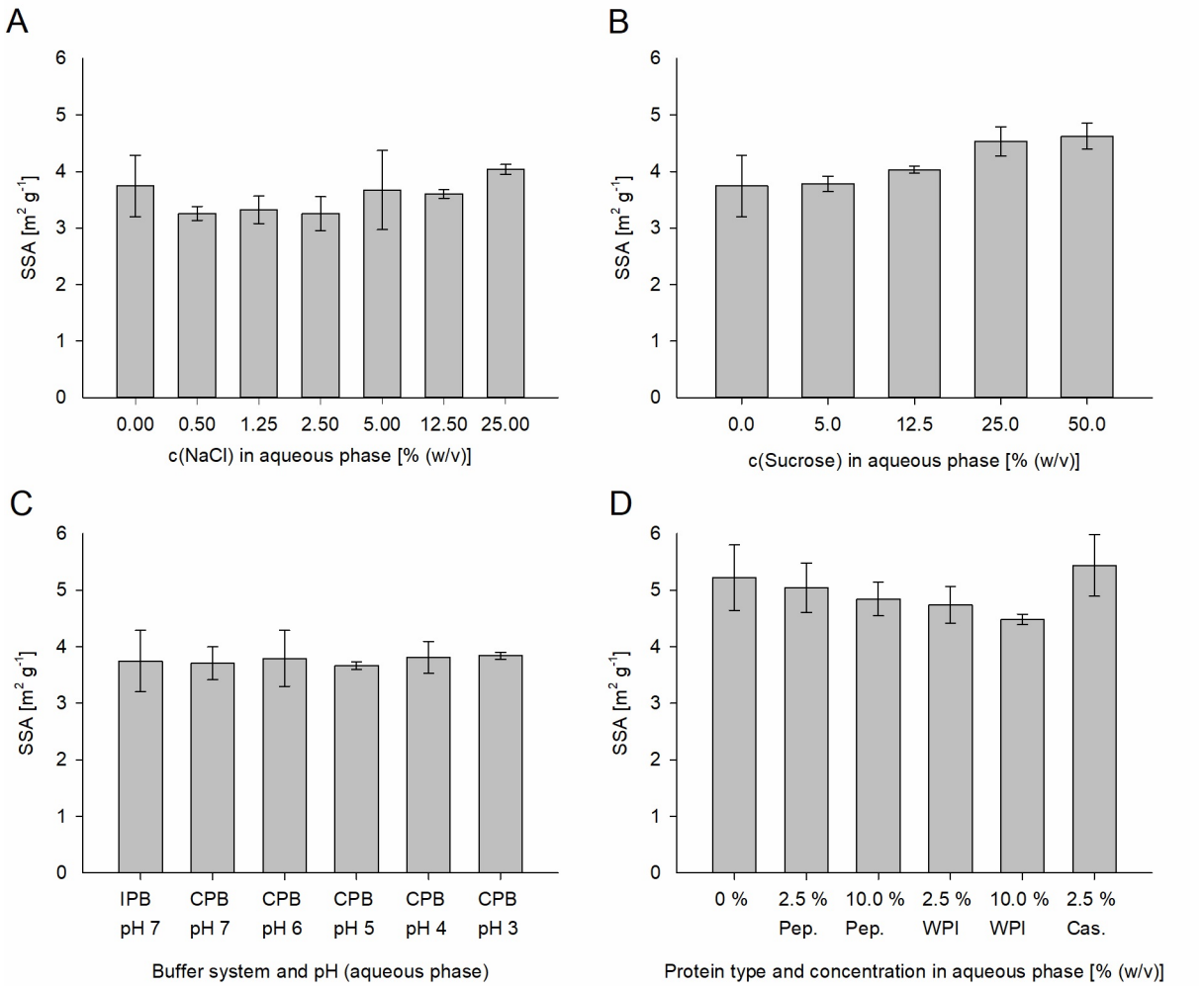

Figure 1: Specific surface area of prepared O/W emulsions. O/W emulsions with varying (A) NaCl concentration, (B) sucrose concentration, (C) pH value and (D) protein type and concentration (Pep. = Peptone; Cas. = Casein) were prepared by two-step homogenization using appropriate homogenization pressures. The specific surface area (SSA) was determined using a laser particle size analyzer. The presented values are the means of at least three replicates. Error bars represent the standard deviation.

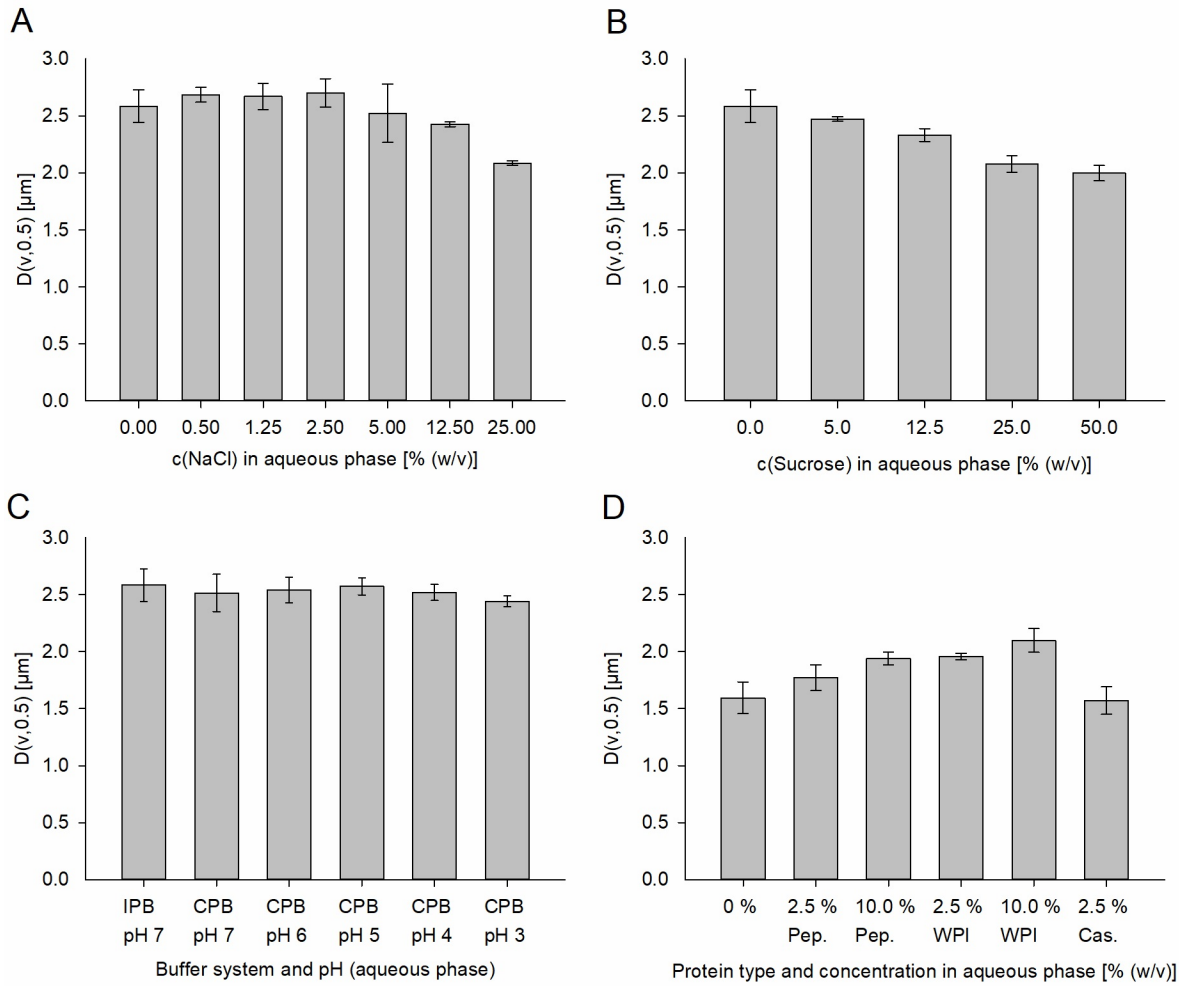

Figure 2: Median droplet size of prepared O/W emulsions. O/W emulsions with varying (A) NaCl concentration, (B) sucrose concentration, (C) pH value and (D) protein type and concentration (Pep. = Peptone; Cas. = Casein) were prepared by two-step homogenization using appropriate homogenization pressures. The median droplet size D(v,0.5) was determined using a laser particle size analyzer. The presented values are the means of at least three replicates. Error bars represent the standard deviation.

3.1.2 Storage stability

In order to ensure that the prepared emulsions still show the initially determined characteristics (droplet size, oil-water contact area) at the time of conduction of the HHP inactivation experiments, their stability was assessed with respect to creaming and coalescence.

3.1.2.1 Creaming

Creaming is especially relevant for emulsions in which the droplets of the dispersed phase are loosely packed and able to move freely within the continuous phase. Due to the lower density of fat compared to aqueous solutions, the oil droplets in O/W emulsions tend to move upwards and accumulate in the upper part of the emulsion, leading to a heterogeneous distribution of the two phases within the emulsion. The creaming rate of the prepared emulsions was assessed by

multi-sample analytical centrifugation using a LUMiFuge® device (LUM GmbH, Berlin, Germany) at conditions (2,474 rpm, 25 °C, 700 s) (see Figure 3 and Appendix Table 17).

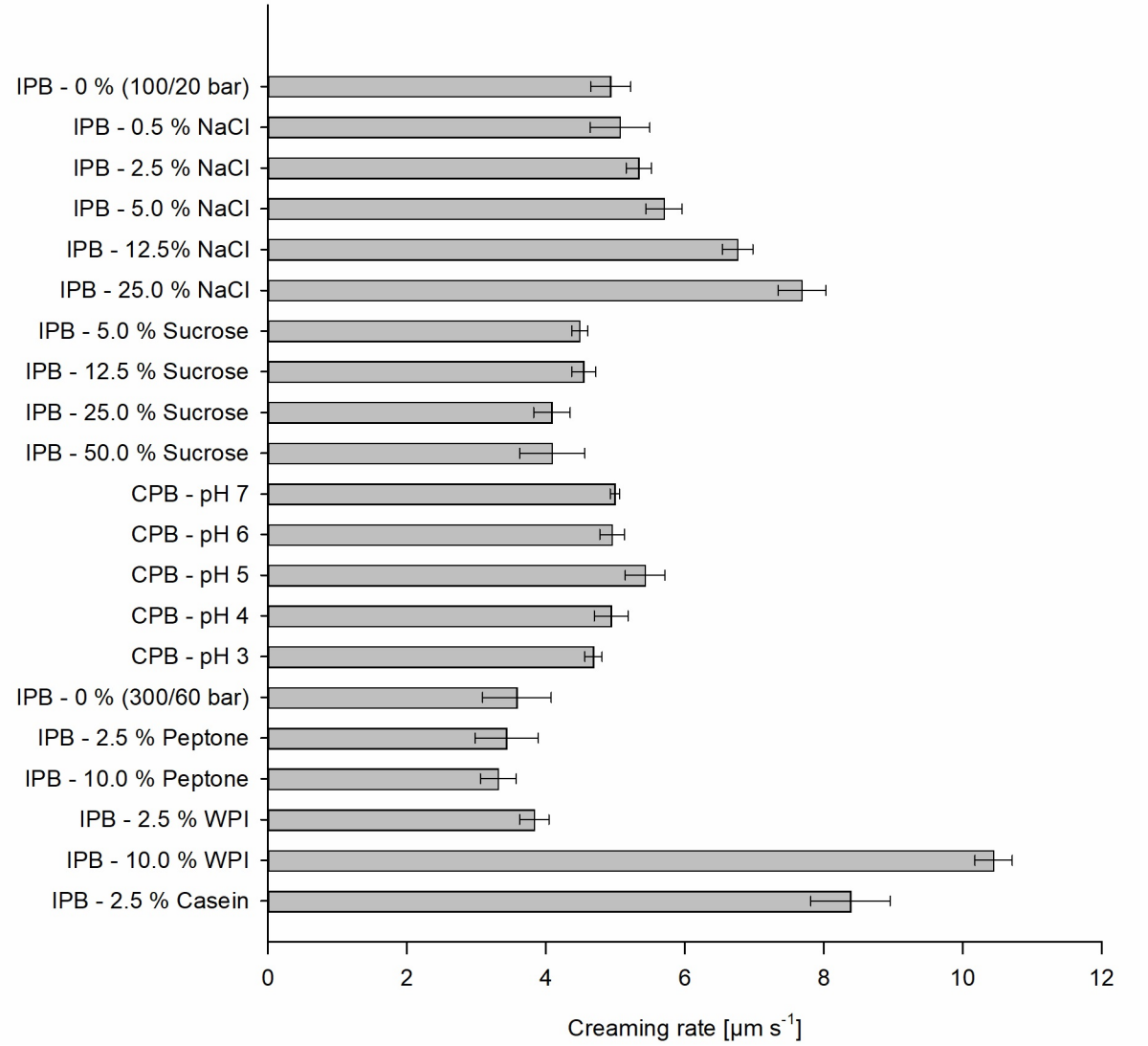

Figure 3: Creaming rate of prepared O/W emulsions. The creaming rates of O/W emulsions were determined using analytical centrifugation immediately after preparation. The buffer system and the type and concentration of solute in the aqueous phase are indicated on the left. The presented values are the means of at least three replicates. Error bars represent the standard deviation.

While the standard emulsion (0 % solute, 100/20 bar) showed a creaming rate of 4.93 µm s^{-1}, the addition of increasing amounts of NaCl caused an increase in creaming rate up to 7.69 µm s^{-1} with 25 % NaCl, showing that NaCl may contribute to emulsion destabilization. Sucrose, in contrast led to a slight reduction in creaming rate (4.09 µm s^{-1} with 50 % sucrose). While changing the buffer solution to CPB and lowering the pH value had no effect on creaming (4.68 – 5.53 µm s^{-1}), emulsions with different protein types and concentrations showed great

variation: Peptone and the low WPI concentration of 2.5 % showed creaming rates (3.32 – 3.83 µm s^{-1}) similar to those of the corresponding standard emulsion prepared with 300/60 bar (3.58 µm s^{-1}), whereas 10 % WPI and 2.5 % Casein dramatically enhanced creaming, leading to rates of 10.44 and 8.38 µm s^{-1}, respectively.

3.1.2.2 Coalescence

Re-evaluation of SSA and D(v,0.5) values after storage at 4 °C for 9 days and comparison with values obtained immediately after preparation showed that the droplet size did not change over the mentioned time frame in any of the emulsions (Appendix Table 18), conforming that no coalescence had occurred. Visual inspection of the emulsions after storage at 4 °C for a period of 9 d (Appendix Figure 47 - Figure 50) revealed no visible change of any of the emulsions except for those with 20 % WPI and 2.5 % casein. The fact that the observed phase separation was reversible by agitation (Appendix Figure 51) confirmed that creaming, and not coalescence, had occurred. These results confirmed the stability of the prepared emulsions for at least 9 days. Nevertheless, emulsions were used only during the week of preparation in order to avoid microbial spoilage, since the homogenization process was performed under non-sterile conditions.

3.1.3 Pressure stability

In order to obtain significant results from HHP inactivation studies it had to be ensured that the examination system is stable even under the high-pressure conditions applied during the experiments. Therefore, the prepared emulsions were subjected to HHP treatment using the maximum pressure conditions (500 MPa, 25 °C, 5 min) applied in the subsequent inactivation study, and the droplet size and the creaming rate were measured after HHP treatment and compared to the values obtained immediately after preparation (3.1.1).

3.1.3.1 Effect of HHP on droplet size

For most of the examined emulsions, HHP treatment caused no alterations in oil-water interface (Figure 4) and median droplet size (Figure 5). However, the emulsions with 1.25 % to 5 % NaCl and with 25 % and 50 % sucrose experienced a slight reduction of SSA with a concomitant increase in D(v,0.5), indicating a slight coalescence of the dispersed phase under pressure. The strongest effect was observed with the 1.25 % NaCl emulsion, as the SSA decreased from 3.32 to 2.32 m^2 g^{-1} and D(v,0.5) increased from 2.67 to 3.13 µm. Visual evaluation revealed slight gel formation in emulsions containing WPI and casein (data not shown). This effect was stronger with the higher (10 %) compared with the lower (2.5 %) WPI concentration. However, evaluation

of the SSA and D(v,0.5) values provided no evidence for coalescence of oil droplets. The observed changes in the respective emulsions were taken into account during interpretation of the results of HHP inactivation experiments.

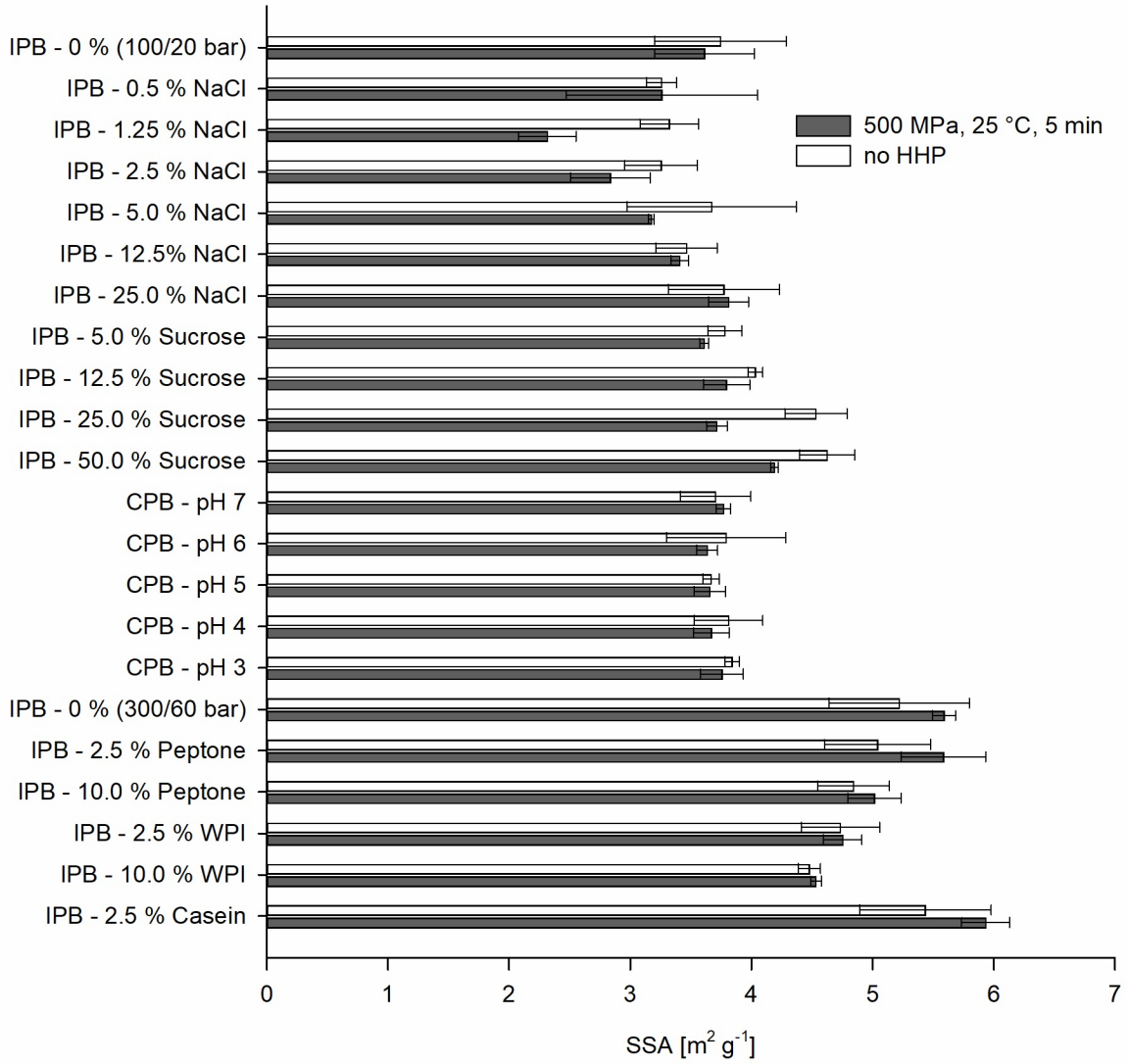

Figure 4: Specific surface area of prepared O/W emulsions before and after HHP treatment. O/W emulsions were treated with HHP (500 MPa, 25 °C, 5 min). Afterwards, specific surface area was determined using a laser particle size analyzer and compared to untreated samples of the same emulsions. The presented values are the means of at least three replicates. Error bars represent the standard deviation.

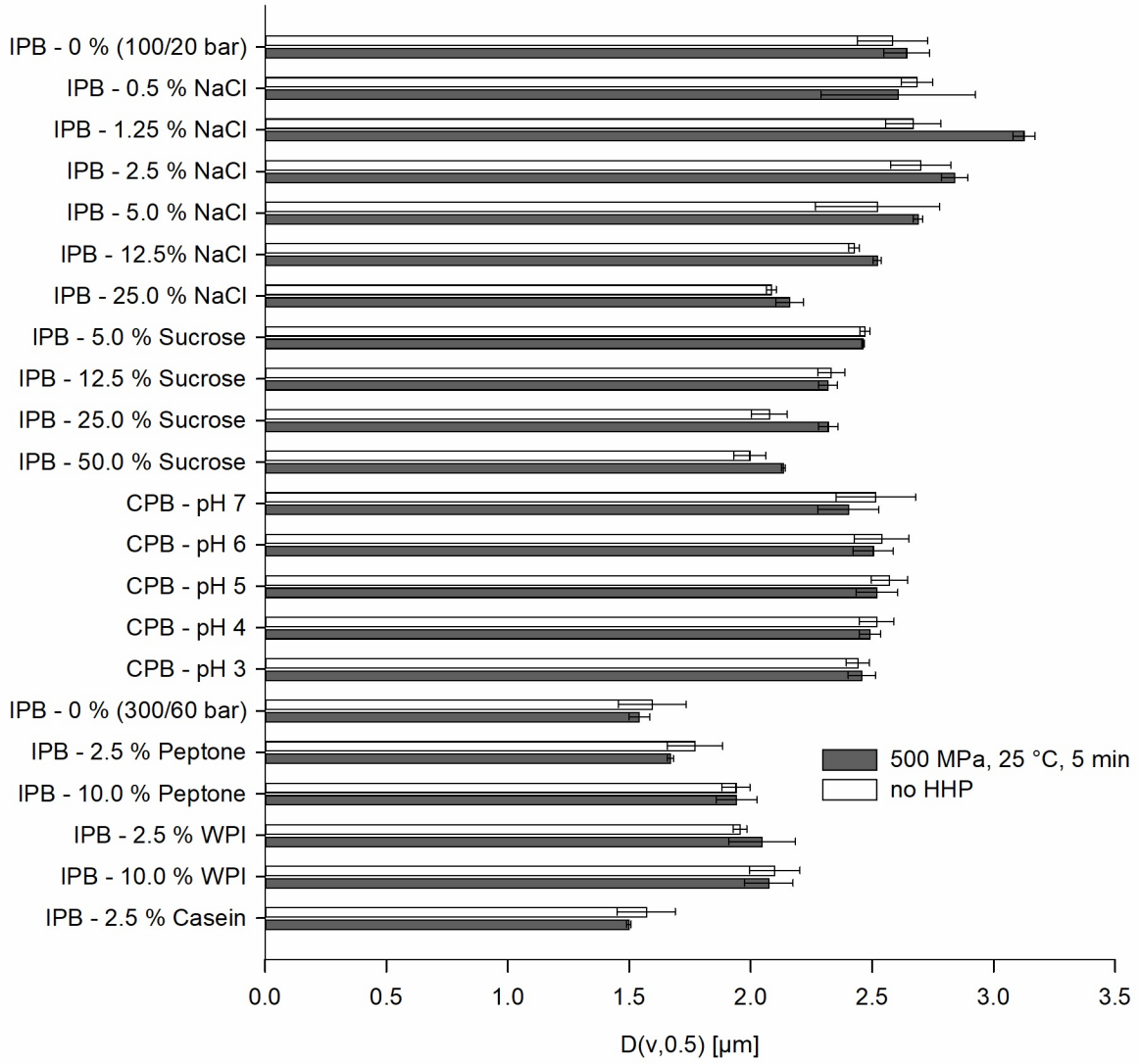

Figure 5: Median droplet size of prepared O/W emulsions before and after HHP treatment. O/W emulsions were treated with HHP (500 MPa, 25 °C, 5 min). Afterwards, median droplet size was determined using a laser particle size analyzer and compared to untreated samples of the same emulsions. The presented values are the means of at least three replicates. Error bars represent the standard deviation.

3.1.3.2 Effect of HHP on creaming behavior

As shown in Figure 6 the application of HHP (500 MPa, 25 °C, 5 min) in general had no effect on the creaming rate. Only for the emulsions with 50 % sucrose and 10 % WPI creaming rate was slightly reduced from 4.09 to 3.10 µm s^{-1} and from 10.44 to 8.85 µm s^{-1}, respectively. Since the creaming rate was not increased by HHP in any of the investigated emulsions, stability under HHP with respect to the creaming behavior was demonstrated.

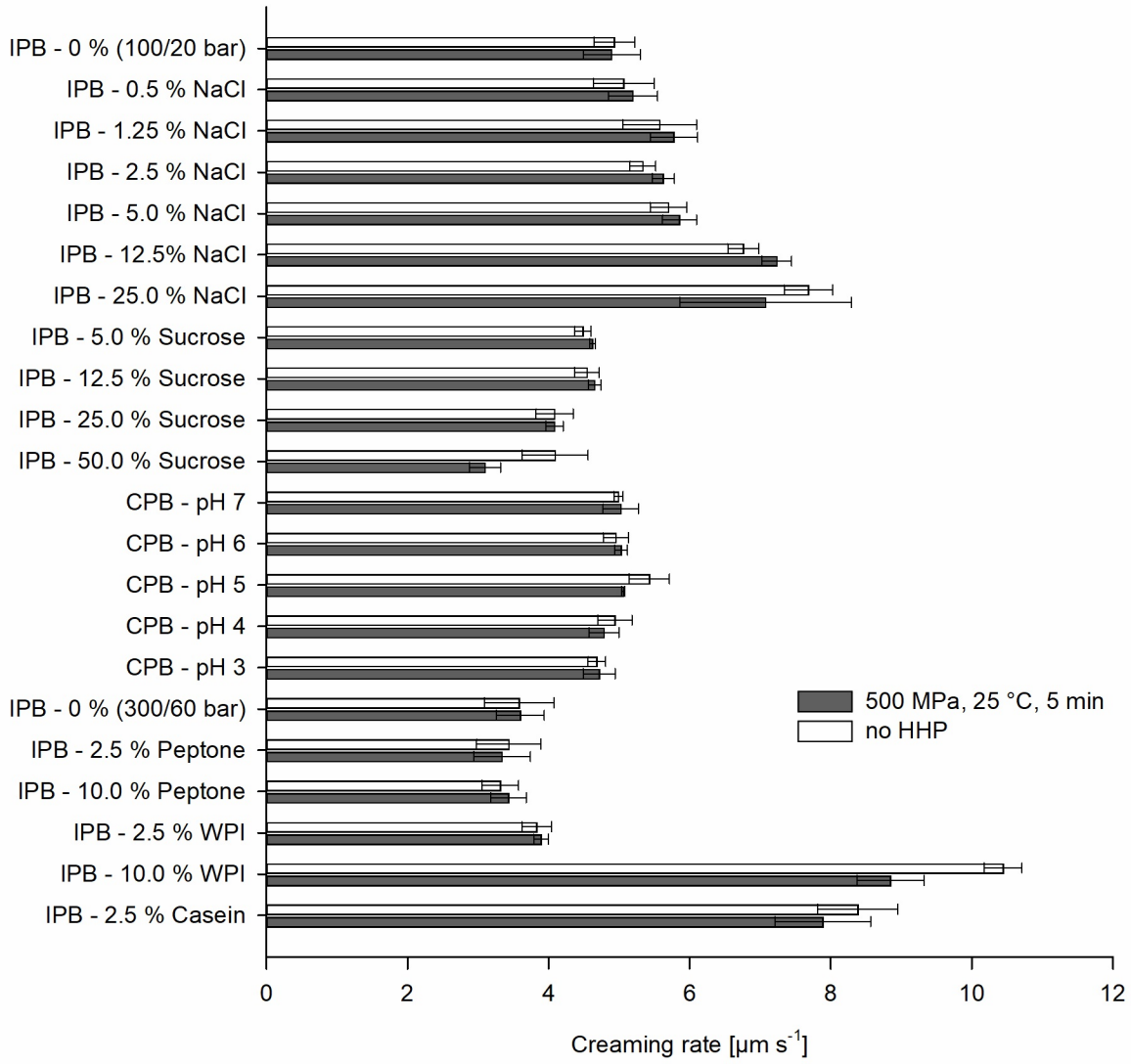

Figure 6: Creaming rate of prepared O/W emulsions before and after HHP treatment. O/W emulsions were treated with HHP (500 MPa, 25 °C, 5 min). Afterwards, the creaming rate was determined by analytical centrifugation and compared to untreated samples of the same emulsions. The presented values are the means of at least three replicates. Error bars represent the standard deviation.

3.2 Strain selection

Initially, 38 strains belonging to one of the three *Lactobacillus* species, *L. plantarum*, *L. sakei* and *L. fructivorans* were selected from the strain collection of the Chair of Technical Microbiology Weihenstephan (TMW) based on their isolation source and characteristics discovered in previous studies indicating relevance for food-spoilage. This section describes a preparatory screening with the aim of selecting two strains with high spoilage potential, preferably differing in their surface characteristics with respect to hydrophobicity or, more precisely, affinity to organic, hydrophobic surfaces.

3.2.1 Cell surface hydrophobicity of *Lactobacillus* strains

3.2.1.1 Screening for *Lactobacillus* strains with different surface characteristics

Since fat is insoluble in aqueous systems, the lipid phase can exist as finely dispersed droplets like in O/W emulsions, but still remains separate on the molecular level. Therefore, the effect of fat on suspended microorganisms may depend on their localization within the matrix, i.e. whether or not a direct contact to the lipid phase exists. The affinity to fat may depend on CSH, which varies greatly on species as well as on strain level. To select strains differing in CSH, the set of 38 pre-selected strains was screened using the MATH test (see 2.4.2). In this assay, an aqueous cell suspension is agitated with an organic liquid. Cells with hydrophobic surfaces tend to adhere to the organic liquid, thus being removed from the aqueous suspension, leading to a reduction in optical density. The extent of CSH is then determined by comparison of the optical density before and after agitation. As shown in Figure 7, the two *L. fructivorans* strains TMW 1.452 and TMW 1.59 had strongly hydrophobic cell surfaces, as the OD_{600} was reduced to 3 % and 2 % of the initial value, respectively. The third *L. fructivorans* strain, TMW 1.1856, showed intermediate hydrophobicity with a relative OD_{600} of 60 %. In contrast, almost all tested *L. sakei* strains showed strongly hydrophilic cell surfaces. The widest range of intra-species variation was observed within the species *L. plantarum*: Some strains of this species showed a reduction in OD_{600} by ~90 % (TMW 1.25 and TMW 1.277), whereas others, such as TMW 1.1, TMW 1.64 and TMW 1.1308, almost completely remained suspended in the aqueous phase, as shown by a reduction of the OD_{600} by less than 5 %. In addition, a considerable fraction of *L. plantarum* strains showed intermediate CSH with relative OD_{600} values between 40 and 60 %.

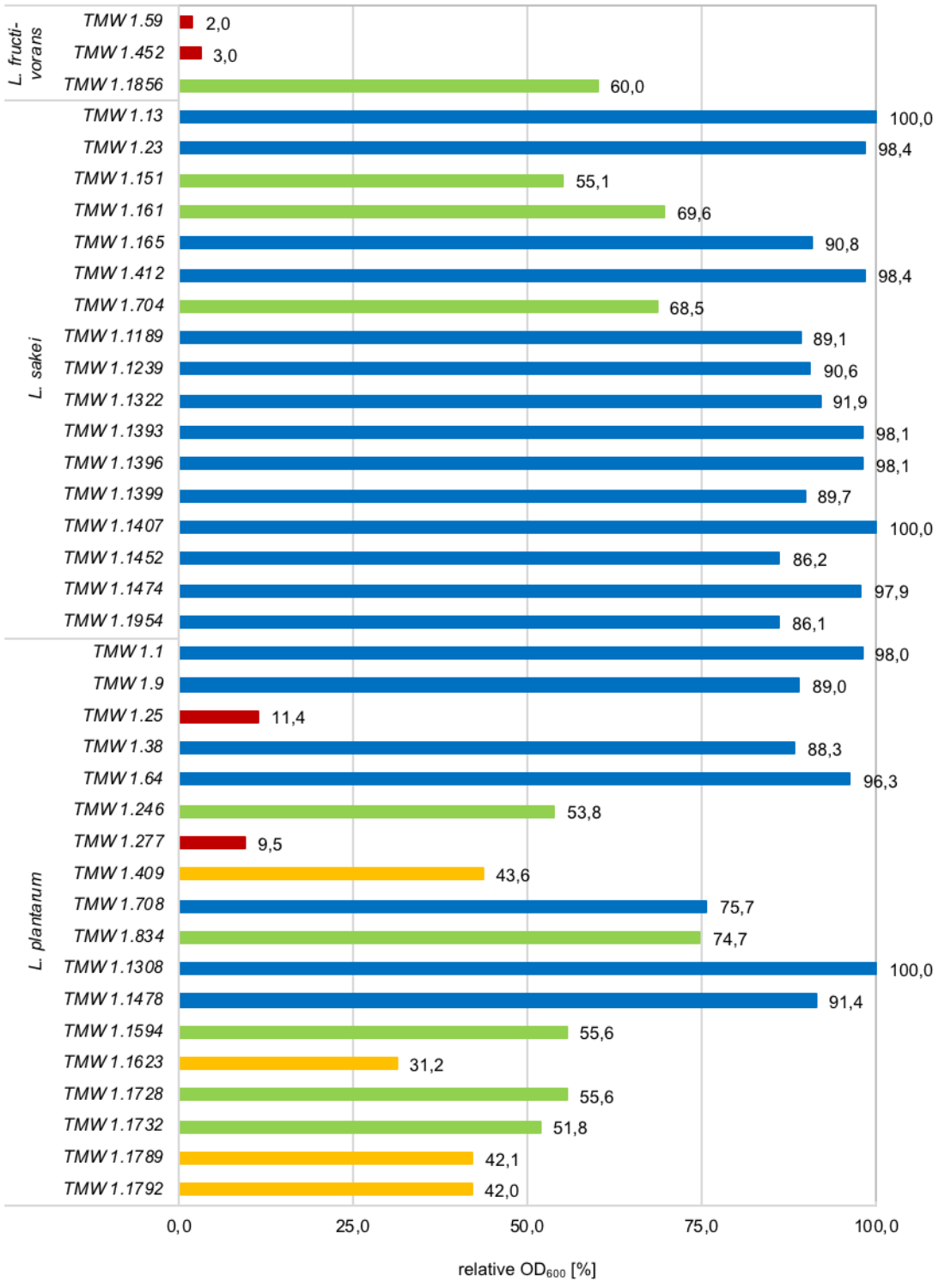

Figure 7: CSH of pre-selected *Lactobacillus* strains in exponential growth phase. The extent of cell adhesion to the organic phase was determined by comparison of the optical density of the cell suspensions before and after agitation in the presence of n-hexadecane. Strains were classified and color-coded in the diagram according to the relative optical density into highly hydrophobic (0.0 – 25.0 %; red), moderately hydrophobic (25.1 – 50.0 %; yellow), moderately hydrophilic and (50.1 – 75.0 %; green) and highly hydrophilic (75.1 – 100.0 %; blue) strains.

3.2.1.2 Effect of growth phase on CSH

The cell envelope of bacteria changes depending on culture age and growth phase (see 1.1.4.2.3). To test whether the growth phase also influences CSH and to be able to select the appropriate culture age for further experiments, subsets of at least two strains per species with strongly different surface characteristics (3.2.1.1) were selected and subjected to MATH test after harvesting in exponential or stationary phase. The results depicted in Figure 8 (see also Appendix Table 20) show that in general CSH changes only marginally from exponential to stationary phase with surface characteristics being slightly more pronounced in stationary than in exponential phase. The strongest enhancement was observed for *L. plantarum* strain TMW 1.708. A slight weakening of the (hydrophobic) surface properties was observed only for *L. fructivorans* strain TMW 1.59.

These results show that cells with hydrophilic or hydrophobic surface characteristics mostly retain these properties throughout different growth phases, confirming that both exponential and stationary phase cells are suitable for HHP inactivation studies.

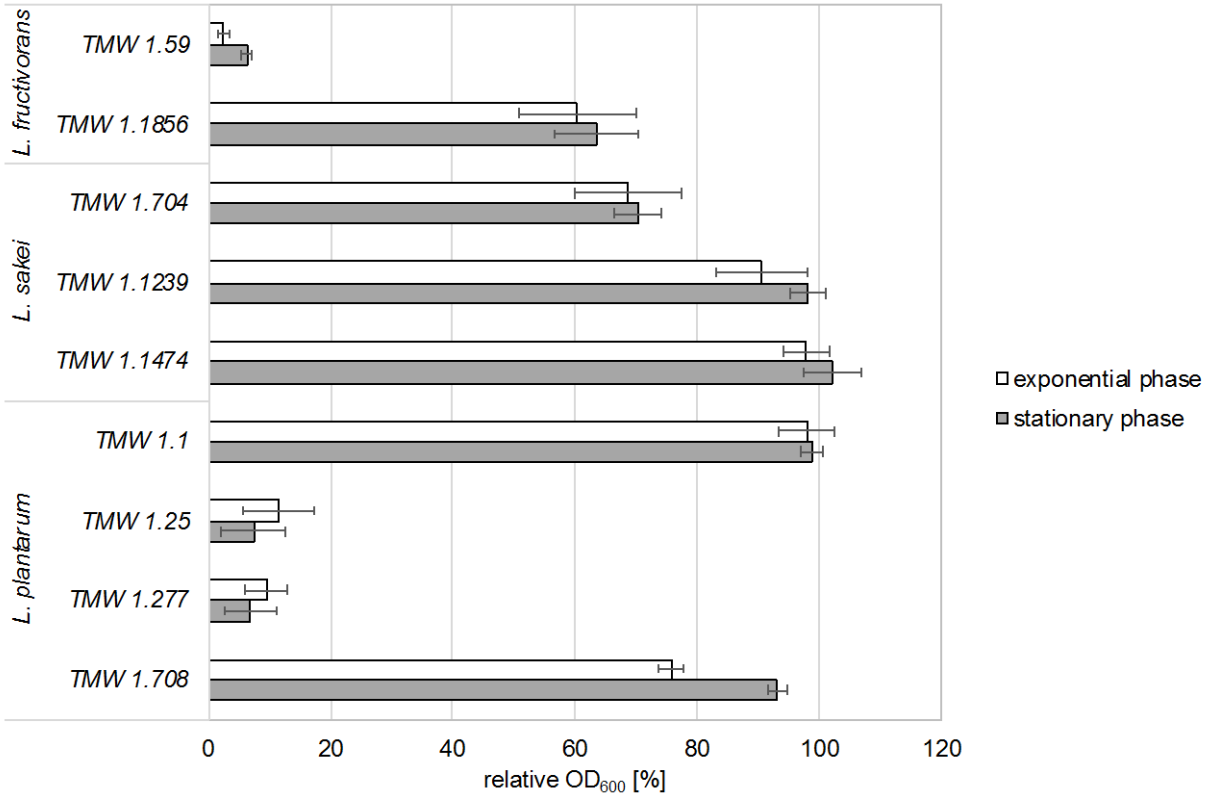

Figure 8: CSH of pre-selected *Lactobacillus* strains in exponential and stationary phase . MATH test was performed with cells harvested in exponential (white bars) and stationary (gray bars) growth phase. Bars indicate the relative OD_{600} after agitation with n-hexadecane compared to the OD_{600} before agitation. Presented values are the means of at least three independent experiments. Error bars represent the standard deviation.

3.2.2 Biodiversity of *Lactobacillus* strains on proteomic level

On the cell surface of Gram-positive bacteria there are a variety of molecular structures, including proteins. Since proteins vary widely in terms of hydrophobicity, they are assumed to influence bacterial cell surface characteristics. It was therefore hypothesized that specific surface proteins are responsible for the development of hydrophobic or hydrophilic surface characteristics and that the presence or absence of these proteins manifests in the proteomic profile of the respective strains. To examine a possible correlation between CSH and the proteomic composition of the cells, MALDI-TOF-MS analysis was performed with the 38 pre-selected *Lactobacillus* strains grown to stationary phase, recording specific mass spectra based on small peptide fragments. Differences in these spectra were visualized by high-throughput multidimensional scaling (HiT-MDS) and are shown as the Euclidean distance in a 2-dimensional plane. When comparing the spectra of all 38 strains, the three species appeared clearly distinguished from each other, demonstrating that the proteomic profiles within one species show much higher similarity than among different species (Figure 9). Consequently, the impact of species-specific proteomic properties was much stronger than the impact of the present proteins, possibly associated with CSH.

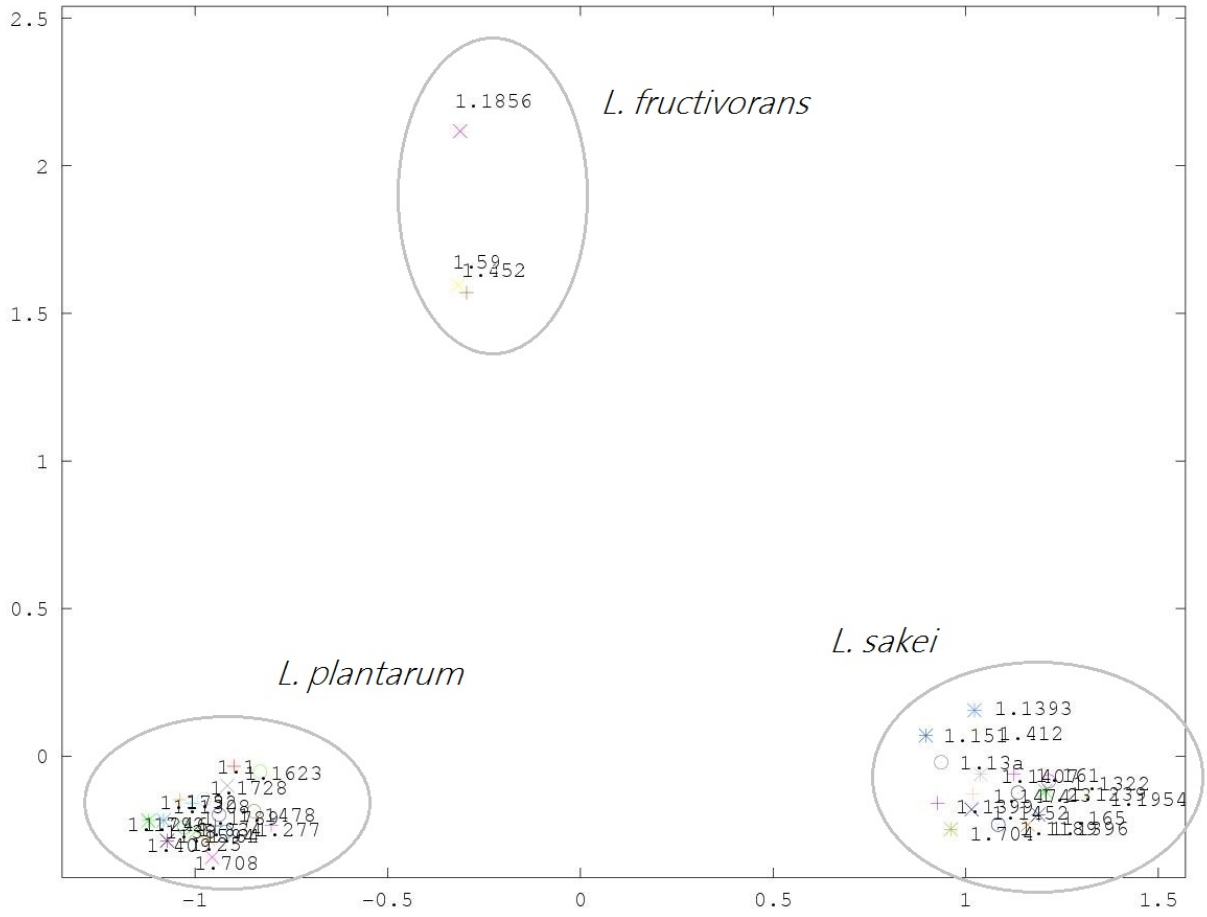

Figure 9: Multidimensional scaling of *Lactobacillus* strains according to MALDI-TOF MS mass spectra. Mass spectra of stationary phase cells of the 38 pre-selected strains of the species *L. plantarum*, *L. sakei* and *L. fructivorans* were investigated concerning mutual distance by multidimensional scaling. Each strain is marked with a different symbol accompanied by its TMW number. The clusters obtained for the different species are defined by elliptical framing.

To gain a more detailed insight into biodiversity within each species and to test for a correlation between CSH and the proteomic profile on strain level, the spectra of strains belonging to individual species were subjected to MDS. The fact that only three *L. fructivorans* strains were considered, makes an interpretation of the MDS impossible (see Appendix, Figure 52). MDS of *L. sakei* strains led to a central cluster containing most of the strains and one or two outliers in each direction (Figure 10A). The three strains with moderately hydrophilic cell surfaces were found evenly distributed and closer to highly hydrophilic strains than to each other. Likewise, most *L. plantarum* strains clustered in the center with one or two outliers in each direction and no evidence for correlation of CSH and proteomic data (Figure 10B). However, the only two highly hydrophobic *L. plantarum* strains, TMW 1.25 and TMW 1.277, show similar protein mass spectra that markedly differ from the other *L. plantarum* strains. In this case, CSH might be related to the proteomic profile, but no correlation between the two features was observed in general.

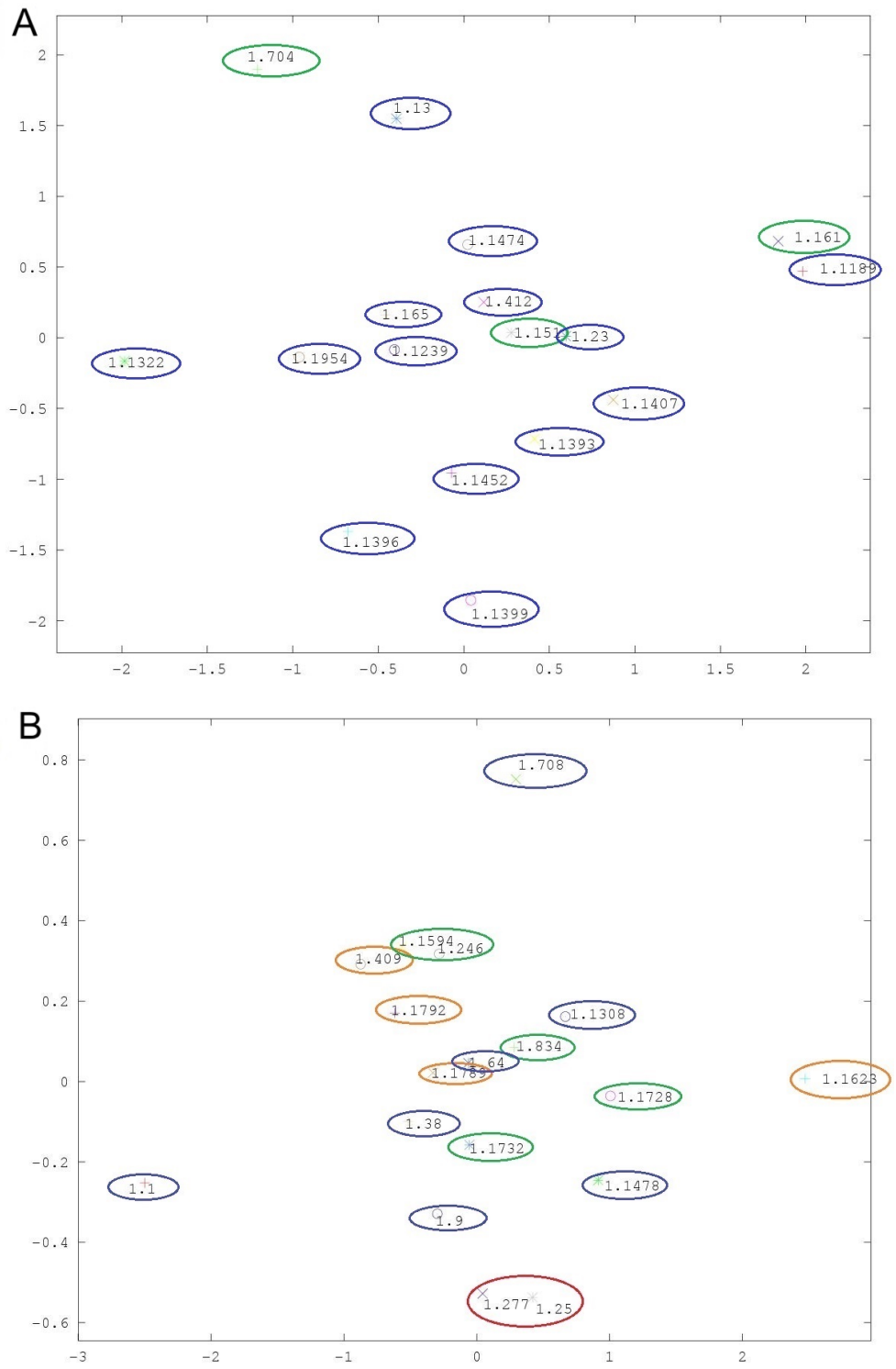

Figure 10: Multidimensional scaling of *L. sakei* and *L. plantarum* strains according to MALDI-TOF MS mass spectra. Mass spectra of stationary phase cells of the pre-selected *L. sakei* (A) and *L. plantarum* (B) strains were investigated concerning mutual distance by multidimensional scaling. Each strain is marked with a different symbol accompanied by its TMW number. The degree of CSH, as determined in 3.2.1.1, is indicated by colored markings corresponding to Figure 7 (red: highly hydrophobic; yellow: moderately hydrophobic; green: moderately hydrophilic; blue: highly hydrophilic).

3.2.3 Strain selection based on CSH, proteomic biodiversity and isolation source

Considering the results from determination of CSH (3.2.1), the proteomic profile (3.2.2) and, if applicable, information on the origin, fourteen strains out of the initial 38 were selected for further investigation (see Table 14). Of each species, strains with strong, intermediate and weak CSH were chosen, and it was ensured that the selection contained both strains with highly characteristic proteomic profiles, i.e. form the margin of the MDS cluster, and strains representative for the respective species from the center of the MDS clusters. In general, strains with a known, food-relevant source of isolation were favored.

Table 14: Overview of 14 *Lactobacillus* strains selected for further investigation. The strains were selected based on CSH, proteomic profile and, if applicable, the source of isolation.

Species	Strain (TMW #)	Cell surface characteristics (MATH)	Biodiversity on proteomic level (Position in MDS 2-D plane)	Source of isolation
L. fructivorans	1.59	Highly hydrophobic	n/a	Unknown
	1.1856	Moderately hydrophilic	n/a	Whey
L. sakei	1.151	Moderately hydrophilic	Central	Unknown
	1.704	Moderately hydrophilic	Marginal (top)	Sourdough
	1.1239	Highly hydrophilic	Central	Sourdough
	1.1322	Highly hydrophilic	Marginal (left)	Unknown
	1.1474	Highly hydrophilic	Central	Unknown
L. plantarum	1.1	Highly hydrophilic	Marginal (left)	Raw sausage
	1.25	Highly hydrophobic	Marginal (bottom)	Raw sausage
	1.277	Highly hydrophobic	Marginal (bottom)	Palm wine
	1.708	Highly hydrophilic	Marginal (top)	Raw sausage
	1.834	Moderately hydrophilic	Central	Unknown
	1.1478	Highly hydrophilic	Central	Honey
	1.1623	Moderately hydrophilic	Marginal (right)	Breast milk

3.2.4 Compatibility with the examination system

To ensure the integrity of the O/W emulsions used as examination system during the HHP inactivation experiments of this study, strains with the potential to destabilize the O/W emulsions by degradation of emulsion constituents had to be eliminated. Furthermore, it was necessary to ensure that organic components, alone and combinations thereof, added to the aqueous phase of O/W emulsions, do not trigger growth of suspended bacteria in order to prevent possible alterations in the physiological state, the cell number and HHP sensitivity. For this, the degradation, uptake or metabolization of organic components of the O/W emulsions, i.e. fat, the emulsifier Tween 80 and the different components of the aqueous phase like sucrose and protein by the selected 14 strains (see 3.2.3) was assessed.

3.2.4.1 Lipolytic activity

Many bacterial strains produce extracellular lipases to make lipid compounds in their environment accessible. Lipolytic activity was determined on the basis of degradation of tributyrin, a triglyceride consisting of glycerol esterified with three butyric acid moieties, thus sharing the basic structure with the molecules present in edible fats, only differing in the fatty acid chain length. Lipolytic strains are identified by transparent halos in the otherwise turbid, tributyrin-containing substrate that are formed around the growing colonies due to tributyrin degradation resulting from the esterase activity of extracellular lipases. Figure 11: Bacterial growth on mMRS-TB agar shows one representative strain of each of the three *Lactobacillus* species investigated and of *B. subtilis* strain TMW 2.472 with known lipolytic activity as positive control after growth on agar containing 10 % tributyrin. The remaining *Lactobacillus* strains are depicted in Figure 53: Bacterial growth on mMRS-TB agar (Appendix). In contrast to the positive control *B. subtilis*, none of the *Lactobacillus* strains investigated caused halo formation, demonstrating the absence of significant lipolytic activity that could lead to destabilization of O/W emulsions.

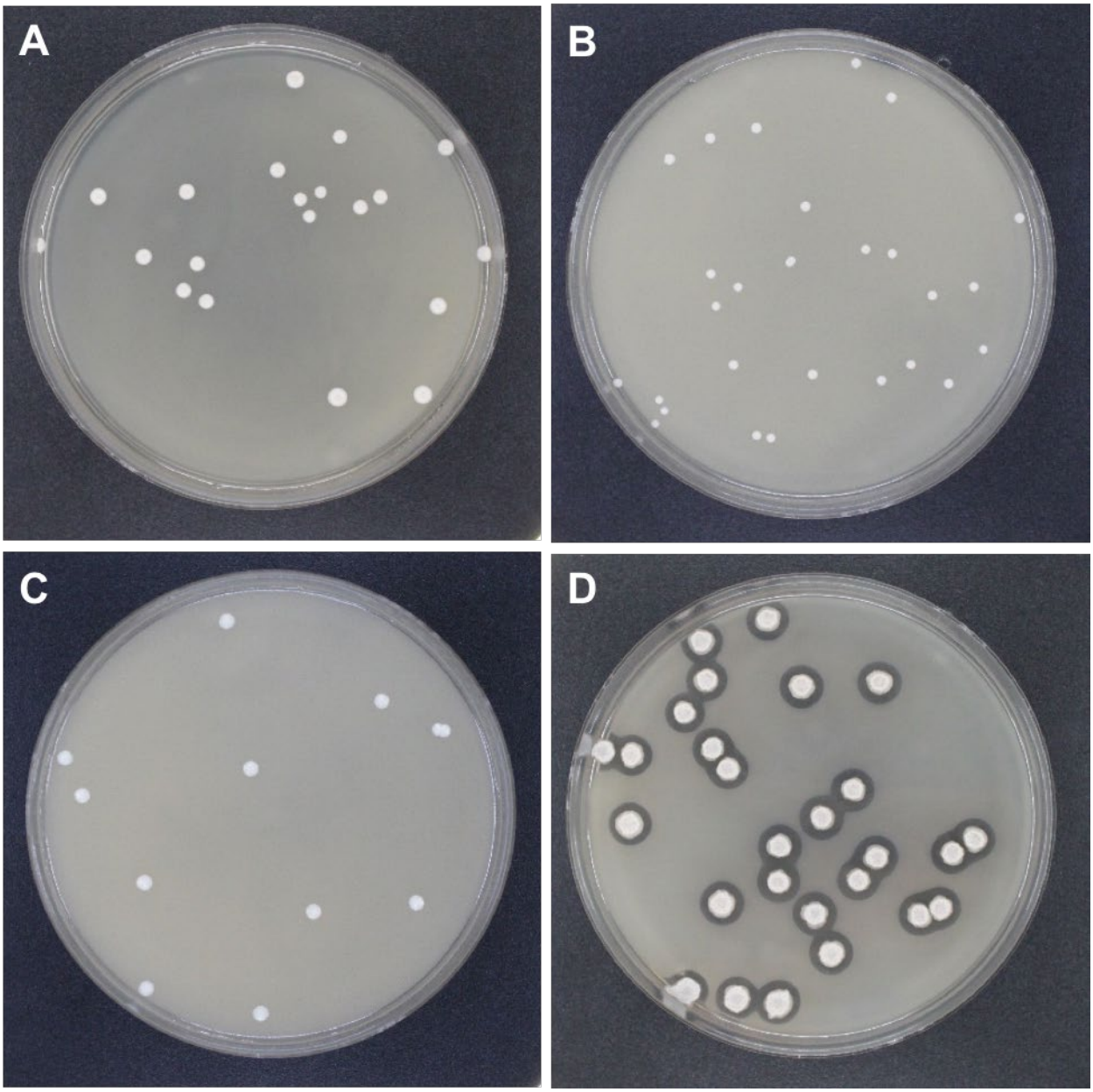

Figure 11: Bacterial growth on mMRS-TB agar. (A) *L. fructivorans* TMW 1.1856, (B) *L. sakei* TMW 1.1474, (C) *L. plantarum* TMW 1.25. and (D) *B. subtilis* TMW 2.472

3.2.4.2 Degradation of emulsifier

The emulsifier Tween 80 used for stabilization of O/W emulsions in this study is widely applied as a growth enhancing agent in bacterial cultivation media, providing an easily accessible source of fatty acids. However, the degradation of the emulsifier by suspended bacteria had to be precluded in order to prevent destabilization of the O/W emulsions and it was hypothesized that in the absence of other essential nutrients cells are unable to degrade Tween 80. To prove this, the selected strains were tested for growth in IPB containing 1 % (w/v) Tween 80

representing the Tween 80 concentration in the O/W emulsions used in this study, by means of optical density on microtiter plates for a period of 15 h, which spaciously covers the contact time with O/W emulsions during the planned HHP inactivation experiments. Growth in pure IPB and mMRS medium was recorded in parallel as negative and positive control, respectively. Comparison of the maximum OD_{600} reached within 15 h incubation with the respective initial OD_{600} values showed that none of the tested strains was able to increase cell density in IPB containing solely Tween 80 (Appendix Table 21). Therefore, there is no evidence for the degradation of Tween 80 in O/W emulsions by any of the selected *Lactobacillus* strains.

3.2.4.3 Degradation of aqueous phase components

A central part of this study deals with the investigation of aqueous phase parameters of O/W emulsions, including the presence and concentration of sucrose and protein-based substances in the form of casein, whey protein isolate (WPI) and peptone. Many bacteria secrete extracellular proteases that cleave proteins in their environment into small peptides to make them ready for cellular uptake. The proteolytic activity of the selected *Lactobacillus* strains was measured to assess their potential to degrade whole proteins like casein and WPI. Being low-molecular organic compounds, both sucrose and peptone constitute potential, readily accessible nutrients for bacteria. The degradation of peptone and sucrose was examined by determination of growth characteristics in IPB supplemented with the solutes in the concentrations found in the O/W emulsions of this study. Even though the presence of one organic compound may not be able to trigger growth, the combination with other potential nutrients might be sufficient. Therefore, the combinations of peptone or sucrose with emulsifier Tween 80 in the relevant concentrations were additionally examined, as these represent the real conditions found in the O/W emulsions.

3.2.4.3.1 Proteolytic activity

The proteolytic activity of the selected strains was assessed through the degradation of fluorescence-labeled FTC-casein. For this, stationary phase cells were incubated in IPB containing FTC-casein, and the maximum fluorescence intensity (FI_{max}) as well as the maximum increase in fluorescence intensity over a certain period of time (ΔFI_{max}) were determined (Figure 12 and Appendix Table 22). *B. subtilis* strain TMW 2.472 with known proteolytic activity and TPCK trypsin were used as positive controls.

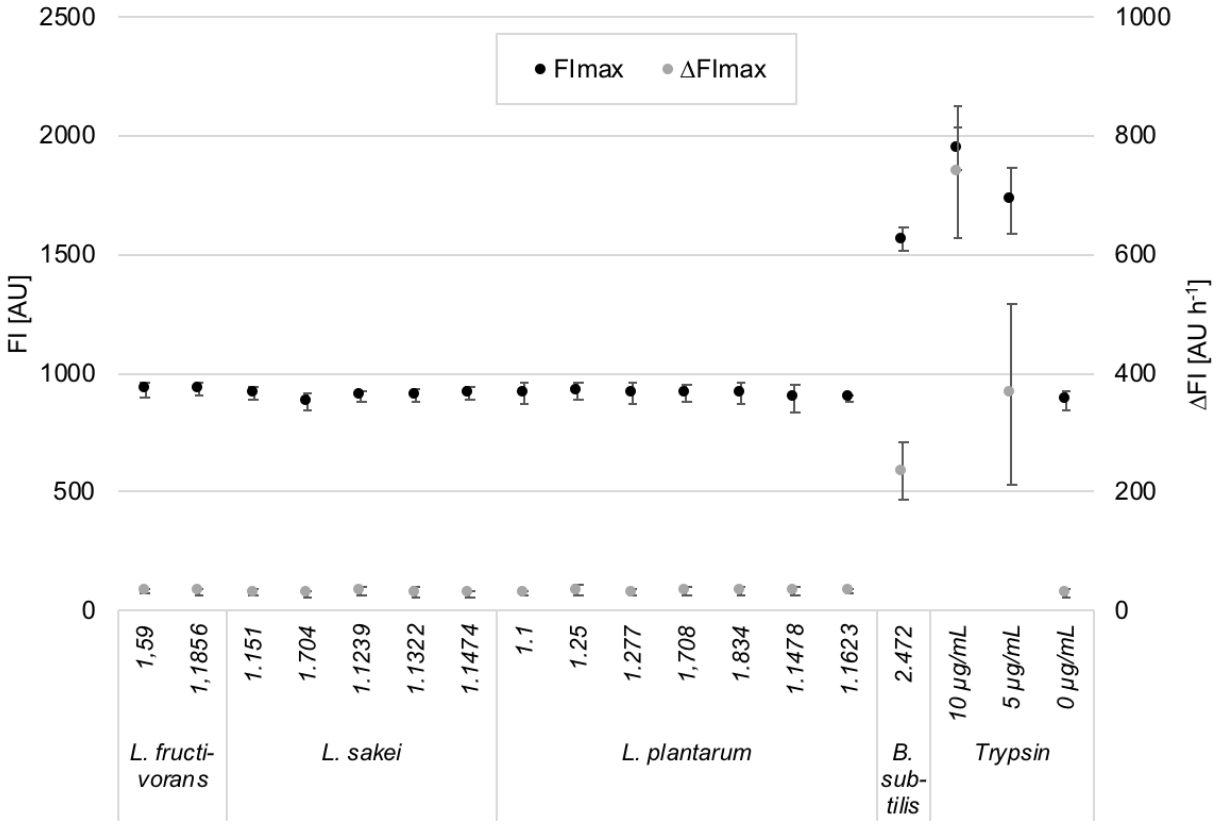

Figure 12: Proteolytic activity of pre-selected *Lactobacillus* strains. Stationary phase cells were suspended in IPB containing FTC casein and incubated for 10 h at 25 °C. Fluorescence intensity (FI) was measured every 5 min during incubation using a filter set with 485/520 nm (excitation/emission) and a gain of 300. Proteolytically active *B. subtilis* strain TMW 2.472 and TPCK trypsin were used as biological and chemical positive control, respectively. IPB containing FTC casein was used as negative control. Pure IPB without FTC casein was used as blank and values were subtracted from all measurement values for correction. FI_{max}: maximum FI value recorded during incubation; ΔFI_{max}: maximum increase in FI (Data were smoothed by calculating the mean of three subsequent values in order to correct for random fluctuations). Error bars represent standard deviation of three independent measurements.

The positive controls TPCK trypsin and *B. subtilis* TMW 2.472 caused a marked increase in fluorescence intensity (FI) over time, reaching FI_{max} values between 1500 and 2000 AU and ΔFI_{max} of 234 AU h^{-1} (TMW 2.472), 739 AU h^{-1} (10 µg mL^{-1} trypsin) and 366 AU h^{-1} (5 µg mL^{-1} trypsin). In contrast, none of the *Lactobacillus* strains exceeded the negative control (FTC casein in IPB), showing FI_{max} values of ~900 AU and ΔFI_{max} values of ~30 AU h^{-1}. These results show that none of the pre-selected *Lactobacillus* strains possesses considerable proteolytic activity.

3.2.4.3.2 Degradation of peptone

The potential of the selected strains to utilize peptone as the sole carbon and energy source was assessed by measuring maximum OD_{600} reached in IPB containing peptone in the concentrations found in the aqueous phase of the O/W emulsions over a period of 6 h, which covers the contact time during the planned HHP inactivation experiments (Appendix Table 23).

OD_{600} values of the *L. plantarum* strains increased from 0.14 - 0.19 and 0.09 - 0.12 to 0.25 - 0.52 and 0.36 - 0.51 in the presence of 2.5 % (w/v) and 10 % (w/v) peptone, respectively. Likewise, *L. sakei* strains showed an increase in OD_{600} from 0.09 - 0.11 (2.5 % peptone) and 0.05 - 0.06 (10 % peptone) to 0.15 - 0.30 and 0.14 - 0.29, respectively. In contrast, the OD_{600} of the two *L. fructivorans* strains did not increase in IPB containing peptone. However, even the positive controls in mMRS medium showed only a slight increase in OD_{600} after 6 h, demonstrating a weak growth behavior of this species in general. These results show that all tested *L. plantarum and L. sakei* strains have the potential to degrade peptone present in the aqueous phase of emulsions and thus may experience physiological alterations during the presence in such environments.

3.2.4.3.3 Degradation of sucrose

In analogy to peptone, the potential to grow on sucrose in the absence of other nutrients was determined based on the maximum OD600 reached in IPB containing sucrose in the concentrations corresponding to those used in HHP inactivation experiments over a period of 6 h. Comparison of the initial and the maximum OD600 values revealed that cell density did not increase during the incubation period for any of the tested strains in the presence of sucrose, providing no evidence for any of the tested strains to degrade sucrose in the O/W emulsions or corresponding buffer solutions used in this study (see Appendix Table 24).

3.2.4.3.4 Growth on mixtures of Tween 80 with sucrose or peptone

The growth behavior of the selected strains on combinations of peptone or sucrose with 1 % Tween 80 was examined as described above for peptone (3.2.4.3.2) and sucrose (3.2.4.3.3) alone. As shown in

Table **25** (see Appendix), none of the tested strains showed growth in IPB containing mixtures of Tween 80 and sucrose, as indicated by maximum OD_{600} values comparable to the initial OD_{600} values and those of the negative control IPB alone. Therefore, there is no evidence for degradation of the used O/W emulsions containing sucrose by any of the selected strains. In the presence of Tween 80 and 2.5 % and 10 % peptone, the OD_{600} of the *L. sakei* strains increased from 0.07 - 0.08 and 0.04 - 0.05 to 0.12 - 0.36 and 0.14 - 0.32, respectively. For *L. plantarum* strains, the increase was even stronger with OD_{600} values rising from 0.12 - 0.17 (2.5 % peptone) and 0.08 - 0.11 (10 % peptone) to 0.24 - 0.54 and 0.23 - 0.54, respectively. For *L. fructivorans* no growth was observed, although it has to be considered that also the positive controls in mMRS showed only a marginal increase in OD_{600} during the 6 h incubation period.

The OD_{600} increase of *L. sakei* and *L. plantarum* was comparable to that observed with 2.5 % and 10 % peptone without Tween 80 (see 3.2.4.3.2), demonstrating that the addition of Tween 80 had no further growth-enhancing effect.

3.2.5 Spoilage potential

Further selection of appropriate strains was done based on their relevance for food spoilage. For this, their ability to cope with different hurdles used in food preservation to prevent bacterial growth and spoilage, including acidity (low pH), low a_w and low temperature, was assessed by determination of growth parameters such as the maximum growth rate (μ_{max}) and maximum cell density ($maxOD_{600}$).

Acid tolerance was examined by growth in mMRS medium buffered with citric acid and aspartic acid (mMRS-CA) and adjusted to different pH values (pH 4.5, 4.0, and 3.5) in a food-relevant range. mMRS-CA adjusted to pH 6.2, the pH value of standard mMRS, was used as positive control. The ability to grow at low a_w was examined by cultivation in mMRS medium with NaCl added in concentrations of 5 %, 6 %, 7.5 % and 10 % (w/v), leading to a_w values of 0.961, 0.955, 0.948 and 0.930, respectively. For the investigation of cold tolerance, strains were cultivated in standard mMRS at 10 °C and 4 °C.

An overview of the determined growth parameters at all investigated conditions is given in Table 26, Table 27 and Table 28 (see Appendix). To allow for direct comparison, the condition of each of the three stress parameters leading to the most striking differences among the strains was chosen for illustration of $maxOD_{600}$ and μ_{max} in Figure 13 and Figure 14, respectively.

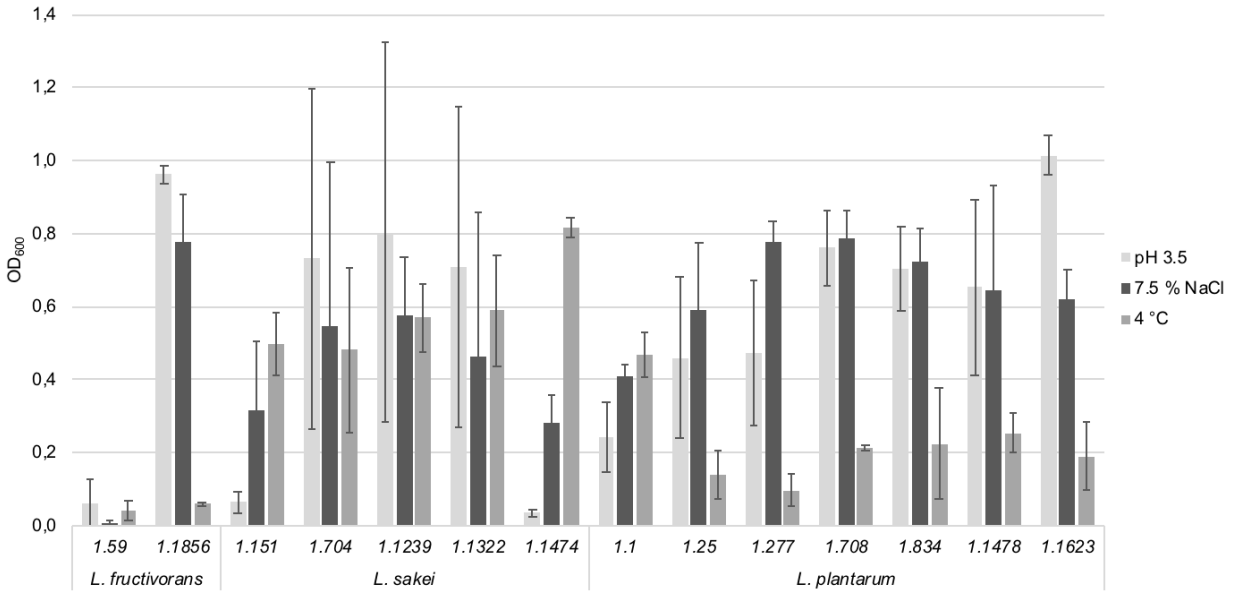

Figure 13: Maximum optical density (OD_{600}) reached by *Lactobacillus* strains at low pH, low a_w and low temperature conditions. Liquid cultures of the strains in mMRS-CA (pH 3.5), mMRS containing 7.5 % NaCl (a_w = 0.948) or standard mMRS were incubated at 30 °C for 96 h with OD_{600} measurement every 30 min (pH 3.5, 7.5 % NaCl) or at 4 °C for 9 days with OD_{600} every 24 h (4 °C), respectively. Presented data are the mean values of at least three independent measurements. Error bars represent the standard deviation.

L. fructivorans strain TMW 1.59 showed maxOD_{600} values below 0.1 and thus only marginal growth at the stress conditions shown. The second *L. fructivorans* strain TMW 1.1856, in contrast, reached the second highest maxOD_{600} values at pH 3.5 (0.96) and 7.5 % NaCl (0.78), but showed weak growth at 4 °C. Strains of the species *L. sakei* were characterized by a high variability in terms of acid tolerance, with strains TMW 1.151 and 1.1474 showing almost no growth at pH 3.5 and TMW 1.704, 1.1239 and 1.1322 reaching maxOD_{600} values between 0.71 and 0.80, however with great variation among different replicates. At 7.5 % NaCl *L. sakei* strains reached maxOD_{600} values between 0.32 and 0.58. maxOD_{600} values between 0.48 and 0.82 at 4 °C and the fact that TMW 1.1474 was the most cold-tolerant of all strains demonstrated the great cold tolerance of *L. sakei* in comparison with the other two species. *L. plantarum* strains also varied widely in terms of maxOD_{600} at pH 3.5: While TMW 1.1 remained at an OD_{600} of 0.24, TMW 1.1623 reached a value of 1.01. With OD_{600} values between 0.41 (TMW 1.1) and 0.79 (TMW 1.708) at 7.5 % NaCl, *L. plantarum* strains were slightly more tolerant to low-a_w environment than *L. sakei*, in general. Remarkably, TMW 1.1, showing the lowest maxOD_{600} in the high acid and low a_w environments, was the most cold-tolerant of all *L. plantarum* strains and the only *L. plantarum* strain reaching a maxOD_{600} (0.47) comparable to those of observed with most *L. sakei* strains at 4 °C, whereas other *L. plantarum* strains only reached values between 0.10 and 0.25.

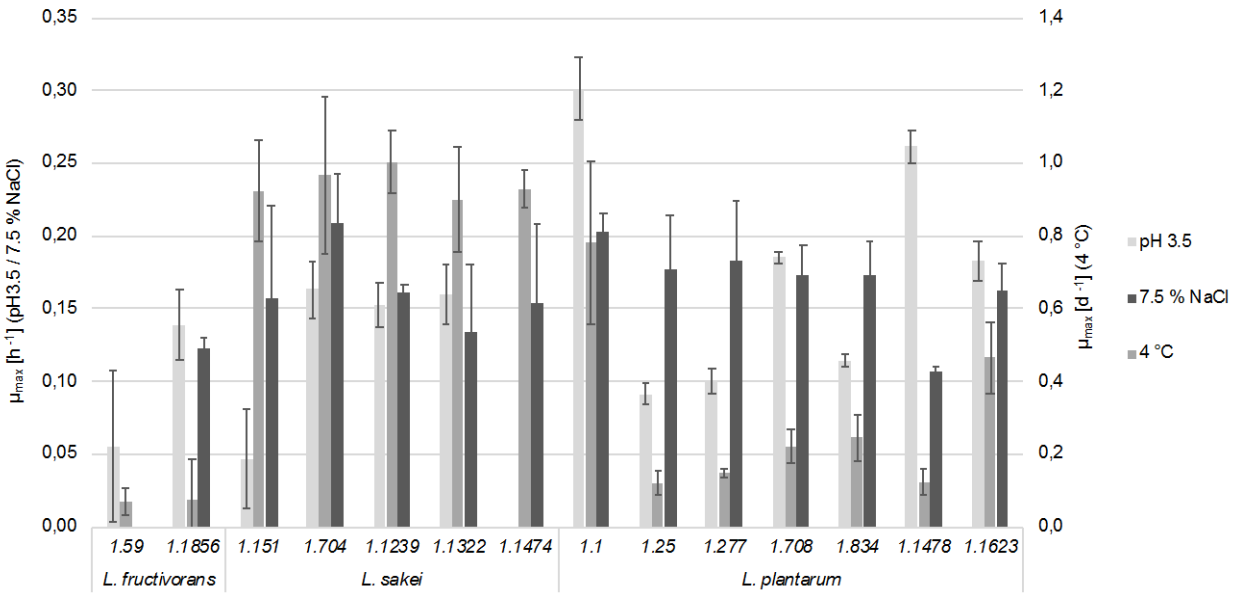

Figure 14: Maximum growth rate (μ_{max}) reached by *Lactobacillus* strains at low pH, low a_w and low temperature conditions. Liquid cultures of the strains in mMRS-CA (pH 3.5), mMRS containing 7.5 % NaCl (a_w = 0.948) or standard mMRS were incubated at 30 °C for 96 h with OD_{600} measurement every 30 min (pH 3.5, 7.5 % NaCl) or at 4 °C for 9 days with OD_{600} every 24 h (4 °C), respectively. Presented data are the mean values of at least three independent measurements. Error bars represent the standard deviation.

Corresponding to the values of the maximum optical density, *L. fructivorans* strain TMW 1.59 showed slow growth at pH 3.5 (μ_{max} 0.06 h^{-1}) and 4 °C (0.07 d^{-1}) and did not grow at all at 7.5 % NaCl. TMW 1.1856, in contrast, showed maximum growth rates of 0.14 h^{-1} at pH 3.5 and of 0.12 h^{-1} at 7.5 % NaCl, but slow growth at 4 °C (0.08 d^{-1}).

L. sakei strains TMW 1.151 and TMW 1.1474 showed very slow or now growth at all at pH 3.5, respectively, whereas maximum growth rates of ~0.15 h^{-1} were detected with the other three *L. sakei* strains. This demonstrates a strong intraspecies variation in terms of acid tolerance. At 7.5 % NaCl maximum growth rates of *L. sakei* strains varied between 0.13 and 0.21 h^{-1}. In line with the observed maximum OD values (Figure 13) *L. sakei* strains showed considerably higher growth rates (0.90 - 1.00 d^{-1}) than the two other species at 4 °C, demonstrating a relatively high cold tolerance of this species. The only *L. plantarum* strain with a growth rate comparable to *L. sakei* at 4 °C was TMW 1.1 with μ_{max} of 0.78 d^{-1}. The other *L. plantarum* strains showed very slow growth at 4 °C (0.12 - 0.47 d^{-1}). At pH 3.5, *L. plantarum* strains varied strongly in terms of μ_{max}, with TMW 1.1 reaching the highest growth rate of all strains (0.30 h^{-1}) and TMW 1.25 exhibiting the slowest growth (~0.09 h^{-1}) of the *L. plantarum* strains. μ_{max} values of *L. plantarum* strains at 7.5 % NaCl varied between ~0.11 h^{-1} (TMW 1.1478) and 0.20 h^{-1} (TMW 1.1), which approximately corresponds to the range observed with *L. sakei*.

3.2.6 Selection of appropriate strains for HHP inactivation studies

Based on the results of the experiments on proteomic biodiversity, CSH, the potential to destabilize O/W emulsions, spoilage potential and additional information, such as the source of isolation, two strains were selected as model organisms for the further experiments. The two selected strains should differ greatly in terms of CSH, but belong to the same species to allow for sound comparison in other respects. They also should be relevant food spoilage organisms with considerable spoilage potential, but should have no destabilization activity towards the components of the O/W emulsions used in the further experiments.

The investigations described in (3.2.4) provide no indication of destabilization of the examination system by any of the selected *Lactobacillus* strains, and none of the strains seemed to be able to start growth and thus undergo physiological changes under the conditions present in the O/W emulsions and corresponding buffer solutions used in this study. The sole exception identified as a potential target for degradation was peptone, which caused slight growth of all tested *L. plantarum* and *L. sakei* strains. Since especially TMW 1.59 as one of the two *L. fructivorans* strains showed the lowest spoilage potential of all pre-selected strains, this species could not be further considered. Thus, potential degradation of peptone-based O/W emulsions was taken into account. Since growth on peptone was still weak compared to the positive control mMRS, a rapid conduction of the experiments with preferably short contact times with the respective matrices was considered sufficient to avoid a significant perturbation of the examination system and physiological state of the bacteria. Nonetheless, the degradation potential for peptone was taken into account during the interpretation of results from HHP inactivation.

Strains of the species *L. sakei* showed the greatest cold tolerance, whereas *L. plantarum* strains seemed to better cope with low-pH and low-a_w conditions. Since all *L. sakei* strains had considerably hydrophilic surfaces, *L. plantarum* strains, showing a great intra-species variation in CSH, were considered for further selection. TMW 1.25 and 1.277 had by far the most hydrophobic, and TMW 1.1, 1.1478 and 1.708 the most hydrophilic cell surfaces. Showing considerably high spoilage potential with lower variability than TMW 1.1 and 1.1478, TMW 1.708 was selected as the hydrophilic model strain. Sharing the source of isolation, namely raw sausage, with TMW 1.708, TMW 1.25 was chosen as its hydrophobic counterpart.

3.3 Localization of *L. plantarum* in oil-water systems

Being two immiscible fluids, oil and water remain separate on the microscopic scale in emulsions, even if they form a macroscopically homogeneous mixture. Therefore, bacterial cells suspended in the aqueous phase of an emulsion are not necessarily in direct contact with fat. However, the effect of fat during HHP treatment may depend on the proximity and/or physical interaction of fat and microorganisms. Therefore, it was tested whether the prediction made on the basis of the results of the MATH test stating that hydrophobic but not hydrophilic cells attach to oil droplets, can be confirmed in the real examination system. Furthermore, it was examined whether the presence of emulsifier, which coats the oil droplets making the lipid phase inaccessible for cells suspended in the aqueous phase, affects the localization of suspended bacterial cells.

3.3.1 Localization in oil-water mixtures

On the basis of the results obtained from MATH test (see 3.2.1), it was hypothesized that only the hydrophobic cells of TMW 1.25, but not those of the hydrophilic strain TMW 1.708, attach to oil droplets dispersed in an aqueous solution. The localization of the two selected strains was investigated microscopically in 1:1 mixtures of rapeseed oil and IPB. As shown in Figure 15, the cells of the hydrophobic strain TMW 1.25 readily adhered to the oil droplets, whereas cells of TMW 1.708 with hydrophilic surfaces showed only casual adhesion.

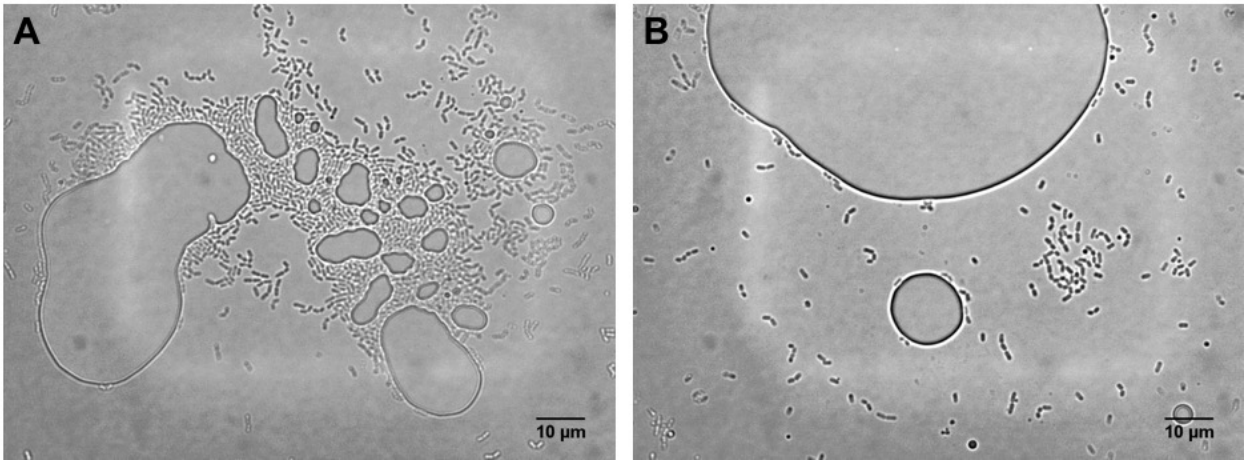

Figure 15: Localization of *L. plantarum* cells in O/W mixtures. Stationary phase cells of (A) hydrophobic strain TMW 1.25 and (B) hydrophilic strain TMW 1.708 were suspended in 1:1 mixtures of rapeseed oil and IPB to a final cell concentration of ~10^8 cfu mL^{-1} and analyzed microscopically. Magnification: 1000x.

These results confirm the prediction made after the MATH test and clearly show that CSH affects the localization of bacterial cells in oil-water mixtures.

3.3.2 Localization in O/W emulsions

In the O/W emulsions used in this study, the oil droplets are covered by Tween 80 molecules, with their oleic acid moiety pointing towards the center of the droplet and the hydrophilic head group facing the aqueous phase including the bacterial cells suspended therein.

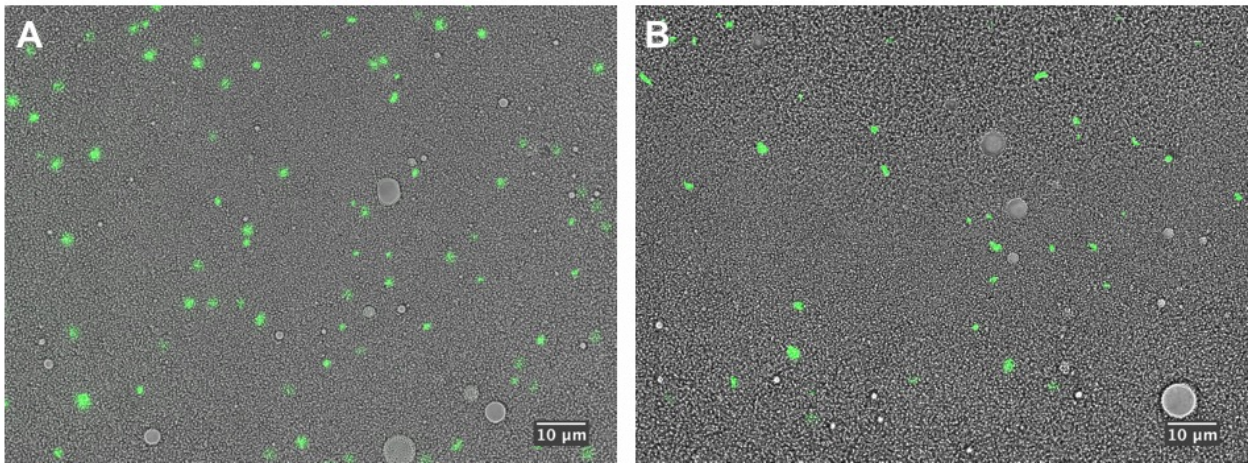

Figure 16: Localization of *L. plantarum* cells in O/W emulsions. Stationary phase cells of (A) hydrophobic strain TMW 1.25 and (B) hydrophilic strain TMW 1.708 were suspended in O/W emulsions with 50 % rapeseed oil in IPB to a final cell concentration of ~10^8 cfu mL^{-1} and analyzed microscopically. Magnification: 1000x.

In contrast to simple O/W mixtures, the oil droplets in the emulsifier-stabilized O/W emulsion were tightly packed to each other such that a clear distinction between aqueous and lipid phase areas was not possible (Figure 16). The bacterial cells were stained with fluorescent dye SYTO®9 for better visualization and were found to be distributed homogeneously within the emulsion. A clear statement about the adhesion of cells to oil droplets covered with emulsifier molecules could not be made from these pictures. However, the cells of both strains were tightly surrounded by oil droplets indicating that cells were forced into direct contact to oil droplets, regardless of the (hydrophobic or hydrophilic) cell surface characteristics. In this condition, the cell surface is probably in tight contact with hydrophilic head group of emulsifier molecules covering the oil droplets.

3.4 Identification of appropriate HHP treatment conditions

To identify potential effects of the investigated influencing factors on the HHP sensitivity of *L. plantarum*, HHP conditions that cause substantial but not complete inactivation of the two selected strains were applied. The pressure holding time was kept constant at an economically reasonable value of 5 minutes, unless otherwise stated. Due to the high temperature-sensitivity of many fat-containing, emulsion-based food products, and to meet worst-case conditions with maximum HHP resistance – microorganisms are generally most pressure resistant when treated at temperatures slightly below their cultivation temperature (Sonoike *et al.*, 1992), which was

30°C in this study – the starting temperature was set to 25 °C. Appropriate pressure intensities were determined by treatment in IPB with pressures between 250 and 600 MPa with 50 MPa increments.

As shown in Figure 17A and Table 30 (Appendix), inactivation levels of TMW 1.25 remained below 1 log up to 300 MPa, then increased dramatically up to 2.37 log for 350 MPa followed by a gradual increase up to 3.48 log at 450 MPa before again showing a great leap with a rise to ~5.18 log at 500 MPa and > 6 log at 550 and 600 MPa. The inactivation curve of TMW 1.708 showed an apparently exponential behavior with log reduction values rising from 0.64 log at 300 MPa over 0.87 (350 MPa), 1.24 (400 MPa) and 2.56 (450 MPa) to 5.95 log at 500 MPa (Figure 17B and Appendix Table 30). As observed for TMW 1.25, treatment with higher pressures led to a reduction of > 6 log.

The pressure at which both strains showed considerable (> 1 log) but not complete inactivation was between 400 and 450 MPa. Since for TMW 1.708 inactivation levels at 450 MPa varied widely among different replicates, impeding proper interpretation of results, a pressure of 400 MPa (25 °C, 5 min) was chosen as the standard pressure for the following inactivation experiments.

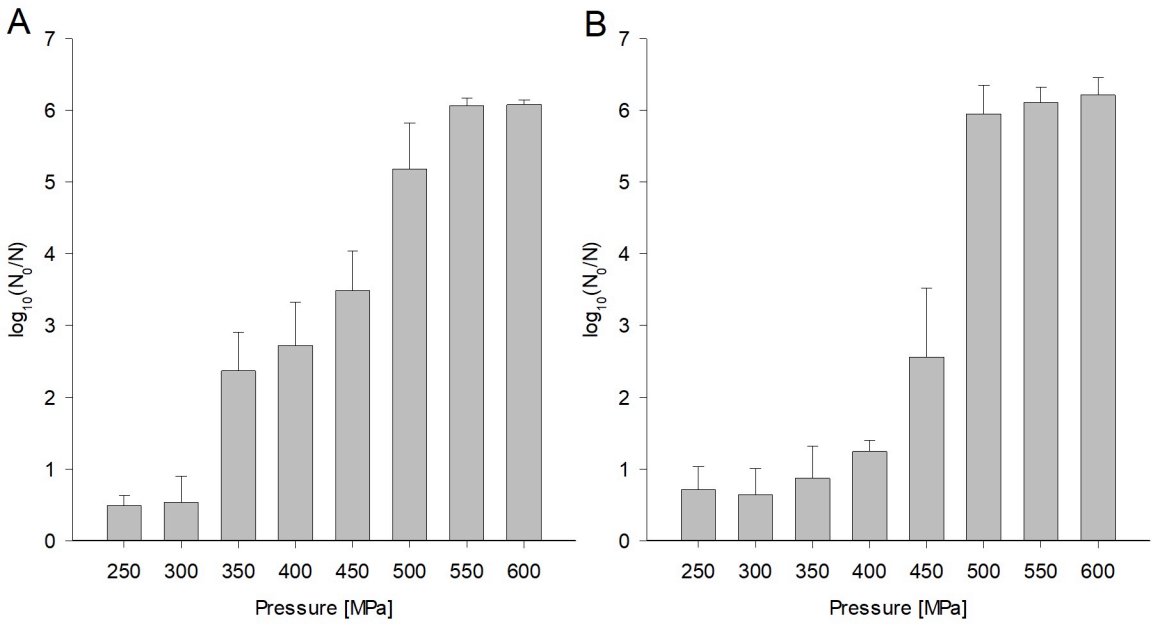

Figure 17: HHP inactivation curves of selected *L. plantarum* strains. Stationary phase cells of (A) TMW 1.25 and (B) TMW 1.708 suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB were treated with the indicated pressure intensities at 25 °C for a holding time of 5 min. Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation.

3.5 Mutual influence of fat and aqueous-phase parameters during HHP inactivation of *L. plantarum* in O/W emulsions

In this section, it was investigated whether there is a mutual influence between fat and other food matrix parameters regarding the effect on bacterial HHP sensitivity, i.e. it was hypothesized that the effect of different parameters based on aqueous environments like NaCl, sucrose concentration, protein content on HHP sensitivity does not change depending on the presence or absence of fat. Furthermore, additional *L. plantarum* strains were used in order to investigate whether observed differences in HHP sensitivity in different matrices correlate with CSH.

3.5.1 Effect of fat on HHP inactivation at different NaCl concentrations

It was hypothesized that the effect of NaCl on HHP inactivation is not affected by the presence of fat. To test this hypothesis, IPB containing NaCl in a food-relevant concentration range from 0 to 25 % (w/v) was used as the aqueous phase of 50 % rapeseed oil emulsions. *L. plantarum* strains suspended in these emulsions or the corresponding pure IPB with the respective NaCl concentration were pressure-treated (400 MPa, 25°C, 5 min). The obtained log reductions are depicted in Figure 18 and Table 31 (Appendix).

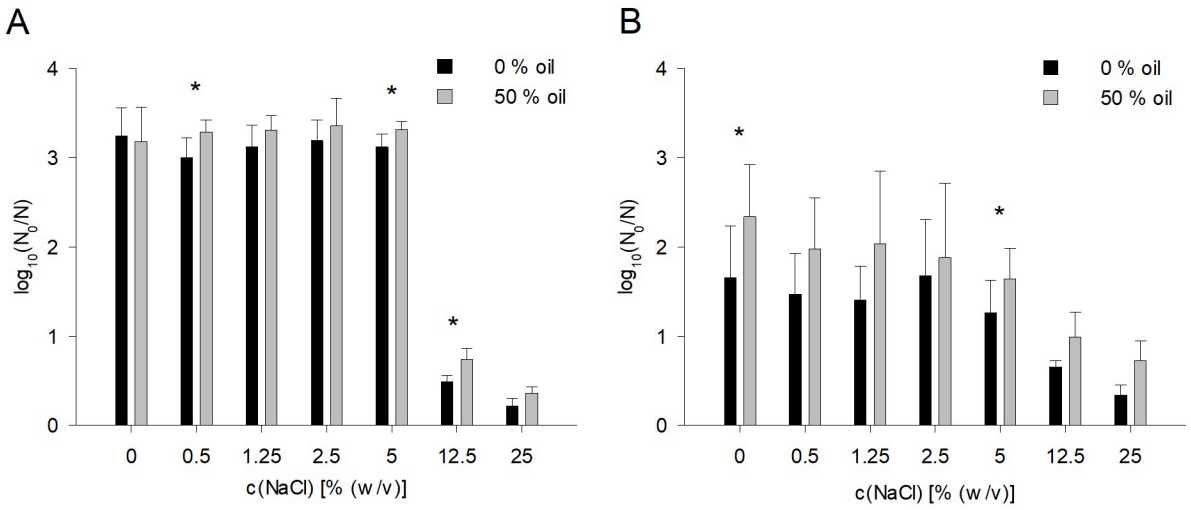

Figure 18: HHP inactivation of selected *L. plantarum* strains under variation of the NaCl concentration in the presence and absence of fat. Stationary phase cells of (A) TMW 1.25 and (B) TMW 1.708 suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB (0 % oil) or O/W emulsions (50 % oil) with the indicated NaCl concentration (in the aqueous phase) were treated with HHP (400 MPa, 25 °C, 5 min). Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation and data pairs (0/50 % oil) with statistically significant differences ($p < 0.05$) are marked with an asterisk.

At NaCl concentrations up to 5 %, log reduction values between 3.0 and 3.5 were observed with hydrophobic strain TMW 1.25. In contrast, NaCl concentrations of 12.5 and 25 % reduced inactivation to < 1.0 log and < 0.5 log, respectively, demonstrating protection from HHP-induced inactivation by higher NaCl concentrations. Inactivation was slightly stronger in emulsions than

pure IPB, but only when NaCl was present, whereas equal inactivation levels were observed in the absence of NaCl. Despite the differences being very weak, these results show that food matrix parameters NaCl and fat can affect each other regarding their impact on HHP inactivation.

Hydrophilic strain TMW 1.708 showed a lower initial inactivation (< 2 log in IPB, 0 % NaCl) than TMW 1.25, and the protective effect of high NaCl concentrations (12.5 and 25 %) was weaker, leading to slightly stronger inactivation than with TMW 1.25. In contrast to TMW 1.25, TMW 1.708 exhibited stronger inactivation in emulsion compared to pure IPB also in the absence of NaCl, demonstrating no interference of the two food matrix parameters fat and NaCl for this strain.

3.5.2 Effect of fat on HHP inactivation at different sucrose concentrations

It was hypothesized that the effect of sucrose on HHP inactivation is not affected by the presence of fat. To test this hypothesis, the sucrose concentration was varied in a food-relevant range from 0 up to 50 % (w/v) in IPB. The resulting buffers were used as the aqueous phase for 50 % rapeseed oil emulsions. *L. plantarum* strains TMW 1.25 (hydrophobic) and TMW 1.708 (hydrophilic) were suspended in emulsion or pure IPB with the respective sucrose concentration and treated with HHP (400 MPa, 25°C, 5 min). The resulting inactivation, expressed as log reduction, is displayed in Figure 19 and Table 32 (Appendix).

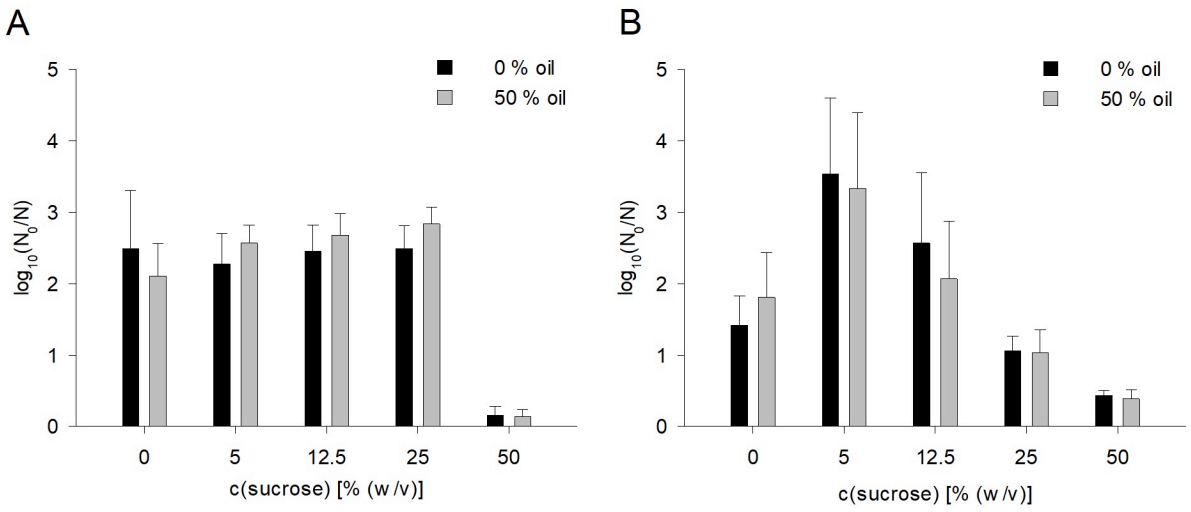

Figure 19: HHP inactivation of selected *L. plantarum* strains under variation of the sucrose concentration in the presence and absence of fat. Stationary phase cells of (A) TMW 1.25 and (B) TMW 1.708 suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB (0 % oil) or O/W emulsions (50 % oil) with the indicated sucrose concentration (in the aqueous phase) were treated with HHP (400 MPa, 25 °C, 5 min). Inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation and data pairs (0/50 % oil) with statistically significant differences ($p < 0.05$) are marked with an asterisk.

Figure 19A shows that under consideration of the standard deviations, low sucrose concentrations up to 25 % had almost no effect on the HHP inactivation of TMW 1.25 in pure IPB (log reductions between 2.0 and 2.5). In emulsions the presence of 5, 12.5 and 25 % sucrose in the aqueous phase enhanced inactivation by > 0.5 log cycles compared to the emulsion without sucrose. Similar to NaCl, low sucrose concentration abolished the slightly protective effect of fat (0 % sucrose) and caused stronger inactivation in the emulsion compared to the corresponding plain buffer solution. 50 % (w/v) sucrose had a strong protective effect, regardless of the presence of fat.

In contrast, the hydrophilic strain TMW 1.708 showed clearly enhanced inactivation at 5 % and 12.5 % sucrose, whereas protection was observed with 25 and 50 %, where no difference between emulsions and plain IPB was observed (Figure 19B). While in the absence of sucrose, inactivation of TMW 1.708 was stronger in the emulsion than in plain IPB, this effect was reversed by the addition of 5 or 12.5 % sucrose, which represents exactly the opposite to the results observed with TMW 1.25. However, due to the great variation among replicates, the observed differences are not statistically significant.

3.5.3 Effect of fat on HHP inactivation at different pH values

It was hypothesized that the effect of the pH on HHP inactivation of *L. plantarum* is not affected by the presence of fat. Since the buffer range of IPB is limited to a narrow window around pH 6-7, the buffer system was switched from IPB to citric acid/phosphate buffer (CPB) according to McIlvaine in order to obtain a pressure-stable buffer within the acidic pH range. CPB with different pH values from 7 down to 3 was used to prepare 50 % rapeseed oil emulsions. To enable the detection of effects of the alternative buffer system by itself, regular IPB (pH 7) and the corresponding emulsion were included in the test series for comparison. Stationary phase *L. plantarum* cells were suspended in these emulsions or in pure CPB or IPB with the respective pH and HHP treated. Since an enhancement of HHP inactivation by low pH values was expected, a second test series at 300 MPa (25 °C, 5 min) in addition to the regular one (400 MPa, 25 °C, 5 min) was performed to increase the probability to obtain considerable but not complete inactivation. The results represented as log reduction are depicted in Figure 20 and Table 33 (Appendix).

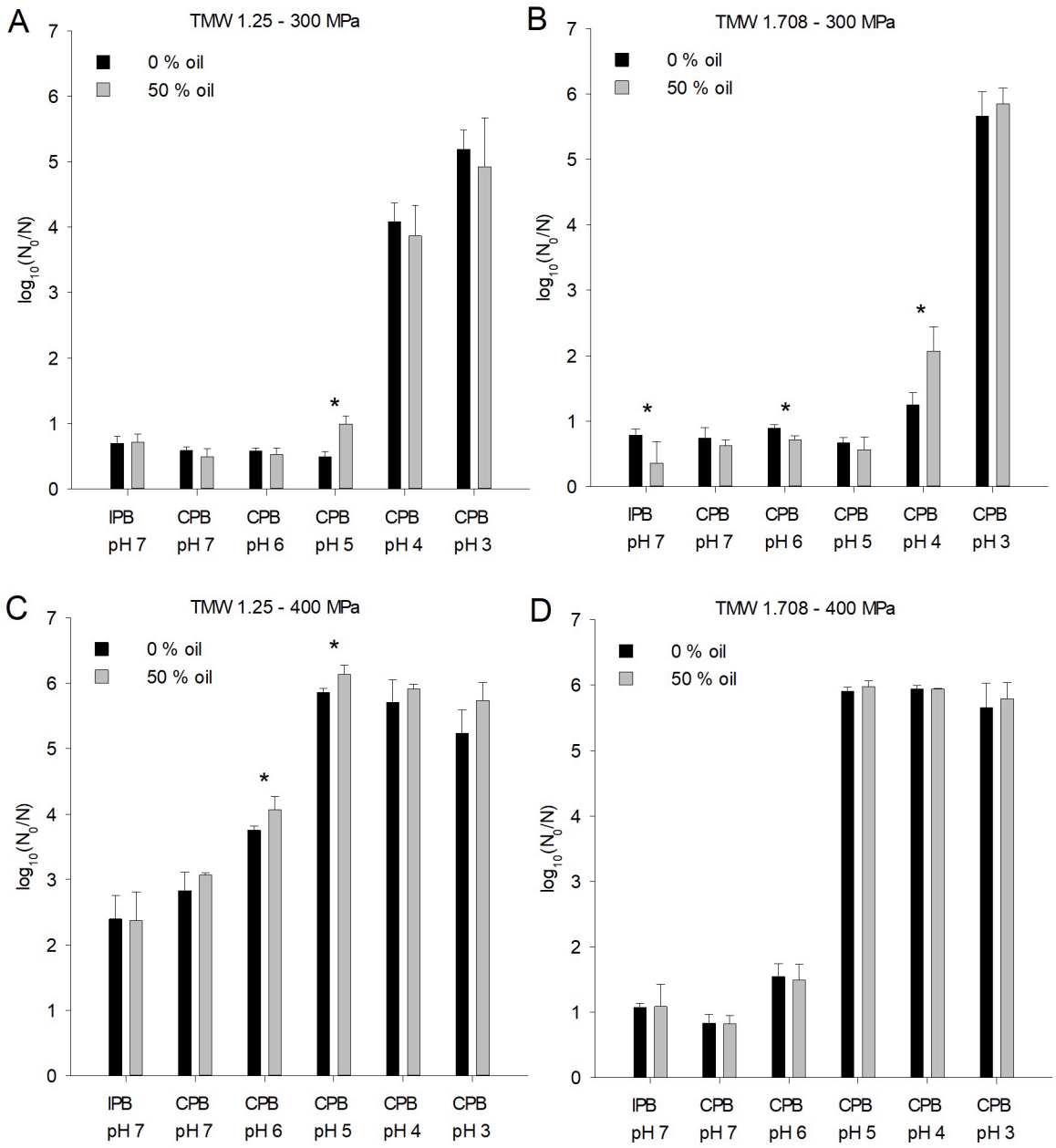

Figure 20: HHP inactivation of selected *L. plantarum* strains under variation of the pH value in the presence and absence of fat. Stationary phase cells of (A, C) TMW 1.25 and (B, D) TMW 1.708 suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB or CPB with the indicated pH values (0 % oil) or O/W emulsions containing the respective buffer as the aqueous phase (50 % oil) were HHP treated with (A-B) 300 MPa or (C-D) 400 MPa (25 °C, 5 min). Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation and data pairs (0/50 % oil) with statistically significant differences ($p < 0.05$) are marked with an asterisk.

Similar inactivation levels in IPB and CPB at pH 7 observed with both strains showed that the change of the buffer system did not affect HHP sensitivity. Lowering the pH resulted in enhanced inactivation for both strains, but the response to pH changes was different:

Inactivation of TMW 1.25 by 300 MPa remained below 1 log down to pH 5, whereas pressurization at pH 4 and pH 3 led to strong inactivation of ~4 and ~5 log, respectively (Figure 20A). At 400 MPa, inactivation of TMW 1.25 gradually increased from ~3 log at pH 7 and ~4 log at pH 6 to ~6 log at pH 5 (Figure 20C).

The hydrophilic strain TMW 1.708 treated with 300 MPa also retained weak inactivation of < 1 log down to pH 5 (Figure 20B). In contrast to TMW 1.25, it showed only low inactivation of ~1 log and ~2 log at pH 4 in the absence and presence of rapeseed oil, respectively. Strong inactivation (~6 log) was observed only at pH 3. Pressurization at 400 MPa caused inactivation levels of < 1 log and < 2 log at pH 7 and pH 6, respectively, followed by a sharp increase at pH 5 (Figure 20D). At most pH/pressure conditions, inactivation of the two strains was not affected by the presence of oil. For TMW 1.25, statistically significant differences were observed at pH 6 and pH 5 and treatment with 400 MPa with stronger inactivation in emulsion than pure buffer. For TMW 1.708, oil had a slightly protective effect at pH 7 in IPB and pH 6 (CPB) during treatment with 300 MPa, while the difference at pH 7 (CPB) and pH 5 were not statistically significant. The greatest difference was observed at two pH-pressure conditions (300 MPa, pH 5 for TMW 1.25 and 300 MPa, pH 6 for TMW 1.708) that represent points with a strong change in HHP sensitivity from the next higher to the next lower pH value. At these conditions, inactivation in emulsions was significantly stronger than in the corresponding plain buffer solution, indicating that in the presence of oil the pH-dependent increase in inactivation starts at slightly higher pH values than in pure CPB.

3.5.4 Effect of fat on the HHP inactivation in the presence of protein

In this section the general effect of proteins on HHP inactivation in emulsions was investigated. In order to cover a wide range of possible protein structures, three types of proteins or protein-associated substances derived from milk protein were investigated. The first type, peptone from casein represents small peptides, i.e. fractions of proteins that are readily water-soluble and can easily be ingested by bacteria. The second type, whey protein isolate (WPI), stands for highly water-soluble, whole proteins, whereas casein represents proteins with very low water solubility. It was hypothesized that the effect of the individual protein types and concentrations should not be affected by the presence of fat. To test this hypothesis, the three substances were incorporated in IPB in food-relevant concentrations of 2.5 and 10 % (w/v) and the IPB-protein mixtures were used to prepare emulsions containing 50 % rapeseed oil. Since the preparation of stable emulsions was not possible with IPB containing 10 % (w/v) casein, only the emulsion with 2.5 % casein was prepared. *L. plantarum* cells suspended in the IPB-protein mixtures and the corresponding emulsions were HHP treated and the log reduction determined (Figure 21 and Appendix Table 34). Due to the reported protective effect of some proteins during HHP treatment (Simpson & Gilmour, 1997, Black *et al.*, 2007, Narisawa *et al.*, 2008), a second test series using 500 MPa (25 °C, 5 min) was run in addition to the regular treatment with 400 MPa to ensure sufficient cell inactivation to allow for the detection of substantial differences between individual conditions.

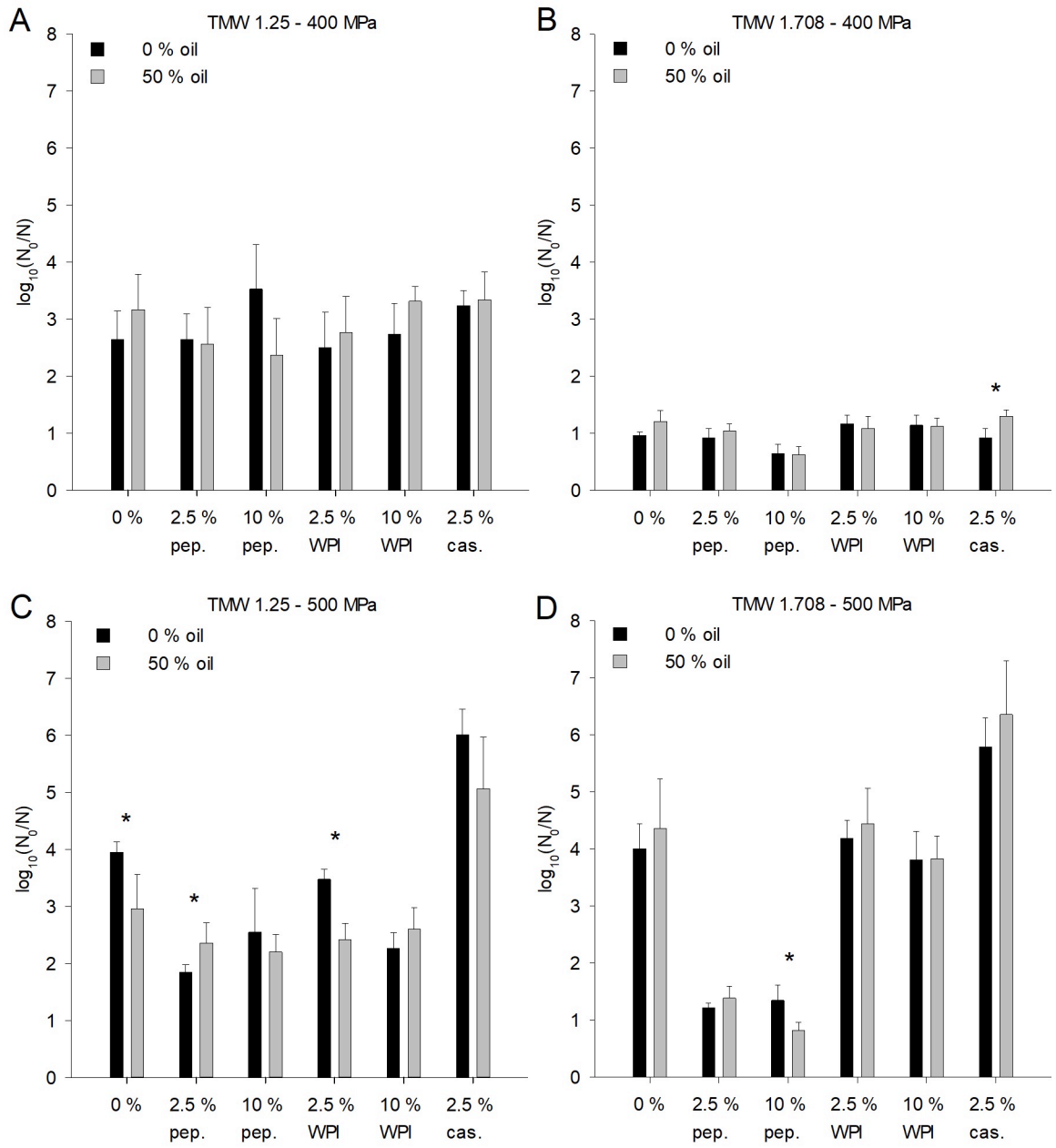

Figure 21: HHP inactivation of selected *L. plantarum* strains under variation of the protein type and concentration in the presence and absence of fat. Stationary phase cells of (A, C) TMW 1.25 and (B, D) TMW 1.708 suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB (0 % oil) or O/W emulsions (50 % oil) containing the indicated protein type (pep. = peptone; WPI = whey protein isolate; cas. = casein) and concentration (in the aqueous phase) were HHP treated with (A-B) 400 MPa or (C-D) 500 MPa (25 °C, 5 min). Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation and data pairs (0/50 % oil) with statistically significant differences ($p < 0.05$) are marked with an asterisk.

After treatment with 400 MPa, only minor effects of protein on HHP inactivation were observed, as inactivation remained nearly stable at ~3 log for TMW 1.25 (Figure 21A) and ~1 log for TMW 1.708 (Figure 21B) among different protein matrices. Inactivation of TMW 1.25 was strongest in

IPB containing 10% peptone and weakest in the corresponding emulsion, but variation among replicates was too strong for the ~1 log difference to be statistically significant. In other protein matrices, inactivation was largely unaffected by oil or even marginally stronger in the emulsion (e.g. 0 % protein, 10 % WPI). While TMW 1.708 experienced a slightly protective effect by 10 % peptone both in plain IPB and in emulsion, 2.5 % casein was the only condition with a significant difference in inactivation between pure IPB and emulsion. Similar to the matrix without protein (0 %), inactivation was slightly stronger in the emulsion than in pure aqueous buffer. During pressurization with 500 MPa, TMW 1.25 was protected by peptone and WPI, whereas casein enhanced inactivation (Figure 21C). The presence of fat reduced inactivation in the protein-free matrix (0 %), 2.5 % WPI and 2.5 % casein by ~1 log and, to a minor extent, in 10 % peptone but led to slightly increased inactivation in 2.5 % peptone and 10 % WPI. This shows that the effect of peptone or WPI on HHP inactivation of TMW 1.25 depends on the presence of oil. In contrast, 10 % peptone and 2.5 % WPI also caused a reduction in inactivation, but the protective effect of fat was retained under these conditions. 2.5 % casein led to enhanced inactivation of up to ~6 and ~5 log for IPB and emulsion. With TMW 1.708, peptone reduced HHP inactivation by ~3 log cycles, whereas WPI had no effect, and casein increased HHP sensitivity by ~2 log cycles (Figure 21D). A weak protective effect of fat was observed in the presence of 10 % peptone, but not with other conditions, as inactivation was even slightly stronger in the emulsion.

3.5.5 Correlation of CSH with matrix-related changes in HHP sensitivity

In a subsequent step it was investigated whether the differences regarding the effect of the surrounding matrix on HHP sensitivity observed between the two strains TMW 1.25 and 1.708 are linked to their CSH. For this, an additional set of six *L. plantarum* strains, three with hydrophobic (TMW 1.277, TMW 1.284, TMW 1.1810) and three with hydrophilic (TMW 1.1204, TMW 1.2089, TMW 1.1808) cell surfaces, was chosen according to CSH determined in previous experiments. Differences in CSH and compatibility with the examination system were confirmed by MATH test (2.4.2) and as described in 2.4.4, respectively (see Appendix Table 21 to Table 25, Table 29 and Figure 53). The strains were subjected to HHP using one or two conditions for each aqueous-phase parameter (NaCl, sucrose, pH, protein) that led to highly different inactivation levels of TMW 1.25 and TMW 1.708. For each condition, treatment was done in the pure aqueous phase and in the corresponding 50 % rapeseed oil emulsion to test whether the presence of fat affects the matrix-dependent changes in HHP sensitivity in a CSH-dependent manner. The log reduction values of TMW 1.25 and 1.708 shown in this section correspond to the values presented in Figure 18 to Figure 21 and are shown with those of the

six additional strains for better comparison. Since inactivation in plain buffer solution (0 % added solutes/pH 7) varied considerably among the used strains (see Appendix Figure 54 and Figure 55, the difference in log reduction between the respective condition and 0 % solute/pH 7 was calculated for better visualization, in addition.

NaCl

For the aqueous phase parameter NaCl, two conditions, 5 % and 12.5 % were selected. While 5 % NaCl showed almost no effect and 12.5 % led to strong protection of TMW 1.25, the difference was much smaller for TMW 1.708 (see Figure 22 and Figure 23). The two hydrophobic strains TMW 1.284 and 1.277 behaved similarly to TMW 1.25, as 5 % NaCl had almost no effect and 12.5 % NaCl strongly reduced HHP inactivation from values of ~2-3 log to < 1 log. TMW 1.1810, in contrast, showed considerably weaker inactivation in general (< 0.5 log). In the 12.5 % NaCl matrix, inactivation of TMW 1.1810 was similar to TMW 1.284 and 1.277, whereas with 5 % NaCl the addition of oil significantly increased inactivation. The three additional hydrophilic strains were generally less HHP sensitive than TMW 1.708 but, as with TMW 1.708 and in contrast to the hydrophobic strains, a protective effect was already observed with 5 % NaCl. Exceptions were TMW 1.1808, which was hardly affected by HHP in general, and TMW 1.1204, which was protected by 5 % NaCl in pure buffer but not in the emulsion. Except for a few cases, especially where inactivation levels were very low, all strains were inactivated more strongly in the emulsion than in the pure aqueous environment.

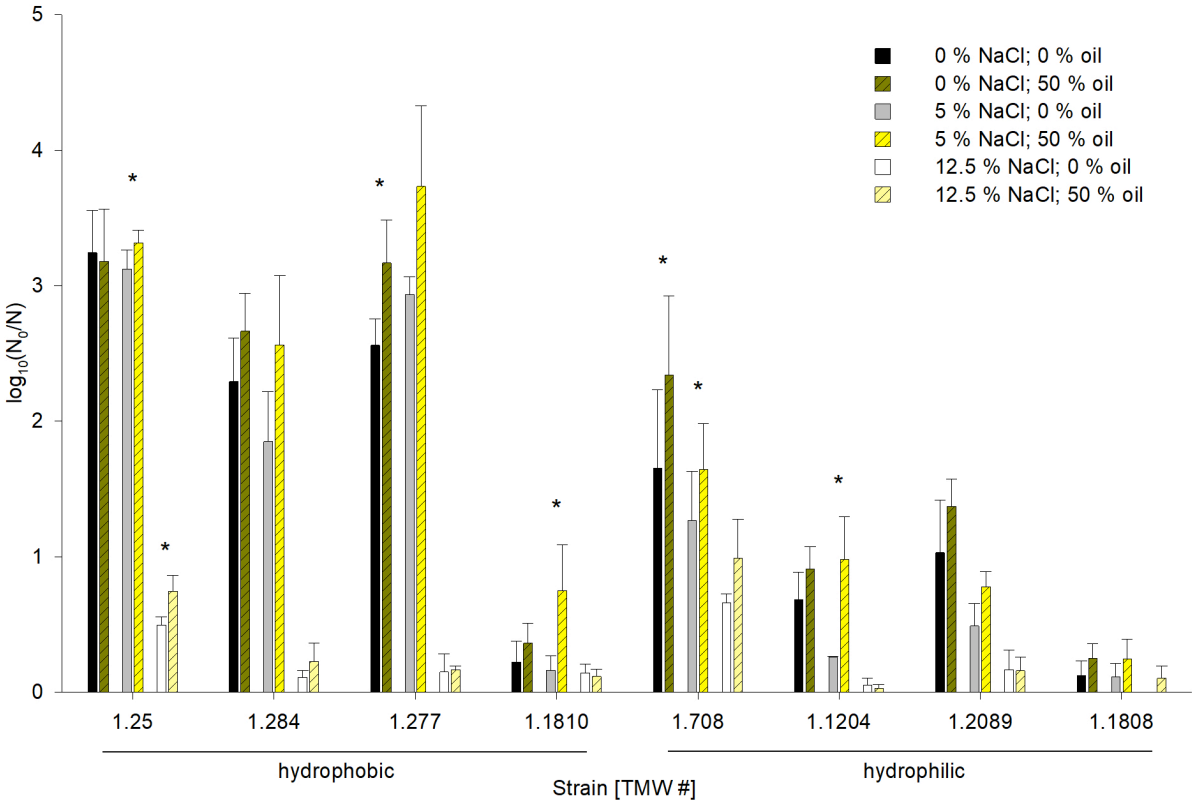

Figure 22: HHP inactivation of *L. plantarum* strains with hydrophobic and hydrophilic cell surfaces at selected NaCl concentration in the presence and absence of fat. Stationary phase cells suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB (0 % oil) or O/W emulsions (50 % oil) with the indicated NaCl concentration (in the aqueous phase) were treated with HHP (400 MPa, 25 °C, 5 min). Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation and data pairs (0/50 % oil) with statistically significant differences ($p < 0.05$) are marked with an asterisk.

The effect of NaCl did not correlate with CSH but seems to be rather strain-specific and may, at least partially, depend on the general pressure sensitivity, which is also strain-dependent. Although the effect of NaCl was mostly unaffected by fat, a few cases were observed where the presence of fat counterbalanced (TMW 1.284, 5 % NaCl; TMW 1.1204, 5 % NaCl) or even inverted (TMW 1.1810, 5 % NaCl) the protective effect of NaCl.

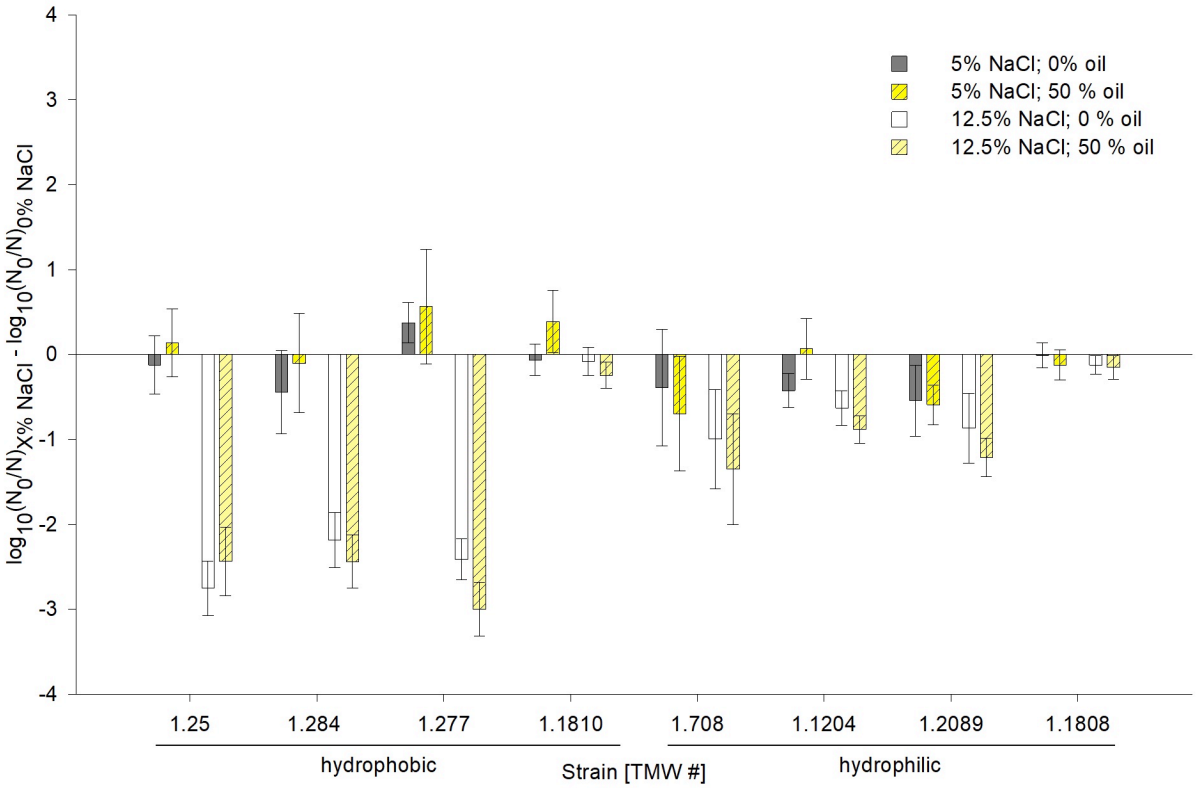

Figure 23: Impact of fat on the NaCl-based effect on HHP sensitivity. The difference between the log reduction (see Figure 22) of *L. plantarum* strains with hydrophobic and hydrophilic cell surfaces observed in pure IPB (0 % oil) or O/W emulsion (50 % oil) with the indicated NaCl concentration and 0 % NaCl are shown. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation. Positive and negative values indicate an enhancement or weakening of inactivation from 0 % to the indicated NaCl concentration, respectively. Error bars represent the square root of the sum of the squares of the standard deviations of the log reduction values considered for calculation.

Sucrose

For the aqueous phase parameter sucrose, two conditions, 5 % and 50 % were selected. Similar to NaCl, 5 % sucrose had no effect and 50 % exerted strong protection on TMW 1.25. TMW 1.708, in contrast, showed increased inactivation in the 5 % sucrose matrix but was also protected by 50 % (see Figure 24 and Figure 25). As with TMW 1.25, inactivation of the additional three hydrophobic strains was largely unaffected by 5 % sucrose, but TMW 1.284 experienced a significantly stronger inactivation in the emulsion containing 5 % sucrose. 50 % sucrose strongly protected the hydrophobic strains, regardless of the presence of fat, as shown by a decline in inactivation by 2-3 log for the three strains with high initial inactivation and an almost complete protection of 1.1810 with high initial pressure resistance. Strong protection by 50 % sucrose was also observed with the hydrophilic strains, as TMW 1.204 and 1.1808 were hardly inactivated and inactivation of TMW 1.708 and 1.2089 remained below 0.5 log. The ~2 log increase in inactivation of TMW 1.708 in the 5 % sucrose matrix was not observed for TMW

1.1204 and TMW 1.2089, but TWM 1.1808 was slightly more sensitive, especially in the emulsion with 5 % sucrose. In general, inactivation was stronger in the emulsion compared to the purely aqueous environment except for the 50 % sucrose matrix where similar inactivation levels were obtained with all strains.

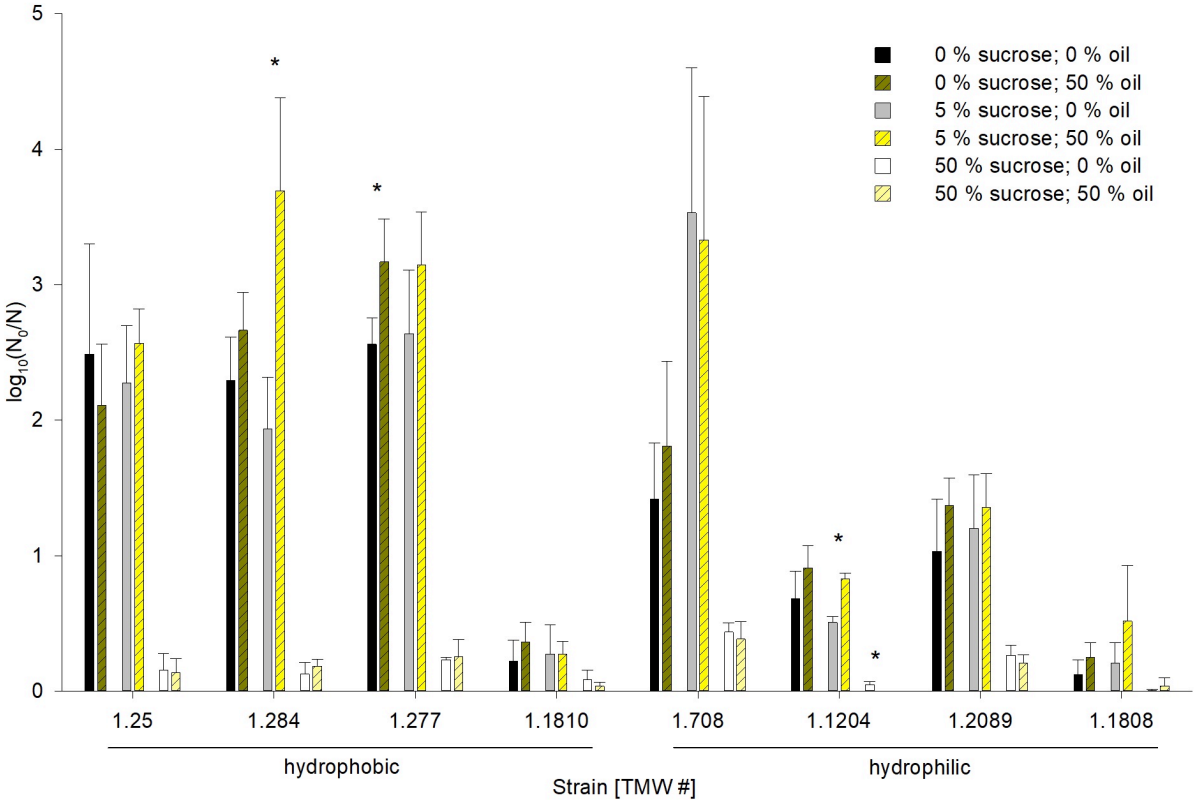

Figure 24: HHP inactivation of *L. plantarum* strains with hydrophobic and hydrophilic cell surfaces at selected sucrose concentration in the presence and absence of fat. Stationary phase cells suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB (0 % oil) or O/W emulsions (50 % oil) with the indicated sucrose concentration (in the aqueous phase) were treated with HHP (400 MPa, 25 °C, 5 min). Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation and data pairs (0/50 % oil) with statistically significant differences ($p < 0.05$) are marked with an asterisk.

Thus, for most strains protection by 50 % sucrose turned out slightly stronger in the emulsion. The effect of 5 % sucrose was inverted by the addition of fat for TMW 1.25 and 1.284, whereas for the other strains, including TMW 1.708 with strongly increased inactivation, the impact of 5 % sucrose was largely unaffected by fat. Like with NaCl, a general correlation between CSH and the behavior in sucrose environment under pressure could not be detected.

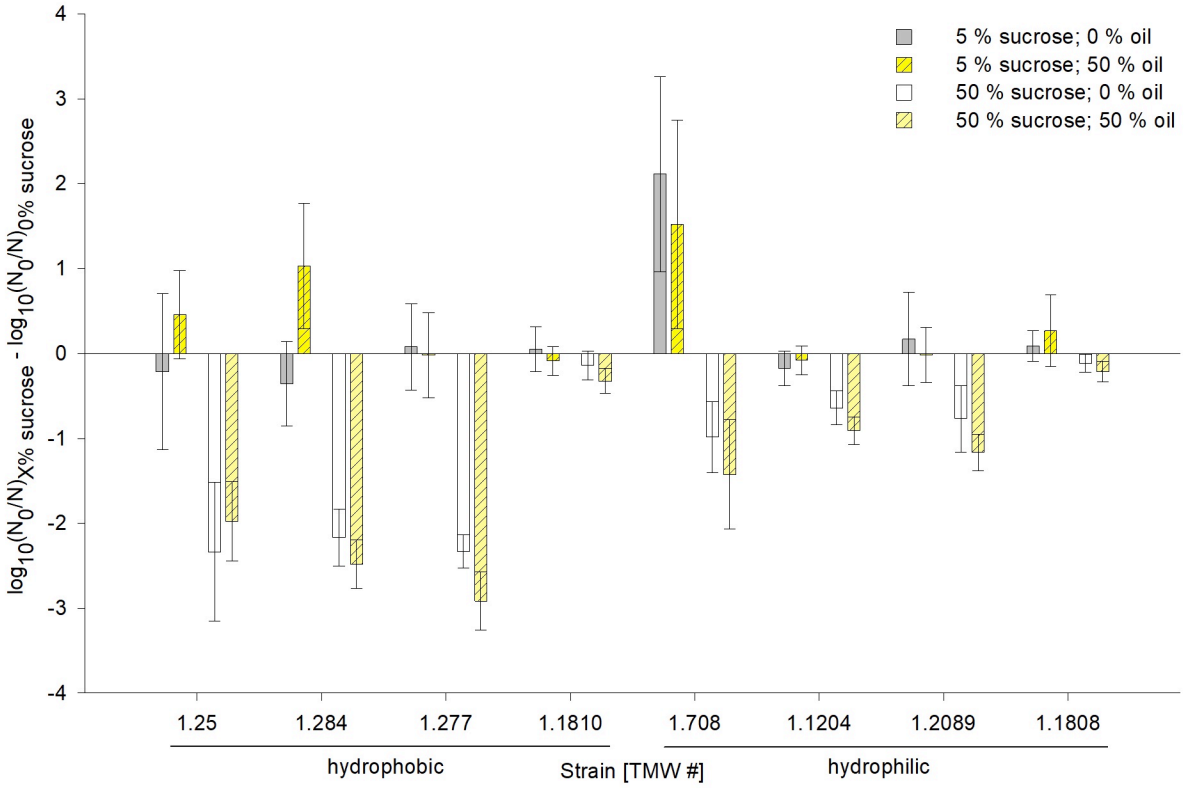

Figure 25: Impact of fat on the sucrose-based effect on HHP sensitivity. The difference between the log reduction (see Figure 24) of *L. plantarum* strains with hydrophobic and hydrophilic cell surfaces observed in pure IPB (0 % oil) or O/W emulsion (50 % oil) with the indicated sucrose concentration and 0 % sucrose are shown. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation. Positive and negative values indicate an enhancement or weakening of inactivation from 0 % to the indicated sucrose concentration, respectively. Error bars represent the square root of the sum of the squares of the standard deviations of the log reduction values considered for calculation.

pH value

The effect of the pH value on HHP sensitivity in emulsions was investigated at two different pressure levels. The pressure/pH combinations 300 MPa/pH 4 and 400 MPa/pH 6 led to the greatest difference in inactivation between TMW 1.25 and TMW 1.708 (see Figure 20) and were therefore selected for this experiment. Inactivation was compared between pH 4 (for 300 MPa) or pH 6 (for 400 MPa) and pH 7 in CPB. To detect possible effects of the buffer system, IPB (pH 7) was included in the test row again (see 3.5.3). At pH 7, inactivation by 300 MPa remained below 1 log for all strains and especially the three additional hydrophilic strains were hardly affected at all (Figure 26A). The buffer system itself had no impact on the inactivation except for TMW 1.284 and TMW 1.708 where a protective effect of fat observed in the IPB environment was not present in CPB and for TMW 1.1810 where the presence of fat enhanced inactivation in CPB but not in IPB. However, especially in the latter case the difference was small and

variability among the replicates was high. HHP treatment at pH 4 considerably increased inactivation of hydrophobic strains TMW 1.25 and TMW 1.277 by about 3.5 log and 4.5-5 log to inactivation levels of ~4 log and ~5 log, respectively. The other six strains were affected to a lesser extent and reached inactivation levels between ~2 and ~3 log. Except for TMW 1.25, but in particular for TMW 1.1810 and all four hydrophilic strains, the inactivation at pH 4 was generally stronger in the emulsion than in pure aqueous buffer. Since inactivation at pH 7 was nearly equal in pure CPB and emulsion, also the difference in log reduction between pH 4 and 7 is therefore greater in the emulsion (see Figure 26A and Figure 27A).

As shown in Figure 26B, HHP treatment with 400 MPa also led to similar inactivation levels in the two buffer systems at pH 7, except for the hydrophilic strains TMW 1.1204 and 1.2089 which experienced stronger and weaker inactivation in CPB than in IPB, respectively. An increase in inactivation by ~0.5-1.0 log from pH 7 (CPB) to pH 6 was observed only for the three hydrophobic strains TMW 1.25, 1.284 and 1.277 and for the hydrophilic strain TMW 1.708. In contrast, with TMW 1.1808 (hydrophobic) as well as 1.2089 and 1.1810 (both hydrophilic) negligible changes were observed. TMW 1.1204 showed a unique behavior as in pure CPB environment the inactivation remained stable at 1 log whereas in the emulsion it was raised by more than 1.5 log leading to a difference of > 2 log between pure buffer and emulsion at pH 6. Unlike at 300 MPa, the increase in HHP from the higher to the lower pH environment was not generally stronger in the emulsion (Figure 26B and Figure 27B). These results show that like with NaCl and sucrose, also the response to changes in the pH value in terms of HHP sensitivity does not correlate with the strains' CSH but rather depends on strain-specific, so far unknown features.

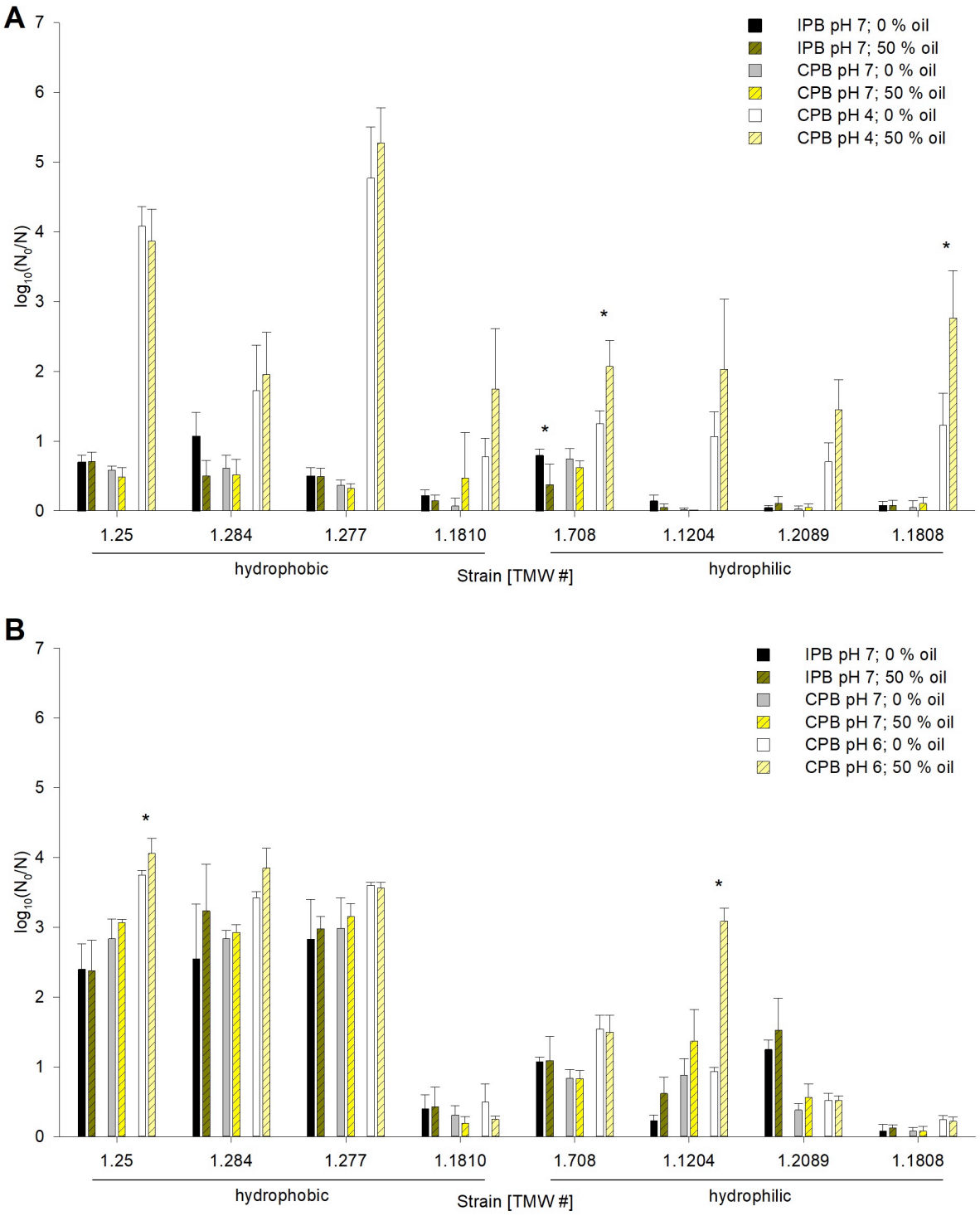

Figure 26: HHP inactivation of *L. plantarum* strains with hydrophobic and hydrophilic cell surfaces at different pH values in the presence and absence of fat. Stationary phase cells suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB or CPB with the indicated pH values (0 % oil) or O/W emulsions containing the respective buffer as the aqueous phase (50 % oil) were treated with (A) 300 MPa or (B) 400 MPa (25 °C, 5 min). Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation and data pairs (0/50 % oil) with statistically significant differences ($p < 0.05$) are marked with an asterisk.

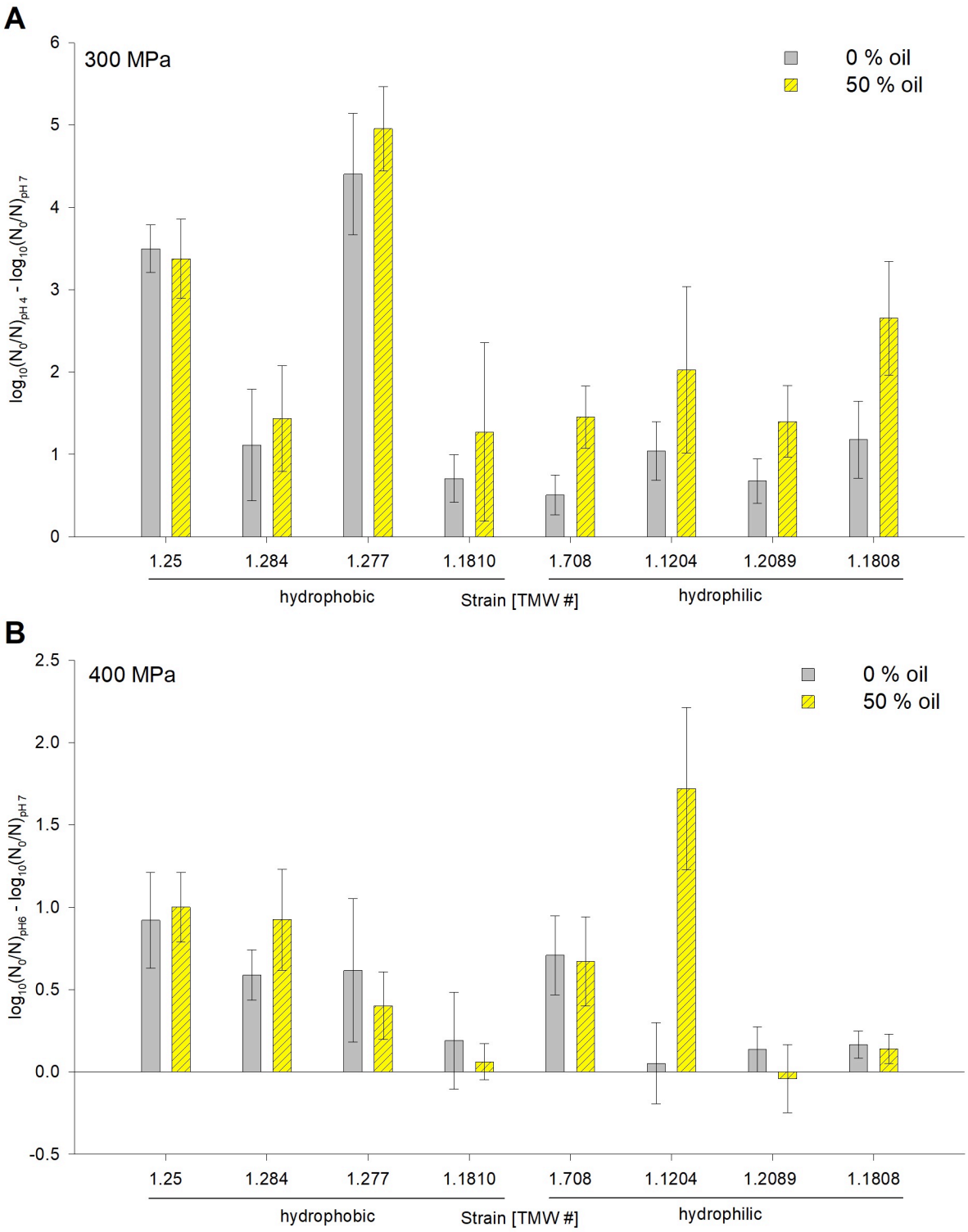

Figure 27: Impact of fat on the pH-based effect on HHP sensitivity. The difference between the log reduction (see Figure 26) of *L. plantarum* strains with hydrophobic and hydrophilic cell surfaces observed in pure CPB (0 % oil) or O/W emulsion (50 % oil) with pH 4 (300 MPa; A) or pH 6 (400 MPa; B) and pH 7 are shown. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation. Positive and negative values indicate an enhancement or reduction of inactivation from pH 7 to the indicated pH value, respectively. Error bars represent the square root of the sum of the squares of the standard deviations of the log reduction values considered for calculation.

Protein

The effect of proteins on HHP sensitivity in emulsions was investigated at two different pressure levels. An appreciable effect of protein was observed only during treatment with 500 MPa where 10 % peptone exerted a stronger protection on TMW 1.708 than on TMW 1.25 and led to the greatest difference between the two strains (see Figure 21). During treatment with 500 MPa, inactivation was stronger or weaker in the emulsion than in pure IPB (up to 1 log difference), depending on the strain (Figure 28). Strains TMW 1.25, 1.277 and 1.2089 were significantly protected by the presence of oil whereas TMW 1.1204 showed a significant increase in inactivation and TMW 1.284, 1.1810, 1.708 and 1.1808 were only marginally affected. The addition of peptone almost always had a protective effect, the extent of which varied strongly among strains and the surrounding matrix, i.e. pure IPB or emulsion. However, with all tested strains except TMW 1.25 and 1.1204, peptone altered the relation between pure buffer and emulsion, i.e. where in the absence of peptone inactivation was stronger in pure IPB, it became stronger in the emulsion when peptone was present (TMW 1.277, 1.1810) or vice versa (TMW 1.284, 1.708). With strains TMW 1.2089 and 1.1808 the protective effect of fat was just eliminated by peptone leading to equal inactivation in IPB and emulsion. The strongest effect was observed with TMW 1.1810, where peptone in the combination with oil enhanced inactivation from ~1.5 log to ~2 log although a reduction from ~1.7 to < 1 log was observed in pure IPB.

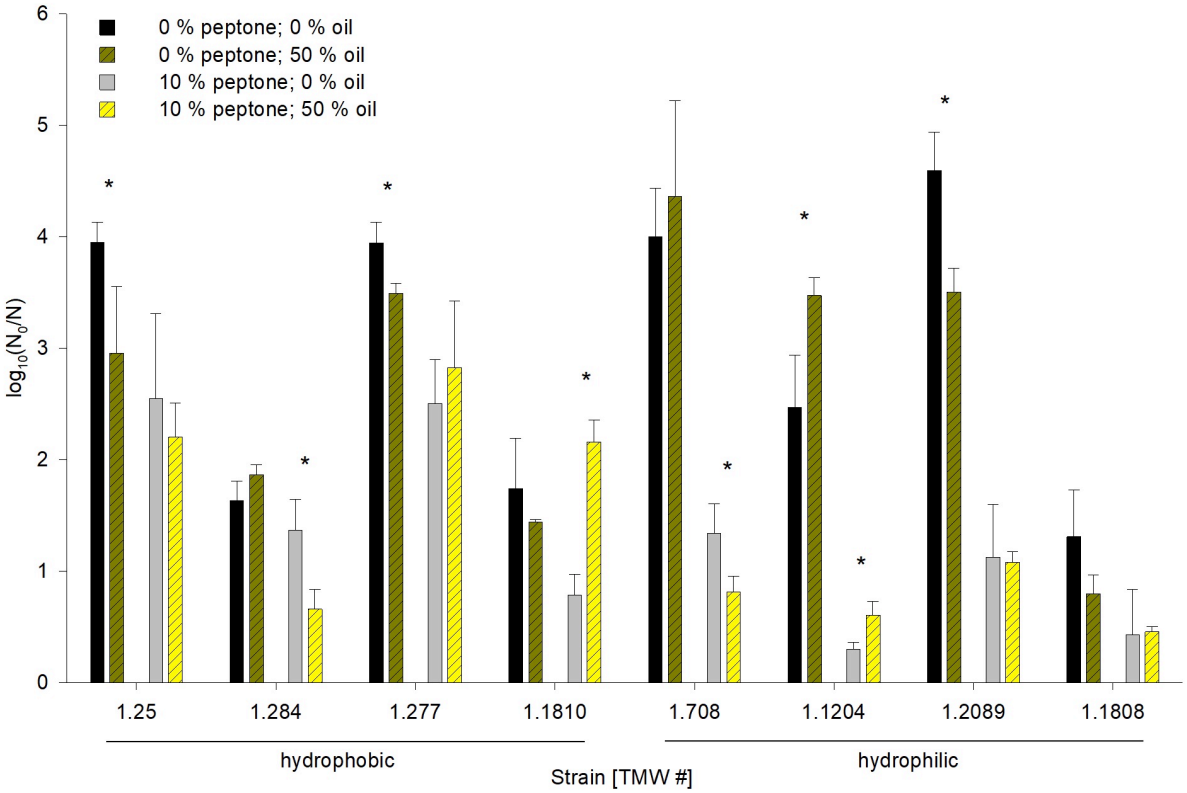

Figure 28: HHP inactivation of *L. plantarum* strains with hydrophobic or hydrophilic cell surfaces in the presence and absence of peptone and fat. Stationary phase cells suspended in IPB (0 % oil) or O/W emulsions (50 % oil) containing 0 % or 10 % (w/v) peptone (in the aqueous phase) to a final concentration of ~10^7 cfu mL^{-1} were HHP treated with 500 MPa (25 °C, 5 min). Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation and data pairs (0/50 % oil) with statistically significant differences ($p < 0.05$) are marked with an asterisk.

Figure 29 visualizes the effect of 10 % peptone on the HHP inactivation in IPB and 50 % rapeseed oil emulsion and demonstrates that there is a complex interplay between peptone and fat, the effect of which is highly dependent on strain-specific features, but does not correlate with CSH.

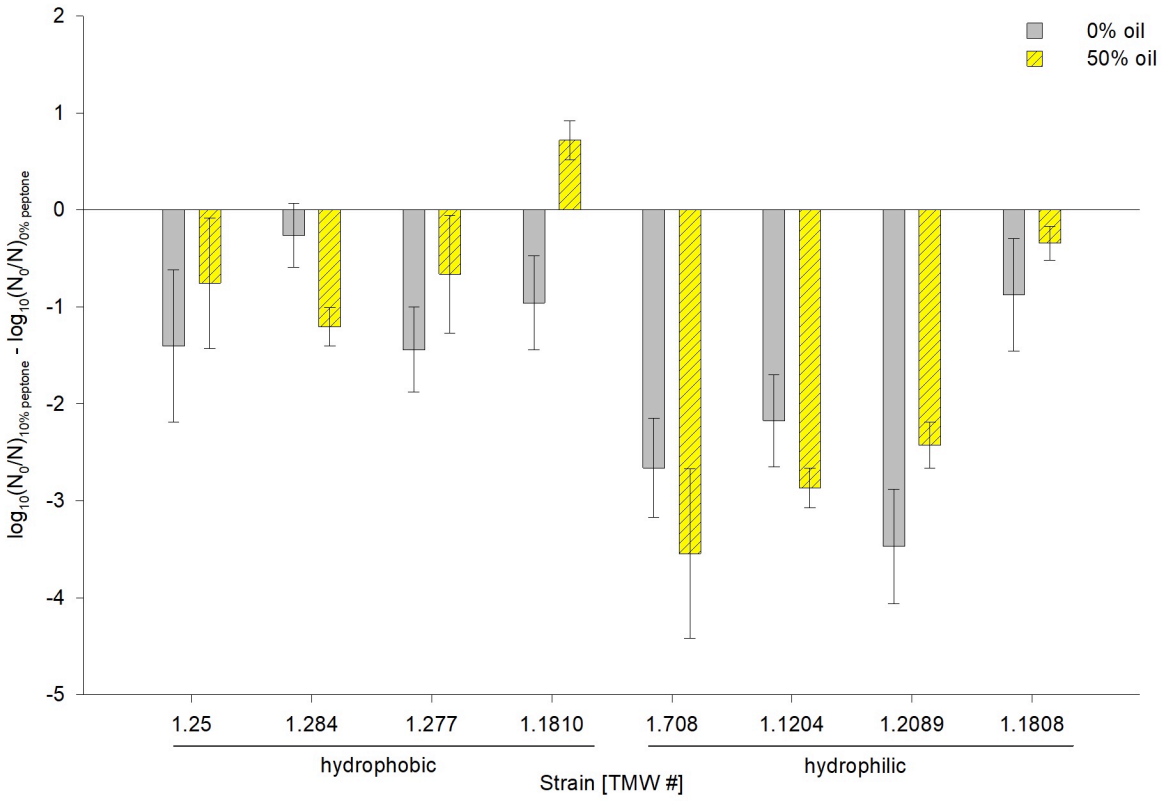

Figure 29: Impact of fat on the peptone-based effect on HHP sensitivity. The difference between the log reduction (see Figure 28) of *L. plantarum* strains with hydrophobic and hydrophilic cell surfaces observed in pure IPB (0 % oil) or O/W emulsion (50 % oil) with 10 % and 0 % peptone are shown. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation. Positive and negative values indicate an enhancement or weakening of inactivation from 0 % to 10 % peptone, respectively. Error bars represent the square root of the sum of the squares of the standard deviations of the log reduction values considered for calculation.

3.6 Cellular response to fat contact and effect on HHP sensitivity – effect of preconditioning

3.6.1 Effect of preconditioning in O/W emulsions on HHP sensitivity of *L. plantarum*

As shown in 3.5, the presence of fat alone in O/W emulsions during HHP treatment has only a minor effect on HHP sensitivity of suspended *L. plantarum* cells. In order to examine whether a prolonged contact time with fat influences HHP sensitivity, cells were incubated for 24 or 48 h in O/W emulsions containing 50 % rapeseed oil prior to HHP treatment at 400 MPa, 5 min, 25 °C. To identify a potential effect of the emulsifier, IPB containing 1 % Tween 80, representing the emulsifier concentration present in the O/W emulsion, was tested in parallel. In addition, the effect of the storage temperature was investigated, comparing storage of the suspensions at room temperature (25 °C) with food-relevant refrigerating conditions (4 °C).

As shown in Figure 30A and Table 35 (Appendix), incubation at 4 °C in pure IPB or IPB with 1 % (w/v) Tween 80 slightly increased HHP inactivation levels of TMW 1.25 from 2.84 log and 2.27 log to 3.34 log and 2.78 log after 48 h, respectively. O/W emulsion (2.55 log at 0 h) caused a stronger increase, reaching 4.34 log reduction after 48 h. Preceding incubation in pure IPB and IPB containing 1 % (w/v) Tween 80 at 25 °C enhanced HHP inactivation of TMW 1.25 to 3.78 log and 3.58 log after 24 h, respectively, and to almost 5 log after 48 h. This effect was even stronger in O/W emulsion, where inactivation levels reached 4.53 log after 24 h and 5.44 log after 48 h. Compared to pure IPB, inactivation was slightly weaker in IPB containing 1 % (w/v) Tween 80 in general, whereas O/W emulsion caused a stronger inactivation after prolonged inactivation.

In line with the results presented in 3.5, TMW 1.708 showed weaker inactivation than TMW 1.25 in general (1.04 – 1.32 log) when treated immediately after cultivation (Figure 30B and Appendix Table 35). As observed with TMW 1.25, the increase in HHP sensitivity of TMW 1.708 over time was weaker at 4 °C than at 25 °C: Incubation at 4 °C in IPB, IPB with 1 % (w/v) Tween 80 and O/W emulsion for 48 h caused inactivation levels of 3.54 log, 3.03 log and 3.80 log, whereas preceding incubation at 25 °C led to log reduction values of 5.72 log, 5.23 log and 5.66 log, respectively. In line with TMW 1.25, IPB with 1 % (w/v) Tween 80 generally led to the weakest inactivation, whereas O/W emulsion did either not change (0 h and 48 h) or enhance (24 h) inactivation compared to pure IPB.

These results show that both temperature and matrix composition during incubation preceding HHP treatment have an impact on HHP sensitivity of *L. plantarum*, with higher temperatures and the presence of fat causing stronger inactivation.

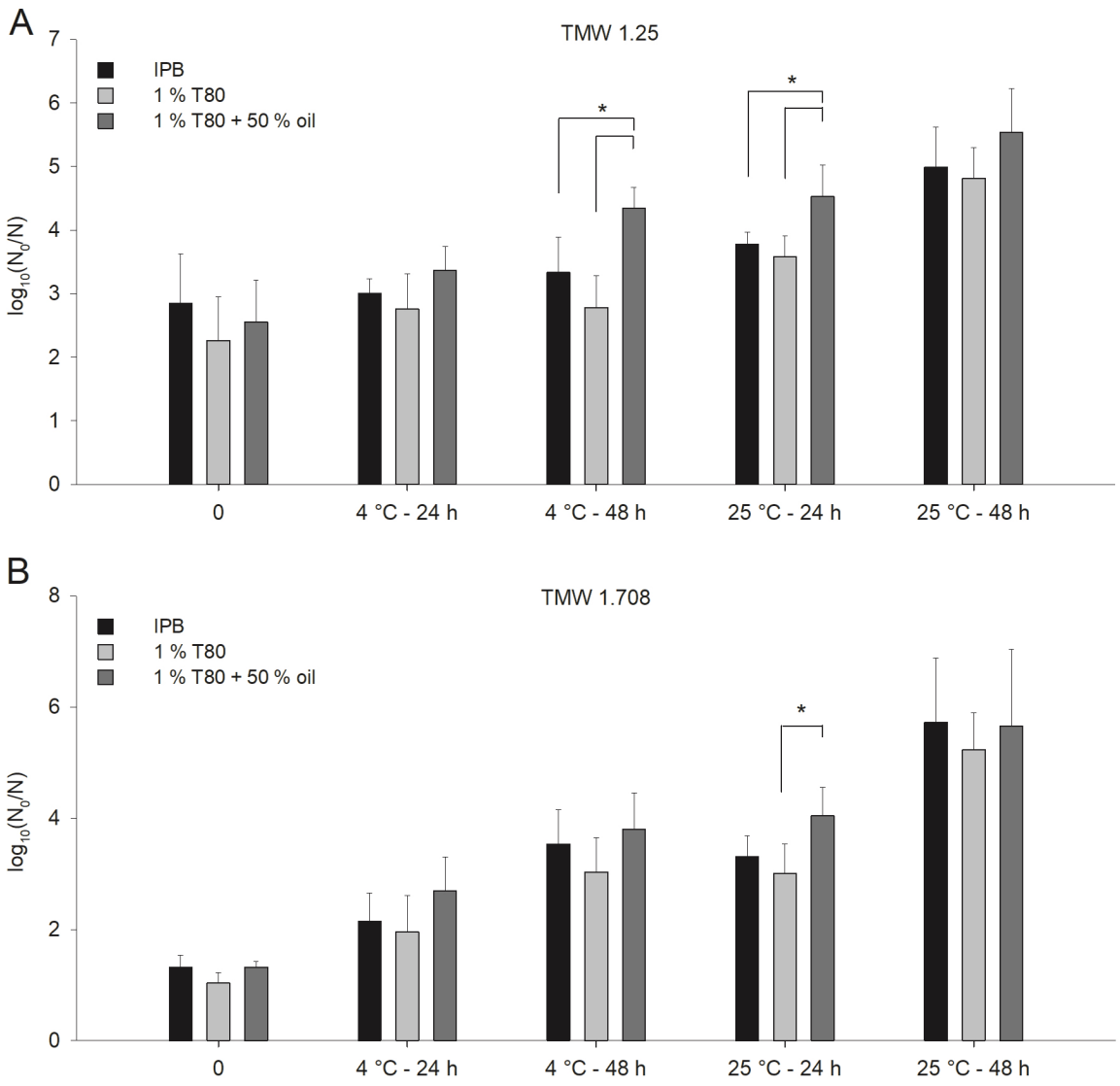

Figure 30: HHP inactivation of *L. plantarum* upon extended incubation time in O/W emulsion at 4 °C and 25 °C. Hydrophobic strain TMW 1.25 (A) and hydrophilic strain TMW 1.708 (B) were cultivated in mMRS for 24 h at 30 °C. Cultures were washed once and resuspended in IPB, IPB containing 1 % (w/v) Tween 80 or O/W emulsion of 50 % (v/v) rapeseed oil in IPB with 1 % (w/v) Tween 80 as emulsifier to a final concentration of ~10^7 cfu mL^{-1}. Suspensions were HHP treated (400 MPa, 5 min, 25 °C) either immediately or after 24 h or 48 h incubation at 4 °C or 25 °C. The values shown are the mean values of at least three independent experiments. Error bars represent the standard deviation. Asterisks (*) indicate statistically significant difference ($p < 0.05$) within individual HHP conditions.

3.6.2 Effect of growth medium supplementation with fat and fat-associated compounds on high-pressure inactivation

Bacteria are strongly influenced by the chemical composition of their environment during growth and proliferation. Cultivation conditions influence the resistance of cells to subsequently applied stress conditions (Zotta *et al.*, 2013). Moreover, the previous experiment (Effect of preconditioning in O/W emulsions on HHP sensitivity of *L. plantarum*) showed that residence in a

fat-containing environment can change HHP sensitivity. In order to test whether the presence of fat or fat-associated compounds during cultivation has an impact on the HHP sensitivity, HHP inactivation of *L. plantarum* was determined after cultivation in growth medium supplemented with oil and other fat-associated compounds, such as Tween or free fatty acids.

3.6.2.1 Effect of rapeseed oil on high-pressure inactivation

The effect of fat during cultivation on the HHP sensitivity of *L. plantarum* was investigated by the addition of rapeseed oil to the growth medium. In order to prevent the uptake of exogenous fatty acids form alternative sources, mMRS medium from which Tween 80 was omitted (mMRS-) was used. 50 % (v/v) rapeseed oil was dispersed using an Ultra Turrax® disperser-homogenizer device to achieve a maximum contact area of medium and oil. The cultures were allowed to grow for 24 h at 30°C with agitation to counteract phase separation and to facilitate the contact of cells to the oil surface. HHP inactivation at different pressure levels from 350 to 450 MPa is shown in Figure 31 and Table 36 (Appendix).

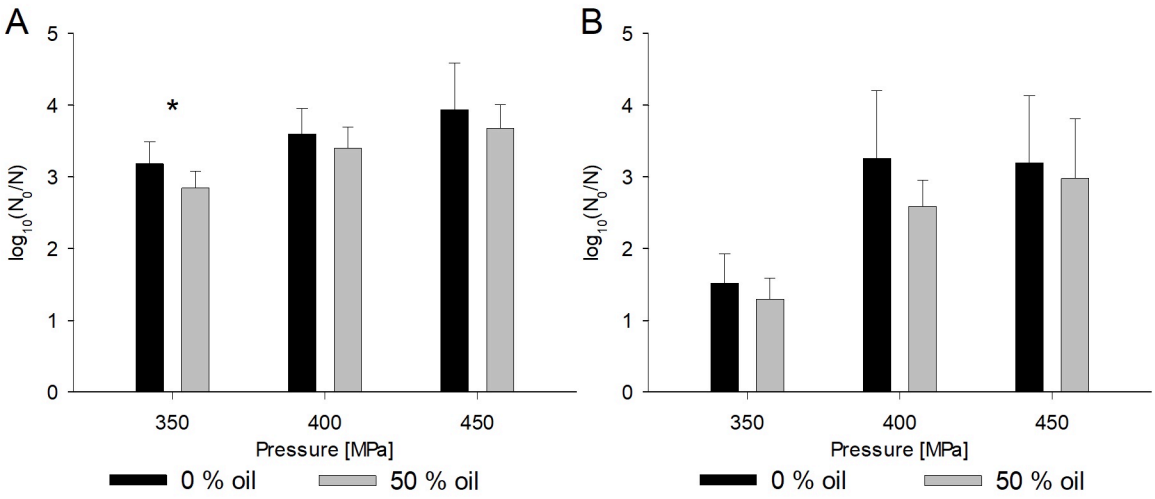

Figure 31: HHP inactivation of *L. plantarum* upon cultivation in the presence of rapeseed oil. Cells of (A) hydrophobic strain TMW 1.25 and (B) hydrophilic strain TMW 1.708 were cultivated in mMRS- mixed with 50 % (v/v) rapeseed oil for 24 h at 30 °C with agitation. Cultures were washed three times and resuspended in IPB to a final concentration of ~10^7 cfu mL^{-1} and finally subjected to HHP (350/400/450 MPa, 5 min, 25 °C). The values shown are the mean values of at least three independent experiments. Error bars represent the standard deviation. Asterisks (*) indicate statistically significant difference ($p < 0.05$) within individual HHP conditions.

HHP inactivation of hydrophobic strain TMW 1.25 was slightly weakened by < 0.5 log when rapeseed oil was present during cultivation. The same trend was observed with TMW 1.708, showing that the slightly protective effect of rapeseed oil is independent of the CSH of the cells. Considering the standard deviations, however, the observed differences were not large enough to be statistically significant.

3.6.2.2 Effect of cultivation with Tween on HHP sensitivity of *L. plantarum*

Oleic acid makes up more than 60 % of the mass of rapeseed oil (as determined in the course of this study by VFG Labor GmbH, see Appendix), and is thus by far the most abundant fatty acid therein. On the other hand, it represents the hydrophobic part of Tween 80, which, besides its function as emulsifier, is a well-known additive of bacterial growth media, providing an easily accessible oleic acid source for bacterial cells. Therefore, the effect of the presence of Tween 80 in the growth medium on the HHP sensitivity was investigated in detail.

3.6.2.2.1 HHP sensitivity upon cultivation with different Tween 80 concentrations

In order to investigate the effect of cultivation at different concentrations of Tween 80 on the HHP sensitivity of *L. plantarum*, cells were either grown in normal mMRS, which contains 0.1 % (w/v) by default, mMRS from which Tween 80 was omitted (mMRS-) or mMRS to which Tween 80 was added to a final concentration of 1 % (w/v) before HHP treatment in pure IPB. The inactivation at different pressure levels is shown in Figure 32 and Table 37 (Appendix).

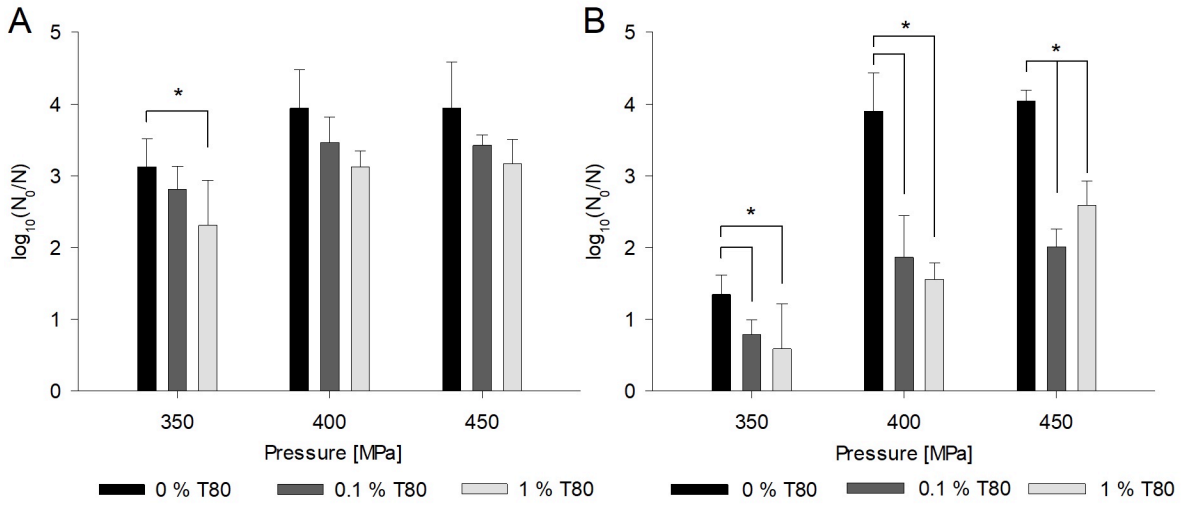

Figure 32: HHP inactivation of *L. plantarum* upon cultivation in mMRS medium supplemented with different concentrations of Tween 80. Cells of (A) hydrophobic strain TMW 1.25 and (B) hydrophilic strain TMW 1.708 were cultivated in mMRS without Tween 80 (mMRS-), standard mMRS containing 0.1 % (w/v) Tween or mMRS supplemented with 1 % (w/v) Tween 80 for 24 h at 30 °C. Cultures were washed once and resuspended in IPB to a final concentration of ~10^7 cfu mL^{-1} and finally subjected to HHP (350/400/450 MPa, 5 min, 25 °C). The values shown are the mean values of at least three independent experiments. Error bars represent the standard deviation. Asterisks (*) indicate statistically significant difference ($p < 0.05$) within individual HHP conditions.

The presence of 0.1 % Tween 80 in the growth medium reduced HHP inactivation of hydrophobic strain TMW 1.25 by ~0.5 log compared to mMRS- at all tested pressure levels. At the higher concentration of 1 % the effect of Tween 80 was even stronger, with inactivation levels reduced by ~1 log.

With hydrophilic strain TMW 1.708 the effect of Tween 80 was even more pronounced, with log reduction levels at 400 and 450 MPa being reduced from ~4 to ~2 by 0.1 % Tween. At 400 MPa, but not at 450 MPa, the higher Tween 80 concentration (1 %) caused a further reduction in inactivation. At 350 MPa, the protective effect of Tween 80 was lower, as inactivation was reduced by ~0.5 and < 1.0 log upon cultivation at 0.1 % and 1 % Tween 80, respectively. However, at these conditions, TMW 1.708 showed a relatively weak inactivation of < 1.5 log even when grown without Tween 80.

3.6.2.2.2 Effect of different Tween types on high-pressure inactivation of L. plantarum

The protective effect conferred by Tween 80 was further investigated to find out which part of the Tween 80 molecule, i.e. the polyoxyethylene moiety or the fatty acid part is responsible for the increased HHP sensitivity observed after cultivation in the presence of Tween 80. Individual Tween types differ in their fatty acid residue with Tween 80 containing oleic acid, Tween 60 being characterized by stearic acid, Tween 40 by palmitic acid and Tween 20 having lauric acid as lipophilic part. It was hypothesized that different Tween types influence HHP sensitivity according to their specific fatty acid. To test this, *L. plantarum* strains TMW 1.25 and TMW 1.708 were grown to stationary phase in mMRS- or in mMRS containing different Tween types, suspended in IPB and subsequently HHP treated with pressure-time-temperature combinations leading to significant but not complete inactivation. Results are shown in Figure 33 and Table 38 (Appendix).

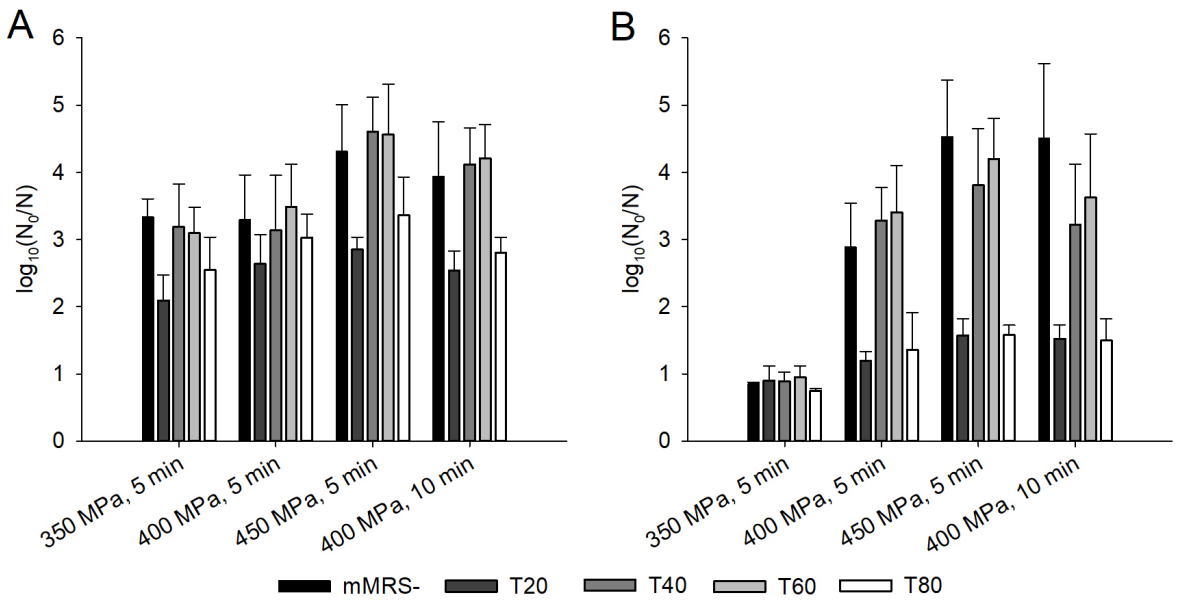

Figure 33: HHP inactivation of *L. plantarum* cultivated in mMRS supplemented with different Tween types. Cells of (A) hydrophobic strain TMW 1.25 and (B) hydrophilic strain TMW 1.708 were grown in mMRS- or mMRS supplemented with 1 g L^{-1} of different Tween 20 (T20), Tween 40 (T40), Tween 60 (T60) or Tween 80 (T80) and treated at different pressure/holding time combinations (350/400/450 MPa, 5 min; 400 MPa, 10 min) at 25 °C. Inactivation levels (log reduction of cfu mL^{-1}) were determined on mMRS agar plates. Means and standard deviations derived from at least three independent replicates are depicted. Statistically significant differences are presented in Table 39 (Appendix).

The HHP-induced inactivation of TMW 1.25 cultivated in mMRS-, was between 3.0 and 3.5 log cycles for 350 MPa and 400 MPa and between 4.0 and 4.5 log cycles at 450 MPa, when pressure holding time was 5 min. Pressure treatment with 400 MPa for 10 min led to inactivation by ~4.0 log cycles. Tween 40 and Tween 60 caused no significant changes, with similar inactivation compared to mMRS-. In contrast, Tween 20 and Tween 80 conferred considerable protection to TMW 1.25 cells, as inactivation was decreased by ~1 or more orders of magnitude at 450 MPa, 5 min and 400 MPa, 10 min. The protective effect of Tween 20 and Tween 80 was smallest during treatment with 400 MPa for 5 min.

Inactivation levels of TMW 1.708 grown in mMRS- generally showed greater variation among different pressure-holding time combinations than those of TMW 1.25 with 350 MPa, 5 min leading to ~1 log reduction and inactivation strongly increasing with higher pressures and longer holding times, reaching levels of ~3 log at 400 MPa, 5 min and ~4.5 log at 450 MPa, 5 min and 400 MPa, 10 min.

Except for the mildest pressure condition applied (350 MPa, 5 min) where similar HHP inactivation levels (~1 log) were observed with all growth conditions, TMW 1.708 cells grown in

the presence of Tween 20 or Tween 80, but not Tween 40 or Tween 60, showed strongly reduced HHP inactivation. The protective effect of Tween 20 and Tween 80 on TMW 1.708 was even stronger than with TMW 1.25, as inactivation levels were reduced by ~2 log (400 MPa 5 min) and ~3 log (450 MPa, 5 min; 400 MPa, 10 min) by both Tween 20 and Tween 80.
These results clearly indicate a role of the specific fatty acids present in the different Tweens to be responsible for changes in HHP sensitivity.

3.6.2.3 Effect of free fatty acids on high-pressure inactivation

On the basis of the results in the previous section, it was hypothesized that the protective effect of Tween 20 and Tween 80 results from the respective fatty acids coupled to the Tween polyoxyethylene backbone, i.e. oleic acid and lauric acid, respectively. In order to test this assumption, TMW 1.25 and TMW 1.708 were grown to stationary phase in mMRS- supplemented with different food-relevant free fatty acids, including those present in the different Tween types, and subsequently HHP treated with pressures between 300 and 500 MPa. To incorporate free fatty acids into mMRS-, they were dissolved in 95 % (v/v) ethanol and subsequently added to the medium. To exclude the possibility of changes in HHP sensitivity due to the fatty acid solvent (95 % ethanol), a sample composed of mMRS- and the amount of 95 % ethanol introduced during fatty acid solubilization was included (FA solvent). Results are shown in Figure 34 and Table 40 (Appendix).

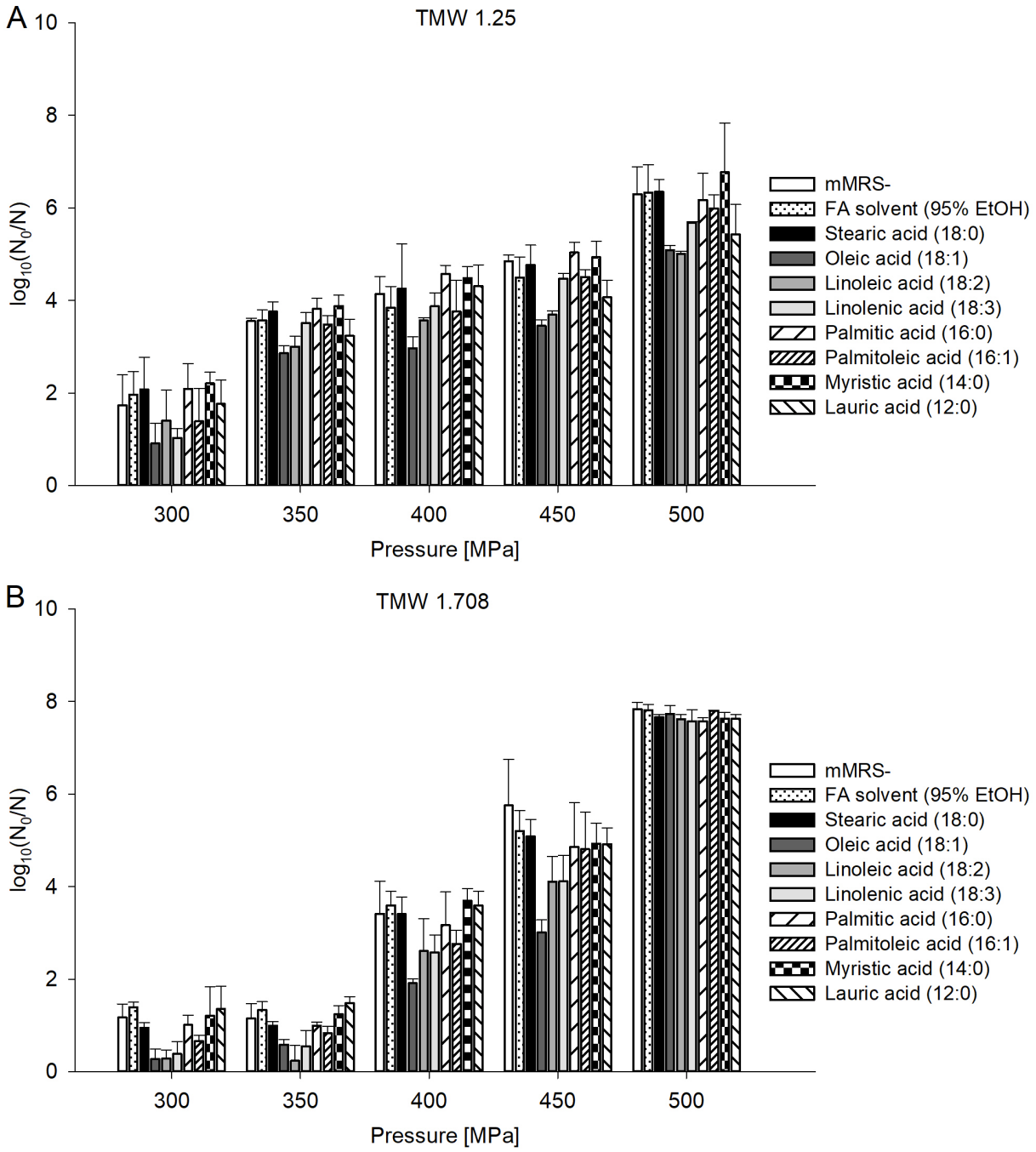

Figure 34: HHP inactivation of *L. plantarum* upon growth in mMRS supplemented with different free fatty acids. Cells of (A) hydrophobic strain TMW 1.25 and (B) hydrophilic strain TMW 1.708 were grown in mMRS- or mMRS supplemented with 50 mM of different free fatty acids at 30 °C for 24 h, harvested, washed and re-suspended in IPB to a final concentration of 10^7-10^8 cfu mL^{-1} and treated with the indicated pressures at 25°C for 5 min. The log reduction was determined on mMRS agar plates. Shown are the means and standard deviations of at least 3 replicates. Statistically significant differences are presented in Table 41 (Appendix).

HHP inactivation of TMW 1.25 grown in mMRS- increased with pressure from < 2 log at 300 MPa to > 6 log at 500 MPa. The fatty acid solvent alone had no impact on HHP inactivation, which means that any differences between fatty acid samples and mMRS- are due to effects of the individual fatty acids.

The unsaturated fatty acids stearic, palmitic, myristic and lauric acid had no effect on HHP sensitivity, although lauric acid slightly reduced HHP inactivation at 450 and 500 MPa. Palmitoleic acid caused a slight reduction in inactivation, but differences were not statistically significant (see Table 41). In contrast, growth in the presence of oleic acid reduced HHP inactivation by about 1 log cycle, regardless of the pressure height. A similar effect, although less pronounced, was observed with linoleic acid, whereas linolenic acid reduced inactivation only at 300 and 500 MPa.

In accordance with the results presented in the previous section, the pressure height had a stronger impact on the inactivation of TMW 1.708 compared to TMW 1.25: Inactivation of TMW 1.708 grown in mMRS- or mMRS- with FA solvent increased from ~1.5 log at 300 and 350 MPa to ~4 log at 400 MPa and ~6 log at 450 MPa and reached the detection limit (almost 8 log) after treatment with 500 MPa. The four saturated fatty acids under investigation, stearic, palmitic, myristic and lauric acid had no effect on HHP inactivation whereas the unsaturated fatty acids linoleic, linolenic and palmitoleic acid exerted a slightly protective effect. As with TMW 1.25, oleic acid had the greatest impact, strongly protecting cells from HHP inactivation. Especially at 400 and 450 MPa, growth with oleic acid reduced inactivation by > 1 and > 2 log cycles, respectively. Treatment of TMW 1.708 with 500 MPa caused inactivation levels close to the detection limit, regardless of the cultivation condition.

With the exception of Tween 20 and lauric acid - Tween 20 reduced HHP inactivation to an extent comparable to that of Tween 80 but lauric acid had almost no effect on HHP sensitivity - the effects of the free fatty acids on HHP sensitivity match with those of the corresponding Tween type. In summary, these results show that the fatty acid moiety of Tween is responsible for its effect on HHP sensitivity. Moreover, it turns out that only oleic acid and related unsaturated C18 FAs can increase HHP resistance. Consequently, the protective effect of Tween 20 might be due to traces of oleic acid, which can occur in Tween 20 (Partanen *et al.*, 2001).

3.6.3 Investigation of the positive effect of Tween 80 on HHP resistance of *L. plantarum*

The effect of cultivation with fat-associated compounds on HHP sensitivity of *L. plantarum* was investigated in more detail using Tween 80 as a model compound. Tween 80 was chosen due to its high food relevance – it provides cells with oleic acid that is present in high amounts in many vegetable oils – and because the impact on HHP sensitivity was greater than with other Tween types, any free fatty acids or genuine rapeseed oil. Other Tween types were included for comparison where appropriate. TMW 1.708 was selected as a model organism due to the wider range of inactivation levels observed upon treatment with pressures between 0 and 500 MPa and because the effect of cultivation with fat and fat-associated compounds on HHP inactivation was stronger than with TMW 1.25.

To gain an insight into the cellular mechanisms underlying Tween 80-mediated HHP tolerance, the effect of Tween 80 on the transcriptomic profile, the cellular fatty acid composition was examined. Furthermore, the extent of HHP-induced sublethal and lethal injury as well as metabolic activity after HHP treatment of TMW 1.708 grown in the presence and absence of Tween 80 was examined. Since the cell membrane is an important target structure for HHP, the effect of Tween 80 on membrane integrity under HHP was investigated in detail.

3.6.3.1 Transcriptomic response to cultivation with Tween 80

Transcriptomic analysis provides detailed insight into the bacterial physiology and metabolic activity under specific conditions and thus enables the identification of the effect of environmental conditions, such as the presence of Tween 80, on bacterial cells on a molecular level.

The transcriptomic response of TMW 1.708 to the addition of Tween 80 to the growth medium was assessed in mid-exponential growth phase where gene expression levels and metabolic activity are generally highest, and changes due to exogenous factors are thus most pronounced and evident.

Shotgun RNA sequencing and bioinformatic analysis in triplicate revealed that < 30 % of TMW 1.708 genes were affected by Tween 80 (corrected p-value < 0.05) and only 11.43 % of these genes showed expression changes greater than two-fold (Figure 35).

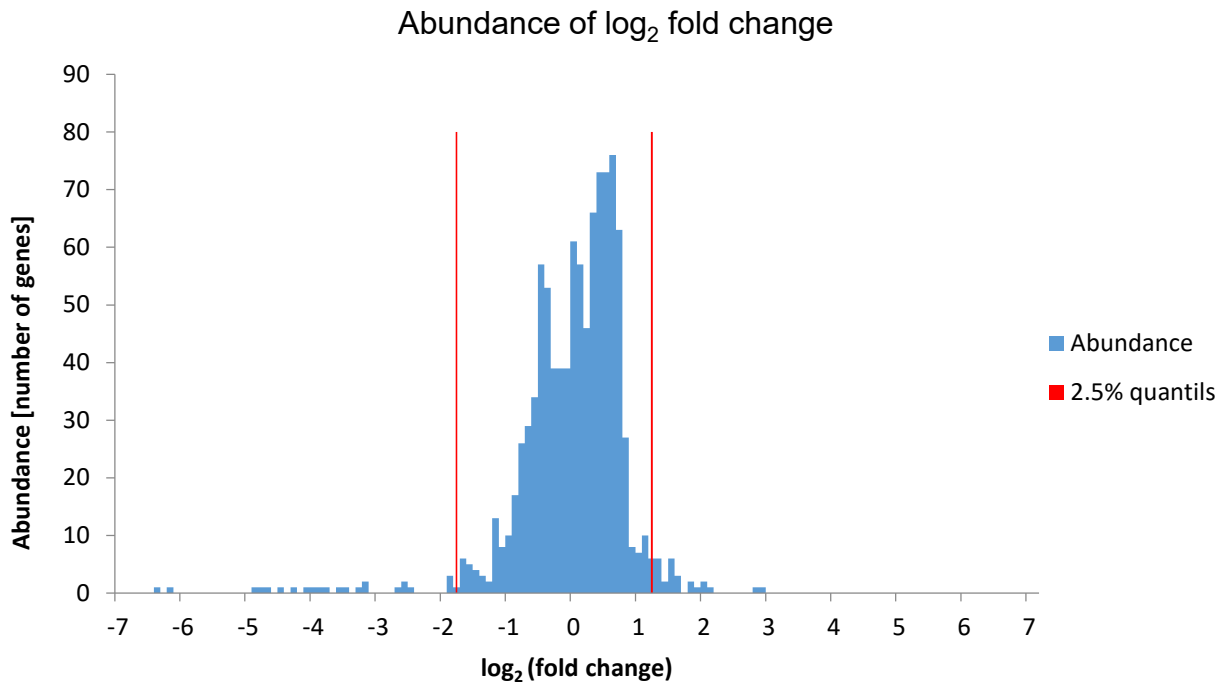

Figure 35: Abundance of changes in gene expression of *L. plantarum* strain TMW 1.708 caused by Tween 80. Tween 80 was added to a culture of the strain in mMRS- in mid-exponential phase to a final concentration of 1 g L^{-1}. Cells were harvested 30 min after the addition of Tween 80 and preparations were sent to GATC Biotech GmbH (Konstanz, Germany) for Shotgun RNA sequencing. Sequencing data were analyzed using Rockhopper software on the basis of the genome of *L. plantarum* TMW 1.708 (Biosample: SAMN05805046) published earlier by Kafka *et al*. The entire process was performed in triplicate. Shown are genes with significant changes in expression level (corrected p-value < 0.05). The vertical red lines mark the lower and upper 2.5 % quantiles.

For detailed analysis, the lower and upper 2.5 % quantiles of genes with significant expression changes were selected, representing the 2.5 % of genes with the strongest decrease (Table 15) or increase (Table 16) in expression in response to medium supplementation with Tween, respectively. The most pronounced effect was observed with key elements of the fatty acid synthesis pathway such as *fabZ*, *fabH* and *fabD* as well as the acyl carrier protein AcpA2 and others, which showed strong downregulation upon the addition of Tween 80 (Table 15). Genes involved in transmembrane transport of oligopeptides (*oppC*) and potassium (*kup1/2*) as well as *gadB* glutamate decarboxylase, which catalyzes the conversion from glutamate into 4-aminobutanoate and CO_2, were also downregulated in response to Tween 80 addition. An increase in mRNA levels upon the addition of Tween 80 was observed for genes playing a role in the uptake of amino acids such as lysine (*lysP*) and glutamine (*glnQ3, glnPH2*) as well as the synthesis of glutamine (*glnA*). Furthermore, genes implicated in the uptake of manganese (*mntH3*, predicted protein) and phosphate (*pstB1*), the ingestion of N-acetylglucosamine (*pts18CBA*), the transmembrane diffusion of uncharged solutes like urea, glycerol, and lactic acid (*glpF4*) were upregulated. In addition, genes involved in pyrimidine metabolism showed increased mRNA levels in mMRST80, which indicates an increase in metabolic activity upon the

addition of Tween 80. These are (i) *pyrP*, a predicted transmembrane permease for uracil uptake (Arsène-Ploetze *et al.*, 2006), (ii) orothate phosphoribosyltransferase *pyrE*, which catalyzes the conversion of orothate to uridine monophosphate (UMP) (Bouia *et al.*, 1990), (iii) *pyrG*, a cytidine triphosphate (CTP) synthase, which is responsible for the synthesis of CTP and glutamate from uridine triphosphate (UTP) and glutamine, and (iv) *pyrR1*, the transcriptional regulator of the biosynthetic pyrimidine *pyrR1-B-C-Aa1-Ab1-D-F-E* operon (Nicoloff *et al.*, 2005). Upregulation was observed also for (i) two genes coding for predicted N-acetyltransferases, (ii) part of the *cps4* gene cluster including genes with predicted glycosyltransferase (*cps4G, cps4I*), polysaccharide polymerase (*csp4H*) and flippase (*csp4J*) function, and (iii) a predicted mannose-specific adhesin (*msa*) and a multiple antibiotic resistance regulator (MarR) family transcriptional regulator probably involved in mannose adhesion.

Table 15: Genes of *L. plantarum* TMW 1.708 with decreased expression levels upon the addition of Tween 80 to the growth medium. The 2.5 % quantile of genes with significantly decreased expression levels (corrected p-value < 0.05) from mMRS- to mMRST80 is shown. Genes are sorted according to their log_2 expression changes. Results are the mean values of three independent experiments.

sseqid	Name	Gene product/function	General functional category	log_2 expression change (mMRS- → mMRST80)
BIZ33_13110	-	Fatty acid-binding protein (DegV family)	Fatty acid metabolism	-6.43
-	-	part of BIZ33_13110	Fatty acid metabolism	-6.25
BIZ33_06635	*fabZ*	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ (EC 4.2.1.59)	Fatty acid biosynthesis, Biotin metabolism	-4.95
BIZ33_06640	*fabH*	3-Oxoacyl-ACP synthase (EC 2.3.1.180)	Fatty acid biosynthesis	-4.80
BIZ33_06645	*acpA2*	Acyl carrier protein	Fatty acid biosynthesis	-4.72
BIZ33_06650	*fabD*	ACP S-malonyltransferase (EC 2.3.1.39)	Fatty acid biosynthesis	-4.50
BIZ33_06655	*fabG1*	beta-ketoacyl-ACP reductase (EC 1.1.1.100)	Fatty acid biosynthesis	-4.38
BIZ33_06660	*fabF*	beta-ketoacyl-[acyl-carrier-protein] synthase II (EC 2.3.1.179)	Fatty acid biosynthesis	-4.14
BIZ33_06665	*accB2*	Acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	Fatty acid biosynthesis	-4.04
BIZ33_06670	*fabZ2*	beta-hydroxyacyl-ACP dehydratase (EC 4.2.1.59)	Fatty acid biosynthesis	-3.99
BIZ33_06675	*accC2*	Acetyl-CoA carboxylase biotin carboxylase subunit (EC 6.3.4.14)	Fatty acid biosynthesis	-3.83
BIZ33_06680	*accD2*	Acetyl-CoA carboxylase subunit beta (EC 6.4.1.2)	Fatty acid biosynthesis	-3.62
BIZ33_06685	*accA*	Acetyl-CoA carboxylase carboxyl transferase subunit alpha (EC 6.4.1.2)	Fatty acid biosynthesis	-3.55

sseqid	Name	Gene product/function	General functional category	log_2 expression change (mMRS- → mMRST80)
BIZ33_06690	*fabI*	Enoyl-[acyl-carrier-protein] reductase (EC 1.3.1.9)	Fatty acid biosynthesis	-3.36
-	-	part of BIZ33_06695		-3.28
BIZ33_06695	-	Phosphopantetheinyl transferase	Fatty acid biosynthesis	-3.22
BIZ33_06620	-	Uncharacterized membrane protein	-	-2.75
BIZ33_13555	-	Uncharacterized protein	-	-2.69
BIZ33_02005	*kup1*	Kup system potassium uptake protein	Potassium uptake	-2.62
BIZ33_13550	-	Membrane-bound cell surface alpha-beta hydrolase	-	-2.58
BIZ33_10730	-	Glycosyl transferase (family 1)	-	-1.94
BIZ33_13185	*kup2*	Kup system potassium uptake protein	Potassium uptake	-1.94
BIZ33_13665	-	Glutamate decarboxylase (EC 4.1.1.15)	Amino acid transport and metabolism	-1.92
BIZ33_06705	-	Transcriptional regulator (LysR family)	transcription regulation	-1.85
BIZ33_05085	*oppC*	Transmembrane oligopeptide ABC transporter (TC 3.A.1.5.1)	Amino acid transport and metabolism	-1.80
BIZ33_12155	-	Extracellular transglycosylase with LysM peptidoglycan binding domain	-	-1.79

Table 16: Genes of *L. plantarum* TMW 1.708 genes with increased expression levels upon the addition of Tween 80 to the growth medium. The 2.5 % quantile of genes with significantly increased expression levels (corrected p-value < 0.05) from mMRS- to mMRST80 is shown. Genes are sorted according to their log_2 expression changes. Results are the mean values of three independent experiments.

seqid	Name	Gene product/function	General functional category	log_2 expression change (mMRS- → mMRST80)
BIZ33_08655	*glnPH2*	Glutamine ABC transporter substrate binding and permease protein	Amino acid transport and metabolism	2.81
BIZ33_08650	*glnQ3*	Glutamine ABC transporter ATP-binding protein	Amino acid transport and metabolism	2.73
BIZ33_04195	*lysP*	Lysine-specific permease	Amino acid transport and metabolism	2.07
BIZ33_01905	*pyrG*	CTP synthase (EC 6.3.4.2)	Pyrimidine metabolism	1.94
BIZ33_09855	*pyrP*	Uracil permease	Pyrimidine metabolism	1.90
BIZ33_10880	*pyrE*	Orotate phosphoribosyltransferase (EC 2.4.2.10)	Pyrimidine metabolism	1.81
BIZ33_13095	-	N-acetyltransferase (GNAT family)	-	1.79

seqid	Name	Gene product/function	General functional category	log_2 expression change (mMRS- → mMRST80)
BIZ33_04925	*glpF4*	Glycerol uptake facilitator protein	Glycerol uptake/metabolism	1.74
-	*msa*	Mannose-specific adhesin, LPXTG-motif cell wall anchor	mannose adhesion	1.58
BIZ33_04935	-	Transcriptional regulator (MarR family)	mannose adhesion	1.55
BIZ33_10915	*pyrR1*	Pyrimidine nucleotide operon transcriptional regulator/Uracil phosphoribosyltransferase (EC 2.4.2.9)	pyrimidine metabolism	1.53
BIZ33_06275	*glnA*	Glutamine synthetase (type I) (EC 6.3.1.2)	Amino acid transport and metabolism	1.50
-	-	hypothetical protein	-	1.47
BIZ33_08600	*cps4I*	Glycosyltransferase (family 2)	-	1.46
BIZ33_06270	-	Transcriptional regulator (MerR family)	Transcription regulation/Amino acid metabolism	1.43
BIZ33_10925	-	Xanthine permease	Xanthine/uracil uptake	1.42
-	*mntH3*	Manganese transport protein	Manganese uptake	1.41
BIZ33_05610	-	N-acetyltransferase (GNAT family)		1.40
BIZ33_08610	*cps4G*	Glycosyl transferase (family 1)		1.30
BIZ33_12780	-	hypothetical protein		1.26
BIZ33_10280	*pts18CBA*	PTS N-acetylglucosamine transporter subunit IIABC (EC 2.7.1.69)	Amino sugar and nucleotide sugar metabolism; Phosphotransferase system (PTS)	1.26
BIZ33_12290	-	Amino acid transport protein	Amino acid transport and metabolism	1.25
BIZ33_08605	*cps4H*	Polysaccharide polymerase	polysaccharide synthesis	1.24
BIZ33_08595	*cps4J*	Polysaccharide repeat unit transporter (Flippase)	polysaccharide synthesis	1.23
BIZ33_12070	-	nucleotide binding protein, universal stress protein UspA	-	1.23
BIZ33_03065	*pstB1*	Phosphate ABC transporter ATP-binding protein (TC 3.A.1.7.1)	phosphate transport	1.20

3.6.3.2 Effect of Tween on growth characteristics

Tween 80 is used as a growth enhancer in bacterial culture media and the results from transcriptomic analysis (see 3.6.3.1) substantiate its growth stimulating effect. To measure the effect of Tween 80 on the growth of *L. plantarum* directly, growth curves were recorded in mMRS- and mMRS supplemented with Tween 80 and from these the growth parameters maximum optical density (OD_{600}max), maximum growth rate (μ_{max}) and duration of the lag phase (TI) were calculated. In order to determine the specific role of the fatty acid part, three additional types of Tween were included. These are Tween 20, Tween 40 and Tween 60, containing lauric acid, palmitic acid and stearic acid, respectively, as the specific fatty acid moiety. As shown in Figure 36 and Table 43 (Appendix), OD_{600}max was highest with Tween 20 and Tween 80. However, the differences were small and only Tween 20 led to a significant increase compared to mMRS- (Figure 36B). Tween 80 was the only Tween type that significantly increased μ_{max} compared to the control (mMRS-) (Figure 36C), whereas a significant shortening of the lag time was observed with all Tween types, although the impact of Tween 60 was even stronger compared to the other three Tween types (Figure 36D). However, it has to be kept in mind that due to pre-cultivation in mMRS- for 24 h, causing the pre-cultures to be in the early stationary phase at the time of inoculation, only minor adaptation of the metabolism was required. Therefore, the lag phase was very short for all samples. In summary, growth analysis confirms the growth enhancing effect of Tween 80 and shows that its effect on bacterial growth is mainly due to the fatty acid part.

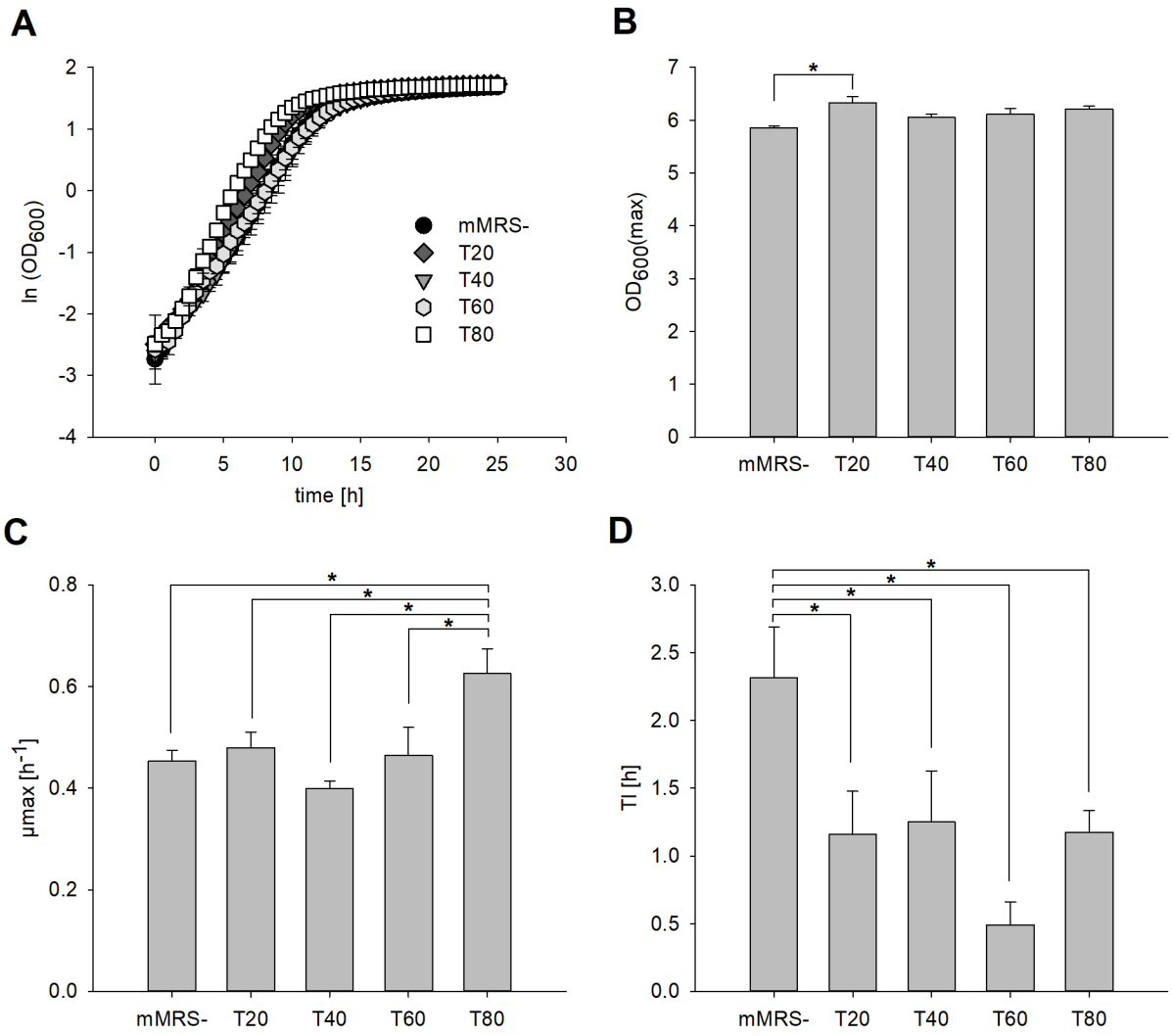

Figure 36: Growth curves and parameters of *L. plantarum* TMW 1.708 grown in mMRS supplemented with different Tween types. Cells from a 24 h pre-culture in mMRS without Tween supplement (mMRS-) were transferred (1 % v/v) in fresh mMRS- or mMRS containing 1 g L^{-1} of either Tween 20 (T20), Tween 40 (T40), Tween 60 (T60), or Tween 80 (T80) and grown at 30 °C for 30 h. Growth curves (A), maximum OD_{600} (B), maximum growth rate μ_{max} (C) and duration of lag phase TI (D) are depicted. The presented values are the means of at least three replicates. Error bars represent the standard deviation. Asterisks (*) mark data with statistically significant difference ($p < 0.05$).

3.6.3.3 Cellular fatty acid profile upon growth in medium supplemented with Tween

The results of transcriptomic analysis of TMW 1.708, showing the downregulation of genes involved in fatty acid synthesis through the addition of Tween 80, indicate that exogenous fatty acids are incorporated directly into the cytoplasmic membrane. Moreover, previous studies revealed that supplementation of bacterial growth media with Tween can have a strong impact on the cellular fatty acid profile (Partanen *et al.*, 2001), which mostly represents the fatty acids

present in the cytoplasmic membrane. Lastly, in this study a significant impact of Tween as well as free fatty acids on HHP sensitivity was shown. At the same time, the cytoplasmic membrane is well-known as one of the main cellular target structures during HHP-induced bacterial inactivation. These observations gave rise to the hypothesis that medium supplementation with Tween changes the fatty acid composition of the cell membrane of *L. plantarum* according to the exogenous fatty acid(s) provided and that a correlation exists between the cellular fatty acid profile and HHP sensitivity.

Therefore, cellular fatty acid profiles of stationary phase cells of TMW 1.708 after growth in mMRS- or mMRS supplemented with different Tween types were determined. The relative abundance of the individual fatty acids is shown in Figure 37A and Table 42 (Appendix).

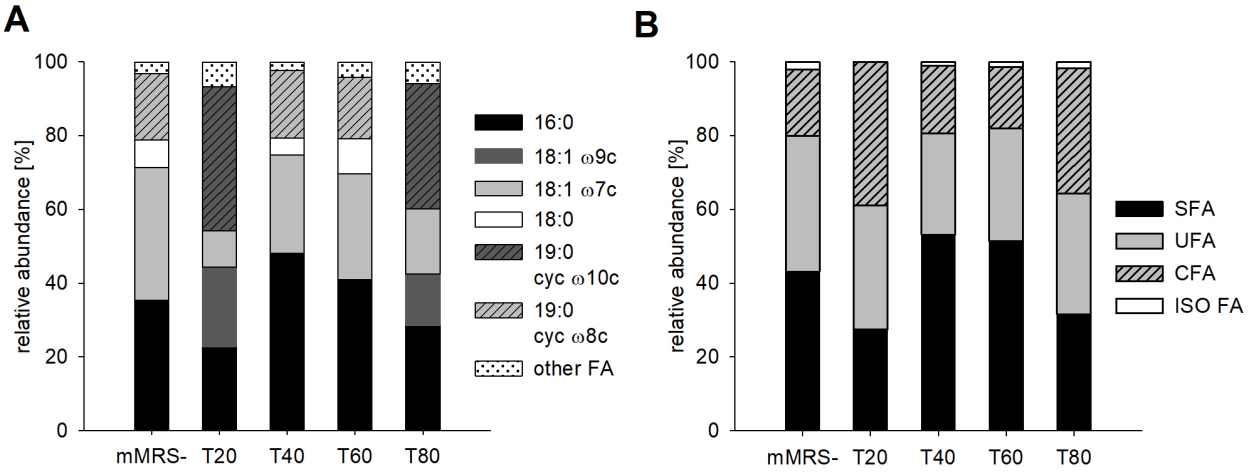

Figure 37: Cellular fatty acid profiles of *L. plantarum* TMW 1.708 after growth in the presence of different Tween types. Cellular fatty acid profiles were determined after 24 h growth in mMRS-, mMRST20, mMRST40, mMRST60 or mMRST80. (A) Relative abundance of specific fatty acids. Fatty acids with relative abundance lower than 3 % are grouped and displayed as “other FA”. (B) Relative abundance of saturated (SFA), unsaturated (UFA), cyclic (CFA) and iso-fatty acids (ISO FA).

The fatty acid profile after growth in mMRS- was mainly characterized by four fatty acids, namely palmitic (16:0) (35.25 %), cis-vaccenic (18:1 ω7cis) (31.11 %), stearic (18:0) (7.44 %) and lactobacillic acid (19:0 cyclo ω8cis) (17.99 %), accounting for > 96 % of the total FA content. The amounts of myristic (14:0), palmitoleic (16:1 ω7cis) acid and a fatty acid containing functional groups like hydroxyl or methyl groups (15:0 iso 2OH) were negligible (Appendix Table 42).

Supplementation with Tween 40 or Tween 60 caused only minor changes in the fatty acid profile, which were mainly attributable to the exogenous fatty acid, palmitic acid or stearic acid, provided by the respective Tween type: Tween 40 led to an increase in the amount of palmitic

acid to 48.14 %, whereas Tween 60 slightly raised the stearic acid proportion from 7.44 to 9.41 %. The increase in these fatty acids took place mainly at the expense of *cis*-vaccenic acid.
Tween 20 and Tween 80 led to considerable proportions of oleic (18:1 ω9cis) (21.49 % with Tween 20 and 13.89 % with Tween 80) and dihydrosterculic (19:0 cyclo ω10cis) acid (39.02 % / 34.02 %), which were not found in the other three fatty acid profiles. At the same time, cis-vaccenic (9.90 % / 17.51 %) and palmitic (22.82 % / 28.64 %) acid levels were reduced, whereas lactobacillic and stearic acid disappeared completely. Contrary to expectations according to its molecular composition with lauric acid as the fatty acid moiety, the fatty acid profiles caused by Tween 20 showed only marginal lauric acid levels (0.22 %) but strongly resembled that of Tween 80.
Comparing the abundance of fatty acid types, i.e. saturated fatty acids (SFA), unsaturated fatty acids (UFA), cyclic fatty acids (CFA) and branched-chain fatty acids of the iso series (ISO FA) revealed a strong overall increase in the abundance of CFA (17.99 % with mMRS-) in the presence of Tween 20 (39.02 %) or Tween 80 (34.02 %), whereas SFA levels decreased from 43.07 % with mMRS- to 27.35 % with Tween 20 and 31.55 % with Tween 80. UFA levels remained nearly constant (mMRS-: 36.84 %; Tween 20: 33.63 %; Tween 80: 32.70 %) (Figure 37B and Appendix Table 42). Supplementation with Tween 40 and Tween 60, in contrast, caused a slight increase in SFA contents from 43.07 % (mMRS-) to 53.14 % (Tween 40) and 51.40 % (Tween 60) at the expense of UFA (Tween 40: 27.44 %; Tween 60: 30.56 %), and left CFA levels almost unaffected (Tween 40: 18.35 %; Tween 60: 16.64 %).
These results clearly demonstrate an interrelation between the effect of Tween on HHP sensitivity and the corresponding fatty acid profile/membrane fatty acid composition and strongly indicate that the protective effect observed with Tween 20 and Tween 80 is based on the corresponding membrane properties.

3.6.3.4 Effect of Tween 80 on sublethal injury upon high-pressure treatment

Microbial inactivation by HHP depends on a variety of factors, such as the cellular state (growth phase, pre-treatment), environmental conditions (matrix) and of course the pressure-time-temperature combination. Depending on these factors, some cells in a pressure-treated population remain unaffected, thus fully viable, whereas others undergo injury that is so severe that there is no possibility of repair and recovery and cell death is inevitable even under favorable post-pressure conditions. Many cells, however, are injured to such an extent that repair is possible under favorable conditions, whereas additional stress, such as high NaCl concentrations, would inhibit recovery. The standard plate count method providing nearly

optimal conditions allows both undamaged and sublethally damaged cells to recover, but the extent of sublethal injury is not assessed. However, the sublethally injured fraction is of particular interest in studying the effect of HHP on viability and the integrity of different cellular target structures. In the present study, amount of sublethal injury was assessed to further investigate the impact of cultivation in the presence of Tween 80 on HHP sensitivity and the physiological state after HHP treatment.

The extent of sublethal injury can be assessed by comparison of colony formation on non-selective and selective agar. Selective agar contains a stress parameter such as NaCl in the maximum non-inhibitory concentration (MNIC) that still allows growth of fully viable cells. The MNIC of NaCl in mMRS was determined for TMW 1.708 by plating stationary-phase cells on agar plates containing mMRS supplemented with 0-10 % (w/v) NaCl. As shown in Table 44 (Appendix), 7 % (w/v) was the highest NaCl concentration that did not reduce the number of colonies formed by more than 20 %, and was thus selected as the MNIC according to Cebrián *et al.* (2014).

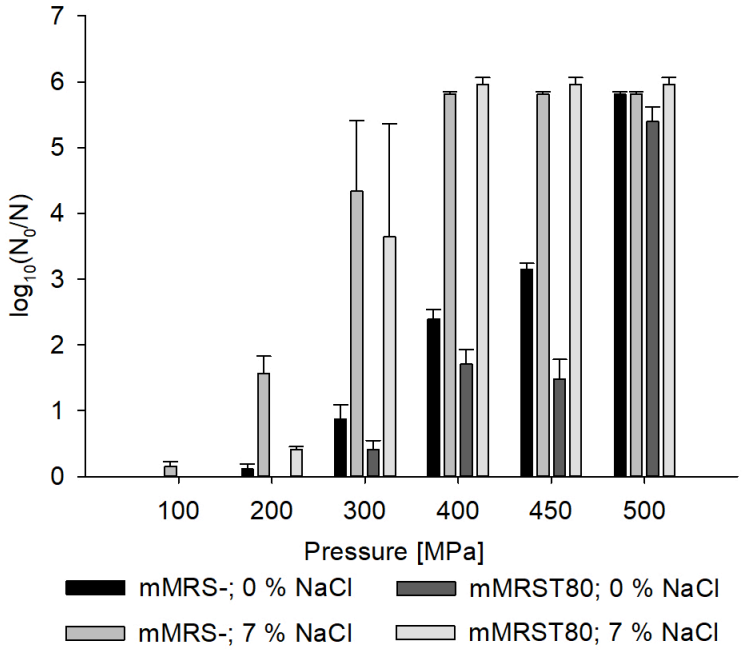

Figure 38: High pressure inactivation of *L. plantarum* TMW 1.708 after growth in mMRS with or without Tween 80 determined by cultivation on selective and non-selective agar. Cells were grown in mMRS- or mMRST80 and treated at different pressure levels (100/200/300/400/450/500 MPa) at 25 °C for 5 min. Log reduction was determined on mMRS (dark columns) or mMRS-NaCl (light columns) agar plates. Shown are the means and standard deviations of at least three independent replicates.

The colony count on non-selective mMRS agar (0 % NaCl) stands for all cells that are able to grow under favorable conditions, i.e. both undamaged and sublethally injured cells.

Consequently, the resulting log reduction values (dark bars) represent the fraction of irreversibly injured cells (see Figure 38).

The colony count on selective mMRS agar (7 % NaCl), by contrast, represents all cells unaffected by the HHP treatment, and the corresponding log reduction (light bars) stands for all cells with either lethal or sublethal damage that allows no growth under such harsh conditions. Thus, the difference between log reductions on selective and non-selective agar indicates the fraction of cells with sublethal injury. As shown in Figure 38 and Table 45 (Appendix), pressure-treatment at 100 MPa caused sublethal damage of a small fraction of cells grown in mMRS-, but had no adverse effect on cells grown in mMRST80. At 200 MPa, a small amount of lethal damage was detected for cells grown in mMRS- (0.11 log reduction) and the number of sublethal injury increased (1.57 log reduction on 7 % NaCl). Growth in mMRST80 protected from lethal damage and reduced sublethal injury compared to mMRS- (0.41 log reduction). At 300 MPa, lethal damage was observed after growth in both mMRS- and mMRST80 with reductions by 0.88 and 0.42 log on non-selective agar, respectively. Log reductions of 4.34 and 3.64 on selective agar revealed a considerable increase in sublethal injury. Upon treatment with pressures of 400 MPa and more, colony counts on selective agar were below the detection limit, showing that (nearly) all cells were either lethally or sublethally injured and all survivors on non-selective agar had undergone sublethal damage. Inactivation of cells grown in mMRS- increased from 2.40 to 3.15 log with pressures of 400 and 450 MPa, respectively. In contrast, for cells grown in mMRST80 inactivation remained below 2 log at both pressure heights (1.71 log and 1.48 log, respectively), showing that growth in the presence of Tween 80 protects against the increase in lethal damage at these pressure conditions. At 500 MPa, the whole population of cells grown in mMRS- was irreversibly inactivated, whereas a small fraction of cells grown in mMRST80 was still able to recover under favorable post-pressure conditions. In summary, medium supplementation with Tween 80 protected TMW 1.708 from sublethal damage at pressures below 300 MPa and inhibited the development of sublethal into lethal injury at higher pressures starting from 400 MPa, either by mitigating HHP-induced damage or by increasing the ability of affected cells to successfully repair and overcome sublethal HHP induced damage.

3.6.3.5 Effect of Tween 80 on metabolic activity upon high-pressure treatment

Using the plate count method, the number of cells able to grow and form colonies is determined. However, some cells may be alive but unable to divide and reproduce even under favorable conditions. Moreover, even non-selective growth media might not offer optimal conditions so that not all possibly viable cells are able to grow. The proportion of these viable-but-non-culturable

(VBNC) cells can be estimated by measuring post-pressure metabolic activity by means of resazurin reduction. The blue dye resazurin is thereby reduced by cellular enzymes to red fluorescent resorufin, which is detected by fluorescence measurement. The reaction runs faster the more metabolically active cells are present in the sample. Thus, the metabolic activity can be determined from the increase of fluorescence intensity over time. Figure 39 and Table 46 (Appendix) show the fluorescence increase with TMW 1.708 grown in the presence or absence of Tween 80 and treated at different pressure levels, expressed as percent of the value of untreated cells.

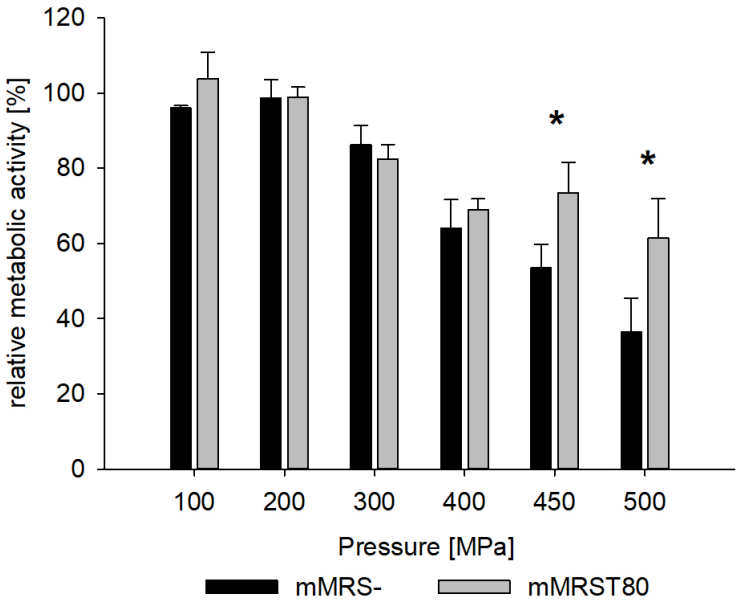

Figure 39: Metabolic activity of *L. plantarum* TMW 1.708 after growth in mMRS- or mMRST80 and HHP treatment at different pressure intensities. Cells were grown in mMRS- or mMRS supplemented with 1 g L^{-1} Tween 80 and treated with different pressures (100/200/300/400/450/500 MPa) at 25°C for 5 min. Metabolic activity after pressure treatment was determined using the resazurin reduction assay. Presented values are the percentage of fluorescence increase at the indicated pressure level compared to the untreated control. Shown are the means and standard deviations of independent 3 replicates. Asterisks (*) indicate statistically significant difference ($p < 0.05$) within data pairs.

Pressure treatments with 100 or 200 MPa had no effect on metabolic activity, 300 MPa slightly reduced metabolic activity to 86.19 % and 82.35 % for cells grown in mMRS- and mMRST80, respectively. Treatment of cells grown in mMRS- with 400, 450 and 500 MPa reduced their metabolic activity to 64.02 %, 53.56 % and 36.50 % of the initial value, respectively. In contrast, cells grown in mMRST80 retained 61.51 – 73.42 % metabolic activity under all the three pressure conditions, demonstrating that the protective effect of Tween 80 is also apparent with

metabolic activity. At the same time, these results indicate a large number of VBNC cells after HHP treatment with 400 MPa and more, as metabolic activity did not fall below 40 and 60 % of the initial value of cells grown in mMRS- and mMRST80, although < 10 % and < 1 % of cells were able to form colonies on non-selective agar (> 1 and > 2 log reduction, see 3.6.3.4), respectively.

3.6.3.6 Effect of Tween 80 on membrane integrity under high pressure

The cytoplasmic membrane is well-known as a target structure for HHP and loss of membrane integrity correlates with HHP-induced cell death (Ulmer *et al.*, 2000, Klotz *et al.*, 2010). The correlation between fatty acid composition and HHP sensitivity shown in previous sections (3.6.3.2 - 3.6.3.5) strongly suggests that the cell membrane plays a key role in Tween 80-mediated HHP resistance. Therefore, HHP-induced membrane damage was determined in TMW 1.708 grown in the absence or presence of Tween 80 by means of release of protein and uptake of fluorescence dye propidium iodide (PI).

3.6.3.6.1 Effect of Tween 80 on HHP induced protein release

In the past, HHP treatment was reported to cause loss of cytoplasmic proteins due to holes in the cell membrane and the release of membrane- and cell-wall bound proteins (Mañas & Mackey, 2004, Klotz *et al.*, 2010, Charoenwong *et al.*, 2011). The extent of protein release depends on the integrity of the cytoplasmic membrane and is thus a measure for its stability under pressure. Therefore, the amount of protein released by HHP treated cells of *L. plantarum* TMW 1.708 grown in mMRS- and mMRST80 was determined by measuring the protein concentration in the supernatant of the cell suspension (Figure 40 and Appendix Table 47). For comparison, cells treated with heat (100 °C, 15 min), showing complete inactivation (confirmed in a separate experiment, data not shown), were included as a control sample.

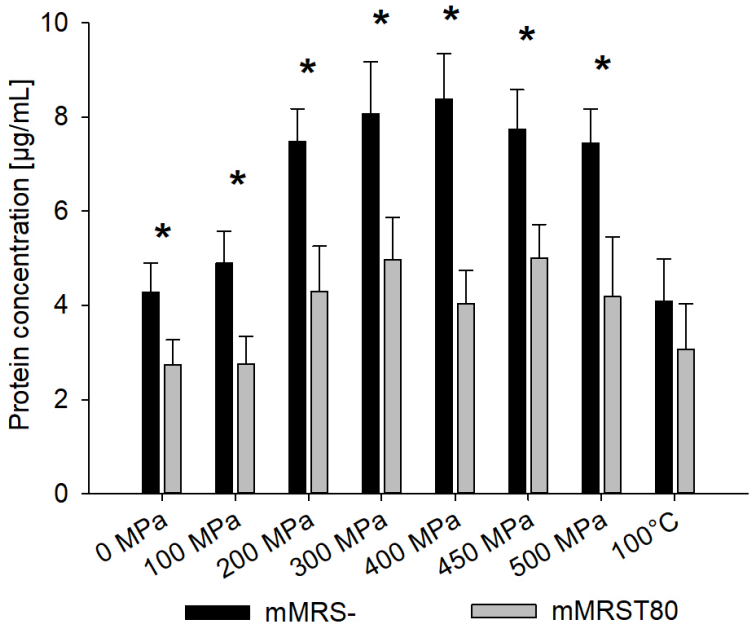

Figure 40: HHP-induced release of proteins by cells of *L. plantarum* TMW 1.708 grown in mMRS- or mMRST80. Cells were grown mMRS medium without and with 1 g L^{-1} Tween 80 and treated at different pressure levels (100/200/300/400/450/500 MPa) at 25 °C for 5 min or with heat (100 °C, 15 min). The protein concentration in the supernatant after pressure treatment was determined using the Bradford method. Shown are the means and standard deviations of at least seven replicates. Data pairs marked with an asterisk (*) show statistically significant difference ($p < 0.05$).

The results in Figure 40 show protein release into the environment by untreated cells, leading to baseline concentrations of 4.27 µg mL^{-1} for cells grown in mMRS- and 2.73 µg mL^{-1} after cultivation in mMRST80. Pressure treatment at 100 MPa slightly increased the concentration of released protein to 4.89 µg mL^{-1} for cells grown in mMRS-, whereas in the supernatant of cells grown in mMRST80 protein concentration remained at the level observed before pressure treatment (2.76 µg mL^{-1}). After growth in mMRS-, concentrations of released protein increased with higher pressures, reaching 7.47 µg mL^{-1} at 200 MPa and 8.07 µg mL^{-1} at 300 MPa until a maximum of 8.37 µg mL^{-1} was reached at 400 MPa. Further pressure increase slightly diminished extracellular protein concentrations to 7.74 µg mL^{-1} and 7.45 µg mL^{-1} after treatments at 450 and 500 MPa, respectively. In contrast, the concentration of protein released from cells grown in mMRST80 reached maximum values of 4.96 and 5.01 µg mL^{-1} after treatment with 300 and 450 MPa, whereas 400 and 500 MPa led to only 4.04 and 4.18 µg mL^{-1}. Unlike pressure treatment, inactivation by heat (100 °C, 15 min) did not result in additional protein release, despite complete loss of viability. These results show that the presence of Tween 80 leads to reduced permeability of the cell membrane under both, atmospheric and high hydrostatic pressure. Although these results indicate that Tween 80 stabilizes the cytoplasmic membrane

and alleviates the effect of HHP, the differences are not great enough to explain the impact of Tween 80 on HHP inactivation levels determined in this study (see 3.6.2.2).

3.6.3.6.2 Effect of Tween 80 on HHP-induced membrane permeability

The integrity of the cytoplasmic membrane was assessed by staining with the red-fluorescent dye propidium iodide (PI). Intact cell membranes are impermeable to PI, but when membrane integrity is impaired due to HHP, PI can enter the cell and intercalate with the DNA. Only in this state it shows its fluorescent properties, consisting in excitation by green (excitation maximum: 535 nm) and emission of red light (emission maximum: 617 nm). Some cell membranes that undergo injury during HHP treatment may be able to reseal upon pressure release while others may be irreversibly damaged. In order to assess the extent of membrane damage during pressure treatment, PI was added to the samples before pressurization, ensuring the presence of the dye under high pressure. Thus, cells appear fluorescent even if their membranes are able to reseal upon pressure release. Permanent membrane permeability, by contrast, was measured by adding PI to the samples after pressure release, so that only such cells with irreversibly permeabilized membranes were stained. The fluorescence intensity (FI) values were corrected for the number of cells (optical density) present in the sample and are depicted in Figure 41 as well as Table 48 and Table 49 (Appendix). Heat inactivated cells (15 min, 100 °C) showing the maximum possible FI values were used as positive control.

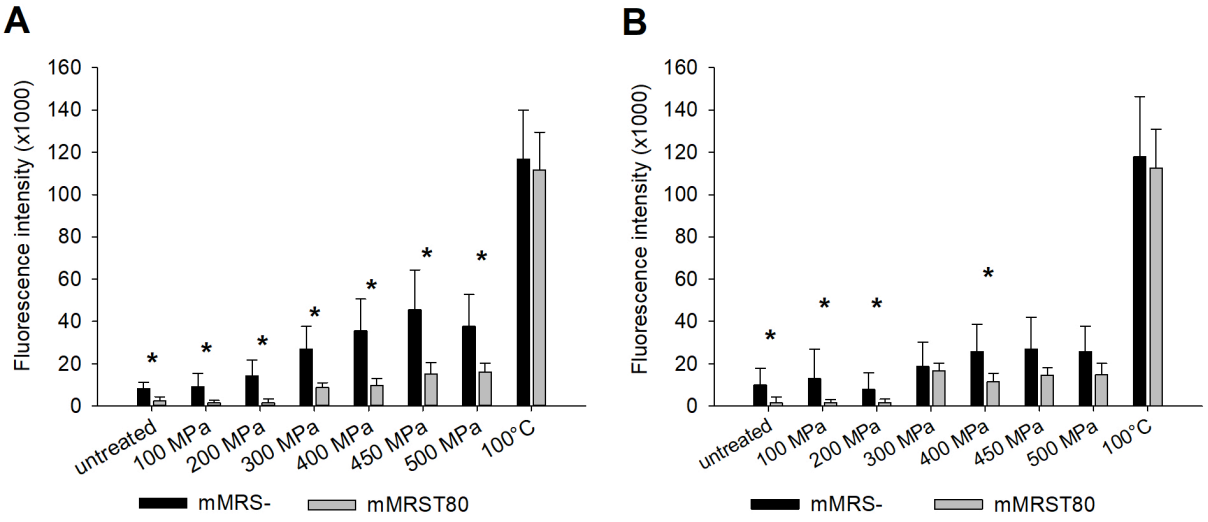

Figure 41: Uptake of PI by *L. plantarum* TMW 1.708 grown in mMRS- or mMRST80 during and after treatment at different pressure levels. The cells were grown in mMRS without or with 1 g L^{-1} Tween 80 and treated at different pressure levels (100/200/300/400/450/500 MPa) at 25 °C for 5 min or with heat (100 °C, 15 min). The cells were stained with 3 µM PI before (A) or after (B) pressure/heat treatments. Shown are the means and standard deviations of at least five replicates. Data pairs marked with an asterisk (*) show statistically significant difference ($p < 0.05$).

Membrane permeability under HHP:

Cultivation in mMRST80 showed lower initial FI (2.43 x 10^3 AU) under atmospheric pressure than growth in mMRS- (8.23 x 10^3 AU). In cells grown in mMRS-, HHP treatment led to an increase in FI from 9.20 x 10^3 AU at 100 MPa up to 45.48 x 10^3 AU at 450 MPa, whereas FI slightly decreased again at 500 MPa to a value (37.77 x 10^3 AU) comparable to the one observed with 400 MPa (35.75 x 10^3 AU). In contrast, cells grown in mMRST80 were unaffected by pressures up to 200 MPa (1.67 x 10^3 AU), and showed significantly lower FI under higher pressures than their counterparts grown in mMRS-. The highest FI values (15.13 x 10^3 and 16.02 x 10^3 AU), observed after treatment with 450 and 500 MPa, respectively, remained below 50 % of the values observed with mMRS-. These results show that the presence of Tween 80 during cultivation reduces the susceptibility of the cell membrane to HHP induced permeabilization. However, FI observed after heat inactivation (mMRS-: 116.77 x 10^3 AU and mMRST80: 111.78 x 10^3 AU) was several times higher than after HHP treatment.

Heat inactivation of a cell suspension used in this experiment (10^8 - 10^9 cfu mL^{-1}) at 100 °C for 15 min resulted in no detectable cell growth (see Appendix), which under consideration of the detection limit means a reduction by at least 6 log cycles, or > 99.9999 % of cells being unable to recover. HHP treatment at 500 MPa for 5 min led to a reduction in viable cell count by > 5 log cycles (see 3.6.3.4), i.e. > 99.999 % of cells were unable to recover.

Assuming that HHP and heat inactivation lead to the same extent of membrane permeability, similar fluorescence signals of samples treated with HHP (500 MPa, 5 min) and heat (100 °C, 15 min) were expected, as the difference between the fluorescence signals of samples containing 99.999 % and 99.9999 % fluorescing cells is far below the detection limit of the PI assay. The fact that even with HHP conditions leading to (nearly) complete inactivation (500 MPa) PI fluorescence intensities remained lower than 50 % of the maximum achievable signal (heat inactivated positive control) demonstrates that the loss of ability to recover by far exceeds membrane permeabilization.

With pressures of 0, 100 and 200 MPa no increase in FI (mMRS-: 10.06 x 10^3; 12.95 x 10^3; 7.99 x 10^3 AU; mMRST80: 1.50 x 10^3; 1.49 x 10^3; 1.61 x 10^3 AU) was observed when PI staining was performed after pressurization meaning that no permanent membrane permeability was induced at these conditions, regardless of the growth conditions. With cells grown in mMRST80, treatment at 300 MPa led to a strong increase in FI, reaching a value (16.76 x 10^3 AU) comparable to that observed with cells grown in mMRS- (18.65 x 10^3 AU). Pressures of 400, 450 and 500 MPa further increased FI in cells grown in the absence of Tween 80 (25.37 x 10^3; 26.93

x 10^3; 25.71 x 10^3 AU), whereas after growth in mMRST80 FI (11.38 x 10^3; 14.69 x 10^3; 14.73 x 10^3 AU) did not exceed the level observed with 300 MPa. This indicates that Tween 80 also inhibits permanent membrane permeabilization. Like with pre-pressure staining, FI values exhibited by heat-inactivated cells (mMRS-: 117.83 x 10^3 AU and mMRST80: 112.59 x 10^3 AU) were several fold higher than those observed after HHP treatment.

Cells grown in mMRS- showed higher FI values when stained before than after pressurization, showing that a substantial fraction of cells underwent transient membrane damage and were able to reseal their membranes after pressure release. In contrast, for cells cultivated in mMRST80 staining before or after HHP treatment resulted in similar FI levels, which means that the complete amount of HHP-induced membrane damage undergone by these cells was of permanent nature. This means that cells grown in the presence of Tween 80 are less susceptible to HHP-induced membrane permeabilization, than those grown in the absence of Tween 80, but once their membranes are damaged under pressure, they are not able to repair them.

Microscopic analysis

As shown above, even with the highest pressure levels of 500 MPa, which led to > 5 log reduction (> 99.999 % of cells inactivated) in colony count (see 3.6.3.4), PI fluorescence intensity was several times lower that after heat inactivation. To test whether (i) PI fluorescence is equally distributed over (almost) all cells, but with lower intensity than with heat-treated samples, or (ii) only few cells in the HHP treated sample have accumulated PI, the distribution of PI fluorescence in HHP treated samples was assessed by fluorescence microscopy of TMW 1.708 cells grown in mMRS- or mMRST80 and stained before or after HHP treatment at 500 MPa. Samples were additionally stained with the green fluorescent dye SYTO®9 (excitation/emission wavelength: 485/498 nm), penetrating also intact cell membranes, to visualize the complete population including cells with intact membranes. Untreated and heat-inactivated samples were used as negative and positive controls, respectively.

Figure 42 – Figure 45 show that in heat-treated samples, the whole population, i.e. all cells that took up green-fluorescent SYTO®9, had accumulated PI, whereas in the HHP treated sample only a small fraction showed red PI fluorescence. This pattern was observed for cells grown in both mMRS- and mMRST80 and with staining before and after HHP treatment. Consequently, differences in PI fluorescence shown above resulted from different numbers of fluorescing cells, but not from different fluorescence intensity of individual cells.

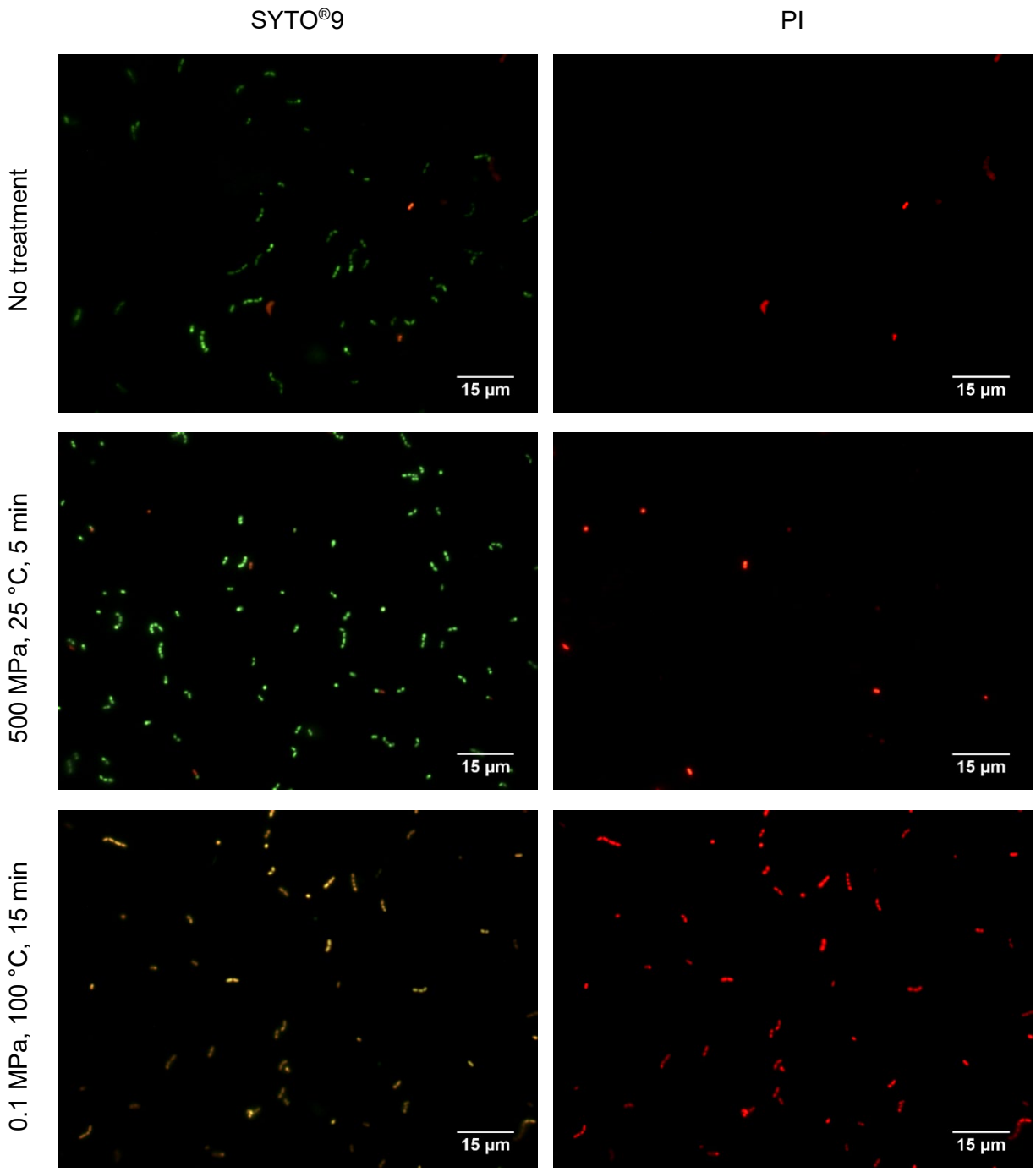

Figure 42: *L. plantarum* TMW 1.708 grown in mMRS- and stained with PI before HHP treatment or heat inactivation. *L. plantarum* TMW 1.708 was grown in mMRS- to stationary phase, stained with 3 µM PI and 3 µM SYTO®9 and then treated with 500 MPa at 25 °C for 5 min or with heat (100 °C, 15 min). Suspensions were analyzed by fluorescence microscopy and compared with untreated cells stained at the same time.

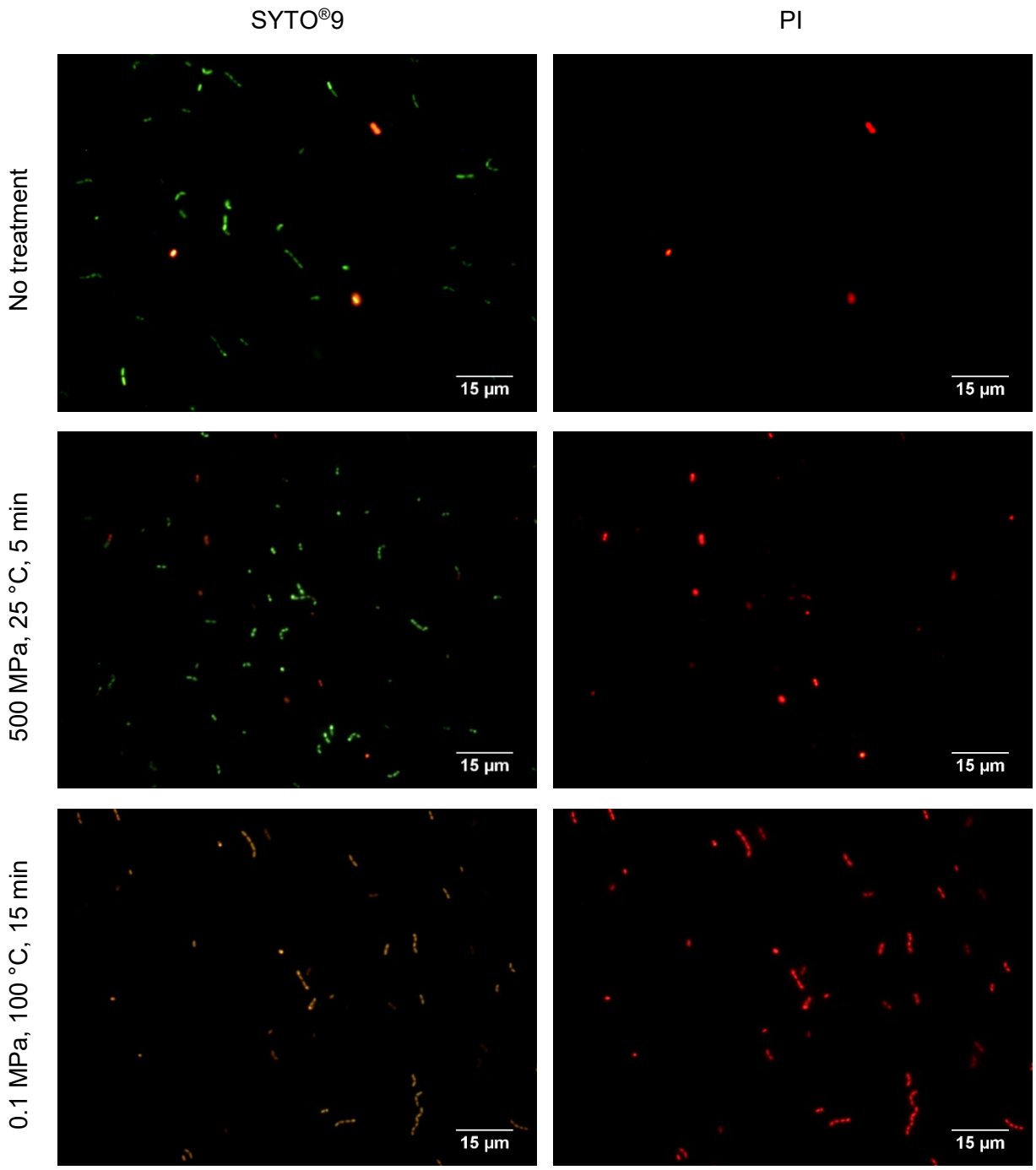

Figure 43: *L. plantarum* TMW 1.708 grown in mMRST80 and stained with PI before HHP treatment or heat inactivation. *L. plantarum* TMW 1.708 was grown in mMRS supplemented with 1 g L^{-1} Tween 80 to stationary phase, stained with 3 µM PI and 3 µM SYTO®9 and then treated with 500 MPa at 25 °C for 5 min or with heat (100 °C, 15 min). Suspensions were analyzed by fluorescence microscopy and compared with untreated cells stained at the same time.

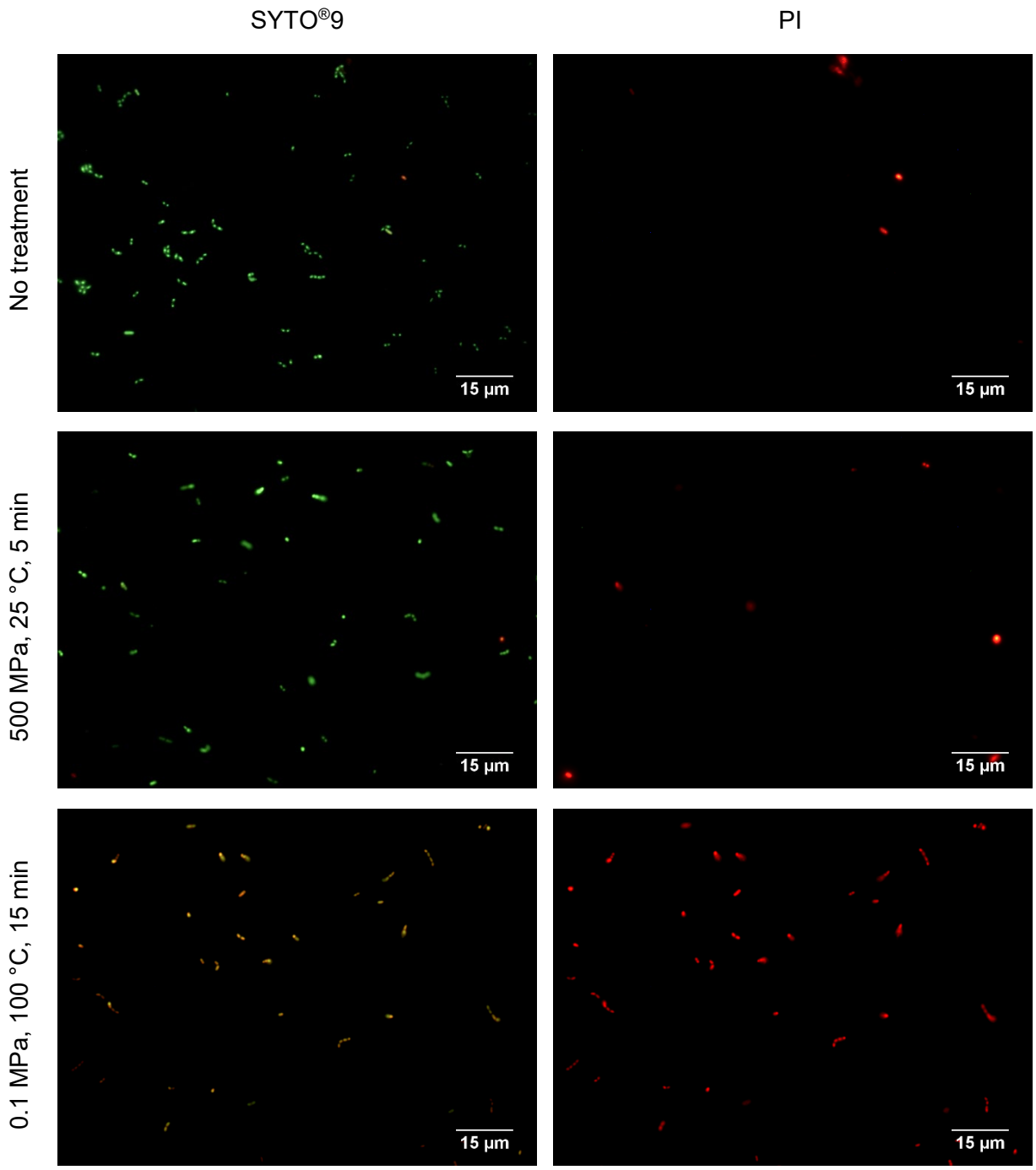

Figure 44: *L. plantarum* TMW 1.708 grown in mMRS- and stained with PI after HHP treatment or heat inactivation. *L. plantarum* TMW 1.708 was grown in mMRS- to stationary phase, treated with 500 MPa at 25 °C for 5 min or with heat (100 °C, 15 min), and afterwards stained with 3 µM PI and 3 µM SYTO®9. Suspensions were analyzed by fluorescence microscopy and compared with untreated cells stained at the same time.

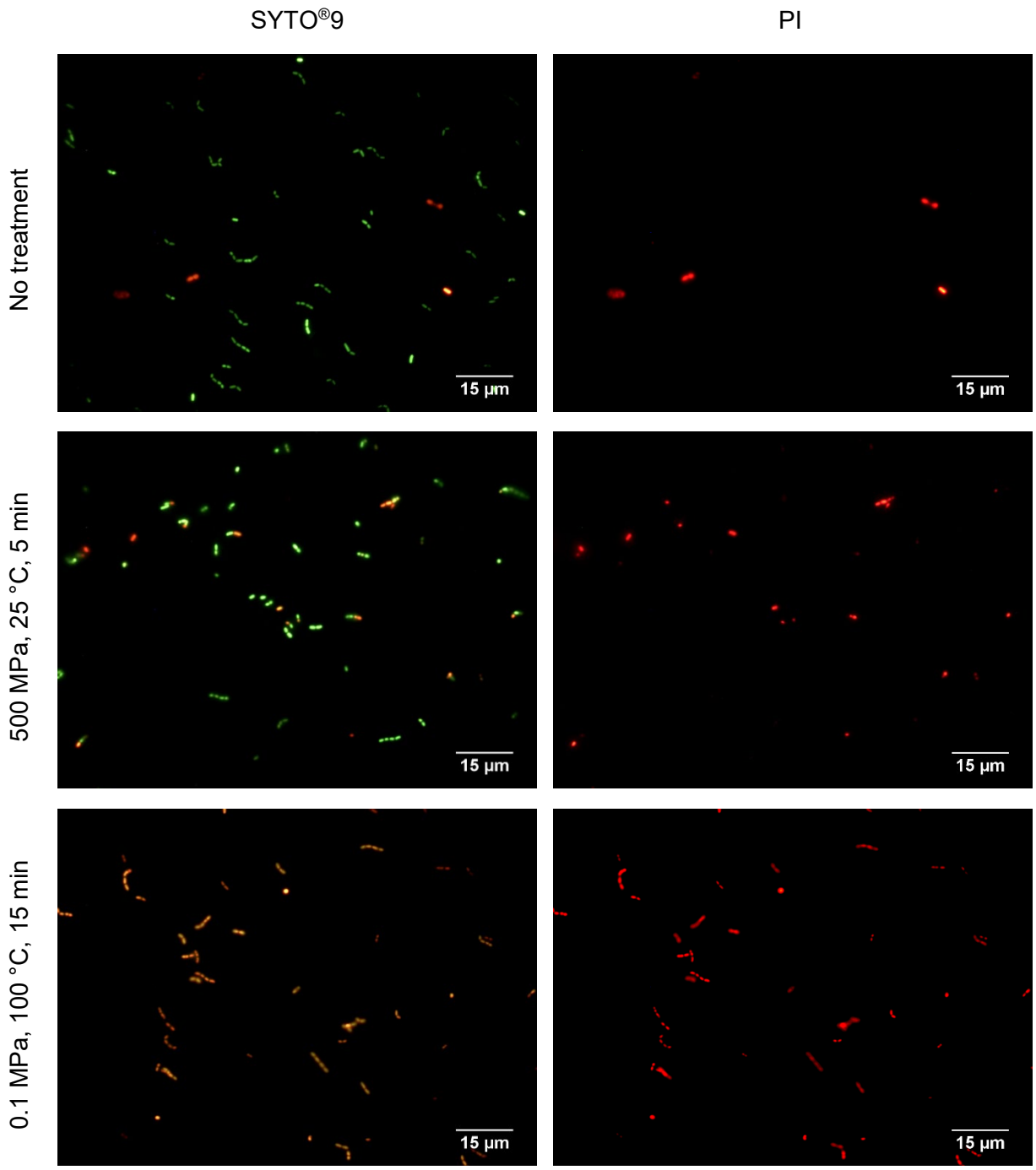

Figure 45: *L. plantarum* TMW 1.708 grown in mMRST80 and stained with PI after HHP treatment or heat inactivation. *L. plantarum* TMW 1.708 was grown in mMRS supplemented with 1 g L^{-1} Tween 80 to stationary phase, treated with 500 MPa at 25 °C for 5 min or with heat (100 °C, 15 min), and afterwards stained with 3 µM PI and 3 µM SYTO®9. Suspensions were analyzed by fluorescence microscopy and compared with untreated cells stained at the same time.

These results demonstrate that the protective effect of growth in the presence of Tween 80 is evident also in terms of membrane integrity. However, HHP-induced loss of membrane integrity cannot account for the concomitant loss of the ability to grow on non-selective agar observed

with the same pressure conditions, showing that membrane permeabilization is not necessary to prevent cell growth under optimal, non-selective conditions after HHP treatment.

3.6.3.6.3 Correlation between PI uptake and loss of metabolic activity

Both residual metabolic activity and membrane integrity exceeded colony formation of HHP-treated *L. plantarum* cells on non-selective agar, indicating a high number of VBNC cells and showing that cells cease to grow even if their membranes are still impermeable for PI. While obviously no correlation existed between colony formation and either PI uptake or loss of metabolic activity, it was hypothesized that the latter two parameters show correlation in HHP treated *L. plantarum* cells. To test this, the relative metabolic activity (referred to the activity of untreated cells) was plotted against the relative transient membrane permeability (referred to the values observed with heat-inactivated cells) observed at different pressure levels (Figure 46).

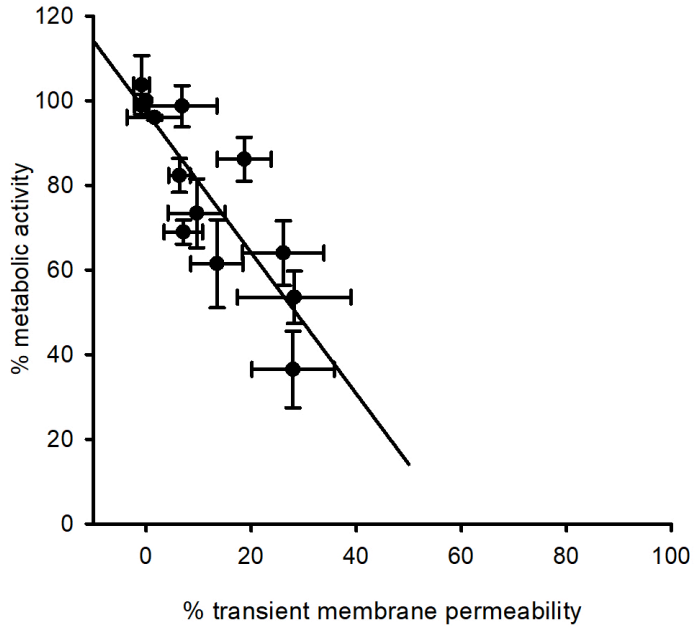

Figure 46: Comparison of metabolic activity and transient membrane permeability of *L. plantarum* TMW 1.708 after HHP treatments. Relative metabolic activity of *L. plantarum* cells (% of untreated cells) is plotted against their relative transient membrane permeability (% PI fluorescence compared with samples treated at 100 °C, 15 min).

By increasing the pressure level from 0 to 500 MPa, metabolic activity decreased from 100 % to < 40 %, whereas membrane permeability remained below 30 % of the maximum achievable value (100 % = heat treated positive control). Despite the seemingly linear relationship, HHP treatment reduces metabolic activity to a stronger extent than membrane integrity and demonstrates that membrane permeabilization is not a prerequisite for the loss of metabolic activity under HHP.

4 Discussion

In this study, the mutual influence of fat and aqueous phase parameters regarding HHP inactivation efficiency of *L. plantarum* was investigated in O/W emulsions. The presence of rapeseed oil increased HHP inactivation only marginally, whereas aqueous phase parameters strongly affected survival. In general, baroprotection was observed with NaCl, sucrose and proteins, while low pH values led to enhanced inactivation. Although there was no general effect of rapeseed oil on the impact of aqueous phase parameters, sporadic matrix- and strain-specific, though CSH-independent, alterations in inactivation efficiency were observed.

Investigation of preconditioning revealed a slightly stronger increase in HHP sensitivity during prolonged incubation in O/W emulsions compared to pure aqueous buffer solution. However, when rapeseed oil or associated compounds such as Tween 80 and oleic acid where present during cultivation, HHP sensitivity of *L. plantarum* was reduced. Medium supplementation with Tween 80 further led to changes in gene expression and caused a characteristic cellular fatty acid profile. Comparison with other Tween types revealed that the fatty acid profile correlates with HHP sensitivity and provided evidence for the cell membrane playing a central role in Tween 80-mediated baroprotection. Indeed, Tween 80 was shown to reduce the HHP-induced loss of membrane integrity and metabolic activity, as well as the development of sublethal into lethal damage. However, the fact that membrane damage and impairment of metabolism were far weaker than the loss of growth capacity indicates that HHP inactivation of *L. plantarum*, and thus also protection against it by Tween 80, is a highly complex process involving various cellular target structures.

Some of the working hypotheses (see 1.4.2) were confirmed by the observations made in the course of this study, whereas others were refuted. On that basis, initial hypotheses can be refined and converted into theses (●), and new theses (○) as well as further speculations (▪) resulting from these can be made. Modifications of initial hypotheses are highlighted in bold. The arguments justifying these theses are given in the subsequent discussion section.

Mutual influence of fat and aqueous-phase parameters during HHP inactivation of bacteria in O/W emulsions

- The presence of fat **can change** the effect of the aqueous phase parameters (I) NaCl content, (II) sucrose content, (III) pH value and (IV) protein type and content on HHP inactivation, as long as the water content in the aqueous phase remains constant.

  - Although there is no general pattern, occasional combinations of fat and aqueous parameters can alter HHP sensitivity sporadically and in a strain-dependent manner.

- The response of treated bacteria to changes in the surrounding matrix in O/W emulsions regarding HHP sensitivity is independent of CSH.
  - General HHP sensitivity varies widely among strains, but does not correlate with CSH. Changes in HHP sensitivity due to individual matrix compositions do not correlate with CSH, but are highly strain-specific.

Effect of preconditioning fat and fat-associated compounds on HHP sensitivity

- Prolonged residence of bacteria in O/W emulsion prior to HHP treatment changes HHP sensitivity, and these changes are independent of CSH, but their intensity depends on the temperature during the contact time.
  - Prolonged contact to both aqueous buffer and O/W emulsion increases HHP sensitivity with the effect being stronger in O/W emulsion.
    - *Unfavorable conditions due to the absence of nutrients increase the HHP sensitivity of bacteria.*
    - *Temperature variations during HHP treatment are stronger in O/W emulsions than in pure aqueous buffer due to differences in adiabatic heating behavior. Contact with unfavorable conditions increases the sensitivity to such temperature changes.*
  - Changes in HHP sensitivity during prolonged contact with O/W emulsions are independent of CSH, since oil droplets are coated with emulsifier molecules, resulting in a loss of their hydrophobic character. As both the coated oil droplets and the aqueous environment are hydrophilic there is no difference in localization between hydrophilic and hydrophobic cells.
  - The increase in HHP sensitivity during residence in O/W emulsions is stronger at higher than at lower temperatures.
    - *Low temperatures decelerate metabolic reactions, leading to reduced consumption of residual nutrients and slower degradation of cellular structures during the stay in nutrient-depleted environments, and thus to a slower increase in HHP sensitivity.*

- The presence of rapeseed oil (without emulsifier) during cultivation changes HHP sensitivity of **both** hydrophobic **and** hydrophilic bacteria **despite the** favored contact to fat **of hydrophobic cells**.
    - The presence of rapeseed oil during cultivation reduces HHP sensitivity of bacteria.
        - *The presence of rapeseed oil cause changes in the physiological state of bacteria that can reduce their HHP sensitivity.*
    - The effect of cultivation in the presence of rapeseed oil reduces HHP sensitivity of both hydrophilic and hydrophobic bacteria, if direct fat contact is ensured, e.g. by continuous agitation.
        - *Direct contact of bacteria to the lipid phase is necessary for changes in HHP sensitivity. Hydrophilic bacteria adhere to the lipid phase in O/W mixtures while hydrophilic ones only experience accidental contact. In matrices where cell-fat interactions are allowed to form spontaneously, a protective effect of fat is stronger with hydrophobic than hydrophilic bacteria. If continuous agitation ensures similar contact times for hydrophobic and hydrophilic cells, no differences are observed.*
        - *Direct contact is not necessary, since rapeseed oil releases small amounts of fatty acids into the aqueous phase which can be ingested by both hydrophobic and hydrophilic bacteria equally.*
- Oleic acid is the most abundant fatty acid in rapeseed oil and constitutes the hydrophobic part of emulsifier Tween 80. Therefore, preconditioning by cultivation in the presence of Tween 80 or free oleic acid causes the same changes in HHP sensitivity as observed with rapeseed oil.
    - Cultivation in the presence of Tween 20, Tween 80 or free oleic acid, but not other Tween types or free fatty acids, reduces HHP sensitivity.
        - *The ingestion of oleic acid, but not of other fatty acids, leads to physiological changes that cause a reduction of HHP sensitivity.*
- Preconditioning by cultivation in the presence of rapeseed oil or associated compounds like Tween 80 provokes a cellular response that accounts for changes in HHP sensitivity. The cellular response to Tween 80 comprises an adaptation of the fatty acid composition

of the cell membrane according to exogenous fatty acids and manifests in changes in the transcriptomic profile. The transcriptomic response to the presence of Tween 80, which is widely used in cultivation of lactobacilli, provides general insight in its growth promoting effect beyond preconditioning towards HHP tolerance.

- The presence of Tween 80 causes the downregulation of fatty acid biosynthesis and upregulation of pyrimidine synthesis and amino acid uptake.
  - *Exogenous oleic acid from Tween 80 is incorporated by bacteria, rendering fatty acid biosynthesis redundant.*
  - *Energy and resource savings due to the presence of exogenous oleic acid lead to enhanced cell growth.*
- The presence of Tween 80, and partially of Tween 20, causes enhanced growth.
  - *Exogenous oleic acid provided by Tween 20 or Tween 80 enhances growth.*
- The addition of Tween 20 or Tween 80 to the growth medium changes the cellular fatty acid profile by introducing oleic acid and increasing CFA levels. This fatty acid profile is associated with reduced HHP sensitivity.
  - *Exogenous oleic acid is incorporated into the cell membrane and partially converted into the corresponding CFA dihydrosterculic acid. The resulting fatty acid profile increases the pressure stability of the cell membrane.*
- Cultivation in the presence of Tween 80 reduces HHP-induced loss of membrane integrity and metabolic activity and impedes the development of sublethal into lethal cell injury.
- The impact of HHP, and with it the protective effect of Tween 80, on growth capacity is much stronger than that on membrane integrity or metabolic activity.
  - *Loss of membrane integrity and metabolic activity is not a prerequisite for the failure to grow under favorable conditions.*
  - *The increased amounts of CFA in the cell membrane increase the pressure stability of the membrane itself and protect important membrane-bound proteins from HHP induced damage, ensuring proper functionality, and thus reducing HHP sensitivity.*

4.1 Preparation and characterization of O/W emulsions

The systematic investigation of the concerted effect of fat and aqueous phase parameters on the HHP inactivation, required O/W emulsions with constant fat and emulsifier concentration and droplet size and varying, but defined aqueous phase parameters. To this end, the processing parameters were adjusted if necessary to account for changes in the physical properties of the mixtures caused by the solutes added to the aqueous phase. While no adjustment of processing parameters was necessary with NaCl, sucrose and the pH value, the addition of protein caused a reduction in droplet size. This is in line with the data of Sünder *et al.* (2001) and the fact that both whey proteins and casein have emulsifying properties and thus are widely used as emulsifiers in the food industry (Dickinson, 1997, Hasenhuettl & Hartel, 2008, Degner *et al.*, 2014). After empiric identification of suitable process parameters, the median droplet size and SSA of the emulsions belonging to the same aqueous phase parameter varied by less than 20 % compared to the respective emulsion without aqueous-phase additives. Therefore, the emulsions were considered comparable and suitable for HHP inactivation studies.

Creaming of the emulsions during a one-week period at gravity was simulated by analytical centrifugation. Most of the prepared emulsions showed similar creaming rates, but with casein and high WPI and NaCl concentrations, the creaming process was remarkably faster. NaCl has been shown previously to increase the creaming rate of O/W emulsions (Sünder *et al.*, 2001). The effect of casein and WPI may be explained with their emulsifying properties. Although generally stabilizing emulsions (Dickinson, 1997), it cannot be excluded that these proteins can also negatively affect the emulsion system, e.g. through interaction with emulsifier molecules. Since creaming is a reversible process with gentle agitation being sufficient to return the emulsions into the initial state, and no coalescence was observed after storage at 4 °C for 9 days, the stability of the emulsions over this period was confirmed. To avoid changes in the chemical composition, e.g. oxidation of unsaturated fatty acids, or microbial spoilage, emulsions were used for experiments only during the week of preparation.

Most of the prepared emulsions were found stable under the harshest pressure conditions used in this study (500 MPa, 25 °C, 5 min), as no significant impact on visual appearance and droplet size was observed. These results corroborate previous studies where vegetable oil-water emulsions or dairy cream were not negatively affected by HHP in the range of several hundred MPa (Karbstein *et al.*, 1992, Dumay *et al.*, 1996, Simpson & Gilmour, 1997, Al-Bandak *et al.*, 2011).

In emulsions containing WPI, HHP caused slight gel formation, which is in line with studies on (I) bovine milk, where whey proteins α-la and β-lg denatured (Huppertz *et al.*, 2006), (II) purified

whey proteins that showed gelation under pressures below 500 MPa under appropriate pH and ionic composition of the matrix (Van Camp & Huyghebaert, 1995, Cheftel & Dumay, 1998, Ipsen *et al.*, 2002, Saalfeld *et al.*, 2015) and (III) whey protein-stabilized O/W emulsions (Dumay *et al.*, 1996). Other authors observed only denaturation and loss of solubility of WPI, but not real gel formation in the pressure and concentration range applied in the present study (Kanno *et al.*, 1998, Krešić *et al.*, 2006). Also the fact that 2.5 % casein showed gelation under HHP stands in contrast to a previous study by Cadesky *et al.* (2017) who found that HHP induced (350-450 MPa) gel formation only with 10 % but not with 2.5 % casein. However, the behavior of milk proteins under pressure strongly depends on the type and purity of the protein preparation, e.g. micellar/non-micellar casein, the surrounding matrix, e.g. ionic strength and pH (Saalfeld *et al.*, 2015), as well as temperature, pressure build-up and release rate and holding time (López-Fandiño, 2006). Variation of these parameters among different studies might explain such apparent contradictions. In line with observations made by Dumay *et al.* (1996), the oil droplet size and creaming rate were not significantly changed by HHP, despite the observed gel formation.

4.2 Selection of *Lactobacillus* strains

For HHP inactivation studies on the combined effect of fat and aqueous phase parameters as well preconditioning with fat-associated compounds, two strains with preferably different CSH but otherwise comparable features, great relevance to food spoilage, and a minimal effect on the stability of the examination system were chosen out of a pre-selection of strains belonging to the three species *L. fructivorans*, *L. sakei* and *L. plantarum*.

4.2.1 Cell surface hydrophobicity

There are several methods for the determination of microbial CSH, including Contact Angle Measurement (CAM) (Ly *et al.*, 2006), Adhesion to Polystyrene and Microbial Adhesion to Hydrocarbons (MATH) which not always provide consistent results (Rosenberg, 2006). The latter was deemed most suitable for this study, as the adhesion of microbial cells to an organic fluid phase, e.g. n-hexadecane, most closely describes the conditions found in O/W emulsions. There are a couple of variations of the MATH assay described in the literature, with different organic solvents, organic to aqueous phase ratios, agitation times and wavelengths for absorbance measurement (Rosenberg *et al.*, 1980, Rosenberg, 2006). For the determination of the CSH of 38 pre-selected *Lactobacillus* strains, the MATH assay originally described by Rosenberg *et al.* (1980) was modified according to the needs of this study.

The CSH of the 38 strains in mid-exponential and stationary phase varied greatly, covering the entire range from highly hydrophobic to highly hydrophilic. Especially *L. plantarum* and *L. fructivorans* showed great variation whereas *L. sakei* strains tended to be generally hydrophilic. These results show that CSH is not necessarily species-specific, which is in accordance with previous reports on lactococci (Ly *et al.*, 2006, Giaouris *et al.*, 2009, Tarazanova *et al.*, 2017). CSH characteristics of selected strains tended to strengthen from the earlier to the later growth stage. This stands in contrast to observations made by Rosenberg *et al.* (1980) and Patel *et al.* (2011), who found *Serratia marescens* more hydrophobic and *E. coli* more hydrophilic in stationary phase, respectively, irrespective of their surface character in exponential phase. These differences might be based on species specificity and the fact that *L. plantarum* is a Gram-positive species whereas the latter ones are Gram-negatives, and thus show a completely different structure of the cell envelope. Since for *L. plantarum* differences in CSH characteristics are largest, and thus possible effects of CSH on HHP inactivation are most pronounced in stationary phase, cells were grown to stationary phase for HHP inactivation studies.

4.2.2 Biodiversity on proteomic level and correlation with CSH

CSH, and thus interaction with external surfaces is mediated by polysaccharides, polypeptides and proteins attached to the cell wall. Especially those of the S-layer, are currently believed to play a major role in determining CSH (van der Mei *et al.*, 2003). Moreover, the development of characteristic surface features probably requires specific anabolic pathways comprising an appropriate set of enzymes. Therefore, it was hypothesized that the CSH should be discernible in the proteomic profile. However, species-specific multidimensional scaling of the proteomic profiles allowed no distinction on the basis of CSH, showing that CSH-specific differences in the proteome were either superimposed by other features or non-existent.

4.2.3 Compatibility with the examination system

To ensure the chemical and physical integrity of buffer solutions and emulsions during contact with suspended bacteria and to avoid changes in the physiological state of the cells due to metabolization of matrix constituents, the pre-selected strains were screened for degradation activity against the organic components of the prepared emulsions, such as fat, emulsifier Tween 80, proteins, sucrose and combinations thereof occurring in the matrices used in this study with the aim of selecting strains without adverse effects on the examination system.

Degradation of the lipid phase – lipolytic activity

To exploit fat in their environment for nutritional purposes many bacteria secrete lipases, which hydrolyze the ester bonds of triacylglycerols forming di- and monoacylglycerols, free fatty acids and glycerol that are then transported through their membranes (Jensen, 1983, Jaeger *et al.*, 1994). Although LAB possess weak lipase activity compared to other bacteria (Meyers *et al.*, 1996), lipase activity of some lactobacilli plays an important role in flavor development during food fermentation (Sanz *et al.*, 1988, Hammes *et al.*, 1990, Silva Lopes *et al.*, 1999, Esteban-Torres *et al.*, 2014). It varies on species as well as strain level (Gobbetti *et al.*, 1996, Katz *et al.*, 2002) and strongly depends on the environmental conditions such as pH, NaCl concentration and temperature (Gobbetti *et al.*, 1999, Silva Lopes *et al.*, 1999). Most lactobacilli possess only intracellular lipases (Meyers *et al.*, 1996), but also extracellular lipase activity has been shown, among others, for a strain of *L. plantarum* (Silva Lopes *et al.*, 1999, Katz *et al.*, 2002, Lopes *et al.*, 2002). To identify and exclude strains able to degrade the lipid phase of emulsions, lipolytic activity was assessed by growth on tributyrin agar. Since none of the *Lactobacillu*s strains offered evidence for lipase activity, which is in line with the commonly accepted assumption of low or absent lipolytic activity in lactobacilli (Meyers *et al.*, 1996, Kenneally *et al.*, 1998, McSweeney, 2004), degradation of the lipid phase in O/W emulsions by the strains used could be excluded.

Degradation of Emulsifier Tween 80

Since the presence and integrity of the emulsifier is crucial for the stability of O/W emulsions, its degradation by suspended microorganisms has to be prevented. Besides its role as emulsifier in food emulsions, Tween 80 is used as fatty acid source in microbiological culture media (De Man *et al.*, 1960) and it is well-known that bacteria are able to degrade Tween 80. Comparison of the optical density of bacterial suspensions in IPB containing Tween 80 before and after a time period covering the contact time during HHP inactivation experiments showed that none of the strains was able to grow solely on Tween 80, ensuring the integrity of the emulsifier. Besides, these results show that despite its growth-enhancing effect, Tween 80 alone, without other nutrients, is not sufficient for cell proliferation.

Proteolytic activity

To exploit proteins present in their environment, e.g. casein in milk, many lactobacilli possess a three-part proteolytic system consisting of (i) extracellular, cell wall-bound proteases that degrade proteins into smaller oligopeptides, (ii) peptide transporters for the uptake of

oligopeptides into the cell, and (iii) intracellular peptidases for degradation into smaller peptides and amino acids (Kunji *et al.*, 1996).

Several studies reported proteolytic activity of strains, especially those used in starter cultures during meat and milk fermentation and in sourdough, belonging to the three species used in this study (Spicher & Nierle, 1984, Fadda *et al.*, 1998, Fadda *et al.*, 1999, Basso *et al.*, 2004, Savijoki *et al.*, 2006). Using an assay based on the cleavage of fluorescence-labeled FTC casein, no proteolytic activity was observed for any of the tested strains, meaning that negative effects on the stability of emulsions containing casein or WPI added to the aqueous phase could be excluded. Although these results indicate that none of the used strains possess the ability to degrade proteins, it has to be considered that the proteolytic activity of lactobacilli depends on growth phase and environmental conditions (Pereira *et al.*, 2001), and that some lactobacilli show proteolytic activity only after growth for an extended time in protein-rich environment (Khalid & Marth, 1990). Therefore, it is possible that some of the strains are generally capable of degrading proteins but not in stationary phase and under the minimal conditions and the short time frame used here.

Degradation of peptone

Peptone consists of small peptides and amino acids that can be easily ingested by microorganisms and is thus used as an amino acid and nitrogen source in many microbiological growth media (De Man *et al.*, 1960, Cohn *et al.*, 1968, Holzapfel, 1992, Reuter, 1992, van Netten & Kramer, 1992, Busse, 1995). Peptones can be obtained from various protein sources by enzymatic digest. In this study, peptone from casein was used, which alone and in combination with Tween 80 caused weak growth of all *L. plantarum* and *L. sakei* strains indicating that these strains are able to utilize peptone as the sole carbon and energy source. Hence, the contact time in the respective matrices during HHP inactivation experiments was kept as short as possible in order to avoid peptone degradation and changes in the physiological state of the cells. The observed growth could be explained by a residual pool of nutrients in the cells carried over from the previous growth medium that in combination with the small peptides from peptone, readily ingested through oligopeptide transport systems (Kunji *et al.*, 1996, Savijoki *et al.*, 2006) may be sufficient for a few reproduction cycles. Moreover, peptone is a complex natural-source product, the composition of which can vary widely among different batches and manufacturers (Reissbrodt *et al.*, 1995, Gray *et al.*, 2008). Therefore, the presence of trace amounts of essential nutrients that may be sufficient to induce weak growth cannot be completely excluded.

Degradation of sucrose

In contrast, sucrose solutions in IPB alone and in combination with Tween 80 did not cause noticeable growth, showing that the prepared O/W emulsions with varying sucrose concentrations in the aqueous phase were stable with respect to bacterial degradation and that changes of the cells' physiological state were not to be expected.

4.2.4 Spoilage potential

To select preferably spoilage-relevant *Lactobacillus* strains, their ability to grow under stress conditions usually applied to prevent microbial growth during food preservation was assessed. These stress parameters included acidification, the reduction of a_w through the addition of NaCl, and low temperatures. Each parameter was varied in a food-relevant range (pH: 6.2 – 3.5; NaCl: 0 – 10 % (w/v); T: 10 °C and 4 °C) and for each parameter, the condition leading to the greatest differences in growth characteristics among the strains (pH 3.5, 7.5 % NaCl (a_w 0.948) and 4 °C) was selected for comparison.

The observation that almost all strains showed growth at the lowest pH value of 3.5 is not surprising due to the generally high acid tolerance of lactobacilli. Producing lactic and other organic acids as their main metabolites during sugar fermentation (Papadimitriou *et al.*, 2016), many *Lactobacillus* strains are able to withstand acidic conditions down to pH 3 (Vermeulen *et al.*, 2007, Sanders *et al.*, 2015), which allows them to grow in and spoil even foods with high acid content (Dakin & Radwell, 1971, Smittle, 1977, Smittle & Flowers, 1982, Meyer *et al.*, 1989, Bjorkroth & Korkeala, 1997). The highest growth rates (μ_{max}) and cell densities ($maxOD_{600}$) at pH 3.5 were observed for strains of the species *L. plantarum*, though with great intra-species variation. Also, the behavior of *L. fructivorans* and *L. sakei* strains varied strongly, with *L. fructivorans* TMW 1.1856 and three *L. sakei* strains reaching remarkable cell densities and the others showing almost no growth. These results closely reflect the literature, describing *L. plantarum* as one of the most acid-tolerant *Lactobacillus* species (McDonald *et al.*, 1990, G-Alegría *et al.*, 2004, Parente *et al.*, 2010, Papadimitriou *et al.*, 2016), even though acid tolerance can vary considerably among *L. plantarum* strains, as well (Cebeci & Gürakan, 2003, Sanders *et al.*, 2015). In general phenotypic variation within one *Lactobacillus* species is often greater than among different species, due to intense adaptation to their natural habitats (Sanders *et al.*, 2015).

At the highest NaCl concentration of 10 % (a_w 0.930) no significant growth was observed with any strain. This is in line with previous observations, where *L. plantarum* was shown to cease growth at a_w values between 0.94 and 0.95 when adjusted with NaCl (Troller & Stinson, 1981,

Sperber, 1983). *L. fructivorans* has been shown to grow in salad dressing with a shown a_w value as low as 0.91 (Meyer *et al.*, 1989), apparently contradicting the present results. However, it has to be kept in mind that the effect of low a_w values strongly depends on the solute used and that a certain a_w achieved with NaCl, leading to growth inhibition may still allow growth when achieved with less harmful solutes, such as compatible solutes. In line with the generally high tolerance of lactobacilli to high osmolarity (Papadimitriou *et al.*, 2016), 7.5 % NaCl (a_w 0.948) allowed growth of almost all strains and at the same time led to the greatest differences among them. All *L. sakei* and *L. plantarum* strains as well as *L. fructivorans* strain TMW 1.1856 exhibited similar maximum growth rates (μ_{max}) between 0.1 and ~0.2 h^{-1}, but varied greatly regarding $maxOD_{600}$, with *L. fructivorans* TMW 1.1856 and some *L. plantarum* strains reaching higher densities than *L. sakei* in general and other *L. plantarum* strains. This stands in partially in contrast to the generally accepted image of *L. sakei* as a comparably salt tolerant species widely applied in meat fermentation where high salt concentrations inhibit the growth of other, undesired microorganisms (Champomier-Vergès *et al.*, 2001, Marceau *et al.*, 2003). *L. plantarum*, in contrast, has been shown to stop growing at NaCl concentrations > 6 % (Siezen *et al.*, 2010), whereas in another one no growth was observed at the conditions used here (Sanders *et al.*, 2015). As with pH tolerance, variations on strain level are probably responsible for seemingly contradicting results.

Besides salt tolerance, *L. sakei* is known as one of the most psychrotolerant *Lactobacillus* species, growing well at refrigerating temperatures and tolerating even the combination of both stresses, as shown by growth at 4 °C in the presence of 4 % NaCl (Champomier-Vergès *et al.*, 2001, Marceau *et al.*, 2004). In contrast, *L. fructivorans* and *L. plantarum* show very slow or no growth at low temperatures (Mejlholm & Dalgaard, 2013). This was confirmed by the data obtained in this study, as the five *L. sakei* strains achieved the highest $maxOD_{600}$ values and showed the highest μ_{max} at 4 °C, with *L. plantarum* TMW 1.1 being the only strain belonging to another species with nearly the same growth behavior.

Except for cold tolerance, where *L. sakei* outperformed the other two species, the intra-species variation was as great as that between different species. This confirms previous observations that spoilage potential is rather a strain-specific than species-specific feature (Sanders *et al.*, 2015).

Especially *L. plantarum*, which is known for its comparably high diversity in genomic, proteomic and metabolic features within the order of LAB (Molenaar *et al.*, 2005, Koistinen *et al.*, 2007, Siezen *et al.*, 2010) showed great variation. The two *L. fructivorans* strains investigated here exhibited highly different growth characteristics even under non-stress conditions in mMRS broth

where TMW 1.59 showed comparably slow growth. This indicates that the chosen medium provided suboptimal conditions for this strain and that a sound comparison with other strains regarding stress tolerance was not possible.

This is not surprising as *L. fructivorans* is one of the most fastidious *Lactobacillus* species with a comparably narrow growth range among lactobacilli (Sanders *et al.*, 2015). Many *L. fructivorans* strains are adapted to extreme habitats such as high-ethanol or low pH environments. This results in various extraordinary requirements, e.g. mevalonic acid and a pH optimum at pH 4.5 – 5.0 of sake-spoiling *L. fructivorans* strains, while others grow best at pH 5.0 – 5.5 (Suzuki et al., 2008).

The observations made in this study further demonstrate that there is no general stress resistance for the tested strains, since none of them showed the strongest growth at all three stress parameters but, on the contrary, many strains with high tolerance to one parameter were outstandingly sensitive to another one, as for example *L. fructivorans* TMW 1.1856, *L. sakei* TMW 1.1474 and *L. plantarum* TMW 1.1478 and 1.1623.

The aim of the strain selection was to find two spoilage-relevant strains, preferably of the same species to ensure proper comparison, one of which had hydrophobic and one hydrophilic cell surface characteristics. While sufficient spoilage potential was shown for most *L. plantarum* and *L. sakei* strains, the two *L. fructivorans* strains were eliminated at this point owing to their weak growth at low temperatures, and since TMW 1.59 was inhibited also under high-NaCl and low-pH conditions. The fact that *L. plantarum* was the only species with strains showing differential, i.e. hydrophobic and hydrophilic, surface characteristics, was the basis for the decision to continue the study with two *L. plantarum* strains instead of *L. sakei* with consistently hydrophilic cell surfaces.

4.3 Localization of *L. plantarum* cells in O/W mixtures/emulsions

Microscopic analysis of the localization of the selected *L. plantarum* strains in emulsifier-free O/W mixtures showed that the hydrophobic strain TMW 1.25 had a stronger affinity for oil droplets than the hydrophilic strain TMW 1.708. This also corroborates previous findings on the attachment of hydrophobic *Lactococcus* cells to fat droplets in dairy cream (Ly *et al.*, 2006) and is in line with the generally observed phenomenon that a hydrophobic cell surface favors attachment to hydrophobic materials (An & Friedman, 1998, Araújo *et al.*, 2010, Krasowska & Sigler, 2014). In emulsifier-stabilized O/W emulsions, however, the oil droplets with a median droplet size of approximately 2.5 µm were very small and tightly packed, such that cells of both the hydrophobic and the hydrophilic strain seemed to be equally entrapped in the small

interstices between the oil droplets. Consequently, it was not possible to identify the effect of the emulsifier on cellular adhesion behavior, i.e. to see whether an increase in the hydrophilic character of the oil droplets due to coverage of the surface prevents adhesion of hydrophobic cells. These results further show that in the present type of O/W emulsions, possible correlations between HHP inactivation and CSH are independent of CSH-mediated localization.

4.4 Mutual influence of fat and aqueous-phase parameters during HHP inactivation of *L. plantarum* in O/W emulsions

Several studies reported a protective effect of the food matrix during HHP inactivation of spoiling microorganisms (Cheftel, 1995, Patterson *et al.*, 1995, Gervilla *et al.*, 2000, Patterson, 2005, Smiddy *et al.*, 2005, Black *et al.*, 2007). However, these results, especially on the role of fat, are often inconsistent and, at least in part, contradictory (Simpson & Gilmour, 1997, Gervilla *et al.*, 2000, Escriu & Mor-Mur, 2009, Ramaswamy *et al.*, 2009, Kruk *et al.*, 2014, Bover-Cid *et al.*, 2015, Bover-Cid *et al.*, 2017). This may be due to the complexity of the food matrix composition, as each of the manifold components may alter the HHP inactivation of microorganisms in a different way, and their effects may influence each other in a synergistic or antagonistic manner. According to a recent study in this research group, there is no evidence for a protective effect of fat per se, but rather enhanced inactivation of vegetative bacteria in O/W emulsions compared to aqueous buffer (Kafka *et al*., 2017). The present study is the first to systematically investigate the effect of fat in combination with food-relevant parameters of the aqueous phase on the HHP inactivation of *L. plantarum*. The results of these investigations are discussed in the following.

General HHP sensitivity of *L. plantarum* strains

Assessing the general HHP resistance of the selected *L. plantarum* strains showed considerable variation among different strains, especially in the range between 300 and 500 MPa (see Figure 17 and Appendix Figure 54 and Figure 55).

This is in line with previous studies showing that HHP sensitivity is highly variable among bacterial strains of the same species (Patterson *et al.*, 1995, Simpson & Gilmour, 1997, Alpas *et al.*, 1999, Benito *et al.*, 1999, Pagan & Mackey, 2000, Cebrián *et al.*, 2016). In accordance with a previous study in this laboratory on the effect of fat in O/W emulsions on HHP inactivation of *L. plantarum*, inactivation in general was slightly stronger in the presence compared to the absence of fat, which may in part be attributed to stronger adiabatic heating of rapeseed oil compared to water (Kafka *et al.*, 2017). According to Kafka (2018), an effect of the emulsifier Tween 80 present in the emulsions but not the corresponding aqueous buffer can be excluded which means that any difference observed between emulsions and IPB alone can be ascribed to

fat. However, several exceptions with reduced inactivation in the emulsion compared to pure IPB especially at higher (e.g. TMW 1.25, TMW 1.277 and TMW 1.2089 at 500 MPa), but also at lower pressures (TMW 1.708 at 300 MPa), show that fat alone can have a varying impact on different strains and even on the same strain at different pressure conditions. Such a variation in the effect of fat at different pressure intensities was reported earlier in the context of *Listeria monocytogenes* in dry-cured ham (Bover-Cid *et al.*, 2015).

NaCl

In line with data on *L. plantarum* and other bacterial species reported previously (Oxen & Knorr, 1993, Simpson & Gilmour, 1997, Molina-Gutierrez *et al.*, 2002, Van Opstal *et al.*, 2003, Molina-Höppner *et al.*, 2004, Gayán *et al.*, 2013), the presence of NaCl in the surrounding matrix had a baroprotective effect on the tested *L. plantarum* strains, provided that the concentration was sufficiently high. Molina-Höppner *et al.* (2004) observed baroprotection of *Lactococcus lactis* by 4 M NaCl, which approximately corresponds to the highest NaCl concentration (25 % (w/v)) used in this study. Gayán *et al.* (2013) reported that *E. coli* was considerably less pressure-sensitive at 4 to 5 M NaCl, and thereby showed reduced membrane permeability during pressure treatment, indicating high salt concentrations to act, at least in part, through protection of the cell membrane.

In general, the extent of baroprotection exerted by high NaCl concentrations was similar in the presence and absence of fat, as changes in log reduction values were comparable.

Whilst the hydrophilic strain TMW 1.708 experienced slightly stronger inactivation in the O/W emulsion than in pure IPB regardless of the NaCl concentration, for the hydrophobic strain TMW 1.25, this was only the case when NaCl was present. Inactivation data in the NaCl-free matrices are in line with the results from a previous study (Kafka *et al.*, 2017). Most importantly, these findings demonstrate that depending on the strain there can be an interplay between the two food matrix parameters fat and NaCl regarding the effect on HHP inactivation of suspended bacteria.

Comparison of the HHP sensitivity of three additional hydrophobic and hydrophilic *L. plantarum* strains at 0 %, 5 % and 12.5 % NaCl showed that this effect does not correlate with CSH. The increase in inactivation of TMW 1.1810 and TMW 1.1204 observed with the combination of 5 % NaCl and fat, although the same NaCl concentration protects from HHP inactivation in the corresponding buffer, is further proof of a considerable mutual influence appearing in a strain-specific manner. Although it has to be considered that at low NaCl concentrations between 1.25

and 5 % HHP caused slight coalescence of the oil droplets in O/W emulsions, the inactivation data obtained provide no evidence for an influence of this event on HHP inactivation.
When evaluating these results with respect to real food applications one also has to consider the growth-inhibiting effect of a_w reduction by NaCl that counteracts its baroprotective effect during HHP processing (Ulmer *et al.*, 2000, Duranton *et al.*, 2012). Hence, the efficiency of HHP treatment of food products containing considerable amounts of salt may be underestimated when microbes are recovered by recovery media immediately after HHP treatment.

Sucrose

Similar to NaCl, high sucrose concentrations of 50 % (and 25 % for TMW 1.708) reduced HHP inactivation in a fat-independent manner. The protective effect observed with high sucrose concentrations is in line with the results from previous studies in this laboratory showing baroprotection of *Lactococcus lactis* by 0.5 M sucrose, corresponding to approximately 17 % (w/v) (Molina-Höppner *et al.*, 2004), as well as 1.0 M (34 % (w/v)) and 1.5 M (51 % (w/v)) (Molina-Gutierrez *et al.*, 2002), and with the work of Van Opstal *et al.* (2003), who reported a baroprotective effect of 10-50 % (w/v) sucrose on *E. coli*, and showed that sucrose also reduces post-pressure inactivation of sublethally injured *E. coli* cells. It also corroborates the results from studies on *Listeria monocytogenes* (Koseki & Yamamoto, 2007), *Cronobacter sakazakii* (Arroyo *et al.*, 2011), bacterial spores (Raso *et al.*, 1998) as well as yeasts (Oxen & Knorr, 1993). Being a compatible solute, sucrose can be accumulated by microorganisms to high intracellular concentrations (Glaasker *et al.*, 1998), stabilizing the native state of proteins and biological membranes (Leslie *et al.*, 1995). This explains the baroprotective effect of sucrose and disaccharides in general.
Lower sucrose concentrations had almost no effect on the HHP inactivation of TMW 1.25 in pure IPB, but slightly enhanced inactivation in O/W emulsion. For TMW 1.708 enhanced inactivation was observed with 5 % and 12.5 % sucrose, regardless of the presence of fat. Data on the effect of lower sucrose concentration are rare in literature. However, at least for *E. coli* 0.1 M (~3.4 % (w/v)) sucrose has been shown to enhance HHP inactivation at 400 MPa, whereas higher sucrose concentrations greater than 0.2 M had a baroprotective effect (Hasegawa *et al.*, 2013), which is in line with the observations from this study for TMW 1.708. These results show that there is a threshold concentration for baroprotection by sucrose and that low concentrations are not only insufficient for protection, but in some cases may even enhance HHP inactivation. The fact that Hasegawa *et al.* (2013) did not observe such an effect with 350 MPa, where inactivation rates continuously decreased with increasing sucrose concentrations, indicates that pressure

intensity plays a crucial role, as well. Comparison of eight *L. plantarum* strains revealed that the effect of low sucrose concentrations strongly depends on the strain treated.

The fact that in the presence but not in the absence of sucrose TMW 1.25 showed slightly stronger inactivation in emulsions than pure IPB demonstrates that, depending on the bacterial strain, an interplay between fat and the two solutes is possible. Another example for strain-specific interrelation of fat and sucrose is the enhanced inactivation of TMW 1.708 at lower sucrose concentrations, where the ratio of log reductions between emulsion and IPB alone was reversed compared to the 0 % sucrose matrix. The increase in inactivation of TMW 1.284 observed with the combination of 5 % sucrose and fat, but not in the corresponding buffer, is further proof of a considerable mutual influence of fat and sucrose appearing in a strain-specific manner. Unlike sucrose concentration, the slight coalescence observed in emulsions with high sucrose concentrations under HHP (see 3.1.3) seemed to have no influence on HHP inactivation.

pH value

The results from this study, showing increased HHP sensitivity at lower pH values for all tested strains, are in line with previous data and the generally accepted view of HHP sensitivity increasing with the acidity of the environment (Stewart *et al.*, 1997, Garcia-Graells *et al.*, 1998, Alpas *et al.*, 2000, Ritz *et al.*, 2000, Molina-Gutierrez *et al.*, 2002, Koseki & Yamamoto, 2006, Ritz *et al.*, 2008, Arroyo *et al.*, 2011, Li *et al.*, 2016). Going from high to low pH values, especially at pH values where the strongest increase in HHP sensitivity of TMW 1.25 and TMW 1.708 was observed, the addition of fat resulted in stronger inactivation. Similar results were observed with TMW 1.1810, 1.1204, 1.2089 and 1.1808 at 300 MPa, and 1.284 and 1.1204 at 400 MPa, although without consideration of the pH course. This enhancement of the pH-dependent increase in HHP sensitivity may be due to an additive or synergistic interplay of acidity and temporarily elevated temperatures in proximity to oil droplets due to adiabatic heating during pressure build-up (Kafka *et al.*, 2017). The inversion of the ratio of log reductions between emulsion and pure IPB observed with TMW 1.708 at 300 MPa, pH 4 and the great difference between emulsion and IPB at particular strain-pressure-pH combinations (e.g. TMW 1.1808, 300 MPa, pH 4 and TMW 1.1204, 400 MPa, pH 6) demonstrates that the mutual influence of pH and fat acts in a strain specific manner, as well. In light of the general enhancement of the acid-dependent increase in HHP sensitivity by fat observed in this study and the fact that many emulsion-based foods such as salad dressings and mayonnaise commonly have a pH value between 3.2 and 4.0 (Smittle & Flowers, 1982), HHP seems suitable

for preservation of such products, as rather gentle HHP conditions suffice to achieve adequate microbial inactivation. When reducing the acid content of this type of foodstuffs, higher pressure levels may be necessary to obtain equivalent and sufficient inactivation levels.

Proteins

Proteins are generally associated with increased HHP resistance of microorganisms (Simpson & Gilmour, 1997, Black *et al.*, 2007, Narisawa *et al.*, 2008). In this study, three types of bovine milk protein or derivatives thereof were used to investigate the effect of protein in the aqueous phase of O/W emulsions on the HHP sensitivity of *L. plantarum*: (i) casein, insoluble in water at neutral pH, (ii) water-soluble whey protein isolate and (iii) peptone from casein, consisting of small peptide fragments. WPI had a slightly protective effect on TMW 1.25 depending on pressure intensity and the presence or absence of fat, but left the inactivation of TMW 1.708 virtually unaffected, whereas casein generally led to enhanced inactivation, especially at 500 MPa. The effect of WPI and casein may in part be related to physical changes in the surrounding matrix under the applied pressure conditions, as pressurization at 500 MPa led to gel formation (see 3.5.4). The impact of gelation on the HHP inactivation of suspended microorganisms has not been described yet. However, according to the results of Narisawa *et al.* (2008), who showed that the coagulated whey fraction from skim milk protects *E. coli* from HHP inactivation and that cells incorporated in protein aggregates showed less injury than cells in planktonic suspension, it can be speculated whether these physical changes contribute to the slightly lower HHP sensitivity of TMW 1.25 observed in the presence of WPI. If such a protective effect exists with casein as well, then it was superimposed by other, sensitizing effects one of which may be the slight pH drop from pH 7 to ~6 observed during incorporation of 2.5 % casein into IPB (data not shown). This could be one reason for the increased inactivation levels observed with casein for both TMW 1.25 and TMW 1.708, which stands in contrast to the data of Ramaswamy *et al.* (2009), who observed baroprotection by casein. Another reason might be the fact that in the present study isolated, non-micellar casein was used. Black *et al.* (2007) observed that only casein bound in micelles with the corresponding minerals, but not non-micellar casein, was able to exert baroprotection on *Listeria innocua*. Therefore, the mineral fraction and the micellar organization seem to be critical for the baroprotection of vegetative microorganisms by casein.

The protective effect of peptone observed with all tested strains during treatment with 500 MPa is in line with previous studies on *E. coli* (Ramaswamy *et al.*, 2009) and *Listeria monocytogenes* (Koseki & Yamamoto, 2006). When evaluating HHP inactivation associated with peptone it has to be kept in mind that peptone solubilized in IPB caused growth of the suspended

microorganisms (see 3.2.4.3.2). Thus the physiological state might have changed compared to the matrices without peptone, as some cells may already have switched their metabolism towards a more active state characterized. Since HHP sensitivity is generally higher in the exponential than in the stationary phase, the actual protection by peptone may be even stronger than observed in this study.

The addition of fat had in part disparate effects, depending on the protein type and content, e.g. significant baroprotection of TMW 1.25 during treatment at 500 MPa in the protein-free and the 2.5 % WPI matrices and enhanced inactivation with 2.5 % peptone. Especially the combined effect of fat and peptone varied considerably, as inactivation of TMW 1.25 and TMW 1.708 by 500 MPa at 2.5 % peptone tended to be higher in the emulsion than IPB alone, whereas with 10 % peptone the opposite was observed. For some strains, fat was able to significantly enhance (TMW 1.1810) or reduce (TMW 1.708, TMW 1.284) inactivation only in combination with 10 % peptone, but not in the peptone-free environment. Furthermore, the addition of fat changed the ratio between the inactivation intensities in the absence and presence of peptone for five out of eight strains. These results show that the effects of peptone and fat are able to influence each other in a strain-dependent but CSH-independent manner and that changes in the food matrix can have an opposite impact on individual strains. In addition, the exact composition and effect of complex matrix components like protein, and especially all natural food ingredients, can vary greatly, as reported by Gray *et al.* (2008) who found that the effect of peptone on bacterial growth differs among different manufacturers and batches. These points make the prediction of HHP inactivation efficiency in complex food matrices difficult and have to be considered when establishing HHP process parameters for food preservation.

Correlation of CSH with response to changes in the surrounding matrix

CSH is able to favor or impair contact to hydrophobic surfaces in complex matrices, such as emulsifier-free oil-water mixtures, which may affect the susceptibility of a bacterial cell to HHP inactivation (Kafka *et al.*, 2017). Due to the tight packing of the oil droplets in the O/W emulsions used in this study, suspended *L. plantarum* cells were localized in the interstices between the oil droplets, regardless of their CSH (see 3.3.2). Therefore, cellular localization in this study was independent of CSH, which allowed for the identification of CSH-related features in a localization-independent manner. In line with previous observations, HHP sensitivity of *L. plantarum* strains per se did not correlate with CSH (Kafka *et al.*, 2017). Moreover, the impact of parameters of the aqueous phase per se as well as modifications of the action of these parameters by the addition of fat were independent of CSH. Thus, cellular features associated

with CSH are not involved in the mechanism conveying resistance to HHP, but changes occurred rather sporadically and strain-specifically.

Conclusion

In conclusion, the results of this study in part demonstrate that fat is able to significantly change, and sometimes even invert, the effect of other food matrix parameters on HHP inactivation of suspended microorganisms. However, a general effect was not observed, but alterations of the impact of aqueous phase parameters induced by fat occurred strain-specifically, and independent of CSH. More research is needed to uncover the mechanisms underlying the mutual influence of different food matrix components and the strain specificity of certain food matrix-related effects on HHP inactivation. However, the present data provide the basis for further investigation and may build awareness for the complexity of the behavior of microorganisms under pressure. To unveil the cellular interrelations and underlying these phenomena will be the subject of future studies.

4.5 Cellular response to fat contact and effect on HHP sensitivity – effect of preconditioning

Most spoilage-causing and pathogenic microorganisms are introduced into food products during the manufacturing process. They can be attached to the surface of specific ingredients, for example spices and herbs, or stem from air, machine surfaces or the manufacturing staff. If HHP treatment is not conducted immediately after preparation, the food matrix might influence introduced microorganisms and thus alter their HHP sensitivity. This might cause a stress response that incidentally increases resistance to HHP. Moreover, they might be able to take up beneficial substances that increase their pressure resistance from the food environment. Some types of fatty acids, for example, have been associated with an increase in pressure resistance in the past (Allen *et al.*, 1999). In case of favorable conditions and long storage times, cells might even be able to grow and divide before pressure treatment, leading to elevated microbial counts. On the other hand, the conditions prevailing in the food matrix might increase pressure sensitivity and thus increase inactivation efficiency. To date, little is known about the cellular response of vegetative bacteria to contact with fat, and it is not known whether this has an impact on HHP sensitivity. Understanding the mechanisms behind potential fat-mediated alterations in HHP sensitivity is necessary for the development of avoidance strategies, the identification of appropriate HHP processing conditions and the design of continuing experiments. Therefore, the second part of this study addresses the question whether contact to

fat or associated substances can trigger a cellular response that has an impact on HHP sensitivity and deals with further investigations of the underlying cellular mechanisms.

4.5.1 Effect of prolonged incubation in O/W emulsions on HHP sensitivity

Extended incubation in IPB or O/W emulsion prior to HHP treatment for up to 48 h led to enhanced inactivation of both tested *L. plantarum* strains, with stronger enhancement at the higher incubation temperature of 25 °C compared to 4 °C. One reason might be that biochemical reactions generally run slower at lower temperatures and, consequently, alterations in the physiological state that cause the observed increase in HHP sensitivity are less advanced at 4 °C compared to 25 °C. In contrast to previous observations (Kafka, 2018), the presence of emulsifier 1 % Tween 80 in IPB slightly protected cells from HHP inactivation. However, the differences were very small and had no statistical significance and the increase in HHP sensitivity over time was similar in IPB with and without 1 % Tween 80. In contrast, incubation in O/W emulsion caused a stronger increase in HHP sensitivity over time compared to non-fat environment. This may depend on one of two different mechanisms: On the one hand, the presence of fat might alter the physiological state of the suspended bacteria, for example by providing compounds like fatty acids, such that the cells become more pressure sensitive. On the other hand, the physiological state achieved after prolonged incubation might render the cells more susceptible to fat-mediated effects like adiabatic heating during HHP treatment.

Although being significantly more pressure-resistant directly after cultivation, TMW 1.708 reached a HHP sensitivity comparable to TMW 1.25 when treated after 48 h. This shows that the pressure resistance can vary considerably depending on the conditions experienced previously and that these conditions have to be considered when comparing different bacterial strains (and species) regarding their HHP sensitivity.

4.5.2 Effect of the presence of fat and fat-associated compounds in the growth medium on HHP sensitivity of *L. plantarum*

Rapeseed oil present during cultivation had a slightly protective effect on both investigated strains of *L. plantarum*. At first glance, this seems contrary to the observations made with O/W emulsions where the presence of rapeseed oil was associated with enhanced HHP inactivation, especially after prolonged incubation in the respective matrices (see 3.6.1). However, it has to be kept in mind that in the latter experiments oil was present during HHP treatment, whereas in the present one cells were suspended in aqueous buffer for HHP treatment. This means that any differences in HHP sensitivity observed here are the result of changes of the physiological state of the cells. Probably, rapeseed oil exerts opposite effects on HHP sensitivity of

L. plantarum, depending on whether it is available during cultivation or HHP treatment. While its presence during cultivation alters the physiological state of the cells such that they become more HHP resistant, it seems to enhance the lethal effect of HHP when present during pressurization, possibly due to adiabatic heating. In real food scenarios, where spoilage bacteria have time to grow before HHP treatment the two effects may superimpose and counterbalance each other. This may be the case in the experiment of this study on prolonged incubation in O/W emulsion before HHP treatment (3.6.1), where the sensitizing effect of fat during HHP treatment was stronger. Furthermore, emulsifier molecules covering the oil droplets may have impeded contact of bacterial cells and rapeseed oil and a subsequent impact on the physiological state. It is also very likely that metabolic activity, which involves uptake of extracellular matter, is necessary for rapeseed oil to influence the physiological state of bacteria. According to their CSH and the resulting affinity to hydrophobic or hydrophilic surfaces, only the hydrophobic strain TMW 1.25, but not the hydrophilic strain TMW 1.708 was expected to be influenced by cultivation with rapeseed oil. The fact that both strains experienced slight baroprotection could be explained by the continuous agitation of the liquid cultures which probably forced both strains to the same extent to come into contact with the lipid phase.

These results indicate that despite their lack of extracellular lipase activity (see 3.2.4.1) the tested *L. plantarum* strains can take up substances from rapeseed oil that render them more baroresistant. Vegetable oils can undergo a process called hydrolytic rancidification consisting in the hydrolytic cleavage of the ester bond linking the glycerol backbone with the fatty acid residues (Osawa *et al.*, 2007). The free fatty acids released in this way can be ingested by non-lipolytic bacteria.

Being the major fatty acid in rapeseed oil (see Appendix Table 19), oleic acid was hypothesized to underlie the baroprotective effect and was thus investigated in more detail. The availability of fatty acids for bacterial cells is increased when they are delivered in the form of polysorbate/Tween, such as Tween 80, which contains mostly oleic acid and is not only used as an emulsifier in the food industry but also as supplement for bacterial growth media (De Man *et al.*, 1960). Comparison of *L. plantarum* cells grown in mMRS medium with or without Tween 80 revealed a clear protective effect of the amphiphilic compound, substantiating the suspicion of oleic acid being responsible for the increase in barotolerance. The fact that the baroprotective effect was much stronger with Tween 80 than with rapeseed oil, despite a much lower concentration, could be related to the increased availability of oleic acid for the cells. As shown in 3.2.4.1, the *L. plantarum* strains used possess no significant extracellular lipase activity and thus struggle to utilize fatty acids bound in triglycerides. Moreover, the access on the

microscopic level is limited due to the lacking miscibility of the lipid and aqueous phase. By contrast, the amphiphilic nature of Tween 80 ensures water solubility and easy absorption by the cells. In addition, the greater proportion of oleic compared to other fatty acids could be responsible for the difference in baroprotection: Tween 80 usually contains only traces (Partanen *et al.*, 2001), whereas rapeseed oil has > 35 % other FA than oleic acid.

To test whether the oleic acid part or the hydrophilic poly-oxyethylenesorbitan moiety of Tween 80 is the decisive factor, four Tween types characterized by different fatty acids, but the same hydrophilic head group, were compared for their effect on HHP sensitivity. The fact that Tween 20, consisting mainly of lauric acid, and Tween 80, but not Tween 40 (palmitic acid) or Tween 60 (stearic acid), reduced HHP inactivation, confirms the critical role of the fatty acid, showing that some fatty acids increase HHP resistance whilst others seem to have no effect at all. In this context it has to be mentioned that the protective effect of Tween 20 is possibly due to traces of oleic acid, since Tweens can contain other fatty acids apart from the main fatty acid, and Tween 20, besides lauric acid, has been shown to contain considerable amounts of oleic acid (Partanen *et al.*, 2001, Hansen *et al.*, 2015). Investigation of HHP inactivation after growth in mMRS medium supplemented with free fatty acids confirmed this presumption and supported the crucial role of oleic acid, which in contrast to all saturated fatty acids tested, including lauric acid, reduced HHP sensitivity. The only fatty acids besides oleic acid conferring slight protection from HHP were the polyunsaturated C18 FA linoleic and linolenic acid. Protection of bacteria from HHP inactivation by poly-UFAs *per se* has not been reported and moreover, they are associated with growth inhibition of lactobacilli (Kankaanpää *et al.*, 2001). Therefore, their baroprotective effect possibly results from intracellular conversion into oleic acid through saturation, which involves four genes (Kishino *et al.*, 2013) that are present in both *L. plantarum* strains used here, according to the available genome sequences (Kafka *et al.*, 2017).

These results show that the HHP sensitivity of spoilage microorganisms can be modified by fat and fat-associated substances, such as oleic acid. Considering that most vegetable oils contain large proportions of oleic acid, and vegetable oils are increasingly used to replace animal-based fat sources, these results may be useful for the food industry in order to optimize processing conditions. Especially long storage times between mixture of ingredients and food preparation on the one hand and pressurization on the other hand should be avoided in order to prevent adaptation and uptake of fat-associated compounds like oleic acid that would increase HHP resistance. To better understand the intracellular mechanisms underlying oleic acid-mediated baroprotection, further experiments were conducted using Tween 80 as a surrogate since the

baroprotective effect was stronger than with free oleic acid. For the same reason, *L. plantarum* TMW 1.708 was used as model organism in the following experiments.

4.5.3 Investigation of the Tween 80-mediated baroprotective effect

To gain insight into the mechanism underlying baroprotection mediated by Tween 80, a detailed analysis of the physiological state and behavior under HHP of *L. plantarum* TMW 1.708 grown in the presence of Tween 80 was performed.

The transcriptomic response of TMW 1.708 to growth medium supplementation with Tween 80 showed a significant downregulation of genes associated with the fatty acid biosynthesis. This is in line with data on *L. casei,* showing that fatty acid synthesis genes were downregulated in the presence of Tween 80, and reflects the decreased demand of fatty acid synthesis due to exogenously available oleic acid (Al-Naseri *et al.*, 2013). The role of Tween 80 as a supplier of exogenous matter might also be involved in the observed downregulation of the *oppC* gene coding for an oligopeptide transporter: In light of the availability of the carbon source Tween 80 the need for peptide uptake might be reduced. Furthermore, the upregulation of glutamine- (*glnPH2, glnQ3*) and lysine-specific (*lysP*) amino acid transporters and as well as transmembrane transport systems for uncharged ions (*glpF4*) and N-acetylglucosamine (*pts18CBA*) indicate that in the presence of Tween 80, cells preferably rely on individual components like free amino acids and sugars instead of entire peptides. The reason for potassium uptake channels to be downregulated may be ascribed to the fact that Tween 80 can bind small ions and carry them through the cell membrane (Thoman, 1986).

Tween 80 was shown to enhance bacterial growth in this as well as previous studies (Jacques *et al.*, 1980, Partanen *et al.*, 2001, Endo *et al.*, 2006). An increased demand for nucleic acid components provoked by this boost in cell growth is probably associated with the concomitant upregulation of genes implicated in the biosynthesis (*pyrE, pyrG*) and uptake (*pyrP*) of pyrimidines and related nucleotides. Especially the elevated mRNA levels of *pyrE*, the product of which catalyzes the formation of the UMP precursor orotidine-5’ monophosphate (OMP) from orotate (Bouia *et al.*, 1990), indicates that UMP production is enhanced in the presence of Tween 80.

The enzymes that catalyze *de novo* pyrimidine synthesis in *L. plantarum* are encoded on the *pyr* operon (Elagöz *et al.*, 1996). The first gene of the *pyr* operon, *pyrR1*, encodes an RNA binding regulator that attenuates transcription of the downstream *pyr* genes after binding to UMP in response to high exogenous pyrimidine levels (Nicoloff *et al.*, 2005). The transcription of *pyrP* is probably regulated likewise (Arsène-Ploetze *et al.*, 2006). Since the pyrimidine concentration in

the surrounding medium was the same in mMRS with and without Tween 80, the increased mRNA levels of *pyrE* as part of the *pyr* operon and *pyrP* probably result either from reduced mRNA degradation or from reduced transcription repression due to rapid pyrimidine consumption accompanying the accelerated cell growth in the presence of Tween 80 (see 3.6.3.2).

In light of the repressor function of *pyrR1*, the fact that this gene was found upregulated in the presence of Tween 80 surprises at first sight. However, one has to keep in mind that *pyrR1* represses transcription only if the ratio of UMP to phosphoribosyl pyrophosphate is high enough, which is not necessarily the case in the present study (Nicoloff *et al.*, 2005). Moreover, PyrR1 itself exhibits minor uracil phosphoribosyl transferase activity, catalyzing the conversion of uracil and phosphoribosylpyrophosphate into uridine monophosphate (UMP), according to its homolog in *Bacillus subtilis* PyrR (Martinussen *et al.*, 1995).

The upregulation of *pyrG* encoding CTP synthase points towards enhanced production of CTP from UTP, since this enzyme catalyzes the amination of the pyrimidine into a cytosine ring using glutamine. *pyrG* expression is regulated by attenuation in response to high intracellular CTP levels in *Lactococcus lactis* and *Bacillus subtilis* (Meng & Switzer, 2001, Jørgensen *et al.*, 2004), and probably also in *L. plantarum*. Thus, also *pyrG* mRNA levels seem to be elevated either due to reduced degradation or increased CTP turnover in the faster-growing cells.

The upregulation of genes coding for glutamine synthetase (*glnA*) and glutamine ABC transporter (*glnQ3, glnPH2*) is probably associated with the increased CTP synthase expression, as these genes are implicated in providing the substrate for CTP synthase. In turn, downregulation of the *gadB* gene with glutamate decarboxylase function in the presence of Tween 80 probably serves to keep glutamate concentrations stable, thus providing sufficient amounts of substrate for the GlnA enzyme. The upregulation of genes encoding a mannose-specific adhesin and glycosyltransferases of the *cps4* gene cluster indicates that the cell surface characteristics change in response to Tween 80. Adhesins are surface proteins mediating bacterial adhesion to extracellular surfaces such as host tissue (Silhavy *et al.*, 2010). The mannose-specific adhesion allows adhesion to mannose-containing receptor on e.g. intestinal epithelial cells. *L. plantarum* has been shown to adhere to mannose via the msa gene product in a highly strain specific manner (Pretzer *et al.*, 2005, Gross *et al.*, 2010). This property is thought to contribute to the probiotic function of these bacteria, as it allows them to successfully compete with pathogenic bacteria for attachment sites in the human intestine (Pretzer *et al.*, 2005). Therefore, *msa* upregulation due to growth medium supplementation with Tween 80 may be exploited in the cultivation of probiotic bacteria in order to obtain cells with improved adhesion in

the human intestine. The primary role of genes encoded on the *cps* clusters is the production of capsular surface polysaccharides (CPS) (Remus *et al.*, 2012), which are polymers linked to the cell surface via covalent bonds to phospholipid or lipid A molecules (Cescutti, 2010). According to Remus *et al.* (2012), the role of cell surface polysaccharides varies from protection from phagocytosis and opsonization of pathogenic bacteria (García *et al.*, 2000) and from innate immune factors in the host intestine (intestinal bacteria) (Lebeer *et al.*, 2011) over signaling between bacteria and host, e.g. by suppressing pro-inflammatory responses in macrophages (Yasuda *et al.*, 2008), to modification of the adhesion behavior to intestinal epithelial cells (Lebeer *et al.*, 2009). Glycosyltransferases are required for the transfer of monosaccharides from nucleotide sugars on the lipid carrier undecaprenyl phosphate to form repeating oligosaccharide units that are subsequently translocated across the cell membrane for polymerization to polysaccharides (De Vuyst & Degeest, 1999, Delcour *et al.*, 1999, Remus *et al.*, 2012). In *L. plantarum*, the *csp4* cluster is especially important for the quantity of produced CPS (Remus *et al.*, 2012). Therefore, the reason for upregulation of Cps4 glycosyltransferases in the presence of Tween 80 is probably also associated with concomitantly increased growth rate. In summary, transcriptomic analysis revealed that Tween 80, probably by providing exogenous oleic acid, reduces the cells' need for fatty acid synthesis. Probably, the concomitant energy and material savings are the reason for enhanced cell growth, manifesting primarily in the upregulation of genes involved in the synthesis of pyrimidine nucleotides, which are needed for *de novo* synthesis of nucleic acids.

Determination of growth parameters in mMRS medium confirmed the growth-promoting effect of Tween 80 indicated by the results from the transcriptome analysis. The fact that various Tween types differed in their effect on growth demonstrates that the fatty acid part rather than the polysorbate head group is crucial for growth enhancement. Visual inspection of the growth curves showed growth promotion only by Tween 80 and Tween 20, which also led to the highest maximum optical density, whereas Tween 80 was the only Tween to significantly raise the maximum growth rate and Tween 60 reduced the lag time most strongly, although lag time was fairly short already in mMRS, making interpretation of the effect difficult. Similar results were obtained earlier with *Lactobacillus delbrueckii*, which showed enhanced growth in the presence of Tween 20 and Tween 80, but not Tween 40 and Tween 60 (Partanen *et al.*, 2001). As with HHP inactivation, the growth promotion observed with Tween 20 could be due to oleic acid present in Tween 20 (Partanen *et al.*, 2001, Hansen *et al.*, 2015), as a similar effect was obtained earlier with free oleic but not lauric acid (Partanen *et al.*, 2001).

In earlier studies, Henderson & McNeill (1966) and Weeks & Wakil (1970) showed that exogenous FA are directly incorporated by *L. plantarum* into cellular lipids, and that mono-UFA like oleic acid, but not SFA, reduce *de novo* FA synthesis with a concomitant growth-promoting effect. In line with this, and corroborating further studies on different lactobacilli (Partanen *et al.*, 2001, Corcoran *et al.*, 2007, Hansen *et al.*, 2015), cellular fatty acid analysis of TMW 1.708 revealed an increase in the amount of oleic acid by medium supplementation with Tween 80, whereas the proportions of saturated lauric (Tween 20), palmitic (Tween 40) and stearic (Tween 60) acid increased to a much smaller extent. Remarkably, Tween 20 led to a similar fatty acid profile as Tween 80, probably owing to traces of oleic acid as mentioned above. Evaluation of these FA profiles shows that TMW 1.708 readily substitutes endogenous cis-vaccenic by exogenous oleic acid, which has the same chemical structure except for the position of the C-C double bond. The reduced need for and subsequent downregulation of *de novo* fatty acid synthesis causing energy and material savings is probably the reason for growth promotion, which reflects in the concomitant up-regulation of pyrimidine synthesis and amino acid uptake.

The reason why exogenous oleic acid, but not SFAs enhance growth may be related to the interconversion of UFA into SFA. SFA can be generated by conversion of the corresponding UFA (Kishino *et al.*, 2013), whereas UFA can only be synthesized during *de novo* FA biosynthesis (Cronan & Thomas, 2009). Therefore, in the presence of exogenous UFA the FA biosynthesis pathway can be downregulated and the cells are still able to maintain an appropriate membrane FA composition, whereas in the presence of exogenous SFA, FA synthesis is still required to ensure the production of UFA, since exogenous SFA cannot be directly converted into UFA.

Tween 20 and Tween 80 further caused an increase in total CFA while reducing SFA levels with stearic acid being completely eliminated. CFA are produced at the onset of stationary phase from *cis*-UFA by CFA synthase, which catalyzes *in situ* methylenation of the double bond (Grogan & Cronan, 1997), in order to cope with an increasingly hostile environment characterized by accumulating metabolites and/or adverse pH conditions. Therefore, high CFA proportions in the membrane lipids are associated with increased resistance of various bacteria to unfavorable environmental conditions such as low pH (Chang & Cronan Jr, 1999) as well as high osmotic pressures (Guillot *et al.*, 2000), temperatures (Suutari & Laasko, 1992, Annous *et al.*, 1999) and alcohol concentrations (Grogan & Cronan Jr, 1986). In this context, cis-vaccenic and oleic acid are transformed into lactobacillic and dihydrosterculic acid, respectively (Polacheck *et al.*, 1966, Smith & Norton, 1980). Thus, the observed appearance of dihydrosterculic acid and the concomitant decreased in lactobacillic acid levels is the result of a

change in the substrate for CFA synthase. The fact that lactobacillic acid had completely disappeared after growth with Tween 20 and Tween 80 although *cis*-vaccenic acid was still present in considerable proportions suggests a preference of CFA for oleic acid.

The fatty acid profiles obtained from growth with different Tween types are similar to previous profiles of various lactic acid bacteria (Johnsson *et al.*, 1995) and correlate with the results from HHP inactivation of TMW 1.708, as Tween 20 and Tween 80, but not Tween 40 or Tween 60, both increased the resistance to HHP and led to a distinctive fatty acid profile characterized by oleic and dihydrosterculic acid instead of cis-vaccenic and lactobacillic acid and increased total CFA levels. In light of the fact that in bacteria, except some species that accumulate lipids for storage, fatty acids are primarily found in the phospholipids of the cytoplasmic membrane (Cronan & Thomas, 2009), these results strongly indicate that the Tween-dependent changes in HHP sensitivity are based upon concomitant alterations in membrane fatty acid composition. This is in accordance with the general view of the cytoplasmic membrane of vegetative bacteria as one of the main target structures for HHP and data from Ulmer *et al.* (2002), who showed that membrane properties can have a strong influence on HHP sensitivity of *L. plantarum*.

Evaluation of the colony count of HHP treated cells of TMW 1.708 on selective and non-selective agar containing NaCl in the maximum non-inhibitory concentration revealed that the large majority of pressure-treated cells were at least sublethally injured. Especially at pressures where, according to colony formation on non-selective agar, Tween 80 exerted a strong baroprotective effect, the entire population of survivors showed sublethal injury. This means that except from very mild pressure conditions HHP treatment leads to damage in all cells.

Consequently, there are two possible ways for Tween 80 to exert its baroprotective effect. The first one would be to increase the ability of the cells to repair and recover from sublethal injury. The second possibility is that Tween 80 protects cells directly by keeping HHP-induced injury at a sublethal level, thus allowing more cells to recover.

Besides the cells that are still able to form colonies, there can be a fraction that is alive but cannot grow and divide any more. These so-called viable-but-non-culturable (VBNC) cells were detected by determination of the metabolic activity using the resazurin reduction assay which revealed that after exposure to 400 MPa cells retained more than 60 % of their initial metabolic activity although the colony count on non-selective agar was reduced by around two log cycles (i.e. around 1 % of cells were able to grow), for example. The difference was even greater at higher pressures where a reduction of the colony counts by around 6 log (1 cell in a million able to grow) came along with a residual metabolic activity of around 40 % for cells grown in mMRS- and 60 % after growth in mMRST80, showing that a large proportion of cells was still viable but

not culturable under the conditions offered. These results are in line with observations made earlier with HHP treated *Lactococcus lactis,* which retained about 25 % of the initial metabolic activity after HHP treatment leading to a 3 log reduction in colony counts (Molina-Gutierrez *et al.*, 2002), and data on *L. plantarum* strain TMW 1.1460, which still showed considerable metabolic activity upon pressure treatments causing a reduction of viable cell counts by more than 4 log, as determined based on its ability to reduce 2-(-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) to insoluble formazan (Ulmer *et al.*, 2000). Moreover, ATP-generating glycolytic enzymes of *L. plantarum* have been shown to retain their activity after HHP treatments resulting in a considerable reduction of viable cell counts, requiring severe HHP treatments causing a reduction of the colony count to 0.4 % for complete inhibition, whereas ATPase activity seemed to be inhibited earlier (Wouters *et al.*, 1998). In concert with these data the results from the present study indicate that the loss of metabolic activity is not a prerequisite for cell inactivation. However, the fact that cultivation with Tween 80 reduced the HHP-induced loss of metabolic activity indicates a protective effect of Tween 80, and presumably the supplied oleic acid, on the functionality of redox enzymes. The exact mechanism of protection, and whether the characteristic fatty acid profile plays a role in this context, still has to be investigated. The bacterial cell membrane is one of the key target structures for HHP-induced cell damage, as it undergoes changes in the phase state and an increase in permeability, which becomes visible in the uptake of extracellular substances and the loss of intracellular matter (Ulmer *et al.*, 2000, Klotz *et al.*, 2010). Especially in *E. coli* the interrelation between loss of membrane integrity and cell inactivation upon exposure to HHP has been well-demonstrated (Klotz *et al.*, 2010, Charoenwong *et al.*, 2011). In Gram-positive bacteria, in contrast, the role of membrane damage is not yet as clear (Smelt *et al.*, 1994, Arroyo *et al.*, 1999, Ananta & Knorr, 2009). However, also for *L. plantarum* increased membrane permeability under HHP has been shown (Wouters *et al.*, 1998). Other studies have emphasized the importance of membrane properties during HHP-induced inactivation of membrane-bound enzymes with critical functions for the survival of *L. plantarum* (Ulmer *et al.*, 2000, Ulmer *et al.*, 2002). The correlation between the fatty acid composition after cultivation with different Tween types and HHP sensitivity observed in the present study led to the hypothesis of the cell membrane to play a key role in the Tween 80-mediated reduction in HHP sensitivity. The assessment of HHP induced membrane damage by determination of protein release revealed a reduced loss of protein with both HHP treated and untreated cells after cultivation with Tween 80. This indicates a generally lower permeability, increased pressure-stability of the membrane. The observation that the amount of released protein increased only up to a certain pressure level of 400 MPa is in line with previous studies

on *E. coli* (Klotz *et al.*, 2010, Charoenwong *et al.*, 2011) and may result either from the formation of intracellular aggregates of denatured proteins, which are unable to penetrate the peptidoglycan network, and/or structural changes of the cell envelope, which prevent proteins from escaping the cell (Vázquez-Laslop *et al.*, 2001, Klotz *et al.*, 2010). Furthermore, it has to be considered that not all released proteins necessarily originate from the cytoplasm, but also membrane-bound protein may be released due to changes in their 3-D configuration or the membrane phase state under pressure, impairing proper interaction with the phospholipid bilayer.

The Tween 80 induced increase in pressure stability of the cell membrane was observed in respect of PI uptake, as well. Likewise, the base permeability of untreated cell membranes was reduced by Tween 80. Comparison of the PI fluorescence after staining before and after HHP treatment showed that the membranes of cells grown without Tween 80 permeabilized under pressure are partially resealable, whereas after cultivation with Tween 80, membrane permeability is generally lower, but no resealing seems to appear at all. Consequently, the effect of Tween 80 is probably based on the stabilization of the fraction of membranes at the transition from an undamaged state to permanent injury, whereas the fraction of strongly injured membranes is not affected by Tween 80.

Analysis by fluorescence microscopy revealed that the high discrepancy in PI fluorescence of heat- and HHP-treated cells with similar inactivation levels (e.g. at 400-500 MPa) results from the fact that HHP treatment causes PI fluorescence only in a minor fraction of cells. Neither membrane integrity nor metabolic activity was impaired to an extent able to explain the inability to grow on non-selective agar after HHP treatment. This lack of correlation is in line with previous studies on lactobacilli (Arroyo *et al.*, 1999, Ulmer *et al.*, 2000, Ananta & Knorr, 2009) and *Lactococcus lactis* (Molina-Gutierrez *et al.*, 2002) and demonstrates that at least in *L. plantarum* strain TMW 1.708 loss of membrane integrity *per se* is not the main reason for HHP-induced inactivation. Observations made by Gayán *et al.* (2013) with *E. coli*, which was able to reseal disrupted membranes after decompression even after lethal HHP treatment, point in the same direction. On the contrary, some *E. coli* strains have been shown to survive membrane leakage (Pagan & Mackey, 2000). A possible explanation for the discrepancy between the strong loss of viability on the one, and the merely slight impairment of membrane integrity and metabolic activity on the other hand might be found in a previous study on *E. coli*, where the HHP treatment induced the disassembly of aggregated misfolded proteins (Govers *et al.*, 2014). Although the cells were viable and metabolically active, these proteins impeded resuscitation until they were re-assembled to proper inclusion bodies.

Despite the lack of correlation, the two parameters loss of membrane integrity and failure to grow on non-selective agar, may still be interrelated, since both were reduced significantly by the cultivation with Tween 80. This might be related to the exogenous oleic acid supply and the cellular fatty acid profile characterized by elevated CFA content. According to Abe (2013), there is evidence for the pressure resistance of microorganisms to depend on their ability to maintain membrane properties like lipid packing and fluidity under a wide pressure range rather than membrane fluidity and phase transition pressure/temperature, in general. Consequently, microorganisms with membranes that undergo smaller volume changes during HHP-induced phase transition are more pressure-resistant than those with large volume changes, as demonstrated in a study on *L. plantarum* where cells with gel phase membranes at atmospheric pressure were more pressure-resistant than cells with membranes in the liquid-crystalline phase (Ulmer *et al.*, 2002, Abe, 2013). One reason for this may be the reduced impairment of membrane-bound proteins due to phase changes. Membrane lipids with a higher degree of unsaturation cause smaller volume changes during liquid crystalline-to-gel phase transition, since the kinks present in unsaturated, and to some extent also in cyclopropanated, acyl chains lead to large free volumes even in the gel phase (Tada *et al.*, 2010, Abe, 2013). From the beginning of stationary growth phase lactobacilli use CFA synthase to convert monounsaturated fatty acids into the corresponding CFA (Polacheck *et al.*, 1966, Grogan & Cronan, 1997), which have been associated with the resistance of lactobacilli to various stress factors, such as cold stress (Smittle *et al.*, 1974), high acidity (Broadbent *et al.*, 2010, Broadbent *et al.*, 2014), bile acids (Hamon *et al.*, 2011) and high metabolite concentrations in stationary growth phase (Grogan & Cronan, 1997, Murínová & Dercová, 2014). Furthermore, CFA increase high pressure resistance in *E. coli*, and CFA synthase knockout mutants of *E. coli* exhibited enhanced membrane permeabilization and loss of viability upon HHP treatment compared to the wild type (Charoenwong *et al.*, 2011, Chen & Gänzle, 2016). According to Poger & Mark (2015), cis-CFA like for example lactobacillic and dihydrosterculic acid have a dual effect on the cell membrane: On the one hand, they increase overall fluidity and diffusion compared to SFA and UFA, which shifts the phase transition from liquid-crystalline to gel phase towards higher pressure levels. As a consequence, crucial membrane-bound enzymes are protected and membrane functionality is assured up to higher pressure intensities, leading to increased survival as observed in this study after cultivation with Tween 80. On the other hand, the acyl chains of CFA exhibit a higher degree of order than those of analogous UFA, resulting in increased rigidity and consequently in reduced permeability to small molecules, such as many toxic compounds and dyes like PI. This might be an explanation for the reduced permeability to PI after growth with Tween 80.

Moreover, CFA might increase cell viability due to the fact that they are inert to oxidation by reactive oxygen species produced under high pressure, as methylenation of UFA, i.e. formation of CFA, has been associated with improved resistance to superoxide, singlet oxygen, ozonolysis and oxidative stress, in general (Grogan & Cronan, 1997).

Conclusion

The results of the second part of this study demonstrate that the contact to fat and fat-associated compounds provokes a physiological response in growing *L. plantarum* cells, which is linked to reduced HHP sensitivity. Based on the observations made, the protective effect of fat during pre-conditioning can probably be ascribed to oleic acid, since other free fatty acids, or Tween types containing other fatty acids, did not affect HHP sensitivity. In practice, this means that foodstuffs prepared with high amounts of vegetable oils, which are often rich in oleic acid, may increase the resistance of vegetable spoilage bacteria through preconditioning, especially if products contain other ingredients that enable microbial growth, such as carbohydrates and proteins. Hence, extended time intervals between the preparation, where most spoilage microorganisms are introduced, and a subsequent HHP treatment should be avoided to reduce the risk of resistance development. Tween 80 added to the growth medium of *L. plantarum* primarily acts through the supply with oleic acid, leading to a shut-down of fatty acid biosynthesis and an incorporation of oleic acid in the cell membrane. As cellular fatty acid profiles associated with reduced HHP sensitivity showed increased amounts of CFA, these fatty acids probably play a major role in the development of HHP resistance. However, the cell membrane per se does not appear to be the primary structure associated with Tween 80/oleic acid-mediated HHP resistance, since the effect of HHP on the capacity for growth was considerably larger than on membrane integrity. Although Tween 80 reduces the pressure-induced loss of metabolic activity and membrane integrity, these events are not a prerequisite for *L. plantarum* to lose its growth capacity. However, it is conceivable that the mentioned fatty acid composition with elevated CFA levels protects the functionality of membrane-bound enzymes under HHP. This study provides the basis for further investigations of the mechanisms underlying inactivation by and resistance to HHP of *L. plantarum*, and Gram-positive bacteria, in general.

5 Summary

Consumers' demand for high-quality, minimally processed food free of artificial preservatives is increasing. At the same time, convenience products enjoy growing popularity. High hydrostatic pressure (HHP) processing is a promising, non-thermal preservation technology that has the potential to fulfill these requirements, as it efficiently inactivates spoiling and pathogenic microorganisms and leaves their organoleptic and nutritional properties largely unaffected. However, the efficiency of inactivation by HHP varies widely among microorganisms and can be strongly affected by the composition of the food matrix. Since systematic data especially on the interplay of different matrix constituents are rare, appropriate HHP processing parameters have to be determined case-by-case for each product. Moreover, there is little knowledge on the connection between the physiological response of microorganisms to different matrix constituents and their intrinsic pressure resistance. Foodstuffs for which HHP processing offers a great alternative in preservation include heat sensitive ready-made meals like sauces, dips and salads on the basis of mayonnaise and related preparations, which are characterized by a complex composition and a considerable fat content. Spoilage of such products is often caused by lactic acid bacteria, such as lactobacilli. Therefore, this study aimed at investigating the interplay between fat and other food matrix parameters during HHP inactivation of *Lactobacillus* strains. Moreover, the effect of preconditioning of lactobacilli by contact to fat or fat-associated substances on their HHP sensitivity was assessed, and the concomitant physiological response was investigated with the goal of identifying the mechanisms underlying potential changes in HHP sensitivity.

In the first part of this study, defined oil-in-water (O/W) emulsions containing rapeseed oil as the lipid phase and Tween 80 as emulsifier were used as a model system to assess the impact of fat on the effect of the aqueous phase parameters sodium chloride (NaCl), sucrose, pH and proteins on the HHP inactivation of *Lactobacillus* strains. To identify a potential interconnection with cell surface characteristics, strains with hydrophobic and hydrophilic cell surfaces were considered. General HHP sensitivity varied widely among the strains. High NaCl and sucrose concentrations generally exerted a baroprotective effect, whereas low-pH environments enhanced inactivation, and the impact of proteins varied according to the type of protein. The presence of fat had no general influence on any of the aqueous phase parameters in its impact on HHP inactivation, but caused sporadic matrix- and strain-specific, however CSH-independent alterations in inactivation efficiency. These data show that fat is able to significantly change the effect of other food matrix parameters on HHP inactivation of suspended microorganisms. The

fact that alterations occurred in a highly strain-specific manner and did not follow an explainable pattern shows that the mechanisms underlying the mutual influence of matrix components and the strain specificity of certain food matrix-related effects are far from understood. These data provide the basis for further investigation and may build awareness for the complexity of the behavior of microorganisms under pressure.

Preconditioning of *L. plantarum* cells by prolonged incubation prior to HHP treatment generally increased HHP sensitivity. The increase was slightly enhanced in O/W emulsions, but reduced by Tween 80 alone, in comparison to plain aqueous buffer solution. Investigating the effect of preconditioning by growth medium supplementation revealed that rapeseed oil, free oleic acid, Tween 20, and Tween 80, both providing cells with oleic acid, reduces HHP sensitivity of *L. plantarum*, whereas Tween 40, Tween 60 and other free fatty acids, show no such effect. Growth in the presence of Tween 20 or Tween 80 led to specific fatty acid profile characterized by elevated oleic acid and cyclopropane fatty acid levels. Transcriptomic analysis during cultivation showed that Tween 80 supplementation causes the downregulation of cellular fatty acid biosynthesis, and indicated increased metabolic activity, which was corroborated by enhanced cell growth compared to other growth conditions. Growth in the presence of Tween 80 reduced the HHP induced loss of metabolic activity and membrane integrity, as determined by protein release and uptake of the fluorescent dye propidium iodide. However, metabolic activity and membrane integrity were reduced to a far lesser extent than the ability to grow and proliferate, showing that these events are not prerequisites for HHP-induced inactivation of *L. plantarum*.

These data demonstrate a protective effect of fat when present during the growth of microorganisms, which, according to the observations made, is most likely caused by exogenously available oleic acid and the accompanying changes in the fatty acid profile. The acquired data indicate that the mentioned protection manifests not only during or soon after pressurization but probably during the recovery process. This knowledge gain provides the basis for further studies and may assist the food industry in determining appropriate process parameters and optimizing process workflows.

6 Zusammenfassung

In modernen Industrienationen steigt die Nachfrage sowohl nach qualitativ hochwertigen und minimal verarbeiteten Lebensmitteln also auch nach leicht zuzubereitenden Fertigprodukten. Hydrostatischer Hochdruck (HHD) als nicht-thermische Technologie zur Konservierung bietet eine vielversprechende Möglichkeit, diesen Bedarf zu bedienen, da sich durch Behandlung mit HHD Verderbs- und pathogene Mikroorganismen effizient inaktivieren lassen, während die organoleptischen und nutritiven Eigenschaften eines Lebensmittels weitgehend erhalten bleiben. Allerdings variiert die Inaktivierungseffizienz durch HHD stark zwischen Mikroorganismen und kann erheblich durch die Zusammensetzung der Lebensmittelmatrix beeinflusst werden. Da bisher wenig systematische Daten, insbesondere zum Zusammenspiel verschiedener Matrixbestandteile existieren, müssen geeignete HHD-Prozessparameter für jedes Produkt separat ermittelt werden. Zudem ist bislang wenig darüber bekannt, inwieweit die intrinsische Druckresistenz von Mikroorganismen von deren physiologischer Antwort auf verschiedene Matrixbestandteile abhängt. Vor allem für hitzeempfindliche Fertigprodukte wie Soßen, Dips und Salate auf Basis von Mayonnaise und ähnlichen Zubereitungen mit komplexer Zusammensetzung und erhöhtem Fettgehalt stellt die HHD-Technologie eine vielversprechende Alternative dar. Für den Verderb solcher Produkte sind oftmals Milchsäurebakterien wie Laktobazillen verantwortlich. Ziel dieser Studie war daher einerseits die Untersuchung des Zusammenspiels zwischen Fett und anderen Matrixparametern bei der HHD-Inaktivierung von *Lactobacillus*-Stämmen. Andererseits wurde untersucht, inwieweit eine Vorkonditionierung der Bakterien durch Kontakt mit Fett oder fettassoziierten Substanzen deren HHD-Sensitivität beeinflusst. Die Untersuchung der damit einhergehenden physiologischen Antwort sollte Aufschluss über die zellulären Mechanismen geben, welche einer möglichen Änderung der HHD-Sensitivität durch Fettkontakt zugrunde liegen. In definierten Öl-in-Wasser (O/W)-Emulsionen mit Rapsöl als Lipidphase und Tween 80 als Emulgator wurde untersucht, inwieweit Fett den Effekt von Parametern der wässrigen Phase wie Natriumchlorid (NaCl), Saccharose, dem pH-Wert und Proteinen auf die HHD-inaktivierung der *Lactobacillus*-Stämme beeinflusst. Um eine mögliche Verbindung mit Oberflächeneigenschaften zu erkennen, wurden Stämme mit hydrophober und mit hydrophiler Zelloberfläche verwendet. Die allgemeine HHD-Sensitivität variierte zwar stark zwischen den einzelnen Stämmen, jedoch konnte festgestellt werden, dass hohe NaCl- und Saccharosekonzentrationen generell baroprotektiv wirken, während Umgebungen mit niedrigem pH-Wert die Inaktivierung verstärken. Der Einfluss von Proteinen war dagegen abhängig von der Art der Proteine. Die Anwesenheit von Fett hatte zwar keine

generelle Auswirkung auf die Parameter der wässrigen Phase bezüglich deren Effekt auf die HHD-Inaktivierung, führte aber zu sporadischen matrix- und stammspezifischen Veränderungen in der Inaktivierungseffizienz, welche unabhängig von der Zelloberflächenhydrophobizität waren. Dies zeigt, dass Fett durchaus in der Lage ist, den Einfluss anderer Matrixparameter auf die HHD-Inaktivierung von Mikroorganismen zu verändern. Das Fehlen eines eindeutigen Musters zeigt, dass die dem gegenseitigen Einfluss von Matrixkomponenten und der Stammspezifität bestimmter Matrixeffekte zugrunde liegenden Mechanismen noch weitgehend unbekannt sind. Diese Daten liefern die Grundlage für weitere Untersuchungen und können ein Bewusstsein für die Komplexität des Verhaltens von Mikroorganismen unter Druck schaffen.

Eine Vorkonditionierung von *L. plantarum*-Zellen durch verlängerte Inkubation vor der HHD-Behandlung führte generell zu einer erhöhten HHD-Sensitivität. Die Zunahme war in O/W-Emulsionen geringfügig stärker und in einer Lösung des Emulgators Tween 80 ohne Fett geringer als in reiner wässriger Pufferlösung. Bei der Untersuchung der Vorkonditionierung durch Zugabe von Fett und fettassoziierten Substanzen zum Wachstumsmedium verringerten Rapsöl, freie Ölsäure, Tween 20 und Tween 80, welche ebenfalls Ölsäure liefern, die HHD-Sensitivität von *L. plantarum*, während ein solcher Effekt bei Tween 40, Tween 60 und anderen freien Fettsäuren nicht beobachtet wurde. Die Kultivierung in Anwesenheit von Tween 20 oder Tween 80 führte zu spezifischen Fettsäureprofilen mit erhöhten Gehalten an Ölsäure- und Cyclopropanfettsäuren. Eine Transkriptomanalyse während der Kultivierung zeigte eine Herunterregulierung der zellulären Fettsäurebiosynthese infolge der Zugabe von Tween 80 und deutete auf erhöhte metabolische Aktivität hin, was durch verstärktes Wachstum im Vergleich mit anderen Wachstumsbedingungen bestätigt wurde. Die Kultivierung mit Tween 80 verringerte den durch HHD verursachten Verlust an metabolischer Aktivität und Membranintegrität, wie Experimente zur Proteinfreisetzung und Aufnahme des Fluoreszenzfarbstoffs Propidiumiodid zeigten. Allerdings wurden die metabolische Aktivität und Membranintegrität weitaus weniger stark beeinträchtigt als die Fähigkeit zu Zellwachstum und Proliferation, was bedeutet, dass diese Ereignisse keine Voraussetzung für die HHD-induzierte Inaktivierung von *L. plantarum* sind. Diese Daten liefern Hinweise auf einen protektiven Effekt von Fett während des Wachstums der Mikroorganismen, welcher höchstwahrscheinlich auf der Aufnahme von exogen verfügbarer Ölsäure und den damit einhergehenden Veränderungen im Fettsäureprofil basiert. Der beschriebene protektive Effekt zeigt sich wahrscheinlich nicht nur während oder kurz nach der HHD-Behandlung, sondern auch während der Erholungsphase. Diese Erkenntnisse liefern die Grundlage für weitere Studien und können der Lebensmittelindustrie bei der Ermittlung geeigneter Prozessparameter und der Optimierung von Prozessabläufen hilfreich sein.

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8 Appendix

8.1 Figures and Tables

Table 17: Emulsion characteristics before and after HHP treatment. O/W emulsions with varying aqueous phase parameters (NaCl concentration, sucrose concentration, pH value, protein type and concentration) were prepared by two-step homogenization using appropriate homogenization pressures and, where applicable, treated with HHP (500 MPa, 25 °C, 5 min). The specific surface area (SSA) and the median droplet size (D(v,0.5)) were determined using a laser particle size analyzer. The creaming rate was determined using an analytical centrifuge (LUMIfige®). Presented values are the means of at least three replicates.

#	Aqueous phase				Homo-genization pressure (step 1/2) [bar]	SSA [$m^2 g^{-1}$]		D(v,0.5) [µm]		Creaming rate [$\mu m\ s^{-1}$]	
	Buffer sys-tem	Solute	Con-cen-tration	pH		No HHP	500 MPa, 25 °C, 5 min	No HHP	500 MPa, 25 °C, 5 min	No HHP	500 MPa, 25 °C, 5 min
1	IPB	-	-	7	100/20	3.74 ± 0.54	3.61 ± 0.41	2.58 ± 0.14	2.64 ± 0.09	4.93 ± 0.29	4.89 ± 0.40
2	IPB	NaCl	0.5 %	7	100/20	3.26 ± 0.13	3.26 ± 0.79	2.68 ± 0.06	2.61 ± 0.32	5.07 ± 0.43	5.20 ± 0.35
3	IPB	NaCl	1.25 %	7	100/20	3.32 ± 0.24	2.32 ± 0.24	2.67 ± 0.11	3.13 ± 0.04	5.58 ± 0.52	5.78 ± 0.34
4	IPB	NaCl	2.5 %	7	100/20	3.25 ± 0.30	2.84 ± 0.33	2.70 ± 0.12	2.84 ± 0.05	5.34 ± 0.18	5.63 ± 0.16
5	IPB	NaCl	5 %	7	100/20	3.67 ± 0.70	3.17 ± 0.02	2.52 ± 0.26	2.69 ± 0.02	5.70 ± 0.26	5.86 ± 0.24
6	IPB	NaCl	12.5 %	7	100/20	3.61 ± 0.08	3.41 ± 0.07	2.43 ± 0.02	2.52 ± 0.02	6.76 ± 0.22	7.24 ± 0.21
7	IPB	NaCl	25 %	7	100/20	4.04 ± 0.09	3.81 ± 0.16	2.09 ± 0.02	2.16 ± 0.06	7.69 ± 0.34	7.08 ± 1.22
8	IPB	Sucrose	5 %	7	100/20	3.78 ± 0.14	3.61 ± 0.04	2.47 ± 0.02	2.46 ± 0.01	4.49 ± 0.11	4.62 ± 0.04
9	IPB	Sucrose	12.5 %	7	100/20	4.03 ± 0.06	3.80 ± 0.19	2.33 ± 0.06	2.32 ± 0.04	4.54 ± 0.17	4.65 ± 0.09
10	IPB	Sucrose	25 %	7	100/20	4.53 ± 0.26	3.72 ± 0.09	2.08 ± 0.07	2.32 ± 0.04	4.09 ± 0.26	4.08 ± 0.12
11	IPB	Sucrose	50 %	7	100/20	4.63 ± 0.23	4.19 ± 0.03	2.00 ± 0.07	2.13 ± 0.01	4.09 ± 0.47	3.10 ± 0.22
12	CPB	-	-	7	100/20	3.71 ± 0.29	3.77 ± 0.06	2.52 ± 0.16	2.40 ± 0.13	4.99 ± 0.07	5.02 ± 0.25
13	CPB	-	-	6	100/20	3.79 ± 0.49	3.64 ± 0.09	2.54 ± 0.11	2.51 ± 0.08	4.95 ± 0.18	5.03 ± 0.09
14	CPB	-	-	5	100/20	3.67 ± 0.07	3.66 ± 0.13	2.57 ± 0.07	2.52 ± 0.09	5.43 ± 0.29	5.06 ± 0.02
15	CPB	-	-	4	100/20	3.81 ± 0.28	3.67 ± 0.15	2.52 ± 0.07	2.49 ± 0.04	4.94 ± 0.24	4.79 ± 0.21
16	CPB	-	-	3	100/20	3.84 ± 0.06	3.76 ± 0.18	2.44 ± 0.05	2.46 ± 0.06	4.68 ± 0.12	4.72 ± 0.23
17	IPB	-	-	7	300/60	5.22 ± 0.58	5.59 ± 0.10	1.60 ± 0.14	1.54 ± 0.04	3.58 ± 0.49	3.60 ± 0.34
18	IPB	Peptone	2.5 %	7	200/40	5.04 ± 0.44	5.59 ± 0.35	1.77 ± 0.11	1.67 ± 0.01	3.44 ± 0.46	3.34 ± 0.40
19	IPB	Peptone	10 %	7	100/20	4.84 ± 0.30	5.02 ± 0.22	1.94 ± 0.06	1.94 ± 0.08	3.32 ± 0.26	3.43 ± 0.25

#	Aqueous phase				Homo-genization pressure (step 1/2) [bar]	SSA [m² g^{-1}]		D(v,0.5) [µm]		Creaming rate [µm s^{-1}]	
	Buffer sys-tem	Solute	Con-cen-tration	pH		No HHP	500 MPa, 25 °C, 5 min	No HHP	500 MPa, 25 °C, 5 min	No HHP	500 MPa, 25 °C, 5 min
20	IPB	WPI	2.5 %	7	150/30	4.74 ± 0.32	4.75 ± 0.16	1.96 ± 0.03	2.05 ± 0.14	3.83 ± 0.21	3.89 ± 0.11
21	IPB	WPI	10 %	7	100/20	4.48 ± 0.09	4.53 ± 0.04	2.10 ± 0.10	2.07 ± 0.10	10.44 ± 0.27	8.85 ± 0.48
22	IPB	Casein	2.5 %	7	150/30	5.44 ± 0.54	5.94 ± 0.20	1.57 ± 0.12	1.50 ± 0.01	8.38 ± 0.57	7.89 ± 0.68

Table 18: Storage stability of prepared O/W emulsions. SSA and D(v,0.5) were determined immediately after preparation and after incubation at 4 °C for 9 days. Since no changes in emulsion parameters were observed with any of the tested emulsions, the experiment was conducted only once without replicates. Standard deviations originate from three consecutive measurements of the same emulsion.

#	Aqueous phase				Homogenization pressure (step 1/2) [bar]	SSA [m² g^{-1}]		D(v,0.5) [µm]	
	Buffer system	Solute	Concen-tration	pH		0 d	9 d	0 d	9 d
1	IPB	-	-	7	100/20	2.81 ± 0.17	2.79 ± 0.00	3.19 ± 0.04	3.19 ± 0.01
2	IPB	NaCl	0.5 %	7	100/20	3.16 ± 0.09	3.36 ± 0.00	2.84 ± 0.02	2.80 ± 0.00
3	IPB	NaCl	1.25 %	7	100/20	3.62 ± 0.30	3.32 ± 0.00	2.67 ± 0.05	2.73 ± 0.00
4	IPB	NaCl	2.5 %	7	100/20	3.47 ± 0.12	3.53 ± 0.00	2.66 ± 0.02	2.65 ± 0.01
5	IPB	NaCl	5 %	7	100/20	3.61 ± 0.21	3.34 ± 0.00	2.58 ± 0.04	2.64 ± 0.00
6	IPB	NaCl	12.5 %	7	100/20	3.68 ± 0.14	3.69 ± 0.01	2.47 ± 0.02	2.47 ± 0.00
7	IPB	NaCl	25 %	7	100/20	4.32 ± 0.28	4.11 ± 0.03	2.15 ± 0.04	2.17 ± 0.00
8	IPB	Sucrose	5 %	7	100/20	3.59 ± 0.06	3.61 ± 0.01	2.54 ± 0.01	2.55 ± 0.00
9	IPB	Sucrose	12.5 %	7	100/20	3.98 ± 0.09	3.89 ± 0.00	2.37 ± 0.02	2.41 ± 0.00
10	IPB	Sucrose	25 %	7	100/20	4.61 ± 0.00	4.55 ± 0.01	2.00 ± 0.00	2.04 ± 0.00
11	IPB	Sucrose	50 %	7	100/20	4.70 ± 0.02	4.73 ± 0.00	1.99 ± 0.00	2.01 ± 0.00
12	CPB	-	-	7	100/20	3.62 ± 0.01	3.66 ± 0.01	2.50 ± 0.00	2.51 ± 0.00
13	CPB	-	-	6	100/20	3.47 ± 0.02	3.52 ± 0.01	2.57 ± 0.00	2.55 ± 0.00
14	CPB	-	-	5	100/20	3.55 ± 0.02	3.69 ± 0.00	2.51 ± 0.00	2.51 ± 0.00
15	CPB	-	-	4	100/20	3.50 ± 0.00	3.65 ± 0.00	2.50 ± 0.00	2.49 ± 0.00
16	CPB	-	-	3	100/20	3.70 ± 0.00	3.63 ± 0.00	2.47 ± 0.00	2.49 ± 0.01
17	IPB	-	-	7	300/60	4.13 ± 0.06	4.36 ± 0.15	2.12 ± 0.01	2.10 ± 0.04
18	IPB	Peptone	2.5 %	7	200/40	5.56 ± 0.02	5.32 ± 0.01	1.69 ± 0.00	1.72 ± 0.00
19	IPB	Peptone	10 %	7	100/20	4.87 ± 0.01	4.85 ± 0.00	1.95 ± 0.00	1.97 ± 0.00
20	IPB	WPI	2.5 %	7	150/30	4.69 ± 0.01	4.77 ± 0.01	1.98 ± 0.00	1.98 ± 0.00
21	IPB	WPI	10 %	7	100/20	4.18 ± 0.00	4.12 ± 0.01	2.32 ± 0.00	2.34 ± 0.00
22	IPB	Casein	2.5 %	7	150/30	5.75 ± 0.00	5.87 ± 0.01	1.50 ± 0.00	1.50 ± 0.00

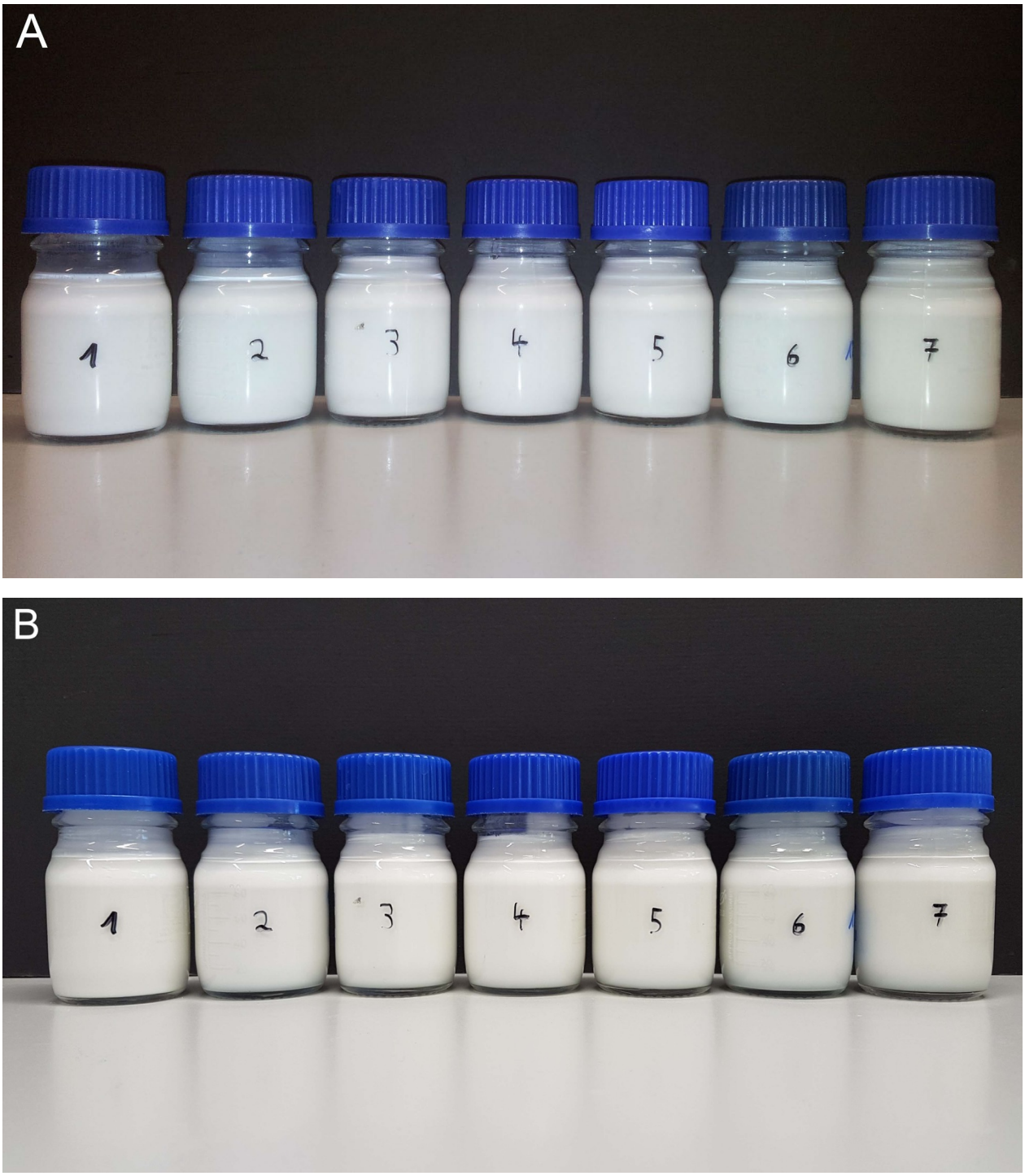

Figure 47: Visual inspection of O/W emulsions with varying NaCl concentration. O/W emulsions were inspected visually for phase separation and creaming immediately after preparation (A) and after storage at 4 °C for 9 d (B). Emulsions are numbered according to the NaCl concentration in the aqueous phase. Numbers in brackets (#) refer to the number coding in Tables 21 and 22: 1: 0 % (#1); 2: 0.5 % (#2); 3: 1.25 % (#3); 4: 2.5 % (#4); 5: 5 % (#5); 6: 12.5 % (#6); 7: 25 % (#7).

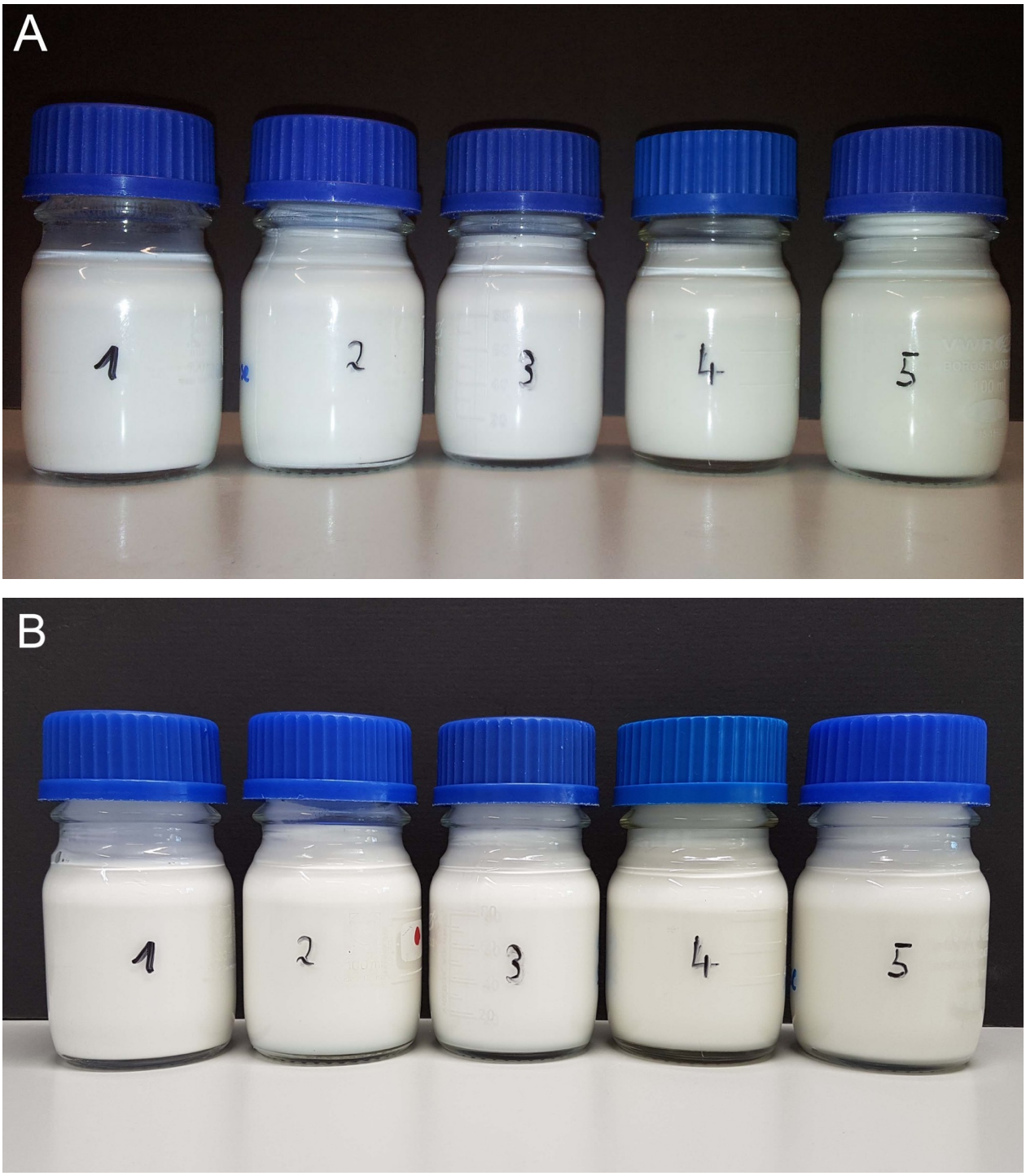

Figure 48: Visual inspection of O/W emulsions with varying sucrose concentration. O/W emulsions were inspected visually for phase separation and creaming immediately after preparation (A) and after storage at 4 °C for 9 d (B). Emulsions are numbered according to the sucrose concentration in the aqueous phase. Numbers in brackets (#) refer to the number coding in Tables 21 and 22: 1: 0 % (#1); 2: 5 % (#8); 3: 12.5 % (#9); 4: 25 % (#10); 5: 50 % (#11).

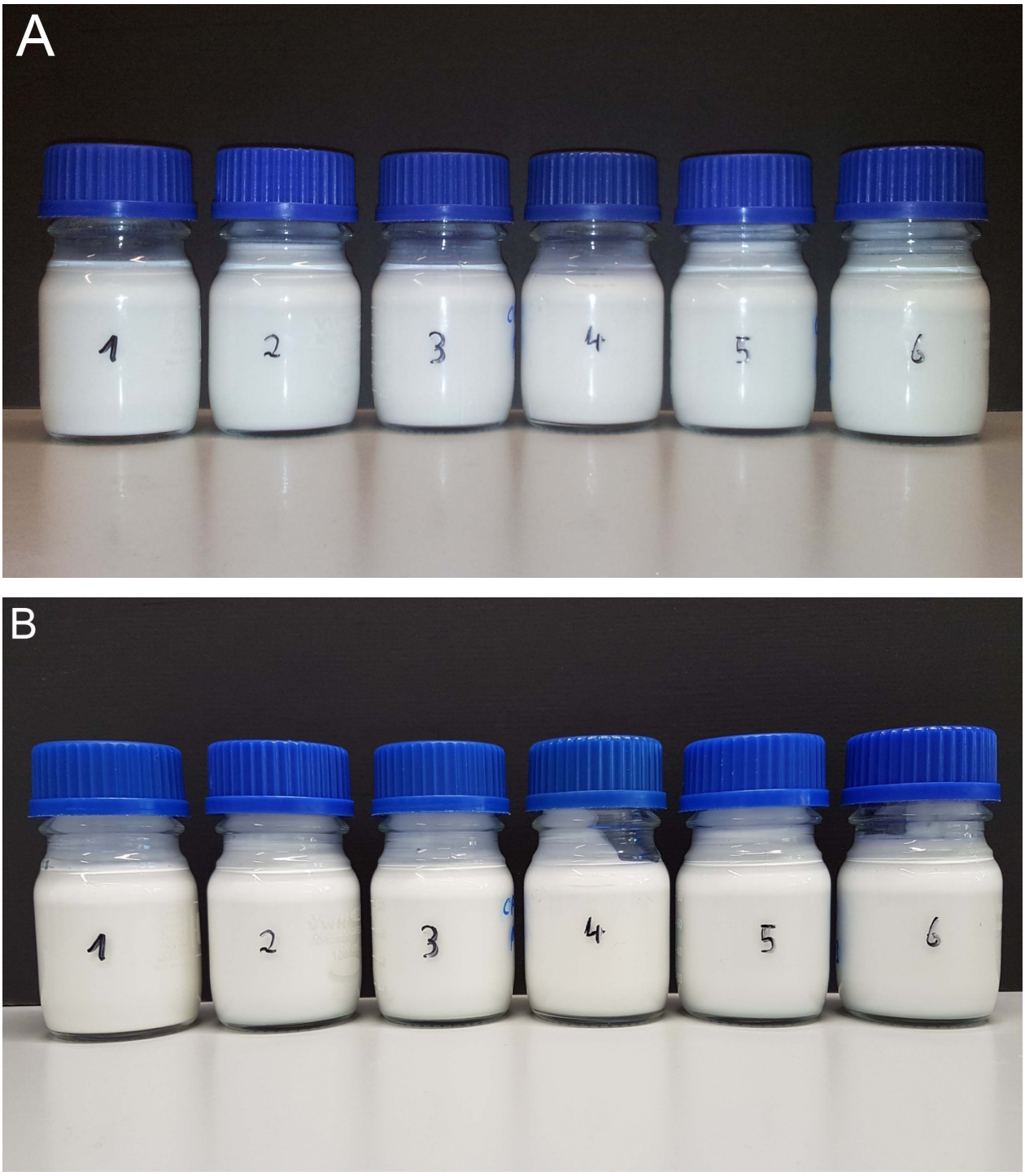

Figure 49: Visual inspection of O/W emulsions with varying pH value. O/W emulsions were inspected visually for phase separation and creaming immediately after preparation (A) and after storage at 4 °C for 9 d (B). Emulsions are numbered according to buffer solution and the pH value in the aqueous phase. Numbers in brackets (#) refer to the number coding in Tables 21 and 22: 1: IPB pH 7 (#1); 2: CPB pH 7 (#12); 3: CPB pH 6 (#13); 4: CPB pH 5 (#14); 5: CPB pH 4 (#15); 6: CPB pH 3 (#16).

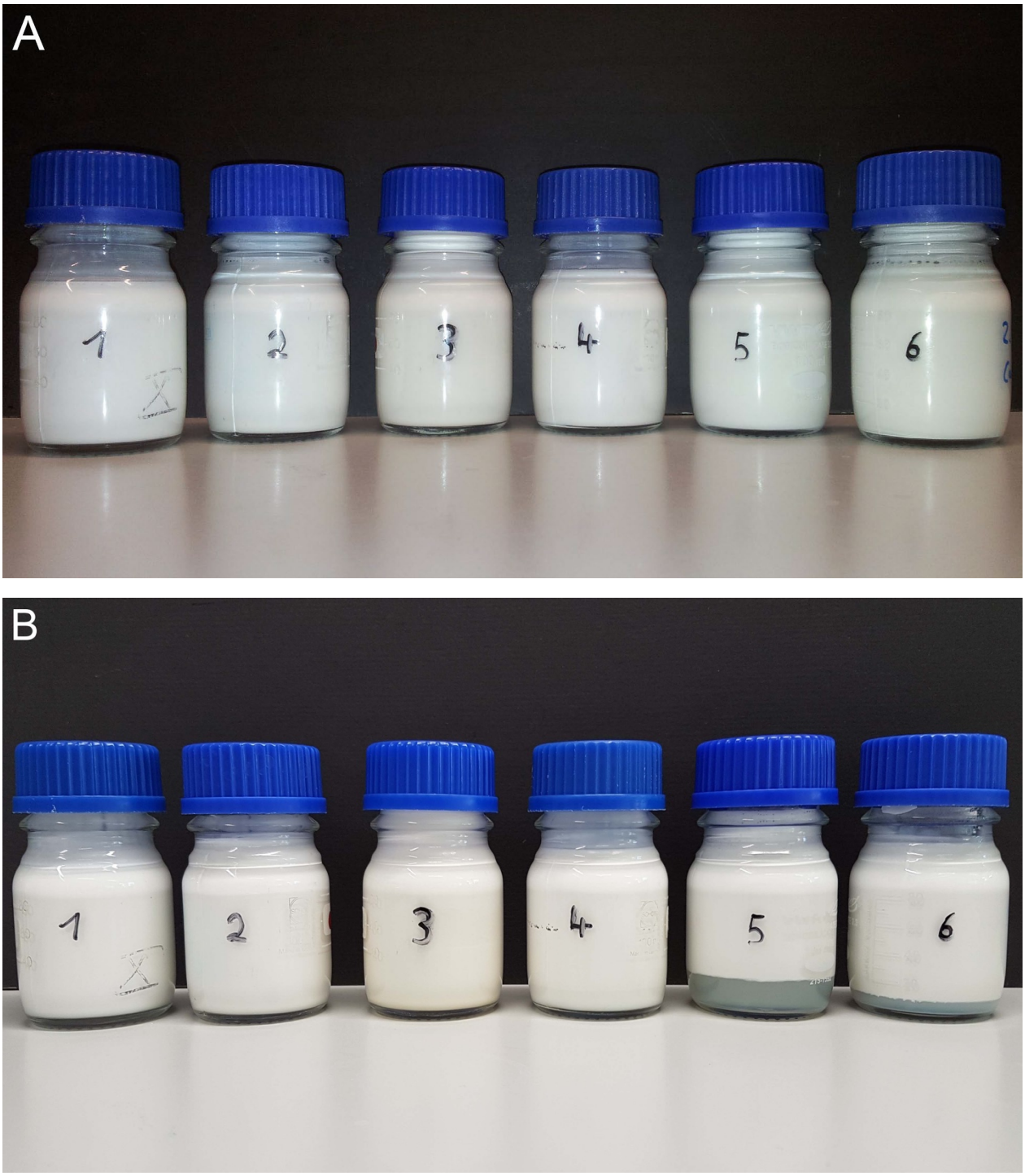

Figure 50: Visual inspection of O/W emulsions with protein type and content. O/W emulsions were inspected visually for phase separation and creaming immediately after preparation (A) and after storage at 4 °C for 9 d (B). Emulsions are numbered according to the protein type and concentration in the aqueous phase. Numbers in brackets (#) refer to the number coding in Tables 21 and 22: 1: 0 % protein (#17) 2: 2.5 % peptone (#18); 3: 10 % peptone (#19); 4: 2.5 % WPI (#20); 5: 10 % WPI (#21); 6: 2.5 % casein (#22).

Figure 51: Visual inspection of O/W emulsions with protein type and content stored at 4 °C for 9 d after manual agitation. After storage at 4 °C for 9 d (see Figure 50B) O/W emulsions agitated manually by gently inverting the flask and afterwards reevaluated for inspected visually for phase distribution. Emulsions are numbered according to the protein type and concentration in the aqueous phase. Numbers in brackets (#) refer to the number coding in Tables 21 and 22: 1: 0 % protein (#17) 2: 2.5 % peptone (#18); 3: 10 % peptone (#19); 4: 2.5 % WPI (#20); 5: 10 % WPI (#21); 6: 2.5 % casein (#22).

Table 19: Fatty acid composition of rapeseed oil. The fatty acid composition of the rapeseed oil used in this study as determined by VFG-Labor GmbH & Co. KG (Versmold, Germany).

Fatty acid group	Fatty acid	Number of C atoms : double bonds	Concentration [g/100 g]
Saturated fatty acids (SFA)	Butyric acid	4:0	< 0.03
	Caproic acid	6:0	< 0.03
	Caprylic acid	8:0	< 0.03
	Capric acid	10:0	< 0.03
	Undecanoic acid	11:0	< 0.03
	Lauric acid	12:0	< 0.03
	Myristic acid	14:0	0.07
	Pentadecanoic acid	15:0	< 0.03
	Palmitic acid	16:0	4.55
	Margaric acid	17:0	< 0.03
	Stearic acid	18:0	1.95
	Arachidic acid	20:0	0.55
	Behenic acid	22:0	0.27
	Lignoceric acid	24:0	0.05
Monounsaturated fatty acids (Mono-UFA)	Tridecenoic acid	13:1	< 0.03
	Myristoleic acid	14:1	< 0.03
	Pentadecenoic acid	15:1	< 0.03
	Palmitoleic acid	16:1	0.25
	Heptadecenoic acid	17:1	0.07
	Oleic acid	18:1	63.20
	Eicosenoic acid	20:1	1.40
	Erucic acid	22:1	0.30
	Nervonic acid	24:1	0.10
Polyunsaturated fatty acids (Poly-UFA)	Linoleic acid	18:2	19.76
	Linolenic acid	18:3	7.40
	Eicosadienic acid	20:2	0.09
	Eicosatrienoic acid	20:3	< 0.03
	Arachidonic acid	20:4	< 0.03
	Eicosapentaenoic acid	20:5	< 0.03
	Docosadienoic acid	22:2	< 0.03
	Docosahexaenoic acid	22:6	< 0.03
Total	SFA	-	7.44
	Mono-UFA	-	65.31
	Poly-UFA	-	27.25

Table 20: CSH of pre-selected *Lactobacillus* strains in exponential and stationary phase. MATH test was performed with cells harvested in exponential and stationary growth phase. The relative OD_{600} after agitation with n-hexadecane compared to the OD_{600} before agitation is shown. Presented values are the mean of at least three independent measurements.

Species	Strain	Relative OD_{600}	
		Exponential phase	Stationary phase
L. fructivorans	TMW 1.1856	60.3 ± 9.6	63.4 ± 6.9
	TMW 1.59	2.5 ± 0.8	6.2 ± 0.8
L. sakei	TMW 1.1239	90.6 ± 7.6	98.1 ± 2.8
	TMW 1.1474	97.9 ± 3.8	102.0 ± 4.8
	TMW 1.704	68.5 ± 8.7	70.2 ± 3.9
L. plantarum	TMW 1.1	98.0 ± 4.6	98.9 ± 1.8
	TMW 1.25	11.4 ± 5.8	7.4 ± 5.3
	TMW 1.277	9.5 ± 3.3	6.8 ± 4.2
	TMW 1.708	75.7 ± 1.9	93.1 ± 1.7

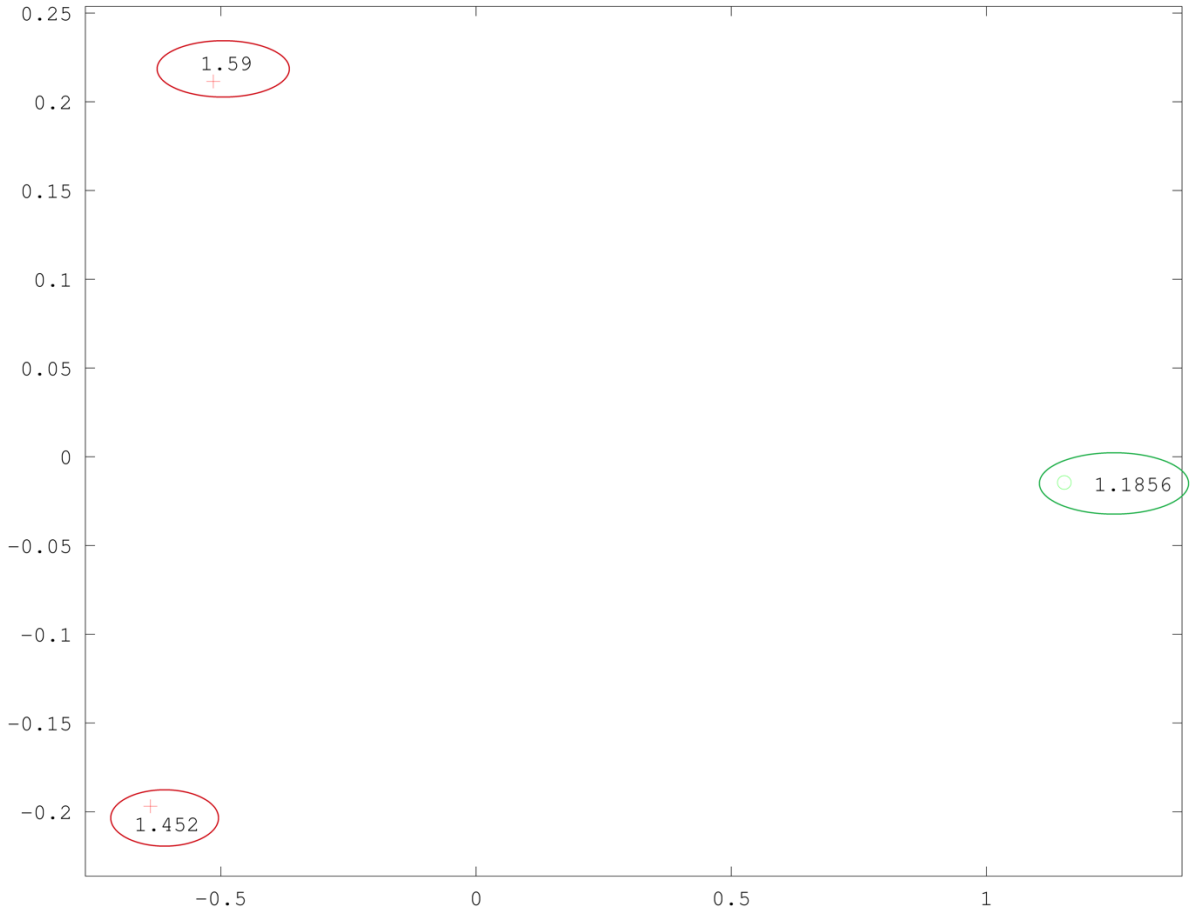

Figure 52: Multidimensional scaling of *L. fructivorans* strains according to MALDI-TOF MS mass spectra. Mass spectra of stationary phase cells of the three *L. fructivorans* strains were investigated concerning mutual distance by multidimensional scaling. Each strain is marked with a different symbol accompanied by its TMW number. The degree of CSH, as determined in 3.2.1.1, is indicated by colored markings corresponding to Figure 7 (red: highly hydrophobic; green: moderately hydrophilic).

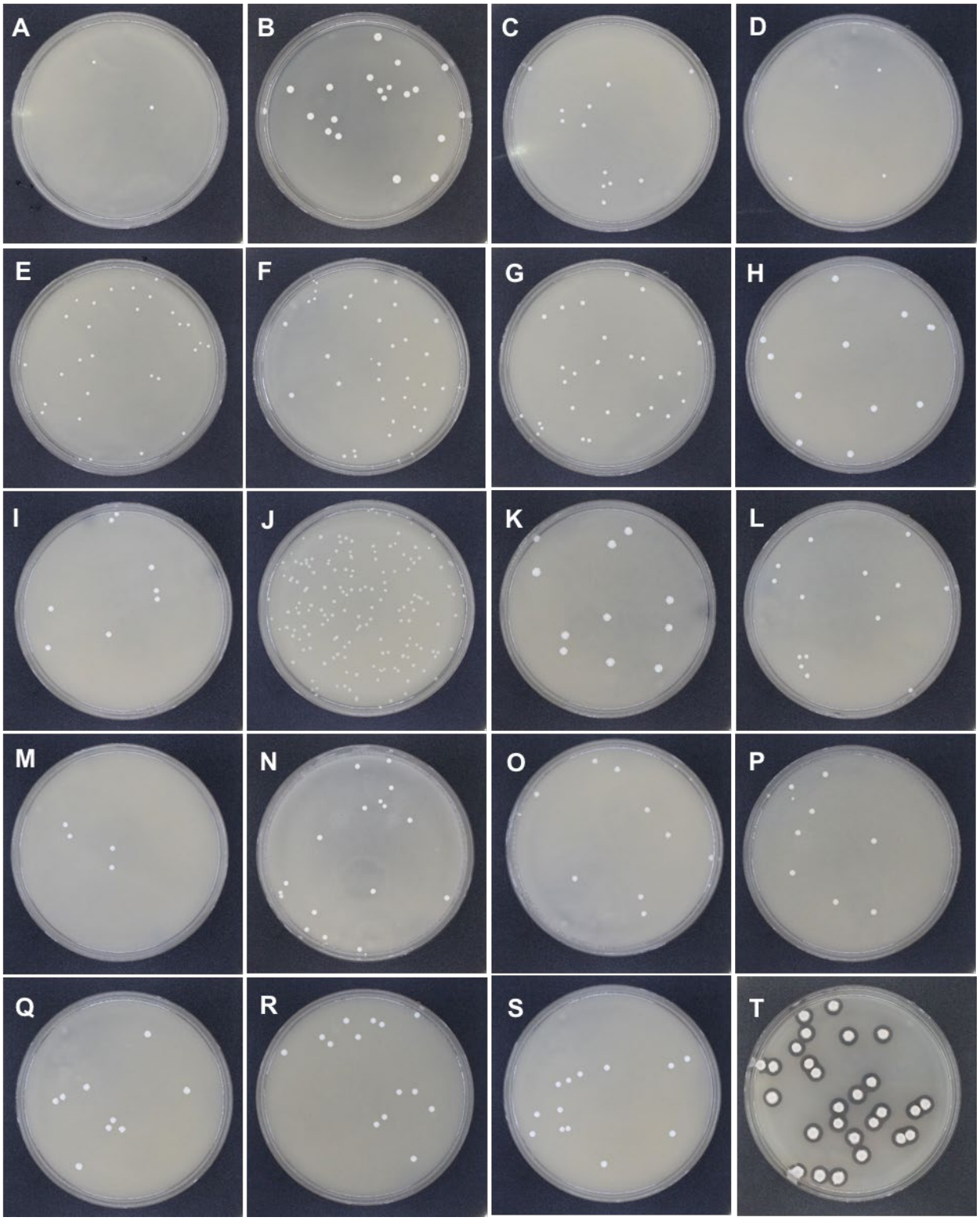

Figure 53: Bacterial growth on mMRS-TB agar. *L. fructivorans*: (A) TMW 1.59, (B) TMW 1.1856; *L. sakei*: (C) TMW 1.151, (D) TMW 1.704, (E) TMW 1.1239, (F) TMW 1.1322, (G) TMW 1.1474; *L. plantarum*: (H) TMW 1.25, (I) TMW 1.1, (J) TMW 1.1478, (K) TMW 1.277, (L) TMW 1.708, (M) TMW 1.834, (N) TMW 1.1623, (O) TMW 1.284*, (P) TMW 1.1810*, (Q) TMW 1.1204*, (R) TMW 1.2089*, (S) TMW 1.1808*; *B. subtilis*: (T) TMW 2.472. (*L. plantarum* strains marked with an asterisk (*) were not part of the initial selection but were included during the course of the study based on their CSH properties.)

Table 21: Growth test of selected *Lactobacillus* strains on Tween 80. *Lactobacillus* strains were suspended in pure IPB (negative control), IPB with 1.0 % (w/v) Tween 80 and mMRS medium (positive control), and incubated at 30 °C for 15 h. The OD_{600} was determined every 5 min over a period of 15 h. The initial OD_{600} and the maximum OD_{600} measured during incubation (max. OD_{600}) are presented.

Species	Strain	IPB		IPB + 1.0 % Tween 80		mMRS	
		Initial OD_{600}	max. OD_{600}	Initial OD_{600}	max. OD_{600}	Initial OD_{600}	max. OD_{600}
L. fructivorans	TMW 1.59	0.10 ± 0.02	0.14 ± 0.04	0.07 ± 0.02	0.11 ± 0.04	0.05 ± 0.03	0.45 ± 0.12
	TMW 1.1856	0.11 ± 0.04	0.12 ± 0.05	0.12 ± 0.02	0.17 ± 0.05	0.06 ± 0.03	0.85 ± 0.40
L. sakei	TMW 1.151	0.07 ± 0.05	0.09 ± 0.07	0.14 ± 0.03	0.16 ± 0.02	0.10 ± 0.02	3.14 ± 0.22
	TMW 1.704	0.13 ± 0.03	0.14 ± 0.02	0.13 ± 0.03	0.14 ± 0.02	0.10 ± 0.03	3.04± 0.15
	TMW 1.1239	0.19 ± 0.08	0.20 ± 0.09	0.17 ± 0.02	0.18 ± 0.03	0.11 ± 0.00	3.45 ± 0.14
	TMW 1.1322	0.17 ± 0.03	0.20± 0.02	0.13 ± 0.03	0.15 ± 0.02	0.12 ± 0.02	3.16 ± 0.38
	TMW 1.1474	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.03	0.16 ± 0.05	0.10 ± 0.01	3.82 ± 0.22
L. plantarum	TMW 1.1	0.24 ± 0.06	0.25 ± 0.06	0.22 ± 0.01	0.24 ± 0.01	0.17 ± 0.06	4.73 ± 0.05
	TMW 1.25	0.21 ± 0.03	0.22 ± 0.02	0.21 ± 0.01	0.21 ± 0.01	0.19 ± 0.04	4.12 ± 0.04
	TMW 1.277	0.19 ± 0.01	0.21 ± 0.03	0.26 ± 0.03	0.30 ± 0.04	0.22 ± 0.03	4.26 ± 0.08
	TMW 1.284*	0,19 ± 0,02	0.21 ± 0.04	0,19 ± 0,02	0.28 ± 0.06	0,14 ± 0,04	2.99 ± 0.07
	TMW 1.708	0.26 ± 0.04	0.29 ± 0.01	0.23 ± 0.02	0.39 ± 0.21	0.21 ± 0.01	4.08 ± 0.08
	TMW 1.834	0.22 ± 0.01	0.23 ± 0.02	0.26 ± 0.02	0.34 ± 0.11	0.24 ± 0.12	4.13 ± 0.08
	TMW 1.1204*	0,21 ± 0,04	0.24 ± 0.01	0,27 ± 0,03	0.44 ± 0.17	0,21 ± 0,04	4.71 ± 0.06
	TMW 1.1478	0.20 ± 0.04	0.22 ± 0.07	0.24 ± 0.03	0.29 ± 0.02	0.16 ± 0.03	5.13 ± 0.03
	TMW 1.1623	0.17 ± 0.04	0.19 ± 0.04	0.23 ± 0.01	0.28 ± 0.03	0.16 ± 0.02	4.38 ± 0.14
	TMW 1.1808*	0,24 ± 0,02	0.21 ± 0.02	0,30 ± 0,05	0.90 ± 0.83	0,14 ± 0,10	4.30 ± 0.44
	TMW 1.1810*	0,20 ± 0,00	0.26 ± 0.03	0,18 ± 0,01	0.23 ± 0.02	0,16 ± 0,06	4.04 ± 0.07
	TMW 1.2089*	0,22 ± 0,02	0.25 ± 0.01	0,25 ± 0,05	0.53 ± 0.34	0,15 ± 0,04	4.50 ± 0.31

*Strain was not part of the initial selection but was included during the course of the study based on its CSH properties.

Table 22: Proteolytic activity of pre-selected *Lactobacillus* strains. Stationary phase cells were suspended in IPB containing FTC casein and incubated for 10 h at 25 °C. Fluorescence intensity (FI) was measured every 5 min during incubation using a filter set with 485/520 nm (excitation/emission) and a gain of 300. Proteolytically active *B. subtilis* strain TMW 2.472 and TPCK trypsin were used as biological and chemical positive controls, respectively. IPB containing FTC casein was used as negative control. Pure IPB without FTC casein was used as blank and values were subtracted from all measurement values for correction. FI_{max}: maximum FI value recorded during incubation; ΔFI_{max}: maximum increase in FI (Data were smoothed by calculating the mean of three subsequent values in order to correct for random fluctuations.)

Sample	FI_{max} [AU]	ΔFI_{max} [AU]
L. fructivorans TMW 1.59	930 ± 35	32 ± 4
L. fructivorans TMW 1.1856	932 ± 26	31 ± 4
L. sakei TMW 1.151	912 ± 27	31 ± 6
L. sakei TMW 1.704	881 ± 37	28 ± 4
L. sakei TMW 1.1239	904 ± 23	32 ± 7
L. sakei TMW 1.1322	907 ± 29	31 ± 8
L. sakei TMW 1.1474	917 ± 29	28 ± 5
L. plantarum TMW 1.1	914 ± 45	29 ± 5
L. plantarum TMW 1.25	923 ± 35	34 ± 9
L. plantarum TMW 1.277	916 ± 41	31 ± 5
L. plantarum TMW 1.284*	896 ± 16	30 ± 4
L. plantarum TMW 1.708	915 ± 38	32 ± 7
L. plantarum TMW 1.834	916 ± 44	33 ± 7
L. plantarum TMW 1.1204*	901 ± 23	28 ± 5
L. plantarum TMW 1.1478	895 ± 57	33 ± 7
L. plantarum TMW 1.1623	895 ± 14	34 ± 4
L. plantarum TMW 1.1808*	900 ± 28	32 ± 9
L. plantarum TMW 1.1810*	913 ± 14	93 ± 7
L. plantarum TMW 1.2089*	893 ± 50	27 ± 5
B. subtilis TMW 2.472	1563 ± 49	234 ± 48
Trypsin (0 µg mL^{-1})	884 ± 36	29 ± 7
Trypsin (5 µg mL^{-1})	1730 ± 139	366 ± 152
Trypsin (10 µg mL^{-1})	1942 ± 89	739 ± 111

*Strain was not part of the initial selection but was included during the course of the study based on its CSH properties.

Table 23: Growth test of selected *Lactobacillus* strains on peptone. *Lactobacillus* strains were suspended in pure IPB (negative control), IPB with 2.5 % (w/v), IPB with 10.0 % (w/v) peptone from casein and mMRS medium (positive control), and incubated at 30 °C for 6 h. The OD_{600} was determined every 5 min over a period of 6 h. The initial OD_{600} and the maximum OD_{600} measured during incubation (max. OD_{600}) are presented.

Species	Strain	IPB		IPB + 2.5 % peptone		IPB + 10.0 % peptone		mMRS	
		initial OD_{600}	max. OD_{600}	initial OD_{600}	max. OD_{600}	initial OD_{600}	max. OD_{600}	initial OD_{600}	max. OD_{600}
L. fructivorans	TMW 1.59	0.11 ± 0.03	0.11 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	0.06 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.20 ± 0.06
	TMW 1.1856	0.06 ± 0.02	0.08 ± 0.02	0.09 ± 0.04	0.11 ± 0.05	0.05 ± 0.03	0.09 ± 0.03	0.08 ± 0.02	0.23 ± 0.09
L. sakei	TMW 1.151	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.20 ± 0.07	0.06 ± 0.01	0.22 ± 0.06	0.10 ± 0.02	3.14 ± 0.22
	TMW 1.704	0.09 ± 0.02	0.10 ± 0.00	0.10 ± 0.01	0.28 ± 0.02	0.06 ± 0.00	0.29 ± 0.05	0.10 ± 0.03	3.04 ± 0.15
	TMW 1.1239	0.06 ± 0.01	0.09 ± 0.00	0.10 ± 0.00	0.30 ± 0.05	0.05 ± 0.00	0.29 ± 0.05	0.11 ± 0.00	3.45 ± 0.14
	TMW 1.1322	0.05 ± 0.03	0.08 ± 0.02	0.11 ± 0.03	0.27 ± 0.05	0.05 ± 0.01	0.20 ± 0.02	0.12 ± 0.02	3.16 ± 0.38
	TMW 1.1474	0.05 ± 0.02	0.08 ± 0.00	0.09 ± 0.00	0.15 ± 0.01	0.05 ± 0.00	0.14 ± 0.02	0.10 ± 0.01	3.82 ± 0.22
L. plantarum	TMW 1.1	0.12 ± 0.06	0.17 ± 0.02	0.18 ± 0.02	0.45 ± 0.01	0.10 ± 0.01	0.45 ± 0.02	0.15 ± 0.01	1.63 ± 0.18
	TMW 1.25	0.12 ± 0.04	0.18 ± 0.03	0.16 ± 0.02	0.40 ± 0.02	0.09 ± 0.02	0.38 ± 0.03	0.15 ± 0.01	1.43 ± 0.21
	TMW 1.277	0.09 ± 0.03	0.17 ± 0.03	0.17 ± 0.03	0.41 ± 0.02	0.10 ± 0.01	0.36 ± 0.02	0.15 ± 0.03	1.37 ± 0.22
	TMW 1.284*	0.07 ± 0.04	0.10 ± 0.02	0.11 ± 0.02	0.16 ± 0.02	0.07 ± 0.00	0.12 ± 0.02	0.11 ± 0.03	0.44 ± 0.08
	TMW 1.708	0.13 ± 0.04	0.15 ± 0.02	0.14 ± 0.03	0.38 ± 0.02	0.10 ± 0.01	0.36 ± 0.01	0.13 ± 0.02	1.25 ± 0.15
	TMW 1.834	0.16 ± 0.06	0.18 ± 0.03	0.19 ± 0.04	0.25 ± 0.02	0.12 ± 0.01	0.24 ± 0.00	0.18 ± 0.00	1.32 ± 0.14
	TMW 1.1204*	0.16 ± 0.02	0.23 ± 0.03	0.20 ± 0.01	0.56 ± 0.01	0.12 ± 0.01	0.53 ± 0.04	0.18 ± 0.03	1.94 ± 0.16
	TMW 1.1478	0.12 ± 0.05	0.16 ± 0.03	0.18 ± 0.02	0.52 ± 0.02	0.12 ± 0.02	0.51 ± 0.00	0.17 ± 0.00	2.32 ± 0.08
	TMW 1.1623	0.11 ± 0.05	0.16 ± 0.01	0.16 ± 0.05	0.48 ± 0.01	0.10 ± 0.01	0.50 ± 0.04	0.18 ± 0.03	1.97 ± 0.26
	TMW 1.1808*	0.16 ± 0.07	0.22 ± 0.03	0.23 ± 0.02	0.65 ± 0.14	0.14 ± 0.01	0.48 ± 0.02	0.19 ± 0.01	1.85 ± 0.21
	TMW 1.1810*	0.09 ± 0.03	0.14 ± 0.02	0.13 ± 0.03	0.48 ± 0.03	0.09 ± 0.01	0.48 ± 0.05	0.12 ± 0.01	1.38 ± 0.12
	TMW 1.2089*	0.10 ± 0.01	0.20 ± 0.02	0.18 ± 0.01	0.49 ± 0.01	0.11 ± 0.01	0.40 ± 0.08	0.15 ± 0.01	1.64 ± 0.19

*Strain was not part of the initial selection but was included during the course of the study based on its CSH properties.

Table 24: Growth test of selected *Lactobacillus* strains on sucrose. *Lactobacillus* strains were suspended in pure IPB (negative control), IPB with 5 %, 12.5 %, 25 % or 50 % (w/v) sucrose and mMRS medium (positive control), and incubated at 30 °C for 6 h. The OD_{600} was determined every 5 min over a period of 6 h. The initial OD_{600} and the maximum OD_{600} measured during incubation (max. OD_{600}) are presented.

Species	Strain	OD_{600} meas.	IPB	IPB + 5 % sucrose	IPB + 12.5 % sucrose	IPB + 25 % sucrose	IPB + 50 % sucrose	mMRS
L. fructivorans	TMW 1.59	initial	0.11 ± 0.03	0.10± 0.03	0.09± 0.01	0.05± 0.00	0.01± 0.00	0.09± 0.01
		max.	0.11 ± 0.02	0.11± 0.03	0.10± 0.01	0.06± 0.00	0.05± 0.00	0.20 ± 0.06
	TMW 1.1856	initial	0.06 ± 0.02	0.08± 0.03	0.07± 0.02	0.04± 0.02	0.03± 0.04	0.08± 0.02
		max.	0.08 ± 0.02	0.09± 0.03	0.08± 0.03	0.05± 0.02	0.08 ± 0.08	0.23 ± 0.09
L. sakei	TMW 1.151	initial	0.11 ± 0.01	0.10 ± 0.00	0.09 ± 0.01	0.05 ± 0.01	0.01 ± 0.00	0.10 ± 0.02
		max.	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.09 ± 0.00	0.06 ± 0.00	3.14 ± 0.22
	TMW 1.704	initial	0.09 ± 0.02	0.08 ± 0.01	0.01 ± 0.01	0.04 ± 0.00	0.01 ± 0.00	0.10 ± 0.03
		max.	0.10 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.06 ± 0.00	0.05 ± 0.01	3.04 ± 0.15
	TMW 1.1239	initial	0.06 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.04 ± 0.02	0.01 ± 0.00	0.11 ± 0.00
		max.	0.09 ± 0.00	0.09 ± 0.00	0.08 ± 0.01	0.05 ± 0.03	0.04 ± 0.00	3.45 ± 0.14
	TMW 1.1322	initial	0.05 ± 0.03	0.09 ± 0.01	0.09 ± 0.00	0.05 ± 0.01	0.01 ± 0.00	0.12 ± 0.02
		max.	0.08 ± 0.02	0.10 ± 0.01	0.11 ± 0.02	0.07 ± 0.01	0.05 ± 0.01	3.16 ± 0.38
	TMW 1.1474	initial	0.05 ± 0.02	0.08 ± 0.01	0.07 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.10 ± 0.01
		max.	0.08 ± 0.00	0.09 ± 0.01	0.09 ± 0.01	0.13 ± 0.10	0.03 ± 0.00	3.82 ± 0.22
L. plantarum	TMW 1.1	initial	0.12 ± 0.06	0.17 ± 0.01	0.13 ± 0.02	0.07 ± 0.00	0.01 ± 0.00	0.15 ± 0.01
		max.	0.17 ± 0.02	0.19 ± 0.00	0.17 ± 0.00	0.11 ± 0.00	0.08 ± 0.02	1.63 ± 0.18
	TMW 1.25	initial	0.12 ± 0.04	0.15 ± 0.01	0.13 ± 0.01	0.06 ± 0.01	0.02 ± 0.01	0.15 ± 0.01
		max.	0.18 ± 0.03	0.18 ± 0.02	0.16 ± 0.02	0.10 ± 0.00	0.04 ± 0.00	1.43 ± 0.21
	TMW 1.277	initial	0.09 ± 0.03	0.14 ± 0.02	0.13 ± 0.01	0.06 ± 0.02	0.01 ± 0.01	0.15 ± 0.03
		max.	0.17 ± 0.03	0.18 ± 0.01	0.17 ± 0.02	0.01 ± 0.00	0.04 ± 0.00	1.37 ± 0.22
	TMW 1.284*	initial	0.07 ± 0.04	0.12 ± 0.02	0.09 ± 0.01	0.04 ± 0.00	0.01 ± 0.00	0.11 ± 0.03
		max.	0.10 ± 0.02	0.12 ± 0.02	0.10 ± 0.01	0.07 ± 0.01	0.04 ± 0.02	0.44 ± 0.08
	TMW 1.708	initial	0.13 ± 0.04	0.14 ± 0.01	0.11 ± 0.02	0.06 ± 0.00	0.02 ± 0.00	0.13 ± 0.02
		max.	0.15 ± 0.02	0.17 ± 0.01	0.15 ± 0.02	0.11 ± 0.01	0.05 ± 0.00	1.25 ± 0.15
	TMW 1.834	initial	0.16 ± 0.06	0.20 ± 0.01	0.16 ± 0.01	0.08 ± 0.01	0.02 ± 0.00	0.18 ± 0.00
		max.	0.18 ± 0.03	0.21 ± 0.01	0.18 ± 0.01	0.12 ± 0.00	0.07 ± 0.02	1.32 ± 0.14
	TMW 1.1204*	initial	0.16 ± 0.02	0.17 ± 0.02	0.15 ± 0.01	0.07 ± 0.00	0.03 ± 0.00	0.18 ± 0.03
		max.	0.23 ± 0.03	0.22 ± 0.01	0.20 ± 0.02	0.13 ± 0.01	0.04 ± 0.00	1.94 ± 0.16
	TMW 1.1478	initial	0.12 ± 0.05	0.17 ± 0.00	0.12 ± 0.01	0.06 ± 0.01	0.01 ± 0.01	0.17 ± 0.00
		max.	0.16 ± 0.03	0.20 ± 0.01	0.18 ± 0.00	0.11 ± 0.01	0.10 ± 0.08	2.32 ± 0.08
	TMW 1.1623	initial	0.11 ± 0.05	0.16 ± 0.01	0.12 ± 0.01	0.06 ± 0.00	0.02 ± 0.00	0.18 ± 0.03
		max.	0.16 ± 0.01	0.19 ± 0.01	0.17 ± 0.02	0.11 ± 0.01	0.07 ± 0.04	1.97 ± 0.26
	TMW 1.1808*	initial	0.16 ± 0.07	0.02 ± 0.01	0.17 ± 0.02	0.09 ± 0.01	0.02 ± 0.01	0.19 ± 0.01
		max.	0.22 ± 0.03	0.23 ± 0.00	0.21 ± 0.03	0.15 ± 0.00	0.06 ± 0.00	1.85 ± 0.21
	TMW 1.1810*	initial	0.09 ± 0.03	0.12 ± 0.02	0.10 ± 0.01	0.05 ± 0.01	0.02 ± 0.00	0.12 ± 0.01
		max.	0.14 ± 0.02	0.14 ± 0.01	0.13 ± 0.01	0.08 ± 0.02	0.04 ± 0.00	1.38 ± 0.12

Species	Strain	OD_{600} meas.	IPB	IPB + 5 % sucrose	IPB + 12.5 % sucrose	IPB + 25 % sucrose	IPB + 50 % sucrose	mMRS
	TMW 1.2089*	initial	0.10 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.07 ± 0.01	0.01 ± 0.01	0.15 ± 0.01
		max.	0.20 ± 0.02	0.20 ± 0.01	0.18 ± 0.01	0.14 ± 0.01	0.07 ± 0.02	1.64 ± 0.19

*Strain was not part of the initial selection but was included during the course of the study based on its CSH properties.

Table 25: Growth test of selected *Lactobacillus* strains on mixtures of Tween 80 with sucrose and peptone. *Lactobacillus* strains were suspended in pure IPB (negative control), IPB with 5 %, 12.5 %, 25 % or 50 % (w/v) sucrose and mMRS medium (positive control), and incubated at 30 °C for 6 h. The OD_{600} was determined every 5 min over a period of 6 h. The initial OD_{600} and the maximum OD_{600} measured during incubation (max. OD_{600}) are presented.

Species	Strain	OD_{600} meas.	IPB	IPB + 1 % T80 + 5 % sucrose	IPB + 1 % T80 + 12.5 % sucrose	IPB + 1 % T80 + 25 % sucrose	IPB + 1 % T80 + 50 % sucrose	IPB + 1 % T80 + 2.5 % peptone	IPB + 1 % T80 + 10.0 % peptone	mMRS
L. fructivorans	TMW 1.59	initial	0.08 ± 0.03	0.05 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.08 ± 0.02	0.04 ± 0.01	0.07 ± 0.02
		max.	0.11 ± 0.01	0.08 ± 0.02	0.07 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.10 ± 0.03	0.08 ± 0.02	0.16 ± 0.10
	TMW 1.1856	initial	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
		max.	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.10 ± 0.05
L. sakei	TMW 1.151	initial	0.06 ± 0.03	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	0.07 ± 0.02	0.04 ± 0.01	0.06 ± 0.00
		max.	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.02	0.06 ± 0.01	0.04 ± 0.01	0.20 ± 0.13	0.18 ± 0.14	0.86 ± 0.65
	TMW 1.704	initial	0.06 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.08 ± 0.02	0.05 ± 0.02	0.07 ± 0.01
		max.	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.02 ± 0.00	0.32 ± 0.12	0.32 ± 0.15	1.67 ± 0.70
	TMW 1.1239	initial	0.05 ± 0.02	0.05 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.07 ± 0.01	0.04 ± 0.00	0.06 ± 0.01
		max.	0.08 ± 0.00	0.07 ± 0.00	0.06 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.36 ± 0.17	0.32 ± 0.15	1.67 ± 0.91
	TMW 1.1322	initial	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.08 ± 0.02	0.05 ± 0.01	0.08 ± 0.01
		max.	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.03 ± 0.01	0.27 ± 0.13	0.23 ± 0.12	1.00 ± 0.56
	TMW 1.1474	initial	0.06 ± 0.03	0.05 ± 0.02	0.04 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.07 ± 0.01	0.04 ± 0.01	0.05 ± 0.02
		max.	0.09 ± 0.02	0.09 ± 0.02	0.07 ± 0.01	0.05 ± 0.01	0.03 ± 0.00	0.12 ± 0.02	0.14 ± 0.05	0.38 ± 0.08
L. plantarum	TMW 1.1	initial	0.08 ± 0.02	0.10 ± 0.02	0.06 ± 0.02	0.03 ± 0.01	0.01 ± 0.00	0.15 ± 0.02	0.08 ± 0.01	0.12 ± 0.03
		max.	0.12 ± 0.03	0.16 ± 0.03	0.12 ± 0.02	0.09 ± 0.01	0.04 ± 0.00	0.40 ± 0.05	0.47 ± 0.20	1.72 ± 1.08
	TMW 1.25	initial	0.13 ± 0.06	0.09 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.01 ± 0.00	0.14 ± 0.01	0.08 ± 0.00	0.15 ± 0.02
		max.	0.17 ± 0.04	0.16 ± 0.00	0.13 ± 0.00	0.10 ± 0.01	0.03 ± 0.00	0. 42 ± 0.01	0.38 ± 0.03	1.16 ± 0.07
	TMW 1.277	initial	0.10 ± 0.03	0.08 ± 0.01	0.06 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.15 ± 0.01	0.09 ± 0.00	0.23 ± 0.08
		max.	0.16 ± 0.04	0.17 ± 0.01	0.14 ± 0.00	0.10 ± 0.01	0.04 ± 0.00	0.39 ± 0.04	0.36 ± 0.04	1.38 ± 0.23

Spe-cies	Strain	OD_{600} meas.	IPB	IPB + 1 % T80 + 5 % sucrose	IPB + 1 % T80 + 12.5 % sucrose	IPB + 1 % T80 + 25 % sucrose	IPB + 1 % T80 + 50 % sucrose	IPB + 1 % T80 + 2.5 % peptone	IPB + 1 % T80 + 10.0 % peptone	mMRS
L. plantarum	TMW 1.284*	initial	0.09 ± 0.04	0.06 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	0.11 ± 0.00	0.06 ± 0.01	0.10 ± 0.01
		max.	0.13 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.07 ± 0.01	0.02 ± 0.01	0.18 ± 0.01	0.12 ± 0.01	0.37 ± 0.05
	TMW 1.708	initial	0.11 ± 0.02	0.10 ± 0.02	0.06 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.17 ± 0.02	0.08 ± 0.03	0.16 ± 0.02
		max.	0.15 ± 0.04	0.18 ± 0.01	0.15 ± 0.01	0.11 ± 0.01	0.05 ± 0.01	0.42 ± 0.03	0.39 ± 0.01	1.21 ± 0.08
	TMW 1.834	initial	0.15 ± 0.11	0.12 ± 0.00	0.08 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.17 ± 0.02	0.11 ± 0.01	0.17 ± 0.02
		max.	0.20 ± 0.09	0.19 ± 0.01	0.16 ± 0.01	0.10 ± 0.01	0.05 ± 0.00	0.24 ± 0.02	0.23 ± 0.01	1.02 ± 0.14
	TMW 1.1204*	initial	0.16 ± 0.07	0.10 ± 0.01	0.08 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.19 ± 0.04	0.10 ± 0.01	0.18 ± 0.01
		max.	0.21 ± 0.04	0.20 ± 0.01	0.16 ± 0.02	0.11 ± 0.00	0.04 ± 0.00	0.67 ± 0.17	0.53 ± 0.06	1.65 ± 0.09
	TMW 1.1478	initial	0.15 ± 0.08	0.10 ± 0.02	0.06 ± 0.02	0.04 ± 0.01	0.01 ± 0.01	0.12 ± 0.04	0.07 ± 0.02	0.08 ± 0.03
		max.	0.16 ± 0.09	0.17 ± 0.04	0.13 ± 0.03	0.09 ± 0.02	0.03 ± 0.01	0.43 ± 0.08	0.54 ± 0.31	1.60 ± 0.21
	TMW 1.1623	initial	0.18 ± 0.09	0.09 ± 0.01	0.06 ± 0.02	0.04 ± 0.01	0.02 ± 0.01	0.15 ± 0.01	0.08 ± 0.03	0.15 ± 0.01
		max.	0.21 ± 0.06	0.18 ± 0.01	0.14 ± 0.01	0.10 ± 0.00	0.05 ± 0.01	0.54 ± 0.10	0.49 ± 0.04	1.54 ± 0.16
	TMW 1.1808*	initial	0.19 ± 0.09	0.14 ± 0.03	0.09 ± 0.02	0.04 ± 0.01	0.01 ± 0.00	0.19 ± 0.03	0.11 ± 0.04	0.17 ± 0.00
		max.	0.23 ± 0.07	0.20 ± 0.01	0.18 ± 0.01	0.13 ± 0.02	0.05 ± 0.01	0.54 ± 0.01	0.40 ± 0.02	1.40 ± 0.03
	TMW 1.1810*	initial	0.07 ± 0.02	0.08 ± 0.01	0.06 ± 0.01	0.03 ± 0.00	0.01 ± 0.00	0.12 ± 0.03	0.07 ± 0.01	0.11 ± 0.01
		max.	0.11 ± 0.01	0.20 ± 0.06	0.14 ± 0.03	0.09 ± 0.01	0.05 ± 0.00	0.48 ± 0.02	0.44 ± 0.06	1.10 ± 0.09
	TMW 1.2089*	initial	0.11 ± 0.04	0.10 ± 0.01	0.07 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.16 ± 0.03	0.09 ± 0.01	0.18 ± 0.02
		max.	0.19 ± 0.06	0.20 ± 0.02	0.18 ± 0.01	0.12 ± 0.00	0.06 ± 0.03	0.49 ± 0.02	0.35 ± 0.04	1.43 ± 0.19

*Strain was not part of the initial selection but was included during the course of the study based on its CSH properties.

Table 26: Growth parameters of pre-selected *Lactobacillus* strains under variation of the pH. Growth curves in mMRS broth were recorded and from these the growth parameters OD_{max} (maximum optical density measured at 600 nm) and μ_{max} (maximum growth rate) were calculated. Presented values are mean values of at least three replicates ± standard deviation.

Species	Strain	OD_{max}				μ_{max} [h^{-1}]			
		pH 3.5	pH 4.0	pH 4.5	pH 6.2	pH 3.5	pH 4.0	pH 4.5	pH 6.2
L. fructivorans	TMW 1.59	0.06 ± 0.07	0.46 ± 0.20	0.95 ± 0.34	1.06 ± 0.26	0.06 ± 0.05	0.23 ± 0.12	0.48 ± 0.23	0.44 ± 0.24
	TMW 1.1856	0.96 ± 0.03	1.37 ± 0.02	1.34 ± 0.05	1.24 ± 0.02	0.14 ± 0.02	0.26 ± 0.04	0.24 ± 0.01	0.23 ± 0.05
L. sakei	TMW 1.151	0.06 ± 0.03	0.06 ± 0.03	0.71 ± 0.19	0.71 ± 0.18	0.05 ± 0.03	0.09 ± 0.05	0.20 ± 0.06	0.30 ± 0.13
	TMW 1.704	0.73 ± 0.47	0.42 ± 0.51	1.06 ± 0.30	1.01 ± 0.29	0.16 ± 0.02	0.23 ± 0.04	0.26 ± 0.03	0.49 ± 0.15
	TMW 1.1239	0.80 ± 0.52	0.85 ± 0.52	1.10 ± 0.33	1.28 ± 0.12	0.15 ± 0.02	0.25 ± 0.03	0.40 ± 0.06	0.43 ± 0.01
	TMW 1.1322	0.71 ± 0.44	0.92 ± 0.58	1.06 ± 0.56	1.32 ± 0.26	0.16 ± 0.02	0.25 ± 0.08	0.37 ± 0.10	0.54 ± 0.08
	TMW 1.1474	0.03 ± 0.01	0.04 ± 0.02	0.74 ± 0.17	1.02 ± 0.03	0.00 ± 0.00	0.00 ± 0.01	0.24 ± 0.05	0.35 ± 0.03
L. plantarum	TMW 1.1	0.24 ± 0.10	0.54 ± 0.14	1.23 ± 0.07	1.33 ± 0.04	0.30 ± 0.02	0.39 ± 0.02	0.44 ± 0.01	0.53 ± 0.04
	TMW 1.25	0.46 ± 0.22	1.06 ± 0.03	1.17 ± 0.05	1.29 ± 0.03	0.09 ± 0.01	0.30 ± 0.02	0.38 ± 0.02	0.50 ± 0.01
	TMW 1.277	0.47 ± 0.20	1.12 ± 0.01	1.23 ± 0.05	1.40 ± 0.04	0.10 ± 0.01	0.30 ± 0.01	0.41 ± 0.04	0.54 ± 0.05
	TMW 1.708	0.76 ± 0.10	1.21 ± 0.01	1.26 ± 0.09	1.28 ± 0.02	0.19 ± 0.00	0.32 ± 0.02	0.37 ± 0.01	0.47 ± 0.03
	TMW 1.834	0.70 ± 0.12	1.09 ± 0.03	1.05 ± 0.06	1.12 ± 0.04	0.11 ± 0.00	0.24 ± 0.02	0.34 ± 0.05	0.44 ± 0.02
	TMW 1.1478	0.65 ± 0.24	1.15 ± 0.03	1.20 ± 0.02	1.42 ± 0.02	0.26 ± 0.01	0.38 ± 0.04	0.53 ± 0.05	0.58 ± 0.01
	TMW 1.1623	1.01 ± 0.05	1.21 ± 0.05	1.27 ± 0.04	1.32 ± 0.02	0.18 ± 0.01	0.33 ± 0.04	0.53 ± 0.02	0.51 ± 0.04

Table 27: Growth parameters of pre-selected *Lactobacillus* strains at low temperatures. Growth curves in mMRS broth were recorded and from these the growth parameters OD_{max} (maximum optical density measured at 600 nm) and μ_{max} (maximum growth rate) were calculated. Presented values are mean values of at least three replicates ± standard deviation.

Species	Strain	OD_{max}		μ_{max} [d^{-1}]	
		4 °C	10 °C	4 °C	10 °C
L. fructivorans	TMW 1.59	0.04 ± 0.03	0.16 ± 0.13	0.07 ± 0.04	0.18 ± 0.07
	TMW 1.1856	0.06 ± 0.01	0.48 ± 0.11	0.08 ± 0.11	0.69 ± 0.27
L. sakei	TMW 1.151	0.50 ± 0.09	0.70 ± 0.18	0.92 ± 0.14	2.11 ± 0.16
	TMW 1.704	0.48 ± 0.23	0.62 ± 0.27	0.97 ± 0.21	2.28 ± 0.29
	TMW 1.1239	0.57 ± 0.09	0.74 ± 0.11	1.00 ± 0.09	1.86 ± 0.43
	TMW 1.1322	0.59 ± 0.15	0.88 ± 0.23	0.90 ± 0.14	1.58 ± 0.30
	TMW 1.1474	0.82 ± 0.03	1.00 ± 0.10	0.93 ± 0.05	2.00 ± 0.29
L. plantarum	TMW 1.1	0.47 ± 0.06	1.21 ± 0.07	0.78 ± 0.22	1.51 ± 0.13
	TMW 1.25	0.14 ± 0.07	0.67 ± 0.17	0.12 ± 0.03	0.73 ± 0.09
	TMW 1.277	0.10 ± 0.05	0.59 ± 0.16	0.15 ± 0.01	0.80 ± 0.09
	TMW 1.708	0.21 ± 0.01	0.65 ± 0.04	0.22 ± 0.05	0.99 ± 0.04
	TMW 1.834	0.23 ± 0.15	0.74 ± 0.15	0.25 ± 0.06	0.88 ± 0.06
	TMW 1.1478	0.25 ± 0.05	1.13 ± 0.07	0.12 ± 0.04	1.27 ± 0.15
	TMW 1.1623	0.19 ± 0.09	1.00 ± 0.12	0.47 ± 0.10	1.18 ± 0.11

Table 28: Growth parameters of pre-selected *Lactobacillus* strains under variation of the NaCl concentration. Growth curves in mMRS broth were recorded and from these the growth parameters OD_{max} (maximum optical density measured 600 at nm) and μ_{max} (maximum growth rate) were calculated. Presented values are mean values of at least three replicates ± standard deviation.

Species	Strain	OD_{max}				μ_{max} [h^{-1}]			
		5 % NaCl	6 % NaCl	7.5 % NaCl	10 % NaCl	5 % NaCl	6 % NaCl	7.5 % NaCl	10 % NaCl
L. fructivorans	TMW 1.59	0.51 ± 0.44	0.27 ± 0.45	0.01 ± 0.01	0.02 ± 0.02	0.05 ± 0.05	0.09 ± 0.10	0.00 ± 0.00	0.00 ± 0.00
	TMW 1.1856	1.04 ± 0.11	0.91 ± 0.13	0.78 ± 0.13	0.04 ± 0.05	0.19 ± 0.04	0.17 ± 0.02	0.12 ± 0.01	0.00 ± 0.00
L. sakei	TMW 1.151	0.57 ± 0.22	0.40 ± 0.09	0.32 ± 0.19	0.05 ± 0.01	0.40 ± 0.05	0.33 ± 0.08	0.16 ± 0.06	0.04 ± 0.01
	TMW 1.704	0.72 ± 0.28	0.67 ± 0.29	0.55 ± 0.45	0.07 ± 0.03	0.40 ± 0.03	0.35 ± 0.03	0.21 ± 0.03	0.04 ± 0.02
	TMW 1.1239	0.84 ± 0.40	0.76 ± 0.39	0.58 ± 0.16	0.07 ± 0.05	0.44 ± 0.03	0.32 ± 0.03	0.16 ± 0.01	0.01 ± 0.00
	TMW 1.1322	0.68 ± 0.55	0.68 ± 0.37	0.46 ± 0.39	0.12 ± 0.12	0.32 ± 0.05	0.25 ± 0.01	0.13 ± 0.05	0.05 ± 0.04
	TMW 1.1474	0.71 ± 0.10	0.55 ± 0.12	0.28 ± 0.07	0.04 ± 0.02	0.38 ± 0.06	0.29 ± 0.08	0.15 ± 0.06	0.00 ± 0.00
L. plantarum	TMW 1.1	1.02 ± 0.05	0.87 ± 0.06	0.41 ± 0.03	0.08 ± 0.01	0.46 ± 0.07	0.36 ± 0.07	0.20 ± 0.01	0.00 ± 0.00
	TMW 1.25	1.01 ± 0.05	0.85 ± 0.03	0.59 ± 0.19	0.05 ± 0.03	0.35 ± 0.03	0.30 ± 0.07	0.18 ± 0.04	0.00 ± 0.00
	TMW 1.277	1.04 ± 0.08	0.90 ± 0.12	0.78 ± 0.06	0.04 ± 0.02	0.36 ± 0.06	0.31 ± 0.08	0.18 ± 0.04	0.00 ± 0.00
	TMW 1.708	1.06 ± 0.10	1.04 ± 0.02	0.79 ± 0.08	0.08 ± 0.04	0.39 ± 0.06	0.31 ± 0.05	0.17 ± 0.02	0.02 ± 0.00
	TMW 1.834	0.85 ± 0.12	0.65 ± 0.14	0.72 ± 0.09	0.10 ± 0.03	0.38 ± 0.08	0.27 ± 0.04	0.17 ± 0.02	0.03 ± 0.01
	TMW 1.1478	1.21 ± 0.12	1.07 ± 0.14	0.65 ± 0.29	0.07 ± 0.03	0.33 ± 0.04	0.26 ± 0.07	0.11 ± 0.00	0.00 ± 0.00
	TMW 1.1623	0.91 ± 0.20	0.84 ± 0.04	0.62 ± 0.08	0.06 ± 0.03	0.36 ± 0.05	0.26 ± 0.02	0.16 ± 0.02	0.00 ± 0.00

Table 29: CSH of additional *L. plantarum* strains used for HHP inactivation studies. MATH test was performed with cells harvested in stationary growth phase. The relative OD_{600} after agitation with n-hexadecane compared to the OD_{600} before agitation is shown. Presented values are the mean of at least three independent measurements.

***L. plantarum* strain**	**Relative OD_{600}**	**Surface characteristics**
TMW 1.25	7.4 ± 5.3	hydrophobic
TMW 1.277	6.8 ± 4.2	
TMW 1.284	6.6 ± 1.0	
TMW 1.1810	17.6 ± 3.4	
TMW 1.708	93.1 ± 1.7	hydrophilic
TMW 1.1204	84.4 ± 5.7	
TMW 1.1808	95.0 ± 5.6	
TMW 1.2089	80.2 ± 8.5	

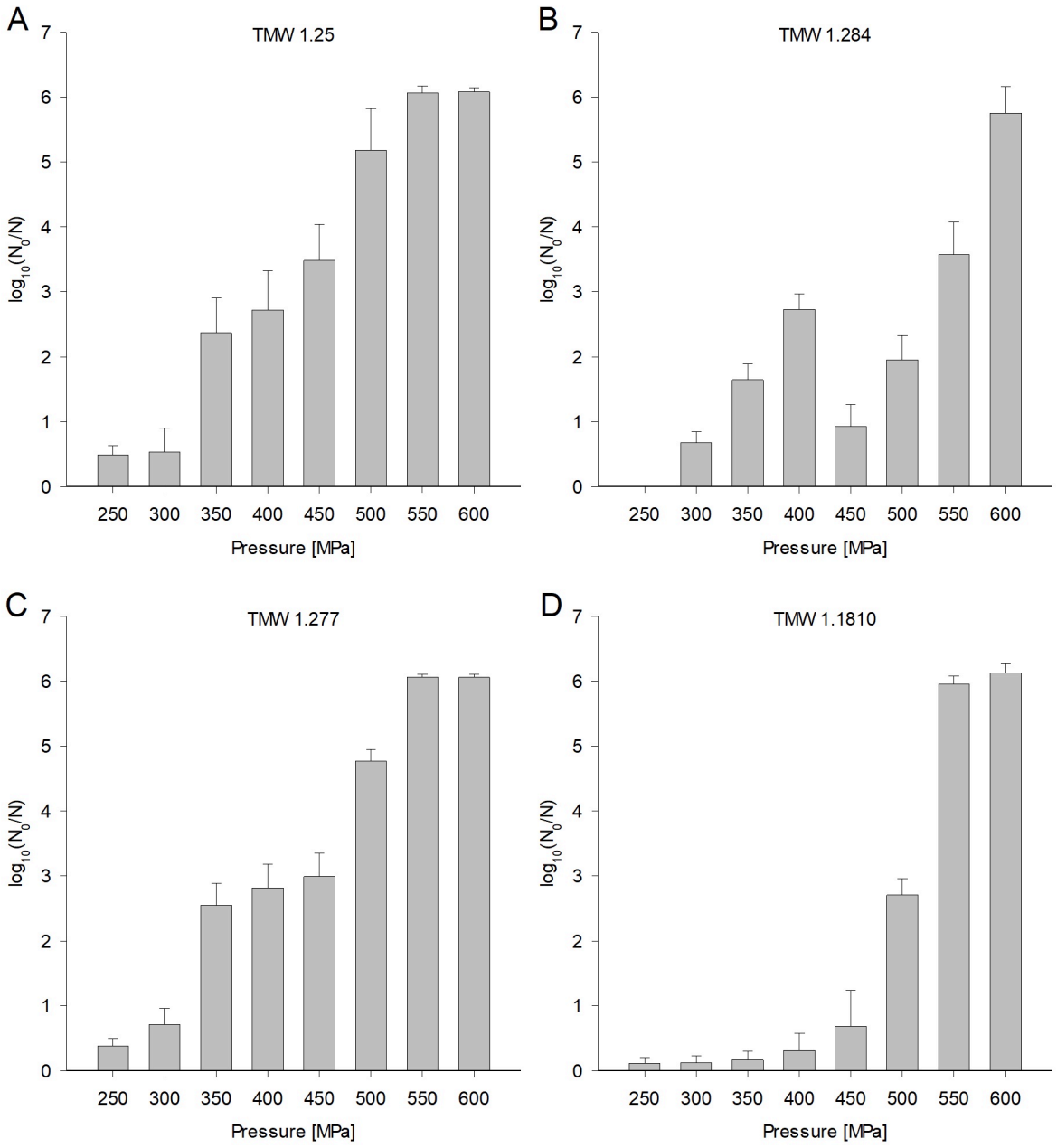

Figure 54: Overview of HHP inactivation of selected *L. plantarum* strains with hydrophobic surface characteristics. Stationary phase cells of TMW 1.25 (A), TMW 1.284 (B), TMW 1.277 (C) and TMW 1.1810 (D) suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB were treated with the indicated pressure intensities at 25 °C for a holding time of 5 min. Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation.

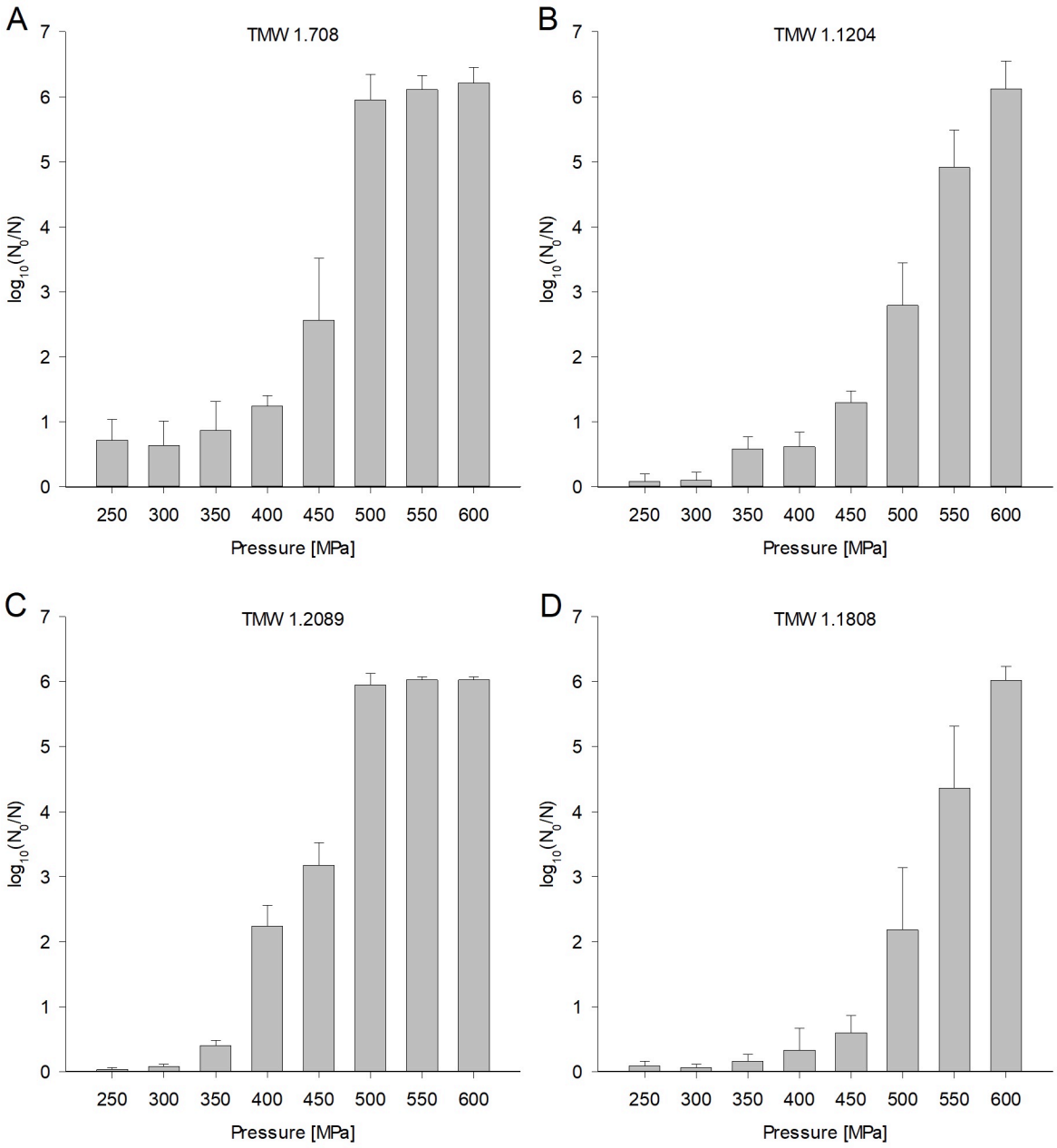

Figure 55: Overview of HHP inactivation of selected *L. plantarum* strains with hydrophilic surface characteristics. Stationary phase cells of TMW 1.708 (A), TMW 1.1204 (B), TMW 1.2089 (C) and TMW 1.1808 (D) suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB were treated with the indicated pressure intensities at 25 °C for a holding time of 5 min. Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments. Error bars represent the standard deviation.

Table 30: HHP inactivation of selected *L. plantarum* strains with hydrophobic and hydrophilic surface characteristics. Stationary phase cells suspended to a final concentration of ~10^7 cfu mL^{-1} in IPB were treated with the indicated pressure intensities at 25 °C for a holding time of 5 min. Microbial inactivation is expressed as log reduction. The presented data are the mean values of at least three independent experiments.

Pressure [MPa]	hydrophobic cell surface				hydrophilic cell surface			
	TMW 1.25	TMW 1.284	TMW 1.277	TMW 1.1810	TMW 1.708	TMW 1.1204	TMW 1.2089	TMW 1.1808
250	0.49 ± 0.14	0.00 ± 0.00	0.38 ± 0.12	0.11 ± 0.09	0.72 ± 0.32	0.08 ± 0.11	0.03 ± 0.03	0.08 ± 0.07
300	0.53 ± 0.36	0.68 ± 0.16	0.71 ± 0.25	0.12 ± 0.11	0.64 ± 0.37	0.10 ± 0.13	0.08 ± 0.04	0.06 ± 0.05
350	2.37 ± 0.54	1.64 ± 0.24	2.55 ± 0.34	0.16 ± 0.14	0.87 ± 0.44	0.59 ± 0.18	0.39 ± 0.08	0.16 ± 0.11
400	2.72 ± 0.61	2.73 ± 0.24	2.82 ± 0.37	0.31 ± 0.27	1.24 ± 0.16	0.62 ± 0.22	2.24 ± 0.32	0.33 ± 0.34
450	3.48 ± 0.55	0.93 ± 0.34	2.99 ± 0.36	0.69 ± 0.55	2.56 ± 0.96	1.30 ± 0.18	3.17 ± 0.35	0.59 ± 0.27
500	5.18 ± 0.64	1.95 ± 0.37	4.77 ± 0.18	2.71 ± 0.25	5.95 ± 0.40	2.79 ± 0.65	5.95 ± 0.18	2.18 ± 0.96
550	6.06 ± 0.11	3.58 ± 0.50	6.06 ± 0.05	5.96 ± 0.12	6.11 ± 0.21	4.91 ± 0.57	6.03 ± 0.04	4.37 ± 0.95
600	6.08 ± 0.06	5.75 ± 0.41	6.06 ± 0.05	6.12 ± 0.15	6.21 ± 0.24	6.12 ± 0.43	6.03 ± 0.04	6.02 ± 0.22

Table 31: HHP inactivation of *L. plantarum* in O/W emulsions with variation of the NaCl content in the aqueous phase upon treatment at 400 MPa, 5 min, 25 °C. Inactivation intensities are given as log reduction values (log_{10} N_0/N). N_0 = cell count of untreated control sample (~10^7 cfu mL^{-1}). N = cell count after HHP treatment. Given values are mean values of at least 3 independent replicates ± standard deviation. Statistically significant differences ($p < 0.05$) between two corresponding 0 % and 50 % samples are indicated by asterisks.

Strain	Fat content [%]	c(NaCl) [%]						
		0	0.5	1.25	2.5	5	12.5	25
TMW 1.25	0	3.24 ± 0.31	3.00 ± 0.22	3.12 ± 0.25	3.19 ± 0.23	3.12 ± 0.14*	0.49 ± 0.06*	0.22 ± 0.09
	50	3.18 ± 0.39	3.29 ± 0.14	3.31 ± 0.16	3.36 ± 0.31	3.32 ± 0.09*	0.74 ± 0.12*	0.36 ± 0.07
TMW 1.284	0	2.29 ± 0.32	-	-	-	1.85 ± 0.37	0.11 ± 0.05	-
	50	2.66 ± 0.28	-	-	-	2.56 ± 0.51	0.23 ± 0.14	-
TMW 1.277	0	2.56 ± 0.20*	-	-	-	2.93 ± 0.13	0.15 ± 0.14	-
	50	3.17 ± 0.32*	-	-	-	3.37 ± 0.60	0.17 ± 0.03	-
TMW 1.1810	0	0.22 ± 0.15	-	-	-	0.16 ± 0.11*	0.14 ± 0.07	-
	50	0.36 ± 0.14	-	-	-	0.75 ± 0.34*	0.12 ± 0.05	-
TMW 1.708	0	1.65 ± 0.58	1.47 ± 0.45	1.14 ± 0.38	1.68 ± 0.62	1.26 ± 0.37	0.66 ± 0.07	0.34 ± 0.11
	50	2.34 ± 0.59	1.98 ± 0.58	2.03 ± 0.82	1.88 ± 0.83	1.64 ± 0.34	0.99 ± 0.29	0.73 ± 0.22
TMW 1.1204	0	0.68 ± 0.20*	-	-	-	0.26 ± 0.00*	0.05 ± 0.05	-
	50	0.91 ± 0.16*	-	-	-	0.98 ± 0.32*	0.03 ± 0.03	-
TMW 1.2089	0	1.03 ± 0.38	-	-	-	0.49 ± 0.17	0.16 ± 0.14	-
	50	1.37 ± 0.20	-	-	-	0.78 ± 0.11	0.16 ± 0.10	-
TMW 1.1808	0	0.12 ± 0.11	-	-	-	0.11 ± 0.10	0.00 ± 0.00	-
	50	0.25 ± 0.11	-	-	-	0.25 ± 0.14	0.10 ± 0.09	-

Table 32: HHP inactivation of *L. plantarum* in O/W emulsions with variation of the sucrose content in the aqueous phase upon treatment at 400 MPa, 5 min, 25 °C. Inactivation intensities are given as log reduction values (log_{10} N_0/N). N_0 = cell count of untreated control sample (~10^7 cfu mL^{-1}). N = cell count after HHP treatment. Given values are mean values of at least 3 independent replicates ± standard deviation. Statistically significant differences ($p < 0.05$) between two corresponding 0 % and 50 % samples are indicated by asterisks.

Strain	Fat content [%]	c(sucrose) [%]				
		0	5	12.5	25	50
TMW 1.25	0	2.49 ± 0.81	2.28 ± 0.43	2.45 ± 0.37	2.49 ± 0.31	0.15 ± 0.12
	50	2.11 ± 0.45	2.57 ± 0.25	2.68 ± 0.31	2.84 ± 0.24	0.13 ± 0.11
TMW 1.284	0	2.29 ± 0.32	1.94 ± 0.38*	-	-	0.12 ± 0.09
	50	2.66 ± 0.28	3.69 ± 0.68*	-	-	0.18 ± 0.05
TMW 1.277	0	2.56 ± 0.20*	2.64 ± 0.47	-	-	0.23 ± 0.02
	50	3.17 ± 0.32*	3.15 ± 0.39	-	-	0.25 ± 0.13
TMW 1.1810	0	0.22 ± 0.15	0.28 ± 0.21	-	-	0.08 ± 0.07
	50	0.36 ± 0.14	0.28 ± 0.09	-	-	0.04 ± 0.03
TMW 1.708	0	1.42 ± 0.41	3.53 ± 1.07	2.57 ± 0.99	1.05 ± 0.21	0.43 ± 0.07
	50	1.81 ± 0.63	3.33 ± 1.06	2.07 ± 0.80	1.03 ± 0.32	0.38 ± 0.13
TMW 1.1204	0	0.68 ± 0.20	0.51 ± 0.04*	-	-	0.05 ± 0.02*
	50	0.91 ± 0.16	0.83 ± 0.04*	-	-	0.00 ± 0.00*
TMW 1.2089	0	1.03 ± 0.38	1.20 ± 0.39	-	-	0.26 ± 0.08
	50	1.37 ± 0.20	1.35 ± 0.25	-	-	0.21 ± 0.06
TMW 1.1808	0	0.12 ± 0.11	0.21 ± 0.15	-	-	0.01 ± 0.01
	50	0.25 ± 0.11	0.52 ± 0.41	-	-	0.04 ± 0.06

Table 33: HHP inactivation of *L. plantarum* in O/W emulsions with variation of the pH value in the aqueous phase upon treatment at 300/400 MPa, 5 min, 25 °C. Inactivation intensities are given as log reduction values (log_{10} N_0/N). N_0 = cell count of untreated control sample (~10^7 cfu mL^{-1}). N = cell count after HHP treatment. Given values are mean values of at least 3 independent replicates ± standard deviation. Statistically significant differences ($p < 0.05$) between two corresponding 0 % and 50 % samples are indicated by asterisks.

Strain	Fat con-tent [%]	IPB, pH 7		CPB, pH 7		CPB, pH 6		CPB, pH 5		CPB, pH 4		CPB, pH 3	
		300 MPa	400 MPa	300 MPa	400 MPa	300 MPa	400 MPa	300 MPa	400 MPa	300 MPa	400 MPa	300 MPa	400 MPa
TMW 1.25	0	0.70 ± 0.10	2.40 ± 0.36	0.58 ± 0.06	2.83 ± 0.28	0.58 ± 0.05	3.75 ± 0.06*	0.49 ± 0.08*	5.86 ± 0.05*	4.08 ± 0.28	5.70 ± 0.35	5.19 ± 0.29	5.23 ± 0.35
	50	0.71 ± 0.13	2.38 ± 0.43	0.49 ± 0.13	3.06 ± 0.04	0.53 ± 0.10	4.06 ± 0.21*	0.99 ± 0.12*	6.13 ± 0.14*	3.87 ± 0.46	5.91 ± 0.07	4.92 ± 0.75	5.74 ± 0.27
TMW 1.284	0	1.07 ± 0.34	2.55 ± 0.78	0.61 ± 0.18	2.83 ± 0.12	-	3.42 ± 0.09	-	-	1.72 ± 0.65	-	-	-
	50	0.50 ± 0.22	3.23 ± 0.67	0.52 ± 0.22	2.93 ± 0.11	-	3.85 ± 0.29	-	-	1.96 ± 0.60	-	-	-
TMW 1.277	0	0.50 ± 0.12	2.83 ± 0.57	0.37 ± 0.08	2.99 ± 0.43	-	3.60 ± 0.05	-	-	4.77 ± 0.73	-	-	-
	50	0.49 ± 0.12	2.98 ± 017	0.32 ± 0.07	3.16 ± 0.18	-	3.56 ± 0.09	-	-	5.28 ± 0.51	-	-	-
TMW 1.1810	0	0.22 ± 0.09	0.40 ± 0.20	0.07 ± 0.11	0.30 ± 0.14	-	0.49 ± 0.26	-	-	0.78 ± 0.27	-	-	-
	50	0.14 ± 0.08	0.43 ± 0.28	0.47 ± 0.65	0.19 ± 0.10	-	0.25 ± 0.04	-	-	1.75 ± 0.86	-	-	-
TMW 1.708	0	0.79 ± 0.09*	1.07 ± 0.07	0.74 ± 0.15	0.83 ± 0.13	0.90 ± 0.05*	1.54 ± 0.20	0.67 ± 0.08	5.91 ± 0.06	1.25 ± 0.19*	5.94 ± 0.05	5.66 ± 0.37	5.66 ± 0.37
	50	0.38 ± 0.30*	1.09 ± 0.34	0.62 ± 0.10	0.82 ± 0.12	0.71 ± 0.07*	1.50 ± 0.24	0.56 ± 0.19	5.98 ± 0.08	2.07 ± 0.37*	5.94 ± 0.02	5.85 ± 0.24	5.79 ± 0.25
TMW 1.1204	0	0.13 ± 0.09	0.23 ± 0.08	0.02 ± 0.01	0.88 ± 0.24	-	0.93 ± 0.06*	-	-	1.07 ± 0.36	-	-	-
	50	0.05 ± 0.05	0.62 ± 0.23	0.00 ± 0.01	1.37 ± 0.46	-	3.09 ± 0.18*	-	-	2.03 ± 1.01	-	-	-
TMW 1.2089	0	0.04 ± 0.04	1.25 ± 0.14	0.03 ± 0.05	0.38 ± 0.09	-	0.52 ± 0.10	-	-	0.71 ± 0.27	-	-	-
	50	0.11 ± 0.10	1.53 ± 0.46	0.05 ± 0.05	0.56 ± 0.20	-	0.52 ± 0.07	-	-	1.45 ± 0.43	-	-	-
TMW 1.1808	0	0.07 ± 0.06	0.08 ± 0.09	0.01 ± 0.10	0.08 ± 0.05	-	0.24 ± 0.06	-	-	1.23 ± 0.46*	-	-	-
	50	0.08 ± 0.07	0.12 ± 0.05	0.11 ± 0.09	0.08 ±0.07	-	0.22 ± 0.06	-	-	2.76 ± 0.68*	-	-	-

Table 34: HHP inactivation of *L. plantarum* in O/W emulsions with variation of the protein type and content in the aqueous phase upon treatment at 400/500 MPa, 5 min, 25 °C. Inactivation intensities are given as log reduction values (log_{10} N_0/N). N_0 = cell count of untreated control sample (~10^7 cfu mL^{-1}). N = cell count after HHP treatment. Given values are mean values of at least 3 independent replicates ± standard deviation. Statistically significant differences ($p < 0.05$) between two corresponding 0 % and 50 % samples are indicated by asterisks.

Strain	Fat content [%]	0		2.5 % peptone		10 % peptone		2.5 % WPI		10 % WPI		2.5 % casein	
		400 MPa	500 MPa	400 MPa	500 MPa	400 MPa	500 MPa	400 MPa	500 MPa	400 MPa	500 MPa	400 MPa	500 MPa
TMW 1.25	0	2.64 ± 0.51	3.95 ± 0.18*	2.65 ± 0.45	1.84 ± 0.14*	3.53 ± 0.78	2.55 ± 0.76	2.50 ± 0.62	3.48 ± 0.17*	2.74 ± 0.55	2.26 ± 0.27	3.24 ± 0.26	6.01 ± 0.45
	50	3.17 ± 0.62	2.96 ± 0.60*	2.57 ± 0.63	2.36 ± 0.36*	2.37 ± 0.64	2.20 ± 0.31	2.76 ± 0.64	2.41 ± 0.29*	3.31 ± 0.25	2.60 ± 0.38	3.34 ± 0.48	5.06 ± 0.92
TMW 1.284	0	-	1.63 ± 0.18	-	-	-	1.37 ± 0.28*	-	-	-	-	-	-
	50	-	1.86 ± 0.09	-	-	-	0.66 ± 0.18*	-	-	-	-	-	-
TMW 1.277	0	-	3.94 ± 0.19*	-	-	-	2.50 ± 0.40	-	-	-	-	-	-
	50	-	3.49 ± 0.09*	-	-	-	2.83 ± 0.60	-	-	-	-	-	-
TMW 1.1810	0	-	1.74 ± 0.45	-	-	-	0.78 ± 0.19*	-	-	-	-	-	-
	50	-	1.44 ± 0.02	-	-	-	2.16 ± 0.20*	-	-	-	-	-	-
TMW 1.708	0	0.97 ± 0.06	4.00 ± 0.43	0.93 ± 0.16	1.21 ± 0.08	0.65 ± 0.16	1.34 ± 0.27*	1.17 ± 0.15	4.19 ± 0.31	1.14 ± 0.17	3.81 ± 0.50	0.92 ± 0.17*	5.79 ± 0.51
	50	1.21 ± 0.19	4.36 ± 0.86	1.04 ± 0.12	1.38 ± 0.21	0.63 ± 0.13	0.81 ± 0.14*	1.09 ± 0.21	4.44 ± 0.62	1.13 ± 0.14	3.83 ± 0.40	1.30 ± 0.11*	6.35 ± 0.94
TMW 1.1204	0	-	2.47 ± 0.47*	-	-	-	0.30 ± 0.07*	-	-	-	-	-	-
	50	-	3.47 ± 0.16*	-	-	-	0.60 ± 0.12*	-	-	-	-	-	-
TMW 1.2089	0	-	4.60 ± 0.35*	-	-	-	1.13 ± 0.48	-	-	-	-	-	-
	50	-	3.50 ± 0.21*	-	-	-	1.08 ± 0.10	-	-	-	-	-	-
TMW 1.1808	0	-	1.31 ± 0.42*	-	-	-	0.43 ± 0.40	-	-	-	-	-	-
	50	-	0.80 ± 0.17*	-	-	-	0.45 ± 0.05	-	-	-	-	-	-

Table 35: HHP inactivation of *L. plantarum* upon extended incubation time in O/W emulsion at 4 °C and 25 °C. Strains were cultivated in mMRS for 24 h at 30 °C. Cultures were washed once and resuspended in IPB, IPB containing 1 % (w/v) Tween 80 or O/W emulsion of 50 % (v/v) rapeseed oil in IPB with 1 % (w/v) Tween 80 as emulsifier to a final concentration of ~10^7 cfu mL^{-1}. Suspensions were HHP treated (400 MPa, 5 min, 25 °C) either immediately or after 24 h or 48 h incubation at 4 °C or 25 °C. The values shown are the mean values of at least three independent experiments. Error bars represent the standard deviation.

Strain	Temperature	-	4 °C		25 °C	
	Incubation time	0 h	24 h	48 h	24 h	48 h
TMW 1.25	IPB	2.84 ± 0.78	3.01 ± 0.22	3.34 ± 0.56	3.78 ± 0.20	4.99 ± 0.63
	IPB + 1 % (w/v) Tween 80	2.27 ± 0.69	2.76 ± 0.55	2.78 ± 0.50	3.58 ± 0.32	4.82 ± 0.48
	O/W emulsion	2.55 ± 0.66	3.37 ± 0.38	4.34 ± 0.33	4.53 ± 0.49	5.44 ± 0.69
TMW 1.708	IPB	1.32 ± 0.22	2.15 ± 0.51	3.54 ± 0.62	3.31 ± 0.37	5.72 ± 1.15
	IPB + 1 % (w/v) Tween 80	1.04 ± 0.19	1.96 ± 0.65	3.03 ± 0.61	3.01 ± 0.53	5.23 ± 0.66
	O/W emulsion	1.32 ± 0.11	2.69 ± 0.61	3.80 ± 0.65	4.04 ± 0.51	5.66 ± 1.38

Table 36: HHP inactivation of *L. plantarum* upon cultivation in the presence of rapeseed oil. Cells were cultivated in mMRS- mixed with 50 % (v/v) rapeseed oil for 24 h at 30 °C with agitation. Cultures were washed three times and resuspended in IPB to a final concentration of ~10^7 cfu mL^{-1} and finally subjected to HHP (350/400/450 MPa, 5 min, 25 °C). The values shown are the mean values of at least three independent experiments. Error bars represent the standard deviation.

Strain	Growth condition	350 MPa	400 MPa	450 MPa
TMW 1.25	mMRS-	3.18 ± 0.31	3.60 ± 0.35	3.94 ± 0.65
	mMRS- + rapeseed oil (50:50)	2.85 ± 0.23	3.40 ± 0.30	3.67 ± 0.33
TMW 1.708	mMRS-	1.52 ± 0.41	3.26 ± 0.94	3.20 ± 0.94
	mMRS- + rapeseed oil (50:50)	1.29 ± 0.30	2.59 ± 0.36	2.98 ± 0.84

Table 37: HHP inactivation of *L. plantarum* upon cultivation in mMRS medium supplemented with different concentrations of Tween 80. Cells were cultivated in mMRS without Tween 80 (mMRS-), standard mMRS containing 0.1 % (w/v) Tween or mMRS supplemented with 1 % (w/v) Tween 80 for 24 h at 30 °C. Cultures were washed once and resuspended in IPB to a final concentration of ~10^7 cfu mL^{-1} and finally subjected to HHP (350/400/450 MPa, 5 min, 25 °C). The values shown are the mean values of at least three independent experiments. Error bars represent the standard deviation.

Strain	Growth condition	350 MPa	400 MPa	450 MPa
TMW 1.25	mMRS-	3.13 ± 0.39	3.94 ± 0.54	3.94 ± 0.65
	mMRS- + 0.1 % Tween 80	2.81 ± 0.32	3.46 ± 0.35	3.42 ± 0.15
	mMRS- + 1 % Tween 80	2.31 ± 0.63	3.12 ± 0.23	3.17 ± 0.34
TMW 1.708	mMRS-	1.34 ± 0.27	3.90 ± 0.53	4.04 ± 0.15
	mMRS- + 0.1 % Tween 80	0.79 ± 0.20	1.86 ± 0.58	2.01 ± 0.25
	mMRS- + 1 % Tween 80	0.59 ± 0.38	1.56 ± 0.17	2.59 ± 0.20

Table 38: HHP inactivation of *L. plantarum* cultivated in mMRS supplemented with different Tween types. Cells were grown in mMRS- or mMRS supplemented with 1 g L^{-1} of different Tween types and treated at different pressure / holding time combinations (350/400/450 MPa, 5 min; 400 MPa, 10 min) at 25 °C. Inactivation levels (log reduction of cfu mL^{-1}) were determined on mMRS agar plates. Means and standard deviations derived from at least three independent replicates are depicted. Statistically significant differences are presented in Table 39.

Strain	Growth condition	350 MPa, 5 min	400 MPa, 5 min	450 MPa, 5 min	400 MPa, 10 min
TMW 1.25	mMRS-	3.34 ± 0.27	3.30 ± 0.67	4.31 ± 0.70	3.94 ± 0.82
	mMRS- + Tween 20	2.09 ± 0.38	2.64 ± 0.43	2.85 ± 0.18	2.54 ± 0.29
	mMRS- + Tween 40	3.19 ± 0.64	3.14 ± 0.82	4.60 ± 0.52	4.12 ± 0.54
	mMRS- + Tween 60	3.10 ± 0.38	3.49 ± 0.64	4.56 ± 0.75	4.21 ± 0.50
	mMRS- + Tween 80	2.55 ± 0.49	3.03 ± 0.34	3.36 ± 0.57	2.81 ± 0.22
TMW 1.708	mMRS-	0.85 ± 0.02	2.89 ± 0.65	4.53 ± 0.84	4.51 ± 1.11
	mMRS- + Tween 20	0.90 ± 0.22	1.20 ± 0.13	1.58 ± 0.24	1.52 ± 0.20
	mMRS- + Tween 40	0.89 ± 0.14	3.28 ± 0.49	3.81 ± 0.84	3.22 ± 0.91
	mMRS- + Tween 60	0.95 ± 0.17	3.41 ± 0.69	4.20 ± 0.61	3.62 ± 0.95
	mMRS- + Tween 80	0.75 ± 0.04	1.36 ± 0.55	1.58 ± 0.15	1.50 ± 0.32

Table 39: Statistical analysis of HHP inactivation rates of *L. plantarum* after supplementation with different Tween types. HHP-induced log reduction values were compared by analysis of variance. Numbers indicate the growth conditions to which the conditions shown on the left have statistically significant difference ($p < 0.05$) at a given pressure/holding time combination (columns). Number coding: 1 = mMRS-, 2 = Tween 20, 3 = Tween 40, 4 = Tween 60, 5 = Tween 80.

Strain	Growth condition	350 MPa, 5 min	400 MPa, 5 min	450 MPa, 5 min	400 MPa, 10 min
TMW 1.25	mMRS-	2	-	2	2, 5
	mMRS- + Tween 20	1	-	1, 3, 4	1, 3, 4
	mMRS- + Tween 40	-	-	2	2, 5
	mMRS- + Tween 60	-	-	2	2, 5
	mMRS- + Tween 80	-	-	-	1, 3, 4
TMW 1.708	mMRS-	-	2, 5	2, 5	2, 5
	mMRS- + Tween 20	-	1, 3, 4	1, 3, 4	1, 4
	mMRS- + Tween 40	-	2, 5	2, 5	2, 5
	mMRS- + Tween 60	-	2, 5	2, 5	2, 5
	mMRS- + Tween 80	-	1, 3, 4	1, 3, 4	1, 4

Table 40: High-pressure inactivation of *L. plantarum* TMW 1.25 and TMW 1.708 upon growth in mMRS supplemented with different free fatty acids. Cells were grown in mMRS- or mMRS supplemented with 50 mM of different free fatty acids at 30 °C for 24 h, harvested, washed and re-suspended in IPB to a final concentration of 10^7-10^8 cfu mL^{-1} and treated with the indicated pressures at 25°C for 5 min. The log reduction was determined on mMRS agar plates. Shown are the means and standard deviations of at least 3 replicates. Statistically significant differences are presented in Table 41.

Strain	Growth condition	300 MPa	350 MPa	400 MPa	450 MPa	500 MPa
TMW 1.25	mMRS-	1.74 ± 0.66	3.56 ± 0.06	4.14 ± 0.37	4.85 ± 0.14	6.30 ± 0.59
	FA solvent (95 % EtOH)	1.97 ± 0.50	3.57 ± 0.22	3.84 ± 0.47	4.50 ± 0.45	6.33 ± 0.61
	Stearic acid (18:0)	2.08 ± 0.70	3.76 ± 0.21	4.26 ± 0.97	4.77 ± 0.43	6.35 ± 0.27
	Oleic acid (18:1)	0.91 ± 0.44	2.86 ± 0.16	2.97 ± 0.26	3.45 ± 0.13	5.09 ± 0.10
	Linoleic acid (18:2)	1.40 ± 0.66	3.01 ± 0.22	3.57 ± 0.05	3.69 ± 0.08	5.01 ± 0.05
	Linolenic acid (18:3)	1.03 ± 0.21	3.51 ± 0.22	3.88 ± 0.29	4.47 ± 0.12	5.68 ± 0.02
	Palmitic acid (16:0)	2.09 ± 0.55	3.82 ± 0.22	4.57 ± 0.19	5.04 ± 0.22	6.17 ± 0.58
	Pamitoleic acid (16:1)	1.39 ± 0.71	3.48 ± 0.20	3.76 ± 0.68	4.51 ± 0.16	5.99 ± 0.29
	Myristic acid (14:0)	2.21 ± 0.24	3.88 ± 0.23	4.48 ± 0.25	4.93 ± 0.34	6.77 ± 1.06
	Lauric acid (12:0)	1.76 ± 0.52	3.23 ± 0.36	4.31 ± 0.46	4.07 ± 0.37	5.43 ± 0.64
TMW 1.708	mMRS-	1.17 ± 0.29	1.15 ± 0.32	3.14 ± 0.71	5.76 ± 1.00	7.84 ± 0.15
	FA solvent (95 % EtOH)	1.39 ± 0.11	1.34 ± 0.18	3.60 ± 0.30	5.20 ± 0.44	7.82 ± 0.12
	Stearic acid (18:0)	0.95 ± 0.12	0.99 ± 0.10	3.41 ± 0.37	5.09 ± 0.36	7.76 ± 0.06
	Oleic acid (18:1)	0.28 ± 0.21	0.58 ± 0.12	1.91 ± 0.09	3.01 ± 0.27	7.73 ± 0.18
	Linoleic acid (18:2)	0.28 ± 0.19	0.24 ± 0.33	2.61 ± 0.70	4.11 ± 0.55	7.62 ± 0.10
	Linolenic acid (18:3)	0.38 ± 0.26	0.55 ± 0.34	2.58 ± 0.38	4.12 ± 0.56	7.58 ± 0.24
	Palmitic acid (16:0)	1.01 ± 0.21	0.99 ± 0.08	3.17 ± 0.72	4.86 ± 0.95	7.58 ± 0.07
	Pamitoleic acid (16:1)	0.66 ± 0.12	0.83 ± 0.15	2.76 ± 0.29	4.81 ± 0.80	7.80 ± 0.02
	Myristic acid (14:0)	1.21 ± 0.63	1.24 ± 0.18	3.69 ± 0.26	4.93 ± 0.44	7.63 ± 0.13
	Lauric acid (12:0)	1.36 ± 0.48	1.48 ± 0.14	3.59 ± 0.31	4.92 ± 0.35	7.63 ± 0.10

Table 41: Statistical analysis of HHP inactivation rates of *L. plantarum* after supplementation with different free fatty acids. HHP-induced log reduction values were compared by analysis of variance. Numbers indicate the growth conditions to which the conditions shown on the left have statistically significant difference ($p < 0.05$) at a given pressure condition (columns). Number coding: 1 = mMRS-, 2 = FA solvent, 3 = Stearic acid, 4 = Oleic acid, 5 = Linoleic acid, 6 = Linolenic acid, 7 = Palmitic acid, 8 = Palmitoleic acid, 9 = Myristic acid, 10 = Lauric acid.

Strain	Growth condition	300 MPa	350 MPa	400 MPa	450 MPa	500 MPa
TMW 1.25	mMRS-	-	4	4	4	-
	FA solvent (95 % EtOH)	-	4	4	4	-
	Stearic acid (18:0)	-	4, 5	4	4	-
	Oleic acid (18:1)	-	1, 2, 3, 6, 7, 8, 9, 10	3, 7, 9, 10	1, 2, 3, 6, 7, 8, 9, 10	9
	Linoleic acid (18:2)	-	3, 7, 9	-	1, 2, 3, 6, 7, 8, 9	9
	Linolenic acid (18:3)	-	4	-	4, 5	-
	Palmitic acid (16:0)	-	4, 5	4	4, 5, 10	-
	Pamitoleic acid (16:1)	-	4	-	4, 5	-
	Myristic acid (14:0)	-	4, 10	4	4, 5, 10	4, 5
	Lauric acid (12:0)	-	4, 9	4	3, 4, 7, 9	-
TMW 1.708	mMRS-	4, 5, 6	4, 5, 6	4	4	-
	FA solvent (95 % EtOH)	4, 5, 6	4, 5, 6	4	4	-
	Stearic acid (18:0)	-	5	4	4	-
	Oleic acid (18:1)	1, 2, 9, 10	1, 2, 8, 9, 10	1, 2, 3, 7, 8, 9, 10	1, 2, 3, 7, 8, 9, 10	-
	Linoleic acid (18:2)	1, 2, 9, 10	1, 2, 3, 7, 9, 10	-	-	-
	Linolenic acid (18:3)	1, 2, 9, 10	1, 2, 9, 10	-	-	-
	Palmitic acid (16:0)	-	5	4	4	-
	Pamitoleic acid (16:1)	-	4, 10	4	4	-
	Myristic acid (14:0)	4, 5, 6	4, 5, 6	4	4	-
	Lauric acid (12:0)	4, 5, 6	4, 5, 6	4	4	-

Table 42: Cellular fatty acid profiles of *L. plantarum* TMW 1.708 after growth in the presence of different Tween types. Cellular fatty acid profiles were determined after 24 h growth in mMRS-, mMRST20, mMRST40, mMRST60 or mMRST80. Relative abundance of specific fatty acids and of total saturated (SFA), unsaturated (UFA), cyclic (CFA) and iso-fatty acids (ISO FA) in percent of the total fatty acid content.

Trivial name	Configuration	Growth medium				
		mMRS-	mMRST20	mMRST40	mMRST60	mMRST80
-	20:1 ω7c	-	-	-	-	-
-	20:2 ω6,9c	-	-	-	-	-
Arachidic acid	20:0	-	1.29	-	-	-
-	19:0 ISO	-	-	-	-	1.09
Lactobacillic acid	19:0 cyclo ω8c	17.99	-	18.35	16.64	-
Dihydrosterculic acid	19:0 cyclo ω10c	-	39.02	-	-	34.02
-	11 me 18:1 ω7c	-	-	0.34	0.52	-
Stearic acid	18:0	7.44	1.41	4.68	9.41	1.82
cis-Vaccenic acid	18:1 ω7c	36.11	9.90	26.54	28.84	17.51
Oleic acid	18:1 ω9c	-	21.49	-	0.35	13.89
-	17:0	-	-	-	0.44	
Palmitic acid	16:0	35.25	22.82	48.14	40.87	28.64
Pamitoleic acid	16:1 ω7c	0.73	2.24	0.56	0.85	1.30
	15:0 iso 2OH	2.10	-	1.07	1.40	0.64
Myristic acid	14:0	0.38	1.61	0.32	0.68	1.09
Lauric acid	12:0	-	0.22	-	-	-
UFA	-	36.84	33.63	27.44	30.56	32.70
CFA	-	17.99	39.02	18.35	16.64	34.02
SFA	-	43.07	27.35	53.14	51.40	31.55
ISO FA	-	2.11	-	1.07	1.40	1.73

Table 43: Growth parameters of *L. plantarum* TMW 1.708 grown in mMRS supplemented with different Tween types. Cells from a 24 h pre-culture in mMRS without Tween supplement (mMRS-) were transferred (1 % v/v) in fresh mMRS- or mMRS containing 1 g L^{-1} of either Tween 20 (mMRST20), Tween 40 (mMRST40), Tween 60 (mMRST60), or Tween 80 (mMRST80) and grown at 30 °C for 30 h. Growth curves (A), maximum OD_{600} (B), maximum growth rate μ_{max} (C) and duration of lag phase TI (D) are depicted. The presented values are the means of at least three replicates.

Growth medium	OD_{600}(max)	μ_{max} [h^{-1}]	TI [h]
mMRS-	5.85 ± 0.04	0.45 ± 0.02	2.13 ± 0.38
T20	6.33 ± 0.13	0.48 ± 0.03	1.16 ± 0.32
T40	6.06 ± 0.06	0.40 ± 0.01	1.25 ± 0.38
T60	6.11 ± 0.11	0.46 ± 0.05	0.49 ± 0.17
T80	6.21 ± 0.06	0.63 ± 0.05	1.18 ± 0.16

Table 44: Determination of the maximum non-inhibitory NaCl concentration for *L. plantarum* TMW 1.708. Cells were grown in mMRS, plated on mMRS agar supplemented with varying NaCl concentrations and incubated at 30 °C. Colonies were counted after 3, 4 and 5 days and compared with the values of 0 % NaCl after 3 days. Shown are the means and standard deviations of at least three independent replicates.

c(NaCl) [% (w/v)]	4 d		5 d		3 d	
	Number of colonies (mean)	Number of colonies (mean)	% survivors	% survivors	Number of colonies (mean)	% survivors
0	106	n/d	n/a	n/d	n/d	n/d
1	111	n/d	105	n/d	n/d	n/d
2	104	n/d	98	n/d	n/d	n/d
3	90	n/d	85	n/d	n/d	n/d
4	98	n/d	93	n/d	n/d	n/d
5	71	n/d	67	n/d	n/d	n/d
6	95	n/d	90	n/d	96	91
7	86	87	82	82	89	84
8	0	0	0	0	30	28
9	0	0	0	0	0	0
10	0	0	0	0	0	0

Table 45: High pressure inactivation of *L. plantarum* TMW 1.708 after growth in mMRS with or without Tween 80 determined by cultivation on selective and non-selective agar. Cells were grown in mMRS- or mMRST80 and treated at different pressure levels (100/200/300/400/450/500 MPa) at 25 °C for 5 min. Log reduction was determined on mMRS (dark columns) or mMRS-NaCl (light columns) agar plates. Shown are the means and standard deviations of at least three independent replicates.

Growth condition		Pressure intensity					
Before HHP	After HHP	100 MPa	200 MPa	300 MPa	400 MPa	450 MPa	500 MPa
mMRS-	0 % NaCl	0.00 ± 0.23	0.11 ± 0.08	0.88 ± 0.70	2.40 ± 0.14	3.15 ± 0.09	5.81 ± 0.04
mMRS-	7 % NaCl	0.16 ± 0.07	1.57 ± 0.26	4.34 ± 1.07	5.81 ± 0.04	5.81 ± 0.04	5.81 ± 0.04
mMRST80	0 % NaCl	0.00 ± 0.06	0.00 ± 0.07	0.42 ± 0.13	1.71 ± 0.22	1.48 ± 0.30	5.40 ± 0.22
mMRST80	7 % NaCl	0.00 ± 0.06	0.41 ± 0.05	3.64 ± 1.72	5.96 ± 0.10	5.96 ± 0.10	5.96 ± 0.10

Table 46: Metabolic activity of *L. plantarum* TMW 1.708 grown in mMRS- or mMRST80 after HHP treatment. Cells were grown in mMRS- or mMRS supplemented with 1 g L^{-1} Tween 80 and treated with the indicated pressure at 25°C for 5 min. Metabolic activity after pressure treatment was determined using resazurin reduction and compared to the values of untreated cells. Shown are the means and standard deviations of 3 replicates. At conditions marked with an asterisk (*) a statistically significant difference ($p < 0.05$) between cells grown in mMRS- and mMRST80 was observed.

Growth condition	Pressure intensity					
	100 MPa	200 MPa	300 MPa	400 MPa	450 MPa*	500 MPa*
mMRS-	96.03 ± 0.59	98.72 ± 4.89	86.19 ± 5.17	64.02 ± 7.65	53.56 ± 6.17	36.50 ± 9.03
mMRST80	103.76 ± 6.99	98.92 ± 2.66	82.35 ± 3.99	68.96 ± 2.84	73.42 ± 8.14	61.51 ± 10.42

Table 47: HHP-induced release of proteins by *L. plantarum* cells grown in mMRS- or mMRST80. Cells were grown mMRS medium without and with 1 g L^{-1} Tween 80 and treated with the indicated pressure at 25 °C for 5 min or with heat (100 °C, 15 min). The protein concentration in the supernatant after pressure treatment was determined using the Bradford method. Shown are the means and standard deviations of at least seven replicates. At conditions marked with an asterisk (*) a statistically significant difference ($p < 0.05$) between cells grown in mMRS- and mMRST80 was observed.

Growth condition	c(protein) (µg/mL)							
	-*	100 MPa*	200 MPa*	300 MPa*	400 MPa*	450 MPa*	500 MPa*	100 °C
mMRS-	4.27 ± 0.63	4.89 ± 0.69	7.47 ± 0.71	8.07 ± 1.11	8.37 ± 0.97	7.74 ± 0.84	7.45 ± 0.72	4.08 ± 0.91
mMRST80	2.73 ± 0.53	2.76 ± 0.59	4.30 ± 0.96	4.96 ± 0.90	4.04 ± 0.70	5.01 ± 0.71	4.18 ± 1.28	3.06 ± 0.98

Table 48: Uptake of PI by *L. plantarum* grown in mMRS- or mMRST80 during HHP treatment. Cells were grown in mMRS without or with 1 g L^{-1} Tween 80 and treated with the indicated pressure at 25 °C for 5 min or with heat (100 °C, 15 min). The cells were stained with 3 µM PI before pressure/heat treatments. Shown are the means and standard deviations of at least five replicates. At conditions marked with an asterisk (*) a statistically significant difference ($p < 0.05$) between cells grown in mMRS- and mMRST80 was observed.

Growth condition	Fluorescence intensity ($x10^3$)							
	-*	100 MPa*	200 MPa*	300 MPa*	400 MPa*	450 MPa*	500 MPa*	100 °C
mMRS-	8.23 ± 3.12	9.20 ± 6.20	14.19 ± 7.65	27.02 ± 10.70	35.75 ± 14.98	45.48 ± 18.86	37.77 ± 14.96	116.77 ± 23.27
mMRST80	2.43 ± 1.96	1.69 ± 1.02	1.67 ± 1.85	8.81± 2.23	9.66 ± 3.31	15.13 ± 5.47	16.02 ± 4.32	111.78 ± 17.73

Table 49: Uptake of PI by *L. plantarum* grown in mMRS- or mMRST80 after HHP treatment. Cells were grown in mMRS without or with 1 g L^{-1} Tween 80 and treated with the indicated pressure at 25 °C for 5 min or with heat (100 °C, 15 min). The cells were stained with 3 µM PI after pressure/heat treatments. Shown are the means and standard deviations of at least five replicates. At conditions marked with an asterisk (*) a statistically significant difference ($p < 0.05$) between cells grown in mMRS- and mMRST80 was observed.

Growth condition	Fluorescence intensity ($x10^3$)							
	-*	100 MPa*	200 MPa*	300 MPa	400 MPa*	450 MPa	500 MPa	100 °C
mMRS-	10.06 ± 7.82	12.95 ± 13.98	7.99 ± 7.77	18.65 ± 11.59	25.73 ± 12.85	26.93 ± 14.92	25.71 ± 12.11	117.83 ± 28.52
mMRST80	1.50 ± 2.74	1.49 ± 1.62	1.61 ± 1.73	16.76 ± 3.56	11.38 ± 4.23	14.69 ± 3.48	14.73 ± 5.55	112.59 ± 18.17

9 List of publications and supervised student theses

9.1 Publications

Peer-reviewed journals

Kafka TA, **Reitermayer D**, Lenz CA & Vogel RF (2017)
High hydrostatic pressure inactivation of *Lactobacillus plantarum* cells in (O/W)-emulsions is independent from cell surface hydrophobicity and lipid phase parameters. High Pressure Research 37, 430–448.

Reitermayer D, Kafka TA, Lenz CA & Vogel RF (2018)
Interrelation between Tween and the membrane properties and high pressure tolerance of *Lactobacillus plantarum*. BMC Microbiology 18: 72.

Reitermayer D, Kafka TA, Lenz CA & Vogel RF (2019)
Interaction of fat and aqueous phase parameters during high-hydrostatic pressure inactivation of *Lactobacillus plantarum* in oil-in-water emulsions. Submitted

Poster presentations

Kafka TA, Reitermayer D, Lenz CA & Vogel RF (2014) Influence of surface hydrophobicity on high-pressure inactivation of *Lactobacillus plantarum*. 8th International Conference on High Pressure Bioscience and Biotechnology, HPBB 2014. 07/2014. Nantes, France.

9.2 Supervised student theses

Schröder SK (2014), Der Einfluss von Kultivierungsbedingungen, insbesondere der Anwesenheit von Fettsäuren, auf die Hochdruckinaktivierung von Lebensmittelverderbsorganismen. Bachelor's thesis

Buchweitz VJ (2015), Einfluss des Fettgehalts von Fleischwaren auf die Inaktivierung verderbsbegründeter *Lactobacillus*-Spezies mittels hydrostatischen Hochdruck. Bachelor's thesis