New approaches in salami manufacture with *in-situ* exopolysaccharide-forming starter cultures

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Hiermit bestätige ich die Richtigkeit des oben beschriebenen Arbeitsanteils des Kandidaten.

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Symbols

h	Hours	(h)
N	Newton	$kg \cdot m/s^2$
rpm	Revolutions per minute	rad/s
t	Time	1/min
T	Temperature	°C

Abbreviations

AiF	Arbeitsgemeinschaft industrieller Forschungsvereinigungen e.V.
CFU	Colony forming units
CLSM	Confocal laser scanning microscopy
EPS	Exopolysaccharide
FEI	Forschungskreis der Ernährungsindustrie e.V.
GRAS	Generally recognized as safe
HePS	Heteropolysaccharide
HPLC	High performance liquid chromatography
HoPS	Homopolysaccharide
IEP	Isoelectric point
LAB	Lactic acid bacteria
MRS	De Man, Rogosa and Sharpe
NCS	Nitrite curing salt
PCA	Plate count agar
TPA	Texture profile analysis

Summary

Lactic acid bacteria have always been of great importance in the production of fermented sausages such as salami, as they contribute not only to microbial stability but also to acidity and flavor profiles of such products. Recently, exopolysaccharide (EPS)-forming starter cultures have attracted the interest of the food industry. EPS have water-binding, gelling, viscosity-increasing, as well as emulsifying properties and, due to these technofunctionalities, can contribute to the improvement of existing products as well as to new product developments. However, compared to hydrocolloids, which have similar functionalities, *in-situ* formed EPS do not have to be legally declared as ingredients on a package. Initial studies looking at the use of such cultures in spreadable, short-ripened raw sausages showed that the use of EPS-forming starter cultures can lead to a significant improvement in the spreadability of fat-reduced tea sausage and deeper acidified onion mettwurst (pH 5.1 instead of 5.6). However, no study to date has comprehensively addressed the use of *in-situ* EPS-forming starter cultures in sliceable, raw fermented sausage products such as salami, which differ significantly from spreadable raw sausage products in terms of product matrix. Since growth kinetics and acidification depend on the microorganism and the food matrix used, the growth and acidification behavior of selected homo- and heteropolysaccharide (HePS)-forming lactic acid bacteria as a function of different sugar concentrations (2.5 - 10 g/kg) was initially investigated. This was done to obtain an indication of the sugar concentration required in the raw sausage mass to achieve a target pH of 4.8-5.3 in the final product. Subsequently, the performance of two HePS-producing strains *L. plantarum* TMW 1.1478, and 1.25; and the two homopolysaccharide-producing lactic acid bacteria *L. curvatus* TMW 1.624 and *L. sakei* TMW 1.411 was investigated in a raw sausage model system (inoculation concentration 10^6 CFU/g), which, in addition to 25% pork back fat, 75% lean pork meat, also contained ascorbic acid (0.5 g/kg), nitrite curing salt (28 g/kg), and dextrose or sucrose (5 g/kg). Thereby, the strains to be used were specifically analyzed with regard to their suitability for EPS-formation under typical fermentation conditions prior to use in salami production. The latter was done qualitatively by confocal laser microscopy (CLSM), followed by semi-quantitative data interpretation using MATLAB. The results showed that all selected strains were able to produce EPS in the raw sausage model matrix. There, EPS were located on the surfaces of the proteins. Since presence of HePS, which are more complex in terms of chemical structure and are often charged, can lead to changes in the organization of protein matrices even when used in very small amounts due to e.g. electrostatic interactions, sausages were subsequently prepared with a HePS-forming (*L. plantarum* 1.1478) and a non-EPS-

forming starter culture (*L. sakei* 1.2037; control). Moreover, the influence of different inoculation concentrations (10^7 and 10^9 CFU/g) on fermentation and associated HePS-formation, as well as their effect on quality parameters of the final products, were investigated. The selection of inoculation concentrations was governed by the hypothesis that higher inoculation concentrations could lead to a higher *in-situ* formed HePS amount in the raw sausage matrix and therefore to enhanced structural and thus organoleptic relevant effects. For this purpose, pork meat and fat-based raw sausages were prepared by adding and mixing spices, 0.5 g/kg Na-ascorbate, 5 g/kg sugar, the appropriate starter culture (10^7-10^9 CFU/g), and in the end 28 g/kg nitrite curing salt. Afterwards, the mass was filled, fermented (24 °C), smoked, and dried to a weight loss of 31%. In addition to pH and bacterial plate counts, the formed EPS were detected by CLSM and the influence of the formed HePS on the texture of the raw sausages was analyzed by texture profile analysis (at 16, 23, 27, and 31% weight loss) and further evaluated in a sensory evaluation for the attributes of consistency and taste. Although no significant differences were found with respect to the detected HePS and the inoculation concentration used, dependencies emerged with respect to product quality. Raw sausages produced with the HePS-producing starter culture *L. plantarum* 1.1478 were significantly ($p < 0.05$) softer than the corresponding control samples. This effect was more pronounced the higher the inoculation concentrations, which was also reflected in the sensory evaluation of samples. Semi-quantitative data interpretation of the CLSM images revealed that the HePS were predominantly formed during the first 72 h of fermentation at 24 °C, until the final pH of 4.95 ± 0.05 was reached. Although there was no clear preference in the sensory analysis performed, raw sausages with a firmer consistency are generally preferred in Germany. Accordingly, the use of an EPS-forming culture could, depending on the market, also have a negative impact on product properties. To gain a better understanding of the observed results and the influence of process conditions on *in-situ* HePS-formation and its effects on the quality of sliceable raw fermented sausages, the temperatures of the fermentation phase were varied in a further study. In addition to the 24 °C already examined, an additional incubation temperature of 16 °C, commonly used in the production of raw sausages, and a low temperature incubation of 10 °C were chosen, since increased stress conditions are often associated with increased EPS formation. Raw sausages inoculated with *L. plantarum* 1.1478 or *L. sakei* 1.2037 (10^8 CFU/mL) were fermented at 10, 16, or 24 °C within the first 7 days and then dried under the same conditions (14 °C, controlled relative humidity) until a weight loss of 31% was reached. Microbial growth, pH, and weight loss development were monitored, EPS detected with CLSM, and products further characterized

by texture profile analysis and a sensory test. Here, texture profile analysis was performed not only from the final product, but also after 21% and 26% weight loss to better understand the influence of the *in-situ* produced HePS. Differences were found depending on the starter culture used as well as on the fermentation temperature. Products manufactured with the non-EPS-forming strain *L. sakei* 1.2037 reached the target weight loss of 31% slightly faster than products manufactured with the HePS-former *L. plantarum* 1.1478. In both products, the final weight loss of 31% was reached faster at an initial fermentation temperature of 24 °C than at the lower fermentation temperatures. A correlation of temperatures with the amount of HePS formed could not be conclusively proven using semi-quantitative data analysis of CLSM images because matrix effects complicated the determination. However, texture profile analysis results showed a difference between products fermented at 24 °C and those fermented at cooler temperatures. In addition, significant ($p < 0.05$) differences were again observed between products with (softer) and without (harder) HePS-forming starter cultures at weight losses at or above 21%. These results were confirmed in the final sensory evaluation of the products (pH 4.89 - 5.01; 31% weight loss). In summary, the results of this thesis show that the use of a HePS-forming starter culture in sliceable raw fermented sausage can induce specific structural and textural changes. HePS-formation and associated quality attributes may be modulated via the inoculation concentration and control of processing parameters such as fermentation temperature. The texture softening observed in the present work, can be positively or negatively associated with the product depending on the target country and market. Taken together, results of this work underline the importance of a suitable starter culture selection for the production of fermented sausages.

Zusammenfassung

Milchsäurebakterien sind von großer Bedeutung bei der Herstellung von fermentierten Wurstwaren wie z. B. Rohwurst, da sie nicht nur zur mikrobiellen Sicherheit, sondern auch zur Bildung charakteristischer Säure- und Geschmacksprofile derartiger Produkte beitragen. In jüngster Zeit haben Exopolysaccharid (EPS)-bildende Starterkulturen das Interesse der Lebensmittelindustrie geweckt. EPS haben wasserbindende, gelierende, viskositätserhöhende, sowie emulgierende Eigenschaften und können aufgrund dieser Technofunktionalitäten sowohl zur Verbesserung bestehender Produkte als auch zu neuen Produktentwicklungen beitragen. Im Vergleich zu Hydrokolloiden, die ähnliche Funktionalitäten aufweisen, müssen *in-situ* gebildete EPS jedoch nicht auf der Verpackung als Inhaltsstoffe deklariert werden. Fermentierte Produkte werden von Verbrauchern als natürlich, sicher und qualitativ hochwertig wahrgenommen. Erste Studien, die sich mit dem Einsatz derartiger Kulturen in streichfähigen, kurz gereiften Rohwürsten beschäftigt haben, zeigten, dass der Einsatz von EPS-bildenden Starterkulturen zu einer signifikanten Verbesserung der Streichfähigkeit von fettreduzierter Teewurst und hochgesäuerter Zwiebelmettwurst (pH 5,1 statt 5,6) führen kann. Bislang beschäftigte sich allerdings noch keine Studie umfassend mit dem Einsatz von *in-situ* EPS-bildenden Starterkulturen in schnittfesten Rohwurstprodukten. Schnittfeste Rohwürste unterscheiden sich grundsätzlich von streichfähigen Rohwurstprodukten. Strukturell bestehen sie aus einem kontinuierlichen Proteinnetzwerk in das Fettpartikel eingelagert sind, Da die Wachstumskinetik und Säurebildung der eingesetzten Mikroorganismen von der Struktur und Zusammensetzung der Lebensmittelmatrix abhängt, wurde zu Beginn der Dissertation das Wachstums- und Säuerungsverhalten von selektierten, Homo- und Heteropolysaccharid (HePS)-bildenden Milchsäurebakterien in Abhängigkeit verschiedener Zuckerkonzentrationen (2,5 – 10,0 g/kg) untersucht. Das Ziel war, Selektionskriterien für die Auswahl geeigneter Zuckerkonzentrationen in der Rohwurstmasse zu erarbeiten, so dass pH-Werte von 4,8-5,3 im Endprodukt erzielt werden können. Darüber hinaus wurden die beiden HePS-bildenden Stämmen *L. plantarum* TMW 1.1478, und 1.25; sowie die beiden Homopolysaccharid-bildenden Milchsäurebakterien *L. curvatus* TMW 1.624 und *L. sakei* TMW 1.411 in einem Rohwurstmodellsystem eingesetzt (Ausgangskonzentration jeweils 10^6 KBE/g), das neben 25% Schweinerückenspeck und 75% magerem Schweinefleisch, auch Ascorbinsäure (0,5 g/kg), Nitrit Pökelsalz (NPS; 28 g/kg), sowie Dextrose oder Saccharose (5 g/kg – basierend auf den Untersuchungen in MRS Medium) enthielt. So konnten die zu verwendeten Stämme vor dem Einsatz in der Salamiproduktion hinsichtlich ihrer Eignung zur EPS-Bildung unter typischen Fermentationsbedingungen bewertet werden. Die EPS Bildung wurde qualitativ

mittels konfokaler Lasermikroskopie (CLSM) charakterisiert, gefolgt von einer semi-quantitativen Dateninterpretation mittels MATLAB. Die Ergebnisse belegten, dass alle ausgewählten Stämme grundsätzlich in der Lage waren, EPS in der Rohwurstmodellmatrix zu produzieren, wobei sich die EPS an den Oberflächen der Proteinpartikel akkumulierten. Da die in ihrer Struktur komplexeren und oftmals geladenen HePS aufgrund von beispielsweise elektrostatischen Wechselwirkungen bereits in sehr kleinen Mengen zu Veränderungen in der Proteinmatrix führen können, wurden anschließend schnittfeste Rohwürste mit einer HePS-bildenden (*L. plantarum* 1.1478) und einer nicht EPS-bildenden Starterkultur (*L. sakei* 1.2037; Kontrolle) hergestellt und der Einfluss verschiedener Inokulationskonzentrationen (10^7 und 10^9 KbE/g) auf die Fermentation und die assoziierte HePS-Bildung, sowie deren Auswirkung auf Qualitätsparameter der Endprodukte bestimmt. Der Auswahl der Inokulationskonzentrationen ging die Hypothese voraus, dass höhere Inokulationskonzentrationen zu einer höheren *in-situ* gebildeten HePS-Menge in der Rohwurstmatrix und somit zu verstärkten strukturellen und somit sensorisch-relevanten Effekten führen. Dazu wurden Rohwurstmassen unter Zugabe von Gewürzen, 0,5 g/Kg Na-Ascorbat, 5 g/Kg Zucker, der zu untersuchenden Starterkultur (10^7-10^9 KbE/g) und 28 g/Kg NPS hergestellt, die Massen in Hüllen gefüllt, fermentiert (24 °C), geräuchert, und bis zu einem Gewichtsverlust von 31% getrocknet. Neben pH und anaeroben Keimzahlen, wurden die gebildeten EPS mit CLSM detektiert und der Einfluss der gebildeten HePS auf die Textur der Rohwürste mittels Texturprofilanalyse (bei 16, 23, 27 und 31% Gewichtsverlust) analysiert sowie in einer sensorischen Evaluierung auf die Attribute Konsistenz und Geschmack hin bewertet. Obwohl keine signifikanten Unterschiede in Bezug auf die detektierten HePS und die verwendete Inokulationskonzentration festgestellt werden konnte, ergaben sich Abhängigkeiten hinsichtlich der Produktqualität. Rohwürste, welche mit der HePS-produzierenden Starterkultur *L. plantarum* 1.1478 hergestellt wurden, waren signifikant *($p < 0,05$)* weicher als die entsprechenden Kontrollproben. Dieser Effekt war im Falle höherer Inokulationskonzentrationen noch stärker ausgeprägt, was sich auch in der sensorischen Bewertung der Produktproben widerspiegelte. Die semiquantitative Dateninterpretation der CLSM-Bilder ergab, dass die HePS überwiegend während der ersten 72 h der Fermentation bei 24 °C, bis zum Erreichen des End-pH-Wertes von 4,95 ± 0,05 gebildet wurden. Auch wenn es in der durchgeführten Sensorik keine eindeutige Präferenz gab, werden in Deutschland allgemein eher Rohwürste mit fester Konsistenz bevorzugt. Der Einsatz einer EPS-bildeten Kultur könnte sich demnach, je nach Markt, auch negativ auf die Produkteigenschaften auswirken. Um ein besseres Verständnis hinsichtlich des Einflusses von

Prozessbedingungen auf die *in-situ* HePS-Bildung und deren Auswirkungen auf die Qualität von schnittfester Rohwurst zu erhalten, wurden die Temperaturen der Fermentationsphase variiert. Neben der bereits verwendeten Fermentationstemperatur von 24 °C wurde eine in der Produktion von Rohwürsten übliche Inkubationstemperatur von 16 °C, und mit 10 °C eine niedrige Temperatur gewählt, da erhöhte Stressbedingungen zu einer gesteigerten EPS-Bildung führen könnten. Die mit *L. plantarum* 1.1478 oder *L. sakei* 1.2037 beimpften (10^8 KbE/mL) Rohwürste wurden innerhalb der ersten 7 Tage bei 10, 16 oder 24 °C fermentiert und anschließend unter gleichen Bedingungen bis zum Erreichen eines Gewichtsverlusts von 31% getrocknet. Das mikrobielle Wachstum, der pH-Wert und der Gewichtsverlust wurden über den Studienzeitraum von 7 Tagen bestimmt, EPS mit CLSM detektiert und die Produkte mittels Texturprofilanalyse und einem sensorischen Test charakterisiert. Eine Texturprofilanalyse wurde nicht nur am Endprodukt, sondern auch in Proben, die einen Gewichtsverlust von 21% und 26% hatten, durchgeführt um die Kinetik der *in-situ* HePS Wirkung zu verstehen. Es konnten sowohl Unterschiede je nach eingesetzter Starterkultur als auch Fermentationstemperatur festgestellt werden. Produkte, die mit dem nicht-EPS bildenden Stamm *L. sakei* 1.2037 hergestellt wurden, erreichten den angestrebten Gewichtsverlust von 31% schneller, als die Produkte, die mit dem HePS-Bildner *L. plantarum* 1.1478 produziert wurden. In beiden Produkten wurde ein Gewichtsverlust von 31% bei einer Fermentationstemperatur von 24 °C schneller erreicht, als bei den niedrigeren Temperaturen. Eine Korrelation zwischen Temperaturen und HePS-Bildungsmenge konnte mittels der semiquantitativen Datenanalyse der CLSM Bilder jedoch nicht herbeigeführt werden, da Bestimmungen der Menge mit großen Schwankungen verbunden waren. Allerdings zeigten die Ergebnisse der Texturprofilanalyse tendenziell einen Unterschied zwischen Produkten die bei 24 °C oder bei geringeren Temperaturen fermentiert wurden. Darüber hinaus konnten signifikante *($p < 0.05$)* Unterschiede zwischen Produkten mit und ohne HePS-bildender Starterkultur bereits bei 21% Gewichtsverlust festgestellt werden. Die Ergebnisse konnten in der abschließenden sensorischen Evaluierung der schnittfesten Rohwürste (pH 4.89 – 5.01; 31% Gewichtsverlust) bestätigt werden. Zusammenfassend zeigen die Ergebnisse dieser Dissertation, dass der Einsatz einer HePS-bildenden Starterkultur in schnittfester Rohwurst spezifische Struktur- bzw. Texturveränderungen hervorrufen kann. Hierbei ist es möglich, über die Inokulationsmenge und die Steuerung der Fermentationstemperatur, Einfluss auf die HePS-Bildung und assoziierte Qualitätsattribute zu nehmen. Die in der vorliegenden Arbeit beobachteten texturellen Veränderungen könnten abhängig von der Präferenz der Verbraucher positiv oder negativ mit dem Produkt assoziiert werden. Die Ergebnisse der Arbeit

unterstreichen damit die große Bedeutung einer geeigneter Starterkulturselektionen für die Herstellung fermentierter Wurstwaren.

CHAPTER 1

Usage of *in-situ* exopolysaccharide-forming lactic acid bacteria in food production: Meat products – a new field of application?

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Abstract

In the meat industry, hydrocolloids and phosphates are used to improve the quality attributes of meat products. However, latest research results revealed that the usage of exopolysaccharide (EPS)-forming lactic acid bacteria (LAB), which are able to produce EPS *in-situ* during processing could be an interesting alternative. The current review aims to give a better understanding of bacterial EPS production in food matrices with a special focus on meat products. This includes an introduction to microbial EPS production (homopolysaccharides as well as heteropolysaccharides) and an overview of parameters affecting EPS formation and yield depending on LAB used. This is followed by a summary of methods to detect and characterize EPS to facilitate a rational selection of starter cultures and fermentation conditions based on desired structure–function relationships in different food matrices. The mechanism of action of *in-situ* generated EPS is then highlighted with an emphasis on different meat products. In the process, this review also highlights food additives currently used in meat production that could in the future be replaced by *in-situ* EPS-forming LAB.

1.1 Introduction

Hydrocolloids derived from plants, microorganisms or seaweeds are widely used in the food industry due to their stabilizing, emulsifying, thickening, and gelling properties (Galle & Arendt, 2014; van den Berg et al., 1995). Since hydrocolloids are added due to the above described technofunctional properties i.e. with a functional purpose in mind, their addition to food products requires a declaration as food additives. However, consumer increasingly reject having lengthy and complex ingredient lists despite the fact that many of the used ingredients are naturally derived such as e.g. alginates or carrageenans, which are derived from brown and red seaweed. For that reason, there is a growing interest in the replacement of commercial hydrocolloids by microbial, *in-situ* formed extracellular polymers, so called exopolysaccharides (EPS) that – to date – do not yet have to be labeled. While one can argue whether it is a good strategy to do away with compounds that have in many cases been shown to function as fibers thereby exhibiting beneficial effects in human nutrition, it is nevertheless interesting to look at alternative ways of providing needed technofunctionalities. A benefit of the microbial approach is for example, that extraction and fractionation procedures are not needed since compounds are directly produced in the target food matrix. The approach lends itself to foods that are anyway produced via a fermentation approach, where addition of starter cultures is part of the typical production process.

Important attributes of microbial EPS is their thickening, emulsifying, gelling and stabilizing ability by binding high amounts of water and/or interacting with other components in the food matrix (de Vuyst & Degeest, 1999; Laws, Gu, & Marshall, 2001). While *in-situ* EPS forming lactic acid bacteria (LAB) have been widely used in dairy- and cereal applications, their application to meat products constitutes a new, more rarely studied approach. Enhancing water binding capacity or reducing fat content though are interesting possible uses that could benefit a variety of meat products such as e.g. cooked ham or spreadable raw fermented sausages.

The current review aims to give a better understanding of bacterial EPS-production in food matrices with a special focus on meat products. This includes an introduction to microbial EPS-production (homopolysaccharides; HoPS and heteropolysaccharides; HePS) and an overview of parameters affecting EPS-formation and yield depending on LABs used. This is followed by a closer look at methods to detect and characterize EPS to facilitate a rational selection of starter cultures and fermentation conditions based on desired structure-function relationships in different food matrices. The mechanism of action of *in-situ* generated EPS in a variety of matrices such as dairy- and bakery products is then highlighted with an emphasis on different meat products. In the process, this review also highlights food additives currently used in meat production that could in the future be replaced by *in-situ* EPS-forming LAB strains.

1.2 *In-situ* EPS formation by lactic acid bacteria

EPS are defined as extracellular polymeric substances (biopolymers) of biological origin that participate in the formation of microbiological aggregates. Proteins, nucleic acids, and amphiphilic compounds including phospholipids and glycoproteins can also be part of EPS (Czaczyk & Myszka, 2007; Wingender, Neu, & Flemming, 2012). Hereafter, the abbreviation EPS is only used for “exopolysaccharides” referring to long-chain, high molecular weight (6 to 30 kDa), branched or linear biopolymers consisting of repeating saccharide units or derivatives linked by glycoside bonds. Many microorganisms that are generally recognized as safe (GRAS) have the ability to produce EPS, including LAB, to protect themselves against environmental stresses such as desiccation, heat or cold exposure, and acidic stresses (Caggianiello, Kleerebezem, & Spano, 2016). Moreover, it was shown that microbial EPS can contribute to surface adhesion, promote cellular signal transduction and improve the probiotic potential of bacteria; a fact that has been attributed to these biopolymers being soluble fibers having associated health promoting effects (Caggianiello et al., 2016; Marco et al., 2017;

Patel, Prajapati, Holst, & Ljungh, 2014). While many EPS cannot be directly utilized by LABs as energy sources, they may contribute to the development of other bacteria in a symbiotic fashion (J. Cerning, 1990). Most microbial EPS are highly soluble in water or in diluted salt solutions. Based on their chemical structures and biosynthesis pathways, they can be classified into homopolysaccharides (HoPS) and heteropolysaccharides (HePS).

1.2.1 Homopolysaccharides (HoPS) & Heteropolysaccharides (HePS)

HoPS formed by LAB are polymers that consist of a single type of monosaccharide, usually glucose or fructose, and are extracellularly synthesized by glycanosucrases or fructansucrases from sucrose or starch (Monsan et al., 2001). HoPS may differ in chain length, molecular radii and weight (> 10^6 Da), and polymer structure i.e. linear or branched (Badel, Bernardi, & Michaud, 2011; Gerwig, 2019). Moreover, they can be categorized based on their glycosidic bonds with α-D-glucans (linear or branched; consisting of α(1→6) linked D-glucosyl units), β-D-glucans (linear or branched; consisting of β(1→3) linked D-glucosyl units), and fructans (consisting of β(2→6) linked fructosyl units). Fructose-containing HoPS such as levan include fructans, whereas glucose-containing HoPS include α- and β-glucans such as dextran (Monsan et al., 2001). Some LAB species such as *Lactobacillus reuteri* were found to be able to form different HoPS including dextran, levan, and reuteran all possessing different molecular weights (Gerwig, 2019). HoPS usually carry no charge which may be attributed to the lack of functional groups such as e.g. sulfates in carrageenans or glucuronic acid groups in xanthan that may be protonated or deprotonated depending on pH (K. M. Lynch, Zannini, Coffey, & Arendt, 2018). HoPS-forming starter cultures are frequently used in cereal products (e.g. sourdough) that contain sucrose as an ingredient (de Vuyst & Degeest, 1999; van den Berg et al., 1995). In contrast to HePS (mg/L), higher amounts of HoPS (g/L) can typically be formed in the respective microbial media or food matrix, which may be attributed to differences in the involved biosynthetic pathways.

In HePS, the involved biosynthesis is more complex and energy-demanding, and is strongly related to cell wall biosynthesis (de Vuyst & Degeest, 1999; Laws et al., 2001). Although the synthesis of HePS has not yet been fully understood it is known that it involves the transport of sugar into the cytoplasm where the repeating units are synthesized whereas polymerization takes place on the outside of the cell. Since biosynthesis of HePS is linked with the primary carbohydrate metabolism of LAB, it has been postulated that EPS synthesis takes place during sugar consumption (Jolly, Vincent, Duboc, & Neeser, 2002). HePS can be produced by mesophilic (e.g. *Lactobacillus plantarum*) and thermophilic (e.g. *Lactobacillus delbrueckii*

ssp. *bulgaricus*) LAB. The involved genes encoding the regulatory proteins and enzymes needed for HePS production are of plasmid origin in the former, and of chromosomal origin in the latter case (de Vuyst & Degeest, 1999; Laws et al., 2001). HePS are usually composed of a backbone of repeating units ranging in size from disaccharides to octasaccharides that contain predominately galactose, rhamnose and glucose in different ratios. Moreover, fucose, ribose, N-acetylated monosaccharides and substituents such as glucuronic acid, glycerol or phosphates can be present (Donot, Fontana, Baccou, & Schorr-Galindo, 2012). This variety together with the differences in ratio, linkage type (α and β present), and degree of branching, leads to HePS having greatly varying molecular weights (10^4 to 10^6 Da), degree of rigidity and charge. This affects both the physicochemical properties of EPS as well as the interactions with other components (e.g. proteins) in the surrounding media, which is quite crucial for many food applications. HePS-forming starter cultures are for instance used in dairy products containing lactose as a possible substrate for EPS-formation (Lin & Chien, 2007; Ian W. Sutherland, 2001; Wingender et al., 2012).

1.2.2 Parameters influencing *in-situ* EPS-formation

As stated above, EPS-production is often stress induced and thus depends on the composition of surrounding media (carbon source, other nutrients, pH, and salts), superimposed processing-, storage- and / or incubation conditions (e.g. temperature, humidity), and on the strain involved (HePS or HoPS-former, mesophilic or thermophilic, growth phase, bacterial concentration).

Growth phase

Several studies revealed that EPS production is not only affected by growth conditions but also by the strain used, with thermophilic LAB often possessing a growth-associated- and mesophilic LAB often showing a growth-independent EPS formation (de Vuyst, Vanderveken, van de Ven, & Degeest, 1998; B. Degeest, Janssens, & De Vuyst, 2001). While both mesophilic and thermophilic LAB play a role in the dairy industry, starter cultures used in the meat industry are usually mesophilic. Growth-associated EPS production typically takes place during the exponential growth phase with the highest yields being reached under optimal growth conditions (de Vuyst et al., 1998). Whereas for non-growth associated EPS-formation, the conditions for optimal growth behavior and EPS formation are different compared to the growth-associated EPS producing strains. EPS formation of strains belonging to the non-growth associated producers can hence be triggered through superimposition of environmental stresses as shown for *Lactobacillus plantarum* or *L. paracasei* (Ana A Bengoa

et al., 2018; de Vuyst & Degeest, 1999; Sanchez, Martinez, Guillen, Jimenez-Diaz, & Rodriguez, 2006; Tallon, Bressollier, & Urdaci, 2003). Those LAB were also found to be able to produce EPS at the beginning, during, or at the end of the exponential phase, and/or during the stationary phase. For instance, EPS formation by *L. pentosus* LPS26 or *L. delbrueckii* subsp. *bulgaricus* was reported to be most pronounced during the stationary phase, which was attributed to isoprenoid phosphate carriers being involved in cell growth during the exponential phase and hence not being available for EPS production (S. Petry, Furlan, Crepeau, Cerning, & Desmazeaud, 2000; Sanchez et al., 2006). Moreover, different strains of the same species were reported to produce EPS during different growth phases. For instance, *L. reuteri* LB 121 and LB 180 were both found to produce EPS during the early exponential growth phase but only LB 180 was also able to produce EPS during the stationary phase (van Geel-Schutten, Flesch, Ten Brink, Smith, & Dijkhuizen, 1998). Independent of the strain used, a degradation of EPS was observed after a prolonged incubation time. One reason for that could be that cell lysis occurred at the end of the stationary phase leading to a release of intracellular glycohydrolases initiating EPS degradation (J. Cerning, 1990). In addition, hydrolytic or eliminase enzymes being present in the surrounding media might have contributed to the observed degradation (de Vuyst et al., 1998; B. Degeest et al., 2001; Pham, Dupont, Roy, Lapointe, & Cerning, 2000).

Availability of carbon and nitrogen

The microbial production of EPS depends on the availability of carbon and nitrogen sources in the growth medium. While the main sources of carbon are carbohydrates, typical nitrogen sources are ammonium salts and amino acids. As stated above, sucrose is a key substrate for HoPS production. Palomba et al. (2012) investigated microbial HoPS-formation in sourdoughs with and without added sucrose. Without sucrose, doughs containing either HoPS or non-EPS forming strains had the same properties while doughs containing a HoPS-forming strain and 5% sucrose were found to have higher viscosities (pseudoplastic behavior). Increasing the amount of available sucrose was shown to increase the biomass as well as the rate of EPS-formation by *L. reuteri* LB 121, K-24, and 35-5 (van Geel-Schutten et al., 1999; van Geel-Schutten et al., 1998). Not only the amount but also the source of carbon was found to influence EPS-formation. For instance, the molecular weight of levans formed by *Weisella* increased from ~10^5 g/mol with fructose to ~10^7 g/mol with glucose (Malang, Maina, Schwab, Tenkanen, & Lacroix, 2015). Moreover, Grobben et al. (1998) studied EPS-synthesis by *Lactobacillus delbrueckii* ssp. *bulgaricus* NCFB 2772 on agar either supplemented with

fructose or with a mixture of fructose and galactose. With fructose, the strain was found to produce 25 mg/L HePS consisting of glucose and galactose (ratio 1:2.4) while the sugar mixture increased the EPS yield to 80 mg/L. At the same time, HePS composition changed to glucose, galactose and rhamnose (ratio 1:7:0.8). However, S. Petry et al. (2000) reported that *Lactobacillus delbrueckii* ssp. *bulgaricus* strains produced very similar EPS with different carbon sources (same monosaccharide proportion). For LAB displaying a growth-associated EPS formation, an optimal ratio of carbon to nitrogen is required to obtain a maximum in HePS formation in the culture medium. This is because nitrogen is needed for cell synthesis and carbon provides energy for metabolic activities (de Vuyst et al., 1998; Bart Degeest, Vaningelgem, & De Vuyst, 2001). For instance, increasing the nitrogen content by supplementing reconstituted skim milk with whey protein concentrate led to an increased EPS production (37 °C, optimal pH) from 406 mg/L to 1029 mg/L by *Streptococcus thermophilus* 1275 (Zisu & Shah, 2003).

Temperature

Optimal cultivation temperatures for EPS synthesis were estimated to range between 26 and 31 °C for most LAB and Bifidobacteria (Gancel & Novel, 1994; Gandhi, Ray, & Patel, 1997; Lory, 1992), whereas a decrease of the cultivation temperature by 10 °C below the optimal growth temperatures was found to inhibit microbial EPS biosynthesis (Czaczyk & Myszka, 2007; Ian W. Sutherland, 2001). For instance, Prasanna, Grandison, and Charalampopoulos (2012) found that *Bifidobacterium longum* ssp. *infantis* CCUG 52486 and *Bifidobacterium infantis* NCIMB 702205 had the highest EPS production at the optimal growth temperature of 37 °C while at 25 °C, no EPS production could be detected. Yilmaz et al. (2015) and D. Li et al. (2016) reported similar results for *Streptococcus thermophiles* displaying a growth-associated EPS synthesis yielding high amounts of EPS under optimal growth conditions. In contrast, suboptimal growth conditions were found to enhance EPS production of mesophilic LAB as shown in a study by van den Berg et al. (1995). There, EPS production by *Lactobacillus sake* 0-1 (CBS 532.92), a strain isolated from fermented Belgian salami sausage, was found to substantially decrease from 700 mg at 10 °C to 280 mg at the optimal growth temperature of 30 °C, respectively. In another study, the amount of EPS produced by *Lactobacillus lactis* subsp. *cremoris* NIZO B40 was reported to be 17% higher when incubated at sub-optimal temperature (T = 25°C instead of $T_{optimal}$ = 30°C) and pH conditions (pH = 5.3-5.8 instead of $pH_{optimal}$ = 6.3) (Looijesteijn, Boels, Kleerebezem, & Hugenholtz, 1999). This was attributed to the competition between cell wall synthesis and microbial growth, both requiring sugar nucleotides and energy. This was also supported by findings of Ian W. Sutherland (2001) who demonstrated that cell wall synthesis slows down under suboptimal conditions, which is in agreement with the study of van den Berg et al. (1995) stating that under optimal growth temperatures, bacteria favor to use sugar for cell wall synthesis.

pH

The optimal pH for EPS production depends on the strain used and might differ from the one needed for optimal growth conditions (Aslim, Yüksekdag, Beyatli, & Mercan, 2005; de Vuyst et al., 1998; Grobben et al., 1998; Looijesteijn et al., 1999; van den Berg et al., 1995). The growth of microorganisms and hence the biosynthesis of EPS is inhibited at very low- (pH 2-3) as well as at very high pH values (pH ≥ 10). Generally, pH values between 5.5 and 6.5 were reported to be optimal for microbial EPS production for *Lactobacillus* species (Czaczyk & Myszka, 2007). For instance, *Lactobacillus reuteri* LB 121 was found to produce higher

amounts of EPS at a pH value of 5.8 as compared to a pH value of 4.8 in a modified MRS media containing 100 g/L glucose (van Geel-Schutten et al., 1998). Moreover, a controlled and constant pH during EPS production was found to increase EPS yield (de Vuyst et al., 1998; Grobben et al., 1998; Mozzi, Savoy de Giori, & Font de Valdez, 2003; S. Petry et al., 2000; Vaningelgem, Zamfir, Adriany, & De Vuyst, 2004).

1.3 Application of *in-situ* EPS-forming lab in food matrices

Application of *in-situ* EPS forming bacteria in dairy and cereal products has a long tradition with fat-reduced products (Di Cagno et al., 2006; Ling Zhang et al., 2015), sourdough-based and gluten-free breads (Kieran M. Lynch, Coffey, & Arendt, 2018; Schwab, Mastrangelo, Corsetti, & Gänzle, 2008), as well as fermented milk drinks (de Oliveira Leite et al., 2013) being some of the more novel products. Recently, the application of EPS-producing LAB to ferment legume protein – rich foods has been found to be highly beneficial regarding both texture and taste (Yan Xu et al., 2019; Y. Xu et al., 2017) and the application of HoPS-forming strains during spreadable raw fermented sausage production was found to enable a reduction of fat content in the product while maintaining its spreadability (Hilbig, Gisder, et al., 2019). **Table 1** provides an overview of dairy, cereal, and meat products that have been produced with *in-situ* EPS forming starter cultures.

1.3.1 Dairy and cereal products

Dairy products

Suitable starter cultures for the application in dairy products are usually those strains that are able to produce EPS in the presence of lactose limiting the selection to HePS-forming bacteria. During the fermentation of milk, lactobacilli play a key role besides other lactic acid bacteria to ensure a proper acidification of the product (Badel et al., 2011). EPS in dairy products have been described to influence texture and rheological properties of products and to decrease syneresis in yogurts (Amatayakul, Halmos, Sherkat, & Shah, 2006; Bouzar, Cerning, & Desmazeaud, 1997; Gentès, Turgeon, & St-Gelais, 2016; Purwandari, Shah, & Vasiljevic, 2007; Lanjun Zhang, Folkenberg, Amigo, & Ipsen, 2016). *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* are commonly used in yoghurt production with both strains being able to produce EPS. It was reported that the amount of EPS synthesized during fermentation ranged from 60 to 150 mg/L for *L. delbrueckii* ssp. *bulgaricus* (42 °C for 10 h) and from 30 to 70 mg/L for *S. thermophilus* (37 °C for 10 h), respectively (Amatayakul et al., 2006; Bouzar et al., 1997; Marshall & Rawson, 1999). The

influence of EPS producing *S. thermophilus* strains was also investigated in the yoghurt drink Ayran (Yilmaz et al., 2015). Scanning electron micrographs showed that the *in-situ* produced EPS formed a network, which resulted in a higher viscosity compared to Ayrans produced with non-EPS producing strains. In cheese production, the application of EPS-forming microorganisms was found to be beneficial with regard to the water holding capacity and the overall texture of the product (Garbowska, Pluta, & Berthold-Pluta, 2016; Nepomuceno, Junior, & Costa, 2016). For instance, Zisu and Shah (2007) investigated the influence of EPS produced by *Streptococcus thermophilus* strains on the texture and baking properties of low-fat Mozzarella cheese. The *in-situ* produced EPS were found to improve moisture retention, springiness and meltability of cheese. Similar results were reported by Perry, McMahon, and Oberg (1997) using a starter culture mix consisting of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. With respect to fermented dairy drinks, Kefir is one of the most popular products fermented by bacteria and yeasts being present in the so called “kefir grains”, with *L. kefiranofaciens* producing the EPS kefiran, a branched glucogalactan (Bengoa, Iraporda, Garrote, & Abraham, 2019).

Cereals (dough, bread)

HoPS-forming starter cultures are frequently used in cereal products such as for example in sourdough since these products usually contain sucrose as carbon source (de Vuyst & Degeest, 1999; van den Berg et al., 1995). For instance, Katina et al. (2009) investigated the influence of EPS (dextran) on wheat sourdough produced *in-situ* by *Weissella confusa* VTT E-90392. The formed HoPS increased the viscosity of the sourdough and the volume of the final bread by up to 10% and improved the crumb softness (25-40%). Similar results were reported for *in-situ* synthesized dextrans produced by *Weissella cibaria* MG1 in gluten-free sourdoughs (buckwheat, quinoa, and teff) (Wolter et al., 2014) as well as for *L. sanfranciscensis* TMW 1.392, *L. curvatus* TMW 1.624, *L. reuteri* 1.106, and *L. animalis* 1.971 in gluten-free breads made of buckwheat and rice flour (Rühmkorf, Rübsam, et al., 2012). Although EPS production in cereal products is dominated by HoPS-forming strains, HePS-synthesizing LAB that have been part of the cereal microflora, can rarely be found in sourdoughs (van der Meulen et al., 2007).

Table 1 Overview of dairy-, cereal, and meat products that have been produced with *in-situ* EPS forming starter cultures.

Species	Strain	DAIRY PRODUCTS 1 EPS Composition	Product	Reference
Lactobacillus delbrueckii ssp. *bulgaricus*	CNRZ 1187	Rhamnose, arabinose, mannose, galactose, and glucose	Yogurt	Bouzar et al. (1997)
Lactobacillus delbrueckii ssp. *bulgaricus*	LY 03	ND	Stirred yogurt	Marshall and Rawson (1999)
Streptococcus thermophilus	SY 102	ND		
Streptococcus thermophilus	ASCC 285	ND	Yogurt	Amatayakul et al. (2006)
Streptococcus thermophilus	ASCC 1275	ND		
Streptococcus thermophilus	ST 285	ND	Set-type yogurt	Purwandari et al. (2007)
Streptococcus thermophilus	ST 1275	ND		
Streptococcus thermophilus	ST1	Galactose, ribose, N-acetylgalactosamine, and glucose	Set and stirred yogurt	Gentès et al. (2016)
Lactobacillus delbrueckii ssp. *bulgaricus*	LB1	Galactose and glucose		
Lactobacillus delbrueckii ssp. *bulgaricus*	LB2	Galactose and glucose		
Lactobacillus bulgaricus	CHCC-10935	ND	Low-fat yogurt	Lanjun Zhang et al. (2016)
Lactobacillus bulgaricus	CHCC-5213	ND		
Streptococcus thermophilus	CHCC-13140	ND		
Streptococcus thermophilus	CHCC-3048	ND		

Species	Strain	DAIRY PRODUCTS 2 EPS Composition	Product	Reference
Streptococcus thermophilus	YOG CY-340 DSL	ND	Ayran	Yilmaz et al. (2015)
Streptococcus thermophilus	YO-MIX 499 LYO 100 DCU	ND		
Streptococcus thermophilus	1275	ND	Low-fat Mozzarella cheese	Zisu and Shah (2007)
Streptococcus thermophilus	285	ND		
Mix of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*	Not specified	ND	Low-fat Mozzarella cheese	Perry et al. (1997)
CHN-19 culture XT-312 culture	Not specified Not specified	ND ND	Reduced-fat Dutch-type cheese	Garbowska et al. (2016)
Streptococcus thermophilus *Lactococcus lactis* ssp. *lactis* *Lactococcus lactis* ssp. *cremoris*	Not specified Not specified Not specified	ND ND ND	Prato cheese	Nepomuceno, Junior, et al. (2016)
Lactobacillus plantarum	LBIO1	ND	Skimmed milk	Bachtarzi, Kharroub, and Ruas-Madiedo (2019)
Lactobacillus plantarum	LBIO28	ND		
Lactobacillus kefiranofaciens	ZW3	ND	Mozzarella cheese	Rehman, Wang, Wang, and Geng (2018)

Species	Strain	CEREAL PRODUCTS 1 EPS Composition	Product	Reference
Lactobacillus animalis	TMW 1.971	ND	Gluten-free sourdough	Rühmkorf, Jungkunz, Wagner, and Vogel (2012)
Lactobacillus reuteri	TMW 1.106	ND		
Lactobacillus curvatus	TMW 1.624	ND		
Weissella confusa	VTT E-90392	Dextran	Wheat sourdough	Katina et al. (2009)
Weissella cibaria	MG1	Dextran	Gluten-free sourdough (buckwheat, quinoa, and teff)	Wolter et al. (2014)
Lactobacillus curvatus	69B2	Dextran	Wheat sourdough	Palomba et al. (2012)
Leuconostoc lactis	95A	Dextran		
Gluconobacter cerinus	DSM 9533	Fructan-like homopolymers		
Neoasaia chiangmaiensis	NBRC 101099	Fructan-like homopolymers		
Kozakia baliensis	DSM 14400	Fructan-like homopolymers		
Lactobacillus sanfranciscensis	TMW 1.392	Levan	Wheat bread	Kaditzky, Seitter, Hertel, and Vogel (2008)
Lactobacillus curvatus	69B2	Dextran	Wheat bread	Torrieri, Pepe, Ventorino, Masi, and Cavella (2014)
Leuconostoc lactis	95A	Dextran		
Lactococcus lactis ssp. *lactis* biovar. *diacetylactis*	Not specified	ND	Low-fat brioche	Gemelas, Degraeve, Hallier, and Demarigny (2018)
Leuconostoc mesenteroïdes	Not specified	ND		

Species	Strain	CEREAL PRODUCTS 2 EPS Composition	Product	Reference
Lactobacillus plantarum	NR_104573.1	ND	Iranian pan bread	Abedfar,

				Hosseininezhad, and Corsetti (2019)
Leuconostoc mesenteroides	DSM 20343	Levan	Fermented fava bean dough	Shi, Hou, Xu, Mørkeberg Krogh, and Tenkanen (2019)

Species	**Strain**	**MEAT PRODUCTS EPS Composition**	**Product**	**Reference**
Lactobacillus plantarum	162 R	ND	Turkish-type fermented sausage sucuk	Dertli et al. (2016)
Leuconostoc mesenteroides	N6	ND		
Lactobacillus plantarum	TMW 1.1478	Heteropolysaccharide	Cooked ham model system	Hilbig, Loeffler, Herrmann, and Weiss (2019a)
Lactobacillus plantarum	TMW 1.25	Heteropolysaccharide		
Lactobacillus curvatus	TMW 1.624	Dextran		
Lactobacillus sakei	TMW 1.411	Dextran		
Lactobacillus plantarum	TMW 1.1478	Heteropolysaccharide	Reconstructed ham	(Hilbig, Loeffler, Herrmann, & Weiss, 2019b)
Lactobacillus sakei	TMW 1.411	Dextran		
Lactobacillus plantarum	TMW 1.1478	Heteropolysaccharide	Fat reduced raw fermented sausage	Hilbig, Gisder, et al. (2019)
Lactobacillus curvatus	TMW 1.1928	Dextran		
Lactobacillus sakei	TMW 1.411	Dextran		

Lactobacillus curvatus	TMW 1.1928	Dextran	Raw fermented sausage	Hilbig, Loeffler, Hildebrandt, Herrmann, and Weiss (2018)
Lactobacillus curvatus	TMW 1.51	Dextran		
Lactobacillus sakei	TMW 1.411	Dextran		

1.3.2 Meat products as a new field of application of *in-situ* formed EPS

State of the art

In the meat industry, hydrocolloids and phosphates are often used to improve the quality attributes of meat products. Hydrocolloids are defined as higher molecular weight biopolymers usually possessing a large number of hydroxyl groups thereby being able to bind substantial amounts of water. Due to their gelling, stabilizing, thickening, film forming, dispersing and texture modifying properties, these biopolymers are widely used in the food industry (Funami, 2011; J.-M. Li & Nie, 2016; Saha & Bhattacharya, 2010). In meat product, hydrocolloids are often used to improve water holding capacity (Andrès, Zaritzky, & Califano, 2006), influence gelling characteristics of meat proteins (Verbeken, Neirinck, van Der Meeren, & Dewettinck, 2005) and/or to improve texture properties of products with a low or reduced fat content (Gibis, Schuh, & Weiss, 2015; Totosaus & Pérez-Chabela, 2009). Hydrocolloids used in the meat industry include starches, gums such as carrageenan or xanthan, and lately also fibers (Choi et al., 2013; Sarteshnizi, Hosseini, Khaneghah, & Karimi, 2015). For instance, in bologna sausage potato and corn starch were found to improve emulsion stability (Aktaş & Gençcelep, 2006; Seo, Kang, Cho, Ba, & Seong, 2015), whereas mixtures of konjac and starch in presence or absence of carrageenan were reported to alter the texture of emulsified sausages (Z. F. Wang, Xu, Wang, & Deng, 2018). Moreover, hydrocolloids are known for their ability to improve yield and juiciness of cooked/reconstructed ham, whereas they contribute to the spreadability and mouthfeel of fat-reduced raw fermented sausages such as teewurst (Sarteshnizi et al., 2015). Besides hydrocolloids, phosphates (E 338-452) are another group of additives that are widely used in the meat industry. Through the addition of phosphates, electrostatic forces in the muscular actin-myosin-complexes are altered, leading to an expansion of void spaces between actin and myosin thereby enabling additional amounts of water to be bound (Lampila, 2013; Torley, D'Arcy, & Trout, 2000; P. Wang, Xu, & Zhou, 2009). Addition of phosphates may increase the products' pH leading to an increased net negative charge contributing to stronger electrostatic and repulsive forces between meat proteins. Moreover, phosphates are known for their ability to inhibit growth of certain microorganisms and prevent lipid oxidation (Feiner, 2006; Lampila, 2013). However, both hydrocolloids and phosphates have to be declared as additives on the package.

While EPS-producing cultures are widely used in the dairy- and cereal industry to improve product characteristics (**chapter 1.3.1**) while at the same time accounting for consumer's

demand for products being cleanly labeled or containing only few additives, the application of such cultures in the meat processing industry constitutes a relatively new field of research.

1.3.3 *In-situ* EPS formation in meat matrices

In meat processing LAB (together with other starter cultures such as *Staphylococcus* ssp.) may be used for the production of (spreadable) raw fermented sausages and cured hams (Gogoi, Borpuzari, Borpuzari, Hazarika, & Bora, 2015; Hammes & Knauf, 1994). In raw fermented sausage production, starter cultures are applied to modify sensory attributes (e.g. texture and taste) and control microbial safety (Cocolin, Dolci, & Rantsiou, 2011; Devine & Dikeman, 2014) of the final products. Microorganisms used are often mixtures of LAB and *Staphylococcus* strains, whereby latter ones predominately contribute to the color formation and aroma profile of the products. Depending on the strain and type of sugar used, LAB may also form EPS which, provided the formed amount is high enough, may lead to structural and hence textural changes of the final products (Yilmaz et al., 2015). Associated benefits of an *in-situ* EPS production in (spreadable) raw fermented sausages could be improvements with respect to sensory characteristics (e.g. mouthfeel and texture) and the possibility to lower the overall fat content of products while maintaining spreadability which is usually realized with the application of hydrocolloids that have to be labeled (Sarteshnizi et al., 2015). Although, the application of LAB during cooked ham manufacturing is currently not part of the actual manufacturing process, the time span between production and heating is quite long and may be sufficient to allow certain LAB to produce EPS *in-situ* while not lowering the pH of the product. In this case, EPS may contribute to an improved water binding capacity and hence an improved juiciness of products.

In-situ EPS production in cooked ham & reconstructed ham

Raw material used for cooked ham production typically consists of lean meat *e.g.* whole muscle such as topside or in case of reconstructed ham also different meat pieces. The materials are treated with 15-20% of brine based on the initial weight of the raw material. Ingredients of the brine are typically nitrite curing salt (10-14%), seasoning agents, sodium ascorbates, phosphates, and sugars. During processing, brine (~ 0 °C) is injected into the meat tissues by an automatic multi-needle injector or by using curing brine pumps with a single or multi-needle device. Nitrite is used as an antimicrobial agent against *Clostridium botulinum* (USDA, 2015) and to ensure proper flavor and color development whereby the latter one is accelerated by sodium ascorbates. As previously mentioned in **chapter 1.3.2**, phosphates may be added to improve the water binding capacity and texture of meat products (Frédéric Leroy

& de Vuyst, 2004; P. Wang et al., 2009). After injection, a tumbling step is performed allowing for an improved salt distribution and facilitating an even solubilization of surface proteins which is later key to binding individual meat pieces together to form a sliceable ham after heating. Tumbling is typically carried out for several hours at low temperatures (-2°C to 4°C) followed by a resting period prior to heating to a core temperature of ~ 70 °C. The duration of the tumbling and resting phase has a strong impact on the quality of the final product since it directly affects the release of dissolved muscle proteins (Devine & Dikeman, 2014; Heinz & Hautzinger, 2009). In order to replace phosphates with *in-situ* EPS producing LAB, Hilbig, Loeffler, et al. (2019a) examined different strains in a cooked ham model system for their ability to produce EPS under typical ham manufacturing conditions (2 °C, 1.92% salt). The authors reported that selected LAB strains such as *L. sakei* TMW 1.411 (HoPS) or *L. plantarum* TMW 1.1478 (HePS) were not able to grow but did produce EPS in the model system without lowering the pH making them generally suitable for cooked ham manufacturing. However, the amount of EPS produced were found to be too low to cause measurable effects with respect to water binding or texture properties, independent of whether authors produced cooked ham from a whole muscle or from different meat pieces (Hilbig, Loeffler, et al., 2019b) . It was postulated that the combined cold (2°C) and salt stress (1.92%) had a negative influence on EPS production, which has often been reported to be highest during the exponential growth phase (Vaningelgem et al., 2004; J. Wang, Zhao, Tian, Yang, & Yang, 2015). Due to the harsh conditions during cooked ham manufacturing, the investigated LAB strains were not able to grow, which may explain why no effect on texture or water binding were observed, even though only low amounts of HePS are usually needed to cause effects (Gerwig, 2019). The influence of cold and salt stress on dextran production by *L. sakei* TMW 1.411was also investigated by Prechtl, Wefers, Jakob, and Vogel (2018a). Authors concluded that the amount, as well as the molecular and macromolecular structure of *in-situ* formed dextrans are markedly influenced by the applied temperature (10°C or 30°C). Moreover, high salt concentrations (9.5%) were found to strongly limit dextran polymerization. Based on the gained results the application of *in-situ* EPS-forming LAB seems to be more promising for the production of e.g. spreadable raw fermented sausages since processing temperatures there may better facilitate microbial growth.

Raw fermented sausages (Sucuk and salami)

Typically, lean pork meat and pork back fat is used for the production of raw fermented sausages but depending on eating habits, the meat and fat composition may vary as e.g. for

suczuk, a raw fermented sausage product that does usually not contain pork but beef and lamb (Bozkurt & Bayram, 2006; Dertli et al., 2016). Besides lean meat and pork back fat, nitrite curing salt, ascorbate, and spices such as pepper, sugar and starter cultures are used, with the addition of nitrite curing salt being the last step in the salami mass preparation. After filling in permeable casings such as e.g. cellulose, sausages are fermented, whereby reported conditions for dry sausages vary in terms of duration and temperature (1-7 days, 12-24 °C, controlled relative humidity). In Germany and Italy, fermentation is usually carried out at relatively high temperatures (18-24°C) (Vignolo, 2010). During fermentation, the added sugar is metabolized by LAB to lactic acid leading to a decrease in pH and hence to a change of texture and taste (Frédéric Leroy & de Vuyst, 2004). Drying is performed until a weight loss of around 30-35% is reached (Germany; product dependent). Moreover, sausages are often smoked. A good overview of dry fermented sausage production from a global, country-dependent perspective has been provided in the Handbook of Meat Processing (Vignolo, 2010). Since LAB are traditionally used for raw fermented sausage production, the application of strains known for EPS-formation does not involve any adaption of the production process. Dertli et al. (2016) examined the influence of EPS-forming *L. plantarum* 162 R and *Leuconostoc mesenteroides* N6 on microstructural, physicochemical, microbiological and textural properties of Turkish sucuk. In comparison to sausages that have been produced without an EPS-forming culture, products appeared to be less adhesive, harder and tougher, which could be attributed to *in-situ* produced EPS leading to web-like structures in the sausage matrix as determined by scanning electron microscopy. An influence of *in-situ* HePS-producing *L. plantarum* TMW 1.1478 (inoculation concentrations used: 10^6-10^7 CFU/g and 10^9 CFU/g) on the properties of salami could also be confirmed by Velasco, Weiss, and Loeffler (2019). There, it was reported that the presence of EPS led to dry fermented sausages (salami) with quality attributes being negatively affected. Although taste was not altered, the texture of the salami was found to be significantly softer. The two different results indicate that screening for suitable strains is of key importance for a prospective industrial application of EPS-producing starter cultures in meat products since the observed effects depend on the meat matrix, processing conditions and strains used. The negative effect observed in the study of Velasco et al. (2019) could however be promising for spreadable, fat-reduced meat products where a softening effect would improve the product quality and may facilitate the production of semi-dried sausages with lower pH values, which could contribute to the shelf life of such products. This softening effect of EPS was also confirmed by Hilbig et al. (2018) who were able to produce spreadable raw fermented onion mettwurst with a lowered pH value using the EPS-producing strain *L.*

sakei TMW 1.411. Usually, a decrease in pH close to the isoelectric point of meat proteins (~5.0) leads to a reduced net charge of the proteins which affects electrostatic repulsion and hence the stability as well as the spreadability of the sausages. The presence of HoPS allows for spreadability at lower pH values (see also **Figure 1**), possibly due to phase separation effects as further described in **chapter 1.4.** Generally, physical phenomena such as molecular protein - polysaccharide interactions are likely to play a key role for the functionality in various meat products.

Spreadable raw fermented sausages

Spreadable raw fermented sausages contain a significant amount of fat. For finely cut spreadable raw sausages the fat content ranges between 35-50%, whereas for coarse-minced mixed raw sausages the content should be >25% to ensure spreadability (Feiner, 2006). Teewurst is a traditional German and Austrian spreadable raw fermented sausage that contains around 35% pork back fat. The structure of the sausage can be described as a highly concentrated suspension of meat and fat particles. The fat acts as a lubrication agent between the meat particles keeping them suspended and the sausage spreadable (Feiner, 2006; Lücke, 2015). As such, below critical fat contents, spreadability may be lost and products become sliceable. The pH-value of the product is adjusted to around 5.3 by adding sugar (3-4 g per kg product) and starter cultures or glucono-δ-lactone as an acidifier (3-4 g per kg product). To achieve a better shelf life, higher concentrations of sugar or glucono-δ-lactone may be added in order to decrease the pH-value of the product to 4.9-5. This, however, typically negatively affects spreadability since repulsive interactions between meat proteins decrease causing aggregation and networking to occur. Additionally, nitrate curing salt, spices, and often ascorbic acid is used for teewurst production (Devine & Dikeman, 2014). After sausage mass preparation, the mass is filled in casings and fermented for around 1-3 days at 18-24 °C. To improve taste and shelf life, products are often slightly smoked using a cold smoke process. Products are dried until a weight loss of around 10% is reached (Feiner, 2006; Toldrá, Hui, Astiasaran, Sebranek, & Talon, 2007). Hilbig, Gisder, et al. (2019) studied the effect of HePS and HoPS-forming starter cultures (initial concentration 10^6 CFU/g) on the spreadability of fat-reduced teewurst. Using *L. sakei* TMW 1.411 or *L. curvatus* TMW 1.1928, spreadable raw fermented sausages with a lower fat content of 20% could be produced that were significantly softer and better spreadable than control samples containing a non-EPS forming LAB. In contrast to that, the HePS-forming strain *L. plantarum* TMW 1.1478 did not improve the quality of those products. Authors attributed these results to differences in the EPS content,

which was found to be high in samples containing one of the HoPS-producing strains (0.46 – 1.03 g/kg) and significantly lower in samples containing *L. plantarum* TMW 1.1478 (0.08 – 0.30 g/kg). Although, HePS are known to be more effective at low concentrations than HoPS, the produced content seemed to be too low to maintain the spreadability of the fat-reduced product.

1.3.4 Qualitative and quantitative approaches used for EPS determination and analysis

There are several ways to identify and analyze EPS, which have been produced *in-situ* in food products (**Table 2**). Qualitative methods for the detection of EPS include confocal laser scanning microscopy (CLSM) and electron microscopy. In order to quantify and further analyze the structure and composition of EPS, colorimetric methods, high performance liquid chromatography (HPLC) or nuclear magnetic resonance (NMR) spectroscopy are frequently used. However, in these cases EPS have to be isolated and purified prior to analysis making qualitative methods the preferred methods to obtain a first insight into the presence and distribution of EPS in food matrices. It should also be noted that a comparison of results obtained by quantitative methods is sometimes difficult, not because the EPS analytical method itself is not accurate, but rather because the extraction method may have had an influence on yield (Metzger, Lankes, Fischpera, & Frimmel, 2009). Initially, food matrices are diluted and homogenized to produce an EPS-containing liquid media that is then centrifuged in order to separate cells and other solid particles from the supernatant containing EPS (P. Ruas-Madiedo & de los Reyes-Gavilán, 2005). The supernatant is then subjected to a precipitation treatment with ethanol to concentrate EPS as needed for further analysis (B. Degeest et al., 2001; Korakli, Rossmann, Gänzle, & Vogel, 2001; Palomba et al., 2012; Rimada & Abraham, 2001; Rühmkorf, Rübsam, et al., 2012; van Geel-Schutten et al., 1999). Acetone or ethanol in combination with acetone have also been reported to be suitable for EPS precipitation (de Vuyst et al., 1998; Faber, Kamerling, & Vliegenthart, 2001; Faber, van den Haak, Kamerling, & Vliegenthart, 2001; Lemoine et al., 1997; Navarini et al., 2001). Additionally, trichloroacetic acid (TCA) and/or proteases may be used to fragment proteins and liberate potentially bound EPS in protein-rich samples including samples from meat and dairy products (Bouzar et al., 1997; Cerning, Bouillanne, Landon, & Desmazeaud, 1992; de Vuyst et al., 1998; Lemoine et al., 1997; Navarini et al., 2001; Pham et al., 2000; Patricia Ruas-Madiedo, Tuinier, Kanning, & Zoon, 2002; C. Xu, Santschi, Schwehr, & Hung, 2009). In some cases, further fractionation or isolation techniques such as membrane filtration, size- or ion-exclusion chromatography are performed (P. Ruas-Madiedo & de los Reyes-Gavilán,

2005). Afterwards, EPS are hydrolyzed using acids such as TCA or hydrochloric acid to determine the type and concentration of the monosaccharides (de Vuyst et al., 1998; Sandrine Petry et al., 2003). Korakli et al. (2001) hydrolyzed the polysaccharides in 1 M H_2SO_4 for 2 h prior investigation.

Table 2 Qualitative and quantitative approaches used for EPS determination and analysis.

Confocal Laser Scanning Microscopy (CLSM)			
Analysis	**Food product**	**Bacteria**	**Reference**
Qualitative	Yogurt	*Lactococcus lactis* ssp. *cremoris* and *L. sakei*	Arltoft, Madsen, and Ipsen (2007)
Qualitative	Yogurt	*Streptococcus thermophilus* CHCC 3534 and 3541	A. N. Hassan, Ipsen, Janzen, and Qvist (2003)
Qualitative	Milk and stirred and unstirred fermented milk	*Lactococcus lactis* CHCC 3367, combination of *Streptococcus thermophilus* CHCC 3534, *Lactobacillus delbrueckii* ssp. *bulgaricus* CHCC 769 and RR	Ashraf N. Hassan, Frank, and Qvist (2002)
Qualitative	Half-fat Cheddar cheese	*Lactococcus lactis* ssp. *cremoris* DPC6532	
Qualitative	Skim milk	*Streptococcus thermophilus* MR-1C and *Lactobacillus delbrueckii* ssp. *bulgaricus* MR-1R	
Qualitative	Fermented soymilk	*Lactobacillus plantarum* 70810, *L. rhamnosus* 6005, commercial starter culture	C. Li et al. (2014)
Qualitative	Yogurt	Commercial starter cultures	Buldo et al. (2016)
Qualitative	Low-fat yogurt	*Lactobacillus bulgaricus* CHCC-10935 and CHCC-5213, *Streptococcus thermophilus* CHCC 13140, and CHCC-3048	Lanjun Zhang et al. (2016)
Qualitative	Cooked ham model system	*Lactobacillus plantarum* TMW 1.1478, *L. plantarum* TMW 1.25, *L. curvatus* TMW	Hilbig, Loeffler, et al. (2019a)

		1.624, and *L. sakei* TMW 1.411	
		Scanning Electron Microscopy (SEM)	
Qualitative	Karish cheese, feta cheese, and fermented milk	*Streptococcus thermophilus* CHCC 3534 and *Lactococcus lactis* ssp. *cremoris* JFR1	A. N. Hassan, Frank, and Elsoda (2003)
Qualitative	Reduced-fat cheddar cheese	*Streptococcus thermophilus* CHCC 3534, *Lactococcus lactic* spp. *cremoris* JFR1, and *S. thermophilus* Slab	A. N. Hassan and Awad (2005)
Qualitative	Half-fat cheddar cheese	*Lactococcus lactis* ssp. *cremoris* DPC6532	Costa et al. (2010)
Qualitative	Set yogurt	*Streptococcus thermophilus* ASCC 285 and *S. thermophilus* ASCC 1275	Amatayakul et al. (2006)
Qualitative	Fresh buttermilk and ultrafiltration permeate with various amounts of added whey protein	*Lactococcus lactic* spp. *cremoris* JFR1	Ayala-Hernandez, Goff, and Corredig (2008)
Qualitative	Iranian pan bread	*Lactobacillus plantarum* NR_104573.1	Abedfar et al. (2019)
Qualitative	Mozzarella cheese	*Lactobacillus kefiranofaciens* ZW3	Rehman et al. (2018)

Phenol-Sulfuric Method			
Quantitative	Whey based medium	*Lactobacillus rhamnosus* RW-9595M	Doleyres, Schaub, and Lacroix (2005)
Quantitative	Wheat sourdough	*Leuconostoc lactis* and *Lactobacillus curvatus*	Palomba et al. (2012)
Quantitative	Stirred yogurt	*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* SY 102	Marshall and Rawson (1999)
Quantitative	Yogurt	*Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 1187 and *Streptococcus thermophilus* CNRZ 389	Bouzar et al. (1997)
Quantitative	Yogurt	*Streptococcus thermophilus* ASCC 285 and *S. thermophilus* ASCC 1275	Amatayakul et al. (2006)
Quantitative	Low-fat mozzarella cheese	*Streptococcus thermophilus* 1275 and *S. thermophilus* 285	Zisu and Shah (2007)
Anthrone Method			
Quantitative	Whey and skim milk	Kefir grains CIDCA AGK1	Rimada and Abraham (2003)
Quantitative	Whey and skim milk	Kefir grains CIDCA AGK1	Piermaria, de la Canal, and Abraham (2008)
Gel Permeation Chromatography			
Quantitative	Yogurt-like products	*Bifidobacterium longum* ssp. *infantis* CCUG 52486 and *B. infantis* NCIMB 702205	Prasanna et al. (2012)

Quantitative	Fermented milks	*Lactococcus lactis* ssp. *cremoris*	Patricia Ruas-Madiedo et al. (2002)
Size Exclusion Chromatography			
Quantification and molecular weight	gluten-free flours (buckwheat, oat, quinoa, and teff)	*Weissella cibaria* MG1	Wolter et al. (2014)
Quantification and molar mass	Skimmed milk	*Lactobacillus plantarum* LBIO1 and *L. plantarum* LBIO28	Bachtarzi et al. (2019)
High Performance Liquid Chromatography (HPLC)			
Composition	Milk medium	*Streptococcus thermophilus* BTC, LY03, 480, and Sfi20	de Vuyst et al. (1998)
Quantification	Gluten-free sourdough	*Lactobacillus animalis* TMW 1.971, *L. reuteri* TMW 1.106, and *L. curvatus* TMW 1.624	Rühmkorf, Jungkunz, et al. (2012)
Quantification	Reconstructed ham	*Lactobacillus plantarum* TMW 1.1478 and *L. sakei* TMW 1.411	(Hilbig, Loeffler, et al., 2019b)
Quantification	Fat reduced raw fermented sausage	*Lactobacillus plantarum* TMW 1.1478, *L. curvatus* TMW 1.1928, and *L. sakei* TMW 1.411	Hilbig, Gisder, et al. (2019)
Quantification	Raw fermented sausage	*Lactobacillus curvatus* TMW 1.1928, *L. curvatus* TMW 1.51, and *L. sakei* TMW 1.411	Hilbig et al. (2018)

High performance Anion Exchange Chromatography (HPAEC)			
Quantification	Wheat sourdough	*Weissella confusa* VTT E-90392	Katina et al. (2009)
Quantification	Fermented fava bean dough	*Leuconostoc mesenteroides* DSM 20343	Shi et al. (2019)
Gas Liquid Chromatography			
Composition	Skimmed milk	*L. delbrueckii* ssp. *bulgaricus* rr	Gruter, Leeflang, Kuiper, Kamerling, and Vliegenthart (1993)
High Performance Liquid Chromatography (HPLC)			
Composition	Milk medium	*Streptococcus thermophilus* BTC, LY03, 480, and Sfi20	de Vuyst et al. (1998)
Quantification	Gluten-free sourdough	*Lactobacillus animalis* TMW 1.971, *L. reuteri* TMW 1.106, and *L. curvatus* TMW 1.624	Rühmkorf, Jungkunz, et al. (2012)
High performance Anion Exchange Chromatography			
Quantification	Wheat sourdough	*Weissella confusa* VTT E-90392	Katina et al. (2009)
Gas Liquid Chromatography			
Composition	Skimmed milk	*L. delbrueckii* ssp. *bulgaricus* rr	Gruter et al. (1993)
Nuclear Magnetic Resonance (NMR) Spectroscopy			
Structure	Reconstructed skimmed milk	*Lactobacillus delbrueckii* ssp. *bulgaricus* 291	Faber, Kamerling, et al. (2001)

Confocal laser scanning microscopy (CLSM)

CLSM is a qualitative method suitable for investigating the presence and distribution of fluorescently labeled EPS in food matrices (Hettinger et al., 2000). This approach has the advantage of imaging structures at discrete levels within an intact biological sample. CLSM is most frequently applied for imaging fixed and fluorescently labeled samples in single and multiple wavelength modes, and can also be applied to investigate living samples *in-situ* (Paddock, 1999). As such, CLSM is able to visualize changes in food microstructures as they occur. Moreover, the samples need no preparation such as slicing, freezing or dehydration which may alter microstructure (Baier-Schenk et al., 2005). The staining of different food components such as proteins, carbohydrates, and fat with different fluorescent substances offers one the possibility to determine the spatial location of one component class relative to another one within the food matrix (Wingender et al., 2012). Techniques such as the environmental scanning electron microscopy and atomic force microscopy are also able to investigate structure of hydrated samples, albeit preparation and measurement procedures may be more involved (Arltoft et al., 2007). The preservation of the microstructure in a hydrated state and the possibility to simultaneously label different food components in order to distinguish between different structures makes CSLM therefore a useful device for the qualitative analysis of EPS in complex matrices including dairy (Arltoft et al., 2007; Buldo et al., 2016; Costa et al., 2012; A. N. Hassan, Ipsen, et al., 2003; C. Li et al., 2014; Perry et al., 1997; Lanjun Zhang et al., 2016) or meat products (Hilbig, Loeffler, et al., 2019a; H. Wang, Ding, Wang, Xu, & Zhou, 2013). For instance, Ashraf N. Hassan et al. (2002) successfully investigated the distribution of *in-situ* formed EPS in milk and stirred/unstirred fermented milk using Concanavalin A (EPS stain) and Calcofluor White Stain (protein stain). In this study, four strains of EPS-producing bacteria, *Lactococcus lactis* CHCC 3367, a combination of *Streptococcus thermophilus* CHCC 3534 and *Lactobacillus delbrueckii* ssp. *bulgaricus* CHCC 769 and *Lactobacillus delbrueckii* ssp. *bulgaricus* RR were used. There, milk proteins could be observed as distinct units, whereas EPS were detected in the pores of the protein network. Similar observations were reported by Hilbig, Loeffler, et al. (2019a) who determined *in-situ* formed EPS in a cooked ham model system. Authors stated that the EPS were located on surfaces and between meat particles. Other methods to localize EPS in food matrices include covalent labeling of the respective polysaccharides or the usage of antibodies (Arltoft et al., 2007; Tromp, van de Velde, van Riel, & Paques, 2001; van de Velde, Weinbreck, Edelman, van der Linden, & Tromp, 2003).

Electron microscopy (EM)

Another imaging technique to qualitatively analyze *in-situ* produced EPS is electron microscopy. The technique allows one to determine structural characteristics in the nanometer range due to the high resolution of generated images (Dohnalkova et al., 2011). The imagining is typically conducted under high vacuum conditions (A. N. Hassan, Frank, et al., 2003), and dehydration of samples is thus necessary, which can cause substantial structural changes in cells. Moreover, treatments with organic solvents can cause cell membrane interruptions, leading to the extraction of cell constituents and morphological alterations (Beveridge, 2005; Graham & Beveridge, 1990). In order to improve the contrast of the image and to preserve structures, a chemical fixation with aldehydes and treatments with heavy metals can be applied (Montesinos, Esteve, & Guerrero, 1983). A suitable solution for the sample preparation challenges associated with conventional EM is the use of cryo-scanning electron microscopy (cryo-SEM) (Sargent, 1988). This method enables imaging of biological material in a nearly fully hydrated state. To prevent the growth of ice crystals, the freezing step must be carried out quickly (Dohnalkova et al., 2011; Glaeser, 2008). This method was for instance applied to observe EPS in dairy products (Amatayakul et al., 2006; Costa et al., 2010; A. N. Hassan & Awad, 2005; A. N. Hassan, Frank, et al., 2003). A final option is the use of environmental scanning electron microscopy (ESEM), which allows samples to be imaged under partial and not full vacuum. The technique has a lower resolution limit than full vacuum SEM though due to partial adsorption of electron beams by water vapor.

Colorimetric method

Colorimetric methods such as the phenol-sulfuric- or the anthrone method are frequently used to determine presence of carbohydrates and hence also the EPS content in samples. These methods are based on reactions between reducing sugars and reducing sugar residues in polysaccharides with a coloring reagent. To determine the total sugar content the polysaccharides have to be hydrolyzed prior analysis (Albalasmeh, Berhe, & Ghezzehei, 2013; Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The phenol-sulfuric method developed by Dubois et al. (1956), forms the basis for the so called sulfuric acid UV method (Albalasmeh et al., 2013) which is faster and more accurate than the former one. This method is often used for the determination of EPS in growth media (Di Cagno et al., 2006; Doleyres et al., 2005; Gancel & Novel, 1994; Jung, Choi, & Lee, 2013; Maalej et al., 2015; Onbasli & Aslim, 2009; Tallon et al., 2003) and was also reported to be the EPS quantification method of choice for some dairy (Amatayakul et al., 2006; Bouzar et al., 1997; Purwandari et al.,

2007; Zisu & Shah, 2007) and cereal products (Palomba et al., 2012). In contrast Rimada and Abraham (2003) and Piermaria et al. (2008) used the anthrone method to determine the amount of EPS (kefiran) in fermented whey and fermented skim milk. The anthrone method was also used to determine the EPS content in active sludge in wastewater processing (Ras, Girbal-Neuhauser, Paul, & Lefebvre, 2008).

Chromatography

With chromatography, both the monomer composition, the molecular weight of the polysaccharides, and the total amount of *in-situ* formed EPS can be analyzed. With size exclusion chromatography (SEC), often also named as gel permeation chromatography it is possible to quantify the amount of EPS provided there is a reference EPS such as e.g. dextran in case of HoPS-forming LAB. The method also allows the molecular weight distribution of formed polysaccharides to be determined (Prasanna et al., 2012; Patricia Ruas-Madiedo et al., 2002; Wolter et al., 2014). A coupled Multi-Angle Laser Light Scatterer (MALLS) and a refractometer allow for additional information regarding masses and sizes of the biopolymers to be obtained (Lin & Chien, 2007; Picton, Bataille, & Muller, 2000). For instance, Patricia Ruas-Madiedo et al. (2002) investigated the amount of EPS produced by *Lactococcus lactis* ssp. *cremoris* in fermented milk at different temperatures using SEC, whereas Wolter et al. (2014) used the method to also determine the molecular weight of formed EPS by *Weissella cibaria* MG1(10^6 - 10^7 Da).

A commonly used method to investigate the amount and composition of hydrolyzed EPS (monomers) is high performance liquid chromatography (HPLC) (de Vuyst et al., 1998; Jakob, Steger, & Vogel, 2012; Kim, Seo, Hwang, Lee, & Park, 2008; Rühmkorf, Jungkunz, et al., 2012; Rühmkorf, Rübsam, et al., 2012; Smitinont et al., 1999). Kim et al. (2008) investigated EPS produced by *Weissella hellenica* SKkimchi3. They illustrated that with increasing sucrose concentration (50 – 300 g/L) the amount of produced EPS increased from around 18 g/L to around 72 g/L after 48 h of incubation. Furthermore, the produced EPS were only composed of glucose. Recently, this method was also successfully applied to quantify and further analyze EPS that were formed in meat matrices (Hilbig, Gisder, et al., 2019). Besides HPLC, high performance anion exchange chromatography (Katina et al., 2009) and gas liquid chromatography coupled with a mass spectrometer (GC-MS) have been successfully used in EPS analytics and quantification (Badel et al., 2011; Gruter et al., 1993; Tallon et al., 2003; Vijayabaskar, Babinastarlin, Shankar, Sivakumar, & Anandapandian, 2011).

Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is an advanced technique allowing one to examine both structure and composition of EPS (Rühmkorf, Rübsam, et al., 2012). NMR facilitates the investigation of anomeric conformations of monosaccharides, linkage types, and substituents. Solid state NMR is also suitable for the investigation of insoluble polymers (Badel et al., 2011). For instance, Faber, Kamerling, et al. (2001) investigated the structure of EPS produced in reconstructed skimmed milk by *Lactobacillus delbrueckii* ssp. *bulgaricus* 291 with 1D/2D NMR (^{1}H and ^{13}C) spectroscopy. They illustrated that the produced EPS consist of branched pentasaccharide repeating units made of glucose and galactose. Not only can the structure of EPS be determined using NMR spectroscopy but also the composition of EPS. Metzger et al. (2009) examined EPS produced by *Pseudomonas putida* DSM 12735 grown on Pseudomonas Isolation Agar plates for 24 h at 35 °C using high-resolution solid state ^{13}C NMR spectroscopy. Authors showed that the extraction of EPS with EDTA can lead to higher EPS yields compared to results gained with other extraction methods which they attributed to EDTA (around 40%) being accumulated within the EPS. Comparing the results reported in literature with regard to structural changes of matrices and EPS composition, some structure – function relationships can be developed and the influence of EPS on the properties of certain food products better understood.

1.4 Mechanistic insights and structure-function relationships

To date, the full functionality of EPS in various food matrices is not fully understood, but the presented studies show that monomer composition of EPS, chain length, degree of branching and/or rigidity, charge density of the exopolysaccharides as well as environmental conditions such as e.g. temperature, pH and ionic strength play all an important role. These factors influence two key phenomena that are of importance to the various quality attributes of foods in which EPS have been generated, namely polymer – solvent and polymer – polymer interactions (van de Velde, de Hoog, Oosterveld, & Tromp, 2015). The question of whether the former or the latter is preferred in a specific food matrix determines which spatial orientation and thus which function the EPS will have.

For example, if polymer – solvent interactions are strongly preferred, EPS and other polymers present in the food matrix may not only act as good water binding agents to stay dispersed in the solvent, they may also undergo a phenomenon referred to as segregation, which is a phase separation (Thongkaew, Hinrichs, Gibis, & Weiss, 2015). This is for example the case for combinations of whey proteins when mixed with carrageenans or pectins at neutral pH

leading to the formation of a protein-rich and a pectin rich phase. Such phase separation phenomena are of thermodynamic origin and have to do with the degree of freedom of orientation and thus entropic contributions to the overall free energy of the system. As such, the formation of EPS occurring *in-situ* in the matrix may cause similar phase separations once the EPS concentration reaches a critical value. These phenomena depend not only on polymer concentration, but on structural properties, and occur for example more readily if polymers have high molecular weights, carry high similar charges and have differ in flexibility and ability to assume different configurations (Zhao, Li, Zhao, & Li, 2017). Studies have shown segregative phase separation phenomenon can be a major contributor to a soft texture and spreadability, since network forming polymers (e.g. proteins) are hindered in their ability to crosslink and form percolating structures. This has for example been observed in emulsified sausages to which pectins were added. Above a critical pectin concentration, the typically solid and sliceable boiled sausages turned into purely viscoelastic materials that could for example be spread on a slice of bread (Zeeb, Herz, Kinne, Herrmann, & Weiss, 2016; Zeeb et al., 2018). This is because pectins had phase separated to form small pectin-rich microdomains in the sausage matrix disrupting the previously continuous protein microstructure. It is likely that similar effects were at work to maintain the spreadability of the previously mentioned teewurst, i.e. formation of EPS may have induced a phase separation with other food polymers in the matrix, first and foremost the aforementioned meat proteins (Hilbig, Gisder, et al., 2019).

If polymer – polymer interactions dominate – a case referred to as the so-called association behavior, then polymers tend to aggregate to form more or less soft clusters with different voluminosities. In foods containing proteins, EPS may form such mixed EPS – protein particulates especially at lower pH values where the proteins are either neutral or slightly positively charged. Since many EPS are negatively charged across the pH spectrum of typical food products, they readily associate with positively charged moieties on the proteins. The formed structures can have various effects on the organoleptic properties of a food product. For example, if voluminosities are high (i.e. if densities are low), these mixed polymer aggregates can contribute to texture such that food products appear more creaminess and/or fatty. If voluminosities are low though, the particles are often perceived as grainy or sandy (Krzeminski et al., 2013; Kücükcetin, 2008). Such particles though may be very good at strengthening other networks, i.e. they can act as inert fillers providing gelled foods with an increased hardness. As such, EPS formation in matrices containing proteins can have many effects, ranging from increased water binding to an increased spreadability, or from providing

a more creamy or fatty mouthfeel to a harder texture, and clear structure-function relationships have yet to be established.

To give a few concrete samples, a number of studies have been performed in the area of dairy products including the ones from Ayala-Hernandez et al. (2008), Ayala-Hernández, Hassan, Goff, and Corredig (2009) and Ayala-Hernández, Hassan, Goff, Mira de Orduña, and Corredig (2008) who showed that negatively charged EPS tend to aggregate with the milk protein phase, which could be confirmed by CLSM pictures illustrating differences in protein strand distribution in dependency of the presence or absence of EPS. Taking a closer look at the microstructure of such milk gels, pores were found to be distributed throughout the protein network containing the starter cultures and the *in-situ* formed EPS in close vicinity hence affecting gel viscosities (Ayala-Hernandez et al., 2008; Kristo, Miao, & Corredig, 2011). A. N. Hassan, Frank, et al. (2003) studied the influence of *in-situ* EPS-producing cultures on the properties and microstructure of yogurt further and stated that the pore size also depends on electrostatic interactions with smaller pores being formed in presence of negatively charged EPS that are attracted to the protein network. While authors have often suggested that the structure and not the amount of EPS is of critical importance with regard to textural changes in food products (Badel et al., 2011; Purwandari et al., 2007). Hilbig, Gisder, et al. (2019) found a contrary relationship when using EPS-forming starter cultures in fat-reduced teewurst, in order to cover fat-free parts on protein particles thereby maintaining spreadability (**Figure 1**). Also in their other studies performed in meat products, HoPS-forming strains were found to be more promising (Hilbig, Gisder, et al., 2019; Hilbig, Loeffler, et al., 2019b; Hilbig et al., 2018). However, Vincent, Faber, Neeser, Stingele, and Kamerling (2001) indicated in their study that side chains of EPS are of key importance with respect to their texturizing properties although these side chains were shown to convert polysaccharides into more compact structures possessing a lower viscosifying capacity (S. W. Cui, 2005). It appears thus that the nature of the food matrix (intrinsic and extrinsic properties, etc.), and not just the EPS structure and/or amount are of importance. When it comes to meat matrices, processing conditions and meat matrix composition should be considered with respect to EPS formation and function as illustrated in **Figure 1**. Since *L. sakei* TMW 1.411 was found to be a promising strain with regard to structural and textural changes in meat products, it may be a good model in the future to study impact of processing conditions and meat matrix composition on EPS production in more depth (Hilbig, Gisder, et al., 2019; Hilbig, Loeffler, et al., 2019b; Hilbig et al., 2018).

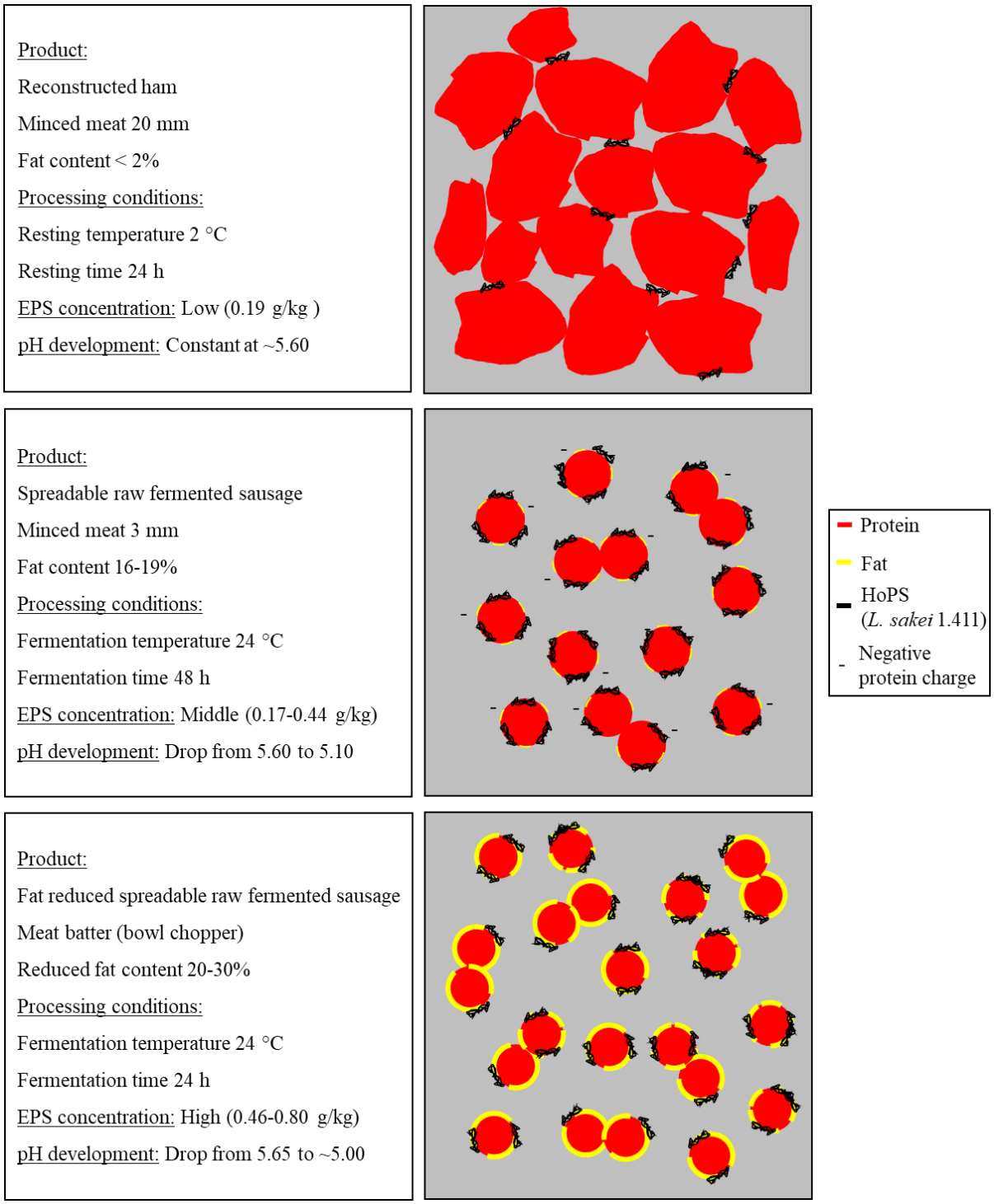

Figure 1 Meat products (reconstructed cooked ham, spreadable raw fermented sausage aka onion mettwurst, and fat-reduced spreadable raw fermented sausage aka teewurst (usually contains 35-40% fat)), that have been produced with the HoPS-forming strain *L. sakei* TMW 1.411 (~10^6 CFU/g). *Left:* Processing conditions, information to matrix composition, pH development and EPS concentration. *Right:* Location & suggested function of HoPS in the different meat matrices.

Further insights into structure - process - function relationships may also be obtained by isolating and possibly fractionating EPS followed by deliberate addition to food matrices subjected to certain processes. However, to date there are only a few studies available comparing the influence of an *in-situ* EPS production to the application of isolated EPS, likely due to the difficulty of isolating large enough quantities of EPS for product and process related investigations. In case of bread made from sourdough containing either *in-situ* EPS producing LAB or comparable amounts of isolated EPS that have been added to the bread formulation, qualitatively better products resulted from doughs containing EPS that were synthesized in the matrix (Di Monaco, 2015). Yan Xu et al. (2019) who investigated the properties and microstructure (CLSM) of fava bean protein concentrate that was either fermented with an EPS-producing starter culture or simply supplemented with dextran and organic acids also showed that the structures formed through fermentation (pH drop and *in-situ* EPS production) could not be mimicked by simply adding the EPS to the matrix. Authors suggested that the *in-situ* formed EPS interacted with fava bean proteins thereby leading to exclusion and phase separations effects as outlined above. In turn, less homogenous protein structures emerged, which altered the viscoelastic behavior of the protein-EPS system leading to quite different textural properties than when EPS were simply mixed back into the matrix. Moreover, kinetic effects may play a role, i.e. the temporal differences in pH development during fermentation may also contribute to the evolution of specific structures. For instance, de Jong, Klok, and van de Velde (2009) reported protein-polysaccharide gels having a coarser microstructure at lower acidification rates since slow gelation kinetics promote phase separation predominating during gel formation. Last but not least the presence of microbial cells may affect structure and hence texture of food products (de Vuyst & Degeest, 1999).

1.5 Conclusions

Although the use of EPS-producing starter cultures seems to be a promising approach in various food products to replace certain food additives such as hydrocolloids and phosphates, or to improve their taste and health benefits, there are still a lot of hurdles that need to be overcome prior a successful application at industrial scale. The lack of clear structure - process - function relationships prove to be a challenge for the screening of suitable EPS-producing starter cultures, since EPS-production and function strongly depends on food matrix composition and processing conditions limiting the application of EPS-producing cultures as demonstrated for e.g. different meat products. Moreover, the aspect of expected standardized product qualities can be challenging for certain applications since EPS amounts

produced may need to be similar or at least always within a certain concentration range at which functionality is guaranteed. This is particular difficult when raw materials are subject to variations and can thus not be standardized. As such, future research should focus on broadening the range of investigated food matrices to better understand which EPS may be best suited for which application. Use of models containing known food polymers at known concentrations and structured in a variety of different ways (e.g. as dispersions, foams or networks) may be helpful to advance the understanding of the action of *in-situ* produced EPS.

Aim and underlying hypothesis of the thesis

The use of lactic acid bacteria with GRAS (generally recognized as safe) status in food production has a long tradition. In recent years, *in-situ* exopolysaccharide (EPS)-producing lactic acid bacteria has become a focal point in research, since EPS formed have excellent technofunctionalities comparable to those known from hydrocolloids, and in contrast are not subject to declaration on the package of the product. Moreover, since EPS are formed *in-situ* and not added during production, different structural changes may result, paving the way for new product developments and the improvement of existing product properties. However, structural changes may also have a negative impact on product quality, and thus a good understanding of the interactions of formed EPS and the target matrix is of great importance. This also includes knowledge of how different process parameters affect the formation of EPS, as this can be used to influence the product properties in a targeted manner. To date, very little is known about the formation and effects of *in-situ* formed EPS in fermented meat products. First studies have shown great potential of EPS-forming starter cultures in spreadable, short-ripened raw sausages. However, there is a lack of knowledge on the use of these cultures in the production of sliceable raw fermented sausages, which have a completely different product matrix. Therefore, the present work aims to explore the microbial formation of EPS in raw fermented sausage matrices, to investigate how their formation can be triggered (inoculation concentration, processing conditions) and to generate a better understanding of how the in situ formation of EPS affects the quality of the final product (salami).

The thesis contains 4 chapters: An extensive literature review (chapter 1, published), a preliminary study on *in-situ* EPS-formation in a raw sausage model system (chapter 2, under review) and 2 studies in which EPS or more precisely the HePS-production by *L. plantarum* 1.1478 has been examined in raw fermented sausages subjected to different processing conditions (chapter 3 and 4, both published).

I. Preliminary study on *in-situ* EPS-formation in a raw sausage model system (**chapter 2**)

   The aim of the first study was to determine starter cultures capable of producing EPS under fermentation conditions (25 °C) in a raw sausage matrix while lowering the pH to the product target value of 4.9-5.3. For this purpose, different strains (homopolysaccharide- and heteropolysaccharide (HePS)-forming lactic acid bacteria) and sugar concentrations (2.5-10.0 g/kg; source depending on strain) were examined.

Growth and acidification kinetics were investigated in a raw sausage model system containing 28 g/kg nitrite curing salt *(induced stress)*, *in-situ* formed EPS then analyzed qualitatively by confocal laser scanning microscopy (CLSM) and results then interpreted using a semi-quantitative approach.

II. Investigations on formation and effects of *in-situ* produced HePS in raw fermented sausages: *Influence of the inoculation concentration* (**chapter 3**)

The aim of this study was to gain a better understanding of the influence of a selected *in-situ* HePS-producing strain (*L. plantarum* TMW 1.1478) on the quality properties of sliceable raw fermented sausage (*salami*) with particular emphasis on texture as a function of the inoculation concentration used (10^7 and 10^9 CFU/g). To determine the influence of the formed HePS on the structural and thus textural changes, the HePS were qualitatively evaluated using CLSM and the results were interpreted using again a semi-quantitative approach. In addition, as in all other analyses, the texture/consistency of products containing the HePS-forming strain *L. plantarum* TMW 1.1478 was compared with products prepared with a non-EPS-forming starter culture (*L. sakei* TMW 1.411). The latter quality attributes were investigated by texture profile analysis and sensory evaluation.

III. Investigations on formation and effects of *in-situ* produced HePS in raw fermented sausages: *Influence of different fermentation temperatures* (**chapter 4**)

The objective of this study was to understand how different fermentation temperatures and thus altered process conditions affect HePS-formation and thus the overall product quality of sliceable fermented raw sausages (salami). For this purpose, three different fermentation temperatures were chosen, two traditionally used in raw sausage production (24 °C and 16 °C) and one far below the optimal fermentation temperature (10 °C), to account for EPS-formation under increased stress conditions. As before, sausages were prepared with *L. plantarum* TMW 1.1478 and with the control strain *L. sakei* TMW 1.411 (10^8 CFU/g), stored for 7 days at the above temperatures, and then dried until a weight loss of 31% was reached (same drying conditions for all sausages). Analyses such as texture profile analysis and sensory analysis were always performed using samples with the same weight loss (different drying time) and the influence of the three fermentation temperatures on HePS-formation was again determined using CLSM and a semi-quantitative interpretation approach.

With the present work we wanted to test the following hypothesis:

- Raw sausage model system (**chapter 2**):

  The selected homo- and heteropolysaccharide-producing lactic acid bacteria are capable of fermenting added sugars to achieve the target pH of a raw sausage under typical fermentation conditions while producing EPS.

- Raw sausage and inoculum concentration (**chapter 3**):

  The HePS-producing strain *L. plantarum* TMW 1.1478 is able to produce sufficient EPS *in-situ* during fermentation to induce structural and thus textural changes in sliceable raw fermented sausages (salami). This effect is more pronounced when the inoculation concentration is increased from 10^7 - 10^9 CFU/g.

- Raw sausage production under different temperature fermentation (**chapter 4**):

  The change in fermentation temperatures has a pronounced influence on *in-situ* HePS-formation by *L. plantarum* TMW1.1478 and thus also on the quality characteristics of raw fermented sausages (salami). HePS-formation is particularly favored by the low fermentation temperature as an additional stress factor (besides the presence of nitrite curing salt).

CHAPTER 2

Screening of Exopolysaccharide-Forming Starter Cultures to be Used in Raw Fermented Sausage Production

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Submitted to Fermentation

Abstract

To date, very few studies have been conducted on lactic acid bacteria and their exopolysaccharide (EPS)-synthesis in meat products. Therefore, the aim of this study was to generate a better understanding of microbial EPS-formation in fermented meat matrices using heteropolysaccharide-forming (*L. plantarum* TMW 1.1478 and 1.25) or homopolysaccharide-forming strains (*L. curvatus* TMW 1.624 and *L. sakei* TMW 1.411), with the aim of selecting a starter culture capable of producing EPS while lowering pH as required in salami manufacture. For this purpose, growth kinetics, associated pH development at 25 °C fermentation temperature (prescreening of different sugar concentrations; 2.5-10.0 g/kg), and EPS formation and distribution in the raw sausage model matrix (25% pork fat, 75% lean pork meat, 0.5 g/kg ascorbic acid, 28 g/kg nitrite curing salt, 10^6 CFU/g starter culture) were investigated. Microbial EPS-formation was qualitatively assessed using confocal laser scanning microscopy, and results further evaluated using semi-quantitative data interpretation (Matlab). The strains studied were able to produce EPS in the raw sausage model matrix, lowering the pH to 4.8-5.3, which is within the typical range for raw fermented sausages. EPS-formation and pH development were dependent on the strain used, with *L. plantarum* 1.1478 seeming to be a promising strain for raw sausage production trials in order to observe EPS-induced effects.

2.1 Introduction

Starter cultures are traditionally used in food production, including in the manufacture of fermented meat products such as raw fermented sausages, where they contribute to the microbial safety, texture and flavor of the products (Laranjo, Potes, & Elias, 2019). In addition to the already known applications, some starter cultures, including lactic acid bacteria, have been further explored in recent years due to their ability to form exopolysaccharides (EPS) in food matrices. EPS are extracellular polymeric substances of biological origin that are produced by many microorganisms in order to protect the cell against environmental stress (Badel et al., 2011; Donot et al., 2012). EPS from microorganisms such as xanthan have been used in food production for decades (Yemenicioğlu, Farris, Turkyilmaz, & Gulec, 2020) and attempts to produce EPS *in-situ* in dairy and bakery products were found to be very successful (Nepomuceno, Costa Junior, & Costa, 2016; Tinzl-Malang, Rast, Grattepanche, Sych, & Lacroix, 2015; Lanjun Zhang et al., 2016). EPS producing strains including lactic acid bacteria (and isolated EPS) were shown to be suitable viscosity- and texture modifiers being able to improve the quality of yoghurt, fat-reduced cheese and sourdough (Dabour, Kheadr, Benhamou, Fliss, & Lapointe, 2006; Galle, Schwab, Arendt, & Gänzle, 2010; Güler-Akin, Serdar Akin, & Korkmaz, 2009). For instance, *L. curvatus* TMW 1.624, which has been isolated from fermented sausages, was found to improve the texture of gluten-free breads due to the formation of sufficient amounts of dextran (Rühmkorf, Rübsam, et al., 2012). With respect to the structure of EPS, one can distinguish between heteropolysaccharides (HePS) consisting of various sugars, and homopolysaccharides (HoPS) that are composed of only one monomer type, usually D-glucose or L-fructose. HePS are often charged and highly branched and are synthesized in an energy-demanding process (de Vuyst & Degeest, 1999; Donot et al., 2012). Due to their more complex structure, less HePS than HoPS are usually needed to cause visible effects in food matrices. For more insights on EPS synthesis we recommend the reviews provided by (Jolly et al., 2002) and Loeffler, Hilbig, Velasco, and Weiss (2020). Recently, *in-situ* EPS-forming strains have become even more popular as they are expected to meet the consumer demand for clean-labelled products and were found to have health-promoting effects (Angelin & Kavitha, 2020; Asioli et al., 2017). The application of *in-situ* EPS-forming strains to meat products is a very new field of research. EPS-forming strains including *Lactobacillus plantarum*, *L. curvatus*, and *L. sakei* are species of technological interest in meat processing (Bredholt, Nesbakken, & Holck, 2001; Cocolin et al., 2011). Depending on the meat product, the presence of EPS may have a different influence on the overall product quality. While for

reconstructed ham, the presence of EPS may enhance the water binding capacity, leading to a juicer product, the situation is different for raw fermented sausages, where EPS may influence the drying behavior or lead to products with different texture properties. To date, however, only very limited research has been conducted that focuses on the influence of microbial *in-situ* EPS formation in meat products. A study done by Prechtl et al. (2018a) showed that some EPS-forming lactic acid bacteria have the ability to tolerate cold- and salt stress when incubated in growth media and are still able to produce EPS under those stress conditions. However, in a study done by Hilbig, Loeffler, et al. (2019a) it was found that the combination of high salt stress and cold temperature (2°C) (cooked ham model system; <2% fat) leads to a remarkable decrease in EPS production. While raw fermented sausages contain nitrite curing salt (salt stress), they are usually fermented and dried at higher temperatures, which may contribute to a more pronounced EPS-synthesis. First studies revealed the potential of EPS-producing strains in spreadable raw fermented sausages (Hilbig, Gisder, et al., 2019). However, very little is known about the use of these cultures in sliceable raw fermented sausages, which have a different product matrix.

For this reason, the growth and fermentation kinetics as well as the EPS-forming ability of different lactic acid bacteria, including the HePS-forming strains *L. plantarum* TMW 1.25 and TMW 1.1478 as well as the HoPS-forming strains *L. curvatus* TMW 1.624 and *L. sakei* TMW 1.411, were analyzed in a raw sausage model system under typical fermentation conditions (25 °C) with the aim of finding strains that appear suitable for raw sausage production. To ensure that the target pH of raw fermented sausages (4.8-5.3) is achieved even though the same sugar source is used for EPS production, the first intermediate objective of this study was to evaluate what sugar concentration is sufficient to achieve an acceptable product pH during fermentation. In addition, the study aimed to visualize the distribution of EPS in the raw sausage meat matrix and to establish a first semi-quantitative relationship between fermentation time and EPS production.

2.2 Materials and Methods

2.2.1 Materials and equipment

2.2.1.1 Microbiological culture medium and chemicals

De Man, Rogosa and Sharpe (MRS) broth and agar (peptone from casein 10.0 g/L, meat extract 10.0 g/L, yeast extract 4.0 g/L; D (+)-glucose 20.0 g/L, dipotassium hydrogen phosphate 2.0 g/L, Tween® 80 1.0 g/L, di-ammonium hydrogen citrate 2.0 g/L, sodium acetate 5.0 g/L, magnesium sulfate 0.2 g/L, and manganese sulfate 0.04 g/L, ± agar-agar 14.0

g/L) and Anaerocult® were purchased from Merck KGaA (Darmstadt, Germany). Peptone water (potassium dihydrogen phosphate* 3.6 g/L, disodium hydrogen phosphate dehydrate 7.2 g/L, sodium chloride 4.3 g/L, and casein peptone 1.0 g/L) was purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Plate count agar (PCA; agar 15.0 g/L, glucose 1.0 g/L, peptones 5.0 g/L, and yeast extract 2.5 g/L) was obtained from AppliChem GmbH (Darmstadt, Germany). Calcofluor White Stain and Concanavalin A were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany.

2.2.1.2 Starter cultures

The HePS- and HoPS-forming strains used in this study were provided by the Technical University of Munich (Department of Technical Microbiology, Freising, Germany) and were selected according to their ability to produce EPS in model media under salt stress conditions [21]. All strains were maintained at -80 °C in 25 wt% glycerol and reactivated on MRS agar prior to the experiments. The strains used and their incubation conditions are listed in **Table 3**.

Ingredients for the raw sausage model system preparation

Lean pork meat and pork back fat were purchased at MEGA (Fach-Zentrum für die Metzgerei und Gastronomie eG. Stuttgart, Germany) and standardized to S II and S VIII. Nitrite curing salt was provided by Südsalz GmbH, (Heilbronn, Germany), ascorbic acid was purchased from Gewürzmüller, (Korntal-Münchingen, Germany), and dextrose as well sucrose was obtained from Südzucker AG (Mannheim, Germany).

2.2.2 Methods

2.2.2.1 Bacterial growth kinetics

In order to reach similar inoculation concentrations in the raw sausage model systems, the growth curves of all strains (**Table 3**) were initially examined in MRS broth over a period of 48 h. Reactivated cultures of *L. plantarum* TMW 1.1478 and 1.25, *L. curvatus* TMW 1.624, and *L. sakei* TMW 1.411 were used to investigate bacterial growth kinetics in MRS broth under optimal incubation conditions (48 h at 30 °C). Bacterial counts were determined at intervals of 3 hours using a spiral platter (Don Whitley Scientific Limited, West Yorkshire, UK). Plates were incubated at 30 °C for 24 h - 48 h under anaerobic conditions, and colonies then counted using an automatic colony counter (Acolyte, Synbiosis, Cambridge, UK). The values obtained for each strain were plotted against the incubation time. Assays were performed in triplicate.

Table 3 Investigated starter cultures.

Bacterial strain	*TMW	EPS	Incubation conditions (MRS broth / model)
Lactobacillus sakei	1.114	HoPS	30 °C / 25 °C
Lactobacillus plantarum	1.25	HePS	30 °C / 25 °C
Lactobacillus curvatus	1.624	HoPS	30 °C / 25 °C
Lactobacillus plantarum	1.1478	HePS	30 °C / 25 °C

*TMW: Technical University of Munich (Department of Technical Microbiology, Freising, Germany)

2.2.2.2 Raw sausage model system preparation

The raw sausage model systems were prepared as follows: 25% pork fat S VIII, 75% lean pork meat SII (partially frozen / grounded; 3 mm), ascorbic acid (0.5 g/kg), sugar (2.5 to 10 g/kg dextrose or sucrose, depending on the strain used), lactic acid bacteria, and finally nitrite curing salt (28 g/kg) were mixed in a bowl chopper (K 64 DC; Seydelmann, Aalen, Germany). The initial concentration of the starter cultures was 10^6 CFU/g. Bacterial strains of *L. plantarum* were diluted before inoculation, whereas the HoPS forming strains could be used without further preparation. The raw sausage mass was then stuffed into plastic containers (~ 125 g) and sealed. The samples were vacuumed three times (19 mbar) using a vacuum machine (BOSS Type RC 63, Helmut Boss Bad Homburg, Germany) to evacuate the air. The raw sausage model systems were then stored at 25 °C and 90-80% relative humidity (RH) for 48 h.

In the initial experiments, we screened for the optimal sugar concentration (2.5 to 10 g/kg dextrose or sucrose) needed to reach a "product" – pH value of around 4.8 – 5.3. All following experiments were then performed with the most suitable sugar concentration (5 g/kg dextrose for HePS- and 5 g/kg sucrose for HoPS-producing strains) in order to further study bacterial growth and EPS formation in the raw sausage model systems.

2.2.2.3 Microbiological analysis of the meat matrix

The microbiological analysis of the raw sausage model systems was conducted at 0, 6, 10, 24, and 48 h of storage at 25 °C. To obtain viable cell counts, 10 g of sample was taken aseptically from the core of the raw sausage model system and mixed with 90 mL of buffered peptone water for 1 min using a Masticator (Laborhomogenisator, IUL Instrument GmbH, Königswinter, Germany). Appropriate dilutions were plated on MRS agar using an automated spiral plater (Don Whitley Scientific, West Yorkshire, UK). In order to get an overview of the meat quality, aerobic cell counts were also determined using the same procedure, but PCA

instead of MRS agar. The plates were then incubated at 30 °C for 24 h under either anaerobic (MRS) or aerobic (PCA) conditions and colonies again counted using an automatic colony counter (Acolyte, Synbiosis, Cambridge, UK).

2.2.2.4 pH measurement

The pH measurement was conducted using a pH meter (WTW Microprocessor pH Meter, WTW GmbH, Weilheim, Germany). For the experiments that aimed to determine the appropriate sugar concentration to reach a final pH of 4.8 – 5.3, the pH was recorded at the same time as the viable cell counts were determined. In the following experiments (focus on EPS production) the pH values of the raw sausage model systems were recorded after 0, 6, 10, 24 and 48 h of storage at 25 °C.

2.2.2.5 Qualitative and semi-quantitative EPS analysis using CLSM and MATLAB

A qualitative assessment of the produced EPS was performed by confocal laser scanning microscopy (CLSM) after 0, 6, 10, 24, and 48 h in the raw sausage model systems that have been prepared with the ingredients listed in section **2.2.2.2** and the selected sugar concentration of either 5 g/kg dextrose (substrate for HePS-forming bacteria) or sucrose (substrate for HoPS-forming bacteria). Samples (0.5 cm high, 1.5 cm wide) were always taken from the core of the raw sausage matrixes, placed on a concave plastic slide and EPS subsequently stained with 10 µL of Concanavalin A (working solution: 1:20 diluted stock solution containing 1 mg of dye and 1 mL of 10 mM phosphate buffer; pH 6). The samples were then kept in the dark at 12 °C for 60 min, followed by a second staining step with 10 µL of Calcofluor White Stain (protein stain). Samples were then examined using a Nikon Eclipse-Ti Inverse Microscope D- Eclipse C1 (Nikon GmbH, Düsseldorf, Germany) equipped with a C1 laser box (10 mW argon ion laser, 2.0 mW helium-neon laser, 25 mW diode laser). A 60-fold magnification lens (Nikon, Plan Flour) with immersion oil (Nikon, Plan Apo VC) was used to examine the stained meat samples. An argon laser, at 488 nm, and a red helium-neon laser, at 638 nm, were used for the excitation of EPS (green) and proteins (blue). At least 6 pictures of each sample were taken and further processed by creating a RGB picture using the EZ-C1 3.70 Imaging Software (Nikon GmbH, Düsseldorf, Germany), followed by a semi-quantitative data interpretation using MATLAB (The Math Works, Inc. version R2013b 8.2.0.701) according to a method developed by Bosse, Gibis, Schmidt, and Weiss (2015). For a better interpretation of the EPS formed, the green channel of the picture was automatically transformed to a black-and-white picture and only pixels with a threshold over 0.08 were

counted to remove noise. In the resulting picture, the percentage of the green area (EPS) compared to the whole area was calculated using **Equation 1**:

$$\text{Green area (\%)} = \frac{\text{green area (pixels)}}{\text{total area (pixels)}} \cdot 100\% \quad (\mathbf{1})$$

2.2.3 Statistical Analysis

All measurements were repeated at least 3 times using duplicate samples. Means and standard deviations were calculated using Excel 2013 (Microsoft, Redmond, WA, USA).

2.3 Results

2.3.1 Growth kinetics of lactic acid bacteria in MRS broth

The growth characterization of the strains used was performed in MRS broth with the aim to standardize the incubation and inoculation procedure for the following studies in raw fermented meat model systems. The growth curves obtained for the four strains are shown in **Figure 2**. The time required for each strain to reach the stationary phase was 24 h. Viable cell counts determined for the two HePS-forming strains *L. plantarum* 1.1478 and 1.25 were approx. 2 log higher than for the HoPS-producing strains *L. curvatus* 1.624 and *L. sakei* 1.411.

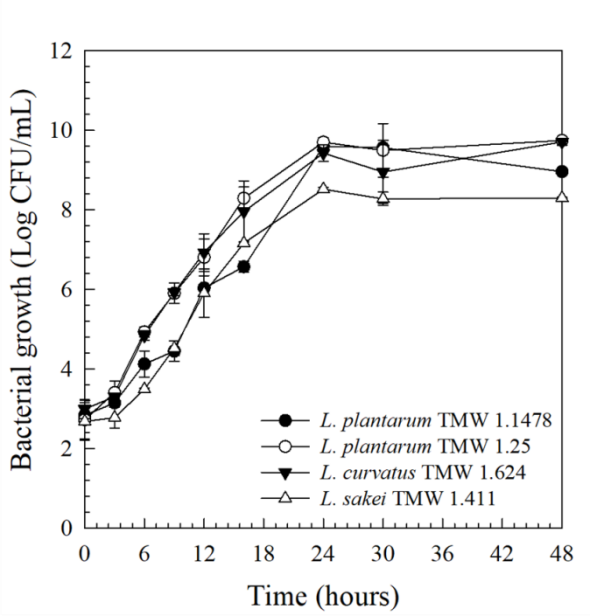

Figure 2 Growth kinetics of *L. plantarum* TMW 1.1478, *L plantarum* TMW 1.25, *L. curvatus* TMW 1.624, and *L. sakei* TMW 1.411 in MRS broth (30 °C) over a period of 48 h; n = 4 measurements.

2.3.2 Influence of sugar content on pH development (preliminary study)

The pH value of raw fermented sausages is crucial with regard to product quality and safety, and should be in the range of pH 4.8-5.3. For this purpose, raw sausage model systems were produced that contained either 2.5 - 10 g/kg dextrose and the HePS-producing strain *L. plantarum* 1.1478, or 2.5 - 10 g/kg sucrose and the HoPS-producing strain *L. curvatus* 1.624. Samples were stored at 25 °C and the pH development recorded over a period of 48 h (**Figure 3 A** and **B**).

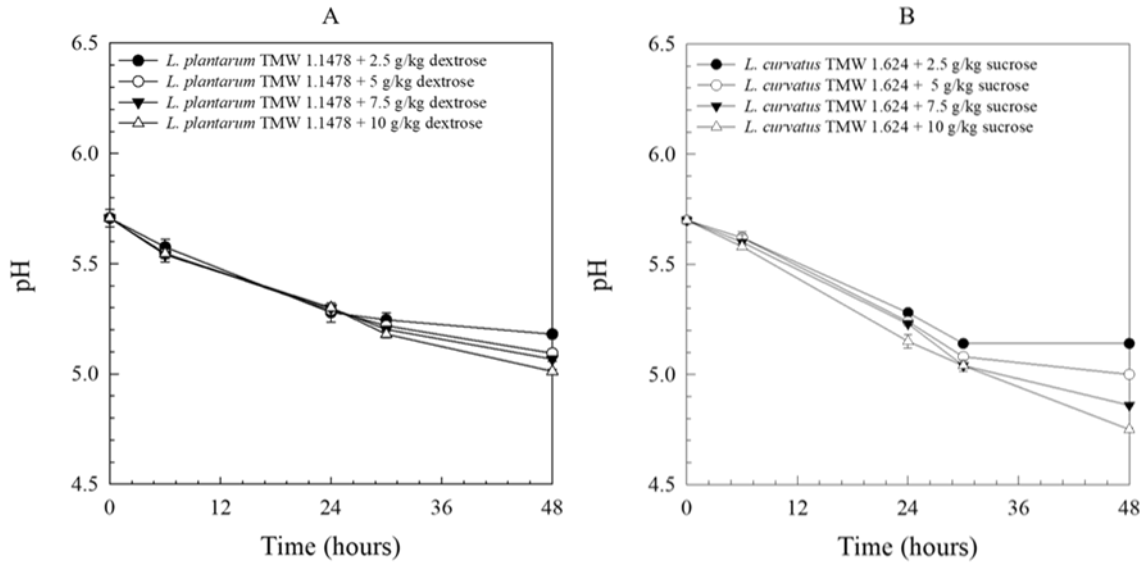

Figure 3 pH values determined in the raw sausage model system containing either *L. plantarum* TMW 1.1478 and 2.5-10 g/kg dextrose (A) or *L. curvatus* TMW 1.624 and 2.5-10 g/kg sucrose (B); n = 4 measurements.

In both cases a sugar concentration of 5 g/kg was sufficient to reach a final pH of 5.09 or 5.00 in samples containing *L. plantarum* 1.1478 or *L. curvatus* 1.624, respectively. This sugar concentration was thus used for all further studies.

2.3.2 Bacterial growth and pH development in the raw sausage model system

Prior to the inoculation with starter cultures, the raw meat quality was assessed by determining viable cell counts on PCA agar. The bacterial counts ranged between Log 2 and Log 3 CFU/g, indicating a good raw material quality. The growth curves of *L. plantarum* 1.1478 and 1.25, *L. curvatus* 1.624, and *L. sakei* 1.411 determined in the raw sausage model systems, containing either 5 g/kg dextrose or sucrose, respectively, are shown in **Figure 4**, and the corresponding pH values after 0, 6, 10, 24, and 48 h of storage at 25 °C are presented in **Table 4**. Bacterial counts determined in the stationary growth phase were found to be very similar among the strains investigated (Log 9.40 – Log 10.10 CFU/g), whereas different pH values were determined in the meat model systems depending on the starter cultures used. The pH values after 48 h of incubation at 25 °C in meat model systems containing *L. plantarum* ssp., *L. curvatus* 1.624 or *L. sakei* 1.411 were 5.36, 5.27 and 5.46, respectively, and thus slightly higher than in the previous experiments.

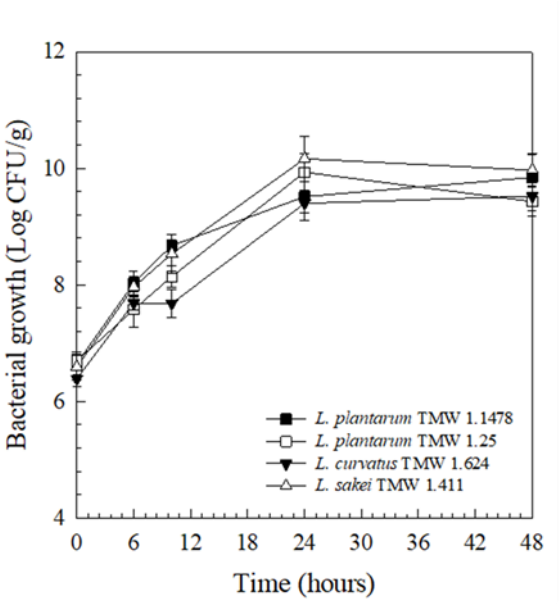

Figure 4 Viable counts of *L. plantarum* TMW 1.1478, *L. plantarum* TMW 1.25, *L. curvatus* TMW 1.624, and *L. sakei* TMW 1.411 determined over 48 h at 25 °C in a raw fermented sausage model system containing 5 g/kg sugar (dextrose or sucrose); initial inoculation concentration: ~10^6 CFU/g; n= 4 measurements.

Table 4 pH development in raw fermented sausage model systems (48 h, 25 °C) in presence of either *L. plantarum* TMW 1.1478 or TMW 1.25 and 5 g/kg dextrose or in presence of *L. curvatus* TMW 1.624 or *L. sakei* TMW 1.411 and 5 g/kg sucrose.

Time (h)	*L. plantarum* TMW 1.1478		*L. plantarum* TMW 1.25		*L. curvatus* TMW 1.624		*L. sakei* TMW 1.411	
	pH							
0	5.69	± 0.04	5.60	± 0.02	5.78	± 0.02	5.59	± 0.04
6	5.63	± 0.04	5.58	± 0.01	5.66	± 0.02	5.57	± 0.01
10	5.64	± 0.01	5.53	± 0.01	5.58	± 0.03	5.52	± 0.02
24	5.61	± 0.02	5.52	± 0.03	5.48	± 0.01	5.53	± 0.02
48	5.37	± 0.02	5.36	± 0.03	5.27	± 0.03	5.46	± 0.01

2.3.4 Detection and quantification of EPS formed in the raw sausage model system

Figure 5 exemplarily shows CLSM images of raw sausage model systems containing *L. plantarum* 1.1478 and 5 g/kg dextrose. The samples were stored over a period of 48 h at 25 °C and investigated for the presence of *in-situ*-produced EPS after 0, 10, 24, and 48 h using CLSM. The EPS (green) were stained with Concanavalin A and the proteins (blue) with Calcoflour White Stain. In a preliminary study, the fat particles were also stained using Nile Red. However, the EPS were found to be located only on the outer edges of the protein surfaces, and therefore further experiments were conducted without staining the fat phase. As

can be seen in **Figure 5** *L. plantarum* 1.1478 was able to produce EPS in the raw sausage model system with amounts of *in-situ* produced EPS increasing from 0 to 24 h of storage. The small amounts of EPS that could be detected directly after production of the model systems (0 h) may be attributed to the presence of the autochthonous meat microflora (Log 2 – Log 3 CFU/g).

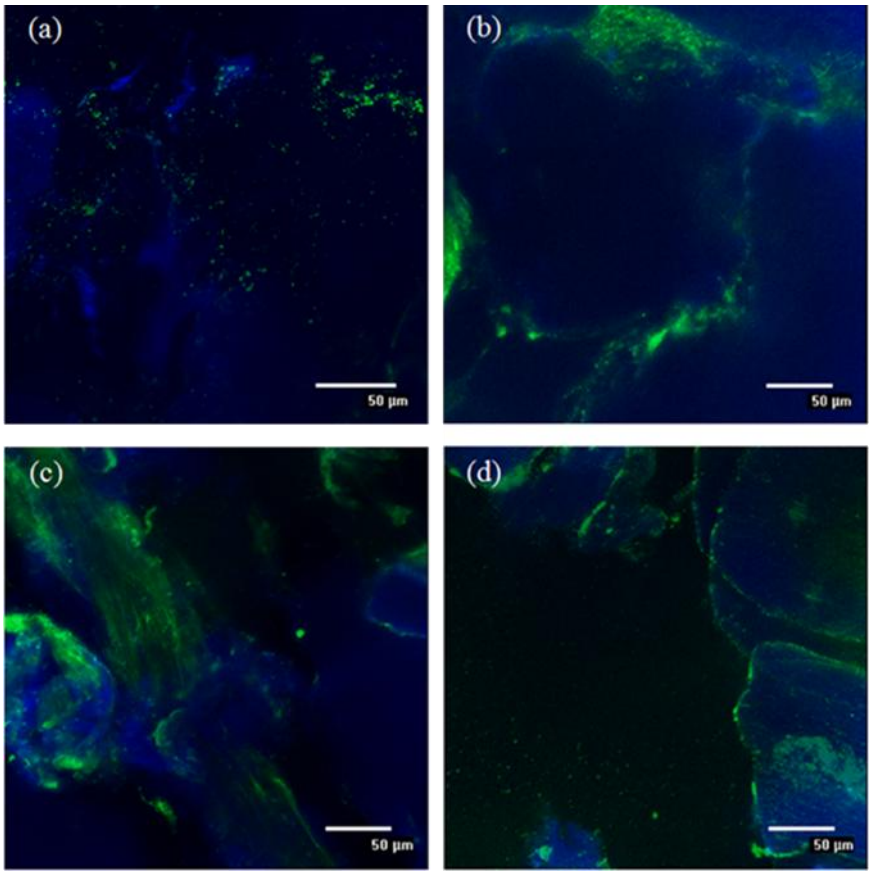

Figure 5 Raw fermented sausage model system containing heteropolysaccharide (HePS)-forming *L. plantarum* 1.1478 (initial inoculation concentration: ~10^6 CFU/g) and 5 g/kg dextrose. Sample (a) is the model system after production (0 h), whereas sample (b), (c), and (d) were stored for 10 h, 24 h, and 48 h at 25 °C, respectively. EPS are stained green (Concanavalin A) and proteins are stained blue (Calcofluor White Stain); n=6 pictures.

Table 5 and **6** summarize the corresponding results of the semi-quantitative MATLAB analyses. The detected amount of EPS expressed as mean green area (%) increased remarkably within the first 10 to 24 h of storage at 25 °C, independent of whether *L. plantarum* or *L. curvatus* was used, which correlates well with the results of the qualitative image analyses. The decrease observed in EPS after 48 h of incubation may be traced back to an enzymatic degradation (B. Degeest et al., 2001; Pham et al., 2000). In contrast to the other strains, the HoPS-forming strain *L. sakei* 1.411 formed EPS only to a very small extent and at a later time, and the pH value determined in the raw sausage model matrix was also somewhat

higher than that of the other lactic acid bacteria at the typical initial sugar concentration of 5 g/kg. For these reasons, *L. sakei* 1.411 appeared to be less suitable for a prospective product trial, at least in the present model system, compared to the other strains studied. However, the results could also be indicative of a less vital strain (at the time of the experiments).

Table 5 Results of the image analysis of CLSM pictures of raw fermented sausage model systems containing *L. plantarum* 1.1478 (initial concentration ~10^6 CFU/g) and 5 g/kg dextrose. Samples were stored at 25 °C over a period of 48 h. The green area represents the amount of detected EPS presented in Figure 5.

Sample	Storage time (h) at 25 °C	Green area picture (%)	Mean green area* (%)	Standard deviation area (%)
Picture A	0	5.06	4.32	1.47
Picture B	10	30.91	14.16	11.23
Picture C	24	29.60	33.35	7.70
Picture D	48	35.23	20.66	18.00

Table 6 Results of the image analysis of CLSM pictures of raw fermented sausage model systems containing either *L. curvatus* 1.624 or *L. sakei* 1.411 (initial concentration ~10^6 CFU/g) and 5 g/kg sucrose or *L. plantarum* 1.25 (initial concentration ~10^6 CFU/g) and 5 g/kg dextrose. Samples were stored at 25 °C over a period of 48 h. The green area represents the amount of detected EPS.

Strain	Storage time (h) at 25 °C	Mean green area* (%)	Standard deviation area (%)
L. curvatus 1.624	0	5.50	3.86
	10	27.67	5.40
	24	18.70	12.78
	48	8.76	7.53
L. plantarum 1.25	0	5.66	1.71
	10	10.31	6.94
	24	12.13	5.53
	48	6.67	4.17
L. sakei 1.411	0	6.97	3.26
	10	3.97	1.22
	24	4.96	2.97
	48	13.96	8.00

2.4 Discussion

In appropriate amounts, *in-situ*-produced EPS have already been found to improve the product properties of dairy- and of bakery products (A. N. Hassan & Awad, 2005; Rühmkorf,

Rübsam, et al., 2012). While the use of EPS-producing strains or isolated EPS is already quite common in these industries, the application of potentially EPS-forming starter cultures, the associated formation of EPS and their influence on the product quality of meat products has just emerged as a promising research area. In the meat industry, hydrocolloids are usually applied to improve the texture properties and quality of certain products, although their usage has to be labeled (Yemenicioğlu et al., 2020). Having a closer look to the potential of microbial *in-situ*-EPS formation in meat matrices may thus not only lead to an improved product quality, or even new product developments, but would also contribute to the green-labeling philosophy, which is demanded by the majority of consumers.

EPS are usually produced under certain stress conditions in order to protect the microorganisms (Czaczyk & Myszka, 2007; Wingender et al., 2012). These include non-optimal pH values and temperature ranges, unfavorable osmotic pressure conditions, as well as an insufficient availability of carbon and nitrogen sources (Hilbig, Gisder, et al.; Loeffler et al., 2020; Torino, Hébert, Mozzi, & Font de Valdez, 2005). In the case of raw fermented sausages, the formation of EPS may be, amongst other things, induced through the presence of nitrite curing salt. For instance, Hufner et al. (2007) studied the influence of curing salt on the growth behavior on *L. sakei*, and concluded that the presence of curing salt induces an increase in the glycolytic enzymes that contribute to EPS synthesis. Moreover, in a study done by Quesada, Béjar, and Calvo (1993), it was shown that the amount of EPS produced by *Volcaniella eurihalina* increased from 0.4 g/L to 3 g/L when the salt content in the malt yeast medium was increased from 0 to 10% wt/vol. In the present study, it was shown that all strains studied, more specifically *L. plantarum* 1.1478 and 1.25, *L. curvatus* 1.624, and *L. sakei* 1.411, are able to grow in a raw fermented sausage model system (**Figure 4**) while reducing the pH (**Table 4**) and producing EPS (**Figure 5; Table 5-6**). Moreover, according to the semi-quantitative data interpretation, the amounts of formed EPS were found to be usually higher than those determined in a cooked ham model system that contained one of the respective strains but was ripened at 2 °C (Hilbig, Loeffler, et al., 2019a). The growth of microorganisms and the biosynthesis of EPS is inhibited at low (pH 2-3) and at high pH values (pH ≥ 10) and optimal between pH 5.5 - 6.5 (Czaczyk & Myszka, 2007). For instance, *Lactobacillus reuteri* LB 121 was found to produce higher amounts of EPS at the pH value of 5.8, as compared to a pH value of 4.8 in a modified MRS media containing 100 g/L glucose (van Geel-Schutten et al., 1998). Minervini et al. (2010) who investigated the potential of *L. curvatus* DPPMA 10 to produce EPS in MRS broth also found this relationship. In the present study, the pH values were found to be in the optimal range for EPS formation and raw

fermented sausage production (pH 4.8 – 5.3) when 5 g/kg of dextrose or sucrose (depending on the strain) were used (**Figure 3; Table 3**).

Different methods have been reported that deal with the qualitative and quantitative determination of EPS. A summary of the commonly applied methods can be found in the review prepared by Loeffler et al. (2020). The combination of a qualitative EPS analysis using CLSM (**Figure 5**) with the semi-quantitative approach using MATLAB (**Table 5-6**) provided a good first impression of EPS-formation and distribution in complex meat matrices. The images and calculated values showed that EPS production is mainly correlated with the exponential growth phase of the studied strains. The results also showed that EPS production depends not only on the food matrix but also on the strain used. The correlation between bacterial growth and EPS formation has also been studied and demonstrated for other Lactobacilli strains (Gamar-Nourani, Blondeau, & Simonet, 2004; van den Berg et al., 1995). The analyses of the raw sausage model systems directly after production indicated a good raw material quality. However, the presence of the autochthonous meat microflora (Log 2 – Log 3 CFU/g) and the applied lactic acid bacteria solution at the beginning of the experiment may explain the appearance of small amounts of EPS directly after production (**Figure 5**). Frequently isolated bacteria species of meat products that are capable of producing EPS in meat matrices include *Pseudomonas* spp. and *Lactobacillus* spp. (Hufner et al., 2007; F. Leroy, Verluyten, & De Vuyst, 2006; Ian W. Sutherland, 2001). Moreover, in a previous study done by Hilbig, Loeffler, et al. (2019a) it was proven, that an increase in *in-situ* formed EPS can only be observed when both an EPS-forming strain (~10^6 CFU/g) and the respective sugar substrate were added to the meat matrix (*proof of concept*). Although the qualitative EPS determination using CLSM gives a very good first impression of *in-situ* EPS production and distribution, the method has some disadvantages that are related to the very complex and heterogeneous meat matrix just allowing derivation of semi-quantitative data (MATLAB) since the EPS are not evenly distributed throughout the samples. Reduced amounts of EPS could often be observed in meat samples that were investigated after 48 h of storage at 25 °C (**Table 5 and 6**). This phenomenon has already been reported in other studies and could be attributed to a potential enzymatic EPS degradation (de Vuyst & Degeest, 1999).

2.5 Conclusion

Overall, the study clearly shows that the investigated strains have the ability to produce EPS in raw sausage model systems and at the same time generate acid to lower the pH to 4.9-5.3, which would be a requirement for the production of raw fermented sausage. Based on the

obtained results, the HePS-producing strain *L. plantarum* 1.1478 seems to be very promising in terms of potential application trials in salami, considering both pH development and EPS formation. Since HePS are often charged and have a more complex structure than HoPS, interactions with the protein matrix and thus structural changes are very likely to occur even at very low levels. The interactions of proteins and polysaccharides could thereby have both positive and/or negative effects on the final products.

CHAPTER 3

Influence of Microbial *In-Situ* Heteropolysaccharide Production on Textural Properties of Raw Fermented Sausages (Salami)

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Abstract

The purpose of the study was to investigate the influence of a heteropolysacchride (HePS)-forming lactic acid bacteria (LAB) on the quality attributes of raw fermented sausages.

Therefore, salamis with the HePS-producing strain *L. plantarum* TMW 1.1478 or the non-EPS-producing strain *L. sakei* TMW 1.2037 (control) were manufactured using two different inoculation concentrations: more precisely, 10^7 CFU/g (typical starter culture concentration) or 10^9 CFU/g. Growth behavior, a_w and pH development were recorded until a weight loss of 31% was reached and *in-situ*-formed EPS detected using confocal laser scanning microscopy. Moreover, the influence of the HePS formed on texture (texture profile analysis; TPA) and sensory attributes (26 panelists, ranking test) was investigated.

The final products containing *L. plantarum* TMW 1.1478 were found to be significantly softer *($p < 0.05$)* than the respective control samples, an effect that was even more pronounced at the higher inoculation level of 10^9 CFU/g. The semi-quantitative data interpretation of the CLSM pictures revealed that the EPS were predominantly formed during the first 72 h of fermentation at 24 °C until the final pH of 4.95 ± 0.05 was reached (stationary phase). The sensory evaluation (consistency) was in accordance with the TPA results and taste was not negatively influenced by the HePS-forming strain. Results clearly indicate that EPS-producing LAB can have a negative influence on the quality of raw fermented sausages. However, these strains (in the present case *L. plantarum* TMW 1.1478) might be interesting for application in the field of spreadable raw sausage manufacturing.

3.1 Introduction

In raw fermented sausage production, starter cultures are applied to guarantee that the final products have a high sensory and microbial quality(Cocolin et al., 2011; Devine & Dikeman, 2014). As a substrate, sugar is usually added during processing, which is then metabolized to e.g. lactic acid during fermentation, leading to a decrease in pH (Frédéric Leroy & de Vuyst, 2004). However, depending on the strain and type of sugar used, lactobacilli may have the ability to also form exopolysaccharides (EPS) which, provided the amount formed is high enough, may lead to structural and, hence, textural changes of the final products (Yilmaz et al., 2015). This phenomenon is well-known in the bakery and dairy industries, where EPS-producing starter cultures are frequently used to improve the product characteristics of sourdough (Ua-Arak, Jakob, & Vogel, 2015), (fat-reduced) yoghurts, and cheeses (Badel et al., 2011; Nepomuceno, Costa Junior, et al., 2016; Lanjun Zhang et al., 2016) and was lately also reported for legume protein-rich foods (Yan Xu et al., 2019). Most microbial EPS are highly soluble in water or in diluted salt solutions and can be divided into two different groups: homopolysaccharides (HoPS) and heteropolysaccharides (HePS). The latter are synthesized out of a variety of different substrates, whereas HoPS are generally synthesized out of sucrose (J. Wang et al., 2015). EPS are often formed under suboptimal or so-called "stress conditions", including temperature or salt stress, in order to protect the microorganisms (Prechtl et al., 2018a; Wingender et al., 2012). The *in-situ* formation, however, does not only depend on the processing conditions, but also on the strain (concentration) used, growth phase, and availability of nutrients as well as their distribution (Kumar, Mody, & Jha, 2007; Prechtl, Wefers, Jakob, & Vogel, 2018b). Very few studies have, as yet, focused on *in-situ* EPS formation in meat matrices, including the one from Hilbig et al. (2019a) who investigated EPS formation in a cooked ham model system. In their study, general requirements of EPS formation in meat matrices were also investigated with a special focus on different sugar sources and processing conditions (simulation of tumbling conditions at 2 °C and a reduced stress level at 15 °C). So far, there are only 2 other studies available in the current literature focusing on *in-situ* EPS production in raw fermented sausages. The one from Dertli et al. (2016), which examined the influence of EPS-producing lactic acid bacteria (LAB) on sucuk, a Turkish–type fermented sausage, and the work done by Hilbig, Gisder, et al. (2019), which focused on the influence of *in-situ* EPS production on the properties of fat-reduced Teewurst. Although, in the first case, the products were found to be tougher, harder, and less adhesive compared to sucuk without an EPS-forming culture, the opposite was true for fat-reduced Teewurst (up to 50%), in which the *in-situ* formed EPS

maintained the spreadability of the products and improved the mouthfeel. This shows that EPS-forming LAB can have very different effects, depending on the strain, processing conditions and meat matrix used. While an earlier hardening of raw fermented products could reduce the production time, a softening effect as seen for fat-reduced Teewurst would lead to a final product with undesirable properties. For this reason, the present study aimed to clarify whether a HePS-forming starter culture (*L. plantarum* TMW 1.1478) is able to produce sufficient amounts of EPS in salami (typical raw fermented sausage), while reducing the pH of the product during fermentation, in order to have an influence on the product properties. In this course, the influence of different inoculation concentrations (10^7 and 10^9 CFU/mL) was also investigated and all results compared to products containing a non-EPS forming culture (*L. sakei* TMW 1.2037). Following the methods of Hilbig, Loeffler, et al. (2019a), it was decided to use a qualitative (information about the location) and semi-quantitative approach for EPS detection via CLSM, since salami contains a lot more fat than cooked ham, which may influence the location and distribution of EPS in the meat matrix.

3.2 Materials

3.2.1 Media and chemicals

MRS agar and broth (peptone from casein 10.0 g/L, meat extract 10.0 g/L, yeast extract 4.0 g/L; D (+)-glucose 20.0 g/L, dipotassium hydrogen phosphate 2.0 g/L, Tween® 80 1.0 g/L, di-ammonium hydrogen citrate 2.0 g/L, sodium acetate 5.0 g/L, magnesium sulfate 0.2 g/L, and manganese sulfate 0.04 g/L, ± agar-agar 14.0 g/L), as well as Anaerocult® were purchased from Merck KGaA (Darmstadt, Germany). Peptone water (pH 7.0±0.2; 5 g/L) was purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Plate Count Agar (agar 15.0 g/L, glucose 1.0 g/L, peptones 5.0 g/L, and yeast extract 2.5 g/L) was obtained from AppliChem GmbH (Darmstadt, Germany). Calcofluor White Stain and Concanavalin A were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

All microbiological media were prepared as specified by the respective manufacturers and autoclaved for 15 minutes at 121 °C.

3.2.2 Starter cultures

Lactobacillus plantarum TMW 1.1478 (HePS-forming strain; henceforth referred to as *L. plantarum* 1.1478) and *Lactobacillus sakei* TMW 1.2037 (control strain; henceforth referred to as *L. sakei* 1.2037) were provided by the Technical University of Freising (Department of Technical Microbiology, Munich, Germany). The bacterial strains were stored in MRS broth

containing 25% (v/v) glycerol at -70 °C and reactivated on MRS agar prior to the experiments followed by cultivation in MRS broth at 30 °C for 48 h. Starter cultures were subsequently used in two different concentrations, more precisely at ~10^7 CFU/g and at ~10^9 CFU/g meat. In order to reach higher inoculation concentrations, the incubated MRS solution was centrifuged (Z32HK, Hermle Labortechnik GmbH, Wehingen, Germany) at 5000 rpm for 10 min and 20 °C and the resulting pellets then suspended in small aliquots of water.

3.2.3 Ingredients for raw sausage production

Lean pork meat (S II) and pork back fat (S VIII) were purchased at MEGA (Fachzentrum für die Metzgerei und Gastronomie eG. Stuttgart, Germany). Nitrite curing salt (NCS) was provided by Südsalz GmbH, (Heilbronn, Germany), ascorbic acid and black pepper were purchased from Gewürzmüller, (Korntal-Münchingen, Germany), and dextrose, as well as sucrose, was obtained from Südzucker AG (Mannheim, Germany).

3.3 Methods

3.3.1 Raw fermented sausage preparation

Four batches of raw sausages were produced using two different starter cultures as well as different initial bacterial concentrations: more precisely, the non-EPS-forming strain *L. sakei* 1.2037 (control; ~10^7 and 10^9 CFU/g) or the HePS-producing strain *L. plantarum* 1.1478 (~10^7 and 10^9 CFU/g). The meat (from different animals to account for differences in the raw material) batter was prepared in a bowl chopper (type K64DC, Seydelmann, Aalen, Germany) by chopping 20% pork back fat (S VIII, -18 °C) and 45% lean pork meat (S II, -10 °C). Afterwards, 3 g/kg black pepper, 5 g/kg sugar (Südzucker AG, Mannheim, Germany), the respective starter culture (either 10^7 or 10^9 CFU/g), and 0.5 g/kg ascorbate were added and mixed. In the next step, 35% ground lean pork meat (3 mm, + 2°C) and, finally, 28 g/kg nitrite curing salt (NCS) was added and mixed to solubilize the meat proteins and ensure a homogenous distribution of NCS. The different sausage batters were filled into cellulose casings (50 mm diameter; Nalo fasser S, Werner Niedenbeger GmbH, München, Germany) and afterwards ripened for approx. 17 days until a weight loss of 31% was reached (determined by differential weighting). In the first 7 days, the fermentation and ripening was performed at 24 °C, followed by a 10-day period at 14 °C. Additionally, the sausages were smoked after 24, 48 and 72 h of fermentation in a smoking chamber (Maurer, Reichenau, Germany) at 24 °C for 15 min. For analysis purposes, duplicate samples were collected after 0, 24, 48, and 72 h, and after 9, 12, 14, and 17 days of ripening.

3.3.2 Microbiological analysis of raw fermented sausages

Microbiological analysis of the raw sausages was conducted at time 0 (raw material ± starter culture), 24, 48, and 72 h, and after 9, 12, 14, and 17 days of ripening. To obtain viable cell counts of the different products, 10 g of sample was always taken aseptically from the core of the respective raw sausage and subsequently mixed for 1 min with 90 mL of buffered peptone water using a Masticator (Laborhomogenisator, IUL Instrument GmbH, Königswinter, Germany). Appropriate dilutions were plated on plate count agar (PCA; raw material quality) and/or MRS agar using an automated spiral plater (Don Whitley Scientific, West Yorkshire, UK) followed by incubation at 30 °C for 24 h – 48 h under either anaerobic (MRS) or aerobic (PCA) conditions. Subsequently, the colonies were counted using an automatic colony counter (Acolyte, Synbiosis, Cambridge, UK).

3.3.3 pH measurement and a_w determination

The pH measurement was conducted using a pH meter (WTW Microprocessor pH Meter, WTW GmbH, Weilheim, Germany) and the pH values of the different products recorded after 0, 24, 48 and 72 h, as well as after 9, 12, 14, and 17 days of ripening. Before the pH of the dried sausages was measured, the salamis were chopped using a Moulinette. The water activity (a_w) was determined at the same time using the Aqua Lab device (CX-2- Decagon Devices Inc., Pullman, USA). Two independent samples were always analyzed in triplicate.

3.3.4 Visualization of EPS using Confocal Laser Scanning Microscopy (CLSM)

A qualitative assessment of the EPS production was performed after 0, 24, 48, 72 h, and 9, 12, 14, 17 days of ripening using the method developed by Hilbig, Loeffler, et al. (2019a). A cylindrical metal pipe was used to take a sample (0.5 cm high, 1.5 cm wide) from the core of the respective raw fermented sausage, which was then transferred to a concave plastic slide. In order to stain *in-situ*-formed EPS (green), 10 µL of a diluted (1:20) Concanavalin A solution (stock: 5 mg lypolized powder in 5 mL phosphate buffer 10 mmol; pH 6) was added to the meat sample. After keeping it in the dark (12 °C) for 60 min, 10 µL of Calcoflour White Stain was added to stain the proteins (blue). The samples were analyzed using a Nikon Eclipse-Ti Inverse Microscope D- Eclipse C1 (Nikon GmbH, Düsseldorf, Germany). A 60-fold magnification lens (Nikon, Plan Flour) with immersion oil (Nikon, Plan Apo VC) was used to examine the stained meat samples. An argon laser, at 488 nm, and a red helium-neon laser, at 638 nm, were used for the excitation of EPS (green) and proteins (blue). Ten pictures of each sample were taken and further analyzed. Scales were inserted using the ImageJ software (Version 1.4.3.67, National Institutes of Health, Bethesda, MD, USA) after creating

a RGB picture using the EZ-C1 3.70 Imaging Software (Nikon GmbH, Düsseldorf, Germany). The semi-quantitative analysis of the EPS was performed using the MATLAB software (The Math Works, Inc., version R2013b 8.2.0.701) following a method developed by Bosse et al. (2015). Here, the green channel of the picture was automatically transformed to a black-and-white picture and only pixels with a threshold over 0.08 were counted to remove noise. In the resulting picture, the percentage of the green area (EPS) compared to the whole area was calculated using **Equation 1**:

$$\text{Green area (\%)} = \frac{\text{green area (pixels)}}{\text{total area (pixels)}} \cdot 100\% \quad (1)$$

3.3.5 Texture profile analysis

In order to determine textural changes in the raw fermented sausages, a textural profile analysis (TPA) was performed as soon as the sausages achieved 16, 23, 27, and 31% weight loss, on day 9, 12, 14, and 17, respectively. After removing the sausage casings, slicing, and storing the samples for 2 h at 12 °C (same experimental conditions), 15 samples were taken from each batch (2 cm high x 1.5 cm wide) and analyzed in a double compression test (50%; 20 s interval between cycles) at a cross-head speed of 50 mm/min using an Instron device (Model 1011, Instron Engineering Corp., Canton, MA, USA) equipped with a 100 N load cell. The hardness of the samples was determined at the first peak of compression.

3.3.6 Sensory analysis

The sensory evaluation of the raw fermented sausages was performed by 26 untrained panelists. Here, sausages containing either the non-EPS-forming strain *L. sakei* 1.2037 (control) or the HePS-forming culture *L. plantarum* TMW 1.1478 were cut into 1.5 cm thick slices with a diameter of about 3.5 cm and served at room temperature. For the evaluation, a ranking test was performed using a 1 to 4 scale for the evaluation of the following attributes: Consistency (1 hardest; 4 softer), consistency based on preference (1 more preferred; 4 less preferred), and taste based on preference (1 favorite; 4 not preferred).

3.3.7 Statistical analysis

Each experiment was conducted twice and all measurements repeated three times. Means and standard deviations were calculated using Excel (Microsoft, Redmond,WA, USA) and SPSS (IMB SPSS Statistics 24, IBM, Germany) was used to statistically evaluate the results gained. A one – way ANOVA with a post – hoc Tukey’s test was performed to interpret TPA results,

while Duncan was used for the EPS results to investigate significant differences between the samples and the control ($p < 0.05$).

3.4 Results and Discussion

3.4.1 Microbial growth behavior and pH development

The viable cell counts of the raw material used (PCA) ranged between 10^2 – 10^3 CFU/g, indicating a good raw material quality. Typical starter cultures for raw fermented sausage production are LAB, usually used at an inoculation level of 10^6 – 10^7 CFU/g meat (Erkkilä et al., 2001). Among the starter cultures known for EPS-production, *L. plantarum* has been widely studied (Dertli et al., 2016; Fontana, Li, Yang, & Widmalm, 2015; Guidone et al., 2014; Prechtl et al., 2018b; Tallon et al., 2003) and was hence selected for the present investigation. In order to examine the influence of a typical (10^6-10^7 CFU/g) and an increased (10^9 CFU/g) inoculation concentration on EPS-formation, and hence on the quality parameters of raw fermented sausages, the HePS-forming strain *L. plantarum* 1.1478 was inoculated accordingly and its growth studied over a period of 17 days (**Figure 6**). As a control, the non-EPS-forming strain *L. sakei* 1.2037 was used. The growth behavior of both strains was found to be very similar, with anaerobic cell counts of *L. plantarum* 1.1478 decreasing at the end of storage (**Figure 6).** The same growth behavior could be observed in the repetition of the experiment (data not shown). LAB need fermentable sugars, which are then metabolized to organic acids leading to a decrease in the product's pH (F. Leroy et al., 2006). This can also be seen in the present study, where the initial pH of 5,66 ± 0.06 decreased to a final pH (31% weight loss reached) of 4.89 ± 0.01 for *L. sakei* 1.2037 and of 5.035 ± 0.01 for *L. plantarum* 1.1478, respectively, independent of the inoculation concentration used (**Figure 6**). The water activity (a_w) of all sausages decreased during the 17 days of observation from 0.93 (raw meat) to 0.85 ± 0.03 (final product; 31% weight loss).

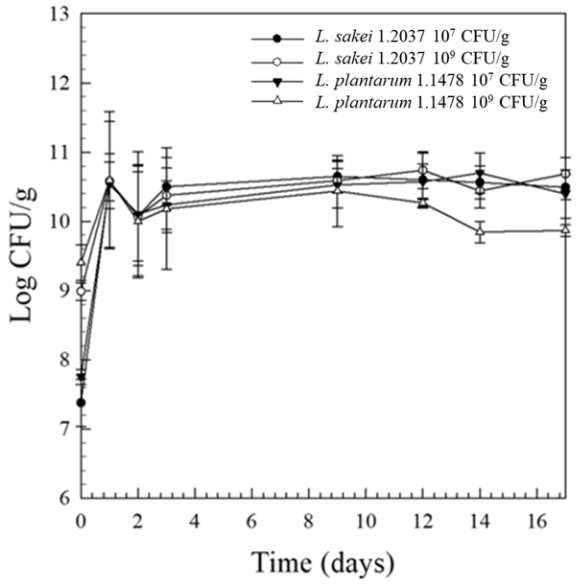

Figure 6 Anaerobic cell counts of raw fermented sausages produced with the non-EPS-forming strain *L. sakei* 1.2037 (control) or the HePS-forming strain *L. plantarum* 1.1478 during 17 days of fermentation and storage (7 days at 24 °C, 10 days at 14 °C). Inoculation concentrations were either 10^7 or 10^9 CFU/g. Error bars are standard deviations from two independent replicates, each examined in duplicate (n=4).

3.4.2 EPS detection

EPS formation of many mesophilic strains appears to be non-growth associated and was found to take place at both the beginning and the end of the exponential growth phase as well as during the stationary phase (Ana A. Bengoa et al., 2018; Pham et al., 2000; J. Wang et al., 2015). The amount of HePS produced by *L. plantarum* ssp. was often found to be increased under suboptimal growth conditions, which was attributed to the fact that a slowing down of the cell wall synthesis results in more isoprenoid glycosyl lipid carriers available as precursor molecules for EPS formation (I. W. Sutherland, 1972). The analysis of the EPS formation was done according to Hassan et al. (2002), who investigated the distribution of EPS in dairy products, including fermented milk, using Concanavalin A and Calcofluor White Stain. **Figure 8** illustrates raw fermented sausage samples after production (0 days), during fermentation (1-3 days), and after reaching the final weight loss of 31% (17 days) that have either been produced with the non-EPS-forming strain *L. sakei* 1.2037 (10^7) or with the HePS-forming strain *L. plantarum* 1.1478 at an initial concentration of 10^7 or 10^9 CFU/g salami, respectively. While only very few EPS could be detected throughout the production for the control samples, which can be attributed e.g. to the autochthone meat microflora, significantly more EPS (< *0.05*; after 24 h of incubation) could be found in the presence of *L. plantarum* 1.1478. This is in accordance with the results of the semi-quantitative data interpretation

(**Table 7**), which followed a modified method from Bosse et al. (2015), who analyzed the distribution of staphylococci in raw ham. The EPS-formation in the present study primarily took place during the exponential, and at the beginning of the stationary, growth phase, which is also reflected in the pH drop because of the sugar metabolism (**Figure 7).** Since the biosynthesis of HePS is linked with the primary carbohydrate metabolism, EPS synthesis is expected to take place during the time of fermentation. This is in accordance with our findings, showing an increase in the quantities of HePS formed in the salami within the first 24 h – 72 h of storage at 24 °C (

Table 7). EPS were only located at the outer parts of the proteins, which is why the fat was not subsequently stained in the present study. However, the HePS formed were found to not be homogenously distributed in the raw sausage matrix (**Figure 8).** The decrease in the overall EPS content at the later stage of the ripening or incubation phase, was already observed in other studies and was attributed to an enzymatic degradation as a result of cell lysis at the end of the stationary phase. There, intracellular glycohydrolases might be released from the cells, initiating EPS degradation (Jutta Cerning, 1990; Vaningelgem et al., 2004).

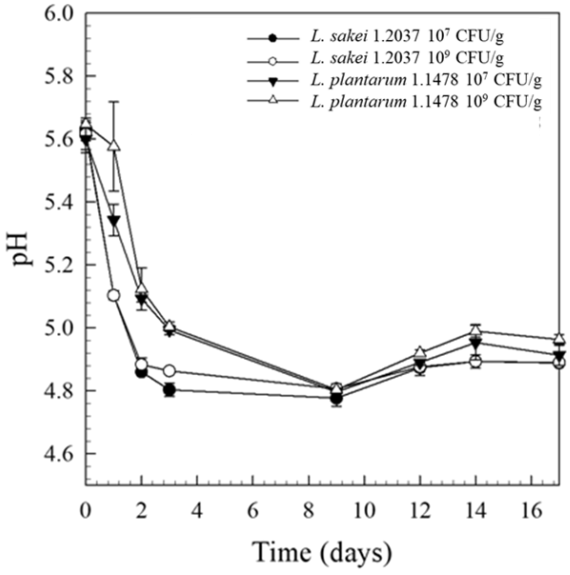

Figure 7 pH values of raw fermented sausages produced with the non-EPS-forming strain *L. sakei* 1.2037 (control) or the HePS-forming strain *L. plantarum* 1.1478 during 17 days of fermentation and storage (7 days at 24 °C, 10 days at 14 °C). Inoculation concentrations were either 10^7 or 10^9 CFU/g.

Table 7 Percentage of EPS formed (mean value) in raw fermented sausages that have been either inoculated with the non-EPS-forming strain *L. sakei* 1.2037 (~10^7 CFU/g and ~10^9 CFU/g), or with the HePS-forming strain *L. plantarum* 1.1478 (~10^7 CFU/g and ~10^9 CFU/g). The EPS are expressed as percentage of green area in pictures obtained using CLSM (n= 10 pictures).

Storage time (days)	*L. s.* 1.2037 ~10^7 CFU/g	*L. s.* 1.2037 ~10^9 CFU/g	*L. p.* 1.1478 ~10^7 CFU/g	*L. p.* 1.1478 ~10^9 CFU/g
A	**EPS [%]**	**EPS [%]**	**EPS [%]**	**EPS [%]**
0	1.67 ± 1.71[a]	11.28 ± 6.34[b]	11.17 ± 2.96[b]	4.01 ± 2.01[a]
1	13.97 ± 10.47[a]	10.25 ± 4.90[a]	50.70 ± 16.55[b]	47.14 ± 13.75[b]
2	5.45 ± 2.68[a]	15.33 ± 12.92[a]	33.88 ± 7.81[b]	55.06 ± 19.16[c]
3	18.93 ± 9.11[a]	18.91 ± 7.51[a]	49.49 ± 18.36[b]	48.74 ± 20.66[b]
9	5.45 ± 3.87[a]	5.78 ± 3.93[a]	19.76 ± 10.42[b]	18.85 ± 9.55[b]
12	5.94 ± 3.94[a]	8.20 ± 4.39[a]	24.03 ± 11.49[b]	30.68 ± 10.40[b]
14	4.58 ± 1.22[a]	9.32 ± 3.27[a]	23.49 ± 12.60[b]	27.74 ± 11.15[b]
17	5.93 ± 2.33[a]	4.34 ± 1,69[a]	24.93 ± 9.58[b]	28.06 ± 18.97[b]

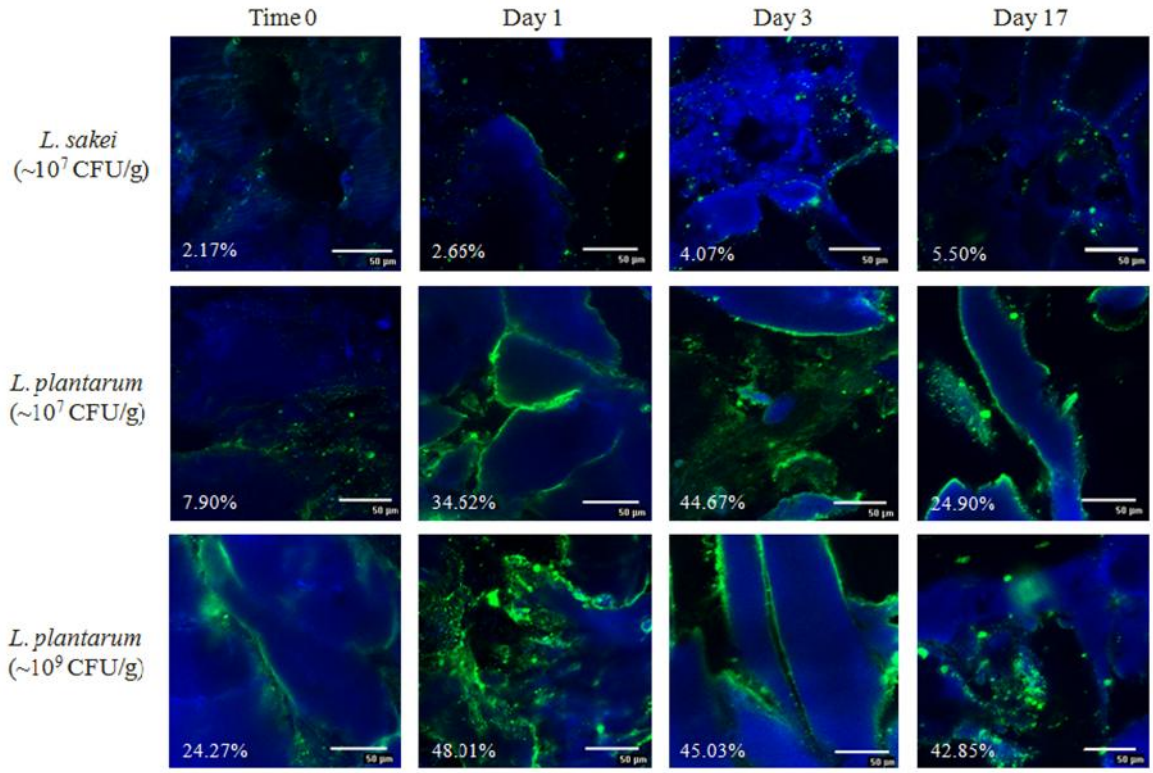

Figure 8 Example of a qualitative analysis of the formed EPS (stained green; proteins stained blue) in raw fermented sausages produced with the non-EPS-forming strain *L. sakei* 1.2037 (control) or the HePS-forming strain *L. plantarum* 1.1478 after production (0 days), during fermentation (1 and 3 days; 24 °C) and in the final product (31% weight loss; 17 days). Inoculation concentrations were either 10^7 or 10^9 CFU/g. Presented values give the relation between the detected EPS and the total area of the picture; n= 10.

3.4.3 Texture profile- and sensory analysis

In-situ produced HePS were found to increase gel strength, water-binding capacity and viscosity of dairy products including fat-reduced cheese and yoghurts (Jutta Cerning, 1990; A. N. Hassan & Awad, 2005) while Dertli et al. (2016) found HePS to increase the hardness of the sucuk. With one exception (23% weight loss, experiment A with *L. plantarum* 1.1478 10^9 CFU/g), the samples containing the HePS-forming strain were always found to be significantly softer *($p < 0.05$)* than the respective control samples (**Table 8**). For instance, products (31% weight loss; final product) containing the control strain *L. sakei* 1.2037 (10^7 CFU/g) had hardness values of 110.13 ± 6.09 and 116.89 ± 8.58, respectively, while those for products containing the EPS-forming strain *L. plantarum* 1.1478 (10^7 CFU/g) were found to have significantly lower values (80.54 ± 5.98 and 84.00 ± 3.11). This effect was, in most of the cases, even more pronounced when the initial bacterial concentration for *L. plantarum* 1.1478 was increased from 10^7 CFU/g to 10^9 CFU/g, although no significant differences could be observed with regard to HePS formation. This leads to the conclusion that the

difference with regard to the HePS amount formed is too small to be covered by the semi-quantitative approach used. This is also indicated by the low amount of HePS detected by Hilbig et al. (2019b) in spreadable raw fermented sausages (teewurst containing 30-40% fat; meat particles are covered by a fat layer) that have been produced with *L. plantarum* 1.1478. Detected HePS-concentrations (HPLC) ranged between 0.08 ± 0.01- 0.30 ± 0.04 g/kg dry matter while products containing the HoPS-producing strain *L. sakei* 1.411 were found to contain significantly higher amounts of EPS (0.46 ± 0.01 - 0.80 ± 0.02 g/kg dry matter). In the present study *L. sakei* 1.2037 has been used as a non-EPS forming control strain and *L. plantarum* 1.1478 as a strain able to form heteropolysaccharides under fermentation conditions. Although these strains do not belong to the same species, several studies showed that lactic acid bacteria contribute to textural changes by acidifying the meat batter leading to a coagulation of muscle proteins, and by forming exopolysaccharides *in-situ* during processing (Laranjo, Elias, & Fraqueza, 2017). In addition, changing the environment to acidic conditions can result in an increased activity of cathepsin D (at low pH), which is involved in muscle proteolysis. (Molly et al., 1997). Changing processing and hence fermentation conditions thus has a remarkable impact on textural properties. In the present study, same fermentation conditions have been used for both strains that showed similar fermentation kinetics leading to products with pH values in the same range after storage for 9 days at 24 °C (**Figure 7**). Since lactic acid bacteria do not display a pronounced lipolytic or proteolytic activity under the processing conditions used (lipases from lactobacilli usually display little or no activity during sausage fermentation) (F. Leroy et al., 2006), the observed differences in texture can be attributed to the formation of EPS, which was also shown in other studies (Hilbig, Gisder, et al., 2019). This is also the reason why most commercial meat starter cultures are combined cultures of lactic acid bacteria and coagulase-negative staphylococci that are known for their ability to stabilize color but also for their strain-dependent proteolytic and lipolytic activity predominantly contributing to aroma formation (Ravyts, De Vuyst, & Leroy, 2012). Although no significant differences could be observed with regard to detected HePS and inoculation concentration used, a correlation between HePS formation and influence on the quality parameters of the salami could still be made, not only with regard to the results gained from the TPA, but also from a sensory point of view: There, products containing the HePS-forming strain *L. plantarum* 1.1478 were rated higher in terms of consistency, which is directly associated with a softer texture (**Figure 9**). However, the taste of the products containing the HePS-forming strain was not negatively affected.

With regard to the quality attributes of raw fermented sausages, the results clearly indicate that using an EPS-forming culture in raw fermented sausage manufacturing might be a real issue, since the texture of the products gets softer, which is often associated with a poorer product quality. However, the effect observed could make a positive contribution in the field of highly-perishable spreadable raw fermented sausage products, including onion mettwurst (meat particles are not covered by a fat layer), having a high product pH of 5.5-5.6 (Feiner 2006). Using EPS-forming LAB in onion mettwurst production may allow for a decrease in pH to 5.1 (close to, but above the IEP of meat proteins) while maintaining the product's spreadability, which could, in turn, improve the shelf life of the product. This assumption is supported by the data results, since the softening effect was seen in raw fermented sausages with a pH of around 5.0 (**Figure 7**). Moreover, the effects observed could even be more pronounced when using HoPS-forming LAB since higher amounts of EPS are usually produced, which may then interact with meat proteins as indicated in **Figure 8**.

Table 8 Hardness of raw fermented sausages produced with the non-EPS-forming strain *L. sakei* 1.2037 (~10^7 CFU/g and ~10^9 CFU/g), or with the HePS-forming strain *L. plantarum* 1.1478 (~10^7 CFU/g and ~10^9 CFU/g).

Weight loss [%]	*L. s.* 1.2037 ~10^7 CFU/g	*L. s.*1.2037 ~10^9 CFU/g	*L. p.* 1.1478 ~10^7 CFU/g	*L. p.* 1.1478 ~10^9 CFU/g
A	***Hardness (N)**			
16	56.63 ± 5.07[a]	55.42 ± 2.82[a]	46.48 ± 2.94[b]	42.59 ± 2.76[c]
23	71.90 ± 4.76[a]	82.09 ± 5.63[b]	56.21 ± 2.68[c]	78.64 ± 3.92[b]
27	87.25 ± 5.35[a]	77.17 ± 4.88[b]	60.67 ± 2.77[c]	57.39 ± 3.88[c]
31	110.13 ± 6.09[a]	116.35 ± 10.35[a]	80.54 ± 5.98[b]	81.62 ± 4.79[b]
B	***Hardness (N)**			
16	82.35 ± 5.05[a]	74.26 ± 5.37[b]	64.78 ± 8.07[c]	56.00 ± 4.52[d]
23	80.92 ± 3.18[a]	78.61 ± 3.39[a]	73.35 ± 3.38[b]	55.69 ± 3.81[c]
27	92.30 ± 6.41[a]	93.54 ±7.14[a]	74.71 ± 5.79[b]	66.37 ± 1.56[c]
31	116.89 ± 8.58[a]	103.32 ± 4.68[b]	84.00 ± 3.11[c]	69.91 ± 4.66[d]

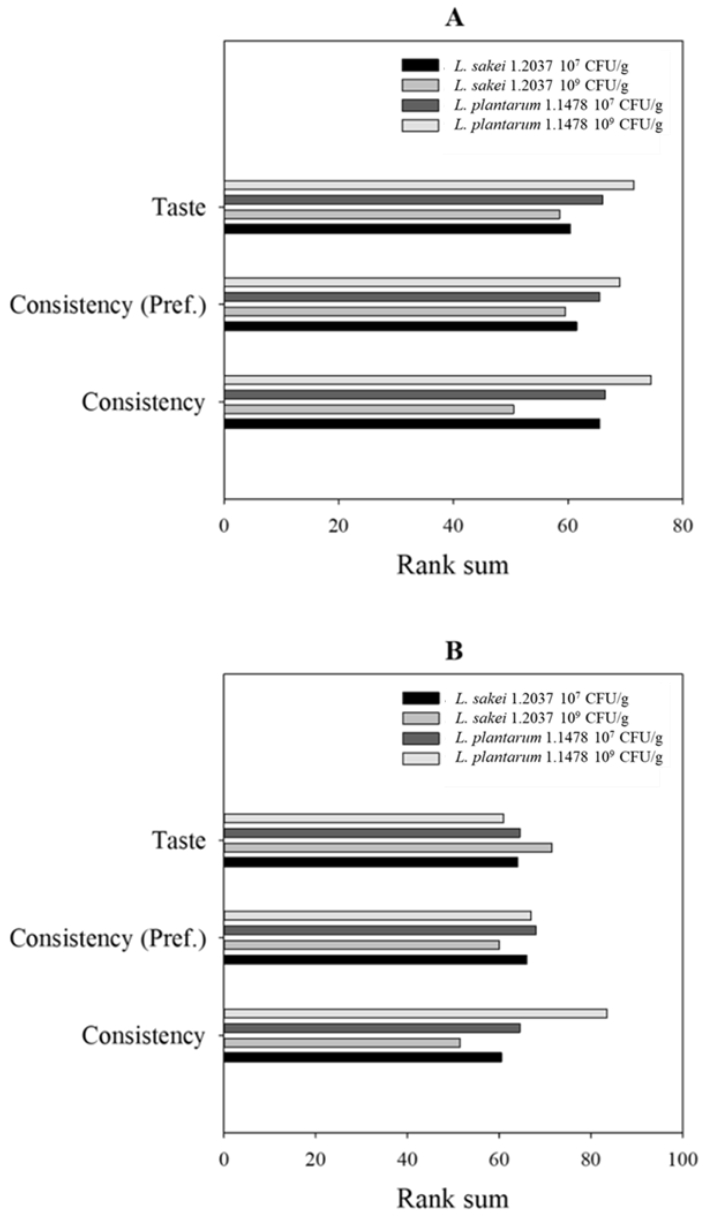

Figure 9 Results of the sensory evaluation (26 panelists) of raw fermented sausages (31% weight loss) produced with the non-EPS-forming strain *L. sakei* 1.2037 (control) or the HePS-forming strain *L. plantarum* 1.1478 of the first experiment (A) and the repetition (B).

3.5 Conclusion

While in the food industry *in-situ* HePS-forming starter cultures are generally associated with a positive influence on product quality, the results of the present study showed the opposite. These starter cultures might, however, be promising in the field of spreadable raw fermented sausage manufacturing (e.g. onion mettwurst), because the softening effect observed in the present study could lead to a better spreadability. This would have the advantage that the EPS-forming culture could be used as an alternative to typically-applied hydrocolloids that

have to be declared on the package. The observed softening effect of *in-situ*-formed EPS could even be more pronounced when a HoPS-producing starter culture is used during raw fermented sausage production, since more HoPS are usually formed due to the HePS-synthesis being much more complex and energy demanding. In future investigations one should consider using EPS-detection methods with a high sensitivity, such as high performance liquid chromatography.

CHAPTER 4

Influence of Fermentation Temperature on *in-situ* Heteropolysaccharide-Formation (*L. plantarum* TMW 1.1478) and Texture Properties of Raw Sausages

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Abstract

This study puts a focus on the influence of microbial *in-situ* heteropolysaccharide (HePS)-formation on the quality of raw fermented sausages (salami). Since exopolysaccharide-production is often triggered by sub-optimal growth conditions, the influence of different fermentation temperatures was also investigated. For this reason, the sausage batter was inoculated with (*Lactobacillus plantarum* TMW 1.1478) or without (*L. sakei* TMW 1.2037; control) a HePS-producing starter culture (inoculation concentration ~10^8 CFU/g), and the sausages fermented at either 10, 16, or 24 °C (7 days), followed by a drying period at 14 °C until the final weight loss of 31% was reached. Microbial growth, pH and weight loss development were monitored and the products further characterized using texture profile analysis and a sensory test. HePS in the salami matrix were determined using confocal laser scanning microscopy and a semi-quantitative data interpretation approach. Sausages containing *L. plantarum* were found to be significantly ($p < 0.05$) softer compared to control samples, which was also confirmed in the sensory analysis. The different fermentation temperatures had an influence on the drying speed. Here, sausages produced with *L. plantarum* needed more time to reach the final weight loss of 31% as compared to control samples, which could be attributed to the presence of exopolysaccharides in the matrix ($p < 0.05$). Using HePS-forming starter cultures in raw fermented sausage manufacturing can lead to products with a softer texture (undesired in Europe) depending on the strain and processing conditions used, highlighting the importance of a suitable starter culture selection in food processing.

4.1 Introduction

Several exopolysaccharide (EPS)-producing lactic acid bacteria are used in food production due to EPS having texturizing, viscosifying, gelling and emulsifying properties (K. M. Lynch et al., 2018). Moreover, *in-situ* produced EPS do not need to be labeled on the package, accounting for the consumers demand for more "natural" products. According to the mechanism of biosynthesis and composition, EPS can be classified into heteropolysaccharides (HePS) and homopolysaccharides (HoPS) (de Vuyst & Degeest, 1999). HoPS such as dextran or levan consist of a single type of monosaccharide and are extracellularly synthesized from sucrose or starch, whereas HePS are usually composed of repeating monosaccharide units ranging in size from disaccharides to octasaccharides and are synthesized in a complex, energy-demanding biosynthesis from different sugars (Donot et al., 2012; Monsan et al., 2001). The ratio of the monosaccharides, the degree of branching as well as the linkage type and presence of other substituents affect both the molecular weight as well as the overall charge of the HePS, all influencing physicochemical interactions with food ingredients. This is also the reason why usually lower amounts of HePS are required to cause structural and to some extend textural changes in food products when compared to HoPS (Wingender et al., 2012). Previous studies showed that EPS-production from mostly mesophilic bacteria can be improved under sub-optimal growth conditions due to e.g. environmental stress (Ana A. Bengoa et al., 2018; Prechtl et al., 2018a). In case of raw fermented sausage production (*salami*), nitrate curing salt may for instance favor microbial EPS-formation by lactic acid bacteria, which are traditionally used in salami production to control microbial safety and sensory properties (Cocolin et al., 2011; F. Leroy et al., 2006).While a lot of research has been done with regard to EPS-formation in dairy or bakery products (Abedfar et al., 2019; Gemelas et al., 2018; Yilmaz et al., 2015), very few studies focused on the usage of EPS-forming starter cultures in meat products although they have been associated with health benefits besides their technofunctional properties. The meat industry is, however, constantly seeking for new starter cultures possessing different fermentation kinetics or other metabolic activities of interest. According to Dertli et al. (2016) who examined the influence of HePS-producing *L. plantarum* 162 R and *Leuconostoc mesenteroides* N6 on the physicochemical, microbiological, microstructural and textural properties of lamb and beef-based Turkish sucuk, products containing the EPS-producing strains were harder and less adhesive as compared to control samples. In contrast to that Hilbig, Gisder, et al. (2019) reported on fat-reduced teewurst with an improved spreadability when EPS-forming strains (HoPS: *Lactobacillus sakei* TMW 1.411 or HePS: *L. plantarum* TMW 1.1478) had been used during

production. Latter effect would be crucial when it comes to dry fermented sausage production since softer products are usually associated with a less good quality (especially in European countries). These results highlight the importance of an EPS screening under fermentation conditions and the need for a better understanding of microbial *in-situ* EPS-formation in meat matrices and their influence on product properties. The present study therefore aims to (i) get a better understanding of the influence of HePS-production on quality attributes of raw fermented sausages (*salami*), and to (ii) investigate the influence of varying fermentation temperatures as an additional stress besides the present salt content, which may lead to an improved HePS-production. For this reason three fermentation temperatures have been investigated, two that are traditionally used in raw fermented sausage production (24 °C and 16 °C *in variable temperature fermentation*) and one that is far below the optimal fermentation temperature (10 °C) accounting for an EPS-formation under enhanced stress conditions

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Ingredients for raw sausage production

Pork meat and pork fat were purchased from a local wholesaler (MEGA eG, Stuttgart, Germany) and standardized according to the GEHA meat classification system (Prändl, Fischer, Schmidhofer, & Sinell, 1988) to S II and S VIII, respectively. Nitrite curing salt (NCS, 0.5% nitrite) was provided by Südsalz GmbH (Heilbronn, Germany), ascorbic acid and black pepper were purchased from Gewürzmüller (Korntal-Münchingen, Germany) and dextrose as well sucrose was obtained from Südzucker AG (Mannheim, Germany).

4.2.1.2 Microbiological culture medium and chemicals

De Man, Rogosa and Sharpe (MRS) agar and broth (peptone from casein 10.0 g/L, meat extract 10.0 g/L, yeast extract 4.0 g/L; D (+)-glucose 20.0 g/L, dipotassium hydrogen phosphate 2.0 g/L, Tween® 80 1.0 g/L, di-ammonium hydrogen citrate 2.0 g/L, sodium acetate 5.0 g/L, magnesium sulfate 0.2 g/L, and manganese sulfate 0.04 g/L, with (agar) or without (broth) agar-agar 14.0 g/L), as well as Anaerocult® were purchased from Merck KGaA (Darmstadt, Germany). Peptone water (pH 7.0 ± 0.2; 5 g/L) was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Plate Count Agar (PCA; agar 15.0 g/L, glucose 1.0 g/L, peptones 5.0 g/L, and yeast extract 2.5 g/L) was obtained from AppliChem GmbH (Darmstadt, Germany). Anaerocult® was used to assure an anaerobic atmosphere

during the incubation of MRS agar. Preparation: 35 mL of water was distributed over 1 sachet of Anaerocult. Calcofluor White Stain, Concanavalin A and Glycerol (≥99.0%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All microbiological media were prepared as specified by the respective manufacturers and autoclaved for 15 minutes at 121 °C.

4.2.2 Methods

4.2.2.1 Starter culture preparation

Lactobacillus plantarum TMW 1.1478 (HePS-forming strain; henceforth referred to as *L. plantarum* 1.1478), and *Lactobacillus sakei* TMW 1.2037 (non-EPS-forming control strain; henceforth referred to as *L. sakei* 1.2037) were obtained from the Technical University of Munich (Department of Technical Microbiology, Freising, Germany). Bacterial strains were stored in MRS broth containing 20% (v/v) glycerol at -80 °C. Before being used to inoculate the raw sausage mass, both *Lactobacillus* strains were activated in MRS broth for 48 h at 30 °C. To obtain a higher concentration of the initial inoculums (target: 10^8 CFU/g meat), the solutions were centrifuged (Universalzentrifuge Hermle Z32HK, Hermle Labortechnik GmbH, Germany) at 5000 rpm for 10 minutes at 25 ºC and the pellets then suspended by using small amounts of peptone water (c = 5g/L). This had the additional benefit that the taste of the final products was not influenced by the presence of MRS broth.

4.2.2.2 Raw fermented sausage production

The production of salami was performed in the pilot plant of the University of Hohenheim following a standard raw fermented sausage formulation (35% minced pork shoulder S II, 45% frozen pork meat S II, and 20% frozen pork back fat S VIII). After mincing the pork shoulder in a meat grinder with a 3 mm whole plate (Type Wd 114, Seydelmann, Aalen, Germany) it was mixed with the remaining pork meat, back fat and spices (3.0 g/kg black pepper, 5.0 g/kg sucrose, and 0.5 g/kg ascorbic acid) using a vacuum bowl chopper (Type K64 DC, Seydelmann, Aalen, Germany). In total 64 kg of sausage batter was produced and divided into 2 batches. The first batch was inoculated with the non-EPS-forming strain *L. sakei* 1.2037 (control; ~10^8 CFU/g) and the second batch with the HePS-producing strain *L. plantarum* 1.1478 (~10^8 CFU/g). Same quantities but different raw materials (to e.g. account for differences in the raw material quality) were used to perform the repetition of the experiment. After adding and distributing the starter cultures, 28.0 g/kg nitrate curing salt (NCS) was added (short mixing step to homogenously distribute the salt) and the sausage batter then filled (MWF 591, MADO Patron, Dornhan, Germany) into casings (50 mm

diameter; Nalo fasser S, Werner Niedenbeger GmbH, München, Germany). Sausages containing the control strain and those containing *L. plantarum* 1.1478 were further divided into 3 portions to allow for exposure to three different fermentation temperatures (10, 16, or 24 °C) during the next seven days, followed by a ripening (drying) period at 14 °C until all sausages reached 31% of weight loss. Additionally, sausages were smoked after 24, 48 and 72 h of fermentation in a smoking chamber (Maurer, Reichenau, Germany) at 24 °C for 15 min.

4.2.2.3 Microbiological analysis of raw fermented sausages

Microbiological analysis of raw fermented sausages was conducted at time 0 (raw material ± starter culture), after 24, 48, and 72 h, and after 9, 13, and 15 days of production. To obtain aerobic and anaerobic viable cell counts of the different products, always 10 g of sample was taken aseptically from the core of the respective salami, transferred to a sterile filter bag and subsequently mixed for 1 min (6 strokes / second) with 90 mL of buffered peptone water (5 g/L) using a Masticator (Laborhomogenisator, IUL Instrument GmbH, Königswinter, Germany). Appropriate dilutions were plated on plate count agar (PCA; raw material quality) and on deMan, Rogosa and Sharpe (MRS) agar using an automated spiral plater (Don Whitley Scientific, West Yorkshire, UK) followed by incubation at 30 °C for 24 h – 48 h under either anaerobic (MRS) or aerobic (PCA) conditions. Subsequently, the colonies were counted using an automatic colony counter (Acolyte, Synbiosis, Cambridge, UK). Two independent samples were analyzed in triplicate.

4.2.2.4 pH measurement & water activity

The pH values were monitored during fermentation and drying using a pH meter (WTW Microprocessor pH Meter, WTW GmbH, Weilheim, Germany), whereas the water activity was determined using an "Aqua Lab" device (CX-2- Decagon Devices Inc., Pullman, USA). Two independent samples were analyzed in triplicate.

4.2.2.5 Weight loss measurement

The weight loss (target: 31%) of the sausages was gravimetrically monitored and calculated according to **equation 2**:

$$\text{WT loss } [\%] = \frac{M_{initial} - M_{end}}{M_{initial}} * 100$$

[1]

Where $M_{initial}$ is the weight of the sample prior to the fermentation process (time 0; after filling) and M_{end} is the recorded weight after a specific processing time.

4.2.2.6 EPS examination and quantification using Confocal Laser Scanning Microscopy (CLSM)

A qualitative assessment of the EPS-production was performed after 0, 24, 48, 72 h, and during the drying phase using the method as provided by Hilbig, Loeffler, et al. (2019a), which is based on a method developed by Ashraf N. Hassan et al. (2002). A cylindrical metal pipe was used to take a sample (0.5 cm high, 1.5 cm wide) from the core of the respective raw fermented sausage sample, which was then stained with 10 µL of a diluted (1:20) Concanavalin A solution (stock: 5 mg lypolized powder in 5 mL phosphate buffer 10 mmol; pH 6) to determine *in-situ* formed EPS. Proteins were made visible by adding Calcofluor White Stain (10 µL) after 60 min of dark incubation at 12 °C. The samples were analyzed using a Nikon Eclipse-Ti Inverse Microscope D- Eclipse C1 (Nikon GmbH, Düsseldorf, Germany) and a 60-fold magnification lens with immersion oil. A red helium-neon laser at 638 nm and an argon laser at 488 nm were used for the excitation of EPS and proteins. At least nine pictures of each sample were taken and further analyzed. Scales were inserted using the ImageJ software (Version 1.4.3.67, National Institutes of Health, Bethesda, MD, USA) after creating a RGB picture using the EZ-C1 3.70 Imaging Software (Nikon GmbH, Düsseldorf, Germany). The semi-quantitative analysis of formed EPS (expressed as green area) was performed using MATLAB (The Math Works, Inc., version R2013b 8.2.0.701) following a method developed by Bosse, Gibis, Schmidt, and Weiss (2015) who introduced the following equation (**equation 1**), which was also used in the present study:

$$\text{Green area picture } [\%] = \frac{\text{Green area (pixels)}}{\text{Total area pixcels}} * 100\% \qquad [1]$$

4.2.2.7 Texture Profile Analysis

A texture profile analysis (TPA) was performed as soon as the raw sausages achieved 21, 26, and 31%weight loss, respectively to get deeper insights on the influence of EPS- production during drying. Prior to performing the TPA, the sausages were equilibrated (12 °C), sliced, and casings removed. 15 samples were taken from each batch (2 cm high x 1.5 cm wide; in filling direction) and analyzed using a double compression test (50%; 20 s interval between the two compression cycles) with a probe of 2.5 cm diameter, at a cross-head speed of 50 mm/min using an Instron device (Model 1011, Instron Engineering Corp., Canton, MA, USA) equipped with a 100 N load cell. The hardness of the samples was determined at the first peak of compression and the gumminess calculated by multiplying hardness with cohesiveness. The springiness index (deformation during the compression) and cohesiveness (stability of the sausage) were calculated according to **equation 3** and **4.**

$$Springiness\ index\ (-) = \frac{(a-b)}{(c-d)}$$

[3]

With *a* being the distance to the maximal second compression (mm), *b* being the distance to the onset of the second compression (mm), *c* being the distance to the maximal first compression (mm), and *d* being the distance to the onset of the first compression (mm).

$$Cohesiveness\ (-) = \frac{Second\ area\ (J)}{First\ area\ (J)}$$

[4]

4.2.2.8 Sensorial analysis

The sensory evaluation of the raw fermented sausages was performed with a panel of 20 untrained participants who were familiar with the product. Here, sausages containing either the control strain *L. sakei* 1.2037 or the HePS-forming strain *L. plantarum* TMW 1.1478 were cut into 1.5 cm thick slices with a diameter of about 3.5 cm and served at room temperature. For the evaluation, a paired comparison test was done with the control sample corresponding to a score of 5 (scale: 0 – 10). Samples containing the HePS-forming strain had to be rated compared to the control regarding consistency, preference and taste with values < 5 indicating a softer texture/consistency and a less good taste. The sensory test was carried out and recorded with the software Fizz Acquisition 2.51 and Fizz Acquisition 2.50 (both Biosystems, France).

4.2.2.9 Statistical analysis

Each experiment was conducted twice and all measurements repeated three times. Means and standard deviations were calculated using Excel (Microsoft, Redmond,WA, USA). SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA) was used to statistically evaluate the results using the Shapiro-Wilkes test for normality ($p < 0.05$; failed) and the Levene test for equal variance ($p < 0.05$). This was followed by a Kruskal-Wallis test and a multiple comparison procedure (Student-Newman-Keuls test) to determined significant differences ($p < 0.05$) between results gained from the TPA and EPS-analysis, whereas a paired t-Test was performed to analyze differences between results gained from the sensory evaluation.

4.3 Results and Discussion

4.3.1 Microbiological analysis of raw fermented sausage samples

The mesophilic aerobic viable cell counts (PCA) of the minced raw meat ranged between 5.70 $\cdot 10^3$ and 6.05 $\cdot 10^3$ CFU/g meat indicating a good raw material quality (Feiner, 2006). Among lactic acid bacteria known for HePS-formation, *L. plantarum* has been widely studied in food mimicking model systems (Hilbig, Loeffler, et al., 2019a; Prechtl et al., 2018b) as well as in bakery- (Abedfar et al., 2019), kefir- (J. Wang et al., 2015) and lately also in meat-based products (Dertli et al., 2016). Moreover, (Hilbig, Gisder, et al., 2019) also used *L. plantarum* 1.1478 for fat-reduced spreadable raw fermented sausage production. To some extent, this allows for a better data interpretation and presentation of mechanistic insights of the present study, even though the matrix structure of spreadable raw fermented sausages (*protein surrounded by fat*) is different from salami (*fat surrounded by protein*). To get a better understanding of the influence of HePS on product quality, sausages produced with the non-EPS forming *L. sakei* 1.2037 were always compared to sausages containing *L. plantarum* 1.1478. Based on a previous study done by the authors, HePS-production by *L. plantarum* 1.1478 in a salami matrix has been found to be more pronounced when using higher inoculation concentrations, which is why in the present study bacterial suspensions were concentrated prior to application leading to concentrations of almost 10^8 CFU/g at time 0 h (**Figure 10**). Taking the repetition (second independent production) and standard deviations into account, no clear influence of fermentation temperature (10, 16 or 24 °C) on microbial growth behavior could be observed, which might be attributed to the inhomogeneous salami matrix with fat and potentially EPS acting as an additional barrier to colder temperatures (D'Amico, Collins, Marx, Feller, & Gerday, 2006). Moreover, due to inoculation concentrations being very high, no remarkable growth is expected but one can see a slight decrease of bacterial counts after 10 days of production.

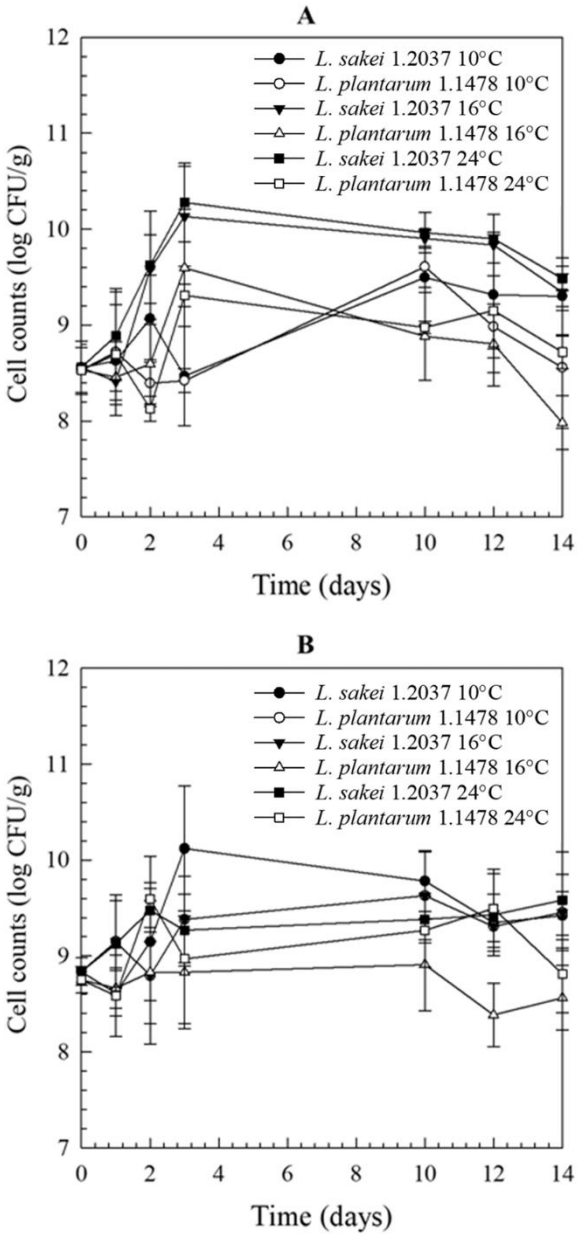

Figure 10 Anaerobic cell counts of raw fermented sausages that have been produced with the non-EPS-forming strain *L. sakei* TMW 1.2037 (control; ~10^8 CFU/g) or the HePS-forming strain *L. plantarum* TMW 1.1478 (~10^8 CFU/g) of the first (A) and second experiment (B; independent sausage production). Measurements were carried out over the fermentation (performed at 10, 16, or 24 °C) and drying phase until 31% weight loss was reached).

4.3.2 pH, a_w and weight loss development in raw fermented sausages

The optimal pH for EPS production can differ from the one associated with optimal growth, especially for strains showing a growth-independent EPS-formation(de Vuyst & Degeest, 1999) with a pH of 5.5 – 6.5 promoting EPS production of many *Lactobacillus strains* (Czaczyk & Myszka, 2007). Lactic acid bacteria such as *L. sakei* 1.2037 and *L. plantarum* 1.1478 are known to contribute to textural changes by acidifying the meat batter leading to a coagulation of muscle proteins (Laranjo et al., 2017). Changing fermentation conditions thus has an influence on textural properties. In the present study, same fermentation conditions have been used for both strains that showed similar fermentation kinetics with *L. plantarum* 1.1478 being slightly slower in the beginning which may be attributed to EPS metabolism. However, both strains led to products with pH values in the same range after storage. The values obtained are represented in **Figure 11**. Independent of the fermentation temperatures used, the pH values of raw sausages inoculated with the HePS-forming strain *L. plantarum* decreased from 5.64 ± 0.01 to 4.89 ± 0.01, 5.09 ± 0.01, and 4.97 ± 0.01 at 10, 16, and 24 °C, respectively. The pH of sausages that have been produced with the control strain *L. sakei* 1.2037 decreased from an initial pH value of 5.64 ± 0.01 to 4.89 ± 0.00 (10 °C), 4.89 ± 0.01 (16 °C), and 4.95 ± 0.01 (24 °C), respectively at the end of production (31% weight loss). The final values are in agreement with the typical pH range for salami products (pH 4.8 – 5.3). Similar results were obtained in the repetition (second independent production) with all values being slightly higher, including the pH right after production.

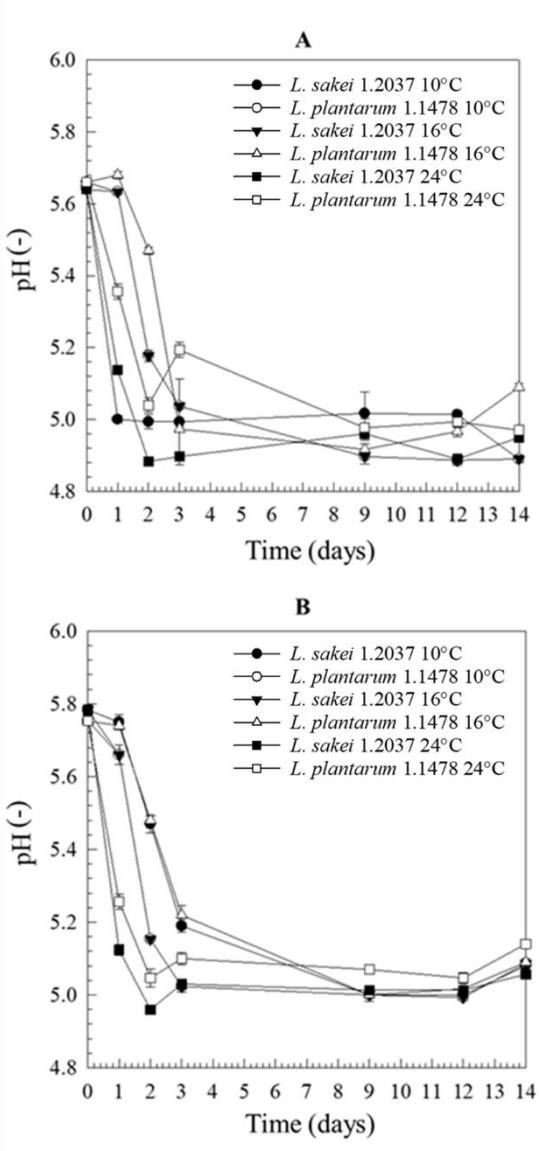

Figure 11 pH – values of the sausages produced with the non-EPS-forming strain *L. sakei* TMW 1.2037 (control; ~10^8 CFU/g) or the HePS-forming strain *L. plantarum* TMW 1.1478 (~10^8 CFU/g) during the fermentation (performed at 10, 16, or 24 °C) and drying phase of the sausages of the first (A) and second production (B; independent sausage production).

The final a_w values for raw fermented sausages from both independent productions were in the same range of 0.89 ± 0.01 and 0.88 ± 0.01, respectively.

Differences could however be seen with regard to the weight loss development of the products (**SD 1**). Samples containing the control strain *L. sakei* 1.2037 reached the final weight loss of 31% after 14 days of storage, provided the initial fermentation temperature was set to 10 or 16 degrees, whereas it took only 11 days when the sausages were exposed to 24 °C during the first seven days of production. In contrast to that, inoculating sausages with the HePS-forming strain *L. plantarum* 1.1478 (~10^8 CFU/g) led to an increase of time needed to

reach the final weight loss of 31% being 15, 14, and 13 days for products stored at 10, 16, and 24 °C during the first seven days of production, respectively. This can be a first indication that *in-situ* HePS-formation influence the properties of salami.

4.3.3 EPS detection, TPA, and sensory analysis

The *in-situ* formed HePS by *L. plantarum* 1.1478 were qualitatively and semi-quantitatively assessed using CLSM and a MATLAB approach following a method modified after Bosse et al. (2015). **Figure 12** shows the EPS development over time in sausages containing the control strain *L. sakei* 1.2037 whereas **Figure 13** presents the EPS development over time in sausages containing the HePS-forming strain *L. plantarum* 1.1478. *L. plantarum* 1.1478 was able to form HePS during fermentation, while amounts stained in the control samples did not change remarkably over time. Since the biosynthesis of HePS is linked with the primary carbohydrate metabolism, EPS synthesis usually takes place during fermentation and partially at the stationary phase. This is in accordance with our findings, showing an increase in the quantities of HePS formed in the salami matrix within the first 48 h of storage at 10, 16, or 24 °C, respectively (**Figure 13**). While significant differences regarding EPS-formation could be detected between the starter cultures, the different temperatures applied during the first seven days of production (10, 16, and 24 °C) had, according to the semi-quantitative data interpretation (**Table 9**), a less pronounced influence on HePS-production. Other studies reported on an enhanced EPS production when EPS-forming strains were exposed to temperatures far below their optimum (van den Berg et al., 1995). The results found in the present study could be attributed to HePS being not homogenously distributed in the complex food matrix making it difficult to see differences between the EPS-results gained at different temperatures. Latter one is additionally supported by the fact that usually very low amounts of HePS are formed. For instance, Hilbig, Gisder, et al. (2019) determined the EPS content in spreadable raw fermented sausages that have been produced with either the HoPS-forming strains *L. sakei* TMW 1.411 or *L. curvatus* TMW 1.1928 or the HePS-forming strain *L. plantarum* 1.1478 (fermentation at 24 °C). Authors found high EPS-concentrations in sausages containing one of the HoPS-producing strains (0.46 – 1.03 g/kg) and significantly lower amounts in sausages containing *L. plantarum* 1.1478 (0.08 – 0.30 g/kg). HePS are known to cause structural changes at much lower concentrations than HoPS, which could explain the differences in weight loss even though the influence of temperature on HePS-production could not be fully proven with the used semi-quantitative approach. This is further supported by the results gained through the texture analysis (TPA), which revealed that sausages produced with the HePS-forming strain were found to be significantly softer ($p <$

0.05) than the corresponding control samples as illustrated in **Figure 14** (illustrated for 21%, 26%, and 31% weight loss). Furthermore, the gumminess, cohesiveness and springiness of the products were influenced by both the present starter culture and the fermentation temperature used (**Table 10**), which could also be seen in the second, independent production (data not shown). These results are also reflected in the sensory evaluation (**SD 2**), during which 20 panelists rated the sausages with HePS softer in terms of consistency. However, taste was not negatively affected.

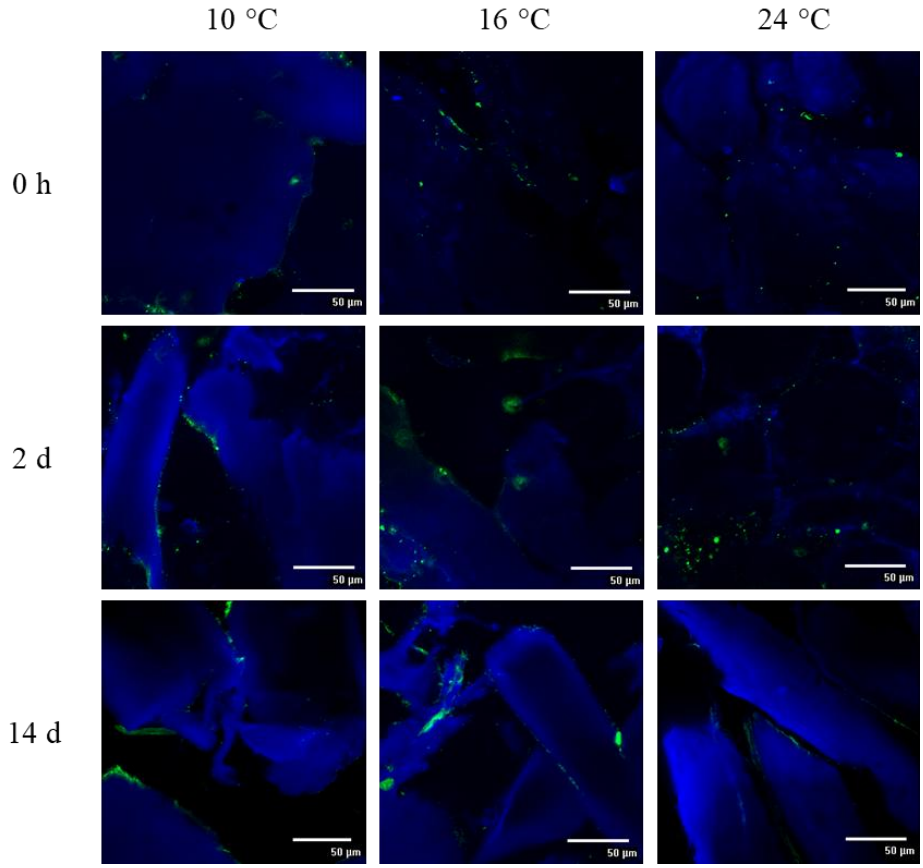

Figure 12 Qualitative EPS determination (CLSM, 600X magnification) in sausages produced with *L. sakei* TMW 1.2037 (control; ~10^8 CFU/g) in dependency of the different fermentation temperatures used (10, 16, or 24 °C). EPS are stained green and proteins are stained blue. The corresponding semi-quantitative data (based on ≥ 9 pictures / sample) are presented in Table 9.

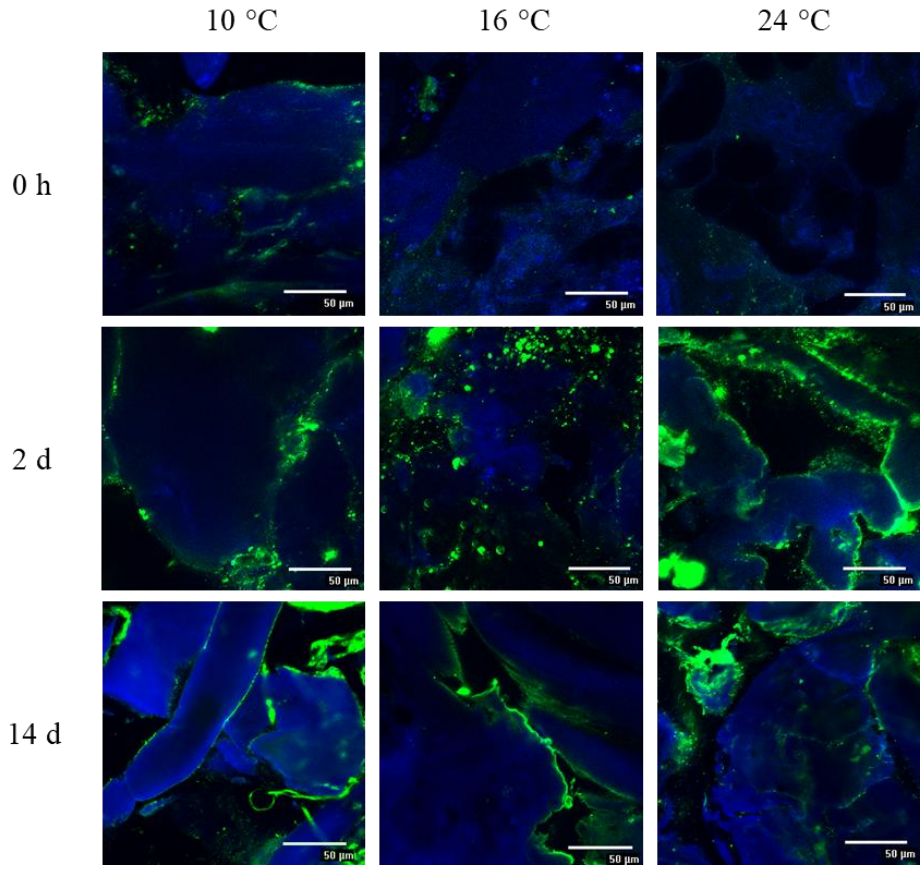

Figure 13 Qualitative EPS determination (CLSM, 600X magnification) in sausages produced with the HePS-forming strain *L. plantarum* TMW 1.1478 (~10^8 CFU/g) in dependency of the different fermentation temperatures used (10, 16, or 24 °C). EPS are stained green and proteins are stained blue. The corresponding semi-quantitative data (based on ≥ 9 pictures / sample) are presented in Table 9.

Table 9 Results of the CLSM image analysis of sausages produced with the non-EPS-forming strain *L. sakei* TMW 1.2037 or the HePS-forming strain *L. plantarum* TMW 1.1478 (~10^8 CFU/g) of the first production.

$T_{Fermentation}$	Day	*L. sakei* 1.2037		*L. plantarum* 1.1478	
	0	7.48 ± 2.87		7.48 ± 2.87	
10 °C	1	3.54[a]	±1.65	23.87[b]	±8.41
	2	4.02[a]	±1.16	20.87[b]	±11.57
	3	3.89[a]	±1.92	27.77[b]	±17.62
	~14	9.71[a]	±3.96	26.86[b]	±15.64
	1	3.36[a]	±1.21	26.55[b]	±12.58

16 °C	2	6.29[a]	±3.24	32.25[b]	±12.70
	3	9.40[a]	±5.22	38.30[b]	±13.96
	~14	8.50[a]	±3.95	14.87[a]	±10.98
24 °C	1	5.28[a]	±2.64	35.00[b]	±12.16
	2	6.91[a]	±3.16	25.14[b]	±7.46
	3	8.30[a]	±3.68	39.05[b]	±17.52
	~14	6.77[a]	±1.24	35.30[b]	±9.90

Note: Measurements were carried out over the fermentation and drying period until 31% weight loss was reached; n ≥ 9 pictures. Values with different letters show significant differences (p < 0.05) within the row; ± is the standard deviation

Table 10 Textural profile analysis parameters of sausages (31% weight loss) produced with the non-EPS-forming strain *L. sakei* TMW 1.2037 or the HePS-forming strain *L. plantarum* TMW 1.1478 (~10^8 CFU/g) and fermented at different temperatures; 1st production.

Fermentation		*L. sakei* 1.2037		*L. plantarum* 1.1478	
Gumminess (N)	10 °C	37.02[a,A]	±1.45	29.97[b,A]	±1.19
	16 °C	36.54[a,A]	±1.97	30.59[b,A]	±1.82
	24 °C	32.47[a,B]	±1.17	26.50[b,B]	±1.37
Cohesiveness (-)	10 °C	0.49[a,A]	±0.01	0.47[b,A]	±0.01
	16 °C	0.42[a,B]	±0.01	0.45[b,B]	±0.01
	24 °C	0.40[a,C]	±0.01	0.45[b,B]	±0.00
Springiness Index (-)	10 °C	0.06[a,A]	±0.02	0.08[b,A]	±0.02
	16 °C	0.07[a,B]	±0.02	0.06[b,B]	±0.02
	24 °C	0.04[a,C]	±0.01	0.07[b,C]	±0.02

Different capital letters show significant differences (p < 0.05) within the column whereas values with different lowercase letters indicate significant differences (p < 0.05) within the row; ± is the standard deviation

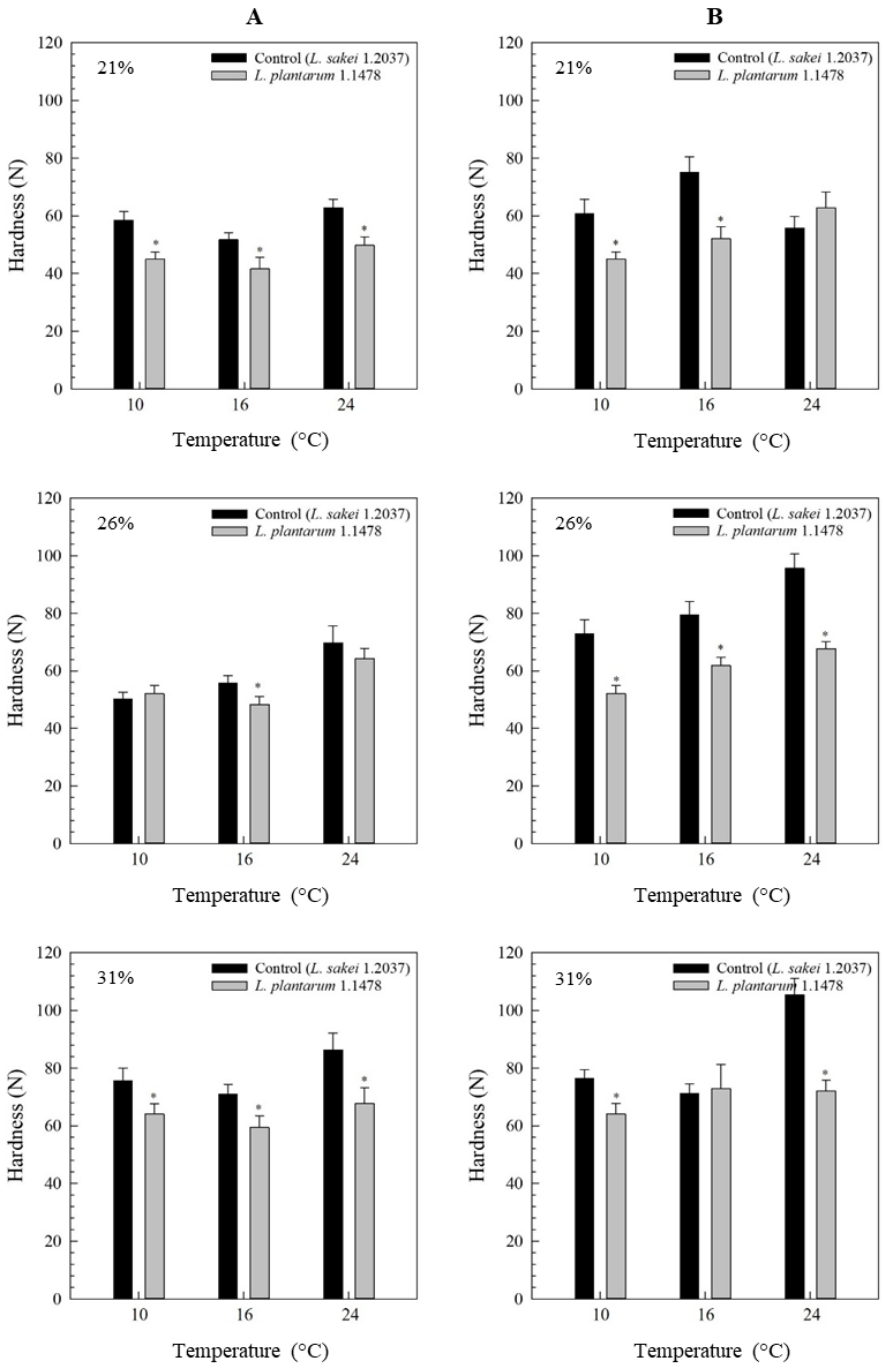

Figure 14 Hardness (N) of sausages produced with the non-EPS-forming strain *L. sakei* TMW 1.2037 (control; ~10^8 CFU/g) or the HePS-forming strain *L. plantarum* TMW 1.1478 (~10^8 CFU/g) of the first (a) and second production (b; independent sausage production). Production was performed at different fermentation temperatures (10, 16, or 24 °C) followed by a drying period at 14°C Above: Sausages reached 21% weight loss; center: Sausages reached 26% weight loss; below: Sausages reached 31% weight loss. An asterisk indicates significant differences to control samples ($p < 0.05$).

To date, the functionality of EPS in various food matrices is yet not completely understood. Known is that monomer composition and ratio, degree of branching, charge density of EPS,

as well as the actual food matrix, and extrinsic/intrinsic conditions during processing influence two phenomena that are of importance with regard to observed EPS functionalities: the polymer - solvent and the polymer – polymer interactions (van de Velde et al., 2015). In sausages with high protein content such as salami (fat particles are surrounded by proteins) latter phenomena are more pronounced, especially at lower pH values since proteins are then often slightly positive charged. Since HePS are often negatively charged they readily associate with positively charged moieties on meat proteins thereby leading to structures (clusters – associative behavior) that influence the organoleptic properties of the final product. Depending on the type and amount of HePS formed this may cause different effects ranging from increased water binding to an increased spreadability as shown for teewurst (pronounced amount of HoPS), or to a harder or softer texture of final products. This is also supported by a study done by Ayala-Hernandez et al. (2008) who proved that bacteria cells are able to bind to protein particles via EPS strands. Moreover, same authors reported that negatively charged EPS tend to aggregate with the milk protein phase as illustrated by CLSM. The formed clusters can then have different effects on the organoleptic properties of food products explaining why for instance Dertli et al. (2016) reported on harder and tougher raw fermented sausages (Sucuk – based on different raw material) while our results showed the exact opposite for salami. However, clear structure-function relationships have yet to be established.

4.4 Conclusion

Using HePS-forming starter cultures in raw fermented sausage manufacturing may lead to products with a softer texture, depending on the strain, matrix, and processing conditions used. Clear structure–function relationships still need to be developed taking not only the target matrix but also processing conditions into account. The present data indicate that one would need to consider the potential ability of starter cultures to produce EPS *in-situ* in meat matrices thereby leading to desired or undesired properties highlighting the importance of a suitable starter culture selection in food processing.

APPENDIX

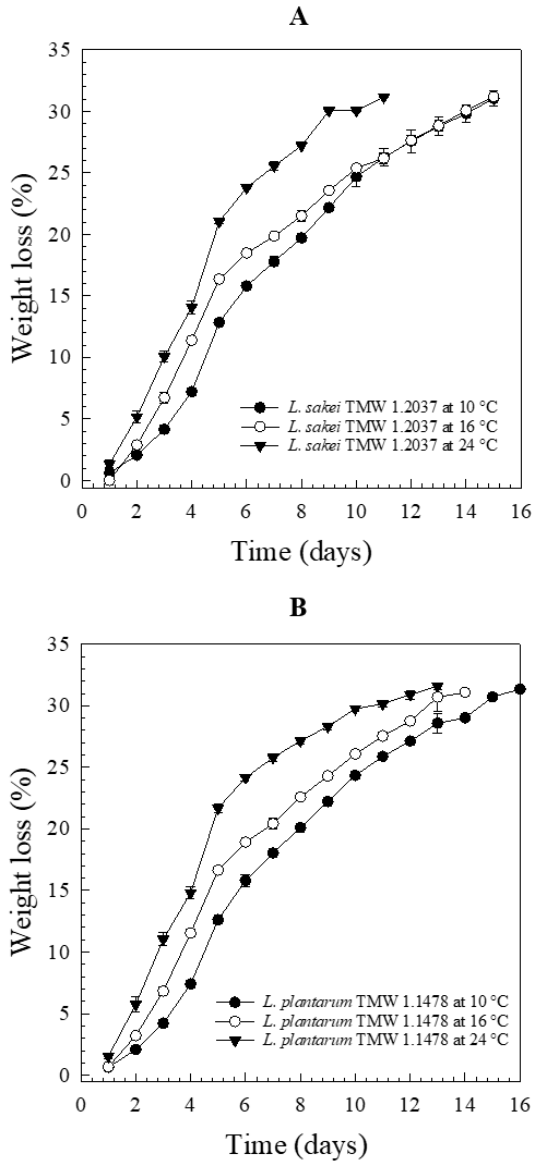

Figure SD 1 Weight loss determination over time of raw fermented sausages that have been produced with the non-EPS-forming strain *L. sakei* TMW 1.2037 (control A; ~10^8 CFU/g) or the HePS-forming strain *L. plantarum* TMW 1.1478 (B; ~10^8 CFU/g) under different fermentation conditions 10, 16 or 24 °C.

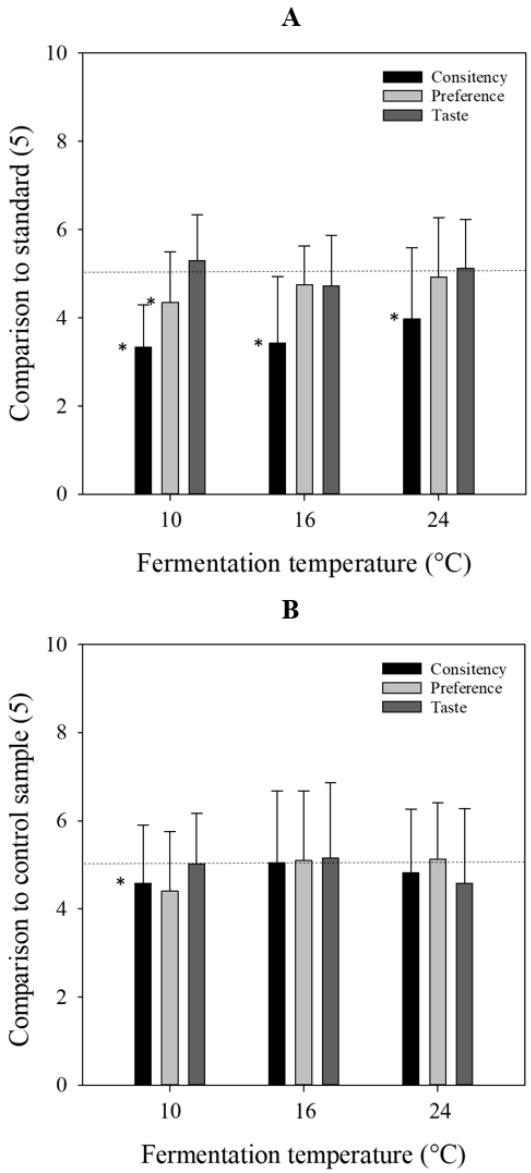

Figure SD 2 Sensory analysis of raw fermented sausages (31% weight loss) that have been produced using different fermentation conditions (10 °C, 16 °C or 24 °C) and either *L. sakei* 1.2037 (control; set as standard with a value of 5, which is indicated by the dotted line) or the HePS-forming strain *L. plantarum* 1.1478 of the first (A) and second (B) independent production. Significant differences between samples are indicated by an asterisk.

Concluding remarks

The present work aimed to generate a better understanding of microbial EPS-formation in meat matrices by investigating the relationship between the stress response (nitrite curing salt and altered processing conditions), initial starter culture concentration and heteropolysaccharide (HePS)-formation in raw fermented sausages (salami). Here, we also aimed to generate a better knowledge of the relationship between microbial HePS-formation, the resulting structural changes in the protein matrix, and the associated changes in quality attributes, such as texture (**chapter 3** and **4**). In order to select a suitable strain for the manufacture of the raw fermented sausages, different lactic acid bacteria, capable of producing EPS were initially examined in a raw sausage model system under typical fermentation conditions and fermentation kinetics studied by investigating different sugar concentrations (**chapter 2**). The formation of EPS was qualitatively and semi-quantitatively assessed using confocal laser scanning microscopy and possible structure function relationships proposed (**chapter 2-4**).

Lactic acid bacteria capable of producing EPS are widely used in the food industry to improve the quality characteristics of food products, especially dairy and bakery products (Y. Cui, Jiang, Hao, Qu, & Hu, 2017; Mende, Rohm, & Jaros, 2016; Ua-Arak et al., 2015). However, there is a growing interest in the use of such cultures in meat products currently produced with hydrocolloids and/or phosphates, as EPS formed *in-situ* appear to have similar technofunctionalities but do not need to be declared (Loeffler et al., 2020). In addition, the use of such starter cultures offers new opportunities in terms of product development, provided that it is possible to generate a better understanding between the manufacturing processes, sufficient microbial EPS formation and interactions with the target meat matrix (varies from product to product) to establish structure-function relationships. Depending on the strain and available sugar source, either homopolysaccharides (HoPS) or HePS are formed, with the latter formation being more complex and energy consuming (Donot, Fontana, Baccou, & Schorr-Galindo, 2012). This is also the reason why higher amounts of HoPS than HePS are usually formed in food matrices. However, unlike HoPS, HePS have a more complex structure and are often charged, which can lead to structural changes even at very low concentrations. EPS formation is generally dependent on many factors and is often enhanced under increased stress conditions (Prechtl, Wefers, Jakob, & Vogel, 2018). Initial studies with meat products showed that HoPS-forming starter cultures appear to be promising for spreadable fermented products where the goal is to reduce fat content and product pH while

maintaining spreadability (Hilbig et al., 2019). These initial results and an overview of conditions that may favor EPS formation in food matrices, as well as a summary of EPS detection methods, were presented in an extensive literature review (**chapter 1**).

To pre-select suitable lactic acid bacteria capable of producing EPS in a meat matrix while lowering the pH to the target value for a raw fermented sausage (4.9-5.2) under typical fermentation conditions (24 °C), a model system already containing the typical ingredients of a raw sausage (including 28 g/kg nitrite curing salt) was used. In addition, the acidification kinetics were studied as a function of different sugar concentrations, with the sugar source being adjusted if HoPS- or HePS-forming strains were studied. A total of 4 different lactic acid bacteria were examined, the HePS-forming strains *L. plantarum* TMW 1.1478 and 1.25, and the HoPS-forming strains *L. curvatus* TMW 1.624 and *L. sakei* TMW 1.411. The formed EPS were then qualitatively analyzed by confocal laser scanning microscopy after staining the EPS and proteins with Concanavalin A and Calcofluor, respectively, followed by semi-quantitative data interpretation (image analysis) (**chapter 2**). After only 10 h of fermentation at 24 °C, increased amounts of EPS were detected and were located on the protein surface. Although all strains studied were able to produce EPS in the matrix while lowering the pH, *L. plantarum* TMW 1.1478 showed the best overall performance. Moreover, due to the complex and often charged structure of HePS mentioned earlier, it is very likely that their formation leads to changes in the salami protein matrix (polymer-polymer interactions). Therefore, *L. plantarum* TMW 1.1478 was subsequently used in the production of raw fermented sausage and the results were compared with those obtained with a non-EPS-forming strain with similar kinetics (*L. sakei* TMW 1.2037) (**chapter 3** and **4**).

Based on the above strain selection, raw fermented sausages containing either *L. plantarum* TMW 1.1478 or *L. sakei* TMW 1.2037 (control) were prepared and the quality attributes of the salami, especially texture (consistency) and taste, were evaluated. HePS formation and its influence on product characteristics were studied as a function of different inoculation concentrations (10^7 CFU/g to 10^9 CFU/g), following our hypothesis that a higher inoculation concentration leads to a stronger formation of HePS and thus to more pronounced structural and, consequently, textural changes (**chapter 3**). In addition, the influence of different fermentation temperatures, more specifically 24 °C, 16 °C and 10 °C, was investigated on the assumption that colder temperatures may further enhance HePS formation due to the additional stress factor (besides nitrite curing salt). The same formulations were used for all productions, with the latter samples being exposed to the respective "fermentation

temperature" within the first 7 days of production. Thereafter, all samples were dried under the same processing conditions until a weight loss of 31% was achieved (**chapter 4**). While changes in inoculation concentration and fermentation temperature were not reflected in the results of the EPS analysis, which was attributed to matrix effects, an inhomogeneous distribution of HePS formed, and the generally low amount of HePS formed during biosynthesis, significant differences were observed with respect to the quality characteristics of the products. In general, products containing the HePS-forming strain *L. plantarum* TMW 1.1478 were found to be softer *($p < 0.05$)* compared to control samples containing the non-HePS-forming strain *L. sakei* TMW 1.2037. This was also true for the products prepared under other fermentation conditions. This softening effect was more pronounced at higher inoculation concentrations and was also reflected in the sensory analysis. Taste was not negatively affected by the presence of the starter culture. However, depending on the target market, the observed softening effect is associated with poorer product quality (salami), as is the case for the German market, for example. Therefore, the use of a HePS-forming strain in dry-fermented sausage does not seem to be recommendable based on the results and findings so far. In addition, the differences in fermentation temperature affected the time required to achieve a 31% weight loss, with the samples containing the HePS-forming strain requiring slightly more time (**chapter 3** and **4**).

In summary, the results presented in this dissertation contribute to establishing a link between microbial *in-situ* HePS-formation and their effects in a complex protein-rich matrix, particularly in raw fermented sausages (salami). To date, the biosynthesis of HePS and how it is influenced by environmental factors remains an active area of research with many questions still unanswered. This thesis provides some insights into HePS-production, especially as it pertains to complex meat matrices, and gives first indications on how it can be modulated by changing critical process parameters such as fermentation temperatures or the initial inoculum levels. At this stage, structure-function relationships need to be further investigated, but the obtained results suggest that polymer-polymer interactions may be of importance in particular in protein-rich matrices, since the charged nature of most HePS promotes pH-dependent electrostatic interactions with meat proteins. These interactions may lead to aggregations or phase separations as observed in dairy products, affecting the structural and hence organoleptic properties of such products. The observed softening effect may be disadvantageous with respect to sliceable dry sausages, but may have potential for other applications, such as spreadable onion sausages. Initial findings on the effect of HoPS-forming starter cultures are available, but should be further investigated in depth

Outlook

The investigations carried out in this thesis have shown that there is a correlation between EPS and, in particular, microbial HePS-formation and quality changes in sliceable raw fermented sausages, especially as it pertains to product texture. These changes can be triggered by appropriate choices of inoculation levels and fermentation temperature thereby offering opportunities for process control. To gain a better understanding of HePS-formation and its effects on product properties, all product applications were performed with the same strains. However, since EPS production is not only matrix- but also strain-dependent, further studies should be carried out to include other HePS- or even HoPS-producing lactic acid bacteria. While the latter have shown great potential in terms of improving the spreadability of selected short-ripened products such as tea sausage, their interactions with the product matrix of a salami might be different. The observed softening effect of salami when using *L. plantarum* 1.1478 has been associated with poorer quality in the German market but could prove to be a benefit in other markets and in other fermented products. Since HePS can vary greatly in structure, further investigations should focus on the influence of process parameters and matrix composition on their formation. This will also help to establish structure-function relationships that may serve as product development guides to the food industry. Additionally, comparing the effects caused by an *in-situ* EPS formation with those caused by an *ex-situ* application of EPS, could further improve the overall understanding in this field of research.

On a final note, the results of this thesis also showed that a correlation of the observed effects with the amounts of EPS formed under the different processing conditions is quite difficult, mostly because the chosen method – while being a useful qualitative method for detecting EPS in meat matrices – is one that is associated with large standard deviations due to the complexity of the food matrix. To obtain a better understanding of EPS-production and a better estimation of the amount of EPS formed, high performance liquid chromatography (HPLC) may be used instead. However, one should be aware that the sample preparation there is quite labor intensive limiting high throughput investigations.

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Education

09.2013 – 09.2014 **Exzellenz -Stipendienprogramm "Ph.D International Program Global Food Security"**

Fakultät Agrarwissenschaften, Universität Hohenheim.

Food Security Center. Stuttgart

- Identifizierung der Hauptfaktoren für unsichere Nahrungsmittel sowie Ernährungssituationen
- Entwicklung von Strategien zur Problemlinderung der Ernährungssicherheit in Entwicklungsländern

09.2009 – 12.2011 **Master of Science - Food Science and Engineering (Ernährungs- und Lebensmittelwissenschaften mit Fachrichtung in Prozess- und Produktwissenschaft)**

Universitat Politécnica de Valencia – Spanien

Masterarbeit: "Opinion and consumer response to cheeses with reduced salt and / or fat content: interest in their consumption and influence of nutritional information on their acceptability"

02.1998 – 10.2008 **Bachelor of Science - Industrielle Mikrobiologie**

Pontifícia Universidad Javeriana, Bogota – Kolumbien

Bachelorarbeit: "Combined effect of High Hydrostatic Pressures (HHP) and Olive Powder in the inactivation of *Bacilllus cereus* in a vegetable beverage"

Beruflicher Werdegang

Seit 09. 2020 **GMP Compliance Manager im Bereich QC**
Biotest A.G. Dreieich

- Eigenverantwortliche Planung, Koordination und Überwachung von Aufgaben, die im Rahmen des Life-Cycle- Managements von GMP relevanten Systemen, Methoden und Geräten anfallen.
- Eigenverantwortliche Betreuung der QC Labore als Schulungsverantwortliche. Überwachung des Schulungsstandes und Pflege der Qualifikationsprofile.
- Unterstützung bei der Planung, Durchführung und Auswertung von Maßnahmen im Rahmen von Gerätequalifizierungen und Methodenvalidierungen zusammen mit den QC Laboren.
- Information und Weiterentwicklung der QC-Labore: Data Integrity, Validierung, Qualifizierung Abweichungen, Chance Control
- Planung von vor- und nachbereitenden Maßnahmen für Audits und Inspektionen in Zusammenarbeit mit den QC-Laboren und deren selbständige Verfolgung und Begutachtung im Rahmen allgemeiner GMP-Richtlinien.

09.2019 – 08. 2020 **Process Analytics Qualitätskontrolle Molekularbiologie**
Biotest A.G. Dreieich

- Laborprozesse Optimierung (Nukleinsäure-Amplifikations-Techniken (NATs) zum Nachweis von Virusnukleinsäuren in Spenderblut)
- Dokumentation von Analysenergebnissen (Rohdaten) in Protokollen und Prüfanweisungen.
- Review von statistischer Analyse.

09.2018 – 07.2019 **Wissenschaftliche Beraterin für funktionelle Lebensmittel Projekte**
Home based, Frankfurt am Main

- Technische Beratung bei neuen Anwendungen von Bakterien in fermentierten Produkten.

01.2018 – 07.2018 **Assistentin der Projektleitung**
Deea Solutions GmbH. Frankfurt am Main

- Data Research im Bereich Solarenergie (Internationale Projekte).
- Unterstützung in der Verarbeitung der technischen Berichte.

10.2013 – 03.2017 **Doktorandin für Lebensmittelwissenschaft**

- Betreuung für Biophysik und Master/Bachelor Studenten im Bereich Mikrobiologie und Fleischherstellung
- Abwicklung von Laborpraktiken und Testaktivitäten auf dem Gebiet der Biophysik (Wechselwirkungen zwischen Polymeren, Emulsionen und Rheologie)
- Untersuchungen zu sensorischen Eigenschaften, Akzeptanz und deskriptive sensorische Analyse

01.2012 – 06.2013 **Assistentin des Projektmanagements / wissenschaftliche Beraterin**
Community Level Environmental Awareness Network Foundation Africa e.V. CLEAN AFRICA, Stuttgart

- Planung, Koordinierung und Anpassung von Projekten mit dem Schwerpunkt der Förderung nachhaltiger Entwicklung in Afrika
- Technische Beratung zu Fragen der Ernährungssicherheit und Ernährung gefährdeter Bevölkerungsgruppen (schwangere Frauen und schulpflichtige Kinder)

10.2008 – 07.2010 **Wissenschaftliche Hilfskraft**
Institut für Agrochemie- und Lebensmitteltechnologie (IATA), Valencia – Spanien
Projekt "Impact of High Hydrostatic Pressures HHP and heat treatment on the inactivation and risk assessment of *Listeria innocua* CECT 910 Y and *E. coli* CECT 433 in different substrates"

Lina Maria Velasco Cucaita
Frankfurt am Main, 23.08.2021

Eidesstattliche Versicherung

Eidesstattliche Versicherung gemäß § 7 Absatz 7 der Promotionsordnung der Universität Hohenheim zum Dr. rer. nat.

1. Bei der eingereichten Dissertation zum Thema *New approaches in salami manufacture with in-situ exopolysaccharide-forming starter cultures* handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

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Die Richtigkeit der vorstehenden Erklärung bestätige ich: Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Frankfurt am Main 23.08.2021

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