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Influence of Yoghurt and Cheese Reformulation Strategies on Microbial Ecology and Product Characteristics

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Abstract

Salt and sugar are important ingredients for the production, microbial safety, shelf life and flavour of quality fermented dairy products. However, these ingredients are also modifiable risk factors for non-communicable diseases, such as coronary heart disease and diabetes, whose prevalence accounts for almost half of the global disease burden. Together with legislative action and consumer education, reformulation of foods to contain less salt and sugar can positively influence individual and population health. The aim of this study was therefore to analyse the effect of different reformulation strategies on the microbial ecology and product characteristics of two fermented dairy products.

In the first subproject, sweetness-enhanced yoghurt was produced using enhanced milk created using a bi-enzymatic modification of the moderately sweet milk sugar lactose to increase its sweetening power by a factor of 2-3. This aimed to ultimately reduce the added sugar needed to achieve the same sweetness in the end product. The growth and acidification of starter lactic acid bacteria (SLAB) was investigated in reformulated yoghurt, demonstrating that SLAB fermented the enhanced milk in an equal manner to regular milk. Testing for potentially pathogenic or spoilage bacteria indicated that the microbial quality of reformulated samples was not affected. Product characteristics such as pH were monitored throughout fermentation and storage, showing no difference between enhanced and regular samples. High-throughput sequencing (HTS) studies demonstrated comparable microbial diversity in sweetness-enhanced and regular yoghurt, and a quick dominance of SLAB during fermentation. In a parallel study, it was demonstrated that a 10 – 20 % reduction in added sugar was possible without affecting the sensory properties of the reformulated yoghurt samples. Further studies to test the scalability of these pilot plant experiments are necessary to establish the industrial potential of this reformulation approach.

In the second subproject, Edam cheese was reformulated to contain < 0.4 % Na by applying a simple NaCl reduction strategy as well as a mineral salt substitution approach during brining. SLAB growth and technological parameters were examined throughout fermentation and a ripening period of six weeks. A 30 % Na reduction was possible through simple reduction, whilst mineral salt substitution achieved a 50 % Na reduction. No difference in SLAB growth or analysed product characteristics such as mineral content and pH was observed between sodium-reduced and control cheeses. A challenge test with the *Listeria monocytogenes* surrogate *Listeria innocua*, as well as testing for potentially pathogenic or spoilage bacteria, indicated no loss of microbial safety through

sodium-reduction. HTS studies showed a significant difference in microbial ecology between production and ripening, yet no difference in microbial diversity between sodium-reduction strategies was found. However, a parallel study of the sensory attributes demonstrated that the reformulated Edam samples displayed a distinctly bitter taste. A production experiment in the research lab of a commercial dairy indicated that these approaches were scalable to larger cheese loaves, yet further studies are needed to confirm industrial applicability and reduce off-flavours.

These two reformulation studies provide a scientific basis for successful reformulation of fermented dairy products, thereby providing avenues for viable approaches to lower salt and sugar with the aim of improving population health if commercialised.

Zusammenfassung

Zucker und Salz sind wichtige Zutaten für die Produktion, die mikrobielle Sicherheit, die Haltbarkeit und den Geschmack von fermentierten Milchprodukten. Sie sind jedoch auch Risikofaktoren für nichtübertragbare Krankheiten wie die koronare Herzkrankheit oder Diabetes, welche etwa die Hälfte der weltweiten Krankheitslast ausmachen. Gemeinsam mit gesetzgeberischen Maßnahmen und Aufklärungskampagnen für Verbraucher kann die Reformulierung von Lebensmitteln, insbesondere durch die Reduktion von Zucker oder Salz, eine positive Auswirkung auf die Gesundheit der Bevölkerung haben. Das Ziel dieser Doktorarbeit war es, sowohl die mikrobielle Diversität als auch ausgewählte Produkteigenschaften von Joghurt und Edamer Käse zu untersuchen, welche durch den Einsatz unterschiedlicher Methoden reformuliert wurden.

Im ersten Teilprojekt wurde anhand eines bi-enzymatischen Systems eine 2-3-fache Steigerung der Süßkraft von Lactose in Milch erreicht, die dann für die Joghurtproduktion eingesetzt werden konnte. Das übergreifende Ziel dabei war es den Anteil an zugesetztem Zucker senken zu können ohne die Süße im Endprodukt zu reduzieren. Die Untersuchungen zeigten, dass sich die Mikroorganismen der Starterkulturen in süßkraftverstärkter Milch und in Kontrollmilch bezüglich ihres Wachstums- und Säuerungsverhaltens während der Fermentation und der anschließenden Produktlagerung sehr ähnlich verhielten. Es konnten keine Hindernisse für den Fermentationsprozess oder die Produktsicherheit des Joghurts festgestellt werden. Mittels Hochdurchsatzsequenzierung (HTS) wurde gezeigt, dass die Diversität der Mikroorganismen im süßkraftverstärkten Produkt vergleichbar mit der des Kontrolljoghurts war. Eine sensorische Studie die parallel zu dieser verlief, zeigte, dass eine Reduktion von 10 – 20 % an zugesetztem Zucker möglich war ohne den Geschmack des reformulierten Joghurts zu verändern. Der nächste Schritt wird daher sein die Versuche im Industriemaßstab durchzuführen um die Möglichkeit der Umsetzung weiter zu beurteilten, da mikrobiell keine Vorbehalte zum Nutzen dieser Reformulierung bestehen.

Im zweiten Teilprojekt wurde mittels einfacher NaCl Reduktion und dem Einsatz einer Mineralsalzmischung in der Salzlake ein reformulierter Edamer Käse mit < 0.4 % Na hergestellt. Die salzreduzierten Käseproben wurden auf Produktcharakteristika und das Verhalten der Starterkulturen untersucht. Dabei konnte einerseits eine 30 % Reduktion des Natrium-Gehaltes durch einfache Reduktion und anderseits sogar eine 50 % Senkung des Natrium-Gehaltes durch den Einsatz von Mineralsalzmischungen erreicht werden. Die eingesetzten Milchsäurebakterien zeigten bei beiden Strategien einen für

Edamer Käse typischen Wachstumsverlauf. Dabei konnte kein erhöhtes Risiko durch ein Vorkommen von Fremdbakterien in Natrium-reduziertem Käse festgestellt werden. In einem Challenge-Versuch mit *Listeria innocua*, einem Surrogatkeim für *Listeria monocytogenes*, verhielten sich diese im salzreduzierten Käse ähnlich wie im Standardprodukt und vermehrten sich während der Käsereifung nur minimal. Mittels HTS zeigte sich, dass die Salzbadzusammensetzung keinen Einfluss auf die mikrobielle Diversität ausübte, jedoch wurde ein signifikanter Unterschied der Biodiversität zwischen der Käseherstellung und der -reifung beobachtet. Eine sensorische Studie die parallel zu dieser verlief, zeigte dass die reformulierten Edamer Proben einen bitteren Geschmack aufwiesen. Ein Großversuch im Technikum eines Milchbetriebes deutete an, dass diese Reformulierungsstrategien auf den größeren Industriemaßstab angepasst werden können. Es sind jedoch weitere Versuche erforderlich, um die Forschungsergebnisse unter praxisüblichen Produktionsbedingungen zu bestätigen und das Vorkommen von Fremdgeschmack in Natrium-reduziertem Käse zu reduzieren.

Diese beiden Reformulierungsprojekte bilden eine wissenschaftliche Grundlage für die mögliche Reformulierung zweier fermentierter Milchprodukte. Sie zeigen, dass die untersuchte Reduktion von Zucker und Salz in diesen Produkten mikrobiell unbedenklich ist und dass die Reformulierung von Joghurt und Käse daher einen Beitrag zu einer gesünderen Ernährung liefern könnte.

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List of Abbreviations

The International System of Units (SI) has been applied as the system of measurement throughout this thesis and individual components are not further elaborated in the following list of abbreviations.

A	adenine
ALOA	agar *Listeria* according to Ottaviani & Agosti
a_w	water activity
BHI	Brain-heart-infusion (agar)
C	cytosine
cDNA	complementary DNA
CFCD	cetrimide fucidin cephalothin (agar) containing Delvocid®
cfu	colony forming units
DGE	Deutsche Gesellschaft für Ernährung
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	nucleotide triphosphate
DVS	direct vat set
G	guanine
HPLC	high-performance liquid chromatography
HS	high sensitivity
HTS	high-throughput sequencing
KAA	kanamycin aesculin azide (agar)
LAB	lactic acid bacteria
MRI	Max Rubner-Institut
mRNA	messenger RNA
MRS	de Man, Rogosa and Sharpe (agar)
NCD	non-communicable disease
NSLAB	non-starter lactic acid bacteria
OTU	operational taxonomic unit
PCR	polymerase chain reaction
RBB+C	repeated bead beating plus column (method)
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SLAB	starter lactic acid bacteria
subsp.	subspecies
T	thymine
TAE	tris-acetate-EDTA
T_m	melting temperature
UF	ultrafiltration
UV	ultraviolet
VRBD	violet red bile dextrose (agar)
WHO	World Health Organization
YGC	yeast extract chloramphenicol (agar)

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1 Introduction

1.1 Influence of salt and sugar on human health

High consumption of dietary salt and sugar are important modifiable risk factors for non-communicable diseases (NCDs).[1–4] NCDs have been identified as the main cause of morbidity and mortality in the world and are responsible for almost half of the global disease burden and roughly 60 % of total deaths worldwide.[5] The risk factors for developing NCDs are, therefore, a major public health concern. From both an individual and a population health perspective, the level of salt and added sugars consumed by the majority of people worldwide is too high, contributing greatly to a rise in the incidence of associated health problems.[6,7]

Despite being risk factors for NCDs, sodium and sugar are vital for the human body to function properly and their exclusion from our diet would be detrimental to our health. The mineral sodium (Na) is commonly ingested in the form of table salt, where it forms an ionic compound with chloride (sodium chloride, NaCl). Sodium is essential for many vital bodily functions such as the regulation and maintenance of blood plasma volume; cell signal transmission including nerve impulse conduction at the synapses; normal cell function including that of ion-linked transporters for amino acids; and acid-base tolerance.[8,9] Physiologically, the human body requires an intake of as little as 200 - 500 mg of Na per day to guarantee proper bodily function. However, both the Deutsche Gesellschaft für Ernährung (DGE) and the US Institute of Medicine consider an intake of 1.5 g of Na per day satisfactory to allow for sufficient uptake of other nutrients and to replace sweat losses.[10–13] A high daily intake of sodium, in combination with low potassium consumption, has been shown to elevate blood pressure levels, while a reduction in sodium intake can decrease blood pressure regardless of baseline sodium intake.[7,10] Elevated sodium intake has been associated with NCDs such as coronary heart disease, kidney failure, gastric cancer, cataracts and osteoporosis.[14]

Sugar is a broad term used to describe sweet-tasting carbohydrates and is often consumed in the form of table sugar, which is a disaccharide of fructose and glucose called sucrose that is commonly extracted from sugar cane or beet. Carbohydrates are a natural component of a wide selection of foods ranging from fruit to milk and honey. Complex sugars such as disaccharides, oligosaccharides and polysaccharides are digested into monosaccharides within the human digestive tract. With the help of the peptide hormone insulin, our body can use these carbohydrates immediately as an important source of

energy to keep the body functioning or, alternatively, can store them as glycogen in liver and muscle tissue.[9] Although there is no exact indication of the daily metabolic requirement of carbohydrates, the human body receives sufficient carbohydrates from the digestion of sugars contained in natural foods. When discussing consumption of this macro-nutrient, the World Health Organization (WHO) distinguishes between "(a) intrinsic sugars contained in fruit and vegetables, (b) milk sugars (lactose and galactose) and (c) free sugars, which are all monosaccharides and disaccharides that are added to foods and beverages during production, cooking or consumption, as well as natural sugars present in honey, syrups, fruit juices and fruit juice concentrates".[6] The term 'added sugar' is generally used to describe all sugars added during processing and preparation as well as sugar added at the table, not including natural sugars.[15–17] The excess consumption of free sugars presents a major challenge to the human body as large amounts of energy are added, yet no further nutrients are gained. Increased sugar intake is closely connected to the occurrence of NCDs such as obesity, diabetes mellitus type II, hypertension, dyslipidaemia and dental caries.[2,15,16]

1.2 Food reformulation

Although sodium and sugar are vital for the healthy functioning of the human body, their excess consumption should be minimised in order to reduce the risk of NCDs and thereby achieve both personal and public health benefits. Interventions for reducing consumption levels are being sought at both national and international levels. Most notably, the WHO has released recommendations for daily salt and sugar consumption for adults and children, and many countries have introduced legislation for salt and sugar reduction in processed foods to reduce the associated morbidity and mortality through public policy.[6,7,17–23] Despite general guidelines providing a solid starting point for reformulation efforts, Afeiche et al. (2018) emphasised the importance of specific, population-level public health initiatives taking differences in food patterns between populations into account.[24]

Reduction of salt and sugar is difficult for individuals with a high proportion of processed foods in their diet, as processed foods are substantial contributors to total sodium and sugar consumption. In Europe and North America, 75 – 80 % of salt consumption is derived from the intake of processed foods such as bread, meat and dairy products as well as from restaurant meals, while 10 – 15 % stems from table salt added by the consumer during cooking and consumption, and up to 10 % is of natural origin in the consumed foods.[13,25,26] In Germany, 10 % of the portion of sodium ingested through processed

foods can be attributed to cheese and other dairy products, 18 % to processed meats, 25 % to bread and baked products, whilst the remaining 47 % make up a wide range of processed foods.[27] The scope of processed foods contributing to added sugar intake varies greatly amongst populations, as does the initial added sugar content for similar products or for the same products marketed in different countries.[17,28,29] For the German population, nutrition studies have shown that 36 – 41 % of added sugar consumed stems from beverages, including sweetened fruit juices, nectar and other sweetened beverages, 35 – 37 % from confectionery and snacks, 13 – 15 % from pastries and bakery products, 7 – 9 % from dairy products, 1 – 2 % from grain and cereal products and 1 % from spreads and sauces.[27,30,31] These statistics are similar to those for the US American population, where these sources of added sugar constitute 68 % of total sugar consumption.[29]

A wide-reaching approach to reducing sodium and sugar intake in the general population is to reformulate processed foods. Reformulation of foods aims to alter the recipes and composition of processed foods, for example to contain less sodium or less sugar, without compromising the taste, texture, palatability, microbiological safety and storage properties of the products.[11,32–34] Food reformulation can contribute to altered consumption levels without calling on consumers to change their dietary habits. This can especially aid the most disadvantaged people who are often limited in choice, which is why in the UK, for example, the most substantial salt reductions have so far been implemented in the cheapest products.[23,35] However, reformulation should always go hand in hand with public health campaigns that educate consumers about modifiable risk factors for NCDs. Furthermore, food labelling systems such as the use of ‘traffic light’ indicators have also proved useful, directly influencing consumer behaviour and aiding informed food choices, whilst simultaneously motivating the food industry to reformulate their products.[23,36] After testing multiple food labelling systems for ease of use and understandability, the German Government announced the introduction of a voluntary, easy-to-use labelling system called Nutri-Score® in September 2019, which uses a combination of letters (A – E) and colours (green to red) to clearly convey a summary of the nutritional quality of a product to consumers.[37]

Food reformulation benefits consumers by helping reduce risk factors for NCDs, yet the food industry is presented with challenges on multiple fronts to make this happen. Both sodium and added sugars are essential for the production, preservation, safety, taste, texture and palatability of processed food.[11,33,38–40] Therefore, simply removing or reducing the salt or added sugar content from recipes or during the production process could lead to significant quality and safety compromises, and ultimately to market failure of a product or, in the worst case, to serious health risks for the consumer. In addition,

technological challenges arise when reformulating processed food. The food industry is also presented with higher research and development costs in order to create healthier foods for the consumer. However, on a population-scale, the decrease in costs to public healthcare systems, such as the costs of drugs, physician visits, laboratory testing and hospital maintenance through reducing NCD prevalence could be substantial, and many countries are therefore increasingly focussing on food reformulation as a part of public policy.[20,23,41,42] As directly changing the eating habits of consumers is very difficult, and previous efforts to do this have been ineffective,[43] population-wide approaches are instrumental to achieving reformulation goals. Although personal health benefits are not always directly evident at an individual level, population health studies have shown the effectiveness of small reductions in salt and sugar consumption on morbidity and mortality levels resulting from NCDs.[3,6,22,43]

In response to the identification of sodium and added sugars as major risk factors for NCDs and an increase in their intake worldwide, the WHO issued 'strong recommendations' for the daily intake of both sodium and free sugars by adults and children in order to decrease global morbidity and mortality.[6,7] These 'strong recommendations' are evidence-based and the positive effects of this guidance prevail over any possible undesirable consequences.[44] The WHO advises a daily intake of up to 2 g Na or 5 g NaCl per day, a value that has been adopted as a target for reduction efforts in many countries.[7] In Germany, the DGE recommends a daily salt intake of less than 6 g NaCl.[4] Amongst European Union countries, daily salt intake currently ranges between 8 – 12 g per day and the figures for the German population lie at 8.4 g NaCl per day for women and 10 g NaCl per day for men.[27,45] For free sugars, the WHO recommends that less than 10 % of the total daily energy intake should be from free sugars, which equates to roughly 50 g of free sugars a day for adults with an average size and calorie intake.[6] In addition, a conditional recommendation was issued stating that further health benefits may arise, if the free sugars intake is reduced to less than 5 % of the daily energy consumption.[6] Worldwide intake habits vary greatly and strong discrepancies in free sugars intake between European countries are present.[28] In Germany, the Nationale Verzehrsstudie II (2005-2007) demonstrated that for German women 13.9 % of their daily energy intake comes from added sugars and for men this figure lies at 13 %.[31]

To date, numerous countries have begun facing the challenge of salt and sugar reduction in a variety of processed foods and on a population-wide level, with the earliest efforts to reduce salt consumption dating back to the 1960s and 1970s in Japan, Finland and the USA.[23] Successful efforts in Japan, showing a reduction of between 1.4 – 4 g NaCl per day and a reduction in blood pressure in the Japanese population over a 10-year period,[23] and in Finland, presenting an 80 % decline in Finnish mortality rates due to

coronary heart disease over a 35-year period,[46,47] set the stage for salt reduction programs worldwide. Consumer education efforts began in the US as early as 1972, with guidelines for reducing sodium intake first introduced in 1980.[20] In recent years, Australia, Canada, France, Ireland, the Netherlands, Sweden and the UK have all implemented reduction, food reformulation and public policy strategies to provide consumers with healthier food and beverage alternatives, ultimately affecting both individual and population health.[23] Different countries have applied varying strategies for reduction and reformulation; however, generally multiple strategies are implemented in parallel.[48] The UK, for example, began a reduction campaign in 2003 with the overall aim of decreasing salt consumption from an average of 9.5 g NaCl to 6 g NaCl per day by applying food reformulation measures and heightening consumer awareness alongside front-of-packaging labelling.[20] In addition, the UK followed the example of countries such as Chile, France, Hungary, Mexico and Norway,[49] by implementing a tax on sugar sweetened beverages that came into effect in April 2018. By putting a levy on the amount of sugar per 100 mL of sweetened drinks, the British government pressured the food industry to reformulate sweetened beverages to contain less sugar and to discourage people from drinking sweetened drinks.[50]

In Germany, a national reduction and innovation strategy for salt, sugar and fat in processed products has been developed and was introduced by the Federal Ministry of Food and Agriculture in December 2018 as part of a more holistic approach to encouraging healthier food habits in the population.[51] This all-encompassing strategy brings together the approaches of committing the food industry to reduction goals, monitoring population health, and continuing Federal support for research and innovation focussed on reduction and reformulation of processed foods.[51] Furthermore, a sugar tax system has been debated but not yet implemented, due to a perceived lack of long-term evidence of the benefits of such a strategy.[51]

1.3 Reformulation strategies

Food reformulation is a time-consuming and expensive process that is best applied to food groups that are substantial contributors to daily, population-wide intake in order to achieve the highest impact on health outcomes. Based on the diverse nature of processed foods, the reformulation approach can vary greatly depending on the product group. Three commonly implemented reformulation strategies are reduction, replacement and removal, and all require substantial industry efforts to ensure effective manufacture, adequate safety, as well as consumer acceptance of the final product.[32,52] The

choice of reformulation strategy will depend on, and be adapted to, each food group accordingly and it should be used in conjunction with other strategies, including public policy and awareness strategies.

1.3.1 Reduction, replacement and removal

Reduction, replacement and removal are key strategies of food reformulation that can be implemented either independently or in parallel. Reduction strategies describe progressive decreases, for example in salt or added sugars, that are implemented in small steps over an extended period of time so that minor changes in taste are not noticed by consumers.[52] This covert reduction of sodium or added sugar in small steps is possible as minor decreases are not detectable by the consumer, and therefore a progressive adaptation to the reformulated products is possible without consumers altering their product choices.[11,52,53] Indeed, in 2003 Girgis et al. showed that a sodium reduction of up to 25 % in bread was not perceived by consumers.[54] However, incremental reduction requires an extended period of time to effectively reduce salt or sugar levels without compromising consumer acceptance. It is important that consumer preferences are gradually adjusted to the lowered salt or sugar levels, to avoid salt or sugar being added again at the table. Further challenges faced during simple reduction are retaining the taste, flavour and palatability of the product, as well as ensuring quality and safety.

Replacement describes the substitution of ingredients of processed foods that have been identified as risk factors for NCDs with less harmful substitutes that exhibit similar technological, preservative and sensory functions.[17,55] Examples of food reformulation through replacement include the use of minerals such as potassium as a substitute for sodium, exchanging the added sugars in products with non-caloric or low-energy sweeteners, or even adding alternative ingredients such as herbs to manufacture products with reduced sodium or added sugar content.[11,17,56] Studies have investigated substitution of sodium with other mineral salts, but this approach is limited as substitution salts often impart off-flavours to the products in addition to the desired salty flavour.[11] For example, it was found that up to 30 % of sodium chloride can be replaced by potassium chloride in a variety of products ranging from cheese to bread to salted mackerel without producing negative sensory qualities.[57–59] However, increased substitution dramatically decreased consumer acceptance. Furthermore, not only salt substitutes, but also low-calorie sugar substitutes such as stevia, have been found to impart off-flavours in dairy products.[60] Despite the drawbacks of replacement strategies, this reformulation method is the most wide-spread to date, and is often implemented in conjunction with the addition of flavour-enhancing compounds or bitterness-blocking molecules.

Complete removal of salt or added sugars without replacement is a third possible reformulation strategy.[17,61] However, the removal of salt or added sugars is only feasible in a select range of products in which removal can be concealed by the addition of other ingredients such as herbs and spices in sauces, condiments or seasoning.[17,62] It must be noted that such reformulation efforts can only be implemented in food groups where salt and added sugar do not have any technological or preservation roles. Reformulation through removal requires completely new recipes to be developed in order to balance the loss of salt or added sugars.

1.3.2 Reformulation of fermented products

Salt and sugar play an essential role in the manufacture of fermented products, where LAB are implemented to ferment intrinsic sugars to generate the desired taste and texture of products, as well as to protect commodities against spoilage bacteria through the production of lactic acid and growth-inhibitors.[63] The LAB are adapted to the specific conditions of each food commodity and the food matrix (e.g. milk) must provide optimal bacterial growth conditions so that the desired products can be manufactured. These specific requirements call for additional attention during reformulation of fermented products compared with other food groups. For this, the implemented LAB for each product must be closely monitored in the reformulated environment and their composition may need to be adjusted or even replaced during research and development efforts to achieve the desired taste, texture, palatability and safety of the fermented products. A particular focus should also be placed on the impact of LAB adjustments on the potential growth of spoilage or pathogenic bacteria. Furthermore, the implementation of functional starters that produce sugar polymers, sweeteners or aroma compounds may be an added advantage during reformulation of fermented dairy products.[64]

1.4 Lactic acid bacteria and their use as starter cultures

Lactic acid bacteria are phylogenetically related microorganisms that play a crucial role in the production of fermented foods. Taxonomically, they belong to the *Firmicutes* phylum and the *Bacilli* class and are further taxonomically and morphologically classified into various genera of the order *Lactobacillales*.[65] LAB are described as a group of gram-positive, catalase-negative, non-respiring yet aerotolerant, and non-sporulating bacteria with a low mol % GC content.[65] Morphologically, they are either rod-shaped (bacilli) or spherical (cocci) and are further characterised both by their ability to produce lactic acid, as well as by the enantiomer of lactic acid produced.[63] Lactic acid production by LAB can

occur either through fermentation of carbohydrates via a homofermentative process, producing primarily lactic acid, or through heterofermentative fermentation to produce lactic acid, carbon dioxide, acetic acid and ethanol as end products.[65] Furthermore, their classification into different genera is influenced by their optimal growth conditions, ranging from 10 °C to 45 °C, and their tolerance of both salt, acids and bases and conditions of low water activity (a_w).[63,66,67]

LAB are naturally present in milk as well as on fruit, vegetables and grains. In these foods, and in their desired growth conditions, they can undergo spontaneous fermentation of the sugars present resulting in naturally fermented products.[68] The lactic acid produced during fermentation creates a growth-inhibitory environment for spoilage or pathogenic bacteria, making fermentation an effective method of food preservation.[33] In addition to indigenous LAB, specific starter cultures with a defined microbial composition based on desired metabolic and functional characteristics can also be added during the manufacturing of fermented foods.[69] This is most commonly done for foods based on fermentation of milk or meat. The addition of starter cultures has the major advantage of allowing reproducibility and standardisation of product characteristics due to knowledge of the composition and properties of the implemented bacteria.[64] When starter cultures are added to fermented products, these are added in such high numbers that starter culture growth outcompetes the growth of the non-starter lactic acid bacteria (NSLAB) and other bacteria naturally present in the foods. The composition of starter cultures will vary depending on the product, yet they are designed so that the mixed culture can ferment the majority of lactose present in the matrix into lactic acid.[70] Most mesophilic mixed dairy starter cultures predominantly contain *Lactococcus* (*Lc.*) strains, whilst thermophilic mixed cultures usually contain of two strains of microorganisms, namely *Streptococcus* (*S.*) *thermophilus* and a *Lactobacillus* (*Lb.*) strain such as *Lb. delbrueckii* subsp. *bulgaricus*, as used for example in yoghurt production. Fermentation using starter cultures can, in conjunction with the addition of fermentable sugars, also be applied to foods that do not undergo spontaneous fermentation, for example meat or fish products, providing a means of preservation and augmenting taste properties.[71]

In addition to the mixed starter cultures described above, many fermented dairy products, such as cheese, also benefit from the addition or presence of adjunct cultures of LAB, NSLAB and other, non-lactic acid producing microorganisms.[72–74] Adjunct cultures are often implemented in the production of cheese, where selected mesophilic or thermophilic bacteria such as specific *Lactobacillus* strains are added to aid flavour formation and, in some instances, produce antimicrobial compounds against unwanted microorganisms.[73,75] In the case of thermophilic adjunct cultures, these organisms can withstand

the temperatures needed during cheese production, but bacterial growth is limited below 25 °C, allowing contribution to flavour production but little addition to acidity.[73] NSLAB that are present in fermented dairy products originate either from the raw milk or from the production process and can contribute to product flavour.[76] Although outnumbered by starter LAB (SLAB) during production, NSLAB grow quickly during ripening and can influence flavour through their metabolic activity and the production of volatile flavour compounds.[73] Furthermore, non-LAB microorganisms can also be implemented in the production of fermented dairy products to achieve distinct product characteristics such as the propionibacteria used in Emmental cheese production.[73] Finally, in addition to the fermentation properties of LAB, some of these microorganisms such as bifidobacteria were found to have beneficial effects on the gut microbiome. Some serve as probiotics, whilst others produce beneficial enzymes upon reaching the intestine, giving them ideal characteristics to function as prebiotics.[76–78] Thanks to these beneficial properties, the consumption of fermented products, including fermented dairy products, has seen a massive increase in popularity amongst health-conscious consumers in recent years, also making them an important target for reformulation efforts.

Starter cultures of LAB for the commercial production of fermented dairy products can be obtained in liquid, deep-frozen or freeze-dried form.[79] Liquid cultures, however, are less stable than deep-frozen or freeze-dried starter cultures, and require multiple sub-culturing steps to achieve an active culture prior to inoculation of the milk.[79] The use of deep-frozen or freeze-dried cultures is becoming more widespread in the dairy industry due to ease of use and reproducibility.[80] The International Organization for Standardization states that starter cultures should contain at least 10^8 cfu/g of viable starter culture bacteria.[81] However, deep-frozen and freeze-dried starter cultures are highly concentrated and generally contain around 10^{10} – 10^{11} cfu/g and 10^{11} – 10^{12} cfu/g of SLAB, respectively.[79] They are often labelled as Direct Vat Set (DVS) cultures as they can be added directly to the milk vat without prior sub-culturing.

1.5 Yoghurt

1.5.1 Production and microbial ecology

Yoghurt is a dairy product that relies on the close interaction of a select few thermophilic SLAB that live in symbiosis and ferment the carbohydrates in milk to produce a thickened, sour milk product. Yoghurt is most commonly produced with pasteurised and homogenised bovine milk, although milk of other origins such as ovine milk can also be

used. The pasteurisation step is performed at either 62.8 °C for 30 minutes (batch pasteurisation) or at 71.7 °C for 15 seconds (high-temperature short-time pasteurisation) and serves to eliminate unwanted microorganisms that could later lead to spoilage of the product or illness of consumers.[82] In addition, it diminishes the overall bacterial count, assisting further processing of the milk.[83] Subsequent high-pressure homogenisation at 100 – 350 bar and 60 – 70 °C to achieve equally distributed fat particles of 0.5 – 1 µm in diameter, as well as standardisation steps, then help create a uniform composition of fat and dry matter in the milk to produce the best possible product characteristics.[83]

Traditional yoghurt is manufactured with the help of specific starter culture strains of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*, which are added to the pasteurised and homogenised milk in *Streptococcus* to *Lactobacillus* ratios ranging from 1:1 to 2:3.[83] The ratio of microorganisms changes many times throughout fermentation and resumes the original ratio towards the end of the fermentation process.[83] These microorganisms symbiotically ferment lactose to produce lactic acid, reducing the pH of the product to 4.2 – 4.5.[70] These two cultures are implemented simultaneously, as their symbiotic relationship increases acid production and decreases overall fermentation time.[84,85] Since *S. thermophilus* is more aerotolerant than its counterpart, it grows rapidly upon inoculation of the milk, producing formate and creating more suitable growth conditions for *Lb. delbrueckii* subsp. *bulgaricus* later in production.[85,86] The growth of *S. thermophilus* also produces carbon dioxide and thereby lowers the oxygen pressure in the matrix, stimulating *Lb. delbrueckii* subsp. *bulgaricus* growth once the pH reaches 5.2.[84,87] The high proteolytic activity of *Lb. delbrueckii* subsp. *bulgaricus* produces peptides and free amino acids from milk protein that *S. thermophilus* can then utilise.[86,87] This proteolytic activity stimulates *S. thermophilus* to produce the aroma compound acetaldehyde, a major flavour component typical for yoghurt.[70,83] As *S. thermophilus* does not grow at pH 4.4, *Lb. delbrueckii* subsp. *bulgaricus* is responsible for late stage and post-acidification of the yoghurt. However, long storage times can lead to excessive acidity and the formation of bitter peptides due to the proteolytic activity of *Lb. delbrueckii* subsp. *bulgaricus*.[87] Through the release of fatty acids, the lipolytic activity of the *Lactobacillus* strain also contributes to aroma formation in yoghurt.[83]

Yoghurt can be left to set directly in yoghurt containers, stirred during production to produce a smooth texture or further homogenised after fermentation to produce drinking yoghurt.[83] Fermentation of yoghurt is typically performed between 43 °C and 45 °C, as the ideal growth temperature for *S. thermophilus* lies between 37 – 42 °C and that of *Lb. delbrueckii* subsp. *bulgaricus* lies between 42 – 45 °C.[83] After acidification to about pH 4.6, post acidification occurs when the product is cooled and stored at 4 °C to reach

an end pH of 4.4.[83] Fermentation at temperatures over 42 °C allows the thermophilic bacteria crucial for yoghurt production to dominate over potentially present mesophile NSLAB originating from the milk.[83]

To produce a more mild-tasting yoghurt, an adapted combination of LAB can be used in which *Lb. delbrueckii* subsp. *bulgaricus* is replaced with other *Lactobacillus* species such as *Lb. acidophilus*.[83] Some work has also been done using *Lb. delbrueckii* subsp. *bulgaricus* mutants to achieve yoghurt with a milder taste.[87] In addition, *Bifidobacterium (B.)* species can be added during production to not only contribute to less acidity, but also to provide additional health benefits to the consumer.[88] The growth optimum of these alternate bacteria lies at 40 – 43 °C and they are less capable of producing acid, hence a longer fermentation time of 6 – 8 hours is needed for fermentation to be complete.[83] The final pH of mild-tasting yoghurt lies above 4.0 and this contributes to the reduced sourness of the final product as well as the reduced formation of bitter peptides.[83] In order to benefit from the health-promoting effects of yoghurt containing bifidobacteria, these microorganisms need to be added in such high concentrations as to guarantee functional levels in the yoghurt until the end of the product use-by dates.[89] Furthermore, prebiotics such as the carbohydrate oligosaccharides can be added to sour milk products to promote the growth of LAB or bifidobacteria in the gut of the consumer.[88] In Germany, the combination of *S. thermophilus* and *Lb. acidophilus* that produces a mild-tasting yoghurt has become very popular amongst consumers and legislation requires all yoghurt produced with these mild-tasting cultures to be labelled accordingly.[90]

1.5.2 Sugar content

A simple comparison of the nutrient composition of yoghurt and sweetened dairy products in the local supermarket performed by the Max Rubner-Institut in 2016 revealed that these products often contain up to 22 g total sugar per 100 g.[31] Of these ca. 20 %, about 4 – 6 % can be attributed to the naturally occurring milk sugar, lactose, in bovine milk.[74] Lactose is a disaccharide consisting of the two monosaccharides D-glucose and D-galactose, which can be hydrolysed by the enzyme β-D-galactosidase to yield its monosaccharide components.[91] As lactose is only moderately sweet, with a relative sweetness of 20 – 40 % that of sucrose, this milk sugar hardly contributes to the overall sweetness of the end product.[92] However, its individual monosaccharide components have considerably higher sweetening power, with glucose exhibiting 60 – 70 % and galactose 50 – 70 % the sweetness of sucrose.[92] In comparison, fructose is about one-third sweeter than sucrose.[92]

Yoghurt is a naturally sour product due to carbohydrate fermentation by LAB and the resulting formation of lactic acid, and hence many consumers prefer sweetened rather than natural yoghurt products. Natural yoghurt is sweetened by manufacturers through the addition of sugars. These nutritive carbohydrate sweeteners such as cane sugar, high fructose corn syrup or dextrose,[93] constitute the majority of the sugar content of sweetened dairy products and reduction of these added sugars is a key target of reformulation efforts.

1.5.3 Reformulation strategy: bi-enzymatic system of lactose conversion

The reformulation strategies reduction, replacement and removal presented earlier are suitable for implementation in all sweetened dairy products to help reduce population-wide added sugar intake. However, due to the natural acidity resulting from the fermentation process carried out by LAB, consumer acceptance of reformulated flavoured yoghurts may be especially difficult to achieve, as large amounts of added sugar are needed to counter the natural sourness of the product. An additional reformulation strategy applicable to sweetened dairy products is to make use of the inherently present lactose as a sweetener. The monosaccharide components of lactose exhibit a considerably higher sweetening power than lactose itself. Thus, hydrolysing this disaccharide into D-glucose and D-galactose using the enzyme β-galactosidase serves to sweeten the milk by utilising the inherent sugars that are naturally sweeter than lactose, rather than adding further sugars to sweeten products. This process has been widely implemented by manufacturers of lactose-free milk products since the mid-1970s, albeit with the primary aim of removing lactose. It has also been investigated for use, for example, in chocolate milk and ice cream to contribute to sweetening these dairy products.[94–96]

Although contributing to sweetness, lactose hydrolysis does not make milk sweet enough to be able to entirely replace the added sugar component of sweetened dairy products whilst retaining a similarly accepted product. Previous studies have investigated the possibility of combining lactose hydrolysis with a subsequent glucose isomerisation step in lactose solutions or in whey to generate fructose, in order to increase the relative sweetness.[97,98] In 2003, Lorenzen et al. investigated the potential of using the glucose-isomerase enzyme to isomerise roughly 50 % of the glucose gained through lactose hydrolysis into fructose in permeates from skim milk.[99] Through this bi-enzymatic process (lactose hydrolysis and glucose isomerisation), the authors were able to enhance the sweetening power of milk by 2 – 3 times relative to unmodified milk.[99] By utilising this process, there is potential to greatly reduce, or ideally even entirely replace, the added sugar component of flavoured yoghurts and other sweetened milk products.

However, this bi-enzymatic system for lactose conversion presents some technological challenges for production of sweetness-enhanced products, in particular during the second enzymatic conversion step. These challenges lie within the calcium sensitivity of the glucose-isomerase enzyme used for the conversion of glucose to fructose, limiting its ability to isomerise the glucose moiety.[99] Lorenzen et al. presented a possible solution to this problem by implementing an ultrafiltration (UF) step during production of dairy products.[99] In this process, raw milk first undergoes a skimming process to remove the cream layer and this skim milk is then ultrafiltered to produce a calcium-free UF-permeate and a UF-retentate in which the calcium, bound to the casein micelles, is removed.[99,100] The UF-permeate is subjected to both lactose hydrolysis and glucose isomerisation to produce a sweetness-enhanced UF-permeate. Finally, the UF-retentate, UF-permeate and the cream layer removed during the initial skimming step are combined to produce sweetness-enhanced milk ready for further standardisation, processing and dairy product production. In an additional production step to further increase sweetness, the UF-retentate can also undergo lactose hydrolysis before being reunited with the UF-permeate and the cream layer.

Although lactose-hydrolysed and glucose-isomerised syrups have been previously studied in the manufacturing of ice cream,[101] the bi-enzymatic system presented by Lorenzen et al.[99] demonstrates the possibility of almost doubling the sweetness of natural yoghurt by implementing enzymatically-modified milk during yoghurt production with minimal impact on technological and sensory properties. However, as yoghurt is a fermented product whose microbial ecology is very distinct, it is crucial that the growth and fermentation ability of LAB implemented for yoghurt production are closely studied during production and storage of the product.

1.6 Cheese

1.6.1 Production of Dutch-type semi-hard cheese

Dutch-type, semi-hard, rennet-coagulated cheeses such as Edam present a common cheese variety with a defined production process.[102] Production begins with the pasteurisation of raw bovine milk (see chapter 1.5.1), to which mesophilic DL-starter cultures, calcium chloride to assist coagulation, potassium nitrate to hinder the growth of clostridia species and, if desired, colouring agents are added at 30 °C.[73,102] After a brief pre-ripening period the rennet, a mix of enzymes containing the proteinase chymosin, is added. Chymosin causes the casein in the milk to coagulate and after allowing the rennet to

curdle the milk for about 25 minutes, the coagulum is cut into 8 – 15 mm pieces.[102] A first draining step removes roughly 50 % of the whey and scalding is then performed by replacing the whey with water at 35 – 45 °C.[102] This stimulates further syneresis where whey is separated from the curd, reducing the size and the lactose content of the curds and further regulating the pH.[73,103] Furthermore, whey removal leads to less chymosin binding the casein in the milk after initial curdling is complete, thereby reducing casein degradation and the development of bitterness, although some rennet is retained in the cheese.[104,105] After a second, partial draining step, the curds are pressed under whey, upon which the remaining whey is fully drained and the cheese curds are pressed into cheese moulds. These are then placed in a NaCl brine containing 16 – 18 % NaCl for 5 – 6 hours, depending on the size of the cheeses. Edam is typically covered in a red wax layer and left to ripen between 5 weeks and 2 years at 12 – 15 °C.[73]

A small number of eyes that should be no larger than a pea in size, can form during later stages of Edam cheese ripening as a result of some starter and adjunct LAB producing CO_2 during citrate metabolism.[73] The occurrence of small, uneven openings in the cheese body of Edam, however, is undesired and is a consequence of incomplete fusion of curd pieces or uneven pressing of curd into the cheese moulds.[106] The fat in dry matter ranges from 40 – 52 % in Edam cheese and can be adjusted by standardising the milk directly after pasteurisation.[107] Edam cheese generally has a final moisture content of 43 %.[73]

1.6.2 Salt content and its complex role in cheese

Salting is one of several traditional methods of food preservation, alongside dehydrating, fermenting, heating, cooling and the addition of sugar.[103] Salt protects the product against the growth of pathogens and spoilage bacteria, most prominently by lowering the a_w in the food matrix, but also by inhibiting germination of microbial spores.[108,109] As LAB are more salt tolerant than other microorganisms, they are able to grow in a higher salt environment, whilst the growth of pathogenic or spoilage bacteria is hindered by salt.[11,110] Microorganisms that are less salt tolerant undergo cell death or suffer from retarded growth due to removal of water from the cells as a result of osmotic shock.[111]

Salt is important for the production of cheese and greatly contributes to the desired sensory properties of the final product. Sodium adds to the overall taste of cheese both directly through imparting a salty flavour, as well as indirectly by modulating LAB activity, which affects the production and the perception of volatile aroma compounds. It affects the enzymes and biochemical processes that play key roles in the manufacturing

process.[111] For example, sodium can influence the activity of proteinases, disturbing coagulation of the milk.[80] Furthermore, salt is essential for syneresis and the salt-in-moisture content is fundamental for achieving the desired microstructure, cooking properties, texture and rheology of the cheese. The rheological properties such as firmness, fracture stress, fracture strain and sensory hardness contribute greatly to the overall mouthfeel of the product and, therewith, to consumer acceptance.[103] Although salt is technologically important in the production of all cheeses, the final salt content varies greatly between varieties, ranging from 0.7 % NaCl in Emmental cheese to roughly 6.0 % and even 8.8 % in the Middle Eastern cheeses Domiati and Nabulsi, respectively.[103,112] Gouda and other Gouda-like cheeses such as Edam typically contain around 2 – 2.4 % NaCl, which is an equivalent of 0.8 – 0.9 % sodium (1 % NaCl = 0.4 % Na).[103,112]

There are three common methods with which cheese varieties can be salted: brining, dry salting and rubbing salt on the cheese surface.[113] Brining involves placing the moulded cheese curds in a salt solution (ranging from 10 – 20 %, typically 16 – 18 % NaCl) for a specific period of time depending on the desired salt content of the final product and the type of cheese. Immersion in brine results in the cheese surface being uniformly exposed to the salt concentration. The range of cheeses that are brined is large and this method of salting is also applied for the production of Edam cheese. It relies on the concept of diffusion, resulting from the difference in osmotic pressure between the cheese moisture on the inside and the brine on the outside.[113] This process leads to roughly two parts water being lost for every part salt gained. The second common method of salting, direct dry salting, is a technique used for example in the production of Cheddar cheese, whereby the cut curd pieces are salted with dry salt crystals before pressing of the curd. This method also functions on the principle of osmosis; however, salt uptake is much quicker due to the larger surface area directly exposed to salt. The major drawback of dry salting is the possibility of an uneven distribution of salt in the final product. Finally, surface salt rubbing is applied during the production of mould ripened cheeses such as blue vein cheese and is performed by rubbing dry salt across the moulded curd over a period of several days. Salt uptake occurs via diffusion of moisture to the surface and salt into the cheese.

1.6.3 Microbial ecology of Dutch-type cheese

The composition of microorganisms is vital for successful cheese production and for the development of cheese flavour. The growth of microorganisms in cheese, including SLAB, adjunct LAB and NSLAB, is regulated by a multitude of factors including a_w, salt concentration, pH, oxidation-reduction potential, nitrate and temperature.[73] For the

production of Dutch-type cheese such as Edam, mixed mesophilic DL-SLAB cultures are typically implemented.[107] These SLAB cultures generally include a combination of strains of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. Adjunct LAB cultures can be added in combination with the SLAB and can include *Leuconostoc* (*Leuc.*) *lactis* or *Leuc. mesenteroides* subsp. *cremoris*, which ferment citrate and produce carbon dioxide leading to small eye formation, and *Lc. lactis* subsp. *lactis* biovar Diacetylactis, which produces the flavour compound diacetyl.[105,107] Lactobacilli such as *Lb. plantarum* or *Lb. rhamnosus* can also be added as adjunct cultures, aiding flavour formation during cheese ripening.[114] The microbiota present in ripened cheese is a combination of predominantly starter cultures added during production that rapidly decline in number during ripening due to autolysis and adjunct LAB cultures that increase in number during ripening. In addition, a small proportion of NSLAB such as facultatively heterofermentative lactobacilli, pediococci and micrococci are present and usually increase slightly in bacterial counts during production. These originate either from the raw milk, although LAB do not generally survive pasteurisation, or have entered the cheese during production.[105]

The lactic acid produced by LAB significantly contributes to the technological processing of cheese by lowering the pH of the milk, whereas the aroma compounds produced by the LAB proteolytic systems and amino acid metabolism pathways contribute to flavour development during processing and ripening.[79] Initial acidification of the milk is essential for successful coagulation of the milk by the rennet. This mixture of proteinases is typically derived from the stomach of ruminants but may also be of microbial origin such as from the microorganism *Mucor miehei*.[105] In rennet of animal origin, 90 % of the proteolytic activity originates from chymosin and the remaining 10 % from pepsin, both of which are aspartic acid proteases.[73] However, not only rennet is responsible for proteolysis during cheese production, but also the proteolytic systems of starter, adjunct and non-starter LAB. These microorganisms contribute to flavour formation during processing and ripening through proteolysis, amino acid catabolism and lipolysis.[115] Proteolysis is a complex biochemical event resulting in the production of short peptides and amino acids, which can be flavour compounds or act as substrates for catabolism reactions to produce flavour compounds.[116] The breakdown of fatty acids from milk through lipolysis occurs to a lesser extent in Dutch-type cheeses, however, also contributes to flavour formation.[117]

Adjunct cultures also contribute to overall proteolysis during cheese ripening. In 2001 Tungjaroenchai et al. demonstrated that the addition of adjunct cultures of *Lb. helveticus* and *Lc. lactis* subsp. *lactis* biovar Diacetylactis in Edam cheese resulted in high aminopeptidase activity as well as high levels of proteolysis.[118] Although proteolysis is important for flavour development, it can also lead to the development of bitter peptides if it is produced in excess, or if the specificity of the proteinase is incorrect.[73] Furthermore,

the production of aroma compounds such as acetate, acetoin, aldehydes, ketones, citrate and diacetyl by adjunct cultures is essential for obtaining the desired cheese flavours of the final product.[75,102] The choice of adjunct cultures can therefore improve the organoleptic qualities of the cheese and their growth can contribute towards minimising growth of spoilage and pathogenic bacteria through the production of antimicrobial compounds.[75]

During ripening, the SLAB population declines due to autolysis and the adjunct and non-starter LAB increase in number. In the early stages of fermentation, SLAB grow from about 10^5 – 10^7 colony forming units (cfu)/mL to 10^8 – 10^9 cfu/g in the cheese curd, at which point Edam cheese will have a pH of roughly 5.7.[73,104,105] In Gouda, another Dutch-type cheese, SLAB populations decline to < 10^3 cfu/g during the first weeks of ripening.[105] The initial counts of NSLAB can be as low as 100 cfu/g but increase significantly during ripening. For example, facultative heterofermentative lactobacilli such as *Lb. paracasei*, *Lb. rhamnosus* or *Lb. plantarum* that can be added as adjunct cultures in Edam cheese can grow up to 10^8 – 10^9 cfu/g during the first months of ripening and remain high.[75,119]

1.6.4 Reformulation strategies for Dutch-type cheese

Strategies to reduce salt content in cheese have been studied in many different cheese varieties. One of the most studied cheeses in the field of salt reduction is Cheddar cheese, where significant salt reductions in NaCl content have been achieved, most prominently through partial substitution with other mineral salts such as potassium chloride (KCl).[120–127] As the taste perception of salt reduction in cheese is closely connected with the fat content, some studies have focussed on combinations of fat and salt reduction on the cheese composition, as well as on microbiological and biochemical characteristics of cheese.[128–131] Despite many studies on salt reduction concentrating on Cheddar cheese, Edam cheese has to date been the target of only a limited number of studies in the field of salt reduction,[118,132–136] although other Dutch-style cheeses such as Gouda cheese have also been investigated.[137]

The most common reformulation strategy applied thus far for salt reduction in cheese has been the partial replacement of NaCl with other salts, most commonly KCl, but also $MgCl_2$, $MgSO_4$ or $CaCl_2$.[138,139] KCl is a mineral salt chemically similar to NaCl that exhibits similar preservative effects in the product.[14] In addition, increased potassium intake has been shown to reduce the effects of hypertension,[140] making it a beneficial choice to counteract the hypertensive effects of sodium. However, there are major product defects resulting from substitution of NaCl with KCl. These are most prominently the occurrence

of a bitter and metallic taste leaving an unpleasant aftertaste, a general loss of the salty flavour characteristic for cheese and a potentially brittle cheese consistency.[55,141] Therefore, KCl cannot be used as an outright substitute for NaCl in cheese, but partial substitution of up to 30 % has shown acceptable organoleptic results, in which case the remaining sodium content can still counteract bitterness.[138]

Partial substitution of NaCl with other mineral salt compositions in cheese has been explored both with and without the addition of flavour enhancers and bitterness-masking compounds.[55,142] As substitute mineral salts are unable to stimulate the sodium-specific cation channels in taste receptor cells that are necessary to experience saltiness,[52] flavour enhancing compounds can be implemented to help increase the saltiness perception of the remaining sodium. Flavour enhancers include glutamates or nucleotides imparting an umami taste, yeast extracts or hydrolysed vegetable protein providing a brothy taste, or amino acids, lactates or food grade acids to enhance saltiness perception through imparting sourness.[14,110] Furthermore, bland taste resulting from a reduction in sodium content can be ameliorated by the addition of herbs and spices in some foods.[110]

Alternatively, bitterness blocking compounds that prevent activation of the cell signalling proteins essential for transmitting bitter taste through receptor cells can be used to offset bitterness resulting from mineral salts.[127,143] Examples of bitter blockers include adenosine-5'-monophosphate as well as glycine, lactose and potassium lactate.[110,127] Often mixtures of substitutes and flavour enhancers are implemented in combination and are available in proprietary mixtures such as Jungbunzlauer's sub4salt® products.[144] Finally, specific adjunct cultures that produce volatile aroma compounds can be added to cheese to help mask off-tastes originating from mineral salts used to substitute sodium. For example, adjunct cultures of lactobacilli could reduce bitter flavour development through aminopeptidase activity.[145] Furthermore, microorganisms such as *Lb. paracasei* and *Lc. lactis* subsp. *lactis* biovar Diacetylactis that produce specific aroma profiles can enhance cheese flavour and subdue bitterness.[75]

Further reformulation strategies for salt reduction in cheese include stepwise reduction, for which studies describing reduction of NaCl in small increments over a period of at least one year have also been documented.[52,55] However, due to the essential role of salt in cheese production and safety, silent reductions of sodium are not only organoleptically, but also technologically limited. Simple reduction of sodium leads to an increase in a_w, acidity and proteolysis, which decreases the hardness of the cheese, and affects the growth of starter and non-starter LAB.[108] Alternatively, there have also been suggestions to modify the structure of NaCl molecules to achieve an increased perception of

saltiness from lower salt content, yet partial substitution of NaCl remains the most promising salt reduction strategy in cheese.[55]

1.7 Culture-independent determination of microbial ecology

Although LAB are essential for the fermentation processes described in the two dairy matrices investigated in this study, other food-associated, potentially spoilage- and disease-causing bacteria may also be present in these products with their origin ranging from contamination of raw milk to storage, processing and packaging facilities. For many thousands of years fermented products have been produced without exact knowledge of the microbiota responsible for creating the acidic characteristics of the products or the associated biochemical processes.[146] As knowledge and technology progressed, many fermentation-associated LAB were identified and characterised using culture-dependent techniques.[147] For many products, including fermented dairy commodities, this knowledge led to the ability to implement specific combinations of LAB in order to achieve defined product characteristics. However, culture-dependency has the inherent but significant drawback of only providing selective insight into the viable, culturable and dominant microbiota present within the tested sample.[148]

The emergence of high-throughput sequencing (HTS) technologies has provided the food industry with the unique opportunity to accurately identify and characterise the microbial composition of food samples in a culture-independent manner, empowering producers with specialised information about their products that can be applied to improve product quality, safety and consistency.[149] It allows the complete microbial ecology to be studied in detail, including the functional properties of microorganisms and the metabolic pathways responsible for favourable and unfavourable product characteristics.[150] As a result, not only can the diversity and distribution of the LAB essential for fermentation processes be established more precisely than ever before, but NSLAB, secondary microbiota and potentially spoilage and disease-causing pathogens can also be identified and monitored throughout production, fermentation and storage.[151] Even small numbers of spoilage or pathogenic bacteria can be detected within samples, which in turn may be traced back to the exact origin for subsequent elimination of contamination sites.[152]

High-throughput sequencing can be used to generate phylobiomics, metagenomics or metatranscriptomics data, each approach providing a range of associated benefits.[151] Phylobiomics, the study of the taxonomy of the microbiota, targets marker genes such as the 16S rRNA gene, which are amplified from genomic DNA by means of a polymerase chain reaction (PCR) and subsequently sequenced.[153] Analysis of 16S rDNA

amplicons can be applied to identify bacteria at the genus level. In contrast, metagenomics and metatranscriptomics analyse the total DNA or cDNA of a complex sample, respectively. Metagenomics, also known as DNA sequencing, is able to decode the composition and diversity of the microbial community present by revealing its spectrum of genes.[154] However, this method cannot differentiate between DNA originating from dead or alive cells within in the sample. Metatranscriptomics (RNA sequencing) on the other hand can depict the full range of active genes in a sample at a specific sampling time by analysing the set of mRNA present in the sample.[155] This method can reveal an overview of the range of genes being expressed in the sample, providing possible insight into when certain genes are expressed and when they are not.

Not only has HTS become increasingly specific in recent years, it has also become progressively more accessible as technological advances and a rise in demand have driven sequencing costs down to more affordable levels.[152] As a result, we see the emergence of large-scale sequencing studies in many fields of research including dairy science. Indeed, the largest number of HTS studies of food matrices has been performed on fermented dairy products, followed by beverages, dough and vegetables.[151] Sour milk products have been the focus of numerous recent HTS studies, whereby analysis of the 16S rRNA gene was the most commonly chosen approach.[151] The majority of these studies focussed on kefir or traditional milk fermentations of bovine, caprine or even yak milk,[156–164] and shed light on the microbial ecosystems of various fermented milk products. However, perhaps due to the well-studied symbiotic relationship between the two starter culture microorganisms implemented in traditional yoghurt production,[165] this fermented dairy product has been the focus of only a few HTS studies. For example, Xu et al. (2015) used HTS to assess the composition of the microbial community of naturally fermented traditional home-made yoghurts.[166] The study of the microbial ecology of yoghurt pertaining to its reaction to enzyme-modified milk in the context of yoghurt reformulation has, to current knowledge, not been published.

The fermented dairy product most abundantly studied by means of HTS methods to date is undoubtedly cheese. The microbial composition of SLAB and NSLAB of a wide range of cheeses made from raw and pasteurised milk has been extensively investigated, allowing for specification of the microbial ecology and detailed analysis of the functional properties of the microorganisms.[155] HTS studies of the cheese microbiome have yielded insight into the microbial composition of and the relationships between SLAB and NSLAB of many different cheeses.[167–170] In addition, HTS has been implemented to analyse the effects of various production parameters on the microbial diversity and distribution during fermentation and ripening.[171–173] For example, in 2012, Quigley et al. were able to use HTS to detect subpopulations of bacteria that had not been associated with artisanal

Irish cheeses prior to that study and also suggested that salt could influence the microbial counts of both *Leuconostoc* and *Pseudomonas* in these cheeses.[172] Furthermore, Porcellato and Skeie (2016) used 16S rRNA gene sequencing in combination with metagenomics to explore technological processing adaptations on the microbiota and metabolism of LAB throughout three months of ripening of Dutch-type cheeses.[171] Further applications of phylobiomics have been to determine manufacturing site-specific microbial changes in artisan cheese samples[174] and to track spoilage-associated bacteria in cheese manufacturing sites and cheese samples.[175] Phylobiomics and metatranscriptomics have also been used to analyse functional microbiome changes due to temperature alterations in the Protected Designation of Origin Caciocavallo Silano cheese.[173]

2 Aims and Objectives

Salt and sugar play a central role in the production, microbial safety and shelf life of fermented dairy products, yet they present major modifiable risk factors for NCDs such as coronary heart disease and diabetes mellitus type II. Reformulation of fermented dairy products can contribute to the amelioration of both individual and population health, and therefore presents an important research focus. As part of two multi-faceted food reformulation projects funded by the German Federal Office for Agriculture and Food, the aim of this study was to reformulate two fermented dairy products by applying different reformulation strategies in order to assess their effects on the microbial ecology and product characteristics. The first reformulation project focussed on the production of yoghurt using enzymatically sweetened milk with the ultimate goal of being able to reduce the added sugar content in sweetened yoghurt products. In particular, the growth and acidification potential of starter and non-starter LAB in reformulated yoghurt prepared with sweetness-enhanced milk and different starter cultures was investigated. Technological parameters were monitored throughout fermentation and storage over a period of 21 days. The second reformulation project aimed to produce sodium-reduced Edam cheese with less than 0.4 % Na to ultimately contribute to reducing salt intake by consumers by targeting a popular fermented dairy product. Sodium-reduction in Edam cheese was investigated by application of a simple NaCl reduction approach, as well as a mineral salt replacement strategy. Bacterial growth and technological parameters were examined in sodium-reduced Edam throughout fermentation and ripening. Culture-independent techniques were implemented to assess the growth of starter and non-starter LAB during both fermentation, as well as storage and ripening of both yoghurt and cheese, respectively. High-throughput sequencing approaches served to study microbial ecology in reformulated product samples. In summary, this study aimed to investigate the effects of the chosen reformulation strategies on microbial ecology and product characteristic aspects using a holistic approach.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals, reagents, kits, instruments, consumables and software

The chemicals, reagents, kits, instruments, consumables and software used to complete this study are listed in the Appendices. The chemicals and reagents can be found in Table A 1, along with the details of their application and manufacturer. Details of the kits applied are specified in Table A 2. The instruments and their manufacturers can be found in Table A 3, whereas consumables and their manufacturers are listed in Table A 4. Finally, the software used to analyse data throughout this study is listed in Table A 5.

3.1.2 Culture media

The composition of all nutrient media used for cultivation and analysis of bacteria is listed in Table 1. Media were prepared using de-ionised H_2O, boiled if agar-agar was a component of the medium, and autoclaved at 121 °C for 15 min, with the exception of Violet Red Bile Dextrose (VRBD) medium, which does not require autoclaving. Freeze-dried supplements were reconstituted in type 1 ultrapure H_2O. The final pH (at 25 °C) of each medium is indicated in Table 1, as provided by the manufacturer or as adjusted to by using 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH) prior to autoclaving.

Table 1: Culture media composition

Culture media	**Components / pH**	
Basal medium for fermentation studies [176]		pH adjusted to 6.3 ± 0.2
	Peptone from casein	10 g
	Yeast extract	4 g
	Tween® 80	1 g
	Potassium dihydrogen phosphate	2 g
	Sodium acetate • 3 H_2O	5 g
	Sodium citrate • 2 H_2O	2 g
	$MgSO_4$ • 7 H_2O	0.2 g
	$MnSO_4$ • 1 H_2O	0.05 g
	Chlorophenol Red	0.08 g
	De-ionised H_2O	1,000 mL
de Man, Rogosa and Sharpe (MRS) agar [176]	Pre-prepared anhydrous medium [a]:	pH 5.7 or adjusted to 5.4
	Enzymatic digest of casein	10 g
	Meat extract	10 g
	Yeast extract	4 g
	Glucose	20 g
	Dipotassium hydrogen phosphate	2 g
	Sodium acetate	5 g
	Triammonium citrate	2 g
	$MgSO_4$ • 7 H_2O	0.2 g
	$MnSO_4$ • 4 H_2O	0.05 g
	Agar-agar	13 g
	Polyoxyethylene sorbitan monooleate (Tween® 80)	1.08 g
	De-ionised H_2O	1,000 mL
MRS broth [176]	Pre-prepared anhydrous medium [a]:	pH 6.4 ± 0.2
	Enzymatic digest of casein	10 g
	Meat extract	10 g
	Yeast extract	5 g
	Glucose	20 g
	Dipotassium hydrogen phosphate	2 g
	Sodium acetate	5 g
	Diammonium citrate	2 g
	$MgSO_4$ • 7 H_2O	0.2 g
	$MnSO_4$ • 4 H_2O	0.05 g
	Tween® 80	1.08 g
	De-ionised H_2O	1,000 mL

Table 1 continued

Bile-MRS [177]	Pre-prepared anhydrous MRS agar [a]	
	Ox Bile	0.15 % (w/v)
LP-MRS [177]	Pre-prepared anhydrous MRS agar [a]	
	LiCl	0.2 % (w/v)
	Sodium propionate	0.3 % (w/v)
M17 agar [178]		pH adjusted to 7.1 ± 0.2
	Phytone™ peptone	5 g
	Polypeptone	5 g
	Yeast extract	2.5 g
	Beef extract	5 g
	Na-ß-glycerophosphate	19 g
	Ascorbic acid	0.5 g
	1M $MgSO_4$ • 7 H_2O	1 mL
	Agar-agar	15 g
	De-ionised H_2O	940 mL
	After autoclaving and cooling to 50 °C, add separately autoclaved solution of:	
	Lactose	5 g
	1M $CaCl_2$ • 2 H_2O	10 mL
	De-ionised H_2O	60 mL
M17 broth [178]	Same as M17 agar medium without agar-agar	
Cetrimide fucidin cephalothin agar containing Delvocid® (CFCD) [179]		pH adjusted to 7.1 ± 0.2
	Glycerol	10 g
	Peptone from gelatine	16 g
	Casein hydrolysate	10 g
	K_2SO_4	10 g
	$MgCl_2$ • 6 H_2O	1.4 g
	Delvocid® Instant	1 g
	Agar-agar	11 g
	De-ionised H_2O	1,000 mL
	After autoclaving and cooling to 50 °C, add:	
	Pseudomonas CFC selective supplement [a] (contains cephalothin sodium and N-Cetyl-N'N'N'-trimethylammonium bromide)	2 vials; reconstitute each vial in 1 mL sterile, ultrapure H_2O + 1 mL 99 % ethanol

Table 1 continued

Violet red bile dextrose (VRBD) agar [179]	Pre-prepared anhydrous medium [b]:	pH 7.4 ± 0.2
	Pancreatic digest of gelatine	7 g
	Yeast extract	3 g
	Bile salts	1.5 g
	Sodium chloride (NaCl)	5 g
	D-glucose	10 g
	Neutral Red	0.03 g
	Crystal Violet	0.002 g
	Agar-agar	13 g
	De-ionised H_2O	1,000 mL
Kanamycin aesculin azide (KAA) agar [180]	Pre-prepared anhydrous medium [b]:	pH 7.1 ± 0.2
	Peptone from casein	20 g
	Yeast extract	5 g
	NaCl	5 g
	Sodium citrate	1 g
	Sodium azide	0.15 g
	Kanamycin sulphate	0.02 g
	Aesculin	1 g
	Ammonium iron (III) citrate	0.5 g
	Agar-agar	15 g
	De-ionised H_2O	1,000 mL
Yeast extract glucose chloramphenicol (YGC) agar [179]	Pre-prepared anhydrous medium [a]:	pH 6.6 ± 0.2
	Yeast extract	5 g
	D-glucose	20 g
	Chloramphenicol	0.1 g
	Agar-agar	15 g
	De-ionised H_2O	1,000 mL
Brain-Heart-Infusion (BHI) broth	Pre-prepared anhydrous medium [c]:	pH 7.4 ± 0.2
	Pig brain infusion	7.5 g
	Pig heart infusion	10 g
	Peptone	10 g
	Glucose	2 g
	NaCl	5 g
	Disodium phosphate	2.5 g
	De-ionised H_2O	1,000 mL

Table 1 continued

Agar *Listeria* according to Ottaviani and Agosti (ALOA) [181]	Pre-prepared anhydrous medium [a]:	pH 7.2 ± 0.2
	Meat peptone	18 g
	Peptone from casein	6 g
	Yeast extract	10 g
	Sodium pyruvate	2 g
	Dextrose	2 g
	Magnesium glycerophosphate	1 g
	$MgSO_4$	0.5 g
	NaCl	5 g
	LiCl	10 g
	Disodium phosphate anhydrous	2.5 g
	5-bromo-4-chloro-3-indolyl-ß-D-gluco-pyranoside	0.05 g
	Agar-agar	12 g
	De-ionised H_2O	1,000 mL
	After autoclaving and cooling to 50 °C add:	
	Listeria selective supplement [a] (contains per vial: 38350 IU polymyxin B, 0.025 g cycloheximide, 0.010 g ceftazidime, 0.010 g nalidixic acid)	2 vials; reconstitute each freeze-dried vial in 6 mL sterile, ultrapure H_2O
	Listeria enrichment supplement [a] (contains 1 g L-α-phosphatidylinositol + 24 mL sterile ultrapure H_2O)	2 bottles

[a] VWR International GmbH, Darmstadt, Germany; [b] Merck, Darmstadt, Germany; [c] Carl Roth, Karlsruhe, Germany

3.1.3 Buffers and solutions

Buffers used for preparation and subsequent analysis of yoghurt and cheese samples are listed in Table 2. Buffers (or their components) and solutions were prepared using de-ionised H_2O, unless otherwise specified, and were autoclaved at 121 °C for 15 min or filtered using a 0.2 µm microfilter if heat sensitive.

Table 2: Composition of buffers

Buffer / solution	**Composition**	**Buffer application**
1 M Tris-HCl	12.11 % (w/v) Tris base 1 M HCl	Yoghurt sample dilutions
Sodium citrate	2 % (w/v) sodium citrate • 2 H_2O	Cheese sample dilutions
NaCl/casein hydrolysate	0.85 % (w/v) NaCl 0.1 % (w/v) casein hydrolysate	Serial dilutions of all samples
50x TAE	40 mM Tris base 40 mM Acetic acid 0.1 mM Ethylenediaminetetraacetic acid (EDTA) pH 8.0	Stock solution for agarose gels (working concentration 1x TAE)
RBB+C lysis	500 mM NaCl 50 mM Tris-HCl pH 8.0 50 mM EDTA pH 8.3 4 % (w/v) Sodium dodecyl sulphate (SDS)	DNA Isolation from yoghurt and cheese samples
DGGE loading	0.05 % (w/v) Bromophenol Blue 40 % (w/v) sucrose 0.1 M EDTA pH 8.0 0.5 % (w/v) SDS	Denaturing gradient gel electrophoresis (DGGE)

For sugar fermentation tests, sugar solutions of D-glucose, D-fructose, D-galactose and lactose monohydrate were prepared at 2.5 % (w/v) in type 1 ultrapure H_2O. All sugars were sterilised via filtration using a 0.2 µm filter.

3.1.4 Starter cultures

The following six commercial yoghurt cultures from Chr. Hansen (Nienburg, Germany) were chosen for analysis in regular and enzyme-modified milk: YoFlex® Premium 1.0, YoFlex® Premium 4.0, YoFlex® Mild 2.0, YF-L812, ABT-6 Probio-Tec® and ABT-100. The composition of LAB contained in these cultures as indicated by the manufacturer in the product data sheets, as well as the corresponding culture media and culture conditions, are outlined in Table 3. However, the exact concentration of each of the LAB in the starter cultures was not provided by the manufacturer to protect their trade secret. All cultures were received as deep-frozen DVS cultures and stored at - 80 °C before usage.

Table 3: Yoghurt starter culture composition and strain culturing conditions

Culture name	**Starter culture strains**	**Culture medium**	**Culturing conditions**
YoFlex® Premium 1.0	*Streptococcus* (*S.*) *thermophilus*	M17	43 °C, aerobic, 48 h
	Lactobacillus (*Lb.*) *delbrueckii* (*d.*) subsp. *bulgaricus*	MRS	37 °C, aerobic, 48 h
YoFlex® Premium 4.0	*S. thermophilus*	M17	43 °C, aerobic, 48 h
	Lb. d. subsp. *bulgaricus*	MRS	37 °C, aerobic, 48 h
YoFlex® Mild 2.0	*S. thermophilus*	M17	43 °C, aerobic, 48 h
	Lb. d. subsp. *bulgaricus*	MRS	37 °C, aerobic, 48 h
YF-L812	*S. thermophilus*	M17	43 °C, aerobic, 48 h
	Lb. d. subsp. *bulgaricus*	MRS	37 °C, aerobic, 48 h
ABT-6 Probio-Tec®	*S. thermophilus*	M17	43 °C, aerobic, 48 h
	Lb. acidophilus	Bile-MRS	37 °C, aerobic, 48 h
	Bifidobacterium sp.	LP-MRS	37 °C, anaerobic, 48 h
ABT-100	*S. thermophilus*	M17	43 °C, aerobic, 48 h
	Lb. acidophilus	Bile-MRS	37 °C, aerobic, 48 h
	Bifidobacterium sp.	LP-MRS	37 °C, anaerobic, 48 h

Four commercial cheese starter cultures were obtained from Chr. Hansen (Nienburg, Germany) for cheese production: FLORA™ C1060, CR-550, LH-32 and CR-BUTTERY01. The composition of LAB contained in these cultures as indicated by the manufacturer in the product data sheets, as well as the corresponding culture conditions of each bacterial strain are outlined in Table 4. However, the exact concentration of each of the LAB in the starter cultures was not provided by the manufacturer to protect their trade secret. All cultures were received as deep-frozen DVS cultures and stored at - 80 °C before usage.

Table 4: Cheese starter culture composition and strain culturing conditions

Culture name	**Starter culture strains**	**Culture medium**	**Culturing conditions**
FLORA™ C1060	*Lactococcus* (*Lc.*) *lactis* subsp. *cremoris*	M17	25 °C, aerobic, 48 h
	Lc. lactis subsp. *lactis*	M17	25 °C, aerobic, 48 h
	Lc. lactis subsp. *lactis* biovar Diacetylactis	M17	25 °C, aerobic, 48 h
	Leuconostoc (*Leuc.*) sp.	MRS pH 5.4	25 °C, aerobic, 48 h
CR-550	*Lactobacillus* (*Lb.*) sp.	MRS pH 5.7	43 °C, aerobic, 48 h
	Lc. lactis	M17	25 °C, aerobic, 48 h
LH-32	*Lb. helveticus*	MRS pH 5.7	43 °C, aerobic, 48 h
CR-BUTTERY01	*Lb. paracasei*	MRS pH 5.7	43 °C, aerobic, 48 h
	Lb. rhamnosus	MRS pH 5.7	43 °C, aerobic, 48 h
	Lc. lactis subsp. *lactis*	M17	25 °C, aerobic, 48 h

3.2 Methods

3.2.1 Starter culture analysis

Yoghurt and cheese starter cultures were obtained from Chr. Hansen (Nienburg, Germany; see Table 3 and Table 4) and were analysed as outlined in the following chapter.

3.2.1.1 Strain isolation from mixed cultures

Individual strains of SLAB were isolated from the mixed-strain cultures for subsequent classification and characterisation, as well as for use as reference strains for microbial ecology studies of product samples (see chapter 3.2.4). The isolation of pure cultures was achieved by dilution streaking three times using the culture medium and incubation conditions outlined in Table 3 and Table 4.

Strain isolates were verified based on colony morphology and microscopy, and then used to inoculate broth media, which was incubated for 24 h. After incubation, 800 µL of broth culture were mixed with 200 µL of 85 % glycerol to create stocks cultures, which were stored at - 70 °C. For subsequent usage of these stock cultures, a sterile loop was used to aseptically scrape off a small portion of the frozen culture for inoculation of fresh broth medium.

3.2.1.2 Identification of starter cultures by 16S rDNA sequencing

For further strain isolate classification, bacterial DNA was extracted from overnight broth cultures of all isolates obtained from the commercial yoghurt and cheese cultures (see chapter 3.2.1.1). For this the 'PeqGOLD Bacterial DNA Mini Kit' was used with 2 mL of log-phase bacteria in broth culture and according to the manufacturer's guidelines for gram-positive bacteria (VWR International GmbH, Darmstadt, Germany). Briefly, the 'PeqGOLD Bacterial DNA Mini Kit' is based on a DNA-binding column technology, whereby bacterial cell walls are first treated with lysozyme (10 mg/mL), proteinase K (20 mg/mL) and RNase A (20 mg/mL), before the DNA of lysed cells is reversibly bound to a column matrix where it undergoes a series of wash steps to remove salt and protein contamination. The DNA is then eluted from the column using the elution buffer provided. The elution step for DNA isolated from all yoghurt and cheese samples was performed twice using 50 µL of elution buffer per elution.

To confirm DNA extraction from bacteria was successful, polymerase chain reaction (PCR) amplification from DNA of all strains isolated from yoghurt and cheese cultures was performed using the 'High efficiency Taq Polymerase with Buffer E' kit (Genaxxon Bioscience GmbH, Ulm, Germany) together with the universal 16S bacterial DNA primers

27f and 1540r (Table A 6). These primers amplify a fragment of approx. 1500 bp, which indicates successful extraction and amplification of bacterial DNA from the samples.

The PCR reaction consisted of a 10x amplification buffer constituted with 2.5 mM $MgCl_2$, 10 mM of each nucleotide triphosphate (dNTP), 10 pmol of each primer, 10 µL of non-adjusted DNA sample (for a 50 µL reaction), 1.5 U of Taq-Polymerase, and made to a final volume of 50 µL with sterile, type 1 ultrapure H_2O. A Touchdown PCR reaction was performed on a PeqSTAR thermocycler with an initial denaturation step at 94 °C for 3 min; followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C (- 1 °C/cycle) for 30 s, and extension at 72 °C for 90 s; followed by 22 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 90 s; completed by a final extension step at 72 °C for 5 min.

Following the initial classification steps of colony morphology and PCR confirmation of the bacterial DNA isolates, Sanger sequencing of 16S rRNA gene PCR amplicons was used to confirm the identity of isolated strains. For this, 5 µL of each PCR product were run on a 1.5 % agarose gel containing a 1:20 dilution of GelRed™ Nucleic Acid Gel Stain for 1.5 h at 100 V in 1x TAE buffer. The gel was visualised under UV light on a BioDocAnalyze gel documentation system. Samples showing successful amplification of the expected 1500 bp fragment length were cleaned using the 'NucleoSpin® Gel and PCR Clean-up Kit' and samples were sent to Eurofins Genomics (Ebersberg, Deutschland) for Sanger sequencing using the company's LightRun Tube sequencing with the universal 16S (27f and 1540r) primers outlined in Table A 6.

3.2.1.3 Carbohydrate utilisation tests

Miniaturised sugar fermentation tests were used to assess carbohydrate fermentation by the yoghurt culture isolates obtained in chapter 3.2.1.1. These tests aimed to determine the ability of different strains of LAB in the yoghurt cultures to metabolise a variety of mono- and disaccharides. They were performed using the basal medium for fermentation studies (Table 1), based on the method proposed by Jayne-Williams in 1975.[182] The colour indicator chlorophenol red contained in this medium helped determine whether the bacteria being tested could successfully ferment the provided sugar, producing acidic compounds. Upon successful fermentation of the added sugar of interest, the basal medium turned from red to yellow. Glucose was used as a positive control whereas sterile, type 1 ultrapure H_2O served as a negative control.

For the sugar fermentation tests, 1 mL of overnight broth culture of the strain to be tested was centrifuged for 10 min at 4,000 rpm in a Heraeus Fresco21 centrifuge and the supernatant was subsequently removed. The pellet was resuspended in 1 mL basal

medium and this suspension was then further diluted 1:50 in basal medium. A 96-well plate was prepared with 25 µL per well of each carbohydrate solution to be tested (see chapter 3.1.3), working under sterile conditions in a class 2 safety cabinet and using 'PCR clean and sterile filter tips'. Thereafter, each well was inoculated with 100 µL of the previously prepared 1:50 bacterial dilution in basal medium. The 96-well plates were incubated for 48 h at the relevant incubation conditions for the individual strains (see Table 3), with initial evaluation after 24 h.

3.2.2 Yoghurt production and analysis

Yoghurt was produced using the starter cultures provided by Chr. Hansen (Nienburg, Germany; refer to Table 3) in enzyme-modified and control milk and analysed as outlined in this chapter. Yoghurt production was first performed in small-scale laboratory production experiments to analyse the growth and acidification potential of starter cultures in sweetness-enhanced milk. These experiments allowed two yoghurt cultures to be chosen for subsequent large-scale pilot plant production experiments to verify the laboratory results. Samples from the pilot plant yoghurt production experiments were used for subsequent studies of microbial ecology. All yoghurt production experiments were performed in biological triplicates and the arithmetic mean and standard error of the mean were calculated using Microsoft Excel.

Enzyme-modification of milk, as well as pilot plant yoghurt production and measurement of technological parameters, was carried out in collaboration with the team of Prof. Dr. Peter Chr. Lorenzen at the Department of Safety and Quality of Milk and Fish Products at the Max Rubner-Institut (MRI) in Kiel as part of the reformulation project 'Enhancing the sweetening power of lactose by enzymatic hydrolysis and partial isomerisation of the glucose moiety' (FZ 2819107616). This project was funded by the German Federal Office for Agriculture and Food.

3.2.2.1 Production of sweetness-enhanced milk

Sweetness-enhanced milk was produced using the bi-enzymatic system of lactose conversion described by Lorenzen et al. in 2013 and outlined for this project in Figure 1.[99] Raw milk was obtained from the MRI research farm in Schädtbek, located near Kiel. The milk was skimmed to separate the cream section from the skim milk. The former was heated to 85 °C and the latter was pasteurised at 72 °C for 20 s to inactivate possible contaminating microorganisms. As the glucose isomerase enzyme that is used during processing is calcium-sensitive, the skim milk section was ultrafiltered at 5,000 Da using a Type HF 1.0-43-PM5-PB membrane to yield a calcium-poor UF permeate and a

calcium-rich UF retentate. The calcium-poor UF-permeate was demineralised using a Purolite® S930 Plus cation exchanger prior to further processing. The UF-permeate then underwent lactose hydrolysis using the β-galactosidase enzyme NOLA™ Fit 5500 and subsequent glucose isomerisation using the soluble glucose isomerase enzyme Gensweet® SGI. In parallel, residual lactose in the UF-retentate was also hydrolysed using the NOLA™ Fit 5500 β-galactosidase enzyme. The hydrolysed and isomerised UF-permeate was then reunited with the calcium-containing, hydrolysed UF-retentate, as well as cream, to produce enzymatically sweetened milk. This milk was adjusted to 3.5 % fat content and 14 % dry matter content and homogenised at 60 °C and 200 bar prior to yoghurt production.

In parallel to production of enzyme-modified milk, portions of cream and pasteurised skim milk were reunited to reconstitute regular milk, which was also adjusted to 3.5 % fat and 14 % dry matter content (Figure 1). This milk was homogenised at 60 °C and 200 bar and used for production of regular yoghurt in the pilot plant experiments (see chapter 3.2.2.3), serving as a control yoghurt.

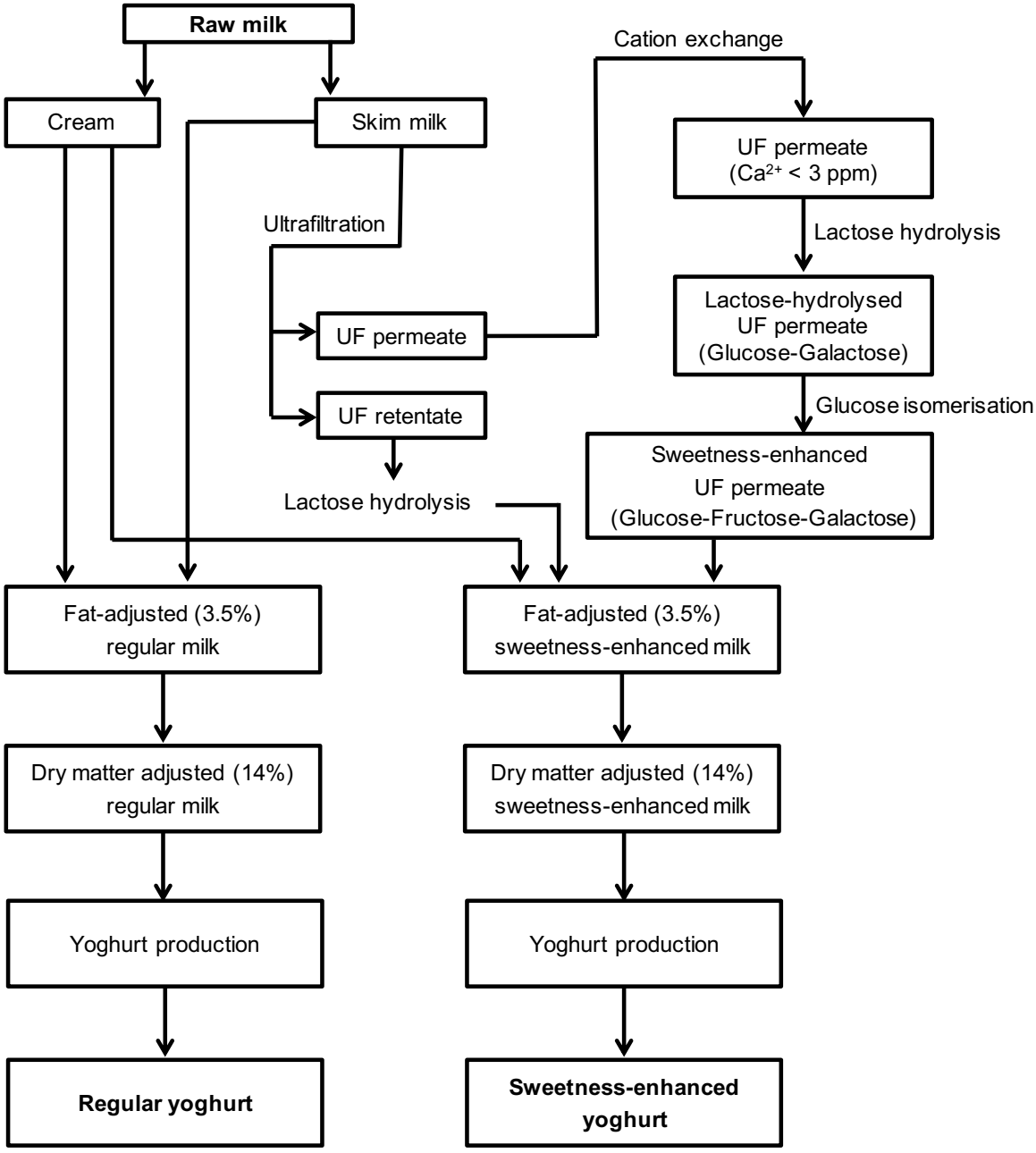

Figure 1: Manufacturing process of regular and sweetness-enhanced yoghurt produced in the MRI pilot plant. Flow-chart kindly provided by Prof. Dr. P. Chr. Lorenzen.

3.2.2.2 Laboratory yoghurt production

The progression of milk acidification and the growth of LAB during fermentation were examined for the six commercial yoghurt starter cultures listed in Table 3 in order to select two yoghurt starter cultures for extended analyses in subsequent pilot plant scale productions (see chapter 3.2.2.3). All milk acidification and bacterial growth analyses were carried out in three independent experiments for each of the six commercial yoghurt starter cultures.

Yoghurt was produced in a total volume of 100 mL, whereby the starter culture dosage was scaled down according to the manufacturer's instructions for laboratory production with DVS ripening cultures (Chr. Hansen, Nienburg, Germany). The exact concentration of LAB contained in the starter cultures was not provided by the manufacturer to protect their trade secret. For inoculation of milk, 250 g of a 50-unit bag of frozen culture pellets were thawed at 25 °C in a water bath and mixed thoroughly prior to inoculation of the milk. A 1:10 dilution of each culture was prepared either in 3.5 % fat, commercially-available regular milk (homogenised, pasteurised and microfiltered to extend shelf life; Milbona Frische Vollmilch, Fude + Serrahn Milchprodukte GmbH & Co. KG, Hamburg, Germany) or 3.5 % enzyme-modified milk (see chapter 3.2.2.1). From the respective dilutions, 0.02 % (v/v) were inoculated into sterile jars containing 100 mL of regular or enzyme-modified milk. The inoculated milk was left to ferment at 43 °C in a water bath until a pH of 4.4 was achieved. Samples were then placed in a 4 °C water bath for monitoring of post-cooling acidification. Acidification at 43 °C and post-cooling acidification at 4 °C was monitored for a total of 18 h using an 8-channel pH meter, with pH measurements documented automatically every 10 min using a GigaLog S Datalogger in conjunction with the GigaTerm software.

Due to the temperature sensitivity of the pH electrodes, a pH measurement error upon cooling of the milk was observed in preliminary experiments. To remove this calculation error during laboratory yoghurt production, a series of three experiments with enzyme-modified milk and commercially-available regular milk that were not inoculated with starter cultures was performed to document the error margin measured by the electrodes upon cooling the milk from 43 °C to 4 °C. A median error of 0.24 pH units was calculated for both milk types and this value was then subtracted from all pH measurements taken after the time point of cooling to 4 °C to monitoring post-cooling acidification. Furthermore, this cooling step required that the measuring equipment be restarted for subsequent measurements at 4 °C.

For the first six hours of acidification, samples for microbiological growth monitoring were taken at intervals of 1.5 h. To this end, 1 mL of sample was diluted using a ten-fold dilution series with NaCl/casein hydrolysate buffer (see Table 2). Volumes of 100 µL were spread-plated onto M17, MRS, Bile-MRS or LP-MRS agar (see Table 3) in technical duplicates and at three different dilutions. Plates were incubated according to the culturing conditions specific for each starter culture strain as outlined in Table 3. After incubation, colonies were counted and colony-forming units per mL (cfu/mL) were calculated using the following formula:

$$\frac{cfu}{ml} = \frac{\sum c}{(1 * n1 + 0.1 * n2)} * d$$

$\sum c$ = sum of all colonies counted

n1 = number of agar plates of the lowest evaluable dilution

n2 = number of agar plates of the second lowest evaluable dilution

d = dilution factor the lowest evaluable dilution (reciprocal value)

3.2.2.3 Pilot plant yoghurt production

After evaluation of milk acidification and bacterial growth of all six yoghurt cultures in laboratory production experiments, two of the cultures (YoFlex® Premium 4.0 and ABT-100) were selected for production at a larger, pilot plant scale. Three independent production experiments for each culture were performed.

Yoghurt was manufactured in 4 L batches using bi-enzymatically modified milk and unmodified (regular) milk as a control (see chapter 3.2.2.1). Inoculation of the milk with starter cultures occurred after transferring pelleted cultures from - 80 °C to - 20 °C for overnight storage, before thawing the 50-unit culture bag in a water bath at 25 °C for 20 min. The culture dosage was calculated according to the manufacturer's instructions for laboratory production with DVS ripening cultures (Chr. Hansen, Nienburg, Germany). A 1:10 dilution of the 50-unit DVS culture in either commercial (Milbona Frische Vollmilch, Fude + Serrahn Milchprodukte GmbH & Co. KG, Hamburg, Germany) or enzyme-modified milk was prepared. From this dilution 0.02 % (w/w; 8 mL) was inoculated into the 4 L batch. After inoculation of the milk (pre-warmed to 45 °C) with starter cultures, the milk was poured into 150 mL yoghurt containers, sealed with aluminium foils using a manual cup sealing device and allowed to ferment at 43 °C in a CRS C+10/350 incubator, with continual pH monitoring in one sample cup using an inoLab pH meter. Once the pH reached 4.5, the yoghurt was cooled to and stored at 4 °C. Post-cooling acidification was monitored at five days and 21 days of storage.

3.2.2.4 Technological parameters

For measurement of technological parameters during preparation of enzyme-modified and regular milk, various methods were applied. These were kindly carried out by the team of Prof. Dr. Peter Chr. Lorenzen, MRI Kiel. Analysis of the fat content in milk was determined using the Gerber Method (VDLUFA C 15.3.2), a German standard method provided by the "Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten" that is used in industry and research.[183] The pH during preparation of enzyme-modified milk was measured using an inoLab pH meter. The dry matter content

was measured gravimetrically using a German standard method (VDLUFA C 35.5), with temperature and time adjusted to 137 °C and 17 min, respectively.[183]

For measurement of carbohydrate distribution at different stages of enzyme-modification, UV-test methods were used. For the determination of lactose and galactose the 'Lactose/D-galactose test kit' was used following the manufacturer's instructions (R-Biopharm AG, Darmstadt, Germany). For glucose and fructose determination, 'D-glucose' and 'D-fructose' reagents for photometric determination of D-glucose/D-fructose in homogenous liquid samples were used according to the manufacturer's instructions (Thermo Fisher Scientific, Darmstadt, Germany). Following calibration with commercially available standard solutions, samples were measured using a Konelab 20i automatic photometer system.

3.2.2.5 Microbiological tests

To monitor bacterial growth and acidification of the starter culture bacteria during fermentation and storage of yoghurt produced in the pilot plant, samples were taken after milk inoculation to determine inoculation levels, as well as at the time point of cooling (when yoghurt reached a pH of 4.5), and after five and 21 days of post-acidification at 4 °C. For all microbiological testing of yoghurt produced in the pilot plant using the YoFlex® Premium 4.0 and the ABT-100 yoghurt cultures, 10 g of sample was placed in a BagFilter® 400 P lab blender bag with a < 250 µm lateral filter, to which 90 mL of sodium citrate buffer (Table 2) was added. The bag was placed in a BagMixer® lab blender for 2 min at the maximum speed. Of the supernatant, 1 mL was diluted in NaCl/casein hydrolysate buffer (Table 2) using a ten-fold dilution series. To determine the bacterial count of SLAB in the yoghurt, volumes of 100 µL of appropriate dilutions were spread-plated in technical triplicates and at three different dilutions onto the appropriate media for each starter culture and incubated accordingly for each medium (Table 3). The bacterial count was determined using the equation outlined in chapter 3.2.2.2.

In addition, the presence of spoilage or opportunistically pathogenic bacteria was tested for using selective agar after 21 days of storage. Possible contamination with enterobacteria using VRBD medium, enterococci using KAA agar, yeast or mould using YGC agar, and pseudomonads using CFCD nutrient media was tested for. These selective agar plates were incubated aerobically at 25 °C for 48 h (YGC, CFCD), 30 °C for 24 h (VRBD) or 37 °C for 48 h (KAA). The acidity of all yoghurt samples was monitored at each sampling time point indicated above, using an MP-220 pH-Meter.

3.2.2.6 Genomic DNA extraction

Genomic DNA was extracted from pilot plant samples of inoculated milk and yoghurt taken at all the time points described in the previous chapter (3.2.2.5) for subsequent molecular biological analyses. The method used for DNA isolation from inoculated milk and yoghurt samples was adapted from the Repeated Bead Beating Plus Column (RBB+C) Method published by Yu and Morrison in 2004 and was performed in a class 2 safety cabinet.[184] For total genomic **DNA isolation from inoculated milk samples**, 20 mL of each milk sample were transferred to a conical 50 mL Falcon® centrifuge tube and centrifuged for 30 min at 6,000 x g at 10 °C in a Heraeus Multifuge. The supernatant was transferred to a new 50 mL Falcon® tube and centrifuged again with the same conditions. This final supernatant was stored at - 20 °C until further batch processing. For genomic **DNA isolation from yoghurt samples**, 20 g of yoghurt were mixed with 20 mL of 1 M Tris-HCl (pH 7.5). Samples were centrifuged for 20 min at 300 x g at 10 °C in a Heraeus Multifuge. The supernatant was transferred to a new 50 mL Falcon® tube and centrifuged again using the same conditions. The final supernatant was then transferred to two conical 50 mL Falcon® centrifuge tubes with a maximum volume of 25 mL per tube and also stored at - 20 °C until further batch processing.

For batch processing, samples of inoculated milk and yoghurt were thawed at room temperature. One volume of RBB+C lysis buffer (Table 2) was added and samples were mixed well by shaking on an Intelli-Mixer RM-2M in the U2-mode at 80 rpm for 10 min. Samples were then incubated in a 70 °C water bath for 20 min with shaking at 196 rpm (max. speed) before centrifuging at 4 °C for 30 min at 6,000 x g in a Heraeus Multifuge. The fat layer that formed on top of the sample was removed with a sterile pipette tip and discarded, whilst the supernatant was transferred to a fresh 50 mL Falcon® tube, taking care to minimise the debris transferred to the next 50 mL Falcon® tube. This centrifugation step was repeated twice until a clear supernatant was obtained.

To each lysate tube, 10 M ammonium acetate amounting to 10 % of the total sample volume was added. After briefly vortexing, the samples were incubated on ice for 15 - 20 min. All tubes were centrifuged at 4 °C for 30 min at 6,000 x g in a Heraeus Multifuge and the supernatant was transferred to a fresh 50 mL Falcon® tube. This centrifugation step was repeated if there were any particles visible in the supernatant. The supernatant was then equally divided into two 50 mL Falcon® tubes and one volume of 2-propanol (pre-cooled to 4 °C) was added to each tube to precipitate the DNA. Samples were mixed well and incubated on ice for 45 min, before being centrifuged at 4 °C for 30 min at 6,000 x g, followed by removal of the supernatant. Nucleic acid pellets were then resuspended in 1 mL of 70 % HPLC gradient grade ethanol and the resuspended

pellet was transferred to 2 mL Eppendorf Tubes®. Samples were centrifuged at 13,000 rpm for 15 min at 4 °C in a Heraeus Fresco21 centrifuge, air-dried in the class 2 safety cabinet and resuspended in 200 µL 10mM Tris-HCl pH 8.0. Where necessary, the pellets were incubated briefly at 37 °C whilst shaking at 800 rpm to facilitate dissolution of the pellet. If the pellet did not dissolve and appeared slimy, an additional centrifugation step at 4 °C of 5 min at 13,000 rpm was performed and the resulting supernatant was then used to continue with the protocol.

Following dissolution of the pellet, an adapted version of the final phase of the RBB+C Method was implemented using the 'QIAamp DNA Stool Mini Kit' (QIAGEN GmbH, Hilden, Germany). In brief, 4 µl of DNAse-free RNase (10 mg/mL) was added and samples were incubated at 37 °C for 15 min. Thereafter, 30 µL of proteinase K solution (20 mg/mL) and 200 µL of 'Buffer AL' from the kit were added per sample and the mixture was incubated at 70 °C for 10 min. Two volumes of 99 % HPLC gradient grade ethanol were added and samples were mixed thoroughly, prior to being transferred to 'QIAamp Mini spin columns' from the kit and centrifuged at 10,000 rpm for 1 min at room temperature in a Heraeus Fresco21 centrifuge. This step was repeated until the entire sample was loaded onto the columns. Subsequent washing steps with Buffers 'AW1' and 'AW2' were done as specified in the RBB+C method. Samples were eluted in 2 x 50 µL of prewarmed 'Buffer AE' with centrifugation at 6,000 rpm for 1 min to maximise the eluted DNA. The concentration of genomic DNA extracted from inoculated milk and yoghurt samples was measured on a NanoDrop™ 2000 spectrophotometer.

3.2.3 Cheese production and analysis

Cheese production was carried out in collaboration with the team of Dr. Wolfgang Hoffmann at the Department of Safety and Quality of Milk and Fish Products at the Max Rubner-Institut (MRI) in Kiel as part of the project 'Strategies for salt reduction in semi-hard cheese' (FZ 2819107716). This project was funded by the German Federal Office for Agriculture and Food. Cheese production was carried out in a set of three independent batches for each experimental condition, and samples were measured in technical triplicates unless otherwise indicated. Arithmetic mean and standard error of the mean calculations were performed using Microsoft Excel. Furthermore, a single scale-up production experiment in a commercial dairy was kindly performed by the team of Dr. Ralf Zink in the research laboratory of the DMK Deutsches Milchkontor GmbH (Edewecht, Germany).

The manufacturing process for Edam cheese at the MRI pilot plant is outlined in Figure 2. The raw milk for cheese production was obtained from the MRI-associated research farm in Schädtbek located near Kiel and was adjusted to 2.45 - 2.70 % fat and 3.30 – 3.55 % protein before being pasteurised at 73 °C for 18 s to inactivate possible contaminating microorganisms and cooled to 8 °C for storage prior to cheese production. Cheese manufacturing was performed at 32 °C in a 180 L cheese vat. The four cheese cultures (Table 4) were added to the milk, together with 0.02 % $CaCl_2$ to lower the pH as well as aid curd formation, and 0.015 % KNO_3 to hinder activation of Clostridia spores. Inoculation of the starter cultures occurred after thawing the cultures in a water bath at 30 °C for 1 h. The culture dosage per 100 L of milk was as follows: 9.4 g of FLORA™ C1060, 18.9 g of CR-550, 2.5 g of LH-32 and 8.9 g of CR-BUTTERY01. The inoculation levels of LAB in the starter cultures was not provided by the manufacturer to protect their trade secret. After 30 min, Hannilase® XP 750 NB rennet was added at 5 mL/100 L milk and the curd was left undisturbed to set for 45 min. The curd was then cut into 5 – 7 mm cubes and the whey was scalded at 40 – 45 °C to allow for increased whey separation. Pressing of the curd into round 1 kg plastic cheese moulds with individual press covers was carried out in a whey bath for a total of 1 h 45 min at room temperature, beginning with 1 bar, followed by a subsequent increases of pressure to 2 and 3 bar after 15 and 45 min, respectively. Each production batch yielded 16 cheese loaves.

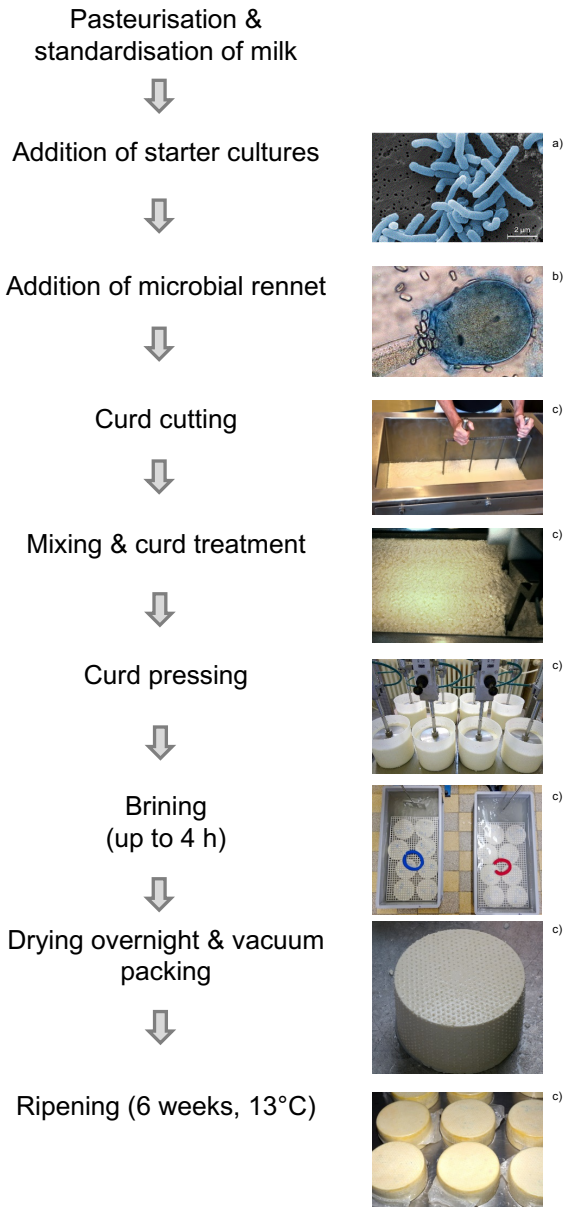

Figure 2: An outline of cheese production in the MRI pilot plant. Photos kindly provided by a) Dr. Horst Neve (shown: *Lactobacillus paracasei*), b) Niels Rösch (shown: *Mucor miehei*) and c) Dr. Wolfgang Hoffmann of the Max-Rubner Institut, Kiel.

Following pressing of the curd into the cheese moulds, the blocks were placed in brine solutions for salting. Three combinations of brine composition and retention times were applied per production batch and each batch was subdivided into one control cheese (4 loaves) and the two salt reduction approaches, namely simple reduction (4 loaves) and mineral salt substitution (8 loaves). The brining conditions are outlined in Table 5. The cheese blocks were dried at 13 °C (80 % humidity) overnight, vacuum wrapped in plastic cheese foil, briefly immersed in hot water to shrink the foil and allowed to ripen at 13 °C for six weeks.

Table 5: Brining conditions for sodium-reduced and control Edam

Cheese variation	Brine composition	Brining time	Number of loaves per batch
Simple NaCl reduction	17 % NaCl 0.13 % Ca†	1 h	4
Mineral salt substitution	20 % sub4salt® ‡ 0.13 % Ca†	1.5 h	8
Control Edam	17 % NaCl 0.13 % Ca†	4 h	4

% = w/w; † added as $CaCl_2 \cdot 2\ H_2O$; ‡ composition of sub4salt® = 24 % Na, 11 % K, 45 % Cl, 18.5 % organic acids and 1.5 % moisture content.

Cheese samples were taken at five time points throughout processing and ripening for a series of microbiological, molecular biological, biochemical and chemical analyses. These time points were as follows: 1) directly after inoculating the pasteurised milk with starter cultures, 2) the curd directly before the pressing process and 3) cheese samples after one week, 4) after three weeks, and 5) after six weeks of ripening at 13 °C.

As the microbiological food safety criteria for the presence of *Listeria (List.) monocytogenes* in ready-to-eat foods in Europe are strict, an additional manufacturing experiment in which the cheese milk was co-inoculated with 1 x 10^5 cfu/mL of *List. innocua*, acting as a surrogate for *List. monocytogenes*, during processing was performed. These challenge tests using *List. innocua* as a surrogate bacteria aimed to provide an indication of whether sodium-reduced Edam samples posed a public health risk from possible pathogenic *List. monocytogenes* contamination. *List. innocua* is non-pathogenic to humans, demonstrates equal growth characteristics to *List. monocytogenes* and is recommended by the 'European Union Reference Laboratory for *Listeria monocytogenes*' as an adequate surrogate for pilot plant-scale challenge tests.[185]

The *List. innocua* strain (MRI culture collection strain number 114a, serovar 6b) was kindly provided by Dr. Philipp Hammer, MRI Kiel and cultured aerobically in BHI broth at 37 °C. This experiment was performed in triplicate under the same production conditions as described above, including the brine composition and brining times presented in Table 5. Samples were taken at the time points as outlined above. In addition, slices of ripened cheese were wrapped in cheese paper after the six-week ripening period and stored at 4 °C to simulate the behaviour of consumers at home. These cheese samples again underwent microbiological testing for possible spoilage or potentially pathogenic organisms after one and three weeks of refrigeration.

Furthermore, using an overnight culture of *List. innocua*, a DNA extraction was performed as outlined in chapter 3.2.1.2 in order to implement this strain as a reference strain for denaturing gradient gel electrophoresis studies (see chapter 3.2.4.1).

In addition to cheese production in the MRI pilot plant, the production parameters for sodium-reduced Edam were scaled up to 15 kg loaves for a single production experiment in the research facility of a commercial dairy (DMK Deutsches Milchkontor GmbH, Edewecht, Germany). The resulting cheeses were only analysed during ripening, as no access to the research lab was possible or allowed during production. Technical triplicates were analysed for all tests.

3.2.3.1 Technological parameters

During Edam production, the pH was monitored using a SenTix™ insertion pH electrode, whilst pH during ripening was measured using an MP-220 pH-Meter. The dry matter and fat in dry matter content, as well as the NaCl, Na and K contents were kindly measured by the team of Dr. Wolfgang Hoffmann, MRI Kiel after six weeks of ripening according to German standard methods (VDLUFA 2003). The German standard method for NaCl determination (the Erbacher method) could not be used to determine the NaCl concentration for cheese samples produced with sub4salt®, as this method measures the Cl content in the sample, from which the NaCl content is then calculated. This method assumes the Cl in the tested cheese samples is only present in the form of NaCl and since sub4salt® contains Cl in the form of KCl as well as NaCl, this calculation is not accurate for cheese samples produced with sub4salt®. The Na and K content was measured using ion-sensitive electrodes (ISE) according to Rabe[186] in a Konelab 20i.

3.2.3.2 Microbiological tests

Microbiological analyses of cheese samples were done at the five time points of processing and ripening as outlined in chapter 3.2.3. For microbiological testing of cheese, 10 g of sample were placed in a BagFilter® 400 P lab blender bag with a < 250 µm lateral filter, to which 90 mL of sodium citrate buffer (Table 2; pre-warmed to 30 °C) was added. The bag was placed in a BagMixer® lab blender for 2 min at maximum speed. Of the filtered supernatant, 1 mL was diluted with NaCl/casein hydrolysate buffer (Table 2) in a ten-fold dilution series. Volumes of 100 µL of appropriate dilutions were spread-plated in triplicate onto the appropriate media for each starter culture and incubated at their respective growth conditions (see Table 4). In addition, samples from the cheese production experiments co-inoculated with *List. innocua* were also spread-plated in triplicate onto ALOA agar plates and incubated at 37 °C for 48 h to determine bacterial counts of *List. innocua*.

In addition to microbiological analysis of the cheese starter cultures, samples taken after six weeks of ripening were also tested for possible contamination with spoilage or opportunistically pathogenic bacteria. Possible contamination with enterobacteria was tested for on VRBD medium, enterococci on KAA agar, yeast or moulds on YGC agar, and pseudomonads on CFCD nutrient media. These selective agar plates were incubated aerobically at 25 °C for 48 h (YGC, CFCD), 30 °C for 24 h (VRBD) or 37 °C for 48 h (KAA). Refrigeration samples of the *List. innocua* co-inoculation experiments were additionally tested for these contaminants after one and three weeks of storage in cheese paper at 4 °C. After incubation of all selective agar plates, bacterial colonies were counted and the cfu/g was calculated using the formula presented in chapter 3.2.2.2.

As a_w and acidity of the food matrix are important factors influencing potential growth of food spoilage or opportunistically pathogenic bacteria, a_w was measured in curd and cheese samples, and pH values was measured in all samples taken throughout cheese manufacture and ripening. Acidity was measured using an MP-220 pH-Meter and a_w was measured using a HygroLab C1 bench-top indicator with digital a_w humidity-temperature probes.

3.2.3.3 Biochemical tests

Biochemical tests were used to analyse the production of various aroma compounds by LAB during lactic fermentation. The level of D- and L-lactic acid and acetic acid production by the starter cultures throughout cheese ripening was analysed for samples taken after one, three and six week of ripening in a single batch for cheese produced with and without *List. innocua* co-inoculation. Samples were prepared using 10 g of sample, placed in a BagFilter® 400 P lab blender bag with a < 250 µm lateral filter, to which 90 mL of sodium citrate buffer (Table 2; pre-warmed to 30 °C) was added. The bag was placed in a BagMixer® lab blender for 2 min at maximum speed 4. The supernatant was centrifuged in a Heraeus Fresco21 centrifuge at 300 x g for 10 min at room temperature and the clear supernatant was applied to the UV-based ‘D-/L-lactic acid test kit’ and ‘acetic acid test kit’ according to the manufacturer’s instructions (R-Biopharm AG, Darmstadt, Germany). Samples were measured in technical duplicates at 340 nm in a Specord 50 spectrophotometer at room temperature.

Proteolysis is an important biochemical process central to achieving a desired cheese flavour and texture, hence a modified colorimetric assay using a Cd-ninhydrin reagent as described by Folkertsma and Fox in 1992 was applied to cheese samples after one, three and six weeks of ripening to measure peptidase activity.[187] This test measured the amount of free amino acids in the samples. The Cd-ninhydrin reagent was adapted from Method C outlined by Doi et al. in 1981 and was comprised of 0.4 g of ninhydrin dissolved

in 40 mL of 99 % HPLC gradient grade ethanol, to which 10 mL of acetic acid and 0.5 g of cadmium chloride, pre-dissolved in 500 µL type 1 ultrapure H_2O, was added.[188]

A standard curve for the proteolysis test was generated using a 10 mg/mL solution of L-alanine at concentrations of 0.010, 0.125, 0.250, 0.400 and 0.600 mg/mL. For measurement of samples, 40 µL of each sample were diluted in 160 µL of 55 mM Tris-HCl (pH 7.5) prior to addition of 1.5 mL of Cd-ninhydrin reagent. Samples were incubated at 84 °C for 5 min on ice and measured on a Specord 50 spectrophotometer at 505 nm and room temperature. Samples were measured in technical triplicates.

3.2.3.4 Genomic DNA extraction

Genomic DNA was extracted from milk, curd and cheese samples for a number of subsequent molecular biology analyses. The sampling time points are outlined in chapter 3.2.3. For **milk samples**, 100 mL of inoculated milk was centrifuged in two 50 mL Falcon® tubes at 6,000 x g for 30 min at 10 °C in a Heraeus Multifuge, the fat layer and supernatant were removed and the pellets (two per sample) were frozen at - 20 °C until further batch processing. For **curd and cheese samples**, 10 g of sample were placed in a BagFilter® 400 P lab blender bag with a < 250 µm lateral filter, to which 90 mL of pre-warmed sodium citrate buffer (Table 2) was added. The bag was placed in a BagMixer® lab blender for 2 min at maximum speed 4. The filtered supernatant was then transferred to 2 x 50 mL Falcon® tubes and centrifuged at 6,000 x g for 30 min at 10 °C. The fat layer and supernatant were removed and discarded whilst the pellets (two per sample) were stored at - 20 °C until batch processing upon completion of each cheese ripening experiment.

The method used for DNA isolation from cheese samples was adapted from the RBB+C method published by Yu and Morrison in 2004 and was performed in a class 2 safety cabinet.[184] Both pellets of each sample were thawed and collectively resuspended in 1 mL of RBB+C lysis buffer (see Table 2; pre-warmed to 60 °C). The samples were transferred to 2 mL Eppendorf Tubes® and 0.4 g of sterile 0.1 mm Zirconium/glass-Beads® per sample were added. The samples were homogenised for 5 – 10 min at maximum speed on an Intelli-Mixer RM-2M bead-beater using the U2-mode at 80 rpm until homogenous. After a 15 min incubation period at 70 °C with shaking at 400 rpm in an Eppendorf Thermomixer, the samples were centrifuged at 4 °C for 5 min at 10,000 rpm in a Heraeus Fresco21 centrifuge and the supernatant transferred to fresh 2 mL Eppendorf Tubes®. Repetition of this centrifugation step was necessary to obtain a clear lysate. Thereafter, 300 µL fresh RBB+C lysis buffer was added to the tubes containing debris and beads from the lysis step, and the samples were again homogenised, incubated at

70 °C with shaking at 400 rpm and centrifuged at 10,000 rpm for 5 min, as described above.

A volume of 10 % of the total sample volume of 10 M ammonium acetate was added to each sample. The samples were incubated on ice for 10 min, followed by centrifugation at 13,000 rpm for 10 min at 4 °C. One volume of 2-propanol, pre-cooled to 4 °C, was added to each sample to precipitate the DNA. Samples were mixed well and incubated on ice for 45 min. Centrifugation for 15 min at 13,000 rpm at 4 °C was then performed, the supernatant removed and discarded, and nucleic acid pellets washed with 70 % HPLC gradient grade ethanol. The pellets were air-dried under a class 2 safety cabinet and subsequently dissolved in 100 µL of 10mM Tris-HCl pH 8.0, with a short incubation period at 37 °C to aid dissolution.

Thereafter, the final phase of the RBB+C Method was adapted and implemented using the 'QIAamp DNA Stool Mini Kit' (QIAGEN GmbH, Hilden, Germany). In brief, 4 µl of DNAse-free RNase (10 mg/mL) was added and samples were incubated at 37 °C for 15 min. Thereafter, 30 µL of proteinase K solution (20 mg/mL) and 200 µL of 'Buffer AL' from the kit were added per sample and the mixture was incubated at 70 °C for 10 min. Two volumes of 99 % HPLC gradient grade ethanol were added and samples were mixed thoroughly, prior to being transferred to 'QIAamp Mini spin columns' from the kit and centrifuged at 10,000 rpm for 1 min at room temperature in a Heraeus Fresco21 centrifuge. This step was repeated until the entire sample was loaded onto the columns. Subsequent washing steps with Buffers 'AW1' and 'AW2' were done as specified in the RBB+C method. Samples were eluted in 2 x 50 µL of pre-warmed 'Buffer AE' with centrifugation at 6,000 rpm for 1 min to maximise the eluted DNA. The concentration of genomic DNA extracted from inoculated milk and yoghurt samples was measured on a NanoDrop™ 2000 spectrophotometer.

3.2.4 Studies of microbial ecology

The microbial ecology of sweetness-enhanced yoghurt and sodium-reduced Edam samples taken throughout fermentation and storage was analysed using culture-independent techniques. These included denaturing gradient gel electrophoresis, 16S rRNA gene high throughput sequencing and whole genome sequencing.

3.2.4.1 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was used to compare the composition of microbial populations in yoghurt samples throughout the fermentation process. The DGGE method relies on a chemical denaturing gradient of urea and formamide to

separate nucleic acids across an acrylamide gel based on their melting temperatures.[189] This method can be used to differentiate single base changes within a pool of DNA fragments, as double-stranded DNA samples have varying melting temperatures (T_m) depending on their nucleic acid composition. Each DNA fragment melts in segments called melting domains and each T_m is unique. After reaching its T_m whilst migrating through a gel with an increasing concentration of denaturing agent, partially melted DNA forms branches as it dissociates, which slows its progression through the polyacrylamide and separates it from other DNA fragments on the gel. DNA samples with sequence differences will move through the gel at different rates based on their T_m. In this way, the bacterial composition of individual samples can be visually differentiated from one another.

In an initial step, the DNA to be analysed on a denaturing gel was amplified by PCR. A 40 bp GC-rich clamp was added to all amplification products via the forward primer (see Table A 6) for better resolution of the fragments across the gel. As the GC-Clamp is long enough to prevent full dissociation of the DNA fragments, single nucleotide differences can be separated across the gradient in each melting domain of the DNA fragment.[190] The PCR targeted the V3 region of the eubacterial 16S rRNA gene (see Table A 6; 338f-GC, 518r +GC-Clamp) and was performed using the 'High efficiency Taq Polymerase with Buffer E' protocol (Genaxxon Bioscience GmbH, Ulm, Germany) with 10x amplification buffer constituted with 2.5 mM $MgCl_2$, 10 mM of each dNTP, 10 pmol of each primer, 3 µL non-adjusted DNA sample for a 50 µL reaction (6 %) and 1.5 U Taq-Polymerase. PCR reactions were performed in a PeqSTAR thermocycler with an initial denaturation step at 94 °C for 3 min; followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 56 °C for 40 s, and extension at 72 °C for 1 min; followed by a final extension step at 72 °C for 7 min. Following PCR, a 5 µL aliquot of the PCR product was run on a 1.5 % agarose gel at 100 V for 1 h to test for successful amplification of products before proceeding with the DGGE gel.

DGGE gels were prepared with a 35 to 70 % gradient using the 'DCode™ Electrophoresis Reagent Kit for DGGE/CDGE' according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., CA., USA; see Table A 7 for gel composition) and run on a BIORAD DCode™ System. The DGGE loading dye used for samples is outlined in Table 2 and was applied 1:1 to samples before loading the gels. The PCR products were separated on DGGE polyacrylamide gels and subjected to electrophoresis at 100 V at 60 °C for 16 h. Subsequent detection of PCR product band profiles was performed by staining the gels with GelRed® Nucleic Acid Gel Stain diluted 1:20 in 1x TAE buffer (Table 2) for 60 min. This was followed by 30 min of de-staining in type 1 ultrapure H_2O for 30 min before visualising gels under UV light using a BioDocAnalyze gel documentation system.

The band pattern visible for each sample, as well as the relative intensity of each band, could then be compared to known samples that were run on the same gel (a mix of the pure bacterial DNA from starter culture isolates outlined in chapter 3.2.1.2), from which the relative abundance of bacterial species within the samples could be estimated. Band comparison and analysis was performed using the BioNumerics (Version 7.6.2) software by applying the Pearson correlation coefficient (*r*) in conjunction with the UPGMA (unweighted pair group method with arithmetic mean) clustering algorithm.[191,192] Gel images were optimised using GIMP 2.10.10. Each biological triplicate for all yoghurt and cheese production experiments was analysed using DGGE.

3.2.4.2 16S rRNA gene high throughput sequencing

16S rRNA gene high throughput sequencing was used to identify and compare the bacteria present in reformulated yoghurt and cheese samples. Library preparation, sequencing and data analysis steps are presented below.

3.2.4.2.1.1 Library preparation and sequencing

The bacterial community composition was determined using tag-encoded, MiSeq™ System-based 16S rDNA high throughput sequencing. Total bacterial genomic DNA was isolated from yoghurt and cheese samples as described in chapters 3.2.2.6 and 3.2.3.4, respectively. Prior to these analyses, the DNA concentration was measured using a Qubit® 3.0 Fluorometer in conjunction with the 'Qubit™ dsDNA BR Assay Kit' following the manufacturer's protocol (Thermo Fisher Scientific, Darmstadt, Germany). The DNA was then diluted to 1.67 ng/µL prior to library preparation.

Specific primers for the 16S rRNA gene V3 and V4 regions with Illumina adapter overhangs were used for amplification of the bacterial community in all samples (16S fw-meta and 16S rev-meta; Table A 6). DNA amplification was carried out following the two-stage PCR protocol (16S Metagenomic Sequencing Library Preparation) provided by Illumina (Illumina Inc., San Diego, CA, USA). A total volume of 25 µL was used for the first-stage PCR, containing 7.5 µL template DNA at a concentration of 1.67 ng/µL, 5 µL of 16S fw-meta and 16S rev-meta primers (1 µM; Table A 6), and 12.5 µL of 'Phusion® High-Fidelity Master Mix with HF Buffer containing Phusion® High-Fidelity DNA Polymerase' (New England Biolabs, Frankfurt am Main, Germany). The PCR reactions were performed in a PeqSTAR thermocycler using the following program: initial denaturation at 95 °C for 3 min; followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final elongation at 72 °C for 5 min. The PCR products were purified using Agencourt AMPure XP magnetic beads and subsequently used as a template for the second-stage, index PCR in order to attach dual indices to the PCR products using the

'Nextera XT Index Kit' (Illumina Inc., San Diego, CA, USA). PCR was performed using the Phusion® High-Fidelity Master Mix with HF Buffer for 8 cycles: denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final elongation step at 72 °C for 5 min. The index PCR products were purified using the Agencourt AM-Pure XP magnetic beads and the final DNA concentration was measured using Qubit® 3.0 Fluorometer.

The size of the 16S rRNA gene PCR products was confirmed by means of automated electrophoresis using the Experion™ Automated Electrophoresis System in conjunction with the 'Experion™ DNA 12K Assay Kit' according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., CA, USA). Considering PCR-product size and final DNA concentration, each sample was adjusted to a 4 nM library with 10 mM Tris-HCl (pH 8.5). Thereafter, 5 µL of normalised DNA from each sample were mixed in order to pool the libraries containing unique indices. The pooled samples were further diluted using the 'Hybridisation Buffer' (HT1) supplied in the 'Nextera XT Index Kit' to a final loading concentration of 6 pM. 16S rDNA sequencing was performed on a MiSeq™ System using the 'MiSeq Reagent Kit v3' following the manufacturer's instructions (Illumina Inc., San Diego, CA, USA).

3.2.4.2.1.2 Bioinformatics: Rhea pipeline

Following 16S rRNA gene sequencing, the raw dataset containing de-multiplexed, pair-ended reads was analysed using the *de novo* analysis pipeline of the IMNGS platform for ecology and diversity studies.[193] The raw fastq files were pre-processed using the Remultiplexor Perl script available through the IMNGS platform and then uploaded to the IMNGS pipeline, which implements the UPARSE algorithm from the USEARCH 8 (32-bit) package.[194,195] The pipeline produced operational taxonomic unit (OTU) tables for each sample, using the Greengenes 16S rRNA gene collection as a reference database.[196] For more extensive phylogenetic classification, the OTU sequences were uploaded to the SINA Arb Silva Aligner to additionally compare OTUs with both the Silva and rdp reference databases.[197,198]

The consolidated and verified OTU tables were used for statistical analysis using RStudio Version 1.1.423 by implementing the set of 'Rhea scripts' published by Lagkouvardos et al. in 2017.[199] The Rhea pipeline encompasses a set of R scripts for the downstream analysis of OTUs. These R scripts were used for 16S rDNA bioinformatics analysis of yoghurt and cheese samples by processing the OTU tables to normalise samples, as well as determine alpha and beta diversity of the samples. Taxonomic binning to visualise the taxonomic composition of samples was performed in Microsoft Excel using the OTU tables generated by the Rhea pipeline.

To compare the alpha diversity of the samples, the species richness and the Shannon diversity index were calculated using the Rhea pipeline. Species richness gives a simple indication of the number of OTUs present in each sample. The Shannon index indicates the diversity of all tested samples, with high values indicating a diverse community, while values close to zero indicate a community with low diversity. For calculation of alpha diversity, normalised OTU counts above 0.5 were implemented in the Rhea pipeline.

Beta diversity describes diversity between samples or groups of samples and was calculated for all yoghurt and cheese experiments. The Rhea pipeline applies the generalised UniFrac distances as suggested by Chen et al. in 2012, measuring the diversity of microbial profiles and providing a middle-ground between weighted and unweighted UniFrac calculations.[200] The Rhea pipeline used multi-dimensional scaling (MDS) to create distance matrix figures and applied a permutational multivariate analysis of variance (PERMANOVA) using distance matrices (vegan::adonis) to calculate the significance (p-values) of differences in microbial composition between groups of OTUs.[199,201]

3.2.4.3 Whole genome sequencing

Whole genome sequencing was used to confirm the identity of cheese strain isolates. Library preparation, sequencing and data analysis steps are presented below.

3.2.4.3.1.1 Library preparation and sequencing

Whole genome sequencing of bacterial DNA isolated from individual cheese starter culture isolates derived from the four cultures implemented in Edam manufacture (see chapter 3.2.1.2 and Table 4) was performed. Prior to metagenomics analyses, the DNA concentration was measured using a Qubit® 3.0 Fluorometer in conjunction with the 'Qubit™ dsDNA HS Assay Kit' following the manufacturer's protocol (Thermo Fisher Scientific, Darmstadt, Germany). Samples were diluted to 2.0 ng/µL prior to library preparation.

Samples were subsequently sheared to 450-bp fragments using an M220 Focused-ultrasonicator™. The fragment size was confirmed using the Experion™ Automated Electrophoresis System using the 'Experion™ DNA 12K Assay Kit' (Bio-Rad Laboratories, Inc.). The fragmented DNA samples were transferred to a 0.5 mL deep-well plate and further sample preparation was performed following the TruSeq® Nano DNA LT Library Prep protocol (Illumina Inc., San Diego, CA, USA). Samples were normalised according to the manufacturer's protocol and 5 µL of each sample were mixed in order to pool the libraries containing unique indices. The pooled samples were diluted using the 'Hybridisation Buffer' (HT1) supplied in the 'Nextera XT Index Kit' to a final loading concentration of 13 pM. Whole genome sequencing was performed on a MiSeq™ System using the

'MiSeq Reagent Kit v2' following the manufacturer's instructions (Illumina Inc., San Diego, CA, USA).

3.2.4.3.1.2 Bioinformatics

Following whole genome sequencing, initial bioinformatics analysis was performed using PATRIC, the Pathosystems Resources Integration Center.[202] Using this online platform, genome annotation was performed using the RAST took kit.[203] Following gene annotation, the Chun Lab's Orthologous Average Nucleotide Identity Tool (OAT) software using the OrthoANI algorithm was used to calculate average nucleotide identities between the strain isolates derived from the cheese starter cultures and type strains of each species claimed to be included in the mixed cultures by the culture manufacturer Chr. Hansen.[204] An exception was made with *Leuc. pseudomesenteroide*s, as the type strain for this species was not originally identified by means of whole genome sequencing and no whole genome sequence of the type strain was available.[205] Therefore, a non-type-strain isolate of *Leuc. pseudomesenteroides* was used for whole genome comparisons.

4 Results

4.1 Yoghurt

In the following chapter, results of reformulation efforts to enzymatically sweeten yoghurt will be presented. Initially, the production process as well as chemical and physical parameters measured throughout milk processing will be presented. This lays the foundation for a detailed exploration of the influence of this reformulation strategy on the microbial ecology of yoghurt produced using a traditional starter culture (containing *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*) as well as a mild-tasting, probiotic starter culture (containing *S. thermophilus*, *Lb. acidophilus* and *B. animalis*). Enzyme-modification of milk, as well as pilot plant yoghurt production and collection of technological data, were carried out in collaboration with the Department of Quality and Safety of Milk and Fish Products at the MRI in Kiel. Parts of this chapter were accepted for publication in the *International Journal of Dairy Technology* in November 2019.[*]

4.1.1 Production of sweetness-enhanced yoghurt

For the production of sweetness-enhanced yoghurt, the bi-enzymatic system of lactose conversion as presented by Lorenzen et al. in 2013 was applied.[99] The production process of regular and sweetness-enhanced yoghurt is depicted in Figure 1. For enzyme-modification, the raw milk was processed to separate the cream from the skim milk, the latter was then ultrafiltered to yield the UF permeate and the UF retentate. The UF permeate was further processed using a cation exchanger to reduce Ca^{2+} levels as the glucose isomerase enzyme is calcium sensitive, before subsequently undergoing lactose hydrolysis and glucose isomerisation. Combining the calcium-rich, lactose-hydrolysed UF retentate with the sweetness-enhanced UF permeate and cream created the sweetness-enhanced milk, which then underwent further fat and dry matter adjustments prior to yoghurt production. Fat and dry matter adjustments were performed in parallel with regular milk prior to fermentation with yoghurt starter cultures to manufacture the regular yoghurt used as a control for the pilot plant experiments.

[*] Luzzi, G., Steffens, M., Clawin-Rädecker, I., Hoffmann, W., Franz, C.M.A.P., Fritsche, J. & Lorenzen, P. Chr. Enhancing the sweetening power of lactose by enzymatic modification in the reformulation of dairy products. *International Journal of Dairy Technology* (accepted for publication on 29th November 2019).

To determine the efficacy of the bi-enzymatic system to produce sweeter tasting carbohydrates from lactose, the distribution of sugars in the UF retentate and UF permeate was measured before and after enzymatic modification of milk, the results of which are shown in Figure 3. The lactose contained in the UF retentate was almost completely converted to glucose and galactose through lactose hydrolysis, whilst hydrolysis of the UF permeate did not completely convert lactose to glucose and galactose. Following isomerisation, the conversion of glucose to the sweeter-tasting carbohydrate fructose was evident.

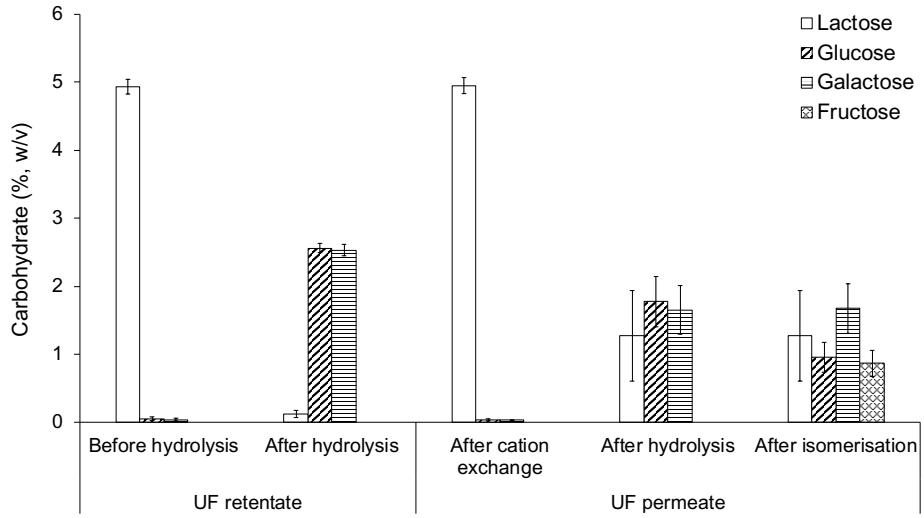

Figure 3: Carbohydrate distribution following lactose hydrolysis and glucose isomerisation in the UF retentate and UF permeate during the production of sweetness-enhanced milk. The β-galactosidase enzyme was heat inactivated after hydrolysis and the level of residual lactose is shown after isomerisation. The arithmetic mean (n = 6 biological replicates, 3 per culture) ± standard error of the mean are shown.

Throughout milk processing in preparation for fermentation with starter cultures, the pH, dry matter and fat were monitored for potential effects of enzyme-modification on these product characteristics (Table 6). Adjustments during processing aimed for a final fat content of 3.5 % (w/w) and dry matter content of 14 % (w/w). In regular milk, fat and dry matter adjustments resulted in milk with an average final fat content of 3.55 % and an average dry matter content of 14.02 %. Final adjustments in sweetness-enhanced milk resulted in an average fat content of 3.59 % and an average dry matter content of 14.85 %.

Table 6: Technological parameters measured during preparation of regular and sweetness-enhanced milk

Matrix	pH	Dry matter (%)	Fat (%)
Raw milk	6.73 ± 0.01	12.91 ± 0.16	3.98 ± 0.15
Skim milk	6.73 ± 0.01	ND	0.07 ± 0.02
Cream	6.72 ± 0.01	45.53 ± 1.68	40.05 ± 1.97
Regular milk after fat and dry matter adjustment	6.67 ± 0.01	14.02 ± 0.01	3.55 ± 0.01
Sweetness-enhanced milk after fat and dry matter adjustment	6.82 ± 0.03	14.85 ± 0.27	3.59 ± 0.01

n = 6 biological replicates ± standard error of the mean; % = w/w; ND = not done

4.1.2 Isolation of starter culture strains from mixed culture

Prior to yoghurt production and screening of traditional and probiotic yoghurt cultures for optimal fermentation in sweetness-enhanced milk, individual strains contained in these mixed starter cultures were isolated to test for the utilisation of different carbohydrates and to use as reference strains for studies of microbial ecology using culture-independent methods. Pure cultures were successfully isolated using dilution streaking on selective agar, verified morphologically and microscopically, and their identity confirmed by sequencing PCR amplicons of the 16S rRNA gene of each strain and comparing these with the NCBI 16S ribosomal RNA database using a Standard Nucleotide BLAST® and the EZ Taxon database using EzBioCloud's Identify services.[206,207] The first (closest) match of the sequences of the 16S rRNA gene amplicons with each database is shown in Table 7. The query cover for each isolate was 100 %. All of the strains indicated on the product data sheet for each culture were isolated and could thus be identified at species level.

Table 7: Comparison of selected 16S rRNA gene amplicons of yoghurt starter culture strains with the NCBI 16S rRNA and EZ Taxon databases

Culture name	Microbial composition acc. to Chr. Hansen	Isolate Nr. (agar used)	Nucleotide BLAST® * (accession number)	Identity (%)	EZ Taxon ** (accession number)	Identity (%)
YoFlex® Premium 1.0	*S. thermophilus* *Lb. delbrueckii* subsp. *bulgaricus*	1 (M17)	*S. thermophilus* (ATCC 19258)	99.93	*S. thermophilus* (ATCC 19258)	100
		2 (MRS)	*Lb. delbrueckii* subsp. *bulgaricus* (NBRC 13953)	99.93	*Lb. delbrueckii* subsp. *bulgaricus* (ATCC 11842)	99.79
YoFlex® Premium 4.0	*S. thermophilus* *Lb. delbrueckii* subsp. *bulgaricus*	1 (M17)	*S. thermophilus* (ATCC 19258)	100	*S. thermophilus* (ATCC 19258)	100
		2 (MRS)	*Lb. delbrueckii* subsp. *bulgaricus* (NBRC 13953)	100	*Lb. delbrueckii* subsp. *bulgaricus* (ATCC 11842)	99.93
YoFlex® Mild 2.0	*S. thermophilus* *Lb. delbrueckii* subsp. *bulgaricus*	1 (M17)	*S. thermophilus* (ATCC 19258)	100	*S. thermophilus* (ATCC 19258)	100
		2 (MRS)	*Lb. delbrueckii* subsp. *bulgaricus* (NBRC 13953)	100	*Lb. delbrueckii* subsp. *bulgaricus* (ATCC 11842)	99.93
YF-L812	*S. thermophilus* *Lb. delbrueckii* subsp. *bulgaricus*	1 (M17)	*S. thermophilus* (ATCC 19258)	100	*S. thermophilus* (ATCC 19258)	100
		2 (MRS)	*Lb. delbrueckii* subsp. *bulgaricus* (NBRC 13953)	100	*Lb. delbrueckii* subsp. *bulgaricus* (ATCC 11842)	99.93

Continued on next page

Table 7 continued

Culture name	Microbial composition acc. to Chr. Hansen	Isolate Nr. (agar used)	Nucleotide BLAST® * (accession number)	Identity (%)	EZ Taxon ** (accession number)	Identity (%)
ABT-6 Probio-Tec®	*S. thermophilus* *Lb. acidophilus* *Bifidobacterium* sp.	1 (M17)	*S. thermophilus* (ATCC 19258)	100	*S. thermophilus* (ATCC 19258)	100
		2 (Bile-MRS)	*Lb. acidophilus* (NBRC 13951)	100	*Lb. acidophilus* (CIP 76.13)	100
		3 (LP-MRS)	*B. animalis* subsp. *lactis* (YIT 4121)	100	*B. animalis* subsp. *lactis* (DSM 10140)	100
ABT-100	*S. thermophilus* *Lb. acidophilus* *Bifidobacterium* sp.	1 (M17)	*S. thermophilus* (ATCC 19258)	100	*S. thermophilus* (ATCC 19258)	100
		2 (Bile-MRS)	*Lb. acidophilus* (NBRC 13951)	100	*Lb. acidophilus* (CIP 76.13)	100
		3 (LP-MRS)	*B. animalis* subsp. *lactis* (YIT 4121)	100	*B. animalis* subsp. *lactis* (DSM 10140)	100

The first sequences producing significant alignments in * the NCBI 16S ribosomal RNA database using a Standard Nucleotide BLAST®, and ** the EZ Taxon database using EzBioCloud's Identify service are shown. Query cover/completeness was 100 % for all isolates.

4.1.3 Screening yoghurt cultures for optimal sugar utilisation

Prior to yoghurt production using sweetness-enhanced milk, preliminary sugar utilisation tests were performed on individual strains isolated from yoghurt cultures to determine whether any of these strains were able to utilise galactose, whilst preferably not metabolising fructose (Table 8). During fermentation, LAB produce lactic acid mainly by metabolising the glucose moiety of lactose as opposed to the galactose moiety, the latter of which is present in yoghurt at 1.5 – 2.5 %.[70] However, incorporating galactose-utilising strains in yoghurt starter cultures would result in increased residual glucose in the matrix and a sweeter product due to the higher sweetness of glucose compared to galactose. In combination with the bi-enzymatic system of lactose conversion, galactose-utilising strains could further contribute to the overall sweetness of yoghurt.

Sugar utilisation screening was performed in biological triplicates as outlined in chapter 3.2.1.3 and was completed for strain isolates from traditional starter cultures. As the mild-tasting, probiotic cultures (ABT-6 Probio-Tec® and ABT-100) were added to the project at a later stage, screening experiments for these cultures commenced directly with laboratory production (see chapter 3.2.2.2). None of the tested starter culture strains were able to utilise galactose, showing no potential advantage of any of the four traditional starter cultures tested with respect to galactose utilisation.

Table 8: Sugar utilisation by starter culture isolates from traditional yoghurt cultures

Name of culture	***S. thermophilus* / *Lb. delbrueckii* subsp. *bulgaricus***			
	Glucose	Galactose	Fructose	Lactose
YF-L812	–/+	–/–	–/+	+/+
YoFlex® Mild 2.0	–/+	–/–	–/+	+/+
YoFlex® Premium 1.0	–/+	–/–	–/+	+/+
YoFlex® Premium 4.0	–/+	–/–	–/+	+/+
ABT-6 Probio-Tec®	ND	ND	ND	ND
ABT-100	ND	ND	ND	ND

n = 3 biological replicates; '+' = utilises this sugar; '–' = does not utilise this sugar; ND = not done

As the positive control, glucose, presented negative results for *S. thermophilus*, it was suspected that these strains did not receive adequate growth nutrients in the basal medium used for fermentation studies (Table 1). Hence, an additional test was performed in which the *S. thermophilus* isolates were grown in modified M17 broth, where the lactose component was replaced by an equal amount of glucose (Table 9). This was compared to growth of the isolates in basal medium with 2.5 % glucose. This test showed that the four isolates of *S. thermophilus* tested were not supplied with adequate nutrients for growth in basal medium. Hence, no definite conclusions about sugar utilisation can be made from the sugar utilisation tests for this microorganism presented in Table 8.

Table 9: Utilisation of glucose by *S. thermophilus*

Name of culture	**Basal medium with Glucose**	**M17 medium with Glucose**
YF-L812	wg	+
YoFlex® Mild 2.0	wg	+
YoFlex® Premium 1.0	wg	+
YoFlex® Premium 4.0	wg	+

n = 1 biological replicate; wg = weak growth

4.1.4 Laboratory yoghurt production

All six yoghurt cultures provided by Chr. Hansen (Table 3) were screened in small scale laboratory production experiments to test for optimal growth and acidification behaviour in sweetness-enhanced milk. These tests were essential to evaluate whether the starter culture LAB were able to ferment the sugars available to them in a similar manner to those accessible in non-modified (regular) milk. The acidification curves determined for sweetness-enhanced and regular yoghurt were compared to each other, but also to those provided by Chr. Hansen in the starter culture product data sheets (see Appendices).

This screening aided in the selection of one traditional yoghurt culture (containing *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*) and one mild-tasting, probiotic yoghurt culture (containing *S. thermophilus*, *Lb. acidophilus* and *B. animalis*), each of which displayed the most similar fermentation behaviour when comparing sweetness-enhanced and regular milk. These two cultures were used for subsequent large-scale production experiments in the MRI pilot plant. For screening of yoghurt cultures in the preliminary laboratory production experiments presented in this chapter, a commercially available regular milk with 3.5 % fat was used. Due to the temperature sensitivity of the pH electrodes, the cooling of samples from 43 °C to 4 °C once they reached pH 4.4 required that the measuring equipment be restarted for subsequent measurements (see chapter 3.2.2.2). Hence, the acidification curves of the laboratory production experiments show a pH measuring gap when the samples reached a pH of 4.4.

Bacterial growth as well as acidification behaviour of the traditional yoghurt culture YF-L812 are shown in Figure 4. Both *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* displayed expected growth progressions for symbiotic fermentation in both regular and sweetness-enhanced milk, whereby initial growth of *S. thermophilus* allowed for the subsequent growth of *Lb. delbrueckii* subsp. *bulgaricus*. The acidification curve of this culture was similar in both sweetness-enhanced milk as well as regular milk, and followed the same progression described by the manufacturer in the product data sheet (see Appendices, Figure A 1). Despite growth and acidification showing expected progressions, this culture was not chosen for subsequent pilot plant scale production as *Lb. delbrueckii* subsp. *bulgaricus* could not be cultivated on selective agar plates during one of the biological triplicate experiments. Time constraints prevented further testing.

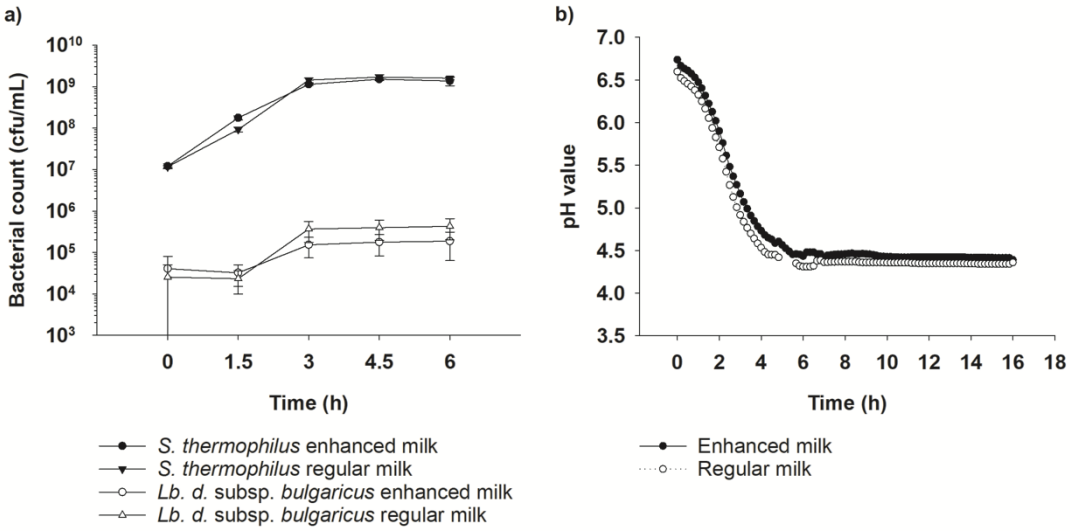

Figure 4: a) Growth of and b) milk acidification caused by starter lactic acid bacteria (*S. thermophilus* and *Lb. delbrueckii* (*d.*) subsp. *bulgaricus*) in the YF-L812 culture during laboratory production of yoghurt at 43 °C using sweetness-enhanced and regular milk. Samples were cooled to 4 °C once a pH of 4.4 had been reached. The arithmetic mean (n = 3 biological replicates) is shown, ± the standard error of the mean for (a).

Subsequently, the bacterial growth and acidification potential of the YoFlex® Mild 2.0 culture was screened. The results of this screening are shown in Figure 5. Whilst *S. thermophilus* showed an expected growth progression that was very similar to all yoghurt cultures screened, the *Lb. delbrueckii* subsp. *bulgaricus* strain contained in the YoFlex® Mild 2.0 culture showed a steep decline in bacterial counts towards the end of fermentation in sweetness-enhanced milk. For this reason, the YoFlex® Mild 2.0 culture was not chosen for further study, despite acidification curves showing an expected progression similar to that described by the manufacturer (see Appendices, Figure A 2).

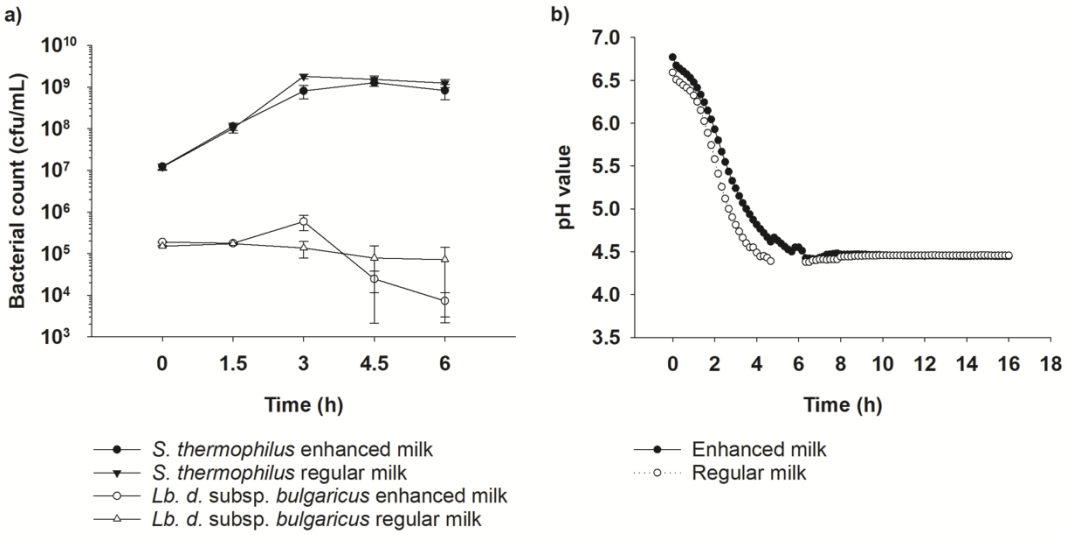

Figure 5: a) Growth of and b) acidification caused by starter lactic acid bacteria (*S. thermophilus* and *Lb. delbrueckii* (*d.*) subsp. *bulgaricus*) in the YoFlex® Mild 2.0 culture during laboratory production of yoghurt at 43 °C using sweetness-enhanced and regular milk. Samples were cooled to 4 °C once a pH of 4.4 had been reached. The arithmetic mean (n = 3 biological replicates) is shown, ± the standard error of the mean for (a).

The YoFlex® Premium 1.0 culture was next tested for bacterial growth and acidification in sweetness-enhanced milk and compared to regular milk (Figure 6). Whilst growth of *S. thermophilus* showed an expected progression, *Lb. delbrueckii* subsp. *bulgaricus* showed only minimal growth throughout fermentation. The inoculation ratio of *Streptococcus* to *Lactobacillus* in this culture differed considerably to that in the other traditional yoghurt cultures screened. The acidification pattern of this culture was similar in both types of milk and showed a progression comparable to that described by the manufacturer in the product data sheet (see Appendices, Figure A 3). Due to the low inoculation level and subsequent slow growth of *Lb. delbrueckii* subsp. *bulgaricus*, the YoFlex® Premium 1.0 culture was also not chosen for pilot plant production.

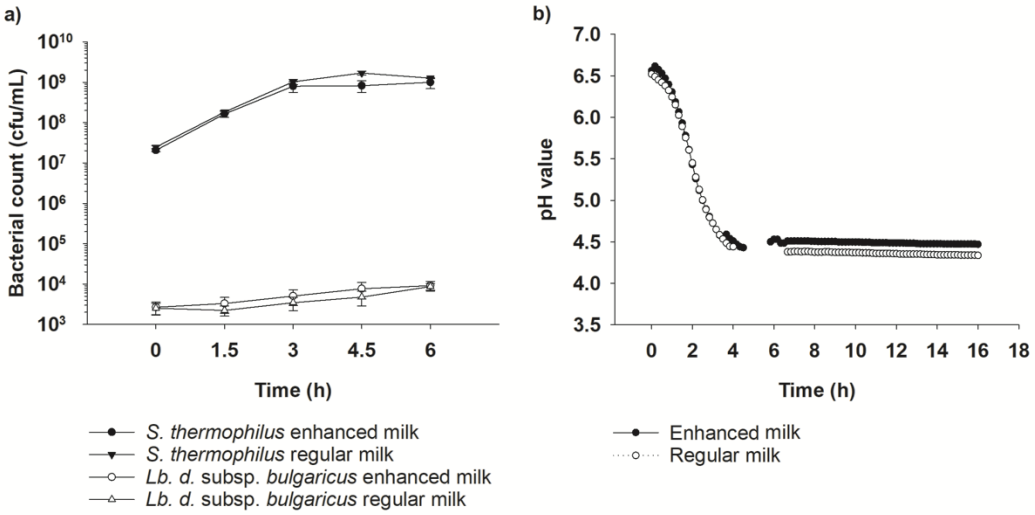

Figure 6: a) Growth of and b) acidification caused by starter lactic acid bacteria (*S. thermophilus* and *Lb. delbrueckii* (*d.*) subsp. *bulgaricus*) in the YoFlex® Premium 1.0 culture during laboratory production of yoghurt at 43 °C using sweetness-enhanced and regular milk. Samples were cooled to 4 °C once a pH of 4.4 had been reached. The arithmetic mean (n = 3 biological replicates) is shown, ± the standard error of the mean for (a).

The fourth and final traditional yoghurt culture screened for bacterial growth and acidification potential in sweetness-enhanced milk was the YoFlex® Premium 4.0 culture (Figure 7). In regular milk, *S. thermophilus* growth lagged unexpectedly in the first 1.5 h of fermentation, which allowed *Lb. delbrueckii* subsp. *bulgaricus* growth to temporarily increase by approx. 1 log cfu/mL in this type of milk. However, similar growth development to those measured for other traditional yoghurt cultures (YF-L812, YoFlex® Mild 2.0 and YoFlex® Premium 1.0) were observed in both starter culture strains in regular milk towards the end of fermentation. Development of the starter cultures in sweetness-enhanced milk progressed as expected throughout fermentation. The acidification pattern of the YoFlex® Premium 4.0 culture was similar in both types of milk and reflected the acidification curve provided by the manufacturer in the product data sheet (see Appendices, Figure A 4). The YoFlex® Premium 4.0 culture was chosen for extended pilot plant production experiments, the results of which are presented in chapter 4.1.5.

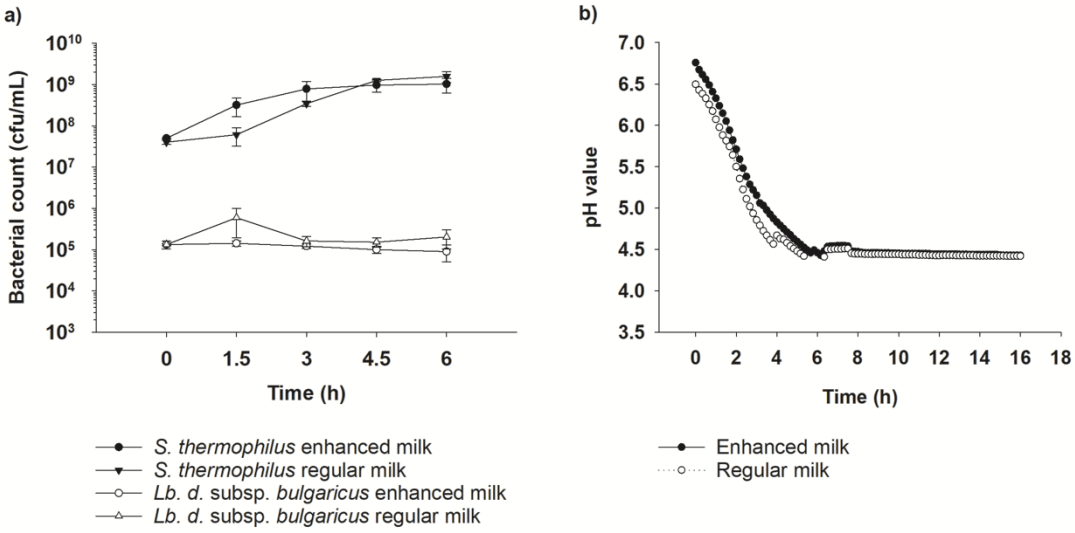

Figure 7: a) Growth of and b) acidification caused by starter lactic acid bacteria (*S. thermophilus* and *Lb. delbrueckii* (*d.*) subsp. *bulgaricus*) in the YoFlex® Premium 4.0 culture during lab production of yoghurt at 43 °C using sweetness-enhanced and regular milk. Samples were cooled to 4 °C once a pH of 4.4 had been reached. The arithmetic mean (n = 3 biological replicates) is shown, ± the standard error of the mean for (a).

In addition to the four previously presented traditional yoghurt cultures screened, two mild-tasting, probiotic yoghurt cultures containing *S. thermophilus*, *Lb. acidophilus* and *B. animalis* were also tested for growth and acidification potential in sweetness-enhanced and regular milk. The growth of these starter culture strains, as well as the acidification curves for the ABT-6 Probio-Tec® culture, are shown in Figure 8. Some discrepancies were observed in the growth of the starter cultures in regular compared to sweetness-enhanced milk. Bacterial counts of *B. animalis* dropped about 1 log cfu/mL towards the end of the fermentation period; however, this was only observed in yoghurt produced with regular milk. In addition, *Lb. acidophilus* displayed growth difficulties in one of the biological triplicates. The acidification progression measured for the ABT-6 Probio-Tec® culture in sweetness-enhanced milk showed an expected curve based on the manufacturer's product data sheet (see Appendices, Figure A 5), yet this culture failed to adequately acidify regular milk in all biological triplicate experiments. Hence, the ABT-6 Probio-Tec® culture was disregarded for further pilot plant studies.

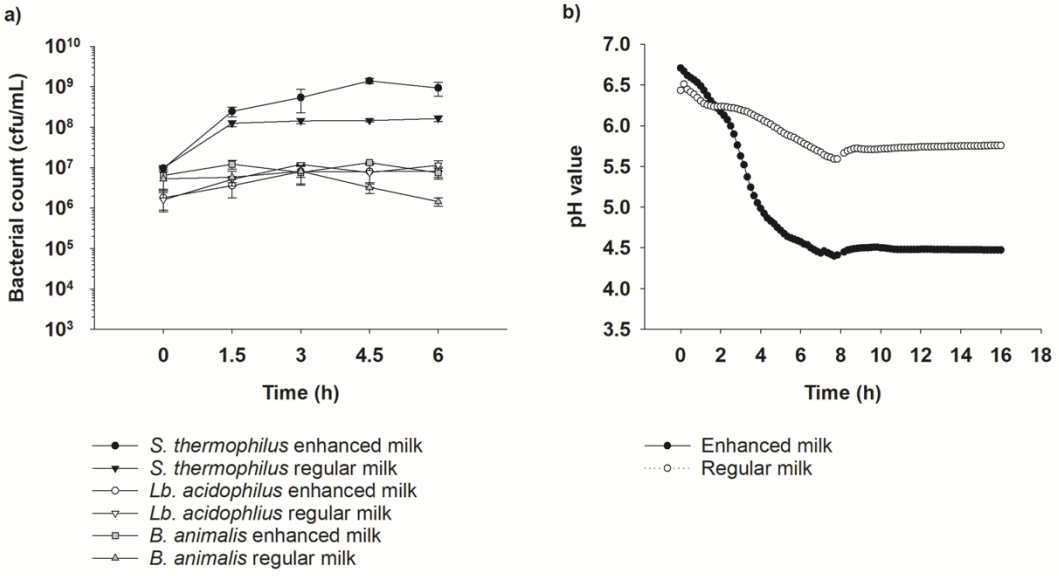

Figure 8: a) Growth of and b) acidification caused by starter lactic acid bacteria (*S. thermophilus*, *Lb. acidophilus* and *B. animalis*) in the ABT-6 Probio-Tec® culture during laboratory production of yoghurt at 43 °C using sweetness-enhanced and regular milk. Samples were cooled to 4 °C once a pH of 4.4 had been reached. The arithmetic mean (n = 3 biological replicates) is shown, ± the standard error of the mean for (a).

The second probiotic yoghurt culture screened for bacterial growth progression and acidity was the ABT-100 culture (Figure 9). The bacterial growth for all three starter culture strains showed a similar progression in sweetness-enhanced milk compared to regular milk. Acidification of both types of milk followed a similar and expected progression based on the curve provided by the manufacturer (see Appendices, Figure A 6). The ABT-100 culture seemed well-suited to fermenting sweetness-enhanced milk as very similar growth and acidification curves were observed in enhanced milk compared to regular milk. This culture was therefore chosen for subsequent larger-scale pilot plant production, the results of which are presented in chapter 4.1.5.

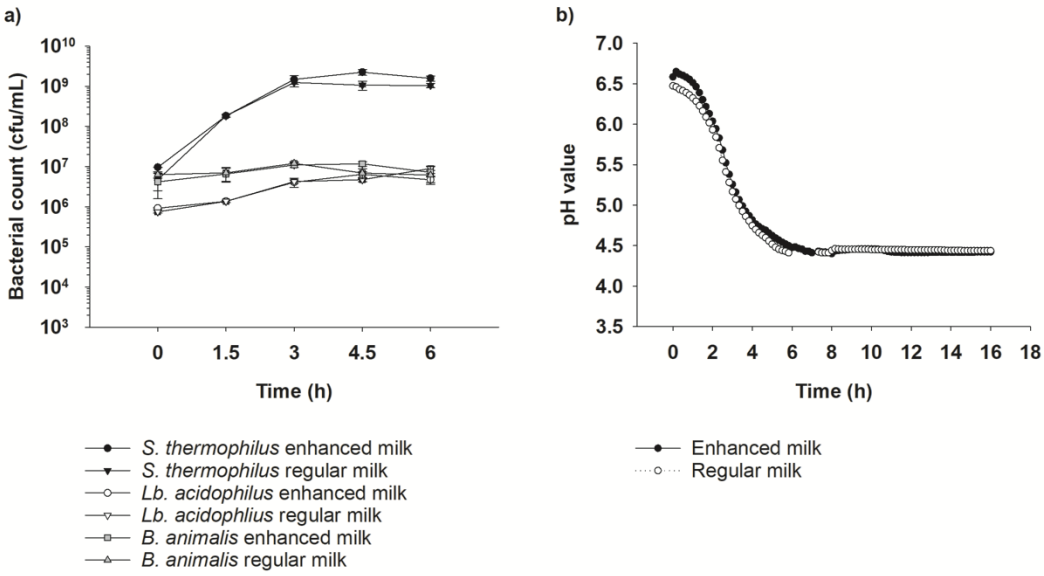

Figure 9: a) Growth of and b) acidification caused by starter lactic acid bacteria (*S. thermophilus*, *Lb. acidophilus* and *B. animalis*) in the ABT-100 culture during laboratory production of yoghurt at 43 °C using sweetness-enhanced and regular milk. Samples were cooled to 4 °C once a pH of 4.4 had been reached. The arithmetic mean (n = 3 biological replicates) is shown, ± the standard error of the mean for (a).

4.1.5 Pilot plant yoghurt production

Following initial screening of six commercial yoghurt cultures for starter culture growth progression and acidification potential in sweetness-enhanced milk (presented in chapter 4.1.4), the traditional yoghurt culture YoFlex® Premium 4.0 and the mild-tasting, probiotic culture ABT-100 were chosen for subsequent large-scale yoghurt production experiments in the MRI pilot plant in Kiel. These pilot plant production experiments aimed to verify the bacterial growth progression and acidification behaviour that was observed in the laboratory production experiments in larger (4 L) batch productions. As the small-scale laboratory production involved substantial downscaling of the commercial starter

culture dosages, potentially misrepresenting the results, pilot-plant productions were important to test the efficacy of this reformulation approach.

For these experiments, regular milk was produced following the production outline shown in Figure 1 and this milk was used as the control milk. Yoghurt was produced in biological triplicates and the growth patterns of starter culture strains are presented in Figure 10. Bacterial growth of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* showed matching progressions in both sweetness-enhanced and regular milk in yoghurt produced with the YoFlex® Premium 4.0 culture (Figure 10a). *S. thermophilus* was inoculated at 5 × 10^7 cfu/mL, grew rapidly to approx. 1 × 10^9 cfu/mL during fermentation, and remained at these levels throughout storage. *Lb. delbrueckii* subsp. *bulgaricus* was inoculated at approx. 1 × 10^5 cfu/mL, increasing to approx. 8 × 10^5 cfu/mL after fermentation and five days of storage, before dropping to approx. 2 × 10^5 cfu/mL after 21 days of storage at 4 °C.

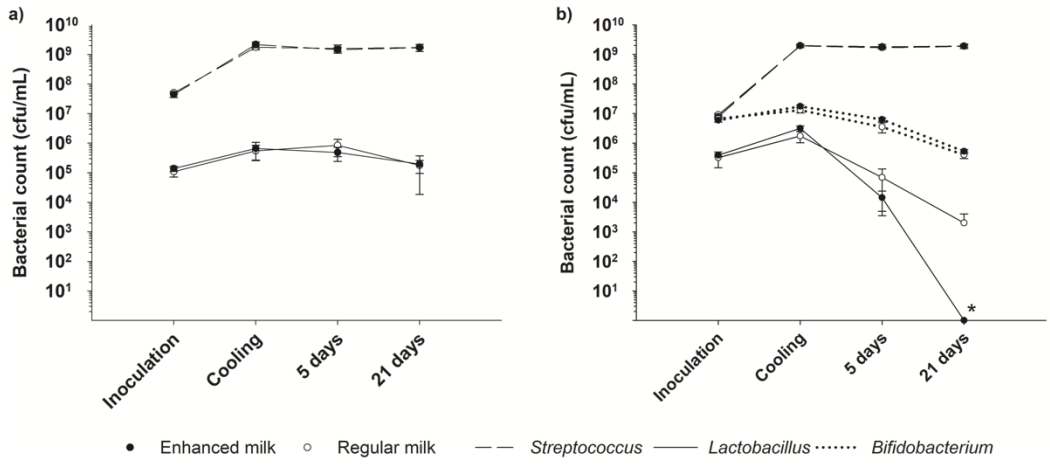

Figure 10†: Starter culture growth during fermentation and storage of yoghurt produced in the pilot plant with a) the traditional YoFlex® Premium 4.0 culture and b) the probiotic ABT-100 culture. Samples were cooled to 4 °C once a pH of 4.5 had been reached, after which they were stored for post-acidification at 4 °C for a total of 21 days. The arithmetic mean (n = 3 biological replicates) ± the standard error of the mean are shown. * indicates below the limit of detection, which was set at 1 x 10^2 cfu/mL.

The ABT-100 culture (Figure 10b), however, showed considerable variation in growth patterns during the pilot plant production when compared to the preliminary laboratory scale production (Figure 9). *S. thermophilus* showed expected growth progressions in this culture, starting with inoculation at approx. 1 × 10^7 cfu/mL and growing rapidly to approx. 1 × 10^9 cfu/mL. However, *B. animalis* displayed a decline in bacterial count from

† This figure was accepted for publication in the *International Journal of Dairy Technology* (Luzzi et al., accepted on 29th November 2019).

approx. 7×10^6 cfu/mL at inoculation to approx. 5×10^5 cfu/mL after five and 21 days of storage at 4 °C. This was not observed in preliminary laboratory-scale production experiments. Finally, *Lb. acidophilus* showed growth difficulties after cooling in both sweetness-enhanced and regular milk. *Lb. acidophilus* was inoculated at approx. 4×10^5 cfu/mL, grew to 3×10^6 cfu/mL during fermentation, before a steep decrease in bacterial counts was observed during storage for both types of milk, ending at 6×10^3 cfu/mL in regular milk after 21 days (Figure 10b). *Lb. acidophilus* could not be cultivated from samples of sweetness-enhanced yoghurt taken after 21 days of storage.

Yoghurt produced with both YoFlex® Premium 4.0 and ABT-100 cultures was tested for possible contamination with spoilage or opportunistically pathogenic bacteria after 21 days of storage at 4 °C. Bacterial counts of enterobacteria, enterococci, yeast or moulds and pseudomonads could not be measured above the limit of detection set at 1×10^2 cfu/mL for this study, indicating that the quality of sweetness-enhanced yoghurt samples was not compromised when compared with yoghurt samples produced with regular milk.

As acid production by LAB is an important aspect of fermentation, the pH of yoghurt samples produced with sweetness-enhanced and regular milk was measured throughout fermentation and storage. The acidification progression of the two cultures YoFlex® Premium 4.0 and ABT-100 during production and post-acidification is shown in Figure 11. Both cultures showed similar acidification in both sweetness-enhanced and regular milk.

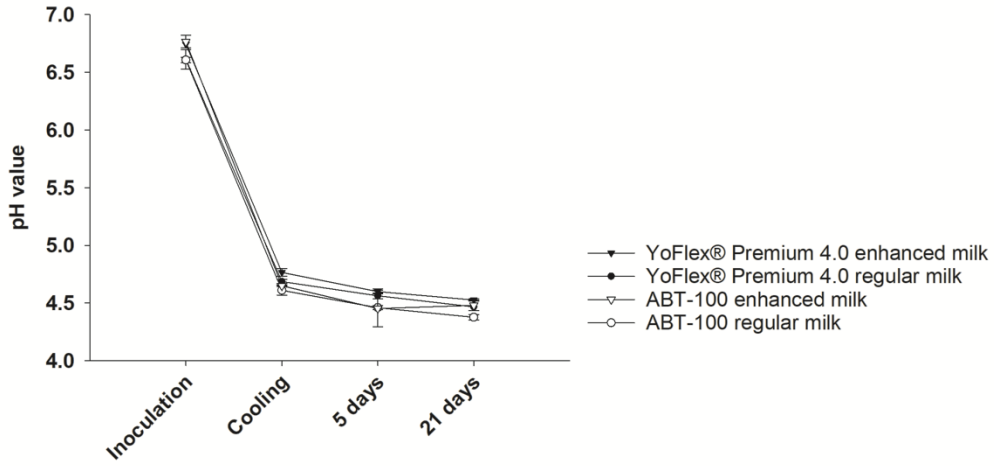

Figure 11: Milk acidification during fermentation and storage of yoghurt produced with YoFlex® Premium 4.0 and ABT-100 cultures using sweetness-enhanced and regular milk. The arithmetic mean (n = 3 biological replicates) ± the standard error of the mean are shown.

4.1.6 Microbial ecology of sweetness-enhanced yoghurt

The microbial diversity of yoghurt produced in the MRI pilot plant production experiments using YoFlex® Premium 4.0 and ABT-100 cultures in sweetness-enhanced and regular milk was initially investigated qualitatively by means of DGGE. The resulting gel images showed a unique molecular fingerprint specific for the mol % GC content of the 16S rDNA PCR product of each bacterium and could therefore be used to assess the microbial diversity of bacteria in yoghurt samples at different stages of fermentation by comparing the amplicons of samples with the amplicons from the individual starter culture isolates (see chapter 4.1.2). An exemplary fingerprint profile for yoghurt produced with the traditional yoghurt culture YoFlex® Premium 4.0 is shown in Figure 12. The microbial diversity of samples at inoculation differed visibly from samples taken after fermentation (at the time point of cooling) and during storage, when starter culture bacteria dominated the samples. At inoculation, distinct bands that did not match those of the starter culture isolates were visible, indicating a higher microbial diversity in the inoculated milk prior to proliferation of the starter culture bacteria during the fermentation.

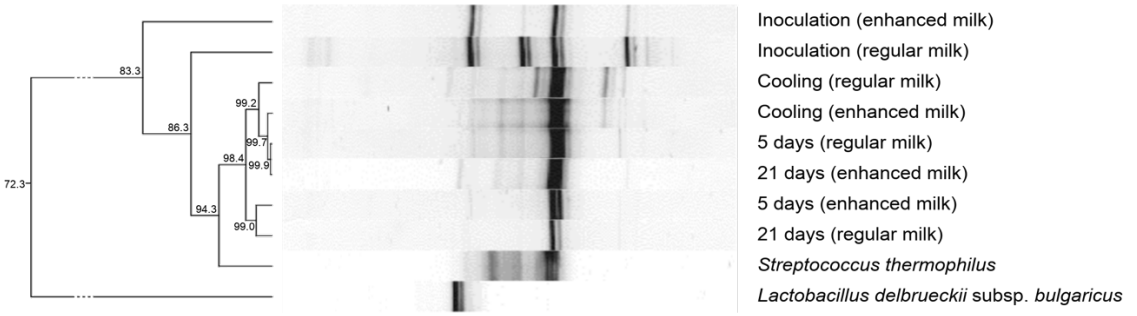

Figure 12: Molecular fingerprints of 16S rRNA gene amplicons derived from the complete bacterial population in yoghurt samples produced with YoFlex® Premium 4.0 in sweetness-enhanced and regular milk throughout fermentation and storage. A representative DGGE with a 35 % / 70 % gradient is shown, where samples are presented alongside amplicons from starter culture isolates. The dendrogram was calculated using BioNumerics and gel images were optimised using GIMP.

A representative DGGE image of the microbial diversity in yoghurt produced with the ABT-100 culture in sweetness-enhanced and regular milk is shown in Figure 13. Similar to the YoFlex® Premium 4.0 culture, a higher microbial diversity was observed in the pasteurised milk at inoculation compared to the sampling time points of cooling, and after five and 21 days of storage at 4 °C. This suggests that the starter culture strains became dominant in the yoghurt samples during fermentation.

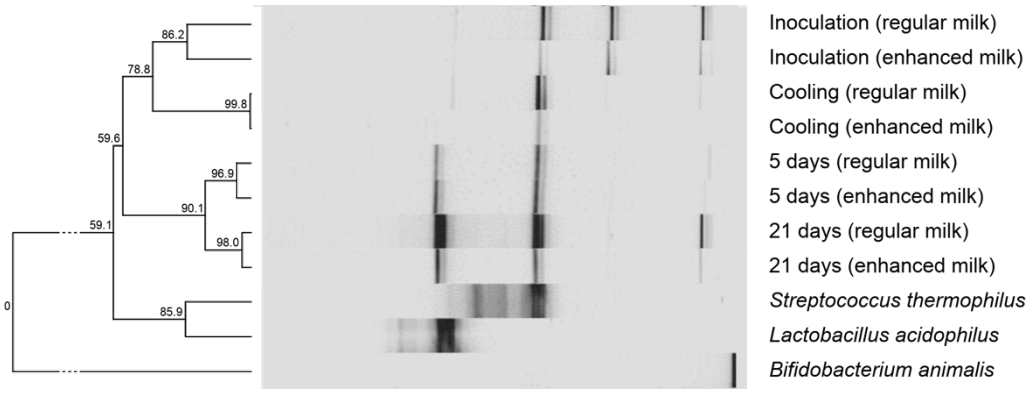

Figure 13: Molecular fingerprints of 16S rRNA gene amplicons derived from the complete bacterial population in yoghurt samples produced with ABT-100 in sweetness-enhanced and regular milk throughout fermentation and storage. A representative DGGE with a 35 % / 70 % gradient is shown, where samples are presented alongside amplicons from starter culture isolates. The dendrogram was calculated using BioNumerics and gel images were optimised using GIMP.

After gaining an initial indication of the microbial diversity in yoghurt produced with regular and sweetness-enhanced milk with the help of DGGE, a 16S rRNA gene high throughput sequencing approach was applied to analyse the microbial biodiversity in these samples. Raw sequence data from 16S rRNA gene sequencing were processed using the IMNGS platform and analysed using a set of R scripts combined in the Rhea pipeline for subsequent downstream analysis of OTUs.[193,199] Both the YoFlex® Premium 4.0 and ABT-100 yoghurt samples were sequenced in a single sequencing run and a combined normalisation step for all samples was performed to account for differences in sequencing depth between samples prior to further analyses of the 16S rRNA gene sequencing data. The rarefaction curves generated during normalisation of the samples were used to assess the sequencing depth for each sample and are shown in Figure 14. The number of reads reached a plateau for the majority of samples, demonstrating that these samples were sequenced with sufficient sequencing depth (Figure 14a). However, further sequencing may have extended the knowledge of sample diversity for the five most under-sampled cases of all 78 samples sequenced, as the number of reads did not reach a plateau for these samples (Figure 14b).

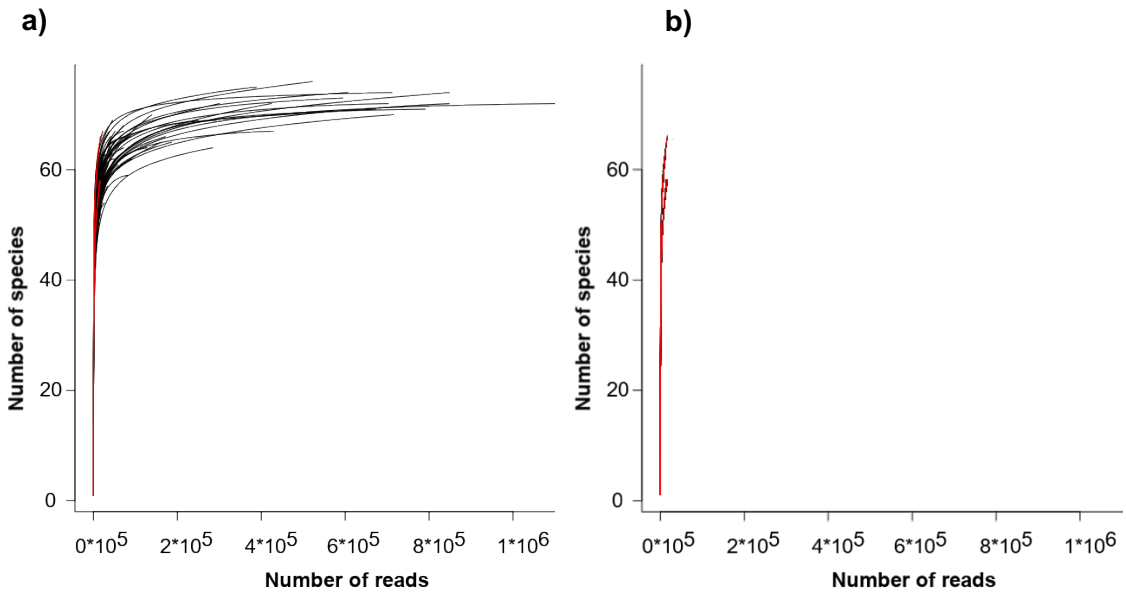

Figure 14: Rarefaction curves of a) all samples and b) the five most under-sampled cases, generated to assess sequencing depth for pilot plant yoghurt samples.

After normalisation, all subsequent analyses of microbial ecology were performed separately on the datasets for each individual culture. The range of OTUs in a sample, also known as the alpha-diversity, was calculated for yoghurt fermented with both the YoFlex® Premium 4.0 and the ABT-100 multiple-species starter cultures. The species richness, as well as the Shannon diversity index (shown as its counterpart, the effective number of species) for both cultures are presented in Table 10. The species richness shows the number of individual species and displayed some variance between yoghurt samples produced with regular and sweetness-enhanced milk. In the YoFlex® Premium 4.0 culture, the species richness was higher at inoculation than when the samples were cooled to 4 °C after fermentation for both types of milk. At cooling there was roughly 15 % difference in richness between regular and sweetness-enhanced milk in this culture, with richness further decreasing and reaching similar amounts in both milk types during storage. This difference was not as marked between regular and sweetness-enhanced milk for the ABT-100 culture. The Shannon effective values were used to compare the number of equally common species in each sample and, as expected, showed higher species diversity at inoculation than after fermentation and during subsequent storage of samples at 4 °C for both cultures.

Table 10: Alpha-diversity of yoghurt samples produced with the YoFlex® Premium 4.0 and ABT-100 cultures in regular and sweetness-enhanced milk

Conditions		**YoFlex® Premium 4.0**		**ABT-100**	
Time point	Milk	Richness	Shannon*	Richness	Shannon*
Inoculation	Regular	57.33 ± 2.60	11.66 ± 0.72	56.33 ± 0.88	9.78 ± 0.31
	Enhanced	62.00 ± 0.58	11.22 ± 0.60	58.33 ± 2.73	9.62 ± 0.68
Cooling	Regular	50.33 ± 1.20	7.15 ± 1.06	57.00 ± 1.00	6.89 ± 0.74
	Enhanced	60.33 ± 2.85	7.22 ± 0.84	60.67 ± 0.88	7.06 ± 0.17
5 days	Regular	54.67 ± 0.33	7.39 ± 0.56	53.67 ± 2.19	8.50 ± 0.14
	Enhanced	53.33 ± 1.86	6.41 ± 1.03	54.00 ± 3.06	7.77 ± 0.67
21 days	Regular	54.33 ± 1.20	7.32 ± 0.44	51.33 ± 1.20	8.12 ± 0.38
	Enhanced	55.33 ± 2.03	6.87 ± 0.30	49.00 ± 2.45	7.50 ± 0.46

* Shannon effective; n = 3 biological replicates ± the standard error of the mean

The beta-diversity, an indication of the similarity between microbial profiles, was calculated based on normalised OTU tables for YoFlex® Premium 4.0 and ABT-100 cultures. This was done to compare the bacterial abundance between yoghurt samples produced with sweetness-enhanced and regular milk. Relative abundances were calculated at genus level and a cut-off was set at 0.05 %, under which abundances are not shown. The relative microbial abundance of samples taken at different times throughout fermentation with the YoFlex® Premium 4.0 culture and subsequent storage at 4 °C for a total of 21 days is shown in Figure 15. The microbial diversity in pasteurised milk just after inoculation with starter cultures showed a higher diversity in both regular and enzyme-modified milk than after fermentation (at the time points of cooling, five days and 21 days). Following fermentation, the starter culture *Streptococcus* (81 – 85 %) dominated the yoghurt samples. Starter culture *Lactobacillus* were present at 2 – 7 %. The higher abundance of *Streptococcus* coincides with the inoculation ratio and growth patterns of both starter culture strains, as shown in Figure 10a.

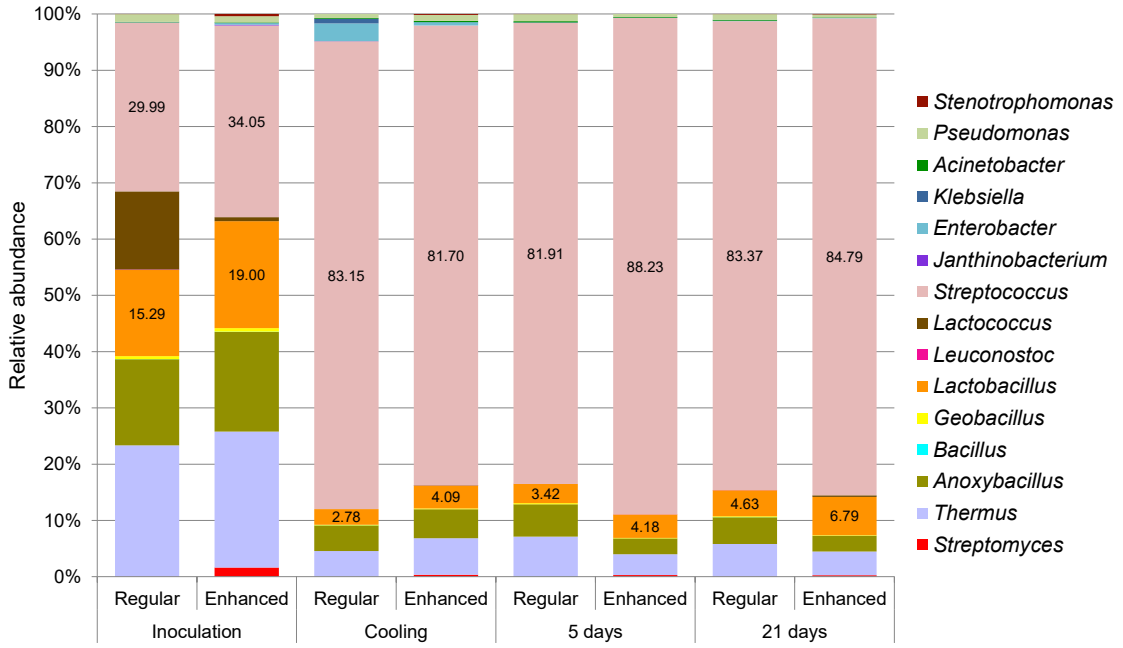

Figure 15: Relative bacterial abundance in yoghurt produced with the YoFlex® Premium 4.0 starter culture in regular and sweetness-enhanced milk. An abundance cut-off was set at 0.05 % and the relative abundance of the starter culture genera (*Streptococcus* and *Lactobacillus*) are indicated numerically. n = 3 biological replicates.

To further visualise the beta-diversity between yoghurt produced with the YoFlex® Premium 4.0 culture in regular and sweetness-enhanced milk, and to see whether differences in diversity between sample groups were significant, MDS plots were calculated and are shown in Figure 16. In the YoFlex® Premium 4.0 culture, no significant difference ($p = 0.401$) was calculated between the microbial ecology of regular and sweetness-enhanced yoghurt samples. However, the microbial diversity changed significantly ($p = 0.001$) between the time point of inoculation and all subsequent samples. There was no significant difference between samples taken at cooling, after five and after 21 days of storage at 4 °C.

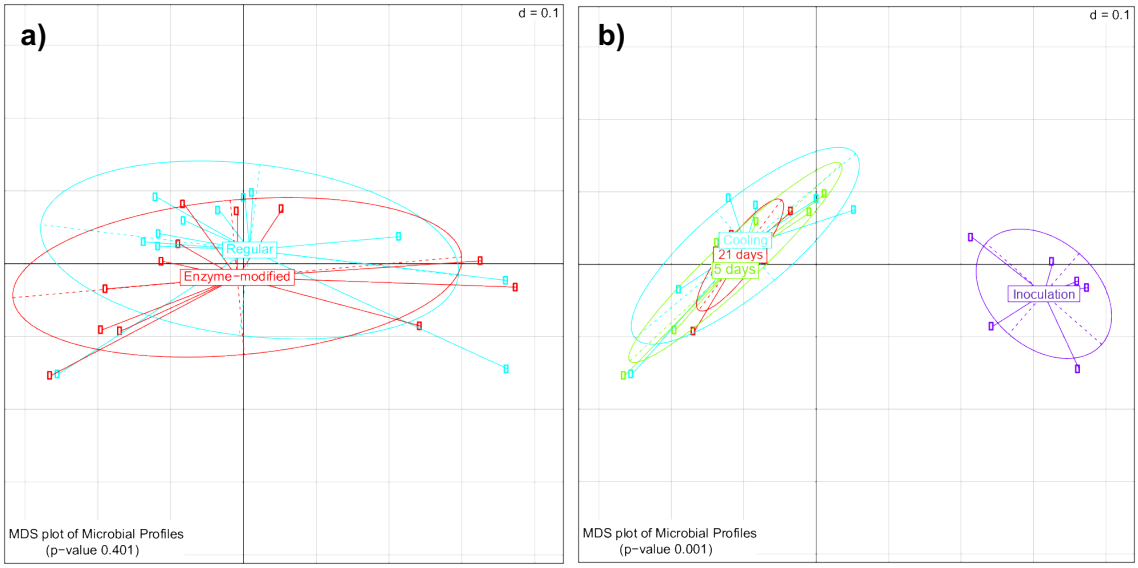

Figure 16: Multidimensional scaling plots showing a) the microbial beta-diversity of yoghurt produced with YoFlex® Premium 4.0 culture in regular and sweetness-enhanced milk and b) the microbial beta-diversity of this yoghurt at different time points during fermentation and storage. d = dissimilarity scale of the grid (d = 0.1 means the distance between two grid lines represents 10 % dissimilarity between samples). n = 3 biological replicates.

Beta-diversity analyses were also performed on samples of yoghurt produced with the probiotic ABT-100 culture taken at different time points throughout fermentation. These results are presented in Figure 17 and showed that the diversity of microorganisms at the time point of inoculation was greater than after fermentation (at cooling and after five and 21 days of storage), when *Streptococcus* species dominated the samples. After five and 21 days of storage at 4 °C, the abundance of *Lactobacillus* and *Bifidobacterium* species from the starter cultures increased and balanced out the initial *Streptococcus* dominance. This increase in abundance of *Lactobacillus* and *Bifidobacterium* species did not parallel culture-dependent bacterial growth curves generated using selective agar, which dropped towards the end of the storage period (Figure 10b).

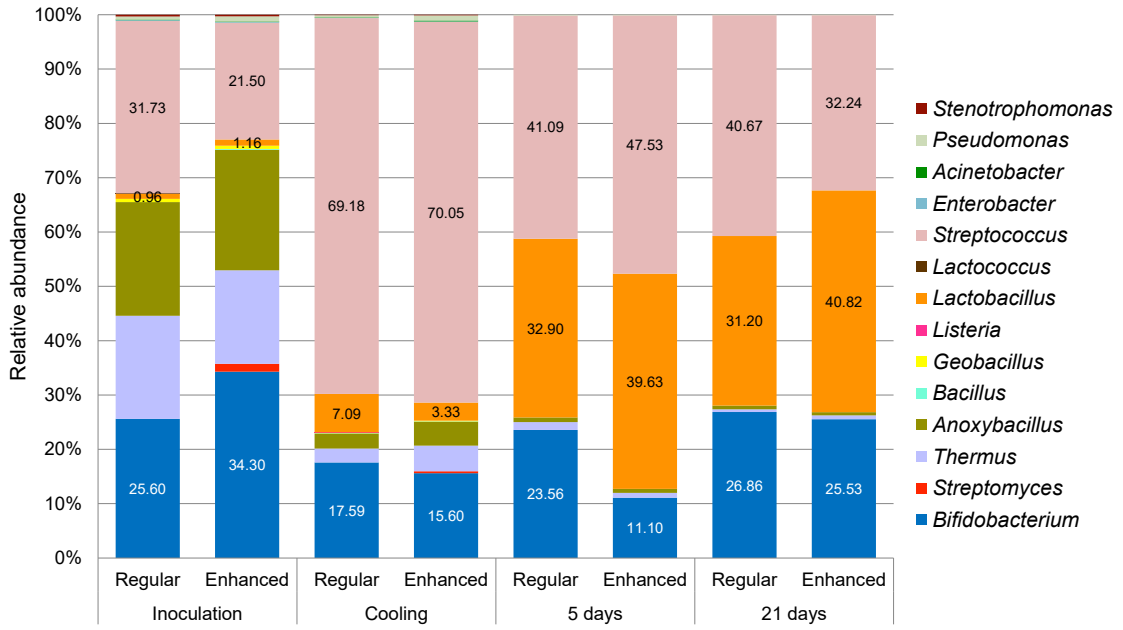

Figure 17: Relative bacterial abundance in yoghurt produced with the ABT-100 starter culture in regular and sweetness-enhanced milk. An abundance cut-off was set at 0.05 % and the relative abundance of the starter culture genera (*Streptococcus*, *Lactobacillus* and *Bifidobacterium*) are indicated numerically. n = 3 biological replicates.

To visualise beta-diversity between yoghurt produced with the ABT-100 culture in sweetness-enhanced milk compared to regular milk, MDS plots were calculated and are shown in Figure 18. No significant difference ($p = 0.657$) was calculated between the microbial ecology of regular and sweetness-enhanced yoghurt samples produced with the ABT-100 culture. However, a significant difference ($p = 0.001$) in microbial diversity was calculated between the sampling time points of inoculation, cooling and both samples taken during storage at 4 °C. There was no difference in microbial diversity between samples taken after five and 21 days of storage at 4 °C.

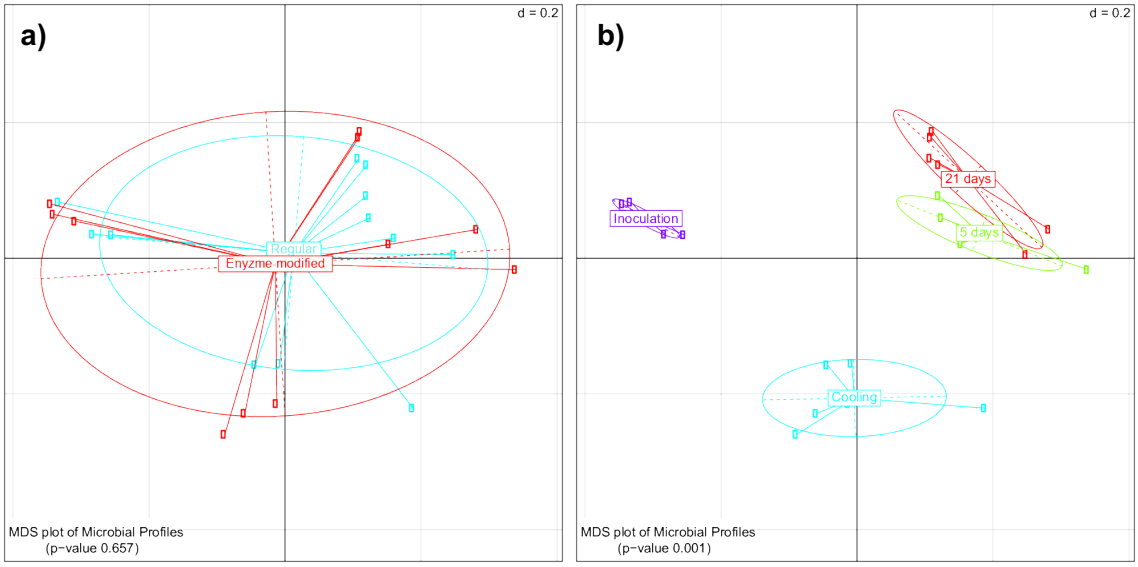

Figure 18: Multidimensional scaling plots showing a) the microbial beta-diversity of yoghurt produced with the ABT-100 culture in sweetness-enhanced milk and regular milk and b) the microbial beta-diversity of this yoghurt at different time points during fermentation and storage. d = dissimilarity scale of the grid (d = 0.2 means the distance between two grid lines represents 20 % dissimilarity between samples). n = 3 biological replicates.

4.2 Cheese

In the following chapter, results of the study to reduce the sodium content of Edam cheese will be presented, beginning with an outline of the technological workflow as well as chemical and physical parameters measured throughout fermentation and ripening, followed by a detailed exploration of the influence of the chosen reformulation strategies on the microbial ecology. Cheese production and collection of technological data were carried out in collaboration with the Department of Quality and Safety of Milk and Fish Products at the MRI in Kiel. Parts of this chapter were accepted for publication in the *International Journal of Dairy Technology* in November 2019.[‡]

4.2.1 Production of sodium-reduced Edam cheese

Edam cheese with < 0.4 % Na content was successfully produced by following the cheese production process outlined in Figure 2. The two reformulation approaches, simple NaCl reduction and substitution with the mineral salt mixture 'sub4salt®', were applied in parallel during the brining process (Table 5). A regular Edam cheese was also produced alongside the reformulated samples so that direct comparisons could be made between the salt reduction approaches and a control cheese.

To evaluate possible effects of the chosen reformulation strategies on the product characteristics of sodium-reduced Edam, technological production parameters were monitored throughout manufacturing and ripening at the MRI pilot plant. The pH values of the inoculated milk during processing, as well as of cheese samples taken at three time points throughout ripening, are shown in Figure 19. Whilst a rapid decrease in pH was observed during the production process, the most substantial acidification occured between curd pressing (pH 6.4) and the first week of ripening (pH 5.6). During this time the LAB are most active in fermenting the milk matrix, causing acidification through the production of lactic acid. However, due to vacuum-wrapping of the pressed and salted curds, the pH was not continuously monitored between curd pressing and the first week of ripening. Furthermore, as *List. innocua* does not contribute greatly to acid production during manufacture and ripening of Edam, no differences in pH progression between Edam produced with and without *List. innocua* co-inoculation were observed.

[‡] Hoffmann, W., Luzzi, G., Steffens, M., Clawin-Rädecker, I., Franz, C.M.A.P. & Fritsche, J. Salt reduction in film-ripened semihard Edam cheese. *International Journal of Dairy Technology*. in print (2019). DOI: 10.1111/1471-0307.12675

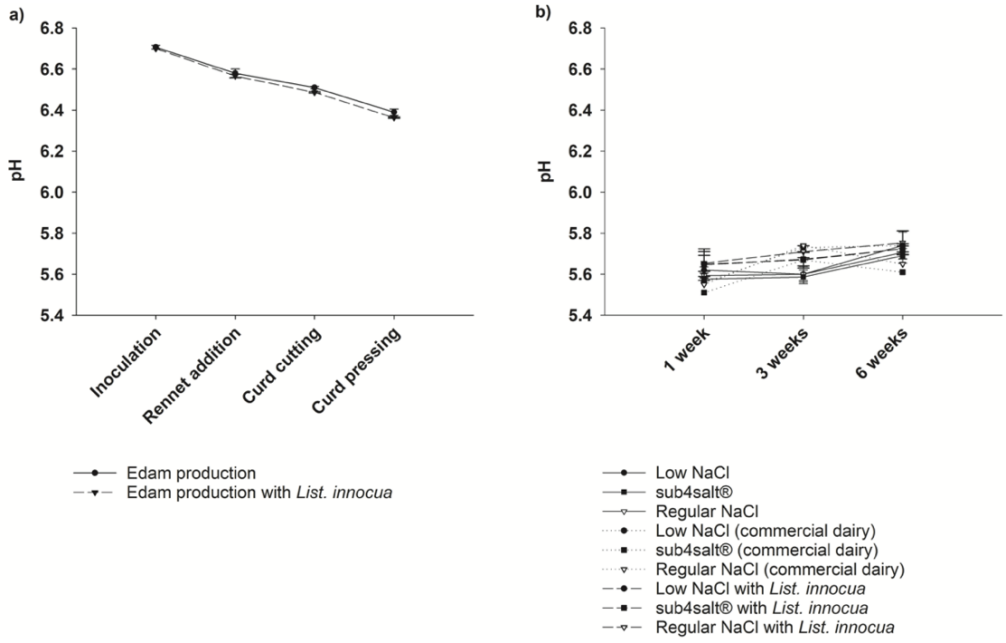

Figure 19: Matrix acidification during a) production and b) maturation of Edam produced with (n = 3 biological replicates) and without *List. innocua* co-inoculation (n = 3 biological replicates) in the MRI pilot plant, and Edam manufactured in the research facility of a commercial dairy (n = 1). Low NaCl cheeses show simple NaCl reduction, whilst sub4salt® shows cheeses produced with this mineral salt substitution product.

Chemical and physical properties of sodium-reduced Edam produced with simple NaCl reduction and mineral salt substitution strategies (Table 11) were tested after six weeks of ripening to assess whether the initial target of < 0.4 % Na content could been achieved and to monitor possible effects of the altered mineral composition on the physical properties of sodium-reduced Edam. Both sodium reduction strategies produced Edam with < 0.4 % Na content. The Na values of cheeses brined in a sub4salt® displayed the lowest sodium values; however, as expected, they also showed the highest potassium values due to the composition of this sodium substitution product (24 % Na, 11 % K, 45 % Cl; see Table 5). Potassium values measured in the low NaCl cheese that was produced via the simple reduction strategy, were similar to those in regular NaCl Edam, suggesting that in comparison to mineral salt substitution, the simple salt reduction strategy produced a cheese most similar to the regular Edam from a salt composition point of view. The NaCl values for sub4salt® cheeses could not be measured as the Erbacher method used for NaCl determination calculates NaCl values based on measurement of chloride ions and the sub4salt® product contains chloride in the form of KCl as well as NaCl (see chapter 3.2.3.1). The protein content of all cheeses was > 3.51 % and the fat content was > 2.68 %. This resulted in a dry matter content of greater than 54 % for all cheeses, exceeding the set value for the minimum dry matter in a German standard Edam cheese with 40 % fat in dry matter.[208]

Table 11: Chemical and physical properties of sodium-reduced Edam after six weeks of ripening

Property (%)	Cheese variation	Low NaCl	sub4salt®	Regular NaCl
NaCl	Edam	0.85 ± 0.07	ND	1.23 ± 0.06
	Edam with *List. innocua*	0.77 ± 0.04	ND	1.19 ± 0.04
	Edam (commercial dairy)	0.84	ND	1.44
Na	Edam	0.29 ± 0.03	0.21 ± 0.00	0.43 ± 0.02
	Edam with *List. innocua*	0.28 ± 0.02	0.23 ± 0.01	0.45 ± 0.03
	Edam (commercial dairy)	0.33	0.29	0.56
K	Edam	0.06 ± 0.00	0.16 ± 0.00	0.06 ± 0.00
	Edam with *List. innocua*	0.06 ± 0.00	0.16 ± 0.00	0.06 ± 0.00
	Edam (commercial dairy)	0.08	0.19	0.07
Dry matter	Edam	55.43 ± 0.11	56.06 ± 0.13	55.77 ± 0.27
	Edam with *List. innocua*	56.84 ± 0.14	56.77 ± 0.25	56.56 ± 0.06
	Edam (commercial dairy)	55.79	54.16	57.56
Fat in dry matter	Edam	43.59 ± 0.52	43.41 ± 0.40	43.03 ± 0.41
	Edam with *List. innocua*	43.69 ± 0.50	43.74 ± 0.47	43.61 ± 0.29
	Edam (commercial dairy)	43.0	43.4	41.7

ND = not done; n = 3 biological replicates ± standard error of the mean for Edam and Edam with *List. innocua*; n = 1 for Edam (commercial dairy)

4.2.2 Microbiology and biochemistry of sodium-reduced Edam

The production and ripening of sodium-reduced Edam was accompanied by microbiological testing using culture-dependent techniques. The selective agar and culturing conditions specific for each starter culture strain contained in the mixed starter cultures are outlined in Table 4. Samples were taken from the pasteurised milk directly after inoculation with the starter cultures to determine inoculation levels, from the curd directly before pressing and from the core of cheese loaves after one, three and six weeks of foil-ripening at 13 °C to monitor the progression of SLAB, acid production and proteolysis.

The bacterial counts of SLAB and adjunct cultures during production and ripening of sodium-reduced Edam are shown in Figure 20 and followed an expected development throughout fermentation as documented in previous studies.[134,137,171] The bacterial count

of *Lactococcus* species increased rapidly from the inoculation levels of approx. 5×10^7 cfu/mL to 5×10^8 cfu/g in the curd, further increasing to approx. 1×10^9 cfu/g after one week before decreasing to approx. 5×10^8 cfu/g and 3×10^7 cfu/g at three and six weeks, respectively. The number of *Leuconostoc* species increased slightly from approx. 1×10^5 cfu/mL at inoculation to approx. 3×10^5 cfu/g in the curd, before rising to and remaining between approx. 5×10^6 cfu/g and 1×10^7 cfu/g for the remainder of the fermentation process. Finally, *Lactobacillus* species counts developed from 6×10^6 cfu/mL at inoculation to approx. 2×10^7 cfu/g in the curd before reaching, and remaining at, approx. 1×10^8 cfu/g from week one through to week six of ripening. All brine conditions showed similar bacterial growth progressions (Figure 20a).

In addition, bacterial counts for *Lactococcus*, *Lactobacillus* and *Leuconostoc* species showed a similar progression independent of co-inoculation with *List. innocua* (Figure 20b). The *Listeria* species counts showed approx. 3×10^5 cfu/mL at inoculation, rising to approx. 8×10^5 cfu/g in the curd, peaking around approx. 1×10^6 cfu/g after one and three weeks, before decreasing slightly to approx. 7×10^5 cfu/g after six weeks. The presence of *List. innocua* did not affect SLAB growth compared to the control cheese and the reduced sodium conditions did not affect bacterial growth patterns of *List. innocua*.

Finally, growth of starter cultures implemented in the scaled-up production of sodium-reduced Edam in the research facility of a commercial dairy was monitored during ripening and is shown in Figure 20c. Due to processing restrictions, bacterial counts during processing could not be monitored in these productions. *Lactococcus* and *Lactobacillus* species counts showed similar trends to those observed in the MRI pilot plant productions. *Lactococcus* species were counted between 1×10^9 cfu/g and 4×10^9 cfu/g after one week and dropped by about 1 log cfu/g by the end of ripening at six weeks. *Lactobacillus* species remained at 1×10^8 cfu/g from week one to week six of ripening, same as was observed at the MRI pilot plant. Finally, *Leuconostoc* species showed a counts of between 6×10^5 cfu/g and 1×10^7 cfu/g in week one to between 7×10^5 cfu/g and 1×10^8 cfu/g in week six.

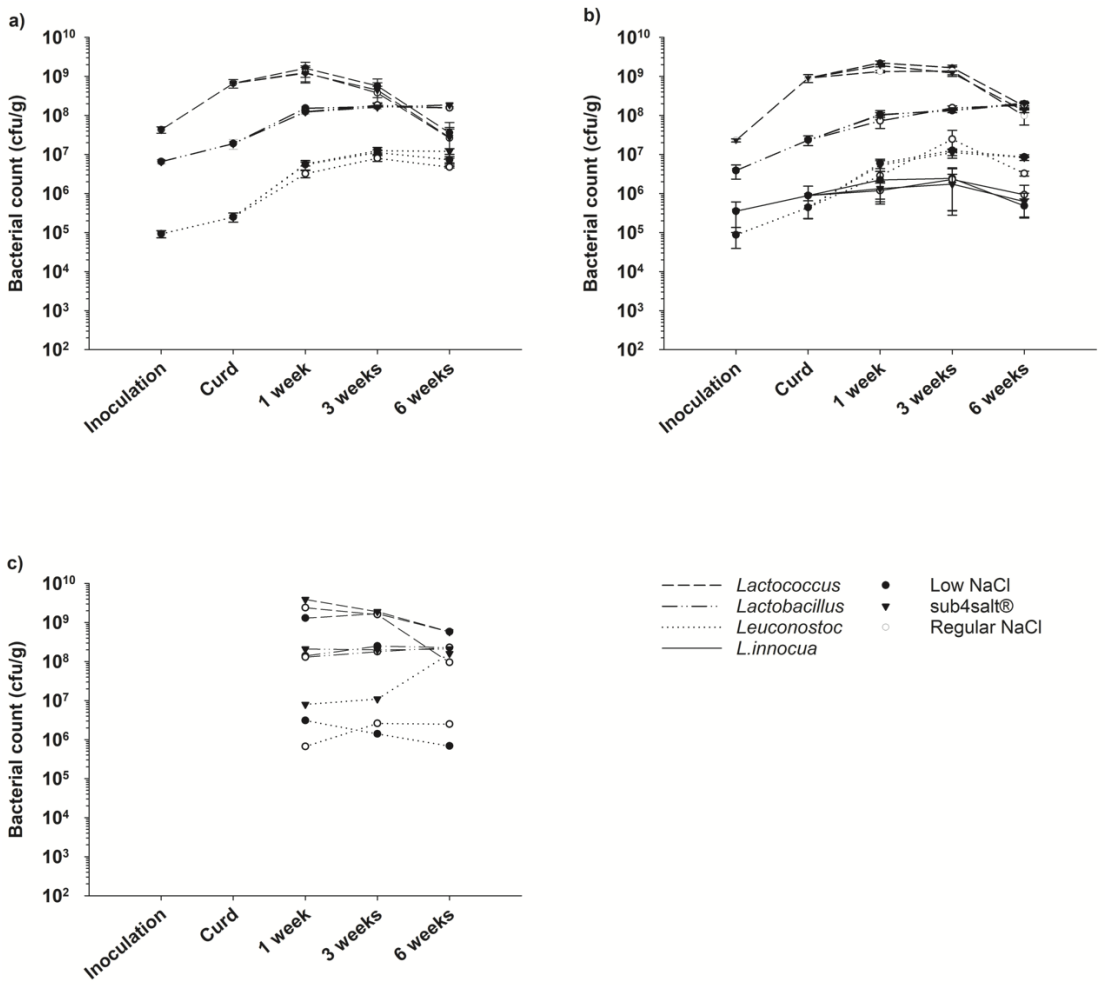

Figure 20[§]: Bacterial counts of starter cultures during production and ripening of sodium-reduced Edam manufactured in the MRI pilot plant a) without and b) with co-inoculation with *List. innocua*, as well as c) manufactured in the research lab of a commercial dairy. Low NaCl cheese shows a simple sodium reduction strategy, whilst sub4salt® shows a mineral salt substitution approach. The MRI data show mean values (n = 3 biological replicates) ± standard error of the mean, and the commercial dairy data document a single production (n = 1).

As a_w can aid the estimation of the growth potential of spoilage or opportunistically pathogenic bacteria, this factor was monitored throughout fermentation and ripening (Figure 21). After six weeks of ripening, a_w values showed comparable levels in cheeses without (Figure 21a) and with *List. innocua* co-inoculation (Figure 21b), despite some fluctuations during earlier stages of ripening. This was observed regardless of the brine conditions and, hence, the sodium content of the cheeses (Table 11). Similar a_w values were also observed in samples ripened for six weeks at the commercial dairy lab (Figure 21c).

[§] Figure 20 a + b were accepted for publication in the *International Journal of Dairy Technology* (Hoffmann et al., in print, 2019).

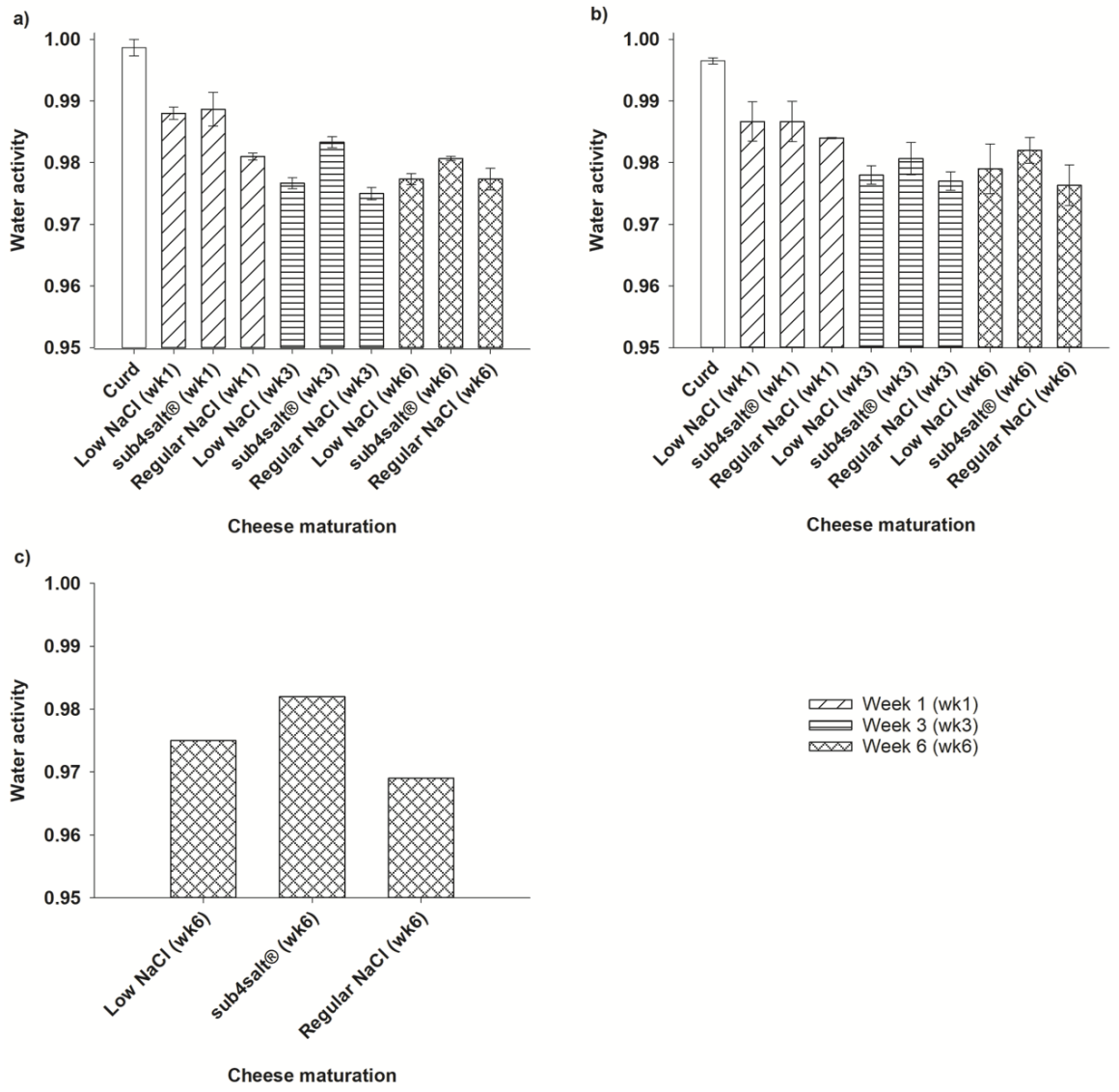

Figure 21: Water activity of sodium-reduced Edam produced in the MRI pilot plant a) without and b) with co-inoculation of *List. innocua* and c) produced in a commercial dairy research lab, measured after six weeks. Low NaCl cheese shows a simple sodium reduction strategy, whilst sub4salt® shows a mineral salt substitution approach. The MRI data show mean values (n = 3 biological replicates) ± standard error of the mean and the commercial dairy data document a single production (n = 1).

Based on a_w values, it was possible to make an initial assessment that increased growth of spoilage or opportunistically pathogenic bacteria in sodium-reduced Edam compared with regular Edam was unlikely. However, to further assess the microbiological risk of sodium reduction in Edam, these findings were complemented by counting bacterial growth of enterobacteria, enterococci, yeasts or moulds and pseudomonads after six weeks of maturation using selective agar. These bacterial counts were below the limit of detection set at 1×10^2 cfu/g for this study, regardless of the brining conditions for sodium-reduced as well as regular Edam, both with and without *List. innocua* co-inoculation. Furthermore, *List. innocua* co-inoculated cheese samples that were wrapped in

cheese paper and stored at 4 °C to mimic the behaviour of consumers at home did not show an increase in bacterial growth of *List. innocua* after one and three weeks of storage.

Monitoring acid progression during cheese production is important as acid content can influence the structure, mineral content and moisture of the final product.[209] Hence, the production of L- and D-lactic acid, as well as acetic acid, by LAB in reduced-sodium Edam was monitored throughout the six-week ripening period (Figure 22a). Whilst D-lactic acid and acetic acid were hardly produced by the LAB, 12 g/kg of L-lactic acid were produced by starter and adjunct cultures added during cheese making, a typical amount for Dutch-type cheeses such as Gouda.[210] The starter and adjunct cultures *Lc. lactis*, *Lb. paracasei* and *Lb. rhamnosus* are known to produce L-lactic acid.[65] Of the mixture of LAB, only *Lb. helveticus* is known to produce D-lactic acid in addition to L-lactic acid, whilst *Leuconostoc* species produce D-lactic acid.[79] The proportion of D-lactic acid production seems hardly to have contributed to overall acid production.

Furthermore, proteolysis was measured throughout ripening, as the proteolytic activity of LAB plays an essential role in flavour and texture development during cheese ripening through producing short peptides and amino acids. The terminal stage of proteolysis, the formation of free amino acids as a result of peptidase activity, was measured directly using a Cd-ninhydrin reagent (see chapter 3.2.3.3). As expected, a steady increase in proteolysis was observed as ripening of Edam cheese progressed (Figure 22b) and similar trends were observed for all experimental parameters relating to salt levels and type.

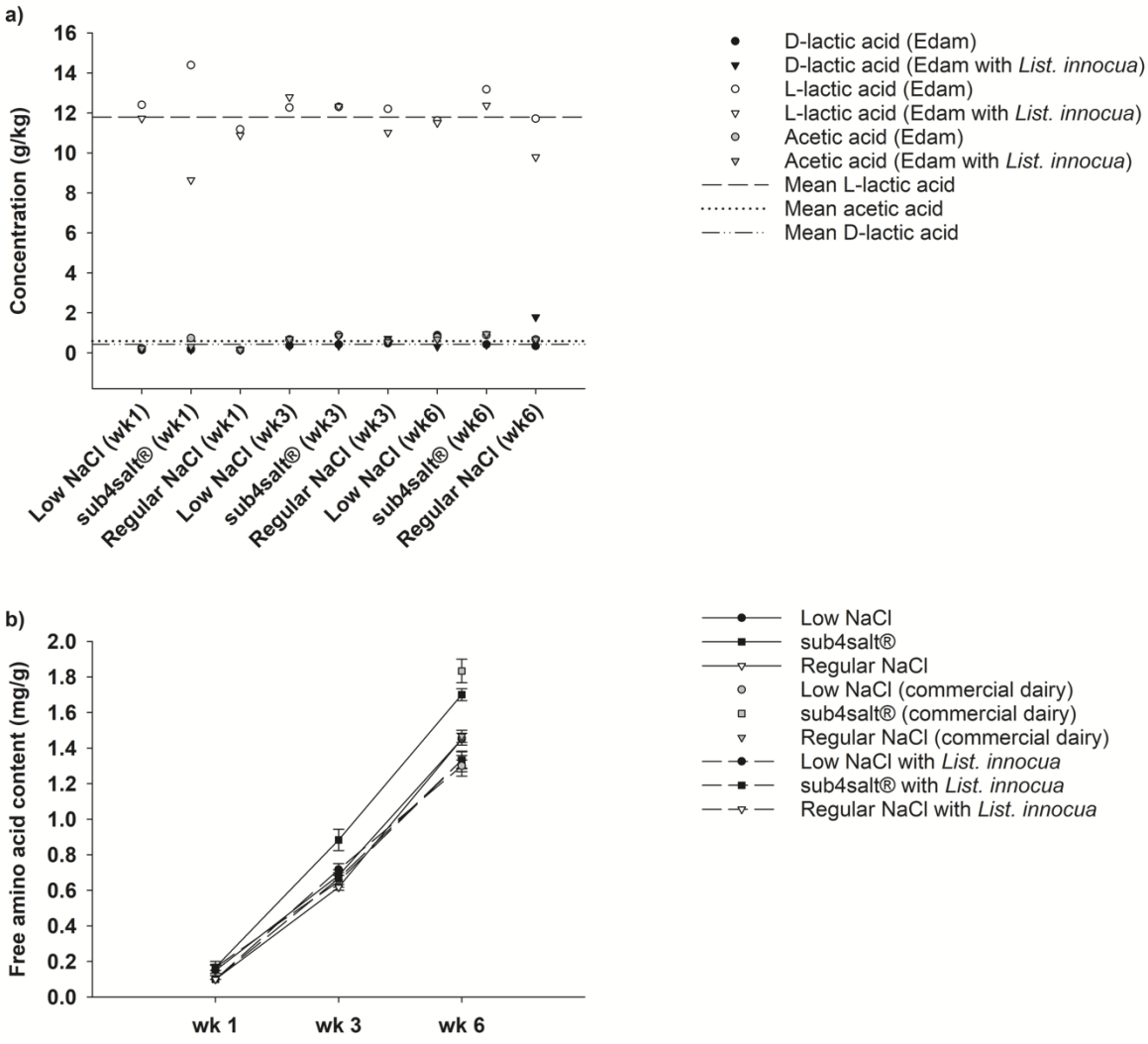

Figure 22: a) D- and L-lactic acid and acetic acid production throughout maturation of sodium-reduced Edam variations produced with and without *List. innocua* co-inoculation (n = 1) and b) free amino acid content measured during ripening (n = 3 biological replicates ± the standard error of the mean for all samples except commercial dairy samples, where n = 1). Maturation is shown in weeks (wk).

4.2.3 Isolation of starter culture strains from mixed culture

As multiple mixed starter cultures were implemented in combination for the production of Edam cheese, the individual strains contained in these starter cultures were isolated for use as reference strains for studies of microbial ecology using culture-independent methods. Strains were successfully isolated using selective agar dilution streaking, verified morphologically and microscopically, and their identity confirmed by means of Sanger sequencing of PCR amplicons of the complete 16S rRNA gene of each strain and comparing these with the NCBI 16S rRNA database using a Standard Nucleotide BLAST® and the EZ Taxon database using EzBioCloud's Identify services.[206,207] The first (closest) match of the PCR amplicons with each database is shown in Table 12. All starter culture strains specified by the manufacturer in the product details (Table 4) were successfully isolated and the majority could be identified at species level. Definite subspecies level identification was not possible due to the size of the 16S rRNA gene and thus its limited resolution for identification to subspecies level.

Table 12: Comparison of selected 16S rRNA gene amplicons of cheese starter culture strains with the NCBI 16S rRNA and EZ Taxon databases

Origin	Microbial composition acc. to Chr. Hansen	Isolate code (agar used)	Nucleotide BLAST® * (accession number)	Identity (%)	EZ Taxon ** (accession number)	Identity (%)
FLORA™ C1060	*Lc. lactis* subsp. *cremoris* *Lc. lactis* subsp. *lactis* *Lc. lactis* subsp. *lactis* biovar Diacetylactis *Leuc.* sp.	C2 (M17)	*Lc. lactis* subsp. *cremoris* (NCDO 607)	100	*Lc. lactis* subsp. *cremoris* (NCDO 607)	100
		B2 (MRS pH 5.4)	*Leuc. pseudomesenteroides* (KCTC 3652)	99.64	*Leuc.* sp. (PS12)	99.93
CR-550	*Lb.* sp. *Lc. lactis*	A1 (M17)	*Lc. lactis* (NBRC 100933)	100	*Lc. lactis* subsp. *lactis* (JCM 5805)	100
		E2 (MRS pH 5.7)	*Lb. paracasei* (R094)	100	*Lb. paracasei* subsp. *tolerans* (JCM 1171)	100
LH-32	*Lb. helveticus*	D1 (MRS pH 5.7)	*Lb. helveticus* (DSM 20075)	100	*Lb. helveticus* (DSM 20075)	100
CR-BUT-TERY01	*Lb. paracasei* *Lb. rhamnosus* *Lc. lactis* subsp. *lactis*	CR_K1 (M17)	*Lc. lactis* (NBRC 100933)	99.93	*Lc. lactis* subsp. *lactis* (JCM 5805)	99.93
		CR_K15 (MRS pH 5.7)	*Lb. paracasei* (R094)	99.93	*Lb. paracasei* subsp. *tolerans* (JCM 1171)	99.93
		NCRK2 (MRS pH 5.7)	*Lb. rhamnosus* (NBRC 3425)	100	*Lb. rhamnosus* (JCM 1136)	100

The first sequences producing significant alignments with * the NCBI 16S ribosomal RNA database using a standard nucleotide BLAST®, and ** the EZ Taxon database using EzBioCloud's Identify service are shown. Query cover/completeness was 100 % for all isolates.

4.2.4 Whole genome sequencing of starter culture isolates

The complete genomes of thirteen isolates from mixed-strain cheese starter cultures were sequenced to confirm the taxonomy of these isolates. This was done to elaborate on the taxonomic classification achieved by Sanger sequencing of PCR amplicons of the 16S rRNA gene as presented in chapter 4.2.3 and thus to obtain an exact identification. To do this, the average nucleotide identity of each strain was compared to those of type strains for the genera specified in the product data sheets of each culture provided by the manufacturer Chr. Hansen. The sequenced isolates, their mixed-strain starter culture origins, and the anticipated isolate identity based on the product specifications are shown in Table 13.

Table 13: Bacterial isolates chosen for whole genome sequencing

Isolate	**Culture of origin**	**Media and respiration conditions used for strain isolation**	**Anticipated isolate identity based on culture specifications**
A1	CR-550	M17, aerobic	*Lc. lactis*
C1	FLORA™ C1060	M17, aerobic	*Lc. lactis*
C2	FLORA™ C1060	M17, aerobic	*Lc. lactis*
C3	FLORA™ C1060	M17, aerobic	*Lc. lactis*
C4	FLORA™ C1060	M17, aerobic	*Lc. lactis*
C5	FLORA™ C1060	M17, aerobic	*Lc. lactis*
CRK1	CR-BUTTERY01	M17, aerobic	*Lc. lactis* subsp. *lactis*
D1	LH-32	MRS pH 5.7, aerobic	*Lb. helveticus*
E2	CR-550	MRS pH 5.7, aerobic	*Lactobacillus* sp.
CRK15	CR-BUTTERY01	MRS pH 5.7, aerobic	*Lb. paracasei*
NCRK2	CR-BUTTERY01	MRS pH 5.7, anaerobic	*Lb. rhamnosus*
B1	FLORA™ C1060	MRS pH 5.7, aerobic	*Leuconostoc* sp.
B2	FLORA™ C1060	MRS pH 5.7, aerobic	*Leuconostoc* sp.

Heatmaps were generated using the OAT software to visualise the relatedness of isolates and the type strains of *Lc. lactis* subspecies. A heatmap generated to visualise the taxonomy of isolated *Lactococcus* strains compared with type strains is shown in Figure 23. The species level taxonomy determined by Sanger sequencing was confirmed and subspecies level matches to type strains could be determined with > 98 % relatedness. Isolates A1 and CRK1 showed 99.99 % relatedness to each other and 98.66 % relatedness to the *Lc. lactis* subsp. *lactis* (JCM 5805^T) type strain. Samples C1 – C5 displayed at least 99.96 % relatedness to each other and 98.83 % relatedness to the *Lc. lactis* subsp. *cremoris* (ATCC 19257^T) type strain.

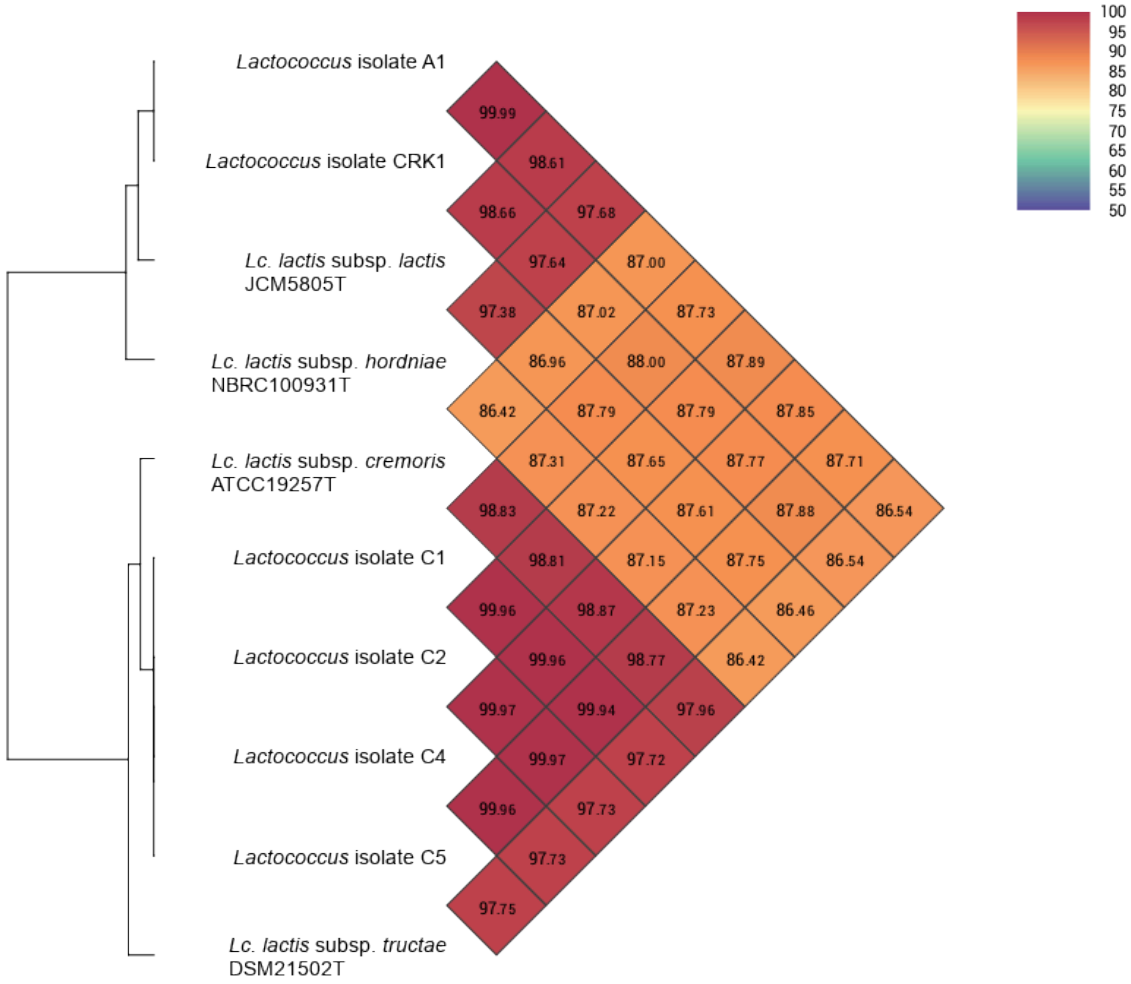

Figure 23: Heatmap showing the relatedness of *Lactococcus* isolates from mixed cheese starter cultures compared to type strains. Generated with OrthoANI values calculated by the OAT software.[204] Isolates were derived from the following mixed cheese starter cultures: A1 from CR-550, CRK1 from CR-BUTTERY01, and C1 – C5 from FLORA™ C1060.

A heatmap was also generated to visualise the relatedness of *Lactobacillus* strains isolated from mixed strain cheese starter cultures to different *Lactobacillus* type strains (Figure 24). The species level taxonomy determined by means of Sanger sequencing of the 16S rRNA gene amplicons was confirmed and further relatedness to the nearest subspecies could be determined. Isolate E2 showed 98.64 % relatedness to sample CRK15 and 98.43 % relatedness to *Lb. paracasei* subsp. *paracasei* (DSM 5622^T). Sample NCRK2 showed 99.74 % relatedness to *Lb. rhamnosus* (DSM 20021^T) and isolate D1 displayed 99.69 % identity to *Lb. helveticus* (DSM 20075^T).

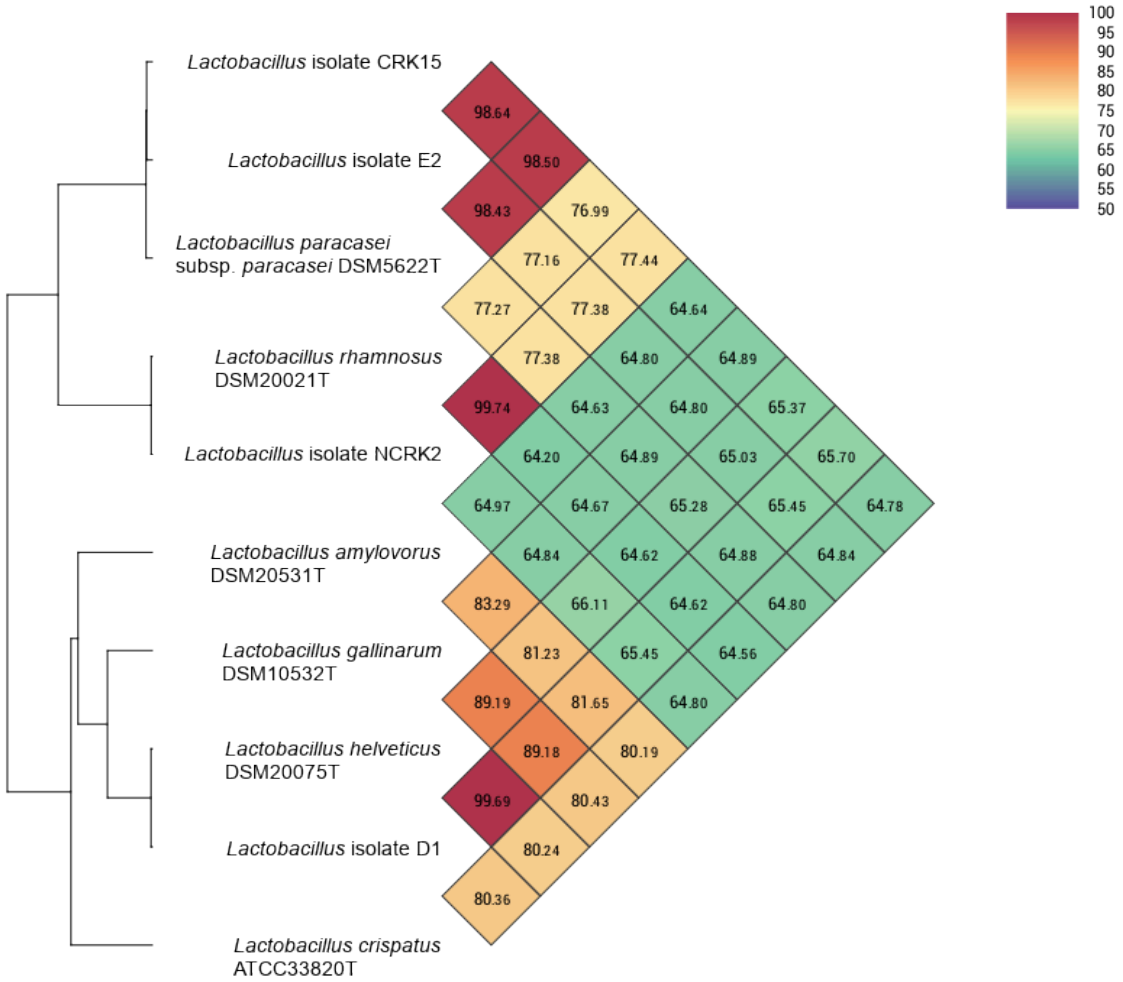

Figure 24: Heatmap showing the relatedness of *Lactobacillus* isolates from mixed cheese starter cultures compared to type strains. Generated with OrthoANI values calculated by the OAT software.[204] Isolates were derived from the following mixed cheese starter cultures: CRK15 and NCRK2 from CR-BUTTERY01, E2 from CR-550, and D1 from LH-32.

Furthermore, a heatmap was also generated for *Leuconostoc* strains isolated from mixed strain cheese starter cultures, comparing these isolates with relevant type strains (Figure 25). Isolates B1 and B2 showed 99.97 % relatedness to each other and B2 showed 92.46 % relatedness to the non-type strain *Leuc. pseudomesenteroides* (CBA 3630). This non-type strain was used to generate the heatmap as the type strain of this organism (DSM 20193) was defined based on the 16S rRNA gene sequence of this organism and not the whole genome sequence.[205]

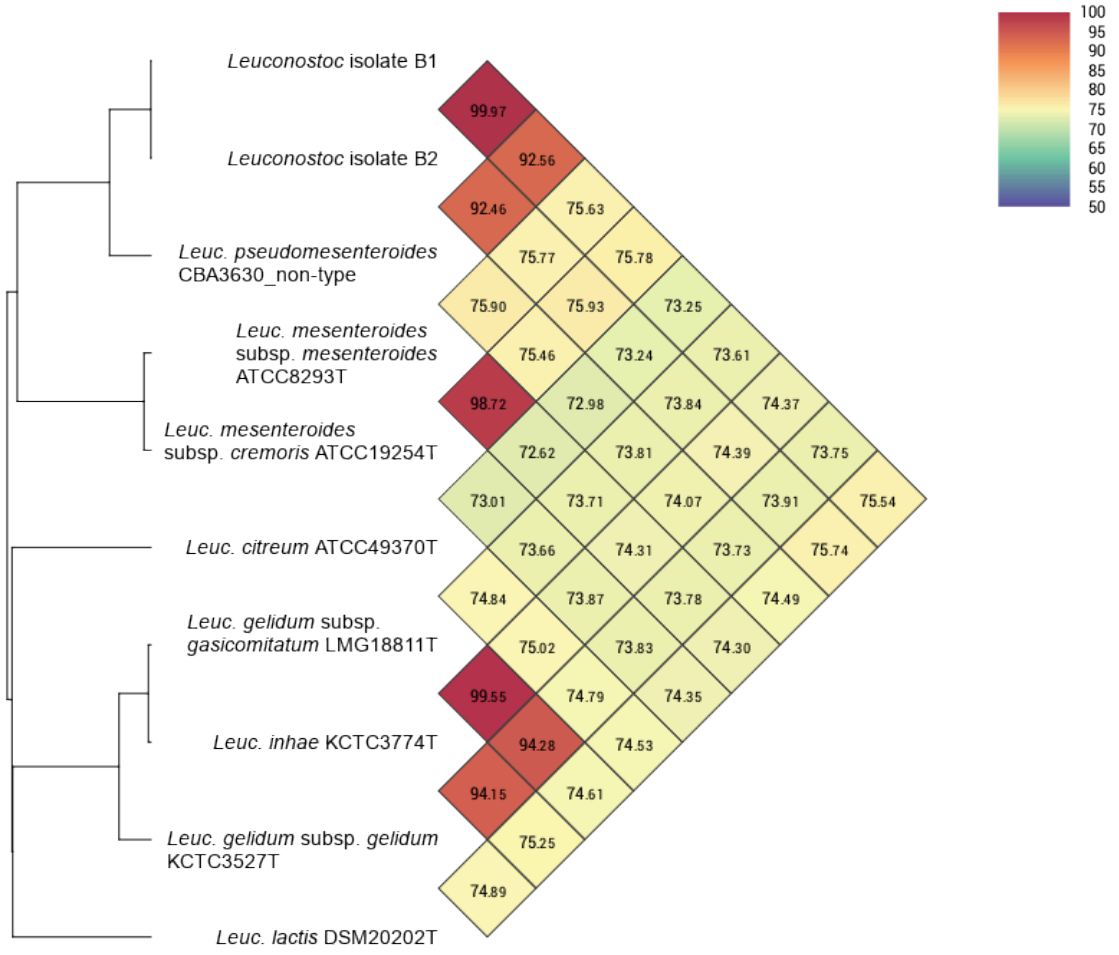

Figure 25: Heatmap showing the relatedness of *Leuconostoc* isolates from mixed cheese starter cultures compared to type strains and the non-type strain *Leuc. pseudomesenteroides* (CBA 3630). Generated with OrthoANI values calculated by the OAT software.[204] Isolates B1 and B2 were both derived from the FLORA™ C1060 culture.

4.2.5 Microbial ecology of sodium-reduced Edam cheese

The microbial ecology of sodium-reduced Edam cheese was investigated by a range of culture-independent methods. Similar to analysis of the microbial ecology of yoghurt samples, DGGEs were used to assess the microbial diversity of DNA isolated from cheese samples at different stages of fermentation. An exemplary fingerprint profile for sodium-reduced Edam production is shown in Figure 26. The microbial diversity changed visibly between the curd sample and cheese samples taken throughout fermentation and although a mixture of SLAB was present, *Lc. lactis* clearly dominated the samples.

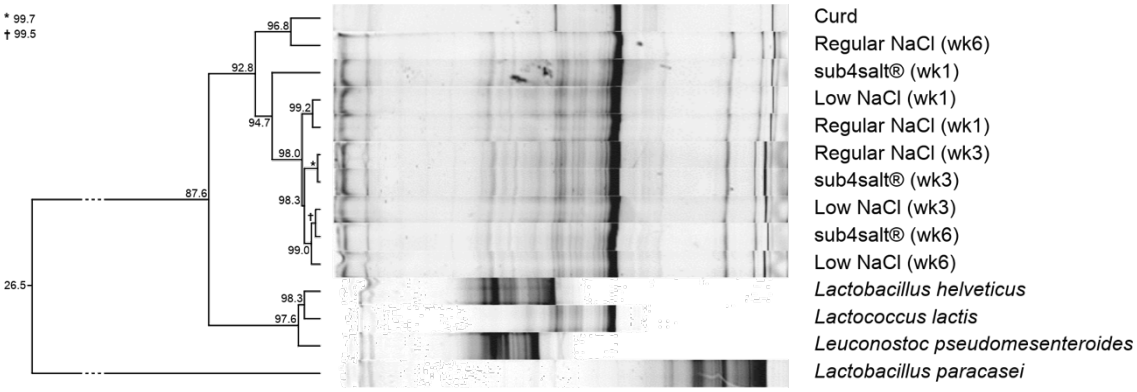

Figure 26: Molecular fingerprints of 16S rRNA gene amplicons derived from the complete bacterial population in sodium-reduced Edam cheese samples taken during production and ripening. An exemplary DGGE with a 35 % / 70 % gradient is shown, where samples are presented alongside amplicons from starter culture isolates. The dendrogram was calculated using BioNumerics and gel images were optimised using GIMP. wk = weeks.

The molecular fingerprints of samples of sodium-reduced Edam produced in the research lab of a commercial dairy are shown in Figure 27. The microbial diversity between cheese samples at different stages of ripening was very similar and was dominated by SLAB, as could be inferred by comparison with starter culture isolates.

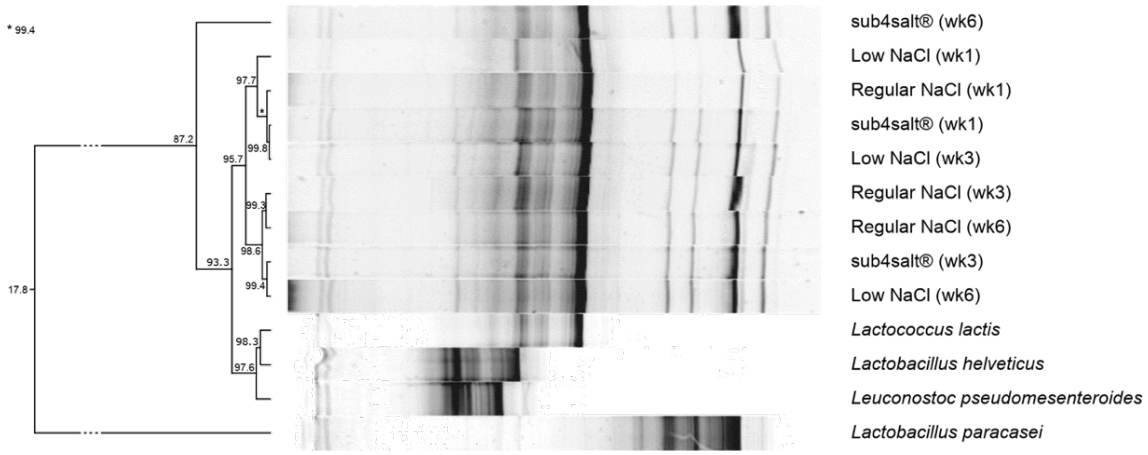

Figure 27: Molecular fingerprints of 16S rRNA gene amplicons derived from the complete bacterial population in sodium-reduced Edam cheese samples produced in the lab of a commercial cheese dairy. A DGGE with a 35 % / 70 % gradient is shown, where samples are presented alongside amplicons from starter culture isolates. The dendrogram was calculated using BioNumerics and gel images were optimised using GIMP. wk = weeks.

A similar pattern of microbial diversity could be observed in the DGGE of sodium-reduced Edam samples co-inoculated with *List. innocua* during production (Figure 28). Again, a dominance of *Lc. lactis* could be observed throughout cheese fermentation and ripening.

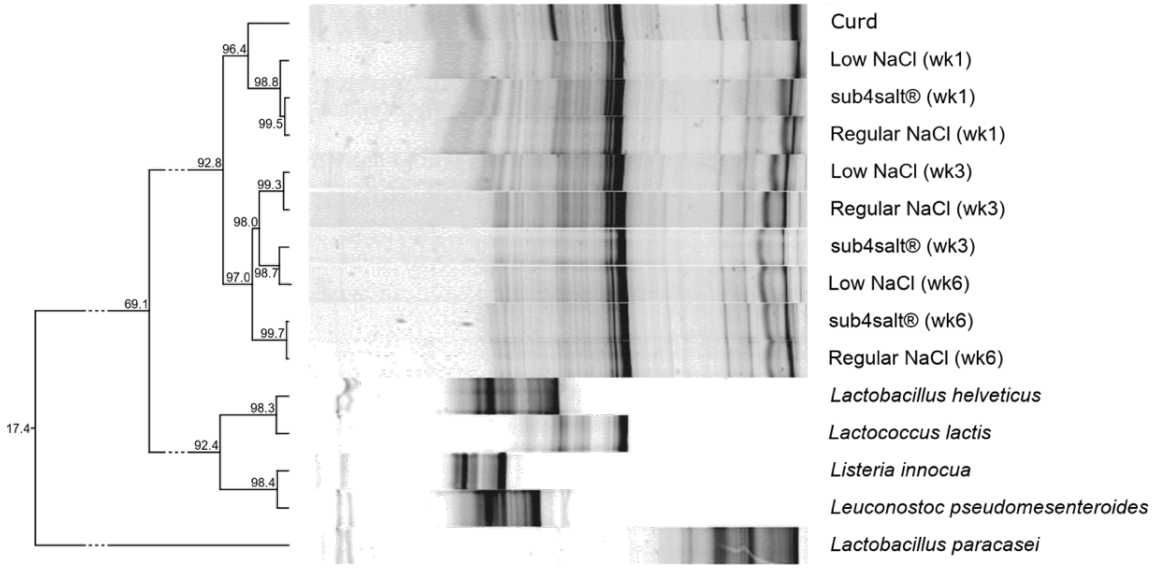

Figure 28: Molecular fingerprints of 16S rRNA gene amplicons derived from the complete bacterial population in sodium-reduced Edam cheese samples co-inoculated with *List. innocua* during production and ripening. An exemplary DGGE with a 35 % / 70 % gradient is shown, where samples are presented alongside amplicons from starter culture isolates. The dendrogram was calculated using BioNumerics and gel images were optimised using GIMP. wk = weeks.

Following the qualitative analysis of microbial diversity in sodium-reduced Edam samples using DGGE, a 16S rRNA gene high throughput sequencing approach was applied for

semi-quantitative analysis of these samples. Raw sequence data from 16S rRNA gene sequencing were processed using the IMNGS platform and analysed using a set of R scripts combined in the Rhea pipeline for subsequent downstream analysis of OTUs.[193,199] As sequencing was performed in a single sequencing experiment for all cheese experiments, these samples were normalised together. The rarefaction curves used to assess the sequencing depth for each sample are shown in Figure 29. The number of species reached a plateau for all samples, hence it could be inferred that the sequencing depth was sufficient for all samples. Further sequencing would not have extended knowledge of sample diversity.

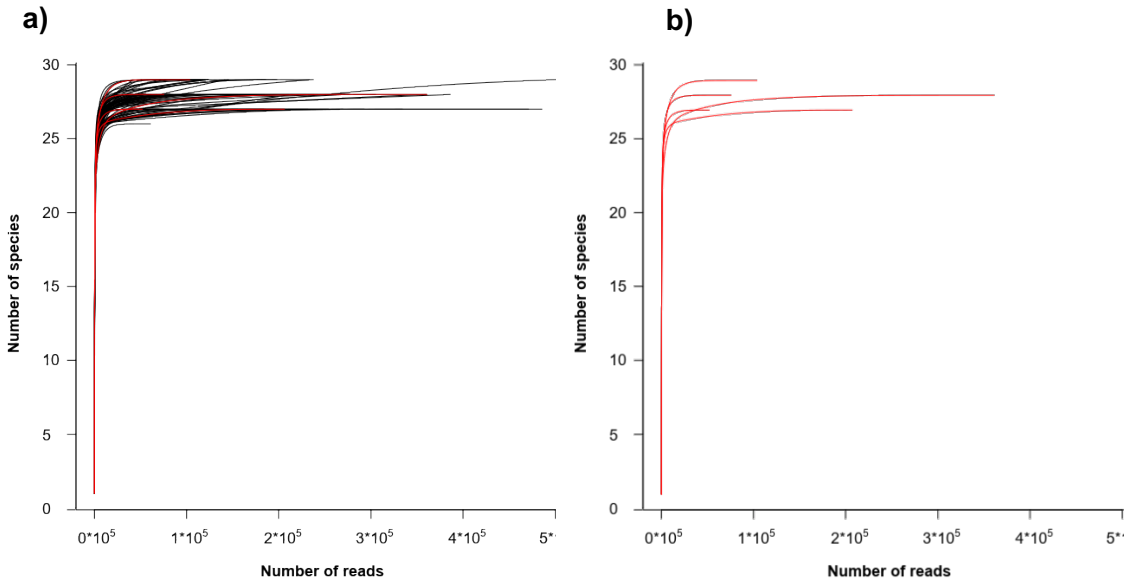

Figure 29: Rarefaction curves of a) all samples and b) the five most under-sampled cases, generated to assess sequencing depth for all sodium-reduced Edam samples prior to normalisation.

Subsequent analyses of microbial diversity in sodium-reduced Edam samples were performed on all individual experimental groups: sodium-reduced Edam produced in the MRI pilot plant, commercial dairy production, and sodium-reduced Edam produced with *List. innocua* co-inoculation in the MRI pilot plant. The range of OTUs in a sample (alpha-diversity) was calculated for each experimental group. The species richness and the Shannon diversity indices for sodium-reduced Edam produced in the MRI pilot plant are presented in Table 14. The species richness was similar for all samples and the Shannon effective values showed slightly higher species diversity at inoculation and in the curd than after one week of ripening at 13 °C. During production the starter cultures grew rapidly to ferment the milk, dominating the community composition. After three and six weeks of ripening, the Shannon effective rose again slightly as the community composition changed.

Table 14: Alpha-diversity of sodium-reduced Edam cheese samples

Sampling time	Sample type	Richness	Shannon effective
Production	Inoculated milk	26.67 ± 0.67	5.70 ± 0.16
	Curd	26.00 ± 0.00	6.05 ± 0.24
Week 1	Low NaCl	26.00 ± 0.00	5.03 ± 0.24
	sub4salt®	26.67 ± 0.33	5.00 ± 0.12
	Regular NaCl	26.67 ± 0.33	5.06 ± 0.21
Week 3	Low NaCl	27.00 ± 0.00	5.46 ± 0.28
	sub4salt®	27.33 ± 0.33	5.45 ± 0.24
	Regular NaCl	26.67 ± 0.33	5.72 ± 0.24
Week 6	Low NaCl	26.00 ± 0.00	5.68 ± 0.05
	sub4salt®	26.33 ± 0.33	5.71 ± 0.25
	Regular NaCl	26.67 ± 0.33	5.89 ± 0.11

n = 3 biological replicates ± standard error of the mean

The alpha-diversity of cheese samples produced in the lab of a commercial dairy is shown in Table 15. In this single production experiment, the Shannon effective values did not show differing species diversity between sampling time points.

Table 15: Alpha-diversity of cheese samples from a single production of sodium-reduced Edam cheese manufactured in the research lab of a commercial dairy

Sampling time	Sample type	Richness	Shannon effective
Week 1	Low NaCl	27	5.06
	sub4salt®	27	5.08
	Regular NaCl	26	4.50
Week 3	Low NaCl	28	5.64
	sub4salt®	27	5.60
	Regular NaCl	28	5.19
Week 6	Low NaCl	27	5.76
	sub4salt®	27	5.41
	Regular NaCl	28	5.10

Finally, the alpha-diversity of samples of sodium-reduced Edam produced with *List. innocua* co-inoculation in the MRI pilot plant was calculated and these values are shown in Table 16. The species richness was similar for all samples and the Shannon effective values showed higher species diversity at inoculation and in the curd than after one, three and six weeks of ripening at 13 °C.

Table 16: Alpha-diversity of sodium-reduced Edam cheese samples co-inoculated with *List. innocua*

Sampling time	Sample type	Richness	Shannon effective
Production	Inoculated milk	28.00 ± 0.58	6.11 ± 0.22
	Curd	27.67 ± 0.33	6.35 ± 0.13
Week 1	Low NaCl	27.67 ± 0.33	4.71 ± 0.43
	sub4salt®	27.00 ± 0.00	4.94 ± 0.18
	Regular NaCl	27.00 ± 0.00	5.08 ± 0.14
Week 3	Low NaCl	28.00 ± 0.00	5.25 ± 0.15
	sub4salt®	28.67 ± 0.33	5.06 ± 0.18
	Regular NaCl	28.00 ± 0.58	5.23 ± 0.10
Week 6	Low NaCl	27.67 ± 0.33	5.57 ± 0.24
	sub4salt®	27.00 ± 0.00	5.65 ± 0.13
	Regular NaCl	27.33 ± 0.33	5.89 ± 0.09

n = 3 biological replicates ± standard error of the mean

The beta-diversity, an indication of the similarity between microbial profiles, was calculated based on normalised OTU tables for all sodium-reduced Edam cheese production experiments. This was done to compare the bacterial abundance between sodium-reduced Edam samples produced with two different reformulation approaches at different stages of fermentation and ripening. Relative microbial abundances were calculated at genus level and a cut-off was set at 0.05 %, under which abundances are not shown.

The relative microbial abundance within samples of sodium-reduced Edam produced in the MRI pilot plant are shown in Figure 30. Throughout production and ripening of sodium-reduced Edam, the *Lactococcus* (58 – 72 %) and *Lactobacillus* (36 – 41 %) species of the starter culture dominated the microbial ecology and the relative abundance of the adjunct *Leuconostoc* species was beneath 0.5 %. As not all microorganisms have been classified to date, < 0.2 % of bacteria present within the samples remained unclassified. When comparing the salt reduction strategies of simple NaCl reduction and mineral salt substitution (sub4salt®) with regular NaCl levels in the control Edam, the starter culture ratios remained similar.

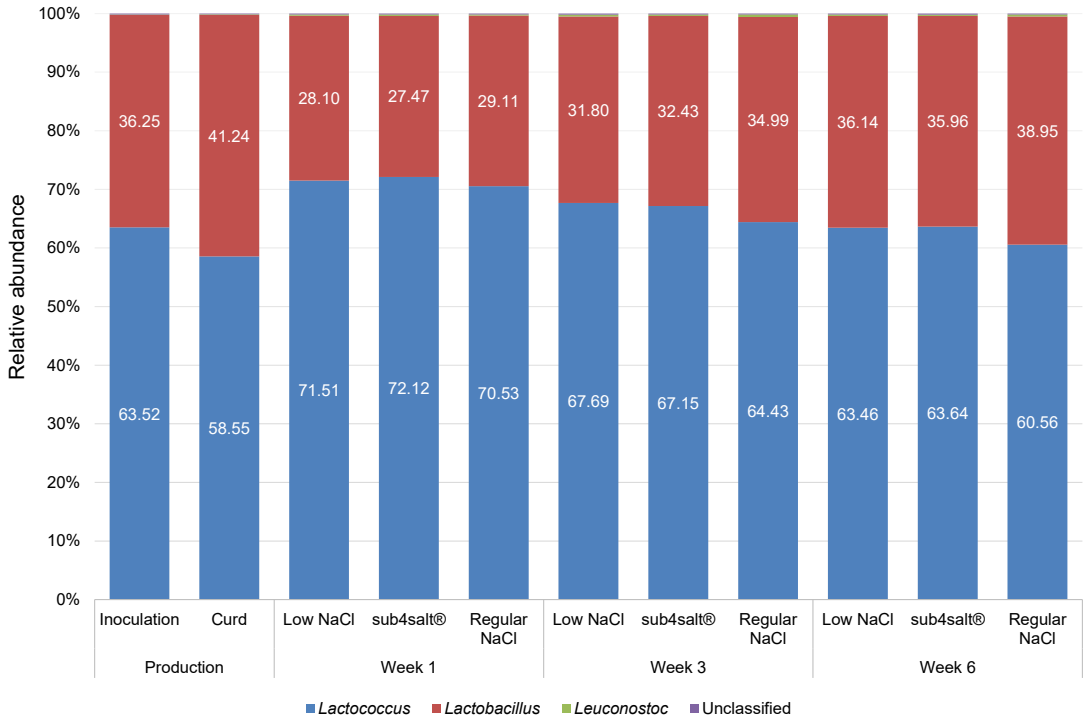

Figure 30: Relative bacterial abundance in sodium-reduced Edam cheese. An abundance cut-off was set at 0.05 %. The relative abundance of *Leuconostoc* species and unclassified bacteria remain under 0.5 % throughout production and ripening. n = 3 biological replicates.

To further visualise beta-diversity between cheese sampling time points as well as between varying brine compositions, MDS plots were calculated and are shown in Figure 31. Whilst a significant difference between the microbial ecology of samples taken at five different time points during cheese production and ripening was calculated ($p = 0.001$), no significant difference in the microbial ecology of cheese samples treated with different brine solutions was observed ($p = 0.884$).

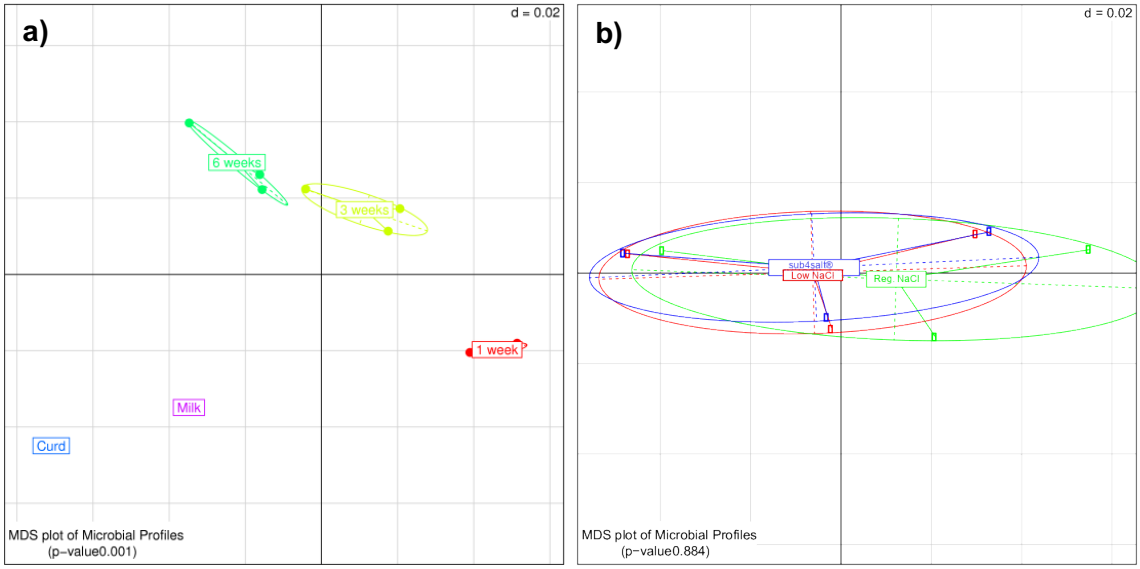

Figure 31: Multidimensional scaling plots showing a) the microbial diversity between sampling time points during production and maturation of sodium-reduced Edam, and b) the microbial diversity between varying brine compositions used to produce sodium-reduced Edam varieties. d = dissimilarity scale of the grid (d = 0.02 means the distance between two grid lines represents 2 % dissimilarity between samples).

The microbial beta-diversity of sodium-reduced Edam cheese produced in a single production experiment at the research facility of a commercial dairy using the parameters applied in the MRI pilot plant productions and scaled-up to industrial proportions is shown in Figure 32. Although only a single production was carried out, similarities with the production experiment performed at the MRI pilot plant in Kiel, which was verified through biological triplicates, were evident (Figure 30). Throughout ripening, the starter *Lactococcus* (63 – 77 %) and *Lactobacillus* (22 – 37 %) species dominated the microbial ecology. The relative abundance of the adjunct *Leuconostoc* species were below 0.15 % and less than 0.2 % of bacteria present within the samples remained unclassified. Starter culture ratios remained similar between brining conditions (low NaCl, sub4salt® and regular NaCl).

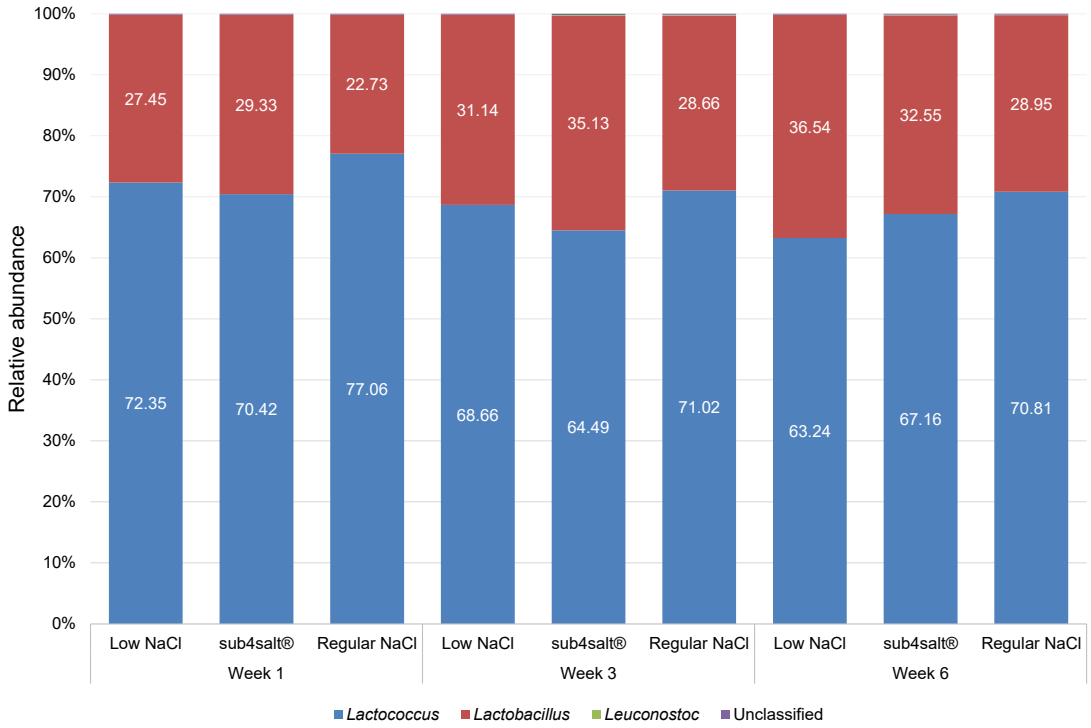

Figure 32: Relative bacterial abundance in sodium-reduced Edam cheese produced in the research lab of a commercial dairy. An abundance cut-off was set at 0.05 %. n = 1 biological replicate.

To visualise beta-diversity between sampling time points as well as between brine composition of cheese samples produced in the scale-up experiment in the lab of a commercial dairy, MDS plots were calculated (Figure 33). Due to the single production, no significant differences in microbial diversity between ripening time points ($p = 0.179$) and brine composition ($p = 0.15$) were calculated.

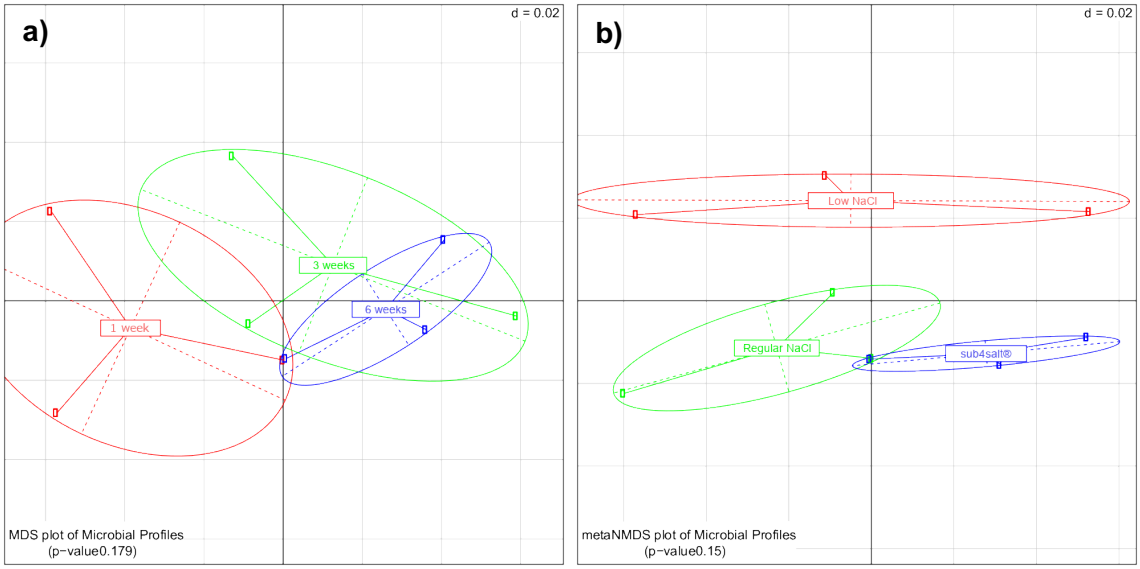

Figure 33: Multidimensional scaling plots showing no significant differences in a) the microbial diversity between sampling time points during maturation of sodium-reduced Edam, and b) microbial diversity between varying brine compositions used to produce sodium-reduced Edam varieties in a scale-up experiment in the lab of a commercial dairy. d = dissimilarity scale of the grid (d = 0.02 means the distance between two grid lines represents 2 % dissimilarity between samples).

The microbial beta-diversity of sodium-reduced Edam cheese samples co-inoculated with *List. innocua* during production to simulate contamination with the related, yet pathogenic, *List. monocytogenes*, is shown in Figure 34. Similar to Edam produced without *List. innocua* co-inoculation, the starter culture *Lactococcus* (55 – 75 %) and *Lactobacillus* (24 – 45 %) species dominated the microbial ecology and the relative abundance of the adjunct *Leuconostoc* species was beneath 0.5 % throughout production and ripening of sodium-reduced Edam. As not all microorganisms have been classified to date, < 0.4 % of bacteria present within the samples remained unclassified. When comparing the salt reduction strategies of simple NaCl reduction and mineral salt substitution (sub4salt®) with regular NaCl levels in the control Edam, the starter culture ratios remained similar.

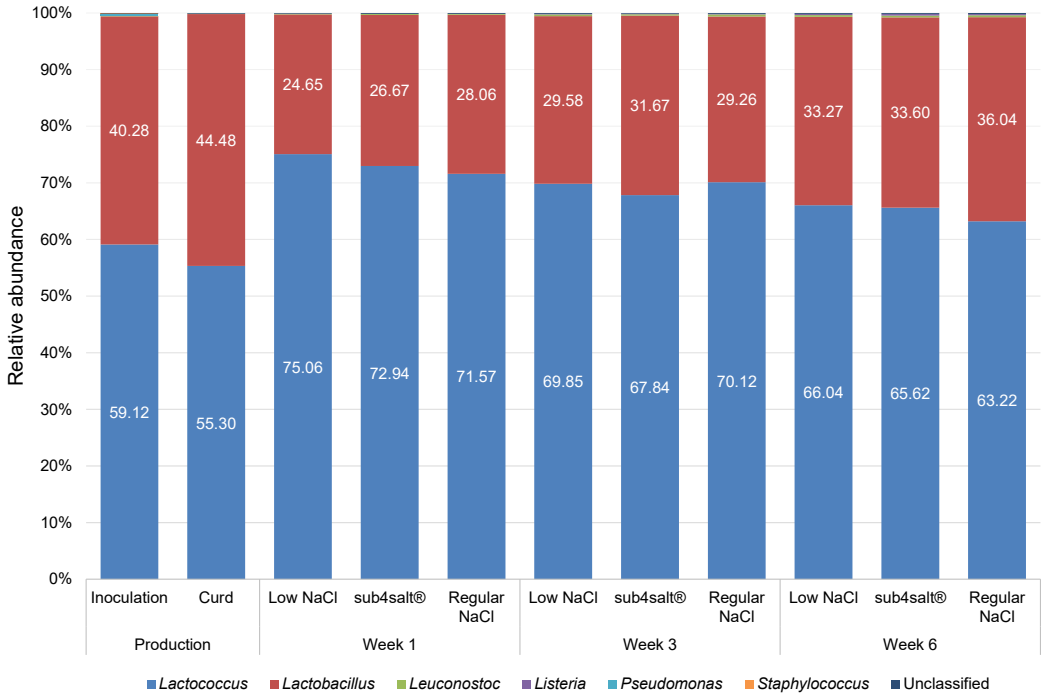

Figure 34: Relative bacterial abundance in sodium-reduced Edam cheese co-inoculated with *List. innocua*. An abundance cut-off was set at 0.05 %. n = 3 biological replicates.

The relative microbial abundances of all microorganisms including the adjunct *Leuconostoc* as well as the *Listeria* species that are shown in Figure 34 are further numerically specified in the adjoining Table 17. Although co-inoculated at 1×10^5 cfu/mL, only minimal relative abundance of DNA from *Listeria* species was detected after three and six weeks of cheese ripening.

Table 17: Relative microbial abundance (%, cut-off: 0.05 %) in sodium-reduced Edam cheese co-inoculated with *List. innocua*

	Production		Week 1			Week 3			Week 6		
	Inoculation	Curd	Low NaCl	sub4salt®	Reg.NaCl	Low NaCl	sub4salt®	Reg.NaCl	Low NaCl	sub4salt®	Reg.NaCl
Lactococcus	59.1	55.3	75.1	72.9	71.6	69.9	67.8	70.1	66.0	65.6	63.2
Lactobacillus	40.3	44.5	24.7	26.7	28.1	29.6	31.7	29.3	33.3	33.6	36.0
Leuconostoc	0.1	0.1	0.1	0.2	0.1	0.3	0.3	0.4	0.3	0.3	0.3
Listeria	-	-	-	-	-	0.1	0.1	0.1	0.2	0.3	0.2
Pseudomonas	0.3	-	-	-	-	-	-	-	-	-	-
Staphylococcus	0.1	-	-	-	-	-	-	-	-	-	-
Unclassified	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2

To visualise beta-diversity between cheese sampling time points as well as between brine composition during the challenge test with *List. innocua* in sodium-reduced Edam, MDS plots were calculated (Figure 35). Whilst a significant difference (p = 0.001) in microbial ecology between each sampling time point was observed, again no significant difference (p = 0.895) in microbial composition of cheese samples treated with different brine compositions could be determined.

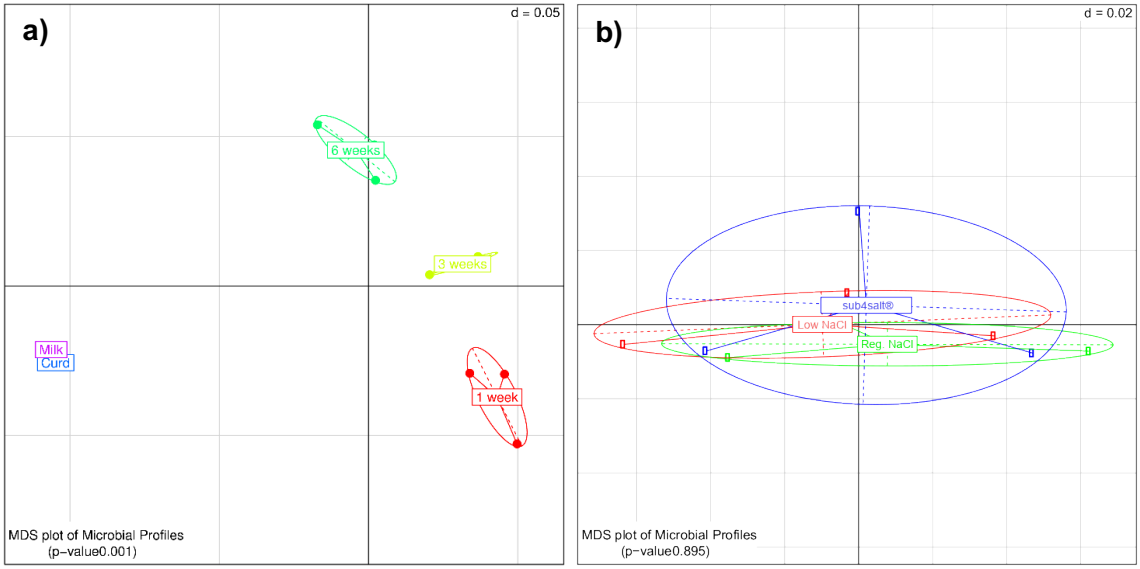

Figure 35: Multidimensional scaling plots showing a) the microbial diversity between sampling time points during production and maturation of sodium-reduced Edam co-inoculated with *List. innocua*, and b) microbial diversity between varying brine compositions used to produce these sodium-reduced Edam varieties. d = dissimilarity scale of the grid (d = 0.05 means the distance between two grid lines represents 5 % dissimilarity between samples).

5 Discussion

Salt and sugar are important ingredients for the production of quality fermented dairy products. However, these ingredients are also major modifiable risk factors for NCDs and therefore a reduction in their consumption is beneficial to both individual and population health. Reformulating fermented dairy foods to contain less salt and less added sugar can help reduce consumption of these nutrients. Extensive research of product-specific reformulation strategies, and knowledge of the behaviour of LAB within these reformulated products, can help assess the applicability of these strategies for industry production. To this end, the behaviour of LAB and the microbial diversity in reformulated yoghurt and cheese samples, as well as selected product characteristics, were analysed in this study.

5.1 Sweetness-enhanced yoghurt

Reformulated yoghurt was produced using a combination of lactose hydrolysis and glucose isomerisation to produce sweetness-enhanced milk, which was then fermented with commercial yoghurt starter cultures to produce sweetness-enhanced yoghurt. The objective of this reformulation approach was to use the inherent milk sugar lactose to sweeten the yoghurt, thereby helping reduce the amount of added sugar needed for the manufacture of sweetened yoghurt products, which are often produced with up to 22 % added sugar. After application of the bi-enzymatic system of lactose conversion for the production of sweetness-enhanced milk, six commercial yoghurt starter cultures were tested for their growth and milk acidification potential to ultimately choose two cultures, a traditional yoghurt culture (with *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*) and a mild-tasting, probiotic yoghurt culture (with *S. thermophilus*, *Lb. acidophilus* and *B. animalis*), for further pilot plant scale productions. Following analysis of product characteristics and culture-dependent microbiology analyses, culture-independent approaches for studying the microbial ecology of sweetness-enhanced yoghurt were applied and compared to samples of regular yoghurt.

Technological parameters were measured during the production of sweetness-enhanced milk, demonstrating the successful conversion of the disaccharide lactose into its sweeter monosaccharide components of glucose, galactose and fructose. Taking the relative sweetness of each monosaccharide into account, the overall increase in sweetness achieved by applying the bi-enzymatic system in this reformulation study confirmed

the findings presented by Lorenzen et al. showing an increase of sweetness by a factor of 2-3.[99] Despite this achievement, additional enzymatic conversions could further increase the sweetness of lactose. Previous studies by Jørgensen et al. and Torres et al. demonstrated the possibility of implementing a β-galactosidase and a glucose isomerase, as used in this study, in conjunction with an L-arabinose isomerase to convert galactose to tagatose, yielding tagatose, galactose, glucose and fructose in the enhanced milk.[211,212] As tagatose has a relative sweetness of 90 % that of sucrose, additional enzymatic conversions such as this one could further increase the sweetness of lactose for use in reformulated products.[213]

Individual strains isolated from the mixed, commercial yoghurt starter cultures were screened for sugar utilisation. The presence of galactose-utilising starter culture strains would increase the residual glucose in the matrix, because these LAB would preferentially ferment galactose instead of glucose, thus leaving more residual glucose in the milk matrix. This could add to the overall sweetness of the matrix. As no such strain was found in the starter cultures analysed, this avenue was not further investigated. Recently, however, starter cultures such as Sweety® T-1 (Chr. Hansen) containing a galactose-utilising *S. thermophilus* strain and Sweety® Y-1 (Chr. Hansen), which in addition also contains a galactose-utilising *Lb. delbrueckii* subsp. *bulgaricus* strain, have been marketed to increase the natural sweetness of yoghurt using only starter cultures.[214,215] The manufacturer claims that a 1 % reduction in added sucrose is possible whilst retaining the same sweetness in the end product.[214] Thus, further reduction of added sugar in yoghurt may be achieved in future by combining galactose-utilising starter culture strains with the bi-enzymatic system of lactose conversion.

Laboratory yoghurt production with sweetness-enhanced and regular milk using six commercial yoghurt cultures was done to select two starter cultures for larger pilot plant yoghurt production, based on their growth and acidification behaviour. All traditional starter cultures with *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* showed similar acidification progressions in regular and sweetness-enhanced milk, and these reflected the acidification curves supplied by the manufacturer Chr. Hansen in the product data sheets. As the sweetness-enhanced milk displayed a slightly higher pH at the beginning of fermentation than regular milk, the acidification progression for sweetness-enhanced and regular milk can be considered comparable. Previous studies of yoghurt produced with lactose-hydrolysed milk, as manufactured for lactose intolerant individuals, have demonstrated increased acidification rates of lactose-hydrolysed milk compared to control milk due to increased efficiency of carbohydrate utilisation by *S. thermophilus* when free glucose is available.[216,217] However, no increased acidification rate was observed during fermentation of the lactose-hydrolysed and glucose-isomerised milk in this study,

as the free glucose generated through hydrolysation is partly converted to fructose by the glucose-isomerase enzyme and is therefore no longer freely available to the micro-organisms. Thus, bi-enzymatically modifying the yoghurt milk cannot decrease fermentation time through accelerated used of carbohydrates.

Following successful laboratory-scale yoghurt production two starter cultures (one traditional and one mild-tasting, probiotic culture) were chosen for larger, pilot-plant scale tests using sweetness-enhanced and regular milk. Milk acidification and the SLAB growth progressions, as determined by culture-dependent techniques, both showed typical developments compared to those well-documented for traditional and probiotic yoghurt in the literature.[83,218,219] In the traditional yoghurt culture containing *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* (YoFlex® Premium 4.0), the LAB showed nearly identical growth progressions in both sweetness-enhanced and regular milk. Slight discrepancies observed between types of milk in the lab scale productions were not verified in the scaled-up, pilot plant experiments, and were possibly only seen in laboratory productions due to the difficulty to scale-down the culture dosage of commercial culture batches to small laboratory productions. The two starter culture bacteria work symbiotically to ferment milk and acidification is more successful when this symbiosis is present compared to growth of the individual strains in milk.[220] *S. thermophilus* grows better at a higher pH than its counterpart *Lb. delbrueckii* subsp. *bulgaricus*, and hence it initiates fermentation of the milk matrix.[83] *S. thermophilus* contributes most prominently to the progression of acidification, releasing essential amino acids and metabolites, and providing ideal growth conditions for *Lb. delbrueckii* subsp. *bulgaricus*. This symbiosis was observed in traditional yoghurt produced with both sweetness-enhanced and regular milk. Therefore, the altered carbohydrate composition, and the ready availability of glucose, galactose and fructose in the sweetness-enhanced milk matrix, did not affect the fermentation rate of these organisms.

In the mild-tasting, probiotic yoghurt culture (ABT-100), *S. thermophilus* and the probiotic organism *B. animalis* also showed comparable growth progressions in both sweetness-enhanced and regular milk samples during pilot plant production. Despite expected growth during fermentation, *Lb. acidophilus* showed a rapid decrease in bacterial counts in both types of yoghurt milk during storage, and an inability to be cultured after 21 days of storage in sweetness-enhanced yoghurt. This was despite successful previous cultivation on the same agar (Bile-MRS) for the purposes of strain identification (isolation from the starter culture) and in preliminary laboratory production (cultivation from yoghurt samples). Owing to the three biological replicates performed for all experiments, it is unlikely that this drop in bacterial counts can be traced back to an error in media preparation or plate incubation conditions of the selective agar. However, the probiotic

organism *Lb. acidophilus* is sensitive to the build-up of dissolved oxygen, organic acids and hydrogen peroxide produced by LAB metabolism, and it has been well documented that, as a result, the viable number of these bacteria in yoghurt declines during storage.[221–223] Shah et al. analysed five commercially available yoghurts claiming to contain viable *Lb. acidophilus* and *B. bifidum* cultures, monitoring bacterial counts starting from two to three days after production for a total of 33 days.[89] The *Lb. acidophilus* counts remained high throughout storage for all yoghurts that displayed counts of around 10^8 cfu/mL shortly after production. However, two of the tested products showed only 10^4 and 10^6 cfu/mL shortly after production and a strong decline in bacterial counts during storage, resulting in no viable counts measured after 21 and 30 days for these samples, respectively. This same tendency was observed in yoghurt produced with the ABT-100 culture, where viable *Lb. acidophilus* counts were measured at 10^6 cfu/mL at the time point of cooling for both types of milk but showed a rapid decline in counts during storage. Gilliland et al. emphasised that *Lb. acidophilus* grows poorly in yoghurt milk unless readily available nutrients are added during production.[223] Hence, further studies with addition of nutrients to the sweetness-enhanced milk may help raise the viable count of *Lb. acidophilus* in yoghurt produced with the ABT-100 culture. However, raising the viable counts of probiotic *Lb. acidophilus* would also contribute to increased acid production, especially during post-acidification, which from a sensory perspective would make the yoghurt more sour, an undesired characteristic for the production of mild-tasting yoghurt.

The second probiotic culture contained in the ABT-100 culture, *B. animalis*, also showed a decline in bacterial counts towards the end of the storage period. This decline followed a very similar progression in both sweetness-enhanced and regular yoghurt. Unlike the *Lb. acidophilus* counts, the *B. animalis* counts were measured around 5×10^5 cfu/mL after 21 days of storage. These results are consistent with previous studies describing an ubiquitous reduction in bacterial counts of probiotic bifidobacteria during yoghurt storage.[89,219,224] The viability of bifidobacteria during yoghurt storage may differ depending on the *Bifidobacterium* strain and may be attributed to the acid sensitivity of the organism, as growth is retarded under pH 5.0, but also to additional factors such as oxygen toxicity, the availability of nutrients and temperature.[89,221] Shah et al. also documented the viability of *B. bifidum* strains in commercial yoghurt products.[89] Many of these products presented low bacterial counts between 10^3 and 10^4 cfu/mL at two to three days after production, and all samples showed a steep decline in bacterial counts throughout 21 days of storage. When compared to the behaviour of the related *B. bifidum* strain as analysed by Shah et al., the *B. animalis* strain contained in the ABT-100 culture was inoculated at

relatively high levels and did not rapidly decline in numbers, so that the final bacterial count was measured around 5×10^5 cfu/mL.

The strong decline in probiotic organisms during storage of yoghurt produced with the ABT-100 culture may be the result of the sensitivity of these organisms to dissolved oxygen within the yoghurt sample, which permeates into the sealed plastic cups during storage. Dave and Shah studied the growth of probiotic organisms in yoghurt stored in glass bottles versus plastic cups and demonstrated that the diffusion of oxygen into yoghurt stored in plastic cups had a significant negative impact on growth of these organisms.[219] The authors established that both *Lb. acidophilus* and bifidobacterial counts showed improved viability in glass bottles whilst technological parameters such as pH and titratable acidity were not affected by packaging conditions.

A wide range of *Lb. acidophilus* and bifidobacterial growth behaviours throughout yoghurt storage have been documented and hence the decline in viable probiotic cultures observed in milk fermented with ABT-100 in this thesis work may not be uncommon. However, as it has been suggested that to provide health benefits, viable probiotic bacteria must be present at $\geq 10^6$ cfu/mL at the time of consumption, it is unlikely that the *Lb. acidophilus* in the ABT-100 culture can provide health benefits to the consumer unless the yoghurt were to be consumed directly after fermentation. Despite this, the viable counts of *B. animalis* retained potentially health-benefitting viable counts during storage but also dropped just below 10^6 cfu/mL shortly before the end of the 21-day storage period. Taking these results into consideration, consumers looking to benefit from probiotics in yoghurt should take care to buy recently processed products, preferably packaged in glass jars, and consume these as soon as possible to ensure ingestion of viable probiotic cultures.

The microbial diversity of yoghurt, measured by means of DGGE and 16S high throughput sequencing, was not significantly different between sweetness-enhanced and regular yoghurt at all sampling time points during fermentation and storage. This held true for both of the yoghurt cultures tested in pilot-plant scale experiments and supports the culture-dependent results showing comparable growth and acidity curves in sweetness-enhanced and regular milk. As expected, the diversity observed in DGGE analysis and the number of individual species detected in alpha diversity studies was higher just after inoculation of the milk with starter cultures than after the subsequent fermentation. This is most likely due to culture-independent methods detecting all bacterial DNA in the samples, including that of dead or injured cells. Hence, milk samples taken just after inoculation with yoghurt starter cultures contain, in addition to the inoculated starter cultures, abundant DNA from the raw milk microbiota that was killed during pasteurisation. During

milk fermentation, the starter cultures undergo rapid growth, and this results in the relative abundance of SLAB DNA dominating over residual DNA from raw milk microbiota, so that these are no longer strongly represented in relative abundance calculations.

HTS results showed that in the traditional culture, *Streptococcus* dominated the microbial ecology of yoghurt after fermentation and throughout storage, making up 81 – 85 % of the relative microbial abundance in sweetness-enhanced and regular milk. However, *Lactobacillus* showed relative abundances of only 2 – 7 % after fermentation and during storage. This abundance profile reflects the bacterial counts obtained through spread-plating, where a 3-log difference between *Streptococcus* and *Lactobacillus* was measured after fermentation. The significant difference in microbial ecology between inoculation and after fermentation confirms the essential growth of starter cultures needed for both acidification of the matrix and protection against the growth of unwanted organisms, not to mention to provide the desired product characteristics.[225]

In the probiotic culture, HTS results confirmed the roughly equal inoculation levels of *Streptococcus* and *Bifidobacterium* (20 – 35 % relative abundance and inoculation of approx. 7×10^6 cfu/mL, each). *Lactobacillus* were inoculated about 1-log lower than *Streptococcus* and *Bifidobacterium*, resulting in a relative bacterial abundance of 1 % in the HTS results. After fermentation (at the time point of cooling), *Streptococcus* (70 %) dominated the microbial profile the yoghurt, but the abundance of *Lactobacillus* and *Bifidobacterium* increased during storage to yield roughly equal thirds of bacterial abundance of each starter culture by the end of storage. The bacterial abundance differed significantly between inoculation and cooling, and also between cooling and sampling after 5 days. The distribution of microbial abundances in the ABT-100 culture did not agree with the culture-dependent results showing a rapid decline in the viable probiotic bacteria, especially in *Lb. acidophilus*. This discrepancy could again be explained by a misrepresentation of microbial DNA due to the accumulation of DNA from dead or injured probiotic bacteria.

In this thesis work, a low abundance of *Pseudomonas* and other Proteobacteria (< 5 %) was detected in yoghurt samples produced with sweetness-enhanced and regular milk, with the Firmicutes phylum clearly dominating in yoghurt produced with both cultures. A study by Zalewska et al. in 2018, who investigated the bacterial composition of yoghurt produced *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* in raw and pasteurised bovine milk using 16S rDNA amplicon sequencing, found a high abundance of Firmicutes (e.g. *Lactobacillales*), but also of Proteobacteria (e.g. *Pseudomonadales*), in yoghurt produced with pasteurised bovine milk immediately after fermentation.[226] An increase in Proteobacteria was observed by the end of the 28-day storage period. In comparison to

the study by Zalewska et al., it is likely that the quick dominance of SLAB observed by the end of yoghurt fermentation in this thesis work contributed to restraining the growth of any spoilage or potentially pathogenic bacteria through the creation of acidic conditions hindering pathogen growth.[225] However, earlier HTS studies by Quigley et al. in 2013 demonstrated that bovine milk contains a diverse microbial ecology and that the microbial composition measured in milk samples, including *Lactobacillales* as well as *Pseudomonadales*, greatly depends on the farm and processing facility.[227] Hence, comparison of microbial abundances between studies must be made with caution. Furthermore, the opportunities for secondary contamination with *Pseudomonadales* after pasteurisation is higher in a commercial milk processing environment than in pilot plant production, as the milk holding tanks and processing equipment provide ample sources of contaminating bacteria.

The absence of spoilage or potentially pathogenic bacteria above the limit of detection after 21 days of storage suggests that the enzymatic modification of yoghurt milk used in this thesis work did not increase the chance of growth of (potentially) pathogenic bacteria. For most pathogens, the minimum number of microorganisms necessary to cause infection in 50 % of the population lies above 10^2 cfu/mL, with most needing between 10^3 to 10^8 cfu/mL organisms to cause infection.[228] Therefore, a limit of detection threshold was set at 1×10^2 cfu/mL to test for microbial quality. Based on the absence of growth of enterobacteria, enterococci, pseudomonads, yeast or moulds above this threshold on selective agar after 21 days of storage, it can be inferred that these organisms did not compromise the microbial quality of sweetness-enhanced yoghurt. Although low abundances of potentially pathogenic Proteobacteria were detected through HTS after fermentation and during storage, this could possibly be explained by the residual presence of DNA from bacteria eliminated during pasteurisation.

This study demonstrated that the bi-enzymatic system of lactose conversion can be applied to the reformulation of yoghurt without influencing the microbial ecology or product characteristics. However, a further essential aspect all food reformulation efforts is to manufacture products with the same sensory qualities as their standard counterparts. Hence, the question arises whether the sweetness-enhanced yoghurt produced in this study can compete with a standard product sweetened with sucrose. A sensory study run in parallel to the MRI pilot plant experiments in this thesis demonstrated that application of the bi-enzymatic system for yoghurt production with both the traditional YoFlex® Premium 4.0 and probiotic ABT-100 cultures enabled an 11.4 % and a 14.6 % reduction in added sugar in the final product, respectively (Luzzi et al., accepted for publication 2019).[229] The bi-enzymatic system was also applied to the production of reformulated pudding products, where a reduction potential of added sugar of up to 21.8 % was

demonstrated (Luzzi et al., accepted for publication 2019).[229] Reformulated yoghurt produced with both starter cultures was perceived to be significantly sweeter and less sour than regular products by a panel of 30 assessors with experience in the descriptive sensory analysis of dairy products. Hence, application of sweetness-enhanced milk for the production of reformulated yoghurt in order to be able to reduce the added sugar component in sweetened yoghurt products was shown to be technically possible, microbiologically safe and sensorially satisfactory.

There are, however, some limitations to the industrial applicability of implementing enzyme-modified milk to reformulate dairy products to contain less added sugar. The most prominent of these is the high cost for the manufacturer, most importantly related to the calcium sensitivity of the glucose isomerase enzyme. In future, research into the production of calcium resistant glucose isomerase enzymes could help reduce these costs and may make this reformulation approach more industrially relevant. Furthermore, there is some debate about whether the use of β-galactosidase enzymes must be declared in lactose-hydrolysed milk.[230] This automatically raises the same question for products made with lactose hydrolysed and glucose isomerised milk. However, according to Article 20 of the Regulation (EU) No 1169/2011 of the European Parliament, food enzymes do not need to be declared in the list of food ingredients if they do not serve a technological function in the finished product.[231] Therefore, by applying an enzymatic approach to the reformulation of sweetened yoghurt products, and the inactivation of the enzymes after usage, manufacturers can benefit from this regulation by not needing to declare the enzymes, thus heightening the appeal of the reformulated products for health-conscientious consumers seeking all-natural products.

5.2 Sodium-reduced Edam cheese

Sodium-reduced Edam cheese was manufactured using a simple NaCl reduction strategy and mineral salt substitution approach. Overall, a 30 % Na reduction was achieved through simple NaCl reduction, whilst a 50 % Na reduction was possible when sub4salt® was used for brining. The production of Edam cheese with a sodium content of < 0.4 % was achieved without compromising the microbial quality of the cheese samples in all production experiments using both reformulation approaches. The two sodium reduction strategies were applied during the brining stage of cheese manufacture; hence any potential influence of salt reduction on the microbial ecology of these cheeses would take effect during ripening of the cheese without affecting the initial fermentation of the cheese milk by SLAB. Alongside the measurement of technological parameters during

production and ripening, culture-dependent techniques were applied to monitor the microbial progression of viable culture counts of the SLAB. Following analysis of product characteristics and culture-dependent microbiology analyses, culture-independent approaches for studying the microbial ecology of reformulated, sodium-reduced Edam were applied.

Salt content, pH and moisture influence the biochemical changes during ripening that play a central role in flavour development, texture and appearance.[106] Therefore, technological parameters including pH, mineral composition and selected physical properties were measured during manufacturing and ripening of sodium-reduced Edam cheese for comparison with a regular NaCl Edam cheese. Measurement of these properties demonstrated that even with a 30 – 50 % sodium reduction compared to the regular NaCl cheese, the pH, dry matter and fat in dry matter content during cheese ripening were not adversely affected. The dry matter for all cheeses, including the regular NaCl Edam, was greater than the minimum dry matter content required for German standard Edam cheeses with 40 % fat in dry matter content.[208] The increase in dry matter content can be attributed to the high protein and high fat content of the cheese milk, as increasing protein and fat content in cheese milk leads to a reduction in moisture content.[232] The pH of all Edam samples taken during ripening was between 5.5 and 5.7, which corresponds to the typical pH of Edam cheese as compiled from various sources by Guinee and Fox.[103] During cheese production the milk acidifies as a direct result of acid production by the SLAB. D- and L- lactic acid and acetic acid measurements taken in a representative Edam production during ripening, showed L-lactic acid levels around 12 g/kg in all samples and effectively no D-lactic acid or acetic acid. The L-stereoisomer is known to be the main enantiomer in the related, Dutch-type Gouda cheese and the L-lactic acid measurement lies within the typical lactate range of this cheese.[210] L-lactic acid is produced by starter *Lactococcus* strains but also partly by *Lactobacillus* strains.[65] *Lactobacillus* and *Leuconostoc* also produce D-lactic acid; however, it is beneficial that this stereoisomer was not produced in large amounts during Edam processing, as D-lactic acid is less soluble than L-lactic acid and its presence can lead to lactate crystal formation in mature cheese, which affects the cheese texture.[210]

Proteolysis is the main biochemical process in Edam cheese, essential for cheese flavour and texture, and is affected by salt content through the stimulation or inhibition of enzymatic activities.[103,233] It is initiated by starter and adjunct cultures during fermentation and in early stages of ripening whilst, as ageing progresses, the production of volatile flavour compounds by NSLAB becomes increasingly important.[116] Proteolysis during ripening of sodium-reduced Edam, as measured by the free amino acid content, showed a steady increase over the course of the six-week maturation period in both sodium-

reduced and regular Edam samples. The mineral salt replacement strategy showed a tendency for slightly higher levels of proteolysis in the MRI pilot plant production without *List. innocua* co-inoculation, as well as in commercial dairy production. However, these data must in future be confirmed or negated through multiple repetitions or by expression studies of proteolytic genes, for example by applying RNA sequencing. A study by McMahon et al. investigating the effect of different cations on proteolysis in Cheddar cheese found that a 40 % potassium content in the salting mixtures did not adversely affect proteolysis.[124] Hence, the potassium contained in the mineral salt substitution mixture used in this thesis may not show a significant effect on proteolysis in sodium-reduced Edam upon future study repetition.

Furthermore, the pH of cheese is important for optimal function of the microbial enzymes influencing proteolysis. The principle proteolytic enzyme of *Lactococcus*, lactocepin, has its pH optimum around 5.5 – 6.5, and is therefore likely to play a central role in proteolysis in the sodium-reduced Edam produced for this thesis work.[116] In addition to *Lactococcus* enzymes, a study by Tungjaroenchai et al. emphasised the important contribution of adjunct cultures such as *Lb. helveticus* and *Lc. lactis* subsp. *lactis* biovar Diacetylactis to proteolysis in Edam cheese, as these authors measured high aminopeptidase activity for these adjunct LAB.[118] Considering that the mixed starter cultures implemented for Edam production in this thesis also contained both of these strains, their aminopeptidase activity is likely to have contributed to the steady increase in proteolysis during ripening.

The influence of proteolysis on flavour development in cheese results mainly from the generation of short peptides and amino acids.[116] In a technological and sensory study, which was run in parallel and which evaluated the same sodium-reduced Edam samples produced in the MRI pilot plant for this thesis, Hoffmann et al. analysed selected peptides by liquid-chromatography mass-spectrometry, revealing a complex peptide pattern that changed throughout maturation but was not affected by salt reduction.[234] Two known bitter peptides (β-CN f193-209 and β-CN f194-209) were identified in all Edam samples including the control Edam, with the most prominent amount of both peptides measured after one week of maturation. No effect of the reformulation strategy (either simple reduction or mineral salt substitution with sub4salt®) on the generation of these bitter peptides was observed. A similar effect was found in a study of semi-hard Prato cheese by Baptista et al., in which a 25 % reduction in sodium did not increase the relative intensity of these same bitter-tasting peptides compared to control cheese. However, these authors also observed that although these bitter peptides were produced, they did not affect the sensory acceptance of the sodium-reduced Prato cheese.[235] Contrary to this study of Prato cheese, Hoffmann et al. found that the sodium-reduced Edam produced using both reformulation strategies tasted bitter in comparison to regular Edam and was

therefore unsatisfactory.[234] This bitter taste may have resulted from the presence of bitter peptides similar to those discussed above. However, in the mineral salt cheese produced by using sub4salt® in the brine, the bitter taste may also be attributed to the higher potassium content of the resulting cheeses. In regular Edam the bitterness originating from the presence of bitter peptides seems to be masked by the higher sodium content. Furthermore, the production of volatile aroma compounds by specific adjunct starter cultures may help mask off-flavours in sodium-reduced Edam. However, Hoffmann et al. demonstrated that the dose of adjunct cultures used in this study was not sufficient to produce adequate amounts of volatile flavour compounds to prevent the detection of off-flavours. In future, the addition of alternate adjunct cultures provides an important approach for further study.

The progression of viable culture counts of SLAB, as determined by culture-dependent techniques throughout fermentation and ripening, demonstrated a typical microbial development similar to that documented in the literature for Edam-type cheese.[119,134,137,171] Both reformulation approaches showed comparable progressions of *Lactococcus*, *Lactobacillus* and *Leuconostoc* starter and adjunct LAB throughout fermentation and ripening to the control cheese, indicating that, as expected, the sodium reduction did not affect bacterial growth compared to the regular NaCl Edam. To ensure adequate acid production during cheese manufacture, large numbers of active bacteria must be present in the starter culture mixtures and these grow rapidly during production.[79] However, as ripening progresses, the composition of starter and adjunct LAB changes, and this can been seen in the culture-dependent bacterial counts of all Edam production experiments. These results are in accordance with a previous study by Porcellato et al., which analysed the dynamics of LAB, adjunct cultures and NSLAB in Dutch-type cheese.[119] These authors observed a dominance of adjunct *Lb. paracasei* and *Lb. rhamnosus* cultures with viable counts of around 10^8 cfu/g after four weeks of ripening.[119] A similar increase was observed for the genus *Lactobacillus* in all salt reduced and regular salt Edam produced in this thesis work; however, this could not be further determined on a species-level due to limitations of both the culture-dependent and culture-independent methods applied. Furthermore, the culture-dependent growth progressions of starter and adjunct LAB monitored during ripening of the commercial dairy Edam samples showed some discrepancy to the MRI productions. This is most likely due to the single repetition of this production experiment, but may also be influenced by so-called in-house flora of the commercial dairy lab.

A challenge test in which Edam cheese was co-inoculated with the *List. monocytogenes* surrogate *List. innocua*, was performed to test for altered growth of this species in sodium-reduced Edam. Inoculation of the cheese milk with 10^5 cfu/g of *List. innocua* did

not affect fermentation or ripening of salt reduced and regular Edam. This is not surprising, as *List. innocua* does not contribute to the fermentation or ripening processes; however, it is reassuring that there were no discrepancies between sodium-reduced and regular Edam samples, and that *List. innocua* numbers did not increase to a greater extent in sodium-reduced Edam during ripening compared with control Edam. In an additional test performed to investigate potential growth of *List. innocua* in ripened, sodium-reduced Edam samples stored at 4°C for three weeks to mimic consumer behaviour at home, again no further increase in growth of *List. innocua* could be observed.

List. innocua was implemented as a surrogate based on its comparable growth conditions, hence it is likely that growth of the pathogen *List. monocytogenes* would not be enhanced by the reduced Na conditions presented in this study. However, further challenge tests with other possible pathogens such as *Escherichia (E.) coli* or *Staphylococcus aureus* strains are needed to complete a full assessment of the microbial safety of sodium-reduced Edam. In 2003, Hystead et al. examined the effects of sodium reduction, as well as KCl supplementation, on the survival of *List. monocytogenes* in Cheddar cheese.[122] These authors analysed different time points of post-processing contamination, and found that sodium reduction of up to 50 % and a 1:1 substitution of NaCl with KCl did not affect *List. monocytogenes* survival; however, this pathogen did survive better when inoculated at later time points post processing. A further study by Shrestha et al. analysed the survival of *List. monocytogenes* introduced as a post-aging contaminant during storage of low-salt (0.7 % NaCl) Cheddar cheese and demonstrated that although this pathogen survived on all samples, and thus good sanitation practices are still necessary, there was no difference in *List. monocytogenes* survival between salt reduced and standard products.[236] A parallel study by these authors, in which the pathogen of interest was replaced with *Salmonella*, found that salt-reduced samples were also equally safe as their full salt counterparts with regards to this pathogen.[237]

To expand the assessment of possible microbial risk resulting from sodium-reduction in Edam cheese, and thereby to provide an indication of the microbial quality of sodium-reduced cheese samples, selective agar tests for enterobacteria, enterococci, yeasts, moulds and pseudomonads were performed on all samples at the end of the six-week ripening period. As all culture-dependent analyses showed bacterial counts below the limit of detection set at 1×10^2 cfu/g, regardless of the brining conditions of the cheese samples, it may be assumed that the microbial quality of the sodium-reduced Edam produced in this study was not compromised by these spoilage and potentially opportunistic pathogens.

As the presence of NaCl in foods directly influences water activity, measurement of the a_w value is an important quality control step for the evaluation cheese. According to data compiled by Guinee and Fox, the water activity of Edam cheese generally lies around 0.96, depending on the length of the maturation period.[103] The a_w value in the sodium-reduced Edam produced in this study exceeded 0.97 in all samples but, as expected, showed a decreasing tendency as ripening progressed due to the inverse relationship between maturation and moisture content.[106] A slight tendency towards higher a_w levels in cheeses brined in the mineral salt mixture containing potassium, compared to simple sodium reduction cheeses, was observed; however further experimental repetitions should be done to allow for statistical significance to be tested. As the minimum a_w value for many spoilage or potentially pathogenic bacteria lies well below 0.96, the a_w value cannot be a sole indicator of microbial safety and the growth potential for individual pathogens must be tested for separately. Although measurement of a_w values constitutes an important part of quality control, the acid produced during milk fermentation by LAB, as well as the cool storage temperature during ripening, help provide effective control of microbial growth.[103]

The microbial composition of Edam cheese, determined by culture-independent methods, exhibited no significant difference amongst salt reduction strategies or in comparison to regular Edam cheese. These results can be collectively summarised for all Edam cheese produced with and without *List. innocua* co-inoculation at the MRI pilot plant. The single production experiment performed with larger cheese loaves (15 kg) in the lab of a commercial dairy showed comparable tendencies. Similar to observations made by Randazzo et al. in artisanal cheese, the microbial diversity estimated through DGGE analysis was visibly different in curd samples compared to the cheese samples taken throughout ripening.[238] This difference was confirmed as significant by MDS analyses of the HTS data. The diversity of microorganisms present in Edam samples analysed in this thesis was clearly dominated by the starter culture *Lactococcus* species (55 – 75 %), with a significant contribution of starter and adjunct *Lactobacillus* strains (22 – 45 %). As the sodium-reduced Edam was only ripened for six weeks, the bacterial abundance of NSLAB strains was almost negligible, as these bacteria play a more prominent role in later stage ripening processes.[239] The milk was pasteurised prior to cheese manufacture, hence NSLAB originating from the raw milk, which is the largest source of NSLAB, did not influence Edam ripening.[240] Although some NSLAB may enter the cheese during the production process, these are unlikely to grow within short ripening periods.[239] However, it is interesting to note that the adjunct *Leuconostoc* cultures that showed expected bacterial progressions in culture-dependent determinations were not detected above 1 % in the HTS studies. Although primer bias may play a role in this result, a more likely

explanation is that the relative abundances of *Lactococcus* and *Lactobacillus* starter culture strains, which were inoculated at 1 – 2.5 log cfu/mL higher than the adjunct *Leuconostoc* cultures, overshadow the relative abundance of *Leuconostoc* cultures in HTS studies. As an adjunct culture, *Leuconostoc* counts are anticipated to increase with longer ripening times, so it is expected that during an extended ripening, the relative abundance of these bacteria would also increase.[240]

Many recent HTS studies of cheese have focussed on raw milk cheeses, where NSLAB play a much more prominent role than in Edam. However, one recent study by Salazar et al. investigated the microbial ecology of samples of commercial Gouda cheese, produced with pasteurised milk, analysing differences in spatial variability within cheese wheels and length of cheese ripening.[241] The authors demonstrated that although samples taken from the core of the round cheese loaves showed some variance in the distribution of microbial abundance, all samples were dominated by *Lactococcus* species (46 – 55 %), likely attributed to the starter cultures. This is comparable to the microbial abundance observed in sodium-reduced Edam in this thesis work. However, Salazar et al. discovered a much smaller percentage of *Lactobacillus* (3 – 5 %) in commercial Gouda cheeses and a large percentage of unidentified members of the Bacillaceae family (40 %), which was not observed in the current study of sodium-reduced Edam. Furthermore, the authors determined that maturation time greatly influenced the species richness in commercial Gouda. A further study by Quigley et al. 2012 used HTS to analyse artisanal cheeses. These analyses also revealed a dominance of *Lactococcus* species in semi-hard cheeses, with LAB abundances of 84 % *Lactococcus*, 7.3 % *Lactobacillus* and 0.5 % *Leuconostoc* species. Especially in cheeses with short ripening times, these studies demonstrate that, although bacterial abundances differ greatly between cheese types and sampling time points, the microbial composition of cheese samples in the first months of ripening is largely biased by the starter culture combinations.

A major limitation of the HTS data obtained for both reformulated yoghurt and cheese produced for this thesis work is the potential confounding of data through the presence of DNA derived from dead or injured cells, which accumulates in the samples during storage and ripening of these products. The starter cultures, which are added in large quantities and grow rapidly during fermentation, die off during storage and ripening. Hence, as the complete DNA of the food samples was isolated and sequenced in this study, it is impossible to accurately analyse the microbial abundances of live bacterial populations within these samples. This urgently needs to be taken into consideration in follow-on studies. A possible solution is to apply DNA-staining of dead or injured cells through implementation of propidium monoazide (PMA) or ethidium monoazide staining before subsequent elimination of the stained cells from the complex microbial

sample.[242,243] Erkus et al. demonstrated successful implementation of PMA to study the community dynamics in Gouda cheese after showing that the combined relative contribution of injured and dead cells to microbial abundances was larger than 70 % in samples taken after four weeks of ripening.[243] This presents a skewed view of community dynamics. Although PMA staining promises to minimise the skewing of results, it may also be affected by the cell wall structure of gram-positive and gram-negative bacteria. In a study by Løvdal et al. it was found that PMA staining underestimated the abundance of viable, gram-positive *List. innocua* during quantification of live bacteria after heat-treatment.[244] Contrary to this, Elizaquivel et al. demonstrated that PMA staining was a suitable method for quantification of gram-negative *E. coli*.[245] In samples comprising a mixture of gram-positive and gram-negative organisms, staining to eliminate DNA from dead or injured cells in complex samples should therefore be applied with caution.

Both sub-studies (sugar reduction in yoghurt and salt reduction in cheese) have introduced plausible approaches to the reformulation of fermented dairy products. However, due to the technological scope of these experiments, only three biological replicates of each production experiment could be produced within the frame of this thesis. In order to be able to test for statistical significance in all tests, further production repetitions are therefore necessary. Nevertheless, the tendencies provided here give a solid indication that these reformulation approaches should be considered for reformulation of yoghurt and semi-hard cheese in future.

Finally, in addition to larger-scale production experiments, a promising avenue for future research lies in the investigation of the effects of the reformulation strategies presented in this thesis on the bacterial metatranscriptome of reformulated dairy products. Insight into the active genes at each sampling time point through sequencing of the mRNA may help to profile the effect of the altered carbohydrate composition in sweetness-enhanced yoghurt, as well as any changes resulting from sodium reduction in Edam, on the expression of technologically important genes. Recently, Bisanz et al. analysed the bacterial metatranscriptome of a probiotic yoghurt by first isolating total RNA and then enriching mRNA in the samples before performing RNA sequencing.[246] In a preliminary experiment alongside this thesis work, this same method was attempted on cheese samples taken throughout ripening of sodium-reduced Edam. Unfortunately, due to the complexity of the cheese matrix, it was not possible to isolate sufficient mRNA from these samples for adequate sequence coverage. Nevertheless, metatranscriptome analyses of reformulated products present an interesting target for further in-depth studies.

6 Conclusions and Outlook

In conclusion, this study investigated the effects of selected reformulation strategies on the microbial ecology and product characteristics of yoghurt and cheese. Research of product-specific reformulation strategies, including the study of microbial ecology, is essential for maintaining the product quality and microbial safety of reformulated fermented products, whilst assessing the applicability of these strategies for manufacturers.

In the first reformulation project, sweetness-enhanced yoghurt was successfully produced with lactose-hydrolysed and glucose-isomerised milk using a traditional and a probiotic yoghurt culture. Analysis of microbial ecology and product characteristics demonstrated that the tested commercial starter cultures can be implemented to produce comparable yoghurt with both sweetness-enhanced and unmodified milk. The growth of starter cultures and their ability to acidify the milk matrix was not affected by the altered carbohydrate composition of enhanced milk, and no microbiological risk from potentially pathogenic or spoilage bacteria was detected in either sweetness-enhanced or regular yoghurt after storage of samples for three weeks. In a parallel study of the same reformulated product samples at the MRI, it was shown that a 10 – 20 % reduction in added sugar is possible when applying this reformulation strategy to the production of sweetened dairy products.

In a second reformulation project, Edam cheese with < 0.4 % sodium was successfully manufactured by applying a simple reduction (achieving a 30 % Na reduction) and a mineral salt substitution approach (achieving a 50 % Na reduction). The chemical and physical characteristics of sodium-reduced Edam were demonstrated to be comparable to those of a regular Edam control cheese and the growth of starter culture bacteria was not affected by either reformulation strategy. No increased microbial risk from potentially pathogenic or spoilage bacteria, or from the pathogen *List. monocytogenes* tested via implementation of a non-pathogenic surrogate organism, was determined in the sodium-reduced cheeses. Both reformulation approaches could be successfully applied to a scaled-up trial production in the lab of a commercial dairy. However, in a parallel project at the MRI analysing the same reformulated cheese samples, sensory tests demonstrated that the reformulated Edam displayed a distinctly bitter taste, making it unsatisfactory for consumers and calling for further research to reduce off-flavours.

Reformulation of these two fermented dairy products using the selected approaches is feasible from both a microbiology and a technology perspective, providing a basis for extended, applied research. The results of this thesis work expand the current knowledge

of reformulation strategies for fermented dairy products, providing a basis for further detailed study. The transfer of the yoghurt reformulation option investigated here to an industrial scale is required as a next step; however, further research particularly into avoiding the calcium sensibility of the glucose-isomerase enzyme is also necessary to reduce costs and increase industrial applicability. Although an scaled-up trial production of Edam using the chosen reformulation strategies was successful in the lab of a commercial dairy and the reformulation approaches applied to Edam cheese do not incur massive cost increases for manufacturers, further experiments of this scale are needed to validate these results and to improve palatability. Continued product-specific research into the applicability of reformulation strategies is essential to present feasible reformulation approaches to manufacturers, ultimately providing a wider range of products with reduced sugar and reduced salt content to consumers. Together with legislative support and efficient public education, reformulation of yoghurt and cheese can contribute to reducing the morbidity and mortality resulting from NCDs on a population level.

Appendices

Extended materials

Table A 1: List of chemicals and reagents

Chemical/reagent	Application	Company
2-propanol	DNA extraction	Carl Roth, Karlsruhe, Germany
40 % Acrylamide/Bis-acrylamide solution (ratio 37.5 : 1)	DGGE gels	Bio-Rad Laboratories, Inc., CA., USA
50x TAE buffer	DGGE gels	Bio-Rad Laboratories, Inc., CA., USA
Acetic acid	Proteolysis test	Merck, Darmstadt, Germany
Agar-agar	Media preparation	Carl Roth, Karlsruhe, Germany
Agarose	Agarose gels	Carl Roth, Karlsruhe, Germany
Ammonium acetate	Media production and DNA extraction	Merck, Darmstadt, Germany
Ammonium persulphate	DGGE gels	Bio-Rad Laboratories, Inc., CA., USA
Ascorbic acid	Media preparation	Merck, Darmstadt, Germany
Beef extract	Media preparation	Carl Roth, Karlsruhe, Germany
Bromophenol blue	Loading buffer	Merck, Darmstadt, Germany
Cadmium chloride	Proteolysis test	Merck, Darmstadt, Germany
Calcium chloride	Media and brine preparation	Merck, Darmstadt, Germany
Casein hydrolysate	Media preparation	Merck, Darmstadt, Germany
Chlorophenol red	Media preparation	Merck, Darmstadt, Germany
D-galactose	Sugar fermentation tests	Merck, Darmstadt, Germany
D-glucose	Sugar fermentation tests	Merck, Darmstadt, Germany
D-glucose reagent for photometric determination	Carbohydrate determination	Thermo Fisher Scientific, Darmstadt, Germany
D- fructose	Sugar fermentation tests	Merck, Darmstadt, Germany
D-fructose for photometric determination	Carbohydrate determination	Thermo Fisher Scientific, Darmstadt, Germany
Delvocid® Instant	Media preparation	DSM Food Specialties, Delft, Netherlands

Table A 1 continued

DNAse-free RNase	DNA extraction	VWR International GmbH, Darmstadt, Germany
Ethylenediaminetetraacetic acid	Buffer preparation	Carl Roth, Karlsruhe, Germany
Formamide deionised	DGGE gels	Bio-Rad Laboratories, Inc., CA., USA
Gensweet® SGI	Glucose isomerisation	DuPont de Nemours GmbH, Neu-Isenurg, Germany
GelRed™ nucleic acid gel stain	DNA staining	Biotium, Inc., Fremont, CA, USA
Glycerol (85 %)	Media preparation and storage of bacteria	Carl Roth, Karlsruhe, Germany
Hannilase® XP 750 NB rennet	Cheese production	Chr. Hansen, Nienburg, Germany
HPLC gradient grade ethanol (99 %)	DNA extraction	Carl Roth, Karlsruhe, Germany
Hydrochloric acid	Buffer preparation	Carl Roth, Karlsruhe, Germany
L-alanine	Proteolysis test	Merck, Darmstadt, Germany
Lactose monohydrate	Media preparation and sugar fermentation tests	Merck, Darmstadt, Germany
Listeria enrichment supplement	Media preparation	VWR International GmbH, Darmstadt, Germany
Listeria selective supplement	Media preparation	VWR International GmbH, Darmstadt, Germany
Lithium chloride	Media preparation	VWR International GmbH, Darmstadt, Germany
Lysozyme	DNA extraction	AppliChem GmbH, Darmstadt, Germany
Magnesium chloride	Media preparation	Merck, Darmstadt, Germany
Manganese sulphate	Media preparation	Merck, Darmstadt, Germany
Magnesium sulphate	Media preparation	Merck, Darmstadt, Germany
Ninhydrin	Proteolysis test	Merck, Darmstadt, Germany
NOLA™ Fit 5500 β-galactosidase	Lactose hydrolysis	Chr. Hansen, Nienburg, Germany
Nucleotide triphosphates	PCR	Genaxxon Bioscience GmbH, Ulm, Germany
Peptone from casein	Media preparation	Carl Roth, Karlsruhe, Germany
Peptone from gelatine	Media preparation	Merck, Darmstadt, Germany

Table A 1 continued

Polypeptone	Media preparation	Carl Roth, Karlsruhe, Germany
Potassium dihydrogen phosphate	Media preparation	Merck, Darmstadt, Germany
Potassium sulphate	Media preparation	Merck, Darmstadt, Germany
Phytone™ peptone	Media preparation	BD Biosciences, Erembodegen, Belgium
Proteinase K	DNA extraction	AppliChem GmbH, Darmstadt, Germany
Pseudomonas CFC selective supplement	Media preparation	VWR International GmbH, Darmstadt, Germany
Ox bile	Media preparation	Merck, Darmstadt, Germany
RNase A	DNA extraction	VWR International GmbH, Darmstadt, Germany
Sodium acetate	Media and buffer preparation	Merck, Darmstadt, Germany
Sodium-β-glycerophosphate	Media preparation	VWR International GmbH, Darmstadt, Germany
Sodium chloride	Buffer and brine preparation	VWR International GmbH, Darmstadt, Germany
Sodium citrate	Media and buffer preparation	Merck, Darmstadt, Germany
Sodium dodecyl sulphate	Buffer preparation	Carl Roth, Karlsruhe, Germany
Sodium hydroxide	Media preparation	Carl Roth, Karlsruhe, Germany
Sodium propionate	Media preparation	Fluka Chemie AG, Buchs, Switzerland
sub4salt®	Cheese production	Jungbunzlauer Ladenburg GmbH, Ladenburg, Germany
Sucrose	Loading buffer	Merck, Darmstadt, Germany
Tetramethylethylenediamine (TEMED)	DGGE gels	Bio-Rad Laboratories, Inc., CA., USA
Tris base	Buffer preparation	Carl Roth, Karlsruhe, Germany
Tween® 80	Media preparation	Merck, Darmstadt, Germany
Urea	DGGE gels	Bio-Rad Laboratories, Inc., CA., USA
Yeast extract	Media preparation	Merck, Darmstadt, Germany

Table A 2: List of kits

Kit name	Application	Company
Acetic acid test kit	Determination of acetic acid in cheese	R-Biopharm AG, Darmstadt, Germany
DCode™ Electrophoresis Reagent Kit for DGGE/CDGE	DGGE	Bio-Rad Laboratories, Inc., CA., USA
D-/L-lactic acid test kit	Determination of D-/L-lactic acid in cheese	R-Biopharm AG, Darmstadt, Germany
Experion™ DNA 12K assay kit	Automated electrophoresis	Bio-Rad Laboratories, Inc., CA., USA
High efficiency Taq Polymerase with Buffer E	PCR	Genaxxon Bioscience GmbH, Ulm, Germany
PeqGOLD Bacterial DNA Mini Kit	DNA extraction	VWR International GmbH, Darmstadt, Germany
Phusion® High-Fidelity Master Mix with HF Buffer containing Phusion® High-Fidelity DNA polymerase	PCR	New England Biolabs, Frankfurt am Main, Germany
Lactose/D-galactose test kit	Carbohydrate determination	R-Biopharm AG, Darmstadt, Germany
MiSeq Reagent Kit v2	Whole genome sequencing	Illumina, Inc., CA, USA
MiSeq Reagent Kit v3	16S high throughput sequencing	Illumina, Inc., CA, USA
Nextera® XT Index Kit	Library preparation	Illumina, Inc., CA, USA
NucleoSpin® Gel and PCR Clean-up Kit	DNA clean up	Macherey-Nagel GmbH & Co. KG, Düren, Germany
QIAamp DNA Stool Mini Kit	DNA extraction	QIAGEN GmbH, Hilden, Germany
Qubit™ dsDNA BR Assay Kit	DNA quantification	Thermo Fisher Scientific, Darmstadt, Germany
Qubit™ dsDNA HS Assay Kit	DNA quantification	Thermo Fisher Scientific, Darmstadt, Germany

Table A 3: List of instruments

Instrument	Application	Company
8-channel pH meter with GigaLog S Datalogger	Parallel pH monitoring	IMC Ingenieurbüro Messelektronik, Chemnitz, Germany
BagMixer® lab blender	Homogenising yoghurt and cheese samples	Interscience for Microbiology, Saint Nom, France
BioDocAnalyze	Agarose gel visualisation	Biometra, Analytik Jena AG, Jena, Germany
BIORAD DCode™ System	DGGE	Bio-Rad Laboratories, Inc., CA, USA
Class 2 safety cabinet (Claire® B-2-190)	Sterile workspace for microbiology and molecular biology work	BERNER International GmbH, Elmshorn, Germany
Eppendorf Thermomixer	All heating steps for 1.5 mL and 2 mL Eppendorf Tubes®	Eppendorf AG, Hamburg, Germany
Experion™ Automated Electrophoresis System	Automated electrophoresis	Bio-Rad Laboratories, Inc., CA, USA
Heraeus Fresco21 centrifuge	Centrifugation of 1.5 mL and 2 mL Eppendorf Tubes®	Thermo Fisher Scientific, Waltham, MA, USA
Heraeus Multifuge 1 S-R (Rotor: 75002005)	Centrifugation of 15 mL and 50 mL Falcon® tubes	Thermo Fisher Scientific, Waltham, USA
HygroLab C1 bench-top indicator with digital a_w humidity-temperature probes	Water activity measurements	Rotronic Measurement Solutions, Bassersdorf, Switzerland
Incubator (APT.line™ KB 115)	Incubation of microbiological agar plates	BINDER GmbH, Tuttlingen, Germany
Incubator (CTS C+10/350)	Incubation of yoghurt during production	Clima Temperatur Systeme, Hechingen, Germany
inoLab pH Level 2	pH monitoring during yoghurt production	WTW GmbH & Co. KG, Weilheim, Germany
Intelli-Mixer RM-2M	Homogenisation of yoghurt and cheese samples	ELMI, Calabasas, CA, USA
Konelab 20i	UV-tests	Thermo Fisher Scientific, Waltham, USA
Purelab flex 2 water purification system	Production of type 1 ultrapure H_2O	Elga LabWater, High Wycombe, UK
M220 Focused-ultrasonicator™	DNA shearing	Covaris, Inc., Woburn, MA, USA

Table A 3 continued

MiSeq™ System	16S rRNA gene and whole genome sequencing	Illumina Inc., San Diego, CA, USA
MP-220 pH-Meter	pH measurements	Mettler Toledo, Greifensee, Schweiz
NanoDrop™ 2000 spectrophotometer	Measuring DNA concentrations	Thermo Fisher Scientific, Waltham, MA, USA
PeqSTAR thermocycler	PCR	VWR International GmbH, Darmstadt, Germany
Precelleys 24 lysis and homogenisation machine	Cell lysis	Bertin Instruments SAS, Montigny-le-Bretonneux, France
Qubit® 3.0 Fluorometer	Measuring DNA concentrations	Thermo Fisher Scientific, Waltham, MA, USA
SenTix™ insertion pH electrode	pH measurements	WTW™, Weilheim, Germany
Specord 50 spectrophotometer	UV-based tests	Analytik Jena AG, Jena, Germany
Ultrafiltration machine (custom-made)	Ultrafiltration of milk	Alfa Laval Mid Europe GmbH, Glinde, Germany
Water bath (F32 refrigerated and heating circulator)	Incubation of yoghurt for laboratory production	Julabo GmbH, Seelbach, Germany

Table A 4: List of consumables

Consumable	Application	Company
0.2 µm filter	Sterile filtration of heat sensitive solutions	VWR International GmbH, Darmstadt, Germany
0.5 mL deep-well plate	Preparation of sequencing libraries	Thermo Fisher Scientific, Darmstadt, Germany
10 x 4 x 45 mm cuvettes	Spectrophotometer measurements	Sarstedt AG & Co. KG, Nümbrecht, Germany
15 mL / 50 mL Falcon® conical centrifuge tubes	Centrifugation of samples	Corning Inc., New York, USA
96-well plate	Sugar utilisation tests	VWR International GmbH, Darmstadt, Germany
AMPure XP magnetic beads	Library preparation	Beckman Coulter, Inc., CA, USA
BagFilter® 400 P lab blender bag with < 250 µm filter	Sample preparation	Interscience for Microbiology, Saint Nom, France
Cheese foil (300 x 400 mm)	Vacuum wrapping of cheese	IP Ingredients, Süderlügum, Germany
Cheese moulds (height and diameter: 14 cm)	Pressing of cheese curd	MilkySky GmbH, Lauben, Germany
1.5 mL / 2 mL Eppendorf Tubes®	Sample preparation	Eppendorf AG, Hamburg, Germany
GELoader Tips (0.5 - 20 µL)	Loading of DGGE gels	VWR International GmbH, Darmstadt, Germany
Oxoid™AnaeroGen™ sachets	Anaerobic incubation	Thermo Fisher Scientific, Darmstadt, Germany
PCR clean and sterile filter tips	All sterile pipetting work	Eppendorf AG, Hamburg, Germany
Purolite® S930 Plus cation exchanger	Cation exchange	Purolite Germany GmbH, Ratingen, Germany
Type HF 1.0-43-PM5-PB membrane	Ultrafiltration of milk	Koch Membrane Systems, Wilmington, Massachusetts, USA
Zirconium/glass-Beads® (0.1 mm)	Genomic DNA extraction	Carl Roth, Karlsruhe, Germany

Table A 5: Software used for data analysis

Software	Application	Company/Author
Basic Local Alignment Search Tool (BLAST®)	Comparison of DNA sequences with online database	Public domain software from the National Center for Biotechnology Information (NCBI), Bethesda, MD, USA https://blast.ncbi.nlm.nih.gov/Blast.cgi
BioNumerics software, Version 7.6.2	DGGE band analysis	Applied Maths, NV, Sint-Martens-Latem, Belgium © 1998-2017
Chromas, Version 2.6.5	Chromatogram analysis	Technelysium Pty Ltd, Brisbane, Australia © 1998-2018
IMNGS, integrated microbial NGS platform	Analysis of prokaryotic 16S rRNA gene amplicon datasets	Lagkouvardos et al. 2016 [193] https://www.imngs.org/
GigaTerm	Data tracking in conjunction with the GigaLog S Datalogger	Controlord, La Farlede, France www.controlord.fr
GIMP 2.10.10	Image processing	Kimball, Mattis and The GIMP Development Team © 1995-2019 https://www.gimp.org/
Microsoft Excel, Version 1808	Data processing and statistics	Microsoft Office Professional Plus 2019, Microsoft Corporation, Redmond, WA, USA
MegAlign™	Alignment of DNA sequences	DNASTAR, Inc., Madison, WI, USA © 1993-2015
Orthologous Average Nucleotide Identity Tool (OAT) software	Heatmap generation	Lee et al. 2015 [204]
PATRIC, Version 3.5.43	Genome annotation	Wattam et al. 2017 [202] https://www.patricbrc.org/
RStudio, Version 1.1.423	Metagenomics analyses	RStudio, Inc., Boston, MA, USA © 2009-2018
SigmaPlot, Version 14.0	Create graphs	Systat Software Inc., San Jose, CA, USA
SINA, Arb Silva Aligner, Version 1.2.11	OTU database comparisons	Pruesse et al. 2012 [197] https://www.arb-silva.de/aligner/

Table A 6: List of primers and probes

Primer name	Sequence (5' - 3')	Source
16S 27f	AGA GTT TGA TCM(A/C) TGG CTC AG	Rademaker et al. 2006[247]
16S 1540r	AAG GAG GTG ATC CAA CCG CA	Ohnishi et al. 2011[248]
338f-GC	GC Clamp[a]-ACT CCT ACG GGA GGC AGC AG	Cocolin et al. 2001[249]
GC Clamp[a]	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C	Rettedal et al. 2010[250]
518r	ATT ACC GCG GCT GCT GG	Cocolin et al. 2001[249]
16S fw-meta	TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG	Klindworth et al. 2013[251]
16S rev-meta	GCT TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C	Klindworth et al. 2013[251]

Table A 7: DGGE gel composition

	Component	Quantity
35 % Gel	40 % Acrylamide/Bis-acrylamide (ratio 37.5 : 1)	5 mL
	50x TAE buffer	500 µL
	Formamide deionised	3.5 mL
	Urea	3.68 g
	Type 1 ultrapure H_2O	Make to 25 mL, then add:
	Tetramethylethylenediamine (TEMED)	55 µL
	Ammonium persulphate (10 %)	95 µL
70 % Gel	40 % Acrylamide/Bis-acrylamide (ratio 37.5 : 1)	5 mL
	50x TAE buffer	500 µL
	Formamide deionised	6.25 mL
	Urea	7.35 g
	Type 1 ultrapure H_2O	Make to 25 mL, then add:
	TEMED	55 µL
	Ammonium persulphate (10 %)	95 µL

Acidification curves (Chr. Hansen)

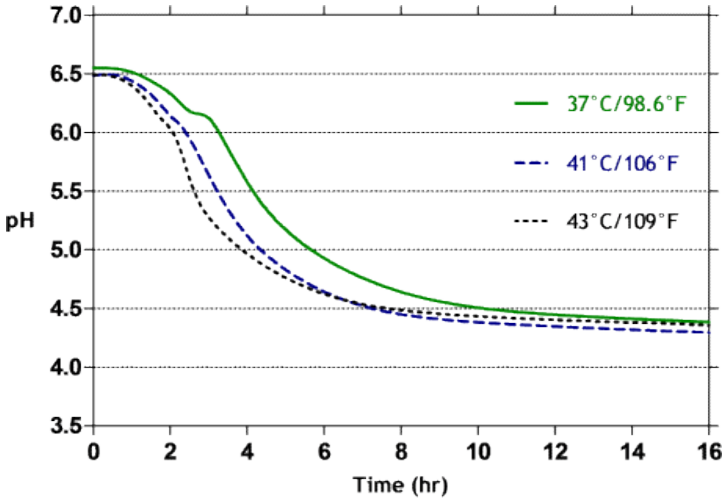

Figure A 1: Acidification curve for the YF-L812 culture provided by Chr. Hansen in the product data sheet.

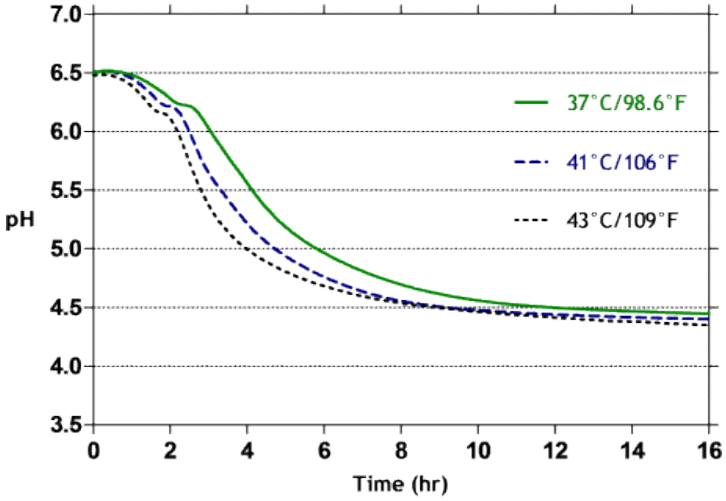

Figure A 2: Acidification curve for the YoFlex® Mild 2.0 culture provided by Chr. Hansen in the product data sheet.

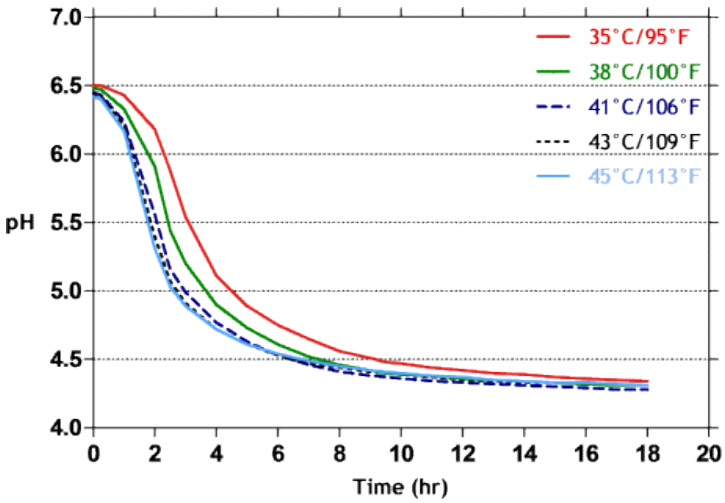

Figure A 3: Acidification curve for the YoFlex® Premium 1.0 culture provided by Chr. Hansen in the product data sheet.

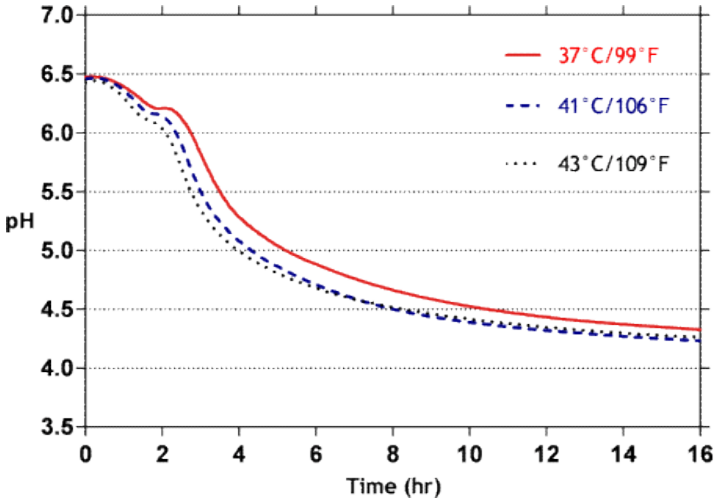

Figure A 4: Acidification curve for the YoFlex® Premium 4.0 culture provided by Chr. Hansen in the product data sheet.

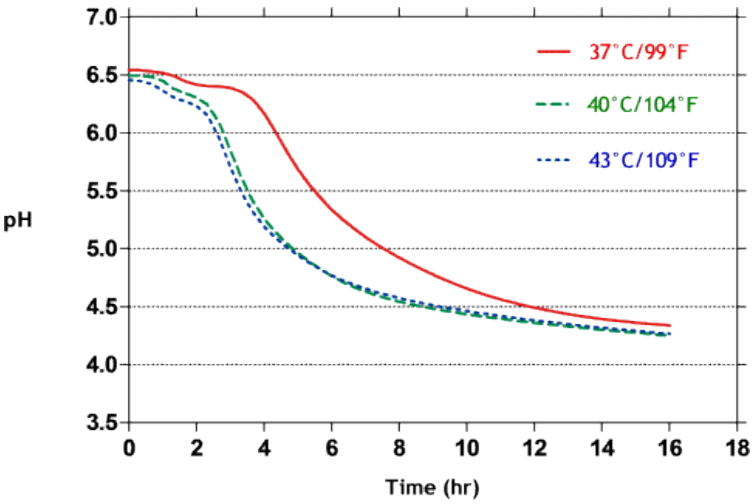

Figure A 5: Acidification curve for the ABT-6 Probio-Tec® culture provided by Chr. Hansen in the product data sheet.

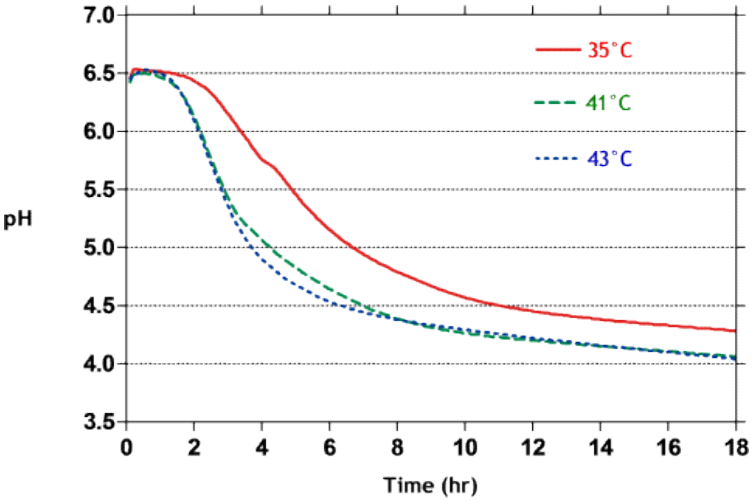

Figure A 6: Acidification curve for the ABT-100 culture provided by Chr. Hansen in the product data sheet.

References

1. He, F. J. & Macgregor, G. A. Salt and sugar: their effects on blood pressure. *Pflügers Arch. - Eur. J. Physiol.* **467**, 577–586 (2015).

2. Rippe, J. M. & Angelopoulos, T. J. Relationship between added sugars consumption and chronic disease risk factors: current understanding. *Nutrients* **8**, 1–19 (2016).

3. He, F. J., Jenner, K. H. & MacGregor, G. A. WASH - World action on salt and health. *Kidney Int.* **78**, 745–753 (2010).

4. Strohm, D. *et al.* Salt intake in Germany, health consequences, and resulting recommendations for action. A scientific statement from the German Nutrition Society (DGE). *Ernährungs Umschau* **63**, 62–70 (2016).

5. World Health Organization. Global status report on noncommunicable diseases. (2014). Available at: https://www.who.int/nmh/publications/ncd-status-report-2014/en/. (Accessed: 25th July 2019)

6. World Health Organization. Guideline: sugars intake for adults and children. (2015). Available at: https://www.who.int/nutrition/publications/guidelines/sugars_intake/en/. (Accessed: 16th April 2019)

7. World Health Organization. Guideline: sodium intake for adults and children. (2014). Available at: https://www.who.int/nutrition/publications/guidelines/sodium_intake/en/. (Accessed: 4th June 2019)

8. Domnariu, C. D., Cucu, A. & Furtunescu, F. L. Public health and management: World Health Organization guidelines on salt intake in adults and children. *Acta Medica Transilv.* **2**, 166–168 (2013).

9. Liebermann, M. & Marks, A. D. *Marks' Basic Medical Biochemistry: A Clinical Approach*. (Lippincott Williams & Wilkins, 2009).

10. World Health Organization. Reducing salt intake in populations: report of a WHO forum and technical meeting. (2007). Available at: https://www.who.int/dietphysicalactivity/reducingsaltintake_EN.pdf. (Accessed: 26th July 2018)

11. Doyle, M. E. & Glass, K. A. Sodium reduction and its effect on food safety, food quality, and human health. *Compr. Rev. Food Sci. Food Saf.* **9**, 44–56 (2010).

12. Deutsche Gesellschaft für Ernährung (ed.). *13. DGE-Ernährungsbericht*. (Köllen Druck + Verlag GmbH, 2016).

13. Brown, I. J., Tzoulaki, I., Candeias, V. & Elliott, P. Salt intakes around the world: implications for public health. *Int. J. Epidemiol.* **38**, 791–813 (2009).

14. Dötsch, M. *et al.* Strategies to reduce sodium consumption: a food industry perspective. *Crit. Rev. Food Sci. Nutr.* **49**, 841–851 (2009).

15. Te Morenga, L., Mallard, S. & Mann, J. Dietary sugars and body weight: systematic review and meta-analyses of randomised controlled trials and cohort studies. *BMJ* **345:e7492**, 1–25 (2013).

16. Johnson, R. K. *et al.* Dietary sugars intake and cardiovascular health: a scientific statement from the American Heart Association. *Circulation* **120**, 1011–1020 (2009).

17. World Health Organization. Incentives and disincentives for reducing sugar in manufactured foods: an exploratory supply chain analysis. (2017). Available at: http://www.euro.who.int/en/health-topics/disease-prevention/nutrition/publications/2017/incentives-and-disincentives-for-reducing-sugar-in-manufactured-foods-2017. (Accessed: 18th June 2019)

18. European Commission. Implementation of the EU salt reduction framework. (2012). Available at: https://ec.europa.eu/health/sites/health/files/nutrition_physical_activity/docs/salt_report1_en.pdf. (Accessed: 11th October 2018)

19. Commission of the European communities. White paper on a strategy for Europe on nutrition, overweight and obesity related health issues. (2007). Available at: https://ec.europa.eu/health/archive/ph_determinants/life_style/nutrition/documents/nutrition_wp_en.pdf. (Accessed: 7th June 2019)

20. Appel, L. J. *et al.* Population-wide sodium reduction: the bumpy road from evidence to policy. *Ann. Epidemiol.* **22**, 417–425 (2012).

21. He, F. J., Campbell, N. R. C. & MacGregor, G. A. Reducing salt intake to prevent hypertension and cardiovascular disease. *Rev. Panam. Salud Pública* **32**, 293–300 (2012).

22. He, F. J. & MacGregor, G. A. Reducing population salt intake worldwide: from evidence to implementation. *Prog. Cardiovasc. Dis.* **52**, 363–382 (2010).

23. He, F. J. & MacGregor, G. A. A comprehensive review on salt and health and current experience of worldwide salt reduction programmes. *J. Hum. Hypertens.* **23**, 363–384 (2009).

24. Afeiche, M. C., Koyratty, B. N. S., Wang, D., Jacquier, E. F. & Lê, K.-A. Intakes and sources of total and added sugars among 4 to 13-year-old children in China, Mexico and the United States. *Pediatr. Obes.* **13**, 204–212 (2018).

25. Mattes, R. & Donnelly, D. Relative contributions of dietary sodium sources. *J. Am. Coll. Nutr.* **10**, 383–393 (1991).

26. James, W. P. T., Ralph, A. & Sanchez-Castillo, C. P. The dominance of salt in manufactured food in the sodium intake of affluent societies. *Lancet* **329**, 426–429 (1987).

27. Max Rubner-Institut. Nationale Verzehrsstudie II, Ergebnisbericht, Teil 2. (2008). Available at: https://www.bmel.de/SharedDocs/Downloads/Ernaehrung/NVS_ErgebnisberichtTeil2.pdf?__blob=publicationFile. (Accessed: 25th September 2018)

28. Azaïs-Braesco, V., Sluik, D., Maillot, M., Kok, F. & Moreno, L. A. A review of total and added sugar intakes and dietary sources in Europe. *Nutr. J.* **16**, 1–15 (2017).

29. Huth, P. J., Fulgoni III, V. L., Keast, D. R., Park, K. & Auestad, N. Major food sources of calories, added sugars, and saturated fat and their contribution to essential nutrient intakes in the U.S. diet: data from the national health and nutrition examination survey (2003-2006). *Nutr. J.* **12**, 1–10 (2013).

30. Alexy, U., Sichert-Hellert, W. & Kersting, M. Associations between intake of added sugars and intakes of nutrients and food groups in the diets of German children and adolescents. *Br. J. Nutr.* **90**, 441–447 (2003).

31. Max Rubner-Institut. Reformulierung von verarbeiteten Lebensmitteln: Bewertungen und Empfehlungen zur Reduktion des Zuckergehalts. (2016). Available at: https://www.mri.bund.de/fileadmin/MRI/Themen/Reformulierung/Reformulierung_Thema-Zucker.pdf. (Accessed: 10th June 2019)

32. van Raaij, J., Hendriksen, M. & Verhagen, H. Potential for improvement of

population diet through reformulation of commonly eaten foods. *Public Health Nutr.* **12**, 325–330 (2009).

33. Sleator, R. D. & Hill, C. Molecular analysis of the microbial food safety implications of food reformulations for improved health. *Foodborne Pathog. Dis.* **5**, 499–504 (2008).

34. Buttriss, J. L. Food reformulation: the challenges to the food industry. *Proc. Nutr. Soc.* **72**, 61–69 (2013).

35. Leroy, P., Réquillart, V., Soler, L.-G. & Enderli, G. An assessment of the potential health impacts of food reformulation. *Eur. J. Clin. Nutr.* **70**, 694–699 (2016).

36. Pietinen, P., Valsta, L. M., Hirvonen, T. & Sinkko, H. Labelling the salt content in foods: a useful tool in reducing sodium intake in Finland. *Public Health Nutr.* **11**, 335–340 (2008).

37. Bundesministerium für Ernährung und Landwirtschaft. Ergebnis der Verbraucherbeteiligung liegt vor: Bundeesministerin Julia Klöckner wird Nutri-Score® einführen (Press release Nr. 197, 30.09.2019). Available at: https://www.bmel.de/SharedDocs/Pressemitteilungen/2019/197-eNWK.html. (Accessed: 18th November 2019)

38. Kloss, L., Meyer, J. D., Graeve, L. & Vetter, W. Sodium intake and its reduction by food reformulation in the European Union - A review. *NFS J.* **1**, 9–19 (2015).

39. Taormina, P. J. Implications of salt and sodium reduction on microbial food safety. *Crit. Rev. Food Sci. Nutr.* **50**, 209–227 (2010).

40. Goldfein, K. R. & Slavin, J. L. Why sugar is added to food: food science 101. *Compr. Rev. Food Sci. Food Saf.* **14**, 644–656 (2015).

41. Murray, C. J. *et al.* Effectiveness and costs of interventions to lower systolic blood pressure and cholesterol: a global and regional analysis on reduction of cardiovascular-disease risk. *Lancet* **361**, 717–725 (2003).

42. Schorling, E., Niebuhr, D. & Kroke, A. Cost-effectiveness of salt reduction to prevent hypertension and CVD: a systematic review. *Public Health Nutr.* **20**, 1993–2003 (2017).

43. Bibbins-Domingo, K. *et al.* Projected effect of dietary salt reductions on future cardiovascular disease. *N. Engl. J. Med.* **362**, 590–599 (2010).

44. World Health Organization. WHO handbook for guideline development - 2nd edition. (2014). Available at: https://www.who.int/publications/guidelines/handbook_2nd_ed.pdf?ua=1. (Accessed: 25th July 2019)

45. Johner, S. A., Thamm, M., Schmitz, R. & Remer, T. Current daily salt intake in Germany: biomarker-based analysis of the representative DEGS study. *Eur. J. Nutr.* **54**, 1109–1115 (2015).

46. Laatikainen, T. *et al.* Sodium in the Finnish diet: 20-year trends in urinary sodium excretion among the adult population. *Eur. J. Clin. Nutr.* **60**, 965–970 (2006).

47. Vartiainen, E. *et al.* Thirty-five-year trends in cardiovascular risk factors in Finland. *Int. J. Epidemiol.* **39**, 504–518 (2010).

48. Trieu, K. *et al.* Salt reduction initiatives around the world - a systematic review of progress towards the global target. *PLoS One* **10**, e0130247 (2015).

49. Baker, P., Jones, A. & Thow, A. M. Accelerating the worldwide adoption of sugar-sweetened beverage taxes: strengthening commitment and capacity. *Int. J. Heal. Policy Manag.* **7**, 474–478 (2018).

50. HM Revenue and Customs. Soft drinks industry levy policy paper. (2016). Available at: https://www.gov.uk/government/publications/soft-drinks-industry-levy/soft-drinks-industry-levy. (Accessed: 5th June 2019)

51. Bundesministerium für Ernährung und Landwirtschaft. Nationale Reduktions- und Innovationsstrategie für Zucker, Fette und Salz in Fertigprodukten. (2018). Available at: https://www.in-form.de/fileadmin/Dokumente/PDF/20181121bmel-strategiepapier-zucker.pdf. (Accessed: 10th June 2019)

52. Liem, D. G., Miremadi, F. & Keast, R. S. J. Reducing sodium in foods: the effect on flavor. *Nutrients* **3**, 694–711 (2011).

53. Mohan, S., Campbell, N. R. C. & Willis, K. Effective population-wide public health interventions to promote sodium reduction. *Can. Med. Assoc. J.* **181**, 605–609 (2009).

54. Girgis, S. *et al.* A one-quarter reduction in the salt content of bread can be made without detection. *Eur. J. Clin. Nutr.* **57**, 616–620 (2003).

55. Bae, I., Park, J.-H., Choi, H.-Y. & Jung, H.-K. Emerging innovations to reduce the salt content in cheese; effects of salt on flavor, texture, and shelf life of

cheese; and current salt usage: a review. *Korean J. Food Sci. Anim. Resour.* **37**, 793–798 (2017).

56. Lê, K.-A., Robin, F. & Roger, O. Sugar replacers: from technological challenges to consequences on health. *Curr. Opin. Clin. Nutr. Metab. Care* **19**, 310–315 (2016).

57. Kamleh, R. *et al.* The effect of partial substitution of NaCl with KCl on the physicochemical, microbiological and sensory properties of Akkawi cheese. *J. Sci. Food Agric.* **95**, 1940–1948 (2015).

58. Charlton, K. E., Macgregor, E., Vorster, N. H., Levitt, N. S. & Steyn, K. Partial replacement of NaCl can be achieved with potassium, magnesium and calcium salts in brown bread. *Int. J. Food Sci. Nutr.* **58**, 508–521 (2007).

59. Park, J. N., Hwang, K. T., Kim, S. B. & Kim, S. Z. Partial replacement of NaCl by KCl in salted mackerel (*Scomber japonicus*) fillet products: effect on sensory acceptance and lipid oxidation. *Int. J. Food Sci. Technol.* **44**, 1572–1578 (2009).

60. Guggisberg, D., Piccinali, P. & Schreier, K. Effects of sugar substitution with Stevia, Actilight™ and Stevia combinations or Palatinose™ on rheological and sensory characteristics of low-fat and whole milk set yoghurt. *Int. Dairy J.* **21**, 636–644 (2011).

61. Yeung, C. H. C. *et al.* Modelling of the impact of universal added sugar reduction through food reformulation. *Sci. Rep.* **7**, 1–8 (2017).

62. Durack, E., Alonso-Gomez, M. & Wilkinson, M. G. Salt: a review of its role in food science and public health. *Curr. Nutr. Food Sci.* **4**, 290–297 (2008).

63. Todar, K. Todar's Online Textbook of Bacteriology. (2012). Available at: www.textbookofbacteriology.net. (Accessed: 6th June 2019)

64. Leroy, F. & De Vuyst, L. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci. Technol.* **15**, 67–78 (2004).

65. von Wright, A. & Axelsson, L. Lactic acid bacteria: an introduction. in *Lactic Acid Bacteria: Microbiological and Functional Aspects* (eds. Lahtinen, S., Salminen, S., Ouwehand, A. C. & Von Wright, A.) 1–16 (CRC Press, 2012).

66. Khalid, K. An overview of lactic acid bacteria. *Int. J. Biosci.* **1**, 1–13 (2011).

67. Adams, M. R. & Moss, M. O. *Food Microbiology*. (The Royal Society of Chemistry, 2008).

68. Cogan, T. M. *et al.* Invited review: advances in starter cultures and cultured foods. *J. Dairy Sci.* **90**, 4005–4021 (2007).

69. Aryana, K. J. & Olson, D. W. A 100-year review: yogurt and other cultured dairy products. *J. Dairy Sci.* **100**, 9987–10013 (2017).

70. Jay, J. M., Loessner, Martin, J. & Golden, D. A. *Modern Food Microbiology*. (Springer Science + Business Media, Inc., 2005).

71. Teusink, B. & Molenaar, D. Systems biology of lactic acid bacteria: for food and thought. *Curr. Opin. Syst. Biol.* **6**, 7–13 (2017).

72. Casey, M. G., Häni, J. P., Gruskovnjak, J., Schaeren, W. & Wechsler, D. Characterisation of the non-starter lactic acid bacteria (NSLAB) of Gruyère PDO cheese. *Le Lait, INRA Ed.* **86**, 407–414 (2006).

73. Fox, P. F., Guinee, T. P., Cogan, T. M. & McSweeney, P. L. H. *Fundamentals of Cheese Science*. (Springer New York, 2017).

74. Doyle, M. P. & Beuchat, L. R. *Microbiology: Fundamentals and Frontiers*. (ASM Press, 2007).

75. Irlinger, F., Helinck, S. & Jany, J. L. Secondary and adjunct cultures. in *Cheese: Chemistry, Physics and Microbiology* (eds. McSweeney, P. L. H., Fox, P. F., Cotter, P. D. & Everett, D. W.) 273–300 (Elsevier Ltd., 2017).

76. Settanni, L. & Moschetti, G. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. *Food Microbiol.* **27**, 691–697 (2010).

77. Hill, D. *et al.* Recent advances in microbial fermentation for dairy and health. *F1000Research* **6**, 1–9 (2017).

78. Ashraf, R. & Shah, N. P. Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt - a review. *Int. J. Food Microbiol.* **149**, 194–208 (2011).

79. Parente, E., Cogan, T. M. & Powell, I. B. Starter cultures: general aspects. in *Cheese: Chemistry, Physics and Microbiology* (eds. McSweeney, P. L. H., Fox, P. F., Cotter, P. D. & Everett, D. W.) 201–226 (Elsevier Ltd., 2017).

80. Freitas, A. C., Rodrigues, D., Duarte, A. C. & Gomes, A. M. The principles of cheese making: an overview. in *Handbook of Cheese in Health: Production, Nutrition and Medical Sciences* (eds. Preedy, V. R., Watson, R. R. & Patel, V.

B.) 55–72 (Wageningen Academic Publishers, 2013).

81. International Organization for Standardization. ISO 27205:2010 [IDF 149:2010] Fermented milk products - bacterial starter cultures - standard of identity. in *67.100.10 Milk and Processed Milk Products* (International Organization for Standardization, 2010).

82. Holsinger, V. H., Rajkowski, K. T. & Stabel, J. R. Milk pasteurisation and safety: a brief history and update. *Sci. Tech. Rev. Off. Int. des Epizoot.* **16**, 441–451 (1997).

83. Spreer, E. *Technologie der Milchverarbeitung*. (B. Behr's Verlag, 2011).

84. Driessen, F., Kingma, F. & Stadhouders, J. Evidence that *Lactobacillus bulgaricus* in yogurt is stimulated by carbon dioxide produced by *Streptococcus thermophilus*. *Netherlands Milk Dairy J.* **36**, 135–144 (1982).

85. Perez, P. F., De Antoni, G. L. & Anon, M. C. Formate production by *Streptococcus thermophilus* cultures. *J. Dairy Sci.* **74**, 2850–2854 (1991).

86. Horiuchi, H. & Sasaki, Y. Short communication: effect of oxygen on symbiosis between *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. *J. Dairy Sci.* **95**, 2904–2909 (2012).

87. Möller, C., Bockelmann, W., Ammann, A. & Heller, K. J. Production of yoghurt with mild taste by a *Lactobacillus delbrueckii* subsp. *bulgaricus* mutant with altered proteolytic properties. *Biotechnol. J.* **2**, 469–479 (2007).

88. Kailasapathy, K. & Chin, J. Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immunol. Cell Biol.* **78**, 80–88 (2000).

89. Shah, N. P., Lankaputhra, W. E. V., Britz, M. L. & Kyle, W. S. A. Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *Int. Dairy J.* **5**, 515–521 (1995).

90. Bundesministerium der Justiz und für Verbraucherschutz. Verordnung über Milcherzeugnisse (MilchErzV) vom 15. Juli 1970. *BGBl* I S. 1150 (1970).

91. Chandan, R. C. & O'Rell, K. R. Principles of yoghurt processing. in *Manufacturing Yogurt and Fermented Milks* (eds. Chandan, R. C., White, C. H., Kilara, A. & Hui, Y. H.) 195–209 (Blackwell Publishing, 2006).

92. Schaafsma, G. Lactose and lactose derivatives as bioactive ingredients in

human nutrition. *Int. Dairy J.* **18**, 458–465 (2008).

93. Frye, C. P. Regulations for product standards and labeling. in *Manufacturing Yogurt and Fermented Milks* (eds. Chandan, R. C., White, C. H., Kilara, A. & Hui, Y. H.) 57–71 (Blackwell Publishing, 2006).

94. Harju, M., Kallioinen, H. & Tossavainen, O. Lactose hydrolysis and other conversions in dairy products: technological aspects. *Int. Dairy J.* **22**, 104–109 (2012).

95. Li, X. E., Lopetcharat, K., Qiu, Y. & Drake, M. A. Sugar reduction of skim chocolate milk and viability of alternative sweetening through lactose hydrolysis. *J. Dairy Sci.* **98**, 1455–1466 (2014).

96. Abbasi, S. & Saeedabadian, A. Influences of lactose hydrolysis of milk and sugar reduction on some physical properties of ice cream. *J. Food Sci. Technol.* **52**, 367–374 (2015).

97. Chiu, C. P. & Kosikowski, F. V. Conversion of glucose in lactase-hydrolyzed whey permeate to fructose with immobilized glucose isomerase. *J. Dairy Sci.* **69**, 959–964 (1986).

98. Abril, J. R. & Stull, J. W. Lactose hydrolysis in acid whey with subsequent glucose isomerisation. *J. Sci. Food Agric.* **48**, 511–514 (1989).

99. Lorenzen, P. C., Breiter, J., Clawin-Rädecker, I. & Dau, A. A novel bi-enzymatic system for lactose conversion. *Int. J. Food Sci. Technol.* **48**, 1396–1403 (2013).

100. Phadungath, C. Casein micelle structure: a concise review. *J. Sci. Technol.* **27**, 201–212 (2005).

101. Arndt, E. A. & Wehling, R. L. Development of hydrolyzed and hydrolyzed-isomerized syrups from cheese whey ultrafiltration permeate and their utilization in ice cream. *J. Food Sci.* **54**, 880–884 (1989).

102. van Hoorde, K. & van Landschoot, A. Gouda-type cheeses. in *Handbook of Cheese in Health: Production, Nutrition and Medical Sciences* (eds. Preedy, V. R., Watson, R. R. & Patel, V. B.) 227–236 (Wageningen Academic Publishers, 2013).

103. Guinee, T. P. & Fox, P. F. Salt in cheese: physical, chemical and biological aspects. in *Cheese: Chemistry, Physics and Microbiology* (eds. McSweeney, P. L. H., Fox, P. F., Cotter, P. D. & Everett, D. W.) 317–375 (Elsevier Ltd, 2017).

104. van den Berg, G. Dutch-type cheeses. *Encyclopedia of Food Sciences and Nutrition* 1129–1135 (2003).

105. Farkye, N. Y. Cheeses: chemistry and microbiology of maturation. *Encyclopedia of Food Sciences and Nutrition* 1062–1066 (2003).

106. Fox, P. F. & McSweeney, P. L. H. Cheese: an overview. in *Cheese: Chemistry, Physics and Microbiology* (eds. McSweeney, P. L. H., Fox, P. F., Cotter, P. D. & Everett, D. W.) 5–21 (Elsevier Ltd., 2017).

107. Düsterhöft, E. M., Engels, W. & van den Berg, G. Dutch-type cheeses. *Encyclopedia of Dairy Sciences* 721–727 (2011).

108. El-Bakry, M. Salt in cheese: a review. *Current Research in Dairy Sciences* **4**, 1–5 (2012).

109. Smoot, L. A. & Pierson, M. D. Inhibition and control of bacterial spore germination. *J. Food Prot.* **45**, 84–92 (1982).

110. Institute of Medicine. *Strategies to Reduce Sodium Intake in the United States*. (The National Academies Press, 2010).

111. Guinee, T. P. & Sutherland, B. J. Cheese: salting of cheese. *Encyclopedia of Dairy Sciences* 595–606 (2011).

112. Ayyash, M., Sherkat, F. & Shah, N. Sodium chloride substitution of cheese. in *Handbook of Cheese in Health: Production, Nutrition and Medical Sciences* (eds. Preedy, V. R., Watson, R. R. & Patel, V. B.) 545–565 (Wageningen Academic Publishers, 2013).

113. Guinee, T. P. Salting and the role of salt in cheese. *Int. J. Dairy Technol.* **57**, 99–109 (2004).

114. Kongo, J. M. & Malcata, F. X. Cheese: chemistry and microbiology. *Encyclopedia of Food and Health* 735–740 (2016).

115. Marino, M., Frigo, F., Bartolomeoli, I. & Maifreni, M. Microbial community composition in cheese using molecular techniques. in *Handbook of Cheese in Health: Production, Nutrition and Medical Sciences* (eds. Preedy, V. R., Watson, R. R. & Patel, V. B.) 517–529 (Wageningen Academic Publishers, 2013).

116. McSweeney, P. L. H. Biochemistry of cheese ripening. *Int. J. Dairy Technol.* **57**, 127–144 (2004).

117. McSweeney, P. L. H. Biochemistry of cheese ripening: introduction and overview. in *Cheese: Chemistry, Physics and Microbiology* (eds. McSweeney, P. L. H., Fox, P. F., Cotter, P. D. & Everett, D. W.) 379–387 (Elsevier Ltd., 2017).

118. Tungjaroenchai, W., Drake, M. A. & White, C. H. Influence of adjunct cultures on ripening of reduced fat Edam cheeses. *J. Dairy Sci.* **84**, 2117–2124 (2001).

119. Porcellato, D., Østlie, H. M., Brede, M. E., Martinovic, A. & Skeie, S. B. Dynamics of starter, adjunct non-starter lactic acid bacteria and propionic acid bacteria in low-fat and full-fat Dutch-type cheese. *Int. Dairy J.* **33**, 104–111 (2013).

120. Grummer, J., Bobowski, N., Karalus, M., Vickers, Z. & Schoenfuss, T. Use of potassium chloride and flavor enhancers in low sodium Cheddar cheese. *J. Dairy Sci.* **96**, 1401–1418 (2013).

121. Rulikowska, A. *et al.* The impact of reduced sodium chloride content on Cheddar cheese quality. *Int. Dairy J.* **28**, 45–55 (2013).

122. Hystead, E., Diez-Gonzalez, F. & Schoenfuss, T. C. The effect of sodium reduction with and without potassium chloride on the survival of *Listeria monocytogenes* in Cheddar cheese. *J. Dairy Sci.* **96**, 6172–6185 (2013).

123. Ganesan, B., Brown, K., Irish, D. A., Brothersen, C. & McMahon, D. J. Manufacture and sensory analysis of reduced- and low-sodium Cheddar and Mozzarella cheeses. *J. Dairy Sci.* **97**, 1970–1982 (2014).

124. McMahon, D. J. *et al.* Effect of sodium, potassium, magnesium, and calcium salt cations on pH, proteolysis, organic acids, and microbial populations during storage of full-fat Cheddar cheese. *J. Dairy Sci.* **97**, 4780–4798 (2014).

125. Murtaza, M. A. *et al.* Texture, flavor, and sensory quality of buffalo milk Cheddar cheese as influenced by reducing sodium salt content. *J. Dairy Sci.* **97**, 6700–6707 (2014).

126. Lu, Y. & McMahon, D. J. Effects of sodium chloride salting and substitution with potassium chloride on whey expulsion of Cheddar cheese. *J. Dairy Sci.* **98**, 78–88 (2015).

127. Khetra, Y., Kanawjia, S. K. & Puri, R. Selection and optimization of salt replacer, flavour enhancer and bitter blocker for manufacturing low sodium Cheddar cheese using response surface methodology. *LWT - Food Sci. Technol.* **72**, 99–106 (2016).

128. Johnson, M. E., Kapoor, R., McMahon, D. J., McCoy, D. R. & Narasimmon, R. G. Reduction of sodium and fat levels in natural and processed cheeses: scientific and technological aspects. *Compr. Rev. Food Sci. Food Saf.* **8**, 252–268 (2009).

129. McCarthy, C. M., Wilkinson, M. G., Kelly, P. M. & Guinee, T. P. Effect of salt and fat reduction on the composition, lactose metabolism, water activity and microbiology of Cheddar cheese. *Dairy Sci. Technol.* **95**, 587–611 (2015).

130. McCarthy, C. M., Kelly, P. M., Wilkinson, M. G. & Guinee, T. P. Effect of fat and salt reduction on the changes in the concentrations of free amino acids and free fatty acids in Cheddar-style cheeses during maturation. *J. Food Compos. Anal.* **59**, 132–140 (2017).

131. Ferrão, L. L. *et al.* Strategies to develop healthier processed cheeses: reduction of sodium and fat contents and use of prebiotics. *Food Res. Int.* **86**, 93–102 (2016).

132. Barth, C. *et al.* Possibilities and limits of reducing the salt content in semi-hard cheese. *Kieler Milchwirtsch. Forschungsberichte* **41**, 105–136 (1989).

133. Prokopek, D. *et al.* Reduction of the salt content in Edam cheese in industrial production. *Kieler Milchwirtsch. Forschungsberichte* **42**, 565–596 (1990).

134. Wachowska, M. [Microbiological changes in Edam-type cheese, brined in a mixture of sodium and potassium chloride during the ripening process]. *Rocz. Panstw. Zakl. Hig.* **61**, 419–424 (2010).

135. Küçüköner, E. & Martin, J. H. Microbial changes on full and reduced fat Edam cheese during maturation. *Food Sci. Technol. Res.* **5**, 262–264 (1999).

136. Küçüköner, E. & Haque, Z. U. Physico-chemical and rheological properties of full fat and low fat Edam cheeses. *Eur. Food Res. Technol.* **217**, 281–286 (2003).

137. Ruyssen, T. *et al.* Characterisation of Gouda cheeses based on sensory, analytical and high-field 1H nuclear magnetic resonance spectroscopy determinations: effect of adjunct cultures and brine composition on sodium-reduced Gouda cheese. *Int. Dairy J.* **33**, 142–152 (2013).

138. Hoffmann, W. Partial substitution of sodium chloride by potassium chloride in natural cheeses. in *IDF Special Issue: The importance of salt in the manufacture and ripening of cheese* (ed. International Dairy Federation) **1401**, 1–14 (2014).

139. Cruz, A. G. *et al.* Cheeses with reduced sodium content: effects on functionality, public health benefits and sensory properties. *Trends Food Sci. Technol.* **22**, 276–291 (2011).

140. Geleijnse, J. M., Kok, F. J. & Grobbee, D. E. Blood pressure response to changes in sodium and potassium intake: a metaregression analysis of randomised trials. *J. Hum. Hypertens.* **17**, 471–480 (2003).

141. Farkye, N. Y. & Guinee, T. P. Low-fat and low-sodium cheeses. in *Cheese: Chemistry, Physics and Microbiology* (eds. McSweeney, P. L. H., Fox, P. F., Cotter, P. D. & Everett, D. W.) 699–714 (Elsevier Ltd., 2017).

142. McGregor, R. The use of bitter blockers to replace salt in food products. in *Reducing Salt in Foods: Practical Strategies* (eds. Kilcast, D. & Angus, F.) 221–230 (Woodhead Publishing Limited, 2007).

143. Ley, J. P. Masking bitter taste by molecules. *Chemosens. Percept.* **1**, 58–77 (2008).

144. Scholten, C. sub4salt® - Jungbunzlauer's way to reduce sodium. *Innovations in Food Technology* 22–23 (2007).

145. Laan, H., Eng, S., Bruinenberg, P., Limsowtin, G. & Broome, M. Aminopeptidase activities of starter and non-starter lactic acid bacteria under simulated Cheddar cheese ripening conditions. *Int. Dairy J.* **8**, 267–274 (1998).

146. Campbell-Platt, G. Fermented foods - a world perspective. *Food Res. Int.* **27**, 253–257 (1994).

147. Cotter, P. D. & Beresford, T. P. Microbiome changes during ripening. in *Cheese: Chemistry, Physics and Microbiology* (eds. McSweeney, P. L. H., Fox, P. F., Cotter, P. D. & Everett, D. W.) 389–409 (Elsevier Ltd., 2017).

148. Giraffa, G. & Neviani, E. DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *Int. J. Food Microbiol.* **67**, 19–34 (2001).

149. Alkema, W., Boekhorst, J., Wels, M. & van Hijum, S. A. F. T. Microbial bioinformatics for food safety and production. *Brief. Bioinform.* **17**, 283–292 (2016).

150. Kelleher, P., Murphy, J., Mahony, J. & van Sinderen, D. Next-generation sequencing as an approach to dairy starter selection. *Dairy Sci. Technol.* **95**,

545–568 (2015).

151. De Filippis, F., Parente, E. & Ercolini, D. Metagenomics insights into food fermentations. *Microb. Biotechnol.* **10**, 91–102 (2017).

152. Solieri, L., Dakal, T. C. & Giudici, P. Next-generation sequencing and its potential impact on food microbial genomics. *Ann. Microbiol.* **63**, 21–37 (2013).

153. van Hijum, S. A. F. T., Vaughan, E. E. & Vogel, R. F. Application of state-of-art sequencing technologies to indigenous food fermentations. *Curr. Opin. Biotechnol.* **24**, 178–186 (2013).

154. Walsh, A. M., Crispie, F., Claesson, M. J. & Cotter, P. D. Translating omics to food microbiology. *Annu. Rev. Food Sci. Technol.* **8**, 113–134 (2017).

155. Jonnala, B. R. Y., McSweeney, P. L. H., Sheehan, J. J. & Cotter, P. D. Sequencing of the cheese microbiome and its relevance to industry. *Front. Microbiol.* **9**, 1020 (2018).

156. Dobson, A., O'Sullivan, O., Cotter, P. D., Ross, P. & Hill, C. High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. *FEMS Microbiol. Lett.* **320**, 56–62 (2011).

157. Garofalo, C. *et al.* Bacteria and yeast microbiota in milk kefir grains from different Italian regions. *Food Microbiol.* **49**, 123–133 (2015).

158. Leite, A. M. O. *et al.* Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. *Food Microbiol.* **31**, 215–221 (2012).

159. Liu, W. *et al.* High-throughput sequencing reveals microbial community diversity of Tibetan naturally fermented yak milk. *Ann. Microbiol.* **65**, 1741–1751 (2015).

160. Liu, X. *et al.* The bacterial community structure of yond bap, a traditional fermented goat milk product, from distinct Chinese regions. *Dairy Sci. Technol.* **95**, 369–380 (2015).

161. Marsh, A. J., O'Sullivan, O., Hill, C., Ross, R. P. & Cotter, P. D. Sequencing-based analysis of the bacterial and fungal composition of kefir grains and milks from multiple sources. *PLoS One* **8**, e69371 (2013).

162. Nalbantoglu, U. *et al.* Metagenomic analysis of the microbial community in kefir grains. *Food Microbiol.* **41**, 42–51 (2014).

163. Walsh, A. M. *et al.* Microbial succession and flavor production in the fermented dairy beverage kefir. *Appl. Environ. Sci.* **1**, e00052-16 (2016).

164. Liu, W. *et al.* High-throughput sequencing for the detection of the bacterial and fungal diversity in Mongolian naturally fermented cow's milk in Russia. *BMC Microbiol.* **15**, 1–12 (2015).

165. Settachaimongkon, S., van Valenberg, H. J. F. & Smid, E. J. Metabolomics as an emerging strategy for the investigation of yogurt components. in *Yogurt in Health and Disease Prevention* (ed. Shah, N. P.) 427–449 (Elsevier Inc., 2017).

166. Xu, H. *et al.* Assessment of the bacterial and fungal diversity in home-made yoghurts of Xinjiang, China by pyrosequencing. *J. Sci. Food Agric.* **95**, 2007–2015 (2015).

167. De Filippis, F., La Storia, A. La, Stellato, G., Gatti, M. & Ercolini, D. A selected core microbiome drives the early stages of three popular Italian cheese manufactures. *PLoS One* **9**, e89680 (2014).

168. Aldrete-Tapia, A., Escobar-Ramírez, M. C., Tamplin, M. L. & Hernández-Iturriaga, M. High-throughput sequencing of microbial communities in Poro cheese, an artisanal Mexican cheese. *Food Microbiol.* **44**, 136–141 (2014).

169. De Pasquale, I. *et al.* Causal relationship between microbial ecology dynamics and proteolysis during manufacture and ripening of Protected Designation of Origin (PDO) cheese Canestrato Pugliese. *Appl. Environ. Microbiol.* **80**, 4085–4094 (2014).

170. Masoud, W. *et al.* Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis and pyrosequencing. *Int. Dairy J.* **21**, 142–148 (2011).

171. Porcellato, D. & Skeie, S. B. Bacterial dynamics and functional analysis of microbial metagenomes during ripening of Dutch-type cheese. *Int. Dairy J.* **61**, 182–188 (2016).

172. Quigley, L. *et al.* High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. *Appl. Environ. Microbiol.* **78**, 5717–5723 (2012).

173. De Filippis, F., Genovese, A., Ferranti, P., Gilbert, J. A. & Ercolini, D. Metatranscriptomics reveals temperature-driven functional changes in microbiome impacting cheese maturation rate. *Sci. Rep.* **6**, 21871 (2016).

174. Bokulich, N. A. & Mills, D. A. Facility-specific 'house' microbiome drives microbial landscapes of artisan cheesemaking plants. *Appl. Environ. Microbiol.* **79**, 5214–5223 (2013).

175. Stellato, G., De Filippis, F., La Storia, A. & Ercolini, D. Coexistence of lactic acid bacteria and potential spoilage microbiota in a dairy processing environment. *Appl. Environ. Microbiol.* **81**, 7893–7904 (2015).

176. De Man, J. C., Rogosa, M. & Sharpe, M. E. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **23**, 130–135 (1960).

177. Vinderola, C. G. & Reinheimer, J. A. Culture media for the enumeration of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in the presence of yoghurt bacteria. *Int. Dairy J.* **9**, 497–505 (1999).

178. Terzaghi, B. E. & Sandine, W. E. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **29**, 807–13 (1975).

179. Merck KGaA. Microbiology Manual 12th Edn. (2010). Available at: https://www.analytics-shop.com/media/Hersteller/Kataloge/merck-de/Merck_Microbiology_Manual_12th_edition.pdf. (Accessed: 23rd January 2019)

180. Mossel, D., Bijker, P. & Eelderink, I. Streptokokken der Lancefield-Gruppe D in Lebensmitteln und Trinkwasser - Ihre Bedeutung, Erfassung und Bekampfung. *Arch. Lebensmittelhyg.* **29**, 121–127 (1978).

181. Ottaviani, F., Ottaviani, M. & Agosti, M. Esperienze su un agar selettivo e differenziale per *Listeria monocytogenes*. *Ind. Aliment.* **36**, 888–895 (1997).

182. Jayne-Williams, D. J. Miniaturized methods for the characterization of bacterial isolates. *J. Appl. Bacteriol.* **38**, 305–309 (1975).

183. Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten. Methodenbuch Band VI Milch. in *Handbuch der landwirtschaftlichen Versuchs- und Untersuchungsmethodik* (VDLUFA-Verlag, 2003).

184. Yu, Z. & Morrison, M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* **36**, 808–812 (2004).

185. European Union Reference Laboratory for Listeria monocytogenes. EURL *Lm* technical guidance document for conducting shelf-life studies on *Listeria*

monocytogenes in ready-to-eat foods. (2019). Available at: https://eurl-listeria.anses.fr/en/system/files/LIS-Cr-201909D2.pdf. (Accessed: 25th September 2019)

186. Rabe, E. Zur Natrium- und Kaliumbestimmung mit ionensensitiven Elektroden. *Zeitschrift für Leb. Untersuchung und Forsch.* **176**, 270–274 (1983).

187. Folkertsma, B. & Fox, P. F. Use of the Cd-ninhydrin reagent to assess proteolysis in cheese during ripening. *J. Dairy Res.* **59**, 217–224 (1992).

188. Doi, E., Shibata, D. & Matoba, T. Modified colorimetric ninhydrin methods for peptidase assay. *Anal. Biochem.* **118**, 173–184 (1981).

189. Bio-Rad Laboratories Inc. The DCode™ Universal Mutation Detection System. (1996). Available at: https://www.bio-rad.com/webroot/web/pdf/lsr/literature/M1709080C.pdf. (Accessed: 7th September 2018)

190. Myers, R. M., Fischer, S. G., Maniatis, T. & Lerman, L. S. Modification of the melting properties of duplex DNA by attachment of a GC-rich DNA sequence as determined by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* **13**, 3111–3129 (1985).

191. Pearson's correlation coefficient. in *Encyclopedia of Public Health* (ed. Kirch, W.) (Springer, 2008).

192. Sokal, R. R. & Michener, C. D. A statistical method for evaluating systematic relationships. *Univ. Kansas Sci. Bull.* **38**, 1409–1438 (1958).

193. Lagkouvardos, I. *et al.* IMNGS: a comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity studies. *Sci. Rep.* **6**, 33721 (2016).

194. Edgar, R. C. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **10**, 996–998 (2013).

195. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460–2461 (2010).

196. DeSantis, T. Z. *et al.* Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* **72**, 5069–72 (2006).

197. Pruesse, E., Peplies, J. & Glöckner, F. O. SINA: accurate high-throughput

multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* **28**, 1823–1829 (2012).

198. Cole, J. R. *et al.* Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* **42**, D633–D642 (2014).

199. Lagkouvardos, I., Fischer, S., Kumar, N. & Clavel, T. Rhea: a transparent and modular R pipeline for microbial profiling based on 16S rRNA gene amplicons. *PeerJ* **5**, e2836 (2017).

200. Chen, J. *et al.* Associating microbiome composition with environmental covariates using generalized UniFrac distances. *Bioinformatics* **28**, 2106–2113 (2012).

201. Anderson, M. J. Permutational multivariate analysis of variance (PERMANOVA). *Wiley StatsRef Stat. Ref. Online* 1–15 (2017).

202. Wattam, A. R. *et al.* Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource center. *Nucleic Acids Res.* **45**, D535–D542 (2017).

203. Brettin, T. *et al. RASTtk*: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci. Rep.* **5**, 8365 (2015).

204. Lee, I., Kim, Y. O., Park, S. & Chun, J. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *Int. J. Syst. Evol. Microbiol.* **66**, 1100–1103 (2016).

205. Farrow, J. A. E., Facklam, R. R. & Collins, M. D. Nucleic acid homologies of some vancomycin-resistant Leuconostocs and description of *Leuconostoc citreum* sp. nov. and *Leuconostoc pseudomesenteroides* sp. nov. *Int. J. Syst. Bacteriol.* **39**, 279–283 (1989).

206. Altschup, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).

207. Yoon, S. H. *et al.* Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* **67**, 1613–1617 (2017).

208. Bundesministerium der Justiz und für Verbraucherschutz. Käseverordnung (KäseV) vom 14. April 1986. *BGBl* I S. 412 (1986).

209. Paquet, J., Lacroix, C. & Thibault, J. Modeling the pH and acidity for industrial cheese production. *J. Dairy Sci.* **83**, 2393–2409 (2000).

210. Düsterhöft, E.-M., Engels, W. & Huppertz, T. Gouda and related cheeses. in *Cheese: Chemistry, Physics and Microbiology* (eds. McSweeney, P. L. H., Fox, P. F., Cotter, P. D. & Everett, D. W.) 865–888 (Elsevier Ltd, 2017).

211. Jørgensen, F., Hansen, O. C. & Hansen, P. Enzymatic conversion of D-galactose to D-tagatose: heterologous expression and characterisation of a thermostable L-arabinose isomerase from *Thermoanaerobacter mathranii*. *Appl. Microbiol. Biotechnol.* **64**, 816–822 (2004).

212. Torres, P. & Batista-Viera, F. Immobilized trienzymatic system with enhanced stabilization for the biotransformation of lactose. *Molecules* **22**, 284 (2017).

213. Tiefenbacher, K. F. Technology of main ingredients - sweeteners and lipids. in *The Technology of Wafers and Waffles: Operational Aspects* 123–225 (Elsevier Inc., 2017).

214. Chr. Hansen. Sweety® Y-1: an innovative culture to reduce added sugar in yogurt and stay sweet. (2019). Available at: https://www.chr-hansen.com/_/media/files/chrhansen/home/food-cultures-and-enzymes/fresh-dairy/sweety-y-1_onepager.pdf. (Accessed: 20th November 2019)

215. Chr. Hansen. SWEETY® cultures: reduce the amount of added sugar in yogurt and keep the sweetness, using only culture. (2019). Available at: https://www.chr-hansen.com/en/food-cultures-and-enzymes/fresh-dairy/cards/product-cards/sweety. (Accessed: 22nd November 2019)

216. O'Leary, V. S. & Woychik, J. H. Utilization of lactose, glucose, and galactose by a mixed culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in milk treated with lactase enzyme. *Appl. Environ. Microbiol.* **32**, 89–94 (1976).

217. Vedamuthu, E. R. Natural (unhydrolyzed) milk versus lactose-hydrolysed milk for cultured dairy products: physiological and practical implications for the starter industry. *J. Food Prot.* **41**, 654–659 (1978).

218. Rul, F. Yogurt: microbiology, organoleptic properties and probiotic potential. in *Fermented Food - Part II: Technological Interventions* (eds. Ray, R. C. & Monet, D.) 418–450 (CRC Press, 2017).

219. Dave, R. I. & Shah, N. P. Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures. *Int. Dairy J.* **7**, 31–41 (1997).

220. Courtin, P. & Rul, F. Interactions between microorganisms in a simple ecosystem: yogurt bacteria as a study model. *Le Lait, INRA Ed.* **84**, 125–134 (2004).

221. Shah, N. P. Probiotic bacteria: selective enumeration and survival in dairy foods. *J. Dairy Sci.* **83**, 894–907 (2000).

222. Ng, E. W., Yeung, M. & Tong, P. S. Effects of yogurt starter cultures on the survival of *Lactobacillus acidophilus*. *Int. J. Food Microbiol.* **145**, 169–175 (2011).

223. Gilliland, S. E. & Speck, M. L. Instability of *Lactobacillus acidophilus* in yogurt. *J. Dairy Sci.* **60**, 1394–1398 (1977).

224. Iwana, H., Masuda, H., Fujisawa, T., Suzuki, H. & Mitsuoka, T. Isolation and identification of *Bifidobacterium* spp. in commercial yogurts sold in Europe. *Bifidobact. Microflora* **12**, 39–45 (1993).

225. Vedamuthu, E. R. Starter cultures for yogurt and fermented milks. in *Manufacturing Yogurt and Fermented Milks* (eds. Chandan, R. C., White, C. H., Kilara, A. & Hui, Y. H.) 89–115 (Blackwell Publishing, 2006).

226. Zalewska, B., Kaevska, M. & Slana, I. Sequence analysis of changes in microbial composition in different milk products during fermentation and storage. *Curr. Microbiol.* **75**, 202–205 (2018).

227. Quigley, L. *et al.* The microbial content of raw and pasteurized cow milk as determined by molecular approaches. *J. Dairy Sci.* **96**, 4928–4937 (2013).

228. LaRocque, R. C. & Calderwood, S. B. Syndromes of enteric infection. in *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases* (eds. Bennett, J. E., Dolin, R. & Blaser, M. J.) 1238–1247 (Saunders, 2015).

229. Luzzi, G. *et al.* Enhancing the sweetening power of lactose by enzymatic modification in the reformulation of dairy products. *Int. J. Dairy Technol.* accepted for publication (2019).

230. McCain, H. R., Kaliappan, S. & Drake, M. A. Invited review: sugar reduction in dairy products. *J. Dairy Sci.* **101**, 8619–8640 (2018).

231. European Parliament. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011. *Off. J. Eur. Union* **L 304**, 18–63 (2011).

232. Panthi, R. R., Jordan, K. N., Kelly, A. L. & Sheehan, J. J. D. Selection and treatment of milk for cheesemaking. in *Cheese: Chemistry, Physics and Microbiology* (eds. McSweeney, P. L. H., Fox, P. F., Cotter, P. D. & Everett, D. W.) 23–50 (Elsevier Ltd., 2017).

233. Fox, P. F. Proteolysis during cheese manufacture and ripening. *J. Dairy Sci.* **72**, 1379–1400 (1989).

234. Hoffmann, W. *et al.* Salt reduction in film-ripened semihard Edam cheese. *Int. J. Dairy Technol.* in print (2019). doi:10.1111/1471-0307.12675

235. Baptista, D. P., da Silva Araujo, F. D., Everlin, M. N. & Gigante, M. L. Reduction of 25 % salt in Prato cheese does not affect proteolysis and sensory acceptance. *Int. Dairy J.* **75**, 101–110 (2017).

236. Shrestha, S., Grieder, J. A., McMahon, D. J. & Nummer, B. A. Survival of *Listeria monocytogenes* introduced as a post-aging contaminant during storage of low-salt Cheddar cheese at 4, 10, and 21°C. *J. Dairy Sci.* **94**, 4329–4335 (2011).

237. Shrestha, S., Grieder, J. A., Mcmahon, D. J. & Nummer, B. A. Survival of *Salmonella* serovars introduced as a post-aging contaminant during storage of low-salt Cheddar cheese at 4, 10, and 21 °C. *J. Food Sci.* **76**, (2011).

238. Randazzo, C. L., Torriani, S., Akkermans, A. D. L., Vos, W. M. De & Vaughan, E. E. Diversity, dynamics, and acitivity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. *Appl. Environ. Microbiol.* **68**, 1882–1892 (2002).

239. Blaya, J., Barzideh, Z. & LaPointe, G. Symposium review: interaction of starter cultures and nonstarter lactic acid bacteria in the cheese environment. *J. Dairy Sci.* **101**, 3611–3629 (2017).

240. Gobbetti, M., De Angelis, M., Di Cagno, R., Mancini, L. & Fox, P. F. Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. *Trends Food Sci. Technol.* **45**, 167–178 (2015).

241. Salazar, J. K. *et al.* Metagenomics of pasteurized and unpasteurized Gouda cheese using targeted 16S rDNA sequencing. *BMC Microbiol.* **18**, 189 (2018).

242. Rudi, K., Moen, B., Drømtorp, S. M. & Holck, A. L. Use of ethidium monoazide and PCR in combintion for quantification of viable and dead cells in complex samples. *Appl. Environ. Microbiol.* **71**, 1018–1024 (2005).

243. Erkus, O. *et al.* Use of propidium monoazide for selective profiling of viable microbial cells during Gouda cheese ripening. *Int. J. Food Microbiol.* **228**, 1–9 (2016).

244. Løvdal, T., Hovda, M. B., Björkblom, B. & Møller, S. G. Propidium monoazide combined with real-time quantitative PCR underestimates heat-killed *Listeria innocua*. *J. Microbiol. Methods* **85**, 164–169 (2011).

245. Elizaquível, P., Sánchez, G. & Aznar, R. Application of propidium monoazide quantitative PCR for selective detection of live *Escherichia coli* O157:H7 in vegetables after inactivation by essential oils. *Int. J. Food Microbiol.* **159**, 115–121 (2012).

246. Bisanz, J. E., Macklaim, J. M., Gloor, G. B. & Reid, G. Bacterial metatranscriptome analysis of a probiotic yogurt using an RNA-Seq approach. *Int. Dairy J.* **39**, 284–292 (2014).

247. Rademaker, J. L. W., Hoolwerf, J. D., Wagendorp, A. A. & Te Giffel, M. C. Assessment of microbial population dynamics during yoghurt and hard cheese fermentation and ripening by DNA population fingerprinting. *Int. Dairy J.* **16**, 457–466 (2006).

248. Ohnishi, A. *et al.* Development of a 16S rRNA gene primer and PCR-restriction fragment length polymorphism method for rapid detection of members of the genus *Megasphaera* and species-level identification. *Appl. Environ. Microbiol.* **77**, 5533–5535 (2011).

249. Cocolin, L., Manzano, M., Cantoni, C. & Comi, G. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. *Appl. Environ. Microbiol.* **67**, 5113–5121 (2001).

250. Rettedal, E. A., Clay, S. & Brözel, V. S. GC-clamp primer batches yield 16S rRNA gene amplicon pools with variable GC clamps, affecting denaturing gradient gel electrophoresis profiles. *FEMS Microbiology Letters* **312**, 55–62 (2010).

251. Klindworth, A. *et al.* Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **41**, 1–11 (2013).

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Declaration

I hereby declare the following:

- that apart from the guidance and advice of my supervisor Prof. Dr. Charles M.A.P. Franz, the content and design of this thesis is all my own, independent work.
- that this thesis has not been submitted either partially or wholly as part of a doctoral degree to another examining body. Parts of this thesis have been accepted for publication in the following papers:
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    - Luzzi, G., Steffens, M., Clawin-Rädecker, I., Hoffmann, W., Franz, C.M.A.P., Fritsche, J. & Lorenzen, P. Chr. Enhancing the sweetening power of lactose by enzymatic modification in the reformulation of dairy products. *International Journal of Dairy Technology*. Accepted for publication on 29th November 2019.
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- that this thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation.
- that an academic degree has never been withdrawn.

Giuseppina Luzzi

Kiel, 5th December 2019