Simultaneous growth and metabolite production by yoghurt starters and probiotics: a metabolomics approach

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Thesis

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Dedication

To my dear Parents and my Family

Thanks for their love, continuing support and encouragement

ขอมอบคุณความดีและประโยชน์ที่พึงมีจากวิทยานิพนธ์ฉบับนี้ แก่ บิดา-มารดา ผู้มีพระคุณสูงสุด

และคณาจารย์ทุกท่านตั้งแต่อดีตจนถึงปัจจุบัน ที่ได้ประสิทธิ์ประสาทวิชาความรู้ให้แก่ข้าพเจ้า

อันนำไปสู่กระบวนการวางแผน คิดวิเคราะห์ และแก้ไขปัญหาในการทำวิจัยให้สำเร็จลุล่วงได้ด้วยดี

Abstract

The main objective of this research was to investigate the simultaneous growth and metabolite production by yoghurt starters and different probiotic strains, i.e. *Lactobacillus rhamnosus* GG, *Bifidobacterium animalis* subsp. *lactis* BB12 and *Lactobacillus plantarum* WCFS1, during set-yoghurt fermentation and refrigerated storage. In this context, the microbial activity was evaluated in terms of bacterial population dynamics, milk acidification and formation of volatile and non-volatile metabolites in set-yoghurt. A complementary metabolomics approach using headspace SPME-GC/MS and ^{1}H-NMR was applied for characterization of biochemical changes associated with the microbial metabolism during fermentation and storage. The results revealed that incorporation of the three probiotic strains did not significantly influence the acidity and concentrations of key-aroma volatile compounds of set-yoghurt. Still, the presence of probiotics substantially contributed to the formation of a large number of volatile and non-volatile metabolites detected at low concentration. Because many probiotic strains are not able to survive well in fermented milk, a strategy to enhance their survival was additionally applied by preculturing the three probiotic strains under sublethal salt and low pH stress conditions prior to inoculation in milk. The results revealed an improved survival of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12, specifically by preculturing at relatively low pH conditions. Moreover, incorporation of sublethally precultured *L. plantarum* WCFS1 significantly impaired the survival of *L. delbrueckii* subsp. *bulgaricus*, which consequently reduced the post-acidification of yoghurt. Metabolomics analyses revealed that the presence of stress-adapted probiotics induced significant changes in the overall metabolite profile of yoghurt. This finding is important, since variations in the relative abundance of various organic acids, aroma volatiles and proteolytic-derived compounds may directly influence the organoleptic quality of product. Finally, multivariate analysis enabled to distinguish yoghurts fermented by different types of starter combinations and different durations of storage according to their metabolite profiles. This research provides new information regarding the impact of probiotics on the metabolome of yoghurt and potential application of stress-adapted probiotics in an actual food-carrier environment.

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Chapter 1

General introduction

1.1 Definition of yoghurt

Yoghurt is one of the most popular fermented milk products and the consumption is increasing worldwide [64]. Based on the CODEX.STAND.243-2003: Standard for fermented milks, yoghurt is obtained by fermentation of milk, which may have been manufactured from products derived from milk with or without compositional modification, by the action of specific microorganisms, i.e. symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which shall be viable, active and abundant in the product until the date of expiration. If the product is heat-treated after fermentation the requirement for viable microorganisms does not apply [12].

1.2 Diversity of yoghurt

Fermented milk has been consumed ever since the domestication of animals. A wide range of products has constituted a vital part of the human diet in many regions around the world [11]. Originally, the primary function of fermenting milk was to extend its shelf-life. The fermentation process generated further advantages including the distinctive consistency, flavor, texture and digestibility of the products [69]. Nowadays, different types of yoghurt have been introduced to the dairy marketplace in response to consumer preferences, changing lifestyles, and health concerns [71]. Yoghurt can be categorized according to manufacturing processes and variations in physical, chemical, flavor and compositional modifications [69]. Examples of various types of yoghurt available in the marketplace are listed in Table 1.1.

1.3 Manufacturing of yoghurt

The fundamental basis of yoghurt manufacturing is the acid coagulation of milk proteins, i.e. mainly caseins, by the activity of starter cultures. The milk coagulation results in a three-dimensional gel network capable to capture the liquid serum phase

[39]. Based on the physical characteristics, three common types of yoghurt are available in the daily marketplace: (i) set-yoghurt, (ii) stirred-yoghurt and (iii) drinking yoghurt [69] (Figure 1.1).

Table 1.1. Examples of various types of yoghurt available in the marketplace

Source of variation	Product category
Milk from various sources	• Bovine's milk yoghurt • Goat's milk yoghurt • Sheep's milk yoghurt • Buffalo's milk yoghurt • Camel's milk yoghurt • Mare's milk yoghurt • Soy-milk yoghurt • Corn-milk yoghurt • Organic-certified milk yoghurt
Manufacturing process	• Set-yoghurt • Stirred- yoghurt • Drinking yoghurt • Concentrated/strained yoghurt • Frozen yoghurt • Dried yoghurt • UHT yoghurt
Flavor fortification	• Plain yoghurt • Fruit flavored yoghurt • Miscellaneous flavored yoghurt
Rate of post-acidification	• Traditional yoghurt • Semi-mild yoghurt • Mild yoghurt
Fat content and composition	• Full-fat yoghurt • Low-fat yoghurt • Non-fat yoghurt • Fat-substitutes yoghurt • Vegetable oil yoghurt
Health-promoting yoghurt	• Low calorie yoghurt • Lactose hydrolyzed yoghurt • Probiotic yoghurt • ABT yoghurt • Bio-yoghurt • Biogarde yoghurt • Omega-3 enriched yoghurt • Cholesterol free yoghurt • Vitamin supplemented yoghurt • Fiber enriched yoghurt

Information compiled from Chandan [11], Varnam & Sutherland [76], A. Y. Tamime & Robinson [69] and Walstra et al. [78].

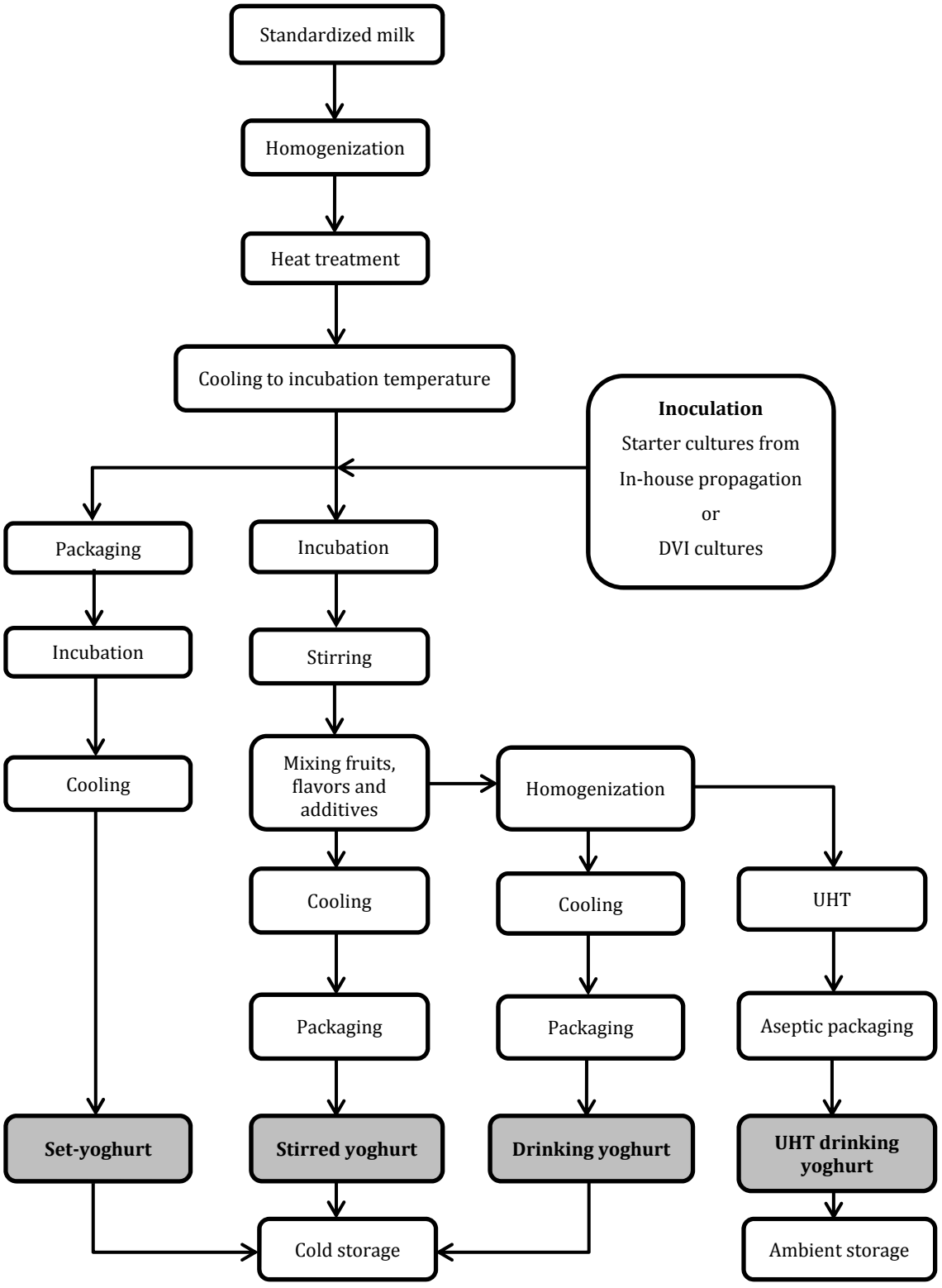

Fig. 1.1. Flowchart outline for the manufacturing of yoghurt. Information adapted from Chandan [11], A. Y. Tamime & Robinson [69], Varnam & Sutherland [76] and Walstra et al. [78].

Set-yoghurt is usually fermented in package; and has a firm gel-like texture and natural flavor associated with its more traditional image. Stirred-yoghurt is sheared after fermentation, which produces a semi-solid pourable product, and then stabilizers, fruit mixtures as well as other ingredients are commonly added. Drinking yoghurt is produced by mixing an ordinary yoghurt with water and/or fruit concentrate, resulting in a low viscosity and drinkable characteristics of the product [39].

Although manufacturing stages of the three types of yoghurt have much in common, the acidification rate greatly differs between set-yoghurt and stirred-yoghurt due to the difference in inoculum size and incubation temperature [78]. This condition reflects substantial changes on the rheological and organoleptic quality of the fermented products. The research described in this thesis concentrates on the simultaneous growth and metabolite production by yoghurt starters and probiotics during set-yoghurt fermentation and refrigerated storage.

1.4 Yoghurt starter bacteria

1.4.1 *Streptococcus thermophilus*

S. thermophilus is widely used for the manufacturing of fermented dairy products and is considered as the second most important species of industrial lactic acid bacteria (LAB) after *Lactococcus lactis* [31]. It is a Gram-positive, catalase-negative, thermophilic, facultatively anaerobic LAB. Young cells of *S. thermophilus* are spherical in shape and occur in chains. This bacterium has an optimum growth temperature of 40 – 45 ^{o}C and an optimum pH near 6.0 – 6.5 [46]. One of the main functions of *S. thermophilus* is to provide a rapid acidification in milk. *S. thermophilus* is homofermentative and generates L(+) lactic acid as the main product from lactose metabolism. It exhibits β-galactosidase (β-gal) and metabolizes only the glucose moiety of lactose [69]. *S. thermophilus* is unable to metabolize galactose and thus expels this sugar from the cell into the medium. Glucose, fructose and mannose can also be metabolized, but the fermentation of maltose and sucrose is strain specific [51]. The rate of acidification is a strain-dependent

metabolic trait that may be influenced by other factors, such as proteolytic system and urease activity [31]. Most of *S. thermophilus* strains display limited proteolytic activity due to the lack of cell-enveloped proteases (PrtS). Normally, the level of free nitrogen sources available in milk is insufficient, and thus supplementation is usually required to support their full growth. One of the strategies used in the manufacturing of yoghurt is co-cultivation the non-proteolytic *S. thermophilus* with a suitable proteolytic culture, i.e. *L. delbrueckii* subsp. *bulgaricus* [28]. Several *S. thermophilus* strains also have capacity to produce aroma volatiles and exopolysaccharides (EPS). These strains are used to facilitate the distinctive flavor and texture characteristic of yoghurt [81]. Recently, the complete genome sequence and functional-genomic analyses of many *S. thermophilus* strains have been extensively published [6, 18, 32, 67].

1.4.2 *Lactobacillus delbrueckii* subsp. *bulgaricus*

L. delbrueckii subsp. *bulgaricus* is one of the economically most important species of LAB, with a worldwide application in yoghurt manufacturing. It is a Gram-positive, thermophilic, facultatively anaerobic, non-motile, non-spore forming LAB. It occurs as single or short chains of rod cells, with rounded ends. This bacterium has an optimum growth temperature in milk between 40 and 45 ^{o}C and an optimum pH near 5.0 – 5.5 [50]. Its basic sugar metabolism is obligate homofermentative. When grown in milk, *L. delbrueckii* subsp. *bulgaricus* transports lactose into the cell in association with the expulsion of galactose similar to that found in *S. thermophilus*. Lactose is hydrolyzed by β-galactosidase with only glucose being metabolized but, in this case, the end product is D(–) lactic acid [50]. This form of lactic acid is less readily metabolized by humans than the L(+) isomer [51]. Unlike *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* is more proteolytic, and thus it can hydrolyse caseins, especially β-casein, by means of cell-enveloped proteases (PrtB) to generate free amino acids and oligopeptides [51]. A number of commercial yoghurt starters contain *L. delbrueckii* subsp. *bulgaricus* strains that produce substantial amount of volatiles and EPS [81]. The complete genome sequences and functional-genomic analyses of certain *L. delbrueckii* subsp. *bulgaricus* strains have been recently published [66, 75].

1.4.3 Interaction between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*

Even though *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are able to grow independently in milk, these bacteria perform a symbiosis interaction known as "proto-cooperation" in mixed culture [14, 69]. The proto-cooperation is based on the exchange of several metabolites which provide growth stimulating effects to each other (Fig 1.2).

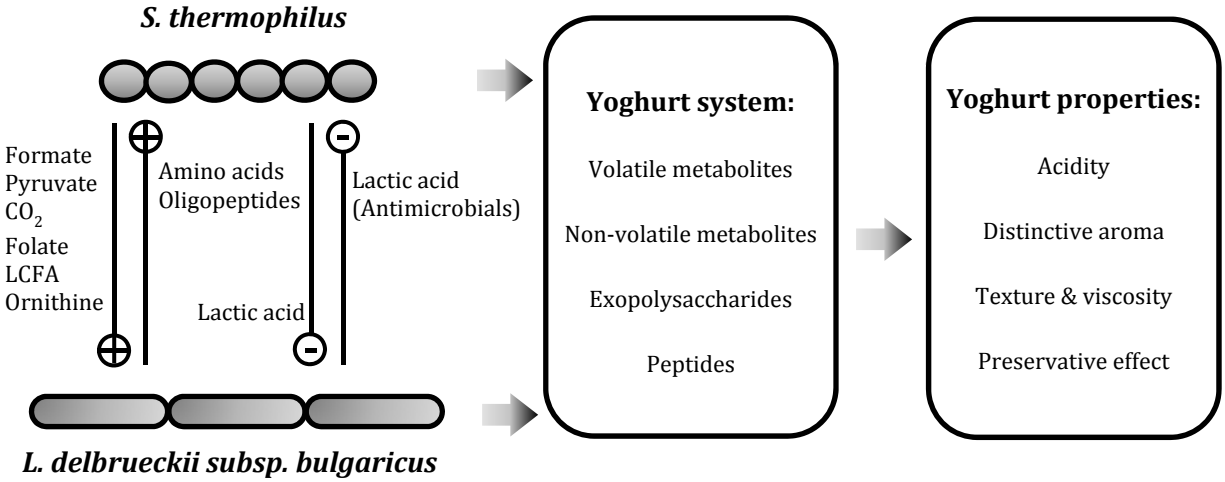

Fig. 1.2. Schematic representation of the proto-cooperation between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* and their contribution to the characteristics of yoghurt. (+) growth stimulatory effect; (–) growth inhibitory effect; LCFA: long-chain fatty acids. Information adapted from Chandan [11], Sieuwerts et al. [65], A. Y. Tamime & Robinson [69] and Walstra et al. [78].

Traditional yoghurt starters consist of weakly proteolytic *S. thermophilus* strain and proteolytic *L. delbrueckii* subsp. *bulgaricus* strain [78]. During the early stage of fermentation, the initial pH of milk (*ca.* 6.7) is more favorable to the growth of *S. thermophilus*. These bacteria develop by using free amino acids and peptides available in milk. However, the contents of these free nitrogen sources are not sufficient to promote their full growth [37], then *S. thermophilus* utilizes amino acids and peptides obtained from the proteolytic activity of *L. delbrueckii* subsp. *bulgaricus*. On the other hand, *S. thermophilus* produces lactic acid which consequently lowers the pH, hence retards its growth, and creates a favorable growth condition for *L. delbrueckii* subsp. *bulgaricus*. Furthermore, pyruvic acid, formic acid, folate, ornithine, several long-chain fatty acids and CO_2 produced by *S. thermophilus* are the growth stimulants of *L. delbrueckii* subsp. *bulgaricus* [2, 65, 70, 81]. The proto-cooperation has an important role on the growth of

the two species, rate of milk acidification and development of distinctive flavor and texture characteristics of the fermented product [11, 81]. Although interaction between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* is often positive, absence of interaction or even negative effects can take place depending on the combination of bacterial strains, type and pre-heating process of base milk and fermentation conditions [14].

1.5 Probiotics

Functional foods are defined as foods that potentially provide health benefits in addition to the nutrients they contain [64]. One way in which foods can be modified to become functional is by addition of health-associating microorganisms referred as probiotics [63]. The term "probiotics" is originated from Greek meaning "for life" [24]. According to the FAO/WHO: Guidelines for the evaluation of probiotics in food, probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [22]. Several criteria need to be considered in the selection of probiotic microorganisms including the clinical safety, functional properties and technological characteristics [54].

Most commercial probiotics incorporated in dairy products are strains belonging to the genera *Lactobacillus* and *Bifidobacterium* [38]. Members of these two genera have a long history of safe use in the manufacture of fermented foods and found as a part of normal microbiota in the human gastrointestinal tract [62]. A number of health benefits is claimed in favor of the consumption of probiotic lactobacilli and bifidobacteria including modulation of immune system, prevention and reduction of gastrointestinal disorders, alleviation of lactose intolerance, prevention of allergy, reduction of the risk associated with mutagenicity and carcinogenicity, inhibition of intestinal pathogens, prevention of inflammatory bowel disease and reduction in serum cholesterol [26, 59, 64, 77]. However, it should be mentioned that the beneficial health effects imparted by probiotics are completely strain specific. Among the probiotic strains incorporated in dairy products, *L. rhamnosus* GG (Valio), *L. casei* Shirota (Yakult) and *B. animalis* subsp.

lactis BB12 (Chr. Hansen) are of extensively studied strains with most clinical-documented effects in humans [63].

1.5.1 General characteristics of the genus *Lactobacillus*

Lactobacillus is the largest genus within the group of LAB. The general characteristics are as described previously in *L. delbrueckii* subsp. *bulgaricus*. Most strains have an optimum growth temperature between 35 – 45 ^{o}C, and the optimum pH ranges from 5.5 – 6.0 [69]. The level of proteolytic activity, EPS and bacteriocins production differs between species of *Lactobacillus* and varies even among the strains of the same species [16, 36]. They are mostly fastidious, chemo-organotrophic, requiring nutrient-rich media to grow [16]. Members of the genus *Lactobacillus* are classified into three distinct groups based on their carbohydrate metabolism: (i) obligate homofermentative, which ferment hexoses almost entirely to lactic acid; (ii) facultative heterofermentative, which ferment hexoses either almost entirely to lactic acid or, under glucose-limiting conditions, to lactic acid, acetic acid, ethanol, and formic acid; and (iii) obligate heterofermentative, which ferment hexoses to lactic acid, CO_2, acetic acid, and ethanol [16]. *Lactobacillus* can be found in diverse environments, such as foods (dairy products, fermented meat, sour dough, fermented vegetable, beverages), respiratory, gastrointestinal and genital tracts of humans and animals as well as in various plant-based materials [23]. Moreover, they have been the most common bacterial species isolated from the human intestine [77]. The safety and functional properties of probiotic lactobacilli, particularly the strains of *L. acidophilus*, *L. casei*, *L. rhamnosus*, and *L. johnsonii*, have been extensively studied [63]. To date, the complete genomes of many probiotic *Lactobacillus* strains have been sequenced and published [1, 34, 43, 80]. This information has notably facilitated to understand the functionality and technological attributes of these bacteria.

1.5.2 General characteristics of the genus *Bifidobacterium*

Bifidobacterium are naturally found as major inhabitants of the gut of a large variety of mammals including humans. They were first isolated from breast-fed infant faeces by Tissier, at the Pasteur Institute in 1900, who discovered a bacterium with a characteristic Y shape and named it as *Bacillus bifidus.* In 1957, Dehnart first realized the existence of multiple biotypes of *Bifidobacterium* and proposed a scheme for the differentiation of these bacteria based on the different carbohydrate metabolism [4]. *Bifidobacterium* are Gram positive, obligately anaerobic, non-motile, catalase-negative and non-spore forming LAB. They have polymorphic branched rod shapes including short curved rods, club shaped rods, and typical bifurcated Y shaped rods that may occur singly, in chains or clumps. The branching nature of bifidobacteria not only depends on the strains but also on the media used for cultivation [49]. Most *Bifidobacterium* strains originating from humans have been reported to grow optimally at a temperature of 36 – 38 ^{o}C. The optimum pH for the growth of bifidobacteria is between 6.0 – 7.0 [63]. Since bifidobacteria are strictly anaerobic LAB, oxygen is considered as a significant factor affecting their survival in fermented milk products [33]. *Bifidobacterium* strains are heterofermentative. All bifidobacteria from human origin are able to utilize glucose, galactose, lactose and fructose as carbon sources [63]. *Bifidobacterium* metabolize hexoses using the "Bifidus pathway" by which acetic and lactic acid (ratio 3:2) are generated from the fermentation of glucose while galactose is metabolized through the Leloir pathway [69]. Specific health-promoting properties of probiotic *Bifidobacterium* strains have been extensively documented [63, 77].

1.5.3 Technological aspects of probiotics in yoghurt

Nowadays, there has been a notable increase in the variety of probiotic dairy products including pasteurized milk, ice cream, frozen desserts, fermented milk, dairy-based beverages, cheeses and infant milk powder [68]. Among the mentioned products, yoghurt is remarkably the most important food-carrier for the delivery of probiotics [60]. Although the primary criteria for selection of probiotic strains are based on the clinical safety and functional properties [77], the following aspects must be considered

from a technological standpoint: (i) the interaction between probiotics with traditional yoghurt starters, (ii) the ability of probiotics to grow in milk, (iii) the robustness of probiotics to withstand the manufacturing process and storage condition and (iv) the influence of probiotics on the sensory characteristics of yoghurt [42, 48, 68].

There are several approaches by which probiotics can be added during the production of yoghurt: (i) probiotics are inoculated as an adjunct direct-vat inoculation (DVI) culture; (ii) probiotics are propagated in one batch of milk or suitable media in order to achieve a high biomass and then mixed together with yoghurt; and (iii) probiotics are used as a starter culture, e.g. *L. casei* Shirota (Yakult), or a part of the starter consortium, e.g. *L. acidophilus* and *B. bifidum* (ABT culture) [68]. Most commonly, however, many probiotic strains grow slowly in non-supplemented milk and the traditional fermentation temperature is often unsuitable for their growth, particularly the strains originating from the human GI tract [78]. Thus, the suitable milk acidification with satisfactory sensory properties is rarely occurs by pure culture of probiotics. Furthermore, the limited growth of probiotics in milk results in the risk of overgrowth of undesirable microorganisms which may cause undesirable flavor and texture in the fermented product [42].

In practice, it is common to used probiotics as an adjunct culture in combination with traditional yoghurt starters. The activity of yoghurt starters can create a favourable growth condition for probiotics. For example, *S. thermophilus* creates an anaerobic environment which subsequently stimulates the growth of bifidobacteria while *L. delbrueckii* subsp. *bulgaricus* sustains the amino acid requirement of probiotics by its proteolytic activity [77]. On the other hand, organic acids and volatile compounds produced by the activity of probiotics may contribute to the organoleptic quality of yoghurt [45]. Thus, the interaction between yoghurt starters and probiotics is another important aspect that must be considered in order to select the most suitable combination regarding the functionality and sensory quality of product [68].

The definition of probiotics underlines that these functional bacteria need to be viable, metabolically active and present in sufficiently high number at the time of

consumption to ensure their beneficial health effects [22]. It is recommended that a probiotic product should contain at least 10^6 cfu/g of viable probiotic cells throughout the entire shelf-life [77]. However, numerous studies have demonstrated that many of probiotic strains are not able to survive well in fermented milk [19, 27, 56, 62]. The survival of probiotics can be adversely affected by certain metabolites including lactic acid, hydrogen peroxide, and bacteriocins produced by yoghurt starters [42]. Besides, various factors accountable for the viability loss of probiotics during yoghurt production and storage; including sensitivity of the strains used, inoculation rate and level, fermentation temperature, level of oxygen permeation through the package, presence of other competitive LAB, and application of food additives, have been extensively reported [42, 48, 63].

1.5.4 Strategies for improving the survival of probiotics in yoghurt

Certain approaches have been applied for improving the survival of probiotics during yoghurt production and storage. The most prevalent of which are the selection of appropriate strains on the basis of their acid and bile tolerances, supplementation of the milk with nutrients, addition of protective compounds, manipulation of starter cultures, selection of appropriate packaging materials, application of oxygen scavengers, performing two-stage fermentation and application of microencapsulation technique [61, 62, 68].

An alternative strategy to improve the survival of probiotics in yoghurt is to enhance their ability to cope with the harsh environments during production and storage. Stress adaptation is one of the strategies to improve the survival of probiotics by pre-treating (preculturing) them in a sublethal stress condition prior to exposure to a more harsh or lethal environment [73]. This approach allows probiotic cells to develop adaptive stress responses, i.e. a genotypic and/or phenotypic reaction to growth inhibition induced by environmental or physiological imbalances [17], leading to an increase in their survival compared to those that are directly shifted into the same lethal stress condition [55]. Adaptive responses on various types of stress, i.e. heat, cold, acid, bile, osmotic, oxygen, high pressure and nutrient starvation, in lactobacilli and

bifidobacteria have been well investigated [15, 53, 72, 74]. These stress conditions are characterized due to the environmental challenges where probiotics are typically encountered, i.e. during human gastrointestinal transit, industrial-scale production and in the food systems [53]. Nevertheless, it should be mentioned that stress responses in LAB are expressed in a very specific process depending on the species, strains and particular types of stress [74]

Recent advances in post-genomics technologies, i.e. transcriptomics and proteomics, have extensively provided novel insights into how probiotics counteract with environmental stresses from a molecular perspective [58]. Adaptive stress responses in probiotics are associated with the expression of a large number of genes, synthesis of stress-response proteins and alteration of various physiological features [35, 53, 57, 74]. As a consequence, stress adaptation not only enables to enhance the survival of probiotics but also induces substantial changes in their performance in a system. This information is important for the application of stress-adapted probiotics in yoghurt since their metabolic activity may influence the biochemical and organoleptic characteristics of the product.

1.6 Metabolomics approach

1.6.1 Metabolomics in food research

The suffix "~omics" derives from the Latin voice "*~omne"* that means everything, entirety or totality [44]. Metabolomics is an emerging field of ~omics research that focuses on comprehensive characterization of small molecular weight metabolites (< 1,000 Da) present in a biological system. The collective set of metabolites found within a system is commonly referred as "metabolome" [79]. The advanced technologies in metabolomics have provided a high-throughput characterization of hundreds of metabolites in a single measurement. Metabolomics technologies are generally employed by means of targeted and non-targeted analyses. The targeted analysis focuses on a group of metabolites that require the specific identification and

quantification process. This analysis is important for assessing the behavior of a particular collection of metabolites under certain conditions. On the other hand, the non-targeted analysis focuses on the detection of as many groups of metabolites as possible to discover the overall profiles or fingerprints of the samples [10]. Recently, metabolomics has been applied to many disciplines including food and nutritional research [9]. This analytical platform provides opportunities to discover and establish new biochemical pathways, metabolite database, molecular profiles, potential biomarkers and bioactive compounds which can be directly correlated to the quality, safety, fermentation, processing, traceability and authenticity of food products [9, 10, 25, 29, 44, 79].

1.6.2 Metabolomics for the study of microbial activity in yoghurt

Metabolomics has widely been applied to investigate the biochemical changes related to microbial activity during fermentation and the possibility to predict the sensory and nutritional quality of fermented food products [44]. From a molecular perspective, yoghurt is considered as a complex food system consisting of hundreds of biomolecules including proteins, lipids, carbohydrates and many other small compounds, such as amino acids, organic acids, nucleic acids, fatty acids, minerals and other aroma volatiles responsible for its distinctive flavor characteristics [69]. Regarding this range of chemical classes, measurement of all metabolites in yoghurt using a single analytical platform is usually unattainable. The application of mass spectrometry (MS)-based and nuclear magnetic resonance (NMR)-based techniques have shown to be very effective for determining a wide range of metabolites in fermented foods [44]. Headspace SPME-GC/MS has been applied in the determination of volatile metabolite profile in liquid milk and yoghurt [13, 21, 30, 40]. ^{1}H-NMR has been used for better understanding the overall biochemical changes associated with the microbial activity in fermented milk and cheeses [3, 7, 8, 44, 47, 52]. The outcome has provided new insights regarding the variation in metabolite profiles of products related to specific type of starter culture, fermentation process and storage condition [47].

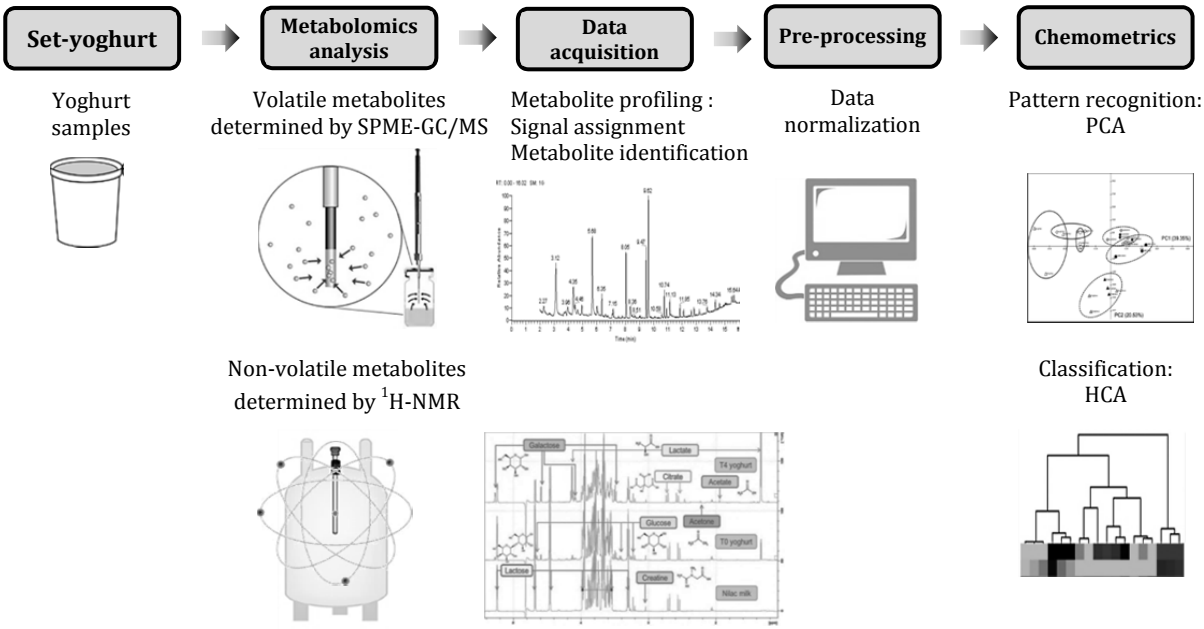

Fig. 1.3. General workflow of the metabolomic-based analytical approach in this research.

1.6.3 Interpretation of metabolomics data

The key success strategies for metabolomics research are challenged by how to (i) collect high-throughput data (metabolome) and (ii) interpretation of multi-dimensional datasets by which essential knowledge in advanced analytical chemistry and chemometrics needs to be combined [5]. The application of multivariate statistical analysis reduces the dimension of metabolomic dataset and enables to identify possible patterns among the samples [41]. In this research, the two most common unsupervised algorithms: (i) principal component analysis (PCA) and (ii) hierarchical cluster analysis (HCA) were applied to distinguish significant patterns from the metabolite profiles of set-yoghurts (Fig. 1.3). The two algorithms reveal the comparative metabolite profiles among yoghurt samples from different perspectives. The PCA enables to visualize the relationships between samples and metabolites by means of two component plots. The score plot illustrates how the samples are distinguished according to their metabolite profiles. The loading plot indicates how much each metabolite is relatively contributed on that specific principal component corresponding to the pattern observed [20]. The HCA classifies metabolite profiles of the samples according to the overall similarities determined by a metric consisting of Pearson's correlation distances with average

linkages. The results are demonstrated in terms of a list of clusters (dendrogram) and their members at each level of the hierarchy [20].

1.7 Objectives and outline of the thesis

The main objective of this research was to investigate the simultaneous growth and metabolite production by traditional yoghurt starters and different probiotic strains in set-yoghurt. A strategy to enhance the survival of probiotics in acidic condition of yoghurt was additionally applied by preculturing these functional bacteria under sublethal salt and low pH stress conditions. This approach would enable probiotics to develop adaptive responses leading to an increase in their survival prior to inoculation in milk. The activity of yoghurt starters and probiotics was investigated by monitoring bacterial population dynamics, milk acidification and changes in the molecular profiles of yoghurt (Fig. 1.4).

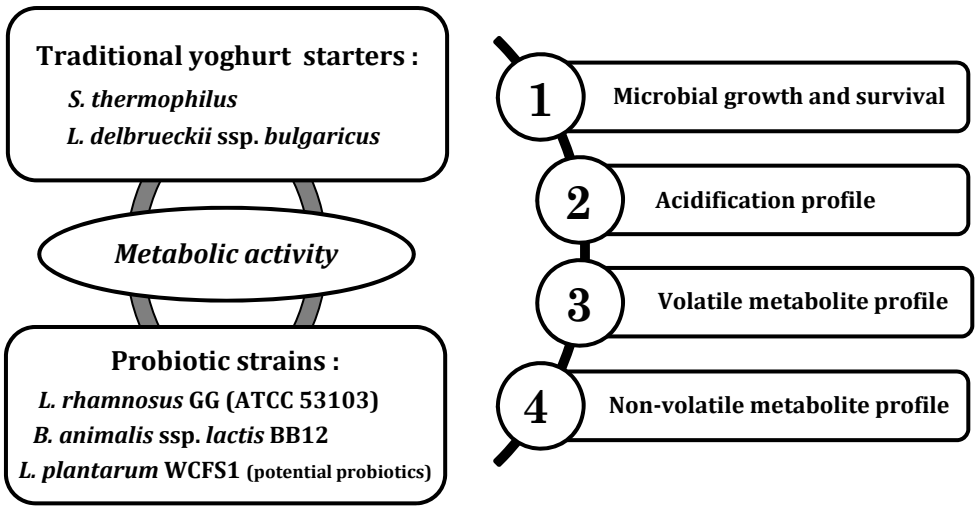

Fig. 1.4. Schematic representation of the approaches used to investigate the activity of yoghurt starters and probiotics in this research.

A complementary metabolomics approach using headspace SPME-GC/MS and ^{1}H-NMR was applied for characterization of biochemical changes related to microbial metabolism during set-yoghurt fermentation and refrigerated storage. Finally, metabolite profiles of yoghurts fermented with different types of starter combinations and different durations of storage were statistically compared by means of multivariate analysis. The outcomes are expected to provide new insights concerning the impact of probiotics incorporation in yoghurt, since their metabolic activity may substantially affect the biochemical and organoleptic characteristics of this product. Understanding the activity of probiotics in milk and yoghurt is an essential step for optimizing their performance yielding in a higher quality product.

The outline of this thesis consists of the following chapters devoted to specific investigations:

- **Chapter 2** focuses on the interaction between different proteolytic strains of *S. thermophilus* in co-culture with *L. delbrueckii* subsp. *bulgaricus* in set-yoghurt. The impact of proto-cooperation on the growth of the two species, milk acidification and changes in volatile and non-volatile metabolite profiles of yoghurt are discussed. The importance of suitable strain selection for achieving the best technological performance regarding the quality of product is underlined.

- **Chapter 3** reveals the impact of probiotics incorporation on the metabolite formation in set-yoghurt. Two commercial probiotic strains, *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12, were co-cultivated with traditional yoghurt starters. The microbial activity during fermentation and storage is discussed in terms of bacterial population dynamics, milk acidification and changes in volatile and non-volatile metabolite profiles of yoghurt.

- **Chapter 4** introduces preculturing under sublethal stress condition as a potential strategy to improve the survival of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 in yoghurt. The two probiotic strains were precultured

under elevated NaCl and low pH stress combinations in a batch fermentor prior to inoculation in milk. Adaptive responses of sublethally precultured probiotics are discussed in terms of significant increase in their survival and substantial impact on the metabolite formation in yoghurt.

- **Chapter 5** continues on the framework of the previous study by evaluating the growth and survival of potential probiotic *L. plantarum* WCFS1 in co-fermentation with traditional yoghurt starters. The influence of sublethally precultured *L. plantarum* WCFS1 on the survival of *L. delbrueckii* subsp. *bulgaricus*, post-acidification and metabolite formation in yoghurt are discussed.

- **Chapter 6** provides a general discussion including technical aspects of the two metabolomic-based analytical platforms and the main findings of the studies described in the earlier chapters. The main conclusions and implications of the studies are addressed, and recommendations for future research are proposed.

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Chapter 2

Influence of different proteolytic strains of *Streptococcus thermophilus* in co-culture with *Lactobacillus delbrueckii* subsp. *bulgaricus* on the metabolite profile of set-yoghurt

Based on:

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Abstract

Proto-cooperation between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* is one of the key factors that determine the fermentation process and final quality of yoghurt. In this study, the interaction between different proteolytic strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* was investigated in terms of microbial growth, acidification and changes in the biochemical composition of milk during set-yoghurt fermentation. A complementary metabolomics approach was applied for global characterization of volatile and non-volatile polar metabolite profiles of yoghurt associated with proteolytic activity of the individual strains in the starter cultures. The results demonstrated that only non-proteolytic *S. thermophilus* (Prt-) strain performed proto-cooperation with *L. delbrueckii* subsp. *bulgaricus*. The proto-cooperation resulted in significant higher populations of the two species, faster milk acidification, significant abundance of aroma volatiles and non-volatile metabolites desirable for a good organoleptic quality of yoghurt. Headspace SPME-GC/MS and ^{1}H-NMR resulted in the identification of 35 volatiles and 43 non-volatile polar metabolites, respectively. Furthermore, multivariate statistical analysis allows discriminating set-yoghurts fermented by different types of starter cultures according to their metabolite profiles. This finding underlines that selection of suitable strain combinations in yoghurt starters is important for achieving the best technological performance regarding the quality of product.

2.1 Introduction

Yoghurt is one of the most popular fermented dairy products and its consumption is increasing worldwide [40]. According to the Codex standard for fermented milks [7], yoghurt is specifically characterized by the presence of symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. During fermentation, these bacteria perform three major biochemical conversions of milk components: (i) conversion of lactose into lactic acid (fermentation), (ii) hydrolysis of caseins into peptides and free amino acids (proteolysis) and (iii) breakdown of milk fat into free fatty acids (lipolysis) [44]. These reactions lead to the production of various metabolites resulting in a decrease of the pH, formation of a semi-solid texture and a distinctive yoghurt flavor [23]. Even though *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are able to grow individually in milk, they can have a symbiotic interaction called "proto-cooperation" in mixed cultures [43]. The interaction is based on the exchange of several metabolites which provide mutual growth stimulating effects [41]. In summary, *S. thermophilus* produces pyruvic acid, formic acid, folic acid, ornithine, long-chain fatty acids and CO_2 which stimulate the growth of *L. delbrueckii* subsp. *bulgaricus*. Lactic acid produced by *S. thermophilus* also reduces the pH of milk to an optimum level for *L. delbrueckii* subsp. *bulgaricus*. Consequently, the growth of *L. delbrueckii* subsp. *bulgaricus* supplies peptides, free amino acids and putrescine that stimulate the growth of *S. thermophilus* [41]. Recent post-genomic studies in mixed culture of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* have revealed new insights in physiology and molecular basis of the interaction [18, 33, 36, 41, 42]. Although interaction between the two species is often positive (proto-cooperation), absence of interaction or even negative effects can take place depending on the combination of bacterial strains, type and heating process of base milk and fermentation conditions [11].

Proteolytic systems in lactic acid bacteria rely on the function of bacterial cell-envelope proteinases, peptide transport systems and intracellular peptidases [30]. Proteolytic activity of one of the species in the mixed cultures (mostly *L. delbrueckii* subsp. *bulgaricus*) plays an important role in proto-cooperation as mentioned

previously. Traditional yoghurt cultures consist of non-proteolytic (Prt-) *S. thermophilus* and high proteolytic *L. delbrueckii* subsp. *bulgaricus* [46]. Thus, the former bacteria take advantage from active proteolytic system of the latter ones to meet their amino acid requirement. Furthermore, metabolism of amino acids generates volatile metabolites responsible for the aroma profile of product [30]. The contributions of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* to aroma volatile formation in fermented milk are well documented [22, 34, 39]. However, the expression of proteolytic activity (Prt+) in several *S. thermophilus* strains allows them to grow independently in milk leading to substantial acidification. This strong impact of proteolytic activity on acidifying capacity of *S. thermophilus* has been reported [10, 12, 15]. Despite extensive publications on the strain selections, mechanisms of amino acid biosynthesis, genome sequences and potential application of proteolytic *S. thermophilus* strains [12, 21, 25], the role of these proteolytic streptococci in mixed culture with *L. delbrueckii* subsp. *bulgaricus* hardy received attention.

The developments in metabolomics allows discovery of a wide range of metabolites in complex biological systems including food matrices [17]. The application of mass spectrometry (MS) and nuclear magnetic resonance (NMR) technique have shown to be very successful in determining a wide range of metabolites related to microbial activity during fermentation, ripening and storage of fermented dairy products [9, 13, 14, 16, 32, 38]. However, to our knowledge, a complementary approach has never been applied to attain information regarding the effects of proteolytic activity of individual strains of *S. thermophilus* in mixed culture with *L. delbrueckii* subsp. *bulgaricus* during yoghurt fermentation on the global metabolite profile of product.

The objective of this study was therefore to investigate the interaction between proteolytic and non-proteolytic strains of *S. thermophilus* in mixed culture with *L. delbrueckii* subsp. *bulgaricus* during set-yoghurt fermentation. Growth of starter cultures, changes in milk pH and titratable acidity were monitored. Biochemical changes related to the interaction between the two species were characterized in terms of volatile and non-volatile polar metabolite profile of yoghurt using headspace SPME-GC/MS and ^{1}H-NMR technique.

2.2 Materials and methods

2.2.1 Milk preparation

The milk was obtained by reconstituting 10% (w/v) Nilac skimmed milk powder (NIZO, Ede, The Netherlands) in milli-Q water (45 °C) to obtain final liquid milk at 9.5% dry matter content. The milk was pasteurized at 90 ^{o}C for 5 min and then was cooled down in a water bath until the temperature of 42 ^{o}C was reached.

2.2.2 Starter cultures

Frozen direct-vat-inoculation pellets of *S. thermophilus* Prt+ (ST-Prt+) strain C38, *S. thermophilus* Prt- (ST-Prt-) strain C44 and *L. delbrueckii* subsp*. bulgaricus* Prt+ (LB) strain C49 were supplied by CSK Food Enrichment (Ede, The Netherlands). The difference in proteolytic activity between the two *S. thermophilus* strains refers to the extracellular protease PrtS targeting milk proteins during yoghurt production. The pellets were stored at -45 ^{o}C and were placed at ambient temperature (20 ± 3 °C) for 15 min before use. Inoculation was performed to obtain an initial viable bacteria level at 10^6 cfu/g. Five different types of single strain and mixed cultures: (i) pure ST-Prt+, (ii) pure ST-Prt-, (iii) pure LB, (iv) mixed ST-Prt+/LB and (v) mixed ST-Prt-/LB were investigated in this study. The combinations of *S. thermophilus* and *L. delbrueckii* subsp*. bulgaricus* were inoculated at the ratio 10^6:10^6 cfu/g because in preliminary experiments, this ratio had demonstrated the best profile (bacterial growth, acidity and texture) for yoghurt (data not shown).

2.2.3 Set-yoghurt fermentation

After inoculation, set-yoghurt fermentation was carried out in a water bath at 42 ^{o}C for 4 h. Samples were taken every hour during fermentation for microbiological and chemical analysis. The enumeration of viable bacteria and determination of pH and titratable acidity were carried out directly after sampling. For ^{1}H-NMR analysis, the samples were stored at -20 °C until the analysis. The fermentation was performed in three replicates for each type of starter culture.

2.2.4 Enumeration of viable bacteria

Viable bacteria in set-yoghurt were enumerated using the standard pour plate technique. Viable counts of *S. thermophilus* were determined on *S. thermophilus* agar after aerobic incubation at 37 °C for 24 h [3]. Viable counts of *L. delbrueckii* subsp. *bulgaricus* were determined on MRS agar pH 5.70 (Merck, Darmstadt, Germany) after anaerobic incubation (Anoxomat™-Mart® Microbiology, Drachten, the Netherlands) at 37 °C for 48 h [3].

2.2.5 Determination of acidification profile

Production of acid during fermentation was expressed by changes in pH and increases in titratable acidity. Yoghurt samples were weighed to 25.0 g and the pH measurements were performed using a laboratory pH meter (InoLab pH720, WTW, Weilheim, Germany). The samples were titrated with 0.1 N NaOH (Merck, Darmstadt, Germany) with continuous magnetic stirring until pH 8.30 was reached. The amount of 0.1 N NaOH (mL) used to titrate 100 g of yoghurt was referred as Titratable acidity (TA). The TA value was expressed as % acid equivalent to lactic acid (% LA) in the sample [24].

2.2.6 Analysis of volatile metabolites by headspace SPME-GC/MS

For headspace SPME-GC/MS analysis, a mimic-scenario of set-yoghurt fermentation was carried out directly in GC vials to avoid loss of these compounds during sample preparation. The inoculated NILAC milk was directly divided (3 mL) into a series of five clear headspace GC vials (10 mL, 46 x 22.5 mm) sealed with 20 mm silicone/PTFE septa and magnetic caps (Grace, Albany, OR, USA). The vials were placed in a water bath at 42 °C for 4 h. The samples were stored at -20 °C until the analysis. In order to ensure that the results were comparable with the yoghurt fermented in section 2.3, the final pH (4 h) from in-vial fermentation was regularly verified (data not shown). The fermentation was performed in three replicates for each type of starter culture.

a) Extraction of volatile compounds by Solid Phase Micro Extraction (SPME)

Frozen samples were thawed and incubated at 60 °C for 1 min. Afterward, volatile compounds in the headspace were extracted at 60 °C for 5 min with a 75 µm Carboxen™–PDMS-SPME fiber (Supelco, Bellefonte, PA, USA) using TriPlus™ autosampler (Thermo Scientific, Austin, TX, USA). Milli-Q water was analyzed as blank sample. This method was based on the method developed by Hettinga et al. [19].

b) Determination of volatile compounds by gas chromatography coupled with mass spectrometry (GC/MS)

The SPME fiber was desorbed for 10 min in the GC injection port. The GC/MS analysis was performed using Trace GC Ultra connected with DSQ II mass spectrometer (Thermo Scientific, Austin, TX, USA). The Stabilwax®-DA-Crossband®-Carbowax®-polyethylene-glycol column with 30 m length, 0.32 mm internal diameter, and 1 µm film thickness (Restek, Bellefonte, PA, USA) was used. The oven temperature was maintained at 40 °C for 3 min, then increased at 15 °C/min to 220 °C and maintained for 1 min. The carrier gas was helium fed with a constant flow rate at 1.5 mL/min. The MS iron source was maintained at 225 °C with full scan. Electron impact mode was at 70 eV with the mass range 33-250 *m/z*. This procedure was modified based on Hettinga et al. [19]. Volatile metabolites were identified using AMDIS software (NIST, Gaithersburg, MD, USA) referred to NIST/EPA/NIH database and library provided by Hettinga et al. [20]. Peaks from column bleed and SPME fiber were corrected using the blank sample. Specific retention time and *m/z* model were used for automated peak integration in the XCalibur software package (Thermo Scientific, Austin, TX, USA).

2.2.7 Analysis of non-volatile polar metabolites by ^{1}H-NMR spectroscopy

a) Sample preparation and ^{1}H-NMR analysis

For ^{1}H-NMR analysis, the samples from two replicates were analyzed. Frozen yoghurt samples were thawed at room temperature and pH was adjusted to 6.0 using 1.0 N NaOH to achieve low variation, i.e. location and shape of peaks, in the spectra obtained [31]. Residual lipids were removed by dichloromethane extraction. The samples were diluted 1:2 (w/w) with dichloromethane (Merck, Darmstadt, Germany),

then were mixed and centrifuged (Multifuge X3R, Thermo Scientific, Austin, TX, USA) at 4,100*g* for 15 min at 4 °C. The clear liquid was collected and ultra-centrifuged (Beckman L60 Ultracentrifuge, Boulevard Brea, CA, USA) at 117,500*g* for 75 min at 4 °C to remove the protein fraction. The clear serum was collected and ultra-filtrated using microcentrifugation (Spectrafuge™ 16M Microcentrifuge, Labnet Int. Inc., Woodbridge, NJ, USA) at 13,800*g* for 20 min at room temperature through a Pall Nanosep® centrifugal device with 3 kDa molecular weight cutoff (Pall life science, Ann Arbor, MI, USA). The filtrate was mixed 1:1 (v/v) with phosphate buffer pH 6.0 (300 mM KH_2PO_4, 10% (w/w) D_2O and 1 mM 3-(Trimethylsilyl) propionic-2, 2, 3, 3-d4 acid sodium salt (TSP)) as internal standard. All chemicals used to prepare the buffer were obtained from Sigma-Aldrich (Steinheim, Germany). The mixture was stabilized at 4 °C overnight and then re-centrifuged at 13,800*g* for 20 min at room temperature for final precipitate removal. Finally, 350 µL of the mixture was transferred into a 4.25 mm NMR tube. NOESY 1D-^{1}H-NMR measurements were performed at 300 K in a 600 MHz NMR spectrometer (Bruker, Rheinstetten, Germany) equipped with a cryogenic probe, using Bruker sample handler (BACS-60) operated under full automation, with similar parameters as described by Lu et al. [29].

b) ^{1}H-NMR spectra processing

The ^{1}H-NMR spectra were baseline-corrected, phase-corrected, aligned and calibrated based on the internal standard (TSP) peak. For each spectrum, chemical shift (δ) across the range of 0.00 - 10.00 ppm was segmented (binning) with an interval of 0.02 ppm [1]. The signal intensity in each bin was integrated and expressed in arbitrary units using AMIX software (Bruker, Rheinstetten, Germany). The bins corresponding with the water region (δ = 4.73 - 4.99 ppm) and methanol (δ = 3.35 - 3.37 ppm) were eliminated from the analysis. Metabolite labels were presumptively assigned to the bins by means of Chenomx NMR suite 7.5 library (Chenomx Inc., Alberta, Canada), Human Metabolome Database version 3.0 [47] and from literature [5, 26, 29]. For unlabeled bins, significant variables were selected based on one-way ANOVA at 95% confidence level.

2.2.8 Statistical analysis

One-way ANOVA with multiple comparisons by Tukey's test were performed using IBM SPSS statistics package version 19 (SPSS Inc., Chicago, IL, USA). A probability at $p < 0.05$ was considered statistically significant. Metabolomics data from GC/MS and ^{1}H-NMR were normalized by median-centering and $\log_2$-scaling before multivariate statistical analysis. Principal component analysis (PCA) and heat-map visualization combined with Pearson's correlation-based hierarchical cluster analysis (HCA) were performed using Multi-Experiment Viewer (MeV) version 4.8 (www.tm4.org/mev/).

2.3 Results and discussion

2.3.1 Bacterial growth profiles

The growth (increase in biomass) of ST-Prt+ with LB (as pure and mixed culture) (Fig. 2.1A) and ST-Prt- with LB (as pure and mixed culture) (Fig. 2.1B) were monitored during fermentation. In pure cultures, the two ST strains grew rapidly during the early part of fermentation (0 – 3 h), while LB remained in the lag-period for at least one hour. This can be explained by the initial pH of Nilac milk (6.5 ± 0.1) which is more favorable for the growth of ST [46] and their effective capacity to use nutrients available in milk. These bacteria initially utilize free amino acids and peptides available in milk. However, the free nitrogen content in milk is very limited, usually not exceeding 100 mg/L depending on the animal breed, milking season, heat-treatment and storage [28], thus only the ST-Prt+ is expected to be able to continue growing with support of its proteolytic activity. Even though the LB in this study is a proteolytic strain, its growth was found to be retarded by a lower optimum pH and higher nutritional requirements [41]. At the end of fermentation, the viable counts of pure ST-Prt+ were significantly higher ($p < 0.05$) than those of pure ST-Prt- and pure LB which were not significantly different from each other. The final numbers increased by an average of 1.5 log cfu/g for pure ST-Prt+ and 1.2 log cfu/g for pure ST-Prt- and for pure LB. These results demonstrate that the ST-Prt+ strain exhibited a significant higher capacity to develop

individually in milk compared to ST-Prt- and LB. This observation is in agreement with the work of Courtin et al. [10].

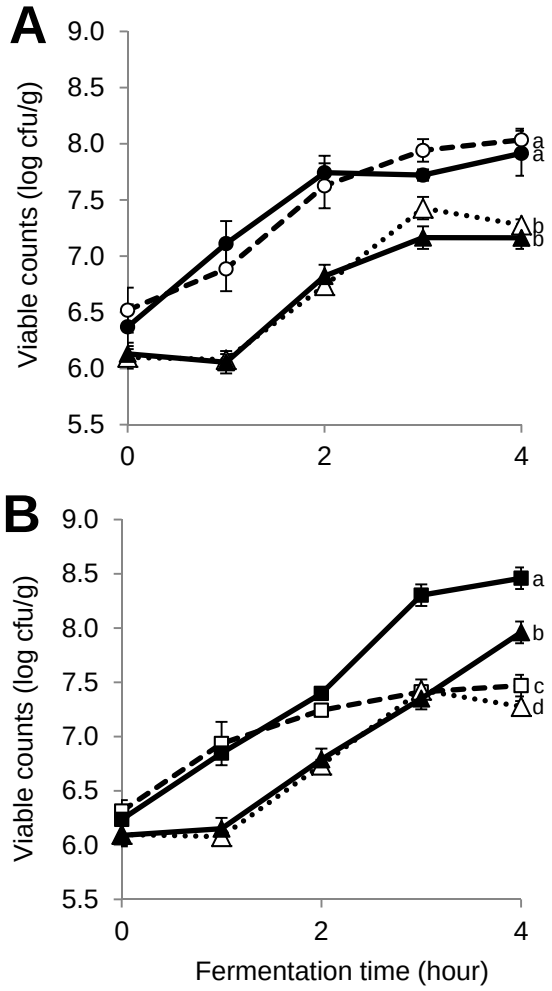

Fig. 2.1. Changes in viable counts during set-yoghurt fermentation with a pure culture of *S. thermophilus* Prt+ (-○-, panel A), pure culture of *L. delbrueckii* subsp. *bulgaricus* (···△··, panel A) and their mixed culture (—●—,—▲—, panel A) compared with pure culture of *S. thermophilus* Prt- (-□-, panel B), pure culture of *L. delbrueckii* subsp. *bulgaricus* (···△··, panel B) and their mixed culture (—■—,—▲—, panel B). Error bars represent standard deviations based on three independent replicates. Letters (a-c) indicate significant differences among mean values ($p < 0.05$) of samples at the end of fermentation (4 h).

In mixed culture with LB, growth of the two ST strains started deviating from each other after 2 h. The viable counts of ST-Prt+ remained virtually constant towards the end of fermentation whereas those of ST-Prt- strongly increased (2 – 3 h), with an average of 0.9 log cfu/g before remaining at a stable level. The results suggested that ST-Prt- took advantage of the proteolytic activity of LB whereas ST-Prt+ did not benefit from this interaction. At the end of fermentation, the viable counts of ST-Prt+ in mixed culture were not significantly different ($p > 0.05$) from the pure culture. In contrast, the viable counts of ST-Prt- were significantly higher ($p < 0.05$) from those of the pure culture. The final numbers of ST-Prt+ and ST-Prt- increased by an average of 1.5 and 2.3 log cfu/g, respectively. The growth of LB in mixed culture with either ST-Prt+ or ST-Prt- took place during 1 – 3 h. However, during 3 – 4 h, the viable counts of LB were constant in mixed culture with ST-Prt+ while the counts in mixed culture with ST-Prt- still increased. This could be related to the continuous growth of ST-Prt- which consequently produces acid and lowers the pH to a level which favors for the growth of LB. Moreover, the LB might be stimulated by several metabolites produced by ST-Prt- as mentioned previously [41]. At the end of fermentation, the viable counts of LB in pure culture and in mixed culture with ST-Prt+ were not significantly different ($p > 0.05$) whereas its counts were significantly higher ($p < 0.05$) in mixed culture with ST-Prt-. The final numbers of LB in mixed culture with ST-Prt+ and ST-Prt- increased by an average of 0.9 and 1.9 log cfu/g, respectively. This observation clearly demonstrates the proto-cooperation between ST-Prt- and LB resulting in significant higher populations of the two species at the end of fermentation. This mutual growth stimulation between ST and LB is in agreement with the results of Courtin & Rul [11] and Herve-Jimenez et al. [18]. However, these results contrast with the findings of Courtin et al. [10] who mentioned that the proteolytic activity of ST has no significant effect either on bacterial growth or final pH of yoghurt in mixed culture with a proteolytic LB. This might be explained by the differences in bacterial strains employed leading to their particular proteolytic profiles and other experimental factors such as type and pre-treatment of base milk as well as fermentation conditions.

2.3.2 Acidification profiles

The changes in pH were monitored every hour during fermentation (Fig. 2.2A). At the end of fermentation, the pH values of all yoghurts fermented by different types of starter cultures were significantly different ($p < 0.05$). Mixed ST-Prt-/LB showed the best capacity to acidify milk followed by mixed ST-Prt+/LB, pure ST-Prt+, pure ST-Prt- and pure LB respectively. The two mixed cultures were able to acidify milk to a pH value below 5.0. However, only mixed ST-Prt-/LB was efficient in lowering pH to a value below 4.6 at which caseins aggregate [46]. Gel formation was only observed in the samples fermented with this mixed culture (data not shown). The similar capacity to lower milk pH by a mixed culture of ST and LB has also been reported by others [10, 18, 37].

Titratable acidity was expressed as % acid equivalent to lactic acid (Fig. 2.2B). In pure cultures, ST-Prt+ showed the best capacity to produce acid with an amount that is two-times higher than ST-Prt- and four-times higher than LB at the end of fermentation. In mixed culture, ST-Prt+ did not show good interaction with LB. The amount of acid produced by mixed ST-Prt+/LB (0.50%) was slightly higher than the sum of acid produced by pure ST-Prt+ and pure LB together (0.41%). This result can be associated with the populations of ST-Prt+ and LB which are not significantly different in pure culture and mixed culture. On the other hand, the amount of acid produced by mixed ST-Prt-/LB (0.64%) was almost three-times higher than the sum of acid produced by pure ST-Prt- and pure LB together (0.22%). This observation is in accordance with the proto-cooperation observed on the growth of ST-Prt- and LB in mixed culture. Obviously, the significant higher populations of the two species are expected to lead to higher acid production.

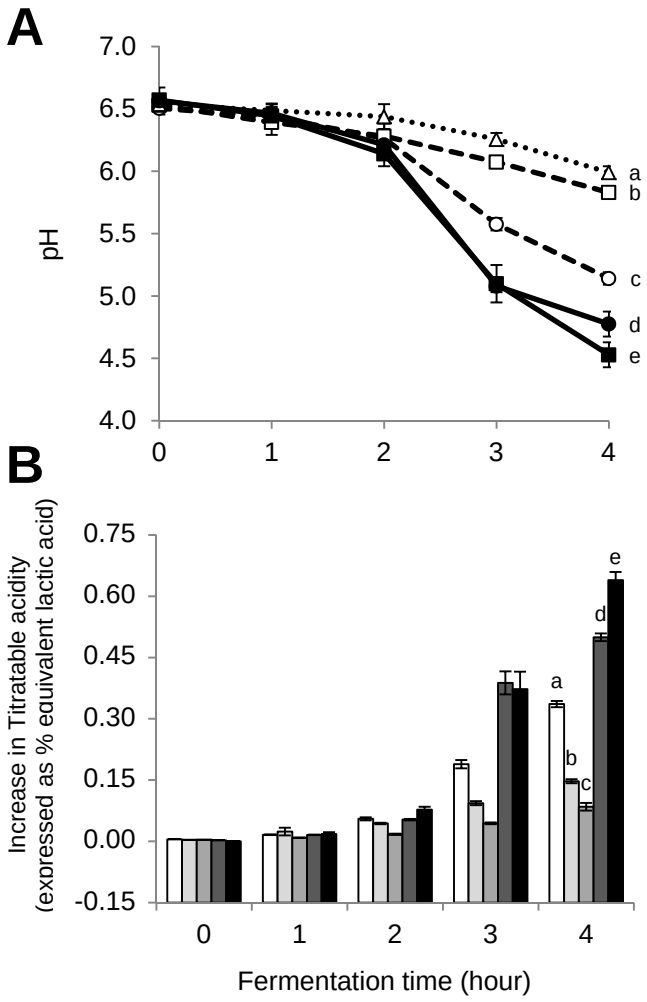

Fig. 2.2. Changes in pH (panel A) and titratable acidity (panel B) during set-yoghurt fermentation by a pure culture of *S. thermophilus* Prt+ (○, □), pure culture of *S. thermophilus* protease Prt- (□, ▒), pure culture of *L. delbrueckii* subsp. *bulgaricus* (△, ▒), mixed culture of *S. thermophilus* Prt+ with *L. delbrueckii* subsp. *bulgaricus* (●, ▒) and mixed culture of *S. thermophilus* Prt- with *L. delbrueckii* subsp. *bulgaricus* (■, ■),. Error bars represent standard deviations based on three independent replicates. Letters (a-e) indicate significant differences among mean values ($p < 0.05$) of samples at the end of fermentation (4 h).

2.3.3 Volatile metabolite profiles determined by headspace SPME-GC/MS

In this study, a total of 35 compounds consisting of alcohols, carbonyl compounds, organic acids, sulfur compounds and heterocyclic compound were identified in Nilac milk and set-yoghurts (Table S2.1). This list is comparable to the volatiles identified in yoghurt using headspace SPME-GC/MS in other studies [8, 14]. The

35 compounds were introduced as variables for multivariate analysis. If necessary, missing values were replaced by the median of respective variables.

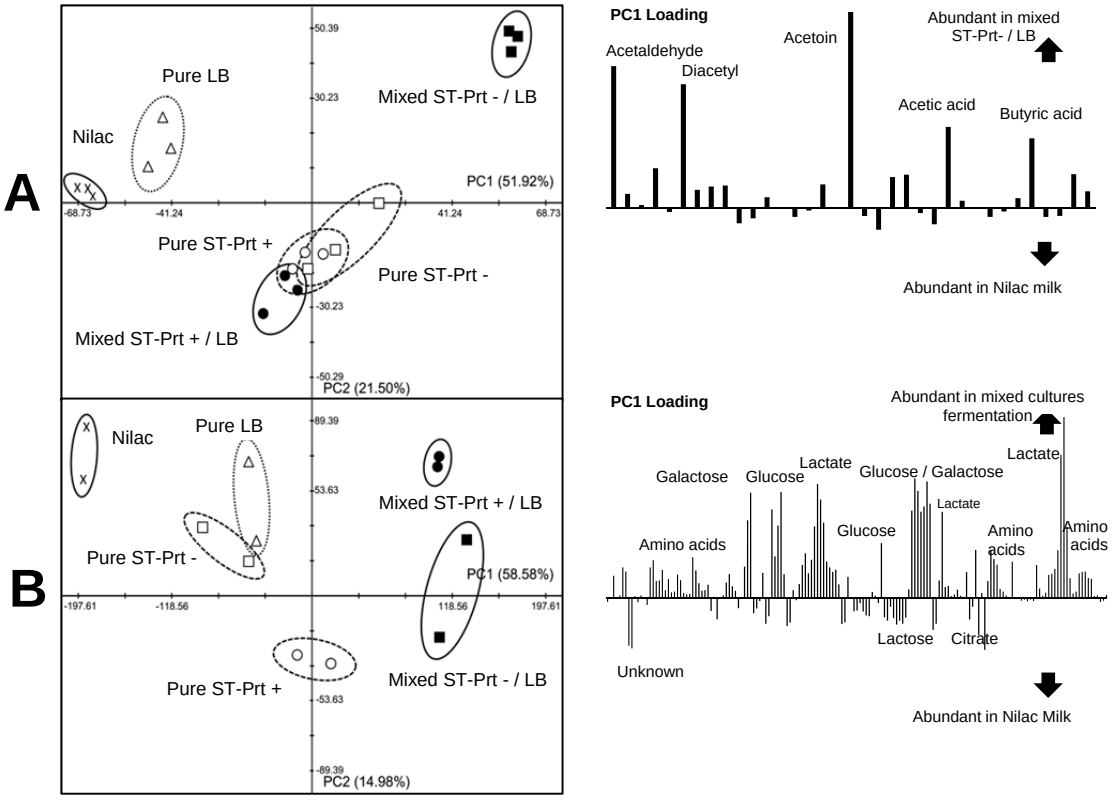

Fig. 2.3. PCA score plots and loadings of PC1 derived from volatile metabolite profiles (panel A) and non-volatile polar metabolite profiles (panel B) of Nilac milk (X) and set-yoghurt fermented by a pure culture of *S. thermophilus* Prt+ (○),pure culture of *S. thermophilus* protease Prt- (□), pure culture of *L. delbrueckii* subsp. *bulgaricus* (Δ),mixed culture of *S. thermophilus* Prt+ with *L. delbrueckii* subsp. *bulgaricus* (●) and mixed culture of *S. thermophilus* Prt- with *L. delbrueckii* subsp. *bulgaricus* (■).

An overall PCA score plot was constructed with a total variance of 73.4% (Fig. 2.3A). The samples fermented with mixed ST-Prt-/LB were clearly separated from Nilac milk and from the samples fermented with pure LB along PC1 (51.9% variance) while the distinction among the samples fermented with pure ST-Prt+, pure ST-Prt- and mixed ST-Prt+/LB was not clearly visible. PC1 loading indicated that acetaldehyde, diacetyl, acetoin, acetic acid and butyric acid mainly accounted for the separation of mixed ST-Prt-/LB from Nilac milk and other yoghurt samples.

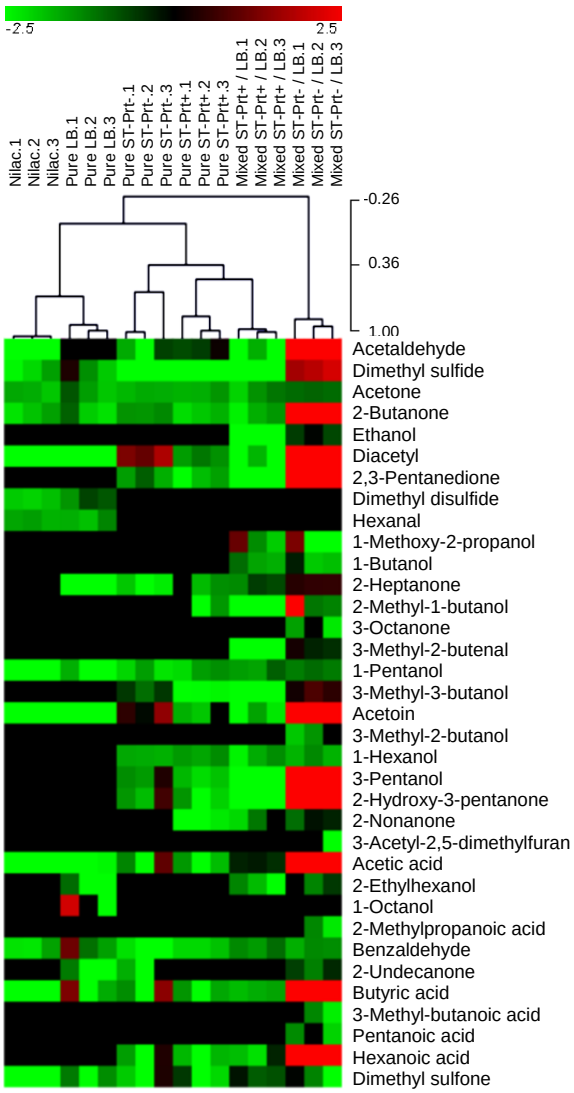

Fig. 2.4. Heat-map and hierarchical clustering of volatile metabolite profiles from Nilac milk and set-yoghurts fermented by different types of starter cultures. Dendrogram represents sample clusters based on Pearson's correlation coefficient with average linkage. Each square in the heat-map expresses normalized volatile content respected to the color range. The red color indicates higher content of the corresponding compound.

Heat-map visualization combined with HCA (Fig. 2.4) demonstrated that acetaldehyde, dimethyl sulfide, 2-butanone, diacetyl, 2,3-pentanedione, acetoin, 3-pentanol, 2-hydroxy-3-pentanone, acetic acid, butyric acid and hexanoic acid were present in high relative abundance (shown in red) in the samples fermented with mixed

ST-Prt-/LB. These compounds are desirable for a good organoleptic quality of yoghurt. The dendrogram showed that Nilac milk and different yoghurt samples could be well grouped according to their volatile metabolite profiles. Unlike PCA, the samples fermented with pure ST-Prt+, pure ST-Prt- and mixed ST-Prt+/LB could be clearly assigned into different clusters.

In terms of technological relevance, all major yoghurt aroma volatiles [6]; i.e. acetaldehyde (fresh, green, pungent), diacetyl (buttery, creamy), acetoin (buttery), 2-butanone (sweet, fruity), 2,3-pentanedione (buttery, vanilla-like) and acetic acid (vinegar, pungent) were detectable in this study. The contributions of ST and LB to aroma volatile formation in fermented milk are well documented [4, 6, 45, 46]. In pure cultures, the total numbers of volatiles identified were almost equal in the samples fermented with pure ST-Prt+ (n = 20) and ST-Prt- (n = 19) but higher compared to those in the samples fermented with pure LB (n = 16) (Table S2.1). Acetaldehyde is the most important compound contributing to typical yoghurt aroma which can be derived from amino acid catabolism [6]. In case of ST, threonine is converted into acetaldehyde by the activity of threonine aldolase [25]. The two pure ST cultures showed high capacity of acetaldehyde production without difference depending on their proteolytic activity. Indeed, the proteolytic activity of ST strains was expected to have an impact on the concentrations of various volatiles derived from amino acid catabolism; e.g. 1-methoxy-2-propanol (Val), 2-methyl-1-butanol (Ile/Leu), 3-methyl-3-butanol (Leu), benzaldehyde (Trp/Phe), 3-methyl-2-butenal (Ile/Leu), 2,3-pentanedione (Ile), 3-methyl-butanoic acid (Leu), 2-methyl-propanoic acid (Val), acetic acid (Thr) and sulfur compounds (Cys/Met) [2, 44]. However, the two pure ST cultures only showed significant differences between each other in concentration of 2-methyl-1-butanol and 3-methyl-3-butanol. Possibly, the formation of these compounds by the two pure ST cultures initially relies on the utilization of free amino acids available in milk. Thus, the impact of different proteolytic activity between the two ST strains was not observed. In mixed cultures, although the total numbers of identified volatiles increased, relatively low numbers were found in the samples fermented with mixed ST-Prt+/LB (n = 25) compared to mixed ST-Prt-/LB (n = 32) (Table S2.1). It was apparent that mixed ST-

Prt+/LB did not show a significant capacity to increase the concentration of major yoghurt aroma volatiles compared to their pure cultures. On the other hand, all major aroma volatiles were detected in significant higher abundance in the samples fermented with mixed ST-Prt-/LB (Fig. 2.4). Furthermore, 3-methyl-2-butanol, 3-octanone, 3-acetyl-2,5-dimethyl-furan, 3-methyl-butanoic acid, 2-methyl-propanoic acid and pentanoic acid were exclusively detected in the samples fermented with this mixed culture. As previously mentioned, these compounds are derived from amino acid catabolism. Because the proteolytic activity of ST-Prt- is low, the formation of these compounds relies on proteolytic activity of LB in the mixed culture. The results demonstrated that interaction between these two strains generated a favorable yoghurt volatile profile resulting in highest numbers of compounds identified with significant abundance of key-aroma compounds desirable for a good organoleptic quality of yoghurt. This finding confirms the proto-cooperation between ST-Prt- and LB as previously observed for bacterial growth and acidification profile.

2.3.4 Non-volatile polar metabolite profiles determined by ^{1}H-NMR

In this study, a total of 43 metabolites including amino acids, carbohydrates, organic acids, lipid derivatives, carbonyl compounds, a sulfur compound and a nucleoside were presumptively identified (Fig. S2.1). The quantification was performed by summation of signal intensities in all bins corresponding to the target metabolite [35]. The integrated intensities were finally expressed in $\log_{10}$ transformed (arbitrary unit) (Table S2.2). For multivariate analysis, it should be noted that the 43 identified metabolites accounted for labeling of 149 bins. A complementary data filtering by one-way ANOVA was performed for selection of the remaining unknowns [27]. Finally, a total of 165 bins were introduced as variables for the analysis.

An overall PCA score plot was constructed with a total variance of 73.6% (Fig. 2.3B). All yoghurt samples could be distinguished according to different types of starter cultures along PC1 (58.6% variance). A complete distinction was observed between Nilac milk and yoghurts fermented with mixed cultures. The distinction between the samples fermented with pure ST-Prt- and pure LB was small but they could still be

separated. Loading of PC1 indicated that lactose, citrate and unknown bins contributed to the separation of Nilac milk from fermented milk samples. The samples fermented with mixed cultures were clearly determined by lactate, glucose, galactose and most of the metabolites in aliphatic and aromatic region including organic acids and free amino acids. These compounds are well known as major products derived from milk fermentation [45, 48]. Thus, the loading plot provides a good indication for metabolic activity of mixed cultures of ST and LB during set-yoghurt fermentation.

Heat-map visualization combined with HCA (Fig. 2.5) demonstrated that the majority of metabolites were present in high abundance (shown in red), with exceptions for citrate and lactose, in the samples fermented with mixed ST-Prt-/LB. Free amino acids were present in high relative abundance, especially in the samples fermented with pure ST-Prt+ and mixed ST-Prt-/LB. Interestingly, these protein-breakdown products were less present when ST-Prt+ was inoculated in mixed culture with LB, although they are both proteolytic strains. The dendrogram showed that metabolite profiles of the samples fermented with pure LB and pure ST-Prt- were less different from Nilac milk, i.e. closely clustered together. This suggests lower metabolic activity of these two cultures during fermentation. Another main cluster consisted of the samples fermented with pure ST-Prt+ which was well separated from mixed ST-Prt+/LB and mixed ST-Prt-/LB. It can be observed that Nilac milk and yoghurt samples are clearly grouped into different clusters according to their non-volatile polar metabolite profiles. This observation corresponds with the results obtained from PCA.

Changes in lactose, galactose and lactic acid concentration in milk directly indicate the primary metabolic activity of ST and LB during yoghurt fermentation [45] (Table S2.2). In pure cultures, a significant decrease in lactose was only observed in the samples fermented with pure ST-Prt+. The concentration of lactate was significantly increased in the samples fermented with pure ST-Prt+ followed by pure ST-Prt- and pure LB respectively. This observation agrees with the acidification profiles. In mixed cultures, the concentration of lactose and citrate significantly decreased while those of glucose, galactose and lactate were significantly increased. Moreover, dynamic changes in several organic acids, e.g. acetate, benzoate, citrate, formate, isobutyrate, orotate and

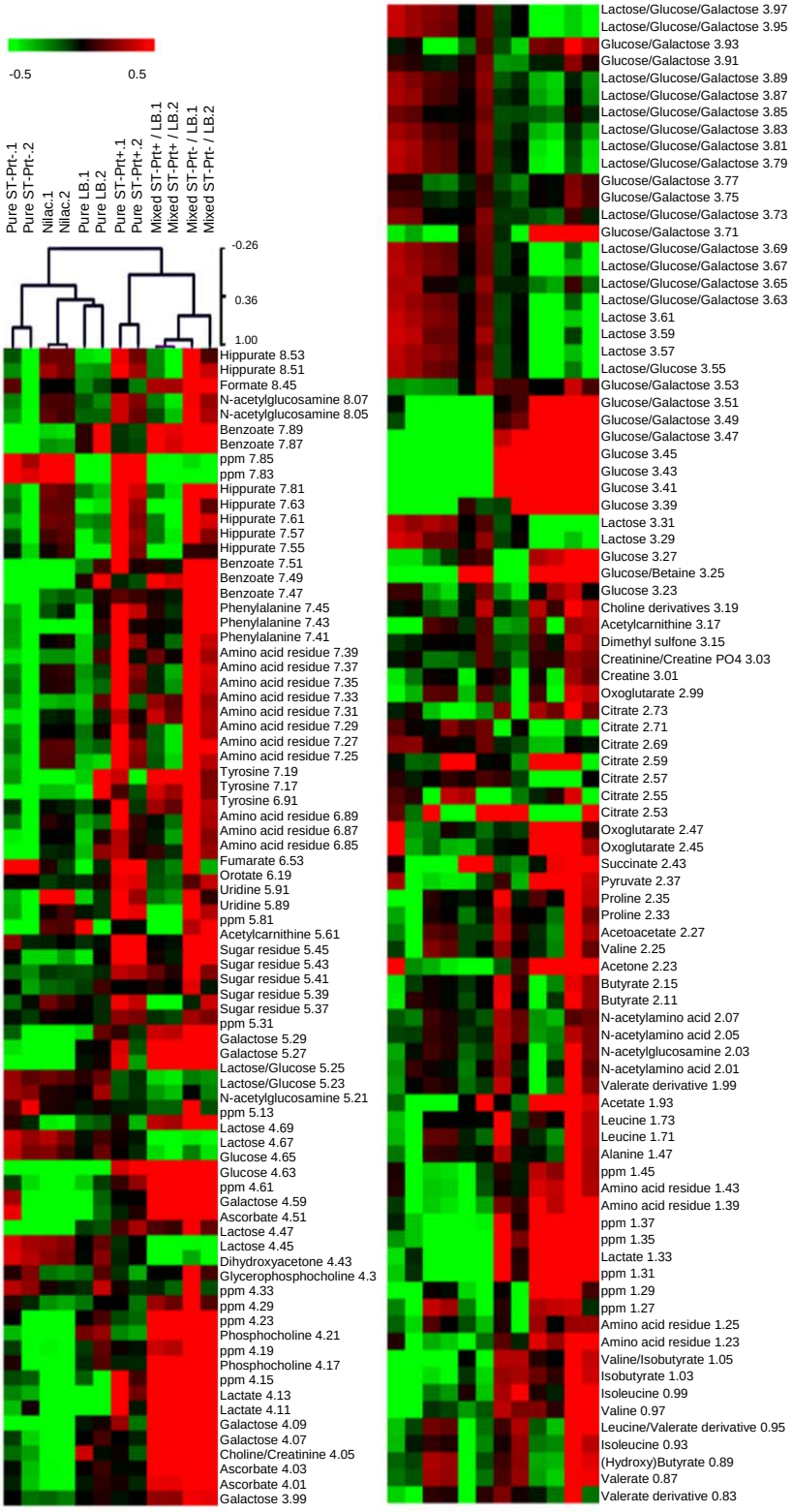

Fig. 2.5. Heat-map and hierarchical clustering of non-volatile polar metabolite profiles from Nilac milk and set-yoghurts fermented by different types of starter cultures. Dendrogram represents sample clusters based on Pearson's correlation coefficient with average linkage. Each square in the heat-map expresses normalized metabolite content respected to the color range. The red color indicates higher content of the corresponding compound.

succinate, were also revealed by ^{1}H-NMR. It should be mentioned that these organic acids also contribute partially to the titratable acidy of yoghurt. The results demonstrate that mixed ST-Prt-/LB shows a higher capacity to generate these compounds compared to mixed ST-Prt+/LB.

The influence of proteolytic activity was characterized by an overall increase in free amino acid concentrations (proteolytic profile) (Table S2.2). In pure cultures, significant increases in N-acetyl amino acids, phenylalanine and valine were observed in the samples fermented with pure ST-Prt+ whereas most of free amino acids were significantly decreased in the samples fermented with pure ST-Prt- and pure LB. This result demonstrates the impact of different proteolytic activity between the two ST strains. Interestingly, the proteolytic profile of samples fermented with mixed ST-Prt+/LB was not significantly different from those observed in pure cultures, although both strains have an extracellular proteolytic activity. On the other hand, the proteolytic profile of samples fermented with mixed ST-Prt-/LB was evidently increased. The concentrations of all free amino acids were significantly increased with an exception for tyrosine. The proto-cooperation between ST-Prt- and LB provides not only growth stimulatory effect on the two species but also exclusively stimulates the proteolytic activity of LB in mixed culture. This assumption is supported by the work of Sieuwerts et al. [42] who reported a considerably higher expression of the proteolytic gene (*prtB-LBUL-1105*) responsible for the extracellular protease activity of LB in mixed culture.

In summary, the samples fermented with mixed ST-Prt-/LB demonstrated a significant higher level of non-volatile flavor compounds (Table S2.2), i.e. lactate, pyruvate, formate, succinate and free amino acids (as precursors for yoghurt aroma formation) for a good organoleptic quality of yoghurt [6]. These results confirm the proto-cooperation between ST-Prt- and LB as observed previously for bacterial growth, acidification and formation of aroma volatile compounds.

2.4 Conclusions

The present study demonstrated that selection of suitable strain combinations between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in yoghurt starters is important for achieving the best technological performance regarding the quality of product. Although *S. thermophilus* Prt+ showed the best capacity to grow individually in milk, it did not interact well in mixed culture with *L. delbrueckii* subsp. *bulgaricus*. On the other hand, proto-cooperation between *S. thermophilus* Prt- and *L. delbrueckii* subsp. *bulgaricus* was evidently observed. The proto-cooperation resulted in significant higher populations of the two species, more efficient milk acidification, significant abundance of aroma volatiles and non-volatile metabolites desirable for a good organoleptic quality of yoghurt. A complementary metabolomics approach using headspace SPME-GC/MS and ^{1}H-NMR resulted in the identification of 35 volatiles and 43 non-volatile polar metabolites, respectively. Furthermore, multivariate statistical analysis allows discriminating set-yoghurts fermented by different types of starter cultures according to their metabolite profiles.

Supplementary data

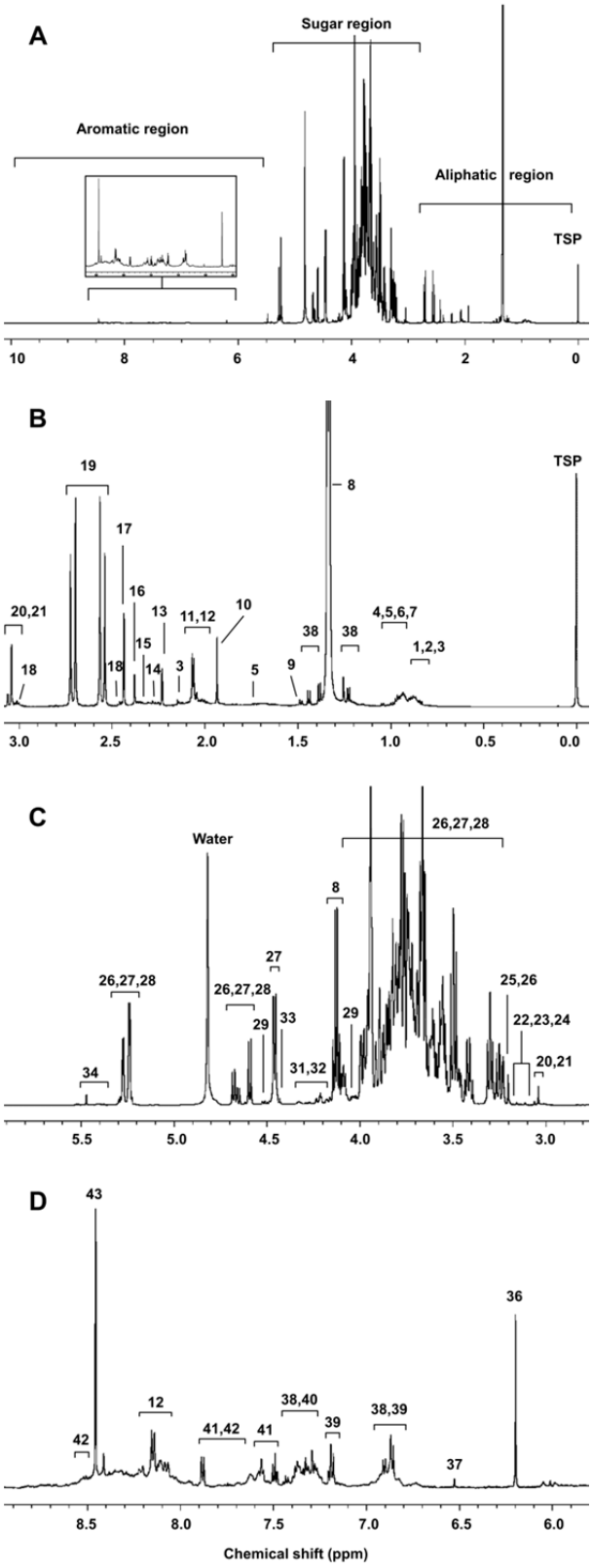

Fig. S2.1. Representative NOESY 1D-^{1}H-NMR spectra of a set-yoghurt sample fermented by mixed culture of *S. thermophilus* protease (-) and *L. delbrueckii* subsp. *bulgaricus* (panel A) and expansions corresponding for aliphatic region (panel B), sugar region (panel C) and aromatic region (panel D) with assigned peaks: TSP: internal standard, 1: valerate derivatives, 2: valerate, 3: butyrate, 4: isoleucine, 5: leucine, 6: valine, 7: isobutyrate, 8: lactate, 9: alanine, 10: acetate, 11: N-acetyl amino acids, 12: N-acetyl glucosamine, 13: acetone, 14: acetoacetate, 15: proline, 16: pyruvate, 17: succinate, 18: oxoglutarate, 19: citrate, 20: creatine, 21: creatinine, 22: dimethyl sulfone, 23: acetylcarnitine, 24: choline derivatives, 25: betaine, 26: glucose, 27: lactose, 28: galactose, 29: ascorbate, 30: choline, 31: phosphocholine, 32: glycerophosphocholine, 33: dihydroxyacetone, 34: sugar residues, 35: uridine, 36: orotate, 37: fumarate, 38: amino acid residues, 39: tyrosine, 40: phenylalanine, 41: benzoate, 42: hippurate, 43: formate.

Table S2.1. Volatile metabolites identified in base milk and set-yoghurts (samples at 4 h) fermented by different types of starter cultures using headspace SPME-GC/MS

Chemical group	Compound	Pasteurized	Starter cultures				
		Nilac milk	Pure ST-Prt+	Pure ST-Prt-	Pure LB	Mixed ST-Prt+/LB	Mixed ST-Prt-/LB
Alcohol	1-Butanol	-[a]	-	-	-	5.44[b] ± 0.06a[c]	5.48 ± 0.15a
	Ethanol	-	-	-	-	5.89 ± 0.10a	6.48 ± 0.06b
	2-Ethyl-hexanol	-	-	-	5.46 ± 0.18a	5.51 ± 0.11a	5.65 ± 0.09a
	1-Hexanol	-	5.60 ± 0.02a	5.56 ± 0.01a	-	5.55 ± 0.08a	5.58 ± 0.04a
	1-Methoxy-2-propanol	-	-	-	-	6.33 ± 0.23a	6.26 ± 0.33a
	2-Methyl-1-butanol	-	4.78 ± 0.09b	-	-	4.41 ± 0.08a	5.43 ± 0.35c
	3-Methyl-2-butanol	-	-	-	-	-	5.51 ± 0.04
	3-Methyl-3-butanol	-	5.18 ± 0.01a	5.50 ± 0.05b	-	5.10 ± 0.04a	5.71 ± 0.05c
	1-Octanol	-	-	-	4.88 ± 0.32	-	-
	1-Pentanol	4.79 ± 0.08a	5.09 ± 0.06ab	5.04 ± 0.06ab	4.91 ± 0.18ab	5.15 ± 0.07b	5.18 ± 0.02b
	3-Pentanol	-	5.37 ± 0.03b	5.60 ± 0.17b	-	5.07 ± 0.12a	6.63 ± 0.07c
Carbonyl compound	Acetaldehyde	4.07 ± 0.08a	6.49 ± 0.08c	6.26 ± 0.17bc	-	6.11 ± 0.13b	7.39 ± 0.04d
	Acetoin	5.30 ± 0.20a	7.32 ± 0.03b	7.77 ± 0.12c	5.06 ± 0.20a	7.25 ± 0.10b	8.41 ± 0.06d
	Acetone	7.29 ± 0.03a	7.31 ± 0.03a	7.29 ± 0.01a	7.35 ± 0.09a	7.33 ± 0.07a	7.42 ± 0.01a
	Benzaldehyde	5.77 ± 0.06ab	5.76 ± 0.02ab	5.69 ± 0.04a	6.09 ± 0.21b	5.90 ± 0.04ab	5.86 ± 0.04ab
	2-Butanone	6.49 ± 0.05a	6.48 ± 0.03a	6.58 ± 0.01a	6.53 ± 0.11a	6.50 ± 0.07a	7.55 ± 0.16b
	Diacetyl	5.25 ± 0.03a	6.49 ± 0.03b	6.98 ± 0.06c	5.53 ± 0.15a	6.30 ± 0.10b	7.38 ± 0.03d
	2-Heptanone	-	5.59 ± 0.04ab	5.47 ± 0.06a	5.37 ± 0.00a	5.73 ± 0.06b	5.97 ± 0.01c
	Hexanal	5.62 ± 0.02a	-	-	5.63 ± 0.05a	-	-
	2-Hydroxy-3-pentanone	-	4.93 ± 0.09ab	5.18 ± 0.21b	-	4.64 ± 0.10a	6.25 ± 0.04c
	3-Methyl-2-butenal	-	-	-	-	4.40 ± 0.07a	5.21 ± 0.06b

	2-Nonanone	-	5.40 ± 0.05a	-	-	5.58 ± 0.08ab	5.78 ± 0.08b
	3-Octanone	-	-	-	-	-	5.88 ± 0.06
	2,3-Pentanedione	-	5.91 ± 0.09b	6.02 ± 0.07b	-	5.38 ± 0.09a	6.88 ± 0.06c
	2-Undecanone	-	-	4.65 ± 0.10ab	4.54 ± 0.24a	-	4.89 ± 0.07b
Heterocyclic compound	3-Acetyl-2,5-dimethylfuran	-	-	-	-	-	5.75 ± 0.32
Sulfur compound	Dimethyl disulfide	5.64 ± 0.02a	-	-	5.82 ± 0.07b	-	-
	Dimethyl sulfide	6.69 ± 0.07ab	6.77 ± 0.03b	6.81 ± 0.03b	6.90 ± 0.18b	6.41 ± 0.16a	6.72 ± 0.15ab
	Dimethyl sulfone	6.21 ± 0.07a	6.45 ± 0.18a	6.52 ± 0.27a	6.43 ± 0.14a	6.64 ± 0.06a	6.55 ± 0.18a
Volatile organic acid	Acetic acid	6.21 ± 0.01a	6.48 ± 0.10ab	6.75 ± 0.24b	6.21 ± 0.25a	6.78 ± 0.02b	7.91 ± 0.08c
	Butyric acid	5.92 ± 0.05a	6.27 ± 0.11b	6.56 ± 0.27b	6.50 ± 0.31b	6.30 ± 0.02b	7.63 ± 0.09c
	3-Methyl-butanoic acid	-	-	-	-	-	5.19 ± 0.08
	2-Methyl-propanoic acid	-	-	-	-	-	5.10 ± 0.08
	Pentanoic acid	-	-	-	-	-	5.63 ± 0.06
	Hexanoic acid	-	5.70 ± 0.11a	5.92 ± 0.21a	-	5.85 ± 0.16a	7.07 ± 0.07b

[a] (-) indicates compound not detected.

[b] Metabolite contents are expressed as $\log_{10}$ [peak area of respective compound in arbitrary unit]. Values are mean ± standard deviation from three independent replicates.

[c] Letters (a-d) indicate significant difference ($p < 0.05$) among sample means within the same row.

Table S2.2. Presumptive polar metabolites identified in base milk and set-yoghurts (samples at 4 h) fermented by different types of starter cultures using NOESY 1D-^{1}H-NMR

Chemical group	Compound	Pasteurized	Starter cultures				
		Nilac milk	Pure ST-Prt+	Pure ST-Prt-	Pure LB	Mixed ST-Prt+/LB	Mixed ST-Prt-/LB
Amino acid and derivatives	Alanine	6.75[a] ± 0.00c[b]	6.78 ± 0.07bcd	6.58 ± 0.10a	6.72 ± 0.01b	6.69 ± 0.03ab	6.89 ± 0.07d
	Creatine and Creatinine	7.39 ± 0.00a	7.37 ± 0.07ab	7.41 ± 0.02ab	7.42 ± 0.07ab	7.44 ± 0.03b	7.52 ± 0.01c
	Isoleucine	7.49 ± 0.01c	7.54 ± 0.05cd	7.39 ± 0.05ab	7.32 ± 0.09a	7.41 ± 0.01b	7.62 ± 0.04d
	Leucine	7.53 ± 0.01c	7.56 ± 0.02c	7.36 ± 0.00a	7.38 ± 0.06ab	7.42 ± 0.04b	7.64 ± 0.03d
	N-Acetyl-amino acids	7.95 ± 0.01d	8.01 ± 0.03e	7.88 ± 0.00b	7.90 ± 0.00c	7.83 ± 0.02a	8.01 ± 0.02e
	Phenylalanine	6.26 ± 0.01a	6.54 ± 0.14c	6.14 ± 0.14a	6.26 ± 0.11ab	6.32 ± 0.03b	6.56 ± 0.07c
	Proline	7.17 ± 0.02b	7.23 ± 0.09bc	6.97 ± 0.09a	7.14 ± 0.03b	7.16 ± 0.04b	7.30 ± 0.06c
	Tyrosine	6.52 ± 0.01a	6.66 ± 0.12ab	6.40 ± 0.18a	6.65 ± 0.14ab	6.74 ± 0.02b	6.82 ± 0.12b
	Valine	7.23 ± 0.01c	7.32 ± 0.03d	7.04 ± 0.10a	7.19 ± 0.01b	7.26 ± 0.03cd	7.43 ± 0.07e
	Amino acid residues	7.78 ± 0.01a	7.86 ± 0.07ab	7.70 ± 0.10a	7.73 ± 0.04a	7.87 ± 0.04b	8.00 ± 0.07c
Carbohydrate and derivatives	Galactose	8.36 ± 0.01a	8.63 ± 0.04c	8.55 ± 0.06b	8.62 ± 0.03c	8.89 ± 0.00d	8.95 ± 0.01e
	Glucose	8.29 ± 0.00a	8.62 ± 0.03d	8.40 ± 0.04b	8.49 ± 0.02c	8.81 ± 0.03e	8.87 ± 0.01f
	Lactose	9.63 ± 0.01c	9.53 ± 0.03b	9.66 ± 0.02c	9.59 ± 0.05bc	9.32 ± 0.03a	9.38 ± 0.07a
	N-Acetylglucosamine	7.38 ± 0.01c	7.40 ± 0.04c	7.33 ± 0.04bc	7.31 ± 0.01b	7.23 ± 0.05a	7.48 ± 0.09c
	Sugar residues	7.22 ± 0.00a	7.34 ± 0.01b	7.20 ± 0.03a	7.23 ± 0.02a	7.21 ± 0.01a	7.39 ± 0.04b
Organic acid	Acetate	6.96 ± 0.02a	7.17 ± 0.05b	7.06 ± 0.11ab	7.35 ± 0.15bc	7.37 ± 0.02c	7.42 ± 0.10c
	Acetoacetate	6.90 ± 0.01c	6.93 ± 0.06cd	6.72 ± 0.09a	6.82 ± 0.00b	6.80 ± 0.04ab	6.99 ± 0.05d
	Ascorbate	7.75 ± 0.01a	7.94 ± 0.01c	7.86 ± 0.06b	7.94 ± 0.02c	8.07 ± 0.00d	8.12 ± 0.02e
	Benzoate	6.51 ± 0.00b	6.68 ± 0.04c	6.32 ± 0.02a	6.77 ± 0.13cde	6.76 ± 0.02d	6.91 ± 0.05e

	Butyrate	7.10 ± 0.01b	7.18 ± 0.08bc	7.03 ± 0.09ab	7.08 ± 0.03ab	6.97 ± 0.10a	7.23 ± 0.05c
	Hydroxybutyrate	7.36 ± 0.01c	7.33 ± 0.03c	7.22 ± 0.01b	7.15 ± 0.10ab	7.16 ± 0.01a	7.41 ± 0.01d
	Citrate	7.72 ± 0.06b	7.57 ± 0.16ab	7.71 ± 0.03b	7.73 ± 0.02b	7.44 ± 0.06a	7.52 ± 0.11a
	Formate	6.45 ± 0.00b	6.41 ± 0.05ab	6.38 ± 0.16ab	6.38 ± 0.03a	6.55 ± 0.00c	6.85 ± 0.01d
	Fumarate	5.65 ± 0.04a	6.35 ± 0.16b	6.32 ± 0.24b	5.51 ± 0.16a	5.59 ± 0.02a	5.70 ± 0.15a
	Hippurate	7.11 ± 0.00b	7.18 ± 0.05c	6.94 ± 0.09a	6.93 ± 0.01a	6.91 ± 0.06a	7.18 ± 0.06c
	Isobutyrate	6.23 ± 0.01b	6.46 ± 0.01d	6.08 ± 0.11a	6.29 ± 0.06b	6.41 ± 0.02c	6.56 ± 0.08e
	Lactate	7.52 ± 0.01a	9.05 ± 0.16d	8.79 ± 0.06c	8.51 ± 0.02b	9.34 ± 0.01e	9.38 ± 0.01f
	Orotate	6.52 ± 0.01b	6.68 ± 0.00c	6.54 ± 0.01b	6.54 ± 0.03b	6.49 ± 0.01a	6.63 ± 0.05c
	Oxoglutarate	7.10 ± 0.00a	7.06 ± 0.04a	7.10 ± 0.10a	7.07 ± 0.06a	7.51 ± 0.07b	7.38 ± 0.16b
	Pyruvate	6.95 ± 0.01a	7.06 ± 0.09b	7.05 ± 0.18abc	7.00 ± 0.06ab	7.40 ± 0.04d	7.26 ± 0.06c
	Succinate	6.70 ± 0.04a	6.91 ± 0.02b	6.88 ± 0.16abc	7.34 ± 0.07d	7.06 ± 0.10c	7.50 ± 0.09d
	Valerate and derivatives	7.61 ± 0.01c	7.61 ± 0.01c	7.47 ± 0.01b	7.45 ± 0.08ab	7.44 ± 0.01a	7.68 ± 0.04d
Lipid derivatives	Acetylcarnitine	6.86 ± 0.01c	6.82 ± 0.01b	6.66 ± 0.01a	6.84 ± 0.05bc	6.79 ± 0.13abc	6.99 ± 0.01d
	Choline and derivatives	7.48 ± 0.02a	7.58 ± 0.04b	7.56 ± 0.01b	7.67 ± 0.02c	7.79 ± 0.02d	7.79 ± 0.04d
	Glycerophosphocholine	7.28 ± 0.02a	7.27 ± 0.03a	7.37 ± 0.02b	7.33 ± 0.09ab	7.24 ± 0.03a	7.37 ± 0.14ab
	Phosphocholine	7.33 ± 0.01a	7.55 ± 0.01b	7.49 ± 0.09b	7.66 ± 0.03c	7.82 ± 0.00d	7.89 ± 0.00e
Carbonyl compound	Acetone	6.75 ± 0.02b	6.87 ± 0.07c	6.94 ± 0.15c	6.66 ± 0.06a	7.11 ± 0.01d	7.24 ± 0.02e
	Dihydroxyacetone	7.16 ± 0.01a	7.17 ± 0.05a	7.29 ± 0.04c	7.23 ± 0.07abc	7.24 ± 0.00b	7.37 ± 0.10c
Miscellaneous	Dimethyl sulfone	6.82 ± 0.00c	6.76 ± 0.01a	6.79 ± 0.01b	6.85 ± 0.04c	6.83 ± 0.04bc	6.92 ± 0.02d
	Uridine	6.10 ± 0.01c	6.06 ± 0.09c	5.72 ± 0.06a	5.81 ± 0.03b	5.74 ± 0.01ab	6.02 ± 0.11c

[a] Metabolite contents are expressed as log10 [sum of intensity of respective metabolite in arbitrary unit]. Values are mean ± standard deviation from two independent replicates.
[b] Letters (a-e) indicate significant difference ($p < 0.05$) among sample means within the same row.

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Chapter 3

The impact of selected probiotic bacteria on metabolite formation in set-yoghurt

Based on:
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Abstract

The objective of this study was to evaluate the influence of *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB12 in co-fermentation with traditional starters on metabolite formation in set-yoghurt. The microbial activity during fermentation and refrigerated storage was investigated by monitoring bacterial population dynamics, milk acidification and overall changes in yoghurt metabolite profiles. A complementary metabolomics approach using SPME-GC/MS and ^{1}H-NMR resulted in the identification of 37 volatile and 43 non-volatile metabolites, respectively. The results demonstrated that the two probiotic strains did not influence acidity and the key-aroma volatile metabolites of set-yoghurt. However, a contribution by the presence of *L. rhamnosus* GG on non-volatile metabolite profile of yoghurt was specifically noticed during storage. Furthermore, multivariate analysis allowed yoghurts fermented by different starter combinations and different durations of storage to be differentiated according to their metabolite profiles. This finding provides new insights regarding the impact of probiotics on the metabolome of yoghurt.

3.1 Introduction

Yoghurt is a product obtained by lactic acid fermentation of milk. Its production can be regarded as one of the oldest methods to preserve milk [41]. According to the Codex standard for fermented milks [8], yoghurt is specifically characterized by the presence of symbiotic cultures of two lactic acid bacteria (LAB), i.e. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. A successful yoghurt manufacture relies on the mutual interaction, also called proto-cooperation, between these two species. Proto-cooperation is not only linked with lactic acid production but also with the formation of distinctive flavor and texture characteristics of the product [11]. Nowadays, societal interest in healthy food products has contributed to the development of functional dairy products that potentially provide health benefits in addition to the nutrients they contain [39]. An example of a functional type of dairy products is yoghurt with probiotic incorporation. These bacteria (i.e. probiotics) are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [15]. In order to ensure their health-promoting effects, a probiotic product should contain at least 10^6 cfu/g to 10^7 - 10^8 cfu/g of viable probiotic cells throughout the entire shelf-life [44]. Most commercial probiotics incorporated in dairy products are strains belonging to the genera *Lactobacillus* and *Bifidobacterium* [23]. Members of these two genera have a long history of safe use in the manufacture of fermented food products and can be found as a part of normal microbiota in the human gastrointestinal tract [38]. Despite high numbers of studies on strain selection, safety concerns, health-promoting properties and technological approaches to improve the survival of probiotics in fermented dairy products [26, 39], the actual metabolic activity of probiotics grown or suspended in milk is not fully understood [32]. This information is important, since the organic acids and volatiles formed by these non-starter bacteria may directly contribute to the organoleptic quality of product [30].

Metabolomics is recognized as an effective tool to investigate the overall chemical composition in complex biological systems including food matrices [17]. The application of mass spectrometry (MS)-based and nuclear magnetic resonance (NMR)-based techniques have shown to be very effective for determining a wide range of metabolites

in liquid milk [6, 20, 24] and fermented dairy products [10, 31, 33]. However, until now, the number of publication revealed metabolomics as a tool for better understanding the activity of probiotics in fermented dairy products is rather limited [4, 27, 33].

The study described in Chapter 2 has demonstrated the influence of different proteolytic activity of starter bacteria, i.e. *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, on biochemical characteristics of set-yoghurt from a metabolomics perspective. As a consequence, the objective of this study was to evaluate the impact of two commercial probiotic strains, *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12, in co-fermentation with traditional starters on metabolite formation in set-yoghurt. Changes in bacterial population, decrease of pH and increase in titratable acidity were monitored during set-yoghurt fermentation and storage. Biochemical changes associated with bacterial metabolism were characterized in terms of volatile and non-volatile polar metabolite profiles using headspace SPME-GC/MS and ^{1}H-NMR technique. Finally, metabolite profiles of different yoghurt samples were statistically compared using multivariate analysis.

3.2 Materials and methods

3.2.1 Yoghurt Starters and probiotic strains

Frozen direct-vat-inoculation (DVI) pellets of *Streptococcus thermophilus* C44, *Lactobacillus delbrueckii* subsp*. bulgaricus* C49 (CSK Food Enrichment, Ede, The Netherlands) and *Bifidobacterium animalis* subsp. *lactis* BB12 (Chr. Hansen, Hørsholm, Denmark) were stored at -45 °C and were defrosted at ambient temperature (20 ± 3 °C) for 15 min before use. A freeze-dried culture of *Lactobacillus rhamnosus* GG (ATCC 53103) was propagated in our laboratory and stored as a 20% (v/v) glycerol stock-culture at -80 °C. The cultures were re-propagated in MRS broth (Merck, Darmstadt, Germany) at 37 °C for 24 h under anaerobic incubation (Anoxomat™ Mart® Microbiology, Drachten, the Netherlands). Then, the cells were collected by centrifugation at 4,000*g* for 15 min at 4 °C, washed twice using peptone-physiological-

salt solution (Tritium microbiology, Eindhoven, the Netherlands) and finally resuspended in milk before use. Set-yoghurts were fermented with three different types of starter combinations: (i) traditional yoghurt starters (Y) consisting equal numbers of *S. thermophilus* C44 and *L. delbrueckii* subsp*. bulgaricus* C49, (ii) co-culture of yoghurt starters with *L. rhamnosus* GG (Y-LGG) and (iii) co-culture of yoghurt starters with *B. animalis* subsp. *lactis* BB12 (Y-BB12). The combination of the two yoghurt starters and probiotic strain were inoculated each at 10^6 cfu/g at the starting point of fermentation (ratio 1:1:1).

3.2.2 Set-yoghurt fermentation

Pasteurized Nilac milk was prepared according to the method described in Chapter 2. After inoculation, set-yoghurt fermentation was carried out in a water bath at 42 °C for 4 h. Yoghurts were then placed in a cold chamber (4 ± 2 °C) for 28 days of storage. Samples were taken hourly during fermentation and weekly during storage. The enumeration of viable bacteria and determination of pH and titratable acidity were carried out immediately after sampling. For ^{1}H-NMR, the samples were stored at -20 °C until the analysis. The fermentation was performed in three replicates for each type of starter combination.

3.2.3 Enumeration of viable bacteria

Viable counts of *S. thermophilus* were determined on *S. thermophilus* agar after aerobic incubation at 37 °C for 24 h [2]. Viable counts of *L. delbrueckii* subsp*. bulgaricus* in yoghurt fermented with (i) Y, (ii) Y-BB12 and (iii) Y-LGG were determined on: (i) MRS agar pH 5.70 (Merck, Darmstadt, Germany) after anaerobic and (ii) modified atmosphere (6% O_2, 7% CO_2) incubation (Anoxomat™ Mart® Microbiology, Drachten, the Netherlands) at 37 °C for 48 h (modified from Ashraf & Shah [2]), and (iii) MRS agar pH 5.7 supplemented with 20 mg/L ciprofloxacin (Sigma-Aldrich, Steinheim, Germany) after anaerobic incubation at 37 °C for 48 h (tested in this study). Viable counts of *L. rhamnosus* GG were determined on MRS agar supplemented with 50 mg/L vancomycin (Merck, Darmstadt, Germany) after anaerobic incubation at 37 °C for 48 h [42]. Viable counts of *B. animalis* subsp. *lactis* BB12 were determined on MRS agar supplemented

with 0.5 g/L cysteine-HCl (Merck, Darmstadt, Germany) and 50 mg/L mupirocin (LGC Standards, Middlesex, UK) after anaerobic incubation at 37 °C for 48 h [2].

3.2.4 Determination of acidification profile

Production of acid during set-yoghurt fermentation and refrigerated storage was expressed by changes in pH and increases in titratable acidity. The pH measurement and determination of titratable acidity were performed according to the methods described in Chapter 2.

3.2.5 Analysis of volatile metabolites by headspace SPME-GC/MS

For headspace SPME-GC/MS analysis, set-yoghurt fermentation was also carried out directly in glass GC vials as previously described in Chapter 2. The fermentation was performed in three replicates for each type of starter combination. Extraction and determination of volatile compounds by headspace SPME-GC/MS were performed according to the method described in Chapter 2. This method was based on the method developed by Hettinga et al. [18].

Volatile metabolites were identified using AMDIS software (NIST, Gaithersburg, MD, USA) referred to NIST/EPA/NIH database and library provided by Hettinga et al. [19]. Specific retention time and *m/z* model were used for automated peak integration in the XCalibur software package (Thermo Scientific, Austin, TX, USA) [37].

3.2.6 Analysis of non-volatile polar metabolites by ^{1}H-NMR spectroscopy

For ^{1}H-NMR analysis, the samples from two replicates were analyzed according to the method described in Chapter 2. Frozen yoghurt samples were thawed at room temperature and pH was adjusted to 6.0 using 1.0 N NaOH to achieve low variation, i.e. location and shape of peaks, in the spectra obtained [25]. NOESY 1D-^{1}H-NMR measurements were performed at 300 K in a 600 MHz NMR spectrometer (Bruker, Rheinstetten, Germany) operated under full automation, with similar parameters as described by Lu et al. [24].

The ^{1}H-NMR spectra were baseline-corrected, phase-corrected, aligned and calibrated based on the internal standard (TSP) peak. For each spectrum, chemical shift (δ) across the range of 0.00 - 10.00 ppm was segmented (binning) with an interval of 0.02 ppm [37]. The signal intensity in each bin was integrated and expressed in arbitrary units using AMIX software (Bruker, Rheinstetten, Germany). Metabolite labels were assigned to the bins by means of Chenomx NMR suite 7.5 library (Chenomx Inc., Alberta, Canada) and from the list of metabolites identified in Chapter 2 [37]. For unlabeled bins, significant variables were selected based on one-way ANOVA at 95% confidence level.

3.2.7 Statistical analysis

One-way ANOVA with multiple comparisons by Tukey's test were performed using IBM SPSS statistics package version 19 (SPSS Inc., Chicago, IL, USA). A probability at $p < 0.05$ was considered statistically significant. Metabolomics data from GC/MS and ^{1}H-NMR were normalized by median-centering and $\log_2$-scaling before multivariate statistical analysis. Principal component analysis (PCA) and heat-map visualization combined with Pearson's correlation-based hierarchical cluster analysis (HCA) were performed using Multi-Experiment Viewer (MeV) version 4.8 as previously described in Chapter 2.

3.3 Results and discussion

3.3.1 Bacterial growth and survival profiles

The Viable cell counts of yoghurt starters and probiotics were enumerated during set-yoghurt fermentation (0, 1, 2, 3, 4 h) and refrigerated storage (7, 14, 21, 28 d) (Fig. 3.1). In the samples fermented with standard yoghurt starters (Y) (Fig. 3.1A), *S. thermophilus* developed rapidly during the early stage of fermentation, especially during 0 – 3 h, while *L. delbrueckii* subsp. *bulgaricus* remained in lag-phase for at least one hour. The growth of *L. delbrueckii* subsp. *bulgaricus* can be observed after 1 h towards the end

of fermentation. Similar growth patterns of the two species of traditional yoghurt starters have been well documented [11, 41].

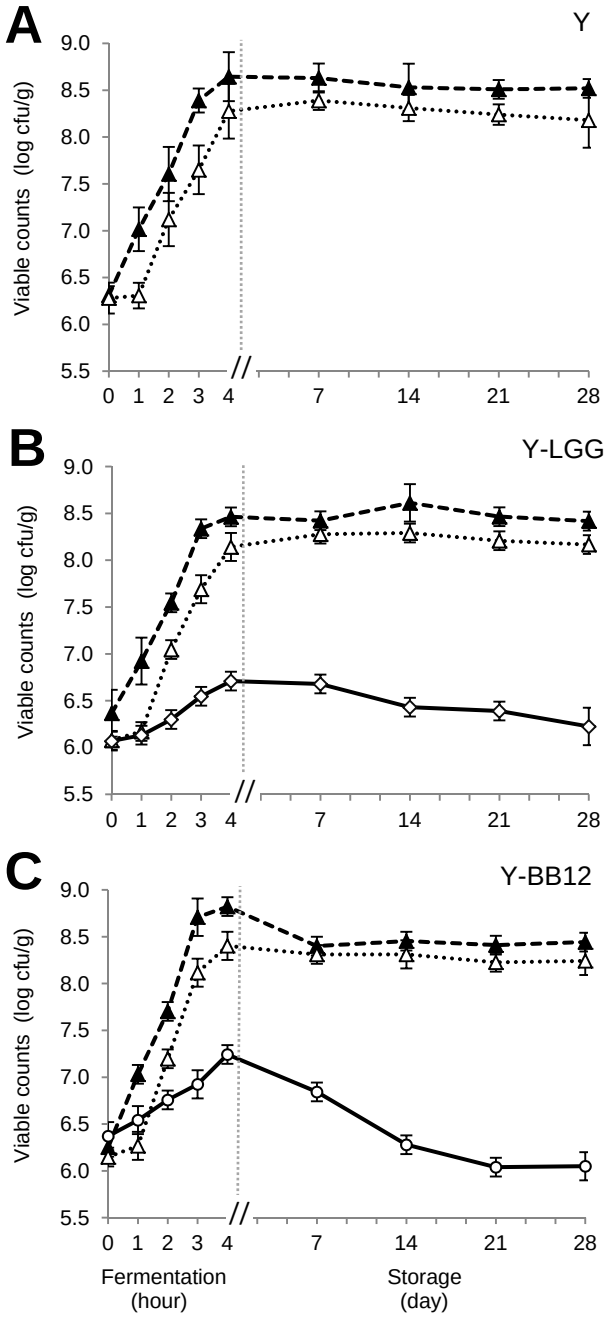


Fig. 3.1. Changes in viable bacteria counts during set-yoghurt fermentation (4 hours) and refrigerated storage (28 days). Samples were fermented with traditional yoghurt starters (panel A) consisting of *S. thermophilus* (▲) and *L. delbrueckii* subsp. *bulgaricus* (△) compared with co-cultures of yoghurt starters with *L. rhamnosus* GG (◇, panel B) and *B. animalis* subsp. *lactis* BB12 (○, panel C). Error bars represent standard deviations based on three independent replicates.

At the end of fermentation, the viable counts of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* increased by 2.3 and 2.0 log units to reach an average value of 8.7 ± 0.2 and 8.3 ± 0.3 log cfu/g, respectively. The viable counts of the two species remained nearly stable (above 8.0 log cfu/g) throughout the 28-day storage period. The high survival of *S. thermophilus* has been well recognized. However, many authors found that the survival of *L. delbrueckii* subsp. *bulgaricus* was strain-dependent and a loss of viable count down to 1.5 log units during refrigerated storage has been reported [12, 28, 35].

In co-culture with *L. rhamnosus* GG (Y-LGG) (Fig. 3.1B) or *B. animalis* subsp. *lactis* BB12 (Y-BB12) (Fig. 3.1C), yoghurt starters showed similar growth and survival pattern as found in the samples fermented without probiotics. The viable counts of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* at the end of fermentation (4 h) and at the end of storage (28 d) in the samples fermented with Y-LGG and Y-BB12 were not significantly different ($p > 0.05$) from those observed in the samples fermented with only Y. These results suggest no obvious interference from the addition of *L. rhamnosus* GG or *B. animalis* subsp. *lactis* BB12 on growth and survival of yoghurt starters. It can be noticed that *S. thermophilus* predominates the microbial population in all tested culture combinations. This observation confirms reports of other researchers [28, 35]. On the other hand, the two probiotic strains exhibited different growth patterns and varied in their survival during storage. The viable counts of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 inoculated individually in milk remained stable during incubation at 42 °C for 4 h (data not shown). The limited capacity of *L. rhamnosus* GG to develop in milk is explained by the lack of ability to ferment lactose. The weak proteolytic activity along with a low redox potential requirement explain the poor growth of *B. animalis* subsp. *lactis* BB12 in milk [29]. In association with yoghurt starters, the growth of *L. rhamnosus* GG was slightly enhanced while the growth of *B. animalis* subsp. *lactis* BB12 was evidently stimulated. In comparison, the viable counts of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 at the end of fermentation increased by 0.6 and 0.9 log units to reach an average value of 6.7 ± 0.1 and 7.2 ± 0.1 log cfu/g, respectively. This is in accordance with observations reported by El-Dieb et al. [13] and Saccaro et al. [35] who also found an evident stimulating effect on the growth of bifidobacteria in milk when co-

fermented with yoghurt starters. The viable counts of *L. rhamnosus* GG slightly decreased (0.5 log reduction) throughout the entire storage period while those of *B. animalis* subsp. *lactis* BB12 drastically decreased (1.2 log reduction) especially during the first two weeks and then remained nearly stable until the end of storage. These results correspond with data from literature indicating a higher stability of probiotic lactobacilli compared to bifidobacteria in fermented milk [16, 23]. The final viable counts of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 at the end of storage reached an average value of 6.2 ± 0.2 and 6.1 ± 0.2 log cfu/g, respectively, which still met the minimum recommended level (not less than 6.0 log cfu/g) to ensure their potential health-promoting effects [39].

3.3.2 Acidification profiles

Changes in pH were monitored during fermentation and refrigerated storage (Fig. 3.2A). Similar acidification patterns were observed in all samples whether they were fermented with or without probiotics. At the end of fermentation, the pH values of all samples were not significantly different ($p > 0.05$) with an average value of 4.4 ± 0.1. The pH values showed an evident decrease especially during the first two weeks of storage (*ca.* 0.3 pH units); later, only a slight decline in pH was observed. At the end of storage, the pH values were reduced to an average value of 4.1 ± 0.1 without significant difference ($p > 0.05$) regarding the presence of probiotics.

To determine acid production, titratable acidity was measured during fermentation and storage expressed as % equivalent lactic acid (w/w) (% LA) (Fig. 3.2B). For better comparison, the titratable acidity was subtracted by its initial value measured in the sample at 0 h. A higher acid production was found in the samples fermented with Y-LGG and Y-BB12 compared to those fermented with Y. At the end of fermentation, the amount of titratable acidity produced by Y-LGG (0.71%) was slightly higher than that produced by Y-BB12 (0.68%) and Y (0.64%), respectively. However, the differences were not statistically significant ($p > 0.05$). A low capacity to acidify milk by different probiotics strains was previously reported [26, 36]. This finding contradicts with the study of Saccaro et al. [35] who found a significant contribution of *L. rhamnosus*

and *B. animalis* subsp. *lactis* to milk acidification when co-fermented with yoghurt starters.

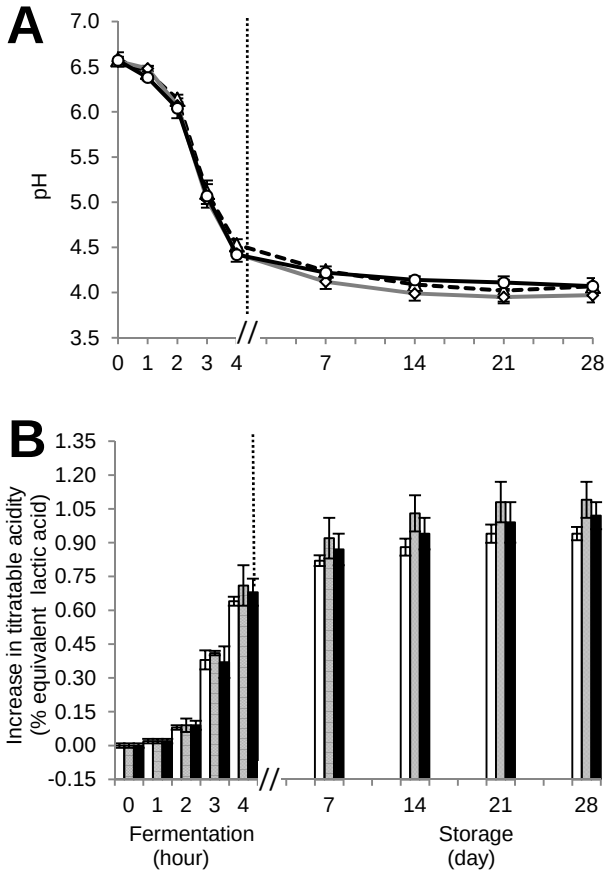


Fig. 3.2. Changes in pH (panel A) and increase in titratable acidity (panel B) during set-yoghurt fermentation (4 hours) and refrigerated storage (28 days) by traditional yoghurt starters (△, □) consisting of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* compared with co-cultures of yoghurt starters with *L. rhamnosus* GG (◇, ■) and *B. animalis* subsp. *lactis* BB12 (○, ●). Error bars represent standard deviations based on three independent replicates.

During storage, it appeared that acid was produced to the same extent in all samples resulting in a total average increase of 0.34%. At the end of storage, the amount of titratable acidity produced by Y-LGG (1.09%) was slightly higher than that produced by Y-BB12 (1.02%) and Y (0.94%), respectively. The difference in final titratable acidity between the samples of Y-LGG and Y was statistically significant (p = 0.03). Decrease in pH and accumulation of organic acids during refrigerated storage of fermented milk are

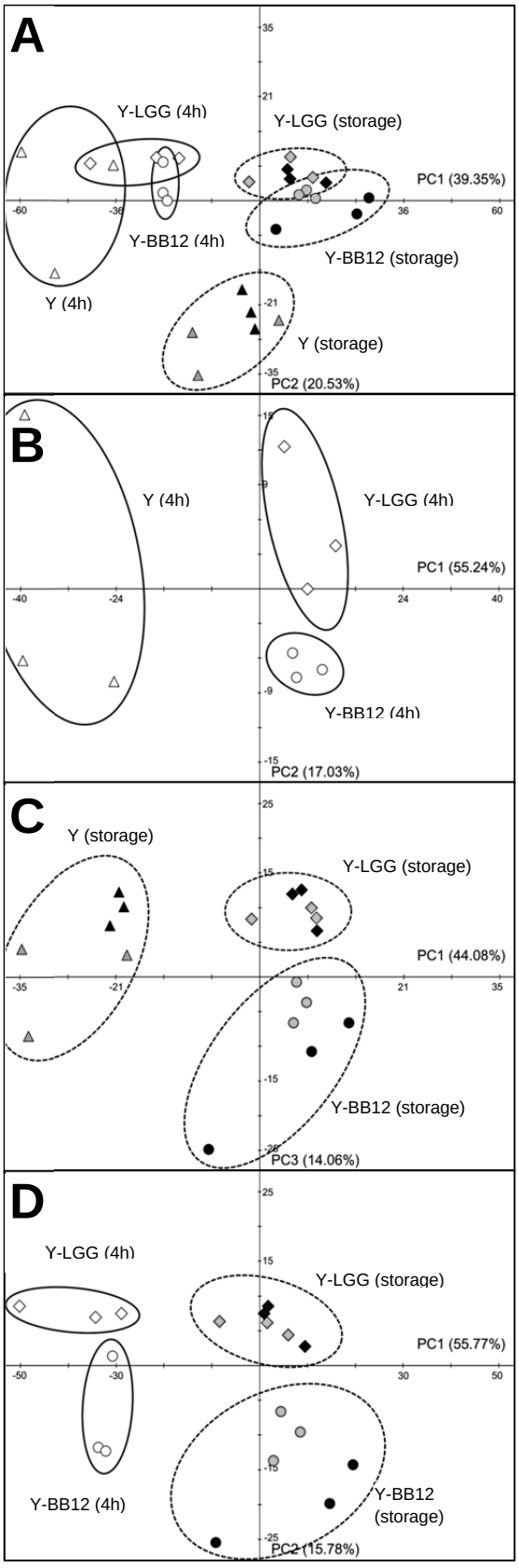
defined as “post-acidification” which is predominantly attributed to the metabolic activity of *L. delbrueckii* subsp. *bulgaricus* [38]. This phenomenon has been identified as one of the most detrimental factors for the stability of probiotics during yoghurt shelf-life [12]. The results confirm a significant negative effect of post-acidification on the survival of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 in set-yoghurt during refrigerated storage. Generally, lactobacilli are reported to be more tolerant to acidic condition of fermented milk than bifidobacteria [12, 13].

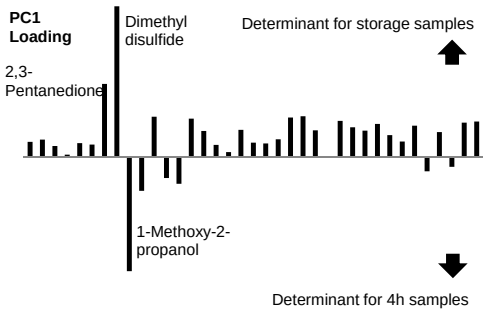
3.3.3 Volatile metabolite profiles determined by headspace SPME-GC/MS

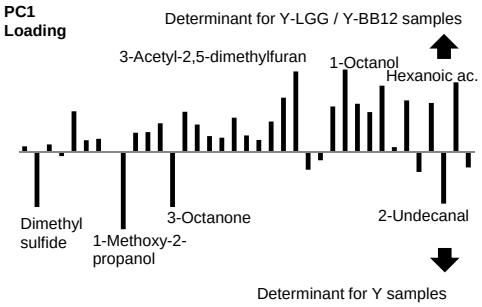
Volatile metabolite profiles of set-yoghurts fermented with Y, Y-LGG and Y-BB12 were evaluated at the end of fermentation (4 h) and every two weeks during storage (14 d and 28 d). In this study, yoghurt samples were directly fermented in GC vials. The advantages of this approach are the small amount of sample required (3 mL) and minimal loss of volatiles during sample preparation. To ensure that the results were comparable with the samples fermented in section 3.2.2, the pH from in-vial fermentation was regularly verified (data not shown). A total of 37 compounds comprising of alcohols, carbonyl compounds, organic acids, sulfur and heterocyclic compounds were identified in this study (Table S3.1). This list is comparable to the volatiles identified in various types of yoghurt using SPME-GC/MS technique by others [9, 14, 22].

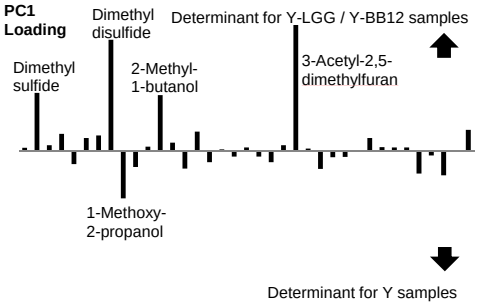
PCA was performed to distinguish the profiles of volatile metabolites among set-yoghurts fermented with Y, Y-LGG and Y-BB12. Samples at the end of fermentation (4 h) and during storage (14 d and 28 d) with three replicates were statistically treated as individual objects (n = 27) in a multivariate analysis. A total of 37 volatile metabolites were introduced as variables. If necessary, missing values were replaced by the median of respective metabolites.

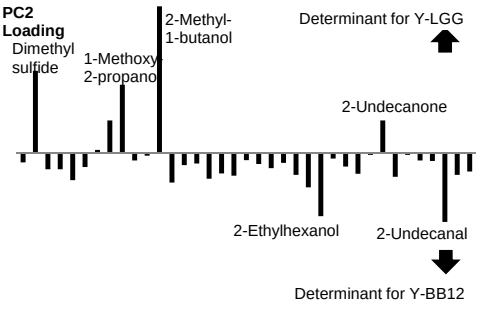
Fig. 3.3. PCA score plots and PC loadings, for overall comparison (panel A), comparison among samples at 4 h (panel B), comparison among storage samples (panel C) and comparison between two probiotic yoghurts (panel D) derived from volatile metabolite profiles of set-yoghurts fermented with traditional yoghurt starters (Y; △), co-cultures of yoghurt starters with *L. rhamnosus* GG (Y-LGG; ◇) and *B. animalis* subsp. *lactis* BB12 (Y-BB12; ○). White, grey and black filled blocks correspond to the samples at 4 hours, 14 days and 28 days, respectively.


A
Y-LGG (4h)
Y-LGG (storage)
PC1 (39.35%)
Y-BB12 (4h)
Y-BB12 (storage)
Y (4h)
Y (storage)
PC2 (20.53%)
B
Y (4h)
Y-LGG (4h)
PC1 (55.24%)
Y-BB12 (4h)
PC2 (17.03%)
C
Y (storage)
Y-LGG (storage)
PC1 (44.08%)
Y-BB12 (storage)
PC3 (14.06%)
D
Y-LGG (4h)
Y-LGG (storage)
PC1 (55.77%)
Y-BB12 (4h)
Y-BB12 (storage)
PC2 (15.78%)


PC1 Loading
Dimethyl disulfide
Determinant for storage samples
2,3-Pentanedione
1-Methoxy-2-propanol
Determinant for 4h samples


PC1 Loading
Determinant for Y-LGG / Y-BB12 samples
3-Acetyl-2,5-dimethylfuran
1-Octanol
Hexanoic ac.
Dimethyl sulfide
1-Methoxy-2-propanol
3-Octanone
2-Undecanal
Determinant for Y samples


PC1 Loading
Dimethyl disulfide
Determinant for Y-LGG / Y-BB12 samples
Dimethyl sulfide
2-Methyl-1-butanol
3-Acetyl-2,5-dimethylfuran
1-Methoxy-2-propanol
Determinant for Y samples


PC2 Loading
Dimethyl sulfide
1-Methoxy-2-propanol
2-Methyl-1-butanol
Determinant for Y-LGG
2-Undecanone
2-Ethylhexanol
2-Undecanal
Determinant for Y-BB12

An overall PCA score plot was constructed with a total variance of 59.9% (Fig. 3.3A). Within the group of 4 h samples, a good separation between Y and Y-BB12 was observed whereas the samples of Y-LGG were overlapped between the two groups. The stored samples of Y showed an identical volatile profile which was clearly isolated from other groups. On the other hand, the stored samples of Y-LGG and Y-BB12 were not clearly separated. Volatile metabolite profiles of the 4 h samples could be distinguished from those of stored samples along PC1 (39.4% variance). The metabolites accountable for separation are indicated in PC1-loading. 1-Methoxy-2-propanol was a determinant for the 4 h samples whereas the majority of volatiles, particularly dimethyl disulfide and 2,3-pentanedione, were accountable for the separation of stored samples. To focus on the incorporation of probiotics, two PCA score plots were constructed for comparison among yoghurt samples at 4 h (n = 9) with a total variance of 72.3% (Fig. 3.3B) and among stored samples (n = 18) with a total variance of 58.2% (Fig. 3.3C). In both cases, volatile profiles of the samples fermented with Y were clearly separated from those fermented with Y-LGG and Y-BB12 along PC1 accounting for 55.2% and 44.1% variance, respectively. PC1-loading in Fig. 3.3B suggests that the presence of probiotics during fermentation facilitates the higher production of volatile metabolites in yoghurt.

As storage time progressed, the numbers of metabolites contributing to separation decreased (PC1-loading in Fig. 3.3C). In other words, the overall metabolite composition of all samples became more similar to each other. Despite low numbers of indicative metabolites in the loading plot, the stored samples of Y, Y-LGG and Y-BB12 were still completely separated. The final PCA score plot was constructed to evaluate the distinction between two probiotic strains (Fig. 3.3D) with a total variance of 71.6%. The samples of Y-LGG and Y-BB12 were clearly separated along PC2 with metabolites accountable for separation indicated in the loading plot. The results demonstrate that volatile profiles of set-yoghurts can successfully be distinguished according to the differences in types of starter cultures and also durations of storage. Besides this, indicative metabolites in the loading plots can be considered as potential biomarkers for detection of specific combinations of starter cultures and probiotics.

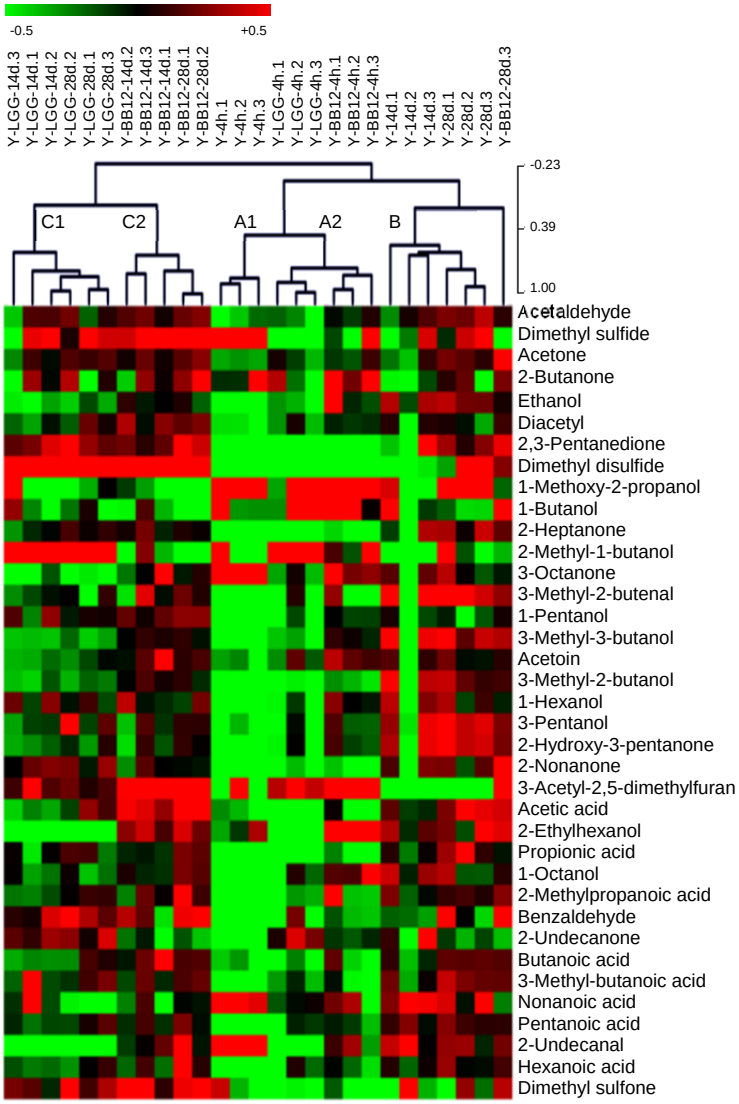


Fig. 3.4. Heat-map and hierarchical clustering of volatile metabolite profiles from set-yoghurts fermented with traditional yoghurt starters (Y), co-cultures of yoghurt starters with *L. rhamnosus* GG (Y-LGG) and *B. animalis* subsp. *lactis* BB12 (Y-BB12). Dendrogram represents sample clusters based on Pearson's correlation coefficient with average linkage. Each square in the heat-map expresses normalized volatile content respected to the color range. The red color indicates higher content of the corresponding compound.

Heat-map visualization combined with hierarchical cluster analysis was used to analyze the quantitative relationships of volatile profiles from different yoghurt samples (Fig. 3.4). Most metabolites are present in high relative abundances (shown in red or black) in the stored samples. The dendrogram shows that samples can be accurately

grouped into different clusters, i.e. samples at 4 h of Y (A1), samples at 4 h of Y-LGG and Y-BB12 (A2), stored samples of Y (B) with an outlier from Y-BB12, stored samples of Y-LGG (C1) and stored samples of Y-BB12 (C2), according to their volatile metabolite profiles. This observation is in accordance with the PCA results in Fig. 3.3A showing that volatile profiles of the samples fermented with Y-LGG and Y-BB12 are rather similar. However, unlike PCA, the overlap between two groups is not observed.

Acetaldehyde, diacetyl, acetoin, 2,3-pentanedione, acetone, 2-butanone and acetic acid were present in high relative abundances in the samples (Fig. 3.5). These volatile metabolites are known as major aroma compounds of yoghurt [7]. Acetaldehyde (fresh, green, pungent) is the most important compound contributing to typical yoghurt aroma which is mainly generated by threonine metabolism of yoghurt starters. Despite the high capacity of acetaldehyde production by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* [5], also bifidobacteria have been reported to contribute to acetaldehyde formation in fermented milk [3, 34]. This study confirmed that Y-BB12 resulted in significant higher acetaldehyde concentration ($p < 0.05$) in the samples at 4 h compared to Y and Y-LGG. Although, this metabolite increased substantially during storage, there was no significant difference in acetaldehyde concentration among yoghurt samples at the end of storage. Diacetyl (buttery, creamy), acetoin (buttery) and 2,3-pentanedione (buttery, vanilla-like) are primarily generated by *S. thermophilus* through pyruvate metabolism [7]. The results showed no significant difference in diacetyl and 2,3-pentanedione among all yoghurt samples at 4h while acetoin was significantly higher ($p < 0.05$) in the samples fermented with Y-BB12. It has been reported that bifidobacteria may convert pyruvate to acetoin instead of organic acids to maintain their internal pH [30]. Acetone and 2-butanone are naturally present in cow's milk [18] but a certain amount can be additionally produced by yoghurt starters [7]. The results showed that these two metabolites remained steady without any significant difference in either type of starter culture or storage duration.

Despite the similar abundance in major aroma volatiles among yoghurt samples, most of the minor carbonyl compounds, volatile organic acids and alcohols were present in significant higher abundance especially in the samples co-fermented with probiotics

(Table S3.1). These metabolites also showed a significant increase during refrigerated storage. They may be generated from catabolism of pyruvate as well as amino acids or derived from other biochemical conversions by the activity of native milk enzymes remained after pasteurization, bacterial metabolism and lipid oxidation [1, 5, 43].

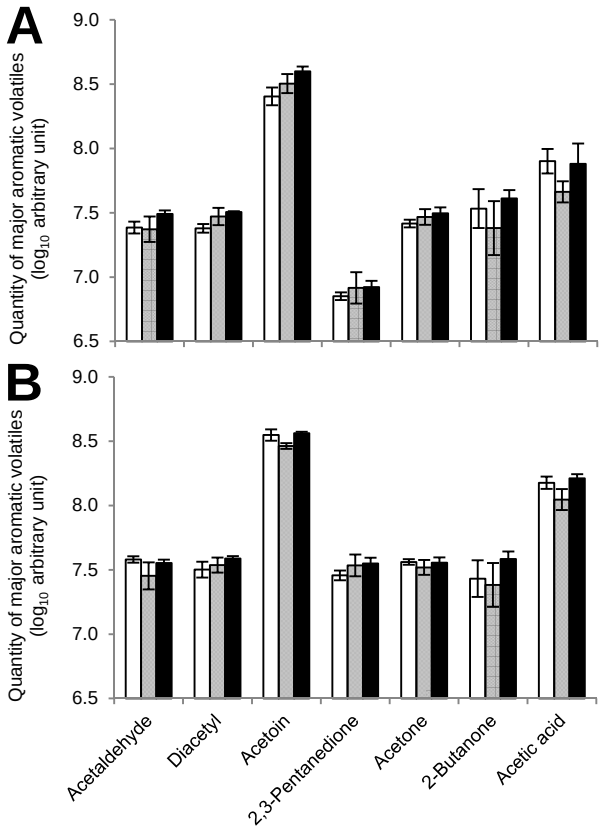


Fig. 3.5. Quantity of major volatile metabolites in set-yoghurt samples determined at the end of fermentation (4 hours; panel A) and the end of storage (28 days; panel B). Samples were fermented by traditional yoghurt starters (□) consisting of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* compared with co-culture of yoghurt starters with *L. rhamnosus* GG (▒) and *B. animalis* subsp. *lactis* BB12 (■). Error bars represent standard deviations based on three independent replicates.

The impact of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 on benzaldehyde, organic acids and ethanol production in fermented milk has been previously reported [29, 34, 46]. Particularly, the capacity to convert lactose into acetic acid and lactic acid in the proportion of 3 : 2 (known as Bifidus pathway) is a remarkable heterofermentative attribute of bifidobacteria [41]. However, the results did not show a

significant increase in acetic acid concentration in the samples fermented with Y-BB12. The effects of high temperature incubation on the formation of acetic acid as well as other volatiles by bifidobacteria have been previously reported [30]. An incubation temperature near the optimum level for growth of bifidobacteria (35-37 °C) combined with a long incubation time (10 - 18 h) was found to link with their capacity of volatile formation [29, 46]. Thus, a relatively high incubation temperature combined with rapid acidification rate by yoghurt starters in this study might explain the low acetic acid production by *B. animalis* subsp. *lactis* BB12, although it was able to develop by approximately one log cfu/g during fermentation. Indeed, an excessive concentration of acetic acid (vinegar, pungent) in yoghurt may be considered as undesirable.

In summary, contributions by *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 are likely pronounced in the formation of minor volatile metabolites present at low concentration, especially volatile organic acids and alcohols, in yoghurt. Therefore, results demonstrate that the incorporation of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 did not significantly influence the major aroma volatile metabolites desirable for a good organoleptic quality of yoghurt. However, the overall volatile metabolite profiles of set-yoghurts could be statistically distinguished by multivariate analysis.

3.3.4 Non-volatile polar metabolite profiles determined by ^{1}H-NMR

For multivariate analysis, it should be mentioned that the 43 identified metabolites accounted for labeling of 149 bins. A complementary data filtering by ANOVA was performed for selection of the remaining unknowns [21]. Finally, a total of 214 bins were introduced as variables for further analyses. An overall PCA score plot was constructed with a total variance of 65.5% (Fig. 3.6A). The distinction among all the 4 h samples was rather small but they could still be separated. It was evident that non-volatile profiles of the samples at 4 h could be distinguished from those of stored samples along PC1 (36.1% variance). The loading plot indicates that the majority of metabolites in the sugar region are determinant for the samples at 4 h whereas those from amino acids and lactate contribute to the separation of stored samples.

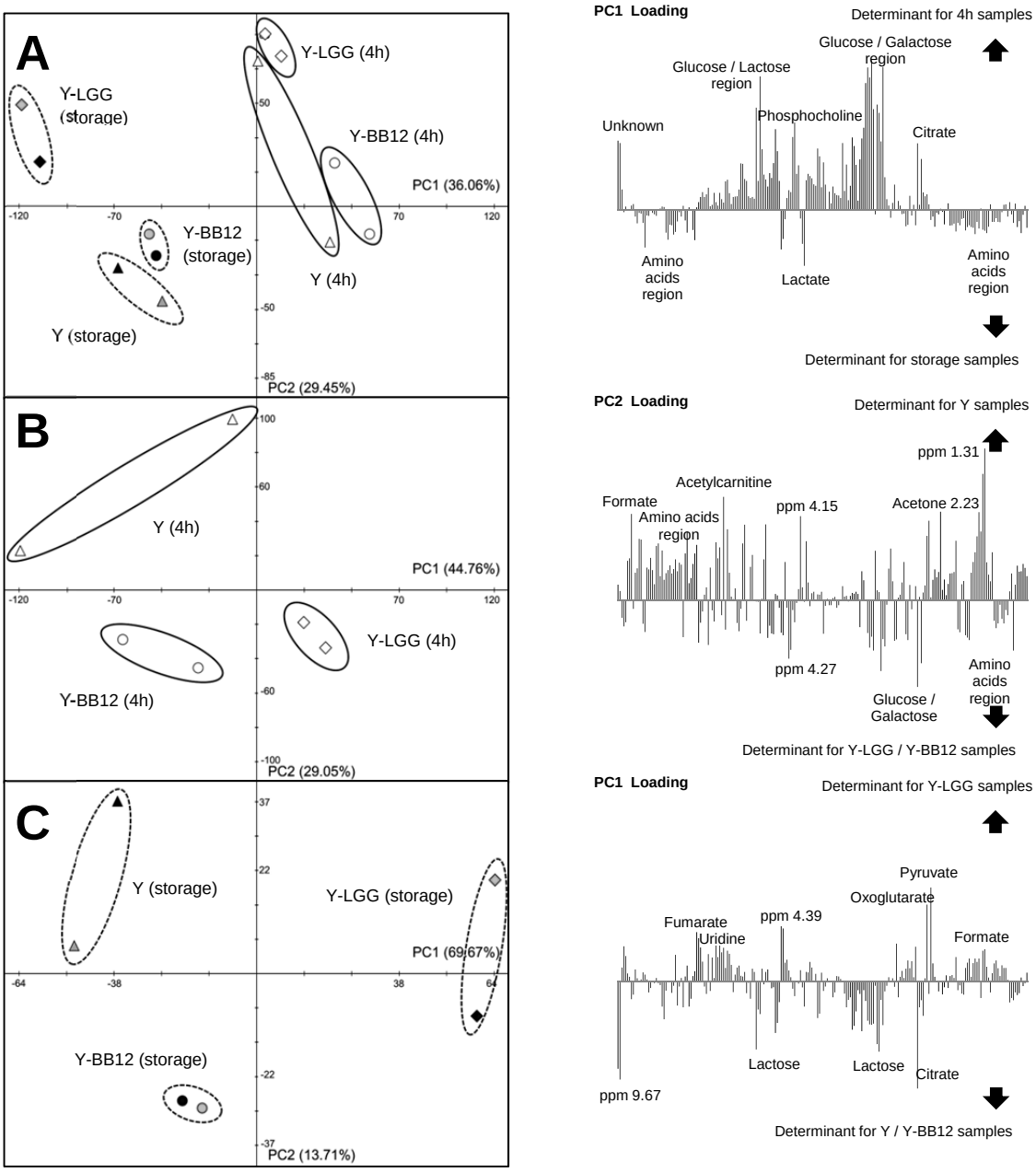


Fig. 3.6. PCA score plots and PC loadings, for overall comparison (panel A), comparison among samples at 4 h (panel B) and among storage samples (panel C), derived from non-volatile polar metabolite profiles of set-yoghurts fermented with traditional yoghurt starters (Y; △), co-cultures of yoghurt starters with *L. rhamnosus* GG (Y-LGG; ◇) and *B. animalis* subsp. *lactis* BB12 (Y-BB12; ○). White, grey and black filled blocks correspond to the samples at 4 hours, 14 days and 28 days, respectively.

To focus on the incorporation of probiotics, two separate PCA score plots were constructed for comparison among yoghurt samples at 4 h (n = 6) with a total variance

of 73.8% (Fig. 3.6B) and among stored samples (n = 6) with a total variance of 83.4% (Fig. 3.6C). In both cases, the samples fermented with Y, Y-LGG and Y-BB12 were clearly separated from each other. It was remarkable that the distinction is larger among stored samples. For instance, a complete separation was found between the samples of Y-LGG and those of Y and Y-BB12 as determined by formate, pyruvate, oxoglutarate, fumarate and uridine along PC1. These metabolites provide a good indication for the presence of *L. rhamnosus* GG contributing to the changes in non-volatile metabolite profile of set-yoghurt during refrigerated storage. The results demonstrate that non-volatile polar metabolite profiles of set-yoghurts can successfully be distinguished according to the differences in types of starter cultures and also duration of storage.

Heat-map visualization combined with hierarchical cluster analysis was used to analyze the quantitative relationships of non-volatile polar metabolite profiles of different samples (Fig. 3.7). The majority of metabolites, especially amino acids and organic acids, are present in high abundance (shown in red) in stored samples. On the other hand, metabolites in the sugar group notably decreased during storage (shown in green). This illustrates ongoing metabolic activity of starter cultures under refrigerated conditions. The dendrogram grouped the samples into two main clusters, i.e. all samples at 4 h (A) and stored samples (B), according to their non-volatile polar metabolite profiles. Pearson's correlation-based linkages showed that non-volatile polar metabolite profiles of the samples fermented with Y-LGG and Y-BB12 at 4 h were relatively close to each other. However, as storage time progressed, non-volatile metabolite profiles of the samples fermented with Y-LGG became well isolated. This observation demonstrates that *L. rhamnosus* GG contributes to a remarkable change in the formation of non-volatile polar metabolites in set-yoghurt during refrigerated storage.

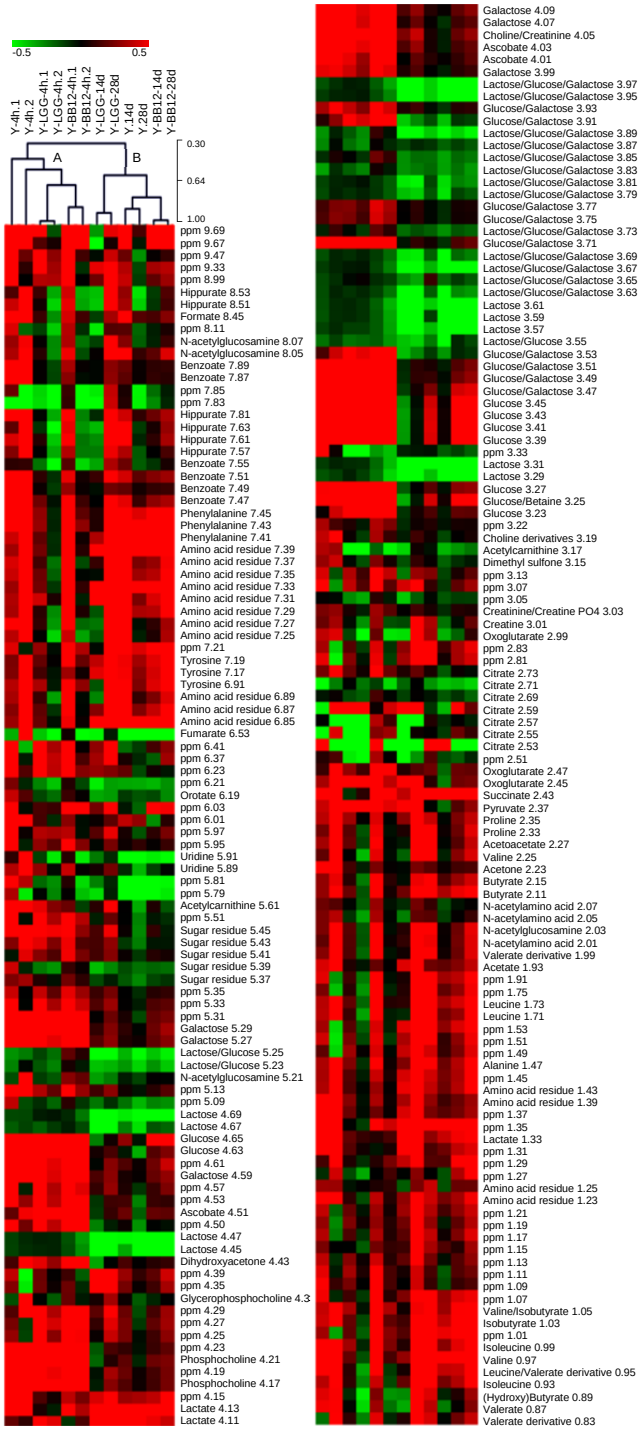


Fig. 3.7. Heat-map and hierarchical clustering of non-volatile polar metabolite profiles from set-yoghurts fermented with traditional yoghurt starters (Y), co-cultures of yoghurt starters with *L. rhamnosus* GG (Y-LGG) and *B. animalis* subsp. *lactis* BB12 (Y-BB12). Dendrogram represents sample clusters based on Pearson's correlation coefficient with average linkage. Each square in the heat-map expresses normalized metabolite content respected to the color range. The red color indicates higher content of the corresponding compound.

During fermentation, the role of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in the biochemical conversion of milk components is well documented [41]. The proto-cooperation between these two species, based on the exchange of several metabolic derived compounds which provide mutual growth stimulating effects to each other [40], leads to the production of various metabolites resulting in a formation of semi-solid texture and typical yoghurt flavor [11]. The primary role of dairy starter cultures is acidification of milk by conversion of lactose into lactic acid. Although, individual cultures of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 exhibit low acidifying capacity in milk, their contribution to acidification may increase from slight to a significant level in co-culture with yoghurt starters [34, 36]. According to the quantification of non-volatile metabolites presented in Table S3.2, the results confirmed that lactate concentration was slightly higher in the samples fermented with Y-LGG and Y-BB12 compared to those fermented without probiotics. Due to the lack of ability to ferment lactose of *L. rhamnosus* GG, this result suggests that this strain might take advantage from the free galactose generated by β-galactosidase activity of yoghurt starters. In contrast to the homofermentative yoghurt starters, *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 are classified as heterofermentative by which certain amounts of lactate, acetate, ethanol and CO_2 can be simultaneously generated from their carbohydrate metabolism [41]. Therefore, an increase in these compounds during fermentation could directly indicate the activity of probiotics. This was confirmed by higher amounts of acetate and ethanol (previously mentioned) detected in the samples fermented with Y-LGG and Y-BB12. Nevertheless, the difference in acetic acid concentration was negligible in their volatile metabolite profiles. Ascorbate, isobutyrate and succinate were present at higher concentrations especially in the samples fermented with Y-BB12. However, contributions by these organic acids were rather small and did not contribute to a significant difference in acidification profiles among yoghurt samples. Pyruvate is a key-metabolite derived from carbohydrate metabolism and can be further converted into various organic acids and volatile compounds [45]. This metabolite was evidently more abundant in the samples fermented with Y-LGG and Y-BB12. The influence of proteolytic activity was characterized by an overall increase in free amino acid concentrations. Growth of bifidobacteria in milk is restricted due to

their low proteolytic activity [34]. However, active growth of *B. animalis* subsp. *lactis* BB12 in co-culture with yoghurt starters found in this study suggests that bifidobacteria might take advantage from free nitrogen sources supplied by the proteolytic activity of *L. delbrueckii* subsp. *bulgaricus*. A small increase in free amino acid concentrations was observed in the samples of Y-BB12, particularly for alanine, phenylalanine, tyrosine and valine.

During refrigerated storage, post acidification is mainly caused by ongoing metabolic activity of *L. delbrueckii* subsp. *bulgaricus* [38]. Decreases in lactose and glucose were observed in all samples and corresponded with an increase in lactate concentration. An excessive acidification is detrimental for the stability of probiotics during yoghurt shelf-life as discussed previously. Moreover, H_2O_2 (analysis not performed in this study) generated by *L. delbrueckii* subsp. *bulgaricus* can be harmful to probiotic cells due to their lack in catalase activity [44]. Increases in organic acids and free amino acids were clearly observed in the samples fermented with Y-LGG. It is possible that proteolysis-derived compounds increased the buffering capacity of milk. This could explain the significant difference in titratable acidy observed between the samples of Y and Y-LGG without significant difference in pH value. The results indicate a substantial metabolic activity of Y-LGG during refrigerated storage which corresponds with a distinctive non-volatile polar metabolite profile demonstrated by multivariate analysis.

3.4 Conclusions

A complementary metabolomics approach using headspace SPME-GC/MS and ^{1}H-NMR was used for characterization of volatile and non-volatile polar metabolite profiles of set-yoghurt during fermentation and storage. *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 did not influence acidity and major aroma volatile metabolites desirable for a good organoleptic quality of yoghurt. On the other hand, a contribution of *L. rhamnosus* GG to non-volatile polar metabolite profile of yoghurt was seen during refrigerated storage. The combination of metabolomic-derived data with multivariate analysis allows discrimination of yoghurt samples statistically according to the difference in types of starter combinations, together with durations of storage. This finding provides new insights regarding the impact of probiotics on the metabolome of yoghurt.

Supplementary data

Table S3.1. Volatile metabolites identified in set-yoghurts (samples at 4 hours, 14 days and 28 days) fermented by different combinations of yoghurt starters and probiotics using headspace SPME-GC/MS

Chemical group	Compound	Starter cultures								
		Traditional yoghurt starters (Y)			Y + *L. rhamnosus* GG (ATCC.53103)			Y + *B. animalis* subsp. *lactis* BB12		
		4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d
Alcohol	1-Butanol	5.45[a] ±0.18a[b]	5.46 ± 0.42a	5.33 ± 0.04a	5.67 ± 0.31a	5.34 ± 0.20a	5.51 ± 0.17a	5.60 ± 0.15a	5.51 ± 0.20a	5.40 ± 0.29a
	Ethanol	6.48 ± 0.06a	6.91 ± 0.09cd	6.93 ± 0.03d	6.73 ± 0.05b	6.72 ± 0.03b	6.77 ± 0.04b	6.83 ± 0.02bc	6.85 ± 0.01c	6.84 ± 0.04bc
	2-Ethyl-hexanol	5.64 ± 0.10c	5.69 ± 0.07c	5.65 ± 0.06c	5.39 ± 0.03b	5.27 ± 0.01a	5.31 ± 0.08ab	5.84 ± 0.01d	5.72 ± 0.04c	5.76 ± 0.04c
	1-Hexanol	5.57 ± 0.04a	5.80±0.17abc	5.83 ± 0.07bc	5.69 ± 0.07b	5.83 ± 0.07bc	5.80 ± 0.03c	5.82 ± 0.05c	5.83 ± 0.08c	5.80 ± 0.06bc
	1-Methoxy-2-propanol	6.02 ± 0.57b	4.94 ± 0.33ab	ND[c]	5.45 ± 0.40ab	4.88 ± 0.26ab	4.97 ± 0.30ab	5.52 ± 0.17b	5.00 ± 0.38ab	4.48 ± 0.51a
	2-Methyl-1-butanol	5.20±0.53a-d	4.60 ± 0.38a	4.95 ± 0.23ab	5.72 ± 0.05d	5.53 ± 0.05c	5.61 ± 0.08cd	5.20 ± 0.18b	4.92 ± 0.30ab	4.95 ± 0.04a
	3-Methyl-2-butanol	5.51 ± 0.05a	5.96±0.22bcd	6.03 ± 0.04d	5.73 ± 0.09b	5.86 ± 0.04c	5.87 ± 0.02c	5.85 ± 0.03c	5.98 ± 0.02d	5.97 ± 0.03d
	3-Methyl-3-butanol	5.71 ± 0.05a	5.96±0.21a-d	6.05 ± 0.06d	5.77 ± 0.90ab	5.81 ± 0.00b	5.83 ± 0.03b	5.93 ± 0.03c	5.94 ± 0.02c	5.97 ± 0.06cd
	1-Octanol	4.45 ± 0.15a	5.16 ± 0.07b	5.09 ± 0.09b	5.03 ± 0.09b	5.04 ± 0.05b	5.03 ± 0.06b	5.12 ± 0.03b	5.06 ± 0.01b	5.14 ± 0.02b
	1-Pentanol	5.18 ± 0.02a	5.40±0.21a-d	5.45 ± 0.07bc	5.39 ± 0.09b	5.55±0.09bcd	5.52 ± 0.02cd	5.50 ± 0.02bc	5.56 ± 0.02d	5.59 ± 0.04d
	3-Pentanol	6.62 ± 0.08a	6.83±0.15a-d	6.95 ± 0.05d	6.67 ± 0.11ab	6.74 ± 0.04b	6.78 ± 0.02b	6.78 ± 0.06bc	6.78 ± 0.07bc	6.84 ± 0.03c
Carbonyl compound	Acetaldehyde	7.39 ± 0.05a	7.49 ± 0.07ab	7.58 ± 0.02c	7.37 ± 0.10ab	7.49 ± 0.09ab	7.45±0.11abc	7.49 ± 0.03b	7.48±0.10abc	7.55 ± 0.03bc
	Acetoin	8.40 ± 0.07a	8.50 ± 0.12ab	8.55 ± 0.04b	8.50 ± 0.07ab	8.44 ± 0.02a	8.46 ± 0.02a	8.60 ± 0.04b	8.55 ± 0.03b	8.56 ± 0.01b
	Acetone	7.42 ± 0.01a	7.46 ± 0.07a	7.56 ± 0.02a	7.47 ± 0.06a	7.49 ± 0.06a	7.52 ± 0.06a	7.50 ± 0.05a	7.48 ± 0.12a	7.56 ± 0.04a
	Benzaldehyde	5.86 ± 0.04a	6.21 ± 0.02b	6.23 ± 0.07b	6.20 ± 0.13bc	6.33 ± 0.06bc	6.40 ± 0.06cd	6.19 ± 0.04b	6.26 ± 0.11bc	6.43 ± 0.01d
	2-Butanone	7.53 ± 0.15a	7.35 ± 0.07a	7.43 ± 0.14a	7.38 ± 0.21a	7.44 ± 0.13a	7.38 ± 0.17a	7.61 ± 0.06a	7.45 ± 0.12a	7.58 ± 0.06a
	Diacetyl	7.38 ± 0.03a	7.44 ± 0.20a	7.50 ± 0.06a	7.47 ± 0.07a	7.48 ± 0.04a	7.54 ± 0.06a	7.51 ± 0.10a	7.58 ± 0.05a	7.59 ± 0.02a
	2-Heptanone	5.99 ± 0.12a	6.35±0.19bcd	6.50 ± 0.06d	6.11 ± 0.08b	6.38 ± 0.04c	6.42 ± 0.06cd	6.20 ± 0.09b	6.42 ± 0.07cd	6.45 ± 0.03d
	2-Hydroxy-3-pentanone	6.25 ± 0.04a	6.61 ± 0.17bc	6.74 ± 0.06c	6.45 ± 0.13b	6.51 ± 0.02b	6.53 ± 0.03b	6.57 ± 0.05b	6.55 ± 0.07b	6.62 ± 0.03b

	3-Methyl-2-butenal	5.08 ± 0.15a	5.64±0.23bcd	5.78 ± 0.03d	5.41 ± 0.22b	5.58 ± 0.04b	5.52 ± 0.12b	5.46 ± 0.22bc	5.62 ± 0.14bc	5.68 ± 0.03c
	2-Nonanone	5.77 ± 0.09a	6.29 ± 0.17bc	6.35 ± 0.06c	6.17 ± 0.04b	6.37 ± 0.04c	6.34 ± 0.05c	6.18 ± 0.02b	6.33 ± 0.05c	6.32 ± 0.01c
	3-Octanone	5.87 ± 0.07d	5.25 ± 0.37ab	5.43 ± 0.08bc	5.35 ± 0.07b	5.29 ± 0.06ab	5.26 ± 0.02a	5.52 ± 0.05c	5.41 ± 0.00b	5.41 ± 0.02b
	2,3-Pentanedione	6.85 ± 0.10a	7.26 ± 0.13b	7.46 ± 0.04c	6.92 ± 0.12a	7.48 ± 0.04c	7.53 ± 0.08bc	6.92 ± 0.05a	7.40 ± 0.06bc	7.55 ± 0.05c
	2-Undecanal	ND	5.64 ± 0.20b	5.53 ± 0.06b	5.14 ± 0.08a	5.22 ± 0.03a	5.23 ± 0.06a	5.49 ± 0.10b	5.49 ± 0.03b	5.62 ± 0.21b
	2-Undecanone	4.88 ± 0.08a	5.31 ± 0.16bc	5.33 ± 0.04b	5.46 ± 0.06c	5.45 ± 0.03c	5.43 ± 0.04c	5.35 ± 0.02b	5.28 ± 0.10b	5.30 ± 0.04b
Heterocyclic compound	3-Acetyl-2,5-dimethylfuran	5.55 ± 0.64ab	5.12 ± 0.27a	5.21 ± 0.12a	6.35 ± 0.04c	6.24 ± 0.02b	6.26 ± 0.02b	ND	ND	ND
Sulfur compound	Dimethyl disulfide	5.26 ± 0.05a	5.64 ± 0.29b	6.19 ± 0.17c	5.19 ± 0.30ab	6.51 ± 0.14c	6.48 ± 0.35c	5.22 ± 0.20ab	6.37 ± 0.20c	6.61 ± 0.42c
	Dimethyl sulfide	6.69 ± 0.20a	6.17 ± 0.57a	6.56 ± 0.10a	6.22 ± 0.13a	6.51 ± 0.20a	6.08 ± 0.83a	6.44 ± 0.26a	6.43 ± 0.48a	6.13 ± 0.94a
	Dimethyl sulfone	6.51 ± 0.24a	6.66 ± 0.37a	6.48 ± 0.28a	6.27 ± 0.18a	6.66 ± 0.05a	6.66 ± 0.04a	6.41 ± 0.21a	6.77 ± 0.10a	6.77 ± 0.04a
Volatile organic acid	Acetic acid	7.90 ± 0.10b	8.05 ± 0.05c	8.18 ± 0.05de	7.66 ± 0.08a	7.95 ± 0.02b	8.05 ± 0.08cd	7.88 ± 0.16bc	8.19 ± 0.05de	8.21 ± 0.03e
	Butyric acid	7.62 ± 0.10a	7.75 ± 0.07ab	7.84 ± 0.00c	7.61 ± 0.10ab	7.70 ± 0.01ab	7.75 ± 0.07b	7.70±0.13abc	7.84 ± 0.04bc	7.83 ± 0.00c
	Hexanoic acid	7.07 ± 0.08a	7.68 ± 0.04b	7.71 ± 0.05b	7.61 ± 0.10b	7.63 ± 0.01b	7.63 ± 0.05b	7.62 ± 0.07b	7.68 ± 0.06b	7.71 ± 0.02b
	3-Methyl-butanoic acid	5.18 ± 0.10a	5.66 ± 0.09bc	5.76 ± 0.03c	5.60 ± 0.05b	5.64 ± 0.03b	5.66 ± 0.04bc	5.56 ± 0.10b	5.70 ± 0.04bc	5.73 ± 0.01c
	2-Methyl-propanoic acid	5.10 ± 0.09a	5.56 ± 0.07bc	5.58 ± 0.01c	5.43 ± 0.05b	5.49 ± 0.02b	5.55 ± 0.03bc	5.47 ± 0.07b	5.57 ± 0.04bc	5.64 ± 0.06c
	Nonanoic acid	5.26 ± 0.05c	5.22 ± 0.06c	5.20 ± 0.12bc	5.06 ± 0.03b	5.06 ± 0.02b	4.79 ± 0.14a	5.06 ± 0.18ab	5.07 ± 0.15ab	5.05 ± 0.04b
	Pentanoic acid	5.57 ± 0.07a	6.06 ± 0.04b	6.07 ± 0.03b	5.95 ± 0.09b	5.98 ± 0.02b	6.01 ± 0.03b	5.97 ± 0.05b	6.04 ± 0.04b	6.06 ± 0.04b
	Propionic acid	5.51 ± 0.06a	6.01 ± 0.05cd	6.07 ± 0.05cd	5.85 ± 0.02b	5.98 ± 0.08c	6.06 ± 0.01d	5.86 ± 0.09bc	6.03 ± 0.04cd	6.07 ± 0.04cd

[a] Metabolite contents are expressed as $\log_{10}$ [peak area of respective compound in arbitrary unit]. Values are means ± standard deviation from three independent replicates.
[b] Letters (a-e) indicate significant difference ($p < 0.05$) among sample means within the same row.
[c] ND indicates compound not detected.

Table S3.2. Presumptive polar metabolites identified in set-yoghurts (samples at 4 hours, 14 days and 28 days) fermented by different combinations of yoghurt starters and probiotics using NOESY 1D-^{1}H-NMR

Chemical group	Compound	Starter cultures								
		Traditional yoghurt starters (Y)			Y + *L. rhamnosus* GG (ATCC.53103)			Y + *B. animalis* subsp. *lactis* BB12		
		4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d
Amino acid and derivatives	Alanine	6.89[a]	7.01	6.90	6.86	6.91	7.06	6.93	6.94	7.01
	Creatine and Creatinine	7.52	7.53	7.38	7.43	7.42	7.60	7.50	7.44	7.49
	Isoleucine	7.62	7.76	7.63	7.54	7.61	7.81	7.63	7.66	7.72
	Leucine	7.64	7.79	7.66	7.53	7.56	7.81	7.65	7.67	7.74
	N-Acetyl-amino acids	8.01	8.01	7.92	7.92	7.92	8.04	8.01	7.96	7.99
	Phenylalanine	6.56	6.73	6.64	6.47	6.49	6.69	6.61	6.64	6.67
	Proline	7.30	7.39	7.26	7.29	7.31	7.46	7.33	7.32	7.38
	Tyrosine	6.82	7.00	6.91	6.86	6.89	7.03	6.95	6.95	6.99
	Valine	7.43	7.65	7.52	7.40	7.44	7.64	7.53	7.55	7.60
	Amino acid residues	8.00	8.09	8.00	7.95	8.01	8.15	8.01	8.04	8.08
Carbohydrate and derivatives	Galactose	8.95	8.90	8.87	9.04	8.95	9.01	9.09	8.94	8.97
	Glucose	8.87	8.77	8.68	9.10	8.64	8.70	9.18	8.80	8.82
	Lactose	9.38	9.19	9.07	9.27	8.91	8.95	9.23	8.99	9.03
	N-Acetylglucosamine	7.48	7.49	7.36	7.36	7.36	7.54	7.44	7.41	7.47
	Sugar residues	7.39	7.25	7.21	7.32	7.27	7.32	7.35	7.26	7.25
Organic acid	Acetate	7.42	7.64	7.63	7.58	7.71	7.77	7.61	7.66	7.69
	Acetoacetate	6.99	7.06	6.90	6.91	6.88	7.09	6.98	6.95	7.01
	Ascorbate	8.12	8.13	8.10	8.25	8.13	8.18	8.31	8.15	8.17
	Benzoate	6.91	6.90	6.82	6.79	6.80	6.93	6.86	6.85	6.86
	Butyrate	7.23	7.33	7.23	7.18	7.21	7.34	7.27	7.26	7.29

	Hydroxybutyrate	7.41	7.44	7.28	7.22	7.22	7.48	7.32	7.30	7.40
	Citrate	7.52	7.54	7.50	7.50	7.51	7.55	7.57	7.52	7.49
	Formate	6.85	6.77	6.71	6.74	6.72	6.80	6.82	6.74	6.76
	Fumarate	5.70	5.67	5.57	5.50	5.48	5.62	5.60	5.56	5.60
	Hippurate	7.18	7.18	7.02	7.03	7.01	7.24	7.11	7.07	7.13
	Isobutyrate	6.56	6.80	6.67	6.57	6.60	6.77	6.72	6.72	6.77
	Lactate	9.38	9.55	9.56	9.45	9.62	9.64	9.45	9.63	9.63
	Orotate	6.63	6.49	6.48	6.47	6.43	6.44	6.55	6.48	6.45
	Oxoglutarate	7.38	7.24	7.09	7.36	7.32	7.38	7.31	7.20	7.23
	Pyruvate	7.26	7.20	7.14	7.50	7.53	7.57	7.65	7.25	7.29
	Succinate	7.50	7.63	7.62	7.45	7.56	7.60	7.63	7.62	7.62
	Valerate and derivatives	7.68	7.71	7.55	7.55	7.54	7.77	7.63	7.62	7.69
Lipid derivatives	Acetylcarnitine	6.99	6.75	6.65	6.63	6.66	6.79	6.68	6.69	6.69
	Choline and derivatives	7.79	7.81	7.73	7.73	7.73	7.82	7.83	7.78	7.80
	Glycerophosphocholine	7.37	7.29	7.27	7.39	7.32	7.34	7.45	7.32	7.32
	Phosphocholine	7.89	7.89	7.83	8.08	7.80	7.89	8.18	7.89	7.91
Carbonyl compound	Acetone	7.24	7.26	7.22	7.22	7.21	7.28	7.25	7.25	7.26
	Dihydroxyacetone	7.37	7.26	7.22	7.25	7.25	7.29	7.32	7.27	7.27
Miscellaneous	Dimethyl sulfone	6.92	6.89	6.85	6.96	6.75	6.84	7.08	6.87	6.88
	Uridine	6.02	5.83	5.77	5.91	5.90	5.95	5.94	5.84	5.85

[a] Metabolite contents are expressed as $\log_{10}$ [sum of signal intensity of respective metabolite in arbitrary unit]. Values at 4 hours are the average from two independent replicates. Values at 14 days and 28 days are represented from one replicate.

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Chapter 4

Effect of sublethal preculturing on the survival of probiotics and metabolite formation in set-yoghurt

Based on:
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Abstract

The objective of this study was to investigate the effect of preculturing of *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB12 under sublethal stress conditions on their survival and metabolite formation in set-yoghurt. Prior to co-cultivation with yoghurt starters in milk, the two probiotic strains were precultured under sublethal stress conditions (combinations of elevated NaCl and low pH) in a batch fermentor. The activity of sublethally precultured probiotics was evaluated during fermentation and refrigerated storage by monitoring bacterial population dynamics, milk acidification and changes in volatile and non-volatile metabolite profiles of set-yoghurt. The results demonstrated adaptive stress responses of the two probiotic strains resulting in their viability improvement without adverse influence on milk acidification. A complementary metabolomic approach using SPME-GC/MS and ^{1}H-NMR resulted in the identification of 35 volatiles and 43 non-volatile polar metabolites, respectively. Principal component analysis revealed substantial impact of the activity of sublethally precultured probiotics on metabolite formation demonstrated by distinctive volatile and non-volatile metabolite profiles of set-yoghurt. Changes in relative abundance of various aroma compounds suggest that incorporation of stress-adapted probiotics considerably influences the organoleptic quality of yoghurt. This study provides new information on the application of stress-adapted probiotics in an actual food-carrier environment.

4.1 Introduction

During the past decades, societal interest in healthy foods has contributed to the development of functional dairy products that potentially provide health benefits in addition to the fundamental nutrients they contain [31]. An example of a functional type of yoghurt is one that carries "probiotics" which are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [4]. The definition underlines that probiotics need to be alive and present in sufficiently high number at the time of consumption to ensure their health-promoting effects. With respect to this, a probiotic product should contain at least 10^6 cfu/g of viable probiotic cells throughout the entire shelf-life [38]. Most commercial probiotics incorporated in dairy products are strains belonging to the genera *Lactobacillus* and *Bifidobacterium* [12]. However, many of these strains exhibit a low capacity to grow in milk during fermentation and are not able to survive well in fermented milk during refrigerated storage [6], mainly due to the reduction of pH and accumulation of organic acids [30].

Stress adaptation is one of the strategies to improve the survival of probiotics by pre-treating (preculturing) them in a sublethal stress condition prior to exposure to a more harsh or lethal environment [35]. This approach allows probiotic bacteria to develop adaptive stress responses leading to an increase in their survival compared to those that are directly shifted into the same lethal stress condition [26]. Adaptive responses towards various types of stress, i.e. heat, cold, acid, bile salts, osmotic, oxygen, high pressure and nutrient starvation, have been well documented for lactobacilli and bifidobacteria [3, 25, 34, 37]. These stress features usually resemble the environmental niches typically encountered by probiotics during human gastrointestinal tract transit, industrial-scale production and in the food matrix [25]. Acid and osmotic stress, as consequences of lactic acid production and application of food additives, are the most predominant stress factors during yoghurt manufacture and refrigerated storage [17]. Recent advances in post-genomics technologies, i.e. transcriptomics and proteomics, have provided novel insights into how probiotics counteract environmental stresses [27]. Despite high numbers of publications focusing on the molecular basis of stress responses in probiotics, there is only a limited number of studies investigating the fate

of stress-adapted bacteria when administered in a real food system such as milk and yoghurt [5, 14, 16, 30]. Particularly, the influence of metabolic activity of stress-adapted probiotics on the biochemical characteristics of the food-carrier has received little attention.

Metabolomics is recognized as an effective tool to investigate the overall chemical composition of complex biological systems including food matrices [7]. The application of mass spectrometry (MS) and nuclear magnetic resonance (NMR) has shown to be successful in determining a wide range of metabolites in fermented dairy products [19, 22, 23, 29]. This approach can be implemented for monitoring the overall biochemical changes associated with the metabolic activity of starter cultures and probiotics during yoghurt manufacture [19, 27]. The outcomes are expected to provide new information concerning the impact of stress-adapted probiotics applied in yoghurt, since their metabolic responses may substantially affect the biochemical and organoleptic characteristics of this product [28].

The objective of this study was to investigate the impact of preculturing of two probiotic strains, *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB12, under sublethal stress conditions (combinations of elevated NaCl and low pH) on their survival and metabolite formation in set-yoghurt. Changes in viable counts of yoghurt starters as well as probiotics and extent of milk acidification were monitored during fermentation and refrigerated storage. Furthermore, biochemical changes associated with bacterial metabolism were characterized by a metabolomics approach using headspace SPME-GC/MS and ^{1}H-NMR technique. Finally, multivariate analysis was applied to analyze volatile and non-volatile polar metabolite profiles of set-yoghurts.

4.2 Materials and methods

4.2.1 Yoghurt Starters and probiotic strains

Frozen direct-vat-inoculation (DVI) pellets of *Streptococcus thermophilus* C44, *Lactobacillus delbrueckii* subsp*. bulgaricus* C49 (CSK Food Enrichment, Ede, the

Netherlands) and *B. animalis* subsp. *lactis* BB12 (BB12) (Chr. Hansen, Hørsholm, Denmark) were stored at -45 °C. A culture of *L. rhamnosus* GG (LGG) (ATCC 53103) was propagated in our laboratory and stored as a 20% (v/v) glycerol stock-culture at -80 °C. Frozen cultures were transferred to ambient temperature (20 ± 3 °C) for 15 min before use. Probiotic strains were re-propagated in MRS broth (0.5 g/L cysteine-HCl supplemented for BB12) (Merck, Darmstadt, Germany) at 37 °C for 24 h under anaerobic incubation (Anoxomat™-Mart®, Drachten, the Netherlands). Then, the cells were collected by centrifugation at 4,000*g* for 15 min at 4 °C, washed twice using peptone-physiological-salt solution (Tritium microbiology, Eindhoven, the Netherlands) and finally resuspended in milk before inoculation. These cultures were defined as control groups, i.e. standard precultured LGG and BB12.

4.2.2 Preculturing of probiotics under sublethal stress conditions

a) Screening for sublethal stress conditions

Suitable sublethal stress conditions, combinations of elevated NaCl concentrations and low pH values, for LGG and BB12 were preliminary determined. For screening of sublethal salt levels, probiotic cells were cultured in NaCl-adjusted MRS broth (0.5 g/L cysteine-HCl supplemented for BB12). NaCl (Merck, Darmstadt, Germany) was added to MRS broth at concentrations ranging from 0.5% to 5.0% (w/v) with a 0.5% interval level. The concentrations which caused 0.5 and 1.0 log reduction of viable probiotic cells compared to those enumerated in unsalted MRS broth after anaerobic incubation (Anoxomat™-Mart®, Drachten, the Netherlands) at 37 °C for 24 h (data not shown) were considered as low and high sublethal NaCl levels, i.e. 2.0%/4.0% (w/v) for LGG and 0.5%/1.5% (w/v) for BB12. Sublethal pH values for LGG and BB12 were assigned at 1.0 pH unit above and below the optimum pH for their growth, i.e. pH 4.5/6.5 (LGG) and pH 5.0/7.0 (BB12). The combinations of sublethal NaCl-pH treatments were finally organized as a 2 X 2 between subjects factorial design (Table 4.1).

Table 4.1. Sublethal stress conditions (combinations of elevated salt and low pH) in modified MRS broth for preculturing of *L. rhamnosus* GG (LGG) and *B. animalis* subsp. *lactis* BB12 (BB12) in a batch fermentor

Probiotics	**Salt stress**	**Acid stress**	
		Low pH	High pH
LGG	Low %NaCl	2.0% NaCl – pH 4.5	2.0% NaCl – pH 6.5
	High %NaCl	4.0% NaCl – pH 4.5	4.0% NaCl – pH 6.5
BB12	Low %NaCl	0.5% NaCl – pH 5.0	0.5% NaCl – pH 7.0
	High %NaCl	1.5% NaCl – pH 5.0	1.5% NaCl – pH 7.0

b) Preculturing of probiotics in a batch fermentor

Preculturing of probiotics was conducted in a 750 mL Multifors-2 Bacterial System Bioreactor fully operated by IRIS-V.5.3 control software (Infors HT, Bottmingen, Switzerland). The fermentor was filled with 350 mL NaCl-adjusted MRS broth and then was equipped with auxiliary devices (tubes, gas-pipes, pumps, reagent bottles, sampling system, pH, optical density and temperature sensors) before sterilization (121 °C for 30 min). For BB12, the medium was supplemented with 0.5 g/L cysteine-HCl after sterilization. The pH of the medium was adjusted and automatically maintained at a desired pre-set value (pH-stat) by adding 1 N NaOH or 1 N HCl. A fresh overnight culture of the probiotics was inoculated at 1% (v/v) into the NaCl-pH adjusted medium. Batch scale preculturing was carried out at 37 °C for 24 h under anaerobic condition created by a continuous N_2-flushing system with a flow rate of 1 L/min through a 0.22 μm filter. The medium was continuously stirred at a constant speed of 100 rpm. After 24 h (stationary phase monitored by optical density; data not shown), sublethally precultured probiotic cells were collected by centrifugation at 4,000*g* for 15 min at 4 °C, washed twice using peptone-physiological-salt solution and the cell pellets were finally resuspended in milk before use. These steps were performed to avoid carryover effect of nutrients from MRS broth which is a nonfood-grade laboratory medium [26]. Sublethally precultured probiotics were subsequently inoculated in co-cultures with traditional

yoghurt starters as described previously in Chapter 3. The preculturing was performed in three batches for each stress combination.

4.2.3 Set-yoghurt fermentation

Pasteurized Nilac milk was prepared and inoculated according to the methods described in Chapter 2 and 3. After inoculation, set-yoghurt fermentation was carried out in a water bath at 42 °C for 4 h. Yoghurts were then placed in a cold chamber (4 ± 2 °C) for 28 days of storage. Samples were taken hourly during fermentation and weekly during storage. The enumeration of viable bacteria and determination of pH and titratable acidity were carried out immediately after sampling. For ^{1}H-NMR, the samples were stored at -20 °C until analysis. The fermentation was performed in three replicates for each type of starter combination.

4.2.4 Enumeration of viable bacteria

Viable counts of *S. thermophilus*, *L. delbrueckii* subsp*. bulgaricus*, *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 were determined according to the methods described in Chapter 3.

4.2.5 Determination of acidification profile

Production of acid during set-yoghurt fermentation and refrigerated storage was expressed by changes in pH and increases in titratable acidity. The pH measurement and determination of titratable acidity were performed according to the methods described in Chapter 2.

4.2.6 Analysis of volatile metabolites by headspace SPME-GC/MS

For headspace SPME-GC/MS analysis, set-yoghurt fermentation was also carried out directly in glass GC vials as described in Chapter 2. The fermentation was performed in three replicates for each type of starter combination. Extraction and determination of volatile compounds by headspace SPME-GC/MS were performed according to the method described in Chapter 2. This method was based on the method developed by Hettinga et al. [8].

Volatile metabolites were identified using AMDIS software (NIST, Gaithersburg, MD, USA) referred to NIST/EPA/NIH database and library provided by Hettinga et al. [9]. Specific retention time and *m/z* model were used for automated peak integration in the XCalibur software package (Thermo Scientific, Austin, TX, USA) [29].

4.2.7 Analysis of non-volatile polar metabolites by ^{1}H-NMR spectroscopy

For ^{1}H-NMR analysis, the samples from two replicates were analyzed according to the method described in Chapter 2. Frozen yoghurt samples were thawed at room temperature and pH was adjusted to 6.0 using 1.0 N NaOH to achieve low variation, i.e. location and shape of peaks, in the spectra obtained [15]. NOESY 1D-^{1}H-NMR measurements were performed at 300 K in a 600 MHz NMR spectrometer (Bruker, Rheinstetten, Germany) operated under full automation, with similar parameters as described by Lu et al. [13].

The ^{1}H-NMR spectra were baseline-corrected, phase-corrected, aligned and calibrated based on the internal standard (TSP) peak. For each spectrum, chemical shift (δ) across the range of 0.00 - 10.00 ppm was segmented (binning) with an interval of 0.02 ppm [29]. The signal intensity in each bin was integrated and expressed in arbitrary units using AMIX software (Bruker, Rheinstetten, Germany). Metabolite labels were assigned to the bins by means of Chenomx NMR suite 7.5 library (Chenomx Inc., Alberta, Canada) and from the list of metabolites identified in Chapter 2 and 3 [29]. For unlabeled bins, significant variables were selected based on one-way ANOVA at 95% confidence level.

4.2.8 Statistical analysis

One-way ANOVA with multiple comparisons by Tukey's test were performed using IBM SPSS statistics package version 19 (SPSS Inc., Chicago, IL, USA). A probability at $p < 0.05$ was considered statistically significant. Metabolomics data from GC/MS and ^{1}H-NMR were normalized by median-centering and $\log_2$-scaling before multivariate statistical analysis. Principal component analysis (PCA) was performed using Multi-Experiment Viewer (MeV) version 4.8 as previously described in Chapter 2.

4.3 Results

4.3.1 Bacterial growth and survival profiles

Viable counts of yoghurt starters and probiotics were enumerated during set-yoghurt fermentation and refrigerated storage (Fig. 4.1). Bacterial populations in the samples co-fermented with sublethally precultured *L. rhamnosus* GG (LGG) and *B. animalis* subsp. *lactis* BB12 (BB12) were compared with those in the samples co-fermented with standard precultured probiotics (control group) of each strain. For comparison, the effect on growth (increase in biomass) and survival (retention of viability) of probiotics were discussed in terms of increase or decrease in $\log_{10}$ transformed units of viable counts. The main effects of the individual preculturing stress factors, i.e. NaCl and pH, and their interaction were statistically determined using two-way ANOVA with 2 X 2 between subjects factorial design (Table 4.2).

In co-cultures with LGG (Fig. 4.1; left panels), growth and survival of yoghurt starters were not significantly affected by the incorporation of sublethally precultured probiotics. At the end of fermentation, the viable counts of *S. thermophilus* (Fig 4.1A) and *L. delbrueckii* subsp. *bulgaricus* (Fig. 4.1C) increased by 2.2 and 2.1 log units to reach an average value of 8.5 ± 0.1 and 8.1 ± 0.1 log cfu/g, respectively. The viable counts of two yoghurt starters remained virtually stable (above 8.0 log cfu/g) throughout the entire duration of storage. Variations in growth and survival of LGG were observed among the control group and their sublethally precultured cells (Fig. 4.1E & Table 4.2). During fermentation, LGG precultured at 2.0% NaCl-pH 6.5 exhibited the highest increase in viable counts (0.8 log increase) while those precultured at 4.0% NaCl-pH 6.5 showed the lowest increase (0.5 log increase). However, none of the preculturing conditions could significantly enhance ($p > 0.05$) the growth of LGG in milk compared to the control group (0.6 log increase). Among the groups of sublethally precultured LGG, the effects of NaCl and interaction between NaCl*pH during preculturing contributed significantly ($p = 0.01$ and 0.02, respectively) to their growth in milk during set-yoghurt fermentation.

The effect of preculturing on the survival of LGG and their sublethally precultured cells during storage was evidently observed. At the end of storage, LGG precultured at pH 4.5 (with either 2.0% or 4.0% NaCl) showed a significant improvement (p = 0.03) on their survival (0.2 and 0.3 log reduction, respectively) compared to the control group (0.5 log reduction). On the other hand, the survival of LGG precultured at 4.0% NaCl-pH 6.5 was significantly impaired (p < 0.01) (1.2 log reduction). Statistical tests demonstrated that only the main effect of pH during preculturing significantly contributed (p < 0.01) on the survival of LGG during storage.

In co-cultures with BB12 (Fig. 4.1; right panels), growth and survival of *S. thermophilus* (Fig 4.1B) were not significantly affected by the incorporation of sublethally precultured probiotics. Their viable counts increased by 2.3 log units to reach and average value of 8.5 ± 0.1 log cfu/g at the end of fermentation and remained stable (above 8.0 log cfu/g) throughout the entire duration of storage. On the other hand, the growth of *L. delbrueckii* subsp. *bulgaricus* (Fig. 4.1D) was impaired by co-cultivation with BB12 precultured at 1.5% NaCl (with either pH 5.0 or 7.0) resulting in significantly lower (p < 0.01) viable counts at the end of fermentation (8.1 ± 0.1 log cfu/g) compared to the control group (8.4 ± 0.1 log cfu/g). Although the survival of *L. delbrueckii* subsp. *bulgaricus* during storage was not affected by co-cultivation with BB12 precultured at 1.5% NaCl (with either pH 5.0 or 7.0), the initially lower viable counts at 4 h subsequently resulted in significantly lower (p = 0.02) viable counts at the end of storage (7.8 ± 0.2 log cfu/g) compared to the control group (8.3 ± 0.2 log cfu/g). Variations in growth and survival of BB12 were observed among the control group and their sublethally precultured cells (Fig. 4.1F & Table 4.2). During fermentation, it was evident that the growth of BB12 precultured at 1.5% NaCl (with either pH 5.0 or 7.0) (0.4 log increase) was significantly impaired (p < 0.01) while the growth of BB12 precultured at 0.5% NaCl (with either pH 5.0 or 7.0) (1.1 log increase) was not significantly affected (p > 0.05) compared to the control group (0.9 log increase). Among the groups of sublethally precultured BB12, statistical tests demonstrated that only the main effect of NaCl contributed significantly (p < 0.01) on their growth impairment during set-yoghurt fermentation.

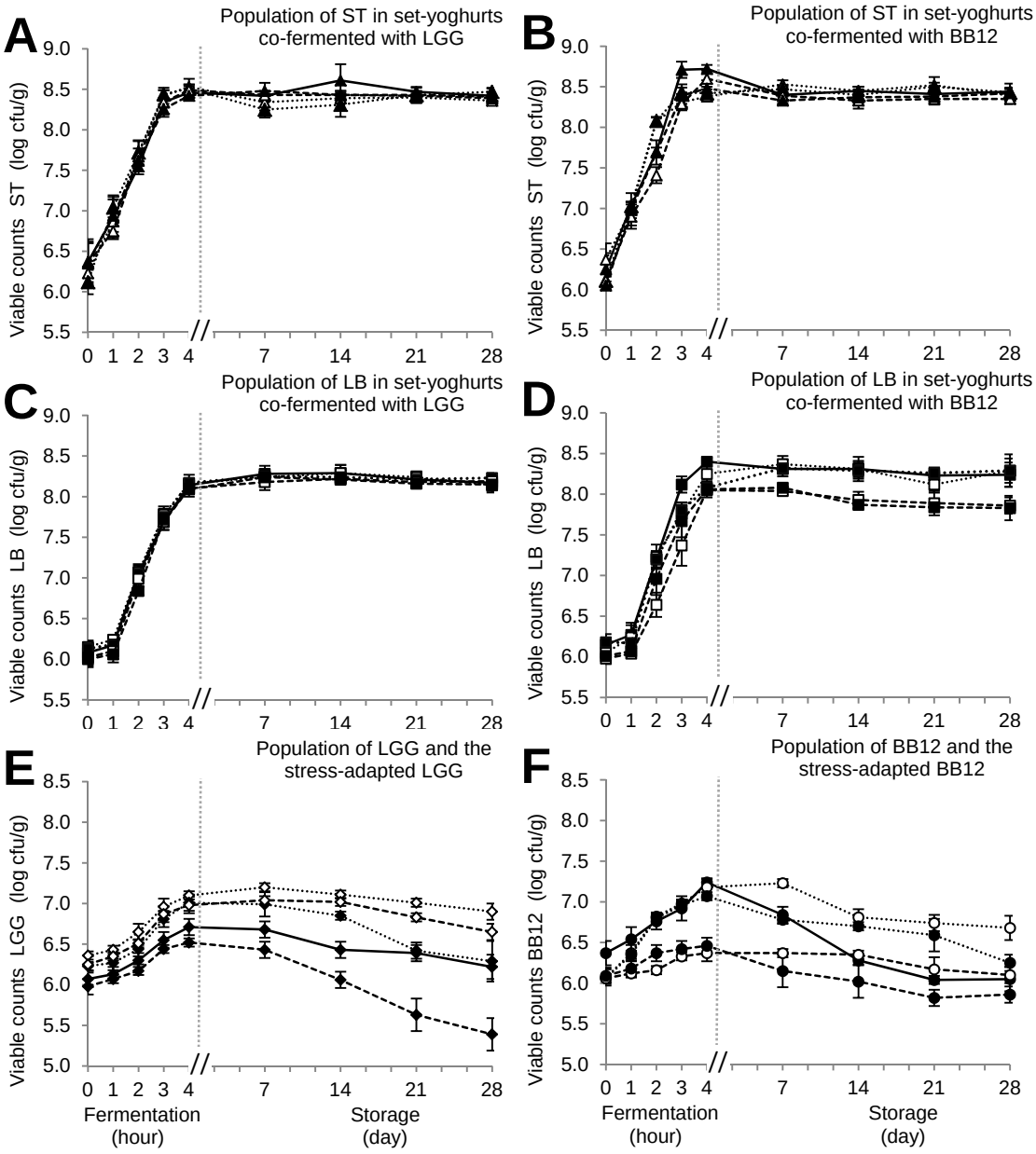

Fig. 4.1. Changes in viable counts of *S. thermophilus* (ST, △; panel A and B), *L. delbrueckii* subsp. *bulgaricus* (LB, □; panel C and D), *L. rhamnosus* GG (LGG, ◇; panel E) and *B. animalis* subsp. *lactis* BB12 (BB12, ○; panel F) during set-yoghurt fermentation (4 hours) and refrigerated storage (28 days). Data are labeled according to the sublethal stress conditions of probiotics of which the bacteria are in co-culture with; i.e. standard precultured (control) group (——; black markers), low-salt-low-pH ('·····'; white markers), low-salt-high-pH ('·····'; black markers), high-salt-low-pH (– – –; white markers) and high-salt-high-pH (– – –; black markers). For information on the sublethal stress conditions of probiotics, the reader is referred to Table 1. Error bars represent standard deviations based on three replicates.

Table 4.2. ANOVA of the main effects of individual stress factors, i.e. NaCl and pH, and their interaction on growth and viability of *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium animalis* subsp. *lactis* BB12 (BB12) in set-yoghurt

Changes in bacterial population	Standard LGG	Sublethally precultured LGG				Test of significance between effects		
	(control)	2.0% NaCl		4.0% NaCl		Main effect		Interaction
		pH 4.5	pH 6.5	pH 4.5	pH 6.5	NaCl	pH	NaCl*pH
Increase in viable counts during fermentation (log cfu/g 4h – 0h)	0.6 ± 0.1ab[a]	0.7 ± 0.0ab	0.8 ± 0.1b	0.7 ± 0.1ab	0.5 ± 0.1a	$p = 0.01$	$p > 0.05$	$p = 0.02$
Decrease in viable counts during storage (log cfu/g 28d – 4h)	-0.5 ± 0.0b	-0.2 ± 0.1a	-0.8 ± 0.3bc	-0.3 ± 0.1a	-1.2 ± 0.3c	$p > 0.05$	$p < 0.01$	$p > 0.05$
	Standard BB12	Sublethally precultured BB12				Test of significance between effects		
	(control)	0.5% NaCl		1.5% NaCl		Main effect		Interaction
		pH 5.0	pH 7.0	pH 5.0	pH 7.0	NaCl	pH	NaCl*pH
Increase in viable counts during fermentation (log cfu/g 4h – 0h)	0.9 ± 0.2b	1.1 ± 0.1b	1.0 ± 0.0b	0.3 ± 0.1a	0.4 ± 0.2a	$p < 0.01$	$p > 0.05$	$p > 0.05$
Decrease in viable counts during storage (log cfu/g 28d – 4h)	-1.2 ± 0.2d	-0.5 ± 0.0b	-0.8 ± 0.1c	-0.3 ± 0.0a	-0.6 ± 0.0b	$p < 0.01$	$p < 0.01$	$p > 0.05$

[a] Letters (a-d) indicate significant difference ($p < 0.05$) among means within the same row.

An effect of preculturing on the survival of BB12 and their sublethally precultured cells during refrigerated storage was also observed. At the end of storage, all sublethally precultured BB12 showed a significant improvement ($p < 0.05$) (max. 0.8 log reduction) on their survival compared to the control group (1.2 log reduction). Interestingly, the viable counts of BB12 precultured at 1.5% NaCl (with either pH 5.0 or 7.0) which were significantly impaired during fermentation decreased relatively slow during storage (0.3 and 0.6 log reduction, respectively). The two main effects of NaCl and pH (without interaction) during preculturing contributed significantly ($p < 0.01$) to the survival of BB12 during storage.

4.3.2 Acidification profiles

Changes in pH were monitored during set-yoghurt fermentation and refrigerated storage (Fig. 4.2). In the samples co-fermented with LGG and their sublethally precultured cells (Fig. 4.2A), similar pH decrease patterns were observed during fermentation throughout the entire duration of storage. The average pH values of all samples were not significantly different ($p > 0.05$) either at the end of fermentation (4.4 ± 0.1) or the end of storage (4.0 ± 0.1). In the samples co-fermented with BB12 and their sublethally precultured cells (Fig. 4.2C), similar pH decrease patterns were observed during fermentation resulting in an average value of 4.4 ± 0.1 at 4 h. During storage, co-fermentation with standard precultured BB12 and BB12 precultured at 0.5% NaCl (with either pH 5.0 or 7.0) showed similar pH decrease patterns with an average value of 4.1 ± 0.1 while a small deviation in pH reduction was observed in the samples co-fermented with BB12 precultured at 1.5% NaCl (with either pH 5.0 or 7.0) resulting in an average pH value of 4.3 ± 0.1 at the end of storage. However, the difference was not statistically significant ($p > 0.05$).

Titratable acidity, expressed as % equivalent lactic acid (w/w) (% LA), was measured during set-yoghurt fermentation and refrigerated storage. For better comparison, the titratable acidity was subtracted by its initial value in the sample at 0 h (unfermented milk) and presented as titratable acidity produced by bacterial activity. In the samples co-fermented with LGG and their sublethally precultured cells (Fig. 4.2B),

there was no significant difference (p > 0.05) in titratable acidity either at the end of fermentation (0.70 ± 0.02%) or the end of storage (1.07 ± 0.05%).

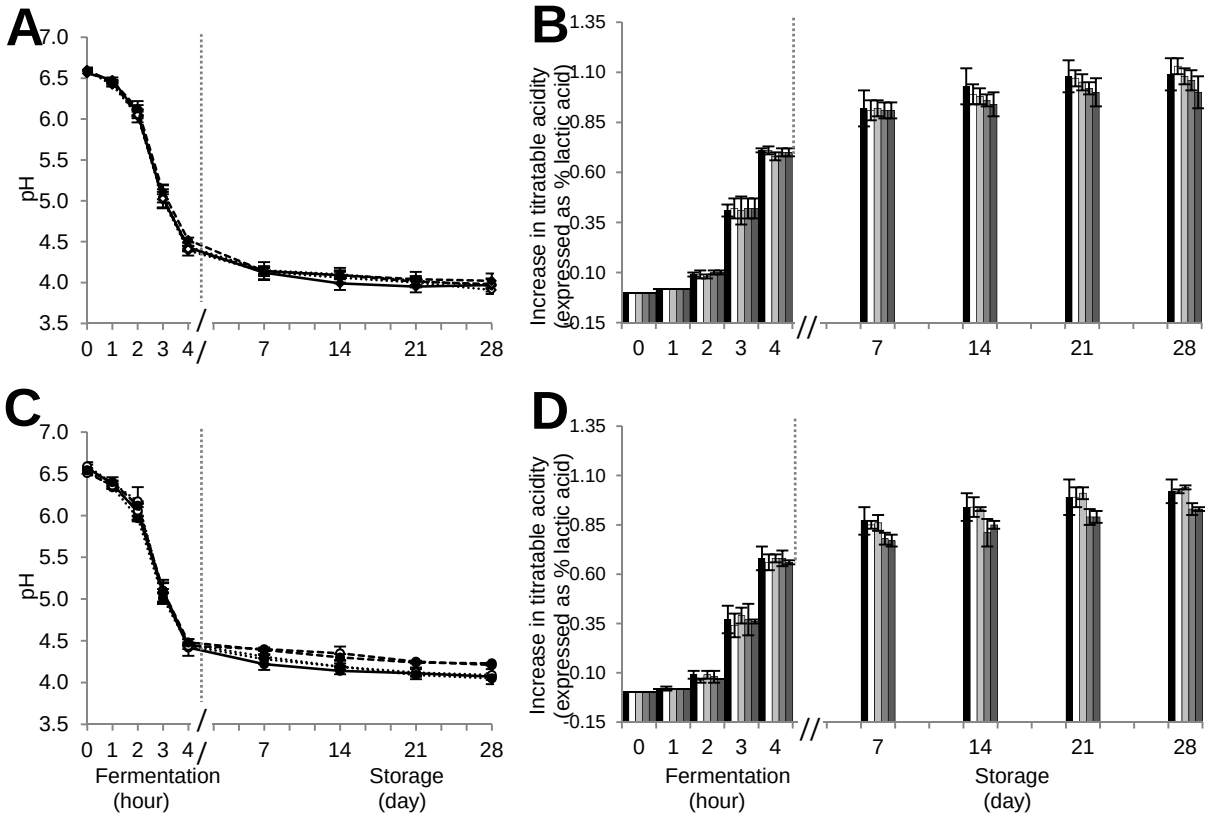

Fig. 4.2. Changes in pH (left) and increase in titratable acidity (right) during fermentation (4 hours) and refrigerated storage (28 days) in set-yoghurts co-fermented with *L. rhamnosus* GG (panel A–B) and *B. animalis* subsp. *lactis* BB12 (panel C–D). Data are labeled according to the sublethal stress conditions of probiotics; i.e. standard precultured (control) group (—; black markers, ■), low-salt-low-pH (‘•••••’; white markers, □), low-salt-high-pH (‘•••••’; black markers, ■), high-salt-low-pH (‘- - -’; white markers, ■) and high-salt-high-pH (‘- - -’; black markers, ■). For information on the sublethal stress conditions of probiotics, the reader is referred to Table 1. Error bars represent standard deviations based on three replicates.

In the samples co-fermented with BB12 and their sublethally precultured cells (Fig. 4.2D), there was also no significant difference in titratable acidity during fermentation (0.67 ± 0.03%). However, a lower acid production during storage was observed in the samples co-fermented with BB12 precultured at 1.5% NaCl (with either pH 5.0 or 7.0). These two cultures resulted in a lower titratable acidity (0.93 ± 0.02%)

compared to the control group (1.02 ± 0.06%). This finding is in accordance with the pH decrease patterns found in these two sublethally precultured BB12 cultures. Although the difference seemed to be negligible, the final titratable acidity of the samples co-fermented with BB12 precultured at 1.5% NaCl-pH 7.0 was significantly different (p = 0.02) from the control group.

4.3.3 Volatile metabolite profiles determined by headspace SPME-GC/MS

Volatile metabolite profiles of set-yoghurts co-fermented with probiotics and their sublethally precultured cells were evaluated at the end of fermentation (4 h) and every two weeks during storage (14 d and 28 d). According to the method described in Chapter 2, set-yoghurt was directly fermented in GC vials. The advantages of this approach are the small amount of sample required (3 mL) together with prevention of volatile loss during sample preparation. A total of 35 volatile metabolites consisting of alcohols, carbonyl compounds, organic acids, sulfur compounds and heterocyclic compound were identified (Table S4.1). These compounds were introduced as variables for multivariate analysis. If necessary, missing values were replaced by the median of respective metabolites. Samples from three replicates of each type of starter combination were statistically treated as individual objects. Principal component analysis (PCA) was performed to distinguish the volatile metabolite profiles of set-yoghurts co-fermented with standard precultured probiotics and their sublethally precultured cells within the same species.

For the samples co-fermented with LGG and their sublethally precultured cells (Fig. 4.3), an overall PCA score plot was constructed with a total variance of 45.5% (n = 45) (Fig. 4.3A). Volatile metabolite profiles of the samples at 4 h could be well distinguished from those of stored samples along PC1 (27.1% variance). The PC-loading indicated which metabolites were accountable for discrimination. It can be seen that 1-methoxy-2-propanol is determinant for the 4 h samples while 2,3-pentanedione, dimethyl disulfide, 2-heptanone, acetic acid and dimethyl sulfone are accountable for discrimination of stored samples. For better comparison, two separated PCA score plots were constructed for distinguishing among samples at 4 h (n = 15) with a total variance

of 58.7% (Fig. 4.3B) and among stored samples (n = 30) with a total variance of 47.1% (Fig. 4.3C).

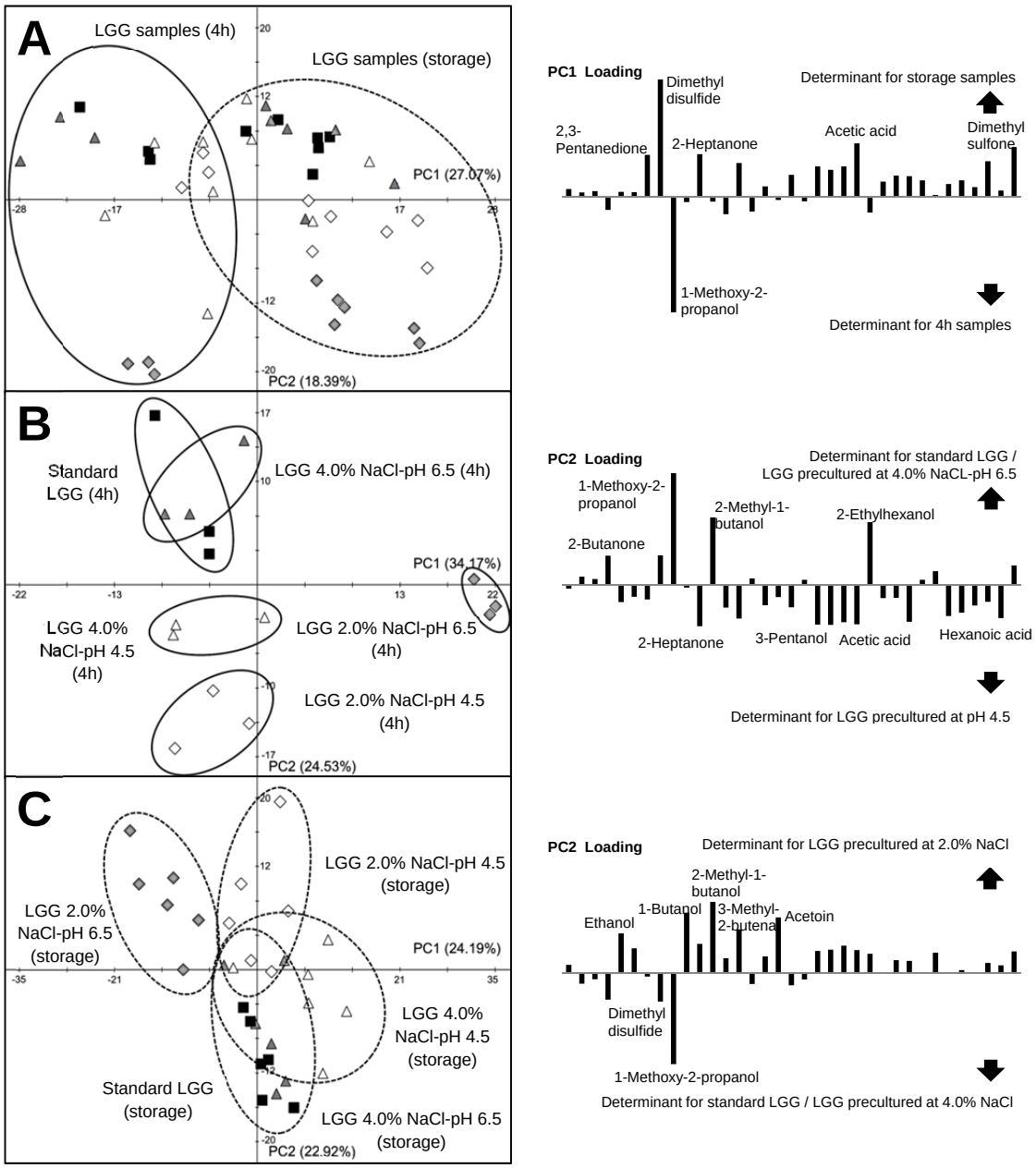

Fig. 4.3. PCA score plots and PC loadings, for overall comparison (panel A), comparison among samples at 4 h (panel B) and among storage samples (panel C), derived from volatile metabolite profiles of set-yoghurts co-fermented with standard precultured (control) *L. rhamnosus* GG (LGG) (■), LGG precultured at 2.0% NaCl-pH 4.5 (◇), LGG precultured at 2.0% NaCl-pH 6.5 (◆), LGG precultured at 4.0% NaCl-pH 4.5 (△) and LGG precultured at 4.0% NaCl-pH 6.5 (▲).

At the end of fermentation, volatile metabolite profiles of the samples co-fermented with sublethally precultured LGG were clearly distinguished from each other as well as from the control group with an exception for those of 4.0% NaCl-pH 4.5 which showed an overlap with the control group. The samples co-fermented with LGG precultured at pH 4.5 (with either 2.0% or 4.0% NaCl) were distinguished from the other groups along PC2 (24.5% variance). The PC2-loading indicated that the majority of volatile metabolites especially 2-heptanone, 3-pentanone, acetic acid and hexanoic acid were accountable for the separation of samples co-fermented with LGG precultured at pH 4.5 (with either 2.0% or 4.0% NaCl) while 2-butanone, 1-methoxy-2-propanol, 2-methyl-1-butanol and 2-ethylhexanol were accountable for the separation of samples co-fermented with standard precultured LGG and LGG precultured at 4.0% NaCl-pH 6.5. During storage, it was remarkable that the volatile metabolite profiles of samples co-fermented with different types of sublethally precultured LGG became less isolated. Nevertheless, the samples co-fermented with LGG precultured at 2% NaCl (with either pH 4.5 or 6.5) were still clearly distinguished from the other groups along PC2 (22.9% variance). The PC2-loading indicated that ethanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-2-butenal and acetoin contributed to the separation of samples co-fermented with LGG precultured at 2% NaCl (with either pH 4.5 or 6.5) while dimethyl disulfide and 1-methoxy-2-propanol accounted for the separation of the other groups.

For the samples co-fermented with BB12 and their sublethally precultured cells (Fig. 4.4), an overall PCA score plot was constructed with a total variance of 64.5% (n = 45) (Fig. 4.4A). Volatile metabolite profiles of the samples at 4 h could be well distinguished from those of stored samples along PC1 (41.8% variance). The PC1-loading indicated that dimethyl sulfide and 1-methoxy-2-propanol were determinant of the samples at 4 h while 2,3-pentanedione, dimethyl disulfide and 2-heptanone were determinant for discrimination of stored samples. For better comparison, two separated PCA score plots were constructed for distinguishing among samples at 4 h (n = 15) with a total variance of 62.1% (Fig. 4.4B) and among stored samples (n = 30) with a total variance of 67.9% (Fig. 4.4C).

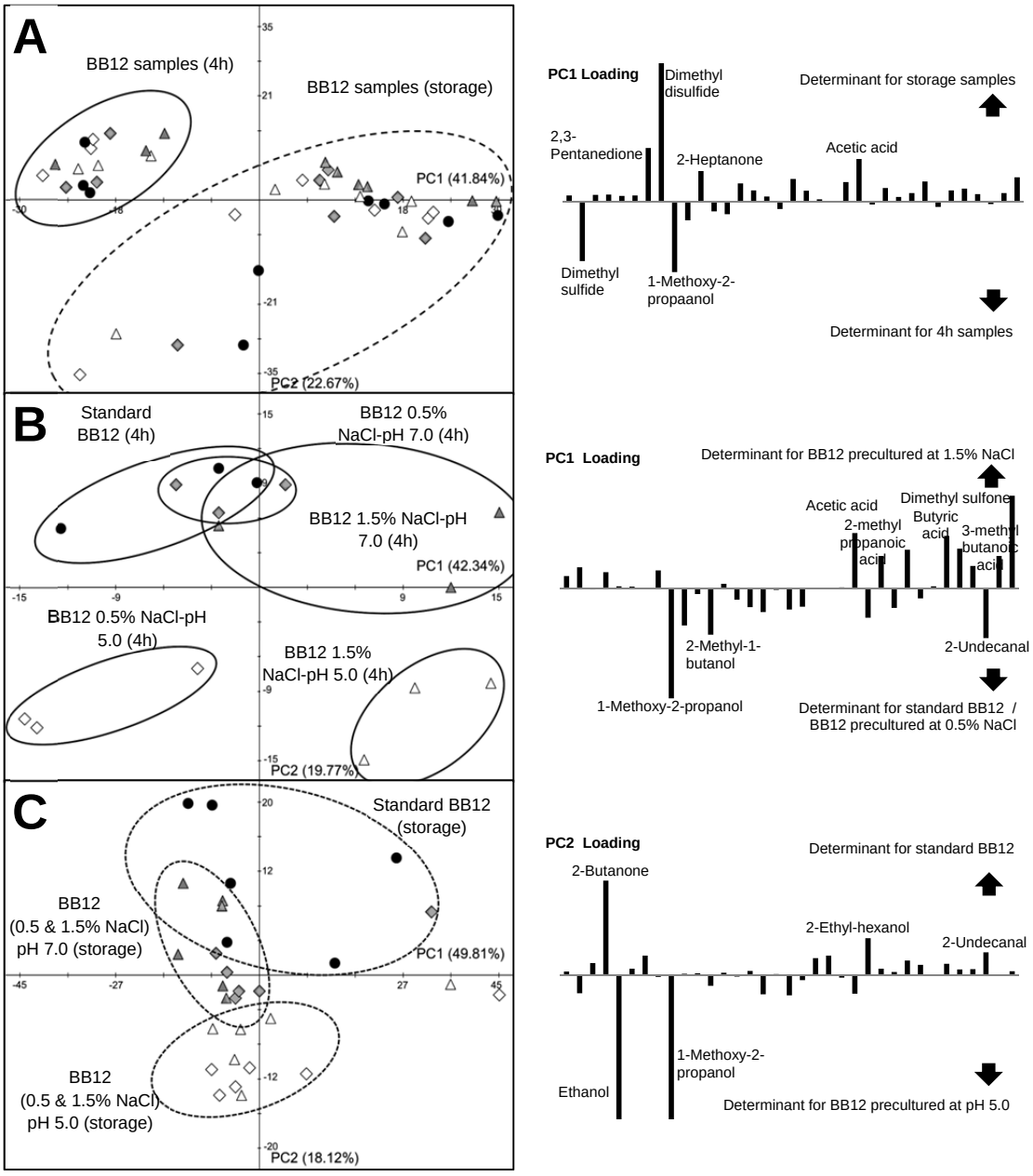

Fig. 4.4. PCA score plots and PC loadings, for overall comparison (panel A), comparison among samples at 4 h (panel B) and among storage samples (panel C), derived from volatile metabolite profiles of set-yoghurts co-fermented with standard precultured (control) *B. animalis* subsp. *lactis* BB12 (BB12) (●), BB12 precultured at 0.5% NaCl-pH 5.0 (◇), BB12 precultured at 0.5% NaCl-pH 7.0 (◆), BB12 precultured at 1.5% NaCl-pH 5.0 (△) and BB12 precultured at 1.5% NaCl-pH 7.0 (▲).

At the end of fermentation, volatile metabolite profiles of the samples co-fermented with sublethally precultured BB12 were clearly distinguished from each

other as well as from the control group. However, the samples co-fermented with BB12 precultured at 0.5% NaCl-pH 7.0 were not clearly separated from the control group. According to the adverse effect on the growth of BB12 in milk, the samples co-fermented with BB12 precultured at 1.5% NaCl (with either pH 5.0 or 7.0) were distinguished from the other groups along PC1 (42.3% variance). The PC1-loading indicated that acetic acid, 2-methyl-propanoic acid, butyric acid, 3-methyl butanoic acid and dimethyl sulfone were the major volatile metabolites accountable for discrimination. During storage, the distinction between sublethally precultured BB12 precultured under the same pH level (with either 0.5% or 1.5% NaCl) moved close to each other. A clear distinction between the samples co-fermented with BB12 precultured at pH 5.0 (with either 0.5% or 1.5% NaCl) and the control group was observed along PC2 while those co-fermented with BB12 precultured at pH 7.0 (with either 0.5% or 1.5% NaCl) were situated between these two groups. The PC2-loading indicated that ethanol and 1-methoxy-2-propanol accounted for the separation of the samples co-fermented with BB12 precultured at pH 5.0 (with either 0.5% or 1.5% NaCl) while 2-butanone, 2-ethylhexabnol and 2-undecanal contributed to those co-fermented with standard precultured BB12.

4.3.4 Non-volatile polar metabolite profiles determined by ^{1}H-NMR

For non-volatile polar metabolite profiling, NOESY-1D-^{1}H-NMR spectra of set-yoghurt were processed according to the method described in Chapter 2. A total of 43 metabolites including amino acids, carbohydrates, organic acids, lipid derivatives, carbonyl compounds, a sulfur compound and a nucleoside were presumptively identified. The quantification was achieved by summation of signal intensities in all bins corresponding to the respective metabolite [21] and expressed in $\log_{10}$ transformed values (arbitrary unit) (Table S4.2). For multivariate analysis, it should be mentioned that the 43 identified metabolites accounted for labeling of 149 bins. A complementary data filtering by ANOVA was performed for selection of the remaining unknowns [11]. A total of 218 (LGG) and 164 (BB12) bins were finally introduced as variables for comparison within the same species of probiotics.

For the samples co-fermented with LGG and their sublethally precultured cells, an overall PCA score plot was constructed with a total variance of 67.7% (n = 20) (Fig. 4.5). Non-volatile polar metabolite profiles of the samples at 4 h could be completely distinguished from those of stored samples along PC1 (43.7% variance). At the end of fermentation, the samples co-fermented with LGG precultured at 2.0% NaCl (with either pH 4.5 or 6.5) and LGG precultured at 4.0% NaCl-pH 4.5 were clearly distinguished from those of standard precultured LGG and LGG precultured at 4.0% NaCl-pH 6.5 along PC2 (24.1% variance). Among stored samples, the same distinction pattern remained, except for the samples co-fermented with LGG precultured at 4.0% NaCl-pH 6.5 which showed an overlap between the two major groups. The PC2-loading indicated that most metabolites in amino acid regions, lactate, citrate, oxoglutarate and pyruvate accounted for the separation of samples co-fermented with standard precultured LGG and LGG precultured at 4.0% NaCl-pH 6.5 while succinate and metabolites in the sugar region contributed to the separation of LGG precultured at 2.0% NaCl (with either pH 4.5 or 6.5) and 4.0% NaCl-pH 4.5.

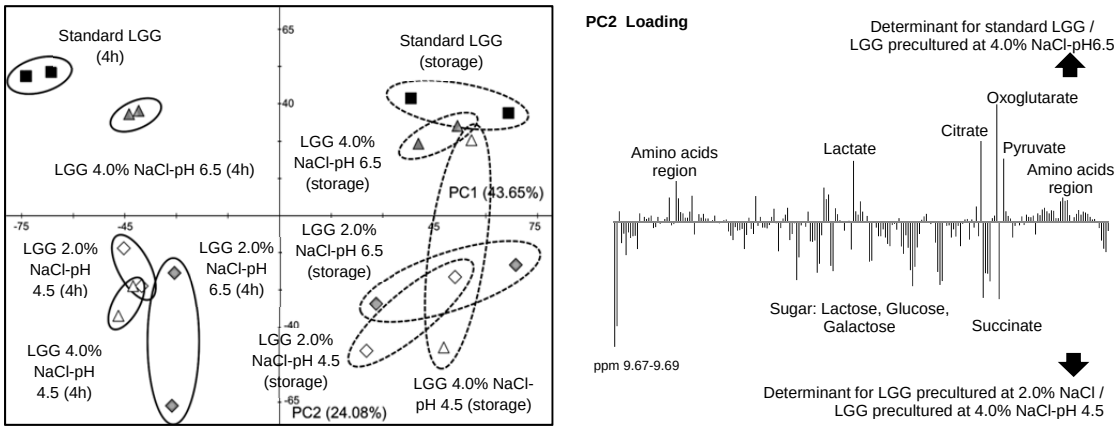

Fig. 4.5. Overall PCA score plot and PC loading derived from non-volatile polar metabolite profiles of set-yoghurts co-fermented with standard precultured (control) *L. rhamnosus* GG (LGG) (■), LGG precultured at 2.0% NaCl-pH 4.5 (◇), LGG precultured at 2.0% NaCl-pH 6.5 (◆), LGG precultured at 4.0% NaCl-pH 4.5 (△) and LGG precultured at 4.0% NaCl-pH 6.5 (▲).

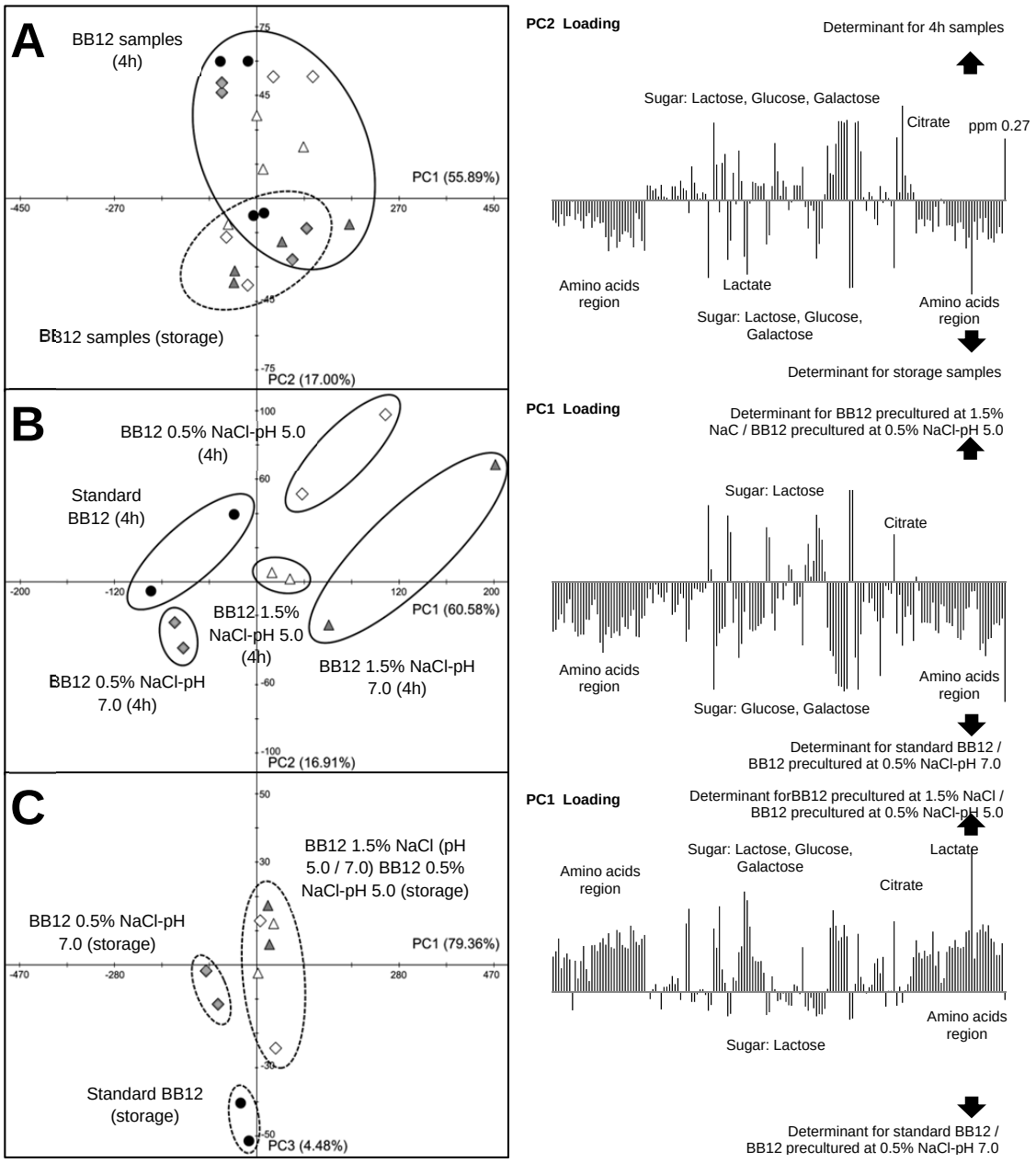

Fig. 4.6. PCA score plots and PC loadings, for overall comparison (panel A), comparison among samples at 4 h (panel B) and among storage samples (panel C), derived from non-volatile polar metabolite profiles of set-yoghurts co-fermented with standard precultured (control) *B. animalis* subsp. *lactis* BB12 (BB12) (●), BB12 precultured at 0.5% NaCl-pH 5.0 (◇), BB12 precultured at 0.5% NaCl-pH 7.0 (◆), BB12 precultured at 1.5% NaCl-pH 5.0 (△) and BB12 precultured at 1.5% NaCl-pH 7.0 (▲).

For the samples co-fermented with BB12 and their sublethally precultured cells, an overall PCA score plot was constructed with a total variance of 72.9% (n = 20) (Fig.

4.6A). Complete separation between non-volatile polar metabolite profiles of the samples at 4 h and stored samples was not observed, although the two groups could be distinguished along PC2 (17.0% variance). For further comparison, two separated PCA score plots were constructed for distinguishing among samples at 4 h (n = 10) with a total variance of 77.5% (Fig. 4.6B) and among stored samples (n = 10) with a total variance of 83.8% (Fig. 4.6C). At the end of fermentation, the samples co-fermented with BB12 precultured at 0.5% NaCl-pH 5.0 and 1.5% NaCl (with either pH 5.0 or 7.0) were clearly distinguished from those of standard precultured BB12 and BB12 precultured at 0.5% NaCl-pH 7.0 along PC1 (60.6% variance). The PC1-loading indicated that most of metabolites in amino acid and sugar regions were accountable for discrimination of the latter two groups. As storage time progressed, it was evident that the distinction among samples co-fermented with different types of sublethally precultured BB12 became less apparent.

4.4 Discussion

The vigorous growth and good retention of survival of *S. thermophilus* C44 and *L. delbrueckii* subsp. *bulgaricus* C49 during yoghurt fermentation and refrigerated storage, respectively, have been discussed previously in Chapter 2 and 3. In case of probiotics, it has been documented that stress responses vary depending on the growth phase, i.e. cells in stationary phase develop more general resistance to various types of stresses [26, 39]. Therefore, the preculturing in this study was prolonged for 24 h to allow attaining stress-adapted probiotic cells from stationary phase. The results demonstrated that preculturing under sublethal stress conditions could not significantly improve the growth of LGG and BB12 in milk during set-yoghurt fermentation. Adversely, the growth of BB12 was significantly impaired by preculturing at high NaCl level (1.5%). On the other hand, it was evident that the viable counts of LGG and BB12 during storage could be successfully improved by preculturing at relatively low pH values. The results showed that the combination of sublethally low-NaCl/low-pH, i.e. 2.0% NaCl-pH 4.5 (LGG) and 0.5% NaCl-pH 5.0 (BB12), delivered the most significant effect on the viability

improvement of LGG and BB12. These conditions resulted in the highest viable counts of the two probiotics at the end of storage without significant impairment on their growth during fermentation. The statistical tests suggested that only the main effect of pH significantly influenced the survival of LGG whereas the effects of both NaCl and pH (without interaction) significantly influenced the survival of BB12 during storage. It should be mentioned that adaptive responses in probiotics are highly strain-dependent and vary largely according to the type of stresses exposed [14, 18, 26]. Interestingly, the viable cells of BB12 precultured at 1.5% NaCl showed a significant improvement in survival during storage, although their growth was significantly impaired during fermentation. It has been reported that alteration in certain cellular protective mechanisms including changes in membrane surface properties, permeability and structural components induced by adaptive stress responses may provide an adverse effect on bacterial growth [37]. This study therefore demonstrated adaptive responses of LGG and BB12 to sublethal NaCl-pH conditions in terms of the viability improvement in yoghurt during refrigerated storage. It should be mentioned that the final viable counts of probiotics in this study, except for those precultured at high-NaCl-high-pH condition, remain well above the minimum recommended level (6.0 log cfu/g) to ensure their potential health-promoting effects [31].

Acidification profiles of set-yoghurts during fermentation and refrigerated storage were not significantly affected by either the different strains of probiotics or the preculturing conditions. However, a small deviation in pH decrease pattern resulting in slightly lower titratable acidity at the end of storage was observed in the samples co-fermented with BB12 precultured at high NaCl level (1.5%). The reduction of pH and accumulation of organic acids during refrigerated storage of fermented milk are defined as “post-acidification” which is mainly attributed to the ongoing metabolic activity of *L. delbrueckii* subsp. *bulgaricus* (Shah, 2000). Accordingly, the slightly lower post-acidification found in yoghurts co-fermented with BB12 precultured at 1.5% NaCl could be associated with the significant impairment on the viable counts of *L. delbrueckii* subsp. *bulgaricus* affected by these two BB12 cultures.

The distinction between volatile metabolite profiles of set-yoghurts determined at the end of fermentation and during refrigerated storage was clearly demonstrated by PCA. At the end of fermentation, co-cultures with sublethally precultured probiotics lead to distinctive yoghurt volatile profiles that could be well distinguished from the control group. This result confirms the impact of stress-adapted probiotics on the biochemical characteristics of product. Incorporation of sublethally precultured probiotics particularly from the conditions adjusted to a low pH level, i.e. 4.5 (LGG) and 5.0 (BB12), resulted in distinctive volatile metabolite profiles of set-yoghurt compared to the other groups. Volatile metabolite profiles of the samples co-fermented with BB12 precultured at high NaCl level (1.5%) might be associated with the lower viable counts of BB12 and *L. delbrueckii* subsp. *bulgaricus* at the end of fermentation. Acetic acid is a primary metabolite generated from heterofermentative carbohydrate utilization by bifidobacteria (Bifidus pathway) [33]. However, acetic acid in the samples co-fermented with BB12 precultured at 1.5% NaCl was detected in higher abundance (PC1-loading in Fig. 4B & Table S1B) without significant influence on pH and titratable acidity. This result suggests metabolic activity of BB12 precultured at 1.5% NaCl, although their growth is significantly impaired by this sublethal condition. As storage time progressed, loading plots showed that ongoing metabolic activities of yoghurt starters and probiotics resulted in higher abundance of many volatile metabolites. Furthermore, it was remarkable that previously distinct volatile metabolite profiles of the samples co-fermented with different types of sublethally precultured probiotics gradually merged during refrigerated storage. Nevertheless, volatile metabolite profiles of the different LGG groups could still be distinguished according to the concentration of NaCl present during preculturing (Fig. 4.3C) while those among the BB12 groups were clearly distinguished according to the pH value during preculturing (Fig. 4.4C). This finding demonstrates that the effect of sublethal stress responses during preculturing on the volatile metabolite profiles of set-yoghurt is species-specific.

In terms of technological relevance, all major aroma volatiles of yoghurt, i.e. acetaldehyde (fresh, green, pungent), diacetyl (buttery, creamy), acetoin (buttery), 2,3-pentanedione (buttery, vanilla-like), acetone (sweet, fruity), 2-butanone (sweet, fruity)

and acetic acid (vinegar, pungent) [1], were detected in high relative abundances in the samples (Table S4.1). The contributions of LGG and BB12 to aroma volatile production in fermented milk have been extensively reported [20, 24, 36, 40]. During storage, the concentration of most aroma compounds remained rather stable while 2,3-pentanedione and acetic acid significantly increased. The contribution of these two compounds for discriminating stored samples was confirmed in loading plots. Furthermore, various carbonyl compounds, volatile organic acids and alcohols also contributed to the discrimination of samples co-fermented with different types of probiotics either at the end of fermentation or during storage. This observation suggests that the incorporation of stress-adapted probiotics may considerably influence the organoleptic quality of product. Therefore, in short-term perspective, additional research focusing on sensory evaluation of yoghurt with trained panelists is recommended.

The list of non-volatile polar metabolites identified in this study was derived from the previous work [29]. This list is comparable to what was found in other ^{1}H-NMR-based studies of liquid milk and cheese [2, 10, 32]. The numbers of significant variables (bin) filtered by ANOVA suggested that non-volatile metabolite profiles among the LGG groups (218 bins) were rather dissimilar compared to those of BB12 (164 bins). This was confirmed by the patterns of non-volatile metabolite profiles demonstrated by PCA. Non-volatile metabolite profiles among the LGG groups were clearly distinguished according to the differences in types of sublethally precultured probiotics as well as duration of storage (Fig. 4.5). Moreover, the separating patterns were quite comparable with those observed for the volatile metabolite profiles, i.e. standard precultured LGG was grouped nearby LGG precultured at 4.0% NaCl-pH6.5 while the other three sublethally precultured LGG were rather separated. A consistent discrimination was observed between the LGG precultured at 2.0% NaCl and the control group. This observation suggests that preculturing at 2% NaCl induces changes in the metabolic activity of LGG resulting in distinctive non-volatile metabolite profiles of set-yoghurt. Unlike LGG, a complete distinction of non-volatile metabolite profiles among the groups of BB12, either in different types of sublethally precultured cells or duration of storage,

was not achieved by the first PCA score plot (Fig. 4.6A). The distinction among the 4 h samples could still be observed while the stored samples were poorly distinguished from each other. Interestingly, non-volatile metabolite profiles of BB12 precultured at 1.5% NaCl were placed nearby (4 h) or grouped together (storage) with those of BB12 precultured at 0.5% NaCl-pH 5.0, although the capacity to grow in milk of the former group was definitely impaired. This result indicates that distinctive patterns of yoghurt metabolite profiles found by PCA do not directly correlate with the viable counts of stress-adapted probiotics.

4.5 Conclusions

The present study demonstrated that preculturing of LGG and BB12 under sublethal salt (NaCl) and pH stress conditions did not significantly enhance their growth during set-yoghurt fermentation. On the other hand, the survival of probiotics during refrigerated storage could be successfully improved specifically by preculturing at relatively low pH value. Preculturing at 2.0% NaCl-pH 4.5 and 0.5% NaCl-pH 5.0 provided the most significant improvement on the survival of LGG and BB12, respectively. A complementary metabolomics approach using SPME-GC/MS and ^{1}H-NMR combined with multivariate analysis revealed substantial impact of preculturing of probiotics on the formation of volatile and non-volatile polar metabolites in set-yoghurt. Moreover, various volatile aroma compounds indicated in loading plots suggested that incorporation of stress-adapted probiotics might considerably influence the organoleptic quality of yoghurt. The results demonstrate that adaptive responses of LGG and BB12 to sublethal salt and low pH stress conditions not only affect their survival during yoghurt production but also lead to substantial changes in the metabolome of the fermented product. This study provides new information on the application of stress-adapted probiotics in an actual food-carrier environment.

Supplementary data

Table S4.1-A. Volatile metabolites identified in set-yoghurts at the end of fermentation (4 hours) and the end of storage (28 days) fermented by co-culture of yoghurt starters with standard *L. rhamnosus* GG (LGG) and their sublethally precultured cells using headspace SPME-GC/MS

Chemical group	Compound	Standard LGG (Control)		Sublethally precultured LGG							
				2% NaCl + pH 4.5		2% NaCl + pH 6.5		4% NaCl + pH 4.5		4% NaCl + pH 6.5	
		4 h	28 d	4 h	28 d	4 h	28 d	4 h	28 d	4 h	28 d
Alcohol	1-Butanol	5.67[a]±0.31ab	5.51 ± 0.17a	5.81 ± 0.16b	5.89 ± 0.10b	6.26 ± 0.08c	6.06 ± 0.15b	5.61 ± 0.26ab	5.65 ± 0.16ab	5.82 ± 0.10b	5.77 ± 0.16ab
	Ethanol	6.73 ± 0.05a	6.77 ± 0.04ab	6.86 ± 0.00b	6.84 ± 0.04b	7.23 ± 0.01c	7.20 ± 0.01c	6.87 ± 0.05b	6.83 ± 0.04b	6.78 ± 0.04ab	6.80 ± 0.04ab
	2-Ethyl-hexanol	5.39 ± 0.03c	5.31 ± 0.08c	5.05 ± 0.09ab	5.07 ± 0.04ab	6.08 ± 0.02e	5.92 ± 0.02d	5.13 ± 0.03b	4.98 ± 0.05a	5.39 ± 0.03c	5.34 ± 0.05c
	1-Hexanol	5.69 ± 0.07b	5.80 ± 0.03c	5.70 ± 0.07b	5.72 ± 0.06b	5.59 ± 0.02a	5.55 ± 0.05a	5.85 ± 0.03c	5.72 ± 0.04b	5.81 ± 0.04c	5.76 ± 0.10bc
	1-Methoxy-2-propanol	5.45 ± 0.40c	4.97 ± 0.30bc	4.86 ± 0.27bc	ND[c]	5.15 ± 0.13c	ND	4.90 ± 0.14bc	4.68 ± 0.32ab	5.24 ± 0.40b	4.36 ± 0.20a
	2-Methyl-1-butanol	5.72 ± 0.05bc	5.61 ± 0.08b	5.24 ± 0.19a	5.91 ± 0.08d	5.66 ± 0.08bc	5.75±0.17bcd	5.77 ± 0.08cd	5.49±0.30abc	5.62±0.14abc	5.38 ± 0.16ab
	3-Methyl-2-butanol	5.73 ± 0.09ab	5.87 ± 0.02c	5.82±0.10abc	5.91 ± 0.03cd	5.81 ± 0.01ab	5.81 ± 0.02ab	5.83 ± 0.02b	5.97 ± 0.03d	5.69 ± 0.12a	5.96 ± 0.12cd
	3-Methyl-3-butanol	5.77 ± 0.90a	5.83 ± 0.03ab	5.87±0.09abc	5.92 ± 0.05bc	5.87 ± 0.02b	5.94 ± 0.02c	5.91 ± 0.04bc	5.87±0.06abc	5.73 ± 0.11a	5.82±0.11abc
	1-Octanol	5.03±0.09bcd	5.03±0.06bcd	5.03±0.07bcd	5.11 ± 0.02d	4.94 ± 0.06b	5.04 ± 0.04c	4.88 ± 0.10ab	5.09 ± 0.04cd	4.83 ± 0.02a	4.99 ± 0.07bc
	1-Pentanol	5.39 ± 0.09a	5.52 ± 0.12a	5.45 ± 0.08a	5.36 ± 0.07a	5.38 ± 0.09a	5.38 ± 0.09a	5.48 ± 0.04a	5.34 ± 0.07a	5.52 ± 0.10a	5.34 ± 0.15a
	3-Pentanol	6.67 ± 0.11ab	6.78 ± 0.02bc	6.79 ± 0.04bc	6.93 ± 0.03d	6.72 ± 0.02b	6.87 ± 0.03cd	6.73 ± 0.07b	6.83 ± 0.02c	6.50 ± 0.09a	6.76 ± 0.07bc
Carbonyl compound	Acetaldehyde	7.37±0.10abc	7.45 ± 0.11bc	7.49 ± 0.02c	7.47 ± 0.03c	7.37 ± 0.00b	7.48 ± 0.05c	7.29 ± 0.08a	7.39 ± 0.07ab	7.25 ± 0.02a	7.28 ± 0.01a
	Acetoin	8.50 ± 0.07b	8.46 ± 0.02b	8.48 ± 0.03b	8.47 ± 0.01b	8.48 ± 0.02b	8.43 ± 0.04ab	8.47 ± 0.02b	8.44 ± 0.05ab	8.37 ± 0.03a	8.37 ± 0.02a
	Acetone	7.47 ± 0.06bc	7.52 ± 0.06c	7.43 ± 0.04bc	7.50 ± 0.05c	7.34 ± 0.03a	7.46 ± 0.02c	7.43±0.07abc	7.43 ± 0.04bc	7.41 ± 0.01b	7.47 ± 0.02bc
	Benzaldehyde	6.20±0.13abc	6.40 ± 0.06c	6.17 ± 0.04a	6.25 ± 0.01b	6.20 ± 0.04ab	6.28±0.07abc	6.13 ± 0.07a	6.18 ± 0.09ab	6.33 ± 0.08ab	6.30 ± 0.08bc
	2-Butanone	7.38 ± 0.21a	7.38 ± 0.17a	7.28 ± 0.12a	7.27 ± 0.12a	7.12 ± 0.05a	7.18 ± 0.13a	7.26 ± 0.10a	7.21 ± 0.12a	7.41 ± 0.07a	7.40 ± 0.11a
	Diacetyl	7.47 ± 0.07bc	7.54 ± 0.06bc	7.45 ± 0.06b	7.67 ± 0.11c	7.64 ± 0.04c	7.51 ± 0.07bc	7.55 ± 0.04bc	7.69 ± 0.13c	7.37 ± 0.02a	7.47±0.10abc
	2-Heptanone	6.11 ± 0.08ab	6.42 ± 0.06c	6.30 ± 0.06c	6.56 ± 0.06d	6.31 ± 0.01c	6.59 ± 0.05d	6.18 ± 0.01b	6.34 ± 0.06c	6.07 ± 0.05a	6.36 ± 0.14c
	2-Hydroxy-3-pentanone	6.45±0.13abc	6.53 ± 0.03b	6.58 ± 0.04bc	6.69 ± 0.02d	6.53 ± 0.02b	6.65 ± 0.03cd	6.52 ± 0.07b	6.61 ± 0.02c	6.29 ± 0.10a	6.54 ± 0.08bc
	3-Methyl-2-butenal	5.41±0.22abc	5.52 ± 0.12ab	5.57 ± 0.03b	5.77 ± 0.03d	5.67 ± 0.03c	5.94 ± 0.06e	5.46 ± 0.06ab	5.51 ± 0.15ab	5.28 ± 0.15a	5.42 ± 0.11ab
	2-Nonanone	6.17 ± 0.04ab	6.34 ± 0.05b	6.39 ± 0.06b	6.53 ± 0.01d	6.51 ± 0.02c	6.62 ± 0.02e	6.27 ± 0.05b	6.33 ± 0.02b	6.13 ± 0.06a	6.33 ± 0.08b
	3-Octanone	5.35 ± 0.07c	5.26 ± 0.02b	5.35 ± 0.07c	5.14 ± 0.12ab	5.26 ± 0.07bc	5.08 ± 0.15ab	5.26 ± 0.08bc	5.16 ± 0.18ab	5.14 ± 0.05ab	5.07 ± 0.08a

	2,3-Pentanedione	6.92 ± 0.12ab	7.53 ± 0.08e	7.02 ± 0.09b	7.33 ± 0.06d	7.15 ± 0.11bc	7.22 ± 0.04c	7.03 ± 0.10b	7.27 ± 0.05cd	6.88 ± 0.09a	7.36 ± 0.08de
	2-Undecanal	5.14 ± 0.08bc	5.23 ± 0.06c	4.90 ± 0.14ab	5.22 ± 0.06c	5.01 ± 0.15b	5.14 ± 0.14bc	5.04 ± 0.11b	4.99±0.30abc	4.59 ± 0.17a	5.18 ± 0.05bc
	2-Undecanone	5.46 ± 0.06ab	5.43 ± 0.04a	5.44 ± 0.06ab	5.59 ± 0.07bc	5.62 ± 0.06bc	5.61 ± 0.02c	5.58 ± 0.03bc	5.57 ± 0.01bc	5.46 ± 0.14ab	5.53 ± 0.04b
Sulfur compound	Dimethyl disulfide	5.19 ± 0.30a	6.48 ± 0.35c	5.21 ± 0.13a	5.90 ± 0.12b	5.13 ± 0.08a	6.22 ± 0.13c	5.22 ± 0.34a	5.57 ± 0.52ab	5.19 ± 0.13a	5.97 ± 0.15bc
	Dimethyl sulfide	6.22 ± 0.13a	6.08 ± 0.83a	6.13 ± 0.02a	5.92 ± 0.31a	5.73 ± 0.06a	6.11 ± 0.04a	6.04 ± 0.20a	5.46 ± 1.11a	6.13 ± 0.10a	6.04 ± 0.33a
	Dimethyl sulfone	6.27 ± 0.18ab	6.66 ± 0.04c	6.30 ± 0.15ab	6.53 ± 0.07b	6.10 ± 0.24a	6.56 ± 0.12bc	6.17 ± 0.12a	6.50 ± 0.08b	6.32 ± 0.21ab	6.53±0.22abc
Volatile organic acid	Acetic acid	7.66 ± 0.08a	8.05 ± 0.08cd	7.93 ± 0.06bc	8.11 ± 0.01d	7.88 ± 0.05b	8.17 ± 0.07d	7.67 ± 0.04a	7.95 ± 0.01b	7.54 ± 0.18a	8.96 ± 0.02bc
	Butyric acid	7.61 ± 0.10ab	7.75 ± 0.07cd	7.82 ± 0.04d	7.78 ± 0.03cd	7.71 ± 0.02b	7.76 ±0.08a-d	7.66 ± 0.02a	7.72 ± 0.03bc	7.57 ± 0.11ab	7.71 ± 0.04bc
	Hexanoic acid	7.61 ± 0.10ab	7.63 ± 0.05ab	7.76 ± 0.03c	7.74 ± 0.02c	7.71 ± 0.02bc	7.72 ± 0.07bc	7.69 ± 0.01b	7.70 ± 0.03bc	7.58 ± 0.03a	7.70 ± 0.06bc
	3-Methyl-butanoic acid	5.60 ± 0.05ab	5.66 ± 0.04bc	5.72 ± 0.03c	5.75 ± 0.03c	5.66 ± 0.04b	5.73 ± 0.10bc	5.64 ± 0.03b	5.66 ± 0.02b	5.51 ± 0.08a	5.69 ± 0.07bc
	2-Methyl-propanoic acid	5.43 ± 0.05a	5.55 ± 0.03bc	5.60 ± 0.03c	5.61 ± 0.01c	5.54 ± 0.06bc	5.62 ± 0.08bc	5.44 ± 0.05a	5.56 ± 0.00b	5.38 ± 0.09a	5.51 ± 0.06bc
	Pentanoic acid	5.95 ± 0.09ab	6.01 ± 0.03b	6.07 ± 0.01c	6.10 ± 0.02c	5.99 ± 0.03b	6.05 ± 0.05bc	5.94 ± 0.02a	6.03±0.07abc	5.91 ± 0.07ab	5.98 ± 0.01b
	Propionic acid	5.85 ± 0.02a	6.06 ± 0.01d	5.97 ± 0.05bc	6.05 ± 0.03cd	5.92 ± 0.06ab	5.97±0.07bcd	5.89 ± 0.05ab	6.01±0.08bcd	5.81 ± 0.17ab	5.96 ± 0.01b

[a] Metabolite contents are expressed as $\log_{10}$ [peak area of respective compound in arbitrary unit]. Values are means ± standard deviation from three independent replicates.
[b] Letters (a-e) indicate significant difference ($p < 0.05$) among sample means within the same row.
[c] ND indicates compound not detected.

Table S4.1-B. Volatile metabolites identified in set-yoghurts at the end of fermentation (4 hours) and the end of storage (28 days) fermented by co-culture of yoghurt starters with standard *B. animalis* subsp. *lactis* BB12 (BB12) and their sublethally precultured cells using headspace SPME-GC/MS

Chemical group	Compound	Standard BB12 (Control)		Sublethally precultured BB12							
				0.5% NaCl + pH 5.0		0.5% NaCl + pH 7.0		1.5% NaCl + pH 5.0		1.5% NaCl + pH 7.0	
		4 h	28 d	4 h	28 d	4 h	28 d	4 h	28 d	4 h	28 d
Alcohol	1-Butanol	5.60[a] ±0.15a[b]	5.40 ± 0.29a	5.52 ± 0.16a	5.50 ± 0.26a	5.67 ± 0.32a	5.48 ± 0.18a	5.33 ± 0.16a	5.53 ± 0.25a	5.29 ± 0.21a	5.42 ± 0.16a
	Ethanol	6.83 ± 0.02a	6.84 ± 0.04a	6.97 ± 0.06bc	7.08 ± 0.03c	6.84 ± 0.05a	6.96 ± 0.01b	7.01 ± 0.06bc	7.05 ± 0.01c	6.84 ± 0.05a	6.97 ± 0.05bc
	2-Ethyl-hexanol	5.84 ± 0.01d	5.76 ± 0.04c	5.54 ± 0.06b	5.43 ± 0.02a	5.53 ± 0.07ab	5.40 ± 0.00a	5.50 ± 0.11ab	5.48 ± 0.02b	5.37 ± 0.08a	5.40 ± 0.05a
	1-Hexanol	5.82 ± 0.05a	5.80 ± 0.06a	5.79 ± 0.01a	5.71 ± 0.02a	5.80 ± 0.05a	5.79 ± 0.03a	5.76 ± 0.09a	5.71 ± 0.02a	5.75 ± 0.05a	5.77 ± 0.04a
	1-Methoxy-2-propanol	5.70±0.42bcd	4.48 ± 0.51a	5.97 ± 0.09d	5.47 ± 0.10c	ND[c]	5.25 ± 0.12b	5.42 ± 0.10bc	5.43 ± 0.26bc	5.15±0.25abc	4.70 ± 0.37a
	2-Methyl-1-butanol	5.33 ± 0.07d	4.95 ± 0.04ab	5.11±0.15a-d	5.06 ± 0.06bc	5.26 ± 0.08cd	5.12 ± 0.08c	5.01 ± 0.01b	4.88 ± 0.08a	4.94 ± 0.04a	5.08±0.11abc
	3-Methyl-2-butanol	5.85 ± 0.03b	5.97 ± 0.03c	5.88 ± 0.03b	6.20 ± 0.02e	5.81 ± 0.09ab	6.09 ± 0.02d	5.80 ± 0.06ab	6.10 ± 0.08de	5.69 ± 0.08a	6.10 ± 0.05d
	3-Methyl-3-butanol	5.93 ± 0.03b	5.97 ± 0.06bc	6.04 ± 0.04cd	6.13 ± 0.06e	5.91 ± 0.04ab	6.02 ± 0.03c	5.92±0.10abc	6.06 ± 0.01d	5.79 ± 0.08a	5.97 ± 0.04bc
	1-Octanol	5.12 ± 0.03a	5.14 ± 0.02a	5.11 ± 0.04a	5.03 ± 0.04a	5.08 ± 0.03a	5.03 ± 0.03a	5.07 ± 0.13a	5.02 ± 0.01a	5.07 ± 0.08a	5.14 ± 0.02a
	1-Pentanol	5.50 ± 0.02c	5.59 ± 0.04d	5.52 ± 0.03cd	5.39 ± 0.04ab	5.50 ± 0.02bc	5.46 ± 0.04b	5.44 ± 0.05ab	5.40 ± 0.06ab	5.42 ± 0.04ab	5.39 ± 0.02a
	3-Pentanol	6.78 ± 0.06bc	6.84 ± 0.03c	6.62 ± 0.02a	6.78 ± 0.02b	6.76 ± 0.03b	6.89 ± 0.02d	6.69±0.13abc	6.81±0.08bcd	6.62 ± 0.02a	6.84±0.07bcd
Carbonyl compound	Acetaldehyde	7.49 ± 0.03a	7.55 ± 0.03a	7.45 ± 0.06a	7.46 ± 0.08a	7.42 ± 0.05a	7.49 ± 0.06a	7.51 ± 0.00a	7.52 ± 0.04a	7.43 ± 0.09a	7.50 ± 0.03a
	Acetoin	8.60 ± 0.04bc	8.56 ± 0.01b	8.55 ± 0.03ab	8.59 ± 0.06bc	8.63 ± 0.04c	8.57 ± 0.03bc	8.70 ± 0.14c	8.53 ± 0.01a	8.53 ± 0.01a	8.54 ± 0.05ab
	Acetone	7.53 ± 0.06a	7.56 ± 0.04a	7.49 ± 0.01a	7.50 ± 0.04a	7.53 ± 0.08a	7.55 ± 0.02a	7.50 ± 0.02a	7.51 ± 0.06a	7.46 ± 0.05a	7.56 ± 0.02a
	Benzaldehyde	6.19 ± 0.04b	6.43 ± 0.01d	6.16 ± 0.04b	6.23 ± 0.08bc	6.18 ± 0.03b	6.32 ± 0.04c	6.09 ± 0.06ab	6.20 ± 0.02b	6.10 ± 0.03a	6.23±0.13abc
	2-Butanone	7.61 ± 0.06d	7.58 ± 0.06d	7.04 ± 0.14ab	6.85 ± 0.09a	7.60 ± 0.15d	7.55 ± 0.05d	7.12 ± 0.11b	6.93 ± 0.07a	7.45 ± 0.06cd	7.42 ± 0.03c
	Diacetyl	7.51 ± 0.01bc	7.59 ± 0.02d	7.41 ± 0.04a	7.60 ± 0.07cd	7.53 ± 0.02c	7.58±0.08bcd	7.47±0.07abc	7.58 ± 0.05cd	7.45 ± 0.04a	7.56±0.06bcd
	2-Heptanone	6.20 ± 0.09a	6.45 ± 0.03b	6.17 ± 0.06a	6.41 ± 0.04b	6.10 ± 0.02a	6.43 ± 0.03b	6.13 ± 0.07a	6.43 ± 0.02b	6.09 ± 0.06a	6.45 ± 0.02b
	2-Hydroxy-3-pentanone	6.57 ± 0.05bc	6.62 ± 0.03c	6.41 ± 0.04a	6.53 ± 0.04b	6.55 ± 0.03b	6.65 ± 0.02c	6.48±0.15abc	6.56 ± 0.06bc	6.39 ± 0.02a	6.60 ± 0.07bc
	3-Methyl-2-butenal	5.46±0.22abc	5.68 ± 0.03c	5.54 ± 0.03b	5.58 ± 0.06b	5.43 ± 0.05a	5.63 ± 0.08bc	5.40 ± 0.08a	5.62 ± 0.08bc	5.40 ± 0.02a	5.57 ± 0.10bc
	2-Nonanone	6.18 ± 0.02b	6.32 ± 0.01d	6.17±0.05abc	6.27 ± 0.05c	6.15 ± 0.03ab	6.32 ± 0.04cd	6.19±0.11abc	6.33 ± 0.03cd	6.13 ± 0.02a	6.33 ± 0.06cd
	3-Octanone	5.52 ± 0.05cd	5.41 ± 0.02b	5.46 ± 0.03bc	5.45 ± 0.08bc	5.59 ± 0.05d	5.40 ± 0.07ab	5.58±0.16bcd	5.33 ± 0.05a	5.38 ± 0.06ab	5.28±0.11abc

	2,3-Pentanedione	6.92 ± 0.05a	7.55 ± 0.05c	6.83± 0.07a	7.29 ± 0.02b	6.89 ± 0.05a	7.33 ± 0.04b	6.77 ± 0.08a	7.34 ± 0.02b	6.87 ± 0.08a	7.28 ± 0.08b
	2-Undecanal	5.49 ± 0.10bc	5.62 ± 0.21c	5.52 ± 0.14c	5.43 ± 0.07bc	5.37 ± 0.16bc	5.44 ± 0.06bc	5.36 ± 0.14bc	5.38 ± 0.03b	5.16 ± 0.05a	5.35±0.19abc
	2-Undecanone	5.39 ± 0.07a	5.30 ± 0.04a	5.47 ± 0.07a	5.36 ± 0.01a	5.44 ± 0.05a	5.40 ± 0.01a	5.44 ± 0.25a	5.40 ± 0.02a	5.35 ± 0.04a	5.40 ± 0.02a
Sulfur compound	Dimethyl disulfide	5.22 ± 0.20a	6.61 ± 0.42c	5.29 ± 0.11a	5.79 ± 0.28b	5.24 ± 0.12a	6.09 ± 0.17bc	5.13 ± 0.09a	5.81 ± 0.28b	5.22 ± 0.12a	6.14 ± 0.12bc
	Dimethyl sulfide	6.44 ± 0.26a	6.12 ± 0.95a	6.55 ± 0.19a	5.97 ± 1.30a	6.49 ± 0.26a	6.14 ± 1.10a	6.68 ± 0.12a	6.24 ± 1.07a	6.59 ± 0.26a	6.95 ± 0.04a
	Dimethyl sulfone	6.41 ± 0.21a	6.77 ± 0.04b	6.23 ± 0.36a	6.79 ± 0.13b	6.48 ± 0.25ab	6.68 ± 0.05b	6.76 ± 0.22b	6.76 ± 0.13b	6.54 ± 0.26ab	6.71 ± 0.14b
Volatile organic acid	Acetic acid	7.88 ± 0.16a	8.21 ± 0.03b	7.90 ± 0.07a	8.44 ± 0.11d	7.87 ± 0.09a	8.18 ± 0.05b	8.30 ± 0.01c	8.29 ± 0.05c	7.94 ± 0.21a	8.22 ± 0.08bc
	Butyric acid	7.70 ± 0.13ab	7.83 ± 0.00b	7.58 ± 0.10a	7.89 ± 0.08bc	7.71 ± 0.06a	7.83 ± 0.04bc	7.98 ± 0.12c	7.81 ± 0.04ab	7.75±0.14abc	7.80±0.08abc
	Hexanoic acid	7.62 ± 0.07ab	7.71 ± 0.02b	7.53 ± 0.09a	7.74 ± 0.06b	7.63 ± 0.03a	7.73 ± 0.02b	7.78 ± 0.10b	7.69 ± 0.03b	7.63 ± 0.08ab	7.70 ± 0.07ab
	3-Methyl-butanoic acid	5.56±0.10abc	5.73 ± 0.01d	5.46 ± 0.06a	5.70 ± 0.07cd	5.57 ± 0.04b	5.71 ± 0.02cd	5.77 ± 0.11cd	5.66 ± 0.04c	5.60±0.10abc	5.71±0.12bcd
	2-Methyl-propanoic acid	5.47 ± 0.07ab	5.64 ± 0.06c	5.37 ± 0.09a	5.57 ± 0.05bc	5.40 ± 0.04a	5.63 ± 0.12bc	5.64 ± 0.14bc	5.55 ± 0.02b	5.50±0.12abc	5.58 ± 0.06bc
	Pentanoic acid	5.97 ± 0.05a	6.06 ± 0.04bc	5.95 ± 0.04a	6.10 ± 0.06bc	6.01 ± 0.03ab	6.10 ± 0.02c	6.13 ± 0.07bc	6.04 ± 0.01b	5.97 ± 0.09ab	6.04±0.10abc
	Propionic acid	5.86 ± 0.09a	6.07 ± 0.04b	5.79 ± 0.10a	6.02 ± 0.05b	5.84 ± 0.08a	6.01 ± 0.07b	6.00 ± 0.10ab	6.02 ± 0.06b	5.88 ± 0.08a	6.01 ± 0.11ab

[a] Metabolite contents are expressed as $\log_{10}$ [peak area of respective compound in arbitrary unit]. Values are means ± standard deviation from three independent replicates.

[b] Letters (a-e) indicate significant difference ($p < 0.05$) among sample means within the same row.

[c] ND indicates compound not detected.

Table S4.2-A. Presumptive polar metabolites identified in set-yoghurts (samples at 4 hours, 14 days and 28 days) fermented by co-culture of yoghurt starters with standard *L. rhamnosus* GG (LGG) and their sublethally precultured cells using NOESY 1D-^{1}H-NMR

Chemical group	Compound	Standard LGG (Control)			Sublethally precultured LGG											
					2% NaCl + pH 4.5			2% NaCl + pH 6.5			4% NaCl + pH 4.5			4% NaCl + pH 6.5		
		4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d
Amino acid and derivatives	Alanine	6.86[a]	6.91	7.06	6.90	6.98	7.07	6.94	6.92	7.02	6.99	7.05	7.12	7.03	7.12	7.12
	Creatine and Creatinine	7.43	7.42	7.60	7.48	7.49	7.53	7.50	7.50	7.50	7.52	7.52	7.56	7.53	7.56	7.55
	Isoleucine	7.54	7.61	7.81	7.61	7.73	7.83	7.65	7.64	7.79	7.72	7.79	7.88	7.74	7.87	7.86
	Leucine	7.53	7.56	7.81	7.60	7.72	7.84	7.64	7.65	7.79	7.72	7.80	7.90	7.76	7.89	7.88
	N-Acetyl-amino acids	7.92	7.92	8.04	7.95	7.99	8.05	8.00	7.99	8.02	8.04	8.04	8.08	8.06	8.10	8.09
	Phenylalanine	6.47	6.49	6.69	6.53	6.64	6.73	6.56	6.53	6.68	6.63	6.70	6.95	6.71	6.83	6.82
	Proline	7.29	7.31	7.46	7.32	7.39	7.48	7.35	7.32	7.43	7.41	7.45	7.51	7.44	7.51	7.50
	Tyrosine	6.86	6.89	7.03	6.93	6.99	7.06	6.94	6.87	7.01	6.99	7.03	7.10	7.01	7.10	7.08
	Valine	7.40	7.44	7.64	7.50	7.60	7.70	7.50	7.49	7.64	7.58	7.65	7.74	7.61	7.73	7.72
	Amino acid residues	7.95	8.01	8.15	8.01	8.09	8.18	8.04	8.03	8.14	8.08	8.13	8.20	8.12	8.19	8.20
Carbohydrate and derivatives	Galactose	9.04	8.95	9.01	9.08	9.03	9.05	9.09	8.93	9.00	9.14	9.02	9.04	9.15	9.07	9.05
	Glucose	9.10	8.64	8.70	9.09	8.84	8.78	9.13	8.84	8.65	9.22	8.81	8.87	9.23	8.93	8.85
	Lactose	9.27	8.91	8.95	9.23	9.21	9.18	9.31	9.32	9.22	9.22	9.19	9.12	9.15	9.10	9.09
	N-Acetylglucosamine	7.36	7.36	7.54	7.41	7.46	7.55	7.47	7.49	7.52	7.51	7.54	7.60	7.54	7.60	7.61
	Sugar residues	7.32	7.27	7.32	7.34	7.31	7.33	7.38	7.34	7.30	7.37	7.33	7.33	7.40	7.34	7.31
Organic acid	Acetate	7.58	7.71	7.77	7.61	7.76	7.81	7.70	7.64	7.81	7.67	7.75	7.79	7.61	7.74	7.75
	Acetoacetate	6.91	6.88	7.09	6.96	7.01	7.11	7.02	7.00	7.06	7.07	7.08	7.16	7.09	7.15	7.15
	Ascorbate	8.25	8.13	8.18	8.24	8.19	8.21	8.26	8.14	8.15	8.31	8.19	8.21	8.31	8.23	8.21
	Benzoate	6.79	6.80	6.93	6.84	6.89	6.94	6.91	6.89	6.93	6.93	6.94	7.06	6.94	6.98	6.98
	Butyrate	7.18	7.21	7.34	7.27	7.36	7.44	7.30	7.27	7.38	7.36	7.41	7.49	7.39	7.49	7.46
	Hydroxybutyrate	7.22	7.22	7.48	7.26	7.38	7.50	7.37	7.38	7.48	7.44	7.48	7.56	7.46	7.55	7.56

	Citrate	7.50	7.51	7.55	7.62	7.52	7.58	7.53	7.53	7.53	7.55	7.54	7.62	7.68	7.61	7.60
	Formate	6.74	6.72	6.80	6.87	6.86	6.92	6.83	6.75	6.84	6.93	6.94	7.00	6.96	7.00	6.95
	Fumarate	5.50	5.48	5.62	5.44	5.42	5.43	5.57	5.97	5.45	5.54	5.53	5.51	5.64	5.52	5.50
	Hippurate	7.03	7.01	7.24	7.07	7.14	7.25	7.16	7.16	7.22	7.22	7.23	7.33	7.25	7.31	7.31
	Isobutyrate	6.57	6.60	6.77	6.67	6.73	6.81	6.64	6.66	6.74	6.72	6.79	6.88	6.78	6.89	6.86
	Lactate	9.45	9.62	9.64	9.49	9.58	9.63	9.41	9.47	9.60	9.46	9.55	9.62	9.50	9.59	9.61
	Orotate	6.47	6.43	6.44	6.54	6.50	6.46	6.54	6.59	6.45	6.57	6.50	6.51	6.58	6.51	6.46
	Oxoglutarate	7.36	7.32	7.38	7.49	7.22	7.34	7.34	7.29	7.28	7.34	7.27	7.54	7.64	7.57	7.58
	Pyruvate	7.50	7.53	7.57	7.29	7.17	7.25	7.33	7.23	7.21	7.36	7.23	7.29	7.37	7.32	7.39
	Succinate	7.45	7.56	7.60	7.46	7.68	7.66	7.61	7.49	7.65	7.61	7.65	7.50	7.21	7.48	7.43
	Valerate and derivatives	7.55	7.54	7.77	7.59	7.67	7.78	7.68	7.68	7.75	7.74	7.76	7.84	7.77	7.83	7.84
Lipid derivatives	Acetylcarnitine	6.63	6.66	6.79	6.74	6.75	6.83	6.81	6.89	6.81	6.76	6.82	6.88	6.82	6.85	6.84
	Choline and derivatives	7.73	7.73	7.82	7.88	7.82	7.84	7.88	7.79	7.78	7.93	7.81	7.85	7.95	7.87	7.86
	Glycerophosphocholine	7.39	7.32	7.34	7.51	7.39	7.41	7.62	7.46	7.40	7.72	7.49	7.44	7.62	7.47	7.45
	Phosphocholine	8.08	7.80	7.89	8.11	7.95	7.95	8.11	7.90	7.85	8.19	7.93	7.98	8.17	8.01	7.97
Carbonyl compound	Acetone	7.22	7.21	7.28	7.35	7.30	7.32	7.32	7.30	7.32	7.32	7.29	7.32	7.40	7.33	7.32
	Dihydroxyacetone	7.25	7.25	7.29	7.45	7.28	7.30	7.44	7.43	7.23	7.49	7.29	7.34	7.49	7.36	7.32
Miscellaneous	Dimethyl sulfone	6.96	6.75	6.84	6.88	6.86	6.89	6.90	6.92	6.86	6.93	6.89	6.94	6.94	6.92	6.91
	Uridine	5.91	5.90	5.95	5.93	5.89	5.97	5.99	5.95	5.96	5.97	5.96	5.98	6.01	5.94	5.96

[a] Metabolite contents are expressed as $\log_{10}$ [sum of signal intensity of respective metabolite in arbitrary unit]. Values at 4 hours are the average from two independent replicates. Values at 14 days and 28 days are represented from one replicate.

Table S4.2-B. Presumptive polar metabolites identified in set-yoghurts (samples at 4 hours, 14 days and 28 days) fermented by co-culture of yoghurt starters with standard *B. animalis* subsp. *lactis* BB12 (BB12) and their sublethally precultured cells using NOESY 1D-^{1}H-NMR

Chemical group	Compound	Standard BB12 (Control)			Sublethally precultured BB12											
					0.5% NaCl + pH 5.0			0.5% NaCl + pH 7.0			1.5% NaCl + pH 5.0			1.5% NaCl + pH 7.0		
		4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d
Amino acid and derivatives	Alanine	6.93[a]	6.94	7.01	6.67	7.03	7.10	6.90	6.78	6.82	6.87	7.09	6.31	6.71	7.06	7.06
	Creatine and Creatinine	7.50	7.44	7.49	7.40	7.53	7.56	7.47	7.38	7.37	7.47	7.54	7.34	7.41	7.53	7.54
	Isoleucine	7.63	7.66	7.72	7.35	7.75	7.83	7.60	7.42	7.54	7.58	7.79	6.93	7.43	7.79	7.81
	Leucine	7.65	7.67	7.74	7.34	7.76	7.87	7.60	7.36	7.53	7.58	7.82	6.98	7.39	7.83	7.85
	N-Acetyl-amino acids	8.01	7.96	7.99	7.85	8.05	8.09	7.98	7.85	7.88	7.97	8.06	7.82	7.90	8.04	8.06
	Phenylalanine	6.61	6.64	6.67	6.33	6.68	6.80	6.56	6.38	6.48	6.53	6.75	5.89	6.27	6.77	6.79
	Proline	7.33	7.32	7.38	7.09	7.42	7.47	7.31	7.14	7.21	7.30	7.43	6.83	7.17	7.42	7.44
	Tyrosine	6.95	6.95	6.99	6.75	7.00	7.08	6.93	6.80	6.84	6.90	7.05	6.05	6.75	7.03	7.05
	Valine	7.53	7.55	7.60	7.27	7.61	7.71	7.49	7.30	7.40	7.47	7.67	6.71	7.26	7.67	7.69
	Amino acid residues	8.01	8.04	8.08	7.83	8.12	8.17	8.00	7.89	7.96	7.98	8.12	7.28	7.91	8.12	8.14
Carbohydrate and derivatives	Galactose	9.09	8.94	8.97	9.01	9.03	9.09	9.11	8.97	8.94	9.13	9.06	8.34	8.88	9.04	9.04
	Glucose	9.18	8.80	8.82	9.02	8.81	8.94	9.13	8.66	8.61	9.18	9.03	8.32	8.65	8.91	8.87
	Lactose	9.23	8.99	9.03	9.23	9.26	9.17	9.17	9.23	9.05	9.29	9.13	9.38	9.38	9.23	9.23
	N-Acetylglucosamine	7.44	7.41	7.47	7.24	7.53	7.58	7.42	7.24	7.31	7.40	7.54	7.14	7.32	7.53	7.54
	Sugar residues	7.35	7.26	7.25	7.25	7.33	7.34	7.33	7.22	7.19	7.35	7.36	7.21	7.25	7.31	7.29
Organic acid	Acetate	7.61	7.66	7.69	7.45	7.67	7.72	7.58	7.61	7.63	7.58	7.64	6.80	7.48	7.67	7.71
	Acetoacetate	6.98	6.95	7.01	6.73	7.05	7.13	6.94	6.68	6.82	6.93	7.10	6.50	6.82	7.09	7.11
	Ascorbate	8.31	8.15	8.17	8.17	8.19	8.24	8.26	8.09	8.08	8.27	8.22	7.70	8.04	8.20	8.20
	Benzoate	6.86	6.85	6.86	6.75	6.90	6.94	6.86	6.75	6.77	6.83	6.90	6.23	6.78	6.91	6.92
	Butyrate	7.27	7.26	7.29	7.04	7.38	7.45	7.27	7.09	7.16	7.24	7.43	6.85	7.05	7.40	7.42
	Hydroxybutyrate	7.32	7.30	7.40	7.02	7.42	7.51	7.27	7.00	7.20	7.24	7.44	6.86	7.17	7.46	7.49

	Citrate	7.57	7.52	7.49	7.41	7.55	7.58	7.51	7.38	7.41	7.49	7.52	7.77	7.51	7.50	7.48
	Formate	6.82	6.74	6.76	6.62	6.95	6.99	6.86	6.70	6.72	6.86	6.96	6.05	6.73	6.92	6.94
	Fumarate	5.60	5.56	5.60	5.40	5.43	5.45	5.42	5.25	5.22	5.43	5.44	5.46	5.38	5.44	5.40
	Hippurate	7.11	7.07	7.13	6.80	7.20	7.28	7.07	6.75	6.92	7.05	7.25	6.78	6.95	7.23	7.25
	Isobutyrate	6.72	6.72	6.77	6.48	6.77	6.89	6.67	6.52	6.57	6.65	6.90	5.68	6.35	6.87	6.87
	Lactate	9.45	9.63	9.63	9.39	9.58	9.60	9.47	9.57	9.56	9.17	9.44	9.48	9.40	9.55	9.57
	Orotate	6.55	6.48	6.45	6.50	6.49	6.50	6.48	6.45	6.40	6.50	6.51	6.63	6.48	6.51	6.50
	Oxoglutarate	7.31	7.20	7.23	7.15	7.49	7.59	7.50	7.17	7.12	7.51	7.56	6.92	7.19	7.50	7.41
	Pyruvate	7.65	7.25	7.29	7.27	7.32	7.36	7.37	7.26	7.30	7.36	7.34	6.67	7.36	7.40	7.44
	Succinate	7.63	7.62	7.62	7.55	7.53	7.47	7.46	7.59	7.57	7.39	7.45	6.69	7.55	7.51	7.58
	Valerate and derivatives	7.63	7.62	7.69	7.35	7.72	7.79	7.60	7.33	7.48	7.57	7.76	7.08	7.48	7.74	7.76
Lipid derivatives	Acetylcarnitine	6.68	6.69	6.69	6.59	6.79	6.79	6.68	6.58	6.57	6.67	6.80	6.81	6.72	6.79	6.77
	Choline and derivatives	7.83	7.78	7.80	7.81	7.85	7.90	7.91	7.75	7.73	7.91	7.87	7.48	7.70	7.86	7.87
	Glycerophosphocholine	7.45	7.32	7.32	7.21	7.36	7.38	7.29	7.21	7.23	7.29	7.34	7.17	7.25	7.34	7.36
	Phosphocholine	8.18	7.89	7.91	8.00	7.93	8.03	8.08	7.77	7.77	8.11	8.06	7.25	7.73	7.98	7.98
Carbonyl compound	Acetone	7.25	7.25	7.26	7.14	7.29	7.32	7.27	7.19	7.17	7.28	7.29	6.26	7.19	7.27	7.28
	Dihydroxyacetone	7.32	7.27	7.27	7.33	7.34	7.38	7.40	7.17	7.15	7.41	7.41	7.13	7.20	7.38	7.35
Miscellaneous	Dimethyl sulfone	7.08	6.87	6.88	6.78	6.88	6.90	6.87	6.75	6.73	6.85	6.88	6.82	6.78	6.89	6.89
	Uridine	5.94	5.84	5.85	5.84	5.95	5.95	5.94	5.86	5.82	5.96	5.98	6.11	5.89	5.94	5.96

[a] Metabolite contents are expressed as $\log_{10}$ [sum of signal intensity of respective metabolite in arbitrary unit]. Values at 4 hours are the average from two independent replicates. Values at 14 days and 28 days are represented from one replicate.

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Chapter 5

Incorporation of precultured *Lactobacillus plantarum* WCFS1 in set-yoghurt: Impact on the survival of yoghurt starters, post-acidification and metabolite formation

Based on:
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Abstract

The objectives of this study were to evaluate the growth of *Lactobacillus plantarum* WCFS1 (LP-WCFS1) in co-culture with yoghurt starters and investigate the impact of preculturing on its survival and metabolite formation in set-yoghurt. The LP-WCFS1 was precultured under sublethal stress conditions (combinations of elevated NaCl and low pH) before inoculation in milk. Adaptive responses of LP-WCFS1 were evaluated by monitoring changes in bacterial populations, acidification, volatile and non-volatile metabolite profiles of set-yoghurts. The results demonstrated that sublethal preculturing did not significantly affect the growth and survival of LP-WCFS1. Alternatively, incorporation of sublethally precultured LP-WCFS1 significantly impaired the survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* which consequently reduced the post acidification of yoghurt during refrigerated. A complementary metabolomics approach using SPME-GC/MS and ^{1}H-NMR combined with multivariate analysis revealed substantial impact of LP-WCFS1 on metabolite profiles of set-yoghurts. This study provides insight in the technological implications of potential probiotic LP-WCFS1, such as its good stability in fermented milk together with the inhibitory effect on post-acidification.

5.1 Introduction

Functional yoghurt variants have been made by incorporating bacterial strains called "probiotics" which are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [8]. Probiotics have been widely employed as adjunct cultures in the production of fermented dairy products [42]. Most commercial probiotics incorporated in yoghurt are strains belonging to the genera *Lactobacillus* and *Bifidobacterium* [19] of which functional and technological attributes have been extensively documented [42].

It is recommended that a probiotic product should contain at least 10^6 cfu/g of viable probiotic cells throughout the entire shelf-life for ensuring their health-promoting effects [42]. However, many of probiotic strains do not survive well in fermented milk [10, 34]. One of the strategies to improve the viability of probiotics is stress adaptation which can be performed by pretreating (preculturing) probiotic cells sublethal stress conditions prior to exposure to a more harsh environment [41]. The study described in Chapter 4 focusing on two dairy probiotic strains, i.e. *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12, demonstrated that this approach allows probiotic cells to develop adaptive stress responses leading to an increase in their survival in set-yoghurt. Furthermore, a complementary metabolomics approach using headspace SPME-GC/MS and ^{1}H-NMR successfully revealed substantial impacts from the metabolic activity of yoghurt starters and probiotics corresponding with distinctive volatile and non-volatile polar metabolite profile of the fermented product (Chapter 2 – 4). This information is technologically relevant since metabolic responses of stress-adapted probiotics may substantially affect the biochemical and organoleptic characteristics of product [32].

L. plantarum is a versatile facultative heterofermentative lactic acid bacterium (LAB) present in plant-based fermented foods as well as meat, fish and dairy products [7, 37]. *L. plantarum* is also encountered as a natural inhabitant of the human gastrointestinal tract with identified candidate probiotic genes and potential health-associating properties [7, 14, 39]. A variety of *L. plantarum* strains, e.g. 299v and Lp01, have been commercialized in the probiotic marketplace [7, 35]. Advances in "~omics"

technologies were instrumental in making *L. plantarum* one of the primary model organisms in LAB research [38]. The complete genome sequence of *L. plantarum* WCFS1, a single colony isolate of *L. plantarum* NCIMB 8826 from human saliva, has been published [14, 39]. In addition, functional-genomics studies have extensively provided new information on how *L. plantarum* responds to various environmental stresses from a molecular perspective [2, 29]. Nevertheless, the information regarding technological aspects of applying *L. plantarum* strains in fermented milk is rather limited [6, 9, 22, 26].

The objectives of this study were (i) to evaluate the growth and survival of *L. plantarum* WCFS1 in co-fermentation with traditional yoghurt starters and (ii) to investigate the impact of preculturing under sublethal stress conditions (combinations of elevated NaCl and low pH) on its survival and metabolite formation in set-yoghurt. Changes in bacterial population dynamics and extent of milk acidification were monitored during fermentation and refrigerated storage. Biochemical changes associated with bacterial activity were characterized using headspace SPME-GC/MS and ^{1}H-NMR technique. Finally, volatile and non-volatile polar metabolite profiles of yoghurt samples were statistically compared using multivariate analysis.

5.2 Materials and methods

5.2.1 Yoghurt Starters and probiotic strains

Frozen direct-vat-inoculation pellets of *Streptococcus thermophilus* C44, *Lactobacillus delbrueckii* subsp*. bulgaricus* C49 (CSK Food Enrichment, Ede, the Netherlands) were placed at ambient temperature (20 ± 3 °C) for 15 min before use. A culture of *L. plantarum* WCFS1 (LP-WCFS1) obtained from NIZO Food Research (Ede, the Netherlands) was propagated in our laboratory and stored as a 20% (v/v) glycerol stock-culture at -80 °C. Frozen LP-WCFS1 culture was re-propagated in MRS broth (Merck, Darmstadt, Germany) at 37 °C for 24 h under anaerobic incubation (Anoxomat™-Mart®, Drachten, the Netherlands). Then, the cells were collected by centrifugation at 4,000*g* for 15 min at 4 °C, washed twice using peptone-physiological-

salt solution (Tritium microbiology, Eindhoven, the Netherlands) and resuspended in milk before use. This culture was defined as control group, i.e. standard precultured LP-WCFS1.

5.2.2 Preculturing of *L. plantarum* WCFS1 under sublethal stress conditions

a) Screening for sublethal stress conditions

Suitable sublethal stress conditions, combinations of elevated NaCl concentrations and low pH values, for LP-WCFS1 were preliminary determined according to the method described in Chapter 4. The concentrations which caused 0.5 and 1.0 log reduction of viable cells compared to those enumerated in unsalted MRS broth after anaerobic incubation at 37 °C for 24 h (data not shown) were determined as low and high sublethal NaCl levels, i.e. 1.5% and 4.5% (w/v), respectively. On the other hand, sublethal pH levels were assigned at 1.0 pH unit above and below the optimum pH for the growth of LP-WCFS1, i.e. pH 4.5 and 6.5. The combinations of sublethal NaCl-pH treatments were finally organized as a 2 X 2 between subjects factorial design (Table 5.1).

Table 5.1. Sublethal stress conditions (combinations of elevated salt and low pH) in modified MRS broth for preculturing of *L. plantarum* WCFS1 in a batch fermentor

Salt stress	**Acid stress**	
	Low pH	High pH
Low %NaCl	1.5% NaCl – pH 4.5	1.5% NaCl – pH 6.5
High %NaCl	4.5% NaCl – pH 4.5	4.5% NaCl – pH 6.5

b) Preculturing of *L. plantarum* WCFS1 probiotics in a batch fermentor

Preculturing of LP-WCFS1 was conducted in a 750 mL Multifors-2 Bacterial System Bioreactor fully operated by IRIS-V.5.3 control software (Infors HT, Bottmingen, Switzerland) as previously described in Chapter 4. The preculturing medium (MRS broth) was adjusted and automatically maintained at a desired pre-set value

(combination of elevated NaCl and low pH). After 24 h (cells in stationary phase monitored by optical density; data not shown), sublethally precultured LP-WCFS1 cells were collected by centrifugation at 4,000*g* for 15 min at 4 °C, washed twice using peptone-physiological-salt solution and the cell pellets were finally resuspended in milk before use. These steps were performed to avoid carryover effect of chemicals and nutrients from preculturing medium (MRS broth) which is considered as nonfood-grade [31] and may significantly influence the metabolomics data derived from ^{1}H-NMR analysis. The preculturing was performed in three batches for each stress combination.

5.2.3 Set-yoghurt fermentation

Pasteurized Nilac milk was prepared according to the method described in Chapter 2. The milk was inoculated with co-cultures of yoghurt starters and different types of LP-WCFS1, i.e. standard precultured (control) and four types of sublethally precultured cells. The initial inoculum of the two yoghurt starter bacteria and LP-WCFS1 were adjusted at 10^{6} cfu/g (ratio 1:1:1). After inoculation, set-yoghurt fermentation was carried out in a water bath at 42 °C for 4 h; then yoghurts were placed in a cold chamber (4 ± 2 °C) for 28 days of storage. Samples were taken hourly during fermentation and weekly during storage. The enumeration of viable bacteria and determination of acidity were carried out immediately after sampling. For ^{1}H-NMR, the samples were stored at -20 °C until the analysis. The fermentation was performed in three replicates for each type of starter combination.

5.2.4 Enumeration of viable bacteria

Viable counts of *S. thermophilus* were determined according to the methods described in Chapter 2. Viable counts of *L. delbrueckii* subsp*. bulgaricus* were determined on MRS agar pH 5.7 (Merck, Darmstadt, Germany) after anaerobic incubation (Anoxomat™-Mart®, Drachten, the Netherlands) at 45 °C for 72 h (selectivity tested in this study). Viable counts of LP-WCFS1 were determined on MRS agar pH 5.7 supplemented with 50 mg/L vancomycin (Merck, Darmstadt, Germany) after anaerobic incubation at 37 °C for 24 h.

5.2.5 Determination of acidification profile

Production of acid during set-yoghurt fermentation and refrigerated storage was expressed by changes in pH and increases in titratable acidity. The pH measurement and determination of titratable acidity were performed according to the methods described in Chapter 2.

5.2.6 Analysis of volatile metabolites by headspace SPME-GC/MS

For headspace SPME-GC/MS analysis, set-yoghurt fermentation was also carried out directly in glass GC vials as described in Chapter 2. The fermentation was performed in three replicates for each type of starter combination. Extraction and determination of volatile compounds by headspace SPME-GC/MS were performed according to the method described in Chapter 2. This method was based on the method developed by Hettinga et al. [8].

Volatile metabolites were identified using AMDIS software (NIST, Gaithersburg, MD, USA) referred to NIST/EPA/NIH database and library provided by Hettinga et al. [9]. Specific retention time and *m/z* model were used for automated peak integration in the XCalibur software package (Thermo Scientific, Austin, TX, USA) [29].

5.2.7 Analysis of non-volatile polar metabolites by ^{1}H-NMR spectroscopy

For ^{1}H-NMR analysis, the samples from two replicates were analyzed according to the method described in Chapter 2. Frozen yoghurt samples were thawed at room temperature and pH was adjusted to 6.0 using 1.0 N NaOH to achieve low variation, i.e. location and shape of peaks, in the spectra obtained [15]. NOESY 1D-^{1}H-NMR measurements were performed at 300 K in a 600 MHz NMR spectrometer (Bruker, Rheinstetten, Germany) operated under full automation, with similar parameters as described by Lu et al. [13].

The ^{1}H-NMR spectra were baseline-corrected, phase-corrected, aligned and calibrated based on the internal standard (TSP) peak. For each spectrum, chemical shift (δ) across the range of 0.00 - 10.00 ppm was segmented (binning) with an interval of 0.02 ppm [29]. The signal intensity in each bin was integrated and expressed in

arbitrary units using AMIX software (Bruker, Rheinstetten, Germany). Metabolite labels were assigned to the bins by means of Chenomx NMR suite 7.5 library (Chenomx Inc., Alberta, Canada) and from the list of metabolites identified in Chapter 2 and 3 [29]. For unlabeled bins, significant variables were selected based on one-way ANOVA at 95% confidence level.

5.2.8 Statistical analysis

One-way ANOVA with multiple comparisons by Tukey's test were performed using IBM SPSS statistics package version 19 (SPSS Inc., Chicago, IL, USA). A probability at $p < 0.05$ was considered statistically significant. Metabolomics data from GC/MS and ^{1}H-NMR were normalized by median-centering and $\log_2$-scaling before multivariate statistical analysis. Principal component analysis (PCA) was performed using Multi-Experiment Viewer (MeV) version 4.8 as previously described in Chapter 2.

5.3 Results

5.3.1 Bacterial growth and survival profiles

Viable counts of yoghurt starters and probiotics were enumerated during set-yoghurt fermentation and refrigerated storage (Fig. 5.1). Bacterial populations in the samples co-fermented with *L. plantarum* WCFS1 (LP-WCFS1), which was precultured under sublethal stress conditions, were compared with those observed in the samples co-fermented with the standard precultured LP-WCFS1 (control group). The main effects of individual stress factors, i.e. elevated NaCl and low pH, and their interaction were determined using two-way ANOVA with 2 X 2 between subjects factorial design (Table 5.2).

Growth (increase in viable count during fermentation) and survival (retention in viable count during refrigerated storage) of *S. thermophilus* were not significantly affected by the incorporation of all cultures of LP-WCFS1 (Fig 5.1A). Their viable counts increased by 2.2 log units to reach and average value of 8.5 ± 0.1 log cfu/g^{-} at the end of fermentation and remained stable (above 8.0 log cfu/g) towards the end of storage.

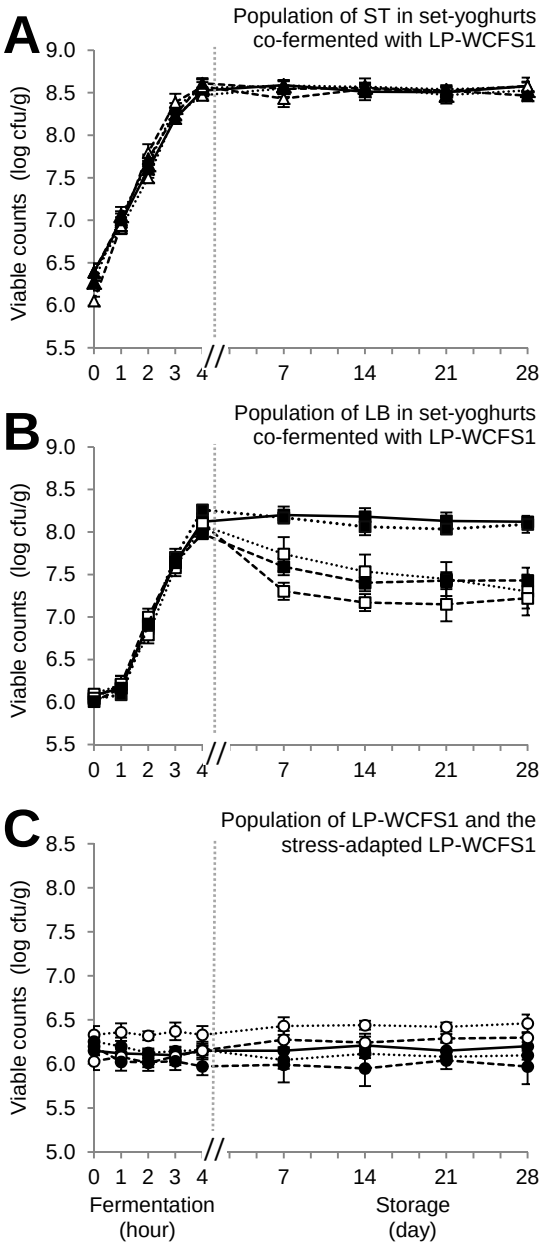

Fig. 5.1. Changes in viable counts of *S. thermophilus* (ST, △; panel A), *L. delbrueckii* subsp. *bulgaricus* (LB, □; panel B) and *L. plantarum* WCFS1 (LP, ○; panel C) during set-yoghurt fermentation (4 hours) and refrigerated storage (28 days). Data are labeled according to the preculturing conditions of LP: standard precultured (control) group (——; black markers), LP precultured at 1.5% NaCl-pH 4.5 (‘‘‘‘‘‘; white markers), 1.5% NaCl-pH 6.5 (‘‘‘‘‘‘; black markers), 4.5% NaCl-pH 4.5 (– – –; white markers) and 4.5% NaCl-pH 6.5 (– – –; black markers). Error bars represent standard deviations based on three replicates.

Table 5.2. ANOVA of the main effects of individual stress factors, i.e. NaCl and pH, and the interaction on the viability of *L. delbrueckii* subsp. *bulgaricus* C49, pH and titratable acidity in set-yoghurts co-fermented with *L. plantarum* WCFS1

Significant parameter	Standard LP[a]	Sublethally precultured LP				Test of significant effects		
at the end of storage (28 days)	(control)	1.5% NaCl		4.5% NaCl		Main effect		Interaction
		pH 4.5	pH 6.5	pH 4.5	pH 6.5	NaCl	pH	NaCl*pH
Viable counts of *L. delbrueckii* subsp. *bulgaricus* (log cfu/g)	8.1 ± 0.1b[b]	7.3 ± 0.2a	8.1 ± 0.1b	7.2 ± 0.2a	7.4 ± 0.2a	$p < 0.01$	$p < 0.01$	$p = 0.01$
pH value	4.1 ± 0.1a	4.3 ± 0.1ab	4.1 ± 0.1a	4.3 ± 0.0b	4.3 ± 0.0b	$p < 0.05$	$p < 0.05$	$p > 0.05$
Titratable acidity (% lactic acid)	0.98 ± 0.02b	0.83 ± 0.03a	0.94 ± 0.01b	0.77 ± 0.02a	0.84 ± 0.04a	$p < 0.05$	$p < 0.05$	$p > 0.05$

[a] *Lactobacillus plantarum* WCFS1

[b] Letters indicate significant difference ($p < 0.05$) among mean values within the same row.

The growth of *L. delbrueckii* subsp. *bulgaricus* during fermentation was not affected by co-cultivation with sublethally precultured LP-WCFS1 resulting in an average value of 8.1 ± 0.1 log cfu/g at the end of fermentation. On the other hand, deviations in the survival of *L. delbrueckii* subsp. *bulgaricus* during refrigerated storage were clearly observed (Fig. 5.1B). The survival of *L. delbrueckii* subsp. *bulgaricus* was significantly impaired ($p < 0.01$) by co-cultivation with LP-WCFS1 precultured at 1.5% NaCl-pH 4.5 and 4.5% NaCl (with either pH 4.5 or 6.5) resulting in a significantly lower average viable counts (7.2 ± 0.2 log cfu/g) compared to the non-precultured group (8.1 ± 0.1 log cfu/g) at the end of storage. The main effects of NaCl and pH as well as their interaction accounted on stress-adapted LP-WCFS1 cells provided an indirectly adverse effect on the stability of *L. delbrueckii* subsp. *bulgaricus* during storage (Table 5.2). Although all cultures of LP-WCFS1 used in this study could not grow in milk during fermentation, these bacteria demonstrated very good stability in set-yoghurt during refrigerated storage (Fig. 5.1C). The viable counts of standard precultured LP-WCFS1 and all sublethally precultured cells remained virtually stable from the beginning of fermentation throughout the entire duration of storage (*ca.* 6.2 ± 0.1 log cfu/g).

5.3.2 Acidification profiles

Changes in pH were monitored during set-yoghurt fermentation and refrigerated storage (Fig. 5.2A). Similar pH decrease patterns were observed during fermentation in all yoghurt samples regardless of the types of preculturing of the LP-WCFS1 culture, resulting in an average pH value of 4.5 ± 0.1 at the end of fermentation. During refrigerated storage, co-fermentation with standard precultured LP-WCFS1 and LP-WCFS1 precultured at 1.5% NaCl-pH 6.5 demonstrated similar pH decrease pattern resulting in a final pH value of 4.1 ± 0.0. On the other hand, deviations in the reduction of pH were observed in the samples co-fermented with LP-WCFS1 precultured at 1.5% NaCl-pH 4.5 and 4.5% NaCl (with either pH 4.5 or 6.5) resulting in an average pH value of 4.3 ± 0.1 at the end of storage. Although this variation appeared to be negligible, statistical tests demonstrated a significant difference ($p = 0.02$) compared to the control group. The main effects of NaCl and pH (without interaction) contributed significantly ($p < 0.05$) on the final pH of yoghurt samples (Table 5.2).

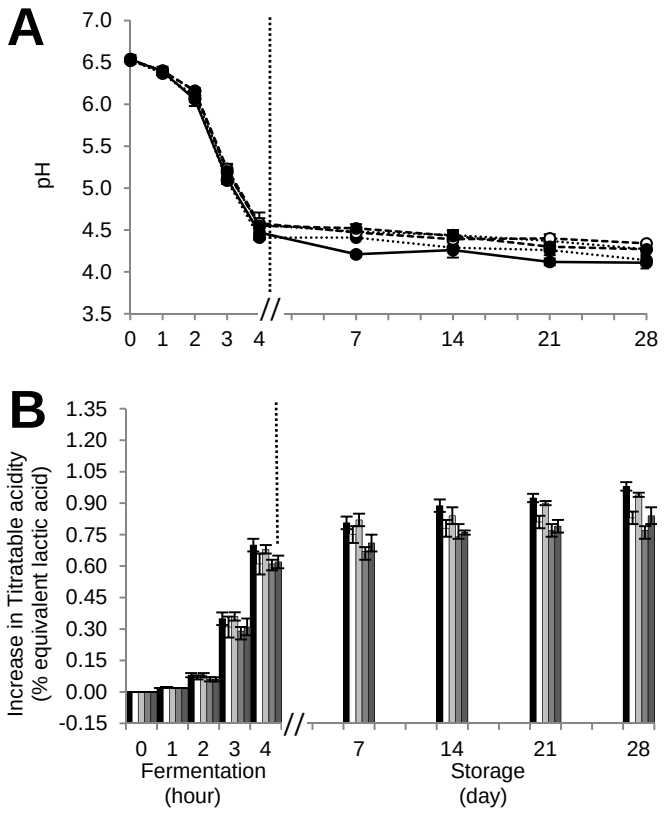

Fig.5.2. Changes in pH (panel A) and titratable acidity (panel B) during fermentation (4 hours) and refrigerated storage (28 days) in set-yoghurts co-fermented with *L. plantarum* WCFS1 (LP) and their stress-adapted cells. Data are labeled according to the preculturing conditions of LP; i.e. standard precultured (control) group (—●—, ■), LP precultured at 1.5% NaCl-pH 4.5 (┈○┈, □), 1.5% NaCl-pH 6.5 (┈●┈, □), 4.5% NaCl-pH 4.5 (╌○╌, ■) and 4.5% NaCl-pH 6.5 (╌●╌, ■). Error bars represent standard deviations based on three replicates.

Titratable acidity measured during set-yoghurt fermentation and refrigerated storage was expressed as % acid (w/w) equivalent to lactic acid (% LA) (Fig. 5.2B). The titratable acidity was subtracted by its initial value in the sample at 0 h (unfermented milk) and discussed as titratable acidity produced by bacterial activity. The result did not show significant difference in titratable acidity among yoghurt samples at the end of fermentation (0.64 ± 0.04%). However, a lower acid production during storage was observed in the samples co-fermented with LP-WCFS1 precultured at 1.5% NaCl-pH 4.5 and 4.5% NaCl (with either pH 4.5 or 6.5). These stress-adapted cultures resulted in a

significant lower titratable acidity (0.81 ± 0.04%) (p = 0.01) compared to the control group (0.96 ± 0.03%). The two main effects of NaCl and pH (without interaction) accounted on stress-adapted LP-WCFS1 cells contributed significantly (p < 0.05) on the difference in titratable acidity among yoghurt samples at the end of storage (Table 5.2). This result is in agreement with the reduction of pH previously observed.

5.3.3 Volatile metabolite profiles determined by headspace SPME-GC/MS

Volatile metabolite profiles of set-yoghurts co-fermented with different types of LP-WCFS1 were evaluated at the end of fermentation and every two weeks during storage. A total of 35 volatile metabolites consisting of alcohols, carbonyl compounds, organic acids, sulfur compounds and heterocyclic compound were identified (Table S5.2). These compounds were introduced as variables for multivariate analysis. Principal component analysis (PCA) was performed to distinguish the volatile metabolite profiles among set-yoghurts co-fermented with different types of LP-WCFS1. Samples from three replicates were statistically treated as individual objects.

An overall PCA score plot was constructed with a total variance of 60.8% (n = 45) (Fig. 5.3). The result demonstrated that volatile profiles of the samples co-fermented with non-precultured LP-WCFS1 were completely distinguished from those co-fermented with (i) LP-WCFS1 precultured at 1.5% NaCl (with either pH 4.5 or 6.5) along PC1 (33.1% variance) and (ii) LP-WCFS1 precultured at 4.5% NaCl (with either pH 4.5 or 6.5) along PC2 (27.7% variance). Loading plots indicated which metabolites were accountable for discrimination. The PC1-loading indicated that dimethyl sulfide, 3-methyl-2-butenal, acetic acid and 2-ethylhexanol were the key determinant for stress-adapted LP-WCFS1 at 1.5% NaCl while the PC2-loading indicated that 2-butanone, 1-butanol, 3-methyl-3-butanol, 3-pentanol, acetic acid, 2-ethylhexanol and nonanoic acid were the key determinant for LP-WCFS1 precultured at 4.5% NaCl. Among the indicative metabolites mentioned, acetic acid (vinegar, pungent) and 2-butanone (sweet, fruity) are two of the major volatile compounds responsible for distinctive aroma profile of yoghurt [3]. These two compounds were detected in significantly higher abundance in

the samples co-fermented with sublethally precultured LP-WCFS1, especially at 4.5% NaCl level (Fig. 5.4).

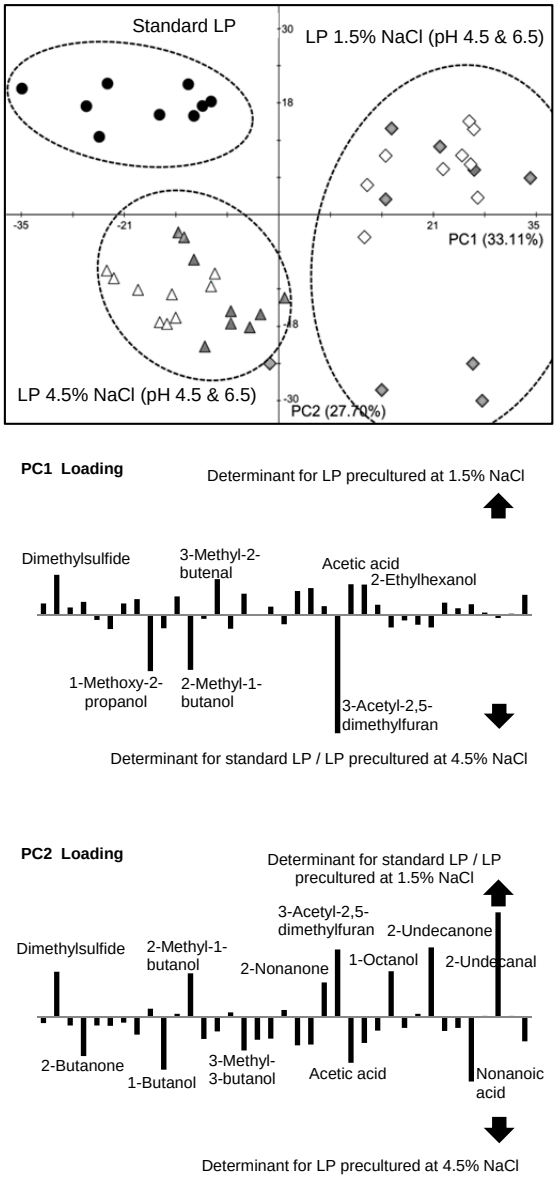

Fig. 5.3. Overall PCA score plot and PC loadings derived from volatile metabolite profiles of set-yoghurts co-fermented with standard precultured *L. plantarum* WCFS1 (LP) (●), LP precultured at 1.5% NaCl-pH 4.5 (◇), 1.5% NaCl-pH 6.5 (◆), 4.5% NaCl-pH 4.5 (△) and 4.5% NaCl-pH 6.5 (▲).

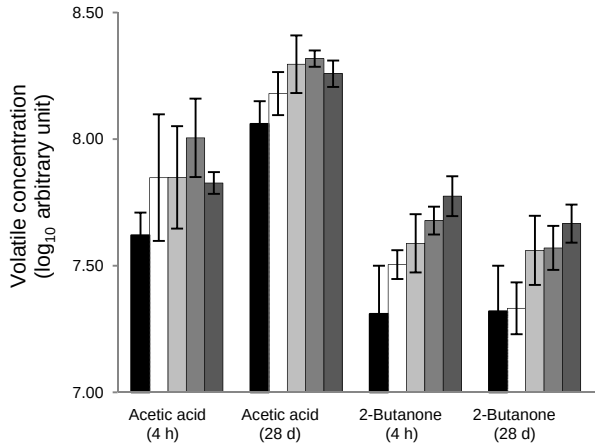

Fig. 5.4. Quantity of acetic acid and 2-butanone present in set-yoghurts co-fermented with standard precultured *L. plantarum* WCFS1 (LP) (■), LP precultured at 1.5% NaCl-pH 4.5 (□), 1.5% NaCl-pH 6.5 (▒), 4.5% NaCl-pH 4.5 (▓) and 4.5% NaCl-pH 6.5 (▓) at the end of fermentation (4 hours) and the end of storage (28 days). Error bars represent standard deviations based on three independent replicates.

5.3.4 Non-volatile polar metabolite profiles determined by ^{1}H-NMR

For non-volatile polar metabolite profiling, NOESY-1D-^{1}H-NMR spectra of set-yoghurts were processed according to the method described in Chapter 2. A total of 43 metabolites including amino acids, carbohydrates, organic acids, lipid derivatives, carbonyl compounds, a sulfur compound and a nucleoside were identified. The quantification was achieved by summation of signal intensities in all bins corresponding to the respective metabolite [27] and expressed in $\log_{10}$ transformed (arbitrary unit) (Table S5.3). For multivariate analysis, it should be noted that the 43 identified metabolites accounted for labeling of 149 bins. A complementary data filtering by ANOVA was performed for selection of the remaining unknowns [16]. Finally, a total of 266 bins were introduced as variables in the analysis.

An overall PCA score plot was constructed with a total variance of 43.0% (n = 20) (Fig. 5.5A). The result demonstrated that non-volatile polar metabolite profiles of the samples co-fermented with LP-WCFS1 precultured at 1.5% NaCl (with either pH 4.5 or 6.5) could be well distinguished from those of non-precultured LP-WCFS1 and LP-

WCFS1 precultured at 4.5% NaCl (with either pH 4.5 or 6.5) along PC1 (30.3% variance). The PC1-loading indicated that the majority of metabolites contributed to the separation of the two latter groups. However, a good distinction between the standard precultured LP-WCFS1 and LP-WCFS1 precultured at 4.5% NaCl was not observed. Thus, an additional PCA score plot was constructed with a total variance of 36.2% (n = 12) (Fig. 5.5B). The result revealed that the samples co-fermented with standard precultured LP-WCFS1 could be distinguished from those of LP-WCFS1 precultured at 4.5% NaCl (with either pH 4.5 or 6.5) along PC3 (9.5% variance).

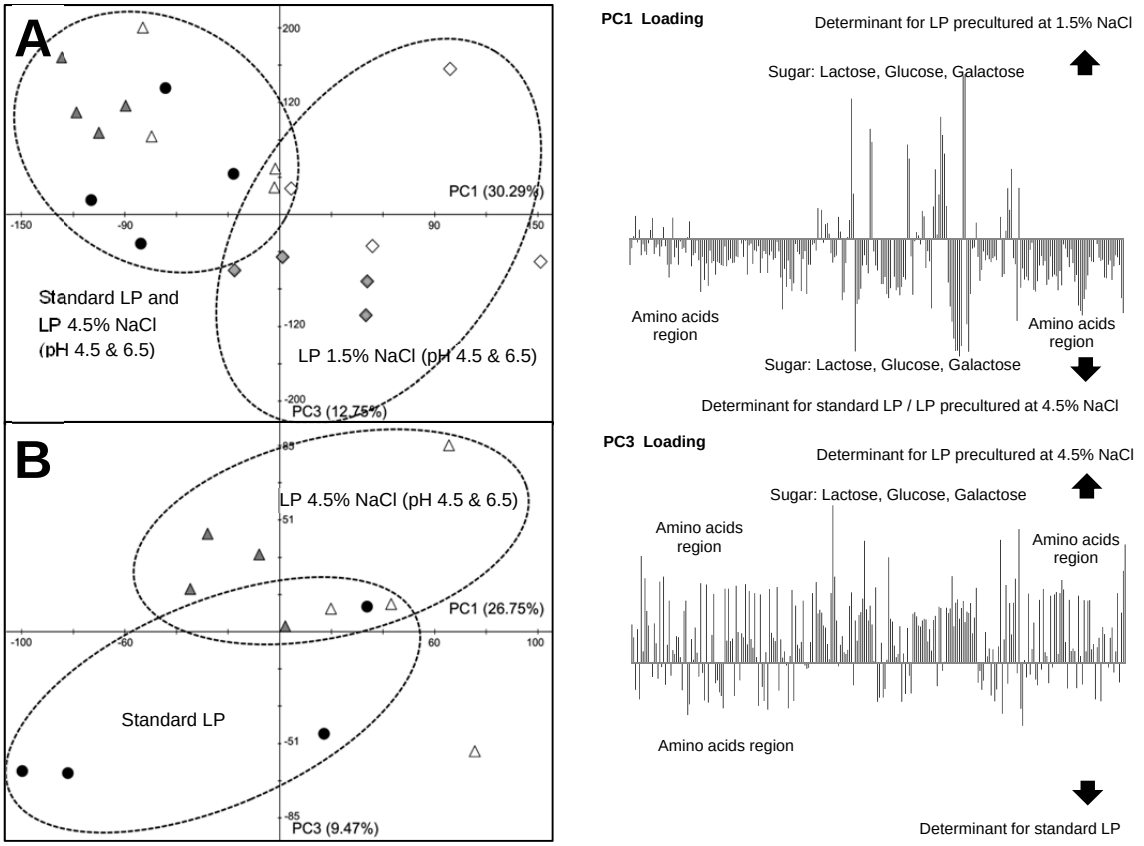

Fig. 5.5. Overall PCA score plot and PC loading derived from non-volatile polar metabolite profiles of set-yoghurts co-fermented with standard precultured (control) *L. plantarum* WCFS1 (LP) (●), LP precultured at 1.5% NaCl-pH 4.5 (◇), 1.5% NaCl-pH 6.5 (◆), 4.5% NaCl-pH 4.5 (△) and 4.5% NaCl-pH 6.5 (▲). Overall comparison among the groups of LP (panel A) and comparison between standard precultured LP and LP precultured at 4.5% NaCl (panel B) are respectively presented.

5.4 Discussion

The present study was aimed to (i) evaluate the growth and survival of *L. plantarum* WCFS1 in co-fermentation with traditional yoghurt starters and (ii) investigate the impact of preculturing under sublethal stress conditions (combinations of elevated NaCl and low pH) on its survival and metabolite formation in set-yoghurt. Besides, it has been reported that stress responses vary depending on the growth phase of LAB, i.e. cells in stationary phase develop more general resistance to various types of stresses [31]. Therefore, the preculturing in this study was prolonged for 24 h to allow attaining stress-adapted LP-WCFS1 cells from the stationary phase (monitored by optical density; data not shown).

The population dynamics and acidifying capacity of *S. thermophilus* C44 and *L. delbrueckii* subsp. *bulgaricus* C49 in set-yoghurt fermented without probiotics have been discussed previously in Chapter 2 and 3. In this study, incorporation of standard precultured LP-WCFS1 did not significantly affect the growth and survival of yoghurt starters as well as acidification profile of product (Table S5.1). In co-cultures with sublethally precultured LP-WCFS1, it was interesting that the survival of *L. delbrueckii* subsp. *bulgaricus* during refrigerated storage was significantly impaired by co-culturing with LP-WCFS1 precultured at 1.5% NaCl-pH 4.5 and 4.5% NaCl (with either pH 4.5 or 6.5). On the other hand, there was no adverse effect observed on the survival of *S. thermophilus*. A proposed explanation for this could be that sublethal preculturing may trigger the synthesis of certain compounds in stress-adapted LP-WCFS1 which provide inhibitory effect on *L. delbrueckii* subsp. *bulgaricus*. Many members of LAB are known to produce peptides or proteins with antimicrobial activity (bacteriocins) to improve their competitiveness against related species [13]. Bacteriocins produced by different strains of *L. plantarum* (plantaricins) have been identified and characterized [25]. It has been documented that environmental factors, e.g. sugar, NaCl, pH and temperature, play an important role in regulation of bacteriocin production in *L. plantarum* [17, 25]. Moreover, induction of bacteriocin production by co-culturing with a range of bacterial strains appeared to be a common feature in *L. plantarum* [21]. The LP-WCFS1 genome provided indications to a region, containing *pln* genes, encoding plantaricin synthesis

[14, 38]. Although the native state of LP-WCFS1 was bacteriocin negative strain, Sturme et al. [40] reported that its bacteriocin production could be induced. Plantaricins produced by LP-WCFS1 showed activity against closely related species which can be found in the same ecological niches [40]. Therefore, the adverse effect of stress-adapted LP-WCFS1 on the survival of *L. delbrueckii* subsp. *bulgaricus* found in this study is an interesting issue that needs to be further investigated.

Regarding the preculturing effect on growth and survival of LP-WCFS1, there was no significant difference observed among the standard precultured LP-WCFS1 and the sublethally precultured cells. None of preculturing conditions applied in this study could enhance the growth of LP-WCFS1 during set-yoghurt fermentation. This observation corresponds with the previous study on *L. rhamnosus* GG and *B. animalis* subsp. *lactic* BB12 (Chapter 4) in which we also did not manage to find a suitable preculturing condition for successful growth improvement of probiotics in milk. In this study, however, all cultures of LP-WCFS1 exhibited good survival in set-yoghurt. Their populations remained virtually stable from the starting point of fermentation throughout the entire duration of storage. Indeed, it should be mentioned that the final viable counts of LP-WCFS1 and the sublethally precultured cells still remain above the minimum recommended level (6.0 log cfu/g) to ensure their potential health-promoting effects [36]. This finding makes LP-WCFS1 a good candidate probiotic strain for yoghurt production. High survival of various strains of *L. plantarum* in fermented milk has been reported [9, 22]. Furthermore, the genome of LP-WCFS1 has provided information on how this LAB strain may have adapted to growth in diverse environmental niches such as fermented foods, plants, and the human gastrointestinal tract [18, 24].

Acidification profiles of set-yoghurts co-fermented with sublethally precultured LP-WCFS1 during fermentation were not significantly different from the control group (Table S5.1). On the other hand, a substantial decline in acid production resulted in a significant higher pH and lower titratable acidity at the end of storage in the samples co-fermented with LP-WCFS1 precultured at 1.5% NaCl-pH 4.5 and at 4.5% NaCl (with either pH 4.5 or 6.5). Although the variation in final pH appeared to be negligible, samples could be categorized into different product segments: (i) mild (pH_{28d} > 4.30) for

those co-fermented with LP-WCFS1 precultured at 1.5% NaCl-pH 4.5 and 4.5% NaCl (with either pH 4.5 or 6.5) and (ii) semi-mild ($4.00 < pH_{28d} < 4.25$) for those co-fermented with standard precultured LP-WCFS1 and LP-WCFS1 precultured at 1.5% NaCl-pH 6.5 according to the information provided by yoghurt starters supplier [5]. The reduction of pH and accumulation of organic acids during refrigerated storage of fermented milk are defined as "post-acidification" which is mainly attributed to the ongoing metabolic activity of *L. delbrueckii* subsp. *bulgaricus* (Shah, 2000). With respect to this, the significantly lower post-acidification observed in set-yoghurts co-fermented with stress-adapted LP-WCFS1 could be potentially associated with the decrease in viable counts of *L. delbrueckii* subsp. *bulgaricus* affected by these stress-adapted LP-WCFS1 cultures.

The primary PCA result showed that incorporation of standard precultured LP-WCFS1 contributed to distinctive volatile metabolite profiles of set-yoghurts compared to those fermented without probiotics (Fig. S5.1A). In the samples co-fermented with various types of LP-WCFS1, the distinction among volatile profiles of set-yoghurts was clearly observed. Co-cultivation with sublethally precultured LP-WCFS1 resulted in distinctive yoghurt volatile profiles compared to the control group (Fig. 5.3). Particularly, the distinction was recognized according to the concentration of NaCl at which the LP-WCFS1 was precultured. Relating to the adverse effect on the survival of *L. delbrueckii* subsp. *bulgaricus* and significant decrease in post-acidification observed previously, a distinction between volatile profiles of the samples co-fermented with LP-WCFS1 precultured at 1.5% NaCl-pH 4.5 and pH 6.5 was expected. However, the PCA result revealed that volatile profiles of the samples co-fermented with these two cultures were relatively close to each other. This observation suggests that only the main effect of NaCl accounted on stress-adapted LP-WCFS1 cells predominantly contributes to the distinctive volatile profiles of set-yoghurts. In conjunction with the study in Chapter 4, it can be concluded that the volatile profiles of set-yoghurts co-fermented with stress-adapted lactobacilli, i.e. *L. rhamnosus* GG and *L. plantarum* WCFS1, are distinguished according to sublethal concentrations of NaCl present during

the preculturing while those co-fermented with stress-adapted *B. animalis* subsp. *lactis* BB12 are distinguished according to levels of pH adjusted during preculturing.

From a technological standpoint, the key yoghurt aroma volatiles, i.e. acetaldehyde (fresh, green, pungent), diacetyl (buttery, creamy), acetoin (buttery), 2,3-pentanedione (buttery, vanilla-like), acetone (sweet, fruity), 2-butanone (sweet, fruity) and acetic acid (vinegar, pungent) [3], were all detected at high relative abundances (Table S5.2). The contribution of traditional yoghurt starters and various *L. plantarum* strains on aroma volatile production in fermented milk has been documented [3, 6, 28, 30]. Loading plots derived from PCA indicated that acetic acid and 2-butanone were two of the major aroma volatiles contributing to discriminate volatile profiles of the samples co-fermented with stress-adapted LP-WCFS1. Nevertheless, it should be mentioned that a number of carbonyl compounds and alcohols also contributed to the discrimination. Indeed, it would be possible that the metabolic activity of LP-WCFS1 may result in an undesirable aroma profile of yoghurt, since this potential probiotic strain was originally isolated from a non-dairy environment [14]. Taking into account the observed beneficial effect on post-acidification, our finding suggests that incorporation of stress-adapted LP-WCFS1 in set-yoghurt may considerably influence the organoleptic quality of product. Therefore, a research focusing on sensory evaluation of yoghurt with trained panelists is additionally required.

The list of non-volatile polar metabolites identified in this study (43 compounds) was derived from our previous study [33] and ^{1}H-NMR-based studies in liquid milk and cheese [1, 4, 15]. The primary PCA result showed that incorporation of standard precultured LP-WCFS1 contributed to distinctive non-volatile metabolite profiles of set-yoghurts compared to those fermented without probiotics (Fig. S5.1B). In the samples co-fermented with sublethally precultured LP-WCFS1, non-volatile metabolite profiles of set-yoghurts could be distinguished according to the concentration of NaCl at which the LP-WCFS1 was precultured (Fig. 5.5). This result was in accordance with the distinction pattern previously observed in their volatile profiles.

5.5 Conclusions

This study provides relevant information on technological implication of the use of stress-adapted LP-WCFS1. Although LP-WCFS1 showed poor capacity to grow in milk, its viable counts remained virtually stable in set-yoghurt throughout the entire duration of refrigerated storage. The presence of LP-WCFS1 did not influence the growth and survival of yoghurt starters as well as acidification profile of product. This finding makes LP-WCFS1 a good candidate probiotics for yoghurt manufacture. Interestingly, application of LP-WCFS1 precultured at 1.5% NaCl-pH 4.5 and 4.5% NaCl (with either pH 4.5 or 6.5) significantly impaired the survival of *L. delbrueckii* subsp. *bulgaricus* during refrigerated storage. This consequently provided a significant reduction of post-acidification.

Supplementary Data

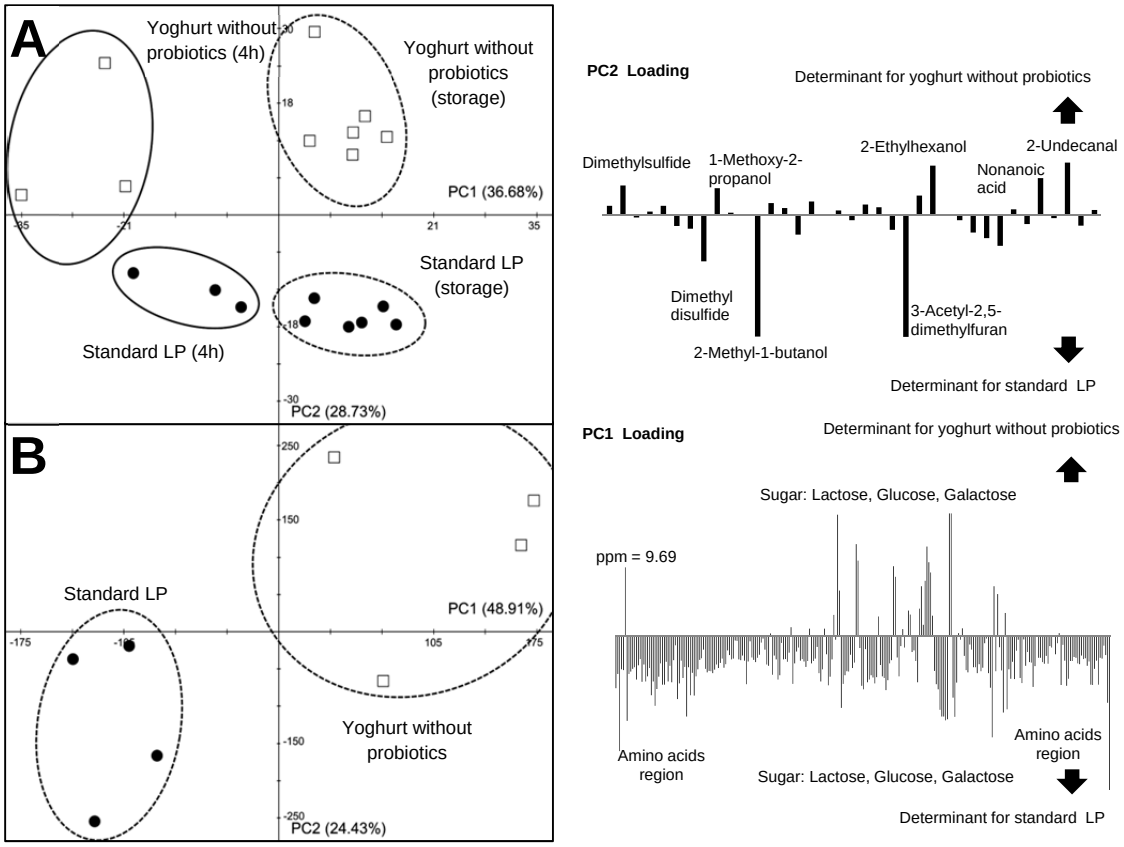

Fig. S5.1. PCA score plots and PC loadings derived from volatile (panel A) and non-volatile polar metabolite profiles (panel B) of set-yoghurts fermented with only yoghurt starters (□) and yoghurt starters with an addition of standard precultured *L. plantarum* WCFS1 (LP) (●).

Table S5.1. Fermentation parameters in set-yoghurts (samples at 4 hours and 28 days) fermented by yoghurt starters and co-culture of yoghurt starters with standard *L. plantarum* WCFS1

Time	Fermentation parameter	Starter cultures	
		Y[a]	**Y-LP**[b]
4 hours	Viable counts of *S. thermophilus* (log cfu/g)	8.7 ± 0.3	8.5 ± 0.1
	Viable counts of *L. delbrueckii* subsp. *bulgaricus* (log cfu/g)	8.3 ± 0.3	8.1 ± 0.1
	pH	4.5 ± 0.1	4.5 ± 0.1
	Titratable acidity (% equivalent lactic acid)	0.64 ± 0.02	0.70 ± 0.03
28 days	Viable counts of *S. thermophilus* (log cfu/g)	8.5 ± 0.1	8.6 ± 0.1
	Viable counts of *L. delbrueckii* subsp. *bulgaricus* (log cfu/g)	8.2 ± 0.3	8.1 ± 0.1
	pH	4.1 ± 0.0	4.1 ± 0.1
	Titratable acidity (% equivalent lactic acid)	0.94 ± 0.03	0.98 ± 0.02

[a] Y = yoghurt starters consist of *S. thermophilus* C44 and *L. delbrueckii* subsp. *bulgaricus* C49
[b] LP = Standard (non-sublethally precultured) *L. plantarum* WCFS1

Table S5.2. Volatile metabolites identified in set-yoghurts (samples at 4 hours and 28 days) fermented by co-cultures of yoghurt starters with *L. plantarum* WCFS1 (LP) and their sublethally precultured cells using headspace SPME-GC/MS

Chemical group	Compound	Standard LP (Control)		Sublethally precultured LP							
				1.5% NaCl - pH 4.5		1.5% NaCl - pH 6.5		4.5% NaCl - pH 4.5		4.5% NaCl - pH 6.5	
		4 h	28 d	4 h	28 d	4 h	28 d	4 h	28 d	4 h	28 d
Alcohol	1-Butanol	5.60[a]±0.34ab[b]	5.56±0.18ab	5.25 ± 0.11a	5.41±0.31ab	5.50±0.30ab	5.35 ± 0.15a	5.54±0.23ab	5.96 ± 0.13b	5.76 ± 0.12b	5.86 ± 0.11b
	Ethanol	6.77 ± 0.06b	6.79 ± 0.02b	6.58 ± 0.02a	6.65 ± 0.02a	6.63 ± 0.06a	6.70±0.04ab	6.62 ± 0.06a	6.72±0.01ab	6.59 ± 0.09a	6.84 ± 0.07a
	2-Ethyl-hexanol	5.30 ± 0.06a	5.30 ± 0.06a	5.67 ± 0.09b	5.61 ± 0.01b	5.68 ± 0.11b	5.66 ± 0.10b	5.61 ± 0.08b	5.63 ± 0.03b	5.62 ± 0.03b	5.59 ± 0.02b
	1-Hexanol	5.69 ± 0.06a	5.84 ± 0.01b	5.72 ± 0.11ab	5.71 ± 0.02a	5.80 ± 0.05b	5.61 ± 0.13aa	5.71 ± 0.02ab	5.69 ± 0.02a	5.82 ± 0.03b	5.74 ± 0.02ab
	1-Methoxy-2-propanol	5.51 ± 0.54b	4.91 ± 0.31ab	4.71 ± 0.31ab	ND[c]	5.37 ± 0.32b	4.17 ± 0.08a	5.32 ± 0.21b	5.15 ± 0.19b	5.55 ± 0.16b	ND
	2-Methyl-1-butanol	5.75 ± 0.08c	5.66 ± 0.07c	4.86 ± 0.13a	4.94 ± 0.48ab	5.08 ± 0.19ab	5.04 ± 0.22ab	5.21 ± 0.08b	5.16 ± 0.11b	5.24 ± 0.07b	5.09 ± 0.15ab
	3-Methyl-2-butanol	5.78 ± 0.10a	5.84 ± 0.03ab	5.76 ± 0.08a	5.94 ± 0.05b	5.85 ± 0.09ab	5.97 ± 0.08b	5.83 ± 0.03ab	6.10 ± 0.06b	5.81 ± 0.05ab	6.10 ± 0.03b
	3-Methyl-3-butanol	5.78 ± 0.10a	5.84 ± 0.02a	5.88 ± 0.08ab	6.00 ± 0.02b	5.96 ± 0.15ab	6.01 ± 0.06b	6.00 ± 0.04b	6.14 ± 0.04b	5.96 ± 0.07ab	6.06 ± 0.01b
	1-Octanol	5.05 ± 0.07b	5.09 ± 0.07b	5.01 ± 0.08b	5.01 ± 0.02b	5.00 ± 0.14b	ND	4.71 ± 0.09a	4.68 ± 0.06a	4.78 ± 0.13ab	4.74 ± 0.08a
	1-Pentanol	5.38 ± 0.05ab	5.51 ± 0.03b	5.29 ± 0.16ab	5.30 ± 0.01a	5.46 ± 0.13ab	5.30 ± 0.07ab	5.44 ± 0.01b	5.36 ± 0.03ab	5.48 ± 0.05b	5.41 ± 0.04b
	3-Pentanol	6.61 ± 0.12a	6.77 ± 0.03a	6.85 ± 0.02b	7.02 ± 0.01c	6.91 ±0.10abc	7.03 ± 0.05c	6.86 ± 0.02b	7.11 ± 0.05c	6.84 ± 0.07ab	7.02 ± 0.02c
Carbonyl compound	Acetaldehyde	7.32± 0.08a	7.43 ± 0.09ab	7.48 ± 0.04ab	7.50 ± 0.08ab	7.48 ± 0.05ab	7.58 ± 0.05b	7.47 ± 0.10ab	7.54 ± 0.05b	7.42 ± 0.06a	7.41 ± 0.05a
	Acetoin	8.54 ± 0.10ab	8.42 ± 0.03a	8.43 ± 0.07a	8.43 ± 0.03a	8.54 ± 0.16ab	8.51 ± 0.07ab	8.58 ± 0.01b	8.61 ± 0.02b	8.66 ± 0.05b	8.61 ± 0.03b
	Acetone	7.47 ± 0.06a	7.58 ± 0.04a	7.49 ± 0.05a	7.53 ± 0.03a	7.53 ± 0.05a	7.60 ± 0.04a	7.51 ±0.07a	7.58 ± 0.05a	7.56 ± 0.08a	7.58 ± 0.01a
	Benzaldehyde	6.15 ±0.14abc	6.36 ± 0.07c	6.11 ± 0.02a	6.18 ± 0.03b	6.29 ±0.14abc	6.23 ± 0.08b	6.10 ± 0.04a	6.19 ± 0.03ab	6.16 ± 0.04ab	6.21 ± 0.07bc
	2-Butanone	7.31 ± 0.19a	7.32 ± 0.18a	7.50 ± 0.06ab	7.33 ± 0.10a	7.59 ± 0.11ab	7.56 ± 0.14ab	7.68 ± 0.05b	7.57 ± 0.09ab	7.77 ± 0.08b	7.67 ± 0.08b
	Diacetyl	7.40 ± 0.07ab	7.57 ± 0.04b	7.33 ± 0.06a	7.39 ± 0.05a	7.43 ± 0.17ab	7.39 ± 0.11ab	7.47 ± 0.06ab	7.51 ± 0.01b	7.52 ± 0.03b	7.64 ± 0.06b
	2-Heptanone	6.13 ± 0.07ab	6.45 ± 0.04bc	6.29 ± 0.05b	6.62 ± 0.05c	6.26 ± 0.03b	6.48 ± 0.12c	6.12 ± 0.06ab	6.45 ± 0.03c	6.03 ± 0.03a	6.34 ± 0.07c
	2-Hydroxy-3-pentanone	6.44 ± 0.10a	6.51 ± 0.04a	6.65 ± 0.03b	6.78 ± 0.01c	6.69 ±0.13abc	6.83 ± 0.05c	6.66 ± 0.03b	6.87 ± 0.05c	6.61 ± 0.06ab	6.74 ± 0.02c
	3-Methyl-2-butenal	5.42 ± 0.22a	5.58 ± 0.13a	5.68 ± 0.04ab	5.91 ± 0.13b	5.71 ± 0.08ab	5.87 ± 0.08b	5.53 ± 0.13a	5.88 ± 0.10b	5.39 ± 0.15a	5.61 ± 0.10ab
	2-Nonanone	6.11 ± 0.05b	6.30 ± 0.03c	6.37 ± 0.04c	6.53 ± 0.06d	6.25 ±0.20abc	6.26 ±0.22abc	5.99 ± 0.03a	6.18 ± 0.04b	5.92 ± 0.05a	6.08 ± 0.05ab
	3-Octanone	5.37 ± 0.08ab	5.27 ± 0.01a	5.33 ± 0.13ab	5.24 ± 0.05ab	5.41 ±0.19abc	5.27 ± 0.13ab	5.46 ± 0.05bc	5.39 ± 0.02b	5.61 ± 0.10c	5.44 ± 0.04bc

	2,3-Pentanedione	6.96 ± 0.12a	7.54 ± 0.09b	7.12 ± 0.04a	7.50 ± 0.03b	7.12 ± 0.05a	7.48 ± 0.08b	7.01 ± 0.05a	7.52 ± 0.04b	6.91 ± 0.07a	7.50 ± 0.03b
	2-Undecanal	5.14 ± 0.09cd	5.26 ± 0.05d	5.00 ± 0.12c	5.14 ± 0.02cd	4.85 ± 0.29bc	4.80 ± 0.29bc	4.22 ± 0.34ab	4.35 ± 0.18ab	4.59 ± 0.06b	4.36 ± 0.08a
	2-Undecanone	5.42 ± 0.04b	5.42 ± 0.03b	5.47 ± 0.07b	5.48 ± 0.04b	5.32 ± 0.30ab	5.13 ± 0.27ab	5.07 ± 0.09a	5.02 ± 0.03a	5.05 ± 0.04a	5.03 ± 0.06a
Heterocyclic compound	3-Acetyl-2,5-dimethylfuran	6.37 ± 0.06e	6.27 ± 0.03e	4.57 ± 0.11b	4.42 ± 0.07b	4.40 ± 0.12b	3.98 ± 0.15a	5.84 ± 0.08d	5.80 ± 0.05d	5.31 ± 0.04c	5.17 ± 0.07c
Sulfur compound	Dimethyl disulfide	5.13 ± 0.32a	6.40 ± 0.45b	5.38 ± 0.10a	5.90 ± 0.44ab	5.61 ± 0.20a	6.47 ± 0.19b	5.54 ± 0.26a	6.03 ± 0.14b	5.54 ± 0.06a	6.08 ± 0.11b
	Dimethyl sulfide	6.24 ± 0.14ab	6.08 ± 0.91ab	6.68 ± 0.17b	6.13 ± 0.73ab	6.61 ± 0.31ab	6.75 ± 0.12b	6.60 ± 0.32b	5.91 ± 0.25a	6.51 ± 0.09b	5.72 ± 0.64ab
	Dimethyl sulfone	6.27 ± 0.19a	6.61 ± 0.03b	6.35 ± 0.25ab	6.67 ± 0.09b	6.41 ± 0.19ab	6.82 ± 0.14b	6.54 ± 0.32ab	6.79 ± 0.03b	6.27 ± 0.18a	6.67 ± 0.22ab
Volatile organic acid	Acetic acid	7.62 ± 0.09a	8.06 ± 0.09c	7.85 ± 0.25ab	8.18 ± 0.09c	7.85 ± 0.20ab	8.30 ± 0.11cd	8.01 ± 0.15bc	8.32 ± 0.03d	7.83 ± 0.04b	8.26 ± 0.05cd
	Butyric acid	7.65 ± 0.08a	7.75 ± 0.08ab	7.71 ± 0.17ab	7.78 ± 0.03b	7.73 ± 0.16ab	7.79 ±0.02b	7.80 ± 0.08b	7.84 ± 0.02b	7.69 ± 0.05a	7.87 ± 0.03b
	Hexanoic acid	7.62 ± 0.08a	7.64 ± 0.04a	7.62 ± 0.09a	7.69 ± 0.02a	7.63 ± 0.13a	7.58 ± 0.08a	7.63 ± 0.03a	7.66 ± 0.03a	7.55 ± 0.04a	7.68 ± 0.02a
	3-Methyl-butanoic acid	5.59 ± 0.03ab	5.62 ± 0.05b	5.61 ±0.10abc	5.70 ± 0.03b	5.63 ±0.14abc	5.73 ± 0.02bc	5.63 ± 0.07ab	5.78 ± 0.03c	5.54 ± 0.04a	5.72 ± 0.02b
	2-Methyl-propanoic acid	5.64 ± 0.04a	5.64 ± 0.06a	5.41 ± 0.13a	5.55 ± 0.02a	5.46 ± 0.20a	5.57 ± 0.06a	5.57 ± 0.17a	5.63 ± 0.10a	5.46 ± 0.21a	5.62 ± 0.07a
	Nonanoic acid	5.04 ± 0.03b	4.79 ± 0.15a	ND	4.44 ± 0.37a	4.97±0.99abc	5.59±0.88abc	5.69 ± 0.23c	5.65 ± 0.19c	5.40 ± 0.11c	5.36 ± 0.09c
	Pentanoic acid	5.91 ± 0.07a	6.01 ± 0.02a	5.94 ± 0.11a	6.02 ± 0.02a	5.99 ± 0.14a	5.98 ± 0.04a	5.97 ± 0.04a	6.01 ± 0.01a	5.95 ± 0.05a	6.02 ± 0.01a
	Propionic acid	5.82 ± 0.02a	5.92 ±0.06bc	5.91±0.18abc	6.01 ± 0.03b	5.93 ± 0.09ab	6.03 ± 0.06bc	5.96 ± 0.05b	6.09 ± 0.05c	5.87±0.03ab	6.02 ±0.04bc

[a] Metabolite contents are expressed as $\log_{10}$ [peak area of respective compound in arbitrary unit]. Values are mean ± standard deviation from three independent replicates.
[b] Letters (a-e) indicate significant difference ($p < 0.05$) among sample means within the same row.
[c] ND indicates compound not detected.

Table S5.3. Presumptive polar metabolites identified in set-yoghurts (samples at 4 hours, 14 days and 28 days) fermented by coculture of yoghurt starters with *L. plantarum* WCFS1 (LP) and their sublethally precultured cells using NOESY-1D-^{1}H-NMR

Chemical group	Compound	Standard LP (Control)			Sublethally precultured LP											
					1.5% NaCl - pH 4.5			1.5% NaCl - pH 6.5			4.5% NaCl - pH 4.5			4.5% NaCl - pH 6.5		
		4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d	4 h	14 d	28 d
Amino acid and derivatives	Alanine	6.99[a]	7.04	7.09	6.87	7.00	7.16	6.93	7.02	6.99	6.91	7.24	7.11	7.02	7.23	7.17
	Creatine and Creatinine	7.50	7.50	7.54	7.49	7.54	7.61	7.50	7.49	7.47	7.51	7.70	7.55	7.55	7.63	7.58
	Isoleucine	7.70	7.79	7.84	7.60	7.73	7.93	7.64	7.74	7.73	7.63	7.93	7.86	7.72	7.94	7.91
	Leucine	7.71	7.81	7.87	7.59	7.79	8.00	7.63	7.77	7.77	7.63	8.00	7.94	7.74	8.01	7.98
	N-Acetyl-amino acids	8.02	8.02	8.07	7.99	8.02	8.12	8.01	8.01	7.98	8.00	8.17	8.07	8.07	8.14	8.10
	Phenylalanine	6.65	6.73	6.78	6.49	6.75	6.94	6.53	6.68	6.68	6.52	6.97	6.91	6.64	6.97	6.95
	Proline	7.41	7.44	7.48	7.31	7.37	7.53	7.35	7.38	7.35	7.33	7.56	7.47	7.43	7.56	7.52
	Tyrosine	6.97	7.01	7.06	6.89	7.01	7.15	6.93	6.97	6.96	6.89	7.21	7.11	6.97	7.19	7.15
	Valine	7.57	7.65	7.70	7.43	7.65	7.84	7.48	7.61	7.60	7.46	7.85	7.79	7.58	7.87	7.83
	Amino acid residues	8.04	8.11	8.17	8.01	8.09	8.21	8.04	8.08	8.08	8.01	8.15	8.25	8.10	8.16	8.21
Carbohydrate and derivatives	Galactose	9.15	9.00	9.05	9.07	9.04	8.92	9.12	8.99	8.98	9.17	9.07	9.07	9.19	9.16	9.09
	Glucose	9.20	8.91	8.88	9.01	8.93	8.73	9.17	8.85	8.80	9.14	9.09	9.03	9.27	9.20	9.08
	Lactose	9.15	9.12	8.75	9.41	9.29	9.20	9.24	9.19	9.07	9.34	9.20	9.15	9.05	8.96	8.91
	N-Acetylglucosamine	7.47	7.52	7.57	7.48	7.49	7.65	7.47	7.49	7.47	7.46	7.71	7.58	7.55	7.65	7.61
	Sugar residues	7.32	7.30	7.31	7.45	7.32	7.35	7.35	7.28	7.24	7.36	7.51	7.32	7.42	7.40	7.34
Organic acid	Acetate	7.55	7.64	7.69	7.51	7.64	7.72	7.56	7.62	7.63	7.52	7.74	7.68	7.55	7.70	7.68
	Acetoacetate	7.03	7.09	7.14	6.99	7.05	7.23	7.01	7.04	7.02	7.01	7.24	7.17	7.09	7.25	7.21
	Ascorbate	8.29	8.16	8.21	8.11	8.19	8.23	8.30	8.16	8.14	8.24	8.34	8.26	8.34	8.32	8.24
	Benzoate	6.86	6.89	6.93	6.89	6.91	7.00	6.86	6.87	6.86	6.87	7.07	6.95	6.91	6.98	6.95
	Butyrate	7.32	7.37	7.42	7.21	7.37	7.53	7.27	7.37	7.35	7.24	7.56	7.48	7.36	7.58	7.52
	Hydroxybutyrate	7.36	7.46	7.53	7.35	7.39	7.60	7.35	7.41	7.41	7.36	7.57	7.53	7.46	7.59	7.55

	Citrate	7.47	7.52	7.48	7.50	7.50	7.56	7.56	7.47	7.48	7.56	8.06	7.56	7.68	7.57	7.51
	Formate	6.83	6.83	6.91	6.81	6.88	6.98	6.85	6.81	6.78	6.85	7.01	6.92	6.88	7.03	6.93
	Fumarate	5.42	5.35	5.40	5.79	5.40	5.45	5.43	5.36	5.27	5.60	5.58	5.40	5.63	5.49	5.40
	Hippurate	7.15	7.23	7.28	7.15	7.19	7.39	7.14	7.18	7.16	7.16	7.41	7.31	7.24	7.40	7.35
	Isobutyrate	6.77	6.84	6.88	6.51	6.87	7.03	6.63	6.76	6.75	6.61	7.10	7.01	6.75	7.10	7.06
	Lactate	9.44	9.51	9.58	9.40	9.51	9.52	9.46	9.50	9.60	9.43	9.50	9.53	9.45	9.51	9.50
	Orotate	6.47	6.44	6.45	6.57	6.54	6.55	6.51	6.48	6.46	6.54	6.66	6.50	6.56	6.55	6.51
	Oxoglutarate	7.43	7.29	7.40	7.27	7.41	7.53	7.46	7.28	7.29	7.48	7.90	7.43	7.62	7.62	7.47
	Pyruvate	7.62	7.43	7.55	7.32	7.26	7.32	7.41	7.30	7.30	7.40	7.52	7.40	7.51	7.45	7.43
	Succinate	7.46	7.58	7.56	7.59	7.61	7.59	7.50	7.58	7.55	7.39	7.01	7.58	7.23	7.51	7.56
	Valerate and derivatives	7.68	7.75	7.80	7.66	7.68	7.87	7.66	7.69	7.67	7.67	7.87	7.80	7.77	7.89	7.84
Lipid derivatives	Acetylcarnitine	6.63	6.81	6.76	6.88	6.75	6.88	6.73	6.74	6.72	6.78	7.00	6.80	6.79	6.82	6.82
	Choline and derivatives	7.98	7.82	7.88	7.75	7.84	7.88	7.94	7.82	7.80	7.88	8.08	7.89	8.00	7.96	7.89
	Glycerophosphocholine	7.31	7.31	7.37	7.34	7.34	7.40	7.34	7.32	7.29	7.33	7.46	7.37	7.37	7.41	7.37
	Phosphocholine	8.07	7.94	7.99	7.83	8.00	8.08	8.17	7.96	7.93	8.09	8.21	8.08	8.18	8.19	8.10
Carbonyl compound	Acetone	7.23	7.24	7.30	7.26	7.26	7.33	7.25	7.25	7.23	7.27	7.38	7.29	7.36	7.35	7.30
	Dihydroxyacetone	7.33	7.30	7.31	7.32	7.40	7.47	7.48	7.34	7.32	7.44	7.69	7.45	7.49	7.51	7.44
Miscellaneous	Dimethyl sulfone	6.87	6.87	6.88	6.85	6.88	6.97	6.89	6.85	6.83	6.87	7.06	6.92	6.93	6.97	6.93
	Uridine	5.96	5.93	5.96	6.35	5.96	5.99	5.95	5.90	5.87	6.00	6.09	5.93	5.98	5.96	5.95

[a] Metabolite contents are expressed as $\log_{10}$ [sum of signal intensity of respective metabolite in arbitrary unit]. Values at 4 hours are the average from two independent replicates. Values at 14 days and 28 days are represented from one replicate.

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Chapter 6

General discussion

6.1 Introduction

The first objective of this research was to investigate the simultaneous growth and metabolite production by yoghurt starters **(Chapter 2)**, and different probiotic strains during set-yoghurt fermentation and refrigerated storage. *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 represent members of the two LAB genera commonly incorporated in fermented dairy products **(Chapter 3 and 4)** [48]. *L. plantarum* WCFS1 is a potential probiotic strain originating from human saliva [24] **(Chapter 5)**. Since many probiotic strains do not survive well in fermented milk [20, 51], the second objective was to investigate the effect of sublethal preculturing of these three strains under elevated salt and low pH stress conditions on their survival in yoghurt **(Chapter 4 and 5)**. In this context, the microbial activity was evaluated in terms of bacterial population dynamics, milk acidification and formation of volatiles and non-volatile metabolites in set-yoghurt. A complementary metabolomics approach using headspace SPME-GC/MS and ^{1}H-NMR was applied for characterization of biochemical changes associated with the microbial metabolism during fermentation and storage. Finally, metabolite profiles of different yoghurt samples were statistically compared by means of multivariate analysis.

This chapter provides an overview of the studies described in this thesis. Different sections focusing on: (i) metabolomics-based analytical approach, (ii) interaction between the two yoghurt starter bacteria, (iii) incorporation of different probiotic strains and (iv) impact of sublethal preculturing on the performance of probiotics in yoghurt are respectively discussed. The main conclusions and implications of these studies are addressed, and recommendations for future research are proposed.

6.2 Metabolomic-based analytical approach

6.2.1 Analysis of volatile metabolites by headspace SPME-GC/MS

In this research, a mimic-scenario of set-yoghurt fermentation was carried out directly in a series of glass GC vials subjected to the same conditions as employed in the normal scale experiments. The difference was only the volume of milk used, i.e. 3 mL instead of 100 mL. The concept of in-vial fermentation was developed based on the method used for detection of volatiles produced by mastitis pathogens [22]. After inoculation, milk was aseptically transferred into a vial, the headspace was completely flushed with N_2 through a 0.22 μm filter. Then, the vial was immediately sealed with a silicone-septa magnetic cap. This step allows adjusting a standard atmosphere in the vial headspace prior to fermentation. The pH of set-yoghurt fermented in the vials was verified at the end of fermentation (4 h) and the end of storage (28 d) to ensure that the results were comparable with those obtained in the normal scale experiment. Extraction and determination of volatile metabolites by headspace SPME-GC/MS were based on the method developed by Hettinga et al. [21]. Volatile metabolites were identified referred to the NIST database and the library of Hettinga et al. [22]. Trial samples were spiked with acetaldehyde and ethanol in order to validate the identification process. Preliminary results revealed that the number of volatile metabolites identified in set-yoghurts fermented in vial (37 compounds) was higher than those detected in the samples prepared from a normal scale experiment (27 compounds). The list is comparable to the volatiles identified in yoghurt using headspace SPME-GC/MS technique in other studies [8, 15]. This observation indicates the advantage of in-vial fermentation, combining low volume milk samples with the prevention of volatile loss during sample preparation.

From a practical viewpoint, it should be mentioned that set-yoghurt was fermented and stored in a tightly-sealed glass vial. This type of material is impermeable and provides different protective properties compared to other packaging materials commonly used in yoghurt manufacture, e.g. laminated carton, polypropylene, polystyrene and polyethylene [57]. The tightly sealed glass vial completely prevents the

loss of volatile components. The impact of packaging materials on aroma volatile compositions and sensory characteristics of yoghurt has been documented [40, 45]. This consideration needs to be taken into account when comparing the results in this research with those in a practical situation.

6.2.2 Analysis of non-volatile polar metabolites by ^{1}H- NMR

From a technical standpoint, it should be mentioned that the pH of the sample strongly influences data acquisition during ^{1}H-NMR measurements due to variation in peak intensities and location across samples [35]. This fact is acknowledged by several authors and careful pH adjustment is generally required to complex samples, such as wine, blood serum or urine [27, 49]. A slight variation in pH might induce changes in the position and the overall shape of the peaks. Deviations in the exact peak positions in the spectra result in a significantly lower reproducibility of metabolite identification and quantification, especially when an automatic integration of the signal intensities is applied [35]. Therefore, pH adjustment and buffering of yoghurt samples were performed to achieve a low variation in the final ^{1}H-NMR samples (defined at pH 6.0) in this research. Furthermore, the presence of biomacromolecules, especially lipids and proteins, produces interference of the ^{1}H-NMR spectra with broad background signals due to their limited rotational diffusion and short relaxation times [23, 56]. This consequently results in a lower sensitivity for peaks identification and quantification [4]. To obtain high quality ^{1}H-NMR spectra, therefore, pre-treatments of yoghurt samples are required. Residual lipid fractions need to be removed by dichloromethane extraction, despite the use of reconstituted Nilac skimmed milk was this research. Large (caseins) and small protein (whey proteins) fractions were removed using ultra-centrifugation and ultra-filtration, respectively. Finally, a clear liquid fraction of yoghurt serum was introduced to the ^{1}H-NMR analysis.

For accurate metabolite profiling, several manipulations regarding different ^{1}H-NMR spectral elucidations (NOESY, JRES, Skyline) and quantification techniques (manual, semi-manual and automatic peaks integration) were evaluated. Finally, an automatic calculation of the signal intensities within specified segments of spectrum

(binning) was selected for quantification of ^{1}H-NMR spectra in this research. The binning technique not only attempts to minimize variations and the time-required for manual peak integration but also to produce suitable datasets for pattern recognition by multivariate statistical analysis [1]. The chemical shift across the range of 0.00 - 10.00 ppm of a spectrum was segmented with an interval of 0.02 ppm (bin). The signal intensity in each bin was calculated by applying automatic integration software. This step allows extracting the ^{1}H-NMR spectral profiles of yoghurt samples from a non-targeted approach. A series of 40 pure reference compound solutions were analyzed for validation of the accuracy of the identification process. Since interactions between various metabolites may occur in the milk serum, 15 reference compounds were spiked in the trial samples for final validation of the ^{1}H-NMR method. Although the overall spectrum was dominated by broad signals from sugars, especially lactose, a total of 43 non-volatile polar metabolites were identified in this research. Relative quantification of a given metabolite was achieved by summation of signal intensities in all bins [38]. At this stage, non-volatile polar metabolite profiles of yoghurt samples were obtained from a targeted approach.

6.2.3 Data processing and statistical analysis

Metabolomic data derived from GC/MS and ^{1}H-NMR were normalized by median-centering and $\log_2$-scaling before subjecting to multivariate analysis. The pre-processing of data was aimed to modify the relative influences (variances) of the scaled variables and to align the entire dataset into a normal distribution [5]. Multivariate analysis reduced the dimension of the dataset [32]. In this research, two algorithms: (i) principal component analysis (PCA) and (ii) hierarchical cluster analysis (HCA) were used to extract significant patterns from the metabolite profiles of yoghurts. The classification potential of PCA and HCA were compared from their corresponding distinction patterns.

The results obtained in this research demonstrated the effectiveness of quantitative metabolite profiling combined with multivariate analysis as a tool to distinguish the molecular profiles of yoghurts. The two algorithms were equally effective in distinguishing metabolite profiles among yoghurt samples fermented with different

types of starter combinations especially when different probiotic strains were incorporated. Indicative metabolites as obtained by loading plots allow recognition of specific combinations of yoghurt starters and probiotics. PCA appeared to be more effective than HCA in the comparison of yoghurts co-fermented with precultured probiotics of the same strain (Chapter 4 and 5). Since HCA classifies samples according to the overall similarity, this algorithm is less suitable when the metabolite profiles of yoghurts become more alike.

6.3 Interaction between different proteolytic strains of *S. thermophilus* in co-culture with *L. delbrueckii* subsp. *bulgaricus*

S. thermophilus and *L. delbrueckii* subsp. *bulgaricus* are recognized as auxotrophs, i.e. lacking the ability to synthesize one or more essential nutrients, in particular for a number of amino acids [26]. Since the concentration of free nitrogen sources in milk is very limited [63], optimal growth of yoghurt starters must depend on their proteolytic capacity. It is well documented that bacterial cell-envelope associated (extracellular) proteases are responsible for the initial step of casein hydrolysis yielding a large number of free amino acids and oligopeptides [26]. Most commonly, yoghurt starters consist of non-proteolytic *S. thermophilus* and proteolytic *L. delbrueckii* subsp. *bulgaricus* [62]. The proper growth of streptococci thus relies on the proteolytic activity of lactobacilli to produce sufficient amino acids [10]. However, the expression of proteolytic activity in several *S. thermophilus* strains allows them to grow independently in milk leading to substantial acid production [9, 11, 16, 29, 52]. In Chapter 2, the interaction between proteolytic (Prt+) and non-proteolytic (Prt-) strains of *S. thermophilus* in co-culture with *L. delbrueckii* subsp. *bulgaricus* during set-yoghurt fermentation is described.

The influence of bacterial proteolytic activity is characterized by an overall increase in concentration of free amino acids in the growth medium. The ^{1}H-NMR measurement reflects the balance between the consumption and production of these compounds in mixed cultures of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Table S2.2). The result revealed that proto-cooperation between *S. thermophilus* Prt-

and *L. delbrueckii* subsp. *bulgaricus* provided not only growth stimulatory effect on the two species but also activated the proteolytic activity of *L. delbrueckii* subsp. *bulgaricus* resulting in significantly higher abundance in the concentration of free amino acids. This statement is supported by the work of Sieuwerts et al. [55] who reported a considerably higher expression of the proteolytic gene (*prtB-LBUL-1105*) encoding the extracellular protease activity of *L. delbrueckii* subsp. *bulgaricus* in a mixed culture.

Furthermore, catabolism of amino acids generates volatile metabolites responsible for the aroma profile of fermented dairy products [2, 34, 59]. For example, acetaldehyde is the most important compound contributing to typical yoghurt aroma [6]. This compound is derived from threonine catabolism by the activity of threonine aldolase in *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* [57]. The results demonstrated that proto-cooperation between *S. thermophilus* Prt- and *L. delbrueckii* subsp. *bulgaricus* generated a favorable volatile profile with significant abundance of all key aroma compounds of yoghurt compared to the mixed culture of *S. thermophilus* Prt+ and *L. delbrueckii* subsp. *bulgaricus* (Fig. 2.4).

The main finding of the study described in Chapter 2 reveals that proteolytic activity is essential for the optimal growth of *S. thermophilus* when present individually in milk. On the other hand, proto-cooperation is exclusively observed between *S. thermophilus* Prt- and *L. delbrueckii* subsp. *bulgaricus* resulting in significant higher populations of the two species, more efficient milk acidification and significant abundance of aroma volatiles and non-volatile metabolites desirable for a good organoleptic quality of yoghurt. These observations indicate that selection of suitable strain combinations between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in yoghurt starters is important for achieving the best quality of the product.

6.4 Incorporation of selected probiotic strains in set-yoghurt

6.4.1 Selective enumeration of probiotic bacteria in combination with yoghurt starters

Yoghurt is one of the most widely marketed dairy products that is used to carry probiotic bacteria [30]. In order to ensure the delivery of potential health-benefits, a probiotic product should contain at least 10^6 cfu/g of viable probiotic bacteria during the shelf life [61]. Important for assessing the functionality of probiotic yoghurt is therefore the enumeration of viable probiotic cells. In practice, selective enumeration of individual probiotic strain in yoghurt is rather complicated due to the presence of multiple and closely related LAB species including yoghurt starters as well as other probiotic strains [44]. The difficulties are mainly caused by the similarity in their growth requirements, biochemical characteristics and antibiotic susceptibilities [3]. Selective media and differential enumeration methods for each individual strain of yoghurt starters and probiotics were adapted from literature [3] and validated with the strains co-cultivated together in this research (Table 6.1).

6.4.2 Effect of inoculation moment on the survival of probiotics in set-yoghurt

A preliminary research was performed to investigate the effect of inoculation moment on the survival of *B. animalis* subsp. *lactis* BB12 and *L. plantarum* WCFS1 in yoghurt during refrigerated storage. Reductions in viable counts of probiotics inoculated at different moments were evaluated: (i) inoculation together with yoghurt starters into milk at the beginning of fermentation and (ii) inoculation into yoghurt at the end of fermentation. Inoculation together with yoghurt starters at the beginning of fermentation resulted in a higher survival of probiotics during refrigerated storage (data not shown). It has been documented that dynamic changes during the fermentation generate a number of harsh conditions such as low pH, substrate breakdown products and cell population density in the environment [50]. These stress conditions may induce broad metabolic adaptations facilitating better survival and different performance of microorganisms involved as was concluded by Van de Guchte et al. [60]. This could indeed also be the case for probiotic strains present in yoghurt during fermentation.

Table 6.1. Media used for selective enumeration of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. rhamnosus* GG, *B. animalis* subsp. *lactis* BB12 and *L. plantarum* WCFS1 in set-yoghurt

Bacterial strain	Medium[a]	Selectivity mediator	Incubation
S. thermophilus	STA	sucrose and aerobic incubation	37 °C for 24 h.
L. delbrueckii subsp. *bulgaricus*	MRS pH 5.7	(i)[b] pH and anaerobic incubation	37 °C for 48 h.
	MRSCi	(ii) ciprofloxacin	37 °C for 48 h.
	MRS pH 5.7	(iii) modified atmosphere[c] incubation	37 °C for 48 h.
	MRS pH 5.7	(iv) incubation temperature at 45 °C	45 °C for 72 h.
L. rhamnosus GG	MRSV	vancomycin	37 °C for 48 h.
B. animalis subsp. *lactis* BB12	BSM	cysteine-HCl and mupirocin	37 °C for 48 h.
L. plantarum WCFS1	MRSV	vancomycin	37 °C for 24 h.

[a] MRS: deMan Rogosa Sharpe, STA: *Streptococcus thermophilus* agar, MRSCi: MRS supplemented with ciprofloxacin, MRSV: MRS supplemented with vancomycin, BSM: Bifidobacteria selective medium

[b] (i): in co-culture with *S. thermophilus*, (ii): in co-culture with *S. thermophilus* and *L. rhamnosus* GG, (iii): in co-culture with *S. thermophilus* and *B. animalis* subsp. *lactis* BB12, (iv): in co-culture with *S. thermophilus* and *L. plantarum* WCFS1

[c] low oxygen (6% O_2, 7% CO_2) condition (Anoxomat™ Mart® Microbiology, Drachten, the Netherlands)

6.4.3 Growth and survival of selected probiotic strains in set-yoghurt

Production of fermented dairy products containing probiotics is a major challenge for the dairy industry, since milk is not considered as a suitable growth medium for these microorganisms [36]. The limited capacity of *L. rhamnosus* GG to develop in milk is explained by the lack of ability to ferment lactose [13]. The weak proteolytic activity explains the poor growth of *B. animalis* subsp. *lactis* BB12 and *L. plantarum* WCFS1 in milk [19, 36].

The study described in Chapter 3 revealed a stimulatory effect of co-cultivation of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 with yoghurt starters on their growth. The growth of *L. rhamnosus* GG was slightly enhanced while that of *B. animalis* subsp. *lactis* BB12 was evidently stimulated (Fig. 3.1). Based on the ^{1}H-NMR results described in Chapter 2, the growth of bifidobacteria might be stimulated by free amino

acids generated from the active proteolytic activity of *L. delbrueckii* subsp. *bulgaricus* in the mixed culture. This observation is in accordance with literature [54]. On the other hand, co-cultivation with yoghurt starters also resulted in an impairment of the survival of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12. The viable count of *L. rhamnosus* GG decreased slightly while that of *B. animalis* subsp. *lactis* BB12 decreased drastically towards the end of storage (Fig. 3.1). The decrease in pH and accumulation of organic acids in fermented milk during refrigerated storage, also called post-acidification, have been recognized as one of the most detrimental factors for the survival of probiotics [12].

Based on the results described in Chapter 5, it is interesting to see that the population of *L. plantarum* WCFS1 in set-yoghurt remains stable from the starting point of fermentation until the end of storage (Fig. 5.1C). The good survival of various *L. plantarum* strains in fermented milk has been previously documented [19, 33]. This observation makes *L. plantarum* WCFS1 a good candidate probiotic strain for yoghurt manufacture. The studies described in Chapter 3 and 5 indicate that the two probiotic lactobacilli are more tolerant to acidic condition of yoghurt than bifidobacteria. This finding is in accordance with literature [12, 14]. However, it should be mentioned that the final viable counts of all probiotic strains in this research still remained above the minimum recommended level (10^6 cfu/g) to ensure their beneficial effects on consumers health [53].

6.4.4 Impact of probiotic incorporation on the metabolite formation in set-yoghurt

Compared to yoghurt starters, there is still limited information regarding the metabolic activity of probiotics in milk [39]. This information is important, since the organic acids and volatile compounds formed by these functional bacteria may directly influence the organoleptic quality of product [37]. In this research, a complementary metabolomics approach, using headspace SPME-GC/MS and ^{1}H-NMR, was applied for characterization of the volatile and non-volatile polar metabolite profiles of set-yoghurts. The studies described in Chapter 3 and 5 revealed that incorporation of *L. rhamnosus* GG, *B. animalis* subsp. *lactis* BB12 and *L. plantarum* WCFS1 did not

significantly influence the acidity and concentrations of key-aroma volatile compounds of set-yoghurt. Still, all probiotic strains had a significant impact on the overall yoghurt metabolite profiles. The presence of probiotics substantially contributed to the formation of a large number of volatile and non-volatile metabolites detected at low concentration.

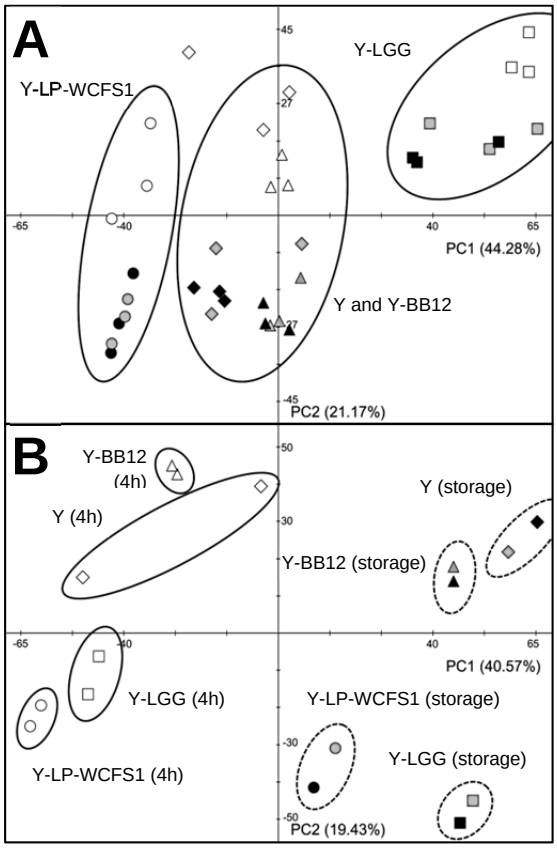

Fig. 6.1. PCA score plots derived from volatile (panel A) and non-volatile polar metabolite profiles (panel B) of set-yoghurts fermented with traditional yoghurt starters (Y; ◇) co-cultures of yoghurt starters with *L. rhamnosus* GG (Y-LGG; □), *B. animalis* subsp. *lactis* BB12 (Y-BB12; △) and *L. plantarum* WCFS1 (Y-LP-WCFS1; ○). White, grey and black filled blocks correspond to the samples at 4 hours, 14 days and 28 days, respectively.

Variation in the overall metabolite profiles of set-yoghurts co-fermented with different probiotic strains could be statistically determined using multivariate analysis. PCA and HCA provided pattern recognition and classification of yoghurt metabolite

profiles. Moreover, loading plots indicated which metabolites were accountable for the separation. The classification potentials of GC/MS and ^{1}H-NMR techniques were compared from their corresponding PCA score plots (Fig. 6.1). A good comparable pattern of the two metabolite profiling platforms was observed. The two approaches successfully enabled to distinguish yoghurts fermented by different starter combinations and different durations of storage according to their metabolite profiles. The result demonstrates that metabolomic datasets acquired by two different analytical approaches support each other in the discrimination of the metabolome of yoghurt. The metabolite profiles of the samples co-fermented with *B. animalis* subsp. *lactis* BB12 are comparatively close to the samples fermented without probiotics. On the other hand, distinct metabolite profiles were clearly observed for the samples co-fermented with *L. rhamnosus* GG and *L. plantarum* WCFS1. This finding suggests a substantial impact on biochemical composition of yoghurt due to the presence of the two lactobacilli, although their capacity to grow in association with yoghurt starters is rather limited.

6.5 Preculturing of probiotics under sublethal stress conditions

6.5.1 Growth and survival of sublethally precultured probiotics in set-yoghurt

One of the key criteria in the development of yoghurt containing probiotics is that these functional bacteria must survive the harsh conditions encountered during fermentation and refrigerated storage [47]. Although *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 are commercially selected based on their appropriate technological performance [42], the study described in Chapter 3 reveals that significant decrease in viable counts of the two probiotic strains still occurs in practice. Regarding to this, a strategy to induce cell-protective mechanisms by preculturing of probiotics under sublethal stress conditions (combinations of elevated NaCl and low pH) prior to inoculation in milk was investigated in Chapter 4 and 5. Such an approach allows probiotics to develop adaptive stress responses leading to an increase in their survival compared to those that are directly subjected under the same stress condition [43].

The study described in Chapter 4 demonstrated that preculturing of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 under sublethal salt and low pH stress conditions did not significantly enhance their growth during set-yoghurt fermentation. On the other hand, their survival during refrigerated storage was successfully improved, specifically by preculturing at relatively low pH (Fig. 4.1). Furthermore, the growth and survival of the yoghurt starters were not affected by the incorporation of sublethally precultured *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12.

The study described in Chapter 5 showed that preculturing under sublethal salt and low pH stress conditions could not significantly enhance the growth of *L. plantarum* WCFS1 in milk but also did not impair its excellent survival in set-yoghurt (Fig. 5.1). Interestingly, the survival of *L. delbrueckii* subsp. *bulgaricus* during refrigerated storage was significantly impaired by co-cultivation with certain types of sublethally precultured *L. plantarum* WCFS1. Indeed, this adverse effect was not observed on the survival of *S. thermophilus*. A proposed explanation for this could be that sublethal preculturing may trigger the synthesis of certain antimicrobial compounds, e.g. bacteriocins, in stress-adapted *L. plantarum* WCFS1 which provide inhibitory effect on *L. delbrueckii* subsp. *bulgaricus*. It has been recognized that environmental factors as well as co-cultivation with a variety of bacterial strains play an important role in the regulation of bacteriocin production in *L. plantarum* [28, 31]. However, additional experiments are needed to confirm the evidence of bacteriocin production in sublethally precultured *L. plantarum* WCFS1.

6.5.2 Impact of sublethally precultured probiotics on the metabolite formation in set-yoghurt

Adaptive stress responses in probiotics are associated with the expression of a large number of genes, synthesis of stress-response proteins and alteration of various physiological features [25, 41, 46, 60]. This complex network of reactions induces variations in the metabolic activities of probiotics which may have a substantial impact on the biochemical characteristics of the product [50]. The studies described in Chapter 4 and 5 revealed adaptive responses of *L. rhamnosus* GG, *B. animalis* subsp. *lactis* BB12 and *L. plantarum* WCFS1 to sublethal salt and low pH stress conditions exposed during

preculturing. The sublethal preculturing not only affects their growth and survival during yoghurt production but also leads to substantial changes in the biochemical characteristics of the final product. Besides the direct impact on the activity of probiotics, the significantly lower post-acidification observed in the samples co-fermented with sublethally precultured *L. plantarum* WCFS1 could probably be associated with the decrease in viable counts of *L. delbrueckii* subsp. *bulgaricus*.

The impact of stress-adapted probiotics on the volatile metabolite profiles of set-yoghurts was clearly revealed by PCA. The volatile profiles of the samples co-fermented with sublethally precultured lactobacilli, i.e. *L. rhamnosus* GG and *L. plantarum* WCFS1, could be distinguished based on the salt concentration (Fig. 4.3C and 5.3) whilst those co-fermented with sublethally precultured *B. animalis* subsp. *lactis* BB12 could be distinguished based on pH levels during preculturing (Fig. 4.4C). This finding demonstrates that the impact of sublethal stress responses on the volatile metabolite profiles of set-yoghurts is species-specific. Furthermore, loading plots indicated that several major aroma compounds, i.e. acetic acid (vinegar, pungent), 2,3-pentanedione (buttery, vanilla-like) and 2-butanone (sweet, fruity), as well as other minor carbonyl compounds, volatile organic acids and alcohols contributed to the separation of samples co-fermented with different types of sublethally precultured probiotics. From a technological standpoint, variations in the concentration of these compounds suggest that incorporation of sublethally precultured probiotics may influence the organoleptic quality of yoghurt [6, 7].

The impact of stress-adapted probiotics on the non-volatile polar metabolite profiles of set-yoghurts was not evident for all strains. Non-volatile metabolite profiles of the samples co-fermented with different types of sublethally precultured *L. rhamnosus* GG and *L. plantarum* WCFS1 were clearly distinguished by PCA according to the concentrations of salt and levels of pH manipulated during preculturing (Fig. 4.5 and 5.5). Indeed, it should be noted that the distinct patterns were in accordance with those previously observed with the volatile metabolite profiles. Unlike for the two lactobacilli, non-volatile metabolite profiles of the samples co-fermented with different types of sublethally precultured *B. animalis* subsp. *lactis* BB12 were rather similar (Fig. 4.6). This

result suggests less change in the metabolic activity of bifidobacteria induced by sublethal preculturing compared to those observed in lactobacilli.

6.6 Main Conclusions

The studies described in this thesis provide new information regarding the impact of interaction between the two yoghurt starter bacteria and incorporation of different probiotic strains on the metabolite formation in set-yoghurt. A complementary metabolomics approach using headspace SPME-GC/MS and ^{1}H-NMR has shown to be very useful for characterization of volatile and non-volatile polar metabolites changed during fermentation and refrigerated storage. This information is important, since the biochemical conversions of milk components related to microbial metabolism are responsible for the sensory characteristics of yoghurt. Based on the main observations described in the previous chapters, the overall conclusions of this research are:

- A complementary metabolomics approach using headspace SPME-GC/MS and ^{1}H-NMR resulted in the identification of 37 volatiles and 43 non-volatile polar metabolites, respectively. The advantages of in-vial fermentation combined with headspace SPME-GC/MS technique are that only a small amount of sample is required; and there is no loss of volatiles during sample preparation. The advantages of ^{1}H-NMR technique are the minimal pre-treatment required and the simultaneous measurement of all non-volatile polar metabolites present in the sample.

- Multivariate analysis enables to recognize yoghurt metabolite profiles according to different types of starter combinations as well as durations of storage.

- Proto-cooperation is exclusively observed between non-proteolytic *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* resulting in a significant higher population size of the two species, a more efficient milk acidification

and a significant abundance of aroma volatiles and non-volatile metabolites desirable for a good organoleptic quality of yoghurt.

- Co-fermentation of yoghurt starters with probiotics stimulates the growth of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 in milk whilst the growth of *L. plantarum* WCFS1 was not affected. During refrigerated storage, the two probiotic lactobacilli exhibit more tolerance to the acid condition of yoghurt than the bifidobacteria.

- An improved survival of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 in yoghurt can be obtained by preculturing under sublethal salt and low pH stress conditions. Specifically, preculturing at relatively low pH condition was effective for a better survival.

- Application of stress-adapted *L. plantarum* WCFS1 significantly impairs the survival of *L. delbrueckii* subsp. *bulgaricus,* which consequently reduces the post-acidification of yoghurt.

- The presence of probiotics and their stress-adapted cultures induces significant changes in the global metabolite profile of yoghurt. Variations in relative abundances of key-aroma compounds may considerably influence the organoleptic quality of product.

6.7 Implications and recommendations

The metabolomics approach applied in this research provides datasets which are particularly useful for unravelling the impact of probiotic incorporation on the overall biochemical composition and organoleptic quality of yoghurt. The application of headspace SPME-GC/MS and ^{1}H-NMR combined with multivariate analysis enables to monitor the overall biochemical changes related to microbial metabolism during yoghurt fermentation and refrigerated storage. Besides this, indicative metabolites suggested by PCA can be considered as potential biomarkers for detection of particular probiotic strains. The unique features of the molecular profiles may also facilitate authentication of yoghurt in the future. The overall findings contribute to a better understanding of the metabolic activity of probiotics in an actual food environment. In terms of technical implication, similar approaches of sample preparation, measurement and data processing can certainly be extended to investigate the molecular profiles of other fermented dairy products.

Development of sublethal preculturing for improving the survival of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 has shown to be very successful at laboratory scale. Accordingly, it would be interesting to investigate whether these results can be extended in the pilot-scale preparation of probiotic cultures. Another important finding is that application of sublethally precultured *L. plantarum* WCFS1 provides significant impairment on the survival of *L. delbrueckii* subsp. *bulgaricus* and adverse effect on post-acidification. This observation is technologically relevant since post-acidification is recognized as the main detrimental factor for the survival of probiotics in fermented milk [51]. Reducing post-acidification might be an interest for future development in the mild-flavor yoghurt. Thus, the mechanism explaining the inhibitory effect of stress-adapted *L. plantarum* WCFS1 on the survival of *L. delbrueckii* subsp. *bulgaricus* requires further investigation. From a sensory perspective, it should be noted that stress-adapted probiotics induce substantial changes in the yoghurt metabolome which may influence the organoleptic quality of product. An additional research focusing on sensory evaluation of yoghurt with trained panelists is therefore recommended. Last but not least, it needs to be mentioned that adaptive stress responses of probiotics are strain

specific; therefore, optimization of preculturing conditions is necessary for each probiotic strain to achieve a notable performance improvement.

Based on the studies described in this thesis, it appears very interesting to broaden the knowledge with follow-up research on a range of probiotic strains. Another relevant extension is the investigation of the metabolite profiles of stirred yoghurts. The lower fermentation temperature combined with longer incubation period is expected to affect the performance of yoghurt starters and probiotics which could have a significant impact on the properties of product. From a technical viewpoint, improving the acquisition and interpretation of metabolomics data from the two analytical platforms should be considered. First, the absolute concentration of key aroma volatiles and non-volatile metabolites responsible for sensory characteristics of yoghurt should be quantified. This will provide more insight whether the amount of these compounds is detected within the same ranges as normally found in literature or commercial products. Based on this information, potential impact on the organoleptic quality of yoghurt could be appropriately predicted. Second, it should be noted that the identification of non-volatile polar metabolites by ^{1}H-NMR is still hampered by the dominating broad signal of lactose. Eliminating either lactose from the sample or lactose peaks from the spectrum would enable to extend the list of identified metabolites. Also the application of a correlation network to elucidate the time-dependent relationships between volatiles and non-volatile metabolites developed during yoghurt fermentation and refrigerated will provide extra useful information.

Furthermore, future research in this field must take advantage of the development of LAB genome projects and functional genomics technologies. A large number of publications and accessible databases generated from transcriptomic and proteomic profiling will facilitate to better understand the molecular mechanisms involved in the interaction between different microorganisms in fermented food environments. Application of genome-scale metabolic models to predict the formation of flavor compounds is one of the most relevant examples [17, 18, 58]. This information may finally contribute to establish an appropriate route toward improving technological and functional properties of fermented dairy products.

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Summary

The activity of starter cultures during yoghurt fermentation is one of the most important factors that determine the fermentation process and sensory characteristics of product. During the past decades, societal interest in healthy foods has contributed to the development of functional yoghurt variants that have been made by incorporation of health-promoting bacterial strains called “probiotics”. Compared to yoghurt starters, there is still limited information regarding the actual metabolic activity of probiotics grown or suspended in milk. Therefore, the main objective of this research was to investigate the simultaneous growth and metabolite production by yoghurt starters and different probiotic strains during set-yoghurt fermentation and refrigerated storage. *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 represent members of the two LAB genera commonly incorporated in fermented dairy products. *L. plantarum* WCFS1 is a potential probiotic strain used as a model, in this research, to study the activity of non-dairy LAB in a dairy-based environment. The microbial activity during fermentation and refrigerated storage was investigated by monitoring bacterial population dynamics, milk acidification and changes in volatile and non-volatile metabolite profiles of yoghurt.

In **Chapter 2**, the interaction between different proteolytic strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* during set-yoghurt fermentation was investigated. A complementary metabolomics approach was applied for characterization of volatile and non-volatile polar metabolite profiles of yoghurt associated with proteolytic activity of the individual strains in the starter cultures. Headspace SPME-GC/MS and ^{1}H-NMR resulted in the identification of 35 volatiles and 43 non-volatile polar metabolites, respectively. The results demonstrated that only non-proteolytic *S. thermophilus* strain performed proto-cooperation with *L. delbrueckii* subsp. *bulgaricus*. The proto-cooperation resulted in significant higher populations of the two species, faster milk acidification, significant abundance of aroma volatiles and non-volatile metabolites desirable for a good organoleptic quality of yoghurt. This finding underlines that selection of suitable strain combinations in yoghurt starters is important for achieving the best technological performance regarding the quality of product.

The study described in **Chapter 3** was aimed to evaluate the impact of two commercial probiotic strains, *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12, in co-fermentation with traditional yoghurt starters on the metabolite formation in set-yoghurt. The results revealed that the two probiotic strains did not influence acidity and the major aroma volatile compounds of yoghurt. However, the presence of *L. rhamnosus* GG contributed to a remarkable change in non-volatile metabolite profile of yoghurt during refrigerated storage. Multivariate analysis allowed distinguishing yoghurts fermented by different starter combinations and different durations of storage according to their metabolite profiles.

Since many probiotic strains are not able to survive well in fermented milk, a strategy to enhance their survival was additionally applied by preculturing the three probiotic strains under sublethal stress conditions (combination of elevated salt and low pH) in a batch fermentor prior to inoculation in milk. In **Chapter 4**, the effect of sublethal preculturing on the survival of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 and metabolite formation in set-yoghurt was discussed. The results demonstrated adaptive stress responses of the two probiotic strains resulting in their viability improvement without adverse influence on milk acidification. PCA revealed substantial impact of sublethally precultured probiotics on metabolite formation demonstrated by distinctive volatile and non-volatile metabolite profiles of yoghurt. This study demonstrates a potential application of stress-adapted probiotics in an actual food-carrier environment.

The study described in **Chapter 5** continued on the framework of the previous studies to evaluate the performance of potential probiotic *L. plantarum* WCFS1 in co-fermentation with yoghurt starters and investigate the impact of sublethal preculturing on its survival and metabolite formation in set-yoghurt. The results demonstrated that sublethal preculturing did not significantly affect the growth and survival of *L. plantarum* WCFS1. Alternatively, incorporation of sublethally precultured *L. plantarum* WCFS1 significantly impaired the survival of *L. bulgaricus* which consequently reduced the post acidification of yoghurt during refrigerated storage. PCA revealed substantial impact of *L. plantarum* WCFS1 on the metabolite profiles of yoghurt. This study provides

insight in the technological implications of *L. plantarum* WCFS1, such as its good stability in fermented milk together with the inhibitory effect on post-acidification.

In conclusion, the studies described in this thesis revealed that incorporation of *L. rhamnosus* GG, *B. animalis* subsp. *lactis* BB12 and *L. plantarum* WCFS1 did not significantly influence the acidity and concentrations of major aroma volatile compounds of set-yoghurt. Still, all probiotic strains had a significant impact on the overall yoghurt metabolite profiles. The presence of probiotics considerably contributed to the formation of a large number of volatile and non-volatile metabolites detected at low concentration. Variation in the overall metabolite profiles of yoghurts co-fermented with different probiotic strains could be statistically determined using multivariate analysis. The outcomes provided pattern recognition and classification of yoghurt metabolite profiles. Moreover, indicative metabolites suggested by PCA can be considered as potential biomarkers for detection of particular probiotic strains. The unique features of the molecular profiles may also facilitate authentication of yoghurt in the future. Development of sublethal preculturing for improving the survival of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 has shown to be very successful at laboratory scale. Another important finding is that application of sublethally precultured *L. plantarum* WCFS1 provides significant impairment on post-acidification. This observation is technologically relevant since post-acidification is recognized as the main detrimental factor for the survival of probiotics in fermented milk. Furthermore, reducing post-acidification might be an interest for future development in the mild-flavor yoghurt. From a sensory perspective, it should be noted that stress-adapted probiotics induce substantial changes in the metabolome of yoghurt. This finding is important, since variations in the relative abundance of various organic acids, aroma volatiles and proteolytic-derived compounds may directly influence the organoleptic quality of product. Thus, an additional research focusing on sensory evaluation of yoghurt with trained panelists is recommended. Finally, similar metabolomic-based analytical approaches can certainly be extended to investigate the molecular profiles of other fermented dairy products.

Samenvatting

De activiteit van starter culturen tijdens yoghurt fermentatie is een van de belangrijkste factoren die het fermentatie proces en sensorische eigenschappen van het product bepaald. De afgelopen decennia heeft de maatschappelijke interesse in gezonde voeding bijgedragen aan de ontwikkeling van functionele yoghurt varianten die gebruik maken van gezondheid bevorderende bacterie streng; de zogenaamde 'probiotica'. In vergelijking tot yoghurt starter culturen, is er maar weinig informatie beschikbaar over de werkelijke metabolische activiteit van probiotica die groeien of gesuspendeerd zijn in melk. Daarom was het hoofddoel van dit onderzoek om de simultane groei en metaboliet productie te onderzoeken van yoghurt starters en verschillende probiotica strengen tijdens standyoghurt fermentatie en gekoelde opslag. *L. rhamnosus* GG en *B. animalis* subsoort *lactis* BB12 zijn twee leden van de LAB genera die veel toegepast worden in gefermenteerde zuivel producten. *L. plantarum* WCFS1 is een potentiële probiotica streng, die als model gebruikt wordt in dit onderzoek, voor de studie naar activiteit van niet-zuivel LAB in een zuivel omgeving. De microbiële activiteit tijdens fermentatie en gekoelde opslag was onderzocht door het monitoren van drie factoren; de bacteriële populatie dynamiek, het verzuren van melk en het veranderen van vluchtige en niet-vluchtige metabolische profielen van yoghurt.

In **Hoofdstuk 2** werd de interactie tussen verschillende proteolytische streng van *S. thermophilus* en *L. delbrueckii* subsoort *bulgaricus* tijdens standyoghurt fermentatie onderzocht. Een aanvullende metabolische aanpak werd toegepast voor de karakterisering van vluchtige en niet-vluchtige polaire metabolische profielen van yoghurt geassocieerd met proteolytische activiteit van de individuele strengen in de starter culturen. Het gebruik van headspace SPME-GC/MS en ^{1}H-NMR resulteerde in de identificatie van respectievelijk, 35 vluchtige en 43 niet-vluchtige polaire metabolieten. Uit de resultaten bleek dat alleen niet-proteolytische *S. thermophilus* streng proto-cooperatie aanging met *L. delbrueckii* subsoort *bulgaricus*. De proto-cooperatie resulteerde in significant hogere populaties van de twee soorten, snellere verzuring van de melk en significante aanwezigheid van vluchtige en niet-vluchtige aroma

metabolieten die gewenst zijn voor een goede organoleptische kwaliteit van yoghurt. Deze resultaten onderstrepen het belang van selectie van geschikte bacterie streng combinaties in yoghurt starters om de beste technologische prestatie te behalen voor product kwaliteit.

De studie die in **Hoofstuk 3** is beschreven, was gericht op het evalueren van de invloed van twee commerciële probiotica strengen, *L. rhamnosus* GG en *B. animalis* subsoort *lactis* BB12, op metaboliet vorming in standyoghurt tijdens co-fermentatie met traditionele yoghurt starters. De resultaten onthulden dat de twee probiotica strengen geen invloed hadden op de zuurgraad en de meest-voorkomende vluchtige aroma componenten in yoghurt. Echter, de aanwezigheid van *L. rhamnosus* GG droeg bij aan een aanzienlijke verandering in het niet-vluchtige metabolische profiel van yoghurt tijdens gekoelde opslag. Met behulp van een multivariabele analyse kon yoghurt die gefermenteerd was met verschillende starter combinaties en verschillende opslag tijden gescheiden worden, gebaseerd op hun metabolische profiel.

Sinds veel probiotica strengen niet kunnen overleven in gefermenteerde melk, werd een strategie toegepast om hun levensduur te verlengen door te pre-cultiveren onder sub-letale stress condities (combinatie van verhoogde zout concentraties en lage pH) in een batch fermentor voor inoculatie in de melk. In **Hoofdstuk 4**, werd het effect van sub-letaal pre-cultiveren op de overleving van *L. rhamnosus* GG en *B. animalis* subsoort *lactis* BB12 en metaboliet formatie in standyoghurt bediscussieerd. De resultaten lieten zien dat adaptieve stress reacties van de twee probiotica strengen resulteerden in een verbetering van hun levensvatbaarheid zonder de verzuring van de melk te beïnvloeden. Het gebruik van PCA liet zien dat er een substantiële invloed van sub-letale gepre-cultiveerde probiotica was op metaboliet formatie, gedemonstreerd door verschillende vluchtige en niet-vluchtige metabolische profielen van yoghurt. De studie liet een potentiële toepassing van stress-geadapteerde probiotica zien in een echte voedsel matrix.

De studie in **Hoofdstuk 5** vervolgde het raamwerk van de eerdere studies met het evalueren van de prestatie van potentiële probiotica *L. plantarum* WCFS1 streng in

co-fermentatie met yoghurt starters en om de invloed van sub-letale pre-cultivering te onderzoeken op hun overleving en metaboliet formatie in standyoghurt. De resultaten lieten zien dat sub-letale pre-cultivering geen significante invloed had op de groei en overleving van *L. plantarum* WCFS1. Maar incorporatie van sub-letaal gepre-cultiveerde *L. plantarum* WCFS1 verhinderde de overleving van *L. bulgaricus* significant, wat vervolgens ook leidde tot reductie van naverzuring van yoghurt tijdens gekoelde opslag. Deze studie geeft inzicht in de technologische implicaties van *L. plantarum* WCFS1, zoals de goede stabiliteit in gefermenteerde melk in samenhang met het beletten van naverzuring.

Tot conclusie, de studies die zijn geschreven in dit proefschrift lieten zien dat incorporatie van *L. rhamnosus* GG, *B. animalis* subsoort *lactis* BB12 en *L. plantarum* WCFS1 geen significante invloed hadden op de zuurgraad en concentraties van veelvoorkomende vluchtige aroma componenten van standyoghurt. Echter, alle probiotica strengen hadden een significante invloed op het gehele metabolische profiel van yoghurt. De aanwezigheid van probiotica droeg aanzienlijk bij aan de vorming van een groot aantal vluchtige en niet-vluchtige metabolieten, die bij een lage concentratie gedetecteerd konden worden. Variatie in de algehele metabolische profielen van yoghurt die geco-fermenteerd was met verschillende probiotica strengen, kon statistisch worden vastgesteld met multivariabele analyses. Bovendien konden sommige metabolieten die waren voorgesteld door PCA worden beschouwd als potentiële biomarkers voor detectie van specifieke probiotica strengen. De unieke eigenschappen van de moleculaire profielen kan ook bijdragen aan het authentiseren van yoghurt in de toekomst. De ontwikkeling van sub-letale precultivering for het verbeteren van de overleving van *L. rhamnosus* GG en *B. animalis* subsoort *lactis* BB12 was op labschaal zeer succesvol. Een andere belangrijke vinding is dat de toepassing van sub-letaal gepre-cultiveerde *L. plantarum* WCFS1 het naverzuring significant belemmert. Deze observatie is technologisch relevant omdat naverzuring wordt gezien als een van de meest nadelige factoren voor de overleving van probiotica in gefermenteerde melk. Daarbij kan het reduceren van naverzuring interessant zijn voor de toekomstige ontwikkeling van milde yoghurt. Vanuit sensorisch perspectief moet worden vastgesteld dat stress

geadapteerde probiotica een substantiële verandering in het yoghurt metaboloom introduceren. Deze vinding is van beland omdat variatie in de relatieve concentratie van organische zuren, vluchtige aroma's en proteolytische componenten direct van invloed kunnen zijn op de organoleptische kwaliteit van het product. Daarom is het adviseerbaar om in vervolgonderzoek te focussen op de sensorische evaluatie van de yoghurt met een getraind panel. Tot slot kan een vergelijkbare metaboliet-gebasseerde analytische aanpak zeker worden uitgebreid voor de analyses van de moleculaire profielen van andere gefermenteerde zuivelproducten.

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Sarn

Inspiration

It is impossible to live without failing at something, unless you live so cautiously that you might as well not have lived at all - in which case, you fail by default.

(J.K. Rowling, British novelist)

มันคงเป็นไปได้ยากที่คนเราจะมีชีวิตอยู่โดยไม่เคยพบเจอกับความล้มเหลว
นอกเสียจากว่าคนคนนั้นจะใช้ชีวิตด้วยความระมัดระวังอย่างถึงที่สุด…
…ซึ่งนั้นก็แทบไม่ต่างจากการไม่ได้มีชีวิตอยู่เลย
ถ้าเป็นเช่นนั้นก็เท่ากับว่าคนคนนั้นล้มเหลวในการใช้ชีวิตไปแล้วโดยปริยาย

(เจ.เค. โรว์ลิง, นักเขียนชาวอังกฤษ)

Study abroad is a perfect opportunity for Thai students to develop their adaptive stress responses.

Self-enduring happiness is a combination of living simply, forgiving more and expecting less.

Curriculum Vitae

Sarn Settachaimongkon was born on March 25, 1981 in Nakhon-Ratchasima, Thailand. He grew up in that province until finishing his high school diploma from Ratchasima Wittayalai in 1998. He continued his BSc degree in Microbiology at Kasetsart University, Bangkok, Thailand and graduated in 2002. During 2002-2003, he took the opportunity to perform international internships in French dairy sectors. He worked in the microbiological laboratory of Syndicat Interprofessionnel du Reblochon, Ecole Nationale des Industries du Lait et des Viandes and Laboratoire Vétérinaire Départemental de la Haute-Savoie where he has developed his knowledge and competence in microbial food safety and quality control. In 2005, he received a full-scholarship offered by the European Commission for continuing a post-graduate study under Erasmus Mundus program. He finished his MSc degree in Vintage from Ecole Supérieure d'Agriculture, Angers, France, with a doubled Máster Universitario degree in Viticulture, Oenology and Wine Industry from Polytechnic University of Valencia, Spain, and a certificate in Oenology from the University of Bologna, Italy. Afterwards, he started his career as a part-time instructor in food science at Mahidol University International College, Salaya, Thailand. In 2009, he received a full-scholarship from the Commission on Higher Education of the Royal Thai Government to perform a doctoral research in Dairy Science and Technology at Wageningen University, the Netherlands. He graduated his PhD degree in 2014 and the outcomes of that project are described in this thesis. Sarn is currently appointed as an instructor at the Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. His interests include microbial physiology, microbial ecology, molecular-based analytical techniques and chemometrics applied in fermented food and dairy research.

List of publications

Peer-reviewed journals

Settachaimongkon S., Nout M.J.R., Antunes Fernandes E.C., Hettinga K.A., Vervoort J.M., van Hooijdonk T.C.M., Zwietering M.H., Smid E.J., van Valenberg H.J.F. (2014). Influence of different proteolytic strains of *Streptococcus thermophilus* in co-culture with *Lactobacillus delbrueckii* subsp. *bulgaricus* on the metabolite profile of set-yoghurt. International Journal of Food Microbiology 177, 29-36.

Settachaimongkon S., Nout M.J.R., Antunes Fernandes E.C., van Hooijdonk A.C.M., Zwietering M.H., Smid E.J., van Valenberg H.J.F. (2014). The impact of selected strains of probiotic bacteria on metabolite formation in set-yoghurt.
International Dairy Journal, in press.

Settachaimongkon S., van Valenberg H.J.F., Winata V., Wang X., Nout M.J.R., van Hooijdonk A.C.M., Zwietering M.H. Smid, E.J. Effect of sublethal preculturing on the survival of probiotics and metabolite formation in set-yoghurt.
(Submitted for publication)

Settachaimongkon S., van Valenberg H.J.F., Gazi I., Nout M.J.R., van Hooijdonk A.C.M., Zwietering M.H. Smid, E.J. Incorporation of precultured *Lactobacillus plantarum* WCFS1 in set-yoghurt: Impact on survival of yoghurt starters, post-acidification and metabolite formation.
(Manuscript in preparation)

Conference abstracts

Settachaimongkon S., Valenberg H.J.F. van, Smid E.J., Nout M.J.R., Hooijdonk A.C.M. van, Zwietering M.H. (2013). Metabolomics approach for understanding the viability and activity of probiotics in set-yoghurt: Determination of volatile and non-volatile polar metabolite profile. The 7th Probiotics, Prebiotics and New Foods Congress. Rome, Italy. (Oral presentation)

Settachaimongkon S., Valenberg H.J.F. van, Nout M.J.R., Hooijdonk A.C.M. van, Zwietering M.H. (2010). Simultaneous growth and production of metabolite compounds by yoghurt starter cultures and probiotics: a metabolomics approach. The 1st China International Conference on Lactic Acid Bacteria and Technological Innovations of Fermented Dairy Products. Hohhot, China.
(Oral presentation)

Overview of completed training activities

Discipline specific activities

Courses

- Advanced Statistics Course: Design of Experiments (2009), WIAS, Wageningen
- Reaction Kinetics in Food Science (2009), VLAG, Wageningen
- Advanced Food Analysis (2010), VLAG, Wageningen
- Genetics and Physiology of Food-associated Microorganisms (2010), VLAG, Wageningen
- Systems Biology: Statistical Analysis for Omics Data (2010), VLAG & EPS, Wageningen
- Nutrient Density of Milk, Milk Genomics and Health Benefits of Dairy (2011), VLAG, Wageningen
- Food Fermentation (2012), VLAG, Wageningen

Conferences

- China International Conference on Lactic Acid Bacteria and Technological Innovations of Fermented Dairy Products (2010), Hohhot, Inner-Mongolia, China
  (Oral presentation)
- 7th Probiotics, Prebiotics and New Foods Congress (2013), Rome, Italy
  (Oral presentation)

General courses

- VLAG PhD Introduction Week (2009), VLAG, Maastricht
- Information Literacy and EndNote Introduction (2009), WGS, Wageningen
- Project and Time Management (2010), WGS, Wageningen
- PhD Competence Assessment (2010), WGS, Wageningen
- Techniques for Writing and Presenting a Scientific Paper (2011), WGS, Wageningen
- Philosophy and Ethics of Food Science and Technology (2012), WGS, Wageningen
- Applied Statistics (2012), VLAG, Wageningen

Optional courses and activities

- Preparation of PhD research proposal (2009)
- MSc course: Dairy Chemistry and Physics (2009), FQD, Wageningen
- DST meetings and Seminars in Food Quality and Design (2009-2014), FQD, Wageningen
- PhD excursion of Laboratory of Food Microbiology to Switzerland (2010)
- PhD excursion of Food Quality and Design Department to the United Kingdom (2012)

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