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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 product. This study provides new information on the application of stress-adapted probiotics in an actual food-carrier environment.

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 (Shiby and Mishra, 2013). 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 (FAO/WHO, 2002). This 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 (Vasiljevic and Shah, 2008). Most commercial probiotics incorporated in dairy products are strains belonging to the genera *Lactobacillus* and *Bifidobacterium* (Lourens-Hattingh and Viljoen, 2001). 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 (Gueimonde et al., 2004), mainly due to the reduction of pH and accumulation of organic acids (Shah, 2000).

Stress adaptation is one of the strategies to improve the survival of probiotics. This is achieved by pre-treating (preculturing) them in a sublethal stress condition prior to exposure to a more harsh or lethal environment (Upadrasta et al., 2011). 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 (Saarela et al., 2004). 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 (De Angelis and Gobbetti, 2004; Ruiz et al., 2011; Tsakalidou

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and Papadimitriou, 2011; Van de Guchte et al., 2002). These stress features usually resemble the environmental niches typically encountered by probiotics during human gastrointestinal tract transit, during industrial-scale production and in the food matrix (Ruiz et al., 2011). 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 (Mohammadi et al., 2012). Recent advances in post-genomics technologies, i.e. transcriptomics and proteomics, have provided novel insights into how probiotics counteract environmental stresses (Sánchez et al., 2013). 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 (Giraffa, 2012; Maus and Ingham, 2003; Mills et al., 2011; Shah, 2000). Particularly, the influence of metabolic activity of stress-adapted probiotics on the biochemical characteristics of the food-carrier received little attention.

Metabolomics is recognized as an effective tool to investigate the overall chemical composition of complex biological systems including food matrices (Herrero et al., 2012). 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 (Mozzi et al., 2013; Piras et al., 2013; Rodrigues et al., 2011; Settachaimongkon et al., 2014a). This approach can be implemented for monitoring the overall biochemical changes associated with the metabolic activity of starter cultures and probiotics during yoghurt manufacture (Mozzi et al., 2013; Sánchez et al., 2013; Settachaimongkon et al., 2014b). 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 (Serrazanetti et al., 2009).

The objective of this study was to investigate the impact of preculturing of two commercial probiotic strains, *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB12, under sublethal stress conditions (combinations of elevated NaCl and adjusted 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.

2. Materials and methods

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) 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 refreshed in MRS broth (1% (v/v) inoculation) (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 4000 × *g* for 15 min at 4 °C, washed twice using peptone-physiological-salt solution (Tritium microbiology, Eindhoven, the Netherlands) and finally resuspended in milk to obtain the cell density at approximately 10^8 CFU/g before inoculation. These cultures were defined as control groups, i.e. standard pre-cultured LGG and BB12.

2.2. Preculturing of probiotics under sublethal stress conditions

2.2.1. 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 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 × 2 between subjects factorial design (Table 1).

2.2.2. 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 4000 × *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 (Saarela et al., 2004). Sublethally precultured probiotics were subsequently inoculated in co-cultures with traditional yoghurt starters as described previously (Settachaimongkon et al., 2014b). The

Table 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	Neutral 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

preculturing was performed in three batches for each stress combination.

2.3. Set-yoghurt fermentation

Reconstituted Nilac skimmed milk (NIZO Food Research, Ede, the Netherlands) was prepared according to the method previously described (Settachaimongkon et al., 2014a). Set-yoghurts were fermented with two types of starter combinations: (i) co-cultures of yoghurt starters with LGG and (ii) co-cultures of yoghurt starters with BB12. The initial inoculum size of the two yoghurt starters and probiotic strains were adjusted respectively at 10^6 CFU/g (ratio 1:1:1). After inoculation, set-yoghurt fermentation and sample collection were carried out according to the methods previously described (Settachaimongkon et al., 2014b). The fermentation was performed in three replicates for each type of starter combination.

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 previously (Settachaimongkon et al., 2014b).

2.5. Determination of acidification profile

Production of acid during set-yoghurt fermentation and storage was expressed by changes in pH and increases in titratable acidity as described previously (Settachaimongkon et al., 2014a).

2.6. Analysis of volatile metabolites by headspace SPME-GC/MS

A model scenario of set-yoghurt fermentation was carried out directly in GC vials (Settachaimongkon et al., 2014a). 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 previously described (Settachaimongkon et al., 2014a). Volatile metabolites were identified using AMDIS software (NIST, Gaithersburg, MD, USA) referred to NIST/EPA/NIH database and the library provided by Hettinga et al. (2009). Specific retention time and *m/z* model were used for automated peak integration in XCalibur software package (Thermo Scientific, Austin, TX, USA).

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

For ^{1}H-NMR analysis, the samples from two replicates were prepared according to the method previously described (Settachaimongkon et al., 2014a). NOESY 1D-^{1}H-NMR measurements were performed in a 600 MHz NMR spectrometer (Bruker, Rheinstetten, Germany) operated with similar parameters as described by Lu et al. (2013). 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 (Settachaimongkon et al., 2014a). 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 by Settachaimongkon et al. (2014a). For unlabeled bins, significant variables were selected based on one-way ANOVA at 95% confidence level.

2.8. Statistical analysis

ANOVA and multiple comparisons by Tukey's test were performed using IBM-SPSS statistics package version 21 (SPSS Inc., Chicago, IL, USA). A probability at $P < 0.05$ was considered statistically significant. Metabolomics data were normalized before multivariate analysis (Settachaimongkon et al., 2014a). Principal component analysis was performed using Multi-Experiment Viewer (MeV) version 4.8 (www.tm4.org/mev/).

3. Results

3.1. Bacterial growth and survival

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. In 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 2×2 between subjects factorial design (Table 2).

In co-cultures with LGG (Fig. 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 1A) and *L. delbrueckii* subsp. *bulgaricus* (Fig. 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. 1E & Table 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 ($P < 0.01$) to the survival of LGG during storage.

In co-cultures with BB12 (Fig. 1; right panels), growth and survival of *S. thermophilus* (Fig 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. 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

Table 2
ANOVA of the main effects of individual stress factors, i.e. NaCl and pH, and their interaction on growth and viability of *L. rhamnosus* GG (LGG) and *B. animalis* subsp. *lactis* BB12 (BB12) in set-yoghurt.

Changes in bacterial population	Standard LGG (control)	Sublethally precultured LGG				Test of significance between effects		
		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 4 h − 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 28 d − 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 (control)	Sublethally precultured BB12				Test of significance between effects		
		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 4 h − 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 28 d − 4 h)	−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.

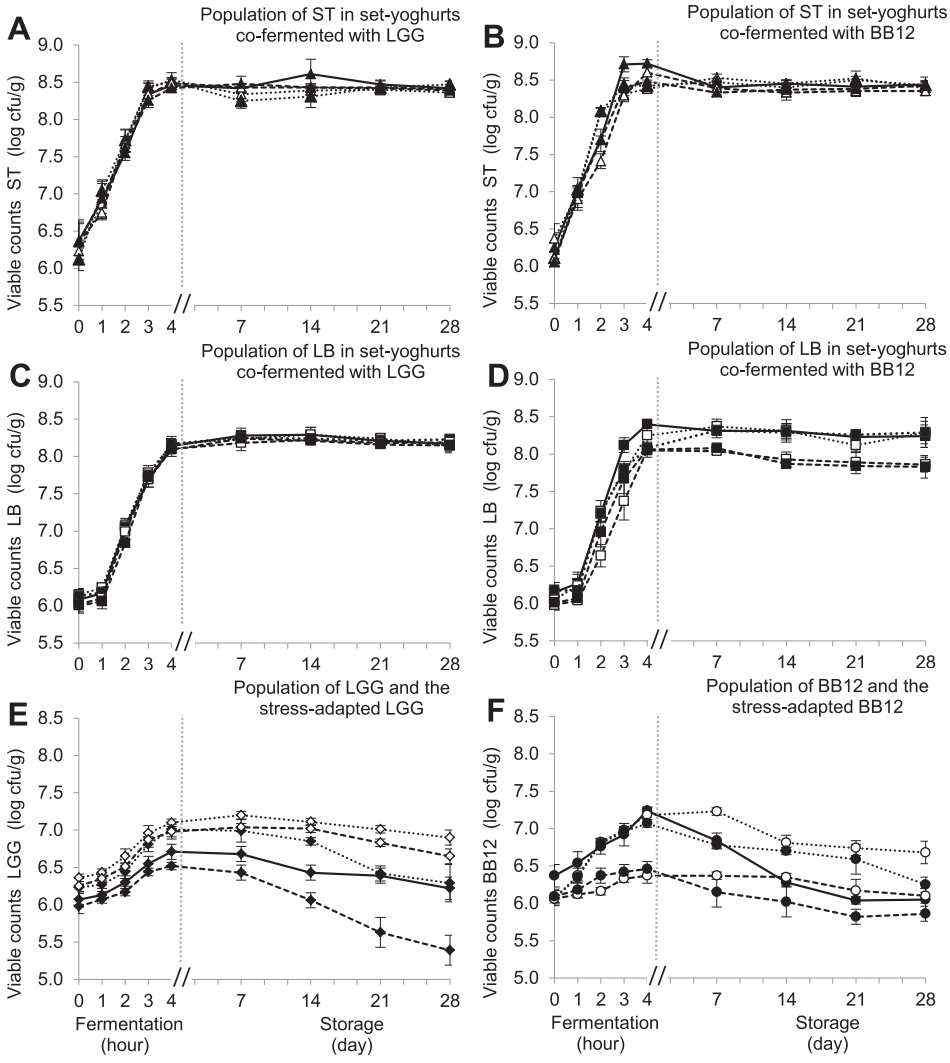

Fig. 1. Changes in viable counts of *S. thermophilus* (ST, △; panel A and B), *L. delbrueckii* subsp. *bulgaricus* (LB, □; panel C and D), *L. rhamnosus* GG (LGG, ◇; panel E) and *B. animalis* subsp. *lactis* BB12 (BB12, ○; panel F) during set-yoghurt fermentation (4 h) and refrigerated storage (28 days). 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-neutral-pH (·····; black markers), high-salt-low-pH (– – –; white markers) and high-salt-neutral-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.

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 (*ca.* 0.1 log reduction) 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. 1F & Table 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 ($P < 0.01$) to their growth impairment during set-yoghurt fermentation. 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) in 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.

3.2. Acidification profiles

In the samples co-fermented with LGG and their sublethally precultured cells (Fig. S1A), 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. S1C), 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 ($P > 0.05$).

The titratable acidity, expressed as % equivalent lactic acid (w/w), 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. S1B), 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%). In the samples co-fermented with BB12 and their sublethally precultured cells (Fig. S1D), 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.

3.3. Volatile metabolite profiles determined by headspace SPME-GC/MS

Volatile metabolite profiles of set-yoghurts 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 our previous study (Settachaimongkon et al., 2014a), 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 S1). 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. 2), an overall PCA score plot was constructed with a total variance of 45.5% (n = 45) (Fig. 2A). 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. 2B) and among stored samples (n = 30) with a total variance of 47.1% (Fig. 2C). 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.

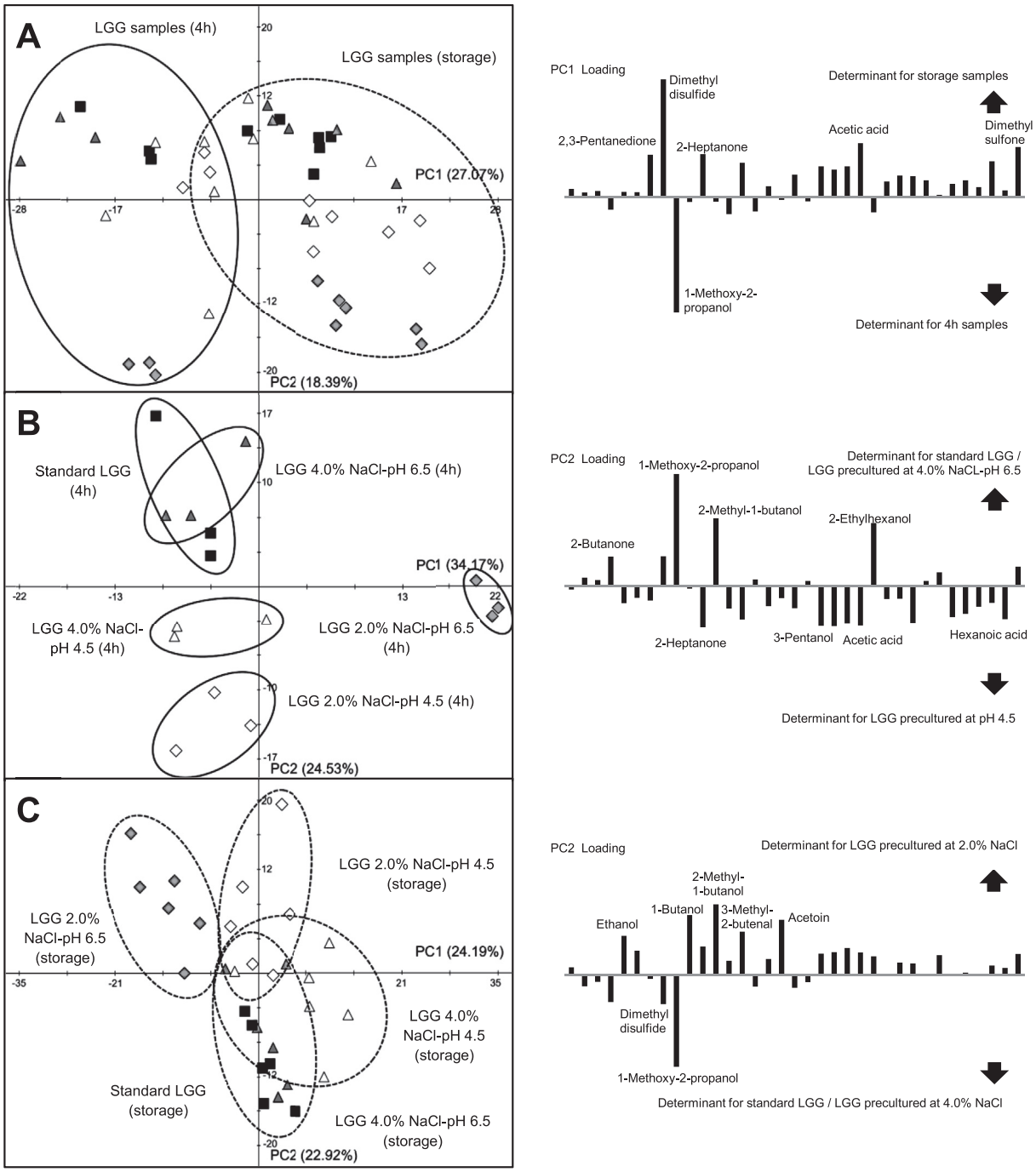

Fig. 2. PCA score plots and PC loadings, for overall comparison (panel A), comparison among samples at 4 h (panel B) and among storage samples (panel C), derived from volatile metabolite profiles of set-yoghurts co-fermented with standard precultured (control) *L. rhamnosus* GG (LGG) (■), LGG precultured 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 (▲).

For the samples co-fermented with BB12 and their sublethally precultured cells (Fig. 3), an overall PCA score plot was constructed with a total variance of 64.5% (n = 45) (Fig. 3A). 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. 3B) and among stored samples (n = 30) with a total variance of 67.9% (Fig. 3C). 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

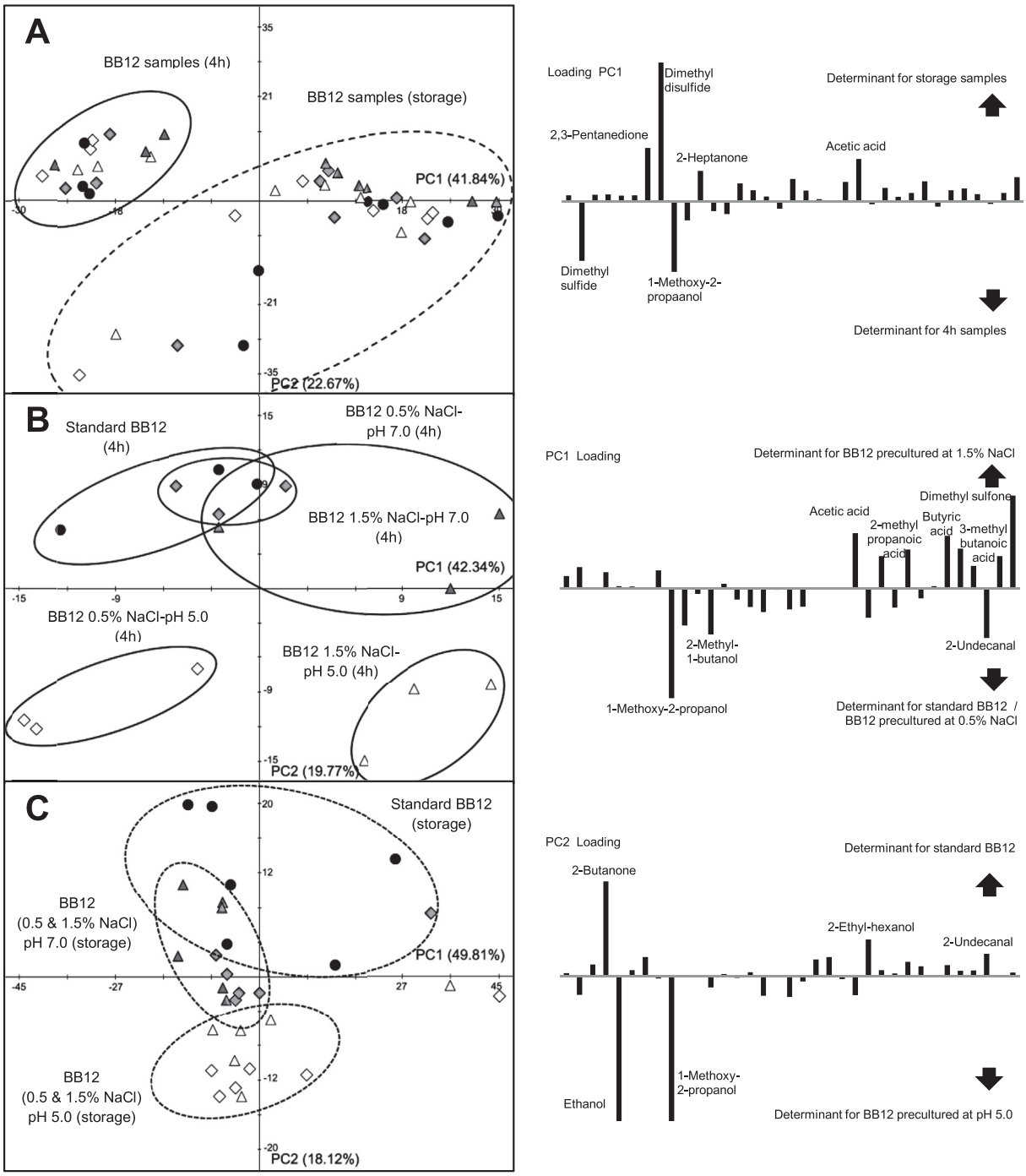

Fig. 3. PCA score plots and PC loadings, for overall comparison (panel A), comparison among samples at 4 h (panel B) and among storage samples (panel C), derived from volatile metabolite profiles of set-yoghurts co-fermented with standard precultured (control) *B. animalis* subsp. *lactis* BB12 (BB12) (●), 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 (▲).

butanoic acid and dimethyl sulfone were the major volatile metabolites accountable for discrimination. The difference in metabolite profiles of sublethally precultured BB12 at the same pH level (with either 0.5% or 1.5% NaCl) largely disappeared during storage. 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-ethylhexanol and 2-undecanal contributed to those co-fermented with standard precultured BB12.

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 our previous study (Settachaimongkon et al., 2014a). A

total of 43 metabolites including amino acids, carbohydrates, organic acids, lipid derivatives, carbonyl compounds, a sulfur compound and a nucleoside were identified. Quantification was achieved by summation of signal intensities in all bins corresponding to the respective metabolite (Park et al., 2013) and expressed in $\log_{10}$ transformed values (arbitrary unit) (Table S2). 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 (Lamanna et al., 2011). 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). 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.

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. 5A). 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. 5B) and among stored samples (n = 10) with a total variance of 83.8% (Fig. 5C). 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. Discussion

The vigorous growth and good retention of survival of *S. thermophilus* C44 and *L. delbrueckii* subsp. *bulgaricus* C49 during set-yoghurt fermentation and refrigerated storage have been discussed previously (Settachaimongkon et al., 2014b). In co-cultures with sublethally precultured probiotics, it was remarkable that the growth of *L. delbrueckii* subsp. *bulgaricus* during fermentation was significantly impaired by co-culturing with BB12 precultured at 1.5% NaCl. It has been documented that bifidobacteria are inhibitory to a wide range of microorganisms due to the production of lactic and acetic acids as a part of their carbohydrate metabolism (Reis et al., 2012). Moreover, various antimicrobial substances such as bacteriocins produced by bifidobacteria have been found to possess potent antimicrobial activities towards closely related species as well as towards lactobacilli (Cheikhyoussef et al., 2008; Martinez et al., 2013). The activity of a bacteriocin produced by *B. animalis* subsp. *lactis* BB12 (known as biflact Bb12) has been reported (Martinez et al., 2013; Saleh and El-Sayed, 2004). A number of environmental factors including composition of the culture medium, nutrient shortage as well as the presence of other competing microorganisms play an important role in regulation of bacteriocin production in bifidobacteria (Martinez et al., 2013). In our study, however, co-culturing with BB12 precultured at 1.5% NaCl resulted in a lower titratable acidity compared to the control group. Possibly, preculturing at 1.5% NaCl triggers the synthesis of certain compounds in BB12 which provide a slight inhibitory effect on the growth of *L. delbrueckii* subsp. *bulgaricus* during set-yoghurt fermentation. This effect is interesting and requires further investigation.

For several probiotic bacteria, it has been documented that stress responses vary as a function of the growth phase, i.e. cells in stationary phase develop more general resistance to various types of stresses (Saarela et al., 2004; Waddington et al., 2010). Therefore, the preculturing period in this study was prolonged for 24 h, allowing the development of adaptive stress responses in the probiotic cells in stationary growth phase. Adaptive stress

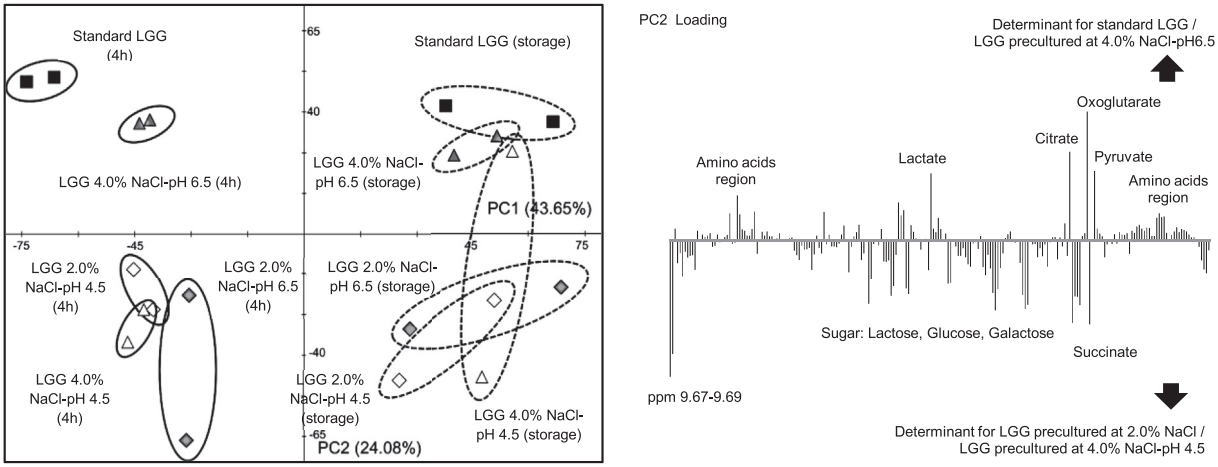

Fig. 4. Overall PCA score plot and PC loading derived from non-volatile polar metabolite profiles of set-yoghurts co-fermented with standard precultured (control) *L. rhamnosus* GG (LGG) (■), LGG precultured at 2.0% NaCl-pH 4.5 (◇), LGG precultured at 2.0% NaCl-pH 6.5 (◈), LGG precultured at 4.0% NaCl-pH 4.5 (△) and LGG precultured at 4.0% NaCl-pH 6.5 (◬).

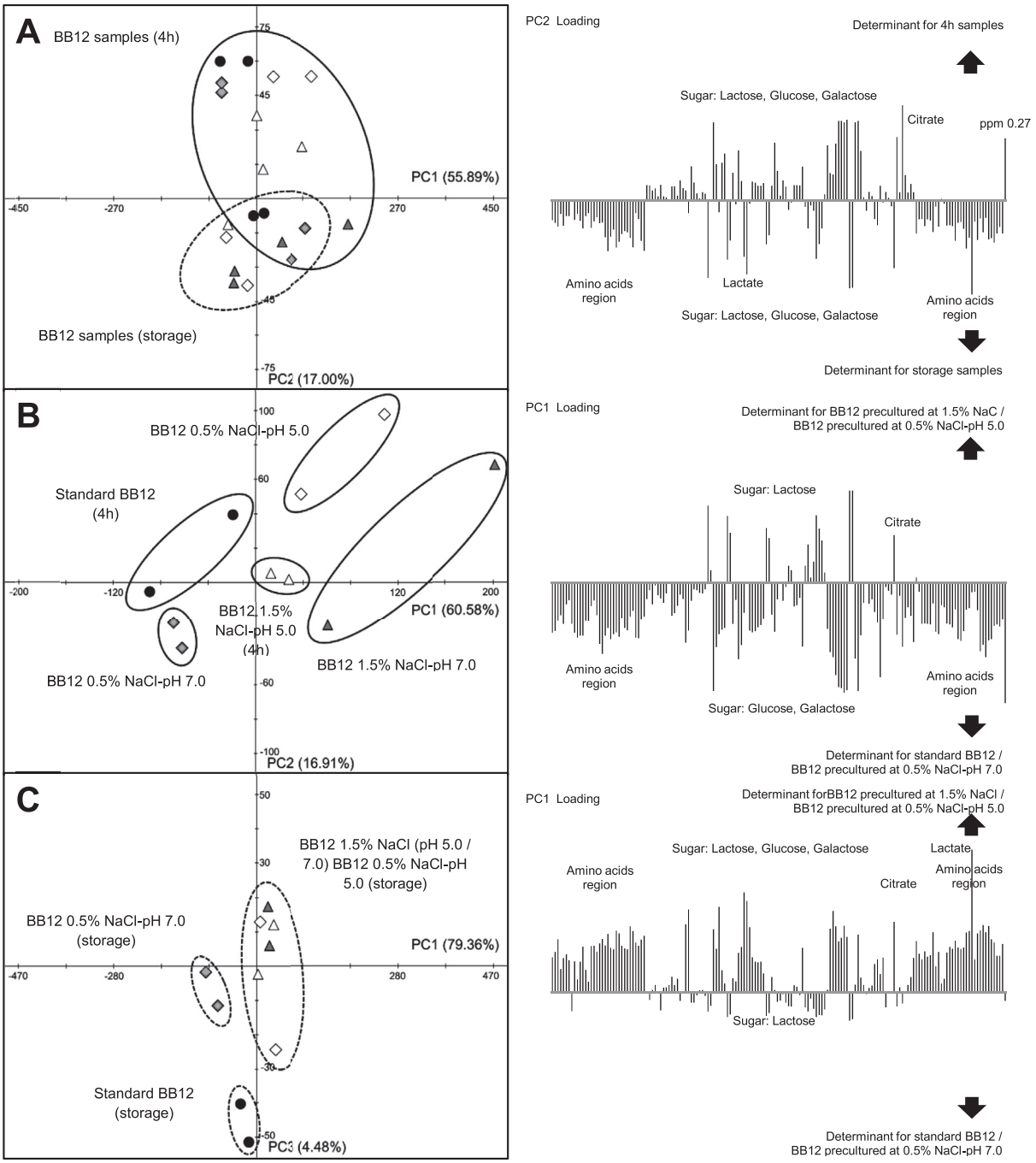

Fig. 5. PCA score plots and PC loadings, for overall comparison (panel A), comparison among samples at 4 h (panel B) and among storage samples (panel C), derived from non-volatile polar metabolite profiles of set-yoghurts co-fermented with standard precultured (control) *B. animalis* subsp. *lactis* 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 (◬).

responses in probiotics are associated with the alteration of various physiological features (Van de Guchte et al., 2002). A better survival of probiotics under acidic conditions is induced by physiological adaptation known as acid tolerance response (ATR) (Van de Guchte et al., 2002). The ATR associated mechanisms primarily include (i) pH homeostasis by proton-translocating F_1F_0-ATPase, (ii) alteration of cell membrane properties by modification in fatty acid composition, (iii) increase of alkalinity of cytoplasm by the activity of arginine deiminase, urease and glutamine decarboxylase and (iv) production of several stress proteins (De Angelis and Gobbetti, 2004; Ruiz et al., 2011; Van de Guchte et al., 2002). The response to osmotic stress results in the accumulation of compatible solutes and activation of membrane associated proteins for maintaining turgor pressure of the cell (Serrazanetti et al., 2009). The results in this study demonstrated adaptive responses of LGG and BB12 to sublethal NaCl-pH conditions, especially at relatively low pH value, in terms of viability improvement in yoghurt during refrigerated storage. This finding supports the hypothesis that pre-adaptation can enhance the survival of probiotics in a food system (Ross et al., 2005; Saarela et al., 2004; Sánchez et al., 2012; Shah, 2000). However, this is in contradiction with the work of Maus and Ingham (2003) who found an equal acid tolerance between

pretreated (combination of temperature, starvation time and pH stress) and untreated cells of *Bifidobacterium lactis* in yoghurt during refrigerated storage. These authors suggested that yoghurt fermentation process may override any previous enhancement in acid-tolerance achieved during preculturing (Maus and Ingham, 2003). Nevertheless, it is well documented that adaptive responses in probiotics are highly strain-dependent and vary largely according to the type of stresses exposed as well as experimental conditions (Maus and Ingham, 2003; Mozzetti et al., 2013; Saarela et al., 2004). 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 induced by adaptive stress responses may provide an adverse effect on bacterial growth (Van de Guchte et al., 2002). At the end of storage, it should be mentioned that the final viable counts of probiotics in this study, except for those precultured at high-NaCl-neutral-pH condition, still remain above the minimum recommended level (6.0 log CFU/g) to ensure their potential health-promoting effects (Shiby and Mishra, 2013).

Acidification profiles of set-yoghurts 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 1.5% NaCl. 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* as discussed previously.

It is well documented that environmental stresses induce alterations in the metabolic activity of probiotics leading to substantial changes in their technological and functional performances (Ruiz et al., 2011; Serrazanetti et al., 2009; Tsakalidou and Papadimitriou, 2011). As a result, a broader variety of metabolites can be formed which may considerably influence the biochemical and organoleptic characteristics of the fermented product (Serrazanetti et al., 2009). PCA results in this study confirm the impact of metabolic activity of stress-adapted LGG and BB12 on the volatile and non-volatile metabolite profile of yoghurt. The distinct patterns suggest that volatile metabolite profiles of the samples co-fermented with LGG can be distinguished according to either acid stress or osmotic stress while those of the samples co-fermented with BB12 can only be distinguished according to acid stress. Furthermore, it was remarkable that distinct volatile metabolite profiles of the samples co-fermented with different types of sublethally precultured probiotics at the end of fermentation gradually merged during storage. An explanation for this could be that the ongoing metabolic activity of starter cultures, mainly by *L. delbrueckii* subsp. *bulgaricus* as discussed earlier in post-acidification, contributed to the production of volatile metabolites during refrigerated storage. This could also be associated with the population size of the yoghurt starters, especially during storage, which were approximately ten to hundred-times higher compared to those of the probiotic adjuncts. Thus, the influence on volatile metabolite profiles caused by the metabolic activity of stress adapted probiotics during fermentation most likely was obscured. Regarding the non-volatile metabolite profiles, 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 clearly confirmed by the patterns of PCA. Non-volatile metabolite profiles of the samples co-fermented with LGG can be distinguished according to osmotic stress. Indeed, it should be noted that the separated patterns are in accordance with those previously observed for their volatile metabolite profiles. Unlike the situation of LGG, non-volatile metabolite profiles of the samples co-fermented with BB12 could not be clearly distinguished. The PCA results demonstrate that the effect of sublethal stress responses during preculturing on the metabolome of set-yoghurt is species-specific. To our knowledge, this is the first time that a combined metabolomics approach followed by multivariate analysis has been applied to understand the relation between pre-adaptation and technological performances of probiotics in food systems.

The contributions of yoghurt starters, LGG and BB12 to the biochemical conversion of milk components, i.e. production of aroma volatile and non-volatile metabolites, during fermentation and refrigerated storage of fermented milk have been extensively reported (Cheng, 2010; Østlie et al., 2003; Tamime and Robinson, 2007; Urbach, 1995) and discussed in our previous study (Settachaimongkon et al., 2014b). Regarding the influence of sublethal preculturing, it has been documented that the ATR in lactobacilli and bifidobacteria is associated with certain metabolic changes, especially the function of enzymes involved in glycolysis and pyruvate metabolism (Ruiz et al., 2011; Sánchez et al., 2012). The results showed that acetic acid, acetoin, 2-butanone and ethanol were accountable for the separation of yoghurt samples co-fermented with sublethally precultured probiotics. An increase in the production of these metabolites could be correlated with a higher yield of ATP for supporting the pH homeostasis by F_1F_0-ATPase (Sánchez et al., 2007). Furthermore, a higher concentration of several enzymes involved in the biosynthesis of branched-chain amino acids as well as sulfur amino acids was reported to be associated with ATR (Sánchez et al., 2007). Our results showed an effect of sublethally precultured probiotics on the content of various volatiles derived from the catabolism of these amino acids in yoghurt; i.e. 1-methoxy-2-propanol (Val), 2-methyl-1-butanol (Ile/Leu), 3-methyl-2-butenal (Ile/Leu), 3-methyl-butanoic acid (Leu), 2-methyl-propanoic acid (Val) and sulfur compounds (Cys/Met) (Ardö, 2006). According to the quantification of non-volatile metabolites (Table S2), a lower concentration of pyruvate and a higher concentration of acetate, formate, isoleucine, leucine and valine were clearly observed in the samples co-fermented with sublethally precultured LGG compared to BB12. This observation could be associated with a good distinction in their metabolite profiles revealed by PCA. In terms of technological relevance, variations in these compounds may considerably influence the organoleptic quality of product (Clark, 2009). For example, an excessive concentration of acetic acid causes a vinegar-like pungent flavor and masks the flavor-notes from other aroma compounds in yoghurt (Clark, 2009). In future research, the absolute concentration of these indicative volatile metabolites should be quantified. This will show whether the concentration of these compounds is still present within the same concentration ranges as normally detected in commercial products. Based on this information, the potential impact on the organoleptic quality of yoghurt could be properly predicted (Settachaimongkon et al., 2014b). Besides this, an additional research focusing on sensory evaluation of yoghurt with trained panelists is also recommended.

5. Conclusions

The present study demonstrated that preculturing of LGG and BB12 under sublethal salt (NaCl) and pH stress 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 volatile and non-volatile polar metabolite formation in set-yoghurt. Moreover, various aroma volatile 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 metabolite composition of the fermented product. This study provides new information on the application of stress-adapted probiotics in an actual food-carrier environment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2015.01.011.

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