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

Sarn Settachaimongkon [a], Hein J.F. van Valenberg [b], Vera Winata [b], Xiaoxi Wang [b], M.J. Robert Nout [c], Toon C.M. van Hooijdonk [b], Marcel H. Zwietering [c], Eddy J. Smid [c, *]

[a] Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[b] Dairy Science and Technology Group, Chair of Food Quality and Design, Wageningen University, P.O. Box 17, 6700 AA, Wageningen, The Netherlands
[c] Laboratory of Food Microbiology, Wageningen University, P.O. Box 17, 6700 AA, Wageningen, The Netherlands

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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

* Corresponding author. Tel.: +31 317 484978; fax: +31 317 482834.
E-mail address: eddy.smid@wur.nl (E.J. Smid).

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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