International Journal of Food Microbiology 145 (2011) 22–27

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Use of lactulose as prebiotic and its influence on the growth, acidification profile and viable counts of different probiotics in fermented skim milk

Ricardo Pinheiro De Souza Oliveira [a,b,*], Ana Carolina Rodrigues Florence [a], Patrizia Perego [b], Maricê Nogueira De Oliveira [a], Attilio Converti [b]

[a] Biochemical and Pharmaceutical Technology Department, Faculty of Pharmaceutical Sciences, São Paulo University, Av Prof Lineu Prestes, 580, Bl 16, 05508-900, São Paulo, Brazil
[b] Department of Chemical and Process Engineering, Genoa University, Via Opera Pia 15, I-16145 Genova, Italy

ARTICLE INFO

Article history:
Received 25 August 2010
Received in revised form 8 October 2010
Accepted 7 November 2010

Keywords:
Probiotic
Prebiotic
Milk
Yoghurt
Lactulose
Co-cultures

ABSTRACT

Lactulose can be considered as a prebiotic, which is able to stimulate healthy intestinal microflora. In the present work, the use of this ingredient in fermented milk improved quality of skim milk fermented by *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus* and *Bifidobacterium lactis* in co-culture with *Streptococcus thermophilus*. Compared to control fermentations without lactulose, the addition of such a prebiotic in skim milk increased the counts of all probiotics, with particular concern to *B. lactis* (bifidogenic effect), the acidification rate and the lactic acid acidity, and concurrently reduced the time to complete fermentation ($t_{pH4.5}$) and the pH at the end of cold storage for 1 to 35 days.

1. Introduction

Significant part of the world population suffers gastrointestinal diseases caused by pathogenic bacteria that invade the human intestine. A few days after the birth, the human intestine is colonized mainly by bifidobacteria which play a very important role in the maintenance of a good health (Olguin et al., 2005). So, in order to solve this health problem, food industry and in particular dairy technology has developed dairy functional products enriched with probiotics like lactobacilli (*Lactobacillus acidophilus*, *Lactobacillus casei*, etc.) and bifidobacteria (Donkor et al., 2007).

The word 'probiotic' was initially used as an antonym of the word 'antibiotic'. It is derived from the Greek words προ and βιοτοσ and translated as 'for life' (Hamilton-Miller et al., 2003). In the past, different definitions of probiotics were given, but, following the recent recommendations of a working group on the evaluation of probiotics in food of FAO/WHO (2002), probiotics are considered as live microorganisms that, when administered in adequate amounts, confer health benefits on the host. Consequently, a wide variety of species and genera could be considered potential probiotics (Holzapfel et al., 1998); commercially, however, the most important strains are lactic acid bacteria (LAB).

Lactic acid bacteria (LAB) are widely used in the production of fermented foods. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are traditionally used as starters for milk fermentation in yoghurt production. In addition to *L. delbrueckii* subsp. *bulgaricus*, which contributes to accelerate lactic acid development in yoghurt as well as to improve flavor and textural properties (Curry and Crow, 2003), some other probiotics are used for this purpose, among which are *Lactobacillus rhamnosus*, *L. acidophilus*, *Lactobacillus johnsonii* and *Bifidobacterium lactis*, because of their ability to grow in milk and to confer functional properties and benefits to the health (Vasiljevic and Shah, 2008).

The importance of certain technological and physiological characteristics of probiotic strains was recognized long time ago (Gordon et al., 1957). To achieve successful outcome of the lactobacilli therapy, the culture must have certain requirements: it should be a normal, non-pathogenic inhabitant of the intestine, capable of efficient gut colonization and present in substantially high concentrations in the product (10^7–10^9 CFU/mL) (Mattila-Sandholm et al., 2002; Ouwehand, et al., 1999).

The dairy industry has been quickly revitalized by the introduction of products characterized not only by their high nutritional value and pleasant taste, but also by their ability to exert positive effects on the consumer's health (Casiraghi et al., 2007). In this context, some non-digestible ingredients, called prebiotics, have received considerable attention, mainly because of their ability to selectively stimulate the growth and/or activity of probiotic bacteria in the colon (Gibson and Roberfroid, 1995; Huebner et al., 2007). However, the ability of

* Corresponding author. Biochemical and Pharmaceutical Technology Department, Faculty of Pharmaceutical Sciences, São Paulo University, Av Prof Lineu Prestes, 580, Bl 16, 05508-900, São Paulo, Brazil. Tel.: +39 0 10 3532593; fax: +39 0 10 3532586.
E-mail address: rpsolive@usp.br (R.P. De Souza Oliveira).

0168-1605/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2010.11.011

probiotic microorganisms to use the prebiotics is strain and substrate specific (Shah, 2001).

To be effective, prebiotics must escape digestion in the upper gastrointestinal tract and be used by a limited number of microorganisms comprising the colonic microflora, mainly lactobacilli and bifidobacteria (Gibson and Roberfroid, 1995; Isolauri, 2004); thus, in the later case they are referred to as bifidogenic factors (Berg, 1998; Macfarlane and Cummings, 1999; Roberfroid, 2000). More rarely, they are reported to mitigate the virulence of pathogenic bacteria like *Listeria monocytogenes* (Park and Kroll, 1993). This family of compounds includes several oligosaccharides (namely fructo-, gluco-, galacto-, isomalto-, xylo-, and soyo-oligosaccharides), inulin, lactulose, lactosucrose, among others (Fric, 2007).

Lactulose is widely used in pharmaceutical industry (Aider and de Halleux, 2007), mainly as an effective drug against diseases like acute and chronic constipation (Tamura et al., 1993). Nevertheless, some promising applications are reported also in the nutraceuticals and food industries because of its beneficial effects on human health (Donkor et al., 2007). It is a synthetic disaccharide obtained by isomerization of lactose, which is present in milk and whey in relatively high content, approximately 4.5% as an average (Zokaee et al., 2002), and contains fructose instead of glucose (Strohmaier, 1998). Since it is not absorbed in the small intestine, it has the potential to function as a prebiotic (Kontula et al., 1999). Moreover, several studies showed the effectiveness of lactulose to stimulate the growth of bifidobacteria (Olano and Corzo, 2009; Sako et al., 1999; Shin et al., 2000).

Taking into account all these considerations, lactulose appears as an important food ingredient that might be additionally explored for the production of functional foods, and one can expect its future large scale production for food and nutraceuticals purposes. To this purpose, the present study explores the effects of this ingredient on the acidification kinetics, post-acidification, growth and metabolism of binary co-cultures of *L. bulgaricus*, *L. acidophilus*, *L. rhamnosus* and *B. lactis* with *S. thermophilus*, as well on their survival during skim milk fermentation.

2. Materials and methods

2.1. Microbial cultures

Strains of pure starter freeze-dried cultures (Danisco, Sassenage, France) were used: *S. thermophilus* TA040 (St) and *L. delbrueckii* subsp. *bulgaricus* LB340 from here onwards called *L. bulgaricus* (Lb) (yoghurt microorganisms); *L. acidophilus* LAC4 (La), *L. rhamnosus* LBA (Lr) and *Bifidobacterium animalis* subsp. *lactis* BL 04.

2.2. Milk preparation

Milk prepared by adding 13 g of skim powder milk (Castroni, Reggio Emilia, Italy) in 100 g of distilled water was used either in the presence (SM) or absence (M) of 4% (w/w) lactulose (trade name: Lactulose 61360) (Sigma Aldrich, Italy). The above solid content of milk corresponds to the average value reported by Restle et al. (2003) for integral cow milk, while the selected lactulose concentration was in the range (3–6% w/w) admitted by Brazilian legislation in yoghurts (ANVISA, 2002). Both milks were then thermally treated at 90 °C for 5 min in water bath, model Grant Y6 (Cambridge, England). The heat treated milks were transferred to 1.0 L sterile flasks, cooled in ice bath, distributed into 250-mL sterile Shott flasks inside laminar flow chamber, and stored at 4 °C for 24 h before using.

2.3. Inoculum preparation

The La, Lb, Lr, Bl and St freeze-dried cultures were prepared by dissolving in 50 mL of milk 10% (w/w) of total solids; autoclaved at 121 °C for 20 min. After blending and activation at 42 °C for 30 min, 1.0 mL of the pre-culture was inoculated into 250 mL of milk. Bacterial counts in these pre-cultures ranged from 6.0 to 6.6 log CFU/mL.

2.4. Fermentations

After inoculation, flask samples were transferred to a water bath, and batch fermentations were performed in triplicate at 42 °C up to pH 4.5, which were selected as the conditions to stop the fermentation. Fermentations were monitored by pH determinations.

2.5. Post-acidification

Once the fermentation of milk had been complete, post-acidification was determined after a) 1 day (D1), b) 7 days (D7) and c) 35 days of cold storage at 4 °C (D35) by pH measurement using a pH meter, model pH 210 Microprocessor (Hanna instruments, Padova, Italy).

2.6. Counts of probiotic bacteria

Bacterial counts were carried out in triplicate either after D1, D7 or D35. One mL of sample was diluted with 9 mL of 1 g/L sterile peptonated water. Afterwards, eight serial dilutions were done, and each bacterium was counted in the three most appropriate dilutions, applying the pour plate technique (Kodaka et al., 2005). Counts were finally presented as mean values. All media were obtained from Merck (Darmstadt, Germany). St colonies were counted in M17 agar (Oxoid) by aerobic incubation at 37 °C for 48 h. Lb, La and Lr counts were carried out in MRS agar medium after pH adjustment to 5.4 by acetic acid addition and aerobic incubation at 37 °C for 48, 72 and 72 h, respectively. Bl was counted in MRS Agar medium containing 50 g/L cysteine without any pH adjustment (IDF, 1996, 1997, 2003).

2.7. Kinetic parameters

From the pH data collected during fermentation, the acidification rate (V_{max}) was calculated as the time variation of pH (dpH/dt) and expressed as 10^{-3} pH units/min. During the incubation period, the following kinetic parameters were also calculated: (i) t_{max} (h), time at which V_{max} was reached; and (ii) $t_{pH4.5}$ (h), time to reach pH 4.5 (i.e., to complete the fermentation).

Once pH 4.5 had been reached, the fermentations were manually interrupted by aseptically agitating the clot by means of a stainless steel rod with perforated disks; the rod was gently moved upwards and downwards for 80 s. The fermented product was quickly cooled in an ice bath, and the fermented products were stored at 4 °C.

2.8. Analytical methods

A high-performance liquid chromatography, model 1100 (Hewlett Packard, Palo Alto, CA), was used for analysis of lactic acid (after D1, D7 and D35) and lactulose (every 1.5 h during the fermentation). The system consisted of a HP-1050 Intelligent Auto Sampler, a HP-1047A Refractive Index Detector (for organic acids), a HP-1050 UV Detector (for sugars) and a HP-1050 Pump. Separation was achieved using a Supelcogel H59304-U column (Sigma Aldrich, Bellefonte, PA) at 50 °C with 0.01 M sulfuric acid as eluent at 0.4 mL/min.

2.9. Statistical analysis

The experimental data of bacterial counts, post-acidification and lactic acid concentration, either after D1, D7 or D35, as well as those of lactulose concentration along the runs were presented as mean values. Variations with respect to the mean values were presented as standard deviations. Mean values of these parameters were submitted to analysis of variance (ANOVA) by the Statistica Software 6.0. They