International Food Research Journal 26(2): 695-703 (April 2019)

Journal homepage: *http://www.ifrj.upm.edu.my*

Bifidobacterium lactis HN019: survival, acid production and impact on sensory acceptance of fermented milk

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Article history

Received: 26 May 2018
Received in revised form: 18 October 2018
Accepted: 14 December 2018

Keywords

Probiotics
Organic acids
Quantitative ^{1}H NMR spectroscopy
Fermented dairy products

Abstract

Bifidobacterium lactis HN019 was monitored for its viability and acid production during milk fermentation and cold storage. Milk was fermented for 72 h at 37°C, and the shelf life of the resulting probiotic milk was evaluated at 4°C for 30 d. Additionally, lactic and acetic acids and lactose contents during the fermentation were measured by *in situ* quantitative proton nuclear magnetic resonance spectroscopy (isq ^{1}H NMR), and the sensorial acceptance of the fermented milk was evaluated by 80 panellists. The pH of the milk fermented by *B. lactis* HN019 reduced from 6.36 to 3.97 after 72 h incubation. The viability of the evaluated strain was maintained above 9.21 up to a decrease to 8.85 log CFU/mL after 48 h fermentation. With respect to metabolism, isq ^{1}H NMR revealed that the acetic and lactic acid contents increased from 0.47 ± 0.06 g/L to 16.36 ± 1.86 g/L and 2.59 ± 0.10 g/L to 9.40 ± 0.18 g/L, respectively, indicating high production of acetic acid by HN019 during the 72 h fermentation. On the other hand, lactose content decreased throughout the fermentation period. In fact, strain HN019 showed a high content of acetate throughout fermentation; however, exhibited the stability required in fermented milks with high viable cell counts and low activity of post-acidification during the shelf life. In sensory evaluation, the panellists preferred the probiotic milk fermented for 8 h because after 72 h the acetic flavour was already perceptible.

Introduction

Fermented milks containing probiotics have been extensively explored. Of these dairy products, products obtained using Bifido strains are an important category worldwide. In fact, *Bifidobacterium* is one of the most important groups of probiotic cultures used in fermented milk production owing to their well-documented health-promoting properties in human host (Urita *et al.*, 2015; Meng *et al.*, 2016). However, the production of fermented milk using bifidobacteria is a great challenge in the dairy industry because milk is not a suitable matrix for the growth of this genus as they lack essential proteolytic activity (Prasanna *et al.*, 2014). Thus, a longer fermentation time is required thereby resulting in some defects, such as sandy or slimy texture, yeasty or vinegary taste, or insufficient aroma (Sreeja and Prajapati, 2015).

Bifidobacterium is not included in the traditional lactic acid bacteria (LAB) group due to its genetic and physiological unrelatedness. However, for practical and traditional reasons, it is considered as belonging to the LAB order (Russel *et al.*, 2011). Moreover, bifidobacteria differ from other colonic genera with respect to the mechanism employed to ferment carbohydrates (Mayo *et al.*, 2010). Bifidobacteria metabolise hexoses using the "bifidus pathway", whose key enzyme is fructose-6-phosphate phosphoketolase (F6PPK), which leads to a theoretical molar ratio of acetate to lactate of 3:2 (Scardovi *et al.*, 1971). Furthermore, it has been suggested that the proportion of the final fermentation products varies considerably from one strain to another, and is dependent on the carbon source used and the growth phase (Oliveira *et al.*, 2009).

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Bifidobacterium lactis HN019 is a commercial probiotic strain with beneficial effects such as prevention against infections (Shu and Gill, 2001; Gopal *et al.*, 2001); positive changes in the intestinal microbiota (Ahmed *et al.*, 2007); decrease in whole gut transit, gut discomfort conditions in adults (Waller *et al.*, 2011); decrease in obesity, blood lipids, and inflammatory markers in patients with metabolic syndromes (Bernini *et al.*, 2016); and immunity enhancement (Sanders, 2006; Bogsan *et al.*, 2014; Miller *et al.*, 2017). Additionally, HN019 strain exhibits interesting characteristics, such as oxygen-, bile-, and acid-tolerance, rendering this microorganism adequate for industrial utilisation in fermented milks (Sanders, 2006). Therefore, this strain is sold as a probiotic culture and is commonly used in association with starter cultures in the formulation of dairy products.

Monitoring the organic acid content in milk fermented using different *Bifidobacterium* strains is important for dairy technology, either as an indicator of fermentation or as a means to evaluate the strain efficiency (Bouteille *et al.*, 2013). Thus, *in situ* quantitative proton nuclear magnetic resonance spectroscopy (isq ^{1}H NMR) has been tested to evaluate the bacterial metabolites in dairy gels (Bouteille *et al.*, 2013). Moreover, NMR is a non-destructive method and a qualitative and quantitative tool that does not require separation or chemical modification (Gowda and Raftery, 2015).

Although numerous reports on *B. lactis* HN019 identity, safety and health effects on the host are available (Meile *et al.*, 1997, Masco *et al.*, 2004; Zhou and Gill, 2005; Dekker *et al.*, 2009), there is still limited published information concerning its metabolism or the technological production in dairy products. Herein, we report the efficiency of the HN019 strain, the viability and acidification during milk fermentation and cold storage, as well as the effect of acid production on sensory acceptance. In addition, lactic and acetic acids production and lactose consumption were also quantified by isq ^{1}H NMR.

Materials and methods

Sample, microorganism and chemicals

Skim milk powder was acquired from Confepar (Londrina, PR, Brazil), and the culture *B. lactis* HN019™ (also known as HOWARU® Bifido) was purchased from DuPont™ Danisco A/S (Copenhagen, Denmark). Analytical standard chemicals: (S)-2-hydroxypropionic acid (L-(+)-lactic acid), acetic acid solution for HPLC, D-Lactose monohydrate (4-O-β-D-galactopyranosyl-D-glucose), TSP [3-(trimethylsilyl) propionic-2,2,3,3-d4 acid], and 98.0% sodium hydroxide were purchased from Sigma-Aldrich (Steinheim, Germany).

Production of fermented milk

Milk was prepared by adding 10% (w/v) skim cow milk powder (Confepar, Londrina, PR, Brazil) to water, thermally treated at 121°C for 15 min, and cooled to 37°C in an ice bath. The freeze-dried culture was added through Direct Vat Inoculation (DVI) with mass percentage of 0.25% p/v (log 11 CFU/mL) (Bernini *et al.*, 2016), and the inoculated milk was incubated at 37°C for 72 h. The viable bacterial count, pH, TA, organic acid and lactose content were determined by NMR in the milk after 0–12 h (every 2 h), 12–24 h (every 4 h), 48 h, and 72 h of incubation. Three replicates of each fermentation were performed, and the experiments were performed in triplicate. From the bacteriological incubator, the samples were collected and then subjected to viable count measurement and chemical analysis. Additionally, three aliquots of each time point were frozen immediately at −18°C (±1°C) in Eppendorf tubes to minimise bacterial activity.

Determination of pH and TA

The pH of the samples was measured using a pH meter (Kasvi, Curitiba, PR, Brazil). The titratable acidity (TA) was determined using the potentiometric method described by AOAC (2012).

Detection of viable cell counts of bifidobacteria

Samples of fermented milk were ten-fold serially diluted with sterile 0.1% (w/v) peptone water until proper dilution. Then, viable cell counts of the probiotic strain HN019 were determined on De Man Rogosa Sharpe (MRS) agar enriched with L-cysteine as the reducing agent as previously described (Casteele *et al.*, 2006) with modifications; specifically, the concentration of L-cysteine was increased to 0.3% because no growth was observed at 0.05%. Petri dishes were incubated for 72 h at 37°C under anaerobic condition (Anaerobac of Probac®). The results were expressed as CFU/mL of fermented milk.

Effect of cold storage on probiotic survival

Based on preliminary results, fermentation was performed in a single bottle cap for 12 h at 37°C. Following fermentation, the product was placed in 50 mL sterile Falcon plastic tubes and stored at 4°C for 30 d. Samples were taken at 7-d intervals, and pH, TA, and viable cell counts were determined.

Sensory analysis

The study involving humans was conducted according to the Declaration of Helsinki and was approved by the Ethical Committee on Human Research of the Universidade Norte do Paraná, PR, Brazil (process number 67602217.1.0000.0108).

The sensory analysis of the probiotic fermented milk was performed after 8 h and 72 h fermentation using individual booths with white light. Fifty millilitre of each fermented milk sample was packed in plastic white cups at 4 ± 1°C and labelled with random three-digit codes. The untrained panellists (n = 80) included students and members of staff of the University Campus. The panellists were asked to evaluate both samples to identify the most acidic sample by means of the paired comparison test (forced choice test). The paired preference test was also performed to determine the preferred sample. Finally, the purchase intent test was conducted using the following five-point scale− 5: "definitely buy", 4: "probably buy", 3: "may or may not buy", 2: "probably not buy", and 1: "definitely not buy".

Analytical method

Calibration curves for isq ^{1}H NMR

The standard solutions of acetic acid, lactic acid, and lactose were prepared in Milli-Q water from authentic and new stock solutions of the highest purity available. Five standard solutions were obtained for each metabolite with concentrations of acetic acid ranging from 0.8 to 46.86 g/L, lactic acid ranging from 0.8 to 16 g/L, and lactose ranging from 2 to 85.4 g/L. These concentration ranges were chosen in accordance with the average concentrations found in milk fermented by bifidobacteria (Østlie *et al.*, 2003; Hu *et al.*, 2007).

isq ^{1}H NMR

Immediately prior to measurements, fermented milk samples from 12 time points (between 0 and 72 h) were thawed and homogenised by thorough vortexing for approximately 1 min. Aliquots of 500 µL of each sample were introduced into 5-mm NMR glass tubes (Sigma-Aldrich Corp., St. Louis, Mo., USA). A sealed glass capillary tube containing a solution of 0.48% TSP in D2O (deuterium oxide; 99.9% atom D; Sigma Aldrich) was also introduced into each NMR tube as the internal chemical shift reference. NMR experiments were performed using a 400.13 MHz Bruker spectrometer (Mod. Avance III, Bruker Biospin, Karlsruhe, Germany) equipped with a 5-mm multinuclear Broadband Inverse Probe (BBI). All spectra were acquired using the same parameters: 16 scans of 32 k data points, spectral width of 15 kHz, recycle delay of 25 s per scan, flip angle of 90°, and requiring 8 min and 19 s per sample. All spectra were also recorded at a temperature of 298 K. The resulting spectra were phased, baseline corrected, and then calibrated for TSP at 0.00 ppm using TopSpin software version 3.5.7 (Bruker Biospin, Karlsruhe, Germany).

Quantitative analysis by integration

Lactic and acetic acids and lactose NMR signals were integrated in TopSpin version 3.5.7. Integration was performed both for the reference spectra with known concentrations of the analytes and for the milk spectra. The resulting data of standard solutions were plotted to produce graphs of integral area versus analyte concentration. The equations for the calibration curves were then developed by least square linear regression so that the sample concentration could be computed directly.

Statistical analyses

The results of pH, TA and viable cell counts were analysed by one-way ANOVA. The samples were compared over different times of fermentation and during various days of cold storage. Mean values were then compared using the Tukey's test at $p < 0.05$ by Statistica software version 10.0 (StatSoft South America, SP, Brazil). For sensory analysis, the directional difference test (forced choice procedure) was used to determine the consumer's ability to detect the difference in acidity between the fermented milk samples and preferred sample (ASTM International, 2008). $p < 0.05$ was considered statistically significant.

Results and discussion

Growth and acidification profile during milk fermentation

After 72 h of fermentation with *B. lactis* HN019, fermented milk had pH values ranging from 6.36 to 3.97, whereas TA increased from 0.22% to 1.92% lactic acid (Table 1). It was observed that the time to complete fermentation, i.e., the time required to reach pH 4.5, was 12 h with TA of 1.1% lactic acid, demonstrating the poor acidification performance of strain HN019 when compared to that of traditional yoghurt starter cultures (Bouteille *et al.*, 2013). Nevertheless, the desired characteristics for fermented milks were reached (pH about 4.5 and titratable acidity higher than 0.6% to 2%) according to the FIL norm no 150:1991 recommended in the Codex Alimentarius.

Table 1. Bacterial load (log CFU/mL), pH and titratable acidity (%) of milk fermented with *B. lactis* HN019 at 37°C. Results are expressed as mean ± standard deviation, $n = 9$.

Time (hour)	log CFU/mL	pH	TA (%)
0	9.21 ± 0.18^{a}	6.36 ± 0.03^{a}	0.22 ± 0.01^{k}
2	9.25 ± 0.29^{a}	5.72 ± 0.10^{b}	0.29 ± 0.02^{j}
4	9.29 ± 0.05^{a}	5.14 ± 0.03^{c}	0.50 ± 0.02^{i}
6	9.33 ± 0.11^{a}	4.91 ± 0.02^{d}	0.65 ± 0.01^{h}
8	9.47 ± 0.11^{a}	4.74 ± 0.01^{e}	0.81 ± 0.02^{g}
10	9.46 ± 0.04^{a}	4.63 ± 0.03^{f}	0.98 ± 0.02^{f}
12	9.49 ± 0.08^{a}	4.52 ± 0.03^{g}	1.10 ± 0.11^{e}
16	9.44 ± 0.18^{a}	4.40 ± 0.01^{h}	1.18 ± 0.05^{e}
20	9.34 ± 0.10^{a}	4.31 ± 0.01^{i}	1.36 ± 0.01^{d}
24	9.36 ± 0.07^{a}	4.25 ± 0.04^{i}	1.50 ± 0.01^{c}
48	9.29 ± 0.09^{a}	4.14 ± 0.02^{j}	1.71 ± 0.09^{b}
72	8.85 ± 0.22^{b}	3.97 ± 0.05^{k}	1.92 ± 0.17^{a}

Means within a column with different superscripts are significantly ($p < 0.05$) different.

Despite the slow growth (Table 1), strain HN019 persisted in the milk matrix without significant multiplication until 48 h ($p > 0.05$), although lactose consumption and the production of the organic acids, characteristics of fermentation occurred (Figure 2), thus, confirming its higher tolerance to acidic conditions (Prasad *et al.*, 1998). In fact, because of this advantageous technological property, the species *B. lactis* is more suitable to be used in fermented dairy products in comparison to other *Bifidobacterium* species (Gueimonde *et al.*, 2004). The final pH of 3.97 after 72 h fermentation and the time required to reach pH 4.5 (approximately 12 h) are in agreement with other reported data of cow milk fermented with *B. lactis* BB12 at 37°C (Østlie *et al.*, 2003). However, another strain, *B. lactis* BL 04, persisted for the longest time to complete fermentation (16.3 h), proving that the metabolism of bifidobacteria is strain-dependent (Oliveira *et al.*, 2009).

The low pH observed after 48 h and the accumulation of organic acids probably decreased the specific growth rate of strain HN019. Usually, milk is not a suitable matrix for the growth of bifidobacteria because amino acids and low molecular weight peptides are only present at low concentrations (Prasanna *et al.*, 2014). Furthermore, other species of bifidobacteria, such as *B. animalis* subsp. *animalis*, invariably require strict anaerobic condition and a low redox potential in the early phase of growth; however, strains of *B. animalis* subsp. lactis exhibit elevated oxygen tolerance, allowing multiplication at a higher rate under non-anaerobic conditions (Meile *et al.*, 1997; Masco *et al.*, 2004).

In this context, *B. lactis* HN019 has showed an excellent survival rate (93.25%) after 72 h, and the concentrations (up to 9 logs) since the first hours were around one logarithmic cycle highest to those reported by other authors who evaluated different *B. lactis* strains at 37°C (Østlie *et al.*, 2003; Prasanna *et al.*, 2012). The observed stationary phase since the beginning and the high probiotic counts could be attributed to the inoculation method (DVI) adopted in the present work, which is similar to the industrial or commercial process. In fact, as bifidobacterial cultures are supplied in a highly concentrated form, usually meant for Direct Vat Set (DVS) application, the use of these DVS cultures avoids the need for propagating this culture at the production site. We herein noted that this DVS method presented highest counts and rate of acidification compared to a previous study by our group using DVI for *B. lactis* HN019 as the pre-culture (10% v/v) added to skim milk (Bernini *et al.*, 2016).

Finally, in order to be functional, bifido milk should contain viable bifidobacteria at high numbers during production and storage (Sreeja and Prajapati, 2015). Although minimal effective doses should be established for each probiotic product, because probiotic effects are strain-specific and might vary depending on the population group and health status (Bertazzoni *et al.*, 2013), *B. lactis* HN019 was detected above the recommended levels in many studies that indicates at least 108 and 109 CFU per serving in the product to be consumed (Shah, 2000; Bernini *et al.*, 2016; Meng *et al.*, 2016).

Cold storage viability and post-acidification

B. lactis HN019 showed high viability over 30 d of cold storage as samples had counts above 9 log CFU/mL throughout the storage period. The strain remained stable throughout 30 d and was not affected by storage time ($p > 0.05$; Table 2). These constant counts recorded during the shelf life are similar to those reported by other studies involving milk fermented with *B. lactis* BB-12 and *B. lactis* BL 04 (Oliveira *et al.*, 2009; Casarotti *et al.*, 2014). According to Russel *et al.* (2011), an important property of a probiotic culture is the ability to survive in the carrier material, as this ensures that the probiotic bacteria remain viable and retain their metabolic activity. *B. animalis* subsp. lactis persisted significantly longer in fermented milk under different conditions compared to *B. longum* and B. infantis (Jayamanne and Adams, 2009; Florence *et al.*, 2016). These data indicate that strains of *B. lactis*, such as HN019, used in the present work, exhibited a high ability to withstand environmental stress conditions, such as acidic environment and low temperatures.

Table 2. Stability [pH, titratable acidity (TA) and viable cell counts] of milk fermented with *B. lactis* HN019 (for 12 h) during cold storage (30-day refrigerated at 4°C). Results are expressed as mean ± standard deviation, n = 9.

Parameter	Storage time (day)					
	0	1	7	14	21	30
Counts (log CFU/mL)	9.28 ± 0.08[a]	9.30 ± 0.10[a]	9.12 ± 0.27[a]	9.24 ± 0.22[a]	9.25 ± 0.11[a]	9.13 ± 0.18[a]
pH	4.49 ± 0.05[b]	4.55 ± 0.05[a]	4.56 ± 0.06[a]	4.51 ± 0.02[a]	4.56 ± 0.06[a]	4.56 ± 0.03[a]
TA (%)	1.07 ± 0.27[a]	1.08 ± 0.26[a]	1.09 ± 0.24[a]	1.15 ± 0.31[a]	1.17 ± 0.33[a]	1.18 ± 0.31[a]

Means within a column with different superscripts are significantly ($p < 0.05$) different.

The pH of the fermented milk remained stable and the acidity was maintained after 30 d of cold storage, as a low rate of post-acidification was observed without statistical significance ($p > 0.05$; Table 2). Post-acidification is an important factor for the shelf life of fermented milks because survival of probiotics is affected considerably by pH and TA of the products (Mortazavian *et al.*, 2010). A very low pH value increases the concentration of undissociated organic acids in fermented products, thereby enhancing the bactericidal effect of these acids (Tripathi and Giri, 2014).

Sensory analysis

Bacteria of the genus *Bifidobacterium* usually produce high concentration of components that might contribute negatively to taste and aroma, e.g., vinegar flavour (Mohammadi *et al.*, 2011). Therefore, we evaluated whether consumers could identify higher acidity with the fermentation time and the effect of acidification on sensory preference of the formulated probiotic milk.

The forced choice test of both probiotic milks after 8 h and 72 h of fermentation showed a significant difference ($p < 0.05$) between the samples. A high number of panellists (88.75%) detected higher acidity as the fermentation time increased (72 h fermentation). Additionally, 62.5% of panellists preferred the less acidic sample (8 h fermentation).

Regarding the purchase intention for the milk fermented for 8 h, 65% of the panellists voted "may or may not buy", "probably buy", or "definitely buy" the product (sum of scores 3, 4, and 5 of purchase intention test, respectively), whereas 35% of the panellists voted as would "probably not buy" and "definitely not buy" for the product (sum of scores 1 and 2). In contrast, for the milk fermented for 72 h, 52.5% of the panellists voted as "probably not buy" and "definitely not buy" for the product. These results indicate that the development of fermented milk by *B. lactis* HN019 for 8 h of fermentation had a higher acceptability than that for 72 h. This could be explained by the fact that prolonged fermentation of milk produced higher acetic acid taste which is not preferable (Figure 2A). Additionally, organoleptic effects of the in-bottle sterilisation treatment used in the present work included a sterilised-caramelised flavour and browning caused by the Maillard reaction. In this case, both effects are desired as the slightly caramelised flavour of fluid fermented milk was appreciated by consumers, and the yellowish colouring would exempt the use of added colorants.

Determination of organic acids and lactose in fermented milk by isq ^1H NMR

The selected proton NMR signals were integrated within the chemical shifts (resonances) ranges of δ 1.20–1.40 (methyl protons of lactic acid), δ 1.85–2.05 (methyl protons of acetic acid), and δ 3.85–3.20 (lactose). The assignments of these chemical shifts are in agreement to the NMR data reported for reference samples of the same compounds (Nord *et al.*, 2004; Bouteille *et al.*, 2013). Figure 1 shows the spectra of milk samples at different times after bifidobacterial inoculation. In these spectra, acetic and lactic acid resonance intensities increased with time, whereas lactose resonance intensities decreased with time.

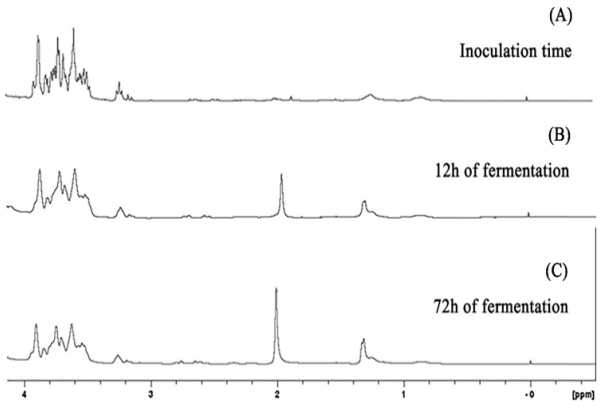

Figure 1. In situ quantitative proton nuclear magnetic resonance spectra of milk fermented by *B. lactis* HN019 at 37°C recorded at inoculation time (A), 12 h of fermentation (B) and 72 h of fermentation (C). The area of the resonance of the doublet at 1.33 ppm (lactic acid) and the area of the singlet at 2.00 ppm (acetic acid) increased with time while the area of the resonance between 4.00 ppm and 3.11 ppm (lactose) decreased with time.

To determine the concentration of these metabolites, calibration curves were prepared, and the areas of the following ^{1}H NMR signals were then used as a function of the concentration of each metabolite for integral calibration: the doublet at 1.32 ppm (lactic acid); the singlet at 1.97 ppm (acetic acid), and the triplet at 3.25 ppm (lactose). Equations were formulated using the linear least square regression method and used for separately computing the concentration of lactic acid, acetic acid, and lactose in fermented milk.

It is important to note that lactic and acetic acids and lactose yielded signals in regions relatively free from other signals and, consequently, allowed their accurate quantification by integration of the spectrum of the milk samples. However, when there were small drifts in chemical shift for a signal between spectra due to, for instance, pH variations, as observed in the fermented milk in the present work, there might be high integration errors if the same integration range was used for all spectra (Bharti and Roy, 2012). We concluded that the best result was obtained when each signal was manually integrated in each individual spectrum.

The calculated quantities of each metabolite are displayed in Figure 2. High correlations were obtained for organic acids (R2 = 0.97), whereas for lactose, the correlation was slightly lower (R2 = 0.92). Both organic acids linearly increased during the examination period. However, lactose consumption reached a plateau at around 48 h fermentation. The shapes of the curves reflected bacterial growth, and the profiles observed closely resembled the profiles previously reported by Bouteille *et al.* (2013) who first quantified lactate and lactose concentrations in dairy gels (yoghurt) by isq ^{1}H NMR.

Acetic acid was the main metabolite produced throughout the fermentation period, ranging from 0.47 ± 0.06 g/L to 16.36 ± 1.86 g/L at the end of 72 h (Figure 2A). To the best of our knowledge, this is the first study to quantify acetate in milk by isq ^{1}H NMR. Our measurements showed a higher production of acetate by *B. lactis* HN019 as compared to that by other bifidobacteria strains evaluated at the same temperature (Chick *et al.*, 2001; Østlie *et al.*, 2003). From the sensory point of view, acetic acid can be an undesirable end product in fermented milk as high amounts result in a "vinegary" flavour. A longer fermentation time and, consequently, additional acetic acid production had a lower acceptability among panellists in our sensory analysis. However, in terms of health, acetate is the main short chain fatty acid (SCFA) in the colon and can be used as a nutrient for the colonic epithelium and as a modulator of colonic

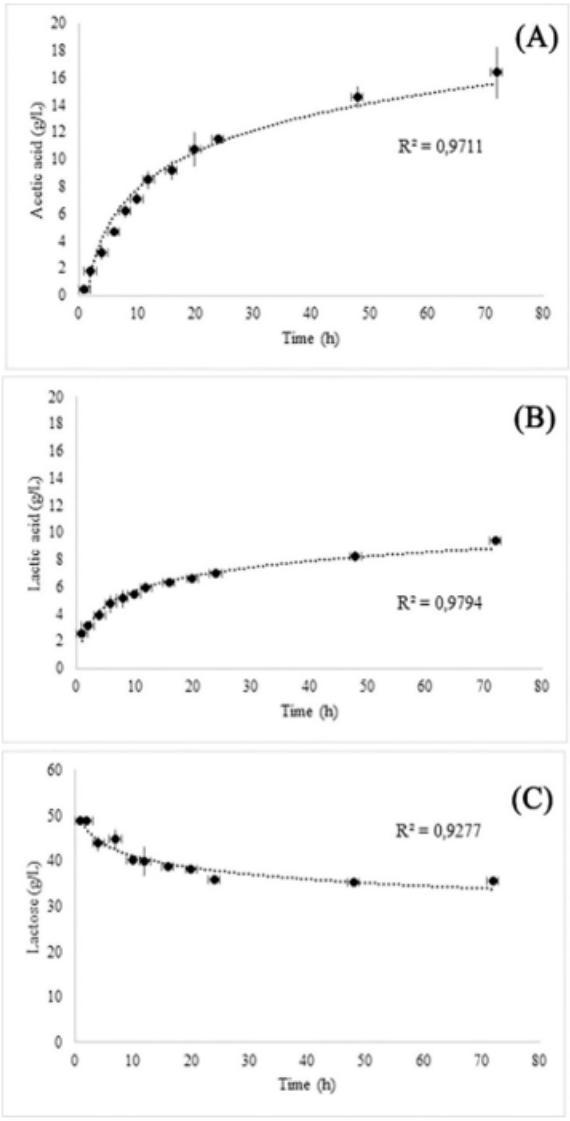

Figure 2. Concentration (g/L) of produced acetic acid (A) and lactic acid (B) and of consumed lactose (C) during milk fermentation with B. lactis HN019 at 37°C for 72 h. Results are expressed as mean ± standard deviation, n = 3.

and intracellular pH, cell volume, and other functions (Hijova and Chmelarova, 2007).

In contrast, lactic acid is a milder, sweeter acid that does not generate objectionable flavour (Nguyen *et al.*, 2012). The initial concentration of lactic acid was 2.59 ± 0.10 g/L (Figure 2B). This lactate content in unfermented skim milk was higher than that typically reported in literature (Østlie *et al.*, 2003; Nguyen *et al.*, 2012). The mean of the final concentrations of lactic acid was 9.40 ± 0.18 g/L. According Østlie *et al.* (2003), a satisfactory fermented probiotic milk product with the appropriate aroma and flavour should contain approximately 8 g/L lactate. However, only after 48 h, *B. lactis* HN019 reached concentrations of 8.24 g/L. In fact, the utilisation of the bifidus pathway by bifidobacteria is less efficient

in producing lactic acid, and as such an extended period of 48 h of fermentation would be inconvenient from the viewpoint of production. Therefore, *Bifidobacterium* strains are often incorporated as co-cultures in fermented dairy products, such as yoghurt, which is fermented by ordinary LAB starters, such as *Streptococcus thermophilus* and *Lactobacillus* delbrueckii ssp. bulgaricus (Prasanna *et al.*, 2014).

The production of organoleptically acceptable and stable probiotic fermented milks could be improved by determining the produced important metabolic products and their concentrations (Østlie *et al.*, 2003). It has been suggested that one of the main mechanisms of inhibition by bifidobacteria is related to the fermentative production of acids, such as acetate and lactate (Makras and Vuyst, 2006). The average ratio of acetate to lactate (ratio A/L) in the present work at the end of 12 h fermentation was 1.48, whereas the A/L ratio at the end of 24 and 72 h were 1.64 and 1.74, respectively. The A/L ratio increased as fermentation continued due to the increase in the rate of acetate production. The A/L ratio at 12 h fermentation was in agreement with the theoretical yield of 1.5 mol acetic acid and 1 mol lactic acid mentioned in the literature (Scardovi *et al.*, 1971). However, this also concurs with previous studies that demonstrated strain-dependence in producing these two main organic acids by bifidobacteria (Palframan *et al.*, 2003; Van der Meulen *et al.*, 2006; Nguyen *et al.*, 2012).

Finally, the content of lactose ranged from 48.9 ± 0.25 g/L to 35.48 ± 0.70 g/L after 72 h fermentation (Figure 2C). These values are in good agreement with lactose measurements by NMR by Hu *et al.* (2007) and Bouteille *et al.* (2013). Lactose is the primary substrate for acid production in milk; however, fermentation is not limited by the amount of lactose available but by the production of organic acids and the concomitant lowering of pH, which increasingly inhibits the microorganisms long before lactose is exhausted (Narvhus *et al.*, 1998). Normally, growth and metabolism mostly cease after 24 h fermentation; however, it has been established that growth and metabolism can be uncoupled (Østlie *et al.*, 2003). Although incomplete utilisation of lactose was observed in the present work, the production of organic acids by *B. lactis* HN019 occurred up to 72 h and cessation of growth was observed after 48 h (Table 1). These results indicate that organic acid production was uncoupled from the growth of strain HN019.

Conclusion

Herein, *B. lactis* HN019 showed a long fermentation time in milk when compared to industrial starter cultures (e.g., S. thermophilus and L. delbrueckii subsp. bulgaricus), with a high content of acetate production throughout fermentation. The level of acetic acid after 8 h fermentation culminated in a product with the best sensory acceptability, whereas long fermentation (72 h) generated defects typical of the metabolism of bifidobacteria with high acidity and vinegary flavour. However, tolerance of the strain toward low pH and end products was high (> log 8.85 CFU/mL) at 72 h fermentation. In addition, the strain showed high survival during cold storage for 30 d and reduced post-acidification ($p > 0.05$). Our findings show the efficiency of *B. lactis* HN019 as a fermenting bacterium through its production of acids. The study of strain metabolism allowed us to better understand the flavour variations in the fermented milk, as the production of acetic or lactic acid determines the acceptance of the fermented product. We also highlighted the importance of controlling the fermentation time because *Bifidobacterium* strains produce different amounts of metabolic products after a certain fermentation period. Although other analytical techniques for acid quantification have already been consolidated, the present work also confirms that isq ^{1}H NMR is a simple and non-destructive method that yielded robust results for lactic and acetic acids and lactose contents.

Acknowledgement

The authors are thankful to the CNPq for the financial support through grant 142385/2014-0. The support from the Confepar Agro-industrial Cooperativa Central in providing skim milk powder is also acknowledged.

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