Annals of Microbiology (2019) 69:1361–1372
https://doi.org/10.1007/s13213-019-01518-y

ORIGINAL ARTICLE

Selection of potentially probiotic *Kluyveromyces lactis* for the fermentation of cheese whey–based beverage

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Received: 7 June 2019 / Accepted: 13 September 2019 / Published online: 1 November 2019

Abstract

Purpose This work aimed to assess the probiotic potential of different *Kluyveromyces lactis* strains isolated from Canastra cheese and to produce a fermented cheese whey beverage added to beetroot juice using the selected strain.

Methods *Kluyveromyces lactis* strains were tested for their resistance to the passage through the simulated gastrointestinal tract, adhesion properties, and functional effects such as inhibition of enteric pathogens, short-chain fatty acids (SCFA) production, and β-galactosidase activity. The selected strain was used to produce a fermented cheese whey beverage added to beetroot juice in different proportions. The produced beverages were characterized using HPLC for sugars, Folin-Ciocalteu for total phenolic content, DPPH for antioxidant activity, and GC-MS for volatiles compounds.

Results Except B51, all strains showed viability above 75% after exposure to the simulated gastric and duodenal juices. The aggregation rates were above 84% in 24 h. Only B9 and C16 strains presented hydrophobicity above 60%. The highest B9 β-galactosidase activities were 2.17 U/g and 2.21 U/g for pH 7 and 9, respectively. The B9 SCFA profile was similar to that found for *Saccharomyces bourllardi*. The fermented cheese whey beverages presented phenolic content ranging from 102.75 to 291.61 μg EAG/mL and inhibition of DPPH ranging from 38.69 to 81.02% after 21 days of storage, besides being lactose free. Esters and acetates were the most abundant compounds.

Conclusions *Kluyveromyces lactis* B9 presented interesting results as a potential probiotic yeast. The produced beverages allowed the delivery of *K. lactis* B9 through innovative product with functional properties.

Keywords β-Galactosidase · Functional beverages · Antioxidant activity · Volatile compounds

Introduction

Probiotics have been increasingly highlighted as a research subject in the last years. They are defined as living microorganisms capable of conferring health benefits to the host when administered in adequate quantities (FAO and WHO 2002). Probiotics exert several benefits to human health, such as helping to maintain intestinal integrity and immunological modulation. Other benefits such as cholesterol reduction, vitamin B production, regularization of intestinal microbiota, and increased bioavailability of nutrients are also associated with probiotics (Cummings and Macfarlane 2002).

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The genera of microorganisms most used as probiotics in the food industry are *Lactobacillus* and *Bifidobacterium*, commonly used in dairy products. Alternatively to bacteria, yeasts have been exploited for their probiotic potential. Genera such as *Kluyeromyces*, *Debaromyces*, *Torulaspora*, *Pichia*, *Cryptococcus*, *Zygosaccharomyces*, *Trichosporon*, and *Candida* have demonstrated probiotic potential, tolerating passage through the gastrointestinal tract (Chen et al. 2010a, b; Rajkowska and Kunicka-Styczynsk 2010; Hatoum et al. 2012; Syal and Vohra 2013; Diosma et al. 2014; Aloglu et al. 2016; Fadda et al. 2017; Amorim et al. 2018).

Yeasts of the genus *Kluyveromyces*, mainly *Kluyveromyces lactis*, have emerged as one of the most important yeast species for research and industrial biotechnology being used for the production of metabolites and proteins, mainly the enzyme β-galactosidase already commercialized for industrial purposes such as the production of lactose-free dairy products (Spohner et al. 2016). They are frequently isolated from milk and cheese

and naturally consumed along with these foods (Andrade et al. 2017; Ceugniez et al. 2017; Fadda et al. 2017). This genus has also shown resistance to the passage through the gastrointestinal tract and potential for adhesion to the intestinal epithelium, as well as functional properties such as short-chain fatty acid production, immune modulation, inhibition of pathogens, and pro-apoptotic activity in cancerous epithelial cells (Kumura et al. 2004; Maccaferri et al. 2012; Ceugniez et al. 2017; Saber et al. 2017). These considerations make evident the importance of the search for new yeasts strains, which have the beneficial effects common to established one and/or other beneficial effects. Besides, the discovery of new probiotics yeast would enable the production and marketing of new types of beverages and probiotic foods.

Fermented beverages are used as vehicles for probiotics in the human diet, mainly milk-based beverages. In this context, cheese whey is an interesting raw material for the production of functional foods with probiotics. It is rich in calcium, bioactive peptides, and essential amino acids such as leucine, valine, isoleucine, and cysteine, which are important agents in metabolism, neural function, and homeostasis (Patel 2015). Since the cheese whey concentration process results in a relatively high cost for the industry, the use of non-concentrated cheese whey may represent an interesting alternative for cost reduction. For this reason, we evaluated the use of non-concentrated cheese whey compared with concentrated cheese in the production of a fermented beverage.

Although dairy beverages are the most frequent beverages used for the delivery of probiotics, the industry has been looking for different probiotic foods that promote health benefits. Besides dairy products, fruits such as pineapple and Cornelian cherry have been recently reported by Costa et al. (2013) and Amorim et al. (2018) as good substrates to produce probiotic beverages. Unlike fruit juices, the vegetable juices are still little explored for the production of probiotic beverages. As vegetables have excellent nutritional properties with a high content of amino acids, vitamins, and the presence of biologically active antioxidant compounds (Kiefer et al. 2004), they are also good substrate to be used in the fermentation with probiotics. One interesting vegetable in this context is beetroot whose juice contains a high level of biologically available antioxidants and many other health-promoting compounds such as potassium, magnesium, folic acid, iron, zinc, calcium, phosphorus, niacin, biotin, and fibers (Wootton-Beard and Ryan 2011). In this work, we used beetroot juice to obtain a fermented beverage added to these health-beneficial components, especially the antioxidant compounds.

The objective of this work was to evaluate the probiotic potential of different *K. lactis* strains previously isolated from Canastra cheese production process and additionally use the selected strain to produce a fermented cheese whey beverage added to beetroot juice.

Materials and methods

Microorganisms used

The eleven studied *K. lactis* strains were previously isolated from the Canastra cheese (Andrade et al. 2017) production process. The strain *Saccharomyces cerevisiae* var. *boulardii* (S1) was got from the commercial product Floratil® AT 250 (Merck KGaA, Darmstadt, Germany) and used in all experiments as a control.

Inoculum preparation

The strains were initially grown at 28 °C for 48 h in 2 mL YPD broth (1% yeast extract, 2% peptone, and 1% glucose) until reaching the 10^7 cell/mL. The obtained cultures were centrifuged at 5000 rpm for 10 min at 4 °C and then washed with (PB) (g/L: sodium chloride 8.0, potassium chloride 0.2, disodium phosphate 1.44, and potassium phosphate 0.24; pH 7). The biomass was used as the initial inoculum for tests described below.

Evaluation of probiotic potential

Passage through the simulated gastrointestinal tract

To evaluate the passage through the upper gastrointestinal tract, the cells were initially exposed to 10 mL of synthetic gastrointestinal juice (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$, 1.2 g/L $NaHCO_3$, 0.3% pepsin, pH 3.0) and incubated at 37 °C under stirring. After 90 min, 17.5 mL of synthetic duodenal juice (6.4 g/L $NaHCO_3$, 0.24 g/L KCl, 1.28 g/L NaCl, 0.1% pancreatin, 4 mL of bile 10%) adjusted to pH 7.4 with 5 M HCl was added to the 10 mL synthetic gastrointestinal juice. To determine the strains survival rate, at 0 (T0), 90 (T1), and 270 (T2) min, samples were plated in YPD with incubation at 37 °C for 48 h (Fadda et al. 2017).

Auto-aggregation test

The auto-aggregation test was performed according to Fadda et al. (2017) with modifications. The cell biomass was resuspended in 3 mL of PBS; the solution was vortexed for 10 s and then subjected to OD reading in a spectrophotometer at of 560 nm. The solution was kept in the cuvette, incubated at 37 °C for further readings at times 2, 4, and 24 h. The percentage of auto-aggregation was expressed by the equation:

$$\text{Auto}-\text{aggregation}\% = (\text{At}/\text{A0}) \times 100$$

where *At* represents the absorbance at time 2 h, 4 h, or 24 h, and *A*0 represents the absorbance at time 0 h.

Hydrophobicity assay

The cell biomass was resuspended in 5 mL PBS and from this, aliquots of 3 mL were placed in contact with 1 mL of n-hexadecane and vortexed for 120 s. The solution was incubated for 1 h at 37 °C to allow complete separation of phases. The aqueous phase was carefully removed, and the absorbance was measured in a spectrophotometer at 560 nm (Fadda et al. 2017).

The decrease in absorbance was taken as a measure of the hydrophobicity of the cell surface, calculated using the equation:

$$[(OD0-OD)/OD0] \times 100$$

where *OD*0 and *OD* are the optical densities before and after the contact with n-hexadecane, respectively.

Functional activities

Activity β-galactosidase

The strains pre-selected based on the above-described tests were used for the determination of their β-galactosidase activity. The inoculum obtained as described previously was transferred to 10 mL of the enzyme production medium (3.0% lactose, 0.7% yeast extract, 0.3% peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.3% K_2HPO_4) (Song et al. 2010) and incubated at 30 °C/200 rpm for 48 h. After, 500 mg of biomass was harvested and used for the cell permeabilization with the addition of 5 mL of isoamyl alcohol, 25 mL of phosphate buffer, and glass bead (1 mm) followed by vortexing for 5 min and incubation under stirring for a further 15 min at room temperature. The substrate for the enzyme was prepared according to Cardoso et al. (2015). Initially, 200 μL of permeabilized cell solution was placed in contact with 800 μL of the ONPG solution (2.5 mg/mL ONPG in 0.1 M phosphate buffer, pH 6); the reaction occurred for 15 min at 37 °C and then was stopped by the addition of 200 μL Na_2CO_3 (mmol/L). The OD was read in a spectrophotometer at 420 nm. For the calculation of the enzymatic activity, an ONPG extinction coefficient of 4.6 mM was used.

Stability β-galactosidase

The strain that showed the best result in the first assay was then submitted to a new test for the characterization of the enzymatic activity at pH 5, 7, and 9 and different temperatures (20 °C, 30 °C, and 45 °C) in order to verify the β-galactosidase stability.

Inhibition of pathogens

Three methods proposed by Ceugniez et al. (2015) were used to evaluate the inhibitory potential against pathogens, with some modifications. In the "late method," 100 μL of yeast culture (10^8 cell/mL) was plated in YPD and incubated at 28 °C for 48 h. The colonies were then removed, and plates were inoculated with 100 μL (10^4 cell/mL) of the pathogen cultures (*Escherichia coli* ATCC 055, *Salmonella enteritidis* ATCC 5190, and *Listeria monocytogenes* ATCC 11778). Plates were incubated at 37 °C for 48 h.

In the "supernatant assay," the TSA plates were flooded with 100 μL of the pathogen cultures which were allowed to dry. After, 7 wells of 5 mm were made on each plate and filled with filtered (0.22 μm) yeast growth medium. The plates were incubated for 2 h at 4 °C to allow the diffusion of the supernatant and then incubated at 37 °C for 48 h.

In the "gas production antagonism test," two compartment plates were inoculated with a yeast culture in one compartment and with the pathogen cultures in another compartment. The plates were incubated at 37 °C for 48 h. For all of these experiments, the presence or absence of zones of inhibition was inspected after the incubation periods.

Fermentation of prebiotic and production of short-chain fatty acids

To evaluate the capacity of selected strain to ferment prebiotic fibers and produce short-chain fatty acids (SCFA), a fermentation was performed using a medium composed by peptone (2%), yeast extract (1%), and as a source of carbon, 2% of commercial prebiotic Tamarine fibers® (inulin, fructooligosaccharide, and polydextrose). The fermentation was carried out at 37 °C in anaerobiosis and samples were withdrawn at 6 h, 12 h, and 24 h. A control fermentation was inoculated with a commercial probiotic mix (Probiatop®) containing the *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis*. This bacterial mix was chosen as a "control" treatment due to the recognized efficiency of bacteria to produce high concentrations of SCFA. The collected samples were analyzed by gas chromatography using a GC-FID (Shimadzu) equipped with a BP21 column (30 m × 0.25 mm × 0.25 μm). The injector and detector were kept at 180 °C and 200 °C, respectively. The oven was maintained at 200 °C and the carrier gas flow was 1.1 mL/min. Butyric, acetic, and propionic acids were identified by comparison of their retention time with the retention times of pure standard. The quantification was performed using external calibration curves.

Elaboration of fermented beverages

Inoculum preparation

The selected strain was reactivated in 2 mL YPD and incubated at 28 °C for 24 h. Afterward, the biomass was transferred to Erlenmeyer flasks containing 100 mL of medium, incubated for 48 h followed by the subsequent transfer of the biomass to 1 L of YPD, which was incubated until the population reached 10^8 cells/mL.

Preparation of raw material

The cheese whey concentration by nanofiltration was performed in a dairy factory in Lavras-MG. The non-concentrated cheese whey was collected right after the cheese mass separation in the production process at the dairy pilot plant of the Food Science Department at the University of Lavras. The beetroot juice was prepared by grinding the vegetable with water in the ratio 1:1 (w:v). The cheese whey and beetroot juice were pasteurized using direct steam in an autoclave for 7 min (Andrade et al. 2017).

Fermentation and addition of beetroot juice

The pasteurized concentrated (CW) and non-concentrated (NW) cheese whey were inoculated with 10^8 cells/mL of potential probiotic *K. lactis* strain followed by incubation at 28 °C until 120 h after the stabilization of Brix. After fermentation, the beetroot juice was added to the fermented cheese whey in the ratios of 1:1 and 1:3 (fermented cheese whey:beetroot juice).

Stability of beverages

The produced beverages were bottled in 250-mL clear bottles, which were kept under refrigeration at 4 °C for 21 days. Every 7 days, a bottle was opened for yeast, psychotropic and mesophilic bacteria counting. Aliquots of the beverages were plated in Plate Count Agar (PCA) (yeast extract 0.25%, peptone 0.5%, glucose 0.1%, agar 1.5%), supplemented with nystatin 0.4 g/L, and the plates were incubated at 37 °C for mesophilic bacteria and 4 °C for psychotropic bacteria. The yeast viability was determined using YPD agar with 1 g/L chloramphenicol followed by incubation at 28 °C for 48 h.

Analysis of sugars by HPLC

The main sugars in the 21 days beverages were analyzed by HPLC which was performed using a Shimadzu chromatograph equipped with a refractive index detector (RID-10A) and Supelcogel 8H column operated at 30 °C. Elution was performed with 5 mM sulfuric acid at a flow rate of 0.4 mL/min. The compound identification was performed by comparing the retention times of the peaks in the samples with those of the pure standards injected under the same conditions. The quantification was performed using the external calibration method (Duarte et al. 2010; Andrade et al. 2017).

Quantification of total phenolics

The total content of polyphenols was determined using the method of Folin-Ciocalteu, according to Wootton-Beard and Ryan (2011) with adaptations. A 0.2-mL aliquot of each beverage was added to 1.5 mL of Folin-Ciocalteu reagent (1:10 v/v). The solution was allowed to equilibrate for 5 min and then mixed with 1.5 m/L of sodium carbonate (60 g/L), followed by incubation in the dark for 90 min. The absorbance of the mixture was read at 725 nm using the respective solvent as blank. The results were expressed in milligram equivalents of gallic acid.

Evaluation of the antioxidant activity of the beverages

The method of Escudero-López et al. (2016) was used with modifications. First, 120 μL of the centrifuged beverages (10,000 rpm, 10 min, 4 °C) and 600 μL of methanol were mixed. Then, 180 μL of a DPPH solution (0.5 mM in ethanol) was added. The reaction was incubated for 60 min at 30 °C in the dark and after that, read in a spectrophotometer at 517 nm. Ethanol was used as blank and the DPPH solution without juice addition, the control. The percentage of DPPH reduction was calculated as follows:

[(Control Absorbance - Sample Absorbance)/Control Absorbance*100]

Analysis of volatile compounds by HS SPME GC-MS

The volatile compounds were extracted from the headspace using 5 mL of sample loaded in 15 mL vials. The extraction was carried out at 60 °C for 25 min with a DVB/Carboxen/PDMS Stable flex SPME (Supelco, Bellefonte, PA, USA) fiber in a manual holder (Andrade et al. 2017). The extracted compounds were analyzed in a GC-MS-QP2010 Plus (Shimadzu) chromatograph equipped with a Rts-5MS (30 m × 0.25 mm × 0.25 μm) column. Thermal desorption was at 270 °C for 100 s and injections in splitless mode (30 s at 25 psi). The system was operated at 35 °C and increment of 4 °C/min until reaching 240 °C using helium at 1.78 mL/min as the carrier gas. Compounds were identified using the NIST library 2011 and the quantification performed using internal calibration with 4-nonanol at a final concentration of 125 μg/L (Duarte et al. 2010).

Statistical analysis

The Sisvar 5.6 software (Lavras-MG) and XLstat 2014.5 software (Addinsoft's, New York, NY) were respectively used for Scott-Knott and principal component analysis. All experiments were performed in triplicate.

Results and discussion

Probiotic potential

To exert their functional effect on the human organism, probiotics must first survive to the adversities encountered during the traversal of the gastrointestinal tract. In our work, all the tested *K. lactis* strains showed resistance above 82% after 90 min of exposure to simulated gastric juice. The best (Scott-Knott $p < 0.05$) survival rate of 93.86% was found for C3 strain (Table 1). These survival rates showed the potential of the studied strains, once the resistance to gastric juice (stomach pH) is the first barrier faced by the microorganisms. The hydrochloric acid excreted by the parietal cells is responsible for the acidity in the organism (which pH ranges from 1.5 to 3.5) and acts as a defense against pathogenic microorganism. To be considered probiotic, a bacterium must survive to pH 2.0 and 3.0 during 3 h (Park et al. 2006). This is also a requirement for probiotic yeasts candidates. The tested *K. lactis* strains were isolated from samples of milk, Canastra cheese, cheese whey, and "pingo" (endogenous starter), which are naturally low pH environments, a condition that may have favored the resistance of the yeasts to the acidity of the stomach.

Table 1 Resistance of yeast strains in simulated gastric (90 min) and intestinal (270 min) conditions

Yeast	Survival rate (%) 90 min	Survival rate (%) 270 min
B7	88.00 ± 0.05^{i}	79.46 ± 0.02^{h}
B9	84.09 ± 0.04^{d}	83.87 ± 0.03^{k}
B34	82.95 ± 0.11^{b}	75.31 ± 0.05^{a}
B35	86.51 ± 0.16^{h}	78.53 ± 0.00^{e}
B51	83.27 ± 0.10^{c}	75.64 ± 0.03^{c}
C1	85.54 ± 0.15^{e}	75.99 ± 0.05^{d}
C3	93.86 ± 0.01^{l}	84.03 ± 0.05^{l}
C5	86.32 ± 0.01^{g}	78.61 ± 0.01^{f}
C16	85.64 ± 0.03^{f}	80.40 ± 0.01^{i}
D19	88.35 ± 0.01^{k}	81.93 ± 0.03^{j}
D22	82.12 ± 0.03^{a}	75.43 ± 0.20^{b}
S. boulardii	88.13 ± 0.07^{j}	79.29 ± 0.06^{g}

Averages followed by the same letter did not differ statistically by Scott-Knott test ($p > 0.05$)

After passing through the simulated gastric juice, the strains were submitted to the pancreatic juice. The pancreatic secretion contains multiple enzymes to digest all three major food groups (proteins, carbohydrates, and fats) (Guyton and Hall 2011). In our work, this pancreatic scenario was experimentally simulated by the addition of commercial pancreatin (Creon®) to the synthetic duodenal juice which contains the lipase, amylase, and protease. After exposure to simulated pancreatic juice (270 min), the survival of the strains ranged from 75.31% (strain B34) to 84.03% (strain C3). The probiotic *Saccharomyces boulardii* showed a survival rate of 79.29%, while six (B9, C3, C5, C16, D19, B7) of the tested strains showed higher (Scott-Knott $p < 0.05$) values (Table 1). It is interesting to note that the decrease in the survival rate did not exceed the values previously reported by Ceugniez et al. (2017) and Fadda et al. (2017) for *Kluyveromyces* strains.

The tolerance to bile salts is an important requirement for probiotic to settle in the intestinal epithelium. The physiological concentration of bile salts in the small intestine ranges from 0.2 to 2.0%. As the bile concentration used in our work was higher than the cited ones found naturally, it is possible to infer that the studied *K. lactis* strains are resistant to bile salts.

After resisting the adversities during the crossing of the upper gastrointestinal tract, the probiotics must be able to adhere to the cells of the intestinal mucosa, once this adhesion prevents their immediate elimination through peristaltic movements (Kos et al. 2003). The studied strains showed auto-aggregation rates ranging from 55.24% (D19) to 80.58% (C16) at 4 h, and from 77.14% (D19) to 94.87% (C16) at 24 h. The values presented by C16 strain were statically higher than those found for *S. boulardii* (Table 2). Only

Table 2 Auto-aggregation of *K. lactis* strains after 0 h, 4 h, and 24 h incubation

Strains	Auto-aggregation (%)		
	0 h	4 h	24 h
B7	32.31 ± 0.02^{e}	63.97 ± 0.01^{h}	93.67 ± 0.01^{j}
B9	35.97 ± 0.03^{g}	66.19 ± 0.13^{j}	89.93 ± 0.00^{g}
B34	25.77 ± 0.02^{b}	58.53 ± 0.00^{d}	87.37 ± 0.00^{f}
B35	49.86 ± 0.01^{k}	68.22 ± 0.01^{k}	84.79 ± 0.02^{d}
B51	16.90 ± 0.02^{a}	45.07 ± 0.01^{a}	34.51 ± 0.02^{a}
C1	47.87 ± 0.01^{j}	64.45 ± 0.01^{i}	84.83 ± 0.01^{e}
C3	29.82 ± 0.01^{d}	63.82 ± 0.00^{f}	92.98 ± 0.01^{i}
C5	37.77 ± 0.01^{i}	57.08 ± 0.01^{c}	83.26 ± 0.01^{c}
C16	67.41 ± 0.01^{l}	80.58 ± 0.00^{l}	94.87 ± 0.00^{l}
D19	36.43 ± 0.01^{h}	55.24 ± 0.01^{b}	77.14 ± 0.00^{b}
D22	32.82 ± 0.01^{f}	62.75 ± 0.01^{e}	91.30 ± 0.00^{h}
S. boulardii	27.6 ± 0.01^{c}	63.97 ± 0.01^{g}	94.85 ± 0.04^{k}

Averages followed by the same letter did not differ statistically by Scott-Knott test ($p > 0.05$)

B51 strain presented unsatisfactory results with 34.51% of auto-aggregation in 24 h (Table 2). The lowest values of hydrophobicity were found for B35, B51, C1, and *S. boulardii*. *Kluyveromyces lactis* strains B9 and C16 presented hydrophobicity rates of 65.80% and 62.48%, respectively (Fig. 1). Inferences on the adhesion capacity of probiotics to intestinal mucosa have been made based on auto-aggregation and hydrophobicity data (in vitro tests). Recently, Ceugniez et al. (2017) and Fadda et al. (2017) showed the probiotic potential of *Kluyveromyces* strain evaluating, among others, auto-aggregation and hydrophobicity parameters. Here, the data for these two parameters evidenced the potential adhesion of the studied strains to the intestinal mucosa.

Functional activities

The studied *K. lactis* strains showed no inhibitory activity against the pathogenic bacteria used in this work. However, in this specific case, this result cannot be extrapolated to in vivo conditions considering the complexity of factors that inhibit pathogens or their pathogenicity factors such as competition for the same receptors and nutrients and other actions such as the inhibition of toxins, which could not be evaluated through the used tests. Among the main antagonistic mechanisms exerted by yeast are competition for nutrients, production of killer toxins, pH changes, and production of compounds (volatile or non-volatile) as organic acids.

Based on statistical differences (Scott-Knott $p < 0.05$) found for survival rates after the passage through the simulated gastrointestinal tract (270 min), auto-aggregation (4 h), and hydrophobicity, the strains coded as B9, C3, and C16 were pre-selected and submitted to a preliminary test for determination of β-galactosidase activity. The enzymatic activity found was 1.08 ± 0.22, 0.80 ± 0.10, and 0.96 ± 0.13 U/g for strains B9, C3, and C16 respectively. The enzymatic activity of yeast B9 was statistically superior (Scott-Knott $p < 0.05$) to the activities found for the other 2 yeasts. *Kluyveromyces lactis* B9 strain was then selected for the enzyme stability test under different conditions. There were no significant differences between the three used temperatures ($p > 0.05$). The general averages for the pH 3 and each temperature, 20, 30, and 45 °C, were respectively 1.76, 1.70, and 1.63 U/g, while the averages of each pH were 0.71 (pH 5), 2.19 (pH 7), and 2.2 U/g (pH 9) (Fig. 2). There was no statistical difference ($p > 0.05$) between the last two values. Therefore, the β-galactosidase produced by strain B9 has optimum activity at neutral and alkaline pH. The results are interesting from the functional point of view, showing that there is enzyme activity in body temperature and intestinal pH, a possible positive effect that may help in the cases of lactose intolerance. The lactose hydrolysis by microorganisms is important considering the benefit in cases of lactose intolerance, whether by the production of β-galactosidase in the intestinal lumen or by the use of microorganism to manufacture free-lactose dairy foods.

Besides β-galactosidase activity, *K. lactis* B9 also showed functional properties through the production of SCFA. Table 3 shows the SCFA (acetate, butyrate, and propionate) produced during the fermentation of commercial prebiotic mix containing inulin, polydextrose, and FOS. Prebiotics are selectively fermented allowing specific modifications in the composition and/or activity of the intestinal microbiota, thus conferring benefits to host health (Gibson et al. 2004). These benefits are derived from the production of metabolic products, especially short-chain fatty acids acetate, propionate, and butyrate. The SCFA profile of *K. lactis* B9 and *S. bourllardi* were similar for the three different times of fermentation, except in the case of acetate (Table 3). However, the commercial mix of probiotic bacteria containing *L. paracasei*, *L. rhamnosus*, *L. acidophilus*, and *B. lactis* produced higher (Scott-Knott $p < 0.05$) concentrations of acetate, reaching 310.81 mmol/L at the end of 24 h. This concentration is higher than that reported in the intestinal lumen which is around 69.1 mmol/L in the cecum (Cummings et al. 1987). Although the acetate production by B9 strain was lower than that produced by the bacteria, it was similar to that produced by *S. boulardii* and reported in studies using, for example, the fecal microbiota fermenting FOS, inulin, and

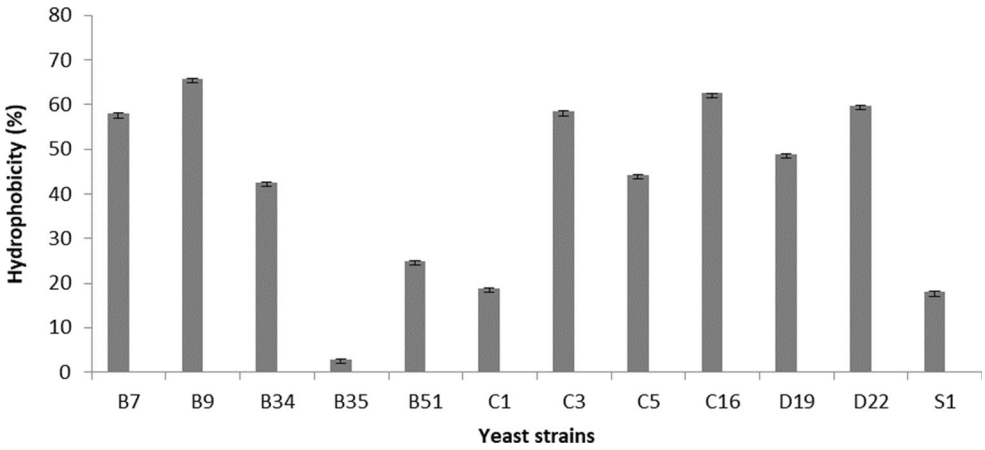

Fig. 1 Hydrophobicity of eleven different *K. lactis* strains. S1, *Saccharomyces boulardii*

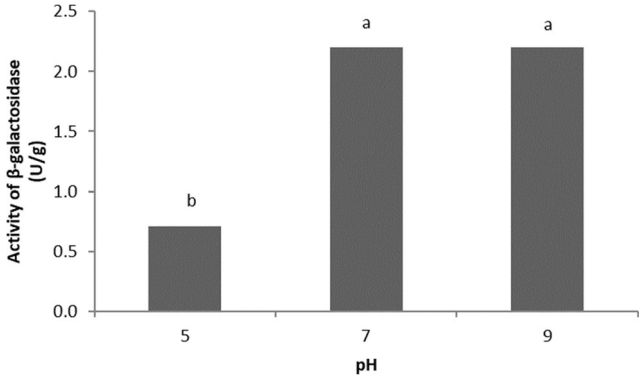

Fig. 2 The β-galactosidase activity of selected *K. lactis* (B9) at different pH. β-Galactosidase activities presented as averages of three different temperatures (20, 30, and 45 °C). Different letters indicate statistical difference by Scott-Knott test ($p < 0.05$)

polydextrose (Mäkeläinen et al. 2007; Beards et al. 2010; Chen et al. 2018; Di et al. 2018). The SCFAs play a crucial role in health as energy source; for example, butyrate is the main energy source used by the colonocytes. In addition, SCFAs have an immunoregulatory effect, with beneficial effects on inflammatory diseases of the intestinal tract and some forms of cancer. They also influence the metabolism of lipids such as cholesterol, contributing to metabolic conditions that promote preservation or recovery of the endothelial functions and reduce the risk of developing or worsening cardiovascular diseases (Richards et al. 2016). Such benefits show that SCFA is an important parameter for the functional effect of strains with probiotic potential, satisfactorily met by B9 strain.

Table 3 Concentrations of SCFA at different fermentation time

	Time (h)	Concentrations of SCFA (mmol/L)		
		K. lactis B9	Bacteria*	*S. boullardi*
Acetate	0	nd	nd	nd
	6	8.84 ± 2.25^{a}	98.47 ± 25.29^{b}	7.87 ± 1.02^{a}
	12	8.24 ± 0.48^{a}	134.66 ± 38.24^{b}	4.60 ± 1.80^{a}
	24	8.76 ± 0.32^{a}	310.81 ± 77.65^{b}	3.75 ± 0.71^{a}
Butyrate	0	nd	nd	nd
	6	0.11 ± 0.05^{a}	0.06 ± 0.01^{a}	0.16 ± 0.08^{a}
	12	0.13 ± 0.04^{a}	0.10 ± 0.03^{a}	0.15 ± 0.02^{a}
	24	0.13 ± 0.01^{a}	0.25 ± 0.04^{a}	0.15 ± 0.09^{a}
Propionate	0	nd	nd	nd
	6	0.21 ± 0.12^{a}	0.11 ± 0.01^{a}	0.20 ± 0.18^{a}
	12	0.25 ± 0.09^{a}	0.24 ± 0.04^{a}	0.21 ± 0.02^{a}
	24	0.26 ± 0.10^{a}	0.52 ± 0.04^{a}	0.30 ± 0.29^{a}

*Bacteria = probiotic mix: *Lactobacillus paracasei*, *L. rhamnosus*, *L. acidophilus*, and *Bifidobacterium lactis*

nd = not detected. Averages followed by the same letter did not differ statistically by Scott-Knott test ($p > 0.05$)

Characterization of produced beverages

Yeast population

The *K. lactis* B9 was selected for the fermentation of concentrated and non-concentrated cheese whey due to its good performance, presenting resistance, adhesion capacity, and high production of β-galactosidase. Non-concentrated (NW) and concentrated (CW) cheese whey were fermented for 120 h and after that added to beetroot juice in proportions of 1:1 or 1:3 (fermented cheese whey:beetroot juice). The fermentation process started with 1.5×10^{8} cells/mL and this population remained stable (7.9×10^{8} cells/mL) during the shelf life test up to 21 days. To exert beneficial effects, probiotic microorganisms must be alive and available in populations around 10^{8} to 10^{9} cells/g (mL) of the product at the time of consumption. In Brazil, the National Agency of Sanitary Surveillance (ANVISA) recommends that probiotic cell count should be between 10^{8} to 10^{9} CFU in the ready-to-eat product, although lower values can be accepted since the effectiveness has been proven. Considering that B9 strain population remained viable throughout the process, we can infer it could exert its probiotic effects when conveyed via the produced beverage. The produced beverages did not present growth of psychrotrophic and mesophilic bacteria during the 21 days of storage under refrigeration.

Sugars

The sugar content of cheese whey, beetroot juice, and produced beverages was measured by HPLC (Table 4). In both cases of diluted (NW) and concentrated (CW) cheese whey, at the end of fermentation, yeast completely consumed the sugar content (Table 4), highlighting the efficient activity of β-galactosidase produced by *K. lactis* B9. This result is in line with the current scenario of the β-galactosidase use, commonly applied in the food industry to reduce the lactose content of dairy products, preventing problems of crystallization of lactose and increasing sweetness, taste, and solubility (Gänzle et al. 2008). In addition, the hydrolysis of lactose allows the production of lactose-free products suitable for people intolerant to lactose. In general, the sugar profiles of the produced beverages were qualitatively similar, being fructose, glucose, and sucrose the main residual sugars. The most significant (Scott-Knott $p < 0.05$) difference was the residual fructose content of beverages produced with non-concentrated cheese whey (NW), which was twice as high as the content of the concentrated cheese whey beverages (CW) (Table 4). This higher fructose content can impact the taste of the beverages since this sugar has a sweetness 2 times greater than, for example, glucose.

Table 4 Sugar concentration in non-concentrated and concentrated cheese whey, beetroot juice, and fermented beverages

Raw material	Lactose	Fructose	Glucose	Sucrose	Galactose
Sweet beet juice	nd	12.00 ± 2.12	nd	17.87 ± 0.18	nd
Concentrated cheese whey	55.45 ± 4.11	nd	58.08 ± 2.67	nd	38.89 ± 1.87
Non-concentrated cheese whey	46.33 ± 2.05	nd	nd	nd	nd
Fermented beverages after 21 days under refrigeration	Lactose	Fructose	Glucose	Sucrose	Galactose
Beverage NW 1:1	nd	1.50 ± 0.10^{d}	2.28 ± 0.30^{d}	nd	nd
Beverage NW 1:3	nd	1.63 ± 0.03^{c}	1.02 ± 0.13^{a}	1.06 ± 0.50^{b}	nd
Beverage CW 1:1	nd	0.87 ± 0.12^{b}	1.28 ± 0.10^{b}	1.29 ± 0.16^{c}	nd
Beverage CW 1:3	nd	0.70 ± 0.17^{a}	1.90 ± 0.92^{c}	0.54 ± 0.01^{a}	nd

NW 1:1, non-concentrated fermented cheese whey added to beetroot juice in a ratio of 1:1 (v:v); NW 1:3 (v:v), non-concentrated fermented cheese whey added to beetroot juice in the ratio of 1:3 (v:v); CW 1:1, concentrated fermented cheese whey added to beetroot juice in the ratio of 1:1 (v:v); CW 1:3, concentrated fermented cheese whey added to beetroot juice in the ratio of 1:3 (v:v). nd, not detected

Total phenolics and antioxidant activity

Total phenolics (Folin-Ciocalteu) and antioxidant activity (DPPH) were determined at 0 and 21 days of beverages storage under refrigeration at 4 °C. The total phenolic content for all beverages decreased between days 0 and 21 days (Fig. 3). Beverages produced with three parts of beetroot juice presented higher (Scott-Knott $p < 0.05$) amounts of total phenolics than the beverages produced with one part of juice (Fig. 3). Similar to that observed for total phenolic content, there was also a decrease in the antioxidant activity between the initial and final days of shelf life. The use of three parts of beetroot juice resulted in beverages with higher antioxidant activity (Scott-Knott $p < 0.05$) than those activities found for the beverages produced with one part of juice beetroot juice (Fig. 4). These reduction profiles were also observed in the beetroot juice used as control (Figs. 3 and 4). In both cases of total phenolics and antioxidant activity, it is possible to note that the results are proportionally to the used amount of beetroot juice. As the main reason for using beetroot juice was to obtain a fermented beverage with high antioxidant activity, the beverages, NW 1:3 and CW 1:3, showed the higher total phenolic content and DPPH inhibition (Figs. 3 and 4). The antioxidant activity from beetroot juice allowed the improvement of the functional potential of the beverages already containing the probiotic yeast. The antioxidant potential of beetroot juice has been widely demonstrated in several studies. Works such as Wootton-Beard and Ryan (2011) reported a rate of DPPH inhibition up to 100% showing that beet juice has higher values than tomato juice and carrot juice. The antioxidant activity of beets comes mainly from compounds such as betalains (Pitalua et al.

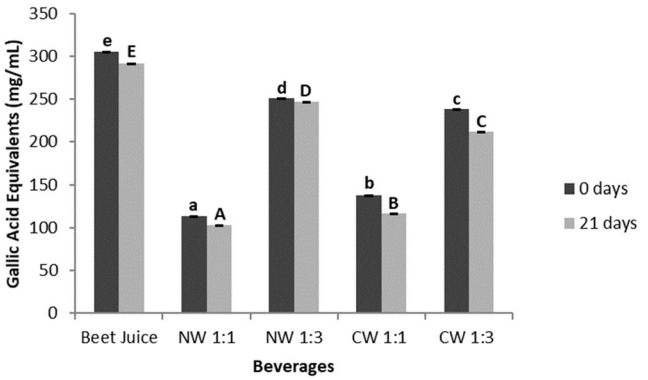

Fig. 3 Total phenolic content of produced beverages at 0 and 21 days of storage under refrigeration at 4 °C. NW 1:1, non-concentrated fermented cheese whey added to beetroot juice in a ratio of 1:1 (v:v); NW 1:3 (v:v), non-concentrated fermented cheese whey added to beetroot juice in the ratio of 1:3 (v:v); CW 1:1, concentrated fermented cheese whey added to beetroot juice in the ratio of 1:1 (v:v); CW 1:3, concentrated fermented cheese whey added to beetroot juice in the ratio of 1:3 (v:v). Different capital letters indicate statistical difference (Scott-Knott test $p < 0.05$) between the beverages at 21 days of storage. Different lowercase letters indicate statistical difference (Scott-Knott test $p < 0.05$) between the beverages at 0 days of storage

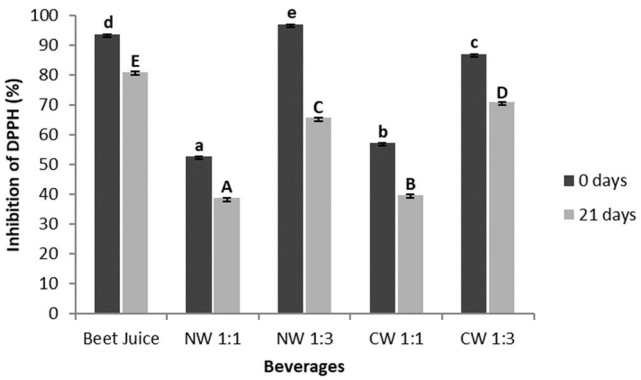

Fig. 4 Antioxidant potential (DPPH) of beverages at 0 and 21 days of storage under refrigeration at 4 ° C. NW 1:1, non-concentrated fermented cheese whey added to beetroot juice in a ratio of 1:1 (v:v); NW 1:3 (v:v), non-concentrated fermented cheese whey added to beetroot juice in the ratio of 1:3 (v:v); CW 1:1, concentrated fermented cheese whey added to beetroot juice in the ratio of 1:1 (v:v); CW 1:3, concentrated fermented cheese whey added to beetroot juice in the ratio of 1:3 (v:v). Different capital letters indicate statistical difference (Scott-Knott test $p < 0.05$) between the beverages at 21 days of storage. Different lowercase letters indicate statistical difference (Scott-Knott test $p < 0.05$) between the beverages at 0 days of storage

2010) and beets are also rich in phenolic acids such as 4-hydroxybenzoic acid, cinnamic acid, vanillic acid, chlorogenic acid, ferulic acid and caffeic acid, and gallic acid (Ravichandran et al. 2012; Wruss et al. 2015).

Beetroot is a source of antioxidants and many other health-promoting components, such as folic acid, iron, magnesium, selenium, potassium, calcium, zinc, phosphorus, biotin, niacin, and β-carotene, as well as vitamins A, B6, and C. The high antioxidant capacity of beetroot along with its other compounds of nutritional value makes beetroot juice a positive component to be added to the diet (Wootton-Beard and Ryan 2011). Cheese whey also has advantageous nutritional properties such as proteins with a high content of essential amino acids, especially branched-chain ones, high calcium content, and bioactive peptides (Haraguchi et al. 2006). Besides these beneficial characteristics of beetroot and cheese whey, the probiotic potential of

Table 5 Concentrations of volatile compounds (μg/L) in fermented cheese whey added to 3 parts of beetroot juice

No.	Compound	Beverage CW 1:3	Beverage NW 1:3	Descriptors
Esters and acetates				
1	Ethyl butanoate	3796.09 ± 10.51	3905.30 ± 5.43	Fruity[a]; papaya, butter, sweetish, apple, perfumed[b]
2	Ethyl pentanoate	10.45 ± 0.24	11.35 ± 0.38	–
3	Ethyl hexanoate	2832.33 ± 1.37	2748.96 ± 5.83	Fruity, green apple[b]
4	Ethyl heptanoate	68.95 ± 3.68	111.41 ± 9.07	–
5	Ethyl benzoate	120.00 ± 5.85	279.67 ± 1.30	–
6	Ethyl octanoate	24756.23 ± 45.91	23259.67 ± 18.07	Apple[b]
7	Ethyl nonanoate	150.51 ± 2.30	293.24 ± 11.80	Fruity[c]
8	Ethyl 9-decenoate	5203.96 ± 42.01	5043.09 ± 21.14	Rose[d]
9	Ethyl decanoate	12302.95 ± 59.41	12886.65 ± 40.51	Fruity, grape[d], woody[e]
10	Ethyl 9-hexadecenoate	178.74 ± 3.83	77.17 ± 5.08	–
11	Ethyl dodecanoate	1605.15 ± 17.23	2737.31 ± 9.49	Fruity, sweet[d]
12	Ethyl 11-hexadecenoate	177.30 ± 2.64	217.54 ± 6.72	–
13	Ethyl tetradecanoate	501.26 ± 9.57	804.66 ± 14.08	–
14	Ethyl undecanoate	30.59 ± 7.67	20.83 ± 1.57	–
15	Ethyl hexadecanoate	745.33 ± 28.49	612.13 ± 1.50	Fruity, apple, wine-like[d]
16	Ethyl linoleate	377.32 ± 2.68	142.59 ± 16.48	–
17	Ethyl Oleate	465.48 ± 1.47	369.30 ± 0.51	–
18	Propyl butanoate	16.26 ± 2.35	31.45 ± 1.04	–
19	2-Methylpropyl butanoate	79.99 ± 0.58	78.93 ± 3.59	–
20	Butyl butanoate	371.00 ± 1.66	nd	–
21	3-Methylbutyl butanoate	2009.78 ± 83.48	1394.87 ± 11.31	–
22	2-Methylbutyl butanoate	494.57 ± 8.19	640.76 ± 4.06	–
23	2-Methylpropyl hexanoate	10.13 ± 1.59	19.86 ± 0.97	–
24	3-Methylbutyl hexanoate	271.12 ± 1.57	253.20 ± 8.24	–
25	2-Methylpropyl octanoate	185.93 ± 3.79	nd	–
26	Phenethyl propionate	90.42 ± 2.97	75.10 ± 1.49	–
27	Phenylethyl butanoate	1978.71 ± 12.58	224.68 ± 22.07	–
28	3-Methylbutyl octanoate	787.01 ± 38.51	465.54 ± 53.43	Pineapple[c]
29	2-Methylbutyl octanoate	153.18 ± 4.39	190.58 ± 3.40	–
30	Propyl decanoate	nd	14.16 ± 0.44	Floral, bitter[f]
31	2-Methylpropyl decanoate	27.91 ± 0.84	25.27 ± 2.00	–
32	3-Methylbutyl decanoate	100.27 ± 5.92	95.58 ± 1.91	–
33	2-Methylbutyl decanoate	34.78 ± 2.48	34.41 ± 3.07	–
34	Isoamyl acetate	58.12 ± 7.34	112.27 ± 19.57	Banana[g]
35	2-Methyl-1-butanol acetate	16.82 ± 0.92	20.57 ± 2.35	–
36	2-Phenylethyl acetate	10902.47 ± 25.94	3214.85 ± 7.80	Flowery[d]; rose[c]
Acids				
37	Octanoic acid	593.37 ± 22.83	570.93 ± 4.46	Rancid, harsh[g]; fatty acids, vegetable oil[4]
38	Decanoic acid	923.01 ± 4.75	812.74 ± 17.00	Fatty[g]; wax, tallow, rancid, soap[b]
39	Dodecanoic acid	50.34 ± 1.49	102.33 ± 12.61	–
40	Hexadecanoic acid	24.59 ± 2.68	32.04 ± 0.14	–
41	Nonanal	52.66 ± 1.08	57.20 ± 2.49	Citrus-like, soapy[a]
42	Decanal	43.88 ± 0.82	17.71 ± 1.04	Orange[c]
Alcohols				
43	3-Methyl-1-butanol and 2-Methyl-1butanol	2430.95 ± 29.07	1599.12 ± 28.56	Fruity, sweet[a]
44	2-Phenylethanol	183.31 ± 2.49	269.96 ± 4.90	Flowery, honey-like[a]

nd, not detected; NW 1:3, non-concentrated fermented cheese whey added to beetroot juice in the ratio of 1:3 (v:v)

CW 1:3, concentrated fermented cheese whey added to beetroot juice in the ratio of 1:3 (v:v)

[a] Czerny et al. (2008), [b] Meilgaard (1975), [c] Soares et al. (2015), [d] Costa et al. (2018), [e] Ledauphin et al. (2003), [f] Alves et al. (2015), [g] Siebert et al. (2005)

yeasts makes the produced beverages promising in the context of functional foods.

Volatile compounds

Based on the phenolic content and antioxidant activity, the beverages added to 3 parts of beetroot juice were submitted to HS SPME GC-MS analysis. Forty-four volatile compounds were identified in the produced beverages (Table 5). For most compounds, the profile and concentrations found were similar in the two beverages (Fig. 5). The principal component analysis shows that the first two components (PC1 and PC2) accounted for 100% of the total variance. Only 2-phenylethyl acetate (compound no. 36) allowed a slight differentiation between the beverages.

The ester and acetate group presented the highest number of compounds (Table 5). The highest production of esters and acetates agrees with the expected, considering the use of *K. lactis*, a known yeast for its efficiency for the production of these compounds. Ethyl octanoate was the most abundant compounds measured in the beverages CW 1:3 and NW 1:3 in concentrations of 24756.23 μg/L and 23259.67 μg/L, respectively (Table 5). The second highest concentration was found for ethyl decanoate with values of 12302.95 μg/L and 12886.65 μg/L in the beverages CW 1:3 and NW 1:3, respectively (Table 5). Ethyl octanoate and ethyl decanoate are associated with descriptors such as "sweet, apple, and fruity" (Meilgaard 1975). Other esters and acetates were also measured in high concentration (Table 5). Most of them were above the odor thresholds commonly reported. The great diversity of esters and acetates and their high concentrations were a positive point in the fermentation of whey by *K. lactis* B9. According to Zheng et al. (2018), in cheese, esters are desirable volatile compounds because they have "fruity" and high volatility. This may help to mask the undesirable flavors, for example, of the acids and other compounds.

The group of acids was composed only by 4 compounds, octanoic, decanoic, dodecanoic, and hexadecenoic acids (Table 5). Decanoic acid was found in concentrations of 923.01 μg/L and 812.74 μg/L in beverages CW 1:3 and NW 1:3, respectively (Table 5). This compound has aroma descriptors "wax, tallow, rancid, soap" (Meilgaard 1975).

The least diverse groups were those of the alcohols and aldehydes (Table 5). 3-Methyl-1-butanol, 2-methyl-1butanol, and 2-phenylethanol were the found alcohols. 2-Phenethylethanol is associated with descriptors such as "flowery and honey-like" (Czerny et al. 2008). The isoamyl alcohols (3-methyl-1-butanol and 2-methyl-1butanol) are important for the aroma of many fermented products since they present descriptors such as "fruity and sweet." Only two aldehydes were found in the beverages CW 1:3 and NW 1:3 (Table 5). Nonanal was the most abundant aldehyde being measured in concentrations of 52.66 μg/L and 57.20 μg/L respectively in the beverages CW 1:3 and NW 1:3 (Table 5). This compound is commonly associated with aroma descriptors "Citrus-like, soapy" (Czerny et al. 2008).

In general, it can be noted that the profile of volatile compounds was characterized by compounds from the fermentation. The addition of beetroot juice after the fermentation of the cheese whey did not strongly affect the composition of aromatic volatiles.

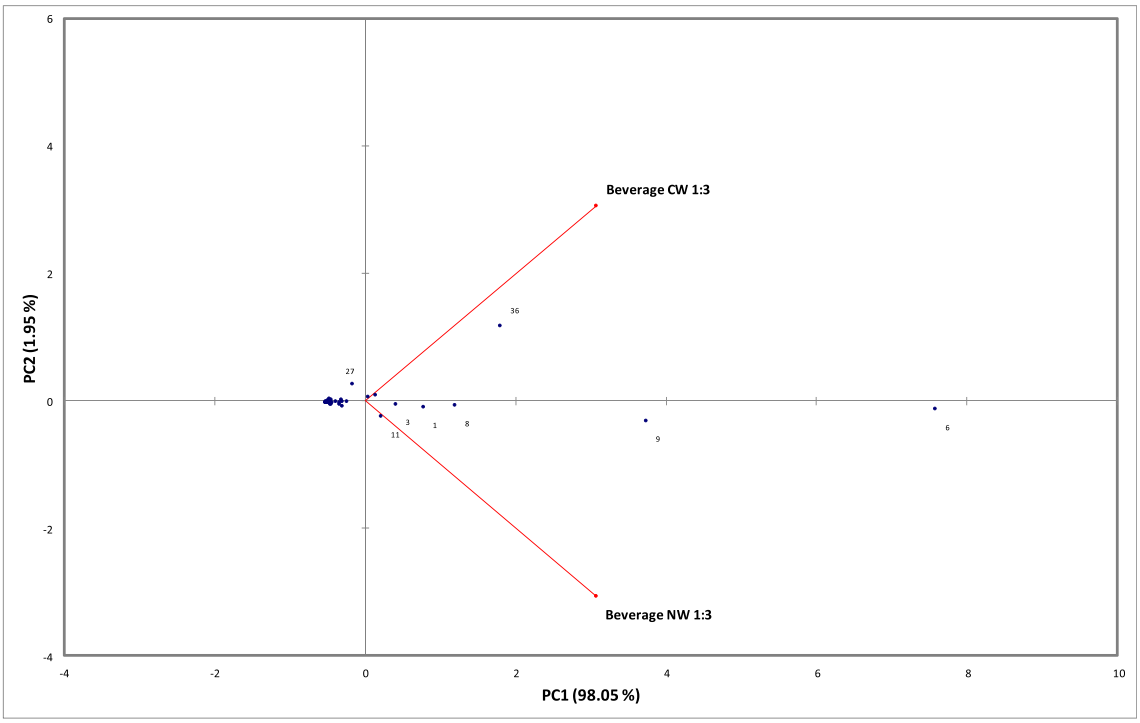

Fig. 5 Principal component analysis of volatile compounds

Conclusions

Due to its survival performance during passage through the simulated gastrointestinal tract, indicative of adhesion to the intestinal epithelium, β-galactosidase activity, production of short-chain fatty, and survival during 21 days of the beverage storage under refrigeration, *K. lactis* B9 is a promising strain for the manufacture of cheese whey–based functional beverages. Another point to be highlighted is the probable innocuity of the used strain. As the yeast was isolated from a cheese made with raw milk, it is already consumed alive in this product. Regarding the produced beverages, they stand out by their antioxidant properties and for being lactose-free without addition of commercial β-galactosidase, which is advantageous from both economic and functional points of view. Considering the fact that both the concentrated and the diluted cheese whey resulted in the generation of fermented beverages with many esters and acetates (whose aroma descriptors are "fruity, sweet, floral, etc."), the use of the diluted cheese whey is an interesting alternative to the concentrated one. The production of the latter implies a relatively high-cost process in the industry.

Funding information This study was financially supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), and Fundação de Amparo à Pesquisa de MG (FAPEMIG).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This study did not involve human participants or animals.

Informed consent The authors mutually agree with the submission of the manuscript to Annals of Microbiology.

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