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Probiotic Potential and Application of Indigenous Non-Starter Lactic Acid Bacteria in Ripened Short-Aged Cheese

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Abstract

There are massive sources of lactic acid bacteria (LAB) in traditional dairy products. Some of these indigenous strains could be novel probiotics with applications in human health and supply the growing needs of the probiotic industry. In this work, were analyzed the probiotic and technological properties of three *Lactobacilli* strains isolated from traditional Brazilian cheeses. In vitro tests showed that the three strains are safe and have probiotic features. They presented antimicrobial activity against pathogenic bacteria, auto-aggregation values around 60%, high biofilm formation properties, and a survivor of more than 65% to simulated acid conditions and more than 100% to bile salts. The three strains were used as adjunct cultures separately in a pilot-scale production of Prato cheese. After 45 days of ripening, the *lactobacilli* counts in the cheeses were close to 8 Log CFU/g, and was observed a reduction in the *lactococci* counts (around -3 Log CFU/g) in a strain-dependent manner. Cheese primary and secondary proteolysis were unaffected by the probiotic candidates during the ripening, and the strains showed no lipolytic effect, as no changes in the fatty acid profile of cheeses were observed. Thus, our findings suggest that the three strains evaluated have probiotic properties and have potential as adjunct non-starter lactic acid bacteria (NSLAB) to improve the quality and functionality of short-aged cheeses.

Introduction

The global cheese market is valued at around $100 bn, and the consumption of this product is expected to increase by more than 13% by 2029 [1]. This high demand and the new consumer needs for healthy and safe dairy foods have challenged the industry to develop new strategies for improving the functional and safety properties of dairy products.

It is widely recognized that the physical and organoleptic characteristics of cheese are related to the microbial ecology present during ripening. The LAB strain used as starter culture is usually involved in the fermentation of lactose, the production of high concentrations of lactic acid, and the increase of curd acidification. On the other hand, the non-starter LAB (NSLAB) cultures present during cheese ripening, influence the biochemistry of curd maturation and contribute to the development of specific sensory characteristics of the cheese [2]. The NSLAB produces diverse enzymes that can affect biochemical events such as proteolysis, lipolysis, which results in the development of cheese flavor, aroma and texture [3]. Thus, the NSLAB has been explored due to its positive effect on cheese flavor development, ripening time reduction, control of adventitious microbiota, and increased casein hydrolysis properties [4–6]. However, most of the research is focused on commercially available probiotic strains, and the works that analyze indigenous strains in both, functional and technological properties as adjunct cultures in cheese models, are scarce.

Prato cheese is an important and highly consumed dairy product in Brazil, is the second in production after Mozzarella cheese and constitutes about 20% of all cheeses produced in the country and is considered a good model of short-aged cheese due to its similarity with Gouda and

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Danbo cheeses [7, 8]. This product is manufactured from cows' milk through the enzymatic coagulation of semi-cooked mass and ripened for at least 25 days [9]. Some recent studies have demonstrated the potential of Prato cheese as a carrier matrix for lactic bacteria with probiotic properties, improving probiotic viability [10], product quality, sensorial acceptability [11], and also promoting health benefits to the consumer such as reduction of oxidative stress [12] and attenuation of renal calculi [13]. Therefore, the addition of NSLAB with probiotic properties into Prato cheese could be a promising alternative, helping to refine the final quality, technological and sensory properties of the product, but also, could be an alternative to add functional properties to this dairy product, due to the health benefits associated to probiotics intake, such as the improvement of the immune system [14] and the strengthening of intestinal immunity [15]. In this sense, the use of commercial probiotic *lactobacilli* and *bifidobacterium* have been explored as adjunct cultures in different types of cheeses, including Cheddar [16, 17], Feta [18], Coalho cheese [19], Cottage [20], Minas Frescal [21], Scamorza [22], Prato cheese [10], Fascal Cheese [23], and others; demonstrating the great potential of cheese as carrier matrix for probiotics, and an excellent model to study, proteolytic and lipolytic effect of novel NSLAB strains.

As new candidates for probiotics are expected to show both, functional and technological properties [24], the present study aimed to evaluate the probiotic features of the Brazilian indigenous NSLAB strains *Lacticaseibacillus paracasei* ItalPN16, *Lactobacillus acidophilus* ItalPN270 and *Lactobacillus acidophilus* ItalTR260, and also, study their effect in the main physicochemical and microbiological properties of Prato cheese during ripening. To this end, the safety, and functional properties of the three strains were tested in vitro. Then, were used separately as adjunct cultures in a pilot-scale production of Prato cheese, to evaluate their survival rate, proteolytic behavior, and possible effects on the fatty acid profile of the cheese after 45 days of ripening.

Materials and Methods

Bacterial Strains and Media

The NSLAB strains *Lacticaseibacillus paracasei* ItalPN16, *Lactobacillus acidophilus* ItalPN270 and *Lactobacillus acidophilus* ItalTR260, belonging to the culture collection of Dairy Technology Center of the Food Technology Institute, were isolated from traditional Brazilian cheeses, and maintained in a frozen stock culture of De Man–Rogosa–Sharpe (MRS) medium (Merck, Darmstrat, Germany) broth containing 20% (v/v) glycerol. The strains were previously identified by 16S rDNA sequence analysis for taxonomic identification and deposited in GenBank under the accession numbers JALGQW000000000, OP019274.1 and OP175047.1 for *L. paracasei* ItalPN16, *L acidophilus* ItalTR260 and *L. acidophilus* ItalPN270, respectively.

For the in vitro tests, the strains were incubated overnight in MRS medium at 37 °C. For cheese making, the cultures were prepared in 200 m of 10% (w/v) sterile skim milk inoculated with the last overnight sub-culturing (1%, v/v) and incubated for 24 h at 37 °C to reach a final concentration of around 10^8 CFU/mL. Lyophilized commercial cultures of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Sacco, São Paulo, Brazil) were reactivated in sterile reconstituted skim milk according to supplier instructions for Prato cheese production.

Safety Assessment

Hemolytic Activity

The hemolytic activity was estimated by analyzing the strain´s growth in blood agar plates containing 5% (v/v) sheep blood (Newprov, Pinhais, Brazil) incubated at 37 °C for 48 h. After the incubation time, the hemolytic activity of each strain was evaluated and classified according to the lysis of the red blood cells around the bacterial colonies: green zones (α-hemolysis), clear zones (β-hemolysis) and without clear zones around colonies (γ-hemolysis) [25]. The positive hemolytic strain *Staphylococcus aureus* ATCC 25923, was used as positive control.

Antibiotic Susceptibility

The three NSLAB isolates were evaluated for antibiotic susceptibility by the disc diffusion technique using MRS agar (Merck, Darmstadt, Germany). First, was prepared a suspension with a density of 0.5 McFarland in buffered saline of fresh overnight cultures and plated on MRS agar plates; then were dispensed the antibiotic discs (Cefar, São Paulo, Brazil) onto the plates and incubated at 37 °C for 24 h in anaerobic conditions. The inhibitory diameter halos were assessed and the resistance or sensibility to each antibiotic was defined according to the criteria of interpretation issued by the Clinical Laboratory Standards Institute [26]. In this work, the strains with a zone of inhibition less than or equal to 14 mm were considered as resistant (R) and those with more than 20 mm diameter as susceptible (S), and those having inhibition zones between 15 and 19 mm as intermediate (I).

Probiotic Features

Cell Auto-Aggregation

Cell auto-aggregation was investigated according to the method described by Collado and co-workers [27]. Briefly, fresh overnight cultures grown in MRS broth at 37 °C were harvested by centrifugation (4000 *g* for 10 min and 4 °C), washed twice with phosphate buffered saline (PBS) buffer (pH 7.0), and resuspended in the same PBS until an OD 600 of 0.25. This suspension was incubated for 4 h at 37 °C, and at the end of this time, one milliliter of the upper phase was carefully removed to measure the OD 600. Finally, the cell auto-aggregation was estimated using the following equation:

$$\begin{aligned} &\text{A}uto-aggregation\,(\%) \\ &= ((Initial\,OD - final\,OD)/Initial\,OD) * 100 \end{aligned}$$

Biofilm Formation Assay

The quantification of biofilm formation by the three NSLABs was performed using a microtiter plate-based crystal violet staining [28], with some modifications. First, overnight MRS growth cultures were harvested by centrifugation and standardized to 10^7–10^8 CFU/mL by adjusting the OD at 600 nm (0.25) with PBS buffer, then 20 µl of these bacterial suspensions were added to each 96-well plate containing 180 µl of MRS broth and incubated at 30 °C for 24 h. After the incubation time, the planktonic cells were eliminated, and the wells were washed with PBS. The biofilm formed in each well was then stained with 200 µl of 0.4% (w/v) crystal violet stain for 30 min. The dye excess was washed with a PBS three times and the plate was then dried in a laminar flow cabinet at room temperature. The crystal violet bound to the cells was solubilized with 200 µl ethanol (95%) per well. Finally, the absorbance was measured at 595 nm in a microplate reader. The analysis was carried out in triplicate and sterile MRS broth was used as a control. The cut-off (ODC) was defined as the mean OD value of the negative control. Based on the OD, strains were classified as non-biofilm producers ($OD \leq ODC$), weak ($ODC < OD \leq 2 \times ODC$), moderate ($2 \times ODC < OD \leq 4 \times ODC$) or strong biofilm producers ($4 \times ODC < OD$; [28].

Acid and Bile Tolerance

To determine the acid tolerance, the three strains were tested as described by Plessas et al. [29] with some modifications. Briefly, the NSLAB cells were grown at 37 °C for 24 h in MRS and then harvested by centrifugation at 6000 *g* and for 10 min at 4 °C. The pellets were washed three times with sterile saline solution (8.5 g/l NaCl) and suspended (10%) in saline solutions containing 0.3% (w/v) pepsin (Dinâmica, Indaiatuba, Brazil) and previously adjusted to pH 2.5 and pH 3.5, and. The bacterial suspensions were then incubated at 37 °C for 90 min. Aliquots of each suspension were taken at 0 and 90 min of incubation and serially diluted in sterile phosphate buffer pH 7.4, first dilution, for return to neutral pH, and then in saline solution. Viable cells were determined on MRS agar plates incubated at 37 °C for 48 h, in anaerobic conditions. The survival percentage of each bacterium was calculated as follows:

$$\begin{aligned} \%\,\text{Survival} &= (\text{CFU at the end of the test} \\ &\quad /\text{Initial CFU inoculated}) * 100 \end{aligned}$$

For the bile salt tolerance assay, the strains were cultivated and centrifuged as described above. Then, the strains (10% inoculum size) were incubated at 37 °C for 4 h in phosphate buffer pH 7.4 containing 0.3% (w/v) of bile salt (Sigma, Dorset, UK). The viable cells were determined at 0 h and 4 h of incubation by the spread plate method in MRS agar plates. The percentage survival of the bacteria was calculated in the same way as it was done for the acid tolerance.

Antagonistic Activity of Isolates

The antagonistic activity was evaluated using the double-layer agar diffusion test (spot on the lawn) as described by Oliveira et al. [30]. Initially, aliquots of 5 µL from *lactobacilli* cultures containing around 10^9 CFU/mL were spotted onto wells (5 mm diameter) made in the MRS agar plates and incubated for 24 h under anaerobic conditions. Then, the production of antibacterial compounds was revealed by overlaying the plate with 4 mL of Brain Heart Infusion (BHI medium, Kasvi, São José do Pinhais, Brazil) soft broth medium (0.75%) inoculated with 10 µL (10^9 CFU/mL) of each indicator bacteria and incubated at 37 °C for 24 h under aerobic conditions. The inhibitory zone diameters were measured when was observed the presence of a clear zone around the *lactobacilli* spot. Test microorganisms were *Escherichia coli* ATCC 25922*, Listeria monocytogenes* ATCC 7644 and *Bacillus cereus* ATCC 14575.

Experimental Pilot Scale Cheese-Making

Pilot-scale Prato cheeses were produced at the Instituto de Tecnologia de Alimentos (Tecnolat, Campinas, Brazil). The experimental Prato cheese was manufactured using 17 L of pasteurized cow's milk by sample, purchased from Agrindus S.A (São Paulo, Brazil). Milk was heated to 33–35 °C and divided into four equal portions. All separated portions of milk were inoculated with commercial starter bacteria *Lactococcus lactis* subsp. lactis and *Lactococcus lactis*

subsp. cremoris (1% v/v, approximately 7–8 log CFU/g). Then, selected strains (*L. paracasei* ItalPN16, *L. acidophilus* ItalTR260 or *L. acidophilus* ItalPN270) were added separately at 0.5% v/v (approximately 6–7 Log CFU/g), resulting in three treatments with the starter strain plus one selected NSLAB and the control containing only the starter culture. Subsequently, calcium chloride (200 mg/l of milk), and coagulant (Ha-La 1175, CHR Hansen Indústria e Comércio, São Paulo, Brazil) were added to the milk for coagulation within 40–50 min. The curd was placed in rectangular plastic molds (0.5 kg) and pressed. The cheeses were further stored at 10–12 °C for 10 h and salted in brine (20% w/v NaCl) for 5 h at 5 °C. Finally, the cheeses were dried for 48 h, vacuum-packed into heat-shrinkable plastic bags and stored for ripening at 11 ± 1 °C for 45 days. Four cheeses were manufactured, the conventional formulation (with starter bacteria only) named "CC" and the other three with the starter bacteria and each one of the selected NSLAB, named as CPN16, CPN270 and CTR260.

Physico-Chemical Analysis

For the analyses, 50 g of each cheese sample was cut, homogenized in a blender, and stored in glass flasks at 4 °C. Proximate composition was determined using traditional methods for moisture and ash content in cheese samples [31]. Total nitrogen was determined using a Kjedahl steam distillation [32], and protein concentration was calculated using the conversion factor of 6.38.

Microbiological Analysis

Cheese samples at 3, 15, 24, and 45 days of ripening were subjected to total lactic acid bacteria enumeration. To carry out this analysis, 25 g of each sample were weighed aseptically and homogenized in 225 mL of sterile sodium citrate solution (2% w/v). Then, the samples were serial diluted in sterile peptone water (0.1%) and 0.1 mL of each dilution was subcultured in M17 agar (for *lactococci* enumeration) and MRS agar (for *lactobacilli* enumeration), respectively. The total counts of the viable were reported as logarithmic colony forming units per gram of cheese (log CFU/g), after incubation at 37 °C by 48 h under aerobic and anaerobic conditions, for *lactococci* and *lactobacilli,* respectively.

Cheese Proteolysis

The evaluation of proteolysis was performed by the determinations of the extent of proteolysis index (DPI) and depth proteolysis index (EPI), using the concentrations of soluble nitrogen in 12% (w/v) trichloroacetic acid (SN-TCA) and soluble nitrogen at pH 4.6 (SN-pH 4.6), respectively. Briefly, 10 g of homogenized sample was completely diluted in a 200 mL solution of 0.5 mol/mL sodium citrate. From these solutions, aliquots were taken to determine the levels of SN-TCA and SN-pH 4.6 by Kjedahl steam distillation [32]. DPI and EPI were calculated by dividing the SN-pH 4.6 and SN-TCA values by the total nitrogen of the sample.

Fatty Acid Profile

For analysis of fatty acid composition, the lipids present in 0.2 g of cheese samples were extracted using 7.2 mL of hexane–isopropanol (3:2 v/v) followed by 4.8 mL of sodium sulfate solution (6.7% w/v) as described elsewhere by Lima Maciel et al. [33]. After solvent evaporation under oxygen-free nitrogen at 40 °C, the extracted lipids (~ 20 mg) were dissolved in hexane and methyl acetate and then transesterified to fatty acid methyl esters (FAME) by alkali catalysis [34]. The FAME in 1.0 mL sample at a split ratio of 1:50 were separated and quantified using a gas chromatograph (model 7820A; Agilent Technologies, Santa Clara, CA, USA) equipped with an FID detector and a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm × 0.20 µm; Agilent Technologies). The equipment was operated as described previously by Cruz-Hernandez et al. [35]. The FAME were identified according to their retention times as compared with reference FAME standards (Sigma-Aldrich®, St. Louis, USA; Larodan AB, Stockholm, Sweden; Luta-CLA® 60, BASF Brasil, São Paulo, Brazil); minor *trans/cis*-18:1 isomers and *trans*-9, *cis*-11 CLA were identified according to their elution order. The FA composition was reported in this work as a percentage (%) w/w of total fatty acids, according to theoretical response factors [36].

The identified fatty acids (FA) were grouped according to the number of carbons present as short-chain saturated fatty acid (SCFA; C4:0-C10:0), medium-chain saturated fatty acid (MCFA; C12:0-C15:1), and long-chain saturated fatty acid (LCFA; > C16:0). Monounsaturated fatty acids (MUFA) levels were calculated considering palmitoleic fatty acid (C16:1) and total C18:1, and polyunsaturated fatty acids (PUFA) levels were calculated considering linolenic (C18:2) and α-linolenic acid (C18:3). The atherogenic index (AI) and thrombogenic index (TI) were determined by using the following equations [37]:

$$AI = (C12{:}0 + 4 \times C14{:}0 + C16{:}0)/[\Sigma\,MUFA + \Sigma\,PUFA(n-6)\,(n-3)]$$

$$TI = (C14{:}0 + C16{:}0 + C18{:}0)/[0.5 \times \Sigma\,MUFA + 0.5 \times \Sigma\,PUFA(n-6) + 3 \times \Sigma\,PUFA(n-3) + (n-3)/(n-6)]$$

Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) and the means were compared by Tukey's test setting the reference level of significance to $P < 0.05$, using the STATISTICA® program (StatSoft. Inc., Tulsa, USA) version 10.

Results and Discussion

Safety Profiling

Probiotic bacteria must be essentially safe strains, unable to cause hemolysis, and must be sensitive or present innate and non-transferable resistance to antibiotics, to not propagate resistance properties to pathogenic bacteria present in the same niche [38]. Thus, the hemolytic activity and antibiotic susceptibility were evaluated. As shown in Table 1, the tree *lactobacilli* evaluated presented γ hemolysis. The hemolytic activity assay is a useful tool for rapid initial assessment of bacterial pathogenicity, thus the absence of α- or β-hemolysis indicates that the strains do not possess hemolytic enzymes.

The strain's susceptibility to 10 different antibiotics was determined by the disc diffusion assay. As results, the three strains were resistant to ciprofloxacin and the strain L. *paracasei* ItalPN16 was also resistant to vancomycin (Table 1), in agreement with these results, a recent work of our research group, found the vancomycin resistance gene (*vanZ*) in the L. *paracasei* ItalPN16 genome, indicating high tolerance to vancomycin and other lipoglycopeptide antibiotics [39]. *Lactobacilli* resistance to vancomycin and ciprofloxacin was reported previously by other authors [40, 41] and is considered intrinsic in this bacterial genus. The LAB vancomycin resistance is related to chromosomally encoded variations in their peptidoglycan assembly pathway, which could result in the change of the d-Ala-d-Ala dipeptide residue in the muramyl pentapeptide cell wall by a high-level (d-Ala-d-lactate) or low-level (d-Ala-d-Ser) resistance residues [42]. In fact, the *lactobacilli* natural resistance to vancomycin and other antibiotics raises concerns regarding the transference of resistance genes to pathogenic bacteria. However, some of these resistance elements are chromosomally encoded, therefore, the resistance is not transferable [43]. Although the three lactobacilli strains share the resistance to ciprofloxacin, they were sensitive to most of the antibiotics used in the test. This information is valuable since both, antibiotic sensitivity and intrinsic antibiotic resistance of *lactobacilli* are relevant for human health and food safety [40], and also indicate that these strains could present low gut survival if the host is treated with antibiotics.

Probiotic Features

Auto-Aggregation and Biofilm Formation

The auto-aggregation is an ability present in probiotic bacteria that allows them to maintain their population in the gut and is also involved in the inhibition of pathogens growth by entrapping them within the aggregates [44]. This bacterial property along with its ability to form biofilms are useful parameters to determine their adhesion ability to epithelial cells in the gastrointestinal tract, which could contribute to the prevention of pathogens colonization [45]. The results of the auto-aggregation assay are presented in Table 2. Among the three *lactobacilli* tested, the *L. acidophilus* ItalTR260 showed the highest auto-aggregation ability (71.08%) after 2 h of incubation, followed by *L. paracesei* ItalPN16 and *L. acidophilus* ItalPN270 with 68.66% and 59.49%, respectively. Similar values of auto-aggregation were observed in 16 LAB strains isolated from the fermented drink Neera, with values ranging from 40% to 78.95% after 2 h of incubation [46].

Regarding the biofilm ability formation, the three NSLAB strains showed to be strong biofilm formers (Table 2). The highest OD (1.79) was observed for *L. acidophilus* ItalTR260, however no significant difference ($P > 0.05$) was found as compared with the other two strains. Recent works reported similar OD values in biofilm-formation assays for the probiotic candidates *L. casei* MYSRD-108 (1.92) and *L.*

Table 1 Antibiotic susceptibility and hemolytic activity of selected NSLAB strains

Strain	Antibiotic tested										Hemolysis
	AM 10	IPM 10	VA 30	CLI 2	TE 30	E 15	LN 30	Cip 5	PEN 10	M 10	
L. paracasei ItalPN16	S	I	R	S	S	S	S	R	S	S	γ
L. acidophilus ItalPN270	S	S	S	S	S	S	S	R	S	S	γ
L. acidophilus ItalTR260	S	S	S	I	S	S	S	R	S	S	γ

Antibiotics: *AM* ampicillin, *IPM* imipenem, *VA* vancomycin, *CLI* clindamycin, *TE* tetracycline, *E* erythromycin, *LN* linezolid, *C* chloramphenicol, *Cip* ciprofloxacin, *PEN* Penicillin, *M* meropenem, *R* resistant, *S* sensitive, *I* Intermediate resistance. Hemolysis: no zones around colonies (γ-hemolysis)

Table 2 Probiotic properties and pathogenic antagonistic activity of the NSLAB strains PN16, PN270, and TR270

Properties	PN16	PN270	TR270
Auto-aggregation in 2 h (%)	68.66 ± 1.42^{b}	59.49 ± 1.54^{a}	71.08 ± 3.37^{b}
Biofilm formation 24 h (Abs 600 nm)	1.712 ± 0.19^{a}	1.568 ± 0.40^{a}	1.794 ± 0.11^{a}
Bile salt tolerance index in 4 h (%)	112.54 ± 1.12^{c}	99.51 ± 3.51^{a}	105.23 ± 0.75^{b}
pH 2.5 + 0.3% pepsin, survival index (%)	67.99 ± 0.29^{a}	65.99 ± 2.05^{a}	70.76 ± 0.06^{b}
pH 3.5 + 0.3% pepsin, survival index (%)	103.98 ± 1.68^{c}	94.80 ± 0.47^{a}	98.03 ± 0.60^{b}
Antagonistic activity	Inhibition halo (mm)		
L. monocytogenes	17.1 ± 0.4^{a}	18.6 ± 0.7^{a}	17.00 ± 1.3^{a}
B. cereus	19.6 ± 0.6^{b}	22.4 ± 0.9^{c}	11.9 ± 0.2^{a}
E. coli	18.3 ± 0.8^{c}	12.7 ± 0.5^{b}	10.4 ± 0.6^{a}

Values are means ± standard deviations of three independent experiments. Different superscript letters in the same line denote significant differences ($P < 0.05$).

plantarum MYSRD-71 (1.62) and around 1.70 for the strains *L. rhamnosus* PTCC1712 and *L. plantarum* PTCC1745 [47].

Survival to Gastrointestinal Conditions

To simulate gastric juice and intestinal conditions, a solution of pepsin-pH 2.5, pepsin-pH 3.5 and bile salts were used. The three NSLAB strains presented survival indexes superior to 65% at pH 2.5 and in the range of 94 to 103% at pH 3.5 (Table 2). Notably, the strain *L. paracasei* ItalPN16 displayed the higher values ($P < 0.05$) for viability to pH 3.5 and bile salt tolerance, 103.9% and 112.5%, respectively. Similar to our results, Lee and coworkers [48], reported bile salt tolerance higher than 100% in the *L. plantarum* KU 200656 and *L. rhamnosus* GG, nonetheless, they also showed a survival rate superior to 99% in artificial gastric acid (pH 2.5). For performing the probiotic functions, growth and fermented prebiotic fibers, LAB can tolerate the low pH in the gastrointestinal tract through several mechanisms, such as neutralizing the media, forming protective biofilms, activating proton pumps and pre-adaptation [49, 50]. Thus, the three strains presented a medium level of tolerance to acidic conditions (Table 2), possibly due to the environment of isolation (cheese), which normally presents a pH of around 5.2. However, other physicochemical conditions present in the cheese, such as the presence of salt (around 2%) and osmotic pressure, could favor the strain's tolerance to bile salts, which is an important feature for potential probiotic LAB.

Antibacterial Activity

Antagonism against pathogens is a relevant property of probiotic bacteria and can involve different strategies such as co-aggregation, production of antimicrobial compounds and competitive exclusion [45]. The antagonistic effect of the strains was tested against three food pathogens in a co-culture assay. The results, presented in Table 2, demonstrated that all three strains exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria with inhibition halos superior to 10 mm. A significant ($P < 0.05$) higher inhibitory values were observed in *L. paracasei* ItalPN16 (18.3 mm) against *E. coli*; and in *L. acidophilus* ItalPN270 against *B. cereus* (22.4 mm). Our results are in agreement with other in vitro studies, that showed that *lactobacilli* isolated from dairy products can exhibit antagonistic activity against Gram-positive and Gram-negative pathogenic bacteria such as *L. monocytogenes E. coli* and *Salmonella* [48–51]. This inhibitory effect is attributed to the production of different metabolites such as bacteriocins, diacetyl, and acetic acid, produced by the strains during LAB fermentation [52]. Thus, the antimicrobial activity exhibited by the three strains could favor the safety of dairy products, inhibiting the presence of pathogenic bacteria. However, more tests must be conducted to elucidate the mechanism of antimicrobial activity and their effects on other pathogens and spoilage microorganisms.

Prato Cheese Production

The proximate analysis of all cheeses is presented in Table 3. Similar amounts of fat, total protein, moisture, and ash were observed between the control and the cheeses containing the selected NSLAB, indicating that the addition of the strains does not influence the basic physicochemical indicators of cheeses. Similar results were reported by Chavez and Gigante [10] and Costa et al. [53], who reported Moisture values around 46%; Fat 28%; FDM 51%; Protein 21%; and aches around 1.9%. After evaluating the proximate composition of Prato cheese produced with probiotic microorganisms as adjunct culture.

Mesophilic Lactobacilli and Lactococci Counts

The enumeration of *lactococci* and *lactobacilli* during cheese ripening (days 4, 10, 24 and 45) was determined

Table 3 Composition of Prato cheese with and without adjunct cultures

Component	CC	CPN16	CPN270	CTR270
Moisture (%)	47.79 ± 0.10^{a}	49.14 ± 0.21^{b}	48.18 ± 0.58^{ab}	47.61 ± 0.22^{a}
Fat (%)	28.32 ± 0.19^{a}	28.50 ± 0.16^{a}	27.48 ± 0.18^{a}	28.56 ± 0.19^{a}
FDM (%)	54.25 ± 0.41^{a}	56.04 ± 0.25^{b}	53.37 ± 0.95^{a}	54.53 ± 0.58^{a}
Protein (%)	18.74 ± 0.10^{a}	18.60 ± 0.10^{a}	18.27 ± 0.14^{a}	18.87 ± 0.10^{a}
Ash (%)	3.50 ± 0.04^{a}	3.50 ± 0.08^{a}	3.84 ± 0.02^{c}	3.65 ± 0.03^{b}

Values are means ± standard deviations of three independent experiments. Different superscript letters in the same line denote significant differences ($P < 0.05$). FDM = fat in dry matter. CC = control cheese (no adjunct culture added). CPN16 cheese containing *L. paracesei* ItalPN16

CPN270 cheese containing *L. acidophilus* ItalPN270, *CTR270* cheese containing *L. acidophilus* ItalTR260

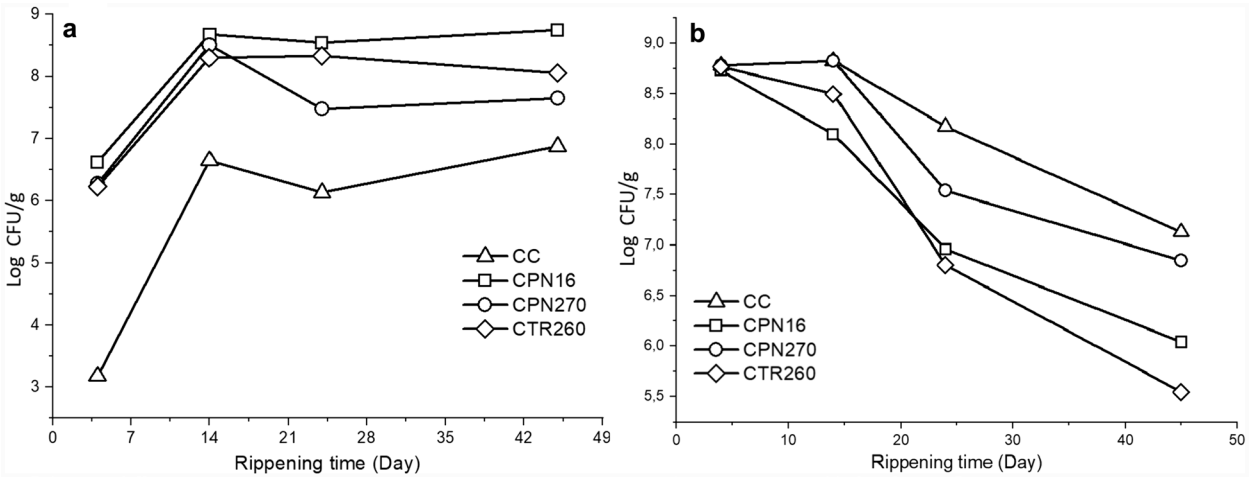

Fig. 1 Changes of viable counts of total mesophilic *lactobacilli* (**a**) and total *lactococci* (**b**) in Prato cheeses during a 45 day-ripening period. The values represent the means obtained after the three trials of cell enumeration on days 4, 14, 25, and 45

by counts in M17 and MRS agar medium, respectively. As result, the number of *lactobacilli* in the cheeses containing the NSLABs was around 6.5 log CFU/g at day 4 of ripening, while the count in control was 3.17 log CFU/g (Fig. 1a). *Lactobacilli* counts in the control presumably come from the LAB pilot plant environment. On day 10 of ripening, the *lactobacilli* count reached their maximum values, around 8.5 and 6.5 log CFU/g for the treatments and the control, respectively. From day 10, until the end of the ripening time (day 45), the cheeses CC, CPN16 and CTR260 presented small changes in their *lactobacilli* counts, in contrast, the cheese CPN270 prepared with strain *L. acidophilus* ItalPN270 showed a decrease of around 1 log CFU/g at the end of ripening time.

On the other hand, the *lactococci* counts presented a decreasing trend in all samples from day 4, until the end of the ripening period (Fig. 1b). The *lactococci* count reduction was higher in the cheeses CPN16 (− 2.7 Log CFU/g) and CTR260 (− 3.2 Log CFU/g) as compared with the control CC (− 1.5 Log CFU/g). Usually, the viability of *lactococci* in cheese is decreased due to lactose depletion, salt addition, and low values of pH and temperature during the ripening [54]. Nonetheless, it was reported that the Operational Taxonomic Units (OTUs) of *Streptococcus* decreased more in the presence of an adjunct *lactobacilli* (especially *L. paracasei*) in Caciocavallo Pugliese cheese after 45 days of ripening, as compared with the control without *lactobacilli* [55]. In addition, the authors found a positive correlation between the increment of OTUs of *lactobacilli* and the concentration of free amino acids (FAA), hydrophilic peptides and several volatile compounds (e.g., alcohols, aldehydes, ketones, esters, and furans) [55]. The production of strain-specific volatile compounds and peptides during cheese ripening, results in an oxidant environment affecting the amino acid conversion by *Lactococcus* and consequently, affecting its viability [56]. Therefore, the notable reduction of *lactococci* counts in the Prato chesses prepared with NSLABs, indicates a possible strain-dependent depletion of lactose and production of volatile compounds, which resulted in different levels of *Lactococcus* inhibition during Prato cheese ripening.

Proteolysis During Ripening

The ratio between pH 4.6 soluble nitrogen and total nitrogen (EPI) was used to estimate the level of primary proteolysis. Primary proteolysis reflects the number of casein-derived peptides with large and medium-sized peptides in the cheese, which are mainly a result of residual coagulant activity [57]. However, the EPI increment during the later stage of cheese ripening is also related to the presence of bacterial proteinases, which are normally released via lysis of microorganisms [58]. In this work, the EPI values increased gradually with the time of ripening, varying from 9 to 17% at the end of ripening, with small differences among the samples (Fig. 2a). Usually, adjunct cultures do not influence the EPI because soluble nitrogenous compounds are formed principally by the coagulant activity (remaining in the cheese curd), milk proteinases (plasmin and cathepsin), and cell membrane-associated proteases of the starter bacteria [59].

Nitrogenous compounds soluble in 12% TCA (depth of proteolysis index—DPI) are peptides with a molecular weight below 3000 Da, amino acids, urea, and ammonia. As shown in Fig. 2b, DPI values increased around two fold until day 45 of ripening in all samples. The cheese CPN16 presents a small increment of depth proteolysis at the end of the ripening time as compared with other treatments and the control (CPN270, CTR260 and CC). This could be due to a higher proteolytic and peptidolytic activity, specific to the *L. paracasei* ItalPN16 strain.

In agreement with our results, de Asanbuja et al., [11] reported no significant differences in DPI and EPI during Prato cheese ripening, produced with the adjunct culture *L helveticus* (B02). In another work, Prato cheese produced with the commercial probiotics *L. acidophilus* La5 and *Bifidobacterium* Bb12 showed no significant difference in EPI as compared with the control. At the same time, an increase in DPI was observed in cheeses containing *L. acidophilus* La5. According to the authors, this effect was probably due to the production of intracellular amino-, di- and tri-peptidases by the La5 strain during the cheese ripening [10]. Similar results were reported by Sato et al. [60], where Prato cheese produced with *L. helvetius* or *L. plantarum* presented a significant difference in the DPI but not the EPI values. Thus, based in our results, the three *lactobacilli* strains do not change in the Prato cheese EPI, and only the *L. paracasei* ItalPN16 produced a slight increase in the DPI proteolysis during the ripening. However, low proteolysis activity is a desirable property in adjunct cultures in Prato cheese, in order to maintain the cheese's physical and sensorial attributes.

Therefore, the NSLAB does not generate relevant proteolysis modifications in the cheeses.

Prato Cheese Fatty Acids Profile

The LAB catabolism of amino acids, lactose fermentation, and lipolysis affect the flavor of mature cheese and are precursors of specific fatty acids, produced during the cheese ripening [61]. However, the fatty acid (FA) profile of cheese depends mainly on milk composition, animal diet, genetics (individual variation and breed), season, physiological factors (e.g., stage of lactation), and production processes [62]. Other factors such as milk lipoprotein lipase, lipases from LAB, molds, yeasts, lipases from rennet paste, and exogenous lipase can also affect the FA profile of cheese [63].

The FA composition of the produced Prato cheeses after a 45-day ripening period is shown in Table 4. All cheeses had

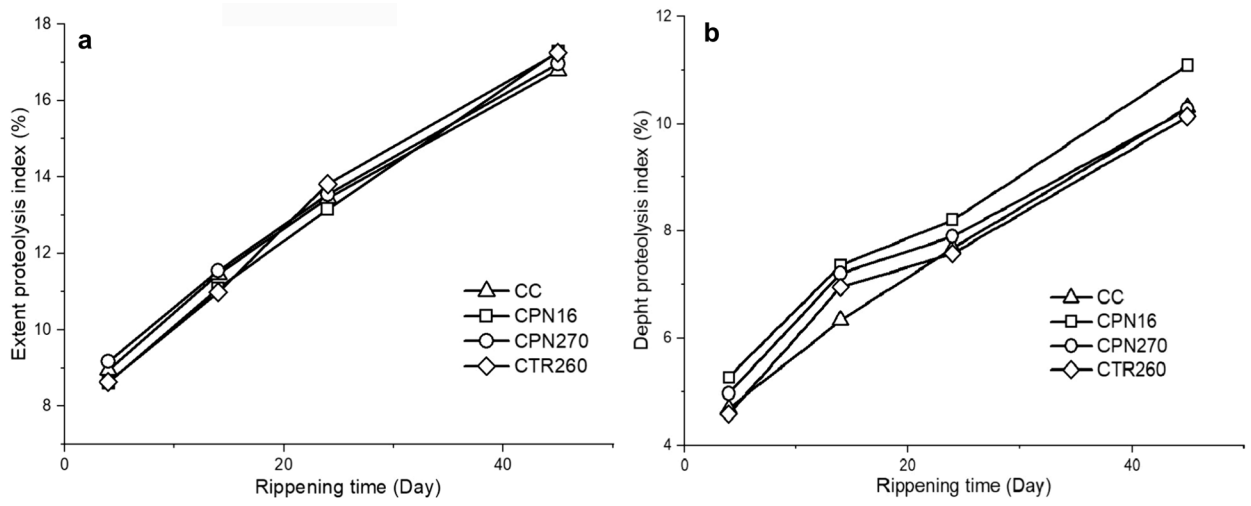

Fig. 2 Extent of proteolysis index (**a**) and depth proteolysis index (**b**) of Prato cheeses during a 45 day-ripening period. The values represent means obtained from three independent experiments

Table 4 The fatty acid composition (g/100 g of total fatty acids) of Prato cheese produced with the addition of different NSLAB strains

Fatty acid	Cheese sample			
	CC	CPN16	CPN270	CTR260
C4:0	3.23	3.45	3.45	3.40
C5:0	0.034	0.034	0.038	0.032
C6:0	2.09	2.20	2.22	2.20
C7:0	0.019	0.019	0.020	0.019
C8:0	1.48	1.54	1.57	1.51
C9:0	0.031	0.026	0.025	0.024
C10:0	2.86	2.89	2.93	2.92
cis-9 C10:1	0.221	0.222	0.219	0.217
C11:0	0.073	0.068	0.072	0.065
C12:0	3.04	3.01	3.04	3.05
cis-9 C12:1 [a]	0.150	0.149	0.151	0.150
iso C14:0	0.086	0.085	0.085	0.086
C14:0	9.73	9.67	9.71	9.76
cis-9 C14:1	0.737	0.761	0.733	0.735
iso C15:0	0.170	0.164	0.167	0.171
anteiso C15:0	0.387	0.383	0.382	0.382
C15:0	0.817	0.883	0.817	0.830
iso C16:0	0.193	0.197	0.192	0.197
C16:0	27.16	27.14	27.11	27.33
trans-9 C16:1[b]	0.323	0.312	0.319	0.320
trans-12 C16:1	0.174	0.167	0.171	0.166
cis-9 C16:1[c] +	1.48	1.47	1.48	1.47
C17:0	0.467	0.447	0.452	0.455
iso C18:0	0.064	0.066	0.062	0.063
cis-9 C17: 1	0.152	0.151	0.153	0.154
C18:0	10.63	10.66	10.60	10.78
trans-4 C18:1	0.037	0.036	0.037	0.032
trans-5 C18:1	0.028	0.026	0.028	0.024
trans-6 + 8 C18:1	0.306	0.282	0.269	0.307
trans-9 C18: 1	0.303	0.262	0.276	0.260
trans-10 C18:1	0.460	0.404	0.397	0.405
trans-11 C18: 1	1.04	1.13	1.13	1.11
trans-12 C18:1	0.463	0.461	0.455	0.469
trans-13 + 14 C18:1	0.665	0.769	0.676	0.800
cis-9 C18: 1	19.47	19.38	19.46	19.38
cis-11 C18:1	0.787	0.825	0.817	0.781
cis-12 C18:1	0.554	0.564	0.564	0.555
cis-13 C18:1	0.139	0.145	0.152	0.132
trans-16 C18:1	0.356	0.358	0.351	0.355
trans-9 + 12 C18:2	0.024	0.026	0.028	0.020
cis-9 trans− 12 C18: 2	0.044	0.053	0.057	0.046
trans-9 *cis* 12 C18:2	0.048	0.032	0.033	0.030
C18:2 n-6	4.01	3.98	3.95	3.94
C18:3 n-6	0.037	0.040	0.039	0.037
C18:3 n-3[e]	0.600	0.604	0.600	0.593
C19:0[d]	0.085	0.098	0.101	0.097
C20:0	0.122	0.120	0.118	0.122
cis-9 *trans*− 11 CLA	0.495	0.495	0.498	0.496
trans-9 *cis* 11 CLA	0.025	0.028	0.030	0.030
trans-10 *cis* 12 CLA	0.010	0.013	0.014	0.014
C21:0	0.0280	0.0295	0.0303	0.024
C20:2 n-6	0.0122	0.0164	0.0194	0.017
C20:3 n6	0.045	0.045	0.045	0.045
C20: 4 n-6	0.210	0.197	0.195	0.187
C20:5 n-3	0.025	0.025	0.027	0.025
C21:0	0.028	0.029	0.030	0.024
C22:0	0.125	0.123	0.123	0.122
C22:5 n-3	0.061	0.061	0.063	0.060
C23:0	0.028	0.030	0.031	0.031
C24:0	0.046	0.045	0.043	0.045
∑ SFA	62.92	63.28	63.30	63.63
∑ MUFA	27.85	27.87	27.83	27.82
∑ PUFA	5.12	5.08	5.06	5.00
∑ SCFA	9.97	10.39	10.48	10.33
∑ MCFA	42.65	42.61	42.56	42.86
∑ LCFA	41.33	41.35	41.26	41.37
∑ OBCFA[F]	1.93	1.99	1.92	1.92
AI	2.14	2.14	2.14	2.16
TI	2.85	2.85	2.85	2.89

ΣSFA, sum of all saturated fatty acids (FA); ΣMUFA, sum of all monounsaturated FA; ΣPUFA, sum of all polyunsaturated fatty acids; SCFA, short-chain fatty acids (C4 to 10); MCFA, medium-chain fatty acids (C12 to C16), LCFA, long-chain fatty acids (≥C18); OBCFA odd- and branched-chain FA

CC control cheese (no adjunct culture added), *AI* atherogenic index, *TI* thrombogenic index, *CPN16* cheese containing *L. paracesei* ItalPN16, *CPN270* cheese containing *L. acidophilus* ItalPN270, *CTR270* cheese containing *L. acidophilus* ItalTR260.

[a]Co-elutes with 13:0

[b]Co-elutes with *iso* C17:0

[c]Co-elutes with anteiso C17:0

[d]Co-elutes with cis-15 C18:1

[e]Co-elutes with *cis*-11 C20:1

[f]Sum of saturated OBCFA, except *iso* C17:0 and *anteiso* C17:0 as they co-eluted with *trans*-9 C16:1 and cis-9 16:1, respectively.

very similar FA profiles, suggesting that the adjunct strains did not exhibit lipolytic activity and did not change the FA composition of Prato cheese, even after a ripening period of 45 days. These results are similar to previous reports, where the addition of probiotic bacteria during cheese processing does not affect the FA profile in cheeses such as Pickle white cheese [64], Argentinian Ovine Cheese [65], and Minas Frescal cheese [66]. This is mainly attributed to the weak lipolytic activity of probiotic bacteria, as well as a short time of ripening [67].

As shown in Table 4, most of the FA found in Prato cheese samples were saturated (~63% of total FA), with palmitic (C16:0), stearic (C18:0), and myristic (C14:0) acids being the predominant ones, which present values typically reported for milk and dairy products [7, 64, 65]. Moreover, were found elevated amounts of medium/long chain fatty acids like myristic (C14:0), palmitic (C16:0), and stearic (C18:0), with values around 10, 27, and 13 g/100 g of total FAs, respectively. These results are similar to those reported for Prato cheese produced with the probiotic bacteria *L. casei*-01 and containing different levels of salt and enhancers [7], indicating that these medium/long-chain fatty acids amounts are characteristic of this kind of cheese. In addition, all samples of Prato cheese contained high amounts of MUFA (around 27 g/100 g of total FAs), with a notable quantity of oleic acid (*cis*-9 C18:1), which represented around 19 g/100 g of total FAs. This fact is important since MUFA consumption has been associated with lower chronic inflammation, a reduction of low-density lipoprotein (LDL), and an anti-atherogenic lipid profile [68].

AI and TI in the Prato cheese samples were calculated from the values of fatty acids composition (Table 4). AI value is associated with saturated fatty acids with pro-atherogenic activity, and the TI expresses the presence of low-density lipids, mainly C18:0 that are related to forming clots inside a blood vessel, restricting blood flow, and leading to thrombus formation [37]. As observed with the fatty acid profile, no differences were observed in the AI and TI values, between the cheeses produced with different NSLAB. Similar values of AI and TI (around 2.14 and 2.85, respectively), were recently reported in different artisanal Brazilian chesses [69]. In contrast, Rodrigues and co-workers [70] reported that the incorporation of probiotic bacteria strains (*Lactobacillus casei*-01, *Bifidobacterium lactis* B94) modified the fatty acid profiles of cheese after 60 days of ripening. In addition, Ziarno and collaborators [71], demonstrated that *L. casei* or *Propionibacterium* reduces the AI and TI indices in cheese, after 6 weeks of ripening. However, the authors of these works reinforce that these effects are strain-specific, and also could vary according to the cheese composition and ripening time.

Conclusions

In the current study, three potential probiotics isolated from Brazilian cheeses were evaluated as adjunct cultures for Prato cheese production. All the isolates showed safety and probiotic properties regarding their tolerance to gastrointestinal conditions, as well as strong pathogen antagonistic and biofilm formation ability. The three strains (*L. paracasei* ItalPN16, *L. acidophilus* ItalPN270, and *L. acidophilus* ItalTR260) were used as adjunct cultures in pilot scale Prato cheese and did not show significative effect on the cheese's physicochemical composition, proteolysis, or fatty acid profile. Conversely, the presence of the adjunct cultures produced changes in the *lactococci* counts during the ripening probably by higher reduction of lactose and the production of inhibitory compounds. These findings are relevant to the dairy industry in its search for strategies to improve the quality and functionality of cheeses. However, despite the in vitro potential probiotic properties observed in the strains, further in vivo studies are needed to confirm their putative health benefits and claim, to a future application in the development of functional dairy products.

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Author's Contribution CMBP and FGE conducted strain reactivation and isolation, formal analysis, and data curation and wrote the original draft. MAdG, ATSA, LME, and AB, were responsible for the project supervision, obtaining financial resources, revision, and editing of the manuscript. All authors read and approved the final version of the manuscript.

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Data Availability Not applicable.

Declarations

Conflict of Interests The authors declare no competing interests.

Ethics Approval and Consent to Participate This article does not contain any studies performed with humans and/or animal models.

Consent for Publication Not applicable.

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