Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Novel bifidobacteria strains isolated from nonconventional sources. Technological, antimicrobial and biological characterization for their use as probiotics

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Keywords
bifidobacteria, biological characteristics, chicken, dairy products, monkey, technological properties.

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2019/0595: received 4 April 2019, revised 23 June 2019 and accepted 26 June 2019

doi:10.1111/jam.14367

Abstract

Aim: To characterize four novel autochthonous bifidobacteria isolated from monkey faeces and a *Bifidobacterium lactis* strain isolated from chicken faeces by evaluating their technological and biological/functional potential to be used as probiotics. Different stressors, including food process parameters and storage, can affect their viability and functionality.

Methods and Results: The resistance to frozen storage, tolerance to lyophilization and viability during storage, thermal, acidic and simulated gastric resistance, surface hydrophobicity and antimicrobial activity against pathogens were studied. *Bifidobacterium lactis* Bb12 and INL1 were used as reference strains. The results obtained demonstrated that the new isolates presented strain-dependent behaviour. Good results were obtained for thermal resistance, frozen storage at −80°C and lyophilized powders maintained at 5°C. Cell viability during refrigerated storage was higher when the strains were resuspended in milk at pH 5·0 than at 4·5. The surface hydrophobicity ranged between 7 and 98% depending on the strain. The simulated gastric resistance was improved for the strains incorporated in cheese. Regarding antimicrobial activity, bifidobacteria isolated from monkey presented higher inhibitory capacity than the reference strains.

Conclusion: This research provides a deeper insight into new strains of bifidobacteria isolated from primates and chicken that have not been previously characterized for their potential use in dairy products and confirm the most robust stress tolerance of *B. lactis*.

Significance and Impact of the Study: The possibility of expanding the available bifidobacteria with the potential to be added to a probiotic food necessarily implies characterizing them from different points of view, especially when considering unknown species. For monkey isolates (which showed higher antimicrobial activity against pathogens), more in-depth knowledge is needed before applying strategies to improve their performance. On the contrary, the chicken isolate *B. lactis* P32/1 showed similar behaviour to the references *B. lactis* strains; therefore, it could be considered as a potential probiotic candidate.

Introduction

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.* 2014). Currently, the most commonly used probiotic micro-organisms belong to the genera *Lactobacillus* (*L. acidophilus*, *L. casei*, *L. rhamnosus*) and *Bifidobacterium* (*B. animalis* subsp. *lactis*, *B.*

longum, B. brevis, B. bifidum) (Foligné *et al.* 2013; Siroli *et al.* 2017; Champagne *et al.* 2018; Ranadheera *et al.* 2018). Bifidobacteria, are natural components of the intestinal microbiota and play an important role in the maintenance of the host health. Reduced levels of bifidobacteria in the natural microbiota of the human gastrointestinal tract (GIT) have been linked to several gastrointestinal diseases, such as irritable bowel syndrome, enterocolitis and colorectal cancer (Arboleya *et al.* 2011; Rajilić-Stojanović *et al.* 2011; O'Callaghan and van Sinderen 2016).

The production of probiotic dairy products using bifidobacteria represents a challenge due to the growth requirements and low tolerance to stresses of these probiotic micro-organisms (Ruiz *et al.* 2011; Florence *et al.* 2016). Some studies suggest that the addition of glucose oxidase to probiotic yoghurts together with the use of packaging systems with different oxygen permeability rates could be a useful technological strategy to minimize the oxidative stress in the food matrix (Cruz *et al.* 2013; Batista *et al.* 2015). Unfortunately, not all strains of bifidobacteria can be easily prepared at large scale since, usually, they present low yields in the growth media or poor survival to freezing or freeze-drying processes (Roy 2005). Nowadays, it is known that the viability of the probiotic must be maintained throughout the processes of preparation, handling and storage of the food product until the end of the shelf-life (Syngai *et al.* 2016; Patrignani *et al.* 2018). Moreover, in addition to surviving technological stresses, probiotics, once they are ingested with food, must resist to the biological barriers (such as digestive enzymes, gastric acid and bile) present in the GIT to reach their place of action and produce the beneficial effect (Ruiz *et al.* 2011). The most critical factors for the success of the inclusion of probiotics to food are the choice of the micro-organism and its amount in the food matrix (Soccol *et al.* 2010).

Currently, the number of bifidobacteria strains used by the food industry is limited due to their poor technological properties (Evivie *et al.* 2017), and the existing ones were mainly isolated from the human GIT. These limitations have led to the collection of new *Bifidobacterium* strains correctly identified and characterized, with not only functional but also technological properties, for the development of new probiotics with the potential to be successfully incorporated into a food product (Pandey *et al.* 2015). Numerous studies refer to bifidobacteria isolated from humans; in contrast, those relating to bifidobacteria from nonhuman sources are scarce (Ushida *et al.* 2010; Endo *et al.* 2012; D'Aimmo *et al.* 2014; Modesto *et al.* 2014; Tsuchida *et al.* 2014; Michelini *et al.* 2015, 2016).

The strains used in this study, *Bifidobacterium aesculapii* MRM3/1 and MRM4/2, *Bifidobacterium aerophilum* TRE26 and *Bifidobacterium avesanii* TREC, are new bifidobacteria species isolated from primates that have been characterized from a morphological, biochemical and molecular point of view (Modesto *et al.* 2014; Michelini *et al.* 2016), while *B. animalis* subsp. *lactis* P32/1 was isolated from chicken faeces (Mattarelli *et al.* 1992).

Thus, this work aimed at carrying out a deeper characterization of the strains by evaluating their technological and biological/functional potential to be added to a food matrix. Bifidobacteria were evaluated for their resistance to frozen storage, tolerance to lyophilization and viability during storage, thermal and acidic resistance in milk, surface hydrophobicity, simulated gastric resistance and antimicrobial activity against Gram-positive and -negative pathogens.

Materials and methods

Strains and growth conditions

Bifidobacterium strains were routinely grown (1% v/v) in de Man, Rogosa and Sharpe (MRS) broth (Biokar, Beauvais, France) supplemented with 0·1% (w/v) L-cysteine hydrochloride (Sigma, Saint Louis, MO, USA) (MRS-C) at 37°C for 20 h, under anaerobic conditions (Anaeropack, Mitsubishi, Japan). The commercial probiotic strain *B. animalis* subsp. *lactis* Bb12 (*B. lactis*) and the human breast milk *B. animalis* subsp. *lactis* INL1 (previously characterized from a technological and functional point of view (Zacarías *et al.* 2011, 2014; Burns *et al.* 2017)) have been used as reference strains. Stocks of the strains were kept at −80°C in MRS-C, containing 20% (v/v) glycerol.

Resistance to frozen storage in milk

Fresh overnight cultures of the strains (20 h, 37°C, anaerobiosis) in MRS-C broth were centrifuged (8000 ***g***, 30 min, 4°C), washed twice with phosphate-buffered saline (PBS), suspended into 20% (w/v) skim milk (Difco, Becton Dickinson and Company, Sparks, MD) and aliquoted in 1·5-ml test tubes (without leaving head-space). Cultures were frozen stored at −20 and −80°C (Zacarías *et al.* 2011). Cell counts (MRS-C agar, 37°C, 48 h, anaerobiosis) were performed after 15 days and then monthly, for 1 year. The assay was performed in triplicate.

Tolerance to lyophilization and viability during storage

Fresh overnight cultures of the strains (20 h, 37°C, anaerobiosis) in MRS-C broth were centrifuged (8000 ***g***, 30 min, 4°C), washed twice with PBS, suspended into

20% (w/v) skim milk (Difco, Becton Dickinson and Company), aliquoted in 2-ml vials, and frozen overnight at −80°C. Cells were freeze-dried in a laboratory scale freeze dryer (Christ Alpha 1-4 LD Plus; Osterode am Harz, Germany). Freeze-drying conditions were 0·002 mBar, −55°C, 20 h (Zacarías *et al.* 2011). Three independent replicates were performed for each strain. Lyophilized powders were stored at 5 and 25°C. Cell counts were performed before and after freeze-drying, and then monthly for 8 months (MRS-C agar, 37°C, 48 h, anaerobiosis). The assay was performed in duplicate.

Thermal resistance

Strains were grown overnight in MRS-C broth, centrifuged (8000 ***g***, 30 min, 4°C), washed twice with PBS and resuspended in PBS and 10% (w/v) skim milk. Cell suspensions were aliquoted (1 ml) and put into a water bath at 50°C. After 10 min, samples were removed and placed in a cold-water bath. Colony counts (MRS-C agar, 37°C, 48 h, anaerobiosis) at time = 0 and after 10 min, were carried out to determine the thermal tolerance of the strains. This determination was performed in triplicate in three different days.

Resistance to lactic acid

Fresh overnight cultures of the strains (20 h, 37°C, anaerobiosis) in MRS-C broth were centrifuged (8000 ***g***, 30 min, 4°C), washed twice with PBS, suspended into 10% (w/v) skim milk (Difco, Becton Dickinson and Company) pH 6·5 (control), or skim milk pH 5·0 or 4·5 (acidified with 85–90% lactic acid; Ciccarelli, Buenos Aires, Argentina), distributed into 12-ml sterile vials (without leaving head space) and stored at 4°C for 28 days. Cell counts (MRS-C agar, 37°C, 48 h, anaerobiosis) were performed at the beginning ($t = 0$) and weekly, for 28 days (Zacarías *et al.* 2011). The assay was performed in triplicate.

Simulated gastric resistance

Strains were grown overnight in MRS-C broth, centrifuged (8000 ***g***, 30 min, 4°C), washed twice with PBS and resuspended in sodium citrate (2% w/v). The gastric resistance was evaluated for the strains as pure cultures and incorporated into a creamy cheese (homogenized with sodium citrate 2% (w/v)). Cell suspensions (pure or into cheese) were mixed (1 : 1) with a 'saliva–gastric'-resembling solution containing $CaCl_2$ (0·22 g l^{-1}), NaCl (6·2 g l^{-1}), KCl (2·2 g l^{-1}), $NaHCO_3$ (1·2 g l^{-1}) (Marteau *et al.* 1997) and 0·6% (w/v) porcine pepsin (Merck, Darmstadt, Germany), acidified at pH 3·0 with 1 mol l^{-1} HCl and maintained at 37°C for 2 h. Cell counts (MRS-C agar, 37°C, 48 h, anaerobiosis) were carried out at the beginning (time 0) and after 30, 60, 90 and 120 min of simulated gastric digestion. The assay was performed in duplicate.

Antimicrobial activity against pathogens

Overnight cultures (37°C, 16 h) of *Escherichia coli* V517, *Salmonella enteritidis* OMS-Ca, *Staphylococcus aureus* 76 (INLAIN collection) and *Listeria monocytogenes* ATCC 15313 grown in Tryptone Soya (TS) (Britania, Buenos Aires, Argentina) or Brain Heart Infusion (BHI) broth (Britania, for *Listeria*) were inoculated (2% v/v) in TS or BHI agar (melted and cooled to 45–50°C) respectively (Zago *et al.* 2012). After mixing, cell suspensions were poured in 160-mm Petri dishes. Six wells (10 mm) were made on each agar plate. Overnight cultures of the bifidobacteria (MRS-C broth, 20 h, 37°C, anaerobiosis) were centrifuged (8000 ***g***, 30 min, 4°C) and the supernatants were recovered. A sample of each supernatant was used as a nontreated control, and the remaining was neutralized (NaOH pellets; Mallinckrodt, New York) and filter sterilized (0·45 mm; Millipore, Cork, Ireland). To different wells, 180 µl of each supernatant, (neutralized or not) was added. Petri dishes were incubated for 24 h at 37°C, and the diameters of the halos of inhibition were recorded. The assay was performed in triplicate.

Surface hydrophobicity

The hydrophobicity of the strains (ability to adhere to n-hexadecane), was assessed according to Vinderola and Reinheimer (2003). The hydrophobicity (% H) was calculated as follows: $\%H = [(OD_i - OD_f/OD_i]/100$, where OD_i and OD_f were the optical densities before and after the n-hexadecane extraction respectively. The assay was performed in triplicate.

Results

Resistance to frozen storage in milk

Among the technological characteristics of the bifidobacteria taken into consideration, the resistance of the strains to frozen storage in milk ((20% (w/v)) was studied for 1 year. The losses of the viability of the strains, inoculated at a level ranging between 8·5 and 9·0 log CFU per ml, expressed as Δ log CFU per ml, after 15, 90, 180, 270 and 360 days of storage are reported in Fig. 1.

As evidenced in Fig. 1, the resistance of the strains to the frozen storage at −80°C was satisfactory. The most

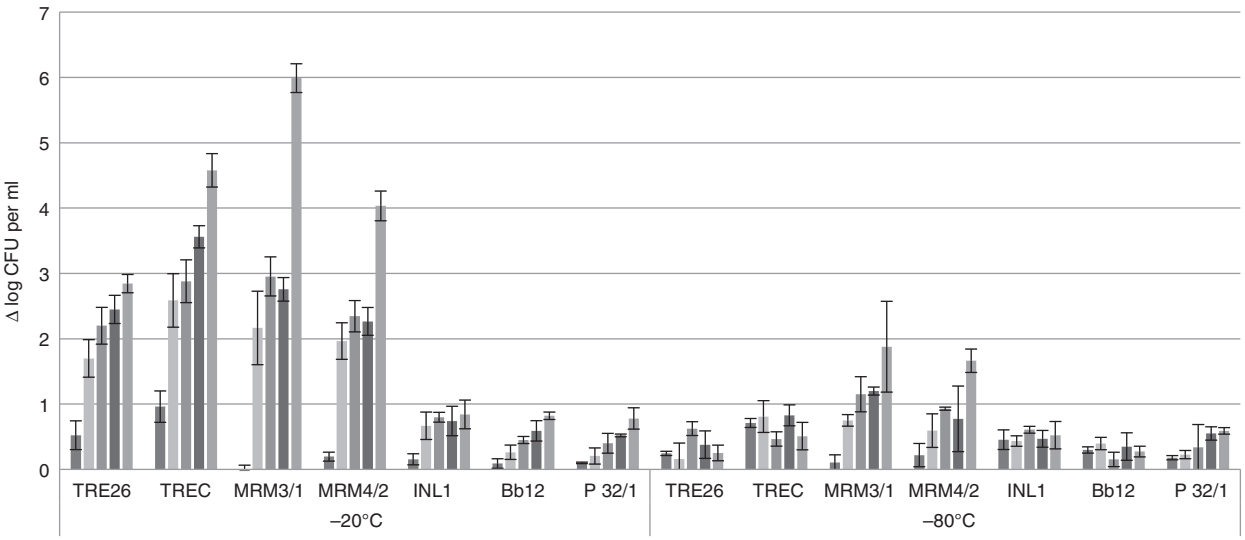

Figure 1 Cell death data expressed as Δ log CFU per ml of the *Bifidobacterium* strains during frozen storage after 15 (■), 90 (■), 180 (■), 270 (■), 360 (■) days at −20 and −80°C.

sensitive strains were *B. aesculapii* MRM3/1 and MRM4/2 with a Δ log CFU per ml of 1·88 and 1·66 after 360 days respectively. All the other tested strains showed limited losses of viability, ranging between 0·25 and 0·52 log CFU per ml, after 360 days of storage resulting similar to the reference strains.

Contrarily, at −20°C the resistance of the four strains isolated from monkey intestine was lower. Only the chicken isolate *B. lactis* P32/1 showed a limited loss of viability, similar to *B. lactis* Bb12 and INL1 (cell death lower than 0·85 log CFU per ml after 360 days of storage) (Fig. 1). Among the strains isolated from monkeys, *B. aerophilum* TRE26 exhibited the most limited loss of viability, showing a Δ log CFU per ml of 2·84 after 360 days of storage. Both *B. aesculapii* strains and *B. avesanii* TREC presented losses of viability higher than 4·0 log CFU per ml after 360 days of storage.

Tolerance to lyophilization and viability during storage

Another technological parameter evaluated was the resistance of the strains, resuspended in skim milk 20% (w/v), to freeze-drying and subsequent storage at two different temperatures (5 and 25°C). Cell death after lyophilization of the different strains (expressed as Δ log CFU per ml) is shown in Table 1. Figure 2(a,b) shows the cell viability of the lyophilized strains stored at 25 and 5°C for 8 months.

As can be observed in Table 1, all the *B. lactis* (P32/1, INL1 and Bb12) and *B. aesculapii* strains (MRM3/1 and MRM4/2) showed satisfactory resistance to the dehydration process with a loss of viability below 0·25 log CFU per ml. However, higher cell deaths were observed for *B. aerophilum* TRE26 and *B. avesanii* TREC.

Table 1 Cell death (Δ log CFU per ml ± SD) of the *Bifidobacterium* strains after freeze-drying in skim milk 20% (w/v)

Strain	Δ log CFU per ml ± SD
B. aesculapii MRM3/1	0·01 ± 0·01
B. aesculapii MRM4/2	0·00 ± 0·00
B. avesanii TREC	1·15 ± 0·13
B. aerophilum TRE26	0·63 ± 0·09
B. lactis Bb12	0·00 ± 0·00
B. lactis INL1	0·23 ± 0·04
B. lactis P32/1	0·00 ± 0·00

The resistance of the lyophilized strains stored at 5°C was very satisfactory for both reference strains for which a limited loss of viability was observed after 8 months of storage. The strains *B. aerophilum* TRE26, *B. aesculapii* MRM3/1 and the chicken isolate *B. lactis* P32/1 presented good resistance to the conditions adopted for the whole period of storage, showing viability losses of 0·28, 0·64 and 0·33 log CFU per ml, respectively, after 8 months of storage. The strains *B. avesanii* TREC and *B. aesculapii* MRM4/2 did not show viability losses in the first 4 months of storage at 5°C, then were found as the most sensitive.

At 25°C the lyophilized strain *B. lactis* P32/1 showed a similar behaviour to the reference ones (*B. lactis* INL1 and Bb12). These strains presented comparable behaviours to those observed at 5°C without viability loss for *B. lactis* Bb12 and limited to 0·37 and 0·22 log CFU per

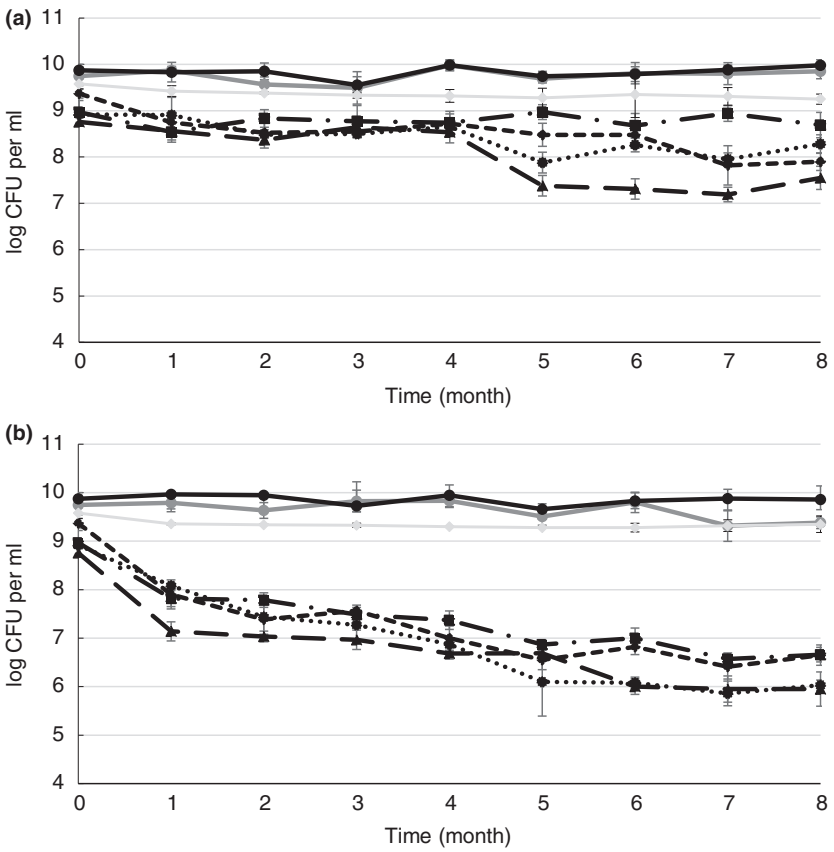

Figure 2 Cell viability data (log CFU per ml) of lyophilized *Bifidobacterium* strains [(B. a. TRE26 (■, full black line), B. a. TREC (▲, full black line), B. a. MRM3/1 (●, dotted black line); B. a. MRM4/2 (◆, dashed black line), B. l. INL1 (●, full grey line), B. l. Bb12 (●, full black line), B. l. P32/1 (●, full grey line)] stored for 8 months at 5°C (a) and 25°C (b).

ml for *B. lactis* INL1 and *B. lactis* P32/1, respectively, after 8 months of storage. On the contrary, the temperature of 25°C was not optimal for the storage of lyophilized bifidobacteria isolated from monkey's faeces.

Thermal resistance

To be successfully introduced into a food product, probiotic bacteria must survive several factors related to food production technology. Among these, high temperatures are a detrimental factor for the maintenance of vitality of probiotics. For this reason, the thermal resistance of the strains, inoculated at a level ranging between 8·0 and 8·5 log CFU per ml and resuspended in PBS buffer and 10% (w/v) skim milk, was determined. The results, expressed as cell viability loss (Δ log CFU per ml), are shown in Fig. 3.

All the strains showed good thermal resistance at 50°C (10 min). In general, a higher survival of the strains in milk compared to PBS was observed. The strains *B. lactis*

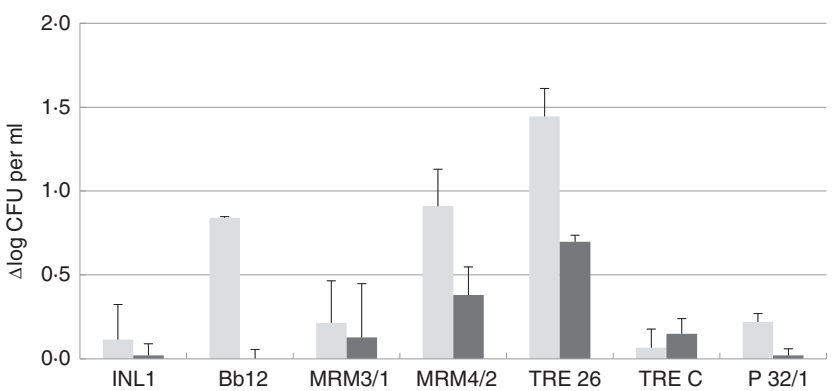

Figure 3 Thermal resistance data, expressed as cell death (Δ log CFU per ml), of the *Bididobacterium* strains at 50°C for 10 min in PBS (■) and 10% (w/v) skim milk (■)

INL1, Bb12 and P32/1, *B. aesculapii* MRM3/1 and MRM4/2 and *B. avesanii* TREC presented the highest thermal resistance showing a loss of viability in milk below 0·5 log CFU per ml after 10 min. Contrarily, the strain *B. aerophilum* TRE26 resulted in the most sensitive to the thermal treatment with a loss of viability of 1·44 and 0·70 log CFU per ml in PBS and milk respectively.

Resistance to lactic acid

Acidity is another stress factor to which probiotic strains may be exposed, and in our study, the viability of the bifidobacteria, inoculated at a level ranging between 7·2 and 7·7 log CFU per ml in a simulated fermented milk (acidified with lactic acid) at different levels of acidity (pH 5·0 and 4·5) for a storage period of 28 days at 4°C was evaluated. Nonacidified milk (pH 6·5) was used as a control. The cell loads of bifidobacteria during storage are reported in Fig. 4(a–c). At all the three pH levels tested, it can be observed that *B. lactis* INL1, Bb12 and P32/1 remained stable without loss of viability during the whole refrigerated storage period. In contrast, the other bifidobacteria showed widely variable results. The most sensitive strain to the three conditions tested was *B. avesanii* TREC, while the strains *B. aesculapii* MRM3/1 and MRM4/2 were the most resistant ones.

For bifidobacteria isolated from monkey faeces, the refrigerated storage in acidified milk did not show satisfactory results, since high losses of cell viability were observed at the lowest pH evaluated (pH 4·5). However, it is important to mention that a pH increase of 0·5 resulted in a better maintenance of the cell viability of the strains considered, particularly for *B. avesanii* TREC and *B. aerophilum* TRE26.

Simulated gastric resistance

The resistance to simulated gastric conditions (pH 3·0) of the strains inoculated at a level of 7·0 log CFU per ml and resuspended in sodium citrate (2% w/v) and creamy cheese was evaluated. The loss of viability (expressed as Δ log CFU per ml) after 30, 60, 90 and 120 min of incubation (37°C) at pH 3·0 is reported in Fig. 5.

Both the reference strains (*B. lactis* INL1 and Bb12) as well as the chicken isolate *B. lactis* P32/1 showed a very limited loss of viability during the whole period of incubation at pH 3·0, independently on the inclusion in a cheese matrix. Bifidobacteria isolated from monkey were more inhibited in sodium citrate than in cheese. The strains *B. aerophilum* TRE26 and *B. avesanii* TREC and resulted in the most sensitive. Thus, the exposure to pH 3·0 of both strains when resuspended in sodium citrate, led to the reduction in the cell loads under the detection limit after 30 and 60 min, respectively. However, the inclusion of these strain in a creamy cheese protected them from simulated gastric conditions. *Bifidobacterium aesculapii* MRM3/1 and MRM4/2 resulted in the most resistant among the strains isolated from monkey. However, for the latter, the protective effect of cheese was evident only after 30 and 60 min of incubation at pH 3·0.

Antimicrobial activity against pathogens

Another functional aspect evaluated was the capability of the bifidobacteria to inhibit Gram-positive and -negative pathogenic micro-organisms associated with food products such as *E. coli*, *L. monocytogenes*, *Salm. enteritidis* and *Staph. aureus*. Results of the inhibition of the cell-free supernatants (not neutralized) are shown in Table 2.

All the strains were able to inhibit, although in different amounts, the pathogenic micro-organisms considered in this study. *B. avesanii* TREC showed the highest antimicrobial activity with inhibition halos ranging between 3 and 5 mm against *E. coli* and *Staph. aureus* and higher than 5 mm against *L. monocytogenes*. In general, bifidobacteria isolated from monkey presented higher antimicrobial activity than the reference strains and *L. monocytogenes* was the most sensitive pathogen. No inhibition activity was observed when the supernatants were neutralized (data not shown).

Surface hydrophobicity

The results of cell hydrophobicity are shown in Table 3. All strains of *B. lactis*, including the chicken isolate P32/1 showed high hydrophobicity (>85%), while bifidobacteria isolated from monkeys showed hydrophobicity values below 25%.

Discussion

Traditionally, the origin of probiotic-marketed bacteria was limited to a rather small number of bacterial species mostly belonging to lactic acid bacteria and bifidobacteria (Douillard and de Vos 2019). *Bifidobacterium* species have historically been considered safe and suitable for human consumption with several published studies addressing their safety (Ouwehand *et al.* 2018). Several studies pointed out the importance of isolating and identifying new strains of the genus *Bifidobacterium* from different animals, including humans, in order to understand how they are mostly distributed. Ecological studies have revealed the presence of bifidobacteria in the gut of a wide variety of animals (e.g. mammals, birds, ungulates, lagomorphs and rodents) and insect pollinators (Michellini *et al.* 2016). The possibility of exploring new

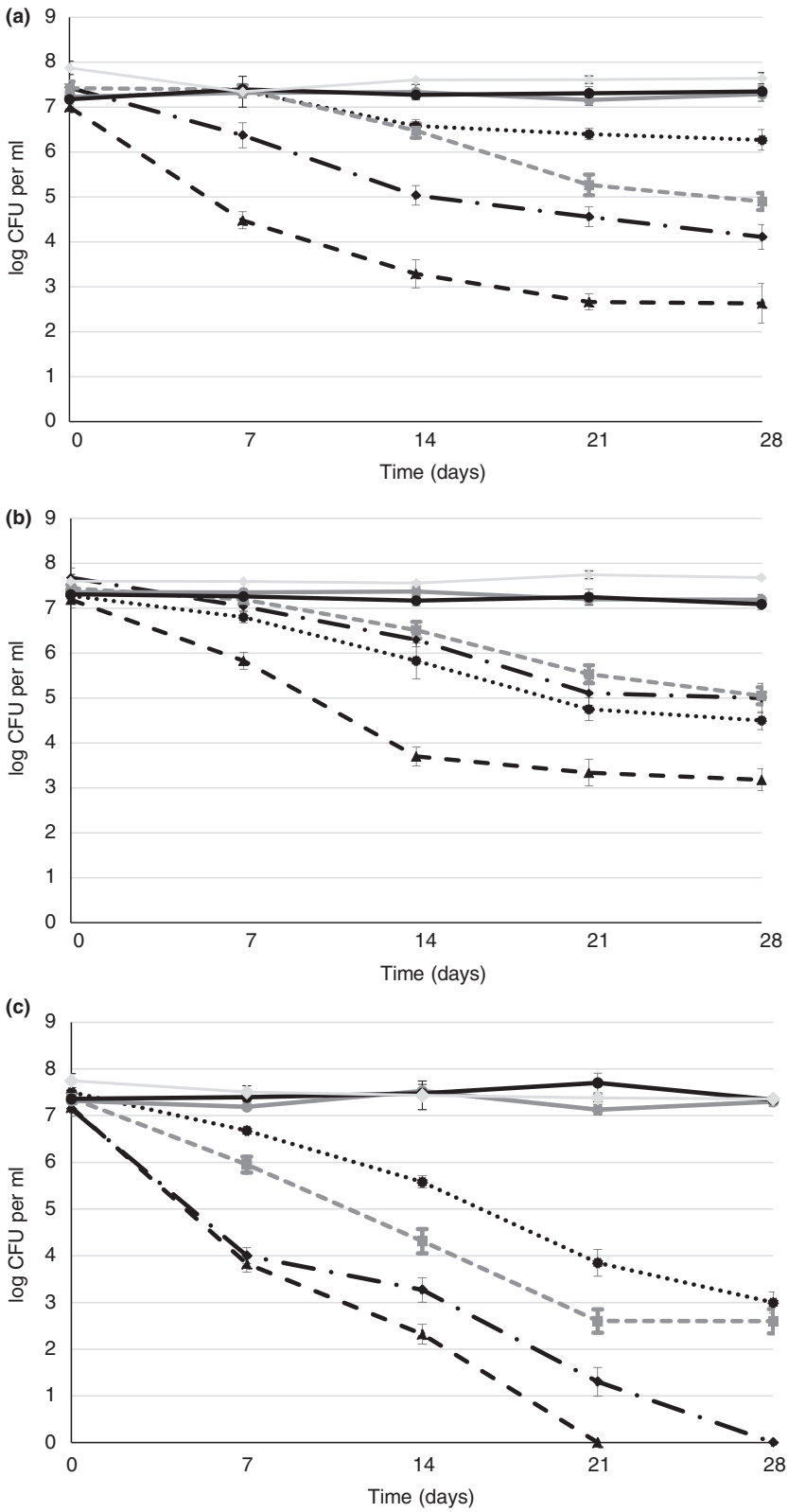

Figure 4 Cell viability data (log CFU per ml) of the *Bifidobacterium* strains [(B. a. TRE26 (◆, full black line), B. a. TREC (▲, dashed black line), B. a. MRM3/1 (●, dotted black line); B. a. MRM4/2 ((■, dashed grey line), B. l. INL1 ((●, full grey line), B. l. Bb12 (●, full black line), B. l. P32/1 (◆, full grey line)] resuspended in milk at pH 6·5 (a), pH = 5·0 (b) and pH = 4·5 (c) during 28 days of storage at 4°C.

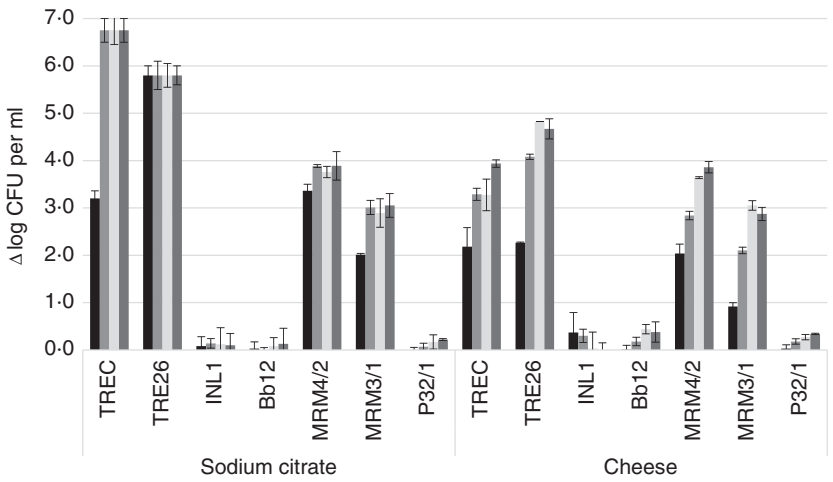

Figure 5 Losses of viability (expressed as Δ log CFU per ml) of the *Bifidobacterium* strains in sodium citrate and cheese after 30 (■), 60 (■), 90 (■) and 120 (■) min of incubation (37°C) at pH 3·0.

Table 2 Antagonistic activity of the *Bifidobacterium* strains against food-related pathogenic micro-organisms

	Escherichia coli V517	*Staphylococcus aureus* 76	*Salmnella enteritidis* OMS-Ca	*Listeria monocytogens* ATCC 15313
B. aesculapii MRM3/1	+	+	+	+
B. aesculapii MRM4/2	+	++	+	++
B. avesanii TREC	++	++	+	+++
B. aerophilum TRE26	+	+	+	++
B. lactis INL1	+	+	+	+
B. lactis Bb12	+	+	+	+
B. lactis P32/1	+	+	+	+

− no inhibition; +: inhibition 1–3 mm; ++: inhibition 3–5 mm; +++: >5 mm.

Table 3 Surface hydrophobicity (%) of the *Bifidobacterium* strains (±SD)

Strain	Surface hydrophobicity (%)
B. aesculapii MRM3/1	13·3 ± 8·5
B. aesculapii MRM4/2	7·1 ± 6·9
B. avesanii TREC	15·1 ± 10·1
B. aerophilum TRE26	23·4 ± 12·6
B. lactis INL1	98·0 ± 1·6
B. lactis Bb12	88·7 ± 5·3
B. lactis P32/1	91·3 ± 2·4

niches and having new strains with probiotic potential is a valuable strategy for expanding the available bifidobacteria.

For probiotic food to guarantee quality and beneficial effects, probiotics should retain viability and integrity through the entire process of biomass and food production, storage and ingestion, when, in fact, multiple environmental challenges (mainly dealing with temperature and oxygen) can affect microbial survival and functionality (Ruiz *et al.* 2011; Fiocco *et al.* 2019). Freezing is a widely used process to preserve cell viability and the technological properties of microbial cultures commonly used at the industrial level. Probiotics are mostly marketed as frozen-concentrated cultures or dehydrated by freeze-drying. Microbial stability can be affected by the freezing process and prolonged frozen storage, depending on the fermentation, stabilization and storage conditions (Zacarías *et al.* 2011). The data obtained showed limited loss of viability of the frozen bifidobacteria studied during 1 year of storage at −80°C.

On the contrary, except for the *B. lactis* strains, the storage at −20°C led to a substantial decrease in cell viability. These results confirm literature data reporting *B. animalis* subsp. *lactis* as the most tolerant *Bifidobacterium* species to stress factors, including frozen storage (Ruiz *et al.* 2011; Andriantsoanirina *et al.* 2013). Moreover, as expected, the resistance of the strains resulted higher at −80°C than at −20°C since the rate of freezing affect the loss of viability of microbial cells. Bigger ice crystals formed during freezing at higher temperatures cause more substantial damage to microbial cells, while faster freezing at lower temperatures allows maintaining the viability of the micro-organisms in the product (Mohammadi *et al.* 2011; Tripathi and Giri 2014).

As previously mentioned, another form of commercialization of micro-organisms is dehydrated by freeze-drying. The strains isolated from monkey showed high resistance to the drying process, except for *B. avesanii* TREC that was the most sensitive. The behaviour during storage was different according to the strain and the storage temperature. In particular, for the bifidobacteria isolated from monkey, the storage temperature of 5°C allowed maintaining a greater vitality of the lyophilized strains, compared to storage at room temperature (25°C), thus being optimal for their preservation. This result agrees with literature data reporting a decline in the viability of several spray-dried *Bifidobacterium* strains stored at 25°C compared to refrigerated storage at 4°C (Simpson *et al.* 2005). On the contrary, the chicken isolate *B. lactis* P32/1 and the reference *B. lactis* strains showed a limited loss of viability as well during the storage at 25°C.

To be successfully incorporated into a food product, probiotic bacteria must be resistant to different stress factors concerning its production technology. In this sense, high temperatures are detrimental to the maintenance of the vitality of probiotics. The results of this study showed that the strains *B. lactis* P32/1, *B. avesanii* TREC and *B. aesculapii* MRM4/2 and MRM3/1 resuspended in milk, maintained satisfactory viability after 10 min at 50°C. This fact is very interesting since temperatures in the range 45–50°C during processing are reported to drastically reduce the survival of probiotics (Simpson *et al.* 2005). Moreover, the survival was higher when the strains were resuspended in milk compared to PBS, which is probably due to the protective effect of milk, rich in proteins and fats, on the microbial cells (Burns *et al.* 2015).

The monkey-isolated *Bifidobacterium* strains showed very variable results regarding the maintenance of cell viability in acidified-fermented milk (pH 4·5 and 5·0). For all of them, the loss of viability observed during storage at pH 4·5 was reduced at pH 5·0. Contrarily, the chicken isolate *B. lactis* P32/1 did not show cell death during the storage at all the considered pH. These results indicate that the viability of the bifidobacteria used could be favoured if they are included in a food product whose matrix could have better protective action, such as cheese, and with a higher pH value compared to fermented milk (Burns *et al.* 2008, 2015). Several authors have reported that the stability of bifidobacteria strains in fermented milk is extremely variable and strain dependent (Zacarías *et al.* 2011). Among bifidobacteria species, *B. animalis* subsp. *lactis* strains are able to survive under more extreme conditions, while others display a low tolerance to stress (e.g. some *Bifidobacterium bifidum* and *Bifidobacterium longum* strains). It is widely reported that bifidobacteria are characterized by a limited acid tolerance except for *B. animalis* (Roy 2005; Ruiz *et al.* 2011; Andriantsoanirina *et al.* 2013). Recently, Patrignani *et al.* (2018) reported that different *B. aesculapii* strains, among them MRM3/1 and MRM4/2, showed good potential to ferment soy milk, but the maintenance of the cell viability, during the refrigerated storage of the fermented product (pH 4·6), varied depending on the strain. Sensory analysis is also a key point to consider when developing a food product (Cruz *et al.* 2010). In this sense, Patrignani *et al.* (2018) confirmed the good quality of the obtained fermented soy milk, using *B. aesculapii* strains.

Gastric acid and bile are important defences of the GIT against ingested micro-organisms, capable of controlling or killing many pathogens. However, in the case of potentially beneficial micro-organisms, this defence mechanism could be harmful (Jungersen *et al.* 2014). The effect of gastrointestinal stress conditions on the viability of probiotics has been widely evaluated, and it has been shown that each bacterium differs in its tolerance levels, so it is important to evaluate them individually (Gueimonde and Salminen 2006). Our results confirmed that the tolerance to simulated gastric conditions is strain dependent. The *B. lactis* strains, including the chicken isolate P32/1, did not show loss of variability. Among the monkey *Bifidobacterium* strains, both *B. aesculapii* MRM3/1 and MRM4/2 were more resistant than *B. avesanii* TREC and *B. aerophilum* TRE26 strains. As expected, the inclusion of the strain in a food matrix such as a creamy cheese increased their survival. Several authors reported cheese matrices as a suitable carrier for *Bifidobacterium* species since the pH, lipid and protein content and oxygen level permitted a long-term survival of bifidobacteria during storage and digestion (Verruck *et al.* 2015; Martins *et al.* 2018).

All the strains, and particularly those isolated from monkey, were able to inhibit the growth of the pathogenic micro-organisms. Furthermore, among bifidobacteria, *B. avesanii* TREC showed strong antimicrobial activity against *L. monocytogenes* and *E. coli*. The antimicrobial effect showed by the *Bifidobacterium* strains can definitively be attributed to the production of organic acids since only the non-neutralized supernatant showed inhibition of the target pathogens. This result agrees with Tejero-Sariñena *et al.* (2012), which attributed the antimicrobial activity of several *Bifidobacterium* species to the production of organic acids.

The binding of probiotic bacteria to intestinal cells is expected to have desirable effects on health, including immunomodulation and the exclusion of pathogens. Nevertheless, the adhesion behaviour of microbial cells is dependent not only on the hydrophobic character of the surfaces but also on the balance of electrostatic and van der Waals interactions (Turpin *et al.* 2012). In the

present study, monkey-isolated bifidobacteria presented lower surface hydrophobicity than *B. lactis* strains. It is known that aggregation and adhesion ability can be conditioned by many other factors (protein factors, fatty acids and EPS) that may positively or negatively affect adherence to other cells (Polak-Berecka *et al.* 2014). Also, technological processes widely applied in the dairy industry, such as high-pressure homogenization treatments performed at sublethal levels (lower than 80 MPa) are reported to increase the hydrophobicity and other functional properties of some probiotic bacteria (Tabanelli *et al.* 2012, 2013, 2015).

The present work contributes to a greater insight into unknown bifidobacteria strains isolated from primates and chicken and confirm the most robust stress tolerance of *B. lactis*. In case of the strains with scarce tolerance to the stressors studied, different strategies such as pre-exposure to sublethal stresses or microencapsulation techniques could be taken into account to increase their viability when incorporated into a suitable food matrix. The chicken isolate *B. lactis* P32/1 showed a good performance to be used in the food industry and could be considered as a potential probiotic candidate. Despite this, further studies are needed, and an *in vivo* assay should be done to know its beneficial effects on health.

Acknowledgements

This work was supported by the Project PICT Jóvenes 2015-0622, Res. 205/15 from de Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and by the Project CAI + D Jóvenes 2016, Res. 48/17 from the Universidad Nacional del Litoral. Sarquis M. A. received a student fellow 'Beca de Iniciación a la Investigación' from the Universidad Nacional del Litoral. Siroli L. received a postdoctoral fellow of the 'Programa de Becas Postdoctorales de Movilidad Internacional (Res. CS. 659/16) from the Universidad Nacional del Litoral.

Conflict of Interest

No conflict of interest declared.

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