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Probiotic potential and biofilm inhibitory activity of *Lactobacillus casei* group strains isolated from infant feces

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ABSTRACT

Probiotic bacteria are receiving growing interest, particularly for the preparation of functional foods. In the present study, eight *Lactobacillus* strains, newly isolated from infant feces, were investigated for the presence of probiotic properties such as antimicrobial susceptibility, hemolytic activity, resistance to simulated gastro-intestinal conditions, bile salts hydrolytic activity, inhibitory ability against biofilm formation by other bacteria, attachment to HT-29 human cancer cells and anti-cancer activity. All the strains tested highlighted interesting properties, but *L. paracasei* DTA93 and *L. paracasei* DTA81 appeared of particular interest. Some properties of these two strains resulted similar, and in some cases superior, to the reference widespread probiotic commercial strain *L. rhamnosus* GG. Strain *L. paracasei* DTA81 possesses amazingly high adherence ability to HT-29 cells, about ten times higher than that of *L. rhamnosus* GG. Moreover, *L. paracasei* DTA93 and *L. paracasei* DTA81 were able to effectively inhibit biofilm formation of *Escherichia coli* and *Listeria innocua*.

1. Introduction

The word "probiotic" is derived from the Greek word meaning "for life". It is usually referred to microorganisms that confer beneficial effects to human and animals by interacting with their gut microbiota (De Vrese & Schrezenmeir, 2008). Lactic Acid Bacteria (LAB) are presently the most studied and characterized bacterial group containing probiotic strains, particularly in the genus *Lactobacillus*. This genus contains more than 170 species and it is considered a taxonomically complex group (Foschi et al., 2017; Goldstein, Tyrrell, & Citron, 2015). Lactobacilli are very widespread in nature and are commonly isolated from several different matrices, such as fermented foods (Pogačić et al., 2010), plant material (Campanaro et al., 2014), soil (Kim et al., 2018) and human gut (Guerra et al., 2018; Zhang et al., 2018). The increase in knowledge on probiotics has led to the development of food products that can provide health benefits beyond basic nutrition. Probiotic foods represent a huge segment of functional food available on the market worldwide, projected to reach a value of US $46.55 billion by 2020 (Singh, Singh, & Singh, 2018). According to the WHO/FAO definition, probiotics can be considered live microbes which confer a health benefit to the host when ingested in adequate amounts. It has been demonstrated that probiotic effects are strains-specific (Motevaseli, Dianatpour, & Ghafouri-Fard, 2017), thus strain identification and evaluation of its safety aspects must be performed prior to connect a specific strain to its own health benefit. Among LAB, the *Lactobacillus* genus includes the highest number of GRAS (Generally Recognized As Safe) species (Klaenhammer & de Vos, 2011). Besides the safety aspects such as resistance to antibiotics and blood hemolytic activity, other important criteria to define a strain as probiotic include survival to the human gastro-intestinal conditions, ability to adhere to the intestinal epithelial surface, which indicates possible antimicrobial activity against pathogens, prevention of colon cancer. In addition, the presence of potentially interesting technological traits would be desirable, such as good sensory properties, phage resistance, viability during processing and stability in production and during storage (Mattila-Sandholm et al., 2002; Ouwehand, Salminen, & Isolauri, 2002). Among all benefits from probiotics, anti-cancer effect has been one of the most interesting characteristic studied during last decade (Kumar & Dhanda, 2017). In

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1990, Kubota reported a correlation between gut microbiota and colon cancer incidence (Kubota, 1990), since illness incidence was lower when the presence of probiotic strains was higher. Lactobacilli can inhibit cancer cells development by inactivate reactive oxygen species (ROS), increase TNF-α, interferon-γ (IFN-γ), downregulating nuclear factor-kappaB (NF-κB) and increase the level of natural killer cell (NK) in the human body (Bruno-Bárcena, Andrus, Libby, Klaenhammer, & Hassan, 2004; Lee, Kim, Yim, & Joo, 2004; Takagi et al., 2008). More recently, Shahid et al. (2018) demonstrated that cell-free culture supernatant (CFCS) of *L. casei* and *L. paracasei* isolated from human breast milk can upregulate the expression of apoptosis-related genes on cervix cancer (HeLa) cells. In this study, we identified and selected some potential *Lactobacillus* probiotic strains isolated from infant feces. We studied the capability to withstand the transit through the gastro-intestinal tract and to hydrolyze bile salts and utilize prebiotic molecules. The absence of hemolytic activity and of transmissible antibiotic resistance were also studied. Finally, we looked for health-related traits, namely ability to attach and contrast the development of HT-29 colorectal cancer cells.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strains used in the present work were isolated from feces of infants aged between 7 and 21 days from different hospitals in Rio de Janeiro (RJ, Brazil). Samples were collected in duplicate in sterile 10-mL plastic tubes and immediately placed on ice inside separate plastic bags. A hand pump was used to remove the air from the bags. Decimal dilutions were performed in Anaerobic Wilkins-Chalgren broth (Oxoid, Basingstoke, United Kingdom) and 100-µl aliquots of each dilutions were plated on Lamvab agar medium (Hartemink, Domenech, & Rombouts, 1997) specific for the isolation of lactobacilli and incubated at 37 °C for 48 h under microaerophilic conditions. Colonies showing typical morphologies were further characterized by Gram staining and catalase test.

2.2. Molecular identification of strains belonging to the Lactobacillus casei group

For DNA extraction one colony was picked from MRS agar plate (Bottari et al., 2017) and transferred into a 0.5 mL Eppendorf tube containing 50 µl of lysis solution (0.25% SDS and 50 mM NaOH). Cell lysis was obtained by incubating the tube at 95 °C for 15 min in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The supernatant was collected by centrifugation at 10,000*g* for 10 min and diluted 1:100 in ultrapure sterile water. DNA yield and purity were assessed by NanoDrop 2000c (Thermo Fischer Scientific, Wilmington, DE, USA).

The multiplex PCR assay was performed using the primer pairs and PCR conditions described previously (Bottari et al., 2017). Results were visualized by gel electrophoresis on SYBR Safe stained 1.5% agarose gel. The type strains *L. paracasei* subsp. *paracasei* DSM 5622, *L. casei* DSM 20011 and *L. rhamnosus* DSM 20021 were used as reference for the species and *L. plantarum* subsp. *plantarum* DSM 20174 was used as negative control.

2.3. Determination of minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration test was performed by the broth microdilution method, in 96-well microtiter plates (Wiegand, Hilpert, & Hancock, 2008). The following antibiotics: ampicillin, ciprofloxacin, erythromycin, gentamycin, kanamycin, penicillin G, tetracycline, and vancomycin (Sigma-Aldrich, Saint Louis, MO, USA) which are recommended by the European Food Safety Authority (EFSA, 2008), were dissolved in MRS plus ISO-Sensitest broth (Sigma-Aldrich) (ratio 10:90) and distributed as 2-fold serial dilutions in the microtiter plate wells. Each microtiter well was then inoculated with 50 µl of bacterial culture to reach 5×10^5 cfu/mL (final concentration). After incubation for 24 h, the MIC was defined as the drug concentration in the first well with no visible growth. The test was performed in triplicate.

2.4. Hemolytic activity test

The hemolytic activity test was performed by streaking fresh cultures of strains to be tested on MRS agar containing 5% (w/v) defibrinated sheep blood (cat. SR0051D, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37 °C. Hemolytic activity was checked after 48 h of incubation. *Staphylococcus aureus* ATCC 6538 and *L. rhamnosus* GG (ATCC 53103) were used as positive and negative control, respectively. The experiment was performed with three technical and three biological replicates (Pieniz, Andreazza, Anghinoni, Camargo, & Brandelli, 2014).

2.5. Resistance to simulated gastrointestinal conditions

The resistance of strains to simulated gastro-intestinal conditions was carried out as previously described (Tarrah, Castilhos, et al., 2018). The basic juice for the gastrointestinal test contained (per liter) calcium chloride, 0.11 g; potassium chloride, 1.12 g; sodium chloride, 2.0 g; potassium dihydrogen phosphate, 0.4 g. The artificial gastric juice contained (per liter) 3.5 g of swine mucin (Sigma-Aldrich) and 0.26 g of swine pepsin (Sigma-Aldrich). The gastric juice was adjusted to pH 2.5 with 1 N HCl, filter sterilized and then added to the basic gastro-intestinal juice. The simulated intestinal juice contained (per liter) 3 g Ox-bile extract (Sigma-Aldrich), 1.95 g pancreatin (Sigma-Aldrich) and 0.1 g lysozyme (Sigma-Aldrich). The pH was adjusted to 8.0 with 1 N sodium bicarbonate and the medium was then filter sterilized. Regarding resistance to gastric conditions, aliquots of 0.1 mL of cell suspension obtained after three subcultures for 24 h in MRS broth was transferred to 0.9 mL of artificial gastric juice (pH 2.5) and incubated for 1 h at 200 rpm agitation at 37 °C. Regarding the intestinal conditions, 1 mL of artificial intestinal solution (pH 8) was added after incubation to gastric juice and incubated at 37 °C at 200 rpm stirring for 180 and 300 min. Microbial viability was evaluated by the micro drop technique. The experiment was repeated three times with three technical replicates each.

2.6. Bile salts hydrolytic activity

Fresh cultures were streaked on MRS agar containing 0.5% taurodeoxycholic acid (Sigma-Aldrich Saint Louis, MO, USA). The hydrolytic activity was checked after 48 h of anaerobic incubation at 37 °C for the presence of a precipitation halo. MRS plates without taurodeoxycholic acid were used as negative controls, whereas *Leuconostoc mesenteroides* SJRP 55 was used as a positive control (Jeronymo-Ceneviva et al., 2014).

2.7. Adhesion to HT-29 cells

The adhesion potential of newly isolated *Lactobacillus* strains to HT-29 cell lines was assessed as previously described (Jacobsen et al., 1999). The adherent strains were counted in 20 random microscopic fields. According to (Jacobsen et al., 1999), strains were scored as non-adhesive if they were fewer than 40, adhesive if the number was between 41 and 100, and strongly adhesive if there were more than 100 bacteria in 20 fields. The test was repeated three times with three technical replicates each.

2.8. Anti-proliferative activity against HT-29 cells

The anti-proliferative activity of the *Lactobacillus* strains on HT-29 cells was assessed by the microculture tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay (Mosmann, 1983). Aliquots of 100 µl of HT-29 cells culture (1.2×10^5 cells/mL) in McCoy's 5A (Sigma-Aldrich) growth medium were inoculated into each well of 96-wells microplates. The strains to be tested were grown for 24 h, then pellets were removed by centrifugation at 5000 rpm for 10 min and pH normalized to 7.0 with 1 N NaOH. The supernatant was then freeze-dried and diluted in McCoy's 5A broth to obtain the following concentrations: 0.125, 0.25, 0.5, 0.75, 1, 2, 4 and 8 mg/mL. When 50% confluence of HT-29 cell was reached, the McCoy's 5A was replaced with 0.1 mL filtered supernatant of strain cultures at different concentrations and cells were incubated in 5% CO_2 atmosphere for 48 h at 37 °C. Then 20 µl of MTT (5 mg/mL diluted in PBS) was added to each well and incubated for further 4 h at 37 °C to allow cells interaction with MTT. Following incubation, the formazan blue crystals formed were dissolved in 100 µl DMSO (Sigma-Aldrich). After 20 min stirring at 200 rpm, the reduction of MTT was measured by reading the absorbance at 570 nm using a microplate reader (Biotek ELx 800, Thermo Fisher Scientific) and viability percentage of HT-29 cells was calculated by using the following formula:

$$\text{Viability } \% = (OD_{570} \text{ of treated cells}/OD_{570} \text{ of cells without treatment}) \times 100$$

Cells incubated with MRS (bacterial culture medium) only and with DMSO 3% (anticancer molecule used as reference) were used as negative and positive controls, respectively. The experiment was repeated two times (biological replicates) in three technical replicates each.

2.9. Biofilm inhibitory activity

The capability of the *Lactobacillus* strains to inhibit biofilm formation by *Escherichia coli* DSM 30083^T and *Listeria innocua* DSM 20649^T was evaluated as previously described (Woo & Ahn, 2013), with some modifications. Biofilm-producing strains were grown on the hydrophobic surface of a 24-wells polystyrene plate with flat bottom. The biofilm inhibitory activity was evaluated in two different conditions, namely competition and exclusion. In the first test the *Lactobacillus* strains were co-inoculated with *E. coli* or *L. innocua*, at a concentration of 10^7 cfu/mL in a 24-wells plate and incubated at 37 °C for 18 h. In the exclusion experiment *Lactobacillus* cell suspensions containing 10^7 cfu/mL were inoculated inside 24 wells plate and incubated for 18 h at 37 °C. The wells were then washed three times with PBS, the wells inoculated with *E. coli* or *L. innocua* cell suspensions at the same concentration (10^7 cfu/mL) and incubated for further 18 h at 37 °C. Wells inoculated with *E. coli* or *L. innocua* alone were used as controls. Following incubation, each well was washed three times to remove non-adherent cells. Biofilms were collected using a sterilized swab and cells were serially diluted using sterile PBS. All dilutions were plated on VRBA (DIFCO, Maryland, USA) for *E. coli* and BHI (DIFCO, Maryland, USA) containing 1.5% LiCl for *L. innocua.* Plates were incubated at 37 °C for 48 h and then colony were counted. The level of inhibition was determined by comparing the values of the co-inoculated cultures with those containing only *E. coli* or *L. innocua*. The experiment was repeated three times.

2.10. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA).

3. Result and discussion

3.1. Molecular identification of Lactobacillus isolates

Thirty-five isolates belonging to this genus, previously isolated from infant stools, were identified at species level by 16 rDNA sequencing and RAPD analysis (Guerra et al., 2018) and grouped into 9 cluster according to a RAPD similarity profile percentage of more than 80%. A molecular method to discriminate species belonging to the *L. casei* group, namely *L. casei, L. paracasei* and *L. rhamnosus*, was recently proposed based on a multiplex PCR assay targeting the *mutL* gene (Bottari et al., 2017). Its application on one strains chosen from each branch of the above mentioned cluster allowed to identify six *L. paracasei* (DTA72, DTA76, DTA81, DTA83, DTA93 and DTA96) and two *L. rhamnosus* (DTA79 and DTA105) (Fig. S1). According to this approach, three strains, namely DTA72 DTA76 and DTA105 were reclassified with respect to their initial attribution. Strain DTA106, that did not give amplification bands, was excluded from this study since it does not belong to the *Lactobacillus* "*casei*" group.

3.2. Hemolytic activity test

According to the European Food Safety Authority (EFSA), the assessment of hemolytic activity is strongly recommended for bacteria intended for food use, even if they are known to be safe or possessing the GRAS or QPS status (WHO/FAO, 2002). In this study, all strains were ɣ-hemolytic, i.e. negative, and none of them showed β-hemolytic activity when grown on MRS agar containing 5% (w/v) sheep blood. *Staphylococcus aureus* ATCC 6538, inserted as positive control, showed β-hemolytic activity.

3.3. Determination of minimum inhibitory concentration (MIC)

To be considered for food use, bacteria should not contain antibiotic resistance genes that could be horizontally transferred to other bacteria, particularly to human pathogens (Ashraf & Shah, 2011). For this reason, the antimicrobial susceptibility tests have become one of the most important assessments needed to evaluate potential probiotic bacteria (Tarrah, Treu, et al., 2018). Although acquired antibiotic resistance is an undesirable characteristic, intrinsic (i.e. non transmissible) resistance could contrary be considered favorable, due to the capability of the probiotic bacteria of withstanding antibiotic treatments on their human host (Charteris, Kelly, Morelli, & Collins, 1998). The results of the Minimum Inhibitory Concentration test on the *Lactobacillus* strains are reported in Table 1. All strains were resistant to kanamycin (K) and vancomycin (VA), while all showed susceptibility to penicillin G ampicillin (AMP), erythromycin (E), tetracycline (TE), ciprofloxacin (CIP) and gentamycin (CN), according to the cut-off values reported by EFSA (EFSA, 2008). Several lactobacilli often display a range of antibiotic resistance but in most cases, this is not transmissible and therefore does not usually represent a safety concern (Saarela, Lähteenmäki, Crittenden, Salminen, & Mattila-Sandholm, 2002). Several species of *Lactobacillus* including *L. paracasei* and *L. rhamnosus* are intrinsically resistant to vancomycin and kanamycin (Blandino, Milazzo, & Fazio, 2008). Resistance towards kanamycin and streptomycin in *L. delbrueckii* is conferred by the occurrence of the *aph*(3′)-IIIa and *ant*(6) genes (Devirgiliis, Zinno, & Perozzi, 2013). In addition, resistance against inhibitors of nucleic acid synthesis, such as trimethoprim was reported to be intrinsic (Ammor, Belén Flórez, & Mayo, 2007). On the other hand, lactobacilli are usually sensitive to inhibitors of protein synthesis such as erythromycin, and tetracycline (Ammor et al., 2007; Coppola et al., 2005; Danielsen & Wind, 2003; Halami, Chandrashekar, & Nand, 2000). According to our Minimum Inhibitory Concentration test results, the resistances found could therefore be considered as natural (intrinsic resistance) and hence do not pose a safety issue on the use of these strains.

Table1
Minimum Inhibitory Concentration (MIC) of 8 antibiotics on *Lactobacillus* strains (μg/mL).

Antibiotic	Strain								
	DTA72	DTA76	DTA79	DTA81	DTA83	DTA93	DTA96	DTA105	GG
Ampicillin	4 (4)	2 (4)	4 (4)	4 (4)	4 (4)	2 (4)	2 (4)	4 (4)	4 (4)
Ciprofloxacin	4 (4)[a]	2 (4)[a]	2 (4)[a]	4 (4)[a]	2 (4)[a]	4 (4)[a]	4 (4)[a]	2 (4)[a]	2 (4)[a]
Erythromycin	0.125 (1)	0.0625 (1)	0.125 (1)	0.125 (1)	0.0625 (1)	0.0625 (1)	0.125 (1)	0.125 (1)	0.125 (1)
Gentamycin	8 (32)	8 (32)	8 (16)	8 (32)	8 (32)	8 (32)	8 (32)	8 (16)	8 (16)
Kanamycin	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*
Penicillin G	1 (4)[a]	0.5 (4)[a]	1 (4)[a]	0.5 (4)[a]	1 (4)[a]	1 (4)[a]	1 (4)[a]	0.5 (4)[a]	0.5 (4)[a]
Tetracycline	1 (4)	1 (4)	1 (8)	1 (4)	1 (4)	1 (4)	1 (4)	1 (8)	1 (8)
Vancomycin	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*

EFSA suggested breakpoints (μg/mL) for each LAB strain are reported in parentheses. Strains with MICs higher than breakpoints are considered resistant and indicated in italics.

[a] According to (Danielsen & Wind, 2003).

3.4. Resistance to simulated gastrointestinal conditions

Resistance to the gastrointestinal juices during passage through stomach and intestine is the key factor for the probiotic strains to reach the gut and benefit the host (Bezkorovainy, 2001). Several studies have assessed the resistance of different species of *Lactobacillus* to the gastrointestinal conditions (Charteris et al., 1998; Fernández, Boris, & Barbes, 2003). The pH in the human stomach can vary from 1.3 to 2.5 during fasting and can increase up to 4.5 soon after a meal (Kong & Singh, 2008). Since the survival of lactobacilli at pH 4.2–4.4, which represents the common value of many fermented dairy products, is well known, pH 2 was selected to evaluate their ability to withstand gastric juice. Several studies reported survival of different *Lactobacillus* strains at that pH (Argyri et al., 2013). In our study, this evaluation was performed in two successive steps. First, the strains were incubated in artificial gastric juice for 60 min and then immediately transferred into the intestinal juice for 180 min (short incubation) followed by further 120 min (prolonged incubation, total 300 min). Cell concentration of bacterial cultures ranged from 8.7 to 9.8 log CFU/mL. All strains exhibited a very good resistance to gastric juice (Fig. 1), as viability decrement was always below 1 log and strains DTA72 and DTA83 showed the lowest viability loss together with the commercial strain GG. Our results on viability of *Lactobacillus* strains in the presence of mucin and pepsin at pH 2 are comparable with data available in the literature (Charteris et al., 1998; Fernández et al., 2003). Besides, considering that most probiotics are used in milk-based products, it is worth mentioning that some studies evidenced how milk proteins can play a significant role in protecting these bacteria (Charteris et al., 1998; Conway, Gorbach, & Goldin, 1987; Fernández et al., 2003).

Regarding incubation in gastro-intestinal juice, after 180 min all strains evidenced a statistically significant reduction, with the sole exception of DTA79, which was interestingly did not show any significant decrease after 1 h of gastric incubation followed by 3 h in gastrointestinal juice.

After prolonged incubation (300 min) in intestinal juice, strains showed a further significant decrease in viability, with the exception of strains DTA96 and DTA 105 that maintained the same level of viability shown after the short incubation.

Although tolerance to intestinal juice is considered to be strain dependent, it has been reported that lactobacilli can progressively adapt to the presence of bile salts and other components of the intestinal juice (Burns et al., 2010; Noriega, Gueimonde, Sánchez, Margolles, & de los Reyes-Gavilán, 2004) due to global cellular responses such as preservation of internal pH, cell membrane integrity/functionality and activation of bile salt efflux pumps (Bustos, Raya, de Valdez, & Taranto, 2011; Wu, He, & Zhang, 2014; Wu, Zhang, Wang, Du, & Chen, 2012). Interestingly, our results showed that all strains had a stronger overall resistance with respect to the commercial strains. Indeed, considering that all cultures started from a cell concentration roughly around 10^9 cfu/mL, all newly isolated strains evidenced a decrease within 2 logs while total *L. rhamnosus* GG decrease was more than 3 logs. Although this strain has great resistance to the gastric juice, it appears to be much more sensitive to intestinal incubation with respect to the lactobacilli tested.

3.5. Bile salts hydrolytic activity

None of the tested *Lactobacillus* strains showed capability to hydrolyze bile salts when grown on MRS agar medium containing 0.5% taurodeoxycholic acid. The meaning of BSH activity in probiotic bacteria has been questioned during last years. Although BSH is somehow connected to the intestinal survival of bacteria and reduction of cholesterol in humans, there are several undesirable effects from de-conjugated bile salts that can induce serious problems to the human body, such as DNA damage, promote colon cancer, diarrhea or inflammation (Berr, Kullak-Ublick, Paumgartner, Munzing, & Hylemon, 1996; Mamianetti, Garrido, Carducci, & Cristina Vescina, 1999). Therefore, we do not consider this capability as a desirable feature for probiotic strains.

3.6. Adhesion to HT-29 cells

The ability to adhere to intestinal cells is another important property for probiotic bacteria to stably colonize the host gut. Results of adhesion test are reported in Table 2 and Fig. 2 shows some images of *Lactobacillus* strains adhesion to HT-29 colorectal cancer cells. Strains DTA93, DTA81 and DTA79 strongly adhered to HT-29 cells whereas strains DTA96 and DTA76 showed normal adhesive characteristic and strains DTA72, DTA83 and DTA105 were non-adhesive. The *in-vitro* adherence ability of probiotic bacteria to HT-29 cells line has been extensively used during the last years (Ahmad, Yap, Kofli, & Ghazali, 2018; Bernet, Brassart, Neeser, & Servin, 1994; Wang et al., 2008). Indeed, there are two different ways by which bacteria can interact with cell surfaces, i.e. specific or non-specific. The latter is a consequence of the physicochemical properties of the cell wall, especially its outer constituents (Schaer-Zammaretti & Ubbink, 2003) and depends on the hydrophobic properties of the surfaces and on the balance of electrostatic interactions (Boonaert & Rouxhet, 2000). Differently, specific adhesion is related to the recognition of a specific site or ligand by a receptor on the bacterial surface (Schaer-Zammaretti & Ubbink, 2003). Many lactobacilli exhibit specific interactions, and the colonization ability of epithelial cells of Lactobacillus strains has been reported (Bouzaine, Dauphin, Thonart, Urdaci, & Hamdi, 2005; Jin, Ho, Abdullah, Ali, & Jalaludin, 1996). In our study, three out of eight strains tested, namely DTA93, DTA81 and DTA79, exhibited strong adherence to HT-29 cells line and particularly *L. paracasei* DTA81 revealed a dramatically strong adherence ability, about ten times higher than that of the commercial strain *L. rhamnosus* GG, thus indicating it as a very

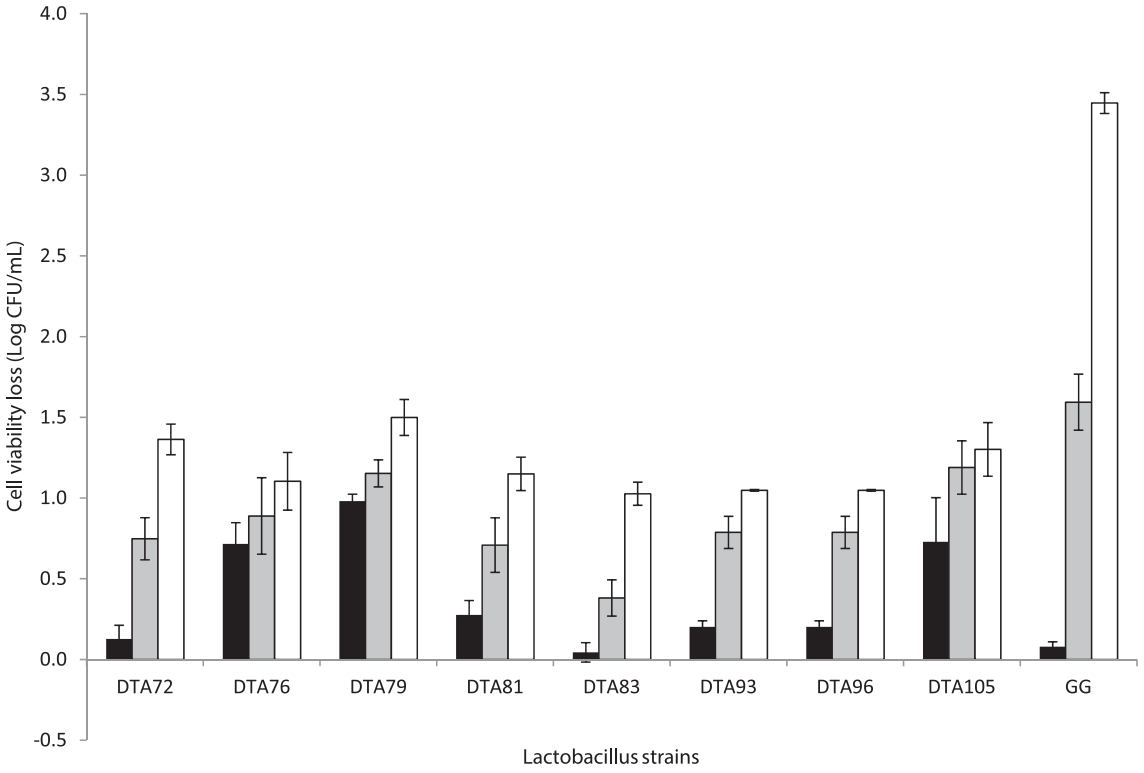

Fig. 1. Cell viability loss of *Lactobacillus* cultures upon exposure to simulated gastrointestinal conditions. Black bars: after 1 h incubation (gastric test); grey bars: after 3 h incubation (short gastrointestinal test); white bars: after 5 h incubation (prolonged gastrointestinal test). Results are expressed as means ± SD (n = 3) of viable cells.

Table 2
Adhesion potential of bacterial strains. Scores are the average number of adhering cells in 20 microscopic fields ± SD (n = 3).

Strain	Adhesion score	Category
L. paracasei DTA72	25.5 ± 2.3	Non-adhesive
L. paracasei DTA76	46.4 ± 4.02	Adhesive
L. rhamnosus DTA79	359.1 ± 7.2	Strongly adhesive
L. paracasei DTA81	4044.0 ± 10.2	Extremely adhesive
L. paracasei DTA83	20.6 ± 1.9	Non-adhesive
L. paracasei DTA93	294.5 ± 5.2	Strongly adhesive
L. paracasei DTA96	41.1 ± 2.7	Adhesive
L. rhamnosus DTA105	28.6 ± 3.5	Non-adhesive
L. rhamnosus GG	420.8 ± 8.1	Strongly adhesive

interesting probiotic candidate.

3.7. Anti-proliferative activity against HT-29 cells

Colorectal cancer is a disease mostly determined by Caco-2 and HT-29 cells that can cause death widely in the world. Strains *L. paracasei* DTA93, DTA96, DTA81 and *L. rhamnosus* DTA79 which had showed good adhesive activity to HT-29 cells and indicated good probiotic potential were tested for anti-proliferative activity. Results (Table 3) indicate that HT-29 cancer cells were significantly inhibited by some lactobacilli supernatants. No significant difference ($P < 0.05$) was found between *L. paracasei* DTA93 and the commercial strain *L. rhamnosus* GG when examined with multiple comparison tests (Tukey's test). The remaining strains, apart from *L. paracasei* DTA96, showed anti-

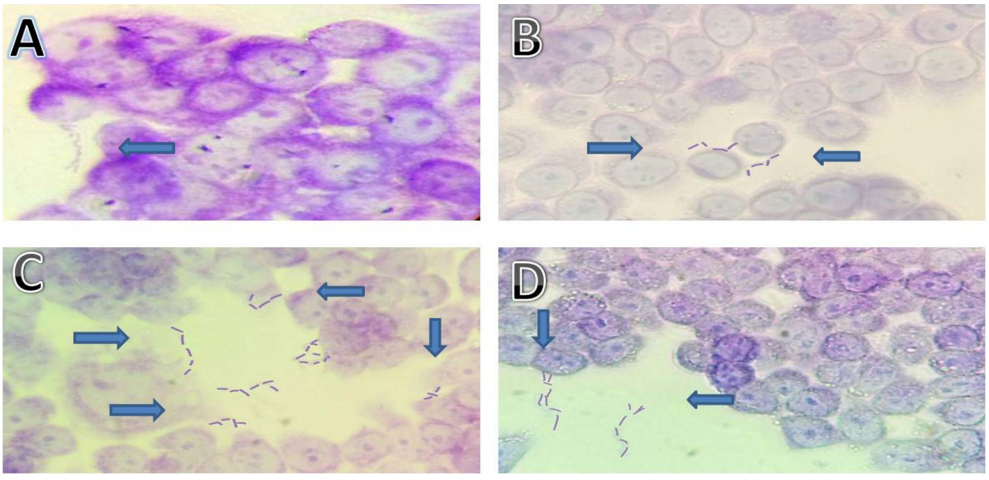

Fig. 2. Adhesion of *Lactobacillus* strains to HT-29 cells observed under optical microscope (1000X). Strains: (A) GG, (B) DTA79, (C) DTA81, (D) DTA93.

Table 3
Antiproliferative effect of lyophilized supernatants collected from lactobacilli cultures on HT-29 cancer cells after 48 h.

Supernatant concentration (μg/mL)	HT-29 cells viability (%)					
	DTA79	DTA81	DTA93	DTA96	GG	MRS medium
125	97.48 ± 0.28	97.81 ± 0.03	70.20 ± 0.01	98.2 ± 0.05	65.8 ± 0.01	90.27 ± 0.10
250	74.94 ± 0.12	97.51 ± 0.05	63.00 ± 0.03	86.9 ± 0.06	63.9 ± 0.09	91.67 ± 0.03
500	65.56 ± 0.07	93.10 ± 0.11	59.98 ± 0.07	88.3 ± 0.11	58.9 ± 0.02	90.03 ± 0.05
1000	57.33 ± 0.05	53.27 ± 0.07	47.42 ± 0.01	84.7 ± 0.06	54.1 ± 0.02	89.47 ± 0.28
2000	46.64 ± 0.03	39.42 ± 0.05	44.79 ± 0.10	89.2 ± 0.05	48.2 ± 0.02	88.61 ± 0.19
4000	37.78 ± 0.00	34.09 ± 0.03	40.48 ± 0.01	90.5 ± 0.05	41.1 ± 0.03	82.63 ± 0.20
8000	31.27 ± 0.07	21.48 ± 0.02	28.56 ± 0.02	88.8 ± 0.24	29.1 ± 0.02	89.44 ± 0.06

Table 4
IC_{50} of probiotic strains against HT-29 cell line (All values are mean ± SD of 2 experiments).

Strains	IC_{50} (mg/mL)[a]
L. rhamnosus DTA79	1.96 ± 0.20
L. paracasei DTA81	1.40 ± 0.25
L. paracasei DTA93	1.30 ± 0.13
L. rhamnosus GG	1.42 ± 0.12

[a] IC_{50}: half minimal inhibitory concentration.

proliferative activity. However, the lowest cell viability was observed in HT-29 cells treated with *L. paracasei* DTA81 supernatant, i.e. 39.4% ± 0.05, 34.1% ± 0.03, 21.5% ± 0.02 at concentrations of 2000, 4000, 8000 μg/mL respectively (Table 3). By considering the half minimal inhibitory concentration (IC_{50}) value related to the strain supernatants (Table 4) it appears that there is no significant difference between *L. paracasei* DTA93, *L. paracasei* DTA81 and the commercial strain *L. rhamnosus* GG. Many lactobacilli, such as *L. acidophilus* and *L. casei* have been reported to possess anti-cancer effects by suppressing the duplication of tumor cells (Lee et al., 2004). Regarding the possible mechanisms of action, in a recent study (Haghshenas et al., 2014) demonstrated that metabolites such as bioactive peptides in the supernatant of several species of lactobacilli could behave as anti-cancer agents, as they can play a crucial role in cytotoxicity by linking to pre-carcinogenic molecules, carcinogenic enzymes or mutagenic compounds or by exerting some immunomodulatory effects (Bermudez-Brito et al., 2012). Moreover, some *L. paracasei* and *L. casei* strains showed effective anticancer activity against cervix cancer (HeLa) cells by upregulating the expression of apoptotic genes *BAX, BAD, caspase3, caspase8*, and *caspase9* and by downregulating the expression of the *BCl-2* gene (Shahid et al., 2018), while other *L. casei* induced up-regulation of TRAIL protein expression (Tiptiri-Kourpeti et al., 2016), known to selectively induce apoptosis in many tumor cell lines without affecting normal cells and tissues, thus appearing as a promising therapeutic drug (Galligan et al., 2005). In another study (Ewaschuk, Walker, Diaz, & Madsen, 2006), the production of Conjugated Linoleic Acid (CLA) as anti-carcinogenic component by different species lactobacilli on HT-29 cell line was demonstrated. Anti-carcinogenic effects of CLA have been also proved by *in-vivo* studies on animals (Liew, Schut, Chin, Pariza, & Dashwood, 1995; Petrik, McEntee, Johnson, Obukowicz, & Whelan, 2000). According to our result, it can be concluded that *L. paracasei* DTA93 and DTA81 which had shown exceptionally good adhesion ability, can be considered as effective strains in anti-proliferative activity against HT-29 cell. Further studies will be needed to understand the mechanics through which the strains exert their activity.

3.8. Inhibition of biofilm formation

The inhibitory activity of the newly isolated *Lactobacillus* strains against biofilm formation by *L. innocua* and *E. coli* was tested by two different approaches. The first strain was chosen because it is physiologically very close to the pathogen *L. monocytogenes*, whose strong ability to form biofilm is well documented and represents a serious problem for surfaces and industrial settings (Klančnik, Toplak, Kovač, Marquis, & Jeršek, 2015). *E. coli* is the most present bacterium in the small intestine and therefore the possibility to displace it represents for a strain a good potentiality to establish in the gut. Fig. 3 shows the results of the biofilm inhibitory activity obtained by inoculating simultaneously one *Lactobacillus* strain together with either *E. coli* (Fig. 3A) or *L. innocua* (Fig. 3B) (competition test) and by inoculating a *Lactobacillus* strain first and subsequently either *E. coli* (Fig. 3A) or *L. innocua* (Fig. 3B) (exclusion test). In the competition assay, all lactobacilli were able to reduce the number of attached *E. coli* and *L. innocua* cells to different extent, but the co-culture with *L. paracasei* DTA81 and DTA93 showed the highest inhibitory effects on both *E. coli* (cells reduction of 0.78 and 0.65 log, respectively) and *L. innocua* (cells reduction of 0.29 and 0.42 log, respectively), comparable or better to the commercial strain *L. rhamnosus* GG (0.59 log on *E. coli* and 0.040 log in *L. innocua*). Similar outcomes were evidenced by the exclusion test where strains DTA81 and DTA93 still evidenced the best inhibition ability (cells reduction of 1.05 and 0.80 log, respectively on *E. coli* and 0.58 and 0.60 log, respectively on *L. innocua*) that resulted higher than that of strain GG on *E. coli* (0.73 log) and slightly lower on *L. innocua* (0.85). Probably, the inhibitory effect of DTA81 and DTA93 can be linked to their strong attachment capability shown in the HT-29 adhesion test. For all lactobacilli, the exclusion effect was always equal or stronger to the respective competition one, with the sole exception of *L. paracasei* DTA83 that clearly inhibited *E. coli* during the competition test (0.52 log decrease) but produced a negligible exclusion effect (0.03 log). A similar behavior was evidenced by DTA83 on another *E. coli* strain, namely ATCC25922 (Guerra et al., 2018), thus allowing to hypothesize that this inhibitory activity could be related to some strain specific antimicrobial effect, such as production of a bacteriocin, rather than to biofilm activity. This idea is also reinforced by the fact that the same effect was not obtained on *L. innocua* and that *L. paracasei* DTA83 showed the worst performance to the adhesion to HT-29 cells test.

4. Conclusions

The results of the present study indicate that the two newly isolated strains *L. paracasei* DTA81 and DTA93 were found to possess *in-vitro* probiotic properties and anti-cancer activity. Some traits resulted very close to and in some cases superior to those of the widespread commercial probiotic strains *L. rhamnosus* GG that we used as reference. Strain *L. paracasei* DTA81 showed an amazingly high adherence ability to HT-29 cells line which resulted about ten times stronger than that of the commercial strain *L. rhamnosus* GG and, to our knowledge, represents the highest level reported to date for this type of cells. Besides, both *L. paracasei* DTA81 and *L. paracasei* DTA93 were able to effectively inhibit biofilm formation by other bacteria. Therefore, further investigation, including *in-vivo* studies, is strongly advisable to evaluate the potential health benefits in the real host. Overall, our study

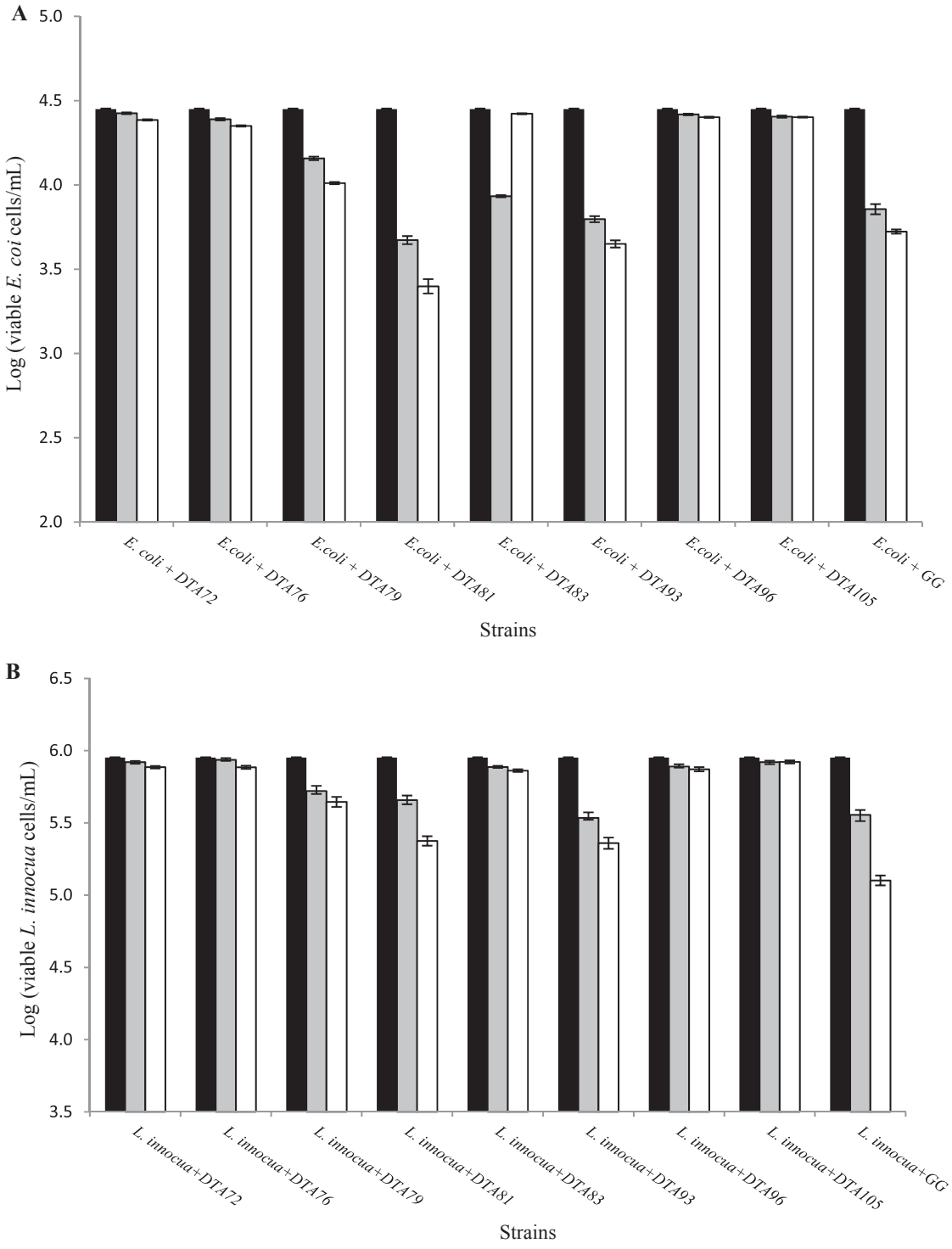

Fig. 3. Biofilm inhibitory activity of *Lactobacillus* strains against *E. coli* (A) and *L. innocua* (B) in competition and exclusion tests. Results are express as mean ± SD (n = 3) of *E. coli* and *L. innocua* viable cells. Black bars: *E. coli* and *L. innocua* population alone; grey bars: *E. coli* and *L. innocua* after competition test; white bars: *E. coli* and *L. innocua* after exclusion test.

confirms what is demonstrated in many other studies regarding the potential of the genus *Lactobacillus* as a very interesting source for the discovery of new health beneficial microbes.

Ethics statement

Our research did not include any human subjects and animal experiments.

Conflict of interest statement

All authors declare there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.02.004.

References

Ahmad, A., Yap, W. B., Kofli, N. T., & Ghazali, A. R. (2018). Probiotic potentials of Lactobacillus plantarum isolated from fermented durian (Tempoyak), a Malaysian traditional condiment. *Food Science & Nutrition, 6*(6), 1370–1377. https://doi.org/10.1002/fsn3.672.

Ammor, M. S., Belén Flórez, A., & Mayo, B. (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology, 24*(6), 559–570. https://doi.org/10.1016/j.fm.2006.11.001.

Argyri, A. A., Zoumpopoulou, G., Karatzas, K.-A. G., Tsakalidou, E., Nychas, G.-J. E., Panagou, E. Z., & Tassou, C. C. (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiology, 33*(2), 282–291.

Ashraf, R., & Shah, N. P. (2011). Antibiotic resistance of probiotic organisms and safety of probiotic dairy products. *International Food Research Journal, 18*(3).

Bermudez-Brito, M., Muñoz-Quezada, S., Gomez-Llorente, C., Matencio, E., Bernal, M. J., Romero, F., & Gil, A. (2012). Human intestinal dendritic cells decrease cytokine release against salmonella infection in the presence of Lactobacillus Paracasei upon TLR activation. *PLoS One, 7*(8), e43197. https://doi.org/10.1371/journal.pone.0043197.

Bernet, M.-F., Brassart, D., Neeser, J.-R., & Servin, A. L. (1994). Lactobacillus acidophilus LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut, 35*(4), 483–489.

Berr, F., Kullak-Ublick, G., Paumgartner, G., Munzing, W., & Hylemon, P. B. (1996). 7 alpha-dehydroxylating bacteria enhance deoxycholic acid input and cholesterol saturation of bile in patients with gallstones. *Gastroenterology, 111*(6), 1611–1620.

Bezkorovainy, A. (2001). Probiotics: Determinants of survival and growth in the gut. *The American Journal of Clinical Nutrition, 73*(2), 399s–405s.

Blandino, G., Milazzo, I., & Fazio, D. (2008). Antibiotic susceptibility of bacterial isolates from probiotic products available in Italy. *Microbial Ecology in Health and Disease, 20*(4), 199–203.

Boonaert, C. J. P., & Rouxhet, P. G. (2000). Surface of lactic acid bacteria: Relationships between chemical composition and physicochemical properties. *Applied and Environmental Microbiology, 66*(6), 2548–2554.

Bottari, B., Felis, G. E., Salvetti, E., Castioni, A., Campedelli, I., Torriani, S., ... Gatti, M. (2017). Effective identification of *Lactobacillus casei* group species: Genome-based selection of the gene mutL as the target of a novel multiplex PCR assay. *Microbiology (United Kingdom)*. https://doi.org/10.1099/mic.0.000497.

Bouzaine, T., Dauphin, R. D., Thonart, P., Urdaci, M. C., & Hamdi, M. (2005). Adherence and colonization properties of Lactobacillus rhamnosus TB1, a broiler chicken isolate. *Letters in Applied Microbiology, 40*(5), 391–396.

Bruno-Bárcena, J. M., Andrus, J. M., Libby, S. L., Klaenhammer, T. R., & Hassan, H. M. (2004). Expression of a heterologous manganese superoxide dismutase gene in intestinal lactobacilli provides protection against hydrogen peroxide toxicity. *Applied and Environmental Microbiology, 70*(8), 4702–4710.

Burns, P., Sánchez, B., Vinderola, G., Ruas-Madiedo, P., Ruiz, L., Margolles, A., ... Clara, G. (2010). Inside the adaptation process of Lactobacillus delbrueckii subsp. lactis to bile. *International Journal of Food Microbiology, 142*(1–2), 132–141.

Bustos, A. Y., Raya, R., de Valdez, G. F., & Taranto, M. P. (2011). Efflux of bile acids in Lactobacillus reuteri is mediated by ATP. *Biotechnology Letters*. https://doi.org/10.1007/s10529-011-0696-3.

Campanaro, S., Treu, L., Vendramin, V., Bovo, B., Giacomini, A., & Corich, V. (2014). Metagenomic analysis of the microbial community in fermented grape marc reveals that Lactobacillus fabifermentans is one of the dominant species: Insights into its genome structure. *Applied Microbiology and Biotechnology, 98*(13), 6015–6037. https://doi.org/10.1007/s00253-014-5795-3.

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Antibiotic susceptibility of potentially probiotic Lactobacillus species. *Journal of Food Protection, 61*(12), 1636–1643.

Conway, P. L., Gorbach, S. L., & Goldin, B. R. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *Journal of Dairy Science, 70*(1), 1–12.

Coppola, R., Succi, M., Tremonte, P., Reale, A., Salzano, G., & Sorrentino, E. (2005). Antibiotic susceptibility of Lactobacillus rhamnosus strains isolated from Parmigiano Reggiano cheese. *Le Lait, 85*(3), 193–204.

Danielsen, M., & Wind, A. (2003). Susceptibility of Lactobacillus spp. to antimicrobial agents. *International Journal of Food Microbiology, 82*(1), 1–11.

De Vrese, M., & Schrezenmeir, J. (2008). Probiotics, prebiotics, and synbiotics. *Food biotechnology* (pp. 1–66). Springer.

Devirgiliis, C., Zinno, P., & Perozzi, G. (2013). Update on antibiotic resistance in food-borne Lactobacillus and Lactococcus species. *Frontiers in Microbiology, 4*, 301.

EFSA (European Food Safety Authority) (2008). Technical Guidance for assessing the safety of feed additives for the environment. *EFSA Journal, 6*(10), 842.

Ewaschuk, J. B., Walker, J. W., Diaz, H., & Madsen, K. L. (2006). Bioproduction of conjugated linoleic acid by probiotic bacteria occurs in vitro and in vivo in mice. *The Journal of Nutrition, 136*(6), 1483–1487.

FAO/WHO (2002). Joint Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food London, Ontario, Canada, April 30 and May 1, 2002.

Fernández, M. F., Boris, S., & Barbes, C. (2003). Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. *Journal of Applied Microbiology, 94*(3), 449–455.

Foschi, C., Laghi, L., Parolin, C., Giordani, B., Compri, M., Cevenini, R., ... Vitali, B. (2017). Novel approaches for the taxonomic and metabolic characterization of lactobacilli: Integration of 16S rRNA gene sequencing with MALDI-TOF MS and 1H-NMR. *PLoS One*. https://doi.org/10.1371/journal.pone.0172483.

Galligan, L., Longley, D. B., McEwan, M., Wilson, T. R., McLaughlin, K., & Johnston, P. G. (2005). Chemotherapy and TRAIL-mediated colon cancer cell death: The roles of p53, TRAIL receptors, and c-FLIP. *Molecular Cancer Therapeutics, 4*(12), 2026–2036. https://doi.org/10.1158/1535-7163.MCT-05-0262.

Goldstein, E. J. C., Tyrrell, K. L., & Citron, D. M. (2015). Lactobacillus species: Taxonomic complexity and controversial susceptibilities. *Clinical Infectious Diseases, 60*(suppl_2), S98–S107. https://doi.org/10.1093/cid/civ072.

Guerra, A. F., Lemos Junior, W. J. F., Santos, G. O. dos, Andrighetto, C., Gianomini, A., Corich, V., ... Luchese, R. H. (2018). Lactobacillus paracasei probiotic properties and survivability under stress-induced by processing and storage of ice cream bar or ice-lolly. *Ciência Rural, 48*(9), https://doi.org/10.1590/0103-8478cr20170601.

Haghshenas, B., Abdullah, N., Nami, Y., Radiah, D., Rosli, R., & Khosroushahi, A. Y. (2014). Different effects of two newly-isolated probiotic Lactobacillus plantarum 15HN and Lactococcus lactis subsp. Lactis 44Lac strains from traditional dairy products on cancer cell lines. *Anaerobe, 30*, 51–59.

Halami, P. M., Chandrashekar, A., & Nand, K. (2000). Lactobacillus farciminis MD, a newer strain with potential for bacteriocin and antibiotic assay. *Letters in Applied Microbiology, 30*(3), 197–202.

Hartemink, R., Domenech, V., & Rombouts, F. (1997). LAMVAB—A new selective medium for the isolation of lactobacilli from faeces. *Journal of Microbiological Methods*. https://doi.org/10.1016/S0167-7012(97)00025-0.

Jacobsen, C. N., Nielsen, V. R., Hayford, A. E., Møller, P. L., Michaelsen, K. F., Paerregaard, A., ... Jakobsen, M. (1999). Screening of probiotic activities of forty-seven strains of Lactobacillus spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology, 65*(11), 4949–4956.

Jeronymo-Ceneviva, A. B., de Paula, A. T., Silva, L. F., Todorov, S. D., Franco, B. D. G. M., & Penna, A. L. B. (2014). Probiotic properties of lactic acid bacteria isolated from water-buffalo mozzarella cheese. *Probiotics and Antimicrobial Proteins, 6*(3–4), 141–156.

Jin, L. Z., Ho, Y. W., Abdullah, N., Ali, M. A., & Jalaludin, S. (1996). Antagonistic effects of intestinal Lactobacillus isolates on pathogens of chicken. *Letters in Applied Microbiology, 23*(2), 67–71.

Kim, H.-J., Lee, H. J., Lim, B., Kim, E., Kim, H.-Y., Suh, M., & Hur, M. (2018). Lactobacillus terrae sp. nov., a novel species isolated from soil samples in the Republic of Korea. *International Journal of Systematic and Evolutionary Microbiology, 68*(9), 2906–2911. https://doi.org/10.1099/ijsem.0.002918.

Klaenhammer, T. R., & de Vos, W. M. (2011). An incredible scientific journey. The evolutionary tale of the lactic acid bacteria. In The 10th LAB symposium. Thirty years of research on lactic acid bacteria (Vol. 24, pp. 1–11). 24 Media Labs.

Klančnik, A., Toplak, N., Kovač, M., Marquis, H., & Jeršek, B. (2015). Quantification of Listeria monocytogenes cells with digital PCR and their biofilm cells with real-time PCR. *Journal of Microbiological Methods, 118*, 37–41. https://doi.org/10.1016/j.mimet.2015.08.012.

Kong, F., & Singh, R. P. (2008). Disintegration of solid foods in human stomach. *Journal of Food Science, 73*(5), R67–R80.

Kubota, Y. (1990). Fecal intestinal flora in patients with colon adenoma and colon cancer. *Nihon Shokakibyo Gakkai Zasshi = The Japanese Journal of Gastro-Enterology, 87*(3), 771–779.

Kumar, R., & Dhanda, S. (2017). Mechanistic insight of probiotics derived anticancer pharmaceuticals: A road forward for cancer therapeutics. *Nutrition and Cancer, 69*(3), 375–380. https://doi.org/10.1080/01635581.2017.1267773.

Lee, J. W., Kim, E. H., Yim, I. B., & Joo, H. G. (2004). Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of *Lactobacillus casei* and Bifidobacterium longum. *Journal of Veterinary Science, 5*(1), 41–48.

Liew, C., Schut, H. A. J., Chin, S. F., Pariza, M. W., & Dashwood, R. H. (1995). Protection of conjugated linoleic acids against 2-amino-3-methylimidazo [4, 5-f] quinoline-induced colon carcinogenesis in the F344 rat: A study of inhibitory mechanisms. *Carcinogenesis, 16*(12), 3037–3043.

Mamianetti, A., Garrido, D., Carducci, C. N., & Cristina Vescina, M. (1999). Fecal bile acid excretion profile in gallstone patients. *Medicina-Buenos Aires-, 59*, 269–273.

Mattila-Sandholm, T., Myllärinen, P., Crittenden, R., Mogensen, G., Fondén, R., & Saarela, M. (2002). Technological challenges for future probiotic foods. *International Dairy Journal, 12*(2–3), 173–182.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*(1–2), 55–63.

Motevaseli, E., Dianatpour, A., & Ghafouri-Fard, S. (2017). The role of probiotics in cancer treatment: Emphasis on their in vivo and in vitro anti-metastatic effects. *International Journal of Molecular and Cellular Medicine, 6*(2), 66–76. https://doi.org/10.22088/acadpub.BUMS.6.2.1.

Noriega, L., Gueimonde, M., Sánchez, B., Margolles, A., & de los Reyes-Gavilán, C. G. (2004). Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross-resistance to bile salts in Bifidobacterium. *International Journal of Food Microbiology, 94*(1), 79–86.

Ouwehand, A. C., Salminen, S., & Isolauri, E. (2002). Probiotics: An overview of beneficial effects. *Antonie van Leeuwenhoek, 82*(1–4), 279–289.

Petrik, M. B. H., McEntee, M. F., Johnson, B. T., Obukowicz, M. G., & Whelan, J. (2000). Highly unsaturated (n-3) fatty acids, but not α-linolenic, conjugated linoleic or γ-linolenic acids, reduce tumorigenesis in ApcMin/+ mice. *The Journal of Nutrition, 130*(10), 2434–2443.

Pieniz, S., Andreazza, R., Anghinoni, T., Camargo, F., & Brandelli, A. (2014). Probiotic

potential, antimicrobial and antioxidant activities of Enterococcus durans strain LAB18s. *Food Control, 37*, 251–256.

Pogačić, T., Samaržija, D., Corich, V., D'Andrea, M., Kagkli, D.-M., Giacomini, A., ... Rogelj, I. (2010). Microbiota of Karakačanski skakutanac, an artisanal fresh sheep cheese studied by culture-independent PCR-ARDRA and PCR-DGGE. *Dairy Science & Technology, 90*(4), 461–468. https://doi.org/10.1051/dst/2010004.

Saarela, M., Lähteenmäki, L., Crittenden, R., Salminen, S., & Mattila-Sandholm, T. (2002). Gut bacteria and health foods—The European perspective. *International Journal of Food Microbiology, 78*(1–2), 99–117.

Schaer-Zammaretti, P., & Ubbink, J. (2003). Imaging of lactic acid bacteria with AFM—Elasticity and adhesion maps and their relationship to biological and structural data. *Ultramicroscopy, 97*(1–4), 199–208.

Shahid, M., Rajoka, R., Zhao, H., Lu, Y., Lian, Z., Li, N., ... Shi, J. (2018). Anticancer potential against cervix cancer (HeLa) cell line of probiotic *Lactobacillus casei* and Lactobacillus paracasei strains isolated from human breast milk. *Food & Function*. https://doi.org/10.1039/c8fo00547h.

Singh, N., Singh, J., & Singh, K. (2018). Small at size, big at impact: Microorganisms for sustainable development. In J. Singh, D. Sharma, G. Kumar, & N. R. Sharma (Eds.), Microbial bioprospecting for sustainable development (pp. 3–28). Singapore: Springer Singapore. https://doi.org/10.1007/978-981-13-0053-0_1.

Takagi, A., Ikemura, H., Matsuzaki, T., Sato, M., Nomoto, K., Morotomi, M., & Yokokura, T. (2008). Relationship between the in vitro response of dendritic cells to Lactobacillus and prevention of tumorigenesis in the mouse. *Journal of Gastroenterology, 43*(9), 661–669.

Tarrah, A., de Castilhos, J., Rossi, R. C., Duarte, V.da. S., Ziegler, D. R., Corich, V., & Giacomini, A. (2018). In vitro probiotic potential and anti-cancer activity of newly isolated folate-producing streptococcus thermophilus strains. *Frontiers in Microbiology, 9*(September), 1–11. https://doi.org/10.3389/fmicb.2018.02214.

Tarrah, A., Treu, L., Giaretta, S., Duarte, V., Corich, V., & Giacomini, A. (2018). Differences in carbohydrates utilization and antibiotic resistance between streptococcus macedonicus and streptococcus thermophilus strains isolated from dairy products in Italy. *Current Microbiology, 1–11*. https://doi.org/10.1007/s00284-018-1528-7.

Tiptiri-Kourpeti, A., Spyridopoulou, K., Santarmaki, V., Aindelis, G., Tompoulidou, E., Lamprianidou, E. E., ... Chlichlia, K. (2016). *Lactobacillus casei* exerts anti-proliferative effects accompanied by apoptotic cell death and up-regulation of TRAIL in colon carcinoma cells. *PLoS One, 11*(2), e0147960. https://doi.org/10.1371/journal.pone.0147960.

Wang, B., Wei, H., Yuan, J., Li, Q., Li, Y., Li, N., & Li, J. (2008). Identification of a surface protein from Lactobacillus reuteri JCM1081 that adheres to porcine gastric mucin and human enterocyte-like HT-29 cells. *Current Microbiology, 57*(1), 33–38.

Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols, 3*(2), 163.

Woo, J., & Ahn, J. (2013). Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens. *Letters in Applied Microbiology, 56*(4), 307–313.

Wu, C., He, G., & Zhang, J. (2014). Physiological and proteomic analysis of *Lactobacillus casei* in response to acid adaptation. *Journal of Industrial Microbiology and Biotechnology*. https://doi.org/10.1007/s10295-014-1487-3.

Wu, C., Zhang, J., Wang, M., Du, G., & Chen, J. (2012). *Lactobacillus casei* combats acid stress by maintaining cell membrane functionality. *Journal of Industrial Microbiology and Biotechnology*. https://doi.org/10.1007/s10295-012-1104-2.

Zhang, D., Zhang, S., Guidesi, E., Zonenschain, D., Sagheddu, V., Soowon, L., Lim, C. Y., & Elli, M. (2018). Isolation and characterization of new probiotic strains from Chinese babies. *Journal of Clinical Gastroenterology, 52*, S27–S34. https://doi.org/10.1097/mcg.0000000000001113.