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Selection of *Lactobacillus* strains newly isolated from Algerian camel and mare fermented milk for their *in vitro* probiotic and lipolytic potentials

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The main objective of this study was the characterization of new lactobacilli probiotic strains belonging to lactic acid bacteria (LAB). Eighty-eight strains were isolated from different Algerian camel and mare fermented milks; three of them were pre-selected for their stability, fast growth and resistance to acidity and bile salts. Cell viability was assessed in simulated gastric and intestinal conditions. On the other hand, cell safety was checked by testing their hemolytic capacity. The *in vitro* tests revealed a good probiotic potential of selected strains. The majority of lactobacilli is resistant to cross-stress and persists beyond 4 h of incubation in contact with simulated gastrointestinal juices; a survival rate of over 80% was observed. All strains showed better lipolytic activity in the presence of natural substrates compared to Tween-80. Lipolysis zones diameters obtained in the presence of butter and olive oil were remarkable (between 20 and 27 mm respectively). Investigation of the cholesterol-lowering and the triglyceride-lowering properties revealed a cholesterol ratio degradation of 54.8% and a triglyceride ratio degradation of 80.3% for *Lactobacillus plantarum* NSC5C.

Key words: Probiotic, camel and mare fermented milks, cholesterol lowering, triglycerides lowering, *Lactobacillus plantarum.*

INTRODUCTION

Hyperlipidemia is the excess of lipids in blood, mainly cholesterol and triglycerides. This physical state is asymptomatic in many people. Nevertheless, it can have adverse consequences on human health. It is one of the most important risk factors associated with cardiovascular disease (Manson et al., 1992). The accumulation of these

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Abbreviations: LAB, lactic acid bacteria; **TG,** triglycerides; ***Lb,*** *Lactobacillus*; ***Lc,*** *Lactococcus*; **CFU,** colony forming unity; **CRD,** cholesterol ratio degradation; **TRD,** triglycerides ratio degradation.

blood lipids is mostly due to bad nutritional balance affecting many western countries resulting in obesity (Ferrières et al., 2004). Dairy products are also an important source of fat, however many studies have shown that some fermented products show a low cholesterol content such as fermented camel and mare milk (Pieszka et al., 2016). These low lipid levels are attributed not only to the composition of the milk but also to the bacterial flora that reside there (Konuspayeva et al., 2008; Raziq et al., 2008; Kamal and Salama, 2009). This flora is principally composed of LAB including lactobacilli; these microorganisms have the capacity to reduce blood lipids (Shah, 2007; Mansoub, 2010). Bacteria with beneficial properties for the organism are considering as probiotics (Lilly and Stillwell, 1965). To be designated as such they must meet several criteria mainly resistance to gastric and intestinal conditions, résistance to antibiotics, antagonism against pathogens, adhesion to intestinal epithelial cells and safety (Salminen et al*.,* 1998; Aarti et al., 2017). The pharmaceutical or agri-food industries are increasingly using probiotics as a dietary supplement (Liao and Nyachoti, 2017), as additives or as alternatives to antimicrobials (Aarti et al., 2018; Alagawany et al., 2018).

New indigenous probiotic strains isolated from dairy sources known for their many health benefits such as components of camel milk (Abdel Gader and Alhaider, 2016) or mare milk (Jastrzębska et al., 2017) could compete with commercial strains while being more effective and less expensive. Fermented milks are widely consumed in Algeria for their health benefits among them camel milk which is known for its cholesterol-lowering and hypotriglyceridemic effects, nevertheless the consumption of fermented raw milk must be very framed. The health of milk-producing animals must be tightly controlled, as must the hygiene of milking tools in order to prevent risks to the health of consumers. These data incited looking for these abilities on a set of lactobacilli from collection of our lab. Three strains were isolated from Algerian camel and mare fermented milks, and were preselected for their resistance to bile salts and acidity. This study was aimed at testing *in vitro*:

1. Strains whose resistance in stress conditions simulates the gastrointestinal conditions,
2. Strains with antagonistic and hemolytic power;
3. The lipolytic power of strains on different lipidic substrates, and finally the search for cholesterol-lowering and triglyceride-lowering power.

MATERIALS AND METHODS

Strains isolation, screening and identification

Different milk samples were collected from each animal, camel or mare, after washing the breast and udder and eliminating the first jets of milk. Samples (100 ml) were placed at 4°C and transported to the laboratory and then incubated at 30°C for 18 h. After an endogenous fermentation, 10 ml of camel or mare fermented milk were homogenized with 90 ml sterile physiological water (0.9% w/v NaCl). Serial decimal dilutions were prepared (from 10^{-1} to 10^{-6}), and 100 µl samples of appropriate dilutions were spread in duplicate on de Man, Rogosa and Sharpe medium plates (MRS, Fluka, Geneva Switzerland). After an incubation of 24 to 48 h at 30°C, distinct colonies were selected randomly and purified by re-streaking on MRS agar plates until only a single type of colonies was observed. The different pure isolates obtained were characterized by Gram staining, catalase production, and cell morphology. Only Gram positive and catalase negative bacilli were selected. Strains were conserved at room temperature after freeze-drying or by storage at -80°C either in 10% skimmed milk or in liquid MRS supplemented with 40% glycerol. All the isolated lactobacilli (88) were tested for their resistance to different acid pH (pH 1-pH6), to different bile salts concentrations (0.25, 0.5, 1, 2 and 10%) (Idoui, 2008), which is one of the most important criteria for the selection of probiotics strains. They were also tested for their lipolytic activity on MRS medium supplemented with butter or olive oil to target strains with liporeductive potential. The three strains presenting the most interesting results for the rest of our research were selected, conserved and then identified using the biochemical galleries API 50CHL (Biomérieux, France).

A molecular identification was also done by the Sanger sequencing of the full length 16S rRNA gene. Total DNA was extracted from overnight culture of the strain using the Phenol-chloroform method (Azcárate-Peril and Raya, 2001). An amplification was done by PCR using primers 16S-27F and 16S-1492R (27F5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-ACGGCTACCTTGTTACGACTT-3′) and also 16S-27F and 16S-19R (27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 19R 5′-GRG TAC CTT TTA TCC GTT G-3′ while R, A or G) (Lane, 1991) in order to amplify V1-V2 16S gene segments for the 3 strains. The PCR conditions were realized with the 5× HOT BIOAmp® Evagreen HRM Mix at 12.5 mM, 2 µl of Enhancer 10X and 4 µl of $MgCl_2$, using 1 µM of forward and reverse primers and 2 µl of genomic DNA template in a total volume of 20 µl. The PCR cycling conditions were as follows: A first denaturation step at 96°C for 12 min, 45 cycles of denaturation at 96°C for 20 s, annealing at 52°C for 20 s, extension at 72°C for 1 min 30 s, followed by an elongation step at 72°C for 5 min. The sequencing was performed in Biofidal laboratories (Lyon, France).

For comparative purposes, two probiotic reference strains *Lactobacillus plantarum* BH14 and *Lactobacillus brevis* CHTD27 isolated from Algerian camel milk of regions of Illizi and Tindouf, respectively were also used. Pathogenic strains used in this study and their origins are presented in Table 1. All strains belong to the LBMB collection (Laboratory of Biology of Microorganisms and Biotechnology, Oran, Algeria).

Resistance to simulated digestive conditions

The survival of the bacterial strains under conditions simulating those encountered during their passage through the digestive tract (stomach and intestines) was tested. This test was carried out in two steps following the method of Bahri (2014).

Resistance to simulated gastric conditions

For the execution of this test, an overnight culture of the LAB strains, obtained after 18 h of incubation in MRS broth at 30°C was used; these cultures were diluted to an optical density of 0.5 to 0.7 under a wavelength of 600 nm. The simulated gastric juice was prepared by mixing pepsin (Sigma) to 0.5% (w/v) NaCl (pH1.5) at a final concentration of 3 g/l. The enzyme was first dissolved in 0.02 M glycine-HCl buffer (pH1.5) and then sterilized using a Millipore

Table 1. Pathogenic strains.

Strains	Origin
Salmonella Thyphimurium	Laboratory of Biology of Microorganisms and Biotechnology (Oran, Es-Sénia)
Proteus mirabilis	
Klebsiella pneumoniae	
Citrobacter freundii	
Enterobacter cloacea	
Enterobacter aerogenes	
Staphylococcus aureus ATCC 25923	
Acinetobacter baumannii	
Pseudomonas aeruginosa ATCC 27853	
Escherichia coli ATCC 25922	
Bacillus cereus	
Staphylococcus aureus (II2) ATCC 433005	

filter (Millipore, MILLEX-GV, 0.22 µm, SLGV0130S). This solution was distributed in tubes at the rate of 9 ml, which have been supplemented with 1 ml of the overnight cultures of LAB strains previously obtained. One hundred microliters of each tube was taken at T_0= 0h, T_1= 2h and T_2=4h, to be counted by the agar plate method on MRS agar after 24 h of incubation at 37°C.

Resistance to simulated intestinal conditions

In order to simulate the hostile conditions of the human small intestine, a solution adjusted to a pH of 8 containing Pancreatin (Nature's plus, Warwickshire, UK) dissolved in buffer (0.013 M Tris-HCl, pH8) at a final concentration of 1 g/l and 0.3% (v/v) of filtered sheep bile (Millipore, MILLEX-GV, 0.22µm, SLGV0130S) was prepared. The prepared simulated intestinal juice was distributed into a tube then inoculated at a rate of 10% (v/v) with a young culture of LAB ($0.5<OD_{600nm}>0.7$, that is 10^9 cells/ml); 0.1 ml was taken from each tube at different exposure time intervals (T_0 = 0 h, and T_1 = 4 h) to inoculate the surface of the MRS agar. The colonies obtained were then counted after incubation at 37°C for 24 h.

Antibacterial activity against pathogenic strains

This antibacterial activity was researched using two methods.

Spot method

The purpose of this test is to determine the inhibitory effect of LAB on some indicator strains according to the method of Fleming et al. (1975). Overnight cultures of all strains (inhibitors and indicators) were inoculated respectively in MRS broth and Luria Bertani (LB) broth for the lactobacilli and pathogenic bacteria, respectively. LAB were inoculated in spots on MRS agar; after 24 h of incubation at 30°C, the obtained colonies were covered with 10 ml of 1% (v/v) soft agar MRS previously seeded with a fresh culture of the indicator strain (pathogens at an OD600 nm ≈ 1)) and then incubated for 24 h at 37°C. The size of the inhibition zones around the spot was measured.

Impregnated disc method

The selected lactobacilli were tested for their antibacterial potency using the impregnated disk method (Savadogo et al. 2004; Tadesse et al., 2004). Fifteen milliliters of LB soft agar were inoculated with 1% (v/v) of fresh pathogenic bacteria culture (OD_{600nm}≈ 1) poured in Petri dish and then allowed to dry at room temperature, 6 mm Whatman filter paper discs were impregnated with 10 µl of a fresh LAB culture and then placed on the surface of the LB soft agar. The size of the inhibition zones around the disks were measured after 24h of incubation at 37°C.

Lipolytic activity

The lipolytic activity of tested strains was investigated on MRS medium supplemented with different natural and artificial lipid substrates. The activity was sought on a solid MRS medium buffered to pH 7 (phosphate buffer Na_2HPO_4/NaH_2PO_4, 0.1 M) containing 1% (v/v) of butter, olive oil or tween 80 as the only lipid source. The medium was pacified by adding 0.5% calcium carbonate ($CaCO_3$) to clearly visualize an eventual lipolytic zone. Overnight cultures LAB strains were spot seeded on the surface of the enriched MRS medium. Two hours of drying at room temperature are necessary before the incubation at 30°C for 24 to 48 h. Lipolysis was then revealed by the appearance of opaque zones around lactobacilli colonies (Guiraud and Galzy, 1980).

Hypocholesterolemic and hypotriglyceridemic *in vitro* activity of lactobacilli

All strains presenting a lipolytic activity were then inspected for their hypocholesterolemic and hypotriglyceridemic properties using the modified method of Guo et al. (2011).

This test was done using MRS broth supplemented with 0.3% (v/v) of sheep bile. Cholesterol and triglycerides were sterilized by filtration (Millipore, MILLEX-GV, 0.22 µm, SLGV0130S, Perkin Elmer, Boston, MA) and then added individually to broth at a final concentration of 200 mg/ml; 500 µl of this solution were transferred to an Eppendorf and supplemented with the same volume of lactobacilli fresh culture (OD_{600nm} ≈1). The final concentration of cholesterol or triglycerides was then 100 mg/ml. This operation was

Table 2. Identification percentages of selected strains using molecular and biochemical methods.

Strain	Taxon	% by molecular identification	% by API 50 CHL identification	Origin
NSC5C	*Lactobacillus plantarum*	99	99.9	Camel milk from Naama, Algeria
NSC10	*Lactobacillus plantarum*	99	99.9	Camel milk from Naama, Algeria
JUMIII4	*Lactobacillus plantarum*	99	99.4	Mare milk from Saida, Algeria

carried out for all the selected lactobacilli. The cells were removed from the culture by centrifugation (12,000 rpm for 10 min at 4°C) after 24 h of incubation at 37°C. The supernatants were recovered, and the cells were washed three times with a volume of MRS broth containing 0.3% (v/v) of bile, identical to the original broth. After each washing, the suspension was centrifuged (12,000rpm for 10 min at 4°C) and the three supernatants were combined and represented the wash solution.

Cells obtained after the third wash step were suspended in MRS broth containing 0.3% of bile plus lysozyme at a final concentration of 4 mg/ml and placed in a water bath at 37°C for 1 h 30 min. Lysis buffer (10% SDS, pH12) was then added at a rate of 100 µl/ml (V buffer/V cells).

The lysed cell solution was centrifuged (12,000 rpm for 10 min) to recover the supernatant containing the cholesterol or triglycerides entrapped in the cells.

In all fractions, the cholesterol or triglyceride concentration was assessed using the colorimetric method described by Rudel et al. (1973) slightly modified.

The ratio of cholesterol degradation (CDR) was calculated from the equation:

$$\mathbf{CDR} = \frac{[\mathrm{C} - (\mathrm{C1} + \mathrm{C2} + \mathrm{C3})]}{\mathrm{C}} \times 100$$

The ratio of triglycerides degradation (TDR) was calculated from the equation:

$$\mathbf{TDR} = \frac{[\mathrm{T} - (\mathrm{T1} + \mathrm{T2} + \mathrm{T3})]}{\mathrm{T}} \times 100$$

Where C and T are the initial substrates concentrations: C1, C2 and C3; T1, T2 and T3 are substrate concentrations of cholesterol and triglycerides, respectively in the supernatant, wash solution, and solution of lysed cells.

Hemolytic activity

The hemolytic activity of lactobacilli was determined by the method of Maragkoudakis et al. (2006). Hemolyticity was examined by seeding strains of lactobacilli on blood agar (Columbia Medium). The type of hemolysis was examined after an incubation of 24 h at 30°C. The result can be α-hemolytic (green around colonies), β-hemolytic (lightening around colonies) or γ-hemolytic (the medium is unaffected).

Data analysis

Data were analysed with Statistica 5.5 software (1999 edition; Tulsa, OK, USA). One-way analysis of variance (ANOVA) with Duncan's multiple range test was performed to compare any significant differences. Values of $P<0.05$ were considered statistically significant. Differences among means were detected by paired Student's test.

RESULTS AND DISCUSSION

Isolation, screening and identification

Eighty-eight lactobacilli were isolated from the different fermented milks; the three most resistant to acidity, bile salts and presenting a good lipolysis activity were selected (NSC10, NSC5C and JUMIII4) to conduct this study in comparison with the two reference probiotic strains. The biochemical identification API 50 CHL revealed the belonging of the 3 selected strains to the *Lb. plantarum* taxon over 99% (Table 2).

Molecular identification

Identification results obtained by the API50 CHL galleries and the sequencing of the 16S gene are indicated in Table 2. Alignment and homology of the PCR amplified sequences were done in NCBI website (http://www.ncbi.nlm.nih.gov) using BLAST Software, which determine identity of the 3 strains NSC5c, JUMIII4 and NSC10 to the taxon *Lactobacillus plantarum.* The phylogenetic tree is represented on Figure 1.

Resistance of lactobacilli to simulated gastrointestinal conditions

Resistance to simulated gastric conditions

The tested lactobacilli had a similar starting concentrations with an optical density ranged between 0.5 and 0.7. Their survival in simulated gastric conditions (3 g/l pepsin, pH 1.5 and 0.5% NaCl) varies according to the strain (Figure 2). It is noted that the number of colonies decreases as soon as the cells are exposed to the solution, which explains the difference of Log10CFU/ml at T_0.

All strains show remarkable resistance after 2 h exposure to simulated gastric conditions with a survival rate of over 80%. After 4 h of gastric stress, NSC5c is the most resistant (3.56Log10CFU/ml at T_0 to 2.64 Log10CFU/ml at T_{4h}), regarding strains, BH14, CHTD27 and JUMIII4, despite a sharp decrease, they were quite resistant to cross-stress and persist even after 4 h of incubation in contact with stressors. The number of cells remained, even so, more important than the most

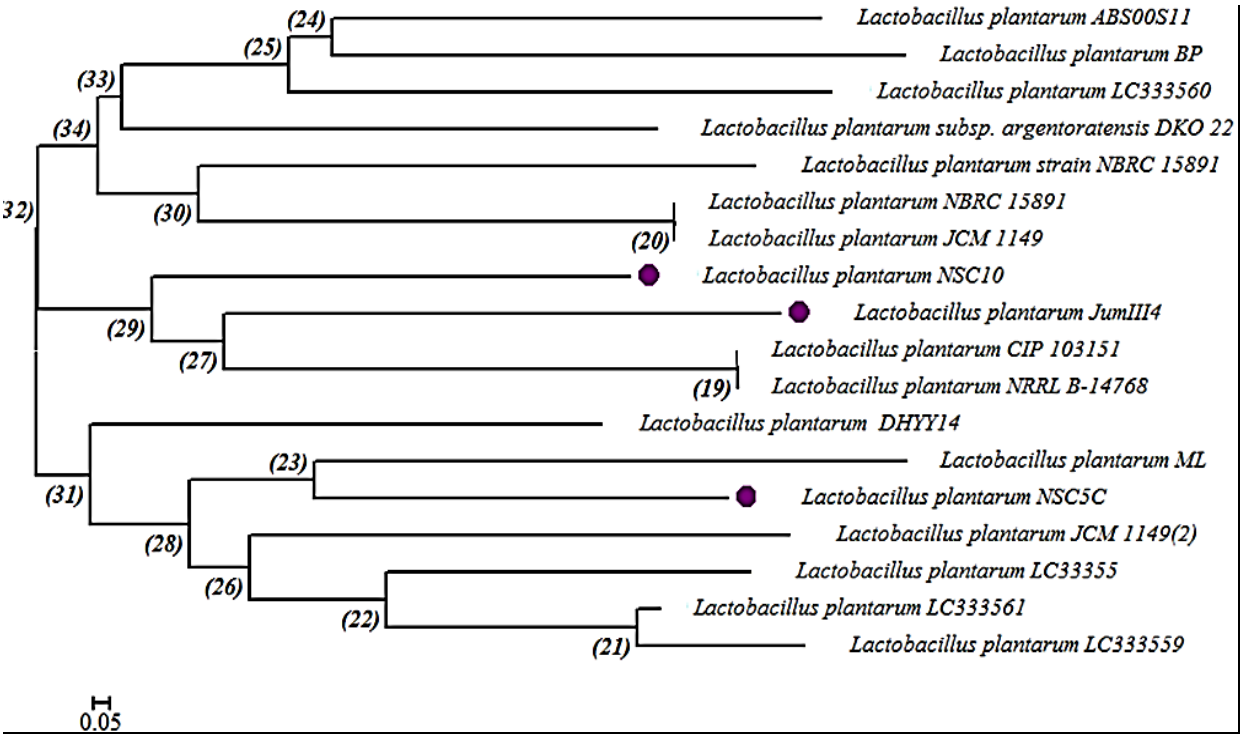

Figure 1. Concatenated phylogenetic tree of *Lactobacillus plantarum* (NSC5c, JUMII4 and NSC10) among neighbouring known species.

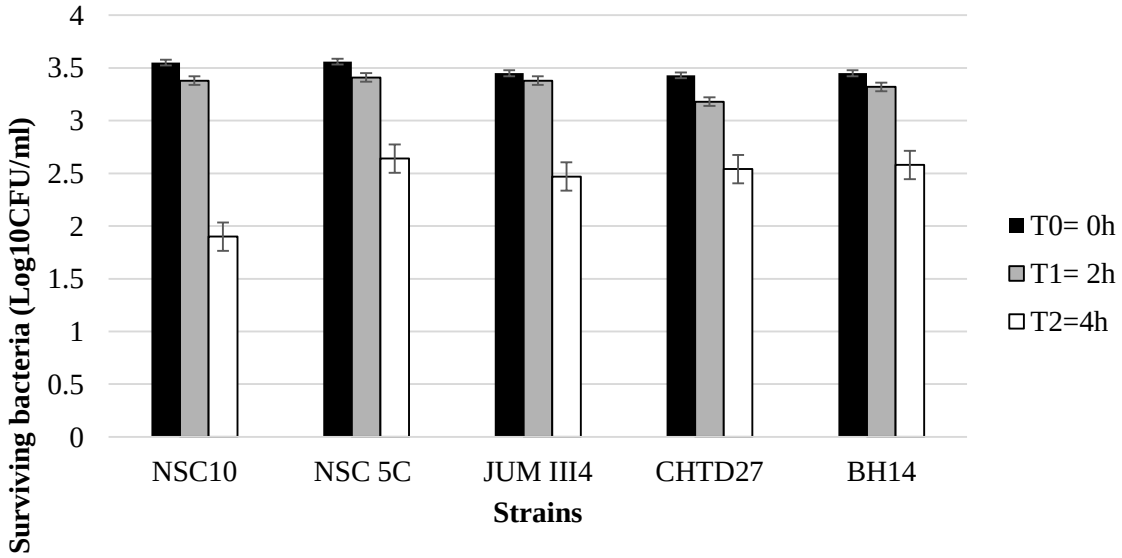

Figure 2. Resistance of lactobacilli in contact with simulated gastric juice.

sensitive strains, such as NSC10 which undergo an important decrease from an average of 3.55 Log10 CFU/ml at T_0 up to 1.9 Log10 CFU/ml after 4h in contact with the simulated gastric juice.

These results are consistent with those obtained by Bahri et al. (2014) who determined the resistance of some strains of *Lactobacillus* including *Lb. plantarum* in similar stress conditions. Maragkoudakis et al. (2005) showed that the tested probiotics resist pH 3 for 3 h, and most have lost their viability in 1 h in pH 1. Akalu et al. (2017)

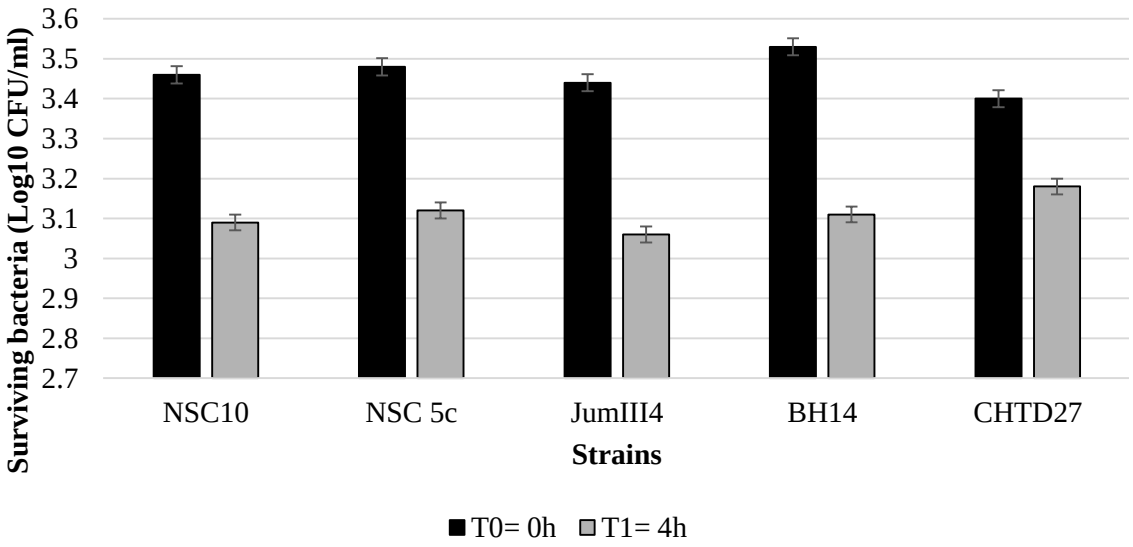

Figure 3. Resistance of lactobacilli in contact with simulated intestinal juice.

showed that 80 to 94% of the tested LAB survives after 6 h at pH 2.5.

Conway et al. (1987) and Lindwall and Fonden (1984) have shown that, unlikely to strains used in the study, *Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus* strains have a very low resistance to acidity and were destroyed very quickly at pH 1, and after about 1 h at pH 3.

Acid stress causes intracellular acidification, which decreases the activity of cytoplasmic enzymes (Even et al., 2002). Transcriptomic and proteomic studies have highlighted that many LAB enhance the levels of glycolytic enzymes under acid, thermal, and osmotic stresses, but without increasing the synthesis of lactic acid (Marceau et al*.,* 2002; Di Cagno et al., 2006a). LAB such as *Lb. plantarum, Lactobacillus. reuteri*, *Lactobacillus rhamnosus* and *Lactococcus lactis* modify pyruvate metabolism at the expense of lactic acid, and they increase the synthesis of basic compounds (e.g., lysine and diacetyl/acetoin) (Heunis et al., 2014; Zuljan et al., 2014). The level of lactate dehydrogenase (Ldh) which is responsible for the synthesis of lactic acid from pyruvate markedly decreases. Acetyl-CoA is rerouted toward the biosynthesis of fatty acids instead of butanoate (Di Cagno et al*.,* 2006b; Koponen et al., 2012), which may enhance the rigidity and impermeability of the cytoplasmic membrane (Cotter and Hill, 2003; Fernandez et al., 2008). Pyruvate oxidase and phosphate acetyltransferase, used to synthesize acetyl-coenzyme A (acetyl-CoA), which are induced in *Lb. delbrueckii subsp. bulgaricus* and *Lb. rhamnosus* under acid stress conditions (Koponen et al., 2012; Zhai et al., 2014).

Resistance to acid stress is an important factor for LAB since they acidify their environment during growth. Lactobacilli are generally more resistant to acid stress than lactococci (Siegumfeldt et al., 2000). In addition, acid-resistant strains also have good resistance to other stresses such as bile salts and NaCl (Collado et al., 2006).

Resistance to simulated intestinal conditions

After passing through the stomach, the bacteria reach the duodenum where the bile is secreted. At this level, some components of bile, especially bile acids such as colic acid, seriously compromise the viability of ingested bacteria. Bile tolerance is also a criterion for *in vitro* selection of probiotic bacteria; it is generally considered necessary to assess their ability to withstand intestinal tract conditions such as pancreatic enzymes and gives them the ability to colonize the intestinal environment (Bron et al*.,* 2006). As well, adaptation to bile can also protect bacteria against other stresses (acid, enzymes or thermal stress) (Saarela et al., 2004; Sanchez et al., 2006).

To investigate the effect of bile stress, *in vitro* experiments were conducted with a solution of 1 g/l of pancreatin and 0.3% (v/v) of sheep bile at pH 8, that is, similar to intestinal conditions. The results are shown in Figure 3. The Log10 CFU/ml of strains at T_0 reaches its maximum for BH14 and NSC5C strains with 3.53 Log10CFU/ml and 3.48 Log10CFU/ml, respectively. Nonetheless, all strains survive even after 4 h in contact with the bile solution. *Lactobacillus plantarum* JUMIII4 has the lowest rate of resistance and presented an important decreasing from 3.44 Log10CFU/ml at T_0 to 3.06 Log10CFU/ml at T_{4h}.

These results express a variable resistance according to the strains; it was reported that bacterial resistance to

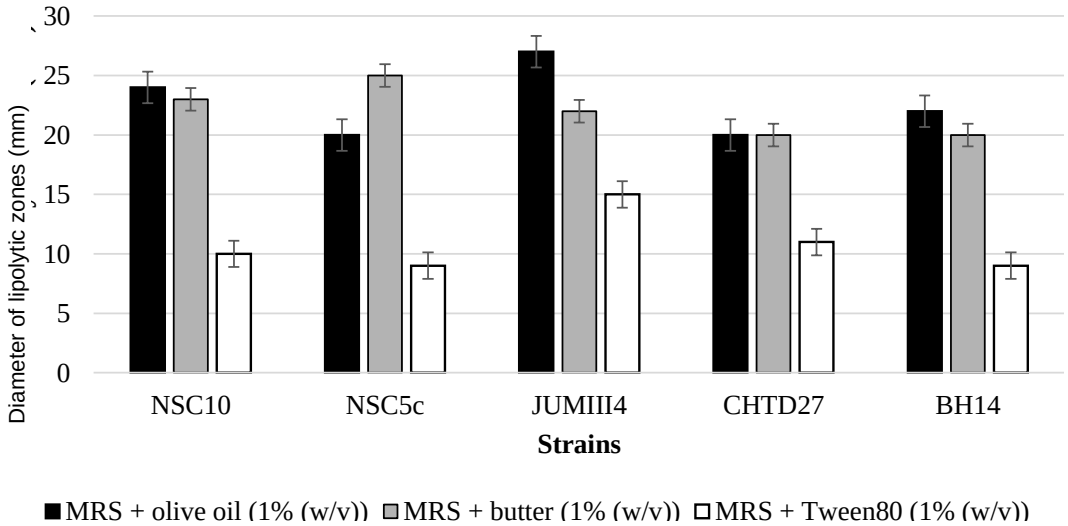

Figure 4. Lipolytic effect of lactobacilli on olive oil, butter and tween 80 (expressed in mm).

bile salts is determined genetically (Fang et al., 2009), so these variations may be explained by a different expression of stress resistance genes and a correlation between acid, saline, biliary and various digestive enzymes.

The stress caused by bile on bacterial cells can corrupt their ability to survive. In contrast to the acidity that fades after gastric passage, the bile that encounters surviving bacterial cells remains in contact with them for a longer time. Marteau and Shanahan (2003) and Izquierdo et al. (2009) clearly demonstrated *in vitro* that bile salts had a bactericidal effect. In the same way as for gastric acidity, their study demonstrated a difference in sensitivity to bile salts between bacterial species. *Lb. bulgaricus* and *Streptococcus thermophilus* have a very low survival percentage compared to *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Bile salts have a detrimental effect on cell membranes resulting from an increase in cell permeability. The resistance to bile salts is likely due to BSH enzymes. Many strains of lactobacilli have the ability to reconvert via these enzymes (BSH, EC 3.5.1.24) (De Smet et al., 1995). According to Reyes-Nava et al. (2016) BSH functions are not yet clearly understood. These authors also concluded that many strains with BSH activity were particularly resistant to bile salts and then had the ability to modulate blood lipids in rats and protect their liver functions.

Wu et al. (2010) found that expression levels of 26 proteins were acutely stimulated and/or regulated by factor of bile salts. Transcription-PCR and bioinformatics analysis showed that the implicated pathways are involved with a complex physiological response under bile salts stress, particularly including cell protection (DnaK and GroEL), modifications in cell membranes (NagA, GalU, and PyrD), and key components of central metabolism (PFK, PGM, CysK, LuxS, PepC, and EF-Tu). Furthermore, Mathipa and Thantsha (2015) concluded that multi-stress pre-adaptation enhances viability of probiotics under simulated gastrointestinal conditions and formulations containing a mixture of multi stress-adapted cells exhibits enhanced synergistic effects against food borne pathogens.

Microencapsulation can be an effective means of increasing the resistance of certain strains used as probiotics to enable them to survive gastrointestinal conditions and reach their target in a viable form (Al-Furaih et al., 2016; Gonzalez-Cuello et al., 2017).

Lipolytic activity

Lipases have a broad spectrum of action on emulsion substrates. LAB which exerts efficient lipase activity could be interesting for use as a probiotic. The tested strains of lactobacilli showed a significant activity in the presence of natural substrates olive oil and butter compared to Tween 80 *(P<0.001).* The majority of the strains show similar results for the degradation of the two natural substrates (Figure 4). Nevertheless, the strain JUMIII4 has preferentially degraded olive oil than butter, with a lipolysis zone of 27 and 22 mm in diameter, respectively, unlike NSC5c that showed better degradation of butter with lysis zone of 25 mm, compared to olive oil with a degradation zone of 20 mm. Dinçer and Kivanç (2018) investigated this activity on 50 strains of LAB isolated from the Turkish pastırma. The lipolytic activity is observed in 25 of the tested strains where *Lb. plantarum* revealed the highest lipolytic activity.

Katz et al. (2002) found a wide variation in activity between strains of *Lb. plantarum, Lb. acidophilus* and

Table 3. Antibacterial activity of lactic acid strains against pathogenic bacteria.

Strain	1	2	3	4	5	6	7	8	9	10	11	12
JUMIII4	+	++	-	++	+++	+	++	-	-	++	-	-
NSC5C	-	-	+++	++	-	-	+++	+++	-	-	++	-
NSC10	++	+	++	+	++	-	+	++	+	+	++	++
CHTD27	+	+	-	++	+	+	+	+	-	+	+	-
BH14	++	++	++	++	++	+	+	+	++	+	++	++

Discs of Whatman papers (6 mm diameter) were soaked with 10 µL of a fresh bacterial suspension. (+++) Inhibition zone>20 mm; (++) Inhibition zone>15 mm; (+) Inhibition zone>10 mm; (−) Inhibition zone<10 mm. 1*: Proteus mirabilis;* 2*: Salmonella Thyphimurium;* 3*: Klebsiella pneumoniae;* 4*: Citrobacter freundi;* 5*: Enterobacter cloacea;* 6*: Staphylococcus aureus;* 7 *:Enterobacter aerogenes* ;8 *: Pseudomonas aeruginosa* ATCC 27853*;* 9*: Escherichia coli 25922*; 10*: Bacillus cereus*; 11*: Staphylococcus aureus* ATCC 433005; 12: *Acinetobacter baumannii.*

Enterococcus feacium. Shahab-Lavasani et al. (2012) also determine that the addition of *Lactobacillus lactis* had a significant (p<0.05) effect on the lipolysis characteristics of *Lighvan* cheese.

These results are in disagreement with those described in several studies, which reported that LAB have a lower lipolitic activity with natural lipids (De Moraes and Chandan, 1982; Kamaly et al., 1988; Papon and Talon, 1989).

Antibacterial activity against pathogenic strains

The presence of inhibition zones is the result of an antagonism exerted by the LAB against the pathogenic strains. Generally, the lactobacilli strains do not present the same spectrum of action towards the pathogens (Table 3). No significant difference was found between the activity of lactobacilli isolated from camel milk and that isolated from mare's milk (JUMIII4), which supports researches of Tremonte et al*.* (2017) who demonstrated that there is no relationship between the intensity of inhibition and the origin of inhibitory strains of *Lb. plantarum*.

Lb. plantarum BH14 inhibited the entire indicator strains tested, these performances are followed closely by the strains CHTD27 and NSC10 which showed a significant inhibitory effect (11 and 10 pathogeneses inhibited, respectively), unlike the NSC5C strains, which inhibited only 7 of the 12 pathogens tested.

Lactobacilli showed relatively similar antagonistic activity against Gram-positive and negative pathogens with a slightly more pronounced activity against Gram-negative pathogens. These results are in agreement with those found by other authors who have shown that LAB are able to prevent the growth of Gram-positive and negative pathogenic bacteria *in vitro* and *in vivo* (Lin et al., 2007; Balcázar and Luna-Rojas, 2007; Mahdhi et al., 2010; Okpara et al., 2014; Anyika et al., 2018; Digo et al., 2017).

Acinetobacter baumannii, Escherichia coli 25922 are the most resistant indicator bacteria, they were inhibited only by 2 LAB out of the 5 tested with a maximum inhibition zone not exceeding 16 mm in diameter. *Enterobacter aerogenes* and *Citrobacter freundii* strains were inhibited by all LAB with inhibition zones ranging between 13 and 21 mm in diameter. Antagonism of lactobacilli was also observed on *Bacillus cereus, Staphylococcus aureus* ATCC 433005 and *Enterobacter cloacae*.

The pathogenic microorganisms tested in this study are involved in toxi-infections or food poisoning such as the following species: *Staph. aureus*, *E. coli, Pseudomonas aeruginosa and Klebsiella pneumonia.* Values obtained for this test coincide for some strains with the work of Belyagoubi and Abdelouahid (2013), where the diameters of the inhibition zones of LAB isolated from Algerian traditional dairy products are of the order of 4 mm up to 34 mm on the same pathogenic bacteria.

García-Cayuela et al*.* (2009) reported that beneficial bacteria, mainly LAB and bifidobacteria, could be a useful and effective strategy for preventing or reducing the incidence of pathogens, thereby improving food safety and protecting consumer health. LAB producing antimicrobial agents have been used as an alternative to antibiotics for the treatment of gastrointestinal diseases (Soomro et al., 2002; Akpinar et al., 2011) and against infections by Candida (Aarti et al., 2018).

The antibacterial activity of a probiotic is essential for the successful colonization of the intestinal mucosa (Tejero-Sarinena et al., 2012). It provides a barrier and defense effect against pathogens (Vaughan et al., 1999). Lactobacilli can produce antimicrobial substances such as organic acids, which are active *in vitro* and *in vivo* on enterovirulent pathogens involved in diarrhea cases (Servin, 2004). Lactic and acetic acids are produced *via* the fermentation of hexoses by lactobacilli. In addition, in acidic medium, the bacterial competitiveness of lactobacilli is favored compared to other bacteria because of their tolerance to acidity (Servin, 2004). Inhibition of pathogens such as *Staph. aureus* and *Bacillus cereus* by LAB is related to several antagonistic factors including decreased pH after lactic acid production, competition for food, production of bacteriocins and hydrogen peroxide (Isolauri et al., 2004; Charlier et al*.,* 2009; Merzoug et al., 2016, 2018).

Table 4. Lactobacilli cholesterol lowering-activity in MRS broth.

Strain	C1 (g/l)	C2 (g/l)	C3 (g/l)	CDR (%)
CHTD 27	0.484	0.011	0.071	43.4
BH14	0.477	0.003	0.011	50.9
NSC5C	0.419	0.022	0.011	54.8
JUMIII 4	0.477	0.007	0.018	49.8
NSC10	0.496	0.003	0.026	47.5

CDR: Cholesterol degradation ratio; C1: Concentration of cholesterol in the supernatant; C2: Concentration of cholesterol in the wash solution; C3: Concentration of cholesterol in fragmented cells solution.

These organic acids can passively diffuse through the bacterial membrane in their undissociated form. They acidify the cytoplasm after dissociation and inhibit the cellular enzymatic activity of acid-sensitive pathogens (Deng et al*.,* 1999). This decrease in pH can therefore affect the viability of bacterial pathogens (Bruno and Shah, 2002; Servin, 2004). This activity is favored under certain *Lactobacillus* culture conditions. Tashakor et al. (2017) showed that the optimum conditions achieved at pH 6.0, 25°C temperature, 1.5% (w/v) Na_2HPO_4 and 0.5% (w/v) peptone. This indicates that the inhibition of pathogens is promoted under controlled conditions *in vitro* rather than in the intestinal tract where the temperature is higher and the nutritional sources variable.

Pathogens can also be inhibited by a nutrient restriction process. It is obvious that the ability of microorganisms to compete for limiting available nutrients is a significant factor in determining the composition of the microbiota. Hence, an increase in the number of lactobacilli obtained during a probiotic treatment would make it possible to reduce the substrates available for the implantation of pathogenic microorganisms (Fooks and Gibson, 2002).

The Fleming et al. (1975) method gave clear results for all the strains tested with significant inhibition diameters (from 15 to 45 mm), but these performances could not be confirmed after reiterations of the test using the same method.

Hypocholesterolemic *in vitro* activity of lactobacilli

Results presented in Table 4 reveal that all strains have a cholesterol-lowering activity. In the presence of bile salts, the cholesterol contained in the culture medium (1 g/l initially) was reduced to more than 50% for 2 strains of the 5 tested. Strains NSC5c is the most effective with a CRD of 54.8% as opposed to the strain CHTD27 which reduced cholesterol only at a ratio of 43.4%. These results are consistent with the studies of Bendali et al. (2017) which reported the effectiveness of LAB in reducing cholesterol *in vitro*. *Lb. pentosus* KF923750 was able to remove 62.4% of cholesterol in the growth medium after 24 h incubation. The hypocholesterolemic power of lactobacillus strains was also revealed by several studies (Mirlohi et al., 2009; Kondo et al., 2010; Huang et al., 2013; Liu et al., 2016; Zhang et al., 2017; Ding et al., 2017).

The concentrations of residual cholesterol in the 3 fractions (C1, C2 and C3) show a higher level in the initial supernatant C1 unlike the wash solution in which the cholesterol level is lower. It expresses that the cholesterol deduced from the supernatant of culture was not adsorbed to the bacterial wall, the low cholesterol level recorded in the fragmented cell solution proves that cholesterol has not been trapped inside the cells either, the hypothesis that can be emitted is that lactobacilli degrade cholesterol extracellularly.

Several hypotheses also have been put forward to explain cholesterol-lowering effect, such as the assimilation of cholesterol by bacteria or the hydrolysis of conjugated bile salts (Zhang et al., 2008). The deconjugation of bile acid by Bile-salt-hydrolase (BSH) was the most supported, the lactobacilli with this activity are preferred over the BSH-negative lactobacilli as selection criteria for probiotic strains with lowering cholesterol properties (Pereira et al., 2003). According to Jaspers et al. (1984), the organic acids produced by its bacteria are presumably cholesterol-lowering agents, hydroxymethyl and orotic acids lower serum cholesterol; on the other hand, uric acid inhibits the synthesis of cholesterol.

Another explanation relates to a decrease in cholesterol level, which would be solely due to the co-precipitation of cholesterol with the deconjugated bile salts, a phenomenon that cannot occur *in vivo* because the pH is higher than in a culture medium acidified by LAB (Desmazeaud, 1996).

Hypotriglyceridemic *in vitro* activity of lactobacilli

Lactobacilli strains showed variable triglycerides reduction (TRD) oscillating between 3% for strain JUMII4 and 80.3% for strain NSC5c (Table 5) which shows that the strains do not have the same abilities to reduce TG. From the observations made by comparing the residual concentrations in the culture supernatants and the fractionated cells solution, it can be seen that, unlike

Table 5. Lactobacilli triglycerides lowering-activity in MRS broth.

Strain	T1 g/l	T2 g/l	T3 g/l	TRD (%)
CHTD27	0.116	0.130	0.696	5.8
BH14	0.492	0.135	0.249	12.4
NSC5C	0.112	0.027	0.058	80.3
JUMIII4	0.192	0.136	0.669	3.0
NSC10	0.189	0.132	0.261	41.8

TDR: Triglycerides degradation ratio, T1: Concentration of triglycerides in the supernatant, T2: Concentration of triglycerides in the wash solution; T3: Concentration of triglycerides in fragmented cells solution.

cholesterol, triglycerides are found in the solution after cell lysis, although very low TRDs for certain strains such as CHTD27 and JUMIII4 (5.8 and 3%, respectively). Their triglycerides levels recorded in the fragmented cell solution (T3) are the highest (0.696 and 0.669 g/l) indicating the capture of triglycerides by these strains within the cells, thereby reducing the concentration of triglycerides in the external medium. NSC5C strain shows the highest TRDs (80.3%) for which the triglycerides concentrations in T3 are very low (0.058 g/l). These results reveal a difference between the mechanisms used by lactobacilli for triglyceride reduction.

Findings are consistent with those obtained by Gao and Li (2018) who also revealed this activity *in vitro* with triglyceride lowering rate for *Lb. acidophilus* L2- 73 and L2-16 and *Enterococcus faecalis* of 38.27% and 41.38% respectively.

As with cholesterol reduction, BSH activity may also be involved in triglycerides reduction *in vitro* and *in vivo*. Huang et al., (2013) results showed that BSH-active *Lb. plantarum* strains could reduce plasma total cholesterol, LDL-cholesterol and triglycerides in rats fed a high cholesterol diet.

When the organism overproduces cholesterol and triglycerides, the surplus is degraded to regulate their rate. Diet is also an important source of these two compounds; lactobacilli tested in this study can help the body reduce this excess in the intestinal lumen before absorption, thus preventing the risk of cardiovascular disease caused by excess lipids. The mechanisms by which triglycerides are degraded are not well known; there are currently very few studies on the elimination of triglycerides by lactobacilli *in vitro* (Gao and Li, 2018) or *in vivo* (Huang et al., 2013).

Hemolytic activity

In this study, none of the lactobacilli strains was able to hydrolyze human blood on Columbia medium, indicating that the strains are non-hemolytic. It means that strains do not possess the phosphatidyl-choline esterase enzyme that allows lysis of red blood cells, which indicates their safety on human health. It is well known that non-hemolytic bacteria are part of the microorganism group Generally Recorganized as Safe (GRAS), which is the case of LAB. Lactobacilli strains do not pose a health risk to animals or humans (Rychen et al., 2017; Olek et al., 2017; Chaves et al., 2017).

Conclusion

Among the 88 LAB isolated from camel milk or mare, only 3 strains of *Lactobacillus* show good *in vitro* probiotic and lipolytic capacities. The NSC5c strain of *Lactobacillus plantarum* isolated from camel milk shows the best performances during *in vitro* tests; indeed this strain is capable of surviving in gastrointestinal conditions, inhibiting pathogenic microorganisms and effectively degrading natural and synthetic lipids. The strain was also able to reduce *in vitro* cholesterol to more than 54% and triglycerides to more than 80%. Further studies are needed to elucidate these bacterial mechanisms in order to predict or specify lipid reduction mechanisms by probiotic strains observed in animal models or in clinical studies. The results certainly contribute to the knowledge of the potential to reduce lipid levels in rare strains of lactobacilli, which is an interesting property for probiotic strains that are candidates for use in food or feed.

This research is now proceeding with an *in vivo* study; they are actually testing the efficiency of the selecting lactic strains on Wistar rats receiving a high fat diet with and without addition of probiotic lactobacilli.

CONFLICT OF INTEREST

The authors Sabrina AMARA, Halima ZADI-KARAM and Nour-Eddine KARAM declare that they have no conflict of interest.

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ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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