Original Article

ISOLATION, IDENTIFICATION AND TECHNOLOGICAL PROPERTIES OF LACTIC ACID BACTERIA FROM RAW COW MILK

ISOLAMENTO, IDENTIFICAÇÃO E PROPRIEDADES TECNOLÓGICAS DE BACTÉRIAS ÁCIDO LÁCTICAS A PARTIR DE LEITE DE VACA CRU

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ABSTRACT: Lactic acid bacteria are used as starter culture for the production of fermented dairy products, and that occur naturally as indigenous microbiota of the raw milk. In this study, lactic acid bacteria were isolated from raw cow milk samples. The serial dilutions of raw milk were made and plated onto LM17 agar and MRS agar adjusted to pH 5.4. The isolates were firstly identified based on cell morphology, reaction to gram stain, catalase production, growth in MRS broth containing 2%, 4%, and 6.5% NaCl, growth ability of different temperatures and formation of gas in MRS broth. Acid formation in 10% reconstituted skim milk and antimicrobial activity against foodborne pathogens *Listeria monocytogenes* ATCC 7644, *Stapylococcus aureus* ATCC 25923, and *Clostridium perfringens* ATCC 13124 were examined using agar well diffusion method. The total of 90 LAB isolates were classified as *Lactobacillus* (37.78%), *Lactococcus* (36.67%), *Enterococcus* (20.00%), *Streptococcus* (4.44%), and *Leuconostoc* (1.11%). Based on technological properties, 56 of 90 isolates (42 cocci, 14 rods) were selected, and further identified at the species level using API 20 Strep and API 50 CH identification system, respectively. The *Lactobacillus* isolates were identified as *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus paracasei* ssp. *paracasei*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii* ssp. *lactis,* and *Lactobacillus rhamnosus*. The enzymatic profiles of the 17 selected isolates were studied with API ZYM system. The *Lactobacillus* spp. strains tested displayed high leucine arylamidase activity. Two *Lactococcus lactis* ssp. *lactis* AKS320.1 and AKS320.2 strains and one *Enterococcus faecalis* AKS424 strain were able to produce bacteriocin. In conclusion, some of these isolates could be considered as potential starter culture candidates for fermented milk products.

KEYWORDS: Lactic acid bacteria. Bacteriocin. Isolation. Identification. Raw cow milk.

INTRODUCTION

Lactic acid bacteria (LAB) are an industrially important group of bacteria and used as starter cultures for the production of fermented milk products (e.g. yoghurt and some cheeses) in the dairy industry. LAB are gram positive, non-sporeforming, catalase negative bacteria, and contain the following genera: *Aerococcus, Alloicoccus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Symbiobacterium, Tetragenococcus, Vagococcus* and *Weissella* (HORVATH et al., 2009). These groups of bacteria have been isolated from the raw materials of food, such as raw milk (CORROLER et al., 1998; RODRIGUEZ et al., 2000; TULINI et al., 2016; ASPRI et al., 2017; PERIN et al., 2017), plants (ALEMAYEHU et al., 2014), meat (EGAN, 1983), vegetables (KELLY et al., 1998), and fruits (CHEN et al., 2017).

Natural habitats, including the indigenous flora of raw milk, can be good source of novel LAB strains with the potential desirable properties for use in the production of novel fermented dairy products (CORROLER et al., 1998; RODRIGUEZ et al., 2000; WOUTERS et al., 2002; DELAVENNE et al., 2012; TULINI et al., 2016; PERIN; NERO, 2014). The most frequently isolated LAB genera from raw milk and dairy products that were made from raw milk were *Enteroccoccus, Lactococcus*, *Lactobacillus, Leuconostoc*, and *Streptococcus* (PERRY; SHARPE, 1960; FRANCIOSI et al., 2009). In another study, lactococcal strains were isolated from raw milk in Camembert cheese area and identified by using both phenotypic criteria (physiological and biochemical tests) and genotypic (RAPD) criteria. The raw milk microflora is also important for the ripening of the cheese (CORROLER et al., 1998). The population of non-starter lactic acid bacteria (NSLAB) in Cheddar cheese produced by commercial manufacturers in the United Kingdom were identified using commercially available identification system (API 50 CH system). *Lactobacillus paracasei* ssp. *paracasei* and *Lactobacillus plantarum* were the most frequently isolated species (WILLIAMS; BANKS, 1997). The major sources of bacteria in

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raw milk are from within the udder, the exterior of the teats and the udder, the milking machine, the storage equipments, the housing, bedding, feed, air and water (QUIGLEY et al., 2013).

The important metabolic activities of LAB used in the manufacture of fermented milk products are mainly the production of lactic acid from milk sugar lactose and other sugars, aroma compounds, and exopolysaccharides. Moreover, LAB produce several substances with the antimicrobial activity: organic acids (lactic acid, acetic acid etc.), hydrogen peroxide, diacetyl, acetaldehyde and bacteriocins (DEEGAN et al., 2006; MAYO et al., 2010; KARAKAS-SEN; AKYOL, 2012). The proteolytic activity of the dairy starter cultures is important for their growth in milk, the formation of flavour in dairy products, and the maturation of cheese (LAW; KOLSTAD, 1983; TULINI et al., 2016).

The collections of dairy relevant microorganisms originating from raw milk environment contain strains with the potential features for the novel dairy product development (WOUTERS et al., 2002). Therefore, the wild-type lactococci isolated from raw milk samples are a potential source of new strains with desired properties. Moreover, the artisanal, starter-free, traditional cheeses made with raw milk ripen faster and develop more intense flavour than cheeses made with pasteurized or microfiltered milk (CORROLER et al., 1998). In another study, wild lactococci strains isolated from raw milk, fermented milk and from non-dairy origin generally produced specific flavour different from those produced by industrial strains (AYAD et al., 1999).

Bacteriocins, ribosomally synthesized antimicrobial peptides produced by various bacteria, display antimicrobial activity against closely related bacterial strains, and are widely used as food preservative in food industry, in agriculture and veterinary medicine as a therapeutic (KARAKAS-SEN et al., 1999; DEEGAN et al., 2006). These peptides are sensitive to specific proteolytic enzymes and can be heat stable. Bacteriocin production ability was widespread among LAB isolated from raw milk (RODRIGUEZ et al., 2000; ASPRI et al., 2017). The strains producing bacteriocin nisin were most abundant among LAB isolated from raw cow, ewes and goat milks. Furthermore, lactococci producing lacticin 481 and enterococci producing enterocin AS-48 have also been found at high incidence (RODRIGUEZ et al., 2000). *Lactococcus lactis* strain producing bacteriocin nisin Z was isolated from a traditional fermented Nigerian dairy product, wara that made from raw cow milk (OLASUPA et al., 1999). Recently, ASPRI et al. (2017) reported the isolation of enterocin producing *E. faecium* strains from raw donkey milk.

It is important to have wild LAB isolates from the raw food materials and the fermented foods possibility to use these isolates as starter cultures in the production of fermented foods. Comparative analysis of LAB genomic data obtained by using next generation sequencing techniques showed a remarkable diversity in LAB group at numerous taxonomic levels, i.e. order, family, group, genus and even species (DOUILLARD; de VOS, 2014).

The objective of this study was to isolate the natural lactic acid bacteria from raw cow milk, identify, characterise and determine some technologically important properties of these natural LAB isolates in order to use as starter culture for the production of fermented milk products. The results show that raw cow milk is a good source of industrially important LAB.

MATERIAL AND METHODS

Isolation of lactic acid bacteria from raw cow milk

Strains of LAB were isolated from raw cow milk samples. A total of 11 raw cow milk samples were aseptically taken into the sterile bottles from bulk tanks at different dairy farms located in Bursa, Turkey. All raw milk samples were kept at 4ºC during transport to the laboratory. Serial dilutions of the milk samples were immediately made in ¼ Ringers solution (Oxoid), and were spread onto MRS agar (Difco) pH adjusted to 5.4 with acetic acid to facilitate the isolation of the genus *Lactobacillus*, and M17 agar (Difco) supplemented with % 0.5 lactose (LM17) to isolate other LAB (DUNCAN et al., 2004). In order to inhibit the growth of yeast and molds, natamax^{TM} (Danisco) was used at 200 mg l^{-1} concentration in the isolation plates. The plates were incubated for 48 h at 30ºC and 45ºC aerobic or under anaerobic conditions in an anaerobic jar (HP0011A, Oxoid) with anaerobic generation kit (BR38, Oxoid). After incubation, the individual colonies having a different morphology were picked and streaked on LM17 or MRS agar plates. Once the isolates were purified, phenotypic and other tests were carried out. The isolates were stored as frozen stocks at -20ºC in MRS broth containing 20% (v/v) glycerol.

Characterisation and identification of lactic acid bacteria isolates to genus level

Gram positive and catalase negative colonies were considered as members of lactic acid

bacteria and further studied. These isolates were firstly identified to the genus level by performing the following tests: cell morphology, growth for 7 d at 10ºC (for cocci), for 7 d at 15ºC (for rods) in a cooling incubator (JSR, Korea), and growth at 45ºC, ability to grow in MRS or M17 broth with 2%, 4% and 6.5% NaCl concentrations for 96 h, carbon dioxide production from glucose by subculturing the isolates in MRS broth with inverted Durham tubes (COGAN et al., 1997; HARRIGAN, 1998). After 48 h incubation at 37ºC, broth media were examined for growth, and the results were recorded. The isolates were also tested for motility in MRS agar in a test tube. Endospor staining was performed by Schaeffer-Fulton's method (SCHAEFFER; FULTON, 1933). Oxidase test was done with the test sticks (Merck, Millipore). The production of ammonia from arginine was tested with the method of HARRIGAN (1998). The ability of an isolate to produce acetoin (acetylmethylcarbinol) and diacetyl was checked by the application of the Voges-Proskauer test (*Barritt's* modification) (HARRIGAN, 1998).

Characterisation and identification of lactic acid bacteria isolates to species level

The isolates identified as the genera of lactic acid bacteria were further characterised at the species level by the use of API 20 Strep or API 50 CH carbohydrate fermentation strips (bioMérieux, France). The identity of the selected 42 lactococci, enterococci or streptococci, and 14 lactobacilli isolates was further assessed by the use of API 20 Strep kit (20600, bioMérieux) and API 50 CH kit using API 50 CHL media (50300, bioMérieux) according to manufacturer's instructions, respectively. The identification of the isolates was obtained using the apiweb^{TM} software (bioMérieux, France).

Acid production ability

Acid production ability was determined in sterile (for 30 m at 110ºC) 10% reconstituted skim milk (Oxoid, UK). A 1% inoculum from an overnight MRS culture was used to inoculate the milk. The cultures were incubated for 24 h at 37ºC and their pH were measured using a pH meter (pH 3210, WTW, Weilheim, Germany). The characteristics of the coagulum (curd firmness, curd breaking, whey amount, presence of gas bubbles) were also recorded by visual inspection (HARRIGAN, 1998).

Proteolytic activity and starch hydrolysis

Proteolytic activity was determined by streaking each strain onto a Milk agar plate which was prepared by addition of 10% reconstituted skim milk to Nutrient agar (Merck, Millipore). Inoculated plates were incubated for 18 h at 37ºC. The clear or strong opaque zone around the growth were evaluated as positive for the protease activity (HARRIGAN, 1998).

Starch hydrolysis was tested using Starch agar (Merck, Millipore). After overnight incubation, the plates were flooded with 5-10 ml iodine solution, and inspected the clear zone for amylase activity (HARRIGAN, 1998).

The antimicrobial activity of LAB isolates against foodborne pathogens

The antimicrobial activities of LAB isolates were tested by agar well-diffusion method (KARAKAS-SEN et al., 1999) using *Listeria monocytogenes* ATCC 7644, *Stapylococcus aureus* ATCC 25923, and *Clostridium perfringens* ATCC 13124 as the indicator strain. For some isolates, the indicator strains *Escherichia coli* EC100, MC1022, *Lb. bulgaricus* 572, which was isolated from Y572 yoghurt culture (Danisco), and the isolate *Lb. fermentum* AKS222.2 were also used.

The indicator strains were grown in Tryptic Soy Broth (TSB) supplemented with 0.3% yeast extract (TSBYE) at 37ºC, otherwise stated. *C. perfringens* was incubated anaerobically in an anaerobic jar. Molten soft TSAYE agar (0.7% agar) at 45ºC was seeded with the fresh overnight culture of the indicator strain. *Lb. bulgaricus* 572 and *Lb. fermentum* AKS222.2 were grown in MRS broth and MRS agar. The wells in agar were made with a sterile cork borer. In order to test antimicrobial activity, the isolated LAB strains were grown for 18 h at 37ºC in MRS broth (Difco). After incubation, the cultures were centrifuged at 10 000*g* for 15 m. The resulting supernatants were adjusted to pH 7.0 with 1 N NaOH and passed through a filter (0.22 µm). The cell free supernatants (75-150 µl) were transferred into the wells. After keeping the agar plate at 4ºC to allow diffusing into agar, the plates were incubated at 37ºC until the growth of the indicator strains. The agar plates seeded with the indicator strain *C. perfringens* were incubated in anaerobic jar. The plates were subsequently examined for zone of inhibition.

The cell free supernatants of three isolates (*Lc. lactis* ssp. *lactis* AKS320.1, AKS320.2, and *E. faecalis* AKS424) were tested for sensitivity to α-amylase, catalase, lipase, proteinase K, pepsin, α-chymotrypsin, trypsin, and papain enzymes (Sigma)

at the concentration of 10 mg/mL using the method of RYAN et al. (1996). The supernatants were incubated with the enzymes for 2 h at 37ºC. Following incubation, the level of antimicrobial activity retained was evaluated via the agar well diffusion method using *L. monocytogenes* ATCC 7644 as the indicator strain. Enzyme solutions were also applied into a well adjacent to wells containing the supernatants made on TSAYE agar plates seeded with the indicator strain. Protease sensitivity was observed as a half moon shaped zone of inhibition.

Enzyme activity

Enzyme activities of the selected 17 strains representing each strain of LAB isolates, were measured using the commercial, semiquantitative API ZYM system (bioMérieux) following the manufacturer's recommendations. Briefly, the sixty-five microliters of cell suspension equal to No:5 McFarland standard was transferred into each well of the API ZYM strips. The test strips were incubated for 4 h at 37ºC in a humid atmosphere, and developed by adding 1 drop of developing reagents ZYM A (Tris-hydroxymethyl-aminomethane 25 g; hydrochloric acid (37%) 11 ml; sodium lauryl sulphate 10 g; distilled water to 100 ml) and ZYM B (Fast blue BB 0.12 g; methanol 40 ml; dimethylsulfoxide (DMSO) 60 ml) to each of the wells. Colour development, indicating positive reactions, was graded with the reference to the API ZYM colour chart.

RESULTS AND DISCUSSION

Isolation and identification of lactic acid bacteria from raw cow milk

Lactic acid bacteria are mostly used as starter culture for the production of fermented dairy products, and that occur naturally as indigenous microbiota of the raw milk (ASPRI et al., 2017; PERIN et al., 2014). Therefore, the raw milk is considered as a good source for the isolation of LAB with technological potential (TULINI et al., 2016).

In this study, the raw cow milk samples were collected from the 11 different dairy farms located in Bursa, Turkey in order to isolate lactic acid bacteria. Gram-positive, catalase-negative, oxidase-negative and non-spore forming isolates were evaluated as lactic acid bacteria. 90 LAB were isolated from raw cow samples, and were identified as *Lactococcus* (36.67%), *Enterococcus* (20.00%), *Streptococcus* (4.44%), *Leuconostoc* (1.11%), and *Lactobacillus* (37.78%) based on physiological and biochemical characterization.

Table 1. Isolated wild lactic acid bacteria from raw cow milk identified at species level by API 20 Strep (for cocci), or API 50 CH (for rods) system

Species identified	Number of isolates	%[a]	%[b]	%[c]
Cocci				
Lactococcus lactis ssp. *lactis*	29	69.05		51.79
Enterococcus durans	5	11.91		8.93
Enterococcus faecalis	3	7.14		5.35
Enterococcus faecium	3	7.14		5.35
Enterococcus avium	1	2.38		1.79
Streptococcus salivarius	1	2.38		1.79
Total (Cocci)	42	100		75.00
Rods				
Lactobacillus acidophilus	1		7.14	1.79
Lactobacillus fermentum	1		7.14	1.79
Lactobacillus paracasei ssp. *paracasei*	5		35.71	8.93
Lactobacillus plantarum	2		14.29	3.57
Lactobacillus delbrueckii ssp. *lactis*	3		21.43	5.35
Lactobacillus rhamnosus	2		14.29	3.57
Total (Rods)	14			25.00
General Total	56		100	100

[a]Percentage of strains over the total of coccal shape strains. [b]Percentage of strains over the total of rod shape strains. [c]Percentage of strains over the total of isolated strains.

In general, the members of *Lactococcus* genus are able to grow a medium containing 4% NaCl, but not a medium containing 6.5% NaCl, and at 45ºC (COGAN et al., 1997). Our results revealed that some of wild-type *Lc. lactis* ssp. *lactis* strains grew weakly in MRS broth containing 6.5% NaCl. This is an agreement with the results of previous studies reporting that *Lc. lactis* ssp. *lactis* strains were isolated from plants (NOMURA et al., 2006; ALEMAYEHU et al., 2014). The ability of the wild lactococci strains to grow in the presence of 6.5% NaCl could be functional for the production of some cheeses, which contain relatively high salt concentrations.

Based on their technological profile, 56 LAB (Table 1) were selected, and further identified at strain level using the API commercial system (API 20 Strep or API 50 CH) (Figure 1). The identified coccal shape strains were *Lc. lactis* ssp. *lactis, E. durans, E. faecium, E. faecalis, E. avium,* and *S. salivarius* (Table 1; Table 2). Our results revealed that the most dominant LAB strain was *Lc. lactis* ssp. *lactis* (51.79%) and the most dominant *Lactobacillus* strain was identified as *Lactobacillus paracasei* ssp. *paracasei* (8.93%). All coccoid shape strains tested, except *Streptococcus salivarius* AKS301.1, fermented ribose. None of the strain fermented inulin. Only few strains tested fermented sorbitol. These LAB isolates were three *E. faecalis* strains (AKS313.1, AKS419, and AKS424), *E. faecium* AKS350, and *E. avium* AKS365. The identified *Lactobacillus* strains were *Lb. acidophilus*, *Lb. fermentum*, *Lb. paracasei* ssp. *paracasei*, *Lb. plantarum*, *Lb. delbrueckii* ssp. *lactis*, and *Lb. rhamnosus* (Table 1; Table 3). In the present study, only three isolates were belonged to heterofermentative group of *Lactobacillus* spp., and one of them was identified as *Lb. fermentum* AKS222.2 (Table 3). This is an agreement with the results of FRANCIOSI et al. (2009) reporting that LAB were isolated from raw cow milk in Italy. In another study, few lactobacilli were isolated from milk samples, and identified as *Lb. plantarum, Lb. casei, Lb. brevis,* and *Lb. fermenti* (PERRY; SHARPE, 1960). DELAVENNE et al. (2012) reported that the raw milk samples from cow, ewe and goat over one-year period, whatever their origin were dominated by Gram-positive catalase-negative cocci (presumptive lactococci and *Leuconostoc*), whereas enterococci and lactobacilli were present in significantly lower concentrations.

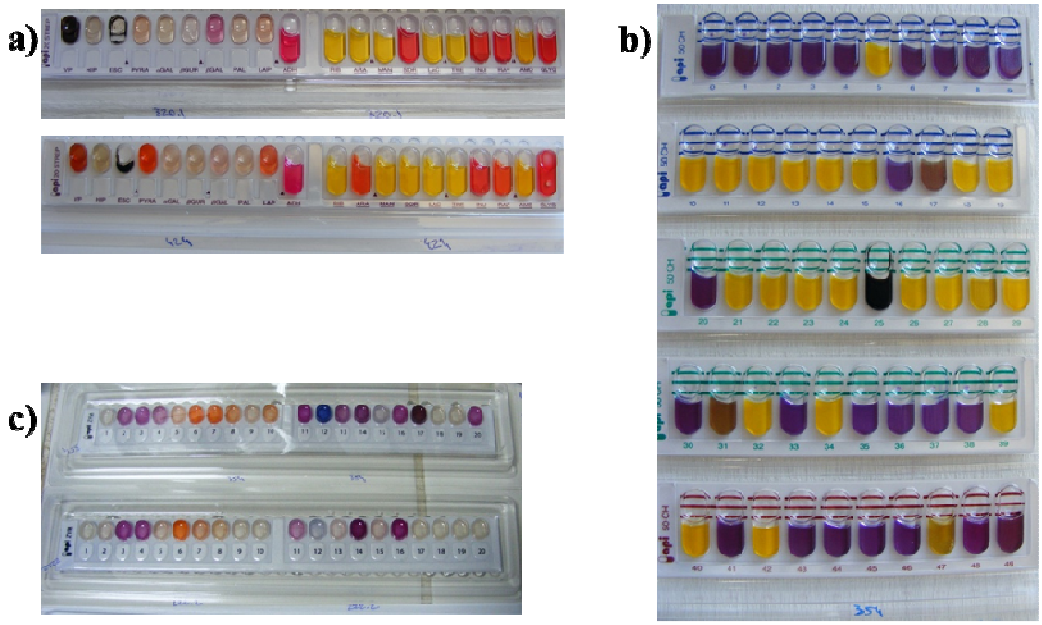

Figure 1. Identification of LAB isolates using **a)** API 20 Strep identification panels, inoculated with the isolates AKS320.1 and AKS424, and **b)** API 50 CH identification panel, inoculated with the isolate AKS354. **c)** API ZYM systems inoculated with the isolates AKS354 and AKS222.2 (see Materials and Methods section).

Technological properties

Acidification activity

Lactic acid bacteria are used as starter culture for the manufacture of fermented dairy products and some cheeses. Lactic acid production from milk sugar lactose is the main function of the dairy starter cultures. LAB play also an important role in the development of flavour and texture of dairy products (KARAKAS-SEN; AKYOL, 2012). Furthermore, LAB produce several antimicrobial substances, which make these bacteria valuable for food biopreservation. These features arouse interest the search of new strains with technological potential (TULINI et al., 2016).

Regarding to the ability to reduce the pH of 10% reconstituted skim milk after 24 h, the majority of *Lactobacillus* isolates were good acid producers than *Lactococcus* and *Enterococcus* isolates (Table 2; Table 3). Most of *Lactobacillus* isolates acidified milk with pH value below 4.00. Strain *Lb. delbrueckii* ssp. *lactis* AKS369 displayed the highest acidifying activity in milk with pH 3.67 (Table 3). Figure 2a shows the growth of some isolates in 10% reconstituted skim milk. Acidification ability in skim milk among the cocci was ranged between pH 4.33 and 5.31 (Table 2). In this respect, *Lactobacillus* isolates could be considered high acidifiers, as reported elsewhere (MONTEAGUDO-MERA et al., 2011).

Proteolytic activity and starch hydrolysis

The proteolytic system of LAB is crucial for the optimal growth in milk via the release of proteolytic enzymes. LAB possess a complex system of proteases and peptidases, which allow them to use milk protein casein as a source of amino acids and nitrogen (MAYO et al., 2010). Our results revealed that *Lactobacillus* isolates tested generally displayed proteolytic activity giving opaque zone on milk agar plates, while few *Lactobacillus* isolates (Table 3) showed clear zone on milk agar plate (Figure 2b). These results are agreement with the previous results reported by TULINI et al. (2016) which the strains were isolated from milks and cheese.

Starch hydrolysis was tested using starch agar plate. None of the strain displayed activity under that assay conditions. However, among *Lactobacillus* strains, only one *Lb. paracasei* ssp. *paracasei* AKS310.1 strain fermented starch when API 50 CH system was used. One possible explanation for the absence of amylase activity of the tested strain on starch agar plate is maybe due to the fact that the amylase activity is very low to be detected on agar plate compared to API kits.

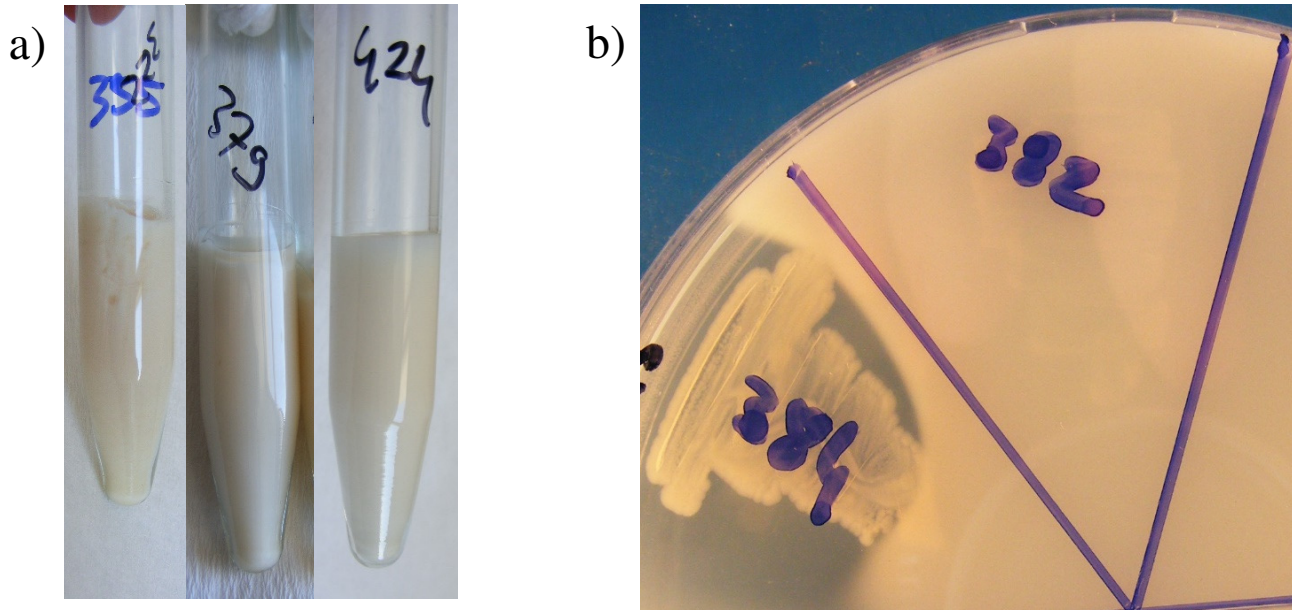

Figure 2. Some technological properties of LAB isolates: **a)** Growth of the isolates AKS355, AKS379 and AKS424 in reconstituted skim milk, **b)** Growth of the isolates AKS382 and AKS384 on milk agar.

Table 2. Some technological characteristics of the coccoid shape wild lactic acid bacteria isolated from raw cow milk

Strain code	Species	Growth in Milk[a] (pH)	Antimicrobial activity against		
	Identification by API 20 Strep		*S. aureus* ATCC 25923	*C. perfringens* ATCC 13124	*L. monocytogenes* ATCC 7644
AKS203.1	*Enterococcus durans*	4.71	+	–	–
AKS207.1	*Enterococcus faecium*	4.72	+	–	–
AKS208.1	*Enterococcus durans*	4.71	–	+	–
AKS216.1	*Enterococcus durans*	4.83	–	–	–
AKS236.2	*Lactococcus lactis* ssp. *lactis*	4.35	–	–	–
AKS240.2	*Enterococcus faecium*	4.74	–	+	–
AKS271.1	*Enterococcus durans*	4.81	–	–	–
AKS273.1	*Enterococcus durans*	4.73	–	–	–
AKS301.1	*Streptococcus salivarius*	4.62	–	+	–
AKS302.1	*Lactococcus lactis* ssp. *lactis*	4.73	–	+	+
AKS303.3	*Lactococcus lactis* ssp. *lactis*	4.50	–	–	+
AKS304.1	*Lactococcus lactis* ssp. *lactis*	4.49	–	–	–
AKS307.1	*Lactococcus lactis* ssp. *lactis*	4.80	–	–	–
AKS312.1	*Lactococcus lactis* ssp. *lactis*	4.54	–	–	+
AKS313.1	*Enterococcus faecalis*	5.31	–	–	–
AKS316.2	*Lactococcus lactis* ssp. *lactis*	4.48	–	–	+
AKS317.2	*Lactococcus lactis* ssp. *lactis*	4.74	–	–	–
AKS318.1	*Lactococcus lactis* ssp. *lactis*	4.52	–	–	–
AKS320.1	*Lactococcus lactis* ssp. *lactis*	4.74	+	+	+
AKS320.2	*Lactococcus lactis* ssp. *lactis*	4.77	+	+	+
AKS323.2	*Lactococcus lactis* ssp. *lactis*	4.63	–	–	+
AKS331.2	*Lactococcus lactis* ssp. *lactis*	4.53	–	+	–
AKS336.2	*Lactococcus lactis* ssp. *lactis*	4.94	–	–	–
AKS342.1	*Lactococcus lactis* ssp. *lactis*	4.44	–	+	–
AKS349.2	*Lactococcus lactis* ssp. *lactis*	4.56	–	–	–
AKS350	*Enterococcus faecium*	4.33	–	+	–
AKS360	*Lactococcus lactis* ssp. *lactis*	4.35	–	–	–
AKS365	*Enterococcus avium*	3.72	–	+	–
AKS402	*Lactococcus lactis* ssp. *lactis*	5.20	–	–	+
AKS406	*Lactococcus lactis* ssp. *lactis*	4.33	–	–	+
AKS409	*Lactococcus lactis* ssp. *lactis*	4.41	–	–	–
AKS410	*Lactococcus lactis* ssp. *lactis*	4.37	–	–	–
AKS411	*Lactococcus lactis* ssp. *lactis*	4.80	–	–	–
AKS413	*Lactococcus lactis* ssp. *lactis*	4.50	–	–	+
AKS418	*Lactococcus lactis* ssp. *lactis*	5.02	–	–	+
AKS419	*Enterococcus faecalis*	5.01	–	+	–
AKS420	*Lactococcus lactis* ssp. *lactis*	5.04	–	–	+
AKS422	*Lactococcus lactis* ssp. *lactis*	4.51	+	–	–
AKS423	*Lactococcus lactis* ssp. *lactis*	4.34	–	–	–
AKS424	*Enterococcus faecalis*	4.92	–	+	+
AKS425	*Lactococcus lactis* ssp. *lactis*	4.37	–	–	–
AKS426	*Lactococcus lactis* ssp. *lactis*	4.59	–	–	–

+, positive; –, negative. All of LAB strains were cocci, gram positive, catalase negative, oxidase negative. [a]Growth in sterile reconstituted skim milk.

Table 3. Some technological characteristics of the rod shape wild lactic acid bacteria isolated from raw cow milk

Strain code	Genus	Species: Identification by API 50 CH	Gas production[a]	Growth in Milk[b] (pH)	Growth on Milk Agar	Antimicrobial activity against: *S. aureus* ATCC 25923	Antimicrobial activity against: *C. perfringens* ATCC 13124	Antimicrobial activity against: *L. monocytogenes* ATCC 7644
AKS209.1	*Lactobacillus*		+	5.79	+[c]	–	–	–
AKS210.2	*Lactobacillus*		–	3.76	ND	–	–	–
AKS212.2	*Lactobacillus*		–	3.81	+[c]	–	+	–
AKS213.1	*Lactobacillus*		+	5.34	+[c]	–	+	–
AKS215.2	*Lactobacillus*		–	3.80	ND	–	+	–
AKS222.2	*Lactobacillus*	*Lb. fermentum*	+	4.94	+[c]	+	+	+
AKS255.1	*Lactobacillus*		–	4.73	ND	+	–	–
AKS310.1	*Lactobacillus*	*Lb. paracasei* ssp. *paracasei*	–	5.06	+[c]	–	+	+
AKS351	*Lactobacillus*		–	3.74	+[c]	–	–	–
AKS352	*Lactobacillus*	*Lb. delbrueckii* ssp. *lactis*	–	5.33	+[o]	–	–	–
AKS353	*Lactobacillus*		–	4.36	+[o]	–	+	–
AKS354	*Lactobacillus*	*Lb. rhamnosus*	–	3.98	+[o]	+	+	+
AKS355	*Lactobacillus*		–	4.19	+[o]	–	–	–
AKS356	*Lactobacillus*		–	6.03	+[c]	+	+	–
AKS357	*Lactobacillus*		–	4.85	–	+	–	–
AKS358	*Lactobacillus*	*Lb. rhamnosus*	–	3.69	+[o]	–	–	–
AKS359	*Lactobacillus*		–	5.97	+[o]	–	–	–
AKS361	*Lactobacillus*		–	3.67	+[o]	–	–	–
AKS362	*Lactobacillus*		–	5.59	–	–	–	–
AKS363	*Lactobacillus*		–	3.83	+[o]	+	–	–
AKS364	*Lactobacillus*	*Lb. acidophilus*	–	3.81	+[c]	–	–	–
AKS367	*Lactobacillus*	*Lb. paracasei* ssp. *paracasei*	–	4.57	+[o]	+	+	–
AKS369	*Lactobacillus*	*Lb. delbrueckii* ssp. *lactis*	–	3.67	+[c]	+	+	+
AKS370	*Lactobacillus*		–	3.76	+[c]	–	–	–
AKS371	*Lactobacillus*		–	3.75	+[c]	+	–	–
AKS372	*Lactobacillus*	*Lb. delbrueckii* ssp. *lactis*	–	3.74	+[c]	–	–	–
AKS379	*Lactobacillus*		–	3.74	+[c]	–	–	+
AKS382	*Lactobacillus*	*Lb. plantarum*	–	3.90	–	+	+	+
AKS384	*Lactobacillus*		–	4.80	+[c]	–	+	–
AKS385	*Lactobacillus*		–	4.40	ND	–	–	–
AKS386	*Lactobacillus*	*Lb. paracasei* ssp. *paracasei*	–	3.80	+[o]	+	+	+
AKS387	*Lactobacillus*	*Lb. paracasei* ssp. *paracasei*	–	3.74	+[o]	+	+	+
AKS389	*Lactobacillus*	*Lb. plantarum*	–	4.10	+[c]	+	+	+
AKS400	*Lactobacillus*	*Lb. paracasei* ssp. *paracasei*	–	3.74	+[o]	+	+	+

+, positive; –, negative. All of LAB isolates were rod shape, gram-positive, catalase negative, oxidase negative and non-spore containing. [a]Gas production assay was conducted in MRS broth with inverted Durham tubes at 37°C for 2 days. [b]Growth in sterile reconstituted skim milk; +[o] opaque zone on milk agar; +[c] clear zone on milk agar. ND, Not Determined.

Antimicrobial activity of the LAB isolates

LAB produce some antimicrobial substances (lactic acid, diacetyl, bacteriocins etc.) active against spoilage and pathogen microorganisms (DEEGAN et al., 2006; KARAKAS-SEN; AKYOL, 2012). Our results revealed that most of LAB isolates showed antimicrobial activity against at least one of the food pathogens tested (Table 2; Table 3; Table 4). The highest percentage (32.22%) of the isolates displayed an inhibitory activity against *C. perfringens* ATCC 13124. This was followed by the antimicrobial activity against *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 with 26.67% and 23.33%, respectively (Table 4).

Table 4. Antimicrobial activity of lactic acid bacteria isolates from raw cow milk

Isolates	Number tested	Antimicrobial activity against					
		***L. monocytogenes* ATCC 7644**		***S. aureus* ATCC 25923**		***C. perfringens* ATCC 13124**	
		No	**%[a]**	**No**	**%[a]**	**No**	**%[a]**
Cocci							
Lactococcus	33	12	36.36	3	9.10	5	15.15
Enterococcus	18	2	11.11	4	22.22	7	38.89
Streptococcus	4	0		0		1	25.00
Leuconostoc	1	0		0		0	
Total	56	14	25.00	7	12.50	13	23.21
Rods							
Lactobacillus	34	10	29.41	14	41.18	16	47.06
General Total	90	24	26.67	21	23.33	29	32.22

[a]Percentage of total isolates showing antimicrobial activity against indicator strain.

Based on the diameter of halos (mm) using agar well assays, 3 LAB isolates (*Lc. lactis* ssp. *lactis* AKS320.1, AKS320.2, and *E. faecalis* AKS424) displayed a high antimicrobial activity were further studied (Figure 3a, b). The nature of inhibitory activities was evaluated with α-amylase, catalase, lipase and some proteases: proteinase K, pepsin, α-chymotrypsin, trypsin and papain. The antibacterial activity of *E. faecalis* AKS424 was completely inactivated by α-chymotrypsin, pepsin and proteinase K (Figure 3b), and partially inactivated by lipase. Heat treatment (for 5 m at 100ºC) slightly reduced the activity. The diameter of zone of inhibition without and with heat treatment was 14.00 mm, and 12.16 mm, respectively. Moreover, the inhibitory activity of the bacteriocin produced by *E. faecalis* AKS424 was evaluated against gram-negative bacteria *E. coli* EC100 and *E. coli* MC1022. Although this bacteriocin was not active against gram-negative bacteria tested, it was active against *Lb. bulgaricus* 572, and the isolate *Lb. fermentum* AKS222.2.

Bacteriocins are antimicrobial peptides and sensitive to proteolytic enzymes. Our results revealed that the antibacterial activities of two *Lc. lactis* ssp. *lactis* AKS320.1, AKS320.2 and *E. faecalis* AKS424 strains were completely inactivated by proteinase K. Therefore, the proteinaeous nature of the antimicrobial substances produced by these three isolates was established their sensitivity to proteolytic enzyme protease K. Similar results have been recorded for bacteriocins produced by *E. faecalis* and *Lc. lactis* (PERIN; NERO, 2014; ASPRI et al., 2017). Our results revealed that amongst the tested LAB, *Lc. lactis* ssp. *lactis* AKS320.1 and AKS320.2 and *E. faecalis* AKS424 demonstrated a good potential for the application as starter or probiotic culture in human and veterinary feeds.

The author EDEN (2014) reported that *E. faecium* and *E. faecalis* strains have been prepared as commercial probiotic supplements by SymbioPharm, Germany and Cerbios Pharma SA, Switzerland, respectively. In addition, *E. faecalis* Symbioflor I had been consumed by humans as a probiotic strain without adverse effects for more than 50 years. The results from another study (MORENO et al., 2006) demonstrated that *E. faecium* SF68 was effective in the prevention of antibiotic-associated diarrhea, and was patented as probiotic. Recently, a new *E. faecium* strain isolated from raw camel milk exhibited important probiotic characteristics such as high cholesterol removal percentages, capability to inhibit pathogens, reasonable acid and bile tolerance, and sensitivity to antibiotics with no haemolysis (AYYASH et al.,

2018). Regarding to the ability of bacteriocin production, LAB may constitute an ecological advantage to be predominant in raw milk (RODRIGUEZ et al., 2000).

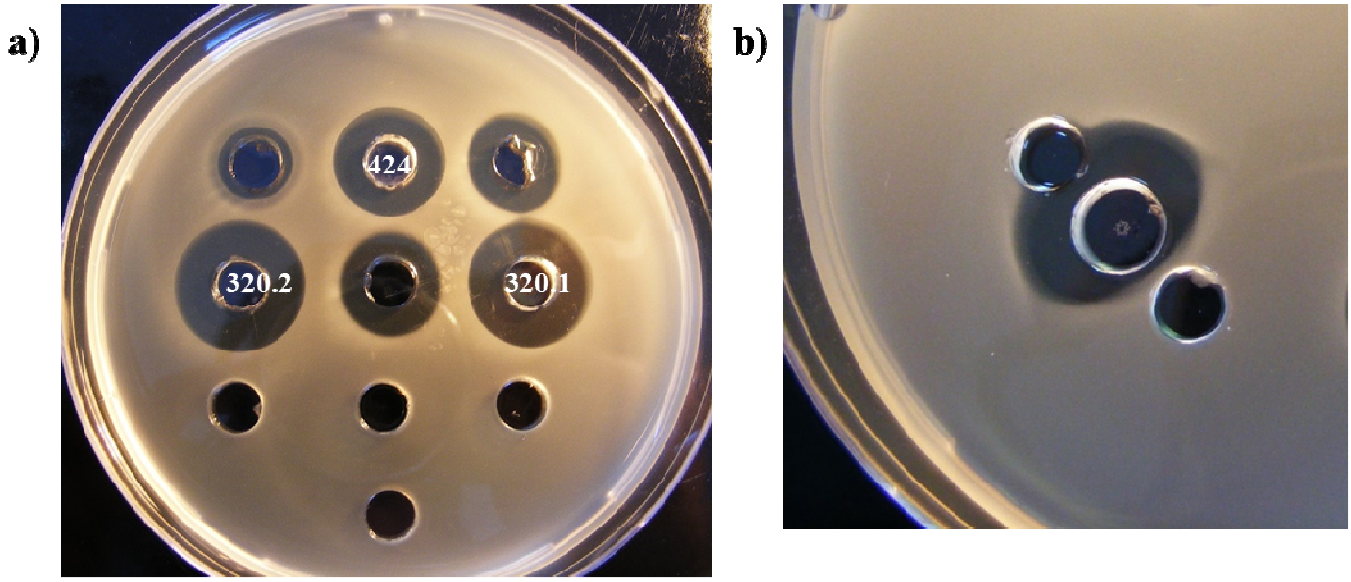

Figure 3. The cell free supernatant activity of LAB isolates as monitored by agar well-diffusion assay using *L. monocytogenes* ATCC 7644 as the indicator strain. **a)** The supernatants of *Lc. lactis* ssp. *lactis* AKS320.1, AKS320.2, and *E. faecalis* AKS424 were marked as 320.1, 320.2, and 424, respectively. **b)** Effect of the protease enzyme proteinase K on the antibacterial activity of the cell free supernatant of *E. faecalis* AKS424.

Enzyme activity

To compare the biochemical properties of the isolated strains, the enzyme activities of the selected 17 strains (8 cocci, 9 *Lactobacillus* spp.) were tested using API ZYM system (Figure 1c). The results showed that the strains tested were positive for 18 of 19 enzymes activities (Table 5). No activity was detected for the enzyme α-mannosidase. Our results revealed that the enzyme activities have varied among the strains and between the same strains. All strains tested showed leucine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase activities. Amongst the 17 LAB strains tested, *Lactobacillus* spp. strains displayed a high leucine arylamidase activity. These findings are in agreement with results reported by other authors for *Lactobacillus* spp. isolated from raw milks (MONTEAGUDO-MERA et al., 2011). The enzyme leucine arylamidase (EC 3.4.11.2) catalyses the removal of an N-terminal leucine from arylamides or p-nitroanilides (MÜLLER et al., 2004). Regard to the development of desirable flavours, aminopeptidase enzyme activity is an important technological characterisation for the strains of LAB planned for use as starter for the production of cheese (MONTEAGUDO-MERA et al., 2011).

The enzyme activities for α-galactosidase, β-galactosidase, β-glucuronidase, and β-glucosidase were only detected for the strains of *Lactobacillus* genus. Amongst *Enterococcus* spp. and *Lactococcus* spp. strains tested, none of them displayed β-glucuronidase activity, while 4 isolates (AKS222.2, AKS212.2, AKS354 and AKS369) of 9 *Lactobacillus* spp. displayed very low β-glucuronidase activity. The authors (GILL; ROWLAND, 2002) reported that β-glucuronidase enzyme has been associated with the stimulation of colon cancer by converting precarcinogens into proximal carcinogens. Therefore, the lack of β-glucuronidase enzyme activity is an asset for the potential use of these strains as dairy starter cultures.

β-Galactosidase activity was found at the highest values in the strains of *Lactobacillus* spp., but it was absent in the other strains tested (Table 5). Our results revealed that amongst LAB tested, the strains *Lb. plantarum* AKS382 and *Lb. plantarum* AKS389 displayed N-Acetyl-β-glucosaminidase enzyme activity. These results are agreement with the results reported by PULIDO et al. (2007) for some *Lb. plantarum* strains isolated from caper berry fermentations.

Table 5. Enzyme activities of the selected lactic acid bacteria strains isolated from raw cow milk determined using the API-ZYM system (bioMérieux, France)

Strain	Enzyme tested[a]																	
	Alkaline phosphatase	Esterase (C 4)	Esterase Lipase (C 8)	Lipase (C 14)	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsin	α-Chymotrypsin	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	α-Galactosidase	β-Galactosidase	β-Glucuronidase	α-Glucosidase	β-Glucosidase	N-acetyl-β-glucosaminidase	α-Fucosidase
Cocci																		
Enterococcus durans AKS273.1	0	4	2	0	4	1	1	0	1	4	1	0	0	0	0	0	0	0
Lactococcus lactis ssp. *lactis* AKS320.2	1	1	2	0	2	0	0	0	0	5	2	0	0	0	0	0	0	0
Lactococcus lactis ssp. *lactis* AKS323.2	1	0	0	0	4	1	1	0	1	5	2	0	0	0	0	0	0	0
Lactococcus lactis ssp. *lactis* AKS331.2	0	1	1	1	3	1	1	0	1	5	2	0	0	0	0	0	0	0
Lactococcus lactis ssp. *lactis* AKS349.2	0	2	2	0	4	0	2	0	2	5	2	0	0	0	0	0	0	0
Enterococcus faecium AKS350	0	4	3	0	3	1	1	0	0	1	3	0	0	0	0	0	0	0
Enterococcus faecalis AKS419	1	4	3	0	2	0	0	0	2	4	3	0	0	0	1	0	0	0
Enterococcus faecalis AKS424	0	3	3	0	3	0	1	0	2	2	2	0	0	0	0	0	0	0
Rods																		
Lactobacillus fermentum AKS222.2	0	4	4	0	5	1	1	0	0	2	1	1	5	1	5	0	0	0
Lactobacillus rhamnosus AKS354	5	5	4	3	5	5	3	1	3	5	5	4	5	1	5	5	0	4
Lactobacillus acidophilus AKS364	0	2	0	0	3	0	0	0	0	3	3	2	1	0	2	2	0	0
Lactobacillus paracasei ssp. *paracasei* AKS367	1	2	2	0	5	5	1	0	0	5	4	0	5	0	5	5	0	0
Lactobacillus delbrueckii ssp. *lactis* AKS369	0	2	1	1	5	2	2	0	0	2	2	1	5	1	1	1	0	0
Lactobacillus delbrueckii ssp. *lactis* AKS372	0	3	1	0	5	2	2	0	2	5	5	0	2	0	5	5	0	0
Lactobacillus plantarum AKS382	3	2	1	1	5	4	4	0	0	5	4	0	5	0	0	5	5	0
Lactobacillus plantarum AKS389	3	2	1	1	5	5	5	0	0	5	4	0	5	0	5	5	5	0
Lactobacillus AKS212.2	3	1	0	1	5	1	1	0	0	5	5	1	5	1	0	0	0	0

[a]Enzyme activities are expressed as colour intensity under assay conditions. 1, 2, 3, 4, and 5 indicates increasing levels of enzyme activity, equivalent to 5, 10, 20, 30 and ≥ 40 Nano moles of hydrolysed substrate after 4 h incubation at 37ºC; 0, no activity detected. No activity was detected for the enzyme α-mannosidase.

L-Fucosidases (EC 3.2.1.51), which are exoglycosidases capable of cleaving-linked L-fucose residues from fucosyloligosaccharides, play the important roles in the adaptation of bacteria to the particular niches (BECKER; LOWE, 2003). L-fucose (6-deoxy-L-galactose) is a monosaccharide that occurs at the nonreducing end of many glycans on mammalian cell surfaces, intestinal mucin, blood group antigens, and human milk oligosaccharides (HMO). The analysis of API ZYM results revealed that only *Lb. rhamnosus* AKS354 strain showed α-L-fucosidase enzyme activity. Our results are agreement with the previous study (BECERRA et al., 2015) reported the characterisation of L-fucose operon a probiotic strain *Lb. rhamnosus* GG. In another study, *Lb. rhamnosus* ATCC 53103 strain showed α-fucosidase enzyme activity that helps the colonization in the intestine (MONTEAGUDO-MERA et al., 2011). Only one strain *Lb. rhamnosus* AKS354 displayed trypsin activity at very low level (Table 5). The lack or low levels of trypsin enzyme activity is a positive aspect for strains which are intended to be used as starter or probiotic cultures (MONTEAGUDO-MERA et al., 2011). The strain *Lb. rhamnosus* AKS354 showing high α-fucosidase and low trypsin enzyme activities may be a good candidate for probiotic strain.

CONCLUSIONS

The diversity of LAB in the raw cow milk indigenous microbiota exhibits a good source of new bacterial strains with interesting technological properties.

Bacteriocin producing strains could be good candidates for the application of milk fermentations, bio-preservation or probiotic organism.

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RESUMO: Bactérias de ácido lático são utilizadas como cultura inicial para a produção de produtos lácteos fermentados e que ocorrem naturalmente como microbiota indígena do leite cru. Neste estudo, as bactérias ácido lácticas foram isoladas a partir de amostras de leite cru de vaca. As diluições em série do leite cru foram feitos e plaqueadas em ágar LM17 e agar MRS ajustado para pH 5,4. Os isolados foram primeiramente identificados com base na morfologia das células, coloração pelo Método de Gram, produção de catalase, crescimento em caldo MRS contendo 2%, 4%, e 6,5% de NaCl, a capacidade de crescimento de diferentes temperaturas e formação de gás em caldo MRS. Foram avaliadas a formação de ácido em 10% de leite desnatado reconstituído e propriedades antagonistas contra os agentes patogênicos de origem alimentar *Listeria monocytogenes* ATCC 7644, *Stapylococcus aureus* ATCC 25923, e *Clostridium perfringens* ATCC 13124, usando o método de difusão em agar. O total de 90 isolados de laboratório foram classificados como *Lactobacillus* (37,78%), de *Lactococcus* (36,67%), *Enterococcus* (20,00%), *Streptococcus* (4,44%), e *Leuconostoc* (1,11%). Com base em propriedades tecnológicas, foram selecionados 56 dos 90 isolados (42 cocos, 14 hastes) e identificados ao nível das espécies usando o sistema de identificação API 20 Strep e API 50 CH, respectivamente. Os isolados de *Lactobacillus* foram identificados como *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus paracasei* ssp. *paracasei*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii* ssp. *lactis* e *Lactobacillus rhamnosus*. Os perfis enzimáticos dos 17 isolados selecionados foram estudados por meio do sistema API ZYM. As cepas de *Lactobacillus* possuem alta atividade de arilamidase leucina. Duas cepas de *Lactococcus lactis* ssp. *lactis* AKS320,1 e AKS320,2 cepas e um *Enterococcus faecalis* AKS424 estirpe foram capazes de produzir bacteriocina. Em conclusão, alguns destes isolados poderiam ser considerados como potenciais candidatos de cultura iniciadora para produtos à base de leite fermentados.

PALAVRAS-CHAVE: Bactérias do ácido láctico. Bacteriocina. Isolamento. Identificação. Leite cru de vaca.

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