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PAGES: 375-379

ORIGINAL PDF URL: <https://dergipark.org.tr/tr/download/article-file/3055149>



Identification and Technological Characterisation of Lactic Acid Bacteria Isolated From Traditional Algerian Cheese “J’ben”

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ARTICLE INFO

Article history:

Received date: 28.02.2022

Accepted date: 30.08.2022

Keywords:

J’ben cheese

Identification

Lactic acid bacteria

Technological properties

ABSTRACT

Lactic acid bacteria have long been utilized in fermented foods and dairy products such as cheese, these bacteria play an important role in food bio-preservation, organoleptic properties development, and quality improvement. The purpose of this research was to determine and assess the biotechnological characteristics of lactic acid bacteria isolated from traditional Algerian cheese "J'ben". Fifteen lactic acid bacteria (gram positive, catalase negative) were molecularly identified according to their 16S rDNA sequences, six belonged to *Enterococcus durans*, three to *Enterococcus faecium*, three to *Lactococcus lactis*, and three to *Leuconostoc mesenterioide*. The strains were evaluated for proteolysis, lipolysis, antibacterial activity, exopolysaccharide synthesis, and safety (hemolytic activity). All studied strains had considerable proteolytic activity but no lipolysis potential, they were also all γ -hemolytic. The antimicrobial activity of the stains against three pathogenic bacteria (*Staphylococcus warneri*, *Serratia plymuthica*, and *Enterobacter aerogenes*) revealed that they were active against at least one of them. Finally, only three organisms produced exopolysaccharide in our study *Enterococcus durans* (KC1); *Leuconostoc mesenterioide* (KC6); and *Lactococcus lactis* (KC15). These findings suggest that the lactic acid bacteria isolated from traditional cheese "J'ben" have significant technological properties, making them suitable for use as starter culture in fermented dairy products.

1. Introduction

The microbial flora, particularly lactic acid bacteria (LAB), is an important element in the production of fermented foods and fermented milk products such as cheese. J’ben is a traditional soft white cheese prepared from non-pasteurised raw milk (sheep, goat, or cow). J’ben is a popular cheese in rural regions due to its unique taste and nutritional properties offered by its indigenous flora (Bousbia et al., 2018; Dahou et al., 2021). Lactic acid bacteria are a group of organisms that are Gram-positive, non-sporulating, cocci, coccobacilli, or rods, catalase negative, and have the common property of producing lactic acid. They are classified into the following genera: *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Leuconostoc*, *Tetragenococcus*, *Aerococcus*, *Carnobacterium*, *Weissella*, *Alloiococcus*, *Symbiobacterium*, and *Vagococcus* (Pfeiler & Klaenhammer, 2007; Kocková et al., 2011; Wedajo, 2015; Toe et al., 2019).

LAB are used to improve organoleptic properties, as probiotic organisms, and as bio-conservation agents (Wedajo, 2015; Karakas-Sen & Karakas, 2018; Göktepe & Elgün, 2020). The most isolated Lactic acid bacteria from raw milk and dairy products are *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Streptococcus* (Franciosi et al., 2009; Karakas-Sen & Karakas, 2018).

The biodiversity of LAB is a crucial factor that has a direct influence on the characteristics and quality of artisanal products (Franciosi et al., 2009). Many variables influence the formation of cheese's organoleptic features, including the milk source and its microbiota, the processes and techniques used to make the cheese, ripening circumstances and others. However, lactic acid bacteria have the most important role in cheese aroma, texture, and flavor by generating substances such as exopolysaccharides (EPS) (Dal Bello et al., 2001; Herreros et al., 2003; Gobbetti et al., 2018).

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The LAB has the desired effect on cheese by developing sensory characteristics that are connected to metabolic processes like proteolysis and lipolysis during maturation (Bruno & Carvalho, 2009; Luiz et al., 2016; Farahani et al., 2017; Asensio-Vegas et al., 2018).

The LAB contributes to the proteolysis of cheese; they have proteinases enzymes associated with their cell wall that can degrade casein into peptides, and these free peptides of casein are hydrolyzed to free amino acids by the action of peptidases. The proteolysis process is important for the growth of LAB themselves, the development of flavor in dairy products, and the maturation of cheese (Tulini et al., 2016; Toe et al., 2019).

Lipolysis is the hydrolysis of triglycerides to produce glycerol, glycerides, and free fatty acids; this metabolic process aids in the development of texture, flavor, and aroma in products such as cheeses (Collins et al., 2003; Esteban-Torres et al., 2014).

Because of the many enzymatic reactions, fermentation is a complicated process (García-Cano, 2019). Furthermore, fermentation reduces pH and produces antimicrobial chemicals such as lactic acid, hydrogen peroxide, diacetyl, acetaldehyde, reuterin, and peptides as a result of lipolysis and proteolytic enzyme activity (Paggianni, 2012; Burgain et al., 2014).

The objectives of this paper were to characterize some technological properties of lactic acid bacteria isolated from traditional Algerian cheese, including their ability to produce exopolysaccharides as well as their proteolytic, lipolysis, haemolytic, and antimicrobial activity for use in fermented dairy products.

2. Materials and Methods

Samples collection and Bacterial isolation

Traditional cheese "J'ben" samples were collected from Naâma region, south-west of Algeria. Samples were aseptically obtained and transported to the laboratory in isotherm container at 4°C under sterile conditions.

For each sample, an initial dilution of 10g and 90 mL of physiological water was made, followed by homogenization in a Stomacher (seward STOMACHER, England). Successive decimal dilutions were made up to 10^{-7} , and 100 µL of each dilution was disseminated onto MRS and M17 agar (pH 6.5). The plates were incubated for 48 to 72 hours at 30°C under both aerobic and anaerobic conditions. Individual colonies with varied morphologies were transferred to M17 or MRS agar plates and were purified by sequential streaking into the same medium. The pure isolates of lactic acid bacteria were kept at 4°C and were renewed every month. The purified isolates were stored at -20°C in MRS or M17 broth containing 20% (v/v) glycerol.

Characterization and molecular identification of lactic acid bacteria isolates

The selected isolates were subjected to preliminary identification of morphology, gram staining, and catalase testing. The isolates that were gram positive, catalase negative, were considered to be lactic acid bacteria.

Fifteen isolates presenting characteristics of lactic acid bacteria were molecularly identified using 16S rDNA amplification and sequencing as follow:

Extraction of total DNA of isolates.

Amplification of the DNA fragments by PCR in a Thermocycler (Biorad, USA) using specific primer (Qbiogene Research Service, Germany) for the 16S rDNA sequences, the following three steps were repeated for 35 cycles: denaturation at 94°C for 3 min, annealing at 53°C for 1 min, and extension at 72°C for 2 min. then the final extension at 72°C for 5 min. The amplified fragment was screened on agarose gel.

Sanger sequencing technique was used to sequence the amplified fragment of DNA. the resulting sequences were matched with GeneBank data, using the NCBI Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>)

Proteolytic and lipolysis activity

The selected stains were screened for their Proteolytic activity on skim milk agar medium (casein 0.5%, yeast extract 0.25%, dextrose 0.1%, and agar 1.5%) supplemented with 10% of reconstituted skim milk. Wells of 6 mm diameter were prepared on the plate. Each well was inoculated by 50 µL of bacterial culture. Plates were then incubated at 30°C for overnight. The existence of a clear or opaque zone surrounding the wells indicated positive protease activity.

Tributyrin agar was used to assess lipolysis activity. Plates were incubated at 30°C for overnight. Positive activity was defined as the occurrence of clean zones surrounding the wells in Tributyrin Agar (Oliveira et al., 2020).

Exopolysaccharide production

Strains were cultivated on modified MRS medium with 50g/L of sucrose as described by Franciosi et al. (2009), and after 72 hours of incubation at 30°C, the plates were tested for the emergence of mucoid properties.

Haemolytic activity

Haemolysis was assessed using Columbia blood agar plates supplemented with 5% sheep blood and incubated for 48 hours at 30°C according to Jikang and Wenxiang, (2019). Plates were examined for the presence of β -haemolysis (clear zones around colonies), α - haemolysis (green-hued zones around colonies), or γ -haemolysis (no zones around colonies).

Antimicrobial activity

The antimicrobial activity of the isolates was tested against three subclinical mastitis pathogen germs (*Staphylococcus warneri*, *Serratia plymuthica* and *Enterobacter aerogenes*) isolated from subclinical cases of intramammary infection on LSTPA lab by Meskini et al. (2021). Agar well diffusion method was used to evaluate the antimicrobial activity of LAB according to Bhola &

Bhadekar, (2019). Briefly, a culture broth of 0.5 OD (McFarland Standard) of LAB and pathogen bacteria was prepared; indicator bacteria were swabbed on agar medium containing TSA+MRS (1:1). Wells of 6 mm diameter were prepared on these pre-swabbed medium and 50 µl of each LAB strain was inoculated in the corresponding well. The plates were then incubated overnight at 37°C then observed for the presence of inhibition halos.

3. Results and Discussion

After preliminary identification, fifteen isolates showing appearances with lactic acid bacteria were chosen at random for further testing. The isolates were all gram positive, catalase negative, and cocci shaped. The 16S rDNA gene sequence data revealed that isolates KC1, KC5, KC7, KC9, KC10, and KC13 were 100% similar to *Enterococcus durans*, whereas isolates KC2, KC3, and KC4 were 100% similar to *Enterococcus faecium*. KC6, KC8, and KC11 showed 100% resemblance to *Leuconostoc mesenteroides*, whereas KC12, KC14, and KC15 had 100% similarity to *Lactococcus lactis*.

Proteolytic and lipolytic activities are highly desired criteria in LAB for use as starter culture in cheese production since these two processes occur and play a vital role during cheese maturation (Bruno & Carvalho, 2009). The proteolytic system is critical in fermentation processes and allows the growth of LAB in milk; this system contains enzymes that hydrolyze casein and provide necessary amino acids to cells (Mayo et al., 2010; Balthazar et al., 2017; Farahani et al., 2017; Karakas-

Sen & Karakas, 2018). The proteolytic system also contributes to the formation of several organoleptic properties in various fermented milk products (Savijoki et al., 2006; Gobetti et al., 2018). In this study, lactic acid bacteria strains were tested for protease and lipolysis activities (Table 1). The results showed that all tested strains exhibited proteolytic activity by giving a proteolysis zone on milk agar plates, 06 strains of *E. durans* presented a diameter zone ranging between 14 and 23.5 mm, 03 strains of *E. faecium* between 12.5 to 14.5 mm, 03 strains of *Lc. lactis* between 12 to 12.5 mm, and 03 strains of *Leu. mesenteroides* between 17.5 to 22.5 mm, while none of the fifteen tested strains exhibited lipolysis activity in Tributyrin agar medium (Table1). Mechai et al. (2014) found that only two strains of the genus *Lactococcus* isolated from fermented milk and traditional cheese had strong proteolytic activity; however, Dahou et al (2021) found that ten *Lactococcus lactis* isolated from traditional Algerian cheese "J'ben" had proteolytic activity. Furthermore, another study found similar characteristic in one *Lactococcus* (*Lc. lactis subsp. Lactis*) and two *Enterococci* (*E. durans*) isolated from raw cow's milk (Franciosi, 2009). All of our selected LAB were classified as having significant proteolytic activity (halo size > 10mm), indicating their usefulness in the development of starter cultures for cheese maturation.

None of our strains had lipolysis activity; according to McSweeney and Sousa (2000) LAB are known to have limited lipolysis activity when compared to other microorganisms such as pseudomonas.

Table 1
Antimicrobial, proteolytic, and lipolysis activities of strains isolated from traditional cheese "J'ben"

Isolates	Activity zone (mm)				
	AA			PA	LA
	<i>Serratia plymuthica</i>	<i>Staphylococcus warneri</i>	<i>Enterobacter aerogenes</i>		
KC1	16 ± 0.00	12 ± 2.00	9 ± 4.50	15 ± 1.00	-
KC2	17.5 ± 7.50	13.5 ± 0.50	7 ± 1.50	14.5 ± 0.50	-
KC3	-	12.66 ± 0.94	7.33 ± 2.00	12.5 ± 0.50	-
KC4	-	15 ± 0.81	7.66 ± 2.50	13.5 ± 0.50	-
KC5	-	14 ± 1.41	7.66 ± 2.50	23.5 ± 1.50	-
KC6	22 ± 3.00	11 ± 1.00	-	11.5 ± 1.50	-
KC7	20 ± 5.00	15 ± 2.00	-	17.5 ± 2.50	-
KC8	11 ± 1.00	14 ± 1.00	-	16.5 ± 2.50	-
KC9	20.5 ± 5.50	15.33 ± 0.47	-	18 ± 2.00	-
KC10	8 ± 0.00	12 ± 3.55	-	21.75 ± 0.25	-
KC11	9 ± 1.00	12.33 ± 3.29	-	15 ± 1.00	-
KC12	16 ± 8.00	15 ± 0.0	-	12 ± 0.00	-
KC13	17.5 ± 7.5	13.5 ± 0.5	-	14 ± 2.00	-
KC14	16 ± 1.00	13.5 ± 1.5	-	12.5 ± 0.50	-
KC15	7 ± 0.00	9.66 ± 0.47	-	12 ± 0.00	-

PA (proteolytic activity) and LA (lipolytic activity): Halo size >10 mm (very high), 3-10 mm (high) and <3 mm (low) for proteolytic and lipolytic activities (Alapont et al., 2015);

AA (Antimicrobial activity); (antimicrobial activity): Halo size >8 mm (strong), 4-8 mm (moderate) and 1-4 mm (weak) for antimicrobial activity (Akabanda et al., 2014);

- (absence of activity);

Enterococcus durans: KC1, KC5, KC7, KC9, KC10, KC13; *Enterococcus faecium*: KC2, KC3, KC4; *Leuconostoc mesenteroides*: KC6, KC8, KC11; *Lactococcus lactis*: KC12, KC14, KC15

In terms of exopolysaccharide synthesis, three strains (KC1, KC6, and KC15) were able to produce EPS when grown on MRS agar medium supplemented

with sucrose (50g/L) as a carbon source. Because of the importance of this chemical compound in the pharma-

ceutical and food sectors, the production of exopolysaccharides is an important feature (Rajoka et al., 2018). EPS influences the stability and organoleptic properties of fermented foods including texture and flavor. Some EPS have a prebiotic impact on human health because they aid in adherence to human mucosa and also have antibiofilm and immunomodulation properties (Dal Bello et al., 2001; Liu et al., 2011; Russo et al., 2012; Rendueles et al., 2013). Three strains (KC1, KC6, and KC15) produced EPS, earning them the right to be chosen as the starter strains.

The existence of a lytic zone on blood agar plate was used to assess the haemolytic activity of our strains. The absence of haemolytic activity is an important indicator of safety when selecting a probiotic strain (Jikang & Wenxiang, 2019). Bacteria with haemolytic activity can produce haemolysin and harm human or animal red blood cells. Our finding was that none of the strains showed a β or α haemolytic activity; instead, they were all γ -haemolytic (no zones surrounding the colonies), showing safety for food or probiotic application.

In this work, antibacterial activity of the isolated LAB was investigated against subclinical mastitis pathogenic bacteria (Table 1).

The agar well diffusion technique exhibited results ranging from 0 to 17.5 mm for various bacteria. Antibacterial activity against *Serratia plymuthica* revealed that 8 LAB strains showed high inhibition (diameter ≥ 16 mm) whereas 3 strains showed no activity, and antibacterial activity against *Staphylococcus warneri* revealed that 14 strains presented inhibition zone (diameter ≥ 12 mm) except for KC15. Finally, for *Enterobacter aerogenes* 10 strains exhibited no inhibition activity, whereas 05 showed mild action.

Lactic acid bacteria are widely used in the food industry as starter cultures and bio-preservatives against pathogenic microorganisms by producing compounds with both technological and antimicrobial properties such as organic acids, diacetyl, reuterin, hydrogen peroxide, and bacteriocins. Many studies have proven the ability of LAB related the *Enterococci*, *Leuconostoc*, and *Lactococcus* groups to inhibit various microorganisms, as well as their utility in the food industry and medical sector. Karakas-Sen and Karakas (2018) revealed that two *Lactococcus lactis* had good antimicrobial activity, while López-Seijas et al. (2020) described an interesting inhibitory effect of *Lactococcus lactis* against the genus *Staphylococcus*; and Musikasang et al., (2009) isolated two *Enterococcus* (*E. faecium* and *E. durans*) that had important probiotic properties including a high antimicrobial activity. Finally, Morandi et al. (2013) identified 35 *Leuconostoc* strains isolated from North Italian typical cheese including *Ln. mesenteroid* with antimicrobial activity against several indicator bacteria.

The antibacterial activity of the traditional cheese J'ben LAB (at distinct genus *Enterococci*, *Leuconostoc*, and *Lactococcus*) identified in this study classified them as interesting bio-conservative agents. The antibacterial

property may be necessary during cheese production to decrease the usage of chemical conservatives.

4. Conclusion

Traditional cheese "J'ben" is a good source with a wide diversity of lactic acid bacteria, and with interesting potential and technological properties. All strains displayed considerable proteolytic activity and their antagonistic effect varied depending on the LAB strain and pathogenic bacteria. LAB of our research exhibited no haemolysis classifying them safe to be used, which is an important characteristic for the selection of probiotic strains and starter cultures. The isolates KC1 and KC6, which exhibited high proteolytic and antibacterial activity, were able to create exopolysaccharides, and were safe (no haemolysis), and can be considered viable candidates for use in fermented dairy products, particularly in the production and maturation of cheese.

All strains had biotechnological properties and the potential for future application as a starter for fermented products or as a probiotic organism; however, more tests will be required to prove the efficiency of these strains and their effect on the quality of fermented products in order to confirm the beneficial role for human and animal health.

5. Acknowledgements

We would like to thank, the Directorate General of Scientific Research and Technological Development "DGRSDT".

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