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Technological Aptitude and Sensitivity of Lactic Acid Bacteria Leuconostoc Isolated from Raw Milk of Cows: From Step-by-Step Experimental Procedure to the Results

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ABSTRACT

We demonstrated several technological aptitudes and sensitivity of lactic acid bacteria (Leuconostoc) isolated from the raw milk of cows. We explained the experiments in detail to get the reader to understand what kinds of technology can be used for testing bacteria. Lactic acid bacteria are used in the production of dairy products to increase their shelf life and improve their organoleptic and nutritional properties. Leuconostocs is a heterofermentative bacterium that produces lactic acid, acetic acid or ethanol, and carbon dioxide. A collection of 138 strains of lactic acid bacteria were isolated from the raw milk of cows in the region of eastern Morocco. 38 strains were identified as Leuconostocs. Their subspecies are 16 Mesenteroides, 11 Dextranicum, and 11 Cremoris. Our study was interested in the technological aptitude of this genus looking for the production of dextran and growth in milk with additional sunflower dye, as well as the acidifying, flavoring, thickening, coagulating, proteolytic activity in solid medium and Lipolytic activity. This work also consisted of studying the profile of the resistance and the sensitivity of these strains to antibiotics.

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1. INTRODUCTION

The microbial microflora of raw milk, composed mainly of lactic acid bacteria, plays an important role in the development of the organoleptic characteristics of fermented dairy products (fermented milk, cheese. The lactic bacteria have the essential technological assets to obtain an optimal bioconversion and a characteristic texture of the fermented and transformed products, like dairy products, the derivatives of agricultural raw materials, alcoholic products, the meats, these assets are in the aptitude to the acidification. Among the most widely used lactic acid bacteria in the dairy industry are the heterofermentative bacteria of the genus Leuconostoc.

In the dairy industry, lactic strains are selected based on their technological properties (lactic acid production, aroma production, proteolytic activity, and growth kinetics), and their functional characteristics (antibacterial activity) (Tamime, 2002; Molin, 2008). This makes it possible to meet the needs of the sanitary point of view in the food industry (Ross *et al*., 2002). Leuconostocs are heterofermentative lactic bacteria, facultative anaerobes, negative catalase, Gram-positive cocci (Ogier *et al*., 2008) asporulated, spherical, mesophilic, have a marked heterofermentative character.

Characterized by the hydrolysis of esculin and the formation of dextran for some species (Ho *et al*., 2007). Leuconostoc metabolizes lactose and citrate, as well as produces lactic acid, acetate, carbon dioxide, ethanol, acetaldehyde, diacetyl, acetoin, and 2,3 butanediol, which contribute to organoleptic (flavor and texture) (Cardamone *et al*., 2011). The growth temperature of this bacterium is between 25 and 30°C. It can grow at different pH conditions (Ho *et al*., 2007). Leuconostocsps species are considered essential technological ingredients in the formation of openings in blue-veined cheese such as Roquefort, due to the production of carbon dioxide that is formed from two distinct substrates: lactose and citric acid. The stability of these openings depends on the kinetics of carbon dioxide production by Leuconostoc.

The acidifying function is the most desired metabolic property of lactic acid bacteria used in food industries. It is manifested by the production of lactic acid from the fermentation of carbohydrates during bacterial growth. The acidity kinetics is one of the essential technological characteristics of lactic bacteria (Hemme *et al.* The production of dextran from sucrose by Leuconostoc allows it to be used as a food additive in dairy technology, as a gelling agent by increasing viscosity, and as a stabilizer by reinforcing the rigidity of the casein network (Hemme & Foucaud Scheunemann, 2004).

The presence of Leuconostocs eliminates the swelling defects found in different varieties of cheese such as Holland (Galesloot, 1950), Cheddar (Overcast & Albrecht, 1952), cottage cheese curds, or late swellings in Saint Nectaire cheese (Devoyod & Poullain, 1988). It is also possible to eliminate the taste defect known as the "green" or "acre" that is usually found in butter and fermented milk. This defect is caused by an overproduction of acetaldehyde compared to diacetyl (Keenan *et al*., 1966). Leuconostocs do not produce ammonia from arginine because they do not possess the enzyme arginine dihydrolase, and are not hemolytic.

The proteolytic activity of lactic acid bacteria is essential for their growth in milk as well as for the development of organoleptic properties of different dairy products (Savoy & Hébert, 2001; Yvon, 2006, Hassaïne *et al*., 2007). This study is a starting point to deepen and characterize the biotechnological properties of the lactic bacteria genus *Leuconostoc* isolated from raw cow milk. We found 38 strains of Leuconostocs namely (*Leuconostoc mesenteroïdes* subspecies

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mesenteroïdes, *Leuconostoc mesenteroides* subspecies *Dextranicum*, and *Leuconostoc mesenteroides* subspecies *Cremoris*). We pre-identified the bacteria using several microbiological tests as well as antibiotic tests to evaluate their resistance and sensitivity to different antibiotics.

2. MATERIALS AND METHODS

Experiments were done as explained in the following:

(i) Isolation of Leuconostoc strains: The strains of *Leuconostoc* were isolated from the raw milk of cows in the city of Taourirt and then subjected to various biochemical and technological tests

(ii) Growth at different temperatures: This test allows us to distinguish thermophilic Leuconostoc from mesophilic Leuconostoc (Carr *et al*., 2002; Badis *et al*., 2004)**.** Isolated bacteria were plated in a liquid medium and then incubated at 15, 30, 37, and 45°C from 24 to 48 hours.

(iii) Thermoresistance**:** This test allows for the selection of thermoresistant species. Colonies of 24 hours on agar of Ln were inoculated in tubes containing broth, then these tubes were placed in a water bath at a temperature of 60°C for 30 minutes. They were then heat-shocked and incubated at 30°C for 48 h (Badis *et al*., 2004).

(iv) Growth in the presence of salt: the medium with 2, 3, 4, and 6.5% of NaCl is inoculated from the bacterial suspension. The incubation was done at 30°C for 24 to 72 hours. Any positive reaction was translated by the presence of disorders (Carr *et al*., 2002; Mathara *et al*., 2004)**.**

(v) Culture on alkaline and acidic media**:** broth with pH adjusted to 9.6, 5, 4, and 3 with NaOH is seeded and incubated at 30°C for 24 to 48 hours (Carr *et al*., 2002; Mathara *et al*., 2004).

(vi) Growth on Sherman's blue milk: The medium used was skim milk contained in 9-mL testing tubes. 1 mL of sample (0.3%) and methylene blue solution (0.1%) was added to each tube. The medium was inoculated with the testing strains and incubated at 30°C for 48 hours (Carr *et al*., 2002; Mathara *et al*., 2004).

(vii) Gas production from glucose: This consisted of demonstrating the formation of carbon dioxide gas. Each young strain (18-24 hours) was cultivated in the modified broth with additional glucose and fitted with a Durham bell. Incubation was done at 30°C for 24 to 48 hours. Gas production was manifested by the appearance of air bubbles. The isolated bacteria that produced gas were noted as heterofermentative, and the other isolated bacteria are homofermentative (Hayward, 1957, Mathot *et al.,* 1994).

(viii) Preparation of the inoculum: The strains of Leuconostoc mesenteroides are hetero lactic strains. The young culture of Leuconostoc (18 hours) was centrifuged at 4600 rpm for 10 minutes to get a pellet. The prepared pellets were washed twice with sterile physiological water.

(ix) Acidifying power: The flasks were for seeding with a lactic culture (V/100V). Incubation was carried out at 30°C. The condition of pH and acidity Dornic were measured in the time intervals of 2, 4, 6, 16, and 24 hours.10 mL of milk was taken and titrated by the soda Dornic NaOH (N/9) in the presence of phenolphthalein (Mchiouer *et al*., 2017).

(x) Dextran production: The production of dextran from sucrose was done on a solid medium (Mayeaux *et al.,* 1962). Dextran-producing strains were characterized by the formation of large, slimy, and sticky colonies.

(xi) Acetoin production: Acetoin (i.e. acetyl-methyl-carbinol) production

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was tested on Clark and Lubs medium. The strains were grown on this medium. After 24 hours of incubation, the Voges-Proskauer reaction was performed (VP). A positive VP means that the strain has a particular metabolic pathway for hexose fermentation and the butylene glycol pathway. The proteolytic activity was investigated in a solid medium added with 2, 5, and 10% of sterile skimmed milk (reconstituted at 10% of the total concentration) (Van Den Berg et *al.,* 1995). Wells of the young culture were filled on the surface of this solid medium. After incubation at 30°C for 48h, the proteolytic activity was revealed by the appearance of a clear halo around each deposited sample.

(xii) Arginine hydrolysis: After incubation at 30°C for 72 hours in a Möeller medium with arginine, growth was manifested by a change in the color of the medium to yellow, (degradation of glucose). If the strain degrades the arginine, it produces an amine that will neutralize the acids which are manifested by the change in color from yellow to purple.

(xiii) Hydrolysis of esculin: The hydrolysis of esculin releases the aglycone which can be detected by a chemical reaction in the presence of iron salt and gives a black color to the culture medium. This test was performed by plating young culture on the agar medium with esculin bile after incubation of cultures is carried out at 30°C for 72 hours.

(xiv) Study of the fermentative profile: The test was performed by cultivating the strains in 10 mL of medium without glucose. We added 40 mg/L of bromocresol purple as a pH indicator (MRS-BCP) (Mannu *et al.,* 2000). Glucose was replaced by the sugar test, filtered through a membrane, and introduced into the solution at a final concentration of 1%. The carbohydrates used were lactose, glucose, sucrose, galactose, sorbitol, mannose, melezitose, raffinose, L-arabinose, D-xylose, melibiose, fructose, cellobiose, maltose, ribose, rhamnose, and mannitol. The fermentation of sugars resulted in the clouding of the medium accompanied by the turning of the pH indicator from purple to yellow (Samelis *et al.,* 1994).

(xv) Growth in sunflower milk**:** The medium was prepared from skimmed milk with an additional 4% of sunflower dye (purple color). The medium was sterilized at 110°C for 15 minutes. This medium was distributed in tubes and seeded with a young culture. The incubation was done at 30°C for 24 hours.

(xvi) Thickening power: This test was carried out by seeding sterile reconstituted cream milk with 12% of sucrose and in the bacterial culture (2V/10V). The incubation was done at 30°C for 24 hours. The thickening power is present if the gel forms have certain viscous rheology.

(xvii) Coagulant power: This test was performed as follows, 100 mL of sterile skimmed milk was inoculated with 3 mL of ferment. After incubation at 30°C for 24 hours, the volume of whey exuded was measured and the appearance of the coagulum obtained was noted.

(xviii) Lipolytic power: Lipolysis was highlighted by a method, on PCa agar added to 1% sterile glycerol, Tween80, and Tween 20. The latter were poured and solidified. Spots of the young culture were deposited on the surface of this agar. After incubation at 30°C for 72 hours, lipolysis was revealed by a lightning area surrounded by a deposit around the spots.

(xix) Hemolytic character: Hemolysis was tested on an agar medium with 5% fresh blood added per flask. After allowing the medium to cool, the sample was taken on the spot, and the

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spot (5%) was added to the medium and mixed carefully then poured onto the plate. After streaking our isolates, we incubated them at 30°C. After incubation, the type of hemolysis was examined.

(xx) Use of citrate: Citrate is the only source of carbon. Its usage results in the alkalinization of the medium. The seeding was done by streaking from a solid medium and incubating for 24 hours at 30°C. The degradation of citrate results in the medium turning from green to blue.

Antibiogram: The susceptibility or resistance of presumptive probiotic strains to antibiotics was evaluated according to the method described by De Almeida Júnior *et al.* (2015). The antibiogram of the strains was determined by the standardized diffusion technique (According to the antibiogram committee of the French society of microbiology, 2018) on agar medium. Starting from an 18-hour culture in a liquid medium, bacterial colonies of each strain were transferred into physiological water and their sample was adjusted to 0.5 Mc Farland. Using a sterile swab, the entire surface of the agar medium was seeded. After drying, the antibiotic discs were placed on the plates (maximum 6 discs on a large petri dish) and incubated at 30°C for 18 to 24 hours.

We studied the behavior of 38 bacterial strains against 18 commercial antibiotics. The antibiotics used were Lincomycin, Cephalothin, Nitroscolin, Norfloscacin, Amplicin, Gentamicin (15 µg), Gentamicin (30 µg); Cefaclor, Amoxicillin+Cavulanic acid, Cefotaxime, Ciprofloxacin, Gentamicin, Trimethoprim+ Sulfamethoxazole (co-trimazole), Cefamandole, Ceftriaxone, Cefazoline, and Rifampicin. After incubation, zones of inhibition can be observed around some discs. The results consisted of measuring the diameter of the zone of growth inhibition caused by the antibiotic. The results of 2 replicates were expressed in terms of resistance when they are in the specific diameters: inhibition zone (≤ 15mm), moderate susceptibility (16-20 mm), or susceptibility (≥ 21mm) (Vlková *et al*., 2006).

3. RESULTS AND DISCUSSION

3.1. Identification of Bacteria

The phenotypic pre-identification of these strains of lactic acid bacteria led us to attribute these isolates to the species *Leuconostoc mesenteroides*. The study of the macroscopic aspect of the strains on solid medium revealed small and round colonies with a regular periphery of a whitish color. The microscopic aspect revealed that the cells were Gram-positive with an ovoid shape associated with pairs or chains. All strains were catalase-negative. All species were mesophilic and were able to grow at a temperature of 10°C (not at 45°C). They resisted the test of thermoresistance at a temperature of 60°C for 30 minutes. They were unable to hydrolyze arginine. They were able to produce carbon dioxide from glucose; thus, they are heterofermentative. The isolates were able to grow at pH = 5 (not at pH = 3).

They are positive tests for all the strains on methyleneblue. All strains of *Leuconostoc mesenteroides* can be classified as subspecies of *Mesenteroides, Dextranicum, and Cremoris.* The production of dextran in sucrose medium, resulting in the formation of large and slimy colonies on the petri dish, is an important criterion to differentiate between species of Leuconostoc. Isolates can hydrolyze sucrose and produce dextran consider as *Mesenteroides* and *Dextranicum*, while *Cremoris weakly* produce dextran (**Table 1**).

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Table 1. Main results for Leuconostoc subspecies.

	Mesenteroides	***Dextranicum***	***Cremoris***
Fermentative tupe	+	+	+
ADH	-	-	-
Esculin test	+	+	-
Acetoin	-	-	+
Use of Citrate	+	+	+
Dextran test	+	+	+-
Hemolytic power	B	B	B

The fermentative profile showed that the three *Leuconostoc* subspecies can not ferment Mannitol. The *Dextranicum and Cremoris are capable* of fermenting lactose. This result is similar to that literature (Bennani *et al*., 2017). *Mesenteroides* can ferment xylose, maltose, galactose, fructose, glucose, lactose, sucrose, and sorbitol. It cannot ferment Mannitol, Arabinose, and cellobiose (**Table 2**). Clustering analysis can classify subspecies of bacteria with similar carbon source metabolism characteristics (Chen *et al*., 2021). The fermentative profile shows that the three Leuconostoc subspecies cannot ferment Mannitol. This result is similar to that reported in the literature (Bhattarai *et al*., 2021). *Dextranicum* and *Cremoris* are capable of fermenting lactose. This result is similar to the literature (Bennani *et al*., 2017). Mesenteroides can ferment xylose, maltose, galactose, fructose, glucose, lactose, sucrose, and sorbitol. They cannot ferment Mannitol, Arabinose, and cellobiose (**Table 2**).

The production of some lactic acid bacteria is presented in **Figures 1 and 2**. We delivered the effect of Dornik acidity (**Figure 1**) and pH condition (**Figure 1**) on the incubation time. The acidity produced by the strains in the milk medium is evaluated by the amount of lactic acid in Dornic degree (°D) (see **Figure 1**). It relates to the growth of bacteria and the fermentation of lactose into lactic acid. After the results were obtained, all strains have remarkable acidifying power. They produced lactic acid progressively as a function of time. This acidity is accompanied by a lowering of the pH of the milk and its coagulation.

Table 2. Sugar fermentation test in Leuconostoc subspecies.

	Mesenteroides	***Dextranicum***	***Cremoris***
Mannitol	-	-	-
L-Xylose	+	-	-
D-Xylose	+ (-)	+	-
L-Maltose	+	+	+
D-Maltose	+	+	-
Galactose	+	+	+
L-Fructose	+ (-)	-	-
D-Fructose	+	+	+ (-)
Glucose	+	+	+
Lactose	+	+	+
Sucrose	+	+	-
Sorbitol	+	-	-
Arabinosis	+	-	-
Cellobiose	-	-	-

+ positive; - negative; **+ (-)** depending on the strain

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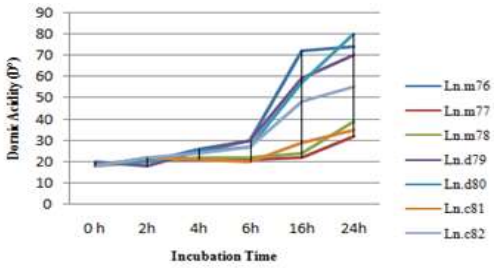

Figure 1. Production of lactic acid by some subspecies (correlation between Dornik Acidity and Incubation time). Samples for *Mesenteroides* are shown in Ln.m76, Ln.m77, and Ln.m78. Samples for *Dextranicum* are shown in Ln.d79 and Ln.d80. Samples for *Cremoris* are shown in Ln.c81 and Ln.c82.

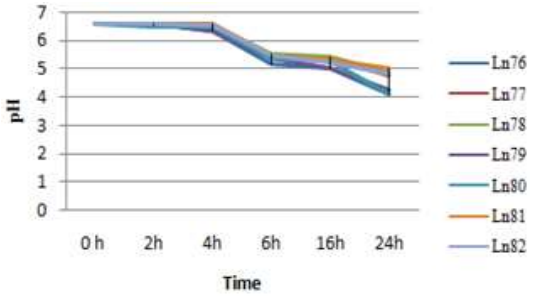

Figure 2. Production of lactic acid by some subspecies (correlation between pH condition and Incubation time). Samples for *Mesenteroides* are shown in Ln76, Ln77, and Ln78. Samples for *Dextranicum* are shown in Ln79 and Ln80. Samples for *Cremoris* are shown in Ln81 and Ln82.

The initial pH of the strains used to perform this test is between 6.53 and 6.61 and their Dornic acidity values vary between 18 and 20°D. After 4 hours of incubation, The Dornik acidity values increase to 21 and 26°D, and the pH decreases between 6.49 and 5.98. After 24 hours of growth, the values of Dornik acidity are between 32 and 80°D, and the pH values vary between 4.82 and 5.5. N'tcha *et al*. (2016) showed in their study that the bacteria were the most acidifying species. In this study, strains of *Dextranicum* are more acidifying, followed by strains of *Mesenteroides* and *Cremoris*

Detailed information on the effect of bile salt concentration on the viability and proliferation of *Leuconostoc mesenteroides* strains is shown in **Table 3**. Varying concentrations (i.e. 0.15 and 0.3%) of bile salt were studied to find out the tolerance of the bacteria after 2, 4, 6, 8, and 10 hours of incubation periods. The proliferation of all strains has been observed to increase with incubation time. Thus, more concentration of bile salt increased resulted in a decrease in the growth rate of strains significantly. *Mesenteroides* are more resistant to bile salt compared to *Crimoris* and *Dextranicum*, which is similar to the literature (Benmechernene *et al*., 2013). This study showed viability and proliferation in all concentrations for 10 hours during the incubation periods. All strains can resist bile salt for 8 hours. *Cremoris* and *Dextranicum* resist bile salt for 6 hours. This can be seen from a loss in viability.

3.2. Thickening Power Test

The results of thickening power showed the appearance of gels formed after fermentation. We noted a thick and dense appearance of coagulums after 24 hours of fermentation in *Mesenteroides* and *Dextranicum. Cremoris* fermented the milk after 72 hours. Strains present a thick and viscous appearance due to the accumulation of Exopolysaccharide (EPS). This viscosity is independent of the number of EPS produced.

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Table 3. The effect of bile salt concentration on the viability and proliferation of *Leuconostoc mesenteroides* strains.

	2h			4h			6h			8h			10h		
	0.0 %	**0.15%**	**0.3 %**	**0.0 %**	**0.15%**	**0.3 %**	**0.0 %**	**0.15%**	**0.3 %**	**0.0 %**	**0.15%**	**0.3 %**	**0.0 %**	**0.15%**	**0.3 %**
Ln.m.m	0.1 85± 0.0 2	0.0 28± 0.0 05	0.0 25± 0.0 05	0.2 53± 0.0 4	0.0 76± 0.0 05	0.0 57± 0.0 06	0.3 97± 0.0 8	0.1 85± 0.0 7	0.1 67± 0.0 2	0.4 89± 0.0 08	0.2 09± 0.0 07	0.1 83± 0.0 02	0.7 58± 0.0 06	0.1 27± 0.0 04	0.0 95± 0.0 03
Ln.*m.d*	0.1 79± 0.0 5	0.0 23± 0.0 02	0.0 21± 0.0 03	0.2 45± 0.0 3	0.0 67± 0.0 07	0.0 64± 0.0 03	0.4 96± 0.0 7	0.1 48± 0.0 1	0.1 39± 0.0 07	0.5 24± 0.0 2	0.1 18± 0.0 05	0.0 95± 0.0 04	0.6 63± 0.0 03	0.0 75± 0.0 05	0.0 45± 0.0 05
Ln.*m.c*	0.1 87± 0.0 7	0.0 26± 0.0 04	0.0 24± 0.0 03	0.2 49± 0 .0 4	0.0 56± 0.0 04	0.0 43± 0.0 02	0.3 89± 0.0 5	0.1 35± 0.0 05	0.0 95± 0.0 04	0.5 34± 0.0 4	0.1 02± 0.0 02	0.0 79± 0.0 07	0.6 68± 0.0 05	0.0 93± 0.0 1	0.0 58± 0.0 05

Note: Ln.m.m is the *Mesenteroides*, Ln.m.d is the *Dextranicum*, and Ln.m.c is the *Cremoris*.

3.3. Temperature and Thermo-Resistant

In this study, all strains were able to grow at a temperature of 10°C (not at 45°C) (Omeonu *et al*., 2022). The more active bacterial enzymes existing correlate to the higher rate of metabolic activities. This could have been responsible for the growth at these temperatures. All strains can resist at 60°C for 30 minutes (Bhattarai *et al*., 2021).

3.4. Heterofermentation and Coagulant Power

According to the result of the milk swirl test (See **Figure 3**), all strains can coagulate milk by extracting glycolytic enzymes that degrade lactose, causing acidification and color reduction. A turn in color from purple to pink indicates acidification (A) due to the attack of lactose. Discoloration of the indicator from the bottom of the tube translates to a reduction (R). The strain can coagulate the milk (C). The majority of strains acidified the milk and coagulated it except for 2 strains of *Cremoris that* reduced the coloring of the milk and then coagulated it. Detailed information is shown in **Figure 3(A)** for the casein hydrolysis with sunflower tincture, **Figure (B)** for the milk appearance after coagulation and gel formation, and **Figure 3(C)** for the aspect of enterococcal surface protein (ESP) production. The results in **Figure 3(B)** showed that all strains have a significant coagulant power. 72% presents a coagulant aspect, having a better odor after 24 hours with a volume of whey varying between 38 and 60 mL. 28% presents a coagulant aspect, having an appreciable odor after 48 hours with a volume of *Leuconostoc* varying between 56 and 58 mL.

Figure 3. Hydrolysis of casein. Figure (A) is the casein hydrolysis with sunflower tincture, Figure (B) is the milk appearance after coagulation and gel formation, and Figure (C) is the aspect of enterococcal surface protein (ESP) production.

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Figure 4 is several products from the fermentation. **Figure 4(A)** is the production of esculin, **Figure (B)** is for the production of CO_2, and **Figure (C)** is for the test of arginine. in this study, all subspecies did not hydrolyze arginine, a result similar to that reported by (Bhattarai *et al*., 2021). In this study, all strains are heterofermentative and produce CO_2, D-lactate, ethanol, and small amounts of acetate from glucose metabolism via the phosphoketolase pathway. The production of CO_2 may alter the texture and cause late blowing in certain cheeses although this often leads to moderate "eye" formation in cheese. It can play an important role in altering the texture of food products (Smid *et al*., 2014). Hexoses (glucose, fructose, mannose) in Leuconostoc can be converted to lactate, CO_2, and ethanol. CO_2 is a product of 6 - P - gluconate degradation, which occurs during the conversion of hexoses to pentoses. Citrate fermentation is an important phenomenon in bacteria since it is closely related to the aromatic activity of these microorganisms (Raynaud *et al*., 2003). All strains give positive results to citrate on Simmons medium by a formation of blue colonies.

3.5. Production of Acetone

The production of acetone is an important test for the identification of subspecies of bacteria and the selection of potential strains for technological interest. Acetone gives a pink-to-red coloration, this coloration appears in *Cremoris*. The results of two subspecies of *Mesenteroides* and *Dextranicum* did not produce acetone, which is similar to the literature (Badis *et al*., 2002; Ghazi *et al*., 2009).

Leuconostoc may further transform diacetyl to acetoin and 2,3-butanediol (Hemme & Foucaud-Scheunemann, 2004). Citrate fermentation is an important phenomenon in *Leuconostoc* since it is closely related to the aromatic activity of these microorganisms (Raynaud *et al*., 2003). All strains give positive results to citrate on Simmons medium by a formation of blue colonies. Several studies demonstrated that the metabolic pathways of *Leuconostoc* species include the conversion of citrate to diacetyl and acetoin, and the production of dextrans from sucrose (Bintsis, 2018; Maina *et al*., 2008). The production of dextran in sucrose medium (resulting in the formation of large and slimy colonies on the petri dish) is an important criterion to differentiate between subspecies of *Leuconostoc*. They can hydrolyze sucrose and produce dextran for *Mesenteroides* and *Dextranicum*, while *Cremoris* *weakly* produce dextran (**Table 1**).

3.6. Lipolytic and Proteolytic Power

The results show that not all strains show lipolytic activity in a medium supplemented with various lipid substrates (Tween 20, Tween 80, and Glycerol), as none of these strains show a hydrolysis zone around the spots. Proteolytic activity is an important property of secondary culture, as it can influence product flavor by providing most of the aroma of precursors (Yvon, 2006). Proteolytic activity in *Leuconostoc* strains shows a medium to low proteolytic result in solid medium supplemented with 2, 5, and 10% sterile reconstituted skim milk.

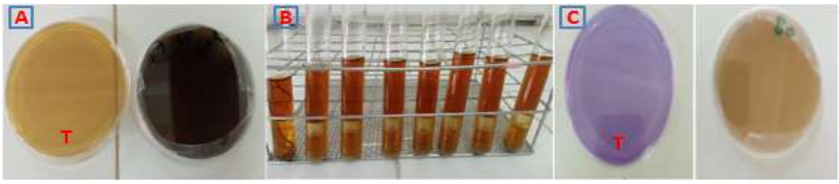

Figure 4. Product. Figure (A) is the production of esculin, Figure (B) is for the production of CO_2, and Figure (C) is for the test of arginine.

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However, it is important to keep in mind that highly proteolytic strains are not always the most suitable for use as starter cultures, since excessive proteolysis can lead to uncontrolled production of bitter peptides and other undesirable compounds, or excessive casein hydrolysis resulting in a final product that is too soft (Buffa *et al*., 2005). In this study, *Mesenteroïdes, Dextrani cum, and Cremoris showed to possess proteolytic properties,* but they *did not show any lipolytic activity.*

3.7. Hemolytic Power

To ensure the absence of pathogenicity and infection of a lactic acid bacterium, its hemolytic activity must be verified (El-Jeni *et al.*, 2016). The results showed that all strains are considered non-hemolytic, which is similar to the literature (Mokdad *et al.*, 2020; Benmechernene *et al.*, 2013). Hemolysis is rarely encountered in the lactic acid bacteria of food origin (Maragkoudakis *et al.*, 2009). The bacteria are not hemolytic species bacteria. They do not completely degrade red blood cells (*β. hemolytic*) (Birri *et al.*, 2013; Devi *et al.*, 2014). Hemolysis is rarely encountered in foodborne lactic acid bacteria (Maragkoudakis *et al.*, 2009).

3.8. Antibiotic Sensitivity

Safety evaluation requirements for LAB strains of technological interest should include the characterization of antibiotic resistance, intermediate, and susceptibility profile (Fraqueza, 2015). In this study, the selected lactic strains were tested using the standardized agar diffusion method. 18 antibiotics were used. The results in **Table 4** showed that all strains were sensitive to antibiotics (i.e. Lincomycin, Gentamicin, and Rifampicin) (Zarour *et al*., 2012). In addition, all strains were resistant to 11 antibiotics (i.e. Cephalothin, Norfloscacin, Amplicin, Cefaclor, Amoxicillin+Cavulanic acid, Cefotaxime,Trimethoprim+Sulfamethoxazole, Cefamandole, Ceftriaxone, Cefazoline, and Vancomycin). Some strains have an intermediate profile that are intermediate to Nitroscolin, Gentamicin, and Ciprofloxacin. The strains present a low resistance to antibiotics of clinical interest (Ogier & Serror, 2008). The majority of lactic strains are resistant to most antibiotics. The resistance of bacteria against antibiotics is generally an intrinsic characteristic. It is related to the presence of pentapeptides with D-lactate bound to the C-terminal instead of D- alanine in the composition of peptidoglycan, preventing the penetration of the antibiotic and consequently the cell lysis (Hemme *et al*., 2004; Zarour, 2012). Resistance to specific antibiotics means that the probiotic can be given at the same time when antibiotic treatment is required. Microflora of the intestine can recover more quickly (Kim & Austin, 2008; Allameh *et al*., 2012). *Leuconostoc* species are intrinsically resistant to Vancomycin (Coelho *et al*., 2022, Carr *et al*., 2002).

4. CONCLUSIONS

From the phenotypic criteria, we have 38 strains of *Leuconostoc*. 16 belong to the M*esenteroides*, 11 to *Dextranicum, and 11 to Cremoris*. All strains are considered non-hemolytic and non-lipolytic. The proteolytic activity in *Leuconostoc* strains shows a medium to low proteolytic result. The kinetic monitoring of growth and evaluation of pH and acidity in the milk medium were done to prove the effectiveness of the strains performing. Regarding the antibiotic test, *Mesenteroid* is 55% resistant, *Dextranicum* is sensitive to 25% of antibiotics, and *Cremoris* is resistant to 50% of antibiotics.

5. AUTHORS' NOTE

The authors declare that there is no conflict of interest regarding the publication of this article. The authors confirmed that the paper was free of plagiarism.

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Table 4. Antibiotic susceptibility testing of some Leuconostoc species.

	Load	Symbol	Ln 76	Ln 77	Ln 78	Ln 79	Ln 80	Ln 81	Ln 82
Lincomycin	15µg	MY	S	S	S	S	S	S	S
Cephalothin	30µg	KF	R	R	R	R	R	R	R
Nitroscolin	20µg	NI	I	S	S	S	I	S	S
Norfloxacin	10µg	NOR	R	R	R	R	R	R	R
Amplicon	10µg	AMP	R	R	R	R	R	R	R
Gentamicin	15µg	CN	S	S	S	I	S	I	I
Cefaclor	30µg	CEC	R	R	R	R	R	R	R
Amoxicillin+Cavulanic acid	30µg	AMC	R	R	R	R	R	R	R
Cefotaxime	5µg	CTX	R	R	R	R	R	R	R
Ciprofloxacin	5µg	CIP	I	I	I	I	I	I	I
Gentamicin	30µg	CN	S	S	S	S	S	S	S
Trimethoprim+Sulfamethoxazole (co-trimoxazole)	25µg	SXT	R	R	R	R	R	R	R
Lincomycin	10µg	MY	S	S	S	S	S	S	S
Cefamandole	30µg	MA	R	R	R	R	R	R	R
Ceftriaxone	30µg	CRO	R	R	R	R	R	R	R
Cefazoline	30µg	KZ	R	R	R	R	R	R	R
Rifampicin	30g	RD	S	S	S	S	S	S	S

Ln 76, Ln 77, Ln78**:** *Lnc. mesenteroïdes* subsp*. mesenteroïdes; Ln 79 and Ln 80: Leuconostoc mesenteroïdes subsp Dextranicum; Ln* 81and *Ln* 82: *Leuconostoc mesenteroïdes subsp. Dextranicum;R:*resistant*;S:susceptible;I:*intermediate

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