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Evaluation and Selection of Autochthonous Lactic Acid Bacteria as Starter Cultures from Goat's Raw Milk

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Abstract: *A total of twenty five indigenous lactic acid bacteria (LAB) isolates were collected from raw goat's milk Oulmes breed in AIT Ichou region (located in the Middle Atlas, Morocco). Among these twenty-five isolates, seven strains of LAB were selected for their significant acidifying activity and were identified using 16S rRNA gene sequencing. The genetic analysis revealed that all the strains were identified as Lactococcus lactis. These strains have shown some interesting technological properties, as a notable proteolytic activity especially C24 (2.37±0.03mMGlycine). All of them have exhibited low autolysis ratio, used citrate and produce (EPS), their safety for human consumption was confirmed relatively to their negative hemolytic activity and negative production of biogenic amine. These bacteria strains also showed an antimicrobial activity against Listeria innocua, Staphylococcus aureus, Pseudomonas aeruginosa ,Klebsiella pneumonia, Escherichia coli and Micrococcus luteus. These findings indicate the richness of the microflora of the goat's milk Oulmes breed in AIT Ichou region with bacteria species that possess promising technological criteria; therefore could being good candidates to develop industrial starter culture.*

Keywords: *Lactic acid bacteria; goat's milk; lactococcus lactis; acidifying activity; starter culture.*

I. Introduction

Goat population occupies 2nd place in farming in Morocco after sheep population. Its workforce is estimated at 5.3 million head, and consists of more than 80% of hardy local goats [1]. Most destiny of its milk production represents only 34 tons annually [2] devoted for consumption and processing into "jben" as an important traditional coagulated milk product . Oulmès-Zaer breed is one of the Moroccan local breeds known pure, it was identified in 1912 and recognized by Ministerial Decree in 1982. Furthermore. The Oulmes region biotope is considered to be a natural reserve of aromatic and medicinal plants, such as *Mentha pulegium, Lavendula stoechas, Lavendula angustifolia, Mentha suaveolens, Nerium oleander, Daphne gnidium, Myrthus cummunis,* etc. [3, 4]. Therefore, goat's milk Oulmes breed undoubtedly will have an incredible health gain through a special local indigenous microflora resulting of the unique climatic conditions of the area.

Dairy products made from locally produced raw milk are still a very important part of daily diet. These products have one feature in common: fermentation by lactic acid bacteria (LAB) is an integral part of their manufacture. The wild lactic acid bacterial flora represents a natural reservoir for cultures that were not exposed to any industrial selection. Some interesting characteristics of these microorganisms are their ability to produce acid at a high and predictable rate, proteolytic activity, synthesize exopolysaccharides (EPS) and to produce antimicrobial compounds, which are essential in fermented milk starter strains [5].

The main objective of this study is the assessment of technological properties of selected pure lactic acid bacteria from goat's milk from Oulmes local breed having the technological properties compared to that of a potential starter for further cheese manufacturing.

II. Materials and Methods

II.1 Sample Collection

Four Goat's milk samples were collected from AIT Ichou region, situated in rural region called Oulmes, located at 150 km at the North-West of Rabat city. This region is set on high mountains of the Middle Atlas and middle hill located to the west, in Berber Tamazight area in Morocco. Raw goat's milk samples were collected in sterile bottles and maintained at 4°C condition during their transport to the microbiology laboratory of regional center of agronomic research- INRA, rabat, for further analysis and search.

II.2 Isolation of Lactic Acid Bacteria

To perform this operation, serial dilutions were done for 1ml of each raw goat's milk sample in0.1% saline peptone solution and the culture was monitored using the Man Rogosa and Sharpe (MRS) (Fluka, Sigma-Aldrich) and M17 (Oxoid) agar media. To conduct the bacteria isolation, plates were incubated during 24 hours at 15º, 32º, 38º and 45ºC followed by a selection of the distinguishable colonies using a sterile loop [6]. A total of 100 strains were isolated whose 25 strains occurring in pairs or chains of size cocci gram positive and catalase negative, were selected and characterized. Technics of Isolation used, in this search work, are those recommended by Van den Berg et al. [7]. For the preservation, the cultures were maintained at -80°C in M17 broth (Oxoid) supplemented with 15% (v/v) glycerol until use. Their regeneration was realized by an overnight incubation at 37°C in M17 broth.

II.3 Technological Characteristics

II.3.1. ***Acidifying Activity***

To evaluate the acidifying power, the concerning 24 strains were initially grown in M17 broth at 37°C for 24 h, and then inoculated at a level of 1% in reconstituted sterile skim milk solution (10% w/v) (Fluka, Sigma-Aldrich). Then, the pH was measured, after 2, 4, 6 and 24 h of incubation at 37°C, by a pH-meter (pH 211 microprocessor pH meter, HANNA Instruments Inc, Italy) previously calibrated using two buffers (pH4.0 and pH 7.0). After each run, the electrodes (HI 1413B, HANNA Instruments) were disinfected with ethanol and wached with sterile deionized water. Moreover, the acidification rate was calculated as pH = pHf (final value) - pH0 (initial value). The experiments were carried out in duplicate.

II.3.2. ***Identification of Isolates by 16S rDNA***

Among the selected bacterial 7 strains showed significant acidifying activity and were identified by 16S rDNA gene sequence analysis in National Centre for Scientific and Technical Research Rabat (CNRST). The resulting 16S rDNA sequences were initially determined using the BLAST program in NCBI GenBank (www.ncbi.nlm.nih.gov/blast) [8].

II.3.3. ***Proteolytic Activity***

In this step of research, the proteolytic activity was determined by the quantity of free amino acids released, according to the method of Church et al., [9]. Bacteria strains were sub cultured twice in reconstituted skim milk (10% w/v), containing yeast extract (0.3% w/v), for 24 h at 37°C and using 1% (v/v) inoculums, the last growth was performed in skim milk (10% w/v) for 24 h at 37°C (1%v/v inoculum). A calibration curve with a glycine concentration ranging between 0.1 and 10 mmol was used. The results were expressed as millimoles of glycine per litre (mmol Gly l^{-1}).

II.3.4. ***Autolytic Activity***

To determine the autolytic activity, after incubation at 37 ° C in the M17 broth, bacterial cultures were centrifuged (5000 × g for 15 min at 4°C). The recovered pellet was washed twice with potassium phosphate buffer (10 mM, pH 7.0) and then resuspended in potassium phosphate buffer (10 mM, pH 5.5).The resulting cell suspension is subjected to a cycle of freezing (-20°C for 22 h) After thawing, the suspension was incubated at 45 ° C for 2h. And then, the autolytic activity was determined as the percentage decrease in the absorbance at 650 nm at different time intervals as described by Boutrou et al. [10], which was defined as follows: (A0 - At) x100/A0 where A0 = initial absorbance and At = absorbance measured after t hours of incubation.

II.3.5. ***Citrate Metabolism***

The flavoring activity was evaluated by the technique of Kempler and McKay. [11]. To evaluate the citrate metabolism, identified lactic acid bacteria were incubated on KMK agar at 37 ° C for 3 to 4 days. Then, blue colonies with a blue or central point represent aromatic strains using citrate and white colonies are strains that do not utilize citrate.

II.3.6. ***Exopolysaccharides (EPS) Production***

Exopolysaccharides production was assessed as reported by Mora et al. [12]. The Overnight cultures were streaked on the surface of plates containing the milk and ruthenium red (10% skimmed milk powder, 1% sucrose, 0.5% yeast extract, 0.08 g / L ruthenium red, 1.5% agar). After incubation at 37°C for 24 hours, exopolysaccharides producing strains colonies were white, whereas the non-producing strains colonies appeared red due to staining of the bacterial cell wall.

II.3.7. ***Haemolytic Activity and Biogenic amine Production***

The hemolytic activity is determined after inoculation of the strain on Columbia agar (OxoidLimited, Basingstoke, England) with 5% sheep blood for 24 h at 37 ° C [13]. Strains were examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green zones around colonies) or γ-haemolysis (no clear zones around colonies).

In addition, their ability to produce biogenic amines was qualitatively determined on an improved screening medium as described by Bover-Cid and Holzapfel [14], using four precursor amino acids: tyramine, histamine, putrescine and cadaverin.

II.3.8. ***Antibacterial Activity Determination***

To evaluate the Antibacterial activity of identified strains was tested against some pathogens by the well diffusion assay using cell culture or cell supernatant [15, 16]. Fresh overnight M17 cultures were centrifuged at 8,000g for 10 minutes, and the cell-free supernatants were used directly or after being filtred aseptically (0.22 µm pore size; Serva, Heidelberg, Germany), neutralized with 1 mol / l NaOH (pH 6.5-7) and treated with catalase (0.5 mg / ml) (Sigma-Aldrich). And the Lactococcus cells was diluted with M17 and used for their antimicrobial activity.

It is noticed that the indicator pathogenic strains included Listeria innocua (LMHAE-LI 107), Staphylococcus aureus (LMHAE-SA 105), Pseudomonas aeruginosa (ATCC 29753), Klebsiella pneumonia (CIP 53153), Micrococcus luteus (ATCC15957) and Escherichia coli (ATCC54127), were tested. They were grown overnight in LB broth at pH 7.0 (Sigma-Aldrich) and diluted with sterile phosphate buffered saline (PBS) (pH 7.2). After dilution, the pathogenic strains were mixed with 5 ml of LB soft agar (0.7% (w / v)) in order to reach a final concentration of 104 CFU / ml, this medium was poured into plates prepared in advance with 10 ml of basal agar containing 2% (w / v) agar. Wells of 5mm diameter were performed using the top of a Pasteur pipette and were filled with approximately 50µl of 108 CFU / ml of identified strains in0.1% saline peptone, of cell-free treated supernatant and cell-free untreated supernatant . The plates were then stored at 4 ° C for 4 hours to allow the radial diffusion of any antimicrobial compound. Following incubation at 37 ° C for 24 h, the plates were monitored for the appearance of clear zones of inhibition. Each test was performed in triplicate.

III. Results and Discussion

III.1 Acidifying Activity

Regarding the acidifying activity of the twenty five selected isolated bacteria from Oulmes region (Fig. 1), seven bacteria showed strong acidity with values &pH6h ranging from 1.24 to 1.56 and &pH24h 1.96 to 2.13. Then, Sixteen bacteria have a medium activity with &pH6h ranging from 0.34 to 0.66 and &pH24h 1.2 to 1.54; One isolate had a weak acidity with an average of 0.11 (&pH6h) and 0.14 (&pH24h) [17].

The decrease of the milk pH below 5.3 after 6h in optimal growth temperature is the most important criterion for selection of starter cultures used in the standardized production of dairy product [18]. It is a crucial factor for coagulation, prevention and reduction of the growth of accidental microflora. Seven bacteria isolates (C8, C10, C11, C12T; C12J; C13; C24) showed a high acidity after 6 hours so they could be used as starter organisms. They can be important starter bacteria for product a typically Oulmes Moroccan fermented dairy, while the poor acidifiers' strains can be used as adjunct cultures depending on their other important properties, e. g., proteolytic and autolytic activity [16].

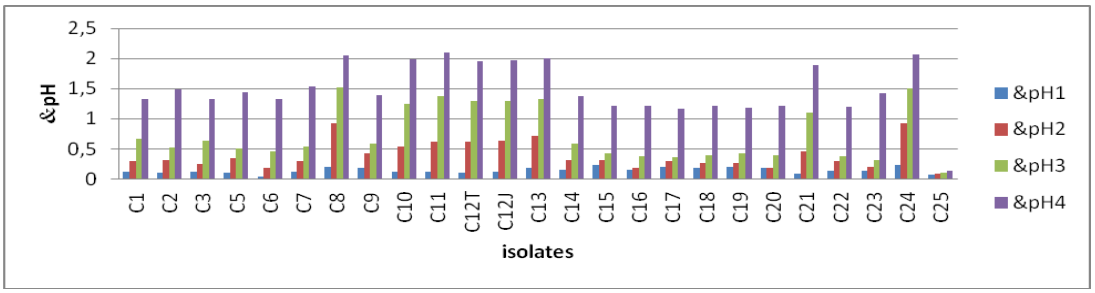

Figure 1 . pH decrease in reconstituted skim milk after 2h '&pH2', 4h '&pH4', 6h '&pH6' and 24h '&pH24' h of incubation at 37°C respectively. Values are mean ± standard deviation.

III.2 Identification of isolates by 16S rDNA

To perform this step of research, all of the isolates were identified to species level by sequencing the 16S rRNA gene, which showed more than 99% similarity to Lactococcus *lactis* reference strains.

III.3 Proteolytic activity

It known that Proteolytic activity is essential for dairy LAB for promoting better growth on milk and for the development of the organoleptic properties of the fermented milk products [19, 20, 21] .

Obtained results of proteolytic activity ranged between 0.91±0.09 and 2.37±0.03 mM Gly (table 1), The lactococci lactis C24 isolate showed the highest activity (2.37±0.03 mM Gly), while lactococci lactis C10 showed the lowest (0.91±0.09 mM Gly). In general, the majority of the isolates exhibited high extracellular proteolytic activity. This can be due to the fact that lactococcus are the microorganisms that develop first time in the milk, providing a high number of bacteria cells and the highest global proteolytic activity. These results are in accordance with those reported by Garacía-Ruíz et al., and Ballesteros et al., [22, 23] in which Lactococcus isolates were the microorganisms that presented the highest proteolytic activity.

It is also observe a good correlation between acidifying and proteolytic activities with bacteria strains that able to coagulate milk fastly being proteolytic too. This results confirmed that obtained by Mayo et al., [24] when have studied technological characterization of strains of L. lactis subsp. lactis strains from Cabrales cheese results In contrast, others [25, 26] reported that the isolates presenting the highest acidifying activity are not always the most proteolytic.

III.4 Autolytic Activity

The ability of the bacteria strains to lyse and to release subsequent of intracellular enzymes can have an impact on the flavor and aroma of the cheese during maturation [27, 28]. All of strains tested in the present resaerch study showed variable autolytic activities (table 1). In general, they exhibited a low autolysis rate as described by Ayad et al. [29]: 1.39±0.02 to 3.61±1.45 % for lactococcus lactis .These results are comparable with those found by ayad [30] who reported that several wild lactococci strains were found to be stable in milk cultures and during cheese ripening in contrast to industrial strains. The autolytic properties of several cheese related microorganisms was reviewed [31]. It was shown that one of the most effective ways to accelerate cheese ripening was addition of special or typical adjunct cultures.

III.5 Citrate Metabolism

All of the identified strains of lactococcus lactis were found able to use citrate (table 1). This metabolism in the majority of lactic acid bacteria results in the synthesis of flavor compounds such as acetate, acetaldehyde and diacetyl [32, 33]. These compounds are involved in sensory quality of cheeses.

III.6 Exopolysaccharides (EPS) Production

Moreover, all strains studied have the ability to produce exopolysaccahride (EPS) (table 1). EPS produced by lactic acid bacteria contribute to the improvement of the texture and the viscosity of the finished product, In general, the presence of exopolysaccharides in fermented products like yogurt, can increase the homogeneity of the product, enhance its presentation, stabilize the yoghurt gel and decrease its tendency to syneresis [34]. The EPS production is a relevant feature in the selection of starter bacteria strains [35].

III.7 Haemolytic Activity and Biogenic amine Production

It is known that, the haemolytic activity constitutes a potential pathogenic trait that can be displayed by lactococcus. However, none of the lactococcus lactis tested produced haemolysin when tested on sheep blood. The absence of such activity should be a criterion for selecting strains that can be used as starter or adjunct cultures in dairy products [36].

The results obtained in this study indicated that none of the strains produced biogenic amines: histamine, tyramine, putrescine and cadaverine, which is not a desirable property of starter cultures [37] and this criterion reinforces their safe use in fermented dairy products.

II.8 Antibacterial Activity Determination

LAB have been used as natural preservatives because of their antimicrobial capacity, On the one hand, Through fermentation products: Antimicrobial activity can be exerted through the reduction of pH or production of organic acids (lactic acid, acetic acid), CO2, reuterin, diacetyl, 2-pyrorelidone, 5-carboxylic acid [38]. Effective starter culture activity can prevent the pathogen and contaminant growth that may occur during cheese making process. and on the other hand, Through bacteriocins can be defined as protein antibiotics of relatively high molecular weight and mainly affecting the same or closely related species. It is known that LAB are generally regarded as safe microorganisms and so are their bacteriocins. Thus, these bacteriocins can potentially be used to control the growth of spoilage and pathogenic organisms in food [39]. Bacteriocin producing lactococcal strains have been used successfully as starter cultures for cheesemaking in order to improve the safety and quality of the cheese. In recent work, 79 wild lactococci have been studied and 32 of theese have been found to be antimicrobially active [40]. In 17 of these strains, the well-known antimicrobial

peptide nisin has been found. Moreover, the use of nisin as an effective preservative in processed cheese has been widely accepted.

According to the results observed in the present work search, none of the lactococcus lactis strains showed bacteriocin activity spectrum against the used indicator organisms since no inhibition was observed when treated supernatants (pH 6.5) was tested. However, it was observed that majority of bacteria cells and untreated supernatant of Lactococcus lactis have an anti-pathogenic effective biological activity (table 2):Untreated Supernatant of C8 and C13 bacteria presents an antibacterial activity against Klebsiella pneumonia, while for the bacteria cells C8 and C10 presents an antibacterial activity against Listeria innocua, also C13 had an antibacterial activity against Staphylococcus aureus. However C8, C10, C12J, C12T and C13 present an antibacterial activity against Pseudomonas aeruginosa and Klebsiella pneumonia too, on the other hand, C8, C10 and C13 presents an antibacterial activity against Escherichia coli, Micrococcus luteus and C12T present an antibacterial activity against Micrococcus luteus too, finally no antibacterial activity was noted for the bacteria strain C24.

The antimicrobial property of these strains can play good role for reducing the number of other undesired microorganisms in milk products as well as perform essential roles in the preservation of product for human consumption [41].

IV. Conclusion

The seven isolates of lactococcus lactis isolated from goat raw milk showed an important property to allow them to be considered as good starter cultures: good acidifying power of milk, ability of procucing exopolysaccharides (EPS), and have a potential metabolic traits related to flavor and aroma development in various fermented foods. Otherwise none of Lactococcus lactis isolated was hemolytic or produced biogenic amines, finally, Lactococcus lactis strains, are the most important organisms needed to in the manufacture and develop typical fermented dairy products such as yogurt, fresh cheeses and many varieties of semi-hard cheeses.

Table 1 : Summary of the results of the biochemical tests carried out on the lactococcus lactis

Strains	Autolysisa (%)	Proteolysisa (mM Gly)	Metabolism of citrate	EPS production
C8	3.65 ±1.45	1.98 ±0.01	+	+
C10	2.57 ±1.06	0.91 ±0.09	+	+
C11	1.39 ±0.20	2.14 ±0.13	+	+
C12T	1.48 ±0.55	2.07 ±0.08	+	+
C12J	1.46 ±0.56	2.08 ±0.09	+	+
C13	1.96 ±0.58	1.97 ±0.23	+	+
C24	3.61 ±1.36	2.37 ±0.03	+	+

Table2: The antimicrobial activity of L. *lactis* against pathogens bacteria

strains	Listeria *innocua* LMHAE-LI 107			Staphylococcus *aureus* LMHAE-SA 105			Pseudomonas *aeruginosa ATCC 29753*			Klebsiella *pneumonia* CIP 53153			Escherichia *coli ATCC54127*			Micrococcus *luteus* ATCC15957		
	S	ST	C	S	ST	C	S	ST	C	S	ST	C	S	ST	C	S	ST	C
C8	-	-	+	-	-	-	-	-	++	+	-	++	-	-	+	-	-	++
C10	-	-	+	-	-	-	-	-	++	-	-	++	-	-	+	-	-	++
C11	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-
C12J	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
C12T	-	-	-	-	-	-	-	-	++	-	-	++	-	-	-	-	-	++
C13	-	-	-	-	-	+	-	-	++	++	-	++	-	-	+	-	-	++
C24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: -. No inhibition; +. inhibition zone between 2 and 6 mm; ++. inhibition zone larger than 6 mm. C: Cells of strains in fresh M17 broth; S: Cell-Free supernatant ; TS: Cell-Free supernatant adjusted to pH 6.5-7 and treated with catalase. Results are averages of three experiments.

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