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# Isolation of lactic acid bacteria strains via goat and camel raw milk and evaluation of their probiotic effects

their probiotic effects

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## Abstract

Probiotic bacteria are of great importance as a friend of the digestive system and are spread in many foods. For this purpose, this study aims to isolate lactobacilli strains with probiotic properties. So, six milk samples were collected from camels and goats in the EL-OUED area, where their quality was evaluated by physicochemical analyzes by measuring electrical conductivity, pH, acidity, density, total solids, ash, sugar and protein concentration. Then, through microbiological analyzes, the lactic acid bacteria strains were isolated and their probiotic properties were studied in the laboratory by tolerating various biological barriers such as acids (pH 2, 3 and 4) and bile salts (0.3%, 0.5% and 1%) and tested for inhibition of microbes against pathogenic bacteria (Explained in **Table 06**) as well as their resistance to antibiotics (explained on **page 30**), and then these probiotic strains were identified by several phenotypic tests as follows: Growth in a medium containing different concentrations of NaCl 2%, 3, 4% 6.5, growth at different pH levels 4.2, 4.8, 4.5, 6.5, 7, 8, growth at different temperatures 10°C, 40°C, 45°C, thermal resistance and fermentation type.

The physicochemical results indicate that camel milk has satisfactory physicochemical quality, while goat milk recorded values somewhat far from international standards in terms of electrical conductivity  $2.63\pm 0.41$  mS/cm, density  $1.04\pm 0.03$ , and total solids  $195.85\pm 6.83$  g/l. As for microbiological analyzes, 7 probiotic strains were isolated belonging to the following families: *Lactococcus* (S5, S4, S1), *Streptococcus* (S10, S9) *Pediococcus* (S7, S2) distributed in the milk as follows, 42.86% of the strains present in camel milk (S10, S9, S7) and 57.14% of the strains are present in goat's milk (S5, S4, S2, S1), as these strains showed the ability to coexist in natural barriers such as acids and bile salts, and they also showed the activity against the pathogen strains that were tested, and a resistant activity against antibiotics. As these selected lactobacilli can be considered as probiotic strains for adoption in nutritional supplements and mainly in prevention and treatment methods with varying proportion of order from the most important strain to the least important as follows (S2, S1, S9, S4, S5, S10 then S7).

**Key Word:** raw milk, acid lactic bacteria, probiotics, physicochemical analyzes, microbiologic analyzes, antibiotics, pathogens bacteria

## Résumé

Les bactéries probiotiques jouent une grande importance en tant qu'amis du système digestif et sont répandues dans de nombreux aliments. Pour ça, l'objectif de cette étude est l'isolement des souches de LAB aux propriétés probiotiques. Dans ce but, six échantillons de lait ont été prélevés à partir des chameaux et des chèvres dans la région d'EL-OUED, où leur qualité a été évaluée par des analyses physicochimiques en mesurant la conductivité électrique, pH, l'acidité, la densité, la matière sèche totale, cendres, la concentration en protéines et le sucre totaux. Ensuite, grâce à des analyses microbiologiques, ont été isolées des souches lactiques et étudiées leurs propriétés probiotiques *in vitro* à partir de la tolérance à diverses barrières biologiques telles que l'acidité (pH 2, 3 et 4) et sels biliaires (0,3%, 0,5% et 1%), leur activité antimicrobienne contre les bactéries pathogènes (présenté dans le **tableau 06**) ainsi que sa résistance aux antibiotiques (présenté dans la **page 30**), puis l'identification de ces souches probiotiques à travers plusieurs tests phénotypiques comme suit: Croissance dans un milieu contenant différentes concentrations de NaCl 2%, 3%, 4%, 6,5%; croissance à différents pH 4.2, 4.5, 4.8, 6.5, 7, 8; teste de croissance à différentes températures 10° C, 40° C, 45 ° C, teste de thermorésistance et de type de fermentation.

Les résultats physico-chimiques indiquent que le lait de chamelle a une qualité physicochimique satisfaisante, tandis que le lait de chèvre a enregistré des valeurs quelque peu éloignées des normes internationales en termes de conductivité électrique  $2.63 \pm 0.41$  mS/cm, densité  $1,04 \pm 0,03$  et de matière sèche totale  $195,85 \pm 6,83$  g/l. Pour les analyses microbiologiques, ont été isolées 7 souches probiotiques appartenant aux familles suivantes: *Lactococcus* (S5, S4, S1), *Streptococcus* (S10, S9), *Pediococcus* (S7, S2) répartis dans le lait comme suit, 42.86% des souches présentes dans le lait de chamelle (S10, S9, S7) et 57,14% des souches sont présentes dans le lait de chèvre (S5, S4, S2, S1), où ces souches ont montré la capacité de résistance aux barrières biologiques telles que les acides et les sels biliaires, et ont également montré une activité antimicrobienne contre les souches pathogènes qui ont été testées, et une résistance aux antibiotiques. Donc ces LAB sélectionnés peuvent être considérés comme des souches probiotiques comme candidats à l'adoption dans les compléments nutritionnels, et principalement dans les méthodes de prévention et de traitement, avec des degrés de proportion variables de la souche la plus importante à la moins importante comme suit (S2, S1, S9, S4, S5, S10 puis S7).

**Mots clés :** lait cru, bactéries lactiques, probiotiques, analyses physicochimiques, analyses microbiologiques, antibiotiques, bactéries pathogènes

## الملخص

تمتلك بكتيريا البروبيوتيك أهمية كبيرة باعتبارها خير صديق للجهاز الهضمي وواسعة الانتشار في العديد من الأغذية. من أجل هذا، تهدف هذه الدراسة الى عزل سلالات بكتيريا لبنية ذات خاصية بروبيوتيك.

لهذا الغرض، تم اخذ ستة عينات حليب من الإبل والماعز في منطقة الوادي حيث تم تقييم جودتها بالتحليل الفيزيوكيميائية وذلك عن طريق قياس الناقلية الكهربائية، الـ pH، الحموضة الدورنية، الكثافة، المواد الصلبة الكلية، الرماد، تركيز السكر والبروتين. ومن ثم، عن طريق التحليل الميكروبيولوجية تم عزل سلالات بكتيريا حمض اللاكتيك ودراسة خصائصها البروبيوتيك في المخبر عن طريق التسامح مع الحواجز البيولوجية المتنوعة مثل الاحماض (درجة الحموضة 2, 3 و4) والاملاح الصفراوية (0.3%, 0.5%, 1%) واختبار تثبيطها للميكروبات ضد البكتيريا الممرضة (موضحة في الجدول 06) كذا مقاومتها للمضادات الحيوية (موضحة في الصفحة 30). و من ثم تحديد هذه السلالات البروبيوتيك عن طريق عدة اختبارات فينوتيبك كالاتي: النمو في وسط يحتوي على تراكيز مختلفة من كلوريد الصوديوم 2%، 3%، 4%، 6.5%، النمو في درجات حموضة مختلفة 4.2، 4.5، 4.8، 6.5، 7، 8، اختبارات نمو في درجات حرارة مختلفة 10، 40، 45 درجة مئوية، دراسة المقاومة الحرارية و اختبار نوع التخدير.

تشير النتائج الفيزيوكيميائية الى ان حليب الابل ذو جودة فيزيوكيميائية مرضية بينما حليب الماعز سجل قيما بعيدة نوعا ما عن المعايير العالمية من ناحية الناقلية الكهربائية 2.63±0.41 ملي سيمناس/ سم والكثافة 1.04±0.03 و المواد الصلبة الكلية 195.85±6.83 غ/ل. اما عن طريق التحليل الميكروبيولوجية، تم عزل 7 سلالات بروبيوتيك تنتمي الى العائلات التالية (*Lactococcus* (S5, S4, S1), *Streptococcus* (S10, S9) *Pediococcus* (S7, S2) متوزعة في الحليب كالاتي، 42.86% من السلالات متواجدة في حليب الإبل (S10, S9, S7) و 57.14% من السلالات متواجدة في حليب الماعز (S5, S4, S2, S1). حيث أظهرت هذه السلالات القدرة على التعايش في الحواجز الطبيعية مثل الاحماض والاملاح الصفراوية، كما أظهرت نشاطا مضادا ضد السلالات الممرضة التي تم اختبارها و نشاطا مقاوما ضد المضادات الحيوية، اذ يمكن اعتبار هذه البكتيريا اللبنة المختارة كسلالات بروبيوتيك مرشحة لاعتمادها في المكملات الغذائية و بشكل رئيسي في الوقاية و الطرق العلاجية بدرجات متفاوتة مرتبة من السلالة الأهم الى الأقل أهمية كالاتي) (S7, S10, S5, S4, S9, S1, S2).

**الكلمات المفتاحية:** حليب طازج، بروبيوتيك، بكتيريا لبنية، خصائص فيزيوكيميائية، خصائص ميكروبيولوجية، مضادات حيوية، بكتيريا ممرضة

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## List of abbreviations

**°C:** Celsius

**°D:** Dornic degrees

**µl :** Microliter

**µm :** Micrometer

**AFMO :** Association Française de Médecine Orthomoléculaire

**AFNOR :** Association Française de Normalisation

**ATCC :** American Type Culture Collection

**BC:** Before christ

**BSA:** Bovine serum albumin

**CDM:** Chemically defined medium

**CFU/ml:** Colony-forming unit per milliliter

**DO:** Density optical

**FAO:** Food and Agriculture Organization

**FPK:** Fructose-6-P phosphoketolase

**g/l:** Gram per liter

**g:** Gram

**GI:** Gastro-intestinal

**GRAS:** Generally recognized as safe

**h:** hour

**H<sub>2</sub>O:** water

**H<sub>2</sub>O<sub>2</sub>:** hydrogen peroxide

**HcL:** Chlorhydric acid

**HMF:** hydroxy-methyl furfural

**IBD:** Inflammatory bowel disease

**IgA:** Immunoglobulin A

**ISO:** International Standard Organization

**LAB:** Lactic acid bacteria

**Log:** Logarithme

**M17:** Terzaghi and Sandine

**mg:** Milligram

**min:** Minute

**ml:** Milliliter

**mm:** Millimeter

**ms/cm:** Millisiemens per centimeter

**N:** normality

**NaCl:** Sodium chloride

**NaOH :** Hydroxyde sodium (soude)

**NF :** Norme française

**nm:** Nanometer

**O<sub>2</sub>:** Oxygen

**PBS:** Phosphate buffered saline

**Ph :** Potentiel d'Hydrogène

**PTS :** Phosphotransférase système

**rpm:** Rotation per minute

**S:** Strain

**SD :** Standard deviations

**T° :** Temperature

**tr :** Tour

**WGO:** World Gastroenterology Organization

**WHO:** World Health organization

**ZOI:** Zone of inhibition

# ***Introduction***

## **Introduction**

We often hear that eating better contributes to good health. Besides, around 400 BC Hippocrates already said, "That your food is your medicine". For this, a healthy and balanced diet provides all the nutrients necessary for the human body (**BIGO, 2017**).

Raw milk is one of the sources benefits for human health. It occupies a strategic place in the daily diet of man, due to its balanced composition in basic nutrients (proteins, carbohydrates and lipids) and its richness in vitamins and minerals, especially in dietary calcium. Nowadays, the needs for milk are more and more important since this product can be consumed in the fresh state but also in pasteurized form, sterilized or transformed into derived products (**SIBOUKEUR, 2007**).

Several microorganisms (bacteria, yeast and mold) are present in raw milk and forming the complex microbial ecosystem. Bacteria can be naturally present in milk or accidentally by manipulation (**HENNANE, 2011**).

The microbial microflora of raw milk, mainly composed of lactic acid bacteria (**BOULLOUF, 2016**), which belong to a group of beneficial bacteria, in virtues are similar, and which produce lactic acid as the final product of the fermentation process. They are widespread in nature, and are also found in the digestive system of humans (**MAGHNIA, 2011**) which have several beneficial actions for health and they are recognized as (probiotics) (**FESSARD, 2017**).

Bacteria in the gastrointestinal tract occupy 1/3 of the stool volume and represent around 400 different bacterial species living naturally in the organs making up the digestive tract (**DUPIN, 1992**).

Recent scientific work such as the work described by **FAO/WHO 2001** on the properties and functionality of microorganisms in food suggests that, probiotics play an important role in immunological, digestive and respiratory functions and they can help fight infectious diseases in children and other high risk groups (**FAO, 2006**).

During their passage through the digestive tract, probiotic microorganisms are able to interact with the gut microbiota, epithelial cells and/or host immune cells. Today, many health effects are associated with probiotic microorganisms (lactose digestion, prevention of

gastrointestinal infections, stimulation of the immune system, treatment of IBD ...) **(LEMETAIS, 2014)**.

This is why in recent years, the idea of positively modulating the microbiota has developed, thanks to the administration of living microorganisms now called probiotics **(DOMITILLE, 2018)**.

It is now possible to select, identify and cultivate these germs for all kinds of uses: food (fermentation starters), medical (probiotics) or biotechnology (production of molecules: bacteriocins, polysaccharides) **(MATAMOROS, 2008)**.

In light of these data, the original idea of our work is based on the realization of two following complementary aspects:

- ✓ The first part: study the physicochemical analyzes of goat and camel raw milk.
- ✓ The second part: the isolation and purification of lactic acid bacteria having probiotic properties.

***First part***

***Bibliographic***

***synthesis***

# ***Chapter I***

## ***Goat and camel milk***

## **I. Definition**

The Codex Alimentarius of 1999 defines milk as the normal mammary secretion of milking animals obtained from one or more milkings, without adding or subtracting anything, intended for consumption as liquid milk or for further processing (**ROUDAUT and LEFRANCQ, 2005**).

The International Congress for the Repression of Fraud in Geneva in 1908 defined milk as being: an integral product of the total and uninterrupted milking of a healthy dairy female; well-nourished and not overworked. Raw milk must be freshly collected cleanly and not contain colostrum (**BENHEDAN, 2012**), not heat treated or subjected to any other sanitation or preservation treatment other than refrigeration (**LATYR FALL, 1997**). The sale deadline corresponds to the day after the day of milking (**JEANET *et al.*, 2008**).

## **II. Organoleptic and physico-chemical characteristics**

### **II.1. Organoleptic characteristics**

Raw milk is a white, opaque liquid, with a slightly pronounced odor, a slightly sweet taste and a viscosity equal to twice that of water (**ALAIS, 1984**). Due to the absence of  $\beta$ -carotenes, goat and camel milk is whiter than cow's milk (**CHILLIARD, 1997**). Goat milk has a slightly sweet taste (**DUTEURTRTE *et al.*, 2005**). It is characterized by a particular flavor and a stronger flavor than cow's milk (**ZELLER, 2005**).

While camel milk has a sweet or salty taste, depending on the type of fodder ingested and the availability of water, the ingestion of fodder such as alfalfa gives it a sweet taste, while the ingestion of certain halophyte plants such as Atriplex, Salosa and Acacia makes it salty (**AL-HAJ and AL-KANHAL, 2010**).

### **II.2. Physico-chemical characteristics**

The main physico-chemical properties of interest to the dairy industry are density, freezing point, acidity and pH (**GUIMA, 2013; VIGNOLA, 2002**).

**Table 01: Physico-chemical characteristics of camel milk and goat milk**

	pH at 20 ° C	Acidity ° D	Density	Reference
<b>Camel milk</b>	6.51	18.6	1.0230	<b>(Kamoun, 1995)</b>
<b>Goat milk</b>	6.45-6.60	14-18	1.027-1.035	<b>(FAO, 1990)</b>
<b>Cow milk</b>	6.60 - 6.80	15-17	1.028-1.030	<b>(FAO, 1990)</b>

### III. Composition of milk

Milk is a complete food that meets the physiological needs of the newborn; it has common characteristics (composed of water, fat, lactose, caseins and other proteins, mineral salts, especially calcium, vitamins). However, their composition varies, qualitatively and quantitatively, depending on the species (**Table 02**) (**BENSALAH, 2010**).

Its average compositions of the main constituents are varied according to a multiplicity of factors: animal breed, diet and state of health of the animal, lactation period, as well as during milking. The fact remains that the exact composition of a milk sample can only be obtained after physicochemical analyzes (**LARAB, 2014**).

**Table 02: Average compositions of the main constituents of goat and camel milk**  
(**KABIR, 2015**)

	Water (%)	Fat (%)	Proteins (%)	Carbohydrates (%)	Minerals (%)
<b>Camel milk</b>	87.6	5.4	3	3.3	0.7
<b>Goat milk</b>	87	3.8	2.9	4.4	0.9
<b>Cow milk</b>	87.5	3.7	3.2	4.6	0.8

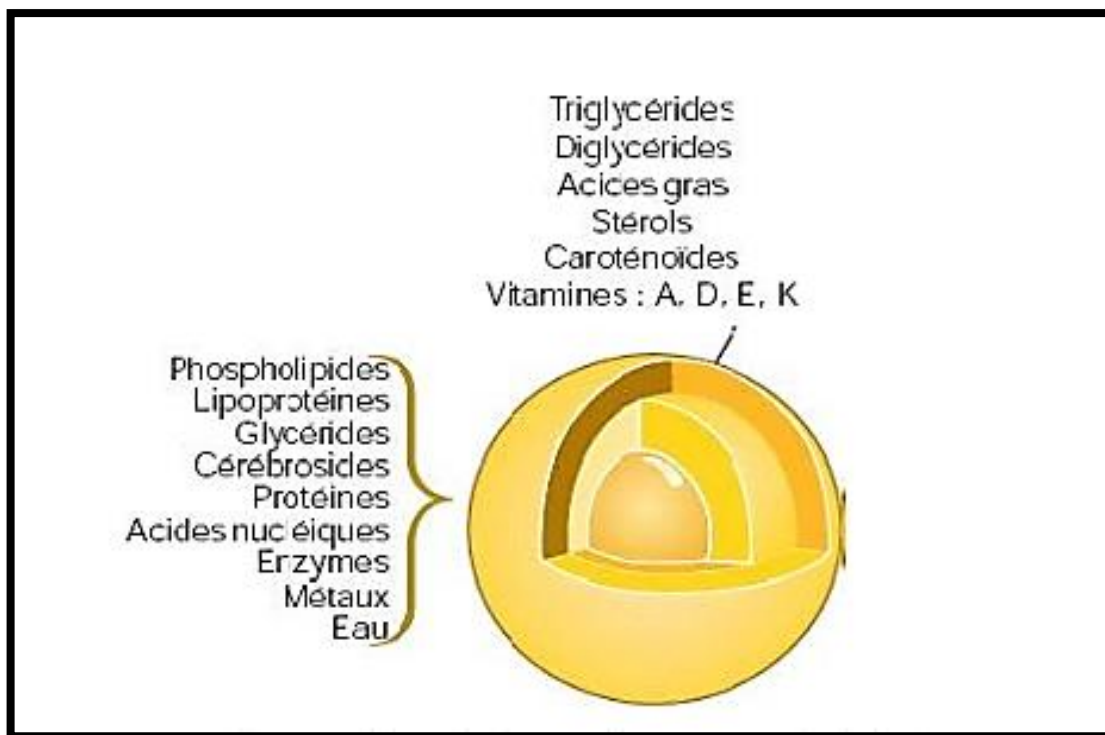
#### III.1. Water

Water is the most important quantitative element: 900 to 910 g per liter (81-87 % of milk), in which are dispersed all the other constituents of milk (**MATHIEU, 1998**).

#### III.2. Fat

Fat consists mainly of 98.5% glycerides (fatty acid and glycerol esters), 1% polar phospholipids and 0.5% liposoluble substances cholesterol, hydrocarbons and vitamins A, D, E, and K (**GOURSAUD, 1985**).

Milk fat has the shape of small spherical globules that are invisible to the naked eye. The size of the fat globules is approximately 0.1 to 20  $\mu\text{m}$ . It should be noted that the size of the fat globules varies according to the species (the globules are smaller in goat's milk (3 to 6  $\mu\text{m}$ ) and camel milk (1.5 to 9  $\mu\text{m}$ )) (**MEHAIA, 1995**).



**Figure 01: Composition of milk fat (HENNANE, 2011)**

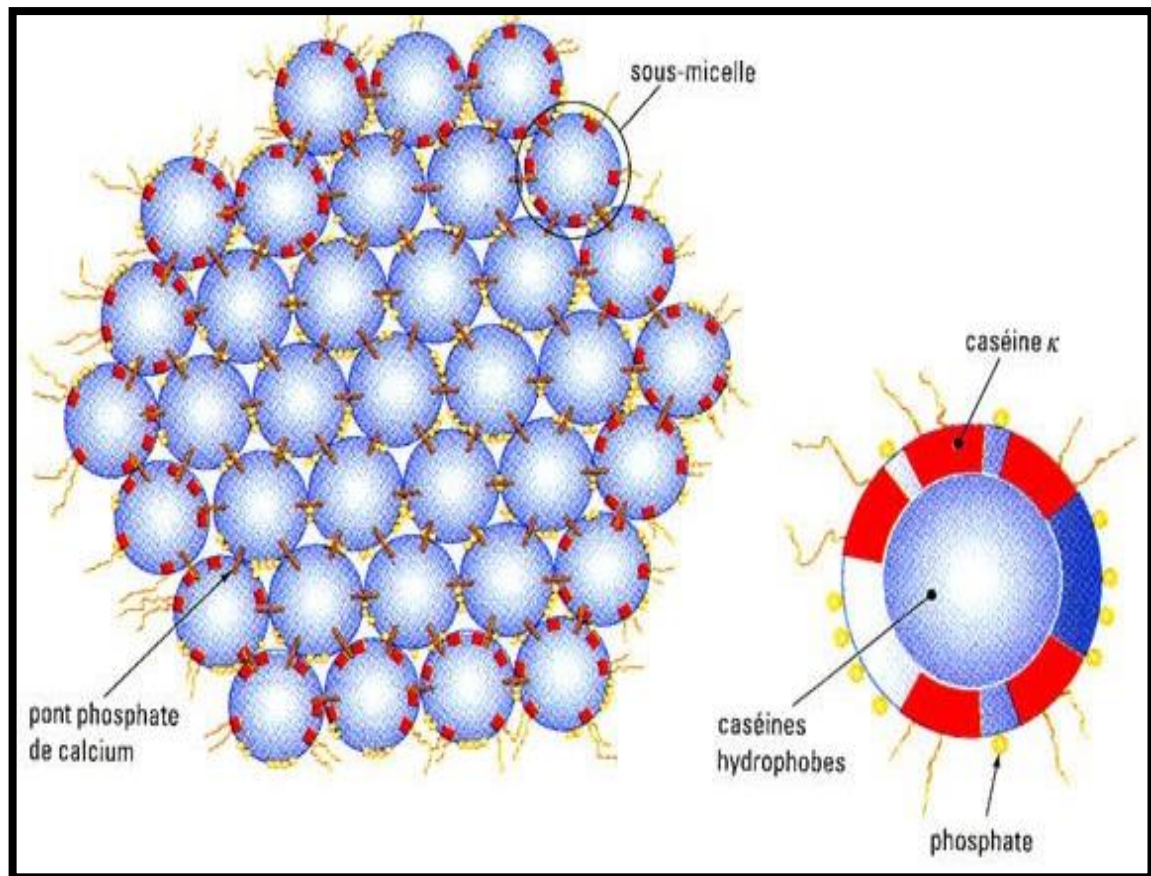
### III.3. Nitrogenous matter

Milk nitrogen includes two groups, protein and non-protein which account respectively for 95% and 5% of milk nitrogen (**BENHEDANE, 2012**).

Proteins are divided into two phases: a micellar phase and a soluble phase. The micellar phase represents the total casein (about 80% of milk proteins) in milk. It is formed by four individual proteins: - Alpha-casein or casein  $\alpha_1$  36% and  $\alpha_2$  10% - Beta-casein or casein  $\beta$  34% - Kappa-casein or casein  $\kappa$  13% - Gamma-casein or casein  $\gamma$  7% (products proteolysis of  $\beta$ -casein) (**GOY *et al.*, 2005**).

A casein micelle contains about 92 to 93% protein, caseins, and 8% minerals. The mineral part of the micelle contains 90% calcium phosphate and 10% citrate and magnesium ions (2.9% Ca, 0.1% Mg, 4.3% phosphate ions, 0.5 % citrate ions) (**CAYOT and LORIENT, 1998**). The presence of casein-linked calcium phosphate is one of the forces responsible for the stability of the casein micelle structure (**MARCHIN, 2007**).

An important property of micelles is that they can be destabilized by the acid or enzymatic route and allow coagulation. It constitutes the basis for the transformation of milk into cheese and fermented milks (**RAMET, 1985**). The other protein fraction (around 17%) of milk is present in whey. The two main serum proteins are  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin (**BENHEDANE, 2012**).



**Figure 02: Micelle and submicelle structure of casein (KABIR, 2015)**

### III.4. Lactose

Lactose and/or carbohydrate, considered the most important milk since it constitutes about 40% of total solids. Its content is on average 50g per liter (LUQUET, 1985). Other carbohydrates may be present in small quantities, such as glucose and galactose which would come from the hydrolysis of lactose (JUILLARD and RICHARD, 1996).

### III.5. Minerals

Milk minerals are found in two main forms, especially in the form of ionized salts which are soluble in serum and in insoluble micellar form. The major basic elements like calcium, potassium, magnesium and sodium form salts with the acid constituents that are proteins, citrates, phosphates and chlorides. In addition, calcium, magnesium, citrates and phosphates are found in colloidal form in the micelles of caseins (JUILLARD and RICHARD, 1996).

### III.6. Vitamins

Milk is among the foods that contain the greatest variety of vitamins, however, the contents are often quite low (**JUILLARD and RICHARD, 1996**).

Goat milk contains almost twice as much vitamin A as cow's milk. It is found exclusively in the form of retinol (**DEBRY, 2001**). While **FARAH (1993)**, **MEHAIA (1994)** and **STAHL *et al.*, (2006)** report that camel milk compared to cow's milk contains lower levels of vitamins A (retinol) and E (tocopherol) and certain group B vitamins such as vitamin B2 (riboflavin), B5 (pantothenic acid) and B9 (folic acid).

Camel milk is characterized by high concentrations of heat-resistant vitamins which make this milk a real food for dietetic purposes. Indeed, this milk is distinguished by its relative richness in vitamin C (**HANOU, 2018**), the contents reported according to (**STAHL *et al.*, 2006**) are on average 3 to 5 times higher than those present in bovine milk.

# ***Chapter II***

## ***Lactic acid bacteria***

## I. Generalities

Lactic acid bacteria, a very old microorganisms used for the fermentation of food for more than 4000 years, without understanding the scientific basis of their use (**DRIDER and PREVOST, 2009**).

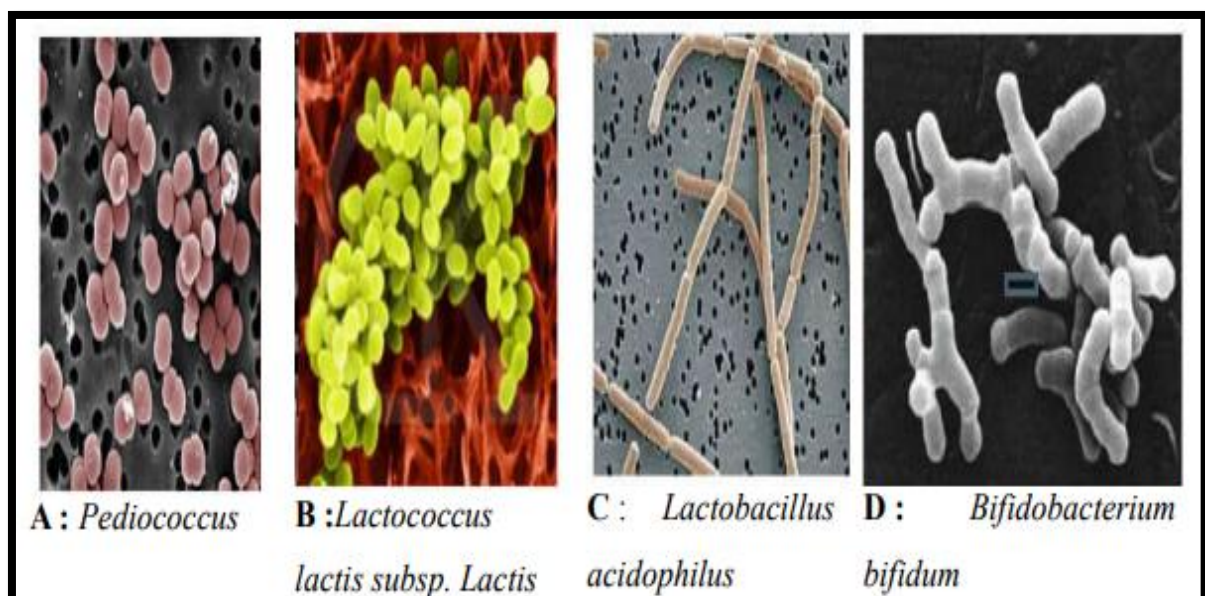
It was not until the end of the 19<sup>th</sup> century, time of great discoveries in microbiology, that the group of lactic bacteria or lactic acid bacteria was defined, more precisely in 1919 by Orla-Jensen. It has brought together several genera characterized by their ability to ferment carbohydrates by producing lactic acid (**TREDEZ and LOUISE, 2008**).

## II. Characteristics of lactic acid bacteria

Lactic acid bacteria (LAB) are defined as living organisms, heterotrophic prokaryotes and chemo-organotrophic (**BELARBI, 2011**). They produce lactic acid as the main end product during the fermentation of carbohydrates (**KANDLER and KUNARTH, 1983**). They were isolated for the first time from milk (**CARR *et al.*, 2002**). They are usually known as microorganisms GRAS (Generally Reconized As Safe) (**CINTAS *et al.*, 2001**).

Lactic acid bacteria are Gram positive, generally immobile, non-spore forming, aero-anaerobic (**SETTANNI and MOSCHETTI, 2010**). They have complex nutritional requirements: amino acids, peptides, vitamins, salts, fatty acids and fermentable carbohydrates (**BADIS *et al.*, 2005**).

They can have different shapes: spherical (shells: Streptococcus, Lactococcus), sticks (bacilli: Lactobacillus) or ovoid (cocci: *Leuconostoc sp*) (**GALVEZ *et al.*, 2011**). In general these bacteria have neither catalase, nor nitrate reductase, nor cytochrome oxidase. They are also unable to ferment glycerol (**SALMINEN *et al.*, 2004**).



**Figure 03: Lactic acid bacteria under electronic microscope (MAGHNIA, 2011)**

### III. Origin and habitat

Lactic acid bacteria are ubiquitous germs, found in different environments, capable of growing on different substrates other than milk (ZERGOUG, 2018). Their presence is revealed in several ecological niches among other plants (plants, fruits, vegetables, and cereals), animals and humans (digestive tract, oral and urogenital cavities) (DELLAGLIO *et al.*, 1994).

Even if they thrive in a variety of habitats, lactic acid bacteria require fermentable carbohydrates, amino acids, fatty acids, salts and vitamins for their growth (ZERGOUG, 2018).

In general, lactic acid bacteria are present everywhere there are high concentrations of carbohydrates, protein degradation products, vitamins and little oxygen (MARTEAU, 2007).

### IV. Classification

Morphological, biochemical and physiological properties was established in 1919 by Orla-Jensen; cited by (BELARBI, 2011).

Modern taxonomy based on phenotypic and genotypic characteristics has resulted in the reclassification of many species of lactic acid bacteria. The classification of lactic acid bacteria is carried out according to their morphology, their type of fermentation and their optimal growth temperature (CINTAS *et al.*, 2001).

According to the edition of Bergey's manual of systematic bacteriology in (2009), lactic acid bacteria are classified in the *Phylum of Firmicutes*, the class of Bacilli and the order of *Lactobacillals* containing thirty-five genera distributed over six families (Table 3).

Among these genera, only twelve are used in food biotechnology, these are: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. Among all these genera mentioned, only five (*Aerococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Pediococcus*) meet the general characteristics of a typical lactic bacteria (SALMINEN *et al.*, 2004).

**Table 03: Families and main genera of lactic acid bacteria (BRENNER *et al.*, 2005).**

Families	Main genus
<i>Lactobacillaceae</i>	<i>Lactobacillus sp., Pediococcus sp.</i>
<i>Leuconostocaceae</i>	<i>Leuconostoc sp., Oenococcus sp., Weissella sp.</i>
<i>Streptococcaceae</i>	<i>Streptococcus sp., Lactococcus sp.</i>
<i>Carnobacteriaceae</i>	<i>Carnobacterium sp.</i>
<i>Enterococcaceae</i>	<i>Enterococcus sp., Tetragenococcus sp., Vagococcus sp.</i>
<i>Aerococcaceae</i>	<i>Aerococcus sp.</i>

Bifidobacterium bacteria are sometimes considered to be part of the lactic acid group thanks to the similarity of their physiological and biochemical properties and their presence in the same ecological habitat. However, these microorganisms belong to the phylum Actinobacteria and are therefore phylogenetically distant from lactic acid bacteria (TRIAS, 2008).

## V. Fermentation routes of lactic acid bacteria

The sugars used by lactic acid bacteria are mainly fermented into lactic acid. To enter the cell, these sugars must first cross the cell membrane. Two active sugar transport systems are present in lactic acid bacteria: the dependent phosphotransferase-phospho-enol-pyruvate (PTS) system, which couples the transport and phosphorylation of the carbohydrate (cascade phosphorylation), and the Energy-Dependent Permease system, which induces carbohydrates in the form of free sugars (CORRIEU and LUQUET, 2008).

There are two main ways of hexose fermentation which are used to classify homofermentative and heterofermentative lactic acid bacteria.

### V.1. Embden-Meyerhof route (Homofermentaries)

Homofermentative species, such as *Pediococcus*, *Streptococcus*, *Lactococcus* and some *Lactobacillus*, produce mainly lactic acid by fermentation of sugars (glycolysis) (CAPLICE and FITZGERALD, 1999).

## V.2. Dickens-Horecker route (Heterofermentaries)

This group of lactic acid bacteria uses the pentose phosphate route (SALMINEN, 2004). As for heterofermentative species, such as *Leuconostoc* and *Weissella*, they produce, in addition to lactic acid, several metabolites such as other organic acids (acetate), ethanol, acetoin, carbon dioxide and compounds aromatics (such as diacetyl, acetaldehyde) (CAPLICE and FITZGERALD, 1999).

The metabolism of the genus *Bifidobacterium* is quite specific; indeed it would be the bifid fermentation pathway or the fructose-6-P phosphoketolase (FPC) pathway. Fructose-6-P is split by fructose-6-phosphate phospho-ketolase into erythrose-4-phosphate and into acetyl phosphate and glyceraldehyde-3-phosphate to form acetyl phosphate and glyceraldehyde-3-phosphate (Figure 4) (DRIDER *et al.*, 2009).

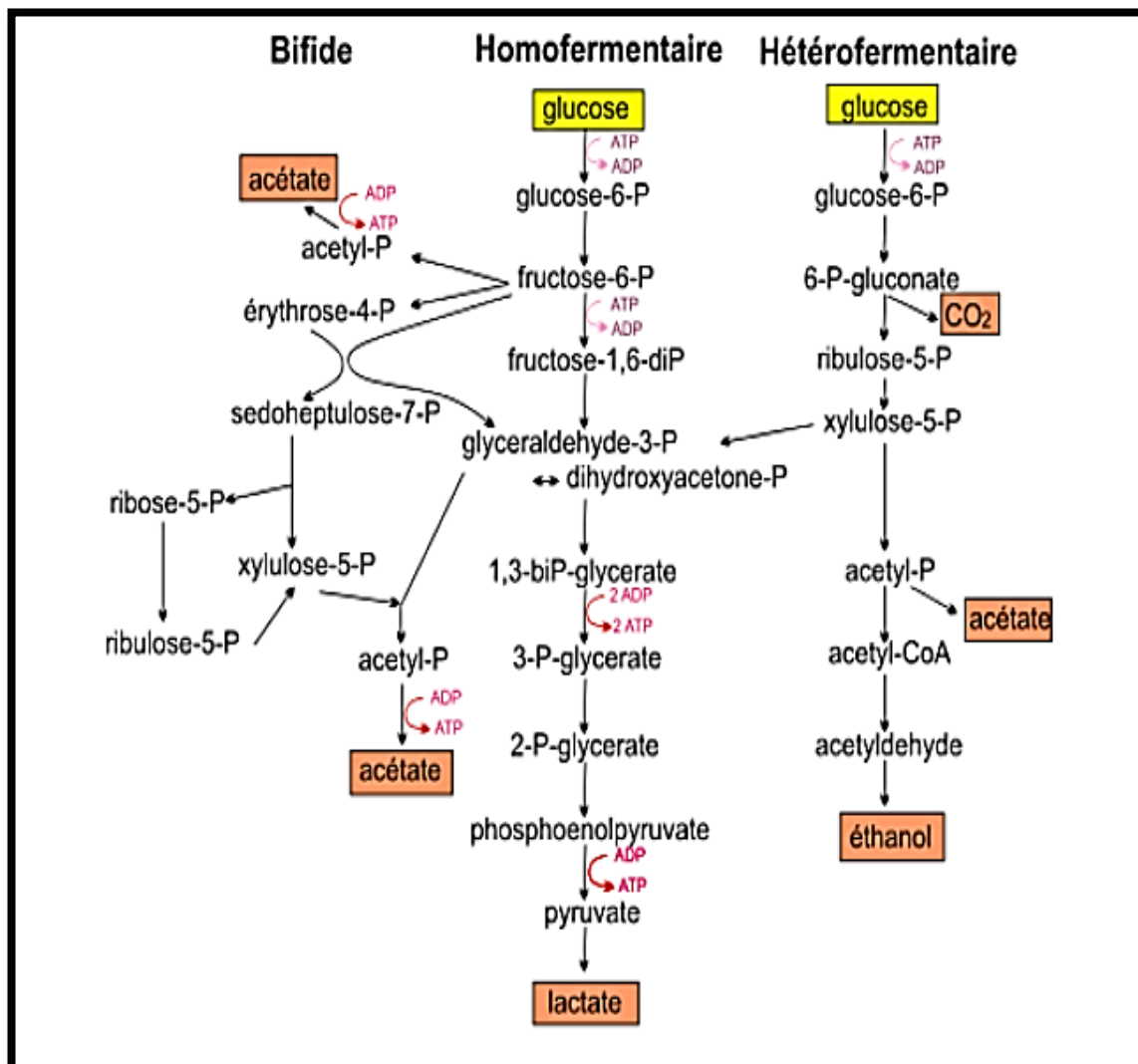


Figure 04: Homofermentative, heterofermentative and bifid metabolic pathways of glucose degradation (DRIDER *et al.*, 2009)

## **VI. Nutritional requirements of lactic acid bacteria**

LB have a low biosynthetic ability and are very demanding from a nutritional point of view. For their growth, they require not only nitrogen, carbon and phosphate substrates but also growth factors such as vitamins and trace elements (**DESMAZEAUD, 1983**).

### **VI.1. Amino acid requirements**

BL require the exogenous supply of amino acids for their growth, because they are unable to synthesize them from a single nitrogen source (**DESMAZEAUD, 1992**).

The nutritional requirements of lactic acid bacteria have been determined by their ability to grow in a chemically defined medium (MCD). These amino acid requirements are, however, variable from one strain to another. Generally, *Streptococcus thermophilus* is the demanding monk species (6 amino acids maximum) while lactobacilli are auxotrophic for a very large number of amino acids (**MONNET *et al.*, 2008**).

### **VI.2. Vitamin requirements**

They are unable to synthesize certain vitamins essential for their growth. These must be provided by the culture medium. The requirements are very variable, even within the same species. A distinction is made between vitamins whose needs are absolute, those which stimulate growth and those which have little effect on growth. Generally, BL requirements often relate to group B vitamins such as niacin, riboflavin and panthothenic acid (**MONNET *et al.*, 2008**).

### **VI.3. Nitrogen source requirements**

In certain strains of lactococci, the production of acid in milk can be stimulated by the mixture: adenine, guanine, uracil and xanthine. In synthetic media, lactobacilli require the presence of adenine, cytosine, deoxyguanosine, guanine, thymidine and uracil (**LAW and KOLSTAD, 1988**).

In general, in lactobacilli, the nitrogen base requirements are very variable according to the strains; the addition of these compounds can even, in certain strains, lead to growth inhibition phenomena (**DESMAZEAUD, 1992**).

### **VI.4. Influence of cations**

BL have a remarkable need for ion. Indeed, the mineral elements play an essential role in the growth of BL and precisely in the bacterial enzymatic activities. Certain species such as *Leuconostoc mesenteroides* cannot grow in the absence of mineral ions, which indicates the

absolute requirement for these elements (FOUCAUD *et al.*, 1997). Magnesium is the main divalent cation of living cells. This ion is essential for the growth of *Lb helveticus* (DESMAZEAUD, 1992).

The mineral ion requirements may vary from species to species.  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Na^+$ ,  $K^+$  and  $Cl^-$  were essential components in the culture medium of *L. plantarum* (WEGKAMP *et al.*, 2010). While  $Zn^{2+}$ ,  $Fe^{2+}$  and  $Mg^{2+}$  had a significant effect on the growth of *Lactococcus lactis* (ZHANG *et al.*, 2009).

## VII. Interest of lactic acid bacteria

Lactic acid bacteria play an important role, whether in the food industry or in the therapeutic field.

### VII.1. Food industry

The involvement of lactic acid bacteria in the fermentation and bio-conservation of food has been known since time immemorial. Thus, the strains of *Lactobacillus bulgaricus*, *Sterptococcus thermophilus* are used for the production of yogurt, cheeses and fermented milks (YATEEM *et al.*, 2008). Wine, fish, meats, cold meats, sourdough bread are among others fermentation products of lactic acid bacteria (BADIS *et al.*, 2005).

The purpose of using these latter is to improve the organoleptic characteristics of fermented products and therefore increase the duration of their conservation without using chemical conservation, thanks to the antimicrobial substances they produce (DORTU and THONART, 2009).

The strains used in the food industry must meet certain criteria: absence of pathogenicity or toxic activity, ability to improve organoleptic characteristics, safety, ease of culture, conservation, and maintenance of desirable properties during storage (MARTH and STEELE, 2001).

### VII.2. Therapeutic area

Being probiotics, lactic acid bacteria bring benefits to the host by conferring a balance on the intestinal microflora, and also playing an important role in the maturation of the immune system (YATEEM *et al.*, 2008).

Various studies have demonstrated the preventive as well as curative role of these bacteria on several types of diarrhea (MKRTCHYAN *et al.*, 2010). Others have cited their ability to reduce food allergies through their proteolytic activity (EL-GHAISH *et al.*, 2011). UEHARA *et al.*, (2006) have demonstrated the capacity of strains of *Lactobacillus crispatus*, used in the form of suppositories to prevent colonization of the vagina by pathogenic bacteria and thus prevent relapse in women who suffer from frequent and repeated inflammation of the bladder.

### VII.3. Industrial domain

Lactic acid bacteria have fairly diverse metabolic activities and an ability to adapt to different environments. This diversity is responsible for their wide range of applications on an industrial scale (STREIT *et al.*, 2007).

In the food industry, these microorganisms allow the conversion of a wide variety of raw materials, thus leading to many products: sausages, fermented milks, cheeses, butter, fermented olives and certain wines. Among these applications, the dairy industry is undoubtedly the largest user of commercial lactic ferments (STREIT *et al.*, 2007).

Lactic acid bacteria are also used in the chemical industry (production of lactic acid), in the medical field (in particular for the treatment of intestinal dysfunctions) and in the industry of food additives (production of exopolysaccharides). They are also used for the production of bacteriocins and therapeutic proteins (RODRIGUEZ *et al.*, 2003).

# ***Chapter III***

## ***Probiotics***

## I. Historical

At the beginning of the 20<sup>th</sup> century, **Elie Metchnikoff** (Russian scientist, Nobel laureate and professor at the Institut Pasteur in Paris) discovered in 1908 that the longevity of Bulgarian farmers was linked to their consumption of milk fermented by Lactobacilli. After his research, the scientist claimed that bacteria synthesizing lactic acid offer health benefits leading to greater longevity. Elie Metchnikoff obtained a Nobel Prize for his research.

The research then continued; a Bifidobacterium was first isolated by a researcher at the Institut Pasteur from a breastfed child, and he called it *Bacillus bifidus communis*. This researcher claimed that bifidobacterium reduced the amount of proteolytic bacteria (*Clostridium difficile*), which causes diarrhea and he recommended the administration of bifidobacteria bacteria to children suffering from this symptom (**WGO, 2008**).

Metchnikoff and Tissier are first to put forward out to emit the idea of administering living exogenous microorganisms in order to compensate for a possible imbalance of the intestinal ecosystem. The concept of probiotics was born and by 1917, yogurt was developed industrially. It was sold exclusively in pharmacies and its consumption was then recommended in digestive disorders (**EZZARIGA, 2015**).

## II. Definition

### II.1. Probiotic

The term "probiotics" was introduced into the literature in 1965 (pro: which is positive and bios: life, as opposed to antibiotics) (**KECHAOU, 2012**). The most used definition is that proposed by a committee of experts, which defines probiotics as living microorganisms which, when administered in sufficient quantities, confer a health benefit on the host (**FAO/WHO, 2002**). It is most often bacteria or yeasts which can be incorporated into different types of products, including food (especially fermented dairy products, particularly bifidobacteria or lactobacilli), medicinal substances and food supplements (**BEAUSOLEIL and FORTIER, 2007**).

They can survive in the digestive tract of the host and they survive the acidity of the stomach to be able to act in the intestine, where they are likely to exert various beneficial actions (**BENBOUZIANE, 2014**).

## II.2. Prebiotics and symbiotic

Prebiotics, unlike probiotics, are not microorganisms but fermentable substances, in other words substances resistant to the different stages of digestion. These are generally small sugars (fructo, galacto-oligosaccharides, etc.) but also fibers, inulins, lactuloses, polyols, etc. (LAFFARGUE, 2015). Their fermentation stimulates the growth and activity of certain microbial species (FRAYSSINHES, 2017). They increase the number of bifidobacteria and lactic acid producing bacteria, since these bacterial groups could be beneficial for the host (Berta *et al.*, 2003).

Symbiotics are a combination of prebiotics and probiotics: Prebiotics increase the growth and activity of probiotics (CLERC and PINNA, 2007; WGO, 2008). They are the substrate of probiotics (TAKEMURA *et al.*, 2010).

## III. Classification

We mainly find lactic bacteria (*lactobacilli*, *bifidobacteria*) or yeasts (*Saccharomyces boulardii*) that we find in milk foods or food supplements (HEYMAN and HEUVELIN, 2006).

### III.1. Bacteria or lactic ferments

#### III.1.1. Lactobacilli

Lactobacilli form a large part of lactic acid bacteria (LAND *et al.*, 2005), they are Gram-positive, anaerobic, non-spore-forming and non-mobile bacteria, presented in the form of long and thin sticks, or very short, or curved or even ovoids. Most lactobacilli multiply in a range of temperatures between 15 ° C and 42 ° C (TAILLIEZ, 2004). Among the most widely used commercial strains, we find the strains of *L. acidophilus*, *L. casei*, *L. paracasei* and *L. johnsonii*, because these three species offer good resistance to gastric acidity and exhibit strong adhesion to intestinal cells (AFMO, 2007).

#### III.1.2. Bifidobacteria

Bifidobacteria are Gram-positive bacteria, strict anaerobes, immobile, of variable morphologies, the most characteristic of which is a Y shape, often containing ramifications (BUTEL and WALIGORA-DUPRIET, 2015; MELANIE, 2007). Bifidobacteria form the predominant bacterial group of the human intestinal flora (MITSUOKA, 1990), they are mostly used as probiotics especially by the food industry because of their many health benefits. This is the case with the commercial strain *B. animalis* ssp. *lactis* bb12 (MOHAN *et al.* 2006; KABEERDOSS *et al.* 2011).

#### III.1.3. Other microorganisms belonging to lactic acid bacteria

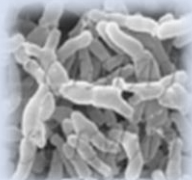
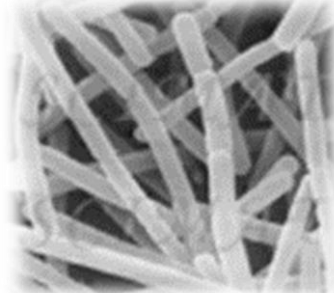
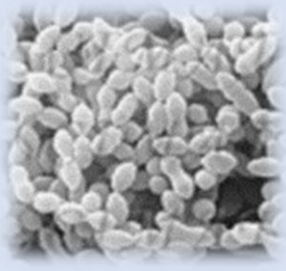
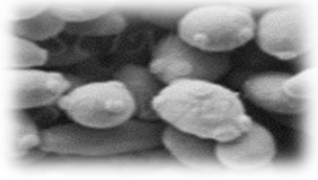
These bacteria have a shell morphology arranged in chains of variable length like lactococci, *streptococci* and *enterococci* (CARMONA, 2016; PIQUEPAILLE, 2013).

### III.2. Yeasts

Yeasts are part of the family of single-celled fungi, used in the food industry for the production of alcoholic beverages but also for baking (RAMPAL, 1996).

Yeasts, especially of the *Saccharomyces* type, are not part of the human intestinal flora but are used as probiotics. The best known is the non-pathogenic: *Saccharomyces cerevisiae* ssp *bouardii*, mainly known for its preventive and curative effects in diarrhea post antibiotic. *Saccharomyces bouardii* is characterized by its ability to tolerate high temperatures and an acidic pH (BULTEL, 2017).

**Table 04: Main species used as probiotics and examples of strains sold (IZEQUERDO ALEGRE, 2009).**

Genus	Species
<p><b><i>Bifidobacterium</i></b></p> 	<p><i>Bf.longum</i> BB536  <i>Bf.breve</i>  <i>Bf.lactis</i> Bb12  <i>Bf.Lactis</i> HN019  <i>Bf.Animalis</i> DNI 73010  <i>Bf.Infqntis</i> 35264</p>
<p><b><i>Lactobacillus</i></b></p> 	<p><i>Lb.acidophilus</i> La5  <i>Lb.casei</i> DNI 14001  <i>Lb.renteri</i> ATCC55730  <i>Lb.delbreckii</i> suhsp. <i>bulgaricus</i> 2038  <i>Lb.gasseri</i> K7  <i>Lb.johnsonni</i>  <i>Lb.paracasei</i> F19  <i>Lb.paracasei</i> 299V  <i>Lb.rhamnosus</i> GG</p>
<p><b><i>Other lactic acid bacteria</i></b></p> 	<p><i>Streptococcus thermophilus</i> 1131  <i>Enterococcus faecalissymbioflor</i>  <i>E.faecium</i> SF68  <i>Pediococcusacidolactici</i></p>
<p><b><i>Non-lactic microorganisms</i></b></p> 	<p><i>Bacillus subtilis</i>  <i>B. cereus</i>  <i>Saccaromyces bouardii</i>  <i>S.cerevisiae</i></p>

#### IV. Sources of probiotics

Many commonly consumed foods also contain live bacteria that are considered to be beneficial to health and could be considered probiotics.

- ✓ Yogurt
- ✓ Cheese
- ✓ The sauerkraut
- ✓ Kefir grains
- ✓ Others: Soy-based preparations, pickles, olives, cold cuts, fish, crustaceans, fruits, green vegetables, sourdough bread, brewer's yeast, beer for keeping ... naturally combine different strains essential for them obtaining (ROFES, 2018).

#### V. Mechanisms of action of probiotic bacteria

Several mechanisms by which certain probiotics exert protective or therapeutic effects have been proposed (Figure 05) (YAHILA, 2017).

##### V.1. Inhibition of adhesion of pathogens: competition / exclusion phenomenon

Probiotics exert a direct antimicrobial action by opposing the invasion of pathogenic microorganisms in the digestive tract while preventing their adhesion to the intestinal walls (VANDERPOOL *et al.*, 2008).

Indeed, there is a direct competition between the probiotic strains and the infectious germs to occupy the sites of adhesion to the walls of the intestine. Some probiotics have a capacity to adhere to the digestive tract and can colonize it a prolonged period. This property could constitute an ecological advantage favoring their implantation at the level of the intestinal walls and consequently, the inhibition of the fixing of pathogenic germs. Thus, probiotics play a role a physical barrier against pathogenic microorganisms. This phenomenon has been observed in certain lactobacilli which adhere to the intestinal villi and inhibit the fixation of enteropathogenic *Escherichia coli* (ROSELLI *et al.*, 2006; COLLADO *et al.*, 2007).

##### V.2. Production of antimicrobial substances

Probiotics could also limit the growth of pathogens by exerting an indirect antimicrobial action. The latter is achieved through the production of various antimicrobial

compounds such as bacteriocins, organic acids, hydrogen peroxide (KLAENHAMMER, 1993).

### V.3. Stimulation the activity of the intestinal immune system

The interaction of probiotics with the immune system increases the host's immune response against enteropathogens. In fact, probiotics are involved in stimulating adaptive immunity, such as the production of IgA-type antibodies (SHU and GILL, 2002), as well as innate immunity, such as the production of macrophages, monocytes, etc. Therefore, probiotics act as adjuvants by modulating a rapid response of the intestinal mucosa and thereby strengthening the intestinal immune system (OELSCHLAEGER, 2010).

### V.4. Reinforcement the barrier effect of the intestinal epithelium

The intestinal epithelium plays an essential role in the defense and protection of the digestive tract. Indeed, it constitutes a real continuous barrier making it possible to prevent the invasion of the enteric flora as well as the different constituents of lumen in the organism (GROSCWITZ and HOGAN, 2009).

However, certain endogenous or exogenous events can alter the protective functions of this barrier, resulting in the passage of microorganisms through the intestinal mucosa and thus triggering inflammatory reactions and intestinal disorders (HOOPER *et al.*, 2001).

Several studies have shown that probiotics are involved in strengthening and maintaining the intestinal barrier by promoting better integrity of the intestinal mucosa. The barrier function of the intestinal mucosa is mediated by the increased expression of the genes involved in the functioning of tight junctions of the intestine (ANDERSON *et al.*, 2010). Probiotics also increase the barrier function of the intestinal mucosa by promoting the production of mucus and certain IgA-type antibodies (OHLAND and MACNAUGHTON, 2010).

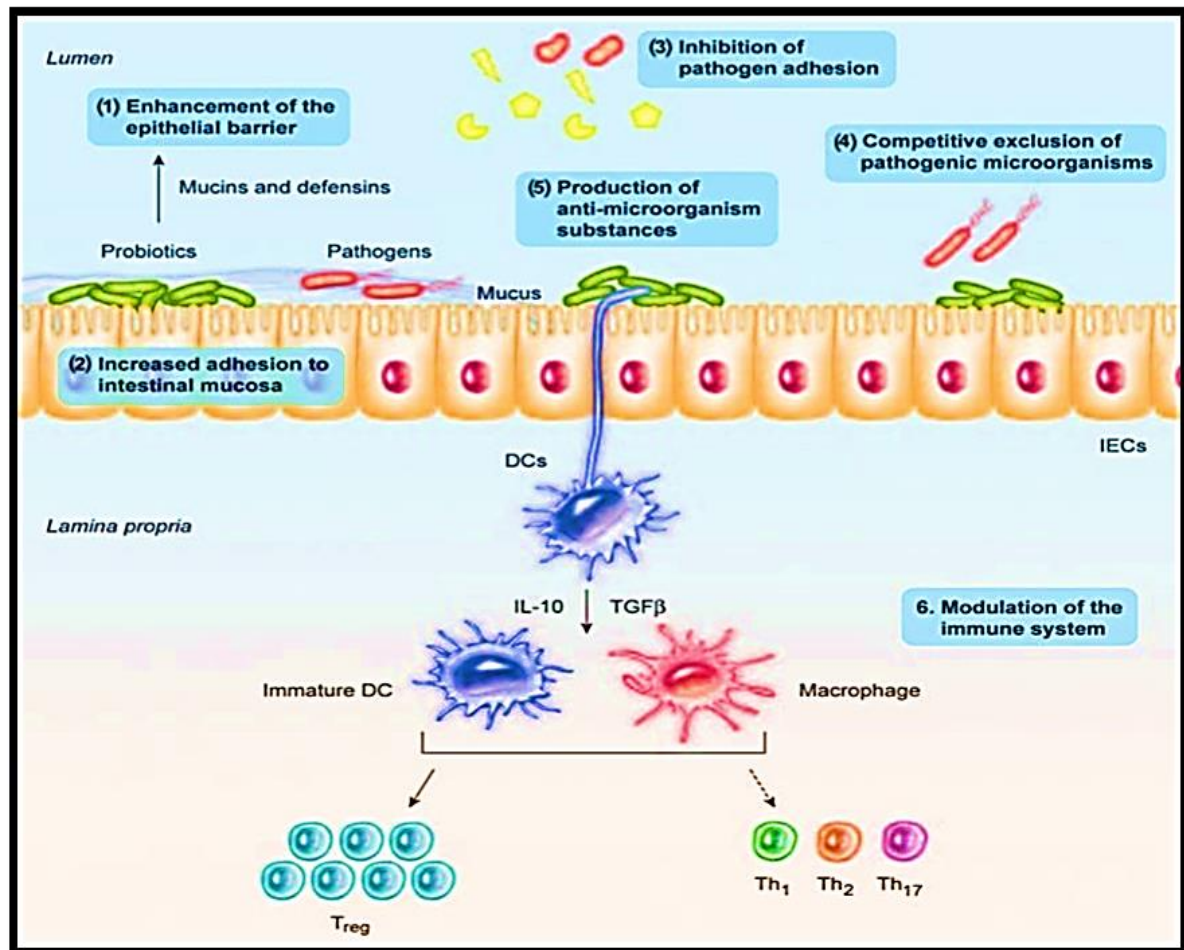


Figure 05: Main mechanisms of action of probiotics (BERMUDEZ-BRITO *et al.*, 2012)

## VI. Current use of probiotics

Probiotics are currently the subject of a certain consensus in the scientific community thanks to their beneficial effects on the health of the host (NAÏMI, 2014). The main effects reported in the literature are:

- ✓ The decrease in diarrhea associated with antibiotics.
- ✓ Lower blood cholesterol or
- ✓ Decrease in the risk of colon cancer
- ✓ Improved digestibility of proteins, lactose and nutrients .
- ✓ Synthesis of certain vitamins.
- ✓ Modification of intestinal écologie.
- ✓ Immune system stimulation and treatment of inflammatory bowel disease (IBD) (IZQUIERDO ALEGRE, 2009; ANN and FERGUS, 2006).

***Second part***

***Experimental study***

# ***Chapter I***

## ***Material & methods***

Our study is based on the evaluation of a few physicochemical parameters involved in the variation of the quality and quantity of the elements composing raw milk of goat and camel on a hand, and the isolation of bacteria with probiotic properties from these milk samples on the other. This work was carried out in the quality and compliance analysis and control laboratory FATILAB in El Oued, Al-Mordjane Lab for medical analyzes, Echahid Hamma Lakhdar University in EL OUED.

## I. Material

### I.1 Biological material

#### I.1.1 Raw milk

Raw milk samples were taken from different healthy animals living in different regions of the wilaya of El Oued (Nakhla, Guemar) during the month of December 2019. The milking of animals (goat, camel) is done in the morning under aseptic conditions.

A total of 06 samples were collected from a manual sample after washing with soapy water, rinsing with bleach and drying the udder, the first 2 jets are eliminated and the milk is collected in sterile vials of 180 ml brought the same day to the laboratory to avoid any kind of contamination.

**Table 05: Characteristics of the animals used for sampling the various raw milk**

Characteristics		Region	Race/Population	Color	Way of life
Animals					
Goat	E1	Nakhla	Cherki	Brown	Intensive
	E2	Nakhla	Cherki	Brown / white	Intensive
	E3	Nakhla	Cherki	Black / white	Intensive
Camel	E1	Guemar	Targui	Brown	Semi intensive
	E2	Guemar	Targui	Brown	Semi intensive
	E3	Guemar	Targui	White	Semi intensive

### I.1.2 Indicator bacterial strains (pathogens)

To evaluate the antimicrobial activity of lactic acid strains with probiotic properties, five indicator bacteria were used:

**Table 06: Indicator pathogenic strains used**

Strain	Gram	Reference	Origin
<i>Staphylococcus aureus</i>	Positive	ATCC 44300	Institute Pasteur - Algeria-
<i>Bacillus subtilis</i>		ATCC 6633	
<i>Pseudomonas aeruginosa</i>	Negative	ATCC 9027	
<i>Escherichia coli</i>		ATCC 25922	
<i>Salmonella enterica serovar Typhi</i>		ATCC 14028	

## II. Methods

The different stages of the methodology followed in this study are shown schematically in the figure below:

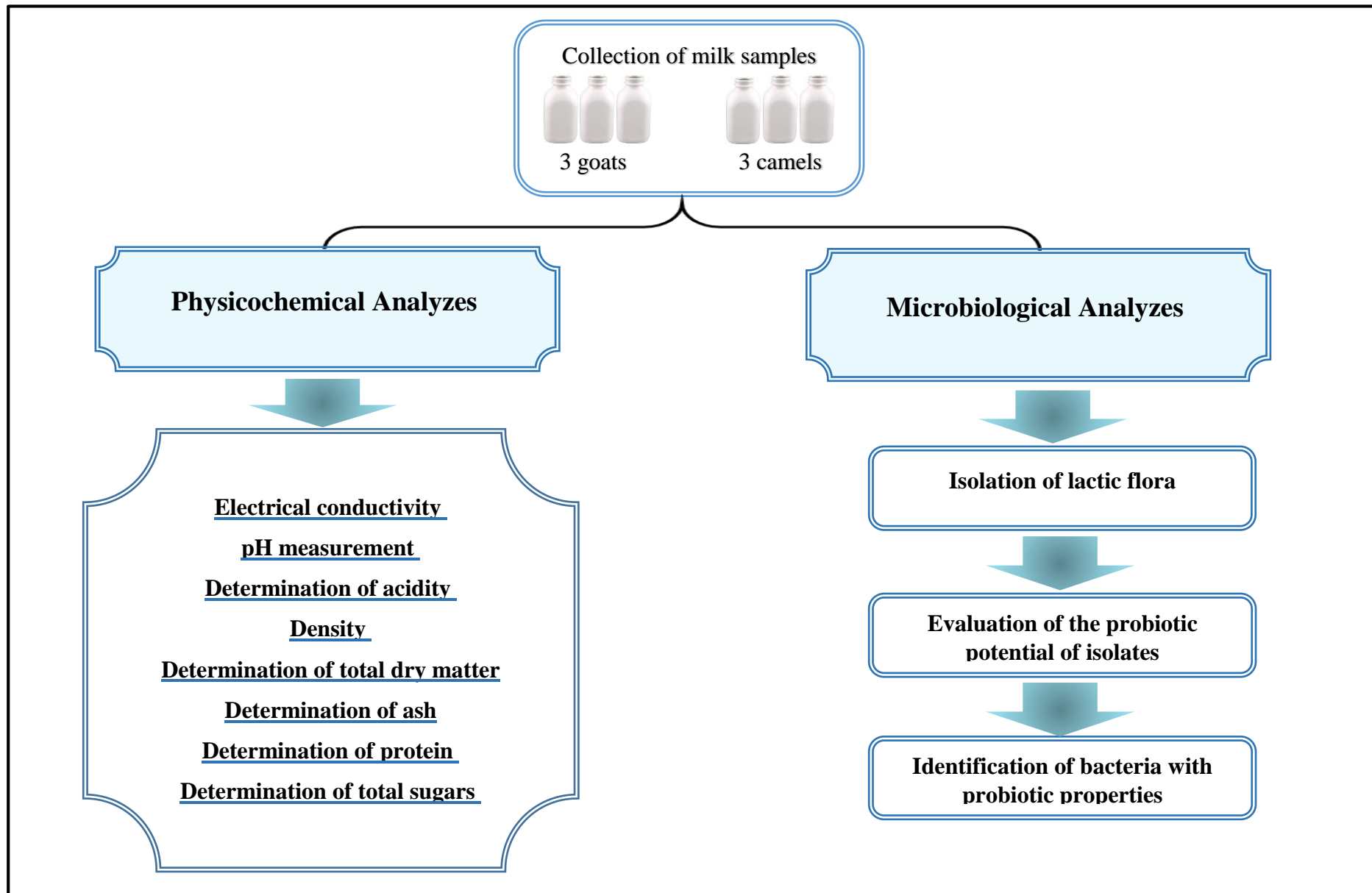


Figure 06: Procedure of different stages of the methodology

## II.1. Milk samples analysis

### II.1.1. Physicochemical analyzes

#### a. Electrical conductivity

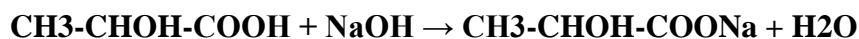
Electrical conductivity reveals the property of a body or a substance to transmit electric current due to ions (mainly chlorides, phosphates, citrates, carbonates and bicarbonates of potassium, sodium, calcium and magnesium). It is measured directly using a conductivity-meter in millisiemens per centimeter (mS/cm) (JACQUINET, 2009).

#### b. pH measurement

PH by definition is the measure of the activity of the H<sup>+</sup> ions contained in a solution. The pH measurement provides information on the acidity of the milk. The latter is determined using a pH meter (KIZI and MAKDOUD, 2014).

#### c. Determination of acidity

This test is demonstrated according to (NF V04-206 (January 1969)). It is based on the titration of lactic acid with sodium hydroxide ((NaOH: 1 / 9N) in the presence 0.1ml of phenolphthalein (1% in 95% alcohol), as a colored indicator, which indicates the limit of neutralization by color change (pale pink).



This acidity is expressed in degree Dornic (° D) where: 1 ° D represents 0.1 g of lactic acid in a liter of milk and given by the following formula:

$$A = V.10$$

Where:

V: volume in ml of sodium hydroxide solution.

#### d. Density

The density of milk is a dimensionless quantity, which designates the ratio between the mass of a given volume of milk at 20° C and the mass of the same volume of water (POINTURIER, 2003). The density measurement is carried out by thermo-lactodensimeter which is provided with a scale on its upper part indicating graduations.

**e. Determination of total dry matter**

The term “dry matter” of milk is understood to mean the product resulting from the drying of the milk in an oven set at  $103 \pm 2^\circ \text{C}$ , after evaporation of the water by means of a boiling water bath. The protocol described by the standard (NF V 04-207 of September 1970).

In the dried and tared capsule weighed beforehand, 5 ml of milk are introduced using a micropipette then the capsule is placed for thirty minutes in the boiling water bath, in an oven set at  $103 \pm 2^\circ \text{C}$  for 3 hours. Then place the capsule in the cooling apparatus and allow cooling to room temperature. The residue is then weighed using an analytical balance.

It is expressed in grams per liter or as a percentage in mass as follows:

**In grams per liter:**  $(M2 - M0 / V) \times 1000$

**Percentage % :**  $(M2 - M0 / M1 - M0) \times 100$

**Where:**

**M0:** is the mass in grams of the dry or calcined empty capsule.

**M1:** is the mass in grams of the capsule with its contents.

**M2:** is the mass in grams of the capsule with its contents after drying.

**V:** is the volume in milliliters of the milk test portion.

**f. Determination of ash**

The term “milk ash” is understood to mean the product resulting from the incineration of the dry residue of milk at  $525 \pm 25^\circ \text{C}$  in a muffle furnace (NF V 04-208 of October 1989).

It is expressed in grams per liter or as a percentage by mass.

**In grams per liter:**  $(M2 - M0) \times 1000 / V$

**Percentage % :**  $(M2 - M0) \times 100 / (M1 - M0)$

**Where:**

**M0:** is the mass in grams of the empty capsule.

**M1:** is the mass in grams of the milk sample.

**M2:** is the mass in grams of the capsule and the ash.

**V:** is the volume in milliliters of the milk test portion.

**g. Determination of protein content**

The protein content (total protein, serum protein and caseins) is determined by the method of (**LOWRY, 1951**).

The principle is based on the development of a dark blue color following the addition to the protein solution a copper salt in an alkaline medium, then of the Folin-Ciocalteu reagent. The coloration results from the reaction of copper with peptide bonds and the reduction of phospho-tungstomolybdic acid by tyrosine, tryptophan and cysteine.

The reduced species absorb light at 750 nm. The dosage protein is performed using a visible spectrophotometer. The protein concentration of the sample analyzed is determined by referring to a calibration curve established using bovine serum albumin (BSA) (**CHETHOUNA, 2011**). The protocol is described in **Annex 02**.

**h. Determination of total sugars**

The total sugar is determined according to the method of (**DUBOIS *et al.*, 1956**), the principle of which is based on the following reaction: concentrated sulfuric acid causes, when hot, the departure of several water molecules from the oses. This dehydration is accompanied by the formation of a hydroxy-methyl furfural (HMF) in the case of hexose and of a furfural in the case of a pentose. These compounds condense with phenol to give colored complexes (yellow-orange).

The intensity of the coloring is proportional to the concentration of the ose. The optical density is measured at 488 nm using a spectrophotometer. The sugar concentration of the sample analyzed is determined by referring to a calibration curve established using glucose (**BENAOUIDA, 2008**). The protocol is described in **Appendix 03**.

## II.1.2. Microbiological analyzes

### II.1.2.1. Isolation and purification of lactic acid bacteria

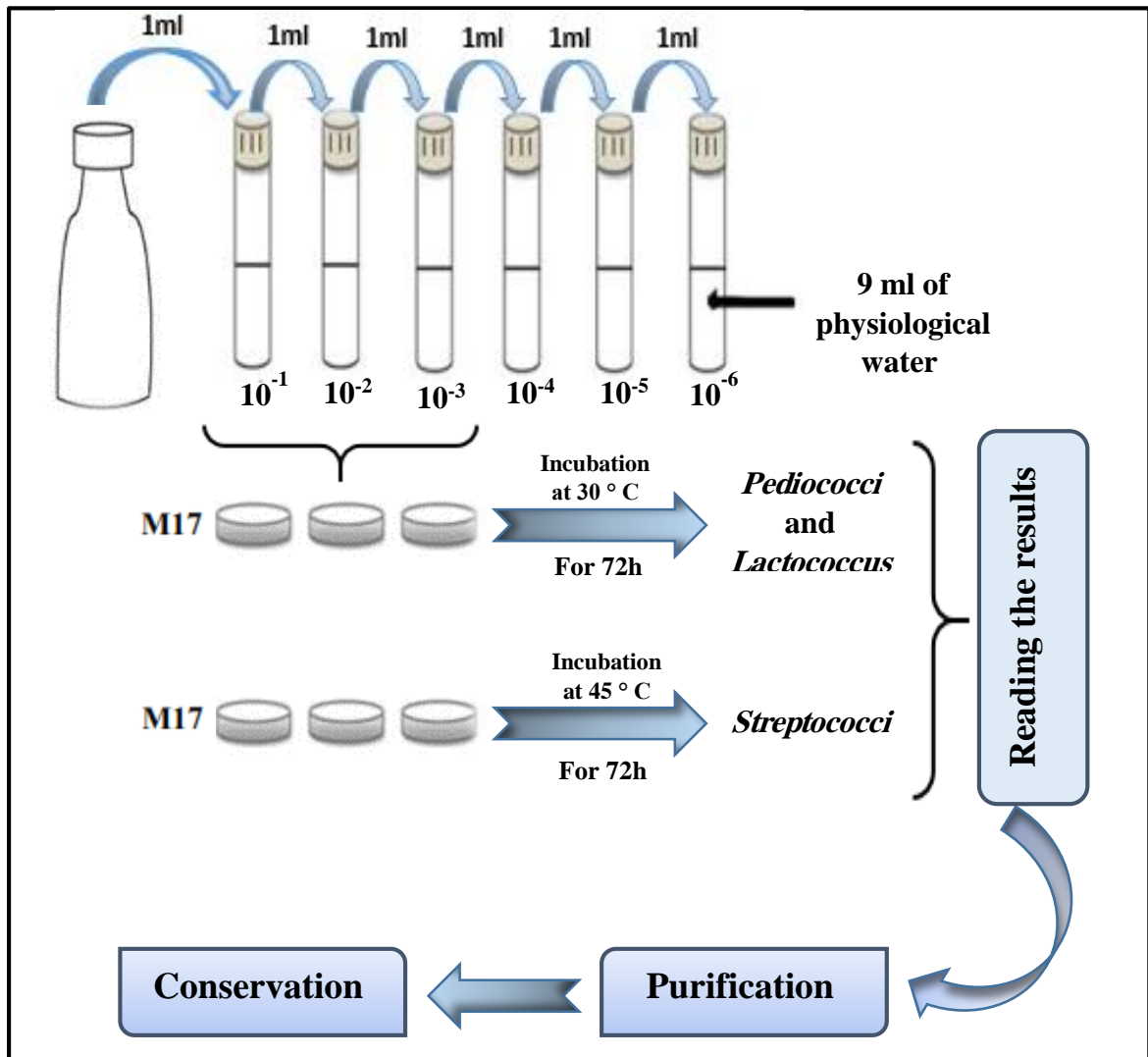
The selective isolation of lactic acid bacteria (LAB) by culture on several media was carried out according to the methods described by the International Milk Federation 1996 (**BADIS et al., 2005**).

Before undertaking the search for the different germs, a series of decimal dilutions should be prepared from each sample according to the method described by (**ISO 6887-6 (2013)**). After vigorous homogenization of the milk samples, 1 ml of the mother solution was added to 9 ml of physiological water (NaCl at 0.9%), a series of decimal dilutions was prepared ( $10^{-1}$  to  $10^{-6}$ ) and homogenized (**Figure 07**). After several tests, only the  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$  dilutions are retained for then inoculated with streaks on the surface of M17 agar. These dilutions make it possible to obtain sufficiently separated colonies. Incubation takes place at 30° C /72h for *Pediococci* and *Lactococcus* and at 45° C /72h for *Lactic Streptococci* (**BADIS et al., 2005**).

In order to purify the strains, successive subcultures are carried out on M17 agar. After the incubation, colonies of different morphological aspects (size, color, surface, depth, etc.) are successively reseeded on M17 agar medium by the streaking method in order to ensure the purity of the cultures until obtaining very distinct and homogeneous colonies having the same aspect, the same color, the same size and the same shape (**GUIRAUD and ROSEC, 2004**).

#### II.1.2.1.1. Strain conservation

For short term conservation, pure strain is performed on solid slant medium. After growth at the optimum temperature, the cultures are maintained at 4° C and the renewal of the strains is carried out by subculturing every 4 weeks (**BENYOUCEF, 2019**). For long term conservation, the strains are kept at -20° C.



**Figure 07: Protocol for the isolation, purification and conservation of lactic acid strains**

### II.1.2.2. Probiotic properties of isolates

For the determination of probiotic properties of isolates in vitro, these major selection criteria were chosen: tolerance to low pH, tolerance against bile salt, antimicrobial activity, antibiotic susceptibility (GUESH *et al.*, 2019).

#### a. Acid resistance

Resistance to pH 3 is often used in vitro assays to determine the resistance to stomach pH. Because the foods are staying during 3 hs, this time limit was taken into account (PRASAD *et al.*, 1998). For this purpose, active cultures (incubated for 16-18 h in M17 broth) were used. Cells were harvested by centrifugation for 10 min at 2500 rpm.

Pellets were washed twice in phosphate-saline buffer (PBS at pH 7.2). Then cell pellets were resuspended in sterile BPS (0.5 mL) which pH was adjusted with 1N HCl to 2, 3, 4 and incubated at 37° C (**HOSSEINI et al., 2009**).

Viable microorganisms were enumerated after exposure to the acidic state for 0, 3 h according to denombrement and incubation at 37° C for 48h (**Annex 04**). This process is repeated three times. LAB counts were expressed in log colony-forming units per milliliter (log CFU / ml) (**BENYOUCEF, 2019**).

### **b. Bile tolerance**

The GI bile concentration is around 0.3% and the staying time of food in small intestine is suggested to be 4 h (**CHARLES and SAMEDY, 2019**). For this purpose, to estimate bile tolerance, bile salt was used to perform the bile salts tests at different percentages.

The cell pellets were harvested by centrifugation, are washed twice and resuspended in the phosphate-saline buffer (PBS at pH 8) supplemented with 0.3%, 0.5% and 1% bile salt and incubated at 37 ° C (**HOSSEINI et al., 2009**). Viable microorganisms were enumerated after incubation at the 0.4 h at 37 ° C for 48h. This process is repeated three times. LAB counts were expressed in (log CFU/ml) (**BENYOUCEF, 2019**).

### **c. Antimicrobial activity**

Antibacterial activity of the LAB strains against selected strains pathogens (**Table 06**) was determined using the agar-disk diffusion method. Volume of mueller-hinton medium agar is poured into sterile petri dishes. After solidification of the medium, the sterile petri dishes are inoculated on the entire surface by the suspension of the strain pathogen using a sterile cotton swab, then the WHATMAN paper discs are filled with 60 to 80 µl of filtered supernatant and neutralized, obtained after centrifugation at 4000 rpm for 15 min of a culture of the lactic strain appropriate (**LABIOUI, 2005**).

After drying, the discs are then deposited on the culture medium; the diffusion of antimicrobial agents in the agar medium is improved by incubating the dishes at 37° C for 24 h. After incubation, the plates were observed for a zone of inhibition (ZOI) around the discs (**ACHEMCHAM and ABRINI, 2005**).

The diameter of the inhibition zone was measured by calipers in millimeters, and a clear zone of 1mm or more was considered positive inhibition. The measure of the inhibition diameter  $Z_i$  is performed according to the following formula (TABAK, 2007).

$$\text{ZOI in (mm)} = \text{diameter of the } Z_i \text{ obtained (mm)} - \text{diameter of disc (6mm)}$$

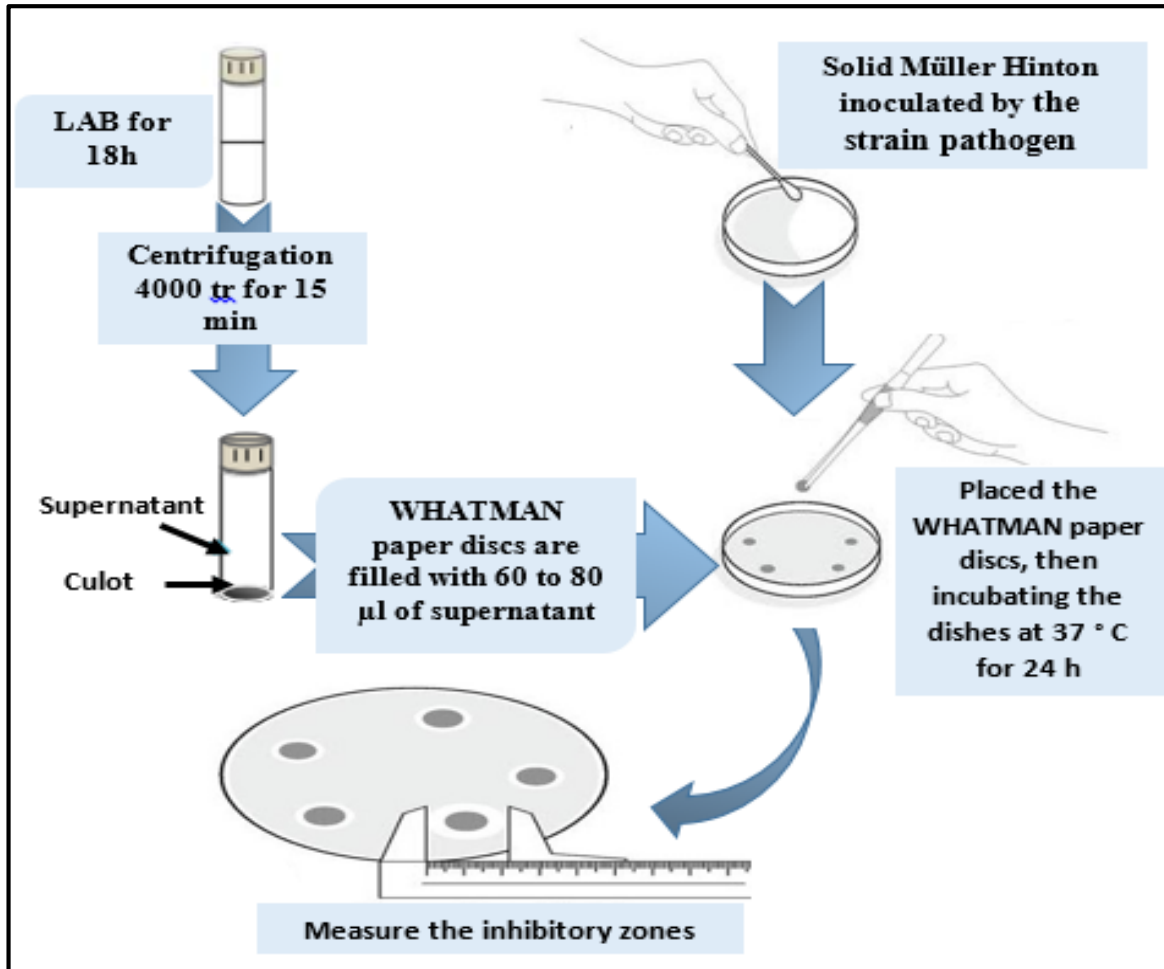


Figure 08: Diagram of the discs-agar diffusion method

#### d. Antibiotic susceptibility tests

Antimicrobial susceptibility of each LAB was determined by using the disc diffusion method as described by (ZHANG *et al.*, 2016) against some antibiotics that included ofloxacin (5µg / ml), gentamicin (10µg / ml), erythromycin (15µg / ml), amoxicillin (25µg / ml), and amoxiclav (30µg / ml).

Thus, a volume of 100 µl of actively growing cultures was swabbed evenly over the surface of nutrient agar plates with a sterile cotton swab. After drying, the antibiotic discs were placed on the solid agar surface, and the plates were left aside for 30min at 4 ° C for the diffusion of antibiotics and incubated at 37 ° C for 24 to 48h. The diameters of

inhibition zones were measured using calipers (VLKOVA *et al.*, 2006); the zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S ( $\geq 21$ mm); intermediate, I (16– 20mm), and resistance, R ( $\leq 15$ mm) (GUESH *et al.*, 2019).

### II.1.2.3. Phenotypic identification of lactic acid strains

The probiotic LAB isolates were identified according to their morphological, physiological, and biochemical characteristics based on Bergey's Manual (NAIR and SURENDRAN, 2005).

#### II.1.2.3.1. Morphological study

This study is based on macroscopic and microscopic observation.

##### a. Macroscopic examination

This test makes it possible to demonstrate the colony morphology obtained on solid media, based on observation with the naked eye to determine the appearance, size, shape and color of the colonies (BENYOUCEF, 2019).

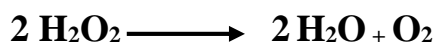
##### b. Microscopic examination

Microscopic examination was carried out after gram staining (Appendix 05), to subsequently determine the shape and arrangement of bacterial cells; as well as their Gram type (Gram + for lactic acid bacteria) (BENYOUCEF, 2019).

#### II.1.2.3.2. Physiological and biochemical tests

##### a. Catalase test

Catalase is an enzyme which has the property of breaking down hydrogen peroxide with the release of oxygen depending on the reaction: (BOURGEOIS *et al.*, 1980).



To demonstrate this enzyme, part of the suspect colony is diluted in a drop of hydrogen peroxide on a sterile slide. The evolution of gas bubbles indicates the presence of catalase (TABAK, 2007).

**b. Growth at different temperatures**

This test is very important because it makes it possible to distinguish mesophilic lactic acid bacteria from thermophilic lactic acid bacteria (BOULLOUF, 2016). After inoculation of the M17 broth by the pure cultures, the tubes are incubated at 10° C for 5 to 7 days for *Streptococcus* and *Lactococcus*, at 40° C and 45° C for 24 to 48 h for *Pediococcus*, *Streptococcus* and *Lactococcus* (BADIS *et al.*, 2005).

**c. Growth in the presence of different concentrations of NaCl**

Growth in the presence of different concentrations of sodium chloride (NaCl) gives valuable information for identification. The cultures to be tested were inoculated on broths hypersaline at 2% and 4% NaCl for *Streptococcus*, *Lactococcus* and 2% 3% 4% and at 6.5% of NaCl for *Pediococcus*. After incubation at 30° C for 24 to 72 hours, the growth of these bacteria is manifested by a cloudiness of the medium (GUIRAUD and GALZY, 1980; BADIS *et al.*, 2005)

**d. Growth in different pH**

The test tubes containing the M17 medium at pH 4.2, 4.5, 4.8, 6.5, 7 and 8 by the addition of the solutions of NaOH and lactic acid (1N) are inoculated using a looped platinum loop by the bacteria to be tested. The media added to pH 4.5, 6.5 for *Streptococcus*, *Lactococcus* and to pH 4.2, 4.8, 7 and 8 for *Pediococcus*. The culture is incubated at 30° C for 48 hours. Growth results in the appearance of a cloudiness in the tube (BEKHOUCHE, 2006; BOURGEOIS *et al.*, 1996; BADIS *et al.*, 2005).

**e. Heat resistant**

Tubes containing 10 ml of liquid M17 are inoculated with the isolated strains, then the tubes are placed in a water bath at 63.5° C for 30 minutes, after sudden cooling under a jet of tap water, they are incubated at 30° C.  $\pm$  1° C for 48 to 72 hours. A positive result results in a cloudiness (RAHLI *et al.*, 2015).

**f. Fermentation type**

Heterofermentation is the ability of bacteria to produce molecules other than lactate such as CO<sub>2</sub>, acetate, ethanol... from sugar. To distinguish homofermentative lactic acid bacteria from heterofermentative lactic acid bacteria, a heterofermentation test is realised (DJADOUNI, 2013). It consists of inoculating the strains in tubes of M17 broth containing

the Durham bells, then incubated at 30° C/24h. The production of gas in the bell indicates the strain is heterofermentative, otherwise it is homofermentative (**BOUMEDIEN, 2013**).

## **II.2. Statistical analyzes**

The results are given in the form of means and standard deviations (SD) of 3 essays. The statistical evaluation is carried out by the student's T test. So, we use MINITAB and EXCEL software which helps us to do the tests.

# ***Chapter II***

## ***Results & discussion***

## I. Physico-chemical analyzes of milk samples

The results of the physico-chemical analyzes of milk are shown in the following table:

**Table 07: physico-chemical analyzes of camel and goat raw milk**

	Camels	Goats
<b>Electrical conductivity (mS/cm)</b>	4.57 ± 1.34	2.63 ± 0.41
<b>pH</b>	6.36 ± 0.18	6.51 ± 0.14
<b>Titrateable acidity °D</b>	18 ± 2.61	16.5 ± 1.02
<b>Density</b>	1.0365 ± 0.01	1.04 ± 0.03
<b>Total dry matter g/l</b>	106.56 ± 7.4	195.85 ± 6.83
<b>Ashes g/l</b>	8.21 ± 0.9	7.85 ± 1.12
<b>Proteins g/l</b>	27.666 ± 5.13	21.666 ± 3.1
<b>Total sugars g / l</b>	43.2 ± 4.3	41.55 ± 3.05

### I.1. Electrical conductivity

**Table 07** shows that the mean values of the electrical conductivity of the camel and goat milk collected is equal to 4.57 mS/cm ±1.34 and 2.63 mS/cm±0.41 respectively. For camel raw milk, the electrical conductivity is within the range of the standard described by (**LEYMARIOS, 2010**) (4-5.5 mS/cm); but for goat's milk is lower.

The value obtained for camel milk is similar to other studies such as (**BOUSBIA *et al.*, 2018**) is 4.49 mS/cm ±0.50.

Studies have shown that the stage of lactation significantly influences the electrical conductivity of milk from healthy quarters, which drops sharply in the first few weeks after calving and then rises more gradually throughout the remainder of lactation (**JACQUINET, 2009**).

**SHELDRAKE *et al.*, (1983)** show that, the conductivity of milk from a healthy quarter drops from 5.3 mS/cm at 35 days postpartum to 5.9 mS/cm at the end of lactation.

## I.2. pH

According to the results presented in **Table 07**, we notice that the average pH values of the camel and goat milk collected is equal to  $6.36 \pm 0.18$  and  $6.51 \pm 0.14$  respectively. It is known that camel milk would be slightly more acidic than human and bovine milk which have respective pH equal to 7.01 and 6.6 (**SIBOUKEUR, 2007**).

These values are close to the pH reported by (**HANOU, 2018**) and (**SIBOUKEUR, 2007**) with a pH at ( $6.35 \pm 0.04$ ,  $6.31 \pm 0.15$  respectively) for camel milk, and to those of milk from other countries such as (**BORNAZ, 2009**) in Tunisia and (**IMRAN, 2008**) in Pakistan with a pH ( $6.51$ ,  $6.59$  respectively) for goat milk.

The richness of milk in certain constituents, more particularly in phosphates, citrates and caseins (**MATHIEU, 1998**), and according to the nature of the fodder and the availability of water (**GORBAN and IZZELDIN, 1997**), the pH of the milk varies from one species to another and depends, for a given species.

According to (**YAGIL, 1985**) and (**SALEY, 1993**) the low pH of camel milk can be attributed to the high concentration of volatile fatty acids and the relatively high content of vitamin C in camel milk.

## I.3. Titratable acidity

The samples of camel and goat raw milk analyzed present a titratable acidity of the order of  $18 \text{ }^\circ\text{D} \pm 2.61$  and  $16.5 \text{ }^\circ\text{D} \pm 1.02$  respectively (**Table 07**). These values comply with the standard (**AFNOR, 1985**), set between 16 and  $18 \text{ }^\circ\text{D}$ .

The results obtained are similar to that reported by other authors in other countries such as (**KHASKHELI et al., 2005**) in India is  $18 \text{ }^\circ\text{D}$ , (**MEILOUD et al., 2011**) in Mauritania is  $16 \text{ }^\circ\text{D}$ , (**KESKIN et al., 2004**) in Turkey, is  $17 \text{ }^\circ\text{D}$ .

The titratable acidity of milk depends on the number of moles of acids present in this product; it is inversely proportional to its pH (**MATHIEU, 1998**).

It is linked to the climate, the stage of lactation, the season and the management of breeding, in particular food and water intake (**AGGAD et al., 2009**). The acidity of the milk can be an indicator of the quality of the milk at the time of delivery because it makes it

possible to assess the quality of acid produced by bacteria or possible fraud (**JOFFIN AND JOFFIN, 1999**).

#### **I.4. Density**

The density content is equal  $1.0365 \pm 0.01$  and  $1.04 \pm 0.03$  in camel and goat raw milk respectively (**Table 07**); the density of camel's milk is close to the norm (**AFNOR, 1985**) (1.028-1.032), while goat's milk has a higher density.

According to these results, the density values of the analyzed samples of raw milk are greater than the results found by (**DAGET and LHOST, 1995**), (**SIBOUKEUR, 2007**), (**ALLOUI-LOMBARKIA et al., 2007**), respectively equal at 1.0260; 1.023; 1.029.

The density depends directly on the dry matter content; poor milk will have a low density (**LUQUET, 1985**). This is clearly visible in the case of goat's milk, which records the highest dry matter rate ( $195.85 \text{ g/l} \pm 6.83$ ) correlated with the highest density ( $1.04 \pm 0.03$ ) (**Table 07**).

#### **I.5. Total dry matter**

The total dry matter content of the samples of camel and goat raw milk respectively is equal to  $106.56 \text{ g/l} \pm 7.4$ ;  $195.85 \text{ g/l} \pm 6.83$  (**Table 07**). For camel milk, it seems lower compared to that of bovine (128 g/l according to **ALAIS, 1984**) and human (129 g/l according to **SIBOUKEUR, 2007**) milk; but the result of goat raw milk is higher.

The results obtained from camel raw milk are within the range of the work of (**RAHLI, 2015**) in the Ouled sidi cheikh population, with values between 98.4 and 119.05 g/l and close to (**SIBOUKEUR, 2007**) with  $113.11 \text{ g/l} \pm 10.58$  and differs from that reported by (**HADDADIN et al., 2008**) in Jordan with 123 g/l.

Several authors have shown that the variation in the total solids content was due to various factors such as the quality of the water and its availability for the animals (**KHASKHELI et al., 2005**). In summer, the water content of milk increases and therefore its dry matter decreases further under the effect of water stress. For this (**HADDADIN et al., 2008**) found that the total dry matter rate reached its maximum in mid-winter and its minimum in summer. This content also varies depending on the stage of lactation (**BENGOUMI et al., 1994**).

## I.6. Ashes

Table 07 shows that the ash contents of the analyzed samples of camel and goat raw milk are equal to 8.21 g/l  $\pm$ 0.9, 7.85 g/l  $\pm$ 1.12 respectively. It therefore seems lower than that of bovine milk (9 g/l) and higher than that of human milk (2.02 g/l) (SIBOUKEUR, 2007).

The ash values obtained in our study are comparable to the results obtained by other authors as (HADDADIN *et al.*, 2008) in Jordan with 8.2 g/l. They are therefore higher than those reported by (LARSSON-RAZNIKIEWWICZ and MOHAMED, 1994) with a value of 6 g/l and (SBOUI *et al.*, 2009) in Tunisia with a value of 7.5 g/l, but they are lower than those obtained by (BOUDJENAH *et al.*, 2012) as 9.39g/l.

The ash (mineral) content of camel milk decreases in case of water deprivation. It also varies depending on the stage of lactation (SIBOUKEUR, 2007).

## I.7. Total protein content

The average total protein content of the samples analyzed of raw camel (27.666 g/l  $\pm$ 5.13) and goat milk (21.666 g/l  $\pm$ 3.1) shown in (Table 07), this is close to those of bovine milk (32 g/l) and is higher compared to that of human milk (12 g/l) (SIBOUKEUR, 2007).

The results obtained are within the range of the results obtained by (MOHAMED *et al.*, 1989) and (GNAN *et al.*, 1994) namely; 46 g/l and 21.5 g/l respectively, and they are lower than that obtained by (KAMOUN, 1994) 34.3 g/l  $\pm$ 4.4; (SBOUI *et al.*, 2009) 34.15 g/l and (SHAMSIA, 2009) 34.6 g/l.

The protein content varies according to the stages of lactation. According to (KAMOUN, 1994), the first two months of lactation are characterized by a decrease in the protein and fat content of camel milk.

The latter reach a minimum value coinciding with the peak of lactation, then find, at the end of lactation, a level comparable to that at the start (DELABY and PEYRAUD, 1994), show that a wheat-based diet induced a moderate increase the protein level of cow's milk compared to a diet based on preserved or grazed grass.

## I.8. Total sugar content

According to the results compiled in (**Table 07**), the average total sugar contents of camel and goat raw milk are equal to 43.2 g/l  $\pm$ 4.3 and 41.55 g/l  $\pm$ 3.05 respectively. This content appears to be comparable to that of bovine milk (44.13 g/l), but it is lower than that of human milk (70 g/l) (**SIBOUKEUR, 2007**).

These results are within the range of works reported by many authors, namely (**MEHAIA *et al.*, 1995**) (44 g/l, 44.3 g/l and 44.4 g/l respectively) and (**KARUE, 1994**) 36.5 g/l. They are similar to those reported by (**KIHAL *et al.*, 1999**) (45.1 g/l  $\pm$ 3) and greater than (**GORBAN and IZZELDIN, 1997**) 25.6 g/l  $\pm$  1.0.

The lactose content of camel milk seems to depend not only on the breed but also on the stage of lactation and the state of hydration. It is weak during the first hours after calving and undergoes a 36% increase in initial content 24 hours later. A reduction of 37% of the initial content was observed in the event of dehydration of the camels. These changes in the lactose content are at the origin of the variations in the flavor of camel milk (**YAGIL and ETZION, 1980**).

## II. Microbiological analyzes

### II.1. Isolation of lactic acid bacteria

From 06 raw milk samples (03 camels and 03 goats), we isolated 20 strains of which 11 stains of them were found to be Gram positive and catalase negative so were kept for the rest of the study. On M17 medium, 08 strains (72.73%) at 30° C are obtained (06 strains from goat milk noted S1, S2, S3, S4, S5, S6 and 02 strains from camel milk S7, S8), and 03 strains (27.27%) at 42° C (S9, S10 from camel's milk and S11 from goat's milk).

### II.2. Probiotic properties of isolates

#### a. Resistance to low pH

The study of the prolonged exposure of our strains to acid conditions similar to those of the stomach was carried out by exposing to different pH (2, 3 and 4) for 3 hours. The results obtained are illustrated in **Table 08**.

These results indicate that, among the 11 isolates, 7 isolates (S1, S2, S4, S5, S7 S9, S10) (63.63%) grow after exposure to pH 2.3 and 4 for 3 h (there is no significant difference between 0 and 3h ( $p > 0.05$ )), 3 isolates (S3, S8, S11) (27.27%) show a highly significant ( $p < 0.01$ ) and very highly significant decrease ( $p < 0.001$ ) between 0.3 h in the number of viable cells after exposure to pH 3, 2 respectively, but grow to pH 4 after 3 h of exposure, and 1 isolate (S6) (9.09%) does not resist all pH after 3 h of exposure.

**Table 08: Effect of acidic pH on strain viability in (log CFU/ml)**

	pH 2		pH 3		pH 4	
	0h	3h	0h	3h	0h	3h
<b>S1</b>	8.73±0.3	8.2±0.2	8.24±0.03	8.1±0.015	8.27±0.1	7.88±0.002
<b>S2</b>	7.26±0.01	6.94±0.11	7.66±0.15	7.23±0.22	9.02±0.3	8.93±0.05
<b>S3</b>	6.14±0.2	1.18±0.14 <sup>b</sup>	7.21±0.04	5.4±0.13 <sup>a</sup>	6.95±0.2	6.32±0.01
<b>S4</b>	8.37±0.001	8±0.2	8.15±0.01	7.98±0.01	9.34±0.002	9.01±0.02
<b>S5</b>	9.45±0.2	9.03 0.3	9.21±0.002	9±0.2	8.77±0.1	8.43±0.03
<b>S6</b>	8.47±0.13	0	8.55±0.2	0	8.85±0.04	0
<b>S7</b>	7.64±0.03	7.22±0.04	7.8±0.03	7.53±0.01	7.94±0.003	7.35±0.01
<b>S8</b>	7.38±0.11	1.42±0.04 <sup>b</sup>	7.55±0.3	5.39±0.2 <sup>a</sup>	8.75±0.002	8.22±0.21
<b>S9</b>	9.20±0.002	8.87±0.04	9.02±0.04	8.7±0.003	7.82±0.14	7.23±0.06
<b>S10</b>	9.28±1.04	8.78±0.72	8.58±0.63	9.01±1.2	9.23±1.03	9.1±0.42
<b>S11</b>	9.92±0.03	3.14±0.02 <sup>b</sup>	9.32±0.12	6.27±0.1 <sup>a</sup>	8.14±0.03	7.84±0.15

Mean values (n = 3) ± Standard Deviation (SD). a :highly significant difference ( $p < 0.01$ ). b: very highly significant difference ( $p < 0.001$ )

### b. Bile salt tolerance

Resistance to bile salts is one of the important criteria for the selection of probiotic strains, since the small intestine and the colon are the first niches of colonization of the host organism by the probiotic strains (BENYOUCEF, 2019).

The results obtained are demonstrated in **Table 09** show that, all the strains are resistant to different concentrations of bile salts with a variable proportion (there is no significant difference ( $p > 0.05$ )) except that the strains S3, S6, S8 are dead and the S4, S11 strains recorded a very highly significant decrease ( $p < 0.001$ ) in the 1% concentration.

**Table 09: Effects of different concentrations of bile salts on strains (log CFU/ml)**

	0.3%		0.5%		1%	
	0h	4h	0h	4h	0h	4h
<b>S1</b>	8.32±0.2	8±0	8.11±0.01	7.8±0.22	8.20±0.13	7.53±0.3
<b>S2</b>	7.45±0.01	7.11±0.3	7.67±0.12	7.31±0.03	7.61±0.2	6.98±0.01
<b>S3</b>	8.26±0.3	7.94±0.92	8.22±0.2	7.91±0.1	7.98±0.3	0
<b>S4</b>	9.16±0.05	8.89±1.02	8.74±0.03	8.53±0.003	8.12±0.02	2.31 <sup>b</sup> ±0.05
<b>S5</b>	7.61±0.04	7.72±0.6	7.62±0.04	7.23±0.13	7.22±0.04	6.51±0.04
<b>S6</b>	8.29±0.2	8.1±1.01	7.75±0.001	7.82±0.04	7.48±0.01	0
<b>S7</b>	6.54±0.01	6.2±0.7	6.85±0.2	6.19±0.12	6.35±0.4	5±0.03
<b>S8</b>	8.71±0.1	8.12±0.03	8.60 ± 0.01	8.22 ± 0.3	8.32±0.11	0
<b>S9</b>	9.23±0.05	8.83±0.8	8.76±0.002	8±0.02	8.86±0.2	7.86±0.15
<b>S10</b>	6.88±0.03	6.42±0.45	7.01±0.15	6.57±0.15	6.92±0.01	5.20±0.04
<b>S11</b>	7.2±0.002	6.87±0.87	7.47±0.2	7.2±0.3	7.41±0.03	2.1 <sup>b</sup> ±0.13

Mean values (n = 3) ± Standard Deviation (SD). **b**: very highly significant difference (p <0.001)

### c. Antimicrobial activity

The inhibitory capacity in vitro of LAB vis-à-vis pathogenic germs demonstrates a good probiotic property, as when it can play a role in the preservation of the hygienic quality of foodstuffs (AMMOR *et al.*, 2006). The measurement of the zones of inhibitions by our strains against the indicator bacteria by the agar-disc diffusion method has been demonstrated in **Table 10**.

**Table 10: Spectrum of antimicrobial activity of lactic acid strains (mm)**

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
<b>EC</b>	4	9	7	5	5	0	8	3	4	5	0
<b>St</b>	6	16	4	3	5	6	15	5	9	8	6
<b>Her</b>	5	3	0	4	4	0	7	0	9	3	5
<b>Bs</b>	8	18	4	9	6	3	7	6	14	11	5
<b>Pa</b>	4	8	0	5	5	0	3	0	5	9	0

**EC**: Escherichia coli; **St**: Staphylococcus aureus; **Her**: Salmonella enterica serovar Typhi;

**Bs**: Bacillus subtilis; **Pa**: Pseudomonas aeruginosa

The results presented in (**Table 10**) show that most isolates possess antibacterial activity against indicator bacteria, but strains S2, S7, S9, S10 show the highest inhibition for all indicator strains while strains S3, S6, S8, S11 showed the lowest inhibition.

The presence of zones of inhibition when using a whole lactic acid culture may be due to the metabolism of lactose to lactic acid which lowers the pH and creates an unfavorable environment for the development of pathogenic bacteria (**FLEMING *et al.*, 1975; BAREFOOT and KLAENHAMMER, 1983; TABAK *et al.*, 2007**), with hydrogen peroxide (**KLAENHAMMER, 1984**), or bacteriocins (**KLAENHAMMER, 1993; VINOD KUMAR *et al.*, 2006**).

#### d. Susceptibility to antibiotics

Antibiotic resistance is considered a prerequisite for the selection of a probiotic strain. The susceptibility and resistance of LAB to various antibiotics vary among species (**MATHUR and SINGH, 2005; DANIELSEN and WIND, 2003**). The results of resistance and susceptibility to different antibiotics used in this study are presented in **Table 11**.

**Table 11** shows that some strains have partial resistance (intermediate susceptibility) to some antibiotics as Gentamicin; while, most LAB are susceptible to most antibiotics with the exception of the antibiotic Amoxilav which resisted by all LAB.

**Table 11: Antibiotic susceptibility profiles from LAB to selected antibiotics (mm)**

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
<b>Erythromycin</b>	S	R	S	S	S	I	S	S	S	R	S
<b>Gentamicin</b>	S	S	I	S	R	S	I	S	I	I	S
<b>Amoxilav</b>	R	R	R	R	R	R	R	R	R	R	R
<b>Amoxycillin</b>	R	S	S	S	I	S	S	I	S	S	I
<b>Ofloxacin</b>	S	I	S	R	S	S	I	I	R	S	S

**S:** susceptible, **R:** resistance, **I:** intermediate susceptibility

Based on the results presented in the previous tables, we can classify the strain, retained through our study, according to their probiotic performance in decreasing order: (2, 1, 9, 4, 5, 10 and 7).

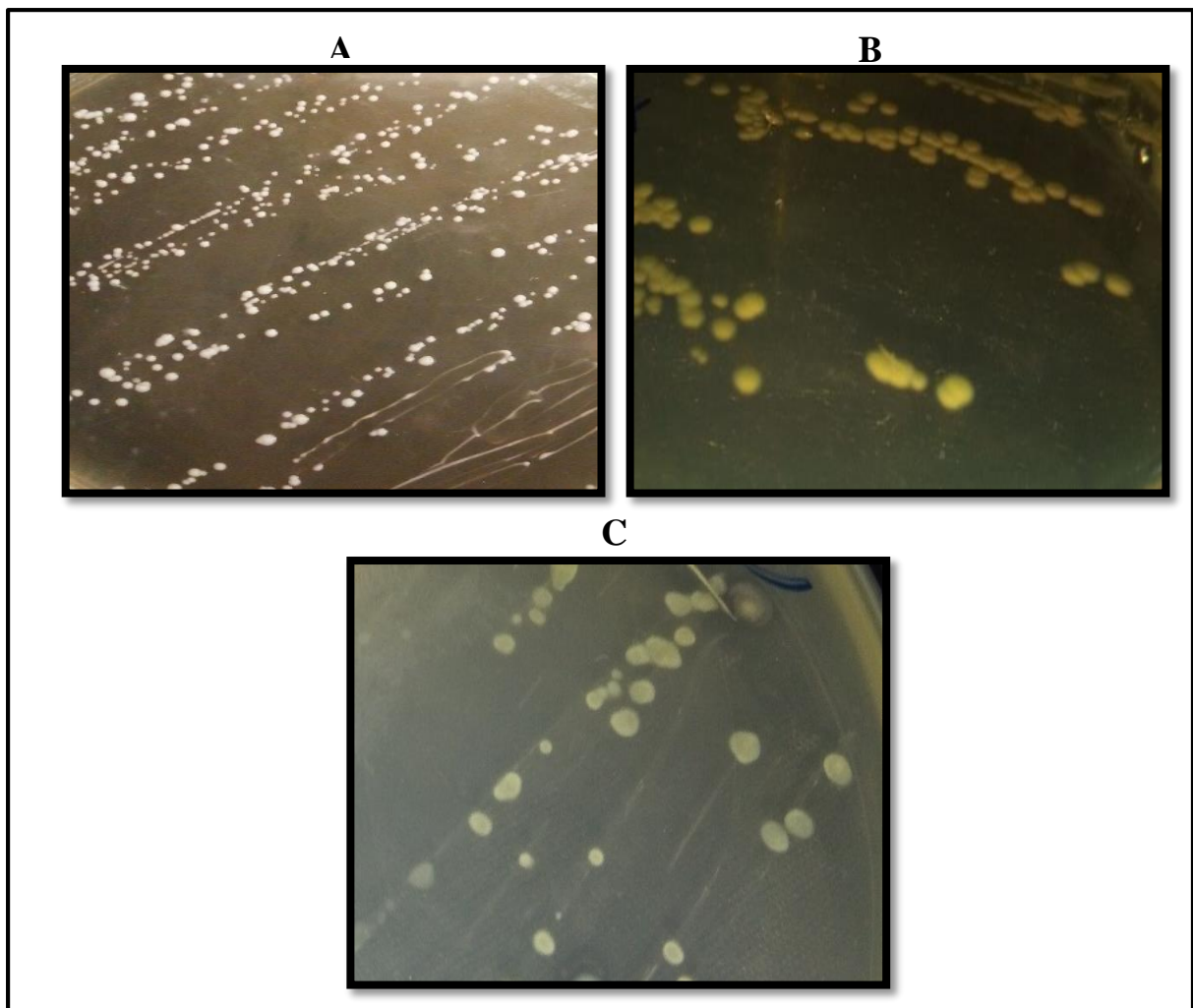
### II.3. Phenotypic identification of lactic acid strains

The 07 strains considered as probiotics (S1, S2, S4, S5, S7, S9, S10) were identified according to morphological criteria, physiological and biochemical.

#### II.3.1. Morphological criteria

##### a. Macroscopic aspects

Macroscopic characterization makes it possible to describe the appearance of the colonies obtained on solid M17 medium at pH 7.2 after 2-3 days of incubation at 30° C/45° C and to determine the criteria relating to these lactic colonies (size, pigmentation, contour, appearance, viscosity). For the isolates tested, we observed small colonies of about 1 to 2 mm in diameter, circular or lenticular and grayish whitish or milky color.



**Figure 09: Macroscopic appearance of lactic acid strains (A: culture of streptococci, B: cultures of lactococci, C: culture of pediococci)**

### b. Microscopic aspects

Microscopic observation after Gram stain made it possible to select bacteria, which represent a bacillary or spherical shape (HASSAINE, 2013). Our isolates tested are Gram positive and are presented in cocci form deposited in pair or in short chain.

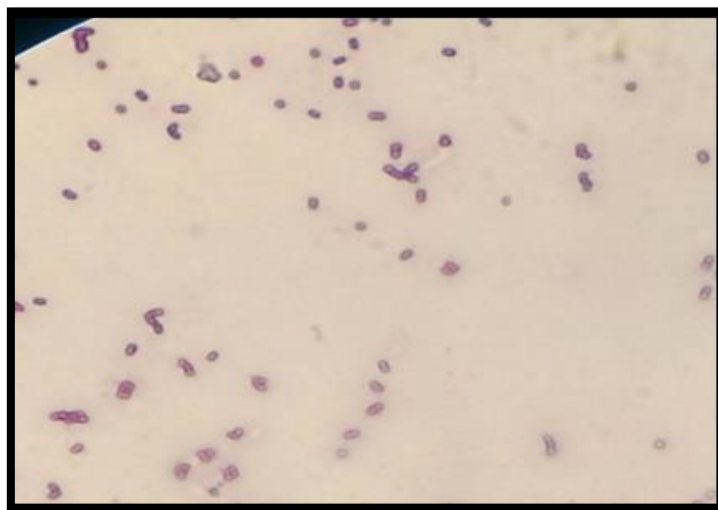


Figure 10: Microscopic appearance of Cocci colonies (Gx100).

### II.3.2. Physiological and biochemical criteria

Table 12: Physiological and biochemical criteria of lactic acid bacteria isolated from raw milk

The strains isolated		S1	S2	S4	S5	S7	S9	S10
T °	10 ° C	+	/	+	+	/	-	-
	40 ° C	-	+	-	-	-	+	+
	45 ° C	-	+	-	-	-	+	+
pH	4.2	/	+	/	/	+	/	/
	4.5	+	/	+	+	/	+	+
	4.8	/	+	/	/	+	/	/
	6.5	+	/	+	-	/	-	+
	7	/	+	/	/	+	/	/
	8	/	-	/	/	+	/	/
NaCl	2%	+	+	+	+	+	-	+
	3%	/	+	/	/	+	/	/
	4%	-	-	+	-	+	+	-
	6.5%	/	-	/	/	+	/	/
RES		-	+	-	-	+	-	+
GAS		-	-	-	-	-	-	-

RES: heat-resistant to 63.5° C for 30 min. GAS: gas production from glucose

### a. Growth at different temperatures

The results obtained in **Table 12** showed that, only the strains S1, S4, S5 grow at 10° C but strains S2, S9, S10 grow at 40° C, 45° C.

### b. Growth in the presence of different concentrations of NaCl

The results obtained showed that all the isolated strains grew at 2% NaCl with the exception of the S9 strain. However, 28.57% of the strains (S2, S7) grow at 3% and 42.86% (S4, S7, S9) at 4% NaCl, and only the strain S7 (14.26%) grows at 6.5% NaCl which has a capacity growth in wide range of NaCl.

### c. Growth in different pH

**Table 12** shows that 71.43% of the strains (S1, S4, S5, S9, S10) grow in pH 4.5, (42.85%) of the strains (S1, S4, S10) grow at pH 6.5. However, (28.57%) of the isolated strains (S2, S7) grow at pH 4.2, pH 4.8 and pH 7, and only strain S7 (14.26%) grows at pH 8 which has a capacity growth in wide range of pH.

### d. Heat resistant

After exposure to a temperature of 63.5° C for 30 min followed by 48h incubation at 30° C  $\pm$  1° C, only 3 strains (S2, S7, and S10) revealed a positive result (**Table 12**). The results show that these 3 strains are heat-resistant strains, unlike the rest of the strains which do not have this character.

### e. Fermentation type

**Table 12** shows that, all the isolated bacteria do not produce gas during the fermentation of glucose, that is to say they are homofermentative. Our identification results of genus of bacteria strains are consistent with (**BADIS *et al.*, 2005**).

**Table 13: Morphological and physiological characters of the putative genera of LAB**

Medium agar	T °	Macro morphology	Micro morphology	Fermentation type	Genus of bacteria strains
M17	30° C	White, round or lenticular colonies	Cocci, diplococci and chain	Homo	<i>Lactococcus</i> (S1, S4, S5)
M17	30° C	Smooth rounded, greyish or whitish colonies	Cocci in tetrad	Homo	<i>Pediococci</i> (S2, S7)
M17	42° C	White, round or lenticular colonies	Cocci, diplococci and chain	Homo	<i>Streptococci</i> (S9, S10)

# ***Conclusion***

## Conclusion

Milk is considered one of the most important food sources for human nutrition, as it represents a medium rich in complex chemical and physical components.

This source is also considered to be a medium rich in beneficial LAB, so bacteria in general are not always harmful, despite their association with disease, but many types are in particular, those in the human body are beneficial bacteria that protect us from many diseases, and some of them have been given as food supplements in capsule form to help improve and regulate the digestive process. Through this study, the first context was a physicochemical study and the second context was a microbiological study of different milk samples.

The results of the physicochemical analysis showed that for all the milk samples analyzed, the majority of the results comply with company standards, with the exception of the electrical conductivity, acidity and dry matter of goat milk. Which presents a simple difference to the norm, and the concentration of its compositions vary according to the diet, the stage of lactation as well as the environmental conditions.

For microbiological analysis, the results show a very varied distribution of species in the six milk samples belonging to the three goats and three camels, 11 strains were isolated were the majority of it strains are present in the raw milk of goat (63.64%). Among the 11 isolates, 7 isolates are considered as probiotic strains belonging to three genera: *Streptococci*, *Lactococci*, *Pediococci* and the most dominant bacterial genus is *Lactococcus* (42.86% of species), followed by *Pediococcus* and *Streptococcus* (28.57%).

The probiotic profile study suggests that LAB strains could be exploited as a probiotic due to its antimicrobial properties and resistance to various antibiotics and tolerance to various biological barriers such as ph acids (2, 3 and 4); bile salts (0.3%, 0.5% and 1%) confirmed its ability to survive in extreme conditions of the digestive tract.

In this study to conclude that, both types of milk are rich in probiotics and we notice the majority of probiotics are present in raw goat milk (57.14%). The identification of the strains isolated in this study required the use of several phenotypic and biochemical techniques, these do not give reliable and secure identification, and molecular identification is still needed.

In perspective, in-depth studies should complement this work such as:

- ✓ A biochemical and genetic identification to complete the morphological and physiological identification
- ✓ In vivo evaluation of the probiotic profile
- ✓ Evaluation in vivo and vitro of antioxidant effect

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# ***Annexes***

## Annex 01: Technical material

Table 14: Lists of culture media, reagents and chemicals, laboratory equipment and small materials used

<b>Culture media</b>	<b>Nutrient agar, M17 broth, M17 agar, Mueller-Hinton agar</b>
<b>Reagents and chemicals</b>	<b>Solvents:</b> Concentrated sulfuric acid, 95 ° alcohol, distilled water, ethanol
	<b>Salts and buffers:</b> Anhydrous sodium carbonate, Hcl, NaCl, NaOH, phosphate buffer saline, potassium phosphate dibasic (K <sub>2</sub> HPO <sub>4</sub> ), potassium phosphate monobasic (KH <sub>2</sub> PO <sub>4</sub> ), copper sulfate, sodium and potassium tartrate
	<b>Specific dyes and reagents:</b> Bromocresol purple, folin-ciocalteu, fuschine, lugol, phenol, phenol phthaline, gentian violet
	<b>Biological products:</b> Glucose, standard proteins (BSA)
<b>Laboratory apparatus and small material</b>	<b>Apparatus:</b> Autoclave, water bath, analytical balance, heating blanket, centrifuge, conductivity meter, freezer, muffle furnace, incubator, lactodensimeter, the top, optical microscope, pH meter, refrigerator, spectrophotometer, glassware (test tube-bicher-sight tube ...), vortex
	<b>Small material:</b> Platinum loop, petri dishes, antibiotics disc, WATMAN paper disc, swabs, micropipette

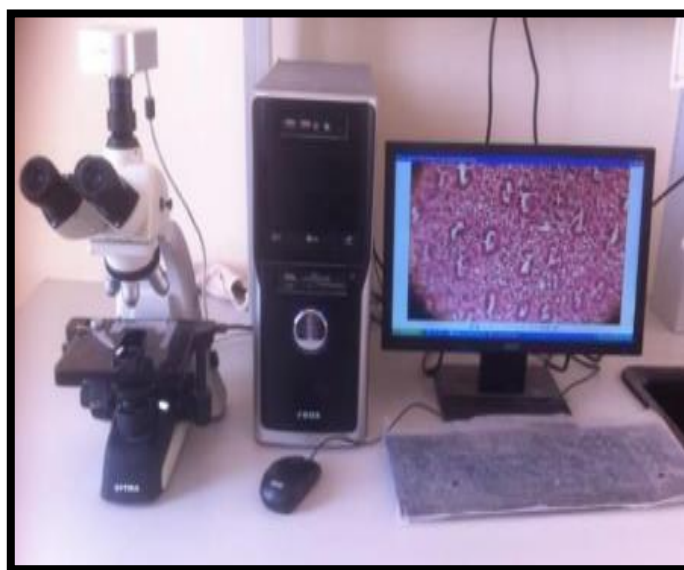


Figure 11: Optical microscope linked to a camera



**Figure 12: Type LIB-060 M bacteriological incubator**



**Figure 13: Centrifuge (SIGMA, Made in Germany)**



**Figure 14 : Analytical balance (Shanghai Sunrise Instrument precision 0.01g)**



**Figure 15: Pinternational type autoclave**

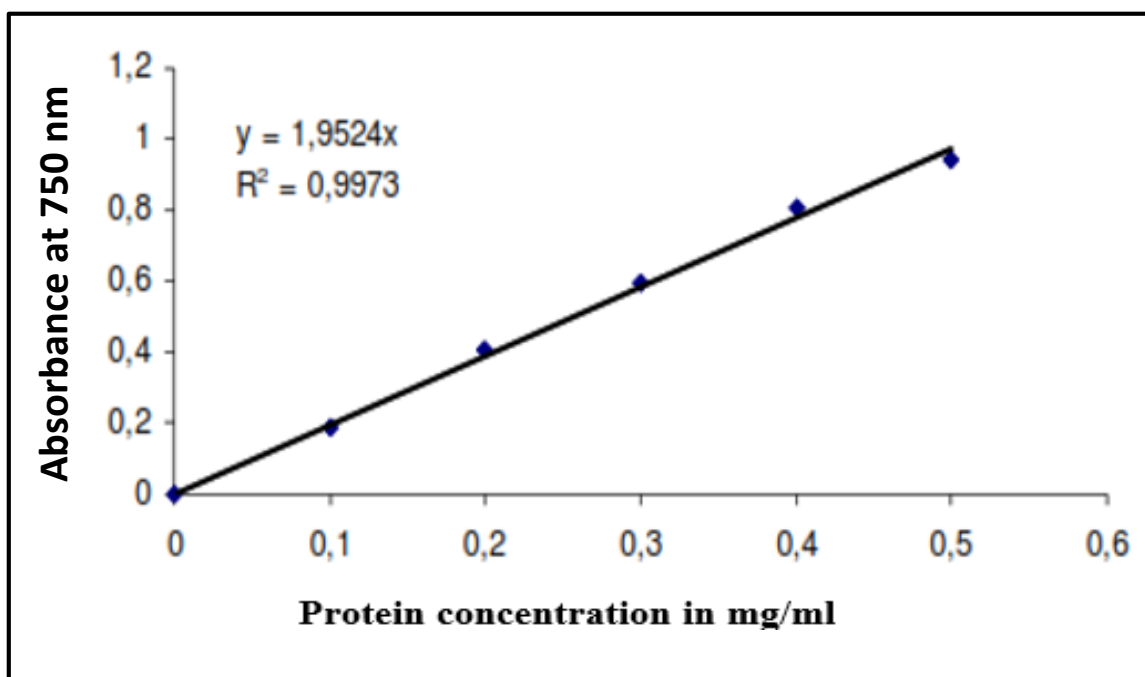
**Annex 02: Determination of Protein by the method of LOWRY 1951****Reagents:**

Alkaline solution (A)	Copper solution (B)	Solution (C)
500 ml of 0.1 N soda (02 g / 500 ml) + 10 g anhydrous sodium carbonate	02 ml of copper sulphate (0.32 g / 100ml) + 02 ml of sodium and potassium tartrate (01 g / 100ml)	50 ml of solution (A) + 01 ml of solution (B)

**Protocol:**

- ✓ Take 01 ml of sample
- ✓ Add 05 ml of solution (C)
- ✓ Leave for 10 min at room temperature
- ✓ Add 0.5 ml of folin-ciocalteu reagent
- ✓ Leave for 30 min in the darkness
- ✓ Read the Do at 750 nm using a UV visible spectrophotometer

Bovine serum albumin is used to plot the calibration curve  $D_o = F(C)$



**Figure 16: Protein calibration curve**

**Annex 03: Determination of total sugars by method of Dubois *et al* 1956.****Reagents:**

- ✓ Phenol 5% in distilled water.
- ✓ Concentrated sulfuric acid at 95% purity and density  $d = 1.84$

**Protocol:**

- ✓ Add to 1 ml of sample, 1 ml of phenol and 5 ml of concentrated sulfuric acid.
- ✓ Shake and let stand for 10 min at room temperature.
- ✓ Incubate in a water bath at 30 ° C for 20 min.
- ✓ Measure the yellow-orange color at 488 nm.

The values obtained are translated into glucose concentrations by reference to a previously established calibration curve.

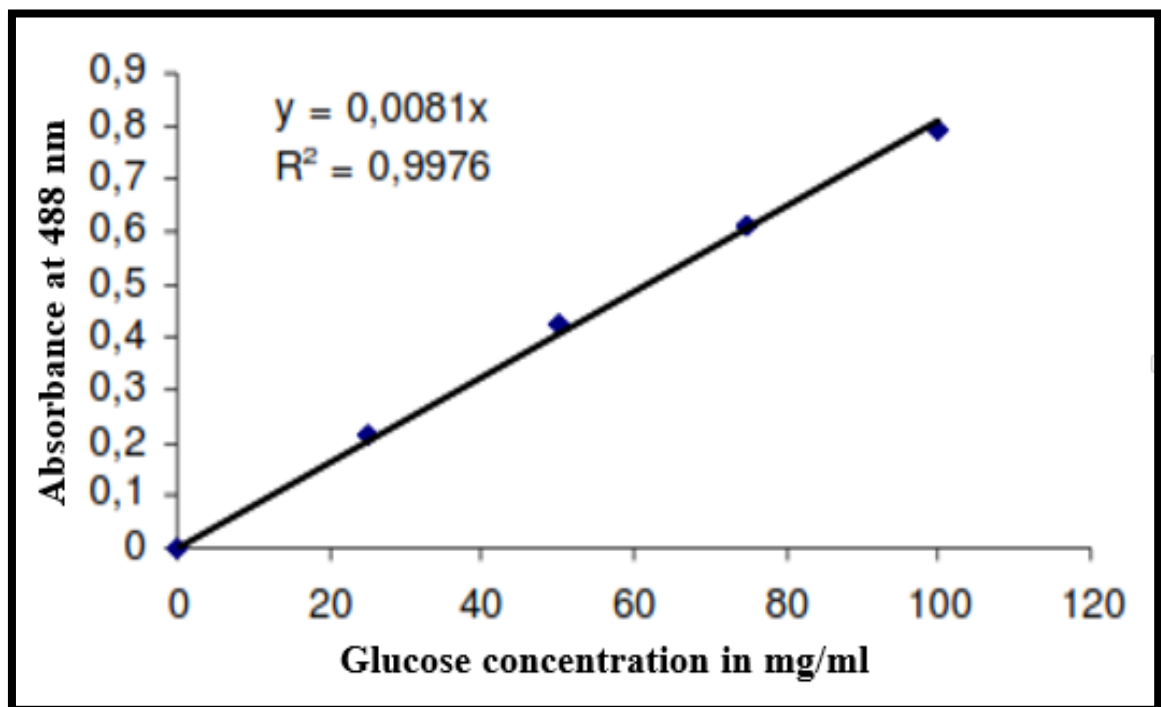


Figure 17: Glucose calibration curve

**Annex 04: Enumeration of bacteria**

A decimal dilution series used ( $10^{-1}$ ;  $10^{-2}$  and  $10^{-3}$ ), 0.1 ml of each dilution was plated on M17 media. After incubation, only the petri dishes containing between 30 and 300 colonies are counted to determine the number of CFU / ml using the following formula (MARTINS, 2012; OUADGHIRI, 2009; ZADI-KARAM *et al.*, 2006).

$$\text{UFC /ml} = \text{number of colonies. (1/V). (1/D)}$$

**Where:**

**V:** volume transferred

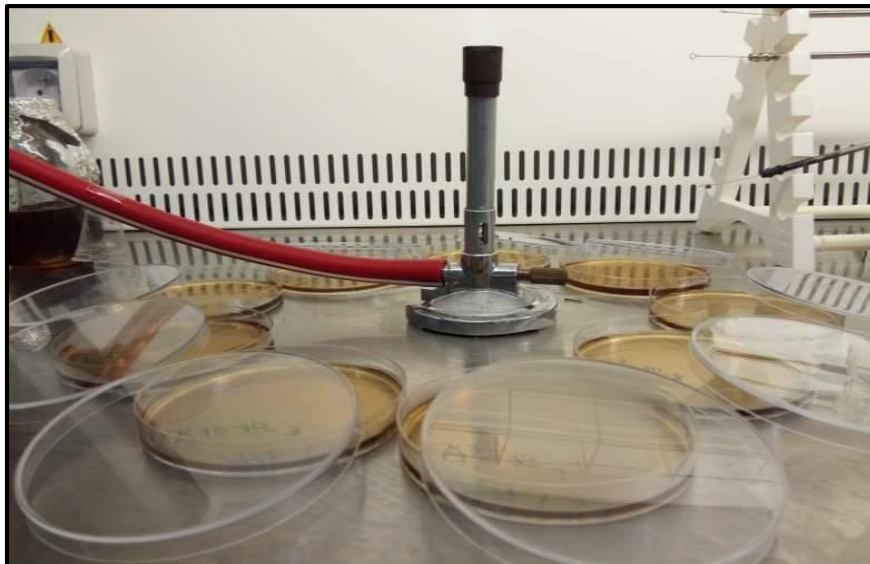
**D:** the dilution

#### **Annex 05: Gram stain**

Gram stain was performed according to the following technique:

- ✓ On a slide, hot fix the bacterial culture;
- ✓ Cover the slide with the gentian violet solution for one minute;
- ✓ Add lugol for 30 seconds;
- ✓ Decolorize with 95 ° alcohol, then rinse with water;
- ✓ Counter-stain using fuschine and leave to act for 20 to 30 seconds;
- ✓ Wash with water;
- ✓ After drying, submit the slide to an immersion microscopic observation (x100). Gram-positive bacteria appear in purple and Gram-negative bacteria in pink.

#### **Annex 06: pictures of some results**



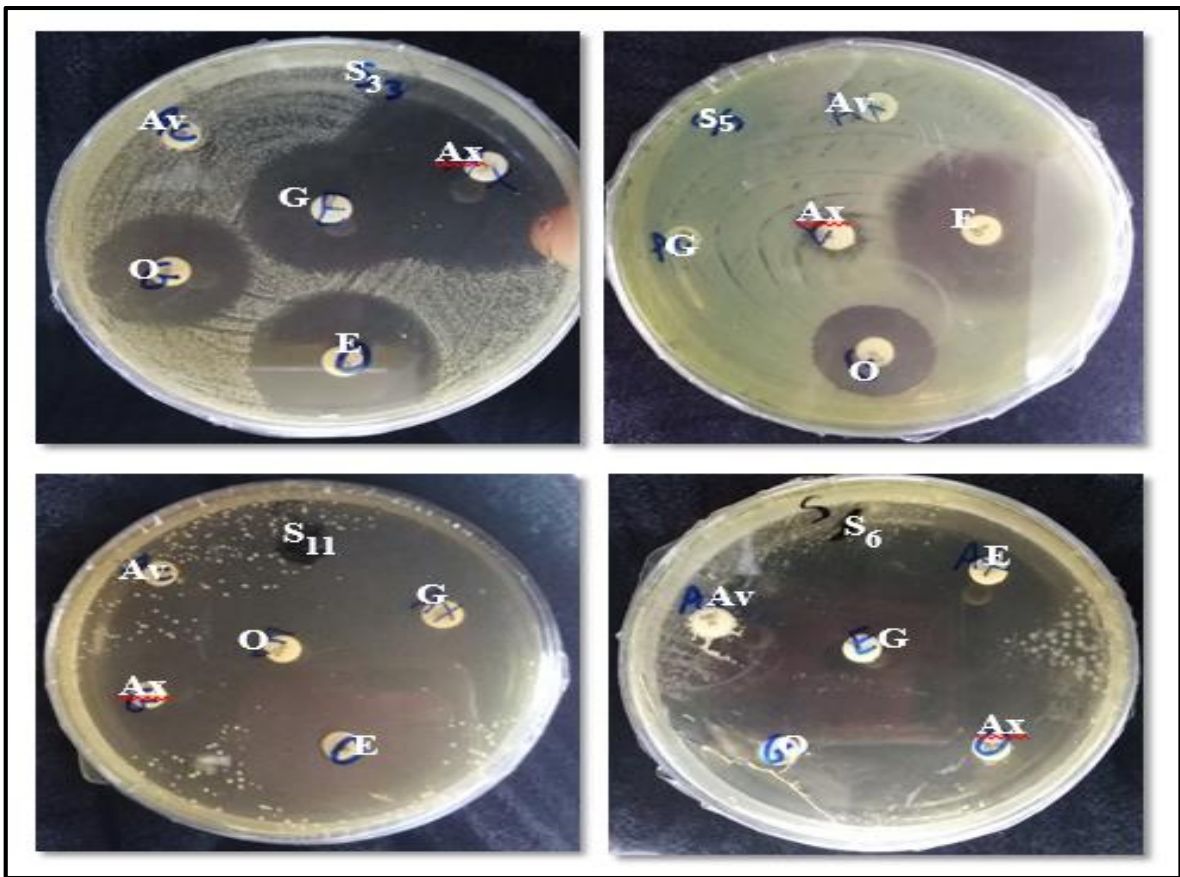


Figure 18: Antibiotic susceptibility profiles from certain LAB to selected antibiotics (Av: Amoxilav; Ax: Amoxycillin; G: Gentamicin; E: Erythromycin; O: Ofloxacin)

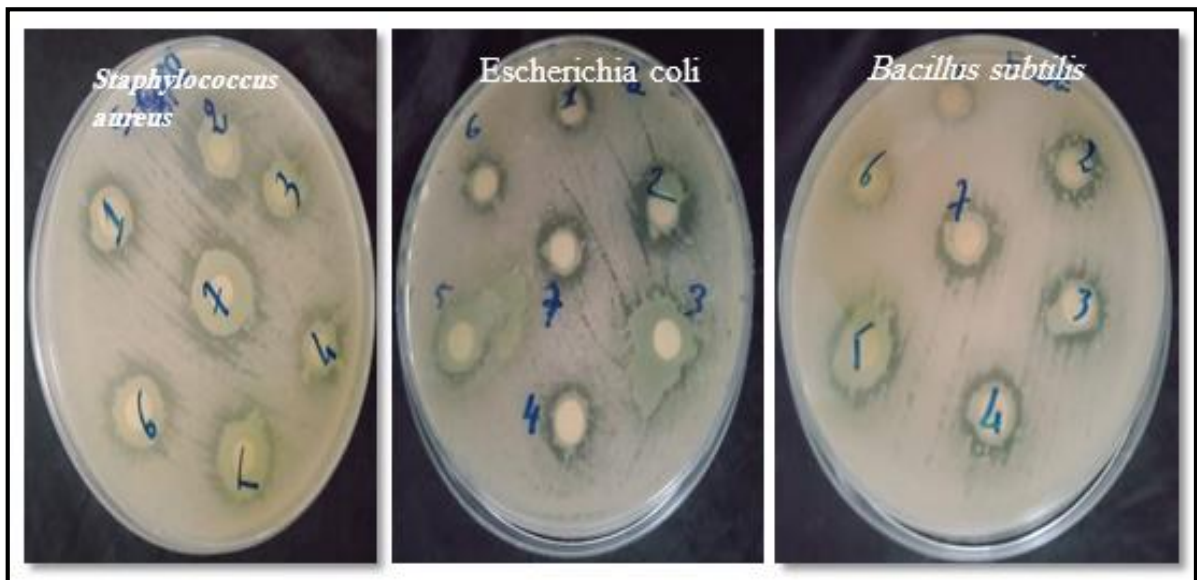


Figure 19: Spectrum of antimicrobial activity of certain LAB against certain pathogen bacteria