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Topic

Emerging probiotics, parabiotics and postbiotics derived from lactic acid bacteria based on their resilience on stressful conditions and ability to form biofilm

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"الحبيب المصطفى صلوات الله عليه"

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الى الايادي الطاهرة التى ازالت من طريقي اشواك الفشل "عائلتي"

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الى زميلات الدفعة اللاتي قاسمنني الذكريات"عمارية وفاء شريفة جهان فريال ازدهار اسماء سارة حياة نور اكرام حكيمة اناس خولة سماح"

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فدوى

اهداء

(وَآخِرُ دَعْوَاهُمْ أَنِ الْحَمْدُ لِلَّهِ رَبِّ الْعَالَمِين)

الحمدلله الذي بنعمته تتم الصالحات في طياتها من الصعوبات والمشقة والتعب اليوم نقطف ثمرها، الحمدلله الذي هدانا لهذا وما كنا لنهتدي لولا أن هدانا الله

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LIST OF ABBREVIATIONS

A

ATCC: American Type Culture Collection

C

CFU: colony forming units

E

E. coli: *Escherichia coli*

F

FRAP: Ferric Reducing Antioxidant Power

G

GI: gastrointestinal
GIT: gastrointestinal tract

H

HSP: heat shock proteins

L

L. plantarum: Lactobacillus plantarum

M

MRS: Man, Rogosa, Sharpe
MH: Muller Hinton

O

ODs: optical densities

P

PBS: Phosphate Buffered Saline

R

RPM: rotations per minute

S

S. aureus: Staphylococcus aureus

S. thermophilus: Streptococcus thermophilus

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

For more than thirty years, it has been understood that the human body harbors a vast number of microbial cells, outnumbering human cells by tenfold. These microorganisms inhabit various regions of the body exposed to the external environment, such as the skin, oral cavity, respiratory system, urogenital tract, and gastrointestinal system **(Gerritsen et *al.*, 2011).** The predominant manifestation of this microbial community is notably observed within the gut, referred to as the intestinal microbiota **(Iacob et *al.*, 2019).** In this context, the gut microbiota, a diverse and dynamic ecosystem consisting of over 1000 species, each specific to different segments of the gastrointestinal (GI) tract, is widely recognized as crucial for maintaining the body's physiological balance and promoting human health **(Piqué et *al., 2019)*.**

In this regard, probiotics are employed as adjuncts to the indigenous microbiota, conferring protection against diverse enteric pathogens. They exhibit promising attributes such as bolstering gut barrier integrity, attributed to their ability to competitively adhere to the intestinal epithelium, thereby enhancing colonization while impeding the proliferation of pathogenic counterparts **(George Kerry et *al.*, 2018).** "*These are living microorganisms, usually consisting of various types of lactic acid bacteria **(LAB),** which when consumed in adequate amounts, confer health benefits to the host such as improving digestive health"* **(Paul et *al.*, 2023).**

Typically, probiotics, predominantly sourced from the gut microbiota of healthy individuals or dairy items, are primarily represented by strains of *Bifidobacterium* or *Lactobacillus* species. Additionally, they encompass organisms from genera such as *Streptococcus*, *Bacillus*, and *Enterococcus*, along with the yeast Saccharomyces, which has been utilized as a probiotic for an extensive period **(Piqué et *al.*, 2019).**

Despite their technological potential, *Enterococcus*, as prominent genera within the LAB group, may encompass strains recognized as opportunistic microorganisms capable of inducing multiple human diseases, attributed to the presence of virulence genes **(Ben Braïek & Smaoui, 2019).**

Critical criteria for lactic acid bacteria **(LAB)** to serve as probiotics include their safety, ability to maintain viability through processing and storage, capacity to counteract pathogens, survival within the intestinal environment, and ability to adhere to the host's intestinal epithelium **(Anwar A et *al.*,*2014*).**

Extensive research into probiotics has yielded promising outcomes, prompting a paradigm shift in microbial biotherapy. It has been acknowledged that certain beneficial effects of probiotics extend beyond live microorganisms. As a result, new concepts such as postbiotics and parabiotics have emerged, representing novel categories of compounds capable of eliciting biological responses similar to those observed in a healthy microbiota **(Capponi et *al.*, 2022)**

However, it was in 2013 that the definition of "postbiotic" was formally articulated "*any factor resulting from the metabolic activity of a probiotic or any released molecule capable of conferring beneficial effects to the host in a direct or indirect way"* **(Cuevas-González et *al.*, 2020).**

on the other hand, *"Parabiotics are the intact, inactivated microbial cells or cell lysates of probiotics containing cell components such as teichoic acids, peptidoglycan-derived muropeptides, pili, fimbriae, flagella, polysaccharides teichoic acids, and more"* **(Capponi et *al.*, 2022).**

Various postbiotic and parabiotic molecules exhibit diverse effects on numerous diseases, alongside the specific gastrointestinal advantages offered by probiotics, such as cholesterol reduction **(Adams, 2010; Capponi et *al.*, 2022)**. Furthermore, these compounds exhibit potent antioxidant activity. As a result, probiotics are viewed as a promising natural strategy for maintaining and enhancing health **(Paul et *al.*, 2023).** Moreover, the emergence of the novel concepts of postbiotics and parabiotics aligns with this family of terms, representing significant microbial-derived tools for health promotion **(Salminen et *al.*, 2021).**

In this study, the characterization of probiotics derived from **LAB** strains, with emphasis on *L. plantarum 299v* and *S. thermophilus*, and their respective health-promoting effects as probiotics, postbiotics, and parabiotics were evaluated.

EXPERIMENTAL SECTION

MATERIAL AND METHODS

1. Location and period of work

the research took place at the faculty of Nature and Life Sciences, specifically within the microbiology and biochemistry laboratories over a two-month period from February to April.

2. Hypothesis

I. *S. thermophilus* and *L. plantarum* 299v could have a probiotic potential.

II. Both viable and non-viable cells of *S. thermophilus* and *L. plantarum* 299v, as well as their postbiotics (byproducts), may harbor antioxidant properties and ability to reduce cholesterol levels.

3. General objective

The aim of this study is to leverage locally sourced probiotics, isolated from their natural environments, to develop a product tailored for industrial and probiotic applications.

4. Specific objectives

I. Isolation of *S. thermophilus*.

II. Identification of both LAB strains.

III. Evaluation of probiotic potential of *L. plantarum* 299v and *S. thermophilus*.

IV. Investigation into the Health-Promoting Benefits of Probiotics, Parabiotics, and Postbiotics Derived from *L. plantarum* 299v and *S. thermophilus*: antioxidant effect and reducing cholesterol levels

5. Experimental Approach

The outlined steps of the experimental approach ensure the effective execution of the work are indicated in the figure01:

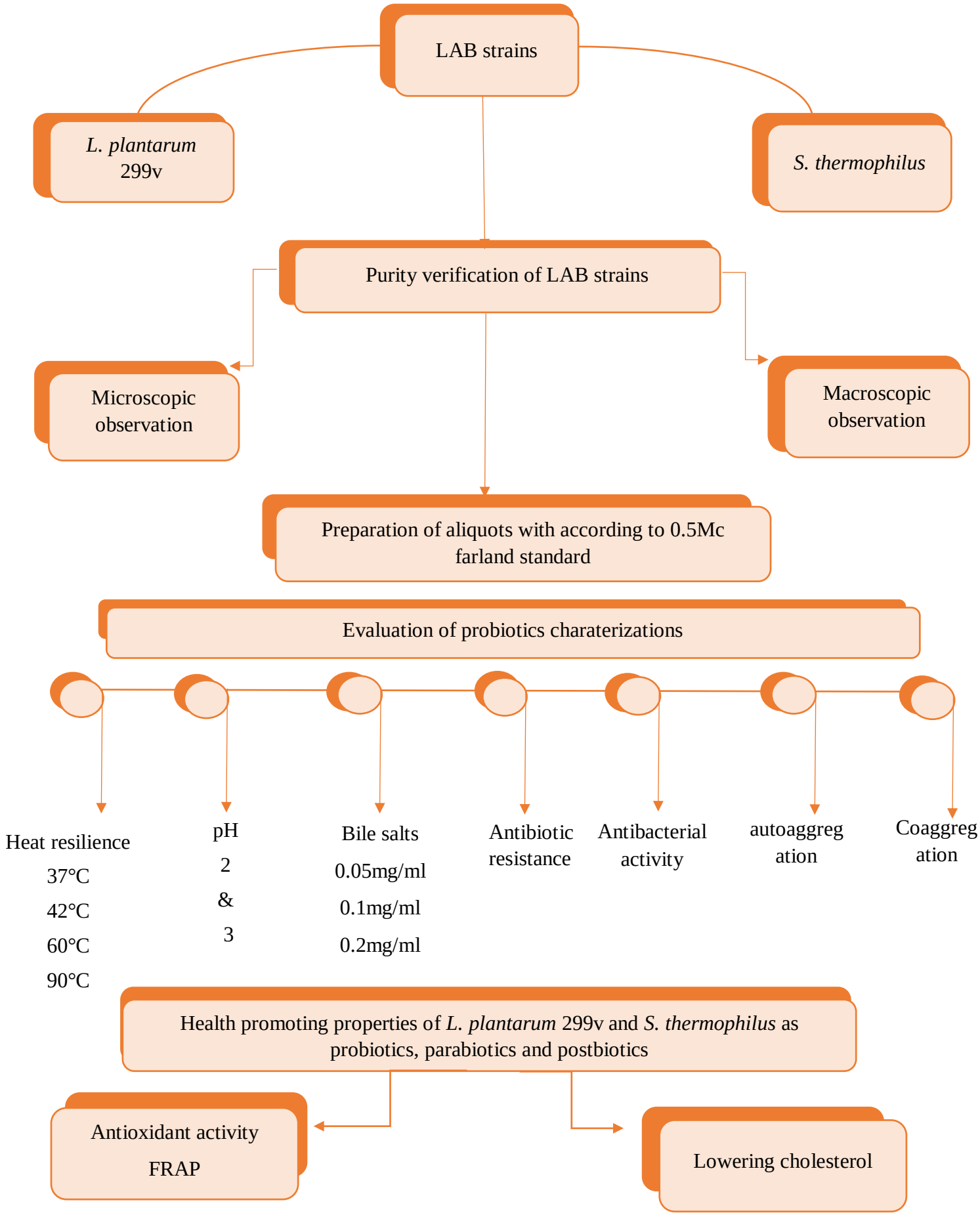


Figure01 : Experimental Protocol

6. MATERIAL

6.1.Biological material

For this study, a total of two strains of LAB were investigated *Streptococcus thermophilus*, isolated from natural yogurt, and a commercial probiotic *Lactobacillus plantarum* 299v.

6.2.Motivation for choosing strains

Lactobacillus plantarum and *Streptococcus thermophilus*, common strains of lactic acid bacteria **(LAB)** with inherent functional characteristics, are extensively utilized in industrial fermentation processes due to their established technological properties. Additionally, owing to their explored health-promoting attributes, selected strains are also marketed as probiotics **(García-Cayuela et *al.*, 2014; Taj et *al.*, 2022).**

6.3.Chemical products and equipment

The figure below illustrates the main equipment and products used to conduct the experimental procedures:

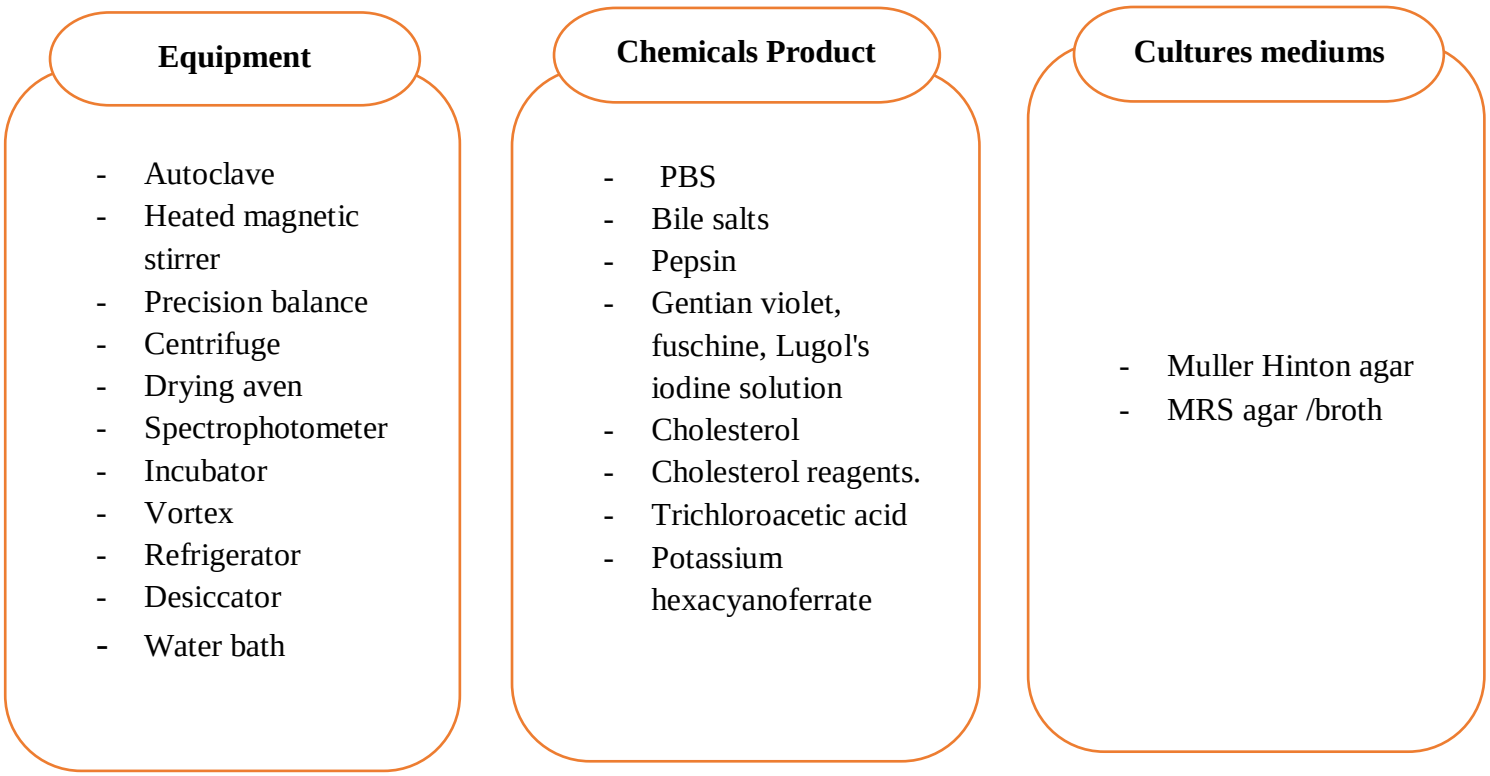


Figure02: Key Elements of the Experimental Approach: Equipment and Products used.

7. METHODS

7.1.Bacterial isolation

The LAB strains used in this study, *L. plantarum* 299v a commercially available LAB strains, while *S. thermophilus* was isolated from natural yogurt samples by our main supervisor, which were collected and stored in a laboratory freezer until they were ready for further processing.

7.2.Strains confirmation

A Gram stain was performed on both strains to confirm their morphology under microscope, additionally; the strains were observed under a contrast microscope to examine their morphology in fresh condition.

7.3.Inocula standardisation

After culturing the both LAB strains on MRS agar for 24 hours, colonies from the cultures were transferred into sterile MRS broth using a Pasteur pipette, the broth was adjusted to an absorbance of 0,11 to 0.13 at 570 nm, corresponding to 10^8CFU/mL **(Boubakeur et *al*., 2021).**

7.4.Evaluation of probiotics characterization

7.4.1. Heat resilience

The optimal growth temperatures and thermotolerance of the two bacterial strains were assessed using the method outlined by **Boubakeur et *al.* (2021),** with certain modifications. A suspension containing 10^8(CFU/mL) from a freshly cultured 18-24 h culture was added to a series of tubes containing MRS broth. These tube series were then placed in incubators set at different temperatures: +37°C and +42°C for 120 min for *L. plantarum* 299v and *S. thermophilus* strains respectively. Tubes designated for the thermoresistance test were subjected to incubation at 60°C and 90°C for both LAB strains, for durations of 120 and 30 minutes respectively. Absorbance readings were taken at 570 nm.

7.4.2. Tolerance of the GIT conditions

a. Acid pH effect

This test was performed according to the method described by **Boubakeur et *al.* (2021)** to prepare a solution similar to gastric juice, 0,3% of pepsin was suspended in 0,5% sterile saline. The mixture's pH was adjusted to pH 2 and pH 3. Colonies of two LAB strains *L. plantarum* 299v and *S. thermophilus* were put into MRS broth. Centrifugation of the suspension was carried out at 6000 g for 20 min. The debris were then removed from the medium by washing with a PBS of 3 ml. This process was repeated three times. The optical densities were measured at a wavelength of 570 nm both before and after a 3 hours incubation at room temperature.

b. Tolerance to bile salt

The ability of both strains to withstand the presence of bile salts was assessed by following a modified protocol based on **Boubakeur et *al.* (2021)**. 1ml inoculum of 10^7 CFU/ml was added to MRS medium supplemented with 0,05%, 0,1% and 0,2% bile salts. After 24h of incubation at 37°C and at 42°C for *L. plantarum* 299v and *S. thermophilus* respectively, the ODs were measured at 570 nm.

7.4.3. Antibiotic resistance

The resistance of the two strains *L. plantarum* 299v and *S. thermophilus* of LAB to five and six antibiotics respectively was performed according to the disc diffusion method detailed by **Boubakeur et *al.* (2021)**.The colistin (CT10; 10µg), Cefepime (FEP30; 30µg), Metronidazole (MT5; 30µg), Gentamicin (CN10; 10µg), tetracycline (TE30; 30µg) and Chloramphenicol (C30; 30µg) discs. The antibiotic discs were dispensed on MH agar spread by LAB strains *L. plantarum* 299v and *S. thermophilus*. The diameters of inhibition zone (mm) were measured after 24 h of incubation at 37°C.

7.4.4. Antagonistic effect

The antagonistic effect of LAB strains was assessed against two pathogenic strains, *E. coli* ATCC25922 and *S. aureus* ATCC6528 using the cross-streak technique described by **Boubakeur et *al.* (2021).** The LAB strains were streaked at the center of the agar plate, and the pathogenic strains. Were streaked perpendicularly to the central streak. After incubation, the antibacterial interactions were analyzed by measuring the diameter of the inhibition percentage was calculated using the formula:

$$\%\boldsymbol{inhibition\ percentage} = \left(\frac{\boldsymbol{inhibitory\ diameter(mm)}}{\boldsymbol{petri\ dish\ diameter}}\right) \times \mathbf{100}$$

7.4.5. Adhesion capacity

a. Auto-aggregation

Auto-aggregation, was assessed using the method described by **Boubakeur *et al.* (2021)** with some modifications. The two strains of LAB were cultured in MRS broth for18-24 h at 37°C and 42°C for both bacterial strains *L. plantarum* 299v and *S. thermophilus* respectively. Cells were harvested by centrifugation at 5000 g for 15 min. and washed three times by PBS. The bacterial pellets were suspended in 3 mL of PBS to achieve (10^8 CFU/mL) at 570 nm and distributed in two tubes series at 4 mL. The bacterial suspension tubes were decanted for 5h. The absorbance at 570 nm was measured for 01mL aliquots taken from the surface of each tube containing the bacterial suspension at 1 h, 2 h, 3 h, 4 h and 5 h.

Auto-aggregation % was calculated according to the following equation:

$$\boldsymbol{Auto-aggregation\,\%\;=\;[1-At/A0]\times 100}$$

Where **At** indicate to absorbance at time **t** after decantation and **A0** indicate to absorbance at **t=0.**

b. Co-aggregation

Coaggregation was evaluated using a modified version of the method described by **Boubakeur et *al. (2021)*.** Both strains of LAB, specifically *S. thermophilus* and *L. plantarum* 299v, were cultured in MRS broth for a period of 18 to 24 h. The cultures were maintained at temperatures of 42°C and 37°C for both LAB respectively. The cells were collected through centrifugation at 6000 g for 15 min followed by three washes with PBS. The bacterial pellets were then mixed with 3ml of PBS to achieve a concentration of 10^8 CFU/ml at 570nm, we mixed 3 ml of *S. thremophilus* with 3ml of *L. plantarum* 299v.The bacterial suspension tubes were decanted for 3h. The absorbance at 570 nm was measured for 1ml samples taken from the top of each tube containing the suspension after 1 h, 2 h and 3 h.

Co-aggregation **%** was calculated according to the following equation:

$$\boldsymbol{Co-aggregation\,\%=\left[1-\frac{A0}{At}\right]\times 100}$$

Where **At** indicate to absorbance at time **t** after decantation and **A0** indicate to absorbance at **t=0.**

7.5.Exploring the health promoting benefits of probiotics, parabiotics and postbiotics derived from LAB strains

7.5.1. Lowering cholesterol

The ability of the viable and dead LAB strains and their postbiotics remove cholesterol was assessed according to **Uyen *et al.* (2021).** with certain adjustments. Each of the two strains of LAB was grown overnight in two 10 mL MRS broth tubes namely R1 et R2. The next day, cell pellets from the R1 and R2 cultures were individually collected by centrifugation at 10000 rpm at 4 ºC for 15 minutes and then washed twice with PBS. For the viable cells in R1 tubes, they were suspended in 10 mL of PBS containing 0.3% bile salts and 100 mg/mL of water-soluble cholesterol. As for the heat-killed cell preparation, the pellets from R2 tubes were suspended in 10 mL of PBS, autoclaved at 90 ºC for 30 minutes, and then supplemented with 0.3% bile salts and 100 mg/mL of water-soluble cholesterol. The postbiotics were obtained by adding 0.3% bile salts and 100 mg/mL of water-soluble cholesterol to the supernatant from the centrifugation of the R1 tubes. The samples were incubated for 24 h and 48 h at 37°C under anaerobic conditions. Cholesterol assimilation by both growing

and dead LAB cells, as well as their postbiotics, was determined by calorimetric identification of the remaining cholesterol in the cultures after bacteria removal. Absorbance readings were taken at 550 nm.

7.5.2. Antioxidant effect

a. FRAP

Ferric reducing antioxidant power (FRAP) or the reducing power of iron was determined according to **Amezouar *et al.* (2013)** method. In test tubes 4 dilutions was made from 10^8 to 10^5 containing 0,5ml of the two LAB strains as probiotics, postbiotics and parabiotics, these concentrations were added to MRS medium. Postbiotics were obtained by centrifugation at 1000 g for 15 minutes, while parabiotics were obtained by autoclaving at 90°C for 30 minutes. Afterwards, 2,5ml of PBS was added, then 2,5 ml of potassium hexacyanoferrate. [K3Fe (CN)6] at 1% in distilled water. The entire mixture was then heated to 50°C in a water bath for 20 min. Afterward, 2,5 ml of trichloroacetic acid (10%) was added. and the mixture was centrifuged at 5000 g for 15 min. The resulting supernatant (2,5 ml) was mixed with 2,5 ml of distilled water and 0,5ml of freshly prepared 1% FeCl3 solution in distilled water. The reading was taken at 700 nm. In this method, the higher absorbance, the higher reduction power.

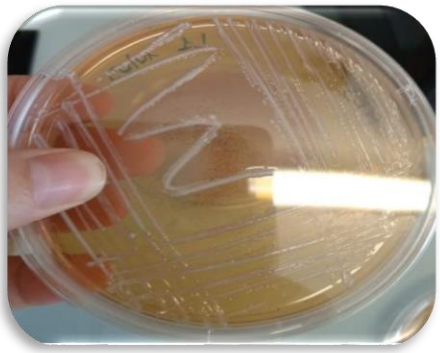
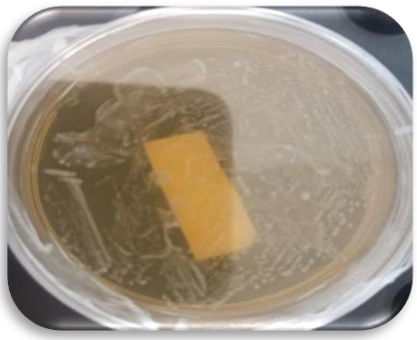
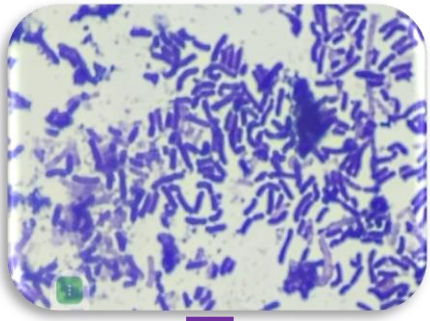
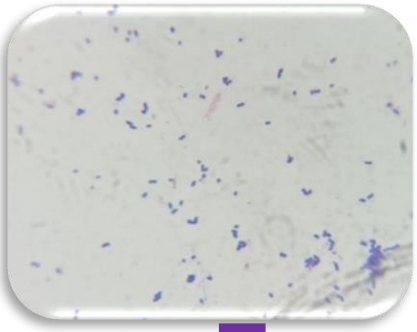
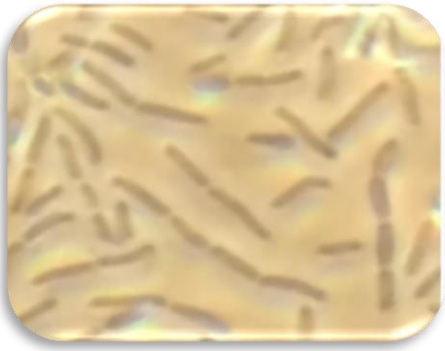
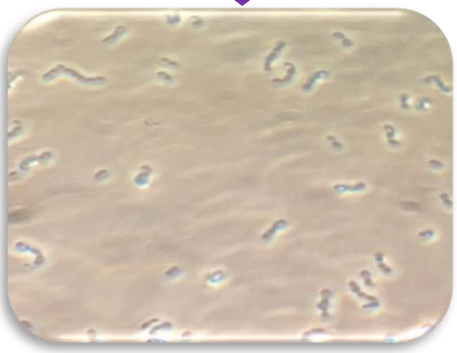
RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

1. Purity verification of both LAB strains and phenotypic identification

The results of the purity verification for both lactic strains are presented in the table N°01.

Table N°01: Morphological Characteristics of *Lactobacillus plantarum* 299v and *Streptococcus thermophilus.*

Characteristics	***L. plantarum* 299v**	***S. thermophilus***
Macroscopic Aspect	Smooth colony morphology, off-white/cream color, round colony shape.	
		
Microscopic Aspect « Gram staining»	Gram-positive& purple cells color	
		
Contrast microscopic treatment		
		
Association Mode	Short chains of bacilli	Long chains of Cocci

2. Evaluation of probiotics characterization of *L. plantarum* 299v and *S. thermophilus*

2.1.Heat resilience

Figure 03 illustrates the impact of temperature on the growth and survival of both LAB strains. The optimal temperatures for the growth of *L. plantarum* 299v and *S. thermophilus*, as demonstrated, are +60°C and +42°C, yielding cell concentrations of 7.776 log CFU/mL and 7.547 log CFU/mL, respectively. A minor yet decrease in growth rates was observed for both *L. plantarum* 299v and *S. thermophilus* after 2 hours of incubation at +42°C and +60°C, resulting in cell concentrations of 7.658 log CFU/mL and 6.862 log CFU/mL, respectively. Additionally, following a 30 minute incubation at +90°C, both LAB strains exhibited reduced viability, with cell concentrations of 7.444 log CFU/mL and 5.884 log CFU/mL for *L. plantarum* 299v and *S. thermophilus*, respectively.

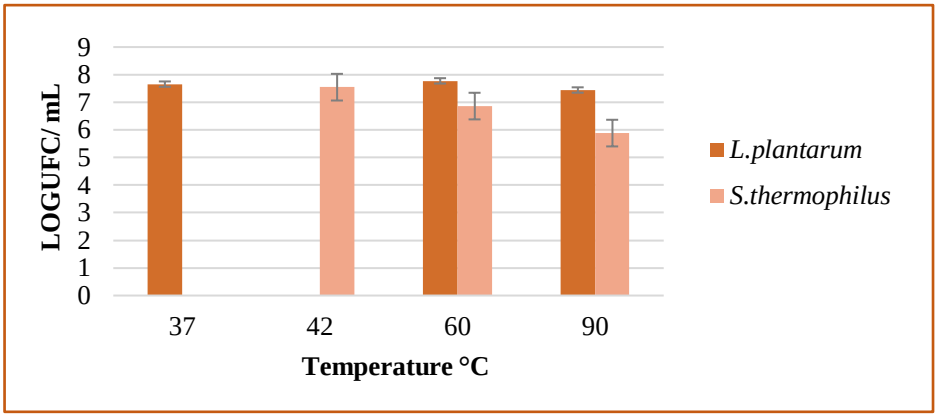


Figure03: The impact of temperature on the growth and survival of both LAB strains

Ferrando et *al.* (2016) showed that the heat resistance of the probiotic strain of Lp had been previously compared to other potential probiotic strains from species of *Lactobacillu*s genus. This previous research indicated that Lp 8329 has relatively high resistance to the stress factor of temperature. While there have been many studies on the cell stress resistance of lactic acid bacteria **(LAB)** in general, most of these have focused on a limited number of strains. Additionally, only a few studies have reported on the resistance of LAB against a diverse range of stress factors.

Although, Previous research has found that *Streptococcus thermophilus* species, despite being confined mainly to dairy environments, displays a surprisingly large diversity in its tolerance to different stress factors **(Parente et al., 2010).**

Under adverse conditions, bacterial cells frequently experience protein misfolding, a phenomenon with significant repercussions on cellular functionality. This misfolding can impede proper metabolic processes, leading to partial or complete metabolic blockade. To counteract this threat, bacteria employ a sophisticated network of molecular chaperones and proteases, which collectively facilitate correct protein folding and degradation of misfolded proteins. This tightly regulated system, orchestrated by various molecular effectors, including chaperones, ensures cellular homeostasis under stress conditions. In response to specific stressors such as heat, bacteria upregulate the synthesis of Heat Shock Proteins (HSPs), a subgroup of chaperones, to bolster their protein folding and degradation machinery. This adaptive response underscores the remarkable resilience of bacterial organisms in navigating hostile environments **(Ferrando et al., 2016).**

2.2.Tolerance to GIT conditions

a. Acid pH effect

LAB used as adjuvants possess a significant characteristic of being able to tolerate bile and resist the effects of gastric juices **(Boubakeur et *al.*, 2021).** While the exact level of tolerance required for optimal growth in the gastrointestinal tract (GIT) has not been determined, it is considered prudent to select species that exhibit enhanced resistance to acidic conditions. When an individual is in a fasting state, the generally accepted pH value of gastric juice is approximately 2. As a result this pH value has been adopted as a standard for conducting *in vitro* tests to assess the survival of probiotic cultures in the human stomach. The results showed that the survival of *Streptococcus thermophilus* and *Lactobacillus plantarum* 299v before and after a 3h incubation period at pH 2 and pH 3. The survival rates of both bacterial strains are higher at pH 2 and pH 3.

Table N°02: The growth of both **LAB** strains under acidic ph.

	Time (h)	***S. thermophilus*** **(Log CFU/mL)**	***L. plantarum* 299v** **(Log CFU/mL)**
Digestion in a solution simulating gastric juice			
PH 2	0	7	7,724
	3	6,69	7,748
pH 3	0	7,322	7,255
	3	7,602	7,278

Our findings agree with those of **Taj et *al.* (2022),** who showed that only six of the ten strains of *S. thermophilus* that were chosen were found to be acid tolerant at both pH levels (2 and 3). According to **khalil (2009)** study, *S. thermophilus* CHCC3534 was found to be non-resistant at pH 1.5 but resistant at pH values

higher than 2.

Numerous investigations have revealed that strains of *S. thermophilus* could not flourish at low pH values **(Haller et *al.*, 2001; khalil 2009; Mahmood et *al.*, 2013; Tuncer & Tuncer)**. According to these investigations, *S. thermophilus* was more susceptible to pH 1 than pH 3, but even at pH 3, cell viability decreased and more than 99,99% of the cells were inhibited.

L. plantarum strains were found to survive for up to six hours at pH 2 after incubation, according to **Taj et al. (2022).** Few strains were able to survive after being exposed to pH 2.0 for two hours, according to these results, which also indicated that most strains had a good resistance to pH 2.5 (> 75% of strains).

In pH 3 medium, all four strains of *Lactobacillus* designated as *L. plantarum* BM7.13, *L. acidophilus* BM10.8, *L. plantarum* BM29.7, and *L. rhamnosus* BM 30 were able to survive, according to **Uyen et *al.* (2021).s**

b. Bile salts

LAB which are widely employed in the production of dairy products and vegetable-based foods, are considered the foremost representatives of probiotics. A vital attribute associated with these bacteria acting as probiotics, is their ability to tolerate bile, which is crucial for their growth and survival **(Boubakeur et *al.*, 2021).** Based on the graph 04, it can be observed that both *S. thermophilus and L. plantarum* exhibit a certain level of tolerance towards bile salts, it is clearly evident that both LAB strains were able to survive even at the highest concentration (0,2%).

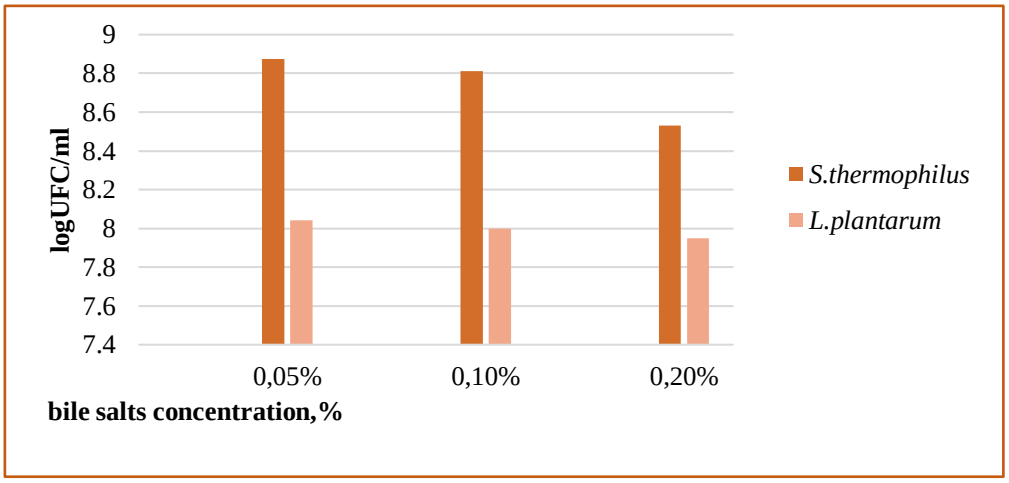


Figure04: Growth Behavior of Two LAB Strains under Various Bile Salt Concentrations

The bile salt tolerance of *S. thermophilus* was found to be 2% of bile salt, as shown by **Mahmood et *al.*, (2013).**

When exposed to bile salts, *Streptococcus thermophilus* strains exhibited poor survival effects, as

demonstrated by **Aslim & Alp. (2009).** However, immobilized strains of *S. thermophilus* demonstrated better survival than free strains. When comparing the culture solution containing 0.3% bile salts to the control sample (which did not contain bile salts), the viability of free strains was significantly lower. But compared to free cells, immobilized A21 and W22 cells had a higher level of resistance to bile salts. More precisely, immobilized *S. thermophilus* W22 had the highest viability.

According to **Pisano et *al.*, (2010)**, the *L. plantarum*-group strains 64FS and 61FS, isolated from Fiore Sardo, and 31C and 143C, isolated from Caciotta cheese made from raw and HPH treated milk, seem particularly intriguing because they were able to survive a 2-hour incubation in the presence of 0.3% bile.

As stated by **Adzuan et *al.*, (2022)** isolates of *H. itama* (HIT 5 and HIT 6) could not survive in 0.3% of bile salt after 4 hours of incubation, while the highest percentage of survival for bile salt tolerance was observed as 92.98% with 5.77 ± 0.52 CFU/mL from TLA 3.

The key attribute of a probiotic lies in its ability to endure low pH levels and high concentrations of bile salts, encased within a protective extracellular polymeric substance (EPS), the bacterium becomes more resilient against the harsh medium of stomach acid and bile salts. The efficacy of this mechanism is contingent upon the EPS's resistance throughout its transit in the intestine **(Boke et *al.*, 2010).**

2.3. Antibiotic resistance

The table N°03 illustrated the resistance of both **LAB** strains to the spectrum of antibiotics under investigation:

Table N°03: the antibiotic resistance and susceptibility of the two LAB strains.

Antibio-resistance/Sensibility						
Strains	FEP30	CT10	MT5	CN10	TE30	C30
S. thermophilus	R	R	R	R	MS	S
***L. plantarum* 299v**	R	R	MS	R	R	/

It is evident that both **LAB** strains exhibit similar behavior towards three antibiotics cefepime, colistin, and gentamicin along with metronidazole, to which the *S. thermophilus* strain demonstrates significant resistance. Regarding tetracycline and chloramphenicol, *S. thermophilus* exhibited moderate susceptibility and significant susceptibility respectively. As for *L. plantarum* 299v, it showed a significant resistance and moderate sensitivity towards tetracycline and metronidazole respectively.

Moghimi et *al.* (2023) showed that *S. thermophilus* strains harbored substantial resistance against a panel of six antibiotics, including tetracycline and gentamicin.

Furthermore, previous research has documented even higher rates of antibiotic resistance among *Streptococcus thermophilus* strains isolated from fermented dairy products. These findings collectively underscore the need for further investigation and clarification regarding the emergence and propagation of antibiotic resistance genes within this bacterial species **(Temmerman et *al.*, 2003; Zhou et *al.*, 2012) .**In contrast to the previous reports, investigations into the antibiotic resistance profiles of *Streptococcus thermophilus* strains have revealed that the majority of these bacterial isolates were not found to exhibit resistance against the tested antibiotic compounds **(Akpinar et *al.*, s. d.).**

Furthermore, members of the *Lactobacillus* genus were observed to display resistance against six antibiotics, including gentamicin, which is consistent with the known intrinsic resistance profile of this bacterial group. Conversely, studies have reported that *Lactobacillus plantarum* strains were found to be susceptible to the antibiotic ampicillin. **(Lee et *al.*, 2014; Li et *al.*, 2019) .**

The data suggests that *Lactobacillus spp.* and *Streptococcus thermophilus* strains may serve as a significant reservoir for antibiotic resistance genes **(Li et *al.*, 2019).**

2.4. Antibacterial activity

The table N°04 illustrates the antibacterial effect of the used strains:

Table N°04: Antibacterial effect of the two **LAB** strains against *E. coli* ATCC25922 and *S. aureus* ATCC6528.

lactic strain	**Antibacterial activity (Intensity according to diameter -mm)**	
	***S. aureus* ATCC6528**	***E. coli* ATCC25922**
S. thermophilus	+++(10%/9mm)	No inhibition
***L. plantarum* 299v**	No inhibition	No inhibition

These results demonstrate significant antagonistic activity of *S. thermophilus* against *S. aureus* ATCC6528, the inhibition percentages were 10%, we did not observe any antibacterial action of *S. thermophilus* against *E. coli* ATCC25922, as is the case for *L. plantarum* 299v against both pathogens bacteria *S. aureus* ATCC6528 and *E. coli* ATCC25922. Lactic acid bacteria have long been utilized in food preservation due to their ability

to produce antimicrobial substances. Through fermentation, carbohydrates are converted into various low molecular low weight organic molecules, such as lactic acetic, propionic acids, and ethanol.

The *E. coli* ATCC25922 was found to be less sensitive to cell-free supernatants from all tested strains of *S. thermophilus* (RIY, RIH4, and RIH 3), with no detectable zone of inhibition or antibacterial activity, according to the results of **Taj et *al.* (2022)**. These outcomes matched what we found.

The antimicrobial activity of *S. thermophilus* strains SL4 and SY2 was demonstrated by **Boubakeur et *al.* (2021)** against all tested bacteria, including *Escherichia coli* and *Staphylococcus aureus*. A bacteriocin known as thermophilin is produced by certain *S. thermophilus* strains, and it works well against a variety of bacteria that cause food spoiling. This bacteriocin has potential applications as a strong bio preservative due to its biochemical and technological value.

Yu et *al.* (2013) The isolated strains of *L. plantarum* exhibited varying degrees of antimicrobial activity against potential pathogens. Specifically, they showed greater activity against *E. coli* O157 and *S. flexneri* CMCC(B), while they showed less activity against *S. aureus* AC1 and *S. typhimurium* S50333. Previous research by **Essid et *al.* (2009)** demonstrated that 17 *L. plantarum* strains isolated from salted meat had varying levels of inhibitory activity against *Pseudomonas aeruginosa, S. aureus, S. arizonae, a*nd *E. coli*. Certain *Lactobacillus* strains isolated from dairy products were found to inhibit Gram-negative pathogens by producing organic acids. Bacteriocins, similar bacteriocins, and other inhibitory metabolites were also produced by *lactobacilli*, exhibiting distinct antimicrobial activity spectra.

2.5.Adhesion capacity

The ability of probiotic bacteria to adhere to the intestinal mucosa is viewed as an important factor in the selection process for potential probiotic candidates. This property is believed to enhance the longevity of probiotics within the gastrointestinal tract, thereby enabling them to effectively exert their beneficial influences. In this context, the capacity of probiotic bacteria to form cellular aggregates through self-association (auto-aggregation) or by clumping with genetically distinct cells (co-aggregation) is generally considered a desirable characteristic. These aggregation capabilities are thought to contribute to the probiotic's ability to persist and function effectively within the intestinal environment **(García-Cayuela et *al.*, 2014).**

a. Auto-aggregation and Coaggregation

The ability of probiotic bacteria to form cellular aggregates through self-aggregation (auto-aggregation) or by clumping with genetically distinct cells (co-aggregation) is regarded as a desirable characteristic. This property can enhance the adherence of probiotics to the intestinal mucosa, thereby increasing their persistence

within the gut and enabling them to exert their intended beneficial effects more effectively **(Paul et *al.*, 2023).** The figure **05** and **06** indicate the auto-aggregation and the co-aggregation percentages of the two LAB strains over a 5-hour period at room temperature.

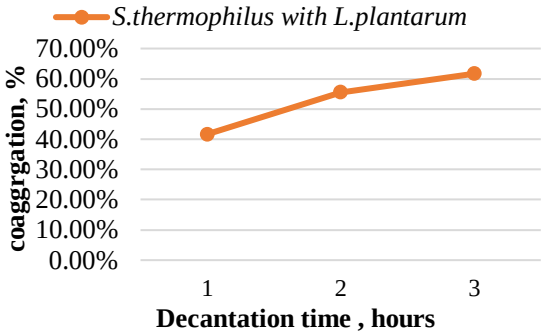


figure05: coaggregation capacity of the two LAB strains

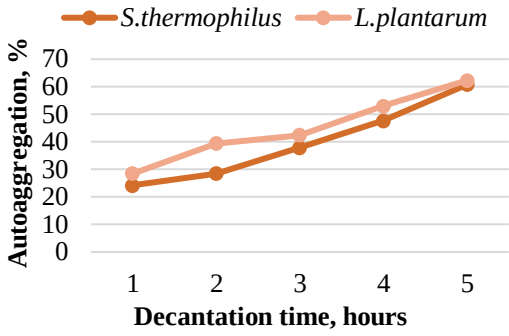


figure06: Adhesion level of the two LAB strains

The results indicated a consistent increase in auto-aggregation percentages with prolonged decantation time, suggesting a direct correlation between the two. For both *S. thermophilus* and *L. plantarum* 299v, auto-aggregation percentages ranged from 24.24% to 61.36% and from 28.46% to 62.3%, respectively, demonstrating similar capacities for aggregation over the duration of the test.

Interestingly, as auto-aggregation increased over time, so did coaggregation between the two LAB strains. The percentage of coaggregation rose from 41.66% to a peak of 61.66% after 3 hours of incubation at room temperature.

The observed simultaneous enhancement in both auto-aggregation and co-aggregation capabilities of probiotic bacteria suggests a potential interdependence between these two phenomena for instance, a study by **Janković et *al*. (2012),** demonstrated that three strains of *L. plantarum* S1, A, and B exhibited desirable auto-aggregation properties. . In contrast, the work of **García-Cayuela et *al.* (2014)** showed that cultures of *L. plantarum* IFPL207 displayed the lowest auto-aggregation values (≤30%) in a clear supernatant. Additionally, **Taj et *al.* (2022)** reported a medium percentage with 49.55 ± 6.24% auto-aggregation for the *S. thermophilus* strain, although some LAB strains have been observed to exhibit weaker aggregation properties, as reported by properties by **Paul et *al.* (2023).**

The study by **Paul et *al.* (2023)** found that over time, the bacterial isolates demonstrated an increased ability to both self-aggregate (auto-aggregation) as well as aggregate with other bacterial species (coaggregation). Notably, the coaggregation ability was generally higher than the auto-aggregation ability across the isolates examined.

The elevated auto-aggregation rate observed in the PBS broth can be attributed to the more favorable growth conditions provided by the liquid medium, as indicated in previous research, such as the study conducted by **Janković et *al.* (2012).** It implies that as the LAB strains aggregate among themselves, they also exhibit a propensity to co-aggregate with each other, indicating a dynamic relationship between the two processes.

3. Exploring the health promoting benefits of probiotics, parabiotics and postbiotics derived from LAB strains

3.1. Lowering cholesterol

In Figure07, cholesterol assimilation levels within a 24-hour period are illustrated for both viable and non-viable cells of LAB strains and their postbiotics. Both isolated strains, *L. plantarum* 299v and *S. thermophilus*, demonstrated a remarkable ability for reducing cholesterol.

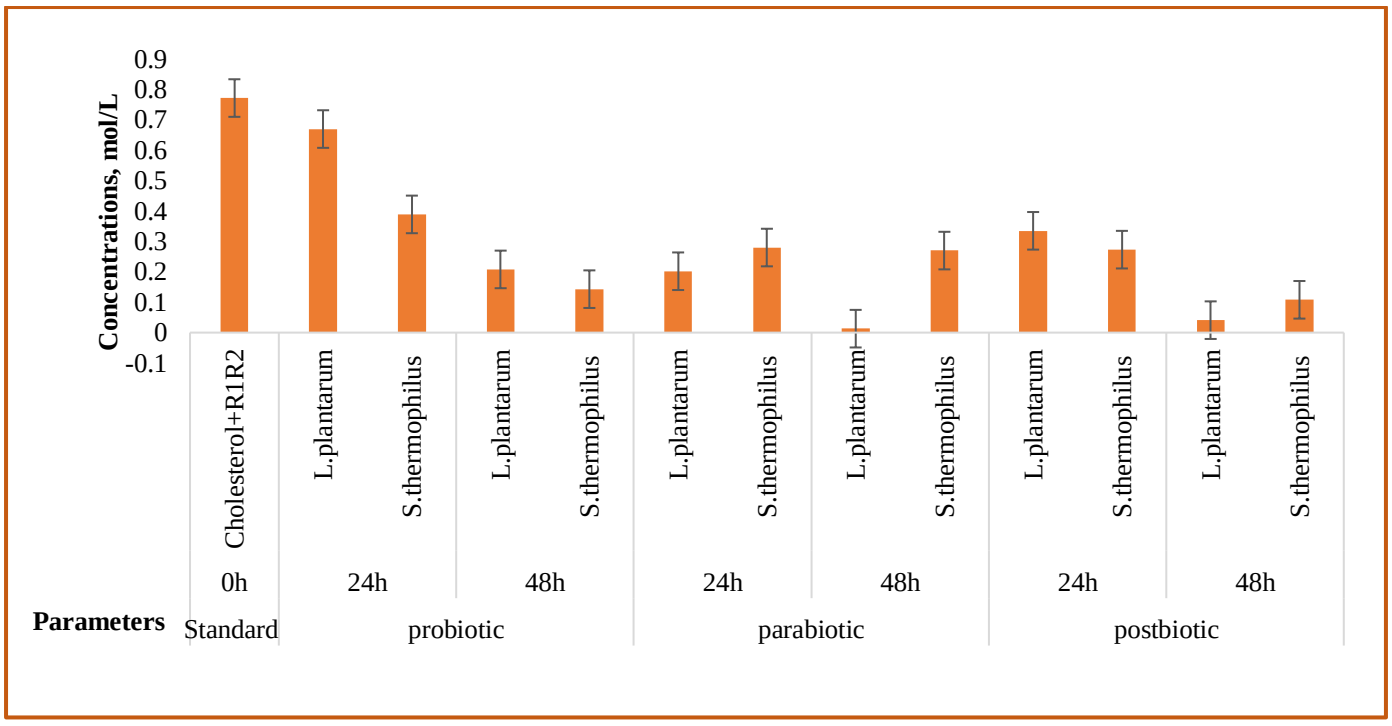


figure07: Cholesterol assimilation of both LAB strains as probiotics, parabiotics and postbiotics during 24 h and 48h at 37°C**.**

In the first 24 hours, the cholesterol concentrations in the medium in the presence of the two LAB strains decreased from 0.772 mol/L (initial concentration before treatment) to 0.67 mol/L, to 0.202 mol/L, and to 0.335 mol/L, respectively for pro, para and postbiotic effect of *L. plantarum* 299v. Conversely, for the effect of *S. thermophilus* on the cholesterol concentrations the reduced cholesterol is decreased in the medium from 0.772 mol/L to 0.389 mol/L, to 0.28 mol/L, and to 0.273 mol/L for the pro, para and postbiotic effect respectively. Both strains exhibited a significant reduction during this initial period.

After 24 hours, the cholesterol concentration for both viable LAB strains decreased, from 0.67 mol/L to 0.208 mol/L and from 0.389 mol/L to 0.143 mol/L for *L. plantarum* 299v and *S. thermophilus* respectively.

Furthermore, for non-viable cells there was a sustained decrease in cholesterol concentrations. Specifically, *L. plantarum* 299v exhibited a significant reduction from 0.202 mol/L to 0.013 mol/L, while *S. thermophilus* showed a minor decrease from 0.280 mol/L to 0.270 mol/L.

Moreover, the postbiotics displayed reductions almost comparable to those of probiotics. For *L. plantarum* 299v, the concentration decreased from 0.335 mol/L to 0.041 mol/L, indicating a substantial reduction. While, postbiotics from *S. thermophilus* showed a decrease in concentration from 0.273 mol/L to 0.108 mol/L, reflecting a moderate reduction.

the study by **Uyen et *al.* (2021)** found that among the LAB strains with the ability to assimilate cholesterol, the *Lactobacillus plantarum* strain demonstrated the greatest degree of this activity. According to the findings reported by **Tarique et *al.* (2022)** members of the bacterial genera *Enterococcus*, *Lactobacillus*, *Lacticaseibacillus*, and *Streptococcus*, isolated from diverse sources, exhibited comparable strain-specific cholesterol-lowering effects. This was in contrast to the relatively lower cholesterol removal activity observed among the remaining bacterial isolates examined in the study.

The study by **Uyen et *al.* (2021)** demonstrated that non-viable cells of *Lactobacillus* species possessed the capability to remove cholesterol. Additionally, the research conducted by **Tok & Aslim. (2010)** revealed that the amount of cholesterol removed by actively growing *Lactobacillus* cells was significantly greater ($p < 0.01$) compared to the cholesterol removal observed in heat-killed cells of the same species.

In this study, the most significant cholesterol removal was observed with both viable strains.

Various mechanisms have been suggested to explain the cholesterol-lowering capabilities of lactic acid bacteria. These putative mechanisms include the adhesion of cholesterol to the bacterial cell wall, the

enzymatic reduction of cholesterol mediated by cholesterol reductase enzymes, and the incorporation of cholesterol into the cell wall structure of the bacteria **(Tarique et *al*., 2022).**

No studies specifically addressing the cholesterol-lowering effects of our strains postbiotics were found. The results obtained in this study regarding the reduction of cholesterol by postbiotics are consistent with the proposal of **Salminen et *al*. (2021),** who defined a postbiotic as a *"preparation of inanimate microorganisms and/or their components that confers a health benefit on the host."* Therefore, the findings from this study align with this definition, illustrating the beneficial effects of postbiotics on cholesterol reduction.

3.2. Antioxidant activity

a. FRAP

In figure 08, highlights the variations in concentrations of the two lactic strains, *L. plantarum* 299v and *S. thermophilus*, known for their significant antioxidant properties, particularly due to their strong iron-reducing capabilities. These bacterial concentrations across three different states: probiotic, parabiotic, and postbiotic which represent varying degrees of microbial transformation and processing.

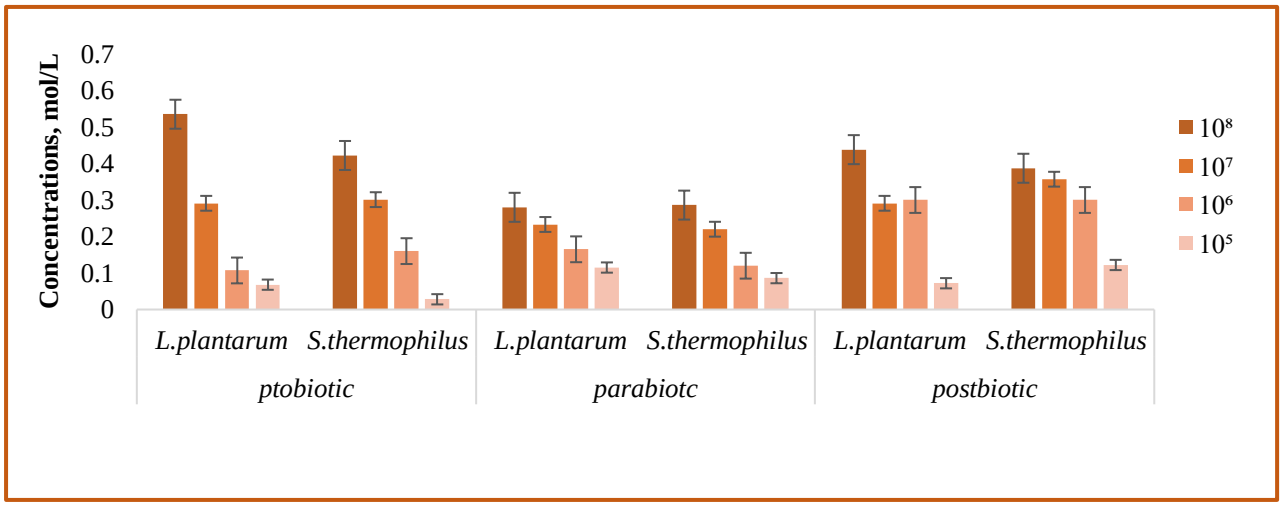


Figure08: antioxidant potential of the two LAB strains as probiotics parabiotics and postbiotics.

In the probiotic state, the concentrations of reduced iron reached to 0,422 mol/L and to 0,535 mol/L at 10^8, respectively for *S. thermophilus and L. plantarum* 299v. The two concentrations are the highest, and the concentrations decrease down to 0,028 mol/L and 0,068 mol/L at 10^5 of the two LAB strains. These results indicate that the two lactic acid bacteria have a strong iron-reducing capacity.

In the parabiotic state, the concentrations of reduced iron, at 10^8 *S. thermophilus,* achieved to 0,286 mol/L, and to 0,28 mol/L at 10^8 of *L. plantarum* 299v, the concentrations decreased to 0,086 mol/L and 0,115 mol/L at 10^5 of both strains.

In which concern the postbiotic effect, the concentrations of reduced iron, in presence of *S. thermophilus-free* supernatant, attained 0,387 mol/L, and 0,438 mol/L. in presence of *L. plantarum* 299v- *free* supernatant, the concentrations decreased to 0,122 mol/L and 0,072 mol/L in the presence of cell-free supernatant of 10^5 of both strains.

The obtained results suggests that the two lactic acid bacteria strains have an antioxidant effect and can reduce iron levels, but this depending on their metabolic state probiotic, parabiotic, and postbiotic.

According to **Lobo et *al.* (2019) ,** It seems like you're referring to a study or an article discussing the antioxidant potential of EPS (Exopolysaccharides) and its comparison with Vitamin C in terms of their antioxidant capacities. The results indicate that EPS demonstrated dose-dependent antioxidant activity within a certain concentration range (0.5–8.0 mg/mL). However, despite this activity, the antioxidant capacity of EPS was found to be lower than that of Vitamin C, suggesting that EPS possesses a moderate level of antioxidant capacity. Additionally, the transformation of Fe3+ to Fe2+ in the presence of EPS samples was investigated using reductive ability as a parameter. EPS1190, produced by a LAB strain, was specifically examined in comparison to Vitamin C, which is known for its strong reducing power, serving as a benchmark for evaluation.

As illustrated by **Tian et *al.* (2022),** the antioxydant capacity of *L. plantarum* KM1 was assessed. The findings indicated that the antioxidant capacity varied depending on the component being examined, including intact cells, cell-free extracts, and fermentation supernatant. The *L. plantarum* KM1 cell-free extracts had a better reducing capacity (310.67 ± 12.019 μmol/L) than the intact cells (271.778 ± 4.006 μmol/L).
The existing literature regarding the antioxidant potential of parabiotics has been notably constrained, despite their demonstrated health-promoting effects, as elucidated in our research.

Antioxidants are chemical compounds that play roles in preventing or reducing oxidative damage caused by free radicals. These unstable molecules are produced during normal metabolic processes, but in excess, they can damage cells and contribute to aging and various diseases. LAB strains produce various components, including EPS, vitamins, and antioxidant enzymes, which have shown their ability to neutralize free radicals and protect cells against oxidative damage **(Son et *al.*, 2018).**

CONCLUSION

CONCLUSION

The objective of this study was to characterize and determine the probiotic properties of the two LAB strains *Streptococcus thermophilus* and *Lactobacillus plantarum* 299v. Before proving their probiotic potential, the two LAB strains were preidentified phenotypically, involving macroscopic and microscopic examination of their morphology, additionally Various tests were conducted to assess their probiotic properties, including resistance to various stress conditions such as high temperatures, low pH, different concentrations of bile salts, adhesion capacity, resistance to antibiotics, and antibacterial activity. This study marks the initial utilization of these isolates as probiotic products, as specific criteria for selecting strains were established.

Also, the findings of this study demonstrate the beneficial effects of *Streptococcus thermophilus* and *Lactobacillus plantarum* 299v and their parabiotic and postbiotics derivatives on reducing iron levels and cholesterol levels. The two LAB strains showed promising potential as probiotics with the ability to modulate iron absorption and cholesterol metabolism with the best concentrations levels in both tests. These results suggest that the use of these probiotic strains and their postbiotics and parabiotics could be a valuable strategy in managing conditions related to iron overload and elevated cholesterol levels. but more in-depth studies should be carried out by testing other protocols (such as free radical scavenging, enzyme inhibition and *in vivo* studies) to better define the antioxidant and anti-cholesterolemic activity of the two strains tested.

S. thermophilus and *L. plantarum* 299v are well-studied probiotic bacteria with a long history of use in various fermented dairy products. In addition to the benefits provided by the probiotic cells; the metabolites, cellular components, and other bioactive compounds derived from these bacteria known as parabiotics and postbiotics have also emerged as promising health promoting agents. Parabiotics and postbiotics may offer advantages over traditional probiotics, such as increased stability and potential for targeted delivery of specific bioactive compounds. The diverse range of beneficial effects attributed to *S. thermophilus*, *L. plantarum*, and their derived biomolecules highlights their significant potential for applications in functional foods, nutraceuticals, and therapeutic interventions. although more detailed *in vitro*, *in vivo* and clinical studies should be carried out to ensure that all the results are obtained and that the strains can be used as probiotics to control oxidative stress and reduce cholesterol levels.

ABSTRACT

This study examinates certain probiotic properties and two biologicals activities of two bacterial strains, *Streptococcus thermophilus* isolated from natural yogurt, and *Lactobacillus plantarum* 299v a commercially available strain. The results indicate that the two LAB strains have demonstrated considerable probiotic potential. They exhibited high temperature resistance and strong survivability under stimulated gastrointestinal conditions, tolerating low pH levels of 2 and 3 for 3h. They also showed good tolerance to bile salts at different concentrations (0,05%,0,1% and 0,2%), Regarding the antibiotic resistance, *S. thermophilus* and *L. plantarum* 299v were resistant to 4 antibiotics tested and considerable antibacterial activity of *S. thermophilus* against *S. aureus* ATCC6528. The strains displayed also favorable adhesive properties, including high auto-aggregation ability (62,30% for *S. thermophilus*, 61,36% for *L. plantarum* 299v), and high degree of co-aggregation (61,66%). as regards biological activities, the results also showed that the live and killed bacteria and their postbiotic derivatives have demonstrated a good iron and cholesterol reduction. This research may represent a first step in assessing the probiotic potential of the bacterial strains tested. With further supporting tests, these strains may prove to be a promising approach to managing oxidative stress indicators and cholesterol levels.

Key words: probiotics, parabiotics, postbiotics, *S. thermophilus*, *L.plantaum 299v*, lactic acid bacteria, cholesterol reduction, antioxidant activity.

الملخص

تهدف هذه الدراسة الى تقييم بعض خصائص البروبايوتيك اضافة الى تقييم النشاط البيولوجي للسلالتين البكتيريتين *S. thermophilus* المعزولة من اللبن الزبادي الطبيعي و *L. plantarum* 299v السلالة المتوفرة تجاريا. النتائج توضح بان سلالتي حمض اللاكتيك أظهرت قدرة بروبايوتيكية مهمة, حيث أنها تميزت بمقاومتها لدرجات الحرارة العالية و بقدرتها القوية في تحملها للبقاء على قيد الحياة في ظل الظروف المعوية, و تحملا كبيرا لدرجات الpH المنخفضة المقدرة ب 2و3 درجات لمدة 3ساعات. بالاضافة الى قدرتها الجيدة في تحمل الاملاح الصفراوية عند ثلاث تراكيز مختلفة (0.05% , 0.1% و 0.2%). فيما يتعلق بمقاومة السلالتين للمضادات الحيوية *S. thermossphilus* و *L. Plantarum* 299v اظهرتا مقاومة لاربع مضادات حيوية, كما اظهرت النتائج نشاط مضاد بكتيري معتبر ل *S. thermophilus* ضد *S. aureus*. كما اعرضت السلالتين خاصيات مهمة في قابلية التراص, بما في ذلك استطاعتها العالية في التراص المتجانس بنسبة 30.62% ل *S. thermophilus* و61.36% ل *L. Plantarum* 299v و كذا درجات عالية من التراص الغير متجانس بنسبة 61.66% . فيما يخص النشاطات البيولوجية, اظهرت النتائج بان البكتيريا الحية و المقتولة و مشتقاتها لها فاعليه مهمة في خفض مستويات كلا من الحديد و الكوليسترول في الوسط المغذي. قد يمثل هذا البحث خطوة اولى مهمة جدا في تقييم الامكانيات البروبايوتيكيه للسلالتين البكتيريتين المعمول عليهما انما و من خلال جراء المزيد من التجارب المدعمه للدراسة, خاصة تجارب مقاومتها للمضادات الحيوية, قد تشكل هاتين السلالتين نهجا واعدا لمراقبة و مكافحة مؤشرات الاجهاد التاكسدي و مستويات الكوليسترول المرتفعة.

مفاهيم البحث الرئيسية : بروبايوتيك, بارابيوتيك, بوستبيوتيك, *L. plantarum* 299v, *S. thermophilus* , انخفاض الكوليسترول, النشاط التاكسدي.

ANNEXES

Annexes

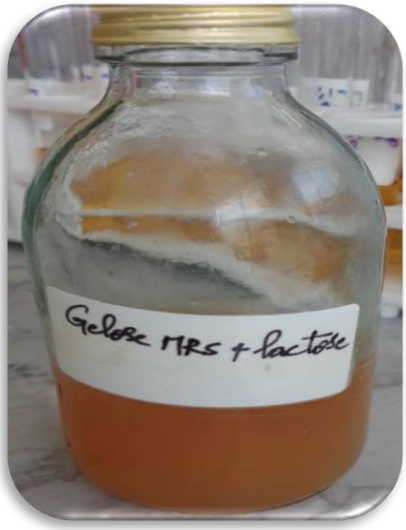
Annex N°01

Culture mediums preparation

MRS medium

MRS: Man, Ragosa, Sharpe

MRS+Glucose:	Concentrations:	MRS+Lactose:
MRS	52,25g/L	MRS
Glucose	5g/L	Lactose



In 250ml of distilled water:

1. **Added 13.06g of MRS + 1.25g of (Glucose or Lactose).**
2. **Agitation using agitator.**
3. **Sterilization of culture medium at 90°C during 30min.**

MH medium

MH: Muller Hinton

Concentration:

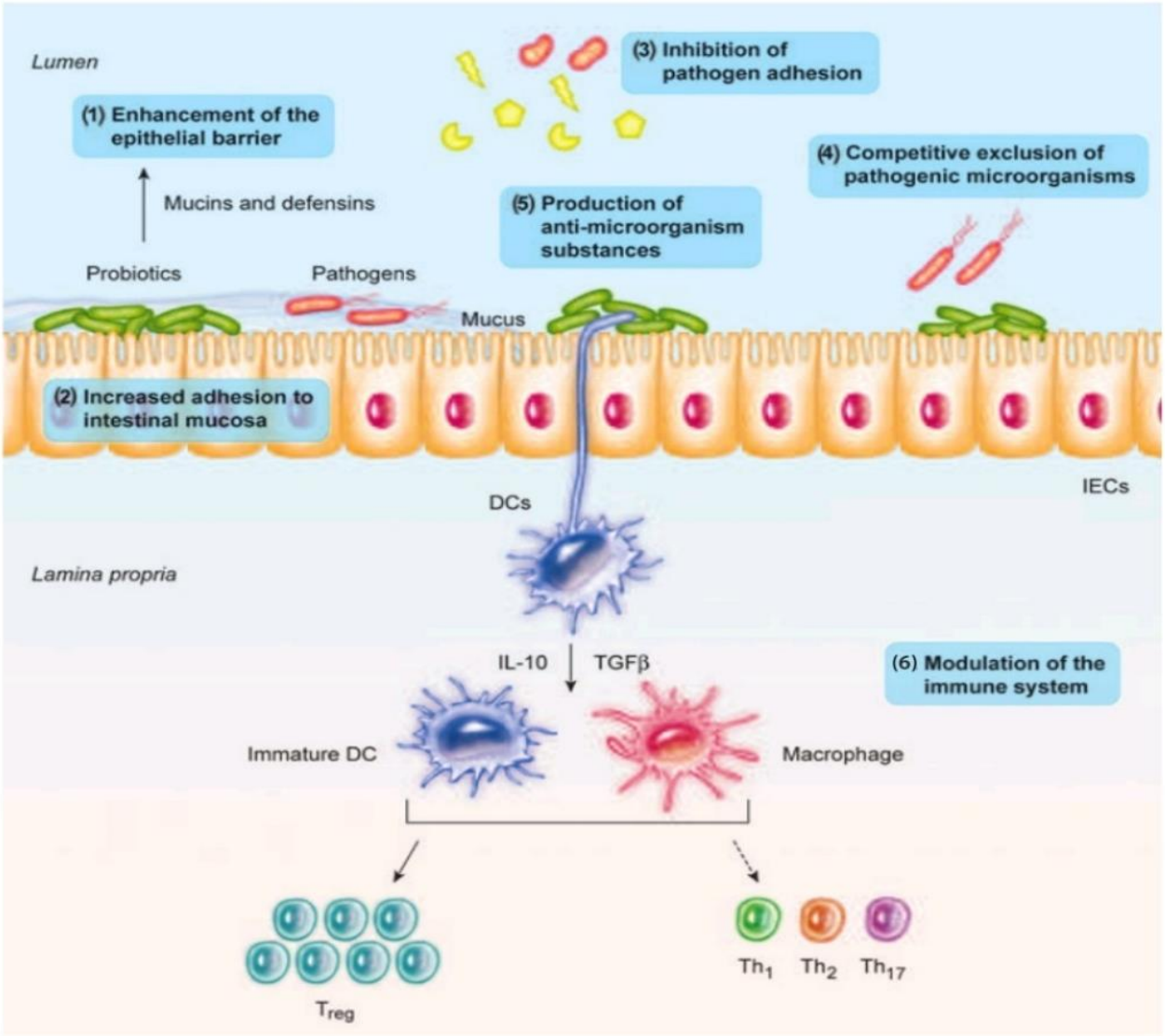
38,0g/L

In 250ml of distilled water:

1. **Added 09,5g MH.**
2. **Agitation using agitator.**
3. **Sterilization of culture medium at 90°C during 30min.**

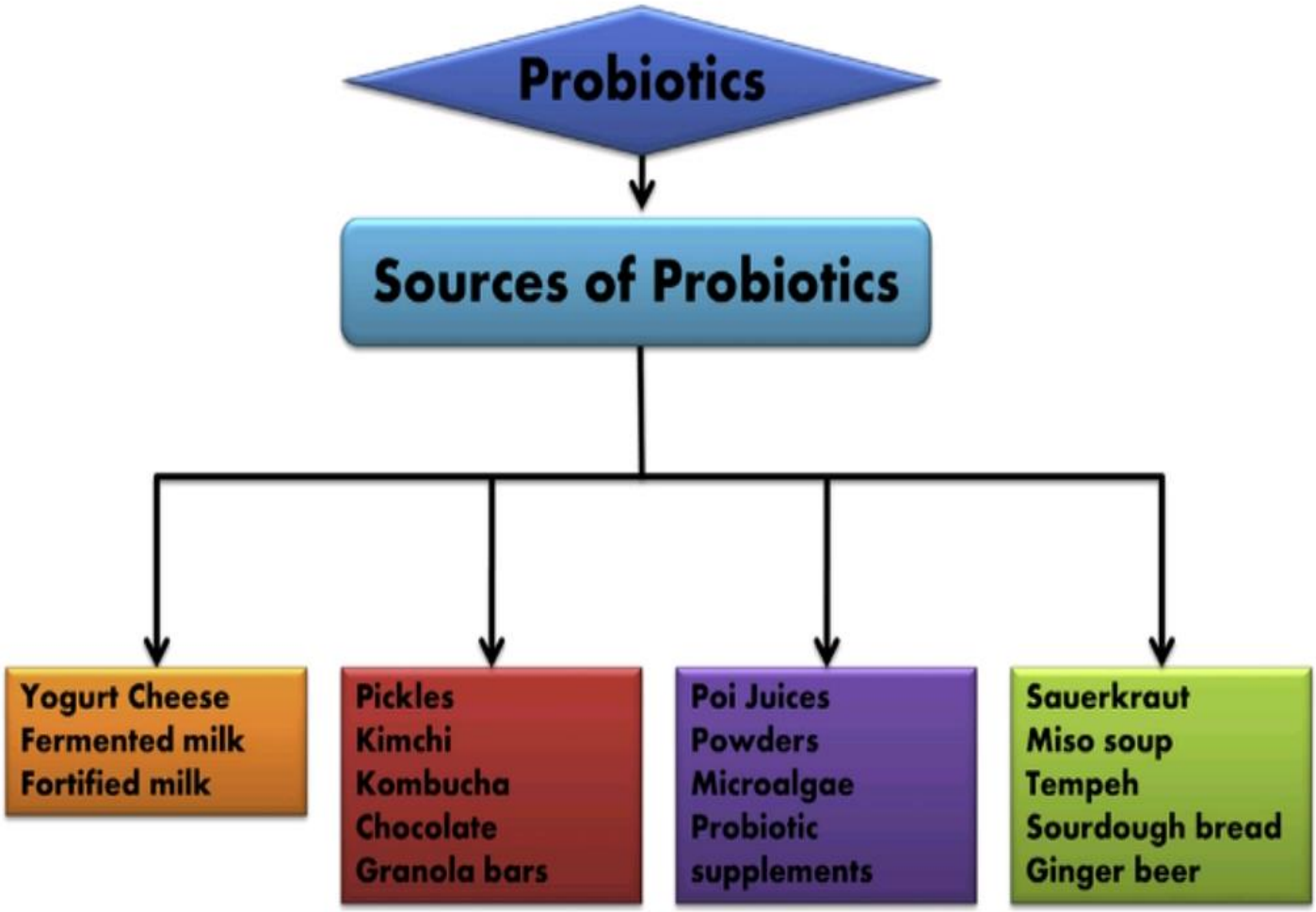
Annex N°02

Primary mechanisms of probiotics action **(Piqué et *al.*, 2019)**



Annex N°03

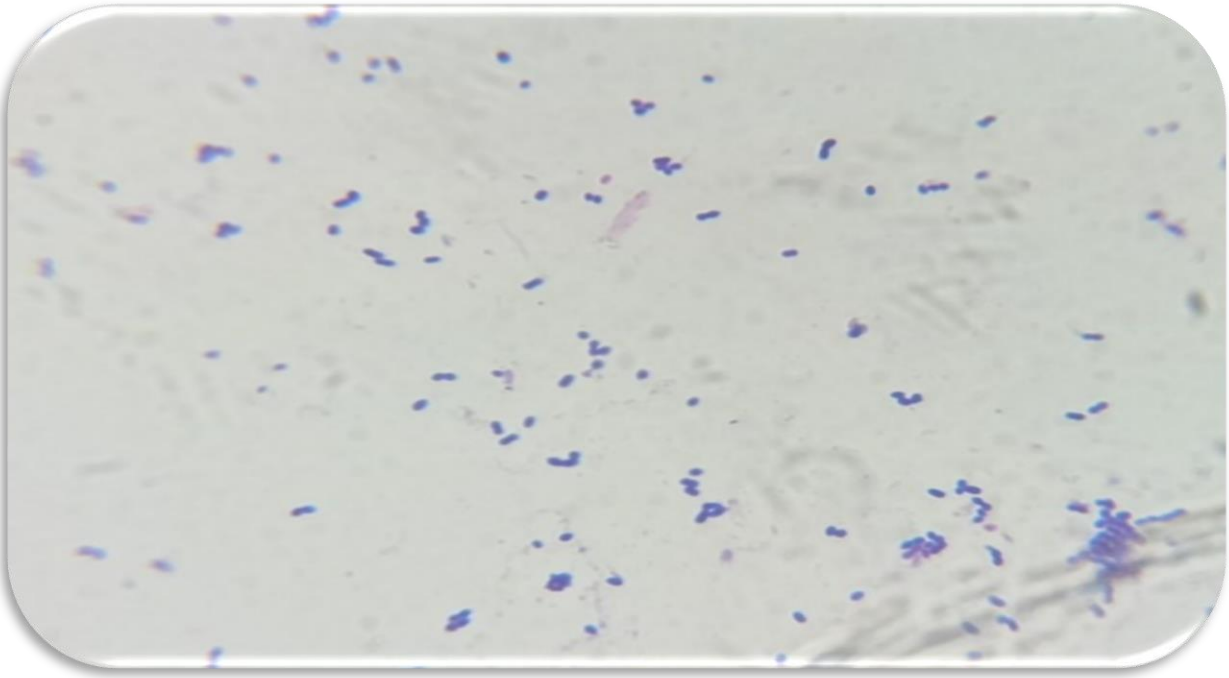
Various sources of probiotics according to **George Kerry et *al.* (2018)**



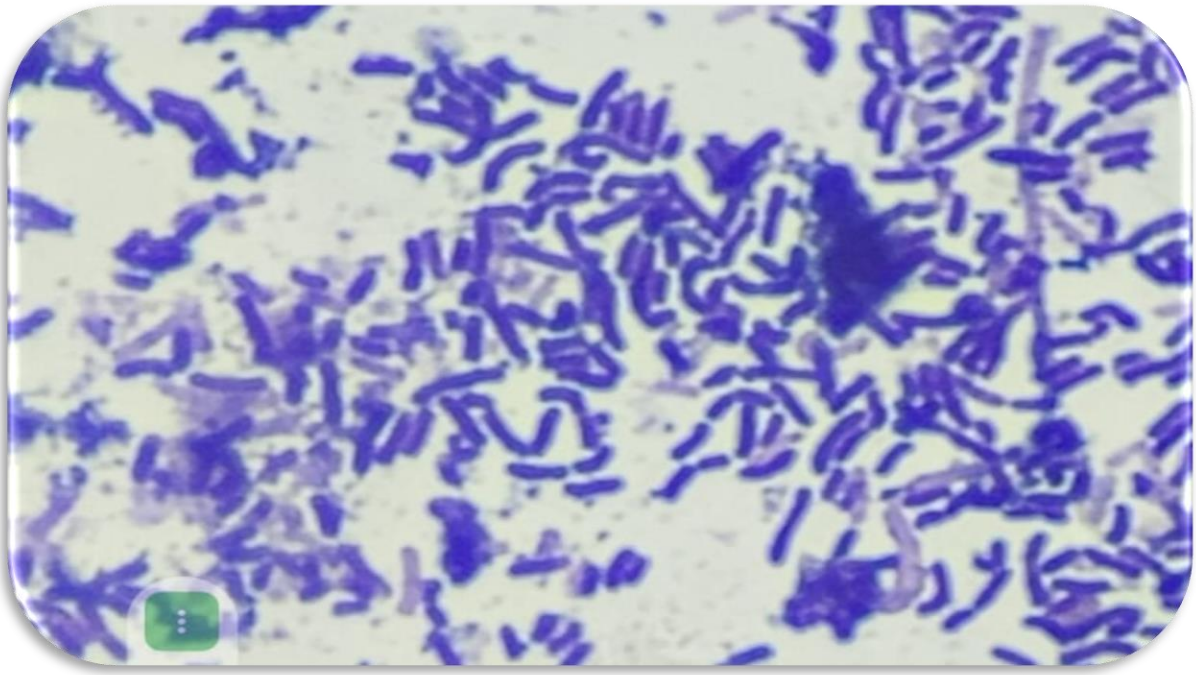
Annex N°04

The morphology of the two LAB strains under microscopic observation

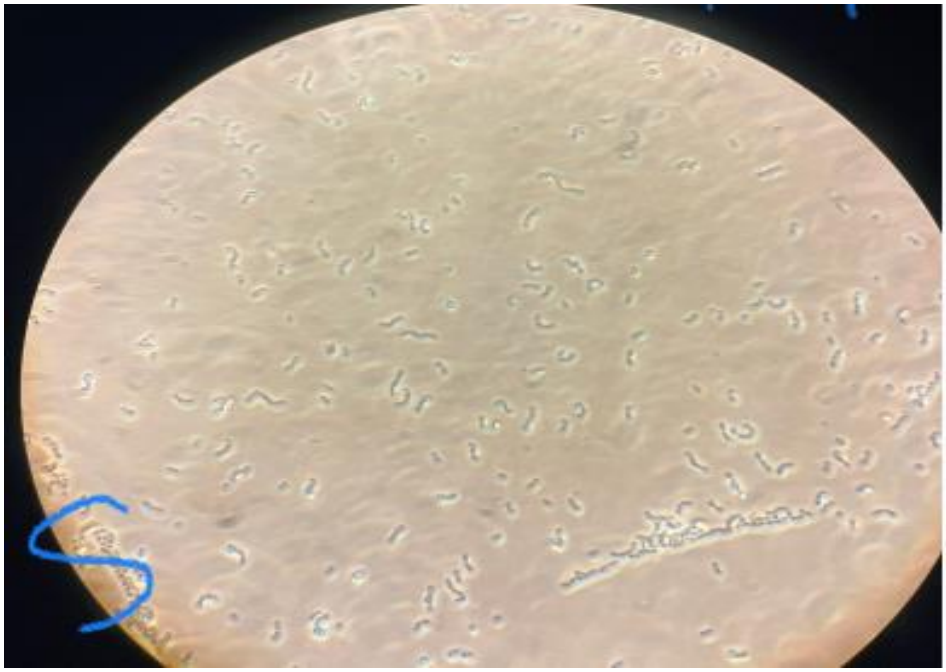
S. thermophilus



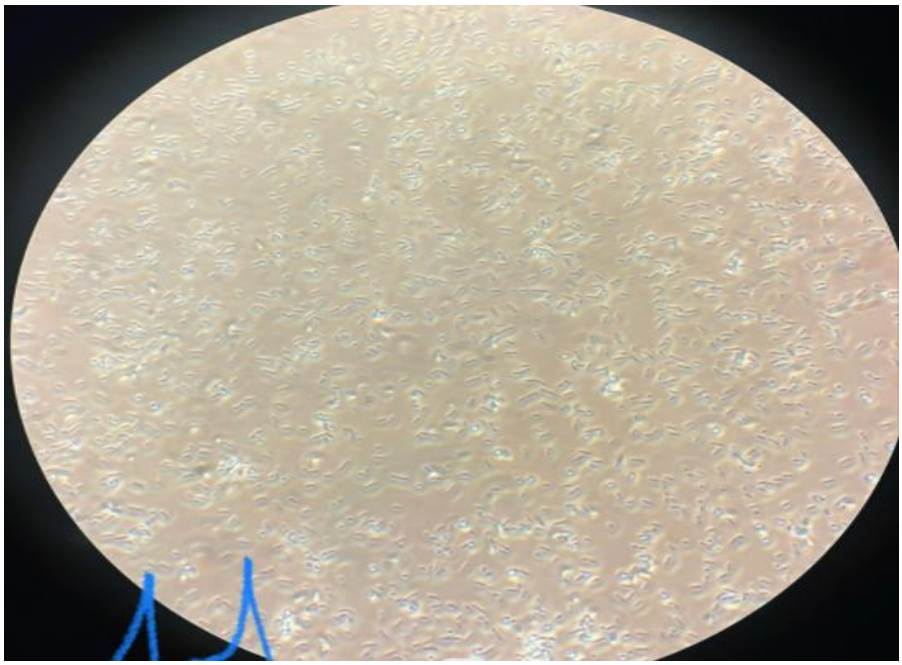
L. plantarum 299v



S. thermophilus

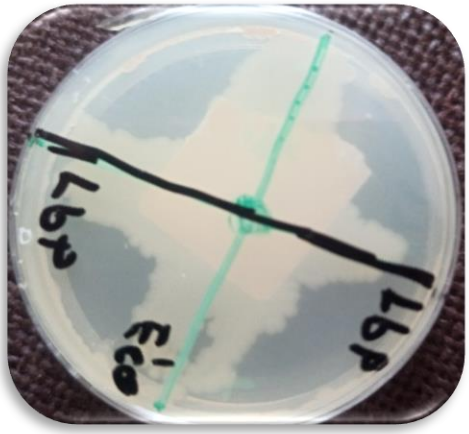
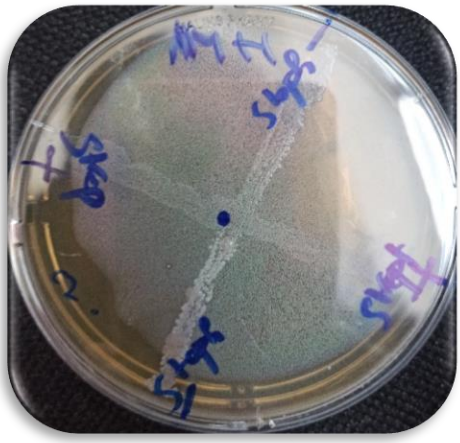


L. plantarum 299v



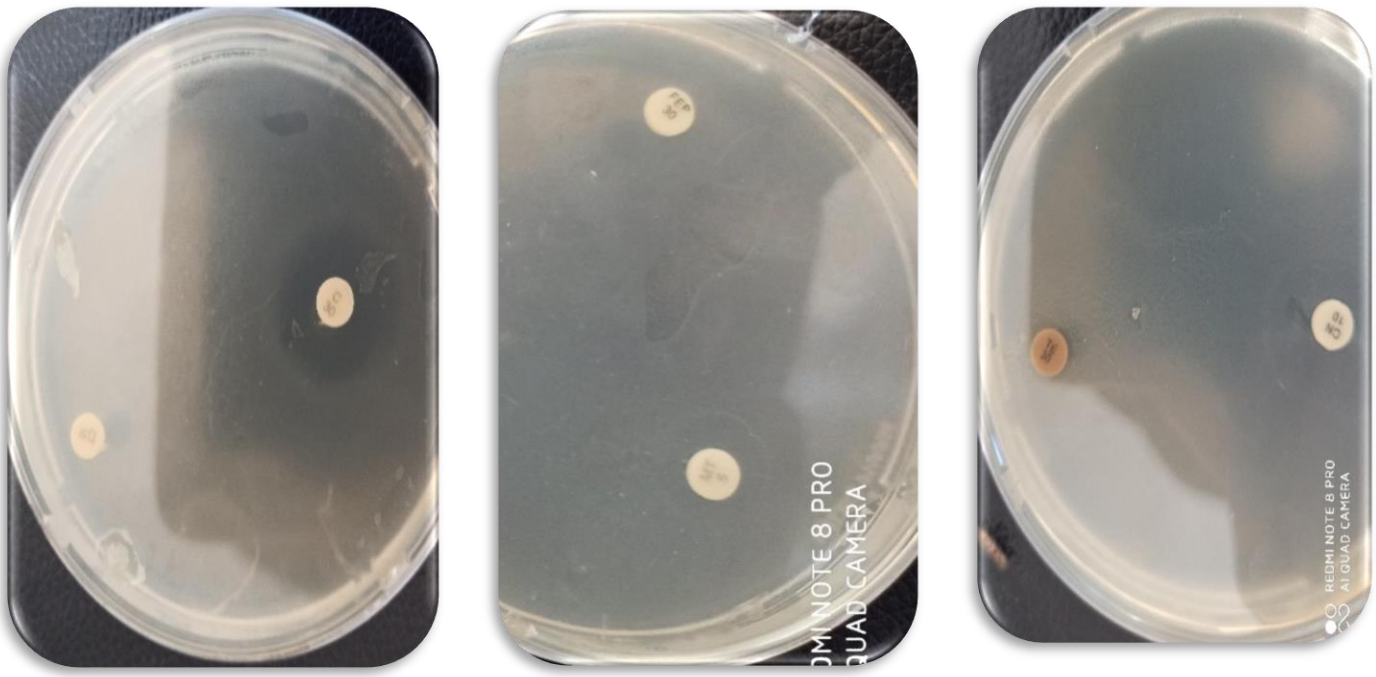
Annex N°05

Antibacterial activity of both **LAB** strains

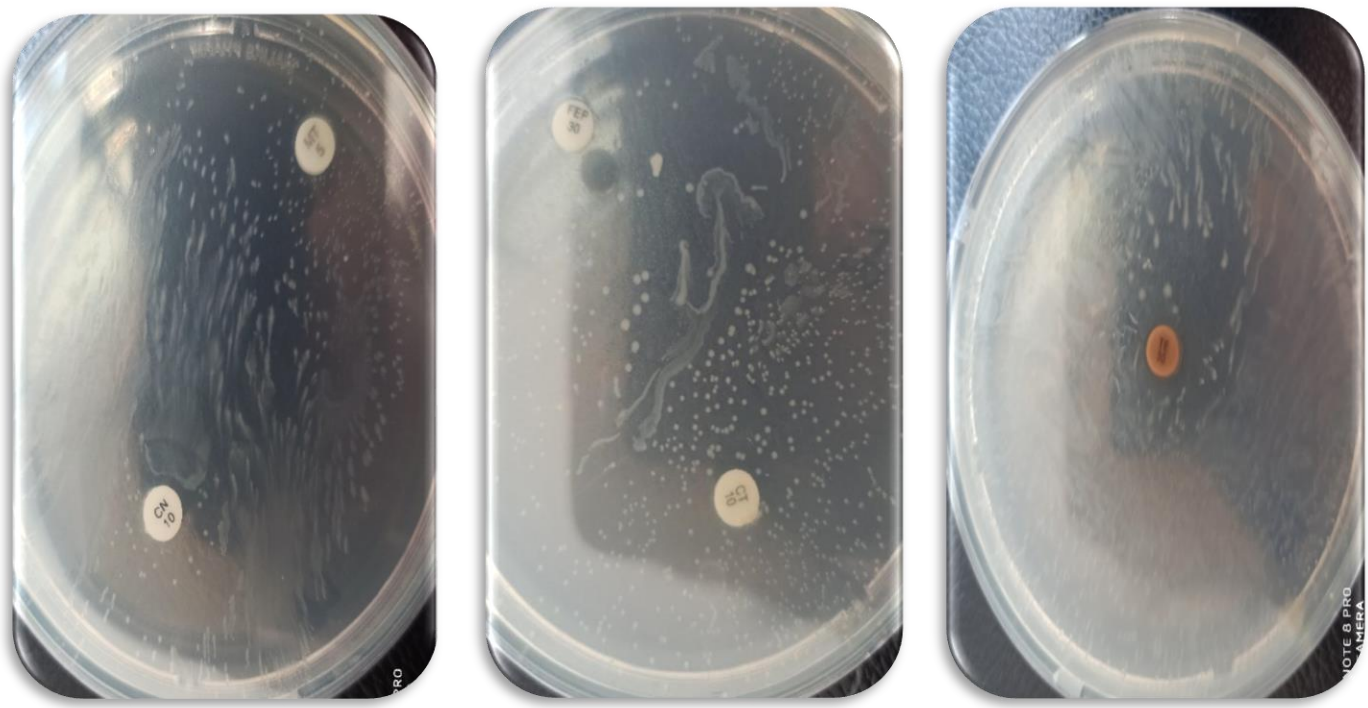
Strains	***L. plantarum* 299v**	***S. thermophilus***
***E. coli* ATCC25922**		
***S. aureus* ATCC6528**		

Annex N°06

Resistance and susceptibility of *S. thermophilus* to a spectrum of six antibiotics



Resistance and susceptibility of *L. plantarum* 299v to a spectrum of five antibiotics



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