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Isolation and Characterization of Probiotic Bacteria From the Digestive Tract of Helix *aspersa Müller*

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Dedication

In the shimmering light of my accomplishments, I humbly dedicate my success to you, my younger self at the tender age of four. You should be filled with immense joy and pride, for it is through your innocent spirit and unwavering determination that I have become the person I am today.

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Abstract

The objective of the present study consists to isolate and identify lactic acid strains from the gastrointestinal tract of the garden snail, *Helix aspersa Müller*. After isolation, the strains underwent characterization and identification using physiological and biochemical methods. A total of ten isolated strains were assessed for their inhibitory properties against five pathogenic microorganisms. The obtained results revealed that the lactic acid strains isolated from *Helix aspersa Müller* gastrointestinal tract, exhibited significant inhibitory activity. These strains demonstrated the ability to inhibit the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*, and *Candida albicans.*

Keywords: lactic acid bacteria, *Helix aspersa Müller*, pathogenic microorganisms, gastrointestinal tract, inhibitory activity.

Résumé

L'objectif de la présente étude consiste à isoler et identifier des souches de bactéries lactiques isolées du tractus gastro-intestinal de l'escargot des jardins, *Helix aspersa* Müller. Après l'isolement, les souches ont été caractérisées et identifiées à l'aide des méthodes physiologiques et biochimiques. Un total de dix souches isolées ont été évaluées pour leurs propriétés inhibitrices contre cinq micro-organismes pathogènes. Les résultats ont révélé que les souches de bactéries lactiques isolées à partir du tractus gastro-intestinal de *Helix aspersa Müller* présentaient une activité inhibitrice significative. Ces souches ont démontré leur capacité à inhiber la croissance d'*Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus* et *Candida albicans*.

Mots-clés: bactéries lactiques, *Helix aspersa Müller*, micro-organismes pathogènes, tractus gastro-intestinal, activité inhibitrice.

ملخص

الهدف من الدراسة هو عزل و تحديد سلالات حمض اللاكتيك المستخلصة من الجهاز الهضمي لحلزون الحدائق *Helix aspersa müller*

حيث تم توصيف و تحديد السلالات باستخدام الطرق الفيزيولوجية و الكيميائية و التكنولوجية بتقييم عشر سلالات معزولة لخصائصها المثبطة ضد خمس بكتيريا ممرضة.

أظهرت نتائج هذه الدراسة أن سلالات حمض اللاكتيك المعزولة من *Helix aspersa Müller* تتمتع بقوة مثبطة جيدة حيث أظهرت القدرة على تثبيط نمو بكتيريا *Escheria coli* و *Pseudomonas aeruginosa* و *Bacillus cereus* و *Staphylococcus aureus* و *Candida albicans* .

الكلمات الدلالية: بكتيريا حمض اللاكتيك، *Helix aspersa müller* ، بكتيريا ممرضة ، الجهاز الهضمي ، القدرة المثبطة

List of abbreviations

* ATCC: American Type Culture Collection.

* CFU: Colony Forming Unit.

* CMS: Content Management System.

* DCs: Dendritic Cells.

* IgA: Immunoglobulin A.

* LA: Lactic Acid.

* Lab: Lactic Acid Bacteria.

* OD: Optical Density.

* S : Strains.

* VP: Voges Proskauer.

List of Tables

List of figures

List of Maps

Table of Contents

Chapter III: Material And methods

- Growth test at different temperatures (15°C and 37°C)
- Growth test in the presence of 2.5, 4, and 6.5 NaCl
- Growth test in a hyperalkaline medium 27

- CO_2 production test from glucose
- CO_2 production test from citrate

General Introduction

General Introduction

Helix aspersa Müller snails hold a significant position within the world's nutrition landscape, providing a valuable source of essential nutrients and associated health benefits. These remarkable gastropods offer a diverse nutritional profile that contributes to overall well-being. With their high protein content, *Helix aspersa Müller* snails serve as an excellent source of quality protein, essential for tissue repair and growth. Additionally, they are abundant in essential amino acids, which act as the fundamental building blocks of proteins, supporting various physiological functions. These snails also possess a noteworthy array of vitamins and minerals, including omega-3 fatty acids that promote cardiovascular health and brain function. Furthermore, their iron, calcium, and magnesium content contribute to maintaining strong bones, teeth, and optimal muscle performance. Despite their modest size, *Helix aspersa Müller* snails provide a sustainable and eco-friendly solution for meeting nutritional needs while simultaneously offering a delightful culinary experience.

In recent years, researchers have shown an increasing interest in exploring a novel facet of *Helix aspersa Müller s*nail biology, particularly focusing on their gastrointestinal tract and its bacterial composition. This emerging field of study aims to investigate the intricate relationship between the snail's digestive system and the diverse community of microorganisms that inhabit it. By scrutinizing the bacterial composition of the snail's gut, researchers aspire to unravel valuable insights into the ecological and physiological roles of these bacteria, thereby shedding light on the intricate workings of this unique organism.

The gastrointestinal tracts of animals undergo adaptations based on their dietary requirements and physiological characteristics. Herbivorous animals that consume lignocellulosic feedstocks commonly exhibit two distinct features: an enlarged digestive tract and a gut microbiota. The elongated digestive tract typically consists of various anatomical regions, including the esophagus, crop, rumen, caecum, and rectal paunch. The gut microbiota confers a specific set of necessary enzymes to the host for the effective digestion of plant materials. In herbivores that predominantly consume lignocellulosic-rich plant materials, their gastrointestinal tracts function as natural bioreactors for the breakdown of plant biomass, thus serving as efficient sources of industrially significant bacteria. The digestion of plant biomass holds immense importance for energy acquisition in numerous herbivores and omnivores. Consequently, the bacterial flora present in the gastrointestinal tract of these animals may play a crucial role in facilitating digestion. These functionally specialized regions within the gastrointestinal tract may represent distinct microenvironments capable of hosting unique bacterial communities **(Dar et al., 2017)**.

The intestinal tract of *Helix aspersa Müller* is indeed rich in a wide variety of bacterial species, including aerobes, strict anaerobes, and facultative anaerobes. Among these, lactic acid bacteria are present. Research findings have shed light on the normal microbiota of *Helix aspersa Müller*, predominantly composed of bacterial species from the *Lactobacillus*, *Lactococcus*, and *Pediococcus* genera. Yeasts, specifically *Candida norvegensis*, *Candida guilliermondii*, and *Cryptococcus humicola*, have also been identified as part of the snail's microbiota. However, in cases where snails exhibit enteropathogenic symptoms, there is a notable decrease in the presence of *Lactobacillus* and *Lactococcus*, while species such as *Klebsiella*, *Pantoea*, *Citrobacter*, and *Enterobacter* show a higher prevalence **(Caullan et al., n.d.)**.

Lactic acid bacteria (LAB) are an important component of the gut microbial communities in various animals, including pigs, fowls, rodents, chickens, horses, gastropods, and insects. These bacteria play a crucial role in maintaining the ecological balance among the different species of microorganisms present in these environments. They are essential for the host's well-being as they contribute significantly to the fermentation of food, thereby providing energy to the host **(Dar et al., 2017).**

Currently, lactic bacteria are predominantly utilized for their probiotic properties in various applications. Probiotics refer to live microorganisms that confer health benefits when administered in adequate amounts. These microorganisms play a vital role in establishing and maintaining a balanced oral and intestinal microbiota. By doing so, they help inhibit the growth and colonization of pathogenic bacteria, thus mitigating the risk of infectious diarrhea and other gastrointestinal disorders. Probiotics also contribute to the overall digestive process by aiding in the breakdown and absorption of nutrients.

Overall, the utilization of lactic bacteria as probiotics continues to be an area of active research and development, holding promise for future advancements in promoting digestive health and preventing gastrointestinal disorders.

The objective of this modest work is to isolate, characterize, and identify lactic acid strains from the gastrointestinal tract of *Helix aspersa Müller* and to evaluate their antimicrobial activity against certain pathogenic bacteria.

This work begins with a literature review that summarizes general information about lactic acid bacteria and probiotics in the first chapter, followed by a second chapter on the biology of the snail *Helix aspersa Müller* . It continues with a description of the materials and methods used in the third chapter.

In the fourth chapter we present our results and their discussion. Our work concludes with a conclusion.

Chapter I:

Lactic acid bacteria and Probiotics

CHAPTER I: LACTIC ACID BACTERIA

I.1.1 Background

During the early 20th century, the term "lactic acid bacteria" (LAB) was initially used to describe microorganisms responsible for the souring of milk. While similarities were observed between these milk-souring organisms and other bacteria that produce lactic acid, the classification of LAB was established based on the monograph written by Orla-Jensen in 1919. Orla-Jensen's criteria, which included cellular morphology, glucose fermentation, temperature ranges for growth, and sugar utilization patterns, remain crucial for the classification of LAB. However, modern taxonomic tools, particularly molecular biological methods, have significantly expanded the number of LAB genera beyond the original four recognized by Orla-Jensen **(Wright & Axelsson, 2019)**.

Traditionally, lactic acid bacteria have been associated with food and feed fermentations and are generally regarded as beneficial microorganisms. Certain strains are even considered health-promoting (probiotic) bacteria. Nevertheless, some genera such as Streptococcus, *Lactococcus*, *Enterococcus*, and *Carnobacterium* also include species or strains that are recognized as human or animal pathogens. Therefore, a comprehensive understanding of the taxonomy, metabolism, and molecular biology of LAB is necessary to fully exploit their technological, nutritional, and health-promoting potentials while minimizing potential risks **(Vinderola et al., 2019)**.

Lactic acid bacteria (LAB) belong to a group of gram-positive, non-spore forming bacteria that primarily produce lactic acid through carbohydrate metabolism. They typically exhibit a rod-shaped or spherical morphology, with cell dimensions ranging from 0.5 to 2.0 μm in diameter and 2 to 10 μm in length.

The cell wall of LAB is composed of peptidoglycan and teichoic acids, providing structural support and protection.

The cytoplasmic membrane consists of phospholipids and proteins, regulating the transport of nutrients and waste products across the cell membrane. Common genera of LAB include *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*. LAB play a significant role in food fermentation by converting lactose and other sugars into lactic acid, which contributes to the characteristic sour taste of fermented foods and aids in preservation **(Vinderola et al., 2019)**.

I.1.2 The Habitats of Lactic Acid Bacteria

Lactic acid bacteria have a wide growth range and can thrive in temperatures below 15 °C and above 45 °C. They are known for their adaptability and can survive in environments with pH levels ranging from 3 to 11.

Lactic acid bacteria are indeed widely distributed in nature and can be found in various habitats such as plant materials, fermented foods, fruits, soil, water, and the oral, genital, intestinal, and respiratory tracts of humans and animals. Their ability to survive in diverse conditions contributes to their prevalence in these environments.

In fermented dairy products, the main genera of lactic acid bacteria are *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus*, with *Lactobacillus* being the dominant bacteria. Sourdough contains Lactobacillus brevis, Lactobacillus plantarum, *Lactobacillus sanfranciscensis*, *Lactobacillus crustorum*, *Lactobacillus paralimentarius*, *Lactobacillus mindensis*, *Staphylococcus pentose*, and *Enterococcus faecium*. Sauerkraut primarily contains *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Lactobacillus*. Fermented meat products consist of *Lactobacillus*, *Enterococcus faecium*, Leuconostoc *mesenteroides*, *Lactobacillus brevis*, and *Streptococcus*.

In the oral cavity, the main lactic acid bacteria genera are *Lactobacillus and Enterococcus*, with Streptococcus being the dominant bacteria. In the stomach, the main lactic acid bacteria are *Lactobacillus*, Streptococcus, and *Bifidobacterium*. The small intestine contains *Streptococcus equinus*, *Streptococcus sanguinis*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Lactobacillus ruminis*, *Pediococcus acidilactici*, and *Bifidobacterium pseudocatenulatum*. The large intestine harbors *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Bifidobacterium*. The female genital tract mainly contains *Lactobacillus* and *Enterococcus*. On the human skin surface, the predominant lactic acid bacteria are *Propionibacterium* and *Streptococcus*. Lactic acid bacteria can also be found in other organs of humans, aside from the digestive and urogenital tracts and body surface.

Previously, it was believed that the lungs were sterile. However, with advancements in molecular biology and sequencing technologies, it has been discovered that the lungs contain a certain number of microbes. These include *Prevotella*, *Sphingomonas*, *Pseudomonas*, *Acinetobacter*, *Fusobacterium*, *Megasphaera*, *Veillonella*, *Staphylococcus*, and *Streptococcus*

(Tian, 2019).

I.1.3.The Current Taxonomy of Lactic Acid Bacteria

During the late 19th and early 20th centuries, an increasing number of lactic acid bacteria were isolated and identified. These microorganisms were primarily known for their ability to acidify milk. Due to their similar physiological characteristics and cell morphology, it became crucial to classify them in a scientific and systematic manner.

It is important to note that the term "lactic acid bacteria" lacks strict taxonomic significance, resulting in potential taxonomic heterogeneity among its members. Through phylogenetic analysis, lactic acid bacteria can be categorized into two phyla: *Firmicutes* and *Actinobacteria*. This classification encompasses a total of 41 genera, including the following: *Bacillus, Halolactibacillus, Saccharococcus, Brochothrix, Listeria, Sporolactobacillus, Gemella, Abiotrophia, Aerococcus, Alkalibacterium, Carnobacterium, Desemzia, Isobaculum, Marinilactibacillus, Trichococcus, Enterococcus, Melissococcus, Tetragenococcus, Vagococcus, Lactobacillus, Paralactobacillus, Pediococcus, Leuconostoc, Oenococcus, Weissella, Lactococcus, Lactovum, Streptococcus, Lachnobacterium, Aeriscardovia, Alloscardovia, Bifidobacterium, Metascardovia, Parascardovia, Scardovia, Atopobium,* and *Olsenella*. Additionally, there are some new genera within the lactic acid bacteria group, namely *Fructobacillus, Lacticigenium, Pilibacter,* and *Sharpea,* which were not included in the 2015 edition of Bergey's Manual of Systematics of Archaea and Bacteria **(Stefanovic et al., 2017)**.

It is worth mentioning that *Bifidobacterium* differs significantly from other lactic acid bacteria based on phylogenetic analysis using the 16S ribosomal ribonucleic acid (16S rRNA) sequence **(Stefanovic et al., 2017)**.

The principal genera of lactic acid bacteria are summarized in **Table 1** below:

Table 1: Principal Genera of Lactic Acid Bacteria**(Stefanovic et al., 2017)**

Genus	Cell morphology	Fermentation	Lactate isomer	DNA (mole % GC)
Lactobacillus	Rods	Homo/hetero	DL, D, L	**32–53**
Lactococcus	Cocci in chains	Homo	L	**33–37**
Leuconostoc	Cocci	Hetero	D	**38–41**
Pediococcus	Cocci	Homo	DL	**34–42**
Streptococcus	Cocci in chains	Homo	L	**40**
Bifidobacterium	**Rods**	**Hetero**	**L**	**46–67**

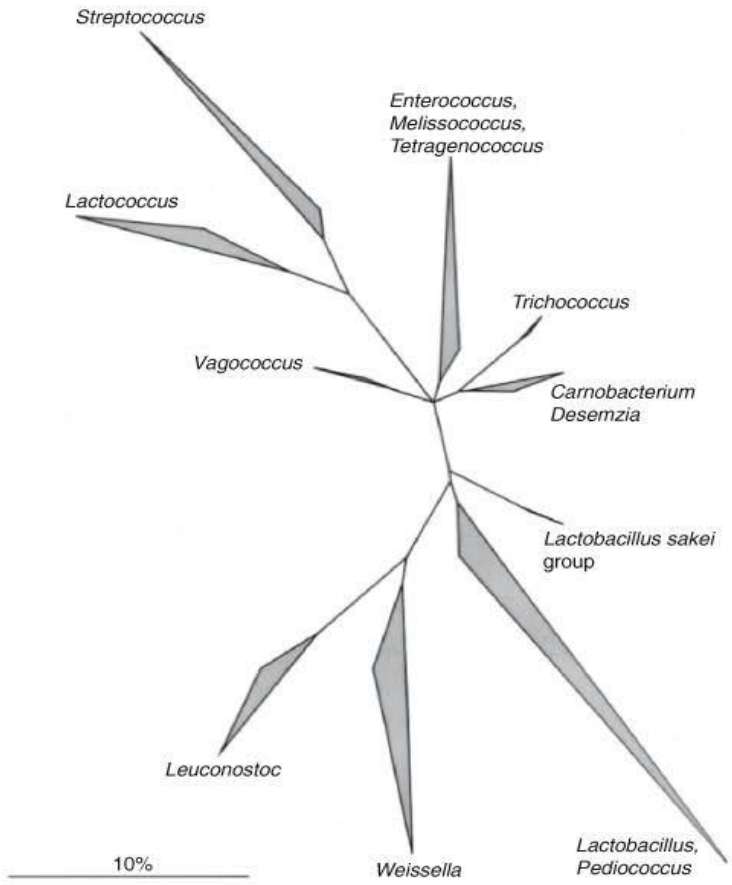


Figure 1: Phylogenetic tree depicting the position of LAB genera in relation to each other. The consensustree is based on maximum parsimony analyses of all available, at least 90% complete 16S rRNA sequences of gram-positive bacteria. Note that the family *Lactobacillaceae* (lower part; *Lactobacillus*, *Pediococcus*, *Weissellla*, and *Leuconostoc*) also includes the genera *Oenococcus* and *Fructobacillus*. The bar indicates 10%estimated sequence divergence (Hammes and Hertel., 2016).

I.1.4. The Safety of Lactic Acid Bacteria

The safety testing of lactic acid bacteria involves several important aspects:

1. The lactic acid bacteria strain must be clearly identified and classified correctly, and it should be deposited in an internationally recognized culture collection. This allows manufacturers, scientists, and regulators to monitor the organisms for any genetic changes and compare them with clinical isolates.

2. Novel strains originating from species with potential pathogenic or adverse properties need to undergo rigorous evaluation. This includes systematic screening for antibiotic resistance and the potential for transfer of resistance genes.

3. The immunomodulatory effects of lactic acid bacteria should be assessed in specific target populations to understand their impact on the immune system.

4. Clinical studies should follow the gold standard of randomized, double-blind, placebo-controlled designs to ensure reliable and unbiased results.

5. Lactic acid bacteria used in animal feed additives or veterinary products must be evaluated for safety in the human food chain, considering potential risks associated with consumption.

6. The labeling of lactic acid bacteria products should provide accurate information regarding the content, shelf life, claimed attributes, and recommended dosage.

7. After the introduction of novel lactic acid bacteria, data on intake patterns should be collected, particularly for long-term consumption, to monitor potential effects on health.

8. Epidemiological surveillance should be implemented to monitor any adverse effects associated with lactic acid bacteria, especially in relation to infections.

9. Clinical isolates should be characterized to compare them with endogenous strains and probiotics, as this is crucial for confirming the safety of lactic acid bacteria **(Chen & Narbad, 2019).**

I.1.5. The Metabolism of lactic acid bacteria

Lactic acid bacteria (LAB) are gram-positive microorganisms widely recognized as safe producers of lactic acid (LA) on an industrial scale. They utilize the glycolysis pathway to produce Lactic Acid under anaerobic conditions, converting hexoses and pentoses through various metabolic pathways, as depicted in **figure (2)** The production efficiency and rate of LA depend on factors such as pH (3.5-9.6), temperature (5-45°C), presence of nutrients (amino acids, peptides, nucleotides, and vitamins), and the specific LAB strains used. Notably, strains belonging to *Leuconostoc, Lactococcus, Lactobacillus, Pediococcus, Enterococcus, Streptococcus, Vagococcus, Aerococcus, Carnobacterium, Tetragenococcus, Oenococcus,* and *Weissella* genera have been employed .

In addition to their role as LA producers, LAB species such as Lactobacillus, *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Pediococcus* find applications as starter cultures in industrial food fermentations. *Lactobacillus* strains, in particular, are highly valuable due to their acid tolerance, high yield, and productivity. Moreover, they can be genetically engineered for selective production of either L- or D-lactic acid **(Abedi & Hashemi, 2020).**

There are two main pathways of lactic acid bacteria (LAB) fermentation :

A) Homofermentative LAB pathway:

Homofermentative LAB possess the aldolase enzyme and primarily convert glucose into lactic acid (LA). They predominantly utilize hexose and pentose sugars through the Embden-Meyerhof pathway (glycolysis) and pentose phosphate pathway. Homofermentative LAB produce two molecules of LA as the major end-product per mole of glucose consumed, with a theoretical yield. Experimental yields may vary depending on the carbon source used. For commercial production of lactic acid (at concentrations above 100 g/L), only homofermentative LAB is used due to its high yield (close to the maximum theoretical value), productivity, and the production of highly pure lactic acid (>99%). Examples of homofermentative LAB include *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, and some *Lactobacillus* species such as *Lb. delbruckii subsp. bulgaricus*, *Lb. acidophilus*, *Streptococcus salivarius subsp. thermophilus*, and Lb. *helveticus*. Additionally, studies have reported that *Enterococcus mundtii* QU 25 and engineered *Lactobacillus plantarum* can also metabolize pentose sugars into lactic acid through homofermentation.

B) Heterofermentative LAB pathway:

Heterofermentative LAB have the ability to metabolize glucose into lactic acid, acetic acid, formate, ethanol, diacetyl, acetoin, and carbon dioxide (CO_2 gas detection is used to distinguish heterofermentation from homofermentation). Heterofermentative LAB can utilize the phosphogluconate pathway (theoretical yield of 0.5 g/g) and the phosphoketolase pathway (theoretical yield of 0.6 g/g) when metabolizing hexose and pentose sugars, respectively. The use of heterofermentative LAB as dairy starter cultures is less common due to the release of CO_2 and simultaneous production of lactic acid and other organic acids. These byproducts are considered defects that can cause issues such as bloated packaging and cracks in dairy products and hard cheeses. Heterofermentative LAB species mainly include *Oenococcus*, *Leuconostoc*, and some Lactobacillus species such as *Lb. brevis*, *Lb. fermentum*, and *Lb. reuteri* **(Abedi & Hashemi, 2020).**

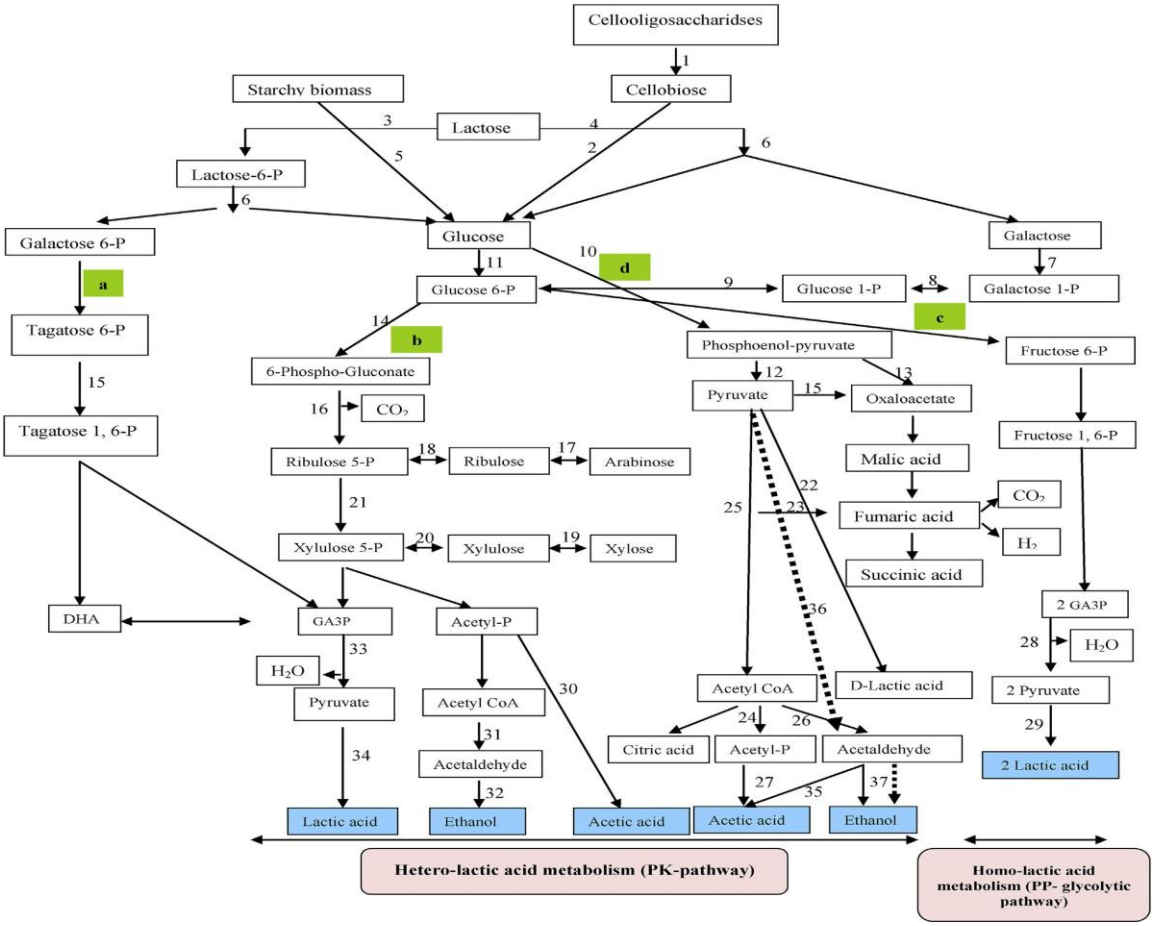


Figure 2: Pathways of lactic acid production from agro-industrial residues **(Abedi & Hashemi, 2020)**.

Number on arrow catalyzed by enzyme and other reaction.

1: Exo $\beta_{1,4}$ Glucanase, 2: β-Glucosidase, 3: lactose phosphotransferase system (*Lac*-PTS), 4: permease, 5: Amylase, 6: β-galactosidase, 7: ATP→ADP, 8: galactose-1-phosphate uridylyltransferase,9: phosphoglucomutase, 10: NAD→ NADH, 11: ATP→ADP, 12: ATP→ADP, 13: Phosphoenolpyruvate carboxylase, 14: ATP→ADP, 15: ATP→ADP, 16: $NAD^þ$→NADH, 17: arabinose isomerase, 18: ribulokinase and ATP→ADP, 19: xylose reductase and xylitol dehydrogenase, 20: ATP→ADP, 21: ribulose 5-phosphate 3-epimerase, 22:D-lactic acid Dehydrogenase, 23: Pyruvate-fomarate lyase, 24: Pta, 25: Pyruvate dehydrogenase complex, 26: Aldehyde dehydrogenase, 2NADH→ $2NAD^þ$, 27: Acetate kinase, 28: 4 ADP→ 4ATP, 2 $NAD^þ$→2NADH, 29: 2NADH→$2NAD^þ$, 30: ADP→ ATP, 31: NADH→ $NAD^þ$, 32: NADH→ $NAD^þ$, 33: 2ADP→ATP, $NAD^þ$→NADH, 34:Lactate dehydrogenase, NADH→$NAD^þ$, 35: Acetaldehyde dehydrogenase, 36: Pyruvate decarboxylase. 37: Alcohol dehydrogenase. GA3P: glyceraldehyde-3-P, DHAP: Dihydroxyacetone-P.

A route: D-tagatose 6-phosphate pathway. B route: Pentose phosphoketolase (PK) pathway: for Hetero lactic acid metabolism. C route: Embden- Meyerhof-Parnas (EMP) pathway: for Homo lactic acid metabolism. D route: Glycolysis pathway in *E. coli, K. lactis* and *S. Cerevisiae*.

I.1.6. The antimicrobial effect of lactic acid bacteria

Lactic acid bacteria (LAB) are known to produce a variety of antimicrobial compounds, as illustrated in **figure 3**. These compounds include organic acids (lactic, citric, acetic, fumaric, and malic acid), hydrogen peroxide, CO_2, diacetyl, ethanol, reuterin, acetaldehyde, acetoin, ammonia,

bacteriocins, bacteriocin-like inhibitory substances (BLIS), and other metabolites. Figure 3 visually depicts the diverse range of antimicrobial substances that LAB can generate.

These antimicrobial compounds play a crucial role in inhibiting the growth and survival of microorganisms. LAB's ability to produce organic acids, such as lactic acid, creates an acidic environment that is unfavorable for the growth of pathogens. Additionally, substances like hydrogen peroxide and bacteriocins exhibit direct antimicrobial activity by disrupting the cellular structures of target microorganisms. The production of these antimicrobial compounds enables LAB to exert a strong antagonistic effect against various pathogens.

In summary, figure 3 highlights the wide array of antimicrobial compounds synthesized by LAB. These compounds, including organic acids, hydrogen peroxide, and bacteriocins, contribute to the antimicrobial activity of LAB and aid in their competition with pathogenic microorganisms **(Agriopoulou et al., 2020).**

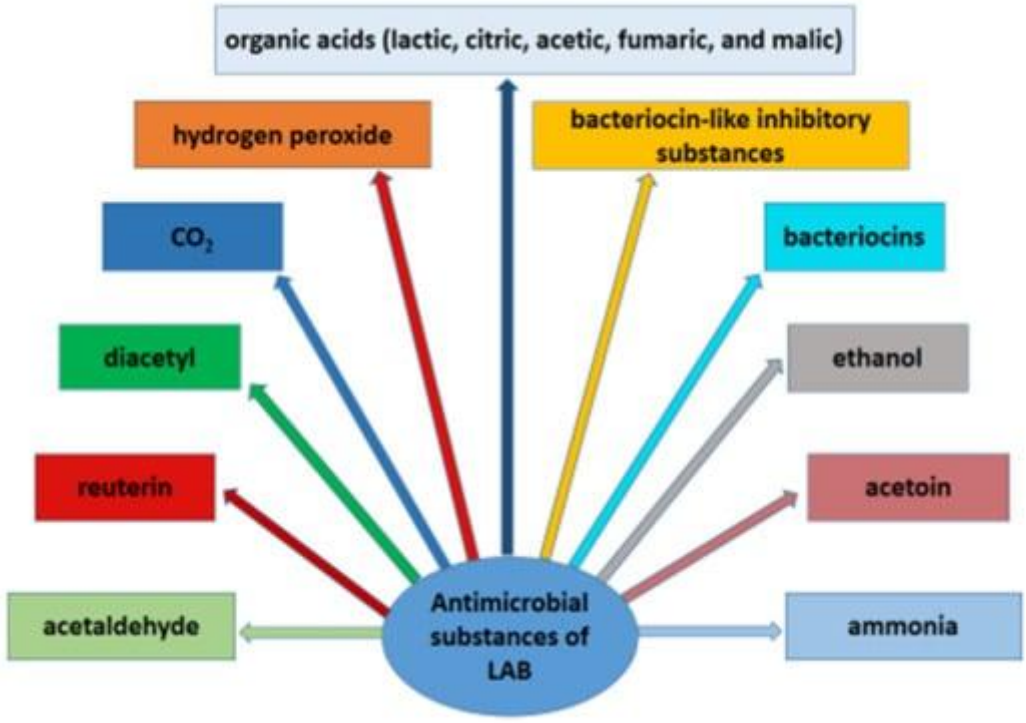


Figure 3: Antimicrobial substances produced by lactic acid bacteria (**Agriopoulou et al., 2020)**.

I.1.7. Lactic Acid Bacteria: Enhancing Immunity and Preventing Cancer

The immune system is comprised of the acquired and innate immune systems, working together to combat invading viruses and pathogens. Recent studies have emphasized the crucial role of dendritic cells (DCs) in connecting innate and adaptive antiviral immunity. DCs are particularly abundant in epithelial surfaces, such as the skin and mucosal linings of the digestive, respiratory,

and urogenital tracts, serving as the first line of defense against pathogens, especially viruses. When these protective barriers are breached, DCs capture and activate pathogens, subsequently migrating to lymphoid organs to initiate specialized immune responses. Mucosal immunity refers to the ability to induce a protective immune response within the mucosal surfaces, where pathogens enter and initiate infections. Both animals and humans can elicit systemic and mucosal immunity by recognizing pathogens as foreign entities requiring neutralization. The distinctive feature of mucosal immunity is the production of secretory immunoglobulin IgA (sIgA), which exhibits greater resistance to protease enzymes . To establish protective mucosal immunity, the involvement of various mucosal immune cells is crucial for the production of protective IgA antibodies. This process can be divided into two main stages: the entrance sites, where pathogens adhere to the mucosal surface, and the effector sites, where plasma cells generate antibodies, triggering a local immune response as depicted in figure 4 **(Rajoka et al., 2016).**

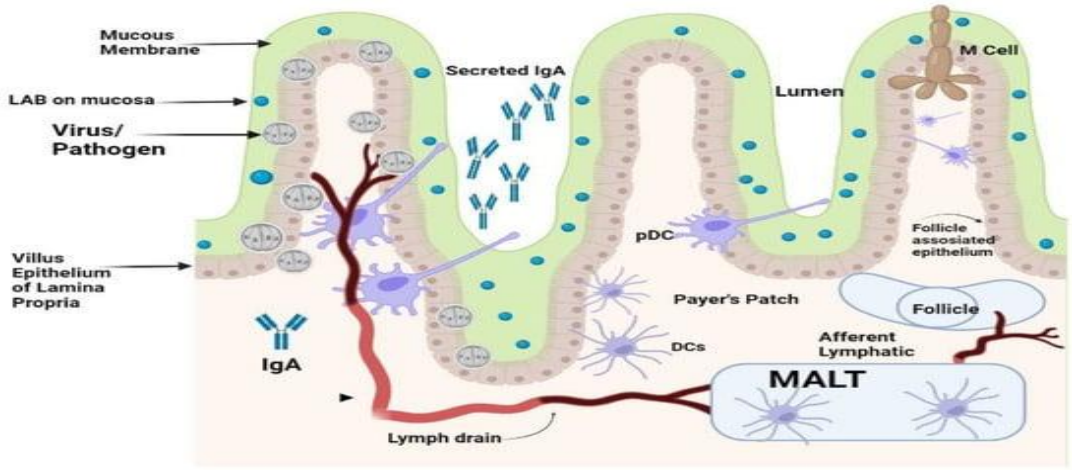


Figure 4: Effect of orally administered LAB on activation of gut-induced mucosal immunity (Rajoka et al., 2016).

Lactic acid bacteria possess the ability to enhance immunity and prevent cancer. Their capacity lies in augmenting the immune system and regulating the production of tumor necrosis factors and interleukins. Through modulating the cell walls via cytokine pathways, these bacteria mediate the host cell immune response. Incorporating highly active lactic acid bacteria strains as supplements offers substantial potential in boosting the host's immunity and providing protection against various diseases, including certain cancers **(Rajoka et al., 2016)**.

I.1.8. Lactic bacteria with the role of 5-hydroxytryptophan synthesis regulation alleviates the symptom of depression

Gut microbiota, consisting of a vast number of bacterial cells (ranging from 10^{14} to 10^{15}) across over 1000 species, plays a crucial role in maintaining a balanced microecology within our bodies. However, disruptions in this delicate ecosystem have been linked to various diseases, particularly psychiatric and neurological disorders like autism, neurodegenerative diseases, and schizophrenia. Interestingly, these imbalances often coincide with gastrointestinal issues and gut microbial dysbiosis.

Research, both in animals and clinical studies, has provided compelling evidence supporting the significant influence of gut microbiota on mood regulation.

Taking advantage of this knowledge, dietary manipulation aimed at targeting the gut microbiota has emerged as an innovative approach to alleviate mood disorders. One such example involves the administration of fructo-oligosaccharides and galacto-oligosaccharides, which have shown the ability to alleviate abnormal behaviors in mice induced by chronic stress. This effect is achieved through the regulation of gut microbiota and the modulation of gene expression related to neurotransmission in the brain.

Directly nurturing the gut through the use of lactic bacteria, specifically strains belonging to the *Lactobacillus* and *Bifidobacterium* genera, has also demonstrated promising anxiolytic and antidepressant effects. The mechanisms underlying these effects involve various factors, including neurotransmission, inflammation, intestinal permeability, hypothalamic-pituitary-adrenal (HPA) axis function, and gut microbiota composition. Some specific strains, such as Lactobacillus helveticus *R0052* and *Bifidobacterium* longum *R0175*, with documented clinical efficacy, have been successfully introduced to the market.

The researchs suggests that the manipulation of diet to target the gut microbiota presents a novel and promising approach for alleviating mood disorders. Whether through the administration of specific oligosaccharides or probiotic strains, the modulation of gut microbiota has shown potential in influencing mood-related behaviors and could offer new therapeutic possibilities in the future as portrayed in figure 5 **(Tian et al., 2019)**.

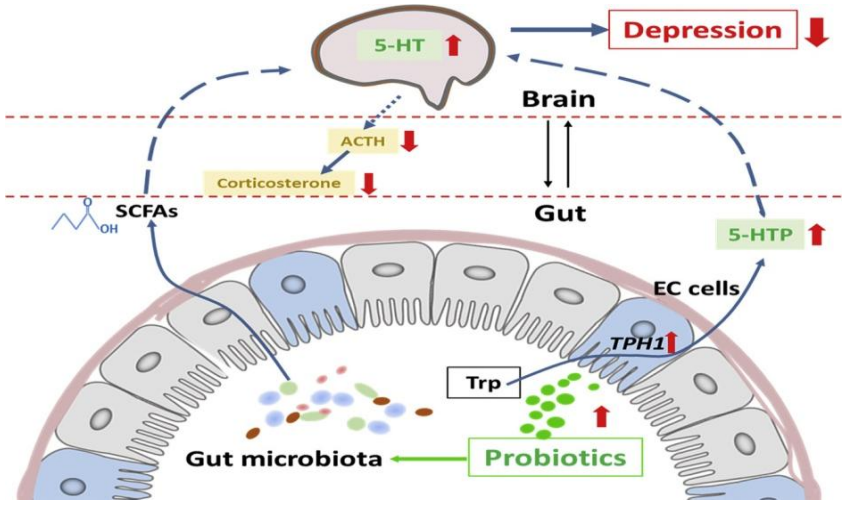


Figure 5: Effect of lactic bacteria to the neurogical system **(Tian et al., 2019)**.

I.2. Probiotics

I.2.1.Background

The term "probiotic" originates from Greek and translates to "for life." It was initially introduced in 1965 by Lilley and Stillwell to describe substances produced by one microorganism that stimulate the growth of another. In 1974, Parker modified this definition to encompass "organisms and substances that contribute to the balance of intestinal microbes" . The present definition of probiotics, as stated by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), refers to " live microorganisms that, when administered in adequate amounts, confer a health benefit to the host".

For probiotic organisms to exert their maximum therapeutic effects, they must possess specific characteristics. Some of these characteristics are considered crucial for probiotics to exhibit therapeutic efficacy. These include the ability to withstand the acidic conditions of the stomach and the presence of bile salts, the capacity to adhere to the intestinal mucosa, and the capability to colonize the intestinal tract **(Rao, 2016)**.

I.2.2. The origin of probiotics

Probiotics, which are beneficial microorganisms, can be obtained from various sources such as fermented non-digestible carbohydrates, food supplements, dairy-based compounds, non-dairy fermented foods, and non-intestinal sources. These strains can be isolated, screened, identified, and characterized from a wide range of natural substrates. Over the years, the sources of potent probiotic strains have significantly expanded and continue to grow.

Recent research conducted by a team of scientists has provided additional support for this concept. Furthermore, individuals have shown a growing interest in consuming live cell-containing foods due to their ability to enhance the nutritive quality and bioavailability of micronutrients, as well as their antioxidative properties. The antioxidative properties of these foods assist in combating oxidative stress, strengthening the host's antioxidative defense mechanisms, and delaying the aging process. Similarly, the improved bioavailability of micronutrients and their antioxidative properties contribute to these beneficial effects.Therefore, incorporating probiotic foods into one's diet can effectively meet the needs and interests of individuals of all ages, as they offer a multitude of benefits related to nutrition, antioxidative defense, and the promotion of healthy aging.This concept is supported by **Abatenh et al. (2018)**.

I.2.3. Characteristics of a good probiotics

In various research studies, probiotics have been identified to possess unique potential properties. To be considered suitable and selected as probiotics, they must meet certain criteria encompassing safety, technological aspects, and functional characteristics. These criteria include:

1. Beneficial Effect: Probiotics should have the ability to confer beneficial effects on the host animal by enhancing resistance to diseases.

2. Human Origin: Probiotics should originate from human sources.

3. Viability: Probiotics must exhibit high cell viability, ensuring their survival and effectiveness.

4. Non-pathogenic and Non-toxic: Probiotics should be non-pathogenic and non-toxic to ensure safety.

5. Immune Modulation: They should be capable of interacting with the immune system and exert immune modulatory activity.

6. Metabolic Activity: Probiotics should possess the capacity to influence local metabolic activity.

7. Gastrointestinal Survival: Probiotics should be able to survive and function in the gastrointestinal environment, including resistance to low pH and organic acids.

8. Stability: Probiotics must be stable, safe, and effective, with the ability to maintain viability during storage and under field conditions.

9. Microbiota Restoration: They should have the potential to restore and replenish the intestinal microflora.

10. Additional Benefits: Probiotics may exhibit additional beneficial activities such as anti-carcinogenic and anti-mutagenic effects, cholesterol-lowering properties, maintenance of mucosal integrity, and enhancement of bowel motility.

11. Digestive Tract Interaction: Probiotics should be able to facilitate and colonize the digestive tract.

12. Resistance to Digestive Juices: They must possess the ability to withstand the effects of gastric juices and bile acid, enabling successful oral administration.

13. Adhesion: Probiotics should be capable of adhering to mucosal and epithelial surfaces, aiding immune modulation, competitive exclusion of pathogens, and prevention of pathogen adhesion and colonization.

14. Antimicrobial Activity: Probiotics should exhibit antimicrobial activity against pathogenic bacteria.

15. Bile Salt Hydrolase Activity: They may possess bile salt hydrolase activity.

16. Antibiotic Resistance: Probiotics may exhibit antibiotic resistance, enabling survival in the presence of administered drugs and other antimicrobial compounds.

17. Colonization: They should demonstrate the ability to multiply rapidly and colonize either temporarily or permanently within the gastrointestinal tract.

18. Microflora Stabilization: Probiotics should contribute to the stabilization of the intestinal microflora and should not be pathogenic.

19. Gastrointestinal Survival: They should survive passage through the gastrointestinal tract, enduring low pH and exposure to bile **(Abatenh et al., 2018).**

I.2.4. Mechanism of action

The precise mechanisms through which probiotics exert their beneficial effects have not been extensively documented. Nonetheless, there are several proposed mechanisms that account for many of their favorable actions as depicted **in the figure 6.**

One such mechanism involves competition for adhesion sites, whereby probiotics contend for cellular attachments. Effective colonization by various pathogenic organisms often necessitates their association with the epithelium of the gastrointestinal (GI) tract . However, specific strains of *bifidobacteria* and *lactobacilli* can adhere to the epithelium and act as "colonization barriers," impeding the attachment of pathogens to the mucosa . Studies have demonstrated this effect using *Lactobacillus rhamnosus* strain GG and *Lactobacillus plantarum 299v*, which both exhibited the ability to inhibit the attachment of Escherichia coli to human colon cells **(Abatenh et al., 2018).**

Another potential mechanism of action involves the modification of the microbial flora by synthesizing antimicrobial compounds. Many types of *lactobacilli* and *bifidobacteria* produce bacteriocins and other antimicrobial compounds. Bacteriocins are defined as "compounds produced by bacteria with a biologically active protein component and bactericidal properties". Lactic acid bacteria also release other biologically active compounds such as hydrogen peroxide, diacetyl, and

short-chain fatty acids. The release of these compounds by probiotic organisms leads to beneficial alterations in the microflora. However, it is important to note that not all strains of lactobacilli or bifidobacteria produce antimicrobial compounds, and some produce compounds that have fairly nonspecific activity, potentially impacting beneficial bacteria along with pathogenic organisms.

Additionally, probiotics have been observed to stimulate the immune response. This immune response may manifest as increased secretion of immunoglobulin-A (IgA), elevated numbers of natural killer cells, or enhanced phagocytic activity of macrophages. The increased secretion of IgA may lead to a decrease in the number of pathogenic organisms in the gut, thereby improving the composition of the microflora. Due to these immunomodulating effects, some researchers speculate that probiotics may not only combat intestinal and urogenital pathogens but also prove beneficial for conditions such as inflammatory bowel disease (IBD), pouchitis, food allergy, and as an adjuvant to vaccination. Probiotics may also compete for nutrients that would otherwise be utilized by pathogens . This scenario occurs with Clostridium difficile, a potentially pathogenic organism that relies on monosaccharides for its growth. Probiotic organisms in sufficient quantities can consume most of the available monosaccharides, thereby inhibiting the growth of C. difficile .The action mechanism of probiotics can be summarized in three steps:

- They stimulate and modulate the immune response.
- They normalize the intestinal microflora by promoting colonization resistance and controlling irritable bowel syndrome and other inflammatory bowel diseases.
- They also exert metabolic effects such as bile salt deconjugation and secretion, lactose hydrolysis, and reduction in toxigenic and mutagenic reactions in the gut, while supplying nutrients to the colon epithelium **(Abatenh et al., 2018).**

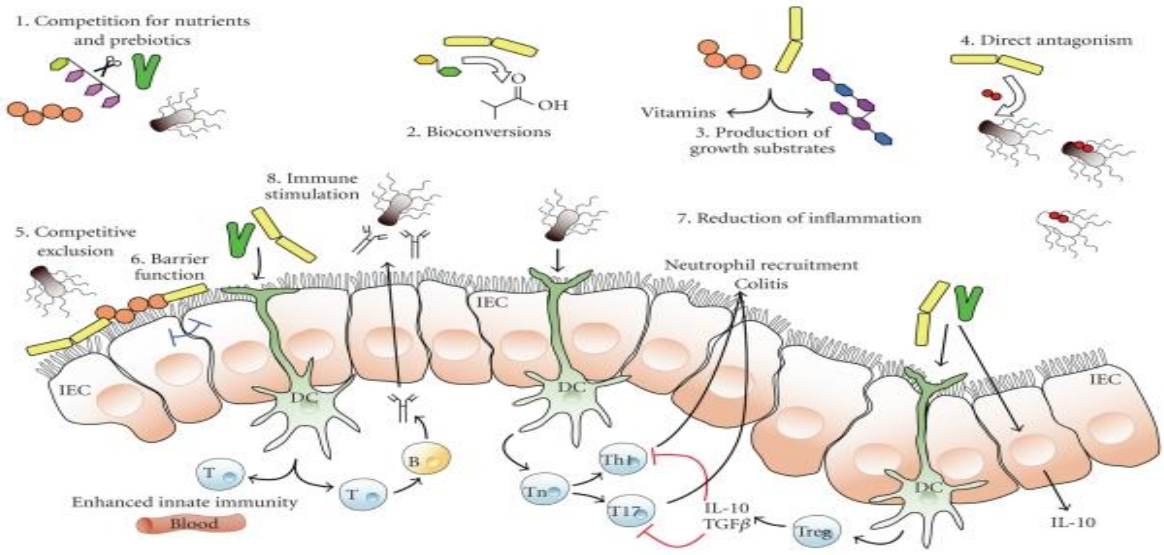


Figure 6: Schematic diagram illustrating potential or known mechanisms whereby probiotic bacteria might impact on the microbiota. These mechanisms include (1)

competition for dietary ingredients as growth substrates, (2) bioconver- sion of, for example, sugars into fermentation products with inhibitory properties, (3) production of growth substrates, for example, EPS or vitamins, for other bacteria, (4)direct antagonism by bacteriocins, (5) competitive exclusion for binding sites, (6) improved barrier function, (7) reduction of inflammation, thus altering intestinal properties for colo- nization and persistence within, and (8) stimulation of innate immune response (by unknown mechanisms). IEC : intra epithelial cells, DC: dendritic cells, T:T-cells **(Rao, 2016)**.

I.2.5. Classification

There are many different microorganisms currently used as probiotics (**table 2**). Tobetter understand how bacteria are named and classified, the following discussion may be helpful. Genus is the first name of a bacterium (e.g., *Lactobacillus*). It is somewhat general and refers to a grouping of organisms based on similarity of qualities, such as physical characteristics, metabolic needs, and metabolic end products.

Species is a bacterium's second name (e.g., *acidophilus*). It is a much more narrow classification based on shared common characteristics that distinguish them from other species. Strain is an even more specific classification that divides members of the same species into subgroups based on several properties that these bacteria have in common that are distinct from other members of the species (e.g., strain LA5) **(Rao, 2016)**.

Table 2: Common probiotic microorganisms **(Rao, 2016).**

Lactobacillus spp.	*acidophilus*
	plantarum
	rhamnosus
	paracasei
	fermentum reuteri
	johnsonii brevis
	casei
	lactis
	delbrueckii gasseri
Bifidobacterium spp.	*breve*
	infantis longum
	bifidum thermophilum
	adolescentis animalis
	lactis

Bacillus spp.	*Coagulans*
Streptococcus spp.	*Thermophilus*
Enterococcus spp.	*Faecium*
Saccharomyces spp.	*Cerevisiae*

I.2.6. Commercial forms

Probiotic organisms can be ingested in two main forms: fermented foods and supplements. Fermented foods include yogurt (dairy origin) and sauerkraut (vegetable origin) and are commonly known examples. Probiotic supplements are available as lyophilized (freeze-dried) bacteria, offered in powder, capsule, or tablet form. To achieve therapeutic effects, it is essential for probiotic products to contain live organisms in sufficient numbers. Both fermented foods and supplements fulfill this requirement. **Table 3** compares the advantages and disadvantages of common probiotic delivery systems **(Rao, 2016)**.

Table 3: The pros and cons of different probiotic delivery systems **(Rao, 2016)**.

Delivery system	Pros	Cons
Fermented dairy	-Affordability and easy Availability -Ease of incorporation into daily patterns -Additional nutritional benefits -Enhanced bacterial survival through upper GI tract (100× less bacteria can be given per dose) -Effective in the upper GI tract	-Contains dairy proteins and lactose -Taste can be issue -Not suitable when travelling -Not suitable for vegans
Capsules	-Ease of administration -Contain no binders	-Not therapeutic in upper GI tract (unless opened or chewed) -May contain allergenic excipients -Higher cost
Tablets	-Ease of administration -Effective in the upper GI tract	-May contain allergenic or otherwise problematic binders and excipients (e.g., gluten) -Higher cost
Powders	-Effective in the upper GI tract -Dosages can be easily adjusted -Can be incorporated into foods or drinks -Contain no binders	-Higher cost

I.2.7. Health Benefits of Probiotics

Probiotics, including dairy strains of lactic acid bacteria (LAB), have been widely utilized throughout history. Humans have been consuming LAB, such as various types of *Lactobacillus* and *Enterococcus* species, on a daily basis since the advent of fermented milk as a dietary staple. It's important to note that the effects of probiotics are specific to each strain. Therefore, the benefits attributed to one strain cannot be directly generalized to others, as each individual probiotic strain offers its own distinct health advantages. These strains have been associated with numerous beneficial effects in relation to various disease conditions. Probiotics hold significant importance and find application in the control of various types of microbial infections. They are applicable to enhancing human health, managing infections, and treating diseases (as indicated in **table 4**) **(Abatenh et al., 2018).**

Table4: . Role of probiotics in health improvement, infection control and disease treatment **(Abatenh et al., 2018).**

Probiotic Strains	**Types of diseases or disorder**	**Probiotic outcomes/results**
Oxalobacter formigenes Lactobacillus and Bifidobacterium species, (Lactobacillus plantarum PBS067, Lactobacillus acidophilus LA-14, Bifidobacterium breve PBS077, Bifidobacterium longum PBS078)	Kidney/Urinary stones	**(i)Modify or utilize several types of urinary stone. (ii) Act as a key tool to manipulate, metabolize and degrade a toxic compound.**
Lactobacillus GG, L. rhamnosus Lactis, Lactobacillus fermentum, Bifidobacterium bifidum, Bifidobacterium lactis, L. acidophilus, L. casei, L. salivarius and Lactococcus lactis	Atopic Diseases	**(i) Atopic eczema reduction is observed, and skin condition also improved. (ii) Atopic dermatitis symptoms are removed from infants who found in moderate-to severe condition. It is mainly depends on the selection of specific probiotic strains, time of administration (on set time), duration of exposure, and dosage.**
L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B.infantis, L. delbrueckii subsp. Bulgaricus, L. reuteri DSM 17938	Colic	**Very effective in reducing colic in breastfed infants and children.**
Lactobacillus, Bifidobacerium* and *L. johnsonii	*Helicobacter pylori* infection	**Destruction the adverse effects of H. pylori through the release of bacteriocins, production of organic acids, and competitive colonization in epithelial or mucosal cells. At that time can hinder its growth, adhesion and bacterial load.**

Lactobacillus rhamnosus, L. rhamnosusGG, B. animalis subsp. lactis alone or in combination with S treptococcus. thermophilus,and L. reuteri, L. rhamnosus (not GG), and L. acidophilus, Saccharomyces boulardii. Lactobacillus casei	**Acute and antibiotic-associated diarrhea**	**(i)Competitive blockage of receptor site signals regulating secretory and motility defenses. (ii) Enhancement of the immune response, and production ofsubstances that directly inactivate the viral particles. (iii)Inhibit the growth by preventing adhesion and invasion of pathogens.**
Lactobacillus rhamnosus, Lactobacillus reuteri,Propionibacterium freudenreichii	***Candida* infection**	Used as a therapeutic option to combat fungal pathogen.
Bifidobacterium species, Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus casei, Lactobacillusrhamnosus, Streptococcus thermophiles, Lactobacillus acidophilus, Lactobacillus bulgaricus	Constipation	**(i)Altering microflora and restoring disturbed community in side GIT, (ii) Participating and solving undesired gastro intestinal problems. (iii)Improving/managing whole gut transit time, stool frequency and consistency.**
L. acidophilus, L. plantarum, L. casei, B.lactis, S cerevisiae	Irritable bowel syndrom	(i) Reduction of irritable bowel syndrome symptoms. (ii) Effective in alleviatingand managing symptoms of this unpleasant condition.
L. GG, L. casei Shirota, L. acidophilus, B. bifidum, L. rhamnosus, L. plantarum* and *L. paracase, Streptococcus salivarius, Bifidobacterium animalis subsp. Lactis	Acute viral upper respiratory infections	**(i)Colonizing epithelial cells and keep away from adherence of pathogens. (ii)Create adhesion, binding sites, nutrients and space competition lastly able to avoid risk of upper respiratory track completely.**
B. animalis* subsp. *Lactis, L. lactis* subsp. *Lactis	Modulation of gut – brainaxis	(i) Modulation of brain activity and Provide mental health.(ii) Maintaining the functionality of the central nervous system through metabolic, neuroendocrine and immune pathways.(iii) Contribute to the early development of normal social and cognitive behaviors. (iv) Useful strains having positive direct effect on central nervous system and also solve disorders.
Lactic acid bacteria	Colon Cancer	(i) Comprise modification of the metabolic activities of intestinal micro flora and alteration of physicochemical conditions in the colon as well as binding site. (ii) Biodegradation of potential carcinogens. (iii) Production of anti-tumorigenic or mutagenic compounds due to ability to decrease the activity of enzyme called βglucuronidase. (iv) Increasing the host immune response, by alteration in pro-cancerous enzymatic activity of colonic microorganisms.
***Lactobacillus acidophilus* NCFM, *Lactobacillus gasseri* SBT2055, *L. rhamnosus* CGMCC1.3724**	**Diabetes and Obesity**	**(i) Decrease the risk of type two diabetes mellitus and insulin resistance. (ii) By improving and maintaining the metabolic equilibrium of the host then actualweight loss is observed significantly.**

I.2.8. Future Perspectives of Probiotics

In today's era, technological innovations have emerged as effective solutions to address the challenges of probiotic stability and viability. Maintaining the pure and active viability of probiotic cells is crucial for their efficacy in food processing and successful transit through the gastrointestinal tract to reach their intended destination. However, probiotics often experience a loss of function and viability due to exposure to the low pH environment of the stomach and high levels of bile salts in the intestines **(Abatenh et al., 2018).**

To overcome these hurdles, various strategies have been developed, including the induction of sublethal stress, encapsulation techniques, and incorporation into food matrices or carriers. Encapsulation involves creating a protective barrier around probiotics through mechanical or physicochemical processes. This shields them from external conditions and safeguards their viability. Microencapsulation technologies have made significant progress in protecting probiotics from environmental damage by providing a protective outer coating. This advancement allows for the storage of viable bacteria at room temperature and facilitates their inclusion in a wide range of food products.

Popular encapsulation methods such as spray-drying, emulsion, and extrusion techniques have been extensively utilized for producing microcapsules containing probiotics. These methods effectively preserve the viability of probiotic strains and enhance their stability.

Looking ahead, genetic engineering shows promise for further improving the overall characteristics of probiotic strains and achieving desired traits. This approach holds potential for enhancing probiotic performance through targeted genetic modifications **(Abatenh et al., 2018).**

CHAPTER II:

Biology of *Helix aspersa Müller* Snail

CHAPTER II: Biology of *Helix aspersa Müller* Snail

II.1. Background

Helix aspersa Müller, commonly known as the Common Garden Snail, is a pulmonated gastropod belonging to the Phylum Mollusca. This species has successfully expanded its population from its original habitat in Europe to various continents worldwide. Its remarkable adaptability to diverse environments can be attributed to several factors. When faced with hot or dry periods, the snail enters a state of aestivation to prevent dehydration. Similarly, during cold conditions, it undergoes hibernation. To create a favorable microclimate within its shell and survive unfavorable environmental circumstances, *Helix aspersa Müller* secretes a mucus epiphragm **(Ballard et al., 2021).**

Moreover, *Helix aspersa Müller* exhibits simultaneous hermaphroditism, enabling it to exchange both eggs and sperm concurrently during mating. This unique reproductive characteristic significantly increases the likelihood of finding a mating partner, as it can engage in reproductive activities with any other member of its species. In a single breeding season, a snail of this species can engage in mating up to six times and continue reproducing in the following season. These adaptive traits and reproductive capabilities make *Helix aspersa Müller* a highly successful and resilient species in various habitats **(Ballard et al., 2021)**.

II.2. Habitat

Helix aspersa, commonly known as the garden snail, prefers sheltered habitats and is typically found in areas with alkaline soils, such as hedge banks, sea cliffs, quarries, graveyards, urban gardens, and neglected waste grounds. It can also be found in deciduous woodlands **(Cowie, 2015)**.

II.3. Nutrition

Helix aspersa with its radula mouthparts, is a versatile grazer that consumes a wide range of food. Its digestive enzymes enable the breakdown of cellulose, hemicellulose, and xylane, while gut microflora further aids digestion. While it primarily selects green plants, it also grazes on flowers, fruits, dead animal tissue, and paper products. Feeding primarily occurs at night with sufficient humidity. The snails' diet varies with the season and plant availability, influenced by taste and chemoreception. Calcium-rich plants are preferred, while plants high in metals are avoided. Humus consumption and diet preferences change as the snails progress from hatchlings to adults. In snail farms, a dry food composed of cereal flour, vitamins, and calcium is commonly used, and a laboratory mix of calcium carbonate, dried milk powder, and breakfast cereal is effective for rearing snails **(Cowie, 2015).**

Table.5: *Helix aspersa Müller* species profile: Terrestrial Invertebrates Species Profiles - Museums Field Guide

Brief description	**Round shell with spiral, soft body.**
Description	Body grey-brown. Shell brown with dark brown or black lines and yellow streaks, circular with 4-5 whorls. Shell up to 4 cm across.
Biology	Garden Snails are hermaphrodites but reproduce sexually. They lay clutches of up to 80 eggs at a time and may lay up to six clutches a year under favourable conditions. Garden Snails were introduced into Tasmania to provide food for birds, such as the European thrush and blackbird. They feed on a wide variety of plants, including fruit trees and vegetable crops as well as garden flowers and are considered major pests by many gardeners. Some are caught for the restaurant escargot trade.
Habitat	Among groundcover plants in damp areas.
Native status	Introduced to Australia
Diet	Herbivore
Colours	brown grey
Distribution	Europe, Africa, North and South America, New Zealand. Eastern mainland Australia and Tasmania.
Habitat types	Terrestrial
Commercial species	True

II.4. Description

The adult shell of *Helix aspersa* typically measures between 25 and 40mm in diameter and 25 to 35mm in height. It is a hard shell with four or five whorls, displaying various shades of dark brown and yellow with flecks or streaks **(CABI, 2020)**. In terms of weight, adults usually weigh around eight to 12 grams, with the preferred weight for commercial purposes being 10 grams **(Snails Pace report)(Figure7)**.

While the exact lifespan of the garden snail is not extensively documented, it is believed to range from three to five years in the wild and up to 10 years in controlled environments **(CABI, 2020)**.

The physiology of this species consists of a head, a foot, and a visceral mass. The head is equipped with two pairs of tentacles: a larger pair containing the eyes and a smaller pair used for tactile interaction with the environment. Below the tentacles lies the mouth, and the genital orifice is situated on the right-hand side behind the head. The foot serves both as a means of locomotion and as support for the weight of the shell. The snail's slime, also known as "filtrate," aids in body protection and facilitates movement. Within the visceral mass, various organs are responsible for respiration, reproduction, digestion, and circulation.

Helix aspersa has the ability to seal the shell opening using a dried mucous membrane called an epiphragm. This adaptation allows the snail to hibernate in cold conditions or aestivate in dry conditions. The epiphragm helps retain moisture and provides protection against predation. In adverse conditions, *Helix aspersa* can enter a state of hibernation or aestivation for several months until conditions improve.

The snail can move at a speed of up to 1.3 centimeters per second, which is approximately 47 meters per hour. It is important to note that the energy cost associated with locomotion, known as the cost of locomotion, is significant for gastropods like *Helix aspersa* and can limit their dispersal ability **(CABI, 2020)**. As a result, unless necessary, these snails do not venture far from suitable food sources.

Nevertheless, human activities, such as the transportation of nursery stock and other plant materials, can inadvertently transport *Helix aspers*a over significant distances. Additionally, this species has been deliberately introduced into many regions around the world for the purpose of snail meat (escargot) and snail caviar production, as well as by hobbyists who collect snails **(CABI, 2020)**.

Figure 7: *Helix aspersa Müller* Snail **(Turmo Gort, 2023).**

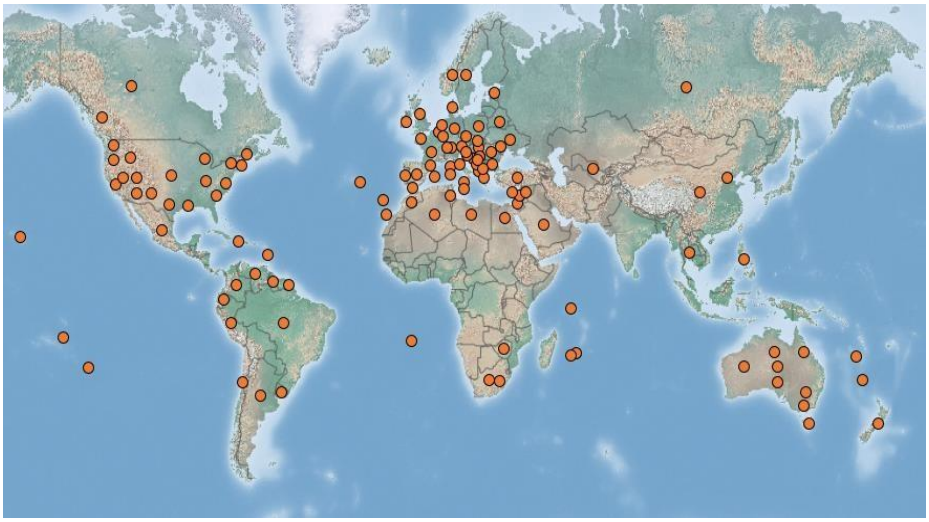
II.5. Taxonomy

- Scientific Name: *Helix aspersa* (Müller, 1774)
- Family: *Helicidae*
- Genus: *Cornu*
- Species: *Cornu aspersum* (Müller , 1774)
- Common Names: Common garden snail, Brown garden snail, European brown garden snail.
- Synonyms: *Cornu aspersum (Müller, 1774); Cantarues aspersea (Müller , 1774); Cantareus asperses or Cryptomphalus asperses; Cantareus aspersus* **(GISD, 2020, UCIPM, 2017)**

II.6. Distribution and Endemism

Helix aspersa is believed to be native to North Africa, with its range extending into Europe and Western Asia. It has distinct eastern and western lineages. The Western lineage is considered the ancestral form of most *Helix aspersa* populations found in Europe.

During the 1800s, live *Helix aspersa* individuals were transported globally due to their significance as a food source. There is also a possibility that snails were unintentionally translocated along with plants and intentionally by collectors **(CABI, 2020)**. *Helix aspersa* has successfully established populations in various human-disturbed landscapes and is recognized as a significant agricultural pest in regions where it has been introduced and become naturalized **(refer to Map 1, CABI, 2020)**.



Map. 1: Distribution map from CABI (Centre for Agriculture and Bioscience International) 2020.*Helix aspersa.*

II.7. Uses List

- General > Laboratory use
- General > Pet/aquarium trade
- General > Research model
- General > Sociocultural value
- Materials > Cosmetics
- Medicinal, pharmaceutical > Source of medicine/pharmaceutical
- Human food and beverage > Eggs
- Human food and beverage > Meat/fat/offal/blood/bone (whole, cut, fresh, frozen, canned, cured, processed or smoked) **cowie2015**.

II.8. *Helix aspersa* as food

Snails, specifically gastropods, have been utilized by humans for both culinary purposes and as a treatment for various ailments. Archaeological evidence, such as fossil remains found in caves, suggests that snails have been enjoyed as a delicacy for thousands of years. Cultivating

snails is relatively easy, as they consist mainly of edible muscle tissue. These creatures are a valuable source of protein and contain essential amino acids necessary for our health.

Throughout history, snails have been highly regarded as a sought-after food. They are particularly favored as a food source in certain regions of Africa, Asia, and South America. In more recent times, their consumption has gained popularity in European countries. Unfortunately, the increased demand for snails as food has led to a decline in their natural populations. This decline has exposed them to predation and the introduction of pathogens, which have had detrimental effects on their productivity.

It is worth noting that inedible parts of snails also find use in the preparation of animal feed, as depicted in **figure 8 (Dar et al., 2017).**

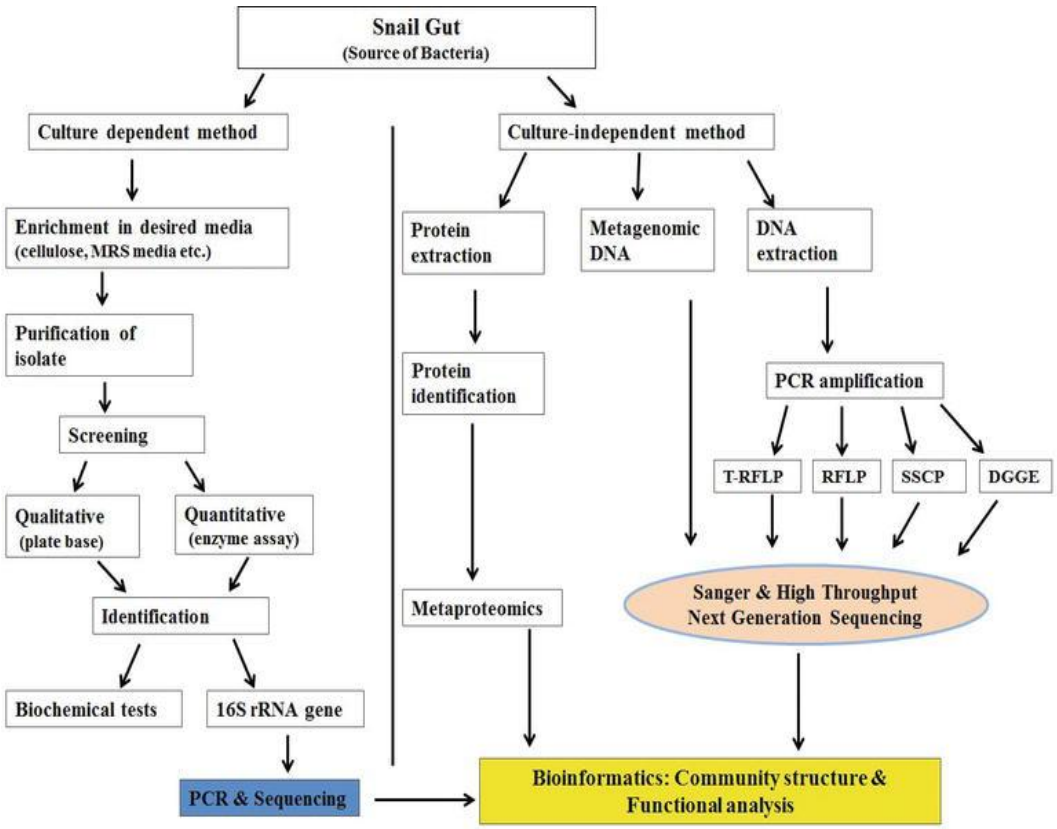


Figure 8: Different methods involved in the isolation and identification of microbes of the snail gut **(Dar et al., 2017)**.

II.9. Role of the gut bacteria in the garden snail

Gut bacteria play a crucial role in the digestive process of snails. Snails that are herbivores and feed on lignocellulosic plant material have certain adaptations in their gastrointestinal tracts. These adaptations include an enlarged digestive tract and a diverse gut microbiota. The digestive tract of these snails is long and consists of various regions such as the esophagus, crop, rumen, caecum, and rectal paunch **(Dar et al., 2017)**.

The gut microbes present in these snails provide them with a specialized set of enzymes that are necessary for the digestion of plant materials. The guts of these herbivorous snails act as natural bioreactors, facilitating the breakdown of plant biomass. This makes them efficient sources of industrially important bacteria. The digestion of plant biomass is vital for these snails to obtain energy. Therefore, the bacterial flora present in their gastrointestinal tract plays a crucial role in the digestion process. The specialized regions of their GI tract create unique microenvironments that can harbor distinct bacterial communities. These bacteria are adapted to the specific conditions within the snail's gut and contribute to the efficient digestion of plant material **(Dar et al., 2017)**.

II.10. Abundance of bacterial symbiont in snail

In the last century, scientists have focused their attention on studying microbes that produce enzymes capable of breaking down cellulose. They have isolated cellulases from the gut of a land-dwelling snail called *H. pomatia*, thanks to the pioneering work of Seillière. Other researchers have studied cellulases, and others discovered that chitinases derived from microbes in *H. pomatia* played a significant role in digesting plant components in all plant-eating snails.

In 2006, Charrie and his group of reseachers observed that the density of bacteria in the distal intestine of snails like *C. aspersum* and *H. pomatia* was as high as 5.10^9 colony-forming units (CFU) per gram of fresh tissue, while in the proximal region, it was 10 to 1000 times lower. *H. pomatia* had the lowest colonization by bacteria. *Helix aspersa*, when fed with carboxymethyl cellulose (CMC), harbored approximately 10^7 bacteria per gram, whereas those fed with native cellulose contained 106 bacteria per gram . In another study conducted by the same authors under aerobic and anaerobic conditions, it was found that gram-positive bacteria numbered around $1.57 \times 10^9 \pm 0.10 \times 10^9$ CFU per gram in the intestine. Gram-negative aerobic bacteria accounted for $5.77 \times 10^8 \pm 1.35 \times 10^8$ CFU per gram in the intestine but represented only 27% of the total bacterial load in *H. aspersa* . However, Simkiss (1985) observed only 0.71×10^6 CFU per gram of body weight in *H. aspersa* . In a similar report, researchers noted less than 10^6 bacteria growing on sterile paper. In the intestine of *Tegula funebralis*, the number of culturable bacteria was only 25×10^5 **(Dar et al., 2017).**

Various strains capable of growing on chitin have been isolated from different snail species such as *C. gillenii, B. agrestis, B. noackiae, and E. malodoratus*. Researchers reported the presence of *chitinolytic* bacteria in *H. pomatia,* with bacterial density ranging from 10^6 CFU per gram of tissue. **Pawar et al. (2012)** used a culture-dependent method and found bacterial counts ranging from 103

to 106 CFU in the entire gastrointestinal tract of *A. fulica* (**Koleva et al.,2017)**, while studying the gut bacteria of *H.aspersa*, found that bacterial diversity varied during different stages of the life cycle, with a maximum count of 1.6×10^9 CFU per ml of gut extract during the active stage. It's important to note that more than 95% of bacteria in any environment, including animal guts, are not easily cultured, and their composition and community structure cannot be fully understood through culture-dependent methods. While most of these studies have utilized culture-dependent approaches, they may not have fully revealed the bacterial composition and community structure in the snail's gastrointestinal tract. Further research is needed to study the bacterial diversity in snails using advanced computational and metagenomic approaches that can tap into the vast microbial diversity present in snail guts. Only a few studies have employed metagenomic methods to analyze bacterial populations in snails. The complete details of the processes and protocols involved in isolating and identifying gut microbes are beyond the scope of this chapter, but a brief overview of these methodologies is provided in **figure 9 (Dar et al., 2017)**.

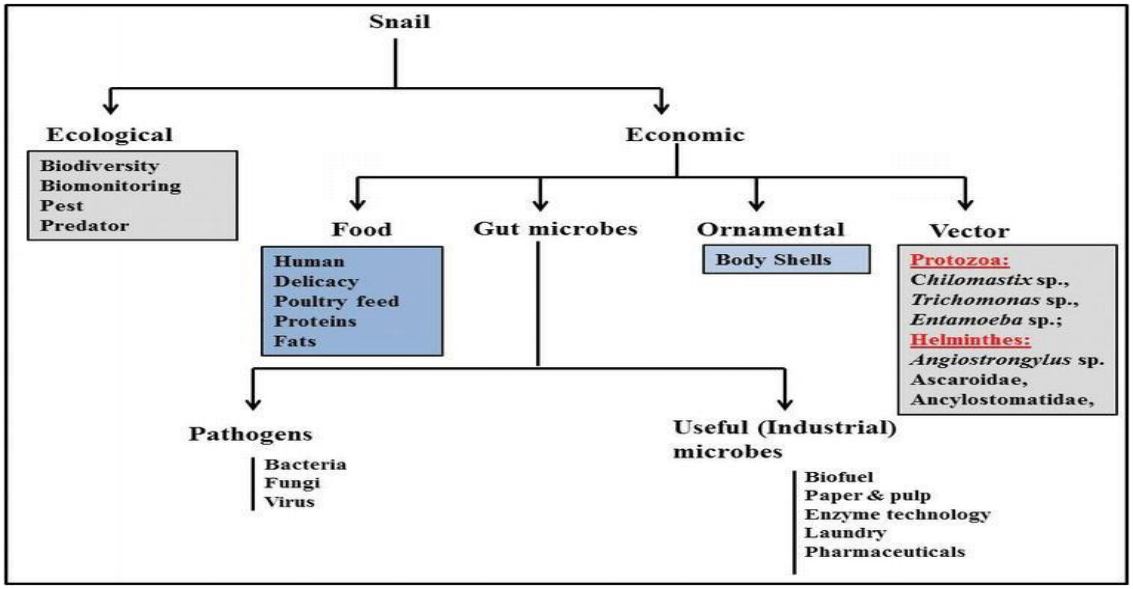


Figure.9: Ecological, economic and industrial utility of snails (**Dar et al., 2017)**.

CHAPTER III:

Materials and Methods

CHAPTER III: Materials and Methods

III.1. Objective and Workplace

This work was carried out at the Laboratory of Beneficial Microorganisms, Functional Foods & Health (LMBAFS) of the university during the period between February and June. The study aimed to investigate and evaluate the survival of different strains of lactic acid bacteria (S1, S2, S3, S4, S5, S6) isolated from the intestine of freshly caught *Helix aspersa Müller* Snails obtained from the market in the city center of Mostaganem, Algeria.

III.2. Sampling

In this study, samples of *Helix aspersa* snails intestines were collected following thorough washing and aseptic wiping. One gram of these intestinal samples was then processed by grinding them in a sterile flask with 9 ml of peptone water and homogenizing the mixture using vortexing. The homogenized mixture was subsequently left at room temperature for 24 hours, allowing for the assessment of any potential bacterial growth or survival.

Figure 10: The common garden snail: *Helix aspersa Müller*.

Figure 11: Digestive tract in a Petri dish

Figure 12 : Mother solution

III.3. Dilution preparation

1 ml of the mother solution was taken and dissolved in 9 ml of peptone water. Then, a dilution series was prepared from the homogenate (10^{-1}) to a dilution (10^{-7}).

III.4. Culture media used

In our work, we used two types of media for the isolation and identification of strains, solid and liquid media:

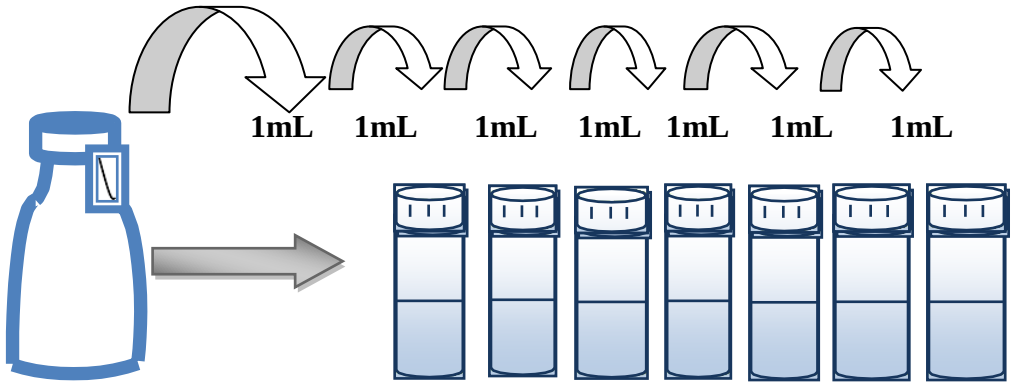
• **MRS agar:** used for the culture and enumeration of *lactobacillus* bacteria in various tests.

• **MRS broth:** used for the reactivation of *lactobacillus* bacteria.

• **Nutrient broth:** used for the reactivation of pathogenic strains.

• **Peptone water:** used for the enrichment of strains.

The composition and preparation of these media are described in the Appendix.

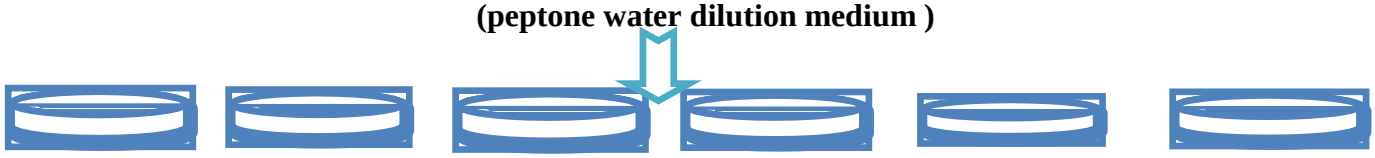


Decimal dilution

9ml of pepton 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} water

+ 1 g of *Helix aspersa* snail intestine in peptone water dilution medium. Incubate at room temperature (24 hours) for enrichment.

(peptone water dilution medium)



- **MRS (incubation at 37°C under anaerobic conditions for 24 to 72 hours) with pH=6 and pH=5.4**
- **M17 Medium .**

- Observation of results.
- Purification and conservation.
- Identification.

Figure 13: A Diagram presenting the protocol for the isolation of lactic acid strains **(Najjari et al., 2007).**

III.5. Isolation of lactic acid bacteria

Once the dilution is made, one hundred microliters (100 uL) of the dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) are taken and spread onto the surface of the Petri dish containing MRS agar at pH 6.2. Incubation is done in an anaerobic jar at 30°C for 24 to 72 hours.

III.6. Purification

After performing the catalase test and Gram staining on the obtained bacteria, only those that were Gram-positive and catalase-negative were kept. One colony was taken from each dish and inoculated in the same medium (MRS agar) by the streaking method. Incubation is done anaerobically at 30°C for 24 to 48 hours.

The purity of the strains is confirmed by homogenous colonies with the same external appearance.

III.7. Identification of strains

III.7.1. Pre-identification of isolates

III.7.1.1. Macroscopic appearance

This study is based on the observation of the culture of isolates on MRS agar to characterize the size, shape, and color of the colonies **(Badis et al., 2005)**.

III.7.1.2. Catalase test

Catalase is a high-molecular-weight enzyme present in all aerobic bacteria, which enables them to live in the presence of oxygen. In addition to the cytochrome respiratory chain, there is a short accessory chain in aerobic bacteria that fixes hydrogen to oxygen, resulting in hydrogen peroxide, which is known for its high toxicity to bacteria. Catalase allows the decomposition of hydrogen peroxide according to the reaction:

$$2\ H_2O_2 \rightarrow 2H_2O + O_2$$

The search for this enzyme is performed simply by bringing a colony in contact with a drop of 10V hydrogen peroxide (H_2O_2). An abundant gas release indicates the presence of catalase. Lactic acid bacteria are catalase negative **(Hassaine, 2012)**.

III.7.1.3. Microscopic Aspect

Microscopic observation at a magnification of (G ×100) allows for the classification of

bacteria according to their Gram stain, cell morphology, and mode of association **(Mami, 2012)**.

III.7.1.4. Gram Staining

This type of staining allows bacteria to be separated into two major categories: Gram-positive bacteria and Gram-negative bacteria. Bacteria that retain gentian violet after washing are called Gram-positive, while those that are decolorized and then take up the color of a second dye are called Gram-negative. This method is based on the difference in the cell wall between the two major groups of bacteria, with a high proportion of lipids in Gram-negative bacteria and a low proportion in Gram-positive bacteria **(Moumene, 2015)**.

A colony was taken from a culture on a plate and spread onto a glass slide. The slide was then air-dried, passed over a flame to fix the sample, and allowed to cool in the open air. After fixation, the slide was placed on a slide holder and stained with crystal violet for 1 minute before being rinsed

with Lugol's solution. The preparation was then covered with Lugol's solution for 1 minute, rinsed with distilled water for about 5 seconds, and then subjected to decolorization with ethanol for about 15 seconds until the spread took on a gray-blue color. The slide was then rinsed with distilled water for about 5 seconds before being stained with safranin for 1 minute, rinsed again with distilled water, and then dried. The slides are examined under an optical microscope (objective 100/ magnification × 100) to differentiate their morphology, arrangement, and Gram type **(Makhloufi, 2011)**.

III.8. Strain Preservation

Two types of preservation were applied, one for short-term and one for long-term storage.

III.8.1. Short-Term Preservation

Short-term preservation of pure strains is performed on an inclined solid medium. After growth at the optimal temperature, cultures are kept at 4°C, and strains are renewed by subculturing every 4 weeks **(Badis et al., 2005)**.

III.8.2. Long-Term Preservation

For long-term preservation, starting from 18-hour cultures (liquid medium), the preservation medium contains 70% bacterial suspension solution and 30% glycerol. Cultures are stored in Eppendorf tubes at -20°C. Prior to use, a frozen culture was reactivated and subcultured on its medium for two successive nights **(Boldus et al., 2006)**.

III.9.Identification of selected strains

- **Physiological, biochemical, and technological tests.**

III.9.1. Physiological test

- **Growth test at different temperatures (15°C and 37°C)**

This test is performed for cocci and bacilli, and it allows differentiation between thermophilic and mesophilic strains. The test is conducted in MRS broth, and growth is assessed by turbidity of the medium after 24 to 72 hours (up to one week) at 15 and 37°C, compared to an unseeded control tube **(Hassaine, 2013)**.

- **Growth test in the presence of 2.5, 4, and 6.5% NaCl**

This test determines whether bacteria have the ability to grow in a high-salt environment, and it can differentiate between enterococci and cocci. The method involves inoculating these bacteria into tubes of MRS medium with 2.5, 4, and 6.5% NaCl and incubating them at 30°C for 24 to 72 hours. After incubation, bacterial growth is indicated by turbidity of the medium, compared to an unseeded control tube **(Hassaine, 2013)**.

III.9.2. Biochemical tests

- **Glucose fermentation test for CO_2 production**

This test allows differentiation between homofermentative and heterofermentative lactic acid bacteria **(Guiraud, 2003)**. It involves detecting the production of gas (CO_2). Young strains that have been prepared beforehand are inoculated into tubes containing MRS broth, along with a Durham tube. After incubation at 37°C for 24-48 hours, the presence or absence of gas in the Durham tube indicates the type of fermentation **(Hariri et al, 2009)**.

Homofermentative strains will produce 90% lactic acid and only 10% CO2, while heterofermentative strains will produce lactic acid and CO2 in equal proportions **(Carr et al, 2002)**.

- **CO_2 production test from citrate**

A series of sterile skim milk tubes (10%) is prepared. 0.5ml of a 10% sodium citrate solution is added to each tube. After shaking, the tubes are allowed to rest for 30 minutes. Each tube is inoculated with 0.1 ml of a young culture and 4 ml of melted and cooled white agar is added. After mixing and solidification, the tubes are incubated at 37°C for at least 3 days. Gas production is indicated by the fragmentation of the agar in the tube **(Sahnouni, 2013)**.

III.9.3. Technological Tests

- **Acetoin Production**

The detection of acetoin is tested using the Voges Proskauer (VP) reaction after 24 hours of culture at 37°C on Clark and Lubs medium (see appendix). Add 5 drops of the VP1 reagent (16% NaOH solution in distilled water) and the same volume of the VP2 reagent (6% alpha-naphthol in 95% alcohol). Shake the tubes carefully and wait for a maximum of 10 minutes. The presence of acetoin is indicated by a pink color on the surface, which can diffuse throughout the medium **(Belarbi, 2011)**.

- **Exopolysaccharide production**

The production of dextran from sucrose is demonstrated on a hypersaccharose gel medium. Dextran-producing strains are characterized by the formation of large, viscous, and sticky colonies. This test is also considered as a key identification tool, allowing for differentiation between dextran-producing and non-producing Leuconostocs **(Mami, 2012)**.

- **Heat resistance test**

Tubes containing 10 mL of liquid MRS are inoculated with isolated strains, then placed in a water bath at 63.5°C for 30 minutes. After rapid cooling, they are incubated at 37°C for 48 to 72 hours. A positive result is indicated by turbidity **(Mahi, 2010)**.

- **Growth test in the presence of methylene blue**

This test indicates the ability of bacteria to grow in the presence of methylene blue, which is blue in highly oxidizing environments and colorless in reducing environments. Each culture to be tested was inoculated in skim milk with methylene blue at 0.1% and 0.3%. After incubation at 37°C for 24 to 48 hours, observations were made regarding the reduction of methylene blue and milk coagulation. *Lactococci* reduce methylene blue with coagulation, while thermophilic streptococci are sensitive to this dye **(Menad, 2017)**.

III.10. Searching for Antimicrobial Activity

a) Direct Method

This method allows testing the inhibitory effect of lactic culture by direct contact with pathogenic strains (table 6). The inhibitory cultures are grown in liquid MRS medium, while the indicator strain is grown in nutrient broth and incubated for 18 hours at 37°C. 21 mL of Mueller-Hinton agar medium is poured into sterile Petri dishes. After solidification of the medium, the dishes are inoculated with the indicator (pathogenic) culture. Then, sterile Wattman paper discs with a diameter of 06 mm are impregnated with MRS broth of the inhibitory strain and placed on the agar.

The cultures are incubated at 37°C for 24 hours, and a positive result is indicated by the presence of inhibition zones formed around the wells **(Zergoug, 2017)**.

b) Indirect Method: Preparation of Active Supernatant

After preparing the lactic pre-culture, it is centrifuged at 5000 rpm for 10 minutes to obtain the supernatant, which is then filtered using a 0.45 µm Millipore filter. The obtained supernatant is divided into two volumes, the first of which is neutralized with 1N NaOH to obtain a pH of 6.50, while the second is left at the initial pH.

The plates are covered with the pathogenic strain initially prepared (OD = 0.08 at 625nm), and sterile Wattman paper discs with a diameter of 06 mm are impregnated with the filtered supernatant and placed on the agar, which was previously inoculated with the pathogenic strain (filtered supernatant, filtered and neutralized supernatant). The prepared plates are incubated at 37°C for 24 hours.

Table.6: List of studied bacterial and fungal strains

Strains	**Code**	**Family**
Escherichia coli	ATCC 25922	*Enterobacteriaceae*
Staphylococcus aureus	ATCC 33862	*Staphylococcaceae*
Bacillus cereus	ATCC 10876	*Bacillaceae*
Pseudomonas aeruginosa	ATCC 27853	*Pseudomonadaceae*
Candida albicans	ATCC10231	*Saccharomycetaceae*

ATCC: American Type Culture Collection.

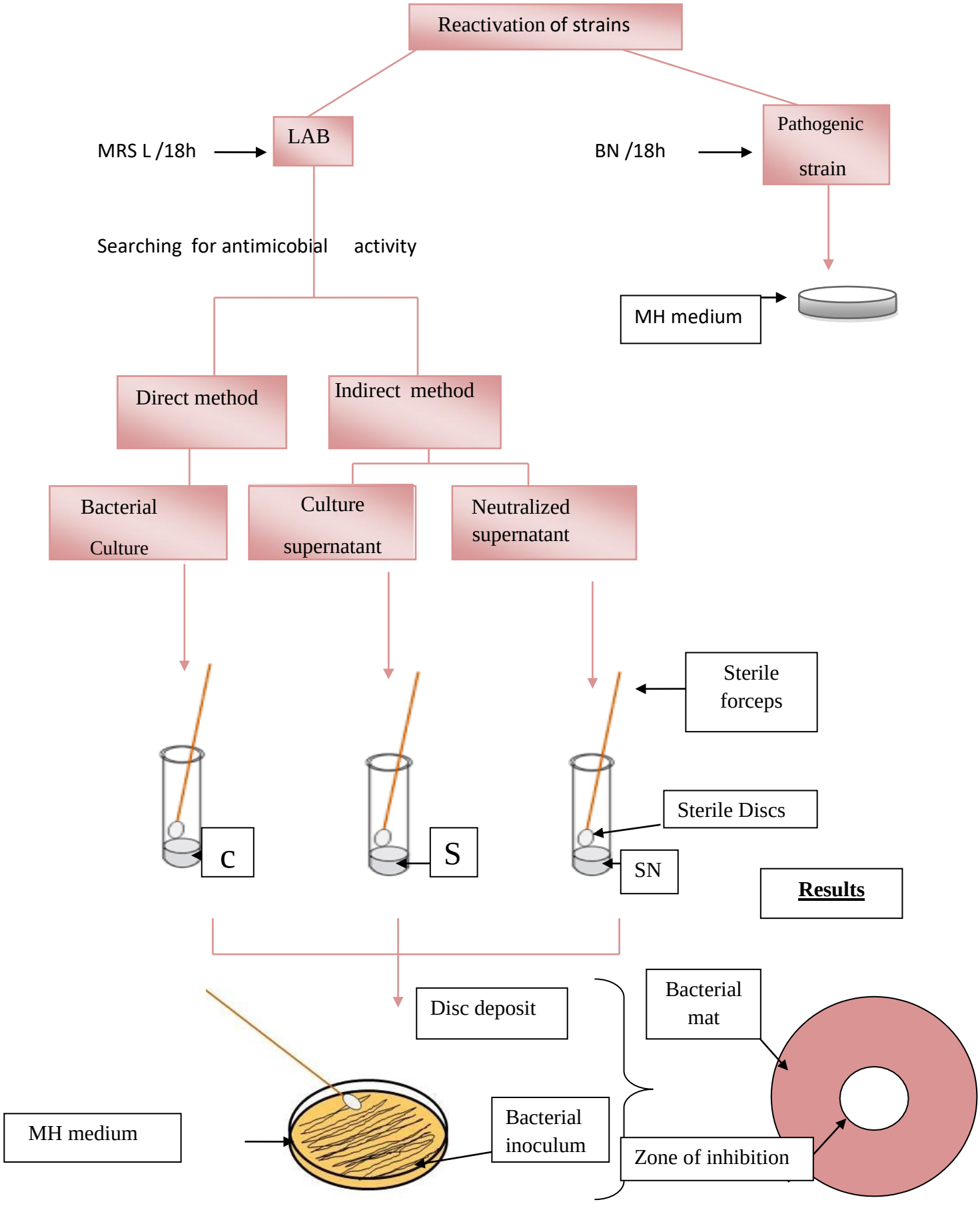


Figure 14: Major steps in the search for antimicrobial activity **(Menad, 2017)**

CHAPTER IV:

Results and Discussions

CHAPTER IV: Results and Discussions

IV.1. Preliminary Identification of Lactic Acid Bacterial Isolates

In this study, I identified strains isolated from the intestine of *Helix aspersa* Müller using conventional phenotypic procedures based on morphological, physiological, and biochemical tests. Further investigations were conducted on isolates demonstrating Gram-positive staining and negative catalase activity.

IV.1.1. Morphological criteria

- **Macroscopic appearance of strains**

During macroscopic examination on solid MRS medium, distinct circular colonies were observed. The colonies appeared convex with a smooth texture and displayed a pristine white color, ranging in diameter from approximately 1mm to 2mm. Another colony variant exhibited an intriguing irregular and eroded morphology, characterized by a creamy hue and a diameter spanning from 1mm to 4 mm. Additionally, a third colony type showcased an appealing convex shape, with a slightly darker cream color and a translucent appearance.

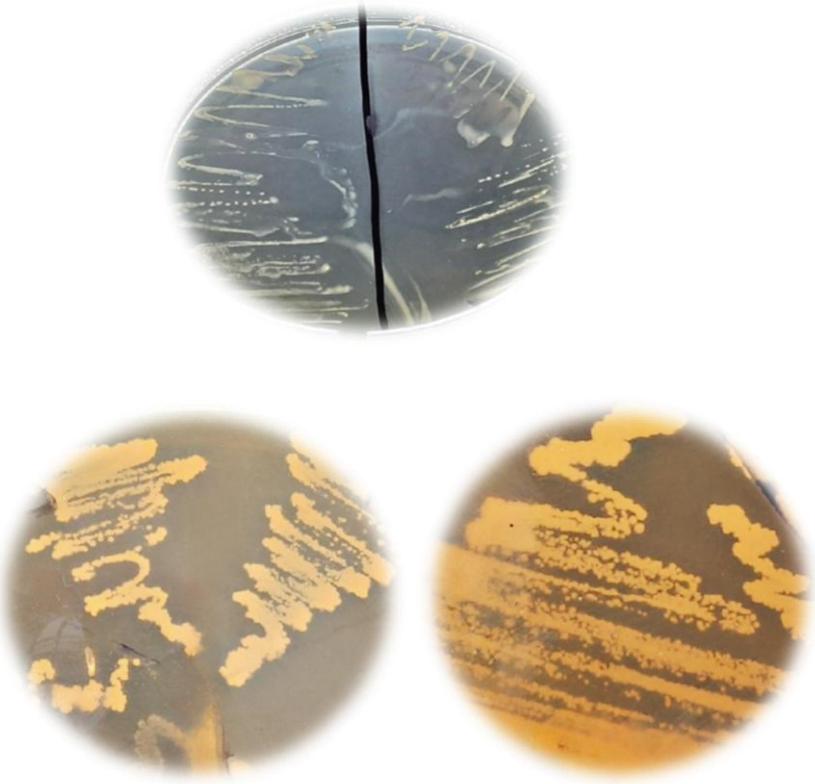
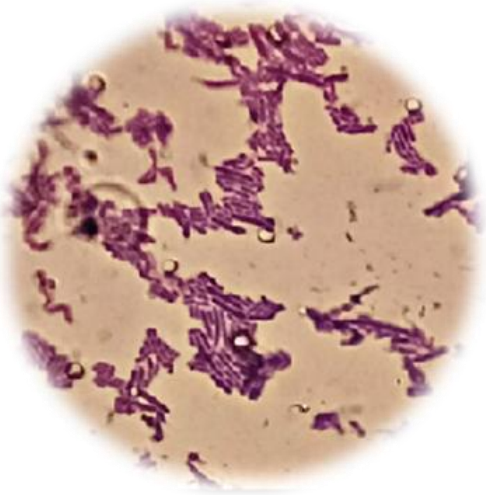


Figure15: Macroscopic appearance of solid MRS medium inoculated with lactic acid bacteria strains isolated from the intestine *Helix aspersa Müller* at 30°C.

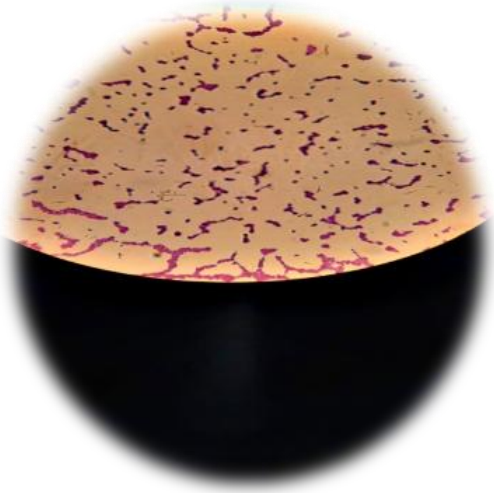
- **Microscopic appearance of strains**

After Gram staining, the cell morphology was revealed: the bacteria exhibited cocci-shaped cells arranged in pairs (diplococci), short chains, and clusters. The results are illustrated in **figure 16** and **table 7.**

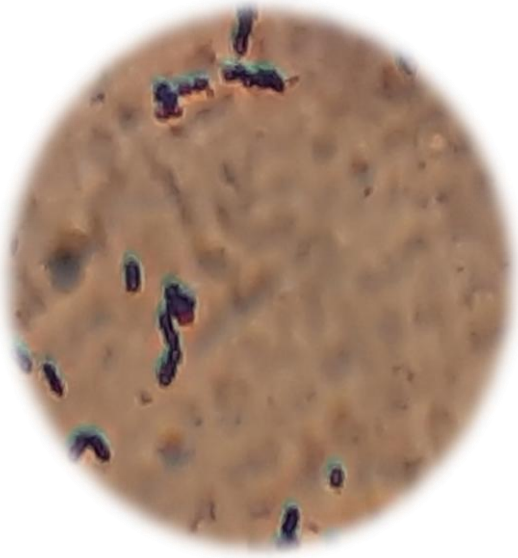
"S1"



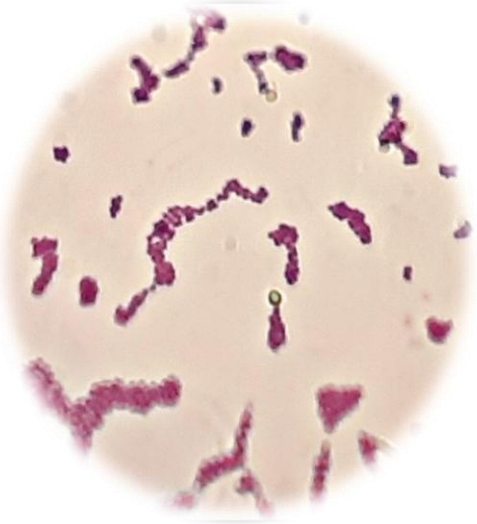
"S2"



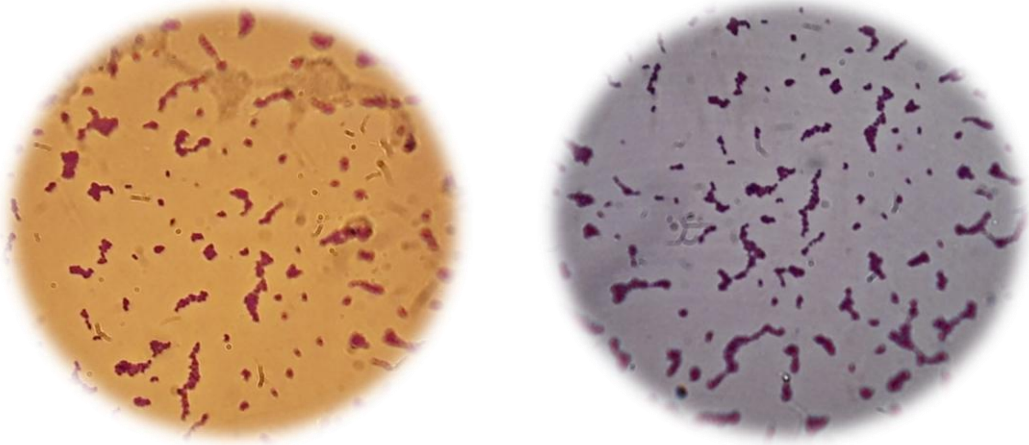
"S3"



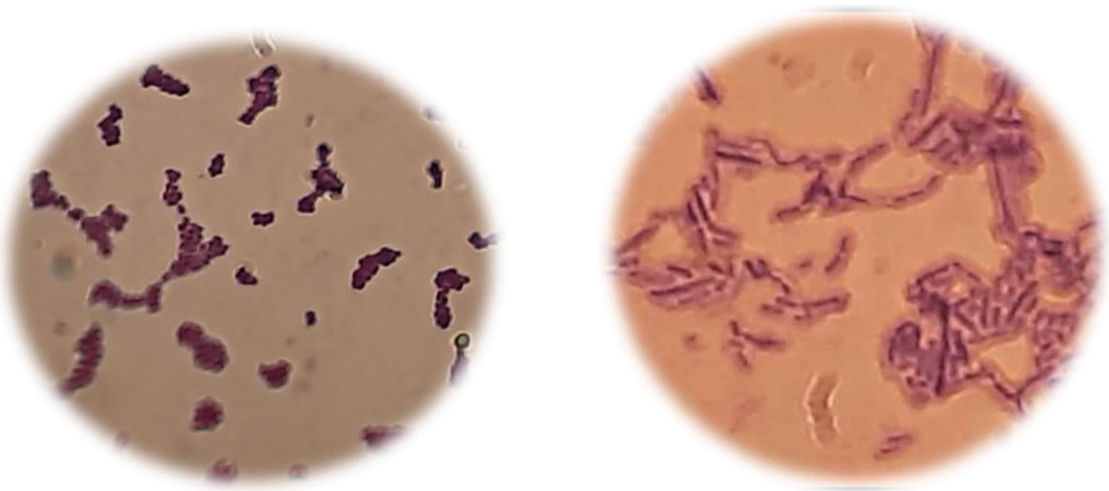
"S4"



“S5” **“S6”**



“S7” **‘‘S8’’**



“S9” **“S10”**

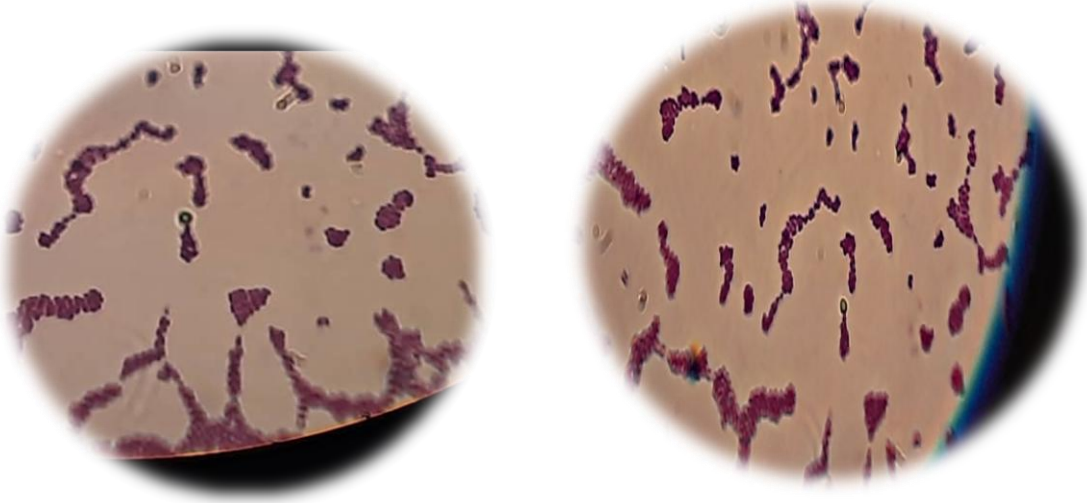


Figure16: Microscopic observations of strains isolated from the digestive tract of *Helix aspersa* (Gx100).

Table 7: Summary of microscopic observation of strains isolated from the digestive tract of *Helix aspersa*.

Lactic acid bacteria	Form	Arrangement	Gram
S1	Streptobacilli	Rod-shaped	Positive
S2	Coccus	Isolated Pairs In chains	Positive
S3	Diplococci	Arranged in pairs, appearing as two cocci attached together.	Positive
S4	Diplococci	Arranged in pairs, appearing as two cocci attached together.	Positive
S5	Coccus	Isolated Pairs In chains	Positive
S6	Coccus	Isolated Pairs In chains	Positive
S7	Coccus	Isolated Pairs In chains	Positive
S8	Diplobacilli	Arranged in pairs, appearing as two cocci attached together.	Positive
S9	Coccus	Isolated Pairs In chains	Positive
S10	Coccus	Isolated Pairs In chains	Positive

- **Catalase test**

The result of the catalase test was negative for all strains (no gas production), suggesting that these bacteria are lactic acid bacteria **(Figure 17)**.

Figure 17: Negative catalase.

IV.2. Identification of isolates

Preliminary identification tests of the isolates were based on the physiological and biochemical characteristics of these bacteria (table 8).

IV.2.1. Physiological tests

IV.2.1.1. Growth under harsh conditions

- **Growth test at different temperatures (15°C and 37°C)**

This study was conducted at 15°C and 37°C for 24 hours and 48 hours **(Badis et al., 2004)**. This test helps differentiate between thermophilic and mesophilic flora.

All isolates exhibited good growth at both 15°C and 37°C after 24 hours and 48 hours of incubation **(figure 18 and figure 19)**. Therefore, all strains are mesophilic in nature **(Carr et al., 2002)**.

Figure18: Bacterial Growth results at 15°C.

Figure19: Bacterial Growth results at 37°C.

- **Growth test in the presence of NaCl**

Culturing the strains in the presence of 2.5%, 4%, and 6.5% NaCl allowed us to assess their ability to grow under different concentrations.

The recorded results demonstrated that all isolated strains were capable of growing in MRS broth with NaCl concentrations of 2.5%, 4%, and 6.5% **(figure 20,21 and figure 22)**.

The growth results obtained at 6.5% NaCl exhibited similarity to *Leuconostoc* strains, where bacterial growth was visually observed as turbidity. My findings are consistent with those reported by **Zadi and Karam (2006)** regarding the confirmation of identification of salt-resistant *lactococci* belonging to the *Leuconostoc* species.

Figure 20: Bacterial Growth at 2.5% NaCl.

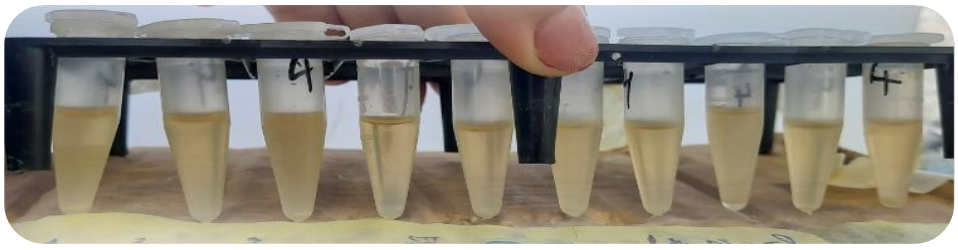


Figure 21: Bacterial Growth at 4% NaCl.

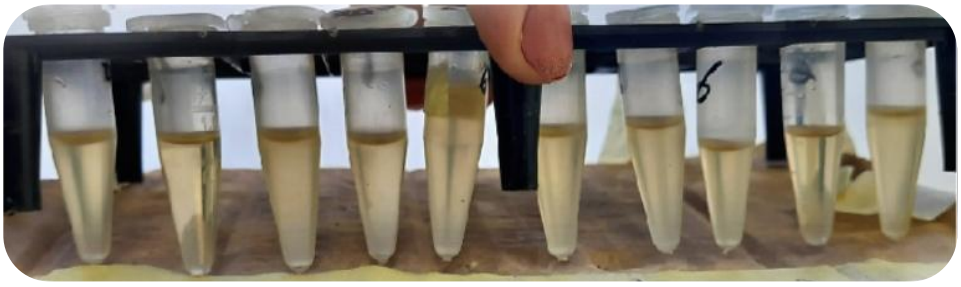


Figure 22: Bacterial Growth test at 6.5% NaCl.

- **Growth test in a highly alkaline medium**

Growth on MRS medium at pH 9.6 resulted in visible bacterial turbidity. All strains demonstrated growth on this medium. The results are presented in **table 8** and **figure 23**.

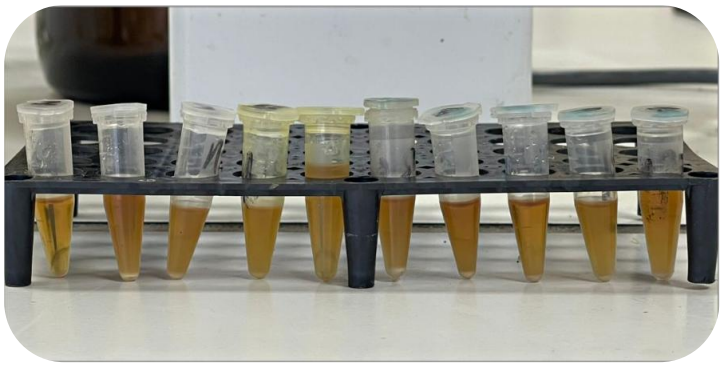


Figure 23: Bacterial Growth in hyperalkaline medium of lactic acid isolates from *Helix aspersa.*

IV.2.2. Biochemical tests

- **CO_2 production from glucose:**

This test allowed us to differentiate between homofermentative and heterofermentative isolates. No gas (CO_2) production was observed in all the strains , indicating their homofermentative metabolism **(figure 24)**.

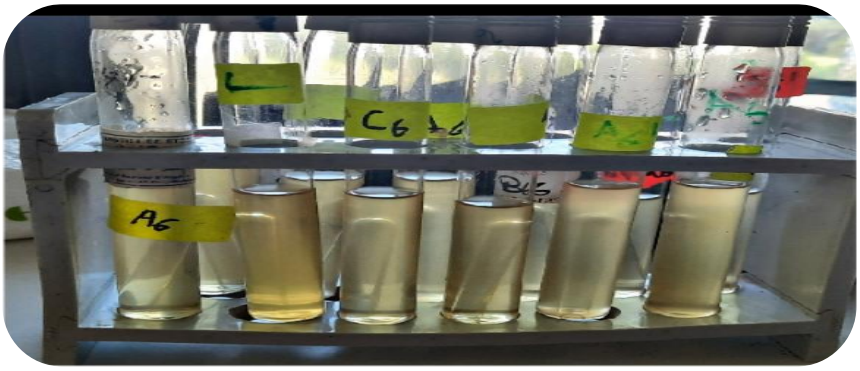


Figure 24: The test of Production of gas by lactic acid isolates showing no production of gas

- **CO_2 production test from citrate:**

We observed that only the strain 6 exhibited agar fragmentation **(figure25)**, while no agar fragmentation was observed in the tubes for the remaining strains**(figure 26)**.

Figure26 Production of CO_2 from citrate by lactic acid isolates (negative result).

Figure 25: Production of CO_2 from citrate by lactic acid isolates (positive result).

IV.2.3. Technological tests

- **Exopolysaccharide production**

Several strains of lactic acid bacteria produce exopolysaccharides, which can be located either in the capsule, closely associated with the bacterial cell wall, or excreted into the external environment in the form of dextran.

The production of exopolysaccharides from sucrose was observed in strains S1,S3,S5,S7 and S8, which have the ability to degrade sucrose and produce dextran. This is manifested by the appearance of large, viscous, and sticky colonies, as shown in **figure 27**. In contrast, strains S2, S4, S6,S9 and S10 are unable to degrade sucrose and produce dextran.

This characteristic is consistent with those mentioned by **(Carr et al., 2002; Bjorkroth et al., 2006; Philippon et al., 2008; Ghazi et al., 2009)**.

Leuconostoc strains exhibit small, convex colonies that strongly adhere to the agar surface, as shown by **(Carr et al., 2002; Badis et al., 2005)**. These observations are also in line with those of **(Du Vuyst et al., 2001)**, who noted that *Lactobacilli* are capable of producing EPS (exopolysaccharides). According to Walling et al. **(2001)**, EPS production by lactic acid bacteria is an industrially significant characteristic.

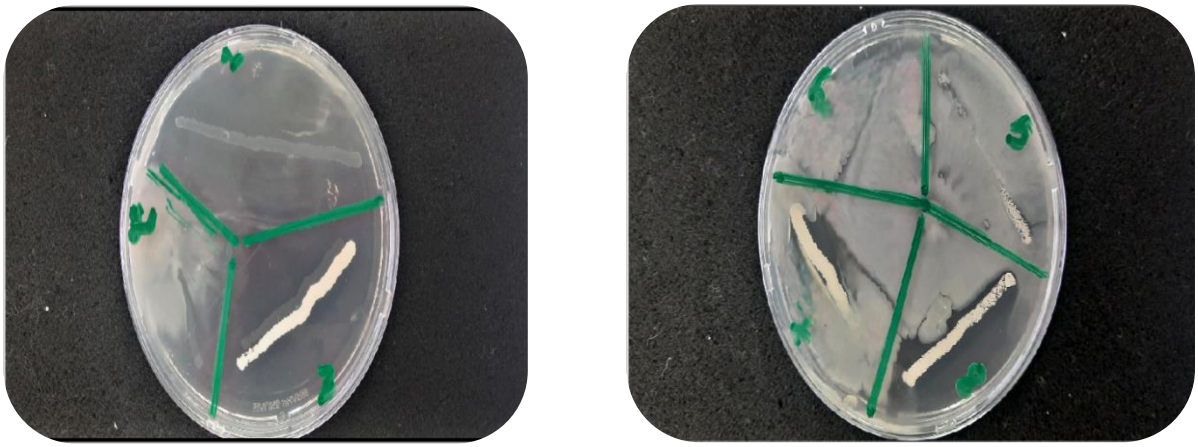


Figure27: Macroscopic observation of strains on high-sugar medium isolated from *Helix aspersa*.

- **Acetoin production test**

Acetoin production was tested using Clark and Lubs medium. All of my isolates produced acetoin. The obtained results are shown in **figure 28**.

Figure 28.: Acetoin production by lactic acid isolates (positive results)

- **Heat resistance test**

This study enables differentiation between thermophilic and mesophilic flora. A positive result is indicated by turbidity **(Rouisset et al., 2006)**. Based on the results obtained, the tested strains did not exhibit resistance to 63.5°C . Expect the first strain. The heat resistance test at 63.5°C is illustrated in **figure 29**.

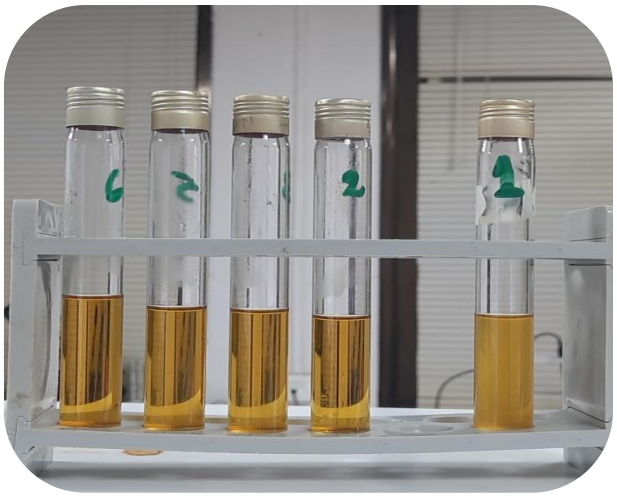


Figure 29: The result of the Heat resistance of the lactic acid isolates from *Helix aspersa*.

- **Growth test in the presence of methylene blue**

Subsequently, the results obtained in sterilized skim milk containing 0.1% methylene blue showed that all the strains were able to grow in the presence of methylene blue. The result is illustrated in **figure 30.**

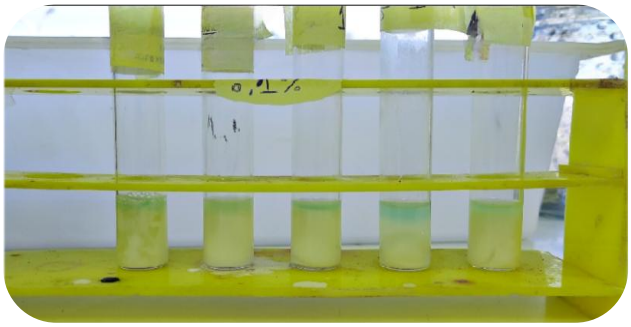


Figure 30: Reduction of methylene blue at a concentration of 0.1% by the isolates.

The result for 0.3% Sherman's milk shows that all the strains are capable of growing in the presence of 0.3% methylene blue **(figure 31)**.

Figure31: Reduction of methylene blue at a concentration of 0.3% by the isolates.

Table8 : Results of physiological identification tests of lactic acid strains isolated from *Helix aspersa.*

Lactic acid isolates.	Growth at different temperatures .		Heat resistance	Hyperalkaline medium.	Growth in the presence of NaCl.			Production of CO2 from:		Growth in the presence of methylene blue.		ACT	Dextran
	15 °C	37° C			2.5 %	4 %	6.5 %	Glu	Citrate	0.1%	0.3%		
S1	+/-	+	-	+	+	+	+	-	+	+	+	+	+
S2	+	+	-	+	+	+	+	-	-	+	+	+	-
S3	+	+	-	+	+	+	+	-	-	+	+	+	+
S4	+	+	-	+	+	+	+	-	-	+	+	+	-
S5	+	+	-	+	+	+	+	-	-	+	+	+	+
S6	+	+	-	+	+	+	+	-	-	+	+	+	-
S7	+	+	-	+	+	+	+	-	-	+	+	+	+
S8	+	+	-	+	+	+	+	-	-	++	+	+	+
S9	+	+	-	+	+	+	+	-	-	++	+	+	-
S10	+	+	-	+	+	+	+	-	-	+	+	+	-

+ : Positive reactions, - : Negative reactions, +/- : Variable response depending on the species.

ACT: Acetoin production, DXT: Dextran production, Glu: Glucose.

IV.3. Demonstration of antimicrobial activity

The solid medium diffusion method was employed to assess the antimicrobial activity of the 10 isolated strains. This method is characterized by the formation of a halo surrounding the inoculated strain on the solid medium. The interpretation of results involves measuring the diameter of the inhibition zone formed by the indicator strain. An inhibition is considered positive if the zone diameter exceeds 6mm.

The pathogenic strains utilized in this study include *Candida albicans, Pseudomonas aeruginosa, Escherichia coli, Bacillus cereus, and Staphylococcus aureu*s. The obtained results are presented partially in **figures 32, 33,34,35** and **36** and summarized in **table 9**.

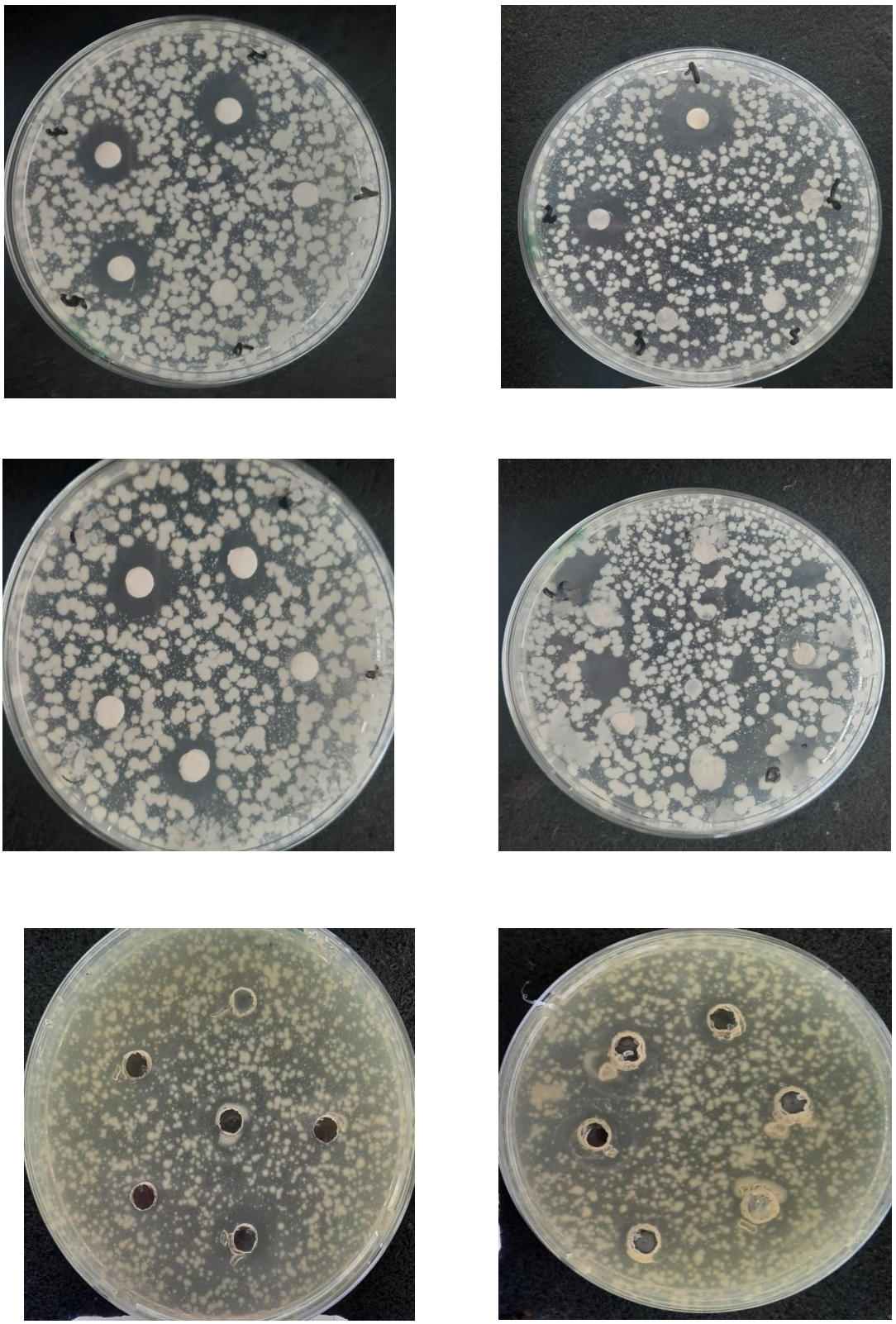


Figure 32 : Inhibition of the indicator strain *Bacillus cereus* by lactic acid bacteria strains.

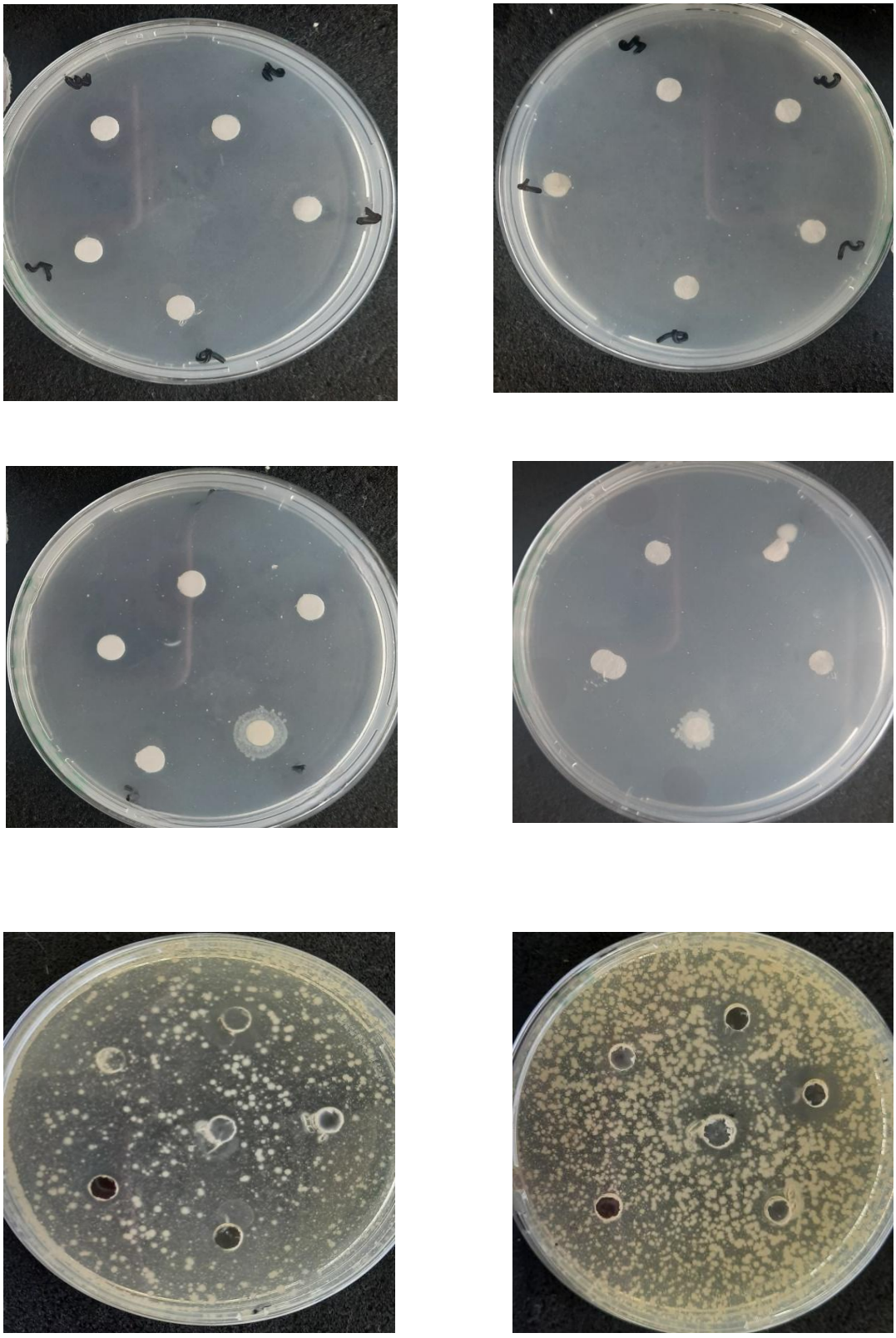


Figure 33: Inhibition of the indicator strain *Pseudomonas aeruginosa* by lactic acid bacteria strains.

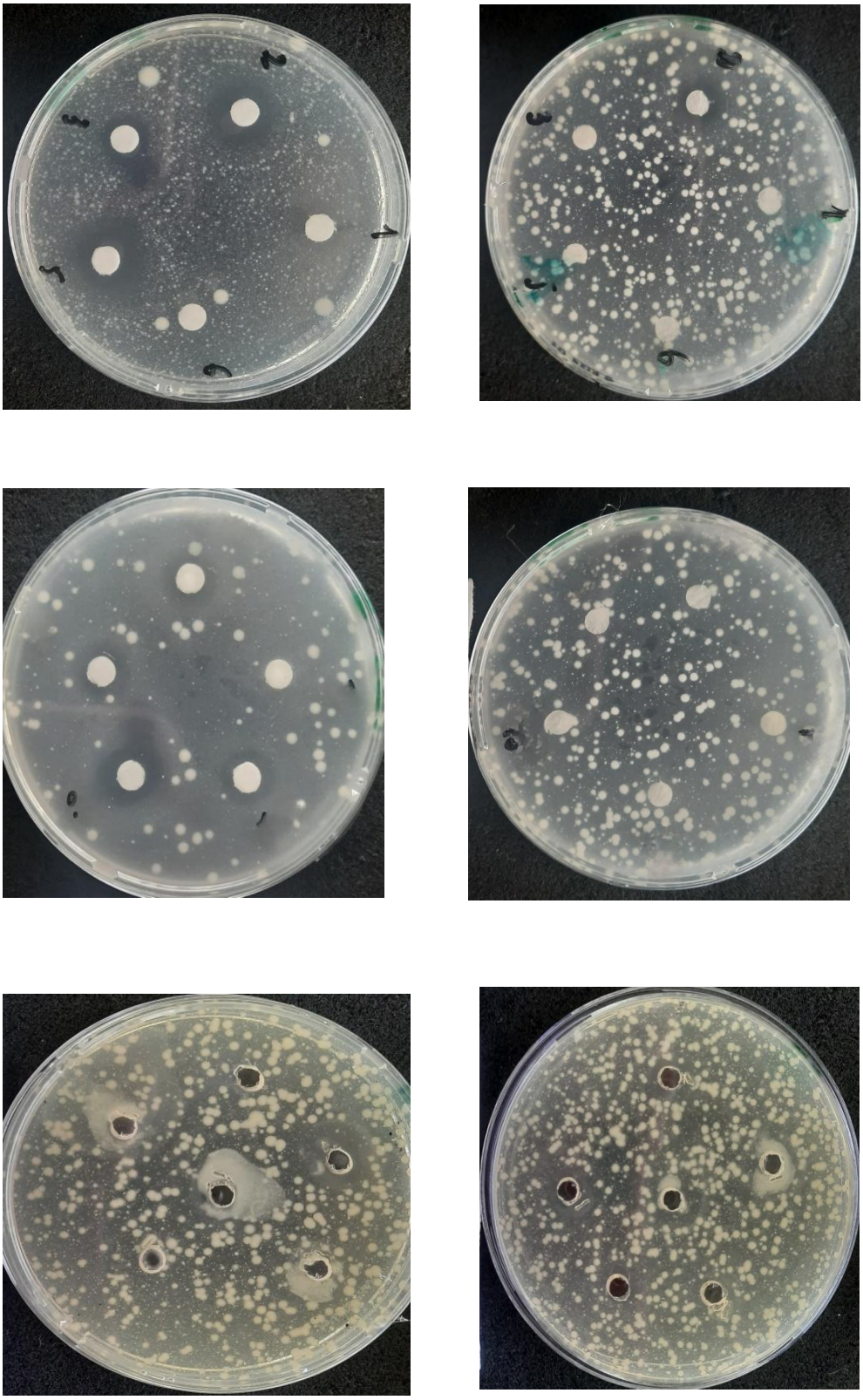


Figure 34: Inhibition of the indicator strain *E.coli* by lactic acid bacteria strains.

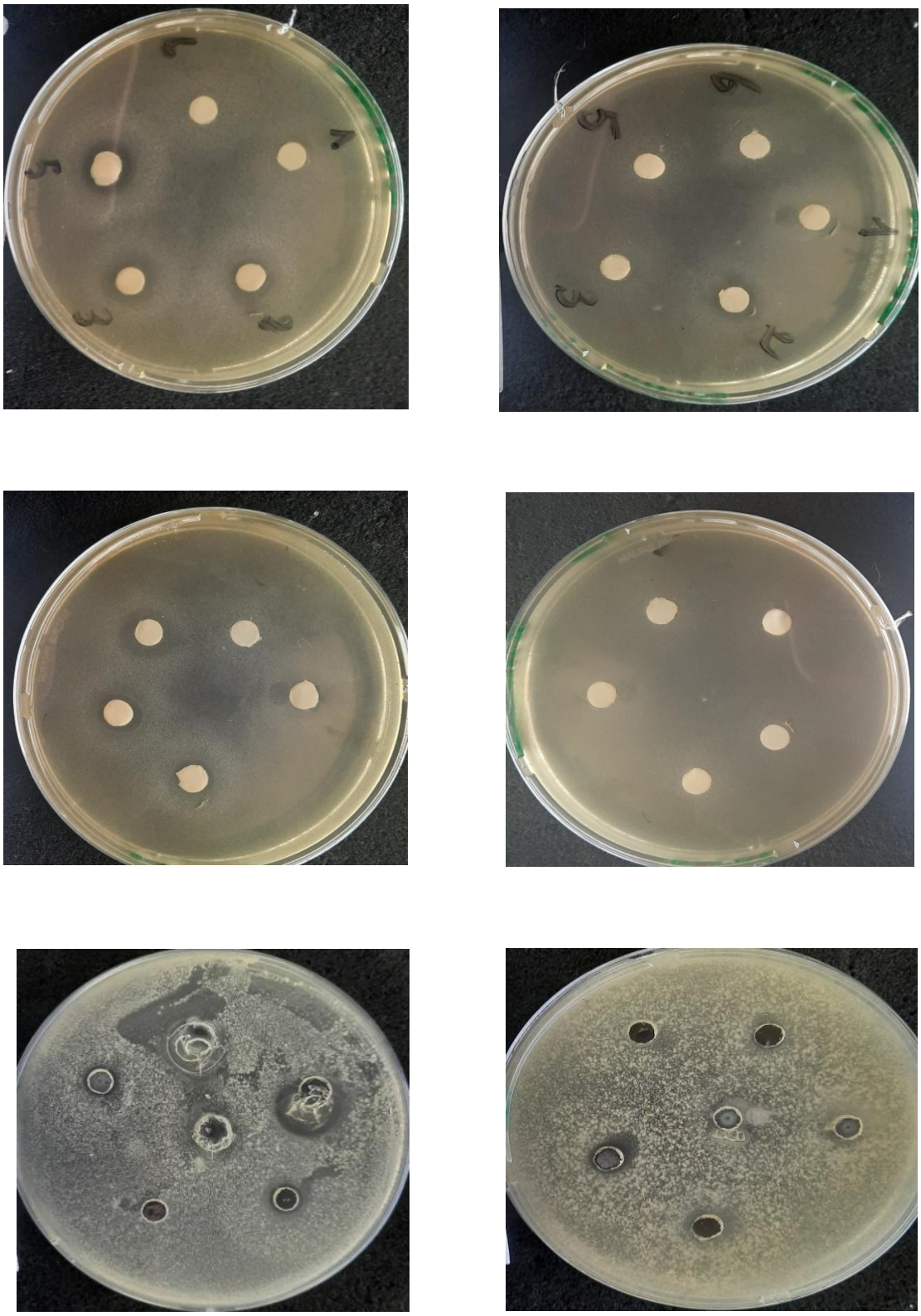


Figure 35: Inhibition of the indicator strain *S.aureus* by lactic acid bacteria strains.

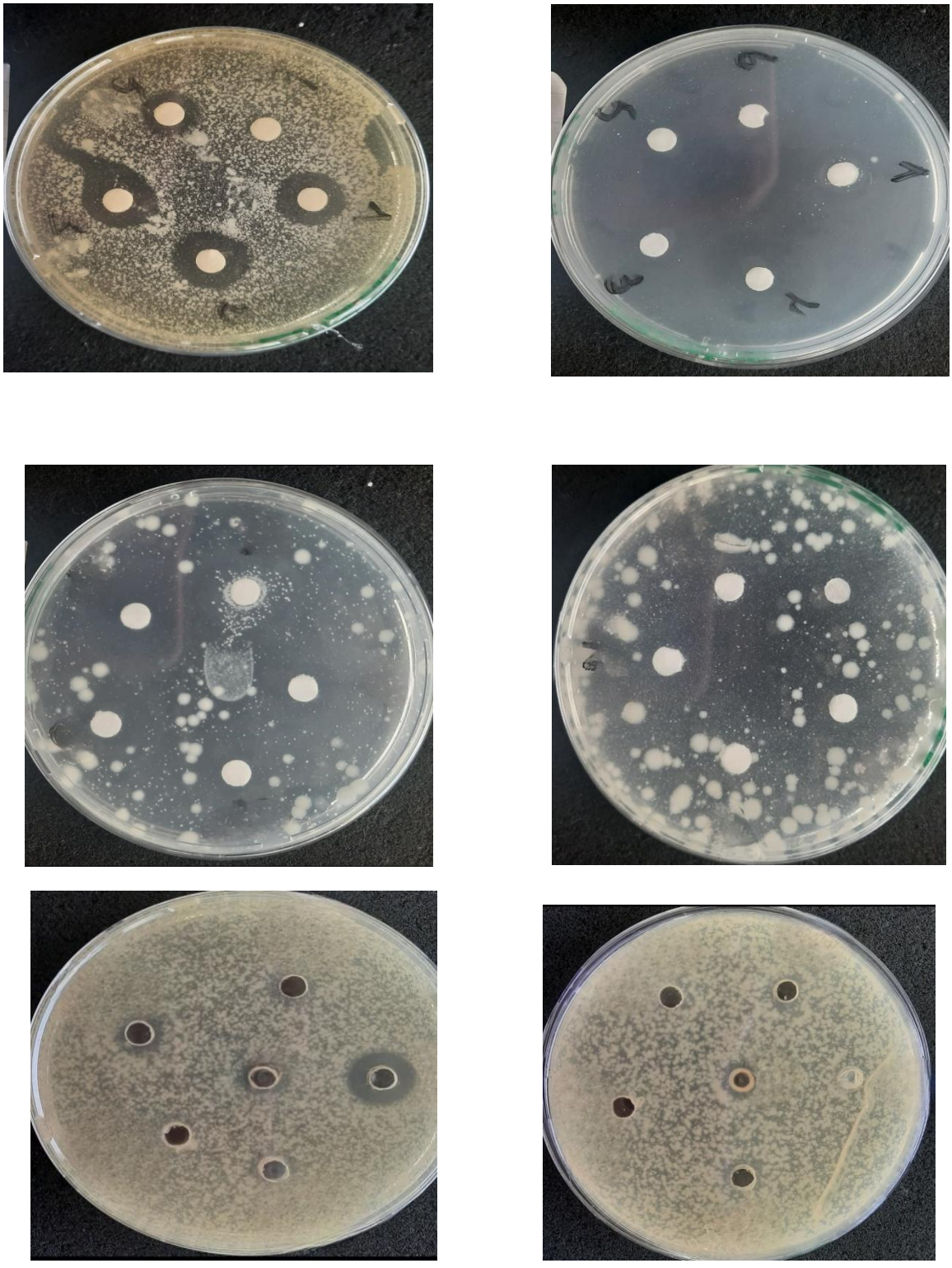


Figure 36: Inhibition of the indicator strain *Candida albicans* by lactic acid bacteria strains

Table 9: Inhibitory activity of 10 lactic acid isolates.

	Direct method										Indirect method																			
	Bacterial culture										Supernatant of the culture										Neutralized supernatant									
Indicator Strains	YZ1	YZ2	YZ3	YZ4	YZ5	YZ6	YZ7	YZ8	YZ9	YZ10	YZ1	YZ2	YZ3	YZ4	YZ5	YZ6	YZ7	YZ8	YZ9	YZ10	YZ1	YZ2	YZ3	YZ4	YZ5	YZ6	YZ7	YZ8	YZ9	YZ10
Candida albicans	16mm	10mm	9mm	12mm	8mm	-	-	-	-	-	16mm	17mm	15mm	12mm	7mm	8mm	8mm	10mm	10mm	7mm	10mm	15mm	10mm	7mm	12mm	15mm	16mm	7mm	16mm	12mm
P. aeruginosa	14mm	11mm	-	9mm	-	12mm	-	10mm	-	-	12mm	14mm	15mm	9mm	6mm	11mm	10mm	15mm	11mm	12mm	13mm	14mm	11mm	10mm	10mm	8mm	6mm	10mm	10mm	9mm
E.coli	13mm	-	9mm	-	7mm	-	9mm	9mm	9mm	8mm	10mm	9mm	11mm	11mm	9mm	12mm	9mm	14mm	10mm	9mm	13mm	14mm	12mm	12mm	7mm	6mm	7mm	7mm	8mm	8mm
B.cereus	13mm	-	-	15mm	-	13mm	-	-	12mm	-	7mm	16mm	17mm	15mm	7mm	8mm	11mm	16mm	7mm	10mm	21mm	16mm	6mm	8mm	7mm	8mm	9mm	7mm	7mm	6mm
S.aureus	12mm	10mm	9mm	6mm	6mm	-	-	9mm	11mm	11mm	10mm	12mm	13mm	11mm	7mm	9mm	7mm	10mm	12mm	8mm	12mm	10mm	8mm	11mm	10mm	10mm	8mm	8mm	10mm	7mm

- absence of inhibition

.

According to the results compiled in Table 9, our isolates exhibited highly variable inhibitory capacities. It is noteworthy that all 10 isolates demonstrated inhibition against all 5 tested pathogenic bacteria.

The lactic acid bacterial strains from our isolates effectively inhibited the growth of the pathogenic strains, with inhibition zones exceeding 20mm in diameter against Bacillus cereus, over 17mm against *Candida albicans*, and more than 14mm against *Pseudomonas aeruginosa.* The observed inhibition zones were consistently pronounced, indicating the strong inhibitory potential of these lactic acid bacterial isolates.

However, the inhibitory effect of these isolates was comparatively less significant against *Escherichia coli.* Based on these results, it is evident that the pathogenic strain most sensitive to our isolates is *B. cereus*, as it was inhibited by all the isolates. The pathogenic strains *P. aeruginosa* and *E. coli* were inhibited by all isolates too, while *Staphylococcus aureus* was inhibited by 2 isolates.

Notably, the halos was observed against the *Candida albicans* pathogenic strain. The reported findings of this study demonstrate that the majority of lactic acid bacterial strains isolated from *Helix aspersa* exhibit significant inhibitory effects on both Gram-positive and Gram-negative pathogenic bacteria, with varying diameters of inhibition zones.

The solid medium diffusion method is employed for the detection of inhibitions. This test involves culturing and bringing bacterial strains into contact, allowing for the detection of negative interactions resulting from the metabolism of lactic acid bacteria against pathogenic bacteria. It also allows for the assessment of the observed inhibitions by measuring the size of clear halos **(Benreguieg, 2015)**.

My results align with those obtained by **Chemlal (2013),** who demonstrated that the inhibitory power of lactic acid bacteria is often associated with their acidifying properties, particularly involved in inhibiting pathogenic flora and spoilage organisms. These inhibitory properties are attributed to the production of organic acids, especially lactic and acetic acids. The inhibitory effect of these acids is closely related to the decrease in pH of the medium, reaching values below pH 5 at the end of incubation. The preservation properties result from the inhibitory properties of lactic acid bacteria, which include competition for nutrients and physicochemical changes in the environment, such as acidification and the production of antimicrobial metabolites. Lactic acid bacteria have the ability to produce numerous antimicrobial substances, such as organic acids (lactic acid), hydrogen peroxide, CO_2, diacetyl, acetaldehyde, and bacteriocins. Several reviews and research studies have reported the potential of lactic acid bacteria used as adjunct cultures to inhibit pathogenic

microorganisms present in food, including the inhibition of *L. monocytogenes* in the presence of lactic acid bacteria in smoked salmon **(Tahiri, 2007)**.

Conclusion

Conclusion

The study of lactic acid bacteria holds significant importance in the field of nutrition. For over four thousand years, humans have utilized these lactic strains for food fermentation, recognizing their ability to produce antibacterial compounds that enable their preferential growth in various ecosystems and their application as natural preservatives.

The primary objective of this study was to explore the different strains of lactic acid flora found in the garden snail intestine. The specific objectives were to isolate and biochemically and technologically characterize acid lactic strains obtained from the intestine of *Helix aspersa*, as well as select those exhibiting antimicrobial activity against a range of pathogenic bacteria typically associated with meat and/or seafood spoilage, as well as foodborne illnesses.

The first phase of this study successfully isolated, characterized, and identified six strains of lactic acid bacteria, primarily belonging to the Streptococcus spp and *Enterococcus spp* families. The results clearly demonstrate that the digestive tract of *Helix aspersa* serves as an ecological niche for lactic strains, emphasizing the need for further attention and in-depth research on these microorganisms.

Through direct cell-to-cell and indirect interactions between the isolated lactic strains and the pathogenic strains, the ability of lactic acid bacterial strains to produce inhibitory substances against target strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 10876, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231) was determined.

Based on the results obtained, it can be concluded that among the purified strains (S1, S2, S3, S4, S5, S6,S7,S8,S9,S10) isolated from the digestive tract of *Helix aspersa*, five out of five tested pathogenic bacteria were inhibited.

In conclusion, this study highlights the presence of lactic acid bacteria in the intestine of *Helix aspersa.* However, further research is warranted to delve into the genetic and technological characterization of these isolated strains and to evaluate their safety and effectiveness in specific food applications.

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Annex

Isolation media

- ☞ MRS medium **(De Man Rogosa and Sharp, 1960)** for 1 L of medium:

• Polypeptone 10.00g

• Meat extract 10.00g

• Yeast extract 5.00g

• Glucose..... 20.00g

• Dipotassium phosphate..... 2.00g

• Sodium acetate 5.00g

• Ammonium citrate..... 2.00g

• Magnesium sulfate..... 0.20g

• Manganese sulfate.....0.05g

• Bacteriological agar 15.00g

• Distilled water .....1000 ml

pH = 6.8 ± 0.1 Autoclave at 120°C for 20 minutes

- ☞ MRS liquid Contains all the ingredients of solid MRS medium, but without agar.

☞ M17 Medium (**Terzaghi and Sandine, 1975**) For 1 liter of medium:

- Tryptone..... 2.50 g
- Meat peptone (pepsin digest).....2.50 g
- Soy peptone (papain digest)..... 5.00 g
- Yeast extract..... 2.50 g
- Meat extract.....5.00 g
- Lactose.....5.00 g
- Sodium glycerophosphate.....19.00 g
- Magnesium sulfate..... 0.25 g
- Ascorbic acid..... 0.50 g
- Bacteriological agar..... 15.00 g
- Distilled water.....1000 ml
    - pH = 7.1 ± 0.2 Autoclave at 120°C for 20 minutes.

☞ M17 Liquid (all ingredients from M17 Solid, but without Agar)

Identification Media

☞ **Clark and Lubs medium:**

• Peptone..... 5g

• Dipotassium phosphate..... 5g

• Glucose..... 5g

• Distilled water..... 950ml

pH=7.4. Autoclave at 120°C for 20 minutes.

☞ **Skimmed milk medium:**

• Skimmed milk powder..... 10g

• Yeast extract.....0.5g

• Distilled water..... 1L

Autoclave at 110°C for 10 minutes.

☞ **Hypersaline broth:**

- Meat extract 5g
- Glucose..... 5g
- Peptone..... 15g
- NaCl 25/40/65g
- Distilled water.....1L

pH=7.2. Autoclave at 120°C for 20 minutes.

☞ **Muller-Hinton medium (Muller and Hinton, 1941):**

- Beef meat infusion 3000ml
- Casein peptone.....17.5g
- Corn starch 1.5g
- Agar-agar 17g
- Distilled water.....1L

pH=7.4. Autoclave at 120°C for 20 minutes.

☞ **Peptone physiological water:**

- Sodium chloride.....8.5g
- Peptone.....0.5g
- Distilled water.....950ml

pH=7. Autoclave at 120°C for 20 minutes.

☞ **White agar:**

- Agar-agar.....16g
- Distilled water.....1L

Autoclave at 120°C for 20 minutes.

☞ **Nutrient broth:**

- Meat extract.....1g
- Yeast extract.....2g
- Peptone.....5g
- Sodium chloride.....5g
- Distilled water.....1000ml

pH=7.4. Autoclave at 120°C for 20 minutes.

☞ **Sherman's milk (Bourgeois and Leveau, 1991):**

• Skimmed milk powder.....10g

• Yeast extract.....0.5g

• Distilled water.....1000ml

pH=6.8

To obtain a milk solution with 0.1% methylene blue, add 1ml of 1% methylene blue solution that has already been sterilized at 120°C for 20 minutes. And to have a milk solution with 0.3% methylene blue, add 1ml of 3% methylene blue solution at the time of use.

☞ **Hypersaccharose medium:**

• Meat extract 10g

• Yeast extract.....3g

• Peptone.....2.5g

• Sucrose.....150g

• K_2HPO_4..... 2g

• Na Cl 1g

• $MgSo_4$ $7H_2O$.....15g

• Distilled water.....1000ml

Autoclave at 120°C for 20 minutes.