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Antimicrobial activity of bacteriocinogenic lactic acid bacteria and the effectiveness of essential oils of *Artemisia herba* and *Ruta montana* against ESBL producing *Enterobacteriaceae* (resistant to antibiotics)

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بسم الله الرحمن الرحيم

ملخص

تنتج البكتيريا اللاكتيكية مجموعة متنوعة من المركبات ذات الفعالية المضادة للميكروبات مثل الأحماض العضوية وبيروكسيد الهيدروجين والدياسيتيل والبكتيريوسين.اين تلعب هذه الجزيئات دورًا رئيسيًا في حفظ الأطعمة الحيوية. يركز هذا العمل على دراسة النشاط المضاد للبكتيريا اللاكتيكية المنتجة للبكتيريوسين المعزولة من أنواع مختلفة من الجبن المصنوع تقليديا.

(Lactobacillus paracasei, Enterococcus durans, Pediococcus pentosaceus, Enterococcus faecium, Lactococcus lactis subsp. lactis و Enterococcus durans)

ضد مجموعة مختلفة من السلالات البكتيرية و التي تتضمن سلالات متعددة المقاومة حيث اثبتت نتائج هذه التجارب ان الانشطة المضادة للميكروبات التي تنتجها السلالات السبعة المختارة لها طيف واسع النطاق موجه ضد البكتيريا الموجبة و السالبة لصبغة غرام بما في ذلك البكتيريا الممرضة المقاومة للأدوية المتعددة اين تم تسجيل مناطق تثبيط للنمو متفاوتة الاقطار والتي تتراوح . بين 10ملم و 23 ملم ضد البكتيريا إيجابية الجرام و بين 8 ملم و 36 ملم ضد البكتيريا سلبية الجرام.

بالمقابل، تم الحصول على زيوت أرتيميزيا هيربا وروتا مونتانا الأساسية بواسطة التقطير بالماء للأجزاء الهوائية (الأوراق) التي تم حصادها من منطقة العقلة (تبسة، الجزائر). بعد دراستنا التحليلية، تم إجراء تجربة بيولوجية لتقييم الخصائص المضادة للبكتيريا لكلا الزيتين ضد أربع سلالات بكتيرية غرام سلبية باستخدام طريقة انتشار القرص. أظهرت النتائج التي تم الحصول عليها أن زيت أرتيميزيا هيربا يمتلك نشاطًا مضادًا للميكروبات ضد جميع البكتيريا الممرضة المختبرة والذي اثبت من خلال المردود الزيتي الوفير و الذي وصل الى 1.67% بينما روتا مونتانا أظهرت نشاطًا أضعف ضد نفس السلالات مع مردود لا يزيد عن0.8% . وبالتالي يمكن أن يختلف هذا النشاط اعتمادًا على السلالة المختبرة و كذا التركيبة الكيميائية للمستخلص الزيتي المتطاير.

الكلمات المفتاحية بكتيريا حمض اللاكتيك– النشاط المضاد للميكروبات– البكتيريا متعددة المقاومة– زيت أساسي– زيت أرتيميزيا هيربا الاساسي– زيت روتا مونتانا الاساسي

Summary:

Lactic acid bacteria produce a variety of compounds with antimicrobial action, such as organic acids, hydrogen peroxide, diacetyl, and bacteriocins. These molecules play a key role in the bio-preservation of food.

This study focuses on the investigation of the antagonistic activity of bacteriocin-producing lactic acid bacteria (LAB) isolated from various types of artisanal cheese (*Lactobacillus paracasei, Enterococcus durans, Pediococcus pentosaceus, Enterococcus faecium, Lactococcus lactis subsp. lactis, and Enterococcus durans*) against a wide range of Gram-positive and Gram-negative human pathogens, including antibiotic-resistant strains (ESBL) such as *E. coli1* (S8E14 Merg), *Klebsiella pneumoniae ssp ozaneae* (S19 E21 Merg), *Raoultella terrigena* (S15 E23 milk), *Serracia odorefira1* (S43 E9 AS). The results of in vitro antimicrobial assays especially the agar overlay method, has revealed a broad-spectrum of antimicrobial activity produced by the selected strains where the zones of inhibition (ZOI) varied between 10mm and 23mm against Gram positive strains, whereas Gram negative ones varied between 8mm and 36mm.

On the other hand, *Artemisia herba* and *Ruta montana* essential oils were obtained by hydro-distillation of aerial parts (leaves) harvested from El Ogla region (Tebessa, Algeria) .Further to our analytic study, a biological trial was carried out with the purpose of assessing the antibacterial properties of both *EOs* against four Gram-negative bacterial strains using disk diffusion method. The results obtained revealed that the *Artemisia herba* essential oil exerts a better antimicrobial activity against all tested pathogenic bacteria with a yield of 1.67 %. However, *Ruta montana,* displayed a weaker yield of 0.8%, hence a minimal activity was recorded against the same strains. Consequently, this activity may vary depending on the tested strain along with the applied volatile extract.

Keywords: Lactic acid bacteria - ESBLs - antimicrobial activity - essential oil - *Artemisia herba* - *Ruta montana*.

Résumé:

Les bactéries lactiques produisent une variété de composés à action antimicrobienne tels que les acides organiques, le peroxyde d'hydrogène, le diacétyl et les bactériocine. Ces molécules jouent un rôle de premier plan dans la bio-préservation des aliments.

Le présent travail est axé sur l'étude de l'activité antagoniste des bactéries lactiques productrices de bactériocines isolées à partir de différents types de fromage artisanaux (*Lactobacillus paracasei, Enterococcus durans, Pediococcus pentosaceus, Enterococcus faecium, Lactococcus lactis subsp. lactis* et *Enterococcus durans*) contre un large éventail de pathogènes humains Gram positifs et Gram négatifs, y compris des souches multi-résistantes aux antibiotiques ; *E. coli1* (S8E14 Merg), *Klebsiella pneumoniae* ssp *ozaneae* (S19 E21 Merg), *Raoultella terrigena* (S15 E23 lait), *Serracia odorefira* (S43 E9 AS VP).Les résultats des essais antimicrobiens in vitro ont révélé une activité antimicrobienne à large spectre produite par les souches sélectionnées contre différentes souches bactériennes ou les zones d'inhibition trouvées se varient entre 10mm et 23mm concernant les bactéries Gram positives et celles des bactéries Gram négatives entre 8mm et 36mm.

D'autre part, les huiles essentielles *d'Artemisia herba* et de *Ruta montana* ont été obtenues par hydrodistillation des parties aériennes (feuilles) récoltées dans la région d'El Ogla (Tébessa, Algérie). Suite à notre étude analytique, un essai biologique a été réalisé dans le but d'évaluer les propriétés antibactériennes des deux huiles essentielles contre quatre souches de bactéries Gram-négatives en utilisant la méthode de diffusion sur disque. Les résultats obtenus ont révélé que l'huile essentielle *d'Artemisia herba* exerce une forte activité antimicrobienne contre toutes les souches pathogènes testées avec un rendement de 1.67 %. Alors que, *Ruta montana* a montré une activité plus faible contre les mêmes souches (0.8%). Par conséquent, cette activité peut varier en fonction de la souche testée ainsi que de l'extrait volatil appliqué.

Mots clés : Bactéries d'acide lactique - BLSE - activité antimicrobienne - huile essentielle - *Artimisia herba* - *Ruta montana*

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Dedication

*First and foremost, I would like to thank **Allah (SWT)** for always giving me the will to evolve and for his constant blessings. I also dedicate this work to:*

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This thesis is dedicated to all those who have played a part, big or small, in my academic and personal growth. Your support, encouragement, and belief in me have been invaluable. Thank you from the bottom of my heart.

May this thesis contribute to the advancement of knowledge and serve as a reminder of the collective effort that has made it possible.

Louiza

Dedication

I am dedicating this work to people who have meant and will mean so much to me.

My mother Nacira , *the source of tenderness and the beat of my heart, who can take the place of all others but whose place no one else can take .*

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My best partner and Friend Louiza .

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Thank you for always believing in me, even when I didn't believe in myself, and for making me what I am today.

Soulef

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List of Abbreviations

LAB:	Lactic acid bacteria
ESBLs:	Extended-spectrum β-lactamases
AH-EO:	*Artemisia herba* essential oil
RM-EO:	*Ruta montana* essential oil
GRAS:	Generally Recognized as Safe'
NADH:	Nicotinamide adenine hydroxyperoxydase dinucleotide
H_2O_2:	Hydrogen peroxide
H_2O:	Water molecule
O_2:	Oxygen
OH:	Hydroxyl radicals
$OSCN^-$:	Hypothiocyanite
O_2SCN^-:	Higher oxyacids
CO_2:	Carbon dioxide
β-hydroxypropionialdehyde:	Reuterin
MW:	molecular weight
QPS system:	The presumption status of safety
FDA:	US Food and Drug Administration
E234:	Lantibiotic nisin
WHO:	World Health Organization.
MAH:	Microwave assisted hydro distillation
CH :	Clevenger hydro distillation
EOs:	Essential oils
MAP:	Medicinal and aromatic plant
MRS:	De Man, Rogosa and Sharpe agar
MH:	Mueller Hinton Agar medium
NB:	Nutrient Broth
HDC:	Clevenger-type apparatus
NCCLS:	National Committee for Clinical Laboratory Standards
EUCAST:	European Committee on Antimicrobial Susceptibility Testing
ZOI:	Zone of inhibition

CFS:	Cell-free supernatant
BLIS:	Bacteriocin-like-inhibitory substance
E-coli:	*Escherichia coli*
S.aureus:	*Staphylococcus aureus*
K. pneumoniae :	*Klebsiella pneumoniae spp ozaena*
R. terrigena :	*Raoultella terrigena*
S.odorifera1 :	*Serratia odorifera1*
S. epidermidis :	*Staphylococcus epidermidis*
S. aureus :	*Staphylococcus aureus*
B. subtilis :	*Bacillus subtilis*
%:	Percentage.
°C:	Degree Celsius
µl :	Microlitre.
µm:	Micromètre.
h :	Hour
MIC:	Minimum inhibitory concentrations
MBC:	Minimum bactericidal concentration

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LITERATURE REVIEW

Lactic acid bacteria, frequently termed “the Lactics” were gradually accepted in the beginning of the 20th century (Carol et Leaon,2010) after perusing other terms as “milk souring” and “lactic acid producing” bacteria . Yet ,the general description of these microorganisms was later defined by Orla-Jensen (1919) as a “great natural group” thoroughly divided into seven genera based on various factors such as morphology (cocci or rods, tetrads), end-product formation from glucose fermentation (homo or heterofermentation) and growth at certain temperatures (Lars, 2000) .These various genera, species and even strains of LAB inhabit and cope with specific environments in order to exert dedicated or multiple specific functions as in converting carbohydrate substrates into organic acids (mainly lactic acid) and producing a wide range of metabolites according to their structural determinants and/or metabolic pathways (Fanny et al., 2018).

Lactic acid bacteria may be characterized as Gram-positive, aerobic to facultatively anaerobic, non-spore-forming rods and cocci (Khalid et Khalisanni, 2011) also oxidase, catalase, and gelatinase negative (Frank et Don, 2002) often capable of producing lactic acid as the sole, major or important end-product of carbohydrate catabolism by sugar fermentation with a non-respiring metabolism (Axelsson, 1998), along with synthesizing some growth inhibition substances such as bacteriocins, hydrogen peroxide and diacyls that prevent the proliferation of food spoilage bacteria and pathogens (De Vuyst et al., 2007; Alakomi et al.,2000). It is often added that these organisms are known as highly fastidious with high tolerance for low pH, generally non-motile and incapable of reducing nitrate under standard conditions (Lars, 2000). They are also incapable of synthesizing cytochromes and porphyrins (components of respiratory chains) which makes acid lactic bacteria devoid of “true” catalase and cytochrome molecules while growing in a laboratory growth media with no hematin or related compounds included (Alessandro et al., 2010). Accordingly, under these following conditions, lactic acid bacteria may rely on carbohydrates fermentation using endogenous carbon sources as the final electron instead of creating a proton gradient acceptor to generate ATP since they do not possess the mechanism of an electron transport chain (König et Fröhlich, 2009). Thus, this function may lead us to one important character of lactic bacteria which elaborates in their ability of growing under anaerobic conditions, yet they can also be defined as aerotolerant anaerobes (Michaela et al., 2009) since they are protected from oxygen by-products such as hydrogen peroxide by peroxidases (König et Fröhlich, 2009). Other different characteristics of LAB can be presented respectively by some genera, species, or strain as in secreting effector proteins, producing exopolysaccharides (EPS) generating biofilms, and adhering on abiotic and/or biologic surfaces, now that they greatly differ in morphology, optimal growth and tolerance temperature, salt and pH tolerance, metabolism and secreted molecule. (Lebeer et al., 2010;Gänzle et al., 2015).

Additionally, lactic acid bacteria can be classified as homofermentative or heterofermentative organisms based on their ability to ferment carbohydrates (Mokoena et al., 2017). Therefore two main sugar fermentation pathways can be distinguished, including Glycolysis (Embden-Meyerhof pathway) that results give almost exclusively lactic acid (<85%) as the sole end product of glucose fermentation under standard conditions of yielding two molecules of lactates from one glucose molecule, hence the metabolism is referred to as homolactic fermentation between some homofermentative species such as *Lactococcus* and *Streptococcus* (Derek et al.,2009). Whereas the pentose phosphate pathway alternatively, referred to as the phosphoketolase or phosphogluconate pathway, results in producing significant amounts of other end products besides lactic acid such as ethanol, acetate, and carbon dioxide CO_2 (Zúñiga et al., 1993; Mokoena et al., 2016). Subsequently, this metabolism is referred to as heterolactic fermentation, mainly performed by heterofermentative lactic acid bacteria such as *Leuconostoc*, *Wiessella*, and some *lactobacilli* (Salminen et al.,1998). However, the metabolic-consensus based on multiple growth conditions may significantly alter the production of multiple end-products, especially within hostile environments of a high pH scale that precedes pH 5.5–5.8, largely known as the growth optimum for LAB. Moreover, these microorganisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates which can attribute to an altered pyruvate metabolism and/or the use of external electron acceptors such as oxygen or organic compounds (Khalid et Khalisanni, 2011).

In this context , Lactic acid bacteria constitute a ubiquitous bacterial group sparsely widespread in nature within different niches and multiple ecosystems on earth (Liu et al., 2014), thus displaying dynamic interactions within animal and plant kingdoms in respect with other microbes such as mutualism, symbiosis, commensalism, or even parasitism-like behavior with their hosts. These highly heterogeneous phylogenetic groups can be dominant or sometimes in minority by dint of their physiological adaptation flexibility and the selective pressure exerted by these environments, which may present a key driver in the genomic diversity among LAB strains derived from distinct habitats (McAuliffe et al., 2018), hence they can exhibit multifaceted ecological and functional properties (Fanny et al., 2018). However some resident LAB permanently inhabit environments with a rich nutrition supply as in decomposing plant material and fruits, in dairy products, fermented meat and fish, beets, potatoes, mash, sauerkraut, sourdough, pickled vegetables, silage, beverages, plants, water, juices, sewage and in cavities (mouth, genital, intestinal and respiratory tract) of human and animals (Konig et al., 2017).

Studies on the microbial diversity of unexplored niches and environments have led to the isolation of an endless number of novel bacterial species, which may display special or unique technological and/or health-promoting properties (Di Cagno et al., 2006; Endo et al., 2017; Olofsson et al., 2014) , since they are regarded as generally recognized as safe (GRAS) due to their ubiquitous use in food and their unique role in the healthy micro flora of human mucosal surfaces (Fanny et al., 2018) .In part, the species of the genus *Lactococcus* are isolated from milk or vegetables, also being the natural reservoirs of most of its species (Bergy's, 2009) .Moreover, the majority of *Lactobacillus* species have been isolated from the gastrointestinal tract of humans and can also be found in several different environments as in milk and cheese (*Lb. casei subsp. casei, Lb. plantarum, Lb. curvatus, and Lb. brevis*), in fermented milk (*Lb. kefir, Lb. brevis, and Lb. fermentum*), in fermented vegetable products, marinades, silage, wine, and fresh or fermented meat (*Lb. brevis, Lb. Curvatus, Lb. buchneri, and Lb. san francisco*) (Demazeaud et al., 1996). Other species of the genus *Streptococcus*, are isolated from pasteurized milk, dairy equipment, and artisanal cultural starters. Species of the genus *Leuconostoc* are isolated from milk, dairy products, fruits, vegetables (especially beetroot), fermented vegetables (such as sauerkraut), bakery products, and viscous sugar solutions in sugar refineries. Species of the genus *Pediococcus* are mainly present in decomposing vegetables, sometimes in alcoholic beverages, milk, various types of cheese (Parmesan and Italian cheese), and culinary preparations such as sausages, salted anchovies, or soy sauce (Bekhouche et al., 2006).

With regard to this, an unequivocal definition of the term lactic acid bacteria, previously described as milk- souring bacteria, may not possibly purvey the totality of its phonotypical and physiological characteristics when it comes to subjecting an accurate classification. However, the ambiguity prevailing in the systematics at that time has retreated somewhat after the publication of a classic monograph "The Lactic Acid Bacteria (1919)" written by Orla-Jensa, where he regarded LAB bacteria as a "great natural group" and subsequently laid an enormous belief that the lactic group is phylogenetically related yet separated from other groups (Khalid, 2011). His assertion was proved through some adequate descriptions of practically all known species of this crucial group including some new species. Furthermore, these latter were classified in an admirably comprehensive system which facilitated the identification of these acid bacteria (Lars, 2000).

This first taxonomic outline given by Orla-Jensen was based on certain characteristics that entailed the following; cell morphology (cocci or rods, tetrad formation), mode of glucose fermentation (homo- or heterofermentation), growth at optimum temperature range (e.g., 10°C and 45°C), and the capacity to ferment sugar. This classification system thus recognized seven genera: *Betabacterium, Betacoccus, Microbacterium,*-

- *Streptobacterium,Streptococcus,Tetracoccus* and *Thermobactenum*, whereas only *Streptococcus* remains as a valid name until present (Lars, 2000). Nevertheless, the boundaries of these genera have been subject to some controversy between scientists, which eventually settled down into a common agreement emphasizing a core group that consists of four genera; *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Khalid, 2011). Notably, recent taxonomic revisions have proposed several new genera and the remaining group now comprises around 20 genera: *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactococcus, Oenococcus, Tetragenococcus, Vagococcus*, and *Weissella. Lactobacilli*, *Carnobacteria* and some *Weissella* are rods while the remaining genera are cocci. This reclassification was needed to fulfill the extent diversity of the original genus, which made it very arduous to classify, also distinguish between different lactobacilli. Thus, the new genera are *Lactobacillus*, *Paralactobacillus* and the 23 novel genera (Zheng et al., 2020). Yet, these genera had a firm standing in the systematics of lactic acid bacteria until the advent of molecular taxonomy in the 1970s and 1980s. From 1984 onwards, major taxonomic revisions have resulted in a further division of these genera. In addition, new genera related to the lactic group have been described (for thorough reviews on the current taxonomy of LAB, see Pot et al., 1994; Wood et Holzapfel, 1995). To sum up, the classification of lactic acid bacteria is mainly based on their morphology along with their mode of glucose fermentation and substrate spectrum which help identifying 3 distinct groups:

a. **Homolactic LAB:** This group includes bacteria that primarily produce lactic acid as the main metabolic product. The most common ones are *Lactobacillus* and *Streptococcus*.
b. **Heterolactic LAB:** This group includes bacteria that produce lactic acid along with other metabolic products such as ethanol, acetic acid, and carbon dioxide. The most common ones are *Leuconostoc*, *Weissella*, and *Oenococcus* as well as some species belonging to the genus *Lactobacillus*.
c. **Heterofermentative LAB:** This group includes bacteria that produce a range of metabolic products such as lactic acid, ethanol, acetic acid, and carbon dioxide .That may incorporate some species belonging to the genus Lactobacillus and the majority of species belonging to the genera *Enterococcus*, *Lactococcus*, and *Streptococcus*. This latter, which occupies an intermediate position between groups I and II, contains species capable of being either homo- or heterofermentative depending on environmental conditions (McLeod et al., 2008).

The conventional approach to lactic acid bacteria classification was based on physiological and biochemical characteristics. However, more recently, a large number of

formers chose to adapt miscellaneous molecular techniques in order to enable a consistent identification of individual strains. (Buddhiman et al., 2008).In fact, the current knowledge on the phylogenetic relatedness of LAB bacteria is mainly based upon comparative sequence analysis of 16s ribosomal ribonucleic acid, random amplified polymorphic DNA profiling, PCR-based fingerprinting, and soluble protein patterns. Yet, the availability of 16S rRNA sequences is believed to be more accurate in defining relationships and phylogenetic positions, since the sequence contains both well-conserved and less conserved regions. That may also emerge in designing some species' specific oligonucleotide probes which were previously meant to be exemplified through the descriptions of new genera (Seppo et al., 2004) .Still, it is imperative to keep in mind that the analyses of these different molecular factors may sporadically lead to obtaining similar phylogenetic trees owing to 16s rRNA derived tree, which happen to reflect the organismal phylogeny and not only the history of a single gene (Karl et al., 1995).With this technique, a clearer picture of phylogeny of lactic acid bacteria is emerging, and the ideas of Orla-Jensen can be examined with more accuracy.

Consequently, Woese and Fox introduced molecular phylogeny in 1977 based on the sequence of ribosomal RNA. This method revolutionized the taxonomy of bacteria, and the preliminary classification of LAB was profoundly modified (Bechachh et Bouderhm, 2020). Other genotypic methods (based on nucleic acids) are also used in classification, such as GC percentage or DNA/DNA hybridization which were applied extensively to differ between groups of closely related strains and to clarify much of the confusion in the systematics prevailing at that time (Mechai, 2009). Accordingly many DNA homology values tend to confer the basis for the definition of species as the basic unit of bacterial taxonomy. This range of classification criteria is quite representative of the diversity of lactic acid bacteria. As early as 1974, according to the Bergey's Manual, lactic acid bacteria were divided into two families: *Streptococcaceae* and *Lactobacillaceae*. In 1985, Schleifer proposed the division of streptococci into 4 genetically distinct genera: *Streptococcus, Enterococcus, Vagococcus,* and *Lactococcus* (Makhlouf et al., 2018). However the genera that, in most respects, fit the general description of the typical LAB are *Aerococcus* , *Lactobacillus* , *Leuconostoc* , *Pediococcus,* and *Streptococcus,* where massive revisions were anticipated in Bergey's Manual of 1986 since this edition represent various continuum with the historical tradition dating back to the work of Orla-Jensen .Yet, revisions after 1986 are basically supported by extensive chemotaxonomic and genetic data (Seppo et al., 2004).

In essence, phylogenetic trees derived from comparative sequence analyses of other conserved macromolecules such as 23s rRNA are constantly in good agreement with the conclusions drawn from the rRNA data previousy provided. Based on these data the bacterial world can be divided into at least 11 major lines of descent or phyla whereas the Gram-

positive bacteria including LAB offer two subdivisions of relevant descent .The so called *Clostridium* subdivision with a DNA base composition of generally less than 50% mol guanine plus cytosine (G+C).It is within this subdivision that most lactic acid bacteria are found (Firmicutes phylum) , However the *Actinomycetes* subdivision groups Gram-positive bacteria with high-GC content (Avagodo, 2004), along with *Btyidobacterium*, *Propionibacterium* and *Brevibacterium*. In fact, the typical LAB belong to the low G + C phylum and the GC% percentage of their DNA gives a fairly similar composition for the *Lactococcus* (34.46%), *Leuconostoc* (36.43%), *Pediococcus* (34.42%), and *Bifidobacterium* (67%) genera, while the *Lactobacillus* genus is characterized by great heterogeneity (32.53%). Except for *bifidobacteria*, all the genera mentioned above belong to the phylum with low G/C content (50%) (Mahi, 2010).

Lactic acid bacteria are known for their immense capability of preventing growth of numerous pathogens along with possessing some relevant probiotic traits that could be used in a large set of technological and functional properties within the food industry (Agnieszka et Barbara, 2022), which could enhance microbial safety and improve the sensory attributes of various foods (Naidu et al., 1999;Todorov et al., 2009). However, in recent years, a paucity of literature has showed more scientific attention to the ability of some LAB strains of inhibiting foodborne pathogens including bacteria, yeast and filamentous fungi, especially during the current era where a lot of consumers are tending to be more health conscious when it comes to their food choices. In this regard, the current brand new technologies are starting to enable bio prospecting for LAB strains with some robust antimicrobials agents in order to prevail an opportunity to maximize their contribution in food and nutrition settings (Mokoena et al., 2021).

Additionally , LAB can largely contribute in bacterial antagonism during their growth and fermentation process since they are considered to be safe (GRAS), along with their ability to impede the growth of Gram-negative and Gram positive bacteria, as well as yeast and molds in several food products .Therefore, they are largely known to produce a wide range of antimicrobial substances, including hydrogen peroxide, lactic acid, acetic acid and low molecular weight substances (diacetyl, fatty acids, reuterin, reutericyclin), antifungal compounds (phenyl lactate, propionate, hydroxyphenyl lactate) and bacteriocins (Castellano et al., 2017). In this regard, organic acids may be referred to as the main products of LAB metabolism with non-specific antimicrobial effects, generally produced by two significant pathways based on the assimilation of pyruvate molecules. This latter may lead to the formation of only lactic acid in homofermentative bacteria as the sole main-product, yet a wide range of lactic acids may be relevant when it comes to heterofermentative strains such as acetic acid, propionic acid, formate and succinate, mainly synthesized by some species of

Acetobacter aceti, Propionibacterium, and Lactobacillus (Liu, 2003). The antimicrobial properties of these organic acids had been attributed to dissociated molecules that are deprotonated upon entry into cellular membranes, causing cellular injury due to the concerted effect of both dissociated ions and non-dissociated molecules. As a result we can elaborate their action mode in further contexts (Pradhan et Kadyan, 2020) mainly based on various trials as in:

- The destabilization of the membrane.
- The inhibition of the synthesis of cell wall enzymes.
- The interference of proton gradients.
- The induction of the formation of reactive oxygen species, thus increasing oxidative stress within the cell.

However, the principal action of LAB lactic acids against pathogenic microflora is mainly based on decreasing the pH of the medium in which they multiply in to some considerable amounts by inhibiting a part of the flora that develops there. Indeed, their competitiveness is gradually improving given their high tolerance to low extra- and intracellular pHs , where other strains convey an antagonistic effect against the development of bacteria, yeasts and filamentous fungi by producing a wide range of organic acids, which results from the action of their non-dissociated form that can passively cross the membrane and acidify the cytoplasm by releasing the proton, thereby affecting its cellular metabolism by inhibiting certain functions (Klaenhammer et al., 1993; Brul et al., 1999; Cotter et al., 2003)**.** Between all these vulnerable organisms we can identify *Salmonella spp* which are intolerant of low pH, and their optimal growth remains in the 4.0–9.0 range (Daliri et al., 2020).

Furthermore, organic acids are one of the classic food preservation agents (Brul et Coote, 1999) and are recognized as food additives. Commonly used acids are benzoic, sorbic, acetic, fumaric, propionic, and lactic acids as a way of preventing or delaying the growth of food-degrading bacteria. Be that as it may, the main challenge resulting from their use is the high concentration required to inhibit pathogenic or undesirable bacteria, which is sometimes unacceptable to the consumer. Indeed, the minimal inhibitory concentration, which is the smallest amount of acid that can prevent the growth of a microorganism, must be determined under some specific conditions of pH, as well as water activity and temperature (Zouari et Benarfa,2018). Still, it may vary between each microorganism to be largely inhibited, where we can deduce the fact that an acetic acid concentration of 0.105 g/L would statistically inhibit the growth of *Bacillus subtilis* at a pH of 5.3, while a concentration of 27.5 g/L is required to inhibit *Lactobacillus plantarum* under the same conditions. Moreover, *Listeria monocytogenes* can be inhibited by lactic acid at 9.0 g/L and a pH of 3.7, while hydrochloric

acid convey a larger effect at a pH of 3 under the same concentration (Gravesen et al., 2004). Likewise, pathogenic bacteria can develop certain resistance mechanisms called "acid tolerance response" in response to exposure to low pHs. These mechanisms are also useful for them to survive in the intestinal transit like *L. monocytogenes* that can survive to an emit exposure of a pH concentration up to pH 3 for straight 60 minutes (Brul et al., 1999; Gahan et al., 1999; Cotter et al., 2003).

Equally important, we can distinct many other small molecules produced by lactic acid bacteria LAB that can exhibit antimicrobial effects, such as diacetyl, hydrogen peroxide (H_2O_2), and reuterin (Hertzberger et al., 2014). For instance, cellular compounds including the membrane proteins in Gram positive and Gram-negative bacteria are known to be destroyed by highly effective antimicrobial compounds, mainly generated in the presence of (H_2O_2) and lactoperoxidase enzymes in raw milk due to a plain oxidation of the SH group which is also known as the stimulation of the lactoperoxidase-thiocyanate system (brahim et Ayivi, 2021). The antimicrobial agent generated of this process is called hypothiocyanite anion, yet its bacteriostatic activity can be more pronounced when it is combined synergistically with heat (Nakajima et al., 2003). In this context, LAB may synthesize hydrogen peroxide (H_2O_2) under aerobic conditions in the absence of intracellular catalase, pseudocatalase, or peroxidase (brahim et Ayivi, 2021), mainly through the action of the flavoprotein oxidase nicotinamide adenine hydroxyperoxydase dinucleotide (NADH). This reaction is conducted by transforming molecular oxygen (O_2) into excited superoxide, hydrogen peroxide (H_2O_2), or water (H_2O) by using some specific enzymes, usually found in strains of *Streptococcus, Lactococcus, Lactobacillus, Leuconostoc,* and *Pediococcus* (Condon et al., 1987). In fact, the antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturation of a number of enzymes and peroxidation of membrane lipids, thereby triggering an increase in membrane permeability (Kong et Davison, 1980). Over and above that, hydrogen peroxide can also act as a precursor of bactericidal free radicals production such as superoxide (O_2) and hydroxyl radicals (OH) that can damage DNA molecules. On the other hand, H_2O_2 has the ability to activate the lactoperoxidase system in raw milk, producing hypothiocyanite ($OSCN^-$), higher oxyacids (O_2SCN^-) and (O_3SCN^-), as well as other intermediate oxidation products that are considered as inhibitors of a wide range of $Gram^+$ and $Gram^-$ bacteria. Furthermore, H_2O_2 can accumulate in large amounts in order to inhibit the action of some microorganisms (Condon, 1987). This accumulation results from an imbalance between synthesis and degradation pathways of lactic acid bacteria. Likewise, it has been lately proven that the production of H_2O_2 by *Lactobacillus* and *Lactococcus* inhibits the growth of *Staphylococcus aureus, Pseudomonas sp* and various psychrotrophic microorganisms (Davidson et al., 1983) by the mediation of some strong oxidation effects on

both membrane lipids and cellular proteins (Lindgren et Dobrogosz, 1990). After all, the amount of hydrogen peroxide produced by lactic acid bacteria depends largely on the strain and the availability of oxygen in the environment (Helander et al., 1997).

Being primarily produced by hetero fermentative lactic acid bacteria, CO_2 can also be considered as a robust antimicrobial agent. Although, the exact mechanism is still unknown and need further research by some professional experts, it is mostly claimed that CO_2 fulfills an antimicrobial function by creating an anaerobic environment that prevents enzymatic decarboxylation along with inducing a major dysfunction of permeability in the outer membrane of numerous bacteria due to its constant accumulation in the specific growth medium (Eklund et al., 1984). This highly potent bio preservative can also prevent the growth of many spoilage microorganisms, particularly Gram-negative psychrotrophic bacteria through a delicate degree of inhibition that varies considerably among species. Thereby, it has been postulated from this standpoint process that an exact level of 10% could decrease bacterial population by 50%, also can precede an antifungal activity in most cases (Lindgren et Dobrogosz , 1990).

Not to mention, reuterin (β-hydroxypropionialdehyde) is a low-molecular-weight, non proteinaceous, soluble, pH-neutral compound synthesized as an intermediate metabolite during the anaerobic fermentation of glycerol by certain strains of heterofermentative *Lactobacillus*, mainly *l.ruteri* (Kim et al., 2008) along with other non-lactic bacterial genera such as *Bacillus, Klebsiella, Citrobacter, Enterobacter, and Clostridium.* Therefore, the fermentation of glycerol may occurs in two leading steps , briefly recapped in dehydrating this molecule by a "glycerol dehydratase" to form reuterin, which is then reduced to 1,3-propanediol by an oxidoreductase until reuterin can finally accumulates in the producing microorganism (Zouari et Benarfa, 2018). At high concentrations, it is excreted into the medium due to its toxicity against the producing cell limits; still some species can be more resistant to these unfavorable conditions, such as *Lactobacillus reuteri*, by synthesizing reterine as an antimicrobial compound with a broad spectrum of inhibitory activity against species of Gram-negative and Gram-positive bacteria including *Salmonella, Shigella, Clostridium, Staphylococcus, Listeria, Candida and Trypanosoma* as well as other organisms as in yeast, fungi and protozoa (Michael et al., 1988; Yasin et al., 1996) which shows its firm activity against foodborne pathogens and spoilage microorganisms (Axelson et al., 1989) . To be clear, the use of reuterin to control Gram-positive and Gram-negative foodborne pathogens has been previously examined in milk and dairy products (El-Ziney et al.,1998; Arqués, 2008) where various productive strains are able to generate this main proxy in cheese and yogurt after being added as steady adjuncts to the cultural starter (Arqués et al., 2008).Interestingly, reuterin bears several properties notably, being water soluble also capable of resisting an

array of proteolytic and lipolytic enzymes (Rasch et al., 2007), where it maintains its antimicrobial activity at a broad working pH range (Asare et al., 2020) and has been therefore studied as a food preservative or auxiliary therapeutic agent (Vollenweider et al., 2004; Axelson et al., 1989; El-Ziney et al., 1999). Reuterin is also believed to play a role in the probiotic effects of *Lb. reuteri* SD2112, against a representative panel of intestinal bacteria that may cause some detrimental effects of certain microbiological, chemical and physical stressors (Casas et Dobrogosz, 2000). However, its mode of action on microbial growth has not been elucidated due to the high complexity of the HPA-system chemistry (Vollenweider et Lacroix, 2004). It has been assumed that reuterin might inhibit the activity of a bacterial enzyme that catalyzes the first step in DNA synthesis, also known as ribonucleotide reductase, by competing with either ribonucleotides for the binding sites, or else by pursuing a selective reaction (3-HPA) with unstable sulfhydryl groups of ribonucleotide reductase or with thioredoxin which is required for enzymatic activity. The inhibition of the conversion of ribonucleotides to deoxyribonucleotides would explain the broad-spectrum activity of reuterin (Dobrogosz et al., 1989). Markedly, it has also been suggested that the mechanism of action of reuterin might be directed towards Sulfhydryl enzymes, based on the assumption that the tolerance of bacteria to reuterin might be associated with intracellular levels of LMW-SH such as glutathione (Valentine et al., 2007).

Another group of potent antimicrobial peptides synthesized by selected LAB species is called bacteriocins. They are defined as small cationic molecules of about 30–60 amino acids strictly forming amphiphilic helices and rather stable at a high temperature of 100C for 10 min straight, which makes them different in their overall properties as in the spectrum of activity, mode of action, molecular weight (MW), genetic origin and some biochemical traits. Indeed, these weaponry compounds are primarily active against closely related organisms, mostly Gram-positive bacteria to gain competitive advantage for nutrients in the environment after being innocuous to the industrial producing strains. Thereby, various industries have been trying to exert bacteriocins as bio control agents to prevent the deterioration of minimally processed food items with the benefit of shelf life extension and prevention of economic loss (Sidhu et Nehra, 2019) since they can selectively pursue an antimicrobial defense against copious bacterial food pathogens on a nanomolar scale, thus implying their QPS status to guarantee the safety of consumers (Field et al., 2018; Ozel et al., 2019). The QPS system , also known as the presumption status of safety, bear some generic aspects mainly elaborated through a strict evaluation which declare four cardinal points of some safety profiles that explain the reasons why LAB byproducts are considered ideal to be used within the food industry. In this context, we can pin point the following reasons:

- They are categorized by the FDA as GRAS (Administration, 1988).
- They are odorless and colorless.
- They do not impact the organoleptic and sensorial characteristics of food.
- Unlike traditional antibiotics, bacteriocins are cleared by proteolytic enzymes since they have the potential to be bioengineered (Perez et al., 2014).

Consequently, numerous types of bacteriocins with potential industrial applications have been isolated and characterized, namely lantibiotic nisin (E234) and pediocin PA-1/AcH which have been approved by the FDA to be commercialized in the food supply chain as preservative agents ,especially when the cold chain falters (Pérez et al., 2021). Albeit, a bunch of experts in the 1990s have aimed to fetch for other active molecules used against foodborne pathogens by conducting some considerable trails on homemade fermented vegetable (Narvhus et Axelsson, 2003). As a result, an antibacterial substances has been identified after noticing a robust activity against both Gram-positive and importantly, Gram-negative common foodborne bacterial pathogens. This broad spectrum of inhibition suggests that these LAB strains have an interesting potential as natural bio preservatives in various food products (Mokoena et Mduduzi, 2017) due to their nontoxicity towards human organisms, thermal stability, protein nature and antagonistic effect towards the majority of opportunistic and pathogenic bacteria including antibiotic-resistant strains (Ayivi et al., 2020; Ananou et al., 2007) . In this regard, several bacteriocins have showed their synergy with antibiotics in order to reduce the ingested concentration that may bear some strict negative side effects in the digestive system. Moreover, a synergistic activity with other biomolecules such as citric acid and nisin has also been applied against *Listeria monocytogenes* and *Staphylococcus aureus* (Soltani et al., 2021) where they showed a noticeable resistance to bacteriocins, still it is always considered minimal compared to the conventional antibiotics' resistance.

Alternatively, a larger number of bacteriocins molecules that are ribosomally synthesized after post-translational modification are generally referred to as thermostable peptides substances featuring a myriad of antimicrobial properties that have been thoroughly characterized with the focus on their antibacterial activity against closely related microbial strains. Per contra, the genetics of their biosynthesis has been constantly elucidated since dubitable theories are still being established to date. It is presumed that the upshot of bacteriocins is predominantly based on the binding of phosphate residues on the cell membranes of target cells, thus, creating pores that permit the activation of autolysin which degrades the bacterial cell walls (Papagianni et Anastasiadou, 2009). Yet, bacteriocins' synthesis is practically based on a ribosomal machinery of LAB bacteria, where encoding genes are located in operons in plasmids, chromosome and other genetic organelles (Hernández et al., 2021).This expression is either regulated by external induction factors,

usually secreted by the producer strain itself (Dimov et al., 2005) causing a change in transmembrane pH gradient along with a full on dissipation of the cytoplasmic membrane potential that intrigues the cell membrane's integrity. Regardless of the taken approach, the consequence would embody cell content loss and eventually cell death. Still, the mechanisms of other bacteriocins are still ambiguous; hence further research is required in the field (Zimmerman et al., 2021). Classification of bacteriocins is based on their primary structures, molecular weights, post-translational modifications and genetic characteristics (Mokoena et Mduduzi, 2017). However, there is no universally adopted classification scheme. Originally, four classes were recognized, and were later revised into three (Liu et al., 2014). Lantibiotics represent class I bacteriocins including nisin and lactocin as main substitutes that enhance their post-translational modification (Parada et al., 2007). Class II bacteriocins consists of small (<10 kDa) heat-stable and non-modified cationic peptides, sub-divided into Class IIa and Class IIb as active proxies (Perez et al., 2014). Individual Class III bacteriocins are defined as heat-labile proteins (>30 kDa), whereas the former Class IV bacteriocins are constituted of variant complexes of proteins that has been de-established, which leaves as at only three classes of bacteriocins based on the genetic and biochemical characteristics of their members (Güllüce et al ., 2013).

Early work on LAB was mainly concentrated on their great contribution in enhancing microbial safety and improving the sensory attributes of various food raw materials within the dairy industry, thereby many species have evolved as a novel wave of antagonists against some foodborne viruses (rotaviruses, noroviruses, caliciviruses, and coronaviruses) either through the mediation of their metabolites or competitive inhibition of the viral cycle (Fang et al., 2019; Daliri et al., 2020).In light of this, LAB have been extensively used as starter cultures, probiotics, and in the production of interesting compounds (i.e., nutraceuticals) due to their versatile metabolism that helped them play a significant role as predominant microbiota in preservation and fermentation processes. (Naeem et al., 2012; Emerenini et al., 2013; Ruiz et al., 2017).From bygone eras, lactic acid bacteria (LAB) have underlined a wide range of beneficial applications that date back to 6000 BC, along with distinct nutritional and therapeutic properties that coupled with adaptive features, which substantially enabled the bacteria to easily thrive in disparate environments such as in dairy-based foods, fermented foods, vegetables as well as in the human gut (Bintsis et al., 2018). During fermentation, a carbon source is dissimilated by microorganisms after yielding ATP energy without net oxidation in order to synthesize primary end products of microbial fermentation such as alcohols and organic acids (Sieuwerts et al., 2016) .These chief metabolites may prevent food spoilage and impact certain organoleptic properties that may increase the digestibility of raw materials subject to the process of consumer preferences, especially when it comes to

undertaking a fully balanced diet based on the sustainable use of natural ingredients as preservatives instead of chemicals (Asioli et al., 2017) hence the need to validate their potential as starter cultures. This latter is defined as a microbial preparation of large numbers of cells added to a raw material in order to produce a fermented food, thus consequently enhancing the overall flavor and texture of the final product (Hati et al., 2013).Moreover, Lactic acid bacteria have been extensively used in food preservation commonly referred to as bio-preservation after showing a pivotal role in prolonging the shelf life of perishable raw materials through active metabolites. For instance, bacteriocins may serve as inhibitory agents against foodborne pathogens, thus ensuring food safety on a general basis.

Conversely, later research on lactic acid bacteria has ratified the probiotic properties possessed by some specific strain where the advent of functional food places more emphasis on seeking alternatives to limit the use of medications thus promoting the regular consumption of fermented foods. In accordance with the WHO (World Health Organization) definition, probiotics are stated as live organisms that confer a positive effect on the host's organism when administered in adequate amounts (Agnieszka et Barbara., 2022). In this sense, they are known to exhibit a favorable impact on the human immune system, thereby reducing the risk of carcinogenesis and other disease conditions including gastrointestinal problems by preventing pathogenic microbes from adhering to the intestinal epithelium. Furthermore, they participate in the biosynthesis of vitamins, and the metabolites produced to regulate the homeostasis of the gastrointestinal system (Zommiti et al., 2020; Srednicka et al., 2021). To date, LAB represents the preponderance of the available commercial probiotics, mainly of *bifidobacteria* and the *Lactobacillus* genera. Yet, only a limited number of *leuconostocs, pediococci, lactococci, enterococci,* and *streptococci* have emerged in the industrial field as probiotics.

Since ancient times, natural compounds have been the basis of health care in the world and still draw attention in the traditional and contemporary attempts (Albayrak et Silahtarlıoğlu, 2020). It is still unknown whether the essential oils were practically used as healing proxies or only restricted for domestic use, but numerous studies prevailed that their sensory acceptability emphasizes a global status as in 'Generally Recognized as Safe' (GRAS) (Lambert et al., 2021; Roller et al., 2002; Rodríguez et al., 2009; Zinoviadou et al., 2009).This latter may aid in the impartation of essential oil antimicrobial benefits without negative flavor profile modification (Zinoviadou et al., 2009) along with presuming a wide selection of potent compounds , mainly characterized by their therapeutic or odoriferous properties that have been exploited for medicinal uses and food preservation for several years. Consequently, essential oils are defined as highly concentrated oily liquids, also known as intense volatile aromatic compounds, extracted from various organs of natural raw plants

including their leaves, stems, roots, seeds, barks, resins, or fruit rinds. Each of these complex precious liquids is obtained from a particular plant species, originating in certain regions with particular environmental conditions and neighboring fauna and flora (Melese et al., 2022). However, unlike fatty oils, the amount of volatile essential oils found in these plants can be anywhere from 0.01 percent to 10 percent of the total (Liu et al.,2009) which brings a bunch of researchers to upgrade the aromatic herbs extraction from relayed traditional methods as in hydro distillation, steam distillation, or solvent extraction, to less energy and solvent consuming physical techniques since they can induce thermal degradation, hydrolysis and water solubilization of some fragrance constituents (Guan et al., 2007).This latter can be briefly resumed in three chief processes, basically referred to as distillation (steam, steam/water and water), expression (also known as cold pressing), or dry distillation of natural materials (Ferhat et al., 2007) along with microwave assisted hydro distillation (MAH) and Clevenger hydro distillation (CH) which have been widely used for commercial scale production due to their important yield activity.

Essential oils represent a potent source of numerous bioactive compounds both polar and non-polar, commonly known for their antimicrobial (Andrade et Barbosa, 2014), insecticidal (Abd-Elhady,2012), and antioxidant (Gürsoy et al., 2012) properties , thereby serving as therapeutic constituents in a wide range of areas including medicine, food, pharmacology and animal husbandry .The most prominent feature of these components giving the herbaceous odor and taste, is to be volatile and fragrant at room temperature (Grassmann et Elstner, 2003), which leaves us at a full spectrum of sole agents that acts as homogeneous matrixes, such as hydrocarbon and oxygenated sesquiterpenes (De Falco et al., 2013) and, in some instances, aliphatic terpenes (2,4-dimethyl hexane) as in *Ruta* type (Zellagui et al., 2012). Apart from the bioactive molecules, essential oils are generally composed of tolerable terpenoids similarly to alcohols and aldehydes (Ali et al., 2015), along with toxic terpenes like ketones (Knobloch et al., 1989). Indeed, a variety of ketones with different structures are present in variable proportions in the chemical compositions of essential oils of some species like *Absinthe* (Rezaeinodehi et Khangholi., 2008), *Artemisia* (Mohammadhosseini et al., 2017), *Salvia officinalis L* (Khalil et Li, 2011), *Peppermint* (El-Ghorab et al.,2006), and *Ruta* (Boutoumi et al., 2009). Overall, the difference in the chemical composition of Eos would practically emphasize variant biological properties, for instance many of these oils are most effective against Gram-positive organisms, though some are more antagonistic towards Gram-negative species (Ouattara et al.,1997; Skandamis et al., 2002 ; Holley et al., 2005). Additional derivatives of these oils may be non-phenolic in nature but still exhibit profound antimicrobial activity, such as allylisothiocyanate, which may be used against Gram-negative bacteria and fungi (Kyung et Fleming, 1997; Lin et al., 2000). Henceforth, a lot of food

industries are exhibiting a wide spectrum of usage when it comes to essential oils since they are considered as ideal additives to food formulations in order to assure the quality and shelf-life of a clean label. However, these compounds are yet to be evaluated for their activity against common food spoilage organisms such as lactic acid bacteria, which favored their adoption on a large manufacturing basis (Laurel et al., 2016). Accordingly, few studies have regarded the biological effects of these extracted molecules in a wide variety of tasks especially in the pharmaceutical field, including the following:

a. **Anti-inflammatory and anti-oxidant activity:** essential oils can widely serve as some potential natural antioxidants, mainly used to prevent lipid oxidation in food systems due to their specific structures that happen to mimic the one of phenols molecules. This function can be explained by several modes of action like the prevention of chain initiation, free radical scavengers, reducing agents, termination of peroxides, prevention of continued hydrogen abstraction as well as quenchers of singlet oxygen formation and binding of transition metal ion catalysts (Phakawat et Soottawat, 2014).
b. **Anti-bacterial activity:** Strong evidence in vitro suggests that essential oils can prevent a wide range of pathogenic bacteria, including *Listeria monocytogenes*, *Listeria innocua*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Shigella dysenteria*, *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella typhimurium* (Lammari et al., 2020).
c. **Anti-microbial activity and wound healing:** the presence of certain components, notably sesquiterpenes, specifies the antibacterial activity of essential oils against a wide spectrum of strains along with accelerating the process of wound healing (Pradeep et Smita, 2022).
d. **Role of Essential Oils as Food Additives:** Eos are known to act as natural additives. Yet due to the unique smell associated with the volatiles, their use is exclusively limited in some types of foods since it may alter their typical smell and flavor (Phakawat et Soottawat, 2014).

On that account, researchers have envisaged the use of essential oils alone or in combination with other traditional or emerging food preservation technologies (e.g., salting, acidification, mild heat, pulsed electric fields, high hydrostatic pressure, ultrasound and cold plasma) to ensure the desired microbial control in foods, since it was to found to have some potential practical application for food industry especially as safe food preservatives that happens to be assured from regulatory authorities to be commercially employed on a continuous basis (Evandro et al., 2021).

Finally, the main objective of this research is to evaluate the potential of antimicrobial substance-producing lactic acid bacteria LAB along with exploring the inhibitory effect of some medicinal plants (*Ruta montana*, *Artemisia herba*) against extended-spectrum beta-lactamase-producing *Enterobacteriaceae* strains, commonly referred to as ESBLs. It also aims to:

- Investigate the antimicrobial activity of antimicrobial substance-producing lactic acid bacteria against multidrug-resistant Gram-negative strains (ESBL) and reference Gram-positive strains.
- Revealing the spectrum of action of antimicrobial molecules produced by lactic acid bacteria.

EXPERIMENTAL PART

MATERIALS AND METHODS

1. Study framework and objectives:

This study is based on the description of various experimental materials and methods conducted in vitro over a period of four months, at the microbiology laboratory, Department of Applied Biology, Larbi Tebessi University -Tebessa- where a bunch of trail essays were conducted on a wide range of bacterial strains in order to assess the antimicrobial activity of antimicrobial substance-producing lactic acid bacteria (LAB), along with exploring the inhibitory properties of *Artemisia herba* and *Ruta montana* essential oil against multidrug-resistant Gram-negative strains (ESBLs).

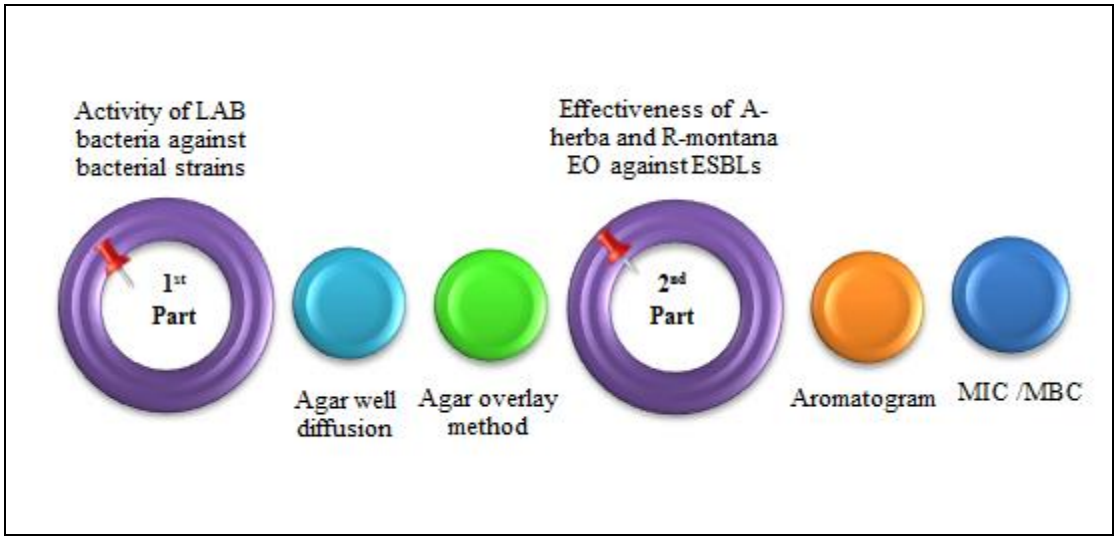

Figure 1: Resuming scheme of the study plan conducted at the laboratory.

2. Materials and methods:

2.1. Material:

2.1.1. Non-biological materials:

a. Culture Media:

- De Man, Rogosa and Sharpe agar (MRS).
- Mueller Hinton Agar medium (MH).
- Mueller Hinton Broth (MH).
- Nutrient Broth (NB).

b. Laboratory Equipment:

- Incubator (37°C).
- Autoclave.
- Refrigerator.

c. Extraction of Essential Oils:

- Clevenger-type hydrodistillation apparatus.

- Heating flask.
- Round-bottom glass flask (1 liter).
- Condenser.
- Glass jars.

d. Antibacterial Assays:

- Petri dishes.
- Paper discs.
- Microwell plates (96 wells).
- Sterile swabs.
- Micropipettes.

2.1.2. Biological materials:

a. Plant material:

Within the study process, an amount of 1 kg of *Artemisia herba* and *Ruta montana* leaves were collected in the region of El Ogla (Tebessa. Algeria) (Figure 2) during the month of May and then dried in the shade for a week at room temperature so as to ultimately obtain a biological product ready for extraction of both essential oil with the hydro-distillation method, in order to conduct the coming up assay trials of our study.

Figure 2: Location map of sampling and the harvest of the wormwood leaves *(Artemisia herba)* and Montain Rue *(Ruta Montana)* from a homogeneous population.

- **Description of *Artemisia herba*:**

Artemisia herba, the white wormwood, is a perennial shrub in the genus Artemisia that grows commonly on the dry steppes from the upper semi-arid to the lower Saharan of the Mediterranean regions in Northern Africa (Saharan Maghreb), Western Asia (Arabian Peninsula) and Southwestern Europe (Abu-Darwish et al., 2015). It is characterized by a wide range of morphological and phytochemical variability as in strongly aromatic leaves covered with fine glandular hairs that reflect sunlight giving a grayish aspect to the shrub, along with sessile head flowers with different tapering

bases (Pottier et al., 1979), mainly associated with different geographical origins of the samples. Hence, the wide traditional use of this herb for diverse diseases as an antiseptic and antispasmodic may be explained in the next test trials in vitro.

- **Description of *Ruta montana* :**

Ruta montana is a medicinal and aromatic plant (MAP) which consists of an evergreen shrub with broad leaves and capsule fruit that can grow up to 0.30 meters tall. It is mainly harvested from the wild for local use as a source of materials and also as an antiseptic agent in folk medicine, especially in North Africa, to treat digestive, infectious, respiratory, neurological, gynecological, and diabetic diseases. Hence this current study aims to review the antimicrobial activity of its essential oil in order to provide data support for further investigations through in-depth in vivo biological studies and assay trials, which are subsequently needed to transmute the traditional applications of RM-EO into scientific-based information.

Table 1: Presentaion of herbal plants used for the hydrodistillation extraction.

Botanical name	Common name	Family	Organ used
Artemisia herba	▪ **Arabic:** "shīeḥ" ▪ **French:** "Armoise herbe blanche" ▪ **English:** "Desert or white wormwood"	Asteraceae	Leaves
Ruta montana	▪ **Arabic**: "Fidjel, Fidjela ,djebeli" ▪ **French**: "Rue des montagnes" ▪ **English** : "Mountain Rue"	Rutacea	Leaves

b. Bacterial Strains and their Origins:

The indicator strains (strains on which the inhibitory action of bacteria producing antibacterial factors is tested) were isolated and identified by the doctoral student Ms Amra Amel at the Molecular Bioactive Compounds and Applications Research Laboratory; *E. coli1* (S8E14 Merg) isolated from merguez, *Klebsiella pneumoniae ssp ozaneae* (S19 E21 Merg) isolated from merguez, *Raoultella terrigena* (S15 E23 milk) isolated from raw milk, *Serracia odorefira*1 (S43 E9 AS VP) isolated from chicken meat. These strains are also multidrug-resistant and produce extended-spectrum beta-lactamases (ESBLs). However, the reference strains used are *Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 35984, and *Bacillus subtilis* ATCC 9372 that belong to our laboratory's collection.

c. Origin of Lactic Acid Bacterial Strains and their Identification:

The seven lactic acid strains (LAB) used in our study were isolated and identified by Ms Metrouh Roumaissa as part of a doctoral thesis in science (LMD) under the supervision of Prof.

Mechai Abdelbasset and Dr. Debabza Manel. Importantly, these lactic acid strains are identified at the species and strain level using the API 50 CHL biochemical gallery and the Maldi-Tof Ms molecular technique.

Table 2: Origin and identification of lactic acid bacteria (LAB)

Nb	Old code	New code	Isolation origin	Presumed species.
01	126 R	JMQ24	Klila	*Lactobacillus paracasei*
02	100 R	KMQ31	Jben	*Enterococcus durans*
03	136R	JMZ02	Klila	*Pediococcus pentosaceus*
04	138 R	ULZ15	Jben	*Pediococcus pentosaceus*
05	EnK107	KMZ 10	Jben	*Enterococcus faecium*
06	LcJ45	QMZ 11	Klila	*Lactococcus lactis sp lactis*
07	EnD119	BZQ 36	Jben	*Enterococcus durans*

Table 3: ESBL strains and their distinct antibiogram profile.

S: sensitive, **R:** resistant, **I**: intermediate.

Antibiogram profile																							
Bacterial strain	**B-Lactam**													**Aminosides**		**Quinolones**			**Others**				**phenotype of resistance**
	AMX	**PRL**	**TIC**	**TTC**	**CL**	**FOX**	**AMC**	**CAZ**	**CTX**	**FEP**	**ATM**	**IPM**	**ETP**	**GM**	**AK**	**NA**	**OFX**	**CIP**	**C**	**NIT**	**FOS**	**COT**	
Escherichia coli (S8.E14.Merguez)	R	R	R	R	R	S	R	R	R	R	R	S	S	S	S	R	R	R	R	S	S	R	Extended-spectrum beta-lactamases (ESBLs)
Klebsiella pneumoniae spp ozaena (S19.E21. Merguez)	R	R	R	R	R	S	S	R	R	R	R	S	S	S	S	S	R	S	S	S	S	R	Extended-spectrum beta-lactamases (ESBLs)
Raoultella terrigena (S15.E23.Milk)	R	R	R	R	R	S	R	R	R	R	R	S	S	R	S	S	R	R	S	S	R	R	Extended-spectrum beta-lactamases (ESBLs)
Serratia odorifera1 (S43.E9..Chicken meat)	R	R	R	R	R	R	R	R	R	R	R	I	R	R	S	R	R	R	S	S	S	R	ESBLs + carbapenemases

AMX: Amoxicilline ,**PRL:** Piperacillin, **TIC:** Ticarcillin, **CL:** Cephalexin,**FOX:** Cefoxitin,**AMC:** Amoxicillin + clavulanic acid,**CAZ:** Ceftazidime, **CTX:** Cefotaxime,**FEP:** Cefepim,**ATM:** Aztreonam,**IPM**: Imipenem,**ETP:** Ertapenem,**GM:** Gentamicin.**AK:** Amikacin,**NA:** Nalidixic acid.**OFX:** Ofloxacin,**CIP:** Ciprofloxacin,**C:** Chloramphenicol.**NIT:** Nitrofurantoin,**FOS:** Fosfomycin,**COT:** Cotrimoxazole

2.2 Methods:

2.2.1 Thawing frozen Lactic acid bacteria (LAB) strains:

Thawing frozen lactic bacteria requires a careful process to ensure the viability and effectiveness of the bacteria while maintaining sterile conditions to minimize the risk of contamination. Therefore, the protocol for thawing LAB bacterial strains typically follows the next steps (Lucy, 2016):

- Prepare a water bath or heat block set to a temperature slightly above the freezing point of the storage medium (37°C for LAB bacterial strains).
- Retrieve the frozen vial containing the bacterial strain from the freezer and ensure it remains frozen until ready to thaw.
- Quickly transfer the frozen vial to the water bath or heat block, ensuring that the vial is tightly sealed to prevent water ingress.
- Allow the vial to thaw completely in the water bath or heat block. Gently swirl or shake the vial occasionally to ensure even thawing.
- Once thawed, remove the vial from the water bath or heat block and wipe it dry with a disinfectant solution.
- Prepare 7 tubes of MRS broth (3ml per each), according to the manufacturer's instructions, and sterilize it by autoclaving.
- Inoculate the MRS broth with a small amount of the lactic acid bacterial strain of interest by transferring 8 drops of the culture from the previously grown cultures of each vial.
- The tubes were subsequently incubated at the optimal temperature and conditions for the LAB bacterial strain (37°C for 24 hours) to allow the revived bacteria to grow.
- Monitor the growth of the bacteria over time, which can be done by observing the turbidity of the culture or by taking periodic samples for analysis.

2.2.2. Evaluation of the antibacterial activity of LAB bacteria:

a. The agar overlay method (direct detection method):

According to (Fleming et al., 1975), this method involves culturing both strains in the same medium using an agar overlay technique. Thus , a bunch of young cultures of lactic acid bacteria strains (a spot of the strain to be characterized in the exponential growth phase) are cultivated on buffered solid MRS (de Man, Rogosa, Sharpe) medium and accordingly incubated at 30°C for 24 hours. In this regard, a defined amount of 1ml of pathogenic bacteria ; *E. coli1* (S8E14 Merg), *Klebsiella pneumoniae ssp ozaneae* (S19 E21 Merg), *Raoultella terrigena* (S15 E23 milk) and *Serracia odorefira1* (S43 E9 AS VP) was added to 9ml of semi-solid MH (containing 0.7% agar-agar) and then poured over the first agar layer following the double-layer method. After solidification of the medium, Petri dishes were incubated at 28°C for 24 hours. Indeed, the interpretation of the results

involves determining the presence of a clear halo around the inoculated strains and measuring their dimensions.

b. The agar well diffusion method (Indirect detection method):

Purified strains are tested for the production of antibacterial substances other than organic acids using the agar well diffusion method (indirect method) described by Barefoot and Klaenhammer (1983) (Figure 03). An MRS broth is inoculated with the test strain at $1/10^3$ dilution from an 18-hour culture at 37°C (end of the exponential phase) to obtain 102 to 103 CFU/ml, and then incubated for 18 hours at 37°C. The resulting culture is centrifuged at 4500 for 20 minutes at 4°C where the supernatant is adjusted to pH 6 with 1M sodium hydroxide (Merck), then filtered (mixed cellulose ester filter, 0.45 µm, Costar). The obtained filtrate represents the culture extract. In parallel, Petri dishes are prepared as follows: the dishes are coated with 10 ml of MH agar seeded with an indicator strain. After solidification, 6 mm diameter wells are created in the agar using a sterile tube. Subsequently, the dishes are dried for 20 minutes before filling the wells with culture extract (100 µl). After complete diffusion of the extract in the agar (1 to 2 hours at room temperature), the dishes are incubated for 18 hours at 37°C and then examined for the presence of inhibition zones (clear zones in a turbid lawn formed by the growth of the indicator bacteria) around the wells.

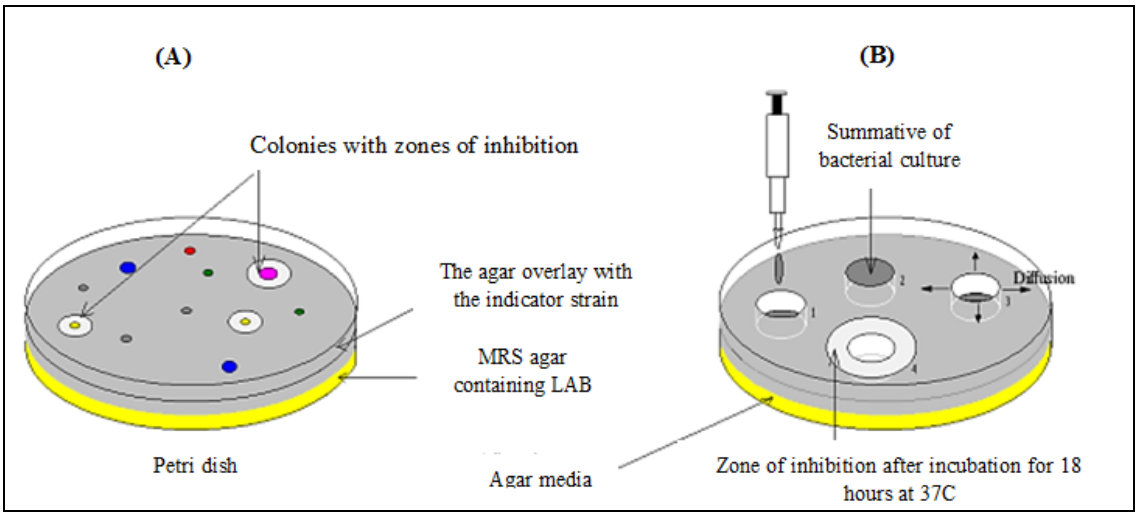

Figure 3: Exhibited methods for antimicrobial substances research.

(A) The agar overlay method **(B)** The agar well diffusion method

2.2.3 Herb Samples and Essential oil Extraction:

The essential oil extraction of *Artemisia herba* and *Ruta montana* was carried out at the Research Laboratory of Bioactive Compounds and Applications of the Faculty of Exact and Natural Sciences at the University of Echahid Cheikh Larbi Tebessi University- Tebessa. This process was performed through Hydro-distillation using a Clevenger-type apparatus (HDC) that was cleaned prior to use with alcohol and rinsed with distilled water to remove any dust or grease that might have been

present, just to prevent any contamination of the fragrant herbal oil. The oil samples were then transferred to a dark glass bottle and stored at a temperature of 4°C before analysis and bioassays tests, and the picture below (Figure 4) shows the essential oil extraction process.

Figure 4: Extraction of essential oil by Hydro-distillation method.

- **Principle of Hydro-distillation technique:**

Hydro diffusion consists of extracting bioactive compounds from plant material through the application of steam distillation. This conventional process is regularly performed via a setup recognized as the Clevenger apparatus, mainly made up of a flask, a condenser, and a collection vessel where the hydrated sample is heated to vaporize volatile constituents just for the steam to rise in a column containing more or less finely ground plant that are carried away through a condenser in order to obtain the distillate. This latter consists of two layers (aqueous and oil-rich) representing subsequently the EO along with excessive water, commonly referred to as 'hydrosol' that is made to dispense out through an opening in the condensing apparatus.

- **Technique procedures:**

The essential oils were extracted by hydro-distillation method by means of a Clevenger device, which depends on the ability of water vapor to carry the essential oil of the target plant (*Artemisia herba* and *Ruta montana*). Hence, the following steps were carried out:

a. The plant material is cut into small pieces and then subjected to hydro distillation by placing a quantity of 50g of dry ground plant sample (plant leaves dried in the shade) in a 1000 ml glass flask, to which a quantity of distilled water (500 ml) was added until the entire sample was immersed.

b. The opening tube of the Clevenger apparatus is inserted in the flask after placing it on the heating mantle. Subsequently, the condenser must be supplied with water, to so as to establish

a balance between the water volumes present in the apparatus that may ensure oil condensation.

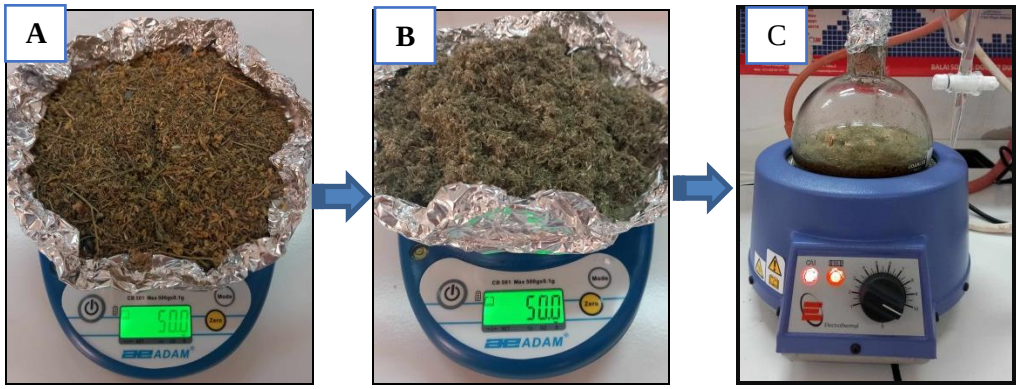

Figure 5: The first preliminary steps for the extraction of sample plants.

c. After boiling under the influence of a heat source, the water vapor becomes saturated with the essential oil of the plant and is transferred through vertical tubes that pass through a cooling device where the process of condensation occurs so that small droplets are formed and later accumulated in a tube containing distilled water.

d. The extraction process is carried out for two hours straight to obtain sufficient EO. Consequently the essential oil is collected in a tightly closed and opaque glass bottle wrapped in aluminum, then kept away from light at a 4°C temperature before analysis and bioassays tests. The figures below show the essential oil extraction process.

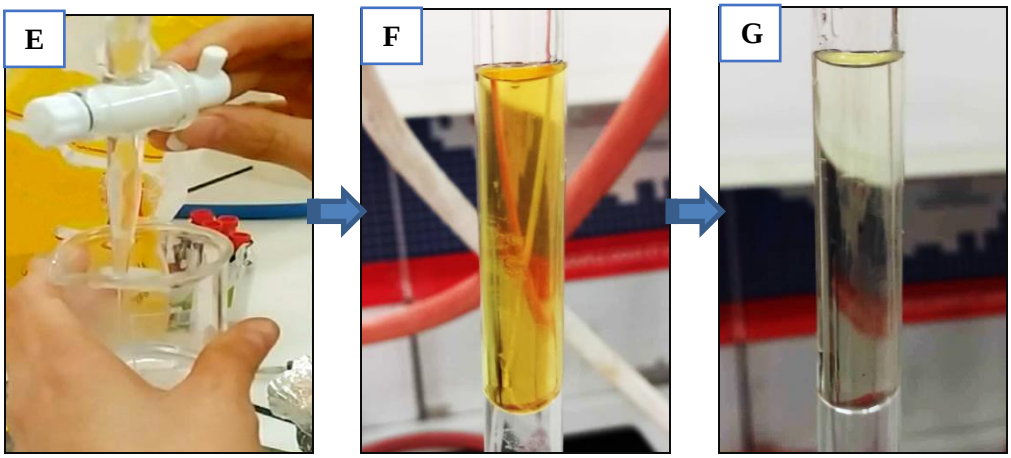

Figure 6: The second preliminary steps for the extraction of sample plants.

E: opening the valve to release the EOs. **F:** The extracted EO of *Artemisia herba*.

G: The extracted EO of *Ruta montana*.

2.2.4 Evaluation of the antibacterial activity of EOs:

a. Target microorganisms:

Microorganisms isolates subjected to antimicrobial susceptibility testing must be isolated in pure culture and should have been identified at the genus and species level. Hence, four Gram-negative bacterial strains, also referred to as Extended-spectrum beta-lactamases (ESBLs) that confer resistance to most beta-lactam antibiotics, were respectively chosen : *Klebsiella pneumonia ssp ozaneae (*S19 E21 Merg), *Escherichia coli* (S8E14 Merg), *Raoultella terrigena* (S15 E23 milk) and *Serratia odorifera1*(S43 E9 chicken meat , carbapenemase positive).These bacteria were acquired from the Molecular Bioactive Compounds and Applications Research Laboratory, Department of Microbiology, Faculty of Biology. Larbi tebessi university (Tebessa, Algeria).

b. Disc diffusion method (Aromatogram):

The antimicrobial activity of essential oils of *Artemisia herba* and *Ruta montana* against ESBL-producing *Enterobacteriaceae* strains ; *E. coli1*(S8E14 Merg), *Klebsiella pneumoniae ssp ozaneae (*S19 E21 Merg), *Raoultella terrigena* (S15 E23 milk) and *Serracia odorefira1* (S43 E9 AS VP), was tested by the agar disk diffusion method also called Vincent's method or the Aromatogram technique developed by Schroeder and Messing in 1949.This method is done in the same way as an antibiogram where the antibiotics are replaced by aromatic essences, previously selected (Bachiri at al., 2016) which is believed to permit the examination of the sensitivity and resistance of bacteria to essential oils, in a reliable and reproducible manner (Yashphe et al., 1979) according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 2001).

- **Principal of the method:**

In this method, Young bacterial cultures for ESBL-producing *Enterobacteriaceae* strains were suspended in physiological saline (0.9% NaCl) at concentrations of 108 CFU/ml, which equals the McFarland turbidity standard of 0.5.These inoculums were spread with sterile swabs on the surface of Petri dishes, containing Mueller-Hinton Agar. Subsequently, sterilized 6 mm (φ6 mm) filter paper discs (Wattman n°4) were saturated with a filtered sterilized plant extract of the desired concentration (80 µl). The impregnated discs are then placed on the surface of a suitable solid agar medium and the petri dishes, allowed to dry at 4°C for 2h just to be incubated at 37°C for 18-24 h. (Da Silva Dannenberg et al., 2019; Chebaibi et al., 2016). The test was carried out in triplicate for each strain which helped determine the viability of the trials by the detection the average of inhibition zones.

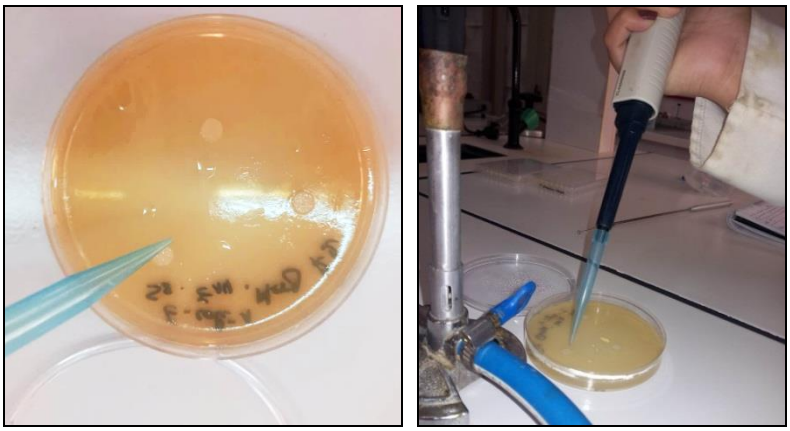

Figure 7: Placement of the impregnated discs and the EOs on the surface of MH agar.

- **Reading results:**

After incubation, the absence of microbial growth is mainly manifested by a translucent halo around the disk, similar to the sterile agar, and the diameters of the inhibition zones (in mm) which were measured using a transparent ruler including the disk diameter in millimeters and expressed as means standard deviation of three replicates. According to the range proposed by (Ponce et al., 2003; Mouas et al., 2017), the strains will be classified based on the inhibitory effect of EOs as follows:

- **Not sensitive (-):** if the inhibition diameter does not exceed 8mm (6 mm ≤ Ø ≤ 8 mm).
- **Sensitive (+):** if 9 < diameter ≤ 14mm.
- **Highly sensitive (++):** if 15 < diameter ≤ 19mm.
- **Extremely sensitive (+++):** if the diameter exceeds 20mm (Ø ≥ 20 mm).

c. Determination of the minimum inhibitory concentration (MIC):

The determination of antimicrobial susceptibility can be performed in either a quantitative or qualitative fashion (Matthew, 2015). The former involves determination of the minimum inhibitory concentration (MIC) of the organism being tested which is commonly defined as the lowest concentration of an antimicrobial compound that will inhibit the visible growth of microorganism after overnight incubation. This period is generally extended for organisms such as anaerobes, which require prolonged incubation for growth (Jennifer, 2001).

- **Principal of the method:**

Determination of Minimum Inhibitory Concentration (MIC) was carried out by the broth dilution method that denotes the performance of the susceptibility test using 96-well microtitration plate format with a capacity of ≤500 µL per well (EUCAST, 2003). Subsequently, a series of doubling dilutions of the EOs meant to be tested are inoculated in nutritive MHB in order to obtain a standardized bacterial suspension that equals to 0.5 McFarland, allowing the microorganism strains in

question (ESBLs) to be inoculated separately into each dilution. The meaning of MIC value itself is interpreted against published standards yielding a qualitative result of resistant, intermediate, or sensitive where the lowest dilution that exhibits visible inhibition of growth is recorded as the MIC (Matthew et al., 2015); henceforth it may help establish the level of resistance of a particular bacterial strain and substantially affect the decision to use certain antimicrobial agents (Acharya, 2022).

d. Minimum Inhibitory Concentration (MIC) value determination assay:

- **Broth microdilution method:**

A liquid microdilution of the essential oils to be tested was performed in 96-well microplates following the protocol described by (Oumaskour et al., 2021) which was determined only with micro-organisms that displayed inhibitory zones. In this context, the culture medium used consisted of Mueller Hinton broth with 0.5% Tween 80 that was incorporated into the agar after autoclaving to enhance oil solubility and also to assure a steady emulsification in order to promote contact germ-compound.20 µl of essential oil was added to the first well containing 170 µl of Mueller-Hinton broth (Tween 80: 0.5%), while the other wells already contained 95 µl of Mueller-Hinton broth (Tween 80: 0.5%). After homogenizing, a serial 11-fold dilutions was executed in the remaining wells of each row by moving 95 µL from well to well until the excess 95 µl from the row's final was discarded (a 2-fold geometric dilution).

Then, each well was inoculated with 5 µl of the bacterial suspension to be tested (3.5 × 107 CFU/ml) previously prepared by diluting a broth culture or by emulsifying four or five colonies of a pure culture in broth or saline overnight to avoid selecting an atypical variant. For either method, the standardization of the bacterial cell number used for susceptibility testing is of critical importance for obtaining accurate and reproducible results. Wells containing Mueller-Hinton broth (Tween 80: 0.5%) inoculated with the test strain were used as positive controls, while uninoculated plates containing essential oil and Mueller-Hinton broth (Tween 80: 0.5%) served as negative control. After 18 hours of incubation at a temperature of 37°C, the results were visually observed and the MIC of the essential oil was defined as the lowest concentration at which the tested microorganism showed no visible growth in the MH broth. (Kengne et al., 2019 ; Oumaskour et al., 2021).Following the allotted time, the plate is removed and checked for bacterial growth. If the broth became cloudy or a layer of cells formed at the bottom, then bacterial growth has occurred. The results of the broth microdilution method are reported in Minimum Inhibitory Concentration (MIC) which reports the lowest concentration of antibiotics that stopped bacterial expansion. For a better assessment thereof; the test was carried out with two repetitions for each germ and the value of the MIC was therefore the average of the two trial tests.

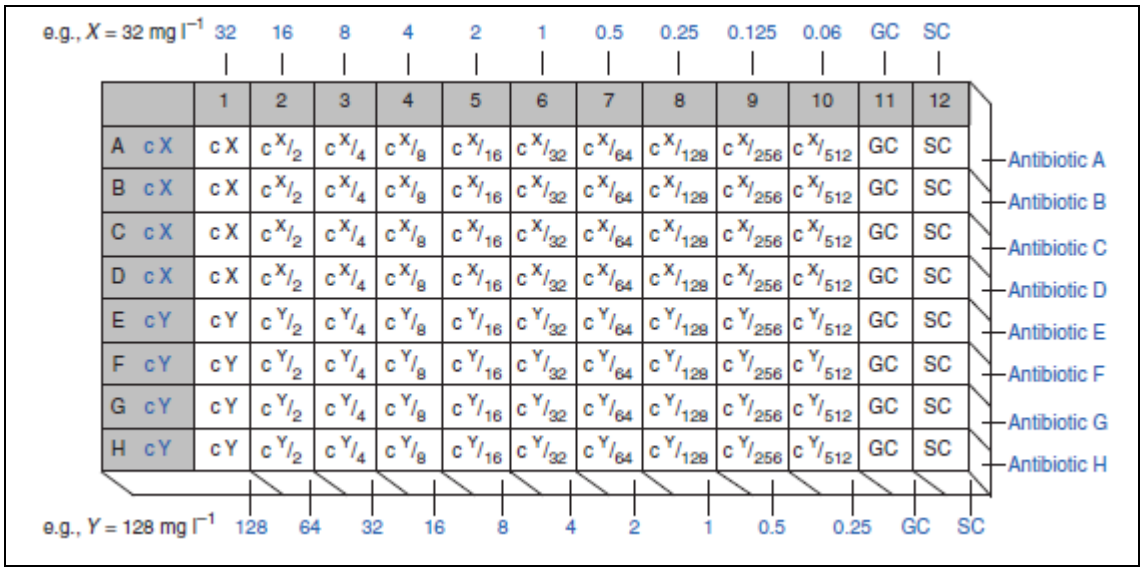

Figure 8: Outline of the setup of a microtiter plate for antimicrobial susceptibility testing with doubling dilutions of different antimicrobial agents in two different concentrations anges with labeling suggestions in blue.

- **Reading results:**

Results must only be read when there is sufficient growth of the test organism (i.e. obvious button or definite turbidity in the positive growth control), no growth in the uninoculated or negative growth control (where present) and when a purity plate shows that the test organism was pure. The amount of growth in each tube or well is compared with that in the positive growth control and the MIC recorded as the lowest concentration of the agent that completely inhibits the growth of ESBLs bacterial strains.

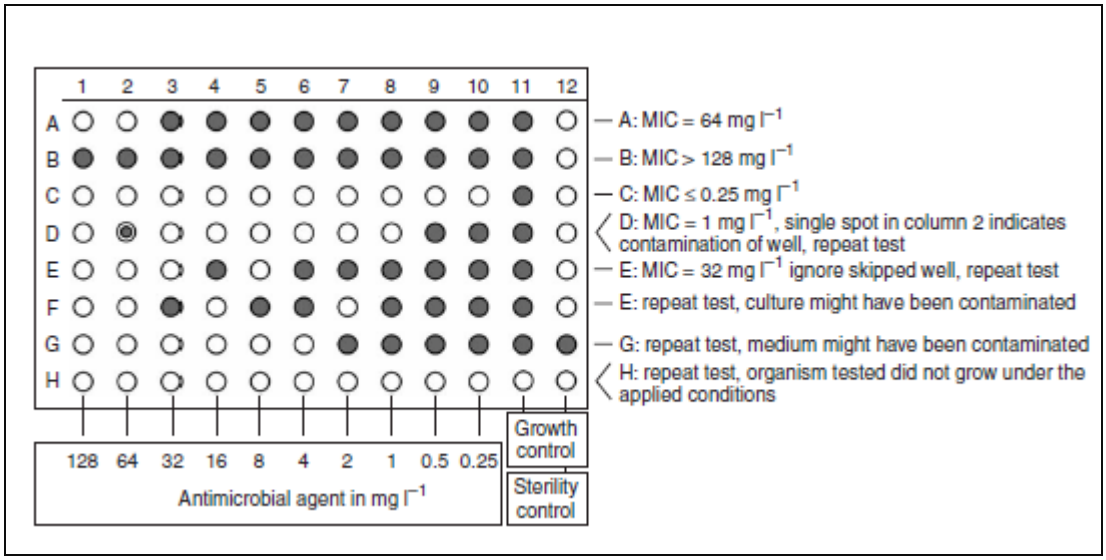

Figure 9: Interpretation of possible growth patterns in MIC microtiter plates.

e. Assessment of the Minimum Bactericidal Concentration (MBC):

Minimum Bactericidal Concentration (MBC) refers to the minimum concentration of an antimicrobial agent necessary to achieve a bactericidal effect. Ergo, it causes 3-logarithmic decrease (99.9% killing) in the size of the standard inoculum. This test was determined by streaking a 10 µL aliquot on solid culture Mueller–Hinton medium from the last wells that demonstrated inhibition of bacterial growth in the MIC assay. The plates were incubated at 37 ◦C for 18 h and MBC was defined as the lowest concentration that stopped bacterial growth (no colonies on the plate). Yet, it is imperative to note that all experiments (MIC and MBC) were running in duplicate throughout the whole bioassay (Maria at al., 2023).

In general, the MBC/MIC ratio is calculated to determine the antibacterial power of the essential oil. Thus, when this ratio is less than or equal to 4, the extract is regarded as bactericidal, and when it is greater than 4, the extract is classified as bacteriostatic (Bouharb et al., 2014). Sometimes, the MBC of some drugs is very close to its MIC, also if the MBC of the tested drug against the tested microorganism is ≧ 32 times the MIC, it can be determined that the microorganism has developed resistance to the tested drug.

- ..

RESULTS AND DISCUSSION

3. Results of the antimicrobial activity of selected LAB:

3.1 The agar well diffusion method:

The agar well diffusion method was used to assess the antimicrobial activity of the selected LAB isolates namely JMQ24, KMQ31, JMZ02, ULZ15, KMZ 10, QMZ 11and BZQ 36 isolated from varied cheese types. Their antimicrobial properties were tested against four major food-borne pathogenic bacteria namely *S. epidermidis*, *S. aureus*, *B. subtilis* and *Aspergillus fumigatus*. Results show that the spectrum of inhibition was different for the isolates tested. Table 04 gives the results for the antimicrobial activity of the isolates in terms of diameter of the zone of inhibition. A diameter >1mm around the well was considered as a positive result. It was assumed that greater the diameter of the inhibition zone (ZOI), greater was the antimicrobial activity of the isolate. Figure 10 shows petri plates with a lawn of an indicator pathogenic strain and a ZOI around the well containing the culture-free isolate.

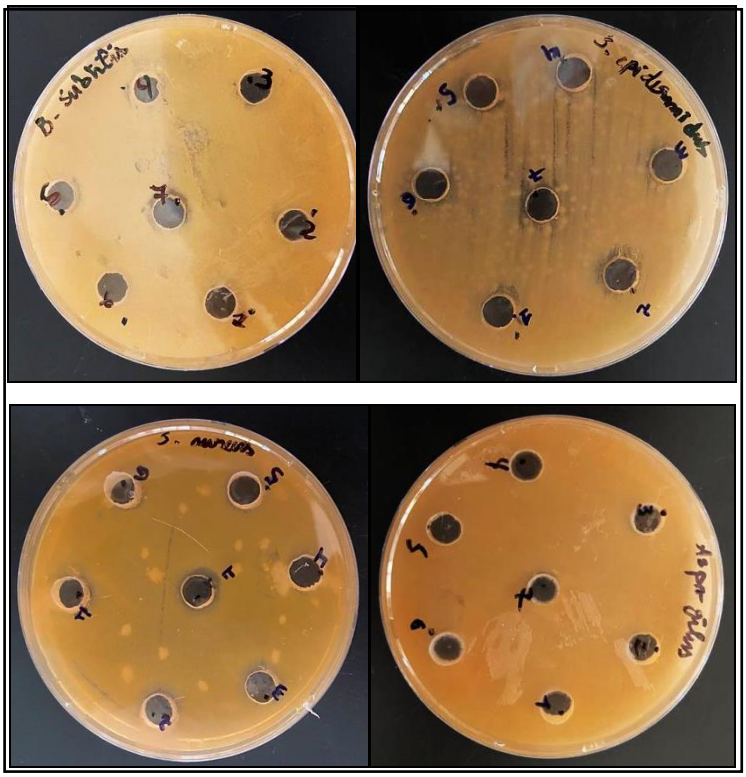

Figure 10: The agar well diffusion results on Gram-positive bacterial strains and fungi.

Table 4: Antimicrobial activity spectrum of lactic acid strains using agar well diffusion.

Reference strains	KMZ 10	BZQ 36	QMZ 11	KMQ31	JMQ24	JMZ02	ULZ15
Gram positive ZOI (mm)							
Staphylococcus aureus **ATCC 25923**	04	05	05	05	04	04	06
Staphylococcus epidermidis **ATCC 35984**	06	06	04	04	04	05	05
bacillus subtilis **ATCC 9372**	-	-	-	05	05	-	07
Fungi ZOI (mm)							
Aspergillus fumigatus **LB22**	-	-	02	-	-	03	03

Results indicate that isolate ULZ15 had the highest antimicrobial property, against all the indicator pathogens tested amongst the seven isolates. Its activity was highest against *B.subtilis* ATCC 9372 with a ZOI of 7 mm and least for *Aspergillus spp* (3 mm). Isolate JMQ24 has also showed an antimicrobial property against the majority of the tested pathogens, being at its highest against *B.subtilis* ATCC 9372 (5 mm), yet no activity was registered against *A. fumigatus* which was in direct contrast to isolate QMZ11. The degree of antimicrobial property among the isolates was in the order of ULZ15> KMQ31> JMQ24> JMZ02> QMZ11> BZQ36> KMZ10. It is however difficult to comment on the reason for this variability in the antimicrobial property amongst the isolates since each one was obtained from different food sources. Interestingly, isolate KMZ10 and BZQ36 were equally inactive against both *B.subtilis* ATCC 9372 and *A. fumigatus* pathogenic microorganisms which may elaborate their weak antimicrobial activity against these strains.

3.2 The agar overlay method:

The agar overlay method was employed to determine the ability of the viable lactic acid bacteria strains to inhibit the growth of the seven indicator pathogens, both Gram positive *(Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC 35984,

Bacillus subtilis ATCC9372 and Gram negative ESBLs *(Escherichia coli1* S8.E14.Merguez, *,Klebsiella pneumoniae spp ozaena* S19.E21.Merguez *,Raoultella terrigena,* S15.E23.Milk *and Serratia odorifera1* S43.E9.AS.Chicken meat), isolated from variant food sources.

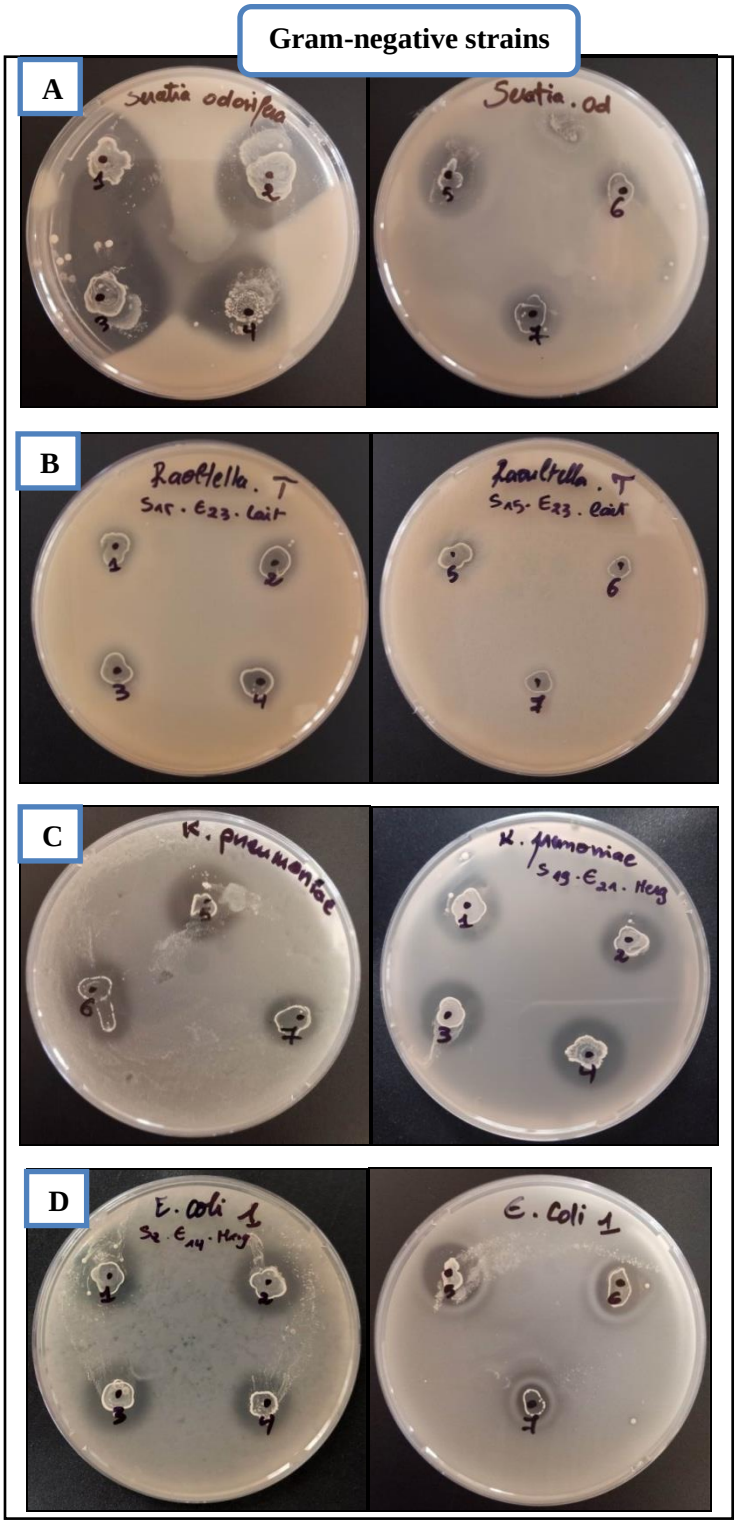

Figure 11: Antagonistic activity of selected LAB against Gram- pathogens (ZOI in mm)

Lactic bacteria has also showed a major activity against Gram-negative strains, usually referred to as multidrug resistant ESBL producing bacteria, which can be mainly observed within a rather large inhibitory zone performed by KMZ 10 against *Serratia odorifera1*(S43.E9.AS.Chicken meat) (28mm). JMZ02 has also showed the highest activity (22mm) against *Klebsiella pneumoniae spp ozaena* (S19.E21. Merguez).However, the different Lab strains has revealed the highest activity against *Serratia odorifera1* (S43.E9.AS.Chicken meat) that prevailed mostly in a larger zone with QMZ11 (36mm), thus easily outweighing the previous results. On the other hand, the lowest antibacterial activity of the tested LAB isolates was recorded against *Raoultella terrigena* (S15.E23.Milk) with the least large inhibitory zones that range between (8-14mm).

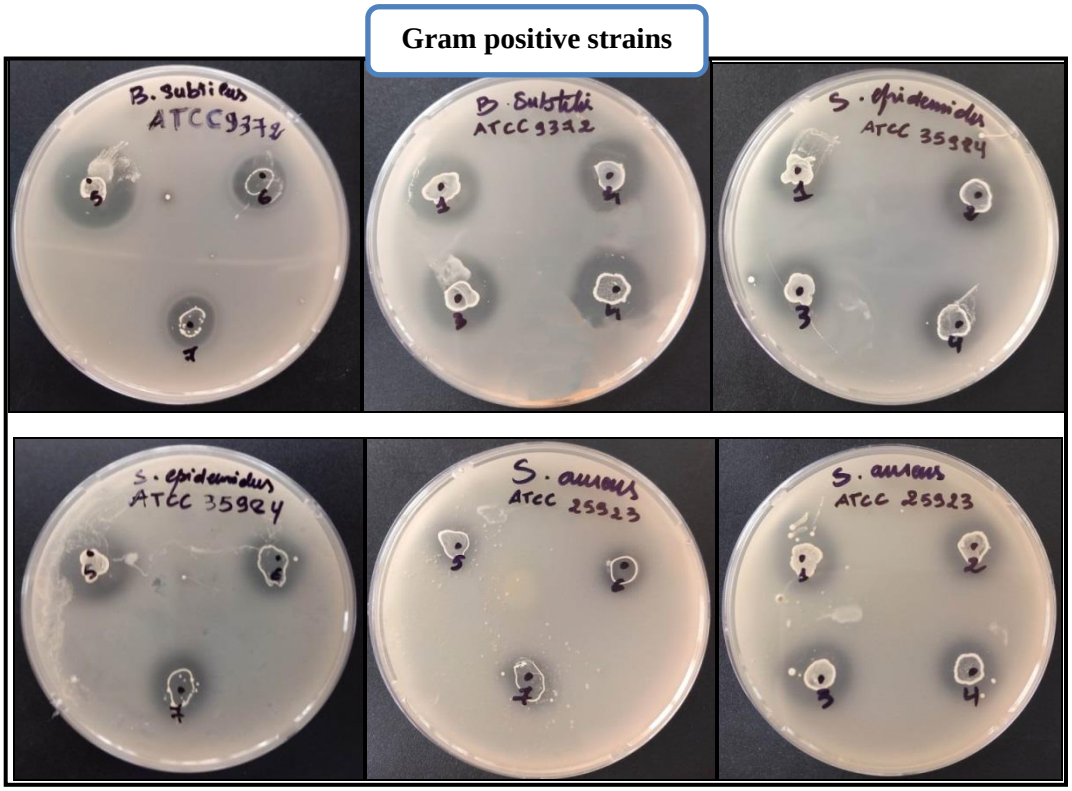

Figure 12: Antagonistic activity of selected LAB against Gram+ pathogens.

Subsequently, the antimicrobial activity of LAB bacteria against the designated Gram-positive strains has revealed some important results. KMZ10 showed the highest activity (23 mm) against *Staphylococcus aureus* ATCC25923 while JMQ24 showed the highest activity (21mm) against *Bacillus subtilis* ATCC9372 followed by JMZ02 and ULZ15,where both revealed a noticeable inhibitory zone around the same strain . The highest zones of inhibition by LAB against *Bacillus subtilis* ATCC9372 was shown by JMQ24 (21mm).However, The lowest antibacterial activity against the three antimicrobial strain of *Staphylococcus aureus* ATCC25923(10mm) ,*Staphylococcus epidermidis* ATCC 35984

(13mm) and *Bacillus subtilis* ATCC9372 (13mm) was shown by JMZ02, QMZ 11 and ULZ15 respectively.

Table 5: Antimicrobial activity of selected LAB against pathogens (ZOI in mm)

Reference strains (LAB)	KMZ 10	BZQ 36	QMZ 11	KMQ31	JMQ24	JMZ02	ULZ15
Gram positive (ZOI in mm)							
Staphylococcus aureus ATCC25923	23	13	16	14	12	10	15
Staphylococcus epidermidis ATCC 35984	15	15	13	14	17	16	16
Bacillus subtilis ATCC9372	19	16	19	18	21	15	13
Gram negative ESBLs (ZOI in mm)							
Escherichia coli1 (S8.E14.Merguez)	20	18	16	14	17	16	12
Klebsiella pneumoniae spp ozaena (S19.E21. Sausage)	19	15	17	19	20	22	15
Raoultella terrigena (S15.E23.Milk)	11	14	13	12	14	08	09
Serratia odorifera1 **(**S43.E9.AS.Chicken meat)	28	31	36	23	23	15	17

The agar overlay method showed that all of the isolates have varying antibacterial activity against the test pathogens which imply a relative comparison in order to deduce the most susceptible strains against the reported LAB bacteria. Based on the results shown in the graph below, it is possible to consider *S.odorefira1* (S43.E9.AS.Chicken meat) as the most vulnerable bacterial strain.

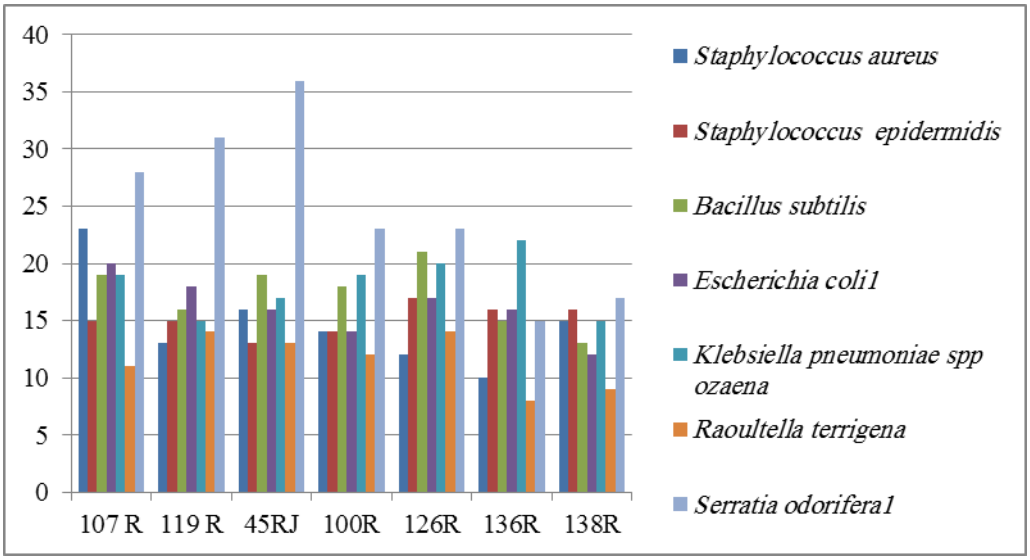

Figure 13: Graph represents the antimicrobial activity of LAB bacteria on different strains.

The inhibitory activities of LAB isolates against some bacterial strains were compared with well diffusion and agar overlay method. In both of methods, tested strains exhibit significant inhibitory activity against indicator microorganisms mostly in our study, in agar overlay method, *S.aureus* ATCC25923 and *S.epidermidis* ATCC25923 were determined as the most sensitive tested bacteria followed by *B.subtilis* ATCC9372 and *Aspergilus fumigatus* LB22 that showed no susceptibility against some tested isolates (Table 5). On the other hand, in well diffusion assay, *B.subtilis* ATCC9372 was the most sensible to indicator microorganisms. Although *S.aures* ATCC25923 and *S.epidermidis* ATCC25923 are inhibited firstly in well diffusion method (4-6mm) the value of the inhibition is lower than agar overlay method (10-23mm) .In spite of *B.subtilis* ATCC9372 was one of the most resistant strains in well diffusion method, it was inhibited 19mm in the other method with *E.faecium.* According to Schillinger and Lucke (Schillinger et al., 1989), agar overlay method is more effective method than well diffusion method for measuring antimicrobial activity. As a result, the inhibitory activity of LAB on tested bacteria under overlay test could be due to all metabolites; lactic acid, acetic acid, di-acetyl, bacteriocin etc. Therefore, the inhibition zone which had been seen around wells in the well diffusion method could be a result of bacteriocin. We conclude that agar overlay assay has several advantages towards well diffusion method by means of the efficiency of the inhibition and the facility of the application of the method.

In general, it was found that the inhibitory effects of potential probiotic strains against Gram-positive pathogenic bacteria are more common than Gram-negative pathogenic bacteria (except its high growth inhibitory effect on *E. coli* S8.E14.Merguez). This result is quite understandable considering the relative inherent resistance of most Gram-negative bacteria

against acidic conditions as well as against bacteriocins produced by *lactobacilli* (Steinbuch et Fridman, 2016). *Lactobacilli* exert protective or therapeutic effects by producing antimicrobial compounds, lowering pH, competing at binding sites against pathogens, stimulating immune-modulatory cells, and competing with pathogens in food (Abotsi, 2011).The role of inhibiting the cell-free supernatant (CFS) growth of probiotic *lactobacilli* has been proven in many previous studies which are consistent to our results. (Aminnezhad et al., 2015 ; Beristain et al., 2016 ; Koohestani et al., 2018 ;Moradi et al., 2019 ; Yang et al., 2021). Moreover, the common feature of most *Lactobacillus* probiotic strains is the ability of these bacteria to produce metabolites and various antimicrobial compounds, including small peptides, bacteriocins, and organic acids, such as butyric, acetic, and lactic acid (Jena, 2013).

Indeed, the production of the above compounds, both *in vitro* and *in vivo*, can cause growth inhibitory effects against pathogenic microbial strains. Based on the results, this strain showed the inhibitory effect against all seven tested pathogenic microbes. For instance, another study revealed that an exposure of pathogenic test organisms to active culture supernatant of *L. paracasei ssp* resulted in a strong inhibition of growth of those test organisms, which suggested the presence of BLIS in the supernatant. Hence, the immediate and drastic reduction in optical density of test pathogens indicated a potent bactericidal effect by BLIS secreted from *L. paracasei ssp. paracasei-1*.which may prove to a certain extent that *L. paracasei* can produce active molecules as in bacteriocins, known as BLIS, to inhibit both Gram-positive and Gram-negative.

Same deduction can be applied on *Lactococcus lactis sp lactis* antimicrobial activity against the tested bacteria since it's known to produce a variety of active molecules, including bacteriocins that may vary in their molecular composition, thus conferring a specific ability for *Lactococcus* genra to antagonize both Gram-positive and Gram-negative pathogens (Gamal et al., 2013). Henceforth the interest in *Lactococcus lactis* has been due to its application as starter culture for yoghurt and soft cheese making as well as its probiotic use. *Lactococcus lactis* is used as a probiotic bacterium because it was shown to improve lactose digestion and absorption in lactose intolerant people. It also has more potential and safety in developing vaccine in human (Dantas et al., 2016).

Respectively, *Enterococci durans and Enterococcus faecium* exhibited antagonistic activity against most of the tested Gram-positive and Gram-negative foodborne pathogens and contaminant bacteria. All the zones of inhibition were clear and from 11 to 23mm in diameter in both spot assay and agar well diffusion test. The largest zones (23 mm) were produced against *Staphylococcus aureus* ATCC25923. This inhibitory activity may be explained by the fact that these genera are very common for bacteriocinogenic activities. In this context,

bacteriocin-like antagonism displayed by *Enterococci* was first reported by Kjems (Kjems et al., 1995). In recent years, several *E. faecium* and *E. faecalis* strains from dairy products (McKay et al., 1990; Giraffa et al., 1994) displaying antimicrobial activity have been isolated, exposing a trait that adds interest to this bacterial group in food preservation. The antimicrobial effects of a broad spectrum of substances from *E. faecium* proved to be effective against both Gram-negative and Gram-positive bacteria (Laukova et al. 1993). Moreover, (Tagg et al ., 1976) stated that not only Gram-positive bacteria but several Gram-negative bacteria were also inhibited by Gram-positive bacteriocin-like substance producer. Our results agree with the above mentioned authors. However, one of the main drawbacks of many antimicrobial agents is their ability to inhibit not only the undesirable bacteria, but also lactic starter and nonstarter cultures when used for preservation of fermented foods. Yet, our antimicrobial producer *Enterococci* may be of interest because of their spectrum of activity, as it would allow relatively selective inhibition of some food-borne pathogens, whereas most of the LAB would not be affected.

In this study, the test strain *Pediococcus pentosaceus* exhibited a broad antimicrobial spectrum, capable of being active against Gram-positive and Gram-negative representatives including food pathogens (maximum zone presented by *k.pneumoniae* (S19.E21. Merguez) being 22mm) and fungi that gave away a minimum inhibitory zone (3mm) in agar well diffusion assay yet an antimicrobial activity can still be noted. These findings were already achieved by many researchers who emphasized the effective role of *Pediococcus* bacteriocins in foods and feeds (Moreno et al., 2006; Izquierdo et al., 2008) .This strain, a member of LAB, has been shown to be effective in the production of antimicrobial peptides (bacteriocins) with applications in the food and health industries.

Numerous studies have been published on the production of bacteriocins, their use in biopreservation processes, and more recently, their use in human and veterinary medicine (Svetoslav et al., 2022). Moreover, the second most widely used bacteriocin in the scientific community is pediocin PA-1, which is produced by various *Pediococcus* strains (Garsa et al, 2014) and used by the food industry as a bio-preservative especially to prevent and control the contamination by Gram-positive pathogens, which explains why ESBLs strains displayed more susceptibility toward these bacterial strains (Cabo et al., 2009). In fact, Pediocin PA-1, produced by several *Pediococcus spp* strains, was the first thoroughly characterized class IIa bacteriocin (Chikindas et al., 1993). Moreover, its anti-bacterial activity and the presence of constitutive amino acid motif became a landmark in the classification and study of bacteriocins. The class IIa bacteriocins, often designated as pediocin-like bacteriocins, constitute the most prevalent group of antimicrobial peptides produced by LAB (Drider et al.,

2006) which can be used to argue that this antimicrobial has more than just killing characteristics as in a bactericidal effect , but may be involved in *quorum sensing* intracellular communications (Chikindas et al., 2018). Therefore, it has a potential for application as a bio preservative in the dairy milk products where lactic acid fermentation is employed mostly for development of products, for flavor and taste of the fermented products, for enhancing the microbial quality and for the safety of processed foods.

To date, knowledge on the susceptibility of LAB to antimicrobial agents is rather limited in view of the large numbers of genera and species encountered in this group of bacteria apart from variances in the resistance spectra .Transfer of resistance to antimicrobial agents is an essential mechanism if the lactic bacteria would adapt and survive in specific environment. Hence in view of the above, before a strain of LAB can be used as a feed additive it must undergo antibiotic resistance screening to ensure its safe application.

3.3 EOS Analysis:

The extraction of essential oils of *Artemisia herba* and *Ruta montana* was carried out by the technique of hydro-distillation under well-defined conditions where the yield of their EOs corresponds to the ratio between the mass of EOS (m EOS) obtained and the mass of plant material (m M_D) used for the extraction (AFNOR, 2000). The in vitro antimicrobial activity of these essential oils against the microorganisms employed and its activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, zone diameters and MIC, MBC values. Following the applied extraction process, we obtained a quantity of both essential oils presented in figure 14 below.

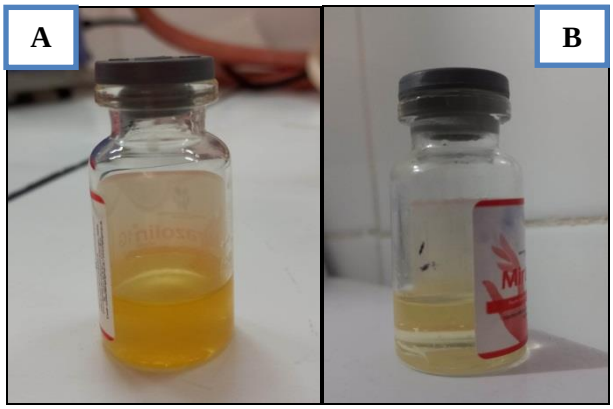

Figure 14: Extracted esential oil by Hydro-distillation method

A: *Artemisia-herba* EO **B:** *Ruta montana* EO

3.3.1 Quantitative Analysis:

a. Yield of essential oils:

Essential oils from *Artemisia herba* and *Ruta montana*, both harvested from El Ogla area, province of Tebessa, Algeria were obtained in yields of 1.67 % and 0.8% respectively after 6-9 h of extraction using hydro distillation process, via a Clevenger-type apparatus according to the European Pharmacopoeia. The mean values of several extraction yields in percentage are mainly reported based on the following formula; Here, Y_{EO} is the yield of essential oil (%), M_{EO} is the mass of the EO (g), and M_D is the mass of dry plant matter (g).

$$Y_{EO}(\%) = \frac{M_{EO}}{M_D} \times 100$$

- **Y_{EO}:** Essential oil yield in %.
- **M_{EO}:** Mass of essential oil in grams.
- **M_D:** Mass of fresh plant material used in grams

❖ **AH-EO yield (%):**

$$\mathbf{AH} - \boldsymbol{Y_{EO}}(\%) = \frac{M_{EO}}{M_D} \times 100$$

$$\boldsymbol{AH} - \boldsymbol{Y_{EO}}(\%) = \frac{2.5}{150} \times 100$$

$$\boldsymbol{AH} - \boldsymbol{Y_{EO}}(\%) = 1.67\%$$

❖ **RM-EO yield (%):**

$$\boldsymbol{RM} - \boldsymbol{Y_{EO}}(\%) = \frac{M_{EO}}{M_D} \times 100$$

$$\boldsymbol{RM} - \boldsymbol{Y_{EO}}(\%) = \frac{1.6}{200} \times 100$$

$$\boldsymbol{RM - herba\, Y_{EO}}(\%) = 0.8\%$$

3.3.2 Qualitative Analyses:

a. Organoleptic Control:

According to (Afnor,2000), essential oils are usually liquid at room temperature and volatile, they are more or less colored and their density is generally lower than that of water. The essential oil of *Artemisia herba* is a yellowish-green liquid with a strong and pleasant odour of white mugwort. These organoleptic properties are related to the climatic and soil conditions, the study region, the plant's state, species, and the harvest period. These organoleptic properties, such as color, odor, and viscosity can also be relevant for judging the quality of oil. The organoleptic control consists of controlling the organoleptic characteristics of the essential oils obtained from *Artemisia Herba* and *Ruta montana* respectively: smell; color; and appearance. These indications make it possible to assess the quality of the oil studied (Vahid *et al.*, 2013).

Table 6: Organoleptic characteristics of herbal plants essential oils.

Essentielle oil	Odor	Aspect	Color
Artemisia herba	strong characteristic herbaceous odor	Limpid liquid.	Pale yellow
Ruta montana	Strong aromatic odor	Liqud Crystal clear.	Light yellow

The AH-EO extracted in this study presented yellow color with a strong penetrating pleasant herbaceous odor characteristic of the plant (Table 6). The average yield of AH-EO obtained from 150 g of dried leaves of the plant corresponds to 1.67% which is relatively higher than other medicinal plants as for example *Salvadora persica* (0.6 %) (Alali et al., 2005), *Matricaria recutita* that gave 0.55% ± 0.045 (Chaouia et al., 2018) and even than the same species like the *Artemisia herba-alba* grown in Matmata Tunisia (0.65%) (Akrout et al., 2004), Djelfa in Algeria (0.80) (Lakehal et al., 2016), Libya (0.90%) (Janaćković et al., 2015), Batoum-Souk Ahras in North-east of Algeria (1.48%) (Delimi et al., 2018), Boussada in North-west of Algeria (0.76%) (Belhattab et al., 2012) and also that of Taforalt in Morroco (1.00 %) (Imelouane et al., 2010). These results are different from those reported in other regions of Algeria which shows the interest of this study ,yet the yield stated by the literature of other countries [0.1–4.9% (v/w)], makes our outcome much more comparable to those found in Eastern regions of Morocco (between 1.3 and 3.3 %) (Paolini et al., 2010; Amor et al., 2019) and southern Tunisia (between 1.20% and 4.86%) (Boukrich et al., 2010) where it can be relatively lower than the AH-EO obtained from Buseirah, south Jordan (3%) (Abu-Darwish et al., 2015) and other intraspecific variation of dried weight of samples noted in Spain (0.41% - 2.30%) for 16 samples from four provenances (Salido at al., 2004) after conducting a constant hydro distillation, which remains the most coveted EO extraction method in current practice (Goudjil et al., 2015). Therefore, EOs yield may depend on several factors like climate conditions and edaphic conditions of the study region, the plant's condition, cultural practices, geographical regions, harvested date, and the method used for extraction (Abdelaziz et al., 2021).The main conditions required for profitable essential oil production are good plant material, plant variety, soil, distillation equipment, and climate (Smallfield et al., 2001).

The steam-hydrodistillation of *R. montana* aerial parts yielded 0.8% of yellowish essential oil with a strong and penetrating odor. This yield was in the same range as those reported in the literature (0.38 - 6.1%) (Kambouche et al., 2008; Boutoumi et al., 2009; Bouzidi et al., 2012; Zellagui et al., 2012; Ferhat et al., 2014; Khadhri et al., 2014; Hammami et al., 2015; Hazzit et al., 2015; Daoudi et al., 2016; Bennaoum et al., 2017; Fekhar et al., 2017; Mohammedi et al., 2020) nevertheless, it is considered as weak, which can be explained by the fact that the region where the harvest was carried out is, at once, subject to topographic influence (mountainous stage) and the influence of the inconsistent climate where it may result a low temperature and a strong atmospheric humidity. These conditions generate a decrease of the production of the essential oils especially that the harvest was carried out in the middle of the winter. In fact, the yield of essential oil is under the dependence of the geographic origin, phonologic stadium and environmental factors such as the temperature and

the quality of the ground (Salima et al., 2013). The essential oil of the *R-montana* leaves has aqueous aspect, yellow color blade, aromatic odor and it is less dense than water.

3.3.3 Antimicrobial activity of essential oils:

The extracted essential oils were found to be different in terms of specific characteristics, especially in odor and color. The values of the essential oil yields varied from one species to another with highly significant differences. Therefore, it can be noticed that the highest yield of EOs was recorded for *A. herba* with a value of 1.67%, followed by *R. montana* (0.8%) as shown in Figure 15 below.

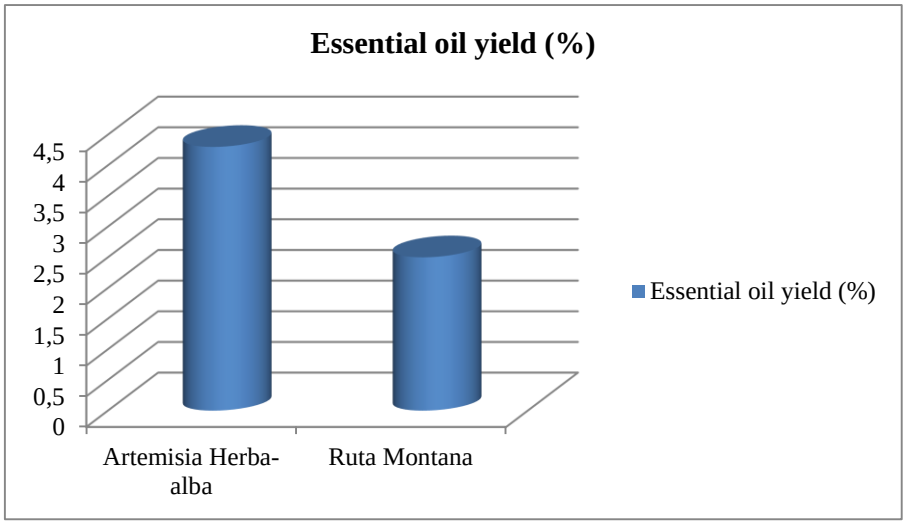

Figure 15: Essential oil yield of the studies plants.

In this context, a review of the existing literature on *A. herba* essential oils afforded a large number of studies, particularly in the last decade. Although the isolation procedure was similar in most cases, the plant parts, the physiological stage, the plant status (fresh or dry), and the geographical origin were quite diverse, in addition to the use of collective samples and to the possibility of existing different subspecies. Only seldom studies have used individual plants, and even in those cases no clear correlation between plant oil types and environmental conditions was established (Rachid et al., 2014)

3.3.3.1 Aromatogram (Test of activity):

The antibacterial activity of tests strains can be evaluated through a vitro test of activity that consists of a disk diffusion method in order to test the sensitivity of bacteria to *A-herba* and *R-montana* essential oils. Results are shown in figure 16 below.

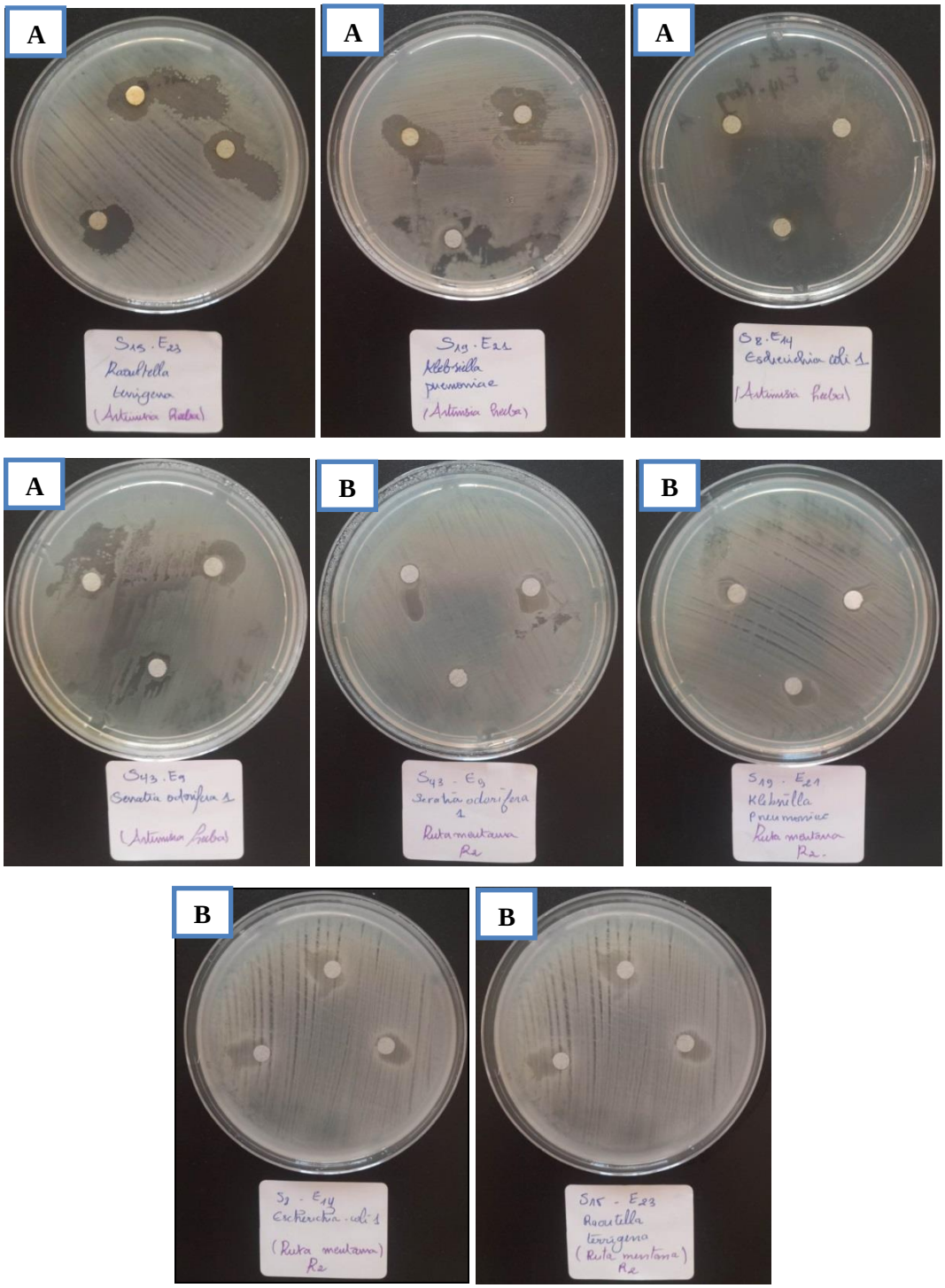

Figure 16: Results of the Aromatogram test against ESBL strains.

A: Inhibitory effect of AH-EO **B:** Inhibitory effect of RM-EO

The Aromatogram test of the essential oils of *Artemisia herba* and *Ruta-montana* from the El Ogla region (Tebessa, Algeria) is shown in table 7. The inhibitory effects of both essential oils (EOs) against the ESBLs employed and their activity potentials were qualitatively and quantitatively assessed by the measurements of inhibition zones which were noted in triplicate in order to define the final average for each strain along with their MIC values. Consistent with the result, a comparison of the antibacterial activity of the volatile extracts of *Artemisia herba* and *Ruta-Montana* was carried out at the same pure concentration. Indeed, a variation in the inhibitory effect was shown depending on the type of germ and the oil presented.

Table 7: The inhibitory activity of both EOs against ESBLs (Aromatogram).

Eos of a herbal plant	***Artemisia Herba EO***				***Ruta Montana EO***			
ESBLs bacterial strains	**Zone of inhibition measured in mm**			**The average**	**Zone of inhibition measured in mm**			**The average**
	R_1	**R_2**	**R_3**		**R_1**	**R_2**	**R_3**	
Escherichia coli1 (S8.E14.Merguez)	22	25	21	22.66	07	08	-	5
Klebsiella pneumoniae spp ozaena (S19.E21. Merguez)	24	22	-	15.33	13	13	11	12.33
Raoultella terrigena (S15.E23.Milk)	16	27	37	26.66	13	15	16	14.66
Serratia odorifera1 **(**S43.E9.AS.Chicken meat)	14	10	17	13.66	11	18	-	9.66

Consequently, both volatile extracts can practically inhibit the growth of the four bacterial strains to a certain extent .In fact, relative results could be obtained based on strict measurements of halos of inhibition's averages, thus revealing a major antimicrobial activity of *A-herba* EO against ESBLs compared to *R-montana* EO, which is characterized by a relative action withdrawal based on the results summarized in table 7. In this context, AH-EO has shown away the best antimicrobial activity against the multi resistant bacterial strains with the predominance of *Raoultella terrigena* (S15.E23.Milk) average that equals 26.66mm.The same strain has revealed an inhibitory zone under the inhibitory effect of RM-EO yet with rather a lower average (14.66mm).Similarly *Klebsiella pneumoniae spp ozaena* (S19.E21. Merguez) disclosed a better sensitivity to AH-EO (15.33mm) in contrast with the

one recorded with RM-EO (12.33mm).These results can be also applicable to both *Serratia odorifera1* (S43.E9.AS.Chicken meat) and *Escherichia coli1* (S8.E14.Merguez) when it comes to AH-EO that can prevail a distinct antimicrobial activity against both strains respectively. Yet RM-EO can be considered weak and rather inactive toward both bacteria due to the drastic decrease of their halo's averages, mainly the one measured around *E.coli1* S8.E14 Merguez (5mm) .On that account, all of the analyzed results may indicate a significant difference between the inhibitory effects of both essential oils on the four ESBL-producing strains.

According to the obtained results, *Artemisia herba* showed the highest activity (26.66mm) against *Raoultella terrigena* (S15.E23.Milk), succeeded by *E.coli1* (S8.E14.Merguez) and *Klebsilla pneumoniae spp ozaena* (S19.E21. Merguez) which displayed an average of 22.6mm and 15.33mm respectively. Yet, we can record the least activity of AH-EO against *Serratia odorifera1* (S43.E9.AS.Chicken meat).This analysis revealed a significant difference between both essential oils.

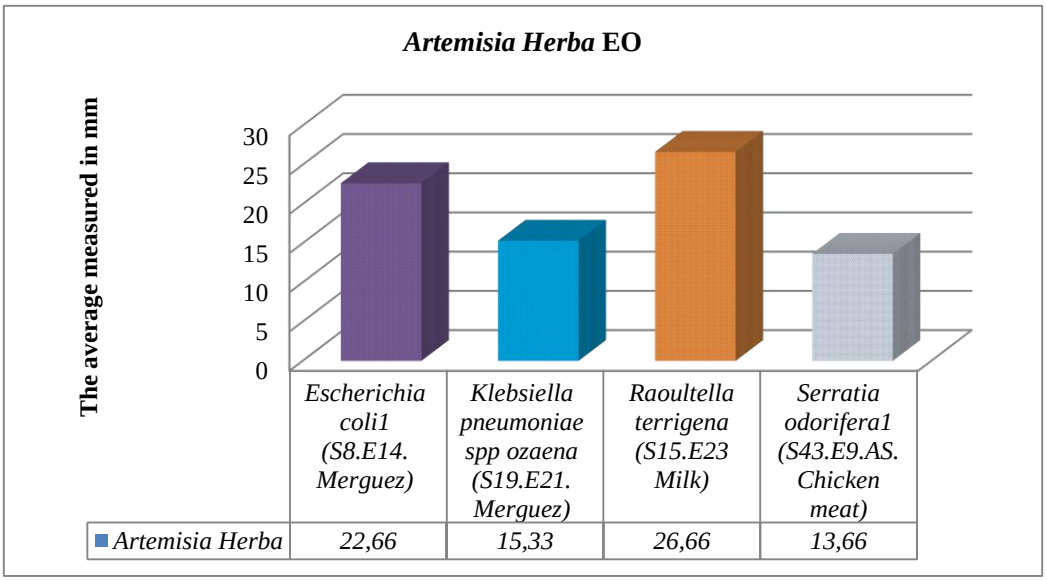

Figure 17: Highest zones of inhibition by *Artemisia herba* EO against the test pathogens.

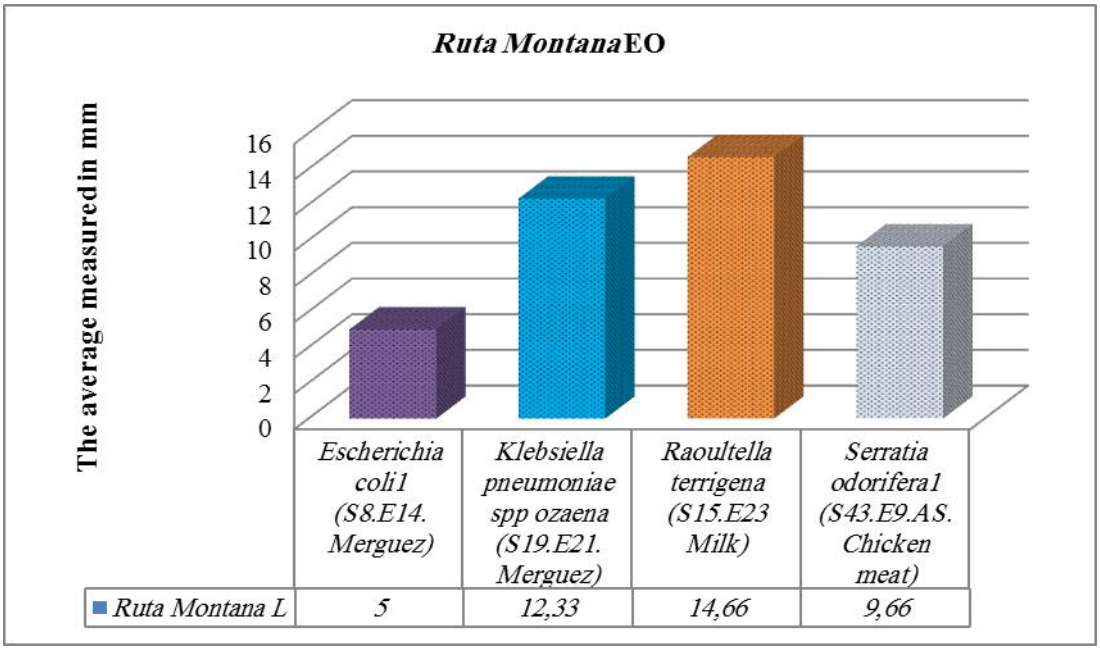

Figure 18: Highest zones of inhibition by *Ruta montana* EO against the tested ESBLs.

The demand for essential oils derived from natural plants has increased over the years owing to their use as medicines against many severe diseases and their low toxicity. For this purpose, we examined the antimicrobial properties of EOs from *Ruta montana* indigenous to Tebessa city. The results of the antibacterial activity of RM-EO against the four selected pathogenic bacteria (ESBLs) are compiled in figure 18. Compared to antibiotics for which all the tested bacteria showed a large significant resistance RM-EO was found to be more or less ineffective against all the tested strains. Moreover, the extraction of RM-EO that we carried out allowed us to obtain a yield of approximately 0.8% ,which falls into the same range as those reported in the literature (0.38 - 6.1%) yet it is always considered as weak compared to the rates, previously noted in other studies. We have noticed that the yield changes as a function of the use of the leaves or flowers, the site of culture, the origin of the seeds, and the moment of the harvest. Importantly, the variation of chemical compounds is generally influenced by the drying conditions, harvest season, geographical location, fertilization, soil pH, as well as the chemo type, the part of the plant used, genotype, or extraction method (Akrout et al., 2003).

In fact, these factors influence the plant's biosynthetic pathways and, consequently, the relative proportion of the main characteristics, both minor and major, compounds. Indeed, a study conducted on EOs extracted from eleven samples belonging to three species of the genus *Ruta* has suggested that the main factors able to influence the chemical composition of RM-EO was the harvested period and the geographical origin (Bennaoum et al., 2017). The EOs extracted from plants harvested in spring and winter, as in our case, were characterized

by the predominance of ketones, whereas those harvested on summer and autumn seasons were characterized by the predominance of sesquiterpenes and monoterpenes (Azzeddine et al., 2021) .

Generally, RM-EO has less to no activity on *Escherichia coli* (5mm). Regarding the antibacterial activity, it can be attributed to the high content of the essential oil in an oxygenated compound as in piperitone. In fact, this activity always concerns the major compounds of the essential oil, involving either a high concentration of piperitone or the synergistic effects of the other constituents that represented the key component in other studies. Yet, the experiment yielded inconclusive results where further investigation is truly required (Mahboubi et al., 2008) The findings revealed, however, that the origin and the plant species have a significant effect on the antibacterial activity of EOs against ESBL-producing bacterial strains isolated from different food sources. Lab-scale produced AH-EO had a higher value of MIC compared to the RM-EO one. Moreover, significant differences were observed among the plant species. The most effective EO was found to be *A. herba*- EO with the highest average of 37mm against *Raoutella terrigena* (S15.E23.Milk), while the least was found to be *Ruta montana* EO, representing a weak antimicrobial activity overall, that can attend to an average of 5mm against *E.coli1* (S8.E14.Merguez), which is always smaller than the one recorded with AH-EO against *Serratia odorifera1*(S43.E9.AS.Chicken meat) with ZOI of *(*10mm) mentioned in figure 19 below.

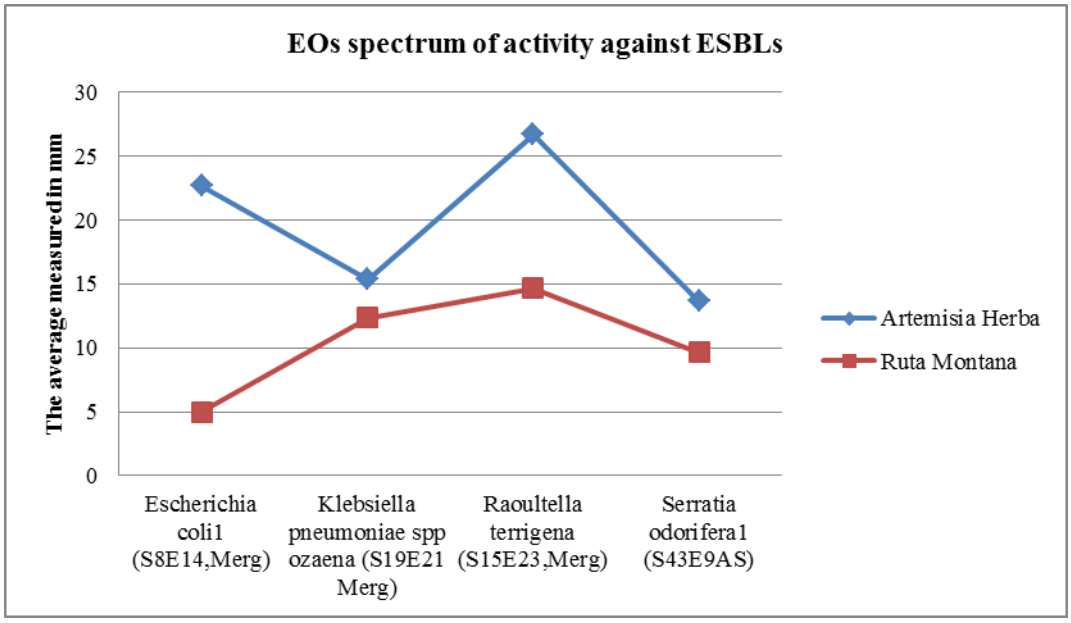

Figure 19: The spectrum of activity of both essential oils against ESBLs.

3.3.3.2 The minimum inhibitory concentration (MIC):

The minimal inhibitory concentration (MIC) was defined as the lowest concentration of an AH-EO and RM-EO that will inhibit the visible growth of microorganism after 24h of incubation .MIC was determined only with ESBLs that displayed inhibitory zones and results are shown in figure 20 below.

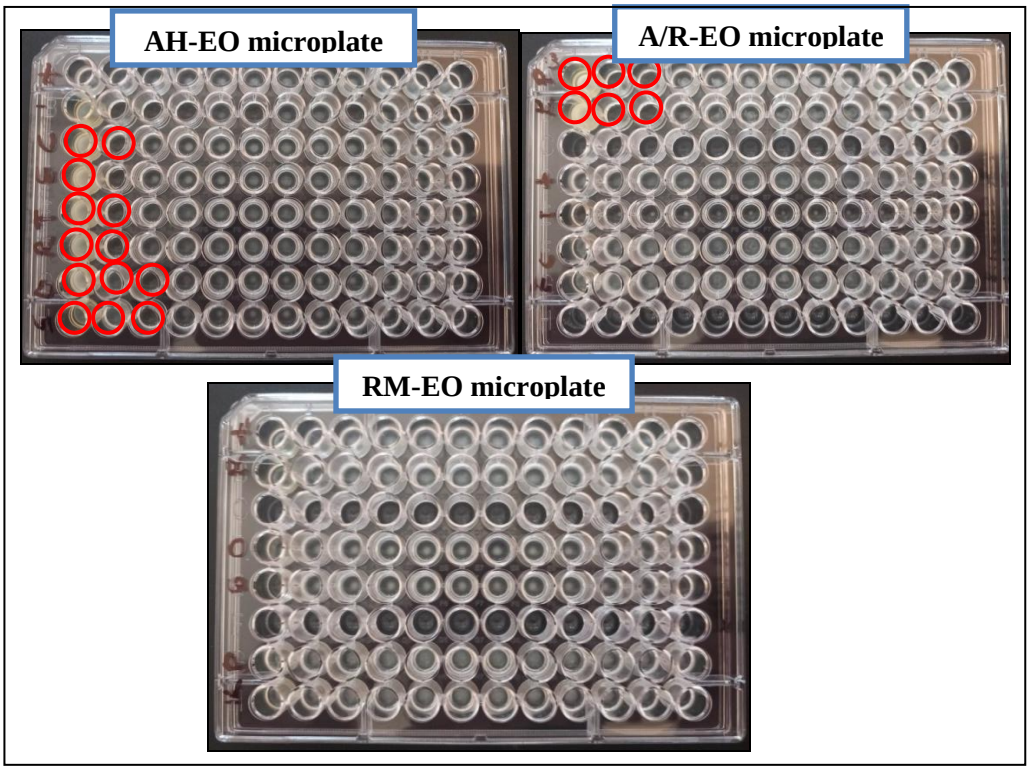

Figure 20: Results of AH-EO and RM-EO activity tested on microtiter plates.

Table 8: Minimum inhibitory concentrations (MIC) results of *AH*- EO on ESBLs.

Extracted plant	***Artemisia Herba* essential oil**							
ESBLs strains	***Escherichia coli1*** (S8.E14.Merguez)		***Klebsiella pneumoniae spp ozaena*** (S19.E21. Merguez)		***Raoultella terrigena*** (S15.E23.Milk)		***Serratia odorifera1*** (S43.E9.Chicken meat)	
10 µl/ml	-	-	-	-	-	-	-	-
5 µl/ml	-	+	-	-	-	-	-	-
2.5 µl/ml	+	+	-	+	+	+	-	-
1.25 µl/ml	+	+	+	+	+	+	+	+
0.625 µl/ml	+	+	+	+	+	+	+	+
0.312 µl/ml	+	+	+	+	+	+	+	+
0.156 µl/ml	+	+	+	+	+	+	+	+
0.078 µl/ml	+	+	+	+	+	+	+	+
0.039 µl/ml	+	+	+	+	+	+	+	+
0.019 µl/ml	+	+	+	+	+	+	+	+
0.0095 µl/ml	+	+	+	+	+	+	+	+
0.0047 µl/ml	+	+	+	+	+	+	+	+
Positive	+	+	+	+	+	+	+	+
Negative	-	-	-	-	-	-	-	-

+ : Positif **; -** : négatif

Table 9: Representation of the average of MIC's for each ESBL strain.

Extracted plant	***Artemisia Herba* EO**							
ESBLs strains	***Escherichia coli1*** (S8.E14.Meguez)		***Klebsiella pneumoniae spp ozaena*** (S19.E21. Merguez)		***Raoultella terrigena*** (S15.E23.Milk)		***Serratia odorifera1*** **(**S43.E9.Chicken meat)	
MIC (µl/ml)	05	10	2.5	05	05	05	2.5	2.5
Average (µl/ml)	7.5		3.75		05		2.5	

Interpretation of sensitivity tests in Aromatogram:

- **MIC < 12 µl.ml-1:** very excellent inhibition power.
- **12 µl.ml-1 < MIC < 48 µl.ml-1:** excellent inhibition power.
- **48 µl.ml-1 < MIC< 96 µl.ml-1:** medium or intermediate inhibition power.
- **MIC > 96 µl.ml-1 :** low inhibitory power.

The activity of essential oils was evaluated in vitro by a broth micro dilution method using a panel of micro-organisms where antimicrobial activity was expressed as Minimum Inhibitory Concentration (MIC). Subsequently, the different inhibition spectra obtained for each volatile extract tested are shown in figure 20. Each of these oils has variable activities which are closely related to their nature and to the bacterial strain present. The essential oil of *Artemisia herba* plant reacted positively to the tested microbial strains with a very excellent inhibition power since the total of MIC values recorded by different strains are MIC < 12 µl.ml-1 ,hence large inhibitory properties of the oil were observed within a range of concentrations ranging from 2.5 to 7.5 µl.ml-1. *S.odorefira1* (S43.E9.AS.Chicken meat) has demonstrated the highest susceptibility toward AH-EO with MIC of 2.5 µl.ml-1 followed by *K.pneumoniae spp ozaena* (S19.E21. Merguez) and *R.terrigena* (S15.E23.Milk), with an MIC of 3.75 µl.ml-1 and 5 µl.ml-1 respectively. On the other hand, *E.coli1* (S8.E14.Merguez) seemed to be less sensitive toward the investigated oil with a MIC of 7. 5 µl.ml-1

The numerical results and graphs proved that the *Artemisia herba* essential oil have a significant antibacterial activity with all ESBL bacterial strains. These results are consistent with those obtained in several studies conducted on *Artemisia herba* in Algeria (Delimi et al., 2018), Lebanon (Hatem et al., 2018), Morocco (Amor et al., 2019) and also Tunisia (Zouari et al., 2014). Based on these results, *Artemisia herba* essential oil can be used as a natural antimicrobial agent for the treatment of many infectious diseases caused by these pathogenic germs resistant to antibiotics.

The antibacterial activity of the essential oil was tested by the disc diffusion assay revealed that Gram-negative bacteria, also referred to as ESBLs, were pretty susceptible to *A-herba* EO. Related to the inhibition of growth, significant differences were detected among these cited strains, since all of them showed an interesting sensitivity for the tested concentration of oil (10ul) with inhibitions zones that range of 13.66-22.66mm. Subsequently, the most sensitive bacteria was *R.Terrigena* (S15.E23.Milk), followed by *E.coli1* (S8.E14.Merguez) and *k.pneumoniae* (S19.E21. Merguez), while *S.odorefira*1 (S43.E9.AS.Chicken meat) was the least susceptible. In this regard, some available literature reports the antimicrobial activity of AH-EO against *E. coli* (S8.E14.Merguez). Moreover, several studies showed a great potential of this oil as an antibacterial agent against *Klebsiella*

pneumoniae spp ozaena (S19.E21. Merguez) and various ESBL strains. Thus, these differences in the sensitivity of microorganisms against the essential oil of *A. herba* can be explained by the quantity and quality of bioactive molecules or the nature and composition of the cell wall as well as the potency of the enzymatic system of the cell which controls its metabolism (Bouzidi,2016).

In order to explain the EOs mechanism of action against ESBL pathogenic bacteria, several studies tried to correlate the antibacterial activity with specific compounds present in EOs. It is known that the biological activity of an essential oil depends on its chemical composition and synergy between the various components both majority and minority (Farah, 2008). By analogy with other studies, AH-EO is composed of molecules belonging to the groups of terpenes and oxygenated molecules (aldehydes, phenols, alcohols, ketones) (Freeman et Carel, 2006) along with sesquiterpenes fractions that may interfere with the integrity and function of the cell membrane, changing the membrane potential, causing loss of cytoplasmic material, as well as on mitochondria leading to deficits of energy and respiratory metabolism of pathogen bacteria (Burt,2004). However, the antibacterial mechanism of EOs can be correlated by the proportions of minor components that may act synergistic or in antagonistic with the major components. Indeed, a study carried out by Viljoen showed that camphor alone exhibited no antibacterial activity, whereas it acts synergistically with other components possessing antimicrobial activity in AH-EO (Viljoen et al., 2003).

The antibacterial properties of EOs can also be attributed to their lipophilic nature, which allows them to effectively infiltrate into bacteria cells. In this regard, it was found that hydrocarbons in EOs (43.03% of AH-EO) are predominantly accumulated in cytoplasmic membranes, which leads to impaired membrane permeability and, ultimately, the rapid death of microorganisms (Amrati et al., 2021).In addition, EOs rich in phenolic components show a great antimicrobial activity such as artemisinins that might show the properties to enhance the susceptibility of *Escherichia coli* to β-lactam antibiotics, as it is mentioned in Arsalan Khan study (Khan, 2022) .However, this property is not related to the effect on the production or activity of β-lactamase but it is more associated with an increase of the accumulation of antibiotics in the cell due to the blockage of the AcrAB-TolC efflux pump system (Wondrack et al., 1996).

Meanwhile, a recent study of the anti-biofilm activity of essential oils from three Tunisian medicinal plants from the genus *Artemisia* showed that *A-herba* essential oil is rich in sesquiterpenes and seem to have a strong antibacterial activity against *E.coli1* (S8.E14.Merguez) that revolves around the inhibition of biofilm formation by phytochemicals , which has been previously described (Di Pasqua et al., 2006; Borges et al, 2014) indicating

that a general mechanism may apply rather than the ability of these compounds to bind to a specified molecular target such as the fimbriae that are critical for biofilm development in *E. coli* (Rodrigues et al., 2009). These results show that different species of the genus *Artemisia* can be used against persistent infections caused by *E. coli* since the formation of biofilms is mandatory for the survival of bacteria under hostile environmental conditions which may also explain the sensitivity of this strain toward AH-EO in our study.

All of these current results agreed with those reported by (Habibi et al., 2013) who showed that AH-EO had significant antibacterial capacity against bacterial strains, particularly versus *Escherichia coli*. In addition, our findings are in accordance with the findings of a number of previous research efforts, such as the one conducted by (Radulović et al., 2013), who reported that the compound *Artemisia* ketone possessed antibacterial power vs. pathogenic microorganisms. Some studies indicated that Gram-positive bacteria are more sensitive to the effect of Eos (Lafraxo et al., 2022). The findings of the current research, on the other hand, suggested that the difference in the cell walls of Gram-positive and Gram-negative bacteria was not a significant factor in determining the antibacterial activity of AH-EO, since *Escherichia coli* was the one of the sensitive strains to AH-EO with a zone of inhibition of 22.66 mm and minimum inhibitory concentration of 5.34 µg/mL. These results may be explained by the broad-spectrum activity of the essential oil. Yet, even though our research was conducted on ESBL bacterial strains only, which gave away a large susceptibly against AH-EO strains. It is known in the literature that Gram–negative bacteria are less sensitive to essential oils and plant extracts than Gram-positive ones (Karaman et al., 2003). This is due to structural differences in their outer membranes (Inouye et al., 2001), where chemical compounds exert their antimicrobial activity on microorganisms by disrupting membrane integrity (Knobloch et al., 1989). The penetration of active compounds present in EOs is therefore different (Kheyer et al., 2014). In Gram-negative bacteria, the outer membrane constitutes an effective permeability barrier, rich in lipopolysaccharides whose negative surface charges prevent the diffusion of hydrophobic molecules (Nikaido, 2003), however, some low molecular weight phenolic compounds can adhere to these bacteria by binding to membrane proteins and lipopolysaccharides using their functional groups and sneaking up to the more vulnerable inner membrane (Dorman et al., 2000). In other words, hydrophobic compounds are able to disrupt the plasma membrane and the outer membrane of Gram-bacteria by inducing its permeability and cell death (Wang et al., 2012), Gram-positive bacteria are less protected against antibacterial agents since the peptidoglycan only hinders the diffusion of molecules larger than 50 kDa (Nikaido et al., 1985).

Table 10: Minimum inhibitory concentrations (MIC) results of *RM*-EO on ESBLs.

Extracted plant	***Ruta montana* EO**							
ESBLs strains	***Escherichia coli1*** (S8.E14.Meguez)		***Klebsiella pneumoniae spp ozaena*** (S19.E21.Merguez)		***Raoultella terrigena*** (S15.E23.Milk)		***Serratia odorifera1*** **(**S43.E9.Chicken meat)	
10 µl/ml	+	+	+	+	+	+	+	+
5 µl/ml	+	+	+	+	+	+	+	+
2.5 µl/ml	+	+	+	+	+	+	+	+
1.25 µl/ml	+	+	+	+	+	+	+	+
0.625 µl/ml	+	+	+	+	+	+	+	+
0.312 µl/ml	+	+	+	+	+	+	+	+
0.156 µl/ml	+	+	+	+	+	+	+	+
0.078 µl/ml	+	+	+	+	+	+	+	+
0.039 µl/ml	+	+	+	+	+	+	+	+
0.019 µl/ml	+	+	+	+	+	+	+	+
0.0095 µl/ml	+	+	+	+	+	+	+	+
0.0047 µl/ml	+	+	+	+	+	+	+	+
Positive	+	+	+	+	+	+	+	+
Negative	-	-	-	-	-	-	-	-

In line with this, the evaluation of MIC and RM-EO (table 10) showed no antibacterial activity with MICs for the recorded bacterial strains. These results are consistent with some previous studies, which reported that essential oils of *Ruta* genus displayed no or less antibacterial activity (Merghache et al., 2008;Bnina et al., 2010; Haddouchi et al., 2013). This weak antibacterial activity could be attributed to the high percentage of ketones in the oils (Gibka et al., 2009 ; Haddouchi et al., 2013). Indeed, the antimicrobial activity of 2-undecanone, the most abundant ketone in this oil, is known to be weak against ESBLs bacterial strains. On the other hand, (Zellagui et al ., 2012) and Bouzidi et al., 2012) reported that the essential oil of *R. montana* has a strong antibacterial activity against all tested bacterial strains (*E. coli, S. aureus, Klebsiella pneumoniae,),* with an inhibition diameter that increases as the concentration of the oil extract increases. This activity appears to be related to the relative amount of hydrocarbon and alcohol terpenes (Haddouchi et al., 2013). It is widely

accepted that essential oils rich in aldehydes or phenols have the highest antibacterial activity, followed by those containing alcoholic terpenes. Essential oils containing high levels of ketones or esters have much weaker activity, while those containing terpene hydrocarbons are often inactive (Bassolé et al., 2012). In total, volatile oil composition of *Ruta montana* are mainly considered as a rich source of ketones and esters which may help identify the reason behind the inactivity of the designed extract within our study. However, in our opinion, this deduction should be approached with caution, as all these strains are classified as ESBL hence, their resistance potential against RM-EO can be associated, not only with the presence of hydrolyzing beta-lactamases, but also with the reduced permeability of the outer membrane due to loss or modification of porins (Pagès et al., 2008; Khalifa et al., 2021).

3.3.3.3 The minimum bactericidal concentration (MBC):

The broth microdilution method was used to determine the minimal inhibitory and minimal bactericidal concentration (MIC and MBC) of AH-EO. Furthermore, the bactericidal power (ratio MBC/MIC) was calculated to determine whether the EO has exhibited bactericidal or bacteriostatic against the tested ESBLs bacterial strains.

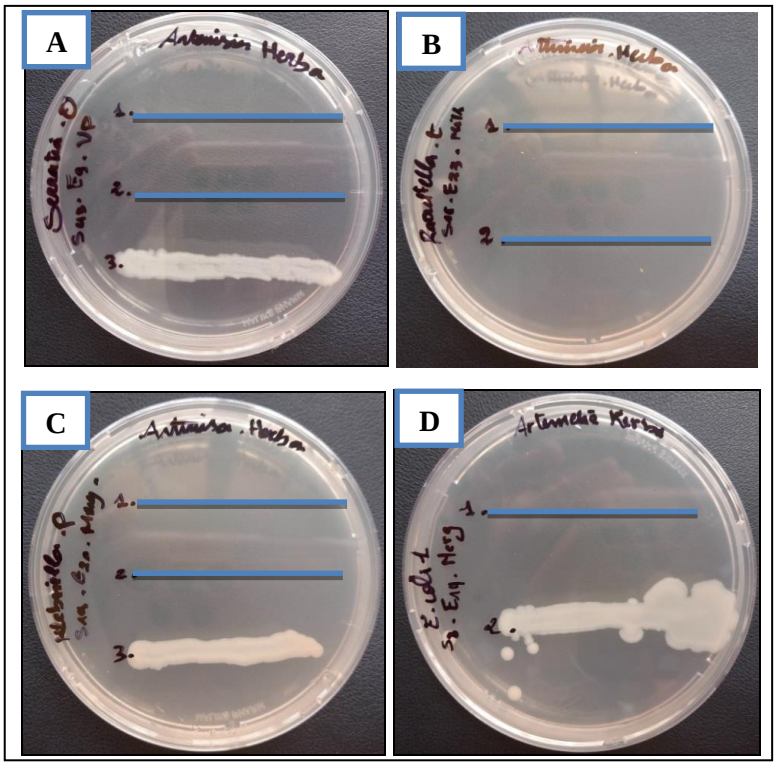

Figure 21: Results of the minimum bacterial concentration (MBC).

A: *Serratia odorifera1* **(**S43.E9.AS.Chicken meat); **B:** *Raoultella terrigena* (S15.E23.Milk)

C: *Klebsiella pneumoniae* (S19.E21. Merguez); **D:** *Escherichia coli1* (S8.E14.Merguez)

Table 11: The minimum Bactericidal Concentrations (MBC) of AH-EO on ESBLs.

Extracted Plant	**Artemisia Herba-alba EO**			
Bacterial strains (ESBLs)	***Escherichia coli1*** *(S8.E14.Merguez)*	***Klebsiella pneumoniae spp ozaena*** *(S19.E21. Merguez)*	***Raoultella terrigena*** *(S15.E23.Milk)*	***Serratia odorifera1*** *(S43.E9.Chicken meat)*
10 µl/ml	-	-	-	-
05 µl/ml	+	-	-	-
2.5 µl/ml	+	+	+	+
1.25 µl/ml	+	+	+	+

+ : Positif **; - :** Négatif

Table 12: The minimum Bactericidal Concentrations of AH-EO for each ESBL strain.

Extracted Plant	***Artemisia Herba* EO**			
Bacterial strains (ESBLs)	***Escherichia coli1*** (S8.E14.Merguez)	***Klebsiella pneumoniae spp ozaena*** (S19.E21. Merguez)	***Raoultella terrigena*** (S15.E23.Milk)	***Serratia odorifera1*** **(**S43.E9.Chicken meat)
MBC	10 µl/ml	05 µl/ml	05 µl/ml	05 µl/ml

After analysis of the results, it appears that the values of the minimum bactericidal concentrations (MBCs) of the four species studied vary from 5 to 10 µl.ml-1. the MBC/MIC ratio points out that these natural species are generally bactericidal with respect to the bacterial strains tested. This effect was observed for the bacterial strains of *K.pneumonia ssp ozaneae* (S19 E21 Merg), *R.terrigena* (S15 E23 milk), *S.odorifera 1*(S43 E9 chicken meat, carbapenemase positive) *and E.coli1* (S8E14 Merg) respectively to *A-herba* essential oil (figure33). In fact, according to the ratio MBC/MIC, If the ratio MBC/MIC ≤4, the effect is considered as bactericidal but if the ratio MBC/MIC > 4, the effect is defined as bacteriostatic (Levison et al., 2004; Benjamin et al., 2012). The MBC/MIC ratios are displayed in table 13.

Table 13: Determination of the ratio MIC/MBC of *A-herba* essential oil.

ESBL strains	Minimum Inhibitory Concentrations (MIC)	Minimum Bactericidal Concentrations (MBC)	MBC/MIC Ratio	Nature of the effect
Escherichia coli1 (S8.E14.Sausage)	7.5 µl/ml	10 µl/ml	1.33 µl/ml	bactericidal
Klebsiella pneumoniae spp ozaena (S19.E21. Sausage)	3.75 µl/ml	05 µl/ml	1.33 µl/ml	bactericidal
Raoultella terrigena (S15.E23.Milk)	05 µl/ml	05 µl/ml	1 µl/ml	bactericidal
Serratia odorifera1 (S43.E9.Chicken meat)	2.5 µl/ml	05 µl/ml	2 µl/ml	bactericidal

The results of this study showed an interesting antibacterial activity compared to those reported in other areas (Btisam et al., 2016;Amin et al., 2019). A study conducted by Amor and his group in the Azzemour region (Southwest Morocco) showed that AH-EO was inactive against *E. coli1* (S8.E14.Merguez) at a concentration of 10 µL (Amor et al., 2019) which is consistent to our study results. However, AH-EO collected in the province of Boulemane (Middle Atlas, Morocco) showed inhibition zones between 12.2±0.6 mm and 25.2±2.6 mm, and MIC between 2.5 and 20 µL/mL (Zoubi et al. 2018), hence both bactericidal and bacteriostatic effect were deduced after determining the MBC/MIC ratio in that study, contrary to ours, which revealed a strong bactericidal activity of Artemisia herba essential oil against the tested ESBL bacterial strains.

In general, the Gram-negative bacteria display the least sensitivity towards the extracts and fractions, and most of the plant extracts (except ethanol extract of barks) with an unvaried activity against *Escherichia coli1* (S8.E14.Merguez), indicating the resistance of this bacterium to the plant extract. This was to be expected, as Gram-negative bacteria offer a much more complex barrier system against permeation of foreign substances (in this case, the antimicrobial agent). This is attributed to the specialized cell wall structure and especially the presence of the outer envelope resulting in the impermeability of these micro-organisms to biocides and antibiotics, and at times, resulting in regulation and prevention of their passage to the target region (Denyer et Maillard, 2002). Resistance to the plant extracts is, thus, exhibited to a far greater extent by the Gram-negative bacteria than by Gram-positive bacteria

(Wink et al.,2012). The lipophilic or hydrophilic nature of compounds also plays a role in the activity, or lack thereof, against the microorganisms. Compounds considered to be more effective against Gram-negative bacteria are considerably less lipophilic. This is because of the structure of their cell wall which also has higher lipid content (Peters et Pasvol,2007).The outer layer of the Gram-negative outer membrane is composed of lipopolysaccharide molecules that provides hydrophilic environment that gives protection against hydrophobic molecules (Diniz et al., 2009). This could explain why the extracts of *A-herba* showed more inhibition against *E.coli1* (S8.E14.Merguez)

CONCLUSION

Conclusion:

Foodborne diseases and food spoilage enteric pathogens including species *Klebsiella, Escherichia coli, Pseudomonas, Salmonella* and *Staphylococcus aureus* continue to exert negative impacts on public health as well as the food industry. Hence foodborne disease outbreaks have resulted in a high rate of mortality, along with the high financial burden stemming from healthcare costs. Moreover, the soaring numbers of confirmed cases of these illnesses are very alarming, despite the availability of the hazard analysis and critical control point (HACCP) system.

Subsequently, a recurring theme in antimicrobial resistance has become readily apparent. Namely, the production of extended-spectrum β-lactamases (ESBLs) that impedes the antimicrobial treatment of mortal infections caused by *Enterobacteriaceae*, and also threatens the currently available antibiotic armory. Therefore, the introduction of strong antibacterial substances to the market is initially associated with an exuberant use, partially being a result of fairly uniform susceptibility of a range of targeted pathogens, which is followed by the emergence of resistance and rapid clonal spread. The rapidity of the development and spread of resistance is a complex process that is influenced by selective pressure, pre-existence of resistance genes and use of infection control measures.

In this context, the interest on the antibacterial activity of lactic acid bacteria along with their novel biological preservation methods has been increasing during recent years, supported by research indicating that antagonistic LAB microorganisms and their antimicrobial metabolites may have some potential use as natural preservatives as a way not only to control the growth but also to inactivate undesired microorganisms in food. Bio preservation using lactic acid bacteria (LAB) and/or their antimicrobial metabolites represents an alternative for improving food safety. These antimicrobial properties of LAB were derived from competition for nutrients and the production of one or more antimicrobial active metabolites such as organic acids (mainly lactic and acetic acid), hydrogen peroxide and also other compounds, such as bacteriocins and antifungal peptides. The important contribution of probiotic LAB in food preservation has been attracting much attention because of the nutritional qualities of the raw material through an extended shelf life of food and their ability to inhibit spoilage and foodborne pathogens, which is interesting for the food industry.

In this present study, seven lactic acid strains isolates (JMQ24, KMQ31, JMZ02, ULZ15, KMZ 10, QMZ 11, BZQ 36) that were previously isolated and identified by Miss Metrouh Roumaissa by using the API 50 CHL biochemical gallery and the Maldi-Tof Ms molecular technique, were evaluated based on their exhibited antimicrobial properties towards a large spectrum of both Gram-positive and Gram-negative pathogen strains.

The evaluation of the inhibitory activity of lactic acid bacteria using the agar well diffusion and the agar overlay method showed that the 07 isolates exerted inhibitory activity against the tested Gram-positive and Gram-negative indicator strains, and this activity varied depending on the tested strain. Hence, our results revealed that *Pediococcus pentosaceus* (ULZ15) had the highest antimicrobial property against all the indicator pathogens tested when it comes to the agar well diffusion method. Its activity was highest against *B.subtilis (*ATCC 9372), showing a relative susceptibility with a ZOI of 7 mm, yet the least inhibitory activity was registered against *Aspergillus fumigatus* (3 mm).Consequently, the antimicrobial activity of LAB bacteria against the designated Gram-positive strains via the agar overlay method has revealed more important results. *Enterococcus faecium* showed the highest activity (28 mm) against *Serratia odorifera1* (S43.E9.AS.Chicken meat) and least one was noted toward *Staphylococcus aureus* (ATCC25923) after the introduction of *Pediococcus pentosaceus.*

These inhibitory outlines of LAB on tested bacteria under both direct and indirect methods might be explained by the presence of a wide range of antimicrobial metabolites as in; lactic acid, acetic acid, di-acetyl and bacteriocin. Therefore, we conclude that agar overlay assay has several advantages towards well diffusion method by means of the efficiency of the inhibition and the facility of the application of the method.

The substitution of essential oils for traditional food preservatives is promising. Given the abundant literature regarding essential oils against foodborne pathogens, it is evident that enthusiasm exists for essential oils as effective food additives. In this regard, the antibacterial activity of tests strains was evaluated through a vitro test of activity that consists of an Aromatogram in order to test the sensitivity of bacteria to *A-herba* and *R-montana* essential oils. Consequently, both volatile extracts can practically inhibit the growth of the four bacterial strains to a certain extent. However, AH-EO displayed a major antimicrobial activity against the multi resistant bacterial strains with the predominance of *Raoultella terrigena* (S15.E23.Milk) average that equals 26.66mm, yet the best activity of RM-EO only attained a major extent of 14.66mm against the same strain, which was approved by their MIC and MBC concentration that enabled us later to conduct a ratio to reveal the bactericidal effect of *Artemsia herba* essential oil. There has been evidence that inhibitory or stimulatory effects of these essential oils on these beneficial microorganisms could vary according to the type of essential oil, tested dose, target microorganism and examined microorganism but most importantly the chemical composition of the volatile extracts themselves which explains the major difference of both EOs against the same bacterial strains.

To sum up, the analysis of all these results highlights the effective effects of the essential oil along with the antibacterial activity of lactic acid bacteria (LAB) against the growth of ESBLs which may open up some future prospects such as:

- Carry out a more in-depth characterization and purification of the inhibitory substances produced by the LAB strains.
- Determine the physicochemical properties of the produced molecules (thermostability, sensitivity to proteolytic, glycolytic, and lipolytic enzymes, activity at different pH levels, sensitivity to detergents).
- Develop and optimize a purification protocol using chromatographic techniques and estimate their molecular weights.
- Perform a chromatographic analysis of the *Artemisia herba* essential oil in order to identify their complex mixture of naturally occurring aromatic chemicals, known as its components.
- Explore other biological activities of the essential oil of *Artemisia herba* and *Ruta montana.*

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ANNEXES

Annexes

Culture media used

The compositions are given per liter of medium.

1. **Muller Hinton agar (MHA)**

A solid medium originally designed for the isolation of pathogenic *Neisseria* species, now widely used for antibiotic susceptibility testing (including sulfonamides).

- **Composition:**
  - Beef infusion (2.0 g)
  - Solid starch (1.5g)
  - Casein hydrolysate (17.5 g)
  - Agar (17g)
  - Final pH 7.3 +/- 0.2 at 25°C

- **Preparation** :

Suspend 38 g in 1 liter of distilled water, bring to the boil to dissolve the medium completely and sterilize by autoclaving at 121°C for 15 minutes. Store prepared media below 8°C, protected from direct light. Also store dehydrated powder, in a dry place, within tightly-sealed containers at 2-25°C.

2. **Mueller Hinton Broth (MH Broth):**

A liquid medium for antibiotic susceptibility studies (MIC-determination).

- **Composition:**
  - Beef infusion (2.0g)
  - Solid starch (1.5g)
  - Casein hydrolysate (17.5g)
  - Final pH 7.4 +/- 0.2 at 25°C
- **Preparation:**

Dissolve 21 g in 1 litre of distilled water. Sterilize by autoclaving at 121°C for 15 minutes precede to storing prepared media below 8°C, protected from direct light. Also store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.

3. **Nutrient agar:**
   - Peptones (15.0g)
   - Yeast extract (3.0g)
   - Sodium chloride (6.0g)
   - D(+)glucose (1.0g)

- Agar-agar (12g)
- Final pH: 7.5 ± 0.2 at 25 ℃.

- **Preparation:**

Suspend 37 g Standard I Nutrient, autoclave (15 min at 121 ℃). The prepared media are clear and yellowish-brown.

4. Nutrient broth:

Nutrient Broth is a liquid medium used for the cultivation of a wide variety of organisms from clinical specimens and other materials. This medium can be enriched with other ingredients such as blood, serum, sugars, etc., for special purposes.

- **Composition:**
  - Beef Extract 1.0
  - Peptone 5.0
  - Yeast Extract 2.0
  - Sodium Chloride 5.0
  - Final pH 6.8 ± 0.2 at 25°C

- **Preparation:**

Suspend 13 g of the powder in one liter of distilled or deionized water. Mix well and heat to boil shaking frequently until completely dissolved. Sterilize in autoclave at 121°C for 15 minutes.

5. MRS Agar:

- Proteose Peptone No.3 (10.0 g)
- Beef Extract (10.0 g)
- Yeast Extract (5.0g)
- Dextrose (20.0 g)
- Polysorbate 80 (1.0g)
- Ammonium Citrate (2.0 g)
- Sodium Acetate (5.0g)
- Magnesium Sulfate (0.1 g)
- Manganese Sulfate (0.05 g)
- Dipotassium Phosphate (2.0 g)
- Agar (15.0 g)

6. MRS Broth:

This media consists of the same ingredients without the agar, which is adjusted and/or supplemented as required to meet the performance criteria

- **Preparation:**
  - Suspend the powder in 1 L of purified water along with Difco™ Lactobacilli MRS Agar (70 g) and Difco™ Lactobacilli MRS Broth (55 g).
  - Mix thoroughly.
  - Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
  - Autoclave at 121°C for 15 minutes.
  - Test samples of the finished product for performance using stable, typical control cultures.