PEOPLE'S DEMOCRATIC REPUBLIC OF ALGERIA
MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH
YAHIA FARES UNIVERSITY OF MEDEA



Departement of **S**ciences- **O**uzera campus

MASTER'S THESIS

Specialty: Applied Microbiology

Determination of antioxidative activity and exopolysaccharide level production by lactic acid bacteria isolated from fermented meat

Presented by

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Presented before the jury on 26/06/2024

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Promotion 2023/2024

Dedication



In the name of Allah, the Most Gracious, the Most Merciful.

First and foremost, I thank Allah for granting me the strength, patience, and wisdom to complete this thesis. Alhamdulillah, without His blessings and guidance, none of this would have been possible.

I extend my deepest gratitude to my parents for their unwavering support and encouragement. Your love and belief in me have been my anchor through this journey, and I am forever grateful for your sacrifices and endless prayers.

To my supervisor, BOUBAKRI Kamel, thank you for your invaluable guidance, patience, and insightful feedback. Your expertise and support have been instrumental in shaping this work, and I am profoundly grateful for your mentorship.

My heartfelt appreciation goes to my best friend, Naila, who has been a constant source of strength and joy in my life. Your friendship and encouragement have been a beacon of light, and your help throughout this journey has been immeasurable. Thank you for being the best friend anyone could ever ask for.

I am also deeply grateful to my colleague, HAFSI Manel. Your collaboration, dedication, and insightful discussions have greatly enriched my research experience. Thank you for being a reliable and supportive colleague throughout this endeavor.

Without forgetting my dear friends at university, Wafaa, Majda, Wiem, and Chahla, your camaraderie and support have made this journey memorable and enjoyable. Thank you for being there through the highs and lows, and for making my university years truly unforgettable.

Lastly, I extend my gratitude to the entire university team for their support and for providing an environment conducive to learning and growth. Your collective efforts have played a significant role in the completion of this thesis.



Maria

Dedication



بسم الله الرحمن الرحيم

{يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ}(صدق الله العظيم)

الحمدلله الذي جعل لنا من العلم نورا نهتدي به

أهدي تخرجي هذا الى حورية قلبي وحبيبة روحي ، داعمتي الاولى ، الى الانسانة العظيمة التي لطالما تمنت ان تقر عينها في يوم كهذا

الى جمال الروح ،رفيقي و سندي . أعطيتني و أحببتني من دون مقابل وبك للنجاح سرت ولا زلت أواصل ، بالأمس حملت حقيبتي المدرسية لأحمل اليوم قبعة التخرج الجامعية

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الى قرة عيني ، الى الغاليتين اخواتي "لبنى" ، "صبرينة" ، الى أخي الغالي" عماد "و زوجته "مروة

بدون ان انسى صديقتي الجميلة ماريا التي سرنا معا هذا الدرب سويا بكل مراحله و حالاته

الى صديقات الدرب و رفيقات السنين إيناس ، مجدة ، وئام ، نهلة

الى من أفاضني بمشاعره و نصائحه المخلصة ، إليكم عائلتي

أهديكم هذا النجاح لطالما تمنيته ، ها انا اليوم أكملته و أتممته بفضل الله سبحانه وتعالى فالحمدلله على ما وهبني و الحمدلله عن البدء والختام

أخيرا أهدي تخرجي الى أهلنا و إخواننا في غزة

الحمدلله الذي بنعمته تتم الصالحات



Manel

Acknowledgement

بسم الله الرحمان الرحيم

At the end of this project, we would like to express our heartfelt gratitude to Allah, the Almighty, for granting us the courage and guidance to overcome the challenges and successfully complete this work.

We would like to thank our dear supervisor Dr. BOUBAKRI Kamel as well as Dr. Bouchibane for supporting us throughout this tough year and kept motivating us to give the best we could.

We would also like to extend our sincere appreciation to the members of the jury -KHAMES MAAMER- and -TOUZOUT Nabil for their esteemed evaluation, which we believe reflects their thoughtful consideration and respect.

Furthermore, we would like to acknowledge the contributions and efforts of the entire faculty of the University of Dr. Yahia Fares, who have played a crucial role in our academic journey.

Through these lines of gratitude, we wish to express our deepest appreciation to all those who have supported us through their presence, encouragement, availability, and counsel, making it possible for this project to come to fruition.

Abstract:

Lactic acid bacteria (LAB) are known for their fermentative activities which enhance the hygienic and sensory qualities of fermented meat products. This study investigates some bio-functional properties such as antioxidative activity, exopolysaccharide (EPS) production and biosurfactant activity of selected LAB known for their their low spoilage, antimicrobial and safety characteristics including *Enterococcus (E.) faecium* (K11 and K19), *Latilactobacillus (Lat.) sakei* K25, *Lat.sakei subsp. sakei* (K22 and K26), *Lactiplantibacillus (Lpb.) paraplantarum* (K7, K8, K18 and K21), *Pediococcus (P.) acidilactici* (BK 14 and BK17), *Weissella (W.) cibaria* (BK2 and BK19), *W. confusa* (BK6 and BK11) and *W.paramesenteroides* BK8 strains isolated from traditional Algerian *Kaddid* meat. The strains *Lat. sakei subsp. sakei* K22 and *W. cibaria* BK19 exhibited the highest DPPH activity with scavenging rates of 97%. While for hydroxyl radical scavenging activity the strains *Lpb. paraplantarum* K18 and K21, *P. acidilactici* BK17 and *W. confusa* BK6 exhibited the highest activity ranging from 86% to ~100%. Furthermore, anti-lipid peroxidation rate was found low-to-moderate varies between 31.43% to 42.857%, However, 1mg/L of ascorbic acid used as control positive exhibited a value of 52.85% .Beside, the spoilage character of some overproducers LAB strains such as *W.cibaria* BK19 which can reach EPS level 2464.625 mg/L which may impact negatively the texture of dry-fermented meat. In the other hand, low-to-moderate EPS LAB producers were determined particulary in the case of *E. faecium* K11, *Lat. sakei* K25 and *Lpb. paraplantarum* K7, emphasizing the need for a careful selection and optimization of EPS (-) future starters. The biosurfactant activity was revealed by all the strains by drop-collapse tests, Thereby, the emulsification index was measured and no significative variations between LAB nor with SDS (1%) used as positive control. Based on those findings and the previous functional screenings studies, *Lpb. paraplantarum* (K18 and K21), *Lat. sakei* K25 and *Lat.sakei subsp. sakei* (K22 and K26) could be suggested as bio-functional LAB starters to be used for preparing safe, stable and healthy fermented meat products.

Key words: Lactic acid bacteria, *Lpb. paraplantarum,* Antioxidation, Exopolysaccharides Biosurfactants, bio-functional.

Resumé:
Les bactéries de l'acide lactique (LAB) sont connues pour leurs activités fermentaires qui améliorent les qualités hygiéniques et sensorielles des produits à base de viande fermentée. Cette étude examine certaines propriétés bio-fonctionnelles telles que l'activité antioxydante, la production d'exopolysaccharides (EPS) et l'activité biosurfactante de LABs sélectionnés Connus pour leur faible taux de détérioration, leurs caractéristiques de securité et antimicrobiennes , y compris *Enterococcus (E.) faecium* (K11 et K19), *Latilactobacillus (Lat.) sakei* K25, *Lat.sakei subsp. sakei* (K22 et K26), *Lactiplantibacillus (Lpb.) paraplantarum* (K7, K8, K18 et K21), *Pediococcus(P.) acidilactici* (BK 14 et BK17), *Weissella (W.) cibaria* (BK2 et BK19), *W. confusa* (B K6 et BK11) et *W. parenteroides* BK8 souches isolées de la viande traditionnelle algérienne *Kaddid*. Les souches *Lat. sakei subsp sakei* K22 et *W. cibaria* BK19 ont présenté l'activité DPPH la plus élevée avec des taux d'inhibition de 97 %, pour les souches *Lpb. paraplantarum* K21, *Lpb. paraaplantarum* K18, *P. acidilactici* BK17 et *W. confusa* BK6 ont montré la plus haute activité, allant de 86 % à 100 %, tandis que le taux de peroxydation anti-lipidique des souches testées varie entre 31,43 % et 42,857 %, ce qui est considéré comme faible à modéré par rapport au contrôle positif (acide ascorbique 1 mg/L).Cependant, la recherche a également mis en évidence la surproduction d' (EPS) par certaines souches de LAB telles que *W. cibaria* BK19 avec une concentration de 2464.625mg/L, ce qui peut avoir un impact négatif sur les produits à base de viande en provoquant des dégâts. Une production modérée d'EPS a été observée chez plusieurs souches *E. faecium* K11, *Lat. sakei* K25 et *Lpb. paraplantarum* K7 avec des concentrations respectivement de 453.375, 258.375 et 138,375mg/L, soulignant la nécessité de sélection attentive et d'optimisation des souches de LAB pour une application efficace en tant que cultures de démarrage pour l'industrie de la viande fermentée. L'activité biosurfactante est évaluée à l'aide des tests d'indice d'émulsion et d'effondrement. Les résultats indiquent que certaines souches de LAB présentent des activités antioxydantes et biosurfactantes importantes, ce qui suggère leur aptitude en tant que démarreurs bio-fonctionnels dans les processus de fermentation de la viande. Ces résultats contribuent à la compréhension de la relation entre l'activité antioxydante, EPS et la production de biosurfactants, offrant des informations sur l'amélioration de la sécurité, de la stabilité et des avantages nutritionnels des produits à base de viande fermenté.
Mots clés : Bacteries lactique, *Lpb. paraplantarum*, Antioxidation, Exopolysaccharides Biosurfactants, bio-fonctionel

الملخص

تُعرف بكتيريا حمض اللاكتيك بأنشطتها التخمرية التي تعزز الصفات الصحية والحسية لمنتجات اللحوم المخمرة. تبحث هذه الدراسة في بعض الخصائص الوظيفية الحيوية مثل النشاط المضاد للأكسدة، وإنتاج عديد السكاريد الخارجي (EPS) والنشاط الخافض للتوتر السطحي الحيوي لبكتيريا حمض اللاكتيك المختارة بما في ذلك بكتيريا حمض اللاكتيك المعوية. معروفون بانخفاض نسبة التلف، وخصائصهم المضادة للميكروبات، وسلامتهم ,الفايسيوم K11) و(K19 ، و *Latilactobacillus (Lat.)* ساكي K22) و(K26 ، و*لاكتيبلانتيباسيليوس (Lactiplantibacillus)* بارابلانتاروم K7) و K8 و K18 و(K21 ، والمكورات اللبنية BK 14) *acidilactici* (P.) و(BK17 ، وويسيللا سيباريا (W.) سيباريا BK2) و(BK19 ، و *W. confusa* (BK6 و(BK11 وسلالات *W.paramesenteroides* BK8 المعزولة من لحم القديد الجزائري التقليدي. أظهرت سلالتا Lat. *sakei subsp. sakei* K22 و W. cibaria BK19 أعلى نشاط لـ DPPH بمعدلات كسح بلغت 97%. بينما بالنسبة لنشاط الكسح الجذري للهيدروكسيل، أظهرت السلالات *Lpb. paraplantarum* K18 و K21 ، *P. acidilactici* BK17 و *W. confusa* BK6 أعلى نشاط يتراوح بين 86% إلى 100% تقريبًا. علاوة على ذلك، تم العثور على معدل بيروكسيد الدهون منخفض إلى متوسط يتراوح بين 31.43% إلى 42.857%، ومع ذلك، أظهر معدل منخفض إلى متوسط من حمض الأسكوربيك المستخدم كعنصر تحكم إيجابي بنسبة 52.85%. بجانب ذلك، فإن خاصية التلف لبعض سلالات LAB المفرطة الإنتاج مثل *W.cibaria* BK19 التي يمكن أن تصل إلى مستوى EPS 2464.625 ملجم/لتر مما قد يؤثر سلبًا على قوام اللحوم المخمرة الجافة. من ناحية أخرى، تم تحديد منتجي EPS LAB منخفضة إلى معتدلة في حالة *E. faecium*. من ناحية أخرى، تم تحديد منتجي EPS منخفضة إلى معتدلة من EPS LAB بشكل خاص في حالة E. *faecium K11*، و Lat. *sakei* K25 و*Lp. paraplantarum* K7، مما يؤكد الحاجة إلى الاختيار الدقيق وتحسين البادئات المستقبلية .(-) EPSوقد تم الكشف عن النشاط الخافض للتوتر السطحي الحيوي من قبل جميع السلالات عن طريق اختبارات الانهيار بالقطرة (-)، وبالتالي تم قياس مؤشر الاستحلاب ولم تظهر أي اختلافات ذات دلالة إحصائية بين الكريات الحبيبية ولا مع SDS (1%)المستخدم كعنصر تحكم إيجابي. بناءً على هذه النتائج ودراسات الفحص الوظيفي السابقة، يمكن اقتراح Lpb. paraplantarum (K18 و (K21 و Lat. sakei K25 و Lat.sakei subsp. sakei (K22 و (K26 كمبتدئات للكلاب الحيوانية الوظيفية لاستخدامها في تحضير منتجات اللحوم المخمرة الآمنة والمستقرة والصحية

الكلمات المفتاحية: بكتيريا حمض اللاكتيك. *Lpb. Paraplantarum*. مضادات الاكسدة. خافض للتوتر السطحي الحيوي . عديد السكاريد الخارجية . وظيفة حيوية

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III. List of abbreviations

CFS: Cell free supernatant

DPPH: 1,1-diphenyl-2-picrylhydrazil

EI: Emulsification index

EPS: Exopolysaccharide

HRs: Hydroxyl radicals

LAB: Lactic acid bacteria

MRS: de Man, Rogosa & Sharpe

PBS: Phosphate buffered saline

PUFA: Polyunsaturated fatty acid

ROS: Reactive oxygen species

RSA: Radical scavenging activity

SDS: Sodium dodecyl sulfate

SD: Standard deviation

SS: Saline solution

Introduction

Introduction

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS) microorganisms. They play a crucial role in meat fermentation, improving the hygienic and sensory quality of the final product. Their fermentative activities, such as the production of lactic acid and the suppression of competing microorganisms and contribute to the ripening process of fermented meat.

The production of exopolysaccharides (EPS) by LAB has gained special interest over the last decade due to functional properties of these biopolymers as well as their generally recognized as safe (GRAS) status **(Dertli et *al*., 2016)**. Under specific growth conditions, some meat-borne LAB species generate a wide range and diversity of EPS structures that can be used as potential application in the food and pharmaceutical industry and/or considered as spoilage issue **(Donot et *al*., 2012**; **Silva et *al*., 2019)**.

As a consequence, LAB are responsible for some unusual alterations in meat: off-flavors, discoloration, gas production, a decrease in pH and slime formation due to the LAB secreting long-chain, high-molecular-mass, viscosifying or gelling exocellular mpolysaccharides, determining the spoilage of the products and reduction in shelf-life **(Samelis et *al*., 2000)**.

Oxidative stress arises from an imbalance between the production and detoxification of reactive oxygen species (ROS) and free radicals in the body. This imbalance leads to the accumulation of ROS and free radicals, which in turn cause damage to cellular components such as DNA, proteins, lipids and small molecules. The resulting cellular damage contributes to the development of various chronic diseases, including cancer, diabetes, atherosclerosis, cardiovascular diseases, inflammatory conditions, and other degenerative diseases in Humans **(Pizzino et *al*., 2017**).

Meat-borne LAB have been found to have excellent antioxidant activities, including DPPH scavenging activity, OH scavenging activity and anti-lipid peroxidation capacity. These bacteria can be used as antioxidant starters in the meat industry to enhance the quality of fermented meat products **(Lin X *et al*., 2018).** Additionally, LAB can mitigate oxidative damage and stress-related diseases caused by external sources through oral route.

Several processing strategies are used to preserve and improve the stability, shelf-life and quality of meat and meat products, from which natural preservative agents are gaining interest. Among these natural preservatives, biosurfactants are emerging molecules. Their natural origin and biodegradability make them appealing for use in the food industry. In meat research, biosurfactants are of great interest as antimicrobial, anti-biofilmogenic and antioxidant agents to reduce meat spoilage and wastage as well as for improving the shelf-life of the products **(Boulahlib et *al*., 2024).**

The aim of this study is to evaluate *in vitro* some bio-functional characteristics of previously selected LAB strains isolated from traditional Algerian *Kaddid* meat, by assessing some antioxidant tests, to quantify the EPS production levels and evaluating their ability to produce biosurfactants taking into consideration the compatibility of these tests with meat characteristics and processing environment. thus helping to improve the final product's safety, stability, and nutraceutical healthcare benefits

I. Bibliographic synthesis: Functional properties of lactic acid bacteria for meat products

I.1. Meat-borne lactic acid bacteria (LAB)

Meat and meat products are recognized as essential for growth and health due to their rich nutritional content, including significant amounts of proteins, fatty acids, vitamins, minerals and other bioactive compounds. Recently, consumers worldwide have become increasingly focused on enhancing the marketability of meat and meat products while also ensuring their health benefits **(Ben Slima et *al*., 2017)**.

Probiotic microorganisms including lactobacilli, *Pediococcus, Bifidibacterium, Bacillus* and *Enterococcus* that receive market interest as functional food biological additives have an extremely important role in food conservation since they are successfully applied in meat products such as fermented sausages **(Ben Slima et *al*., 2017)**.

I.2. Spoilage in meat products

Spoilaging of meat products occurs generally in three types: sliminess (Fig. 1), souring and greening. The predominant slimy and greening bacteria in these products are yeasts, lactobacilli, *Streptococcus (St.), Bronchothrix (B.) thermosphacta*, and *Weissella* (*W.*) *viridescens*. Slime formation (Fig. 1) is favored by a moist surface and usually occurs on the outside of casing. Slime production is influenced by the specific conditions of packaging and storage temperature and is linked to biofilm formation, stress resistance and sucrose utilization of responsible strains. LAB can produce EPS, contributing to slime formation and various physiological functions **(Iulietto et *al*., 2015)**.

The main sausage and fermented-cured meat products spoilage and deteriorations are the excessive growth of bacteria in-side and/or outside the product. The excessive growth of molds on the surface, the oxidative deterioration of fat causing product rancidity and the excessive dehydration. These four forms of spoilage and deterioration can vary widely. Processing under unhygienic conditions as well as the poor hygienic quality of raw materials may cause color

changes, souring, gas formation, and off-odors due to growth of unwanted microorganism **(Erkmen et *al*., 2016)**.

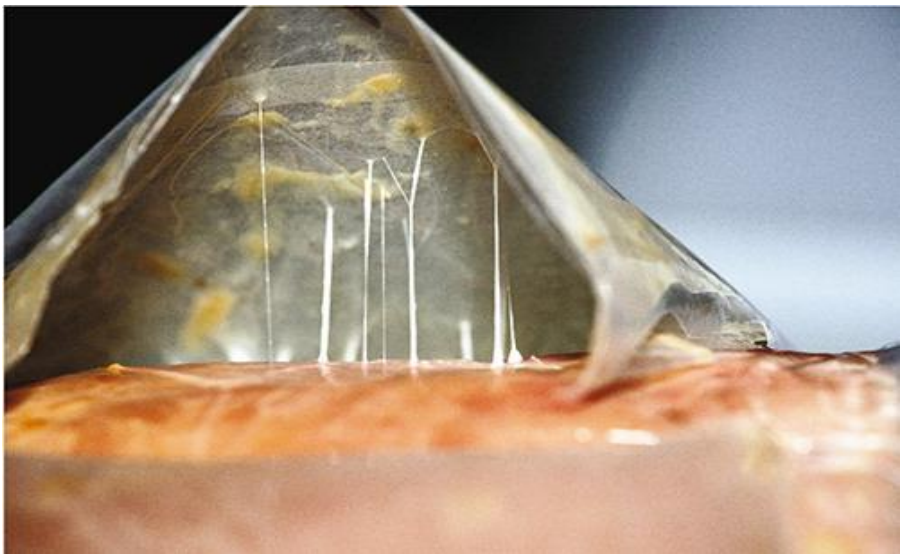


Figure 1. Stretchy filaments on the surface of cooked turkey breast (reprinted from **Iulietto et *al*., (2015)**).

I.3. Preservation of dry-fermented meat

While fermenting meat products, LAB not only add unique flavor substances to the products, but also improve the safety profile of fermented foods **(Wang et *al*., 2022)**. LAB are used in the fermentation of meat products due to their ability to degrade nitrites, provide an antioxidant activity and inhibit the growth of pathogenic bacteria **(Kumar et *al*., 2017)**. The excessive oxidation produces unpleasant color and odor, resulting in the loss of nutritional value, shortening the shelf-life and even producing toxic compounds. LAB can slow down the oxidation of meat products through different antioxidant systems **(Wang et *al*., 2022).**

The formation of exopolysaccharides (EPS) (Fig. 1) indicates the biofilm's irreversible adhesion to the surface and permits it to develop **(Rabin et *al*., 2015)**. When the biofilm reaches a certain mass, it begins to aggressively spread, forming new biofilms elsewhere. Passive dispersal refers to dispersion induced by external sources such as biocides, antibiotics or biosurfactants. Sessile bacteria's gene expression and growth rate are affected by EPS generation, increased cell density of diverse bacterial communities and surface attachment.

I.4. Biosurfactant activity

Biosurfactants, also called microbial surfactants, are surface-active chemicals produced by microorganisms that breakdown hydrocarbons and exhibit a wide range of structural diversity. Various industrial procedures rely on the use of biosurfactants, which can be either low-or high-molecular-weight polymers. Low-molecular-mass biosurfactants include glycolipids, lipopeptides and phospholipids, whereas polymeric and particle surfactants can stabilize emulsions. The biosurfactants are widely used in food industry, however, their utilization in meat research is very limited **(Nitschke and Costa, 2007)**.

Table 1. Major classes of Biosurfactants and microorganisms that produce them (Reprinted by **Banat et *al*., 2014**)

Biosurfactant Class	**Microorganisms**	**Molecular Structure**
Glycolipids	-*Pseudomonas spp*. (Rhamnolipids) -*Rhodoccoccus spp,Nocardia spp.*, *Gordonia spp.*,*Mycobacteruim spp* .(Trehalose lipids) -*Candida bombicola* , *Candida apicola* (Sophorolipids)	-Rhamnolipids: Rhamnose sugars linked to fatty acids -Trehalose lipids : Trehalose (disaccharide) linked to fatty acids -Sophorolipids : Sophorose (disaccharide) linked to fatty acides
Lipopeptides	-*Bacillus subtillis* (Surfactin) -*Bacillus licheniformis* (Lichenysin)	-Surfactin : Cyclic lipopeptide with β-hydroxy fatty acid -Lichenysin: Cyclic lipopeptide with β-hydroxy fatty acid
Phospholipids	-*Acinetobacter calcoaceticus*	-Phospholipids with fatty acids
Fatty acids/ Neutral lipids	-*Corynebacterium lepus*	-Fatty acids, neutral lipids
Lipoproteins	-*Candida antarctica* (Mannosylerythritol lipids) -*Kurtzmanomyces sp*.	-Mannosylerythritol lipids: Mannosylerythritol linked to fatty acids
Polymeric biosurfactant	-*Acinetobacter calcoaceticus* -*Pseudomonas aeruginosa*	-High molecular weight polysaccharide, proteins, lipopolysaccharides

I.4.1. Biosurfactant from LAB

Microbial surfactants are differentiated by their surface activity, tolerance over pH, temperature, and ionic quality, biodegradability, low toxicity, emulsifying/demulsifying capability and antimicrobial action (**Chandran and Das, 2010)**.

Biosurfactants derived from various LAB are complex mixtures that prevent pathogenic microbes from adhering to biotic and abiotic surfaces. However, their chemical composition has not been fully reported and only a few have been partially described. Better understanding of biosurfactant composition through LAB is crucial for identifying active components and optimizing their qualities. Biosurfactants generated from LAB are multicomponent complexes made composed of protein, polysaccharides and maybe phosphate groups, *St. thermophilus* B's biosurfactant consists of polysaccharides and glycolipids. A biosurfactant generated from *St. thermophilus* B effectively reduces water's surface tension to 35 mN/m, found that biosurfactants generated by two *S. mitis* strains exhibit similar structural features due to their glycolipid composition and low protein content. Biosurfactants released by two *St. mitis*, identified as rhamnolipid-like molecules, reduced surface tension to 35 mN/m at a concentration of 1 mg/ml. The isolated biosurfactant derived from *Lacticaseibacillus (Lcb.) paracasei* has been characterized as multicomponent biosurfactant **(Sharma et *al*., 2016).**

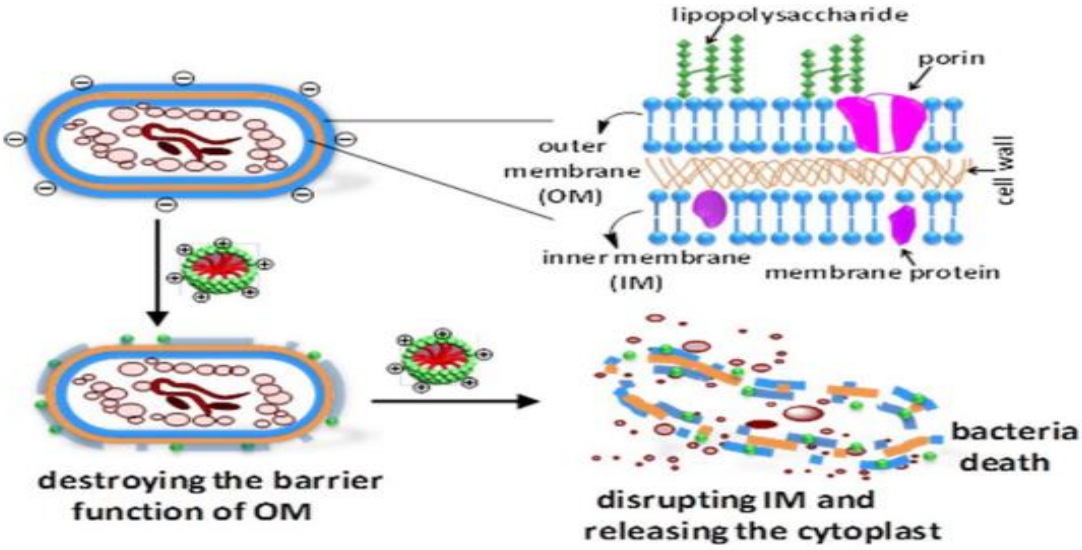


Figure 2. Schematic antibacterial mechanism of surfactant micelles against *Escherichia (E.) coli* (Reprinted from **Zhou et** *al* **., (2016)**).

I.4.2. Antimicrobial activity of biosurfactants

Meat rotting is caused by various bacteria, including *Salmonella*, *Campylobacter jejuni*, *E. coli*, *Listeria monocytogenes*, *Clostridium*, *Pseudomonas*, *Acinetobacter*, *B. thermosphacta*, *Enterobacter*, lactobacilli, molds and yeasts, causing epidemics threatening public health and the economy (**Ji et** *al***., 2023**; **Gautam et** *al***., 2023)**. Research on biosurfactants has shown their potential as antimicrobial (Fig. 2), fungicidal, fungistatic and antibiofilm agents, enhancing preservation procedures and quality control measures for food safety **(Mouafo** *al***.,2020; Kourmentza et** *al***., 2021; Shao et** *al***., 2021; Liu et** *al***., 2023)**.

I.4.3. Antibiofilm activity

Biosurfactants are a sustainable alternative to chemical substances because of their anti-adhesive and antibiofilm qualities. According to research, biosurfactants from *Lcb. casei* strains can inhibit *Staphylococcus (S.) aureus***,** decrease biofilm cells and prevent biofilm formation of six pathogen strains isolated from braised fish. They alter the surface physiochemical characteristics, limiting adhesion and biofilm development (**Merghni et** *al***., 2017**).

I.4.4. Antioxidant activity of biosurfactants

Biosurfactants are natural antioxidant substances utilized to reduce oxidation in muscle meals (**Manessis et** *al*., **2020).** Some biosurfactants have demonstrated substantial benefits, such as reducing lipid oxidation and total volatile basic nitrogen in fresh ground goat meat (**Mouafo et** *al***.,2020)**. Lipopeptide biosurfactants had a considerable influence on lipid oxidation in beef samples and ground beef patties **(Hmidet et** *al***.,2020).** Sophorolipid biosurfactants have proven to be more effective than gelatin-based antioxidant packaging (**Jemil et** *al***., (2020)**. Biosurfactants also have DPPH scavenging action, which neutralizes free radicals by converting protons to electrons (**Abdollahi et** *al***., 2020),** or hydrogen atoms **(Ben Ayed et** *al***., 2015)**. The inclusion of hydrophobic amino acids with the acyl chain of beta-hydroxy fatty acids inhibits lipid peroxidation, hence enhancing the peptide's solubility in hydrophobic media **(Ben Ayed et** *al***., 2015; Abdollahi et** *al***.,2020)**.

I.4.5. Potential use of biosurfactants in meat emulsification

Emulsion-type meat products, such as sausages, frankfurters and bologna are made by finely slicing meat from a variety of sources to form a stable combination that binds water and holds fat. When cooked, this unusual emulsion imparts textural features **(de Souza et** *al***., 2018)**, transforming it into an oil-in-water emulsion (**Santhi et** *al***., 2017).** However, worries about food quality and health are growing, prompting the reformulation of meat products to improve their nutritional profile. Using various emulsifying agents, researchers have attempted to reduce fat content, improve fat profile and convert liquid oil into a semi-solid system (**Guo et** *al***., 2023)**. **Serdaroğlu et** *al***., (2016)** found that replacing some beef fat with gelled emulsion improved cooking yield and texture without affecting water holding capacity or emulsion stability.

I.5. Exopolysaccharide (EPS)

Exopolysaccharides (EPSs) are high-molecular-weight, biodegradable polymers (Fig. 3). They are biosynthesized by a variety of bacteria. LAB can also create EPSs producer microorganisms do not employ bacterial EPSs as energy sources. EPSs have been employed in the preparation of a variety of fermented foods, including thickeners, stabilizers, emulsifiers and gelling or water binding agents **(Sanalibaba and Çakmak., 2016)**.

Two forms of EPS are produced by LAB: capsular polysaccharide (CPS) if they remain attached to the cells or unattached and released into the environment as exopolysaccharides (EPS) **(Iulietto et *al*., 2015).**

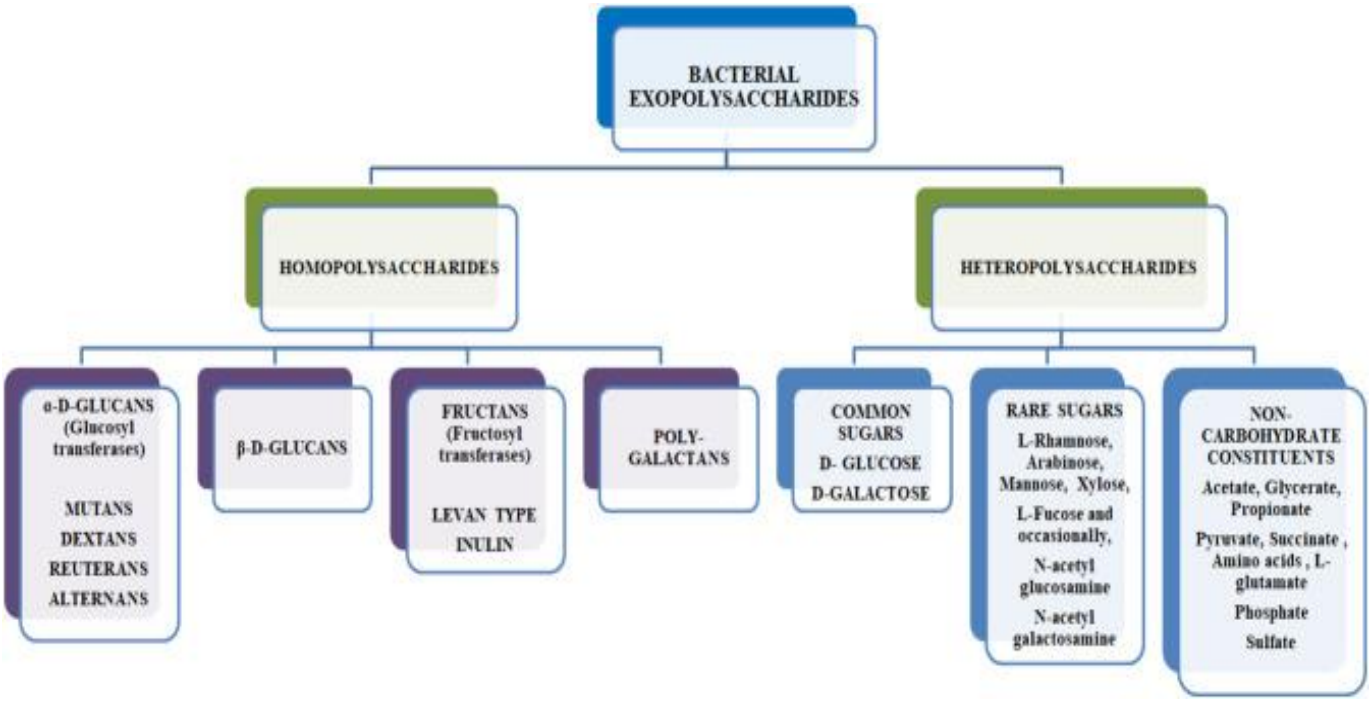


Figure 3. Classification of bacterial exopolysaccharides (reprinted from **Angelin et *al*., (2020**)).

I.5.1. Biosynthetic pathways

Biosynthesis of EPS in probiotic bacteria occur in two ways, intra and extracellular (Fig. 4). homopolysaccharides (HoPs) are typically created *via* an extracellular biosynthetic pathway, whereas heteropolysaccharides (HePs) can be synthesized *via* either intracellular or extracellular pathways (Fig. 3 and 4). During the polymerization phase, unique extracellular glycosyl and fructosyl transferases catalyze the extracellular production of HoPs. Sugar residues are broken into monomeric units outside the cell, which are then assembled extracellularly into a polymer by glycosyl transferases. Sucrose is used as a particular substrate and is cleaved into glucose and fructose before being polymerized into glucan and fructan by glucan sucrase and fructan sucrase. While fructan sucrases are separated into levan-sucrases (levan) and inulin-sucrases (inulin), respectively, glucan-sucrases are further classified into dextran-sucrases (dextran), mutan-sucrases (mutan), reuteran-sucrases (reuteran) and alternan sucrases (alternan). The extracellular environment is immediately exposed to these polymerized **(Dertli et *al*., 2013)**.

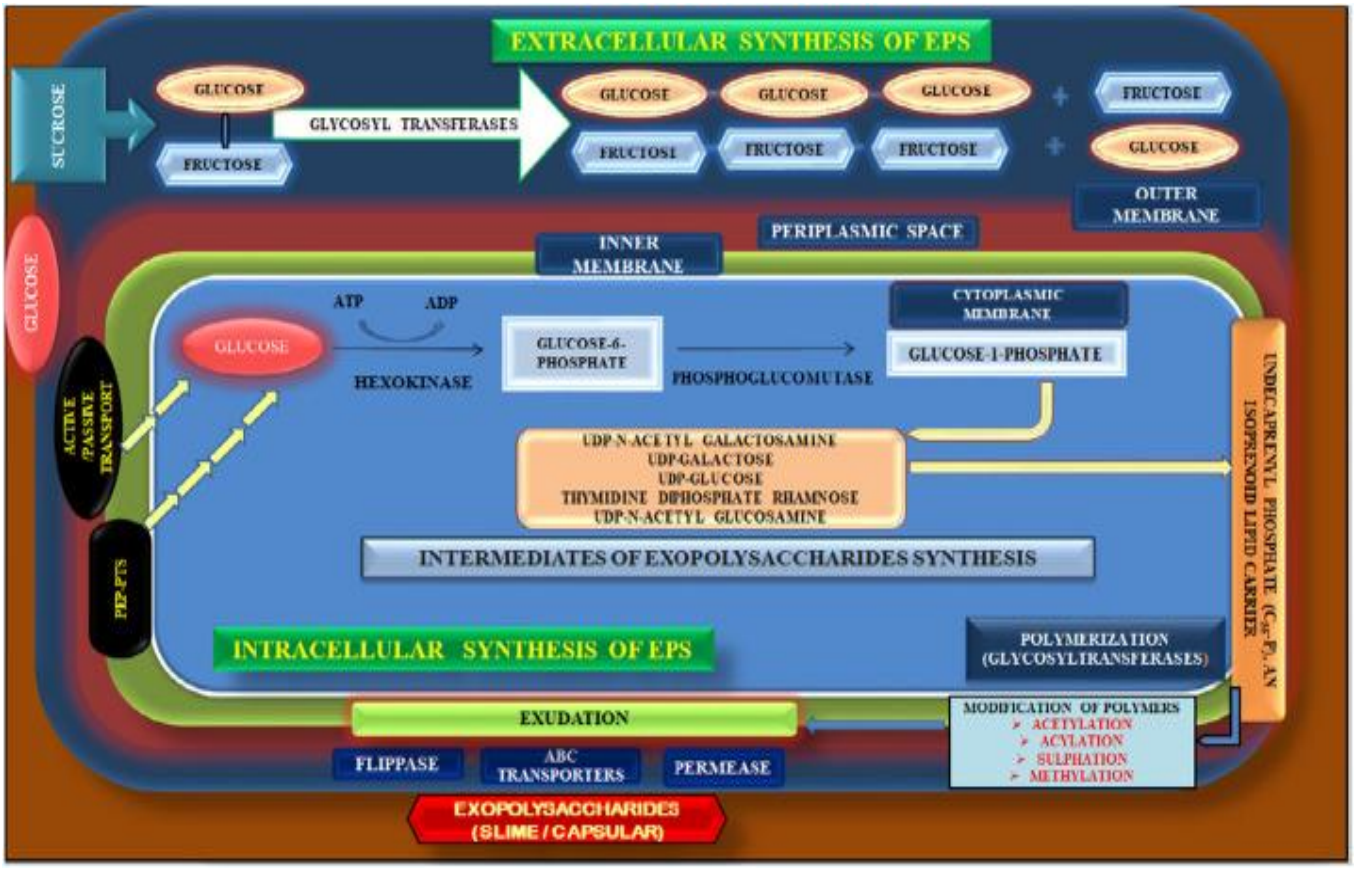


Figure 4. Biosynthetic pathways of EPSs (reprinted from **Angelin et *al*., (2020)**).

I.5.2. Conditions of production

Since EPS production is frequently stress-induced, it is dependent upon the strain involved (HePS or HoPS-former, mesophilic or thermophilic, growth phase, bacterial concentration as well as the composition of the surrounding media (carbon source, other nutrients, pH and salts etc.), as well as any superimposed processing, storage and/or incubation conditions such as temperature and humidity **(Loeffler et *al*., 2020)**.

- Growth Phase: EPS production varies with the LAB strain and its growth phase. Thermophilic LAB often produces EPS independently of growth, whereas mesophilic LAB usually produces EPS during growth, particularly in the exponential phase. Environmental stress can trigger EPS production in non-growth-associated strains, such as *Lactiplantibacillus (Lpb.) plantarum* or *Lcb. paracasei*.

- Availability of carbon and nitrogen: The availability and type of carbon and nitrogen sources influence EPS production. Sucrose is crucial for HoPS synthesis, with increased availability enhancing EPS yield. Different carbon sources can affect the molecular weight and composition of EPS. The optimal carbon-to-nitrogen ratio is necessary for maximizing HePS production in growth-associated strains.
- Temperature: Optimal temperatures for EPS synthesis in most LAB and Bifidobacteria range between 26°C and 31°C. A decrease of 10°C below the optimal growth temperature can inhibit EPS production, highlighting the importance of maintaining appropriate cultivation temperatures.
- pH: The optimal pH for EPS production varies by strain but generally lies between 5.5 and 6.5 for lactobacilli species. Extreme pH levels (below 3 or above 10) inhibit growth and EPS production. Maintaining a controlled and consistent pH can enhance EPS yield. **(Loeffler M et *al*., 2020).**

I.5.3. Structure of EPS

EPSs consist mainly of a limited number of different monosaccharide types or their derivatives and present great diversity through various combinations of monosaccharide units arranged in linear or branched configurations linked by glycosidic bonds **(Nicolaus et *al*., 2010)**.

The number of different chemical structures of bacterial EPS is very high and they are generally heteropolysaccharides with three but also four different monomers organized in groups of a set of 10 or less to give the repetitive units. The monosaccharides that create exopolysaccharides can be pentoses, hexoses, amino acids or uronic acids **(Suresh Kumar et *al*., 2007)**. The most common skeletal bonds of non-saccharidic sequences are β-1,4 or β-1,3 bonds, which have characteristic structural rigidity, as in the xanthan and cellulose skeleton of *Xanthomonas campestris*, while other bonds such as α-1,2 or α-1,6 bonds are considered flexible structures, such as the bonds found in many dextran **(Nicolaus et *al*., 2010)**. The possibility of different bonds in polysaccharides and the variation in monomer arrangements led to a wide range of shapes and structures **(Suresh Kumar et *al*., 2007)**.

I.5.4. Screening methods of EPS production

Exopolysaccharide (EPS) polymers have a high molecular weight and are made up of sugar residues **(Nicolaus et *al*., 2010)**. Colorimetric tests invented by **Dubois et *al*. (1956)** based on the reducing sugars and polysaccharides have been known for a considerable time, Phenol in the presence of sulfuric acid can be used for the quantitative colorimetric micro determination of sugars and their methyl derivatives, oligosaccharides, and polysaccharides. This method is particularly useful for the determination of small quantities of sugars separated with solvents which are volatile-e.g., butanol, ethanol-water, ethyl acetate-acetic acid-water, or methyl ethyl ketone-water. The method is simple, rapid, and sensitive and gives reproducible results. The reagent is inexpensive and stable, and a given solution requires only one standard curve for each sugar. The color produced is permanent and it is unnecessary to pay special attention to the control of the conditions.

I.5.5. Application of EPS in meat fermented products

EPSs formed by LAB on meat products are traditionally regarded as spoiling factors and their potential applications in processed meat are unknown. Found EPSs may improve the textural qualities of sausages, resulting in a harder, less sticky and tougher product. Recent research has examined the utilization of in situ EPS-forming LAB in meat products, describing their beneficial effect in various meat matrices, including low-fat fermented sausages. It has been revealed that in situ generated EPSs influenced the quality of fermented meat products, broadening the possibilities for their application, particularly in fat-reduced meat products, where consumption is now increasing. Numerous research investigations have demonstrated that LAB-produced EPSs can be used to improve the texture of meat products, especially low-fat ones. However, to ensure their quality, the concentration of EPSs in meat products must be within a well-defined range **(Abarquero et *al*., 2021).**

I.5.6. Correlation between EPSs and antioxidant effect

The potential to exert good anti-oxidant activity is prevalent in LAB-derived EPSs, as demonstrated by *in vitro* and *in vivo* studies. EPS-Ca6 from *Lactobacillus (Lb.)* sp.ca 6 demonstrates concentration-dependent 1,1-diphenyl-2-picrylhydrazyl radical 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) reported hydrazyl radical (DPPH) scavenging, β-Carotene bleaching

reduction, reductive and metal chelating activities. EPS from *Lb. gasseri* FR4 shown strong DPPH scavenging, superoxide and hydroxyl radical scavenging, and reductive activities, which are also concentration-dependent **(Zhou et *al*., 2018).**

Furthermore, despite its outstanding cholesterol-lowering properties, EPS from *Lpb. plantarum* BR2 had roughly 40% more DPPH-scavenging activity than ascorbic acid. It is possible that EPS's antioxidant molecular mechanism *in vitro* is based on the hydrolysis of these biomacromolecules when exposed to an acidic environment, producing massive active hemiacetal hydroxyls while also conveying electrons to radicals in the reacting system, facilitating the conversion from free radicals to stable substances and eventually decreasing the concentration of radicals **(Zhou et *al*., 2018).**

I.6. Antioxidative activity of LAB

I.6.1. Oxidative stress and free radicals

Reactive oxygen species (ROS) are non-radical derivatives of oxygen and chemically reactive oxygen radicals. ROS are species containing a minimum of one oxygen atom in each molecule but possess enhanced activity compared with molecular oxygen. ROS include free radical species (singlet oxygen, hydroxyl radical, and superoxide) and non-radical species (hydrogen peroxide H_2O_2) **(Rauf, A et *al*., 2024).**

Oxidative stress is a physiological condition characterized by an imbalance between the production of ROS and the ability of cells to detoxify them. It plays a significant role in the disruption of normal cellular function and can lead to inflammation, tissue injury and organ dysfunction **(hong et *al*., 2024)**. Excessive formation of ROS is responsible for disrupted redox homeostasis which in turn leads to oxidative stress and ROS-mediated damage to all important biomolecules including DNA, proteins, and membranes **(Liguori et *al*., 2018)**. Some lactobacilli have been shown that possessing antioxidative activity, and are able to decrease the risk of accumulation of ROS during the ingestion of food**. (Zhang et *al*., 2011)**.

I.6.2. Importance of antioxidants for health

In the gut, overproduction of ROS results in oxidative stress conditions, which enhance cellular damage through modifications in the function of proteins and the peroxidation of lipids.

Intestinal epithelial cells (IECs), macrophages and neutrophils generate nitric oxide and superoxide through NOX and iNOS activation, respectively. **(Rauf et *al*., 2024).**

During the fermentation of food products, LAB produce small molecules of bioactive peptides and other components, which can exert antioxidant functions through the Keap1-Nrf2-ARE signaling pathway. In the normal state, Nrf2 in the cytoplasm is bound to its inhibitory protein Keap1. When stimulated through oxidation, the cysteine residues of the Keap1 protein are modified and Nrf2 passes through Keap1 before Nrf2 enters the nucleus, Nrf2 heterodimerizes with muscloaponeurotic fibrosarcoma protein (Maf) on CREB binding proteins (CREB) and binds to antioxidant response elements on DNA (ARE), inducing the expression of various antioxidant enzyme genes such as superoxide dismutase (SOD) and NADPH quinone dehydrogenase **(Wang et *al*., 2022).**

I.6.3. Screening methods of antioxidants activity

There are numerous antioxidant tests that directly assess the transfer of hydrogen atoms or electrons from antioxidants to free radicals. The methods for evaluating antioxidant activities have seen significant advancements recently. Initially, these methods gauged the effectiveness of antioxidants by measuring the formation of certain oxidation products, which involved monitoring lipid peroxidation. Over time, various chemical methods have been developed to evaluate antioxidant activity, incorporating highly automated and sensitive detection technologies. These methods include measuring the removal activity against different types of reactive oxygen species or free radicals, assessing reducing potency, metal chelation and more. The idea of antioxidant capacity originated as a chemical concept and was later applied to fields like medicine, biology, food science and epidemiology **(İlhami and Alwasel, 2023).**

It is very important to know the antioxidant profiles of these products in order to avoid loss of commercial and nutritional value during processing and preservation of foods and pharmaceutical products. Therefore, determining the potential antioxidant capacity of foods and pharmaceutical products requires the development of a fast and simple method.

As is known, most of these methods use similar principles and techniques. Measuring the ability of these antioxidant techniques is based on a suitable standard spectrophotometer measurement **(Taslimi et *al*., 2020)**.

I.6.3.1. DPPH radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazil (DPPH) radical was discovered 100 years ago by Goldschmidt and Renn in 1922 **(Goldschmidt et *al*., 2022)**. The principle of the method with the DPPH radical is based on the reduction of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical to 2,2-diphenyl-1-picrylhydrazine (DPPH-H) (Fig. 5) **(Gulcin et *al*., 2023)**. DPPH can accept an electron or hydrogen radical to become a stable, diamagnetic molecule, it can be oxidized only with difficulty, and then irreversibly, the absorption vanishes as the electron pairs off. The resulting decolorization is stoichiometric with respect to the number of electrons taken up. The alcoholic solutions of 0.5 mM are densely colored and at this concentration, the Lambert-Beer law is obeyed over the useful range of absorption **(Kedare et *al* 2011)**. In the presence of an antioxidant, the color changes from purple to yellow. As a result of the reaction, the color intensity decreased in proportion to the antioxidant content. It is one of the most frequently used methods, relatively simple and not requiring a long time to prepare the solutions **(Gulcin et *al*., 2023)**.

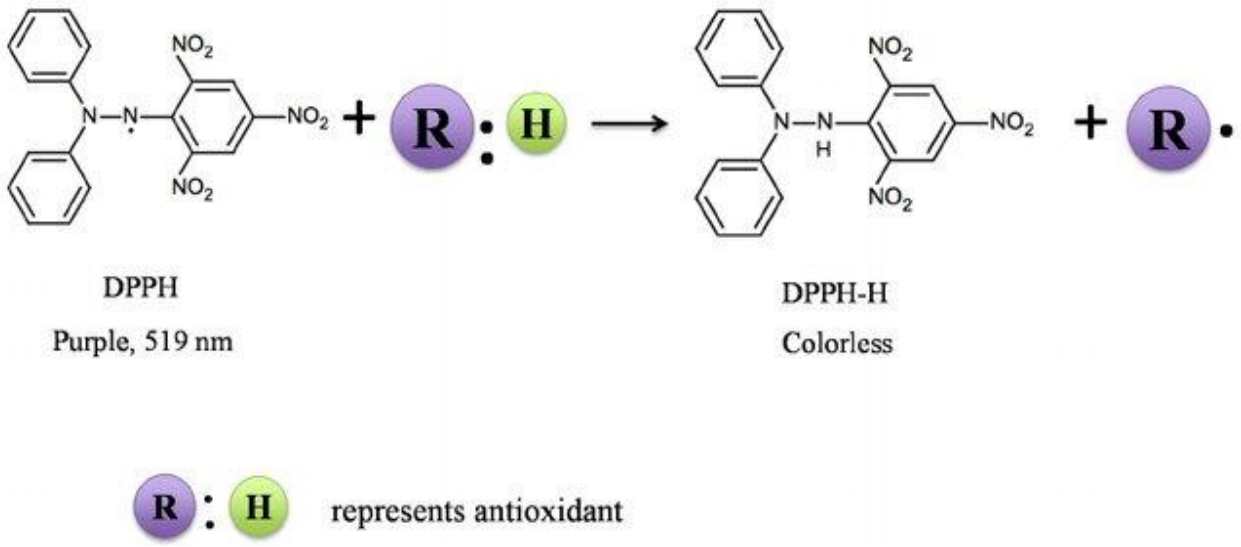


Figure 5. Reaction mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant. H: antioxidant radical scavenger; R: antioxidant radical.

I.6.3.2. Hydroxyl radical scavenging (HRS) activity:

Hydroxyl radicals ($OH^{\cdot}$), being highly reactive forms of hydroxide ions (OH^{-}), are short-lived as they readily transform into hydroxyl groups. On average, a human cell encounters these radicals

and other reactive species around 100,000 times daily, leading to oxidative stress. These radicals can be produced as secondary effects of immune responses, especially by microglia and macrophages when they encounter specific pathogens and bacteria. Hydroxyl radicals (HR) are the most reactive of these species. They can form in the body through high-energy irradiation that causes the splitting of water molecules or from endogenous hydrogen peroxide (H_2O_2) in metal-catalyzed processes. Although UV light cannot split water, it can break down H_2O_2 to produce hydroxyl radicals. Due to their extreme reactivity, these radicals act immediately at their point of origin. The damaging effects of $OH^{\cdot}$ have been linked to several neurological autoimmune disorders when overactive immune cells attack nearby healthy cells **(Gulcin et *al*., 2020).**

HRs are considered to be the most reactive oxygen radicals, which can react with all biomacromolecules functioning in living cells and can induce severe damage to cells. Therefore, scavenging of hydroxyl radical plays a critical role in reducing oxidative damage. In this study, Fenton reaction system was used to determine the HRS capacity of the intact cells (**Rwubuzizi** et *al*., **2023).**

$$Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH^{-} + OH^{\cdot} \text{ -------Fenton reaction.}$$

I.6.3.3. Anti-lipid peroxidation

Lipid peroxidation is a primary reason for the deterioration of meat and meat products, giving undesirable odors, rancidity, texture modification, loss of essential fatty acids and/or toxic compound production. Moreover, lipid oxidation products implicate several human pathologies (atherosclerosis, cancer, inflammation, or aging processes) **(Pereira and Abreu, 2020)**.

Lipid peroxidation is a natural process that mainly impacts cell membranes. This occurs when unsaturated or polyunsaturated fatty acids (PUFAs) undergo peroxidation due to the weakening of double bonds, creation of conjugated dienes, addition of oxygen, formation of peroxyl radicals and loss of hydrogen ions from the lipids. Since cells lack mechanisms to eliminate these byproducts, lipid peroxidation is deemed irreversible **(Milei et *al*., 2007).**

Lipid peroxidation generally involves the degradation of PUFAs and the production of secondary decomposition products, including carbonyls and hydrocarbon compounds. The oxidative stability of meat depends on the balance of anti- and pro-oxidants and the composition of oxidizable substrates, including PUFAs, cholesterol, proteins, and pigments **(Pereira and**

Abreu, 2020). LAB have been found to exhibit antioxidant properties, which can help prevent lipid peroxidation by neutralizing free radicals and reducing oxidative stress **(Rwubuzizi et *al*., 2023)**

I.6.4. Practical application on meat fermentation

LAB strains can significantly enhance the antioxidant activity of sausage extracts, improving the sensory characteristics and safety of the sausages. The antioxidant activities of two LAB strains (*Lactobacillus curvatus* (SR6) and *Lactobacillus paracasei* (SR10-1)) were tested in a sausage model, the strains demonstrated the changes in the quality characteristics and antioxidant activities of fermented sausages. where they rapidly became the dominant microflora and improved the sensory characteristics and safety of the sausages by reducing moisture content, water activity, nitrite, and pH. These findings suggest that both strains can be used as antioxidant starters in the meat industry to enhance the quality of fermented meat products. **(Zhang Y et *al*.,2017).** Other studies have also investigated the antioxidant properties of LAB. For example, a study evaluated the antioxidant activities of 15 LAB strains from fermented foods and feces, screening for strains with strong antioxidant capacity using various tests such as DPPH, hydroxyl radical assays. The study identified several strains with high antioxidant activities, including LAB strains from fermented Chinese food jiangshui (J2-4, J2-5, J2-9), therefore, these strains could be used as potential antioxidants for functional food such as fermented meat and health care **(Hu et *al*., 2023).**

Material and methods

II. Material and methods

This research was conducted at the pedagogical laboratories of Biology (PLB), Faculty of Natural and Life Sciences at Yahia Fares University, Campus of Ouzera-Medea city, Algeria, during the period from March to Juin 2024.

The aims study is to investigate the biosurfactant and exopolysaccharide (EPS) production and to evaluate the antioxidative activity by meat-borne lactic acid bacteria (LAB). The biosurfactant activity was tested by *Drop-collapse* and *emulsification index* tests. Furthermore, the study seeks to determine the capacity of selected LABs strains to produce exopolysaccharides (EPS) by phenol–sulfuric acid method. In addition, the antioxidative activity was assessed for its potential to scavenge reactive oxygen species (ROS) and mitigate oxidative stress. Through this investigation, we aim to shed light on the relationship between antioxidative activity, EPS and biosurfactant production by *Kaddid*-borne selected LAB, elucidating the potential mechanisms underlying their bio-functional starter for fermented meat products, thereby contributing to enhance the final product safety, stability and nutriceutical healthcare benefits.

II.1. Culture medium

The growth LAB medium used in is MRS “de Man Rogosa and Sharpe”, the media was prepared as standard and/or modified. Standard and modified MRS were prepared according to de Man, J.C., Rogosa, M. and Sharpe (1960). The composition of standard MRS broth is listed in table 2.

Table 2. standard MRS broth composition

Ingredients	g/L
Peptone	10
Meat extract	10
Yeast extract	5
Glucose	20
Sodium acetate	5
Magnesium Sulfate	0,1
Manganese sulphate	0,05
Dipotassium hydrogen phosphate	2
Ammonium Sulfate	2
Tween 80	1
Final pH (at 25°C)	6,5 ±0,2

II.2. LAB strains growth conditions and standardization

Sixty (16) LAB strains, previously isolated from *Kaddid* meat and selected for their low spoilage, antimicrobial and safety characteristics (Boubakri et al.,2022, Paper in press) are listed in Table 2. Stock of LAB cultures were maintained at -25 °C in de Man Rogosa Sharpe (MRS) broth (Britania, Argentina) contained 25% (w/v) glycerol (Britania, Argentina). The colonies' purity was verified using both the catalase-negative reaction and the Gram-positive staining. Following confirmation, new stocks of pure strains were generated and conserved in both +4 and -80°C for future applications. Before use, the stock strains were sub-cultured for three consecutive times (1%, v/v) at 30 °C for 18 h under static conditions. LAB viable count (Log CFU/mL) at 18 h the time of mid-exponential phase used for all the experiences of this study was calculated as following, fresh culture from the 3rd subculturing was measured by optical density (OD_{600}). Simultaneously the same culture was harvest by centrifugation (3000×g for 10 min at 20 °C), the obtained cells were washed twice and suspended in saline solution (SS) [0.85% NaCl], then serial decimal dilutions was prepared with the same SS. The count of the viable cells' concentration was carried out by pouring the adequate dilutions in MRS agar and

Table 3. Lactic acid bacteria strains used in this study.

LAB strains	Accession number	Origine	Relevant features
Enterococcus (E.) faecium K11	AP019394.1	Laboratory-prepared *Kaddid*	-Antimicrobial and bacteriocins -Anti-*Listeria* producers
E. faecium K19	AP019394.1		
Latilactobacillus (Lat.) sakei K25	JQ653151.1	Laboratory-prepared *Kaddid*	-Antimicrobial and bacteriocins Anti-*Listeria* producers; -Lack of spoilage; -Safety criteria.
Lat. sakei subsp. sakei K22	NR_042443.1		
Lat. sakei subsp. sakei K26	NR_042443.1		
Lactiplantibacillus (Lpb.) paraplantarum K7	CP053337.1	Laboratory-prepared *Kaddid*	-Antimicrobial activity
Lpb. paraplantarum K8	CP053337.1		
Lpb. paraplantarum K18	CP053337.1	Laboratory-prepared *Kaddid*	-Antimicrobial and bacteriocins Anti-*Listeria* producers; -Lack of spoilage; -Safety criteria; -GABA producer.
Lpb. paraplantarum K21	CP053337.1	Laboratory-prepared *Kaddid*	-Antimicrobial and bacteriocins Anti-*Listeria* producers -Lack of spoilage; -Safety criteria;
Pediococcus (P.) acidilactici BK14	CP050079.1	traditional South-Western Algerian *Kaddid* samples (Boubakri et al., 2022)	-Antimicrobial activity
P. acidilactici BK17	CP050079.1		
Weissella (W.) cibaria BK2	MN994365.1	traditional South-Western Algerian *Kaddid* samples (Boubakri et al., 2022)	-Antimicrobial activity -Safety criteria -Technological characteristics
W. cibaria BK19	MK503640.1		
W. confusa BK6	MT012260.1		
W. confusa BK11	MK503640.1		
W. paramesenteroides BK8	MT012260.1		

incubated at 30 °C for 48 h. the colonies were counted and the concentration level for each LAB subculture at 18h were noted (OD_{600} and viable count).

II.3. Biosurfactants activity

Fresh LAB subculture in standard MRS broth medium containing 5% of soy oil (*elio-cevital*, Bejaia- Algeria) soy oil incubated with shaking 120rpm at 30°C for 72 h. After that, cell-free-supernatants (CFSs) of the cultures were obtained by centrifugation at 10,000 g for 10 min at 4ºC. The CFSs were used to screen the biosurfactant producing LABs **(Rodrigues et *al*., 2006)**.

II.3.1. Qualitative methods: Drop collapse test

The qualitative Drop-collapse test was performed following the protocol of **Bodour** and **Maier (1998)**. To perform this test, 2 µL of soy oil (*elio-cevital*, Bejaia- Algeria) was added to the surface of a glass plate and 5 µl of the culture CFSs was added and observed for 1 min. The CFS that made the oil drop-collapse were consider positive; those drops that remained beaded were scored as negative compared SDS and distilled water as a positive and negative control respectively.

II.3.2. Quantitative method: emulsification index (EI)

The emulsifying activity of LAB-CFSs towards soy oil was carried out as following, 4 mL of soy oil and 4 mL CFS were mixed in test tube and vigorously homogenized using a tube-vortex (Selecta, Spain) for 2 minutes. The emulsification activity was determined after one minute and 24h after vertexing and the percentage (%) of EI_{24} were given by dividing the yielded height of the emulsified layer (mm) after vertexing to the total height of the liquid in test tube (mm), then multiplying by 100. The results were compared with 1% of SDS (1%) as positive control and sterile MRS broth as negative control (**Nagea and Zaiton, 2018**).

II.4. EPS level production

II.4.1. Extraction of EPS

LAB Fresh subcultures of 18 h were inoculated at 1% into 50 mL modified MRS medium containing 100 g/L sucrose. After static incubation at 30 °C for 72h, the samples were

centrifugated at 6000 g for 30 minutes to collect the supernatants **(van Geel-Schutten G et *al*., 1998)**.

To extract EPS, the technique used by **Geel-Schutten** G (1998) were applied with minor modifications, each supernatant was mixed with twofold volumes of cold ethanol (4 °C). The supernatant-ethanol mixture was kept for one night at 4 °C. The mixture (supernatant-ethanol) was centrifuged at 2000 g for 15 min to obtain EPS. The supernatant was removed and EPS was afterwards suspended again with two-folds volumes of ethanol and precipitated by centrifugation at 2000 g for 15 min. EPS rinsing with absolute ethanol step was repeated to eliminate any culture medium residues. Precipitates were dried at room temperature.

II.4.2. Quantification of EPS

A standard curve was prepared using glucose solutions with concentrations ranging from 0 to 1000 mg/L (annex 15). The absorbance (optical density) at 490 nm was measured for each concentration **(Dubois M et *al*., 1956)**. The EPS solution was prepared by dissolving a known quantity of dried EPS in deionized water until reach final concentration of 5 mg/mL. Then, 500 µL of the EPS solution (5 mg/mL) was pipetted into a test tube. Following that, 500 µL of phenol (2.5%; w/v) was added to the test tube, along with 2.5 mL of sulfuric acid (Carlo erba, France). The contents of the test tube were gently swirled to ensure thorough mixing. The test tube was then incubated in a water bath at 30 °C for 30 minutes before being cooled at room temperature. Finally, the absorbance of the solution at 480 nm was measured using a spectrophotometer. The absorbance value of the EPS solution was compared to the standard curve to determine the concentration of EPS in mg glucose/L **(Zaghloul et *al*., 2022)**.

II.5. Antioxidative activity

To perform this test, LAB cells from fresh subculture at 18h were harvest by centrifugation (3000×g for 10 min at 20 °C), the obtained cells were washed twice and suspended in SS, the cells concentration of were adjusted until reach ~11Log CFU/mL basis on the standardization technic described in **II.2**.

II.5.1. DPPH Scavenging Activity

For the evaluation of the DPPH radical scavenging activity was performed according to **(Rwubuzizi et *al*., 2023)**. Briefly, LAB cells as previously prepared were mixed with 1 mL of freshly prepared DPPH solution (0.05 mMol/L, in ethanol) and samples stored in darkness for 1 h. In the meanwhile, the blank was the sterile saline solution and ascorbic acid (Biochem Chemopharma /France) of 1 mg/mL in distilled water served as positive control. The mixture DPPH/cells (10, 000×g for 10 min at 20 °C) was harvest by centrifugation and the supernatants OD_{600} was measured using microplate reader (SAFAS MP 96, Monaco) equipped with SAFAS software. The estimated DPPH radical scavenging activity (%) of tested strains was calculated as follows: Scavenging activity (%) = $[1 - ((A_{sample} - A_{blind})/A_{blank})] \times 100$, where blind used was sterile SS.

II.5.2. Hydroxyl radical scavenging activity (Hydroxyl RSA)

This test was performed according to **Wang et *al*., (2015)**, LAB cells as prepared above were suspended in 1 mL of SS. After that each LAB suspension were transferred to glass test tubes previously filled with 1 mL brilliant blue (0.435 mMol/L), 2 mL $FeSO_4$ (0.5 mMol/L), 1.5 mL H_2O_2 (3%, w/v) and incubated at 30 °C for 1 h. 1 mg/mL of ascorbic acid was used as positive control, while SS without cells was used as negative control. The samples were harvest by centrifugation (3000×g for 10 min at 20 °C) and absorbance of the obtained supernatants were measured at OD_{600} using microplate reader.

For calculations of the hydroxyl radical scavenging effect by LAB cells, the formula prepared by **Wang et *al*., (2015)** was applied:

Hydroxyl RSA (%) = $[(A_0 - A_1)/ (A - A_1)] \times 100$, where A_0 was the absorbance of the solution with LAB sample and A_1 refers to the absorbance of the solution without LAB. A refers to the absorbance of the solution without research sample and the Fenton reaction system prepared only with brilliant blue (0.435 mMol/L).

II.5.3. Anti-lipid peroxidation

For the determination of anti-lipid peroxidation, the method described by **Rwubuzizi et *al*., (2023)** was carried out as following. Briefly, Fresh egg was washed and disinfected with absolute

ethanol, then egg yolk was obtained and transferred into sterile beaker and supplemented to an equal volume of PBS (200 mmol/L, pH 7.2), After that, the whole was homogenized in a magnetic stirrer. The obtained suspension was further diluted with PBS (egg yolk: PBS; 1:25; v/v).

In Eppendorf tubes, 200 µL egg yolk suspension was mixed with 100 µL of LAB sample adjusted to 11 log CFU/mL as mentioned above, 200 µL of PBS and 200 µL of iron sulphate (25 mMol/L) were added.

The tubes were vortexed and stirred in shaker at 30 °C for 15 min. After that, 200µL trichloroacetic acid (20%, w/v) were added to the previous suspension and kept static for 10 min. The tube was then centrifugated (3000×g for 10 min, 20 °C). Then, 600µL was carefully obtained in new Eppendorf tubes, followed by adding 400µL of Thiobarbituric acid (0.6%, w/v) and the mixture heated by stirring in thermo-agidator for 10 min and cooled in laboratory temperature. The samples were centrifugated (3000×g for 10 min, 20 °C) to obtain the supernatant. The absorbance of each sample measured at OD_{600} using microplate reader. The blank (A_0) comprised 100 µL PBS to replace the LAB strains sample in the reaction (A_s).

Lipid peroxide inhibition rate (%) = $[(A_0 - A_s)/A_0] \times 100$. While 1 mg/mL of ascorbic acid served as positive control **(Rwubuzizi et *al*., 2023)**.

II.6. Statistical analysis

Statistical analyses and figures were performed with GraphPad Prism 7.04 Software using one-way ANOVA (and nonparametric) followed by *Dunnett's* and *Tukey* tests multiple comparison with the positive control and between the samples respectively in which $P < 0.05$ was considered significant unless otherwise indicated. All the values were the means of at least two independent trials ± standard deviation with the convenable technical repetitions. The Figure 5 (matix plot) was plotted using Minitab statistical software 22 (Minitab Inc.).

Results and discussion

III. Results and discussion

III.1. Evaluation of EPS level production

There are many microorganisms reported as EPS producers. Since most LAB species have been generally recognized as safe (GRAS) status, species that can produce EPS attract attention. The fact that EPS producer LAB isolates improve the texture properties of meat products and delay color and oxidation **(Kamiloğlu., 2022**), **Trabelsi et *al*. (2018)** show that EPS production is also an important factor technologically.

EPS production levels was determined after 72 h incubation of *Kaddid*-borne LAB strains in modified-MRS medium containing 100g/L of saccharose in place of 20g/L of glucose. The results of LAB-EPSs were shown in Figure 6.

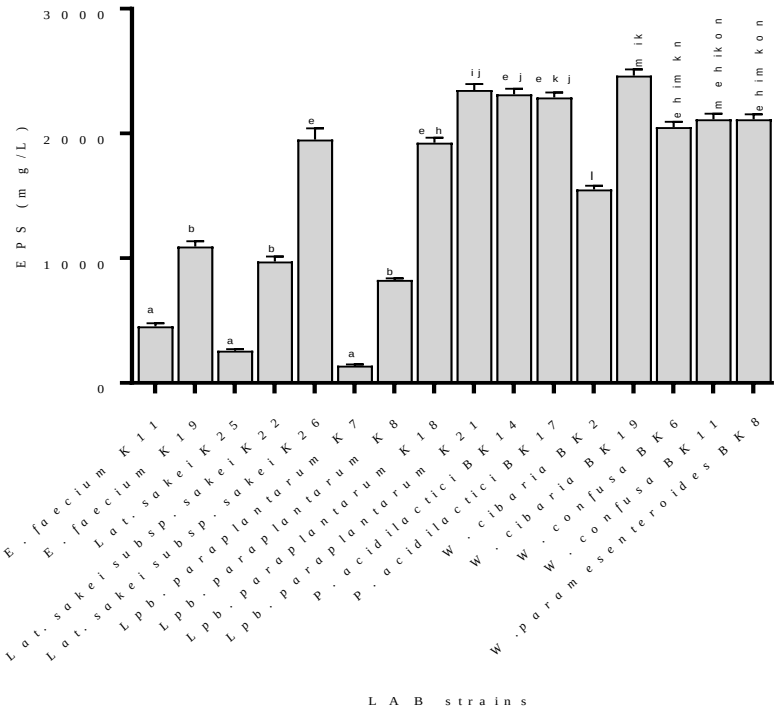


Figure 6. Evaluation of exopolysaccharides (EPS) production (mg/L) by *Kaddid*-borne LAB strains from saccharose (100g/L). Each bar represents the mean ± SD from duplicate performed in 3 technical repetitions. According to One-way ANOVA (nonparametric) followed with *Tukey*

test multiple comparison between groups (P<0.05) with 99.9% confidence interval, bars not sharing similar letters differ significatively (P-value <0.0001).

According to the results the stains *E. faecium* K11, *Lat. sakei* K25 and *Lpb. paraplantarum* K7 exhibited the lowest EPS concentration of 453.375, 258.375 and 138.375mg/L respectively. While *W. cibaria* BK19 exhibited the highest produced concentration of EPS with 2464.625mg/L, which not surprising since this species are widely reported as EPS over producers, such as *W. cibaria* NC516.11 and *W. cibaria* SY003 **(Wang et *al*., 2023)**. The results of this study show that some species produce high levels of EPS regarding close species from other fermented food. Indeed, **Kamiloğlu (2022)** reported that *Lpb. plantarum* KS-11 and *Lpb. pentosus* KS-27 isolated from *Sucuk* a Turkish dry-fermented sausages exhibited production with 991.75±94.81 and 991.75±37.55 mg/L respectively. In the meanwhile, the strains P. acidilactici KS-20, W. hellenica KS-24, L. sakei.KS-30 strains produced the lowest levels. Also, Mıdık et *al.* (2020) demonstrated that strains of *Lpb. plantarum, P. ethanolidurans* and *Levilactobacillus (Lev.) namurensis* isolated from pickles produce EPS concentrations between 215.67 to 408.53 mg/L of EPS from modified MRS medium with 100g/L sucrose

Even thought, the production of EPS by certain LAB can also considered as spoilage indicator for repined meat products particularly leading to undesirable changes in the meat's texture and appearance, therefore, the high levels of EPS can cause a slimy texture and off-flavors (**Gram et *al*., 2002**). However, some other studies reveled that some EPS-producing LAB strains are often sought for their beneficial roles in food fermentation (**Gram et *al*., 2002**).

III.2. Evaluation of antioxidative activity

III.2.1. DPPH scavenging activity (DPPH-SA)

DPPH radical scavenging activity is one of the most preferred methods for determining antioxidative activity of LAB **(Kamiloğlu et *al*., 2022)**, higher percentage of inhibition indicates a stronger antioxidant capacity of the LAB strain. A higher inhibition % means that the LAB strain has a greater ability to scavenge the DPPH radicals. The antioxidant activity raises in ratio with the removal of purple color produced when the DPPH radical was added to the medium **(Rwubuzizi et *al*., 2023).**

The ability to scavenge the DPPH radical for *Kaddid*-borne LAB strains is a strain-dependent feature and varied from 52.5 to 97.23% at the dose of ~11Log CFU/ml, the results were represented in Figure7

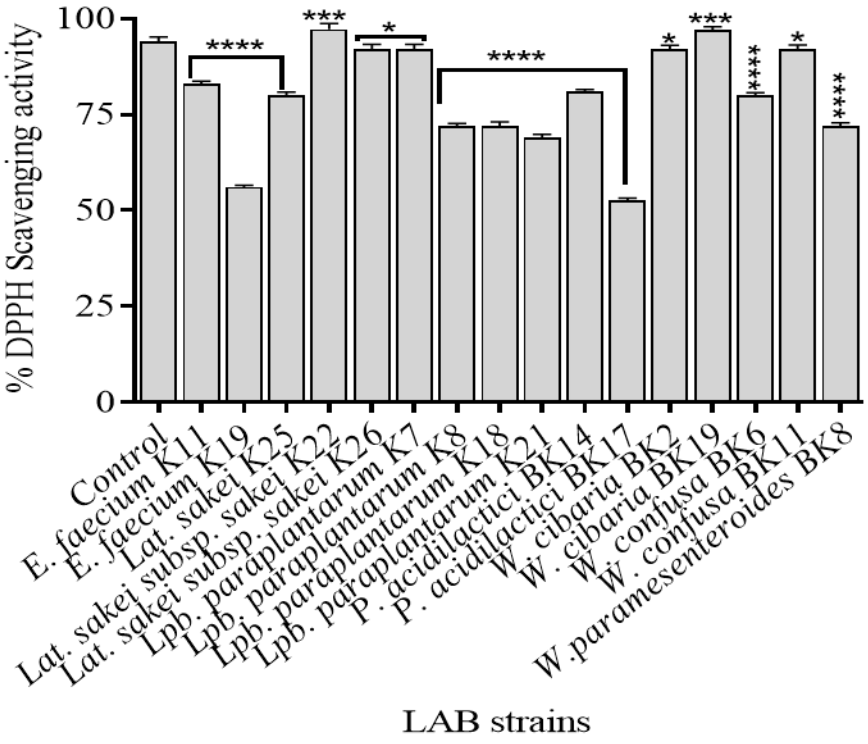


Figure 7. Evaluation of the DPPH activity for *Kaddid*-borne LAB strains. Control: ascorbic acid (1mg/ml), Results represent the mean ± SD of at least 2 repetitions. According to One-way ANOVA (nonparametric) followed with *Dunnett's* multiple comparison test to the control positive (P<0.05) with 99.9% confidence interval, bars superscript asterisks differ significantly (P-value: *0.0332; ***0.0002; *****<0.0001).

Some *Kaddid*-borne strains show a great ability to scavenge of DPPH free radical as observed with the strains *Lat. sakei subsp. sakei* K22; *W. cibaria* BK19 with 97% of scavenging rate, followed by *Lat. sakei subsp. sakei* K26; *Lpb. paraplantarum* K7, *W. cibaria* BK2; *W. confusa* BK11 with 92% scavenging rate. In the meanwhile, ascorbic acid (1 mg/mL) used as control

positive reach 94% of scavenging rate, demonstrating that this LAB have a high scavenging effect comparing to ascorbic acid. In the other hand while *E. faecium* K19 and *P. acidilactici* BK17 showed the lowest activity rate 56% and 52% respectively. Similar results were observed by Rwubuzizi **et** *al.* **(2023)**, where *St. salivarius* strain ST59HK exhibited the highest activity rated with 85.24% and *E. faecium* ST651ea showed the lowest activity of 66.44%, also **Duz et al. (2020)** found that the antioxidant effects of *Lpb. plantarum*, *Lat. sakei* and *Lat. curvatus* strains isolated from fermented Turkish *Sucuk* sausages differed between 58.38% to 90.34%.

III.2.2. Hydroxyl radical scavenging activity (HRSA)

Hydroxyl radicals (HRs) are primarily formed biologically through the Fenton reaction, which involves the interaction of iron (II) ions (Fe^{++}) and hydrogen peroxide (H_2O_2). *In vitro* conditions, the Fe^{++}/H_2O_2 system is also used to generate hydroxyl radicals. The scavenging activity of antioxidants against these radicals is often measured. However, some antioxidants can also act as metal chelators, which can influence the activity of Fe^{++} and potentially inhibit the formation of HRs. For example, certain antioxidants have been shown to inhibit the formation of HRs by chelating iron ions **(Duz et** *al***., 2022)**.

In this study, Fenton reaction system was used to determine the hydroxyl radicals scavenging capacity of 16 *Kaddid*-borne LAB strains as shown in the Figure 8, standing with the fact that ascorbic acid (1mg/ml) used as control positive result high-moderate HRSA with a value of 72%. Thereby, several *Kaddid*-borne LAB whole cells exhibited a high HRSA activity. In which *Lpb. paraplantarum* K21 was fount the most active stains with exhibited value ~100% HRSA, followed by *Lpb. paraplantarum* K18 (95%), *P. acidilactici* BK17 (86.02%), *W. confusa* BK6 (83.38%), *P. acidilactici* BK14 (83%), *Lpb. paraplantarum* K7 (78.88%); *W.paramesenteroides* BK8, (72.36%). While, *Lpb. paraplantarum* K8, *W. cibaria* BK2, *W. confusa* BK11 and well as *E. faecium* K11 exhibited the lowest scavenging activity.

The high HRSA was also observed by **Rwubuzizi et** *al***. (2023)** in *St. salivarius* ST59HK isolated from soybean paste and oral cavity of health volunteers with a value ~ 98.6%. Also, **Duz et** *al***. (2020)** were found that the selected lactobacilli from Turkish *Sucuck*, the strain *Lat.*

cruvatus IH1L, *Lat. sakei* IH15L and *Lpb. plantarum* IH16L exhibited values of 78, 82.20 and 82.25% respectively.

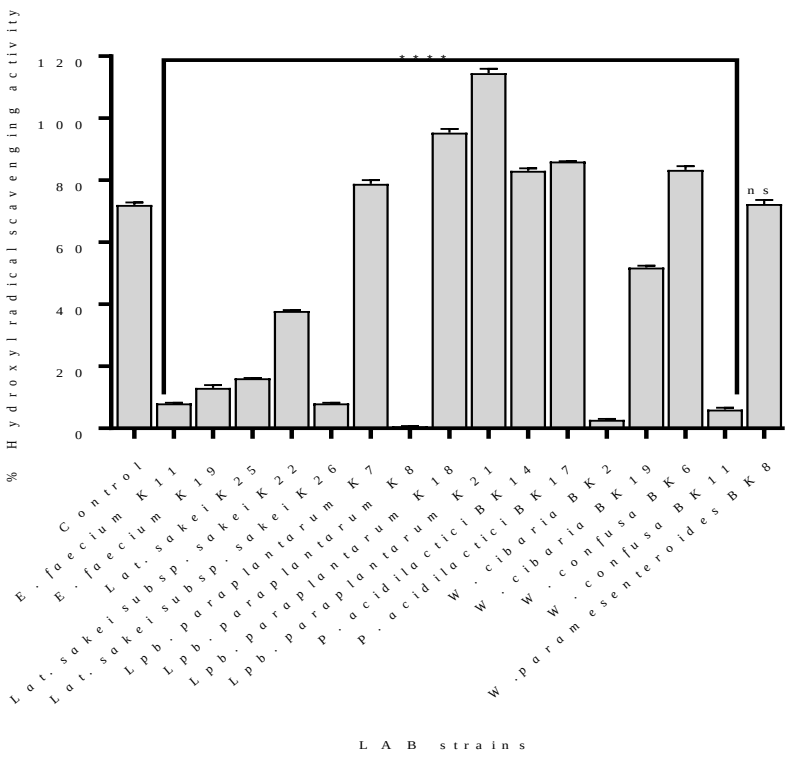


Figure 8. Evaluation of the hydroxyl radical scavenging activity for *Kaddid*-borne LAB strains. Control: ascorbic acid (1mg/ml), Results represent the mean ± SD of at least 2 repetitions. According to One-way ANOVA (nonparametric) followed with *Dunnett's* multiple comparison test to the control positive (P<0.05) with 99.9% confidence interval, bars superscript asterisks differ significantly (P-value: ns: 0.9992; *****<0.0001).

The results of these study demonstrated significant variability in HRSA among *Kaddid*-borne LAB strains, since some strains observed with very low activity like *Lpb. paraplantarum* K8 with HRSA of only 0.62%, however, other strains with very high activity indicating the diverse metabolic capabilities of the *Kaddid*-borne LAB strains in scavenging hydroxyl radicals. LAB-starters with HRSA could be used to prevent lipid peroxidation, reducing rancidity and maintaining the flavor, color and overall quality of dry-fermented meat products.

III.2.3. Anti-lipid peroxidation (ALP)

The oxidation of cellular membrane phospholipids into peroxide derivatives is described as lipid peroxidation, The detection of lipid peroxidation is typically done by measuring the levels of malondialdehyde (MDA), a three-carbon compound formed after the scission of peroxidized polyunsaturated fatty acids (PUFAs). MDA is detected using methods such as the thiobarbituric acid (TBA) test, which reacts with aldehydes produced during lipid oxidation **(Pereira and Abreu, 2020).**

Egg yolk (rich in PUFAs) was applied as the lipid source in the demonstration of the anti-lipid peroxidation activity, Inhibition of lipid peroxidation is a general method used to determine antioxidative activity. In this study, anti-lipid peroxidation rate of the 16 *Kaddid*-borne LAB strains varies between 31.43% and 42.857%, which considered as low-to-moderate comparing with ascorbic acid (1mg/L) with a value of 52.85%, in which *P. acidilactici* BK14 and *W. cibaria* BK19 were found the most active strains (Fig.9).

Duz et *al*. (2020) reported lipid peroxidation inhibition rate ranged between 40.18 to 63.29% and *L. cruvatus* GH1L exhibited the highest inhibition rate. In the same line **Rwubuzizi et *al*. (2023)** were found that LAB strains isolated from the oral cavity of a healthy volunteer and Korean traditional fermented soybean past in which *Lactobacillus (Lb.) gasseri* ST16HK can reach 69.43 % of antilipid peroxidation, while *E. faecium* ST7319ea registered 26.15%.

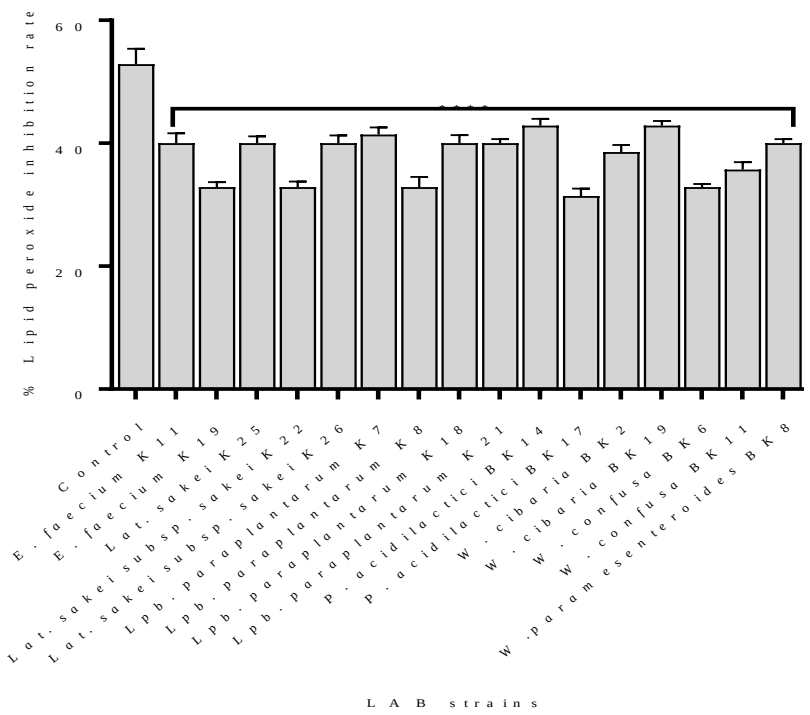


Figure 9. Evaluation of lipid peroxide inhibition rate for *Kaddid*-borne LAB strains. Control: ascorbic acid (1mg/ml), Results represent the mean ± SD of at least 2 repetitions. According to One-way ANOVA (nonparametric) followed with *Dunnett's* multiple comparison test to the control positive (P<0.05) with 99.9% confidence interval, bars superscript asterisks differ significantly (P-value: *****<0.0001).

III.2.4. Correlation between DPPH-SA/HRSA/ALP

The Matrix plot analysis (fig.10) was applied to determine the correlation between the tested antioxidant activity. Since each test indicate the scavenging of a specific pro-oxidant inductors: (DPPH-SA/R˙), (HRSA/OH˙) and (ALP/PUFAs). Therefore, Person correlation analysis were found low correlation between (Peroxidation inhibition/HRSA; r=0.339) and between (DPPH-SA/HRSA; r=0.338). However, almost no correlation between (Peroxidation inhibition/DPPH-SA; r=0.055).

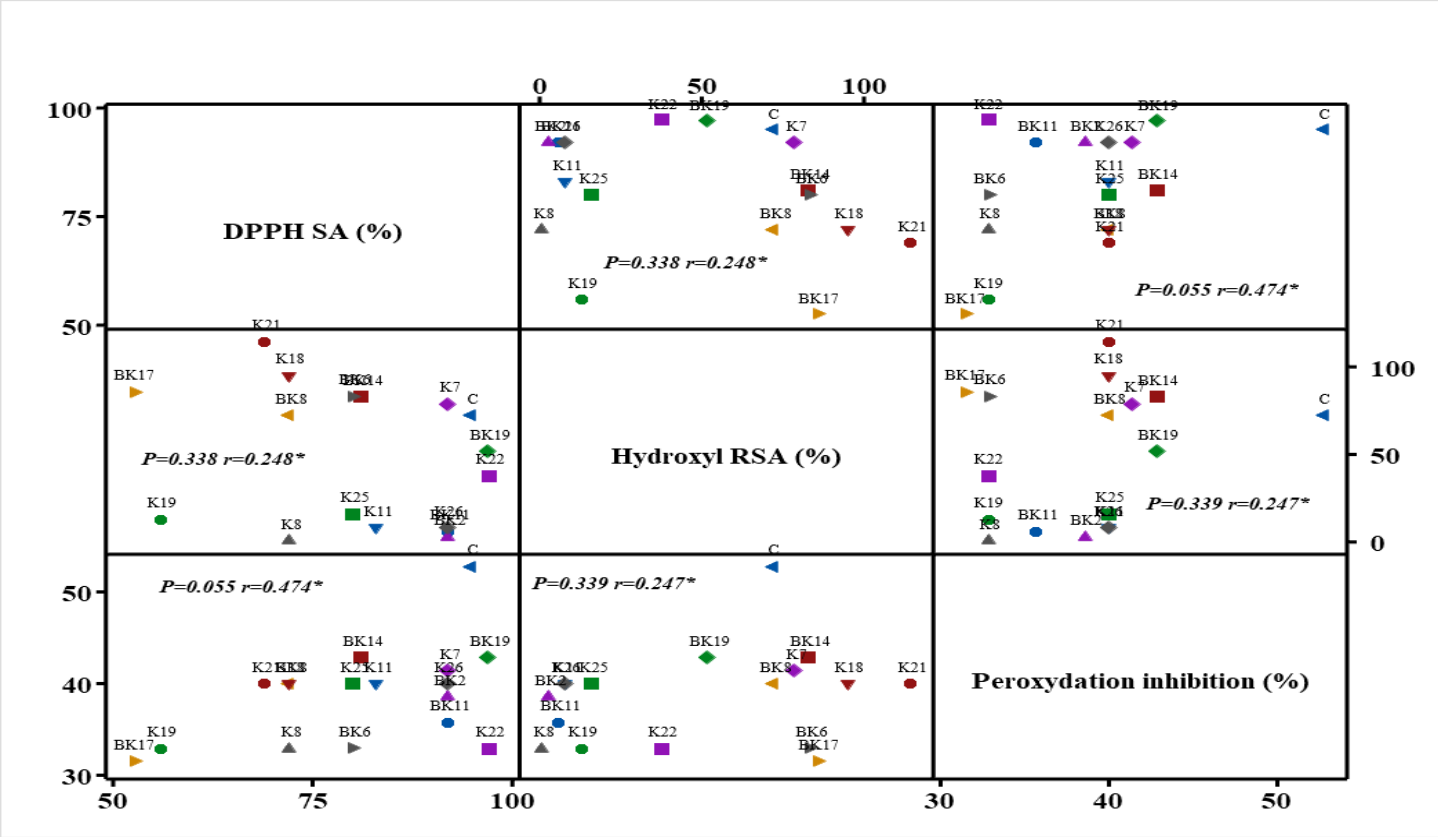


Figure 10. Matrix plot of antioxidant activity of *Kaddid*-borne LAB strains of lactobacilli: DPPH, Hydroxyl Radical scavenging and peroxidation inhibition activity. *E. faecium* (K11, K19), *Lat. sakei* K25, *Lat. sakei subsp. sakei* (K22, K26), *Lpb. paraplantarum* (K7, K8, K18, K21), *P. acidilactici* (BK14, BK17), *W. cibaria* (BK2, BK19), *W. confusa* (BK6, BK11), *W. paramesenteroides* BK8. * *P* and Pearson correlation (*r*) values.

Since red meat also is main source of essential PUFAs and Iron for human nutrition, thus two elements are the main indicators of fat oxidation. During meat fermentation and repining lipid oxidation is the issue of deterioration. Nonetheless, Hydroxyl radicals (OH˙) initiate the peroxidation of lipids, which results in the formation of malondialdehyde and other aldehydes. These compounds are responsible for rancidity, leading to off-flavors, color change and a decrease in the sensory quality of the meat **(Kanner, 1994)**. In addition, it can lead to the formation of toxic compounds such as 4-hidroxinonena **(trabelsi et *al.*, 2018)**. Selected function LAB starters can prevent oxidation and reduce the formation of toxic compounds.

III.3. Biosurfactant activity

III.3.1. Drop collapse test analysis

A qualitative preliminary detection for the presence of biosurfactant in CFS of *Kaddid*-borne LAB were carried out by using *Drop collapse test*, The CFS will cause a drop of oil to collapse and spread when it is added to the oil by which the force between the liquid drops and hydrophobic surface of oil is reduced **(Harkings et *al*., 1959)**.



Figure 11. Drop collapse assay. Collapsed droplets of soy oil after adding CFS of *Kaddid*-borne LABs.

Similar study conducted by **Nagea et *al*., (2018)** in 20 LABs isolates from different sources of food including dairy products, breast milk, fermented shrimp and fruits samples in Malaysia were tested by drop collapse test to detect their ability to produce biosurfactant, including 13 strains were able to produce biosurfactant, however 7 strains were failed the test.

All the 16 tested LAB strains showed positive reaction (data not shown) for the drop collapse test using soy oil as shown in the Figure 11, which indicates in somehow the production of biosurfactant similar to 1% of SDS used as control positive which is known as surface tension-active agent. As control negative, there was no activity observed when using a sterile MRS broth. biosurfactants have gained popularity due to their biodegradable and non-toxic properties. They are becoming increasingly popular as novel functional additives for the food sector. Moreover, the rising awareness of replacing synthetic items with natural products reaches customers who

demand that more dangerous products be replaced with natural ones that satisfy the same standards.

III.3.2. Emulsification index analysis

This method involves mixing a specific volume of oil with a known volume of LAB-CFS. The emulsification index (%) is calculated as the percentage of oil that remains emulsified after a specified time. A higher emulsification index indicates better emulsification capabilities. This method is quantitative and provides a precise measure of the biosurfactant's emulsification properties **(Nagea et *al*., 2018)**.

In this study, the emulsification index is an important parameter in the evaluation of biosurfactant production also to determine the effectiveness of biosurfactant produced by *Kaddid*-borne LAB strains. The results at 0 and after 24h are shown in figure 12 Beside the emulsification index results at 0h (EI_0) were up to 70%, even higher than the SDS (1%). However, it was observed a decrease in the emulsification index at 24h (EI_{24}). In the meanwhile, the EI_{24} of SDS show more stability.

There are no significative differences between the positive control and LAB samples ($P<0.05$). Nevertheless, several strains registered EI_{24} higher than SDS (> 63.41%), such as *E. faecium* K11, *Lpb. paraplantarum* K8 and K21 and *W. paramesenteroides* BK8, indicating those strain could be considered for this feature. Comparing with other studies **Nagea et *al*. (2018)** revealed significant variations in EI_{24} among 20 LAB-CFS. Thereby, 60% of LAB isolates exhibited activity against diesel, 20% against motor oil and 60% against crude. In contrast to our findings, EI_{24} obtained from 1% SDS for diesel, motor and crude were 93.15%, 95.32% and 81.04% respectively, nettle high to our results, indicating the tensio-activity of SDS toward soy oil is inferior regarding fuels.

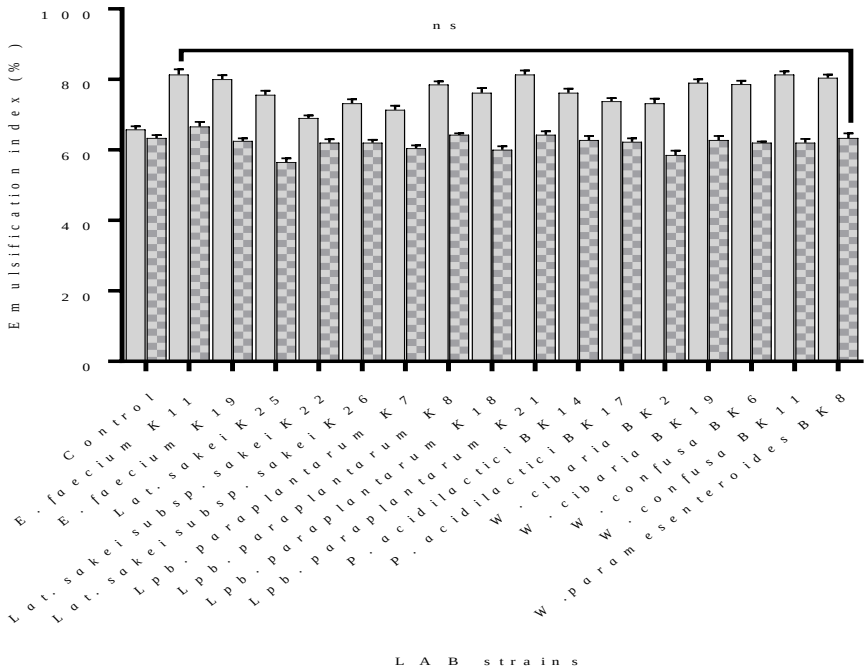


Figure 12. Evaluation of emulsification index by *Kaddid*-borne LAB strains. Control: SDS (1%). Bars without and with fill pattern representing day one and two respectively. Each bar represents the mean ± SD from duplicate performed in 3 technical repetitions. According to One-way ANOVA (nonparametric) followed with *Dunnett's* multiple comparison test to the control positive (P<0.05) with 99.9% confidence interval, bars no differ significantly (P-value=0.9997(ns)).

Most published data reported that bacteria with high potential of emulsifying activity of 50 to 60% are promising microbial candidates for biosurfactant production. There are reports of the action of biosurfactants as emulsifiers for the processing of raw materials, with application in bakery products, influencing the rheological characteristics of flour and meat products in the emulsification of fat. Emulsification plays an important role in consistency and texture formation as well as phase dispersion and flavor solubilization. Also, LAB biosurfactant could prevent biofilm-forming pathogen bacteria able to adhere and form persistent biofilms on food-contact surfaces and meat processing environment (Camargo et al., 2015).

Conclusion

In conclusion, the study successfully demonstrated the functional capabilities of LAB strains isolated from *Kaddid* meat. In relation with the previous studies showcasing their potential as natural preservatives to enhance the quality and shelf-life of fermented meat products.

The *in vitro* tests revealed significant DPPH and hydroxyl radical scavenging activities, along with anti-lipid peroxidation properties, indicating the efficacy of these strains in mitigating oxidative stress. Strains such as *Lat. sakei subsp. sakei* K22 and *W. cibaria* BK19 exhibited the highest DPPH activity with scavenging rates of 97%, closely followed by strains like *Lat. sakei subsp. sakei* K26, *Lpb. paraplantarum* K7, *W. cibaria* BK2 and *W. confusa* BK11 with 92% scavenging rates. For hydroxyl radical scavenging activity, strain *Lpb. paraplantarum* K21 stood out with a remarkable >100% of scavenging rate, while strains like *Lpb. paraplantarum* K18, *P. acidilactici* BK17 and *W. confusa* BK6 also showed high activity ranging from 86% to 95%. Additionally, certain strains demonstrated strong lipid peroxidation inhibition, crucial for rancidity prevention and maintaining the sensory quality of meat products.

However, the research also highlighted the overproduction of exopolysaccharides (EPS) by some LAB strains, which can negatively impact meat products by causing spoilage. Moderate EPS production was observed in several strains, emphasizing the need for careful selection and optimization of LAB strains for effective application as starter cultures for fermented meat industry.

Furthermore, all tested LAB strains showed positive results in the drop collapse test, indicating the production of biosurfactants, which can have significant applications in food and other industries due to their surface-active properties in preventing biofilm-forming pathogens to adhere meat processing surfaces.

The variable screenings of this study lead to select the strains, *Lactiplantibacillus paraplantarum* K18 and K21, *Weissella cibaria* BK2 and *Latilactobacillus sakei* K25 that exhibited high antioxidative activity alongside low-to-moderate EPS producers as well as biosurfactant

production, these characteristics make these strains suitable candidates for their use as bio-functional starter cultures for meat fermentation.

This study opened new perspectives for future researches:

- Extraction and identification of biosurfactant from selected LAB strains;
- Investigation of the optimal conditions for EPS production to balance its beneficial and detrimental effects as spoiler;
- *in situ* study of the effect of the selected strains as staters during meat fermentation and conservation;
- Long-term *in vivo* studies to assess the health benefits and safety of the selected starters when consumed regularly as part of the diet.

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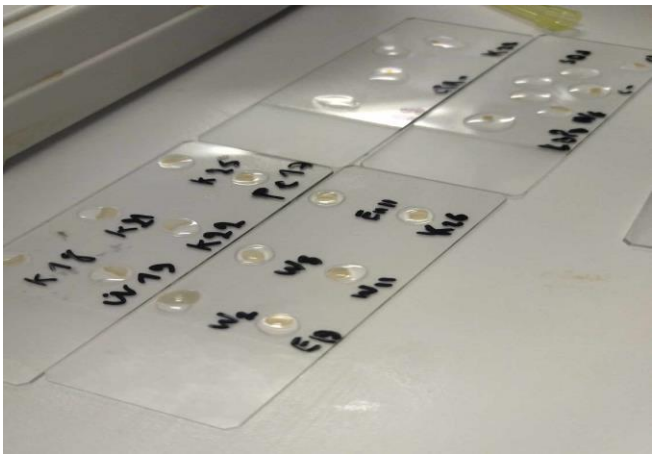
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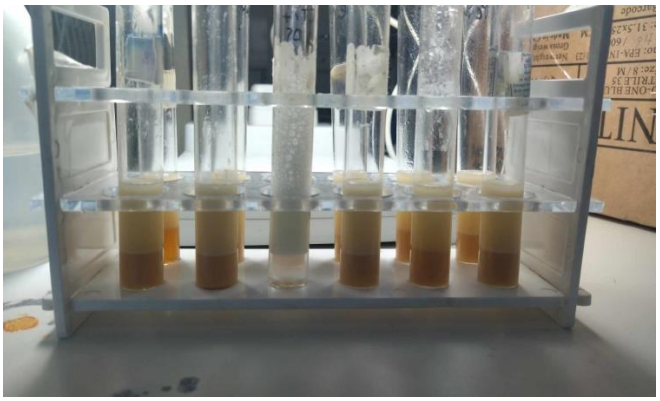
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Annexes



Picture 1. Drop Collapse essay of 16 LAB strains



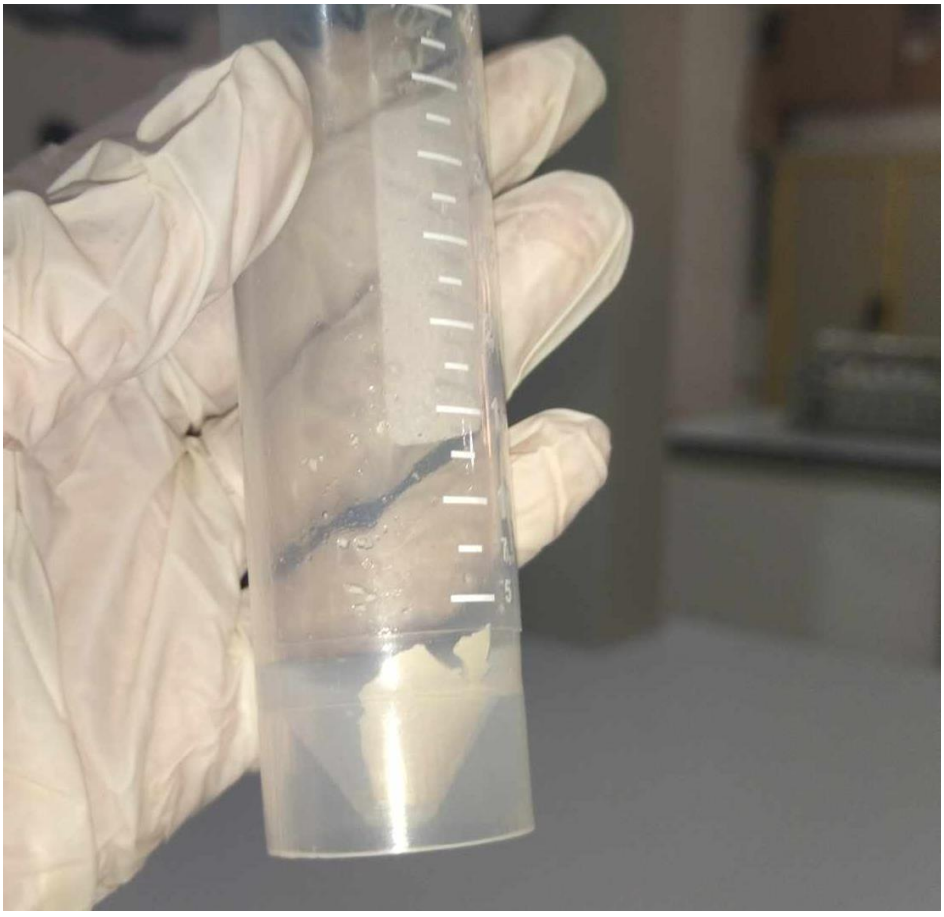
Picture 2. Emulsification Index essay of 16 Kaddid-borne LAB minutes after vortexing



Picture 3. Emulsification Index essay after of LAB strains 24h



Picture 4. Precipitation of EPS by the addition of two folds of ethanol and kept one night at 4°C



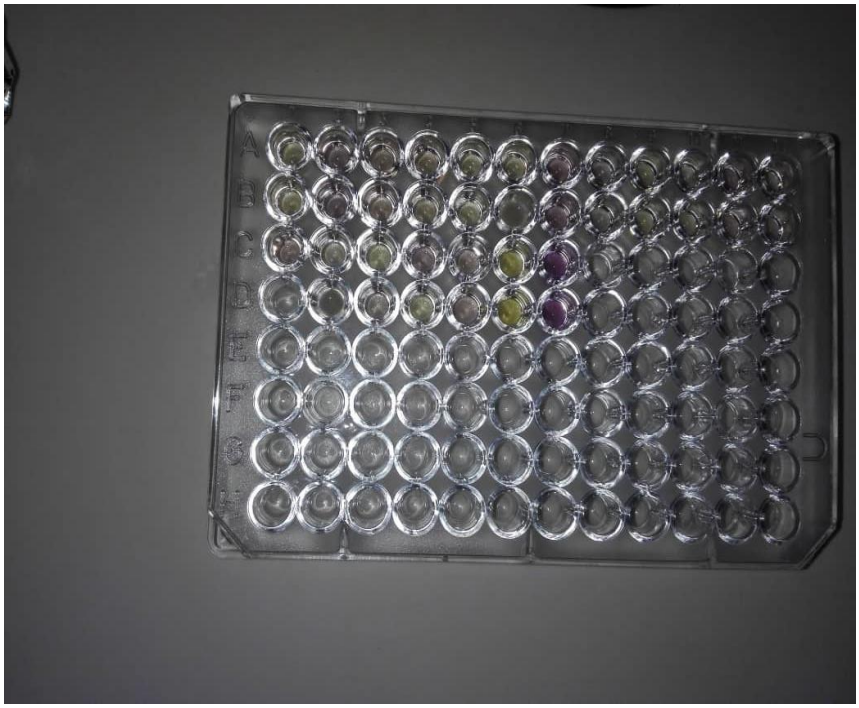
Picture 5. Dried precipitates of EPS at room temperature



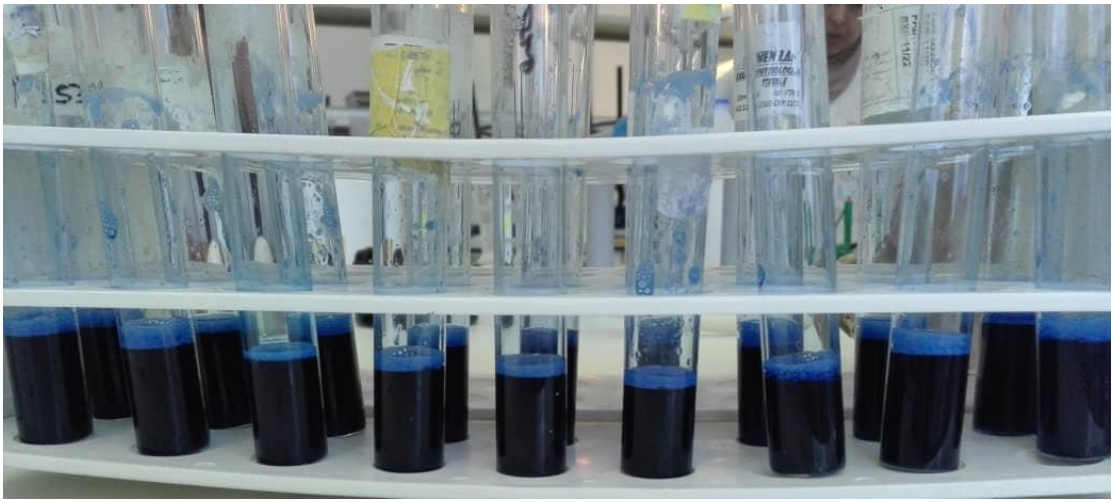
Picture 6. Colometric method by using phenol-sulfuric acid in order to quantify the EPS



Picture 6. Incubation of LAB strains in DPPH solution for one hour



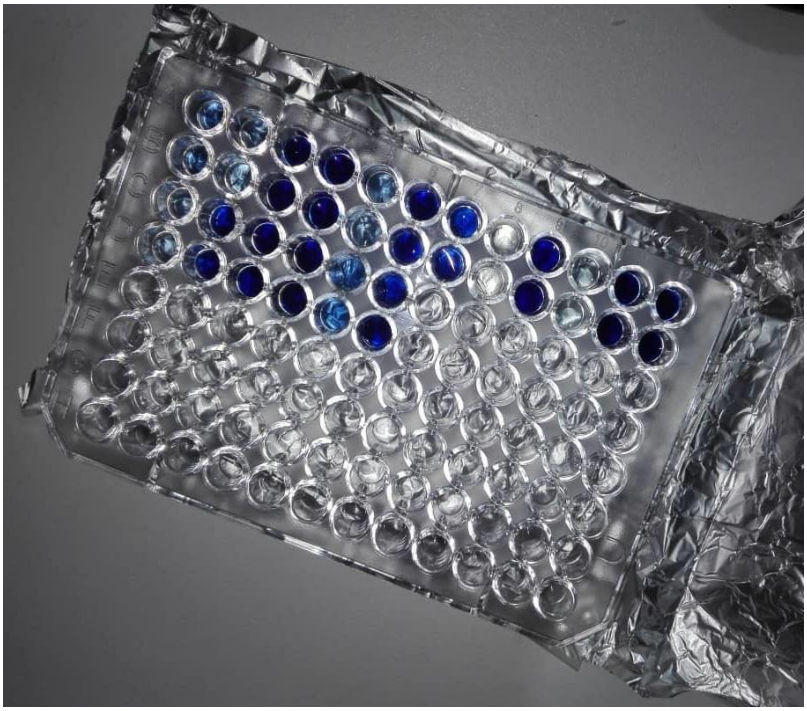
Picture 6. Examination spectrophotometrically of the supernatent obtained by cenrifugation at OD_{600}



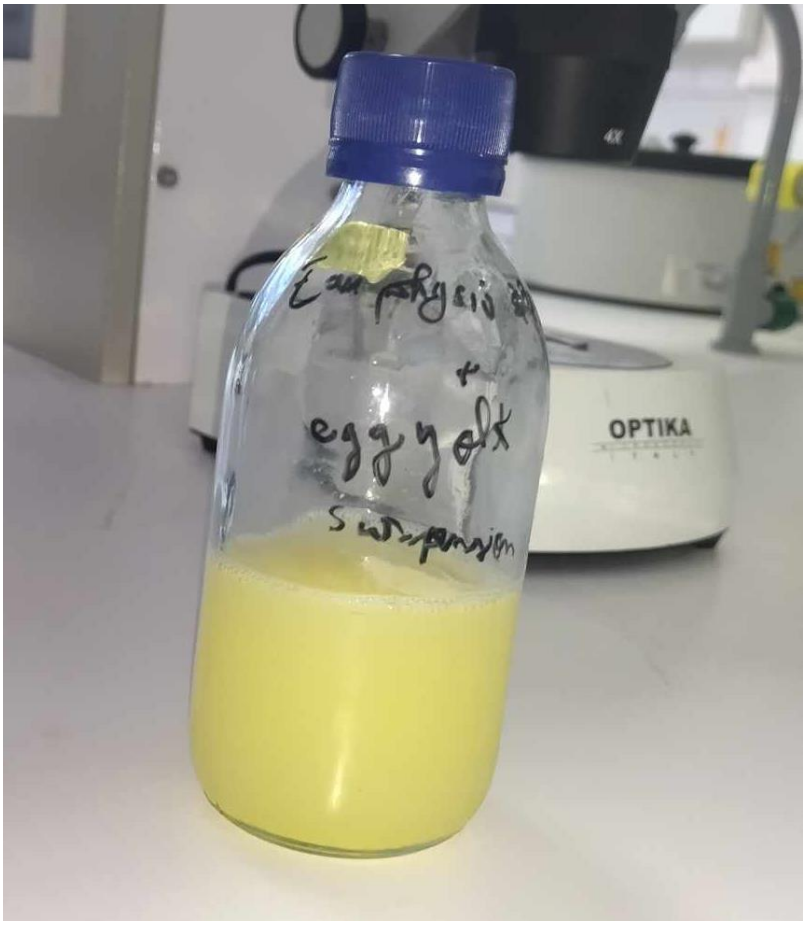
Picture 9. Incubation of strains in Fenton reaction



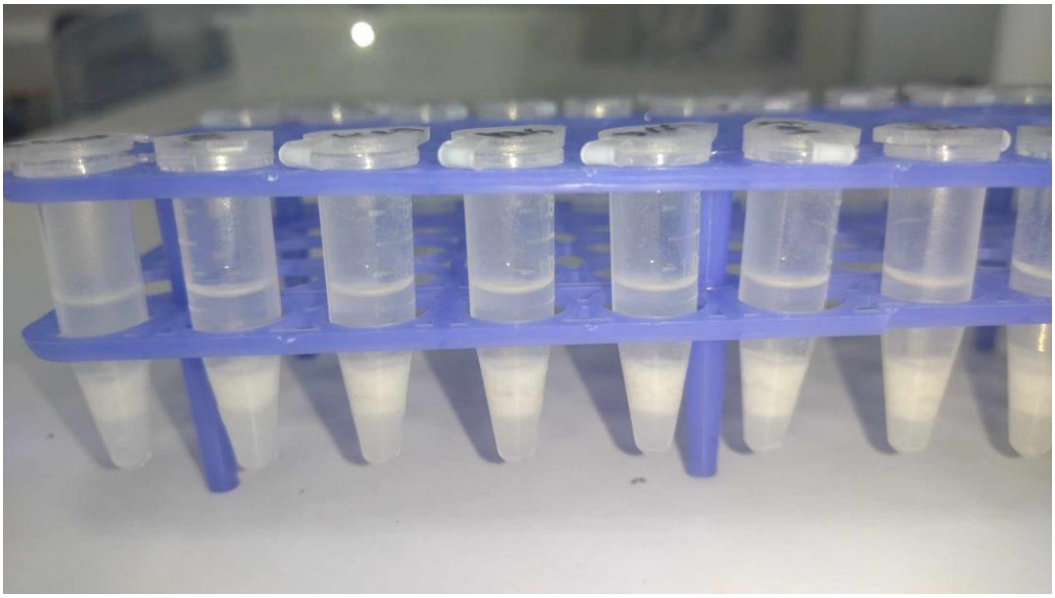
Picture 10. LAB strains after incubation in Fenton reaction



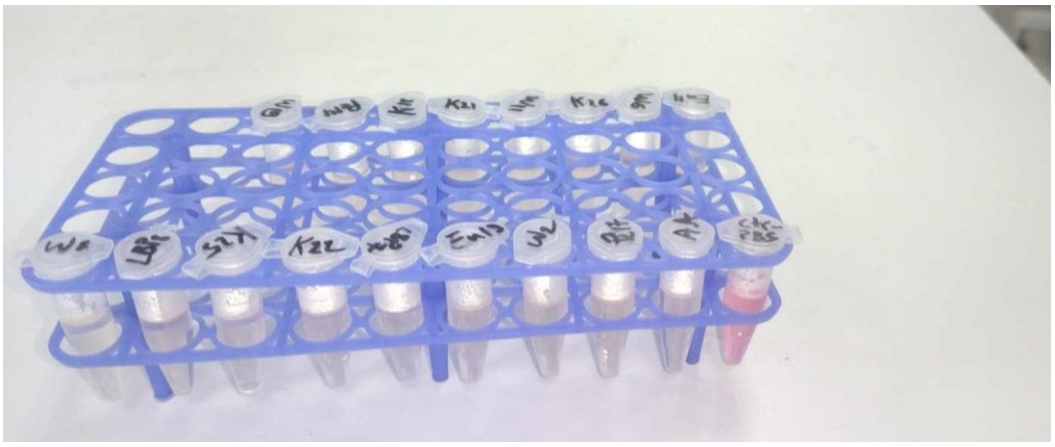
Picture 11. Application in microplate after centrifugation , the absorbance was measured at OD_{600}



Picture 12. Preparation of Egg yolk suspension (egg yolk: PBS; 1:25; v/v).



Picture 13. Denaturation of proteins after Trichloroacetic acid addition



Picture 14. The mixture after adding Thiobarbituric acid (2ml ;0.8%,W/V) and heated by stirring in Thermo-Agidator for 10min

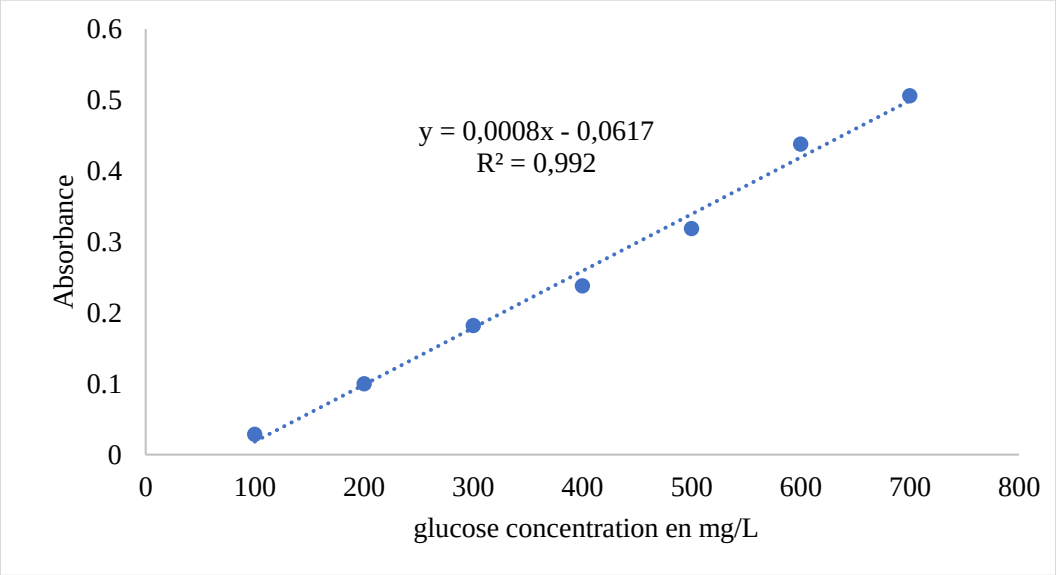


Figure 1. standard curve of glucose