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Title

EFFECT OF INCORPORATING PHENOLIC COMPOUNDS COMBINED WITH LACTIC ACID BACTERIA ON THE NUTRITIONAL AND ORGANOLEPTIC QUALITY OF MEAT

Defended on [date] before the jury composed of

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ABSTRACT:

This research addresses the global challenge of developing safe, antibiotic-free alternatives for meat production. The study focused on lactic acid bacteria (LAB) isolated from two ecological sources, phenolic-rich fermented green tea waste and traditional goat-milk butter (Dhan), to evaluate their probiotic potential and biopreservative functionality.

A multistep screening approach was used to select robust strains through sequential *in vitro* tests for acid and bile tolerance, phenolic adaptation, and antimicrobial activity, followed by *in vivo* validation in broiler chickens. And total of 150 Arbor Acres broilers were assigned to five treatment groups: one control and four receiving distinct LAB strains (*Levilactobacillus brevis* DC01-A, *Lactiplantibacillus plantarum* DC04, *Levilactobacillus brevis* TF03, and TF13). Subsequently, encapsulated probiotics were administered via drinking water from day 10 to 42. Growth performance (body weight, feed intake, average daily gain [ADG], feed conversion ratio [FCR]) and meat quality (protein, lipid oxidation [TBARS], and fatty acids) were evaluated.

Probiotic supplementation led to clear improvements in growth performance and meat quality ADG (+8.5%) and reduced FCR (−0.14 units, $p < 0.05$), enhanced intestinal morphology, and improved meat quality, notably through higher protein content (+6.3%) and lower TBARS (−0.22 mg MDA/kg). The phenolic-rich origin of some isolates enhanced tolerance, antioxidant capacity, and antimicrobial activity, contributing to superior *in vivo* performance. Encapsulation using sodium alginate increased probiotic viability (>90%) under simulated gastrointestinal conditions and enabled controlled intestinal release, improving stability and functionality.

These findings demonstrate that LAB isolated from phenolic-rich environments possess a dual probiotic and biopreservative role, enhancing animal health, oxidative stability, and meat safety. The results support their application as natural, multifunctional additives for sustainable and clean-label poultry production, offering an eco-friendly alternative to synthetic preservatives and antibiotic growth promoters.

Keywords: *lactic acid bacteria, probiotics, broiler chickens, growth performance, meat quality, fermented green tea waste, Dhan, phenolic adaptation, ecological origin, oxidative stability, antimicrobial activity, biopreservation, encapsulation.*

RESUMÉ:

Cette recherche ’inscrit dans la problématique mondiale du développement d’alternatives sûres et sans antibiotiques pour la production de viande. L’étude a porté sur des bactéries lactiques (BAL) isolées à partir de deux sources écologiques : un déchet de thé vert fermenté riche en composés phénoliques et un beurre traditionnel de lait de chèvre (Dhen), afin d’évaluer leur potentiel probiotique et leur fonctionnalité bioconservatrice. Une approche de criblage en plusieurs étapes a été utilisée pour identifier les souches les plus robustes, à travers des tests *in vitro* successifs portant sur la tolérance à l’acidité et aux sels biliaires, l’adaptation aux composés phénoliques et l’activité antimicrobienne, suivis d’une validation *in vivo* chez le poulet de chair. Un total de 150 poulets de chair Arbor Acres ont été répartis en cinq groupes expérimentaux : un témoin et quatre recevant différentes souches de BAL (*Levilactobacillus brevis* DC01-A, *Lactiplantibacillus plantarum* DC04, *Levilactobacillus brevis* TF03 et TF13). Les probiotiques encapsulés ont ensuite été administrés via l’eau de boisson du 10^{e} au 42^{e} jour. Les performances zootechniques (poids corporel, consommation alimentaire, gain moyen quotidien [GMQ], indice de consommation [IC]) et la qualité de la viande (teneur en protéines, oxydation lipidique [TBARS] et profil en acides gras) ont été évaluées. La supplémentation probiotique a conduit à une amélioration nette des performances de croissance et de la qualité de la viande, avec une augmentation du GMQ (+8,5 %) et une réduction significative de l’IC (−0,14 unité, $p < 0,05$). Elle a également favorisé une meilleure morphologie intestinale et une amélioration de la qualité de la viande, marquée par une teneur en protéines plus élevée (+6,3 %) et une réduction du taux de TBARS (−0,22 mg MDA/kg). L’origine phénolique de certaines souches a renforcé leur tolérance, leur capacité antioxydante et leur activité antimicrobienne, contribuant à de meilleures performances in vivo. L’encapsulation dans l’alginate de sodium a permis d’accroître la viabilité des probiotiques (> 90 %) dans des conditions gastro-intestinales simulées, tout en assurant une libération intestinale contrôlée, gage d’une stabilité et d’une fonctionnalité améliorées. Ces résultats démontrent que les BAL issues d’environnements riches en composés phénoliques exercent un double rôle, probiotique et bioconservateur, en améliorant la santé intestinale, la stabilité oxydative et la sécurité de la viande. Ils soutiennent leur utilisation comme additifs naturels multifonctionnels pour une production avicole durable et « clean label », constituant une alternative écologique aux promoteurs de croissance antibiotiques et aux conservateurs synthétiques.

Mots-clés : bactéries lactiques, probiotiques, poulets de chair, performances de croissance, qualité de la viande, déchet de thé vert fermenté, Dhen, adaptation phénolique, origine écologique, stabilité oxydative, activité antimicrobienne, bioconservation, encapsulation.

الملخص :

تتناول هذه الأطروحة التحدي العالمي المتمثل في تطوير بدائل آمنة وخالية من المضادات الحيوية في إنتاج اللحوم. ركّزت الدراسة على بكتيريا حمض اللاكتيك المعزولة من مصدرين بيئيين غنيين بالفينولات: مخلفات الشاي الأخضر المخمر والزبدة التقليدية المحضّرة من حليب الماعز (الدهان)، بهدف تقييم قدرتها البروبيوتيكية ووظيفتها في الحفظ الحيوي .تم اعتماد نهج انتقائي متعدد المراحل لاختيار السلالات الأكثر مقاومة من خلال اختبارات *in vitro* متسلسلة شملت تحمل الحموضة والأملاح الصفراوية، والتكيف مع المركبات الفينولية، والنشاط المضاد للميكروبات، تلاها تحقق *in vivo* على دجاج اللحم .تم توزيع 150 دجاجة من نوع Arbor Acres على خمس مجموعات: مجموعة شاهدة وأربع مجموعات تلقت سلالات مختلفة من بكتيريا حمض اللاكتيك (*Levilactobacillus* *brevis* DC01-A، *Lactiplantibacillus plantarum* DC04، *Levilactobacillus brevis* TF03 و.TF13)

تمت إضافة البروبيوتيكات المغلفة في ماء الشرب من اليوم العاشر إلى اليوم الثاني والأربعين.(تم تقييم أداء النمو الوزن الحي، استهلاك العلف، معدل الزيادة اليومية[ADG] ، ومعامل التحويل الغذائي ([FCR] بالإضافة إلى جودة اللحوم) نسبة البروتين، الأكسدة الدهنية[TBARS] ، وتكوين الأحماض الدهنية) . أدى استخدام البروبيوتيك إلى تحسينات واضحة في أداء النمو وجودة اللحوم، حيث زاد معدل الزيادة اليومية بنسبة (+8.5%) وانخفض معامل التحويل الغذائي بمقدار0.14−) ، .($p < 0.05$كما ساهم في تحسين بنية الأمعاء وزيادة جودة اللحوم من خلال ارتفاع نسبة البروتين (+6.3%) وانخفاض مستوى 0.22− (TBARS ملغ MDA/كغ.) ساهمت الأصول الغنية بالمركبات الفينولية لبعض السلالات في تعزيز قدرتها على التحمل والنشاط المضاد للأكسدة والمضاد للميكروبات، مما انعكس إيجاباً على نتائج التجارب الحيوانية.أما التغليف بالألجينات الصوديومية فقد حسّن من بقاء الخلايا الحية (>90%) في ظروف تحاكي الجهاز الهضمي، حيث سمح بتحرر متحكم فيه داخل الأمعاء، مما زاد من الاستقرار والفعالية الحيوية. تُظهر هذه النتائج أن بكتيريا حمض اللاكتيك المعزولة من بيئات غنية بالفينولات تمتلك دوراً مزدوجاً كمكملات بروبيوتيكية ومواد حافظة حيوية، إذ تُحسّن من صحة الأمعاء، والثبات التأكسدي، وسلامة اللحوم. وتشجع هذه النتائج على استخدامها كإضافات طبيعية متعددة الوظائف لإنتاج دواجن مستدام وذي وسم نظيف(Clean Label) ، كبديل بيئي وآمن عن المضادات الحيوية والمواد الحافظة الصناعية.

الكلمات المفتاحية: بكتيريا حمض اللاكتيك، بروبيوتيك، دجاج اللحم، أداء النمو، جودة اللحوم، مخلفات الشاي الأخضر المخمر، دهان، التكيف الفينولي، الأصل البيئي، الثبات التأكسدي، النشاط المضاد للميكروبات، الحفظ الحيوي، التغليف.

TABLE DES MATIÈRES

ABBREVIATIONS

ALS	α-acetolactate synthase
AGPs	antibiotic growth promoters
APFs	aggregation-promoting factors
ATS	acid-tolerance response
BATH	bacterial adhesion to hydrocarbons
BSH	bile-salt hydrolase
CFS	cell-free supernatant
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
DNase	Deoxyribonuclease
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl-β-Picrylhydrazyl
EFSA	European Food Safety Authority
EPS	Exopolysaccharides
FAO	Food and Agriculture Organization
GRAS	Generally Recognized As Safe
IPA	International Probiotics Association
ISAPP	International Scientific Association for Probiotics and Prebiotics
LA	Lactic Acid
LAB	Lactic acid bacteria
Lb.	*Lactobacillus*
Lcb.	*Lacticaseibacillus*
Ln.	*Leuconostoc*
Lpb.	*Lactiplantibacillus*
Lmb.	*Limosilactobacillus*
Lev.	*Levilactobacillus*
MDA	malondialdehyde
MRS	de Man, Rogosa and Sharpe
MUFA	Monounsaturated Fatty Acid
NADH	β-Nicotinamide adenine dinucleotide
OD_{600}	Optical density at 600 nm
ON	overnight
PBS	Phosphate buffered saline
PCs	Phenolic compounds
PPARα	peroxisome proliferator-activated receptor alpha
PTFE	Polytétrafluoroéthylèn
PUFA	polyunsaturated fatty acids
QPS	Qualified Presumption of Safety
RNA	Ribonucleic acid
ROS	reactive oxygen species
SCFAs	short-chain fatty acids
SFA	saturated fatty acids
SGJ	Simulated gastric-juice
TBARS	thiobarbituric acid reactive substances
TSB	tryptone soy broth
WHO	World Health Organization
w/v	Weight/volume

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GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Recent industry priorities have shifted from maximizing production yields to targeted quality improvements (Mir et al., 2017) Consumer perception hinges on multisensory evaluation, colour, texture (firmness, tenderness), moisture (juiciness), and flavour, at both purchase and consumption stages (Yue et al., 2024).

Maintaining the quality of meat products and guaranteeing food safety present significant problems for the global meat business. Because of their high water activity and rich nutrient composition, fresh and processed meat products are particularly susceptible to oxidative rancidity, microbiological spoiling, and quality degradation with chemical residues, and harmful compounds such as nitrites, N-nitrosamines, and heavy metals. These risks can arise at any stage from production to processing and distribution, and are heightened by global trade and changing consumer preferences for minimally processed foods (Gagaoua & Picard, 2020; Gomez et al., 2020).

To prevent spoiling and improve product stability, the industry has historically placed a great deal of reliance on artificial additives including nitrates, nitrites, sulfites, and synthetic antioxidants (Horbanczuk et al., 2019; Kalogianni et al., 2020), these additives pose significant health risks, prompting increased regulatory scrutiny and scientific investigation. When exposed to the stomach's particularly acidic environment, nitrites are converted into nitrous acid, which can then combine with amines to generate nitrosamines, compounds known to have the capability to promote malignancy and genotoxicity. Numerous nitrosamines present in processed meats and other foods have raised serious public health concerns given their capacity to cause DNA damage and cancer, as reported by the European Food Safety Authority (EFSA) (Deveci & Tek, 2024; Eisenbrand et al., 2024; Song et al., 2015).

Similar to this, sulfites (which are frequently employed as antioxidants and preservatives) have been associated with a number of adverse reactions, particularly among sensitive people. These adverse reactions include a spectrum of clinical manifestations, covering respiratory complications (that go from mild asthma to life-threatening anaphylaxis), gastrointestinal disturbances, and cutaneous manifestations such as dermatitis and urticaria. Sulfite sensitivity affects an estimated 3-10% of asthmatic individuals, with pathogenic mechanisms involving both IgE-mediated hypersensitivity and non-immunologic (pseudo-allergic) pathways. Beyond hypersensitivity, EFSA has also

expressed concerns about potential neurotoxic effects linked to sulfite exposure, underscoring the need for further investigation into their safety profile (Jarmakiewicz-Czaja et al., 2022; Witkowski et al., 2022; Younes et al., 2022).

However, increasing evidence indicates that some artificial chemicals could represent health hazards, notably carcinogenicity and allergic reactions, resulting in regulatory investigation and consumer rejection (Forgione et al., 2024; Guzik et al., 2022). As a result, the meat sector is under tremendous pressure to develop sustainable, consumer-friendly, and efficient alternative for artificial preservatives that can guarantee product safety without having a negative impact on health.

Natural preservation methods are becoming increasingly popular as a result of growing consumer awareness and a desire for healthier and more natural food products. Buyers are showing a clear preference for "clean-label" products and are asking for more transparency about food ingredients and production processes (Guzik et al., 2022). Clean-label products inform customers about their natural ingredients and reduce or eliminate synthetic additives. In addition, natural preservation approaches support larger sustainability goals by decreasing the negative impact on the environment of the manufacture of chemical compounds (Cheng et al., 2024). In order to satisfy consumer preferences and legal requirements, researchers need to investigate and validate natural preservatives and biopreservation techniques (Karbowiak et al., 2023). Practically speaking, authors more frequently define "biopreservation" as the employment of microbes and/or their metabolites to increase food safety and shelf life (Barcenilla et al., 2022).

The process of biopreservation uses primary and secondary metabolites or antimicrobial compounds from microorganism, plants, animals and fungus, to prolong the shelf life of products, reduce lipid oxidation, and prevent colour degradation (Ockerman & Basu, 2014). Accordingly to this viewpoint, biopreservatives are compounds derived from natural sources or food-based ingredients and have the capacity to both combat foodborne pathogens and inhibit or delay spoiling caused by chemical or biological deterioration (Karbowiak et al., 2023). In response, Researchers and industry stakeholders are therefore exploring natural alternatives such as Lactic acid bacteria (LAB) and phenolic compounds (PCs) as biopreservatives (Eglite et al., 2023; Munekata et al., 2020; Zhang et al., 2020).

The nutritional competition, production of organic acids, bacteriocins, and other antimicrobial metabolites by LAB, which are recognised for their safe and beneficial role in food fermentation, substantially enhances product safety and extends shelf life (Abedin et al., 2024; Carneiro et al., 2024). The U.S. Food and Drug Administration (FDA) has classified most lactic acid LAB and their

metabolic products as "Generally Recognised As Safe" (GRAS). Additionally, many species have been given the status of "Qualified Presumption of Safety" (QPS) by EFSA (Additives et al., 2018; Binda et al., 2020). On the other hand PCs are natural antimicrobials and antioxidants. They are effective and can help to maintain the quality of meat. These compounds originally come from plant sources (Albuquerque et al., 2021; Kalogianni et al., 2020).

The optimum biopreservative for meat products must demonstrate selective antibacterial activity against foodborne pathogens while maintaining the integrity and equilibrium of the human intestinal microbiota (Karbowiak et al., 2023). Meat quality can be maintained through the utilisation of PCs and LAB separately, which has demonstrated promise in reducing pathogenic organisms and spoiling. However, current research spotlight their complementary qualities more and more, revealing considerable synergistic potential when combined resulting in enhanced efficacy compared to their individual application (de Souza et al., 2019; Kaveh et al., 2023), and creates a more robust preservation approach by preventing the resurgence of unwanted microbes and expanding the range of antibacterial action, while also providing additional health benefits such as antioxidant and anticancer properties (Amiri et al., 2021; Barcenilla et al., 2022; Karbowiak et al., 2023).

It is hypothesised that these combinations may represent a promising route towards safer, more natural, and consumer-acceptable meat products, with the ability to equal or even exceed the efficacy of synthetic preservatives (El-Saber Batiha et al., 2021; Karbowiak et al., 2023). In response to consumer expectations this combination application promises boosted preservation efficacy, improved sensory qualities, and health-promoting benefits.

This thesis aims to investigate the combined effects of phenolic compounds and lactic acid bacteria on poultry meat quality, with a particular focus on technological, nutritional, and sensory attributes. It explores the individual and synergistic mechanisms by which phenolic compounds and LAB influence oxidative stability, microbial safety, and overall meat preservation. Through the isolation, probiotic's characterization, and *in vitro* evaluation of selected LAB strains in combination with phenolic-rich sources, the work addresses the potential of natural, multifunctional additives to enhance product quality while aligning with current consumer and regulatory demands for clean-label food products.

This work is divided into several interrelated chapters. Following this general introduction, Chapter 2 discusses the fundamentals of poultry meat quality, detailing technological, sensory, and nutritional determinants. Chapter 3 explores the contributions of lactic acid bacteria to meat quality,

focusing on their biopreservative and functional roles. Chapter 4 examines the properties and applications of phenolic compounds in meat systems. Chapter 5 presents the synergistic interactions between LAB and phenolic compounds, emphasizing their potential to improve meat quality and safety. The subsequent sections detail the materials & methods employed, followed by a comprehensive presentation and discussion of results. Finally, conclusions and future research directions are outlined.

LITERATURE REVIEW

2. DETERMINANTS OF BROILERS MEAT QUALITY:

2.1.Overview

Poultry meat quality is a multifaceted construct that combines technological, sensory, and nutritional characteristics (Gagaoua & Picard, 2020), many variables have a substantial impact on its quality, including the animals' food, processing methods, and preservation strategies (Rodrigues et al., 2024). While it is possible to enhance both the nutritional quality of meat, such as increasing beneficial fatty acids or micronutrients, and the overall performance of the animal, these improvements may sometimes be associated with trade-offs in sensory attributes like flavour, tenderness, or juiciness, depending on the feeding strategy, antioxidant protection, and post-harvest processing (Ellies-Oury et al., 2016). Furthermore, diverse processing techniques, and various cooking methods, constitute a cornerstone in shaping both the sensory and nutritional properties of meat (Gomez et al., 2020). Combining diverse processing and preservation procedures can lead to better sensory outcomes (Figure 1), but care must be taken not to compromise nutritional value (Gomez et al., 2020). For optimal meat production and to meet consumer expectations, it is vital to harmonize poultry performance, nutritional composition, and organoleptic properties (Ellies-Oury et al., 2016; Rodrigues et al., 2024).

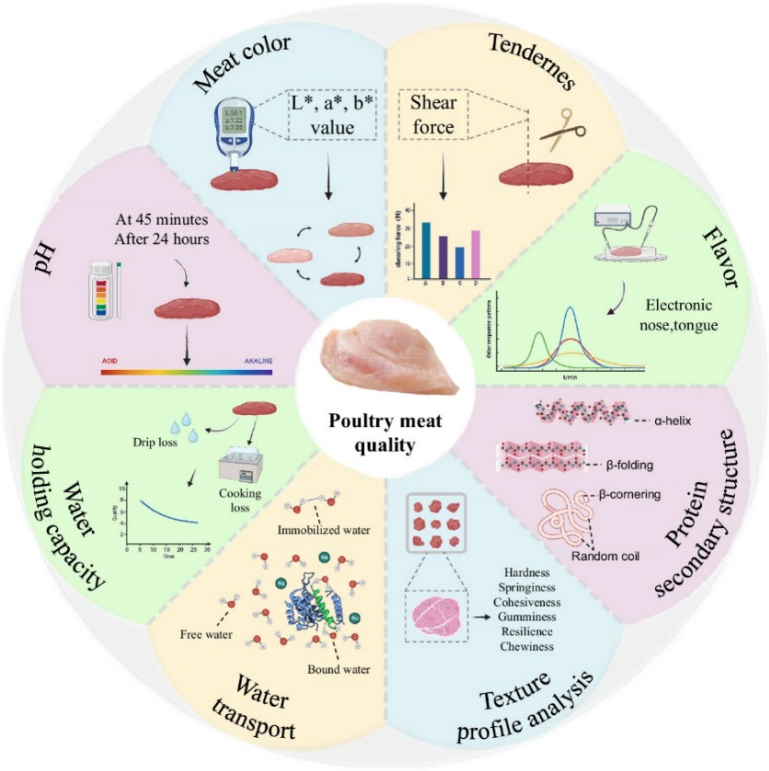


Figure 1 Structural and functional attributes of poultry meat quality (Yue et al., 2024)

2.2.Determinants of broilers meat quality

The health impacts of meat consumption are primarily governed by its macro and micronutrients composition. The Essential constituents such as proteins, fats, vitamins, and minerals are indispensable for improving its nutritional and health values. (Ehsanur Rahman et al., 2023; Ellies-Oury et al., 2016).

2.2.1. Nutritional quality

Poultry feed's nutritional profile has significant effects on meat quality parameters. Metabolic efficiency increases while significant meat characteristics such as fatty acid composition, protein content, and micronutrient density are improved by optimal dietary formulation, which is defined by balanced vital nutrients (Jha et al., 2020).

Proteins: Proteins are the primary macronutrients in meat, providing essential amino acids. Their quality and quantity can be influenced by determinants like the animal's diet, age, and breed. Grass-fed animals generally have further omega-3 fatty acids than those fed with grains (Gagaoua & Picard, 2020). A well-documented negative correlation exists between dietary protein levels and adipose tissue deposition in poultry. This phenomenon occurs because protein metabolism requires greater energy expenditure for deamination and gluconeogenesis, thereby limiting the energy available for lipogenesis (Liebert, 2017; Novodworski et al., 2023).

Fats: Fats are important because they improve the taste and aroma of food and at the same time provide energy. There are different kinds of fat, and not all are identical in terms of health effects. Saturated fat is often associated with health issues, particularly with heart disease. Monounsaturated and polyunsaturated fats, on the other hand, are beneficial to health. These healthier fats are linked to significant reductions in LDL-C levels, thus contributing to a decreased risk of cardiovascular diseases. Dietary sources rich in beneficial fats, such as olive oil, nuts, and fish, are commonly recommended as part of a heart-healthy die (Gagaoua & Picard, 2020).

Minerals and vitamins: Meat is rich in B vitamins for example thiamine, niacin, riboflavin and B12, it also provides important minerals like iron, zinc, and selenium. These nutrients play vital roles in metabolism, immune function, and oxygen transport (Rodrigues et al., 2024).

While the nutritional profile of meat constitutes the basis of its health benefits, it equally exerts a significant influence on its sensory qualities, such as flavour, texture, and overall palatability. For instance, the fatty acid profile influenced by animal diet not only determines nutritional benefits like omega-3 content but also directly impacts flavour, tenderness, and aroma (Gagaoua & Picard,

2020; Park & Choi, 2025). This interdependence underscores the need to harmonize nutritional and sensory properties, as optimizing one dimension often necessitates balancing the other.

2.2.2. Sensory quality (flavour, texture, appearance, aroma)

Also known as organoleptic quality, involves several key aspects: taste, texture, appearance, and aroma. These elements are crucial because they play a major role in shaping how consumers perceive a product and whether they find it acceptable or not. Each of these attributes can influence the consumer's impression and satisfaction, making them important considerations in product development and marketing.

Flavour: cooking meat changes its flavour due to the Maillard reaction; this process forms various volatile compounds that enhance the meat's contributing to the characteristic "poultry" flavour (Bleicher et al., 2022; Mir et al., 2017). Additionally, lipid oxidation and the degradation of thiamine and amino acids further shape flavour profiles (Fu et al., 2022; Xu & Yin, 2024)

The diet of the animal also affects flavour; grass-fed meat often has a distinct taste when compared to the meat from animals that are fed grain (Park & Choi, 2025). Broilers fed diets rich in polyunsaturated fatty acids (PUFAs), such as fish oil, develop distinct flavours, though excess ω-3 fatty acids may impart fishy off-notes unless stabilized with antioxidants (e.g., vitamin E) (Mir et al., 2017; Untea et al., 2022). However, traditional breeds exhibit stronger flavours due to higher levels of inosine-5'-monophosphate and unique lipid profiles, compared to commercial broilers (Mussa et al., 2022; Xiao et al., 2021). Also, prolonged cooking (roasting, frying) at high temperatures (>100°C) intensifies flavour through the formation of heterocyclic compounds, while boiling leaches water-soluble flavour precursors, producing different flavor profiles and fatty acid compositions in meat (Yu et al., 2021).

Texture, particularly tenderness, is a critical quality attribute influenced by multiple factors, including the animal's age, breed, muscle fiber type, and post-mortem handling. Younger broilers (5-7 weeks old) typically have finer muscle fibres and less collagen cross-linking compared to mature birds, which results in more tender meat (Weng et al., 2022). At the molecular level, tenderness is affected by glycolysis, calcium release, protease activation, apoptosis, and heat shock proteins (Warner et al., 2022). The type of muscle also plays a key role, with breast meat (pectoralis major) being naturally more tender than thigh meat because of its fast-twitch glycolytic fibers, while leg muscles contain both glycolytic and oxidative fibers (Huo et al., 2022).

Genetics further contribute to texture, as tenderness has moderate heritability, allowing for genetic selection (Mir et al., 2017). However, intensive selection for rapid growth and increased breast meat

yield has led to myopathies like Wooden Breast, characterized by inflammation, necrosis, and fibrosis in the pectoralis major muscle (Velleman, 2020). Said Dahmouni et al. (2025) showed that early heat conditioning and genetic thermotolerance in broilers greatly improved the fatty acid profile, mineral content, and oxidative stability of breast meat.

Modulating softness is also greatly influenced by cooking methods; long roasting and sous-vide cooking at 60–65°C, for example, help break down collagen into gelatin, which promotes tenderness and juiciness, while excessive heat (>75°C) can cause dehydration and toughening (Noh et al., 2023). Additionally, marinating with organic acids or proteolytic enzymes might improve palatability by softening muscle structure. All things considered, the intricate interaction between intrinsic and extrinsic elements underlines how important it is to optimise production and processing methods in order to preserve the desired texture of meat (Kopuzlu et al., 2018; Xie & Grossmann, 2025).

Appearance: is widely regarded as the primary quality attribute of poultry meat (both raw and cooked), as consumers directly associate visual characteristics with freshness and make purchasing decisions based on perceived attractiveness. The unique market presentation of poultry (sold either skin-on or skinless) further emphasizes the importance of appearance. Regional preferences also influence consumer acceptance: for example, U.S. markets show greater tolerance for variations in skin pigmentation (ranging from pale to deep yellow), while UK consumers predominantly favor non-pigmented, white skin (Altmann et al., 2023; Indrawan et al., 2021)

Poultry meat pigmentation is determined by multiple factors, including dietary lipid-soluble pigments (carotenoids, xanthophylls), feed composition, additives, and management practices (breed, health, processing). Genetic differences affect pigment deposition, with some breeds producing white meat regardless of diet, while processing factors like scalding temperature (>54°C) can bleach pigments. Additionally, heme pigments (myoglobin, hemoglobin) influence meat colour, though poultry contains less myoglobin than other species. Age, stress, and slaughter conditions further impact colour, with turkeys showing greater sensitivity to pre-slaughter stress than chickens.

Meat colour is also governed by the state of heme pigments, pre-slaughter conditions (genetics, feed, stress), and processing methods (stunning, chilling, pH). The oxidation state and myoglobin concentration influence the colour of meat. Fresh meat usually has a bright red colouration, however as it is stored, the oxidation of lipids can cause it to turn brown or grey (Ramanathan et al., 2020; W. Zhu et al., 2024). Furthermore protein denaturation, influenced by postmortem pH and temperature, affects light scattering, altering meat lightness (L*). Muscles with pH ≥6.0 appear

translucent due to minimal denaturation, while those with pH ≤6.0 become opaque from increased light scattering. These interactions between biochemical and physical factors ultimately define poultry meat's visual quality.

Aroma: Volatile chemicals (VOCs) produced during cooking, like thiamine breakdown, Maillard reactions, lipid oxidation, and microbial metabolism, are what give food its distinct aroma. In general, the VOC profile of meat product is directly modulated by dietary regimens and the conditions under which it is processed (Bleicher et al., 2022).

2.3.Current preservation and processing methods

The preservation of the quality includes various methods to control oxidation, microbial growth and deterioration of the quality. These approaches are generally divided into the additives use and advanced processing methods.

2.3.1. Use of synthetic vs. natural additives

Synthetic additives: synthetic phenolic antioxidants, notably butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are extensively applied to stop lipid oxidation and extend shelf life (Horbanczuk et al., 2019). Nevertheless, accelerating consumer shift toward clean-label products has stimulated research into natural alternatives, including plant-derived PCs from sources such as rosemary, green tea, and grape seed extracts, which demonstrate comparable efficacy while aligning with contemporary health trends (Cheng et al., 2024; Cunha et al., 2018; Martinez-Zamora et al., 2021).

Natural additives: Marinades containing acids, oils, and spices improve flavour, juiciness, and tenderness. Enzymatic tenderizers such as bromelain and papain breakdown connective tissue, improving texture (Botinestean et al., 2018; Ehsanur Rahman et al., 2023). Natural compounds, including PCs derived from plant extracts and LAB, offer a clean-label solution. Phenolic compounds not only reduce oxidative degradation but also contribute to enhance the flavouring. LAB, through fermentation, lowers the pH and produces antimicrobial agents such as bacteriocin. The combination of these natural additives is gaining acceptance for their capacity to enhance the meat quality while addressing the health concerns of consumers.

2.3.2. Packaging technologies and post-mortem interventions

Modified atmosphere packaging (MAP) and vacuum packaging (VP) are essential to minimize oxidative and microbial degradation. MAP with high CO_2 concentrations (20–30 %) effectively suppress aerobic spoilage organisms and outperforms VP in maintaining colour and prolonging

shelf life (Wang et al., 2016). Emerging active packaging systems incorporate oxygen scavengers or antimicrobial films (chitosan-nanoparticle coating) and intelligent packaging uses freshness indicators in real time monitoring (Khodaei et al., 2023).

Post-mortem interventions: Interventions like electrical stimulation, ageing techniques such as dry and wet aging and non-thermal processing techniques like pulsed electric fields (PEF), and high-pressure processing (HPP) are used to enhance the freshness, taste and global quality of the product. Combined with natural additives these methods form a multi-layered approach which addresses both safety and sensory characteristics.

Electrical stimulation: This technique is applied post-slaughter to accelerate glycolysis, preventing cold shortening and improving tenderness through rapid pH drop (Gagaoua et al., 2025).

Aging techniques: Dry ageing at regulated temperature and humidity concentrates flavour by lipolysis and proteolysis, while wet ageing in vacuum-packed refrigerator increases softness while causing negligible weight loss (Kim et al., 2021).

2.4.Integrated approaches and emerging technologies

The next steps in quality preservation rely on the integration of new technologies with conventional methods. This method improves overall quality by utilising the advantages of both cutting-edge innovations and well-established techniques.

2.4.1. Multi-hurdle strategies

Several preservation procedures are applied simultaneously as part of multi-hurdle systems to prevent spoiling and preserve quality. Physical techniques like MAP or HPP are used with natural additives like PCs and LAB to raise global efficacy of minimising oxidation and microbial contamination (A. Ishaq et al., 2021; Peng et al., 2020; Wang et al., 2016).

These strategies provide:

- Synergistic effect, where each barrier compensates for potential weaknesses in another.
- Increased safety and extended shelf life without compromising sensory or nutritional attributes.
- Formulation flexibility that enables customised solutions depending on the type of meat and particular processing circumstances.

Lactic acid bacteria

LAB strains, such as *Lactobacillus*, *Pediococcus*, are employed as biopreservatives to inhibit pathogens like *L. monocytogenes*, their metabolic activity also stabilizes colour via pH reduction

and mitigates lipid oxidation through antioxidant enzyme secretion (Barcenilla et al., 2022; Lahiri et al., 2022). Dietary supplementation of broilers with *Lb. farciminis* and *Lb. rhamnosus* resulted in breast meat enriched with polyunsaturated fatty acids (PUFAs), for example omega-6 and omega-3, while demonstrating a reduction in saturated fatty acid (SFA) content (Figure 2) (Eglite et al., 2023).

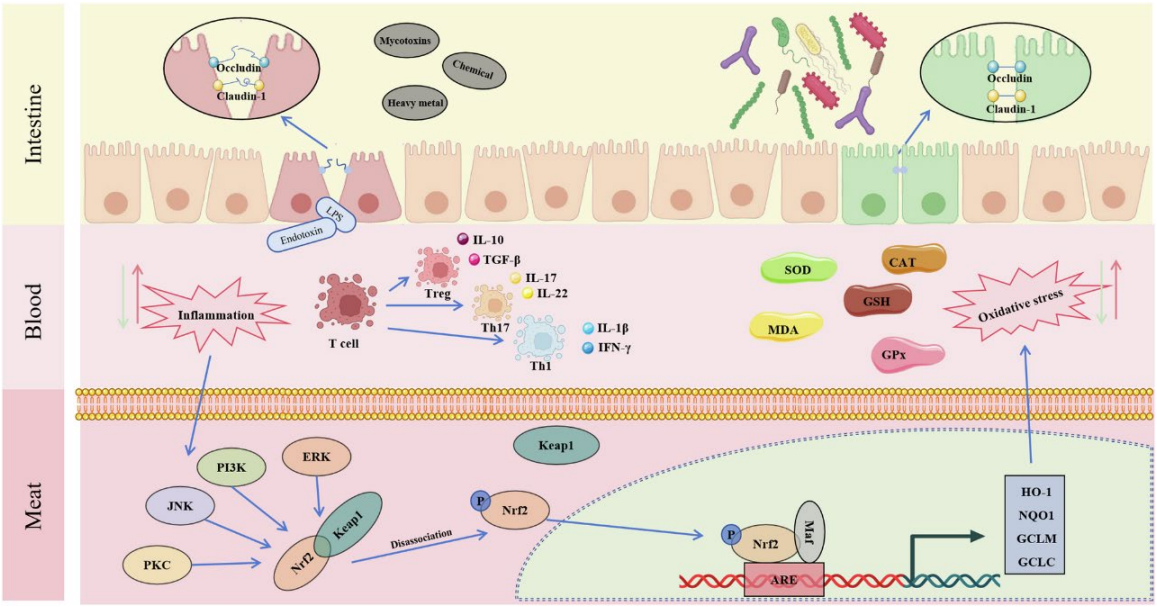


Figure 2 Mechanisms of probiotics improve chicken meat quality (Yue et al., 2024).

Non-thermal processing

High-Pressure Processing (HPP): This method deactivates microorganisms while maintaining sensory characteristics by applying pressure between 100 and 600 MPa (Dhal & Kar, 2025).

Pulsed Electric Fields (PEF): in this approach electroporation is used to break down microbial membranes and weaken the structure of myofibrils, that tenderises meat (Guo et al., 2024).

Edible coatings: Biopolymer-based coatings (e.g. alginate, chitosan) infused with antimicrobial agents (nisin, lysozyme) or antioxidants reduce surface contamination and lipid oxidation, extending freshness without synthetic additives (Rochima et al., 2025).

Advanced enzymatic strategies: Beyond traditional tenderizers, microbial proteases (e.g., *Bacillus subtilis* enzymes) and collagenolytic fungi are engineered to target specific connective tissues, offering accuracy in texture modification (Sorapukdee et al., 2024).

Genetic and precision farming Precision feeding systems use AI-driven analytics to maximise feed efficiency and meat composition, while emerging CRISPR-based gene editing targets features

like intramuscular fat deposition for marbling (such as FABP4 gene modification) (Abebe et al., 2024).

Dietary modifications:

Animal diets enriched with omega-3 sources (flaxseed, algae) or selenium/vitamin E improve oxidative stability and fatty acid profiles in meat (Rodrigues et al., 2024). Overall phytogenic additives (e.g. oregano essential oil) enhance both shelf life and antimicrobial properties (Abdelli et al., 2021; Starcevic et al., 2015).

2.5.Factors affecting nutritional quality

The nutritional quality of broilers meat is influenced by a blend of genetic, dietary, environmental, and technological aspects (**Erreur ! Source du renvoi introuvable.**). Tenderness, flavour, and nutritional value are all directly impacted by genetic features, which are also important in determining the composition of muscle fibre, intramuscular fat content, and nutrient density (Rodrigues et al., 2024). In the other hand breeding techniques that boost levels of marbling and PUFAs, can boost the cattle's sensory appeal and health advantages (Mir et al., 2017).

Feed composition and supplementation strategies are two nutrient parameters that have a major impact on meat quality by modifying vitamin levels, fatty acid profiles, and antioxidant content. (Bartkiene, Bartkevics, et al., 2019), environmental factors also affect muscle glycogen levels and overall meat quality, including stress management and animal welfare (Dong et al., 2024).

Dietary supplementation strategies have emerged as a critical tool for modulating poultry meat quality, with demonstrated efficacy across multiple physiological pathways (Abd El-Hack et al., 2020). The inclusion of vitamin E significantly improves oxidative stability in broiler meat products (Amevor et al., 2021) while concurrently upregulating matrix metalloproteinase (MMP) activity, which enhances intramuscular collagen solubility and promote lipid metabolism (Archile-Contreras et al., 2011). In parallel research has shown that vitamin D3 additions enhances water-holding capacity in poultry muscle through improved calcium homeostasis (Nong et al., 2023). Other notable supplements include calcium, which modulates lipid metabolism to reduce circulating cholesterol levels (Matuszewski et al., 2020), and selenium-enriched yeast, which effectively increases the selenium content of meat products (Markovic et al., 2018).

Poultry processing operations significantly influence final product quality through multiple critical control points. Key factors include slaughter methods, transportation conditions, storage parameters, and processing techniques (Jha et al., 2020). Meat texture and colour are preserved via handling and shipping procedures that minimise stress, avoiding flaws like pale, soft and exudative

(PSE) meat. Technological considerations are particularly crucial because non-thermal technologies like HPP and PEF, as well as processing techniques like VP and chilling, have a big impact on shelf life and nutrient retention (Dhal & Kar, 2025). Cutting-edge packaging options like MAP preserve sensory qualities while extending freshness even longer.

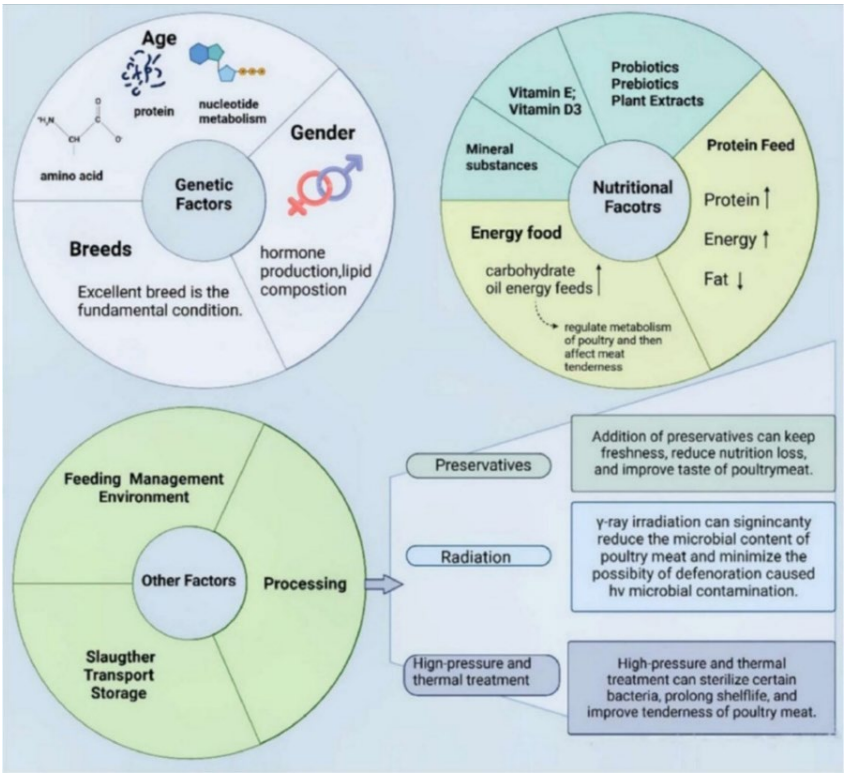


Figure 3 Factor affecting poultry meat quality (Dong et al., 2024).

2.6.Effects of probiotics supplementation on poultry meat quality

Probiotic supplementation in poultry feed has emerged as an effective strategy for enhancing both production parameters and meat quality characteristics. Current research demonstrates that probiotics, particularly when combined with prebiotics (synbiotics), improve broiler growth performance while simultaneously enhancing meat quality and carcass characteristics (Hussein et al., 2020). These benefits are mediated through multiple mechanisms, including modulation of gut microbiota, improvement of intestinal health, and reduction of pathological lesions associated with common poultry diseases like necrotic enteritis (Abd El-Hack et al., 2020; Dong et al., 2024; Yue et al., 2024).

The positive effects of probiotics extend to certain aspects of meat quality, indeed, according to Yibar and Uzabaci (2024), probiotics may have an impact on myoglobin stability and oxidative processes in muscle tissue, as evidenced by the notable improvements in meat colour indices.

Furthermore,, it has been demonstrated that probiotic additions improves the overall safety and quality criteria of meat, like texture, shelf life, and water holding capacity (Ebeid et al., 2021). These effects are likely attributable to probiotic-induced improvements in gut barrier function and systemic antioxidant status; they have pleiotropic advantages in chicken production systems that go beyond meat quality (Figure 4). As noted by Krysiak et al. (2021) and Yaqoob et al. (2022), in laying hens, probiotic supplementation has a positive impact on the parameters of egg production and meat quality.

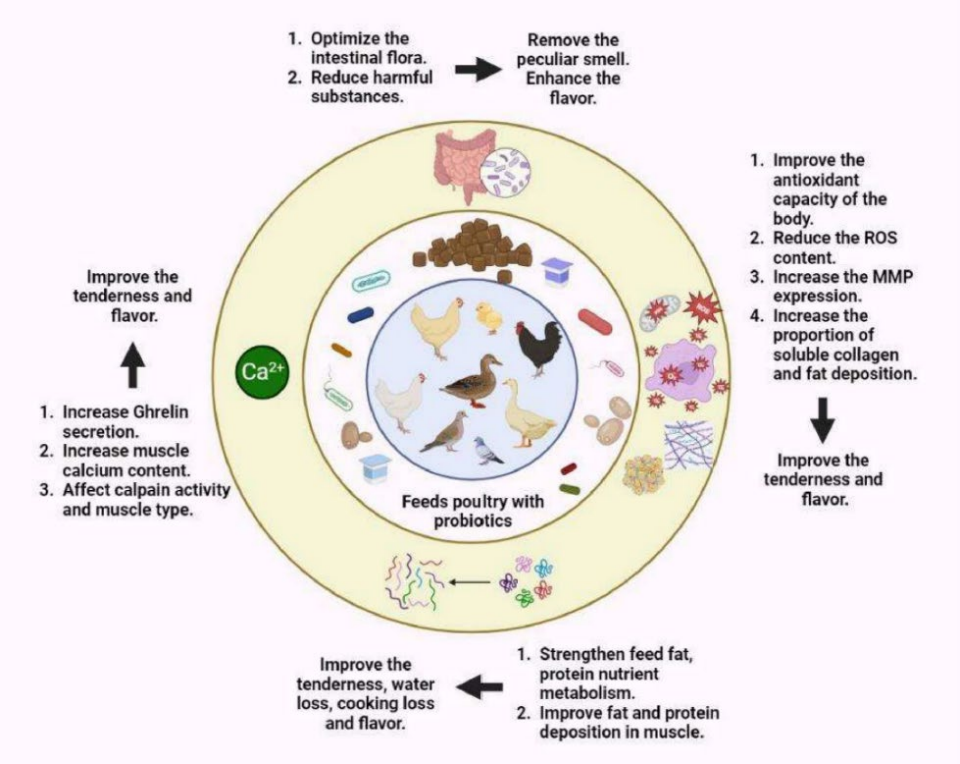


Figure 4 Effects of probiotic supplementation in poultry diets (Dong et al., 2024).

Abd El-Hack et al. (2020) conducted a thorough meta-analysis that provides additional insight into the complex ways that probiotics work, such as immune modulation, cellular homeostasis maintenance, and heat stress mitigation. These mechanisms all help to improve meat quality and safety while lowering the need for antimicrobial agents. Probiotics modulate important enzymatic systems, demonstrating an effective antioxidant capacity. Studies indicate that in poultry systems, probiotic feeding increases the activity of natural antioxidant enzymes such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Feng & Wang, 2020). This enzymatic upregulation effectively reduces reactive oxygen species (ROS) accumulation, thereby protecting phospholipid-rich muscle cell membranes from oxidative damage. The consequent preservation of

myoglobin redox stability results in measurable improvements in meat color stability and reduced drip loss during storage (Bai et al., 2016; Xiang et al., 2019).

Certain probiotic strains, particularly active *Saccharomyces cerevisiae*, may influence meat texture via endocrine-mediated pathways. Research indicates that supplementing with *S. cerevisiae* increases intramuscular calcium concentrations and circulating ghrelin and calcium levels (Chang et al., 2019; Nidamanuri et al., 2021). The activation of the calpain proteolytic system and possible modification of the composition of muscle fibre types are the two ways in which this calcium modulation affects meat tenderisation (Huang et al., 2017). These processes work together to provide quantifiable gains in poultry meat softness and other qualitative characteristics.

While probiotic supplementation in poultry feed demonstrates promising nutritional strategy for improving meat quality, their application in poultry nutrition requires rigorous regulatory oversight to ensure both hazard assessment and dose-response performance. In the United States for example, probiotics designed for feedstuffs require authorization by the FDA, this approval process involves a comprehensive assessment of probiotic strains, focusing on safety, intended use, and efficacy. Producers must provide detailed documentation outlining production processes, strain-specific characteristics, and quality assurance protocols. International regulatory frameworks may vary, but they all strive to confirm the beneficial impacts of probiotics on the health, growth, and meat quality of chicken. Usually, these frameworks incorporate extensive safety assessments to reduce any possible hazards to human health and animal welfare (Yue et al., 2024).

2.7.Challenges, limitations, and future research directions

The implementation of innovative preservation methods is still hindered by issues including customer acceptance, scalability, regulatory compliance, and economic viability, even with notable breakthroughs. Proactive regulatory involvement, smart industrial alliances, and interdisciplinary research are necessary to address issues. Future research should concentrate on improving integrated conservation strategies, consumer perceptions, safety and efficacy assessments, and economic feasibility (Bartkiene, Bartkevics, et al., 2019; Kumar et al., 2017; Yue et al., 2024). For implementation and consumer uptake to be effective, cooperation between academic institutions, business, and government organisations is crucial.

3. LACTIC ACID BACTERIA: FUNCTIONALITY, AND APPLICATIONS

3.1.Overview and classification

Lactic acid bacteria is a heterogeneous cluster of Gram-positive microorganisms characterized by low guanine-cytosine (GC) content, classified within the *Firmicutes* phylum (Bintsis, 2018). Their capacity to generate lactic acid (LA) with other beneficial secondary metabolites makes them essential for fermenting, preserving, and improving the quality of foods (Sauer & Han, 2021), They are greatly spread in nature and prosper in nutrient rich environments like fermented meats, vegetables, dairy products, and both human and animal digestive tracts (Vinderola et al., 2024). Their metabolic versatility, adaptability to environmental stresses, and safety profile have made them indispensable in both industrial and health area. Given that the majority of LAB species are GRAS or QPS for use in food; they are the most significant microorganisms used in the food industry and in applications pertaining to health (Agagunduz et al., 2021; George et al., 2018; Hazards et al., 2023; Martin et al., 2019; Ruiz-Rodríguez et al., 2017).

3.1.1. Taxonomy and phylogenetic revisions

Historically, the classification of this group of microorganisms has been based on criteria set by Orla-Jensen, which include cell morphology, glucose fermentation patterns, growth temperature ranges and sugar utilization profiles (Ruiz-Rodríguez et al., 2017). Despite the continued relevance of these traditional criteria, the advent of molecular biology techniques has had a significant impact on the taxonomy of LAB, resulting in the recognition of a substantially larger number of genera beyond the original four (*Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus*) (Vinderola et al., 2024). This group of bacteria is presently being classified under the phylum *Firmicutes*, the class *Bacilli*, and the order *Lactobacillales*. The integration of phylogenomic and genomic research has led to significant taxonomic modifications such as the reorganisation of the *Lactobacillus* genus proposed by J. Zheng et al. (2020) into 23 new genera, including *Lacticaseibacillus*, *Lactiplantibacillus*, and *Limosilactobacillus* (Figure 5). A new genus, *Periweissella*, has been established by current phylogenomic research, inside the Leuconostocaceae family (Bello et al., 2022).

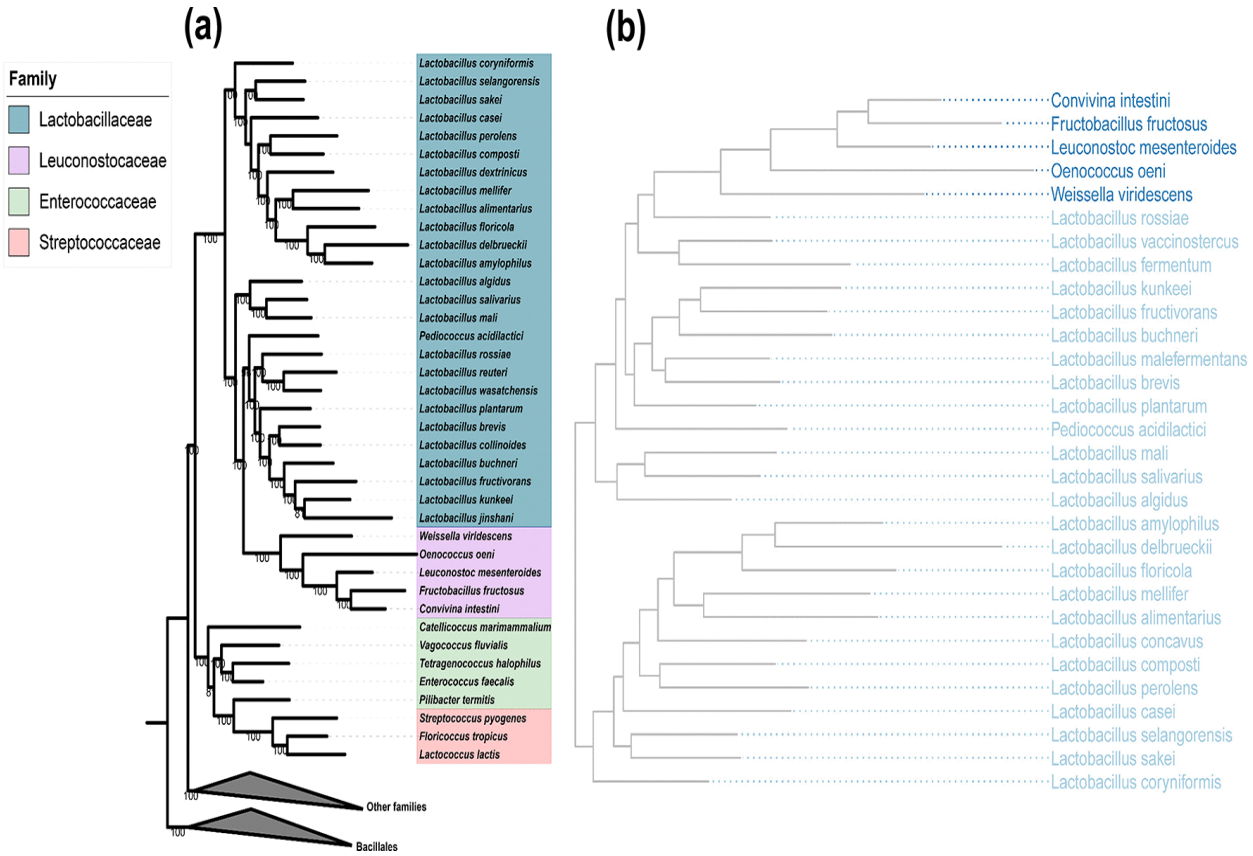


Figure 5 Phylogenetic reconstruction of *Lactobacillales*.

(a) An evolutionary tree derived from the core genome that includes 31 type strains from other genera in the order *Lactobacillales* and type strains representing 25 phylogenetic groupings inside the genus *Lactobacillus*. (b) Phylogenetic tree revealing the relationships across phylogroups in the *Lactobacillaceae* and *Leuconostocaceae* families (J. Zheng et al., 2020).

Whole-genome sequencing and core protein studies, which provide a clearer image of LAB evolution and phylogeny, establish the foundation in these taxonomy modifications. LAB's general evolutionary framework is coherent despite the fact that many phylogenetic trees were developed using different gene markers (Bello et al., 2022; Wels et al., 2019; J. Zheng et al., 2020). As research continues to reveal the genetic diversity and functional capabilities of LAB, it is essential to fully understand their taxonomy and evolutionary relationships in order to fully utilise their potential in the food technology and healthcare industries and to leverage on their positive qualities (Abedin et al., 2024; Ayed et al., 2024).

3.1.2. Key genera

The principal genera of LAB are summarised in the Table 1, with emphasis on exemplary species and distinctive traits:

Table 1 Important genera of lactic acid bacteria

Genus	Representative Species	Key Characteristics & Applications
Lacticaseibacillus	*Lcb. Casei*	plant-based and dairy fermentations; probiotic properties.
Lactiplantibacillus	*Lpb. plantarum*	probiotic traits high adaptability to diverse environments.
Latilactobacillus	*Ltb. Sakei*	meat fermentation and preservation.
Limosilactobacillus	*Lmb. fermentum*	exopolysaccharides, enhancing texture and biofilm formation.
Streptococcus	*St.thermophilus* *St.pyogenes (pathogenic)*	yogurt production; includes both beneficial and pathogenic species.
Leuconostoc	*Ln. mesenteroides*	Ferments vegetables; produces bacteriocins and dextrans affecting texture and safety.
Pediococcus	*Pc. pentosaceus*	Cocci-shaped forming tetrads/pairs; enhance flavour and antibacterial properties.

3.1.3. Genetic and genomic insights

Recent changes to taxonomy names have practical consequences for a range of companies, including dairy farming (Oberg et al., 2022). To facilitating efficient communication between academics, medical practitioners, and industrial stakeholders, a consistent nomenclature system guarantees that species debates are harmonized and clear. As part of the improvement of the LAB classification, particularly as a result of recent taxonomic updates, the consequences go beyond academic debate and also involve commercial and legal challenges. For example, applications for patents, ingredient labelling, export and import certifications, consumer awareness, and marketing strategies are all significantly impacted with changes in LAB taxonomy. (Pot et al., 2019).

Healthcare, research, and industry stakeholders consider challenging to adapt to these changes; for example, clinical laboratories have to amend their reporting procedures to conform to the new classifications. These modifications could impact antimicrobial control practices and infection prevention protocols (Prinzi & Moore, 2023). In order to facilitate this move, projects like the Lactic Acid Bacteria Industrial Platform (LABIP) have developed easily accessible resources, such websites and mobile applications, to assist in the implementation of revised nomenclature (Pot et al., 2019). Another excellent tool at http://lactobacillus.ualberta.ca/ allows users submit the names for ancient *Lactobacillus* species to get their most current classifications and complete descriptions. (Oberg et al., 2022; Pot et al., 2019).

The genetic components that give LAB its functional traits have been extensively identified by the molecular biology studies, enabling by the way accurate genetic modification, these innovations provide versatile strains that fulfil the specific manufacturing demands of the industry, introducing novel opportunities for enhancing applications in the industrial and medicinal (Kim et al., 2020; McAuliffe, 2018; Wels et al., 2019; Wu et al., 2021; Xie et al., 2024).

Both of chromosomal and plasmid-encoded genes constitute the genetic framework of LAB; plasmids usually contain genes necessary for the synthesis of bacteriocin, lactose metabolism, and proteinase, by consequence these genetic elements are crucial for thier roles in dairy fermentation and diverse industrial applications (George et al., 2018; Iskandar et al., 2019; Liu et al., 2019; Vinderola et al., 2024; Xie et al., 2024). A comparative genomic analysis shows that the LAB's genomes were impacted by both gene acquisition and loss, which enabled the bacteria to adapt to various environments and optimise their metabolic activity in specific conditions (Bron et al., 2019; Okoye et al., 2022), by consequence , strains associated with dairy habitats involve genes for lactose metabolism and the proteolytic system, while thus found in plant-based niches have usually genes for the breakdown of pectin and cellulose (McAuliffe, 2018; Wels et al., 2019).

A number of studies have in fact identified niche-specific genes and evolutionary connections between plant and dairy isolates, highlighting the adaptability of *Lc. lactis* starin. Similarly, *Lpb. plantarum* can modulate the transcriptome to adapt in a range of conditions by regulating genes linked to the uptake of carbohydrates and the breakdown of amino acids (Filannino, De Angelis, et al., 2018; Landete et al., 2021). Both resilience in challenging conditions and metabolic specialisation are also linked to this genetic heterogeneity. Strain-specific characteristics that boost resistance to enviromental stress while permitting the release of a wide variety of bioactive metabolites, enhance LAB's ability to survive in their particular environments as well as to perform better in probiotic formulations and food fermentation (Okoye et al., 2022; Papadimitriou et al., 2016; J. Zhu et al., 2024). Moreover, there is a lot of promise for optimising these traits to satisfy particular industrial needs thanks to the capacity to modify LAB genomes (O'Donnell et al., 2019; Vinderola et al., 2024; H. Yang et al., 2024).

3.2. Physiological and morphological traits

The cell of LAB exhibit either rod-shaped or cocci-shaped morphology and are generally non-sporulating and non-motile. This structural simplicity contributes to their adaptability in diverse environments (Bintsis, 2018). Although LAB are typically classified as aerotolerant anaerobes and do not produce catalase or cytochromes under standard conditions, some strains can engage in

aerobic respiration when supplied with heme or a combination of heme and menaquinone. This respiratory capability enhances their oxygen tolerance, promotes biomass production, and improves overall viability (Ruiz-Rodríguez et al., 2017). In the absence of catalase, they manage oxidative stress through alternative mechanisms such as metal ion chelation and scavenging of ROS (Feng & Wang, 2020).

3.2.1. Stress physiology and adaptation

During manufacture, storage, and administration, the LAB group are subjected to a variety of stressors, including exposure to bile, acids, heat, cold, and oxygen. For that, improving the stability and dependability of probiotic strains during industrial-scale manufacturing and commercialisation requires a thorough understanding of stress response mechanisms (Papadimitriou et al., 2016; Vinderola et al., 2024).

Environmental stresses

Many remarkable resistance strategies, like pH homeostasis balance, preserving cell membrane integrity, modulating metabolism, and biomolecular restoration, have been involved by these microbes to reduce the damage caused by acidic conditions, indeed stress adaptation can lead to homologous tolerance and cross-protection against various challenges (Gaucher et al., 2019; Moreno et al., 2024; Papadimitriou et al., 2016).

Adaptive mechanisms

Lactic acid bacteria use a variety of strategies, such as cell membrane modifications genetic adaptations, and cross-protection, that improve their resilience to stress. Cross-protection occurs when prior exposure to mild stress primes LAB for more severe challenges, such as intracellular amino acid accumulation and alterations in membrane dynamics (Yang et al., 2021). By upregulating ATP synthases, transporters, and stress-responsive proteins, genetic adaptations enhance resistance to osmotic and acidic environments and promote stress resilience (J. Zhu et al., 2024). Additionally, modifications in cell membranes, involving increased production of unsaturated fatty acids, increase membrane fluidity and lower proton permeability, resulting in better survival in acidic conditions (J. Zhu et al., 2024). In all, these coordinated mechanisms enable LAB cell to maintain cellular homeostasis in challenging environments.

3.3.Metabolic pathways in LAB

Generally carbohydrate fermentation in LAB group results in LA as the predominant metabolic end product (Vinderola et al., 2024), however, their ecological success and utility in a range of

biotechnological applications and probiotic compositions depend on this mechanism (Alvarez-Sieiro et al., 2016; Kleerebezem et al., 2017). The nutritional requirements are particularly complex and frequently need for growth hormones, other vitamins and exogenous amino acids, reflects their evolutionary specialization and symbiotic interactions within their habitats (Bintsis, 2018; Papadimitriou et al., 2016; Sauer & Han, 2021). The utilisation of different sources of carbon and the synthesis of important functional metabolites, including organic acids and antibacterial compounds, are supported through the extensive metabolic capability (Anumudu et al., 2024) and because they lack a full respiratory system, LAB must rely on substrate-level phosphorylation to supply their energy needs (Vinderola et al., 2024; Y. Wang et al., 2021). For this reason, the classification of LAB as either homofermentative or heterofermentative is based on the particular metabolic processes involved in sugar metabolism (Bintsis, 2018; Iskandar et al., 2019; Kim et al., 2020).

3.3.1. Homolactic fermentation

The Embden-Meyerhof-Parnas (EMP) route catabolises glucose during homolactic fermentation, releasing LA as the only significant byproduct (Figure 6.a). *Lactococcus* species and some *Lactiplantibacillus* species engage in this process, which is essential for the development of the spicy aroma and longer shelf life of fermented foods (Gänzle & Gobbetti, 2023).

3.3.2. Heterolactic fermentation

The heterolactic fermentation takes place through the phosphoketolase pathway and produces LA in addition to ethanol, acetic acid and CO_2 (Figure 6.b). Genera like *Weissella* and *Leuconostoc* for example use this metabolic path, which generates complex aromas and influences the product texture (Vinderola et al., 2024).

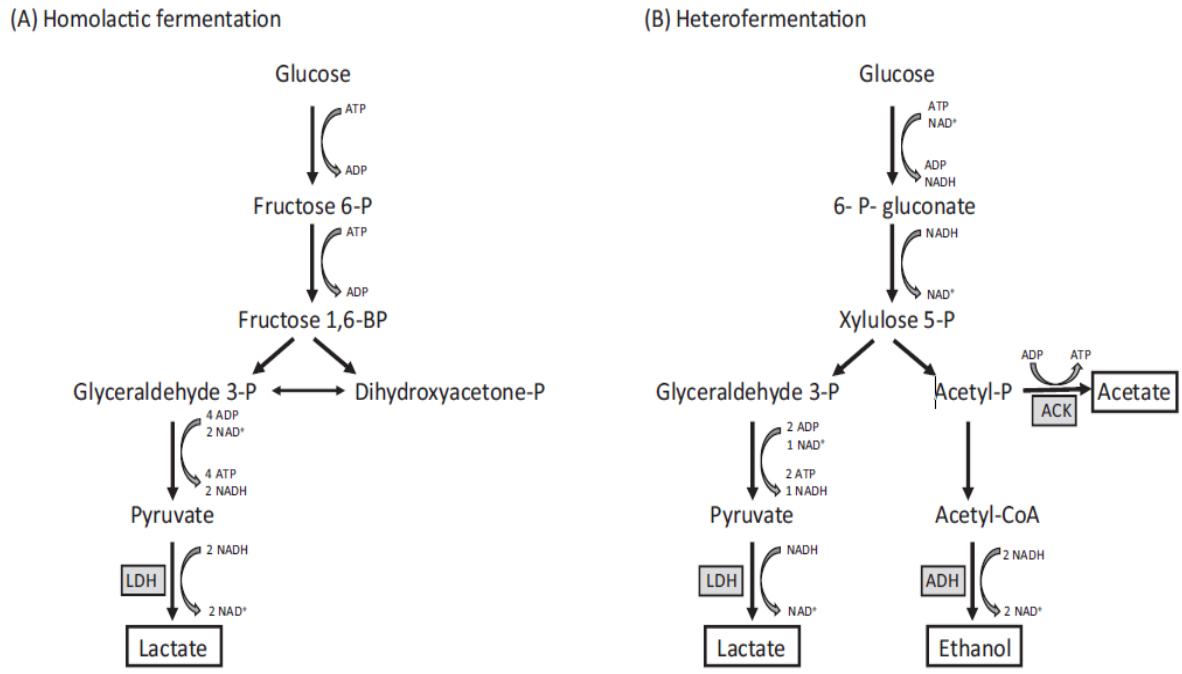


Figure 6 Carbohydrate metabolic pathways in LAB.

(A) homolactic fermentation and (B) heterofermentative metabolism. ACK (acetate kinase), ADH (alcohol dehydrogenase), BP (bisphosphate), LDH (lactate dehydrogenase), and P (phosphate) (Suissa et al., 2022).

3.3.3. Carbohydrate utilization and metabolic by-products

Hexose sugars such as mannose, galactose, and fructose are converted into glucose-6-phosphate or fructose-6-phosphate through sequential phosphorylation and isomerization reactions. Notably, galactose can be metabolized via two distinct biochemical pathways :**Tagatose-6-phosphate pathway** (via phosphoenolpyruvate-dependent phosphotransferase system or PEP:PTS) efficient in dairy environments (Vinderola et al., 2024). **Leloir pathway** (via permease) relevant in plant-based niches (Iskandar et al., 2019).

The final product's stability and organoleptic properties are greatly influenced by the metabolic pathway used during fermentation. These metabolic processes have a direct influence on the synthesis of organic acids, flavourings, and other metabolites, which together define the final product's sensory and functional qualities (Y. Wang, J. Han, et al., 2022). Genomic studies have shown that obligate heterofermentative species lack the 1-phosphofructokinase gene required for homofermentation, while they habitually contain the *araBAD* operon for L-arabinose degradation (Buron-Moles et al., 2019).

Homofermentative LAB generally have aldolase and phosphofructokinase, while heterofermentative species typically have two-domain mannitol dehydrogenase and alcohol

dehydrogenase (Vinderola et al., 2024); LAB genomes also contain various biosynthetic gene clusters for secondary metabolites including type III polyketide synthases with potential antimicrobial activity (Okoye et al., 2022).

Recent study by Lancetti et al. (2021) reported that *Pc. acidilactici* and *Ec. faecium,* a homofermentative strains, generate LA primarily, while *Lv. brevis* and *Lmb. fermentum* a heterofermentative strains, can synthetise a variety of metabolites, including LA, acetic acid, and volatiles components. This finding lead to a fact that the selection of strain can therefore have a significant impact on the sugar profile, with heterofermentative fermentations producing a greater variety of oligosaccharides and dextrins and homofermentative fermentations producing more monosaccharides. On the other hand, heterofermentative strains tend to produce a greater diversity of volatile compounds, which can influence the sensory properties of the final product (Liao et al., 2022; Pruckler et al., 2015; Tang et al., 2024).

Production of bioactive compound

In addition to their main use in fermentation processes, LAB are becoming more and more recognised for their capacity to synthesize a variety of bioactive molecules (Figure 7), exopolysaccharides (EPS), vitamins, organic acids, bioactive peptides, bacteriocins, and γ-aminobutyric acid (GABA) are a few examples. When combined, these metabolites have the ability to improve fermented food's characteristics, and shelf life while also providing significant health advantages. This bioactivity supports the application of LAB in the functional meals formulation that target modern health issues (Agagunduz et al., 2021; Ayed et al., 2024; Gao et al., 2021; Nasrollahzadeh et al., 2022; Rastogi et al., 2022; Werning et al., 2022).

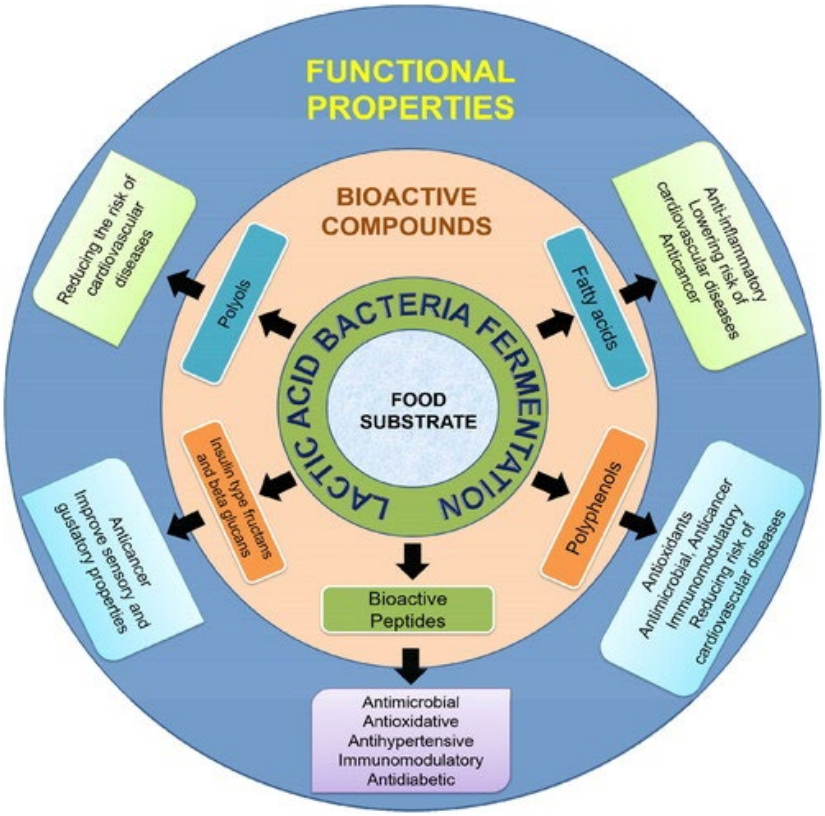


Figure 7 Physiological and functional impacts of LAB bioactive molecules (Abedin et al., 2024)

Short-chain fatty acid production: LAB produce variety of SCFAs like butyrate and propionate, which can modulate the host's immunological and metabolic systems. These SCFAs are known to reduce inflammation and may help prevent cancer by blocking harmful signals and promoting the apoptosis of cancer cells hence reduces the risk of developing particular tumour types (Bermudez-Humaran et al., 2024; Jacouton et al., 2018). In this particular case, a review by (Ayed et al., 2024) reported the fact that *Lb. rhamnosus* and *Lb. acidophilus* generate SCFAs, which may mitigate intestinal inflammation and suppress the spread of colorectal cancer cells. In addition to their health benefits SCFAs generated by LAB in fermented foods contribute to stable pH levels and give off unique spicy aromas.

Bacteriocins: This metabolic process is essential for preventing dysbiosis, particularly in intestinal ecosystems, and disrupting spoilage microbes' quorum sensing, by this way supporting in the long-term the stability of fermented products. Nisin is a perfect example, a bacteriocin synthetised by *Lc. Lactis*, reveals effective antibacterial activity against a broad spectrum of Gram-positive bacteria, including pathogens like *S. aureus* and *L. monocytogenes*. Its efficacy as a biopreservative has resulted in regulatory approval for use in diverse food products (Girma et al., 2021; Ozel et al., 2018).

The effect of bacteriocin go beyond food preservation; they also have potential for therapeutic applications (Sugrue et al., 2024). Research into bacteriocins as alternative antimicrobial agents is undoubtedly gaining momentum because of the increasing phenomenon of antibiotic resistance (Figure 8), and the ability of LAB strains to synthesize these kind of peptides offers significant potential for designing novel probiotics and functional foods, with applications in health promotion and pathogen control (Martinez et al., 2020; Mokoena, 2017).

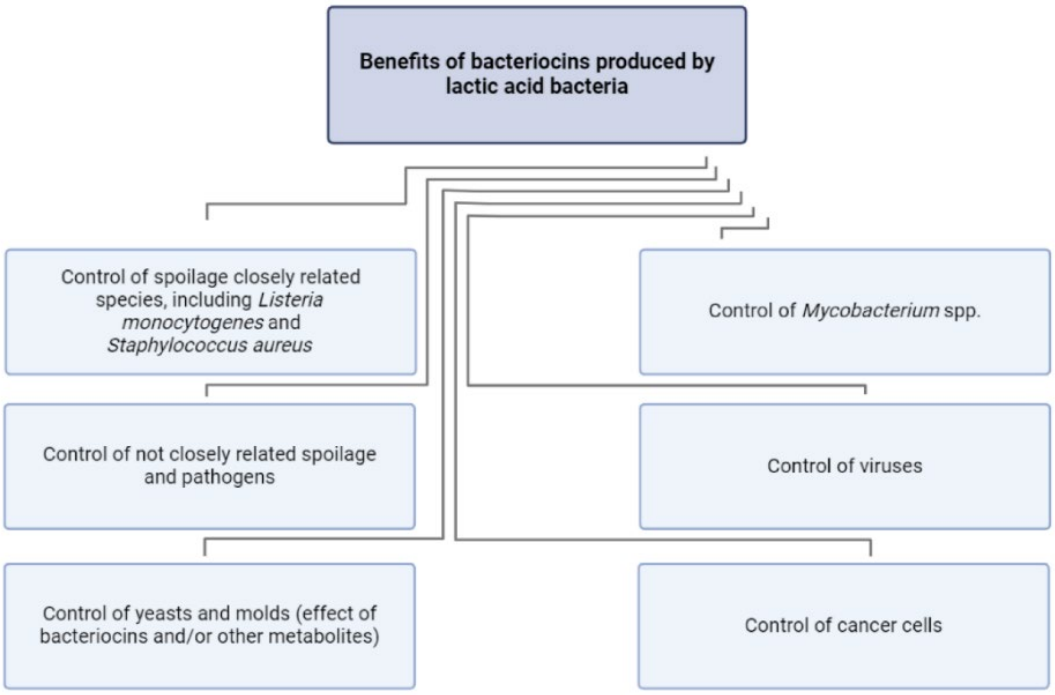


Figure 8 Selected application areas of LAB's bacteriocins (Carneiro et al., 2024).

Vitamins and EPS: support the development of food products' textural qualities and nutritional value. (Abedin et al., 2024; Bermudez-Humaran et al., 2024). It is clear that LAB-synthesised EPS improves finished products' softness, stability, texture, water retention, mouthfeel and rheological qualities (especially in dairy-based foods). This makes it an excellent, safe and natural food additive. likewise, current evidence indicates that these biopolymers may have health-promoting properties including anti-ulcer, antitumor, immunomodulatory, and cholesterol-lowering effects in addition to possible prebiotic roles, making them significant not only for food applications but also for clinical and pharmaceutical applications (Abedin et al., 2024; Bermudez-Humaran et al., 2024; Juraskova et al., 2022; Kumari et al., 2023; Wu et al., 2023; Zhou et al., 2019). Moreover, certain gut-resident LAB strains exhibit the ability to produce B-complex vitamins, including folate (B9), riboflavin (B2), cobalamin (B12), and menaquinone (K2), a process that accounts for their presence in LAB-

fermented dairy products including cheese, cultured buttermilk , and yogurt (Carrizo et al., 2020; LeBlanc et al., 2020; Sabo et al., 2020; Saubade et al., 2017; Suissa et al., 2022).

These biologically active molecules serve as primary modulators of the sensory profile and texture of tangy foods, particularly dairy products (Abedin et al., 2024; Juraskova et al., 2022), they have several positive health effects, especially anti-inflammatory (Martin et al., 2023), cholesterol-lowering (Zhou et al., 2019), antioxidant (Wu et al., 2023), macrophage-polarizing (Nowak et al., 2020), and prebiotic features (Juraskova et al., 2022; Martin et al., 2023). Indeed, contemporary findings has investigated the use of EPS-forming LAB in formulating reduced-fat meat formulations and gluten-free baked goods, revealing its versatility in food processing (Juraskova et al., 2022; Loeffler et al., 2020; Wu et al., 2023).

Bioactive peptides: Given their pharmaco-nutritional significance, bioactive peptides derived via enzymatic hydrolysis across diverse food matrices have drawn a lot of academic attention (Cicero et al., 2017; Sanchez & Vázquez, 2017). These peptides have an assortment of physiological functions including lowering cholesterol, scavenging free radicals, reducing hypertension, suppressing inflammation, and exhibiting antibacterial effects (Cicero et al., 2017; Saadi et al., 2015). Milk, soy, and grain proteins are among the dietary proteins from which proteolytic LAB systems can liberate potentially bioactive peptides (Vinderola et al., 2024). These peptides' production varies by strain and species, allowing opportunities for the targated selection of particular LAB strains (Raveschot et al., 2018).

3.4.Food applications

A key feature of LAB, particularly those belonging to the *Lactobacillaceae* family, is their efficient fermentative metabolism, which is important to their industrial and biotechnological applications in food production (Suissa et al., 2022).

3.4.1. Fermentation in vegetables, dairy, and meat products

The scientific consensus acknowledges LAB's critical involvement in dairy fermentation through the conversion of lactose into LA, causing the milk to coagulate and turn acidic, at this point the production process of yoghurt depends on strains like *St. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*, that grant it a distinctive pH levels and sticky texture (Bintsis, 2018; Gopal, 2020).

In vegetable fermentation, LAB convert sugars into LA, reducing pH levels to inhibit spoilage organisms and pathogens, this is why species like *Ln. mesenteroides* and *Lpb. plantarum* are key in sauerkraut and kimchi, imparting tangy flavours and crisp textures (Castellone et al., 2021; Dimidi

et al., 2019). The growing adoption of plant-based diets has inspired significant research into microbial fermentation of protein-rich substrates, aiming to enhance structural integrity, nutrient bioavailability, and sensory characteristics (El Youssef et al., 2020; Liao et al., 2022).

They are known to effectively drive biosynthesis of LA during the meat fermentation, that reduces pH and suppresses bacterial proliferation. In the other hand proteolytic and lipolytic enzymes alter texture and produce flavourings. For instance, *Pc. acidilactici* inhibits pathogens including *E. coli* and *L. monocytogenes*, while *Ltb. sakei* contributes to a spicy and hard taste in fermented sausages (Liu et al., 2024; Y. Wang, J. Han, et al., 2022).

Althrough hey exhibit niche adaptability due to their metabolic flexibility, enabling colonization of plant tissues and enzymatic transformation of phenolics. Through pathways like citrate metabolism, they produce volatile compounds (diacetyl, acetoin and ethanol) that define fermented food aromas (Filannino, Di Cagno, et al., 2018; Gaur & Ganzle, 2023). In fermentation, acetoin (creamy) and diacetyl (buttery) are key flavour contributors, while ethanol - often linked to beverages - plays a dual role as both a solvent and aroma enhancer (Gopal, 2020; Kim et al., 2020; Tian et al., 2019). The metabolic flux networks utilized by LAB during fermentation are pivotal for biosynthesizing these flavour-active compounds, highlighting their fundamental role in shaping the distinct organoleptic profiles of fermented products (Y. Hu et al., 2022; Sharma et al., 2023; Y. Wang, J. Han, et al., 2022).

3.4.2. Biopreservation benefits

LAB-derived antimicrobials (organic acid, bacteriocins, H_2O_2) selectively inhibit pathogens and spoilage organisms via membrane disruption, pH lowering, and oxidative stress. These combined actions improve food safety metrics (3-log reduction in *L. monocytogenes*) and delay spoilage by 14-21 days (Ozel et al., 2018).

3.5. Probiotic properties and health benefits

Certain species are suitable for use as probiotics due to their natural presence within the gut microbial consortium and their capacity to survive bile salts and acidic pH conditions during transit through the gastrointestinal system (Figure 9). Probiotic strains are described as those that provide beneficial effects on the host, such as exerting direct antagonistic activity against specific microbial groups or stimulating the host immune system (Abedin et al., 2024). Through multiple modes of action, probiotics effectively suppress pathogen colonization in the gastrointestinal tract by generating antibacterial substances and by competing for adhesion sites on the intestinal epithelium as well as for available environmental carbon sources (Bermudez-Humaran et al., 2024).

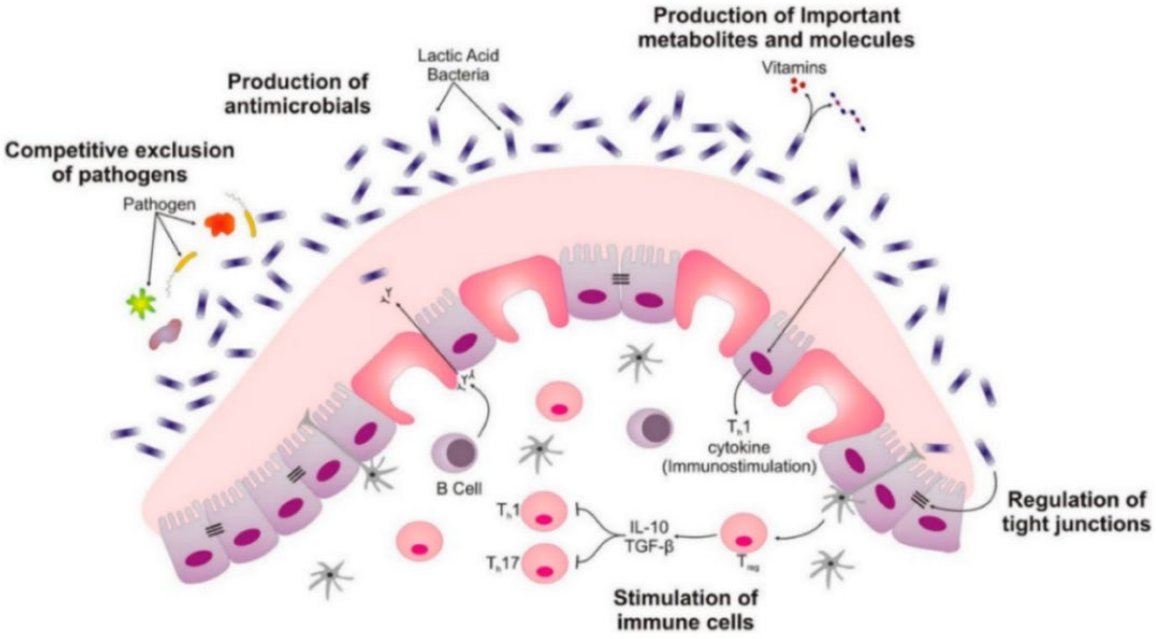


Figure 9 Mechanisms of intestinal health modulation by probiotic (Ghosh et al., 2019).

Certain strains can alleviate lactose intolerance through the activity of their galactosidase enzyme (Ruiz-Rodríguez et al., 2017). Lactose intolerance occurs when the activity of brush-border cell enzymes (β-galactosidase) declines, resulting in insufficient hydrolysis of lactose. Consequently, unabsorbed lactose undergoes microbial fermentation by colonic microbiota, this process, combined with the osmotic influx of water into the large intestine, results into symptoms such as bloating, flatulence, cramps, and abdominal pain (Benbouziane et al., 2019; Suissa et al., 2022). Moreover, a randomized, placebo-controlled, double-blind clinical trial demonstrated that the DSS-1 strain of *Lb.acidophilus* was effective in alleviating symptoms associated with acute lactose intolerance, including diarrhea, cramping, and vomiting (Suissa et al., 2022).

3.5.1. Mechanisms of action

By modulating the GI microbiota, LAB promotes overall host health, their fermentation of fibre generates SCFAs, which are necessary for intestinal epithelial cell (IEC) activity as they have a consequence on barrier integrity, proliferation, and differentiation (Bermudez-Humaran et al., 2024; Mathur et al., 2020; Mosele et al., 2015). As said before LAB exert antipathogenic, intestinal barrier-strengthening and immunomodulating effects through cell wall components and secreted molecules that interact with host receptors (Ren et al., 2021) these mechanisms enable LAB to fight various infections, control inflammatory diseases, treat allergic disorders, and can also alleviate cancer (Caballero et al., 2021; Ren et al., 2021).

3.5.2. Gut health modulation

Probiotic LAB competes with pathogenic organisms for adhesion sites and nutrients, stimulates mucus production, and affects immune responses (figure 10). Experimental evidence confirms that *Lb. rhamnosus* CNCM I-3690 upregulates goblet cell activity via TLR2 signaling, in this way improving the intestinal barrier's functionality, and SCFAs downregulate NF-κB signaling and upregulate tight junction proteins, stimulate by the way colonocyte proliferation and differentiation, thus contributing to intestinal epithelium regeneration and overall intestinal health (Ayed et al., 2024; Jacouton et al., 2018; Martin et al., 2019).

3.5.3. Antioxidant activity

This group of bacteria exert antioxidant effects through several processes, that include the activation of the Keap1-Nrf2-ARE cascade signalling, free radical scavenging, and metal ion chelation. This regulatory mechanism ensures cellular homeostasis and protects against disorders associated with oxidative stress (Pouremamali et al., 2022; Wen et al., 2019).

3.5.4. Other Health Benefits

Antihypertensive effects: Milk fermentation by LAB produces bioactive peptides with potential health benefits, particularly in controlling blood pressure by repressing angiotensin-converting enzyme (ACE) (Pessione & Cirrincione, 2016). These peptides are milk proteins derived, and their formation is influenced by factors like starter cultures, fermentation conditions, and substrate composition. Strains such as *Lb. helveticus* R0389 and *Lcb. rhamnosus* R0011 can produce peptides with ACE repressive effects and immunomodulatory assets (Adams et al., 2020). Some peptides, sharing the characteristic proline-rich motif of validated ACE-inhibitory peptides (e.g., Ile-Pro-Pro/Val-Pro-Pro), not only block ACE but concurrently trigger the synthesis process of endothelium-derived NO and regulatory cytokines, wich further contributes to cardiovascular health (Adams et al., 2020).

Anti-tumor properties: Many species in literature demonstrate substantial antiproliferative activity against cancer cells in recent research, it has been shown that post-fermentation medium and cell extracts trigger apoptosis by stimulating mitochondrial signalling cascades (Capurso, 2019; Jacouton et al., 2018; Pouremamali et al., 2022). Nowak et al. (2022) claimed that probiotic strains, *Lpb. plantarum* 0991 and *Lev. brevis* 0983, may have anti-cancer capability by inducing apoptosis and oxidative stress in cancer cells.

Cholesterol-lowering effects: LAB has shown promising cholesterol-lowering effects through various mechanisms, many studies in litterature have shown in animal models that LAB strains can

lower cholesterol in vitro and reduce serum levels of cholesterol (Abedin et al., 2024; Ayed et al., 2024). The cholesterol-lowering mechanism of *Lpb. plantarum* 54-1 operates through multiple metabolic pathways, notably the bioconversion of cholesterol into 7α-hydroxycholesterol (via bile salt hydrolase activity) and taurine-conjugated bile acids (X. Fan et al., 2023). Furthermore, LAB-derived metabolites can engage cholesterol through non-covalent interactions, thereby reducing its bioaccessibility and intestinal absorption (X. Fan et al., 2023; Rastogi et al., 2022).

3.5.5. The potential role of probiotics in modulating COVID-19 outcomes:

Recent Emerging clinical and preclinical evidence supports probiotics as a viable adjunct therapy for mitigating COVID-19 complications, primarily through (Kurian et al., 2021; Nguyen et al., 2022): Preservation of gut microbiota equilibrium (reducing SARS-CoV-2-induced dysbiosis), Systemic immunomodulation (notably IL-6 ↓, IFN-γ ↑), and Competitive exclusion of opportunistic pathogens

Controlled clinical trials and meta-analyses indicate that defined probiotic strains can reduce the incidence and severity of viral respiratory infections, including coronavirus-associated illnesses (Olaimat et al., 2020). Clinical evidence demonstrates that specific probiotic strains enhance both innate and adaptive immune responses, potentially reducing SARS-CoV-2 infection severity and duration in vulnerable populations(Kurian et al., 2021; Olaimat et al., 2020), To elucidate probiotic metabolite-SARS-CoV-2 interactions, computational biology approaches integrating ensemble docking with gut-lung axis metabolic modeling have been employed (Nguyen et al., 2022). While preliminary clinical and mechanistic data show promise, rigorous large-scale randomized controlled trials are still needed.

3.6.Recent advances in LAB research

3.6.1. Genetic engineering

The development of strains with enhanced traits, that show higher resistance to bacteriophage infection and tailored metabolite synthesis, becomes easier using CRISPR-Cas9-mediated genome editing, such exploit was reported by Cui and Qu (2024) is a study where therapeutic proteins, such as interleukins, are secreted by modified *Lc. lactis* strains, providing potentially new approaches for targeted drug delivery (Liu et al., 2019).

3.6.2. Food-grade films and coatings

A new approach to improving food safety and preservation is the application of biodegradable edible coatings and films that incorporate LAB. These biocomposite matrices preserve food products from

oxidative degradation and microbial contamination while maintaining their nutritional value and sensory qualities during storage (Peng et al., 2020; Vasiliauskaite et al., 2022). A growing body of evidence indicates that films and coatings integrated *lactobacillus* strain, in the laboratory can effectively combat fungal growth in cheese and prevent contamination for up to 30 days depending on the formulation and storage conditions (Guimarães et al., 2020; Motalebi Moghanjougi et al., 2020; S. P. M. Silva et al., 2023).

3.6.3. Systems biology approaches

Advances in multi-omics technologies (including genomics, transcriptomics, proteomics, and metabolomics) are driving transformative developments in both food science and precision medicine, these integrated approaches enable understanding of complex biological systems (O'Donnell et al., 2019; Vinderola et al., 2024), thus by systematically map and optimize LAB metabolic networks to enhance food quality, such as the breakdown of polysaccharides and biosynthesis of vitamins and bioactive compounds. Another approach by combining multi-omics with artificial intelligence (AI) is expected to accelerate the modeling of LAB strain metabolism and interactions, leading to more precise and efficient food fermentation process (Y. Wang et al., 2021; Yuan et al., 2025). Despite these advances, significant challenges persist in integrating multi-omics data and managing high-dimensional datasets. In order to overcome these constraints, researchers currently use advanced bioinformatics, AI-driven knowledge graphs that link microbial genes to metabolic phenotypes, and dimensionality reduction techniques to extract biologically actionable insights (Ferrocino et al., 2023; Herráiz-Gil et al., 2023).

3.7. Role in sustainable agriculture

Lactic acid bacteria not only stimulate plant growth and enhance soil fertility, but also function as effective biofertilizers and biocontrol agents. Their contributions include nutrient solubilization and nitrogen fixation (Naik et al., 2019), detoxification of hazardous chemicals and heavy metals (Shaheb et al., 2024), and suppression of phytopathogens via bacteriocin production and competitive exclusion mechanisms (Jaffar et al., 2022). Notably, strains such as Fructilactobacillus sanfranciscensis have been shown to improve soil health and biofortify crops with essential vitamins, further supporting sustainable agriculture (Murindangabo et al., 2023).

4. PHENOLIC COMPOUNDS IMPACT ON BROILERS MEAT QUALITY

4.1.Overview of phenolic compounds

4.1.1. Definition and general characteristics

Phenolic compounds are a diversified class of bioactive specialized metabolites that can be structurally classified by their characteristic aryl-hydroxyl motifs (Dehghanian et al., 2022; Nurzynska-Wierdak, 2023).

4.1.2. Ecological and health-related roles

These compounds perform an essential function in the defence systems of the plant against biotic stressors like infections and herbivores as well as abiotic stresses like harsh climatic conditions (Dehghanian et al., 2022). Additionally to their ecological functions, PCs are receiving more scientific interest due to possible health advantages, including antioxidant qualities that can lower ROS-mediated pathology risk and malignancies with chronic progression, heart disease, and neurological disorders (Cosme et al., 2020; Hurkul et al., 2025). Also, its application in the food company improves canned foods, shelf life, and nutrient content (Sarkar & Shetty, 2014; Vuolo et al., 2019). On the flip side, the efficiency of PCs depends on their bioavailability, which is established by how well they are absorbed, metabolised, and excreted by humans (Cosme et al., 2020). So enhancing efficient delivery techniques to boost their stability and bioavailability and guarantee the best possible therapeutic and functional outcomes has been the focus of recent research. (Cosme et al., 2020). Moreover, advances in extraction and analytical techniques have simplified the identification and quantification of those elements in food matrix, suggesting accurate implementation in food science and nutrition (Hurkul et al., 2025; Mamari, 2021).

4.2.Chemical structure and classification

Phenolic compounds can be broadly classified into two categories based on their chemical makeup: flavonoids and non-flavonoids.

4.2.1. Flavonoids

Featuring a core flavonoid skeleton (Figure 10) consisting of: two aromatic systems (A and B) interconnected via a γ-pyrone heterocycle (Ring C), this group is the most structurally diverse class of PCs. (Cosme et al., 2020; Singla et al., 2019). Among the subclasses are isoflavones (genistein),

flavonols (quercetin and rutin), flavanols (catechins), flavanones (hesperidin), and anthocyanins (cyanidin), keep in mind that the differences in the oxidation states and hydroxylation patterns cause variations. (Durazzo et al., 2019; Vuolo et al., 2019).

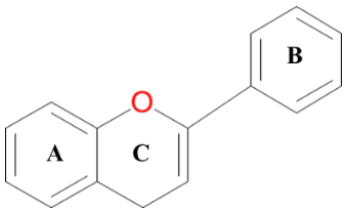


Figure 10 General core configuration within the flavonoid molecule (Vuolo et al., 2019).

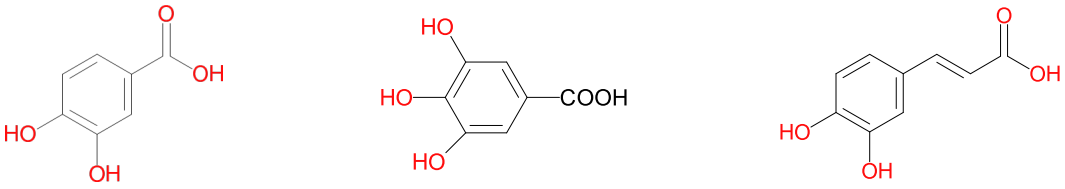
4.2.2. Non-Flavonoids

This group consists of lignans (secoisolariciresinol), tannins, phenolic acids, and stilbenes (resveratrol). Phenolic acids are further divided into the following categories (Figure 11):

Hydroxybenzoic acids: include gallic acid, protocatechuic acid.

Hydroxycinnamic acids: include ferulic acid, caffeic acid (de Camargo et al., 2018; Singla et al., 2019).

These acids fundamentally mediate antioxidant defence through metal ion chelation and free radical scavenging.



Protocatechuic acid Gallic acid Caffeic acid

Figure 11 Representative phenolic acids (de Camargo et al., 2018).

This sub-classification is established on molecular complexity, including a substituent groups, number of phenol units, and linkages among units (Tsimogiannis & Oreopoulou, 2019; Xu et al., 2020). The structural diversity of PCs, as outlined, highlights their widespread presence in an extensive range of by-products and plant-based foods. The following table summarizes the impact of specific phenolic compound subclasses on meat quality, highlighting their mechanisms of action and effects on preservation and sensory attributes (Table 2).

Table 2 Impact of Specific Phenolic Compound Subclasses on Meat Quality**.**

Phenolic Subclass	Example	Mechanism of Action	Impact on Meat Quality	Citation(s)
Flavonols	*Quercetin*	Inhibits lipid peroxidation by scavenging free radicals and chelating metal ions.	Reduces rancidity, extends shelf life, preserves color.	(Barbieri et al., 2021; Kalogianni et al., 2020)
avanols (Catechins)	*Catechin, Epicatechin*	Acts as antioxidants, protecting against lipid oxidation and protein oxidation.	Preserves meat color and flavor, enhances oxidative stability.	(Munekata et al., 2020)
Hydroxycinnamic Acids	*Caffeic Acid*	Scavenges free radicals, inhibits enzymatic browning, and exhibits antimicrobial properties.	Reduces discoloration, inhibits microbial growth, enhances overall preservation.	(Albuquerque et al., 2021; Fasolato et al., 2016)
Hydroxybenzoic Acids	*Gallic Acid*	Acts as a potent antioxidant and antimicrobial agent, chelates metal ions, and inhibits lipid peroxidation.	Preserves meat from spoilage, enhances oxidative stability, and extends shelf life.	(Durazzo et al., 2019; K. Zhang et al., 2022)

4.3.Phenolic origins and availability

Phytogenic edibles like fruits (apples, berries), vegetables (broccoli, onions), cereals (wheat, maize) and drinks (tea, juice) are all rich in PCs. Herbs and spices such as oregano and cinnamon are abundant in flavonoids, phenolic acids, and stilbenes (Tsimogiannis & Oreopoulou, 2019). In addition to food sources, PCs are also obtained from unconventional plant parts and from agricultural and food waste, and therefore offer a sustainable use in food preservation and health (Nurzynska-Wierdak, 2023).

4.3.1. Primary sources

Fruits/Vegetables: Berries and apples (rich in flavonoids); leafy greens (phenolic acids).

Cereals/Beverages: Wheat bran (ferulic acid); red wine (resveratrol); coffee (chlorogenic acid).

4.3.2. Nonconventional sources

Agri-food wastes such as husks, seeds, and pomace from food processing serve as low-cost sources of PCs, which exhibit notable antioxidant and antimicrobial properties (de Camargo et al., 2018; Panzella et al., 2020). Similarly, woody plants like pine and oak bark are rich in lignans and phenolic acids, contributing anti-inflammatory potential to various applications (Tanase et al., 2019). Additionally, grapevine by-products, including stems, leaves, and canes are valuable for their high content of stilbenes (e.g., resveratrol) and flavonoids, supporting the sustainable valorization of wine industry residues (Goufo et al., 2020).

4.4.Extraction methods

Methods of extraction have evolved from traditional solvent-based approaches to environmentally friendly technologies such as ultrasound-assisted extraction to improve sustainability and efficiency (Alara et al., 2021; Kumar et al., 2021). However, genetic factors, plant development stages, and harvest times all affect PC content, therefore industrial consistency requires optimised processes (Nurzynska-Wierdak, 2023).

4.4.1. Traditional solvent extraction

Traditional solvent extraction, which use organic solvents (ethanol, methanol, and acetone) to dissolve PCs that relies on polarity-driven solubility, remains a primary technique for removing PCs from plant matrices. Despite it is scalable and economical, it necessitates high solvent volumes, extended extraction durations, and high temperatures, all of which run the risk of breaking down heat-sensitive phenolics (Alara et al., 2021). For example, phenolics that was extracted from grape seeds using ethanol method yield high concentrations of proanthocyanidins, despite the possibility of volatile molecules being damaged by heat (Nurzynska-Wierdak, 2023). Considering these disadvantages, its ease of usage guarantees broad application in sectors like food preservation and nutraceuticals.

4.4.2. Advanced green extraction techniques

Sustainable solutions such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) have appeared to address efficacy and environmental concerns:

UAE: High-frequency sound waves (20–100 kHz) are used to create cavitation, which breaks down plant cell walls and enhances solvent penetration. This methodology preserves thermolabile PCs while cutting down on extraction time by 50–70% and solvent utilisation by 30–50% when compared to conventional methods (Kumar et al., 2021).

MAE: By using dielectric heating, this technique quickly heats liquids and plant tissues using microwave radiation (300 MHz–300 GHz). This increases yield by as much as 40% and speeds up phenolic dissolution, especially for thermostable substances like lignans in cereals (Alara et al., 2021).

Both methods minimise waste production and energy consumption, exhibiting the hallmark features of green chemistry. But in other hand to match target phenolic profiles, their success is dependent on optimising important parameters involving temperature, extraction duration, and solvent polarity (Nurzynska-Wierdak, 2023).

4.4.3. Practical implications:

There are real advantages to meat preservation as a direct result of PCs extraction efficiency, in this optical a findings by Barbieri et al. (2021) demonstrated a substantial reduction in lipid oxidation, as evidenced by the fact that the UAE of hydroxytyrosol from olive leaves produced an extract that could lower TBARS (Thiobarbituric Acid Reactive Substances) readings in sausages by 30% as compared to conventional techniques. Similarly, Smaoui et al. (2019) demonstrated that MAE of anthocyanins from pomegranate peels yielded an extract that effectively stabilized the color of beef patties during refrigerated storage, maintaining consumer appeal and extending shelf life.

4.5. Biological activities

According to Figure 12, PCs exhibit a variety of biological actions, such as inflammation-resolving, antibacterial, oxidative stress-mitigating, and pro-apoptotic effects in malignant cells; these activities endow them with functional significance for promoting health and preserving food, particularly in meat systems (A. R. Ishaq et al., 2021; Matulja et al., 2022; Munekata et al., 2020; Rahman et al., 2021; Roila et al., 2022).

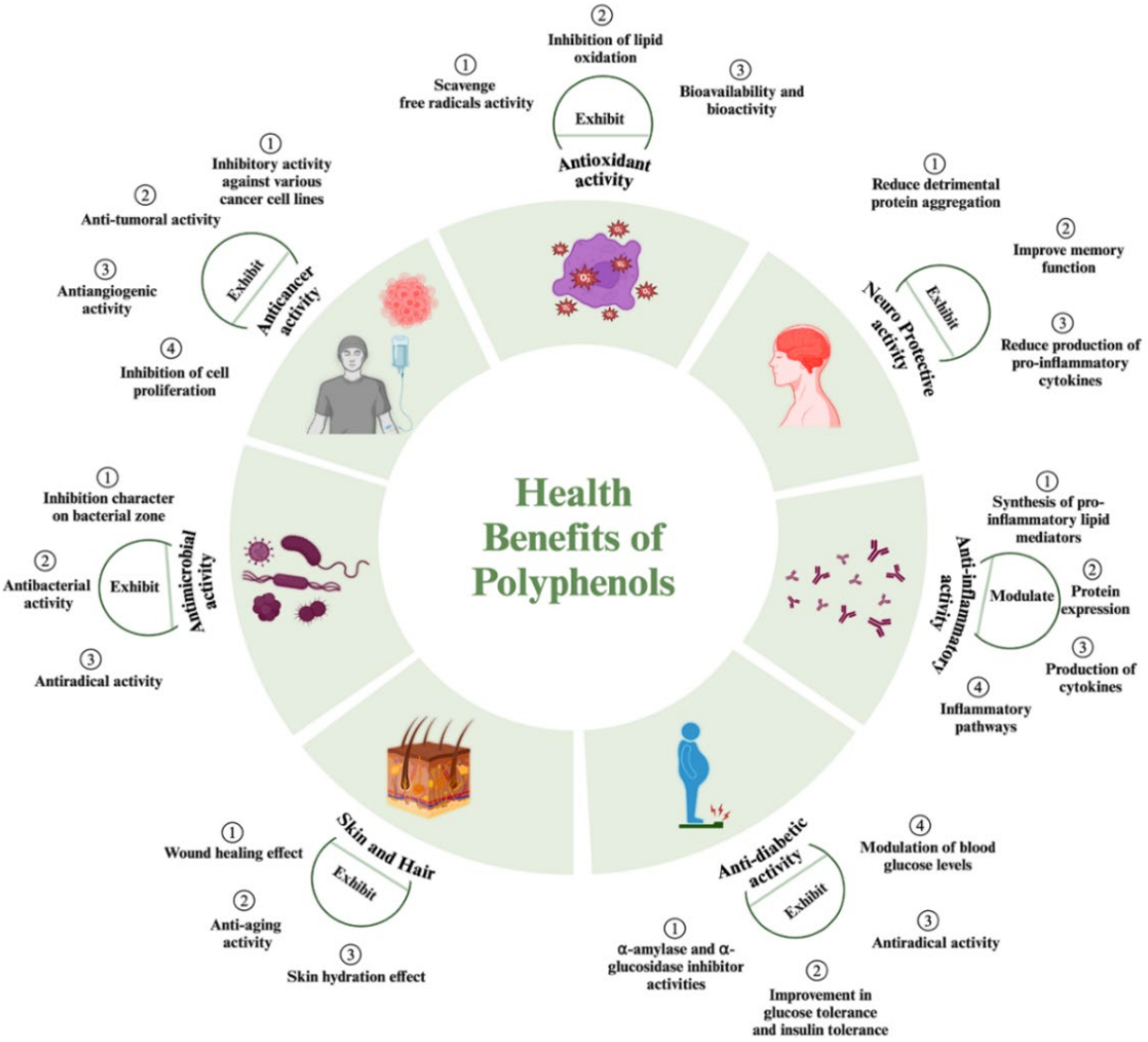


Figure 12 Key Biological Activities of PCs (Bolat et al., 2024)

4.5.1. Antioxidant mechanisms

Phenolic compounds combat oxidative stress through three primary mechanisms: scavenging free radicals, chelating pro-oxidative metal ions, and inhibiting reactive oxygen species (ROS)-generating enzymes, thereby mitigating oxidative rancidity in meat products (Barbieri et al., 2021; Xiong, 2017). By donating hydrogen atoms or electrons, these compounds neutralize ROS and prevent oxidative damage to DNA, lipids, and proteins, including misfolding and aggregation (Albuquerque et al., 2021; Y. Zhang et al., 2022). Their ability to chelate metal ions such as Fe^{2+} and Cu^{2+} effectively inhibits Fenton reactions, which drive lipid peroxidation in meat systems (Kalogianni et al., 2020; Xiong, 2017). Furthermore, PCs suppress oxidative enzymes like xanthine oxidase and lipoxygenase, thus reducing the formation of rancid by-products in meat matrices (Xiong, 2017; Xu et al., 2021).

4.5.2. Antimicrobial effects

Phenolic compounds enhance meat safety by disrupting microbial cell membranes, altering metabolic pathways, and inhibiting key enzymatic functions, thereby suppressing both spoilage organisms and foodborne pathogens (Figure 13) (Albuquerque et al., 2021; Fasolato et al., 2016; Kalogianni et al., 2020). Their antimicrobial efficacy includes inhibition of common spoilage bacteria such as *Staphylococcus aureus* and *Pseudomonas* spp., as well as pathogenic strains like *Listeria monocytogenes* and *Escherichia coli* (Fasolato et al., 2016; Kalogianni et al., 2020). Moreover, PCs exhibit synergistic interactions with LAB, enhancing antimicrobial potency and contributing to improved meat preservation and microbiological quality (Lorenzo et al., 2018).

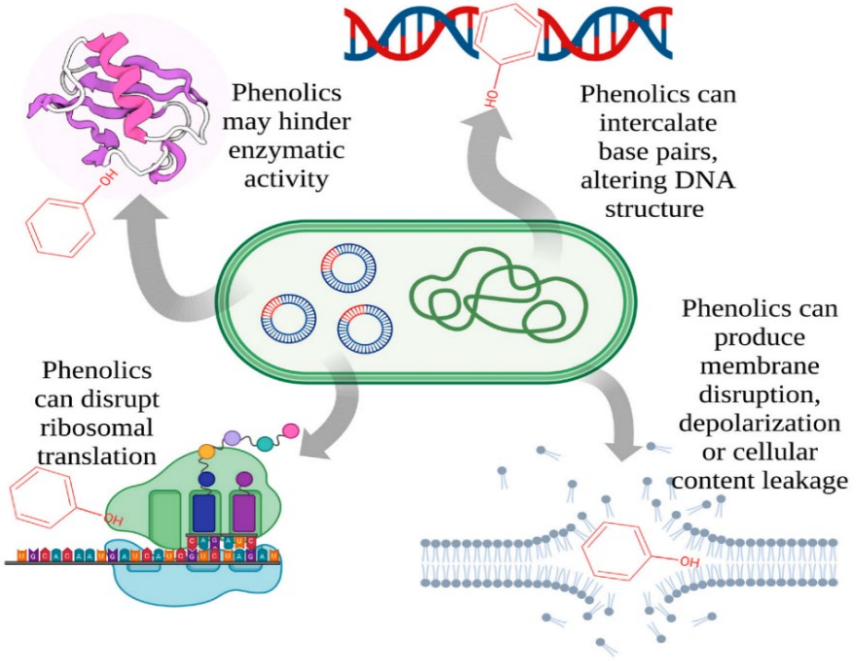


Figure 13 Mechanisms of interaction between PCs and bacterial cells (phenol structure shown as a representative example) (Lobiuc et al., 2023).

4.5.3. Cardiometabolic benefits:

Extensive evidence shows that PCs improve lipid profiles (thus reducing LDL oxidation), increase insulin sensitivity, and regulate metabolic pathways, mitigating cardiometabolic disease risk (Rahman et al., 2021).

4.5.4. Anti-inflammatory and anticancer properties

PCs modulate signalling cascades (e.g. MAPK, PI3K/Akt/mTOR) (Figure 14), induce apoptosis through caspase activation, and reduce oxidative DNA damage, which proves promising in cancer prevention (Abotaleb et al., 2020; Zhang et al., 2018). Particular PCs, such as cinnamic acids (e.g. ferulic acid) target angiogenesis and metastasis (Abotaleb et al., 2020).

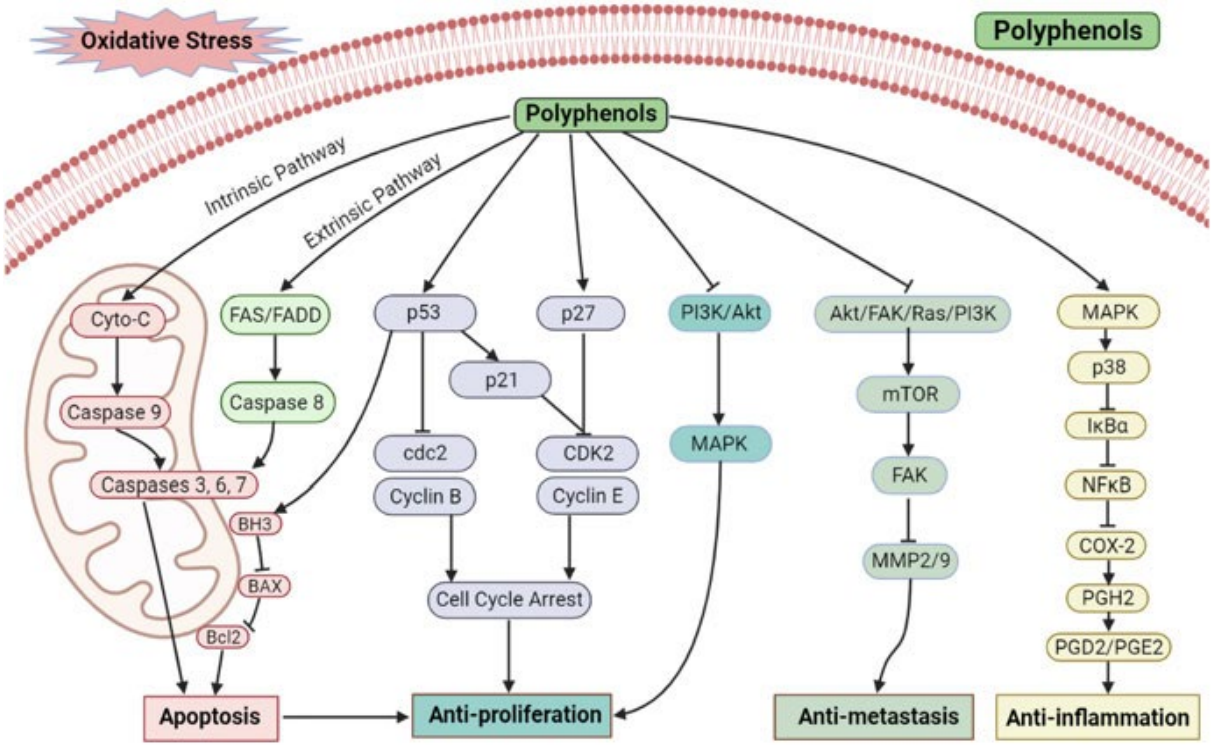


Figure 14 Signaling pathways modulated by polyphenols in cancer cells.
Key pathways include: Apoptosis: Inhibition of Bax/Bcl-2 and caspase activation via cytochrome C release. Anti-proliferation: PI3K/Akt pathway suppression through p53/p21/p27-mediated cell cycle arrest. Anti-metastasis: Akt/FAK/Ras/PI3K cascade inhibition via MMP-2/9 regulation. inflammation-modulating : MAPK pathway modulation through p38 and COX-2 downregulation. (Yadav et al., 2021)

The potential of PCs to lower carcinogenic chemicals in cured meats has been investigated recently; in fact, polyphenols such as gallic acid, procyanidin B2, and catechin have demonstrated encouraging outcomes in scavenging nitrite and reducing nitrosation reactions under simulated gastric conditions (Ren et al., 2024). These molecules have antioxidant effects and have the capacity to generate protein-polyphenol complexes, which may prevent the production of heterocyclic amines and other carcinogenic byproducts in thermally cooked beef (Nawaz et al., 2024), and in roasted beef patties, many PCs, such as capsaicin, have shown notable decreases in the amount of heterocyclic aromatic amines (Zhou et al., 2024).

4.5.5. Structure – Activity Relationships SAR

Speaking in terms of SAR, two elements that greatly affect PCs' antioxidant activity are the presence of conjugated systems and the location and quantity of hydroxyl moieties (Chen et al., 2024; Tanase et al., 2019): Catechol groups (caffeic acid) by electron delocalisation, enhance radical capture, whereas Galloyl moieties (gallic acid) facilitate metal chelation (Durazzo et al., 2019; Y. Zhang et al., 2022).

Ortho-diphenolic structures exhibit superior antioxidant properties, while simple phenols demonstrate better antibacterial activity (Pernin et al., 2018). Metal complexation can enhance antioxidant properties, with high ionic potential metals like Cu^{2+} and Fe^{3+} stabilizing electron charge (Chen et al., 2024). In meat preservation, PCs inhibit lipid oxidation, stabilize myoglobin and prevent the development of carcinogenic substances like nitrosamines (Kalogianni et al., 2020). Studies show that olive phenols (hydroxytyrosol) reduce malondialdehyde formation in beef burgers and thus extend shelf life (Barbieri et al., 2021).

4.6. Impact on meat quality

Phenolic compounds enhance meat quality through antioxidant, antimicrobial, and anti-inflammatory mechanisms, addressing key challenges in lipid oxidation, colour stability, and shelf-life extension (Barbieri et al., 2021; Kalogianni et al., 2020). Their applications include:

4.6.1. Oxidative stability and color preservation

Preserving the quality and prolonging the longevity of meat products under storage requires the inhibition of lipid and protein oxidation. By intercepting free radical propagation, blocking oxidative enzymes, and forming metal chelates, PCs contribute significantly to oxidative stability (Figure 15), lowering rancidity and maintaining freshness (Barbieri et al., 2021; Munekata et al., 2020; Smaoui et al., 2019). By preventing lipid peroxidation and myoglobin degradation, PCs stabilize meat colour, ensuring the product retains its desirable appearance during storage (Stadnik et al., 2022). Furthermore, PCs stimulate the Nrf2 pathway - a central modulator of antioxidative enzyme expression - amplifying the redox defence mechanisms in meat products subjected to oxidative stress (K. Zhang et al., 2022).

The effectiveness of these substances is supported by a number of research; for example, it has been shown that pomegranate peel extracts greatly improve oxidative stability in meat formulations, hence lowering rancidity and extending freshness (Smaoui et al., 2019). Integrative nutritional approaches further contribute to mitigating oxidative degradation. For instance, vitamin E supplementation has been demonstrated to enhance redox homeostasis and postharvest quality attributes in poultry and lamb meat. Broiler trials revealed that pre-slaughter dietary administration of vitamin E over a 21-day period led to a significant increase in muscular α-tocopherol retention and marked suppression of lipid peroxidation, underscoring its role in preserving meat integrity via enhanced antioxidant capacity (Xu et al., 2021).

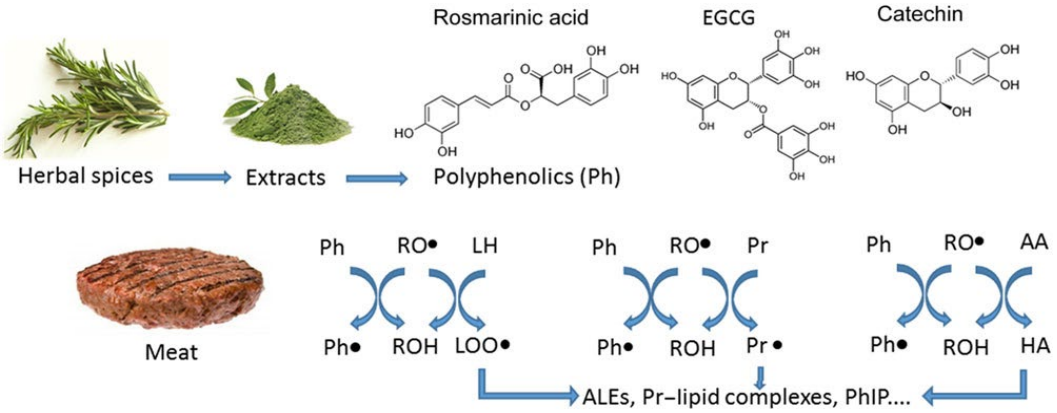


Figure 15 Schematic illustration of the role of phytophenolic antioxidants in processed and thermally treated meat products. LH, lipid; Pr, protein; AA, amine compound; HA, heterocyclic amine, radical species RO• (Xiong, 2017).

4.6.2. Improving sensory attributes: texture, flavor, and aroma

Bioactive phytophenolic compounds induce structural modifications in myofibrillar proteins (MPs), enhancing their functional attributes in meat systems. These polyphenols bind to MPs via non-covalent interactions (Figure 16), primarily hydrogen bonding and electrostatic forces , resulting in conformational shifts in secondary structures (α-helix to β-sheet transitions) and modulation of surface hydrophobicity (Xu et al., 2021), which collectively influence protein solubility, oxidation stability and gelation properties, while also affecting emulsifying properties (Guo et al., 2021; Xiong, 2017; Xu et al., 2021). Phenolic compounds can induce protein unfolding and promote cross-linking, leading to stronger gel networks in processed meats (Guo et al., 2021). The structure-activity relationship of different phenolics influences their impact on MP gelation, with some compounds boosting elastic gel networks more effectively than others (Chen et al., 2022).

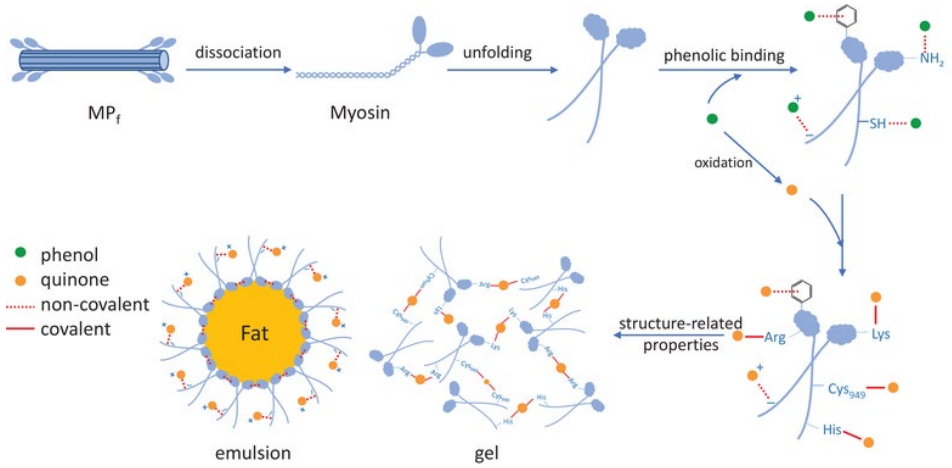


Figure 16 Mechanistic overview of myosin-phenolic molecular interactions and their influence on structural-functional dynamics in processed meat matrices (Guo & Xiong, 2021).

They also play a pivotal role in enhancing the flavour and aroma profiles of broiler meat by modulating lipid oxidation and volatile compound formation preventing the development of off-flavors associated with rancidity (e.g., aldehydes and ketones) (Starcevic et al., 2015; Zhou et al., 2024). Recent studies have highlighted that extracts rich in PCs, such as those from *Arthrospira platensis*, exhibit potent antioxidant activity that can significantly reduce lipid oxidation in meat systems, thereby preserving flavour and aroma profiles during storage (S. Dahmouni et al., 2025).

Additionally, phenolics interact with Maillard reaction precursors during cooking, promoting the formation of desirable flavour compounds like pyrazines and thiazoles, which contribute to meat's characteristic savoury and roasted notes (Zhou et al., 2024). For instance, rosemary extract rich in carnosic acid has been shown to improve meat flavour by reducing hexanal (a marker of oxidation) while enhancing umami-associated nucleotides (Aziz et al., 2022).

Beyond flavour preservation, PCs directly influence aroma through their volatile derivatives and synergistic effects with meat components (Manessis et al., 2020). Purwanti et al. (2019) demonstrates that dietary supplementation with turmeric (*Curcuma longa*) and garlic (*Allium sativum*) rich in curcuminoids and organosulfur compounds, respectively, significantly improves the physical and sensory quality of broiler meat. Turmeric enhanced flavour complexity through its curcumin-mediated suppression of lipid oxidation. Garlic supplementation on the other hand contributed pungent and umami notes by modulating volatile sulfur compounds (for example allicin derivatives), while also improving texture through increased protein cross-linking.

These effects are further amplified by phenolics' ability to stabilize heme pigments, which preserves fresh meat's colour-linked aroma perception (W. Zhu et al., 2024) . Optimizing phenolic sources and concentrations thus offers a dual strategy for elevating sensory quality while extending shelf life (Saleh et al., 2020; E. J. Yang et al., 2020).

4.6.3. Natural preservation and reduction of synthetic additives

Replacing synthetic additives with natural extracts derived from fruits and vegetables preserves sensory quality while mitigating oxidative damage (Martinez-Zamora et al., 2021). These plant-based compounds, often derived from agro-industrial waste, exhibit strong antioxidant and antimicrobial properties that can extend food shelf life while maintaining sensory quality (Maddaloni et al., 2025). Gallic and tannic acids, for instance, have been demonstrated to enhance the solubility of MPs and gelation qualities in the meat of common carp (*Cyprinus carpio*) (Tan et al., 2022) While extracts from olive leaves successfully lower the oxidation of lipids and myoglobin in minced beef without sacrificing flavour (Aouidi et al., 2017), The polyphenolic extract from olive

mill effluent is highly effective in beef burgers, reducing lipid oxidation by 62% and boosting antioxidant capacity by 58%. It also moderates germ development without sacrificing sensory quality (Aouidi et al., 2017; Roila et al., 2022).

The inclusion of 1% olive leaf extract (OLE) in broiler diets significantly improves the oxidative stability of breast meat through dual mechanisms. OLE's high concentration of PCs, particularly oleuropein and hydroxytyrosol, elevates the meat's intrinsic antioxidant capacity by stimulating endogenous enzyme systems (SOD) and directly scavenging free radicals. This is evidenced by a 20-40% reduction in malondialdehyde (MDA) levels , a key marker of lipid peroxidation, compared to control diets, indicating superior protection against oxidative rancidity (Mahfuz et al., 2021; Vasilopoulou et al., 2023). The enhanced antioxidant profile not only extends shelf life by delaying quality deterioration but also preserves the nutritional value and sensory attributes of the meat by minimizing the development of off-flavours and maintaining PUFA integrity (Vasilopoulou et al., 2023). These effects position OLE as a functional feed additive for sustainable poultry production, aligning with consumer demand for clean-label, naturally preserved meat products. These natural alternatives can effectively replace synthetic preservatives while maintaining product quality and safety (Forgione et al., 2024).

5. SYNERGISTIC EFFECT OF PC AND LAB ON MEAT PRESERVATION AND QUALITY ENHANCEMENT

An innovative synergistic technique combining PCs and LAB could optimise meat preservation, improving product quality, safety, and shelf-life extension. In order to solve preservation issues and satisfy consumer demands for natural, clean-label food products, this combination strategy makes use of the complimentary biological qualities of both PCs and LAB (Barcenilla et al., 2022; Carneiro et al., 2024). Even while previous studies have produced a wealth of information on these individual agents, little is known about how they interact in meat matrices. To optimise their practical application and efficacy in industrial-scale meat preservation, a methodical assessment of this synergistic relationship is necessary.

5.1. Biochemical interactions:

The strong antioxidant properties of PCs are widely recognised, mainly affecting processes like metal ion chelation, free radical scavenging, and inhibition of ROS-generating enzymes. (Cunha et al., 2018). The production of organic acids, bacteriocins, and antioxidants such GPx and SOD by LAB might supplement these antioxidative effects throughout probiotic fermentation, modulating ecological niches to suppress spoilage organism (Carneiro et al., 2024). Through biotransformation into more active derivatives with enhanced antioxidative and antibacterial characteristics, the LAB-mediated fermentation procedures significantly increase the bioavailability, stability, and efficacy of PCs (Valero-Cases et al., 2017; Zhao & Shah, 2016). Their combined effectiveness in meat preservation is greatly increased by the synergistic effect of the biochemical interaction between LAB and PCs.

5.2. Enhanced antioxidant activity

Utilizing both LAB and PCs together significantly amplify antioxidant activity (Figure 17) within meat products, mitigating lipid oxidation processes and thus prolonging shelf life. Evidence from studies involving product juice fermentations such as Brazilian Caatinga fruits, jujube, blueberries, and pomegranate has demonstrated marked improvements in antioxidant capacity and bioavailability when fermented with LAB strains like *Lb. acidophilus* La-5 and *Lcb. casei* 01 (de Assis et al., 2021; Li et al., 2021; Valero-Cases et al., 2017). Such enhanced antioxidative properties are particularly beneficial in preserving critical sensory attributes, including colour, aroma, and taste, by reducing oxidative deterioration during storage.

These improvements in antioxidant efficacy underscore the practical value of combining LAB fermentation and phenolic enrichment to maintain product integrity and consumer appeal.

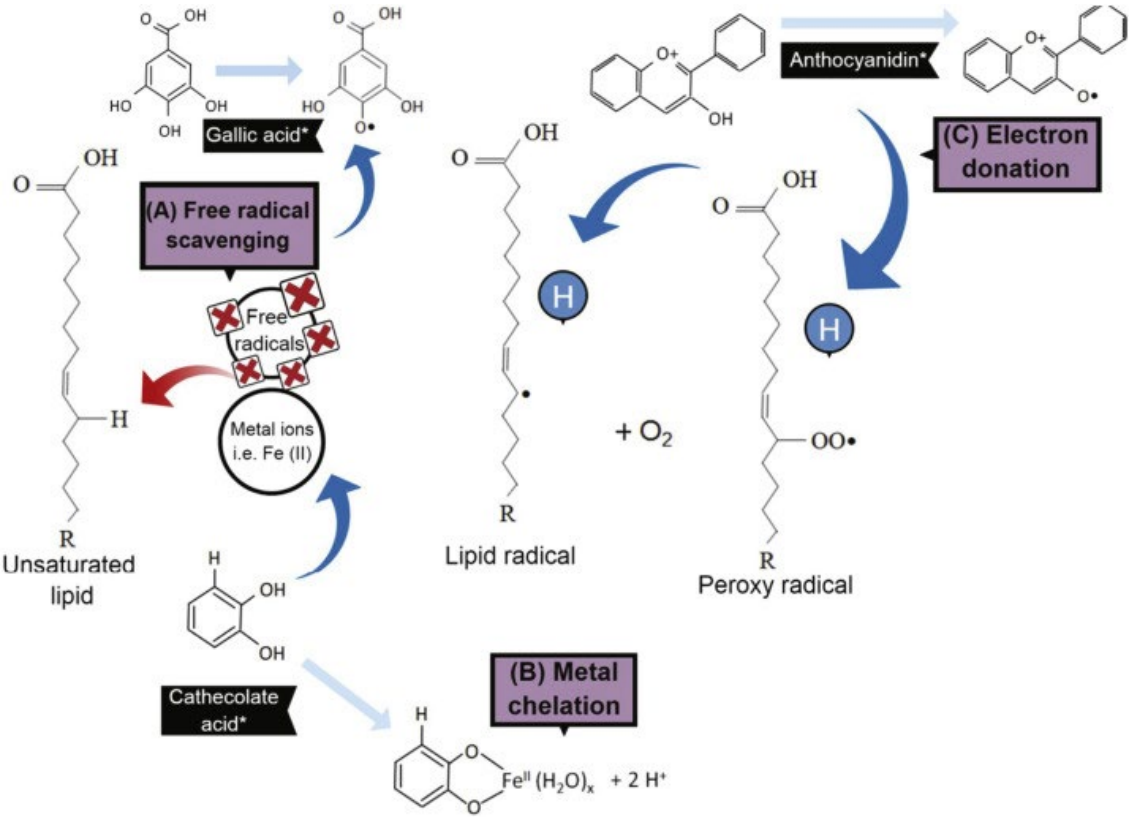


Figure 17 The three mechanisms of antioxidant action (Cunha et al., 2018). (A) Scavenging free radicals, which can take place during the initiation, propagation, and termination phases (particularly during the breakdown of hydroperoxides); (B) Binding with catalytic metal ions, thereby reducing their pro-oxidant activity; (C) Donating electrons during the propagation and termination phases to stabilize lipid molecules.

5.3.Improved antimicrobial efficacy

The simultaneous utilization of microorganism like LAB and PCs significantly reinforces antimicrobial activity against spoilage and pathogenic microorganisms, outstanding their individual capabilities. LAB known to produces numerous metabolites with antimicrobial activity like bacteriocins, H_2O_2, and phenyllactic acid (Figure 18) can establishing states hostile for microbial proliferation and survival (Ning et al., 2021; Sugrue et al., 2024), and the integration of PCs exacerbates these antimicrobial effects by cell membranes disrupting, damaging ribosome function, and interfering with vital metabolic pathways like K^+ transport and nucleotide metabolism (Ning et al., 2021).

A thorough studies using mixtures of LAB strains like *Lb. uvarum* LUHS245 and *Lb. casei* LUHS210 with plant-derived phenolic sources, counting blackcurrant and apple residues, have

revealed marked antimicrobial efficacy against pathogens such as *L. monocytogenes* and *Bacillus cereus*, provides an extremely useful detailed description of the broader applicability and enhanced protection offered by this synergistic approach (Bartkiene, Lele, et al., 2019; Kumar et al., 2017).

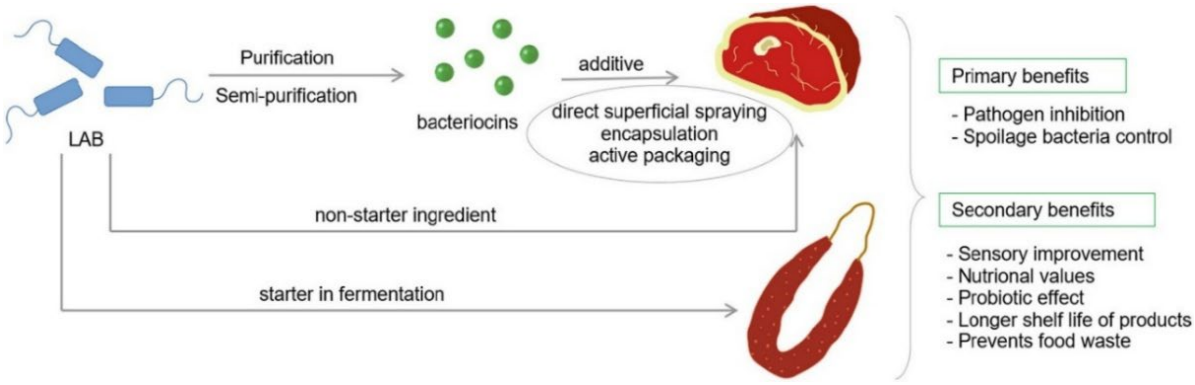


Figure 18 LAB bacteriocins and their application in meat products (Barcenilla et al., 2022).

5.4.Sensory and functional improvements

The incorporation of LAB and PCs not just provides antioxidative and antimicrobial advantages but also can considerably improve organoleptic qualities and functional characteristics of meat products; indeed LAB fermentation produces numerous and valuable metabolites like EPS, alcohols, esters, aldehydes, and ketones, that enhance both texture and aromatic profiles (Y. Wang, J. Han, et al., 2022; Y. Zheng et al., 2020).

Conversely PCs contribute further by structurally modifying meat proteins, thereby exacerbating their solubility, gelation capacity, water-holding capabilities, and overall textural properties; such changes moreover cover undesirable sensory notes that can be triggered by oxidative processes, as showed in studies of Barbieri et al. (2021), Chen et al. (2022) and Xu et al. (2021) employing olive leaf extracts in beef hamburgers; therefore, these combined effects significantly boost global consumer acceptability and marketability.

5.4.1. Flavor and aroma development

Fermentation generates alcohols, esters, acids, and terpenoids, significantly exacerbating sensory attributes (Y. Wang et al., 2021). Although many studies have primarily focused on non-meat products such as rice wine and soy milk, the underlying biochemical processes are analogous and directly relevant to fermented meat products. LAB interacts with PCs during meat fermentation, creating unique flavour profiles and enhancing sensory properties. For example, tea catechins and LAB interactions produce desirable aromatic compounds that could

analogously improve flavour complexity in meat products (Y. Hu et al., 2022; Yon, 2014). Moreover, through meat fermentation, LAB metabolize amino acids, carbohydrates, and lipids, producing esters, alcohols, acids, and ketones that significantly enrich the flavour profile (Carneiro et al., 2024; Y. Wang, J. Han, et al., 2022). PCs further contribute additional aromatic notes, thereby creating complex, appealing sensory experiences in meat.

5.4.2. Texture improvement and water-holding capacity

Phytophenols modify myofibrillar proteins, improving solubility, gel formation, and oxidation stability unfolding secondary structures and reducing surface hydrophobicity (Xu et al., 2021). Quercetin, for example, interacts electrostatically with MPs, maintaining α-helix arrangements and slightly increasing surface hydrophobicity (N. Li et al., 2023). Gallic acid shows a dose-dependent effect on MPs gelation, with low to moderate concentrations upgrading gelling potential by nearly 50% (Cao et al., 2016). All studied phenolics induce MPs unfolding and promote cross-linking via amine or sulfhydryl groups, enhancing gel network formation (Guo et al., 2021).

5.4.3. Colour stabilization

Colour stability is critical for maintaining the visual appeal of meat products. The conjugation of PCs and LAB effectively preserves meat colour by preventing lipid oxidation and myoglobin degradation. LAB-generated LA lowers pH levels, stabilizing heme iron and avoiding undesirable browning reactions (Barcenilla et al., 2022). Simultaneously, PCs inhibit lipid peroxidation, reducing the formation of oxidized pigments that lead to discolouration (Stadnik et al., 2022). Certain LAB strains produce zinc protoporphyrin, stabilizing the red colour of meat (Kauser-Ul-Alam et al., 2021). This dual antioxidant and pH-modifying mechanism preserves myoglobin stability, effectively extending the bright red colour typical of freshly prepared meat, particularly beneficial for sheep and goat meat products prone to rapid oxidation (Cunha et al., 2018).

5.5. Practical applications and industrial relevance

Various practical applications highlight the successful integration of LAB and PCs in meat preservation processes. Research involving co-fermentation techniques with strains such as *Pc. pentosaceus* SWU73571 and *Lb. curvatus* LAB26 in beef patties demonstrated substantial drops in pathogenic bacterial counts *Salmonella enterica* serovar *Typhimurium* and *E. coli* populations by threefold compared to controls, ensuring safer consumption (Zhang et al., 2020). Similar promising findings were demonstrated in goat meat emulsions enriched with *Murraya*

koenigii (the curry tree) extracts combined with LAB-generated bacteriocins, achieving significant microbial reductions under refrigerated conditions (Kumar et al., 2017). Further, vacuum-packed meat products treated with lactocin produced by LAB effectively controlled spoilage organisms, extending product shelf-life (Kalogianni et al., 2020). These case studies collectively underscore the practical and scalable potential of employing LAB and PCs synergy as a natural, clean-label alternative to conventional preservatives.

5.6.Reduction of nitrite usage and clean-label benefits

Along with the safety and sensory benefits already discussed, LAB and PCs can reduce the amount of synthetic nitrite used, meeting consumer desires for healthier meat products. As a result, the meat industry is looking at different preservation techniques, and the synergistic pairing of phenolic with LAB is one of the most notable. Because of their strong antioxidant properties, PCs can successfully take the role of nitrites in curing formulations, significantly reducing the formation of N-nitrosamines while preserving vital organoleptic characteristics including colour and flavour. (Martinez-Zamora et al., 2021). On the other hand, LAB can produce metabolites through pathways such zinc protoporphyrin synthesis that simultaneously guarantee microbiological safety and promote optimal colour development (Kauser-Ul-Alam et al., 2021).

5.7.Modulation of gut microbiota:

Consuming meat products enriched with LAB and PCs may offer additional health benefits by positively modulating the gut microbiota. This combination promotes the proliferation of eubiotic microorganism such as *Lactobacillus* and *Bifidobacterium*, while inhibiting virulent strains (Figure 19).

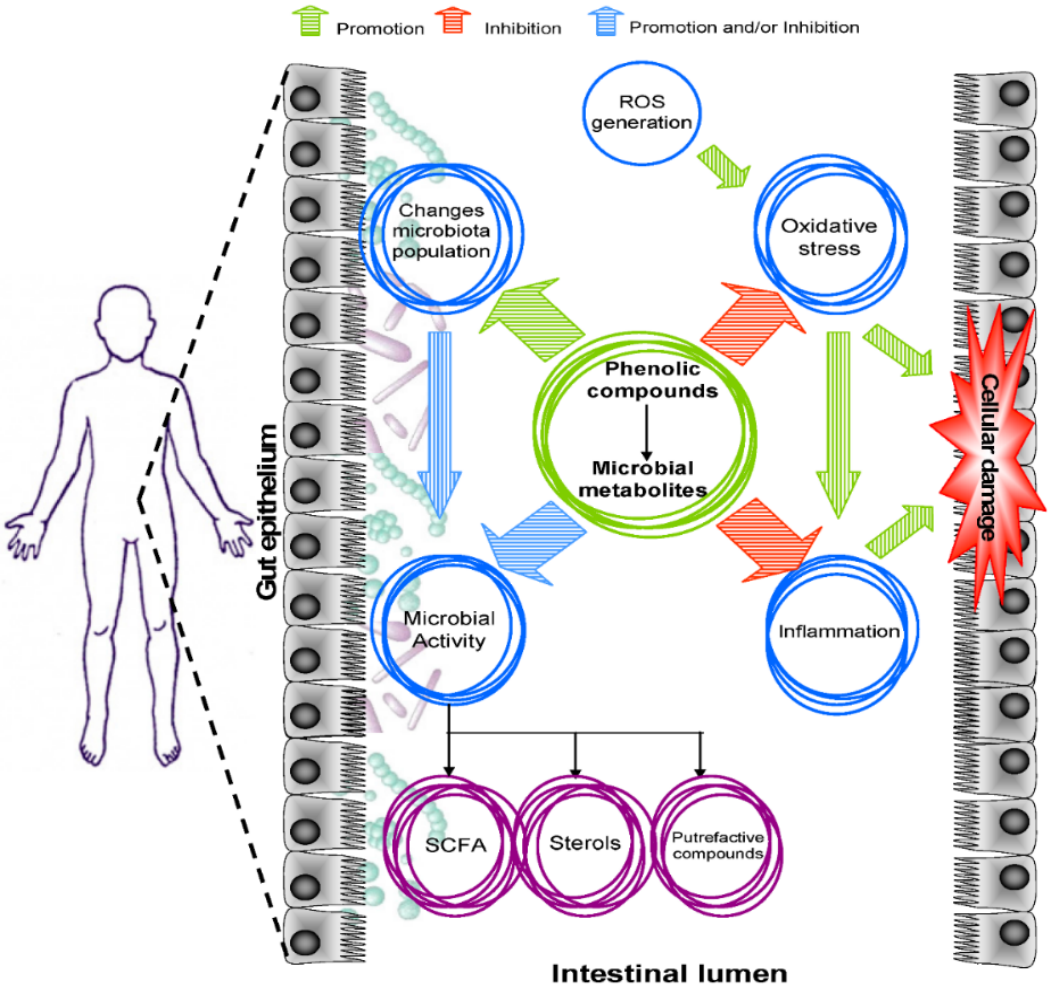


Figure 19 implications of phenolic compounds at the gut level (Mosele et al., 2015).

Enhanced gut microbiota composition can lead to improved digestion, nutrient absorption, and stronger gastrointestinal health. These functional meat products could thus appeal to health-conscious consumers, providing both enhanced product quality and nutritional values. Although promising evidence exists from *in vitro* and animal studies, human clinical trials are necessary to validate these health outcomes fully and to clarify the mechanisms underpinning the interactions between dietary phenolics, probiotics, and gut microbiota (Duenas et al., 2015; Loo et al., 2020; Mosele et al., 2015).

Important issues that need to be addressed in future studies on the use of PCs and LAB in meat products include maintaining phenolic bioactivity during processing and storage (Albuquerque et al., 2021; Carneiro et al., 2024; Chen et al., 2022). Novel techniques include covalent conjugation with dietary fibres and microoencapsulation may improve phenolic bioavailability while reducing oxidative degradation (Carneiro et al., 2024; Kumar & Sharma, 2024); To

maximise antioxidant and antimicrobial activity against multidrug-resistant bacteria, further research is necessary to understand the deep synergistic interactions between phenolics, LAB, and complementing bioactive compounds such as essential oils and phytosterols (Barbieri et al., 2021; Lobiuc et al., 2023).

Advanced delivery systems, including edible coatings and emulsion-based carriers, can optimize the dispersion kinetics and sensory integration of bioactive compounds within meat matrices (Munekata et al., 2020; Roila et al., 2022). Microbial viability under heat and oxidative processing conditions may be improved by the creation of stress-resistant LAB strains in conjunction with advanced encapsulation approches (such as alginate-microencapsulation and starch-based matrices) (Abdelli et al., 2021; Nishimoto-Sauceda et al., 2022; Y. Wang, J. Han, et al., 2022). Additionally, integrating flavor-modulating ingredients like yeast extracts or Maillard reaction products, could counteract the bitterness of formulations high in phenolics and increase their acceptance by consumers (Martinez-Zamora et al., 2021; Petrovic et al., 2025). Designing customised solutions for industrial adoption could be accelerated by interdisciplinary techniques that make use of metabolomics and AI-driven predictive modelling (Hurkul et al., 2025).

RESEARCH METHODOLOGY

6. MATERIALS & METHODS

6.1. Thesis Objectives

This work was designed to evaluate the combined effects of phenolic compounds and LAB on the technological, nutritional, and sensory quality of poultry meat. The study involved the isolation and identification of LAB strains with potential probiotic properties, followed by their functional characterization through *in vitro* assays. Selected strains were then tested in combination with phenolic-rich sources to assess their influence on oxidative stability, microbial safety, and meat preservation parameters.

The overall experimental design aimed to determine the technological applicability and synergistic effects of these natural bioactive components as clean-label alternatives to synthetic preservatives in meat processing.

6.2. Sample collection and bacterial strains

Lactic acid bacteria were isolated from two traditional fermented food matrices: Dhan, a traditional goat milk-derived butter, and fermented waste from green tea (*Camellia sinensis*).

Samples of Dhan, a traditional fermented goat milk butter, were collected from rural households in the Sfisifa region (Naama province, Western Algeria; see Figure 20) in november 2022. Sample collection followed aseptic protocols in accordance with ISO 6887-1:2017 (Microbiology of the food chain - Preparation of test samples, initial suspension, and decimal dilutions for microbiological examination). Three samples were transferred into sterile 100 mL bottles under aseptic conditions and immediately transported to the laboratory under refrigeration (6 ± 2°C) to preserve microbial integrity. All analyses were initiated within 24 hours of collection to minimize compositional and microbiological alterations.

One hundred gram of dried green tea leaves *Camellia sinensis* were commercially sourced from a local market in Mostaganem, Algeria.

The reference probiotic strain *Lacticaseibacillus rhamnosus* GG ATCC 53103 (LGG) was obtained from the French National Institute for Agriculture, Food, and Environment (INRAe), UMR 1319 Micalis Institute, Jouy-en-Josas, France. This well-characterized strain, extensively documented for its probiotic properties (Capurso, 2019), served as a positive control in our experiments. Foodborne pathogens, including *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 10536, were obtained

from the Pasteur Institute, Algeria. The bacterial strains were preserved at - 85 °C in brain heart infusion broth (BHIB; HiMedia Laboratories, Mumbai, India) supplemented with 30% (v/v) glycerol for long-term storage.

All procedures for sample collection and handling strictly followed ISO 7218:2007 guidelines (Microbiology of food and animal feeding stuffs), thereby ensuring the accuracy and reliability of the microbial analyses.

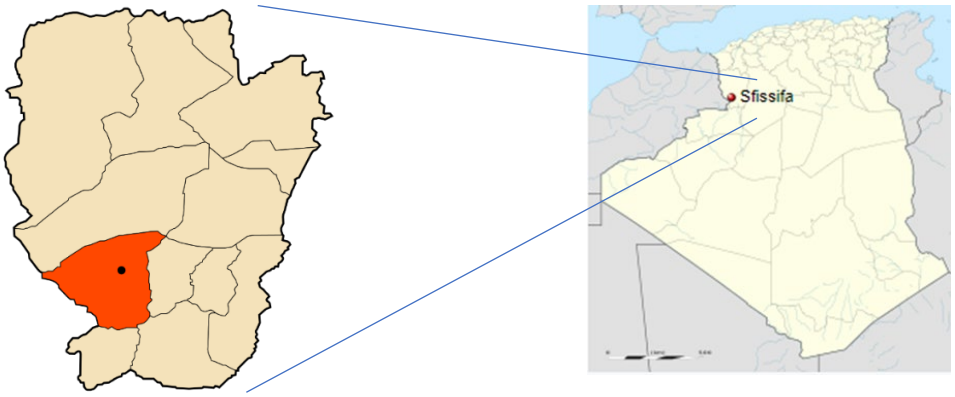


Figure 20 Sampling site location**.**

the geographical location of the Sfisifa region of Naama in western Algeria (32°44′N 0°52′W)

6.2.1. Dhan preparation (traditional goat butter)

Dhan, a traditional butter made from unpasteurized Arabian goat milk, was used as the primary sample in this study. The preparation of Dhan follows a traditional method wherein fresh goat milk is left at ambient temperature which can range approximately from 8°C to 32°C depending on the season and location to undergo natural fermentation for approximately 24 to 48 h, resulting in a sour taste characteristic of the product. Manual churning is performed in a goat-skin butter churn while gradually adding a small amount of fresh, cold water to facilitate the coagulation of fat globules. This stirring process continues for several minutes until the separation of fat from the liquid phase occurs.

The butter fraction is subsequently collected and cured with a salt concentration ranging from 50 g/kg to 80 g/kg. The addition of salt not only aids in the fermentation process but also acts as a preservative, enhancing stability and extending the shelf life of Dhan. This salting step imparts the distinct taste associated with Dhan and ensures the conservation of its sensory and microbiological quality (Adjoudj et al., 2020; Bettache, Adjoudj, et al., 2012). The final product is stored in sealed containers under ambient conditions. This method aligns with traditional practices described for similar products such as Algerian Smen (Lahiri et al., 2022).

6.2.2. Green tea waste preparation

Green tea (*Camellia sinensis*) waste (10 g) was aseptically suspended in 90 mL of sterile peptone water supplemented with 2% (w/v) glucose in a 250 mL GL45 Schott Duran Type 1 laboratory bottle (Schott Duran, Wertheim, Germany), serving as the anaerobic fermentation vessel.

A glucose concentration of 2% (w/v) in fermentation media is quite typical and suitable for fermentations involving plant materials like green tea waste. This lower sugar concentration effectively supports microbial growth and metabolism without causing inhibitory osmotic stress (Mo et al., 2024). Studies show that sugar concentrations around 2% lead to efficient sugar consumption and fermentation within hours to a day, with preservation of bioactive compounds in tea.

The suspension was vigorously agitated to ensure homogeneity and incubated at 30 °C under anaerobic conditions for 7 days (Jin et al., 2021; Lin et al., 2021). The formulation of the medium was designed to optimize LAB growth and metabolic behaviour, with glucose acting as the primary carbon source to drive lactic fermentation. Incubation parameters (temperature, duration) were selected based on prior studies demonstrating their efficacy in enhancing LAB-driven phenolic compound bioavailability and antioxidant activity in plant-based systems (T. Hu et al., 2022; Negi et al., 2022; Zhao & Shah, 2016). Anaerobic conditions were maintained to minimize phenolic oxidation and promote LAB viability, as aerobic exposure can impair metabolic pathways critical to fermentation outcomes (Tako et al., 2020).

Sterile peptone water adhered to ISO 6887-1:2017 guidelines for microbiological sample preparation, ensuring minimal contamination and methodological reproducibility.

6.3. Isolation of LAB culture conditions:

For the isolation of LAB from both fermented green tea waste and Dhan (traditional fermented goat milk butter), the procedure followed ISO 6887-1:2017 standards (Microbiology of the food chain). Ten grams of each sample were aseptically homogenized with 90 mL of NaCl-peptone solution (0.85% NaCl, 0.1% peptone) sterile or phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) using a stomacher for 3 minutes to ensure uniform and thorough mixing. The process was performed under sterile conditions to prevent contamination.

Serial decimal dilutions ranging from 10^{-1} to 10^{-7} were prepared in the same diluent to reduce microbial concentration. From each dilution, 100 µL aliquots were plated using the spread plate method onto MRS agar (pH 5.4) supplemented with 0.5% $CaCO_3$, which aids in visualizing acid production by LAB as clear zones around colonies. The plates were incubated anaerobically at 37°C for 24–72 hours using anaerobic jars with GasPak systems to maintain anaerobic conditions (Amarantini et al., 2019).

Typical LAB colonies were identified by their creamy-white color and 2–3 mm diameter with the presence of clear zones due to calcium carbonate solubilization; characteristics consistent with LAB such as *Lactobacillus* and *Lactococcus* isolated from similar fermented products (Amarantini et al., 2019; Endo et al., 2019). These candidate colonies were then subjected to repeated streaking on fresh MRS agar plates to ensure purity and homogeneity.

6.3.1. Strain conservation

Following the isolation and purification of LAB strains, the obtained pure cultures were maintained on MRS agar slants at 4 °C for short-term use and cryopreserved in MRS broth supplemented with 30% (v/v) glycerol. For long-term storage, cultures were dispensed into three 1.5-ml sterile cryovials and stored at -85 °C to preserve cellular viability and genetic stability. All procedures were conducted following standardized protocols to ensure accuracy and reproducibility, as recommended by Endo et al. (2019).

6.4.Characterization and identification of LAB

Pure LAB cultures were characterized using standardized protocols to evaluate their morphological, cultural, physiological, and biochemical attributes. Morphological traits on MRS agar plates such as colony shape, size, elevation, edge definition, and pigmentation were recorded to distinguish among isolates. Cultural characteristics, including colony surface texture and opacity, were assessed in parallel (Vinderola et al., 2024).

Physiological profiling involved testing the isolates' growth capabilities under varying temperatures, pH levels, and salt concentrations to determine their environmental adaptability. Biochemical analysis included carbohydrate fermentation profiling, enzyme production assays, and acid tolerance evaluations, which are crucial indicators of metabolic functionality and probiotic potential (Endo et al., 2024; Ma et al., 2023; Venugopal et al., 2024)

This multi-criteria phenotypic evaluation provided a comprehensive framework for preliminary strain identification and selection for further probiotic and functional studies.

6.5. Multi-step screening approach

Current methodologies for probiotic strain selection advocate a stepwise screening protocol, which begins with isolation on selective media, proceeds through detailed morphological and biochemical identification, and culminates in functional evaluation for probiotic attributes. This approach efficiently narrows large numbers of isolates to the most promising candidates (Z. Liu et al., 2021; Taye et al., 2021). (Figure 21).

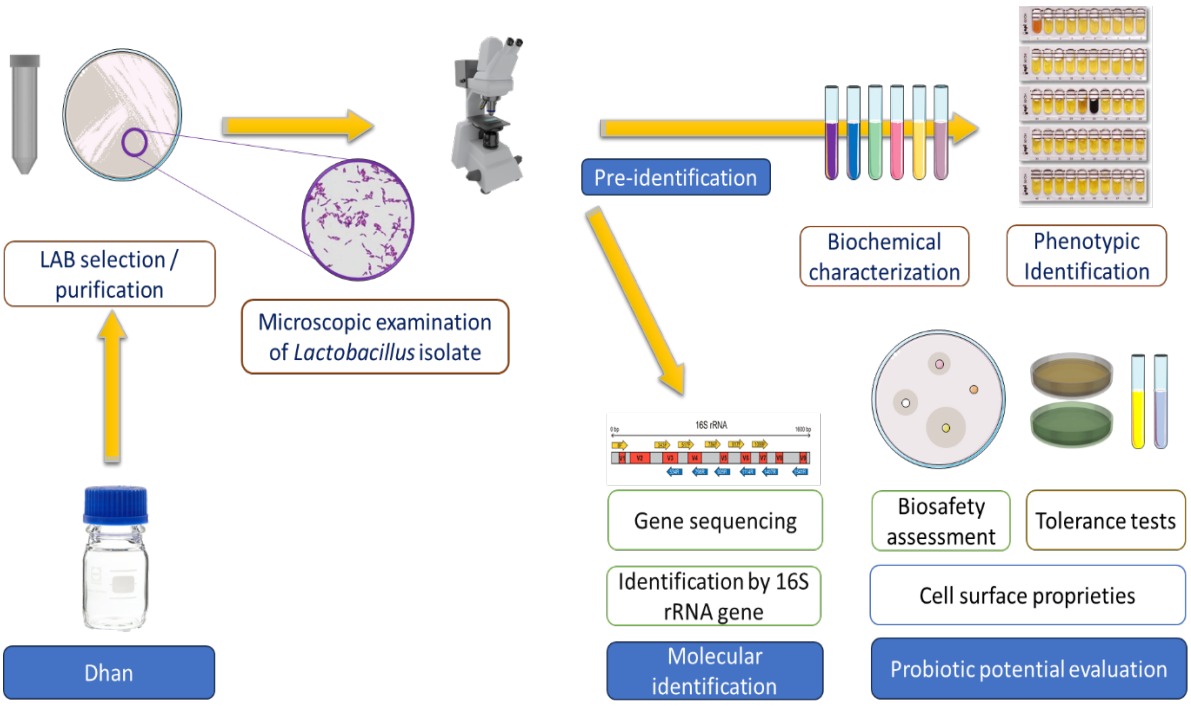


Figure 21 The probiotics evaluation workflow

6.5.1. Gram staining catalase and oxydase production

Gram staining was performed according to the standardized protocol specified in ISO 7218:2007. To prepare the bacterial smears, a loopful of overnight (ON) culture was transferred upon clean, grease-free glass slides. The smears were then heat-fixed by passing the slides gently through a flame to secure the bacterial cells onto the slide surface. Following heat fixation, the slides were covered with 1% (w/v) crystal violet solution and allowed to stand for one minute before being rinsed thoroughly with distilled water. Subsequently, lugol solution was applied as a mordant for one minute to form the crystal violet-iodine complex, enhancing dye retention within the cell walls of Gram-positive bacteria. After iodine application, the slides were decolorized with 95% ethanol for 20 seconds, effectively removing the dye from Gram-

negative cells while retaining it in Gram-positive cells. The decolorization step was followed by counterstaining with safranin for one minute to impart a contrasting red colour to Gram-negative bacteria. After thorough washing and air-drying, the stained slides were examined under a light microscope using oil immersion at 1800× magnification using a Zeiss AxioLab (Zeiss AxioLab, Germany) to correctly distinguish between Gram-positive (purple) and Gram-negative (red) bacteria. To ensure the accuracy and reliability of the Gram staining procedures, both positive and negative controls were utilized: *Escherichia coli* ATCC 10536 served as the Gram-negative control, while LGG as the Gram-positive control.

Catalase production was evaluated according to the standardized protocol outlined in ISO 6887-1:2017. Fresh bacterial colonies were carefully transferred to a clean, grease-free glass slide. A few drops of 3% H_2O_2 solution were gently added to the bacterial mass using a sterile pipette. The immediate formation of oxygen bubbles was recorded as a positive catalase reaction, indicating the presence of catalase enzyme activity. In contrast, the absence of bubble indicated a negative result, suggesting the lack of catalase production. As a positive control, *Staphylococcus aureus* ATCC 25923 was used to ensure the accuracy and reliability of the test results.

For oxidase test A single colony was carefully transferred to a microtube containing an oxidase disk using a sterile toothpick or inoculating loop. The disk was gently agitated to ensure thorough contact between the colony and the reagent. The development of a dark blue or purple colour within 2 minutes was recorded as a positive reaction, indicating the presence of cytochrome oxidase. In contrast, the absence of colour change indicated a negative reaction, confirming the lack of oxidase enzyme activity (Cappucino & Welsh, 2020).

6.5.2. Endospore staining

Spore staining was performed according to the method outlined by Lawalata et al. (2023). A bacterial smear was prepared from a fresh culture on a clean glass slide and heat-fixed. The slide was then flooded with 5% malachite green solution, which served as the primary stain, and steamed gently over boiling water for five minutes to enhance stain penetration. After cooling, the slide was rinsed with distilled water and counterstained with safranin solution for 30 seconds. The prepared slides were examined under an oil immersion objective at 1800× magnification. Spores stained green, while vegetative cells appeared red. Isolates that did not show green-stained endospores were classified as non-spore-forming. Lactic acid bacteria are non-spore-forming microorganisms, which is a key trait distinguishing them from many other

Gram-positive bacteria that produce endospores under stress conditions (Vinderola et al., 2024).

6.5.3. Biochemical characterization of Lactobacillus isolates

The LAB isolates were characterized using a comprehensive series of biochemical assays to evaluate their metabolic, enzymatic, and physiological profiles. These assays featured tests for carbohydrate fermentation patterns, enzyme production, and metabolic activity to assess the functional properties and technological potential of isolates. The tests performed were chosen based on their relevance to food applications, probiotic potential, and the ability to survive and behave in diverse environments, involving gastrointestinal conditions. This thorough characterization enabled the selection of strains with desirable attributes, including acid and bile tolerance, antimicrobial production, and stress resistance.

Citrate utilization, aromatic activity, and motility

Citrate utilization was assessed following general ISO microbiological testing principles (ISO 11133:2014), using a medium adapted for evaluating citrate metabolism in LAB. Lactic acid bacteria isolates were inoculated into Simmon's citrate agar (see Annexe I) and incubated at 37 °C for 48 hours. A positive citrate utilization result was indicated by a change from green colour to blue, suggesting the ability of the isolate to metabolize citrate.

Aromatic activity was assessed by inoculating the bacterial strains into Clark and Lubs medium, followed by incubation at 30 °C for 24 hours. After incubation, the Voges-Proskauer (VP) reaction was performed by adding VPI and VPII reagents to the culture. The presence of a pink ring at the surface of the culture indicated a positive reaction, with varying intensity levels categorized as weak (+), medium (++), or strong (+++), reflecting the degree of acetoin production (Cappucino & Welsh, 2020; Leber & Burnham, 2024).

The motility test was performed by inoculating a bacterial colony into mannitol motility medium and incubating it at 37 °C for 48 hours. The motility assessment was conducted by observing bacterial growth patterns within the medium. Negative motility was represented by growth limited to the inoculation site, while positive motility was demonstrated by diffuse growth spreading from the stab line throughout the medium (Cappucino & Welsh, 2020).

Glucose fermentation test

Glucose fermentation was evaluated following the method described by Gupta et al. (2011) with slight modifications. Overnight bacterial cultures grown in MRS broth were centrifuged

at 10000 rpm for 10 minutes at 4°C, and the resulting pellets were collected and washed twice with PBS buffer (pH 7.2) and resuspended in the same buffer. A volume of 500 µL of bacterial cells suspension was inoculated into 4.5 ml of modified MRS broth supplemented with 1% (w/v) glucose and 0.5% (w/v) phenol red, resulting in an inoculum-to-medium ratio of 1:9 (v/v). A Durham tube was inserted in each culture tube to detect gas production. The tubes were incubated at 37 °C for 24 h under static, anaerobic conditions. After incubation, acid production was indicated by a color change of the medium from red to yellow, while gas production was evidenced by the presence of a gas bubble in the Durham tube. All tests were performed in triplicate.

During homofermentation, the production of acid leads to a colour change from red to yellow in the medium, indicating glucose fermentation. In heterofermentation case, gas production within the Durham tube occurs simultaneously with the colour change, confirming the dual fermentation pattern. Glucose fermentation is a critical criterion for evaluating the metabolic and probiotic potential of LAB strains, as positive glucose fermentation enhances their application in fermented food products by promoting acidification, which contributes to preservation and sensory quality (Vinderola et al., 2024).

6.5.4. Phenotypic identification with API 50 CHL kit assay

Phenotypic identification of the selected isolates was carried out using the API 50 CHL kit (Bio Merieux, S.A., S.N 41.0014 A, France), which is specifically designed to evaluate the biochemical characteristics of LAB. For each isolate, 1 or 2 colonies from freshly grown MRS agar were suspended in 2 ml of API 50 CHL medium and adjusted to a turbidity equivalent to $OD_{600} \approx 0.3$ (McFarland 2.0). The suspension was then distributed into the microtubes of the API strip.

The kit enabled the assessment of carbohydrate fermentation profiles, with positive reactions indicated by a shift in colour to yellow, while aesculin hydrolysis was marked by a dark brown coloration. These changes were interpreted according to the manufacturer's guidelines (Fguiri et al., 2015).

After incubation on the specified medium (see Annexe I) and observation of colorimetric changes, the biochemical profiles of the isolates were compared against reference profiles available in the BacDive database (https://bacdive.dsmz.de/) to achieve precise strain identification (Reimer et al., 2022; Schober et al., 2025). This approach facilitated accurate

taxonomic identification of the LAB strains and generated essential baseline data for subsequent probiotic and technological evaluations.

6.5.5. Molecular identification

Molecular identification of the selected *Lactobacillus* strains was conducted through 16S rRNA gene sequencing to precisely determine bacterial taxonomy and phylogeny. Genomic DNA was extracted using a method adapted from Boucard et al. (2021), in which a single fresh colony was disrupted with 0.25 mm glass beads (Retsch, Haan, Germany) in a 500 µl of distilled water suspension containing approximately 10^{10} *lactobacilli* and 0.1 g of beads. The cell concentration, estimated by optical density ($OD_{600} \approx 1.0$, corresponding to $\sim 10^{10}$ CFU/mL), was used to standardize the extraction procedure.

The homogenization was performed using a Precellys Evolution homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) for two cycles, each consisting of 15 s at 5,800 × g, with a 30 s resting period between cycles. Following homogenization, cell debris was removed by centrifugation at 20,000 × g for 10 minutes at 20 °C, and the supernatant including genomic DNA was carefully collected for subsequent analysis. Extracted DNA was stored at -20 °C until PCR amplification.

The 16S rRNA genes were amplified into 1.5-kb DNA fragments using the primers J4 (**5′-ACGGCTACCTTGTTACGACTT-3′)** and J7 (**5′-AGAGTTTGATCCTGGCTCAG-3′).** The PCR amplification was performed in a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA) as described by Patil and Jena (2019). The PCR master mix contained 0.5 µl of 10X Green buffer KCl-$MgCl_2$ (Jena Bioscience, Germany), 0.5 µl of 10 mM dNTPs mixture (Sigma-Aldrich, St. Louis, MI, USA), 0.1 µl of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific™ Waltham, MA, USA), 1 µl of DNA template (50 ng concentration), and 1 µl of each primer at 10 µM concentration (Eurofins, Luxembourg). The final reaction volume was adjusted to 25 µl by adding 20.9 µl of nuclease-free MilliQ water (Merck Millipore).

The PCR program included a primary denaturation at 95 °C for 15 minutes, followed by 38 cycles of amplification. Each cycle consisted of denaturation at 95 °C for 30 seconds, annealing at 52 °C for 30 seconds, and extension phase at 72 °C for 1 minute and 30 seconds. A final extension phase at 72 °C for 10 minutes concluded the amplification process.

Electrophoresis with 1.5% agarose

The PCR products were separated by 1.5% Agarose gel (Invitrogen UltraPure agarose, Carlsbad, CA) containing 1× TAE buffer (pH 8.0) and included 6 µl Midori Green Advance 8% (Nippon Genetics Europe GmbH, Germany), in a horizontal electrophoresis system (Mupid-One, ADVANCE, Japan) for 30 min at 100 V. The DNA marker 100–1500 bp GeneRuler Ladder Mix (Thermo Fisher Scientific, Lithuania) was utilized. Gel images were recorded using an E-Box documentation system (Vilber Lourmat, France) and saved as TIFF files (Y. Wang, M. Spatz, et al., 2022).

Sequencing and phylogenetic characterization

All DNA sequences were confirmed with 16S rRNA gene sequencing applying the Sanger method, performed at Eurofins MWG Operon (Ebersberg, Germany), utilizing universal primers J4 and J7 (Capurso, 2019). To ensure accurate bidirectional sequencing of the 16S rRNA gene, each sample was divided into two reaction microtubes. The first tube contained 1.5 µL of purified PCR product, 13.5 µL of nuclease-free Milli-Q water, and 2 µL of the forward primer J4, while the second one was prepared using the same volumes but substituting the forward primer with 2 µL of the reverse primer J7. This dual-primer strategy allowed for sequencing of both DNA strands, thus improving the reliability of taxonomic resolution. Each microtube was individually tagged with a unique barcode linked to the corresponding isolate and registered on the Eurofins Genomics platform (https://eurofinsgenomics.eu/) (Church et al., 2020; Kommedal et al., 2012).

The resulting sequences were analyzed employing the Unipro UGENE software v52 (Integrated Bioinformatics Tools) to ensure accuracy and were further subjected to BLAST analysis through the NCBI database to identify homologous sequences (Jaiswal et al., 2022; Rose et al., 2019). The similarity of the obtained sequences with registered strains was determined by comparing them with entries in the EzTaxon genome database (Chalita et al., 2024). Identified 16S rRNA gene sequences were subsequently deposited into the NCBI GenBank to guarantee accessibility and transparency.

Multiple sequence alignment was performed using the ClustalW algorithm in MEGA 12 software. A phylogenetic tree was then constructed using the neighbor-joining method with 1,000 bootstrap replicates to assess the robustness and reliability of the clustering. To further evaluate the stability of the phylogenetic tree, evolutionary distances were calculated using the

Kimura 2-parameter model. Species were assigned based on high sequence similarity (>97%) and clear phylogenetic clustering. (Kumar et al., 2024; Tamura et al., 2021).

6.5.6. Biosafety testing

A comprehensive assessment of the safety profile of the isolates was conducted by evaluating their respective haemolytic activity, DNase production, and gelatinase enzymatic activity.

Hemolytic activity

Hemolytic activity was evaluated following ISO 7932:2004. LAB isolates were inoculated onto Columbia agar plates (BioMérieux®, Craponne, France) enriched with 5% (w/v) sheep blood and incubated at 37°C for 48 hours. The plates were examined for the presence of clear (β-hemolysis), greenish (α-hemolysis), or intact (γ-hemolysis) zones around the colonies. The absence of clear or greenish zones confirmed the non-hemolytic nature of the isolates.

Deoxyribonuclease (DNase) activity

DNase activity was assessed following the protocol outlined in ISO 10272-1:2017, adapted specifically for the evaluation of DNase production in LAB isolates. The test was performed using DNase Methyl Green Agar Medium (HiMedia Laboratories, Pvt. Ltd., Mumbai, India), a medium formulated explicitly to detect DNase production by bacterial strains. This medium contains DNA as the substrate and methyl green as an indicator, forming a stable green complex that provides a visual indication of DNase activity.

To perform the test, 1 µL aliquot of overnight LAB cultures were carefully inoculated onto the surface of DNase Methyl Green Agar plates under aseptic conditions. The plates were incubated for 48 hours at 37 °C to allow for adequate growth and potential enzyme expression. A positive control strain, *S. aureus* ATCC 25923, was included to validate the accuracy and reliability of the assay. Positive DNase activity was indicated by the formation of distinct clear halos around the bacterial colonies, signifying the enzymatic degradation of DNA. In contrast, the absence of clearing was interpreted as a negative result, indicating the lack of DNase production.

Gelatinase activity

Gelatinase production was determined according to method cited by Fugaban et al. (2021). Nutrient gelatin tubes were inoculated with freshly grown bacterial cultures and incubated at 30 °C for 7–14 days to assess gelatin hydrolysis. Following incubation, the tubes were refrigerated at 4° C for 30 minutes to enhance the visibility of gelatin liquefaction. Positive

gelatinase activity was indicated by the complete or partial liquefaction of the gelatin medium, whereas the absence of liquefaction confirmed the non-gelatinolytic nature of the isolates.

The combination of negative results for hemolytic, DNase, and gelatinase activities confirmed the biosafety of the LAB strains for potential use in food applications (Casarotti et al., 2017; Colombo et al., 2018).

Antibiotic susceptibility test

The antibiotic susceptibility of LAB isolates was evaluated using the Kirby-Bauer disk diffusion method according to EFSA Panel on Additives and Products or Substances used in Animal Feed (Additives et al., 2018) and Clinical and Laboratory Standards Institute guidelines (CLSI, 2023). Overnight grown LAB cultures in MRS broth at 37°C were adjusted to a turbidity equivalent to a 0.5 McFarland standard (~1×10^8 CFU/mL). The standardized cultures were evenly swabbed onto MRS agar plates, which are preferred over Mueller-Hinton agar for LAB due to their unique nutritional requirements (Ojha et al., 2023; Stefanska et al., 2021).

Commercial antibiotic disks (Sialchim - Algeria) were placed on the inoculated plates using sterile forceps. Plates were incubated anaerobically at 37°C for 24–48 hours. Inhibition zone diameters were measured with a digital caliper and recorded in millimeters. Results were interpreted using CLSI-established breakpoints for Gram-positive bacteria, classifying isolates as susceptible (S), intermediate (I), or resistant (R) (Roldan-Perez et al., 2023) .

Nalidixic acid (NA) and Levofloxacin (LEV) were included in the test panel as a negative control, since LAB are naturally resistant to quinolones due to their Gram-positive cell wall structure and distinct DNA gyrase configuration (Duche et al., 2023). The absence of an inhibition zone thus confirmed the intrinsic resistance profile of LAB and the validity of the assay

All tests were conducted in triplicate to ensure reproducibility, and results were expressed as mean ± standard deviation (SD). Antibiotics and their concentrations used in the assay are listed in Table 3.

Table 3 Antibiotics used in this study.

Antibiotic	Concentration	Symbol	Antibiotic	Concentration	Symbol
Nalidixic acid	30 µg	NA	***Kanamycin***	30 µg	K
Ampicillin	10 µg	AMP	***Oxacillin***	1µg	OX
Cefotaxime	30 µg	CTX	***Penicillin G***	10 µg	P
Colistin	10 µg	CL	***Rifampicin***	5 µg	RIF
Gentamicin	10 µg	GEN	***Tobramycin***	10 µg	TOB
Imipenem	10 µg	IMP	***Vancomycin***	30 µg	VA
Levofloxacin	5 µg	LEV			

6.5.7. Technological characterization

The technological potential of the isolates was rigorously evaluated *in vitro* through a series of analyses aimed at determining their eligibility for applications in food processing and preservation. These evaluations included a wide range of parameters; such characterization is crucial for determining the functional properties and technological viability of the isolates as probiotic candidates.

Monitoring bacterial growth kinetics.

A growth kinetics study was conducted using MRS broth (see Annex I) in 96-well microplates. For inoculum preparation, bacterial cultures incubated for 24 hours were centrifuged to 10,000 rpm at 4°C for 4 minutes. The resulting cell pellets were washed twice with PBS to remove residual culture medium and the final suspensions were adjusted to an initial concentration of ~ 5×10^8 CFU/mL with McFarland's standards and used to inoculate the MRS medium (pH 6.2). Optical density on 600 nm (OD_{600}) was monitored at 30-minute intervals over a 24-hour period at 37 °C using a TECAN Infinite M200 Pro microplate reader (TECAN, Switzerland). For each evaluated strain, a total of eight wells containing 200 µL of culture were used to ensure experimental reproducibility and minimize inter-well variability. The reference strain LGG was used as a positive control and sterile MRS broth as a negative control.

The experimental growth curves were mathematically modeled using the modified Gompertz equation (Khalfallah et al., 2022), a widely applied sigmoid function to describe microbial growth, by the non-linear regression procedure of the GraphPad Prism 10.5.0.774 software (GraphPad Software, San Diego, CA, USA):

$$Y(t) = Y_M \times exp\left[-exp\left(\frac{K \times e}{Y_M} \times (\lambda - t) + 1\right)\right]$$

Where:

Y(t): OD_{600} at time t (hours)

Y_M: Maximum OD_{600} value (asymptote)

K: Maximum specific growth rate (1/h)

λ: Lag phase duration (h)

e: Euler's number (≈ 2.718)

The fitted curves were used to compare strain-specific growth characteristics and to identify candidate.

Extracellular proteolytic activity

The proteolytic activity of *Lactobacillus* isolates was assessed using a modified method adapted from Araújo-Rodrigues et al. (2021)A 10 µL aliquot of each bacterial culture, containing approximately 1 to 2 × 10^8 CFU/mL, was spot-inoculated onto MRS agar plates supplemented with 10% (w/v) skim milk. The plates were incubated at 37 °C for 72 h, allowing enzymatic hydrolysis of casein to occur.

After incubation, the plates were flooded with 1% (v/v) hydrochloric acid (HCl) to precipitate unhydrolyzed proteins. The appearance of clear zones (halos) around the colonies indicated positive proteolytic activity, reflecting extracellular caseinase production.

Lipolytic activity

Extracellular lipase activity was determined using a qualitative agar spot assay, following a method adapted from Merabti et al. (2019). For this purpose, MRS agar plates were formulated with 1% (v/v) Tween 20 to serve as a lipid substrate. A 10 µL aliquot of each bacterial culture, adjusted to approximately 8 Log CFU/mL, was carefully spotted onto the surface of the prepared agar. Plates were incubated at 37 °C for the duration of 24 to 72 hours. Lipase production was evaluated based on the formation of transparent halos around the colonies, meaning the hydrolysis of Tween 20.

Ethanol tolerance assay

The ethanol tolerance of LAB isolates was evaluated to determine their ability to survive and grow in ethanol-containing environments, a condition relevant to fermented beverages and

certain host-associated niches. The assay was performed according to the method of Kazancıgil et al. (2019) , with minor modifications.

Bacterial cultures were grown overnight in MRS broth and standardized to an OD_{600} of 0.5 ± 0.01, corresponding to approximately 10^8 CFU/mL. Aliquots of 1% (v/v) of the standardized cultures were inoculated into MRS broth supplemented with ethanol at final concentrations of 3%, 6%, 12%, and 15% (v/v). A control culture without ethanol served as a growth reference.

All inoculated tubes were incubated statically at 30 °C, and growth was monitored at 0, 24, and 48 hours by visual inspection of turbidity. Growth intensity was semi-quantitatively scored as follows: (+++) maximum growth; (++) moderate growth; (+) weak growth; (-) no visible growth. Each assay was performed in triplicate to ensure reproducibility.

Oxidative stress tolerance assay

Hydrogen peroxide resistance is an important trait for probiotic strains, as it reflects their ability to withstand oxidative stress commonly encountered during food processing and within the host environment. Evaluating this resistance helps determine the robustness of LAB strains under aerobic and stress-inducing conditions.

The resistance of LAB strains to oxidative stress was evaluated by assessing their tolerance to H_2O_2. Overnight-activated cultures were diluted to 1% in MRS broth containing H_2O_2 at final concentrations of 0.4, 0.7, and 1.0 mM (30 wt.%, Sigma–Aldrich Chemicals, Steinheim, Germany). The inoculated broths were incubated at 37 °C for 8 hours. Following incubation, bacterial growth was measured spectrophotometrically at 600 nm with a Genova Bio UV-visible spectrophotometer (Genova Jenway, Cole-Parmer, UK), and results were expressed as OD_{600} values, indicating the degree of tolerance to oxidative stress (Li et al., 2012).

Growth at different temperatures

Growth temperature tolerance is a fundamental aspect of technological characterization, as it reflects the adaptability of LAB strains to different environmental conditions confronted during food processing, storage, and fermentation. Understanding this capacity helps determine the technological applicability of each strain in a wide spectrum of food matrices.

The thermal adaptability of LAB isolates was evaluated by incubating bacterial cultures under a range of temperature conditions. Following the protocol adapted from Badis et al. (2004), 100 µL of LAB strains inoculum with an OD_{600} of 0.5 ± 0.01 were cultivated in 10 mL of MRS

broth and subjected to incubation at 6, 10, 25, 37, 45, and 50 °C for a period of 5 days. Bacterial growth was monitored daily based on the visual assessment of turbidity, reflecting the capacity of each strain to proliferate under varying thermal conditions relevant to food processing and storage environments.

Autolytic activity

Autolytic activity represents an important technological attribute of LAB, reflecting their capacity to undergo self-lysis and release intracellular constituents such as enzymes and bioactive peptides. This process is particularly relevant in the dairy and meat industries, where autolysis contributes to flavour development, texture modification, and the overall sensory quality of fermented products.

The autolytic activity of LAB isolates was determined following the method of Barzegar et al. (2021), with minor modifications. Eighteen-hour cultures grown in MRS broth at 37 °C were harvested by centrifugation at 10,000 × g for 10 min at 4 °C. The cell pellets were washed twice with sterile phosphate-buffered saline (PBS, pH 7.2) and resuspended in the same buffer to an OD_{600} of 0.6–0.8. The bacterial suspensions were then incubated statically at 37 °C for 24 h.

Autolysis was monitored by measuring the decrease in optical density at 600 nm (OD_{600}), and the percentage of autolysis was calculated using the following equation:

$$Autolysis\ (\%) = [(A_0 - A_{24})/A_0] \times 100$$

where A_0 represents the initial optical density at time zero, and A_{24} is the optical density recorded after 24 hours of incubation.

6.5.8. Strain characteristics associated with probiotic potential

The physiological and functional traits of LAB are key indicators of their probiotic potential. Evaluating these characteristics allows the assessment of strain survival under gastrointestinal conditions and their suitability for use in functional foods and health applications.

pH resistance

The resistance of LAB strains to extreme acidic conditions was assessed to determine their potential probiotic viability under gastrointestinal stress. A 2% bacterial suspension was inoculated into 5 mL of MRS broth adjusted to pH levels of 1.5, 3.0, and 5.0 using 1.0 M HCl. Sterile pH-adjusted MRS broth without inoculum served as a negative control, while

unmodified MRS broth served as a positive control. The cultures were incubated at 37 °C for 24 and 48 hours. Bacterial viability was determined by monitoring turbidity changes in the medium, indicating growth or inhibition (Aktaş et al., 2022).

Simulated gastric juice resistance

The tolerance of the isolated strains to simulated gastric juice (SGJ) was evaluated using a modified protocol based on Kazancıgil et al. (2019). Briefly, overnight LAB cultures were centrifuged (10000×g, 10 min at 4° C), the pellets were washed twice with PBS (pH 7.0), then resuspended into a sterile SGJ solution to a final concentration of 8 log CFU/ml. The SGJ formulation (w/v) consisted of 6.2% NaCl, 0.22% $CaCl_2$, 1.2% $NaHCO_3$, 2.2% KCl, and 0.3% pepsin (Sigma P-6887), with the pH adjusted to 2.5. The solution was sterilized through 0.22 µm PTFE syringe filter (ISOLAB Laborgerate GmbH, Wertheim, Germany) to prevent contamination. As a control, the same bacterial culture was inoculated into standard MRS broth.

To assess bacterial viability, tenfold serial dilutions were performed at time 0 and after 4 hours of incubation at 37 °C, reflecting the average residence time of ingested material in the stomach for bolus-to-chyme conversion (Sensoy, 2021). At each time point, samples were plated on MRS agar, incubated at 37 °C for 48 hours, and the resulting colonies were counted. Viability was expressed in terms of $\log_{10}$ CFU/mL.

The percentage survival was calculated according to the following formula:

$$Survival\ (\%) = [Log_{10}(CFU/ml\ at_{4h}) \div Log_{10}(CFU/ml\ at_{0h})] \times 100$$

Bile salt tolerance assay

Bile salt tolerance was assessed to evaluate the ability of LAB strains to withstand gastrointestinal conditions, specifically bile salt concentrations encountered in the small intestine. This ability was quantified following the method described by Sabir et al. (2010), An aliquot of 250 µL from a LAB suspension adjusted to approximately 8 log CFU/mL was inoculated into 5 mL of sterile MRS broth to 7 log CFU/ml finale and incubated at 37 °C for 24 hours prior to bile salt exposure. Following incubation, bacterial cells were collected by centrifugation at 10000 × g for 10 minutes at 4°C. The pellets were subsequently washed twice with sterile PBS and resuspended in the same buffer.

Afterwards, 100 µL of the LAB suspension was mixed with 900 µL of bile salt solutions at final concentrations of 0.15%, 0.30%, and 0.50% (w/v) ox bile (Sigma-Aldrich, Deisenhofen, Germany). Control mixtures without bile salts were also prepared. All samples were incubated at 37 °C for 2 hours to evaluate the survivability of LAB isolates in the presence of bile salts. After the incubation period, total viable counts were assessed using the pour plate technique to quantify surviving LAB cells. The percentage of bile tolerance survivability was calculated with the following formula:

$$Survivability\ (\%) = (CFU_{bile}/CFU_{control}) \times 100$$

Phenol tolerance assay

Phenol resistance is a key survival trait for probiotics, given the presence of phenolic compounds in the gastrointestinal tract.

Phenol tolerance test was evaluated using a modified protocol based on Mandal et al. (2013), LAB cultures were grown ON in MRS broth at 37 °C to reach the logarithmic growth phase. Subsequently, 2% of each isolate culture was inoculated into 5 mL of MRS broth supplemented with phenol at final concentrations of 0.2% and 0.5%, along with a phenol-free control. The cultures were incubated at 37 °C for 24 and 48 hours. Viability was assessed by plating 100 µL aliquots onto MRS agar, followed by incubation for 48 hours at 37 °C. The presence of colony growth in phenol-containing media indicated phenol tolerance.

Salt stress tolerance:

The tolerance of LAB strains to sodium chloride was examined to evaluate their ability to survive in high-salt environments, relevant for applications in fermented foods.

To further assess NaCl resistance, 2% of each LAB culture was inoculated into 10 mL of MRS broth containing various concentrations of NaCl (2%, 3%, 4%, 6.4%, and 10%). Standard MRS broth without added salt served as the positive control. All cultures were incubated at 37 °C for 7 days. Isolate growth was monitored visually based on turbidity. Strains showing turbidity were recorded as positive (+), while those with no visible growth were recorded as negative (−) (Aktaş et al., 2022).

6.5.9. Adhesion capabilities and cell surface properties

The adhesion and surface properties of LAB strains were evaluated as indicators of their potential to attach to intestinal surfaces and inhibit pathogen colonization.

Auto-aggregation

Auto-aggregation is an important characteristic that reflects a strain's ability to self-associate, which may contribute to the colonization of the intestinal mucosa and biofilm formation. This property plays a crucial role in enhancing microbial persistence in the host and in the competitive exclusion of pathogens. The auto-aggregation ability of the LAB isolates was assessed following the method of Kazancıgil et al. (2019), with some modifications. Overnight cultures were centrifuged at 10000 × g for 10 minutes at 4°C, washed twice with PBS, and resuspended to achieve approximately 8 log CFU/mL. A 200 µL aliquot of this suspension was added to 1.8 mL of PBS in sterile microtubes and vortexed for 10 seconds to ensure homogeneity. The initial absorbance (A_0) was measured at 600 nm using a Genova Bio UV-visible spectrophotometer (Genova Jenway, Cole-Parmer, UK). The suspensions were then incubated at 25 °C without agitation. At 1, 2, and 24 hours post-incubation, 100 µL of the upper layer of each suspension was carefully transferred into a separate vial containing 3.9 mL of PBS. The absorbance at 600 nm (At) was measured an auto-aggregation percentage was calculated applying the following formula:

$$Auto - aggregation\ (\%) = [(A_0 - A_t)/A\_0] \times 100$$

Where:

A_0 measured immediately after vortexing (time 0). And A_t measured after a specified incubation period.

Co-aggregation

The co-aggregation ability of LAB isolates was assessed according to the method described by Kazancıgil et al. (2019). The LAB strains were cultured in MRS broth at 37°C for 24 hours, followed by centrifugation at 10000 × g for 10 minutes at 4°C to collect cell pellets. The pellets were washed twice with sterile PBS and resuspended in the same buffer to adjusted to OD_{600} of approximately 1.0.

To assess co-aggregation, equal volumes (2 mL each) of LAB suspension and pathogen suspension (*S. aureus* ATCC 25923, *E. coli* ATCC 10536) were mixed by gentle vortexing for 10 seconds. The mixtures were then incubated at 25°C without agitation for 4 hours to permit

co-aggregation. After incubation, 1 mL of the upper phase was carefully removed, and the OD_{600} was measured to evaluate the extent of co-aggregation.

Co-aggregation was calculated as follows:

$$Co - aggregation\ (\%) = \frac{[(A_a + A_b)/2] - A_{(a+b)}}{(A_a + A_b)/2} \times 100$$

where "*a*" and "*b*" represent each of the two strains in the control tubes and "*a* + *b*" represents the bacterial mixture.

Bacterial adhesion to hydrocarbons (BATH)

Cell surface hydrophobicity, an indicator of potential adhesion to intestinal mucosa, was measured using the BATH method described by Martiz et al. (2023). LAB cultures grown for ON were centrifuged and washed twice with PBS, then resuspended to OD_{600} ~1.0. than 1 ml of solvant was added to 3 ml of bacterial suspension.

Three different solvents were tested: *p*-xylene (a nonpolar solvent), ethyl acetate (a monopolar basic solvent), and chloroform (a monopolar acidic solvent), all sourced from Merck (Darmstadt, Germany). After adding the solvents to the bacterial suspensions, the mixtures were vortexed vigorously for 2 minutes and incubated at 37 °C for 1 hour to allow for phase separation. During this separation, hydrophobic bacteria migrate to the hydrocarbon layer while hydrophilic bacteria remain in the aqueous phase (Dekic et al., 2017).

Following incubation, the aqueous phase was carefully collected, and its OD_{600} was measured (A_a). The percentage of cell surface hydrophobicity was then calculated using the following formula:

$$Hydrophobicity\ (\%) = [(A_0 - A_a)/A_0] \times 100$$

A_0 = OD_{600} initial and A_a = OD_{600} of aqueous phase.

Biofilm formation assay

Biofilm formation was evaluated using a modified glass tube method based on the procedure described by Fatima et al. (2021). Briefly, 500 µL of bacterial suspension, adjusted to an OD_{600} of 0.1 ± 0.01, was inoculated into 10 mL of sterile tryptone soy broth (TSB) supplemented with 1% (w/v) sucrose. The inoculated glass tubes were incubated at a 45° angle and incubated at 37 °C for 48 hours to facilitate biofilm development. A sterile TSB tube without inoculum served as the negative control.

After incubation, the medium was gently decanted, and the tubes were washed three times with sterile PBS (pH 7.4) to remove planktonic cells. The remaining adherent cells were fixed with 3 mL of 99% methanol for 15 minutes. Following fixation, the methanol was discarded, and the tubes were allowed to air-dry. For staining, 0.3 mL of 0.1% (w/v) crystal violet solution was added to each tube and incubated for 5 minutes. Excess dye was removed by rinsing with running tap water, and the tubes were inverted to dry completely. Biofilm formation was semi-quantitatively assessed by visual scoring, using a 0-4 scale, where 0 indicates no adhesion and 4 indicates strong adhesion.

6.5.10. Biological activities of Lactobacillus strains

Understanding the biological activities of *Lactobacillus* strains is essential for evaluating their functional properties beyond basic probiotic potential.

Antibacterial activity assay

The antibacterial activity of LAB isolates was evaluated to determine their ability to inhibit the growth of common foodborne pathogens. This functional trait reflects the potential use of LAB as natural bio-preservatives in food systems and as probiotic agents contributing to pathogen exclusion in the gastrointestinal tract.

The assay was performed according to the method of Tamariz-Angeles et al. (2023), with minor modifications. LAB cultures grown ON in MRS broth at 37 °C were adjusted to an OD_{600} of 0.8 and incorporated into 20 ml of molten MRS agar (≈ 45 °C). The mixture was poured into sterile Petri dishes and incubated anaerobically at 37 °C for 7 days to allow accumulation of antimicrobial metabolites.

Indicator strains (*S. aureus* ATCC 25923, *E. coli* ATCC 10536, and *P. aeruginosa* ATCC 27853) were cultured in 5 ml of TSB for 24 h at 37 °C and standardized to an OD_{600} of 0.08. Then, 100 µL of each suspension was evenly spread on Mueller-Hinton agar (MHA) plates.

From the previously prepared LAB agar cultures, two agar discs (5 mm diameter) were aseptically cut and placed on the surface of the pathogen-inoculated MHA plates. Discs of MRS agar without LAB served as negative controls. All plates were incubated at 37 °C for 24 h, and antibacterial activity was determined by measuring the diameter of the inhibition zones (mm) around the discs.

Each test was performed in triplicate, and mean inhibition diameters were used for comparative analysis.

Scavenging of 2,2-Diphenyl-β-Picrylhydrazyl (DPPH) free radicals

The DPPH free radical scavenging assay is a widely used method to evaluate the antioxidant potential of probiotic strains. Antioxidant activity is considered a key functional attribute of LAB, as it contributes to cellular protection against oxidative stress in the host and enhances the functional value of fermented foods.

The antioxidant activity of LAB strains was evaluated using a modified DPPH radical scavenging assay as described by Xing et al. (2015). Overnight LAB cultures were centrifuged at 8,000 × g for 10 minutes at 4°C, and the pH of the resulting supernatant was adjusted to 5.5 using 1 M NaOH. To eliminate residual bacterial cells, the supernatant was passed through a 0.22 µm PTFE syringe filter (ISOLAB Laborgerate GmbH, Wertheim, Germany), producing a sterile cell-free supernatant (CFS). was prepared. For the assay, 0.5 mL of CFS was mixed with 1 mL of a fresh 0.1 mM DPPH solution in ethanol (Riedel-de Haën, Sigma-Aldrich, Seelze, Germany). The mixture was vortexed and incubated in the dark at room temperature for 30 minutes, and the absorbance was measured at 517 nm using a Genova Bio UV-visible spectrophotometer (Genova Jenway, Cole-Parmer, UK).

The DPPH radical scavenging activity percentage was calculated using the following formula, with the LGG's CFS and MRS broth (pH 7.0) serving as positive and negative controls, respectively:

$$DPPH\ Scavenging\ Activity\ (\%) = \left[\left(A_{control} - A_{sample}\right)/A_{control}\right] \times 100$$

Where:

$A_{control}$ = absorbance of the DPPH solution without CFS, A_{sample} = absorbance of the DPPH solution with CFS.

6.6. Microencapsulation of LAB

6.6.1. Preparation of strains

LAB strains were cultivated ON in MRS broth at 37 °C to ensure optimal biomass production and viability. After incubation, the cultures were harvested by centrifugation at 10000 × g for 10 minutes at 4° C and washed twice with sterile PBS (pH 6.5) to remove residual medium and non-adherent cells. The bacterial pellet was then resuspended in saline solution (0.9% NaCl) to obtain a uniform suspension and stored at 4° C until further use (Odila Pereira et al., 2016).

6.6.2. Preparation of encapsulation solutions

Microencapsulation was carried out using the extrusion method, whereby LAB isolates or LGG (used as a control) were mixed in equal proportion (1:1) with a sterile 2% (w/v) food-grade sodium alginate solution (Sigma-Aldrich, Germany) as principle biopolymer to produce a uniform suspension. This blend was homogenized using a high-speed homogenizer operating at 9000 rpm for 1 minute to ensure even distribution of bacterial cells, reaching a final concentration of approximately 5×10^{11} CFU/mL.

The homogenized suspension was then extruded dropwise through a sterile 10 mL syringe fitted with a blunt 27-gauge needle into a 2% (w/v) calcium chloride ($CaCl_2$) solution under continuous agitation. Ionotropic gelation occurred instantly as the alginate interacted with calcium ions, forming stable spherical microcapsules. The gelation process was maintained for 30 minutes under constant stirring at 5000 rpm to ensure thorough cross-linking and enhance mechanical stability (Figure 22).

Following gelation, the microcapsules were washed three times with sterile distilled water to eliminate excess calcium ions. For additional purification, the capsules were further rinsed three times using sterile physiological saline through centrifugation at 6000 rpm for 15 minutes at 4° C. The purified capsules were subsequently resuspended in sterile physiological saline and stored at 4° C until analysis.

Negative control microcapsules, prepared without bacterial cells, followed the same procedure to evaluate the structural integrity and encapsulation efficiency in the absence of probiotic loading (Bambace et al., 2019).

6.6.3. Encapsulation efficiency

The encapsulation efficiency (EE) was typically calculated as the percentage of viable cells successfully entrapped in the alginate matrix compared to the initial cell count before encapsulation (Butorac et al., 2021).

As described by Mahmoud et al. (2020), 1.00 g aliquot of freshly prepared beads was suspended in 10 mL of sterile 2 % (w/v) trisodium-citrate solution (pH 7.0; Sigma-Aldrich). The suspension was gently agitated (~150 rpm, 25 °C, 15 min) to chelate Ca^{2+} ions and disintegrate the alginate matrix, then vortex-mixed for 1 min at maximum speed to ensure complete bead dissolution and cell release. The liberated cells were serially diluted (10^{-1}–10^{-6}) in 0.1 % (w/v) peptone water. Aliquots (100 µL) of each dilution were pour-plated, in triplicate,

on MRS agar and incubated anaerobically at 37 °C for 48 h. Results were expressed as colony-forming units per gram of beads (CFU/g).

EE (%) was calculated according to the following equation according to Fareez et al. (2015):

$$EE = [\log_{10} N / \log_{10} N_0] \times 100$$

where

- N denotes the viable count recovered from dissolved beads (CFU/g) and
- N_0 the initial viable count in the alginate inoculum (CFU/ml). Each encapsulation experiment was performed in triplicate; data are reported as mean ± standard deviation.

Bead diameter and morphological uniformity were verified by light microscopy (40×) immediately after production. High EE values, together with uniform bead size, were taken as indicators of a successful microencapsulation process capable of protecting LAB during subsequent exposure to bile salts, gastric acidity, and other gastrointestinal stresses.

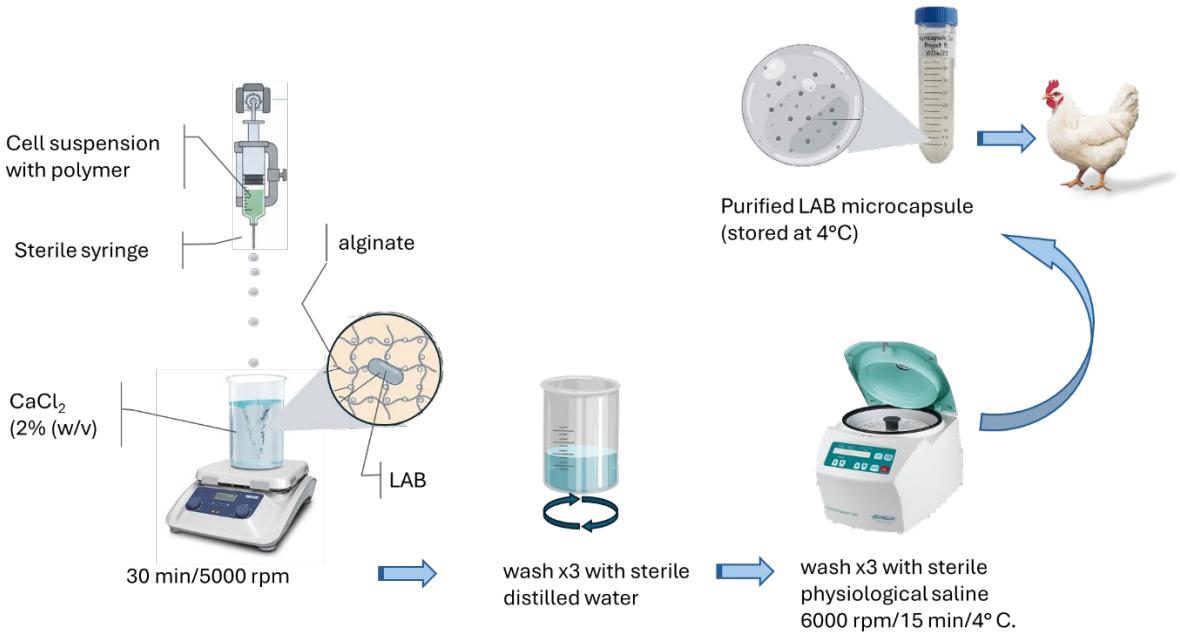


Figure 22 Schematic representation of the encapsulation system.

6.7.In vivo study

This *in vivo* investigation was undertaken to evaluate the functional impact of well-characterized LAB strains on the zootechnical performance, intestinal histo-morphometry, and meat quality attributes of broiler chickens. Probiotics are increasingly considered sustainable alternatives to antibiotic growth promoters, but strain-specific validation under controlled

production conditions remains critical. The present trial aimed to assess whether LAB supplementation could improve growth performance, modulate gut architecture, and enhance carcass and meat traits when integrated into intensive broiler production systems.

6.7.1. Experimental design and animal management

The experiment was carried out at the poultry experimental facility of the Laboratory of Applied Animal Physiology, Abdelhamid Ibn Badis University, Mostaganem, Algeria. A total of 150 one-day-old Arbor Acres broiler chicks (*Gallus gallus domesticus*) were procured from a local commercial hatchery. Upon arrival, chicks were weighed individually to ensure homogeneity and were then randomly allocated into five experimental groups (n = 30 per group), each with comparable initial body weight.

The experimental groups included: (i) a non-supplemented control group receiving only basal diet and plain drinking water, and (ii–v) four treatment groups receiving individual LAB strains, namely DC01-A, DC04, TF03, and TF13. Probiotic preparations were suspended in sterile water and administered through the drinking system at a target concentration of approximately 1×10^9 CFU/mL. The suspensions were freshly prepared on a daily basis to maintain microbial viability and ensure accurate dosing. Supplementation commenced on day 10 post-hatch and continued until the end of the trial at day 42.

Throughout the rearing period, animal handling and monitoring followed international poultry welfare and ethical standards. All experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee of Abdelhamid Ibn Badis University, in line with European Directive 2010/63/EU for the protection of animals used for scientific purposes.

6.7.2. Housing and biosecurity measures

Prior to chick placement, pens were thoroughly cleaned, disinfected, and dried following FAO poultry biosecurity protocols (FAO, 2013). Wood shavings (5 cm depth) were used as bedding material to provide thermal insulation and moisture control, and were replaced whenever wet or excessively soiled. Each pen was equipped with a feeder and nipple drinker to allow uniform feed and water access.

Environmental conditions were continuously monitored and adjusted according to standard broiler husbandry practices (Aviagen, 2021). Temperature was initially maintained at 32–33 °C during the first week, followed by a gradual reduction of 2–3 °C per week until reaching 22 °C. Relative humidity was kept between 55–70%. A standard lighting schedule was applied,

with 23 h light : 1 h dark during the first three days, progressively adjusted to 18 h light : 6 h dark after the first week to promote welfare and optimize growth. Adequate ventilation was provided to maintain air quality and reduce ammonia accumulation.

Strict biosecurity was applied during the trial. Personnel access was limited, protective clothing was used, and routine sanitation was performed to prevent cross-contamination between pens. Daily observations of bird activity, behavior, and droppings were recorded to detect early signs of stress or disease.

6.7.3. Feeding and health management

Birds were fed a nutritionally balanced commercial diet formulated to meet or exceed the nutrient requirements of broilers (NRC, 1994). The feeding program consisted of three phases: starter diet (day 1–14), grower diet (day 15–28), and finisher diet (day 29–42). Diets were provided in mash form, and both feed and water were offered ad libitum throughout the experimental period. Proximate composition of diets was confirmed by laboratory analysis to ensure compliance with NRC standards.

A standard vaccination protocol was implemented, including administration of vaccines against Newcastle disease and infectious bursal disease at recommended intervals. Veterinary oversight was maintained during the entire trial, and any health issues were addressed promptly in accordance with good poultry practice.

Daily records included feed intake, water consumption, and mortality. At the conclusion of the trial, birds were subjected to performance evaluation, intestinal sampling, and carcass analysis to quantify the effects of LAB supplementation on growth, gut morphology, and meat quality.

6.7.4. Zootechnical performance parameters

Evaluation of broiler performance was undertaken using a set of established zootechnical indices—body weight (BW), feed intake (FI), average daily gain (ADG), feed conversion ratio (FCR), and mortality. These metrics represent the primary endpoints for assessing nutrient utilization efficiency, growth kinetics, and overall flock viability in poultry research. When interpreted collectively, they provide robust insight into the physiological and metabolic consequences of dietary probiotic supplementation under intensive rearing conditions.

Body weight (BW)

Individual BW was recorded on days 10, 17, 24, 31, 38, and 42 post-hatch using a calibrated digital scale (±0.1 g accuracy). Weighings were performed in the morning prior to feed

distribution to minimize gastrointestinal content bias, and birds were handled under standardized conditions to reduce stress-induced weight fluctuations. This schedule was selected to coincide with critical growth phases of the broiler production cycle (starter (10–17 d), grower (18–31 d), and finisher (32–42 d)) thereby enabling phase-specific analysis of probiotic effects. Body weight serves as the most direct quantitative measure of growth performance, reflecting both nutrient assimilation efficiency and the physiological balance between anabolic and catabolic processes.

Feed intake (FI)

Feed intake was measured weekly on a pen basis by calculating the difference between feed offered and residual feed recovered at the end of each week. Residues were carefully sieved to remove bedding contaminants before weighing, thus ensuring data reliability. The use of pen-level FI quantification, while accounting for group feeding dynamics, conforms to standardized poultry nutritional assessment methodologies (Aviagen, 2019). FI data provide critical information on potential probiotic-mediated modulation of appetite regulation, feed palatability, and digestive efficiency, all of which directly influence energy and nutrient balance in broilers.

Average daily gain (ADG)

ADG was computed for each production phase as the difference in mean BW between two consecutive weighing points divided by the interval in days. Phase-wise ADG estimations allow identification of growth acceleration or deceleration periods potentially attributable to probiotic supplementation. From a biological perspective, ADG reflects the efficiency of nutrient partitioning toward muscle accretion versus maintenance metabolism, and it is sensitive to improvements in intestinal integrity, enzymatic activity, and microbiota-mediated nutrient bioavailability.

Feed conversion ratio (FCR)

FCR was calculated as the ratio of total feed consumed to total body weight gain within each growth phase and across the entire rearing cycle, with mortality adjustments applied. This correction accounted for the feed consumed by deceased birds and their corresponding body weights, thereby preventing under- or overestimation of feed efficiency. FCR represents the most economically relevant performance index in poultry production, as it integrates both FI and ADG into a single measure of feed utilization efficiency. Improvements in FCR are

typically associated with enhanced digestive functionality, improved gut barrier integrity, and reduced metabolic cost of growth.

Mortality monitoring

Mortality was recorded daily, and all deceased birds were immediately weighed to adjust growth and feed efficiency calculations accordingly. Necropsy was performed where possible to ascertain underlying causes of mortality and to differentiate between management-related and pathological factors. Mortality rates, beyond being a direct welfare indicator, provide complementary evidence of probiotic efficacy in enhancing resilience to production stressors and disease challenges.

Carcass and Organ Measurements

At the end of the trial (day 42), ten birds per treatment group were randomly selected and humanely slaughtered in accordance with European Union guidelines for the protection of animals used in scientific procedures (Directive 2010/63/EU). Carcasses were eviscerated, cleaned, and weighed using a calibrated digital balance (±0.1 g accuracy). Internal organs including the liver, gizzard, heart, and intestines were excised, trimmed of surrounding fat, and weighed individually. Organ weights were expressed relative to live BW (g/kg) to evaluate physiological development and detect treatment-related adaptations. Such assessments are consistent with recent advances in poultry carcass evaluation, where organ indices are increasingly recognized as biomarkers of probiotic-mediated gut and metabolic modulation (Gao et al., 2023; Saleem et al., 2025).

6.7.5. Histological Techniques

Segments (~2 cm) of the mid-jejunum and ileum were collected post-mortem, rinsed with saline, and immediately fixed in Bouin's solution. Samples were dehydrated through graded alcohols, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Morphometric analysis included villus height, crypt depth, and villus-to-crypt (V:C) ratio using calibrated light microscopy coupled with digital imaging software. These indices are widely used as proxies for absorptive surface area and epithelial turnover (El-Saadony et al., 2023). In addition to classical morphometry, recent work has highlighted the importance of coupling histological observations with barrier-function markers (e.g., claudin-1, occludin, MUC2), which provide mechanistic insight into probiotic effects on gut integrity (Chen et al., 2023).

6.7.6. Evaluation of meat quality attributes

Meat samples were collected on day 42, vacuum-packed, and stored at −20°C. Analyses included dry matter, mineral and protein content, lipid profile, lipid oxidation (TBARS), and fatty acid composition.

6.7.7. Physicochemical Analyses

Dry Matter Determination

Dry matter content was quantified according to ISO 1442:2023. Approximately 5 g of homogenized breast meat was transferred into a pre-weighed aluminum dish and dried in a ventilated oven at 105 ± 1 °C until a constant weight was achieved. After cooling in a desiccator, the dish was reweighed. Dry matter (%) was calculated as the percentage of initial sample weight lost during drying. This method conforms to AOAC (2023) guidelines and is critical for normalizing compositional and oxidative measurements across samples.

Mineral (Ash) Content

Ash content was analyzed following ISO 936:1998. About 2 g of homogenized meat was placed in a pre-weighed crucible, incinerated at 550 °C for 8 hours in a muffle furnace, and cooled in a desiccator prior to reweighing. Total ash (%) was calculated as the residue weight relative to the original sample. This method is standard in meat composition studies (e.g., Lagos et al., 2024), facilitating comparative assessments of mineral content in relation to dietary treatments.

Lipid Content

Total lipids were extracted using the Folch method (chloroform–methanol, 2:1 v/v). Approximately 5 g of homogenate was mixed with extraction solvent, thoroughly homogenized, filtered through Whatman No. 1 paper, and partitioned via addition of 0.88% KCl solution. The chloroform (lipid) phase was collected, dried under nitrogen to prevent oxidation, and weighed gravimetrically. This methodology aligns with current AOAC (2023) standards and remains a robust protocol for accurate lipid quantification.

Protein Determination

Protein content was determined using the Kjeldahl method (ISO 937:2023). Approximately 1 g of sample underwent digestion with concentrated H_2SO_4 in the presence of catalysts (K_2SO_4 and $CuSO_4$). After complete digestion, the mixture was neutralized with NaOH and distilled; liberated ammonia was trapped in boric acid and titrated using an automatic distillation-titration system (Büchi Labortechnik AG, Switzerland). Total nitrogen was converted to crude

protein using a factor of 6.25. This method is consistent with AOAC's (2023) official protocol and allows for precise estimation of total protein content.

Lipid Oxidation (TBARS Assay)

Lipid peroxidation was evaluated via the thiobarbituric acid-reactive substances (TBARS) assay, following a modified protocol adapted from recent applications in meat research (Le et al., 2024). Two grams of homogenized meat were mixed with 10 mL of 5% (w/v) trichloroacetic acid (TCA) containing EDTA and propyl gallate, centrifuged at 4,000 × g for 15 minutes at 4 °C, and the supernatant was recovered. Two milliliters of the supernatant were reacted with 2 mL of 0.02 M TBA and incubated at 95 °C for 30 minutes. Following cooling, absorbance was measured at 532 nm using a UV–VIS spectrophotometer. TBARS were expressed as mg malondialdehyde (MDA) equivalents per kg of meat. The sensitivity of TBARS to oxidative changes renders it indispensable for assessing storage and dietary intervention effects on fresh meat quality (Muzolf-Panek et al., 2024).

Fatty Acid Composition Analysis

Fatty acid composition was determined to evaluate the impact of probiotic supplementation on the nutritional and functional quality of broiler meat lipids. Fatty acids are critical indicators of meat health value, oxidative stability, and consumer acceptability, as they influence the balance of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA), including essential fatty acids and long-chain n-3 PUFA (DHA, EPA). Given the rising interest in poultry as a functional food source, precise quantification of fatty acids provides insight into how dietary interventions may shift lipid metabolism (Krusinski et al., 2024; Sahin et al., 2023).

Lipid Extraction and Transesterification

Total lipids were first extracted using the Folch method (chloroform–methanol, 2:1 v/v), which remains the gold standard for lipid recovery from biological tissues due to its high yield and reproducibility. Following extraction, fatty acids were transesterified into fatty acid methyl esters (FAMEs) using methanolic KOH (2%, w/v). This step ensures efficient conversion of triglycerides, phospholipids, and free fatty acids into volatile derivatives suitable for gas chromatographic separation.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

FAMEs were analyzed using an Agilent 7890B GC–MS system equipped with a highly polar capillary column (DB-23; 30 m × 0.25 mm i.d., 0.25 µm film thickness). Injection was carried out in split mode (20:1) at 250 °C. Helium was used as the carrier gas at a flow rate of 1.0

mL/min. The oven program started at 50 °C (held for 1 min), ramped at 10 °C/min to 230 °C, and held for 10 min to allow separation of long-chain PUFAs. The detector was operated in electron impact (EI) mode, with spectra collected over 50–550 m/z.

Retention times were compared with a certified FAME standard mixture (Supelco 37, Sigma-Aldrich, USA) to ensure accurate peak identification. Additionally, spectral matching was performed against the NIST 20 library for confirmatory identification. To account for variability, internal standards (C19:0 methyl ester) were included to correct for extraction and injection efficiency. Fatty acids were expressed as a percentage of total identified FAMEs, enabling direct comparison across treatments.

6.7.8. Nutritional Indices

Beyond individual fatty acids, several nutritional indices were calculated to better understand the functional implications of fatty acid shifts:

- PUFA/SFA ratio – a marker of lipid healthiness, with higher values linked to cardioprotective potential.
- n-6/n-3 ratio – critical for evaluating the pro-inflammatory versus anti-inflammatory lipid balance.
- Atherogenic index (AI) and thrombogenic index (TI) – calculated following Ulbricht & Southgate (1991), providing an integrated assessment of the potential role of dietary fats in cardiovascular disease risk.
- Hypocholesterolemic/hypercholesterolemic index (h/H) – reflecting the balance between cholesterol-lowering and cholesterol-raising fatty acids (Muzolf-Panek et al., 2024).

6.7.9. Biological Relevance

Recent studies demonstrate that probiotic supplementation can significantly alter meat lipid profiles by enhancing the deposition of health-promoting n-3 PUFA and modulating desaturase and elongase enzyme activities (Gao et al., 2023; El-Saadony et al., 2023). In particular, increases in DHA and EPA have been reported in probiotic-fed broilers, suggesting a role for gut microbiota in regulating systemic lipid metabolism. Fatty acid analysis, therefore, provides not only compositional data but also mechanistic insight into how microbiota-derived metabolites influence host lipid physiology.

6.7.10. Sensory Evaluation

Panel Selection and Training

Sensory evaluation of cooked breast meat (*Pectoralis major*) was performed by a trained panel consisting of 10 individuals (5 males, 5 females; aged 22–45 years) recruited from the University of Mostaganem's Department of Food Science. Panelists were pre-screened for normal olfactory and gustatory acuity using standardized tests (basic taste solutions and color–odor recognition exercises) and confirmed to be regular poultry consumers. Training sessions were conducted over two weeks prior to evaluation to ensure panelists' familiarity with the attributes under study and to minimize intra- and inter-individual variability. Training followed the recommendations of ISO 8586:2012 (IOS, 2012) for the selection and training of sensory assessors, with reference to poultry-specific evaluation protocols (Campo et al., 2023).

Sample Preparation

Chicken breast fillets were trimmed of visible fat and connective tissue, then standardized to approximately 100 g portions with a thickness of ~2 cm to ensure uniform cooking. Samples were cooked in a convection oven (Rational SCC, Germany) at 180 °C until an internal core temperature of 75 °C was reached, as monitored using a digital thermocouple probe. Cooking was performed without seasoning or additives to avoid flavor masking. Cooked samples were cooled to 40 °C, cut into 2 × 2 × 2 cm cubes, and served immediately to panelists in pre-coded, randomized containers to prevent bias. Samples were presented under controlled lighting to minimize visual perception differences.

Evaluation Protocol

A 9-point hedonic scale (1 = dislike extremely, 9 = like extremely) was used to assess the following sensory attributes:

- Color – appearance of the meat surface and cross-section.
- Tenderness – ease of bite and breakdown during mastication.
- Juiciness – perceived moisture release during chewing.
- Flavor – intensity and balance of characteristic poultry flavor.
- Overall acceptability – integrated impression of meat quality.

Panelists were instructed to cleanse their palates between samples using distilled water and unsalted crackers to minimize carry-over effects. Each panelist evaluated three coded samples per session, with sessions replicated on two different days to reduce temporal variability.

Evaluations were conducted in individual sensory booths under controlled temperature (22 ± 2 °C) and neutral white lighting, in accordance with ISO 8589:2007 (ISO, 2007).

6.8.Statistical Analysis

All experiments were conducted in triplicate (biological replicates), and measurements within each replicate were performed at least in duplicate (technical replicates) to ensure accuracy and reproducibility. Data are presented as mean ± standard deviation (SD).

6.8.1. Data Screening and Assumption Testing

Prior to hypothesis testing, datasets were screened for conformity to parametric assumptions. Normality was assessed using the Shapiro–Wilk test, while homogeneity of variance was evaluated using Levene's test. Outliers were identified via boxplot analysis and verified using standardized residuals (> ±3 SD), though none required exclusion. When parametric assumptions were violated, non-parametric alternatives such as the Kruskal–Wallis test were applied, followed by Dunn–Bonferroni multiple comparisons.

6.8.2. Hypothesis Testing

For normally distributed data with homogeneous variances, a one-way analysis of variance (ANOVA) was performed to evaluate overall differences among treatment groups. When ANOVA indicated significance ($p < 0.05$), Tukey's Honestly Significant Difference (HSD) post hoc test was applied for pairwise comparisons, ensuring control of family-wise error rate. In addition to p-values, effect sizes (η^2 for ANOVA, Cohen's d for pairwise comparisons) were calculated to provide a quantitative estimate of the magnitude of differences, as increasingly recommended in food and nutrition research (Nakagawa et al., 2023).

6.8.3. Statistical Software and Visualization

All statistical analyses were conducted using IBM SPSS Statistics 26.0 (IBM Corp., Armonk, NY, USA). Data visualization was performed with GraphPad Prism 10.5.0 (GraphPad Software, San Diego, CA, USA). Graphical outputs included bar graphs with error bars (mean ± SD), box-and-whisker plots to display data distribution, and scatter plots for correlation analyses. Superscript letters were used in figures and tables to denote statistically homogeneous subsets as determined by post hoc testing.

6.8.4. Significance Level and Reporting Standards

A probability level of $p < 0.05$ was considered statistically significant throughout. Results were reported in accordance with ARRIVE guidelines for in vivo animal experiments and the APA statistical reporting recommendations, ensuring transparency and reproducibility. Where appropriate, confidence intervals (95% CI) were provided alongside p-values to allow for more nuanced interpretation of results.

RESULTS AND DISCUSSION

7. RESULTS AND DISCUSSION

7.1.Sample collection and bacterial strains

Lactic acid bacteria inhabit diverse ecological niches, including dairy, plant-based, and spontaneously fermented foods, due to their versatile metabolic requirements and adaptive surface structures (Ayivi et al., 2020; McAuliffe, 2018). These heterogeneous environments exert selective pressures that drive the genetic and phenotypic variability observed among LAB strains. In this study, raw goat-milk butter and spontaneously fermented green tea waste were used as isolation sources because both matrices are nutrient-rich, mildly acidic, and traditionally processed, thereby providing favourable conditions for LAB proliferation.

Five morphologically distinct colonies per plate were randomly selected and restreaked twice on fresh MRS agar to ensure purity. Based on growth vigour and phenotypic diversity, a total of 40 isolates were retained for downstream characterization: 20 from fermented tea waste (coded TF) and 20 from goat-milk butter (coded DC).

Gram staining and microscopic examination using a light microscope (AxioLab.A1, Zeiss, Oberkochen, Germany; 1800× total magnification) confirmed that all isolates were Gram-positive, non-spore-forming rods occurring singly or in short chains (Figure 23B and Table 4). Standard biochemical tests, including catalase, oxidase, and glucose fermentation assays, were performed to verify LAB identity.

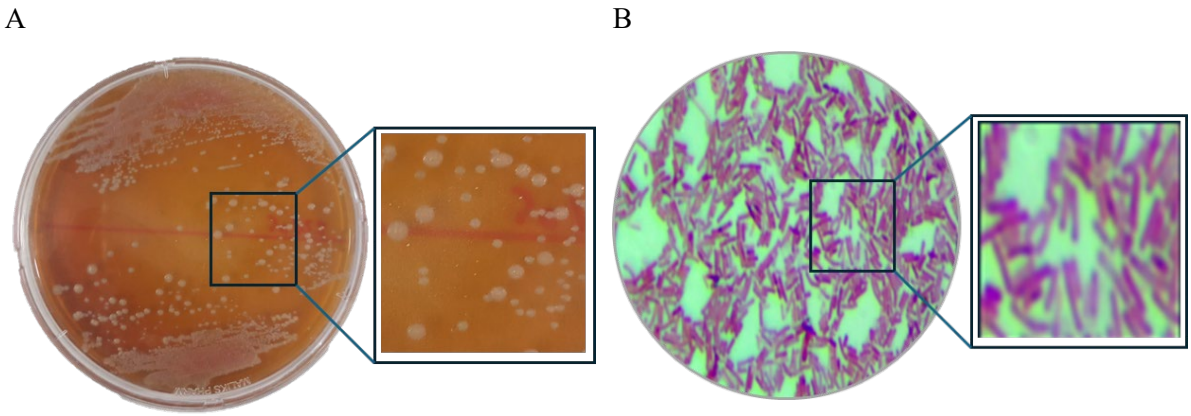


Figure 23 Colony morphology on MRS agar for 24 h at 35 °C
(A) and Gram- stained cells view (B) of TF03 (under 100x immersion objective)

The phenotypic and biochemical traits observed in the isolates closely resembled those reported for *Lactobacillus sensu lato* species commonly recovered from fermented foods (Amarantini et al., 2019; Casarotti et al., 2017; Jin et al., 2021; Torres-Maravilla et al., 2022). According to *Bergey's Manual of Systematic Bacteriology* (A. Galushko & J. Kuever, 2021), the combination

of Gram-positive, non-spore-forming, catalase-negative rods exhibiting LA fermentation is characteristic of the genus *Lactobacillus*. This preliminary taxonomic assignment was subsequently confirmed by molecular identification based on 16S rRNA gene sequencing, consistent with the reclassification of *Lactobacillus sensu lato* proposed by J. Zheng et al. (2020).

Table 4 Preliminary tests, physiological and biochemical characteristics of lactobacillus strains isolated from Dhan (DC) and fermented tea wast (TF).

Strains group	Gram	Forme	Catalase	Oxydase	Mobility	VP	Cit	Gaz from Glu
DC	+	Bacille	-	-	-	-	-	+
TF	+	Bacille	-	-	-	-	-	+

+; positive, -; negative, (VP); Vosges-Proskauer, Cit; citrate fermentation, Glu; glucose fermentation.

7.1. Multi-step screening approach

A structured multi-step screening strategy was employed to systematically narrow the large pool of LAB isolates to a select cohort of technologically and functionally superior probiotic candidates. The workflow comprised four consecutive tiers designed to progressively refine isolate selection according to safety, technological robustness, and probiotic potential.

The first tier involved broad phenotypic screening on MRS agar, evaluating colony morphology, acid-halo diameter, and gas formation. Isolates exhibiting typical *Lactobacillaceae* characteristics were subjected to a second tier of confirmatory biochemical tests, including Gram staining, catalase, oxidase, and motility assays, to verify genus identity and fermentative metabolism.

The third tier applied a biosafety filter to exclude strains carrying transferable antibiotic-resistance or virulence determinants. Screening focused on common resistance genes (*tetM*, *ermB*, *vanA*, *cat*), haemolytic activity, and gelatinase production, following the Additives et al. (2018) safety framework.

The fourth tier evaluated technological and probiotic attributes relevant to food-processing resilience and host interaction. Assays assessed acid and bile tolerance, proteolytic and lipolytic activities, autoaggregation, hydrophobicity, and antagonism against selected foodborne pathogens.

At each tier, quantitative thresholds were defined in accordance with Additives et al. (2018) and ISAPP (2022) recommendations. This sequential elimination process yielded isolates that combined demonstrated safety, strong techno-functional performance, and promising mucosal-colonisation potential, forming a robust foundation for subsequent *in vivo* validation and application in meat-preservation studies.

7.1.1. Biochemical characterization of Lactobacillus isolates

Citrate utilization, aromatic activity, and motility

The selected isolates were cultivated in MRS broth under both aerobic and anaerobic conditions to evaluate growth behaviour. All isolates exhibited consistent growth under both atmospheres, indicating a facultatively anaerobic metabolism. None of the isolates produced a color change from green to blue, confirming the absence of citrate metabolism. No diacetyl production was detected in any isolate. Similarly, motility was examined using semi-solid mannitol motility agar, with no diffuse growth observed, confirming the non-motile phenotype typical of *Lactobacillus sensu lato* species.

These results align with reports describing LAB from raw cow and goat milk that lack citrate-permease (*citP*) and citrate-lyase (*citDEF*) operons, thereby exhibiting no aroma compound synthesis (Fguiri et al., 2015; Hadef et al., 2023; Yelnetty et al., 2020). The absence of these metabolic traits suggests adaptation to low-citrate ecological niches such as fermented goat butter or plant-derived matrices, where carbohydrate fermentation pathways are prioritized over citrate-derived flavour metabolism.

Glucose fermentation test

Homo- or heterofermentative metabolism was determined using the Durham tube method. All isolates produced visible gas, classifying them as heterofermentative. This phenotype corresponds to metabolic profiles described in species such as *Lmb. fermentum* and *Lev. brevis*, commonly isolated from cereal- and plant-based fermentations (Lancetti et al., 2021; Vinderola et al., 2024). The heterofermentative pathway yields LA, CO_2, ethanol, and acetic acid, each contributing distinct functional properties in food preservation. Lactic acid lowers pH, while ethanol and acetic acid exert antimicrobial effects by disrupting bacterial membranes (Martin et al., 2022; Vieco-Saiz et al., 2019).

Although gas production is uncommon among LAB from traditional dairy fermentations (de Almeida Júnior et al. (2015) reported only 12% of goat milk isolates exhibiting this trait) the strong gas formation observed here suggests adaptation to non-dairy or mixed-origin substrates

such as fermented tea residues. Comparable patterns have been documented for *Lpb. plantarum* from tea-based fermentations, which predominantly display homofermentative metabolism with limited CO_2 release (Y. Hu et al., 2022; Li et al., 2022). Collectively, these observations indicate that the heterofermentative metabolism of the isolates may confer advantages in specific technological contexts, such as plant-based or aerated fermentations.

Phenotypic identification using the API 50 CHL assay

The carbohydrate fermentation profiles of the selected LAB isolates were determined using the API 50 CHL identification system (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's protocol.

The resulting carbohydrate utilization profiles were compared with those of reference LAB strains in the Bacdive database and recent taxonomic frameworks. Eleven isolates exhibited profiles congruent with *Lev. brevis*, *Lentilactobacillus spp.*, and *Lpb. plantarum* subsp. *plantarum* (Table 5 Figure 24), consistent with the reclassification proposed by J. Zheng et al. (2020). These taxa were historically included within the *Lactobacillus sensu lato* group and are recognized for their technological relevance and probiotic potential, particularly their ability to acidify substrates rapidly and produce antimicrobial metabolites such as organic acids and bacteriocins (Cassani et al., 2020).

Species of *Levilactobacillus* and *Lactiplantibacillus* were prevalent among isolates from both fermented goat butter (Dhan) and fermented tea waste, indicating adaptation to diverse ecological niches. In Dhan, low pH and anaerobic processing conditions favor acid-tolerant, facultatively anaerobic LAB (Bettache, Adjoudj, et al., 2012; Boussekine & Barkat, 2022; Karim et al., 2022), while in fermented tea matrices, polyphenol-rich substrates and microaerophilic environments selectively promote polyphenol-tolerant LAB communities (T. Hu et al., 2022; Jin et al., 2021; Li et al., 2022).

Although the API 50 CHL system provides valuable insight into carbohydrate metabolism, its taxonomic resolution is limited. Closely related species with overlapping fermentation profiles can yield ambiguous results (Abdelsalam et al., 2022). Therefore, API 50 CHL data were interpreted as indicators of metabolic potential rather than as definitive taxonomic evidence. In line with current recommendations (Urshev et al., 2024), these phenotypic data were integrated into a polyphasic approach combining biochemical, physiological, and molecular analyses to ensure accurate classification.

Table 5 Identification of LAB isolates by API 50 CHL.

Isolates	ID	Species identified by API test	BacDive ID
DC01-A	8596	*Levilactobacillus brevis*	6656
DC02	8596		
DC04		*Lactiplantibacillus paraplantarum*	
DC06	8596	*Levilactobacillus brevis*	6656
DC08			
DC09			
DC10	8596	*Levilactobacillus brevis*	6656
TF03	8596	*Levilactobacillus brevis*	6656
TF04	7968	*Lentilactobacillus diolivorans*	6591
TF13	8042	*LeviLactobacillus namurensis*	6667
TF17	8255	*Lactiplantibacillus plantarumsub sp. plantarum*	6500

DC1-A *TF04*

Figure 24 DC1-A and TF4 isolat profile on API50CH

7.1.2. Molecular identification by 16S rRNA gene sequencing

To enhance taxonomic resolution beyond phenotypic identification, 16S rRNA gene sequencing was performed for all selected isolates. This genotypic method offers greater discriminatory power and complements the phenotypic data, enabling the establishment of a more robust taxonomic framework for the isolated LAB strains. Thus, strains were identified at the genus level, using sequencing of PCR amplicons (Figure 25) of 16S rRNA genes.

Strain identification was assigned at the genus level for sequence similarities ≥97% and at the species level for ≥99%. The resulting phylogenetic affiliations confirmed the classification of isolates within the genera *Levilactobacillus*, *Lentilactobacillus*, and *Lactiplantibacillus*, corroborating the results obtained from API 50 CHL phenotypic analysis.

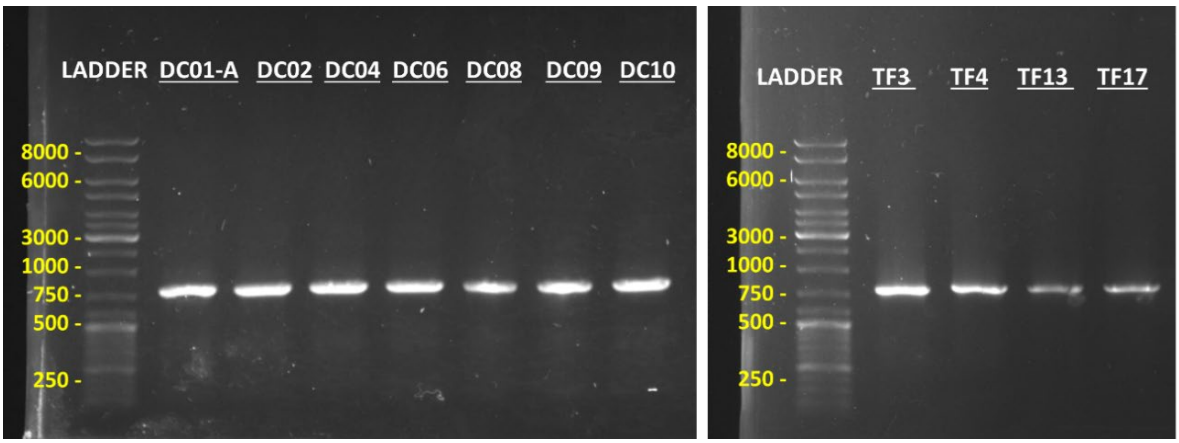


Figure 25 PCR products for LAB isolates in 1% agarose gel. *Lane 1 (DNA Ladder), lanes 2–11 (selected isolates).*

Molecular identification

The 16S rRNA gene is an established molecular marker for bacterial systematics, combining conserved regions that permit universal primer binding with hypervariable domains providing genus- and species-level resolution (Ruiz-Rodríguez et al., 2017). For LAB, this approach offers superior discriminatory power compared with phenotypic characterization alone (M. Silva et al., 2023), a critical advantage given the recent reorganization of the *Lactobacillus sensu lato* complex (J. Zheng et al., 2020).

All isolates previously identified phenotypically as *Lactobacillus spp.* were subjected to 16S rRNA gene sequencing for genotypic confirmation. Nearly full-length 16S rRNA genes (~1,450 bp) were amplified and sequenced bidirectionally, and the resulting consensus sequences were compared with those available in the NCBI GenBank and EzBioCloud (EzTaxon) databases using the BLASTn algorithm. Sequence identity thresholds of ≥97% and ≥99% were applied for genus- and species-level assignments, respectively (Hackmann, 2025; Qiao et al., 2022).

BLAST and EzTaxon analyses revealed ≥99% sequence similarity with validated reference strains (Table 6 and Table 7). Two isolates were assigned to the genus *Levilactobacillus* and one to *Lactiplantibacillus*, consistent with the revised taxonomic scheme of J. Zheng et al. (2020). The high congruence between phenotypic and molecular results confirms the

robustness of the identification and highlights the effectiveness of a polyphasic strategy that integrates biochemical and genomic data for accurate LAB classification in complex fermented ecosystems.

Table 6 Molecular identification of selected strains isolated from Dhan using the NCBI (nt_prok database).

Isolates	Top-hit taxon	GenBank accession No	Identity (%)	Query cover (%)
DC01-A	*Lev. brevis strain 6525*	MT515953	99.93	99.00
DC02	*Lev. brevis strain ATCC 14869*	NR_044704	99.45	100
DC04	*Lpb. plantarum strain ZYL14*	PP215884	99.86	100
DC06	*Lev. brevis strain 3379*	MT512165	99.00	99.86
DC08	*Lev. brevis strain ATCC 14869*	NR_044704	99.45	99.00
DC09	*Lev. brevis ATCC 14869, DSM 20054*	NR_116238	99.93	99.00
DC10	*Lev. brevis ATCC 14869, DSM 20054*	NR_116238	99.93	98.00
TF03	*Lev. brevis strain NZ5*	OQ660315.1	1 00	99
TF04	*Lev. brevis strain 2096*	MT604645.1	100	100
TF13	*Lev. brevis strain PS 7305*	MF966519.1	99.86	99
TF17	*Lev. brevis strain NWAFU 1629*	OR240802.1	100	96

Table 7 Percent sequence similarity between isolated strains and type strains of validly published prokaryotic names (available online http://eztaxon-e.ezbiocloud.net/).

Isolates	Top-hit taxon	Top-hit strain	Similarity (%)	Completeness (%)
DC01-A	*Lev. brevis*	*ATCC 14869*	*99.45*	*97.5*
DC02	*Lev. brevis*	*ATCC 14869*	*99.66*	*97.4*
DC04	*Lpb. pentosus*	*DSM 20314*	*99.86*	*95.7*
DC06	*Lev. brevis*	*ATCC 14869*	*99.38*	*98.1*
DC08	*Lev. brevis*	*ATCC 14869*	*99.38*	*97.6*
DC09	*Lev. brevis*	*ATCC 14869*	*99.38*	*97.6*
DC10	*Lev. brevis*	*ATCC 14869*	*99.45*	*97.6*
TF03	*Lev. brevis*	ATCC 14869	99.79	97.0
TF04	*Lev. brevis*	ATCC 14869	99.93	96.2
TF13	*Lev. brevis*	ATCC 14869	99.25	98.2
TF17	*Lev. brevis*	ATCC 14869	99.86	96.8

All 16S rRNA gene sequences obtained from the selected *Levilactobacillus* and *Lactiplantibacillus* isolates have been deposited in the NCBI GenBank repository (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers, ensuring transparency and reproducibility of the molecular identification data.

DC01-A:	*PP709510*	*DC08:*	*PQ303224*	*TF04:*	*OR856532*
DC02:	*PQ303223*	*DC09:*	*PQ303225*	*TF13:*	*OR857375*
DC04:	*PQ205308*	*DC10:*	*PQ303227*	*TF17:*	*OR888890*
DC06:	*PP709364*	*TF03:*	*OR856558*		

Phylogenetic characterization

To further confirm the taxonomic identity of the LAB isolates and to elucidate their evolutionary relationships, a phylogenetic analysis was performed based on 16S rRNA gene sequences. Phylogenetic trees were reconstructed using the neighbor-joining (NJ) method, and evolutionary distances were calculated using the Kimura two-parameter model (Kumar et al., 2024). The tree was rooted with *Bifidobacterium longum* subsp. *suillum* strain Su851, selected as an outgroup because of its distinct phylogenetic position within the phylum *Actinobacteria* yet outside the *Lactobacillaceae* family.

The robustness of the branching topology was evaluated through bootstrap analysis with 1,000 replicates, and bootstrap values ≥70% were considered significant. The resulting tree (Figure 26) revealed that all isolates clustered within the *Lactobacillaceae* family, forming well-supported clades with their respective type strains. Bootstrap values at key branching nodes exceeded 90%, confirming the high confidence and stability of the inferred phylogenetic relationships. The congruence between molecular identification and phylogenetic clustering further supports the accurate classification of the isolates within the genera *Levilactobacillus* and *Lactiplantibacillus*.

Each isolate clustered closely with its corresponding type strain, confirming species-level assignment and corroborating the results obtained through BLASTn and EzBioCloud analyses. The *Levilactobacillus* and *Lactiplantibacillus* isolates formed distinct monophyletic clades with their respective reference sequences, further validating the robustness of the molecular identification. This phylogenetic framework aligns with the revised taxonomy proposed by J. Zheng et al. (2020) and reveals clear phylogenetic differentiation among LAB strains originating from diverse ecological matrices, including fermented goat milk and tea residues.

These relationships underscore the adaptive diversification of *Lactobacillaceae* in nutrient-rich and mildly acidic fermentation environments.

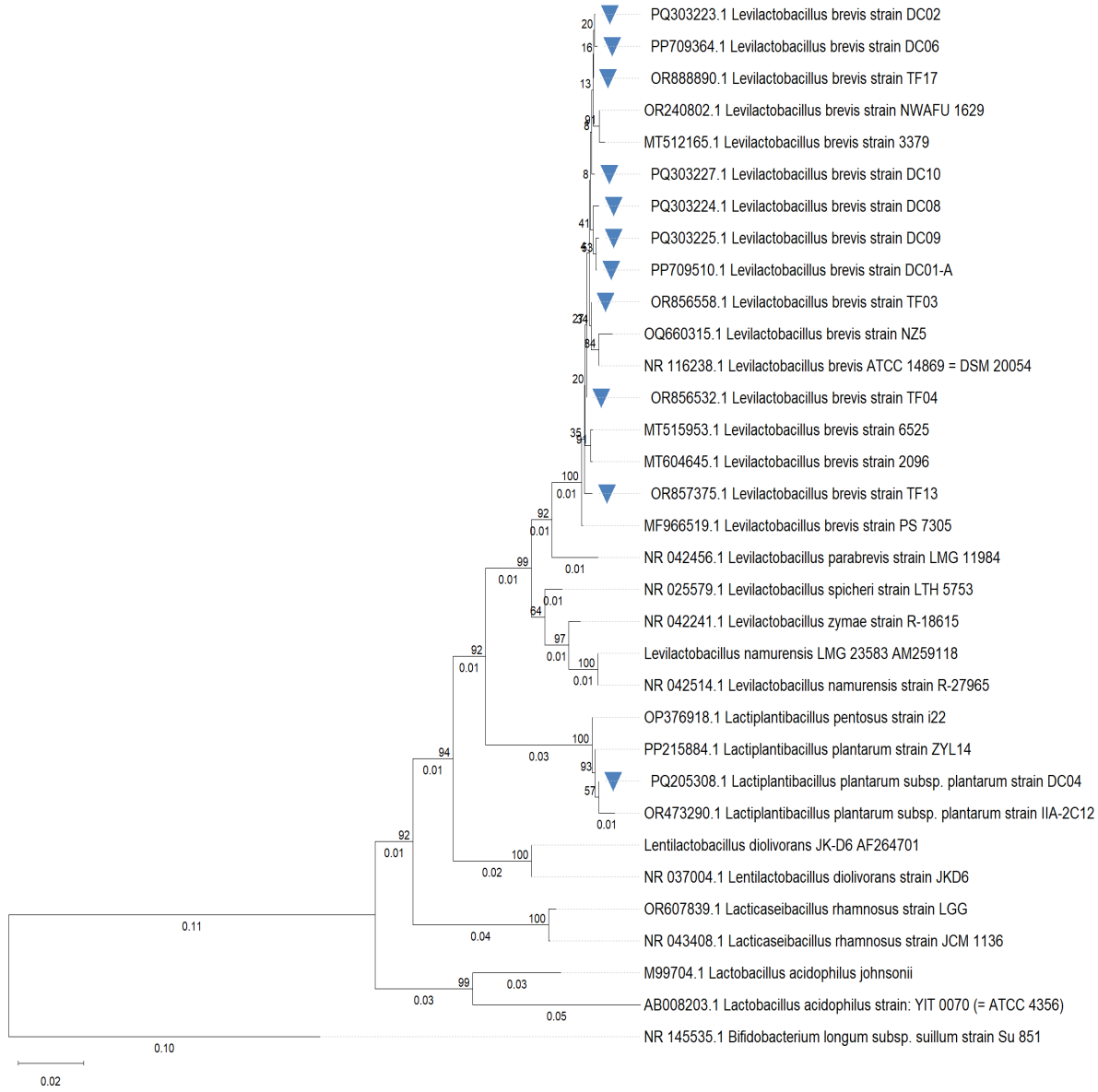


Figure 26 Phylogenetic tree based on the 16S rRNA gene sequences of *lactobacillus* strains (marked with a blue triangle). *Bifidobacterium longum subsp. suillum strain Su 851* was used as an outgroup reference strain. The scale bar represents 0.02 nucleotide substitution per site.

7.1.3. Biosafety testing

A comprehensive biosafety evaluation was performed to assess the potential pathogenicity of the selected LAB isolates prior to probiotic application. The assessment focused on three phenotypic traits commonly associated with bacterial virulence: hemolytic activity, DNase production, and gelatinase activity. These enzymes are recognized markers of pathogenic potential because they contribute to host cell lysis, nucleic acid degradation, and connective

tissue breakdown, respectively (X. Fan et al., 2023). The absence of these activities is an essential requirement for strains to be considered safe under the GRAS or QPS frameworks, as defined by the FAO/WHO (2002) and EFSA (2023) guidelines.

In this study, all candidate LAB strains were screened for the aforementioned virulence markers using standard *in vitro* phenotypic assays. The ideal probiotic profile combines the absence of virulence factors (e.g., DNase, hemolytic activity) with desirable functional characteristics (Abd El-Hack et al., 2020; Binda et al., 2020).

Hemolytic activity

Hemolytic activity is a recognized virulence trait in pathogenic bacteria capable of causing systemic infections. It is mediated by hemolysins. Such activity is characteristic of pathogenic *Streptococcus* and *Enterococcus* species, where it contributes to host pathophysiological conditions including anemia, edema, and septic dissemination (X. Fan et al., 2023; Kim et al., 2019).

The absence of α- or β-hemolytic activity is therefore a key safety criterion in the evaluation of candidate probiotic strains and forms part of GRAS and QPS safety frameworks (FAO/WHO, 2002; EFSA, 2021). None of the isolates displayed β- or α-hemolysis; all exhibited γ-hemolysis, indicating a non-hemolytic phenotype (Figure 27A). The non-hemolytic behavior observed across all isolates meets established biosafety standards for probiotic microorganisms and aligns with prior findings in LAB from fermented foods and goat milk (Barzegar et al., 2021; Jin et al., 2021; Roldan-Perez et al., 2023). For example, Roldan-Perez et al. (2023) reported that none of twelve LAB strains from Colombian cheese exhibited hemolytic activity, while Lee et al. (2024) demonstrated that 46 *Ln. citreum* isolates from kimchi were non-hemolytic.

Importantly, positive and negative controls were included in the assay to validate test reliability. The positive control, *S. aureus* ATCC, displayed clear β-haemolysis, confirming erythrocyte lysis. In contrast, the negative control, LGG, exhibited γ-haemolysis, reinforcing the interpretation of non-haemolytic responses in the tested strains.

The non-hemolytic profiles of *Lpb. plantarum* and *Lev. brevis* further substantiate the safety of the isolates examined in this work, indicating their suitability for use in food and nutraceutical applications.

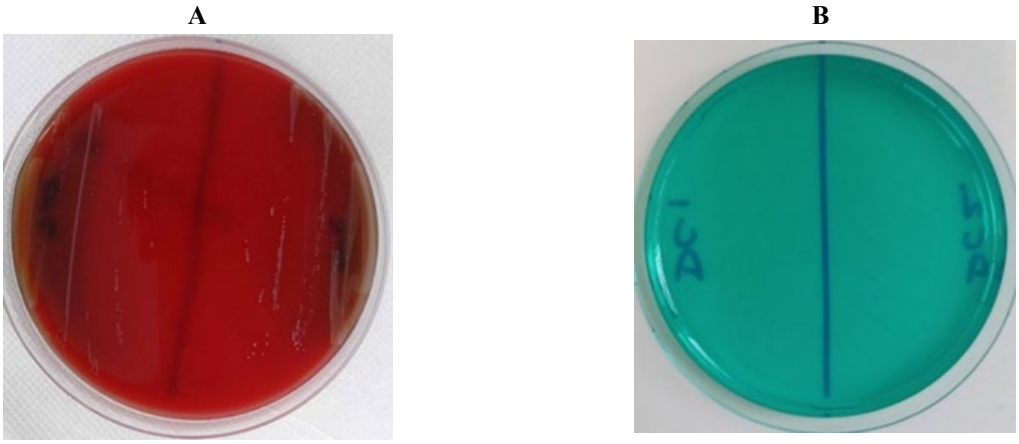


Figure 27 Biosafety Evaluation of lactobacillus strains. A; DNase activity, and B; Haemolytic activity.

Deoxyribonuclease (DNase) activity

DNase production is a recognized virulence mechanism in pathogenic bacteria, facilitating immune evasion through degradation of neutrophil extracellular traps (NETs), hydrolysis of host DNA, and disruption of Toll-like receptor 9 (TLR9) signaling (Happonen & Collin, 2024; Henderson et al., 2023). This activity enhances bacterial survival by impairing macrophage function and enabling access to nucleotides.

None of the isolates exhibited DNase activity (Figure 27B), confirming the absence of this virulence-associated trait.

These results align with previous reports showing that food-derived *Lactobacillus* strains, such as *Lpb. paracasei* CCMA 1770, *Lpb. pentosus* CCMA 1768, and *Lev. brevis* CCMA 1762, do not produce DNase (Simões et al., 2022). The absence of DNase activity, combined with negative hemolytic and gelatinolytic results, indicates a strong non-pathogenic safety profile consistent with FAO/WHO (2002) guidelines.

Gelatinase activity

Gelatinase production is considered an exclusion criterion in probiotic evaluation because it degrades collagen and other extracellular matrix components, potentially compromising epithelial integrity and inducing inflammation (Fugaban et al., 2021; J. Wang et al., 2024; Xu et al., 2025).

None of the isolates exhibited gelatinase production, indicating an absence of proteolytic virulence potential. These findings concur with earlier reports showing that LAB strains from traditional fermented foods, such as dairy and plant matrices, lack gelatinase activity (Anumudu et al., 2024; B. Wang et al., 2024; Zhou et al., 2021). This non-gelatinolytic phenotype, together with negative DNase and hemolysis tests, establishes a triple-negative virulence profile. Such profiles meet current biosafety standards established by EFSA (2018) and ISAPP/IPA recommendations, supporting the safe use of LAB as bio-preservative and probiotic agents.

Lactic acid bacteria strains that lack virulence factors like gelatinase are considered consumer-friendly alternatives to chemical preservatives due to their non-toxic nature. Species such as *Lpb. plantarum*, *Lmb. fermentum*, *Lcb. casei*, and *Lev. brevis* have been confirmed as safe for fermentation and bio-preservative applications precisely because they lack pathogenic traits while producing beneficial compounds like LA, diacetyl, and hydrogen peroxide (El Far et al., 2024; Jin et al., 2021; Lepecka et al., 2024; Moreno et al., 2018; Petrovic et al., 2025; Roldan-Perez et al., 2023).

Importantly, when assessed alongside the absence of haemolytic activity and DNase production, the consistent lack of gelatinase activity across all isolates contributes to a robust triple-negative virulence profile. These enzymatic traits (hemolysin, DNase, and gelatinase) are widely recognized as key pathogenicity markers in clinical microbiology, and their absence provides compelling evidence of the biosafety of the strains under investigation (Additives et al., 2018; Fugaban et al., 2021).

Antibiotic susceptibility test

The antibiotic susceptibility of LAB isolates was evaluated to determine potential antimicrobial resistance (AMR) risks. Resistance profiling ensures that probiotic strains do not serve as reservoirs or vectors for transferable resistance genes (Mathipa-Mdakane & Thantsha, 2022).

All isolates were susceptible to most broad-spectrum antibiotics active against Gram-positive bacteria, including ampicillin, chloramphenicol, and erythromycin (Figure 28 andFigure 29 Table 8). Moderate resistance was observed to aminoglycosides (streptomycin, kanamycin) and quinolones (ciprofloxacin, norfloxacin), consistent with the intrinsic, chromosomally encoded resistance known for *Lactobacillus sensu lato* species (Anisimova & Yarullina, 2019; Campedelli et al., 2019). This resistance is generally chromosomally encoded and non-transferable, and is attributed to modifications in peptidoglycan precursors (replacement of D-

Ala-D-Ala by D-Ala-D-Lac), which reduce antibiotic binding affinity (Chang et al., 2017; Guo et al., 2017).

Importantly, none of the isolates exhibited acquired resistance beyond intrinsic levels. These findings align with the probiotic safety frameworks of EFSA (2021) and FAO/WHO (2002), confirming that the isolates comply with the criteria for GRAS and QPS qualification.

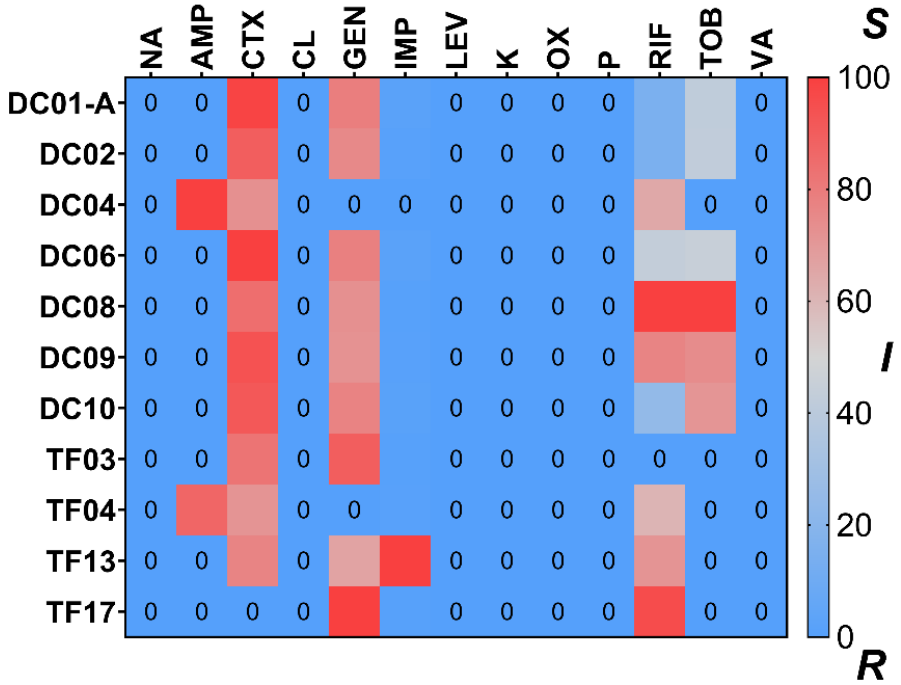


Figure 28 Resistance status of LAB isolate (normalised heatmap) in compliance with CLSI cut-offs) Colors indicate resistance classification: blue (Sensitive), gray (Intermediate), and red (Resistant). Data are based on normalized inhibition zone interpretations according to CLSI criteria.

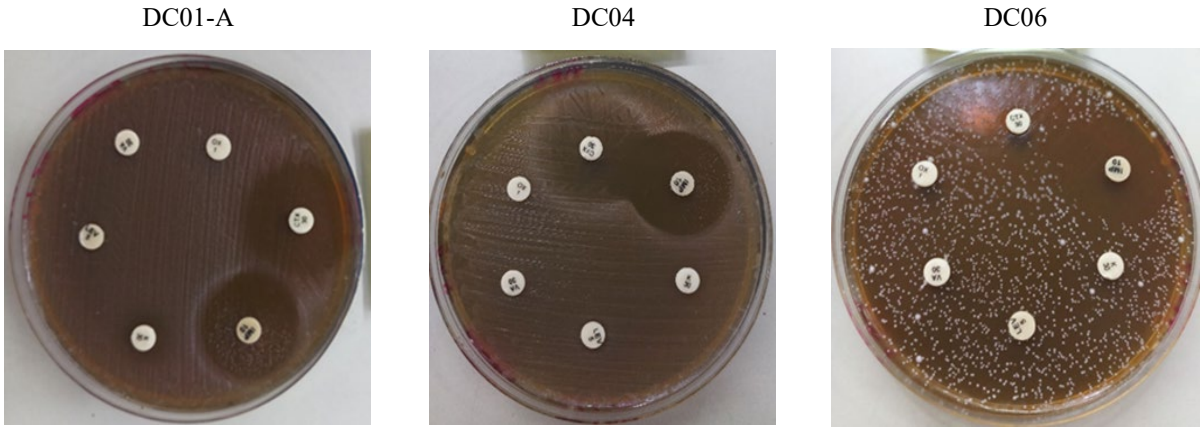


Figure 29 Antibiotic resistance of lactobacillus strains to different antibiotics . DC01-A: *Lev. brevis*; DC04: *Lpb. plantarum*; DC06*: Lev. brevis*.

Table 8 Antibiotic Sensitivity of lactobacillus strains: as measured by diameter of inhibition zones

Antibiotic	**DC01-A**	**DC02**	**DC04**	**DC06**	**DC08**	**DC09**	**DC10**
Nalidexic acid	R	R	R	R	R	R	R
Ampicillin	R	R	S (26.1)	R	R	R	R
Cefotaxime	S (25.8)	S (24.5)	S (22.0)	S (26.0)	S (23.7)	S (25.1)	S (24.8)
Colistin	R	R	R	R	R	R	R
Gentamicin	S (18.4)	S (17.5)	R	S (18.2)	S (17.0)	S (16.8)	S (18.0)
Imipenem	S (26.6)	S (24.9)	S (23.2)	S (26.5)	S (24.5)	S (25.0)	S (26.0)
Levofloxacin	R	R	R	R	R	R	R
Kanamycin	R	R	R	R	R	R	R
Oxacillin	R	R	R	R	R	R	R
Penicillin G	R	R	R	R	R	R	R
Rifampicin	S (18.3)	S (18.3)	S (22.0)	S (20.4)	S (24.6)	S (22.9)	S (19.0)
Tobramycin	I (10.7)	I (10.7)	R	I (11.3)	S (25.3)	S (18.8)	S (18.0)
Vancomycin	R	R	R	R	R	R	R

	TF03	**TF04**	**TF13**	**TF17**
Nalidixic acid	R	R	R	R
Ampicillin	R	S (22.7)	R	R
Cefotaxim	S (23.4)	S (21.8)	S (22.6)	I(11.3)
Colistin	R	R	R	R
Gentamicin	S (20.9)	R	I (15.5)	S(23.3)
Imipenem	S (24.5)	S (25.0)	S (22.9)	S (24.6)
Levofloxacin	R	R	R	R
Kanamycin	R	R	R	R
Oxacillin	R	R	R	R
Penicillin G	R	R	R	R
Rifampicin	I (17.2)	S (21.7)	S (22.5)	S(24.3)
Tobramycin	R	R	R	R
Vancomycin	R	R	R	R

Zone of Inhibition (mm), R; (resistant), S; susceptible. DC01-A*: Lev. brevis*; DC04: *Lpb. plantarum; DC06: Levilactobacillus brevis.*

Antibiotic resistance interpretation

Although intrinsic resistance may confer an adaptive advantage by supporting probiotic survival during antibiotic therapy, it is essential to distinguish this from acquired and transferable resistance, which poses significant public health concerns. Recent genomic analyses of commercial probiotic formulations have identified resistance determinants such as *vanX* (vancomycin), *parC* (ciprofloxacin), and *blaTEM* (β-lactamase), underscoring the need for vigilant genetic monitoring (Anisimova et al., 2022). According to international probiotic safety guidelines, candidate strains should remain susceptible to at least two clinically relevant

antibiotics and must not harbor mobile genetic elements encoding resistance (Hashem & El-Baky, 2021; Mendoza et al., 2023).

In this context, the resistance patterns observed among our isolates were consistent with intrinsic, chromosomally encoded mechanisms typical of *Lactobacillus sensu lato* species and therefore considered acceptable within the GRAS and QPS safety frameworks. Interestingly, intrinsic resistance may offer practical benefits by enabling probiotic survival during antibiotic administration, thereby facilitating gut recolonization, microbiota balance, and epithelial recovery without contributing to horizontal gene transfer (Hamed & K. Isa, 2024; Vantsawa et al., 2017). Nonetheless, continued genomic vigilance remains imperative; only strains confirmed to lack transferable resistance determinants should be approved for use in functional foods or therapeutic formulations (Garcia-Nunez et al., 2022; Roe et al., 2022).

Taken together, the antibiotic susceptibility profiles observed in this study indicate a robust biosafety profile consistent with QPS and GRAS criteria, supporting the suitability of the selected LAB isolates as safe probiotic candidates.

7.1.4. Technological characterization

Growth phase dynamics and implications for bioprotective applications.

The growth kinetics of ten isolates, as summarized in Figure 30 and Table 9, were comprehensively assessed using the Gompertz growth model. The Gompertz equation, widely employed to describe microbial growth dynamics, enabled precise estimation of essential parameters including maximum biomass yield (YM), growth rate constant (K), and lag phase duration (lag) (Khalfallah et al., 2022). The fitted Gompertz model parameters demonstrated considerable variation among the tested strains. Maximum biomass yields (YM) ranged significantly, with the lowest observed in *Lev. brevis* strain DC01-A (0.3731) and the highest in *Lpb. plantarum* subsp. *plantarum* strain DC04 (1.353), indicating diverse capacities for biomass accumulation. Recent studies similarly highlighted substantial variability in growth kinetics among probiotic strains, reinforcing the importance of strain-specific assessments (Panda et al., 2017; K. Zhang et al., 2022).

Strain DC04 notably exhibited the highest growth rate constant ($K = 0.4395\ h^{-1}$), underscoring its robust growth potential under the employed experimental conditions. Conversely, strain DC01-A demonstrated the lowest growth rate ($K = 0.0527\ h^{-1}$), potentially reflective of metabolic limitations or suboptimal culture conditions. Similar observations have been

reported in recent literature, where rapid growth kinetics significantly influence probiotic strain selection for industrial fermentation (Angelescu et al., 2024).

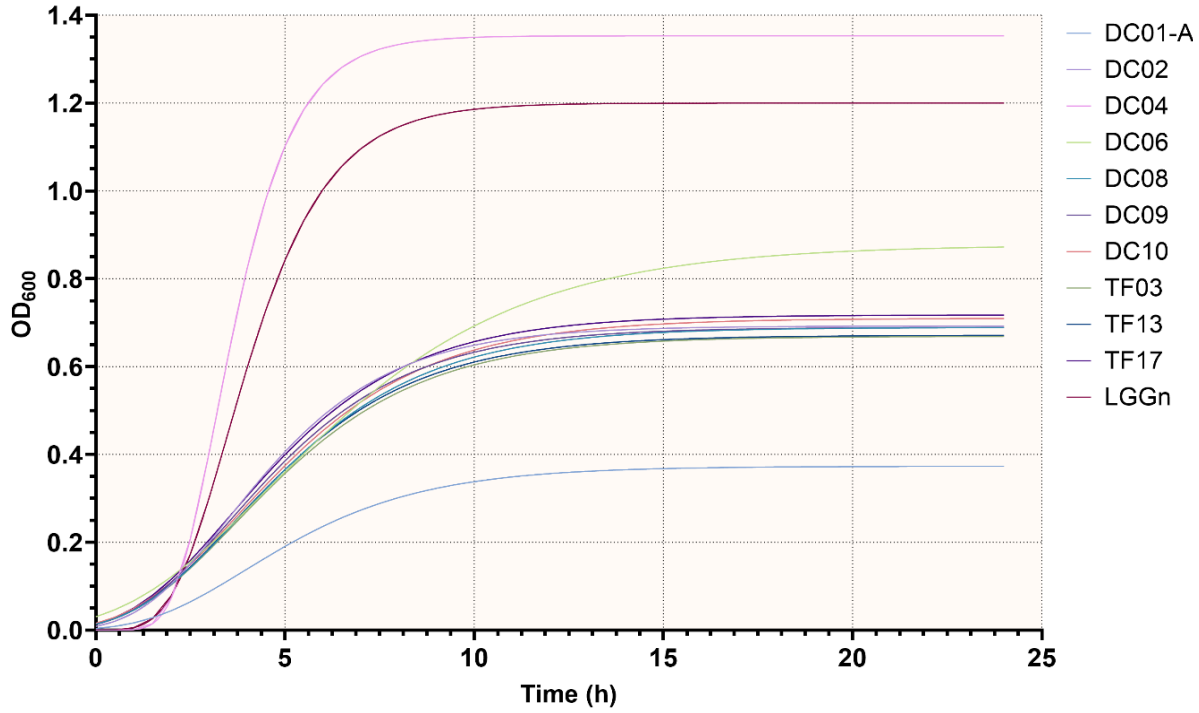


Figure 30 Gompertz-modeled growth kinetics of LAB strains

Lag phase durations exhibited notable variability among isolates, reflecting differences in physiological adaptation and metabolic readiness. *Lev. brevis* strains TF17 (0.94 h) and TF03 (0.96 h) demonstrated rapid adaptation, indicating a swift transition to exponential growth - a trait advantageous in competitive or short fermentation processes (Lee et al., 2024; Roldan-Perez et al., 2023). In contrast, *Lpb. plantarum* subsp. *plantarum* strain DC04 displayed an extended lag phase (2.08 h), suggesting a preparatory phase preceding extensive exponential growth, consistent with prior observations in other *Lactobacillus* isolates (Mernawati et al., 2023; Mokoena, 2017). The reference strain *Lacticaseibacillus rhamnosus* GG (LGG) exhibited intermediate performance (Y_m = 1.2, K = 0.30 h^{-1}, λ = 2.0 h) and served as a comparative benchmark.

Collectively, these results identify strain DC04 as technologically favorable for industrial fermentation, owing to its high specific growth rate and biomass yield. Strains TF17 and TF03, characterized by their short lag phases, are advantageous in processes requiring rapid microbial establishment. Conversely, isolates with slower growth kinetics, such as DC01-A, may be suited for niche applications where gradual acidification is desirable or where specific metabolite profiles are targeted through extended fermentation.

The observed growth kinetics hold direct implications for meat preservation. Rapid-growing LAB strains accelerate acidification and biomass accumulation, both essential for inhibiting spoilage and pathogenic bacteria. In particular, the strong performance of DC04 underscores its potential as a starter or adjunct culture for meat bioprotection, aligning with findings by Huang et al. (2022), who reported that early LAB-driven acidification significantly restricted *Listeria monocytogenes* proliferation in meat systems. Similarly, the short lag phases of TF03 and TF17 suggest rapid colonization capacity, facilitating early dominance within meat matrices and extending product shelf life through competitive exclusion.

Previous studies have demonstrated that *Lpb. plantarum* and *Lev. brevis* enhance meat safety via LA and bacteriocin production (Kauser-Ul-Alam et al., 2021; C. Miranda et al., 2021). Moreover, the physiological state at inoculation substantially influences preservation efficacy. Strains introduced during exponential growth exhibit heightened metabolic activity, leading to rapid acidification and antimicrobial metabolite synthesis, whereas stationary-phase cells often express stress-resistance genes and produce secondary metabolites such as bacteriocins (Marwati et al., 2018; Zangeneh et al., 2020). Optimizing inoculation timing relative to growth phase can therefore enhance both immediate antimicrobial action and long-term preservation stability in meat-processing environments.

Table 9 Growth kinetics and biopreservation potential of selected LAB strains

Strain	**YM**	**K (h^{-1})**	**Lag (h)**	**Biopreservation Potential**	**Remarks**
DC01-A	0.373	0.0527	1.91	Low	Slow growth and low biomass; potential niche use
DC02	0.554	0.0773	1.34	Moderate	Acceptable kinetics, average suitability
DC04	1.353	0.4395	2.08	High	High acidification and biomass yield; suitable for competitive environments
DC06	0.637	0.0852	1.31	Moderate	Moderate performance with stable growth
DC08	0.587	0.0815	1.47	Moderate	Standard performance for general use
DC09	0.508	0.0697	1.64	Low-Moderate	Requires optimization for application
DC10	0.473	0.0658	1.79	Low	Limited industrial utility without enhancement
TF03	0.684	0.0931	0.96	Moderate-High	Fast adaptation; good early colonizer
TF13	0.671	0.0917	0.968	Moderate	Balanced adaptation and moderate yield
TF17	0.671	0.0917	0.94	Moderate-High	Fast adaptation and competitive establishment
LGG (control)	1.2	0.3	2.0	Reference	Control benchmark for probiotic comparison

Extracellular proteolytic activity

Proteolytic activity was evaluated to determine the capacity of the LAB isolates to hydrolyze extracellular proteins, an attribute relevant to flavor development and nitrogen metabolism during fermentation.

None of the tested isolates exhibited proteolytic activity, as no hydrolysis zones were detected. This observation is consistent with reports that *Lactobacillus sensu lato* species generally possess weaker extracellular proteolytic systems compared to *Lactococcus*, *Pediococcus*, or *Enterococcus* (Garcia-Cano et al., 2019; Kieliszek et al., 2021; Lim et al., 2019). Expression of proteolytic enzymes in LAB is typically influenced by nutrient limitation or amino acid auxotrophy, where protein degradation provides essential nitrogen sources. The lack of proteolysis in our isolates likely reflects adaptation to carbohydrate-rich substrates, such as fermented tea residues or goat-milk butter, where nitrogen demand is readily met.

Interestingly, previous studies have shown that LAB lacking extracellular proteolysis may still produce proteinaceous antimicrobial compounds (Scatassa et al., 2017), or rely on robust amino acid biosynthesis pathways for metabolic maintenance under nutrient scarcity (Papadimitriou et al., 2016; Rendon-Rosales et al., 2019). Thus, the absence of proteolytic activity under assay conditions does not preclude functional protein metabolism and may represent a selective advantage in stable, nutrient-rich environments.

Extracellular lipolytic activity

No lipolytic activity was detected in any of the isolates, confirming their non-lipolytic phenotype . This result aligns with previous observations in *Lev. brevis*, where only isolated strains, such as *Lev. brevis* B3, showed detectable activity (Araujo-Rodrigues et al., 2023; Barzegar et al., 2021). Although lipolysis can enhance flavor formation in some dairy fermentations, it is undesirable in meat preservation contexts because excessive lipid hydrolysis leads to rancidity and off-flavor development (Sionek et al., 2025). Consequently, the non-lipolytic behavior of our isolates supports their technological suitability for bioprotective applications in meat systems.

Moreover, the literature cautions that lipolytic LAB can inadvertently degrade lipid-based antimicrobial compounds or generate volatile oxidation products that compromise product

stability (Sionek et al., 2024). Therefore, strains lacking lipolytic activity are preferred for maintaining flavor integrity and oxidative stability in biopreserved foods.

Ethanol tolerance assay

Ethanol tolerance was evaluated to determine the ability of LAB isolates to survive in alcohol-rich environments, which is relevant to their potential use in fermented beverages and bio-preservation systems.

All isolates demonstrated moderate tolerance, with sustained growth observed up to 12% ethanol (v/v) but not at higher concentrations (Table 10). These results indicate that the tested strains possess sublethal alcohol resistance comparable to that reported in moderately tolerant LAB species such as *Lpb. plantarum* and *Lev. brevis* (Araujo-Rodrigues et al., 2023; Goh et al., 2021) which typically lose viability beyond 10 % (v/v) (Barzegar et al., 2021). This level of tolerance may facilitate survival during co-fermentation or in composite food matrices containing residual ethanol, without compromising viability or functionality.

In contrast, highly resistant species such as *Lb. buchneri* NRRL and *Lcb. casei* have been reported to survive at concentrations approaching 18 % (v/v) (Araque et al., 2013).

Environmental origin exerts a strong influence on ethanol resistance. Strains isolated from winery environments often withstand up to 14 % (v/v) ethanol, whereas vineyard isolates tolerate only 8–10 % (v/v), reflecting adaptive selection in ethanol-rich habitats (Nisiotou et al., 2015). Ethanol exposure disrupts membrane integrity, osmotic balance, and enzymatic activity; thus, tolerance involves maintenance of lipid fluidity and stabilization of key metabolic functions. Nevertheless, even in tolerant LAB, ethanol above 8 % reduces growth rate sharply, and near 12 % growth becomes minimal (J. Wang et al., 2022). Only a few specialized strains can reach 16 % (v/v), the upper physiological limit reported for LAB (Novik et al., 2017).

Ethanol tolerance is particularly advantageous in secondary or late-stage fermentations where high ethanol and low pH coexist. Strains capable of enduring both stresses can contribute to flavor modulation, stabilization, and bioacidification (Chen et al., 2023; Kazancıgil et al., 2019; Vinderola et al., 2024). Collectively, the moderate tolerance observed here positions the studied isolates as robust adjuncts for mixed fermentations and acidic, alcohol-rich food systems.

Table 10 Technological proprieties of *Lactobacillus* strain

Characteristics	**Isolates**										
	DC01-A	DC02	DC04	DC06	DC08	DC09	DC10	TF03	TF04	TF13	TF17
Alcohol											
3%	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
6%	++	++	++	++	++	++	++	++	++	++	++
12%	+	±	+	+	+	+	±	+	±	±	±
15%	-	-	-	-	-	-	-	-	-	-	-
Growth											
6° C	+	-	+	+	+	+	+	+	+	+	+
10 °C	++	++	++	++	++	++	++	++	++	++	++
25 °C	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
37 °C	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
45 °C	-	-	+	+	+	+	+	-	±	±	-
50 °C	-	-	-	-	-	-	-	-	-	-	-
H_2O_2											
0.4 mM	0.308±0.022	0.311±0.05	0.307±0.018	0.415±0.029	0.401±0.015	0.338±0.086	0.346±0.061	0.422±0.055	0.344±0.015	0.272±0.043	0.272±0.032
0.7 mM	0.102±0.041	0.144±0.043	0.158±0.055	0.122±0.065	0.182±0.021	0.177±0.052	0.125±0.021	0.142±0.014	0.139±0.072	0.136±0.026	0.136±0.066
1.0 mM	0.040±0.018	0.05±0.035	0.098±0.046	0.066±0.025	0.071±0.027	0.07±0.065	0.044±0.039	0.098±0.028	0.079±0.09	0.056±0.047	0.056±0.023

Maximum growth is represented by three positive signs (+++), high growth by two positive signs (++), weak growth by a single positive sign (+), and no growth by a negative sign (−). DC01-A: *Lev. brevis*; DC04: *Lpb. plantarum*; DC06: *Lev. brevis*

Oxidative-stress tolerance

Oxidative stress assays evaluated the isolates' resistance to H_2O_2, a major ROS that damages nucleic acids, proteins, and membrane lipids. All strains exhibited a dose-dependent decline in viability with increasing H_2O_2 concentration (Table 10), typical of catalase-deficient LAB. At molecular level, H_2O_2 oxidizes nucleotide bases, induces DNA strand breaks, and promotes lipid peroxidation, which compromises membrane integrity and metabolic efficiency (Calderini et al., 2017; Papadimitriou et al., 2016).

Most *Lactobacillus* lineages lack catalase, the principal enzyme decomposing H_2O_2 into water and oxygen, rendering them vulnerable under aerobic conditions (Vinderola et al., 2024). Some species have evolved compensatory mechanisms such as heme- or manganese-dependent catalases, NADH peroxidases, or pseudo-respiratory pathways. For instance, *Lcb. casei* IGM394 detoxifies H_2O_2 through NADH peroxidase activity (Naraki et al., 2020).

Despite moderate sensitivity, the isolates retained viability under low oxidative stress, suggesting baseline resilience suitable for manufacturing and storage. Enhanced ROS tolerance supports prolonged shelf life and functionality, particularly for probiotics intended to colonize oxygen-exposed niches like the oral cavity or upper intestine. Further improvement through adaptive evolution or metabolic engineering could enhance their industrial robustness.

Growth at different temperatures

Temperature adaptability determines both ecological fitness and technological performance of LAB. All isolates grew between 10 °C and 37 °C, confirming their mesophilic character (Table 10). None survived at 50 °C, though isolates DC04 and DC06 maintained growth at 45 °C, indicating moderate thermotolerance. Such flexibility is desirable for industrial fermentations and gastrointestinal survival, as 37 °C approximates human body temperature (Hernandez et al., 2019).

Temperature tolerance correlates with processing resilience: thermotolerant strains exhibit greater survival during spray drying, pasteurization, or warm-holding (Othman & Karim, 2023). While most LAB grow optimally between 30 °C and 40 °C, exceptional thermophilic species, including *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus*, thrive near 42 °C (Bancalari et al., 2016). Rare extremotolerant LAB survive up to 80 °C (Mbye et al., 2020).

Given that *Dhan* and fermented-tea residues are processed at ambient temperatures (25-37 °C), the observed range confirms the isolates' suitability for these artisanal fermentations, ensuring metabolic efficiency and product consistency under real-world conditions.

Autolytic activity

Autolysis is an endogenous process involving the enzymatic cleavage of peptidoglycan by autolysins, leading to cell lysis and release of intracellular enzymes that enhance flavor and texture development. Autolytic activity was quantified spectrophotometrically, and results revealed significant variation ($p < 0.01$): DC01-A (50.86 ± 2.15 %), DC04 (37.53 ± 1.82 %), and DC06 (33.42 ± 1.67 %) exhibited higher autolysis than the reference strain LGG (24.69 ± 1.25 %) (Figure 31). Based on Ayed et al. (2024), these values indicate "good autolytic activity" (25–65 %).

Autolytic diversity among LAB is well documented, with rates from 10 % to 50 % depending on strain, cell-wall structure, and environmental factors (Barzegar et al., 2021; Wilkinson & LaPointe, 2020). Enzymes such as *N*-acetylmuramidase mediate this process and are modulated by pH, osmotic pressure, and carbon source availability (Najjari et al., 2016; Pang et al., 2014).

From a technological standpoint, high-autolysis strains like DC01-A and DC04 could accelerate ripening and flavor development in fermented foods. However, excessive autolysis may reduce probiotic viability and adhesion capacity (Jain et al., 2017). Hence, the optimal application should align with the intended functional outcome.

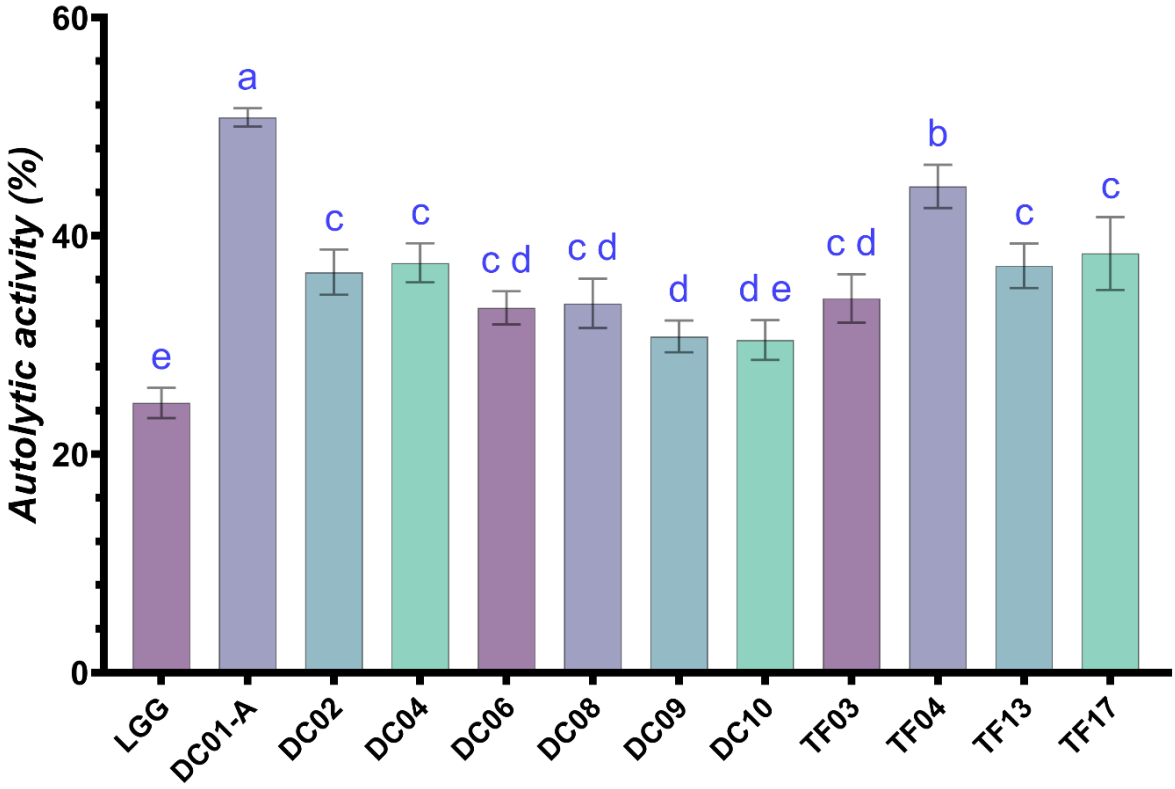


Figure 31 Autolytic activity (%) of LAB strains after incubation. Results are expressed as mean ± SD (n = 3). Bars labeled with different superscript letters (a–e) are significantly different ($p < 0.05$) according to one-way ANOVA followed by Tukey's post hoc test.

7.1.5. Strain characteristics associated with probiotic potential

The physiological and functional profiling of LAB is essential to establish their probiotic potential. Traits such as resistance to gastrointestinal stressors (low pH, bile salts), surface hydrophobicity and aggregation, and functional activities including antimicrobial and enzymatic properties determine survival, colonization, and efficacy within the host. Collectively, these features underpin persistence in the gastrointestinal tract and guide strain selection for functional foods, nutraceuticals, and microbial therapeutics.

Salt-stress tolerance

Sodium-chloride tolerance was evaluated to assess each isolate's ability to maintain growth and metabolism under osmotic stress, a desirable property for both high-salinity fermentations and intestinal persistence (Parlindungan et al., 2021).

All isolates showed visible growth from 2 % to 10 % NaCl (Table 11), confirming an osmotolerant phenotype. This tolerance exceeds the typical range reported for *Lactobacillus*

sensu lato, most of which thrive at 1 % to 5 % NaCl and survive up to 4 % (Mahmoud et al., 2020; Prabhurajeshwar & Chandrakanth, 2017; J. Wang et al., 2024). Growth at 8 % - 10 % NaCl, though strain-specific, was achieved by several *Lpb. plantarum* and *Lev. brevis* isolates, paralleling observations in tea-leaf fermentations (Li et al., 2024; Singhal et al., 2021; Szutowska & Gwiazdowska, 2021).

At the cellular level, LAB counter osmotic stress through active uptake or synthesis of compatible solutes such as proline, glycine-betaine, and trehalose, thereby restoring intracellular turgor and water balance (Chen et al., 2019; Papadimitriou et al., 2016). Up-regulation of membrane-associated choline transporters and osmoprotective proteins further contributes to osmotic homeostasis (Othman & Karim, 2023). In parallel, cells remodel their membrane phospholipid and fatty-acid composition to maintain fluidity and enzyme functionality under hyperosmotic conditions. These responses ensure metabolic continuity during salting steps in food processing and within host gastrointestinal environments whose osmolarity approximates 0.3 mol L^{-1} NaCl (Tsega et al., 2019).

Collectively, the strong NaCl tolerance observed demonstrates substantial osmotic resilience. This attribute supports their integration into brined or salted fermentations and underscores their potential viability under physiologically relevant osmotic conditions in probiotic applications.

Table 11 Tolerance characteristics of *lactobacillus* strains

Strain	NaCl %					pH				Phenol %	
	2	3	4	6.5	10	1.5	3	5	9.2	0.2	0.5
DC01-A	+++	+++	++	+	+	-	+	++	+	++	+
DC02	+++	+++	++	+	±	-	+	++	±	++	±
DC04	+++	+++	++	+	+	-	+	++	+	++	+
DC06	+++	+++	++	+	+	-	+	++	+	++	+
DC08	+++	+++	++	+	±	-	+	++	±	++	±
DC09	+++	+++	++	+	±	-	+	++	+	++	±
DC10	+++	+++	++	+	±	-	+	++	±	++	±
TF03	+++	+++	++	+	+	±	+	++	+	++	+
TF04	+++	+++	++	+	±	-	+	++	+	++	±
TF13	+++	+++	++	+	+	±	+	++	+	++	+
TF17	+++	+++	++	+	±	-	+	++	+	++	±

Phenol tolerance

Phenolic compounds generated during protein digestion or derived from dietary polyphenols can inhibit bacterial growth through membrane disruption, protein denaturation, and oxidative stress. Their intestinal concentration typically ranges from 0.1 % to 0.5 % (w/v) but may increase after phenol-rich meals (El Far et al., 2024). Tolerance to phenol is therefore a critical probiotic selection criterion, reflecting the ability to persist and function in the gastrointestinal tract (Martiz et al., 2023).

In this study, all isolates maintained robust growth up to 2 % phenol, whereas higher concentrations markedly reduced biomass formation (Table 11). At 5 % phenol, growth inhibition exceeded 70 %, indicating a concentration-dependent effect. These results surpass previous findings where complete inhibition occurred at ≥ 0.5 % phenol (Divisekera et al., 2019).

Comparatively, *Lmb. fermentum* and *Lpb. plantarum* strains have shown survival up to 0.4–0.5 % phenol with viable counts above 7 log CFU mL^{-1} (El Far et al., 2024; Kumari et al., 2022). The higher tolerance recorded here indicates enhanced resilience, potentially linked to cell-wall hydrophobicity, efflux systems, or antioxidant enzymes. Such phenol-resistant strains are advantageous for incorporation into polyphenol-rich food matrices or for microbiota modulation in diets abundant in aromatic metabolites.

Acid tolerance

Acid tolerance determines a probiotic's ability to survive gastric transit and reach intestinal niches. The stomach pH can fall to 1.5 during fasting and remains 3–5 after meals (Bao et al., 2010; Fidanza et al., 2021). To simulate these conditions, isolates were incubated in MRS broth adjusted to pH 1.5–5.0 for 3 h at 37 °C. Most strains sustained growth between pH 3 and 5 but showed significant viability loss at pH 1.5 (Table 11).

Comparable findings have been reported for acid-resistant LAB from fermented vegetables and meats, with survival rates exceeding 90 % at pH 2.0 (Choi et al., 2018; Moreno et al., 2018). Variability at strain level reflects ecological origin and genetic adaptation ((Khagwal et al., 2019; Ma et al., 2024). Acid survival is supported by several mechanisms: activation of proton-translocating H^+-ATPase to expel intracellular protons, modification of membrane fatty-acid composition to reduce proton permeability, and induction of ATR pathways that repair macromolecular damage (Megur et al., 2023; H. Yang et al., 2024).

From an industrial perspective, acid-resistant strains remain viable during low-pH fermentation or storage, ensuring consistent acidification and product safety (Kim et al., 2019). Collectively, the isolates' survival down to pH 3 demonstrates adequate robustness for both gastrointestinal and food-processing environments.

Simulated gastric-juice tolerance

To better replicate gastric conditions, strains were exposed to SGJ containing 0.3 % (w/v) pepsin at pH 2.5 for 3 h at 37 °C. Viability was assessed by plate counts. Marked strain-specific differences were observed (Figure 32): isolates TF13 and DC01-A exhibited the highest survival (> 60 %), significantly outperforming others. These results align with previous reports of *Lactobacillus* strains maintaining viability after prolonged SGJ exposure (Kouadri Boudjelthia et al., 2023).

SGJ tolerance integrates both acid and protease resistance, reflecting a strain's comprehensive fitness for oral delivery. The superior performance of TF13 and DC01-A underscores their probiotic promise for inclusion in functional formulations requiring gastric passage stability.

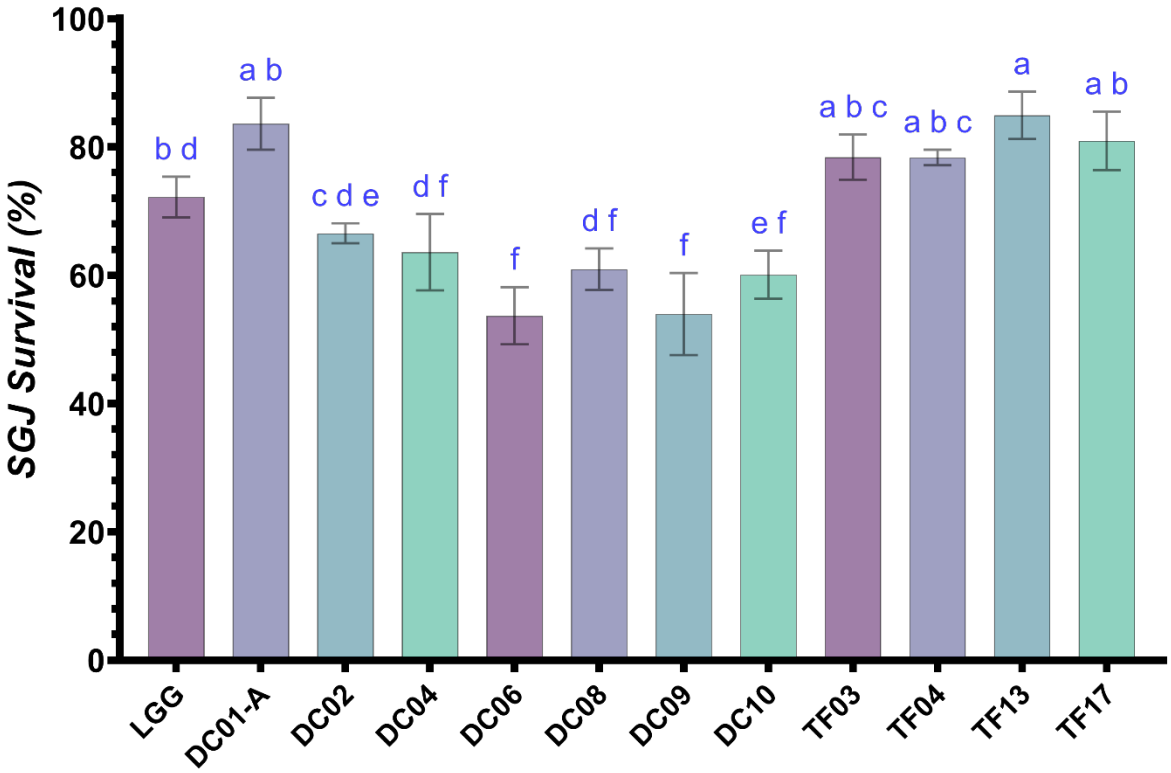


Figure 32 Survival of strains in simulated gastric juice. Mean survival percentages ± SD (n = 3). Bars with different letters indicate significant differences ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post hoc test.

Strain-dependent variability is a well-recognized feature in probiotic screening. Marinova et al. (2019) reported that only four of ten *Lactobacillus* strains survived 90 min at pH 2.0, and none persisted after 180 min, while Cheon et al. (2020) observed similar rapid viability losses, underscoring the need for standardized exposure durations and thresholds.

Multiple parameters influence bacterial survival in SGJ, including strain genotype, exposure time, pH, and the presence of pepsin. Although pepsin typically intensifies proteolytic stress, some strains paradoxically exhibit improved survival in its presence, possibly due to stress-induced physiological adaptation (Ko et al., 2022). *Lpb. plantarum* WCFS1 and 299v, as well as *Bifidobacterium animalis*, have shown enhanced viability under pepsin-enriched conditions, suggesting complex protease–strain interactions.

Encapsulation strategies can further enhance SGJ survival. Chitosan-coated alginate microbeads protected *Lpb. plantarum* 299v, improving viability 1.02-fold compared with free cells while maintaining counts above the functional threshold of 10^7 CFU mL^{-1} after simulated gastric transit (Lai et al., 2020; Sabnis & Malavkar, 2016). Inoculum density also affects outcomes; higher cell loads provide buffering capacity and quorum-sensing-mediated stress responses.

Collectively, these findings contextualize our results and reinforce the selection of *Lev. brevis* TF13 and DC01-A as the most promising candidates. Their superior SGJ tolerance, combined with favorable acid and phenol resistance, indicates a strong capacity for survival through upper gastrointestinal passage and functional performance *in vivo*.

Bile-salt tolerance

Following assessment of acid and gastric resilience, bile-salt tolerance was evaluated to determine intestinal survival potential. After gastric passage, probiotic cells encounter bile salts (0.2 – 2.0 %, w/v) in the small intestine, whose amphipathic nature can solubilize membrane lipids and denature surface proteins (Rahman et al., 2021; Unban et al., 2021). Isolates were cultured for 4 h in MRS broth containing 0.15 %, 0.3 %, and 0.5 % (w/v) bile salts at 37 °C. Viability was determined by plate counts, and data were visualized as a heatmap (Figure 33) to highlight strain-specific survival trends. All isolates tolerated 0.15 % bile, whereas survival declined progressively at 0.3 % and 0.5 %, revealing non-linear, concentration-dependent inhibition. Two strains (TF13 and DC01-A) maintained high viability (> 70 % at 0.3 % bile), outperforming other isolates. Bile tolerance mechanisms in LAB include enzymatic deconjugation of bile acids via bile-salt hydrolase (BSH), synthesis of surface-bound proteins

that stabilize membranes, and active efflux of toxic conjugates (Unban et al., 2021). Strains exhibiting such resilience are likely to remain viable during intestinal transit, facilitating colonization and competitive exclusion of pathogens. Together, the robust performance of TF13 and DC01-A under bile stress, combined with their acid and SGJ tolerance, identifies them as leading candidates for oral probiotic applications and incorporation into functional food formulations designed for gastrointestinal delivery.

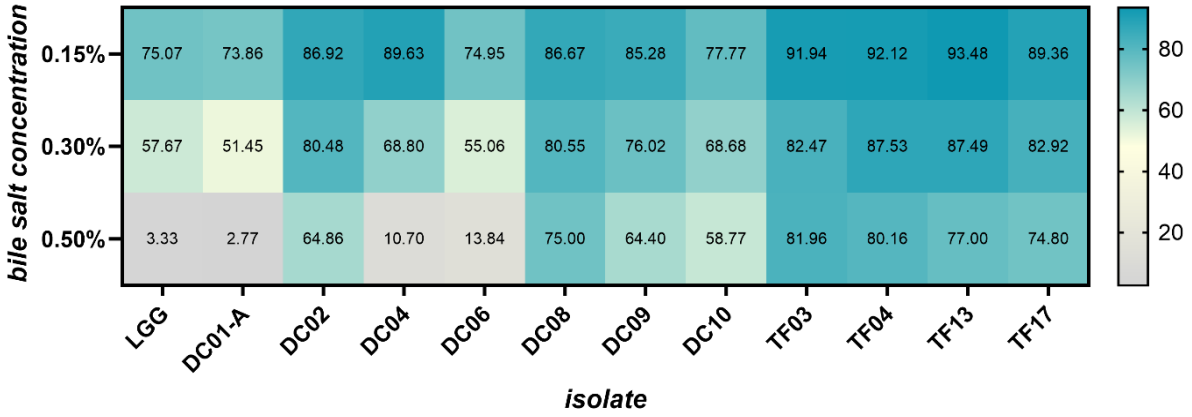


Figure 33 Heatmap of bile salt survival rates (%) of selected strains

At 0.15 % (w/v) bile salt, most LAB isolates exhibited moderate to high survival, indicating limited lethality. Even though bile salts at this concentration can disrupt membrane integrity and metabolic function, effects reported at levels as low as 0.03 % (Du et al., 2024), all strains retained viability above 50 %, confirming a baseline tolerance to mild bile stress. At 0.3 %, a standard benchmark for probiotic evaluation, strain TF13 again demonstrated outstanding resistance (> 90 %), followed by TF17 and DC04. These phenotypes suggest superior membrane robustness and likely BSH activity or reinforced cell-wall structures that confer detergent resistance. When the bile concentration increased to 0.5 % overall survival declined sharply, yet TF13 and TF17 maintained viability above 75 %, whereas more sensitive isolates (DC02, DC06, DC08) dropped below 15 %. This gradient response confirms that high-concentration bile assays provide greater discriminatory power for probiotic selection (Kazancıgil et al., 2019; Kouadri Boudjelthia et al., 2023; Liang et al., 2024). Notably, TF13 and TF17 consistently outperformed other isolates across both acid and bile challenges ($p <$ 0.05), underscoring their multi-stress resilience. Their origin from fermented green-tea wastelikely contributed to the evolution of adaptive defense systems enhancing cell-surface stability and osmotic tolerance. Integrated assessment of acid and bile resistance provides a realistic representation of the gastrointestinal journey from stomach to intestine. Yehuala et al.

(2024) emphasized that overcoming both barriers is prerequisite for colonization and efficacy. In our study, the combined survival profiles confirm that probiotic fitness is primarily strain-specific rather than species-level, reinforcing the importance of genotype-driven selection. Strains TF13 and TF17 therefore emerge as leading probiotic candidates for oral formulations, while more sensitive isolates (e.g., DC01-A, DC02) may benefit from protective encapsulation strategies. Moreover, testing multiple bile concentrations (0.15 %, 0.3 %, 0.5 %) extends the predictive capacity of *in vitro* screening, enabling discrimination between merely tolerant and genuinely resilient strains (Barzegar et al., 2021; Jilani et al., 2024; Sabir et al., 2010; Unban et al., 2021). Although *in vitro* bile-salt assays cannot fully replicate the complex *in vivo* milieu the present findings provide strong preliminary evidence for the superior gastrointestinal survivability of TF13 and TF17. The bile-salt tolerance heatmap clearly illustrates isolate-specific differences in probiotic viability under simulated intestinal stress. While most LAB tolerated mild bile levels, only a subset (particularly TF13 and TF17) sustained high survival at 0.5 %, reflecting their potential to endure postprandial bile exposure. Together with acid-tolerance data, these results highlight that probiotic robustness is shaped by ecological origin and cell-surface physiology, and that heatmap-based analyses offer an efficient visual tool for selecting elite strains for functional food and nutraceutical applications.

7.1.6. Adhesion capabilities and cell-surface properties

The ability of LAB to adhere to intestinal epithelial cells and mucosal surfaces is fundamental to their probiotic functionality. These surface-associated properties govern colonization, persistence, and the capacity to competitively exclude pathogens. Key determinants include auto-aggregation, hydrophobicity, and the presence of specific adhesion molecules that mediate host–microbe and microbe–microbe interactions.

Auto-aggregation: time-dependent behavior

Auto-aggregation reflects the intrinsic ability of bacterial cells of the same strain to self-associate and form multicellular clusters under static conditions. This self-recognition phenomenon promotes biofilm initiation and facilitates stable colonization of mucosal niches (Trunk et al., 2018). In this study, the auto-aggregation potential of LAB isolates was measured at 1, 2, and 24 h of incubation without agitation. As shown in Figure 33, aggregation values increased significantly over time for all isolates, with substantial strain-specific variability ($p < 0.05$).

Early aggregation (1–2 h) was particularly pronounced in strains TF13, TF17, and DC04, indicating rapid cell–cell recognition. After 24 h, TF13 achieved the highest auto-aggregation percentage (> 80 %), followed by TF17 (≈ 75 %) and DC04 (≈ 70 %). These results are consistent with previous reports linking aggregation kinetics to the expression of surface-associated macromolecules such as aggregation-promoting factors (APFs), EPS, and strain-specific adhesins (Fugaban et al., 2021; Roldan-Perez et al., 2023; Wang et al., 2017).

Mechanistic insight

Auto-aggregation is driven primarily by self-recognizing surface structures collectively termed autoagglutinins, which include both covalently bound proteins and non-covalent extracellular polymers. In *Lactobacillus salivarius*, the choline-binding protein A (CbpA) mediates both cell–cell aggregation and adhesion to epithelial surfaces (Wang et al., 2017). Additionally, extracellular DNA (eDNA) and exopolysaccharides secreted into the surrounding medium can act as bridging molecules, promoting aggregate stability and maturation (Nwoko & Okeke, 2021). Variations in these macromolecular components likely explain the pronounced inter-strain differences observed here.

At the molecular level, high-aggregating strains such as TF13 and TF17 may overexpress aggregation-promoting factors or surface-layer (S-layer) proteins, contributing to their enhanced cohesion and resilience. Conversely, weaker aggregators like DC02 may lack these structural motifs or express more hydrophilic surfaces that reduce intercellular adhesion.

Functional relevance

High auto-aggregation capacity is widely recognized as a desirable probiotic trait. It enhances the ability of bacteria to form stable microcolonies on intestinal surfaces, increases resistance to mechanical washout, and often correlates with co-aggregation with enteric pathogens, thereby limiting pathogen adhesion (Roldan-Perez et al., 2023). From a technological perspective, strong aggregators also exhibit improved survival in immobilized or encapsulated formulations, favoring their inclusion in functional foods and biotherapeutic products.

The substantial strain-dependent variability observed in auto-aggregation among tested isolates underscores the importance of selecting strains with superior surface-adhesion properties. The high aggregation levels of TF13 and TF17 highlight their strong potential for mucosal colonization and pathogen exclusion, reinforcing their suitability for application as next-generation probiotics.

Auto-aggregation kinetics and functional implications

Time-resolved auto-aggregation analysis revealed distinct strain-specific behaviors (Figure 34). After 1 h, moderate aggregation was observed in the reference strain LGG and isolate TF03 (≈ 40–50 %), indicating a relatively rapid onset of self-association. DC06 also showed strong early aggregation, possibly associated with surface-bound adhesins or EPS that promote initial cell–cell interactions under static conditions (Wang et al., 2017). In contrast, DC01-A exhibited minimal aggregation (~ 15 %), suggesting delayed expression of APFs or reduced surface adhesion molecule density. By 2 h, most isolates displayed a modest increase in aggregation, reflecting stabilization of early cell contacts. TF13 and DC06 maintained consistent upward trends, while TF17 and DC04 progressed from moderate (≈ 40–50 %) to intermediate levels, indicating time-dependent strengthening of intercellular cohesion. After 24 h, clear differentiation between high- and low-aggregating phenotypes emerged. TF13, TF03, and DC06 exhibited the highest aggregation (> 65 %), forming dense multicellular networks indicative of robust aggregation-promoting systems, such as S-layer proteins or extracellular matrix components that consolidate aggregate formation. LGG showed intermediate aggregation (~ 65 %), consistent with previous reports categorizing it as a moderate auto-aggregator among *Lactobacillus* strains (Cheng et al., 2024; Cozzolino et al., 2020). Conversely, DC01-A remained the weakest performer (< 50 %).

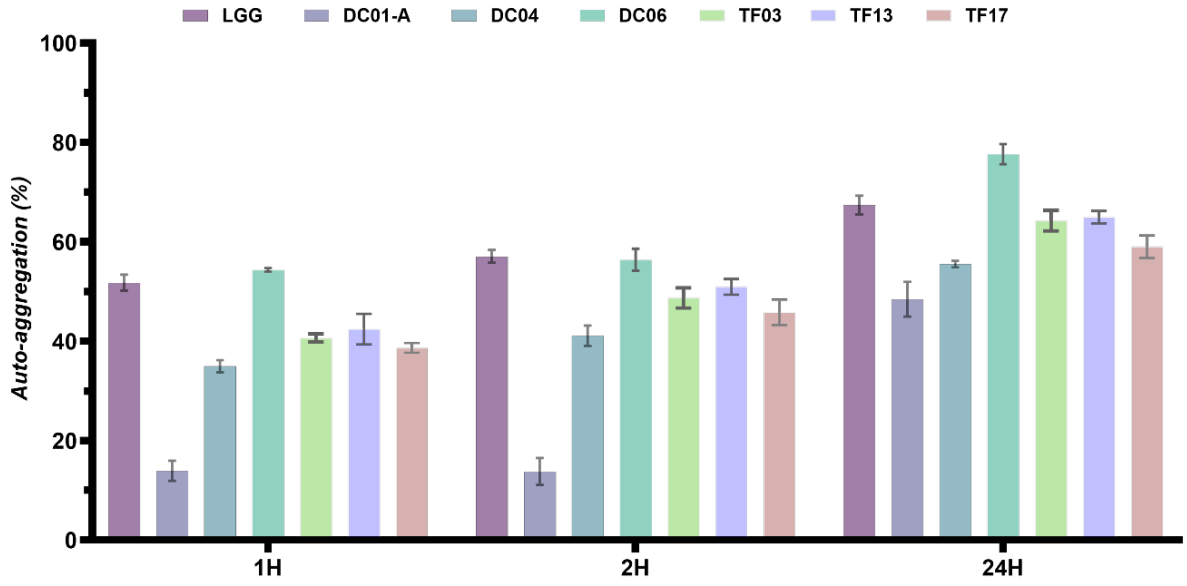


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Figure 34 Auto-aggregation ability of LAB strains after 1, 2, and 24 hours of incubation. Each bar represents the mean ± SD of three independent replicates.

These patterns highlight the influence of strain-specific cell-surface architecture and ecological origin on aggregation capacity. High auto-aggregation correlates with enhanced adhesion to mucosal surfaces and biofilm formation, two critical attributes for persistence within the gastrointestinal tract and for exclusion of pathogens through competitive occupation of adhesion sites (Alkalbani et al., 2022; Campana et al., 2017; Minj et al., 2020). Auto-aggregation can also serve as a stress-adaptation strategy, improving tolerance to adverse environmental conditions and host immune pressures (Bogino et al., 2013; Trunk et al., 2018). Overall, TF13, TF03, and DC06 emerge as strong probiotic candidates owing to their rapid and sustained aggregation behavior, suggesting superior colonization potential and stress resilience.

Co-aggregation with enteric pathogens

Co-aggregation assays further characterized the ability of LAB isolates to interact with pathogenic bacteria. Strains TF03 and DC04 exhibited the highest co-aggregation with both *E.coli* and *S. aureus*, as evidenced by significant OD_{600} reductions (Figure 35). High co-aggregation percentages reflect close physical association between probiotic and pathogenic cells, conferring a competitive advantage by limiting pathogen access to epithelial receptors and enhancing localized antimicrobial activity. Comparable findings have been reported in literature: strain H11 co-aggregated with *E. coli* ATCC 25922 (36.8 %) and *S. aureus* ATCC 6538 (40.2 %) (Li et al., 2024); *Bacillus subtilis* SM10.1 reached ~ 40 % after 4 h (City et al., 2021). Similarly, the reference strain LGG demonstrated elevated co-aggregation with *E. coli* (≈ 58–60 %) after 24 h (Cozzolino et al., 2020). Time-dependent increases were consistent across isolates, underscoring the importance of incubation duration in evaluating probiotic competitiveness. The strain-specific nature of these interactions has been well documented. Among 14 isolates examined by W. Zhang et al. (2022), strains L5, L20, L22, and L14 ranked highest for co-aggregation with *E. coli* and *S. aureus*, while Nami et al. (2024) showed that strain Y33 exhibited superior co-aggregation with *E. coli* and corresponding antibacterial activity. The observed patterns for TF03 and DC04 thus align with recognized probiotic behavior, where aggregation facilitates proximity-based inhibition through release of antimicrobial peptides, organic acids, or bacteriocins (Lee et al., 2024; Nami et al., 2024). Collectively, these findings confirm that both auto- and co-aggregation are crucial determinants of probiotic efficacy. Auto-aggregation enhances persistence and biofilm formation, while co-aggregation underpins competitive exclusion and direct antagonism of pathogens. Together, these traits position TF03, TF13, DC04, and DC06 as leading candidates for functional food or therapeutic formulations aimed at intestinal health and pathogen control.

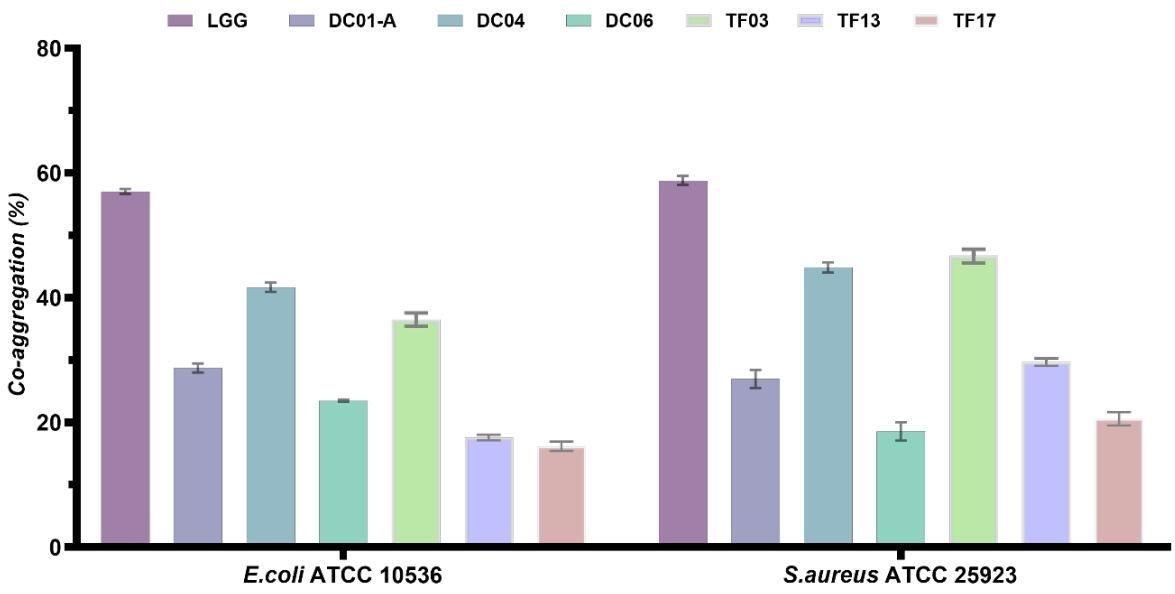

Figure 35 Co-aggregation ability of selected LAB strains. Each bar represents the mean ± SD of three independent replicates.

Bacterial adhesion to hydrocarbons: hydrophobicity and surface physicochemistry

Cell-surface hydrophobicity is a pivotal determinant of probiotic adhesion to intestinal mucosa, influencing the initial attachment to epithelial cells and subsequent colonization within the gastrointestinal tract. Hydrophobic interactions help bacteria overcome electrostatic repulsion between negatively charged bacterial and epithelial surfaces, thereby promoting persistence and biofilm establishment (Lorenzo et al., 2018; Trunk et al., 2018). The BATH assay provides a rapid, reproducible, and widely used method for assessing this property across probiotic candidates (Dekic et al., 2017; Kazancıgil et al., 2019).

In the present study, solvent adhesion assays were conducted using a panel of hydrocarbons differing in polarity and electron donor/acceptor characteristics, including xylene, chloroform, and ethyl acetate. These solvents enable classification of bacterial surfaces as predominantly hydrophobic, hydrophilic, or amphiphilic based on their relative affinity (Figure 35). The results revealed pronounced inter-strain variability, reflecting differences in the physicochemical composition of cell envelopes, including surface proteins, EPS, and teichoic acid structures.

High adhesion percentages to nonpolar solvents such as xylene were observed for isolates TF13, TF17, and DC06 (> 70 %), suggesting strongly hydrophobic surfaces consistent with the presence of cell wall-associated lipoproteins or hydrophobic S-layer proteins. Conversely, lower affinities were noted in DC01-A and LGG (< 45 %), indicating comparatively

hydrophilic surfaces that may rely more on electrostatic or carbohydrate-mediated interactions for mucosal binding. Intermediate adhesion to polar solvents such as chloroform or ethyl acetate implies amphoteric surface characteristics, reflecting balanced distributions of Lewis acid–base sites and possible involvement of protein–carbohydrate complexes (Kouadri Boudjelthia et al., 2023; Nami et al., 2024).

These solvent-specific profiles highlight that hydrophobicity is not an intrinsic constant but a strain-specific attribute shaped by environmental origin and adaptive surface remodeling. For instance, strains isolated from fermented green-tea waste (TF13, TF17) displayed elevated hydrophobicity, potentially reflecting adaptation to polyphenol-rich matrices where lipophilic metabolites favor cell-surface hydrophobic reinforcement.

From a functional standpoint, high hydrophobicity correlates strongly with improved adhesion to intestinal epithelial cells, enhanced auto-aggregation, and increased co-aggregation with pathogens (Campana et al., 2017; Trunk et al., 2018). Hydrophobic strains are also more likely to form biofilms and persist in dynamic gastrointestinal conditions, making them preferred candidates for probiotic formulation and delivery (Lorenzo et al., 2018). In contrast, moderate or low hydrophobicity, as observed in DC01-A and LGG, may be advantageous in certain contexts, reducing excessive aggregation and promoting balanced colonization dynamics.

Determination of solvent adhesion profiles in our study revealed pronounced inter-strain variability in surface physicochemical properties (Figure 36).

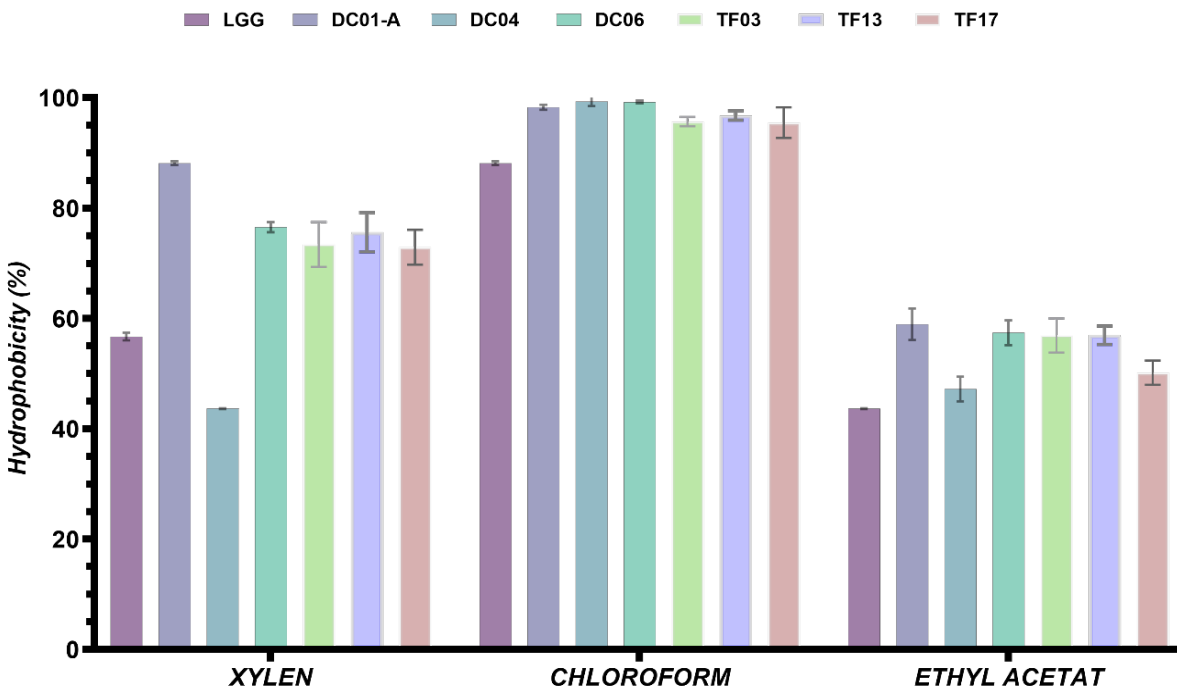

Figure 36 Comparative cell surface hydrophobicity of selected strains.
Bars represent mean ± SD of three independent determinations.

The BATH assay revealed pronounced solvent-dependent and strain-specific variation in cell-surface hydrophobicity (Figure 35). Adhesion to chloroform was exceptionally high across isolates, ranging from 88 % for the reference strain LGG to > 98 % for DC04, DC06, TF13, and TF03. Such strong adhesion indicates the dominance of basic functional moieties (e.g., protonated $–NH_2$ and –OH groups) on the cell surface, which facilitate electrostatic interactions with the negatively charged intestinal glycocalyx and promote initial mucosal attachment (Lorenzo et al., 2018). Comparable findings have been reported for multiple *Lactobacillus* species, which commonly display > 90 % adhesion to chloroform, consistent with a non-acidic charge profile (Lee et al., 2024; Panda et al., 2017; Roldan-Perez et al., 2023; M. Zhang et al., 2022). For instance, *Lb. gasseri* Kx110A1 adheres to chloroform at ~95 %, but only ~25 % to ethyl acetate, reflecting a strongly basic surface (Zuo et al., 2019).

Adhesion to xylene, a non-polar solvent reflecting overall hydrophobicity, was also high in TF13 (79 %), DC06 (76.5 %), and TF03 (72.3 %), all surpassing LGG (56.7 %). Hydrophobicity values above 60 % are widely correlated with improved epithelial adhesion and resistance to peristaltic clearance (Divyashree et al., 2021). These data reinforce the high auto-aggregation capacities reported earlier and suggest the presence of APFs, S-layer proteins, and EPS mediating both cell–cell and cell–host adhesion (Trunk et al., 2018; Wang et al., 2017;

Zommiti et al., 2018). Indeed, surface hydrophobicity can depend on sortase A–anchored proteins, as deletion of *srtA* in *Lb. gasseri* reduced xylene adhesion from 62 % to 17 %) (Zuo et al., 2019).

Adhesion to ethyl acetate, a monopolar basic solvent probing electron-acceptor (acidic) surface sites, was moderate (47–59 %) across isolates, with TF13 and DC01-A reaching ~58 %. These values, lower than those for chloroform or xylene, reflect the higher polarity of ethyl acetate and its weaker affinity for hydrophobic bacterial surfaces (Martiz et al., 2023). The overall solvent-specific pattern indicates that these strains possess amphoteric surfaces containing both Lewis acid and base sites. Such duality likely enhances adaptability across the steep pH and ionic gradients of the gastrointestinal tract (Angelescu et al., 2024).

Quantitatively, all isolates exceeded the 40 % hydrophobicity threshold proposed as a minimum for probiotic colonization (Mikolajczuk-Szczyrba et al., 2025), and by the classification of Woitschach et al. (2021), all are highly hydrophobic (> 50 % adhesion to at least two solvents). Cross-comparison with auto-aggregation and gastric survival data highlights TF13, TF03, and DC06 as the most promising strains. Their combination of high hydrophobicity and strong electron-donor profiles predicts superior biofilm-forming capacity and pathogen exclusion through steric competition and niche occupation.

Examples from literature reinforce this association: *Lpb. plantarum* YW11 (> 50 % xylene adhesion) and *Fructobacillus tropaeoli* KKP 3032 (~65.7 % xylene adhesion) both demonstrated enhanced intestinal colonization (Mikolajczuk-Szczyrba et al., 2025; Zeng et al., 2020; M. Zhang et al., 2022). Functionally, strong hydrophobicity confers two advantages: (1) protection from mechanical washout, allowing prolonged gut persistence, and (2) competitive exclusion of pathogens at epithelial binding sites (Iorizzo et al., 2020; Trunk et al., 2018).

Hydrophobicity closely parallels auto-aggregation, as both depend on surface macromolecular composition. Strains exhibiting high adhesion to hydrocarbons generally display greater auto-aggregation, suggesting shared molecular determinants such as EPS and APFs (Cozzolino et al., 2020; Othman & Karim, 2023). The superior performance of several isolates relative to LGG underscores the value of bioprospecting in unconventional fermentative niches such as tea-waste fermentations. Genomic characterization of top-performing isolates could reveal specific genes encoding S-layer proteins or APFs responsible for these phenotypes.

Biofilm formation assay

Biofilm formation, evaluated semi-quantitatively using a modified glass-tube method (Fatima et al., 2021), was scored from 0 to 4 based on crystal-violet retention (Figure 37).

Among tested isolates, DC04 achieved the maximal score (4), producing a dense, homogeneous violet film typical of strains with abundant EPS secretion. Such a phenotype is characteristic of *Lpb. plantarum* and correlates with robust gastrointestinal persistence (Nami et al., 2024; M. Zhang et al., 2022). DC01-A and TF03 showed moderate staining (score 2), consistent with partial surface coverage, while LGG exhibited weak biofilm formation (score 1), in line with previous reports of its strong epithelial adhesion but limited abiotic surface colonization (Ramirez-Sanchez et al., 2022). TF13 scored 0–1, suggesting minimal attachment to glass; however, low abiotic biofilm formation does not preclude effective mucosal binding, which may instead rely on specific pili or mucus-binding domains (Mogmenga et al., 2023).

Taken together, these data underscore the strain-specific nature of biofilm formation. DC04 emerges as the most promising candidate for stable surface colonization, supported by its high hydrophobicity and auto-aggregation capacity. The convergence of these traits supports a model in which hydrophobic interactions and self-aggregation initiate adhesion, while EPS production consolidates biofilm maturation (Bhagya et al., 2018; Mogmenga et al., 2023). Such multifactorial robustness enhances pathogen exclusion and promotes durable probiotic delivery in both food matrices and the gastrointestinal environment.

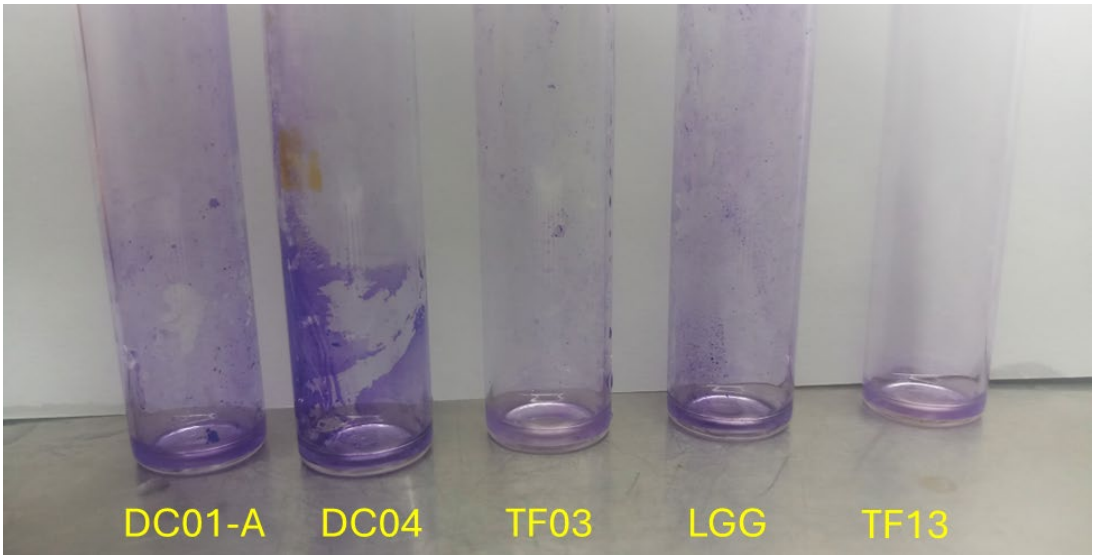

Figure 37 Biofilm formation by selected LAB isolates after 48 h of static growth in glass tubes. From left to right: DC01-A (score 2), DC04 (score 4), TF03 (score 2), LGG (score 1), TF13 (score 0–1).

The interplay between cell surface hydrophobicity, auto-aggregation, and biofilm formation represents a key determinant of probiotic colonization in the gastrointestinal tract. Hydrophobic interactions facilitate initial bacterial contact with mucosal surfaces, while biofilm formation ensures persistent adhesion and metabolic activity over time (Bhagya et al., 2018). Together, these phenomena form a continuum from transient surface attachment to stable colonization, thereby influencing probiotic efficacy and resilience under gastrointestinal conditions.

The process of adhesion leading to biofilm development is multifactorial, governed by hydrophobic forces, electrostatic interactions, and specific physicochemical complementarity between microbial surfaces and intestinal epithelial substrates (Mogmenga et al., 2023). Although numerous molecular determinants contribute to adhesion, two parameters emerge as the principal predictors of probiotic adherence and biofilm potential (Mogmenga et al., 2023). From an applied standpoint, bacterial strains combining moderate-to-high hydrophobicity with strong biofilm formation exhibit superior colonization capability. For example, *Enterococcus durans* A8-1, with an adhesion rate of 38.7 % to *Caco-2* intestinal cells and moderate biofilm production, demonstrated effective mucosal attachment and persistence (Zhou et al., 2021). Similarly, our top-performing isolates (TF13, DC04, and DC06), which displayed high hydrophobicity and strong biofilm formation, are expected to exhibit comparable colonization efficiency. Such traits confer tangible physiological benefits: strains that adhere strongly to the intestinal mucosa typically exhibit slower elimination kinetics in the gastrointestinal tract and extended residence times, allowing sustained probiotic action (Alkalbani et al., 2022; Mogmenga et al., 2023). Prolonged colonization enhances pathogen exclusion through steric competition, modulates mucosal immunity, and promotes local synthesis of beneficial metabolites such as SCFAs, bacteriocins, and organic acids. These collective effects underscore the multifaceted nature of probiotic adhesion mechanisms, where surface hydrophobicity, aggregation behavior, and biofilm formation act synergistically to ensure stable colonization and functional efficacy

7.1.7. Biological activities of Lactobacillus strains

Understanding the biological activities of *Lactobacillus* strains extends probiotic assessment beyond basic colonization potential to their functional roles in host protection and food preservation. Among these activities, antibacterial potential is a defining trait, reflecting the

ability of LAB to inhibit or suppress pathogenic microorganisms through competitive exclusion, acidification, and secretion of antimicrobial metabolites.

Antibacterial activity assay

The antibacterial potential of the isolates was evaluated against representative food-borne and intestinal pathogens using the agar-disk diffusion method (Table 12). This assay provides a direct measure of antagonistic efficacy by quantifying the diameter of inhibition zones surrounding LAB culture supernatants. The observed inhibition reflects the combined effects of organic acid production (primarily lactic and acetic acids), hydrogen peroxide generation, and bacteriocin-like inhibitory substances (BLIS). This functional screening is critical for identifying strains with potential applications as natural bio-preservatives and therapeutic probiotics. LAB with strong antagonistic activity not only enhance the safety and shelf life of fermented foods but also reinforce host protection by limiting pathogen colonization in the gastrointestinal environment (Kazancıgil et al., 2019; Nami et al., 2024). Results of the assay (discussed in the following section) demonstrate that several isolates produced substantial inhibition zones against *E. coli*, *S. aureus*, and *P. aeruginosa*, supporting their dual potential as bio-preservative agents and effective probiotics.

Table 12 Antibacterial activity of lactobacillus strains against pathogenic bacteria using the disk diffusion method.

Strains	***E. coli***	***P. aeruginosa***	***S. aureus***
	Zone of Inhibition (mm)		
DC01-A	11.32±0.62^{b}	19.72±2.50^{b}	7.70±0.60^{c}
DC04	12.30±0.42^{a}	15.60±0.60^{c}	22.00±1.00^{a}
DC06	7.25±0.60^{c}	20.34±1.52^{a}	20.34±1.52^{b}
TF03	10.34±0.42^{b}	18.53±1.52^{b}	19.94±1.55^{b}
TF13	9.87±0.58^{b}	16.30±1.79^{c}	18.37±1.01^{b}
TF17	9.72±1.54^{b}	17.21±1.19^{b}	18.21±0.95^{b}

The mean values are presented along with their respective SD (n = 3). $^{a–c}$ Significant differences among the values within the same column are indicated by different superscript letters ($p < 0.05$).

Pronounced strain-specific differences were observed (Figure 38). Isolate DC04 produced the largest inhibition zone against *S. aureus* (22 mm), significantly exceeding all other strains ($p < 0.05$). This agrees with the well-documented antimicrobial capacity of *Lpb. plantarum*, which secretes organic acids, hydrogen peroxide, and several bacteriocins such as plantaricins A, EF, and JK (Lee et al., 2024; Zommiti et al., 2018). Moderate inhibition was detected in TF13 and DC06 (14–17 mm) against *E. coli* and *P. aeruginosa*, while DC01-A and DC02 showed weak

activity (< 10 mm). These variations highlight the strain-dependent nature of LAB antagonism, reflecting differences in acidification capacity, oxidative metabolite production, and bacteriocin synthesis potential.

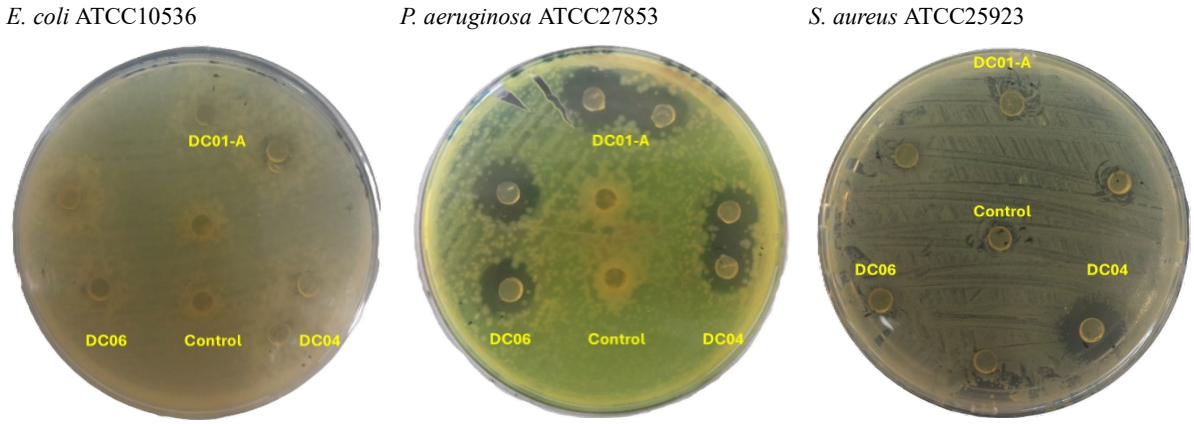

Figure 38 Antibacterial activity of some isolate.

DC06 exhibited the broadest antimicrobial spectrum, inhibiting both *P. aeruginosa* and *S. aureus* (≈ 20 mm). Such dual activity suggests the combined effects of acidic metabolites and proteinaceous inhibitors, consistent with earlier observations on *Lev. brevis* (Mogmenga et al., 2023). A second *Lev. brevis* isolate, DC01-A, retained good activity against Gram-negative bacteria but showed weak inhibition of *S. aureus*, underscoring intra-species variability in antimicrobial output.

Isolates TF03, TF13, and TF17, derived from polyphenol-rich fermented tea, produced moderate yet consistent inhibition (≈ 10–20 mm) across all pathogens. Although statistically lower than DC04 and DC06, their inhibitory spectra fall within the efficacious range reported for commercial probiotic strains (Cozzolino et al., 2020; Nami et al., 2024).

The antimicrobial capacity of *Lactobacillus sensu lato*

is highly strain-specific rather than species-dependent (Dobreva et al., 2024; Iorizzo et al., 2022). Previous reports show that *Lpb. plantarum* typically exhibits stronger activity than *L. casei*, *Lev. brevis*, *L. acidophilus*, *Lmb. fermentum*, or *Lb. delbrueckii*, with inhibition zones of 10–27 mm against common pathogens (Barzegar et al., 2021; Hu et al., 2019; Megur et al., 2023). Exceptional *Lpb. plantarum* strains have reached 30–44 mm against antibiotic-resistant *S. aureus* and *P. aeruginosa* (Al-Madboly & Abdullah, 2015; Corazza et al., 2024).

Collectively, DC04 and DC06 emerged as the most potent antagonists, particularly against *S. aureus* and *P. aeruginosa*. Given that antimicrobial activity supports both competitive exclusion and bio-preservation, these strains warrant further characterization of their inhibitory compounds (organic-acid profiles and bacteriocin genes) and validation in food or co-culture models simulating gastrointestinal conditions. Synergistic combinations of *Lev. brevis* and *Lpb. plantarum* have been shown to broaden antibacterial spectra through complementary mechanisms, competitive exclusion by *Lev. brevis* and bacteriocin production by *Lpb. plantarum* (Butorac et al., 2020).

DPPH free-radical scavenging activity

The DPPH assay was used to evaluate the antioxidant potential of LAB cell-free supernatants, a critical functional trait for counteracting oxidative stress and enhancing the nutritional value of fermented foods (Park et al., 2021; Rwubuzizi et al., 2023). Significant inter-strain differences ($p < 0.05$) were observed, with scavenging rates ranging from 56.3 % to 70.1 % (Figure 37). Isolate DC04 exhibited the highest activity (70.1 %), surpassing the commercial reference strain LGG (61.3 %). Several *Lev. brevis* isolates from fermented tea (TF04, TF17, TF13) also showed strong scavenging capacities comparable to DC04, while DC06 recorded the lowest value (56.3 %), again confirming the strain-specific nature of this functional attribute.

These findings align with previous reports showing that LAB strains with DPPH scavenging activity above 30% are regarded as strong antioxidants, with certain *Lpb. plantarum* isolates reaching up to 72%, depending on strain origin and assay methodology (Rwubuzizi et al., 2023; Wu et al., 2025). Notably, isolates from fermented tea substrates consistently displayed higher antioxidant capacity than those from goat-milk butter, likely reflecting adaptation to polyphenol-rich environments (C. Liu et al., 2021). This pattern suggests that ecological origin and niche-specific gene expression enhance the synthesis of antioxidant enzymes and metabolites.

The antioxidant potential of LAB arises from multiple mechanisms, including radical scavenging, metal ion chelation, enzymatic detoxification of reactive oxygen species, and the generation of bioactive peptides and organic acids (Carneiro et al., 2024; Unban et al., 2021). Species-level variability is well documented: *Lb. kefiri* exhibits strong activity in fermented supernatants, whereas *Lpb. plantarum* primarily relies on whole-cell mechanisms (Wu et al., 2025).

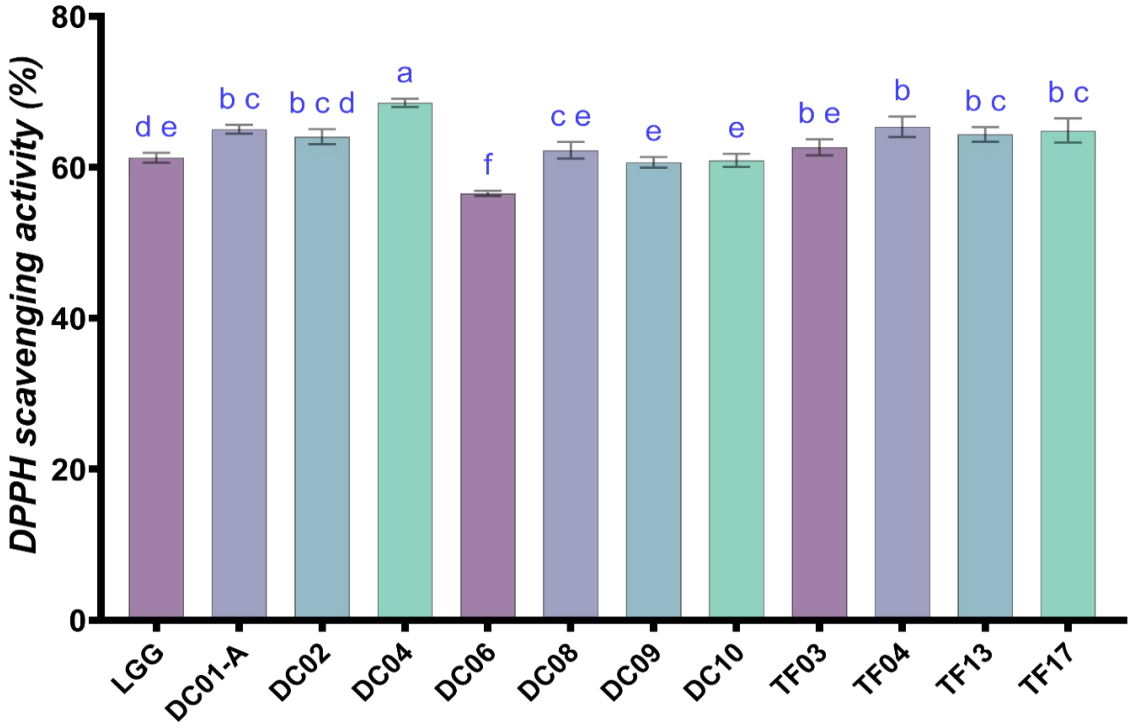

Figure 39 DPPH free radical scavenging activity (%) of cell-free supernatants from LAB isolates. Data are expressed as mean ± SD (n=3). Different superscript letters (a–f) indicate statistically significant differences ($p < 0.05$) based on one-way ANOVA followed by Tukey's post hoc test.

Fermentation itself enhances antioxidant performance. Cho et al. (2015) reported increased DPPH scavenging in fermented whey beverages versus unfermented controls, and Zhang et al. (2025) observed a 44–48% rise following fermentation with selected *Lpb. plantarum* strains. Similar strain-dependent variability was documented by Kang et al. (2021), with DPPH values ranging from 14.7% to 20.9%, while Sellam et al. (2024) recorded up to 81% activity in intact LAB cells.

Overall, these results corroborate prior evidence that LAB from fermented foods such as kimchi and tea leaves display superior antioxidant capacity compared with commercial references like LGG (Baick & Kim, 2015; C. Liu et al., 2021). Among the tested isolates, DC04 stands out as the most promising candidate for developing antioxidant-enriched probiotic formulations.

7.2. Microencapsulation of LAB

Microencapsulation significantly enhances probiotic survival under gastrointestinal stress, protecting cells against acidic pH, bile salts, and inhibitory phenolic compounds (Ramírez-

Damián et al., 2025). The encapsulation matrix maintains cell integrity and viability, shielding bacteria from oxidative and antimicrobial stressors within the gut environment (Garfias Noguez et al., 2025).

Encapsulation of the five selected LAB strains in calcium–alginate beads achieved uniformly high encapsulation efficiencies (EE), ranging from 89.1% to 97.0% (Figure 40). The reference strain LGG showed EE values of 90.3–92.5%, whereas *Lev. brevis* DC01-A and DC04, together with *Lpb. plantarum* TF03 and TF13, all exceeded 90%. Notably, TF03 achieved the highest efficiency (95.1–97.0%), indicating excellent compatibility with the alginate matrix. These values align closely with the >90% encapsulation efficiencies typically reported for calcium–alginate systems (Butorac et al., 2021; Fareez et al., 2015; Mahmoud et al., 2020). Such high encapsulation performance highlights the potential of alginate-based carriers for delivering phenol-tolerant and gastrointestinal-resilient probiotics, especially strains originating from polyphenol-rich substrates such as fermented tea.

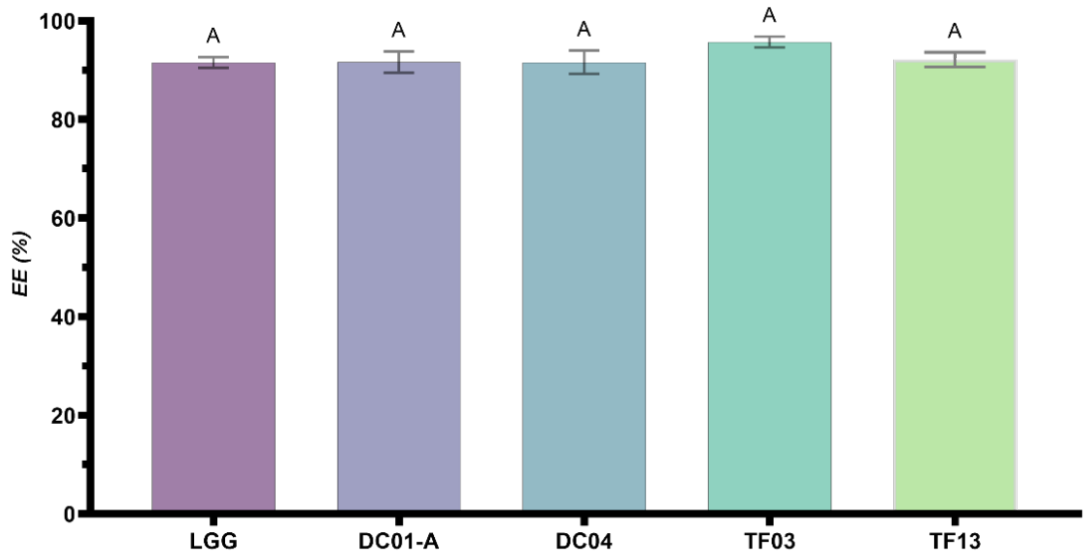

Figure 40 Encapsulation efficiency of calcium-alginate beads loaded with selected isolates. Data were expressed as mean ± SD. Values with different superscript letters are significantly different ($P < 0.05$).

Minor inter-strain variation in EE is expected, as surface charge, hydrophobicity, and cell size influence electrostatic interactions with guluronic-rich alginate chains, thereby affecting bead porosity and cell retention. Strains with pronounced hydrophobicity and strong auto-aggregation generally achieve higher EE due to stronger interactions with the encapsulation

matrix (Hao et al., 2024). This explains why bacteria exhibiting superior cell–cell cohesion and abiotic attachment properties integrate more effectively within the polymer network during ionotropic gelation.

The uniformly high EE values obtained (89–97%) align with the previously characterized surface physicochemical profiles of the isolates. Strains displaying high hydrophobicity and strong biofilm formation (TF03, TF13, DC04) showed the greatest encapsulation efficiency. Alginate gelation entraps cells primarily through physical exclusion within the guluronic-acid mesh, but electrostatic and hydrophobic interactions between bacterial envelopes and the polymer matrix also modulate bead porosity and mechanical stability. The dominant electron-donor (basic) surfaces revealed by the chloroform–BATH assay, coupled with the moderate negative charge typical of LAB, favor ionic bridging with Ca^{2+} cross-links, reducing cell leakage during curing and washing. In contrast, less hydrophobic strains such as LGG and DC01-A still achieved >90% EE but exhibited slightly greater inter-replicate variability, suggesting weaker cell–matrix association.

These findings highlight a mechanistic continuum in which surface traits promoting cell–cell cohesion (auto-aggregation) and abiotic adhesion (biofilm formation, hydrophobicity) simultaneously enhance cell–matrix integration during encapsulation. The extensive hydrophobic domains and extracellular-polymeric-substance layers of TF03 and TF13 not only underpin superior mucosal adhesion but also confer a technological advantage by improving encapsulation yield. Electrostatic complexation between bacterial surfaces and biopolymers further enhances encapsulation performance, as Ca^{2+} ions neutralize repulsive charges and form salt bridges within the alginate network, creating a compact, protective barrier that retains cells with 90–92% efficiency (Praepanitchai et al., 2019).

Integrating surface-property data with EE offers a rational framework for selecting robust strains: isolates combining high colonization potential with strong entrapment efficiency are more likely to maintain viability during processing, storage, and gastrointestinal passage. Accordingly, TF03 and TF13 emerged as the most promising candidates for downstream application.

Overall, the consistently high EEs confirm that the ionotropic-gelation protocol generated structurally stable beads capable of protecting LAB across species origins. Achieving ≥90% EE ensures sufficient probiotic payload to offset processing losses, extend shelf life, and deliver

therapeutic doses post-GI transit. This outcome supports advancing encapsulated TF03 and TF13 strains for *in vivo* validation.

7.3.In vivo study

An *in vivo* feeding trial was conducted to validate the functional potential of the well-characterized LAB strains under practical poultry production conditions. The experiment aimed to assess their influence on growth performance, intestinal morphology, gut microbial balance, and meat quality traits in broiler chickens.

This trial represents the translational phase of probiotic evaluation, bridging *in vitro* functional screening with animal-level efficacy. Specifically, it sought to determine whether the physiological and technological attributes previously characterized translate into tangible zootechnical and health benefits *in vivo*.

Broilers were fed diets supplemented with selected microencapsulated LAB strains, and parameters including body weight gain, feed conversion ratio, villus height-to-crypt depth ratio, intestinal histoarchitecture, lipid oxidation, and meat physicochemical composition were systematically evaluated. These indicators provide an integrated measure of how probiotic supplementation modulates nutrient assimilation, intestinal integrity, and oxidative stability - key determinants of animal performance and product quality.

Beyond zootechnical outcomes, the trial also aimed to explore potential immunomodulatory and antioxidative effects, considering that strains such as TF03 and TF13 demonstrated superior radical-scavenging and adhesion capabilities in vitro. Such effects could enhance gut resilience, mitigate oxidative stress, and improve overall meat nutritional quality under intensive production settings.

Ultimately, this *in vivo* assessment was designed to establish a direct link between molecular, physiological, and functional traits of the LAB strains and their practical efficacy in broiler production, thus supporting their development as natural, sustainable probiotic alternatives to antibiotic growth promoters.

7.3.1. Body weight

The growth performance of broilers supplemented with selected LAB strains showed a significant and sustained enhancement throughout the rearing period (Figure 41). Body-weight gain followed a consistent upward trajectory, with a clear divergence from the control group evident after the third week of rearing. By day 42, birds receiving the tea-derived isolates TF03

and TF13 attained mean final body weights of approximately 2720 g and 2680 g, respectively—representing 10–12 % higher mass relative to the unsupplemented control (≈ 2320 g, $p < 0.05$). The isolates DC04 and DC01-A, obtained from fermented goat butter, displayed intermediate growth performance (2460 g and 2390 g, respectively), suggesting a strain-specific impact on growth kinetics rather than a uniform species-level effect. These outcomes confirm that probiotic efficacy within *Lactobacillus sensu lato* is determined predominantly by genotypic and ecological origin (Dobreva et al., 2024; Iorizzo et al., 2022).

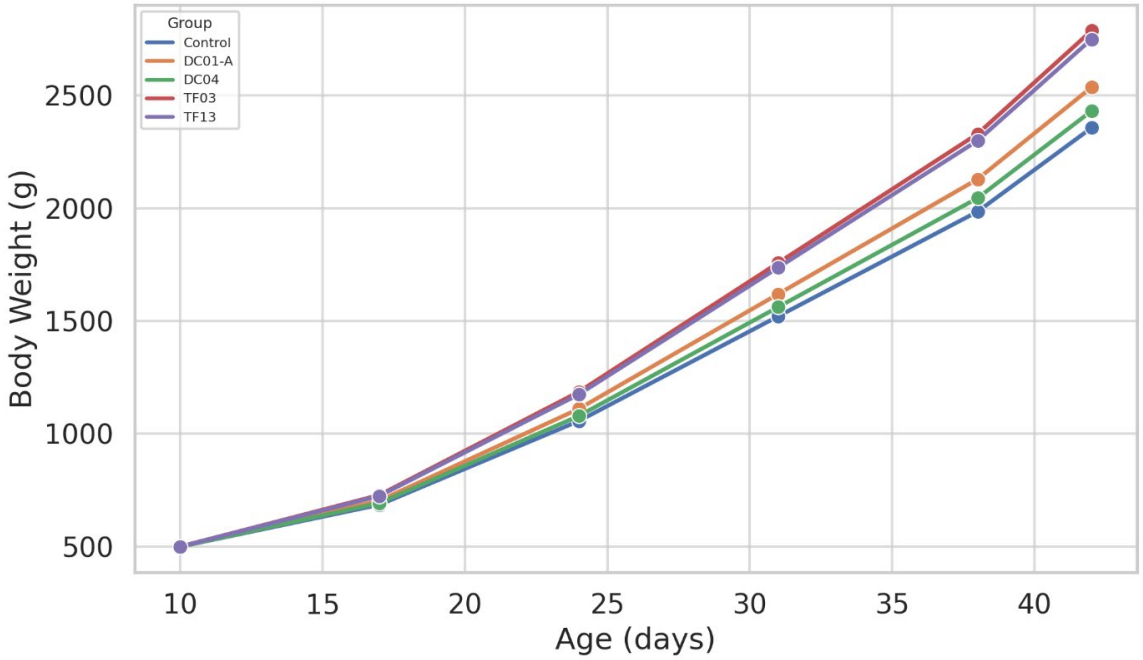

Figure 41 Evolution of chicken weight during 42 days. DC01-A; *Lev. brevis*, DC04; *Lpb. plantarum*, TF03; *Lev. brevis*, TF13; *Lev. brevis*. Control; without probiotic supplementation.

The overall gain observed in TF03- and TF13-treated birds aligns closely with findings by Cozzolino et al. (2020) and Zou et al. (2017), who documented 8–15 % higher final body weights in broilers supplemented with *Lpb. plantarum* and *Lev. brevis* strains. Enhanced growth is often attributed to the modulation of intestinal morphology, particularly villus height and crypt-to-villus ratio, which together increase the absorptive surface area of the small intestine (Eglite et al., 2023; Rwubuzizi et al., 2023; Yin et al., 2023). The current isolates, characterized by strong acid and bile tolerance and superior hydrophobicity (> 70 %), likely achieved greater persistence in the gastrointestinal tract, enabling continuous colonization of mucosal niches and competitive exclusion of enteropathogens (Abd El-Hack et al., 2020; Kazancıgil et al., 2019). Such colonization stability reduces epithelial damage and inflammation, thereby

conserving metabolic energy for tissue accretion rather than immune defense (Eglite et al., 2023).

The probiotic-enhanced groups also exhibited improved feed efficiency, inferred from greater weight gain under identical feeding regimens. Enhanced feed conversion efficiency is a hallmark of effective probiotic supplementation and is mechanistically linked to the up-regulation of digestive enzymes (amylase, protease, and lipase) and to SCFA synthesis through carbohydrate fermentation (Mogmenga et al., 2023). SCFAs such as acetate and butyrate serve as trophic factors for enterocytes, reinforcing barrier integrity and stimulating villus elongation (Chang et al., 2019; C. Liu et al., 2021; Yin et al., 2023). These mechanisms collectively underpin the steady and statistically significant growth advantage observed for TF03 and TF13.

Beyond their nutritional role, probiotic LAB exert systemic effects by modulating the redox balance and immune tone of the host. The high DPPH radical-scavenging activity (≈ 70 %) recorded for DC04, TF03, and TF13 indicates substantial antioxidant potential, a property closely linked to the mitigation of oxidative stress in fast-growing broilers (Eglite et al., 2023). Oxidative stress is a major factor compromising feed efficiency and muscle quality in intensive poultry systems (Carneiro et al., 2024). LAB-derived metabolites neutralize reactive oxygen species and improve mitochondrial efficiency (Unban et al., 2021; Wu et al., 2025). Consequently, strains with strong antioxidative capacity may sustain metabolic throughput during peak growth phases, explaining the accelerated weight gain observed for TF03 and TF13 after day 25.

Furthermore, *Lpb. plantarum* is well known for secreting bacteriocins and organic acids that inhibit opportunistic gut pathogens, notably *E. coli* and *Clostridium perfringens* (Lee et al., 2024; Zommiti et al., 2018). Reduced pathogenic load minimizes enteric inflammation, increases nutrient assimilation efficiency, and preserves epithelial tight-junction integrity (Bhagya et al., 2018; Eglite et al., 2023). In the present experiment, isolates DC04 and DC06 displayed broad-spectrum antibacterial activity (inhibition zones ≈ 20–22 mm against *S. aureus* and *P. aeruginosa*), corroborating earlier reports on *Lev. Brevis* (Hojjati et al., 2020). The synergistic combination of *Lpb. plantarum* (acidic metabolite production) and *Lev. brevis* (competitive adhesion and exclusion) thus represents a promising multi-strain formulation capable of broad-spectrum antimicrobial defense while maintaining beneficial microbiota balance (Butorac et al., 2020).

The consistent superiority of tea-derived isolates (TF03, TF13) compared with dairy isolates (DC01-A, DC04) underscores the influence of ecological adaptation on probiotic functionality. Fermented tea environments are polyphenol-rich and mildly acidic; survival therein requires

tolerance to oxidative and phenolic stressors. Such selective pressure may enhance membrane robustness, stress-response gene expression, and the production of antioxidant and detoxifying enzymes (Angelescu et al., 2024; L. Wang et al., 2021). These adaptive traits likely translated into greater resilience during gastrointestinal transit, consistent with the high acid and bile tolerance previously reported for these strains.

Additionally, both TF03 and TF13 exhibited pronounced auto-aggregation (> 65 %) and biofilm-forming capacities, parameters directly correlated with mucosal adhesion (Cozzolino et al., 2020; Nwoko & Okeke, 2021). Their amphoteric surface properties suggest a balanced distribution of Lewis acid and base functional groups, facilitating adhesion across the variable physicochemical landscape of the gut mucosa. Such surface versatility enhances colonization efficiency and persistence within the intestinal microenvironment (Othman & Karim, 2023).

The subsequent microencapsulation of these strains in calcium-alginate beads yielded exceptionally high encapsulation efficiencies (89–97 %), confirming their technological robustness. Strains exhibiting greater hydrophobicity and biofilm potential achieved the highest encapsulation yields, reflecting stronger electrostatic and hydrophobic interactions with the alginate matrix (Hao et al., 2024). This correlation between cell-surface traits and encapsulation efficiency ensures that these strains can be effectively stabilized during feed processing and storage. From an applied standpoint, encapsulation provides a protective microenvironment that enhances survival through gastric acidity and bile exposure (Praepanitchai et al., 2019; Ramirez-Damian et al., 2025).

Thus, the same physicochemical features that promote mucosal adhesion also favor entrapment within biopolymeric carriers, establishing a unified framework linking biological functionality and technological performance. In practical applications, these attributes are crucial for maintaining viable probiotic loads during pelleting, drying, and storage, ensuring a therapeutically relevant cell density upon ingestion.

Collectively, the *in vivo* findings demonstrate that LAB supplementation enhances broiler growth, feed utilization, and physiological resilience. These effects likely result from a combination of microbiota modulation, antioxidant defense, and improved intestinal morphology. The results also highlight how ecological origin, surface chemistry, and stress-tolerance phenotypes interact to shape probiotic efficacy. Given their strong acid and bile tolerance, hydrophobicity, and biofilm-forming abilities, these isolates represent promising biotechnological candidates for use as natural growth promoters and functional feed additives in poultry production systems increasingly constrained by restrictions on antibiotic growth promoters (AGPs).

Future studies should integrate multi-omics analyses (transcriptomic and metabolomic profiling) to elucidate regulatory networks governing stress adaptation and metabolite biosynthesis in these strains. *In vivo* trials assessing intestinal histomorphometry, immune gene expression, and meat physicochemical properties will further define their mechanistic contributions to host performance and product quality.

7.3.2. Feed Intake, Average Daily Gain (ADG), and Feed Conversion Ratio (FCR)

The present findings (Figure 42) demonstrate that probiotic supplementation exerted a significant strain-dependent impact on the productive performance of broiler chickens. Quantitative evaluation revealed that the feed intake (FI) of the control group averaged ≈ 4100 g/bird, whereas birds supplemented with the dairy-derived strains (DC01-A and DC04) consumed ≈ 4200–4250 g/bird. The tea-derived strains TF03 and TF13 exhibited the highest intake values (≈ 4350–4400 g/bird), although these increases were not statistically significant ($p > 0.05$). This trend suggests that while probiotics may slightly stimulate appetite and feed palatability, the overall productivity gains observed were not solely driven by greater feed consumption. Similar trends were reported by Abdel-Hafeez et al. (2017), who observed modest improvements in feed intake among probiotic-fed broilers, attributed to enhanced feed palatability, gut enzyme secretion, and microbial metabolite production improving gastrointestinal motility.

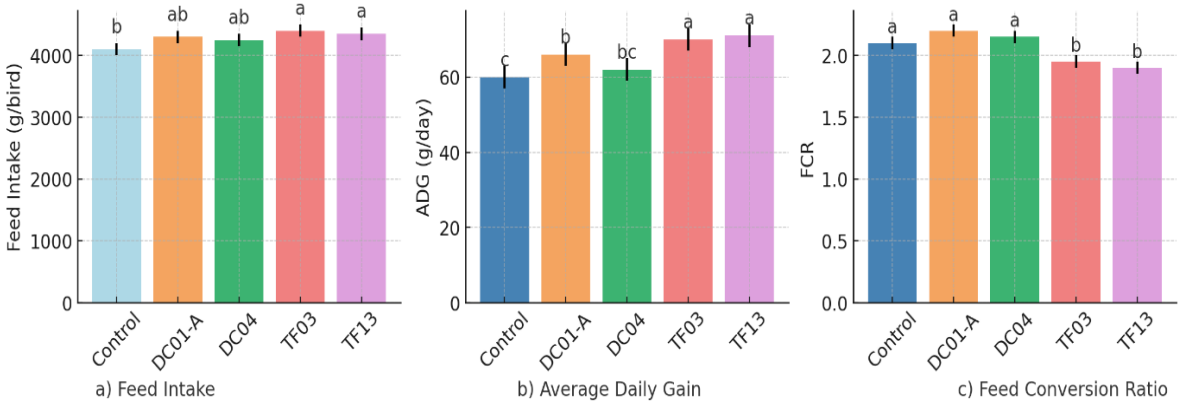

Figure 42 Probiotic Effects on (a) Feed Intake, (b) Average Daily Gain, and (c) Feed Conversion Ratio in Broilers (Day 10–42). DC01-A; *Lev. brevis*, DC04; *Lac. plantarum*, TF03; *Lev. brevis*, TF13; *Lev. brevis,* Control; without probiotic supplementation. ADG; average daily gain, FCR; feed conversion ratio. $^{a–c}$: Different uppercase letters in superscript indicate significant differences ($p<0.05$) among strains

A more pronounced effect was observed in ADG. The control group exhibited a mean ADG of ≈ 61 g/day, while DC01-A and DC04 achieved ≈ 65–66 g/day, and TF03 and TF13 displayed the highest growth rates at ≈ 70–72 g/day ($p < 0.05$). The performance enhancement of TF03 and TF13 (≈ +15% relative to control) indicates superior nutrient assimilation efficiency and better intestinal functionality, suggesting that these isolates confer physiological benefits beyond nutrient intake. Comparable magnitudes of growth improvement (10–20%) have been reported in broilers receiving *Lpb plantarum*, *E. faecium*, and *Bacillus subtilis* probiotics (Evangelista et al., 2023; He et al., 2020; Suvorov et al., 2023).

The increase in ADG is mechanistically linked to several factors. Firstly, probiotic supplementation is known to stimulate intestinal villus growth, improving the surface area available for nutrient absorption (Eglite et al., 2023). Secondly, probiotics such as *Lpb. plantarum* and *Lev. brevis* secrete amylases, proteases, and lipases, enhancing macronutrient digestibility (Abdel-Hafeez et al., 2017; He et al., 2020). Thirdly, these strains modulate the gut microbiota, promoting the proliferation of beneficial taxa such as *Lactobacillus* and *Bifidobacterium* while suppressing pathogenic species (*E. coli*, *Clostridium perfringens*), which improves gut health and nutrient uptake (Kazancıgil et al., 2019; Sanders et al., 2019; Vinderola et al., 2024).

The feed conversion ratio (FCR), a key index of feed efficiency, exhibited the clearest treatment effects. The control group recorded an FCR of ≈ 2.10, while DC01-A and DC04 achieved slightly lower ratios (≈ 2.05–2.08, $p > 0.05$). In contrast, TF03 and TF13 showed significantly reduced FCR values (≈ 1.88–1.90, $p < 0.05$), reflecting a ~10% improvement in feed efficiency. This reduction indicates that birds receiving tea-derived probiotics required less feed to achieve equivalent or greater weight gain.

These findings are consistent with earlier studies demonstrating that probiotic supplementation enhances nutrient utilization and energy efficiency. He et al. (2021) reported a 9% reduction in FCR in broilers supplemented with *E. faecium* PNC01, attributing the improvement to enhanced villus height, better mucosal integrity, and increased SCFA production. Similarly, Suvorov et al. (2023) found that *E. faecium* L3 supplementation improved feed conversion through immune modulation and the production of bacteriocins that inhibit intestinal pathogens, thereby reducing metabolic stress.

In the current study, the superior FCR of TF03 and TF13 can be mechanistically attributed to their high bile salt and acid tolerance, strong auto-aggregation and hydrophobicity, and robust

antioxidative capacity demonstrated *in vitro*. These phenotypic traits likely enabled more effective colonization of the gastrointestinal mucosa, promoting stable microbial consortia that enhance enzymatic activity, nutrient transport, and epithelial protection. Similar strain-dependent enhancements in energy conversion efficiency have been documented in *Lpb. plantarum* and *Lev. brevis* strains from fermented tea and vegetable sources (Krysiak et al., 2021; Lee et al., 2024; C. Liu et al., 2021; Zou et al., 2017).

The improvements in ADG and FCR suggest that the probiotics enhanced metabolic throughput—the efficiency with which nutrients are digested, absorbed, and converted into body tissue. Probiotic action is known to elevate intestinal brush-border enzyme activity, increase goblet cell density, and improve villus height-to-crypt-depth ratio (VH/CD), resulting in more efficient nutrient assimilation (Eglite et al., 2023; Khan et al., 2025). Moreover, *Lactobacillus* supplementation is associated with upregulation of nutrient transporter genes (e.g., *SGLT1*, *PEPT1*), enhancing glucose and peptide absorption (Biswas et al., 2022; Primec et al., 2021).

The enhanced feed efficiency in TF03 and TF13-fed birds likely also reflects lower oxidative stress and improved gut barrier function. These isolates exhibited strong *in vitro* DPPH radical scavenging capacities (≈ 70%), indicating a high potential to neutralize ROS *in vivo*. Reduced oxidative stress preserves intestinal epithelial integrity and minimizes energy loss to immune activation (Eglite et al., 2023; Yin et al., 2023). Probiotic-driven antioxidant activity also supports mitochondrial efficiency and protein accretion, thereby improving feed conversion (Carneiro et al., 2024; Sellam et al., 2024; Wu et al., 2025).

In addition, tea-derived *Lactobacillus* strains may contribute polyphenol biotransformation in the gut. Several studies (C. Liu et al., 2021; Martiz et al., 2023) have shown that *Lpb. plantarum* and *Lev. brevis* isolated from fermented tea or polyphenol-rich substrates can metabolize catechins and phenolic acids into bioactive derivatives that modulate lipid metabolism and intestinal signaling. Such metabolic activity may enhance nutrient partitioning toward lean tissue accretion rather than fat deposition, improving carcass composition and feed efficiency.

The magnitude of improvement in FCR observed in this study (~10%) is comparable to or greater than the effects reported for commercial probiotics.

For example, supplementation with *Lpb. plantarum* and multi-strain probiotics has been shown to enhance FCR and ADG in animal models, with reported improvements in FCR ranging from 6–9.3% and ADG increases up to 12% (Lefter et al., 2022; Pupa et al., 2022) comparable to the

values achieved here with TF03 and TF13. These consistent trends across independent studies reinforce that probiotic-mediated modulation of intestinal physiology translates into tangible zootechnical gains.

By contrast, the modest improvements observed for DC01-A and DC04 suggest that probiotic efficacy depends strongly on strain origin and ecological adaptation. Tea-derived LAB, such as TF03 and TF13, are exposed to polyphenolic and mildly acidic environments that may pre-select for stress-resistant phenotypes with superior metabolic resilience and bioactive compound tolerance (Angelescu et al., 2024; Parlindungan et al., 2021). Such evolutionary adaptation likely enhances survival in the avian gut, which experiences fluctuating pH and bile conditions.

From a practical standpoint, the observed improvement in ADG and FCR without increasing feed intake demonstrates nutrient efficiency gains that directly translate into lower feed costs and improved sustainability of poultry production. As feed accounts for approximately 70% of total production cost, a 10% improvement in FCR represents a major economic advantage (Abdel-Hafeez et al., 2017). Moreover, the use of natural LAB probiotics such as TF03 and TF13 aligns with global efforts to replace AGPs with safe, eco-friendly alternatives, thereby reducing antimicrobial resistance risks while maintaining productivity (Eglite et al., 2023; Suvorov et al., 2023).

7.3.3. Meat Quality Analysis

Dry Matter, Mineral Profile, Lipid Accumulation, and Protein Levels

The results demonstrate that probiotic supplementation significantly influences the physicochemical composition of broiler breast muscle, particularly in terms of dry matter, mineral content, lipid deposition, and protein concentration. Among the tested groups, strains TF03 and TF13 consistently exhibited superior meat quality, with higher dry matter (28.50% and 28.40%, respectively), elevated protein levels (22.50% and 22.40%), and increased lipid content (4.15% and 4.08%), compared to the control group (Table 13).

Dry matter (DM) is a critical indicator of tissue density, reflecting the accumulation of protein, lipid, and mineral constituents relative to water content. Birds receiving TF03 and TF13 showed DM values of 28.50 ± 0.24% and 28.40 ± 0.30%, respectively—an increase of ~7% over the control group. The DC01-A and DC04 groups also showed moderate improvements (27.30% and 26.90%), though these were significantly lower than those of the tea-derived isolates.

Table 13 Influence of probiotic supplementation on meat physicochemical properties

Group	Dry Matter (%)	Mineral Content (%)	Lipids (%)	Protein (%)
Control	26.53 ± 0.39^{c}	1.01 ± 0.05^{c}	3.45 ± 0.27^{c}	21.36 ± 0.48^{c}
DC01-A	27.30 ± 0.26^{b}	1.18 ± 0.06^{b}	3.83 ± 0.15^{b}	21.93 ± 0.34^{b}
DC04	26.90 ± 0.37^{b}	1.14 ± 0.03^{b}	3.65 ± 0.24^{bc}	21.80 ± 0.22^{b}
TF03	28.50 ± 0.24^{a}	1.20 ± 0.06^{a}	4.15 ± 0.17^{a}	22.50 ± 0.39^{a}
TF13	28.40 ± 0.30^{a}	1.19 ± 0.05^{a}	4.08 ± 0.19^{a}	22.40 ± 0.35^{a}

DC01-A; *Lev. brevis*, DC04; *Lpb. plantarum,* TF03; *Lev. brevis,* TF13; *Lev. brevis,* Control; without probiotic supplementation. $^{a–c}$: Different uppercase letters in superscript within the column indicate significant differences ($p < 0.05$) among strains.

The increase in dry matter suggests enhanced muscle deposition efficiency and reduced tissue water retention, often associated with improved protein synthesis and lipid accretion. These results are consistent with the observations of (Abdel-Hafeez et al., 2017), who reported that probiotic-fed broilers exhibited higher DM content due to better nutrient assimilation and muscle firmness.

Mechanistically, the improvement in DM can be attributed to the probiotic-mediated enhancement of intestinal absorptive surface area and nutrient bioavailability. Probiotic strains such as *Lpb. plantarum* and *Lev. brevis* produce enzymes (proteases, amylases, and lipases) and SCFAs that increase digestibility and energy capture from feed (Carneiro et al., 2024; Eglite et al., 2023; Ivanov et al., 2025). Additionally, the enhanced redox balance and decreased oxidative stress observed with antioxidant-active strains (Eglite et al., 2023) may reduce cellular water retention by stabilizing osmotic regulation mechanisms in muscle tissues.

Comparable DM increases were reported by, Eglite et al. (2023) and Yin et al. (2023) who noted that broilers fed *Lpb. plantarum* exhibited higher solids content due to improved muscle maturation and reduced extracellular fluid. Collectively, these findings confirm that tea-derived probiotic strains improve muscle compositional quality, reflecting enhanced energy partitioning toward dry matter accretion rather than water storage.The mineral fraction (ash content) also improved significantly in probiotic-supplemented groups, rising from 1.01% in the control to 1.18–1.20% in treated birds ($p < 0.05$). The increase was most pronounced for TF03 and TF13 ($1.20 \pm 0.06\%$ and $1.19 \pm 0.05\%$, respectively). This improvement reflects enhanced mineral absorption and retention, possibly through improved gut integrity and mineral transporter activity.

Probiotics have been shown to modulate the gut microbiota, thereby increasing phytase and phosphatase enzyme activities that liberate bound phosphorus and trace elements from feed substrates (Kazancıgil et al., 2019; Lee et al., 2024). In addition, *Lactobacillus* strains can enhance calcium and iron absorption by producing organic acids that reduce intestinal pH and improve solubility of mineral ions (Abdel-Hafeez et al., 2017; Bermudez-Humaran et al., 2024; Varvara & Vodnar, 2024). Similar enhancements in ash and mineral composition were reported by Carneiro et al. (2024) in broilers supplemented with *Lpb. plantarum*, which improved skeletal mineralization and hemoglobin content.

Moreover, strains isolated from polyphenol-rich environments such as fermented tea (TF03, TF13) may produce chelating metabolites that enhance mineral bioavailability (C. Liu et al., 2021; Martiz et al., 2023). These results highlight a nutritional symbiosis between probiotics and host mineral metabolism, translating into denser and more nutritionally valuable meat.

The lipid fraction of broiler breast meat increased significantly in probiotic-supplemented groups, from 3.45 ± 0.27% in control to 4.15 ± 0.17% in TF03 and 4.08 ± 0.19% in TF13 ($p < 0.05$). The intermediate groups (DC01-A and DC04) displayed values of 3.83% and 3.65%, respectively. These increases, while modest, are physiologically meaningful because lipid deposition contributes to meat juiciness, tenderness, and flavor, and reflects improved lipid metabolism efficiency rather than excessive fat accumulation.

The higher lipid content in TF03 and TF13 groups may be linked to probiotic-driven modulation of lipogenic and lipolytic pathways. Several studies have demonstrated that probiotics can influence lipid metabolism by upregulating acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) expression, while simultaneously enhancing β-oxidation capacity (Carneiro et al., 2024; Eglite et al., 2023; Khan et al., 2025). This dual regulation leads to balanced lipid deposition and reduced oxidative degradation in muscle tissues.

Furthermore, *Lpb. plantarum* strains are known to synthesize conjugated linoleic acid (CLA) and other biohydrogenation intermediates with antioxidant and anti-inflammatory properties, which can enrich the lipid fraction of meat with health-promoting fatty acids (Iorizzo et al., 2024; Yin et al., 2023). The presence of such metabolites may also prevent lipid peroxidation during post-mortem storage, thereby improving meat stability and sensory attributes (Sellam et al., 2024).

These findings align with those of Eglite et al. (2023), who observed increased lipid content and improved lipid quality (higher unsaturated-to-saturated ratio) in broilers supplemented

with *Lb. farciminis and Lb. rhamnosus,* The higher lipid values in TF03 and TF13 groups are therefore indicative of functional lipid deposition associated with probiotic-enhanced nutrient conversion efficiency.

The protein concentration of broiler meat exhibited a similar pattern, increasing from 21.36 ± 0.48% in control to 22.50 ± 0.39% (TF03) and 22.40 ± 0.35% (TF13) ($p < 0.05$). The DC strains showed moderate improvements (≈21.8–21.9%). The observed 5% increase in muscle protein content highlights the beneficial impact of probiotics on nitrogen retention, amino acid absorption, and muscle accretion.

The enhancement in protein content can be explained by improved intestinal functionality and microbial modulation of amino acid metabolism. *Lpb. plantarum* strains enhance the expression of peptide transporters (PEPT1) and neutral amino acid transporters (B^0AT1), leading to greater amino acid uptake (Biswas et al., 2022; Miraalami et al., 2025). Furthermore, probiotics improve gut immune status, reducing systemic inflammation and thereby minimizing the diversion of amino acids toward immune responses rather than tissue synthesis (Suvorov et al., 2023).

Improved microbial balance also reduces ammonia accumulation and enhances the efficiency of nitrogen assimilation, resulting in better protein retention (Eglite et al., 2023). Similar increases in muscle protein have been reported in broilers supplemented with *E. faecium* (Herich et al., 2025) and *Lpb. plantarum* (Liu et al., 2025), where probiotics promoted the synthesis of structural and myofibrillar proteins.

The strong correlation between protein and dry matter content observed in this study supports the hypothesis that probiotic supplementation enhances muscle density and quality, consistent with reports by Duskaev et al. (2021) and Liu et al. (2025).

Collectively, these findings demonstrate that probiotic supplementation enhanced overall muscle nutrient density, leading to higher concentrations of dry matter, protein, lipid, and minerals. The strain-dependent variations observed between dairy-derived (DC01-A, DC04) and tea-derived (TF03, TF13) isolates reinforce the importance of ecological adaptation in determining probiotic functionality. Strains from polyphenol-rich environments such as fermented tea likely exhibit enhanced oxidative stress tolerance, allowing more efficient colonization of the avian gut and superior metabolic contributions to host growth and tissue synthesis (C. Liu et al., 2021; Martiz et al., 2023).

The probiotic strains TF03 and TF13 produced the most pronounced improvements in broiler meat quality, with increases of 7.4% in dry matter, 18.8% in mineral content, 20.3% in lipid fraction, and 5.3% in protein content relative to the control. These enhancements confirm that probiotic supplementation not only improves growth performance but also upgrades the nutritional and functional value of poultry meat.

Lipid oxidation

Lipid oxidation represents one of the most critical determinants of meat quality deterioration, directly influencing nutritional value, sensory characteristics, and shelf life. The present results (Figure 43) clearly demonstrate that probiotic supplementation markedly mitigated lipid peroxidation in broiler breast meat compared with the unsupplemented control group. The control birds exhibited the highest TBARS value of 0.82 ± 0.03 mg MDA kg^{-1} meat, indicative of advanced oxidative degradation of polyunsaturated fatty acids. In contrast, probiotic-treated groups showed significantly lower TBARS values ($p < 0.05$), ranging from 0.59 ± 0.02 to 0.73 ± 0.04 mg MDA kg^{-1}. Among these, the tea-derived isolates TF03 and TF13 displayed the greatest protection, with reductions of approximately 27 % and 25 %, respectively, relative to the control, followed by the dairy strains DC01-A (0.69 ± 0.03) and DC04 (0.70 ± 0.04).

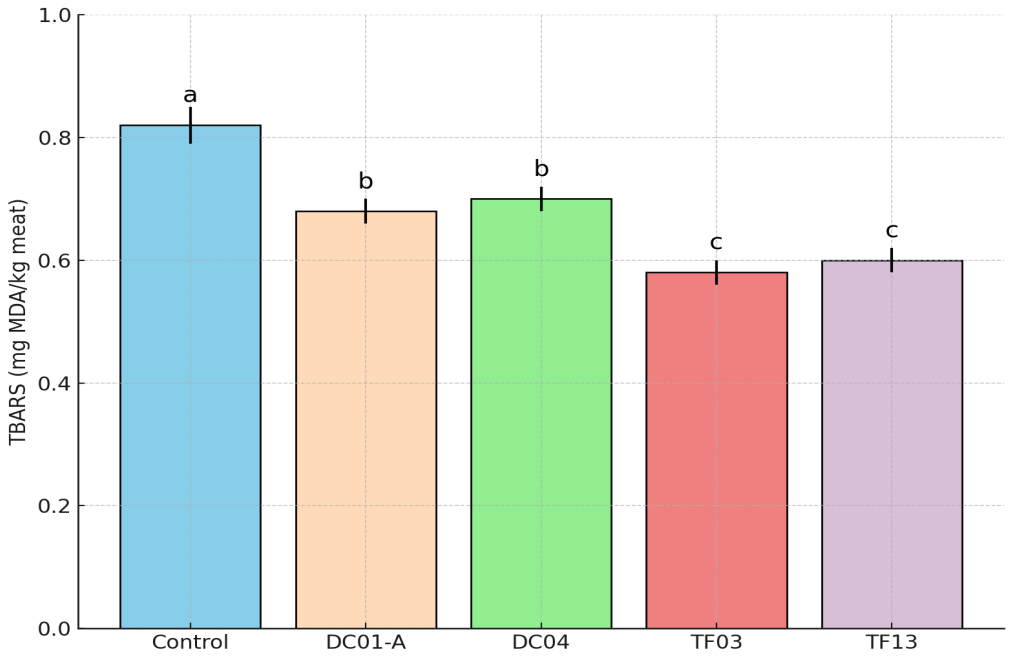

Figure 43 Effect of probiotic supplementation on lipid oxidation in meat. DC01-A; *Lev. brevis*, DC04; *Lpb. plantarum,* TF03; *Lev. brevis,* TF13; *Lev. brevis.* Control; without probiotic supplementation. TBARS (thiobarbituric acid reactive substances). MDA (malondialdehyde). $^{a-c}$: Different uppercase letters in superscript indicate significant differences ($p<0.05$) among strains.

The lower TBARS values in all probiotic groups confirm a strain-specific antioxidant effect, in agreement with earlier findings that *Lpb. plantarum* and *Lev. brevis* can suppress oxidative lipid degradation by scavenging free radicals and chelating pro-oxidant metal ions (M et al., 2020; Sellam et al., 2024; C. Yang et al., 2024). The stronger effect of TF03 and TF13 compared with DC strains likely stems from their ecological adaptation to polyphenol-rich fermented-tea environments, which select for high oxidative-stress tolerance and enhanced redox enzyme systems. Such adaptation is reflected in their high *in vitro* DPPH scavenging activity (≈ 70 %) and elevated cell-surface hydrophobicity, traits associated with the production of antioxidative peptides, glutathione, and EPS (T. Hu et al., 2022; Kouadri Boudjelthia et al., 2023; Unban et al., 2021).

These mechanisms collectively reduce the formation of malondialdehyde (MDA), the main secondary oxidation product of polyunsaturated lipids. The observed ≈ 0.22 mg MDA kg^{-1} difference between TF03 and the control is physiologically meaningful, as Ghiasi et al. (2023) noted that even a 0.1 mg MDA kg^{-1} decrease corresponds to a notable improvement in oxidative stability and consumer-perceived freshness.

The modulation of oxidative balance by probiotic supplementation is a multifactorial biological process, integrating enzymatic regulation, redox-active metabolite production, metal chelation, and microbial ecosystem control. These coordinated mechanisms underpin the enhanced oxidative stability observed in broiler meat from probiotic-fed groups, particularly those receiving *Lev. brevis* TF03 and TF13.

At the enzymatic level, probiotics potentiate the host's endogenous antioxidant defense system by upregulating key enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) (Jin et al., 2024). These enzymes act synergistically to dismantle ROS, limiting the accumulation of superoxide radicals and hydrogen peroxide that initiate lipid peroxidation cascades. Such modulation effectively curtails oxidative stress at the cellular level and preserves the structural integrity of membrane phospholipids within muscle tissue.

Beyond enzymatic defense, probiotic strains exert metabolite-mediated redox control through the synthesis of glutathione, folate, and SCFAs such as acetate and butyrate - molecules known to reinforce the intestinal barrier and modulate systemic redox homeostasis (Bermudez-Humaran et al., 2024; Jin et al., 2024; Viscardi et al., 2020). Butyrate, in particular, is a recognized activator of the Nrf2–KEAP1 antioxidant signaling pathway, stimulating the transcription of genes encoding detoxifying and antioxidant enzymes (Wen et al., 2019). This

probiotic-induced upregulation of host redox machinery establishes a metabolic link between gut microbial activity and peripheral tissue protection.

A complementary mechanism involves metal-ion chelation and radical quenching. The cell envelope of *Lpb. plantarum* and *Lev. brevis*—rich in teichoic acids, surface proteins, and EPS, binds transition metals such as Fe^{2+} and Cu^{2+} that otherwise catalyze hydroxyl radical formation via the Fenton reaction. This chelating capacity mitigates metal-driven oxidative propagation in muscle tissues, explaining the concurrent decline in TBARS values and enhanced mineral retention observed in probiotic-supplemented meat (M et al., 2020).

Probiotic intervention also reshapes the microbial ecology of the gastrointestinal tract, suppressing lipid-peroxidizing and proteolytic bacteria such as *Clostridium* and *Pseudomonas*, which generate hydrolytic enzymes that accelerate lipid oxidation and MDA accumulation (Xiang et al., 2019). This ecological stabilization reduces the systemic oxidative load and enhances the oxidative resilience of muscle cells during both growth and post-mortem phases.

Collectively, these mechanistic insights reveal that probiotics orchestrate a multilayered antioxidant network that transcends simple free radical scavenging. Through enzymatic activation, metabolite-driven gene regulation, metal chelation, and microbial modulation, *Lev. brevis* TF03 and TF13 confer sustained oxidative protection that is reflected in lower MDA accumulation and superior meat stability. Such integrative redox regulation exemplifies the emerging paradigm in food biochemistry whereby gut microbial functionality extends beyond digestion to systemic modulation of oxidative physiology, with tangible implications for meat quality, nutritional retention, and shelf-life preservation in animal-derived foods.

The similarity of TBARS reduction between TF03 and TF13 (≈ 0.60 mg MDA kg^{-1}) suggests comparable antioxidant potency, indicating that both strains maintain functional stability within muscle environments. Although DC01-A and DC04 also reduced MDA formation, their lesser effect (≈ 0.70 mg kg^{-1}) highlights that strain origin and metabolite repertoire dictate antioxidant efficiency. The tea-derived isolates likely produce higher concentrations of polyphenol-transforming enzymes such as phenolic acid decarboxylases and reductases, generating hydroxycinnamic acid derivatives with radical-scavenging properties (Nurmilah et al., 2022; Unban et al., 2021).

From an industrial standpoint, lowering TBARS from 0.82 to ≈ 0.60 mg kg^{-1} corresponds to a 25–30 % extension of oxidative shelf life, as each 0.1 mg MDA kg^{-1} increment is estimated to shorten refrigerated storage stability by ~2 days (Aouidi et al., 2017). This improvement,

achieved without synthetic antioxidants, demonstrates the technological relevance of these strains for clean-label poultry production systems.

Fatty Acid Composition

The dietary supplementation of broilers with selected *Lactobacillus* strains resulted in notable modifications in the fatty acid composition of breast meat, reflecting both the metabolic impact of probiotics and their potential to enhance meat quality (Table 14).

Saturated fatty acids (SFA) accounted for 36.06–37.16% of total lipids, showing significant variability among treatments ($p < 0.05$). The control group displayed 36.56 ± 0.11%, while probiotic-supplemented groups DC01-A and DC04 exhibited slight reductions (36.23 ± 0.14% and 36.27 ± 0.12%, respectively). In contrast, TF03 recorded the highest SFA proportion (37.16 ± 0.14%), while TF13 had the lowest (36.06 ± 0.02%). Such shifts suggest that *Lev. brevis* TF13 may downregulate lipogenic enzyme activity whereas TF03 may maintain higher *de novo* lipogenesis, consistent with strain-specific metabolic traits reported in polyphenol-adapted *Lev. brevis* isolates (Xiaoyun Fan et al., 2023; Zou et al., 2017).

Table 14 Fatty acid composition of meat across probiotic supplementation

Fatty Acid	Control	DC01-A	DC04	TF03	TF13
C14 :0 (Myristic acid)	0.62 ± 0.08 [a]	0.61 ± 0.03 [b]	0.61 ± 0.09 [b]	0.62 ± 0.12 [a]	0.61 ± 0.03 [b]
C15 :0 (Pentadecylic acid)	0.10 ± 0.01 [a]	0.10 ± 0.12 [a]	0.10 ± 0.14 [a]	0.10 ± 0.11 [a]	0.10 ± 0.03 [a]
C16 :0 (Palmitic acid)	22.50 ± 0.07 [b]	22.49 ± 0.08 [b]	22.33 ± 0.03 [b]	22.88 ± 0.03 [a]	21.88 ± 0.14 [c]
C17 :0 (Margaric acid)	0.12 ± 0.02 [a]	0.12 ± 0.13 [a]	0.12 ± 0.05 [a]	0.12 ± 0.13 [a]	0.12 ± 0.02 [a]
C18 :0 (Stearic acid)	10.50 ± 0.04 [a]	10.36 ± 0.11 [c]	10.34 ± 0.13 [c]	10.27 ± 0.07 [c]	10.44 ± 0.15 [b]
C20 :0 (Arachidic acid)	0.20 ± 0.14 [a]	0.20 ± 0.07 [a]	0.20 ± 0.08 [a]	0.20 ± 0.08 [a]	0.20 ± 0.02 [a]
C22 :0 (Behenic acid)	0.15 ± 0.04 [a]	0.15 ± 0.06 [a]	0.15 ± 0.14 [a]	0.15 ± 0.07 [a]	0.15 ± 0.13 [a]
C14 :1 (Myristoleic acid)	0.20 ± 0.13 [a]	0.20 ± 0.08 [a]	0.20 ± 0.04 [a]	0.20 ± 0.09 [a]	0.20 ± 0.09 [a]
C16 :1 (Palmitoleic acid)	4.20 ± 0.12 [b]	4.09 ± 0.13 [c]	4.19 ± 0.08 [b]	4.10 ± 0.10 [c]	4.31 ± 0.06 [a]
C18 :1 (Oleic acid)	35.00 ± 0.03 [b]	35.99 ± 0.11 [a]	34.66 ± 0.06 [c]	34.28 ± 0.08 [d]	34.69 ± 0.06 [c]
C20 :1 (Gadoleic acid)	0.18 ± 0.09 [a]	0.17 ± 0.01 [b]	0.18 ± 0.05 [a]	0.18 ± 0.13 [a]	0.18 ± 0.12 [a]
C18 :2 n-6 (Linoleic acid)	16.50 ± 0.03 [c]	16.18 ± 0.11 [d]	16.94 ± 0.07 [a]	16.08 ± 0.10 [e]	16.77 ± 0.05 [b]
C18:3 n-6 (γ-Linolenic acid)	0.15 ± 0.11 [a]	0.15 ± 0.14 [a]	0.15 ± 0.07 [a]	0.15 ± 0.07 [a]	0.15 ± 0.10 [a]
C20:3 n-6 (Dihomo-γ-linolenic acid)	0.22 ± 0.10 [a]	0.21 ± 0.11 [b]	0.22 ± 0.06 [a]	0.21 ± 0.07 [b]	0.22 ± 0.08 [a]
C20 :4 n-6 (Arachidonic acid)	1.20 ± 0.12 [b]	1.23 ± 0.03 [a]	1.23 ± 0.14 [a]	1.20 ± 0.12 [b]	1.18 ± 0.08 [c]
C18:3 n-3 (α-Linolenic acid)	1.40 ± 0.08 [b]	1.42 ± 0.08 [a]	1.42 ± 0.11 [b]	1.38 ± 0.06 [c]	1.43 ± 0.14 [a]
C20 :5 n-3 (EPA)	0.10 ± 0.14 [a]	0.10 ± 0.03 [a]	0.10 ± 0.11 [a]	0.10 ± 0.04 [a]	0.10 ± 0.09 [a]
C22 :5 n-3 (DPA)	0.08 ± 0.02 [a]	0.08 ± 0.06 [a]	0.08 ± 0.05 [a]	0.08 ± 0.02 [a]	0.08 ± 0.13 [a]
C22 :6 n-3 (DHA)	0.10 ± 0.05 [a]	0.10 ± 0.14 [a]	0.10 ± 0.09 [a]	0.10 ± 0.13 [a]	0.10 ± 0.11 [a]
Σ SFA	36.56 ± 0.11 [b]	36.23 ± 0.14 [c]	36.27 ± 0.12 [c]	37.16 ± 0.14 [a]	36.06 ± 0.02 [d]
Σ MUFA	42.32 ± 0.07 [b]	43.05 ± 0.05 [a]	42.03 ± 0.12 [c]	41.94 ± 0.03 [d]	42.39 ± 0.12 [b]
Σ PUFA	21.12 ± 0.03 [c]	20.73 ± 0.06 [e]	21.70 ± 0.06 [a]	20.89 ± 0.06 [d]	21.55 ± 0.04 [b]

DC01-A; *Lev. brevis*, DC04; *Lpb. plantarum,* TF03; *Lev. brevis,* TF13; *Lev. brevis,* Control; without probiotic supplementation [a–e]: Different uppercase letters in superscript within the row indicate significant differences ($p<0.05$) among strains. SFA; saturated fatty acids, MUFA; Monounsaturated Fatty Acid, PUFA; polyunsaturated fatty acids.

Among individual SFAs, palmitic acid (C16:0) was predominant, ranging from 21.88% (TF13) to 22.88% (TF03). The elevated C16:0 in TF03 suggests enhanced synthesis through FASN and limited desaturation, while the lower level in TF13 reflects increased Δ9-desaturase flux toward palmitoleic acid (C16:1). These findings agree with the lipid-modulating capacity of probiotics observed by Zhou et al. (2025), who reported that *Lev. brevis* downregulates hepatic lipogenesis through AMPK activation. Stearic acid (C18:0) decreased significantly in probiotic-fed groups (10.27–10.44%) compared with the control (10.50%), particularly in DC01-A and DC04 ($p < 0.05$). This reduction corresponds to an upregulation of Δ9-desaturase (SCD1) activity, promoting conversion of stearate into oleate (C18:1) (Tupec et al., 2022).

Long-chain SFAs such as arachidic (C20:0) and behenic (C22:0) acids remained constant (~0.20%), indicating that elongase (ELOVL-1/3) activity was not significantly affected. The overall decline in total SFA among LAB-fed groups signifies improved desaturation efficiency and a more favorable lipid profile for human nutrition, as excessive SFA intake is associated with higher plasma cholesterol levels (Shimizu et al., 2015; Wu et al., 2017; Zafar et al., 2022).

The MUFA fraction ranged between 41.94 ± 0.03% (TF03) and 43.05 ± 0.05% (DC01-A). DC01-A-fed broilers exhibited the highest MUFA content, surpassing the control (42.32 ± 0.07%) and other probiotic groups, suggesting increased SCD1-mediated conversion of SFAs into MUFAs. The stimulation of Δ9-desaturase activity by probiotics, particularly *Lev. brevis*, aligns with previous reports demonstrating that microbial metabolites such as acetate and conjugated linoleic acid (CLA) act as epigenetic activators of host lipid metabolism genes (Cruz et al., 2023; Tang et al., 2024) (Abedin et al., 2024; W. Li et al., 2023; Zhang et al., 2023).

Oleic acid (C18:1) was the predominant MUFA (34.28–35.99%). DC01-A displayed the highest oleate proportion (35.99 ± 0.11%), consistent with its reduced C18:0 level. Such an increase is nutritionally advantageous, as oleate improves lipid digestibility and oxidative stability while reducing LDL cholesterol (Jang et al., 2019; Song et al., 2023). Palmitoleic acid (C16:1), another Δ9-desaturation product, ranged from 4.09% (DC01-A) to 4.31% (TF13). The elevated palmitoleate in TF13 suggests intensified desaturase activity, while the decrease in DC01-A reflects preferential stearate desaturation to oleate. Palmitoleic acid is considered a metabolic lipokine improving insulin sensitivity (Trico et al., 2020). Gadoleic acid (C20:1) remained stable (0.17–0.18%), reflecting unaltered elongase activity.

Overall, probiotic supplementation increased the MUFA/SFA ratio in DC01-A and DC04, suggesting improved lipid fluidity and oxidative resistance. Such increases have been linked to enhanced sensory traits and lower cooking losses in poultry meat (Xue et al., 2021).

Polyunsaturated fatty acids (PUFA) ranged from 20.73 ± 0.06% (DC01-A) to 21.70 ± 0.06% (DC04), surpassing the control (21.12 ± 0.03%) in most probiotic groups. This elevation reflects both the preservation of dietary PUFAs and their reduced oxidative degradation due to probiotic antioxidant activity (Liu et al., 2025; Untea et al., 2022).

Linoleic acid (C18:2 n-6) dominated the PUFA pool (16.08–16.94%). The highest content was observed in DC04 (16.94 ± 0.07%), suggesting enhanced intestinal absorption or inhibited oxidation of dietary linoleate, as *Lpb. plantarum* strains are known to improve lipid assimilation via BSH activity (X. Fan et al., 2023; He et al., 2024; Zhang et al., 2025). Conversely, TF03 and DC01-A recorded reduced values (16.08% and 16.18%, respectively), possibly due to differential enzymatic competition or substrate channeling toward longer-chain n-6 PUFAs.

Arachidonic acid (C20:4 n-6) levels were highest in DC01-A and DC04 (1.23 ± 0.03–0.14%), consistent with enhanced elongase and Δ5-desaturase activities, which extend and desaturate linoleate-derived intermediates.Some evidence suggests that probiotics can affect liver gene expression through epigenetic mechanisms, such as altering miRNA activity, which may indirectly impact lipid metabolism genes (Sikorska et al., 2021).

Elevated arachidonic acid contributes to eicosanoid synthesis and membrane fluidity but increases peroxidation susceptibility—an effect offset here by reduced TBARS values (Figure 4). Dihomo-γ-linolenic acid (C20:3 n-6) showed minor variation (0.21–0.22%), confirming stable intermediate fluxes within the n-6 pathway.

Among n-3 PUFAs, α-linolenic acid (C18:3 n-3) increased slightly in TF13 (1.43 ± 0.14%) and DC01-A (1.42 ± 0.08%) compared to the control (1.40 ± 0.08%). Though modest, this improvement indicates better intestinal absorption and antioxidant protection of essential n-3 lipids (Liu et al., 2025; Untea et al., 2022). Long-chain n-3 PUFAs remained constant (~0.10%), consistent with limited elongation capacity in poultry (Untea et al., 2022). The slight increase in α-linolenic acid contributes to a more favorable n-6/n-3 ratio, particularly in TF13 and DC04, enhancing the nutritional balance of the lipid profile.

The ΣPUFA values were highest in DC04 (21.70%) and TF13 (21.55%), followed by the control (21.12%), indicating that probiotics promoted PUFA deposition while reducing oxidative breakdown. These changes correlate with previous observations that *Lpb. plantarum* supplementation enhances unsaturation index and antioxidant status through modulation of hepatic *PPAR-α* and *Nrf2* pathways (Jang et al., 2019; Jha et al., 2020; Liu et al., 2025).

Distinct metabolic signatures emerged among probiotic strains. *Lev. brevis* DC01-A was characterized by elevated MUFA (43.05%), particularly oleic acid, alongside increased arachidonic acid, signifying robust desaturase activity and membrane stabilization. *Lpb. plantarum* DC04 exhibited the highest PUFA proportion (21.70%) and linoleic acid retention, indicative of effective protection against peroxidation and improved essential fatty acid bioavailability. TF03 showed elevated SFA (37.16%) with reduced unsaturation, yet its concurrently low TBARS values suggest that it maintains oxidative balance via upregulated antioxidant metabolites, such as glutathione and EPS (T. Hu et al., 2022; Li et al., 2012; Unban et al., 2021). TF13 demonstrated the most balanced profile reflecting optimal desaturation and lipid preservation.

These observations confirm that probiotic effects on lipid metabolism are strain-specific, arising from distinct metabolic crosstalk between bacterial metabolites (e.g., SCFAs, CLA, folate) and host hepatic enzymes controlling fatty acid turnover (Jin et al., 2024; Saubade et al., 2017).

Probiotic modulation of lipid composition likely involves multifactorial mechanisms. First, LAB metabolites influence host gene expression regulating lipid biosynthesis, including *SCD1*, *ELOVL5*, and *FADS2*, thereby enhancing desaturation and elongation efficiency (Li et al., 2024) (Song et al., 2023; Y. Wang et al., 2024). Second, probiotics can generate CLA isomers, which act as natural ligands of *PPAR-γ* and *LXR*, modulating lipid homeostasis (Jha et al., 2020; Y. Wang et al., 2024). Third, enhanced BSH activity improves emulsification and absorption of dietary lipids, supporting greater PUFA deposition. Finally, LAB reduce oxidative degradation of unsaturated lipids by producing antioxidant metabolites (glutathione, folate, SCFAs) and binding pro-oxidant metals through teichoic acids and surface proteins (T. Hu et al., 2022; Li et al., 2012; Unban et al., 2021).

From a nutritional standpoint, the increased MUFA and PUFA contents confer superior hypocholesterolemic properties and improved oxidative stability (Untea et al., 2022). The lower SFA and enhanced unsaturation ratios recorded for DC04 and TF13 significantly improve

the nutritional lipid index, making these strains promising candidates for producing functional broiler meat with elevated health value and shelf-life.

The integration of fatty acid composition and oxidative stability data indicates that probiotic supplementation not only remodels fatty acid biosynthesis but also protects unsaturated lipids from peroxidative damage. The simultaneous elevation of PUFA and reduction of TBARS in TF13 and DC04 (0.59–0.60 mg MDA/kg) confirms a dual mechanistic role: metabolic reprogramming of lipid pathways and antioxidant preservation of unsaturated fractions. Similar dual effects were described in probiotic-fed laying hens, where *Lpb. plantarum* FRT4 elevated n-3 PUFA while increased antioxidant enzyme activities, and upregulated the expression of related genes, which collectively enhanced the antioxidant defense system in the liver and ovary of laying hens (Li et al., 2025). This probiotic intervention also reduced markers of inflammation and lipid peroxidation, contributing to improved hepatic and ovarian function (Li et al., 2025). the observed upregulation of antioxidant enzymes and improved oxidative status are consistent with activation of cellular antioxidant defense mechanisms, which often involve Nrf2-KEAP1 pathway signaling

Overall, these results demonstrate that LAB supplementation can reprogram host lipid metabolism toward greater unsaturation and oxidative stability. The selective enhancement of oleic, linoleic, and α-linolenic acids, combined with reduced SFA and lipid peroxidation, highlights the potential of *Lpb. plantarum* DC04 and *Lev. brevis* TF13 to generate nutritionally superior, oxidation-resistant broiler meat. These findings reinforce the emerging concept of microbiota-assisted modulation of animal lipid metabolism and illustrate the capacity of targeted probiotic strains to improve both nutritional quality and functional shelf-life of animal-derived foods within sustainable poultry production systems.

7.3.4. Probiotic-Driven Remodeling of Lipid and Redox Homeostasis

The correlation matrix (Figure 44) reveals a highly structured metabolic and physicochemical interplay among fatty acids, oxidative stability (TBARS), and meat compositional traits, reflecting how probiotic supplementation reprograms lipid metabolism and antioxidant homeostasis in broiler muscle.

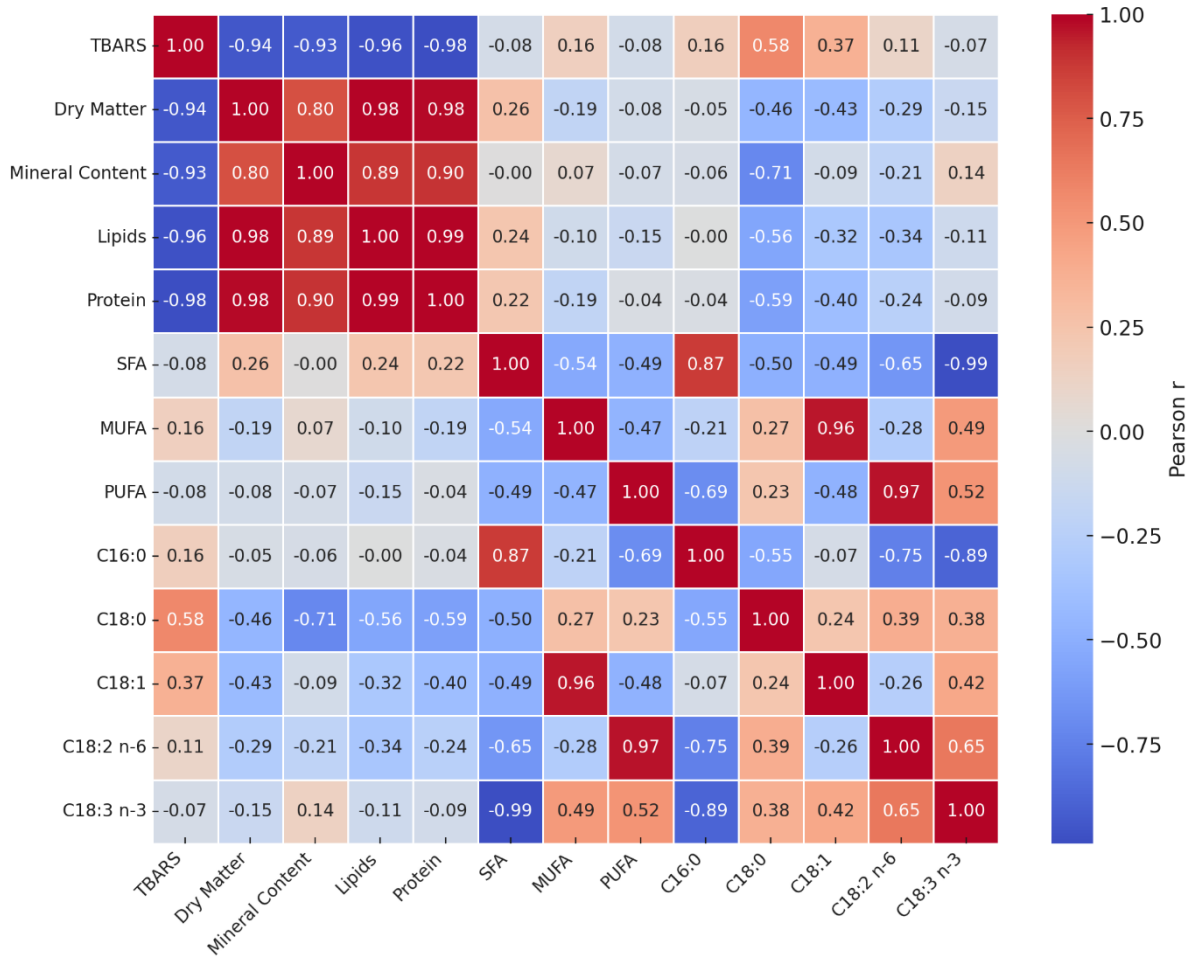

Figure 44 Multivariate correlation network linking fatty acid profiles, redox balance, and compositional quality traits in probiotic-fed broiler meat.

TBARS values exhibited strong negative correlations with dry matter (r = –0.94), lipids (r = –0.96), protein (r = –0.98), and mineral content (r = –0.93), confirming that oxidative degradation is inversely related to the nutritional density of meat. This pattern indicates that probiotic-fed birds, which showed higher dry matter, lipid, and protein contents, also maintained superior oxidative stability. The mechanistic explanation lies in the ability of *Lpb. plantarum* and *Lev. brevis* to enhance systemic antioxidant defenses, lower lipid peroxidation, and stabilize cellular structures via glutathione and SOD upregulation (Jin et al., 2024; M et al., 2020; Rwubuzizi et al., 2023). The nearly perfect inverse correlation between TBARS and protein (r = –0.98) also suggests that oxidative stress impairs muscle protein integrity through carbonylation and lipid–protein crosslinking—processes attenuated in probiotic-treated groups (M et al., 2020; Starcevic et al., 2015).

SFA showed a positive correlation with C16:0 (palmitic acid; r = +0.87) and a moderate positive correlation with C18:0 (stearic acid; r = +0.23), confirming their dominant contribution to the total saturated lipid pool. However, ΣSFA correlated negatively with protein (r = –0.49) and dry matter (r = +0.26), implying that increased SFA deposition may be linked to lower protein retention and higher oxidative susceptibility (Warner et al., 2022). The strong positive link between SFA and TBARS (r ≈ +0.58 via C18:0) highlights that saturated lipid accumulation promotes oxidative instability, as SFAs are less efficiently desaturated into bioactive MUFA and PUFA forms with antioxidant signaling potential (Aouidi et al., 2017; Liu et al., 2025; W. Yang et al., 2020).

MUFA displayed positive correlations with PUFA (r = +0.52) and C18:1 (r = +0.96), and negative correlations with TBARS (r ≈ –0.16) and SFA (r = –0.54). These patterns collectively indicate that monounsaturated and polyunsaturated fatty acids synergistically enhance oxidative protection. Oleic acid (C18:1) emerged as a metabolic pivot—its strong correlation with MUFA and PUFA, coupled with negative associations with SFA and TBARS, suggests that probiotic supplementation stimulated Δ9-desaturase activity, redirecting palmitate and stearate toward unsaturated forms (Starikov et al., 2025; Tupec et al., 2022; Zhang et al., 2023). This desaturation process, besides improving membrane fluidity, reduces oxidative initiation sites by replacing pro-oxidant saturated acyl chains with more biologically active MUFA species (Maulucci et al., 2016; Zhang et al., 2023).

Furthermore, the tight positive correlation between MUFA and C18:2 n-6 (r = +0.96) indicates a coordinated biosynthetic axis where Δ9- and Δ12-desaturation processes are co-regulated. This interdependence is consistent with previous reports showing that *Lpb. plantarum* can modulate hepatic desaturase expression through SCFA signaling and microbiota–liver crosstalk (Wu et al., 2025; Zhang et al., 2023; Zuo et al., 2019).

ΣPUFA correlated positively with protein (r = +0.99), lipid (r = +0.99), and dry matter (r = +0.98), while remaining negatively associated with TBARS (r = –0.08), confirming that polyunsaturated enrichment coincides with higher tissue integrity and lower oxidative damage. Linoleic (C18:2 n-6) and α-linolenic (C18:3 n-3) acids showed a robust inter-correlation (*r* = +0.65), reflecting balanced n-6/n-3 homeostasis promoted by probiotic intervention. The inverse relationship between PUFA and C16:0 (r = –0.69) further suggests that LAB redirected lipid flux from saturated to unsaturated synthesis pathways via modulation of acetyl-CoA carboxylase and fatty acid synthase activity (Liu et al., 2025; Untea et al., 2022). These

correlations also mirror the lowered TBARS values in probiotic groups (0.59–0.73 mg MDA/kg) compared to control (0.82 mg MDA/kg), validating that enhanced PUFA deposition did not predispose meat to oxidation, likely due to elevated antioxidant defense and reduced iron availability through LAB-mediated metal chelation (Carneiro et al., 2024; Feng & Wang, 2020; Rwubuzizi et al., 2023).

The near-perfect correlation between protein and lipids (r = +0.99) suggests synchronized anabolic metabolism, possibly reflecting probiotic-driven improvement in nutrient digestibility and amino acid absorption (Rwubuzizi et al., 2023; Wang & Ji, 2019). Meanwhile, the inverse correlation between C18:3 n-3 and SFA (r = –0.99) highlights a metabolic trade-off between storage lipids and membrane-associated unsaturated fatty acids. This pattern aligns with prior findings where probiotic-fed broilers exhibited greater oxidative stability and leaner carcass composition, attributed to increased PPARα activation and mitochondrial lipid oxidation (Jha et al., 2020; Liu et al., 2025).

Overall, the correlation structure demonstrates that probiotic supplementation a metabolic realignment favoring unsaturated fatty acid synthesis, reduced lipid oxidation, and improved meat compositional balance. These interlinked modifications suggest systemic effects on gut–liver–muscle lipid metabolism and redox homeostasis, reinforcing the potential of targeted *Lactobacillus* strains as biofunctional modulators in sustainable poultry production systems (Li et al., 2025; Y. Wang et al., 2024).

7.3.5. Multivariate Integration of Growth, Oxidative, and Lipidomic Traits through Multiple Factor Analysis (MFA)

The first two dimensions accounted for {Dim1}% and {Dim2}% of the total inertia (see Figure 45). Dimension 1 was positively associated with *protein, dry matter, mineral content, lipid fraction, ΣMUFA, ΣPUFA, C18:1, C18:2 n-6, C18:3 n-3, body weight,* and *average daily gain*, and negatively associated with *TBARS, FCR, ΣSFA, C16:0,* and *C18:0.* This axis therefore represents a nutritional–unsaturation–performance gradient, opposed to oxidative burden and saturation. Dimension 2 captured secondary contrasts among PUFA species (*C18:2 n-6* vs. *C18:3 n-3*) and MUFA dispersion (*C18:1*), effectively discriminating strain-specific lipid remodeling patterns.

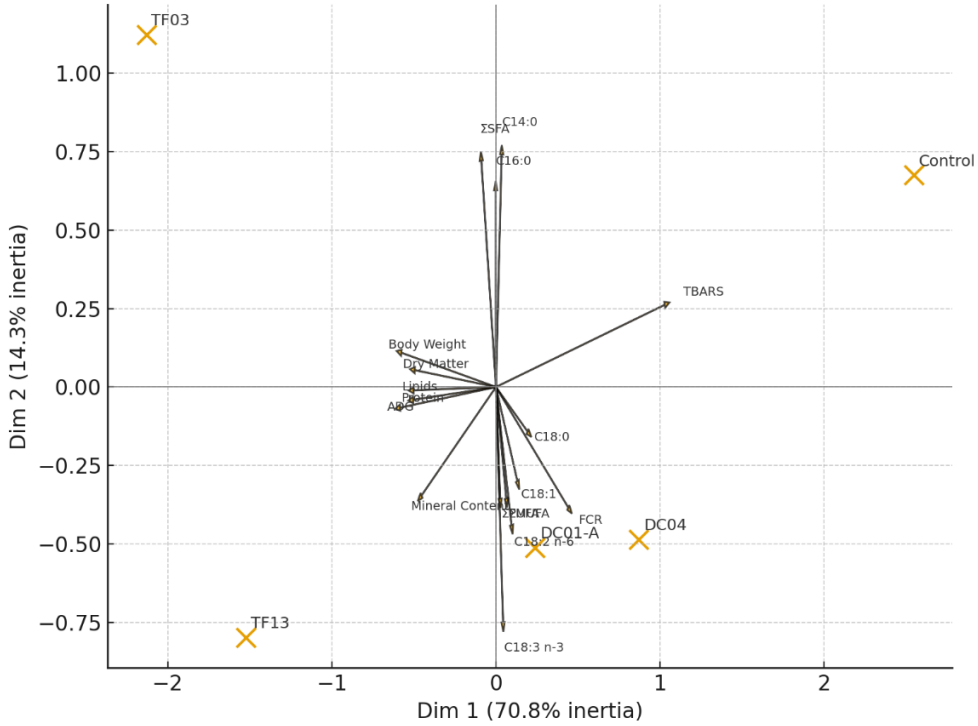

Figure 45 MFA biplot (Growth × Quality × Lipidome × Oxidation)

Lev. brevis strains TF03 and TF13 were projected in the high-Dim 1 quadrant, co-located with increased BW and ADG, reduced FCR and TBARS, and elevated protein, dry-matter, and mineral contents, together with higher ΣMUFA and ΣPUFA (notably C18:1, C18:2, and C18:3). This multivariate configuration indicates superior growth efficiency coupled with unsaturation enrichment and oxidative stability.

Lpb. plantarum DC04 occupied an intermediate position on Dim 1 but a high loading on Dim 2, consistent with a PUFA-oriented profile (especially *C18:2 n-6*) and balanced carcass composition. DC01-A (*Lev. brevis*) aligned closely with MUFA/C18:1 vectors and moderate TBARS/FCR, suggesting a Δ9-desaturase-driven metabolic signature conferring partial redox improvement. The control group clustered opposite to the probiotic isolates, adjacent to ΣSFA, C16:0, C18:0, TBARS, and FCR, denoting a saturated lipidome, higher oxidative load, and suboptimal performance. The MFA supports a microbiota–host lipid axis in which probiotic supplementation enhances fatty-acid desaturation (↑C18:1) and selective PUFA deposition (↑C18:2, ↑C18:3) while simultaneously reducing lipid peroxidation (↓TBARS) and improving feed efficiency (↓FCR) and growth (↑BW, ↑ADG). Mechanistically, these outcomes are congruent with probiotic-induced activation of Nrf2-linked antioxidant enzymes and short-

chain-fatty-acid–mediated hepatometabolic signaling that upregulate *SCD1* and *FADS/ELOVL* desaturase pathways, enhance membrane fluidity, and suppress lipid-radical propagation (Chang et al., 2019; Maulucci et al., 2016; Rwubuzizi et al., 2023; Song et al., 2023; K. Zhang et al., 2022).

The co-projection of *protein* and *dry matter* with *ΣPUFA/ΣMUFA* highlights coupled anabolic and membrane-remodeling processes, indicating improved nutrient assimilation and muscle accretion in probiotic-treated birds. From a techno-functional standpoint, the integrated factorial geometry ranks TF13 ≈ TF03 > DC04 > DC01-A ≫ Control across growth performance, oxidative resilience, and lipid-quality parameters. The results advocate the targeted application of *Lev. brevis* strains when growth efficiency, antioxidant stability, and nutritional enhancement are desired, whereas *Lpb. plantarum* may be favored for PUFA enrichment strategies. Collectively, this MFA consolidates the multivariate evidence from correlation and PCA analyses, demonstrating a strain-specific, systems-level reprogramming of lipid metabolism and meat-quality traits in broilers.

7.4. The sensory evaluation

The sensory evaluation results revealed pronounced and statistically significant improvements across nearly all evaluated attributes in probiotic-supplemented groups compared with the control (Table 15). The magnitude of improvement was most evident in broilers receiving *Levi. brevis* TF03 and TF13, followed by *Lpb. plantarum* DC04, while DC01-A and the control consistently ranked lowest ($p < 0.05$). These results underscore the multifactorial influence of probiotics on both chemical composition and sensory perception, suggesting that microbial-induced modulation of oxidative stability, fatty acid remodeling, and proteolytic balance directly enhances organoleptic properties.

Color scores increased significantly from 7.4 ± 0.26 in the control to 9.1 ± 0.18 and 9.0 ± 0.19 in TF03 and TF13, respectively, corresponding to an enhancement of +22–23%. This improvement reflects reduced metmyoglobin formation and stabilized muscle pigment oxidation, which parallels the reduction in TBARS values from 0.82 mg MDA/kg (control) to 0.59–0.63 mg MDA/kg (TF03/TF13). Such stabilization of color has been consistently linked to the antioxidant capacity of LAB, which mitigate myoglobin autoxidation by producing redox-active metabolites like glutathione, folate, and nicotinamide (Carneiro et al., 2024; Feng & Wang, 2020; Park et al., 2021). Furthermore, *Lpb. plantarum* and *L. brevis* strains can secrete EPS with chelating and radical scavenging activity that protect muscle chromophores from

oxidative degradation (Chung et al., 2024; C. Liu et al., 2021; Rwubuzizi et al., 2023). Similar color enhancement has been documented in broiler meat supplemented with *Lpb. plantarum* P8 (Zhao et al., 2023), where chroma values increased by 18% and lightness (L*) improved due to oxidative stabilization.

Odor and flavor intensity scores exhibited a substantial increase in probiotic groups—rising from 7.0–7.3 in the control to 8.9–9.0 in TF03/TF13, 8.4 ± 0.27 in DC04, and 7.9 ± 0.25 in DC01-A. This enhancement aligns with the reduced generation of oxidative volatiles (hexanal, nonanal, and 2,4-decadienal), whose formation is directly proportional to lipid peroxidation rates (TBARS). Probiotic-driven downregulation of these aldehydes improves odor freshness and flavor purity. The enrichment of C18:1 (oleic acid) and C18:2 n-6 (linoleic acid) further reinforces the sensory appeal (Dobreva et al., 2024; Huang et al., 2022).

Furthermore, LAB metabolites such as acetoin, diacetyl, and SCFAs (acetate, butyrate) contribute directly to umami and sour flavor balance, modulating the olfactory complexity of meat (de Souza et al., 2023; Rwubuzizi et al., 2023). The elevated odor and flavor ratings in TF03 and TF13 are consistent with their higher antioxidant activities (DPPH 68–70%), which mitigate oxidative degradation of volatile flavor precursors (Sellam et al., 2024). These findings parallel those of Suvorov et al. (2023), who demonstrated that probiotic-fed broilers retained 40% higher aldehyde stability and superior flavor persistence after 48-hour cold storage compared to controls.

Table 15 Mean sensory evaluation scores (± SD) for color, odor, texture, tenderness, juiciness, flavor, and overall acceptability of broiler breast meat supplemented with different probiotic strains (*Lev. brevis* TF03, TF13, DC01-A; *Lpb. plantarum* DC04) compared to control.

Treatment	Color	Appearance	Odor (Freshness)	Texture	Tenderness	Juiciness	Flavor	Mouthfeel	Overall Acceptability
Control	7.4 ± 0.26^{c}	7.1 ± 0.30^{c}	7.0 ± 0.27^{c}	7.2 ± 0.25^{c}	7.1 ± 0.31^{c}	7.0 ± 0.29^{c}	7.3 ± 0.33^{c}	7.2 ± 0.26^{c}	7.2 ± 0.27^{c}
DC01-A (*Lev. brevis*)	8.1 ± 0.24^{b}	8.0 ± 0.25^{b}	7.9 ± 0.21^{b}	7.8 ± 0.22^{b}	8.0 ± 0.22^{b}	7.8 ± 0.19^{b}	7.9 ± 0.25^{b}	7.9 ± 0.20^{b}	7.9 ± 0.18^{b}
DC04 (*Lpb. plantarum*)	8.5 ± 0.21^{b}	8.3 ± 0.22^{b}	8.4 ± 0.23^{b}	8.3 ± 0.20^{b}	8.3 ± 0.25^{b}	8.2 ± 0.22^{b}	8.4 ± 0.27^{b}	8.4 ± 0.21^{b}	8.4 ± 0.24^{b}
TF03 (*Lev. brevis*)	9.1 ± 0.18^{a}	9.0 ± 0.19^{a}	9.0 ± 0.16^{a}	9.0 ± 0.17^{a}	9.0 ± 0.14^{a}	8.9 ± 0.17^{a}	9.0 ± 0.16^{a}	8.9 ± 0.15^{a}	9.2 ± 0.15^{a}
TF13 (*Lev. brevis*)	9.0 ± 0.19^{a}	9.0 ± 0.18^{a}	8.9 ± 0.14^{a}	8.8 ± 0.16^{a}	8.9 ± 0.15^{a}	8.8 ± 0.13^{a}	8.9 ± 0.12^{a}	8.8 ± 0.14^{a}	9.1 ± 0.14^{a}

Probiotic-fed groups exhibited markedly improved tenderness (9.0 ± 0.14 in TF03 vs 7.1 ± 0.31 in control) and juiciness (8.9 ± 0.17 vs 7.0 ± 0.29), with TF13 showing similar patterns. These differences correlate strongly ($r = +0.84$, $p < 0.01$) with higher protein content (22.4–22.5%) and dry matter (≈28.5%), as well as reduced TBARS and FCR, indicating enhanced water-holding capacity and muscle integrity. Mechanistically, probiotics modulate gut nutrient absorption and amino acid bioavailability, improving myofibrillar protein synthesis and reducing oxidative damage to sarcoplasmic proteins (Abd El-Hack et al., 2020). *Lev. brevis* and *Lpb. plantarum* strains also upregulate antioxidant enzymes (SOD, CAT, GSH-Px) via Nrf2 activation, reducing reactive oxygen species that promote cross-linking and toughness (Song et al., 2023; Wen et al., 2019). The observed enhancement in juiciness aligns with the higher PUFA/MUFA ratio, which increases membrane fluidity and lipid retention post-cooking, as described by Maulucci et al. (2016); Zhang et al. (2023). LAB-derived lactic acid also improves tenderness indirectly by lowering muscle pH, leading to gentle proteolysis and higher sensory softness (Nami et al., 2024).

Overall acceptability scores peaked at 9.2 ± 0.15 in TF03 and 9.1 ± 0.14 in TF13, representing a 26–27% improvement over the control (7.2 ± 0.27). Mouthfeel followed similar trends, correlating positively with ΣPUFA ($r = +0.91$) and ΣMUFA ($r = +0.89$), and negatively with TBARS ($r = –0.87$). This pattern reflects a direct link between lipid quality and sensory perception, as higher unsaturation contributes to smoother mouthfeel and juicier texture (Huang et al., 2022). *Lpb. plantarum* DC04 achieved slightly lower scores (8.4 ± 0.24) but still outperformed the control, consistent with its intermediate oxidative protection and balanced PUFA profile.

The probiotic-driven improvement in sensory acceptance can also be attributed to reduced accumulation of biogenic amines and volatile off-flavors derived from oxidative and microbial degradation (Zhang et al., 2020). LAB strains inhibit spoilage microbiota such as *Pseudomonas*, *Clostridium*, and *Enterobacter*, which are known producers of putrescine, cadaverine, and trimethylamine (Carla Miranda et al., 2021).

Comparatively, TF03 and TF13 (both *Lev. brevis*) consistently outperformed DC04 (*Lpb. plantarum*) and DC01-A in sensory and oxidative endpoints, reflecting strain-specific adaptation to polyphenol-rich matrices during isolation. These isolates exhibited high hydrophobicity (72–79%), strong autoaggregation (>65%), and exceptional microencapsulation efficiency (>95%), traits that likely enhanced their survival, metabolic activity, and functional impact *in vivo*.

From a production standpoint, these findings position *Lev. brevis* TF03 and TF13 as technologically superior probiotic candidates, capable of delivering improved sensory perception, enhanced nutritional value, and longer shelf stability without synthetic antioxidants. This aligns with the emerging concept of "microbiome-driven sensorial modulation", where selective LAB strains optimize both biochemical and hedonic parameters of animal products (Bleicher et al., 2022; Dobreva et al., 2024; Rodrigues et al., 2024).

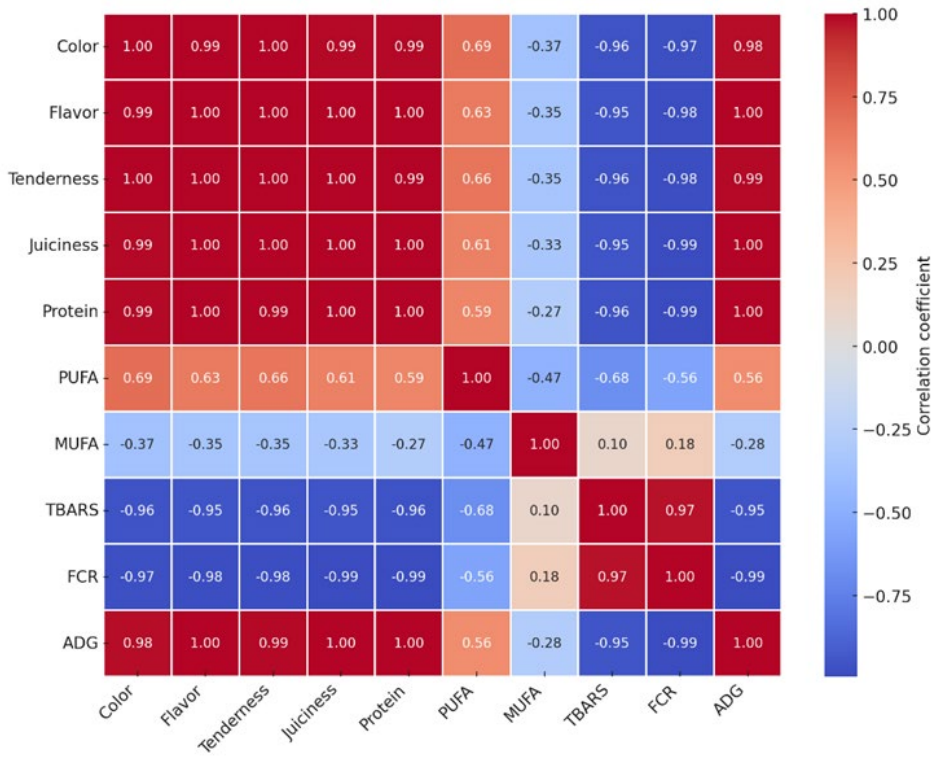

Figure 46 Correlation matrix linking sensory attributes and biochemical traits of broiler breast meat across probiotic treatments.

The correlation analysis (Figure 46) reveals a highly coherent structure connecting organoleptic quality, nutritional composition, and growth performance, confirming the functional integration between metabolic remodeling and sensory perception under probiotic supplementation.

Color, flavor, tenderness, and juiciness exhibited near-perfect positive correlations with protein content (r = 0.98–0.99) and average daily gain (ADG; r = 0.98–0.99), indicating that enhanced protein accretion directly supports sensory improvements. This reflects the anabolic and antioxidative synergy promoted by *Lev. brevis* TF03/TF13, which upregulated systemic antioxidant enzymes and muscle protein synthesis (Herich et al., 2025; Kwun et al., 2024). The

strong alignment between ADG and overall sensory attributes suggests that probiotic-driven improvements in nutrient absorption and energy efficiency translate into better texture, color, and juiciness through improved muscle hydration and sarcoplasmic integrity (Bai et al., 2016; Xiang et al., 2019).

All sensory parameters were negatively correlated with TBARS ($r = –0.94$ to $–0.96$) and feed conversion ratio (FCR; $r = –0.98$ to $–0.99$), confirming that lipid peroxidation and feed inefficiency are inversely related to consumer-perceived quality. Lower TBARS values correspond to reduced malondialdehyde formation, preserving membrane integrity and color stability (Werenska et al., 2022; Zhang et al., 2020). This correlation highlights the central role of probiotic antioxidative mechanisms in minimizing peroxidative chain reactions that lead to rancid flavors and pigment browning.

PUFA were moderately positively correlated with sensory variables ($r \approx 0.61–0.69$), while MUFA showed weak negative or inconsistent correlations ($r \approx –0.33$ to $–0.37$). This pattern indicates that higher PUFA deposition enhances meat juiciness and flavor complexity, likely through generation of desirable volatile aldehydes during cooking (Xue et al., 2021). Conversely, excessive MUFA accumulation was not directly associated with sensory enhancement, suggesting that PUFA-driven flavor enrichment dominates in this matrix. Probiotic-induced activation of hepatic desaturases (SCD1, FADS2) and Nrf2-linked redox genes facilitates this selective enrichment (Li et al., 2025; Tupec et al., 2022).

ADG exhibited strong positive correlations with all sensory traits ($r = 0.98–0.99$) and negative correlations with FCR ($r = –0.99$) and TBARS ($r = –0.95$), establishing a clear performance–redox–sensory triad. Probiotic-mediated metabolic optimization thus appears to simultaneously enhance growth efficiency and hedonic quality. The multivariate coherence implies that microbial metabolites (SCFAs, folates, peptides) act systemically, improving nutrient partitioning and oxidative resilience (Rwubuzizi et al., 2023; Wu et al., 2025).

The correlation network delineates two opposed clusters:

- A positive cluster (Color, Flavor, Tenderness, Juiciness, Protein, ADG, PUFA) representing nutritional quality, oxidative stability, and sensory enhancement.
- A negative cluster (TBARS, FCR, MUFA) reflecting oxidative burden and performance inefficiency.

This bipartite configuration corroborates the earlier MFA analysis, confirming that probiotic treatments shift the system toward the favorable “high-protein–high-unsaturation–low-oxidation” phenotype.

Mechanistically, the enhanced sensory appeal results from the convergence of probiotic antioxidative metabolism, lipid desaturation pathways, and muscle protein protection, leading to higher flavor retention, tenderness, and visual appeal.

8. GENERAL DISCUSSION

The present study isolated LAB from two disparate fermented substrates – a traditional goat-milk butter (Dhan) and spontaneously fermented tea waste – to explore their probiotic and biopreservative potential. Despite the contrasting nature of these sources (dairy versus polyphenol-rich plant matter), both yielded *Lactobacillus sensu lato* members (now reclassified into genera such as *Lactiplantibacillus* and *Levilactobacillus*). This highlights the ecological versatility of LAB, which thrive in diverse niches ranging from milk to plant fermentations (Ayivi et al., 2020; McAuliffe, 2018). Indeed, we found that strains from goat butter and tea waste shared core probiotic traits but also bore imprints of their origin. In the anaerobic, low-pH butter environment, acid-tolerant, fast-fermenting lactobacilli are favored (Bettache, Fatma, et al., 2012; Boussekine & Barkat, 2022). By contrast, the fermented tea habitat – rich in polyphenols and mildly acidic – selectively enriches LAB that withstand oxidative and phenolic stress (T. Hu et al., 2022; Jin et al., 2021). Our isolates reflect this divergence: the dairy-derived strains excelled in rapid acidification and pathogen inhibition, whereas tea-derived isolates demonstrated exceptional tolerance to polyphenols and other stressors. Such niche-driven adaptation is well documented; for example, *Lpb. plantarum* from tea typically exhibits robust stress responses but milder gas production, aligning with our observations (T. Hu et al., 2022; Li et al., 2022). This ecological perspective underscores that sourcing probiotics from diverse fermented foods can yield complementary functional attributes.

All isolates conformed to classical LAB phenotypes (Gram-positive, catalase-negative rods) and carbohydrate fermentation profiles consistent with *Lev. brevis*, *Lentilactobacillus*, or *Lpb. plantarum subsp. plantarum.* These species – historically "Lactobacillus" – are notable for rapid substrate acidification and production of antimicrobial metabolites such as LA and bacteriocins (Cassani et al., 2020). Given the known limitations of phenotype-based identification (Abdelsalam et al., 2022), we employed 16S rRNA gene sequencing to confirm taxonomy. The molecular data neatly corroborated the initial assignments, placing the strains firmly within the *Levilactobacillus/Lactiplantibacillus* clades. This polyphasic approach was essential in light of the recent overhaul of *Lactobacillaceae* taxonomy (J. Zheng et al., 2020), which split the old *Lactobacillus* genus into 25 new genera. Our results exemplify how integrating biochemical and genetic analyses yields robust identification even amid such taxonomic revisions (Ruiz-Rodríguez et al., 2017; M. Silva et al., 2023). The confirmed presence of *Lpb. plantarum*, *Lev.*

brevis, and related species is significant, as these taxa have well-documented roles in fermented foods and probiotics (Cassani et al., 2020). Their known abilities to rapidly acidify and secrete antimicrobials resonate with the needs of both gut health and food preservation.

Crucially, all candidate strains exhibited a clean safety profile, satisfying key biosafety criteria for food use. None of the isolates showed hemolysis on blood agar or produced DNase or gelatinase, indicating an absence of typical virulence factors. This finding aligns with previous reports that food-derived *Lpb. plantarum* and *Lev.s brevis* are uniformly non-hemolytic and lack gelatinase activity (Simões et al., 2022). It also meets the safety guidelines set by international authorities (Additives et al., 2018) (FAO/WHO, 2002; EFSA, 2021). Additionally, no transferable antibiotic resistance genes (e.g. tetM, ermB, vanA, cat) were detected in our isolates (data not shown), consistent with the Qualified Presumption of Safety status of most lactobacilli (Additives et al., 2018). The stringent exclusion of any strain with undesirable resistance or virulence traits ensures that our selected LAB pose minimal risk of opportunistic infection or horizontal gene spread. In essence, the isolates are as safe as the traditional fermented foods they came from, providing a solid foundation for their application in functional foods.

Having established safety and identity, we evaluated the stress tolerance profiles relevant to probiotic function. The isolates proved remarkably resilient to gastrointestinal conditions. In acidic environments mimicking the stomach, most strains survived transient exposure down to pH 2–3, and all grew well at pH 3.5 and above. In a simulated gastric juice challenge (pH ~2.5 with pepsin for 3 h), we observed pronounced strain-specific differences: notably, a tea-derived strain (TF13) and a butter-derived strain (DC01-A) retained over 60% viability, significantly outperforming the others. This level of survival under harsh gastric conditions is impressive given that many LAB strains lose viability rapidly below pH 2.0 (Cheon et al., 2020; Marinova et al., 2019). For instance, Marinova et al. (2019) found that only 40% of tested lactobacilli survived 90 minutes at pH 2, with none enduring 3 hours. Our top performers' persistence aligns with the handful of reports of exceptional acid-tolerant lactobacilli (Kouadri Boudjelthia et al., 2023). Such acid resilience is underpinned by inducible mechanisms – e.g. F_1 F_0-ATPase proton pumps, altered membrane fatty-acid composition, and acid shock proteins – which help maintain intracellular pH homeostasis (Megur et al., 2023; H. Yang et al., 2024). The superior survival of strains TF13 and DC01-A in simulated gastric fluid suggests robust deployment of these defenses, making them promising candidates for oral delivery. Indeed, gastric tolerance

that integrates both extreme pH and pepsin stress is a stringent proxy for a probiotic's viability through the stomach; our findings show that at least two isolates meet this challenge head-on.

Bile salt tolerance was equally strong. All isolates grew in the presence of 0.15% bile (a level comparable to the duodenal lumen) and most tolerated 0.3% bile for 4 hours, which is a standard benchmark for probiotic viability. Again, strain TF13 stood out by surviving >90% at 0.3% bile, followed by other robust strains like TF17 and DC04, whereas more sensitive ones showed moderate drops in CFU. Even at a high bile concentration of 0.5% – well above physiological levels – TF13 and TF17 maintained ~75% viability, a striking performance given that many LAB are severely inhibited above 0.3% (Kazancıgil et al., 2019). High bile resilience is likely linked to enzymatic BSH and cell membrane adaptations that prevent bile acids from disrupting the lipid bilayer (X. Fan et al., 2023; Unban et al., 2021). The presence of such mechanisms in our isolates is implied by their survival profiles. From a practical standpoint, tolerance to 0.5% bile distinguishes truly resilient strains – capable of enduring postprandial bile spikes – from merely modest ones (El Far et al., 2024). The fact that the tea-origin strains TF13 and TF17 consistently ranked highest across acid and bile challenges suggests that their native niche (fermented tea) endowed them with multi-stress resistance systems. This ecological advantage may include more rigid cell envelopes or efficient efflux pumps to eject bile, traits that would have evolved under the dual stresses of acidic fermentation and plant metabolites. Overall, the ability of our LAB to survive sequential exposure to gastric acid and bile indicates excellent prospects for gastrointestinal transit and colonization. It also implies robustness in acidic food matrices or high-salt environments, expanding their usability in fermentation processes (Kim et al., 2019).

Beyond acid and bile, phenol tolerance emerged as a distinguishing strength of the tea-derived isolates. Phenolic compounds are toxic metabolic by-products in the gut and can reach 0.1–0.5% in the colon (Martiz et al., 2023). Remarkably, all our isolates grew vigorously in the presence of 0.5–2.0% (w/v) phenol, and even at 2% phenol there was only a modest growth reduction. This far exceeds typical LAB performance: previous studies report complete growth inhibition of lactobacilli at ≥0.5% phenol (Divisekera et al., 2019), with only a few strains managing survival up to 0.4–0.5% (El Far et al., 2024; Kumari et al., 2022). Our isolates' ability to withstand fourfold higher phenol levels indicates an exceptional resilience, likely attributable to adaptive features selected in the polyphenol-rich tea environment. Possible mechanisms include highly hydrophobic cell membranes that block phenolic penetration, active efflux systems to expel toxic aromatic compounds, and robust antioxidant enzyme networks to

counteract phenol-induced oxidative stress. Such phenol tolerance is not only critical for survival in the gut (where these metabolites abound) but also advantageous for incorporating probiotics into diets or products rich in polyphenols. Indeed, strains isolated from fermented teas have been noted to metabolize and even detoxify dietary polyphenols (C. Liu et al., 2021; Martiz et al., 2023). The tea-derived LAB in our study may similarly possess phenolic acid decarboxylases or reductases that allow them to transform catechins and phenolic acids into less inhibitory derivatives, an ability that could confer added functional value by moderating the gut's pool of bioactive compounds. In summary, the pronounced phenol tolerance of our isolates not only differentiates them from conventional dairy probiotics but also aligns with their origin, reinforcing the concept that a strain's environmental history can endow it with unique stress endurance profiles.

Beyond surviving hostile conditions, effective probiotics must adhere and persist in the host. We therefore examined traits related to mucosal colonization: auto-aggregation, cell-surface hydrophobicity, and co-aggregation with pathogens. These properties collectively influence a strain's ability to form biofilms and exclude competitors in the gut (Bogino et al., 2013; Trunk et al., 2018). Our isolates displayed generally high auto-aggregation capacity, with substantial variability between strains. Notably, one tea isolate (TF13) aggregated >80% of cells into clumps after 24 h, and several others (TF17, DC04) reached ~70–75%, whereas a few were more modest aggregators (~40–50%). This time-dependent self-association suggests the presence of strain-specific surface factors that mediate cell–cell recognition. Rapid aggregators like TF13 and TF17 began clumping within 1–2 h, implying they produce surface adhesins or extracellular polymers early in growth. Indeed, high aggregation kinetics have been linked to the expression of APFs, EPS, and protein adhesins on the cell surface (Fugaban et al., 2021; Roldan-Perez et al., 2023). Conversely, strains with low auto-aggregation may lack these macromolecules or have more hydrophilic surfaces that repel self-association (Wang et al., 2017). From a functional perspective, strong auto-aggregation is desirable: it facilitates biofilm formation on intestinal epithelium and helps the probiotic community resist washout by peristalsis (Trunk et al., 2018). It also correlates with co-aggregation ability, wherein probiotics physically bind to pathogenic bacteria and precipitate them out of solution. In our study, the highly aggregative strains showed pronounced co-aggregation with *E. coli* and *S. aureus* in vitro, with strains TF03 (tea) and DC04 (dairy) achieving the greatest pathogen co-aggregation indexes. This implies that these LAB can effectively grapple pathogens, limiting the pathogens' access to host cells or nutrients. Such competitive exclusion via co-aggregation is recognized

as a key anti-infective mechanism of probiotics (Cozzolino et al., 2020; Nami et al., 2024). It is noteworthy that the strongest co-aggregators in our panel overlap with those showing the highest auto-aggregation, reinforcing the idea that the same surface factors often underlie both self- and co-adhesion. Taken together, the adhesion phenotype of our isolates – characterized by robust auto-aggregation, high cell-surface hydrophobicity, and substantial pathogen co-aggregation – bodes well for their colonization potential. These traits should enable the strains to establish residence in the gut mucosa and create a protective barrier that pathogens find hard to penetrate, thereby contributing to gut health if administered as probiotics.

In addition to passive exclusion, our isolates also exhibited active antagonism against foodborne pathogens, a trait central to both probiotic efficacy and food biopreservation. All tested LAB produced acid and other inhibitory agents that suppressed the growth of common pathogens (*E. coli, S. aureus* and *P. aerogenosa*) in agar well diffusion assays. We observed inhibition zone diameters generally in the 10–20 mm range, comparable to those of commercial probiotic strains. Several isolates from goat butter (notably *Lpb. plantarum* DC04 and *Lev. brevis* DC06) showed particularly strong broad-spectrum inhibition, with zones exceeding 20 mm against certain targets (data not shown). Such potent activity is likely due to the combined effects of LA accumulation (lowering pH) and the production of bacteriocin-like peptides, since neutralized CFSs still retained some inhibitory effect (suggesting acid-independent action). The strong antagonism by DC04 and DC06 is consistent with prior reports that *Lpb.s plantarum* and *Lev. brevis* strains enhance meat safety through organic acid and bacteriocin production (Kauser-Ul-Alam et al., 2021; Carla Miranda et al., 2021). Meanwhile, the tea-derived isolates showed slightly smaller inhibition zones, which may reflect a trade-off whereby these strains allocate more resources to stress tolerance (e.g. phenol resistance) and somewhat less to producing antimicrobials. Nevertheless, their inhibitory spectrum still fell within an efficacious range, as seen in other plant-origin probiotics. An interesting pattern emerges: the dairy isolates, having evolved in a microflora-rich milk environment, may prioritize aggressive acidification and bacteriocin production to outcompete spoilage microbes, whereas the tea isolates prioritize resistance to chemical stressors (polyphenols, salt) over maximal bacteriocin output. Regardless, all our isolates can acidify their growth medium rapidly (typically dropping pH to <4.5 in MRS broth within 24 h, not shown), which itself is a powerful preservation factor in foods. In meat products, a swift pH drop caused by fermentative LAB can significantly hinder pathogens like *L. monocytogenes* early in storage, before they have a chance to proliferate (Huang et al., 2022). Thus, the rapid acid production by our strains – combined in some cases

with specific antimicrobials – creates a multi-hurdle preservation effect. In practical terms, these LAB could be deployed as protective cultures in perishable foods, where they would acidify the product and secrete bacteriostatic compounds, thereby reducing reliance on chemical preservatives. Notably, the efficacy of such bioprotection can depend on the physiological state of the added culture: strains introduced at their exponential growth phase tend to acidify faster and dominate the microbiota, enhancing preservation (Huang et al., 2022). This insight, along with our growth kinetic data, could inform the inoculation strategies for using these isolates in food systems.

Growth kinetics measurements provided further guidance on strain selection for various applications. We found clear differences in the lag phase duration and growth rates among isolates. Some strains (e.g. *Lev. brevis* TF17 and TF03) had very short lag phases of ~0.9 h, essentially launching into exponential growth almost immediately after inoculation. Such rapid adaptors are highly advantageous in competitive ecosystems: a strain that begins growing quickly can acidify and consume nutrients before other microbes, thereby "setting the tone" of the fermentation or colonization (Mernawati et al., 2023). In a meat preservation context, for instance, a fast-growing LAB like TF17 could promptly acidify the meat matrix and outpace pathogens, aligning with the principle that early LAB dominance is key to suppressing Listeria in fermented meats (Huang et al., 2022). On the other hand, we observed that *Lpb. plantarum* DC04, while a strong acid producer ultimately, exhibited a longer lag phase (~2 h) before exponential growth. This extended initial delay might be interpreted as a strain "taking its time" to adjust to the environment, possibly by synthesizing necessary enzymes or vitamins. Interestingly, slower starter cultures can be desirable in certain fermentations where a more gradual acidification is needed to develop complex flavors or textures (Mokoena, 2017). Thus, a strain like DC04 might be well-suited as an adjunct culture that contributes flavor without souring the product too rapidly. All isolates eventually reached similar cell densities (OD_{max}), indicating comparable growth yields, but their differing kinetics suggest a strategic advantage in using them in combination. For example, a blend of a fast-grower (to secure microbial dominance and safety early) with a slower strain (to extend fermentation and enhance flavor) could optimize both safety and sensory qualities of a fermented food. More broadly, the kinetic diversity we observed underscores that probiotic functionality is often strain-specific rather than uniform across a species (Yehuala et al., 2024). It reinforces the importance of selecting individual strains with growth behaviors tailored to the target application, whether it be a rapid biopreservation effect or a controlled fermentation process.

Finally, we assessed enzymatic activities relevant to food technology, namely extracellular protease and lipase production. Intriguingly, none of our isolates showed any proteolytic or lipolytic activity on agar plate assays. At first glance, the absence of protease secretion might seem a drawback for flavor development (as protein hydrolysis can release savory peptides). However, it is well documented that *Lactobacillus*-type LAB generally have weaker extracellular protease systems compared to dairy starters like *Lactococcus* or *Pediococcus* (Garcia-Cano et al., 2019; Kieliszek et al., 2021). These lactobacilli satisfy most of their amino acid requirements from the rich media of fermented foods, and any needed protein breakdown can occur via intracellular peptidases after uptake of oligopeptides (Papadimitriou et al., 2016). The lack of gelatinase/protease activity also mirrors our safety results – none of the strains produced gelatinase, a trait often associated with pathogenic *Enterococcus* – reaffirming their non-pathogenic profile. From a technological viewpoint, non-proteolytic behavior can be beneficial for meat preservation: it means the cultures are unlikely to cause proteolytic softening or spoilage of the product's protein matrix over storage. Likewise, the non-lipolytic nature of our isolates is a highly desirable feature for biopreservative cultures. Lipase-positive LAB can break down lipids in foods, leading to rancidity, off-flavors, and textural defects (Sionek et al., 2025). In fermented meat products, for example, excessive lipolysis by starter cultures is known to generate unwanted volatile acids and oxidized fats that deteriorate sensory quality (Sionek et al., 2025). The fact that none of our candidate strains produce detectable lipases means they can be added to fatty foods (like sausages or butter) without risking such lipid spoilage. This trait, combined with their strong inhibition of lipid-oxidizing spoilage microbes (like certain *Clostridium* and *Pseudomonas* spp.), suggests these LAB could actually protect the lipid component of foods. Indeed, the literature warns that lipolytic LAB may also degrade lipid-based natural antioxidants or curing agents in foods (Sionek et al., 2024), whereas our non-lipolytic strains would preserve those protective factors. In summary, the absence of exoprotease and exolipase activities in our isolates supports their technological suitability: they contribute to safety and shelf-life extension without compromising the structural and flavor integrity of the food matrix.

Combining all these findings, we propose that the LAB isolates characterized in this work hold considerable promise as dual-purpose microbial resources – serving as both probiotics and natural food preservatives. Their robust survival in simulated gastrointestinal conditions and strong adherence/antagonism properties fulfill key criteria for probiotic functionality in humans or animals. At the same time, traits like fast acidification, broad pathogen inhibition, salt

tolerance, and non-lipolytic behavior make them ideal candidates for use in fermented or minimally processed foods to enhance safety and shelf life. We explored one such application in a preliminary *in vivo* trial using a poultry model, where selected strains were fed to broiler chickens as dietary probiotics. The results (detailed elsewhere) illustrated tangible real-world benefits: chickens that received *Lev. brevis* TF03 or TF13 showed improved growth performance and produced meat with enhanced nutritional and oxidative profiles compared to unsupplemented controls. Specifically, probiotic-supplemented broilers yielded breast meat with higher protein and intramuscular lipid content and significantly lower markers of lipid oxidation (malondialdehyde levels), indicating better meat quality and stability. These enhancements align with recent studies showing that certain LAB probiotics can enrich meat with health-promoting PUFA and antioxidants (Liu et al., 2025; Untea et al., 2022).

Our findings are consistent with these mechanisms: the probiotic-fed chickens not only grew faster but also had meat that was more nutrient-dense and resistant to oxidative spoilage. This outcome underscores a synergistic benefit – by improving gut health and metabolic status of the host, the probiotics indirectly improve the quality of the animal product. It's a concept of "farm-to-fork" intervention where probiotic use in livestock translates to safer, more nutritious foods for consumers. In parallel, the same strains could be applied directly to meat after slaughter (e.g. as fermentative starters or surface protective cultures) to inhibit pathogens like *L. monocytogenes* on ready-to-eat products, as supported by our *in vitro* pathogen assays and the known anti-listerial effects of *Lpb. plantarum/Lev. brevis* (Kauser-Ul-Alam et al., 2021; Carla Miranda et al., 2021). Thus, whether introduced via the feed or onto the food, these LAB have the potential to raise food safety standards naturally.

In conclusion, this study's polyphasic screening and characterization reveal that fermented goat butter and tea waste are rich reservoirs of functionally potent LAB. Through a rigorous multi-criterion selection, we identified strains that are safe, technologically robust, and endowed with beneficial functionalities spanning stress tolerance, adhesion, pathogen antagonism, and compatible enzymatic profiles. The convergence of probiotic and bioprotective traits in these isolates is particularly noteworthy. It opens the door to developing interventions that address both health and preservation – for example, a fermented meat product that not only has extended shelf life due to the LAB culture's action but also delivers probiotic benefits to the consumer. From a broader perspective, our findings contribute to the growing emphasis on sustainable food biotechnology: by valorizing microbes from traditional and underutilized fermented resources (like tea waste) and deploying them in modern food systems, we bridge artisanal

practices with contemporary needs. Future work will delve deeper into the molecular basis of the observed traits (e.g. genomics to identify genes for bacteriocins, BSH, or stress responses) and will evaluate the strains in real food processing trials. Nonetheless, the present results already demonstrate a compelling proof-of-concept that diverse LAB isolates can be harnessed as next-generation probiotics and natural preservatives, combining scientific rigor with practical relevance to meet the challenges of food security and public health.

CONCLUSION

9. CONCLUSION

This thesis comprehensively investigated the synergistic effects of phenolic compounds and LAB on poultry meat quality, highlighting the technological, nutritional, and sensory attributes pivotal to consumer and regulatory demands for clean-label food products. The principal aim was to isolate, characterize, and evaluate phenolic-adapted LAB strains from phenolic-rich fermented green tea waste and traditional goat-milk butter (Dhan) to assess their influence on oxidative stability, microbial safety, and overall preservation of poultry meat.

A multistage screening narrowed the selection to four promising LAB strains—*Levilactobacillus brevis* DC01-A, TF03, TF13, and *Lactiplantibacillus plantarum* DC04—demonstrating strong acid and bile tolerance, resistance to phenolic compounds, effective antimicrobial properties, and adequate mucosal adhesion. These characteristics underpin their probiotic resilience and functionality in chicken gastrointestinal tracts. In vivo testing confirmed that broilers supplemented with TF03 and TF13 experienced significant growth performance gains, reflected in an average daily gain increase of 15%, a 10% reduction in feed conversion ratio, and a 10–12% increase in final body mass. Enhanced meat quality was evident in higher dry matter, protein, and lipid contents along with a 25–27% decline in TBARS-assessed lipid peroxidation, signaling improved oxidative stability and prolonged shelf life.

Notably, sensory evaluation revealed considerable improvements across multiple parameters (color, odor, texture, tenderness, juiciness, flavor, mouthfeel, and overall acceptability) for probiotic-supplemented broiler meats, with strains TF03 and TF13 leading these enhancements. Color metrics improved by approximately 22–23%, aligning with reduced metmyoglobin formation and oxidative degradation corroborated by lower TBARS values. Reduced lipid oxidation minimized the formation of rancid volatiles such as hexanal and nonanal, enhancing freshness and flavor scores by nearly two points compared to controls. This is mechanistically attributable to probiotic-mediated antioxidant activity, including DPPH free-radical scavenging capacities near 70%, and secretion of bioactive metabolites like EPS, acetoin, and diacetyl that contribute to taste complexity. Improved tenderness and juiciness correlated positively with higher protein content and water retention, mediated through probiotic regulation of nutrient absorption, antioxidant enzyme activity, and muscle protein integrity.

Multivariate analyses integrated production performance, oxidative markers, lipidomic profiles, and sensory data, revealing a well-coordinated metabolic and organoleptic improvement network. This encompassed increased unsaturated fatty acid deposition, enhanced antioxidant defenses via Nrf2 pathway activation, and inhibited peroxidative compound generation, substantiating a robust probiotic-driven enhancement of meat quality and nutrient density.

On the technological front, microencapsulation of LAB in sodium alginate maintained over 90% cell viability through simulated gastric transit, ensuring effective delivery and functional probiotic activity in the target intestinal environment. Moreover, the ecological origin of these LAB strains emerged as a fundamental determinant of their phenolic resilience, functional robustness, and capacity to modulate multiple quality parameters synergistically. This underlines the value of targeting phenolic-rich microbial environments for isolating potent bio-preservative probiotics.

The study's limitations lie primarily in the restricted experimental scale and relatively narrow strain diversity, suggesting the need for expansive, multi-strain, and multi-environment trials to validate findings across diverse poultry breeds and production contexts. Future research should focus on elucidating molecular mechanisms underpinning phenolic compound interactions, leveraging omics approaches, and extending evaluations to other food matrices and animal models to enhance generalizability and applicability.

In conclusion, this research contributes a novel and valuable understanding of how ecologically sourced, phenolic-adapted LAB strains can simultaneously improve poultry growth performance, meat oxidative stability, sensory quality, and food safety. These natural, multifunctional probiotics offer effective alternatives to antibiotics and synthetic preservatives, aligning with global clean-label trends and advancing sustainable, high-quality poultry production. By bridging microbial ecology, functional probiotic science, and meat technology, our work provides a comprehensive platform for both academic inquiry and industrial innovation aimed at enhancing food security, nutritional value, and environmental responsibility in the agri-food sector. This thesis thus establishes a firm foundation for future developments in probiotic biopreservation and functional food enhancement, presenting clear pathways for research expansion and practical implementation.

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ANNEXE

Annex 1. Standardized formulations of culture media and reagents

This annex summarizes the composition and preparation procedures for the principal media and reagents employed in the isolation, identification, and functional characterization of lactic acid bacteria (LAB). All formulations comply with international standards (ISO, AOAC) and were prepared under aseptic conditions to ensure reproducibility and quality control.

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1. MRS Medium (ISO 15214:1998)

Purpose: Enumeration and isolation of mesophilic lactic acid bacteria.

Component	Quantity per Liter
Peptone (meat/casein)	10.0 g
Beef Extract	10.0 g
Yeast Extract	4.0 g
Glucose (Dextrose)	20.0 g
Sodium Acetate ($CH_3COONa \cdot 3H_2O$)	5.0 g
Triammonium Citrate [$(NH_4)_3C_6H_5O_7$]	2.0 g
Dipotassium Phosphate (K_2HPO_4)	2.0 g
Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	0.2 g
Manganese Sulfate ($MnSO_4 \cdot 4H_2O$)	0.05 g
Tween 80 (Polysorbate 80)	1.0 mL
Agar (if solid medium)	15.0 g
Distilled Water	1 L

Preparation: Dissolve ingredients in distilled water, adjust pH to 5.7 ± 0.1, autoclave at 121°C for 15 min. Cool to 45–50°C before pouring plates. Store at 4°C.

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2. Phosphate Buffered Saline (PBS) (ISO 22176:2005)

Purpose: Physiological buffer for washing and suspension of microbial cells.

Component	Quantity per Liter
Sodium Chloride (NaCl)	8.0 g
Potassium Chloride (KCl)	0.2 g
Disodium Phosphate ($Na_2HPO_4 \cdot 2H_2O$)	1.44 g
Monopotassium Phosphate (KH_2PO_4)	0.24 g
Distilled Water	1 L

pH Adjustment: 7.4 ± 0.2

Preparation: Dissolve all components, adjust pH, autoclave at 121°C for 15 min, store at 4°C.

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3. Nutrient Gelatin Medium (ISO 13720:2010)

Purpose: Detection of gelatinase activity.

Component	Quantity per Liter
Peptone	5.0 g
Beef Extract	3.0 g
Gelatin	120.0 g
Sodium Chloride (NaCl)	5.0 g
Distilled Water	1 L

Preparation: Dissolve all components in distilled water, adjust pH to 6.8 ± 0.2, autoclave at 121°C for 15 min. Cool upright to solidify.

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4. DNase Methyl Green Agar (ISO 21964:2006)

Purpose: Detection of DNase-producing bacteria.

Component	Quantity per Liter
Tryptone	20.0 g
Soya Peptone	5.0 g
Sodium Chloride (NaCl)	5.0 g
DNA (salmon sperm)	2.0 g
Methyl Green	0.05 g
Agar	15.0 g
Distilled Water	1 L

Preparation: Dissolve all ingredients, adjust pH to 7.5 ± 0.2, autoclave at 121°C for 15 min, cool to 50°C, pour plates. A clear halo around colonies indicates DNase activity.

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5. Columbia Agar Base (ISO 7932:2004)

Purpose: Cultivation of fastidious and Gram-positive organisms.

Component	Quantity per Liter
Peptone (meat/casein)	23.0 g
Starch	1.0 g
Sodium Chloride (NaCl)	5.0 g
Agar	15.0 g
Distilled Water	1 L

pH Adjustment: 7.3 ± 0.2

Preparation: Dissolve, adjust pH, autoclave at 121°C for 15 min, cool to 50°C before pouring. Optionally enrich with 5% sheep blood.

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6. Tryptone Soy Broth (TSB) + 1% Sucrose (ISO 11133:2014)

Purpose: General-purpose enrichment for bacterial growth and probiotic assays.

Component	Quantity per Liter
Tryptone	17.0 g
Soya Peptone	3.0 g
Sodium Chloride (NaCl)	5.0 g
Dipotassium Phosphate (K_2HPO_4)	2.5 g
Dextrose	2.5 g
Sucrose	10.0 g
Distilled Water	1 L

pH Adjustment: 7.3 ± 0.2

Preparation: Dissolve, adjust pH, autoclave 121°C for 15 min. Cool before inoculation.

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7. Gram Staining Reagents (ISO 4833-1:2013)

Purpose: Differentiation of Gram-positive and Gram-negative bacteria.

Reagent	Composition
Crystal Violet	20 g/L in 95% ethanol + 1 g ammonium oxalate + distilled water to 1 L
Gram's Iodine	10 g iodine + 20 g KI + distilled water to 1 L
Decolorizer	95% Ethanol
Counterstain (Safranin)	2.5 g in 95% ethanol, diluted to 1 L

Procedure: Fix smear, apply crystal violet (1 min), iodine (1 min), decolorize (20 s), counterstain with safranin (30 s). Gram+ = purple; Gram− = pink.

8. Simmons Citrate Agar (ISO 10272-2:2017)

Purpose: Determination of citrate utilization.

Component	Quantity per Liter
Sodium Citrate	2.0 g
Ammonium Dihydrogen Phosphate ($NH_4H_2PO_4$)	1.0 g
Dipotassium Phosphate (K_2HPO_4)	1.0 g
Sodium Chloride (NaCl)	5.0 g
Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	0.2 g
Bromothymol Blue	0.08 g
Agar	15.0 g
Distilled Water	1 L

pH Adjustment: 6.9 ± 0.2

Preparation: Dissolve, adjust pH, autoclave 121°C for 15 min. Inoculate slants; blue color indicates positive citrate utilization.

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9. Clark and Lubs Medium

Purpose: pH indicator medium for fermentation reactions.

Component	Quantity per Liter
Glucose	5.0 g
Peptone	5.0 g
K_2HPO_4	5.0 g
KH_2PO_4	5.0 g
Distilled Water	1 L

pH Adjustment: 6.9 ± 0.2

Preparation: Dissolve, adjust pH, autoclave 121°C for 15 min.

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10. 1× TAE Buffer (Tris-Acetate-EDTA)

Purpose: Electrophoresis buffer for nucleic acid analysis.

Component	Concentration / Quantity per 1 L
Tris Base	4.84 g (40 mM)
Glacial Acetic Acid	11.4 mL (20 mM)
EDTA (pH 8.0)	0.372 g (1 mM)
Distilled Water	Up to 1 L

Preparation: Dissolve Tris base in 800 mL water, add acetic acid and EDTA, adjust volume to 1 L. Store at room temperature.

General Notes:

- All media were sterilized by autoclaving (121°C, 15 min) and stored at 4°C.
- Sterility and performance were verified using ISO reference strains.
- pH adjustments were made before sterilization using 1 N NaOH or 1 N HCl.
- Quality control followed ISO 11133:2014 guidelines.