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IMPACT OF STARTER CULTURES ON LIPID OXIDATION AND FATTY ACID PROFILE OF PORK MEAT MATURATION

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ABSTRACT

The preservation and proper maturation of pork meat remain critical challenges for the food industry, requiring effective "hurdle technologies" to inhibit pathogens and prevent quality deterioration. This study evaluates the impact of different starter cultures – specifically *Lacticaseibacillus rhamnosus* 20-12, *Lactobacillus plantarum* 66 (MDC 9619) and the commercially used BactoFlavor® BFL-T0 on lipid oxidation, fatty acid (FA) profile, and the safety of matured pork meat. Experimental results demonstrated that *L. rhamnosus* 20-12 significantly improved the lipid profile, reaching a peak of oleic acid (C18:1) concentration up to 44.27% during maturation period. Whole-genome sequencing (WGS) was employed to provide a molecular foundation for these technological benefits. Genomic analysis via KEGG's GhostKOALA revealed a complete repertoire of fatty acid biosynthesis enzymes (modules M00082 and M00083), while a manual search identified a redundant antioxidant defense system comprising multiple peroxidases and six distinct NADH oxidases. Furthermore, BAGEL4 and antiSMASH analyses identified a sophisticated antimicrobial arsenal, including a Class II multi-peptide bacteriocin cluster (containing Enterocin X, LSEI_2386, and Carnocin_CP52) and a RiPP-like biosynthetic gene cluster on contig 58. The accumulation of beneficial oleic acid and the maintenance of oxidative stability are supported by a robust genetic investment in lipid metabolism, while ensuring potent Anti-Listerial activity suggesting a genomic potential for these observed technological benefits. Additionally, Type III Polyketide Synthase (T3PKS) and terpene-precursor clusters suggest potential roles in the production of flavor precursors and oxidative protection. The accumulation of beneficial oleic acid and the maintenance of oxidative stability are supported by a robust genetic investment in lipid metabolism (KEGG modules M00082 and M00083) and a redundant antioxidant defense system comprising multiple peroxidases and NADH oxidases. This multidisciplinary study demonstrates that integrating traditional food science with WGS analysis offers a definitive framework for selecting high-performance starter cultures to produce safer, "clean-label," and nutritionally enriched matured pork meat products.

Keywords: pork meat, fatty acids, lipid oxidation, starter cultures, *Lacticaseibacillus rhamnosus*, whole genome

INTRODUCTION

The consumption of meat and its various products have historically been a fundamental component of the human diet, providing essential nutrients vital for promoting health. Because meat is highly susceptible to contamination, food preservation has been a necessity for human survival since prehistory (**Gram *et al.*, 2002; Grigoryan, Gayane Marmaryan, *et al.*, 2025a**). Early techniques relied on the empirical inactivation of spoilage microorganisms through drying, heating, salting, or fermentation. Despite modern advancements, it is estimated that 25% of all food produced globally is still lost post-harvest or post-slaughter due to microbial spoilage (**Gram *et al.*, 2002**).

To address these challenges, the most effective approach is "hurdle technology", which intelligently combines multiple preservative factors – such as controlled temperature, reduced water activity (WA), and the use of competitive microorganisms – to disturb the homeostasis of pathogens (**Leistner, 2000**). Lactic acid bacteria (LAB) are particularly critical in this framework, serving as starter cultures for fermented products like ham and sausages. LAB may contribute to the sensory profile by potentially enhancing flavor, color, and texture, while simultaneously decomposing large molecules into easily absorbed aromatic substances (**Grigoryan, 2025; Grigoryan, Marmaryan, *et al.*, 2025b**).

Beyond flavor, LAB act as natural biopreservatives by producing organic acids and antimicrobial bacteriocins (**Tkhruni, *et al.*, 2025; Grigoryan, 2025; Grigoryan, Marmaryan, *et al.*, 2025a; Grigoryan, Marmaryan, *et al.*, 2025b**). These metabolites lower the food's pH and restrict the development of spoilage organisms and harmful pathogens like *Listeria monocytogenes* (**Grigoryan, Marmaryan, *et al.*, 2025a**). Furthermore, accurate selection of LAB starter cultures can enhance the product's biological value and oxidative stability, allowing for a shorter maturation period while maintaining high safety standards (**Grigoryan, Marmaryan, *et al.*, 2025a; Grigoryan, 2025**).

The application of whole-genome sequencing (WGS) serves as a definitive foundation for strain identification and the characterization of essential food-processing traits, particularly within meat matrices. Genomic evidence reveals that *Lacticaseibacillus rhamnosus* strains often harbor extensive proteolytic complexes – comprising over 21 distinct proteases – alongside peptidases, antioxidant enzymes, and bacteriocin-producing genes (such as lactococcin), which facilitate accelerated maturation and nutritional enrichment. These multifaceted attributes, combined with a demonstrated low frequency of undesirable markers like virulence factors or antibiotic resistance, support the classification of this species as a robust candidate for use as a food enhancer, probiotic, and producer of bioactive or prebiotic compounds (**Grigoryan, Marmaryan, *et al.*, 2025a).**

While foodborne pathogens and their persistent biofilms present a severe risk to human health throughout the food chain, the current repertoire of bacteriocin resources to combat these threats remain limited. To meet industrial demands, advanced alternating tangential flow (ATF) perfusion-based technology has been successfully implemented for the large-scale preparation of high-density probiotic cultures. For instance, ATF perfusion has been shown to remarkably increase the viable cell counts of *L. rhamnosus* CLK 101 to 11.93 ± 0.14 log CFU/mL, enabling the purification of CLK_01, a novel, low-molecular-mass (701.49 Da) bacteriocin with broad-spectrum activity and potent antibiofilm capabilities (**Chen *et al.*, 2023**).

Bioprospecting from traditional sources, such as naturally fermented yak yoghurt, has further expanded these resources with strains like *L. rhamnosus* XN2. This strain demonstrates a high antibacterial activity of 3200 AU/mL against a wide array of pathogens, including *Bacillus cereus*, *Staphylococcus aureus*, and *Listeria*

monocytogenes. Notably, these bacteriocins exert dual activity by both disrupting cell membrane integrity and inhibiting virulence factors, such as α-haemolysin secretion, through the regulation of bacterial Quorum Sensing (QS) systems (**Wei *et al.*, 2022**).

The rising demand for natural preservatives has intensified research into bacteriocin-based biopreservation as a viable alternative to chemical additives (**Sarika *et al.*, 2019; Wu *et al.*, 2022**). Studies on Bacteriocin GP1 have confirmed its efficacy in maintaining the sensory, chemical, and bacteriological quality of highly perishable products, such as fish filet, during refrigerated storage (**Sarika *et al.*, 2019**). Simultaneously, novel peptides like *Bacteriocin* ZFM216 (11,851.9 Da), purified through sophisticated chromatographic protocols, exhibit remarkable thermal and pH stability, confirming their potential as versatile and safe antimicrobial agents for advanced food safety applications (**Wu *et al.*, 2022**).

Oxidative processes in meat are primary drivers of quality deterioration, leading to significant changes in flavor, color, texture, and nutritional value. To counteract this damage, living cells utilize complex defense mechanisms involving endogenous antioxidants, specifically enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px). SOD is particularly critical for neutralizing superoxide anion radicals, while catalase and GSH-Px serve as the principal peroxide-removing enzymes within the cytosol. Despite their vital role, information regarding the various factors – such as species, muscle type, and genetic background – that influence the activity of these enzymes in meat remains limited **(Chan *et al.*, 1994; Hernández *et al.*, 2004)**.

Beyond oxidative stability, endogenous lipolytic and proteolytic enzymes, such as lipases and cathepsins, are fundamental to meat quality, driving the structural modifications and the release of flavor precursors required for a superior final product (**Hernández *et al.*, 2004**). Building upon previous research regarding the influence of starter cultures on the pork curing process, this study evaluates the evolution of fatty acid profiles and metabolic markers in pork treated with diverse starter cultures during ripening **(Grigoryan, Marmaryan, *et al.*, 2025a)**.

The strain *L. rhamnosus* 20-12 was selected for exhaustive characterization because of its exceptional technological performance, including shortening the pork maturation period from 8 days to 5 days while providing potent antimicrobial and antioxidant activities. To elucidate the mechanisms behind these functional benefits, the whole-genome sequence of this strain was comprehensively analyzed to identify genes associated with its extensive proteolytic complex (comprising more than 21 proteases) **(Grigoryan, Marmaryan, *et al.*, 2025a)**, as well as genes related to lipolytic and antioxidant functions, bacteriocin production, and important secondary metabolite clusters, which we will display in this publication. Based on the identified genomic potential of L. rhamnosus 20-12 and L. plantarum 66 for lipid restructuring and antioxidant defense, the research hypothesis implies that the application of these targeted genomic-selected starter cultures will result in a significantly accelerated maturation rate (measured by pH stabilization) and a higher accumulation of monounsaturated fatty acids (specifically oleic acid) compared to both traditional salting and standard industrial cultures, while simultaneously suppressing lipid oxidation through enhanced microbial antioxidant activity. The research can contribute to the development of biopreservation technology of pork production, compared to the starter culture used in production

To test this hypothesis, the aims of this study were to: (1) compare the impact of different starter cultures on the fatty acid methyl ester (FAME) profiles of pork during ripening, (2) evaluate the comparative efficiency of these cultures in reducing maturation time, and (3) provide a molecular foundation for these phenotypic traits through whole-genome sequencing (WGS) and biosynthetic gene cluster analysis.

MATERIAL AND METHODS

The experimental material used in this study was pork meat derived from the hind leg of 7-month-old crossbred (Local x Landrace) sow, specifically the Local saw was subjected to artificial insemination with Landrace semen, imported from the Russian Federation. The animal breed in the Kotayk Province, was kept under intensive rearing conditions and reached 95 kg at 7- months of age at slaughter.

The pork leg was obtained from a supermarket and N5 approval document is available according to the required regulations https://www.arlis.am/DocumentView.aspx?docid=118821. The availability of such documents confirms the safety of manufacturing and transportation of the raw meat produce. Handling and transportation of the raw meat are regulated by Regulation 034/2013 "On Safety of Meat and Meat Products" Technical Regulations of the Customs Union (TR CU) https://mineconomy.am/page/444.

The maturation process was conducted in a climate-controlled chamber at 4–6 °C with a constant relative humidity of 75 ± 5%. To simulate traditional maturation while controlling for lipid oxidation, samples were stored under aerobic conditions (wrapped in oxygen-permeable food-grade film') to allow for natural gas exchange during the ripening period.

Fermenting strains were selected for salt tolerance (3.5% NaCl) and psychrophilicity (4–6 °C). Based on these criteria, the following starter cultures were chosen: the commercially used BactoFlavor® BFL-T0 culture (Hansen, Denmark), which contains *Pediococcus pentosaceus* and *Staphylococcus carnosus*; *Lactobacillus plantarum* 66 (MDC 9619) and *Lacticaseibacillus rhamnosus* 20-12 (MDC 9631) was originally isolated from traditional Armenian brine-salted cheese. This ecological origin is consistent with the strain's demonstrated salt tolerance (up to 12% NaCl) and ability to remain metabolically active at refrigerated temperatures (4–6 °C), both of which are critical for the meat maturation process investigated here.

The mentioned starter cultures were compared with traditional salting. The latter two strains (*L. plantarum* 66 and *L. rhamnosus* 20-12, formerly *Lactobacillus rhamnosus* MDC 9631) were obtained from the Microbial Depository Center (MDC) of the ArmBiotechnology Scientific and Production Center of NAS RA. The initial microbial load of the inoculated meat was approximately 10^7 CFU/g. By the end of the maturation period (Day 5 for L. rhamnosus 20-12 and Day 8 for the control), the final Lactic Acid Bacteria (LAB) counts reached 10^8–10^9 CFU/g, confirming the dominance of the starter cultures throughout the process. All lactic acid bacteria were stored in sterilized milk with 40% glycerol at −20 °C; viability was maintained for up to six months. Strains were maintained on de Man, Rogosa and Sharpe (MRS) agar at 4–6 °C and subcultured every 1–2 months. Salt sensitivity was tested in MRS broth containing 2–12% NaCl at 37 °C for 24 hours. The pork leg was split into four segments, each weighing 150 grams. For each trial, distinct meat segments (150g each) were processed and inoculated separately, representing independent biological fermentation units. For the purpose of salting, brine (a saline solution) was prepared (50 ml) with the salt concentration being 11,13%, the density being 1,077, at room temperature +15±2^{0}C (in line with *GOST* state standards for meat products).

Fatty acid analysis by GC–MS

Fatty acid composition (oleic acid C18:1, palmitic acid C16:0, and stearic acid C18:0) in pork samples was determined by gas chromatography–mass spectrometry (GC–MS) following lipid extraction and fatty acid methyl ester (FAME) preparation. FAME analysis was performed on a Shimadzu GCMS-QP2010 system (Shimadzu, Japan) equipped with a DB-5MS capillary column. Helium was used as the carrier gas at a constant flow rate of 1.2 mL/min. The oven temperature was programmed from 80 °C to 290 °C at 4 °C/min. Injector, interface, and ion source temperatures were set to 250 °C, 230 °C, and 200 °C, respectively. Mass spectrometric detection used electron impact (EI) ionization at 70 eV with full-scan acquisition over m/z 50–550. Calibration was performed using a 37-component FAME standard mix (e.g., Supelco, Bellefonte, PA, USA). Identification was based on a dual-criteria approach: (1) comparison of retention times with known standards, ensuring a reproducibility of <0.2% RSD, and (2) mass spectral matching against the NIST 11 library with a similarity index (SI) threshold of >85%. The Limit of Detection (LOD) and Limit of Quantification (LOQ) for the major fatty acids (oleic, palmitic, and stearic) were determined to be 0.05 mg/100g and 0.15 mg/100g, respectively, based on a signal-to-noise ratio of 3:1 and 10:1. Quantification was performed by peak-area integration using external calibration curves ($R^2 > 0.99$) (**Retrato *et al.*, 2023**).

Lipid extraction

Homogenized pork meat (1.0 g) was weighed on an analytical balance (Mettler Toledo AB204-S) and suspended in distilled water to a final volume of 20 mL. Lipids were extracted using the method of Folch *et al.* (**Folch *et al.*, 1957)** with chloroform/methanol (2:1, v/v) and incubated at room temperature for 24 h with periodic mixing. The mixture was centrifuged at 3,900 rpm for 10 min (Sigma 2-6E, Sartorius), and the supernatant was collected. A second extraction was performed using chloroform/methanol/water (1:2:0.8, v/v/v). After centrifugation, the supernatants were combined and phase separation was induced by adding chloroform and water (1:1, v/v). The lower organic phase was collected, and lipids were concentrated using a rotary evaporator (Büchi R-210) at 30 °C with a water bath (Büchi B-491). The dried lipid extract was dissolved in chloroform/methanol (1:1, v/v).

FAME preparation

Fatty acids were converted to methyl esters using a modified method of **Ichihara and Fukubayashi (2010)** prior to GC–MS analysis. Briefly, 5 mg of lipid extract was dissolved in toluene, then 2 mL of 1% sulfuric acid in methanol was added. The mixture was incubated at 80 °C for 2 h. After cooling to room temperature, 5 mL of 5% NaCl solution was added. FAMEs were extracted twice with hexane (2 × 5 mL), the combined hexane extracts were washed with 2% $NaHCO_3$ solution and dried over anhydrous Na_2SO_4. The solvent was evaporated under a gentle stream of nitrogen.

Genomic characterization of *L. rhamnosus* 20-12 with focus on meat fermentation traits

The lipid metabolism of *L. Rhamnosus 20-12* was analyzed using KEGG's GhostKOALA (**Kanehisa *et al.*, 2016**). A manual search for antioxidant defense proteins – including peroxidases, superoxide dismutases (SOD), reductases (specifically thioredoxin and glutathione reductases), glutathione S-transferases,

and NADH oxidases – was performed using the annotated *L. Rhamnosus 20-12* genome file («PROKKA_05292026.gbk», available via Mendeley Data).
The *L. Rhamnosus 20-12* bacteriocin gene cluster was identified using BAGEL4 (**Heel *et al.*, 2018**), based on the «assembly_12_13_Lambda.fasta» file generated via Flye assembly (available in the Mendeley data repository).
The bacterial version of antiSMASH (v8.0.4) was used to identify secondary metabolite biosynthetic gene clusters in *L. rhamnosus* relevant to meat fermentation traits (**Blin *et al.*, 2025**).

Statistical analysis

All experiments were performed in biological independent triplication, totally having 12 samples. To ensure statistical robustness, three independent maturation trials were conducted for each treatment group (n=3). The results are presented as Mean ± Standard Deviation (SD). Technical replicates (multiple measurements of the same biological sample) were performed to ensure instrument precision but were averaged prior to statistical analysis to ensure the 'n' reflects true biological independence. Statistical analyses were conducted using Microsoft Excel (**Microsoft Excel, 2018**). The obtained differences were evaluated using one-way ANOVA and statistically significant differences are indicated at $p < 0.05$.

RESULTS AND DISCUSSION

GC–MS Analysis of Fatty Acid Methyl Esters (FAMEs)

The primary objective of this study was to evaluate how different starter cultures influence the fatty acid (FA) composition and oxidative stability of pork leg meat during maturation. Our results demonstrate that the choice of microbial culture significantly alters the lipid profile. To address the relative contribution of microbial metabolism versus endogenous meat enzymes, we compared the fatty acid dynamics of culture-treated samples with the traditional salting group (control), which relies solely on the meat's internal enzymatic activity. The evolution of the fatty acid profile during maturation is a complex interplay between endogenous meat enzymes and microbial metabolic activity. Endogenous lipases and cathepsins in the pork muscle initiate the breakdown of triacylglycerols, a process evident in the traditional salting group which reached 43.31% oleic acid by Day 8. However, the introduction of *L. rhamnosus* 20-12 significantly accelerated this accumulation, reaching 44.27% by Day 5.
This suggests that while endogenous lipolytic activity provides a baseline for lipid restructuring, the microbial machinery – specifically the fatty acid biosynthesis modules (KEGG M00082 and M00083) and exogenous lipases – acts as a catalyst. Furthermore, the starter culture may modulate the activity of endogenous enzymes by rapidly lowering the meat pH, creating an environment that favors specific native lipolytic pathways while inhibiting others through antioxidant protection. Therefore, the final lipid profile is a result of a synergistic contribution from both the meat matrix's internal enzymes and the metabolic output of the starter culture.
The genomic evidence supported these observations; the *in silico* analysis of the *L. rhamnosus* 20-12 proteome revealed a robust investment in lipid metabolism, specifically the presence of complete KEGG modules M00082 (initiation) and M00083 (elongation). The identification of multiple paralogs of 3-oxoacyl-[acyl-carrier protein] reductase (K00059) and the full acetyl-CoA carboxylase complex indicates that this strain possesses the necessary metabolic machinery for *de novo* fatty acid synthesis and lipid restructuring, providing a molecular foundation that likely supports the observed FA variations in Table 1.
While lipid oxidation is a primary driver of quality deterioration in meat, our results showed that starter cultures – particularly *L. plantarum* 66 and *L. rhamnosus* 20-12 – effectively managed oxidative stress. Interestingly, *L. rhamnosus* 20-12 showed the lowest levels of linoleic acid (5.279%), which may be a result of metabolic conversion or protection against oxidation. Experimental results from our previous study demonstrated that *L. rhamnosus* 2012 MDC 9631 exhibited strong antioxidant potential, reaching 40.6% radical scavenging activity on the 5th day of maturation.
The genomic characterization of antioxidant defense proteins (Table 4) suggests a molecular basis for this stability. *L. rhamnosus* 20-12 harbors a redundant system for detoxifying reactive oxygen species (ROS), including thiol peroxidase (100% homology), NADH peroxidases, and glutathione peroxidase (BsaA). Furthermore, the identification of six distinct NADH oxidases suggests a high efficiency in scavenging molecular oxygen. Although a manganese-dependent SOD was not found—a common trait in *Lactobacilli* that utilize intracellular Mn^{2+}—the abundance of other oxidoreductases ensures metabolic stability during the 5-day maturation period. Additionally, the T3PKS cluster (Contig 21) identified via antiSMASH may produce phenolic antioxidants, providing a secondary layer of protection against lipid rancidity.
The accelerated maturation period, shortened from 8 to 5 days by strain 20-12 **(Grigoryan, Marmaryan, *et al.*, 2025a)** requires rigorous pathogen control to ensure safety. Our BAGEL4 and antiSMASH analyses confirmed that *L. rhamnosus* 20-12 is equipped with a sophisticated antimicrobial "arsenal." The identification of a Class II multi-peptide bacteriocin cluster containing Enterocin X, LSEI_2386, and Carnocin_CP52 is highly significant. Carnocins, in particular, are known for their potent activity against *Listeria monocytogenes*, a critical pathogen in pork processing.

Table 1 Effect of different starter cultures on fatty acid composition (%) of matured pork meat

Fatty acid			Type of salting (M±SD)			
			Traditional	Bacto Flavor	*L. plantarum* 66	*L. rhamnosus 20-12*
Saturated fatty acid, SFA		C8:0 Caprylic acid	0.18±0.012	0.19±0.009	0.16±0.012	0.33±0.023
		C10:0 Capric acid	0.09±0.006	0.11±0.005	0.07±0.008	0.11±0.008
		C11:0 Undecylic (Undecanoic) acid	0.24±0.016	0.22±0.015	0.18±0.012	0.36±0.025
		Lauric acid C12:0	0.07±0.005	0.07±0.005	-	-
		C13:0 Tridecylic (Tridecanoic) acid	0.21±0.014	0.18±0.012	0.15±0.010	0.32±0.022
		C14:0 Myristic acid	1.03±0.072	1.14±0.079	0.98±0.068	1.08±0.075
		C15:0Pentadecylic (Pentadecanoic) acid	0.13±0.009	0.10±0.007	0.10±0.007	0.18±0.012
		C16:0 Palmitic acid	**23.44±0.28**	**24.49±0.33^{ns}**	**22.44±0.30^{ns}**	**24.56±0.34^{ns}**
		Margaric (Heptadecanoic) acid C17:0	0.29±0.014	0.25±0.017	0.23±0.016	0.20±0.014
		Stearic acid C18:0	**12.13±0.20**	**13.02±0.23^{ns}**	**12.03±0.22^{ns}**	**12.75±0.23^{ns}**
		C20:0 Arachidic acid	0.15±0.007	0.17±0.011	0.18±0.012	0.17±0.011
		C22:0 Behenic acid	0.25±0.017	0.18±0.014	0.22±0.015	0.25±0.017
		C24:0 Lignoceric acid	0.09±0.006	0.08±0.005	0.19±0.009	-
Unsaturated fatty acids	Mono-unsaturated fatty acids (MUFAs)	Palmitoleic acid C16:1 n-7	2.08±0.14	2.83±0.19	2.18±0.15	2.84±0.19
		C18:1 trans-9 Elaidic acid	0.08±0.005	0.13±0.009	0.16±0.011	0.11±0.007
		C18:1 cis-9 Oleic acid	**43.31±0.20**	**42.82±0.52^{ans}**	**43.89±0.50^{ns}**	**44.27±0.58^{a}**
	Polyunsaturated fatty acids (PUFAs)	C18:2 trans-9,12 Linolelaidic acid	0.15±0.007	0.11±0.006	0.16±0.008	0.16±0.008
		C18:2 cis-9,12 Linoleic acid	**9.61±0.18**	**7.42±0.17^{b}**	**10.12±0.2^{b}**	**5.28±0.15^{b}**
		C18:3 n-3 α-Linolenic acid	0.81±0.048	0.08±0.048	0.50±0.031	0.56±0.033

*Data are represented as Mean±SD; a,b - indicates statistically significant difference in the same rose between the samples with *L. rhamnosus, L. plantarum* compared with Bacto Flavor at $p < 0.05$ according to one-way ANOVA and Tukey's post-hoc test; ns, indicates not significant correlation at $p \geq 0.05$ in the same rose between the samples with *L. rhamnosus and L. plantarum* compared with Bacto Flavor;

The presence of the RiPP-like cluster (Contig 58), which coincides with the bacteriocin findings, further highlights the strain's biopreservative potential. The co-location of these structural genes with a dedicated ABC transporter (ctg44_90) and MFS transporter (ctg44_105) ensures the efficient processing and secretion of these peptides. This genetic framework is consistent with the strain's demonstrated ability to maintain safety and suggests a molecular mechanism for its anti-Listerial activity.

A potential confounding factor in this study is the variation in maturation duration (5 to 8 days). To isolate the effect of the starter culture from the effect of storage time, it is noteworthy that L. rhamnosus 20-12 reached the highest oleic acid concentration (44.27%) by Day 5, significantly surpassing the Traditional control (43.31%) which required 8 days to reach its peak. This inverse relationship where a shorter duration yielded a more favorable lipid profile—indicates that the observed changes are driven primarily by the metabolic activity of the inoculated LAB and the accelerated enzymatic restructuring of the meat matrix, rather than by passive endogenous changes over time

Beyond safety, the secondary metabolite clusters (T3PKS and Terpenes) likely play a "technological" role in sensory optimization. The terpene-precursor clusters on contigs 21 and 48, regulated by the Rgg-family transcriptional regulator, suggest that the strain can modulate its metabolic output based on population density (quorum sensing). These metabolites, combined with the extensive proteolytic complex (>21 proteases) contribute to the release of flavor precursors and aromatic substances. Based on established literature (**Grigoryan *et al.*, 2025b**), these metabolic pathways are hypothesized to enhance the sensory profile of the matured pork products, although direct sensory evaluation was not within the scope of this study.

The study on the effect of different starter cultures on the fatty acid composition (%) of matured pork meat, compared with traditional salting is presented in **Table 1.**

As can be seen from the given results, oleic, palmitic, stearic and linoleic acids were the predominant fatty acids in matured pork samples among all salting conditions. It should be emphasized that there are not significant differences in the concentration of palmitic and stearic acids in matured pork samples with the application of both LAB strains compared with the BactoFlavor® (Hansen) culture used in industry, while significant correlations ($p < 0.05$) were observed in the concentration of oleic and linoleic acids between the samples with *L. rhamnosus* 20-12 and BactoFlavor. Although significant differences were recorded, the opposite trend was observed- if the concentration of oleic acid is higher than that in the L. *rhamnosus*- treated sample, the opposite trend was detected for linoleic acid. While linoleic acid concentrations fluctuated across all ripened samples (Table 1), the *L. plantarum 66* strain maintained the highest levels of this polyunsaturated fatty acid by the end of the maturation period.

Table 2 displays that the concentrations of the predominant fatty acids increased progressively in parallel with maturation, although the extent of accumulation varied depending on the starter culture used and the duration of the maturation process Specifically, pork is considered maturated when pH is reached approximately 5.0. With this regard, in our study the meat samples treated by traditional salting method reached maturation, therefore, the target pH on the 8th day, samples with the BactoFlavor® (Hansen) culture on the 7th day, for *L. plantarum_* 66 - 6th and for *L. rhamnosus* 20-12. on the day 5th day.

It should be noted that oleic acid (C18:1) was the major fatty acid in all types of treatments and exhibited the most significant increase during the maturation period ($p < 0.05$). With regard to the traditionally salted samples, their concentration increased from 22.44% at the beginning of maturation up to 43.31% by day 8. Similar trends were observed in samples treated with starter cultures, the highest oleic acid content was achieved by the *L. rhamnosus* 20-12 group, which reached its peak maturation significantly faster than all other treatment groups. As shown in Table 2, this strain optimized the monounsaturated fatty acid profile within five days, outperforming both the industrial standard and the traditional control in both speed and final concentration. These findings suggest that starter cultures may enhance lipolytic activity and contribute to the release or preservation of monounsaturated fatty acids during maturation.

Table 2 displays the effect of different starter cultures on trend of fatty acid profile in pork meat during maturation process, compared with salt-curd in traditionally and commercially.

Table 2 Impact of different starter cultures on trend of the predominant fatty acids of pork meat during maturation (%)

Type of salting	Maturation phase*	Fatty acids M±SD**			
		C16:0 (Palmitic acid)	C18:0 (Stearic acid)	C18:1 (Oleic acid)	C18:2 cis-9,12 (Linoleic acid)
Traditional salting	start	17.21± 0.25^{a}	10.26±0.18^{a}	22.44±0.18^{a}	5.28±0.12^{a}
	4th day	21.58±0.30^{a}	11.05±0.2^{2a}	31.38±0.22^{a}	7.50±0.1^{5a}
	Matured 8th day	23.44±0.28^{a}	12.13±0.20a	43.31±0.20^{a}	9.61±0.18^{a}
BactoFlavor	start	17.88±0.24^{b,ns}	10.98±0.19$^{b,\ ns}$	20.75±0.32^{b}	4.10±0.11$^{b,\ ns}$
	4th day	23.51±0.31^{ns}	11.80±0.21^{ns}	30.25±0.38^{b}	5.90±0.14^{ns}
	Matured 7th day	24.49±0.33^{b}	13.02±0.23^{b}	42.82±0.52^{b}	7.42±0.17^{b}
***L. plantarum* 66**	start	18.00±0.26^{c}	10.56±0.18^{ns,c}	23.89±0.36^{c}	5.80±0.13^{c}
	4th day	20.52±0.29^{c}	10.98±0.20^{ns}	33.14±0.42^{c}	8.10±0.16^{c}
	Matured 6th day	22.44±0.30^{c}	12.03±0.22^{c}	43.89±0.56^{c}	10.12±0.20^{c}
***L.rhamnosus* 20-12**	start	17.85±0.25^{d}	10.44±0.18^{d}	27.15±0.38^{d}	3.00±0.10^{d}
	4th day	22.19±0.30^{d}	12.00±0.21^{d}	38.55±0.45^{d}	4.20±0.12^{d}
	Matured 5th day	24.56±0.34^{d}	12.75±0.23^{d}	44.27±0.58^{d}	5.28±0.1^{5d}

* Sampling was performed at the functional endpoint of maturation for each group, defined by a target pH of 5.0. The superior lipid profile of the *L. rhamnosus* 20-12 group at Day 5 compared to the Traditional group at Day 8 underscores the culture-specific enhancement of the maturation process**. Data are represented as Mean±SD; Different letters -a,b,c.d indicate statistically significant differences within the column for treatment groups in parallel with maturation at $p < 0.05$; ns, indicates not significant differences at p≥0.05 within the column for each salting type in parallel with maturation.

Overall, the obtained data indicate that maturation period and the applied starter cultures significantly influence on the fatty acid composition. The tendency of fatty acids content increasing was observed in parallel with meat maturation phase, which is more prone to oleic acid with the confidence interval ranging 0.98-0.99. Based on the literature review, sensory paratability of meat positively correlated with oleic acid proportion and this fatty acid increases the sensory quality, texture, and nutritional value in fermented meats (**Hwang, Seon-Tea Jao, 2017; Obana *et al.*, 2026; Chan *et al.*, 1994**). These findings suggest a potential for enhanced quality in the final product. Furthermore, the dominance of unsaturated oleic acid over saturated fatty acids suggests the development of a relatively favorable lipid profile during maturation.

Lipid metabolism in *L. rhamnosus* 20-12

The functional classification of the *L. rhamnosus* 20-12 annotated proteome based on KEGG GhostKOALA annotation is presented in **Supplementary Figure S1**.

These data show that 41 annotated enzymes involved in lipid metabolism represent 2.32% of the annotated proteins and 1.15% of the total proteome sequences. Annotated enzymes of *L. rhamnosus 20-12* relevant to fatty acid biosynthesis are listed in **Table 3**.

In silico analysis of the *L. rhamnosus* proteome revealed a robust genetic investment in lipid metabolism. As detailed in **Table 3**, a complete repertoire of enzymes assigned to KEGG modules M00082 (Fatty acid biosynthesis, initiation) and M00083 (Fatty acid biosynthesis, elongation) were identified. Specifically, the genome encodes the full acetyl-CoA carboxylase complex and the essential enzymes for the elongation cycle, including multiple paralogs of 3-oxoacyl-[acyl-carrier protein] reductase (K00059). These findings indicate that *L. rhamnosus* 20-12 possesses the metabolic machinery required for de novo fatty acid synthesis, a key factor in maintaining cellular membrane integrity under varying environmental conditions.

Table 3 Characterization of annotated coding sequences (CDSs) of *L. rhamnosus* involved in fatty acid biosynthesis

Fatty acid biosynthesis modules	Prokka annotated CDS	Function and nomenclature	KO*	Reference
M00082, initiation	FFIONCCO_03598	[acyl-carrier-protein] S-malonyltransferase [EC:2.3.1.39]	K00645	(Zhang *et al.*, 2003)
	FFIONCCO_03601	3-oxoacyl-[acyl-carrier-protein] synthase III [EC:2.3.1.180]	K00648	(Tsay *et al.*, 1992)
	FFIONCCO_03592	acetyl-CoA carboxylase, biotin carboxylase subunit [EC:6.4.1.2 6.3.4.14]	K01961	(Sun *et al.*, 1997)
	FFIONCCO_03590	acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2 2.1.3.15]	K01962	(Bilder *et al.*, 2006)
	FFIONCCO_03591	acetyl-CoA carboxylase carboxyl transferase subunit beta [EC:6.4.1.2 2.1.3.15]	K01963	(Bilder *et al.*, 2006)
	FFIONCCO_03594	acetyl-CoA carboxylase biotin carboxyl carrier protein	K02160	
M00083, elongation	FFIONCCO_00131, FFIONCCO_01505, FFIONCCO_03164, FFIONCCO_03532, FFIONCCO_03597	3-oxoacyl-[acyl-carrier protein] reductase [EC:1.1.1.100]	K00059	(Rawlings and Cronan, 1992)
	FFIONCCO_03599	enoyl-[acyl-carrier protein] reductase II [EC:1.3.1.9]	K02371	(Marrakchi *et al.*, 2003)
	FFIONCCO_03593, FFIONCCO_03603	3-hydroxyacyl-[acyl-carrier-protein] dehydratase [EC:4.2.1.59]	K02372	(Heath and Rock, 1996)
	FFIONCCO_03595, FFIONCCO_03596	3-oxoacyl-[acyl-carrier-protein] synthase II [EC:2.3.1.179]	K09458	(Zhang *et al.*, 2005)

* KEGG Orthology (KO) assignment by GhostKOALA.

Antioxidant defense in *L. rhamnosus* 20-12

Oxidative stress-relevant enzymes in *L. rhamnosus 20-12* were manually identified from the annotated genome (PROKKA_05292026.gbk, available via Mendeley Data). The search focused on peroxidases, superoxide dismutases (SOD), reductases (specifically thioredoxin and glutathione reductases), glutathione S-transferases, and NADH oxidases. The identified enzymes are summarized in **Table 4**.

Table 4 Enzymes involved in the oxidative stress response of *L. rhamnosus* 20-12

Protein	Contig	CDS, locus_tag	BLAST homology hit	BLAST homology, %	Accession
NADH peroxidase	1	Complement (197779..198324), FFIONCCO_00218	FAD-dependent oxidoreductase, partial [*Lacticaseibacillus rhamnosus*]	99.45	WP_064558702.1
NADH peroxidase	1	Complement (198317..199150), FFIONCCO_00219	NAD(P)/FAD-dependent oxidoreductase, partial *[Lacticaseibacillus rhamnosus]*	97.78	WP_191982129.1
Thiol peroxidase	26	85176..85673, FFIONCCO_01304	thiol peroxidase, partial *[Lacticaseibacillus rhamnosus]*	100.00	WP_064558239.1
Dye-decolorizing peroxidase	7	85372..86313, FFIONCCO_02824	Dyp-type peroxidase *[Lacticaseibacillus rhamnosus]*	99.04	WP_049174945.1
Glutathione peroxidase BsaA	8	197054..197527, FFIONCCO_03137	glutathione peroxidase *[Lacticaseibacillus rhamnosus]*	99.36	WP_270768526.1
Thioredoxin reductase	8	253632..254579, FFIONCCO_03209	Thioredoxin-disulfide reductase *[Lacticaseibacillus paracasei]*	95.56	WP_262865268.1
Glutathione amide reductase	55	Complement (6941..7615), FFIONCCO_02522	dihydrolipoyl dehydrogenase family protein *[Lacticaseibacillus rhamnosus]*	98.21	WP_188434244.1
NADH oxidase	1	16245..16460, FFIONCCO_00023	NADH oxidase, partial *[Lacticaseibacillus rhamnosus MTCC 5462]*	98.53	EGF48653.1
NADH oxidase	1	16794..17021, FFIONCCO_00024	FAD-dependent oxidoreductase *[Lacticaseibacillus rhamnosus]*	97.22	WP_032964123.1
NADH oxidase	1	Complement (23190..24551), FFIONCCO_00034	FAD-dependent oxidoreductase *[Lacticaseibacillus rhamnosus]*	99.56	WP_032964123.1
NADH oxidase	8	59821..60774, FFIONCCO_02965	NADH oxidase, partial *[Lacticaseibacillus rhamnosus]*	88.61	KMO59803.1
NADH oxidase	8	60737..61132, FFIONCCO_02966	NAD(P)/FAD-dependent oxidoreductase *[Lacticaseibacillus rhamnosus]*	96.95	WP_049177766.1
NADH oxidase	9	Complement (134240..135565), FFIONCCO_03621	FAD-dependent oxidoreductase *[Lacticaseibacillus rhamnosus]*	99.55	WP_176817420.1

Genomic analysis of *L. rhamnosus 20-12* (**Table 4)** revealed a comprehensive repertoire of enzymes dedicated to antioxidant defense, suggesting a high capacity for adaptation to oxidative stress. The presence of multiple peroxidases – including NADH peroxidase, thiol peroxidase (100% homology), and glutathione peroxidase (BsaA) – indicates a redundant and robust mechanism for the detoxification of reactive oxygen species (ROS). Furthermore, the identification of six distinct NADH oxidases suggests that this strain is highly efficient at scavenging molecular oxygen and regenerating NAD+, a trait that likely supports its survival and metabolic stability in both the gastrointestinal tract and food fermentation environments. Notably, while peroxidases and oxidases were abundant, a manganese-dependent superoxide dismutase (SOD) was not identified in this strain. The absence of SOD is common among *Lactobacillus* species, as they often utilize high concentrations of intracellular manganese (Mn^{2+}) to chemically neutralize superoxide radicals, thereby bypassing the need for the SOD enzyme (**Bruno-Bárcena *et al.*, 2004**).

Bacteriocin Clusters in *L. rhamnosus*

It was shown that *L. rhamnosus 20-12* strain produced two bacteriocins - BCN1 and BCN 2. Determination of molecular weight demonstrates that molecular weight (Mw) of BCN 1 and BCN 2 are 1.427 Da, 602.6 Da respectively (**Tkhruni *et al.*, 2015**). Mass reconstruction of the bacteriocins BCN 1 and BCN 2 indicate that they have peptide bonds (**Tkhruni *et al.*, 2020).** Obtained bacteriocins possessed antimicrobial activity against Gram-negative and Gram-positive pathogens, such as Salmonella sp., E. coli, Proteus mirabilis, Pasteurella spp., Clostridium spp, Streptococcus spp., Staphylococcus aureus, Shigella spp. E. cloacae as well as antibiotic resistant pathogens **(Tkhruni *et al.*, 2023).**

The *L. rhamnosus* 20-12 bacteriocin gene cluster was identified using BAGEL4 from the assembly file "assembly_12_13_Lambda.fasta," generated with Flye (available in the Mendeley Data repository). A general overview of the area of interest (AOI) for this unique bacteriocin cluster is shown in Figure 1, and an expanded, annotated version of the gene cluster is presented in Supplementary Table S1. The identifier "contig_58.14.AOI_01" indicates the cluster is located on segment 14 of contig 58. The genomic map reveals a complex multi-peptide cluster, characteristic of Class II bacteriocin systems in which multiple structural genes are co-localized with specialized transport and regulatory elements.

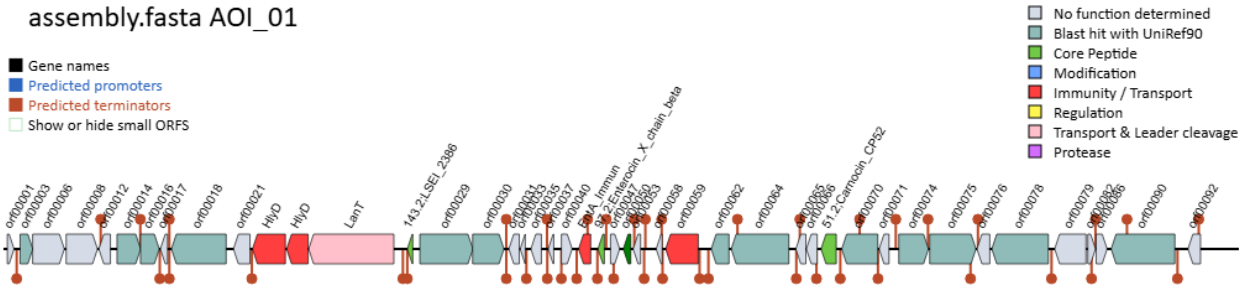

Figure 1 Architecture of the single identified Class II bacteriocin gene cluster in *Lactobacillus rhamnosus* (AOI: contig_58.14.AOI_01). The bacteriocin structural genes are presented as light and dark green arrows.

BAGEL4 identified four distinct structural genes (green arrows in **Fig. 1**) likely encoding antimicrobial peptides. BLAST hits for three of these bacteriocins against the KEGG GENES database (a non-redundant subset of KEGG gene/protein sequences) are shown in Supplementary Figure S2 (available in the Mendeley Data repository). Amino acid sequences of all BAGEL4 identified bacteriocins are presented in Fig. S2A (Enterocin_X_chain_beta) shows features consistent with a Class IIb (two-peptide) or Class IIc bacteriocin. The GGI motif indicates a Gly–Gly cleavage site, a hallmark of non-lantibiotic bacteriocin secretion **(Hu *et al.*, 2010)**. In Fig. S2B (LSEI_2386), the double-glycine motif (visible as "FVGG" in the alignment) suggests a leader peptide that is cleaved during export (**Kuo *et al.*, 2013**). **Fig. S2C** (Carnocin_CP52) is notable because carnocins are reported to have anti-Listeria activity, which is relevant to the sensory optimization and safety of matured meat products discussed in our article.

The third peptide, orf00050 (Fig. 1 and Table S1), was identified by the GG motif as Bacteriocin_IIc, confirming the presence of a Class II bacteriocin precursor; this peptide is poorly characterized.

The BLAST hits of all BAGEL4-identified bacteriocin sequences against the NCBI database are presented in **Table 5**. These sequences were also found on the complementary strand of contig 58 in the same order as in AOI: contig_58.14.AOI_01 among Prokka-annotated CDS. Notably, the LSEI_2386 and Enterocin-X sequences are identical between the Prokka and BAGEL4 annotations. The orf00050 Prokka variant contains an additional asparagine at the C-terminus. The Carnocin_CP52 Prokka variant is extended at the C-terminus by a GGGH motif, whereas the Carnocin_CP52 BAGEL4 variant is extended at the N-terminus by the sequence MVAGSIVLGGTV.

Table 5 BLAST hits of identified *L. rhamnosus* 20-12 bacteriocines against the NCBI database

Polypeptide: Prokka, BAGEL4	Contig	CDS, locus_tag	BLAST homology hit	BLAST homology, %	Accession
Hypothetical protein, LSEI_2386	58	Complement (79046..79180), FFIONCCO_02675	Bacteriocin-like peptide, LSEI_2386 family *[Lacticaseibacillus rhamnosus]*	97.73	WP_005716471.1
Hypothetical protein, Enterocin-X	58	Complement (83763..83921), FFIONCCO_02681	Class IIb bacteriocin, lactobin A/cerein 7B family *[Lacticaseibacillus rhamnosus]*	100.00	WP_005692236.1
Hypothetical protein, orf00050	58	Complement (84390..84575), FFIONCCO_02682	bacteriocin *[Lacticaseibacillus rhamnosus]*	100.00	WP_005686851.1
Putative carnobacteriocin-B2 immunity protein, Carnocin_CP52	58	Complement (89284..89625), FFIONCCO_02689	bacteriocin immunity family protein *[Lacticaseibacillus rhamnosus]*	97.35	WP_270767342.1

Secondary metabolite clusters in *L. rhamnosus* 20-12

Secondary metabolite biosynthetic gene clusters (BGCs) in the L. rhamnosus 20-12 genome were identified from the assembly file assembly_12_13_Lambda.fasta (generated using Flye and available in the Mendeley Data repository) with the antibiotics & Secondary Metabolite Analysis Shell (antiSMASH 8.0) pipeline, which integrates the MiBIG (Minimum Information about a Biosynthetic Gene Cluster) database for comparative analysis of experimentally characterized BGCs (**Blin *et al.*, 2025**). AntiSMASH analysis identified four putative secondary metabolite biosynthetic clusters: a Type III polyketide synthase (T3PKS) cluster and a terpene-precursor cluster on contig 21, an additional terpene-precursor cluster on contig 48, and a ribosomally synthesized and post-translationally modified peptide-like (RiPP-like) cluster on contig 58. These clusters and the corresponding gene annotation color scheme are presented in Supplementary Figure S4.

The genomic analysis of *L. rhamnosus* 20-12 revealed four distinct biosynthetic gene clusters (BGCs) that may contribute to the strain's ecological fitness, antimicrobial potential, stress tolerance, and technological performance during meat fermentation. The Type III polyketide synthase (T3PKS) cluster located on contig 21 contains key enzymes involved in isoprenoid- and polyketide-related metabolism, including HMG-CoA synthase (ctg9_14) and HMG-CoA reductase (ctg9_13). Type III PKS pathways in bacteria are known to produce structurally diverse secondary metabolites, including phenolic compounds and specialized lipids with antioxidant, antimicrobial, and stress-protective properties (Lim *et al.*, 2016; Liu & Li, 2022). Such metabolites may contribute to reducing lipid oxidation during pork fermentation, thereby improving oxidative stability and product quality. The presence of pyruvate oxidase (ctg9_16) further suggests the ability to generate acetate and low levels of hydrogen peroxide under aerobic conditions, potentially enhancing microbial competitiveness and oxidative stress adaptation (**Bruno-Bárcena *et al.*, 2004**).

Two terpene-precursor biosynthetic clusters identified on contigs 21 and 48 encode polyprenyl synthetase family proteins (ctg9_196 and ctg33_4), indicating the capacity for terpene precursor biosynthesis. In bacteria, terpene biosynthetic pathways are commonly associated with the production of carotenoids and other

isoprenoid-derived molecules that stabilize cellular membranes and protect against oxidative and environmental stresses (**Yamada *et al.*, 2015; Kakumu *et al.*, 2025**). These functions may suggest the robustness of L. rhamnosus 20-12 during meat fermentation and contribute indirectly to oxidative stability. Furthermore, the identification of an Rgg-family transcriptional regulator (ctg33_10) could explain that expression of this cluster may be coordinated through quorum sensing, enabling adaptive regulation in response to cell population density during fermentation.

The RiPP-like biosynthetic cluster detected on contig 58 may represent an additional source of antimicrobial peptide production. RiPP biosynthetic pathways are widespread among Gram-positive bacteria and frequently encode bacteriocin-like molecules that enhance competitive fitness and inhibit foodborne spoilage or pathogenic microorganisms (van Heel *et al.*, 2018). This finding is consistent with the previously reported antimicrobial activity of *L. rhamnosus* 20-12 and supports its potential application as a protective starter culture in fermented meat products. NCBI BLASTP annotations for the biosynthetic, biosynthetic-additional, and transport-related genes identified within the four secondary metabolite biosynthetic clusters are summarized in **Table 6.**

Table 6 BlastP hits of biosynthetic, biosynthetic-additional, and transport genes for clusters identified by antiSMASH.

Cluster	Identifier	Length, AA	Function	NCBI Blast hit	BLAST hom	Protein
Contig_21, T3PKS	ctg9_12	393	biosynthetic-additional	acetyl-CoA C-acyltransferase [Lacticaseibacillus rhamnosus]	98.73	WP_061713463.1
	ctg9_13	414	biosynthetic-additional	hydroxymethylglutaryl-CoA reductase, degradative [Lacticaseibacillus rhamnosus]	97.34	WP_005685404.1
	ctg9_14	390	biosynthetic	hydroxymethylglutaryl-CoA synthase [Lacticaseibacillus rhamnosus]	97.69	WP_213950236.1
	ctg9_16	529	biosynthetic-additional	pyruvate oxidase [Lacticaseibacillus paracasei]	93.65	WP_195340802.1
Contig_21, terpene-precursor	ctg9_189	355	biosynthetic-additional	MULTISPECIES: M24 family metallopeptidase [Lacticaseibacillus]	91.83	WP_087912259.1
	ctg9_196	283	biosynthetic	polyprenyl synthetase family protein [*Lacticaseibacillus rhamnosus]*	98.59	WP_032963685.1
	ctg9_206	198	biosynthetic-additional	bifunctional phosphopantothenoylcysteine decarboxylase/phosphopantothenate--cysteine ligase CoaBC [Lacticaseibacillus rhamnosus]	99.49	MFQ9107517.1
contig_48, terpene-precursor	ctg33_4	265	biosynthetic	polyprenyl synthetase family protein [*Lacticaseibacillus rhamnosus]*	97.82	WP_005686160.1
	ctg33_7	172	biosynthetic-additional	metallophosphoesterase family protein *[Lacticaseibacillus rhamnosus]*	88.95	WP_308051521.1
	ctg33_10	294	biosynthetic-additional	Rgg family transcriptional regulator *[Lacticaseibacillus rhamnosus]*	100.00	WP_014571554.1
contig_58, RiPP-like	ctg44_84	206	biosynthetic-additional	aldo/keto reductase *[Lacticaseibacillus rhamnosus]*	89.42	MSC09759.1
	ctg44_85	154	biosynthetic-additional	aldo/keto reductase, partial *[Lacticaseibacillus rhamnosus]*	97.40	KMO63942.1
	ctg44_90	730	biosynthetic	peptide cleavage/export ABC transporter *[Lacticaseibacillus rhamnosus]*	99.45	WP_064520302.1
	ctg44_100	61	biosynthetic	bacteriocin *[Lacticaseibacillus rhamnosus]*	100,00	WP_005686851.1
	ctg44_103	268	biosynthetic-additional	CPBP family intramembrane glutamic endopeptidase [Lacticaseibacillus rhamnosus]	100.00	WP_005692230.1
	ctg44_105	475	transport	MFS transporter [Lacticaseibacillus rhamnosus]	99.79	WP_277755192.1

The RiPP-like cluster (Contig 58) represents specialized antimicrobial machinery. It includes a bacteriocin precursor (ctg44_100), a peptide cleavage/export ABC transporter (ctg44_90), and an MFS transporter (ctg44_105). This serves as a classic "biosynthetic factory" for a ribosomally synthesized and post-translationally modified peptide (RiPP). The combination of a dedicated ABC transporter and a CPBP-family intramembrane glutamic endopeptidase (ctg44_103) confirms that the strain is equipped to process and secrete active antimicrobial peptides. This cluster coincides with the BAGEL4-derived bacteriocin cluster and is likely a primary driver behind the strain's ability to inhibit pathogens, such as *Listeria*, in fermented pork products.

To provide a transparent overview of the multidisciplinary logic of this study, **Table 7** summarizes the associations between our experimentally validated pork phenotypes and the underlying genomic potential of *L. rhamnosus 20-12*. By separating these into distinct categories, we distinguish between 'ground-truth' biochemical observations and 'genome-based hypotheses' (predicted mechanisms). This framework illustrates how the identified biosynthetic gene clusters (BGCs) and metabolic modules provide a molecular foundation that is consistent with the observed acceleration in maturation, enrichment of the fatty acid profile, and enhanced oxidative and microbiological stability of the final meat product.

Table 7 Conceptual summary mapping experimentally observed pork phenotypes to genomic potential.

Observed Pork Phenotype **(Experimentally Validated)**	Genome-Based Hypothesis **(Genomic Potential)**	**Key Genetic Evidence (WGS Analysis)**
Accelerated Maturation: Target pH (~5.0) reached in 5 days vs. 8 days.	Accelerated protein and lipid restructuring via bacterial metabolic activity.	>21 distinct proteases, peptidases, and full acetyl-CoA carboxylase complex.
FA Profile Optimization: Oleic acid (C18:1) peak at 44.27% (Day 5).	*De novo* fatty acid biosynthesis and lipid metabolism support.	KEGG modules M00082 (initiation) and M00083 (elongation).
Oxidative Stability: Reduced lipid oxidation and radical scavenging (40.6%).	Redundant antioxidant defense systems for ROS detoxification.	Thiol peroxidases, multiple NADH oxidases, and T3PKS antioxidant clusters.
Enhanced Safety: Potent anti-Listerial activity and pathogen suppression.	Biopreservation through production of bacteriocins and RiPPs.	Class II multi-peptide bacteriocin cluster (Enterocin X, Carnocin_CP52).

CONCLUSION

This study demonstrates that the selection of specific starter cultures is a decisive factor in controlling lipid oxidation, fatty acid profiles, and the overall safety of pork meat during maturation. Our findings highlight *Lacticaseibacillus rhamnosus* 20-12 as an exceptional candidate for meat biopreservation and technological optimization.

Genomic integration through whole-genome sequencing (WGS) provided a clear molecular basis for the observed experimental benefits. The accumulation of beneficial oleic acid (C18:1) and the maintenance of oxidative stability are supported by a robust genetic investment in lipid metabolism (KEGG modules M00082 and M00083) and a redundant antioxidant defense system comprising multiple peroxidases and NADH oxidases. Furthermore, the identification of a complex Class II multi-peptide bacteriocin cluster and a RiPP-like biosynthetic assembly could explain the strain's potent antimicrobial activity, particularly its potential to inhibit pathogens such as *Listeria monocytogenes*.

Notably, the use of *L. rhamnosus* 20-12 allowed for a significant reduction in the maturation period – from 8 days to 5 days – without compromising the sensory or chemical quality of the product. This multidisciplinary approach, combining fatty acid profiling with advanced biosynthetic gene cluster (BGC) analysis, provides a new framework for selecting high-performance starter cultures. Future research should focus on the expression levels of these identified clusters under diverse industrial conditions to further exploit the potential of *L. rhamnosus* 20-12 in producing "clean-label," safe, and nutritionally enriched fermented pork products. Although direct sensory analysis was not conducted, the genomic presence of terpene-precursor clusters and the significant accumulation of MUFAs provide a strong basis for the hypothesis that *L. rhamnosus 20-12* improves the aromatic profile of pork. Future studies employing Electronic Nose (E-nose) technology and trained sensory panels are required to validate this potential.

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