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Enhancing the microbial quality of fermented sausage using *Lactobacillus plantarum* cell free supernatant (CFS)

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ABSTRACT

Fermented sausages are vulnerable to microbial contamination, which poses significant risks to meat safety if beneficial bacteria from starter cultures fail to inhibit pathogens during production and storage. So, the use of cell-free supernatant (CFS) from *Lactobacillus plantarum* (*L. plantarum*) offers an effective strategy to enhance microbial safety. Therefore, the current study was conducted to assess the impact of *L. plantarum* CFS (1.5 and 3.0% v/w) on the chemical stability, i.e., pH values, and the microbiological quality of fermented sausage during refrigeration. Results revealed a rapid raising of pH values of the control group, where it reached 6.16 on the 27th day of refrigeration, whereas it was 5.70 and 5.56 in the treated groups with 1.5% and 3.0% CFS, respectively. A rapid initial reduction in the bacterial counts, represented by the aerobic plate count, coliform, staphylococcus, and psychrotrophic bacteria, followed by a gradual increase was recorded; however, it was still lower than the control group. On the same line, treated groups showed longer shelf life (up to 48 days in the refrigerator) in relation to the control group. It is worth noting that the antimicrobial effect of *L. plantarum* CFS was directly correlated to the concentration used, where higher concentrations used had higher antimicrobial potency. Based on the results, *L. plantarum* CFS showed potential to be used as a meat additive, especially in fermented sausage

1. INTRODUCTION

Meat is a fundamental component of the human diet, providing a rich source of high-quality protein, essential amino acids, vitamins, and minerals that play a crucial role in maintaining body functions, supporting growth, and enhancing immune health. Globally, meat consumption reflects cultural preferences and economic factors, and it remains a vital food item for millions of people (Geiker et al., 2021). Fermented meat products, such as fermented sausages, play a significant role in nutrition and food technology while offering extended shelf life and enhanced safety. Moreover, fermented meat products contribute to food safety by reducing spoilage losses and enabling storage with limited reliance on synthetic preservatives (Tabanelli et al., 2022).One promising natural preservation approach involves the incorporation of metabolites produced by Lactobacillus species, a group of lactic acid bacteria known for their antimicrobial properties, including organic acids, hydrogen peroxide, and bacteriocins, which can inhibit the growth of spoilage and pathogenic microbes in meat products. The use of Lactobacillus metabolites aligns with the growing demand for clean-label food additives and offers a safer alternative to synthetic preservatives, contributing to improved shelf life and safety of meat products without compromising their nutritional value (Anumudu et al., 2024).Among the microbial-derived preservatives, bacteriocins produced by lactic acid bacteria have gained significant attention due to their potent antimicrobial activity and safety profile. *Lactobacillus plantarum* produces bacteriocins that are effective against foodborne pathogens and spoilage organisms by disrupting the microbial cell membranes, thereby inhibiting their growth and proliferation in meat products (Darbandi et al., 2022).The use of *L. plantarum* bacteriocins in meat preservation offers multiple advantages, i.e., they are natural, biodegradable, and generally recognized as safe, making them suitable for application in food systems. Additionally, their specificity reduces the risk of disturbing beneficial microbiota, and they can be integrated into various preservation techniques such as packaging films, marinades, or direct incorporation into meat formulations. This bio preservation approach not only enhances food safety but also aligns with sustainable food production practices (Bhattacharya et al., 2022). Although fermented sausages rely heavily on starter cultures for safe and effective preservation, contamination risks still exist. Starter cultures, primarily composed of lactic acid bacteria, are essential for lowering pH and inhibiting spoilage and pathogenic microbes; however, their performance can be compromised by factors such as residual antibiotics in the meat, which inhibit bacterial activity and disrupt fermentation. This disruption can allow harmful pathogens to survive and multiply, increasing the risk of foodborne infections. Additionally, if the initial microbial contamination is high or if the starter culture itself is contaminated or expired, the protective effect diminishes, potentially leading to fermentation failures and unsafe products (Holck et al., 2017 and Agüero et al., 2020).Therefore, the current study aimed to evaluate the efficacy of *L. plantarum* cell-free supernatant (CFS) direct

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addition to minced meat used in the preparation of fermented sausage on its sensory, bacteriological, and chemical attributes of shelf life during refrigeration.

2. MATERIAL AND METHODS

2.1. Preparation of L. plantarum CFS

Lactobacillus plantarum strain M2 (PP_788561), that was obtained from Food Hygiene Department, Animal Health Research Institute (AHRI), was propagated into de Man, Rogosa, and Sharpe (MRS) broth (TM media, TM 147) for 24h at 37oC, followed by centrifugation at 8000 RPM for 3 min, followed by filtration through a 0.22 µm filter (hydrophilic PTFE, United States). pH of the obtained CFS was adapted to 5.5-6.0 using 1M NaOH. Working solution (1.5 and 3.0%) was prepared from the obtained CFS using sterile DW as described by Topisirovic et al. (2006) and Boulares et al. (2012).

2.2. Production of fermented semi-dry beef sausage (Lücke, 2003)

Fresh lean beef meat and fat in a ratio of about 70:30 was collected from local butcher in Benha city, Qalyubia governorate, Egypt. The meat and fat were finely ground, then mixed thoroughly with 2–3% salt, 1–2% sugar (to support fermentation), and a blend of spices such as black pepper, garlic powder, and red paprika. A starter culture containing 6 log10 CFU/g *L. plantarum* was added to initiate fermentation. After which, mixture was divided into three portion; where 1st portion was kept plain as control untreated group (G1), while 1.5 and 3.0% v/w of *L. plantarum* CFS was added to the mixture represents 2nd (G2) and 3rd (G3) group, respectively.

The mixture is stuffed into natural casing, followed by fermentation process at 28±2°C with 90–95% relative humidity for 48 hours until the pH falls below 5.3, followed by drying at 12–18°C and 75–80% humidity until 20–30% weight loss is achieved.

Sausage samples were kept in refrigerator during the conducted microbiological examinations every three days for the 1st week of storage, and then every 7 days until appearance of gross signs of spoilage.

2.3. Microbiological examinations

After preparation of tenth-fold serial dilutions according to ISO 6887-1 (2017) using 0.9% normal saline, sausage samples were subjected to the following microbial examinations:

Aerobic plate count (APC, OXOID CM0325B) using plate count agar and incubation at 30 ℃ for 72h according to ISO 4833 (2013).

Coliform bacteria using violet red bile agar (OXOID CM0107B) and incubation at 37 ℃ for 24h according to ISO 4832 (2006).

Staphylococcus bacteria using Baird Parker agar (OXOID CM0275B) according to ISO 6888-1 (2021).

Psychrotrophic bacteria using plate count agar (OXOID CM0325B) and incubation at 5±1 ℃ for 5-7 days according to ISO 17410 (2019).

Total fungal count using dichloran rose-bengal chloramphenicol agar (DRBC, OXOID CM0727B) and incubation at 25 ℃ for 5-7 days according to ISO 21527-2 (2008), and sulfite reducing bacteria according to ISO 15213-1 (2023) using tryptose sulfite agar and incubated anaerobically at 37 ºC for 24h.

Initial counts were recorded at zero day of storage, and were repeated every three days for the first week of storage and then every 7 days until signs of spoilage appear.

Examination were repeated three times at the same preparation and storage conditions for confirming the effect of the treatment.

N.B. it is worth noted that pH was monitored along the experimental period using calibrated pH meter (Adwa, AD1200) dipped in 50g of well-mixed beef sample.

2.4. Statistical analysis

The obtained data was statistically treated by one-way ANOVA using SPSS software for Windows (Version 16). Duncan's post hoc analysis was used to compare the final results, with a p-value of 0.05 being regarded statistically significant. On the other hand, independent T-test was used to compare means between two groups. The values represent Mean ± SD of three experiments, means within the same row (ABCD) followed by different superscript letters are significantly different ($P \leq 0.05$), Means within the same column (abcd) followed by different superscript letters are significantly different ($P \leq 0.05$), *- Superscript star between two groups within the same row are significantly different ($P \leq 0.05$).

3. RESULTS

The examined samples were studied using pH as a chemical stability indicator (Table, 1). Samples revealed significant variation between the control and treated groups, where gradual increase in the pH values was recorded. pH reached 6.16, 5.72 and 5.56 within 27th day of refrigerated storage for control, and treated groups with 1.5% and 3.0% CFS, respectively. Moreover, treated groups kept their gross acceptability up to 48 days of storage indicating longer shelf life .

Table (1): Average values of pH in the minced beef groups during refrigerated storage (4±1°C)

Groups	Control	1.5% conc.	3.0% conc.
Zero day	5.12 ± 0.10^{Ad}	5.1 ± 0.10^{Af}	5.1 ± 0.10^{Af}
3rd day	4.97 ± 0.21^{Ae}	4.92 ± 0.11^{Bg}	4.91 ± 0.2^{Bg}
6th day	5.00 ± 0.17^{Ad}	4.97 ± 0.14^{Bg}	4.95 ± 0.2^{Bg}
13th day	5.36 ± 0.25^{Ac}	5.13 ± 0.2^{Bf}	5.03 ± 0.11^{Cf}
20th day	5.76 ± 0.25^{Ab}	5.24 ± 0.11^{Be}	5.13 ± 0.14^{Ce}
27th day	6.16 ± 0.25^{Aa}	5.31 ± 0.24^{Bd}	5.25 ± 0.21^{Cd}
34th day	S.	$5.43\pm0.19^{*c}$	$5.32\pm0.1^{*c}$
41st day	S.	$5.60\pm0.21^{*b}$	$5.44\pm0.12^{*b}$
48th day	S.	$5.72\pm0.12^{*a}$	$5.56\pm0.24^{*a}$

S.: Apparently spoiled

Regarding aerobic plate count (APC), as a hygiene indicator, Table (2) presented rapid reduction in the plate count in the first few days of treated groups storage, followed by gradual increase in the mean counts up to 48 days of storage; however, it still significantly lower than the control group.

Table (2): Average values of APC (log_{10} CFU/g) in fermented sausage groups at cold storage (4±1 ℃).

Groups	Control	1.5% conc.	3.0% conc.
Zero day	3.28 ± 0.1^{Af}	3.20 ± 0.1^{Af}	3.12 ± 0.1^{Be}
3rd day	3.36 ± 0.1^{Ae}	3.04 ± 0.1^{Bg}	3.00 ± 0.1^{Ce}
6th day	3.50 ± 0.1^{Ad}	3.10 ± 0.1^{Bfg}	3.03 ± 0.2^{Ce}
13th day	3.72 ± 0.1^{Ac}	3.18 ± 0.2^{Bf}	3.08 ± 0.1^{Ce}
20th day	3.98 ± 0.1^{Ab}	3.30 ± 0.1^{Be}	3.10 ± 0.1^{Ce}
27th day	4.22 ± 0.1^{Aa}	3.56 ± 0.1^{Bd}	3.18 ± 0.1^{Cd}
34th day	S.	$3.78\pm0.1^{*c}$	$3.39\pm0.1^{*c}$
41st day	S.	$3.88\pm0.1^{*b}$	$3.64\pm0.1^{*b}$
48th day	S.	$4.08\pm0.1^{*a}$	$3.86\pm0.1^{*a}$

S.: Apparently spoiled

Regarding coliform bacteria (Table, 3), as fecal contamination indicators, significant retardation in the bacterial growth and multiplication in relation to the control untreated group was recorded. Reduction in the bacterial

counts appeared since day zero of the experiment in the treated groups where it directly related to the used concentration of CFS.

Table (3): Average values of coliform count (log_{10} CFU/g) in fermented sausage groups at cold storage (4±1 °C).

Groups	Control	1.5% conc.	3.0% conc.
Zero day	1.26 ± 0.1^{Ae}	1.14 ± 0.1^{Be}	1.03 ± 0.1^{Ce}
3rd day	1.38 ± 0.1^{Ae}	1.04 ± 0.1^{Be}	1.05 ± 0.1^{Be}
6th day	1.54 ± 0.1^{Ad}	1.06 ± 0.1^{Be}	1.06 ± 0.1^{Be}
13th day	1.73 ± 0.1^{Ac}	1.16 ± 0.2^{Be}	1.08 ± 0.1^{Ce}
20th day	1.88 ± 0.1^{Ab}	1.36 ± 0.1^{Bd}	1.16 ± 0.2^{Ce}
27th day	2.10 ± 0.1^{Aa}	1.50 ± 0.1^{Bc}	1.22 ± 0.1^{Cd}
34th day	S.	$1.70\pm0.2^{*b}$	$1.34\pm0.1^{*c}$
41st day	S.	$1.80\pm0.1^{*b}$	$1.56\pm0.1^{*b}$
48th day	S.	$2.02\pm0.1^{*a}$	$1.72\pm0.1^{*a}$

S.: Apparently spoiled

Regarding staphylococcus count (Table, 4), results revealed that the staphylococcus mean count at zero time was 2.04 and 2.02 log CFU/g in control and 1.5% CFS treated group, respectively; whereas, in 3.0% CFS treated group, staphylococcus was not detected. Subsequently, gradual increase in the staphylococcal count was recorded; however, in the treated groups, it was significantly lower than control group that showed signs of spoilage after 27th day of refrigerated storage.

Table (4): Average values of staphylococcus count (log_{10} CFU/g) in fermented sausage groups at cold storage (4±1 °C).

Groups	Control	1.5% conc.	3.0% conc.
Zero day	2.04 ± 0.1^{Ae}	2.02 ± 0.1^{Ad}	<2
3rd day	2.06 ± 0.1^{Ae}	2.00 ± 0.1^{Ad}	<2
6th day	2.14 ± 0.1^{Ad}	2.08 ± 0.1^{Bd}	2.04 ± 0.1^{Bc}
13th day	2.22 ± 0.2^{Ac}	2.12 ± 0.1^{Bc}	2.02 ± 0.04^{Cc}
20th day	2.36 ± 0.1^{Ab}	2.18 ± 0.1^{Bc}	2.06 ± 0.1^{Cbc}
27th day	2.52 ± 0.1^{Aa}	2.26 ± 0.2^{Bc}	2.10 ± 0.1^{Cb}
34th day	S.	$2.38\pm0.1^{*b}$	$2.16\pm0.1^{*b}$
41st day	S.	$2.44\pm0.1^{*b}$	$2.24\pm0.1^{*a}$
48th day	S.	$2.50\pm0.1^{*a}$	$2.30\pm0.1^{*a}$

S.: Apparently spoiled

Table (5) showed that *L. plantarum* CFS had a significant anti-psychrotrophs multiplication appeared as significant reduction in the bacterial counts in the treated sausage groups in relation to the control untreated group.

Table (5): Average values of psychrotrophs count (log_{10} CFU/g) in fermented sausage groups at cold storage (4±1 °C).

Groups	Control	1.5% conc.	3.0% conc.
Zero day	1.48 ± 0.1^{Ad}	1.28 ± 0.1^{Be}	1.12 ± 0.1^{Ce}
3rd day	1.58 ± 0.1^{Ac}	1.16 ± 0.1^{Bf}	1.06 ± 0.1^{Ce}
6th day	1.66 ± 0.1^{Ab}	1.08 ± 0.1^{Bg}	1.04 ± 0.1^{Be}
13th day	1.84 ± 0.2^{Aa}	1.16 ± 0.1^{Bf}	1.08 ± 0.1^{Ce}
20th day	2.00 ± 0.1^{Aa}	1.28 ± 0.1^{Be}	1.22 ± 0.1^{Cd}
27th day	2.18 ± 0.2^{Aa}	1.58 ± 0.1^{Bd}	1.50 ± 0.2^{Bcd}
34th day	S.	$1.74\pm0.1^{*c}$	$1.60\pm0.1^{*c}$
41st day	S.	$1.92\pm0.3^{*b}$	$1.78\pm0.1^{*b}$
48th day	S.	$2.10\pm0.1^{*a}$	$1.92\pm0.2^{*a}$

S.: Apparently spoiled

Regarding the detection of fungal and sulfite-reducing bacterial population, they were not detected along the storage time.

4. DISCUSSION

Meat products play a vital role in human nutrition and global food security due to their dense content of high-quality protein, essential amino acids, vitamins, and key minerals (Stadnik, 2024). Beyond their nutritional importance, meat products, especially those that are fermented or processed with care, offer enhanced safety and extended shelf life by leveraging preservation techniques such as salting, curing, and microbial fermentation (Sawant et al., 2025) .

Fermented meat products, in particular, introduce beneficial microbes (probiotics), improve digestibility, and develop unique flavors and textures that enrich culinary traditions worldwide. The importance of meat products thus spans nutrition, culture, food technology, and public health, making them indispensable in diets and economies across the globe (Dhiman et al., 2025).

Cell-free supernatants derived from Lactobacillus strains, particularly L. plantarum, offer significant benefits for the microbiological safety and overall quality of meat products. These extracts contain metabolites such as organic acids and bacteriocins that exhibit potent antimicrobial activity against a broad spectrum of spoilage and pathogenic bacteria through disruption of bacterial cell membranes and inhibition of cell proliferation, leading to bacteriostasis or cell death. Additionally, *L. plantarum* CFS maintains the sensory and physicochemical qualities of meat products without adversely affecting attributes such as flavor, color, or texture at optimal concentrations (Wang et al., 2023).

Regarding the recorded results of the present study, during 48 days of refrigerated storage, the pH of fermented sausage samples treated with CFS generally demonstrates a characteristic trend of initial rapid decline, followed by relative stability or slow increase over prolonged storage. Upon treatment, the pH typically drops quickly within the first days; that may be attributed to the production of organic acids by residual active compounds in the CFS, often reaching values (4.97 and 4.95) in 1.5% and 3.0% treated groups, respectively, which have been considered inhibitory to most spoilage and pathogenic microorganisms (Mani-López et al., 2024). This acidified environment remains mostly stable throughout the initial phase of storage. Over the course of extended refrigeration, the pH slowly rises due to proteolytic activity and gradual degradation of proteins or buffering effects from amino acids but usually remains below the safety threshold, maintaining effective microbial control and product safety (Campaniello et al., 2020).

Regarding the microbiological quality of the treated groups, fermented sausage samples treated with CFS generally maintained superior microbiological quality compared to untreated controls that may be attributed to the antimicrobial action of organic acids and bacteriocins present in the extract (Sirini et al., 2022). Total aerobic plate count (APC) typically remains under 4 log CFU/g, with initial readings after fermentation of 3.20 and 3.12 log CFU/g for 1.5 and 3.0% CFS-treated groups, and only gradual increases during storage, often not exceeding 4.5 log CFU/g by day 48. Additionally, coliforms remained below 2 log CFU/g throughout storage, indicating strong inhibition of enteric contaminants. Moreover, staphylococcus species were significantly suppressed and often stayed below 3 log CFU/g, reflecting the efficacy of the CFS in reducing pathogen risk.

Furthermore, psychrotrophic bacteria, responsible for spoilage under cold storage, showed low initial counts and remained controlled such that they did not exceed 3 log CFU/g even at the end of storage. On the other hand, fungal growth was undetectable along the storage period, suggesting effective prevention of mold growth under the influence of *L. plantarum* metabolites; in addition, sulfite-reducing clostridia, which can be an indicator of potential spoilage or pathogenic presence, were typically absent or well below detection limits (e.g., <1 log CFU/g) throughout the 48 days of storage.

Cell-free supernatant (CFS) of *L. plantarum* contains a complex mixture of active principles, including organic acids (lactic, acetic), saturated fatty acids, dicarboxylic acids, bacteriocins, and other small antimicrobial

metabolites (Rezgui et al., 2023). The antimicrobial effect of *L. plantarum* CFS is primarily attributed to the combined action of these compounds, where organic acids lower the pH and create an acidic environment that inhibits the growth and survival of many foodborne microorganisms. In addition, bacteriocins—small, ribosomally synthesized peptides—interfere with the integrity of bacterial cell membranes, leading to pore formation, leakage of cellular contents, and ultimately, cell death. Additional metabolites like hydrogen peroxide and certain fatty acids have further inhibitory effects on microbial proliferation (Kuley et al., 2018). The mode of action involves both direct antagonism—through membrane disruption, enzyme inhibition, and interference with metabolic processes—and the indirect enhancement of food safety by shifting the microbial ecosystem towards beneficial lactic acid bacteria, thereby outcompeting pathogens (Thakur and Kaur, 2025).

The obtained results came in line with Wang et al. (2023), who used *L. plantarum* (0.5% v/w) in ground beef preservation and recorded that the treated minced beef samples revealed an extended shelf life of 8 days at 4°C; inhibited total microbial growth; stable pH with unaffected color and texture; and Elyass et al. (2017), who used *L. plantarum* bacteriocin on Sudanese fermented beef and recorded that CFS showed antimicrobial activity against Gram-positive/negative bacteria in vitro; *L. plantarum* CFS contributed to inhibition of Salmonella, E. coli, and Staphylococcus aureus; and proposed potential as a biopreservative for fermented meat.

Although potent antimicrobial effects of the used starter culture in fermented sausage production are predicted due to acidification and lower water activity (Martín et al., 2021), several factors can contribute to the failure of starter cultures during the production of fermented sausages, often necessitating the use of cell-free extracts as an alternative. Among the major causes is the presence of inhibitory substances in the meat, such as residual antibiotics or high salt concentrations, which can suppress or kill beneficial lactic acid bacteria within the starter culture, thus stalling the acidification process crucial for safety and flavor development. Besides that, unsuitable fermentation conditions—including improper temperature, humidity, or oxygen levels—can also hinder starter culture activity, leading to inadequate pH reduction and uncontrolled microbial growth (Shao et al., 2024). Additionally, competition with the natural, non-starter microbiota can prevent the starter culture from becoming dominant, especially if the raw materials have a high initial microbial load or the starter strain lacks sufficient competitiveness (Montanari et al., 2021). Poor handling or storage of the starter culture itself, such as exposure to moisture, heat, or prolonged storage time, may further result in loss of viability and fermentative potential (Owusu-Kwarteng et al., 2020). When these issues cause starter culture failure, the fermentation process becomes unpredictable, increasing the risk of spoilage and pathogenic contamination. In such cases, the use of cell-free extracts—containing antimicrobial metabolites and acids produced by probiotics like L. plantarum—offers a targeted method to control undesirable bacteria and ensure product safety and quality, compensating for the shortcomings of compromised or nonfunctional starter cultures.

The antimicrobial effect of *L. plantarum* cell-free supernatant (CFS) in fermented meat products is distinctly concentration-dependent, with higher concentrations providing more robust inhibition of foodborne microorganisms. Studies on ground beef gels (Wang et al., 2023), for example, showed that supplementation with increasing percentages of *L. plantarum* CFS—specifically 0.5%, 1%, 2.5%, and 5%—progressively extends product shelf life and suppresses total aerobic microbial counts throughout refrigerated storage. While 0.5% and 1% CFS delayed the microbial load from exceeding acceptable levels for a few days, only 2.5% and 5% CFS additions maintained microbial counts below spoilage thresholds for the entire storage period. This dose-response relationship is attributed to the escalating presence of organic acids and bacteriocins in higher concentrations, which act synergistically to disrupt cell membranes and inhibit the growth of various bacteria. Thus, the application of higher concentrations of *L. plantarum* CFS emerges as a highly effective, clean-label strategy to enhance the microbial safety and shelf life of fermented meat products.

5. CONCLUSIONS

The incorporation of *L. plantarum* CFS into fermented sausage formulations significantly improves microbiological quality and extends shelf life during refrigerated storage. The treated groups showed lower bacterial counts and longer shelf life in comparison with the control untreated group, which showed signs of spoilage after twenty-seven days of refrigerated storage. Additionally, *L. plantarum* extracts slowed pH rise, keeping an acidic environment that helped in keeping the shelf life and acceptability of the treated groups. Moreover, a direct relationship between the CFS concentration and the keeping quality and microbial counts was recorded.

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