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Novel synergistic combination of lactic acid and acetic acid bacteria cell-free extracts for enhanced food biopreservation

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Abstract

Introduction and Objective. The aim of the study is to evaluate the synergistic biopreservative potential of cell-free extracts (CFEs) obtained from lactic acid bacteria (LAB: *Lacticaseibacillus paracasei* B1, *Lactiplantibacillus plantarum* O24) and acetic acid bacteria (AAB: *Gluconobacter oxydans* KNS32, *Komagataeibacter saccharivorans* KOM1), and to assess their antimicrobial, antioxidant, and overall combined efficacy in food preservation.

Materials and Method. CFEs were analysed for organic acid and polyphenol content, and tested for antioxidant capacity using DPPH and ABTS assays. Antimicrobial activity was assessed by agar well-diffusion and minimum inhibitory concentration (MIC) assays against Gram-positive and Gram-negative indicator strains. Synergistic effects were evaluated by checkerboard microdilution. The combinations were applied to chicken meat inoculated with *Salmonella enterica* subsp. *enterica* serovar Enteritidis and stored for 7 days at 37 °C and 4 °C.

Results. LAB CFEs contained lactic, gluconic, and acetic acids, whereas AAB CFEs contained gluconic, acetic, and citric acids. MIC values ranged from 3.1 – 100 AU/mL, and several LAB–AAB combinations exhibited synergistic antibacterial activity. In the chicken model, CFEs reduced *S*. Enteritidis counts below the detection limit, and decreased total viable counts by more than 1 log CFU/g after 7 days.

Conclusions. The synergistic combination of LAB and AAB CFEs exhibited strong antimicrobial and antioxidant activities, indicating high potential as natural, microbially derived preservatives for safe food biopreservation.

Key words

antioxidants, lactic acid bacteria, food safety, acetic acid bacteria, hurdle technology, synergistic antibacterial activity, microbial-derived preservatives

INTRODUCTION

Despite the progress made in the food industry in recent years, some food-borne pathogens continue to pose significant public health challenges on a local, national, and international scale. In the European Union (EU), 77,486 salmonellosis cases were reported in 2023, corresponding to an EU notification rate of 18.0 cases per 100,000 people. This resulted in the highest number of hospitalizations (14,801 cases) among reported food-borne gastrointestinal infections [1]. On the other hand, increasing consumer health awareness and demand for safer and more natural food preservatives make new alternatives urgently necessary and desirable [2]. Biopreservation strategies align with these market trends by utilizing naturally derived antimicrobials. While much of the research has focused on microbial interactions and their inhibitory effects on spoilage and pathogenic bacteria, recent advancements have expanded to include novel bioprotective cultures, bacteriophages, postbiotics, organic acids, and plant-derived compounds, all of which are gaining significant attention for their potential in food preservation [3].

Bacterial preparations refer to biologically active substances derived from microbial cultures commonly used for their antimicrobial, antioxidant, and preservative properties. These preparations can include cell-free supernatants, heat-killed bacterial cells, and neutralized supernatants, each exhibiting distinct bioactive profiles [4]. The supernatant is obtained by culturing bacteria in a nutrient medium, followed by centrifugation or filtration to remove the cells, leaving behind a liquid fraction rich in antimicrobial metabolites, such as organic acids, bacteriocins, and enzymes [5]. Unlike postbiotics, which are defined as preparations of inanimate microorganisms and/or their components that confer a health benefit on the host [6, 7], bacterial preparations may not necessarily contain inactivated cells, but instead, focus on extracellular metabolites with antimicrobial potential. Their application in biopreservation is particularly valuable as they offer a natural and sustainable alternative to synthetic preservatives, helping to inhibit foodborne pathogens, reduce spoilage, and extend shelf life [8, 9].

Bacterial preparations are primarily produced by lactic acid bacteria (LAB), often considered probiotics due to their health benefits [10]. LAB-derived preparations are valued for

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their antimicrobial, antioxidant, and immunomodulatory effects, making them promising for food preservation and human health [11]. Their antimicrobial activity results from bioactive compounds, including organic acids (lactic and acetic), bacteriocins, hydrogen peroxide, and diacetyl, which inhibit pathogens by lowering the pH, disrupting cell membranes, or interfering with essential metabolic processes [12]. LAB-derived bacterial preparations exhibit strong inhibitory effects against various microorganisms, including food-borne pathogens, as well as psychrophilic bacteria, yeasts, and moulds responsible for food spoilage [8, 9]. Beyond antimicrobial activity, they enhance food stability and quality through antioxidant effects that prevent lipid oxidation, modulation of microbial communities to extend shelf life and maintain sensory properties, and potential immunomodulatory effects that support functional food applications [13–15].

Another group of microorganisms with promising health-promoting properties are acetic acid bacteria (AAB) [16, 17]. AAB are Gram-negative strict aerobes, capable of incompletely oxidizing carbon sources into corresponding organic compounds, such as ethanol to acetic acid [18]. Additionally, AAB are capable of producing polysaccharides, of which bacterial cellulose and levan are the most widespread, and synthesizing many pro-health organic acids and miglitol [17, 18]. Due to their unique properties, AAB are used in many branches of the food industry, i.e., Kombucha and vinegar production. The utilization of AAB preparations as a cocktail of antimicrobial substances to ensure the safety and quality of food remains poorly studied [17].

Despite the growing demand for natural biopreservatives in the food industry, many existing options face limitations in terms of efficacy, stability, and the spectrum of antimicrobial activity. Traditional biopreservatives, such as bacteriocins and organic acids from well-characterized lactic acid bacteria, are often strain-specific and may not provide broad protection against diverse food-borne pathogens and spoilage microorganisms [19]. Additionally, while LAB-derived antimicrobial compounds, including lactic acid, hydrogen peroxide and diacetyl, have demonstrated efficacy in various food systems, their performance can be influenced by food composition, storage conditions, and interactions with native microbiota [12]. Similarly, the potential of acetic acid bacteria in food preservation remains under-explored, particularly in terms of their compatibility with LAB-based approaches [20, 21]. Furthermore, most studies focus on single-strain applications, which may limit the overall robustness of biopreservation strategies [22]. Research exploring multi-strain or cross-genus interactions remains scarce, despite the potential for synergistic effects between different microbial groups to enhance antimicrobial efficacy and broaden the spectrum of inhibition [21, 23]. Addressing these limitations is crucial for advancing natural biopreservation methods that are both effective and adaptable across various food matrices.

To the best knowledge of the authors, no studies have investigated the antimicrobial effectiveness of combining LAB and AAB preparations. Previously, only pure lactic and acetic acids have been tested together for food preservation [24–27]. Lactic acid has well-established inhibitory effects on various food-borne pathogens, and acetic acid from AAB offers complementary antibacterial properties that potentially enhance overall bioprotective efficacy [28]. On the other hand, in a study by Xia et al. (2022) [29], *in situ* and *in vitro* approaches were employed to investigate the interaction between LAB and AAB during the solid-state fermentation of cereal vinegar. However, research remains limited to the combined use of LAB and AAB metabolites, particularly in real food matrices. A key advantage of the cocktail over individual organic acids lies in its enhanced efficacy, resulting from a combination of diverse organic acids, bacteriocins, hydrogen peroxide, proteins, peptides, and other molecules [19, 29].

Inspired by the unique health benefits of Kombucha, which features a distinctive consortium of acetic acid bacteria and lactic acid bacteria, this study aimed to evaluate the antimicrobial effects of bacterial extracts derived from environmental strains of LAB – *Lacticaseibacillus paracasei* and *Lactiplantibacillus plantarum*), and AAB – *Gluconobacter oxydans* and *Komagataeibacter saccharivorans* for innovative food biopreservation. The assessment was conducted individually and in pairs through *in vitro* experiments and within a food matrix. Additionally, the study evaluated the polyphenol content, antioxidant potential, and organic acids content. To our knowledge, no previous research has examined the efficacy of combining bacterial extracts from environmental strains of LAB and AAB, neither *in vitro* nor within a food matrix.

MATERIALS AND METHOD

Origin of lactic acid and acetic acid bacteria. Two strains of *Lacticaseibacillus paracasei* B1 and *Lactiplantibacillus plantarum* O24 were selected. The *L. paracasei* B1 strain was isolated from the Polish regional sheep's milk cheese 'Bundz' (GenBank Accession Nos: CP161807 and CP161808). According to previous research, this strain exhibits selected *in vitro* probiotic effects, and its properties are protected by patent (Patent No. P.426002) [30]. The *Lactiplantibacillus plantarum* O24 strain was isolated from traditionally fermented cucumber dill pickles (GenBank Accession No: CP157747-CP157755); its selected *in vitro* probiotic properties have been described previously [30–32]. Strains were stored at -80 °C with 20% glycerol v/v in MRS broth (deMan, Rogosa, and Sharpe, Neogen, Lansing, MI, USA).

Two strains of acetic acid bacteria were used in this study. The strain *Gluconobacter oxydans* KNS32 was isolated from a local Kombucha beverage (GenBank Accession No: OQ597203). The potential postbiotic properties and *in vitro* anticancer activity of the supernatant obtained from this strain were previously described by Neffe-Skocińska et al. [20]. Local artisanal honey vinegar was utilized as the source of the strain *Komagataeibacter saccharivorans* KOM1 (GenBank Accession No: OQ594827). The strains were stored at -80 °C with 20% glycerol (v/v) in GC broth (Glucose Calcium Carbonate), prepared according to Neffe-Skocińska et al. (2022) [33].

The bacterial strains are maintained in the internal collection of microorganisms at the Department of Food Gastronomy and Food Hygiene, Institute of Human Nutrition Sciences, at the University of Life Sciences in Warsaw, Poland.

Indicator strains. The following indicator strains were used for the evaluation of antimicrobial activity: *Listeria monocytogenes* ATCC 19111, *Staphylococcus aureus* ATCC 25923, *Bacillus spizizenii* ATCC 6633, *Enterococcus faecalis*

ATCC 51299 (Gram-positive), and *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, *Escherichia coli* ATCC 10536, *Pseudomonas fluorescens* PCM 2123 (Gram-negative). The strains were stored in glycerol stocks at -80 °C. To revive the strains, each one was plated onto appropriate agar media: *Listeria monocytogenes* was streaked on PALCAM agar (LabM, Heywood, UK), *Staphylococcus aureus* on Baird-Parker egg yolk tellurite agar (Oxoid, Basingstoke, UK), *Bacillus spizizenii*, *Enterococcus faecalis,* and *Pseudomonas fluorescens* on Nutrient Agar (Oxoid, Basingstoke, UK), *Salmonella enterica* subsp. *enterica* serovar Enteritidis on XLD Agar (Oxoid, Basingstoke, UK), and *Escherichia coli* on TBX agar (Oxoid, Basingstoke, UK). Plates were incubated at 37 °C for approximately 24 hours. After incubation, single colonies of each indicator strain were selected and transferred to Mueller-Hinton (MH) broth (Oxoid, Basingstoke, UK). The cultures were incubated aerobically at 37 °C for 24 hours, either with gentle shaking or in a stationary incubator, depending on the strain. Once growth reached the desired turbidity (early stationary phase), the cultures were standardized to an optical density of 0.1 at 600 nm to ensure consistent inoculum sizes for antimicrobial testing.

Preparation of LAB and AAB cell-free extracts. The LAB strains were grown anaerobically in fresh MRS broth (De Man, Rogosa, and Sharpe broth) at 37 °C for 16–18 h to achieve a final concentration of approximately 1×10^9 CFU/mL. Anaerobic conditions were established by placing inoculated MRS broth tubes in anaerobic jars equipped with AnaeroGen™ sachets (Oxoid, Basingstoke, UK). Half of the LAB cultures were separated and placed in a water bath at 80 °C for 20 min. Next, both types of bacterial cultures (crude and heat-killed) were centrifuged at 3341 × g for 15 minutes (Eppendorf SE, Hamburg, Germany) to remove the bacterial cells. The supernatants were then sterilized using 0.22 µm syringe filters (AlfaChem, Poznań, Poland). Part of the LAB cell-free crude extract was left with its original pH value. The neutralized cell-free extract was obtained by adding 1 M NaOH to adjust the pH to approximately 6.0 ± 0.2. This adjustment neutralizes the organic acids produced, eliminating their antagonistic effects. Finally, 3 types of LAB preparations were assembled: cell-free crude extract (CFE), cell-free extract of heat-killed cells (hkCFE), and neutralized cell-free extract (nCFE).

The AAB strains were cultured aerobically at 28 °C for 48 h in Hestrin and Shramm (HS) ($1–5 \times 10^7$ CFU/mL) composed of 2 g/L glucose w/v (Neogen, Lansing, MI, USA), 0.5 g/L casein peptone w/v (BTL, Łódź, Poland), 0.5 g/L yeast extract w/v (Merck Life Science, Darmstadt, Germany), 0.27 g/L Na_2HPO_4 w/v (Merck Life Science, Darmstadt, Germany), and 0.15 g/L citric acid w/v (Merck Life Science, Darmstadt, Germany). Based on preliminary studies indicating that AAB extracts lose their antibacterial activity following thermal inactivation and pH neutralization, only one preparation, cell-free crude extract (CFE), was obtained for this group of microorganisms, following the methodology described above. Samples were stored at -20 °C for a maximum period of 14 days for further analysis. Table 1 summarizes the abbreviations applied in this manuscript for the tested strains and their corresponding cell-free extracts (CFEs) variants.

pH value, organic acids and sugar content of CFEs. All analyzed samples were diluted 10 times in standard MilliQ water and centrifuged (Eppendorf Centrifuge 5804 R, Hamburg, Germany) (16,323 × g for 5 min). After that, 1 mL of each sample was filtered through a 0,22 µm syringe PES filter into the vials (qpore-Bionovo, Legnica, Poland). Organic acids and sugars were analyzed with an HPLC system (Shimadzu, USA Manufacturing Inc, USA, 2 LC-20AD pumps, a CBM-20A controller, a CTD-20AC oven, a SIL-20AC autosampler, a RID-10A detector, and a UV/Vis SPD-20AV detector). An Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) at 40 °C with a flow rate of 0.5 mL/min, using a mobile phase of 10 mM H2SO4 (Poch, Gliwice, Poland), was used to separate the compounds. Quantification was based on the detection at a 210 nm wavelength using UV/Vis, RI, and external standard curves, with a range of 0.12–40 µg per injection. Triplicate analyses were conducted. Orion Star™ A211 pH meter (Thermo Fisher Scientific, Newington, NH, USA) was used to measure all pH values.

Table 1. Abbreviations for cell-free extracts (CFEs) used in the study and their variants

B1 CFE	Crude CFE from *L. paracasei* B1
B1 hkCFE	Heat-killed CFE from *L. paracasei* B1
B1 nCFE	Neutralized CFE from *L. paracasei* B1
O24 CFE	Crude CFE from *L. plantarum* O24
O24 hkCFE	Heat-killed CFE from *L. plantarum* O24
O24 nCFE	Neutralized CFE from *L. plantarum* O24
KNS32 CFE	CFE from *G. oxydans* KNS32
KOM1 CFE	CFE from *K. saccharivorans* KOM1

Total phenolic content (TPC). Total phenolic content in samples was assessed using a colorimetric method with Folin-Ciocalteu reagent. Samples were diluted in MilliQ water (HPL 20UV; Hydrolab; Straszyn, Poland), then mixed with Folin-Ciocalteu's phenol reagent (Chempur, Piekary Śląskie, Poland) and 7.5% w/v sodium carbonate solution (Chempur, Piekary Śląskie, Poland) in a 96-well plate (NEST Biotechnology, Wuxi, China). The mixture was incubated at room temperature (25 ± 2 °C) in the dark for 30 minutes with shaking at 300 × g. Absorbance at 750 nm was then measured using a SpectraMax iD3 microplate reader (Molecular Devices, San Jose, CA, USA). Results were expressed as gallic acid equivalent (GAE) in mg/100 mL, with a conversion based on a standard curve ($R^2 = 0.999$). Eight biological replicates were analyzed.

ABTS•+ radical scavenging activity. The antioxidant capacity was determined using the ABTS•+ radical cation. ABTS•+ (Merck Life Science, Darmstadt, Germany) was prepared by mixing 7 mM ABTS•+ stock solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) solution (Merck Life Science, Darmstadt, Germany). The mixture was incubated in the dark at room temperature for 24 hours to allow the generation of ABTS•+ radicals. Before testing, the ABTS•+ solution was diluted with phosphate-buffered saline (PBS) (Merck Life Science, Darmstadt, Germany) to achieve an absorbance of 0.7 ± 0.02 at 734 nm. Sample solutions were prepared by diluting them in MilliQ water, and 100 µL of each sample was added to 100 µL of ABTS•+ solution in a microplate well. The plate was incubated at room temperature for 6 minutes, with gentle shaking. The absorbance at 734 nm was then measured using a SpectraMax iD3 microplate

reader (Molecular Devices, San Jose, CA, USA). Results were expressed as ascorbic acid equivalent (VCEAC), calculated based on a standard curve ($R^2 = 0.998$). A total of 8 biological replicates were analyzed.

DPPH radical scavenging activity. The DPPH method was used to assess antioxidant activity by measuring the reduction of DPPH radical (Merck Life Science, Darmstadt, Germany) to its non-radical form (DPPH-H). The DPPH stock solution was prepared by dissolving DPPH in methanol (Chempur, Piekary Śląskie, Poland) to a concentration of 0.1 mM. The DPPH solution was then diluted with methanol to achieve an absorbance of 1.1 ± 0.05 at 517 nm. For the assay, sample solutions were prepared by diluting them in MilliQ water, and 100 µL of each sample was added to 100 µL of the DPPH solution in a 96-well microplate. The plate was incubated at room temperature for 30 minutes in the dark, with gentle shaking. After incubation, the absorbance at 517 nm was measured using a SpectraMax iD3 microplate reader (Molecular Devices, San Jose, CA, USA). Results were expressed as ascorbic acid equivalent (VCEAC), based on a standard curve ($R^2 = 0.998$). A total of eight biological replicates were performed.

Antibacterial properties. The antimicrobial effectiveness of CFEs and their various combinations (1:1 mix) was verified visually using the agar-based well-diffusion method. A mixture of 20 mL MH agar (Merck Life Sciences, Darmstadt, Germany) and an 18-h culture of each indicator strain (1% inoculum), with a concentration of 10^6, were poured separately into sterile Petri dishes. After solidification, wells of 5.5 mm diameter were cut out and filled (100 µL) with CFEs. Plates were incubated at 37°C for 18 hours, and the diameters of the inhibition growth zones were measured using a Vernier caliper. According to the following formula ($\bar{x}$ = D-d), antimicrobial activity ($\bar{x}$) was calculated, where D represents the inhibition zone diameter and d is the well diameter [34].

Minimal inhibitory concentrations (MICs). The methodology was based on EUCAST guidelines [35] with minor modifications. Overnight cultures of each indicator bacterium were subcultured in MH broth until an optical density at 600 nm (OD_{600}) of 0.5 was obtained, as measured with a SpectraMax iD3 reader (Molecular Devices, San Jose, CA, USA). The bacterial cells were standardized by inoculating 980 µL of broth with 20 µL of culture and aseptically transferring the suspension into each well of a 96-well polystyrene titration plate (NEST Biotechnology, Wuxi, China).

Each antimicrobial extract was prepared as a two-fold serial dilution of the solution of a given extract in sterile MH broth, starting at an initial concentration of 50%, and serially diluted down to 0.049%. To determine the minimum inhibitory concentration, 100 µL of each serially diluted extract was added to the wells. This resulted in a total volume of 200 µL per well, which also contained 100 µL of bacterial suspension. In the next step, the antibacterial impact of CFEs was evaluated using the checkerboard microdilution assay, as described by Bellio et al. (2021) [36], with minor modifications. The resulting checkerboard on the plate contained a combination of a concentration gradient of 2 antimicrobial extracts in a 1:1 v/v ratio. Each well contained 100 µL of the combination, achieving a total volume of 200 µL per well. The plates were sealed and incubated aerobically for 18–24 hours at 37 °C, and the optical density at 600 nm (OD_{600}) was measured.

The MIC of the tested individual extracts and their various combinations was defined as the lowest concentration of the given extract (in Arbitrary Units, AU/mL) that restricted indicator strain growth to an optical density <0.05. Positive and negative control assays were conducted. Positive control assays confirmed the antimicrobial activity, while negative controls ensured that observed effects were due to the activity of the extracts.

Fractional inhibitory concentration (FIC) and synergistic activity. MIC data of the tested combinations and MIC data of the individual extracts themselves were converted into fractional inhibitory concentration (FIC). The FIC for each antimicrobial extract is determined by dividing the MIC of each extract when used in combination by the MIC of the same extract when used alone [37]. Then, as a measure of synergy, the fractional inhibitory concentration index (FICI) was calculated, i.e., the sum of the FIC of each tested antibacterial extract used in combination. FICI was reckoned as follows:

$$FICI = \frac{MIC\ of\ compound\ A\ in\ combination}{MIC\ of\ compound\ A\ alone} + \frac{MIC\ of\ compound\ B\ in\ combination}{MIC\ of\ compound\ B\ alone}$$

where A refers to the LAB CFE and B stands for the AAB-derived CFE.

Traditionally, synergism is defined as a FICI of 0.5 or less, additive interactions occur when FICI is in the 0.5–1.0 range, no interaction is observed in the 1.0–4.0 range, and antagonism is interpreted as a FICI of 4.0 or greater [37].

Model food trials with a chicken meat sample. *S.* Enteritidis ATCC 13076 was used to evaluate inhibitory activity under real-life conditions. The strain was cultured in MH broth at 37 °C for 16 h under aerobic conditions, then diluted in fresh buffered peptone water (Bio-Rad, Marnes-la-Coquette, France) to obtain a final concentration of 1×10^5 CFU/mL. For the *in vivo* test, fresh chicken breast fillets (Morliny, Animex, Kutno, Poland) were aseptically cut into ~5 g portions and placed on sterile Petri dishes. Each portion was inoculated with 10 µL of the bacterial suspension, evenly spread using a sterile loop, resulting in an initial *S.* Enteritidis count of ~1×10^3 CFU/g. The inoculated meat was left for 5–10 min to allow absorption. All previously tested CFEs were prepared in a final volume of 10 mL. Based on prior results demonstrating superior activity of the combined extracts, all 12 mixtures were included in the *in vivo* experiments. To prevent direct contact between the extract and meat, sterile tissue paper (LLG Labware, Meckenheim, Germany) was placed over the inoculated surface using sterile tongs. Subsequently, 200 µL of the tested extract mixture was applied dropwise onto the tissue and left for 30 s to allow absorption. Negative controls included Milli-Q water (Hydrolab, Straszyn, Poland), MRS broth, and HS broth. Samples were incubated at 37 °C for 24 h (optimal for *S.* Enteritidis growth), and at 4 °C for 7 days

(typical refrigeration temperature for meat storage). Viable cell counts were determined by plate counting on Plate Count Agar (Merck Life Science, Darmstadt, Germany) for total bacteria and Xylose Lysine Deoxycholate Agar (XLD) (Oxoid, Basingstoke, UK) for selective enumeration of *S.* Enteritidis. Each test was performed in triplicate.

Statistical analysis. To conduct the statistical analysis, Statistica 13.1 software (TIBCO Software Inc., Palo Alto, CA, USA) was utilized. As part of the analysis, the Brown–Forsythe test was conducted to determine the homogeneity of variance, and the Shapiro–Wilk test was used to confirm the normality of the data. One-way analysis of variance (ANOVA) was performed. Based on Tukey's test, significant differences ($p < 0.05$) were found between the data sets. Data were visualized in R software version 4.3.3 (R Core Team, Vienna, Austria).

RESULTS

Organic acids and sugar. Table 2 shows the concentrations of sugar and organic acids in the CFEs and the pure control media broths used for their cultivation. Lactic acid concentrations in the LAB extracts ranged from 19.9–21.9 mg/mL, with statistically significant differences observed between the control media and CFEs ($p<0.05$). Additionally, hkCFEs exhibited significantly higher concentrations of propionic acid, but lower levels of gluconic, lactic and acetic acids, compared to the crude CFEs. For the AAB extracts, the bacteria significantly consumed citric acid and glucose during growth, as their concentrations were markedly higher in the HS control broth compared to the CFEs ($p<0.05$). The CFEs contained 2 distinctive organic acids – gluconic and acetic acids. The highest concentration of gluconic acid (15.4 mg/mL) was observed in KNS32 CFE. Acetic acid levels ranged from 1.9 to 2.4 mg/mL across the CFEs, with the highest concentration also recorded in KNS32 CFE ($p<0.05$).

Total polyphenol content and antioxidant activity. Table 3 illustrates the total polyphenol content and antioxidant activity of the CFEs. The total polyphenol content ranged from 27.7–120.0 GAE µg/mL, with the highest concentrations observed in B1 CFE (120.0 GAE µg/mL) and O24 CFE (118.6 GAE µg/mL). LAB CFEs exhibited significantly higher total polyphenol levels compared to AAB extracts ($p < 0.05$).

Table 3. Total polyphenol content, ABTS•+ radical scavenging activity, and DPPH radical scavenging activity in CFEs

Extracts	Total polyphenol content	ABTS•+ radical scavenging activity	DPPH radical scavenging activity
	GAE µg/mL	VCEAC/mL	
O24 hkCFE	91.2 ± 4.2^{b}	6.0 ± 0.1^{b}	0.7 ± 0.0^{ab}
O24 CFE	118.6 ± 9.0^{c}	5.9 ± 0.1^{b}	0.6 ± 0.0^{ab}
B1 hkCFE	98.9 ± 5.6^{b}	5.5 ± 0.2^{ab}	0.8 ± 0.1^{ab}
B1 CFE	120.0 ± 10.9^{c}	5.9 ± 0.0^{b}	1.0 ± 0.1^{b}
KOM1 CFE	29.7 ± 1.7^{a}	1.3 ± 0.0^{ab}	0.1 ± 0.0^{a}
KNS32 CFE	27.7 ± 1.7^{a}	1.2 ± 0.0^{a}	0.1 ± 0.0^{a}

Values are expressed as mean ± standard deviation; means followed by different letters (a-c) are statistically significantly different ($p<0.05$)

The antioxidant activity, measured as the ability to scavenge ABTS•+ and DPPH radicals, ranged from 1.2–6.0 VCEAC/mL and 0.1–1.0 mg VCEAC/mL, respectively. Among the tested samples, the B1 CFE demonstrated the highest DPPH radical scavenging activity, significantly exceeding the values observed in AAB extracts ($p < 0.05$). A similar trend was noted for ABTS•+ radical scavenging, where LAB extracts out-performed AAB ones.

Interestingly, the O24 hkCFE displayed the highest ABTS•+ radical scavenging capacity, significantly greater than that of KNS32 CFE ($p < 0.05$). These findings highlight the superior antioxidant potential of LAB-derived extracts.

Antibacterial properties. The CFEs and their various combinations exhibited different pH values and distinct antagonistic activities against the indicator strains in the agar well-diffusion assay (Tab. 4). Among the Gram-positive bacteria, *L. monocytogenes* exhibited the highest susceptibility to growth inhibition, with inhibition zones ranging from 9.7–18.8 mm. In contrast, *E. faecalis* was the most resistant, with inhibition zones ranging from 7.7–13.3 mm. Statistically significant differences ($p < 0.05$) were observed between the antibacterial activity of individual extracts and their mixtures. Crude and heat-killed extracts showed stronger antimicrobial effects than those that had been neutralized, which were largely inactive. Moreover, AAB-derived extracts demonstrated greater activity than

Table 2. Concentration of sugar and organic acids in CFEs and the media broth (as a reference)

Extracts	Glucose	Citric acid	Gluconic acid	Acetic acid	Propionic acid	Pyruvic acid	Lactic acid	Formic acid
	mg/mL							
MRS	15.1 ± 0.1^{a}	5.3 ± 0.1	9.7 ± 0.0^{a}	10.5 ± 0.0^{a}	1.8 ± 0.0^{a}	0.8 ± 0.0^{a}	4.2 ± 0.0^{a}	1.0 ± 0.0^{a}
O24 hkCFE	0.5 ± 0.1^{b}	nd	8.7 ± 0.1^{b}	8.7 ± 0.2^{b}	2.0 ± 0.0^{a}	0.9 ± 0.0^{a}	19.9 ± 0.2^{b}	1.2 ± 0.1^{a}
O24 CFE	0.7 ± 0.2^{b}	nd	9.2 ± 0.2^{c}	9.8± 0.4^{c}	1.1 ± 0.1^{b}	0.8 ± 0.0^{a}	21.1 ± 0.2^{c}	1.1 ± 0.1^{a}
B1 hkCFE	0.7 ± 0.2^{b}	nd	8.8 ± 0.2^{b}	9.1 ± 0.2^{b}	2.0 ± 0.1^{a}	0.9 ± 0.0^{b}	21.2 ± 0.0^{c}	1.0 ± 0.0^{a}
B1 CFE	0.7 ± 0.1^{b}	nd	9.4 ± 0.3^{c}	9.7 ± 0.2^{c}	1.1 ± 0.0^{b}	0.9 ± 0.0^{b}	21.9 ± 0.3^{c}	1.1 ± 0.01^{a}
HS	26.7 ± 0.4^{a}	8.3 ± 0.0^{a}	nd	1.4 ± 0.1^{a}	nd	nd	nd	nd
KOM1 CFE	16.9 ± 0.1^{b}	2.6 ± 0.0^{b}	10.3 ± 0.1^{a}	1.9 ± 0.2^{b}	0.8 ± 0.2^{a}	nd	nd	nd
KNS32 CFE	12.5 ± 0.2^{c}	3.1 ± 0.0^{c}	15.4 ± 0.1^{b}	2.4 ± 0.1^{c}	0.8 ± 0.1^{a}	nd	nd	nd

Values are expressed as mean ± standard deviation. Means followed by different letters (a-c) are statistically significantly different ($p<0.05$); LAB CFEs and AAB CFEs were statistically analyzed and compared separately.
MRS - deMan, Rogosa, and Sharpe broth; HS - Hestrin and Shramm broth; nd - not detected;

Table 4. Inhibitory effect of CFEs against indicator strains

Amount		Extracts	pH value	Gram-positive				Gram-negative		
				Lm 19111	*Sa* 25923	*Bs* 6633	*Ef* 51299	*SE* 13076	*Ec* 10536	*Pf* 2123
				Mean diameter of inhibition zones [mm] for indicator strains						
Individual, 100 µL	B1	CFE	4.10±0.04	12.2±1.0^{bcA}	8.5±3.1^{cdC}	10.8±0.8^{cdB}	9.5±1.3^{deBC}	10.2±1.0^{dB}	12.7±1.9^{cA}	11.2±0.3^{eB}
		hkCFE	4.23±0.05	12.7±2.5^{bcA}	8.8±3.6^{cdC}	11.0±1.3^{cB}	9.7±0.8^{dBC}	10.8±0.3^{dB}	12.8±2.0^{cA}	11.7±0.3^{deAB}
		nCFE	5.84±0.15	9.7±0.6^{cA}	6.7±1.2^{dC}	9.8±1.5^{dA}	7.7±1.0^{eBC}	8.2±1.4^{eB}	9.5±0.9^{dAB}	9.7±1.4^{eA}
	O24	CFE	3.87±0.06	11,8±1.3b^{cAB}	7.3±0.3^{cdC}	10.3±0.6^{cdB}	8.3±1.4d^{eBC}	8.7±0.8^{deBC}	12.5±2.2^{cdA}	11.8±0.6^{deAB}
		hkCFE	4.02±0.08	12.8±1.0^{bcA}	8.5±0.5^{cdC}	10.8±0.8^{cdB}	9.7±0.9^{dBC}	9.3±1.2^{deBC}	12.7±0.6^{cA}	12.0±0.9^{dAB}
		nCFE	5.91±0.13	10.2±1.9^{cAB}	6.3±0.3^{dC}	9.7±0.3^{dB}	8.2±1.5^{eBC}	8.5±1.0^{eBC}	9.3±0.8^{dBC}	10.7±1.4^{eA}
	KOM1	CFE	3.08±0.03	13.5±0.5^{bcA}	9.8±3.3^{cC}	9.5±0.5^{dC}	10.2±0.3^{cdBC}	11.2±0.8^{cdB}	12.8±1.0^{cB}	11.7±0.8^{deAB}
	KNS32	CFE	3.24±0.03	13.8±1.0^{bcA}	10.0±2.7^{cC}	10.7±1.2^{cdBC}	10.5±0.9^{cdBC}	11.8±1.6^{cdB}	13.3±1.5^{bcAB}	11.8±0.3^{deB}
Combination, 50 µL + 50 µL (50% v/v)	B1 + KOM1	B1 CFE + KOM1 CFE	3.87±0.03	17.0±1.0^{abA}	12.8±0.8^{bBC}	11.8±0.8^{bcC}	11.2±1.3^{cC}	12.8±1.0^{bcBC}	14.2±0.3^{bB}	15.2±0.8^{aAB}
		B1 hkCFE + KOM1 CFE	4.02±0.04	17.0±1.7^{abA}	12.8±2.0^{bC}	12.2±1.0^{bC}	12.2±0.8^{bC}	13.3±1.2^{bBC}	14.8±0.3^{abAB}	13.7±2.0^{bcB}
		B1 nCFE + KOM1 CFE	4.88±0.07	14.2±0.3^{bA}	10.3±0.8^{bcC}	11.5±1.3^{bcB}	11.2±1.6^{cBC}	11.2±1.3^{cdBC}	13.5±1.5^{bcAB}	13.5±2,8^{cAB}
	B1 + KNS32	B1 CFE + KNS32 CFE	3.88±0.02	16.8±0.6^{abA}	13.5±1.3^{aBC}	12.7±0.3^{bC}	12.8±0.3^{abC}	13.7±1.2^{bBC}	14.3±1.2^{bB}	15.3±1.8^{aAB}
		B1 hkCFE + KNS32 CFE	4.11±0.02	16.3±0.6^{bA}	12.7±2.3^{bC}	12.3±1.0^{bC}	13.2±0.8^{aBC}	14.8±1.3^{aB}	15.3±0.3^{aAB}	14.5±2.0^{abB}
		B1 nCFE + KNS32 CFE	5.04±0.07	14.3±0.3^{bA}	10.5±0.9^{bcC}	11.2±1.0^{bcC}	11.3±1.2^{cC}	11.7±1.5^{cBC}	13.3±0.6^{bcAB}	12.7±1.8^{cdB}
	O24 + KOM1	O24 CFE + KOM1 CFE	3.68±0.01	18.5±1.5^{aA}	13.5±0.5^{aBC}	12.8±1.0^{abC}	11.8±2.3^{bcC}	13.8±1.3^{abB}	14.5±0.9^{bAB}	13.8±1.8^{dB}
		O24 hkCFE + KOM1 CFE	3.78±0.02	18.8±2.0^{aA}	12.7±2.3^{bC}	13.0±0.0^{abBC}	12.0±2.3^{bC}	13.8±1.3^{abB}	14.8±1.0^{abAB}	14.7±1.6^{bAB}
		O24 nCFE + KOM1 CFE	4.37±0.04	16.2±1.0^{bA}	10.2±1.4^{bcC}	11.0±0.9^{cC}	11.3±1.8^{cBC}	11.5±1.8^{cBC}	12.3±0.6^{cdB}	13.2±2.8^{cdAB}
	O24 + KNS32	O24 CFE + KNS32 CFE	3.61±0.03	18.0±0.9^{aA}	13.0±1.0^{abC}	13.2±1.6^{aC}	13.3±0.6^{aBC}	14.0±1.0^{abB}	14.7±1.5^{bAB}	14.2±2.4^{cAB}
		O24 hkCFE + KNS32 CFE	3.91±0.10	18.5±0.9^{aA}	13.2±1.6^{abBC}	13.0±0.9^{aC}	13.0±0.5^{abC}	14.3±0.6^{aB}	14.8±0.3^{abAB}	14.2±2.4^{bB}
		O24 nCFE + KNS32 CFE	4.48±0.09	16.3±0.3^{bA}	10.5±3.9^{bcC}	11.0±1.8^{cC}	10.5±1.0^{cdC}	11.7±2.0^{cBC}	13.3±0.3^{bcAB}	12.8±3.0^{cdB}

Values are expressed as mean ± standard deviation; means followed by different letters (a-d) for each column are statistically significantly different (p<0.05); means followed by different letters (A-C) between the indicator strains are statistically significantly different (p<0.05);
Lm 19111 - *Listeria monocytogenes* ATCC 19111; *Sa* 25923 - *Staphylococcus aureus* ATCC 25923; *Bs* 6633 - *Bacillus spizizenii* ATCC 6633; *Ef* 51299 - *Enterococcus faecalis* ATCC 51299; SE 13076 - *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076; *Ec* 10536 - *Escherichia coli* ATCC 10536; *Pf* 2123 - *Pseudomonas fluorescens* PCM 2123

LABs when tested individually. For Gram-positive bacteria, significant synergistic effects ($p < 0.05$) were observed when combining LAB and AAB extracts. For example, the mixture of O24 CFE and KOM1 CFE exhibited greater inhibition of *E. faecalis* than O24 CFE alone. Likewise, for *B. spizizenii*, the combination of LAB extracts with KOM1 CFE significantly ($p < 0.05$) enhanced antibacterial activity compared with the KOM1 CFE alone.

In the case of Gram-negative bacteria, the synergistic effect was even more pronounced. Many different combinations exhibited significantly ($p < 0.05$) stronger inhibitory activity against *S.* Enteritidis than the individual LAB CFEs. Similarly, for *E. coli*, mixtures containing neutralized LAB extracts and AABs significantly ($p < 0.05$) enhanced growth inhibition compared to LAB CFEs alone. Regarding the pH analysis, pair-wise combinations of bacterial extracts showed pH values similar to those of the individual extracts, indicating limited interaction effects on acidity (Tab. 4). A moderate increase was noted only in mixtures containing the neutralized nCFEs.

Minimal inhibitory concentrations (MICs) and fractional inhibitory concentration index (FICI). The susceptibility of the studied indicator strains to LAB and AAB extracts was determined using the microdilution method. Table 5 presents the minimum inhibitory concentration (MIC) of individual LAB and AAB CFEs.

At relatively low MIC values, hkCFE B1 and hkCFE O24 exhibited inhibitory effects particularly against *L. monocytogenes* and *E. faecalis*. Similarly, AAB CFEs

Table 5. Minimum inhibitory concentration (MIC) of individual CFEs

Indicator strain	B1 CFE	B1 hkCFE	B1 nCFE	O24 CFE	O24 hkCFE	O24 nCFE	KOM1 CFE	KNS32 CFE
	MIC [AU/mL]							
Lm 19111	3.1	6.3	3.1	6.3	6.3	6.3	6.3	12.5
Sa 25923	12.5	12.5	12.50	25.0	25.0	50.0	12.5	12.5
Bs 6633	25.0	12.5	50.0	12.5	12.5	100.0	6.3	12.5
Ef 51299	3.1	6.3	3.1	6.3	6.3	6.3	6.3	12.5
SE 13076	50.0	6.3	100.0	6.3	6.3	25.0	3.1	6.3
Ec 10536	6.3	6.3	12.5	12.5	6.3	25.0	3.1	3.1
Pf 2123	25.0	12.5	12.5	25.0	6.3	25.0	3.1	6.3

Lm 19111 – *Listeria monocytogenes* ATCC 19111; *Sa* 25923 – *Staphylococcus aureus* ATCC 25923; *Bs* 6633 – *Bacillus spizizenii* ATCC 6633; *Ef* 51299 – *Enterococcus faecalis* ATCC 51299; SE 13076 – *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076; *Ec* 10536 – *Escherichia coli* ATCC 10536; *Pf* 2123 – *Pseudomonas fluorescens* PCM 2123; MIC – minimum inhibitory concentration

Table S1. Minimum inhibitory concentration (MIC) of LAB CFEs and KOM1 CFE when used in combination, determined using the checkerboard method

Indicator strain	KOM1 CFE	B1 CFE	KOM1 CFE	B1 hkCFE	KOM1 CFE	B1 nCFE	KOM1 CFE	O24 CFE	KOM1 CFE	O24 hkCFE	KOM1 CFE	O24 nCFE
	MIC [AU/mL]											
Lm 19111	1.6	1.6	0.8	1.6	1.6	3.1	1.6	1.6	3.1	1.6	1.6	3.1
Sa 25923	12.5	6.3	6.3	6.3	25.0	12.5	12.5	3.1	12.5	3.1	6.3	12.5
Bs 6633	12.5	1.6	1.6	1.6	12.5	3.1	6.3	3.1	3.1	3.1	25.0	6.3
Ef 51299	0.8	1.6	0.8	1.6	1.6	3.1	1.6	1.6	3.1	1.6	1.6	3.1
SE 13076	6.3	1.6	1.6	0.8	12.5	1.6	1.6	0.8	1.6	0.8	12.5	0.8
Ec 10536	1.6	0.8	1.6	0.8	3.1	1.6	6.3	1.6	1.6	1.6	3.1	1.6
Pf 2123	3.1	1.6	3.1	0.8	12.5	3.1	3.1	1.6	3.1	0.8	12.5	3.1

Lm 19111 - *Listeria monocytogenes* ATCC 19111; *Sa* 25923 -*Staphylococcus aureus* ATCC 25923; *Bs* 6633 - *Bacillus spizizenii* ATCC 6633; *Ef* 51299 - *Enterococcus faecalis* ATCC 51299; SE 13076 - *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076; *Ec* 10536 - *Escherichia coli* ATCC 10536; *Pf* 2123 - *Pseudomonas fluorescens* PCM 2123; MIC - minimum inhibitory concentration

Table S2. Minimum inhibitory concentration (MIC) of LAB CFEs and KNS32 CFE when used in combination, determined using the checkerboard method

Indicator strain	KNS32 CFE	B1 CFE	KNS32 CFE	B1 hkCFE	KNS32 CFE	B1 nCFE	KNS32 CFE	O24 CFE	KNS32 CFE	O24 hkCFE	KNS32 CFE	O24 nCFE
	MIC [AU/mL]											
Lm 19111	1.6	3.1	1.6	1.6	1.6	6.3	1.6	3.1	3.1	1.6	6.3	6.3
Sa 25923	25.0	6.3	25.0	3.1	25.0	12.5	12.5	12.5	25.0	6.3	3.1	12.5
Bs 6633	12.5	3.1	6.3	1.6	12.5	3.1	6.3	3.1	6.3	3.1	25.0	6.3
Ef 51299	3.1	3.1	3.1	0.8	3.1	6.3	3.1	3.1	3.1	1.6	6.3	6.3
SE 13076	6.3	1.6	1.6	1.6	25.0	1.6	3.1	0.8	1.6	0.8	12.5	0.8
Ec 10536	3.1	1.6	1.6	1.6	6.3	1.6	3.1	1.6	1.6	1.6	6.3	1.6
Pf 2123	12.5	1.6	3.1	0.8	3.1	1.6	6.3	1.6	3.1	1.6	12.5	3.1

Lm 19111 – *Listeria monocytogenes* ATCC 19111; *Sa* 25923 – *Staphylococcus aureus* ATCC 25923; *Bs* 6633 – *Bacillus spizizenii* ATCC 6633; *Ef* 51299 – *Enterococcus faecalis* ATCC 51299; SE 13076 – *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076; *Ec* 10536 – *Escherichia coli* ATCC 10536; *Pf* 2123 – *Pseudomonas fluorescens* PCM 2123; MIC – minimum inhibitory concentration

Table 6. FIC indexes and interaction types (FICI)

Indicator strain	Extracts	B1 CFE	B1 hkCFE	B1 nCFE	O24 CFE	O24 hkCFE	O24 nCFE
Lm 19111	KOM1 CFE	0.8	0.4	1.0	0.5	0.8	0.8
	KNS32 CFE	0.8	0.4	1.0	0.5	0.6	0.6
Sa 25923	KOM1 CFE	1.5	1.0	3.0	0.8	0.8	1.1
	KNS32 CFE	2.5	2.3	3.0	1.5	1.5	1.1
Bs 6633	KOM1 CFE	0.8	0.4	0.8	1.0	0.8	1.3
	KNS32 CFE	0.8	0.6	0.5	0.8	0.8	0.8
Ef 51299	KOM1 CFE	0.5	0.4	1.0	0.5	0.8	0.8
	KNS32 CFE	1.3	0.6	1.5	0.8	0.6	1.5
SE 13076	KOM1 CFE	0.6	0.5	0.6	0.5	0.5	0.8
	KNS32 CFE	0.4	0.5	0.5	0.6	0.4	0.6
Ec 10536	KOM1 CFE	0.5	0.5	0.8	1.0	0.8	0.6
	KNS32 CFE	1.0	0.8	1.0	0.8	0.8	0.8
Pf 2123	KOM1 CFE	0.6	0.5	2.0	0.6	0.8	1.5
	KNS32 CFE	0.8	0.4	0.5	0.5	0.8	1.0

FICI – fractional inhibitory concentration index; FICI ≤ 0.5 – synergistic; FICI > 0.5-1.0 – additive; FICI > 1.0-4.0 – no interaction; FICI > 4.0 – antagonistic; *Lm* 19111 – *Listeria monocytogenes* ATCC 19111; *Sa* 25923 – *Staphylococcus aureus* ATCC 25923; *Bs* 6633 – *Bacillus spizizenii* ATCC 6633; *Ef* 51299 – *Enterococcus faecalis* ATCC 51299; SE 13076 – *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076; *Ec* 10536 – *Escherichia coli* ATCC 10536; *Pf* 2123 – *Pseudomonas fluorescens* PCM 2123

demonstrated strong antimicrobial activity, especially against *E. coli* and *S.* Enteritidis, with MIC values reaching the lowest levels among the tested extracts. In contrast, the highest MIC values were observed for *B. spizizenii* and *S. aureus*, requiring significantly higher concentrations to inhibit growth. Among all tested extracts, KNS32 CFE was the most effective, exhibiting the lowest MIC values against multiple indicator strains, which highlights its strong antimicrobial potential. Further studies investigating the effects of LAB combined with AAB extracts were conducted using the checkerboard method (Tab. S1 and Tab. S2). Table 6 presents the fractional inhibitory concentration index (FICI) values, indicating the interactions between AAB and LAB extracts. The results suggest a range of interactions, from synergism to no interaction, depending on the combination and the indicator strain. Synergistic effects (FICI ≤ 0.5) were observed in certain combinations, such as B1 hkCFE with AAB extracts against *L. monocytogenes* and *S.* Enteritidis, as well as O24 hkCFE with KOM1 CFE for *S.* Enteritidis. Additive interactions (FICI > 0.5–1.0) were more frequently

noted, particularly for *E. faecalis* and *E. coli*, while no interaction (FICI > 1.0–4.0) was mostly recorded for *S. aureus* and *B. spizizenii*. Antagonistic effects (FICI > 4.0) were not observed in the tested combinations. Notably, the greatest number of cases of synergism between LAB and AAB extracts was observed against the pathogen *S.* Enteritidis, prompting further trials on a food model.

Model food trials with a chicken meat sample. Figure 1 presents the results of total viable counts and *S.* Enteritidis growth under the influence of the tested antimicrobial extracts on the surface of the chicken meat samples. The results demonstrate that the antimicrobial treatments effectively reduced the total bacterial load on chicken breast samples compared to the controls. After 24 hours of incubation at 37 °C, control samples treated with water, MRS broth and HS broth, exhibited higher total aerobic viable counts, with averages of approximately 5.67, 6.25, and 5.97 log CFU/mL, respectively. In contrast, samples treated with combinations of LAB and AAB extracts showed considerably lower counts, ranging from 4.00–4.96 log CFU/mL (Fig. 1A). After 7 days of storage at 4 °C, the controls still maintained relatively high counts (around 3.87–4.19 log CFU/mL), whereas the antimicrobial treatments further reduced the total viable counts, in some cases decreasing below the detection limit and not exceeding approximately 3.46 log CFU/mL (Fig. 1C). Similarly, *S.* Enteritidis counts were significantly lower in treated samples compared to controls. At 24 hours at 37 °C, the controls displayed *S.* Enteritidis counts averaging around 5.21–5.44 log CFU/mL, while B1 CFE together with KOM1 CFE reduced these counts to a range of approximately 0–3.92 log CFU/mL (Fig. 1B). Notably, after 7 days at 4 °C, no viable *S.* Enteritidis were detected in any of the antimicrobial-treated samples, in stark contrast to the controls, which maintained averages of approximately 4.03–4.19 log CFU/mL (Fig. 1D). Overall, these findings demonstrate that the antimicrobial treatments significantly inhibited total aerobic bacterial growth and *S.* Enteritidis survival, with a particularly pronounced effect observed after extended refrigerated storage.

DISCUSSION

The study evaluated the antimicrobial properties of bacterial extracts obtained from LAB (*Lacticaseibacillus paracasei* B1, *Lactiplantibacillus plantarum* O24) and AAB (*Gluconobacter oxydans* KNS32, *Komagataeibacter saccharivorans* KOM1), assessing their efficacy individually and in combination. While previous research has explored the antimicrobial activity of lactic and acetic acids in food preservation [26, 27], the current study extends to-date knowledge by investigating the biopreservative potential of non-living bacterial extracts. These treatments constitute complex mixtures of antimicrobial metabolites that extend beyond organic acids, including bacteriocins, peptides, and phenolic derivatives, as well as possible synergistic interactions between LAB- and AAB-derived components.

The study also identified organic acids, total phenolic content, and antioxidant properties of bacterial supernatants. Lactic acid was the most abundant substance in the LAB supernatants, which was the primary substance responsible

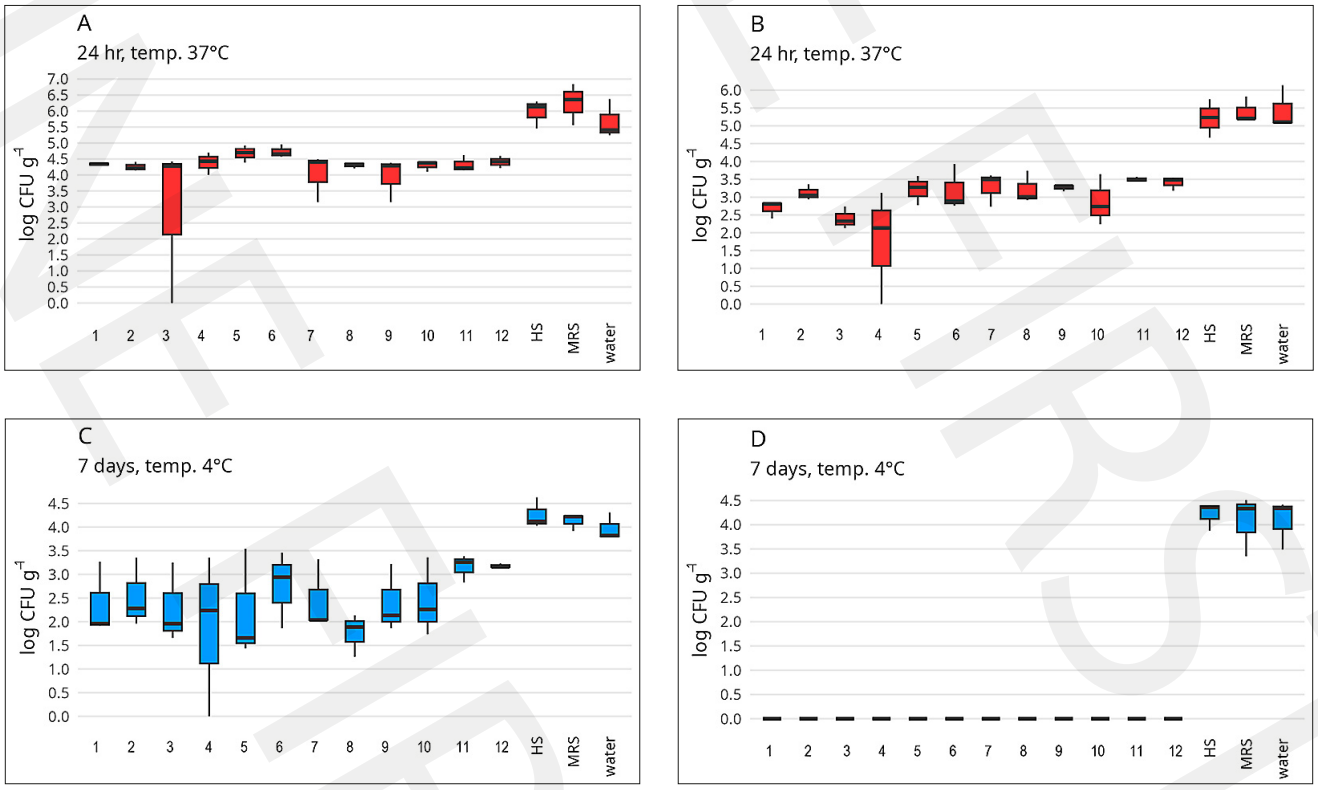

Figure 1. Viable counts on chicken breast samples treated with mixtures of antimicrobials and water, MRS broth, and HS broth (as controls) after 24 hours at 37 °C and 7 days at 4 °C (A and C – total aerobic viable counts; B and D – *S.* Enteritidis viable counts);
Abbreviations: The combinations tested in this study are numbered from (1) to (12) as follows: (1) B1 CFE + KOM1 CFE; (2) B1 CFE + KNS32 CFE; (3) B1 hkCFE + KOM1 CFE; (4) B1 hkCFE + KNS32 CFE; (5) B1 nCFE + KOM1 CFE; (6) B1 nCFE + KNS32 CFE; (7) O24 CFE + KOM1 CFE; (8) O24 CFE + KNS32 CFE; (9) O24 hkCFE + KOM1 CFE; (10) O24 hkCFE + KNS32 CFE; (11) O24 nCFE + KOM1 CFE; (12) O24 nCFE + KNS32 CFE

for lowering the pH value of the broth medium, in line with other findings [38–40]. Similarly, AAB produce a diverse array of organic compounds during their metabolic processes. As a result of AABs extraordinary capacity to oxidize a variety of sugars, polyols, and alcohols, antioxidant products of AAB metabolism mainly comprise gluconic acid, l-sorbose, bacterial cellulose, and levan [41]. Analysis in the current study identified acetic and gluconic acids as the main metabolites in AAB-derived extracts. The metabolism pathway of *G. oxydans* relies on its ability to selectively oxidize a wide range of carbohydrates and alcohols. The *Komagataeibacter* genus is particularly noteworthy for its ability to synthesize organic acids [42, 43]. Additionally, a relatively low concentration of propionic acid was detected. Notably, although AAB do not typically produce propionic acid as a primary oxidative fermentation product, their metabolic activity may indirectly contribute to its formation, or that of its derivatives [44–46].

Phenolic compounds were identified in high concentrations in all tested cell-free extracts. LAB fermentation is known to enhance the production of phenolics and flavonoids, which contribute to antioxidant activity [47, 48]. The antioxidant properties of LAB extracts are attributed to bioactive compounds, including exopolysaccharides, bioactive peptides, antioxidant enzymes, and manganese ions [49]. The results obtained confirmed that LAB cell-free extracts exhibit strong antioxidant potential, with varying abilities to scavenge ABTS•+ and DPPH radicals. Research on LAB strains isolated from food has previously demonstrated their ability to enhance antioxidant properties, particularly in fermented beverages, in which selected strains have been shown to boost phenolic content and increase the capacity to neutralize free radicals [50, 51]. Although AAB extracts displayed lower antioxidant potential than LAB, they play a key role in modulating phenolic compounds through conjugation, thereby enhancing their bioavailability and stability in food products [17, 18, 52]. AAB also contribute to polyphenol stabilization and can improve antioxidant activity through aerobic oxidative fermentation [53]. However, rather than synthesizing phenolic compounds *de novo* when grown in laboratory media, AAB are more likely to modify and transform existing phenolic structures [18]. While the Folin-Ciocalteu assay is widely used for assessing total phenolic content, it is not entirely specific to phenolic compounds, as it can also react with other reducing substances, including peptides and sugars [54]. Despite this limitation, the observed trends suggest that both LAB and AAB extracts contain bioactive compounds that contribute to antioxidant activity. Further characterization using more specific analytical techniques, such as mass spectrometry, would be beneficial to confirm the precise composition of these extracts. Nevertheless, the results of the current study highlight the promising antioxidant potential of LAB and AAB extracts for applications in food technology.

In the agar-based well-diffusion test, all LAB and AAB extracts, as well as their combinations, exhibited antimicrobial properties, although their effectiveness varied between samples. The greater susceptibility of *L. monocytogenes* compared to *E. faecalis* suggests that Gram-positive bacteria may vary in their response to the metabolic compounds produced by LAB and AAB. This variation may be attributed to differences in cell wall composition and stress response mechanisms [55]. Neutralizing the pH of the LAB extracts reduced, but did not completely eliminate, the antagonistic activity. The observed reduction in antimicrobial activity upon neutralization underscores the crucial role of organic acids in inhibiting pathogens, as they can penetrate bacterial membranes and disrupt glycolysis by inhibiting pH-sensitive enzymes [56]. These findings are consistent with previous reports indicating that neutralization diminishes antagonistic effects, confirming organic acids as key contributors to antimicrobial activity [57]. Interestingly, heating the bacterial culture to 80 °C enhanced the antibacterial activity of hkCFE, rather than reducing it. Results obtained in previous research indicate that the bacterial thermal inactivation process enables the maintenance of antimicrobial properties [58].

The current study also identified 2 types of interactions among LAB and AAB extracts – synergism and additivity – with synergistic combinations demonstrating the most potent antibacterial effects. These interactions may result from enhanced membrane permeability, metabolic disruption, or inhibition of microbial enzymes and pathways, thereby preventing the development of resistance mechanisms [19]. Notably, crude CFE and hkCFE combinations exhibited superior inhibitory effects at lower concentrations compared to individual strains, suggesting a broader antimicrobial spectrum with a lower risk of resistance development. Such findings align with studies showing that co-fermenting bacteria can produce a more diverse array of antimicrobial compounds than single-strain extract [59]. Given the challenge of antimicrobial resistance in foodborne pathogens, the combined use of LAB and AAB-derived extracts presents a promising strategy for food preservation. Multi-strain formulations not only enhance efficacy but also contribute to food safety and extended shelf life, offering a natural alternative to synthetic preservatives [60]. These results emphasize the potential of LAB-AAB mixtures as an innovative approach in food processing, warranting further exploration in real-world applications.

Recent studies have shown that many LAB extracts, in the form of cell-free supernatants, are effective in inhibiting or eliminating unfavourable microbiota in meat, and they possess properties that can be utilized as food [61–63]. For AAB, acetic acid is the main metabolite utilized in food preservation as a bactericidal compound [64]. The present study evaluated combinations in real-life conditions to determine their potential for use in meat preservation. Chicken breast meat was selected as the matrix for the analysis, as, according to the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), it is a common source of salmonellosis outbreaks. *S.* Enteritidis was used as the indicator strain at a concentration of 1×10^3 CFU/g, representing realistic contamination levels that remain detectable by standard plating methods. This concentration was chosen to balance practical detectability with biological relevance [65]. The results of the model food trial indicated that antimicrobial mixtures effectively inhibited the foodborne pathogen *S.* Enteritidis in the chicken breast after 24 h of incubation. Notably, all tested combinations exhibited bactericidal effects against *S.* Enteritidis after 7 days of refrigerated storage. Additionally, the longer the contaminated meat was exposed to bacterial preparations, the stronger the antimicrobial effects observed. Recent research has demonstrated that a combination of reuterin and lactic acid exhibits strong

bactericidal effects against *Salmonella enterica* subsp. *enterica* serovar Enteritidis, reducing its viable counts to zero within 10 minutes of contact [66]. Similarly, the combination of essential oils with phage-derived endolysin (LysPB32) significantly reduced *Salmonella enterica* subsp. *enterica* serovar Typhimurium counts in cooked ground beef to below the detection limit after 12 h of incubation, primarily through membrane disruption [67]. Additionally, Elbarbary et al. (2024) [68] and Łepecka et al. (2023) [69], found that acetic acid treatments in the form of apple cider vinegar significantly decreased *Salmonella* spp. populations in beef and wild boar loins, respectively. These findings align with the present study, reinforcing the efficacy of LAB- and AAB-derived preparations in controlling *S.* Enteritidis contamination in meat products.

The undoubted advantage of using bacterial preparations is that they contain a blend of antimicrobial substances. The utilization of single antimicrobial preparations, however, has some limitations. LAB-derived bacteriocins show limited activity against Gram-negative bacteria, and their purification is costly and labour-intensive. Their efficacy may also be reduced by interactions with food components or proteolytic enzymes [19]. Similarly, the use of organic acids, by-products of carbohydrate or oxidative fermentation, also has certain limitations. At higher concentrations, they can alter the sensory properties of food, and some microorganisms can develop tolerance to acidic conditions [70]. Compared with biopreservation methods that use a single antimicrobial agent, the utilization of a cocktail of bacterial preparations, especially their combination, is a promising strategy to overcome those obstacles.

Notwithstanding the promising results, further studies are required to confirm the effectiveness of the tested combinations. Previous research by Karbowiak et al. (2025) [71] demonstrated that *Lacticaseibacillus paracasei* B1 and *Lactiplantibacillus plantarum* O24 harbour multiple bacteriocin gene clusters located on both chromosomes and plasmids, producing proteinaceous antimicrobial compounds. Proteomic and spectroscopic analyses (SDS-PAGE, LC-MS/MS, and FT-IR) revealed novel bacteriocin-like proteins of approximately 10–15 kDa. These findings suggest that the bioactive effects observed in the present study may be associated with similar proteinaceous substances, emphasizing the need for further metabolomic and functional analyses to elucidate their underlying mechanisms.

Although strong inhibition of *S.* Enteritidis was demonstrated, further validation against a broader range of foodborne pathogens is necessary to confirm applicability across different food matrices. Another challenge lies in scaling up these antimicrobial preparations for industrial use, considering factors such as production cost, regulatory approval, and formulation stability. The potential impact on food quality must also be assessed since shelf life depends on the activity of spoilage microorganisms, enzymatic activity, and physicochemical changes. Future research should evaluate how these mixtures affect sensory properties, such as taste, texture, colour, and aroma. Given the potential sensory effects of organic acids and bacterial metabolites, consumer acceptance will be a crucial factor in determining the success of these products. Beyond meat preservation, the technology could also be applied to dairy and plant-based products. To optimize practical use, various delivery methods (e.g., spraying, dipping, or immersion) and encapsulation or controlled-release systems should be explored to enhance stability and efficacy in food applications. [72]. Addressing these challenges will be crucial for fully harnessing the biotechnological potential of LAB and AAB-derived extracts in sustainable food preservation.

CONCLUSIONS

The study provides new insights into mixtures of LAB (*Lacticaseibacillus paracasei* and *Lactiplantibacillus plantarum*) and AAB (*Gluconobacter oxydans* and *Komagataeibacter saccharivorans*) preparations, particularly regarding their antimicrobial properties. To date, no evidence has supported the use of combined LAB and AAB preparations in biopreservation, therefore this study helps to fill that knowledge gap. The findings revealed significant and novel outcomes that advance understanding of their potential applications, offering promising avenues for their use in food biopreservation. Post-cultivation preparations obtained from LAB and AAB exhibited notable antioxidant activity and can be regarded as sources of phenolic compounds. In addition, these preparations contained several organic acids, with lactic, acetic, and glucuronic acids identified as the most abundant. The results of *in vitro* and meat model tests indicate that combining the tested extracts in pairs (1:1) allowed for obtaining synergistic antimicrobial combinations at a lower dose than when used individually, reducing *S.* Enteritidis to below the detection limit within 7 days of chilling storage. No abolition or inhibition of their action was observed. Specifically, hkCFEs and extracts from AAB strains combined exhibited exceptional efficiency.

Future research should focus on identifying the specific bioactive compounds responsible for antimicrobial activity, validating the effectiveness of these preparations against a broader range of pathogens, optimizing application methods, and assessing their impact on food quality, including sensory attributes and industrial scalability.

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