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Enhanced Bioactivity and Anti-microbial Properties of *Lactobacillus plantarum* Fermented Purple Onion (*Allium cepa* L.) Extracts Against Selected Poultry Microbes

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Abstract

This study aimed to optimize the fermentation process of purple onion extract (POE) using *Lactobacillus plantarum* 1582 and evaluate the biological properties of the fermented product (FPOE). The *L. plantarum* 1582 strain, selected for its high survivability in POE, was fermented under optimal conditions of 30% POE concentration for 24 hours, resulting in significant bacterial growth. Post-fermentation analysis revealed an increase in total flavonoid content (P = 0.023) and decreases in phenolic, thiol, and allicin levels by 15.4% ($P < 0.05$), 11.4% ($P < 0.05$), and 12.6% ($P < 0.05$), respectively. Using LC-MS, two new sulfur-containing volatile compounds—2-vinyl-4H-1,3-dithiine and γ-glutamyl-S-2-propenyl-cysteine—were identified in FPOE. These compounds were not present in fresh POE. Antioxidant activities of FPOE, assessed through ABTS and DPPH assays, significantly decreased after fermentation. This study provides essential data on the biochemical changes and novel compound formation during the fermentation of POE with *L. plantarum* 1582, suggesting its potential for applications in the feed or functional food industries.

Introduction

The poultry industry is experiencing rapid growth on a global scale, necessitating large quantities of antibiotics to sustain production. This widespread use of antibiotics in livestock farming has led to significant concerns regarding antibiotic resistance (Nhung *et al*., 2017; Klein *et al*., 2018). Antibiotic-resistant pathogens pose a serious threat to both animal and human health, resulting in treatment failures and economic losses (Osman *et al*., 2018). Various alternatives to antibiotics have been investigated, including phytogenic additives, prebiotics, probiotics, symbiotics, organic acids, antimicrobial peptides, bacteriophages, antibodies, and feed enzymes (Suresh *et al*., 2018). However, these alternatives alone do not fully meet the efficacy of antibiotics in poultry production.

Plants from the *Allium* genus, such as garlic and onions, exhibit potent antibacterial activity due to their rich organosulfur compounds, phenolics, and saponins (Pan *et al*., 2015; Kim *et al*., 2016). For example, garlic has shown effectiveness in poultry feed, specifically against multidrug-resistant bacteria, such as *Salmonella typhimurium* and *Escherichia coli* O78 (Salem *et al*., 2017; Elmowalid *et al*., 2019). Purple onion (*Allium cepa* L.), commonly used in Vietnamese cuisine, is traditionally fermented with native lactic acid bacteria (Yu *et al*., 2023). This natural lactic acid fermentation improves the quality of vegetables and fruits, enhancing flavors through the production of volatile aroma compounds (Di Cagno *et al*., 2013). The process also releases amino acids from proteins, impacting product flavor (Ye *et al*., 2014). Previous studies have indicated that fermentation with lactic acid bacteria enhances the biological functions of *Allium* plants through bioactive component transformations, increasing antibacterial activity (Lee *et al*., 2004).

Spontaneous fermentation, however, may lead to the formation of toxic substances, such as biogenic amines, mycotoxins, and pathogenic bacteria (Xiang *et al*., 2019). Controlled fermentation with probiotic

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strains, such as those from indigenous sources, is preferred to ensure optimal adaptation and safety (Reuben *et al*., 2019). In contrast, heat treatments, while effective in eliminating microorganisms, often compromise biological properties (Ye *et al*., 2019). Notably, heat has been shown to reduce the antibacterial activity of certain *Allium* species (Kyung, 2012).

To date, there has been limited research on the use of fermented purple onion as a potential poultry feed additive. This study investigates the probiotic potential of *L. plantarum*-fermented purple onion extract as a means of reducing antibiotic reliance in poultry, leveraging the antimicrobial properties of both the probiotic strain and the *Allium* substrate. By selecting *L. plantarum* isolated from native chickens, we aimed to develop a host-specific probiotic capable of controlling gastrointestinal pathogens and improving overall productivity in poultry.

Materials and Methods

Bacterial strains

Fifty-seven probiotic strains of the genus *Lactobacillus* were isolated from the manure of indigenous chickens raised in a semi-free-range system without probiotic supplementation. These strains were screened based on morphological and biochemical characteristics. Seven strains were identified as *Lactobacillus plantarum* using the Microbact 12A Kit (Oxoid, Thermo Fisher Scientific) (Hai *et al*., 2024).

Six pathogenic bacterial strains were isolated from fecal samples of native chickens exhibiting diarrhea, suspected of *Escherichia coli* or *Salmonella* spp. infection; these strains include *E. coli* FG31-1, *E. coli* ExPEC_A338, *S. pullorum* NCTC10705, *S. typhimurium* FC13827, *S. typhimurium* DA34837, and *S. gallinarum* RKS5078, with corresponding GenBank ID CP142680.1, CP142559.1, UGWX01000002.1, MK886517.1, MN704402.1, and CP003047.1, respectively, and all carrying the virulence genes *stx1* (for *E. coli*) and *stn* (for *Salmonella* spp.). All bacterial strains were maintained at the Microbiology Laboratory, Faculty of Animal Husbandry and Veterinary Medicine, University of Agriculture and Forestry, Hue University.

Purple onions (*Allium cepa* L. var. *aggregatum* - NCBI GenBank ID: NC_057575.1), grown for 4–5 months under biosafety conditions according to the VietGAP TCVN 11892-1:2017 standard in Dien Mon, Phong Dien, Thua Thien Hue, were used in this study. The extraction process was adapted from Yadav *et al.* (2015). Briefly, after washing and removing damaged bulbs, the onions were sterilized by soaking in a 5% NaCl solution for 120 minutes, followed by UV irradiation (250 nm; 30 mW/cm² for 15 minutes). One hundred grams of onion was crushed, and the extract was filtered through two layers of gauze and centrifuged at 2800 × g for 15 minutes to remove insoluble particles. The resulting supernatant was collected as sterile purple onion extract (POE).

Following the method described by Fadare *et al*. (2022), 100 µL of each *L. plantarum* suspension (1 × 10^8 CFU/mL) was inoculated into an MRS medium with and without 12.5% (v/v) onion extract. Cultures were incubated at 37°C for 24 hours, and growth efficiency was determined using the plate count method. The survival rate (%) of each *L. plantarum* strain in the presence of onion extract was calculated as follows:

Survival rate (%) = 100 × (log CFU of test sample/log CFU of control sample)

Based on their survival rate in the presence of 12.5% onion extract, one *L. plantarum* strain was selected for genetic identification through 16S rRNA gene sequencing. A 1500 bp fragment of the 16S rRNA gene was amplified using the forward primer 27F (AGAGTTTGATCMTGGCTCAG) and the reverse primer 1492R (TACGGCTAC CTTGTTACGACTT) (Shokryazdan *et al*., 2014). PCR reactions were performed in a 20 µL volume containing 20 µL of NZYTaq 2× Green Master Mix, 0.5 µL of each primer, 6 µL of DNase-free water, and 2 µL of DNA template. Amplification products were visualized using the ImageMaster system (Pharmacia Biotech, UK). The 1.5 kb PCR product was purified and sequenced using Sanger sequencing. The resulting sequence was compared to the NCBI GenBank database using BLAST to confirm the strain's identity.

Purple onion extract was diluted with sterile distilled water to concentrations of 10%, 20%, 30%, 40%, and 50% (v/v). Each diluted extract was inoculated with the selected *L. plantarum* strain (10^8 CFU/mL) at a ratio of 100:1 (v/v). Liquid MRS medium was used as a control. The growth of *L. plantarum* in the different POE dilutions was assessed according to the method described by Bin Masalam *et al*. (2018).

At 0, 6, 12, 18, 24, and 48 hours after inoculation, 1 mL of each culture was sampled, homogenized, and plated onto MRS agar. Plates were incubated at 35 ± 1°C for 48 hours. Colony counts were performed at dilutions yielding 30–300 CFU, and the average values were calculated. The optimal POE concentration and fermentation time for *L. plantarum* growth were determined based on these results.

Fermentation of POE was then carried out using the optimized conditions to obtain the fermented POE extract (FPOE) for subsequent analyses.

The total phenolic content of POE and FPOE extracts (10 mg/mL) was determined according to the method described by Suh *et al*. (2016). Briefly, 20 µL of each sample was mixed with 100 µL of Folin–

Ciocalteu phenol reagent in a 96-well microtiter plate and incubated in the dark for 5 minutes. Then, 80 µL of 7.5% (w/v) Na_2CO_3 solution was added, and the mixture was incubated at room temperature for 30 minutes. Absorbance at 750 nm was determined with a microplate reader, and the outcomes were reported as milligrams of gallic acid equivalents (GAE) per gram of extract.

The total flavonoid content of POE and FPOE extracts was estimated using the method described by Suh *et al*. (2016) with minor modifications. In a 96-well microtiter plate, 20 µL of sample extract (50 mg/mL) was mixed with 180 µL of 90% diethylene glycol and 20 µL of 1 N NaOH. The mixture was incubated at room temperature for 60 minutes, absorbance at 405 nm was recorded using a microplate reader, with the results presented as milligrams of quercetin equivalents (QE) per gram of extract.

Thiol and allicin content in the POE extract were measured colorimetrically as described by Han *et al*. (1995) and Yang *et al*. (2014). For thiol determination, 100 µL of sample (10 mg/mL) was mixed with 100 µL of 1.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) solution in a 96-well microtiter plate and incubated at room temperature for 10 minutes. The absorbance was measured at 412 nm using a microplate reader and expressed as micromoles of cysteine equivalents per gram of POE. Total allicin content was determined indirectly by measuring the remaining cysteine concentration after reacting allicin with L-cysteine. For this, 100 µL of each sample was mixed with 250 µM L-cysteine and incubated for 10 minutes. Then, 100 µL of this mixture was added to 100 µL of 1.5 mM DTNB solution and incubated for another 10 minutes. The absorbance was measured at 412 nm. The allicin content was calculated using the following formula: Allicin content (µM) = C − (b − a) / 2; where: a is the thiol content in the sample, b is the thiol content after reaction with L-cysteine, C is the initial amount of L-cysteine added.

A high-resolution liquid chromatography-mass spectrometry (LC-MS) system, the microTOF-QII from Bruker Daltonics (Germany), equipped with an electrospray ionization (ESI) source, was employed to identify pharmaceutical substances. The system configuration includes an ion filter with a Dual Ion Funnel, a Hexapole, an Analytical Quadrupole mass isolator, an internal collision-induced dissociation (CID) ion source, a high-resolution time-of-flight (TOF) mass separator, and a multichannel ion detector. Chromatographic separation was performed using the Agilent 1200 ultra-high-pressure liquid chromatography (UHPLC) system (USA), featuring a high-pressure double pump for mixing flow, an automatic sample injector, and a column oven. Direct sample injections were executed with a KdScientific syringe pump (USA). The separation utilized a reversed-phase liquid chromatography method with an ACE3-C18 column (4.6 × 150 mm, 3.5 µm) maintained at 40°C. The mobile phase was set to a flow rate of 0.3 mL/min. Calibration for ion m/z accuracy was conducted using an ESI-L tuning mix, with direct sample injections at 200 µL/hr, as illustrated in Figure 1. Data processing was conducted on Bruker's Data Analysis software (Germany).

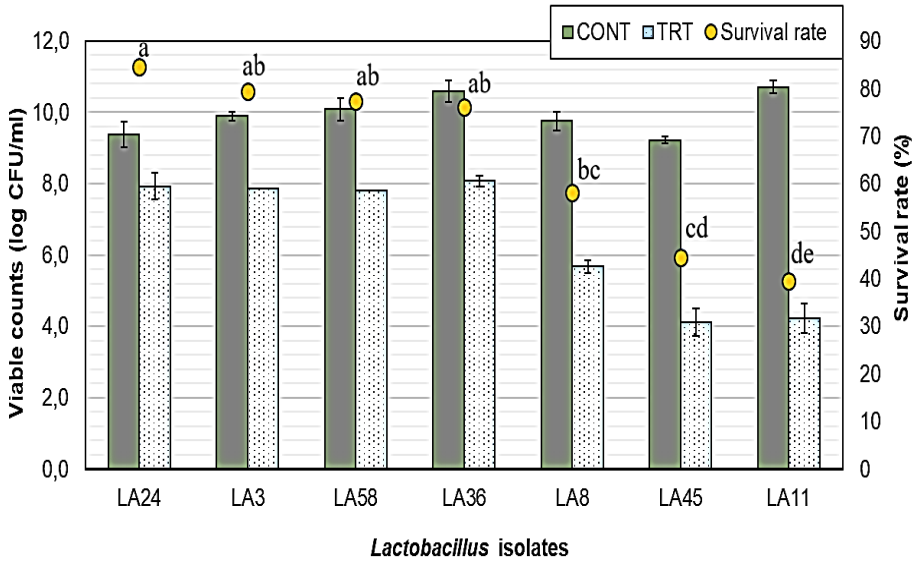

Figure 1. Survival rates of test isolates in POE medium (TRT) compared with the control group (CONT). The error bar is standard deviation.

The antibacterial activity of POE and FPOE against the pathogenic bacterial strains was evaluated using the agar well diffusion method as described by Aujoulat *et al*. (2011). Briefly, Muller Hinton agar (MHA) plates were overlaid with a 0.5 OD_{630} suspension of each pathogen. After 15–20 minutes, six wells (30 mm apart) were punched into the agar. Each well was filled with 100 µL of POE or FPOE (adjusted to 0.5 OD_{630}). The plates were allowed to diffuse at 4°C for one hour and then incubated at 37°C for 24 hours. The diameter of the inhibition zone (DIZ, mm) was measured for each well, and antibacterial activity was calculated using the following formula: DIZ = Diameter of the inhibition zone – Diameter of the well. Antibacterial activity was considered significant when DIZ ≥ 10 mm (Georgieva *et al.,* 2015).

The antiviral activity of POE and FPOE against the low-pathogenic influenza A(H1N1) pdm09 virus was evaluated using a hemagglutination (HA) assay in pathogen-free embryonated chicken eggs, adapted from the method described by Ding (2008). Sample extracts were prepared at 5 to 50 mg/mL concentrations and incubated with the H1N1 virus (1:1) at 4°C for 30 minutes. Aliquots (0.2 mL) of the POE/H1N1 mixture, FPOE/H1N1 mixture, or PBS/H1N1 mixture (positive control) were injected into the yolk sac cavity of 10-day-old embryonated eggs (five eggs per treatment). The injection sites were sealed with paraffin wax, and the eggs were incubated at 35°C for 5 days. After incubation, 0.2 mL of allantoic fluid was collected from each egg, and HA titers were determined using 1% (v/v) chicken erythrocytes at room temperature. Virus titers were expressed as egg infectious dose 50 (EID_{50})/mL.

The antioxidant activities of POE and FPOE were assessed using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay (Suh *et al*., 2016) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Lee *et al*., 2020) with modifications.

For the ABTS assay, a working solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate and incubating in the dark at room temperature for 16 hours. The solution was then diluted with water until the absorbance at 734 nm reached approximately 0.7 ± 0.02. In a 96-well plate, 20 µL of POE or FPOE extract (5 mg/mL) was added to 180 µL of the ABTS working solution. Following a six-minute incubation at 37°C in the dark, the absorbance of the mixture was measured at 734 nm using a BioTek microplate reader. An 80% methanol solution served as the reference.

In the DPPH assay, 20 µL of POE or FPOE extract (5 mg/mL) was combined with 180 µL of 0.2 mM DPPH solution prepared in 80% (v/v) methanol within a 96-well plate. The mixture was kept in the dark at room temperature for 30 minutes, and absorbance was recorded at 517 nm using a microplate reader.

The ABTS and DPPH radical scavenging activities were calculated using the following equation: Radical scavenging capacity (%) = $[1 - (A_s / Ac)] \times 100$; where A_s is the absorbance of the sample and Ac is the absorbance of the control.

Data analysis

All experiments, including phytochemical quantification (TPC, TFC, allicin, and thiol), LC/MS analysis, and biological activity assays (antibacterial, antiviral, and antioxidant), were performed in triplicate. The data were analyzed using IBM SPSS Statistics software (Version 25, IBM, USA). Results are expressed as mean ± standard deviation (SD). Group differences were assessed through one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for pairwise comparisons. Statistical significance was determined at $\alpha = 0.05$.

Results

Selection of *L. plantarum* strain for onion extract fermentation

The survival rates of test isolates cultured in POE medium (TRT) were compared with those in the control group (CONT), with statistically significant differences indicated by different letters ($P < 0.05$). The effect of POE on both co-cultured and single-cultured populations of two enteric pathogens was evaluated using submerged fermentation, based on viable bacterial counts and survival rates. In the POE medium, seven strains (LA3, LA8, LA11, LA24, LA36, LA45, and LA58) demonstrated survival counts of approximately 9-11 log CFU/mL, comparable to the control group at around 8 log CFU/mL. Among these, strains LA11 and LA45 exhibited significantly lower survival rates (approximately 40-45%; $P < 0.05$) compared to the other strains.

The strain LA24, displaying a high survival rate of 88.2% against POE's antibacterial activity, was selected for further analysis. Sequencing of its 16S rRNA gene revealed a 99.86% similarity with *L. plantarum* 1582 (GenBank No. MT597487.1), as shown in Figure 2. *L. plantarum*, a heterofermentative lactic acid bacterium, is known for its prominent role in the fermentation of various fruits and vegetables (Martino *et al*., 2016).

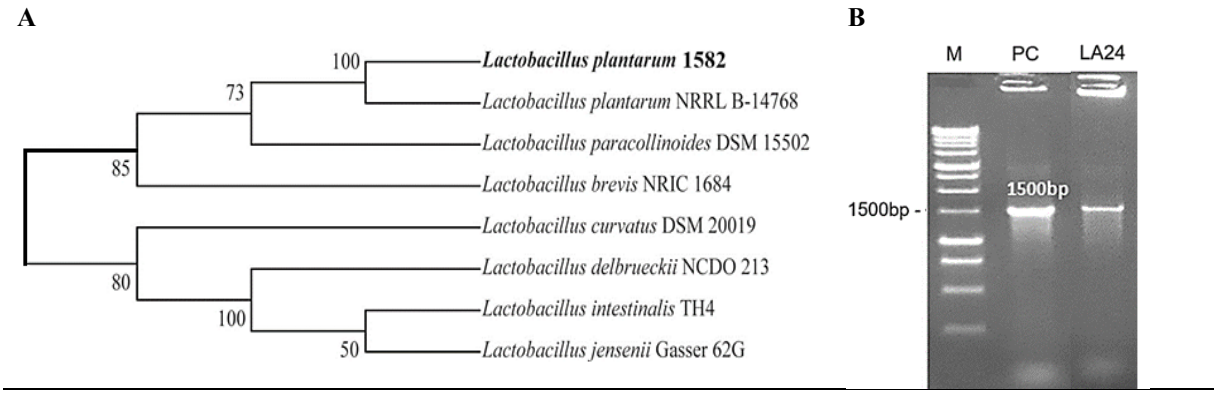

Figure 2. Phylogenetic analysis and agarose gel electrophoresis of *L. plantarum* strain 1582. **A**. Phylogenetic tree constructed from 16S rRNA gene sequences of *L. plantarum* strain 1582 and related strains within the genus *Lactobacillus*, with bootstrap values (from 1000 replicates) indicated at branch nodes. The scale bar denotes 0.01 substitutions per nucleotide position. **B**. Agarose gel electrophoresis results for PCR products following 16S rRNA amplification: lane M represents the 1 kb molecular marker, lane PC indicates the positive control and lane LA24 corresponds to the positive *Lactobacillus* strain at 1500 bp.

Evaluation of fermentation of purple onion using *L. plantarum*

To establish the optimal POE concentration for fermentation changes in pH and the growth of *L. plantarum* strain 1582 were monitored across various POE concentrations over 24 hours. Despite the known antibacterial properties of onion (Lee *et al*., 2004), *L. plantarum* 1582 exhibited robust growth (7.83–8.42 log CFU/mL) in the range of 10–50% POE concentrations (Figure 3A). However, viable cell counts declined marginally at 40% POE and significantly (P < 0.05) at 50% concentration compared to 10–30% (24 hours after inoculation). Additionally, the pH value at 50% concentration (4.5) was substantially higher (P < 0.05) than that observed at 10–40% concentrations (4.0–4.2).

A concentration of 30% POE was selected for subsequent studies. Figure 3B illustrates the progression of viable cell counts and pH over a 48-hour lactic acid fermentation at this concentration. The viable cell count rose sharply during the initial 12 hours, peaking at 24 hours (8.82 log CFU/mL), followed by a notable decline (P < 0.05) at 48 hours (8.21 log CFU/mL). Concurrently, the pH dropped significantly (P < 0.05) from 6.2 to 4.5 within the first 12 hours, stabilizing at approximately 4.0 from 24 to 48 hours. These findings suggest that a 24-hour incubation period is optimal for fermentation and will be applied in future studies.

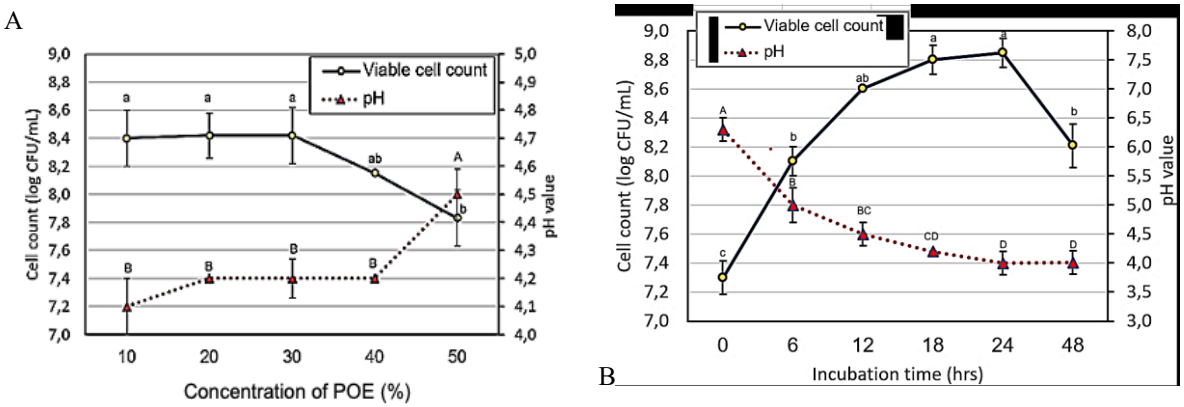

Figure 3. Growth of *L. plantarum* and pH variation during fermentation with different concentration of POE (A) and incubation time (B). Statistically significant differences (P < 0.05) in treatments are indicated by different letters (a-d) for growth and (A-D) for pH.

Changes in active compounds of medicinal herbs

Figure 4 reveals that after 24 hours of fermentation with the probiotic *L. plantarum*, the total phenolic content and organic sulfur compounds, including thiols and allicin, in FPOE significantly decreased by 15.4%, 11.4%, and 12.6%, respectively (P < 0.05) compared to POE. Conversely, the total flavonoid content significantly increased (P = 0.023) post-fermentation.

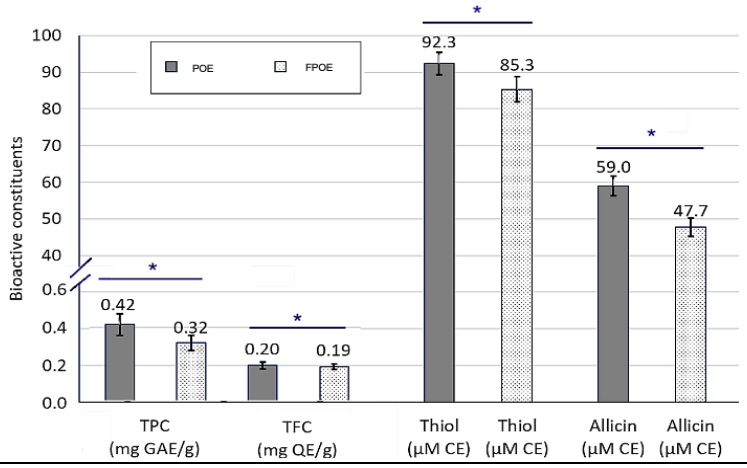

Figure 4. Modifications in the chemical composition of POE following fermentation with L. plantarum. *Indicates a significant difference ($P < 0.05$).

Using GC-MS analysis of extracts from both fresh and POE (Tables 1), five sulfur-containing compounds were identified: isoalliin ($C_6H_{11}NO_3S$), 2-vinyl-4H-1,3-dithiine ($C_6H_8S_2$), methyl-1-propenyl disulfide ($C_4H_8S_2$), diallyl disulfide ($C_6H_{10}S_2$), and γ-glutamyl-S-2-propenyl-cysteine ($C_{11}H_{18}N_2O_5S$). Results showed a higher concentration of sulfur compounds in the POE compared to fresh samples under identical extraction conditions. Notably, fermentation induced the formation of new sulfur compounds, namely 2-vinyl-4H-1,3-dithiine and γ-glutamyl-S-2-propenyl-cysteine, while major sulfur compounds such as isoalliin, methyl-1-propenyl disulfide, and diallyl disulfide exhibited only minor changes in molecular weight. These biochemical alterations reflect specific transformations occurring during the fermentation process.

Table 1. Sulfur-containing volatile compounds present in POE and FPO

Name	Molecular formula	RT (min)	*m/z* value
Isoalliinn	$C_6H_{11}NO_3S$	3.771	178.05312
Methyl-1-propenyl disulfide or Allyl methyl disulfide	$C_4H_8S_2$	4.394	121.06480
Diallyl disulfide	$C_6H_{10}S_2$	9.372	147.04775
2-vinyl-4H-1,3-dithiine	$C_6H_8S_2$	8.4	145.03256
γ-glutamyl-S-2-propenyl- cysteine	$C_{11}H_{18}N_2O_5S$	13.374	291.10047

Biological activity changes in POE following fermentation with *L. plantarum* Antibacterial activity

The antibacterial effects of POE and FPOE on 6 poultry pathogens associated with diarrhea are illustrated in Figure 5A. Both POE and FPOE exhibited inhibitory activity against these bacterial strains, with inhibition zones exceeding 10 mm (DIZ > 10 mm). Notably, FPOE demonstrated significantly enhanced inhibitory activity ($P < 0.05$) compared to POE, particularly against *Escherichia coli* (16.9–17.6 mm vs. 14.2–16.1 mm). Additionally, both extracts were more effective against *E. coli* strains than *Salmonella* strains (14.2–17.6 mm vs. 10.7–15.4 mm, $P < 0.05$).

Antiviral activity

An *in-ovo* model was utilized to assess the antiviral efficacy of FPOE and POE against low pathogenic avian influenza virus H1N1. As depicted in Figure 5B, the hemagglutination assay showed no detectable viral titers in the chicken embryos, indicating potential antiviral activity of POE. FPOE displayed significant antiviral efficacy at concentrations below 10 mg/mL, surpassing POE at equivalent concentrations ($P < 0.001$). Conversely, lower extract doses did not exhibit antiviral activity in chick embryos. Furthermore, both POE and FPOE demonstrated no cytotoxicity up to a concentration of 50 mg/mL, or 1 to 10 mg per chick embryo (data not shown).

Antioxidant activity

The antioxidant potential of POE and FPOE was assessed using ABTS and DPPH free radical scavenging assays. As illustrated in Figure 5C, the free radical scavenging activities of FPOE against 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were significantly reduced by approximately 37% and 28%, respectively, compared to POE ($P < 0.01$).

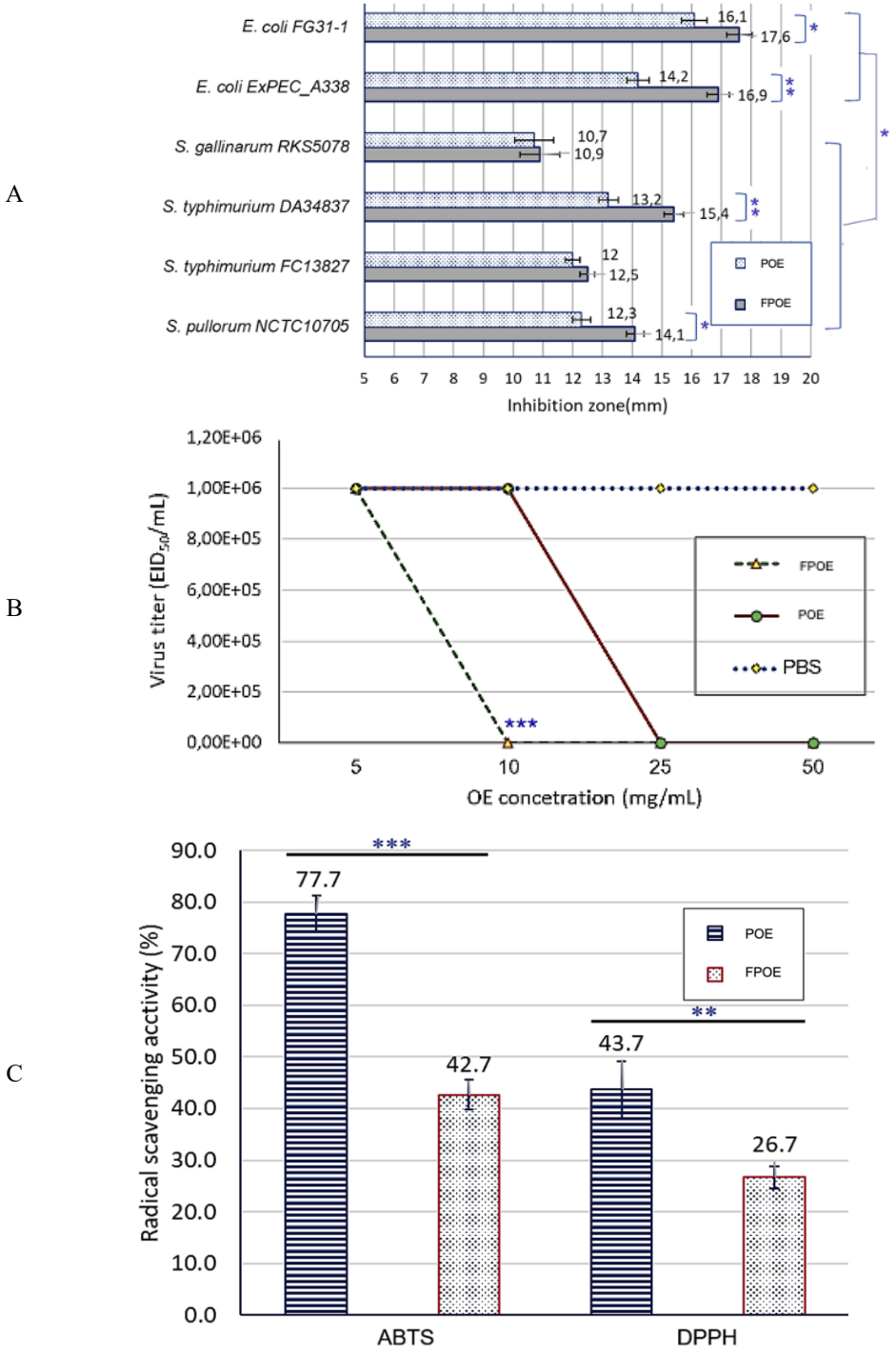

Figure 5. Alterations in biological activity of POE following 24 hours of fermentation with *L. plantarum.* A. Antibacterial activity of POE and FPOE against poultry pathogens measured by agar well diffusion; B. *In ovo* antiviral activity of POE and FPOE against avian influenza virus H_1N_1, assessed by hemagglutination assay. EID50/mL: 50% egg infective dose per mL; C. Antioxidant activity assessed by ABTS and DPPH free radical scavenging assays. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The error bar is standard deviation.

Discussion

The utilization of probiotic strains isolated from native hosts represents a promising approach for producing fermented products with enhanced quality and safety (Torres *et al*., 2020; Hai *et al*., 2019). Recognizing the potential of purple onion, we selected a lactic acid bacterium strain from the digestive tract of native free-range chickens for purple onion fermentation. Our findings indicate that *Lactobacillus plantarum* 1582 exhibited robust

survival during POE fermentation, even amidst the antibacterial properties of onions. Despite these properties, *L. plantarum* displayed normal growth at 10–30% POE concentrations, with viable cell counts ranging from 7.83 to 8.42 log CFU/mL. Cell counts significantly decreased at higher POE concentrations (50%), and pH values increased, suggesting a stronger antibacterial effect at elevated POE concentrations. This outcome aligns with previous studies indicating that while *L. plantarum* can ferment various plants, its survival depends on the plant's antibacterial characteristics.

A 30% POE concentration was found to be optimal for purple onion fermentation, as this concentration resulted in the highest viable cell count and an ideal fermentation pH. During the initial 12 hours, a rapid pH reduction from 6.2 to 4.2 indicated an acidification favorable for *L. plantarum* growth. Compounds like isoalliin and disulfides (methyl-1-propenyl disulfide and diallyl disulfide) remained stable through fermentation, contributing to the distinctive sulfur profile of POE. Certain sulfur compounds, such as those detected in this study, are known for their antibacterial, antioxidant, and anticancer properties (Rahman & Billington, 2000). The formation of new sulfur compounds, such as 2-vinyl-4H-1,3-dithiine and γ-glutamyl-S-2-propenyl cysteine, likely results from enzymatic processes during fermentation, with potential antioxidant and antibacterial activities.

The transformation of allicin into diallyl disulfide and other compounds was also observed, consistent with the literature (Lee *et al*., 2016). *L. plantarum* can synthesize sulfur compounds like γ-glutamyl-S-2-propenyl-cysteine, enhancing the sulfur compound diversity in POE (Rhee *et al*., 2011). The acidic environment created during fermentation promotes reactions among sulfur compounds, facilitating the formation of compounds such as 2-vinyl-4H-1,3-dithiine. As a well-known starter culture, *L. plantarum* enhances the sensory attributes of fermented foods by releasing key metabolites (Ming *et al*., 2018; Brizuela *et al*., 2019).

Fermentation resulted in a reduced total phenolic content, likely due to microbial utilization, which also corresponded with diminished antioxidant activity in FPOE compared to POE. Similar findings have been reported, such as the depletion of phenolic compounds during olive fermentation (Othman *et al*., 2009). The total flavonoid content, however, significantly increased, likely due to the release of bound phenols (Park *et al*., 2019). Flavonoid and polyphenol content do not necessarily correlate with biological activities; instead, specific metabolites may provide more accurate indicators. The reduction in thiols and allicin likely results from their volatility and utilization during fermentation (Yabuki *et al*., 2010; Filocamo *et al*., 2012).

The decrease in allicin content aligns with other studies (Yang *et al*., 2014), such as garlic fermentation, which reduces γ-glutamyl peptide levels, a precursor in flavor biosynthesis (Kothari *et al*., 2019). This reduction in pungent thiols and allicin may improve flavor, enhancing livestock acceptance. Allium herbs exhibit broad-spectrum antibacterial activity due to sulfur compounds that can disrupt bacterial membranes and metabolic functions (Casella *et al*., 2013; Putnik *et al*., 2019). However, these compounds' instability can reduce antibacterial efficacy over time (Millet *et al*., 2012). FPOE's superior antibacterial activity can be attributed to *L. plantarum*, which has demonstrated efficacy against poultry pathogens in vitro and in vivo (Dec *et al*., 2014; Wang *et al*., 2017).

POE fermentation with *L. plantarum* also reduced the pH to approximately 3.95 after 24 hours, achieving a viable cell count of ~9 log CFU/mL. The lower pH likely contributed to pathogen inhibition and product preservation (Torino *et al*., 2013; Wouters *et al*., 2013). The capability to inhibit pathogenic growth is crucial in selecting probiotics as antibiotic alternatives. *L. plantarum*'s antibacterial properties also contribute to competition with spoilage bacteria, improving product stability. In addition, flavonoids like quercetin, kaempferol, myricetin, rutin, and isorhamnetin, which are common in *Allium* species, possess biofilm-inhibiting properties (Al-Yousef *et al*., 2017).

This study's *in ovo* model demonstrated antiviral activity by POE and FPOE, reducing hemagglutination in allantoic fluid, which implies reduced viral loads in chick embryos. *L. plantarum*'s improved antiviral efficacy was more potent at lower concentrations than POE, likely due to increased flavonoid content (Ghoke *et al*., 2018). This enhanced antiviral effect in fermented POE may result from multiple metabolites' synergistic actions.

While antioxidants in medicinal herbs mitigate free radical damage, FPOE's reduced antioxidant activity aligns with decreased total polyphenol content due to microbial consumption. However, flavonoid increases may partially offset these effects, as fermentation can release bound flavonoids (Yang *et al*., 2023).

Conclusion

This study identified *L. plantarum* 1582, isolated from chicken gastrointestinal tracts, as an effective fermenting agent for POE. Under optimal conditions (30% POE, 24-hour fermentation), FPOE exhibited significant biological properties, with enhanced antibacterial effects against *E. coli*, *Salmonella* spp., and antiviral activity against avian influenza H_1N_1. Chemical composition changes included increased flavonoids and two new sulfur compounds, 2-vinyl-4H-1,3-dithiine, and γ-glutamyl-S-2-propenyl-

cysteine. These findings suggest that FPOE could serve as a probiotic alternative to antibiotics in poultry farming. However, additional *in vivo* studies are required to confirm its practical efficacy.

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