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Lactiplantibacillus plantarum fermentation boosts antioxidant and antibacterial effects in *Allium* species, potentially replacing chicken antibiotics

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ABSTRACT: This study aimed to enhance the antioxidant and antibacterial properties of extract of purple onion (OE, *Allium cepa* L.) and chive (CE, *Allium schoenoprasum*) bulbs through *Lactiplantibacillus plantarum*-mediated fermentation regarding their antioxidant capacity, antibacterial activity. Among seven isolates from local free-range chicken feces, LA24 was selected for its highest survival rate (89%) against the antibacterial activities of both OE and CE. It was identified as *L. plantarum* 1582 through 16S rRNA gene sequencing and was utilized for the CE and OE fermentation study. Fermentation significantly increased the total phenolic content (TPC) of both extracts. Specifically, the TPC of fermented purple onion extract (FOE) increased from 4.16 to 11.63 mg gallic acid equivalents (GAE)/g. while the TPC of fermented chive extract (FCE) increased from 8.51 to 23.45 mg GAE/g. Furthermore. both FOE and FCE exhibited enhanced antioxidant activities. The DPPH radical scavenging activity. measured by IC50 values. improved from 17.14 to 11.21 mg/mL for FOE and from 4.63 to 3.18 mg/mL for FCE. Both extracts also demonstrated increased reducing power following fermentation. Importantly, FCE showed stronger antibacterial activity against *Escherichia coli* and *Salmonella* spp., key pathogens associated with diarrhea in broiler chickens, compared to FOE. *In vivo* studies in chickens confirmed the transient colonization potential of *L. plantarum* 1582 in the chicken gut. These findings suggest that fermented *Allium* extracts, particularly FCE, hold promise as natural antibiotic alternatives in poultry feed, contributing to improved antioxidant status and disease resistance.

Keyword: *Allium genus; Antibacterial activity; Lactic fermentation; Lactiplantibacillus plantarum; Poultry*.

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INTRODUCTION

Currently, *Escherichia coli* and *Salmonella* spp. are common Gram-negative bacteria responsible for gastrointestinal diseases in poultry, leading to significant economic losses in the livestock industry (Newman et al., 2021). Importantly, these diseases can be transmitted from animals to humans (zoonoses), primarily through contaminated food, posing serious public health issues (Mertz, 2016). In fact, both *E. coli* and *Salmonella* have been listed by the World Health Organization (WHO) as pathogens requiring antibiotic susceptibility testing prior to treatment (WHO, 2023). Consequently, there is an urgent need to explore natural alternatives to antibiotics.

The *Allium* genus (*e.g.*, purple onion and onions) has demonstrated promising antibacterial activity due to the high levels of organosulfur compounds, phenolics, and saponins, among other bioactive compounds (Pan et al., 2015; Kim et al., 2016). Recently, purple onion has been shown to enhance poultry feed as an effective antimicrobial agent, exhibiting targeted toxicity against pathogens, particularly multidrug-resistant bacteria such as *Salmonella typhimurium* (Salem et al., 2017) and *Escherichia coli* O78 (Elmowalid et al., 2019). Among various *Allium* species, purple onion and chive are widely available herbs used in Vietnamese cuisine and have potential as poultry feed additives.

Pickled onions, a traditional Vietnamese dish, are produced through natural fermentation involving native lactic acid bacteria (LAB). This process primarily involves LAB present in the environment, contributing to the production of fermented foods (Yu et al., 2023). During fermentation, amino acids may be released from proteins through LAB-mediated proteolysis, influencing the flavor profile of the product (Ye et al., 2014). Probiotic fermentation of plants can alter the composition of bioactive compounds such as antioxidants, vitamins, and fiber, enhancing the biological activity of the products (Millet et al., 2012). Additionally, fermented plant-based foods can serve as a source of probiotics, providing health benefits (Peres et al., 2012).

To our knowledge, there is limited information regarding the functional properties of purple onion and chive extracts fermented with LAB for potential development as poultry feed additives. Furthermore, *Lactiplantibacillus plantarum* is well-documented as the dominant LAB species in plant fermentation (Di Cagno et al., 2013). Isolating probiotic strains from the natural host is preferable, as these strains are better adapted to the host's gastrointestinal environment (Reuben et al., 2019). To our knowledge, there is limited information regarding the functional properties of purple onion and chive extracts fermented with LAB for potential development as poultry feed additives. Previously, our group identified seven *Lactobacillus* isolates from local free-range chicken feces as a suitable strain for fermenting purple onion extract, demonstrating its survival and antibacterial potential against *Salmonella* spp. in chickens (Hai et al., 2024). Building on this, the current study evaluates the antioxidant and antibacterial activities of both fermented purple onion and chive extracts, focusing on their potential as natural antibiotic alternatives in poultry feed.

MATERIALS AND METHOD

Bacterial strains used in the study

Pathogenic isolates included *Salmonella enterica* serovar Pullorum NCTC10705 (GenBank ID: UGWX01000002.1), *S. enterica* serovar Typhimurium FC13827 (MK886517.1), *S. enterica* serovar Typhimurium DA34837 (CP029568.1), *Escherichia coli* FG31-1 (CP142680.1), *E. coli* ExPEC_A338 (CP142559.1), and *E. coli* EGI30 (MN704402.1). These isolates, carrying virulence genes *stx1* (*E. coli*) and *stn* (*Salmonella* spp.), were obtained from feces of local chickens with suspected *E. coli* or *Salmonella* infections.

Seven *Lactobacillus* isolates (LA3, LA8, LA11, LA24, LA36, LA45, LA58) were isolated from feces of local free-range chickens (Hai et al., 2024). These isolates demonstrated inhibitory activity against the pathogenic isolates, producing clear zones >10 mm in diameter in agar well diffusion assays. All isolates were preserved at the Microbiology Laboratory, College of Agriculture and Forestry, Hue University, Vietnam.

Lactiplantibacillus

Preparation of purple onion and chive extracts

Purple onion (*Allium cepa* L. var. *aggregatum* - GenBank ID: NC_057575.1, 4-5 months) and chive (*Allium schoenoprasum* var. *pumilum* - GenBank ID: HE687252.1, 4-5 months) were cultivated under organic standards (VietGAP TCVN 11892-1:2017) in Dien Mon, Phong Dien, TT-Hue, Vietnam. Bulbs were washed, damaged ones discarded, and soaked in 5% NaCl solution for 120 minutes for disinfection. One hundred grams of bulbs were homogenized using a Panasonic MJ-SJ01WRA blender, filtered through double-layered muslin cloth, and

centrifuged at 5000 rpm for 15 minutes to remove insoluble particles. The resulting liquid was then stored at -20°C.

Microbial analysis

Changes in microbial counts were performed by routine plate counting. Specifically, microbial suspensions were diluted with appropriate serum and spread onto de Man, Rogosa and Sharpe (MRS) agar (Oxoid Ltd., Basingstoke, England) to count *Lactobacillus* spp. following the method described by Harrigan (1998). The MRS agar plates were incubated anaerobically at 37°C for 24 hours (85% N_2, 10% H_2, and 5% CO_2).

Fermentation of purple onion and chive

The production process for the fermented product was modified according to Mangisah et al. (2021). The selected pure *Lactobacillus* strain was cultured on MRS agar and incubated anaerobically at 37°C for 2 days. Skimmed milk was autoclaved at 121°C for 15 minutes, then diluted in distilled water at a ratio of 1:14 (v/v) and stirred until homogeneous to create a growth medium for the bacteria. *Lactobacillus* (from two plates) was added to skimmed milk (200 mL) and incubated at 37°C for 2 days under anaerobic conditions. To produce the product, OE or CE was mixed and placed in an anaerobic chamber, dissolved in skimmed milk containing *Lactobacillus* at a ratio of 1:2 (v/v), and stirred until homogeneous. The fermentation process occurred at 30°C for 18 hours with shaking at 100 rpm to obtain the fermented purple onion (FOE) and chive (FCE) extracts. This selected isolate, based on its highest survival rate against the antibacterial activities of both OE and CE, was then identified and confirmed as *Lactiplantibacillus plantarum* through 16S rRNA gene sequencing and used for further studies.

Determination of total phenolic content

The total phenolic content in the samples was determined using the Folin–Ciocalteu method as described by Dziri et al. (2012) with some adjustments. Specifically, 1 mL of the extract was diluted appropriately and mixed with 2 mL of Folin–Ciocalteu reagent (10-fold diluted). This mixture was allowed to react for 6 minutes, followed by the addition of 3 mL of 10% (w/v) sodium carbonate solution. The reaction mixture was kept in the dark for 30 minutes, and the absorbance was measured at a wavelength of 725 nm using a VersaMaxTM Tunable microplate reader. The total phenolic content was calculated based on a standard curve using gallic acid as a standard. Each sample was analyzed in triplicate, and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract (mg GAE/g).

Determination of DPPH free radical scavenging activity

The activity of the samples against the free radical 2.2-diphenyl-1-picrylhydrazyl (DPPH) was measured according to the method of Juan and Chou (2010), with some modifications. Specifically, a 0.1 mM DPPH solution in ethanol was prepared, and 1 mL of DPPH solution was mixed with 1 mL of the sample at various concentrations. After incubation in the dark at room temperature for 30 minutes, the absorbance was measured at 517 nm. DPPH scavenging activity was calculated using the formula: DPPH Scavenging Activity (%) = [(Absorbance of control − Absorbance of sample) / Absorbance of control] × 100. Antioxidant activity was expressed as IC50 concentration, which is the concentration required to reduce 50% of the DPPH radical.

Reducing power assay

The reducing power of the samples was determined using the method of Juan and Chou (2010) with some modifications. One mL of the sample was mixed with 2.5 mL of phosphate buffer solution (200 mM, pH 6.6) and 2.5 mL of 1% potassium ferricyanide (K_3FeCN_6). This mixture was incubated at 50°C for 20 minutes. Following incubation, 1 mL of 10% trichloroacetic acid was added, and the sample was centrifuged at 3000 × g for 10 minutes. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride ($FeCl_3$), and the absorbance was measured at 700 nm. Higher absorbance reflects greater reducing power.

Antibacterial activity against pathogenic gastrointestinal bacteria in chickens

The antibacterial activity of *Lactiplantibacillus* isolates against pathogenic isolates (*S. pullorum*, *S. typhimurium*, *E. coli* strains, as presented previously) *Lactiplantibacillus* was assessed using the agar well diffusion method as described by Hai et al. (2024). Muller Hinton Agar (MHA) plates were inoculated with a 0.5 OD_{630} bacterial suspension. After 15–20 minutes, six wells (spaced 30 mm apart) were punched into the MHA. Each well contained 100 µL of an overnight culture of the selected *Lactiplantibacillus* strains (adjusted to 0.5 OD_{630}). The culture was allowed to diffuse into the agar for one hour at 4°C. The plates were then incubated at 37°C for 24 hours to assess the antibacterial activity. The

evaluation formula used was: Diameter (mm) = Diameter of the sterile zone - Diameter of the well.

Assessment of bacterial viability in the intestinal tract of chickens

In this study, all procedures involving the use of chickens were conducted in accordance with the ethical standards and approvals of the Animal Welfare Committee, Hue University, Vietnam, under certificate number HUVNO39, approved on May 20th, 2024. The survival of *Lactobacillus* spp. in the chicken intestinal tract was evaluated as described by Hai et al. (2024). Briefly, a total of 54 one-day-old healthy chicks were obtained from 3FViet Co. (Vietnam), divided into three groups, each with three replicates (6 chicks per replicate). Two experimental groups were administered 1 mL of the fermented extract containing 10^9 CFU/mL *Lactiplantibacillus*, while the control group received 1 mL of distilled water. The chicks were housed in metal cages (0.9 x 0.5 x 0.5 m) under continuous light and maintained at 35°C throughout the experiment (1-3 days old). Prior to the experiment, the cage system and floor were sterilized using heat (gas torch) and disinfectants (Povidine 10%). The chicks were fed a diet composed of locally sourced ingredients such as rice bran, corn meal, peanut meal, and soybean meal, meeting the standards set by the Ministry of Agriculture and Rural Development of Vietnam (10 TCN 661-2005). Before use, the feed and water were sterilized using UV light (300 µW-s/cm², 30 minutes) and provided ad libitum to the chicks. At 24, 48, and 72 hours, three chicks from each group (one per replicate) were randomly selected and euthanized, and samples were collected from the duodenum, cecum, and colon. The number of *Lactobacillus* isolates was determined at the designated time points. Results were expressed as the average isolate count per gram in the duodenum, cecum, and colon.

Statistical analysis

All tests were performed in triplicate, and data were expressed as mean ± standard deviation (SD) and percentage (%).Statistical analysis was conducted using IBM.SPSS software (Version 22), with significance compared and analyzed by one-way ANOVA, followed by Tukey's post hoc test and considered significant at a significance level of $P<0.05$.

RESULTS

Selection of *Lactobacillus* strains with potential for fermentation

The effects of a 50%:50% mixture of purple onion extract (OE) and chive extract (CE) (v/v, 12.5% concentration) on the survival of seven *Lactobacillus* isolates were assessed using a submerged fermentation method (Figure 1). Strains LA3, LA8, LA24, LA36 and LA58 exhibited survival rates (76-89%), significantly higher ($P<0.05$) than the 2 strains LA 11 and LA 45 (40 - 45%).

The bacterial isolate LA24 with the highest sur-

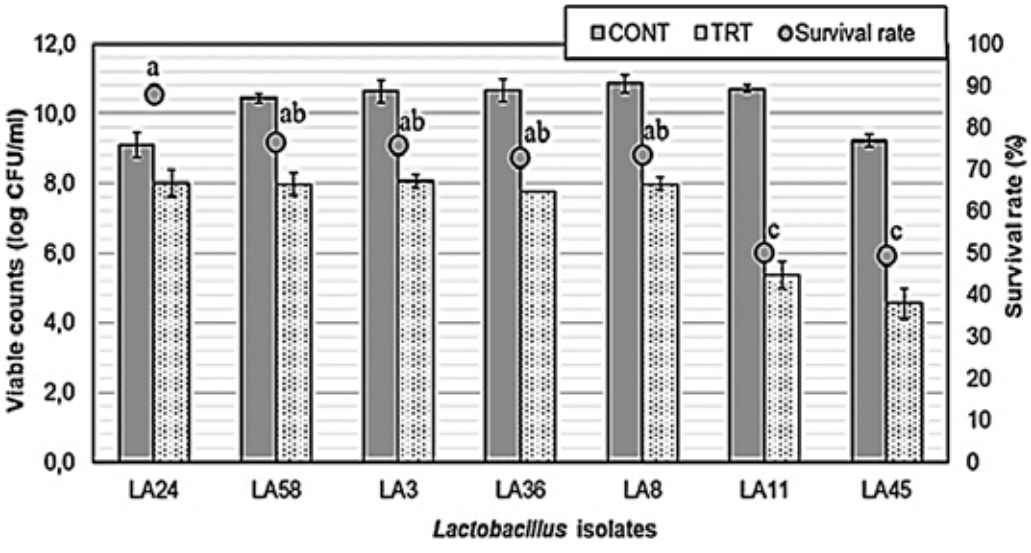

Figure 1. Survival of isolated *Lactobacillus* strains in 50% purple onion extract (OE) and 50% chive extract (CE) (v/v) over 24 hours. Values with different superscript letters ($^{a-c}$) indicate statistically significant differences ($P<0.05$).

vival ability (89%) against the antibacterial activity of OE was selected and sequenced for its 16S RNA gene. Phylogenetic analysis showed that this strain had a high degree of similarity (99.86%) with *L. plantarum* 1582 (GenBank Accession no MT597487.1) (Figure 2).

Antioxidant activity of purple onion and chive before and after fermentation

The results from Figure 3A show that, after fermentation with *L. plantarum*, the total phenolic content (TPC) in FOE and FCE increased nearly 3 times, from 4.16 to 11.63 mg GAE/g and from 8.51 to 23.45 mg GAE/g, respectively. Notably, FCE exhibited significantly higher TPC ($P<0.05$) than FOE both before and after fermentation.

CE extract had the highest DPPH scavenging activity with the lowest IC50 of 4.63 mg/mL, while OE had the highest IC50 of 17.14 mg/mL (Figure 3B). This showed that fermentation increased the DPPH scavenging ability ($P<0.05$). In addition, Figure 3B shows that the DPPH scavenging activity of the chive extract tended to increase with concentration.

Similarly, the reducing power assay (Figure 3C) showed significantly increased absorbance ($P<0.05$) after fermentation, particularly for FOE, the remaining 3 extracts also increased, but no statistically significant difference was detected ($P>0.05$).

Antimicrobial activity of purple onion and chive before and after fermentation

Figure 4 shows the antibacterial effects of the extracts before and after fermentation on six pathogens (*S. enterica* serovar Pullorum NCTC10705, *S. enterica* serovar Typhimurium FC13827, *S. enterica* serovar Typhimurium DA34837, *E. coli* FG31-1, *E. coli* ExPEC_A338, *E. coli* EGI30) causing diarrhea in chickens. Post-fermentation, inhibition zones significantly increased ($P<0.05$), with FOE's inhibition zone against *S. enterica* serovar Pullorum NCTC10705 rising more than 2 times, from 10.04 ± 0.87 mm to 22.12 ± 1.20 mm, and FCE's against *E. coli* ExPEC_A338 increasing from 12.35 ± 0.95 mm to 25.67 ± 1.45 mm (Figure 4).

Survival of *Lactobacillus* in the intestinal tract of chickens supplemented with fermented extracts

During 72 hours of monitoring after feeding chickens with FOE or FCE (1g/chicken, 9.0 log CFU *Lactobacillus*), the chickens did not show any abnormal clinical symptoms, and their ability to receive food was guaranteed.

The graph in Figure 5 shows the survival ability through the number of probiotics and their percentage compared to the initial supplementation for chickens (9.0 log CFU/g) *L. plantarum* 1582 in the fermented extract of chives or purple onions in the ileum, cecum and colon of chickens after 24, 48 and 72 hours of supplementation. In general, the survival of *Lactobacillus* in both treatments in the chicken intestine decreased over time after supplementation, although both survived relatively well in the first 24 hours (36.13 – 38.58%, equivalent to a density of 4.09 – 4.41 log CFU/g) but decreased significantly after 48 hours (30.28 – 36.12%, equiv-

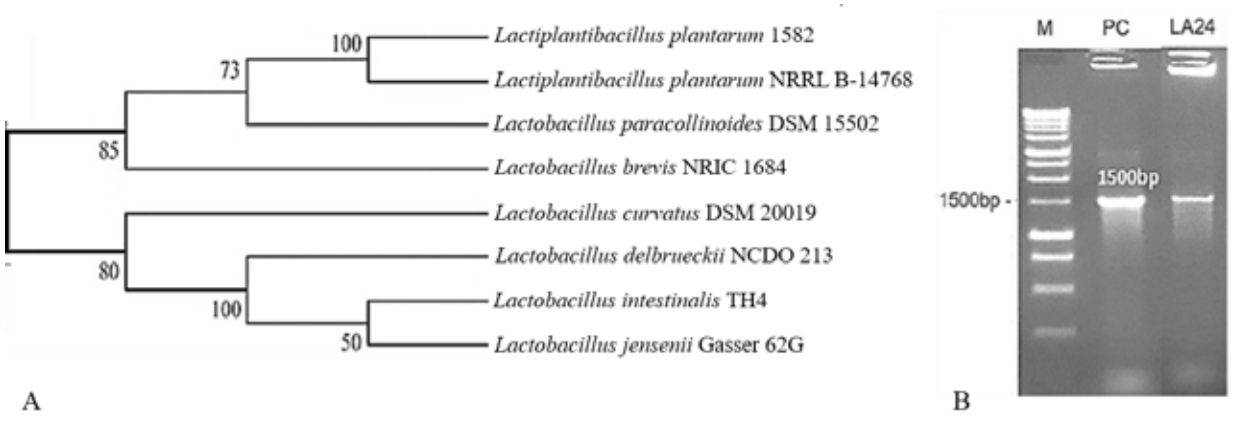

Figure 2. A. Phylogenetic tree based on the 16S rRNA gene sequence of *L. plantarum* 1582 and other strains within the *Lactobacillus* genus. Bootstrap values (from 1000 replications) are displayed at the nodes. The scale bar represents 0.01 substitutions per nucleotide position. B. Agarose gel electrophoresis of the PCR products after 16S rRNA amplification: lane M (1 kb marker), lane PC (positive control), and lane of positive *Lactiplantibacillus* strain (LA24) at 1500 bp.

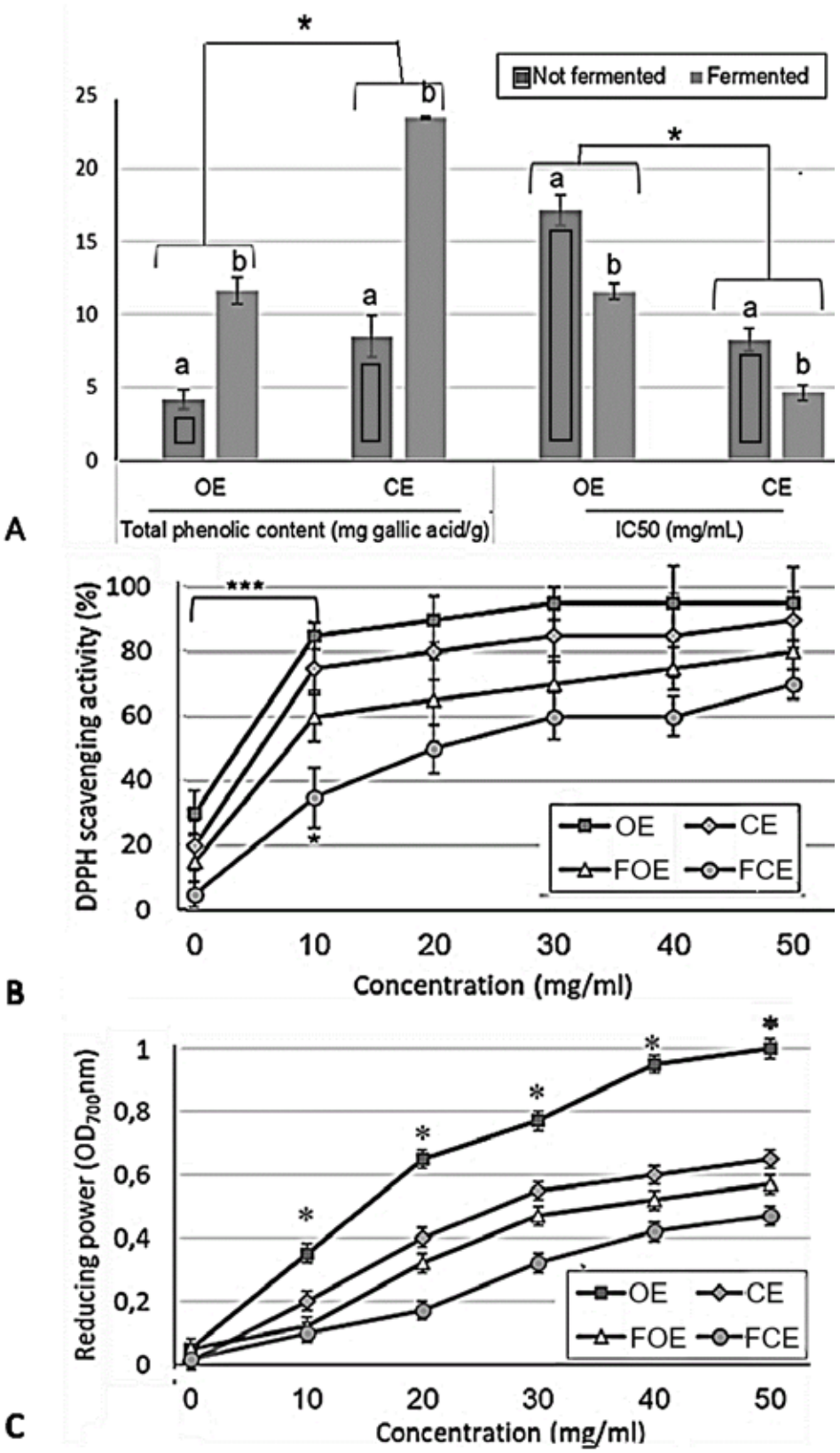

Figure 3. The antioxidant activity of purple onion and chive extracts before and after fermentation. Values with different superscript letters ([a,b]) and *indicate statistically significant differences ($P<0.05$).

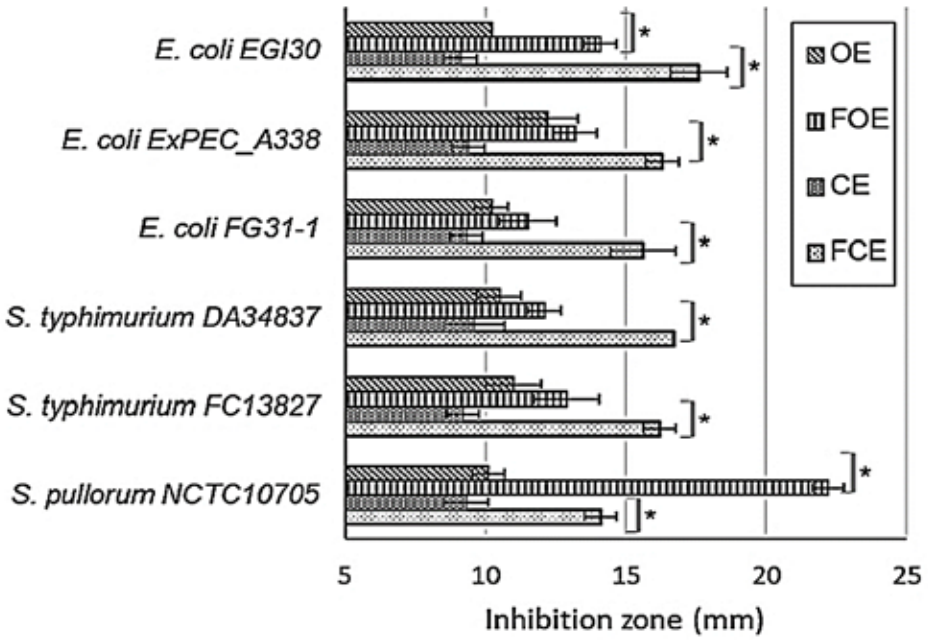

Figure 4. Antibacterial ability of purple onion and chive extracts before and after fermentation against six pathogenic isolates. Brackets indicate comparisons between pre- and post-fermentation inhibition zones, with * indicates statistically significant differences ($P<0.05$).

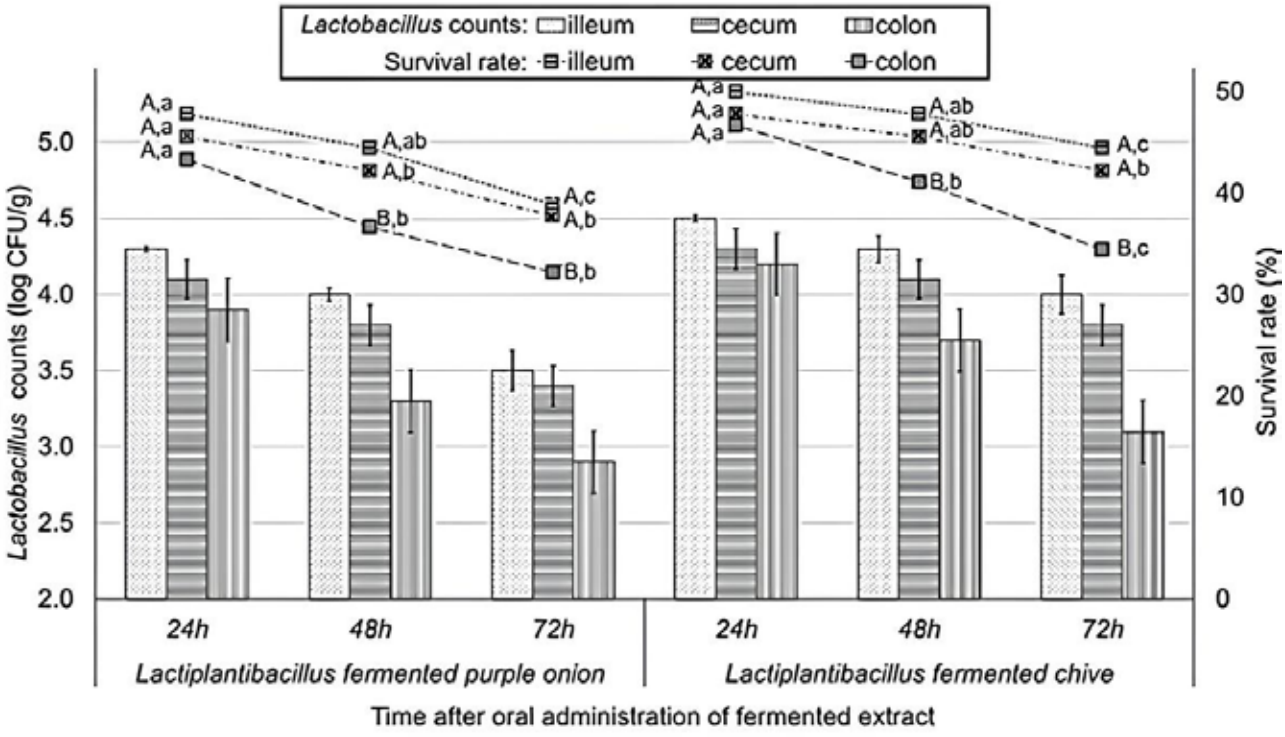

Figure 5. Survival of *Lactobacillus* in the ileum, cecum, and colon of chickens after administration of fermented purple onion or chive extracts (Control data not shown). Values with different superscript letters ($^{a\text{-}c}$) indicate statistically significant differences ($P<0.05$).

alent to a density of 3.36 – 4.20 log CFU/g) and 72 hours (27.05 – 35.78%, equivalent to a density of 2.88 – 3.96 log CFU/g).

DISCUSSION

The results demonstrated that some *Lactobacillus* strains, especially LA24 isolate, have significant potential for use in the fermentation of purple onion extracts, with this strain showing high survival rates and phylogenetic similarity to *Lactiplantibacillus plantarum* 1582. This beneficial bacterium was the most potent native strain adapted to the local conditions and possessing the inherent antibacterial properties of the *Allium* extract, making it suitable as a starter culture for fermentation. Overall, the results of the study showed enhanced antioxidant properties of FOE and FCE after fermentation. The total phenolic content of plant-based foods plays an important biological role and fermentation can alter the TPC content (Hur et al., 2014) in FOE and FCE. In addition, cellulases and tannases produced during fermentation can break down the plant cell wall structure, thereby releasing phenolic compounds (Rodríguez et al., 2009). The increase in phenolic content after fermentation can be explained by these factors, suggesting that fermentation is an effective method to enhance the phenolic content in both purple onion and chive. In the DPPH assay, in purple onion and chive, after fermentation, IC50 is the concentration of antioxidant required to reduce the optical density of the DPPH solution by about 30-50% after a certain reaction time. The 50% inhibition concentration (IC50) reflects the ability of the extracts to scavenge DPPH radicals. A lower IC50 value indicates a more potent antioxidant, as it requires a lower concentration to exert its free radical inhibitory effect. The reducing power of a substance represents its ability to donate electrons, acting as an antioxidant (Kedare and Singh, 2011). The higher reducing capacity of both extracts, especially OE after fermentation, may be due to the fermentation process increasing the content of phenolic compounds and flavonoids, which are known to have strong antioxidant properties; among them, flavonoids can act as antioxidants by donating electrons or hydrogen atoms, or by forming chelates with metal ions (Miller and Paganga, 1996). FOE showed the highest reducing capacity, which may be due to the higher allicin and total flavonoid content in purple onion compared to chives.

The increase in antibacterial activity after fermentation suggests that fermentation may enhance the bioavailability or change the structure of bioactive compounds in onions and chives, improving their efficacy against pathogens. Research by Bhatwalkar et al. (2021) also showed that fermented purple onion extracts as well as several other fermented herbs have the ability to inhibit the growth of a variety of pathogenic bacteria, including *E. coli* and *Salmonella.* Ebrahimi and Pure (2016) evaluated the antibacterial activity against *E. coli* ATCC 1395 and *S. typhimurium* ATCC 1596 and found that kombucha-fermented purple onion had a larger zone of inhibition (21.7 mm) than vinegar-fermented purple onion (17.9 mm). Regarding the antibacterial mechanism, previous studies have shown that plants of the genus *Allium* (such as purple onion and onions) have antibacterial activities due to saponins, phenolics, and sulfur compounds (Yang et al., 2012).

The bioactive compounds in purple onion and chive, which can be enhanced or transformed through fermentation, have the ability to inhibit bacterial growth through various mechanisms such as disrupting cell membranes, inhibiting protein or DNA synthesis, or interfering with bacterial metabolism. In addition, the effect of *Lactiplantibacillus* produced during medicinal fermentation can inhibit the growth of pathogenic bacteria through various mechanisms, including the production of lactic acid from carbohydrate fermentation, thereby reducing the pH of the environment to inhibit bacterial growth (O'Shea et al., 2012). Furthermore, *Lactobacillus* have the ability to produce antibacterial active substances such as bacteriocin, hydrogen peroxide and other organic acids such as acetic acid, propionic acid (Makras and De Vuyst, 2006).

According to Fuller (1989), in some cases, chickens may experience some side effects when they first start using probiotics or when they overdose, such as diarrhea, decreased appetite, and slightly soft stools. This proves that the use of FOE and FCE in this study is safe for chickens.

The results of the study showed that in the ileum, the level of bacterial survival decreased the fastest, possibly due to the rapid movement of food through the small intestine and the natural protective factors of the small intestine such as digestive enzymes and bile. Meanwhile, in the cecum and colon, the bacteria survived longer than in the small intestine, possibly due to a more stable environment and the support of indigenous bacteria in creating favorable conditions for *Lactiplantibacillus* to survive. The above results have contributed to affirming that *Lactiplantibacillus*

exist and circulate in the digestive tract of chickens, and will be excreted in the feces. The results show that this bacteria strain is only temporary organism, only present in the intestine for a short time, they are not considered intestinal microorganisms of the host. Therefore, this beneficial bacterium is recommended to be supplemented regularly with appropriate density to stabilize activity.

CONCLUSION

This study demonstrated that submerged fermentation using *L. plantarum* 1582 significantly enhances the antioxidant and antibacterial activities of both OE and CE. The fermented extracts, particularly the FCE, hold potential for application as probiotic supplements in poultry feed, serving as an alternative to antibiotics. FOE and FCE not only inhibit common intestinal pathogens but also contain high levels of free amino acids, beneficial for digestion and nutrient absorption. Although *L. plantarum* 1582 behaves as a transient inhabitant of the chicken gut, rather than establishing permanent colonization, regular supplementation is necessary to maintain its efficacy. *In vivo* studies on the effects of this formulation on poultry productivity and health are essential to optimize its application in poultry farming.

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Conflict of interests

The authors declare no conflicts of interest.

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