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RESEARCH ARTICLE - BIOLOGICAL SCIENCES

Feeding with Single Strains Versus Mixed Cultures of Lactic Acid Bacteria and *Bacillus subtilis* KKU213 Affects the Bacterial Community and Growth Performance of Broiler Chickens

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Abstract

This study sought to isolate lactic acid bacteria (LAB) from chicken intestines and caeca and apply them as probiotics in broilers. Out of the 247 isolates, 14 LAB were selected based on their tolerance to pH 3 and 0.5% bile salt conditions and were tested against *Salmonella* serovars using two assay methods: (1) bacterial cells and double layers and (2) cell-free supernatants and agar well diffusion. The chicken isolates CA4, CH24 and CH33 strongly inhibited *Salmonella* Typhimurium ATCC13311 and *S*. Enteritidis. The selected strains were identified via 16S rDNA sequencing as *Enterococcus faecium* CA4, *Enterococcus durans* CH33 and *Lactobacillus salivarius* CH24. Only CH33 survived in simulated gastric juice and intestinal juice with survival rates of 90 and 18%, respectively. All three chicken LAB strains as well as food-originating *Pediococcus acidilactici* SH8 and bacteriocin-producing *Bacillus subtilis* KKU213 were tested in broilers. Single strains and mixed cultures of KKU213 and the four LAB strains were orally fed to 1-day-old male Cobb broilers, which were then raised for 45 days. Broilers fed LAB strains demonstrated higher numbers of LAB than the groups fed only *B. subtilis* KKU213 or mixed cultures. Among all treatments, the broilers fed *B. subtilis* KKU213 on days 1 and 3 and LAB on day 5 (T8) had the highest body weights and high-density lipid levels and the lowest uric acid levels. Therefore, a combination of bacterial species originating from various sources exhibits potential as a probiotic mixture to promote health in broilers.

Keywords Lactic acid bacteria (LAB) · *Bacillus subtilis* KKU213 · Probiotics · Broiler chicken

1 Introduction

Currently, organic farms and foods are important to humans because the excessive use of antibiotics for the treatment of diseases and animal husbandry has led to drug resistance in infectious agents, raising interest in products derived from nature to promote human and animal health. Probiotics are bacteria that often reside in and modulate the gastrointestinal system and serve as alternative to antibiotics. They are beneficial to the host, affect bacterial balance in the intestine, confer resistance to acids and bile salts, and produce antimicrobials to inhibit pathogens [1–3]. The Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Working Group [4] described probiotics as “living microorganisms which, when administered in adequate numbers, confer benefits to the host”.

Lactic acid bacteria (LAB), which are gram-positive, non-spore forming, non-motile, oxidase-negative and catalase-negative cocci or rods in the order Lactobacillales, are known probiotics. LAB grow within a temperature range of 2–50 °C and produce antimicrobial activity against pathogens, prevent spoilage due to bacteria or fungi [5,6] and produce biogenic amines [7]. LAB have long been applied to food products, including fermented foods and dairy products, to increase health benefits and facilitate food preservation [8]. LAB have previously been isolated from fermented foods, for example, five isolates of *Lactobacillus* spp. and two isolates of *Pediococcus* spp. were obtained from Omegisool, a traditional food in Korea [9]. Eleven LAB strains were previously isolated from wine [10], and LAB were also isolated from a

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traditional meat product, pastirma, in Turkey by Oz et al. [11]. Specifically, *Lactobacillus sakei* represented approximately 27.4% of total LAB in pastirma. Eleven different species of LAB were isolated from Turkish sourdough [12]. In addition, LAB are known to inhibit pathogens. One hundred LABs and two propionibacteria strains inhibited *Eurotium repens*, *Penicillium corylophilum*, *Aspergillus niger*, *Wallemia sebi* and *Cladosporium sphaerospermum* as well as spoilage-inducing fungi in bakery products [6]. According to Sakaridis et al. [13], 92 LAB strains among a group of *Lactobacillus* spp. and *Pediococcus* spp. isolated from chicken carcasses produced antimicrobials against *Salmonella* spp. and *Listeria monocytogenes*. Six LAB isolates isolated from camel milk inhibited pathogens such as *Escherichia coli* 0157:H7 1934, *Salmonella* Typhimurium 02-8423, *Listeria monocytogenes* ATCC 7644 and *Staphylococcus aureus* ATCC15923 [14]. Furthermore, the supernatant of *Lactobacillus pentosus* UTNFa8.2 is effective against the foodborne pathogens *E. coli* 0157 and *S*. Typhimurium [15].

Chicken meat is an option for meat-loving consumers because it has high protein levels and is cheap in comparison with other meats, such as beef and pork. However, notable problems associated with chicken are its high risk of disease and bacterial infection, including that caused by *Salmonella* spp. *Salmonella enterica* serovar Enteritidis and Typhimurium are dominant *Salmonella* serovars that contaminate eggs or poultry meat products [16,17]. These bacteria cause many symptoms, such as diarrhoea, abdominal cramps and fever [18]. Thorough cooking of poultry products is a major additional defence mechanism against human disease caused by *Salmonella* spp. Thus, farmers frequently apply antibiotics to prevent such problems; however, antibiotics used during chicken rearing remain in the chicken meat and may affect consumers. Probiotics are an alternative to feed antibiotics and, importantly, are safe for the consumer.

Supplementation with either single *Lactobacillus* cultures or mixtures of *Lactobacilli* and other bacteria in broiler diets has resulted in variable outcomes, both positive and negative, on broiler growth performance [19,20] or had no significant effects [21]. According to Olnood et al. [22], four Lactobacillus probiotic candidates (*Lactobacillus johnsonii, Lactobacillus crispatus, Lactobacillus salivarius* and an unidentified *Lactobacillus* spp.) had no adverse effects on general health but altered gut development and microbial immunity in broilers. The varying effects of probiotics on broiler growth performance may be ascribed to differences in species, strains, original sources and delivery routes of these dietary supplements [22,23].

The aim of this study was to isolate and characterize the probiotic properties of native LAB strains derived from the intestines and caeca of domestic fowl and broilers, respectively. Antibacterial activity was tested against *Salmonella* Typhimurium ATCC13311, broiler-origin *Salmonella* Enteritidis and *Salmonella* Braenderup H9812. LAB were identified by 16S rRNA sequencing. Finally, single and mixed cultures of LAB and bacteriocin-producing *B. subtilis* KKU213 probiotics were tested via oral feeding in broiler chickens, and their effects on broiler growth, blood parameters and the microbial community were investigated.

2 Materials and Methods

2.1 Isolation of LAB

Domestic fowl (native) and broiler chicken intestines were used to isolate LAB. After removing all fat, intestines and caeca were washed twice and soaked for 5 min in 0.85% NaCl. Samples were weighed and added to normal saline (10-g sample: 90 mL of 0.85% NaCl) and blended for 5 min using a sterile blender. Samples were serially diluted with 0.85% NaCl and spread on de Man, Rogosa and Sharpe (MRS) agar containing 1% $CaCO_3$ and 0.03% Bromocresol purple. Plates were incubated at 45 °C for 24–48 h. Colonies surrounded by a yellow zone were Gram-stained and tested for catalase and oxidase activities.

2.2 Characterization of LAB

2.2.1 Acid and Bile Salt Tolerance

LAB was cultured in MRS broth and incubated at 37 °C for 48 h.

A 2% (*v*/*v*) LAB dilution was pipetted into MRS broth adjusted to pH 2, 3, 4 or 5 and containing 0.5, 1, 2 or 3% bile salt (*w*/*v*). Samples were examined by streak plating every 1, 2, 3 and 4 h. Plates were incubated at 37 °C for 24–48 h.

2.2.2 Haemolysis Activity

LAB were cultured on MRS broth and incubated at 37 °C for 48 h. Then, cultures were dropped on blood agar base (Columbia) containing 1.2% casein peptone, 1.1% meat peptone, 0.15% starch, 0.5% NaCl and 5% sterile defibrinated Sheep's blood (Scharlau, Barcelona, Spain) and incubated at 37 °C for 48 h.

2.2.3 Chitosan and Rice Bran Utilization

LAB were cultured on MRS broth at 37 °C for 48 h. The bacteria were then streaked on agar medium containing 0.3% beef extract, 0.5% peptone, 2% rice bran or chitosan and 1.5% agar. The plates were incubated at 37 °C for 48 h, and growth was examined.

2.3 Antibiotic Susceptibility Assay

The following antibiotics were used for susceptibility tests: chloramphenicol (C30) at 30 µg, erythromycin (E15) at 15 µg, oxytetracycline (OT30) at 30 µg and streptomycin (S10) at 10 µg. LAB were cultured in MRS broth and incubated at 37 °C for 48 h. Then, LAB were swabbed on MRS agar. Finally, antibiotic discs were dropped onto the plates and incubated at 37 °C for 48 h. The plates were examined for clearance zones.

2.4 Antibacterial Activity of LAB

LAB were tested against the following bacterial indicators: *Salmonella* Typhimurium ATCC13311, *Salmonella* Enteritidis and *Salmonella* Braenderup H9812. The double layer inhibition test method, previously described by Sakaridis et al. [13], was applied. *Salmonella* spp. were grown on LB broth and incubated at 37 °C for 24 h. LAB were cultured in MRS broth and incubated at 37 °C for 48 h. Then, 10 µL of LAB were dropped onto MRS agar, incubated at 37 °C for 24 h and overlaid with 10 mL of soft LB agar. *Salmonella* spp. were swabbed. Plates were incubated at 37 °C for 24 h and then examine for clearance zones.

LAB supernatants were used to carry out agar well diffusion tests. Briefly, LAB were cultured in MRS broth, incubated at 37 °C for 48 h and centrifuged at 8000 rpm for 5 min. The lyophilized pellets were suspended in water, filtered with a 0.22-µm membrane and divided into four aliquots (original supernatant; pH adjusted to 7; boiled at 60 °C for 15 min; and boiled at 100 °C for 15 min). *Salmonella* spp. were grown in LB broth and incubated at 37 °C for 24 h. *Salmonella* spp. were swabbed onto LB agar and into wells created using a cork borer. Then, 10 µL of each supernatant was added to wells and incubated at 37 °C for 24 h. The agar was checked for clearance zones, indicating inhibition.

2.5 Identification of LAB Using 16S rRNA Analysis

LAB were cultured on MRS broth and incubated at 37 °C for 48 h. Chromosomal DNA was extracted from cultures using the phenol-chloroform DNA extraction method [24]. 16S rRNA sequences were amplified by polymerase chain reaction (PCR) using the universal primers 20F and 1500R. Each 25-µL PCR reaction contained 2.5 µL of 10× buffer S, 0.5 µL of 10 mM dNTPs, 1.25 µL of 10 µM 20F, 1.25 µL of 10 µM 1500R, 1 U of Taq DNA polymerase, 2 µL of DNA template and 17 µL of diH_2O. The following PCR conditions were used: 34 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, extension at 72 °C for 1.30 min and a final extension at 72 °C for 10 min. PCR products were purified using a GenepHlow^{TM}Gel/PCR Kit (Geneaid Biotech Ltd.) and verified by performing agarose gel electrophoresis. Purified PCR products were sequenced using the 20F, 920R and 1500R primers (1st BASE, Malaysia). DNA sequences were identified via BLAST analysis.

2.6 Probiotics Properties

2.6.1 Resistance to Simulated Gastrointestinal Conditions

The following method was modified from Babot et al. [25]. LAB were cultured in MRS broth and incubated at 37 °C for 48 h. A 2% (*v*/*v*) culture was pipetted into phosphate buffered saline (PBS) buffer containing 125 mM NaCl, 7 mM KCl, 45 nM $NaHCO_3$and 3 g/L pepsin that was adjusted to a pH of 2.0 using HCl; this solution was used to simulate gastric juice (SGJ). Samples were incubated at 45 °C for 2 h, and cells were counted. Then, the samples were centrifuged, and supernatants were discarded. Simulated intestinal juice (SIJ), which contained 0.5% (*w*/*v*) bile salt and 2 mg/mL trypsin at pH 7.0, was added to cell pellets and incubated at 45 °C. The cells were counted every hour for 3 h.

2.6.2 Cell Surface Hydrophobicity Test

This test was modified from Pringsulaka et al. [3]. Briefly, LAB were cultured in MRS broth and incubated at 37 °C for 48 h. Then, LAB cultures were centrifuged at 8,000 rpm for 5 min. The cell pellets were washed twice with PBS at pH 7.2 and diluted to 10^7-10^8 CFU/mL with PBS buffer ($OD_{640} = A1$). One millilitre of cell suspension was separated into three tubes, and 1 mL of hexadecane was added to each tube and vortexed for 5 min. Cell suspensions were analysed using a spectrometer at OD_{640} ($A2$). The hydrophobicity index (HPBI) was calculated using the following equation: $\%\text{HPBI} = \left[\frac{A1-A2}{A1}\right] \times 100$.

Isolates with HPBI values greater than 70% were classified as highly hydrophobic. An isolate with an HPBI of 50–70% was classified as moderate, and an isolate with an HPBI lower than 50% was classified as having low hydrophobicity.

2.7 Effects of Orally Feeding Single and Mixed Cultures of LAB and *B. subtilis* KKU213 to Male COBB Broilers

All strains were cultured in broth medium (0.3% yeast extract, 0.5% tryptone and 2% glucose) and were incubated at 45 °C for 48 h for LAB and 18 h for *B. subtilis* KKU213. All bacteria were adjusted to 1×10^8 CFU/mL. These fresh cultures were then used for oral feeding to male COBB broilers.

Male Cobb broilers were used in this experiment, with six broilers per treatment and a total of nine treatments: CT, con-

trol feed with sterile distilled water; T1, feed with KKU213; T2, feed with SH8; T3, feed with CA4; T4, feed with CH24; T5, feed with CH33; T6, feed with mixed culture containing *B. subtilis* KKU213 and LAB (SH8, CA4, CH24 and CH33); T7, feed with *B. subtilis* KKU213 on day 1 and LAB on days 3 and 5 ($KKU213^1 + LAB^{3,5}$); and T8, feed with *B. subtilis* KKU213 on days 1 and 3 and LAB on day 5 ($KKU213^{1,3} + LAB^5$).

One-day-old broiler chickens (male COBB) were orally fed 1 mL of bacteria, and feeding was repeated on days 3 and 5. Weights were checked every 7 days for a duration of 42 days. Cholesterol, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and uric acid levels were examined in blood samples from 45-day-old chickens [26]. Total bacteria, LAB and *Salmonella* spp. were counted in the caecum using MRS agar for LAB, Luria-Bertani (LB) agar for total bacteria and xylose lysine deoxycholate agar (XLD agar) for *Salmonella* spp. The broiler experiment is summarized in Fig. 2. To reduce bias in our measurements, the evaluators were blinded to the treatment group assignment for the chickens. This animal experiment was performed according to the guidelines of the Animal Use Committee of Khon Kaen University in Thailand, and the local animal experimentation and husbandry regulations conform to internationally accepted standards.

2.8 Statistical Analysis

Data variance was analysed using Statistix 8 software (Statistix 8, 2003) with a completely randomized design (CRD). Duncan's multiple range test (DMRT) was used for multiple comparisons between treatments at $p < 0.05$ (Bricker 1989).

3 Results

3.1 Isolation and Characterization of LAB

A total of 247 isolates derived from the intestines (CH) and caeca (CA) of the chickens changed the colour of their growth on de Man, Rogosa and Sharpe(MRS) medium (supplemented with 1% $CaCO_3$ and 0.03% Bromocresol purple) from purple to yellow, which indicates the presence of acid produced by LAB. Preliminary LAB properties were assessed, including positive Gram staining and negative catalase and oxidase tests. Forty-four isolates grew under low-oxygen condition. Twenty-four isolates were grown on medium containing chitosan and rice bran and demonstrated gamma haemolysis on blood agar. Sixteen isolates tolerated pH 3, and thirteen isolates tolerated 0.5% bile salt for 4 h. Fourteen isolates were selected based on their tolerance to pH 3 and 0.5% bile salts and were tested for susceptibility to the following antibiotics: 10 μg of streptomycin (S10), 30 μg of oxytetracycline (OT30), 15 μg of erythromycin (E15), and 30 μg of chloramphenicol (C30). Their resistance levels were characterized as resistant, intermediate and sensitive; only one isolate was resistant to all tested antibiotics (Table 1).

Table 1 Antibiotic susceptibility of LAB isolated from broiler (CA) and domestic fowl (CH)

Isolates	Antibiotics			
	S10	OT30	E15	C30
CA4	R	R	R	S
CA22	R	S	I	S
CA23	R	R	S	S
CA44	R	S	S	S
CA69	R	S	I	S
CH12	R	R	I	S
CH13	R	R	S	S
CH14	R	S	S	S
CH16	R	S	R	R
CH24	R	I	S	R
CH25	R	R	R	S
CH29	R	R	R	S
CH33	R	S	R	R
CH34	R	R	R	R

R resistant, *I* intermediate, *S* sensitive

3.2 Antibacterial Activity Against *Salmonella* spp

Salmonella spp. are contaminating bacteria in chickens that cause salmonellosis. Thus, it is important to identify probiotics that inhibit *Salmonella* spp. The selected 14 isolates described above were tested against three *Salmonella* serovars: *S*. Typhimurium ATCC13311, *S*. Braenderup H9812 and *S*. Enteritidis. As determined using the drop plate and double layer methods, all isolates inhibited two or three serovars. Most isolates inhibited all serovars, but all caecum isolates (CA4, CA22, CA23, CA44 and CA69) strongly inhibited *S*. Typhimurium ATCC13311 and *S*. Enteritidis (Table 2 and Fig. 1a).

Based on 16S rRNA sequence identification with 100% similarity, the CA4, CA22, CA23, CA44 and CA69 isolates from broiler caeca were all identified as *Enterococcus faecium*. Among the isolates obtained from domestic fowl, CH14 and CH33 were identified as *Enterococcus durans*, and CH24 was identified as *Lactobacillus salivarius* (Table 3).

E. faecium CA4 and *E. durans* CH14 were cocci and demonstrated effective inhibition of *Salmonella*; *L. salivarius* CH24 was rod-shaped and grew under anaerobic conditions; and *E. durans* CH33 was a coccus and was less effective against *Salmonella.* Due to differences in these species and certain properties, these LAB isolates were cho-

Table 2 Antibacterial activity of LAB isolates against *Salmonella* spp. determined with the drop plate and double layer methods

Isolates	Bacterial indicator		
	S. Typhimurium ATCC13311	*S*. Braenderup H9812	*S*. Enteritidis
CA4	++	−	++
CA22	++	−	++
CA23	++	−	++
CA44	++	−	++
CA69	++	−	++
CH12	++	+	+
CH13	+	+	+
CH14	++	++	++
CH16	++	++	++
CH24	+	+	+
CH25	+	+	+
CH29	+	+	+
CH33	+	+	+
CH34	+	+	+

− No inhibition, ++ colony diameter divided by the diameter of colonies and a zone range of 0–0.5 mm, + colony diameter divided by the diameter of colonies and a zone range of 0.6–1.0 mm

sen for further study to investigate the secreted substances or metabolites that play roles in *Salmonella* inhibition. Cell-free supernatants were divided into four treatments: untreated, adjusted to pH 7, boiled at 60 °C for 15 min, and boiled at 100 °C for 15 min. These supernatants were tested against all three *Salmonella* serovars in an agar well diffusion assay. The untreated, 60 and 100 °C samples from the CA4, CH14, CH24, and CH33 isolates inhibited all serovars. pH 7 treatment affected the substances produced by CA4, CH14 and CH33, and these isolates lost their inhibitory activity. Interestingly, heat and pH did not affect the metabolites in the CH24 supernatant, indicating their stability under various treatment conditions as well as their activity, which was the highest against *S*. Typhimurium ATCC 13311 (Table 4 and Fig. 1b).

3.3 Resistance to Simulated Gastrointestinal Conditions

Two important properties of an effective probiotic are tolerance to gastrointestinal conditions and the ability to survive in the intestines. Thus, a metabolite may act as a probiotic. The tolerance of fresh cultures of *E. faecium* CA4, *L. salivarius* CH24 and *E. durans* CH33 was tested in SGJ (pepsin and pH 2) for 2 h and SIJ (bile salt and trypsin), and only CH33 was tolerant to gastrointestinal conditions. Initially, CH33 cells were present at a concentration of 9.85×10^8 CFU/mL, but this number was reduced to 9.75×10^7 CFU/mL (90% survival) after treatment with gastric juice. The cell concentration further decreased to 1.79×10^7 CFU/mL (18% survival) after passing through SIJ. In contrast, CA4 and CH24 were not able to survive and were not detected after SGJ treatment.

3.4 Cell Surface Hydrophobicity Test

The hydrophobicity test is an indirect measure of the ability of bacterial cells to attach to the lining of the intestinal tract. CA4, with 83.33% HPBI, and CH24, with 70.37% HPBI, were classified as highly hydrophobic, and CH33 with 27.08% HPBI was classified as having low hydrophobicity.

3.5 Effects of Single Strains or a Mixture of LAB and *B. subtilis* KKU213 on the Microbial Community in Broilers

In this experiment, male Cobb broilers were divided into nine treatments, with six broiler chickens per treatment. Fresh

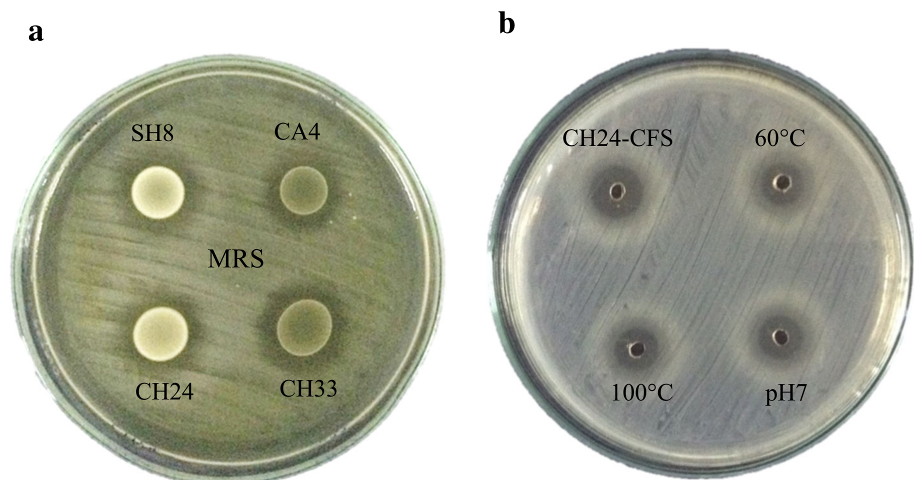

Fig. 1 Antibacterial activity of LAB cultures and supernatants. **a** Inhibition zones of SH8, CA4, CH24 and CH33 cultures against *S*. Enteritidis determined by a drop plate/double layer method in which MRS served as a control dropped with MRS broth. **b** Inhibition zone of untreated cell-free supernatants of CH24 (CH24-CFS), heated at 60 and 100°C and adjusted to pH 7 against *S*. Braenderup H9812 using the agar well diffusion assay

Table 3 Properties of LAB and *B. subtilis* KKU213 strains used in broiler experiments

Species (16S rRNA)	Strain	Isolation source	NCBI accession	Properties
Enterococcus faecium	CA4	Caecum of broiler	MF066894	Inhibits *Salmonella*
				Highly hydrophobic
Lactobacillus salivarius	CH24	Intestines of domestic fowl	MF066895	Inhibits *Salmonella*
				Highly hydrophobic
Enterococcus durans	CH33	Intestines of domestic fowl	MF066896	Resistant to SIJ
Pediococcus acidilactici	SH8	Fermented shrimp	MF061302	Inhibits gram-positive and gram-negative bacteria
				Salt tolerant
Bacillus subtilis	KKU213	Soil	KF220378	Bacteriocin producer
				Carbohydrate fermentation
				Produces various extracellular enzymes

Table 4 Antibacterial activity of LAB isolate supernatants against *Salmonella* determined through an agar well diffusion assay

Isolates	Diameter of the inhibition zone (mm) [a]		
	S. Typhimurium ATCC13311	*S.* Braenderup H9812	*S.* Enteritidis
CA4-untreated	9.5	9	8
CA4-60 °C	9	9	8
CA4-100 °C	8.5	9	8
CA4-pH 7	0	0	0
CH14-untreated	6	3	8
CH14-60 °C	6	2.5	7.5
CH14-100 °C	6	2.5	8
CH14-pH 7	0	0	0
CH24-untreated	12.5	8.5	10.5
CH24-60 °C	11.5	8	9.5
CH24-100 °C	11	8.5	8.5
CH24-pH 7	12	7.5	9
CH33-untreated	9	10	9
CH33-60 °C	8	10	9
CH33-100 °C	8.5	10	9.5
CH33-pH 7	0	0	0

[a] Well diameter is 4 mm

cell suspensions were orally fed to 1-day-old broilers. All feeding treatments and experimental processes are summarized in Fig. 2. Weights were measured every week for 42 days. Caecum and blood samples were taken at 45 days. Blood parameters were determined according to the methods reported by Khochamit et al. [26]. Total LAB and bacteria in the caecum were counted on MRS and LB agar, respectively.

When LAB were counted on MRS agar containing 1% $CaCO_3$ and 0.04% Bromocresol purple, LAB numbers were significantly highest at 8.66 ± 0.66 log CFU/g for T4 (CH24), followed by T2 (SH8) and T3 (CA4) at 7.90 ± 0.12 log CFU/g and 7.54 ± 0.15 log CFU/g, respectively. Overall, total numbers of LAB in chickens fed a single LAB species were higher or similar compared to those of the control (7.63 ± 0.25 log CFU/g) and chickens fed T1 (*B. subtilis* KKU213) at 7.50 ± 0.065 log CFU/g. The exception was T5 (CH33) with 7.54 ± 0.15 log CFU/g, which did not differ from that of the control. In T6, T7, and T8, which were fed mixed *B. subtilis* KKU213 and LAB cultures, LAB numbers were approximately 6.57 ± 0.08 log CFU/g, 6.77 ± 0.11 log CFU/g and 6.55 ± 0.04 log CFU/g, respectively, which were obviously lower than bacterial numbers in the control and in groups fed a single species at $p < 0.01$. Feeding KKU213 together with or followed by LAB did not have much effect on the number of LAB in the caecum (Fig. 3).

For total bacteria counted on LB agar at 50 °C, which was used to select for KKU213 and other thermophiles, the highest numbers were observed for T6 ($KKU213^1 + LAB^1$); these numbers were significantly higher than those for other combinations in T7 and T8 (Fig. 4), demonstrating that feeding broilers specific bacteria at different times likely affects the non-LAB bacterial population in the caecum. It remains to be determined whether KKU213 was the major detected bacteria. The combination of different bacterial species clearly demonstrated that some interactions positively or negatively affect the growth of other species. However, the total bacteria in all groups fed a single species were lower than those groups fed LAB species. High numbers of both total bacteria and LAB in T5 (CH33, the strain that was relatively resistant to simulated intestinal juice) suggested its diminished ability to survive and reduced efficacy in inhibiting other bacteria (Figs. 3 and 4). Low numbers of total bacteria in groups fed a single strain indicated their ability to produce certain metabolites that inhibit other bacteria (Table 4). The very low numbers of total bacteria observed for the T7 and T8 treatments might be explained by the delayed administration of the LAB mixture. Thus, these bacteria may inhibit other bacteria in the caecum. No putative *Salmonella* spp. colonies were found on XLD agar plates for all treatments. Therefore, we are unable to conclude that orally administered bacteria inhibit *Salmonella* in vivo.

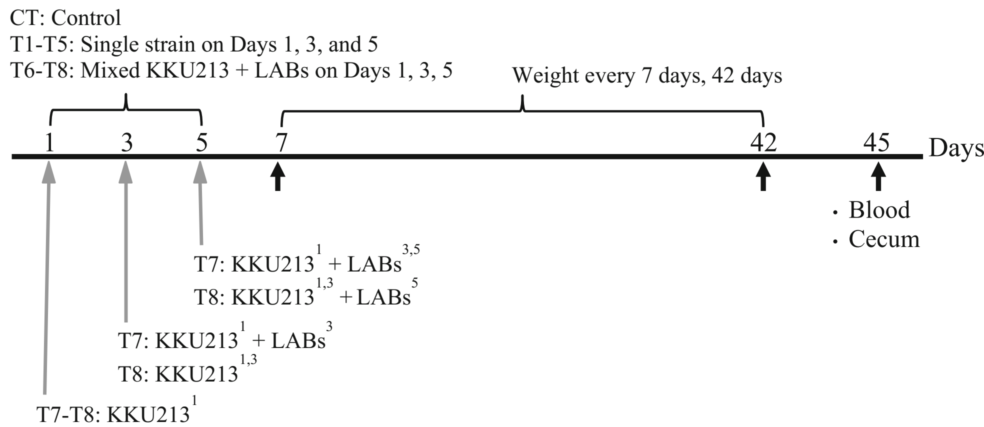

Fig. 2 Timeline of the broiler experiment utilizing single strains and mixtures of *B. subtilis* KKU213 and LAB

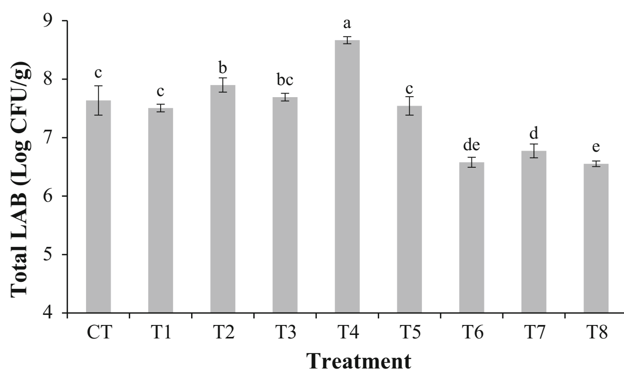

Fig. 3 Total LAB from the caeca of 45-day-old broiler chickens. LAB were counted on MRS agar. The following feeding treatments were applied: CT-sterile distilled water, T1-KKU213, T2-SH8, T3-CA4, T4-CH24, T5-CH33, T6-mixed culture of *B. subtilis* KKU213 and LAB, T7-*B. subtilis* KKU213 on day 1 and LAB on days 3 and 5, and T8-*B. subtilis* KKU213 on days 1 and 3 and LAB on day 5

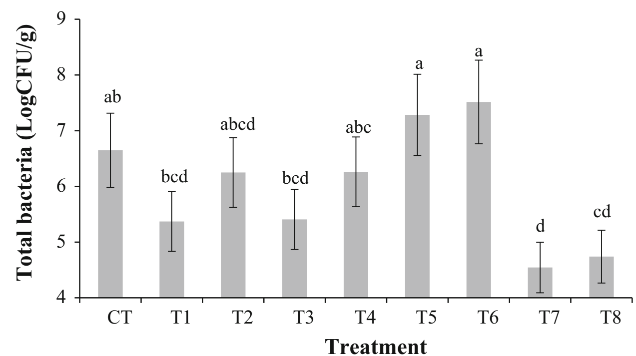

Fig. 4 Total bacteria from the caeca of 45-day-old broiler chickens. Bacteria were counted on LB agar. The following feeding treatments were applied: CT-sterile distilled water, T1-KKU213, T2-SH8, T3-CA4, T4-CH24, T5-CH33, T6-mixed culture of *B. subtilis* KKU213 and LAB, T7-*B. subtilis* KKU213 on day 1 and LAB on days 3 and 5, and T8-*B. subtilis* KKU213 on days 1 and 3 and LAB on day 5

3.6 Effects of a Single Strain versus a Mixture of LAB and *B. subtilis* KKU213 on Broiler Weights and Blood Parameters

Bacteria exerted significant effects on broiler weight from 21–42 days at $p < 0.05$ (Table 5). The experimental codes are as follows: control (CT), feeding of single strains (T1-T5), and feeding of mixed cultures of KKU213 and LAB (T6-T8) at different times as previously described in Sect. 2.7 and Fig. 2. Feeding with a single strain had less of an effect on weight and was similar to that of the control, with the exception of T4 (CH24). At 42 days, all treatments with mixed bacteria were most effective in terms of weight gain, and T8 broilers (KKU213^{1,3} + LAB^{5}) had the highest weights with an average of 1,053.0 g, which was 987.5, 980.0, 970 g and 832.4 g higher than the weights of T6 (KKU213^{1} + LAB^{1}), T7 (KKU213^{1} + LAB^{3,5}), T4 (CH24), and the control (CT), respectively. Thus, broilers fed many strains likely experienced greater benefits than treatment with a single strain, likely attributable to digestive system efficacy. Further study of appropriate timing and species may confer higher benefits.

Table 5 Body weights of broilers at ages of 7–42 days (units in g)

Treatment	7 days	14 days	21 days	28 days	35 days	42 days
CT (H_2O)	110.0 ± 13.2	260.7 ± 29.0	353.6 ± 54.8c	500.0 ± 94.3c	759.3 ± 70.9cd	831.4 ± 107.0c
T1 (KKU213)	111.7 ± 10.3	262.5 ± 48.5	380.0 ± 51.9bc	545.0 ± 88.0bc	667.5 ± 104.9d	906.0 ± 163.3bc
T2 (SH8)	114.2 ± 8.6	261.7 ± 37.6	371.7 ± 29.4bc	548.3 ± 54.9bc	815.0 ± 77.3abc	864.2 ± 81.3bc
T3 (CA4)	115.0 ± 16.7	255.8 ± 53.2	347.5 ± 41.4bc	540.0 ± 88.8bc	768.3 ± 76.5bc	901.7 ± 62.8bc
T4 (CH24)	117.5 ± 17.8	275.8 ± 57.8	378.3 ± 80.8bc	545.0 ± 163.6bc	807.5 ± 133.9abc	970.0 ± 156.5ab
T5 (CH33)	110.0 ± 6.3	260.8 ± 19.6	368.3 ± 29.3bc	533.3 ± 68.3bc	774.2 ± 88.7abc	824.2 ± 114.9c
T6 (KKU213 + LAB)	125.0 ± 18.4	244.2 ± 18.8	478.3 ± 99.5a	655.0 ± 134.7ab	764.2 ± 68.1bcd	987.5 ± 120.8ab
T7 ($KKU213^1$ + $LAB^{3,5}$)	131.7 ± 22.2	245.8 ± 41.5	432.5 ± 80.7ab	546.7 ± 133.7bc	872.5 ± 72.8a	980.0 ± 103.7ab
T8 ($KKU213^{1,3}$+ LAB^5)	130.7 ± 16.9	276.4 ± 42.6	475.0 ± 67.4a	690.0 ± 111.7ab	857.1 ± 71.6abc	1053.0 ± 58.5ab
%C.V.	12.9	15.6	15.9	19.2	11.0	12.1
$p < 0.05$* or 0.01**	ns	ns	**	*	**	*

ns Non-significant difference, *significant difference at $p < 0.05$; *significant difference at $p < 0.01$

Blood parameters were also investigated at 45 days and were indicative of broiler health. Triglyceride and LDL levels did not differ among all treatments. There were some variations since because six broilers were analysed. However, the experiment revealed certain trends suggesting differences in CHL, HDL, and uric acid. CHL was highly variable among all treatments but was lowest in the control. HDL, a good lipid, was highest in T8 ($KKU213^{1,3}$ + LAB^5) and T4 (CH24) at 74.4 and 67.5 mg/dL, respectively, compared to 53.2 mg/dL in the control. HDL was also higher in most treatments compared to the control. Uric acid, a parameter of gouty arthritis, was lower in most treatments compared to the control (3.5 mg/dL) and lowest in T8 at 1.5 mg/dL (Table 6). Overall, HDL and uric acid levels at $p < 0.01$ indicated satisfactory health benefits for both broilers and the consumer.

4 Discussion

In this study, we isolated native LAB strains from chicken intestines and caeca to apply as probiotics in broiler chickens. Most LAB were observed in the caecum, followed by the small intestine and large intestine (data not shown). The caecum is a chicken tissue where bacterial numbers and activity are highest because this location is the final place where bacteria play a major role in absorbing leftover nutrients in the intestines [28]. We preliminarily selected LAB isolates based on the tolerance of fresh LAB cultures to pH 3 and 0.5% bile, which are the conditions present in the chicken stomach and small intestine. pH 2 was also used for screening purposes, but none of the isolates survived. We also investigated the ability of strains to inhibit three serovars, *S*. Typhimurium ATCC13311, *S*. Braenderup H9812 and *S*. Enteritidis, which are representative species of major pathogens that cause infections in chickens. Direct cell contact and the drop plate technique were first used to evaluate anti-*Salmonella* activity under microaerophilic conditions. The observed inhibition was quite weak and similar among most isolates. Thus, metabolites secreted into the supernatant were further investigated to elucidate their direct effects on *Salmonella* in an agar well diffusion assay. LAB commonly produce many organic acids, such as acetic acid, lactic acid propionic acid, succinic acid [29], or bacteriocins, which possess antimicrobial activity [30,31]. Antimicrobial activity of LAB strains, including *P. acidilactici* KTU05-7, *P. pentosaceus* KTU05-9 and *L. sakei* KTU05-6, against many foodborne pathogens has been reported [32].

Based on the observed inhibition of *Salmonella* by cell-free supernatants treated with heat and neutral pH, we speculate that this activity is derived from the bacteriocin that is secreted by LAB strains. We confirmed this hypothesis based on heat resistance results (Table 4) which are consistent with several observations in heat-stable bacte-

Table 6 Blood parameters of 45-day-old broiler chickens

Treatment	CHL	TG	HDL	LDL	Uric
CT ($H_2 0$)	132.1 ± 16.2d	57.7 ± 10.7	53.2 ± 7.6d	67.8 ± 4.6	3.5 ± 0.9a
T1 (KKU213)	147.6 ± 8.4abcd	51.8 ± 6.4	65.2 ± 12.5bc	80.4 ± 7.4	2.7 ± 0.5abc
T2 (SH8)	142.0 ± 11.4bcd	53.0 ± 10.4	58.8 ± 4.4cd	71.2 ± 8.9	1.9 ± 0.6bc
T3 (CA4)	149.1 ± 17.5abc	51.1 ± 11.3	61.5 ± 6.4bc	72.0 ± 7.9	1.9 ± 0.4bc
T4 (CH24)	157.0 ± 17.4ab	55.3 ± 14.4	67.5 ± 7.8ab	78.3 ± 10.5	2.1 ± 0.6bc
T5 (CH33)	137.0 ± 8.5cd	47.6 ± 5.3	60.4 ± 6.6bcd	67.0 ± 4.6	2.0 ± 0.5bc
T6 (KKU213[1] + LAB[1])	147.1 ± 11.3abcd	44.0 ± 5.8	64.7 ± 7.0bc	72.8 ± 6.4	1.9 ± 0.7bc
T7 (KKU213[1] + LAB[3,5])	143.8 ± 11.9abcd	44.3 ± 12.0	64.0 ± 3.9bc	70.2 ± 7.0	2.6 ± 0.8ab
T8 (KKU213[1,3]+ LAB[5])	160.7 ± 20.3a	41.5 ± 5.5	74.4 ± 3.6ab	78.0 ± 9.5	1.5 ± 0.7c
%C.V.	10.0	19.7	11.2	10.7	29.1
$p < 0.05*$ or $0.01**$	*	ns	**	ns	**

All numbers are in mg/dL

CHL cholesterol, *TG* triglyceride, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *ns* non-significant difference.

*Significant difference at $p < 0.05$; **significant difference at $p < 0.01$

riocins produced by *Bacillus* and LAB. Several examples include subtilosin A from *Bacillus* and nisin and pediocin from LAB [26,27,33]. Bacteriocin activity remained when the pH was adjusted to neutral, indicating that low pH derived from organic acids was not responsible for anti-*Salmonella* activity.

The gastrointestinal tract is the digestive organ that stretches from the mouth to the cloaca in poultry. There is low pH from the crop to the gizzard and high pH in the intestine due to the presence of bile salts [34]. Thus, tolerance to simulated gastrointestinal conditions is required of effective probiotics. Although only the CH33 strain met this requirement, other non-tolerant strains may still survive inside the intestines due to the presence of mucus that helps protect bacterial cells from deleterious conditions. To test probiotic properties in broilers, three different species isolated from chickens, specifically *E. faecium* CA4, *L. salivarius* CH24 and *E. durans* CH33, were selected because they originated from different sources. In addition, two other strains were included in this experiment. *P. acidilactici* SH8 was originally isolated from fermented food, but it inhibited several bacterial pathogens and demonstrated certain probiotic properties in broilers (unpublished data). Soil-derived *B. subtilis* KKU213 also qualifies as probiotic and as a single feeding strain, exhibiting health benefits in broilers [26].

As observed for single strains, bacterial origin had no impact on the microbial community and on broiler health performance. Compared with single strain feeding, a combination of various LAB strains and KKU213 was better able to promote broiler growth performance, including body weight gain, increased HDL and decreased uric acid. Thus, *B. subtilis* KKU213 and mixed LAB are a good combination and may interact synergistically in vivo. All bacterial species tested (*B. subtilis, E. faecium, P. acidilactici, L. salivarius,* and *E. durans*) were previously characterized [26,33,35]. *B. subtilis* KKU213 is a bacteriocin (including subtilosin)-producing strain, but it does not inhibit LAB [26] and produces several extracellular enzymes. The CA4, CH24 and CH33 strains demonstrated anti-*Salmonella* activity that might be attributable to bacteriocins. Thus, KKU213 may facilitate and improve the broiler digestive system [28]. The cell surfaces of CA4 and CH24 were highly hydrophobic, and CH33 was tolerant to simulated intestinal conditions. Taken together, these potential strains might survive in the broiler intestines and induce competitive exclusion (CE) by inhibiting the attachment of other microbes or pathogens to the gastrointestinal tract [36,37]. Their positive effects on broiler health are interesting, and further physiological and immunological investigations should be performed to determine how the bacteria induce such effects. The immunomodulatory activity of LAB probiotics has been reported in several studies [35,37,38].

The positive effects of feeding bacteria without antibiotics showed promising results and could possibly be applied to the farmer. According to our results, the body weight of experimental broilers was slightly lower than the average body weight of commercial chickens. These observations might be due to differences in housing and the environment. However, raising chicken without antibiotics is a cost-effective method that can be further developed to meet the demands and requirements of economical farming practice.

Co-feeding or double feeding may not be necessary because these treatment groups only demonstrated obvious differences in total bacterial content in the caecum. Of the two, co-feeding may be more feasible. Species competition is a possibility, and in theory, strains isolated from chickens should demonstrate greater advantage. However, the strains that effectively colonize the broiler gastrointestinal tract must

be elucidated. In conclusion, the combination of *B. subtilis* KKU213 and LAB represents a potential probiotic in broilers that confers several advantages, including improved economic value, chicken meat quality and consumer health.

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