Evaluating the efficacy of an avian-specific probiotic to reduce the colonization of Campylobacter jejuni in broiler chickens

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ABSTRACT Campylobacteriosis is the most frequent zoonotic disease in humans worldwide, and the contaminated poultry meat by Campylobacter jejuni can be considered one of the important sources of enteric infections in humans. The use of probiotics, which can help to improve the natural defense of animals against pathogenic bacteria, is an alternative and effective approach to antibiotic administration for livestock to reduce bacterial contamination. In vitro experiments showed that Enterococcus faecium, Pediococcus acidilactici, Lactobacillus salivarius, and Lactobacillus reuteri isolated from healthy chicken gut inhibited the growth of C. jejuni. To demonstrate this effect in vivo, 1-d-old broiler chicks received 2 mg/bird per day of a multispecies probiotic product via the drinking water. Controls received no probiotic treatment, and all chicks were infected with C. jejuni orally. Results showed that the cecal colonization by C. jejuni was significantly reduced by probiotic treatment at both 8 and 15 d postchallenge. To confirm this effect, in a second in vivo experiment, 1-d-old broiler chicks received the same dose of the same probiotic via the drinking water and controls received no probiotic, and all chicks were infected with C. jejuni orally. Similarly, probiotic treatment reduced (P = 0.001) cecal colonization by C. jejuni at both 8 and 15 d postchallenge. The results of our in vivo experiments conclude that probiotic administration reduced the colonization of C. jejuni in broiler chickens.

Key words: probiotic, Campylobacter jejuni, colonization, food-borne illness, broiler


INTRODUCTION

Campylobacteriosis is the most frequently reported zoonotic disease in humans in the European Union (EU) in recent years (Hugas et al., 2009; Westrell et al., 2009), and the bacterial species most frequently implicated is Campylobacter jejuni (Keener et al., 2004; Humphrey et al., 2007). The European Food Safety Authority (EFSA) has concluded that approximately 180,000 cases occur annually in the EU (EFSA, 2009). Human infections are most commonly associated with C. jejuni. It was shown that broiler chicken meat is frequently contaminated with C. jejuni and is considered to be a major source of human campylobacteriosis (Hänninen et al., 2000; EFSA, 2005; Wingstrand et al., 2006). Consequently, it becomes necessary to control the occurrence of Campylobacter in poultry meat (EFSA, 2008). The frequent contamination of broiler chicken meat with Campylobacter is associated with the fact that Campylobacter exists widely in nature, and therefore the digestive tracts of broiler chickens are the principal reservoirs at farm level. Campylobacter is considered to be a commensal organism in many avian species, including those grown commercially, and the spread of Campylobacter spp. among chickens is very rapid (Keener et al., 2004). Reducing the proportion of Campylobacter-infected poultry flocks or reducing the number of Campylobacter in live poultry will consequently lower the risk to consumers considerably (Keener et al., 2004; Westrell et al., 2009). Therefore, several approaches have been conducted to reduce the number of Campylobacter in poultry over the past few years. For example, vaccination (Noor et al., 1995; Widders et al., 1996; Rice et al., 1997; Wyszyńska et al., 2004), or passive immunization of chickens (Sahin et al., 2003), or feeding of chickens bacteriophages (Wagenaar et al., 2005), bacteriocins (Line et al., 2008), organic acids
ing a strain (Mountzouris et al., 2007) or their derivatives (Hilmärsson et al., 2006), or medium-chain fatty acids (Hermans et al., 2010). Nonetheless, to date, there is no effective, reliable, and practical intervention measure available to reduce colonization of the broiler gut with *Campylobacter* (Lin, 2009). A possible way to reduce *Campylobacter* contamination in poultry is to develop new actions at the primary production level. As a consequence, it has become necessary to develop alternatives such as beneficial microorganisms (probiotics).

Probiotics can be defined as live microbial feed supplements that beneficially affect the host animal by improving its intestinal health (Fuller, 1989). A probiotic feed additive can be composed of one or several different species of microorganisms, including bacteria and yeast (Patterson and Burkholder, 2003). The probiotic colonization characteristics of bacterial species can differ (Isolauri et al., 2004). Additionally, different strains of the same species of probiotic can have unique biological activities, such as different sites of adhesion, specific immunological effects, and fermentation characteristics (Isolauri et al., 2004). The efficacy of probiotics may be potentiated by several methods: the selection of more efficient strains, gene manipulation, and the combination of several strains. This approach of bacterial strain mixture seems to be one of the best ways of potentiating the efficacy of probiotics and is widely used in practice. The proposed mechanisms for the beneficial effects of probiotics are (1) to increase nutrient utilization through improved intestinal health, resulting in greater intestinal enzyme activities and nutrient availability (Nahashon et al., 1996), and (2) to help in maintaining a beneficial microflora in the gastrointestinal tract by inhibiting the growth of pathogenic microorganisms (Jin et al., 1996).

In the last years, several efforts have been dedicated to define targeted microbial mixtures that could have preventive activity in poultry against *Salmonella* infections. *Lactobacillus* strains have been demonstrated to protect chickens from this pathogen (Pascual et al., 1999; Van Coillie et al., 2007; Vicente et al., 2007) and to have a protective effect on raw chicken meat contaminated with *Listeria monocytogenes* and *Salmonella enteriditis* (Maragkoudakis et al., 2009). A reduction of necrotic enteritis due to *Clostridium perfringens* was evidenced upon administration of *L. johnsonii* FI9785 (La Ragione et al., 2004) and a multispecies probiotic based on *Enterococcus, Pediococcus, Lactobacillus,* and *Bifidobacterium* (McReynolds et al., 2009). Use of bifidobacteria in poultry feeding is, to our knowledge, less widespread with respect to lactobacilli administration, although bifidobacteria are an important component of the chicken gut microbiota (Amit-Romach et al., 2004) and have been shown to exert positive effects when administered to other animals, such as piglets (Shu et al., 2001; Modesto et al., 2009). In vivo trials have regarded the use of a multispecies probiotic preparation containing a *Bifidobacterium* strain (Mountzouris et al., 2007) and the administration of a symbiotic mixture containing galacto-oligosaccharides and *B. lactis* (Jung et al., 2008), demonstrating an increase of bifidobacteria in the poultry gut; the only experiment with a selected strain focused on the administration of a commercial *B. bifidum* strain to poultry, resulting in a reduction of cellulitis in broiler chickens (Estrada et al., 2001).

To date, only a few studies have evidenced a possible role of probiotics in preventing the shedding of *C. jejuni* at the level of primary production, although in vitro studies reported a strong antimicrobial activity of several probiotic strains toward this pathogen (Fooks and Gibson, 2002; Chaveerach et al., 2004a). Morishita et al. (1997) reported a 70% reduction in the frequency of *C. jejuni* in chicks with the use of a commercial probiotic containing *L. acidophilus* and *E. faecium*. Willis and Reid (2008) showed that *C. jejuni* was present at a lower level in broiler chickens fed with a standard diet supplemented with a probiotic formulation containing *L. acidophilus, L. casei, B. thermophilus,* and *E. faecium* (10^8 cfu/g) with respect to the control. Recently, a *B. longum* strain (isolated from human intestine) showed a marked antimicrobial activity against *Campylobacter* strains both in vitro and in vivo (Santini et al., 2010). However, little is known about the efficacy of avian-specific probiotic microorganisms on the colonization of *C. jejuni* in chicken intestine. Therefore, more research is needed in finding new probiotic strains with inhibiting activity against *C. jejuni* with the final aim of reducing the contamination of the intestinal pathogen at the farm level and in the chicken meat. In this study, an in vitro experiment was conducted to investigate avian-specific probiotic bacteria (*Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis, Lactobacillus salivarius,* and *Lactobacillus reuteri*) isolated from chicken gut for their antimicrobial activity against *C. jejuni*. As well as the in vitro inhibition of a multispecies probiotic product, a combination of these microorganisms against *C. jejuni* was also investigated. Furthermore, 2 in vivo experiments were conducted to evaluate the efficacy of a multispecies probiotic product containing *Enterococcus, Pediococcus, Lactobacillus,* and *Bifidobacterium* (the same strains used in vitro) to reduce the colonization of *C. jejuni* in the cecum of broiler chickens infected experimentally with *C. jejuni*.

**MATERIALS AND METHODS**

The antimicrobial activity of *Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis, Lactobacillus salivarius,* and *Lactobacillus reuteri* microorganisms (isolated from healthy chicken gut) against *Campylobacter jejuni* were investigated in vitro. These strains are the same strains as in the multispecies probiotic product PoultryStar sol (BIOMIN GmbH, Herzogenburg, Austria). Furthermore, the efficacy of the probiotic product to inhibit the growth of *C. jejuni* in vitro and to reduce its colonization in vivo was also evaluated.
**In Vitro Experiment**

To assess the inhibition of growth of *Campylobacter jejuni* by probiotic bacteria, co-culture experiments were performed to investigate the antimicrobial activity of *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* microorganisms (isolated from healthy chicken gut) against *Campylobacter jejuni*.

A co-cultivation agar plate assay was used for in vitro testing of the ability of *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* microorganisms isolated from the gastrointestinal tract of chickens to inhibit the growth of *C. jejuni* (strain CCUG 25903) which was obtained from the Culture Collection University Goteborg (Sweden). The test for each isolate was carried out in triplicate.

The genera *Lactobacillus*, *Enterococcus*, and *Pediococcus* were precultured in de Man, Rogosa, Sharpe medium (MRS; CM0359, Oxoid) under anaerobic conditions (100% N2) for 24 h at 37°C. Afterward, 10 μL (corresponding to approximately 1 × 10⁵ cells) of the pure preculture for each of these strains was transferred to the middle of MRS agar (CM0361, Oxoid) plates using a sterile Eppendorf Reference pipette (a single channel pipette to dose accurately small volumes; 2–20 μL, Eppendorf, Hamburg, Germany).

The genus *Bifidobacterium* was precultured in complex Bifidus medium under anaerobic conditions (100% N₂) for 72 h at 37°C. The complex Bifidus medium was composed of the following ingredients: caseine peptone 10 g/L, inulin 10 g/L, yeast extract 2.5 g/L, glucose 10 g/L, meat extract 5 g/L, cysteine-HCl 0.5 g/L, Tween 80 1 mL/L, K₂HPO₄ 2 g/L; MgSO₄·7H₂O 0.6 g/L, ZnSO₄·7H₂O 0.25 g/L, FeCl₃ 0.05 g/L, and CaCl₂ 0.05 g/L. pH was 7.0, dissolved in distilled water and autoclaved at 121°C for 15 min. Then 10 μL of the pure preculture (corresponding to approximately 1 × 10⁵ cells) was transferred to the middle of complex Bifidus agar plates. The agar was made using the same composition as the liquid medium with the addition of 12 g/L of agar.

*Campylobacter jejuni* was cultivated in brain heart infusion (BHI; CH1135, Oxoid) under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) generated by using a gas package (Campygen, Oxoid) for 72 h at 37°C. After adequate growth of the test strain, the agar plate was overlaid with 10 mL of semisolid agar containing approximately 10⁸ cells of the precultured *C. jejuni* strain. After 72 h of co-cultivation at 37°C under anaerobic conditions, the agar plate was evaluated for an inhibition zone around the tested strain. The diameter of the inhibition zone and the growth zone of the tested strain was measured, and from these data an inhibition index [diameter of inhibition zone (cm)/diameter of tested strain (cm)] was calculated.

The multispecies probiotic product (PoultryStar sol) containing the same *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* strains was tested in vitro for its inhibitory effects against the growth of *C. jejuni* (strain CCUG 25903). The test was also carried out in triplicate, with the same method as described previously. Briefly, *C. jejuni* was cultivated in BHI under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) generated by using a gas package (Campygen, Oxoid) for 72 h at 37°C. Then, 20 mg of the commercial product was dissolved in 10 mL of sterile saline, and 10 μL of this solution (corresponding to approximately 1 × 10⁵ cells) was transferred to the middle of MRS agar plates using a sterile Eppendorf reference pipette. After short drying in the laminar air cabinet, the agar plates were incubated upside down under anaerobic conditions (100% N₂) for 24 h at 37°C. After adequate growth of the test product, the agar plate was overlaid with 10 mL of semisolid agar containing approximately 10⁸ cells of the precultured *C. jejuni* strain. After 72 h of co-cultivation at 37°C under anaerobic conditions, the agar plate was evaluated for an inhibition zone around the tested strain. The diameter of the inhibition zone and the growth zone of the tested strain was measured, and from these data an inhibition index [diameter of inhibition zone (cm)/diameter test strain (cm)] was calculated.

Furthermore, the multispecies probiotic containing *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* microorganisms isolated from healthy chicken gut (the same strains tested in vitro) was evaluated in vivo for the efficacy to reduce *C. jejuni* colonization.

**The In Vivo Study Design**

Two in vivo experiments were conducted to investigate the efficacy of a multispecies microbial feed additive (PoultryStar sol) to reduce the cecal colonization of *C. jejuni* in broiler chicks. In the first set (experiment 1), a single dose of the probiotic product (2 mg/bird per day) was used. However, in the second set (experiment 2), 2 different doses of the probiotic product (2 mg/bird per day and 20 mg/bird per day) were investigated. The multispecies probiotic product (PoultryStar sol) contains *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* microorganisms.

One-day-old broiler chicks (Ross 308, males and females) were procured from a commercial hatchery with certificate of origin and health. The birds were housed in pens with wood-shaving floor and received a standard nonmedicated corn-soy-based starter diet. Feed and water were available ad libitum. Temperature,
heating, and ventilation followed commercial recommendation.

Before the experimental infection and addition of probiotic microorganisms and to ensure that the birds were free from Campylobacter spp., 10 birds (1 d old) in each experiment were randomly selected and euthanized, and their ceca were harvested for culture of Campylobacter.

**Experimental Challenge with C. jejuni**

In the first experiment, the direct challenge method was used. A freshly isolated field strain of C. jejuni from a broiler chicken flock, reference 3015/2010, was used to infect all birds of the 2 treatment groups on the first day of life. The strain was stored frozen at −80°C and it was reconstituted in Campylobacter Blood-free agar (CBFA; CM0739, Oxoid, Hampshire, UK), and plates were incubated microaerophilically at 41.5 ± 1°C for 44 ± 4 h. Using the McFarland standards, a bacterial suspension in PBS (BR0014G, Oxoid) was prepared and serial dilutions were made to achieve the target challenge population. An inoculum containing approximately 10^5 cfu/mL was prepared and each bird received 0.1 mL of the inoculum orally with a micropipette. The challenge dose was 10^4 cfu for each bird.

In the second experiment, the contact bird challenge method was performed. A freshly isolated field strain of C. jejuni (reference 3015/2010) was used to infect 4 stimulus birds in each group. An inoculum containing 10^5 cfu/mL was prepared and each bird received 0.1 mL of the inoculum orally with a micropipette. The challenge dose was 10^4 cfu for each stimulus bird. The inoculated birds were marked with a black spot on the head and were not sampled.

**Experiment 1**

Forty-four 1-d-old broiler chicks were randomly assigned to 2 experimental treatments: 1) positive control group (22 chicks) received blank water without probiotic product and was challenged with a freshly isolated field strain of C. jejuni from a broiler chicken flock, reference 3015/2010, on the first day of life, 2) birds were infected (22 chicks) with C. jejuni and received a probiotic product (PoultryStar sol) in a dose of 2 mg/bird per day via drinking water.

**Experiment 2**

Seventy-eight 1-d-old broiler chicks were randomly assigned to 3 experimental treatments: 1) positive control group (26 chicks) received blank water without probiotic product and was challenged with a freshly isolated field strain of C. jejuni from a broiler chicken flock, reference 3015/2010 on the first day of the experiment, 2) birds infected with C. jejuni (26 chicks) received a probiotic product (PoultryStar sol) in a dose of 2 mg/bird per day via drinking water, 3) birds infected with C. jejuni (26 chicks) received a probiotic product (PoultryStar sol) in a dose of 20 mg/bird per day via drinking water.

**Collection of Samples for Bacteriology**

In both experiments 1 and 2, 10 birds per group were euthanized and their ceca were harvested for individual quantitative culture of C. jejuni at d 8 of the experiment. Similarly, at d 15 of the experiment, another 10 birds per group were euthanized and their ceca were harvested for individual quantitative culture of C. jejuni.

**Bacteriological Analysis: Presence of Campylobacter spp. Test**

Before experimental infection and addition of probiotic product in both experiments, 10 birds were randomly selected at the first day of life and were euthanized, and their ceca were harvested for culture of Campylobacter. A pool of cecal contents of 10 birds was diluted 1:10 (wt:vol) in PBS, (BR0014G, Oxoid) and the mixture was homogenized using a vortex mixer. Then the homogenate was direct-plated on CBFA. The plates were incubated microaerophilically at 41.5 ± 1°C for 36 to 48 h; after incubation, typical Campylobacter spp. colonies were confirmed.

**Quantitative Culture of Campylobacter spp. Test**

Chicks were killed by cervical dislocation, and individual cecal samples were subjected to quantitative culture of Campylobacter spp. test. Cecal contents were collected in centrifuge tubes, weighed, and diluted 1:10 (wt:vol) in PBS, and the mixture was homogenized using a vortex mixer. Then 10-fold dilutions were made from the stock suspension, and each dilution was directed-plated on CBFA. The plates were incubated microaerophilically at 41.5 ± 1°C for 36 to 48 h; after incubation, typical Campylobacter spp. colonies were counted and confirmed. The direct counts were converted to log_{10} colony-forming units per gram of cecal contents. The detection limit for Campylobacter was <1 × 10^2 cfu/g of cecal contents.

**Statistical Analysis**

Statistical program SPSS (version 17; SPSS GmbH, SPSS Inc., Munich, Germany) was used for data analysis. The individual logarithms of the 10 birds per group either at d 8 or d 15 of each experiment were used for statistical analysis in both experiments 1 and 2. The Kolmogorov Smirnov test was used to test the normal distribution of the data. Mann Whitney test was performed to find the significant difference in the first in vivo experiment. A Kruskal Wallis test was performed between the 3 groups of the second in vivo experiment.
followed by the Mann Whitney test to find the significance between groups. The probability values of 0.05 ($P \leq 0.05$) were considered significant.

RESULTS

Antimicrobial Activity Against Campylobacter jejuni

The in vitro study using a co-cultivation agar plate assay showed that isolates derived from the avian alimentary tract exhibited the ability to inhibit $C. jejuni$ in vitro. The $Enterococcus faecium$, $Pediococcus acidilactici$, $Lactobacillus salivarius$, and $Lactobacillus reuteri$ strains antagonized the growth of $C. jejuni$, presenting a growth inhibition zone with inhibition indexes ranging from 1.27 to 1.50 (Figure 1). The inhibition index is calculated according to the following formula: inhibition index = diameter of inhibition zone (cm)/diameter of test strain (cm).

Those strains showed high inhibition indexes against $Campylobacter jejuni$ in a triplicate co-cultivation agar plate, with good repeatability of results, suggesting that they are promising microorganisms for probiotic application to reduce the $C. jejuni$ colonization. The inhibition index of the multispecies probiotic product was 1.7 (Figure 1), suggesting that the product could be used to control $C. jejuni$ in vivo.

Campylobacter jejuni Colonization in the First In Vivo Experiment

The bacteriological analysis of cecal samples collected from ten 1-d-old broiler chicks before $C. jejuni$ challenge demonstrated that the broiler chicks obtained for the study did not naturally contain cecal $Campylobacter$ spp.

At 8 d postchallenge, the control group had a mean colonization level of 6.67 log cfu/g, with a range between 5 and 8 log cfu/g, but the treated group had a significant reduction in colonization, with less than 3 log cfu/g. Similar results were found at 15 d after the challenge (Table 1). The pathogen colonization of the control group was higher than 8 log cfu/g. Compared with controls, the probiotic-treated group had a significant ($P = 0.001$) reduction of 6-log pathogen colonization (Table 1).

Campylobacter jejuni Colonization in the Second In Vivo Experiment

The bacteriological analysis of cecal samples collected from ten 1-d-old broiler chicks before $C. jejuni$ challenge demonstrated that the broiler chicks obtained for the study did not naturally contain cecal $Campylobacter$ spp.

At 8 d postchallenge, one hundred percent of cecal samples from both probiotic-treated groups had a pathogen colonization count below 2 log cfu/g, but the control group had a mean value of $7.81 \pm 0.52$ log cfu/g with a range between 6 and 9 log cfu/g. Compared with controls, birds receiving the probiotic product showed a significant ($P = 0.001$) 6-log reduction in the cecal colonization of $C. jejuni$ (Table 1). Moreover, there was no significant difference obtained between both probiotic-treated groups.

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**Figure 1.** Antimicrobial activity of probiotic bacterial strains ($Enterococcus faecium$, $Pediococcus acidilactici$, $Lactobacillus salivarius$, and $Lactobacillus reuteri$) derived from the gastrointestinal tract of chicken against $Campylobacter jejuni$ in the co-cultivation agar test expressed by inhibition index [diameter inhibition zone (cm)/diameter test strain (cm)].
Similarly, *C. jejuni* colonization at 15 d postchallenge in the probiotic groups was below 2 log cfu/g, but the mean colonization level was log 7.85 cfu/g in the control group with a range between 7 and 8 log cfu/g. Compared with controls, birds of both probiotic groups had a significant (*P* = 0.001) reduction of 6-log pathogen colonization (Table 1). Additionally, no significant difference was observed between both probiotic-treated groups at 15 d postchallenge (Table 1).

**DISCUSSION**

*Campylobacter* spp. is a classical food-borne pathogen occurring all over the world (Nielsen et al., 2000; Nachamkin et al., 2002; Miller et al., 2005; Unicom et al., 2006). The main symptoms of *campylobacteriosis* in humans are diarrhea, cramping, abdominal pain, and fever. *Campylobacter jejuni* is one of the most common causes of human gastroenteritis in many countries (Fallon et al., 2003; Hämminen et al., 2003). *Campylobacter* infections are associated with consumption of contaminated broiler chicken meat (Pacheco et al., 1999; Stein- hauserova and Fojtikova, 1999; EFSA, 2008).

Broiler chicken is an asymptomatic *Campylobacter* carrier. The most important primary contamination site of *Campylobacter* is at farm level, because *Campylobacter* exists widely in the outside environment (Jacobs-Reitsma, 2000) and colonizes primarily in the ceca and small intestine (Rowe and Madden, 2000). Approximately 80 to 90% of human gastroenteritis cases are caused by *C. jejuni* (Vandamme, 2000) that colonized on the cecum in large amounts, ranging from 10^6 to 10^8 cfu/g (Saleha, 2002).

Over the last 2 decades, only a few studies have evidenced a possible role of probiotics in preventing the shedding of *C. jejuni* at the level of primary production. Moreover, little information is available regarding the antimicrobial effect of avian-specific probiotic microorganisms and their efficacy on the colonization of *C. jejuni* in broiler chickens. Therefore, the present study was carried out to investigate the antimicrobial activity of strains from the genera *Enterococcus, Pediococcus*, *Lactobacillus*, and *Bifidobacterium* (isolated from chicken gut) against *C. jejuni* and their efficacy as a probiotic product to reduce the colonization of *C. jejuni* in the ceca of broiler chickens.

The inhibitory effects of *Enterococcus faecium*, *Pediococcus acidilactici*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* against *C. jejuni* in vitro suggested that these strains showed promising probiotic properties and could have potential to reduce *C. jejuni* in poultry. Similarly, an in vitro growth inhibition of *C. jejuni* by *Lactobacillus* bacteria isolated from chickens was reported (Chaveerach et al., 2004b). Chang and Chen (2000) reported in vitro inhibition of *C. jejuni* by a mixture of 4 *Lactobacillus* species.

The bactericidal effect against *Campylobacter* probably results from the production of organic acids, as already evidenced by Chaveerach et al. (2004b). Antimicrobial activity of probiotic bacteria against pathogens is explicable by their ability to produce organic acids, hydrogen peroxide, carbon dioxide, diacetyl, and bacteriocins (Ouwehand and Vesterlund, 2004). Schillinger and Lucke (1989) and Annuk et al. (2003) reported that bacteriocins produced by probiotics have specific inhibition activity against gram-negative bacteria, including *Campylobacter* spp., which are more sensitive to organic acids.

In the co-culture experiments, co-cultivation of probiotic bacteria and pathogens is performed on agar plates. The mechanism of using agar plates lies in the fact that the antagonistic effect of probiotic bacteria is based on their metabolic compounds that are absorbed into the agar medium. For example, the antagonistic effect of bacteriocin excreted into solid media can be measured by the formation of a growth inhibition zone around the well.

Antimicrobial activity of probiotic bacteria was assayed against *C. jejuni*. Some of the explanations for antagonism are competition for nutrients and attachment on adhesion sites, beneficial changes in growth environment, or combinations of these processes.

In the present study, the in vitro inhibition activity of *Enterococcus faecium*, *Pediococcus acidilactici*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* against *C. jejuni* could explain the mechanism of action of the probiotic product in vivo. Administration of the multispecies probiotic product containing *Enterococci, Pediococci, Lactobacillus, and Bifidobacterium* showed a significant reduction of *C. jejuni* colonization in vivo. Administration of the multispecies probiotic product in vivo. Administration of the multispecies probiotic product containing *Enterococci, Pediococci, Lactobacillus, and Bifidobacterium* showed a significant reduction of *C. jejuni* colonization in vivo. Administration of the multispecies probiotic product containing *Enterococci, Pediococci, Lactobacillus, and Bifidobacterium* showed a significant reduction of *C. jejuni* colonization in vivo.

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<tr>
<th>Item</th>
<th>Control (n = 10)</th>
<th>PoultryStar sol (2 mg/bird/day) (n = 10)</th>
<th>PoultryStar sol (20 mg/bird/day) (n = 10)</th>
<th>SEM</th>
<th>P-value</th>
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<td>First experiment</td>
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<tr>
<td><em>C. jejuni</em> (log cfu/g) (8 d postchallenge)</td>
<td>6.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>—</td>
<td>0.51</td>
<td>0.001</td>
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<tr>
<td><em>C. jejuni</em> (log cfu/g) (15 d postchallenge)</td>
<td>8.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.50&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.23</td>
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<td>Second experiment</td>
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<tr>
<td><em>C. jejuni</em> (log cfu/g) (8 d postchallenge)</td>
<td>7.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;1.00&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><em>C. jejuni</em> (log cfu/g) (15 d postchallenge)</td>
<td>7.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;1.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;1.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.51</td>
<td>0.001</td>
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<sup>a,b</sup>Means within the same row with different superscripts are significantly different (Mann Whitney test was performed for the first experiment, n = 10/treatment and Kruskal Wallis test followed by Mann Whitney test for the second experiment, n = 10/treatment).

<sup>1</sup>Data presented as means of logarithms of 10 cecal samples per group (log cfu/g).
coci, Lactobacilli, and Bifidobacteria reduced the cecal colonization of C. jejuni in broiler chickens. The inhibition activity of the multispecies probiotic (a combination of E. faecium, B. animalis, P. acidilactici, L. salivarius, and L. reuteri) against C. jejuni in vitro could also be confirmed by the results of the in vivo experiments. This indicates that the reduction of C. jejuni colonization in vivo is due to the antimicrobial activity of Enterococcus faecium, Pediococcus acidilactici, Lactobacillus salivarius, and Lactobacillus reuteri against C. jejuni. This result is of great relevance, considering that C. jejuni is the Campylobacter species mainly responsible for campylobacteriosis in humans derived by consumption of poultry meat (Keener et al., 2004). Similarly, Morishita et al. (1997) orally administered a mixture of L. acidophilus and Streptococcus faecium (isolated from chicken gut) to chickens in the drinking water for the first 3 d of life. Six hours following the first treatment, they orally challenged them with Campylobacter jejuni. They found that the chickens that received the treatment were significantly less colonized with C. jejuni than the chickens in the control group. Our results are also in agreement with the findings of Willis and Reid (2008), which showed a lower level of C. jejuni in broiler chickens fed with a standard diet supplemented with a mixed probiotic containing also a Bifidobacterium strain.

In conclusion, the Enterococcus faecium, Pediococcus acidilactici, Lactobacillus salivarius, and Lactobacillus reuteri strains with interesting probiotic properties have been characterized in this study. Their marked antimicrobial activities against C. jejuni suggest that they are excellent candidates for being employed as probiotic supplements for broiler chickens for the reduction of food-borne campylobacteriosis in humans. Moreover, the administration of the multispecies probiotic product containing avian-derived Enterococcus, Pediococcus, Lactobacillus, and Bifidobacterium microorganisms to broiler chickens reduced the cecal colonization of C. jejuni and may change their gut microflora in a way that is beneficial to the health of consumers by reducing the number of potential food-borne pathogens.

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