Epithelial cell adhesion and gastrointestinal colonization of Lactobacillus in poultry

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ABSTRACT Administration of probiotic Lactobacillus cultures is an important alternative to the use of antibiotic growth promoters and has been demonstrated to improve animal health, growth performance, and preharvest food safety in poultry production. While gastrointestinal colonization is thought to be critical to their probiotic functionality, factors important to Lactobacillus colonization in chickens are not well understood. In this study we investigate epithelial cell adhesion in vitro and colonization of Lactobacillus in vivo in broiler chickens. Adhesion of Lactobacillus cultures to epithelial cells was evaluated using the chicken LMH cell line. Lactobacillus cultures were able to adhere effectively to LMH cells relative to Bacillus subtilis and Salmonella Typhimurium. Epithelial cell adhesion was similar for Lactobacillus crispatus TDCC 75, L. crispatus TDCC 76, and Lactobacillus gallinarum TDCC 77, and all 3 were more adherent than L. gallinarum TDCC 78. However, when colonization was evaluated in the ileum and cecum of broiler chicks, L. crispatus TDCC 75 and L. gallinarum TDCC 77 were more persistent than L. crispatus TDCC 76 and L. gallinarum TDCC 78. The reduction of growth in medium supplemented with oxgal was greater for L. gallinarum TDCC 78 than L. gallinarum TDCC 77, suggesting that whereas adhesion was similar for the 2 strains, the difference in colonization between L. gallinarum strains may be due in part to their bile sensitivity. This study demonstrates that whereas adhesion to epithelial cells may be important in predicting gastrointestinal colonization, other factors including bile tolerance may also contribute to the colonization of Lactobacillus in poultry. Additionally, the chicken LMH cell line is expected to provide a platform for investigating mechanisms of Lactobacillus adhesion to epithelial tissue and evaluating the probiotic potential Lactobacillus in poultry.

Key words: Lactobacillus, probiotic, adhesion, colonization, LMH cell

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INTRODUCTION

Lactobacillus species are common inhabitants of the gastrointestinal tract and are often used as probiotics due to their health promoting properties (Klaenhammer et al., 2008). Probiotics, sometimes called direct-fed microbials when used in livestock animals (Sanders, 2008), are often seen as alternatives to the use of antibiotic growth promoters in poultry production (Patterson and Burkholder, 2003; O’Bryan et al., 2008; Huynghaert et al., 2011). The administration of probiotic lactobacilli has been demonstrated to stimulate immune responses (Haghighi et al., 2005; Sato et al., 2009; Brisbin et al., 2011), improve digestive health (Gusils et al., 1999; Kim et al., 2012), and improve growth performance (Loh et al., 2010; Shim et al., 2012; Askelson et al., 2014) in poultry. Lactobacillus administration has also been shown to reduce colonization by Campylobacter (Ghareeb et al., 2012; Neal-McKinney et al., 2012), Clostridium (La Ragione et al., 2004), and Salmonella (Chen et al., 2012; Ghareeb et al., 2012), improving the microbial food safety of poultry.

Colonization and persistence in the gastrointestinal tract are thought to be critical for the realization of probiotic benefits (Bernet et al., 1994; Mack et al., 1999) and serve as important criteria for the selection of probiotic microorganisms (Klaenhammer et al., 2005). Understanding how Lactobacillus species colonize chickens will be important in the selection of more effective probiotic cultures and their application in poultry production. While the ability of probiotic lactobacilli to colonize the gastrointestinal tract of poultry is multifactorial, adherence to epithelial cells is thought to contribute to colonization.

The ability of Lactobacillus species to adhere to epithelial tissues and colonize poultry has been reported (Jin et al., 1996; Edelman et al., 2002; Bouzaine et al., 2005). However, microbial factors important to gastrointestinal persistence of Lactobacillus in poultry are not
well characterized. Aggregation, cell wall hydrophobicity, and adhesion to extracellular components including intestinal mucus, fibronectin, and basement membrane matrix have been used as in vitro measures of the adherence of *Lactobacillus* strains to poultry epithelia (Edelman et al., 2002; Ehrmann et al., 2002; Gusils et al., 2003; Bouzaine et al., 2005; Taheri et al., 2009; Rocha et al., 2012). Additionally, dissected tissue sections (Fuller and Brooker, 1974; Edelman et al., 2002), primary chicken intestinal epithelial cells (Jin et al., 1996; Garriga et al., 1998; Lin et al., 2007), and the HEP-2 (Ehrmann et al., 2002) and Caco-2 (Messaoudi et al., 2012) human epithelial cell lines have been used to evaluate the potential of *Lactobacillus* cultures as poultry probiotics. Whereas cultured epithelial cell lines are the most widely applied models for evaluating the adhesion of microorganisms (Ouwehand and Salminen, 2003), adherence of *Lactobacillus* cultures to a poultry-specific epithelial cell line has not been reported previously. The lack of a species-specific cell culture model has been an obstacle to investigations of *Lactobacillus* adhesion and its contribution to gastrointestinal colonization in poultry.

The chicken LMH epithelial cell line (Kawaguchi et al., 1987), derived from a hepatocellular carcinoma, represents a cell line potentially useful for the investigation of probiotic functionality in poultry. The LMH cell line has been used previously as an in vitro model to investigate mechanisms of *Campylobacter jejuni* colonization in poultry. Competitive inhibition between *C. jejuni* F38011 and *C. jejuni* 02–833L for adherence to gastrointestinal epithelia was demonstrated using the chicken LMH epithelial cell line and verified in broiler chicks in vivo by Konkel et al. (2007). Additionally, LMH cells were used to demonstrate that the adhesins CadF and FlpA contribute to *C. jejuni* colonization in broiler chicks by mediating adherence to epithelial cells (Flanagan et al., 2009), that autoinducer-2 expression contributes to *C. jejuni* colonization by regulating adhesion to host tissues (Quinones et al., 2009), and that the cytokine response to *C. jejuni* in chicken compared with humans contributes to its commensalism in poultry (Larson et al., 2008). The LMH cell line has also been used to identify genes important in cellular invasion by *Salmonella* Enteritidis (Shah et al., 2012) and characterize the association of *Clostridium perfringens* NetB toxin to necrotic enteritis in chickens (Keyburn et al., 2008). Thus, utility of the chicken LMH epithelial cell line for the investigation of host-microbe interactions in the gastrointestinal tract of poultry has been well established.

In this study, we evaluated the adhesion of poultry-derived strains of *Lactobacillus crispatus* and *Lactobacillus gallinarum* to the LMH epithelial cell line in vitro and their colonization in vivo in broiler chicks to gain a better understanding of bacterium-host interactions in chickens.

**MATERIALS AND METHODS**

**Bacterial Strains**

The bacterial strains used in this study are listed in Table 1. *Lactobacillus* strains were cultured using deMan, Rogosa, and Sharpe (MRS) medium (Difco, Franklin Lakes, NJ) in 10% CO\(_2\) at 37°C with rifampicin (Rif; EMD Chemicals Inc., San Diego, CA) added when appropriate. *Salmonella* Typhimurium and *Bacillus subtilis* were cultured using tryptic soy medium (TSA/TSB; Difco) aerobically at 37°C. For adhesion assays, 18 h cultures of bacteria were harvested by centrifugation, washed 3 times with PBS, and subsequently resuspended in PBS to achieve the desired concentration for inoculation onto LMH cells.

**Selection of Rifampicin-Resistant *Lactobacillus* Cultures**

To generate rifampicin resistant (Rif\(^r\)) cultures for use in in vitro adhesion and in vivo colonization assays, *Lactobacillus* cultures were serially transferred in MRS broth supplemented with up to 100 μg·mL\(^{-1}\) Rif and subsequently plated onto MRS agar supplemented with 100 μg·mL\(^{-1}\) Rif. Following incubation in 10% CO\(_2\) at

<table>
<thead>
<tr>
<th>Table 1. Bacterial strains used in this study</th>
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<tr>
<td><strong>Strain</strong></td>
<td><strong>Relevant characteristic</strong></td>
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<tr>
<td><em>Lactobacillus crispatus</em></td>
<td></td>
</tr>
<tr>
<td>ST1</td>
<td>Chicken crop isolate</td>
</tr>
<tr>
<td>JCM 5810</td>
<td>Chicken fecal isolate</td>
</tr>
<tr>
<td>TDCG 75</td>
<td>Rif(^r) variant of <em>L. crispatus</em> ST1</td>
</tr>
<tr>
<td>TDCG 76</td>
<td>Rif(^r) variant of <em>L. crispatus</em> JCM 5810</td>
</tr>
<tr>
<td><em>Lactobacillus gallinarum</em></td>
<td></td>
</tr>
<tr>
<td>ATCC 33199(^2)</td>
<td>Chicken crop isolate, type strain</td>
</tr>
<tr>
<td>JCM 8782</td>
<td>Chicken fecal isolate</td>
</tr>
<tr>
<td>TDCG 77</td>
<td>Rif(^r) variant of <em>L. gallinarum</em> ATCC 33199</td>
</tr>
<tr>
<td>TDCG 78</td>
<td>Rif(^r) variant of <em>L. gallinarum</em> JCM 8782</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> ATCC 23857</td>
<td>Strain 168, genome sequenced variant</td>
</tr>
<tr>
<td><em>Salmonella</em> Typhimurium</td>
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</table>

\(^1\)JCM = Japan Collection of Microorganisms.  
\(^2\)ATCC = American Type Culture Collection.  
\(^3\)ARS = Agricultural Research Service.
37°C, resistant colonies were selected and their Rif resistance was confirmed before use in this study.

**Culture of LMH Cells**

Chicken LMH hepatocellular carcinoma epithelial cells (ATCC CRL-2117) were cultured in flasks coated with 0.1% gelatin (Sigma Chemical Co., St. Louis, MO) using Waymouth’s MB 752/1 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). The cells were maintained in a humidified 5% CO2 incubator at 37°C.

**Lactobacillus-LMH Cell Binding Assay**

Adhesion of *Lactobacillus* cultures to LMH cells was evaluated using methods adapted from Konkel et al. (2007). Unless otherwise stated, assays were performed as follows. Gelatin coated (0.1%) 24-well tissue culture plates were seeded with 1.5 × 10^5 LMH cells per well and incubated for 18 h at 37°C in a humidified 5% CO2 incubator. The LMH cells were rinsed 3 times with PBS and inoculated with approximately 1.5 × 10^7 cfu bacteria suspended in PBS (100:1 bacteria per LMH cell). Plates were centrifuged at 600 × g for 5 min at 20°C to promote bacterium-host cell contact and incubated for 30 min at 37°C in a humidified 5% CO2 incubator. Non-adherent bacteria were removed by rinsing the wells 5 times with PBS, and LMH cells were lysed with 0.1% Triton X-100 (Sigma Chemical Co.) in PBS. Bacterial suspensions were enumerated using MRS agar supplemented with 0.0, 0.1, 0.25, 0.5, and 1.0% (wt/vol) oxgall (Difco). These concentrations were selected to emulate the 0.1 to 1% bile concentration range found in the poultry gastrointestinal tract, which have been reported to be approximately 0.25% in the ileum and 0.1% in the cecum (Green and Kellogg, 1987; Lin et al., 2003; Iwata et al., 2013). Wells containing sterile medium (uninoculated) were used for blank correction. The cultures were incubated under microaerobic con-

**Lactobacillus Broiler Chick Colonization Assay**

*Lactobacillus crispatus* TDCC 75 (ST1-Rif<sup>+</sup>), *L. crispatus* TDCC 76 (JCM 5810-Rif<sup>+</sup>), *L. gallinarum* TDCC 77 (ATCC 33199-Rif<sup>+</sup>), and *L. gallinarum* TDCC 78 (JCM 5810-Rif<sup>+</sup>) were cultured in MRS broth supplemented with Rif (100 µg·mL<sup>−1</sup>) and incubated for 18 h in 10% CO2 at 37°C. Cultures were centrifuged at 700 × g for 5 min at 20°C, washed 3 times with PBS to remove Rif, and resuspended in PBS. On day of hatch and 3 d posthatch, broiler chicks were administered 0.5 mL of *Lactobacillus* (10<sup>8</sup> cfu) culture suspended in PBS by oral gavage. A mock inoculated group was administered sterile PBS. After inoculation, the remaining bacterial suspensions were enumerated using MRS agar supplemented with Rif (100 µg·mL<sup>−1</sup>) to confirm the count (cfu) of *Lactobacillus* in each dose. At 4, 6, and 8 d posthatch, 5 chicks from each group were euthanized by CO2 asphyxiation and necropsied to evaluate gastrointestinal colonization of poultry. At each sampling, a section of ileum and a cecum were dissected from each chick and placed in separate Whirlpak bags. Gastrointestinal specimens were weighed, thoroughly homogenized, diluted serially, and plated onto MRS agar and MRS agar supplemented with Rif (100 µg·mL<sup>−1</sup>) for enumeration of total lactic acid bacteria (*LAB*) and Rif<sup>+</sup> *Lactobacillus*, respectively. The number of viable bacteria recovered was reported as mean log<sub>10</sub> cfu·g<sup>−1</sup> ± SEM gastrointestinal contents from 5 broiler chicks. Gastrointestinal samples in which Rif<sup>+</sup> *Lactobacillus* was not detected were assigned the lower limit of detection, 2 log<sub>10</sub> cfu·g<sup>−1</sup>. *Lactobacillus* colonization over time was analyzed using a 2-factor ANOVA for strain and day. Significantly different means (P ≤ 0.05) were separated using Duncan’s multiple range test.

**Bile Tolerance Assay**

Growth curve analysis was used to evaluate bile tolerance (O’Flaherty and Klaenhammer, 2010) of *L. crispatus* TDCC 75 (Rif<sup>+</sup> ST1), *L. crispatus* TDCC 76 (Rif<sup>+</sup> JCM 5810), *L. gallinarum* TDCC 77 (Rif<sup>+</sup> ATCC 33199), and *L. gallinarum* TDCC 78 (Rif<sup>+</sup> JCM 8782). *Lactobacillus* cultures were inoculated into triplicate wells of a 96-well plate containing 200 µL of MRS broth supplemented with 0.0, 0.1, 0.25, 0.5, and 1.0% (wt/vol) oxgall (Difco). These concentrations were selected to emulate the 0.1 to 1% bile concentration range found in the poultry gastrointestinal tract, which have been reported to be approximately 0.25% in the ileum and 0.1% in the cecum (Green and Kellogg, 1987; Lin et al., 2003; Iwata et al., 2013). Wells containing sterile medium (uninoculated) were used for blank correction. The cultures were incubated under microaerobic con-
ditions at 37°C, and the absorbance (optical density at 600 nm; OD_{600nm}) was recorded at 15-min intervals for 24 h using a Spectrafluor Plus (Tecan Systems Inc., San Jose, CA) microtiter plate reader. Maximum growth rate ($\mu_{\text{max}}$) was determined by fitting blank-corrected growth curves to a modified Gompertz model (Zwietering et al., 1990). Growth rates were reported as the mean $\mu_{\text{max}} \pm$ SEM from 3 independent wells. Growth rate of Lactobacillus cultures was analyzed using a 2-factor ANOVA for strain and treatment. Significant differences ($P \leq 0.05$) in growth rate between strains from the same species for each treatment were determined using a t-test with Bonferroni correction.

**RESULTS**

**Evaluation of Factors Affecting the Adhesion of Lactobacillus to Chicken LMH Cells**

Lactobacillus crispatus ST1 was used to evaluate the effect of the number of postincubation washes (Figure 1A) and dose (cfu bacteria per LMH cell; Figure 1B) on the number of adherent bacteria. After incubating L. crispatus ST1 with LMH cells, individual wells were washed 0, 1, 3, 5, or 8 times before being enumerated. The number of viable-adherent bacteria decreased with each successive wash up to 5 total washes but did not significantly decrease from 5 to 8 washes. Thus, 5 postincubation washes were sufficient to remove the nonadherent bacteria from chicken LMH cells. Lactobacillus cultures were co-incubated for with LMH cells at ratios of 100:1, 250:1, 500:1, and 1,000:1 cfu bacteria per LMH cell. The number of adherent bacteria increased with the count (cfu) per cell. However, the percentage of viable adherent bacteria did not change with increasing dose. Thus, the adhesion of L. crispatus ST1 to LMH cells was determined to be dose dependent and linear within the range evaluated. Based on these data, Lactobacillus cultures were incubated with LMH cells at a ratio of 100 cfu per LMH cell and washed 5 times before enumeration to determine the number of viable adherent bacteria in the subsequent assays. These represent a standard set of conditions for in vitro adhesion assays using the chicken LMH epithelial cell line.

**Adhesion of Lactobacillus Cultures to Chicken LMH Cells**

The adhesion Lactobacillus cultures to chicken LMH cells was evaluated (Figure 2). In preliminary assays evaluating the adhesion of B. subtilis and Salmonella Typhimurium to LMH cells, these cultures were consistently observed to adhere at approximately $10^5$ and $10^7$ cfu per well, respectively (data not shown). Thus, B. subtilis was selected as a low-adherent and Salmonella Typhimurium was selected as a high-adherent control culture. Lactobacillus cultures were more adherent to LMH cells than B. subtilis and less adherent than Salmonella. Lactobacillus crispatus TDCC 76 was the most weakly adherent of the lactobacilli, but no difference in the number of viable adherent bacteria was observed between the remaining Lactobacillus cultures. Within each species, L. crispatus TDCC 75 adhered more effectively to the LMH cells than L. crispatus TDCC 76, whereas there was no difference between the 2 L. gallinarum strains.

**Lactobacillus Colonization of Broiler Chicks**

Colonization of broiler chickens by L. crispatus TDCC 75(Rif$^\text{R}$ ST1), L. crispatus TDCC 76 (Rif$^\text{R}$ JCM 5810), L. gallinarum TDCC 77 (Rif$^\text{R}$ ATCC 33199), or L. gallinarum TDCC 78 (Rif$^\text{R}$ JCM 8782) was evalu-
ated. The recovery of viable Rifr \textit{Lactobacillus} in our time course study showed the administered \textit{Lactobacillus} cultures transiently colonized the ileum and cecum of broiler chickens (Figure 3). The numbers of Rifr \textit{Lactobacillus} recovered from the cecum and intestine of the inoculated groups was similar at 4 d posthatch and decreased over the time-course of our study. \textit{Lactobacillus gallinarum} TDCC 77 was the most persistent colonizer, followed by \textit{L. crispatus} TDCC 75, having been recovered both from the gastrointestinal contents of a greater number of birds and at higher numbers than \textit{L. crispatus} TDCC 76 and \textit{L. gallinarum} TDCC 78 at each time point. There was no difference observed between \textit{L. crispatus} TDCC 76 and \textit{L. gallinarum} JCM TDCC 78. Although there were some differences at individual time points, administration of \textit{Lactobacillus} cultures did not appear to affect the number of total LAB recovered from the gastrointestinal specimens of broiler chickens overall (Table 2). However, \textit{L. crispatus} TDCC 75 and \textit{L. gallinarum} TDCC 77 did comprise a larger proportion of the total LAB at 6 d posthatch in the intestine and 6 and 8 d posthatch in the cecum as compared with the other lactobacilli. Additionally, counts of Rifr \textit{Lactobacillus}, when recovered, and total LAB were generally higher in the cecum than the ileum. When Rifr LAB were recovered from mock inoculated broiler chicks, they were recovered near the limit of detection (2 log_{10} cfu g^{-1}), representing the natural background level of Rif resistance. Thus our containment procedures were sufficient to prevent cross-contamination over the course of the study.

\textbf{Bile Tolerance of \textit{Lactobacillus} Cultures}

Bile tolerance was evaluated by determining the $\mu_{\text{max}}$ of \textit{Lactobacillus} strains cultured in MRS broth supplemented with increasing concentrations of oxgall. The growth of all 4 \textit{Lactobacillus} strains was reduced as oxgall concentration increased. Although the growth rate of the \textit{L. crispatus} strains was greater than the \textit{L. gallinarum} strains, there was no difference in growth rate between stains of the same \textit{Lactobacillus} species when cultured in MRS broth alone. However, when the lactobacilli were cultured in 0.1 and 0.25% oxgall, the growth rate of \textit{L. crispatus} TDCC 75 was greater than

\begin{figure}[h]
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\includegraphics[width=\linewidth]{figure2.png}
\caption{Adhesion of \textit{Lactobacillus} cultures to chicken LMH epithelial cells. The adherence of Rifr \textit{Lactobacillus crispatus} ST1 (TDCC 75), \textit{L. crispatus} JCM 5810 (TDCC 76), \textit{Lactobacillus gallinarum} ATCC 33199 (TDCC 77), and \textit{L. gallinarum} JCM 8782 (TDCC 78) to chicken LMH epithelial cells was evaluated. \textit{Bacillus subtilis} ATCC 23857 and \textit{Salmonella} Typhimurium were used as low- and high-adherent controls, respectively. The geometric mean ± 95% CI adherent bacteria (bars) per well and percent adherent bacteria ± SEM (■) of inoculum from 4 replicate wells from 3 independent assays are shown. Different letters (a–d) indicate that means (adhesion) are significantly different ($P \leq 0.05$).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\linewidth]{figure3.png}
\caption{Gastrointestinal colonization of poultry by \textit{Lactobacillus}. Viable Rifr \textit{Lactobacillus} were enumerated from the (A) ileum and (B) cecum of broiler chicks administered \textit{Lactobacillus crispatus} TDCC 75 (●), \textit{L. crispatus} TDCC 76 (○), \textit{Lactobacillus gallinarum} TDCC 77 (■), \textit{L. gallinarum} TDCC 78 (□), or a mock inoculation (▲). Tissue samples were collected at 4-, 6-, and 8 d posthatch. \textit{Lactobacillus} colonization of broiler chicks is reported as the mean log_{10} ± SEM cfu·g^{-1} recovered from 5 chicks sampled at each time point (limit of detection, 2 log_{10} cfu·g^{-1}). Bracketed data points with different letters (a,b) at each time point indicate that means are significantly different ($P \leq 0.05$).}
\end{figure}
L. crispatus TDCC 76 (Figure 4A), and the growth rate of L. gallinarum TDCC 77 was greater than that of L. gallinarum TDCC 78 (Figure 4B). There was no difference in growth rate between strains of the same species at 0.5% oxgall or greater. Because the reduction in growth rate was greater at lower oxgall concentrations for L. crispatus TDCC 76 and L. gallinarum TDCC 78, they are more sensitive to bile than the other strain from their respective species.

**DISCUSSION**

The goal of this study was to investigate epithelial cell adhesion and its potential contribution to gastrointestinal colonization of *Lactobacillus* in poultry. Although previous studies have evaluated cellular adhesion of *Lactobacillus* cultures for use in poultry using human cell lines (Ehrmann et al., 2002; Lin et al., 2007; Messaoudi et al., 2012), we are unaware of any studies in which the binding potential of *Lactobacillus* cultures has been assessed using an epithelial cell line of chicken origin. Although the LMH chicken epithelial cell line was derived from the liver (Kawaguchi et al., 1987), its utility and in vivo relevance as an in vitro model for host-microbe interactions in the gastrointestinal tract of poultry has been established previously in studies of *C. jejuni* colonization and commensalism (Konkel et al., 2007; Larson et al., 2008; Flanagan et al., 2009; Quinones et al., 2009) and pathogenesis of *C. perfringens* (Keyburn et al., 2008) and *Salmonella* Enteritidis (Shah et al., 2012) in poultry. Finally, the LMH cell line was selected to better reflect the behavior of *Lactobacillus* cultures in the digestive tract of chickens (Konkel et al., 2007).

*Lactobacillus crispatus* ST1 was originally isolated from the crop of a chicken (Edelman et al., 2002) and has been demonstrated to adhere to frozen epithelial tissue sections from the gastrointestinal tract of chickens and to inhibit adhesion of avian pathogenic *Escherichia coli* to intestinal surfaces and ileal mucus of chickens (Edelman et al., 2003). The availability of a complete genome sequence (Ojala et al., 2010) has allowed the characterization of several potentially important adhesive proteins in vitro. *Lactobacillus crispatus* ST1 *Lactobacillus* epithelium adhesin (LEA) has been shown to bind stratified squamous epithelium of frozen chicken crop sections and mediate binding of *L. crispatus* ST1 to the VK2/E6E7 human vaginal cell line (Edelman et al., 2012). Additionally, the extracellular enzymes enolase, glucose-6-phosphate isomerase, glutamine synthetase, and glyceraldehyde-3-phosphate dehydrogenase have been demonstrated to bind murine and human extracellular matrix proteins (Hurmalainen et al., 2007; Kainulainen et al., 2012), suggesting they may serve as moonlighting proteins to promote adhesion to epithelial tissue. Thus, *L. crispatus* ST1 was used to evaluate the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lactobacillus crispatus TDCC 75</th>
<th>L. crispatus TDCC 76</th>
<th>Lactobacillus gallinarum TDCC 77</th>
<th>L. gallinarum TDCC 78</th>
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<tr>
<td>Ileal specimen</td>
<td>Total LAB (log$_{10}$ cfu·g$^{-1}$ ± SEM)$^1$</td>
<td>7.60 ± 0.24</td>
<td>8.00 ± 0.28</td>
<td>7.20 ± 0.37</td>
</tr>
<tr>
<td>d 4</td>
<td>8.85 ± 0.22$^a$</td>
<td>7.67 ± 0.30$^b$</td>
<td>9.67 ± 0.22$^a$</td>
<td>8.66 ± 0.53$^a$</td>
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<tr>
<td>d 6</td>
<td>8.51 ± 0.89</td>
<td>7.35 ± 0.37</td>
<td>7.47 ± 0.35</td>
<td>8.23 ± 0.23</td>
</tr>
<tr>
<td>d 8</td>
<td>26.4 ± 0.8$^b$</td>
<td>73.3 ± 8.7$^a$</td>
<td>68.3 ± 8.2$^a$</td>
<td>82.4 ± 2.8$^a$</td>
</tr>
<tr>
<td>Rif$^r$ of total LAB</td>
<td>23.7 ± 0.9$^b$</td>
<td>55.9 ± 5.0$^a$</td>
<td>29.5 ± 2.8$^a$</td>
<td>65.9 ± 4.3$^a$</td>
</tr>
<tr>
<td>Rif$^r$ LAB-positive chicks$^3$ (n)</td>
<td>24.6 ± 2.8</td>
<td>37.3 ± 3.5</td>
<td>28.3 ± 1.8</td>
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<td>d 4</td>
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<td>d 6</td>
<td>3</td>
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<td>d 8</td>
<td>0</td>
<td>3</td>
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<td>5</td>
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<tr>
<td>Cecal specimen</td>
<td>Total LAB (log$_{10}$ cfu·g$^{-1}$ ± SEM)</td>
<td>9.83 ± 0.22</td>
<td>9.42 ± 0.14</td>
<td>9.02 ± 0.29</td>
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<tr>
<td>d 4</td>
<td>9.15 ± 0.24$^a$</td>
<td>7.88 ± 0.24$^b$</td>
<td>8.98 ± 0.13$^a$</td>
<td>7.78 ± 0.24$^b$</td>
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<tr>
<td>d 6</td>
<td>8.42 ± 0.17$^c$</td>
<td>8.77 ± 0.21$^b$</td>
<td>8.88 ± 0.46$^a$</td>
<td>9.41 ± 0.06$^b$</td>
</tr>
<tr>
<td>d 8</td>
<td>23.4 ± 1.6$^b$</td>
<td>74.8 ± 6.8$^a$</td>
<td>76.5 ± 3.6$^a$</td>
<td>88.4 ± 7.5$^a$</td>
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<tr>
<td>Rif$^r$ of total LAB</td>
<td>24.8 ± 3.4$^b$</td>
<td>85.7 ± 9.1$^a$</td>
<td>36.8 ± 5.7$^b$</td>
<td>99.0 ± 2.7$^a$</td>
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<td>Rif$^r$ LAB-positive chicks (n)</td>
<td>23.8 ± 0.5$^b$</td>
<td>53.0 ± 8.9$^a$</td>
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<td>d 8</td>
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$^a$Different superscripts within rows indicate that means differ significantly ($P \leq 0.05$).

$^1$Limit of detection, 2 log$_{10}$ cfu·g$^{-1}$.

$^2$Days posthatch.

$^3$Number of chicks from which Rif$^r$ LAB were recovered ≥2 log$_{10}$ cfu·g$^{-1}$. L. gallinarum TDCC 77 was greater than that of *L. gallinarum* TDCC 78 (Figure 4B). There was no difference in growth rate between strains of the same species at 0.5% oxgall or greater. Because the reduction in growth rate was greater at lower oxgall concentrations for *L. crispatus* TDCC 76 and *L. gallinarum* TDCC 78, they are more sensitive to bile than the other strain from their respective species.
The effect of various experimental factors on the adhesion of _Lactobacillus_ to the LMH epithelial cell line to establish a standard reproducible set of conditions that could be used to evaluate probiotic adhesion and potentially mimic their colonization in chickens.

The effect of buffer pH on in vitro adhesion of _Lactobacillus_ cultures (Ouweland and Salminen, 2003) has been demonstrated with adhesion being enhanced in acidic buffers (Greene and Klaenhammer, 1994; Lehto and Salminen, 1997) and moonlighting surface proteins becoming detached at neutral to basic pH (Kainulainen et al., 2012). However, when working with tissue culture cells the adhesion buffer should have neutral pH due to the negative effect of low pH on the viability of tissue culture cells and the potential aberrant exposure of potential binding sites due to pH dependent changes in surface proteins of tissue culture cells (Greene and Klaenhammer, 1994; Lehto and Salminen, 1997). Additionally, because the gastrointestinal pH has been reported to be 6.3 to 6.4 in the ileum and 7.0 or greater in the cecum of chickens (Denbow, 2000), PBS (pH 7.2) is appropriate for in vitro adhesion assays.

It was determined that 5 washes were sufficient to remove nonadherent lactobacilli before disruption of LMH cells for enumeration of bacteria (Figure 1A). Previous studies evaluating adherence of _Lactobacillus_ isolates to human epithelial cell lines report the use of 3 to 5 postincubation washes (Kapczynski et al., 2000; Medellin-Peña and Griffiths, 2009; Duary et al., 2011), whereas studies performed using epithelial tissues dissected from poultry reported using 3 washes (Gusils et al., 1999; Ben Salah et al., 2012). The use of 3 postincubation washes has also been reported previously in the evaluation of _C. jejuni_ adherence to LMH cells (Konkel et al., 2007; Larson et al., 2008; Flanagan et al., 2009). Because this bacterium is known to be a particularly strong colonizer of the poultry gastrointestinal tract, it is likely that there are fewer nonadherent bacteria as a percentage of the inoculum than for _Lactobacillus_.

Adhesion of _Lactobacillus_ ST1 to chicken LMH cells was determined to be dose dependent, increasing with the count (cfu) per LMH cell ratio from 100:1 to 1,000:1 (Figure 1B). However, the percentage of the inoculum which adhered to the LMH cell was unchanged. Thus, 100:1 cfu per LMH cell was selected for subsequent assays in this study. Ratios of bacteria (cfu) to cells ranging from 100:1 to 2,000:1 have been reported in studies of _Lactobacillus_ adhesion to human cell lines (Ehrmann et al., 2002; Lin et al., 2007; Medellin-Peña and Griffiths, 2009; Duary et al., 2011). A study evaluating _Lactobacillus_ adhesion to dissected tissue sections from poultry reported a cfu per cell ratio of 200:1 (Ben Salah et al., 2012). A multiplicity of infection of 100:1 is commonly used in studies of _C. jejuni_ adhesion to chicken LMH cells (Konkel et al., 2007; Flanagan et al., 2009). Although we did not investigate whether adherence was saturable at cfu per cell ratios greater than 1,000:1, the dose-dependent nature of _Lactobacillus_ adherence to the chicken LMH cell line suggests a mechanism of adherence dependent upon specific interactions of cell surface factors (Konkel et al., 2010). The adhesive properties of several surface and extracellular proteins of _L. crispatus_ ST1 have been characterized previously (Hurmalainen et al., 2007; Edelman et al., 2012; Kainulainen et al., 2012). However, their role in vivo in mediating adherence to epithelial tissues or gastrointestinal colonization in poultry has not yet been demonstrated.

The _Lactobacillus_-LMH cell binding assay was used to evaluate the adhesion of _Lactobacillus_ cultures to a poultry-derived epithelial cell line (Figure 2). In addition to _L. crispatus_ ST1, 3 additional poultry isolates, _L. crispatus_ JCM 5810, and _Lactobacillus gallinarum_ (ATCC 33199 and JCM 8782), were selected to serve
as model probiotic Lactobacillus cultures. Lactobacillus crispatus JCM 5810 and L. gallinarum ATCC 33199 have been demonstrated previously to inhibit the growth of C. jejuni in vitro and reduce gastrointestinal colonization of C. jejuni in broiler chickens (Neal-Mckinney et al., 2012). Because they would be used to facilitate differentiation of the administered Lactobacillus cultures from the background LAB in the in vivo colonization studies, adhesion assays were performed using Rif² derivative cultures of each Lactobacillus strain as well. All Lactobacillus cultures were found to adhere effectively to the chicken LMH cells when compared with B. subtilis and Salmonella Typhimurium, which were used as low- and high-adherent controls, respectively. Lactobacillus crispatus TDCC 75 and the 2 L. gallinarum strains adhered to the chicken LMH cells at similar levels, whereas adhesion of L. crispatus TDCC 76 was lower than the other strains. The number of adherent Lactobacillus was found to be 1.5 to 3.5% of the cultures used to inoculate the chicken LMH cells. Studies evaluating Lactobacillus adherence to human epithelial cell lines report adherence of 1 to 14% of the inoculum (Ehrmann et al., 2002; Messaoudi et al., 2012).

Lactobacillus gallinarum TDCC 77, followed by L. crispatus TDCC 75, were found to colonize broiler chicks more persistently and at greater levels than the other lactobacilli in our time course study (Figure 3). Although we are hesitant to speculate on the role of tissue-specific tropism on the greater persistence of L. crispatus TDCC 75 and L. gallinarum TDCC 77, it is interesting to note that their wild-type parent strains were both reported to be isolated from the crop of chickens, whereas the other strains were reported to be isolated from chicken feces (Table 1). In addition to differences in gastrointestinal persistence of Lactobacillus cultures, our study demonstrates the transient nature of colonization by allochthonous bacteria (Walter, 2008). The inability of probiotic cultures to establish permanent colonization in the gastrointestinal tract has been well documented (Jacobsen et al., 1999; Tannock et al., 2000; Frese et al., 2012). These data suggest that continued repeated administration is required to maintain the presence of probiotic cultures in the gastrointestinal tract.

The similar in vitro adhesion of L. crispatus TDCC 75, L. gallinarum TDCC 77, and L. gallinarum TDCC 78 suggested that their colonization of broiler chicks might also be comparable. However, colonization of L. gallinarum TDCC 78 was similar to the less adherent L. crispatus TDCC 76. L. gallinarum TDCC 78 was determined to be more sensitive to bile than L. gallinarum TDCC 77, suggesting that although adhesion of the L. gallinarum strains was comparable, the differences in persistence may be due to reduced survivability of L. gallinarum TDCC 78. Bile tolerance has been suggested previously as an additional factor likely to be important in gastrointestinal persistence and has been used previously as a criterion for the selection of Lactobacillus cultures as probiotics for use in humans (Azcarate-Peril et al., 2008; Klaenhammer et al., 2008) and livestock animals (Ehrmann et al., 2002; Shin et al., 2002; Brashears et al., 2003; Taheri et al., 2009).

In this study, we characterized adhesion to epithelial cells and gastrointestinal colonization of 4 model probiotic Lactobacillus cultures in poultry. We have demonstrated the value of the chicken LMH cell line for use in cell culture models of Lactobacillus adhesion to chicken epithelial tissues. However, its relevance to gastrointestinal colonization is limited due to the complexity of the gastrointestinal environment and the multifactorial nature of colonization. Evaluating the adhesion of Lactobacillus cultures to other substrates including intestinal mucus and extracellular matrix components will continue to be important given the limitations of the LMH cell line. Additionally, our results demonstrate that factors unrelated to adhesion such as bile tolerance also contribute to the ability of lactobacilli to persist in the gastrointestinal tract of poultry. Studies using isogenic mutant strains differing in cellular adhesion, bile tolerance, and other properties are needed to elucidate the role of these factors in the gastrointestinal persistence and colonization of probiotic Lactobacillus cultures in poultry (Ouweland and Salminen, 2003). The availability of a chicken-specific cell culture model will contribute to these future studies. Finally, the LMH cell line has also been demonstrated previously to be useful for the investigation of host-microbe interactions of human foodborne pathogens in poultry, including Campylobacter, Clostridium, and Salmonella. This suggests the utility of this cell culture model for investigation of Lactobacillus-mediated competitive exclusion and virulence inhibition of pathogenic microorganisms in poultry. These studies are expected to contribute to a mechanistic understanding of probiotic functionality in chickens, the development of more effective screening tools for the selection of probiotic cultures, and the more effective application of probiotics in poultry production.

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