**Lactobacillus Flora in the Cloaca and Vagina of Hens and Its Inhibitory Activity Against Salmonella enteritidis In Vitro**

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**ABSTRACT** Lactobacilli in the cloaca and vagina of 40 normal laying hens were investigated quantitatively and qualitatively, and their ability to inhibit growth of *Salmonella enteritidis* (SE) was examined using a spot-the-lawn technique. All samples of cloacal contents and half the samples of vaginal mucus were positive for lactobacilli. The means ± SD of total *Lactobacillus* counts in the cloaca and those in the vagina were log$_{10}$ 5.5 ± 1.1 and 2.5 ± 2.6 cfu/g, respectively. In the cloaca, *Lactobacillus acidophilus* was isolated from 92.5% of hens, and *Lactobacillus salivarius* was isolated from 85.0% of hens, whereas *Lactobacillus fermentum* was isolated from only one hen. In the vagina, *L. acidophilus* and *L. salivarius* were isolated from 42.5% of hens. In the inhibition assay in vitro, all strains of *Lactobacillus* from cloacal contents and vaginal mucus inhibited growth of SE. There was a wide range of the inhibitory activity even in the same species. No difference of the growth inhibition zone was observed between lactobacilli from cloaca and those from vagina. The present study suggested that lactobacilli in the cloaca and vagina of hens might have a protective effect against SE colonization.

*(Key words: Salmonella enteritidis, Lactobacillus, ascending infection, layer, egg contamination)*

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**INTRODUCTION**

Outbreaks of human salmonellosis caused by *Salmonella enteritidis* (SE) have dramatically increased during the past two decades and have become an important international public health and economic issue (Rodrigue et al., 1990; Duguid and North, 1991). Epidemiological analyses suggest contaminated eggs or egg products as the major source of the infection (St. Louis et al., 1988; Hansenson et al., 1992). Many strategic plans to prevent the incidence of SE food poisoning outbreaks have been proposed and performed. Despite these efforts, no perfect preventive methods have been found.

An understanding of the mechanism contributing to SE colonization through eggs is essential to reduce the public health risk associated with consumption of infected eggs. Our previous experiments (Miyamoto et al., 1997, 1998) suggested that SE colonized and proliferated in the cloaca, then ascended from the cloaca into the vagina, and colonized in the vagina, which resulted in an increased production of SE-contaminated eggs. Furthermore, Reiber and Conner (1995) reported that virgin Leghorns had a much higher degree of colonization of SE in the ovary, oviduct, and eggs after insemination with contaminated semen than did mated breeder hens. They suspected that one reason for these results was the competitive exclusion of SE in the ovary and oviduct. Therefore, the relation between the production of SE-contaminated eggs and the ascending SE infection from the cloaca into vagina should be investigated to minimize SE-contaminated eggs. In inhibition of the ascending infection of the genital organs in mammals, microflora, especially lactobacilli, in the vagina have been reported to play an important role (Redondo-Lopez et al., 1990; Reid et al., 1990; Hudault et al., 1997). However, little information is available on the normal microflora of the genital organs in poultry (Harry, 1963; Jacobs et al., 1978; Aguire et al., 1992) and their role involving SE infection. We previously reported (Miyamoto et al., 1998) the possibility that lactobacilli are associated with a protective effect against the ascending infection with SE in hens. *Lactobacillus* is one of the most predominant resident organisms in the normal cloaca and vagina, and alteration of the *Lactobacillus* population is observed after intraocular inoculation with SE. We hypothesize that lactobacilli in the cloaca and vagina of hens have a protective effect against SE colonization.

The purpose of the present study was twofold: 1) to provide quantitative and qualitative information on lactobacilli in the cloaca and vagina of normal laying hens, and...
2) to test the ability of lactobacilli isolated from the cloaca and vagina to inhibit the growth of SE in vitro.

**MATERIALS AND METHODS**

**Experimental Hens**

Forty, 30- to 35-week-old White Leghorn, Dekalb-TX35 hens were obtained from the local commercial layer flock. Hens were raised in individual wire-floored cages with controlled artificial illumination and were provided ad libitum access to water and antibiotic-free layer-breeder ration. All experimental hens were at peak egg production.

**Qualitative and Quantitative Examination of Lactobacilli from Cloacal Contents and Vaginal Mucus**

Cloacal contents and vaginal mucus were taken separately by swabbing the mucosal surface of the organs with preweighed sterile cotton swabs. To take mucus from the vagina, its terminal portion was made to protrude outside the cloaca with the same method as in artificial insemination. The terminal portion of the vagina was held gently with sterile forceps to prevent it from receding. The preweighed sterile swabs were carefully inserted into the vagina, and the vaginal mucus was sampled at a depth of about 2 cm. The swabs were weighed again and placed in tubes containing 2 mL of sterile physiological saline solution. Four serial 10-fold dilutions of the specimens were made. Each 0.02 mL from undiluted suspension, second and fourth dilutions of the suspension were withdrawn and spread onto one-third part of Lactobacillus-selective agar with 1.32 mL acetic acid/L of medium. The Lactobacillus-selective agars were incubated at 37 C in an anaerobically sealed jar for 72 h. The different types of colonies were isolated and identified to species level on the basis of their mortility, morphology, Gram staining, and carbohydrate fermentation pattern by the API 50 CHL system. Numbers of colonies on each plate were recorded, and carbohydrate fermentation pattern by the API 50 CHL system. All experimental hens were at peak egg production.

**Bacterial Strains and Growth Media for Inhibition Assay**

Forty-two strains of Lactobacillus isolated from 10 hens that were randomly selected from the above experiment were used in the inhibition assay. Four strains of Escherichia coli and four strains of Bacteroidaceae isolated from cloaca of the experimental hens, which were the predominant organisms in the cloaca and vagina of healthy hens (Miyamoto et al., 1998), were used to compare the inhibition activity of lactobacilli. A strain of SE phage Type 4 used in this study was kindly supplied by T. Tsukamoto, of the Osaka Prefectural Institute of Public Health, Osaka 537-0025, Japan. This strain was originally isolated from a fecal sample from a food-poisoned patient.

The following growth media were used in the inhibition assay: deMan Rogasa Sharpe agar for Lactobacillus; desoxycholate hydrogen sulfide lactose (DHL) agar for Escherichia coli; neomycin-brilliant green-taurocholate-blood (NBGT) agar, composed of 5.8 g blood liver agar base, 20 mg neomycin, 100 mg sodium taurocholate, and 5 mL sheep blood per 100 mL of medium, for Bacteroidaceae; trypto-soya broth to enrich E. coli and SE; Gibco anaerobic medium (GAM) to enrich Lactobacillus and Bacteroidaceae in samples.

**Inhibition Assay**

The spot-the-lawn technique reported by Oyarzabal and Conner (1995) was used. Briefly, lactobacilli and Bacteroidaceae were grown in GAM broth anaerobically at 37 C for 48 h, and E. coli was grown in trypto-soya broth aerobically at 37 C for 24 h. Petri dishes containing approximately 15 mL each of deMan Rogasa Sharpe agar, DHL agar, and NBGT agar were prepared. A sterile paper disk (0.6 cm in diameter) was placed in each plate. Ten µL of each culture broth was dispensed on the top of each disk. Bacterial counts of the broth were calculated by the serial dilution method. Average counts of Lactobacillus, E. coli, and Bacteroidaceae were 10^7 cfu/mL, 10^8 cfu/mL, and 10^9 cfu/mL, respectively. Each plate was seeded with one kind of bacterium. Plates with disks containing Lactobacillus and Bacteroidaceae were incubated anaerobically at 37 C for 48 h, and plates with disks containing E. coli were incubated aerobically at 37 C for 24 h. Next, 9 mL of molten (45 C) trypto-soya agar inoculated with 0.5 mL from 24-h SE culture broth containing 10^8 cfu/mL, was poured over the seeded plates. Overlaid plates were incubated aerobically at 37 C for 24 h. Inhibition of SE growth was determined by measuring the radius of clear zones (in cm) around the paper disks. Four replicates were prepared for each bacterium. Mean and SD of the zones of inhibition were calculated.

For controls, 10 µL of untreated GAM broth or GAM broth adjusted to pH 4.0, 4.6, 5.2, or 5.8 was dispensed on the disk, and the same procedures were performed. The same assays were conducted using 10 µL of cell-free supernatant obtained by centrifugation and filter sterilization of the cultures from five Lactobacillus strains exhibiting the largest zones of inhibition in the above experiments.

**RESULTS**

**Lactobacilli of Cloacal Contents and Vaginal Mucus**

Table 1 shows the isolated lactobacilli and the counts of lactobacilli in the cloacal contents and vaginal mucus.

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1Chubukakinsangyo, Gifu 509-6251, Japan.
2Nihonhaigo-shiryo Co., Ltd., Aichi 478, Japan.
3BBL, Cockeysville, MD 21030.
4BioMérieux, Marcy-l’Etoile 09280, France.
6Nissui, Toshima, Tokyo 170, Japan.
7SB. A. Flory, Etpile 09280, France.
8Etpile 09280, France.
of 40 hens. All cloacal samples were positive for lactobacilli, and the total number of lactobacilli (mean ± SD) was log_{10} 5.5 ± 1.1 cfu/g. In the cloaca, *Lactobacillus acidophilus* (92.5%; log_{10} 4.8 ± 1.7 cfu/g) and *Lactobacillus salivarius* (85.0%; log_{10} 4.5 ± 2.2 cfu/g) were frequently isolated, whereas *Lactobacillus fermentum* (2.5%; log_{10} 5.1 cfu/g) was isolated from only one hen.

Half of the vaginal samples (50.0%) were positive for lactobacilli; the total number of lactobacilli (mean ± SD) was log_{10} 2.5 ± 2.6 cfu/g, *Lactobacillus acidophilus* (42.5%; log_{10} 1.9 ± 2.4 cfu/g) and *L. salivarius* (42.5%; log_{10} 2.1 ± 2.5 cfu/g) were isolated from the vagina.

### Inhibition Assay

All strains of *Lactobacillus* from cloacal contents and vaginal mucus inhibited SE, as evidenced by distinct zones of inhibition (maximum 1.6 cm, minimum 0.2 cm) (Table 2). Several strains of *L. salivarius* isolated from the cloacal contents had larger inhibition zones (1.2 cm in radius) than those of *L. acidophilus*. However, there was a wide range of the inhibition zones even in the same species. No difference in the inhibition zones was observed between lactobacilli from cloaca and those from vagina.

No strain of *E. coli* or *Bacteroidaceae* produced zones of inhibition of SE. Untreated GAM broth; GAM broth adjusted to pH 4.0, 4.6, 5.2, or 5.8, and culture supernatants from five strains of *Lactobacillus* also did not inhibit SE.

### DISCUSSION

Few reports have examined the normal microflora of the cloaca or vagina in poultry (Harry, 1963; Jacobs et al., 1978; Aguire et al., 1992). We reported in our previous study (Miyamoto et al., 1998) that the mean of total bacterial counts was log_{10} 7.7 cfu/g in the cloaca and log_{10} 5.7 cfu/g in the vagina. Anaerobic and aerobic bacteria were equally recognized in the cloaca, whereas in the vagina, anaerobic bacteria were predominant. *Lactobacillus* and *Bacteroidaceae* were isolated frequently from the cloaca and vagina, but *E. coli* was isolated frequently only from the cloaca. In our previous study (Miyamoto et al., 1998), however, lactobacilli were not identified at the species level, and the roles of microflora including lactobacilli of cloaca and vagina were not investigated. Much research has been conducted on the microflora of the digestive tract in chickens (Timms, 1968; Barnes et al., 1972; Salanitro et al., 1978), where lactobacilli are predominant. *Lactobacillus* flora were composed mainly of *L. acidophilus*, *L. salivarius*, and *L. fermentum* but rarely included other species (Morishita et al., 1971). In the present experiment, *L. acidophilus*, *L. salivarius*, and *L. fermentum* were isolated from the cloaca, and *L. acidophilus* and *L. salivarius* were isolated from the vagina. Composition of *Lactobacillus* flora in the cloaca and vagina of hens appears to be similar to that in the intestine.

Many studies (Juven et al., 1991; Hinton et al., 1992; Chateau et al., 1993; Oyarrzabal and Conner, 1995; Jin et al., 1996) have reported that lactobacilli inhibit the growth of various pathogens, including *Salmonella* spp. Lactobacilli isolated from dairy products have been investigated, but the suppressive activity of lactobacilli isolated from the cloaca and vagina of hens against pathogens has not been documented. Our *in vitro* experiment showed that lactobacilli isolated from cloaca and vagina of hens can inhibit the growth of SE. A large variety of the inhibition zones were found in the same species, and several strains of *L. salivarius* isolated from the cloaca made larger inhibition zones than those of *L. acidophilus*. Cosby et al. (1997) reported that *E. coli* produced significant inhibitory activity against the *S. typhimurium* strains tested *in vitro*. However, the inhibitory activity against SE by *Bacteroidaceae* and *E. coli* was not observed in this experiment.

The mechanisms that lactobacilli use to inhibit the growth of various other bacteria *in vitro* are believed to be production of hydrogen peroxide, production of organic acids such as lactic and acetic acids to decrease pH, and production of specific proteins such as bacteriocins (Hinton et al., 1992; Chateau et al., 1993). In the present study, hydrogen peroxide production was unlikely to be the cause of the observed inhibition because all lactobacilli were grown anaerobically. The inhibition may also not

### TABLE 1. Species and counts of lactobacilli in cloacal contents and vaginal mucus of 40 hens

<table>
<thead>
<tr>
<th>Source</th>
<th>Isolates</th>
<th>Frequency of isolation (%)</th>
<th>Counts¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloaca</td>
<td>Total lactobacilli</td>
<td>100</td>
<td>5.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus</em></td>
<td>92.5</td>
<td>4.8 ± 1.7</td>
</tr>
<tr>
<td></td>
<td><em>L. salivarius</em></td>
<td>85.0</td>
<td>4.5 ± 2.2</td>
</tr>
<tr>
<td></td>
<td><em>L. fermentum</em></td>
<td>2.5</td>
<td>5.1</td>
</tr>
<tr>
<td>Vagina</td>
<td>Total lactobacilli</td>
<td>50.0</td>
<td>2.5 ± 2.6</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus</em></td>
<td>42.5</td>
<td>1.9 ± 2.4</td>
</tr>
<tr>
<td></td>
<td><em>L. salivarius</em></td>
<td>42.5</td>
<td>2.1 ± 2.5</td>
</tr>
</tbody>
</table>

¹Log_{10} mean cfu/g ± SD.

### TABLE 2. Inhibition zones of SE by lactobacilli isolated from the cloacal contents and vaginal mucus of hens

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>Mean radius of inhibition zone in each strain (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloaca</td>
<td><em>L. salivarius</em></td>
<td>1.2, 1.2, 1.2, 1.2, 1.1, 1.1, 1.0, 1.0, 1.0, 0.9, 0.8, 0.7</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus</em></td>
<td>1.1, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 1.0, 0.9, 0.9, 0.9, 0.8, 0.8</td>
</tr>
<tr>
<td></td>
<td><em>L. fermentum</em></td>
<td>1.2</td>
</tr>
<tr>
<td>Vagina</td>
<td><em>L. salivarius</em></td>
<td>1.1, 1.0, 0.9, 0.9</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus</em></td>
<td>1.1, 1.0, 1.0, 1.0, 0.9, 0.9</td>
</tr>
</tbody>
</table>
be attributed to the acidification because GAM broth adjusted to low pH did not produce any inhibition of SE. Although the culture supernatant from *Lactobacillus* strains did not produce any inhibition of SE, bacteriocins still could not be ruled out as the cause of this inhibition, because the concentration of bacteriocins in the culture supernatant was possibly not high enough to inhibit the growth of SE. Lewus and Montville (1991) reported that only a few of the strains that showed positive results using the spot-the-lawn technique gave positive results in the well-diffusion assays. In that study (Lewus and Montville, 1991), they speculated that aggregation, non-diffusible bacteriocins, and concentration effects lead to false-negative results with the well-diffusion assay. More work is necessary to characterize the nature of this inhibition.

*Lactobacilli* are usually predominant in the human vagina, and their presence is considered crucial for maintaining the ecological balance of the vaginal microflora (Reid et al., 1990; Hudault et al., 1997). Because *Lactobacillus* is one of the most prevalent and numerous organisms in the normal cloaca and vagina, and lactobacilli inhibit the growth of SE in *vitro*, lactobacilli may be associated with a protective effect against the ascending infection of SE in poultry. The SE may cause infection of lower reproductive organs and subsequently produce contaminated eggs, after interactions with the indigenous microflora (Miyamoto et al., 1997, 1998). More research is needed to determine the role of the microflora, especially lactobacilli, in the cloaca and vagina of poultry.

The autochthonous microflora are beneficial to a wide variety of animals, in both disease resistance and physiological functions. In chickens, to exclude *Salmonella* from the intestine or to suppress *Salmonella* infection in the intestine, competitive establishment of nonpathogenic microflora has been investigated (Baba et al., 1991; Juven et al., 1991). In the human, *Lactobacillus* preparations have been used by several groups in attempts to treat or prevent both bacterial and fungal vaginitis (Bruce and Reid, 1988; Herthelius et al., 1989; McGroarty, 1993), although further work remains to be done before this can become a prophylactic alternative. It is also conceivable that artificial implantation of indigenous bacteria, such as lactobacilli, into the cloaca or vagina would be beneficial in attempts to prevent SE colonization in the cloaca and the ascending infection in the reproductive organs in poultry. Further studies on the microflora in the cloaca and vagina in poultry should be studied to apply this new competitive exclusion method in the future.

**ACKNOWLEDGMENTS**

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