Bacterial Flora of the Respiratory Tracts in Chickens with a Particular Reference to *Lactobacillus* Species

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ABSTRACT. The effects of three different types of breeding such as isolator, floor, and cage breedings on the bacterial flora of the respiratory tracts (nasal cavity, tongue, pharygolarynx, trachea and air sac) in chickens were determined. Total viable bacterial numbers on the nasal mucus of chickens in the isolator breeding as control group (Group A) aged of 14 days were $10^{4.6}$ / g of autopsy specimen (wet weight), $10^{5.7}$ / g of sample in the cage breeding (Group B) aged of 28 days, and $10^{7.0}$ / g of sample in the floor breeding (Group C) aged of 28 days. Staphylococci and micrococci were predominant bacteria in the nasal cavities of all groups. Total viable numbers of tongue and pharygolarynx were from $10^{5.4}$ to $10^{6.5}$ / g of autopsy specimen. Lactobacilli were the predominant bacteria in pharyngolarynx of chickens. The incidence of staphylococci and micrococci in trachea was lower than those in the another regions. Staphylococci and micrococci dominated in the air sacs of two groups (B and C), but the number and incidence of lactobacilli in the air sacs of chickens were lower than those in the another respiratory tracts. The only clostridia isolation in the air sacs of Group A was observed. A total of 75 strains of *Lactobacillus* species was isoalted from all respiratory organs and intestine of chickens. These strains were divided into 19 groups. *Lactobacillus salivearius* subsp. *salivarius* was the predominant lactobacilli isolated from tongue and pharyngolarynx. Most of isolates from the chicken intestines were mainly identified as the *L. acidophilus* group and *L. reuteri*. These findings show that lactobacilli were predominantly isolated from nasal cavity, tongue, and pharyngolarynx of chickens, but not from trachea and air sac and the difference at the species level of *Lactobacillus* is present.—KEY WORDS: chicken, lactobacilli, respiratory microflora.

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The economics of poultry production, along with increasing construction and equipment costs of housing, mandates high bird densities in commercial operations. High bird density in turn increases the possibility of disease spread by airbrone microorganisms and accentuates problems of dust and air contamination. Therefore, many chances might be present in the pathogenic bacteria occupied in the respiratory organs of chickens. Although bacterial flora in the digestive tracts plays an important role on the bird productions [1, 20, 23], there is a few reports about the respiratory microflora of chickens associated with several disease [3, 19]. However, the normal respiratory microflora were not determined. Normal microflora in the respiratory tracts as well as intestine of chickens, especially *Lactobacillus* species, may also play an important role to protect the respiratory infection by other microorganisms.

The present study was undertaken to investigate the bacterial flora in the respiratory organs including nasal cavity, tongue, pharyngolarynx, trachea, and air sac by three different types of chicken breedings, such as isolator, cage, and floor, and to compare the compositions of *Lactobacillus* species isolated from the respiratory tracts and feces of chickens as a special reference.

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MATERIALS AND METHODS

Chickens used: Thirty-nine chickens hatched in gnotobiotic isolator for two weeks were divided into three groups, such as Group A; seven chickens in the isolator breeding, Group B; 16 chickens in the cage breeding, and Group C; 16 chickens in the floor breeding. All the chickens were given a feedstuff without antibiotics.

Analysis of bacterial flora in the respiratory organs of chickens: After all chickens were sacrificed under anesthesia with ethylether, the biopsy specimens from nasal cavity, tongue, pharyngolarynx, trachea, and right and left air sacs were collected by using a sterile wab and weighed. Whole trachea were divided into two regions of the upper part and the lower part. These autopsy specimens were homoginized with 3 m*l* of an anaerobic diluent [14]. Sampling schedules were performed Day 14 after the birth of chickens for Group A and Days 28 and

42 for Groups B and C. After through mixing, the autopsy specimens were serially diluted from 10^{-1} to 10^{-8} with the anaerobic diluents [16]. From each dilution, 0.05 m*l* aliquots were spread on the surface of agar plates including Trypticase soy agar (BBL, U.S.A.) with 5% horse blood [16], DHL (Eiken, Tokyo) for enterobacteria [16], and TATAC agar for enterococci [16] and aerobically at 37°C for 48 hr. Two medium of blood-liver agar for anaerobic bacteria [14] and LBS agar (BBL, U.S.A.) for lactobacilli [16] were incubated at 37°C for 72 to 90 hr by using the anaerobic steel wool jar method [18]. After incubation, each plate was examined for colonies. The identification of isolates was performed with colonial and cellular morphologies, Gram staining, spore formation, and aerobic growth. For the bacterial species identified, the bacterial number per gram of wet feces was counted and converted into a logarithmic equivalent.

Enumeration of airborne microbes in the chicken house: The enumeration of airborne microbes in the chicken house was carried out by using two plates of TS agar for aerobes [15] and PEES agar for staphylococci and micrococci [15]. After opening the lids of agar plates in the chicken house of Groups B and C for 6 hr, two media were incubated at 37°C for 48 hr. The numbers of colony appeared on the surface of each medium were counted.

Characterization of lactobacilii from the respiratory organs of chickens: Several strains of lactobacilli were isolated from nasal cavity, tongue, pharygolarynx, and rectum contents by using BL and LBS agar plates. The identification of all the lactobacilli isolates in the present study were performed by using Mitsuoka's method [14]. Gas formation from glucose broth, growth at 15°C, and sugar fermentation were carried out. After an inoculation of lactobacilli tested to each tube with sugar, the characteristics of these strains were examined after 3 days and 7 days of incubation.

Since *L. acidophilus* could not differentiate from *L. gasseri, L. crispatus*, and *L. amylovorus* on the basis of carbohydrate fermentations [8, 12], they were designated as the *L. acidophilus* group in this study. Since the intestinal heterofermentative lactobacilli formerly identified as *L. fermentum* is named *L. reuteri* [10], the name of this microorganism used as *L. reuteri*.

RESULTS

The comparison of bacterial flora in the nasal cavities of chickens is shown in Table 1. Total viabale counts and the numbers of coryneform bacteria, staphylococci and micrococci, and streptococci from autopsy specimens of the floor-breeding chickens (Group B) were significantly higher than those of the cage breeding (Group C) and the isolator breeding (Group A) chickens. Staphylococci and micrococci were the most predominant bacterial group in all of these groups. Streptococci and coryneform bacteria were also isolated from all birds used in this study.

Table 1 also shows the composition of bacterial flora on the chicken tongues. Staphylococci and micrococci were also the most predominant bacterial groups as well as the nasal cavities. The total viable bacteria among three groups were a similar number whereas the counts of these microorganisms in Group C were significantly higher than those of Group B. Three bacterial groups of lactobacilli, streptococci, and enterobacteria were also detected from the autopsy specimens of three groups.

The microflora of pharyngolarynx mainly consisted of lactobacilli, streptococci, enterobacteria, and staphylococci and micrococci. The total viable counts were a similar level among three experimental groups whereas a significant increasing number of streptococci was found in Group C.

Only staphylococci and micrococci were detected from the tracheas in Groups B and C by Day 28. These microorganisms were never isolated from the autopsy specimens in Group A. No difference of numbers of staphylococci and micrococci was present in the upper and lower part of tracheas.

The bacterial flora of the air sacs in Groups B and C comprised staphylocci and micrococci. Clostridia were isolates from them only in Group A. The lower number and incidence of lactobacilli in Group C was observed.

A total of 75 strains of lactobacilli were isolated from the biopsy specimens of three experimental groups. These isolates were divided into 19 groups by using the carbohydrate fermentation, gas formation, and growth at 15°C as shown in Table 2. Two groups of homofermentative lactobacilli were identified as *L. salivarius* susbsp. *salicinius* and *L. salivarius* subsp. *salivarius*, respectively. Four homofermentative groups (biovars A ~ D) were identified as the *L. acidpohilus* group and four

Table 1. Composition of bacterial flora in the respiratory organs of chickens[a)]

Bacterial flora	Group A	Group B		Group C	
	Day 14	Day 28	Day 42	Day 28	Day 42
			Nasal cavities		
Total bacteria[b)]	4.6±0.6(6/7)	5.7±0.6[c)](8/8)	5.6±0.8[c)](8/8)	7.0±0.5[c,d)](8/8)	6.9±0.8[c,e)](8/8)
Lactobacilli	3.5 (1/7)	3.8±1.0(7/8)	4.3±1.0(7/8)	5.6±1.2(4/8)	5.4±0.8[e)](8/8)
Staphylococci and Micrococci	4.4±0.7(5/7)	5.7±0.8[c)](8/8)	5.4±1.0[c)](8/8)	7.0±0.5[c,d)](8/8)	6.9±0.8[c,e)](8/8)
Streptococci	3.9 (1/7)	4.1±0.8(5/8)	4.2±1.2(6/8)	5.3±0.7[d)](8/8)	5.0±1.1(7/8)
Coryneform bacteria	3.7 (1/7)	3.8±1.0(7/8)	4.3±1.0(7/8)	5.6±1.2(4/8)	5.4±0.8(8/8)
Enterobacteria	5.4 (1/7)	3.5±0.9(2/8)	3.9 (1/8)	3.1 (1/8)	2.9±0.2(3/8)
Aerobic gram-negative rods	—	3.1 (1.8)	4.5±1.6(4/8)	4.5 (1/8)	5.0±0.8(3/8)
Bacilli	3.4±0.2(2/7)	3.5±0.1(2/8)	—	—	2.8 (1/8)
Yeasts	3.3 (1/7)	3.2 (1/7)	—	—	—
Clostridia	3.4 (1/7)	3.4 (1/8)	—	—	3.7±0.9(4/8)
Bacteroides	—	—	—	—	3.3±0.2(4/8)
Eubacteria and Bifidobacteria	—	—	—	—	4.4±0.6(2/8)
			Tongues		
Total bacteria	5.4±0.5(7/7)	5.8±0.4(8/8)	5.9±0.7(8/8)	6.1±0.3(8/8)	6.5±0.4(8/8)
Lactobacilli	5.2±0.5(6/7)	5.4±0.6(8/8)	4.8±1.1(8/8)	5.4±0.8(5/8)	5.2±0.8(8/8)
Staphylococci and Micrococci	4.4±1.1(5/7)	4.4±0.9(8/8)	5.3±0.7(8/8)	5.8±0.6[d)](8/8)	6.2±0.5[e)](8/8)
Streptococci	3.9±0.9(6/7)	4.1±1.2(5/8)	4.7±1.3(8/8)	4.2±0.78(8/8)	5.6±0.7(7/8)
Coryneform bacteria	—	2.7 (1/8)	4.0±0.8(3/8)	4.2±1.0(4/8)	5.0±0.7(8/8)
Enterobacteria	4.5±0.6(3/7)	4.8±1.0(7/8)	4.7±1.2(7/8)	4.0±0.5(5/8)	4.7±0.6(8/8)
Aerobic gram-negative rods	—	—	3.7 (1/8)	—	4.0±0.3(3/8)
Bacilli	4.2 (1/7)	3.4 (1//8)	—	2.9 (1/8)	3.7 (1/8)
Yeasts	—	—	—	—	2.9 (1/8)
Clostridia	—	—	4.6 (1/8)	—	—
Bacteroides	—	—	4.6 (1/8)	—	—
Eubacteria and Bifidobacteria	—	—	—	—	4.4±0.6(2/8)
			Pharyngolarynxs		
Total bacteria	5.7±0.4(7/7)	6.0±0.5(8/8)	5.8±0.5(8/8)	6.0±0.3(8/8)	6.0±0.8(8/8)
Lactobacilli	5.4±0.6(7/7)	5.5±0.7(8/8)	4.8±1.5(8/8)	5.0±0.9(7/8)	5.2±0.9(7/8)
Staphylococci and Micrococci	3.6±0.9(4/7)	3.7±0.9(8/8)	3.6±0.5(7/8)	4.3±0.9(8/8)	4.3±1.0(8/8)
Streptococci	4.6±1.0(5/7)	3.3±0.5(5/8)	3.8±0.7(8/8)	4.6±1.1[d)](7/8)	4.7±1.1[e)](8/8)
Coryneform bacteria	—	—	3.3±0.9(3/8)	4.0 (1/8)	—
Enterobacteria	4.8±0.5(7/7)	5.1±1.5(8/8)	5.1±0.7(/8)	5.3±1.0(8/8)	5.7±1.0(8/8)
Aerobic gram-negative rods	—	—	2.4 (1/8)	—	2.9±0.2(2/8)
Bacilli	3.1 (1/7)	—	3.3 (1/8)	—	—
Yeasts	—	—	—	—	—
Clostridia	—	—	—	—	4.7±0.2(2/8)

Bacteroides	—	—	—	—	5.5 (1/8)
Eubacteria and Bifidobacteria	—	4.7 (1/8)	—	5.8 (1/8)	6.1 (1/8)
			Tracheas[f]		
Total bacteria	—	4.5±0.3(2/8)	—	4.5±0.1(2/8)	3.9±0.2(2/8)
	—	3.9±0.7(3/8)	—	3.8±0.3(2/8)	—
Staphylococci and Micrococci	—	4.5±0.3(2/8)	—	4.5±0.1(2/8)	3.9±0.2(2/8)
	—	3.9±0.7(3/8)	—	3.8±0.3(2/8)	—
			Air sacs		
Total bacteria	2.9±0.2(2/7)	4.1±1.5(6/8)	3.1±0.3(6/8)	3.5±0.8(8/8)	4.0±0.4(8/8)
Lactobacilli	—	—	—	—	2.3±0.2(2/8)
Staphylococci and Micrococci	—	4.0±1.6(6/8)	2.9±0.2(6/8)	3.5±0.9(8/8)	3.9±0.5(8/8)
Streptococci	—	2.3±0.1(2/8)	2.7±0.4(2/8)	2.4±0.1(3/8)	2.8±0.4(6/8)
Coryneform bacteria	—	—	3.7 (1/8)	2.7 (1/8)	2.7±0.4(2/8)
Enterobacteria	—	—	—	—	2.3 (1/8)
Aerobic gram-negative rods	—	3.4 (1/8)	2.6±0.2(4/8)	—	3.7 (1/8)
Yeasts	—	2.6 (1/7)	—	—	—
Clostridia	2.9±0.2(2/7)	—	—	—	3.0±0.1(2/8)
Bacteroides	—	—	—	—	2.5 (1/8)

a) Data are expressed as mean±standard deviation of $\log_{10}$ per gram (wet weight) of autopsy specimen (Frequency of occurrence is expressed as number of subject(s) with the microorganisms detected/number of subjects examined).
b) The viable counts of ≦2.0 were not detected.
c) Statistically significant at the $p<0.05$ level when compared with the numbers obtained in Group A.
d) Statistically significant at the $p<0.05$ level when compared with the numbers obtained in Group B (Day 28).
e) Statistically significant at the $p<0.05$ level when compared with the numbers obtained in Group B (Day 42).
f) The numbers of microoragnisms isolated from the upper and lower parts of thrachea were counted.

Table 2. Distribution of *Lactobacillus* species isolated from autopsy specimens of chickens

Species	Mitsuoka's biovar. [14]	No. of isolates	Nasal cavity	Tongue	Pharyn-golarynx	Rectal contents
Homofermentatives						
L. salivarius subsp. *salicinius*	IIb	1				1
L. salivarius subsp. *salivarius*	IIIb	28	2	10	11	5
L. acidophilus group						
biovar. A	IIIa	2				2
biovar. B	IVa	2				2
biovar. C	IVb	10		1	3	6
biovar. D	VIa	6		2	1	3
Lactobacillus sp. A		2		1		1
Lactobacillus sp. B		2				2
Lactobacillus sp. C		1				1
Lactobacillus sp. D		1			1	
Lactobacillus sp. E		1				1
Lactobacillus sp. F		1			1	
Heterofermentatives						
L. reuteri						
biovar. A	Ia	10		2	3	5
biovar. B	Ib	1				1
biovar. C	IIb	2				2
biovar. D	IVa	2				2
Lactobacillus sp. G		1				1
Lactobacillus sp. H		1				1
Lactobacillus sp. I		1	1			

Table 3. Characterization of *Lactobacillus* species which could not be identified to species level by using currently identification protocols and presently recognized species

Characteristics	*Lactobacillus* species								
	A	B	C	D	E	F	G	H	I
Gas from glucose	−	−	−	−	−	−	+	+	+
Growth at 15C	−	−	−	−	−	+	−	−	+
Acid from:									
Arabinose	−	A	−	−	−	−	−	−	−
Xylose	−	A	−	−	−	−	−	−	A
Rhamnose	−	−	−	−	−	A	−	−	−
Ribose	A	A	−	−	−	−	−	−	A
Cellobiose	A	−	A	A	−	−	−	−	A
Trehalose	A	−	−	−	−	A	−	−	−
Melibiose	A	A	A	A	A	A	A	A	−
Raffinose	A	A	A	A	A	A	A	A	−
Melezitose	A	−	−	−	−	−	−	−	−
Starch	A	A	A	−	−	−	−	−	−
Mannitol	−	−	A	A	A	A	A	−	−
Sorbitol	−	−	A	−	−	A	−	A	−
Esculin	A	−	A	A	−	−	−	−	−
Salicin	A	−	A	A	−	A	−	−	A
Amygdalin	A	−	A	−	−	−	−	−	A

Symbol: + and A; positive reaction, −; negative reaction. all strains of Lactobacillus species produced acid from glucose, mannose, fructose, galactose, sucrose, maltose, and lactose.

heterofermentative groups (biovars A ~ D) were identified as *L. reutri*. Most species and biovars of the lactobacilli isolates were detected in the retum contents of birds. The high isolation rate (48%) of lactobacilli in the intestines of chickens was recognized whereas only *L. salivarius* subsp. *salivarius* was frequently detected from tongues and pharyngolarynxs. Table 3 shows the characterization of *Lactobacillus* species which could not be identified to species level by using currently identification protocols and presently recognized species. These lactobacilli were divided into nine groups consisting of six homofermentatives and three heterofermentatives.

In the poultry house of Group B, the viable counts of the airborne microbes were 0.9/cm^2/min at two weeks and 4.4/cm^2/min at four week. On the other hands, those in the poultry house of Group C were 576/cm^2/min at two weeks and 1128/cm^2/min at four weeks.

DISCUSSOIN

Bacterial flora of the respiratory tracts in chickens was influenced by housing conditions [22]. Sauter *et al.* [21] demonstrated that the most commonly bacteria of the airborne microflora in chicken house were *Bacillus, Micrococcus, Proteus, Pseudomonas,* and *Staphylococcus*. The bacterial numbers in house dust of the experimental room in Group C were higher than those in Group B. The results in the present study show that the bacterial flora of nasal cavity, tongue, and pharyngolarynx were also differed by the breeding methods. Total viable bacterial numbers, and the numbers of staphylococci and micrococci, streptococci, and coryneform bacteria in the nose of Group C were significantly higher than those in Groups A and B. Staphylococci and micrococci in the tongue and pharyngolarynx and streptococci in the pharyngolarynx were also higher in Group C than in Groups A and B. These findings indicate that the stability of normal bacterial flora of respiratory tracts in chickens could be controlled by the numbers of airborne microoragnisms.

Dho and Mouline [4] reported that the aerobic flora of the chicken trachea consisted of *Eschericha coli, Streptococcaceae,* and *Micrococcaceae*. In this study, only staphylococci and micrococci were detected from the tracheas, but the frequency of occurrence of these microorganisms were relatively lower. Gordon and Gibbons [7] demonstrated that the most common isolate on the cultivable flora of human tongue was gram-positive facultative cocci (probably *Micrococcaceae* and *Streptococcus*).

Viable numbers of staphylococci and micrococci detected from the air sacs of chickens in Groups B and C were $10^{2.9\text{-}4.0}$ / g of the autopsy specimen. Eight different types of microorganisms were isolated from the air sacs of Group C by Day 42 whereas four different types of bacteria in Group B by Day 42. Four bacterial groups of lactobacilli, enterobacteria, clostridia, and bacteroides in Group B by Day 42 were never isolated. Smibert *et al.* [22] also reported that micrococci were isolated from air sacs of the 12-months-old chickens, but not from those of 5-weeks-old birds. The results in the present study agreed with their finding described previously [22]. No isoaltion of microorganism except for staphlococci and micrococci from trachea may be due to the functions of microvilli of epitherial cells and the smallness of the mucosal epitherial square in the tracheas.

Coloe *et al.* [2] noted that the normal intestinal flora was stabilized by 40 days after hatching. The development of the respiratory bacterial flora is not known. Since the respiratory flora are influenced by numbers of microorganisms in the air dust, however, it may be difficult to determine a composition of the normal respiratory flora.

The predominant bacteria of the intestinal flora in chickens is lactobacilli [5, 6, 11]. The intestinal microflora in animals is influenced by an ingredient of feedstuffs and their physiological peroperties [15]. Miles *et al.* [13] also reported the effect of a feedstuff supplemented with lactobacilli on the egg production. Therefore, the presence of lactobacilli in the bird intestines may be beneficial to the host bird. It is known that lactobacilli is present in the respiratory organs as an indigenous flora [9]. A higher frequent isolation of *Lactobacillus salivarius* subsp. *salivarius* from the autopsy specimens in respiratory organs of chickens was detected in our study. This microorganism was also isoalted form the intestines. Mitsuoka [14] indicated that biovars of *L. salivarius* which isolated at the highest isolation rate from chicken intestines was IVa (52.7%). In the present study, however, *L. salivarius* IVa was never detected from the respiratory organs and feces of chickens. On the other hand, the intestinal lactobacilli [6, 14] such as the *L. acidophilus* group and *L. reuteri* were also isoalted from tongue and pharyngolarnx of chickens. These findings show that some of *Lactobacillus* species on the respiratory organs of birds are derived from intestines of birds. The distribution of *Lactobacillus* sp. in the respiratory tract may be closely related to the intestinal adhesions with lactobacilli since there is a close association of certain strains with a particular animal [17].

Within the unidentified *Lactobacillus* groups, Groups A~F were homofermentative lactobacilli and Groups G~I were heterofermentative lactobacilli: Groups A, B, D, F, and I were isolated from the respiratory organs of chickens. The other unidentified groups were detected from the only intestines.

Biovars of IV, VI, and VII of *L. acidophilus* and biovar II b of *L. fermentum* were mainly detected from the bird intestines. Distribution of biovars of the *lactobacillus* species in this study was a similar to Mitsuoka's report [14].

In conclusions, the bacterial flora of nasal cavity, tongue, pharyngolarynx, trachea, and air sacs mainly consists of staphylococci, micrococci, lactobacilli, streptococci, and enterobacteria. Particularly, *Lactobacillus salivarius* subsp. *salivarius* was the predominant lactobacilli isolated from tongue and pharyngolarynx. Most of isolates from the bird intestines were mainly identified as the *L. acidophilus* group and *L. reuteri* .

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