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Enhancement of Chicken Resistance Against Escherichia coli Infection by Oral Administration of Bifidobacterium thermophilum Preparations

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SUMMARY. Three types of *Bifidobacterium thermophilum* extract were prepared and fed to 2-wk-old chickens to evaluate their usefulness in enhancing the defense activity of the chickens against pathogenic *Escherichia coli.* All three preparations resulted in significant reduction ($P < 0.05$) of *E. coli* numbers in the lungs of the treated chicken groups compared with the control nontreated group. Besides, improvement in the survival rate was observed in the treated chicken groups, especially the one administered the enzyme-digested *B. thermophilum* extract sample. Concanavalin A–stimulated lymphocytes from the latter group demonstrated significantly higher proliferation activity compared with those from the control group. These results suggest that oral administration of *B. thermophilum* preparations may be used to enhance the resistance of chickens against *E. coli* infection.

RESUMEN. Aumento de la resistencia contra la infección por Escherichia coli mediante la administración oral de preparaciones de Bifidobacterium thermophilum.

Se prepararon tres tipos de extractos de *Bifidobacterium thermophilum* que fueron administrados a pollos de 2 semanas de edad con el objeto de evaluar su aumento en la actividad defensora de los pollos contra cepas patógenas de *Escherichia coli.* Todas las tres preparaciones resultaron en una reducción significante ($P < 0.05$) en el número de *E. coli* presente en los pulmones de los pollos de los grupos tratados, comparada con los grupos controles no tratados. Se observó además un aumento en la sobrevivencia de los pollos tratados, especialmente en los que recibieron la muestra del extracto de *B. thermophilum* digerido con enzimas. Los linfocitos de las aves de este grupo, estimulados por la concanavalina A, demostraron una actividad proliferante significantemente mayor, comparada con la del grupo control. Estos resultados sugieren que la administración oral de las preparaciones de *B. thermophilum* pueden usarse para aumentar la resistencia de los pollos a la infección por *E. coli.*

Key words: *Bifidobacterium thermophilum, Escherichia coli,* chicken, lymphocyte proliferation

Abbreviations: Con A = concanavalin A; OD = optical density; PBS = phosphate-buffered saline; SPB = Sorensen phosphate buffer

Avian colibacillosis represents a severe problem for the poultry industry. Until now, the disease has been controlled by antibiotics as feed additives. Prolonged use of antibiotics, however, often results in the appearance of resistant bacterial strains and is hazardous to human health because of the drug residue. Therefore, safe and effective methods for controlling the disease should be considered.

Certain lactic acid bacteria and their components have immunopotentiating effect in humans and animals when administered orally. The immunogenicity of oral rotavirus vaccine was improved when administered together with *Lactobacillus casei* GG (1). Various *Bifidobacterium* species have been shown to enhance the nonspecific protection against pathogenic bacteria in mice (8,10,12,13), guinea pigs (2), and piglets (3,4,9). These facts suggest that it is possible to use preparations from lactic acid bacteria for prevention and treatment of colibacillosis in chickens. The purpose of this study was to investigate if oral administration of *Bifidobacterium thermophilum* preparations could pro-

tect chickens from pathogenic *Escherichia coli* infection.

MATERIALS AND METHODS

Chickens. Two-week-old white leghorn chickens, strain Hyline W36 (GHEN Corporation, Gifu, Japan), were used in this study. All the chickens were confirmed to be serologically negative to *E. coli* of serogroup O1. Chickens were kept in an isolator and fed with standard experimental diet (SPL 1; Nippon Haigo Siryo Co., Aichi, Japan), which did not contain any antibiotic or insect repellent.

Bacteria. *Escherichia coli* strain IRIG1 belonging to serogroup O1 was isolated from the lungs of a dead layer chicken. The strain was stored at −70 C. An aliquot of the strain was grown on brain–heart infusion agar (Difco Laboratories, Detroit, MI) at 37 C for 16 hr. One colony that showed strong agglutination with an antiserum against *E. coli* outer membrane proteins (Denka Seiken Co., Tokyo, Japan) was picked up and grown aerobically at 37 C in Luria broth (Difco Laboratories) for 18 hr with shaking (200 rpm). The bacterial cells were harvested by centrifugation at 12,000 × *g* for 20 min. The pellet was washed twice with sterile phosphate-buffered saline (PBS) and resuspended in the same buffer. This bacterial suspension was used as inoculation for chickens.

Bifidobacterium thermophilum strain CL (GHEN Corporation) was grown in anaerobic bacterial culture medium broth (Eiken Chemical Co., Tokyo, Japan) under anaerobic conditions for 18 hr at 37 C and harvested by centrifugation at 12,000 × *g* for 20 min. The pellet was washed three times with sterile Sorensen phosphate buffer (SPB), pH 6.2, resuspended in the same buffer, and used for the preparation of extract samples.

Preparation of *B. thermophilum* extract samples. Three types of *B. thermophilum* extracts were prepared according to the following protocols.

Sample A (heat-killed bacteria). Bifidobacterium thermophilum suspensions were boiled at 100 C for 15 min and freeze-dried.

Sample B (disrupted bacteria). The bacteria were pelleted, suspended in nine volumes of SPB, pH 6.8, and disrupted with a high-pressure homogenizer (MINI-RAB 8.30H; APV Homogenizers AS, Copenhagen, Denmark). Complete disruption was confirmed by observation of samples under a light microscope.

Sample C (enzyme-digested lysate). Enzyme-digested lysate of *B. thermophilum* was prepared as described previously (9) with some modification. Briefly, the bacterial suspensions were disrupted as described above. The disrupted bacteria were washed by centrifugation at 14,000 rpm for 30 min. The pellet was suspended in 1/10 volume of SPB and digested with 0.01% egg white lysozyme (Wako Pharmaceutical Inc., Tokyo, Japan) and 0.05% pronase (Wako Pharmaceutical) at 37 C for 48 hr. Then the digested lysate was freeze-dried.

Experimental procedures. *Administration of B. thermophilum extract samples.* A total of 24 chickens were randomly separated into four equal groups. Three groups, A, B, and C, were given standard feed containing 0.025% of samples A, B, and C, respectively. The control group was given the same diet without *B. thermophilum* samples added. All groups were given diet and water *ad libitum* for 1 wk before challenge with *E. coli.*

Escherichia coli challenge. The procedure described by Piercy and West (6) was followed with some modifications. The bacterial inoculum (1×10^6 colony-forming units per chicken) was injected into the air sac on the right side with a 1-ml syringe with a 27-gauge needle.

Isolation of E. coli from lungs. Four days after inoculation of *E. coli,* chickens were anesthetized with ultrashort-acting barbiturate (Sankyo Co., Tokyo, Japan). The whole lung on the right side was recovered and weighed. After that, each lung was homogenized in nine volumes of cold PBS with a glass homogenizer and diluted with cold PBS. The homogenates were then cultured on DHL agar (Eiken Chemical Co.) for 18 hr at 37 C and *E. coli* colonies were counted. The numbers of viable *E. coli* were determined per 1 g of lung.

Lymphocyte proliferation assay. For this trial, 24 chickens were separated into four groups and fed in the same way as described in the experimental procedures section. Seven days after the start of feeding, peripheral blood was collected from each chicken, and the lymphocytes were isolated by slow centrifugation on a Lymphoprep gradient of 1.077 g/ml (Pharmacia Biotech, Uppsala, Sweden). The cells were washed twice with RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan), supplemented with 100 μg/ml of streptomycin, 100 IU/ml of penicillin, and 15% fetal bovine serum. The lymphocytes were resuspended in the same medium, adjusted to 3×10^6 cells/ml, and dispensed to a 96-well, flat-bottom plate in triplicate (100 μl/well). A second plate was prepared in the same manner but 20 μl of 0.1% concanavalin A (Con A) solution (Sigma Co., Ltd., St. Louis, MO) was added to each well. After that, the plates were incubated at 41 C in a 5% CO_2 incubator. On the fourth day, 20 μl of 0.5% 3-(5,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT reagent solution) (Sigma Co.) was added per well and the mixture was incubated further for 6 hr. Then the plates were subjected to low-speed centrifugation. The supernatant of each well was removed by aspiration and the cells were resuspended with 200 μl of a solution containing 50% ethanol and 10% sodium dodecyl sulfate. The

Table 1. Effect of oral administration of *B. thermophilum* samples on chicken resistance against challenge with *E. coli.*

Group	Number of *E. coli* (log_{10})[A]	Survival rate
Control	5.8 ± 1.6	3/6 (50%)
A	1.9 ± 2.2*	4/6 (66.6%)
B	1.9 ± 2.3*	4/6 (66.6%)
C	1.9 ± 2.5*	6/6 (100%)

[A]Number of viable *E. coli* from 1 g of lungs. Data are presented as the mean ± SD. * $P < 0.05$ relative to the control group (Student *t*-test).

optical density (OD) at 590 nm was determined with a microplate reader model MR5000/7000 (Dynatech Laboratories Inc., Chantilly, VA). The OD value of each sample was the average of that from triplicate wells.

Statistical analysis. The numbers of isolated *E. coli* were presented as the mean and standard deviation. Differences in data between groups were analyzed by Student *t*-test.

RESULTS

The results of the *E. coli* challenge experiment are shown in Table 1. On the fourth day postchallenge, three of six chickens (50%) in the control group survived. In groups A and B, four chickens survived (66.6%), but the difference between the latter groups and the control group was not significant. All chickens in group C survived until the day they were euthanatized. On the other hand, the numbers of *E. coli* isolated from the lungs of the control group chickens were significantly higher than those from the *B. thermophilum* sample–administered groups ($P < 0.05$). There was no significant difference among the three treated groups in the numbers of isolated *E. coli.*

As shown in Fig. 1, the lymphocytes from the chickens in all groups demonstrated about the same level of proliferation *in vitro* when the cells were propagated in a medium without mitogenic Con A. When Con A was added, the lymphocytes from the treated groups proliferated faster than those from the control group. However, a statistically significant difference in lymphocyte proliferation rate was observed only in group C in comparison with the control group ($P < 0.05$).

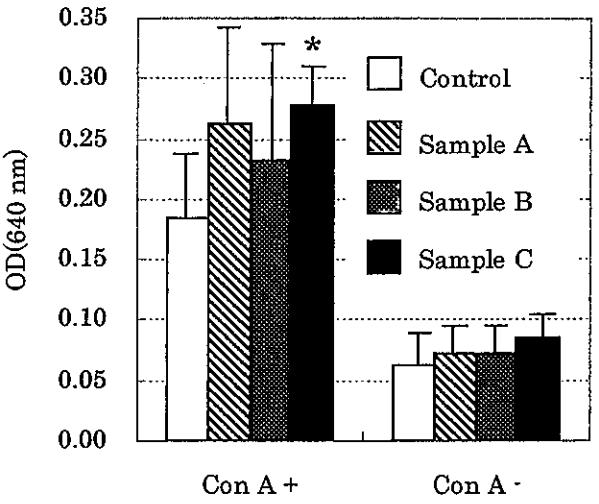

Fig. 1. Comparison of proliferation activity of Con A–stimulated lymphocytes from chickens of the *B. thermophilum* sample–administered and control groups. Each lymphocyte sample was cultured with or without Con A and the average OD was determined. * $P < 0.05$ relative to the control group as determined by Student *t*-test.

DISCUSSION

The role of *Bifidobacterium* species in augmenting nonspecific protective capability of humans and other animals has been well documented (3,4,7,8,9,10,11,12,13,14,15). In an attempt to develop a simple method for controlling *E. coli* infections in the poultry industry, we prepared three different extracts from *B. thermophilum* and fed them to chickens for 1 wk before challenge with a pathogenic *E. coli* strain. The most important finding of the present study is the fact that all three preparations significantly inhibited the growth of the inoculated *E. coli* in chickens. Because the effect of *B. thermophilum* extracts is nonspecific, we expect that they can be used for the augmentation of chicken defense against other pathogenic microbes as well.

The oral administration of *B. thermophilum* preparations also provided some improvement in chicken survival. Although the survival rate on the fourth day of the experiment was not statistically different among the four groups, we expect that the survival rate of the treated groups, especially group C chickens, might have become significantly higher than that of the control group if the experiment had been continued for a few more days. In a previous study, Sasaki *et al.* (8) reported that the protec-

tive effect of peptidoglycan from *B. thermophilum* was dose dependent and only the dose of 500 μg/mouse resulted in full survival of mice challenged with *E. coli*. Therefore, studies should be done to determine the most effective doses of *B. thermophilum* extracts for chickens.

The fact that oral administration of *B. thermophilum* extracts resulted in significantly lower growth of *E. coli* inoculated into the air sacs, a different route, suggests that *B. thermophilum* and its components enhanced the general antibacterial activity of the chickens. In this regard, our study results are in agreement with those of a previous study by Sasaki *et al.* (8), who reported that oral administration of peptidoglycan from *B. thermophilum* significantly reduced *E. coli* numbers in various organs including blood, liver, spleen, and kidney of specific-pathogen-free mice challenged with the bacterium intraperitoneally or intravenously. Because the antibacterial effect of *B. thermophilum* preparations has been reported to be due to their capability to enhance cell-mediated immunity in animals (2,3,7,9,10), we compared the proliferation activity of Con A–stimulated lymphocytes from all groups. We found that, of the three groups administered *B. thermophilum* extracts, only group C demonstrated significantly higher lymphocyte proliferation. This result may be explained by the size of particles in the *B. thermophilum* samples. The enzyme-digested sample, which consisted of small-sized particles and molecules, was absorbed easily through the intestinal blood vessels and was distributed throughout the whole body; thus, it activated lymphocytes more efficiently than the other two samples. The fact that the heat-killed and disrupted bacterial preparations reduced the number of *E. coli* equally well is not contradictory to the above explanation because the latter may have activated other immunity cells such as macrophages. Materials such as bacteria are taken up by microfold cell and transferred to lymphoid follicles of Peyer path where they encounter macrophages and lymphocytes and then cause an immune response (5,12,13,15).

As a conclusion, the findings of this study indicate that it is possible to develop a simple method for controlling *E. coli* infections in chickens with the use of *B. thermophilum* preparations as feed additives. Because the antibacterial effect of these preparations is nonspecific, and thus not long lasting, more studies should be conducted to determine the appropriate treatment frequency as well as the proper concentrations at which these preparations enhance chicken defense activity most effectively.

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