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Biological Activities of Thermo-tolerant Microbes from Fermented Rice Bran as an Alternative Microbial Feed Additive

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Abstract To evaluate the commercial potential of new microbial feed additive, *Issatchenkia orientalis* Y266 and *Bacillus subtilis* B266 from commercial fermented rice bran were tested for their tolerance or resistance to pH, bile, oxgall, and temperature. It was found that the strains grew very well up to pH 3.0 and resistant to relatively high concentrations of bile salt and oxgall. *I. orientalis* and *B. subtilis* are extremely tolerant in range of 70–90°C in solid medium. *B. subtilis* B266 also has excellent tolerant property up to 90°C in liquid medium. The health indexes (the microflora in the small intestines and the antibody titer to Newcastle disease virus) of chicks were significantly improved in the fermented rice bran with these strains (0.25% addition to diet) in comparison with the Avilamycin (20 mg/kg diet)-fed group ($p<0.05$). The fermented rice bran-fed group showed a better microbial flora in the small intestines. Accordingly, it would appear that the fermented rice bran with these strains may be a potential candidate for an alternative microbial feed additive.

Keywords *Bacillus* sp. · *Issatchenkia orientalis* · DFMs · Bile acid · Thermo tolerance

Introduction

Antibiotic growth promoters (AGPs) were used to help the suppression of subclinical disease and improvement of physical performance [1]. The ban of AGPs is encouraging livestock producers to seek alternatives for enhancing animal production to a higher level than that obtained with AGPs. A strategy is aimed at using microorganisms or their

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fermented products for altering circumstance of animal's gastrointestinal tract. More attention may be paid to the use of microbial feed additives as an alternative. The effect of microbial feed additives for the improvement of health and production of livestock has been studied for many years [2, 3].

The direct-fed microbials (DFMs) have been employed to describe microbial-base products [4, 5]. Many microorganisms, or their fermented products, have been used in direct-fed microbial formulation. Only a few species have been developed for commercial use in some industries due to the difficulties in elucidation of physiological activity. The most common organisms found in DFMs or their fermented products include *Saccharomycetes* sp., *Bacillus* sp., *Aspergillus* sp., and lactic acid bacteria [6–9].

Some earlier studies have documented the positive effects of feeding DFM formulation or their fermented products to animals, although further studies on the mechanism of how DFMs improve animal production are still required. A number of studies significantly increase in animal productivity from DFM supplement diets [9, 10]. In addition, fermented rice bran may use a new nutritional food adjunct for animals in Korea, since it contains many essential amino acids and other nutritional value [11, 12]. Several criteria have been used for screening microorganisms for DFMs with resistance to acid, bile, and oxgall [13–15]. In addition, the tolerance of the microorganisms to high temperature is also important, since some feedstuffs are processed in the range of above 60°C [16].

At this experiment, we investigated the effect of fermented rice bran with *Issatchenkia orientalis* Y266 and *Bacillus subtilis* B266 on health indices including antibody response to Newcastle disease virus vaccine and microflora in the small intestine. Also, the present experiment was aimed to assess the physiological activities of these microbes from fermented rice bran to develop a new animal feed additive.

Materials and Methods

Microorganism, Medium, and Cultural Conditions

Issatchenkia orientalis Y266 and *Bacillus subtilis* B266, used for the production of commercial product (Superfeed) as an animal feed additive, were gifts from NEL Biotech Co. (Kyonggi, Korea). Their sugar utilization was tested by using an API test kit (Biomerieux Co.); the data are shown in Table 1. Stock cultures of tested strains maintained

Table 1 Some sugar utilization characteristics of *I. orientalis* and *B. subtilis*.

Sugar	*Issatchenkia orientalis* (strainY266)	*Bacillus subtilis* (strain B266)
Sucrose	−	+
D-Glucose	+	+
D-Fructose	+	+
D -Galactose	−	−
L-Arabinose	−	−
D-Xylose	−	−
D-Ribose	−	−
Raffinose	−	−
Sorbitol	−	+
N-Acetyl-glucosamine	+	+

in glycerol stock (−70°C) were thawed and transferred twice to a proper medium before use. The media for counting were YM agar (Difco Laboratories, Detroit, MI, USA) with 10 mg% of chloramphenicol for yeast, and plate count agar (Difco Laboratories) for *Bacillus* sp. After incubation at 37°C for 24–48 h, the colonies on plates were enumerated as viable microbes. Bile salt and oxgall were from Difco Laboratories. All other reagents were of analytical grade.

Effect of Temperature on Microbial Growth

To estimate the use of microbial feed additives that would grow or survive well under conditions of high temperature—solid or liquid medium, tolerance to temperature was tested in media accompanied with heat treatment at 50, 60, 70, 80, and 90°C in temperature-controlled water bath. Rice bran medium was used as solid medium, and YM or plate count broth as liquid medium for cultivating. The rice bran medium contained 70% rice bran, 10% molasses, 0.12% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.04% KH_2PO_4, and 0.01% K_2HPO_4, in addition to water. This medium was inoculated with a lot of cultural broth of strain Y266 or strain B266, respectively. After cultivating with the strain, viable cells were estimated by using serial dilution method. Tolerance to temperature was regarded as population of viable cell count on agar plate and expressed as colony forming unit (CFU) per gram or milliliter.

Effect of pH, Bile and Oxgall on Microbial Growth

Resistances to acidic pH, bile and oxgall were tested in each broth medium inoculated with strain Y266 or strain B266. The pH levels of the broth medium were adjusted with 1.0 N HCl within a range of pH 2.0–4.0. Bile resistances were measured by counting viable colony on agar plate, after reacting in broth medium, which contained 0.0%, 0.3%, 0.6%, and 0.9% (*w*/*v*) of bile salt [17]. In case of resistance to oxgall, the strains were tested in broth medium containing 0.0–1.0% oxgall (*w*/*v*). The broth media for culture of the strains were YM broth for yeast and plate count broth for *Bacillus* sp. These media were inoculated with a lot of broth culture of strain Y266 or strain B266, respectively. Resistance was regarded as population of viable cell count on agar plate and expressed as colony forming unit (CFU) per gram or milliliter.

Preparation of Fermented Rice Bran

The rice bran was inoculated with strain Y266 or strain B266 to make fermented rice bran (FRB). The rice bran medium was not autoclaved and inoculation was carried out with each 0.1% (*v*/*w*) cultural broth of strain Y266 (2×10^7 cells mL^{-1}) and strain B266 (2×10^7 cells mL^{-1}). Initial incubation temperature was started at 25°C. During fermentation for 5 days, the fermentation temperature was not controlled by heating, and regulated below 65°C by supplying fresh air. After finishing fermentation, moisture content of the fermented rice bran was controlled with a drum-type dryer.

Animals

Forty-five commercial 1-day-old broiler chicks (Cobb strain, male) were held at 23±2°C for 5 weeks with lighting 24 h a day. They had free access to food and water during the experimental periods. The chicks were divided into one control group and two test groups,

and each group consisted of three treatments; three replicates per treatment, and five chicks per replicate.

Corn–soybean meal basal diet (Table 2) for broiler chicks was set to the chemical composition of the standard nutritional requirement [18]. One test group was fed on the above diets including 20 mg Avilamycin [19] per kilogram and another test group was fed on the above diets including 0.25% of FRB prepared the above methods. To investigate the microflora of the content of the small intestine of each chick, more than 1 gram of content was collected in sterilized tubes, as soon as the chicks were sacrificed by cervical dislocation, and their small intestines were exposed on sterilized sheets. One gram of wet content was diluted with saline buffer, and the properly diluted samples were inoculated on suitable media at 37°C for 24–48 h and then the colony forming units were counted. The bacteria that grew on *Salmonella* and *Shigella* agar and regrew on Bismuth sulfite agar (Difco Laboratories) were regarded as *Salmonella* sp., those that grew on Mac-Conkey agar as *Escherichia coli*, and those that grew on Rogosa SL agar as *Lactobacillus* sp. These agar are products from Difco Laboratories.

Antibody Response to Newcastle Disease Virus (NDV) Vaccine

To evaluate antibody response to inactivated Newcastle disease virus (NDV) B1 vaccine from Daesung Microbiological Labs (Kyonggi, Korea), the first vaccination was enforced on 2-week-old chicks by injection into muscle with 2.5 ml of the NDV vaccine, and the second vaccination was done on 4-week-old chicks with the same quantity of material. For the 5-week-old chicks, 5 ml of the blood of each chick was collected from a vein. Each blood serum sample was separated by centrifugation at 1,100×*g* for 20 min at 4°C and treated for 30 min at 56°C to inactivate anti-complementary factors of the serum. Antibody (AB) titer was measured by the Hemagglutination Inhibition (HI) test of Beard [20].

Table 2 Corn-soybean meal basal diet for broiler chicks.

Ingredients	Starter	Finisher
(%)		
Corn	61.25	66.48
Soybean meal	24.76	24.78
Corn gluten meal	8.30	3.70
Soybean oil	2.00	2.00
Limestone	0.98	1.14
TCP	1.75	1.18
Salt	0.47	0.34
Lysine	0.19	0.12
DL-methionine	0.12	0.05
Vitamin premix*	0.10	0.10
Mineral premix**	0.10	0.10
Chemical composition		
ME (kcal/kg)	3,100	3,100
CP (%)	21.50	19.00
Methionine (%)	0.50	0.38
Lysine (%)	1.10	1.00
Ca (%)	1.02	0.90
P (%)	0.45	0.34

* Provided per kilogram of diet: vit. A, 5,500 IU; vit. D_3, 1,100 IU; vit. E, 11 IU; vit. B_{12} 0.0066 mg; riboflavin, 4.4 mg; niacin, 44 mg; pantothenic acid, 11 mg (Ca-pantothenate, 11.96 mg); choline, 190.96 mg (choline chloride 220 mg); menadione, 1.1 mg(menadione sodium bisulfite complex, 3.33 mg); folic acid, 0.55 mg; pyridoxine, 2.2 mg (pyridoxine hydrochloride, 2.67 mg); biotin, 0.11 mg; thiamin, 2.2 mg (thiamine mononitrate, 2.40 mg); ethoxyquin, 125 mg.

** Provided the milligram per kilogram of diet; Mn, 120; Zn, 100; Fe, 60; Cu, 10; I, 0.46; Ca, min: 150 max: 180.

Statistical Analysis

Data were expressed as the mean ± S.D. The difference between control and test groups in these experiments was investigated for statistical significance by Student's *t* tests (SAS program). A value of $p<0.05$ was considered to indicate statistical significance.

Results and Discussion

Tolerance to Temperature

Generally, the direct-fed microbials were fed to animals as the types of powder, granule, pellet, tablet, and paste with treatment of heat by 60–80°C [16]. Results of tolerance to temperature are shown in Table 3 and Fig 1. As shown in Table 3, *I. orientalis* (strainY266) and *B. subtilis* (strain B266) were tolerant up to 90°C of heat treatment for 60 min in solid state of rice bran medium. Strain B266 in solid state was not sensitive to temperature of 50–90°C. Then strainY266 in solid state was less resistant at above 70°C. In case of testing in liquid medium, strainY266 did not survive at above 60°C and was weakly alive at 50°C. The survival numbers of strainY266 were significantly affected by heat treatment of above 50°C.

Thermophilic bacteria can survive at above 50°C as optimum temperature and some isolates of them can tolerate at temperatures of 55– 60°C [21]. Accordingly, two strains can be regarded as thermophilic microbes. Also, because *I. orientalis* showed good ethanol productivity in synthetic media, yeast is classified in alkalophiles, acidophiles, thermo-acidophiles, thermotolerants, and ethanol-tolerant species.

Resistance to pH, Bile salts, and Oxgall

The direct-fed microorganisms in microbial feed additives should have acid-resistant properties because they need to survive and remain metabolically active during the passage from the mouth to the small intestine through the stomach. Using 5% (*v*/*v*) inoculum, the pH effects (2.0–4.0) on the survival of thermo-tolerant microbes at 37°C for 3 and 6 h were examined as can be seen in Table 4. StrainY266 and B266 showed good resistant properties at pH 4.0, while survival of strain B266 was inhibited weakly at pH 2.0, but strainY266 did

Table 3 Effect of temperature on survival ratio of thermo-tolerant during heat treatment for 60 min.

Heat Treatment(°C)	Survival ratio in broth condition (%)		Survival ratio in solid condition (%)	
	Bacillus subtilis (strain B266)	*Issatchenkia orientalis* (strain Y266)	*Bacillus subtilis* (strain B266)	*Issatchenkia orientalis* (strain Y266)
50	97.0	63.7	100	86.9
60	93.5	0	100	–
70	92.4	0	100	64.4
80	96.5	0	97.8	–
90	95.2	0	96.0	34.8

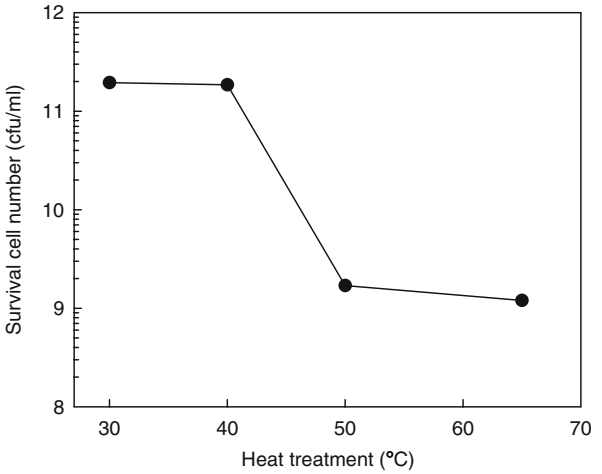

Fig. 1 Survival cell number of thermo-tolerant *Bacillus subtilis* (strain B266) after heat treatment for 24 h

not survive at pH 2.0–3.0. As a result, the growth of strainY266 was significantly decreased by acidic pH (below pH 3.0).

The bile- and oxgall-resistant factors are indispensable for a direct-fed microbe as feed additive. The importance of bile tolerance was established by Gilliland et al. [22]. The effect of bile salts within a range 0.0–0.9% (*w*/*v*) on the growth for 24 h is shown in Fig. 2. As shown in Fig. 2, the two strains that have been tested exhibited bile-tolerant characteristics with up to 0.9% bile slats, which is much higher than that found in the small intestine of most of human and animals [13]. More resistance-to-oxgall tests were also carried out for the two strains, as shown in Fig 3. Also, the resistance-to-oxgall test was also carried out for the two strains as shown in Fig. 3. When oxgall was added to the culture broth medium within a range of 0.0–1.0% (*w*/*v*), significant growth inhibition was not examined. Accordingly, these results mean that two strains have a capacity for direct-fed microbes as feed additive.

Changes of Population of Thermo-tolerant Microbes During Solid Fermentation

Microorganisms of microbial feed additive should have temperature-tolerant properties because they need to survive and grow during solid fermentation or processing at high

Table 4 Effect of acidity on survival ratio of thermo-tolerant microbes.

pH Treatment	Survival ratio of *Bacillus subtilis* (strain B266) (%)		Survival ratio of *Issatchenkia orientalis* (strain Y266) (%)	
	for 3 h	for 6 h	for 3 h	for 6 h
4.0	96.2	88.8	97.1	92.3
3.0	85.8	86.2	0	0
2.5	84.1	79.5	0	0
2.0	80.1	73.4	0	0

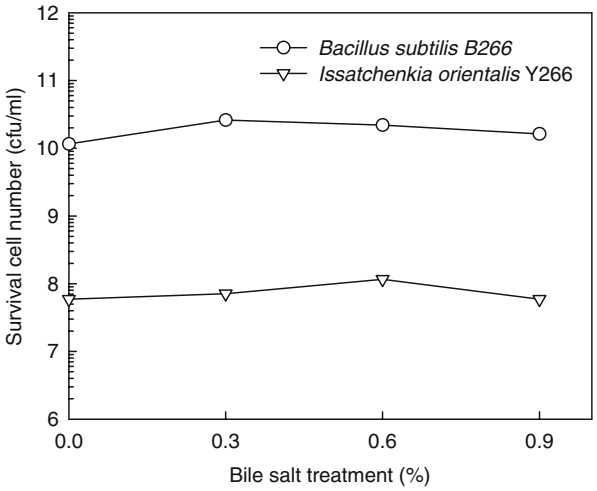

Fig. 2 Survival cell numbers of thermo-tolerant *Bacillus subtilis* (strain B266) and *Issatchenkia orientalis* (strain Y266) after bile salt treatment for 24 h

temperature. As shown in Table 5, viable microbial populations of yeast and *Bacillus* sp. were increased from about 10^4 to 10^7 cfu g^{-1} on the first day in solid fermentation with rice bran, and decreased to about 10^6 cfu g^{-1} on the fifth day of fermentation. After drying at 85°C, the living microorganisms were decreased to about 10^5 cfu g^{-1}. The pH and acidity were dropped according to fermentation time. The pH level was changed from 6.6 to 6.0 and acidity from about 1.0 to 2.8.

Therefore, strainY266 and strain B266 were identified as the strains with the higher tolerance to temperature, bile salts, and oxgall for making direct-fed microbial feed. These strains turned out to be the better potential for the future development of a microbial feed additive.

The exact mechanism through which *Bacillus* strains may alter the type of microflora in the gastrointestinal tract remains uncertain. One explanation might be associated with the

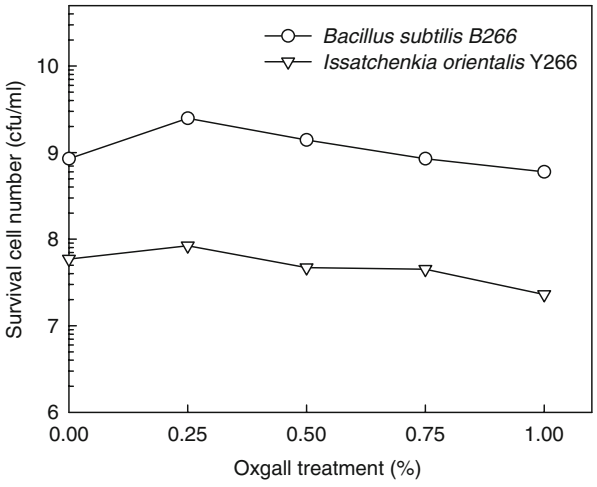

Fig. 3 Survival cell numbers of thermo-tolerant *Bacillus subtilis* (strain B266) and *Issatchenkia orientalis* (strain Y266) after oxgall treatment for 24 h

Table 5 Changes of cell number of thermo-tolerant microbes, pH, and acidity of fermented rice bran cultured with mixed thermo-tolerant microbes during fermentation.

Process	Time	Temperature (°C)	Microbial Population (cfu/g)		pH	Acidity*
			Bacillus subtilis (strain B266)	*Issatchenkia orientalis* (strain Y266)		
Solid Fermentation	0 day	25	2.2×10^4	1.9×10^4	6.65	0.96
	1 day	35	5.4×10^7	1.1×10^7	6.40	1.62
	3 day	55	2.5×10^6	5.4×10^6	6.12	2.64
	5 day	65	8.5×10^5	1.8×10^6	5.96	2.76
Drying		85	2.5×10^5	7.5×10^5	6.01	2.82

* Calculated as percentage of acetic acid content in 100 g of sample

decreased oxidation–reduction potential cause by the germination of spores in the intestine, which has been shown to benefit the growth of *Lactobacillus* sp. [23]. In addition, *Bacillus* strains could produce some metabolites that inhibit pathogens, since some *Bacillus* species used in commercially available products have the ability to produce antimicrobials, such as aminocumaim A [24] and bacteriocin [25, 26].

The yeast utilizes the lactic acid produced by bacteria, thereby increasing the pH of product [27]. Yeast can also stimulate the growth of the lactic acid bacteria through excretion of growth factors and metabolites. Borregaard and Arneborg [28] reported similar interactions between *Lactococcus* sp. and *Issatchenkia* sp. in milk fermentations. Consequently, the presence of yeast in feed may enhance the growth and survival of probiotic microorganisms by increasing the pH.

Although various microbial feed additives have already been commercially available, many improvements in strain selection and technology are still desired to reduce production costs. The current study provided evidence that *I. orientalis* (strain Y266) and *B. subtilis* (strain B266) have the potential to be developed as a microbial feed additive.

Microflora in the Small Intestine

Salmonella sp. and *E. coli* are well-known as harmful bacteria, but *Lactobacillus* sp. are utilized as helpful bacteria in the intestinal tracts of human and animals [29–31]. Increasing the population ratio of *Lactobacillus* sp. brings good conditions in the intestinal tracts [31].

Table 6 Some bacterial populations of the content of the small intestine in broiler chicks after feeding Avilamycin and FRB.

Treatments/kg of diet	Bacterial populations (10^7 cfu g^{-1} of content)				
	Salmonella sp.	*E. coli*	*Lactobacillus* sp.	S/L*	E/L*
Control	6.71±0.65	7.29±0.35	7.68±0.50	0.873	0.949
20 mg Avilamycin	6.66±0.96	7.16±0.64	6.99±0.60[a]	0.953	1.024
2.5 g FRB**	6.81±0.63	7.36±0.42	7.99±0.22[b]	0.852	0.921

* Ratio of *Salmonella* sp. or *E. coli* to *Lactobacillus* sp.

** FRB means fermented rice bran.

[a,b] Values with different superscripts within the same column of a subpart are significantly different at $p<0.05$.

Table 7 Changes of antibody titer against Newcastle disease virus vaccine in broiler chicks after feeding diets containing Avilamycin and FRB (Unit: log2).

Treatments/kg of diet	Control	20 mg Avilamycin	2.5 g FRB[a]
ND value	8.1±0.15[a]	7.27±0.09[b]	8.89±0.08[c]

[*] FRB means fermented rice bran.

[a,b,c] Values with different superscripts within the same low are significantly different at $p<0.05$.

The population of *Salmonella* sp. and *E. coli* in the content of the small intestine for the fermented rice bran (FRB)-fed test group was not significantly lower than that for the Avilamycin group (Table 6). Values for populations of *Lactobacillus* sp. in the content of the small intestine for each group were 7.99×10^7 cfu g^{-1} for the FRB-fed group and 6.99×10^7 cfu g^{-1} for the Avilamycin fed group, and there were significantly different in populations of *Lactobacillus* sp. among two groups. The populations of harmful bacteria–helpful bacteria were expressed as the ratio of *Salmonella* sp. to *Lactobacillus* sp. (*S*/*L*) and *E. coli* to *Lactobacillus* sp. (*E*/*L*) in Table 6. Both ratios, *S*/*L* and *E*/*L*, of each group were 0.953 and 1.024 for the Avilamycin group and 0.852 and 0.921 for the FRB-fed group, respectively. The *S*/*L* and *E*/*L* of the FRB-fed group were lower than those of the Avilamycin-fed groups because of high populations of *Lactobacillus* sp. in the FRB-fed group. There were significant differences in the value of *S*/*L* and *E*/*L* between the FRB-fed group and the Avilamycin-fed group. The values of *S*/*L* and *E*/*L* of control were similar to those of the FRB-fed group, but were a little high.

It is well-known that lactic acid bacteria secrete substances such as lactic acid and bacteriocin that inhibit pathogenic bacteria and so have beneficial effects on the chick intestinal microflora. Numbers of *Lactobacillus* sp. were normally higher than coliforms in healthy pigs, and the reverse in animals suffering from diarrhea [31, 32]. It can be postulated that there were not only indirect interactions between suppression of harmful bacteria and activation of lactic acid bacteria, but also effects on immune modulating activities through both Peyer's patches and macrophage in vivo as suggested by the results of some researchers [6, 33]. This thereby results in a decrease in the populations of *Salmonella* sp. and *E. coli* and in an increase in *Lactobacillus* sp. in the small intestine of broiler chicks.

Antibody Response to Newcastle Disease Virus Vaccine

Newcastle disease is highly contagious, prevalent worldwide, and causes severe economic loss to the poultry industry [34]. Therefore, the importance of locally produced antibodies in the defense of mucosal surfaces against viral infections has been well documented in humans and animals [35, 36].

When NDV antibody (AB) titers were checked for 5-week-old chicks, the AB titers of the FRB-fed group were far higher than those of the Avilamycin-fed groups as shown in Table 7. The value of the AB titers of control were 8.1±0.15, those of the FRB- and Avilamycin-fed group were 8.89±0.08 and 7.27±0.09, respectively.

The AB titers of the FRB-fed group were increased significantly more than those of the Avilamycin-fed group ($p<0.05$). Thus, it can be predicted that the FRB-fed group will have a stronger defensive system against a virulent field strain of NDV than the Avilamycin-fed group or control group.

References

1. Kamphues, J. (1999). *Berliner und MuÉnchener TieraÉrztliche*, *112*, 370–379.
2. Joshi, V. K., Gupta, K., Devrajan, A., Lal, B. B., & Arya, S. P. (2000). *Journal of Food Science and Technology*, *37*, 609–612.
3. Saha, S. K., Senani, S., Padhi, M. K., Shome, B. R., Shome, R., & Ahlawat, S. P. S. (1999). *Current Science*, *77*, 696–697.
4. Kung, L. (1998). In S. Muirhead (Ed.), *Direct-fed microbial, enzyme & forage additive compendium, vol.4: Direct-fed microbial and enzyme feed additives* pp. 15–19. Minnesota, USA: Miller.
5. Martin, S. A., & Nisbet, D. J. (1992). *Journal of Dairy Science*, *75*, 1736–1744.
6. Koh, J. H., Yu, K. W., & Suh, H. J. (2002). *Letters in Applied Microbiology*, *35*, 47–51. doi:10.1046/j.1472-765X.2002.01131.x.
7. Reynolds, D. L. (1998). In S. Muirhead (Ed.), *Direct-fed microbial, enzyme & forage additive compendium, vol 4: An overview of basic microbiology* pp. 9–14. Minnesota, USA: Miller.
8. Yang, S. Y., Song, M. D., Kim, O. H., & Kim, C. W. (2001). *Korean Journal Applied Microbiology and Biotechnology*, *29*, 110–114.
9. Kung, L., Kreck, E. M., Tung, R. S., Hession, A. O., Sheperd, A. C., Cohen, M. A., et al. (1997). *Journal of Dairy Science*, *80*, 2045–2051.
10. Paek, N. S., Lee, Y. B., & Kim, Y. M. (2001). *Korean Journal Applied Microbiology and Biotechnology*, *1*, 56–61.
11. Akita, T., & Kuwahara, A. (1987). *Kochi Joshi Daigaku Kiyo Shizen Kagakuhen*, *35*, 45–58.
12. Kim, K. M., Yu, K. W., Kang, D. H., Koh, J. H., Hong, B. S., & Suh, H. J. (2001). *Bioscience, Biotechnology, and Biochemistry*, *65*, 2294–2296. doi:10.1271/bbb.65.2294.
13. Agarwal, N., Kamra, D. N., Chaudhary, L. C., Sahoo, A., & Pathak, N. N. (2000). *Letters in Applied Microbiology*, *31*, 220–27310. doi:10.1046/j.1472-765x.2000.00826.x.
14. Cho, M. K., Kim, C. H., Lee, T. K., & Kim, K. Y. (2000). *Korean Journal Applied Microbiology and Biotechnology*, *28*, 279–284.
15. Jones, C. D., & Thomas, C. N. (1987). In T. P. Lyons (Ed.), *Biotechnology in feed industry: The maintenance of strain specificity and bile tolerance when producing stable bacteria* pp. 157–166. Kentucky: Alltech.
16. Havenaar, R., Brink, B. T., & Huis, J. H. J. (1992). In R. Fuller (Ed.), *Probiotics, the scientific basis: Int'l veld selection of strains for probiotic use* pp. 209–224. London, England: Chapman & Hall.
17. Lee, J. H., Lim, Y. B., Koh, J. H., Baig, S. Y., & Shin, H. T. (2002). *Journal of Microbiology and Biotechnology*, *12*, 162–165.
18. KFIC (1985). *Nutritional requirement and breeding system for domestic animals in Korea*. Seoul, Korea: Dongsin.
19. Watkins, K. L., Shryock, T. R., Dearth, R. N., & Saif, Y. M. (1997). *Veterinary Microbiology*, *54*, 195–200. doi:10.1016/S0378-1135(96)01276-X.
20. Beard, C. W. (1980). In S. B. Hitchner, C. H. Domermuth, H. G. Purchase, & J. E. Williams (Eds.), *Isolation and identification of avian pathogens* pp. 129–135. Pennsylvania, USA: American Association of Avian Pathology.
21. Ahlam, A. W. S. S. (2005). Ph. D. Thesis. Pakistan: University of the Punjab.
22. Gilliland, S. E., Stahly, T. E., & Bush, L. J. (1984). *Journal of Dairy Science*, *67*, 3045–3051.
23. Vervaeke, I. J., Van Nevel, C. J., Decuypere, J. A., & Van Assche, P. F. (1973). *The Journal of Applied Bacteriology*, *36*, 397–405.
24. Pinchuk, I. V., Bressollier, P., Verneuil, B., Fenet, B., Sorokulova, I. B., Megraud, F., et al. (2001). *Antimicrobial Agents and Chemotherapy*, *45*, 3156–3161. doi:10.1128/AAC.45.11.3156-3161.2001.
25. Zheng, G., & Slavik, M. F. (1999). *Letters in Applied Microbiology*, *28*, 363–367. doi:10.1046/j.1365-2672.1999.00545.x.
26. Cladera-Olivera, F., Caron, G. R., & Brandelli, A. (2004). *Letters in Applied Microbiology*, *38*, 251–256. doi:10.1111/j.1472-765X.2004.01478.x.
27. Subramanian, P., & Shankar, P. A. (1983). *Journal of Food Science and Technology*, *20*, 181–183.
28. Borregaard, E., & Arneborg, N. (1998). Interactions between *Lactococcus lactis* subs. *lactis* and *Issatchenkia orientalis* at milk fermentation. *Food Technology and Biotechnology*, *36*, 75–78.
29. Butaye, P., van Damme, K., Devriese, L. A., van Damme, L., Baele, M., Lauwers, S., et al. (2000). *International Journal of Food Microbiology*, *54*, 181–187. doi:10.1016/S0168-1605(99)00198-1.
30. Fujiwaru, S., Hashiba, H., Hirota, T., & Forstner, J. F. (2001). *International Journal of Food Microbiology*, *67*, 97–106. doi:10.1016/S0168-1605(01)00432-9.
31. Kwon, N. H., Kim, S. H., Bae, W. K., Kim, J. K., & Lim, J. Y. (2001). *Journal of Hygiene Safety*, *16*, 264–273.

32. Barrow, P. A. (1992). In R. Fuller (Ed.), *Probiotics, the Scientific basis: Probiotics for chickens* pp. 225–257. London: Chapman and Hall.
33. Kato, T., & Owen, R. L. (1994). In P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee, & J. Bienenstock (Eds.), *Handbook of mucosal immunology: Structure and function of intestinal mucosal epithelium* pp. 11–26. San Diego, USA: Academic.
34. Alexander, D. J. (1997). In B. W. Calneck (Ed.), *Diseases of poultry, 10th edition: Newcastle disease and other avian Paramyxoviridae infections* p. 54125. Iowa, USA: Iowa State University Press.
35. Heremans, J. F. (1974). In F. Milgrom, & E. Neter (Eds.), *The immune system and infectious diseases: Proceedings. 4th International Convocation Immunology: The secretary immune system. A critical appraisal* pp. 376–385. Buffalo, N.Y. June 1974.
36. Mueller, A. P., Sato, K., & Glick, B. (1971). *Cellular immunology*, *2*, 140–152. doi:10.1016/0008-8749(71)90033-5.