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Effect of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 on Ammonia Reduction and Laying Performance

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Abstract Livestock industry requires alternatives of antibiotics to prevent environmental pollution and to maintain public health. We herein report on an effective method to reduce ammonia from livestock manure, and confirmed environmentally-friendly livestock production by adding three types of yeast probiotics, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59, into the feed stuff, separately and/or mixed, and these three types of yeasts were administered to the Hy-line brown layers for 8 weeks. Compared with control, the laying performance, the egg quality, and the number of intestinal lactic acid producing bacteria of the treated group were improved and/or increased significantly. *Pichia anomala* SKM-T potently reduced ammonia production from poultry manure, and the other strains were also able to reduce the ammonia from it. The optimum condition for the reduction of ammonia with *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 was obtained by using the augmented centroid-simplex design. The ratio of optimum condition was *Pichia farinosa* SKM-1:*Pichia anomala* SKM-T:*Galactomyces geotrichum* SJM-59=0.295:0.209:0.080, and the estimate was - 123.36 ($p=0.0138$). An ability to reduce the ammonia production from livestock manure was maintained at 30°C for 15 weeks.

Key words: *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, *Galactomyces geotrichum* SJM-59, laying performance, ammonia production, augmented centroid-simplex design, optimum condition

The livestock industry is composed of cow and/or cattle industry, hog raising industry, and broiler, layer and eggs industry, which have a long history in Korea as well as in Europe. Recently, two major problems have arisen; the treatment of livestock manure and the misuse of antibiotics [7, 16, 17, 20, 23]. Overproduction of livestock manure was related to the increasing of cage breeding and/or the per capita consumption of meat, chicken, and eggs. To make an organic compost is one of the best economic methods to treat livestock manure. Livestock manure causes severe environmental pollution [4, 11, 15]. Reliable regulations are found in Europe; for example, the total amount of livestock manure production has been regulated by Manure Production Rights (MPR) in Netherlands.

Generally, a suitable composition of wastewater for biological treatment is known as BOD:N:P=100:5:1, but actual nitrogen and phosphate concentrations are shown as BOD:N:P=100:20-100:40 in livestock urine, and the levels are too high to apply to the biological treatment [24]. The effort to regulate phosphate has been conducted with phytase treatment in many European countries [3], where MPR controls only phosphate concentration. Nitrogen compounds are stronger pollutants than phosphate compounds. NOx, especially NO_2, is related to the Greenhouse effect, in which ammonia is a major offensive odor compound derived from livestock manure, especially in the poultry industry, where it has incurred the enmity of the people [22]. In addition, ammonia is a major cause of conjunctivitis, pneumonia, New Castle disease, and weight loss in poultry and swine industry [1, 3, 5, 12]. Therefore, the International Environment Convention and Clean Air Act was established to decrease the production of ammonia and nitrogen compounds from livestock manure.

Poultry and hog breeding have been conducted in large facilities rather than small-sized farms, and in this case, livestocks are raised in cages; therefore, methods to control infectious disease and reduce cost of animal production are needed. If signs of epidemic disease arise, antibiotics are

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used for the animals to prevent the disease, but such antibiotics can not be used to regulate residues in foods and livestocks [8, 9, 18]. Some antibiotics and/or growth-promoting agents have been administered at a low concentration for nonprevention of disorders; for example, bacitracin, gentamicin, cephalosporin, and lincomycin-spectinomycin increase the poultry productivity. Such an abuse of antibiotics creates a severe problem, where antibiotics-resistant bacteria appear via horizental gene flux and/or transposon.

The livestock industry needs alternatives to antibiotics to prevent environmental pollution and, more importantly, to maintain public health. Hence, we report herein an effective method to reduce ammonia from livestock manure, and confirmed environmentally-friendly livestock production with no hazardous effect on the host by adding three strains of yeasts.

MATERIALS AND METHODS

Organisms

Pichia farinosa SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 were isolated and identified from human feces by our research team. These three strains of yeasts were cultured in a potato dextrose broth (Difco) at 30°C. When the yeasts reached logarithmic phase, they were harvested and lyophilized. Powdered yeasts were mixed individually or together with the feed stuff at various levels.

The nutrient composition of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 was analyzed by the methods of AOAC [2], and contents of the organic acids were analyzed by HPLC (μ Bondapak C18 reverse-phase column; 3.9x300 nm, methanol-18 mM of phosphoric acid; 1.5 mL/min, UV 238 nm).

Breeding Condition

Pichia farinosa SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 were administered to the 42 layers (21-29 weeks, Hy-line brown) individually or mixed at various concentrations. Layers were raised in cages, and feed stuff and water were provided *ad libitum*, and the lighting was maintained for 14 h a day, according to the duration of sunshine received during summer, in Korea. The composition of diet is presented in Table 1.

Determination of Laying Performance

To investigate the effect of yeasts administration on the laying performance, daily egg production, feed efficacy, Haugh unit, egg-shell intensity, and thickness were determined during the experimental periods.

Table 1. Formula and chemical composition of experimental diet.

Ingredients	Composition, %
Yellow corn	66.53
Soybean meal	15.00
Fish meal	5.00
Wheat bran	2.00
Tricalcium phosphate	1.07
Salt	0.20
Limestone	7.20
Animal fat	1.95
Vitamin-mineral mixture*	0.60
DL-methionine	0.15
Sand**	0.30
Total	100.00
Analytical values	Chemical composition
Energy, kcal/Kg	2,900.00
Crude protein, %	15.00
Calcium, %	3.41
Phosphorous, %	0.32
Methionine, %	0.34
Lysine, %	0.75

*; Vitamin mixture contains the following per kg: vitamin A, 1,600,000 IU; vitamin D3, 300,000 IU; vitamin E, 800 IU; vitamin K3, 130 mg; vitamin B2, 1,000 mg; vitamin B12, 1,200 g; choline chloride, 35,000 mg; Fe, 4,000 mg; Cu, 500 mg; I, 250 mg; niacin, 2,000 mg; Ca pantothenate, 800 mg; folacin, 60 mg; DL-methionine, 6,000 mg; B.H.T, 6,000 mg; Mn, 12,000 mg; Zn, 9,000 mg; Co, 160 mg.

**; The ratio of sand was regulated by the addition volume of probiotics.

Changes of Intestinal Microorganisms

To determine the changes of intestinal microorganisms, the intestine was removed from the layers at the end of the experiments and then homogenized in sterilized saline. The intestine was spread on potato dextrose agar plate, nutrient agar plate, and MRS agar plate, and all the plates were incubated at 30°C or 37°C under aerobic condition or anaerobic condition in anaerobic jar (Difco).

Determination of Ammonia

Lyophilized yeasts were mixed with feed up to 0.3% and administered to layers. Poultry manure was collected after 16-24 h from the administration, and placed in a bottle. The bottle was closed tightly and incubated at 30°C for 24 h, and ammonia concentration was determined (Gastech, 3La, Japan). To obtain the optimum condition for the reduction of ammonia production from poultry manure, augmented simplex-centroid design was used (Design Expert, ver. 6.0).

RESULTS

Nutrients and Organic Acid Composition of Yeasts

Nutrient composition of the mixture of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces*

Table 2. Feeding effect of the yeast mixture, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59, on the performance of 21-week-old Hy-line brown layers for 8 weeks.

Item	Yeast mixture concentration, %		
	0	0.1	0.3
Total laying efficacy, %	93.90^{a}	97.30^{b}	98.70^{b}
Normal laying efficacy, %	92.50^{a}	94.80^{b}	97.70^{c}
Mean of egg weight, g	58.20	58.50	58.40
Hen-day production, %	55.50^{a}	56.10^{a}	57.90^{b}
Feed intake, g/bird/day	119.50	117.50	116.40
Feed/egg weight	2.19^{a}	2.09^{b}	2.04^{b}

$p<0.05$.

geotrichum SJM-59 was crude protein 12.77%, crude fat 3.5%, crude fiber 6.1%, ash 10.18%, thiamin 8.74 mg, riboflavin 1.77 mg, and pyridoxine 3.51 mg. It was expected that the concentration of protein and vitamin B complex were quite high.

The organic acid contents in the mixture of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 were citric acid 1.66%, succinic acid 0.96%, lactic acid 553.24 ppm, malic acid 823.55 ppm, acetic acid 423.74 ppm, and tartaric acid 396.32 ppm.

Laying Performance and Intestinal Microorganisms

As shown in Table 2, the productivity rate of layers fed yeasts was increased compared with the control. The laying efficacy of yeasts-treated groups was significantly different from that of the control. The normal laying efficacy was also significantly increased. Hen-day production was different between the control and 0.1% yeast-treated group, but not significantly. While the hen-day production showed a high number in the 0.3% yeast-treated group, the mean of the egg weight was not different in all the experimental groups. Feed efficacy also significantly improved in the yeast-feeding groups.

Table 3 shows the effect of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 on the egg quality, shell thickness, shell breaking strength and Haugh unit, showing substantial difference between the control and 0.3% yeast-treated group. However, there was no significant difference between the 0.1% yeast-treated group and the control. Therefore, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 have the ability to improve the productivity and egg quality rates in the layer industry.

Table 3. Feeding effect of yeast mixture, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59, on the egg quality.

Item	Yeast mixture concentration, %		
	0	0.1	0.3
Shell thickness, μm	389.00^{a}	419.00^{ab}	430.00^{b}
Shell breaking strength, kg/cm^{2}	3.63^{a}	4.02^{ab}	4.26^{b}
Shell density, mg/cm^{2}	87.70	89.10	89.80
Haugh unit	80.70^{a}	84.30^{ab}	85.20^{b}

$p<0.05$.

Table 4. Feeding effect of yeast mixture, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59, on the changes of intestinal microorganisms in layers.

Microorganisms	Yeast mixture concentration, %		
	0	0.1	0.3
Lactic acid producing bacteria	8.128^{a}	8.897^{b}	9.957^{b}
Yeast*	9.636	9.650	10.297
Facultative anaerobes	11.299^{a}	12.166^{ab}	12.204^{b}

$p<0.05$.

*; total yeast from the poultry manure as well as administered yeasts.

At the end of the experiments, three layers were sacrificed from each group and then intestinal microorganisms were determined. As shown in Table 4, the number of lactic acid producing bacteria was widely different between the control and yeast-treated group. The number of yeast and facultative anaerobes were also increased in the yeast-treated groups, but not in yeast.

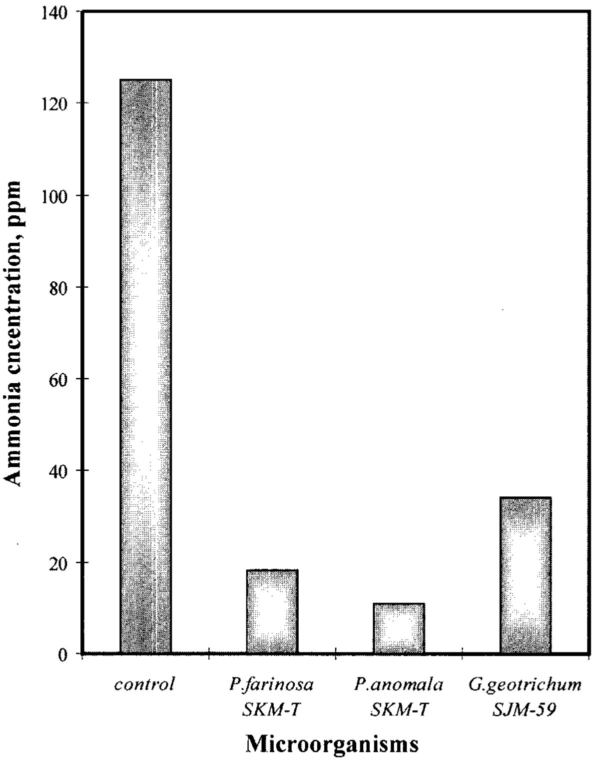

Fig. 1. Reducing effect of ammonia production in poultry manure.

Control; non-administered probiotics, *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59 were administered at 0.3%.

Reduction of Ammonia

Each strain of yeast was administered to layers at 0.3% concentration for seven days to examine the ability of reducing the ammonia production in the manure. As shown in Fig. 1, all tested strains appeared to reduce ammonia production. For *Pichia anomala* SKM-T, 11 ppm of ammonia was detected on the average, indicating a powerful ability to reduce the ammonia production from the manure. On the other hand, the other strains showed feeble effect on the ammonia reduction. Thus, the three strains were mixed with feed stuff at various concentration levels (Table 5) to optimize the condition for ammonia reduction in the manure.

Table 5 shows the experimental design to obtain the optimum condition for the three strains described above, and the results are shown in Fig. 2 and 3. Figure 2 shows

Table 5. Augmented simplex-centroid design of optimum condition for ammonia reduction from the poultry manure.

Trt.	X1		X2		X3		Experimental value				
	C	A	C	A	C	A	1st	2nd	3rd	4th	5th
1	0.50	135	0.00	0	0.00	0	135	155	120	115	130
2	0.00	0	0.00	0	0.50	135	170	120	140	140	135
3	0.00	0	0.00	0	0.00	0	125	130	135	120	135
4	1.00	270	0.00	0	0.00	0	160	145	140	130	135
5	0.10	27	0.60	162	0.10	27	175	165	155	125	165
6	0.25	68	0.25	68	0.25	68	170	205	160	130	145
7	0.25	68	0.00	0	0.25	68	200	170	170	170	205
8	0.20	54	0.20	54	0.20	54	155	130	115	120	165
9	0.00	0	0.50	135	0.00	0	130	130	125	110	155
10	0.00	0	0.50	135	0.50	135	135	140	120	124	125
11	0.50	135	0.00	0	0.00	0	130	140	110	115	145
12	0.00	0	0.00	0	0.33	89	150	150	135	110	175
13	0.33	89	0.00	0	0.33	89	130	135	155	120	150
14	0.00	0	0.00	0	0.00	0	115	120	140	130	120
15	0.00	0	0.00	0	0.00	0	130	130	145	105	150
16	0.50	135	0.50	135	0.00	0	130	140	105	130	130
17	0.25	68	0.25	68	0.00	0	125	105	125	105	135
18	0.00	0	0.00	0	1.00	270	150	130	200	130	125
19	0.33	89	0.33	89	0.33	89	155	140	130	170	200
20	0.33	89	0.33	89	0.00	0	130	105	150	155	165
21	0.50	135	0.50	135	0.00	0	105	120	120	110	145
22	0.25	68	0.25	68	0.25	68	130	105	110	135	155
23	0.00	0	0.33	89	0.00	0	140	120	130	105	115
24	0.33	89	0.00	0	0.33	89	190	140	125	170	150
25	0.00	0	0.00	0	0.50	135	135	150	140	145	145
26	0.10	27	0.10	27	0.60	162	165	130	135	150	120
27	0.10	27	0.10	27	0.10	27	150	115	95	130	150
28	0.00	0	0.25	68	0.25	68	180	140	160	155	160
29	0.00	0	1.00	270	0.00	0	135	160	135	150	135
30	0.50	135	0.00	0	0.00	0	165	120	140	135	160
31	0.50	135	0.00	0	0.50	135	120	95	110	130	140
32	0.00	0	0.50	135	0.00	0	-	40	40	70	95
33	0.60	162	0.10	27	0.10	27	105	125	130	110	140
34	0.33	89	0.33	89	0.00	0	140	115	120	85	140
35	0.33	89	0.00	0	0.00	0	140	140	120	120	140
36	0.00	0	0.33	89	0.33	89	120	110	95	75	110
37	0.10	27	0.10	27	0.10	27	115	115	125	100	140
38	0.00	0	0.00	0	0.50	135	145	120	135	120	180
39	0.00	0	0.33	89	0.33	89	155	125	145	125	135
40	0.00	0	0.50	135	0.00	0	150	145	150	125	150
41	0.00	0	0.00	0	0.50	135	190	185	150	140	155

C, coded value; A, actual addition volume (mg); X1, *Pichia farinosa* SKM-1; X2, *Pichia anomala* SKM-T; X3, *Galactomyces geotrichum* SJM-59.

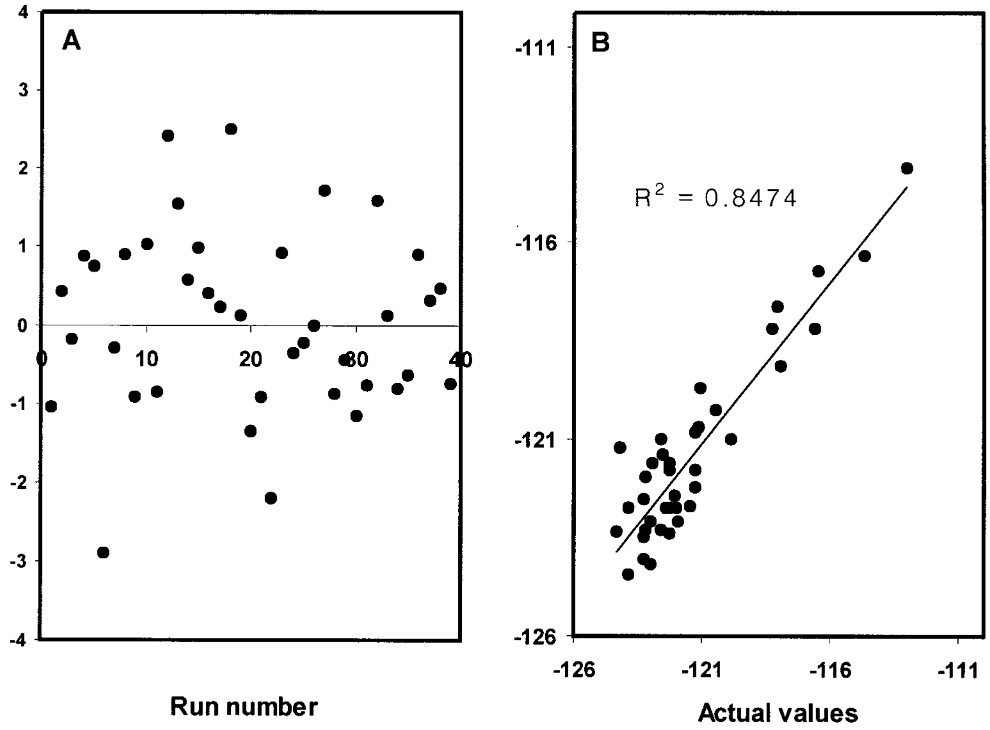

Fig. 2. The validity of the augmented simplex-centroid design and the experiment.
A, outlier T versus actual experimental values; B, predicted value versus actual value.

the validity of the experimental model and results; R-square of the experimental model is 0.8974 and p-value is 0.0137, thus the experimental model is regarded more significant. Figure 2A shows how the results follow the normal distribution; since there is no outlier, the results are considered to follow the normal distribution with skewness of the three. Figure 2B shows the external validity of the experimental model and, according to the results obtained from the experiments, there was no distinct difference between predicted values and actual values, thus the experimental model and the results do not have any constant error.

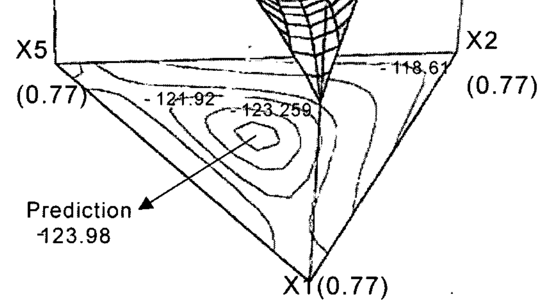

Fig. 3. Response surface diagram and contour map of ammonia reduction in the poultry manure when feeding probiotics with a mixture of *Pichia farinosa* SKM-1:*Pichia anomala* SKM-T: *Galactomyces geotrichum* SJM-59=0.295:0.209:0.080 (p=0.0138).

The equation for the optimum condition of ammonia reduction from the poultry manure was obtained (Y= - 122.46A- 117.67B- 113.98C- 3.7AB- 17.94AC- 27.6BC+ 142.02ABC). As shown in Fig. 3, the optimized ratio of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 to reduce the ammonia reduction from the poultry manure was *Pichia farinosa* SKM-1:*Pichia anomala* SKM-T:*Galactomyces geotrichum* SJM-59=0.295:0.209:0.080, and the estimate was - 123.36 (p=0.0138).

After feeding the mixture of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 into the feed stuff by using optimized composition, the reducing durability of the ammonia production in poultry manure was determined (Fig. 4). As shown in Fig. 4, the ability to reduce the ammonia production in poultry manure was maintained for 15 weeks, even when relative inhibition slightly decreased.

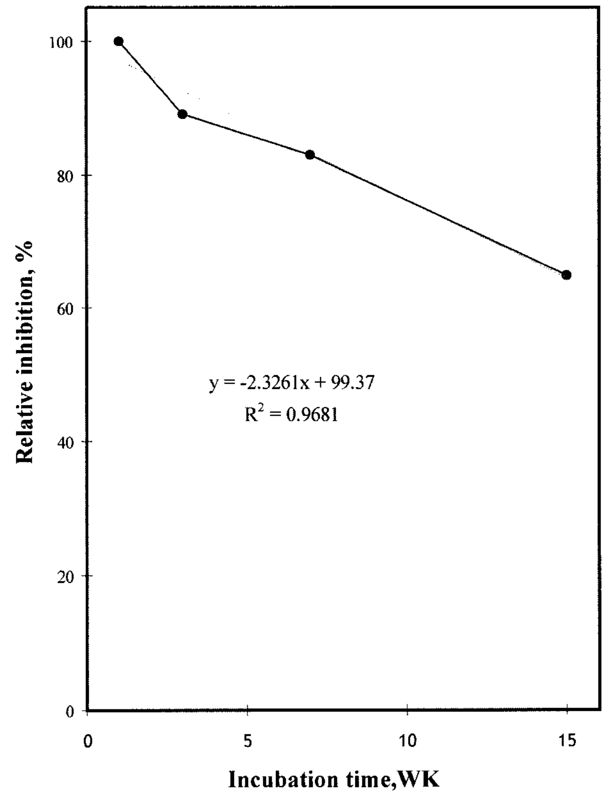

Fig. 4. Durable effect of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 on the ammonia reduction in the poultry manure.

DISCUSSION

Pichia farinosa SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 proved to be effective in improving the laying performance and egg quality, and they were powerful in reducing the ammonia production from the livestock manure. Futhermore, no adverse effect on the experimental animals has been detected during and/ or after experiments, thus these three yeast strains are regarded to be safe, although we did not present the results on the safety test of the strains. Also, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 have an ability to increase the intestinal lactic acid producing bacteria. These results provide important useful characteristics of these probiotics that are safe for the host as well as being efficient [6, 19].

Hen-day production (%) and all laying performance were increased (Table 2) in the treated group (0.3% concentration). It was considered that the immune system of the layers should be strengthened, but the mechanism remains to be elucidated. As shown in Table 3, the egg quality also improved, when *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 were administered to the layer, and that the nutritional state of layers was improved. Since a large amount of protein, vitamin B complex, and minerals are contained in the mixture of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59, the health state of the host should be improved. Moreover, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 produce various kinds of organic acids. In particular, succinic acid is known to contribute to palatability, and the taste of feed stuff also has to be improved. Also, the major organic acids, including citric acid and succinic acid, of the mixture of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 are intermediates of the TCA cycle, and they should contribute in generating energy easily, thus the energy metabolism and efficiency were considered to be improved.

The number of lactic acid producing bacteria in the intestine was increased, depending on the addition volume, where it was regarded to have a direct effect on the mixture of the three strains of yeasts to improve intestinal microorganisms. Furthermore, it is well known that live yeast culture promotes propagation of intestinal lactic acid producing bacteria and improves the productivity of the livestock, while inhibiting the propagation of coliform bacteria as a competitive exclusion [14] of oxygen consumption between yeast and harmful bacteria in the small intestine [21].

Figure 1 shows the effect of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 on the ammonia reduction in the poultry manure. Ammonia elimination in the environment takes place by two mechanisms. The first mechanism is nitrification, generally performed by auxotrophic or mixotrophic microorganisms [10]. Ammonia is oxidized in two steps *via* nitrite to nitrate. Conventional ammonia oxidation occurs by the action of enzyme, and ammonia monooxygenase which is known to be a rate-limiting enzyme. This oxidation of ammonium is generally attributed to *Nitromonas europae*, and the oxidation of nitrite to *Nitrobacter aglis* [25]. The second metabolism is assimilation, which is generally carried out by yeast. In *Saccharomyces cerevisiae*, NADP-dependent glutamate dehydrogenase occupies a key position in the anabolic nitrogen metabolism; *i.e.*, converts ammonia into glutamate by NADP-dependent glutamate dehydrogenase [13]. *Pichia anomala* SKM-T has the best ability to reduce the ammonia generation, and most yeasts contain some glutamate dehydrogenase activity. Therefore, it is considered that *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 have ammonia assimilating ability. These abilities are maintained, when the manure is incubated at 30°C for 15 weeks. However, the manure is not incubated in such a high temperature in the actual livestock industry. The mixture of the optimized composition should be better than other composition of

Pichia farinosa SKM-1 and *Pichia anomala* SKM-T, while *Galactomyces geotrichum* SJM-59 is expected to maintain the activity and ability of ammonia reduction in the field.

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