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Full Length Research Paper

Effect of feeding inulin oligosaccharides on cecum bacteria, egg quality and egg production in laying hens

Park, Sang-Oh and Park, Byung-Sung*

Department of Animal Biotechnology, Kangwon National University, Chuncheon 200-701, Republic of Korea.

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Inulin is reported to improve the egg quality and production of laying hens. In the present study, we investigated the dietary effects of microcapsulated inulin oligosaccharide (INO) which is manufactured from Jerusalem artichoke *(Helianthus tuberosus* L.) on the cecum bacteria, egg quality and production of laying hens. 400 laying hens were randomly allocated to one of the following four treatment groups for 10 weeks: T1 (control without INO or inulin), T2 (200 mg INO/kg diet), T3 (250 mg INO/kg diet) and T4 (300 mg INO/kg diet). Egg production, Haugh unit, egg shell thickness and breaking strength were significantly higher in the T3 and T4 groups than in the T1 and T2 groups (P<0.05). The level of egg cholesterol was highest in the T1 group and decreased in the INO addition groups from 5.68 to 8.46% (P<0.05). When compared with the T1, triglycerides in the blood and total cholesterol decreased significantly in the T2, T3 and T4 groups by 11.75 to 13.45% and 9.41 to 9.85%, respectively (P<0.05). The growth of cecum *Bifidobacterium* and *Lactobacillus* was stimulated in the T2, T3 and T4 when groups compared with the T1 group, while the growth of *Escheria coli* and *Salmonella* was clearly inhibited (P<0.05). The results of this study demonstrate that the addition of microcapsulated inulin oligosaccharide (250 mg/kg) into a laying hen's diet can promote the multiplication of beneficial cecum bacteria and simultaneously improve egg production and quality.

Key words: Jerusalem artichoke, inulin oligosaccharides, egg quality, cecum bacteria.

INTRODUCTION

A recent review demonstrated that indissoluble oligosaccharide, inulin extracted from the Jerusalem artichoke can be used as a prebiotic, which acts as an antibacterial promoter for the poultry diet (Rehman et al., 2008). Prebiotics can reach the lower gastrointestinal tract such as the colon and cecum without being hydrolyzed in the upper gastrointestinal tract of humans and animals, inhibiting the growth of pathogenic bacteria such as *Salmonella*, and simultaneously providing a carbohydrate substrate to promote the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* strains (Zsolt et al., 2011; Gibson and Rastall, 2006; Patterson and Burkholder, 2003). Inulin is a linear fructose polymer in which the fructose units are joined by a β (2→1) glycosidic linkage. More than 80% of inulin reaches the large intestine without being hydrolyzed by animal gastric fluid and digestive enzymes, and they are used as a growth substrate for intestinal bacteria. Inulins are known as prebiotics because they selectively stimulate the growth of beneficial *Lactobacillus* and *Bifidobacterium* and have bifidogenic effects (Rehman et al., 2008). They have also been identified to promote gastrointestinal mineral absorption and modulate lipid metabolism (Azorin-Ortuno et al., 2009).

It has been established that inulin alone is partially broken down by acid in the upper gut. Inulin is also vulnerable to degeneration by air during commercial distribution. To address these problems, the production of inulin oligosaccharide (INO) using microcapsules has been developed (Park, 2008; Dorotea and Maris, 2005). These INO are reported to improve the immune capability of broilers and the growth capability of intestinal beneficial bacteria (Park and Park, 2011a), as well as to promote increased quality of chicken meat and longer storage life (Park and Park, 2011b). Frank (1999) reported that

*Corresponding author. E-mail: bspark@kangwon.ac.kr. Tel: 82-33-250-8615. Fax: 82-33-251-7719.

Table 1. Formula and chemical composition of the standard diet for the laying hens (% as-fed).

Item	Basal diet
Corn grain	51.42
Soybean meal	21.80
Corn gluten meal	6.30
Wheat bran	9.70
Soybean oil	1.50
Limestone	7.70
Dicalcium phosphate	0.80
Sodium chloride	0.30
DL-methionine (50%)	0.10
L-lysine (80%)	0.08
Vitamin-mineral mix.[1]	0.30
Total	100
Chemical composition	
Crude protein (%)	18.78
ME (kcal/kg)	2,900

[1]Provided per kilogram of diet: vitamin A (retinyl palmitate), 1,200 IU; vitamin D_3, 2,500 IU; vitamin E(dl-α-tocopheryl acetate), 20 IU; vitamin K_3, 4.0 mg; thiamin, 1.5 mg; riboflavin, 50.0 mg; pantothenic acid, 17 mg; niacin, 34 mg; pyridoxine, 4.0 mg; choline chloride, 250 mg; folic acid, 0.5 mg; biotin, 0.18 mg; vitamin B_{12}, 0.1 mg; iron, 24 mg; zinc, 40 mg; manganese, 50 mg; copper, 17 mg; iodine, 0.60 mg; selenium, 0.13 mg; cobalt, 0.70 mg.

dietary inulin in layers improved the egg shell intensity by reducing the egg cholesterol through increased mineral absorption from the gut. However, there are virtually no reports regarding the effects of INO's addition and feeding on laying hens.

Therefore, this study was conducted to examine the effects of adding varying levels of INO to the layer diet on egg production, egg quality, blood lipid and changes in cecum bacteria.

MATERIALS AND METHODS

All experimental procedures were approved by the IACUC (Institutional Animal Care and Use Committee of Kangwon National University, Republic of Korea).

Preparation of inulin oligosaccharides

Inulin with a mean degree of polymerization of 26 was extracted from Jerusalem artichokes *(Helianthus tuberosus* L.) using the hot water and cooling extraction method suggested by French (1989), and was subsequently freeze dried. 15 ppm of vitamin E and inulin were mixed with 70°C warm water, and a high pressure homogenate was obtained with a high pressure homogenizer (T25 Basic, IKA, German). Ultrafine powder coating materials (high pressure homogenate 9: Sureteric 1) were manufactured by shooting Sureteric (Colorcon, UK) in the form of an intestine-soluble film coated tablet, into the high-pressure homogenate with compressed air. INO containing more than 90% inulin with a particle size of 100 to 200 μm was manufactured by drying with a spray dryer (B-191, Buchi, Sweiss) (Park and Park, 2011a, b).

Experimental design

400 laying hens (Hyline brown) that were 29 weeks old were randomly divided into four treatment groups with four replicates of 25 animals per group in wire cage. Treatment groups were categorized as T1 (control without INO or inulin), T2 (200 mg INO/kg diet), T3 (250 mg INO/kg diet) and T4 (300 mg INO/kg diet). The addition levels of INO were determined by results from the previous literature (Park and Park, 2011a,b; Park, 2008) and preliminary experiments.

Animal feeding and management

All scientific procedures involving experimental animals complied with the scientific and ethical procedures suggested by Swanson (2008). Experimental diets for treatment groups either satisfied or slightly exceeded the nutrient requirements recommended by the NRC (1994). Experimental diets mainly consisted of corn and soybean meal, and the different addition levels of INO were replaced by the same amount of corn (Table 1). Lighting during the laying period was adjusted to 17 h, and the experimental diet was provided *ad libitum* and they had access to water freely.

Egg production and quality

With egg production and weight recorded daily and dietary intake examined weekly, all the data were expressed as the mean values of the entire experimental period. Evaluation of egg quality measured from 20 eggs was selected through the average weight per treatment every week. Haugh unit (HU), egg shell thickness, egg shell breaking strength and egg yolk color was measured immediately in collected eggs using the egg multi tester EMT-5200 (Robotmation Co. Ltd. Tokyo. Japan).

Cholesterol and fatty acid of eggs

Lipids from the egg yolk were extracted according to the method developed by Folch et al. (1957). Egg cholesterol and fatty acid composition measured from 20 eggs were selected through the average weight per treatment every week. The gas chromatographic system (model GC-15A, Shimadzu Corp., Kyoto, Japan), which is equipped with a flame ionization detector, auto feeder (model AOC-17 Shimadzu Corp.) and the chromatography data system (model Class-VP Shimadzu Corp.) was used. For cholesterol, the fused silica capillary column (15 m × 0.32 mm i.d), SPB-1 coated (Supelco Inc, Bellefonte, PA, USA) with film thickness of 1.0 μm was used. The cholesterol standard and 5α-cholestane purchased from the U.S.A were used as an internal standard (Sigma Chemical Co, St, Louis MO). Lipid methylation was accomplished by the modified Morrison and Smith (1967) method. For fatty acid, the SP™-2560 Capillary GC Column (L×I.D. 100 m × 0.25 mm, df 0.20 μm Omegawax 320 capillary column. USA) was used. U.S. Supelco products (37 Component FAME Mix, Sigma Aldrich Co., St. Louis, MO) were used, and as an internal standard, nonadecanoic acid (19:0) was used.

Blood lipid

Eight chickens were randomly selected from each treatment group

Table 2. Characteristics of egg production in laying hens fed the experimental diets.

Item	Group				PSE	P-value
	T1	T2	T3	T4		
Egg production (%)	92.03[b]	91.72[b]	93.72[a]	93.80[a]	0.3438	0.0210
Egg weight (g)	62.13[b]	62.19[b]	64.03[a]	64.12[a]	0.2983	0.0001
Feed consumption (g/day)	118.4[b]	119.1[b]	123.3[a]	123.5[a]	0.7797	0.0030

T1, Unsupplemented INP (control); T2, 200 mg INP kg/diet; T3, 250 mg INP kg/diet; T4, 300 mg INP kg/diet; PSE, pooled standard error of mean values. [a,b]Mean values within the same row with different superscript letter are significantly different (P<0.05).

Table 3. Levels of TAG, TC, HDL·C and LDL·C in plasma from laying hens fed the experimental diets.

Item (mg/dl)	Group				PSE	P-value
	T1	T2	T3	T4		
TAG	119.29[b]	119.19[b]	105.27[a]	103.24[a]	2.2959	0.0001
TC	101.37[b]	101.85[b]	91.83[a]	91.38[a]	1.5363	0.0001
HDL·C	21.84[c]	23.41[a]	22.72[b]	22.72[b]	0.3571	0.0001
LDL·C	72.20[a]	72.79[a]	68.98[b]	68.67[b]	0.5955	0.0010

TAG, Triacylglyceride; TC, total cholesterol; HDL·C, high density lipoprotein cholesterol; LDL·C, low density lipoprotein cholesterol; PSE, pooled standard error of mean values. [a,b,c]Mean values within a same row with unlike superscript letter are significantly different (P<0.05).

once a week. 1 ml of blood was collected from the carotid artery of each laying hen using a syringe with a Hamilton 25 gauge needle into heparinized tubes (Becton Dickinson, Franklin Lakes, NJ USA), and the plasma was separated. Triglycerides, total cholesterol, high density lipoprotein cholesterol and low density lipoprotein cholesterol were analyzed by a commercial enzyme kit (Sigma Co. Ltd., USA) using the hematology Autoanalyzer (7150, Hitachi, Tokyo, Japan).

Cecum bacteria

20 laying hens were randomly selected from each treatment group at the end of the experiment and sacrificed by cervical dislocation, in accordance with recommendations for the euthanasia of experimental animals (Close et al., 1997). Cultures for cecum bacteria were obtained using sterilized selective plate media; *Lactobacillus* sp. (MRS agar, Oxoid, Basingstoke, UK); *Bifidobacterium* sp. (*Bifidobacterium* selective agar, BIM-25 medium) *Salmonella* sp. (SS agar Difco, CM0099); *Escherichia coli* sp. (McConkey Purple agar). *Salmonella* and *E. coli* were cultured at 37°C for 24 h under aerobic conditions, and *Lactobacillus* and *Bifidobacterium* were stationary cultured at 37°C for 48 h under anaerobic conditions in sealed anaerobic jars equipped with AnaeroGen sachets. All the numbers of bacterial colonies were presented in the common log as CFU (colony forming unit/g of wet of cecum content).

Statistical analysis

The statistical treatment of all analyzed data involved calculating the mean and standard error of each treatment group using the GLM procedure from SAS (2005), conducting ANOVA, and then verifying the statistical significance between treatment mean values using the Duncan's multiple range test at the 95% level (P<0.05). The statistical analysis indicated the degrees of freedom with four repeated measurements.

RESULTS

The effects of addition of different levels of INO on egg production, egg weight and dietary intake are presented in Table 2. Egg production, egg weight and dietary intake were significantly higher in the T3 and T4 groups than in the T1 and T2 groups (P<0.05), and there were no statistically significant differences between the T3 and T4 and between T1 and T2 groups. The results of this study confirm that supplementing the diet for laying hens with INO (250 mg INO kg/diet) can greatly increase egg production and weight.

The effects of adding different INO levels to the laying hen diet with regards to plasma lipids are presented in Table 3. Plasma triglycerides, total cholesterol and low density lipoprotein cholesterol content were significantly lower in T2, T3 and T4 groups than in the T1 group, and the ranges of decrease in the plasma triglycerides, total cholesterol and low density lipoprotein cholesterol in the INO addition group were 11.75 to 13.45, 9.41 to 9.85 and 4.46 to 4.89%, respectively (P<0.05).

The effects of the INO supplemental diet on HU, egg shell thickness, egg shell breaking strength and egg yolk color are shown in Table 4. HU, egg shell thickness and egg shell breaking strength were significantly higher in

Table 4. Haugh unit, egg shell thickness, egg shell breaking and egg yolk color in laying hens fed the experimental diets.

Item	Group				PSE	P-value
	T1	T2	T3	T4		
Haugh unit (HU)	79.38[b]	79.46[b]	89.32[a]	89.49[a]	1.5330	0.0001
Egg shell thickness (mm)	0.28[b]	0.30[b]	0.37[a]	0.38[a]	0.0137	0.0001
Egg shell breaking (kg/cm^2)	1.71[b]	1.72[b]	2.03[a]	2.04[a]	0.0509	0.0001
Egg yolk color	9.46	9.46	9.52	9.54	0.2695	0.5460

[a,b]Mean values within a same row with different superscript letter are significantly different (P<0.05).

Table 5. Cholesterol content of egg yolk from laying hens fed the experimental diets.

Item	Group				PSE	P-value
	T1	T2	T3	T4		
Egg yolk (g)	14.29	14.23	14.17	14.20	0.0369	0.2310
Total cholesterol						
mg/g of yolk	17.01[a]	16.92[a]	16.18[b]	15.67[b]	0.0547	0.0070
mg/60 g of egg	243.07[a]	240.77[a]	229.27[b]	222.51[b]	1.1939	0.0031

[a,b]Mean values within the same row with different superscript letter are significantly different (P<0.05).

Table 6. Fatty acid composition of egg yolk lipids from laying hens fed the experimental diets.

Fatty acid (% of total fatty acid)	Group				PSE	P-value
	T1	T2	T3	T4		
14:0	0.44[b]	0.59[ab]	0.22[c]	0.22[c]	0.0488	0.0001
16:0	28.10[a]	28.14[a]	25.73[b]	25.32[c]	0.3947	0.0001
16:1n-7	4.15[b]	4.09[b]	4.50[a]	4.51[a]	0.1062	0.0080
18:0	7.25[a]	7.31[a]	5.40[b]	5.66[b]	0.0397	0.0018
18:1n-9	44.66[b]	44.81[b]	47.71[a]	47.80[a]	0.4488	0.0001
18:2n-6	15.40[b]	15.06[b]	16.44[a]	16.08[a]	0.1956	0.0001
18:3n-6	-	-	-	-	-	-
18:3n-3	-	-	-	-	-	-
20:1n-9	-	-	0.41	0.41	-	-
20:5n-3	-	-	-	-	-	-
22:6n-3	-	-	-	-	-	-
SFA	35.79[a]	36.04[a]	31.76[b]	31.20[b]	0.4565	0.0001
UFA	64.21[b]	63.96[b]	68.24[a]	68.80[a]	0.3017	0.0001

SFA, saturated fatty acid; UFA, unsaturated fatty acid; (-), not detected. [a,b,c]Mean values within the same row with different superscript letter are significantly different (P<0.05).

the T3 and T4 groups than in the T1 and T2 groups (P<0.05), and there are no statistically significant differences between the T3 and T4 groups and between T1 and T2. Also, there was no statistically significant difference in the egg yolk color among treatment groups.

The composition of cholesterol and fatty acid in the eggs of laying hens fed INO are presented in Tables 5 and 6. The cholesterol content in eggs was highest in the T1 group followed in decreasing order of T4, T3 and T2 groups. There were statistically significant differences between treatment groups (P<0.05). As compared to the control group, the decreased rate of cholesterol for eggs in the INO addition group ranged from 5.68 to 8.46%. The composition of saturated fatty acid in eggs was lower in

Table 7. Cecum bacterial population (log_{10} CFU/g content) in laying hens fed the experimental diets.

Item	Group				PSE	P-value
	T1	T2	T3	T4		
Bifidobacterium	6.24[b]	6.22[b]	8.21[a]	8.15[a]	0.2945	0.0001
Lactobacillus	6.33[b]	6.23[b]	6.78[a]	6.71[a]	0.3746	0.0001
E. coli	9.97[a]	9.72[b]	7.55[c]	7.49[c]	0.3524	0.0001
Salmonella	9.44[a]	9.33[a]	6.33[b]	6.38[b]	0.4599	0.0001

[a,b,c]Mean values within the same row with different superscript letter are significantly different (P<0.05).

T3 and T4 groups as compared to T2 and T1 groups, while unsaturated fatty acid levels were significantly higher (P<0.05). There were no statistically significant differences between T3 and T4 and between T2 and T1.

The effects of different levels of INO addition on the changes of bacteria in the cecum content of laying hens are presented in Table 7. The growth of cecum beneficial bacteria *Bifidobacterium* and *Lactobacillus* was higher in the T2, T3 and T4 groups as compared to T1, while that of harmful *Escherichia coli* and *Salmonella* was clearly inhibited (P<0.05).

DISCUSSION

In our results, we verified that a feeding diet with INO can further improve the egg production as compared to the control diet without INO or inulin. There are some reports that inulin increases the absorption rate of nutrition in the upper gut of animals and provides an excellent substrate for *Bifidobacterium* and *Lactobacillus*, leading to improved immune capabilities (Absolonne et al., 1995; Roberfroid, 1993). It is estimated that egg production is lower in the control group without INO or inluin than in the INO groups due to the reasons described above.

Inulin is known to decrease the level of blood lipid in humans and animals (Fiordaliso et al., 1998). Causey et al. (2000) reported similar results involving a chicory inulin supplement for humans with hypercholesterolemia that reduces blood triglycerides, total cholesterol and low density lipoprotein cholesterol. Inulin reduces blood triglycerides because short chain fatty acids generated from inulin decrease the synthesis of *de novo* fatty acid, inhibiting the entire lipogenic enzymes' gene expression (Delzenne and Kok, 1999). Inulin reduces blood cholesterol because the short chain fatty acids inhibit the biosynthesis of cholesterol in the liver, and propionic acids influence its colonic absorption path and the modulation of carbohydrate and lipid metabolism (Wolever et al., 1995). Moreover, blood cholesterol is also removed in part by the *Bifidobacterium* and *Lactobacillus* in fermentation products (Van and Schaafsma, 1996). Another way in which inulin reduces blood cholesterol is by inhibiting the liver's cholesterol synthesis, through both the increased excretion of bile acids and the inhibition of activity of HMG-CoA reductase, a restriction enzyme associated with cholesterol synthesis (Kim and Shin, 1998). The reduction of blood lipid in the INO addition group is considered a part of this particular mechanism.

It is estimated that the egg HU, eggshell thickness and eggshell breaking strength are higher in the INO addition group (250 mg INO kg/ diet) than in the control group because of the increased absorption rate of nutrients of inulin and minerals such as calcium, a main component of eggshells (Azorin-Ortuno et al., 2009; Lopez et al., 1998). In addition, inulin is reported to improve the mineral absorption rate through the synergistic action of multiple *Bifidobacterium* and short chain fatty acid in the human colon (Mitsuoka, 1990), which mirrors the results of this study. HU, eggshell thickness, eggshell breaking strength and egg yolk color are important factors in determining overall egg quality. As consumers are increasingly pursuing physical well-being, internal and external egg quality become increasingly important for enhancing the egg's marketing value, and HU in particular can be used as a standard for internal egg quality (Lesson and Summers, 1991).

In this study, it is estimated that a greater cholesterol decrease in eggs of the INO addition group reflects the lowest total cholesterol level in the blood of laying hens (Table 3). It is well known that cholesterol in animals is immediately transported through the blood to body tissues and eggs and then accumulated *in vivo*. Also, the results of this study are supported by the report that inulin has the effect of inhibiting lipid synthesis and promoting lipid degeneration (Causey et al., 2000). The lower saturated fatty acid and higher unsaturated fatty acid in the INO addition group (250 mg INO kg/diet) may be related to the biosynthesis and metabolism of short chain fatty acids generated in the lower gastrointestinal tract by inulin inflow into the liver through the hepatic portal vein (Scheppach, 1994).

The number of cecum beneficial bacteria is higher and the number of harmful bacteria is lower in the INO addition group because the microencapsulated INO bypasses the stomach and the small intestine of laying hens, reducing the decomposition rate and absorption rate of inulin. Meanwhile, most of the INO are simultaneously transported to the cecum, and inulin eventually dissolved in the cecum is utilized as a

substrate for the growth of *Lactobacillus* and *Bifidobacterium* (Gong et al., 2002). Park (2008) and Rada et al. (2001) support this study's results by reporting that inulin supplements for broilers and laying hens significantly increase *Bifidobacterium* in the cecum. The reduction of *E. coli* and *Salmonella* in the INO group is due to the significant increase of *Bifidobacterium* and *Lactobacillus* in the cecum and the strong antibacterial activity of inulin (Ahn et al., 2007; Park, 2008).

Conclusion

This study demonstrates that supplementing the layer diets with microcapsulated inulin oligosaccharides resulted in selective stimulation of beneficial *Bifidobacterium* and *Lactobacillus* growth and inhibition of harmful bacteria in the cecum and also improved egg production and quality.

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