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Effect of Selenium-enriched Japanese Radish Sprouts and *Rhodobacter capsulatus* on the Cholesterol and Immune Response of Laying Hens

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ABSTRACT : Immune response and yolk cholesterol are crucial factors for commercial chicken producers. The objectives of this study were to investigate the effect of selenium-enriched Japanese radish sprouts (Se-enriched JRS) and *R. capsulatus* synergistically on immune response and cholesterol in laying hens. A total of 50 laying hens (20-wk old) were assigned to 5 dietary treatment groups, and fed diets supplemented with 2.5 μg/kg, 5 μg/kg, 10 μg/kg Se-enriched JRS and 5 μg/kg Se-enriched JRS+*R. capsulatus* (0.02%). Egg production and yolk color were significantly improved by the supplementation of Se-enriched JRS+*R. capsulatus* in the layer diet ($p<0.05$). Compared to the control, serum cholesterol concentration and triglyceride levels were decreased by all the treatments ($p<0.05$). After 8-wk of the experiment, supplementation of 5 μg/kg, 10 μg/kg and Se-enriched JRS+*R. capsulatus* significantly reduced yolk cholesterol and triglycerides, while the greatest reduction was observed when *R. capsulatus* was incorporated with Se-enriched JRS. Spleen, bursa and thymus weight were significantly increased by both the 5 μg/kg and 10 μg/kg Se-enriched JRS. Compared to the control, supplementation of 5 μg/kg and 10 μg/kg Se-enriched JRS significantly increased serum IgG and yolk IgY concentration and foot web index activity by Newcastle Disease Virus ($p<0.05$). After 4-wk and 8-wk of supplementation, the highest number of leukocytes was observed with Se-enriched JRS+*R. capsulatus* ($p<0.05$). The highest concentration of serum and yolk Se was found in Se-enriched JRS plus *R. capsulatus* treatment. Combined dietary supplementation of Se-enriched JRS and *R. capsulatus* might be beneficial for better health, disease protection and overall production performance. (**Key Words :** Selenium-enriched Japanese Radish Sprout, *Rhodobacter capsulatus*, Cholesterol, Immune Response, Laying Hens)

INTRODUCTION

Immune response, disease resistance and hypercholesterolemia are the crucial factors for commercial chicken producers, and there is no breed or strain with superior immunocompetence status. Recently, a strong relationship between hypercholesterolemia and the immune status of the host animal has been identified in mice (Martens et al., 2008). Chickens merely transfer antibodies to their offspring by depositing antibodies in the egg (Brambell, 1970). This deposited antibody in egg is pivotal for raising immunity of its newborn offspring. The impact of hypercholesterolemia on inflammation and atherosclerosis has received considerable attention, but much less is known about its potential effects on protective immunity. It therefore seems highly desirable to modulate the susceptibility of chicken to infectious challenges and decrease cholesterol by using nutritional adjuvants.

Most studies on micronutrient supplementation by natural ingredients have focused on the preventive or beneficial effects on poultry diseases (Bollengier-Lee et al., 1999). Dietary selenium (Se) supplementation holds promise as a means of treating inflammatory conditions, rejuvenating the aging immune system (Brown et al., 2000), protecting the organism from pathogens, and anti-carcinogenic activity (Yamanoshita et al., 2007). Se has frequently been used in poultry diets via inorganic sources such as sodium selenite (Na_2SeO_3) for boosting immune indices. Organic Se seems more bioavailable than Se in the sodium selenite from the standpoint of immunity; it results in less Se transferred to the environment through feces, and more Se deposited into body tissues and eggs (Cantor and

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Tarino, 1982). The Se content of selenium-enriched Japanese radish sprouts (JRS) is over 600 ppm of Se/g of dry matter. It was reported that JRS have cancer-prevention potentials in rats (Yamanoshita et al., 2007). Se is thought to have cholesterol-lowering ability. Clinical, epidemiological, and experimental studies indicate a strong relationship between the progression of cardiovascular system disease and Se status of the animal.

Probiotics have attracted a lot of attention for their beneficial effects in human and animal health, prevention and treatment of certain diarrheal diseases, improvement of growth, enhancement of immune response, reduction of serum cholesterol (Guarner and Malagelada, 2003) and inhibition of cancer (Hooper et al., 2001) and some pathogenic bacteria (Reid and Jeremy, 2002). We previously reported that *Rhodobacter capsulatus*, a commensal photosynthetic bacteria reduced egg yolk cholesterol in layer, broiler, and Japanese quail (Salma et al., 2007a,b,c), and in rats and pigs (Tsujii et al., 2007, 2008). Commensal bacteria present in the gut microbiota are in close contact with cells of the immune system and have an influence on the development of the immune response. Though our research was conducted with the notion of cholesterol metabolism, we interestingly observed that there was no disease infestation with the *R. capsulatus* supplemented birds.

Considering these factors, we thought that it might be worthwhile to explore whether the administration of both Se-enriched JRS and *R. capsulatus* together would have a synergistic effect on disease prevention together with a hypocholesterolemic action. Thus, it might be possible that Se-enriched JRS and *R. capsulatus* synergistically interact with cells within the chicken gut-associated lymphoid tissues, as well as play a role in the development of immune response with hypocholesterolemic actions.

Thus, the objectives of this study were to investigate the synergistic effect of organic Se and *R. capsulatus* on immune response and cholesterol metabolism in laying hens.

MATERIAL AND METHODS

Birds, management, and diets

A total of 50 Boris Brown ready-to-lay pullets with almost the same live weight were collected from a local commercial flock. They were reared in accordance with the Faculty of Agriculture, Shinshu University guidelines for animal experimentation. The 20-wk-old laying hens were divided into 5 treatment groups and the experiment was conducted for 8-wk. Control birds were fed a commercial diet and experimental groups were supplemented with 2.5 μg/kg, 5 μg/kg, 10 μg/kg Se-enriched JRS and 5 μg/kg Se-enriched JRS+*R. capsulatus* (0.02%). *R. capsulatus* cells were grown in outdoor culture under natural illumination as previously described (Kobayashi and Kurata, 1978). Briefly, the cells of *R. capsulatus* were collected by centrifugation and spray-dried. For the Se-enriched JRS production, Japanese radish sprouts (*Raphanus sativas*) were produced by using Se-added fertilizer (MI Tech Co., Ltd., Nagano, Japan) (Yamanoshita et al., 2007). After harvesting, Se-concentrations analyzed by microwave induced plasma mass spectrometry (PIM-MS) were 600 ppm Se by dry weight. Organic Se was obtained from MI Technology, Nagano, Japan. The hens were immunized against Newcastle disease virus (NDV) with a live attenuated vaccine (1:100) by the oral route after 4-wk of Se-enriched JRS supplementation. The composition of the basal diet and other management practices are as previously described (Salma et al., 2007a).

Sample collection

The laying hens were weighed individually prior to blood collection at the beginning and at the end of each 4-wk feeding period. Daily feed intake, egg production, and egg weight were recorded, and feed conversion efficiency was calculated during the 8-wk feeding period. Eggs from each group were collected at the beginning and at the end of each 4-wk of the feeding period for measurement of egg quality (egg weight, shell weight, shell thickness, albumen weight, albumen height, yolk weight, yolk index, yolk color, and Haugh unit) according to the previously described protocol (Salma et al., 2007a).

Blood, liver and muscle sample preparation

Blood samples of about 2 ml were collected from the brachial wing vein of all birds at the beginning and at the end of each 4-wk of the feeding period. After 1 h standing at room temperature, serum was isolated by centrifugation at 1,150×*g* for 10 min. At the end of the 8-wk feeding period, the laying hens were decapitated, and liver, breast (*Pectoralis major*) and thigh (*Biceps femoris*) muscles were collected. Muscle was dissected free of surface (non-intrinsic) fat. Lipid was extracted from each egg-yolk, liver and muscle sample according to our previously described protocol (Salma et al., 2007a). All the samples were then stored at -80°C until analysis.

Determination of serum IgG and yolk IgY

The levels of total IgG and IgY in serum and yolk were determined using quantitative ELISA kits (Bethyl Laboratories, Montgomery, TX) following the manufacturer's instruction with slight modification. Each plate had its own set of standards and samples were analyzed in triplicate. Reagents and buffers were prepared in the laboratory following the specifications of the

manufacturer (Bethyl Laboratories). The working dilution of detection antibody used was 1:10,000 for IgG and 1:20,000 for IgY. The samples were incubated with tetramethylbenzidine for 30 min and reaction was stopped by 2 M H_2SO_4 after 30 min. The plates were read at 450 nm of primary wavelength using an ELX 800 universal micro plate reader (Bio-Tek Instriments Inc., Winooski, VT).

Lymphoid organs and cutaneous hypersensitivity

At the end of the experiment, four birds from each replicate of the treatment were selected randomly and killed to determine the relative weight of the lymphoid organs and the liver. The thymus tissue was carefully dissected from each side of the neck to ensure complete removal. Organ relative weights were measured to the nearest 0.1 mg.

The foot web index (FWI) was used as an index of the cell-mediated immune response. At the end of the experiment, four separate birds from each treatment were selected and 0.1 ml PHA-P mitogen (1 mg/ml PBS) was injected intradermally into the left foot web. Sterile PBS (0.1 ml) was injected into the right foot web to serve as the control. Measurements were done with a constant tension caliper at 0 and 24 h after the injection, as described by Seema (2002). Foot web swelling was calculated by subtracting skin thickness at 24 h post-injection from that at 0 h pre-injection.

Se and SOD concentration

Se concentration in the diet, yolk and leg muscle of each layer were analyzed by atomic absorption spectrometry with a hydride generator system (Norheim and Haugen, 1986) using a Varian SpectrAA-30 with a VGA-76 vapour generation accessory. Before analysis, each sample was prepared by oxidative digestion in a mixed solution with concentrated nitric and perchloric acids, using an automated system with Tecator 1012 Controller and 1016 Digester heating unit. Plasma samples were assayed for SOD using a commercial SOD kit (Ransod; Randox, Crumlin, UK) as described by Mahmoud and Hijazi (2007).

White blood cell differentiation

The total number of leukocytes in the blood was assessed by hemacytometry. Approximately 100 μl of citrate-stabilized blood was analyzed in a Cell-Dyn 3500 hemacytometer (Abbott Laboratories, Abbott Park, IL) using a specialized configuration for chicken blood. The apparatus was standardized daily using Cell-Dyn22 controls. The leukocytes were measured as cells $\times 10^9$/L. The cell deposits consisting of 98% or more lymphocytes were spread on a slide by the direct blood smear method and stained with Wright's or May-Grunwald-Giemsa stain, and then completely scanned for atypical cells. Through the use of a fixed size wire loop (1.0 mm in outer diameter, Brown & Sharpe Gauge No. 24), four loopfuls of cell deposits were used to deliver approximately 5.0×10^5 cells for each smear.

Enzymatic analysis for cholesterol, triglycerides and bile acids

Cholesterol, triglycerides and high density lipoprotein-cholesterol (HDL-C) in the serum and yolk were determined enzymatically using commercially available kits (Wako cholesterol 439-17501, Wako triglycerides 432-40201 and Wako HDL-C 431-52501 respectively, Wako Pure Chemical Industries Ltd., Tokyo, Japan). For fecal and hepatic bile acids determination, samples were lyophilized, and 50 mg of the lyophilized sample was put into a centrifuge tube containing a 1 ml mixture of t-butanol of water (1:1/v/v) (van der Meer et al., 1985). The tubes were vortexed, incubated for 20 min at 37°C, and centrifuged at 1,000×*g* for 2 min. Supernatant was decanted into another test tube. Fecal and hepatic bile acids were then determined spectrophotometrically using a reagent kit (Wako TBA, 431-15001) according to the manufacturer's instructions.

Fatty acid determination

Total lipid extracts of yolk samples from all the treatments were separated by using a gas chromatograph (Simadzu, GC14B, Kyoto, Japan). The detailed method is described in our previous report (Salma et al., 2007c). The weight of each fatty acid in all detected fatty acids was adopted as a measurement value.

Statistical analysis

Results were expressed as mean±SEM for all observations. The statistical significance of the differences among the treatments was evaluated using the protected Fisher's least significant difference test. The experiment was conducted under a completely randomized design. Percentages were subjected to arc sin transformation prior to analysis. The NCSS (Number Crunchier Statistical System, Kaysville, UT, USA) Version 5.01 computer software package was used for all statistical analysis. Differences were considered significant at ($p<0.05$).

RESULTS

The effect of different dietary levels of Se-enriched JRS on performance of layers is shown in Table 1. Egg production was significantly improved by the supplementation of Se-enriched JRS+*R. capsulatus* in the layer diet ($p<0.05$). Egg mass was improved by both the 5 μg/kg Se-enriched JRS and *R. capsulatus*+Se-enriched JRS treatments, whereas, feed efficiency was improved by both the 10 μg/kg Se and *R. capsulatus*+Se-enriched JRS treatments.

Table 1. Effect of Se-enriched JRS on performance of layers

Parameter	Treatments				
	Control	2.5 μg Se	5.0 μg Se	10.0 μ g Se	Se+PSB
Egg production (%)	85.0±2.0[a]	87.5±1.5[ab]	89.0±2.0[ab]	88.8±1.9[ab]	91.1±1.7[b]
Egg weight (g)	56.0±0.9	56.7±1.1	59.0±1.0	58.9±1.0	59.0±1.6
Egg mass[1]	48.7±1.58[a]	48.9±1.98[ab]	53.9±0.92[b]	52.5±1.7[ab]	53.3±1.24[b]
Feed consumption	123.7±1.7[a]	119.8±1.5[ab]	119.6±1.5[ab]	118.7±1.4 [b]	119.5±1.1[ab]
Feed efficiency[2]	2.56±0.18[a]	2.33±0.09[ab]	2.32±0.12[ab]	2.22±0.12[b]	2.18±0.09[b]
FBW (kg)	1.55±0.08[a]	1.59±0.03[ab]	1.60±0.03[ab]	1.68±0.09[b]	1.68±0.03[b]
Damaged egg (%)	1.26±0.10	1.13±0.07	1.05±0.05	1.22±0.08	1.22±0.06

[a-b] Values with different superscripts differ significantly ($p<0.05$) in the same row.
Values are mean±SEM for 10 laying hens per group.
[1] Egg mass = (egg production×egg weight)/100. [2] Feed efficiency = Feed intake: egg mass (g:g).
PSB = *Rhodobacter capsulatus*; Se = Selenium-enriched Japanese radish sprouts; FWB = Final body weight.

Table 2. Effect of Se-enriched JRS on egg quality

Parameter	Treatment				
	Control	2.5 μg Se	5.0 μg Se	10.0 μg Se	Se+PSB
Yolk color	7.6±0.3[a]	7.6±0.2[a]	9.1±0.1[ab]	8.0±0.2[a]	11.3±0.5[c]
Shell weight (g)	5.7±0.1	5.9±0.3	5.8±0.2	5.5±0.1	5.7±0.2
Albumen weight (g)	37.5± 0.9	37.6±1.3	36.5±1.2	38.4±1.3	38.4±1.3
Albumen height (mm)	9.0±0.4	9.0±0.4	8.4±0.3	7.9±0.6	9.0±0.5
Haugh unit	127.8±0.9	128.8±1.4	126.2±2.4	123.8±2.9	124.9±2.8
Yolk index	0.49±0.01[ab]	0.52±0.02[a]	0.49±0.03[ab]	0.45±0.02[b]	0.47±0.03[ab]
Yolk weight (g)	13.6±0.4	13.4±0.4	12.8±0.3	13.0±0.3	12.9±0.3

[a-b] Values with different superscripts differ significantly ($p<0.05$) in the same row.
Values are mean±SEM for 10 laying hens per group.
PSB = *Rhodobacter capsulatus*; Se = Selenium-enriched Japanese radish sprouts.

The effect of different dietary levels of Se-enriched JRS on egg quality is shown in Table 2. When Se-enriched JRS with *R. capsulatus* was added to the diet, the level of egg yolk color was significantly improved ($p<0.05$).

The effect of different dietary levels of Se-enriched JRS on serum and egg yolk cholesterol and triglycerides is shown in Table 3. Compared to the control, serum cholesterol and triglycerides concentrations were decreased by Se-enriched JRS+*R. capsulatus* ($p<0.05$). After 8-wk of experiment, supplementation of 5 μg/kg, 10 μg/kg and Se-enriched JRS+*R. capsulatus* significantly reduced yolk cholesterol and triglycerides, while the greatest reduction was observed when *R. capsulatus* was incorporated with Se-enriched JRS in the diet. A marked degree of HDL-C was observed with Se-enriched JRS+*R. capsulatus* supplementation.

The effect of different dietary levels of Se-enriched JRS on liver and fecal cholesterol and bile acids is shown in Table 4. Liver cholesterol was significantly ($p<0.05$) decreased by the 5 μg/kg, 10 μg/kg and Se-enriched JRS+ *R. capsulatus* treatments. Liver triglycerides were significantly ($p<0.05$) decreased only by the Se-enriched JRS+*R. capsulatus* treatment. Liver bile acids were significantly ($p<0.05$) increased by both the 10 μg/kg and Se-enriched JRS+*R. capsulatus* treatments. Fecal cholesterol was significantly ($p<0.05$) increased by the 10/μg/kg and Se-enriched JRS+*R. capsulatus* treatments, while fecal bile acids were significantly ($p<0.05$) increased by the 5 μg/kg, 10 μg/kg and Se-enriched JRS+*R. capsulatus* treatments.

The effect of different dietary levels of Se-enriched JRS on yolk fatty acid composition is shown in Table 5. Palmitic and stearic acids decreased while linoleic acid was significantly increased with the supplementation of the 10 μg/kg Se-enriched JRS and Se-enriched JRS+*R. capsulatus* treatments. Linolenic acid was increased by almost all the treatments ($p<0.05$).

Lymphoid organ weight after supplementation of Se-enriched JRS is shown in Table 6. Compared to the control, spleen and thymus weight were significantly increased by both the 5 μg/kg and 10 μg/kg Se-enriched JRS treatments and bursa by the 5 μg/kg and Se-enriched JRS+*R. capsulatus* treatments.

Table 3. Effect of Se-enriched JRS on serum and yolk cholesterol, triglycerides and serum HDL-C concentration (mg/100 ml serum)

Periods	Treatment				
	Control	2.5 μg Se	5.0 μg Se	10.0 μg Se	Se+PSB
Serum cholesterol					
0 wk	162.5±10.6	163.7±11.4	161.0±10.0	166.0±13.5	163.7±14.5
4 wk	159.2±13.2^{a}	158.0±8.5^{a}	151.2±12.0^{ab}	151.5±7.4^{ab}	145.5±12.2^{b}
8 wk	164.7±9.2^{a}	146.5±9.2^{ab}	140.5±7.0^{b}	141.2±8.3^{ab}	136.7±4.6^{b}
Serum triglycerides					
0 wk	1,067.5±31.0	1,043.0±37.9	1,075.5±38.6	1,045.5±39.5	1,065.0±23.5
4 wk	1,028.2±31.0	1,031.0±20.0	1,013.0±17.4	1,016.5±25.6	1,011.5±20.6
8 wk	1,051.7±24.3^{a}	928.0±31.9^{b}	937.5±33.1^{b}	934.0±34.3^{b}	922.5±30.3^{b}
Serum HDL-C					
0 wk	37.0±3.4	39.0±3.8	37.7±2.1	38.2±2.4	38.5±3.3
4 wk	37.6±2.5	42.0±3.5	42.6±3.3	47.2±3.1	48.5±3.61
8 wk	42.0±3.8^{a}	53.0±3.4^{b}	50.2±4.0^{b}	55.2±3.6^{b}	60.1±3.4^{b}
Yolk cholesterol					
0 wk	13.8±0.8	13.7±0.5	13.3±0.6	14.1±0.9	13.8±0.4
4 wk	13.5±4.5	12.5±4.5	12.6±7.3	12.5±7.6	12.0±5.3
8 wk	13.8±0.4^{a}	12.4±0.4^{ab}	12.3±0.5^{b}	12.0±0.4^{b}	11.9±0.4^{b}
Yolk triglycerides					
0 wk	212.0±6.6	210.7±6.2	213.5±6.3	213.2±5.6	213.3±5.5
4 wk	210.5±4.5	205.5±4.5	202.2±7.3	200.5±7.6	200.7±5.3
8 wk	210.2±6.6^{a}	193.7±8.2^{ab}	186.7±5.3^{b}	187.7±7.2^{b}	182.5±9.1^{b}

$^{a-b}$ Values with different superscripts differ significantly ($p<0.05$) in the same row.
Values are mean±SEM for 10 laying hens per group. HDL-C = High density lipoprotein-cholesterol.
PSB = *Rhodobacter capsulatus*; Se = Selenium-enriched Japanese radish sprouts.

Table 4. Effect of Se-enriched JRS on liver bile acids, fecal cholesterol and bile acids concentration

Parameter	Treatment				
	Control	2.5 μg Se	5.0 μg Se	10.0 μg Se	Se+PSB
Liver					
Cholesterol (mg/g)	4.0±0.20^{a}	3.8±0.29^{ab}	3.2±0.20^{b}	2.9±0.18^{b}	2.9±0.17^{b}
Triglycerides (mg/g)	15.5±1.06^{a}	13.0±1.06^{ab}	13.1±1.29^{ab}	12.3±1.1^{ab}	11.3±0.85^{b}
Bile acid (μmol/g)	61.2±3.1^{a}	67.2±4.2^{ab}	69.2±3.5^{ab}	73.9±4.1^{b}	73.0±3.3^{b}
Fecal					
Cholesterol (mmol/g)	0.56±0.04^{a}	0.68±0.06^{ab}	0.70±0.05^{ab}	0.71±0.04^{b}	0.72±0.05^{b}
Bile acids (μmol/g)	25.8±3.5^{a}	34.7±3.3^{ab}	38.2±3.2^{b}	46.4±4.5^{b}	45.9±3.4^{b}

$^{a-b}$ Values with different superscripts differ significantly ($p<0.05$) in the same row.
Values are mean±SEM for 10 laying hens per group.
PSB = *Rhodobacter capsulatus*; Se = Selenium-enriched Japanese radish sprouts.

Table 5. Effect of Se-enriched JRS on yolk fatty acids profile (mg/g yolk)

Treatment	Palmitic acid	Palmitoleic acid	Stearic acid	Linoleic acid	Linolenic acid
Control	7.66±1.08^{a}	0.89±0.26^{a}	3.63±0.44^{a}	10.28±2.20^{a}	2.99±0.52^{ab}
Se 2.5μg/kg	7.53±1.08^{a}	0.84±0.07^{a}	2.92±0.58^{b}	10.14±1.27^{a}	3.89±0.60^{ab}
Se 5.0μg/kg	7.80±1.85^{ab}	0.77±0.23^{ab}	3.05±0.70^{ab}	11.12±2.95^{ab}	4.26±1.08^{b}
Se 10.0μg/kg	5.51±1.17^{b}	0.90±0.21^{b}	2.41±0.60^{b}	13.87±2.01^{b}	4.97±0.71^{b}
Se +PSB	5.16±1.45^{b}	1.02±0.09^{b}	2.33±0.62^{b}	16.40±1.98^{b}	5.81±0.79^{b}

$^{a-c}$ Values with different superscripts differ significantly ($p<0.05$) in the same column.
Values are mean±SEM for 10 eggs per group.
PSB = *Rhodobacter capsulatus*: Se = Selenium-enriched Japanese radish sprouts.

Table 6. Effect of supplementing dietary Se-enriched JRS on the lymphoid organs weight (gm/kg BW) in laying hens

Parameter	Treatment				
	Control	2.5 μg Se	5.0 μg Se	10.0 μg Se	Se+PSB
Liver	29.3±2.0	32.1±1.7	32.6±2.0	32.8±2.5	33.2±2.1
Spleen	2.02±0.28^{a}	2.30±0.36^{ab}	3.67±0.66^{b}	3.42±0.44^{b}	3.40±0.38^{b}
Bursa	1.87±0.12^{a}	2.07±0.31^{ab}	2.87±0.39^{b}	2.57±0.25^{ab}	3.20±0.31^{b}
Thymus	4.15±0.53^{a}	4.60±0.41^{ab}	6.27±0.72^{b}	6.22±0.75^{b}	5.15±0.65^{ab}
Thyroid	0.20±0.02	0.24±0.02	0.25±0.01	0.20±0.01	0.20±0.02
Adrenal	0.18±0.02	0.22±0.03	0.16±0.02	0.18±0.01	0.22±0.01

^{a,b} Mean values within a row that have different letters are different at p≤0.05.
Values are mean±SEM for 10 laying hens per group.
PSB = *Rhodobacter capsulatus*; Se = Selenium-enriched Japanese radish sprouts.

Table 7. Effect of Se-enriched JRS on serum IgG, yolk IgY, SOD concentration (mg/ml) and FWI in laying hens

Parameter	Treatment				
	Control	2.5 μg Se	5.0 μg Se	10.0 μg Se	Se+PSB
Serum IgG	3.70±0.26^{a}	5.0±0.63^{ab}	6.10±0.51^{b}	5.50±0.42^{b}	6.90±0.24^{b}
Yolk IgY	1.35±0.18^{a}	1.80±0.30^{ab}	2.37±0.20^{b}	2.27±0.26^{b}	2.80±0.30^{b}
FWI (mm)	0.43±0.03^{a}	0.45±0.04^{ab}	0.68±0.07^{b}	0.60±0.04^{b}	0.76±0.01^{b}
SOD	91.52±0.69	91.39±1.39	90.21±1.35	90.86±2.09	87.15±3.74

^{a,b} Mean values within a row that have different letters are different at p≤0.05.
Values are mean±SEM for 10 laying hens/eggs per group.
PSB = *Rhodobacter capsulatus*; Se = Selenium-enriched Japanese radish sprouts; FWI = Foot Web Index.

The effect of dietary Se-enriched JRS on serum IgG and yolk IgY concentration and FWI and SOD activity is shown in Table 7. Compared to the control, all the supplementations except 2.5 μg/kg significantly increased serum IgG and FWI, and yolk IgY concentrations SOD activity was not influenced by any of the treatments.

The effect of different dietary levels of Se-enriched JRS on differential leukocyte counts is presented in Table 8. After 4-wk of supplementation, effects of these treatments were variable, highest amount of leucocytes was found in the Se-enriched JRS+*R. Capsulatus*, lymphocytes were significantly increased by the 2.5 μg/kg, 10 μg/kg and Se-enriched JRS+*R. capsulatus* treatments, heterophils were significantly decreased by the 2.5 μg/kg, 10 μg/kg and Se-enriched JRS+*R. capsulatus* treatments, basophils were significantly decreased only by the 10/μg/kg treatment, and

Table 8. Effect of Se-enriched JRS on total and differential leucocyte counts of laying hens

Treatment	Types of white blood cells					
	Leucocytes/μl	Lymphocytes (%)	Heterophils (%)	Eosinophils (%)	Basophils (%)	Monocytes (%)
After 4-wk						
Control	16,750.0±50^{a}	66.7±13.3^{a}	17.3±5.3^{c}	3.8±1.4^{ab}	5.8±2.6^{bc}	5.4±2.5^{a}
Se (2.5 μg/kg)	17,337.5±193^{ab}	74.7±1.9^{bc}	9.4±1.1^{a}	3.4±0.0^{ab}	5.8±0.1^{bc}	6.7±2.1^{ab}
Se (5.0 μg/kg)	21,087.5±123^{c}	68.6±5.2^{a}	17.4±3.9^{c}	3.2±1.8^{ab}	4.8±1.3^{b}	6.0±2.3^{ab}
Se (10 μg/kg)	18,750.0±187^{b}	76.3±0.9^{c}	12.8±2.3^{ab}	1.2±0.3^{a}	1.8±0.4^{a}	7.9±1.9^{b}
Se+PSB	21,887.5±187^{c}	70.3±1.6^{b}	14.6±2.5^{b}	2.4±1.0^{ab}	3.5±1.1^{ab}	9.2±2.3^{c}
After 8-wk						
Control	17,087.5±137^{a}	67.5±10.5^{a}	18.2±7.2^{c}	2.9±1.0^{ab}	7.2±1.4^{c}	4.2±2.2^{a}
Se (2.5 μg/kg)	22,875.0±80^{c}	70.0±5.0^{b}	9.5±2.4^{a}	4.8±1.2^{b}	9.5±2.4^{c}	6.2±2.4^{ab}
Se (5.0 μg/kg)	19,287.5±161^{b}	75.9±3.0^{c}	9.5±0.5^{a}	2.9±0.9^{ab}	4.8±0.9^{b}	6.9±2.5^{ab}
Se (10 μg/kg)	21,800.0±115^{c}	78.5±5.1^{c}	10.0±4.1^{ab}	3.1±0.3^{ab}	3.3±1.0^{ab}	5.1±2.1^{a}
Se+PSB	23,985.0±92^{c}	78.9±1.3^{c}	10.2±2.4^{ab}	1.4±0.0^{a}	2.3±6.0^{ab}	7.2±2.0^{b}

$^{a-c}$ Values with different superscripts differ significantly (p<0.05) in the same column.
Values are mean±SEM for 10 laying hens per group.
PSB = *Rhodobacter capsulatus*; Se = Selenium-enriched Japanese radish sprouts.

moncytes were significantly decreased at the 10 μg/kg treatment, and increased by the Se-enriched JRS+*R. capsulatus* treatment. After 8 wks the responses of differential counts to these treatments continued to be variable. Leucocytes and lymphocytes were significantly increased in all the treated birds, heterophils significantly decreased at the 2.5 μg/kg, 5 μg/kg, 10 μg/kg and Se-enriched JRS+*R. capsulatus* treatments, there were no significant effects of these treatments on eosinophils and basophils, and monocytes were significantly increased at the 5 μg/kg and Se-enriched JRS+*R. capsulatus* treatments, and were significantly decreased by the 10 μg/kg treatment.

The effect of dietary Se-enriched JRS on serum, yolk and meat Se concentration is shown in Table 9. Se levels in the serum were significantly ($p<0.05$) in creased by the 10 μg/kg and Se-enriched JRS+*R. capsulatus* treatments. Se levels in the yolk were variable with a significant decrease seen at the 2.5 μg/kg treatment, and a significant increase seen at the Se-enriched JRS+*R. capsulatus* treatment. Se levels in meat were also variable with a significant increase seen at the 2.5 μg/kg and 5 μg/kg treatments while a significant decrease was seen at the Se-enriched JRS+*R. capsulatus* treatment.

DISCUSSION

A number of nutritional factors like Se are known to influence the ability of an animal to invoke optimum immune responses but few reports are available on the combined effect of Se and probiotics (*Lactobacilli*). Probiotics could protect broilers against pathogens (Pascual et al., 1999) and stimulate the systemic immune responses (Quere and Girard, 1999). This study reports on the use of Se-enriched JRS with or without *R. capsulatus* on the immune response as well as cholesterol metabolism in adult laying hens which were immunized against the NDV. We observed that both the serum IgG and yolk IgY were enhanced by the supplementation of 5 μg/kg Se-enriched JRS in the diet. A similar trend of IgG was observed when Se was supplemented in the broiler diet (Khan et al., 2008). Thus, our study is in agreement with the broiler results of Khan et al. (2008). Further, Se-deficient mice produced lower concentrations of IgG and IgM (Ongele et al., 2002). In chickens, as in mammals, it has been well established that IgY is the antibody isotype that is transferred from the dam to her offspring (Brambell, 1970). The current study reaffirmed that concept. We discovered that a great amount of IgY was transferred to yolk when chickens were treated with Se-enriched JRS and *R. capsulatus*. Se enriched Lactobacillus enhanced serum IgG and IgA in broiler chickens (Huang et al., 2004). Similarly, *Lactobacillus acidophilus* resulted in a significant enhancement of systemic antibody response, mostly of the IgM isotype, in sheep red blood cells (Haghighi et al., 2005). In accordance with previous individually reported affects of Se or *R. capsulatus*, the present study reveals that the combination of Se-enriched JRS and *R. capsulatus* can enhance the immune response in adult laying hens. However, administration of Se-enriched JRS plus *R. capsulatus* did not significantly enhance serum IgG. This phenomenon has yet to be studied further.

The results of the present study reveal that supplementation of Se-enriched JRS increased the weight of lymphoid organs such as the spleen, bursa, thymus and thyroid. The beneficial effect of Se on the weight gain of lymphoid organs was reported by Kukreja and Khan (1997). In general, erythrocytes and lymphoid organs originate from the common pluripotent stem cells. When Se was supplemented in the diet, weights of the bursa and spleen increased in broiler chickens (Singh et al., 2006). The spleen of chicken is a mixed lymphatic tissue, having both the T- and B-cell rejoins, and these data suggest that these rejoins were enhanced by Se-enriched JRS. The thymus is the important lymphoid organ involved in the development and differentiation of T lympocytes (Eerola et al., 1987). The increase in bursal weight after supplementing the diet with 5 μg/kg Se-enriched JRS, along with the increased production of circulatory immunoglobulins and immune complexes, suggests that there may be a greater proliferation of bursal B cells, possibly due to decreased

Table 9. Effect of Se-enriched JRS on serum, yolk and meat selenium concentrations

Treatment	Selenium content		
	Serum (mg/L)	Yolk (mg/kg)	Meat (mg/kg)
Control	0.10±0.0034[a]	0.50±0.0085[b]	0.23±0.0063[b]
Se (2.5 μg/kg)	0.11±0.0045[ab]	0.45±0.0059[a]	0.26±0.0186[c]
Se (5.0 μg/kg)	0.13±0.0026[ab]	0.50±0.0182[b]	0.26±0.0082[c]
Se (10 μg/kg)	0.15±0.0050[b]	0.55±0.0089[bc]	0.23±0.0174[b]
Se+PSB	0.16±0.0049[b]	0.57±0.0067[c]	0.20±0.0062[a]

[a,c] Mean values within a column that have different letters are different at $p\leq0.05$.

Values are mean±SEM for 10 laying hens per group.

PSB: *Rhodobacter capsulatus*; Se: Selenium-enriched Japanese radish sprouts.

oxidative stress, with enhanced production of immunoglobulins and improved antibody responses. One possibility is that Se-enriched JRS is associated with membrane fluidity of lymphoid cells, which in turn has an effect on the immune response mechanism. Thus, the modulation of immune response by *R. capsulatus* might be at the immunoglobulin and cellular level rather than at the lymphoid organ level. This warrants further investigation.

Glutathione peroxidase (GPx) was the first mammalian protein shown to incorporate Se in the form of selenocysteine into its catalytic site and was assumed to be associated with the antioxidant activity and immunity of Se (Koller et al., 1979). GPx obtain electrons from NADPH to catalyze the reduction of hydrogen peroxide and organic hydroperoxides, thus protecting cells from oxidative damage. TrxR and GPx are well characterized as major components of the antioxidant defense, with roles in many cellular processes. It was reported that Se-enriched JRS influenced GPx and glutathione S-transferase (GST) activities in the livers, kidneys and lungs of rats in an organ-specific manner (Hama et al., 2008). A similar trend of GPx and GST activities which are directly associated with the immune system might be functional in the present study. Se-supplementation acts not only to elevate antiviral immunity, but to prevent genetic adaptations in the viral genomic RNA that lead to increased virulence and cardiac pathology (Hoffmann, 2007).

In this study, we demonstrated that supplementation of Se-enriched JRS (5.0 μg/kg) or a combination of Se-enriched JRS and *R. capsulatus* modulates leucocyte population in laying hens. In several species of animals, Se is known to influence the immune response via the activation of phagocytosis by neutrophils, increased antibody production, and enhanced lymphocyte proliferation (Spears, 2000; Panousis et al., 2001). It was suggested that Se protects the leucocytes from self-destruction during the phagocytic activity. Therefore, the leucocytes are retained at the site of infection, protecting the host against the pathogenic effects of the parasite. A remarkable change in lymphocyte and monocyte concentrations was observed with the Se-enriched JRS supplementation. Se-deficient lymphocytes are less able to proliferate in response to mitogen, as well as in macrophages and leukotriene B4 synthesis, which is essential for neutrophil chemotaxis. The combination of Se-enriched JRS and *R. capsulatus* in the present study showed a tendency to increase leucocyte population. The main reason for this phenomenon is not clear, but it is conceivable that several parameters are involved in determining the efficiency of *R. capsulatus* in the stimulation of the immune response, and the immune-enhancing effects of *R. capsulatus* may not be generalized. In contrast to this, *Lactobacillus acidophilus* supplementation boosted lymphocyte population in chickens (Yurong et al., 2005). In a different study, egg layer and broiler chickens treated with probiotics responded differently to TNP, with layer chickens mounting a significantly higher antibody response than broiler chickens, indicating that the genetic background of chickens plays an important role in the mediation of immunomodulatory activities of probiotics (Koenen et al., 2004).

Hypercholesterolemia and cardiovascular disorders have been shown to be associated with Se deficiency (Kang et al., 2000). Se has a crucial role in controlling the effects of thyroid hormone on fat metabolism. Inversely, a low serum Se level is associated with a decrease in liver microsomal activity and serum HDL-C concentration (Masukawa et al., 1983). In this study, the combination of Se-enriched JRS and *R. capsulatus* caused a significant reduction in serum and yolk cholesterol and triglycerides. In connection to this, an earlier study reported that atherosclerosis was reduced by 49% with a subsequent serum cholesterol reduction when Se was incorporated in rabbit diet (Schwenke and Behr, 1998). Thus, the result of our study is in agreement with that of Schwenke and Behr (1998). A significant decrease in liver triglycerides and LDL-cholesterol by the supplementation of Se with vitamin C was reported (Asha and Indira, 2004), whereas in our study, hypocholesterolemic activity of Se-enriched JRS and *R. capsulatus* might be due to a sparing effect of Se-enriched JRS for *R. capsulatus*. Though the optimum level of cholesterol was reduced by the combination of Se-enriched JRS and *R. capsulatus*, fecal bile acid excretion was best observed with 10 μg/kg Se-enriched JRS. This untoward data suggests that the synergistic influence of Se-enriched JRS and *R. capsulatus* on reducing cholesterol is regulated by both bile acid excretion and suppressed cholesterol synthesis. Nassier et al. (1997) reported that hypercholesterolemia associated with Se deficiency was related to the increased 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in liver microsomes. Further work will be needed to elucidate the complete mechanism(s) by which Se-enriched JRS synergistically with *R. capsulatus* render hypocholesterolemic activities.

In this study, combined treatment of Se-enriched JRS and *R. capsulatus* caused a marked increase in egg production and feed efficiency in laying hens. The addition of Se-enriched JRS with *R. capsulatus* to the hen's diet proved to exhibit a positive effect on yolk color. A higher level of carotenoids (4.2%) (Kobayashi and Kurata, 1978) in *R. capsulatus* improved yolk color in laying hens (Salma et al., 2007a). It might be speculated that carotenoids from *R. capsulatus* can be well absorbed and transferred into yolk, markedly increasing yolk color. Layer fed Se-enriched JRS and *R. capsulatus* had a tendency to increase egg quality parameters, which indicates that Se-enriched JRS may not

have toxic effects, at least not at the inclusion level fed in this study. In this study, all the fatty acids measured in egg yolk were altered by the dietary supplementations. Supplementation of Se-enriched JRS with *R. capsulatus* caused a significant decrease in palmitic acid and an increase in linoleic acid. A similar trend of fatty acid alteration with *R. capsulatus* was observed in our previous study (Salma et al., 2007c). There are numerous literature reviews and research articles documenting the importance of eggs as the vehicle for supplying omega-3 unsaturated fatty acids in the human diet. It was indicated that modulating maternal dietary n-6 and n-3 fatty acids may alter leukotriene production in chicks, which could lead to less inflammatory-related disorders and increase immunity in poultry (Hall et al., 2007). Thus, our aim to produce Se and unsaturated fatty acid-enriched eggs was achieved in this study.

Regarding the mechanism, elemental Se absorbed from the Se-enriched JRS was incorporated into the body's proteins. It is reduced to selenide before being transported in the blood, bound to a- and c-globulins, to various organs and target tissues. In this study the Se concentration was 10-fold higher than that used in the previous study (Yamanoshita et al., 2007), which led to more bioavailable in egg yolk and meat. Meat and yolk Se might then incorporate into specific selenoproteins as selenocysteine, and non-specifically as selenomethionine. Se-enriched probiotics were the better choice for raising the concentration of blood Se over sodium selenite (Pan et al., 2007). In our study, a high amount of Se in serum and egg yolk was deposited by the Se-enriched JRS plus *R. capsulatus*. Organic Se actively absorbs and can be directly incorporated into body protein (Combs and Combs, 1986) which is then transferred to chicken eggs (Payne et al., 2005), which is similar to our study. The relationship between Se status and immunity is turning out to be more complex than the simplified notion of Se being in general an immunity "booster," which highlights the critical need for determining the mechanisms by which Se-enriched JRS and *R. capsulatus* synergistically affect the immune system. Ultimately, mechanistic studies will need to involve specific selenoproteins and the roles they play during inflammation and immune responses.

Overall, our cholesterol and immunity data lead to the conclusion that combined dietary supplementation of Se-enriched JRS and *R. capsulatus* are beneficial to the health, disease protection and overall production performance of laying hens. These findings support the notion that crosstalk between immunity and lipid metabolism may influence host defense as well as cardiovascular disease. An understanding of the role of Se-enriched JRS and *R. capsulatus* in the modulation of immunological responses in laying hens will contribute to the development of treatment strategies for various maladies of man and animals.

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