INTRODUCTION

Under intensive aquaculture conditions, fish are predisposed to stress and subsequent infection by pathogens. Aimed at overcoming diseases, the industry is increasingly turning towards the use of prophylactics as well as therapeutic drugs. However, the use of antibiotics in aquaculture leads to the development of drug-resistant strains of pathogens.1 In mammals and birds, immune responses have been shown to increase with the addition of certain nutrients in the diet.2,3 Disease resistance in fish too has been found to increase with the addition of certain nutrients in the diet.2,3 Disease resistance in fish too has been found to increase with the addition of certain nutrients in the diet.4,5 Immunoenhancement by dietary manipulation may therefore offer a viable alternative to the use of drugs in aquaculture. Carotenoids are natural fat-soluble pigments which are widespread and structurally quite diverse. Some of these substances like β-carotene, are important sources of vitamin A for animals.6 Because of its role in intermediary metabolism, carotenoids are even considered as essential nutrients by some7,8 for aquatic animals. In recent years the immunomodulating actions of vitamin A have been investigated in fish.9,10 While evidence is overwhelming that vitamin deficiencies cause immunosuppression, supplementation above requirements for normal growth rarely results in immunoenhancement. This may also be true for carotenoids. Thompson et al., in a study with vitamin A and its interaction with astaxanthin, suggested that these substances have a limited potential as immunostimulants in practical fish diets.11 However, the level used in the trial may have been low to demonstrate a pronounced effect. Furthermore, there could be differences in the immunoenhancing ability of various carotenoids. For example, β-carotene has been found to increase spleen lymphocyte proliferation in parrotfishes.12 The present study therefore investigated the influence of a wide range of levels of β-carotene in semipurified diets on growth and immune response in rainbow trout.

SUMMARY: We attempt to assess the impact of different levels of dietary β-carotene on immune function in rainbow trout. Semi-purified diets containing 0, 40, 200, and 400 mg β-carotene/kg dry diet were fed for 12 weeks to fish with average weight of 45 g. In addition to the humoral and cellular immune parameters, growth and feed utilization were examined. There were no marked differences in growth and feed utilization showing that β-carotene was not particularly efficient in enhancing growth of rainbow trout. Of the immune parameters measured, total immunoglobulin was significantly highest for the 200 mg β-carotene fed group. Serum complement activity (alternate pathway) at 200 and 400 mg β-carotene supplementation was significantly higher than that of the unsupplemented group. An increasing trend in lysozyme activity was observed, however, the differences among the groups were not significant. Phagocytic activity was similar among diet groups except at the highest level of supplementation where it was the maximum. Oxygen radical production by peripheral blood leukocytes appeared to be lower at higher levels of carotenoid supplementation. Overall, dietary β-carotene clearly enhanced immune response parameters in rainbow trout such as serum complement activity and total plasma immunoglobulin but did not show a definite influence for the other factors examined in the present study.

KEY WORDS: β-carotene, diets, immune response, rainbow trout.

**Original Article**

**Effects of dietary β-carotene on the immune response of rainbow trout *Oncorhynchus mykiss***

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**SUMMARY:** We attempt to assess the impact of different levels of dietary β-carotene on immune function in rainbow trout. Semi-purified diets containing 0, 40, 200, and 400 mg β-carotene/kg dry diet were fed for 12 weeks to fish with average weight of 45 g. In addition to the humoral and cellular immune parameters, growth and feed utilization were examined. There were no marked differences in growth and feed utilization showing that β-carotene was not particularly efficient in enhancing growth of rainbow trout. Of the immune parameters measured, total immunoglobulin was significantly highest for the 200 mg β-carotene fed group. Serum complement activity (alternate pathway) at 200 and 400 mg β-carotene supplementation was significantly higher than that of the unsupplemented group. An increasing trend in lysozyme activity was observed, however, the differences among the groups were not significant. Phagocytic activity was similar among diet groups except at the highest level of supplementation where it was the maximum. Oxygen radical production by peripheral blood leukocytes appeared to be lower at higher levels of carotenoid supplementation. Overall, dietary β-carotene clearly enhanced immune response parameters in rainbow trout such as serum complement activity and total plasma immunoglobulin but did not show a definite influence for the other factors examined in the present study.

**KEY WORDS:** β-carotene, diets, immune response, rainbow trout.
MATERIALS AND METHODS

Diet formulation, feeding and analysis

Existing information on the nutritional requirement of *Oncorhynchus mykiss* was used for formulation of the diets.13,14 Casein-based semipurified diets were prepared to contain 0 (DC, control), 40 (AC), 200 (EC), and 400 (HC) mg β-carotene/kg dry diet (Table 1). Among the β-carotene supplemented diets, HC did not contain vitamins A, C, and E included in the mix. The diet was intended to clarify whether the high level of β-carotene could actually compensate for some of the anti-oxidant functions impaired by the removal of the aforementioned vitamins. The basal components constituted about 93% of the diet (Table 2).

Twenty-four fish were reared in duplicates, in well-aerated rectangular tanks supplied with partly recirculating water. Water temperature was maintained at 16 ± 2°C with a flow rate of 400 mL/min. Rainbow trout with an average weight of 45 g were fed the diets to apparent satiation twice a day, 6 days/week, and proper husbandry was followed. Feed consumption was recorded weekly. After 12 weeks of feeding, the fish were sampled to assess growth response and determine the feed utilization. The diets were analyzed for proximate composition as described by Watanabe.16

Total carotenoids from the diet were determined spectrophotometrically using absorbance values and extinction coefficients as described by Schierle et al.17 with slight modifications. Five grams of finely ground feed were mixed with 20 mL distilled water and 5 mg trypsin (Sigma-Aldrich Japan K.K., Tokyo, Japan) and the mixture was digested in an ultrasonic bath at 50°C for 30 min. The pigment was then extracted with 20 mL dichloromethane (Kokusan Chemical Works, Ltd, Tokyo, Japan) by shaking over a period of 10 min and centrifuged at 1000 g for 7 min before drawing a 2.5 mL aliquot from the lower phase which was dried under nitrogen and dissolved in an equal volume of hexane (Wako Pure Chemical Industries, Ltd, Tokyo, Japan). The absorbance was measured by a Beckman DU 640 spectrophotometer (Beckman Instruments Inc., CA, USA) at \( \lambda_{\text{max}} \) and the carotenoid content was calculated according to the formula given below, incorporating adjustments for dilution and sample weight,

\[
C = \frac{(A \times 10,000)}{A_{\lambda_{\text{max}}^{1\%}}},
\]

where \( C \) is the concentration in mg/kg, \( A \) is the absorbance at \( \lambda_{\text{max}} \), \( A_{\lambda_{\text{max}}^{1\%}} \) is the extinction coefficient of β-carotene in hexane.

Preparation of plasma/serum samples and head kidney cells

The fish were anesthetized with 300 p.p.m. 2-phenoxyethanol (Wako) and using heparinized or non-heparinized syringes, blood from individual fish was collected from the caudal vein. The plasma and serum samples were separated later and stored at –80°C until analysis.

Using aseptic techniques, the anterior kidney was removed and the cells were pushed through stainless steel mesh into L-15 medium (Leibovitz; Sigma) containing 2% fetal calf serum (FCS; Gibco BRL, NY, USA), and 1% penicillin/streptomycin (P/S; Sigma). The cells were washed twice in L-15 medium before being layered onto 34% and 51% Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gradient. The cells were centrifuged at 400 g for 20 min to enrich for macrophages. Cells at the 34–51% interface were collected, washed, pelletized, and resuspended in the medium. The process was repeated at least twice to obtain high purity cell suspensions which were finally adjusted to the required cell number for the assay that followed immediately.

| Table 1 | Composition of the experimental diets |
|---|---|---|---|---|
| | DC | AC | EC | HC |
| Carotenoid level (mg/kg) | | | | |
| Ingredients (g/kg) | | | | |
| Basal components | 929.5 | 929.5 | 929.5 | 929.5 |
| Vitamin mix | 10.0 | 10.0 | 10.0 | 10.0* |
| Rovimix (β-carotene 10%)† | – | 0.4 | 2.0 | 4.0 |
| Cellulose | 60.5 | 60.1 | 58.5 | 56.5 |
| Analytical values | | | | |
| β-carotene (mg/kg) | 1.3 | 36.6 | 182.4 | 321.0 |

* Vitamins A, C, and E deleted.
† Roche Vitamin Japan K.K., Tokyo, Japan.

| Table 2 | Basal components of the diet (g/kg) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Vitamin-free casein | Gelatin | Dextrin | Starch | Oil mix* | Mineral mix† | Cystine | Arginine | Choline chloride |
| | 450.0 | 40.0 | 165.0 | 92.0 | 120.5 | 50.0 | 2.0 | 6.0 | 4.0 |

* Two parts pollack liver oil and three parts soybean oil.
† As described in Ogino et al. 1979.15
Serum β-carotene

Total carotenoid concentration in the serum was determined following the method of Schuep et al. Briefly, a 250 µL aliquot of the thawed serum was diluted with an equal amount of high pure water and the protein was precipitated with 500 µL ethanol. The pigment was extracted with 1 mL hexane. The sample was subjected to mechanical shaking for 10 min, and centrifuged before collecting a 400 µL aliquot from the supernatant which was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved completely in 250 µL hexane and a 20 µL sample was employed for the separation and determination by high performance liquid chromatography (HPLC; LC 10 AD, Shimadzu, Kyoto, Japan). A mixture of acetone (Wako) and hexane (2 : 8) served as the mobile phase at a flow rate of 1 mL/min at a column temperature of 35°C using a 4.6×150 mm LiChrosorb SI-60 (GL Sciences Inc., USA) column. The peaks were detected by a photodiode array (SPD-M10A; Shimadzu) at wavelengths of 450 and 470 nm for yellow and red carotenoids, respectively, employing a software package Microsoft Class LC 10 version 1.41E for yellow and red carotenoids, respectively, employing a software package Microsoft Class LC 10 version 1.41E

ACH50 (units/mL) = \frac{1}{K} \times \frac{r}{2},

where $K$ is the amount of serum giving 50% hemolysis, $R$ is the reciprocal of the serum dilution, and 1/2 is the correction factor. The assay was performed on a 1/2 scale of the original method.

Serum lysozyme activity

A turbidimetric assay was used to determine the lysozyme activity. In brief, dilutions of the hen egg white lysozyme (HEWL; Sigma) standard prepared in 0.1 M phosphate citrate buffer (pH 5.8) and of the test plasma (25 µL) were placed into the wells of a microtiter plate in triplicate. One hundred and seventy-five microliters of a 0.075% (w/v) Micrococcus lysodeikticus (Sigma) suspension prepared in the same buffer was then added to each well. After rapid mixing, the change in turbidity was measured at 30 s intervals for a 5 min duration at 450 nm employing a Multiskan microplate reader (Labsystems Oy, Helsinki, Finland). The equivalent units of activity of test sera as compared to the HEWL were worked out with the help of a computer application (Deltasoft3–139F; Biometallics Inc., NJ, USA).

Plasma total immunoglobulin

Plasma total immunoglobulin (Ig) was assayed following the method of Siwicki and Anderson. This assay employs biuret method for protein determination (Sigma Chemicals, Diagnostic method 690) in plasma prior to and after precipitating down the immunoglobulin molecules employing a 12% solution of Polyethylene glycol (PEG; Sigma). The difference in the protein content is considered as the plasma immunoglobulin content. Although the method does not give an accurate estimate of the immunoglobulin content (the precipitate contains around 10% non-immunoglobulin components (Kiron V and Fukuda H, unpubl. data, 1999), it has been adopted here as only comparative values are aimed at.

Production of reactive oxygen species

The oxygen radical production by peripheral blood leukocytes was also determined following the method of Siwicki and Anderson with minor changes. One hundred microliters of freshly collected heparinized blood sample was placed into duplicate microcapillary tubes (Hematlon L; Minato Medical Corp., Tokyo, Japan) and was centrifuged at 1000 g for 5 min at 4°C.
The buffy coat was washed along with the plasma into a microtube using 50 μL of RPMI-1640 (Sigma). The cell suspension was mixed well and 15 μL were transferred into each of the tubes containing 15 μL of RPMI, and 0.2% nitroblue tetrazolium (NBT; Sigma) in RPMI. The tubes were incubated for 60 min at 18°C, after which, 400 μL N,N,N′-dimethyl formamide (Wako) was added, mixed properly and centrifuged at 1500 g for 5 min at 4°C. From the supernatant, 250 μL was transferred into a glass microplate (Hellma, Germany) and the absorbance was read at 540 nm in a microplate reader. Wells containing only cell suspension served as the blank. Oxygen radical production was directly proportional to the amount of NBT reduced.

**Phagocytic activity**

A spectrophotometric method described by Seeley et al.\(^\text{22}\) was used for the measurement of phagocytosis with minor alterations. This method has the advantage of speed and eliminates the need for time-consuming and highly subjective microscopic quantification.\(^\text{22}\) The head kidney cell suspension prepared as described earlier was adjusted to 2 × 10⁶ cells/mL with L-15 medium supplemented with 0.1% FCS and 1% P/S. Yeast cells (Saccharomyces cerevisiae; Sigma) stained with Congo red (Sigma) and inactivated were suspended in HBSS (Sigma) to contain 4 × 10⁷ cells/mL. Two milliliters of this suspension was mixed with 1 mL of the macrophage-rich suspension to give a yeast cell:macrophage ratio of 40:1. The mixture was incubated at room temperature for 60 min with gentle mixing at frequent intervals to prevent cells from getting sedimented at the bottom of the tube. At the end of incubation, 1.5 mL of 51% Percoll was carefully delivered into the bottom of the tube confirming in the process that there were no settled cells. The samples were centrifuged at 850 g for 3 min to separate the cells from free yeast cells. The cells located at the Percoll–media interface were harvested, washed in L-15 medium and spun down. The cell pellet was resuspended in 1.2 mL trypsin-EDTA solution (1.5 g/L trypsin and 0.4 g/L EDTA (Dojindo, Kumamoto, Japan) in phosphate buffered saline (PBS; Gibco) to solubilize the macrophages. After incubating overnight at 37°C the absorbance was read with a Beckman spectrophotometer at 510 nm against a trypsin-EDTA blank. The absorbance was proportional to the number of stained yeast cells that had been phagocytized.

**Statistical analysis**

Results were compared by analysis of variance. Differences between treatment means were compared by Student’s \(t\)-test at a significance level of \(P = 0.05\).

**RESULTS AND DISCUSSION**

**Growth and feed utilization**

No marked differences in growth (SGR, 0.21–0.23%/day) and feed utilization (F/G ratio, 0.84–0.90) were detected (Table 3). Growth did not differ among diet groups suggesting that \(b\)-carotene was not particularly efficient in enhancing growth of rainbow trout. In another study, astaxanthin supplementation also did not markedly improve growth and feed conversion in rainbow trout.\(^\text{11}\) Similarly, in rats, supplementation with canthaxanthin did not affect growth rate even after 20 weeks of feeding.\(^\text{23}\) In contrast, some studies have reported growth enhancement in rainbow trout\(^\text{24}\) and Atlantic salmon.\(^\text{25}\) Feed intake in the present study was not influenced by carotenoid levels and thus feed utilization did not vary. In salmonids, carotenoids are more important as pigmenters rather than as promoters of growth. Well-pigmented fish are considered to be of good quality. At dietary concentrations ranging from 0 to 190 mg/kg, coloration varied but growth was fairly equal among the groups.\(^\text{26}\) In yellowtail as well as in penaeids, carotenoids serve to improve the quality of eggs and nauplii.\(^\text{27,28}\)

<table>
<thead>
<tr>
<th>Diet code</th>
<th>DC</th>
<th>AC</th>
<th>EC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta)-carotene (mg/kg)</td>
<td>0</td>
<td>40</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>Initial weight (g)</td>
<td>45.4±0.6</td>
<td>46.3±0.3</td>
<td>46.4±1.0</td>
<td>43.4±1.1</td>
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<tr>
<td>Final weight (g)</td>
<td>117.3±6.6</td>
<td>118.5±0.7</td>
<td>120.4±2.6</td>
<td>112.5±12.1</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>71.9±7.1</td>
<td>72.1±0.4</td>
<td>74.0±3.7</td>
<td>69.1±11.1</td>
</tr>
<tr>
<td>TFC/fish (g)</td>
<td>60.50±0.67</td>
<td>61.09±0.39</td>
<td>61.72±0.52</td>
<td>58.53±2.63</td>
</tr>
<tr>
<td>F/G ratio</td>
<td>0.88±0.02</td>
<td>0.90±0.05</td>
<td>0.84±0.05</td>
<td>0.90±0.03</td>
</tr>
<tr>
<td>SGR</td>
<td>0.22±0.07</td>
<td>0.21±0.01</td>
<td>0.22±0.05</td>
<td>0.23±0.08</td>
</tr>
</tbody>
</table>

*Not significantly different (\(P > 0.05\)). Data are means of two tanks ± SD.*
Serum carotenoid

Serum β-carotene content reflected the dietary levels except for the diet deficient in vitamins A, C, and E (Fig. 1). The low value obtained with this diet may indicate that in the absence of these vitamins, β-carotene in the serum is rapidly mobilized into the tissues to make up for the lost anti-oxidant function. This mechanism needs to be verified. In rats, the conversion of β-carotene to retinol is so effective that no carotenoid can be detected in the serum at any time point during the 72 h experimental period. In another study with rats, plasma vitamin A levels were slightly higher when the diet contained β-carotene than when it did not, while the liver vitamin A level was fivefold higher. On the other hand, plasma vitamin E level was reduced to half in the presence of β-carotene and canthaxanthin. The decrease in plasma and/or hepatic concentrations of vitamins A or E may indicate that dietary β-carotene competed with dietary vitamins A and E for binding sites on the lipoproteins resulting in decreased absorption and transport of these vitamins into the blood and tissues. Since the tissue vitamin levels were not measured in the present study we are unable to discuss this further.

Serum complement activity

The serum complement activity (ACH50) increased with increasing levels of β-carotene. In the unsupplemented (control) diet the activity was low (240 units/mL). At 40 mg/kg the enhancement was not significant (295.1 units/mL), but at 200 and 400 mg/kg it was significantly higher than the control (307 and 314.5 units/mL, respectively; Fig. 2). However, values among the supplemented groups were not significantly different suggesting that the optimum level is above 40 but not as high as 400 mg/kg. There are no previous reports on enhancement of serum complement activity by β-carotene in fish. Neither vitamin A nor astaxanthin had any effect on complement activity. Even in homeotherms including humans, data available so far do not show a conclusive relation between dietary β-carotene levels and complement activity. However, alternative serum complement activity has been shown to be more important in fish than in mammals, the levels in fish being several times higher in carp, 58.9; rainbow trout, 345; tilapia, 574; yellowtail, 1020 units/mL compared to pig, 13.6; rat, 14.5; sheep, 15.4; human; 18.4 units/mL.

Serum lysozyme activity

Serum lysozyme activity also increased with graded levels of dietary β-carotene. However, no significant difference was found among different groups, the values ranging from 14.2 mg/mL for group HC to 16.7 mg/mL for group EC (Fig. 3) confirming earlier observations with astaxanthin and vitamin A. The lower value in HC can be attributed to the deletion of vitamins A, C, and E. In other studies with rainbow trout, serum lysozyme levels were found to be influenced by factors such as stress or dietary protein levels.

Plasma total immunoglobulin

Among the immune parameters assessed, plasma total immunoglobulin levels were found to increase with...
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can protect the cell membrane and receptors required for antigen recognition from damage by peroxidation of membrane lipids. It has also been found that β-carotene incorporated into liposome models prevent them from being oxidized under physiologic oxygen partial pressures. The apparent decrease in absorbance values at the higher β-carotene levels, indicating lesser oxidative radical production, may be explained by the fact that as an antioxidant which is rated to have a Trolox-equivalent antioxidant capacity (TEAC) similar to vitamin E and lycopene, high levels of β-carotene in the present study quenched excess reactive oxygen species (ROS) in addition to the activity of endogenous enzymes such as superoxide dismutase and glutathione.

Production of reactive oxygen species

Oxygen radical production of the leukocytes as measured by the reduction of the redox dye NBT appeared to be lower at higher levels of carotenoid supplementation (0.29 for HC as against 0.38 for DC; A540). However, the differences among dietary groups were not significant (Fig. 5). Supplemental β-carotene did not affect superoxide generation by neutrophils in humans. In rats, Zhao et al. found a suppressive effect of β-carotene, lutein, and bixin on the respiratory burst of peritoneal macrophages. By quenching free radicals, β-carotene can protect the cell membrane and receptors required for antigen recognition from damage by peroxidation of membrane lipids. It has also been found that β-carotene incorporated into liposome models prevent them from being oxidized under physiologic oxygen partial pressures. The apparent decrease in absorbance values at the higher β-carotene levels, indicating lesser oxidative radical production, may be explained by the fact that as an antioxidant which is rated to have a Trolox-equivalent antioxidant capacity (TEAC) similar to vitamin E and lycopene, high levels of β-carotene in the present study quenched excess reactive oxygen species (ROS) in addition to the activity of endogenous enzymes such as superoxide dismutase and glutathione.

Increasing dietary levels and was highest at 200 mg/kg (10.62 mg/mL). Once again, the absence of vitamins A, C, and E seemed to have a negative influence irrespective of the high level of β-carotene supplemented. The Ig level was lowest in the group that was not provided the supplement (4.23 mg/mL; Fig. 4). In studies with rats, Jyonouchi et al. reported that lutein, astaxanthin, and β-carotene can enhance Ig production against TD antigens in vivo, while Bendich and Shapiro found that β-carotene and canthaxanthin enhanced B-cell and T-cell proliferation in vitro. This may imply that in actual cases of pathogenic invasion, in carotenoid supplemented fish, the T-helper cells may be better activated to stimulate B cells in the antigen–antibody responses. Feeding of β-carotene-supplemented rotifers enhanced mitogen-induced spleen lymphocyte proliferation of Japanese parrotfish larvae and spotted parrotfish larvae. However, in the salmonids, there was negligible or no effect of vitamin A or astaxanthin on antibody induction and secretion.

Fig. 3 The serum lysozyme activity in rainbow trout fed β-carotene supplemented diets. The activity is expressed as equivalent units of hen egg white lysozyme (HEWL) which was used as the standard. Bars represent means ± SD (n = 10 fish). Means are not significantly different (P > 0.05).

Fig. 4 The plasma total immunoglobulin (Ig) in rainbow trout fed β-carotene supplemented diets. Bars represent means ± SD (n = 9 fish). Means with the same letters are not significantly different (P > 0.05).

Fig. 5 The reduction of nitroblue tetrazolium (NBT) by peripheral blood leukocytes from rainbow trout fed β-carotene supplemented diets. Oxygen radical production is directly proportional to the amount of NBT reduced. Bars represent means ± SD (n = 5 fish). Means are not significantly different (P > 0.05).
peroxidase. The suppression of the respiratory burst by carotenoids in vivo may be a way of protecting host cells and tissues from excess free radicals, thereby enhancing the generation of specific immune responses.

**Phagocytosis**

The phagocytic activity of isolated head kidney cells was similar at the different carotenoid supplementation levels except at 400 mg/kg (0.29, A510; Fig. 6). β-Carotene may increase the activity of macrophages through the production of cytokines by B cell or T cell after stimulation with a mitogen. The increased proliferation of lymphocytes in response to a mitogen such as concanavalin A can result in increased production of macrophage-activating factor (MAF) that can in turn alter the activation state of macrophages. In addition, β-carotene was found to stimulate phagosome–lysosome fusion and enhanced F-actin content in rat peritoneal macrophages, which are associated with changes in the cytoskeleton and fluidity of the lysosomal membrane. At the highest level of β-carotene employed in the present study, more macrophage receptors as well as the plasma membrane were protected from oxidative damage due to the enhanced quenching of excess free radicals, thereby probably increasing the phagocytic ability.

In conclusion, β-carotene levels up to 200 mg/kg diet did not clearly influence the cellular non-specific responses like production of ROS and phagocytosis, but indicated enhancement for immune components like serum complement and total plasma immunoglobulin in the experimental rainbow trout. Moreover, the supplementation of β-carotene at 400 mg/kg diet could not compensate for the removal of vitamins A, C, and E since immune responses were better at lower carotenoid concentrations with the vitamins than at the higher concentration without the vitamins.

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**REFERENCES**