Dietary green tea powder affects the immunologic parameters of broiler chicks

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ABSTRACT
Green tea powder diet supplementation effect on some immunological parameters in broiler chickens has been evaluated. To assess both the effect and the beneficial dose, the diet of 271 day-old Ross 308 male broiler chickens has been supplemented with 0, 0.25, 0.50, 0.75 and 1.00% (w/w) green tea powder in addition to feed. The experiment lasted for 21 and 42 days, respectively, for each value of the green tea supplement and for separate groups of animals. Immune response against Newcastle disease, influenza viruses and sheep red blood cells, has been determined as well as lymphoid organ (bursa of Fabricius, thymus and spleen) weights. The sero-response to avian influenza ranged between 3.7 and 6.3 lg2 (33 days) and 3.9–3.8 lg2 (42 days) for all the animals treatment groups. The total sero-response to sheep red blood cells was moderate, in a range from 1.7 to 1.5 lgT after 21 and 42 days, respectively. The plots of the weight of immune organs did not show any relation between organ weight and immunoglobulin titre. While additional data would be needed to assess the optimum supplement feed dose, it can be concluded that green tea feed supplementation is not likely to be able to induce any negative effects on immunological parameters.

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Introduction
Green tea is made from the leaves of the Camellia sinensis, a plant which has a long history of stemming from China over 4000 years ago. It has been used for centuries in different parts of the World by people as an anti-aging herb. Green tea catechins have many biological and biochemical effects mainly attributed to their high antioxidant capacity, specifically epigallocatechin-3-gallate, the most biologically active ingredient in green tea. Green tea has many health benefits including, among the others, antiobesity, blood glucose control, antihypercholesterolaemia and blood pressure improvements (Liao et al. 2001).

In addition to human consumption and health benefits for humans, low grade green tea has been used as an ingredient in animal feed. Diets supplemented with green tea powder has been studied on broiler chickens (Kaneko et al. 2001; Cao et al. 2005), and positive effects of green tea on animal performance have been reported. Feed supplementation induced a decrease in feed intake and body weight gain with a higher dose, but tended to improve feed conversion ratio. Dressing percentage was not affected, though proportions of some parts of the carcase were influenced. Proportion of thigh meat was increased by feeding 1.5% green tea powder while that of wing meat decreased; at the same time the quantity and percentage of abdominal fat decreased significantly. Levels of liver cholesterol, liver fat and blood serum cholesterol were significantly reduced (Biswas & Wakita 2001).

More recent studies on green tea powder supplementation in the daily ration of broilers have shown effects on selected parameters of immunity and on productivity parameters such as growth and carcase characteristics, e.g. lower abdominal fat and neck percentages in birds as well as in higher heart and jejunum percentages (Alimohammadi-Saraei et al. 2014; Seidavi et al. 2014; Alimohammadi-Saraei et al. 2015). It has also been reported that the in ovo administration, even before hatching, of active compounds...
such as betaine and choline appear to have effects on hatchability, growth, carcase characteristics and broiler chickens immune response (Gholami et al. 2015). Feed supplementation with soya bean proteins (Nahavandinejad et al. 2012), orange peel extract (Pourhossein et al. 2015) and dietary fat (Poorghasemi et al. 2015) have also been reported as capable to exhibit an effect on immunity parameters of broiler chiken. A short-term feed restriction followed by re-feeding also affected immunity (Shabani et al. 2015; Rahimi et al. 2015). Similarly, physical characteristics of the feed by alone have been associated with changes in immunity (Nahavandinejad et al. 2012).

Based on a previous study on the effects of green tea powder feed supplementation in the broiler diet, which outlined a positive effect on some immunity parameters (Seidavi et al. 2014), in the present study the effects on immunity parameters using a multi variable design are exploited. Broiler chickens feed has been supplemented with different levels of green tea to assess the effect and the potential beneficial dose on selected immunity parameters.

Materials and methods

Animals and husbandry

Two hundred and seventy-one-day-old Ross 308 male broiler chickens were purchased from a local Iranian commercial hatchery. The chickens were placed in land cages with dimensions of 1.25 × 1.25 m, which provided a floor area of 0.125 m² per bird, in a thermostatically controlled curtain side-wall poultry barn. The cage floors were covered with paper roll litter, and the birds remained in the land cages for the duration of the experiment, which ended at 42 days of age. Each cage of 10 chickens was assigned to a specific dietary treatment group. Ambient temperature within the poultry barn was maintained with supplemental heat from thermostatically controlled gasoline rocket heaters, and humidity was controlled to the barn atmosphere via a water spray to maintain relative humidity between 55–65%. Ambient temperature was controlled at 32 °C at the day of placement of the chicken in the room and thereafter decreased periodically to 24 °C till chicken were 3 weeks old. Thereafter, the temperature was maintained at 24 °C till the end of the study. The study was performed during the year 2014–2015 and the outdoor temperature ranged from 25 °C to 32 °C, with relative humidity varying from 55% to 65%. Illumination was obtained using 23 W fluorescent tubes mounted into the ceiling. Light was kept on for 24 h only on day 1, while on day 2 and onwards till the end of the study light was on for 20 h per day. Ventilation was obtained using three wall-mounted 60 cm diameter fans at one end of the barn, and 160 cm diameter wall-mounted fans on the opposite end of the barn, thus establishing a tunnel ventilation system. The procedures have been approved by the corresponding Authors’ Institution’s Ethic Committee, and maximum care was adopted to minimise the number of animals used.

Feed and water

The feeding programme was designed according to the Ross recommendation (Aviagen 2007). It started with a starter feed given from day 1 to day 21 and a finisher feed given from day 22 to day 42. Details on feed composition and nutrient analysis of the experimental diets used are shown in Tables 1 and 2, respectively. Four dietary green tea powder levels (0.25, 0.50, 0.75 and 1.00%, w/w) were added to feed (see Table 1). The diets met or exceeded Ross 308 catalogue recommendations. Both the feed and water were offered ad libitum. Commercially available green tea has been used for the feed supplementation.

Table 1. Feed composition of starter and finisher diets.

| Ingredient, % | Starter (1st–21st days of age) | Finisher (22nd–42nd days of age) |
|--------------|-------------------------------|----------------------------------|
| Green tea powder | 0 | 0.25 | 0.50 | 0.75 | 1.00 | 0 | 0.25 | 0.50 | 0.75 | 1.00 |
| Maize | 55.70 | 55.55 | 55.39 | 55.23 | 55.08 | 59.80 | 59.61 | 59.47 | 59.33 | 59.17 |
| Soybean meal | 37.09 | 36.98 | 36.89 | 36.8 | 36.69 | 32.33 | 32.25 | 32.14 | 32.01 | 31.92 |
| Soybean oil | 2.82 | 2.82 | 2.82 | 2.82 | 2.82 | 4.02 | 4.04 | 4.03 | 4.03 | 4.03 |
| Di-calcium-phosphate | 1.94 | 1.94 | 1.94 | 1.94 | 1.94 | 1.70 | 1.70 | 1.70 | 1.70 | 1.70 |
| Calcium carbonate | 1.35 | 1.35 | 1.35 | 1.35 | 1.35 | 1.13 | 1.13 | 1.13 | 1.13 | 1.13 |
| Vitamin mixture | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Mineral mixture | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Sodium chloride | 0.19 | 0.19 | 0.19 | 0.19 | 0.19 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Sodium hydrogen carbonate | 0.24 | 0.24 | 0.24 | 0.24 | 0.24 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 |
| α-Methionine | 0.13 | 0.13 | 0.13 | 0.13 | 0.13 | 0.10 | 0.10 | 0.10 | 0.11 | 0.11 |
| L-Lysine-hydro-chloride | 0.04 | 0.05 | 0.05 | 0.05 | 0.06 | 0 | 0 | 0.01 | 0.02 | 0.02 |

*aVitamin A: 5000 IU/g; Vitamin D3: 500 IU/g; Vitamin E: 3 mg/g; Vitamin K3: 1.5 mg/g; Vitamin B2: 1 mg/g.
*bCalcium pantothenate: 4 mg/g; Niacin: 15 mg/g; Vitamin B6: 13 mg/g; Cu: 3 mg/g; Zn: 15 mg/g; Mn: 20 mg/g; Fe: 10 mg/g; K: 0.3 mg/g.
Trial designs and treatments

A total of 270 one-day-old male chicken of the Ross 308 strain (Aviagen, Newbridge, Scotland, UK 35805) were allotted to 27 groups of 10 birds, such that mean group body weights were similar for each group (44.2 ± 1.3 g). Treatments were given according to a 4\times2 factorial arrangement with four dietary green tea powder levels (0.25, 0.50, 0.75 and 1.00%, w/w) and two durations of treatment (21 and 42 days). There were nine treatment groups with three replicates per treatment and one control group. A replicate was one to maximum three birds. The treatments are described in the following:

Treatment 1: Basal diet (control)
Treatment 2: Basal diet plus 0.25% dietary green tea powder for 42 days.
Treatment 3: Basal diet plus 0.25% dietary green tea powder for 21 days of life.
Treatment 4: Basal diet plus 0.50% dietary green tea powder for the first 21 days of life.
Treatment 5: Basal diet plus 0.50% dietary green tea powder for 42 days.
Treatment 6: Basal diet plus 0.75% dietary green tea powder for the first 21 days of life.
Treatment 7: Basal diet plus 0.75% dietary green tea powder for 42 days.
Treatment 8: Basal diet plus 1.00% dietary green tea powder for the first 21 days of life.
Treatment 9: Basal diet plus 1.00% dietary green tea powder for 42 days.

Vaccination programme

Birds were vaccinated using vaccine for the chickens immunisation (Nobilis® N Clone 30, MSD Animal Health) at the 1st, 7th and 20th days of age against Newcastle disease (NCD), and on their 1st and 7th days of age against infectious bronchitis. Influenza and Gumbro vaccinations were given at the first day of age.

Immunity assays

The quality of immune response was quantified by the method described by Pourhossein et al. (2015). In particular, on 10 and 28 days of age, a suspension with sheep red blood cells (SRBC) in 5% phosphate-buffered saline was injected into the breast muscle of six birds per treatment group. In serum obtained at day 24 and at day 42 the total anti antibody titres to SRBC
were determined by haemagglutination assay using U-bottom micro-titre plates. Twofold serial dilutions of heat-inactivated (at 56°C) serum were made with PBS (0.01 mol/L; pH value 7.4) for total antibody. For the determination of immunoglobulin G (IgG) antibody titres, PBS with 1.4% 2-mercaptoethanol was used. Antibody titres were expressed as $2\log$ of the highest dilution of serum that agglutinated an equal volume of a 0.5% SRBC suspension in PBS. The IgM titre was calculated by the difference between total and IgG titre.

Three more birds per treatment group were randomly chosen for blood sampling from the brachial vein for measuring antibody titres against Newcastle disease virus (NDV) and avian influenza (AI) by haemagglutination inhibition test according to Cunningham (1971). Blood sampling was performed on days 17 (for NDV), 33 (for AI) and 42 (for NDV and AI). Serum was separated by centrifugation at 1300 rpm for 15 min following 1 h incubation at room temperature and stored at $-20^\circ$C until the analysis.

**Tissue collection**

At the end of the rearing period (42 days), three birds per treatment were randomly selected and killed by cervical dislocation and the liver, thymus, spleen and bursas of fabricius were removed and their weights were recorded. Data in grams were rounded to integers.

**Statistical analysis**

Since sampled birds per treatment group were small with either three or six, only non-parametric test were performed SPSS software has been used (SPSS 1997). The response to sheep red blood cells was thus analysed and immune response against AI and NCD was only descriptive and not further statistically analysed. The range of the organs weights per treatment were compared by visual analysis.

**Results and discussion**

Table 3 reports the observations after vaccination (influenza and Newcastle disease). Table 4 shows the average immune response after the sheep red blood cells evaluated considering the different green tea powder supplemented to the diet. Little differences can be observed between day 21 and day 42 for the different green tea powder supplementation. Sero-response after first NCD vaccination in the control group was good, but after the second vaccination, two non-responders were found among the three randomly selected birds. Possibly maternal-derived antibodies had interfered with antibody production, since the initial antibody titres were 1:4 to 1:32 in these birds. Moreover, immunity against avian influenza and sheep erythrocytes was normal. One bird in each of the following treatment groups also did not respond after the first NCD vaccination: group 3, 4, 6, 8 and 9. After the second NCD vaccination, one bird each of group 3 and 4 had no detectable NCD antibodies in the serum, whereas all other birds in the treatment groups had mounted titres varying between 1:2 and 1:32. Since sero-response to avian influenza was good

| Trait treatment | Antibody titre against influenza within 33 days after injection (lg2) | Antibody titre against influenza within 42 days after injection (lg2) | Antibody titre against second injection of Newcastle within 22 days after second injection (lg2) |
|----------------|------------------------------------------------------------------------|---------------------------------------------------------------------|---------------------------------------------------------------------------------|
| Usage duration, day | |
| 21            | 3.750                                                                  | 3.917                                                              | 3.000                                                                           |
| 42            | 3.667                                                                  | 3.833                                                              | 2.667                                                                           |
| $p$            | .689                                                                  | .756                                                               | .613                                                                            |
| SEM (Standard error of mean) | 0.144                                                                | 0.186                                                              | 0.456                                                                           |
| Green tea powder level, % | |
| 0.25          | 3.500                                                                  | 4.000                                                              | 2.833                                                                           |
| 0.50          | 3.667                                                                  | 4.000                                                              | 2.167                                                                           |
| 0.75          | 3.833                                                                  | 3.500                                                              | 3.667                                                                           |
| 1.00          | 3.833                                                                  | 4.000                                                              | 2.667                                                                           |
| $p$            | .618                                                                  | .463                                                               | .447                                                                            |
| SEM (Standard error of mean) | 0.204                                                                | 0.264                                                              | 0.645                                                                           |
| Duration (0 days) – level (0%) | 3.667                                                                  | 4.000                                                              | 1.333                                                                           |
| Duration (21 days) – level (0.25%) | 4.000                                                                  | 3.333                                                              | 3.333                                                                           |
| Duration (42 days) – level (0.25%) | 4.000                                                                  | 3.667                                                              | 2.333                                                                           |
| Duration (21 days) – level (0.50%) | 4.000                                                                  | 3.667                                                              | 1.667                                                                           |
| Duration (42 days) – level (0.50%) | 4.000                                                                  | 3.667                                                              | 2.667                                                                           |
| Duration (21 days) – level (0.75%) | 3.333                                                                  | 4.000                                                              | 4.000                                                                           |
| Duration (42 days) – level (0.75%) | 3.667                                                                  | 3.667                                                              | 3.333                                                                           |
| Duration (21 days) – level (1.00%) | 4.333                                                                  | 4.000                                                              | 3.000                                                                           |
| Duration (42 days) – level (1.00%) | 3.667                                                                  | 3.667                                                              | 2.333                                                                           |
| $p$            | .738                                                                  | .969                                                               | .634                                                                            |
| SEM (Standard error of mean) | .0369                                                                | .430                                                               | .096                                                                            |

$^a$Means ($\pm$ standard error of means) within each column of dietary treatments with no common superscript differ significantly at $p < .05$. 

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Table 3. Immune response mean ($\pm$ SEM) after vaccination (influenza and Newcastle) of Ross 308 broilers by four different levels of green tea powder and two different durations of treatment.
After the second vaccination, all but one bird from group 3 showed a suitable sero-response (1:2–1:256). The highest titre, however, was found in a bird of the control group. Anti-sheep IgM response at first vaccination was absent in one bird of the control group and one bird each of group 3 and 6 were non-responders.

Table 4. Immune response mean (±SEM) after injection of sheep red blood cell at Ross 308 broilers affected the four different levels of green tea powder and two different durations of its usage.

| Trait treatment | Total antibody against Sheep Red Blood cell (TSRBC) at 14th day after first injection (lgT) | Immunoglobulin in G antibody against Sheep Red Blood cell (TSRBC) at 14th day after first injection (lgG) | Immunoglobulin in M antibody against Sheep Red Blood cell (TSRBC) at 14th day after second injection (lgM) | Total antibody against Sheep Red Blood cell (TSRBC) at 14th day after second injection (lgT) | Immunoglobulin G antibody against Sheep Red Blood cell (TSRBC) at 14th day after second injection (lgG) | Immunoglobulin M antibody against Sheep Red Blood cell (TSRBC) at 14th day after second injection (lgM) |
|-----------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Usage duration, day | | | | | | |
| 21 | 1.750 | 0.750 | 1.000 | 3.167 | 1.250 | 1.917 |
| 42 | 1.500 | 0.750 | 0.750 | 4.000 | 1.667 | 2.333 |
| p | .531 | 1.000 | .418 | .406 | .215 | .404 |
| SEM (Standard Error of mean) | 0.276 | 0.118 | 0.212 | 0.479 | 0.228 | 0.344 |
| Green tea powder level, % | | | | | | |
| 0.25 | 1.500 | 0.667 | 0.833 | 3.167 | 1.167 | 2.000 |
| 0.50 | 1.833 | 1.000 | 0.833 | 4.167 | 1.667 | 2.500 |
| 0.75 | 1.500 | 0.667 | 0.833 | 2.833 | 1.000 | 1.833 |
| 1.00 | 1.667 | 0.667 | 1.000 | 4.167 | 2.000 | 2.167 |
| p | 0.917 | .418 | .972 | .236 | .151 | .795 |
| SEM (Standard error of mean) | 0.391 | .167 | 0.030 | 0.677 | 0.323 | 0.486 |
| Duration (0 days) – level (0%) | 2.000 | 1.000 | 1.000 | 4.667 | 1.000ab 3.667 | 3.667 |
| Duration (21 days) – level (0.25%) | 2.000 | 0.667 | 0.333 | 4.667 | 1.000ab 3.333 | 3.333 |
| Duration (42 days) – level (0.50%) | 2.333 | 1.000 | 1.333 | 4.333 | 1.667ab 2.667 | 2.667 |
| Duration (42 days) – level (0.75%) | 1.333 | 1.000 | 0.333 | 4.000 | 1.667ab 2.333 | 2.333 |
| Duration (42 days) – level (1.00%) | 1.000 | 0.333 | 0.667 | 3.000 | 1.000ab 2.000 | 2.000 |
| p | .917 | .418 | .972 | .236 | .151 | .795 |
| SEM (Standard error of mean) | 0.391 | .167 | 0.030 | 0.677 | 0.323 | 0.486 |

Table 5. Weight and relative weight mean (±SEM) of organs related with immune system at 42nd days of age in Ross 308 broilers affected the four different levels of green tea powder and two different durations of its usage.

| Trait treatment | Thymus weight, g | Relative weight of thymus, % | Liver weight, g | Relative weight of liver, % | Spleen weight, g | Relative weight of spleen, % | Bursa of fabricius weight, g | Relative weight of bursa of fabricius, % |
|-----------------|------------------|-----------------------------|----------------|-----------------------------|-----------------|-----------------------------|-----------------------------|-------------------------------------|
| Usage duration, day | | | | | | | | |
| 21 | 6.938 | 0.252 | 55.869 | 2.032 | 2.818 | 0.102 | 1.379 | 0.050 |
| 42 | 6.308 | 0.238 | 64.083 | 2.385 | 2.065 | 0.077 | 1.098 | 0.040 |
| p | .526 | .703 | .033 | .005 | .019 | .025 | .054 | .086 |
| SEM (Standard error of mean) | 0.686 | 0.027 | 2.496 | 0.076 | 0.205 | 0.007 | 0.109 | 0.004 |
| Green tea powder level, % | | | | | | | | |
| 0.25 | 6.275 | 0.238 | 63.700 | 2.351 | 2.360 | 0.088 | 1.288 | 0.048 |
| 0.50 | 7.912 | 0.286 | 62.213 | 2.255 | 2.510 | 0.090 | 1.260 | 0.045 |
| 0.75 | 6.618 | 0.239 | 58.252 | 2.100 | 2.590 | 0.093 | 1.227 | 0.044 |
| 1.00 | 5.687 | 0.217 | 55.740 | 2.127 | 2.305 | 0.088 | 1.098 | 0.042 |
| p | .444 | .627 | .392 | .348 | .890 | .348 | .900 | .900 |
| SEM (Standard error of mean) | 0.971 | 0.038 | 3.530 | 0.108 | 0.289 | 0.010 | 0.155 | 0.006 |
| Duration (0 days) – level (0%) | 7.197 | 0.260 | 57.860 | 2.012 | 2.377 | 0.084 | 1.170 | 0.042 |
| Duration (21 days) – level (0.25%) | 6.650 | 0.258 | 55.860 | 2.140 | 2.490 | 0.096 | 1.433 | 0.045 |
| Duration (42 days) – level (0.25%) | 5.900 | 0.217 | 57.720 | 2.563 | 2.230 | 0.080 | 1.143 | 0.041 |
| Duration (21 days) – level (0.50%) | 8.050 | 0.279 | 57.847 | 2.015 | 3.287 | 0.114 | 1.270 | 0.044 |
| Duration (42 days) – level (0.50%) | 7.773 | 0.292 | 66.580 | 2.495 | 1.733 | 0.060 | 1.250 | 0.047 |
| Duration (21 days) – level (0.75%) | 7.607 | 0.271 | 55.710 | 1.986 | 3.153 | 0.113 | 1.700 | 0.061 |
| Duration (42 days) – level (0.75%) | 5.630 | 0.207 | 60.793 | 2.214 | 2.027 | 0.073 | 0.753 | 0.028 |
| Duration (21 days) – level (1.00%) | 5.443 | 0.200 | 54.240 | 1.987 | 2.340 | 0.085 | 1.113 | 0.041 |
| Duration (42 days) – level (1.00%) | 5.930 | 0.235 | 57.240 | 2.268 | 2.270 | 0.090 | 1.083 | 0.043 |
| p | .876 | .948 | .249 | .065 | .212 | .282 | .254 | .302 |
| SEM (Standard Error of Mean) | 1.494 | 0.059 | 4.836 | 0.144 | 0.402 | 0.014 | 0.217 | 0.008 |

*Means (± standard error of means) within each column of dietary treatments with no common superscript differ significantly at p < .05.
one bird in each of group 2, 6 and 7, whereas two birds each in group 3 and 5 had no IgM response. After the second vaccination, still one bird from group 2 and one new bird in group 8 had not produced detectable levels of anti-sheep IgM, but both, however, had responded with a low IgG titre (1:2). After the second vaccination, one bird in the control group and two birds in group 2 had not managed to develop IgG against sheep erythrocytes, but all had low (1:4) IgM titres.

It can be observed that all the sampled birds developed an immune response against sheep erythrocytes 10 days after the last vaccination. There were some individual differences in the onset and magnitude of IgG and IgM response, but regarding the response on avian influenza antigens, none of the sampled birds appeared to suffer from immune-deficiency. Thus, it can be concluded that feed supplementation with green tea powder has no adverse effects on the development of a normal immune response in broilers. The weight of immune organs, namely thymus, bursa of Fabricius bursa, and spleen, is reported in Table 5. For better visualising the results, these have been plotted in Figures 1, 2 and 3, respectively, reporting the weight against the 2log titre of the total of immunoglobulins against sheep erythrocytes after the second vaccination. In all the plots, no relation between organ weight and immunoglobulin titre has been observed, and for this reason no further analysis has been performed. Supplementation with 0.25% green tea powder for 42 days seemed to increase liver weight compared to that without any treatment. Adding a dose of 1.00% to the feed had ambiguous effects on liver weight. When given for 21 days, liver weight was decreased compared to the control, but after 42 days liver weight was mildly increased. Due to sample sizes, these results are likely caused by hazard. Sometimes with no more than three animals per treatment group, statistical inference is not possible. For non-parametric statistic of the data based on six animals per treatment group, if not showing significance, a considerable type 2 failure cannot be excluded.

Conclusions

Taking on board also all the previously obtained results on green tea powder feed supplementation, it is not likely that green tea powder supplementation to the feed could cause any negative effects on broiler...
chickens immunity. It can be concluded, based on our results, that, to avoid the interaction of other substances added or already present in the feed, additional experimental evidences would be necessary for identifying and completely assessing the optimum supplement feed dose.

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