Response of zebrafish to royal jelly supplementation and differences in stocking density


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Abstract

Dietary supplementation with royal jelly (RJ) may improve growth, antioxidant gene expression, and intestinal antioxidant capacity, and possibly mitigate effects of stress induced by high stocking density (HSD) in zebrafish (Danio rerio). Effects of HSD and RJ supplementation on growth performance, antioxidant gene expression, and intestinal antioxidant capacity in zebrafish were investigated. A total of 240 four-month-old zebrafish were used in a 2 x 2 factorial design, with four treatments and three replications. The treatments consisted of normal stocking density (NSD) (1 fish/L) and high stocking density (HSD) (3 fish/L) and an unsupplemented commercial diet (0RJ) and commercial diet supplemented with 10% RJ (10RJ). The experiment lasted 25 days. Daily weight gain, final body length, specific growth rate, and survival were recorded. On day 25, six fish per treatment were euthanised and their intestines analysed for antioxidant capacity, and superoxide dismutase 2 (SOD2) and catalase (CAT) gene expression. There were no effects of diet or SD and diet interaction on growth. High stocking density decreased daily weight gain by 25.31%, body length by 2.70%, and survival by 16.85%. Stocking density (SD) and diet interaction influenced intestinal SOD2 and CAT expression and antioxidant capacity. The HSD/0RJ group had lower antioxidant gene expression and antioxidant capacity than the HSD/10RJ and NSD/0RJ groups. Supplementation with RJ did not mitigate the harmful effects of stocking stress. These results indicate that HSD promotes transcriptional suppression of gene encoding enzymes that form the first line of antioxidant defence, resulting in reduced elimination of toxic substances and occurrence of intestinal oxidative stress.

Keywords: antioxidant capacity, antioxidant gene expression, growth performance, intestinal environment, overcrowding, stress

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Introduction

Animal production efficiency depends on a variety of factors, including the environment in which animals are bred, because genes associated with economically important traits are influenced by the environment (Côté et al., 2007). In fish farming, stocking density (SD) is a highly relevant environmental factor, affecting water quality, animal wellbeing, uniformity, and health (Marques et al., 2004; Ashley, 2007; Dawood et al., 2019). An inadequately high SD may alter feeding behaviour and feed use efficiency, thereby decreasing productive performance (Wocher et al., 2011; Rabbane et al., 2016; Refaey et al., 2018; Long et al., 2019). Highly stocked fish may develop a state of physiological stress in response to increased risk of predation and competition for food (Bolasina et al., 2006; Jia et al., 2016; Long et al., 2019). Under these conditions, metabolic energy is directed towards stress management rather than growth.

Stress can also lead to short- and long-term alterations in gastrointestinal tract functions, exerting negative effects on microflora, intestinal mucosa blood flow, regenerative capacity, and gastrointestinal motility and secretion (Konturek et al., 2011). Previous studies showed that fish raised in highly stocked environments had altered length and bodyweight (Bolasina et al., 2006; Liu et al., 2016) and metabolic changes such as increased cortisol levels (Bolasin et al., 2006; Ramsay et al., 2006; Long et al., 2019) and altered immune response (Montero et al., 1999; Liu et al., 2016; Dawood et al., 2019). Another known effect of high stocking density (HSD) on fish is the occurrence of oxidative stress (Jia et al., 2016; Liu et al., 2016;...
Dawood et al., 2019), caused by an increase in lipid peroxidation and a reduction in the activity of antioxidant enzymes (e.g., superoxide dismutase, catalase, glutathione peroxidase) and levels of the non-enzymatic antioxidant glutathione (Liu et al., 2016; Dawood et al., 2019).

In intensive fish farming, productivity is optimized by using high breeding densities (Bolasina et al. (2012)). An option to minimize the negative effects of stress on fish grown under these conditions is to supplement feed with bioactive ingredients. For instance, royal jelly (RJ), a honeybee secretion, is considered a nutraceutical food with anti-inflammatory (Aslan & Aksoy, 2015), antimicrobial (Coutinho et al., 2018), antiallergic (Guendouz et al., 2017), antioxidant (Nagai et al., 2006), and growth-stimulating properties (Ahangari et al., 2013). This study aimed to investigate the hypotheses that HSD (SD) causes oxidative stress in the intestine of zebrafish (Danio rerio) and that RJ supplementation is able to mitigate the harmful effects of stocking stress on the intestine. To date, this is the first study to simultaneously evaluate the effect of high SD and RJ supplementation on zebrafish performance (weight gain, final body length, specific growth rate, and survival), expression of genes involved in antioxidant defences (superoxide dismutase and catalase genes), and intestinal antioxidant capacity.

Material and Methods

This study was conducted according to the guidelines of the Animal Ethics Committee of the State University of Maringá, Brazil. A total of 240 four-month-old zebrafish (D. rerio) were used with 0.33 ± 0.11 g bodyweight and 3.35 ± 0.20 cm total length. The experiment was conducted at PeixeGen Zebrafish Laboratory of the State University of Maringá, Brazil, following a completely randomized design arranged in a 2 × 2 factorial, with three replications per treatment. Treatments consisted of two SDs, namely NSD (1 fish/L) and HSD (3 fish/L) and two diets, an unsupplemented commercial diet (0RJ) and a commercial diet supplemented with 10% RJ (10RJ). Stocking densities were chosen according to the recommendations of Vargesson (2007) and the observations of Ramsay et al. (2006).

Fish were randomly distributed in 10 L glass aquaria with chlorine-free water and constant aeration under controlled temperature conditions (28.5 ± 1 °C) and a photoperiod of 14 hours light/10 hours dark. Water quality parameters (temperature, ammonia, nitrite, and pH) were monitored weekly. To prevent other sources of stress and ensure water quality, the authors partially replaced water (60%) every two days. Fish were first maintained under conventional conditions for seven days. During this adaptation period, fish were fed commercial feed (without RJ supplementation) three times a day to apparent satiation (at 9h00, 14h00, and 17h00). After the adaptation period, fish were subjected to SD and diet treatments and fed three times a day (at 9h00, 14h00, and 17h00) for 25 days.

A commercial diet was used based on fish meal, pea, fish oil, soy lecithin and fish gelatine (Bernaqua Finest Hatchery Feeds, Belgium). Diet composition is presented in Table 1. The 10RJ diet was prepared by spraying a solution containing 100 mg pure freeze-dried RJ (Apireal, Apis, Flora, Brazil) diluted in 25 mL of distilled water onto the commercial diet. Then, kibbles were dried at room temperature (25 ± 4 °C) in the dark and stored in dark plastic containers at -22 °C until use.

At the beginning and end of the experiment, fish were anaesthetised in 0.0004% benzocaine (Medicinal Farmácia e Manipulação, Brazil) to the point of total loss of equilibrium and weighed. Daily weight gain was determined as the initial bodyweight minus the final body weight divided by 25. Final body length was measured at the end of the experimental period. For this, fish were anaesthetised in benzocaine, and body length (from the beginning of the head to the end of the caudal fin) was measured using a digital calliper (model 366619, MXT, Brazil). Survival and specific growth rate were calculated as described by Gonzalez Jr. (2012).

For analysis of gene expression, three fish from each treatment (n = 3) were euthanized at the end of the experiment by immersion in ice for 5 min. The entire intestine was collected, washed with sterile saline (4 °C), frozen in liquid nitrogen, and stored at -80 °C until analysis. Total RNA was extracted from intestine specimens with TRIzol (Invitrogen, Waltham, Massachusetts, USA) according to the manufacturer’s recommendations, and quantified using a spectrophotometer (Nanodrop 2000c, Thermo Fisher Scientific, USA) at 260 nm. Total RNA purity was determined by the ratio of absorbance readings at 260/280 nm and 260/230 nm, which fell between 1.9 and 2.1. Genomic DNA contamination was eliminated by treating 1 µg of total RNA with the amplification grade DNase I kit (Invitrogen, USA), according to the manufacturer’s instructions. After this procedure, complementary DNA (cDNA) was synthesized using the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen Corporation, Brazil), according to the manufacturer’s instructions. cDNA concentration was measured using a spectrophotometer at 260 nm (Nanodrop 2000c, Thermo Fisher Scientific, USA), and samples were stored at -20 °C until amplification.
Table 1 Composition of experimental diets fed to zebrafish (*Danio rerio*)

<table>
<thead>
<tr>
<th>Composition</th>
<th>Commercial diet</th>
<th>Diet supplemented with 10% royal jelly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Royal jelly (Apireal®) (%)</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Omega-3 highly unsaturated fatty acids (mg/g)</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Docosahexaenoic acid (mg/g)</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (mg/g)</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Fibre (%)</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Vitamin A (IU/g)</td>
<td>2,500.0</td>
<td>2,500.0</td>
</tr>
<tr>
<td>Vitamin D3 (IU/g)</td>
<td>200.0</td>
<td>200.0</td>
</tr>
<tr>
<td>Vitamin C (mg/kg)</td>
<td>75.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Vitamin E (mg/kg)</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Sodium (%)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>14.5</td>
<td>14.5</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Metabolizable energy (kJ/g)</td>
<td>18.6</td>
<td>18.6</td>
</tr>
</tbody>
</table>

Real-time quantitative polymerase chain reaction (qPCR) was performed using 5 µL of cDNA at 40 ng/mL, 0.8 µL of each primer (forward and reverse) at 10 µM (final concentration of 400 µM), 10 µL of PowerUp SYBR GREEN Master Mix (Applied Biosystems, Lithuania), and UltraPure DEPC-treated Water (Invitrogen, USA) to complete a final volume of 20 µL. All analyses were carried out on a StepOne Real-Time PCR System version 2.3 (Applied Biosystems, Lithuania), in duplicate, under thermal cycling conditions, namely activation of uracil-DNA glycosylase at 50 °C for 2 min, activation of Dual-Lock DNA-polymerase at 95 °C for 2 min, 40 cycles of 95 °C for 3 s for denaturation, and annealing and extension at 60 °C for 30 s. Melting curves were obtained from 60 to 95 °C.

Primers for superoxide dismutase 2 (*SOD2*) and catalase (*CAT*) genes of *D. rerio* were designed according to Sarkar *et al.* (2014) on the basis of gene sequences deposited at the National Centre for Biotechnology Information (NCBI) database (NCBI Resource Coordinators, 2018) (Table 2). The β-actin gene was used as endogenous control, and its primers were designed based on sequences deposited at NCBI using the Integrated DNA Technologies platform (www.idtdna.com) (Table 2). All analyses were performed in duplicate. The 2^−ΔΔCT method (Livak & Schmittgen, 2001) was used for relative quantification of gene expression, and results are presented in arbitrary units (AU).
Intestinal antioxidant capacity was determined in samples obtained from three fish per treatment (n = 3) at the end of the experimental period. Animals were stunned by immersion in ice for 5 min, and intestine samples were collected, washed with sterile saline (4 °C), frozen in liquid nitrogen, and stored at -80 °C until analysis. Antioxidant capacity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Sigma–Aldrich, Brazil), according to the protocol described by Brand-Williams et al. (1995), with some modifications. Briefly, intestine samples were added to a test tube containing 1000 µL of methanol and homogenized using a Van Potter homogeniser. The mixture was centrifuged for 10 min at 10,000 × g and 4 °C, and the supernatant (22.50 µL) was added to the wells of a 96-well microplate containing 277.50 µL of a 0.06 mM DPPH solution. Reactions were kept in the dark for 30 min. After this period, samples were read at 515 nm on a microplate reader (VersaMax, Molecular Devices, USA), and the antioxidant capacity (%) of each sample was determined as antioxidant capacity = (1 − (sample absorbance/DPPH absorbance)) × 100.

The Shapiro–Wilk test was used to assess the normality of data distribution. Main and interaction effects of SD and RJ supplementation were investigated by two-way analysis of variance (ANOVA). When significant, the main effect means were compared with Student’s t-test and interaction means by Tukey’s test. The level of significance was set at P < 0.05. All statistical analyses were performed using SAS 2002 (SAS Inst. Inc., Cary, NC, USA).

Results and Discussion

No SD x diet effects were observed on weight gain, final body length, specific growth rate, or survival rate (P > 0.05, Table 3). The main effects of SD on weight gain (P = 0.0285), final body length (P = 0.0475), and survival rate (P = 0.0011) were significant. HSD led to a reduction of 25.31% in weight gain, 16.85% in survival rate, and 2.70% in final body length.

Table 3 Daily weight gains final body length, specific growth rate, and survival of zebrafish (Danio rerio) (1–25 days old) fed diets supplemented or not with royal jelly and reared at different stocking densities

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NSD 0RJ</th>
<th>NSD 10RJ</th>
<th>HSD 0RJ</th>
<th>HSD 10RJ</th>
<th>SD 0RJ</th>
<th>SD 10RJ</th>
<th>SE</th>
<th>SD</th>
<th>RJ</th>
<th>SD × Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>DWG (g)</td>
<td>0.26</td>
<td>0.24</td>
<td>0.19</td>
<td>0.18</td>
<td>0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22</td>
<td>0.21</td>
<td>0.04</td>
<td>0.029</td>
</tr>
<tr>
<td>FBL (cm)</td>
<td>3.77</td>
<td>3.66</td>
<td>3.62</td>
<td>3.61</td>
<td>3.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.66</td>
<td>3.62</td>
<td>0.33</td>
<td>0.048</td>
</tr>
<tr>
<td>SGR (mg/d)</td>
<td>1.76</td>
<td>2.31</td>
<td>1.90</td>
<td>1.78</td>
<td>2.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.83</td>
<td>2.04</td>
<td>0.31</td>
<td>0.267</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>100.0</td>
<td>97.8</td>
<td>78.9</td>
<td>85.6</td>
<td>98.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>89.3</td>
<td>91.6</td>
<td>5.77</td>
<td>0.001</td>
</tr>
</tbody>
</table>

NSD: normal stocking density (1 fish/L); HSD: high stocking density (3 fish/L); SD: stocking density; RJ: royal jelly; 0RJ: unsupplemented commercial diet; 10RJ: commercial diet supplemented with 10% RJ; DWG: daily weight gain; FBL: final body length; SGR: specific growth rate

<sup>a,b</sup>Within a column, means with a common superscript did not differ with probability P < 0.05

Figure 1 shows the CAT and SOD2 expression and total antioxidant capacity in the intestine of zebrafish. There were significant effects of SD x diet on CAT expression (P < 0.0001), SOD2 expression (P = 0.0006),
and antioxidant capacity ($P <0.0001$). Fish fed $0RJ$ and kept at HSD had reduced antioxidant gene expression and antioxidant capacity.

![Figure 1](image)

**Figure 1** Effects of stocking density and royal jelly supplementation on a) catalase and b) superoxide dismutase 2 gene expression and c) antioxidant capacity in the intestine of zebrafish (*Danio rerio*)

Gene expression is presented in arbitrary units (AU). Values are the mean ± standard error of three replicate fish.

Columns headed by a common letter did not differ with probability $P <0.05$

*CAT*: catalase; *SOD2*: superoxide dismutase 2

$0RJ$: unsupplemented commercial diet, $10RJ$: commercial diet supplemented with 10% royal jelly

The current results indicated that the low performance of zebrafish reared at HSD might have occurred because of intestinal oxidative stress from transcriptional suppression of genes involved in antioxidant responses. Highly stocked zebrafish had lower weight gain, body length, and survival rate than zebrafish maintained under normal conditions, as in other studies (Costa *et al.*, 2017; Rabbane *et al.*, 2017; Refaey *et al.*, 2018; Dawood *et al.*, 2019; Long *et al.*, 2019). Stocking density is one of the most relevant environmental factors in the production of zebrafish and other fish, whether under laboratory or natural conditions (Hazlerigg *et al.*, 2012). Intensive fish farming systems are characterized by the use of high
breeding densities, a strategy for yield optimization (Bolasina et al., 2006). However, when SD is inadequately high, important productive parameters may be affected (Costa et al., 2017; Dawood et al., 2019; Long et al., 2019). The negative effects of HSD observed in the current study might have been owing to an increase in competition for food among animals, thereby leading to altered feeding behaviour (Wocher et al., 2011) and nutrient use, ultimately decreasing productive performance. The findings may also have been owing to physiological and metabolic changes in response to stressful situations, such as increased cortisol secretion (Bolasina et al., 2006; Ramsay et al., 2006; Long et al., 2019). High cortisol levels accelerate protein metabolism (van der Boon et al., 1991) and increase the concentration of low molecular weight insulin-like growth factor (IGF)-binding proteins, which may inhibit IGF-1 activity (Peterson & Small, 2005).

When raised at HSDs, fish may develop a state of physiological stress characterized by numerous metabolic changes, including altered antioxidant response (Liu et al., 2016; Dawood et al., 2019). Such an effect can contribute to the establishment of oxidative stress (Dawood et al., 2019). Given the widespread use of increasingly high SDs, it is necessary to find solutions to mitigate the harmful effects of stocking stress on physiological defence systems.

An interesting strategy for nutritional modulation of immune responses is the supplementation of animal feed with nutraceutical foods that have immunostimulatory, anti-inflammatory, and antioxidant properties (Taheri Mirghaeced et al., 2018; Herrera et al., 2019; Bacchetta et al., 2020). Various supplements, including amino acids, fatty acids, vitamins, minerals, and plant extracts, have been used to increase animal resistance to stress by modulating antioxidant and immune defence systems (Hoseinifar et al., 2017; Taheri Mirghaeced et al., 2018; Herrera et al., 2019; Bacchetta et al., 2020). Royal jelly is a honeybee secretion with important anti-inflammatory (Aslan & Aksoy, 2015), antimicrobial (Coutinho et al., 2018), antiallergic (Guendouz et al., 2017), antioxidant (Nagai et al., 2006), and growth-stimulating properties (Ahangari et al., 2013). Despite the potential beneficial effects of this natural food on animal health, few studies have investigated the effect of supplementing animal diets with RJ (Seven et al., 2012; Ferreira et al., 2013; Seven et al., 2016; Babaei et al., 2016). Ferreira et al. (2013) assessed the effects of RJ supplementation on catfish exposed to a highly toxic fungicide and found that RJ and other bee products prevented or reversed oxidative tissue damage. The results were attributed to an increase in superoxide dismutase, catalase, and glutathione transferase activities. Aksakal et al. (2021) evaluated the effects of supplementing with RJ on the expression of several genes. The authors noted that RJ supplementation increased superoxide dismutase and catalase gene expression in the liver, muscle, and kidneys.

In this study, it was hypothesized that a high population density would cause oxidative stress in zebrafish intestines and that RJ supplementation would benefit the intestinal antioxidant defence system. To test this hypothesis, the authors evaluated gut antioxidant capacity and expression of two genes encoding enzymes regarded as the first line of antioxidant defence (SOD2 and CAT). According to Afifi et al. (2017) and Cong et al. (2020) changes in the transcription levels of genes encoding antioxidant enzymes can be used as the primary and most sensitive biomarkers of physiological responses to environmental stress. The results showed that zebrafish subjected to HSD and fed 0RJ had lower SOD2 and CAT expression, and, consequently, limited gut antioxidant capacity.

In the Atlantic cod (Gadus morhua) short-term stocking HDs enhanced expression of genes related to antibacterial activity, metabolism, and antioxidant defence (Caipang, 2012). Long-term overcrowding, on the other hand, seemed to activate chronic stress responses. Liu et al. (2016) observed that juvenile turbot (Scophthalmus maximus) bred for 120 days at a HD had lower glutathione, catalase, and superoxide dismutase levels. Dawood et al. (2019) found that Nile tilapia (Oreochromis niloticus) maintained for 30 days under intensive conditions showed higher lipid oxidation and lower superoxide dismutase, catalase, and glutathione peroxidase activities.

High stocking of un-supplemented zebrafish for 25 days suppressed intestinal antioxidant responses at the transcriptional level, reducing the organ’s capacity to eliminate oxidative and toxic substances. Another interesting result observed in this study was that fish reared at NSD and fed 0RJ showed greater SOD2 and CAT expression and antioxidant capacity than animals reared at NSD but fed 10RJ. This finding suggests that, under normal conditions, genes related to the antioxidant are activated, contributing to the control of reactive oxygen species in tissues and cells, thereby maintaining the organism’s homeostasis. However, when animals are subjected to an environmental challenge, such as HSD, there may be an increase in the production of reactive oxygen species (Bagni et al., 2007; Braun et al., 2010), which can damage DNA, RNA, proteins, and lipids (Sayeed et al., 2003; Kong & Li, 2010), resulting in transcriptional suppression of these genes and oxidative damage that is often irreversible (Kong & Lin, 2010; Nita & Grzybowski, 2016). Unlike other studies that found an increase in the activity of antioxidant enzymes in animals receiving RJ (Kanbur et al., 2009; Silici et al., 2011), we found that dietary supplementation with 10% RJ was not sufficient to maintain and/or increase SOD2 and CAT expression, decreasing the intestinal antioxidant capacity of...
animals. This could alter intestinal functions in the short and long terms, adding to the negative effects of environmental stress on the microflora, cellular regenerative capacity, blood flow in the intestinal mucosa, and gastrointestinal motility and secretion (Konturek et al., 2011). Wang et al. (2018) and Refaey et al. (2018) demonstrated that high population densities cause structural and functional changes in fish intestine, negatively influencing food digestion, nutrient absorption, and, ultimately, productive performance.

Conclusions

In summary, the performance of zebrafish reared at HSD decreased as a consequence of intestinal oxidative stress resulting from transcriptional suppression of genes involved in antioxidant responses. These effects culminated in the reduction of the gut’s capacity to eliminate toxic substances, affecting intestinal function and reducing nutrient absorption and utilisation. Such a reduction might have contributed to the lower antioxidant capacity of the intestine. The authors concluded that commercial feed alone was not sufficient to maintain the normal functioning of the organism if fish were exposed to environmental challenges. Diet supplementation with 10% RJ seemed insufficient to mitigate the harmful effects of stress on the intestine. The inefficiency of RJ observed in this study might also be related to leaching, as RJ was added to feed by spraying, covering only the surface area of the pellet. To date, this was the first study to evaluate the interaction effects of HSD and RJ supplementation on zebrafish. Further studies are needed for improved understanding of the potential of RJ in fish nutrition.

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Authors’ Contributions

BSP, DABM, FRD, MRS, and ASK wrote the manuscript; RPR, LDC, and EG revised the manuscript; BSP, DABM, FRD, MRS, RPR, and MRS conducted animal experiments; BSP, DABM, FRD, MRS, and ASK conducted laboratory analyses; ASK performed the intestinal antioxidant capacity assay; FRD and MRS analysed the data; BSP, DABM, FRD, MRS, RPR and EG secured funding for the project. All authors proofread and approved the final manuscript.

Conflict of Interest Declaration

The authors declare they have no competing interests.

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