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Research article

Evaluation of the potential of extract of seaweed Eucheuma denticulatum as an alternative to antibiotic growth promoter in broiler chickens

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ABSTRACT

The seaweeds are in focus for their immunity and gut health-stimulating potentials in humans and farm animals, but their potential as a gut health-promoting agent and performance booster to replace antibiotic growth promoters (AGP) in broiler chicken-feed remains to be evaluated. *In vivo* feeding experiments were conducted on commercial broiler chickens (1–42 days post-hatch) to evaluate dried aqueous exact of red seaweed *Eucheuma denticulatum* (referred to as PBD 5). Each of the three test diets (basal diet with three dosing regimens of PBD5, 0.25 g kg^{-1} for 0–6 weeks, 0.25 g kg^{-1} for 0–4 weeks or 1.0 g kg^{-1} for 0–2 weeks), along with an AGP supplemented diet (Virginiamycin (V), 20 ppm in basal diet), and a control diet was fed to 13 pen replicates of five chicks in each. PBD5 at 1.0 g kg^{-1} diet for 0–2 weeks improved ($P < 0.05$) cumulative feed efficiency (4.65 % improvement at 28 d, and 3.74 % at 35 d) than the control and comparable to the V group and the trend in improvement persisted up to 42 d. The group fed with PBD5 @ 1.0 g kg^{-1} for 0–2 weeks had significantly ($P < 0.05$) higher serum IgG level, glutathione peroxidase levels, fat digestibility, and expression of occludin and avian beta-defensin 4 gene in the gut and a trend of increased expression of growth hormone receptor gene in the liver as compared to the control with no significant effect on body weight, phytohemagglutinin response or haemagglutination inhibition titer. At d 25 of age, fecal *E. coli* count was significantly ($P < 0.01$) lower in the seaweed extract groups and the V group as compared to the control. It can be concluded that dried aqueous extract of *E. denticulatum* at 1 g kg^{-1} diet for 0–2 weeks can be used as an alternative to antibiotic growth promoter in broiler chickens to improve feed efficiency and reduce gut pathogen load, and the improved performance was associated with increased expression of gut immunity and growth hormone receptor genes.

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1. Introduction

Sustainable poultry meat and egg production are important to provide safe and high-quality protein for human nutrition. The gut of chickens is densely populated with diverse microbiota dominated by bacteria that play a vital role in the digestion and absorption of nutrients, pathogen exclusion, maintenance of the integrity of gut mucosa, and host immune system development [1]. Antibiotic growth promoters (AGP) have been traditionally used in broiler production to prevent diseases and improve growth and feed efficiency. The use of AGPs wherein AGPs are used in sub-therapeutic doses for a longer duration favors the selection of resistance to multiple classes of antimicrobial resistance genes (ARGs) [2,3], and the spread of ARGs among bacterial communities residing in the gut of animals and humans through the food chain or environmental pathways [4]. Considering the seriousness of the threat to human and animal health, some countries have banned the use of AGPs in food animal production. Broiler chickens are aggressively selected for higher growth rate and feed efficiency with limited emphasis on fitness traits or immunity; which is expected to expose them consequently to high oxidative and metabolic stress and make them more susceptible to infectious diseases thus requiring the use of AGPs. Many alternatives to AGPs are being marketed but most of them lack consistency or defined mechanisms, often require high doses, and are not cost-effective. Farmers commonly believe that AGPs may ward off pathogens from the gut at a low cost. Ideal antibiotic alternatives should have the same beneficial effects as AGPs (maintain gut integrity, increase nutrient availability, promote beneficial bacteria growth, and reduce the negative consequences of inflammation caused by enteric infections) and need to be effective in low doses (<1 % in diet) and need to be economical to be widely accepted by the broiler farmers. Among all AGP alternatives, phytochemicals/herbal products can be considered one of the strongest candidates to replace AGPs in poultry because they can stimulate immunity, reduce oxidative stress, increase antioxidant activity in various tissues, modulate the expression of gut immunity, and porosity-related genes [5], increase secretion of digestive enzymes, stimulate liver function, and inhibit viruses and coccidia, besides showing all the beneficial effects of AGP listed above. The macroalgae or seaweeds provide a great variety of metabolites, antioxidants, and bio-active compounds with antimicrobial activity [6], and hence, have attracted the attention of animal and human nutritionists to explore their potential benefits as health supplements to prevent oxidative stress-related diseases and manipulate gut microbiome [7,8]. In another study, Armin et al. [9] reported strong antioxidant activity of marine algae Sargassum sp. and reported that its dietary supplementation improved the stability of broiler meat fatty acids. In another study, it was shown that the marine algae *Spirulina platensis* naturally contained high amounts of carotenoids and can reduce the toxic effects of Aflatoxin in broiler chickens and improve growth performance, immunological functions, and serum biochemical parameters [10]. Seaweeds have the advantage of being a renewable abundant resource that has the potential to contribute to sustainable eco-friendly animal production due to their rich content of bio-active and antioxidant properties. However, there is substantial variation in the bio-active content of different seaweed species or extracts and hence, there is a need to evaluate the potential of different species of seaweed. Further, any alternative to AGP must match the feed efficiency promoting effect of AGPs to become acceptable to farmers. Limited reports are available on the effects of seaweed extracts on feed efficiency, gut pathogen load, and the ability to replace AGPs in broiler chickens. Earlier we observed that performance in broiler chickens was better in groups supplemented with water extracts of red seaweed *Kappaphycus alvarezii* whereas alkaline extracts had no significant effect and the better performance in water extracts supplemented groups was associated with a much higher concentration of antioxidants than that of the alkaline extracts [5]. There is a paucity of information on the efficacy, minimum effective dose, and mode of action of extracts of Eucheuma denticulatum a widely cultivated seaweed species, and its suitability as an alternative to AGP in broiler chickens and also whether limited dosing period in early life can have extended effect on productivity and gut health. The present experiment aimed to evaluate the efficacy of an antioxidant-rich dried aqueous extract of the seaweed species Eucheuma denticulatum on feed efficiency, growth rate, antioxidant status, immunity parameters, and expression of intestinal and growth hormone-related genes in broiler chickens vis-a-vis Virginiamycin, a commonly used AGP.

2. Materials and methods

2.1. Feed additives

2.1.1. Preparation of seaweed extracts

A seaweed extract (PBD5) was prepared by Sea6 Energy Pvt Ltd, Bangalore India using an aqueous extraction method, wherein pulp from red seaweed species *Eucheuma denticulatum* was made into an aqueous solution followed by mechanical separation, concentration, and drying into a powder. This extract is rich in antioxidants, sulfated galactose, and sulfated oligosaccharides (carrageenans). The seaweed used for preparing PBD5 was sourced from Bali, Indonesia.

2.1.2. Other additives

An AGP Virginiamycin was used as a positive control in one experimental group at the dose level recommended by the manufacturers (20 ppm pure Virginamycin in feed).

2.1.3. Animals, treatments, and experimental design

In a completely randomized design, a total of 325 newly hatched male broiler chicks (VenCobb 430Y) obtained from a commercial hatchery (Venkateswara Hatcheries Pvt Ltd, Hyderabad 500,001, India) were assigned to five dietary treatment groups. The birds were randomly divided into 65 pens each measuring 6 ft^2 (stainless steel battery brooder cages) containing 5 birds in each pen and thirteen replicate pens were allotted to each of the 5 dietary treatment groups. The birds were housed in an open-sided poultry house. All the

birds were wing-tagged, weighed, and vaccinated with Marek's disease vaccine on the first day of the feeding trial. All the groups were offered the same basal diet. The control (C) group was not supplemented with either AGP or seaweed extract, whereas the AGP group was fed a diet supplemented with Virginiamycin (V, 20 ppm pure Virginiamycin in feed; as recommended by the manufacturer). The remaining three test groups were supplemented with PBD5 at a dose level of 0.25 g kg^{-1} feed for either 6 weeks i.e. d 1 to d 42 (PBD5_0.25_6wk), or 4 weeks i.e. d 1 to d 28 (PBD5_0.25_4wk) or at a higher dose of 1.0 g kg^{-1} feed for 2 weeks i.e. d1 to d 14 (PBD5_1.0_2wk).

All the chickens were fed the diet ad libitum as per feeding standards recommended by the supplier of the chicken strain (VenCobb 430Y). A detail of diet composition has been presented in Supplementary Table 1. The range of minimum and maximum temperatures in the house during the experiments was 22.2–35.3 °C. Brooding was done at a temperature range of 30–33 °C for up to 21 days with the help of incandescent bulbs. Throughout the trial, all birds had ad libitum access to feed and water. A weighed quantity of feed was offered daily and leftover feed was weighted at weekly intervals. Body weight (BW) was recorded at weekly intervals for each pen and per bird average weight was calculated for each pen. Birds were vaccinated with the New Castle disease Lasota strain vaccine on the 5th and 28th day and with the infectious bursal disease vaccine on the 10th and 16th day. Mortality was recorded daily, and the body weights of the dead bird were recorded. The feed conversion ratio (FCR) was adjusted for mortality and presented as AFCR = feed intake/(weight gain of survivors + weight gain of mortalities).

2.1.4. Metabolism trial

A metabolism trial was conducted during the 4th week of age using the total collection method [11]. Birds from four replicate pens from each group (making a total of 20 birds per treatment) were selected and placed in clean, separate metabolic cages. Adequate but weighed quantity of feed was offered and residual feed was measured dailyTotal excreta were collected, and weighed, and dry matter (DM) content was estimated by drying in a hot air oven at 55 °C for each pen for 3 consecutive days, and the samples were pooled pen-wise for analysis. The nitrogen retention and apparent digestibility coefficients of dry matter (DM) and ether extract (EE) of feed were calculated as per Maynard and Loosli [11].

2.1.5. Chemical analysis of proximate principles

Diets and excreta were analyzed for DM (method 4.1.06; by drying and estimating weight loss), crude protein (method 4.2.03; by Kjeldahl method after acid hydrolysis), ether extract (method 4.8.01; after extraction with petroleum ether by Soxhlet method), and ash (method 4.8.03; by igniting at 550 °C for 3 h in a muffle furnace) contents using the Association of Official Analytical Chemist (AOAC) procedures [12]. The Ca content in diet was measured using an atomic absorption spectrophotometer according to the methods suggested by the manufacturer (AAnalyst 400, PerkinElmer, Shelton, CT, USA). The P content in diet was estimated using a colorimetric procedure [13].

2.1.6. Chemical analysis of seaweed extracts

The phycobilins (phycoerythrin, allophycocyanin, and phycocyanin) content of seaweed extracts was measured as per Aziza et al. [14] with minor modifications as described earlier [5]. Briefly, 400 mg powder of seaweed extract PBD5 was vortexed with 5 mL of phosphate buffer (0.75 mol L^{-1}; pH 7.0) following, which the pigments were separated from residues by centrifugation (2464×*g* at 4 °C for 12 min) and absorbance of the supernatant was measured at 498.5, 614 and 651 nm, respectively and contents of phycoerythrin, allophycocyanin and phycocyanin were calculated as per Kursar et al. [15].

The total phenolic content of the seaweed extracts PBD5 was estimated using the Folin–Ciocalteu reagent as per the method of Ainsworth and Gillespie [16] with minor modifications as described earlier [5].

The antioxidant (free radical scavenging) activity of the seaweed extracts PBD5 was estimated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay method [17] with minor modifications as described earlier [5].

The carrageenan levels in seaweed extracts PBD5 were measured as per Mtolera and Buriyo [18] with minor modifications as described earlier [5].

2.1.7. Cutaneous basophil hypersensitivity test

The cell-mediated immune (CMI) response was assessed during the 34 d of age by a cutaneous basophilic hypersensitivity test using phytohemagglutinin lectin from Phaseolus vulgaris (PHAP) using six birds from each group. Before injection, the injection site (on leg toe web) was marked, thickness of the injection site was measured, and 0.1 mg PHAP in 0.1 ml phosphate-buffered saline was injected intra-dermally. The thickness of the injection site was measured 24 h after injecting the mitogen and the difference in the thickness before and after injection was used as measure of CMI response.

2.1.8. Collection of blood samples and biochemical analysis

Blood samples were taken from the brachial vein of 10 birds/group on d 34 in Vacutainer tubes using a 21-gauge needle and were utilized for the separation of serum. Serum was stored at −20 °C and analyzed for glutathione reductase (as per Bergmeyer et al. [19]), glutathione peroxidase (as per Paglia and Valantine [20]), superoxide dismutase (as per Madesh et al. [21]), and lipid peroxidation (as per Ohkawa et al. [22]). Serum samples were also analyzed for HI titer, IBD-specific antibody titer, and IgG levels as per the procedure given below.

2.1.9. Haemagglutination inhibition (HI) assay

The HI test was carried out on serum samples (10/group) collected at 34 d of age. The ND-specific antibodies were also analyzed in

the serum samples by haemagglutination inhibition (HI) test using 1 % chicken RBCs, as per OIE protocol. The HI titer of the Newcastle disease virus (NDV) antigen (LaSota virus stock) was adjusted by dilution to contain 4 units of haemagglutination activity. The highest dilution of serum samples that inhibited the agglutination of chicken RBCs by NDV was considered as the HI titer.

2.2. IBD-specific antibody titer by ELISA

IBD-specific antibody titers were estimated from serum samples by indirect ELISA by using a commercially available kit (IDEXX Laboratories, USA) following the manufacturer's instructions. Briefly, IBDV antigen pre-coated plates were incubated with 1:1000 diluted serum samples followed by the addition of conjugate and subsequently with the substrate. The color development (OD values) relative to the level of specific antibodies was read at A650 nm. The positive and negative controls provided with the kit were included in the assay. The endpoint titers are calculated with the formula Log 10 titer = 1.09 (Log 10 S/P) + 3.36, wherein S/P is the sample-to-positive ratio. The OD values were converted into titer using the software xCheck Plus (IDEXX Laboratories, USA). The values above the cutoff 396 were considered as the protective titers.

2.2.1. IgG titer

The total IgG antibody titers in serum samples at different time intervals were quantified by using a sandwich ELISA commercial kit by following the manufacturer's instructions (Bioassay Technology Laboratory, UK). Briefly, the chicken IgG antibody precoated ELISA plates were incubated with samples, and subsequently, the biotinylated chicken IgG antibody provided with the kit was added and further added with Streptavidin-HRP. The color development after the addition of substrate was measured at 450 nm. The standards ranging from 4 μg/ml to 64 μg/ml provided with the kit were included and OD values of the standards were used to generate a standard curve. The IgG antibody levels in the samples were calculated from the OD values using the formula derived from the standard curve [$y = 0.4311$ (x) - 0.3586; $R^2 = 0.9376$].

2.2.2. Sample collection, RNA extraction, and qPCR-based mRNA expression analysis

Gene expression analysis in the gut tissue (from jejunum; approximately 1 cm) of chickens was performed for four gene groups as described earlier [5]. Gene expression analysis in hepatic tissues for chicken growth hormone and growth hormone receptors was estimated using primers as described earlier [23]. Briefly, intestinal or hepatic tissues were utilized from 6 birds in each of the groups. Total RNA was extracted using Trizol reagent (15,596,018; Invitrogen Life Technologies) as per the manufacturer's protocol. The quality and quantity of RNA were analyzed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The quality of the extracted total RNA was also checked using Agarose gel electrophoresis.

For each sample, one μg of total RNA was reverse transcribed into complementary DNA (cDNA) using a cDNA synthesis kit (RevertAid first-strand cDNA kit; K1622; ThermoFisher Scientific) as per the manufacturer's recommended protocol. Quantitative Real-time PCR (qPCR) was performed using the Maxima SYBR Green/ROX qPCR master mix, (K0221; ThermoFisher Scientific), and an ABI StepOne qPCR thermal cycler by following the manufacturer's guidelines. Details of qPCR primers and procedures used in this study were the same as reported earlier [5]. The qPCR products were checked for the presence of a single band/product using agarose gel electrophoresis. Expression of two reference (housekeeping) genes, namely glyceraldehydes -3-phosphate dehydrogenase (GAPDH) and beta-actin were also analyzed in each sample using the qPCR protocol.

The relative gene expression levels of each target were calculated using the $2^{-\Delta\Delta}$Ct method [24]. For normalization of the expression levels of the target genes, the geometric mean of two housekeeping genes: glucose-6-phosphate dehydrogenase (GAPDH) and beta-actin (ACTB) were utilized as described by Taylor et al. [25].

2.2.3. Gut content sample collection

Ten healthy chickens (on the 43rd day of age) were selected (one per pen) per group, and euthanized by cervical dislocation.

The gut was cut and opened using sterile scissors and luminal contents from the duodenum to the cloaca including caeca were collected into sterile storage vials, placed at 4 °C and utilized for a culture-based count of *E. coli* and Salmonella within 2–3 h.

2.2.4. Quantification of E.coli and Salmonella by colony counting

The fecal samples were collected from 10 chickens per group and utilized for a culture-based count of *E. coli* and Salmonella within 2–3 h. Similarly, caecal contents collected at 43 d were also utilized for counting *E. coli* and Salmonella.

The weighed quantity of fecal samples or caecal samples was serially diluted in 4.5 ml phosphate-buffered saline at a 1:9 ratio. The diluted fecal samples (50 μl) were plated on agar plates containing specific medium (EMB agar for E. coli and XLD agar for Salmonella). The plates were incubated at 37 °C for 24 h, and colonies were counted and then back-calculated through dilution factors to CFU/g feces.

2.2.5. Statistical analysis

The outlier data points, if any, were removed by the modified Thompson-Tau method [26]. The data on BWG, FCR, slaughter parameters, digestibility, bacterial count, and blood biochemical and immunological parameters were analyzed by one-way analysis of variance using SPSS [27]. Differences between pairs of means were tested using Duncan's multiple range test at $P < 0.05$. Before statistical analysis, all expression data were tested for equal variance (Levane's test) and normality (Shapiro-Wilk test). For statistical analysis, normalized gene expression data were square-root-transformed [28] to satisfy the requirement of the equal variance condition. Expression data were analyzed by using the nonparametric Kruskal-Wallis test followed by the Dunn post hoc test. P-values

were also subjected to FDR adjustment. All expression data presented in Fig. 1 are the geometric mean of untransformed relative mRNA levels [25]. The stability of reference genes was checked using NormFinder [29].

3. Results

Details of the composition of experimental diets have been presented in Supplementary Table 1.

3.1. Chemical composition of seaweed extracts

The seaweed extract contained 135 ± 7.2 mg g^{-1} total phenolics, 2.49 ± 0.003 mg g^{-1} phycoerythrin, 0.525 ± 0.007 mg g^{-1} allophycocyanin, 0.403 ± 0.004 mg g-1, carragenan, 51 ± 0.4 % and 46 ± 0.10 % free radical (DPPH) scavenging activity.

3.2. Effects of additives on growth and feed conversion ratio

From d 1 to d 21, there was no significant effect of treatments on BW or AFCR as compared to the control (Table 1).

However, cumulative AFCR during d 1 to d 28 was significantly lower (birds with a lower value of AFCR are considered more efficient) in the PBD5_1.0_2wk group as compared to the control, whereas in the V group, the AFCR value was statistically comparable but numerically lower than that of the control group. During d 1 to d 35, the AFCR was significantly lower in both the PBD5_1.0_2wk (3.87 %) and the V (2.77 %) groups as compared to the control. The cumulative BWG was not significantly influenced by treatments at 28 or 35th d. During d1 to d 42, there was no significant difference in BWG among groups, but numerically BW was the highest in the V group, followed by the PBD5_1.0_2wk group and the lowest in the PBD5_0.25_6wk. During d1 to d 42, cumulative AFCR values differed significantly among the groups with the highest AFCR value in the PBD5_0.25_6wk group and the lowest AFCR value in the PBD5_1.0_2wk and V groups, whereas the numerical difference in AFCR between the control and the PBD5_1.0_2wk or the V groups was 3.6 %.

3.3. Carcass traits

None of the carcass traits or slaughter variables was affected by dietary treatments. The weight of the liver, spleen, gizzard, and bursa was comparable among the groups (data not presented).

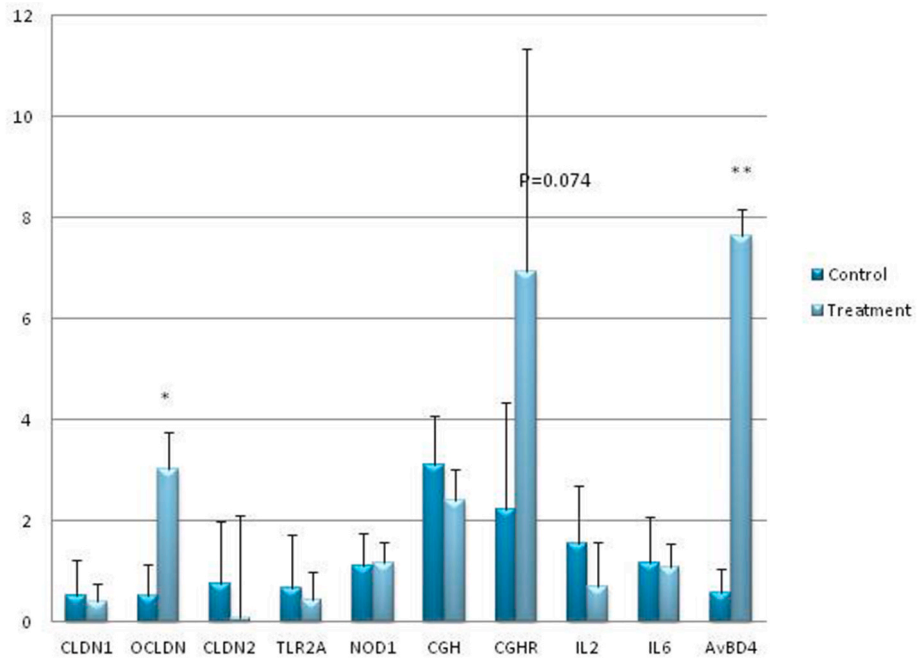

Fig. 1. Effect of supplementing seaweed extract (PBD5 @ 1.0 g kg^{-1} diet for 14 d) on relative normalized gene/mRNA expression in chicken. CLDN1, claudin; OCLDN, occludin; CLDN2, Claudin 2; TLR2A, toll like receptor 2A; NOD1, Nucleotide binding oligomerization domain 1; CGH: chicken growth hormone; CGHR: chicken growth hormone receptor; IL2, interleukin 2; IL6, interleukin 6; AvBD4, avian beta defensin 4. *P < 0.05; **P < 0.01. Error bars indicate standard error mean.

Table 1
Effect of supplementing feed additives on performance of broiler chicken.

	C	V	PBD5_ 0.25_6wk	PBD5_ 0.25_4wk	PBD5_ 1.0_2wk	SEM	N	P-Value
Performance								
1-14 d								
BWG	406	408	407	403	400	2.89	13	0.938
AFCR	1.19	1.19	1.18	1.20	1.19	0.004	13	0.817
1-21 d								
BWG	876	878	883	895	871	6.88	13	0.875
AFCR	1.25	1.22	1.22	1.22	1.23	0.006	13	0.989
1-28 d								
BWG	1540	1560	1537	1557	1541	7.86	13	0.891
AFCR	1.33a	1.30 ab	1.32a	1.32a	1.27b	0.005	13	0.010
1-35 d								
BWG	2192	2245	2191	2218	2222	10.5	13	0.545
AFCR	1.44a	1.40bc	1.44a	1.43 ab	1.39c	0.005	13	0.009
1-42 d								
BWG	2829 ab	2959a	2771b	2825 ab	2872 ab	19.8	13	0.085
AFCR	1.53 ab	1.47b	1.56a	1.53 ab	1.48b	0.008	13	0.008

C, control (basal diet); V, Virginiamycin (20 ppm); PBD5_0.25_6wk, PBD5 at 0.25 kg/ton for entire 6 week; PBD5_0.5_4wk, PBD5 at 0.5 kg/ton for first 4 week; PBD5_1.0_2wk, PBD5 at 1.0 kg/ton for first 2 week BWG:body weight gain; AFCR: feed intake/body weight gain adjusted for mortality; P: probability, N:number of replicates; SEM, standard error of the mean; Means having common superscripts in a row do not vary significantly ($P < 0.05$).

3.4. Immune responses

The HI titer against NDV was significantly lower in group V and PBD5_0.25_6wk as compared to the control. However, HI titer against NDV in the remaining groups was comparable to that of the control (Table 2).

The IBD ELISA titer values were not significantly influenced by treatments. The serum IgG levels were significantly higher in the group PBD5_1.0_2wk as compared to the control. The cell-mediated immune response (skin thickness response against PHAP) was comparable among the groups.

3.5. Serum antioxidant enzymes

The serum glutathione peroxidase level was significantly higher in the PBD5_1.0_2wk and PBD5_0.25_6wk groups as compared to the control (Table 3).

The serum glutathione reductase and FRAP levels were not influenced significantly by the treatments. The serum superoxide dismutase level tended to be lower and lipid peroxidation level tended to be higher in the V group as compared to the control.

3.6. Mortality

Mortality was negligible and random among groups (Table 3).

Table 2
Effect of feeding sea plant extracts on immunity parameters and pathogen count.

Group	IBD ELISA titre at 42 d	IgG (ug/ml) at 34 d	HAtitre (log 2) at 34 d	Faecal Ecoli count 25 d (log10)	Caecal E coli count 43 d (log10)	Faecal Salmonella count 25 d (log10)	Caecal Salmonella count 43 d (log10)	CMI (PHAP response, mm) at 34 d
C	3019	8.91a	4.1b	4.82 d	3.91 ab	0.538	0.478	1.02
V	1915	10.1 ab	2.5a	3.80b	3.57a	0.520	0.490	1.08
PBD5_ 0.25_6wk	2085	9.66a	2.6a	3.36 ab	4.42c	1.58	0.23	0.993
PBD5_ 0.25_4wk	2426	10.6 ab	3.9b	3.40 ab	4.34c	0.919	1.22	0.937
PBD5_ 1.0_2wk	1819	13.7b	3.7b	3.27a	4.21bc	0.888	0.765	1.10
SEM	669	1.29	0.372	0.143	0.13	0.397	0.322	0.038
P-value	0.627	0.043	0.001	<0.001	<0.001	0.192	0.142	0.904
N	10	10	10	10	10	10	10	10

C, control (basal diet); V, Virginiamycin (20 ppm); PBD5_0.25_6 wk, PBD5 at 0.25 kg/ton for entire 6 week; PBD5_0.5_4wk, PBD5 at 0.5 kg/ton for first 4 week; PBD5_1.0_2wk, PBD5 at 1.0 kg/ton for first 2 week BWG; CMI, cell mediated immune response; PHAP, phytohemagglutinin lectin from Phaseolus vulgaris.

Table 3
Effect of additives on serum antioxidant enzyme profile and mortality.

Group	GSPx (U/ml sera)	GSRx (U/ml sera)	SOD (U/ml sera)	FRAP (μmol Fe^{2+} formed per litre sera)	Lipid peroxidation level (nM MDA formed/ml sera)	Mortality
C	726a	1201	7.25 ab	874	3.71 ab	2
V	710a	1374	4.57a	878	5.08b	4
PBD5_ 0.25_6wk	817b	1382	9.78b	1165	2.59a	4
PBD5_ 0.25_4wk	782 ab	1325	9.63b	988	3.74 ab	6
PBD5_ 1.0_2wk	818b	1497	6.83 ab	990	3.73 ab	2
SEM	24.3	149	1.51	97	0.35	
N	10	10	10	10	10	
p-value	0.014	0.725	0.095	0.218	0.092	

C, control (basal diet); V, Virginiamycin (20 ppm); PBD5_0.25_6 wk, PBD5 at 0.25 kg/ton for entire 6 week; PBD5_0.5_4wk, PBD5 at 0.5 kg/ton for first 4 week; PBD5_1.0_2wk, PBD5 at 1.0 kg/ton for first 2 week BWG; GDPx, Glutathione peroxidase; GSRx, Glutathione reductase; SOD, Superoxide dismutase; FRAP, Ferric reducing antioxidant Power; GSPx Unit: amount of GPx enzyme required to cause the oxidation of 1 μmol of NADPH per min; GSRx Unit: amount of GSRx enzyme required for oxidation of 1 micro mol NADPH per min; SOD unit: amount of SOD capable of 50 % inhibition of the MTT reduction reaction/min.

3.7. Gut pathogen count

At d 25 of age fecal *E. coli* count was significantly lower in all the treatment groups (seaweed extracts as well as Virginiamycin) as compared to the control with the lowest count in the PBD5_1.0_2wk group followed closely by the other two seaweed groups (Table 2). However, at 43 d of age, the caecal *E. coli* count in the V and PBD5_1.0_2wk was comparable to that of control but in the groups, PBD5_0.25_6wk and PBD5_0.25_4wk, the count was higher than in control. Salmonella in feces at d 25 and in caecal content at d 43 was very low and not influenced by the treatments.

3.8. Total tract retention response of nutrients

The DM and CP digestibility was not influenced significantly by the treatments, but EE digestibility was significantly higher in all the treatment groups than in control with higher values recorded in the seaweed extract groups than in the Virginiamycin group (Table 4).

3.9. Gene expressions in the chicken gut/hepatic tissue

The relative normalized expression of barrier-forming gene occludin was significantly higher in the PBD5_1.0_2wk group (Fig. 1).

However, the relative normalized expression of the pore-forming claudin 2 gene was not significantly influenced by the seaweed extract. The relative normalized expression of pattern recognition receptor (PRR) genes like toll-like receptor 2A and nucleotide-binding oligomerization domain 1 were also not significantly influenced by the treatment. The relative normalized expression of the host defense peptide gene avian beta-defensin 4 was significantly higher in the treatment group as compared to the control. The relative normalized expression of cytokines interleukin 2 and interleukin 6 was comparable between the treatment and control. The relative normalized expression of the hepatic chicken growth hormone gene was not influenced significantly by the seaweed extract supplementation but the expression of the hepatic growth hormone receptor gene tended to be higher in the seaweed extract group. The AGP had no significant effect on gene expression parameters (data not presented).

Table 4
Effect of diet on total digestive tract nutrient retention percentage.

Group	DM	EE	CP
C	72.1	81.8a	68.6
V	72.8	83.3b	70.1
PBD5_ 0.25_6wk	71.6	85.3c	70.2
PBD5_ 0.25_4wk	71.9	84.9c	68.0
PBD5_ 1.0_2wk	72.4	85.4c	70.2
SEM	1.05	0.689	0.645
p-value	0.732	<0.001	0.139

C, control (basal diet); V, Virginiamycin (20 ppm); PBD5_0.25_6 wk, PBD5 at 0.25 kg/ton for entire 6 week; PBD5_0.5_4wk, PBD5 at 0.5 kg/ton for first 4 week; PBD5_1.0_2wk, PBD5 at 1.0 kg/ton for first 2 week BWG; DM, Dry matter; EE, Ether extract; CP, Crude protein.

4. Discussion

The AMR associated with the feeding of AGPs represents a great global public health concern for poultry production. The present study indicated that the aqueous extract (PBD5) of the red algae Eucheuma denticulatum contained a high concentration of total phenolics, phycobilins (phycoerythrin, allophycocyanin, phycocyanin), carrageenan and had strong antioxidant/free radical scavenging activity (46 % by DPPH method). Harb et al. [33] reported that the concentration of phenolic compounds and phycobilins exhibited a positive correlation with the antioxidant activities of the seaweed (macroalgae) extracts and seaweeds are a potential source for obtaining extracts with high antioxidant properties. Red seaweeds are known to contain sulfated polysaccharides like carrageenan in the range of 360–660 g/kg DM, [34]. The concentration of carrageenan in the aqueous extract (PBD5) of the red algae Eucheuma denticulatum was established at 403 g kg^{-1} in this study.

The present study indicated that the aqueous extract (PBD5) of the red algae had no significant effect on the growth of broiler chickens. Earlier, Mariey et al. [35], Shanmugapriya et al. [36], and Byoung et al. [37] reported that 10 g kg−1 dried microalgae in the diet had a positive effect on BW gain in broiler chickens. Akinyemi and Adewole [38] reported that supplementation of brown seaweed (*Ascophyllum nodosum*) meal in broiler chicken diet at the rate of 2 % in feed increased BW significantly but FCR was not affected. Conversely, 0.5–2 % brown seaweed in broiler chickens' diet did not influence the ABG, FI, or FCR of broiler chickens in the study by Bonos et al. [39]. However, it is noteworthy that due to high fiber and total ash content, intact red seaweeds are difficult to be included in poultry diet directly beyond 1–5 % level due to potential dilution of nutrients and mineral imbalance. The results of these studies cannot be directly compared to the present study and limited reports are available on the effect of extracts of seaweeds on broiler chicken.

In the present study, seaweed extract had a significant effect on AFCR after 21 d of age. However, stimulation of feed efficiency was seen only in the group supplemented with PBD5 @ 1 g kg^{-1} diet but not in the lower dose groups. It is noteworthy that the effect of dosing @ 1 g kg^{-1} for two weeks persisted beyond the dosing period (at least up to 35 d). This may be important for poultry producers from a practical point of view as supplementing only up to 14 d with an adequate dose is expected to be sufficient to maintain a beneficial effect on AFCR for the rest of the production cycle, which in turn will reduce the cost of the supplementation. The present report is the first report to indicate the persistence of the positive effect of seaweed extract on Feed efficiency beyond the dosing period Earlier, Mariey et al. [35] and Evans and Critchley [40] reported that microalgae had a positive effect on FI and FCR. On the other hand, Armin et al. [9] showed that use of intact marine algae Sargassum sp. at the rate of 5 and 10 % in the diet resulted in poor feed efficiency as compared to the control.

In the present study, the humoral immunity in terms of HI titer against NDV and cellular immune response in terms of PHAP response was not influenced by the treatments. However, the IgG level was significantly higher in the group supplemented with PBD5 @ 1 g per kg diet for 14 d. Kang et al. [41] also reported that the microalgae Chlorella have some beneficial effect on immune characteristics, i.e. the number of white blood cells (WBCs), and lymphocytes, and the concentrations of immunoglobulin A (IgA), IgM, and IgG. Earlier, Al-Khaaifah et al. [42] observed that the supplementation of Sargassum sp at 1 and 2 % in diet enhanced IgA titer significantly, whereas Gracilaria sp at 5 % enhanced IgY antibody titers significantly.

There are few references in the literature about the effect of seaweed extracts on antioxidant enzymes. The serum glutathione peroxidase levels increased significantly in some of the seaweed extract groups. Rajauria et al. [43] reported a significant increase in glutathione, SOD, and catalase activities, and reduced values of lipid peroxidation in the serum of piglets fed brown seaweeds. On the other hand, Wan et al. [44] have reported no significant effect of feeding seaweeds on serum lipid peroxidation. An increase in serum glutathione peroxidase as observed in the present study may be attributed to the higher level of antioxidants present in the seaweed extract.

In the present study, significant improvement in EE digestibility was observed in the group fed with the aqueous extract of the red seaweed as compared to the control. Some of the seaweed species were shown not to affect digestibility in pigs, whereas some other species showed a positive effect on N and gross energy digestibility [31]. Sweeney et al. [45] suggested that the improvement in nutrient digestibility is related to the influence of the seaweed carbohydrates and antioxidants, on microbiota and the villous microstructure with an increase in absorptive capacity and nutrient transporters. These effects are also likely to be related to the trophic effect of the volatile fatty acid produced (i.e. butyric acid) on the intestinal mucosal cells in response to changes in microbial composition or activity due to phytoactive compounds present in seaweed extracts.

The present study indicated that seaweed extracts can significantly reduce potential pathogens such as *E. coli* at the 25th day of age and higher doses had higher suppression but such effects of seaweed extract or virginiamycin were nonsignificant at 43 d. Earlier, Del Tuffo et al. [46] reported that supplementation of red seaweed rich in sulfated polysaccharides such as carrageenan inhibited pathogenic bacteria like *E. coli* and *Clostridium difficile*, modulated gut environment, stimulated the innate immune system, and promoted productivity.

Reports on the effect of red seaweed extracts on intestinal gene expression in the chicken are limited. Tight junction proteins play an important role in the regulation of gut barrier function. In this study, expression of barrier-forming gene occludin was significantly higher in the best-performing treatment group (PBD5_1.0_2 wk) as compared to the control. Host defense peptides such as avian beta-defensin 4, also known as antimicrobial peptides are considered frontline immunomodulators for their multifunctional roles in both innate and adaptive immune responses [47]. In this study, increased expression of host defense peptide Avian beta-defensin 4 in broiler chickens on feeding aqueous extract of the red seaweed at the dose level of 1.0 g/kg for 2 weeks in the diet indicated that the red sea-weed extract promoted the innate and adaptive immune responses which could have a significant bearing at field level in combating infection and reducing mortality. A trend of improvement in the expression of hepatic growth receptor genes on seaweed supplementation indicated the possible mechanism of action of seaweed extracts, which involves modulation of GH binding in the

target tissue. However, this aspect requires further detailed study for further clarity. It is noteworthy that gene expression data that pertains to the 43rd d of the experiment which corresponds to 29 d after the dosing period, point out the medium-term effect of seaweed extract well beyond the dosing period, which corroborates with the persistent improvement recorded in feed efficiency beyond the dosing period in the group.

There was no significant difference in carcass cut-up parts and weight of immunity-related organs such as spleen or bursa among the groups. Earlier, Lokaewmanee et al. [48] and Akinyemi and Adewole [38] also reported that there was no significant difference in carcass parameters, cut-up parts, and organ weights after dietary supplementation of seaweed in broiler chickens.

Large variation in response to AGP use has been reported in the literature. In a meta-analysis on AGP involving 174 scientific articles containing 183 experiments on broiler chickens, Maria et al. [49] reported that on average, AGP supplementation resulted in higher weight gain (3.84 %) and lower feed conversion ratio (3.48 %) during the overall experimental period (d 1 to d 42). In an earlier study, we observed that supplementation of broiler chicken diets with AGP did not exert any effects on BW or AFCR except for the 1–21 d period in one of the three feeding trials [2]. In the current study, the AGP had a significant effect on AFCR during d1 to d 35 but the seaweed extract also exerted comparable performance to that of the AGP when used at the dose level of 1 g kg^{-1} for 14 d.

Observed positive effects of the red sea algae extract on feed efficiency, expression of gut immunity and porosity-related genes, gut pathogen density and overall well-being can be largely attributed to its high content of phycobillins, antioxidants, phenolics, and prebiotic carrageenan. Phycobillins have been shown to have numerous health benefits due to their anti-inflammatory, antimicrobial, immunomodulatory, hepatoprotective, nephroprotective, and antioxidant properties [30]. Besides antioxidant effects, most phenolics have also been shown to have a wide variety of biological activities such as antibacterial, antiviral, anti-inflammatory, anti-cancer, cardioprotective, and immune system-promoting effects [31,32]. The prebiotics are known to improve villus height and density of cellulolytic and beneficial bacteria, which improves lactate consumption and pH of the gut and supports probiotic bacteria [10].

This is the first report on effect of the aqueous extract of the red algae Eucheuma denticulatum on growth, feed efficiency, immunity, gut pathogen load and expression of various genes in fast growing broiler chickens. Ealier, some researchers have studied impact of use of different other intact seaweeds on chickens with limited report on expression of gut immunity and porosity related genes. Extracts of the seaweed can be more practical for use as feed additive as compared to intact sea weeds as it does not cause nutrient dilution effect and is effective at very low dose. Moreover, this study has indicated that dosing with the extract for only first two week is adequate as the beneficial effect of the dosing persists for the entire production cycle. An epigenetic modification mechanism might be behind such a sustained effect, which would require further detailed investigation in future.

5. Conclusions

The present study indicated that the antioxidant-rich dried aqueous extracts of the seaweed *E. denticulatum* can improve feed efficiency, EE digestibility, serum IgG levels, and some of the antioxidant enzymes, and reduce the density of potential gut pathogens with some of these effects persisting beyond the dosing period, and the extent of performance improvement was comparable to that of an antibiotic growth promoter. The beneficial effects on performance are likely to be partly attributed to the improvement in digestibility and overall health conditions of animals due to the improvement in antioxidant status or changes in the expression of gut immunity and barrier function genes and GH receptor genes in the liver. Hence, dried aqueous extract of *E. denticulatum* can be used as an alternative to antibiotic growth promoters in broiler chickens.

Data availability statement

All data are available within the manuscript and supplementary material.

Ethics statement

The study protocol was approved by the Institute Animal Ethics Committee of ICAR-Directorate of Poultry Research vide its approval number IAEC/DPR/20/8 dated November 20, 2020.

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CRediT authorship contribution statement

Shyam Sundar Paul: Writing – original draft, Investigation, Data curation, Conceptualization. **Kannaki Thattanthottam Ramasamy:** Investigation, Data curation, Conceptualization. **Hemanth Giri Rao Vantharam Venkata:** Writing – review & editing, Conceptualization. **Savaram Venkata RamaRao:** Writing – review & editing, Conceptualization. **Mantena Venkata Lakshmi Narasimha Raju:** Writing – review & editing. **Sinduja Ramanan:** Writing – review & editing, Conceptualization. **Sri Sailaja Nori:** Writing – review & editing, Conceptualization. **Shrikumar Suryanarayan:** Writing – review & editing, Conceptualization. **Godumagadda Narender Reddy:** Methodology, Investigation, Formal analysis. **Prakki Santosh Phani Kumar:** Investigation, Data curation. **Cadaba Srinivas Prasad:** Writing – review & editing, Conceptualization. **Rudra Nath Chatterjee:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25219.

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