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Exploring the efficacy of a novel prebiotic-like growth promoter on broiler chicken production performance

Zulfqarul Haq [a,*], Ankur Rastogi [b], Ramesh Kumar Sharma [b], Pratiksha Raghuwanshi [c], Maninder Singh [d], Azmat Alam Khan [e], Syed Mudasir Ahmad [f]

[a] Indian Council of Medical Research Project, Division of L.P.M, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, India
[b] Division of Animal Nutrition, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, India
[c] Division of Veterinary Biochemistry and Physiology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, India
[d] Department of Veterinary Public Health and Epidemiology, Guru Angad Dev Veterinary and Animal Sciences University, Rampura Phul, India
[e] Division of LPM, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, India
[f] Division of Animal Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, India

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ABSTRACT

This study attempts to isolate a candidate growth promoter from the ovine paunch waste and scrutinize its effects on the production performance of broiler chickens as compared to mannan-oligosaccharide (MOS), a prebiotic, and lincomycin, an antibiotic growth promoter (AB). The paunch waste collected from slaughtered sheep was processed to remove particulate matter. The clarified liquid was then added to an excess of ethanol (1:9 ratio), and the resultant precipitate {(novel growth-promoting paunch extract (NGPE)} was collected, dried, and stored. *In vitro* increase in cell density for probiotic bacteria *viz. Lactobacillus rhamnosus* and *Enterococcus faecalis* (Log_{10} CFU/ml) were significantly higher ($P < 0.01$) in NGPE supplemented media (2.78 ± 0.11 and 2.77 ± 0.10) as compared to that on MOS (1.28 ± 0.05 and 2.49 ± 0.09) and glucose (1.09 ± 0.04 and 1.12 ± 0.04) supplemented media. In the in-vivo trial of six weeks duration with broiler chickens (Cobb-400), NGPE supplementation resulted in significantly higher growth in weeks IV ($P < 0.05$) and VI ($P < 0.01$) of age in comparison to MOS and AGP supplemented groups, a lower ($P < 0.01$) cumulative feed conversion ratio in comparison to MOS supplemented groups, and a higher ($P < 0.01$) cumulative protein efficiency ratio compared to MOS and AGP supplementation. NGPE supplementation also lowered lipid peroxidation ($P < 0.01$), increased reduced glutathione activity ($P < 0.01$) in chicken erythrocytes, and boosted the lactic acid bacteria count in the cecal contents ($P < 0.01$). This is the first report of the isolation of a paunch waste extract that increased the *in vitro* growth of probiotic bacteria and improved the production performance of broiler chickens.

Introduction

Hindgut microbes and their fermentation end-products have both local and systemic influence over the physiological functions of poultry (Rinttilä & Apajalahti, 2013). Modifying the gut microbiome's physiology has emerged as one of the most promising interventional tools to optimize the performance of poultry birds. This is attempted traditionally via dietary supplementation of antibiotic growth promoters and, more recently, through various microbial feed additives or organic compounds.

Agriculture intensification increased antibiotic usage by more than 36 % in 71 countries, with Brazil, Russia, India, China, and South Africa (BRICS) accounting for more than 75 % of this increase (Tiseo et al., 2020). The European Union banned antibiotics as growth enhancers in food animals in 2006 by implementing Regulation (EC) No. 1831/2003. Almost immediately after that, the FDA (2017) announced that it would implement legislation called the Veterinary Feed Directive (VFD), which restricts the use of antibiotics in livestock production (Abd El-Hack et al., 2020). However, there are no regulations in low- and middle-income countries, wherein widespread use of antibiotics in the animal industry is increasingly linked to the emergence and spread of antibiotic-resistance genes within retail animal products, eventually

Abbreviations: NGPE, Novel growth-promoting paunch extract; MOS, Mannan-oligosaccharide; AB, Antibiotic growth promoter; CO, Basal diet; BWG, Body weight gain; FCR, Feed conversion ratio.

* Corresponding author.
E-mail address: zulfy27@gmail.com (Z. Haq).

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being added to the human microbiome (Murray et al., 2021). Owing to increased consumer awareness and a progressively stringent regulatory framework, researchers and the poultry industry are desperately searching for effective and long-lasting alternatives to antimicrobial growth promoters (Abd El-Hack et al., 2021).

Probiotics, prebiotics, and synbiotics are fast emerging as the most viable alternatives to antibiotic growth promoters. Prebiotics are now well accepted as an effective tool to strengthen the beneficial bacteria community in the gut of poultry while concurrently limiting the population of gut pathogens (Kim et al., 2019). This is generally manifested as an improvement in growth and feed utilization (Micciche et al., 2018) and is achieved through improved immunity and reduced disease instances (Shehata et al., 2022; Zhang et al., 2021).

Gibson and Roberfroid (1995) gave the initial idea of prebiotics as 'non-digestible food ingredients that have a beneficial effect on the host by selectively stimulating already existing bacterial species growth and activity in the colon, therefore attempting to improve host health'. In its present avatar, the prebiotics spectrum also includes non-carbohydrate substances and could deliver health benefits at body sites other than the GIT (El Jeni et al., 2021; Gibson et al., 2017). This widening of the prebiotic umbrella opens up several possibilities for discovering and screening potential prebiotic candidates, focusing on ease of preparation, availability, and cost of production.

The rumen consists of a large number of microbial groups acting synergistically and performing bioconversion of feedstuffs that primarily consist of complex polysaccharides of plant origin. It may be hypothesized that the presence of polysaccharides along with fiber degrading enzymes in rumen would lead to the substantial presence of soluble oligosaccharides at any given point of time in rumen liquor. It is possible to extract these soluble fibers from the rumen liquor with minimal processing. Further, a varied population of commensal microbes exist in the rumen, which preferentially utilize fermentation by-products of co-existing microbial species. Therefore, there is a possibility that supplementation with a soluble fiber-based extract from the rumen liquor may positively alter the hindgut microbial ecosystem of poultry. Harvesting rumen liquor from the slaughterhouse, where it is available as paunch waste, also minimizes the cost of procurement of raw materials.

Material and methods

Ethics statement

The animal experiment was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of SKUAST-Jammu (approval no.16/IAEC-20/2020), which aligns with ARRIVE's guidelines. All experimental protocols and procedures were carried out according to relevant regulations and guidelines established by this committee, and all efforts were made to minimize the suffering of the experimental chickens.

The complete study was divided into three phases described in Table 1:

Table 1
Phase and objectives of the experiment.

Phase	Objective
Phase-I	Extraction of growth-promoting paunch extract (NGPE) from ovine rumen paunch waste collected from the slaughterhouse.
Phase-II	To study the *in vitro* effects of NGPE on pure cultures of *Lactobacillus plantarum, Lactobacillus rhamnosus, Enterococcus faecalis,* and *Escherichia coli* concerning commercially available prebiotic mannan oligosaccharides (MOS).
Phase III	To study the effects of NGPE in broiler chickens as a growth-promoting feed additive compared to commercially available prebiotic MOS and an antibiotic growth promoter (lincomycin).

Phase-I: standardizing the protocol for extraction of growth-promoting extract from paunch waste collected from the slaughterhouse

Paunch waste of ovine origin containing rumen liquor and solids was collected in large plastic containers (20 l capacity) from freshly slaughtered animals at a local slaughterhouse early in the morning. The collected paunch waste from different animals was pooled and transported to the laboratory. At the laboratory, the waste was repeatedly filtered through single, double, and then quadruple layers of muslin cloth to remove the solid undigested fiber and feed. The liquid collected from the straining of paunch waste is then centrifuged to remove finer particulate matter using a tabletop centrifuge for 7 min at 3500 rpm at ambient temperature. Post-centrifugation, the supernatant is collected, and the pellet is discarded. The collected supernatant rumen liquor is then reduced in volume by one-third by evaporating under reduced pressure by using a rotary vacuum evaporator with a temperature set at 78 °C. This step was done to reduce the volume of ethanol used in the next step. The concentrated rumen liquor is mixed with ethanol (extra neutral alcohol, 96.5 % ethanol *v/v* minimum) in a 1:9 ratio (1-part concentrated rumen liquor to 9 parts ethanol). The extra-neutral alcohol was sourced from the State Excise Department under a research license. The mixture is allowed to stand for six hours. After that, the supernatant alcohol is discarded. A diffused precipitate will form immediately. To settle the precipitate, the mixture is allowed to stand for six hours undisturbed. The supernatant is then discarded carefully without disturbing the precipitate. The precipitated mixture is then centrifuged in a tabletop centrifuge for 4 min at 3000 rpm at ambient temperature, and the pellet is collected. The collected pellet is dried overnight at 40 °C in a hot air oven. The dried pellet is then ground to a fine powder to 1 mm sieve size. The powder is ready for use or can be stored in an airtight plastic container at room temperature (Fig. 1). This procedure is now patented under Indian patent no. 439,666.

Phase- II: in vitro efficacy of NGPE over the growth of probiotic bacteria

The prepared NGPE was tested *in vitro* to measure its ability to support or regress the growth of pure cultures of *Lactobacillus plantarum* (MTCC 1407), *Lactobacillus rhamnosus* (MTCC 1408), *Enterococcus faecalis* (MTCC no. 439) and *Escherichia coli* (MTCC no. 443). As compared to commercial prebiotic (Mannan oligosaccharide; MOS) and glucose (control).

The *Enterococcus* and *Lactobacillus* cultures were maintained at −80 °C in MRS Broth (HiMedia Pvt. Ltd., India), and *E. coli* cultures were held at −80 °C in Tryptic Soy Broth [TSB; (HiMedia Pvt. Ltd., India)], followed by incubation at 37 °C for 24–48 h.

The assay was performed by adding 1 % (vol/vol) of an overnight culture of each probiotic bacterial strain, except *E. coli*, to separate tubes containing 10 ml MRS Broth containing 1 % (wt/vol) of glucose

Fig. 1. Novel growth-promoting paunch extract.

(HiMedia Pvt. Ltd., India), MOS (Bio-MOS®Alltech) or NGPE.

For the *E. coli* strain,1 % (vol/vol) of the overnight culture in TSB was transferred to 10 mL of M9 Minimal Medium broth (HiMedia Pvt. Ltd., India) containing 1 % (wt/vol) of glucose (HiMedia Pvt. Ltd., India), MOS (Bio-MOS®Alltech) or NGPE.

All the tubes were then incubated overnight at 37 °C in an ambient atmosphere for 24 h in a BOD shaking incubator. The cell counts as colony-forming units per ml of the media (CFU/ml) were counted using a colony counter by serially diluting the culture and then plating their 10^{-5} and 10^{-6} dilutions on MRS agar plates for *Lactobacillus* and *Enterococcus* and on tryptic soya agar plates for *E. coli* at 0 h (pre-incubation) and 24 h post-incubation. The CFU count per ml was expressed as $\log_{10}$ values. Growth of bacterial cultures was expressed as an increase in cell densities of bacterial cultures (Log_{10} CFU/ml) calculated as a difference in CFU of 0hr and 24 h. Each assay was replicated three times.

Prebiotic activity score (PAS) was calculated based on the growth of probiotic bacteria {*Lactobacillus plantarum* (MTCC 1407), *L. rhamnosus* (MTCC 1408), and *Enterococcus faecalis* (MTCC no. 439)} and intestinal pathogen {*E. coli* (MTCC no. 443)} over MOS or NGPE supplemented media. The PAS for each probiotic bacterial strain was calculated using the following formula (Huebner, Wehling & Hutkins, 2007), wherein NGPE and MOS were taken as the prebiotics.

$$\textbf{PAS} = \frac{(\text{Probiotic on prebiotic @ 24 h} - - \text{0 h})}{(\text{Probiotic on glucose @ 24 h} - - \text{0 h})} - \frac{(\textit{Escherichia coli} \text{ on prebiotic @ 24 h} - - \text{0 h})}{(\textit{Escherichia coli} \text{ on glucose 24 h} - - \text{0 h})}$$

The values used in the formula were log10 CFU/ml for all the bacteria.

Phase III: in vivo trial using NGPE as a feed additive compared to MOS and lincomycin in broiler chickens

Five hundred and twenty commercial, sexed male, day-old Cobb-400 broiler chicks of the same hatch were procured from a commercial hatchery. After two days of common brooding and acclimatization, four hundred and eighty chicks of comparable body weight were selected and equally distributed randomly into four dietary treatment groups *viz.* CO, MOS, NGPE, and AB, as per the completely randomized design with six replicates per group having 20 birds per replicate. The experimental setup is detailed in Table 2.

Brooding of chicks was done for the first ten days by maintaining the temperature of the farm at around 32–35 °C, and a standard protocol for broiler chicken vaccination was followed. Chicks were fed corn-soybean-based pre-starter (fed from 1 to 14 days), starter (fed from 15 to 21 days), and finisher (fed from 21 to 42 days) diets, formulated as per ICAR (2013) (Table 3). The diets for MOS, NGPE, and AB groups were supplemented with the respective feed additives as given in Table 2. CO group chickens were fed unsupplemented diets.

The chicks were kept in deep litter pens with a separate pen per replicate. A drinker and a feeder were provided per replicate. Each bird had an average floor space of about 1 feet. The body weight (g) of the chicks was recorded at the start of the experiment and then at weekly intervals using a digital weighing scale. The difference between weights of consecutive weeks was calculated as weekly body weight gain.

Feed intake per bird was recorded every week by subtracting the left-over feed in the feeder from the total feed offered in that week and then dividing it by the number of birds in the replicate.

The feed conversion ratio was calculated by the formula as feed intake per bird (g)/ body weight gain (g).

Protein efficiency ratio was calculated by dividing crude protein intake per bird (g) {feed intake (g) x (Crude protein percent in feed / 100)} by weight gain.

Table 3
Ingredient composition (%) and Chemical composition (%DM) of broiler mash diets.

Ingredient composition (%) of broiler chicken mash diets			
Ingredient	Pre-starter (Week I–II)	Starter (Week III)	Finisher (Week IV-VI)
Maize grain	59.23	60.20	65.50
Soybean meal	32.00	31.00	25.80
Meat-cum-bone meal	4.80	4.20	3.50
Vegetable oil	2.20	2.80	2.80
De-oiled rice bran	–	–	0.80
Limestone powder	0.60	0.70	0.50
Dicalcium phosphate	0.10	0.10	0.20
Lysine	0.07	0.05	0.10
Methionine	0.25	0.20	0.14
Salt	0.35	0.35	0.35
Choline chloride	0.25	0.25	0.16
Trace minerals mixture[1]	0.10	0.10	0.10
Vitamin premix[2]	0.05	0.05	0.05

Chemical composition (%DM) of the diets fed to experimental broiler chicks			
Attributes*	Pre-starter	Starter	Finisher
Crude Protein	22.22 ± 0.23	21.35 ± 0.10	19.47 ± 0.07
Either Extract	4.91 ± 0.06	5.54 ± 0.14	5.63 ± 0.09
Total Ash	6.06 ± 0.07	6.56 ± 0.11	7.09 ± 0.07
Crude Fiber	3.29 ± 0.17	3.16 ± 0.04	3.08 ± 0.07
Nitrogen Free Extract	63.53 ± 0.07	63.40 ± 0.17	64.74 ± 0.29
Calcium	1.11 ± 0.02	0.95 ± 0.02	0.85 ± 0.04
Phosphorous	0.70 ± 0.02	0.65 ± 0.03	0.61 ± 0.04
Metabolizable Energy (kcal/kg) #	3002.40	3050.94	3100.10

[1] A mix providing (per kg of diet): manganese (MnSO4•H2O), 60 mg; iron (FeSO4•7H2O), 30 mg; zinc (ZnO),50 mg; copper (CuSO4•5H2O), 5 mg; iodine (ethylene diamine dihydroiodide), 0.15 mg; selenium (NaSeO3), 0.3 mg.

[2] A mix providing (per kg of diet): vitamin A, 8818 IU; vitamin D3, 2480 IU; 25-hydroxyvitamin D3, 69 mg; vitamin E, 35 IU; vitamin B12 (cobalamin),15.5 mg; biotin, 0.17 mg; menadione, 1.98 mg; thiamine, 1.87 mg; riboflavin, 7.7 mg; D-pantothenic acid, 13.23 mg; vitamin B6, 3.3 mg; niacin, 44.1 mg; folic acid, 1.1 mg.

* Each value is a mean of three replicates.

Calculated value.

Table 2
Experimental setup.

Replicates	Distribution of chicks in the groups			
	Basal control diet (CO)	Mannan oligosaccharide (MOS) Bio-Mos®Alltech	Novel growth-promoting paunch extract (NGPE)	Antibiotic lincomycin (AB) LINCOMIX®, Zoets
Dose rate	(No Feed additive)	350 g/ton	350 g/ton	40 gm/ton
R1	20	20	20	20
R2	20	20	20	20
R3	20	20	20	20
R4	20	20	20	20
R5	20	20	20	20
R6	20	20	20	20
Total chicks in a group	120	120	120	120
Total chicks in the experiment	480			

Thirty-six birds per treatment group (6 birds each replicate) were slaughtered at the end of the feeding trial (42 days of age) to collect blood and cecal samples.

The collection of whole blood samples was done in microcentrifuge tubes of 2 ml capacity pre-loaded with anti-coagulant (acid citrate dextrose, 150 µl/ml blood) for anti-oxidant assay. The anti-oxidant assay was done in erythrocyte lysate {lipid peroxidation (LPO), superoxide dismutase (SOD) and glutathione-S-transferase (GST)} and whole blood {(reduced glutathione (GSH)}.

A 1 % erythrocyte lysate was prepared in phosphate buffer saline (PBS; pH 7.4) as per Yagi (2012). The LPO activity was determined in terms of MDA (malondialdehyde) production as per Rehman (1984). The activity of SOD was determined by method of Marklund and Marklund (1974). The activity of the GST enzyme was determined by Habig, Pabst and Jakoby (1974) . The concentration of GSH in blood was determined by the method of Beutler (1975).

Immediately after slaughter, ceca of birds were identified and separated from the rest of the intestine. For three birds per replicate, the cecum was cut open longitudinally and contents were transferred in a clean glass beaker using distilled water. The beaker was stirred with a glass rod to mix the contents and pH was recorded immediately using a digital pH meter (Oakton®, Singapore).

For the other three birds per replicate, the cecal contents were transferred into a screw-capped sterilized plastic sample vial and immediately processed for bacterial enumeration. Cecal contents were mixed thoroughly and a 100 mg sample of the digesta was weighed and suspended in 0.9 ml of sterile PBS (pH 7.4). The suspension was serially diluted in 10-fold increments in PBS from 10^{-1} to 10^{-6}. From the last three dilutions, 0.1 ml each was plated on the appropriate medium for the enumeration of microbial populations. Lactic acid bacteria were enumerated on MRS agar (HiMedia Pvt. Ltd., India) post-incubation at ambient atmosphere at 39 °C for 48 h. Lactose-negative Enterobacteria were counted on McConkey agar (HiMedia Pvt. Ltd., India) post-incubation at ambient atmosphere at 39 °C for 24 h. All plates were incubated in the BOD incubator and bacterial numbers were counted using colony counter. The count was expressed as $\log_{10}$ per gm of cecal contents.

Statistical analysis

The data generated were analyzed by one-way analysis of variance (ANOVA) (Snedecor & Cochran, 1994) in SPSS version 23.0 (IBM SPSS Inc.). For *in vitro* tests, different media additives (glucose, MOS, and NGPE for cell densities and MOS and NGPE for PAS) and for *in vivo* trial dietary treatments (CO, NGPE, MOS, and AB) were taken as the independent variables, whereas parameters analyzed were taken as the dependent variables. The value of $P < 0.05$ was taken as the criterion for statistical significance. The mean differences among different treatments were separated by Duncan's multiple range tests (Duncan, 1955).

Results

Composition of novel growth-promoting paunch extract (NGPE)

The average yield of the prepared extract was 6.607 g of dried extract per liter of strained paunch waste. The mean chemical composition of NGPE was 32.02 ± 0.73 % neutral detergent fiber, 0.9 ± 0.004 % acid detergent fiber, 12.40 ± 0.91 % crude protein, and 24.00 ± 1.84 % total ash on a dry matter basis. The NGPE was devoid of the ether extract.

In vitro prebiotic activity assay

The prebiotic activity assay of the NGPE in comparison to commercial prebiotic (MOS) and glucose (control) (Table 4) showed that the *L. plantarum* (cell-densities post 24 h of incubation; log10 CFU/ml) grew significantly ($P < 0.01$) slower on MOS (1.14 ± 0.05) than on NGPE (1.37 ± 0.06) and glucose (1.58 ± 0.08). The increase in cell density for *L. rhamnosus* was significantly ($P < 0.01$) higher in NGPE-supplemented media (2.78 ± 0.11) compared to that on MOS (1.28 ± 0.05) and glucose (1.09 ± 0.04). *Enterococcus faecalis* also showed the highest ($P < 0.01$) growth on NGPE (2.77 ± 0.10) than on MOS (2.49 ± 0.09) and glucose (1.12^{a} ± 0.04). The growth of *E. coli* was comparable ($P > 0.05$) on all three substrates. The prebiotic activity score was significantly higher ($P < 0.05$) for NGPE (−0.11 ± 0.00; 1.59 ± 0.03 and 1.52 ± 0.03) compared to MOS (−0.19 ± 0.01, 0.27 ± 0.01 and 1.34 ± 0.04) for *L. plantarum, L. rhamnosus* and *E. faecalis*, respectively (Fig 2).

Table 4
Increase in cell densities (0 h *vs* 24 h) of bacterial cultures grown on media supplemented with novel growth-promoting paunch extract (NGPE), mannan oligosaccharide (MOS), or glucose.

Bacterial strain	Increase in cell densities (0 h *vs* 24 h) of bacterial cultures (Log_{10} CFU/ml)*		
	Glucose	MOS	NGPE
Lactobacillus plantarum	1.58^{b} ± 0.08	1.14^{a} ± 0.05	1.37^{b} ± 0.06
Lactobacillus rhamnosus	1.09^{a} ± 0.04	1.28^{a} ± 0.05	2.78^{b} ± 0.11
Enterococcus faecalis	1.12^{a} ± 0.04	2.49^{b} ± 0.09	2.77^{c} ± 0.10
Escherichia coli	2.79 ± 0.13	2.53 ± 0.12	2.72 ± 0.13

abc Means bearing different superscripts within a row differ significantly ($P < 0.01$).

* Log_{10} values of colony forming units per ml of media.

In vivo growth trial

Weekly body weight gain

The mean weekly body weight gain (g) of broiler chickens during the growth trial is presented in Table 5. Bodyweight gain (g) was significantly higher ($P < 0.05$) for NGPE-supplemented birds in week IV as compared to CO and MOS group birds, with intermediated values for the AB group. In week V, the highest gain (g) was shown by the MOS-supplemented birds. However, in week VI, the highest weight gain (g) was recorded for the NGPE-supplemented birds. The final body weight of broiler chickens at the end of week VI was 2154.22 ± 53.05, 2358.63 ± 0.23, 2286.99 ± 51.71, and 2202.73 ± 49.09 g for CO, NGPE, MOS, and AB groups, respectively, with NGPE group having the highest ($P < 0.05$) body weight.

Cumulative feed intake, FCR, and PER

The cumulative feed intake (g) for the first four weeks was comparable ($P > 0.05$) among different experimental groups (Table 6). In contrast, it was significantly higher ($P < 0.01$) in MOS fed group (3204.37 ± 14.73 and 4370.37 ± 12.93 g at the end of week V and VI, respectively) and lower in NGPE and AB-fed groups with intermediate values for CO group birds. The cumulative FCR of the first four weeks did not differ significantly ($P > 0.05$) among all four groups. At the end of week V, CO's cumulative FCR was significantly higher ($P < 0.05$) compared to NGPE, MOS, and AB groups. However, the cumulative FCR at the end of the trial was significantly lower ($P < 0.01$) in NGPE (1.79 ± 0.02) followed by AB (1.90 ± 0.05), MOS (1.95 ± 0.05) and was the highest in the CO group (2.02 ± 0.03). The cumulative PER of the first four weeks did not differ significantly ($P > 0.05$) across different groups. Cumulative PER at the end of the week V was significantly lower ($P < 0.05$) in CO compared to NGPE and MOS group with intermediate value for AB group birds. Final PER (at the end of week VI) was significantly lower ($P < 0.01$) in the CO (2.47 ± 0.04) group and higher in NGPE (2.78 ± 0.03), with intermediate values for MOS (2.56 ± 0.06) and AB groups (2.63 ± 0.06).

Erythrocytic antioxidant status indices of broiler chickens

Lipid peroxidation activity (expressed as nmol malondialdehyde

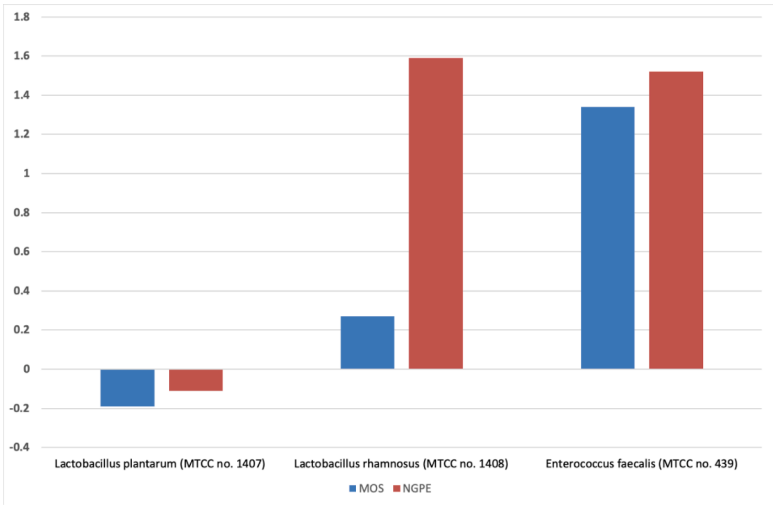

Fig. 2. Prebiotic activity score (as per Huebner et al., 2007) of novel growth-promoting paunch extract (NGPE) and commercial prebiotic (MOS) on various probiotic bacterial strains.

Table 5
Weekly body weight gain (g) of broiler chickens fed unsupplemented or diets supplemented with novel growth-promoting paunch extract (NGPE), mannan oligosaccharides, or antibiotic growth promoter.

Weeks	Dietary treatments*			
	CO	NGPE	MOS	AB
I	112.70 ± 3.85	125.62 ± 2.17	122.63 ± 4.66	120.55 ± 4.27
II	214.85 ± 5.92	219.02 ± 5.89	217.86 ± 5.75	209.72 ± 7.70
III	495.71 ± 17.63	524.29 ± 10.59	506.42 ± 11.32	499.90 ± 26.25
IV	513.15^{a} ± 20.07	597.86^{b} ± 6.56	526.43^{a} ± 26.62	548.34^{ab} ± 21.33
V	395.51^{A} ± 9.74	428.86^{B} ± 4.53	511.98^{C} ± 11.58	423.87^{B} ± 9.45
VI	378.72^{A} ± 9.33	420.24^{B} ± 4.44	358.34^{A} ± 8.10	357.43^{A} ± 7.97

ab Means bearing different superscripts within a row differ significantly ($P < 0.05$).
ABC Means bearing different superscripts within a row differ significantly ($P < 0.01$).
* CO: Control corn–soybean-based basal diet; NGPE: Basal diet supplemented with novel growth-promoting paunch extract (350 g/ton); MOS: Basal diet supplemented with mannan oligosaccharides (350 g/ton); AB: Basal diet supplemented with antibiotic growth promoter, lincomycin (40 g/ton).

produced/ml) in the hemolysate was significantly lower ($P < 0.01$) in NGPE and MOS in comparison to that in CO and AB groups (Table 7). The superoxide dismutase (U/mg Hb) and glutathione-s-transferase (μmole of reduced glutathione-1-chloro-2,4-dinitro benzene conjugate formed/min/mg Hb) levels were comparable ($P > 0.05$) among different dietary treatments. However, the reduced glutathione (nmol/ml) levels were significantly higher ($P < 0.01$) in the NGPE group as compared to birds on other dietary treatments.

Cecal count of lactic acid bacteria and Enterococcus spp. and pH of cecal contents of broiler chickens

The lactic acid bacteria (log cfu/g) count was significantly higher ($P < 0.01$) in the NGPE (8.90) group, followed by CO (8.65), MOS (8.50), and AB (8.22) group. The *Enterobacteria* count (count of *Coliform* and lactose-negative *Enterobacteria*) was comparable ($P > 0.0.5$) among different dietary treatments (Table 8). The values for pH of cecal contents of broiler chickens were not statistically different ($P > 0.0.5$) and ranged from 6.50 to 6.63 among different dietary treatments (Table 8).

Table 6
Cumulative feed intake (g), feed conversion ratio, and protein efficiency ratio of broilers chickens fed unsupplemented or diets supplemented with novel growth-promoting paunch extract (NGPE), mannanoligosaccharides or antibiotic growth promoter.

Weeks	Dietary treatments*			
	CO	NGPE	MOS	AB
Cumulative feed intake (g)				
I	142.23 ± 1.66	153.41 ± 2.88	148.72 ± 3.18	142.76 ± 3.82
II	460.49 ± 8.90	458.28 ± 6.67	455.96 ± 2.79	442.78 ± 8.89
III	1192.06 ± 18.40	1191.83 ± 17.25	1179.62 ± 11.34	1187.50 ± 13.56
IV	2058.42 ± 21.62	2115.79 ± 22.57	2056.20 ± 16.30	2079.37 ± 12.58
V	3146.82^{BC} ± 20.29	3118.97^{AB} ± 28.23	3204.37^{C} ± 14.73	3071.32^{A} ± 10.12
VI	4258.62^{B} ± 37.80	4140.75^{A} ± 35.84	4370.37^{C} ± 12.93	4082.00^{A} ± 10.44
Cumulative feed conversion ratio (g/g)				
I	1.27 ± 0.05	1.22 ± 0.03	1.22 ± 0.06	1.19 ± 0.04
II	1.41 ± 0.05	1.33 ± 0.01	1.34 ± 0.04	1.35 ± 0.05
III	1.45 ± 0.04	1.37 ± 0.02	1.39 ± 0.02	1.44 ± 0.06
IV	1.54 ± 0.03	1.44 ± 0.02	1.50 ± 0.03	1.51 ± 0.03
V	1.82^{b} ± 0.04	1.65^{a} ± 0.02	1.70^{a} ± 0.04	1.71^{a} ± 0.04
VI	2.02^{C} ± 0.03	1.79^{A} ± 0.02	1.95^{BC} ± 0.05	1.90^{AB} ± 0.05
Cumulative protein efficiency ratio (g/g)				
I	3.57 ± 0.13	3.69 ± 0.11	3.73 ± 0.21	3.81 ± 0.13
II	3.21 ± 0.12	3.38 ± 0.03	3.36 ± 0.09	3.37 ± 0.14
III	3.19 ± 0.08	3.36 ± 0.06	3.31 ± 0.05	3.23 ± 0.12
IV	3.13 ± 0.07	3.35 ± 0.04	3.22 ± 0.06	3.20 ± 0.07
V	2.71^{a} ± 0.06	2.99^{b} ± 0.03	2.90^{b} ± 0.07	2.89^{ab} ± 0.07
VI	2.47^{A} ± 0.04	2.78^{C} ± 0.03	2.56^{AB} ± 0.06	2.63^{B} ± 0.06

^{ab}Means bearing different superscripts within a row differ significantly ($P < 0.05$).
^{ABC}Means bearing different superscripts within a row differ significantly ($P < 0.01$).
* CO: Control corn-soybean-based basal diet; NGPE: Basal diet supplemented with novel growth-promoting paunch extract (350 g/ton); MOS: Basal diet supplemented with mannan oligosaccharides (350 g/ton); AB: Basal diet supplemented with antibiotic growth promoter, lincomycin (40 g/ton).

Discussion

Paunch waste extraction

The average yield of the prepared extract was 6.607 g of dried extract per liter of strained paunch waste. The recurring cost (cost of labor, solvent, and energy) and time required for extract preparation were 1.8

Table 7
Erythrocytic antioxidant status indices of broiler chickens fed unsupplemented or diets supplemented with novel growth-promoting paunch extract, mannan oligosaccharides, or antibiotic growth promoter.

Treatments / Attributes	Dietary treatments*			
	CO	NGPE	MOS	AB
LPO[1] (nmol MDA[2] produced/ml)	$4.37^{B} \pm 0.26$	$2.54^{A} \pm 0.20$	$2.93^{A} \pm 0.19$	$4.13^{B} \pm 0.23$
SOD[3] (U/mg Hb[4])	57.78 ± 2.54	56.89 ± 2.83	58.51 ± 1.84	57.74 ± 2.19
GST[5] (μ mole of GSH[6]–CDNB[7] conjugate formed/min/mg Hb)	0.58 ± 0.02	0.59 ± 0.02	0.56 ± 0.01	0.57 ± 0.02
GSH (nmol/ml)	$6.33^{A} \pm 0.40$	$7.80^{B} \pm 0.26$	$6.58^{A} \pm 0.19$	$6.31^{A} \pm 0.30$

[AB]Means bearing different superscripts within a row differ significantly ($P < 0.01$).

* CO: Control corn-soybean-based basal diet; NGPE: Basal diet supplemented with novel growth-promoting paunch extract (350 g/ton); MOS: Basal diet supplemented with mannan oligosaccharides (350 g/ton); AB: Basal diet supplemented with antibiotic growth promoter, lincomycin (40 g/ton).

1 lipid peroxidation.
2 malondialdehyde.
3 superoxide dismutase.
4 haemoglobin.
5 glutathione-s-transferase.
6 reduced glutathione.
7 1-chloro-2,4-dinitro benzene.

Table 8
Enterobacteria and *Lactic acid bacteria* count in cecal contents along with pH of cecal contents of broiler chickens fed unsupplemented or diets supplemented with rumen liquor extract, mannan oligosaccharides or antibiotic.

Treatments / Attributes	Dietary treatments*			
	CO	NGPE	MOS	AB
Lactic acid bacteria (log cfu/g)	$8.65^{C} \pm 0.08$	$8.90^{D} \pm 0.01$	$8.50^{B} \pm 0.02$	$8.22^{A} \pm 0.03$
Enterobacteria^ (log cfu/g)	5.77 ± 0.02	5.80 ± 0.02	5.78 ± 0.02	5.80 ± 0.01
pH	6.62 ± 0.15	6.63 ± 0.16	6.62 ± 0.19	6.50 ± 0.17

^ Enterobacteria are coliform and lactose negative Enterobacteria.
abcd Means bearing different superscripts within a row differ significantly ($P < 0.01$).

* CO: Control corn-soybean-based basal diet; NGPE: Basal diet supplemented with novel growth-promoting paunch extract (350 g/ton); MOS: Basal diet supplemented with mannan oligosaccharides (350 g/ton); AB: Basal diet supplemented with antibiotic growth promoter, lincomycin (40 g/ton).

USD and 14–15 h. per liter of strained rumen paunch waste. The majority of the cost involved is the cost of ethanol used as the solvent, which can be minimized by recovering it through distillation after removal of the precipitate. The recovery rate is high (average 85 %) and therefore the cost of production calculated here can be reduced significantly.

The mean chemical composition of NGPE was 32.02 ± 0.73 % neutral detergent fiber, 0.9 ± 0.004 % acid detergent fiber, 12.40 ± 0.91 % crude protein and 24.00 ± 1.84 % total ash on a dry matter basis. The NGPE was devoid of ether extract. The composition indicates that NGPE is a mixture of precipitable oligosaccharides, minerals, and crude protein, which are either co-precipitated or bound to the fiber component.

The usual procedure of prebiotic production involves enzymatic degradation of a polysaccharide, followed by isolation of the oligosaccharides from the degradation mixture. However, in the production of novel growth-promoting paunch extract (NGPE), the step of enzymatic degradation is bypassed, thereby offering an advantage over other prebiotics. Further, the raw material used for NGPE is paunch waste, which is available globally without any seasonal and regional biases.

In vitro prebiotic activity assay

The positive impact of NGPE supplementation on *L. plantarum, L. rhamnosus,* and *E. faecalis* growth compared to MOS-supplemented culture indicates the strong prebiotic activity of NGPE *in vitro*. The higher growth of *L. plantarum* over glucose compared to MOS and NGPE is in accordance with the observation of (Chapla, Pandit & Shah, 2012), who reported that *Lactobacillus* spp. does not utilize prebiotics preferably over glucose as a carbon source. However, the growth of *L. rhamnosus* observed in the present study over NGPE and MOS compared to glucose was significantly higher, indicating that the substrate preference by the probiotic bacteria is strain-specific (Campana, van Hemert & Baffone, 2017).

The suppression effect of prebiotics over harmful bacteria is mediated by the selective stimulation of beneficial microbes, as prebiotic fermentation yields short-chain fatty acids (SCFAs), which selectively inhibit the growth of pathogens (Boets et al., 2017). However, the suppressing effect of NGPE on *E. coli* was not observed *in vitro*, which might be due to pure cultures, thereby denying the interaction with beneficial microbes.

Nevertheless, the growth stimulation of probiotic bacterial strains on NGPE supplementation as compared to MOS supplementation suggests strong prebiotic activity by NGPE, which was also exhibited by a higher prebiotic activity score of NGPE compared to that for MOS.

Performance of broiler chickens

Weekly body weight gain

The growth-promoting effect of NGPE in broiler chickens is similar to the growth-promoting effects of prebiotics. We know that prebiotics stimulate growth by mechanisms involving increased absorptive surface area in the intestines, reduced intestinal pH, increase in the concentration of favorable metabolic by-products such as SCFAs, improved energy retention, decrease in pathogenic organisms in the intestines, and increased thyroid hormone levels (Zhenping et al., 2013).

Prebiotics are known to increase the proliferation of *Bifidobacteria* and *lactobacilli* in the gut, thereby enhancing the host microbial balance (Shehata et al., 2022). The healthy gut microbiota promotes growth, most likely by enhancing feed ingredient absorption and digestibility as indicated by an increase in nutrient absorption, protein metabolism, energy metabolism, and fibre digestion on prebiotic supplementation in rats (Alvarado-Jasso et al., 2020). Shehata et al. (2022) and Dev et al. (2020) found lower ileum energy and protein content in prebiotic-fed chicks than in control ones, indicating greater energy, protein digestion, and absorption in small intestines.

SCFAs also mediate improved growth. Fermentable carbohydrates alter the microbial ecology and intestinal environment by increasing SCFAs concentration, making them the major luminal anions in the broiler chickens' gut (Shehata et al., 2022). The SCFA concentration rises from negligible levels at birth to its maximum at day 15 in the ceca of chicks (Aljumaah et al., 2020). Higher SCFAs concentration in the intestine with prebiotic supplementation increases the intestinal blood flow, improving tissue oxygenation and nutrition delivery (Fernández et al., 2016).

Similar to the findings of this study, Appelt et al. (2010) also observed that prebiotic supplementation does not affect weight gain during the starter and pre-starter phases (Weeks I–III), whereas, significant effects were observed in the finisher phase (Ribeiro et al., 2018). An increase in the population of targeted microbial species and the resulting balance between commensal and pathogenic microbes may be required for a prebiotic to have an impact on the growth performance of broiler chickens. This could be the probable reason for the delayed observation of its beneficial effects.

Cumulative feed intake, FCR, and PER

This study observed significantly improved FCR on NGPE supplementation indicating better feed assimilation. This could be due to the metabolic interplay between thyroid hormone levels and improved insulin levels, which is also characterized by an increase in the weight of the pancreas (Akter et al., 2021).

Ribeiro et al. (2018) and De Vrese and Schrezenmeir (2008) suggested that prebiotics modulate an improvement in animal performance by optimizing feed digestion and feed intake and triggering the microbiome's evolution to a more favorable construction.

Better nutrient assimilation in NGPE-supplemented birds is indicated by significantly better FCR on similar feed intake compared to control diet-fed birds. Prebiotic supplementation improves growth and reduces abdominal fat deposition in broilers (Zhou et al., 2009). This suggests altered energy utilization in prebiotics-supplemented birds. In addition, Prebiotics and probiotics are known to alter levels of thyroid hormones (Knezevic et al., 2020). Jiang et al. (2020) reported increased thyroid hormone levels in probiotics-supplemented chickens. Talebi et al. (2020) reported that synbiotic supplementation reduces TSH and increases free triiodothyronine levels in hypothyroidism.

An increase in weight gain in prebiotic-supplemented broiler chickens is often observed with higher feed intake. However, similar to the observations of this study, Al-Khalaifa et al. (2019) also found no difference in feed intake between the control and prebiotic-supplemented groups.

Erythrocytic antioxidant status indices of broiler chickens

The intestinal mucosa is the primary defense against feed and non-feed-induced oxidative stress. Prebiotic supplementation is known to exert an antioxidant effect, exhibited as improved SOD levels and reduced MDA levels in serum (Liu et al., 2020).

Prebiotics can exhibit antioxidant effects by stimulating the growth of probiotic bacteria and fermentation by-products. Prebiotics increase Bifidobacteria and Lactobacilli's growth in the gut (Shehata et al., 2022). Bifidobacteria expresses potent antioxidant activity due to the presence of SOD. Their activity is even expressed extracellularly due to the lysis of bacterium by lactobacilli, which is exhibited as a decrease in the MDA levels (Ray et al., 2021). Furthermore, increased fermentation activity in prebiotics-supplemented subjects increases luminal levels of SCFAs, which act as ligands for the G protein-coupled receptors GPRs, reducing the free-radical production (Abbasi, Sheykhsaran & Kafil, 2021). Moreover, improved intestinal integrity or reduced linoleic acid peroxidation could also be why prebiotics can prevent oxidative stress-mediated damage. Additionally, probiotic microbes accumulate selenium, zinc, and copper and incorporate them into organic compounds (Knezevic et al., 2020), which are critical players in antioxidant activity at the cellular level.

NGPE supplementation exhibited enzymatic (GSH) and non-enzymatic (LPO) improvement in antioxidant capability. Reduced oxidative stress is a common finding in other studies exploring prebiotic supplementation effects on chicken. However, improving GSH activity on prebiotic supplementation is not a common finding (Xu et al., 2021). The NGPE could be, therefore, an even better alternative as a growth promoter than MOS and other prebiotics.

Cecal count of lactic acid bacteria and Enterococcus along with pH of cecal contents in broiler

An essential criterion for a compound to qualify as a prebiotic is that it must be selectively fermented by a limited number of beneficial bacteria and stimulate their growth or metabolism. A common observation is increased lactic acid bacteria count on prebiotic supplementation (Shehata et al., 2022; Tarabees et al., 2020). In this study, higher Lactic acid bacteria count in the ceca of NGPE group birds reaffirms this observation under *in vitro* conditions. Soluble fiber prebiotics promotes the fermentation in the large intestine by the beneficial flora and alters the intestinal microbiota by increasing the beneficial flora population, reducing pathogens through competitive exclusion (Tarabees et al., 2020). Increased Lactic acid bacteria population often manifests as improved growth performance in broiler chicken (Zamojska et al., 2021). It is possibly exerted through pathogen exclusion by strengthening the mucosal barrier, immunity modulation, altered inflammatory cytokines expression, and improved gut health. Prebiotics' ability to increase the quantity of LAB in the gut may aid in the competitive exclusion of pathogens from birds' gastrointestinal tracts (Pourabedin & Zhao, 2015).

The hindgut acidosis (reduced cecal pH) is a claimed effect of prebiotics but is not a consistent finding in prebiotics supplementation trials (Al-Khalaifah & Al-Nasser, 2019). Acidic cecal pH indicates anaerobic fermentation by beneficial microbes resulting in the accumulation of fermentation by-products. This study observed acidic pH in all experimental groups irrespective of dietary treatment. This observation may be due to the absence of challenges by pathogenic microbes in all dietary groups, which are often responsible for increasing the hindgut pH (van der Wielen et al., 2000).

Conclusions

The emerging void due to the phasing out of antibiotic growth promoters in the feed of monogastric livestock including broiler chickens is still waiting to be filled by a candidate that will offer the cost-benefit ratio and reliability of antibiotics. Therefore, any prospective feed additive in this regard is to be studied and scrutinized. NGPE requires minimal processing for its preparation and can be prepared from slaughterhouse waste that is available throughout the World. It has shown the ability to alter the growth of probiotic bacteria *in vitro* and improve the growth rate and feed utilization efficiency of broiler chickens. At a similar supplementation level, it has shown higher efficacy than MOS in improving the weight gain during the finisher phase, feed conversion ratio, antioxidant status, and growth of commensal bacteria in the ceca of broiler chickens. However, it cannot be labeled as a prebiotic till its effects over a wide range of commensal bacteria and mode of action are ascertained. Further, as it is being extracted from an unstandardized source, its composition and probably the level of efficacy may also vary between batches.

Ethics statement

The animal experiment was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of SKUAST-Jammu (vide approval no.16/IAEC-20/2020), which aligns with ARRIVE's guidelines. All experimental protocols and procedures were carried out according to relevant regulations and guidelines established by this committee, and all efforts were made to minimize the suffering of the chickens.

No supplementary data

All data generated or analyzed during this study are included in this article.

CRediT authorship contribution statement

Zulfqarul Haq: Conceptualization, Data curation, Formal analysis, Methodology, Visualization. **Ankur Rastogi:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision. **Ramesh Kumar Sharma:** Conceptualization. **Pratiksha Raghuwanshi:** Supervision, Validation. **Maninder Singh:** Investigation, Supervision, Validation. **Azmat Alam Khan:** Writing – original draft, Writing – review & editing. **Syed Mudasir Ahmad:** Supervision, Validation, 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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