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Preparation, characterization and utilization of a novel feed supplement in poultry

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ABSTRACT

This study aimed to prepare and characterize the zinc alginate complexes using SEM-EDAX, XRD, FTIR and evaluate their zinc retention percentage in cockerels. Three complexes were prepared using zinc sulfate (Zn-Alg-CM_1), zinc carbonate (Zn-Alg-CM_2), and zinc nitrate (Zn-Alg-CM_3). Zn-Alg-CM_3 was amorphous with smooth surfaces and showed a shifted absorption spectrum, indicating strong zinc binding compared to Zn-Alg-CM_1 and Zn-Alg-CM_2. Although Zn-Alg-CM_2 had higher zinc composition, Zn-Alg-CM_3 at 45 ppm significantly improved the digestibility of crude protein, crude fibre and zinc retention compared to Zn-Alg-CM_1, Zn-Alg-CM_2, and the control with zinc oxide in cockerels. Zn-Alg-CM_3 exhibited antibacterial activity against *E. coli, Salmonella* and *S. aureus*. Therefore, Zn-Alg-CM_3 showed promising potential as a novel feed supplement in cockerels and further research is essential to fully understand its impact on growth performance, antioxidant activity, and intestinal health in poultry.

Keywords: FTIR, SEM-EDAX, Zinc alginate complex, Zinc retention, XRD

The current target of the poultry industry is to achieve peak performance and optimize feed efficiency. A balanced diet with micronutrients is one factor that influences poultry's growth performance. Zinc is the second most essential trace mineral for poultry, acting as a cofactor for numerous metabolic enzymes, an immune system activator, and an antioxidant. Inorganic zinc added in poultry diets reduces zinc bioavailability, while organic zinc in zinc polysaccharide has been used to enhance bioavailability. Zinc acts as a cofactor for more than 300 metalloenzymes involved in nutrient metabolism (Suttle 2022) and also essential for maintaining bone mineral homeostasis. Moreover, Zn cannot be stored in the body and must be supplemented through diet (Mozhiarasi *et al.* 2024). The natural feed ingredients used in poultry rations do not contain sufficient zinc and dietary supplementation is essential for the growth performance of birds (Jarosz *et al* .2022).

The poultry diets are commonly supplemented with inorganic zinc which is ionized in the GI tract and complexed with other dietary factors to decrease bioavailability of zinc. To overcome these effects, organic zinc supplemented in the form of zinc polysaccharide complexes increased bioavailability and production. Zinc is also essential for gut commensal microflora and dietary supplementation of zinc can restore gut microbiota balance, promoting Firmicutes and decreasing *E. coli* in the intestine (Skalny *et al.* 2021). Metal Polysaccharide complexes are products resulting from the complexing of a soluble salt with a polysaccharide solution declared as an ingredient of the specific metal complex, e.g. zinc polysaccharide complex, etc. (AAFCO 2020). Sodium alginate, a polysaccharide, is combined with zinc to synthesize a zinc alginate complex. Sodium alginate is a FDA-approved natural anionic polysaccharide that cross-linked with divalent cations like Zn^{2+} to form a zinc alginate complex (Makarova *et al.* 2023). The zinc alginate complex increased the digestibility of zinc due to the muco-adhesive property of alginate. In the literature, very few studies investigated zinc alginate complex as feed supplements in poultry. Therefore, the present study was conducted to investigate the preparation and characterization of the zinc alginate complex and to see its effect on the performance of cockerels.

MATERIALS AND METHODS

Production of zinc alginate complex: The zinc alginate complex (Zn-Alg-CM) was prepared in the laboratory using three different zinc salts by adapting standard protocols with slight modifications and chemicals were purchased from SRL, Himedia, and Merck laboratories (Supplementary Fig.1). For Zn-Alg-CM_1, a 0.2 M zinc sulfate monohydrate solution was mixed with a 3% sodium alginate solution and 0.4 M NaOH at 80°C for 4 h, following a modified method of Siddiqui *et al.* (2022),

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resulting in a white precipitate that was filtered, washed, dried, and powdered. Zn-Alg-CM_2 was synthesized from a mixture of 2% sodium alginate and 2% D-(+)-gluconate-δ-lactone added to a 30% zinc carbonate solution, then solidified in a petri dish at room temperature for 48 h before being powdered, following a method adapted from Straccia *et al.* (2015). Zn-Alg-CM_3 was prepared by adding a 5% sodium alginate solution drop-wise to a 5% zinc nitrate solution, continuously stirred for 2 h, followed by drying the resulting hydrogel beads at room temperature and powdering them, based on a method from Abou Oualid *et al.* (2018).

Characterization of zinc alginate complex

Scanning electron microscope with energy dispersive X-ray analysis (SEM- EDAX): The morphology and surface topography of zinc alginate complexes were examined using a Tescan Instruments SEM with TESCAN-VEGA 3 software at National College Instrumentation Facility (NCIF), Trichy. Elemental composition was determined using an Oxford INCA X-Max detector at 5.0 kV. Particle size was measured with Image J software (ver. 1.80, National Institute of Health, MD, USA).

X-Ray diffraction analyser (XRD): The X-ray diffraction patterns were recorded using an Xpert Powder X-Ray Diffractometer (Malvern Panalytical) with HIGH SCORE software at NCIF, Trichy, utilizing Copper Kα radiation.

Fourier transform infra-red (FTIR) spectroscopy: The chemical structure was characterized using an FTIR with ATR (OPUS, BRUKER-ALPHA, Perkin Elmer) at NCIF, Trichy. About 5 mg of each sample was ground and placed on the ATR crystal. Spectra were obtained from 4000 to 400/cm, following the method outlined by Nandiyanto *et al.* (2019).

*Assessment of antimicrobial activity:*The antimicrobial activity of the zinc alginate complex 3 was assessed against *S. aureus, Salmonella,* and *E.coli*. Petri plates containing 20 ml of Muller Hinton agar medium were inoculated with bacterial cultures of *E. coli* and *S. aureus* (culture growth adjusted to McFarland Standard, 0.5%). The wells (10 mm diameter) were cut from the agar using a sterile well cutter, the samples were added into the wells, and plates were incubated at 37°C for 24 h. The antimicrobial activity was determined by measuring the diameter of the zone of inhibition (in mm) against the test microorganisms, following Clinical and Laboratory Standards Institute (2012) guidelines.

Nutrient digestibility and zinc retention study in cockerels: A 21-day metabolic trial was conducted with 12 of 20-week-old adult White Leghorn cockerel birds (1.75±0.25 kg) to determine nutrient digestibility and zinc retention. The experiment was approved (IAEC/14/VCRI-NKL/2023) by the Veterinary College and Research Institute, Namakkal, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Tamil Nadu, India. The cockerels were weighed individually and randomly distributed to four dietary treatments (T_1, T_2, T_3 and T_4) and each treatment consisted of three cockerels. All the experimental birds were reared in separate cages. The experimental diets were formulated as per Bureau of Indian Standards (BIS 2007) specifications and the ingredient and chemical composition of the diets were analyzed following AOAC (2000) standards, as detailed in Table 1.

Table 1. Ingredient (%) and chemical composition of cockerel diets (Per cent DM basis)

Ingredient (%)	T_1	T_2	T_3	T_4
Maize	48	48	48	48
De-oiled rice bran	30	30	30	30
Soybean meal	12	12	12	12
Sunflower oilcake	3.6	3.6	3.6	3.6
Coconut Cake	3.4	3.4	3.4	3.4
Di-calcium phosphate	0.8	0.8	0.8	0.8
Calcite	1.6	1.6	1.6	1.6
Salt	0.3	0.3	0.3	0.3
DL-Methionine	0.03	0.03	0.03	0.03
Trace mineral mixture**	0.1	0.1	0.1	0.1
Vitamin premix*	0.05	0.05	0.05	0.05
Liver tonic	0.03	0.03	0.03	0.03
Coccidiostat	0.05	0.05	0.05	0.05
NSP enzyme[1]	0.04	0.04	0.04	0.04
Total	100	100	100	100
Nutrient composition (per cent DM)[#]				
Crude protein	16.61	16.61	16.61	16.61
Ether extract	1.94	1.94	1.94	1.94
Crude fiber	7.78	7.78	7.78	7.78
Calcium	0.96	0.96	0.96	0.96
Available phosphorus	0.49	0.49	0.49	0.49
Salt as chloride	0.3	0.3	0.3	0.3
Metabolizable energy (kcal/kg)	2512	2512	2512	2512

BIS (2007). **, Trace minerals supplied per kg of diet: Manganese, 60 mg; Iron, 60 mg; Iodine, 1 mg; Copper, 9 mg; Selenium, 0.15 mg. Zinc was supplied in T_1 as zinc oxide at 80 mg, T_2, T_3, and T_4 as zinc alginate complex at 45 mg, respectively. *, Vitamin A, B_2, D_3, K: Each gram contained: Vitamin A, 82,500 IU; Vitamin B_2, 50 mg; Vitamin D_3, 12,000 IU and Vitamin K, 10 mg. [1]NSP enzyme each kg contained: Cellulase, 1,20,00000 IU; Hemi-cellulase, 54,00000 IU; Protease, 24,00000 IU; Amylase, 24,00000 IU and Beta glucanase, 1,06000 IU. [#], Analysed values.

The group supplemented with zinc as inorganic zinc oxide (T_1-control) at 80 ppm, Zn-Alg-CM_1 (T_2), Zn-Alg-CM_2 (T_3) and Zn-Alg-CM_3 (T_4) groups were supplemented with zinc at a level of 45 ppm (80% bioavailability of zinc). The birds were provided with *ad lib.* feed and water. After a two-week adaptation period with the experimental diets, digestibility studies were conducted for seven days. Excreta were collected daily during the last four days and pooled for further analysis. The excreta and feed were analyzed for dry matter, crude protein, and crude fibre content. The zinc content in the feed and excreta was determined by the Atomic Absorption Spectroscopy (AAS) Model-Perkin Elmer PinAAcle 900T with Win lab 32

Table 2. EDX for elemental analysis of zinc alginate complexes prepared using various zinc salts with sodium alginate

Element	Zn-Alg-CM_1		Zn-Alg-CM_2		Zn-Alg-CM_3	
	Weight%	Atomic%	Weight%	Atomic%	Weight%	Atomic%
C K	14.24	22.75	25.34	41.87	29.85	41.38
O K	42.72	51.26	37.86	46.96	51.84	53.96
Na K	17.73	14.80	-	-	-	-
S K	12.27	7.34	-	-	-	-
Zn K	13.05	3.83	36.80	11.17	18.32	4.67
Total	100.00	100.00	100.00	100.00	100.00	100.00

software at AFAQAL, VCRI, Namakkal. The Zn retention was calculated as follows:

Zn retention (%) = [(Ingested Zn − Excreted Zn) / Ingested Zn] × 100 (Yusof *et al.* 2023)

The feed and faecal Zn contents were based on their analyzed values and the digestibility of dry matter, crude protein, and fibre were estimated. Statistical analysis of the collected data was done by using the method of Snedecor and Cochran (1989).

RESULTS AND DISCUSSION

Scanning electron microscopy (SEM): The SEM provides the surface topography image of the prepared zinc alginate complex (Supplementary Fig. 2). The surface of the prepared Zn-Alg-CM_3 was smooth and irregularly spherical indicating that zinc is strongly bound with alginate. Similar to the results of the present study, Urbanova *et al.* (2019) also reported that alginate cross-linked with divalent ions had smooth and homogenous surfaces without cracks. However, Zn-Alg-CM_1 and Zn-Alg-CM_2 showed rough surfaces with numerous cracks and particles that are agglomerated which represents zinc is poorly bound with alginate. Similarly, the zinc oxide nanoparticles showed a rough surface compared to the Fucus vesiculosus-derived alginate coated zinc oxide nano complex (Hamouda *et al.* 2023). The particle size of Zn-Alg-CM_1, Zn-Alg-CM_2, and Zn-Alg-CM_3 ranged from 5.07-34.54 µm, 3.04-20.00 µm and 8.04-38 µm, respectively.

Energy-dispersive X-ray spectroscopy (EDX): The elemental composition of zinc alginate complex was quantified by using EDX (Supplementary Fig. 3) and its composition expressed as atomic and mass percentages are shown in Table 2. The zinc content of Zn-Alg-CM_2 was higher than that of Zn-Alg-CM_1 and Zn-Alg-CM_3. The zinc content in zinc alginate was reported as 14.4% (Said and Hassan 1993) and 20.4% (Alkhayer *et al.* 2020).

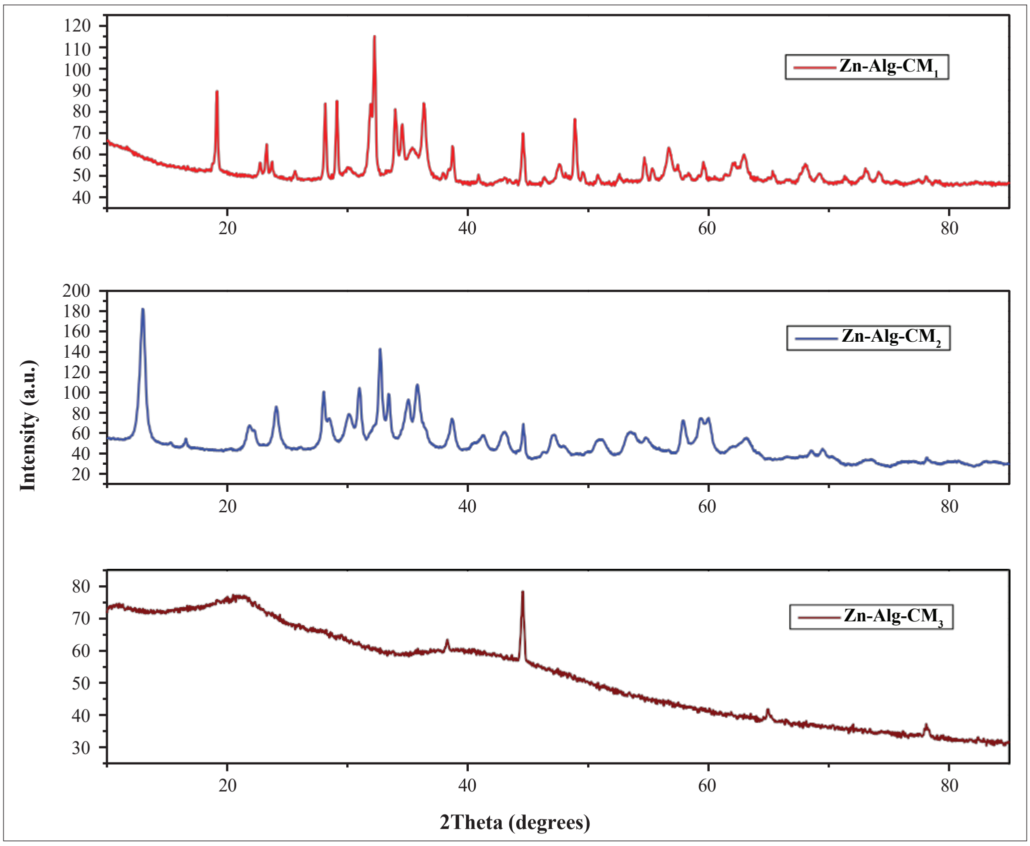

Fig.1. XRD spectrum of zinc alginate complex (Zn-Alg-CM).

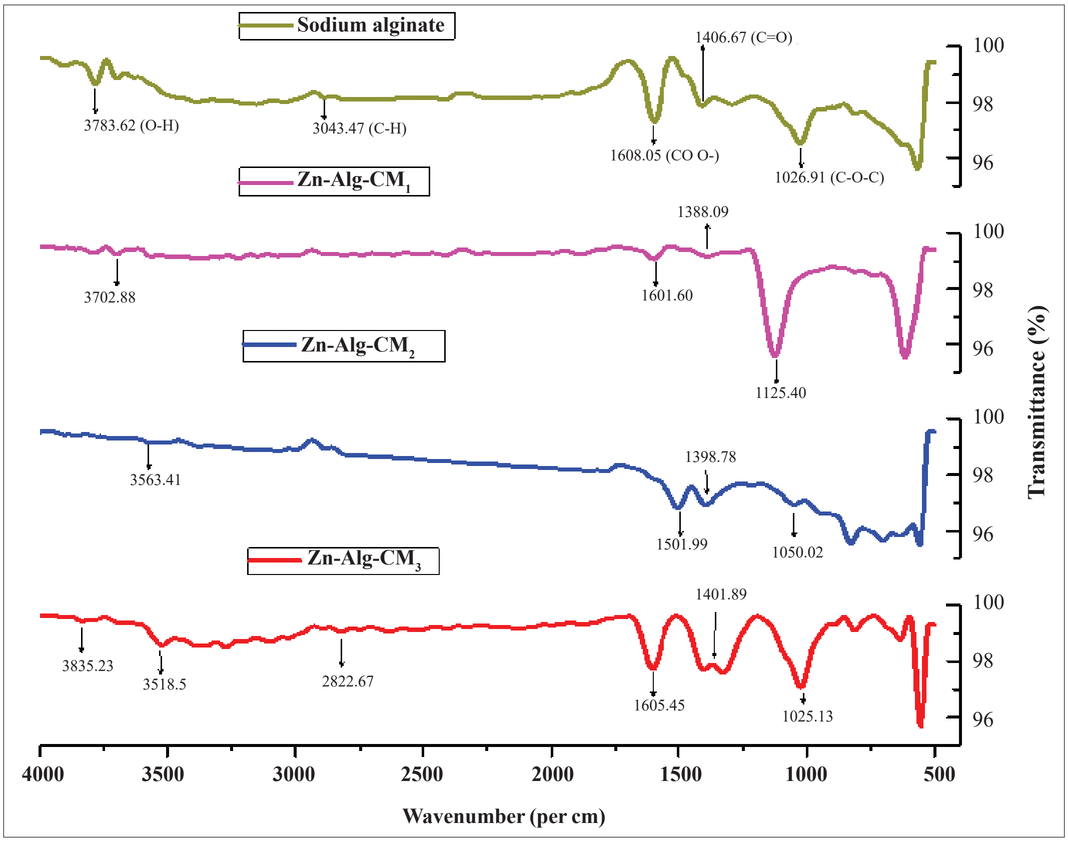

Fig. 2. FTIR spectrum of zinc alginate complex (Zn-Alg-CM).

X-ray diffraction analyser (XRD): The XRD pattern of the zinc alginate complex is shown in Fig. 1. The prepared Zn-Alg-CM$_3$ exhibits broader peaks indicating the material is amorphous. Similar to the result of the present study, Hassan (1993) stated that metal alginate complexes are amorphous by X-ray diffraction. Amorphous materials often exhibit increased apparent solubility and faster dissolution rates in the gastrointestinal tract which can enhance their bioavailability (Yu *et al.* 2018). The amorphous materials offers greater stability compared to crystalline (Castro-Rosas *et al.* 2017).

Further, Zn-Alg-CM$_1$ and Zn-Alg-CM$_2$ showed many sharp peaks indicating materials are crystalline. Similarly, Siddiqui *et al.* (2022) reported that crystalline material exhibited distinct, narrow peaks. The XRD analysis data supports the data that zinc has been effectively bound within the alginate in Zn-Alg-CM$_3$, indicating the success of the complex formation. Moreover, the presence of a crystalline structure in Zn-Alg-CM$_1$ and Zn-Alg-CM$_2$ suggests that the complex formation with alginate was not successful.

Fourier transform infra-red (FTIR) spectroscopy: The FTIR spectral patterns of sodium alginate and zinc alginate complexes are shown in Fig. 2 and Supplementary Table 1. In this study, an absorption peak at 561 and 556/cm can be found in Zn-Alg-CM$_2$ and Zn-Alg-CM$_3$. This is the absorption peak of zinc oxide (Siddiqui *et al.* 2022).

The stretching vibration bands of OH bonds in sodium alginate were in the range of 3000-3800/cm and zinc alginate exhibits in the range of 3100-3750/cm (Alkhayer *et al.* 2020). In this study, sodium alginate showed an absorption peak at 3783/cm due to stretching vibrations of OH bonds. The stretching vibrations of OH bonds of prepared Zn-Alg-CM$_1$, Zn-Alg-CM$_2$, and Zn-Alg-CM$_3$ showed absorption peaks at 3702, 3563, and 3518/cm, respectively. The sodium alginate and zinc alginate complex exhibited absorption peaks in the range of 3000-3800/cm and 3100-3750/cm,

Table 3. Effect of Zn-Alg-CM on nutrient digestibility in cockerels at 20 weeks of age

Treatment group	Digestibility%			Zinc retention %
	Dry matter	Crude protein	Crude fibre	
T$_1$-Control with zinc as zinc oxide (80 ppm)	61.25±1.12	46.61^a±0.60	24.68^b±1.15	28.36^b±1.24
T$_2$-Basal diet + zinc alginate complex 1 (at 80 % bioavailability– 45 ppm)	61.28±0.68	47.22^a±0.45	22.07^a±0.40	21.19^a±0.45
T$_3$-Basal diet + zinc alginate complex 2 (at 80 % bioavailability– 45 ppm)	61.57±0.61	46.27^a±0.36	23.64^{ab}±0.52	25.28^{ab}±0.58
T$_4$-Basal diet + zinc alginate complex 3 (at 80% bioavailability– 45 ppm)	65.24±1.44	53.59^b±0.70	32.76^c±0.15	49.82^c±2.24
P-value	0.065	(P<0.01)	(P<0.01)	(P<0.01)

Each value is a mean of three observations. **, Means having different superscripts in a column differ significantly (P<0.01).

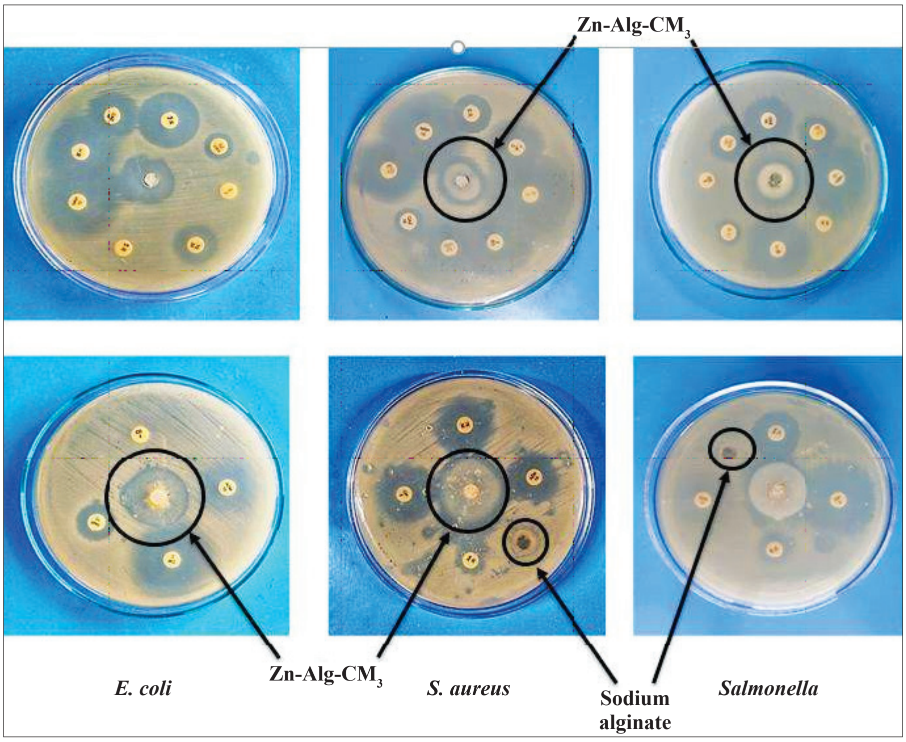

Fig. 3. ABST plates of zinc alginate complex (Zn-Alg-CM_3).

respectively (Alkhayer *et al.* 2020). All the prepared zinc alginate complexes showed absorption peaks within the range of sodium alginate. However, the intensity of the absorption peak of Zn-Alg-CM_3 was similar to that of zinc alginate. The absorption peaks at 1419/cm and 1635/cm are related to the symmetrical and asymmetrical stretching vibrations of COO groups in sodium alginate (Pereira *et al.* 2011). In this study, the absorption peaks of sodium alginate at 1406/cm and 1599/cm are due to symmetric and asymmetric stretching vibrations of COO groups. The prepared Zn-Alg-CM_1, Zn-Alg-CM_2, and Zn-Alg-CM_3 exhibit absorption peaks at 1388 and 1601/cm, 1393 and 1501/cm, and 1401 and 1605/cm, respectively, indicated symmetric and asymmetric stretching vibrations of COO groups. However, Zn-Alg-CM3 has narrow absorption peaks that are nearly identical to those of sodium alginate compared to Zn-Alg-CM_1 and Zn-Alg-CM_2. The stretching vibrations of OH and COO bond to a lower spectrum are observed in Zn-Alg-CM3, which indicated an effective interaction between OH and COO groups alginate with zinc to form a zinc alginate complex. Carboxylate symmetric and asymmetric vibrational frequencies in Zn-Alg-CM_3 are similar to those in sodium alginate. However, the higher spectral difference found in Zn-Alg-CM_3 compared to sodium alginate suggests unidentate coordination (Papageorgiou *et al.* 2010).

Effect of Zinc alginate complex on nutrient utilization: Data (Table 3) on the digestibility of different nutrients in cockerels supplemented with zinc alginate complex at the end of 20 weeks showed that there was no significant effect on dry matter digestibility in cockerels fed with zinc alginate complex. However, crude protein and crude fibre digestibility were significantly ($P<0.01$) increased in Zn-Alg-CM_3 compared to the remaining treatment groups (Zn-Alg-CM_2 and Zn-Alg-CM_1) and control with zinc oxide.

Similarly, Zn-Alg-CM_3 had significantly ($P<0.01$) higher zinc retention compared to other treatment groups including control with zinc oxide.

The high digestibility of Zn-Alg-CM_3 might be because amorphous materials offer greater stability and solubility in the gastrointestinal tract which can increase bioavailability (Yu *et al.* 2018). The polysaccharides are intact in the upper GI tract and release encapsulated material in the intestine (Choudhury *et al.* 2021). Similar to the results of the present study, Rao *et al*. (2023) reported that encapsulated zinc with maltodextrin at 50 ppm significantly ($P<0.05$) increased the ileal digestibility of zinc compared to control (zinc oxide) in broilers. In addition, Ramirez *et al*. (2022) found that the excretion of zinc was significantly low in carbohydrate-encapsulated trace minerals compared to inorganic trace minerals.

Antibacterial activity: From the cockerel study, the Zn-Alg-CM_3 was found to have a significantly higher zinc retention percentage as compared to remaining zinc alginate complexes. Hence, Zn-Alg-CM_3 was further characterized to find an antibacterial sensitivity test (ABST) in comparison with commonly used antibiotics in poultry. The antibacterial activities of the sodium alginate and zinc alginate complex against pathogenic *E.coli, Salmonella,* and *Staphylococcus aureus* are shown in Fig. 3 and Supplementary Table 2.

The sodium alginate does not show any antibacterial activity. However, the zinc alginate complex exhibited the highest zone of inhibition diameter than Nalidixic acid, Azithromycin, Ampicillin, Clindamycin, Amoxycillin, Penicillin, Tetracycline, Vancomycin, Meropenem, Chloramphenicol and Ceftazidime. However, the zinc alginate complex exhibited a lesser zone of inhibition than Ciprofloxacin.

The high antimicrobial activity of the zinc alginate complex was due to the release of Zn^{2+} ions binding to the cell membrane causing membrane destabilization and permeability (Thomas *et al.* 2018). Moreover, the free zinc ions interact with nucleic acids and deactivation of enzymes in the respiratory system of microbes (Fang *et al.* 2006). Similarly, Basha *et al.* (2016) reported that alginate-zinc oxide nanoparticles exhibit antimicrobial activity against *E. coli* and *S. aureus.*

The present research showed that the zinc alginate complex prepared from zinc nitrate is amorphous with a smooth surface and shifting of the absorption spectrum of COO groups indicated zinc is strongly bound with zinc alginate complex which results in better antimicrobial activity and high digestibility in cockerel. The zinc alginate complex from zinc nitrate has promising potential as a novel feed supplement in cockerels and further research is essential to fully understand its impact on growth performance, antioxidant activity, and intestinal health in poultry.

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