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Enhancing nutritional and potential antimicrobial properties of poultry feed through encapsulation of metagenome-derived multi-enzymes

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Abstract

Background The encapsulation of metagenome-derived multi-enzymes presents a novel approach to improving poultry feed by enhancing nutrient availability and reducing anti-nutritional factors. By integrating and encapsulated enzymes such as carbohydrate-hydrolyzing enzymes, protease, lipase, and laccase into feed formulations, this method not only improves feed digestibility but also potentially contributes to animal health and productivity through antimicrobial properties.

Results This study investigates the encapsulation of metagenome-derived enzymes, including carbohydrate-hydrolyzing enzymes, protease, lipase, and laccase, using Arabic and Guar gums as encapsulating agents. The encapsulated multi-enzymes exhibited significant antimicrobial activity, achieving a 92.54% inhibition rate against *Escherichia coli* at a concentration of 6 U/mL. Fluorescence tracking with FITC-labeled enzymes confirmed efficient encapsulation and distribution, while physical characterization, including moisture content and solubility assessments, along with Atomic Force Microscopy (AFM) imaging, validated successful encapsulation. The encapsulated enzymes also effectively hydrolyzed poultry feed, leading to an increase in phenolic content and antioxidant activity, as confirmed by 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays.

Conclusions The encapsulated multi-enzymes improved the overall feed quality by increasing reducing sugars and enhancing physical properties such as solubility and water-holding capacity. The encapsulated multi-enzymes improved the overall feed quality by increasing reducing sugars, antioxidant activity and enhancing physical properties such as solubility and water-holding capacity. Scanning Electron Microscopy (SEM) and Fourier-Transform Infrared Spectroscopy (FTIR) analyses confirmed the enzymatic breakdown of the feed structure. These results suggest that supplementing poultry feed with encapsulated multi-enzymes can enhance its physical, nutritional, and functional properties, leading to improved digestibility and overall feed quality.

Keywords Encapsulation, Metagenome, Multi-enzyme, Antioxidant, Antibacterial activity, Poultry feed

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Background

Enzymatic feed additives are gaining increasing recognition for their crucial role in improving the bioavailability of essential nutrients, especially in poultry [1]. By integrating these enzymes into feed formulations, a dual advantage emerges: a noteworthy economic efficiency through the reduction of high-cost ingredients and a marked improvement in feed nutritional profile [2, 3]. Depending on the mode of action of the enzymes, different types of enzymes can be added to the feed to promote dietary components such as proteins, amino acids, starch, and lipids. The strategic inclusion of an appropriate enzyme can optimize the digestive process, ensuring that poultry derive maximum nutritional benefits from their diet [4]. Proteases are essential feed enzymes that facilitate protein breakdown, enhancing protein digestion and promoting better absorption of minerals and nutrients [5]. In the poultry industry, the use of proteases is considered eco-friendly due to their significant role in reducing nitrogen emissions, thereby minimizing environmental impact [6]. Xylanases target non-starch polysaccharides, whereas amylases assist in the starch breakdown [7]. Additionally, lipases are introduced to enhance lipid digestibility, and the production of fatty acids by the hydrolysis of oils and fats in poultry can improve fat digestibility. Therefore, lipase supplementation can improve feed quality and broiler performance [8].

Ligninolytic enzymes affect polysaccharides in the feed, and by enhancing saccharification, disrupt the rigid structure of carbohydrates and improve the digestibility of cellulosic residues [9, 10]. Despite these advantages, carbohydrate-hydrolyzing enzymes, such as cellulase, xylanase, and amylase, play a vital role in breaking cereal cell walls and liberating antioxidant phenolics, which can improve the oxidative stability of feeds [5, 11, 12]. Laccases are important antimicrobial agents in the poultry industry [13]. Laccases, in particular, exhibit antimicrobial properties, limiting bacterial growth through oxidation and detoxification [13]. Proteases, xylanases, amylases, and lipases play a crucial role in breaking down proteins, non-starch polysaccharides, starch, and lipids, respectively, enhancing nutrient bioavailability and absorption [7].

This study explores the encapsulation of multi-enzymes using natural polysaccharide gums, which enhance enzyme stability and enable controlled release, improving feed effectiveness. The process involves entrapping enzymes within a protective matrix, such as natural polysaccharide gums, which provides several advantages in maintaining enzyme stability and optimizing their release [14]. Gums are organic, hydrophilic compounds that resist organic solvents. When introduced into a medium, they form colloidal solutions or dispersions [15]. Guar and Arabic gums have proven to be ideal encapsulating agents due to their eco-friendly nature and distinctive protective qualities. These gums enhance the stability of encapsulated materials and enable controlled release, making them suitable for various applications from feed to pharmaceuticals [15]. Proper animal nutrition is essential for successful poultry production, and enzyme encapsulation is a key strategy for maximizing feed value, while reducing costs and waste [16, 17]. The use of antimicrobial agents in poultry feed helps manage the gut microbiota and promotes health and growth [18]. Enhancing the antimicrobial properties of poultry feed is crucial for producing disease-free poultry and for improving public health. Given the global challenge of antibiotic resistance, enzymes offer a sustainable alternative for maintaining gut health and reducing the reliance on traditional antimicrobials. Enzyme supplementation in poultry feed not only supports bird health, but also ensures safer poultry products for consumers [19].

One key limitation in poultry production is that using single-activity enzymes may be less effective compared to multi-enzyme mixtures, due to the specific substrate requirements of each enzyme. By designing enzyme cocktails, poultry diets can be significantly improved through synergistic effects that enhance nutrient utilization and overall performance [4]. Using a combination of supplemental feed enzymes or developing a multi-enzyme system boosts digestion and increases the hydrolysis of fats, starches, and nitrogen [4]. However, for optimal degradation of poultry feed, enzymes should exhibit stability across various pH levels, remain effective at ambient temperatures, and resist ions. Unfortunately, enzyme instability under harsh conditions, such as high pelleting temperatures and other industrial challenges, remains a major drawbac [20]. This highlights the urgent need for innovative methods to identify robust enzymes [21, 22]. Metagenomics offers access to environmental microbial species that cannot be cultivated using standard laboratory techniques [23]. Furthermore, advances in bioinformatics and computational tools that allow the analysis of the three-dimensional structure of enzymes [24–26] have led to the discovery of stable enzymes from metagenomic sources for the effective degradation of poultry feed and development of functional and nutritional properties [27, 28].

Although feed enzymes have been a topic of research in poultry nutrition, the need to emphasize their role in increasing potential antimicrobial activity in poultry feed cannot be overstated. This enhancement is pivotal for poultry health and raises concerns regarding bacterial infections in the industry. With a focus on improving potential antimicrobial activity and given the significant function of robust enzymes, especially when combined synergistically in poultry feed hydrolysis, the present

study used metagenome-derived enzymes to develop an enzyme cocktail aimed at elevating feed quality. To improve the activity and efficiency of enzyme mixtures, they were encapsulated in gums. Following this encapsulation, the antimicrobial capabilities of the enzyme mixture were measured and a marked increase in potential antimicrobial activity was demonstrated; therefore, it was selected for further analysis. Subsequent in vitro evaluations provided insights into the higher levels of antioxidant phenolic compounds and improved physical characteristics such as solubility and water-holding capacity. Morphological changes in poultry feed were assessed using the encapsulated enzyme cocktail. This work provides insights into the application of encapsulated enzyme mixtures with potential antimicrobial activity as feed additives to increase the functional, physical, and nutritional features of poultry feed and the wider use of feed enzymes in the poultry industry.

Methods

Enzymes and reagents

The metagenome-derived enzymes used in this study including PersiLipase1, PersiProtease1, PersiAmy3, PersiLac1 and bifunctional PersiCelXyn1 were obtained from the Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj, Iran. Poultry feed with a specific diet composition for broiler chickens after 29–42 days, which was provided by the Animal Science Research Institute of Iran, Karaj, and its composition was determined in the Supplementary Information. Bacterial strains (*Escherichia coli*) were obtained from the Iranian Biological Resource Center (IBRC, Iran). Other materials and chemicals including bovine serum albumin (BSA), potassium ferricyanide, hydrochloric acid (HCl), Sodium hydroxide (NaOH), ferric chloride ($FeCl_3$), Folin reagent, 2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), sodium carbonate (Na_2CO_3), gallic acid, lysogeny broth (LB), kanamycin, ethanol, trichloroacetic acid, Guar gum, Arabic gum, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Fluorescein isothiocyanate (FITC), Dimethyl sulfoxide (DMSO) and 3,5dinitrosalicylic acid (DNS) were of analytical grade and were obtained from Sigma-Aldrich.

Evaluation of multi-enzyme and antimicrobial efficacy

The multi-enzyme comprising PersiAmy3, PersiLipase1, PersiProtease1, PersiLac1, and PersiCelXyn1, as described in prior research, was formulated at a concentration of 3 U/mL [22, 27, 29–31]. To evaluate the antimicrobial efficiency against *Escherichia coli* using multiple enzymes, we employed three distinct methodologies, detailed as follows:

Cultivation and monitoring of Escherichia coli strain

Escherichia coli was cultured using nutrient agar and nutrient broth as growth media. The cultures were incubated under standardized conditions at 37 °C. Bacterial proliferation was quantitatively assessed by determining the optical density (OD) of the culture. Measurements were performed using a spectrophotometer set at a wavelength of 620 nm.

Growth inhibition test by spectrophotometric analysis

The potential antimicrobial activity of the enzymes against *Escherichia coli* was determined using the microdilution method [32]. Briefly, five serial dilutions of the multi-enzyme were prepared and mixed with a standardized amount of *Escherichia coli* suspended in Luria-Bertani (LB) broth. The mixtures were incubated at 37 °C for 12 h with shaking (200 rpm). Additionally, a negative control, consisting of an *Escherichia coli* suspension in LB broth without the enzyme, was also included in the assay. After incubation, microbial growth was determined by measuring the increase in the turbidity of each well at 630 nm using a microplate reader and the percentage of growth inhibition according to the following formula:

$$\text{Growth inhibition } (\%) = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

In addition, the antimicrobial activity against both beneficial and pathogenic bacteria was determined using the well diffusion assay method [33].

Growth inhibition test by agar well diffusion assay

The method adopted was derived from a previous study but featured slight procedural modifications [34]. Initially, *Escherichia coli* was cultured in Luria-Bertani (LB) broth and incubated at 37 °C with continuous agitation at 180 rpm for an hour. The bacterial suspension was then mixed with LB agar (1.5% w/v) to ensure proper diffusion of the test samples. The mixture was poured into sterile Petri dishes and allowed to solidify. Once solidified, four equidistant wells were bored into the agar using a sterilized cavity borer. Autoclaved distilled water served as the negative control and kanamycin (50 mg/mL) was used as the positive control. Two test samples were also introduced into the wells: a 2:1 dilution of the enzyme mixture with autoclaved distilled water (labeled E1) and an undiluted enzyme mixture (labeled E2). The Petri dishes were incubated at 37 °C for 24 h. Antibacterial activity of the multi-enzyme was assessed by measuring the diameter of the inhibition zones formed on the microbial plates [13].

FITC-labeling of multi-enzyme

For multi-enzyme encapsulation, a fluorescence labeling experiment was performed as described in a previous

study [35]. To prepare FITC, 2 mg was dissolved in 2 mL of dimethyl sulfoxide (DMSO) to achieve a uniform solution. The solution was protected from light by wrapping it in foil to maintain its fluorescence intensity. Then, 1 µL of this dye solution was mixed with 50 µL of sesame hydrolysates under conditions that prevented light exposure. This mixture was left for 12 h at 4 °C.

The multi-enzyme solution (5 U/mL) was mixed with a FITC solution and the mixtures were stirred at room temperature for 4 h at 160 rpm, followed by precipitation of the labeled multi-enzymes using acetone several times and centrifugation.

Growth inhibition test by FITC-labeling of multi-enzyme and fluorescence microscope

To prepare FITC, 2 mg was dissolved in 2 mL of DMSO to ensure homogeneity within the solvent. To safeguard the fluorescence intensity and preclude quenching, the solution was shielded with foil to prevent light exposure. Subsequently, 1 µL of the aforementioned dye was combined with 50 µL of the multi-enzyme solution under light-protected conditions. The mixture was incubated at 4 °C for 12 h to ensure stabilization. The labeled multi-enzyme was subsequently introduced into 400 µL of the cultured *Escherichia coli* sample, and the amalgamation was incubated under dark conditions at 37 °C for 1 h. After incubation, the alterations were meticulously examined using fluorescence microscopy.

Preparation multi-enzyme encapsulation powder

To prepare the coating, Guar gum and Arabic gum were independently suspended in 2% (w/w) distilled water and stirred for 1 h at room temperature. The S1 solution was composed of Guar gum, and the S2 solution was prepared from Arabic gum. The next S3 solution was obtained by mixing the solutions of Guar gum and Arabic gum in a ratio of 5:1 (v/v). The ratio of the S1, S2, and S3 solutions to the enzyme mixture was 1:1.

Following thorough homogenization for 2 h at room temperature, the dispersions were spread into Petri dishes in approximately 10 mm layers. They were subsequently frozen at -30 °C for 14 h to yield ice crystals, which were then rapidly plunged into liquid nitrogen at -190 °C in preparation for freeze-drying, ensuring the maximal crystallization of freezable water. Afterwards, the powders were collected, packed in metallized bags, and stored in a freezer at -20 °C until analysis [36]. To verify the encapsulation of multi-enzymes in gums, we assessed the FITC-labeling, solubility, and moisture content of the encapsulated samples, as detailed in the subsequent sections. Finally, the encapsulated samples were observed using a fluorescence microscope (Nikon LHS-H100P-1, Japan).

Solubility

The solubility of the encapsulated sample dry powder was calculated using the method described by Ma et al., with slight modifications [37]. Dried samples (10 mg) were dispersed in 250 µL distilled water, vortexed for 2 min, and centrifuged at 7000 rpm for 3 min at 27 °C. Finally, the amount of protein in the supernatant and the primary solution was measured using the Bradford assay [38]. The UV-visible absorbance of the encapsulated and non-encapsulated samples was determined.

Moisture content

Moisture content was determined according to the method described by Mahfoudhi et [39]. Briefly, 1 g of multi-enzyme mix and microencapsulated enzyme powder were measured immediately after freeze-drying and oven-dried at 70 °C to a constant weight. The moisture content was calculated based on the weight loss before and after drying [39].

$$MC\ (\%) = \frac{sample\ wight}{wight\ loss} * 100$$

Atomic Force Microscopy (AFM) analysis

According to Zuo et al. [40], the surface morphology of the films was assessed using atomic force microscopy (AFM; Brisk model Ara pazhoohesh, Iran). AFM of the mixed enzyme and its encapsulated form was performed at a scan speed of 1.0 Hz and resolution of 500×500 pixels.

Applicability of the encapsulated multi-enzymes for developing the quality of poultry feed

To investigate the capability of encapsulated multi-enzymes to degrade poultry feed, we prepared a solution of poultry feed in potassium phosphate buffer (50 mM, pH 7.0) at a concentration of 10 mg/mL. The feed was homogenized thoroughly, the encapsulated enzyme was added to the reaction mixture at a ratio of 1:10 [41], and the mixture was incubated at 50 °C for 24 h. A control sample was prepared without the addition of the encapsulated multi-enzyme. Following incubation, the reaction mixture was centrifuged at 7,000 rpm for 10 min to separate the liquid fraction, which was then analyzed for reducing sugar, phenolic content, and antioxidant properties. The sediment obtained after centrifugation was used to assess water holding capacity (WHC), solubility, and structural changes [42].

Analysis of released reducing sugars

To evaluate the total reducing sugar content, samples were centrifuged, and the supernatant was collected after 60, 120, 180, and 240 min of incubation. The reducing

sugar liberated due to enzymatic activities were measured by recording the absorbance at 540 nm using a DNS assay [43].

Determination of total soluble phenols

The supernatant obtained after hydrolysis of poultry feed was used to study the effect of encapsulated metagenome-derived enzymes on the release of phenolic compounds from poultry feed. Total phenolic content was measured using Folin-Ciocalteu's colorimetric method, as explained below [44].

To 1 mL of the diluted sample, 5 mL of a 10% (v/v) Folin solution was pipetted and thoroughly mixed. Next, 400 µL of Na_2CO_3 (7.5% w/v) was added and the reaction was kept in the dark for 30 min. The standard used for this assay was gallic acid, which was used to prepare the calibration curve (5–85 µg/mL). The absorbance of each solution was measured at 750 nm and the phenolic content was expressed as µg Gallic Acid Equivalents (GAE) per mL of sample.

Antioxidant assay

ABTS radical cation decolorization

The ABTS* scavenging activity of the samples was determined following the method described in a previous study with some modifications [45]. ABTS radical solution (ABTS*) was prepared by reacting 7.4 mM ABTS with 2.6 mM potassium persulfate in equal quantities in the dark for 12–16 h. One thousand µL of ABTS* solution was diluted with water to get an absorbance of 0.70±0.02 absorbance at 734 nm. Then, 100 µL of samples were incubated with 1 mL of diluted ABTS^{+} solution, and the absorbance readings were taken after 5 min. The percentage quenching of the absorbance at 734 nm was measured.

$$\text{ABTS}^{\star}\ \text{scavenging}(\%) = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

Scavenging activity against DPPH radical

The method described Cuvelier and Berset was used to assay the DPPH radical scavenging capacity of the samples [46]. A DPPH solution (0.1 mM) was prepared in 95% ethanol and mixed with an aliquot of each sample (1:10 v/v). After incubating for 30 min at 25? in the dark, the reduction in absorbance at 517 nm was expressed as follows and compared with the control, which contained water instead of the sample.

$$DPPH\ scavenging(\%) = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

Measurement of reducing capacity

To examine the ability of samples to reduce Fe^{3+} to Fe^{2+}, a previously described method was used [47]. For 0.5 mL of sample solution with a concentration of 1 mg/mL prepared in 200 mM phosphate buffer (pH 6.6), 1.25 mL of potassium ferricyanide (1% w/v) was added and allowed to react with samples at 50 ?. After 20 min, trichloroacetic acid (10% w/v) was added to terminate the reaction, and samples were centrifuged at 3000 g for 10 min. The supernatant was recovered, mixed with $FeCl_3$ (0.1% w/v), and incubated at 25 ? for 10 min. Finally, the absorbance of the solutions was recorded at 700 nm, with a higher absorbance indicating greater reducing power.

Water-holding capacity (WHC)

After centrifugation of the encapsulated multi-enzyme treated feed and control sample, the collected sediments were weighed. According to the weight of the dry poultry feed (W_0), the weight of the tube plus samples (W_2), and weight of the centrifuge tube with the sediment (W_1) the water holding capacity is determined as shown in the following formula [48]:

$$\text{WHC} = \frac{W_2 - W_1}{W_0}$$

Solubility

To measure the solubility of the samples, a suspension of encapsulated enzyme-treated poultry feed in distilled water (100 mg/mL) was stirred for 30 min. The mixture was then centrifuged at 3000x g for 15 min to separate the supernatant. The weight of the sediment after removing the supernatant was determined after lyophilization, and the percentage of solubility was measured as listed below [49]:

$$\text{Solubility}\ (\%) = \frac{W_t - W_{in}}{W_{in}} \times 100$$

Where: W_t is the total weight of the poultry feed, W_{in} is the weight of samples after lyophilization.

Structural characterization of poultry feed

The resulting solid phase after enzymatic hydrolysis was consecutively oven-dried and stored at 4 °C to examine structural changes [50]. Characteristic peaks associated with enzyme activity were studied by Fourier transform infrared (FTIR) spectroscopy. FTIR spectra were recorded between 300 cm^{-1} to 4000 cm^{-1} (model Thermo, AVATART, Germany). The surface morphology of the multi-enzyme treated samples was analyzed using scanning electron microscopy (SEM) images (FEI ESEM QUANTA 200, USA). The dried samples were placed on conducting carbon tape and photographed under an accelerating voltage of 15 kV.

Statistical analysis

In this study, three replicates were performed for all experiments, and means were calculated. Accordingly, the standard deviations were measured using Microsoft Excel software. All data are presented as mean values±standard deviation (SD). Statistical analyses were performed using SPSS Version 20.0, using Duncan's test,

and the results were compared with significance assigned at $P<0.05$.

Results and discussion

Feed enzymes have a significant impact on the quality of poultry feed, and can significantly enhance the digestibility of nutrients in the gut. Carbohydrate-hydrolyzing enzymes, such as amylase, cellulase, and xylanase, are essential in poultry diets because they increase the conversion of cellulose and starch to glucose [51]. It is associated with an effective energy source for birds, improved performance, and reduced pancreatic mass in broilers [52]. Furthermore, these enzymes can degrade substrates and release bound phenolics, which enhances the antiradical capacity of the feed. Therefore, reducing nitrogen emissions associated with broiler production in the environment is of great importance. This can be achieved by supplementing poultry feed with proteases and increasing the protein digestibility [6]. Additionally, the hydrolysis of protein-rich raw materials and production of hydrolysates can improve the functional properties of the feed, such as antioxidant activities [6]. Moreover, using oxidoreductases, such as laccase is a practical way to positively influence the quality of poultry feed because of the antibacterial activities of these enzymes against some bacterial pathogens [13]. Similarly, addition of lipases improves fat digestibility in birds and enhances their performance through fatty acid production. As different enzymes have various targets in poultry feed, developing enzyme cocktails has a greater effect than when enzymes are added to poultry feed separately. Notably, the development of enzyme cocktails containing stable enzymes with improved performance is crucial for utilization during the processing of commercial feeds [3]. Moreover, encapsulation facilitates the controlled release of enzymes, ensuring sustained enzymatic activity over extended periods. The incorporation of encapsulated enzymes into poultry feed represents a significant advancement in poultry nutrition, and supports the health and productivity of poultry. Therefore, as shown in Fig. 1, we developed a metagenome-derived multi-enzyme system and measured its antibacterial activity to ensure its efficiency. Subsequently, the multi-enzyme was encapsulated using Guar and Arabic gums and subjected to FITC-labeling, solubility, and moisture content experiments to validate the effectiveness of the encapsulated multi-enzymes. In the final step, we tested the ability of encapsulated multi-enzyme in poultry feed hydrolysis to develop nutritional and structural features of poultry feed and conducted several experiments to enhance the phenolics, antioxidants, and physical properties of feed.

Potential antimicrobial activity assay of multi-enzyme

Contamination of poultry feed with microbial pathogens is a major risk factor for foodborne illnesses in humans [53]. To reduce this risk, it is essential to ensure that poultry feed is free of bacterial pathogens such as *Salmonella*, *Escherichia coli*, *Campylobacter*, *Clostridium perfringens*, *Listeria monocytogenes*, *and Staphylococcus aureus*. *Escherichia coli* is a common bacterium found in nature, and many strains are part of the normal intestinal flora of humans and animals [54]. Despite its non-pathogenic nature in many cases, *Escherichia coli* is frequently used as a model organism in antimicrobial studies because it allows for safe and controlled assessments of potential antimicrobial properties. This approach can be expanded to include more resistant and pathogenic strains in future studies [55].

The inhibitory effect of the multi-enzyme against *Escherichia coli* was determined using the micro-dilution method. The dilution method is commonly used to measure the minimum concentration of antimicrobial agents. Serial dilutions of a solution make it possible to directly investigate the test material and assay its activity

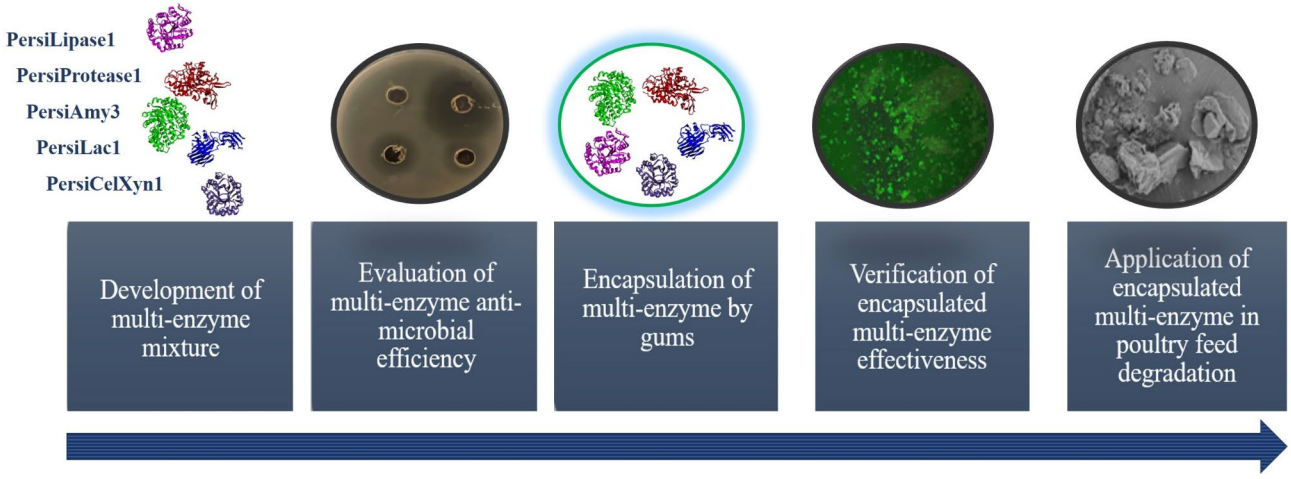

Fig. 1 Different steps were used in this work to investigate the applicability of encapsulated multi-enzyme for the hydrolysis of poultry feed

against several bacterial strains [32]. The absorbance of the samples as well as the inhibition percentage of the control and enzyme-treated poultry are presented in Fig. 2a. According to our results, the control sample demonstrated equal values for various concentrations of multi-enzyme, indicating the growth of bacteria in the medium. As shown in Fig. 2a, the highest percentages of inhibition were observed in the multi-enzyme treated *Escherichia coli* at concentrations between 5 and 6 U/mL of multi-enzyme, indicating values of 88.74% and 92.54%, respectively. This value reduced gently at higher concentrations of multi-enzyme and reached 61.42% at the lowest concentration (1 U/mL). Similarly, the inhibitory effects of the hydrolysates obtained by hydrolysis of proteins have been reported to improve the preservation of feed and food products [56]. In another report, antibacterial peptides were obtained through enzymatic hydrolysis to improve bacteria growth [57]. Our results proved that the degradation of poultry feed by multi-enzyme can lead to a higher growth inhibition of the bacteria, which is a significant factor in the poultry industry.

As shown in the Fig. 2b, the zone of inhibition against *Escherichia coli* was measured. For the two concentrations of multi-enzyme (2 and 4 U/mL), the zones of inhibition were 1.2±0.01 cm and 1.6±0.05 cm, respectively. The control group with distilled water showed no inhibition, while the positive control with Kanamycin (50 mg/mL) displayed a zone of inhibition 2.5±0.02. This indicates the potent antimicrobial activity of the multi-enzymes. These results are in accordance with previous reports that investigated the antimicrobial activities of both enzyme [13]. Another study reported the antimicrobial properties of laccase from *Trametes versicolor* for different applications such as in the food industry [58].

To elucidate the interactions between the multi-enzyme and *Escherichia coli*, the distribution multi-enzyme within the bacteria was monitored using a FITC-labeled variant. As depicted in Fig. 3, within 60 min, the FITC-labeled multi-enzyme is observed to translocate through the *Escherichia coli* cell membrane and accumulate within the cytoplasm. The antibacterial activities of the multi-enzyme against *Escherichia coli* were determined, and the results are illustrated.

Encapsulation analyses of freeze-dried encapsulated multi-enzyme

Encapsulation of enzymes can protect proteins from denaturing substances or bacteria. This technique can also enhance the functionality, longevity, and efficiency of enzymes, which is important for the poultry industry. In the current study, we labeled multi-enzyme using FITC method, and then the multi-enzyme mixture was subjected to encapsulation using Arabic and Guar gums. The efficiency of multi-enzyme labeling by FITC encapsulation was evaluated under fluorescence (green) and visible light (white). As shown in Fig. 4, uniform fluorescence was observed for sample S2, indicating successful encapsulation of this sample. In contrast, samples S1 and S3 exhibited patches and clusters of fluorescence, indicating uneven encapsulation. Additionally, the presence

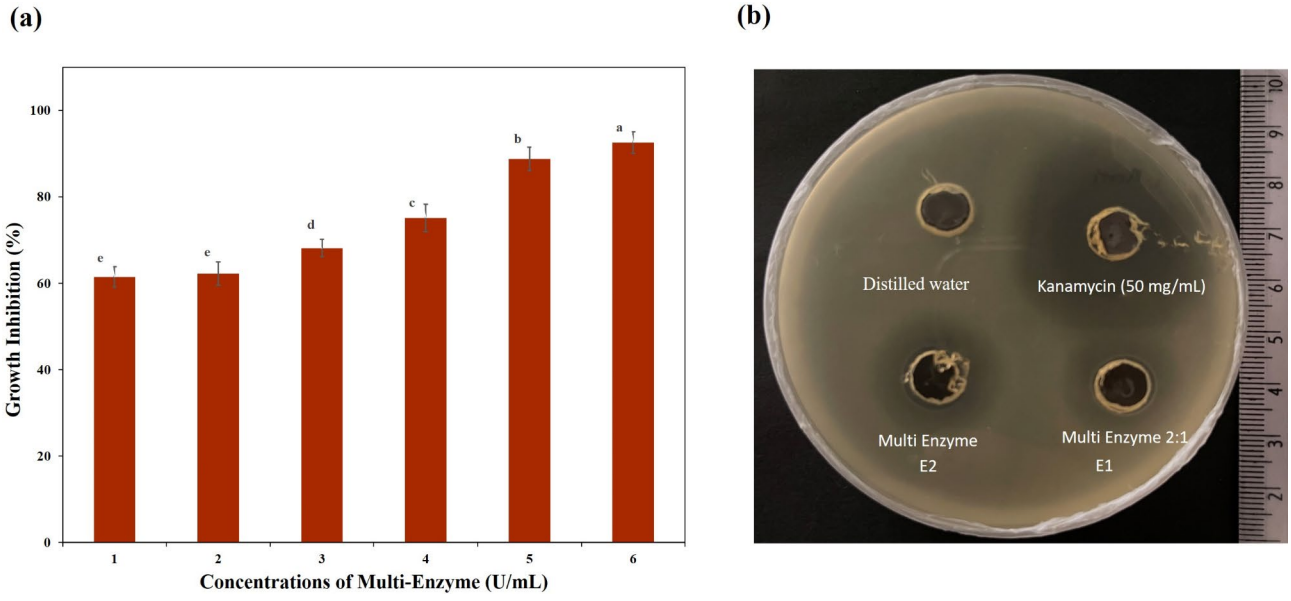

Fig. 2 (**a**) Growth inhibition of *Escherichia coli* using different concentrations of multi-enzyme from 1 to 6 U/mL after incubation at 37 °C for 12 h. A negative control, consisting of an *Escherichia coli* suspension in LB broth without the enzyme, was also included in the assay. Means ± standard deviations ($n = 3$). Values with different letters differ significantly ($p < 0.05$). (**b**) Zone of inhibition diameter against *Escherichia coli* as a result of multi-enzyme activity (diluted enzyme mixture (E1) and undiluted enzyme mixture (E2)) incubated at 37 °C for 24 h. Autoclaved distilled water served as the negative control and kanamycin (50 mg/mL) was used as the positive control

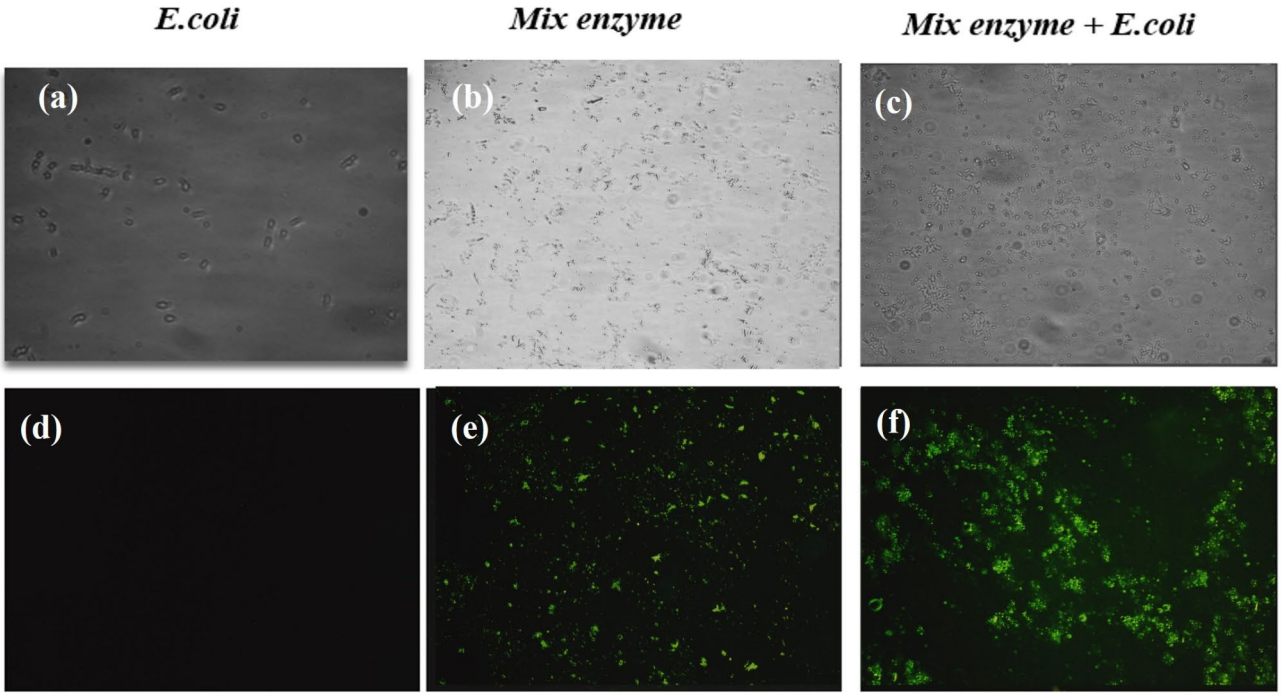

Fig. 3 Fluorescence microscopy images of FITC-labeled multi-enzyme with and without *Escherichia coli* at different magnifications. (**a, b, c**) Light field images (**d, e, f**) Dark field images

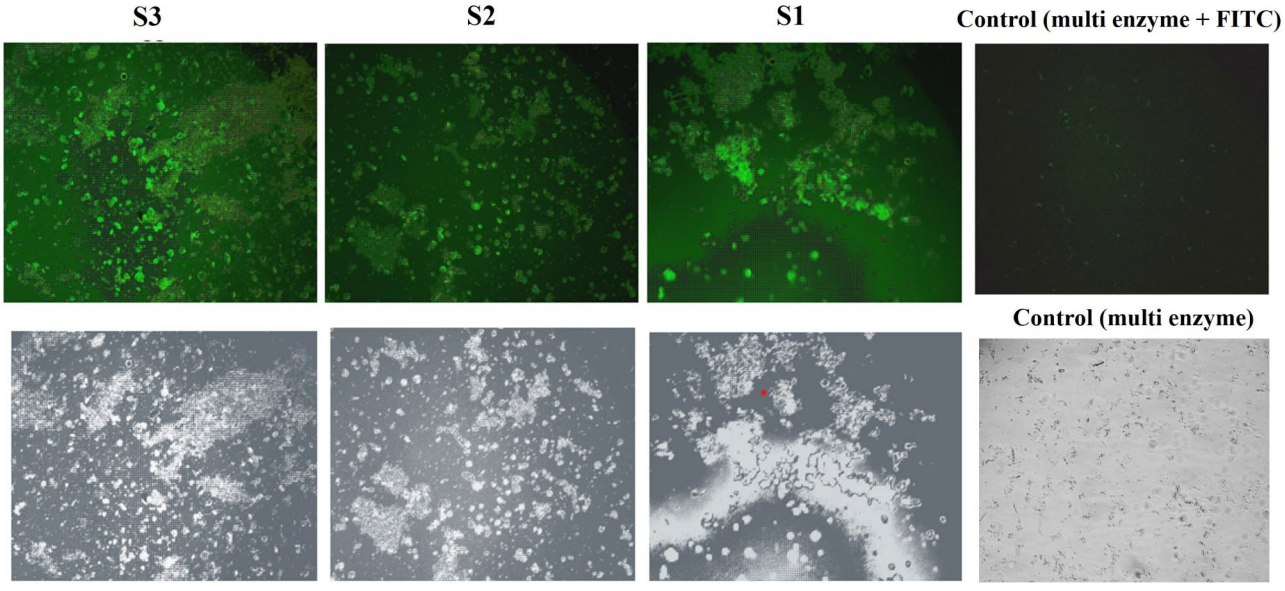

Fig. 4 Fluorescence microscopy images of the encapsulated mixed enzyme using Guar gum and Arabic gum with FITC. The S1 solution consisted of Guar gum and enzyme in a 1:1 ratio, S2 was prepared with Arabic gum and enzyme in a 1:1 ratio, and S3 was obtained by combining Guar gum and Arabic gum in a 5:1 (v/v) ratio, then mixing with the enzyme in a 1:1 ratio. The multi-enzyme alone served as the control

of larger encapsulation structures in samples S1 and S3 revealed undesired encapsulation of multi-enzyme suggesting the aggregation of enzymes, or not favoring the encapsulation process for certain regions over others [59, 60]. The uniform fluorescence observed in sample S2 suggests a consistent spread of the fluorescently tagged substance throughout the sample, implying homogeneous encapsulation, whereas the presence of clusters in samples S1 and S3 indicated that the tagged multi-enzyme was concentrated in specific areas rather than being uniformly spread out [61]. Therefore, the encapsulated multi-enzyme in sample S2, with a homogeneous distribution of gums inside the gums, were selected for further experiments.

Solubility and moisture content

The moisture content of encapsulated enzyme refers to the amount of water present in the enzyme, which is critical due to the sensitivity of enzymes to their surrounding environments. Based on the moisture content analysis, the multi-enzyme had a moisture level of 4.3 g/100 before encapsulation, which decreased to 2.2 g/100 g after encapsulation. It has been suggested that the low moisture content of the encapsulated enzyme results in enhanced stability, and the presence of limited moisture minimizes the potential for microbial growth. Consequently, it has been suggested that products that maintain such conditions often have longer shelf lives and reduced susceptibility to spoilage [62]. In a previous report, using a combination of maltodextrin and Arabic gum, the moisture content of the encapsulated matrix was lower than that of a control sample [63]. This is an indicator of the effectiveness of the encapsulation process and indicates that the multi- enzyme is well protected. Moreover, the solubilities of the encapsulated and non-encapsulated multi-enzyme were recorded. The multi-enzyme exhibited a solubility of 100%, which decreased to 95% after encapsulation. The low solubility of encapsulated multi-enzyme compared with that of the control indicates that the encapsulation process effectively protected the enzyme from external moisture, thereby preserving its stability [64, 65].

Additionally, short-term stability tests conducted after one month of storage demonstrated that the encapsulated enzymes retained their activity, confirming the stability of the enzymes in their encapsulated form. This further supports their potential for long-term use in feed applications.

Owing to the good performance of the encapsulated multi-enzyme in FITC labeling, as well as its favorable moisture content and solubility characteristics, we chose this sample for further experiments. We intended to incorporate it into poultry feed to evaluate its potential benefits and overall suitability for poultry feed application. This decision stems from the belief that its encapsulated form may offer enhanced nutritional and physical properties in poultry feeds.

AFM studies

AFM is a valuable tool for analyzing enzyme mixtures and provides intricate details of their surface topographies. This technique excels in depicting the size, shape, and spatial distribution of enzyme particles, whether in their free or encapsulated states. As shown in Fig. 5, notable differences were observed between free and encapsulated enzyme samples. The AFM image of the free enzyme mixture showed particles that varied widely in size and shape, predominantly adopting a spherical form. This heterogeneity is a hallmark of non-encapsulated enzymes, in which individual molecules freely assume their natural conformation. Additionally, it showed an increase in maximum height, reaching 140 nm.

In contrast, the AFM image of the encapsulated enzyme revealed a more uniform appearance with a reduced maximum height of 119 nm, suggesting a more constrained and consistent structural arrangement due to the encapsulation process. Furthermore, an increased number of particles appeared against the background, which is a clear indication that the encapsulating material enveloped enzyme molecules. These AFM images highlight the profound impact of encapsulation on the enzyme morphology. Encapsulation typically aims to protect enzymes from environmental factors and improve their stability; the structural changes observed via AFM are indicative of such protective measures. Moreover, the reduced height of the encapsulated sample can be attributed to a more compact arrangement. Additionally, the AFM analysis can be interpreted as an indication of a successful encapsulation process. These results are in agreement with the AFM results for polymer-encapsulated enzymes [66] and encapsulating enzymes in polymeric nanoparticles [67].

Influence of encapsulated multi-enzyme in poultry feed degradation

Investigating liberated reducing sugar

Treatment of poultry feed with multi-enzyme positively affected the saccharification and the amount of reducing sugars (Fig. 6). The yield of reducing sugars from the feed was obtained at 178.79 mg/g after 30 min of the reaction, increasing to 228.79 mg/g after 60 min. This value further increased to 328.79 mg/g after 120 min of hydrolysis, reaching a maximum concentration of 346.79 to 358.79 mg/g after 180 to 240 min of incubation. At the end of the reaction, the concentration of liberated reducing sugars increased 2-fold compared to the number of released sugars at the start of hydrolysis. Similarly, enzymatic saccharification of poultry feed initially reached 32.18% and was gradually enhanced with increasing time. In the first 60 min of hydrolysis with the multi-enzyme, 41.18% saccharification was found and enhanced to 59.18% after 120 min to finally reach the highest level of sugar production after 180 to 240 min of incubation (62–64%). Thus, these results suggest the successful degradation of poultry feed by multi-enzyme and its efficiency in supplementing poultry feed. In a previous study, the highest amount of reducing sugar from poultry feed was 81 mg/g after 60 min using xylanase from *Bacillus* [42]. In another study conducted by Alokika et al., xylanase from *Bacillus subtilis* was generated at approximately 100 mg/g of reducing sugar in poultry feed after 48 h of reaction [68]. Another report mentioned the liberation of 346.73 mg/g of reducing sugar from poultry feed by the

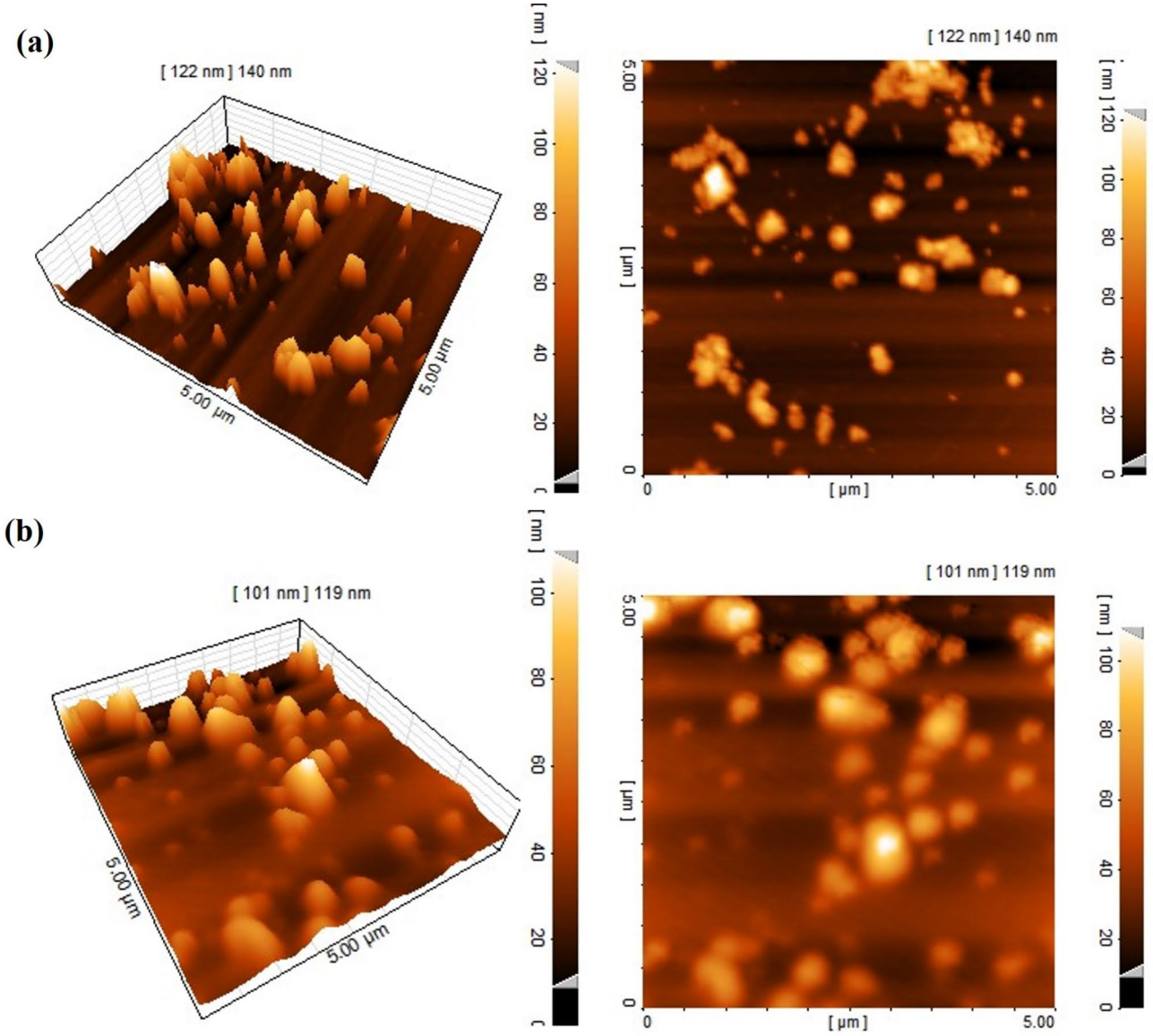

Fig. 5 AFM studies of mixed enzyme (**a**) and its encapsulated form using Arabic gum (**b**)

addition of a cocktail of amylase and xylanase after 72 h of reaction [28]. In contrast, the enzyme cocktail used in this study produced 228.79 mg/g of poultry feed in the first hour of hydrolysis and reached maximum release of reducing sugars (358.79 mg/g of poultry feed) after 240 min. Complete depolymerization of feed requires enzymes with multiple functions that can target various compounds; thus, using a multi-enzyme could promote the degradation of feed and improve the amounts of resulting sugars, indicating its efficiency for feed applications.

Total phenolic content and antioxidant capacities

Enzymes play an important role the release of phenolic compounds associated with antioxidant activity and radical scavenging from substrates during hydrolysis. The presence of phenols in poultry feed has been linked to the reduction of several diseases in birds and to improved animal performance and/or meat sensory properties [69]. Increasing the levels of antioxidant compounds in poultry diets improves the welfare and immune status of chicken [1, 70]. In this study, we investigated the influence of enzymes on the phenolic content and antioxidant activity of poultry feeds. According to the results in Table 1, the encapsulated multi-enzyme treated sample revealed 94.47 µg a phenolic content. This value was three times greater than that of the control, indicating the high capability of the multi-enzyme system for the conversion of poultry feed. Hydrolysis of the poultry feed by multi-enzyme hydrolyzes the starch, polysaccharides,

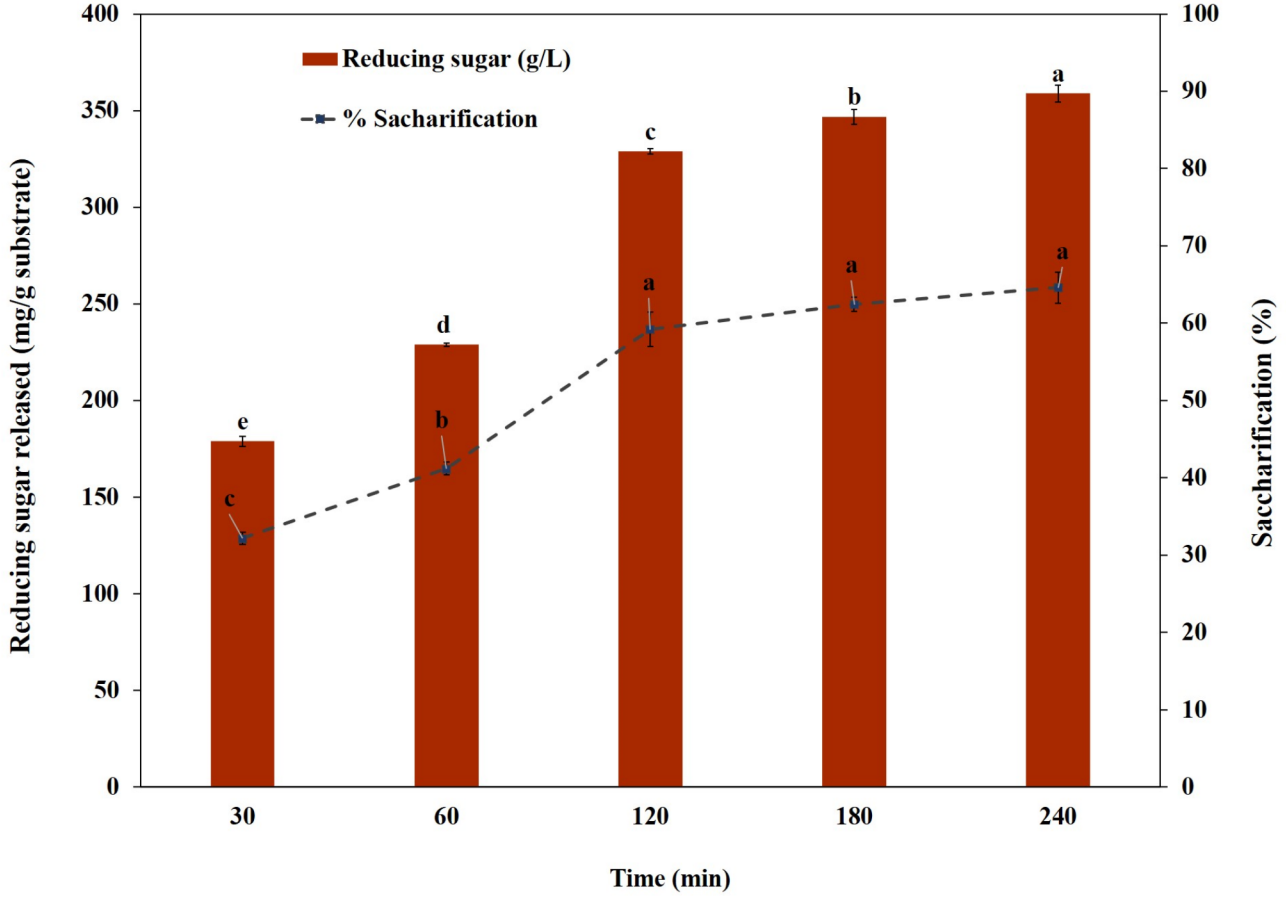

Fig. 6 Effect of multi-enzyme on the concentration of liberated reducing sugar from poultry feed and the percentage of saccharification. Means ± standard deviations ($n = 3$). Values with different letters differ significantly ($p < 0.05$)

Table 1 Phenolic content, radical scavenging rate by ABTS, DPPH, and reducing power of the poultry feed treated with multi-enzyme compared with the control sample

	Untreated poultry feed	Enzyme-treated poultry feed
Phenolic content (μg/mL GAE)	26.50 ± 2.79	94.47 ± 2.77
ABTS radical scavenging (%)	51.66 ± 1.15	90.83 ± 0.99
DPPH radical scavenging (%)	36.17 ± 1.93	47.86 ± 1.70
Reducing power (Absorbance 700 nm)	0.219 ± 0.11	0.320 ± 0.13

and protein structures in the substrate, disrupting the interactions between the phenolics and cell wall components and facilitating the release of phenolics. Additionally, the activity of carbohydrate-hydrolyzing enzymes in the cocktail promotes depolymerization of the substrate and elevates the liberation of phenolic compounds [71].

Moreover, the highest reducing power and free radical (ABTS and DPPH) scavenging activities were observed in the presence of the multi-enzyme, which manifested as 90.83% and 47.86% ABTS and DPPH radical scavenging activities in the presence of the encapsulated multi-enzyme, respectively. A higher absorbance of the samples indicates greater reducing power, as observed at 0.320 (Abs700 nm) after 24 h of hydrolysis by multi-enzyme. In the control sample, the ABTS and DPPH radical scavenging activities were found to be 51.66% and 36.17%, respectively, which were much lower than those of the enzyme-treated poultry feed. The major health benefit of phenolic compounds is their antioxidant capacity. These results indicate the release of antioxidative phenolics owing to the degradation of starch and fiber compounds in poultry feed, highlighting the application of these enzymes in the poultry industry. Previous studies have shown that treating carbohydrate substrates with enzymes modifies their structures by enhancing the amount of released phenolics and increasing their ABTS and DPPH radical-scavenging activities [72, 73]. A previous study reported the release of phenolic acids by cellulases with different antioxidant capacities from rice straw, using enzymes that can be used to produce value-added products [74].

Water-holding capacity (WHC) and solubility determination

Improving the WHC and solubility of poultry feed offers multiple benefits in optimizing bird nutrition and overall farm productivity. Enhanced WHC means that the feed can retain more moisture, ensuring that it remains palatable and facilitating better feed intake by the birds. However, increased solubility ensures that feed components readily dissolve readily in the bird's digestive tract, allowing for a more efficient release of nutrients and their subsequent absorption. WHC and solubility of feed ingredients are two key factors in examining the quality of final products in the industry and influencing their economic value. These functional properties are closely related to the texture of its product owing to the interaction with water. Supplementation with feed enzymes has been reported to influence poultry feed properties, such as water-holding capacity [75, 76]. WHC is defined as the ability of feed to retain water. As shown in Table 2, the WHC values of the encapsulated multi-enzyme treated feed were much higher than those of the controls. The control sample showed a WHC of 150.6%, which increased to 180.36% in the presence of multi-enzyme.

The presence of insoluble fibers in poultry feed is a major obstacle in the feed industry and has a negative impact on poultry because of its low digestibility. Using enzymes as effective additives to poultry feed enhances the number of particles and the surface area by the degradation of starch and proteins present in poultry feed. This is associated with numerous benefits for poultry, such as the promotion of digesta viscosity, physiological development, and improved digestion of feed [77]. Indeed, enzymes reduce substrate size by disrupting the feed structure and developing the digestibility of nutrients in the feed [78]. Based on the results obtained from Table 2, the solubility of poultry feed increased after the addition of the multi-enzyme. This value elevated almost 2 times and indicated the potential of encapsulated metagenome-derived enzymes for the effective biodegradation of poultry feed.

Investigating the structural changes of poultry feed

Enzymes play a vital role in the poultry industry, and many enzymes are used to improve poultry feed quality and digestibility. In this study, we investigated the influence of a multi-enzyme cocktail on the hydrolysis of poultry feed. The results showed that the multi-enzyme generated 5.29 mg/mL of reducing sugar after 24 h of hydrolysis, while this amount was much lower in the control sample (2.29 mg/mL). The enzymatic activities in the cocktail acted synergistically to degrade the substrate and improve feed conversion efficiency. Thus, the development of the enzyme cocktail is an effective way to increase enzymatic activity and its potential in feed applications [29, 79].

Table 2 WHC and solubility of multi-enzyme treated poultry feed in comparison with control

	WHC (%)	Solubility (%)
Untreated	150.6 ± 0.64	22.5 ± 0.97
Multi-enzyme treated	180.36 ± 0.74	42 ± 0.57

Studies have demonstrated the effectiveness of enzymes in the poultry feed industry. Amareh et al. reported the high impact of the xylanase, amylase, and protease in a single or combined form on the performance and digestibility of the poultry feed [80]. In another study, corn-based poultry feed was supplemented with a novel metagenome-derived α-amylase, that enhanced the liberation of reducing sugars [29]. Alokika et al. observed improved nutritional quality and reduced sugar release from poultry feed after treatment with xylanase from *B. subtilis* [68]. These results exhibited the utilization of the laccase enzyme separately and as a cocktail with carbohydrate-degrading enzymes, lipase and protease enhanced the enzyme efficiency in poultry feed hydrolysis.

SEM analysis was performed to study morphological changes in the control and the enzyme-treated samples. As shown in Fig. 7, the untreated sample exhibited a compact structure with a smooth surface and no structural deformation. After enzymatic treatment, the original arrangement of the poultry feed was lost and the structure of the substrate was disrupted into smaller fragments (Fig. 7). Meanwhile, in the presence of multi-enzyme irregular fragments were produced indicating the effectiveness of the enzymes in the hydrolysis of poultry feed. These results implied the considerable impact of metagenome-derived enzymes in the modification of substrate morphology and enhancement of e nutritional properties as compared with untreated poultry feed. Deformation of corn starch and disruption of its structure through enzymatic hydrolysis have been previously reported [81].

The FTIR spectra of the samples are shown in Fig. 8. Major changes in the band intensities were observed at 3000 to 3600 cm^{-1}. Compared with the control sample, the enzyme-treated samples showed a decreased intensity at 3425 cm^{-1}, which is attributed to OH stretching in cellulose, hemicellulose, and lignin [82]. This can be due to the modifications of poultry feed structure and disruption of the cellulose and hemicellulose during hydrolysis by the enzymes. These findings are similar to those of a previous study that investigated microstructural changes in corn starch after enzymatic hydrolysis [83]. In addition, the band at 2800 to 2900 cm^{-1} corresponds to the C-H and CH_2 stretching in cellulose, hemicellulose, and lignin, respectively [84]. This peak in the untreated sample indicated no structural changes, whereas the multi-enzyme exhibited a decrease in transmission (%) in this region. This is evidence of the hydrolysis of the

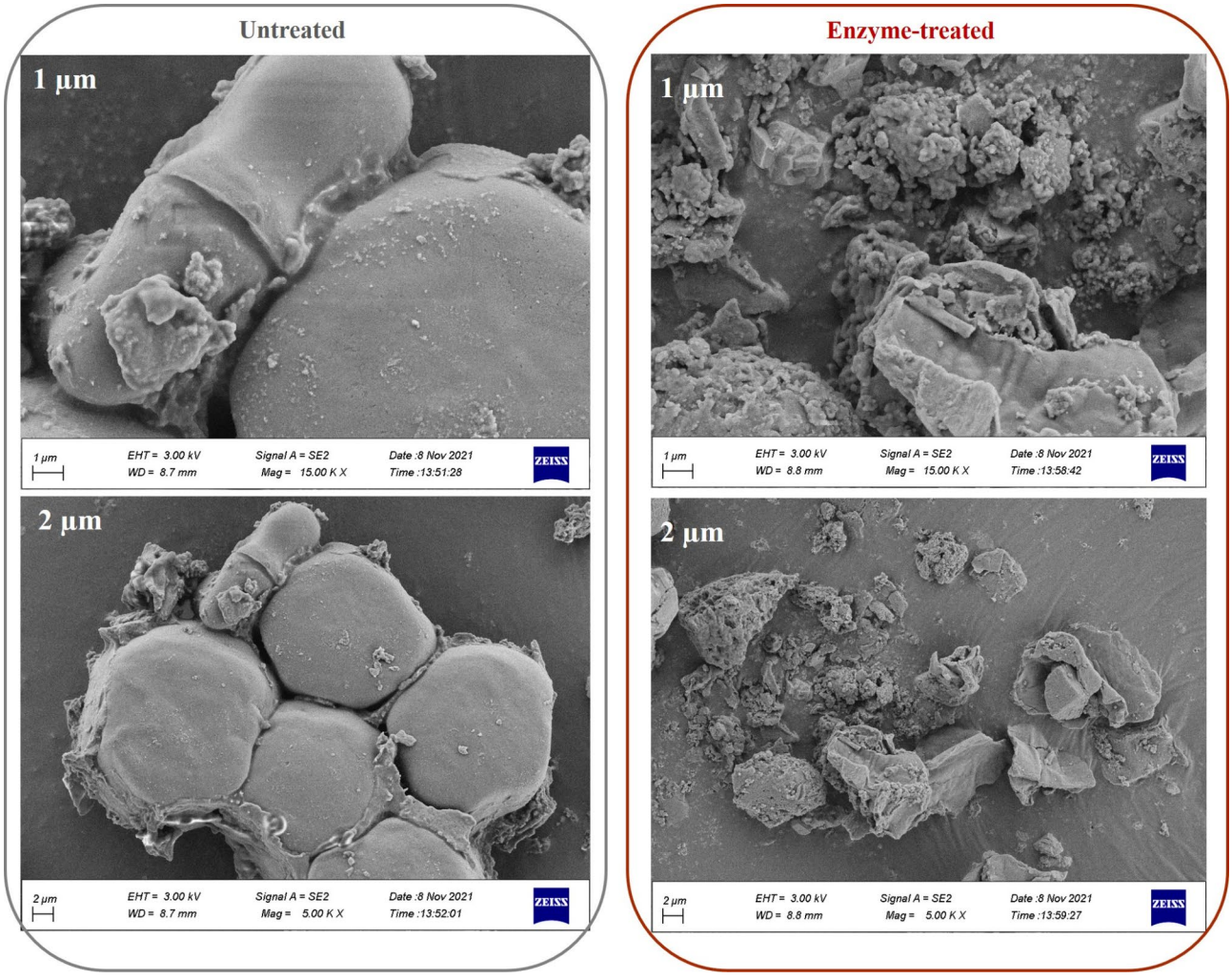

Fig. 7 SEM images (500× and 1500× magnification) of the poultry feed before and after hydrolysis by multi-enzyme

cellulose and hemicellulose bonds in these samples. A band at 1640–1660 cm^{-1} was due to the C=O stretching vibration of acetyl groups in the hemicellulose and un-conjugated and conjugated bands present in hemicellulose [85]. Meanwhile, the decline in these bands in the enzymes-treated poultry feed demonstrated the degradation of the substrate and solubilization of hemicellulose [86]. In this region, the intensity of the 1655 cm^{-1} peak in the presence of the multi-enzyme decreased, indicating the removal of hemicellulose [85]. Generally, after the addition of multi-enzyme, the transmission (%) strongly decreased, suggesting the hydrolysis of hemicellulose and lignin and the release of reducing sugars from the substrate. Moreover, the bands at 1383 cm^{-1} were assigned to C-H bending vibration and C-H stretching in CH_3 in cellulose and hemicellulose [84, 87]. The multi-enzyme showed a slight decrease in this peak intensity compared to the untreated sample, indicating hydrolysis of the cellulose and hemicellulose structures in poultry feed by the multi-enzyme. These findings support the potential of the enzymes for degradation of poultry feed structure and emphasize the enhanced efficiency of the enzymes after cocktail development for depolymerization of poultry feed.

Conclusion

The present study demonstrated that the encapsulation of metagenome-derived multi-enzymes offers a promising approach for improving poultry feed quality. The encapsulated enzymes exhibited potent potential antimicrobial activity against *Escherichia coli*, suggesting their potential to mitigate harmful pathogens in poultry feed. The encapsulation process also enhanced the stability of the enzymes, preserving their activity even after one month of storage, which supports their long-term use in feed applications. The utilization of encapsulated multi-enzymes in poultry feed leads to several key benefits, including enhanced antioxidant activity, increased feed solubility, and improved water-holding capacity. These improvements contribute to better nutrient

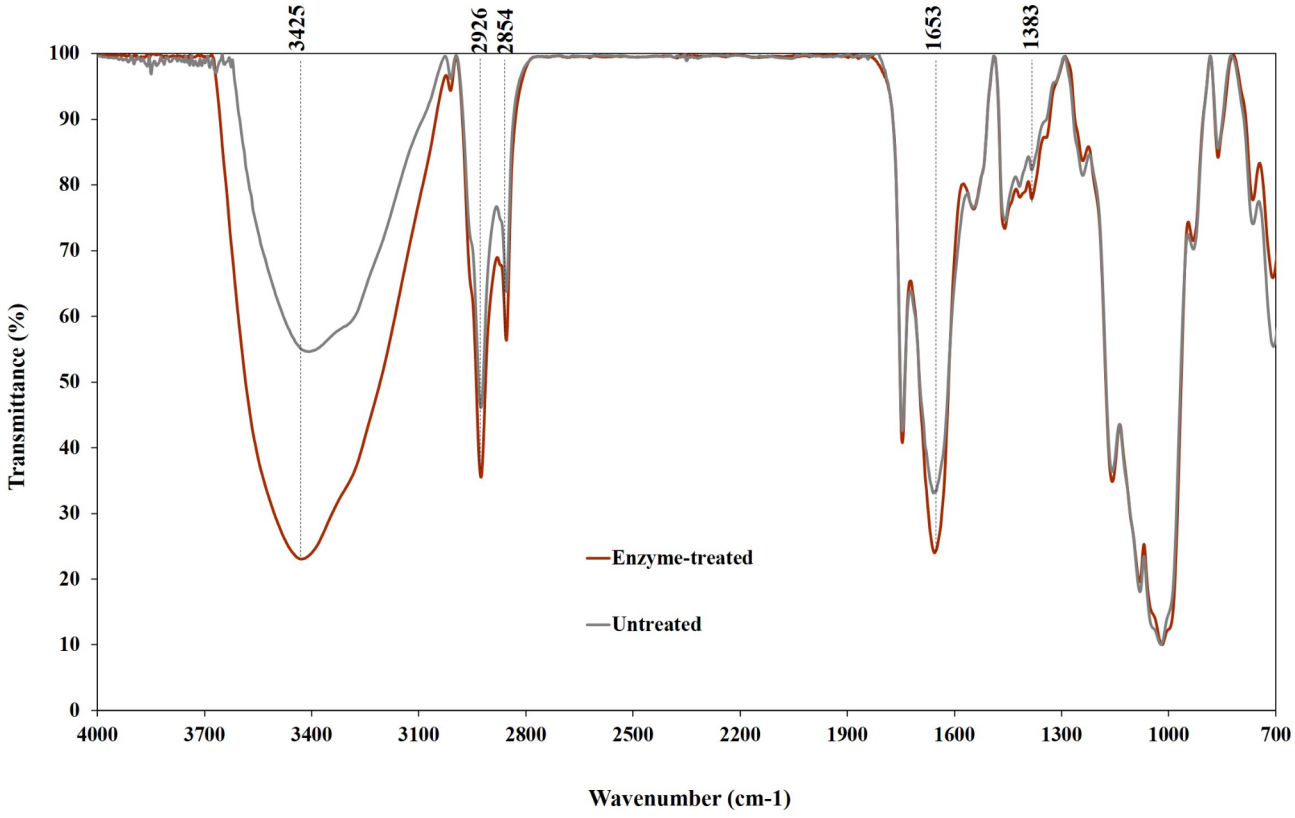

Fig. 8 FTIR spectroscopy analysis of the untreated and enzyme-treated poultry feed by the multi-enzyme

absorption and overall feed quality. Structural analysis further revealed significant morphological changes in the feed, resulting in the release of fermentable sugars and improved digestibility, highlighting the potential of encapsulated multi-enzymes to enhance poultry feed by boosting the nutritional value and controlling microbial contamination. The positive outcomes observed in this study warrant further in vivo investigation to confirm these benefits and assess their broader impact on the poultry industry.

Abbreviations

ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DNS	3,5-Dinitrosalicylic acid
BSA	Bovine serum albumin
SEM	Scanning electron microscope

Supplementary Information

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Supplementary Material 1: **Supplementary Table 1**. **Table S1**. Ingredients and basal diet composition.

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Author contributions
Shohreh Ariaeenejad: Conceptualization, Methodology, Investigation, Writing-original draft, Resources, Project administration. Mehrshad Zeinalabedini: Methodology; Analyzing and interpreting data; writing original draft. Akram Sadeghi: Investigation; Methodology; Microbiological analysis and writing original draft. Sajjad Gharaghani: Methodology and fermentation analysis. Mohsen Mardi: Methodology, Analyzed and interpreted the data.

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Data availability
All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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