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PREPARATION AND IN VITRO PERFORMANCE EVALUATION OF EGG YOLK IMMUNOGLOBULIN-LOADED SODIUM ALGINATE/CHITOSAN/SODIUM ALGINATE MICROCAPSULES FOR FEEDING

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ABSTRACT

Egg yolk immunoglobulin (IgY) is valuable against intestinal infections, however, its activity will be affected by the gastrointestinal fluid of livestock and poultry and the storage environment. Therefore, we prepared IgY-loaded sodium alginate/chitosan/sodium alginate microcapsules (IgY-loaded SA/CS/SA MCs), aiming to improve the poor tolerance of IgY in the gastrointestinal tract of livestock and poultry as well as the instability of its storage performance, so as to improve the performance of its application. In this experiment, IgY was used as the core material, and sodium alginate and chitosan were used as the wall material, and the preparation was carried out by the extrusion method. The results showed that the encapsulation efficiency of the prepared IgY-loaded SA/CS/SA MCs was highest (94.84%) when the IgY concentration was 200 mg/mL, the CS concentration was 0.8%, and the CS solution pH value was 5.0 (P<0.05). After freeze-drying, the shape of the MCs in group T1 was more regular, with an average particle size of 2.159 mm and the highest compressive strength (P<0.05). The stability of the T1 group in simulated gastric fluid (pH 2.0) was significantly enhanced compared to that of the NC, T2, and T3 groups (P<0.05), and the T3 group showed significantly improved antibacterial properties and increased alkalinephosphatase content compared to the T1 and T2 groups (P<0.05). In addition, IgY-loaded SA/CS/SA MCs were stored at room temperature (23±2°C) for 8 weeks, and the IgY activity in the T1 group was maintained at >70% (P<0.05). Therefore, the IgY-loaded SA/CS/SA MCs enhanced the tolerance and storage stability of IgY in simulated gastrointestinal fluids and showed significant inhibition of *E. coli* and *SG* in vitro.

Keywords: Egg yolk immunoglobulin; sodium alginate/chitosan/sodium alginate microcapsules ; preparation process; in vitro evaluation.

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INTRODUCTION

The unregulated use antibiotics leads to drug accumulation, bacterial resistance and other side effects, and many countries have implemented measures to restrict the use of antibiotics (Kovacs-Nolan *et al*., 2012; Stanton, 2013). Egg yolk immunoglobulin (IgY) is an antigen-specific antibody present in the egg yolk of birds and produced by B lymphocytes. It has been widely used as a substitute for antibiotics. It has advantages over other mammalian antibodies such as IgG in terms of ease of preparation, high production efficiency, and cost-effectiveness (Carlander *et al*., 2000). Jin *et al*. showed that IgY can effectively inhibit the colonization of pathogens such as *Escherichia coli* and *Salmonella* in the intestine, thereby playing a role in the prevention and treatment of intestinal diseases (Jin *et al*., 2023). Several in vivo studies have shown that IgY improves productive performance in animals (Li *et al*., 2009a; Xu *et al*., 2020).

The application of IgY is influenced by gastric acidic environments, and the activity of IgY is reduced in animals because of the presence of pepsin in the gastric juice as well as the lower pH environment (Shimizu *et al*., 1992; Chang *et al*., 2010). However, IgY performs its biological activity better in the intestinal fluid (Wang *et al*., 2021). To ensure that orally administered IgY efficiently reaches the small intestine and enhances its biological activity, it is important to consider how IgY

copes with lower gastric acidic environments, trypsin, and other factors that affect its stability during production (Alustiza *et al*., 2016). Therefore, effective methods are required to protect IgY activity (Zhang *et al*., 2020d).

Microcapsules (MCs) can maintain the biological activity of a delivered substance in a complex gastrointestinal (GI) environment and efficiently deliver it to a target site (Li *et al*., 2019). Based on this, microcapsule technology has become a research hotspot in food, medicine, biology and other fields. Studies have shown that microencapsulation protects various biologically active immunoglobulins from passing smoothly through the GI tract and maintains high activity (Li *et al*., 2022). Owing to the differences in the pH of the GI tract and the enzymatic environment, the wall material application of MCs is pH-sensitive and can control the release of core materials (Ren *et al*., 2016).

Sodium alginate (SA), a natural polysaccharide, is an ideal wall material that can make crosslink with Ca^{2+} at room temperature (RT; 23±2°C) (Mostafa *et al*., 2017; Jeong *et al*., 2020), which to some extent can protect bioactive substances from adverse environmental interference. Due to the SA concentration as well as the interaction between ions, the structure of MCs after freeze-drying was thin and loose which can break in a complex gastric acidic environment, resulting in the loss of encapsulated organisms during delivery (Zhang *et al*., 2020c). Therefore, it is necessary using another wall material in combination with SA to further improve MC performance. Chitosan (CS) is the only naturally occurring cationic polysaccharide that has a positive effect on immune stimulation (Silva *et al*., 2021). The cation-rich CS attached to the anion-rich SA gel under the electrostatic reaction between ions and formed a membrane structure through self-assembly, and the combination of the two forms a tighter and less porous network structure. This makes SA/CS MCs more protective and stable than single SA MCs in terms of release through the gastric acid environment and the intestine (Thu *et al*., 1996).

SA/CS-based MCs can be used for the protection and release of bioactive substances such as proteins and probiotics, however, the process of SA/CS MC formation leaves unreacted cations on the MC surface. Some scholars have continued to add SA and CS to a solution with a low concentration of SA to form SA/CS/SA coated MCs and improve their stability (Jiang *et al*., 2014; Cui *et al*., 2018). However, studies on the preparation process of IgY-loaded SA/CS/SA MCs have not been reported. The aim of this study was to prepare IgY-loaded SA/CS/SA MCs by extrusion and to evaluate their properties by characterization and in vitro performance verification. In this study, we further improved the embedding efficiency of IgY on the basis of SA/CS MCs by the creation of SA/CS/SA triple-layer MCs, which provided a new preparation scheme to protect the bioactivity of IgY in the gastrointestinal tract and storage environment.

MATERIALS AND METHODS

Materials: *Escherichia coli K99* (*E.coli*) and *Salmonella Gallinarum KCTC2931* (*SG*) were provided by the Functional Feed Additives Research Laboratory, College of Agriculture, Yanbian University, China. SA (Analytical Reagent, 90%), glycerol, CS (Viscosity 100~200 mpa.s; Deacetylation degree≥95%), Bile salt, $CaCl_2$, NaCl, KCl, Metaphosphate, Pepsin, KH_2PO_4 were purchased from Macklin (Shanghai, China). Korma Brilliant Blue G-250,, de Man, Rogosa, and Sharpe (MRS) broth, MacConkey agar, Bovine Serum Albumin (BSA) and Mucin were purchased from SolarBio (Beijing, China).

Preparation of IgY: This experiment was conducted in the Laboratory of Functional Feed Additives, College of Agriculture, Yanbian University, China in May 2023. IgY was prepared as follows (Hansen, 1998; Tufarelli *et al*., 2020). First, eggs with yolk content >100 mg were selected for the experiment, and IgY was purified from the yolk by aqueous dilution technique after precipitation of the yolk with 40% ammonium sulfate. IgY was purified with precooled ethanol after precipitation from egg yolks, where the purified IgY was placed at -20℃ and excess ethanol was removed. IgY was obtained after precipitation by salting and was heated to yield IgY powder to improve its stability, which was stored at -20℃ until use.

Measurement of IgY purity: IgY concentrations were determined as follows (Deng *et al*., 2011). Dissolve 1 g BSA in 1 L of sterile distilled water and set aside for use. 100 mg of Kaumas Brilliant Blue G-250 was weighed and dissolved in 90% ethanol, subsequently, 100 mL of 85% phosphoric acid was added. The total volume was then brought to 1 L and store at 4℃. BSA standard solutions of 0, 200, 400, 600, 800, and 1000 μg/mL were prepared, and 0.1 mL of each the standard solution was pipetted, 5 mL of Kaumas Brilliant Blue G-250 was added and mixed, and the OD_{595} was measured after 2 min.The regression equation: A=0.0007X+1.3947 (R^2=0.9997) (X: Protein content; A: Absorbance). Using this equation, the purity of IgY was calculated to be 91.52%.

Microencapsulation of IgY by SA/CS/SA: Referring to the method by Cui *et al*. (Cui *et al*., 2018) with slight modifications (Figure 1). Firstly, the prepared IgY and glycerol were mixed with a high concentration of SA, and then microcapsule preparation was carried out. The IgY solution was dropped into 0.1 M calcium chloride solution by extrusion and subsequently SA MCs were formed. Subsequently, SA MCs were stirred thoroughly

in CS solution to form SA/CS MCs. Finally, SA/CS MCs were immersed in 0.1% (w/v) SA solution, and after sufficient reaction, SA/CS/SA MCs were prepared. Lyophilized at 0.06 mbar for 48 h (Alpha 2-4 LSC basic; Christ, Germany).

In this study, the IgY concentration, CS concentration, and pH of the CS solution were the key factors affecting the IgY encapsulation rate. Therefore, different concentrations of materials (IgY concentration: 200 mg/mL, 300 mg/mL, and 400 mg/mL; CS concentration: 0.7, 0.8, and 0.9%; CS solution pH: 5.0, 5.4, and 5.8) were tested in the MC formulation.

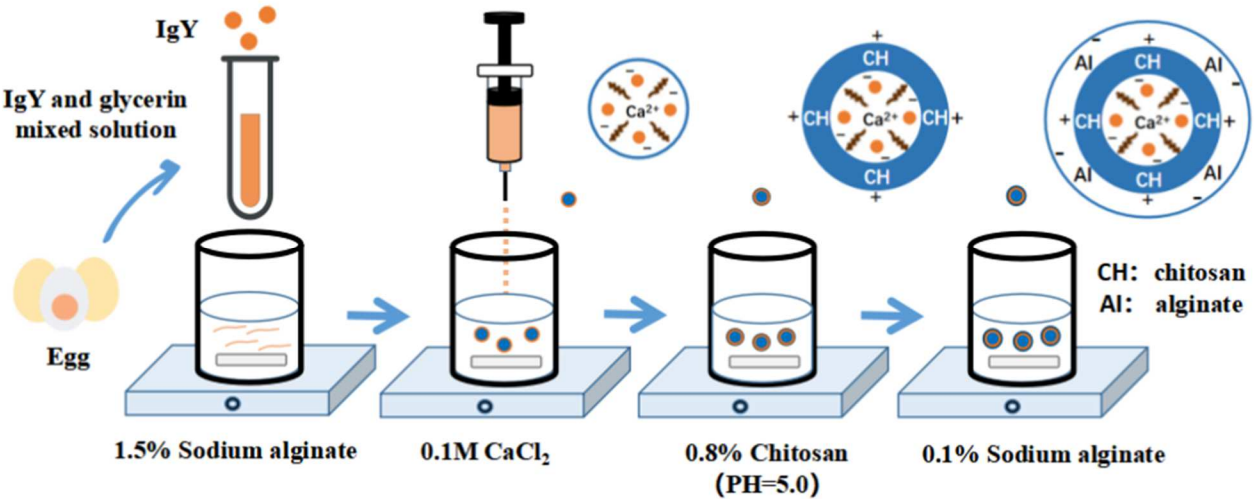

Figure 1 Procedure for preparation of IgY-loaded SA/CS/SA MCs

Encapsulation efficiency and loading content: A quantitative amount of the lyophilized post-MCs (100 mg) was weighed, transferred to 5 mL of a decapsulated solution containing 1 mol/L sodium citrate. The samples were incubated in an oscillating incubator at 170 rpm at 37°C until the MCs were completely dissolved, and then centrifuged at 4000 rpm for 10 min (centrifuge 5910R; Eppendorf, Germany). The supernatant was collected and OD_{595} was measured using a UV spectrophotometer (UV-2450; Shimadzu, Japan). The supernatant of the empty MCs was used as a blank control (De Temmerman *et al*., 2011; Wu *et al*., 2014).The EE (encapsulation efficiency) and LC (loading content) are calculated as follows:

$EE=CV/M_1\times100\%$ (1)

$LC=CV/M_2\times100\%$ (2)

Notes: (1) C: Concentration of IgY in solution (g/mL), V: Total volume of solution (10ml), M_1: Total amount of IgY added (g); (2) M_2: Total mass of MCs (100mg).

Morphological characterization: The external morphology of the MCs was observed using a body microscope (S8APO; Leica, Germany). Those with full moistening and regular spherical shape were regarded as qualified; those with irregular morphology accompanied by severe trailing were regarded as unqualified; 10 MCs were randomly selected, and their particle size dimensions were measured using Vernier calipers. Characterization analysis of MCs was performed by scanning electron microscopy (SU8010; Hitachi, Japan) (Zhang *et al*., 2020a; Zhang *et al*., 2020b). The observation parameters were 3 kv and 5000 ×.

Compressive strength: The maximum pressure that the front side of IgY-loaded SA/CS/SA MCs could withstand was used as compressive strength. Twenty MCs from each of the T1, T2, and T3 groups of SA/CS/SA IgY MCs were randomly selected, glass pieces were placed on top of them, weights were placed on top of the glass pieces, the weight of was increased, the weight was recorded when the capsules ruptured exactly, and the average value was taken.

Simulated gastric fluid and simulated intestinal fluid: The simulated gastric juice was made of sodium chloride (2.0 g/L), calcium chloride (0.11 g/L), potassium chloride (1.12 g/L), metaphoric acid (0.4 g/L), mucin (3.5 g/L), pepsin (0.26 g/L), and the pH was adjusted to 2.0 with hydrochloric acid (Sultana *et al*., 2000). For each group, 100 mg of lyophilized MCs was added to 30 mL of artificial gastric juice in a shaking incubator (ZHTY50N, Zhichu, China) at 37°C with stirring at 170 rpm for 0, 10, 30, 60, and 120 min.

The simulated intestinal fluid was prepared as follows: 0.5 mmol KH_2PO_4 solution was mixed with 100 mg pepsin and 1 mol/L NaOH was used to adjust the pH to 7.0(Ren *et al*., 2016). In each group, 100 mg MCs was added to the simulated intestinal fluid (30 mL), and the IgY concentration was measured after incubation for 0, 2, 4, 6, and 8 h at 37℃ and 170 rpm.

Bacteriostatic test in vitro: The effective IgY content was weighed as 0.3 g of IgY powder and different microencapsulated IgY treatments. *Escherichia coli K99* (*E. coli*) and *Salmonella gallinarium* (*SG*) diluted to 1×10^5 CFU/mL was added to MRS Broth medium and co-cultured with IgY treatment group at 37℃ for 9 h. The number of pathogenic bacteria cells (lg CFU/mL) was determined by plate counting method. (Xu *et al*., 2020).

The above culture solution was centrifuged at 4000 rpm for 10 min. Alkaline phosphatase (AKP) activity in each supernatant was determined using an AKP assay kit (Jiancheng Biological Engineering Company, Nanjing, China).

Storage performance: The method described by Nilsson *et al*. (Nilsson *et al*., 2012) was used with slight modifications. Briefly, IgY was dispensed into 50 mL centrifuge tubes and placed in at 4℃ and 23±2℃ where samples (2 g) were taken at 1, 2, 4, and 8 weeks. MCs were dissolved with 10 mL of 1 mol/L sodium citrate and the concentration of IgY was measured using a UV spectrophotometer (UV-2450; Shimadzu).

Statistical analysis: IBM SPSS Statistics software (version 21.0; IBM Corp., Armonk, NY, USA) performed data analysis. One-way ANOVA was used with Tukey's test to compare significant differences between treatments. All results are expressed as mean±SEM, and $P<0.05$ was considered statistically significant, and data were processed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Optimization of microencapsulated formulations: The effect of the IgY concentration on the EE of the IgY MCs is shown in Figure 2A. When the concentration was 200 mg/mL, the EE was significantly increased to 94.17% ($P<0.05$). Therefore, an IgY concentration of 200 mg/mL was considered optimal in this study.

The effect of CS concentration on the EE of the IgY MCs is shown in Figure 2B. When the CS concentration was 0.8%, the EE of IgY was significantly increased to 87.35 % ($P<0.05$). These results suggest that the CS concentration significantly affects the microencapsulation of IgY. Therefore, a CS concentration of 0.8% was considered as the optimal CS concentration in this study.

The effect of the pH values of the CS solution on the EE of the IgY MCs is shown in Figure 2C. When the solution pH was 5.0, the EE of IgY increased significantly to 94.84% ($P<0.05$). We observed a drug loading of 23.11~24.44% for IgY (Figure 2D); however, no significant effect on LC was seen ($P>0.05$). As shown in Figure 2E, the highest mechanical strength of the MCs was observed when the solution pH was 5.0 ($P<0.05$), the results show that the mechanical strength is best when the pH of CS solution is 5.0.

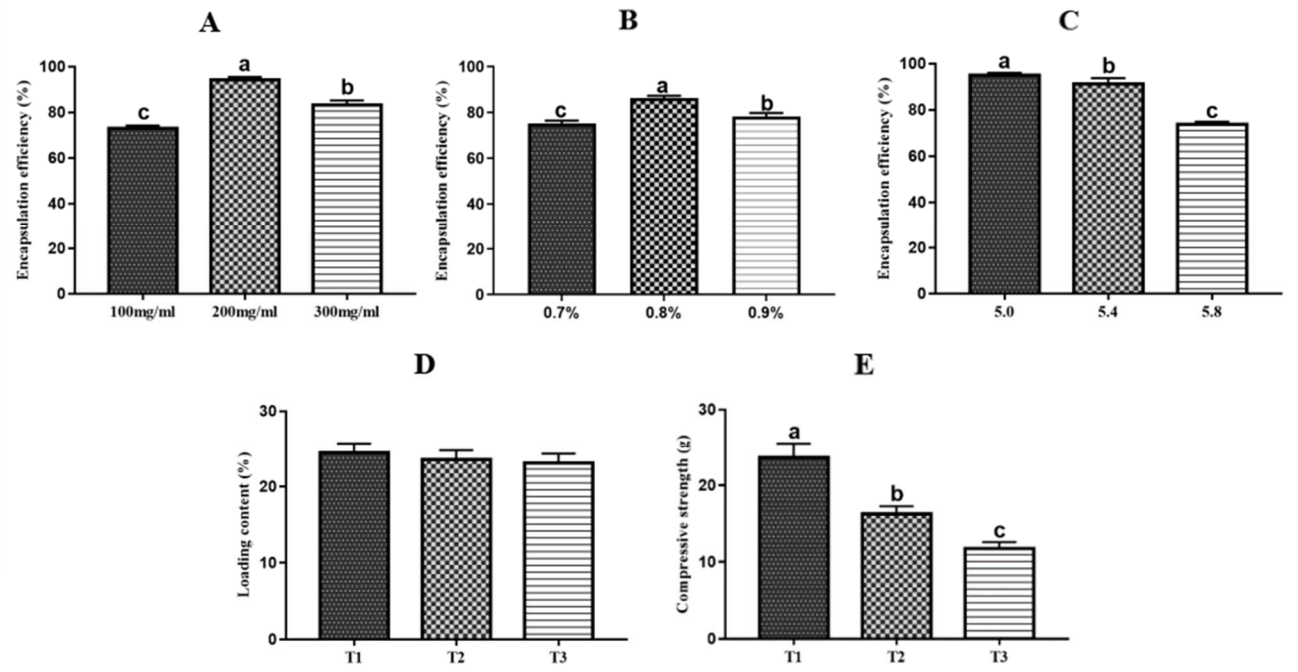

Figure 2 Effect of IgY concentration, chitosan concentration and pH of chitosan solution on IgY-loaded SA/CS/SA MCs preparation

T1 : pH=5.0 ; T2 : pH=5.4 ; T3 : pH=5.8

A-C indicates the effect of IgY concentration, chitosan concentration and pH of chitosan solution on the EE; D indicates the effect of pH of chitosan solution on LC; E indicates the effect of pH of chitosan solution on the compressive strength.

The bars indicate the standard deviation and the different letters above the columns indicate significant differences at the $^{a-c}P<0.05$ (Mean ± SEM, n=10).

Morphological characterization: The particle size and external morphology of the IgY-loaded SA/CS/SA MCs formed at different pH values are shown in Table 1 and Figure 3. The MCs in all groups appeared smooth and spherical to the naked eye, and there was no significant difference in the size distribution of the wet MCs ($P>0.05$). The differences in external morphology (Figure 4) and particle size (Table 1) of the freeze-dried microcapsules were significant ($P<0.05$) when observed using combined body microscopy and SEM. The different pH values of the CS solution changed the surface microstructure of the IgY-loaded SA/CS/SA MCs to some extent. When the pH was 5.0, the surface of the MCs was tight and regularly arranged; when the pH was 5.4, fine voids began to appear on the surface of the MCs; and when the pH was 5.8, cracks and irregular voids appeared on the surface of the MCs.

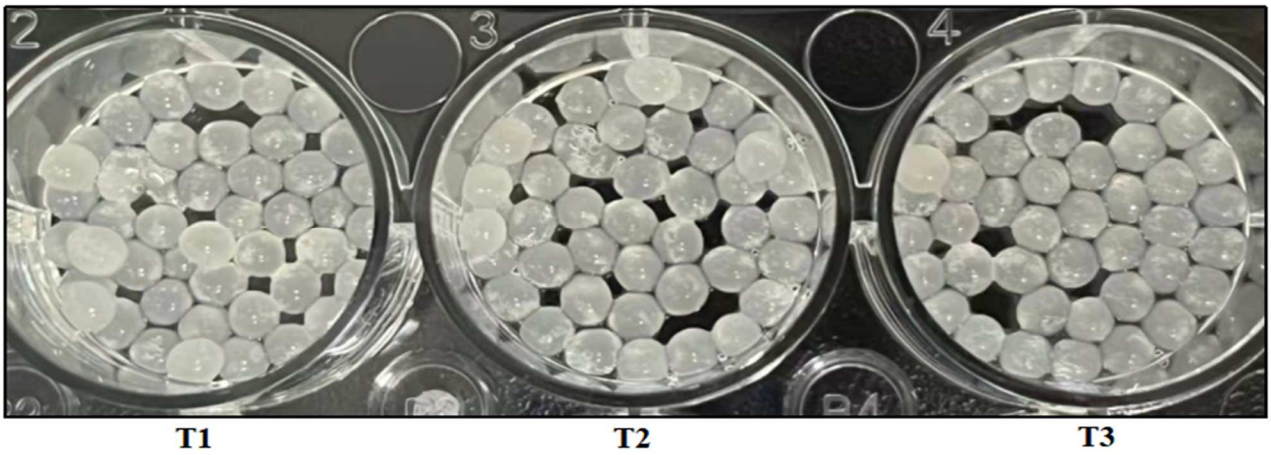

Figure 3 IgY-loaded SA/CS/SA MCs prepared with different pH of chitosan solution
T1：pH=5.0；T2：pH=5.4；T3：pH=5.8

Table 1 IgY-loaded SA/CS/SA MCs particle size before and after freeze-drying.

Groups	Size (mm)	
	Before freeze-drying	after freeze-drying
T1	2.159±0.016	2.009±0.030[a]
T2	2.109±0.027	1.848±0.026[b]
T3	2.112±0.025	1.657±0.020[c]

T1：pH=5.0；T2：pH=5.4；T3：pH=5.8
[a-c] Different letters with same time are significantly different（Mean ± SEM, n=10, $P<0.05$）.

Simulated GI release: The effects of IgY release from the MCs in simulated GI fluids are shown in Figure 5. As shown in Figure 5A, in simulated gastric fluid, the immunoreactivity of non-microencapsulated IgY powder was only 0.49% of the IgY remaining after 2 h of combined action of low pH and pepsin enzymatic digestion. In contrast, the rate of IgY release was significantly different between MCs formed at different CS solution pH values. As the pH increased, the protection rate of microencapsulated IgY gradually decreased. After 2 h, the activity of IgY in MCs formed at pH 5.0 was 72.20%, which was approximately 16% and 22% higher than the protection rate of MCs prepared under other pH conditions, respectively ($P<0.05$). The release effect of MCs in simulated intestinal fluid is shown in Figure 5B, and all of them reached approximately 90% ($P>0.05$) at 8 h. The results showed that IgY-loaded SA/CS/SA MCs could control the release of IgY in the gastric fluid and were immediately released to exert activity in artificial intestinal fluid.

Bacteriostatic activity in vitro: Microencapsulated IgY prepared under different CS solution pH conditions exhibited inhibitory activities against both *SG* and *E. coli*. As shown in Figure 6A-B, the inhibitory effect of IgY MCs against *E. coli* and *SG* was significantly higher than that of the other pH solutions when the pH was 5.0

(P<0.05) but significantly lower than that of non-microencapsulated IgY (P<0.05).

The integrity of the cell wall of the pathogen can be analyzed by measuring AKP activity in the culture medium.As shown in Figure 6C-D , AKP activity was significantly higher in both the microencapsulated and non-microencapsulated IgY-treated supernatants at 9 h than in the NC group (P<0.05). At pH 5.0, the AKP content of the microencapsulated IgY bacteriostatic supernatant was significantly higher than that of the T2 and T3 groups (P<0.05).

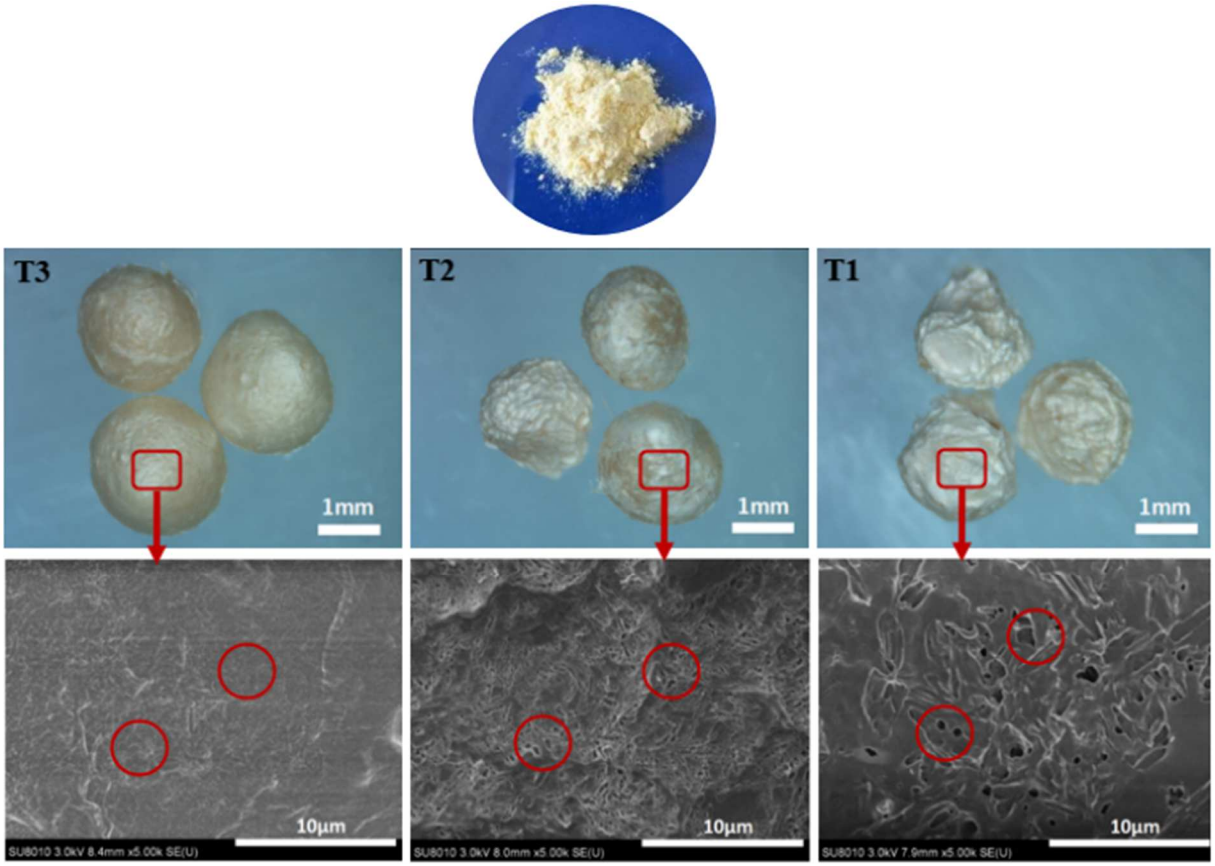

Figure 4 SEM observation of IgY-loaded SA/CS/SA MCs microstructure

NC: IgY powder; T1：pH=5.0；T2：pH=5.4；T3：pH=5.8

A-C: shows the morphological appearance under the observation of body microscope, scale bar: 1 mm. D-F: shows the microstructure under SEM observation, scale bar: 10 μm.

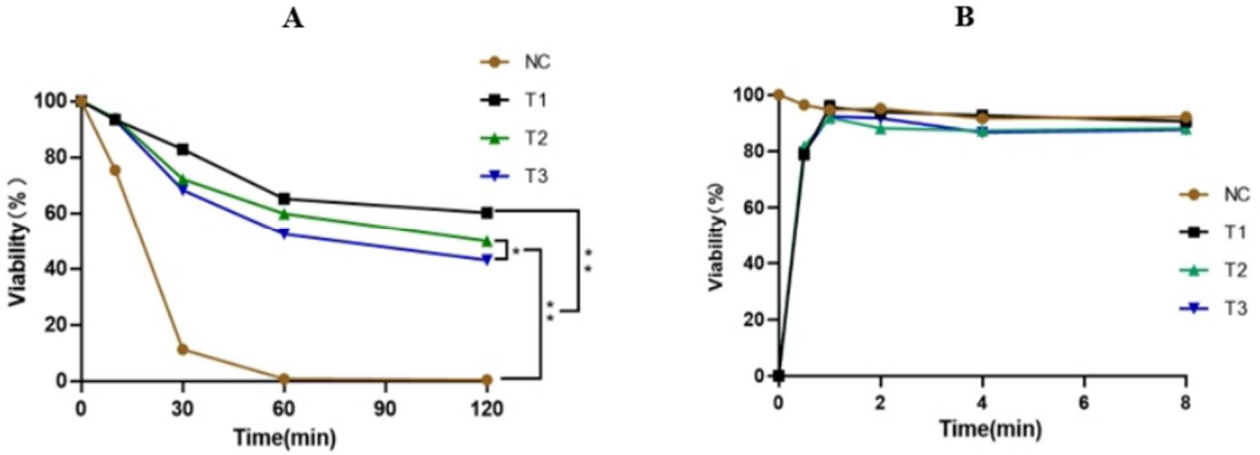

Figure 5 Effect of IgY-loaded SA/CS/SA MCs release in simulated gastric and simulated intestinal fluids

NC: IgY powder；T1：pH=5.0；T2：pH=5.4；T3：pH=5.8

Viability represents the percentage of IgY remaining or released from the microcapsules relative to the initial content. (Mean ± SEM, n=10, $^{*}P<0.05$; $^{**}P<0.01$).

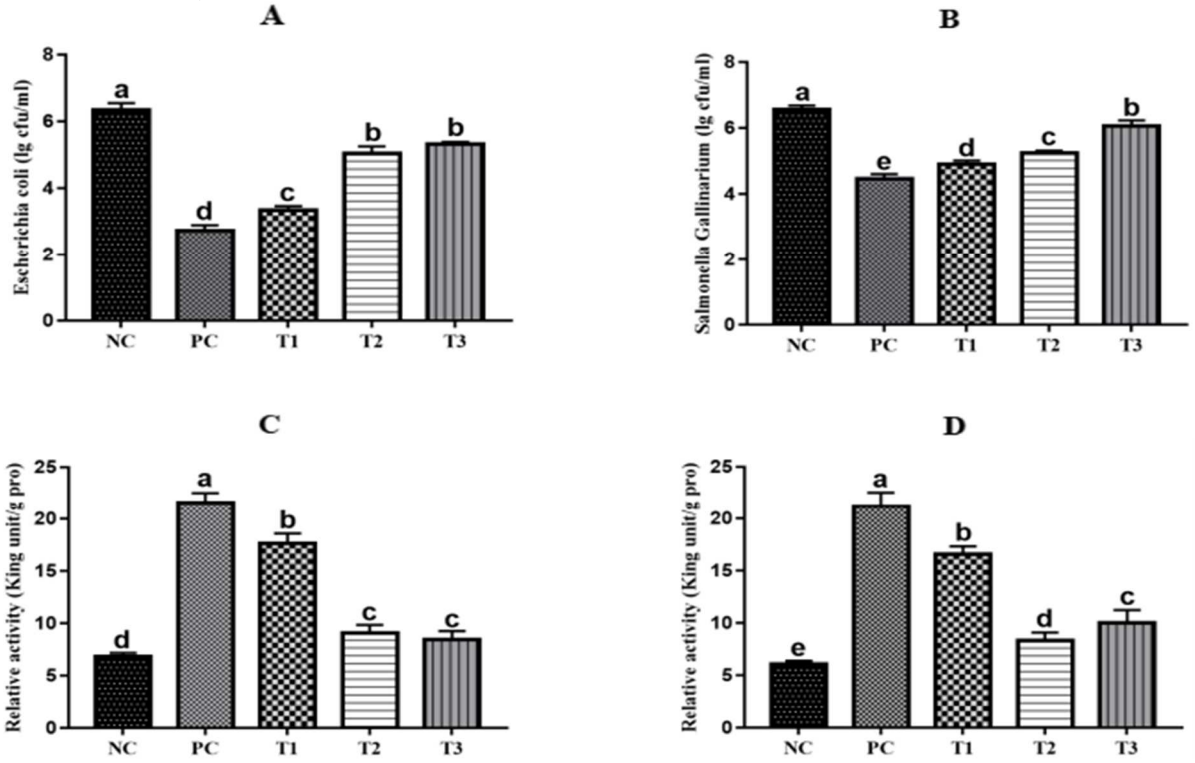

Figure 6 In vitro bacteriostatic properties of IgY-loaded SA/CS/SA MCs

NC: Untreated pathogenic bacteria ; PC : IgY powder ; T1 : pH=5.0 ; T2 : pH=5.4 ; T3 : pH=5.8; (A-B) The number of *Escherichia coli* and *Salmonella gallinarium* in the supernatant. (C-D) Alkaline phosphatase (AKP) activity in the supernatant.
The bars indicate the standard deviation and the different letters above the columns indicate significant differences at the $^{a-e}$ $P<0.05$ (Mean ± SEM, n=10);

Storage stability: The protection rate of IgY in MCs during storage was mainly affected by the microcapsule material and storage temperature. Considering the actual temperatures during production, transportation, and storage, the storage stability of IgY MCs was further evaluated at 1, 2, 4, and 8 weeks of storage at both 4℃ and 23 ± 2℃. The survival of probiotics in different MCs at 4℃ and RT storage conditions is shown in Table 2, and the best storage effect of IgY microencapsulation was formed under the condition of pH 5.0 ($P<0.05$), and the storage performance was gradually reduced with increasing solution pH values.

Table 2 Storage performance at 4°C and room temperature.

Times	Storage performance			
	NC	T1	T2	T3
4℃				
1week	89.02±0.5	93.24±0.2	92.35±0.3	91.38±0.4
k	0^{c}	9^{a}	9^{ab}	7^{b}
2week	81.60±0.5	88.62±0.3	86.88±0.2	82.30±0.4
k	7^{c}	9^{a}	8^{b}	8^{c}
4week	72.91±0.7	86.35±0.3	84.68±0.3	80.18±0.3
k	5^{d}	4^{a}	5^{b}	6^{c}
8week	54.16±1.7	86.13±0.5	83.96±0.2	80.27±0.4
k	8^{c}	0^{a}	6^{a}	8^{b}
23±2 ℃				
1week	84.31±0.6	91.75±0.4	91.38±0.3	89.71±0.2
k	8^{c}	8^{a}	3^{a}	2^{b}
2week	65.39±1.1	86.34±0.4	85.83±0.3	81.71±0.4
k	3^{c}	4^{a}	0^{a}	3^{b}
4week	39.24±1.4	80.88±0.2	74.67±0.3	70.56±0.3
k	1^{d}	8^{a}	4^{b}	0^{c}
8week	12.52±0.5	74.71±0.3	63.93±0.2	59.84±0.4
k	9^{d}	4^{a}	7^{b}	0^{c}

NC: IgY powder ; T1 : pH=5.0 ; T2 : pH=5.4 ; T3 : pH=5.8. $^{a-d}$ Different letters with same time are significantly different（Mean ± SEM, n=10, $P<0.05$)

DISCUSSION

IgY has been shown to be effective in the prevention and treatment of bacterial enteritis (Han *et al*.,

2021). However, IgY is poorly tolerated in gastric acidic environments, which greatly reduces IgY activity. Through microencapsulation, the loss of IgY in gastric juice can be reduced and efficiently released into the small intestine, which improves the therapeutic effect (Zhang *et al*., 2020c). In this study, the preparation process of IgY-loaded SA/CS/SA MCs was optimized, and their in vitro effect was evaluated based on three aspects: the concentrations of IgY and CS as well as the pH of the CS solution.

The EE and LC of the MCs were significantly affected by the core material, CS concentration, and pH of the media solution. It has been shown that the addition of a reasonable amount of core material can improve the EE, which may be because an excessive amount of core material may cause the microcapsule voids to become larger, resulting in spillage of the core material (Lai *et al*., 2021). In our study, the best encapsulation effect was achieved when the IgY concentration was increased to 200 mg/mL, and both 100 mg/mL and 300 mg/mL IgY decreased the encapsulation rate. This was most likely due to a gradual increase in the concentration of the core material in the solution causing the contact between the core material and wall material to increase, leading to an increased encapsulation rate of the MCs. However, the core material concentration is too high, which causes the pores of the membrane structure formed by the carboxyl group in SA and calcium ions to become larger, resulting in the core material overflow and resulting in the decrease of EE.

CS can improve the drug-carrying properties of the MCs. This may be attributed to the fact that CS can be stabilized by electrostatic interactions between the positively charged CS (NH^{3+}) and negatively charged SA (COO^{-}) to form polyelectrolyte complexes, leading to the formation of a more stable network on the surface of the sample (Li *et al*., 2009b). In our study, as the CS concentration increased, the optimal EE was observed when the CS concentration was 0.8% (w/v), and a decreasing trend was observed when the concentration exceeded this concentration. The reason may be that the CS is enlarged, then the formed SA/CS/SA can fully utilize NH^{3+} in solution, and with the addition of a low concentration of SA, it further strengthens the ionic gel network, limits the diffusive loss of proteins during MC formation, and reduces the outward diffusion of IgY (Meera *et al*., 2006). However, the study showed that when the CS concentration exceeded the reasonable range, the polyelectrolyte complexation reaction between IgY and SA and CS increased, resulting in more freeIgY and causing a decrease in EE.

The prepared MCs were subjected to morphological observation and SEM analysis. We found that the MCs before freeze-drying had similar morphological appearances and no significant differences in particle size dimensions. After freeze-drying, the two groups of MCs underwent crumpling, except for those MCs formed at pH 5.0. It was further observed by SEM that the surface of MCs formed at pH 5.0 formed a dense and ordered membrane structure, and voids and cracks gradually appeared with an increase in pH. This may be one of the reasons for the decrease in EE, which caused the loss of core material due to the appearance of voids and cracks, which may be one of the reasons for the decrease in compressive strength.

Many previous studies have shown that the activity of IgY is inhibited by pepsin and gastric acid (pH<3.5), resulting in reduced utility of IgY (Li *et al*., 2022; Hatta *et al*., 1993a; Lee *et al*., 2002). Dusso *et al*. showed that the encapsulation efficiency of *Lactococcus lactis* reached more than 90% by preparing alginate/chitosan microcapsules and protected its activity and storage stability in simulated gastric and simulated intestinal fluids (Dusso *et al*., 2023).In this study, IgY activity was measured by measuring incubation in simulated gastric juice for 2 h, and the activity of non-microencapsulated IgY powder decreased rapidly in simulated gastric juice, with only 0.49% antibody activity remaining after 2 h. However, there was a significant improvement in the stability of IgY after SA/CS/SA encapsulation, and the activity could still reach more than 50%~70%. In simulated intestinal fluid, we found that IgY-loaded SA/CS/SA MCs showed a rapid release trend that was different from that in simulated gastric fluid. It is reported that Cui *et al*. used CS and SA as pH-sensitive wall materials to prepare probiotic-loaded SA/CS/SA MCs to improve the tolerance of probiotics in gastrointestinal fluid (Cui *et al*., 2018). It is also reported that Jiang *et al*. prepared vaccin-loaded SA/CS/SA MCs, which significantly increased the vaccine activity in simulated gastric fluid (pH 2.0) and simulated small intestinal fluid (pH 7.2) by more than 65% and 75%, respectively (Jiang *et al*., 2014). We found that the protection rate of the MCs decreased with increasing pH of the CS solution. The degree of ionization of the two polyelectrolytes could reach approximately 70%~80% at pH 5.0, at which point the two polysaccharides could be fully combined. With the addition of low concentrations of SA, the residual CS cations on the surface of the MCs could be further utilized, resulting in the formation of dense SA/CS/SA membrane structures. However, at a CS solution pH>5.0, the degree of CS ionization is inhibited and CS can form other types of structures (Wang *et al*.,2023). The formation of such structures makes the CS-SA membrane less dense, which increases core loss and decreases simulated gastric fluid protection. The conformations of the membrane structures formed by the different CS solution pH can be observed more visually using SEM.

We found that IgY-loaded SA/CS/SA MCs were rapidly released into artificial intestinal fluid. The disintegration of CS/SA MCs has been reported to be

affected by solution pH (Asadi *et al*., 2023; Anal and Stevens, 2005), where the ability of the MCs to bind to water and undergo swelling is low in a highly acidic environment (pH 1.2), however, In the neutral environment of intestinal fluid (pH 6.8), the anion alginate in the Ca/SA/CS complex could be replaced by hydroxyl ions, leading to the rupture of MCs (Ren *et al*., 2016). The three-layer membrane structure of SA/CS/SA not only strengthens tolerance in the simulated gastric fluid but also provides a slow-release effect.

IgY can effectively inhibit the colonization of pathogens in the intestine (Xu *et al*., 2020; Bustos *et al*., 2021). Studies have shown that IgY can decrease the mobility of pathogens and reduce their ability to freely access nutrients, thus exerting antimicrobial effects (Sunwoo *et al*., 2002). In this study, non-microencapsulated IgY had the most significant antibacterial effect in the in vitro antibacterial culture, which was due to the direct contact of non-microencapsulated IgY with pathogenic bacteria. In addition, we found that MCs prepared at different CS solution pH showed different antibacterial effects, which may be due to the synergistic effect of IgY diffusion and mural enzyme degradation (Vueba *et al*., 2004). The best bacteriostatic effect of the IgY MCs was observed at a pH of 5.0, which emphasizes the importance of the solution pH for the preparation of MCs. Many previous studies have shown that CS itself has natural antibacterial effects (Shirui *et al*., 2014; Luo *et al*., 2020). As a wall material, CS has antibacterial properties, which is related to the cations carried by the CS group, which can attach to the surface of pathogenic bacteria, leading to the damage of the cell membrane structure of pathogenic bacteria (Benltoufa *et al*., 2020). Because of the different pH values of the SA/CS/SA microencapsulation preparation solution in this study, the number of free CS ions increases when the CS solution pH is 5.0, and the increase in the free number of amino groups, which is the group that exerts the bacterial inhibitory efficacy of CS, inevitably leads to an enhancement of the inhibitory performance, resulting in differences in the inhibitory performance. More detailed studies and clearer results are required to demonstrate how SA/CS/SA-microencapsulated IgY affects bacterial biological functions. In addition, the breakage of the cell wall of the pathogen prevents it from colonizing in the intestine and blocks its nutrient exchange pathway, thereby reducing its pathogenicity (Liu *et al*., 2023; Dykes *et al*., 2003).

AKP can be used to reflect the integrity of the bacterial cell wall because it is present between the cell wall and the cell membrane and is released in large amounts when the cell wall is damaged (Gu *et al*., 2023). In this study, different treatments with IgY inhibited both *SG* and *E. coli*. A significant increase in extracellular AKP activity was also observed, suggesting that IgY can disrupt the structure of the bacterial cell wall. Lipid bilayer lipopolysaccharides (LPS) and outer membrane proteins (OMPs) are the main components of the cell wall of Gram-negative bacteria. When pathogenic bacteria invade, the immune system will recognize and respond, thereby inhibiting the activity of pathogenic bacteria (Maiti *et al*., 2011).

The storage stability of IgY-loaded SA/CS/SA MCs was determined by the results showing that there was a significant difference in the IgY protection rate with an increase in the CS solution pH values at the same storage time, indicating that the CS solution pH plays a role in the storage stability of the MCs. At too high and too low an encapsulation pH, the degree of CS ionization was affected, and the degree of binding with SA was reduced, resulting in the formation of a flimsy and fragile membrane structure, which not only resulted in poor EE and compressive strength, but also had a certain impact on storage performance (Li *et al*., 2007). At week 8, the protection rate of microencapsulated IgY was maintained above 80% in all groups at 4°C, and above 60% in all groups at RT. This is similar to previous studies, where Nilsson *et al*. found that IgY could be stored for up to one month at RT and up to 6 months at 4°C (Nilsson *et al*., 2012). Similarly, the microencapsulated IgY prepared by Tai *et al*. showed improved stability (Tai *et al*., 2020). As the SA/CS/SA microencapsulation of IgY reduced the contact with the external environment, the properties of IgY remained stable. In this experiment, after the stabilized structure was formed by SA/CS/SA, a low concentration of SA was used to further crosslink the residual cationic CS to form a more compact structure.

Conclusions: The present study showed that optimal in vitro performance was obtained with the following: the IgY-loaded SA/CS/SA MCs prepared at a concentration of 200 mg/mL of IgY, a high concentration (1.5%) of SA, 0.1 M $CaCl_2$, 0.8% CS, a CS pH of 5.0, and a low concentration (0.1%) of SA. The IgY-loaded SA/CS/SA MCs enhanced the tolerance and storage stability of IgY in simulated gastrointestinal fluids and showed significant inhibition of *E. coli* and *SG* in vitro. Therefore, we prepared them as a novel antibiotic alternative with the aim of providing benefits for livestock performance and intestinal health.

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REFERENCES

Alustiza, F., Bellingeri, R., Picco, N., Motta, C., Grosso, M. C., Barbero, C. A., and Vivas, A (2016). IgY against enterotoxigenic *Escherichia coli* administered by hydrogel-carbon nanotubes composites to prevent neonatal diarrhoea in experimentally challenged piglets. Vaccine. 34(28):3291-3297. https://doi.org/10.1016/j.vaccine.2016.05.004

Asadi, S., Madrakian, T., Ahmadi, M., Aguirre, M. Á., Afkhami, A., Uroomiye, S. S., Ghaffari, F., and Ranjbar, A (2023). Aerosol assisted synthesis of a pH responsive curcumin anticancer drug nanocarrier using chitosan and alginate natural polymers. Sci Rep-UK. 13(1):19389. https://doi.org/10.1038/s41598-023-46904-4

Anal, A. K., and W. F. Stevens (2005). Chitosan-alginate multilayer beads for controlled release of ampicillin. Int J Pharm. 290(1-2):45-54. https://doi.org/10.1016/j.ijpharm.2004.11.015

Benltoufa, S., W. Miled, M. Trad, R. B. Slama, and F. Fayala (2020). Chitosan hydrogel-coated cellulosic fabric for medical end-use: Antibacterial properties, basic mechanical and comfort properties. Carbohyd Polym. 227:115352. https://doi.org/10.1016/j.carbpol.2019.115352

Bustos, C., C. Leiva, M. Gambarotta, N. Guida, and P. Chacana (2021). In vitro Inhibitory Activity of IgY Antibodies Against Salmonella Ser. Newport Isolated from Horses. J Equine Vet Sci. 103:103657. https://doi.org/10.1016/j.jevs.2021.103657

Carlander, D., H. Kollberg, P. E. Wejåker, and A. Larsson (2000). Peroral immunotheraphy with yolk antibodies for the prevention and treatment of enteric infections. Immunol Res. 21(1):1-6. https://doi.org/10.1385/ir:21:1:1

Chang, H., Y. C. Lee, C. C. Chen, and Y. Y. Tu (2010). Microencapsulation Protects Immunoglobulin in Yolk (IgY) Specific against Helicobacter pylori Urease. J Food Sci. 67(1):15-20. https://doi.org/10.1111/j.1365-2621.2002.tb11351.x

Cui, L. H., Yan, C. G., Li, H. S., Kim, W. S., Hong, L., Kang, S. K., Choi, Y. J., and Cho, C. S (2018). A New Method of Producing a Natural Antibacterial Peptide by Encapsulated Probiotics Internalized with Inulin Nanoparticles as Prebiotics. J Microbiol Biotechn. 28(4):510-519. https://doi.org/10.4014/jmb.1712.12008

De Temmerman, M. L., J. Demeester, F. De Vos, and S. C. De Smedt (2011). Encapsulation performance of layer-by-layer microcapsules for proteins. Biomacromolecules. 12(4):1283-1289. https://doi.org/10.1021/bm101559w

Deng, Q., Y. Chen, J. Wu, Y. Li, and W. Song (2011). Preparation of procion brilliant blue-doped silica nanorods and their recognition properties for proteins. Chin J Chromatogr. 29(9):876. https://doi.org/10.3724/SP.J.1123.2011.00876

Dusso, D., and Salomon, C. J (2023). Solving the delivery of Lactococcus lactis: Improved survival and storage stability through the bioencapsulation with different carriers. J Food Sci. 88(4):1495-1505. https://doi.org/10.1111/1750-3841.16538

Dykes, G. A., R. Amarowicz, and R. B. Pegg (2003). An antioxidant bearberry (Arctostaphylos uva-ursi) extract modulates surface hydrophobicity of a wide range of food-related bacteria: implications for functional food safety. Food Control. 14(7):515-518. https://doi.org/10.1016/S0956-7135(02)00110-X

Gu, Y., Dong, J., Li, J., Luo, Q., Dong, X., Tang, G., Zhang, J., Du, X., Pu, Q., He, L., Zhao, K., Han, D., and Xin, J (2023). Antibacterial activity and mechanism of sanguinarine against Staphylococcus aureus by interfering with the permeability of the cell wall and membrane and inducing bacterial ROS production. Front Vet Sci. 10:1121082. https://doi.org/10.3389/fvets.2023.1121082

Han, S., Wen, Y., Yang, F., and He, P (2021). Chicken Egg Yolk Antibody (IgY) Protects Mice Against Enterotoxigenic *Escherichia coli* Infection Through Improving Intestinal Health and Immune Response. Front Cell Infect Mi. 11:662710. https://doi.org/10.3389/fcimb.2021.662710

Hansen, P (1998). Isolation and purification of immunoglobulins from chicken eggs using thiophilic interaction chromatography. J Immunol Methods. 215(1-2):1-7. https://doi.org/10.1016/s0022-1759(98)00050-7

Hatta, H., K. Tsuda, S. Akachi, M. Kim, and T. Ebina (1993a). Oral passive immunization effect of anti-human rotavirus IgY and its behavior against proteolytic enzymes. Biosci Biotech

Bioch. 57(7):1077-1081. https://doi.org/10.1271/bbb.57.1077

Jeong, C., S. Kim, C. Lee, S. Cho, and S. B. Kim (2020). Changes in the Physical Properties of Calcium Alginate Gel Beads Under a Wide Range of Gelation Temperature Conditions. Foods. 9(2):180. https://doi.org/10.3390/foods9020180

Jiang, T., B. Singh, S. Maharjan, H. S. Li, and Y. J. Choi (2014). Oral delivery of probiotic expressing M cell homing peptide conjugated BmpB vaccine encapsulated into alginate/chitosan/alginate microcapsules. Eur J Pharm Biopharm. 88(3):768-777. https://doi.org/10.1016/j.ejpb.2014.07.003

Jin, Y., Lv, H., Wang, M., Cho, C. S., Shin, J., Cui, L., and Yan, C (2023). Effect of microencapsulation of egg yolk immunoglobulin Y by sodium alginate/chitosan/sodium alginate on the growth performance, serum parameters, and intestinal health of broiler chickens. Anim Biosci. 36(8):1241-1251. https://doi.org/10.5713/ab.22.0414

Kovacs-Nolan, J., and Y. Mine (2012). Egg yolk antibodies for passive immunity. Annu Rev Food Sci Technol. 3:163-182. https://doi.org/10.1146/annurev-food-022811-101137

Lai, K., How, Y., and Pui, L. (2021). Microencapsulation of Lactobacillus rhamnosus GG with flaxseed mucilage using co-extrusion technique. J Microencapsul. 38(2):134-148. https://doi.org/10.1080/02652048.2020.1863490

Lee, K. A., S. K. Chang, Y. J. Lee, J. H. Lee, and N. S. Koo (2002). Acid Stability of Anti-Helicobacter pyroliIgY in in Aqueous Polyol Solution. J Biochem Mol Biol. 35(5):488-493. https://doi.org/10.5483/bmbrep.2002.35.5.488

Liu, X., Li, X., Bai, Y., Zhou, X., Chen, L., Qiu, C., Lu, C., Jin, Z., Long, J., and Xie, Z (2023). Natural antimicrobial oligosaccharides in the food industry. Int J Food Microbiol. 386:110021 .https://doi.org/10.1016/j.ijfoodmicro.2022.110021

Li, X., C. Tang, M. Salama, M. Xia, X. Huang, L. Sheng, and Z. Cai (2022). Encapsulation efficiency and oral delivery stability of chitosan-liposome-encapsulated immunoglobulin Y. J Food Sci. 87(4):1708-1720. https://doi.org/10.1111/1750-3841.16116

Li, X. Y., Jin, L. J., Uzonna, J. E., Li, S. Y., Liu, J. J., Li, H. Q., Lu, Y. N., Zhen, Y. H., and Xu, Y. P (2009a). Chitosan-alginate microcapsules for oral delivery of egg yolk immunoglobulin (IgY): in vivo evaluation in a pig model of enteric colibacillosis. Vet Immunol Immunop. 129(1-2):132-136. https://doi.org/10.1016/j.vetimm.2008.12.016

Li, X. Y., L. J. Jin, Y. N. Lu, Y. H. Zhen, and Y. P. Xu (2009b). Chitosan-Alginate Microcapsules for Oral Delivery of Egg Yolk Immunoglobulin (IgY): Effects of Chitosan Concentration. Appl Biochem Biotech. 159(3):778-787. https://doi.org/10.1007/s12010-009-8628-6

Li, X. Y., L. J. Jin, T. A. Mcallister, K. Stanford, J. Y. Xu, Y. N. Lu, Y. H. Zhen, Y. X. Sun, and Y. P. Xu (2007). Chitosan-alginate microcapsules for oral delivery of egg yolk immunoglobulin (IgY). J Agric Food Chem. 55(8):2911-2917. https://doi.org/10.1021/jf062900q

Li, X. Y., M. B. Wu, M. Xiao, S. H. Lu, Z. M. Wang, J. M. Yao, and L. R. Yang (2019). Microencapsulated β-carotene preparation using different drying treatments. J Zhejiang Univ Sci B. 20(11):901-909. https://doi.org/10.1631/jzus.B1900157

Li, X., Tang, C., Salama, M., Xia, M., Huang, X., Sheng, L., and Cai, Z (2022). Encapsulation efficiency and oral delivery stability of chitosan-liposome-encapsulated immunoglobulin Y. J Food Sci. 87(4):1708-1720. https://doi.org/10.1111/1750-3841.16116

Luo, X., and L. Li (2020). Evaluation of single-component chitosan fiber: from advanced materials to contemporary fashion manufacturing. Trxt Res J. 90(2):125-134. https://doi.org/10.1177/0040517519858764

Maiti, B., M. Shetty, M. Shekar, I. Karunasagar, and I. Karunasagar (2011). Recombinant outer membrane protein A (OmpA) of Edwardsiellatarda, a potential vaccine candidate for fish, common carp. Microbiol Res. 167(1):1-7. https://doi.org/10.1016/j.micres.2011.02.002

Meera, George, and, T., Emilia, and Abraham (2006). Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan-a review. J Control Release. 114(1):1-14. https://doi.org/10.1016/j.jconrel.2006.04.017

Mostafa, Bakhshi, Firouz, Ebrahimi, Shahram, Nazarian, Jamil, Zargan, Faeze, and Behzadi (2017). Nano-encapsulation of chicken immunoglobulin (IgY) in sodium alginate nanoparticles: In vitro characterization. Biologicals. 49:69-75. https://doi.org/10.1016/j.biologicals.2017.06.002

Nilsson, E., J. Stålberg, and A. Larsson (2012). IgY stability in eggs stored at room temperature or at +4℃. Br Poult Sci. 53(1):42-46. https://doi.org/10.1080/00071668.2011.646951

Ren, Z., X. Zhang, Y. Guo, K. Han, and N. Huo (2016). Preparation and in vitro delivery performance of chitosan–alginate microcapsule for IgG. Food Agr Immunol. 28(1):1-13. https://doi.org/10.1080/09540105.2016.1202206

Shimizu, M., H. Nagashima, K. Sano, K. Hashimoto, and H. Hatta (1992). Molecular stability of chicken and rabbit immunoglobulin G. Biosci Biotech Bioch. 56(2):270-274. https://doi.org/10.1271/bbb.56.270

Shirui, Liu, Tao, Hua, Xue, Luo, Ngan, Yi, Lam, and Xiao-ming (2014). A novel approach to improving the quality of chitosan blended yarns using static theory. Text Res J. 85(10):1022-1034. https://doi.org/10.1177/0040517514559576

Silva, A., R. Cunha, D. Hotza, and R. Machado (2021). Chitosan as a matrix of nanocomposites: A review on nanostructures, processes, properties, and applications. Carbohyd Polym. 272:118472. https://doi.org/10.1016/j.carbpol.2021.118472

Stanton, T. B (2013). A call for antibiotic alternatives research. Trends Microbiol. 21(3):111-113. https://doi.org/10.1016/j.tim.2012.11.002

Sultana, K., G. Godward, N. Reynolds, R. Arumugaswamy, P. Peiris, and K. Kailasapathy (2000). Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. Int J Food Microbiol. 62(1-2):47-55. https://doi.org/10.1016/s0168-1605(00)00380-9

Sunwoo, H. H., E. N. Lee, K. Menninen, M. R. Suresh, and J. S. Sim (2002). Growth Inhibitory Effect of Chicken Egg Yolk Antibody (IgY) on *Escherichia coli* O157:H7. J Food Sci. 67(4):1486-1494. https://doi.org/10.1007/s11259-007-9029-3

Tai, K., M. Rappolt, L. Mao, Y. Gao, X. Li, and F. Yuan (2020). The stabilization and release performances of curcumin-loaded liposomes coated by high and low molecular weight chitosan. Food Hydrocolloid. 99:105355.1-105355.10. https://doi.org/10.1016/j.foodhyd.2019.105355

Thu, B., P. Bruheim, T. Espevik, O. Smidsrød, P. Soon-Shiong, and G. Skjåk-Bræk (1996). Alginate polycation microcapsules: II. Some functional properties. Biomaterials. 17(11):1069-1079. https://doi.org/10.1016/0142-9612(96)85907-2

Tufarelli, V., N. Musco, I. F. Rehan, M. A. Maky, and Hesham (2020). Egg Yolk IgY: A Novel Trend of Feed Additives to Limit Drugs and to Improve Poultry Meat Quality. Front Vet Sci. 7:350. https://doi.org/10.3389/fvets.2020.00350

Vueba, M. L., L. A. Batista de Carvalho, F. Veiga, J. J. Sousa, and M. E. Pina (2004). Influence of cellulose ether polymers on ketoprofen release from hydrophilic matrix tablets. Eur J Pharm Biopharm. 58(1):51-59. https://doi.org/10.1016/j.ejpb.2004.03.006

Wang, H., Zeng, X., and Lin, J (2021). Ex Vivo Evaluation of Egg Yolk IgY Degradation in Chicken Gastrointestinal Tract. Front Immunol. 12:746831. https://doi.org/10.3389/fimmu.2021.746831

Wang, T., Wang, S., Zhang, L., Sun, J., Guo, T., Yu, G., and Xia, X (2023). Fabrication of bilayer emulsion by ultrasonic emulsification: Effects of chitosan on the interfacial stability of emulsion. Ultrason Sonochem. 93:106296. https://doi.org/10.1016/j.ultsonch.2023.106296

Wu, X., S. Zhao, J. Zhang, P. Wu, and C. Peng (2014). Encapsulation of EV71-specific IgY antibodies by multilayer polypeptide microcapsules and its sustained release for inhibiting enterovirus 71 replication. Rsc Adv. 4(28):14603. https://doi.org/10.1039/c3ra46943c

Xu, L., J. Che, Y. Xu, Y. Chen, Y. Li, B. Murtaza, L. Wang, M. Zhang, and X. Li (2020). Oral administration of microencapsulated egg yolk immunoglobulin (IgY) in turbot (Scophthalmus maximus) to combat against Edwardsiellatarda 2CDM001 infections. Fish Shellfish Immun. 106:609-620. https://doi.org/10.1016/j.fsi.2020.08.024

Zhang, C., S. L. A. Khoo, X. D. Chen, and S. Y. Quek (2020a). Microencapsulation of fermented noni juice via micro-fluidic-jet spray drying: Evaluation of powder properties and functionalities. Powder Technol. 361:995-1005. https://doi.org/10.1016/j.powtec.2019.10.098

Zhang, C., S. L. A. Khoo, P. Swedlund, Y. Ogawa, Y. Shan, and S. Y. Quek (2020b).Fabrication of Spray-Dried Microcapsules Containing Noni Juice Using Blends of Maltodextrin and Gum Acacia: Physicochemical Properties of Powders and Bioaccessibility of Bioactives during In vitro Digestion. Foods. 9(9):1316. https://doi.org/10.3390/foods9091316

Zhang, J., H. H. Li, Y. F. Chen, L. H. Chen, and X. F. Yu. (2020c). Microencapsulation of immunoglobulin Y: optimization with response surface morphology and controlled release during simulated gastrointestinal digestion. J Zhejiang Univ-Sc B. 21(8):611-627. https://doi.org/10.1631/jzus.B2000172

Zhang, Q., D. He, L. Xu, S. Ge, and X. Zhang (2020d). Generation and evaluation of anti-mouse IgG IgY as secondary antibody. Prep Biochem Biotech. 50(8):788-793. https://doi.org/10.1080/10826068.2020.1737940