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ANTIBACTERIAL EFFICACY OF PROTEIN-PHENOLIC ACID COMPLEXES

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Abstract

The understanding interactions between proteins and phenolic compounds is becoming increasingly important in food science, as these interactions might significantly affect the functionality of foods. So far, research has focused predominantly on protein–phenolic interactions, separately, but these components might also form other combinations. Protein (OVA and BLG) and phenolic acids (sinapic acid (SA), gallic acid (GA) and caffeic acid (CA)) were interacted in the combination [(OVA, OVA+SA, OVA+GA, OVA+CA), (BLG, BLG+SA, BLG+GA, BLG+CA)] and the compound tested against food borne bacterial pathogens a) *Staphylococcus aureus* (+ ve), b) *Streptococcus pyogenes* (+ ve), c) *Escherichia coli* (– ve), d) *Klebsiella pneumonia* (– ve). The combination of OVA+GA shows potent antibacterial activity against both Gram-positive and Gram-negative bacteria with Support to the results the difference in susceptibility to polyphenols between Gram-positive and Gram-negative bacteria is a controversial issue.

Keywords: Protein (OVA and BLG), phenolic acids (sinapic acid (SA), gallic acid (GA) and caffeic acid (CA), bacterial pathogens

Introduction

Globally, high rates of morbidity and mortality have been partly linked to microbial infections, with some highly resistant pathogenic bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp*.) frequently implicated. Plant products with antimicrobial properties have obtained emphasis for possible application in food production in order to prevent bacterial and fungal growth (Lanciotti et al., 2004).The functionalization of proteins with phenolic compounds has gained increasing attention. Phenolic compounds and proteins can form phenol-protein via non-covalent physical interactions for e.g. electrostatic, hydrophobic, Van der Waals and hydrogen bonding (Bandyopadhyay. P., *et al*., 2012).Phenolic compounds can be covalently linked with proteins to form phenolic–protein conjugates (Kim. S and Cavaco-Paulo. A 2012). Covalently linked phenolic–protein conjugates are more stable than non-covalent phenolic–protein complexes, although both physical interactions and covalent conjugation can both alter the properties of proteins (Liu. F., et al., 2017; Wei. Z., et al., 2015). Most existing studies have focused on the non-covalent interactions between phenolic compounds and proteins, which is probably because the non-covalent phenolic–protein complexes are more conveniently prepared than covalently linked phenolic–protein conjugates (Bandyopadhyay. P., *et al*., 2012; Jakobek. L.,

2015; Ozdal. T., *et al.,* 2013; Xiao. J and Kai. G., 2012). The structures, biological properties and applications of phenolic-protein are closely related to the conjugation efficiency.

Fig. 1a β-Lactoglobulin (βLG)

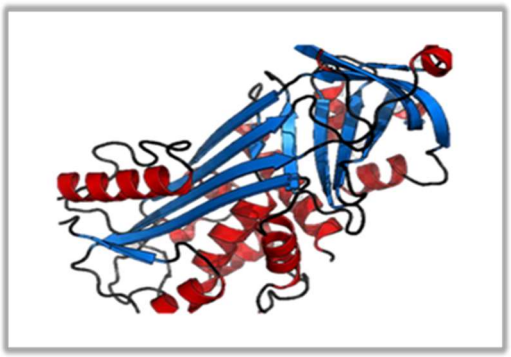

Fig. 1b ovalbumin (OVA)

Materials & Methods

Proteins - Ovalbumin (from chicken egg white, lyophilized powder, ≥ 98%), β-lactoglobulin (βLG) of purity 90% and Phenolic acids - Gallic acid (3,4,5-trihydroxy benzoic acid), Sinapic acid (SA, 3,5-dimethoxy-4-hydroxy-cinnamic acid) (D7927) and Caffeic acid (3,4-hydroxycinnamic acid) were purchased from Sigma-Aldrich Chemical Co, USA) were all used as received. The samples were prepared in ultrapure water (MilliQ Purelab Ultra, Darmstadt, Germany).

Sample preparation

The stock solutions, of OVA and βLG (1×10^{-4} M) were dissolved in 10 ml phosphate buffer solution at the concentration (PBS, 10 mM, pH 7.4) and as the stock solution of caffeic acid (CA), sinapic acid (SA) and gallic acid (GA) were taken in equal volumes at the concentration (1×10^{-5} M) was dissolved in 10 ml ethanol solution. Pipetted-out equal volumes of protein (0.2 $molL^{-1}$) were dissolved in different concentrations of phenolic acids (0.2 $molL^{-1}$ to 1.0 $molL^{-1}$) and each was added to 3 ml of distilled water.

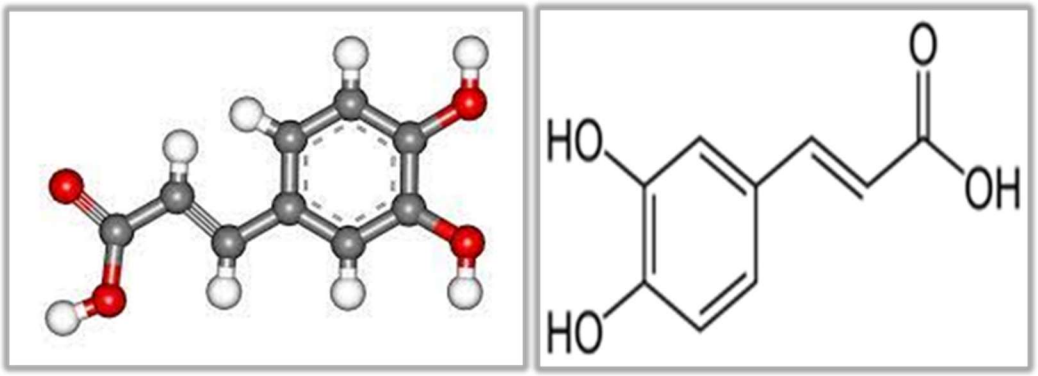

Fig. 2a Structure of caffeic acid (CA)

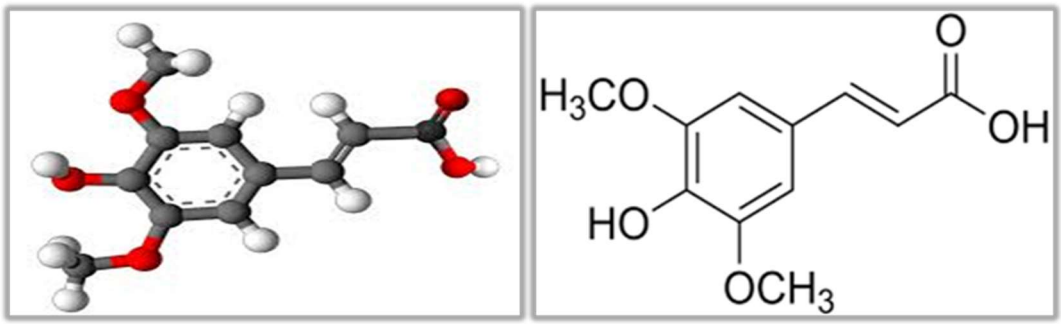

Fig. 2b Structure of sinapic acid (SA)

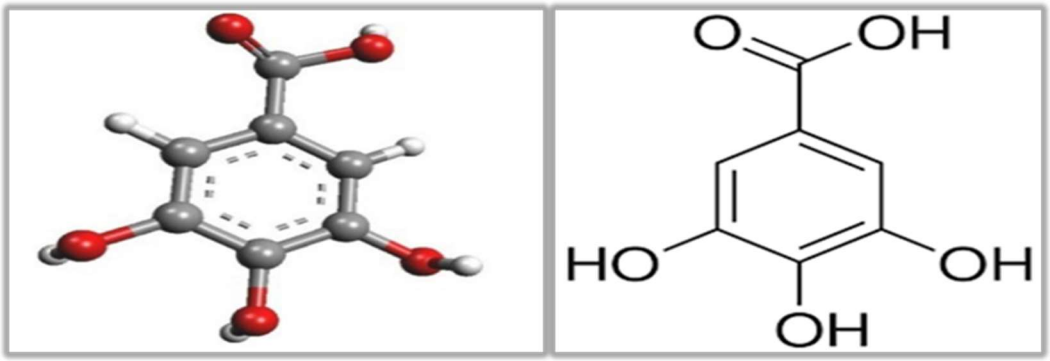

Fig.2c Structure of gallic acid (GA)

Invitro- Agar well diffusion method

The antibacterial activity of selected samples was tested against the bacterial pathogens by agar well diffusion method. For this, bacterial pathogens were inoculated in muller hinton broth and incubated for 12 hrs before antibacterial assay. All the bacterial strains were individually spread on the Muller Hinton agar plates. Wells were made in the plates at 6mm by using cork borer. Samples are dissolved in DMSO. The diluted 100 µl of samples was added on the wells and incubated for 37°C at 24 hours. Chloramphenicol antibiotic was used as a positive control. DMSO used as a negative control. The assay was carried out in triplicates. The zone of inhibition was measured in mm after the completion of the incubation period.

Results & Discussion

Antibacterial activity of β-lactoglobulin with phenolic acids

Antibacterial activity of the protein samples βLG was tested against the four clinical bacterial pathogens: Gram-positive- *staphylococcus aureus*, *Streptococcus pyogenes* and Gram-negative - *Escherichia coli, Klebsiella pneumoniae.* Fig.3 shows the antibacterial activity of the protein sample and the complexes ((i) βLG (ii) βLG +CA (iii) βLG +SA (iv) βLG + GA).

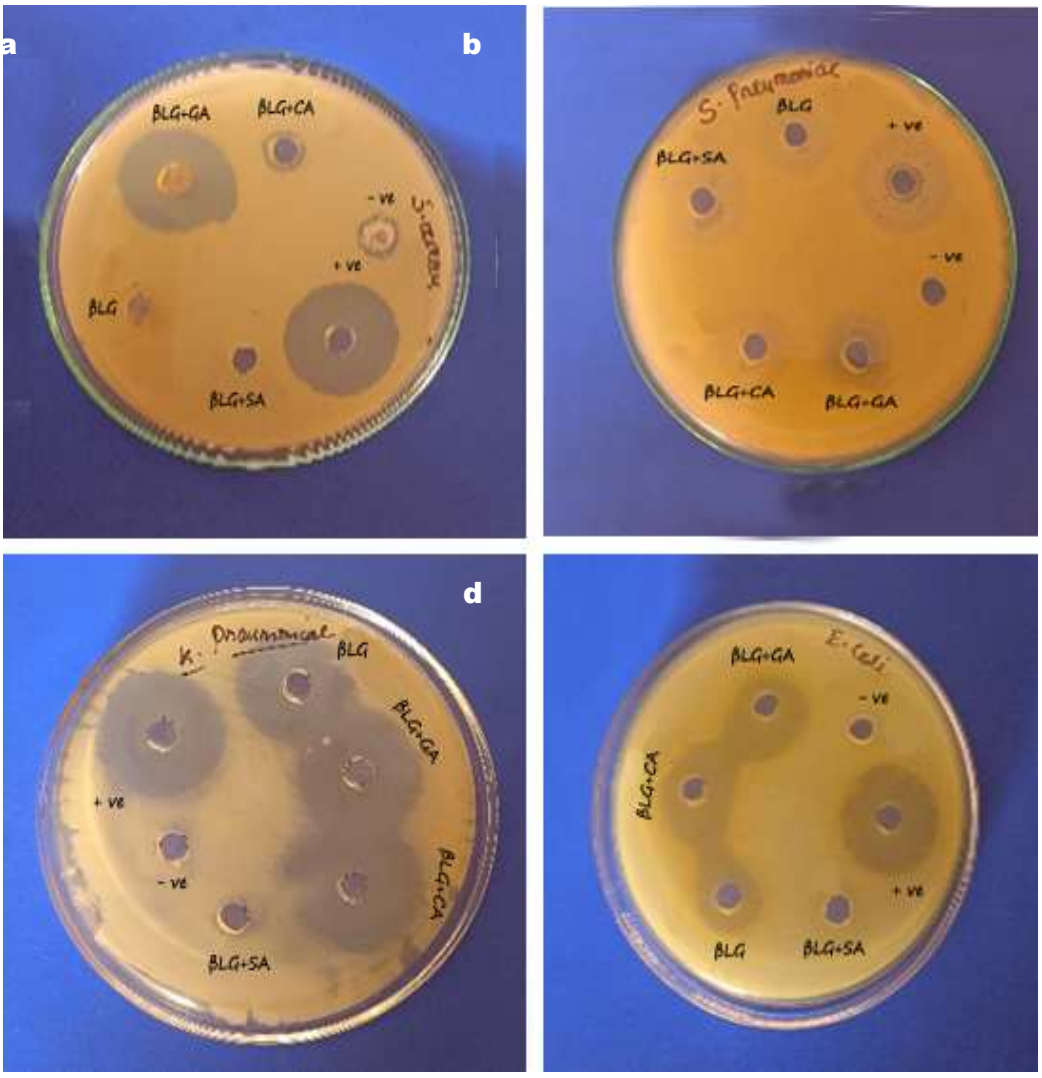

Fig. 3 The antibacterial activity of (βLG, βLG+CA, βLG+SA, βLG+GA)
a) *Staphylococcus aureus* $^{+}$, b) *Streptococcus pyogenes* $^{+}$, c) *Escherichia coli* $^{-}$, d) *Klebsiella pneumonia* $^{-}$

The antibacterial activity elicited by both sinapic acid and p-coumaric acid in this study are more or less significant or consistent with studies on phenolics against bacterial strains (Muniz. D.F., *et al.,* 2021; Santos-Sanchez. N.F., *et al.,* 2019; Zimmermann. S., *et al.,* 2019). Gram-positive and Gram-negative bacteria, and the results are presented in Table 4.12.

Table.1 Antibacterial activity of phenolic acids with βLG

S.No	Bacterial Pathogens	Zone Of Inhibition (mm)				
		βLG	βLG+ CA	βLG+ SA	βLG+ GA	Positive control
1	*Staphylococcus aureus*	13	14	16	20	26
2	*Streptococcus pyogenes*	12	18	14	21	21
3	*Esherchia coli*	16	12	14	18	23
4	*Klebsiella pneumoniae*	18	16	10	24	24

From Table.1 shows that the protein βLG has the potential to inhibit Gram - positive *Staphylococcus aureus* 13mm, but the positive control has the range at 26mm, whereas in combination with phenolic acids βLG+GA has the potency to inhibit 20mm compared to that of other combinations such as βLG+SA (16mm) and βLG+CA (14mm). The maximum inhibition of Streptococcus *pyogenes* and Gram positive bacteria was detected in βLG (12mm) βLG+ CA (18mm) followed by βLG+SA (14mm) βLG+GA (21mm), and compared to that of the positive control 21mm. In the same way the protein βLG and its combination with phenolic acids were subjected to Gram-negative bacteria *Escherichia coli, Klebsiella pneumoniae*. In comparison, βLG+GA (24mm) shows maximum inhibition for *Klebsiella pneumoniae* and βLG+GA (18mm) shows inhibition for *Escherichia coli.*

βLG exhibits antibacterial properties affecting the human immune system by stabilizing cell proliferation (Tai *et al*., 2016). It has been demonstrated that the binding of different ligands to βLG changes their biological activity (Pessato. T.B., *et al*., 2019; Buszewski. B., *et al.,* 2020). Sanhueza. L., *et al*., 2017 demonstrated the antibacterial potential of phenolic acids with MIC values ranging from 300 to 3000 µg/mL and 500 to 4000 µg/mL against S. aureus and E. coli, respectively, while ciprofloxacin maintained an MIC of 1500 µg/mL against both strains. Santos-Sánchez. N. F., *et al*., 2019, on the other hand showed that caffeic acid and gallic acid had MIC values of <1024 mg/ml against *S.aureus.* The antibacterial activity of ciprofaxin against *S.aureus* in this study was in agreement with the report of Zimmermann. S., *et al.,* 2019 showing MIC values in the range of 7.8 to 500 mg/mL. A compound's ability to form hydrogen bonds with amino acid

residues at the active site of the βLG might explain its antibacterial properties (Verma. R., *et al*., 2019; Khade. A.B., *et al*., 2020).

Antibacterial activity of ovalbumin with phenolic acids

Fig.4 shows the antibacterial activity of the protein sample and the complexes ((i) OVA (ii) OVA+CA (iii) OVA+SA (iv) OVA+GA). OVA was tested against the four clinical bacterial pathogens: Among these four bacterial pathogens, two belong to Gram-positive - *staphylococcus aureus, Streptococcus pyogenes* and two belongs to *Gram-negative* - *Escherichia coli* and *Klebsiella pneumoniae*. The combinations of protein and phenolic acids (CA/ SA / GA) were used for antibacterial activity.

The most commonly reported mechanism of action of phenolics against bacteria is based on their accumulation at the surface of bacteria (Negi. P.S., 2012). This accumulation depends on interactions between phenolics and the cell wall of bacteria. Therefore, the surface properties of the 3 strains of bacterial cells were investigated by determining the microbial adhesion to water-solvent interfaces of these cells. These are shown in Table 2

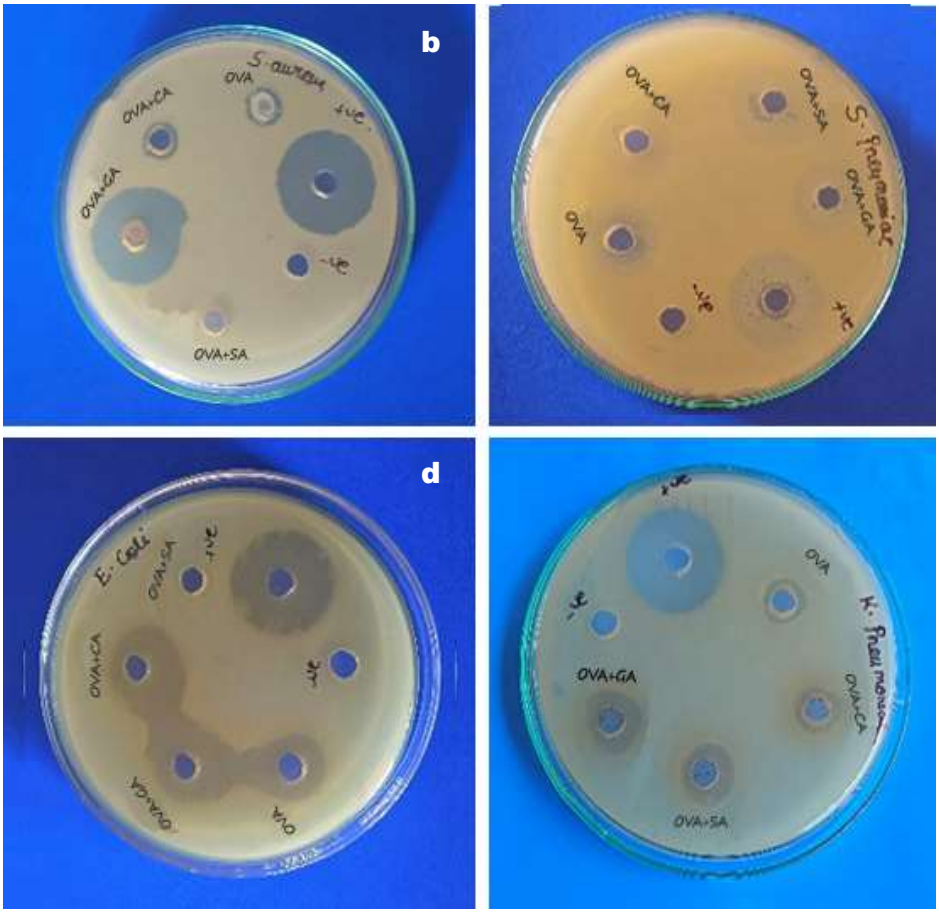

Fig. 4 The antibacterial activity of OVA, OVA + CA, OVA + SA, OVA + GA a) *Staphylococcus aureus* $^{+}$, b) *Streptococcus pyogenes* $^{+}$,

c) *Escherichia coli*$^{-}$, d) *Klebsiella pneumonia*$^{-}$

Table 2 Antibacterial activity of phenolic acids with OVA

S.No	Bacterial Pathogens	Zone Of Inhibition (mm)				
		OVA	OVA+ CA	OVA+SA	OVA+GA	Positive control
1	*Staphylococcus aureus* $^{+}$	20	14	16	24	26
2	*Streptococcus pyogenes* $^{+}$	14	11	12	21	21
3	*Escherichia coli* $^{-}$	11	10	14	18	23
4	*Klebsiella pneumonia* $^{-}$	18	16	12	20	24

From Table 2 shows that the protein OVA has the potential to inhibit Gram - positive *Staphylococcus aureus* 20mm, but the positive control has the range at 26mm, whereas in combination with phenolic acids OVA +GA has the potency to inhibit 24mm compared to that of other combinations such as OVA+SA (16mm) and OVA+CA (14mm). The maximum inhibition of Streptococcus *pyogenes* and Gram positive bacteria was detected in OVA (14mm) OVA + CA (11mm) followed by OVA+SA (12mm) OVA +GA (21mm), and compared to that of the positive control 21mm. In the same way the protein OVA and its combination with phenolic acids were subjected to Gram-negative bacteria *Escherichia coli, Klebsiella pneumoniae*. In comparison, OVA+GA (20mm) shows maximum inhibition for *Klebsiella pneumoniae* and OVA+GA (18mm) shows inhibition for *Escherichia coli.*

To conclude, the combination of OVA+GA shows potent antibacterial activity against both Gram-positive and Gram-negative bacteria with Support to the results the difference in susceptibility to polyphenols between Gram-positive and Gram-negative bacteria is a controversial issue. On one hand, many authors like Inouye. S., *et al.,* 2001 concluded that the antibacterial effect of polyphenols was generally more effective against Gram-positive bacteria than Gram-negative ones. They indicated that Gram-negative bacteria are more resistant to plant secondary metabolites including phenolics due to the cell wall they possess linked to an outer complex membrane, which slows down the passage of chemicals (Inouye. S., *et al*., 2001). Nevertheless,

this outer membrane may also be altered by some polyphenols (Helander, I. M *et al*., 1998). Several mechanisms of action in the growth inhibition of bacteria are involved, such as destabilization of the cytoplasmic membrane, permeabilization of the plasma membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism and deprivation of the substrates required for microbial growth (Puupponen-Pimi. R., *et al*., 2001). It is thus possible that their lipophilicity is less affected by substitutions of the aromatic ring. However, the double bond of the side chain, which is the main difference in the structure of phenolic acids, likely contributes to the antibacterial activity. The number of hydroxyl groups altered the antibacterial activity of the phenolic acids but did not affect the activity of phenolic acids. Likewise, an increase in lipophilicity by substitution of hydroxyl groups with methoxy groups increased the activity.

Spoilage and foodborne pathogenic microorganisms have caused serious economic losses and community-associated infections. The use of natural antimicrobial agents for food preservation is a trend that is followed by both consumers and food manufacturers. Studies on the antimicrobial activity of phenolic-protein conjugates are very limited (Fu. S., *et al*., 2017) and that chlorogenic acid-gelatin conjugate possesses antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogenes* and *Staphylococcus aureus*. The antibacterial activity of chlorogenic acid-gelatin conjugate is quite close to that of free chlorogenic acid. Utilization of the antimicrobial activity of berry phenolics may offer many new applications in the food industry. For example, natural food preservatives targeted at foods that are easily contaminated by bacteria, such as *Salmonella* and *Staphylococcus*, are highly desired (Puupponen-Pimi. R., *et al.,* 2001). Recently Ali. M., *et al.,* 2018 revealed that the antimicrobial activity of rosemary acid-whey protein isolate conjugate obtained by PPO catalysis is higher than that obtained under alkaline conditions. Another type of health-beneficial effect has been proven regarding berry phenolics and pathogenic bacteria. Certain types of cranberries have been shown to aid in maintaining urinary tract health (Howell, A.B., *et al.,* 2005, Williamson. G and Manach. C 2005) with other tannin-containing berries contributing to this effect as well (Kontiokari. T., *et al.,* 2001; Kontiokari. T., *et al.,* 2003). However, the antimicrobial mechanisms of the conjugates are unclear till now.

Conclusion

Protein-phenolic acid interaction can produce various biological effects, which affect the structure, function and quality of proteins. Investigations on protein-phenolic interactions have become the study of interest in many areas of food, nutrition and health. On comparing OVA+GA complexes shows better antibacterial activity. The present study is pave way for valuable products in food processing industry and daily life.

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