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ANTIMICROBIAL ACTIVITY OF CHITOSAN AND COMBINATION WITH ANTIBIOTICS AGAINST MASTITIS-CAUSING PATHOGENS

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ABSTRACT

Bovine mastitis (BM), primarily caused by bacterial pathogens infecting mammary glands, stands as the most prevalent disease in dairy cattle. Traditionally, antibiotics have been the primary choice of treatment, yet their overuse has led to widespread resistance and the presence of antibiotic residues in dairy products. Today, chitosan has emerged as a promising alternative in dairy farming. In this study, we systematically screened and assessed the antibacterial efficacy of five chitosan preparations of different viscosities and components. Additionally, we explored the synergistic antimicrobial potential of the most potent chitosan sample in combination with commonly employed antibiotics, including ampicillin, amoxicillin, oxacillin, and levofloxacin against four prevalent BM-causing pathogens: *Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus uberis* and *Pseudomonas sp.* Agar well diffusion, micro-dilution, and checkerboard techniques were applied to assess the antimicrobial activity and interaction effect. Results indicated that, at a concentration of 1%, low and medium viscosity samples (samples 1, 2, 3) exhibited relatively low activity, compared to very low viscosity ones (samples 4, 5). Notably, sample 5, a combination of chitosan sample 1 with orange and grapefruit essential oils, demonstrated the most potent antibacterial activity with a minimal inhibitory concentration (MIC) of 19.53 mg/L against *S. agalactiae*, *S. uberis* and *S. epidermidis* and 78.13 mg/L against *Pseudomonas sp.*. Furthermore, the combination of this chitosan sample and antibiotics exhibited some synergistic interactions against BM-causing pathogens, as indicated by the fractional inhibitory concentration (FIC) values ranging from ≥ 0.5 to ≤ 1. While these effects were notable, they did not reach the threshold for strong synergism (FIC < 0.5). In summary, our study highlighted the high antibacterial activity of low viscosity chitosan,

particularly in combination with essential oils. Although there were observed synergistic effects with antibiotics against BM-causing pathogens, the strength of these interactions was not robust enough to conclusively categorize them as strongly synergistic. Chitosan, however, emerges as a promising agent in the ongoing exploration of alternatives to antibiotics in the management of BM in dairy farming.

Keywords: Antimicrobial activity; bovine mastitis; chitosan; pathogens.

INTRODUCTION

Bovine mastitis (BM) is a widespread and prominent problem in the dairy industry in Vietnam and various regions worldwide. This illness poses a significant threat to the cattle business, particularly impacting dairy cows. Cows with BM experience a notable 20–30% decline in milk output, coupled with a 15% reduction in lactation production (Fetrow *et al.*, 1991). The primary pathogens responsible for bovine mastitis include a wide range of gram-positive and gram-negative bacteria. These can be categorized as either contagious pathogens (such as *Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma spp.*) or environmental pathogens (including *Escherichia coli, Enterococcus spp.,* and *Streptococcus uberis*). These bacteria reside on the surface of the cow's udder and teats, where they colonize and proliferate, eventually advance into the teat canal (Abebe *et al.*, 2016). These days, antibiotics are widely used to treat bovine mastitis, leading to the development of bacterial resistance and the accumulation of drug residues in milk. Chitosan has been studied and reported to have potential in mastitis management in dairy cows (Cheng *et al.*, 2020). Derived from chitin, which is abundant in the exoskeletons of crustaceans, chitosan is a cationic polysaccharide composed of structural building blocks such as D-glucosamine and N-acetyl D-glucosamine units (Daraghmeh *et al.*, 2011). The molecular weight (MW), the presence of amino groups (NH_2), and the degree of deacetylation (DD) are crucial factors influencing its chemical-physical characteristics and applications (Nadia *et al.*, 2019).

Previous research indicates that chitosan can effectively combat a diverse array of bacteria, fungi, and viruses by disrupting cell membranes and impeding the crucial processes of harmful microorganisms (Rivera *et al.*, 2020). Nevertheless, various hypotheses surround the mechanisms underlying the antimicrobial activity of chitosan. One potential suggestion is that chitosan utilizes its positively charged amino groups to engage with negatively charged components on the cell membrane, resulting in the destruction of cellular structure and leakage of intracellular components (Chung *et al.*, 2004). Additionally, this research also investigated that chitosan is known to selectively form complexes with metal ions on the microorganisms' cell walls, disrupting the cell wall structure and contributing to the inhibition of microbial growth. Besides, chitosan is also believed to inhibit mRNA and protein synthesis due to its ability to traverse the cell membrane, binding to the cell's DNA and thereby preventing mRNA translation, leading to the inhibition of protein synthesis (Sudarshan *et al.*, 1992). Moreover, chitosan could form a chelating metal film on the cell surface to prevent the absorption of vital nutrients and cell

secretion, thereby inhibiting microbial metabolism (Goy *et al.*, 2009; Liu *et al.*, 2001).

In this study, different chitosan preparations were evaluated for their potential activity against common BM pathogens. The samples with high effectivity were then assessed for their synergistic effect with commonly used antibiotics, to see the potential of using chitosan to reduce antibiotic usage in daily life.

MATERIALS AND METHODS

Bacteria species and culture conditions

Four bacterial species, including *Pseudomonas sp.*, *Staphylococcus epidermidis*, *Streptococcus uberis*, and *Streptococcus agalactiae*, were isolated and biochemically identified from mastitis-infected cows at dairy farms in Tay Ninh province, Vietnam and provided by the Vietnam Food Company (VNF Company, Vietnam).

Chitosan preparation

Chitosan samples were prepared from shrimp shell waste (VNF Co. Ltd., Vietnam) and were in the form of liquid. Samples 1-4 were different in viscosity. Sample 5 was prepared by mixing sample 1 with orange and grapefruit essential oils (India). The characteristics of the five chitosan samples used in the study were summarized in Table 1. Chitosan samples were dissolved in acetic acid 1% (v/v). Sample 4 (4.45%) and sample 5 (1.6%) were adjusted using acetic acid 1% (v/v) to 1% chitosan in acetic acid (1%, v/v) before performing an agar-well diffusion and microdilution assay.

Table 1. Characteristics of chitosan samples used in the study.

Features	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Moisture (%)	10.44	10.27	8.76	-	-
Degree of acetyl (%)	92.83	87.05	95.05	87.54	-
Viscosity (cPs)	111.5	186.3	724	3.4	-
Protein (%)	0.44	0.18	0.47	-	-
Ash (%)	0.55%	0.41%	0.79%	-	-
Turbidity (NTU)	4.4	5.38	17	-	11
pH	3.93	3.94	3.92	3.09	5.15
Content (ppm)	10000	10000	10000	44500	16000
Chitosan concentration (%)	1	1	1	4.45	1.60

Solubility in water	-	-	-	Completely	Completely
Ingredients	100% from shrimp shell	100% from shrimp shell	100% from shrimp shell	100% from shrimp shell	Chitosan sample 1, orange and grapefruit essential oils

Agar-well diffusion assay

The assay was performed as previously described (Linh *et al.*, 2020; Thuong *et al.*, 2015). In brief, *Pseudomonas* sp., *S. epidermidis*, and *S. uberis* were cultured on Mueller Hinton Agar (MHA, Himedia, India) plates and *S. agalactiae* on Blood Agar (NamKhoa Biotech Ltd., Vietnam) plates. Cultures were incubated at 37^{o}C for 24 hours. Then, colonies of each species were collected and cultured in Mueller Hinton Broth (MHB, Himedia, India) at 37^{o}C for 24 hours. The overnight cultures were diluted in MHB to have OD_{620nm} of 0.08-0.1. These bacterial suspensions were then used for susceptibility testing. For the disc test, 100 µL of bacterial suspension was spread evenly on the surface of either MHA for *Pseudomonas* sp., *S. epidermidis*, and *S. uberis* or a Blood agar plate for *S. agalactiae* using sterilized glass beads. Wells of 8 mm in diameter were drilled in each plate. Subsequently, 100 µL of five chitosan samples (1% in 1% acetic acid) were loaded into separated wells. A hundred microliters of 1% acetic acid were used as a negative control and cefepime disc of 30 µg (NamKhoa Biotech, Co. Ltd., Vietnam) was used as a positive control. The plates were incubated at 37^{o}C for 24 hours. The inhibition zones were observed and their diameters were measured using a ruler.

Determination of minimum inhibitory concentrations (MIC) by microdillution assay

MIC values were determined using the micro-dilution method described by Chi *et al.* (2017). Briefly, 100 µL Mueller Hinton medium Broth (MHB) was transferred to 96-microwell plates (from wells 1 to 11). The well number 11 was the positive control having 100 µL of MHB medium and 100 µL of bacterial suspension diluted to 1:100 with bacterial suspension ($OD_{620nm:}$ 0.08-0.1), while well number 12 was the negative control having MHB medium only. One hundred µL of samples, which were either antibiotics ampicillin, amoxicillin, oxacillin and levofloxacin or acetic acid 1% or chitosan samples were added to the first well containing 100 µL of MHB, then mixed and serially diluted by two folds until the well number 10 of each row of the 96-well plate. The test range of acetic acid (1%, v/v) and five chitosan samples were from 0.25% to 0.00048%, while the test range of antibiotics was from 4 mg/L to 0.0078 mg/L. Afterwards, 100 µL of a 1:100 bacterial suspension was added to each well. The plates were incubated at 37^{o}C for 24 hours. The MIC values were recorded as the lowest concentration of testing agents where no bacterial growth was observed. The susceptibility was determined using CLSI breakpoints (2021) (CLSI, 2021).

Checkerboard assay

The chitosan sample, which possesses the most effective antimicrobial activity, was used to assess the interaction with antibiotics. Four antibiotics, ampicillin, amoxicillin, oxacillin and levofloxacin, were tested. In the assay, a 96-well plate was loaded with 100 µL of MHB, then two-fold serial dilutions of an antibiotic were applied in the vertical direction and chitosan in the horizontal direction. The concentration testing ranges used in this assay were identical to the MIC assay described above. One hundred µL of bacteria suspension was then added to each well. The plate was then incubated for 24h at 37°C and read at 600 nm, then MIC and FIC were determined following a previous study to conclude on the interaction effect between antibiotics and chitosan (Bellio *et al.*, 2021; Tung, *et al.*, 2024; Lorian, 2005).

The concentration index value is then used to reflect this comparison. The FIC index value considers the drug combination that results in the biggest deviation from the MIC of each antibiotic (Lorian, 2005). The fractional Inhibitory Concentration (FIC) index is used to quantify the interactions between the antibiotics being tested by the equation below:

$$\frac{A}{MIC_A} + \frac{B}{MIC_B} = FIC_A + FIC_B = \sum \text{FIC index}$$

The MIC of each antimicrobial agent in combination (in a single well) are A and B, and the MIC of each agent individually are MIC_A and MIC_B.

The MIC values of each substance alone and in combination have been obtained along with the FIC index by performing the checkerboard method. There are two ways to evaluate the additive, indifferent and antagonistic effects: based on Lorian's work (Lorian, 2005). Lorian (2005) stated that the interaction is synergy when the combination of compounds results in an FIC value of < 0.5, and the inhibitory activity (reduction in MIC) of one or both compounds is increased compared to the individual compounds. The interaction is additive or indifferent if there is no increase in inhibitory activity or a very minor increase due to the additive action of the two compounds combined, this will produce an FIC value ≥ 0.5 and ≤ 4. The interaction is antagonistic when two compounds are combined to produce an FIC value > 4.

Statistical analysis

All experiments were carried out in triplicate. The collected data are analyzed using one-way and two-way ANOVA and statistical comparisons between samples and bacterial species were made using the Duncan Postdoctoral test using a p = 0.05 significance level in IBM SPSS statistics 20 software. All data are presented as means and SD values.

RESULTS AND DISCUSSION

Antibacterial activity of five different chitosan preparations

The obtained results indicated that chitosan samples 1, 2 and 3 showed weak antimicrobial activity compared to chitosan samples 4 and 5 (Table 2, Figure 1). There was no significant difference between

samples 1, 2 and 3 in their antibacterial activity ($p<0.05$). The high antibacterial activity of sample 4 was presented by a large inhibition zone of 13.33 ± 0.58 mm against *S. agalactiae* and *Pseudomonas sp.* and 11.33 ± 0.58 mm against *S. uberis*. Sample 5, even though prepared with the same concentration at 1%, had the highest activity against all four pathogens, *S. uberis*, *Pseudomonas sp.*, *S. epidermidis* and *S. agalactiae*, with a diameter of the inhibition zones of 20.67 ± 1.15 mm, 20.00 ± 1.00 mm, 21. 67 ± 0.58 mm and 16.33 ± 0.58 mm, respectively. However, it should be noted that 1% acetic acid also showed a significant inhibitory effect with all the above-mentioned bacterial species (Figure 1). Therefore, a MIC assay was conducted to exclude the activity of 1% acetic acid and obtain MIC values.

Table 2. Inhibition zones of 5 tested chitosan samples. Data were presented as Mean ± Standard Deviation of three replicates. Different letters indicated significant differences ($p < 0.05$) among chitosan samples for the same bacterial species (lowercase letters) and among bacterial species for the same chitosan sample (capital letters). +: positive control; -: negative control; 1-5: chitosan sample no.1 to no.5.

Bacterial species	Diameter of inhibition zones (mm)						
	1	2	3	4	5	+	-
S. agalactiae	9.00 ± 1.00a A	8.67 ± 0.58a A	8.33 ± 0.58a A	13.33 ± 0.58b A	16.33 ± 0.58c A	20.00 ± 0.00	20.00 ± 1.12
S. uberis	8.67 ± 0.58a A	8.67 ± 0.58a A	8.33 ± 0.58a A	11.33 ± 1.00b B	20.67 ± 1.15c B	27.00 ± 0.00	15.78 ± 0.83
S. epidermidis	8.67 ± 0.58a A	9.67 ± 1.15a A	8.00 ± 0.00a A	8.67 ± 0.58a C	21.67 ± 0.58b B	33.00 ± 0.00	12.44 ± 1.24
Pseudomonas sp.	9.67 ± 0.58a A	9.33 ± 0.58a A	8.00 ± 0.00b A	10.00 ± 1.00a B	20.00 ± 1.00c B	22.00 ± 0.00	14.90 ± 0.78

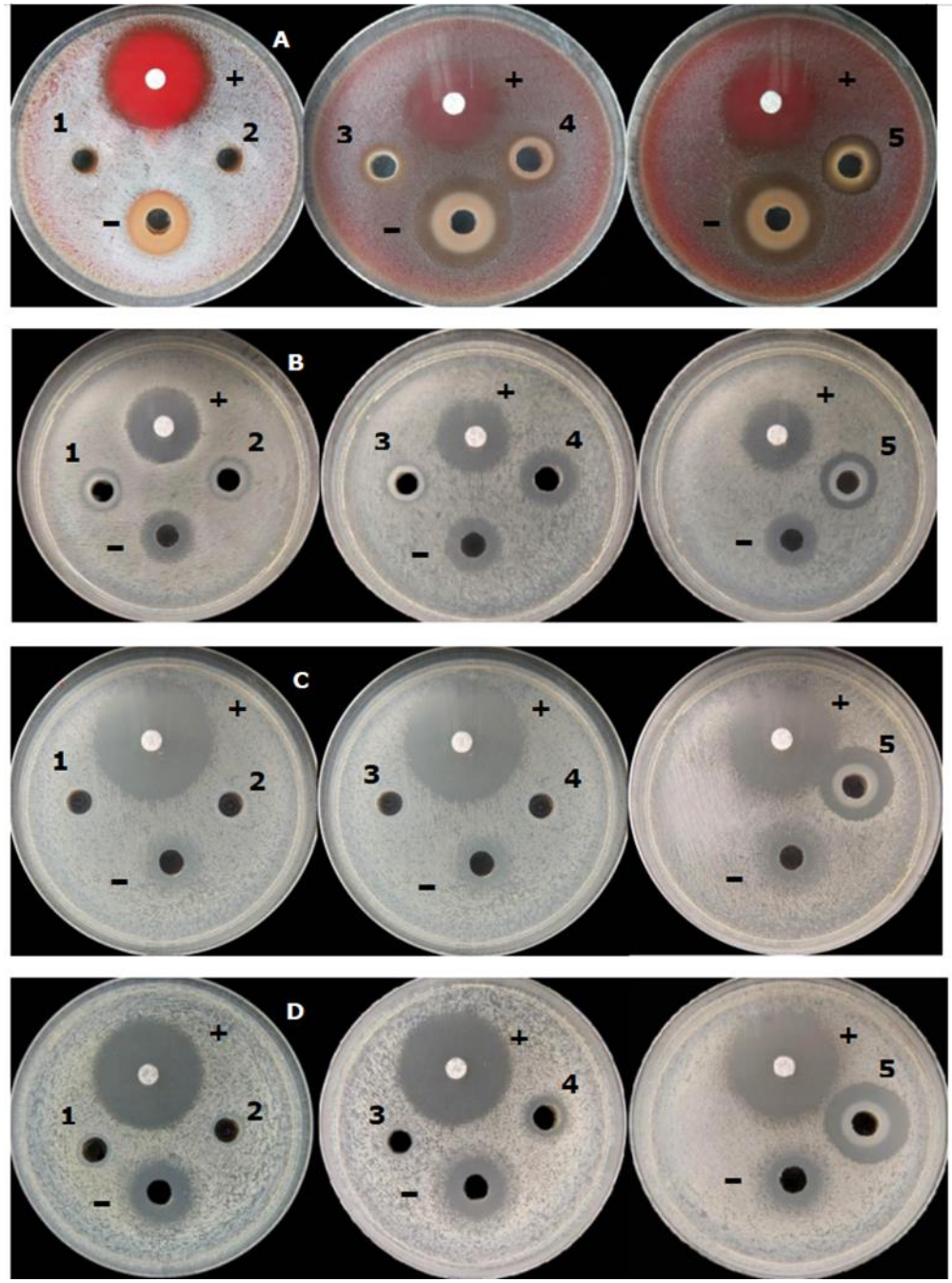

Figure 1. Representative results of the antibacterial activity of 5 chitosan samples. Disc-diffusion assay for A, *S. agalactiae*; B, *S. uberis*; C, *S. epidermis*; D, *Pseudomonas sp.*. Images captured after 24h culture on Blood agar (A) or MHA (B, C, D). (+), cefepime 30 µg disc (positive control); (-) 1% acetic acid (negative control); 1-5: chitosan samples 1 to 5.

Investigation of the minimum inhibition concentrations of 1% acetic acid and five different chitosan samples

The MIC of acetic acid against 4 bacterial species causing bovine mastitis, *S. agalactiae*, *S. uberis*, *S. epidermidis* and *Pseudomonas* sp. was presented in Figure 2. The result indicated that under 0.031% acetic acid, the bacterial growth of all 4 bacterial pathogens was not affected by acetic acid anymore. The MIC value of acetic acid for all tested strains was 0.125% (Figure 2). This outcome was consistent with a previously published study (Pangprasit *et al.*, 2020).

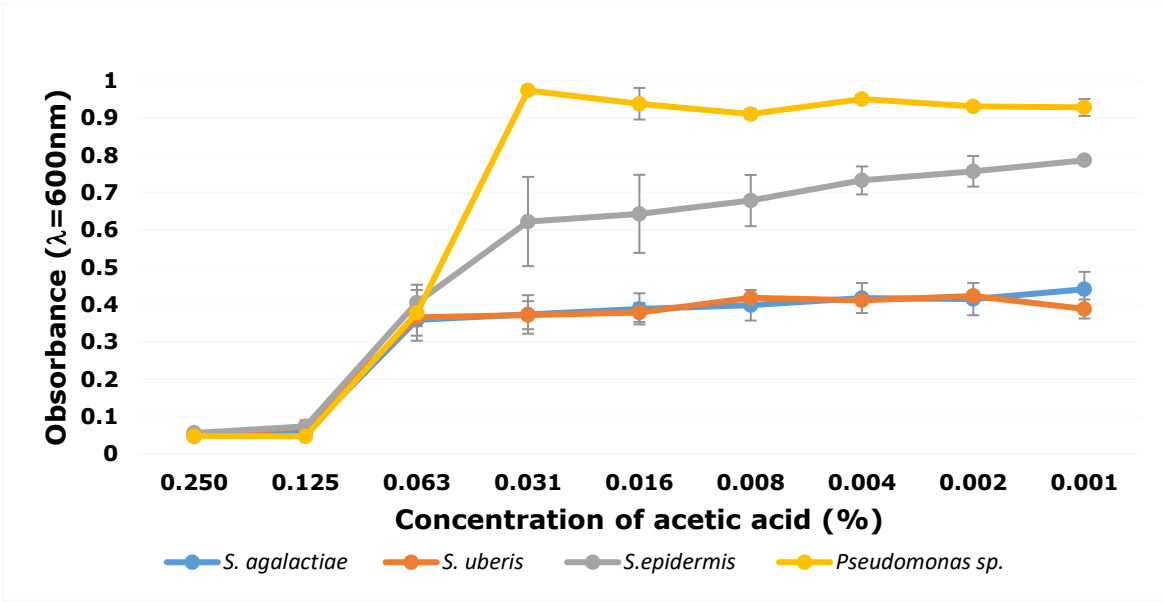

Figure 2. Results of the MIC assay for 1% acetic acid against *Pseudomonas sp., S. epidermidis, S. uberis* and *S. agalactiae*. Data are presented as the mean ± standard deviation of two independent experimental biological replicates.

The MIC of five chitosan samples were summarized in Table 3. The MIC of sample 1 and 2 were out of the tested range (0.25% to 0.00048%) thus being indicated as undetermined (> 0.25% or 2500 mg/L). The MIC of sample 3 was 0.25% (2500 mg/L) for both *S. epidermidis* and *Pseudomonas sp.*. However, its inhibitory effect was also attributed to its solvent. This is because even at a concentration of 0.125%, the solvent, acetic acid, still exhibits antimicrobial properties. Sample 3 showed antimicrobial activity on both *S. agalactiae* and *S. uberis* at the same concentration of 0.008% (78.13 mg/L). With sample 4, it showed antibacterial activity against *S. agalactiae*, *S. uberis* and *S. epidermidis* at 0.125% (1250 mg/L), and against *Pseudomonas* sp. at 0.0625% (625 mg/L). All pathogens were completely inhibited by sample 5. To be more specific, the lowest MIC value was 0.002% (19.53 mg/L) tested on *S. agalactiae*, *S. uberis* and *S. epidermidis*, meanwhile, the MIC value of *Pseudomonas* sp. was 0.008% (78.13 mg/L), tested on *Pseudomonas sp.*.

The main differences between the 5 chitosan samples are their viscosities. The viscosity information can be used to estimate the molecular weight of each chitosan sample. Based on the given ranges from the VNF

company, low molecular weight chitosan has a viscosity of less than 150 cPs, medium molecular weight chitosan has a viscosity ranging from 150 to less than 1000 cPs, and high molecular weight chitosan has a viscosity greater than 1000 cPs. Hence, sample 1 can be classified as low molecular weight chitosan, samples 2 and 3 were medium molecular weight chitosan with different viscosity ranges. Sample 4 was chitosan oligosaccharide with very low viscosity, and sample 5 was a low molecular weight chitosan (sample 1) combined with orange and grapefruit essential oils.

Table 3. Minimum inhibitory concentration (MIC, mg/L) of chitosan in 1% acetic acid against four bacterial species. 1-5: chitosan 1-5; (-): undetermined (> 2500 mg/L or 0.25%).

Samples / **Bacterial species**	**MIC (mg/L)**				
	1	2	3	4	5
S. agalactiae	-	-	78.13	1250	19.53
S. uberis	-	-	78.13	1250	19.53
S. epidermis	-	-	2500	1250	19.53
Pseudomonas sp.	-	-	2500	625.0	78.13

Our data indicated that the antimicrobial activity of chitosan samples varied depending on the characteristics of the chitosan preparation. The antibacterial activity variation obtained in different studies can be explained via the molecular weight (Mw), degree of deacetylation (DD), pH of the chitosan preparation and tested bacterial species (Ardean *et al.*, 2021). Results of the agar well diffusion assay suggested that 1% chitosan samples 1, 2, and 3 even had protective activity rather than antimicrobial activity as the microbial growth was observed in the presence of chitosan samples 1, 2, 3, while it was inhibited in the presence of solvent/ acetic acid 1% only. Some studies reported that at high concentrations, positively charged chitosan due to amino groups may have acted to coat the cellular surface, and the intracellular components of bacteria are blocked in the cell, forming an impermeable layer around the cell. This layer prevents the permeation of acetic acid through the bacterial plasma membrane (Hosseinnejad *et al*., 2016). Chitosan sample 3 exhibited more antimicrobial activity against *Streptococcus* sp. than *Staphylococcus* sp. and *Pseudomonas sp.*. Chitosan sample 4 expressed additional antibacterial activity on *Pseudomonas sp.* with a MIC value of 625 mg/L. This indicated that chitosan sample 4 showed higher antimicrobial activity against gram-negative bacteria than positive bacteria, which was similar to some previous reports (Devlieghere *et al.*, 2004; Chung *et al.*, 2004). Chitosan sample 5 possessed the most effective antimicrobial activity against all four pathogens causing mastitis bovine in both agar well diffusion and MIC assay compared to other samples. Chitosan sample 5 showed high antimicrobial activity against *Streptococcus* sp. (*S. agalactiae* and *S. uberis*) and *Staphylococcus* sp. (*S. epidermidis*), while, the least effective

antimicrobial activity was on *Pseudomonas sp.* On the other hand, this result also supported the observation that chitosan generally showed stronger bactericidal effects for gram-positive bacteria than gram-negative bacteria (No *et al.*, 2002; Fernandez-Saiz *et al.*, 2009). In brief, it appeared that water-soluble chitosan, such as samples 4 and 5, exhibited good antibacterial properties. However, it is important that the chitosan be stored in an acetic acid solvent to prevent the loss of activity, as was observed in a previous report (Qin *et al.*, 2006).

Determination of tested ranges for checkerboard assay using MIC assay

The MIC values of the chitosan sample 5 and four antibiotics to be used for a potential combination were shown in Table 4.

In this testing, the chitosan sample 5 was used in its original form provided by the producer, 1.6% in acetic acid 1% instead of 1.0% in acetic acid 1% as in the previous experiment. We have observed a significant reduction in MIC values of chitosan sample 5 when used in its original form, as 1.6% in 1% acetic acid (Table 4) when compared with 1.0% in 1% acetic acid (Table 3). It is speculated that chitosan should be kept at high concentrations to maintain its antibacterial activity. However, it should be noted that at high concentration, the high viscosity reduces the flexibility of chitosan on bacteria, hence reducing its antimicrobial activity (Jovanović *et al.*, 2016). Thus, high concentrations are recommended for preservation but not for usage. In a previous concentration-testing study for a chitosan sample, 1.5% resulted in the highest activity among 0.5, 1.0, 1.5 and 2.0% (Phượng *et al.*, 2022).

Table 4. MIC values of chitosan sample 5 and four commonly used antibiotics against *S. epidermidis, S. agalactiae, S. uberis,* and *Pseudomonas* sp.. Chitosan sample 5 (1.6% in 1% acetic acid), ampicillin, amoxicillin, oxacillin and levofloxacin were used in the assay. The plates were incubated at 37°C for 18-24 hours.

Antibiotics / Bacterial species	MIC (mg/L)				
	Chitosan	Ampicillin	Amoxicillin	Oxacillin	Levofloxacin
S. agalactiae	7.813	0.008	0.031	0.008	0.25
S. uberis	15.625	0.031	0.25	0.125	0.25
S. epidermidis	7.813	0.031	0.25	0.125	0.25
Pseudomonas sp.	62.5	16	32	512	0.5

Regarding antibiotics, the obtained results indicated that ampicillin, amoxicillin, oxacillin and levofloxacin were still effective against all 3 gram-positive pathogens. On the other hand, only levofloxacin remained effective against the gram-negative pathogen *Pseudomonas* sp..

After screening for MIC values, the combination of chitosan and antibiotics was conducted and the results were presented in

Table 5. The FIC index ranges from 0.563 to 1.0 indicating that the interactions between chitosan and antibiotics resulted in only an additive or indifferent effect (FIC values ≥ 0.5 or ≤ 4) (Lorian, 2005).

However, the combination of ampicillin, amoxicillin, oxacillin and levofloxacin with chitosan showed no synergistic effect as expected, instead they only had additive effects on the mastitis pathogens (Table 5). Among the four tested antibiotics, combinations between amoxicillin and chitosan had the most effect, due to FIC's lowest values among the 4 antibiotics (Table 5).

Table 5. Assessment of synergistic effects between chitosan and antibiotics against *S. epidermidis, S. agalactiae, S. uberis,* and *Pseudomonas sp.*

Ampicillin interaction	**MIC of ampicillin (mg/L)**		**MIC of chitosan (mg/L)**		**FIC index**
	Combination	Alone	Combination	Alone	
S. epidermidis	0.016	0.031	3.906	7.813	1
S. agalactiae	0.004	0.008	3.906	7.813	1
S. uberis	0.016	0.031	3.906	15.625	0.75
Pseudomonas sp.	8	16	31.25	62.5	1

Amoxicillin interaction	**MIC of amoxicillin (mg/L)**		**MIC of chitosan (mg/L)**		**FIC index**
	Combination	Alone	Combination	Alone	
S. epidermidis	0.031	0.125	1.953	7.813	0.75
S. agalactiae	0.016	0.031	1.953	7.813	0.75
S. uberis	0.031	0.25	7.813	15.625	0.625
Pseudomonas sp.	16	32	3.906	62.5	0.563

Oxacillin interaction	**MIC of oxacillin (mg/L)**		**MIC of chitosan (mg/L)**		**FIC index**
	Combination	Alone	Combination	Alone	
S. epidermidis	0.063	0.125	3.906	7.813	1
S. agalactiae	0.004	0.008	3.906	7.813	1
S. uberis	0.063	0.125	7.813	15.625	1
Pseudomonas sp.	128	512	31.25	62.5	0.75

Levofloxacin interaction	**MIC of levofloxacin (mg/L)**		**MIC of chitosan (mg/L)**		**FIC index**
	Combination	Alone	Combination	Alone	

S. epidermidis	0.125	0.25	3.906	7.813	1
S. agalactiae	0.125	0.25	3.906	7.813	1
S. uberis	0.125	0.25	7.813	15.625	1
Pseudomonas sp.	0.25	0.5	7.813	62.5	0.625

The interactions between chitosan and antibiotics resulted in an additive or indifferent effect with FIC index ranging from 0.563 to 1.

Some previous studies showed that chitosan combined with antibiotics enhanced antibiotic efficacy against mastitis pathogens (Breser *et al.*, 2018; Yadav *et al.*, 2022). However, they did not use a checkerboard assay, but simply made conclusion based on the differences in MIC values of antibiotics alone and antibiotics-chitosan combinations. Furthermore, the difference in the types of antibiotics and chitosan also explained the variation. On the other hand, there were studies in line with our study, presenting that combinations between chitosan and ampicillin, amoxicillin and levofloxacin had partial effects against a wide range of bacteria with FIC values > 0.5 and ≤ 1 (Si *et al.*, 2021).

CONCLUSION

All chitosan samples used in the study demonstrated efficacy against BM-causing pathogens. The antimicrobial activity of chitosan varied significantly depending on the chitosan preparation and its application. Interestingly, in certain instances, the presence of chitosan exhibited a protective effect on bacteria, mitigating the harmful impact of acetic acid. Notably, Chitosan sample 5, a low molecular weight chitosan combined with orange and grapefruit essential oils, emerged as the most effective against all the BM-causing pathogens. Furthermore, when chitosan sample 5 was combined with ampicillin, amoxicillin, oxacillin, and levofloxacin, a partial synergistic effect was observed against all tested BM-causing pathogens, *S. epidermidis*, *S. agalactiae*, *S. uberis*, and *Pseudomonas sp.*. Despite its low concentration, chitosan displayed antimicrobial activity and demonstrated some synergistic effects when combined with the tested antibiotics. This suggests the potential use of chitosan in conjunction with antibiotics as a combination therapy for treating mastitis infections.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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