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Research Article

FECAL BACTERIA CRUDE EXTRACTS FROM PHILIPPINE NATIVE CHICKEN (*GALLUS GALLUS DOMESTICUS*) SHOW ANTIMICROBIAL ACTIVITY AGAINST *STAPHYLOCOCCUS AUREUS*

FBR Matias*, JH Pastorano, MBS Salinas

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ABSTRACT: Generally, the study evaluated the *in vitro* antimicrobial activity of fecal bacteria isolated from Philippine native chicken against *Staphylococcus aureus*. Specifically, this study measured the antimicrobial activity of the crude extracts from fecal bacteria cultured at different time intervals, and compared with selected antimicrobials against *S. aureus*. A loopful of isolated fecal bacteria was cultured in nutrient broth at different time intervals (6, 12, 18, and 24 h). After each time interval, the broth culture was centrifuged at 6,000 rpm for 15 min and the cell-free supernatant (crude extract) was collected. Sterile filter paper discs were impregnated with a total of 30 µl crude extracts and placed on spread plate culture of *S. aureus* on Mueller-Hinton (MH) agar, including selected antimicrobials discs (2 µg clindamycin, 5 µg enrofloxacin, and 10 µg penicillin V), then incubated at 37 °C for 18 to 24 h. The zones of inhibition were measured using a Vernier caliper and compared. The crude extracts of fecal bacteria from different breeds of Philippine native chicken have antimicrobial activity against *S. aureus* as shown by various sizes of zone of inhibition. The *Banaba* breed had the greatest number of isolates with zone of inhibition. The crude extracts that produced zone of inhibition were significantly higher compared to selected antimicrobials - clindamycin and penicillin V, but significantly lower compared to enrofloxacin.

Key words: Antimicrobial activity, Fecal bacteria, Crude extract, Philippine native chicken, *Staphylococcus aureus.*

INTRODUCTION

Taxonomically, native chickens belong to the genus *Gallus* of the family Phasianidae (WESVARRDEC 2006). The domesticated chicken (*Gallus gallus domesticus)* has four species that include the red jungle fowl (*G. gallus)*, Ceylonese jungle fowl (*G. layette)*, gray jungle fowl (*G. sonnerati)* and black or green jungle fowl (*G. varius*) (Sawai *et al*. 2010, Lizada *et al*. 2013). They are commonly raised in rural areas. The documented breeds of Philippine native chickens include: *Bolinao* from Pangasinan, *Banaba* from Batangas, *Darag* from Iloilo, *Camarines* from Bicol, *Paraoakan* from Palawan and the newly discovered genetic groups are *Joloanon* from Basilan and *Boholano* from Bohol (Santiago 2018). The important role of native chickens in the Philippine economy lies not on its effect to the gross national income but on serving as a stable and reliable source of protein food for the rural folks and as a direct support for their immediate needs (Lambio 2000).

Aside from this, native chickens being commonly raised in the countryside can adapt, survive, and reproduce under adverse conditions with marginal care and low production inputs (Lopez *et al*. 2014).

The poultry industry is considered to be an important economic asset in the country, and one factor that hinders this industry is the impact of diseases on its production. One common source of disease is from the bacteria that are commonly found in the intestinal tract. The common fecal bacteria in native chickens are *Pepto-streptococcus, Propionibacterium, Bacteroides, Escherichia coli, Salmonella spp. Lactobacillus, Clostridium, Fecali-bacterium, Rumino-coccus, Bacillus, Eubacterium,* and *Fuso-bacterium* (Yu 2014, Stanley *et al*. 2014). One factor that may cause infection by these bacteria is the age-

College of Veterinary Science and Medicine, Central Luzon State University, Science City of Muñoz 3120, Nueva Ecija, Philippines.
**Corresponding author. e-mail: fbrmatias@clsu.edu.ph*

dependent susceptibility to the pathogen, a primary determinant in the bacterial colonization status of the host. For instance, the colonization by *Campylobacter* species appears to be most common in chickens more than two weeks of age, while colonization by *Salmonella* is commonly seen in young chicken with less than two weeks of age (Mohamed *et al*. 2013). Meanwhile, some bacteria can provide protective effects by producing antimicrobial substances (*e.g*., bacteriocins, organic acids as lactic acid, hydrogen peroxide, diacetyl, and carbon dioxide) that inhibit the growth of other bacteria (Lagha *et al.* 2017, Vieco-Saiz *et al*. 2019).

The *Staphylococcus aureus* under the genus Staphylococcus is a microorganism that is present as a commensal organism on the skin, the nose, and mucous membranes of healthy human and animals (Lozano *et al*. 2016, Klimešová *et al*. 2017). However, these bacteria are also opportunistic pathogens that can cause multiple infectious diseases of diverse severity (Lozano *et al*. 2016). According to Smith (2015), *S. aureus* can colonize a variety of animal species. It is the most commonly reported cause of mastitis in dairy-producing animals (including cattle and goats) and bumblefoot in chickens, as well as being identified as a pathogen of farmed rabbits (Smith 2015).

Currently, antibiotics have been continuously used as growth-promotant and prophylaxis against common diseases in livestock animals. Unfortunately, the emergence of antimicrobial resistance among pathogens, especially bacteria, is constantly reported. Thus, regulated uses of antibiotics and/or safer alternatives are being implemented in livestock and poultry raising. Probiotics are live microorganisms that can confer health benefits to their host. These microorganisms together with their benefits in the gut immunity of some species, especially commercial breeds of livestock animals were already reported in several studies. In this study, information on fecal bacteria with antimicrobial activity from Philippine native chicken was explored, thus providing a possible basis on their use in livestock and poultry production.

MATERIALS AND METHODS

Crude extract production and disc preparation

Previously isolated fecal bacteria from Philippine native chickens (Matias *et al.* 2020) were re-cultured in nutrient agar for 24 hours. Based on this study, the fecal samples were collected from 12 chickens (three chickens per each breed). The isolated bacteria were identified based on colony morphology and Gram-staining characteristics. After identification, two colonies per sample were used for further studies, except for sample 2 and 3 of *Darag* breed which have three isolates each.

A loopful of bacteria was cultured in nutrient broth at different time intervals (6, 12, 18, and 24 h). After each time interval, the broth culture was centrifuged at 6,000 rpm for 15 min. The crude extract was transferred to microcentrifuge tubes and stored in - 20 ºC until further used. Labeling of each crude extract is as follows: B- *Banaba*, D- *Darag*, J- *Joloanon*, and P- *Paraoakan* for each breed, followed by the sample number (1, 2, or 3) and isolate number (1, 2, or 3).

A total of 30 µl crude extracts were applied to each filter paper disc (7 mm diameter). Specifically, an initial 20 µl crude extract was first applied on the filter paper disc, then dried. Finally, the 10 µl were added to the filter paper disc after it was placed on the MH agar plated with locally isolated *S. aureus.*

Antimicrobial activity test

A spread plate culture of *S. aureus* on MH agar was prepared. The prepared crude extract discs (with a total of 30 µl crude extracts impregnated) were evenly placed on the plate. The following antimicrobials discs were used: 2 µg clindamycin (Mastdiscs®, Mast Group Ltd, United Kingdom), 5 µg enrofloxacin (Oxoid Ltd, United Kingdom), and 10 µg penicillin V (Oxoid Ltd, United Kingdom), and also placed on the plates. The plates were incubated at 37 °C for 18 to 24 h. The zones of inhibition were measured using a Vernier caliper.

Statistical analysis

The antimicrobial activity was performed in triplicate and the data were presented as mean ± standard deviation (SD) of the triplicate. The data were analyzed using analysis of variance (ANOVA) followed by Tukey's highly significant difference (HSD). The level of significant difference was set at 95% confidence interval and a p-value of < 0.05.

RESULTS AND DISCUSSION

Antimicrobial activity

The study was conducted to evaluate the *in vitro* antimicrobial activity of fecal bacteria isolated from Philippine native chicken against *S. aureus*. This study measured the antimicrobial activity of the crude extracts from fecal bacteria of Philippine native chicken cultured at different time intervals (6, 12, 18, and 24 h) as shown in Table 1, and compared with the selected antimicrobials using modified disc diffusion method as illustrated in Fig. 1.

Among 6 h crude extracts, the highest zone of inhibition was observed in isolate B11, but was still

Table 1. Zone of inhibition produced by the crude extracts of fecal bacteria from Philippine native chicken against *S. aureus.*

Isolates	Zone of inhibition (mm)			
	6 h	12 h	18 h	24 h
Banaba				
B11	9.20 ± 0.92[a]	8.97 ± 1.07[abcd]	8.67 ± 1.08[a]	9.55 ± 0.79[a]
B12	8.33 ± 1.15[a]	9.15 ± 0.49[acd]	8.35 ± 1.35[a]	-
B21	8.98 ± 0.86[a]	8.83 ± 0.15[abcd]	9.22 ± 1.24[a]	-
B22	8.88 ± 1.44[a]	10.10 ± 0.61[a]	9.35 ± 0.79[a]	8.32 ± 1.10[ab]
B31	9.05 ± 0.82[a]	8.03 ± 1.00[bc]	9.13 ± 0.81[a]	9.98 ± 0.38[c]
B32	8.10 ± 0.95[a]	7.92 ± 0.80[bc]	-	-
Darag				
D11	-	-	7.13 ± 0.12[a]	7.27 ± 0.46[b]
D12	7.57 ± 0.98[a]	-	7.33 ± 0.42[a]	-
D21	7.77 ± 1.33[a]	-	-	-
D22	7.87 ± 1.50[a]	-	8.00 ± 1.73[a]	7.28 ± 0.49[b]
D23	-	-	-	-
D31	7.43 ± 0.75[a]	-	7.73 ± 1.27[a]	-
D32	7.43 ± 0.75[a]	-	-	-
D33	-	-	-	-
Joloanon				
J11	-	-	-	-
J12	-	-	7.93 ± 1.62[a]	8.60 ± 0.17[ab]
J21	-	-	-	-
J22	-	-	-	-
J31	-	-	12.60 ± 0.50[a]	7.17 ± 0.29[b]
J32	7.03 ± 0.06[a]	7.07 ± 0.12[b]	7.17 ± 0.29[a]	7.65 ± 1.13[ab]
Paraoakan				
P11	8.75 ± 1.86[a]	-	-	-
P12	9.08 ± 0.46[a]	9.85 ± 2.04[ac]	9.32 ± 1.84[a]	7.10 ± 0.17[b]
P21	9.07 ± 3.58[a]	-	11.90 ± 8.63[a]	-
P22	-	-	-	-
P31	8.93 ± 1.55[a]	7.52 ± 0.89[bd]	8.02 ± 1.06[a]	8.85 ± 2.50[ab]
P32	8.97 ± 1.71[a]	8.08 ± 1.32[bc]	-	-

* The letter in the isolate name indicates the breed of Philippine native chicken (B – *Banaba*, D – *Darag*, J – *Joloanon*, and P – *Paraoakan*) where the fecal bacteria was isolated; the first digit indicates fecal sample number, while the second digit indicates bacterial isolate number.

The data were presented as the mean ± standard deviation (SD) of the triplicate. The value followed by different superscript letters ([abcd]) in a column significantly different from each other at $p < 0.05$.

comparable to all isolates with zones of inhibition. No zones of inhibition were observed in isolates D11, D23, D33, J11, J12, J21, J22, J31, and P22. The breeds of Philippine native chicken that showed the greatest number of isolates with zones of inhibition were *Banaba* followed by *Paraoakan, and Darag* respectively. While, *Joloanon* had the least number of isolates with zone of inhibition.

Among the 12 h crude extracts, the highest zone of inhibition was observed in isolate B22, which was significantly higher than isolates B31, B32, J32, P31, and P32 followed by isolate B12, which was significantly higher than isolate J32. The isolate with the smallest zone

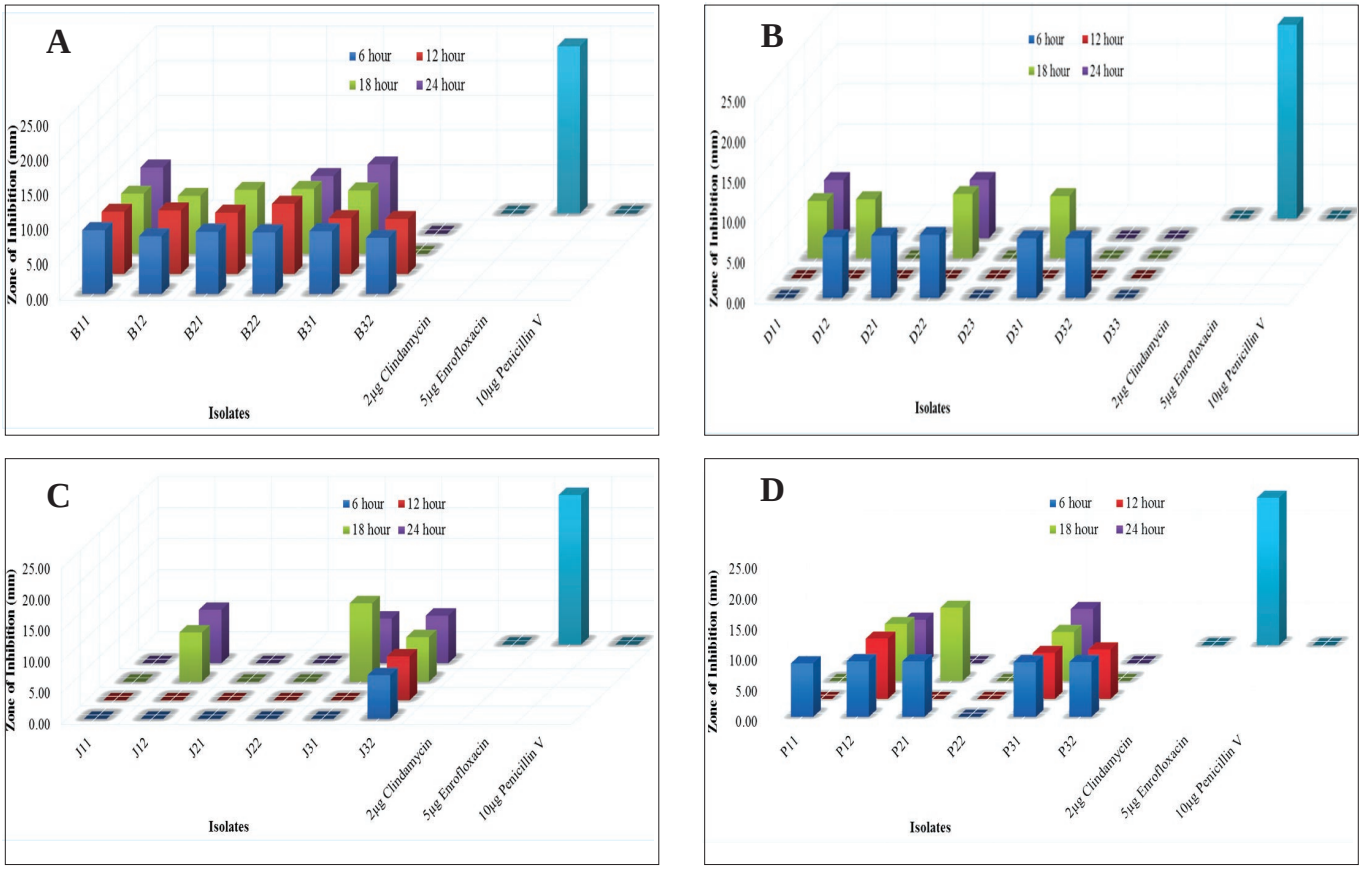

Fig. 1. Antimicrobial activity of the 6, 12, 18, and 24 h crude extracts of fecal bacteria isolated from (A) *Banaba*, (B) *Darag*, (C) *Joloanon*, and (D) *Paraoakan* breeds of Philippine native chicken and selected antimicrobials against *S. aureus*.

of inhibition produced was isolate J32, which was significantly lower than isolates B12, B22, and P12. Isolate P31 was significantly lower to isolate P12. No zone of inhibition was observed in isolates D11, D12, D21, D22, D23, D31, D32, D33, J11, J12, J21, J22, J31, P11, P21, and P22. The breed of Philippine native chicken that showed the greatest number of isolates with zones of inhibition was *Banaba* followed by *Paraoakan, and Joloanon* respectively. While, *Darag* had the least number of isolates with zones of inhibition.

Among the 18 h crude extracts, the highest zone of inhibition was observed in isolate J31, but was insignificant to all isolates. No zone of inhibition was observed in isolates B32, D21, D23, D32, D33, J11, J21, J22 P11 P22 and P32. The breeds of Philippine native chicken that showed the greatest number of isolates with zones of inhibition were *Banaba* followed by *Darag*, and *Paraoakan* respectively. While *Joloanon* had the least number of isolates with zones of inhibition.

Among the 24 h crude extracts, the highest zone of inhibition was observed in isolate B31, which was significantly higher than isolates B11, B22, D11, D22, J12, J31, J32, P12, and P12. The isolate with the smallest zone of inhibition produced was isolate P12, which was significantly lower only to isolates B11 and B31. No zone of inhibition was observed in isolates B12, B21, B32, D12, D21, D23, D31, D32, D33, J11, J21, J22, P11, P21, P22, and P32. The breed of Philippine native chicken that showed the greatest number of isolates with zones of inhibition were *Banaba* and *Joloanon* followed by *Darag*. While, *Paraoakan*, had the least number of isolates with zones of inhibition.

The antimicrobial activity of crude extract from *Banaba* (Fig. 1A) and selected antimicrobials (clindamycin, enrofloxacin, and penicillin V) against *S. aureus*. The crude extracts that produced zones of inhibition were significantly higher compared to clindamycin and penicillin V. However, compared to enrofloxacin, the crude extracts were significantly lower. Similar results were observed from crude extracts from fecal bacteria isolated from *Darag* (Fig. 1B), Joloanon (Fig. 1C), and *Paraoakan* (Fig. 1D). These indicate that crude extracts from the four breeds of Philippine native chicken have antimicrobial activity higher than

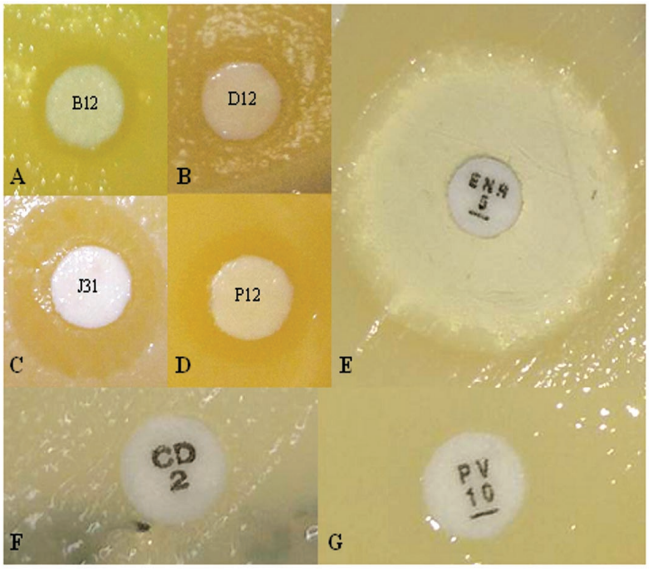

Fig. 2. Zone of inhibitions around filter paper discs containing 18h-crude extracts of fecal bacteria isolated from (A) *Banaba*, (B) *Darag*, (C) *Joloanon*, (D) *Paraoakan* breeds of Philippine native chicken, and antimicrobial discs (E) 5µg enrofloxacin, (F) 2µg clindamycin, and (G) 10µg penicillin V against *S. aureus*.

clindamycin and penicillin but less than enrofloxacin.

Zones of inhibition appear as clear areas surrounding the disc from which the substances with antimicrobial activity diffused (Hudzicki 2009). This area indicates that the bacteria is either killed or inhibited by the antimicrobial agent (Pierce-Hendry and Dennis 2010). According to Thornberry and McDougal (1983), the relative size of zone of inhibition indicates the potency or effectivity of antimicrobial agent. A previous study using the same fecal bacteria isolates but with *E. coli* as indicator bacteria showed zones of inhibition typical of an antimicrobial activity (Matias *et al*. 2020). The zones of inhibition observed in the different crude extract of fecal bacteria isolated from Philippine native chicken, as shown in Fig. 2, thus provide evidences of their antimicrobial activity.

Commensal gut microbe's composition in chicken is dependent on several factors such as environment and diet, which become more complex as the animal grows older (Lan *et al.* 2004). These microbes provide chickens additional metabolic functions such as nutrient utilization and overall well-being of the host animal. However, manipulation of these microbes either through probiotics and antimicrobials or feed and feed additives can alter animal growth, health, and resistance to foodborne pathogens (Wigley 2015). According to Cisek and Binek (2014), these microbes can interact with the host intestinal tract thus influencing the physiological and immunological status of chickens (Binek 2014, Shang *et al.* 2018). In addition, commensal gut microbes can play an essential role in preventing pathogen colonization thru competitive exclusion (Lan *et al.* 2004). Some bacteria have the ability to produce substance that have antimicrobial activity like bacteriocin, reuterin, reutericylin, organic acid (lactic acid and acetic acid), acetaldehyde, acetoine, ethanol, diacetyl, carbon dioxide and hydrogen peroxide (Sivakumar 2012, Veico- saiz *et al*. 2019, Adeniyi *et al*. 2015). These antimicrobial substances exert strong antagonistic activity against many microorganisms, including pathogenic microorganisms (Adeniyi *et al.* 2015). In a previous study, crude extracts of fecal bacteria from Philippine native pig showed antimicrobial activity against *E. coli* (Matias *et al*. 2019) providing a basis of possible innate resistance to gut pathogens.

Bacteriocin is a general term that refers to the protein produced by bacteria with antimicrobial activity (Embaby *et al*. 2014). This substance is usually produced by bacteria when they are exposed to stress conditions such as population increase and nutrient shortage (da Costa *et al*. 2019). Arfani *et al*. (2017) stated that the optimum time production of bacteriocin was determined by the incubation period required. According to Taheri *et al.* (2012) and Costa *et al*. (2018), the production of bacteriocin started as soon as the bacteria entered the exponential phase. Based on the study conducted by Taheri *et al.* (2012), the exponential phase started after two hours of incubation of bacteria. When the bacteria reach the end of exponential phase or start of stationary phase, the bacteriocin activity rose rapidly and the maximum activity was attained after 12 to 16 h of incubation (Taheri *et al.* 2012, Sivaramasamy *et al.* 2014, Danial *et al*. 2016). On the other hand, growth beyond stationary phase (more than 24 h incubation) resulted to decrease in bacteriocin production (Sharma 2014). Bacteria can also produce proteases that have an ability to degrade the bacteriocin. Almost all bacteria that produce bacteriocin can also produce potent proteases (Sivaramasamy *et al.* 2014).

S. aureus is a well-known pathogen of human and animals. Methicillin resistance in this bacterial species represents a threat to human health (Persoons *et al.* 2009). Efforts are continually being made to find new antibiotics and chemotherapeutics drugs to treat the infection as they occur; however, the ultimate goal should be the prevention of staphylococcal infection (Courter and Galton 1962). Clindamycin, enrofloxacin and penicillin were used as tests antimicrobial agents against *S. aureus*. Rayner *et al*. (2005) and Baorto (2019) stated that clindamycin is

one of the treatments of choice for serious case of staphylococcal infection. Enrofloxacin, based on the study conducted by Attili *et al.* (2015) was able to cure staphylococcal mastitis in sheep caused by *S. aureus.* In 1940, *S. aureus* was susceptible to penicillin but afterwards penicillin-resistant *S. aureus* was recognized. This is due to inappropriate use of antibiotics and extensive used as growth promotant in animal feeds (Lowy 2003). According to Rayner *et al.* (2005), most of *S. aureus* strains are now resistant to penicillin due to inappropriate use of antibiotics and extensive use as growth promotant in animal feeds. While clindamycin was used as one of the antibiotics of choice to treat serious case of staphylococcal infection. However, based on the result of the study, *S. aureus* showed resistance to clindamycin.

CONCLUSION

In conclusion, fecal bacteria isolated from different breeds of Philippine native chicken showed antimicrobial activity against *S. aureus* as demonstrated by their various sizes zone of inhibition. In particular, the fecal bacteria from *Joloanon* and *Paraoakan* breeds showed the highest antimicrobial activities, while *Banaba* showed the most numbers of isolates with antimicrobial activities. In addition, the 18 h incubation time interval for bacterial extract production should the most zones of inhibitions. Lastly, some isolates showed antimicrobial activity comparable or even greater than clindamycin and penicillin V. These observations can provide proofs that native animals, in particular, the Philippine native chickens have intrinsic ability that may protect themselves against diseases associated with bacterial pathogens such as *S. aureus*.

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