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Non-Antibiotic Immunomodulatory Combination Treatment for *Staphylococcus aureus* Infections: *In vitro* Preliminary Studies I

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Abstract: Despite advancements in prevention measures, infectious mastitis is a common disease of economic significance in cattle. Clinical mastitis affects 20 to 25% of cows each year. *Staphylococcus aureus* (*S. aureus*) causes an estimated 10-11.7% of clinical bovine infectious mastitis cases in the US. Antibiotic therapy tends to fail 50% of the time and nature and vaccine immunity usually fails, due to *S. aureus*' evasion mechanisms, leading to significant morbidity, chronic mastitis, and culling of such cows. Specific IgYs have been found to inhibit the growth of *S. aureus in vitro*, but such IgYs have been produced especially for such experiments, commercially available presentations have not been compared to the IgY produced by naturally exposed chickens. In the present study, we compare the impact of two sources of IgY. IgY was obtained commercially and purified from the eggs of chickens naturally exposed to *S. aureus* (RIR IgY). Their effect on the *in vitro* growth of *S. aureus* was compared and the commercial chicken anti-SpA IgY (IgY). This was done by mixing tryptic soy broth cultures of *S. aureus* with 5, 25, or 125 µg/mL of IgY or RIR IgY (3 tubes per concentration). The result was evaluated by the plate count method. Next, the treated and control broths were sampled and plated at 4, 8, and 12 h. IgY (commercial) showed a powerful inhibitory effect over *S. aureus*. This effect was sustained for over 12 h when compared to RIR IgY and two respectively to the control. Though the RIR IgY effect was significant; however, it subsided after 8 h. Based on our findings, IgY may have an effective component against *S. aureus* growth which is more observed in the IgY commercially obtained than the IgY purified chickens which are naturally exposed to *S. aureus* (RIR IgY).

Keywords: *Staphylococcus aureus*, IgY, Mastitis

Introduction

Staphylococcus aureus (*S. aureus*) is a ubiquitous opportunistic infectious agent responsible for numerous infections in multiple species, including animals and humans. It is responsible for about 10% of the mastitis cases in dairy cows, 60% of the mastitis cases in sheep, and for various diseases in multiple animal species, which increases its risk as a zoonotic agent (Mørk *et al*., 2007; Tenhagen *et al*., 2009). It also raises concerns about the treatments used on these animals, their impact on human health, and the risk of inadvertently passing resistant bacteria that survived antibiotic treatment from animals to humans (Lee *et al*., 2021). *S. aureus* uses numerous mechanisms to evade the host's immune response, which aren't addressed by antibiotic therapies (Medveďová and Valík, 2012). Antibiotic therapy is the main approach to the treatment of infectious mastitis. Because of this deficiency in the approach, antibiotic treatments often fail in the fight against *S. aureus* (Ruegg, 2021). This often means moving from safer, less toxic, more economical antibiotics, to less safe, more toxic, or more expensive ones. In animals, particularly food animals such as dairy, it means culling, milk waste, and meat waste when these substances persist in the meat (Medveďová and Valík, 2012; Nickerson and Ryman, 2019). These substances are not fully degraded by cooking or pasteurization and may persist in the environment (Alsager *et al*., 2018).

Some of the main mechanisms that lead to the persistence of *S. aureus* in the host despite antibiotic treatment are rapid replication at body temperature, biofilm formation, and antibody sequestration through Sbi

and SpA (Yamada *et al*., 2013; Medveďová and Valík, 2012; Smith *et al*., 2011). Anti-*S. aureus* IgYs have been known to combat the growth and biofilm formation of *S. aureus* (Bachtiar *et al*., 2015). Sbi and SpA are so important to *S. aureus* survival that, in the absence of either, the survival of *S. aureus* is greatly compromised in vivo (Smith *et al*., 2011). The use of an anti-SpA IgY could add potential to counter IgG sequestration to the roster of IgY capabilities and greatly compromise *S. aureus* growth.

One of the limitations of using new technologies for the treatment of infectious diseases is that they have to later be mass-produced and distributed to create an impact, which is costly and often leaves such developments stunted. The current commercial availability of anti-SpA IgY gives it a greater potential for earlier future application of this technology for the treatment of *S. aureus* infections. Similarly, naturally exposed chickens can also produce anti-*S. aureus* IgY which could then be purified and used. Some chicken breeds, such as Rhode Island Red chickens, are often pastured and develop antibodies. The potency of these natural antibodies against *S. aureus* has not been explored but, if significant and comparable (or better than) commercial-specific IgYs, pastured eggs may become a massive, readily available source of IgY and rather rapid expansion of the use of IgY as an adjuvant to the treatment of *S. aureus* mastitis.

In this study, we test the effect of commercially available Exalpha anti-SpA IgY (EA IgY) (Exalpha Biologicals, Inc. 2 Shaker Road, Unit B101 Shirley, MA 01464, catalog #APA-HRP) and the IgY of naturally exposed Rhode Island Red chicken (RIR) IgY on the growth of *S. aureus in vitro*.

Materials and Methods

Tryptic soy agar plates were prepared for plate counting and a sample from them was incubated to check for sterility. A hundred milliliters of tryptic soy broth were prepared. Ten milliliters were inoculated with *S. aureus* (strain) and incubated at 37°C for 24 h. Next, a serial dilution was performed, achieving a concentration of $21 \times 10^{\wedge 2}$ CFU, which was measured by plating the bacteria in tryptic soy agar plates.

Eggs were obtained from pastured Rhode Island Red hens that had been naturally exposed to *S. aureus* and IgY was purified from them using a modified version of the water dilution method described by Hodek *et al*. (2013), and mentioned by Bizanov (2017). Everything was performed as described, except the water dilution, which in our iteration used a 1:10 yolk-to-water ratio before the freezing step, resulting in the RIR IgY. Anti-SpA chicken IgY was commercially obtained from Exalpha (EA).

In a study by Guimarães *et al*. (2009) the maximum used dose of IgY against *S. aureus* was 5 µg/mL, in which a dose-response relationship was established. In this study, the 5 µg/mL dose was used as a baseline and then the dosage was increased to observe whether the dose-response relationship was maintained with these higher doses. Increasing the dosage exponentially was necessary to see the dose-response relationship more clearly. The commercial IgY was purchased with a known concentration of 1mg/ml. The RIR IgY concentration was determined with a NanoDrop assay. The same doses were used with both RIR and EA to try to compare similar IgY amounts. The *S. aureus* broth was inoculated with 5, 25, or 125 µg/mL of either RIR or EA IgY. Two separate exercises were performed: In the first experiment, samples from the broth were plated in tryptic soy agar and incubated for 24 h. This experiment was performed to observe the effect of the concentration of the IgY (alone) on the growth of *S. aureus* colonies. The number of the resulting colonies was counted. A comparison was established between the treatment groups and the control group. In the second experiment, the broth was incubated together with the bacteria and the IgYs and plated every 4 h for 14 h. This experiment was performed to determine the duration of any inhibitory effect as well as the impact of time on the performance of the IgYs. It also served to compare the performance of the EA IgY to that of RIR. The incubation time in this second exercise was determined considering a few factors:

1) Cows in dairy farms are usually milked every 8 or 12 h (depending on the farm). This makes the 8 and 12 h marks preferable, which would be, in the real situation, the most convenient times to treat and sample.
2) A previous study (Kota *et al*., 2020) found effective full inhibition of *S. aureus* growth for up to 8 h with a crude preparation of IgY against two strains of *S. aureus*. The concentrations used in this study were 50 to 200 µg/mL. To better assess whether our study contained a similar inhibition relationship in the form of reduced CFUs, a time point earlier than 8 h was established (4 h). This also allowed the measurement of growth at regular intervals.

The negative control for this study was a culture from the same broth as the treatment groups. The tryptic soy broth was made, checked for sterility, inoculated with a pure culture of *S. aureus*, and serially diluted, CFUs were determined by the plate count method, and one concentration was chosen. The broth with the chosen concentration was split into a control group and treatment groups using equal amounts of *S. aureus* broth.

The effect of the IgY concentration results was determined using the ANOVA (One-way), GraphPad Prism 7 software.

Results

Staphylococcus aureus (*S. aureus*) is an important mastitis agent in humans and animals. Infections are treated with antibiotics. With antibiotic resistance on the

rise, the need for alternatives is made patent. In this study, we explore the effect of commercially produced (EA) and naturally produced (RIR) anti-*Staphylococcus aureus* IgYs in the CFUs of *S. aureus* cultures (ATCC 25923) in tryptic soy broth.

In the first part of this preliminary study, we explore the direct immediate effect of adding 5, 25, or 125 µg/mL of EA or RIR to the *S. aureus* broth culture and plating the mixture into tryptic soy agar plates. The plates were then incubated for 24 h and the number of colonies was counted. The results of this experiment can be observed in Fig. 1.

The next part of this preliminary study (Fig. 2-4) was to observe the relationship between growth and time of exposure of *S. aureus* to anti-*S. aureus* IgY in a liquid medium. The idea was to establish a proxy to the natural environment in the udder, that is, a temperature akin to the bovine and human body temperatures (37°C), the availability of a nutritive liquid medium (represented by the broth), and duration of exposure to the therapeutic agent (8 and 12 h at a time). The times of 8 and 12 h aimed to represent what level of bacterial growth might be found in a condition in which cows, goats, and other dairy animals are milked or treated every 8 h (as in dairy farms that milk 3 times a day), or every 12 h (as in most dairy farms, milking twice a day). This was also to verify the finding by Kota *et al.* (2020) about full inhibition of *S. aureus* for 8 h in the presence of IgY and resumed growth right after. On this aspect, this study differs from Kota's in that plate counting was used as the method of bacterial load determination instead of optical density, because plate counting allows us to determine the CFUs and not biomass (which is what optical density is able to measure). The distinction is important, since samples with similar biomasses may contain very dissimilar numbers of bacteria.

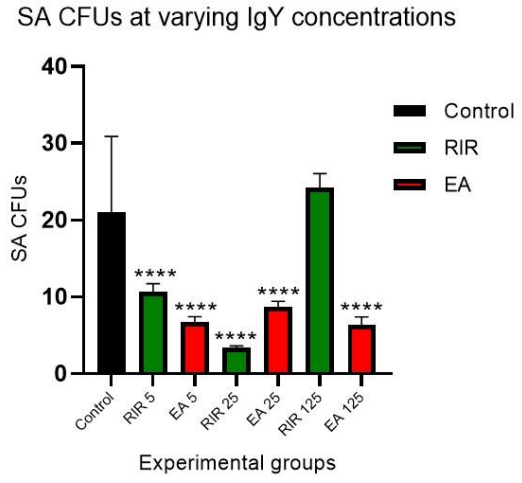

Fig. 1: *S. aureus* (SA) growth expressed as Colony Forming Units (CFUs). Significant inhibition of *S. aureus* was observed at all concentrations of EA IgY. Significant inhibition of *S. aureus* was observed in RIR IgY treatments at 5 and 25 µg/mL only

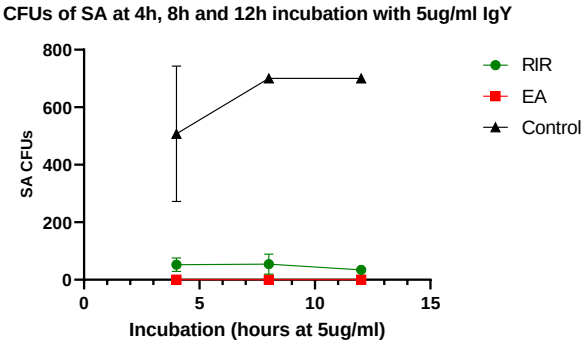

Fig. 2: *S. aureus* (SA) growth (CFUs) when cultured from broth (control), compared to *S. aureus* broth treated with RIR or EA at 37°C for 4, 8, or 12 at 5 µg/mL. The growth of *S. aureus* is similar with either RIR or EA, significantly lower than the control at all the time points. EA-treated *S. aureus* samples showed no growth on the plates

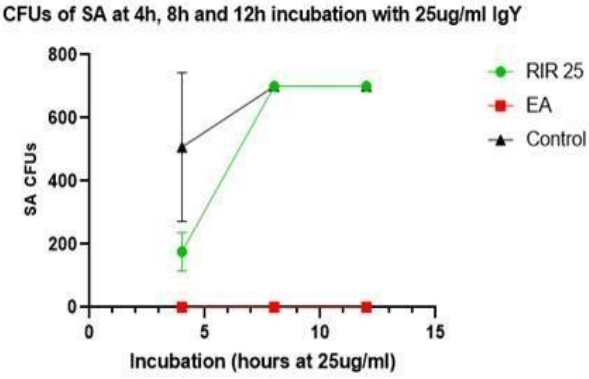

Fig. 3: *S. aureus* (SA) growth (CFUs) when cultured from broth (control), compared to *S. aureus* broth treated with RIR or EA at 37°C for 4, 8, or 12 at 25 µg/mL. The growth of *S. aureus* is similar with either RIR or EA, significantly lower than the control at all the time points. The growth of *S. aureus* with either RIR or EA is significantly inhibited when compared to the control at 4 h, but not significantly different from the control after 8 or 12 h. The growth of *S. aureus* with EA is significantly inhibited at all the time points, showing no growth at all

To determine the inhibition in relation to the duration of incubation, the above-mentioned tubes were incubated for 4, 8, and 12 h respectively at 37°C. After each time point was reached, samples were plated on tryptic soy agar and incubated for 20 h.

The results indicated both EA and RIR IgY can significantly inhibit *S. aureus* growth *in vitro*, EA being superior in its capacity. It is possible that the observed effect was bactericidal.

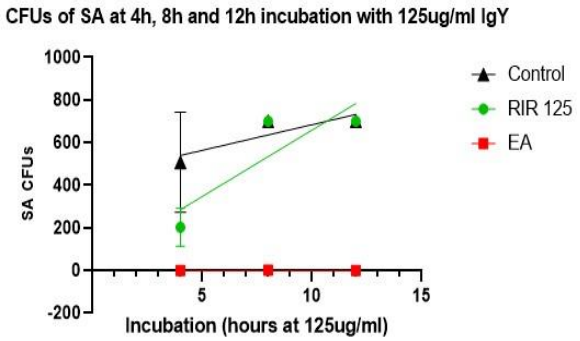

Fig. 4: *S. aureus* (SA) growth (CFUs) when cultured from broth (control), compared to *S. aureus* broth treated with RIR or EA at 37°C for 4, 8, or 12 at 125 µg/mL. The growth of *S. aureus* is similar with either RIR or EA, significantly lower than the control at all the time points. The growth of *S. aureus* with either RIR or EA is significantly inhibited when compared to the control at 4 h, but not significantly different from the control after 8 or 12 h. The growth of *S. aureus* with EA is significantly inhibited at all the time points, showing no growth at all

Discussion

Staphylococcus aureus (*S. aureus*) causes animal and human infections which can frequently be cured using antimicrobial therapy, but the large-scale use of antibiotics leads to the emergence of antibiotic-resistant strains. Especially when used to treat meat and dairy animals, antibiotic use further promotes water and soil pollution, food waste, food safety challenges, and customer distrust.

Antibiotic treatment is not cost-effective for mastitis caused by *Staphylococcus aureus* in cattle. *S. aureus* is the cause of 10-11% of mastitis cases per year in the United States. The most cost-effective solution is culling the affected animals (Schnitt and Tenhagen, 2020) when Methicillin-Resistant *Staphylococcus aureus* (MRSA) is present. Culling accounts for 40% of the cost of mastitis in the United States (Rollin *et al*. 2015). Antibiotic treatment against *S. aureus* mastitis has a 50% failure rate (Nickerson and Ryman, 2019) with antibiotic residues in the milk and wastewater that cannot be removed (Alsager *et al*., 2018). Chronic cases of mastitis lead to fibrosis of the udder, lowering milk production (Bi *et al*., 2020). *S. aureus* persists due to its numerous immune evasion mechanisms and adaptation against antimicrobials which includes inhibition of the autologous IgG and IgM antibodies through the expression of the proteins Staphylococcal protein A (SpA) and Staphylococcal binder of immunoglobulin (Sbi) (Goodyear and Silverman, 2004; Smith *et al*., 2011); it promotes B cell death by SpA (Goodyear and Silverman, 2004) and can form biofilms, all of which protects *S. aureus* from both antibiotics and the immune system of the animal (Wang *et al*., 2011). Finding an effective treatment for mastitis caused by challenging pathogens is rewarding. The development of non-antibiotic, immunity-based approaches to the treatment of infectious mastitis may be the key to the treatment and prevention of antibiotic resistance in multiple species.

Chicken polyclonal IgY has been shown to be effective against *S. aureus in vitro* and in vivo for the treatment of *S. aureus* mastitis, resulting in more effective than penicillin (Zhen *et al*., 2009), with 83.3% of success IgY and 50% success with penicillin in experimentally infected animals, yet only 66.7% of success with IgY and 33.3% success with penicillin in naturally infected animals. The difference in success in experimental infection vs natural infection may be due to differences in the strains of *S. aureus*. However, IgY quality or epitopes may also have an impact. In our study, anti-SpA IgY (EA) is used, which should show efficacy against a wide variety of strains. This IgY is compared to the IgY of chickens that were naturally exposed to *S. aureus* (RIR), which should also have some efficacy against a wide variety of wild strains.

Staphylococcal protein a (SpA) is a surface protein found in *S. aureus* bacteria with multiple effects in the host's body, such as acting as a superantigen against B cells and binding to immunoglobulins such as IgG, inhibiting their capacity to bind to the agents (Goodyear and Silverman, 2004). SpA and Sbi bind to the Fc region of IgM and IgG in the blood incapacitating them (Kim *et al*., 2016; Smith *et al*., 2011), but neither SpA nor Sbi bind to the Fc region of IgY, thus cannot inhibit it, because, while the CH_3 section of the Fc region of IgG is exposed and accessible for being the last portion of the IgG antibody, in IgY the CH_3 section is not exposed, since the last (and exposed) portion is one absent in IgG: The CH_4 region (Kota *et al*., 2020). This means that IgY could be expected to have a therapeutic advantage when compared to endogenous IgG or IgM antibodies.

In this study, we explored the effect of commercially (EA) and naturally (RIR) produced anti-*Staphylococcus aureus* IgY in the CFUs of *S. aureus* cultures (ATCC 25923) in tryptic soy broth. The study demonstrated the growth inhibitory effect of anti-*S. aureus* IgY from EA and RIR on *S. aureus* growth which is consistent with the previous study (Guimarães *et al*., 2009). The difference in the impact of EA and RIR on *S. aureus* growth *in vitro* was notable, due to the total absence of growth of *S. aureus* after incubation with the pathogen for 4, 8, and 12h, at 5, 25, or 125 µg/mL. Though there may be other possible factors leading to the difference in the outcome, however, one can speculate that the reason is that EA is a commercial product under high technological standards and listed for research purposes. Contrarily, RIR IgY is an in-house product from our laboratory, purified from naturally exposed chickens, which inadvertently may have influenced a lesser quality of the antibody compared to the EA IgY. There is a need for further studies to elucidate these findings. Also, the clinical application

such as the persistence of the effect of IgY after the termination of the application and the potential in eradicating an established infection as well as the impact of IgY in concert with other immune-modulatory agents against *S. aureus* growth needs to be studied.

The potential treatment using anti-*S. aureus* IgY as an alternate to an antibiotic will enhance sustainable agriculture, biosafety, food security, and environmental protection since the therapeutic agent is biodegradable**.** In conclusion, the study results indicate, anti-*S. aureus* IgY obtained from EA and RIR may provide an alternative to antibiotic use in the management of *S. aureus* infections in humans and animals. However, the difference in the impact of efficacy between anti-*S. aureus* IgY obtained from EA and RIR may need further studies to understand the differences in their mechanism of action if any at all. Also, the persistence of the effect of IgY after the termination of the application and the potential in eradicating an established infection needs to be further researched.

The study, however, has some limitations. First, only one strain of *S. aureus* was used, while multiple strains can be found in the wild. The SpA may not be present in all wild strains; therefore, the effect of EA may only be relevant for those strains that have SpA. The commercial IgY (EA) is anti-SpA specific, while the RIR IgY is crude IgY from naturally exposed chickens, without further purification for *S. aureus* specificity. While this may be more relevant for wild-type *S. aureus* strains, the concentration of relevant IgYs (anti-*S. aureus* specific IgY) will be less than that in the commercial preparation (EA). Because RIR is from naturally exposed chickens, it may not be representative of other naturally exposed chickens elsewhere, where other strains and environments may be prevalent. The significance of these results for the clinical treatment of *S. aureus* infections cannot be determined by this *in vitro* study. Further studies, including cell culture and in vivo studies are necessary to elucidate the importance of these results.

Conclusion

Based on our findings, IgY may have an effective component against *S. aureus* growth which is more observed in the commercially obtained IgY (EA) than that IgY purified by the water dilution method from chickens which are naturally exposed to *S. aureus* (RIR IgY).

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Author's Contributions

Jatna Isha Rivas Zarete: Study concept, experimental design, data collection, analysis, and interpretation of results drafted and reviewed the paper for publication.

Dominique Lyles: Study concept, experimental design, data collection, analysis, and interpretation of results.

Benjamin Adu-Addai: Study concept, experimental design, data collection, analysis, and interpretation of results drafted and reviewed the paper for publication. Overall supervision of the work.

Ethics

There is no commercial relationship between the authors, or Tuskegee university and exalpha biologicals. The IgY from naturally exposed Rhode Island Red chickens (RIR) mentioned in this study is not a commercial product, but the author's laboratory purification of IgY from eggs.

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