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Research Article

Effect of an IgY-Based Immunomodulatory Combo on the Internalization of *Staphylococcus aureus* by HC11 Cells: Preliminary *in vitro* Studies II

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Abstract: Infectious mastitis is a common disease of cattle of economic significance. Clinical mastitis affects between 20 to 25% of dairy cows each year. *Staphylococcus aureus* (SA) causes an estimated 10-11.7% of bovine infectious mastitis cases in the US, reducing milk production, increasing morbidity, increasing antibiotic use and milk waste, and fueling premature culling. The SA mastitis cure rate varies from 1.1% (spontaneous) to up to 50% (with antibiotics). Hence a need to establish more effective treatments for this disease. SA is internalized by mammary epithelial cells, thus evading most antibiotics and the cellular and humoral immune systems. Once internalized, SA persists subclinically and chronically. Approaches addressing internalization are needed. This study explores the effect of a combination of IgY, vitamin D3, and a novel immunomodulatory peptide, RP185, on the internalization of SA by mouse mammary epithelial cells. HC11 cells were grown in three 96 well plates in RPMI medium with growth factors. The cells were exposed to a gentamicin protection assay, exposing them to SA and DMEM medium containing IgY (at 5 or 10 µg/ml), either by itself, or in combination with vitamin D3 at 20, 50 or 80 nM, or in a further combination of said vitamin D3 concentrations with the novel peptide RP185 (1, 10 or 50 µg/ml). All combinations very significantly reduced internalization of SA by HC11 cells, with the combinations of IgY (10 µg/ml) with vitamin D3 at 50 and 80nM, IgY (10 µg/ml), and with vitamin D3 at 50nM with 1 or 10µg/ml of RP185, resulted consistently in 0 internalized CFU of S. aureus. RP185 did not seem to have contributed to a further reduction in internalization. The impact of RP185 cannot be fully evaluated without an in-vivo study. Both IgY and vitamin D3 at 50nM and 80nM had a positive impact on internalization. These results warrant an in-vivo study of this combination therapy.

Keywords: IgY, Vitamin D3, RP185, Aureus, Internalization, Mastitis, Mammary Epithelial Cells

Introduction

Staphylococcus aureus (*S. aureus*) is a species of bacteria prominent as responsible for numerous mastitis cases in dairy animals, including cows and sheep (Cobirka *et al*., 2020; Vasileiou *et al*., 2019). In dairy cows, *S. aureus* internalizes into the mammary epithelial cells, protecting the agent against the animal's immune response and antibiotics (Báez-Magaña *et al*., 2019; Wang *et al*., 2019). This infection rarely results in spontaneous cure (<2% of cases) and antibiotic treatment fails very often (>45% of cases) (Petersson-Wolfe *et al*., 2010; Nickerson *et al*., 2019). Extended periods of treatment are sometimes necessary for cure with a reduced risk of relapse, but it may not be financially justified, and absent relapse can't be guaranteed (Barkema *et al*., 2006).

As monotherapies, specific IgYs, and cholecalciferol have been found to greatly, but not completely, prevent the internalization of *S. aureus* into the epithelial cells through different and potentially mutually complementary mechanisms (Wang *et al*., 2011; Téllez-Pérez *et al*., 2012) as described in Figure 1. The proposed mechanism of action for the prevention of *S. aureus* internalization by mammary epithelial cells is the downregulation (through an unknown mechanism) of Toll-Like Receptor 2 (TLR2) in by mammary epithelial cells that is dependent on the cholecalciferol concentrations in the media (Alva-Murillo *et al*., 2014).

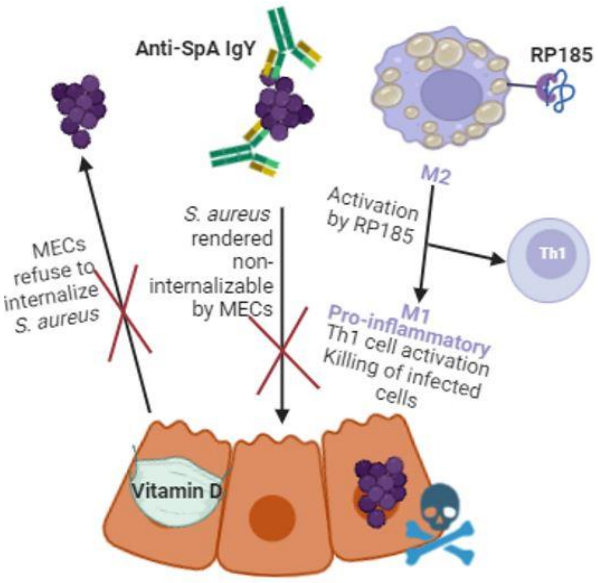

Fig. 1: Mechanisms of action of IgY, cholecalciferol (Vitamin D3), and RP185 to prevent and resolve S. aureus internalization by mammary epithelial cells (MECs). Vitamin D reduces internalization of S. aureus by mammary epithelial cells. IgY blocks internalization, opsonizes and inhibits S. aureus growth. M2 macrophages are activated as M1 by RP185 by binding of the CD206 receptor, with mediation of Th1 cells; M1 macrophages, in turn, kill S. aureus infected cells

Moreover, the peptide RP185 is a synthetic immunomodulatory peptide that has been shown to polarize M2 macrophages to M1 *in vitro* and in vivo by binding to the CD206 receptor found in M2 macrophages (Gazi and Martinez-Pomares, 2009); its effect on milk macrophages, and thus potential as an agent for the treatment of mastitis, are still unknown. However, mammary epithelial cells may behave as an unintended potential target for RP-185, since these cells also express variable levels of the same target receptor CD206 (Fornetti *et al*., 2016).

In this study, we test the effect of a combination treatment of anti-SpA IgY and different concentrations of cholecalciferol on the internalization of mouse mammary epithelial cells. Moreover, we test said combination together with a third agent, RP-185, in exploration of its possible interaction with the mammary epithelial cells in the context of treatment, due to its potential for in vivo therapeutic effect in disease.

Materials and Methods (Figure 2)

Culture of Mouse Mammary Epithelial Cells (mMEC)

HC11 mMEC cells were obtained from ATCC (ATCC® CRL-3062™). HC-11 cells were cultured according to published protocols, (Emerman JT and Pitelka DR, 1977) in RPMI 1640 medium (Thermo Fisher, 11875093) with 10% fetal calf serum, 100U penicillin/ml, 100µg streptomycin/ml (Sigma Aldrich, P4333-20ML) supplemented with 5 µg/ml insulin (Sigma Aldrich, I6634-50 MG) and 10 ng/ml epidermal growth factor (Sigma Aldrich, E5160-100 UG), and passed twice per week, before confluency. Cells were grown in a 5% CO2 atmosphere at 37°C.

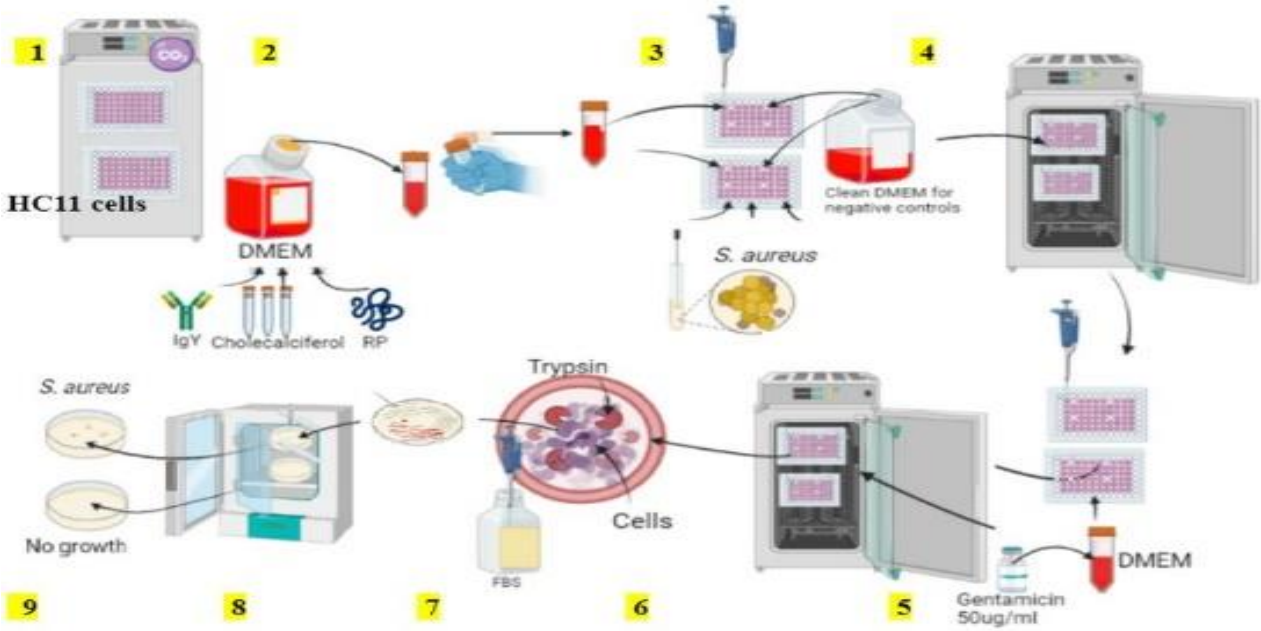

Fig. 2: Gentamicin protection assay. HC11 cells were exposed to *S. aureus* and treatment combinations of anti-SpA IgY, vitamin D3, and RP185 at varying concentrations, followed by lysis and culture to determine internalization

Insulin Preparation

Insulin (Sigma Aldrich, I6634-50MG) comes as a lyophilized powder. It was dissolved according to the manufacturer's instructions.

Epidermal Growth Factor (EGF) Preparation

EGF was diluted in the growth medium with 10% FBS to the desired concentration.

S. Aureus Culture

S. aureus subsp. aureus (ATCC 25923) strain was used in this study. This strain has been shown to be internalized into mMEC (Jiang *et al*., 2017) or bovine mammary epithelial cells (Hyvönen *et al*., 2009). Inoculum is to be prepared from bacteria that are grown at 37 °C overnight in tryptic soy broth (Walmart-Carolina, 788441). The wells were grown in triplicates.

Effect of IgY on S. Aureus 25923 Internalization into mMEC

Commercial chicken anti-SpA IgY was used (5 or 10ug/ml), and the working solutions were dissolved into 2% non-denatured ethanol (Lab Alley, 04-355-450 (EA). In all the experiments, ethanol (vehicle) was used as part of the vehicle. Ethanol has been shown not to produce damage to the cells at the concentrations used (Téllez-Pérez *et al*., 2012).

The IgY concentrations were based on a study conducted by our laboratory (Zarete *et al*., 2022), which demonstrated that anti-SpA IgY completely inhibited *S. aureus* growth for 12 hours at concentrations of 5 µg/mL, 25, and 125 with 5 µg/mL being the most cost-effective among those tested. A concentration of 10 µg/mL was included for comparison.

Effect of IgY + Cholecalciferol on S. Aureus 25923 Internalization into mMEC

Cholecalciferol (Sigma Aldrich, C9756-1G) (vitamin D3) was used together with commercial chicken anti-SpA IgY (5 or 10ug/ml), and the working solutions were dissolved into 2% non-denatured ethanol (Lab Alley, 04-355-450 (EA). In a previous study by Téllez-Pérez *et al*. (2012) concentrations from 1–200 nM were used, but only concentrations up to 50 nM resulted in reduced internalization. For this experiment, concentrations of 20, 50, and 80nM of cholecalciferol were used. In all experiments, ethanol (vehicle) was used as a control. Ethanol has been shown not to produce damage to the cells at the concentrations used (Téllez-Pérez *et al*., 2012).

The rationale for the vitamin D3 concentrations was multifaceted. First, Téllez-Pérez *et al*. (2012) identified 50 nM as the optimal concentration of cholecalciferol to minimize *S. aureus* internalization by bovine mammary epithelial cells. The concentrations tested were also based on the normal physiological plasma concentrations of 25-hydroxyvitamin D in cows (50-125 nM); hence, the 50 nM concentration was included. Second, according to Seldeen *et al*. (2017), normal blood 25-hydroxyvitamin D3 levels in mice are approximately 77.1 nM for obese mice and 81.9 nM for lean mice, making 80 nM a reasonable concentration of cholecalciferol to test.

Also, the data from Téllez-Pérez *et al*. did not include intermediate points between 10 and 50 nM or between 50 and 100 nM. Their findings indicate that at concentrations of 10 nM and lower, as well as 100 nM and higher, internalization is higher than at 50 nM. Finally, to complement the 80 nM concentration (which differs from 50 nM by 30 nM), we also tested 20 nM to help establish a dose-response relationship between cholecalciferol concentration and internalization.

Effect of IgY + Cholecalciferol + RP185 on S. Aureus 25923 Internalization into mMEC

Cholecalciferol (Sigma Aldrich, C9756-1G) (vitamin D3) was used together with commercial chicken *anti-S.* pA IgY (5 or 10 ug/ml), and the working solutions were dissolved into 2% non-denatured ethanol (Lab Alley, 04-355-450 (EA). To this combination, 1, 10 or 50 ug/ml of RP185 were added. The concentrations for RP185 were determined based on concentrations used for this peptide in previous studies about its immunomodulatory effects on cancer studies (unpublished data).

Gentamicin Protection Assay

Cell Growing Conditions

HC11 cells were grown to 50-60% confluency with RPMI prepared according to the manufacturer's instructions, at 5% CO_2 in a CO_2 chamber, at 37°C. The cells were grown in two different plates, avoiding border wells to minimize the border effect. Higher confluency was avoided to avoid the stress of the cells and apoptosis. The risk of well-to-well variability was reduced by vertexing the cells and the treatments before serving them into the wells for each plate. The treatment distribution was also equal between replicates. The cells were from the same batch and grown simultaneously under the same conditions. The degree of confluency was similar for all wells (this was verified).

S. Aureus Exposure and Treatment

Before the experiment, RPMI was removed via pipetting. A dose of 7×10^5 CFU *S. aureus* was deposited in each well. Cells in 3 wells per treatment were covered with 200 µl DMEM combined with the treatment combinations. Negative control wells received sterile DMEM alone. The three wells of the same treatment or control group were not contiguous to one another, but

treatment and control wells were grouped by replication in different parts of the plate, or in a different plate, to help maximize independence and yet the similarity of the experiments. All wells were in the same relative position between the experiments with IgY (5 µg/ml) or IgY (10 µg/ml) to minimize variations due to well position.

The risk of well-to-well variability was minimized by vortexing the cells and treatments before dispensing them into the wells on each plate. Treatment distribution was consistent across replicates. The cells were from the same batch and were grown simultaneously under identical conditions. The degree of confluency was verified to be similar for all wells.

S. Aureus Exposure and Treatment

Before the experiment, RPMI medium was removed. A dose of 7×10^5 CFU of *S. aureus* was added to each well. Cells in three wells per treatment were covered with 200 µL of DMEM combined with the respective treatment combinations. Negative control wells received sterile DMEM alone. The three wells within the same treatment or control group were not contiguous, but treatment and control wells were grouped by replication in different sections of the plate or on separate plates to maximize independence while maintaining experimental consistency.

To minimize variability due to well position, all wells occupied the same relative positions across experiments with IgY (5 µg/mL) and IgY (10 µg/mL)."

Gentamicin Treatment

The cells were incubated for 2 hours at 37°C in a CO_2 chamber with 5% CO_2. After the incubation period, the wells were rinsed with sterile Phosphate Buffered Saline (PBS) and then filled with 100 µl of DMEM containing gentamicin 50 µg/ml. The 96-well plates were incubated for 1 hour, after which the DMEM-gentamicin was removed, and the wells were washed with sterile PBS. To promote cell lysis, 50 µl of sterile double-distilled water was added to each well. Cells were detached by adding 20 µl of trypsin per well for a short period (until detachment was observed using an inverted microscope, typically within less than a minute), followed by the addition of 20 µl of pure Fetal Bovine Serum (FBS) to neutralize the trypsin. A 40 µl sample of lysed cells was taken from each well and cultured on tryptic soy agar plates at 37°C in triplicate for 14 hours.

Data Analysis

The data was analyzed by ANOVA test, using Graph Pad Prism to compare all groups. Non-significant comparisons were excluded. The results were considered significant when $p<0.05$ (). Four asterisks () indicate $p<0.0001$, while three () indicate $p<0.001$. Exploratory analyses were also performed, including comparisons among treatment groups while excluding the positive control group.

Results

This study investigated the effects of anti-SpA IgY, varying concentrations of vitamin D3, and the novel peptide RP185 on the internalization of S. aureus into mouse mammary epithelial cells using HC11 cells. The cells were cultured in RPMI medium and subjected to a gentamicin protection assay in DMEM medium containing different combinations of IgY, vitamin D3, and RP185

Treatments Containing IgY With Vitamin D3

The effect of the treatments on the internalization of *S. aureus* by HC11 cells varied slightly with IgY concentration and treatment combination (Figs. 3 and 4). All treatment combinations resulted in statistically significant ($p<0.001$) reductions in the internalization of *S. aureus*. However, the differences between treatments were not statistically significant. Notably, IgY (10 µg/ml) combined with vitamin D3 at 50 nM and 80 nM resulted in zero internalized S. aureus.

Anti-SpA chicken IgY (5 µg/ml) significantly ($p<0.001$) reduced the internalization of *S. aureus* by HC11 cells, decreasing the average internalization from 18 CFU to 2.7 CFU when used alone. When combined with 20 nM, 50 and 80 nM of vitamin D3, the average internalization further decreased to 1.3, and 0.7 CFU, respectively (Figure 3).

Chicken anti-SpA IgY (10 µg/ml) significantly reduced the internalization of *S. aureus* (Figure 4).

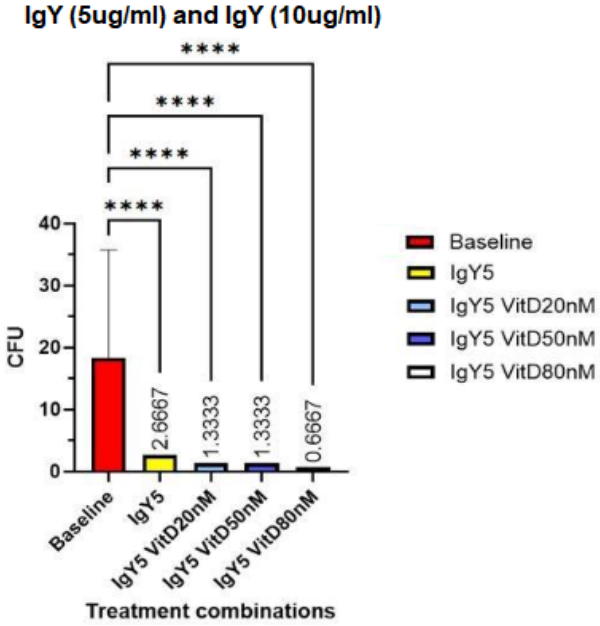

Fig. 3: Internalized CFU of *S. aureus* into HC11 cells treated with IgY (5 µg/ml) and combinations of IgY (5 µg/ml) with different concentrations of vitamin D3 (20, 50 and 80 nM)

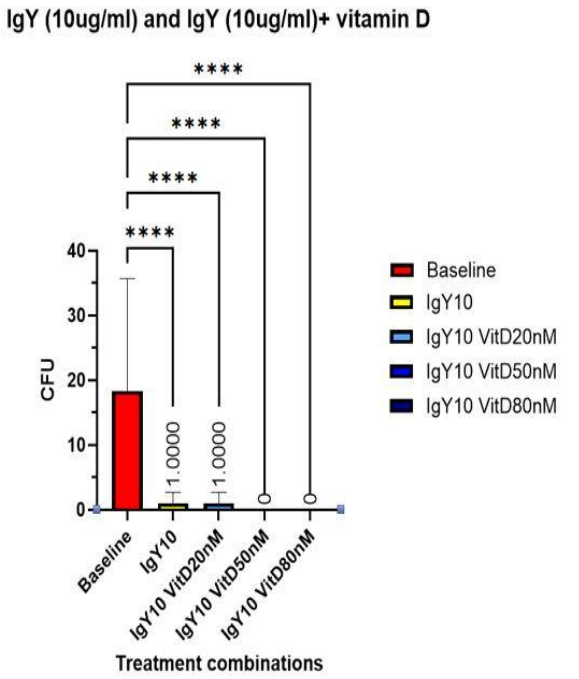

Fig. 4: Internalized CFU of *S. aureus* into HC11 cells treated with IgY (5 µg/ml) and combinations of IgY (10 µg/ml) with different concentrations of vitamin D3 (20, 50, and 80 nM). Combinations with concentrations of 50 and 80 nM resulted in 0 *S. aureus* internalization, while 20 nM seemed to not affect internalization

Untreated cells internalized an average of 18 CFU, with great variability between the wells. Cells treated with 10 µg/ml of anti-SpA IgY, or IgY with 20nM of vitamin D3, internalized an average of 1 CFU, with little variation between wells, including wells with no internalized bacteria. Vitamin D3 concentrations of 50 and 80nM resulted in consistent zero internalizations of *S. aureus* in all wells IgY Combinations with Vitamin D3 and RP185.

The effect of the treatments on the internalization of *S. aureus* by the HC11 cells varied slightly by IgY concentration and treatment combination (Figs. 5 and 6). The differences were not statistically significant between treatments. However, IgY (10 µg/ml) with vitamin D3 at 50 nM with 1 or 10 µg/ml of RP185 resulted in zero internalized CFU. Also, IgY (10 µg/ml) with 80 nM of vitamin D3 and 50 µg/ml of RP185 resulted in zero internalized *S. aureus*.

As seen in Figure 5, cells treated with 10µg/ml of anti-SpA IgY, 20 nM of vitamin D3, and 1, 10 and 50µg/ml of RP185 internalized an average of 2, 1, and 0.7 CFU, correspondingly. When vitamin D3 concentrations were increased to 50nM, and RP185, concentrations of 1, 10 and 50 µg/ml, internalization of *S. aureus* by HC11 cells was an average of 0.3, 2.3, and 1 CFU, correspondingly. When vitamin D3 concentrations were further elevated to 80nM, and RP185 had concentrations of 1, 10 and 50µg/ml, internalizations were an average of 4, 0.3, and 0.7 CFU, correspondingly.

Figure 6 reflects combination treatments involving IgY at 10µg/ml in combination with different concentrations of vitamin D3 and RP185. Cells treated with 10µg/ml of anti-SpA IgY, 20nM of vitamin D3, and 1µg/ml, 10 and 50µg/ml of RP185, internalized an average of 3, 1.7 and 1 CFU, correspondingly. When vitamin D3 concentrations were increased to 50nM, and RP185 had concentrations of 1µg/ml, 10µg/ml and 50µg/ml, internalization of *S. aureus* by HC11 cells was an average of 0, 0 and 0.7 CFU. When vitamin D3 concentrations were further elevated to 80nM, and RP185 had concentrations of 1µg/ml, 10µg/ml and 50µg/ml, internalizations were an average of 0.3, 2, and 0 CFU, correspondingly.

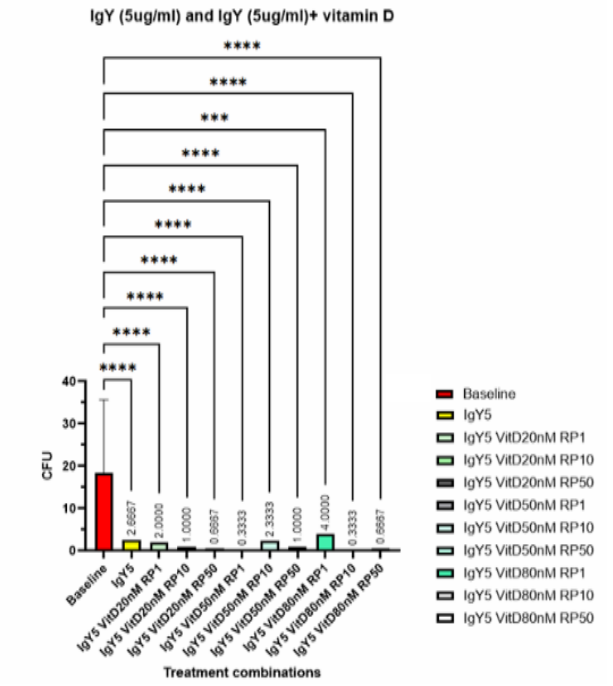

Fig. 5: Internalized CFU of *S. aureus* into HC11 cells treated with IgY (5µg/ml) and combinations of IgY (10µg/ml) with different concentrations of vitamin D3 (20, 50 and 80nM) and RP185 (1, 10, and 50µg/ml). The numbers represent the average number of CFUs found in each well with cells after the Gentamycin Protection Assay. Untreated cells (Baseline) show significantly higher average counts of internalized live *S. aureus* when compared to any of the treated wells. However, none of the treatments yielded an average of 0 internalization, which would suggest potential for relapse in a therapeutic setting

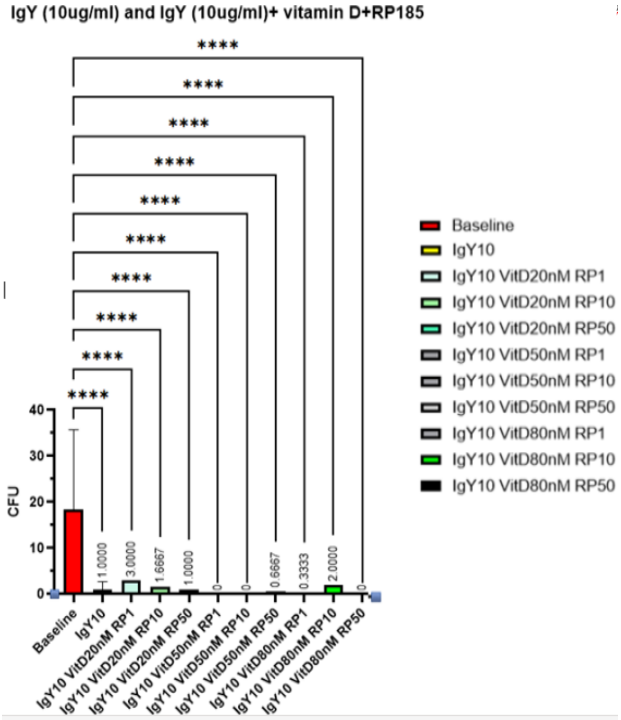

Fig. 6: Internalized CFU of *S. aureus* into HC11 cells treated with IgY (10 µg/ml) and combinations of IgY (10 µg/ml) with different concentrations of vitamin D3 (20, 50 and 80 nM) and RP185 (1, 10, and 50 µg/ml). The numbers represent the average number of CFUs found in each well with cells after the Gentamycin Protection Assay. Untreated cells (Baseline) show significantly higher average counts of internalized live S. aureus when compared to any of the treated wells. Some combinations yielded 0 CFU, showing potential as S. aureus eradication treatments for this cell type

Discussion

In this study, IgY (10 µg/ml) with vitamin D3 at 50 and 80nM, and IgY (10 µg/ml) with vitamin D3 at 50 nM with 1 or 10 µg/ml of RP185, or with IgY (10 µg/ml) with 80nM of vitamin D3 and 50 µg/ml of RP185, respectively, resulted in complete inhibition of internalization of *S. aureus*. All combinations resulting in no internalization notably included 10 µg/ml of anti-SpA IgY, however in comparisons between any of the treatment groups, there were no statistical significance in internalization numbers. This may be due to low statistical power. Even though there was no such statistical difference, such difference can be clinically significant due to the possibility of achieving zero internalization.

Clinically, this approach could minimize the chance of relapse due to subclinical infection in a treated animal that responds similarly *in vivo*, that is, if the healthy cells do not internalize any *S. aureus* after the onset of treatment. *S. aureus* mastitis is infamous for producing chronic mastitis, false negative results due to absence of culturable bacteria in the milk (despite their presence inside the mammary epithelial cells, which require scraping for detection) (Almeida *et al*., 1996), and relapses (Wente *et al*., 2020). Anti-SpA IgY is highly effective in preventing the internalization of *S. aureus* both at 5 ug/ml and at 10ug/ml. Using concentrations of 10 µg/ml of anti-SpA IgY combined with vitamin D3 (50 or 80 nM) has potential as a treatment that reduces internalization of *S. aureus* into mammary epithelial cells. However, the addition of RP185 did not seem to contribute to a reduction in internalization.

Vitamin D3 (cholecalciferol) has been suggested to assist wound healing in the context of antibiotic-resistant infections (Shekhar *et al*., 2019). This may be due to its capacity to help reduce the internalization of bacteria (Téllez-Pérez *et al.*, 2012), and its other immunomodulatory properties. Because its action targets the tissue rather than the pathogen it has potential in the treatment of infections caused by a variety of agents. However, the mechanism of action remains unknown. The anti-phagocytic potential of cholecalciferol on mammary epithelial cells was initially hypothesized to rely by Téllez-Pérez *et al*. (2012) on the cells' ability to produce the enzymes necessary for converting cholecalciferol into 25-hydroxyvitamin D. This conversion, however, did not occur in the study by Téllez-Pérez *et al*., which nonetheless reported a reduction in *S. aureus* internalization by bovine mammary epithelial cells. Our findings support this effect but do not elucidate the underlying mechanism.

Alva-Murillo *et al*. (2014) proposed that the effect may be mediated by the downregulation of TLR2 on the cell membrane of mammary epithelial cells with varying cholecalciferol concentrations.

The receptor CD206 is usually expressed only in mammary epithelial cells that are involuting after lactation, a condition absents in this study; however, the expression of CD206 under conditions of mastitis or *S. aureus* infection is not known, which is why RP185 was still evaluated in this condition. The *in vitro* environment does not fully represent the real-world working conditions for the full expression of RP185's ordinary mechanism of action. No significant effect of RP185 was found in non-professional phagocytosis. Despite the small sample size, the results are consistent with findings from other studies in which vitamin D3 or IgY are used to reduce internalization of *S. aureus* by mammary epithelial cells (in said cases, bovine) (Wang *et al*., 2011; Téllez-Pérez *et al.*, 2012). Therefore, we believe that this study should have good replicability, and the concept may be generalizable to epithelial cells of other mammalian species for which cows and mice are models. While in vivo results may not be identical, the promising outcomes from a previous study using anti-*S. aureus* IgY for the

treatment of bovine mastitis in vivo (Zhen *et al*., 2009) gives us reasons to believe that there is a relationship between the *in vitro* protective effects of IgY and the *in vivo* effects. The *in vivo* effects of cholecalciferol in this context are yet to be determined.

There are significant differences between *vivo* and *in vitro* environments that should be considered for future in vivo studies, especially in large animals. First, the concentration of IgY remains constant in this study, while in a lactating animal treated intramammary, the sample would gradually be diluted during lactation inside the mammary gland, resulting in a gradient of concentrations of the treatments. This reality is difficult to replicate *in vitro*, and thus it would be valuable to replicate this study, using the most effective combinations as a reference, but measuring the effect at different dilutions based on the milk production of the target animal. In in-vivo, however, the small dilution factor due to the milk production of small animals such as mice helps test the efficacy of these treatments with minimal disturbances, making mice a useful intermediate step between large animals in vivo experiments and *in vitro* studies.

Second, the administration of the treatment involves labor costs. Third, temperature control, hormonal changes, immune status, nutritional factors, and genetic variability in lactating animals are not fully represented and could affect the results.

Interestingly, even though Zhen *et al*. (2009) successfully treated cows with *anti-S. aureus* IgY alone, achieving greater success than penicillin, this therapy has not become widespread. Several reasons may explain the lack of adoption:

i. The in-vivo experiments by Zhen *et al*. (2009) were not much more successful (60% recovery) in natural infections than the already available standard of care (Taponen *et al*., 2003)
ii. Anti-*S. aureus* IgY, as such, is not commercially available
iii. Large dairy farmers, which are increasingly the main producers of milk, often prefer culling mastitis cows to maintain low somatic cell counts in the tank, thus obtaining premium prices for milk. For the adoption of IgY technology for mastitis, there has to be a benefit for smaller farmers which have more to lose by culling animals
iv. This work helps address some of those realities by using an already commercially available and mass-produced anti-SpA IgY, reducing the availability limitation and the lack of potential commercial interest from IgY-producing companies. Moreover, it aims to improve outcomes in an attempt to surpass the results of the current standard of care for *S. aureus* mastitis, while incorporating other substances (in this case, vitamin D3) that can be produced organically. This is important because small organic dairy farmers currently lack efficacious treatment for S. aureus mastitis that can be used while maintaining their organic certification. IgY can be obtained at a low cost from chicken eggs, is safe for most people if accidentally consumed, and can be used intramammary

Limitations

This exploratory study has its limitations:

i. The full therapeutic effect of RP185 could not be manifested, since RP185 was designed to act on M2 macrophages. Its inclusion in this paper was purely an exploration of its effects in other contexts
ii. The effect of RP185 on mammary epithelial cells was not studied in isolation from other agents
iii. Only three wells were tested for each combination treatment, which limits the statistical power of this study. Statistical significance was observed due to the large difference in internalization between treated and non-treated wells, but the number of wells used was insufficient to measure statistically significant differences in internalization between treatments

This study was performed in mouse mammary epithelial cells using treatments that had been effective in preventing internalization in bovine mammary epithelial cells. While this demonstrates the potential generalizability of the findings to other species, it may raise skepticism about its efficacy in bovine mammary epithelial cells. However, we considered this step necessary to perform in vivo experiments in mice, which are valid models for both bovine and human mastitis, thus expanding the potential market for the treatment.

Conclusion

Specific anti-SpA IgY significantly helps prevent the internalization of *S. aureus* into mouse mammary epithelial cells. The combination of IgY (10 μg/ml) with Vitamin D3 (50 or 80 nM) may further decrease this internalization, potentially to zero. Although the difference is currently too small to reach statistical significance with the applied methods, it could have great clinical relevance if replicated in vivo, as it may lead to clinical recovery without the risk of subclinical infectious mastitis due to *S. aureus*. This is important, given the substantial losses caused by subclinical mastitis in the dairy industry.

The combination of IgY and vitamin D3 could also be explored in the context of other cell lines that are susceptible to *S. aureus* or other bacterial infections, particularly those that rely on internalization by non-professional phagocytes for intracellular survival or parasitism. This includes its use in preventative care to help prevent ulcers and wound infections by opportunistic pathogens.

The treatments explored in this study must be tested in vivo to determine the efficacy of these approaches in practice. The impact of combining the treatment with RP185 should be investigated in vivo to observe the full effect of this combination, as RP185 targets macrophages. RP185, in this context, may have a small impact on internalization, which can also be explored with other agents. The effect of RP185 alone should also be examined in cells that express CD206 but are not the intended target, to further investigate its effects on potential accidental or incidental targets, thus expanding knowledge about its therapeutic effects and potential side effects.

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Author's Contributions

Jatna Isha Rivas Zarete: Contributed to the study concept and experimental design; performed data curation, formal analysis, and investigation; secured funding; developed methodology; prepared visualizations; drafted the original manuscript; and participated in critical review and editing for publication.

Benjamin Adu-Addai: Contributed to the study concept; performed data curation, formal analysis, and investigation; secured funding; developed methodology; managed project administration; provided validation and supervision; supplied essential resources; drafted the original manuscript; and participated in critical review and editing for publication.

Ethics

There is no commercial relationship between the authors, or Tuskegee University and Exalpha Biologicals.

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