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Article

The Inhibitory Effect of Probiotics on the Growth and Biofilm Formation of *Salmonella* Sp.

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SUMMARY

***Salmonella* is a genus of bacteria responsible for over 90 million cases of intestinal illnesses annually. Like many bacteria, *Salmonella* can create a biofilm matrix which confers stronger resistance against antibiotics. However, there has been relatively little research on the inhibition of *Salmonella* biofilm formation, which is a crucial factor in its widespread growth. This study was conducted to determine the anti-bacterial and anti-biofilm effects of commonly used farm probiotics on *Salmonella*. *Salmonella* species were inoculated in 0.1 A, 0.01 A, 0.001 A concentrations of filtered probiotic supernatants. Bacterial and biofilm growths were measured quantitatively with a UV spectrophotometer using absorbance values and compared qualitatively with crystal violet dye staining. All probiotic filtrate showed some levels of inhibition on *Salmonella* bacterial growth. For most cases, the strongest inhibitory effect was exhibited at the 0.1 A (A=1x10^9 bacteria/ml) concentration. For biofilm inhibition, both autoclaved and non-autoclaved filtrates showed the strongest inhibitory effects at a specific concentration of 0.001 A. In this case, higher concentrations of probiotic filtrate did not appear to correlate with stronger anti-biofilm properties. Differences in the anti-bacterial and anti-biofilm trends of probiotic filtrate suggest that they most likely inhibit growth and biofilm formation through different mechanisms. These findings also bring insight into the species-specific effects of the different probiotic species tested, and provide new information in determining optimal concentrations of probiotics that should be given to farm animals for probiotics to work most effectively against *Salmonella*.**

INTRODUCTION

In the food production industry, the safety and sanitation of food products are of paramount importance. As a result, the growth of bacteria such as *Salmonella* on farm animals and animal products is a cause for great concern. The contamination of farm animals not only represents a threat to consumers, but it also causes heavy economic damage to the pig and poultry industries. *Salmonella* infections are estimated to cause billions of dollars in damages each year from medical costs and productivity losses [1]. Despite their notoriety, efforts to contain *Salmonella* infections have not been able to prevent their growing prevalence in both developed and developing countries.

One of the reasons that *Salmonella* is able to resist human intervention so effectively is because it often exists in biofilms, which are highly resistant to chemical and mechanical stresses, including disinfectants or antibiotics [2]. Biofilms are groups of microorganisms that adhere to each other and to a substratum in a tightly bound extracellular polymeric substance matrix. This polymeric substance is secreted by the microorganisms themselves and provides a thick barrier that restricts the penetration of antimicrobials or exposure to other destructive environmental conditions [3]. In fact, studies have shown that low doses of antibiotics can even cause the reverse of their intended effect and increase the biofilm production of certain microorganisms [4]. As a result, biofilms are able to survive in a large variety of circumstances, making them a common phenomenon.

In sum, the simple restriction of bacterial growth is not sufficient. The deterrence of biofilm growth must also be taken into consideration to most effectively prevent infection. However, while other scientists have conducted much research on both biofilm disruption and *Salmonella*, there has been little research on the disruption of *Salmonella* biofilm formation.

The symptoms of *Salmonella*-related food poisoning, such as diarrhea, are often treated through the use of probiotics, which are a class of live bacteria that are ingested for health benefits [5]. These probiotics are commonly used to balance the gut microbiota with beneficial bacteria. More importantly, while physical or chemical stresses have been found to be less effective against biofilm formation, the use of probiotics has been found to be successful in inhibiting biofilms. In a recent study, five *Lactobacilli* probiotic strains were found to inhibit the growth and biofilm formation of *Streptococcus mutans* [6]. In another study, probiotics were found to have anti-bacterial and anti-biofilm activity against *Streptococcus salivarius* [7]. Therefore, the use of probiotics seems to be a promising method of disrupting biofilm growth.

This study focused on two of the most problematic subspecies of *Salmonella*: *Salmonella enterica* and *Salmonella gallinarum*. *Salmonella enterica* has a wide host range, including humans, and is most commonly associated with “food-poisoning” in humans [1]. *Salmonella gallinarum* is

a major cause of typhoid diseases, causing mortality rates of up to 90% in birds [8]. While this subspecies does not infect humans, *Salmonella* infections of chickens have caused immense financial damage to the poultry industry.

We compared the growth of the two species of *Salmonella* in the absence and presence of the filtrates of probiotics. We chose five probiotic species that are commonly used in farm animals: *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum, Streptococcus thermophilus,* and *Enterococcus faecium.* The aim of this study was to evaluate the biofilm formation of *Salmonella,* observe the anti-bacterial and anti-biofilm effects of commonly used farm probiotics, and investigate the mechanisms behind the probiotic inhibition of biofilm formation and bacterial growth. We hypothesized that higher concentrations of probiotic filtrate would have stronger anti-bacterial and anti-biofilm effects because larger populations of probiotic bacteria would secrete larger amounts of substances that could interfere with the growth and biofilm formation mechanisms of *Salmonella* populations. In addition, as different species of probiotics may affect *Salmonella* differently, five species were investigated to discover which species, or potentially combination of species, could be most effective against *Salmonella* growth and biofilm formation.

Indeed, we found that anti-bacterial effects became more prominent at higher concentrations of filtrate. However, anti-biofilm effects only appeared strongly at a specific 0.001 A concentration of filtrate, indicating that anti-bacterial and anti-biofilm effects may be regulated by different components of probiotic filtrate.

RESULTS

Effect of varying concentrations of probiotic filtrate on antibacterial properties

We used various concentrations of probiotic filtrate to determine whether the concentration of filtrate had an effect on antibacterial properties. The experimental results for *L. casei*, *E. faecium*, and *L. plantarum* showed similar negative effects on the growth patterns of *S. enterica* and *S. gallinarum*, strongly indicating that the concentration of these probiotics has a negative correlation with bacterial growth (**Figure 1**). Most of the probiotic filtrates showed the strongest inhibitory effects at higher concentrations around 0.1 A. With the exception of *L. plantarum*, all probiotic species showed strong inhibitory effects for at least one species of *Salmonella* at the 0.1 A concentration (**Figure 2**). For example, *S. thermophilus* had a strong inhibitory effect on *S. enterica,* while *L. casei* had a strong inhibitory effect on *S. gallinarum* growth. In addition*, L. acidophilus* and *E. faecium* had consistent inhibitory effects for both *Salmonella* species. *L. acidophilus* had the strongest inhibitory effect for both species, with over a 55% reduction in growth rate compared to the control for both *Salmonella* species. These results suggested that the inhibitory effects on bacterial growth had a direct relationship with the concentrations of filtrate in the medium.

Effect of varying concentrations of probiotic filtrate on anti-biofilm properties

Since increasing concentrations of probiotic filtrate had stronger antibacterial properties, We conducted a follow-up experiment to determine whether concentration had a similar effect on anti-biofilm properties. The well plates from the previous experiment with 0.1 A, 0.01 A, and 0.001 A concentration of probiotic filtrate were rinsed and dyed to measure biofilm formation.

In every experimental case, some concentrations of filtrate inhibited biofilm formation while other concentrations facilitated formation in a seemingly random manner (**Figure 3**). Therefore, unlike the case for simple bacterial growth, the majority of experimental cases for biofilm formation had little direct correlation with the filtrate concentration,

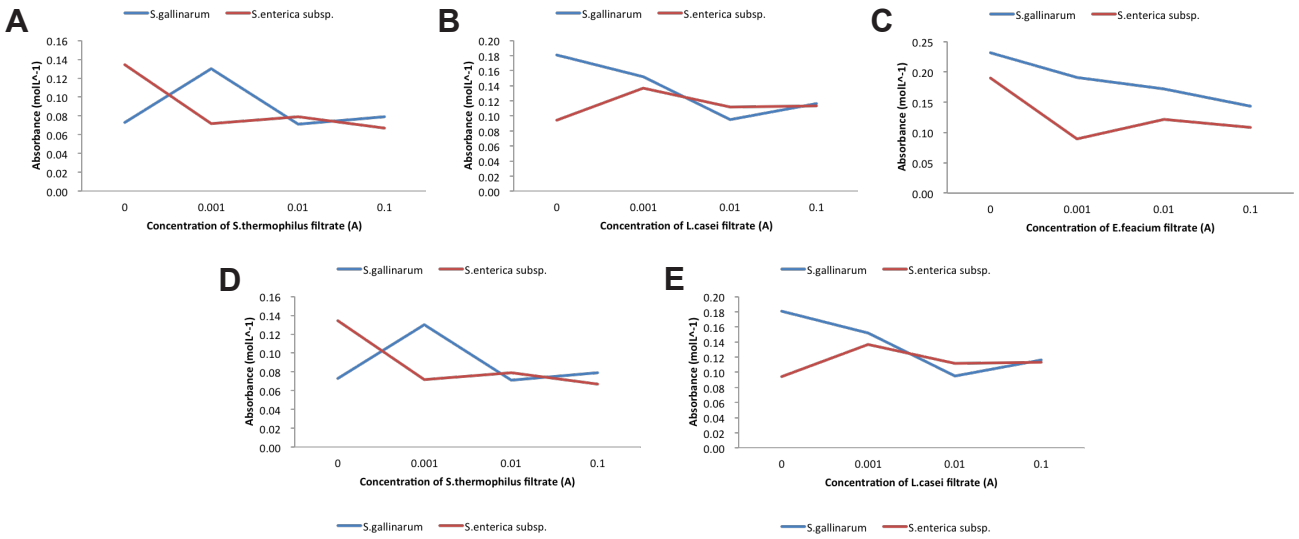

Figure 1. ***Salmonella*** **growth in 0.1 A, 0.01 A, and 0.001 A concentrations of the probiotic filtrates.**

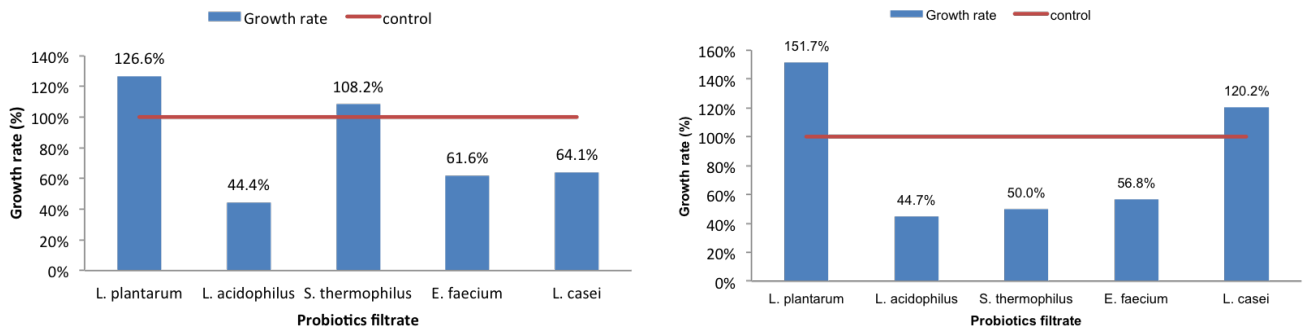

Figure 2. ***Salmonella*** **growth as a percentage of the control in 0.1 A filtrates.**

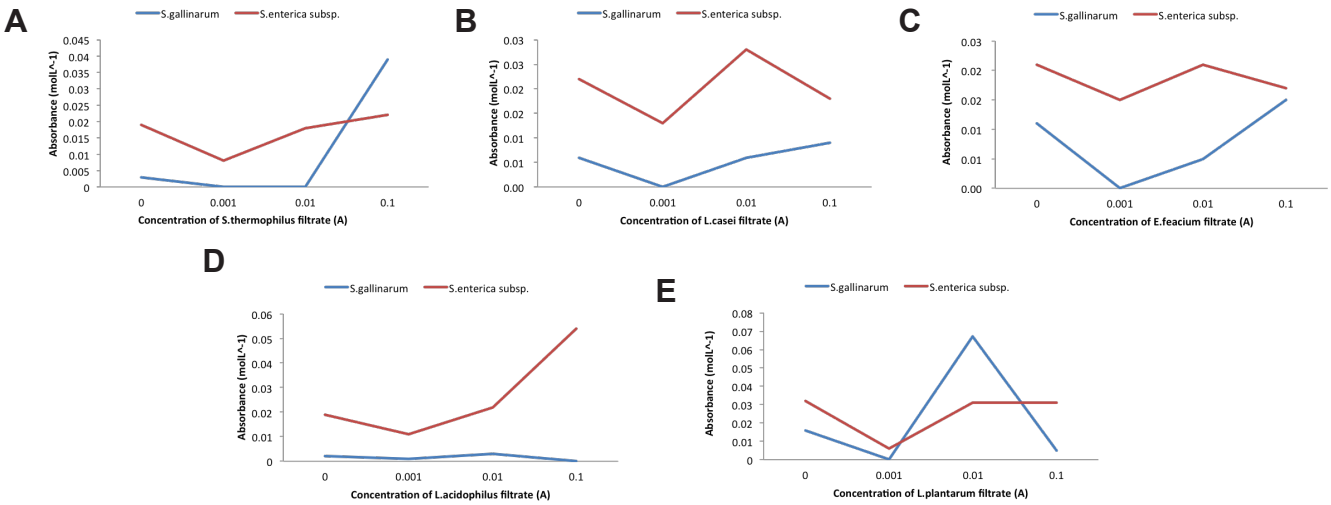

Figure 3. ***Salmonella*** **biofilm formation in 0.1 A, 0.01 A, 0.001 A concentrations of the filtrates.**

suggesting that the component of probiotic filtrate affecting antibacterial properties was not the same as the substance affecting anti-biofilm properties.

However, while a constant trend for each case did not appear, a concentration of 0.001 A of probiotic filtrate inhibited biofilm growth in every experimental case. In the case of *S. gallinarum*, *L. plantarum, S. thermophilus, E. faecium*, and *L. casei* groups completely inhibited biofilm formation (**Figure 4**). For *S. enterica*, the *L. plantarum* medium caused the strongest inhibitory effect, with *S. enterica* forming only 18.8% biofilm in comparison with the control (**Figure 4**). The *E. faecium* medium had the weakest inhibitory effect, with 71.4% biofilm formation in comparison to the control. However, in every case, the 0.001 A concentration of probiotic filtrate displayed strong inhibitory effects, indicating that the substance responsible for anti-biofilm properties may work effectively at specific concentrations around 0.001 A.

Effect of autoclaved probiotic filtrate on anti-biofilm properties

To examine the mechanisms of the anti-biofilm properties of probiotic filtrate, we repeated the experiment with autoclaved probiotic filtrate instead of normal probiotic filtrate. The autoclave process denatures any protein molecules and effectively kills probiotic bacteria within the filtrate. By doing so, the experiment determined whether the component of probiotic filtrate causing the anti-biofilm properties contained protein-based molecules. We prepared each of the autoclaved probiotic extracts in 0.1 A, 0.01 A, and 0.001 A concentrations and we determined the biofilm formation rates of *Salmonella* as in previous experiments.

We quantitatively compared the amount of *Salmonella* biofilm formation using absorbance values. The *Lactobacillus* species *L. casei*, *L. acidophilus*, and *L. plantarum* had high similarity of *Salmonella* biofilm formation rates between the autoclaved probiotic filtrate medium and the regular probiotic filtrate medium (**Figure 5**). On the other hand, the autoclaved and non-autoclaved cases for *S. thermophilus* and *E. faecium* appeared to show less similarity in *Salmonella* growth rates compared to the *Lactobacilli* groups, with differences in growth trends appearing within certain ranges of concentrations (**Figure 6**). However, the high similarity of the results for the *Lactobacillus* probiotic subspecies suggests that the autoclave process does not affect the substance affecting anti-biofilm properties in probiotic filtrates, indicating that these properties may not arise from protein-based components of probiotic filtrate.

Effect of autoclaved probiotic filtrate on chicken skin

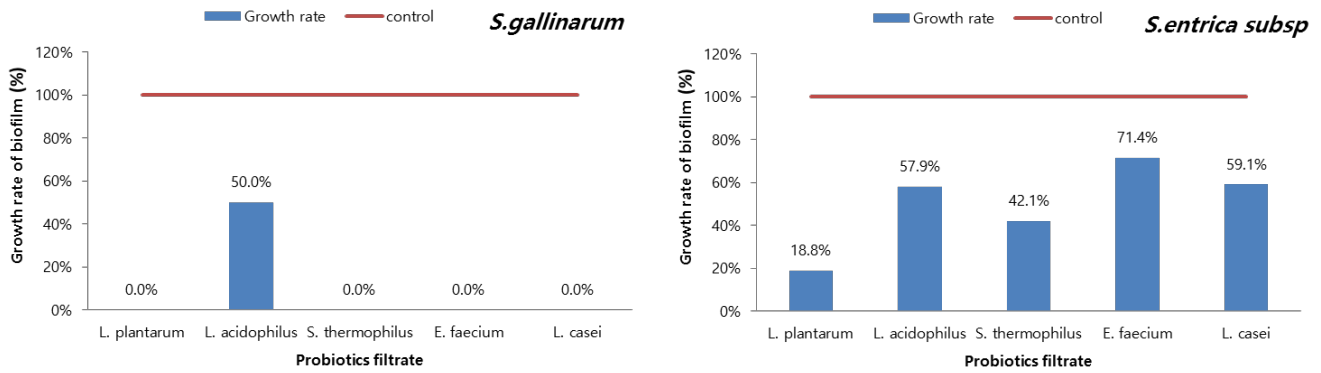

Figure 4. ***Salmonella*** **biofilm formation as a percentage of the control in 0.001 A filtrates.**

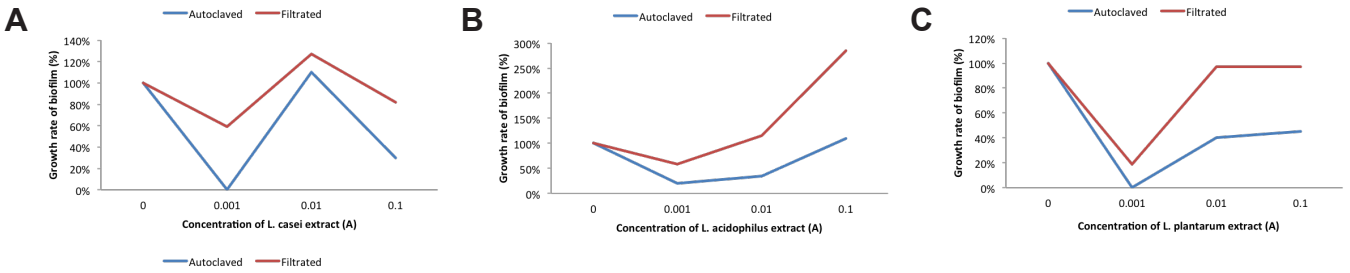

Figure 5. ***Salmonella*** **biofilm formation in autoclaved (blue) v. non-autoclaved (red) probiotic filtrates of A)** ***L. casei*** **B)** ***L. acidophilus*** **C)** ***L. plantarum*****.**

We used autoclaved probiotic filtrate on chicken skin to determine the effectiveness of real-life application. Based on previous experimentation, a concentration of 0.001 A autoclaved filtrate appeared to have the most significant anti-biofilm effect, so we inoculated *Salmonella* with equally shaped pieces of chicken skin in a medium of 0.001 A concentration of autoclaved probiotic extract.

The final masses of the chicken skin increased after the initial weighing because of dye absorption (**Figure 7**). However, assuming that each skin absorbed similar amounts of dye, the relative amount of mass growth for each experimental group can be compared to determine the extent of consumption by the *Salmonella* bacteria. For example, a smaller increase in chicken skin mass would show a higher level of *Salmonella* bacterial growth, because it would indicate a higher level of consumption by the *Salmonella* bacteria. The results show that the control group with no probiotic extract had the least relative growth in mass from the control, indicating that it experienced the most consumption by *Salmonella* (**Figure 7**). This also indicates that the control group experienced the most *Salmonella* growth. The *L. acidophilus* group had the highest relative mass growth, suggesting that it was the least consumed by *Salmonella* and therefore had the lowest *Salmonella* growth, while the *L. plantarum* group showed the least mass growth relative to the other experimental groups, showing that it was the most consumed and had the most *Salmonella* growth. These results are consistent with the first experiment, which suggested that *L. plantarum* had the least inhibitory effect on *Salmonella* bacterial growth and *L. acidophilus* had the strongest inhibitory effect.

The texture of the chicken skin pieces also supported the results. Differences in chicken skin texture reflect the extent of *Salmonella* consumption. For example, chicken skin from the control group and the *L. Plantarum* group became soft and gelatinous after *Salmonella* inoculation (**Figure 8**). On the other hand, chicken skin from the experimental groups in the *S. thermophilus, L. casei, E. faecium*, and *L. acidophilus* filtrates had tough and firm textures. The spongy texture of the skin from the control group and *L. Plantarum* extract group indicate higher levels of damage by the *Salmonella*. In addition, out of the experimental groups, qualitative observations of crystal violet dye staining patterns showed nearly no biofilm formation on the chicken skin pieces from the *L. casei* and *E. faecium* extracts, suggesting that their stronger anti-biofilm properties allowed for less damage from *Salmonella* consumption.

DISCUSSION

Consistent with previous experiments involving probiotics and bacterial species, this study showed that probiotics exhibit both anti-biofilm and anti-bacterial properties, specifically to *Salmonella*. We conducted experimentation to determine if probiotic filtrate had any sort of antibacterial or anti-biofilm properties and whether the concentration of probiotic filtrate would affect the strength of these properties. *L. casei, L. plantarum, L. acidophilus, S. thermophilus,* and *E. faecium* were experimented with, as they are commonly

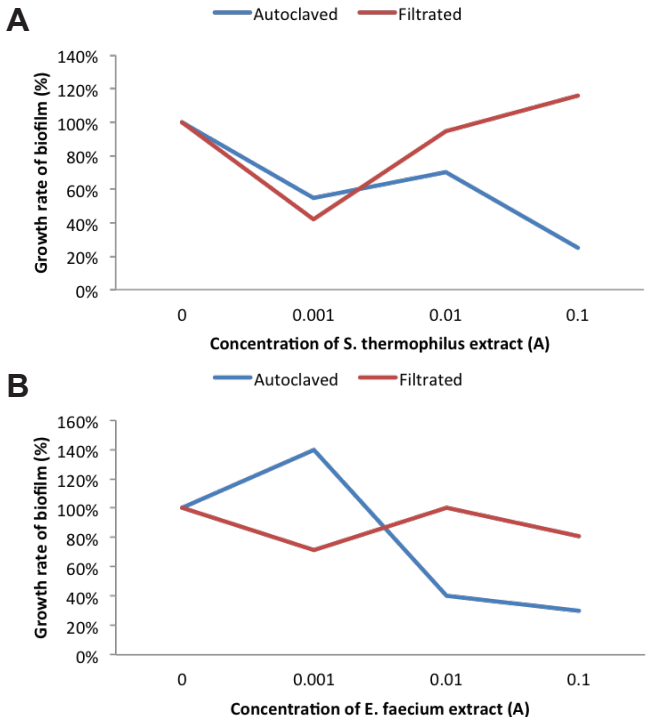

Figure 6. *Salmonella* biofilm formation in autoclaved (blue) v. Non-autoclaved (red) probiotic filtrates of A) *S. thermophilus* B) *E. faecium*.

used species for probiotic medicine on farm animals. For *Salmonella* bacterial growth, the majority of data trended towards the appearance of stronger inhibitory effects at higher concentrations around 0.1 A probiotic filtrate, indicating that the filtrate concentration had a direct relationship with the strength of antibacterial properties. On the other hand, the results for biofilm formation showed no apparent trend between the concentration of filtrate and an inhibitory effect. However, the 0.001 A concentration of probiotic filtrates displayed strong inhibitory effects, indicating that the substance responsible for anti-biofilm properties may work effectively at specific concentrations around 0.001 A.

In addition, *L. acidophilus* had the strongest antimicrobial effect and the weakest anti-biofilm effect while *L. plantarum* had the strongest anti-biofilm effect and the weakest antimicrobial effect. From this data, it can be reasonably concluded that the probiotic factors affecting anti-bacterial properties are different from those affecting anti-biofilm properties because varying concentrations of filtrate affected biofilm formation and bacterial growth differently. In subsequent experimentation, we autoclaved probiotic filtrate to denature any proteins in order to determine whether the substance causing anti-biofilm properties was protein-based.

Results for three of the five probiotic species (*L. casei, L. plantarum, L. acidophilus*) indicated that the autoclaved filtrate and the non-autoclaved filtrate had nearly identical effects on *Salmonella* biofilm production. The high similarity of the results for the *Lactobacillus* species gives strong support for the idea that protein-based molecules are not a major factor

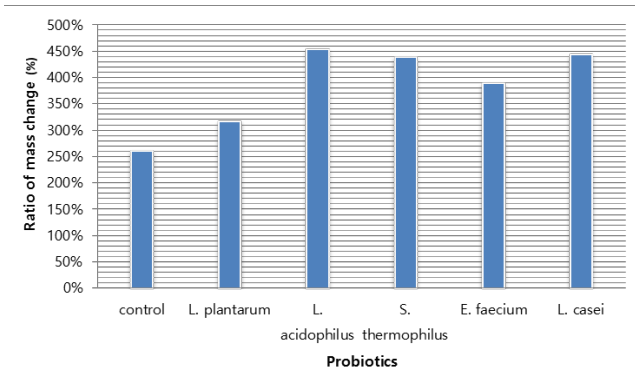

Figure 7. Mass of chicken skin as a percentage of the control after cultivation of *Salmonella* in autoclaved probiotic filtrate media.

in causing anti-biofilm properties. In experiments exposing *Lactobacilli* strains to *Streptococcus mutans,* researchers suggested that the anti-biofilm properties of probiotics might be due to both a change in environmental pH and the production of certain biofilm inhibiting polypeptides [6]. This study suggests that for *Salmonella*, the production of certain polypeptides is not the determining factor in biofilm inhibition. Changes in environmental pH, another factor suggested in the *Streptococcus mutans* study [6], is supported by this study, especially because probiotic filtrates showed strong effects at specific small concentrations, which is characteristic of a pH dependent effect.

One other possible mechanism is that probiotic filtrates disrupt the *Salmonella* quorum sensing process. Quorum sensing is a vital part of social behavior in bacteria because it allows bacterial species to sense the density of the bacterial population surrounding them and change their genetic expression to facilitate cell-to-cell interaction in processes like biofilm formation [9]. Gram-negative bacteria such as *Salmonella* have been found to utilize non-protein-based quorum sensing molecules such as N-Acyl homoserine lactones as part of their quorum sensing mechanism [9]. Probiotic filtrates may cause the disruption of the quorum

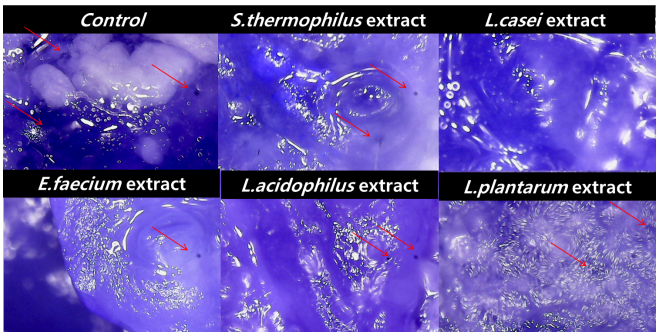

Figure 8. Microscopic images of dyed chicken skin after cultivation with *Salmonella* in autoclaved probiotic filtrate media. Red arrows point to darker spots within the dyed skin, which indicate probable areas of *Salmonella* biofilm formation. On average, autoclaved probiotic supernatants seemed to decrease the number of biofilm formation spots compared to the control group.

sensing process through non-protein-based substances, which would limit biofilm formation by inhibiting the cell-to-surrounding feedback mechanism among *Salmonella* populations.

Finally, experimentation with actual chicken skin supported the previous findings; chicken skin growing in media with probiotic extract showed less damage from *Salmonella.* This shows that probiotic filtrate could potentially be used to inhibit the contamination of *Salmonella* in food substances and reduce cases of food poisoning globally.

Although food contamination is a multifactorial problem, the ingestion of probiotics by livestock or the use of probiotic filtrate coating in unsanitary environments may assist in preventing food contamination. For example, probiotic filtrate coatings could be used on common areas of bacterial biofilm formation such as refrigerator walls or kitchen countertops. In addition, the significant anti-bacterial and anti-biofilm properties of probiotic filtrates support the increased use of probiotics in animal feed. However, because anti-bacterial effects became most prominent at high concentrations, while anti-biofilm effects worked at low concentrations of filtrate, these results may be problematic in determining the concentrations of probiotic filtrate to use in real-life applications. Further studies must be conducted to determine optimal concentrations of probiotic filtrate for effective anti-biofilm and anti-bacterial properties.

Another next step is for experimental trials to be conducted on live farm animals, as the preliminary chicken skin experimentation in this research may not be reflective of the effectiveness of probiotic filtrates in a living and dynamic organism. In addition, many of the results varied depending on the probiotic species. Species such as *S. thermophilus*, which did not follow many of the general trends, should be further investigated to determine whether they are unsuitable for addressing *Salmonella* contamination.

Finally, while the results for the autoclaved and non-autoclaved *Lactobacillus* groups suggest that protein-based molecules are most likely not responsible for anti-biofilm properties, the results for *E. faecium* and *S. thermophilus* filtrates do not necessarily agree with this assumption. While this may be an outlier that could be addressed by more repeated trials, further research should be conducted to pinpoint the exact mechanism of how these antibacterial and anti-biofilm properties arise from probiotic filtrates. By gaining a better understanding of the mechanisms facilitating biofilm formation and other forms of social behavior among harmful bacteria like *Salmonella*, future research can develop more effective means to limit pathogenesis.

METHODS

Preparation of Bacteria

Five species of commonly used probiotics in farm animals were purchased for experimentation. *Lactobacillus acidophilus* (KCTC 3140), *Lactobacillus casei* (KCTC 3260), *Lactobacillus plantarum* (KCTC 21004), *Streptococcus thermophilus* (KCTC 3658), and *Enterococcus faecium* (KCTC 13225) were purchased from the Korean Collection for Type Cultures (KCTC).

Salmonella enterica serovar Typhimurium (KCCM 11806) was purchased from the Korean Culture Center of Microorganisms (KCCM). *Salmonella gallinarum* was obtained from Genomic Information Center of Hankyong National University. Bacterial species were cultured in 20 mL of De Man, Rogosa, and Sharpe (MRS) broth solutions and incubated at 37°C. Before use, all cultures were tested for contamination by streaking.

Preparation of Growth Medium

MRS broth was created by adding 55 g of powder per 1 L distilled water and MRS agar medium was created with 70 g of powder per 1 L of distilled water. Broth solutions were autoclaved and poured into petri dishes to create the agar media. Both the agar media and broths were stored in a refrigerator at 2°C.

Bacterial Filtrates

The probiotic species in MRS broth were cultivated for 72 hours at 37°C inside test tubes. The cultures were mixed with a vortex mixer and filtered into a 10 mL test tube using a 0.2 μm disk filter. Based on the initial concentrations of probiotic species in each culture, filtrate was added to 3 mL of MRS solution inside a well plate to create a solution with the filtrate from a 0.1 A concentration of probiotic bacteria culture (A=1x10^9 bacteria/ml). After evenly mixing, two serial dilutions of 1/10 were made, creating experimental well plates with the filtrates from cultures of 0.1 A, 0.01 A, and 0.001 A concentrations of each probiotic culture in 2.7 mL solutions.

Autoclaved probiotic filtrate was prepared by autoclaving at 121°C and 1.2 atm for 15 minutes. Control sets of undiluted autoclaved solution were prepared for each of the probiotic species. *Salmonella* was added to each experimental set at a concentration of 0.1 A and cultivated for 48 hours.

Observation of Results

To measure bacterial growth, absorbance levels of the solutions were compared with a UV spectrophotometer at Optical Density (OD) 630 nm. To measure biofilm growth, the well plates were gently rinsed with distilled water. Next, 2 mL of a 0.4% crystal violet dye solution was added to the well plates for 48 hours. Again, the well plates were gently rinsed with distilled water. Finally, 2 mL of ethanol was added to each of the well plates, and the absorbance of ethanol was measured with a UV spectrophotometer at OD 590 nm. Absorbance values were used to compare biofilm growth.

Qualitative observations of biofilm formation were also made on borosilicate test tubes. Each of the borosilicate test tubes was wrapped with Parafilm. After gently rinsing the tubes with distilled water and adding 0.4% crystal violet solution, a microscope was used to observe staining patterns

on the sides of the tubes which represented biofilm growth.

Chicken Skin Dyeing

Using sterilized forceps and medical scissors, square pieces of chicken skin weighing around 0.6g were cut out of a raw chicken drumstick. The chicken skin pieces were placed into test tubes of autoclaved *L. acidophilus*, *L. plantarum*, *L. casei*, *S. thermophilus*, and *E. faecium* filtrates of a concentration of 0.001 A in MRS base. Because the chicken skin pieces varied slightly in mass, the amount of solution in each experimental trial was adjusted based on a standard ratio of 20 mL of solution for every 0.6 g of chicken skin. A control set was made on MRS base without probiotic filtrate. *Salmonella* was inoculated into each experimental test tube at 0.1 A concentration. After 12 days of cultivation, the chicken skins were rinsed gently under distilled water and dyed with 10 mL of 0.4% crystal violet solution for 48 hours. The chicken skins were rinsed again with distilled water and excess crystal violet solution was removed with ethanol. The amount of *Salmonella* growth was determined by comparing the initial masses to the final masses after dyeing of the chicken skins.

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REFERENCES

1. T. Roberts, “Salmonellosis Control: Estimated Economic Costs.” *Poultry Science*, Vol. 67, Issue 6, 1988, pp. 936–943.
2. D. Peng. “Biofilm Formation of Salmonella.” *Ch. 12 Microbial Biofilms - Importance and Applications*, 2016, pp. 231-249.
3. R. M. Donlan. “Biofilm Formation: A Clinically Relevant Microbiological Process.” *Clinical Infectious Diseases*, Vol. 33, Issue 8, 2001, pp. 1387–1392.
4. J. Kaplan. “Antibiotic-Induced Biofilm Formation.” *The International Journal of Artificial Organs*, Vol. 34, Issue 9, pp. 737-751.
5. A. Bezkorovainy. “Probiotics: determinants of survival and growth in the gut.” *The American Journal of Clinical Nutrition*, Vol. 73, Issue 2, 2001, pp. 399–405.
6. X. Lin, *et al*. “The effect of five probiotic lactobacilli strains on the growth and biofilm formation of Streptococcus mutans.” *Oral Diseases,* 2015; 21: e128–34.
7. B. Taheur, *et al*. “Anti-bacterial and anti-biofilm activity of probiotic bacteria against oral pathogens,” *Microbial Pathogenesis*, 97, 2016, pp. 213-220
8. C. Sannat, *et al*. “Characterization of Salmonella Gallinarum from an Outbreak in Raigarh, Chhattisgarh.” *Veterinary World*, Vol. 10, Issue 2, 2017, pp. 144–148.
9. T. Rutherford and L. Bassler. “Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control.” *Cold Spring Harbor Persp. in Medicine,* Vol. 2, Issue 11, 2012.