

Determination of the Anti-Adhesive and Anti-Biofilm Capacity of a Wheat Extract on *Staphylococcus Aureus* in Farms

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ABSTRACT

The formation of biofilm by pathogenic microorganisms has become a problem in the livestock industry, since it is considered a potential source of infection for farm animals, while increasing microbial resistance to physical and chemical agents. Some plant extracts, such as soluble wheat extract, have been shown to be effective in inhibiting or destroying biofilm of certain microorganisms under specific conditions. The objective of this study is to evaluate the capacity of the pathogen to form biofilm on different surfaces used in livestock, as well as to evaluate the anti-biofilm capacity of soluble wheat extract against *S. aureus* on these surfaces. The inhibition potential of inhibition or destruction of biofilm was tested *in vitro*. Wheat extract at a concentration of 0.29mg / 100mL showed anti-biofilm activity on *S. aureus*, inhibiting its formation, as well as destroying it greatly after a contact time of 24 hours, on those surfaces where the microorganism presents more adhesion capacity.

Keywords: Microbiology; Biofilm; Anti-biofilm; Wheat Extract; *Staphylococcus Aureus*

Introduction

Microbial contamination and infections have always been a problem and a limiting factor for growth and animal production on fattening farms. To combat and prevent certain livestock infections by certain microorganisms, antimicrobial therapy has been used in animals in recent years. Even so, several studies question this methodology due to the increasingly frequent appearance of resistance to these compounds. Therefore, it is necessary to find new ways to prevent microbial infections in livestock that are independent of substances that can generate resistance [1]. Several studies have proposed alternative products that can be used as additives in animal feed, with the ability to prevent adhesion and intestinal colonization of pathogenic bacteria in animals, which also do not generate resistance by microorganisms [2-4]. Some of the alternative products can be such as prebiotics, probiotics, plant

extracts, organic acids, enzymes, or micro minerals. A different problem from colonization and infection of cattle is the ability of certain microorganisms to adhere to certain types of surfaces, forming films of microbial communities enveloped by extracellular matrix called "biofilms". It is known that microbial growth in nature is found mainly in the form of biofilms since in this way the microorganisms present more resistance to environmental factors and antimicrobial agents than in the free form [5]. In some cases, low doses of antibiotics may even favour the formation and growth of a biofilm, which indicates a natural defence mechanism for microorganisms to avoid the toxic effect of these substances [6].

These structures are therefore much more difficult to combat by physical or chemical methods and can cause livestock infections more easily [7]. Some natural products have been shown to be

effective against the adhesion of certain microorganisms to surfaces, thus presenting new study possibilities to combat the formation of biofilms in fattening farms independently of microbial substances [8-9]. Even so, many of these substances or natural extracts have complex compositions and it is, therefore, difficult to determine their activity and their ability to block the adhesion of bacterial cells to surfaces is in some cases purely empirical [10]. In this work, the ability of a natural extract from rye grain to inhibit the formation and / or break biofilm of the pathogenic microorganism *Staphylococcus aureus* (Friederich Julius Rosenbach, 1884) on different types of surfaces of snail and poultry farms has been tested. The efficacy of this natural extract was previously described against biofilms formed in epithelial tissue of mammary glands in cows, causing mastitis and subclinical infections of the mammary glands [10]. *S. aureus* is a non-sporulated facultative anaerobic microorganism that is Gram-positive cocci and is positive for catalase and coagulase reactions. Despite being a pathogenic microorganism, it is widely distributed in a wide variety of environments and habitats, also forming part of the normal microbiota of the skin and mucosa of warm-blooded animals.

It can cause a wide variety of infections and diseases, either through infection and colonization of tissues, or through the production of toxins, among which several types of enterotoxins and hemolysins stand out. Among the diseases caused by this microorganism, stand out from benign mucosal and skin infections or gastroenteritis caused by enterotoxins to infections of internal tissues, pneumonia, meningitis, endocarditis, or sepsis, among others. The adhesion of *S. aureus* on animal tissue surfaces and the formation of biofilms have been described as important virulence factors [3]. It is also known that the mechanism described as “quorum sensing” intervenes in the regulation and coordination between the different microorganisms responsible for the creation and maintenance of a biofilm [11-12]. In this sense, the plant extract tested in this study has been shown to be a potential inhibitor of biofilm formation due to its anti-adhesive properties against *Enterobacteriaceae* [1,13-14].

The main objective of the work is to demonstrate the effect that a soluble wheat extract has on *S. aureus* biofilm on four different types of surfaces:

- A PVC surface, a metal surface and a cloth surface used as a coating for rooms on snails' farms.
- Plastic material channeling tube used to channel the water used in poultry farm waterers.

For all surfaces, the ability of the wheat extract to prevent the formation of *S. aureus* biofilm was determined, as well as the ability to destroy an already formed biofilm and prevent its reappearance.

Material and Methods

To carry out the different tests, the 4 surfaces were divided into strictly equal parts as follows:

- PVC, metallic and cloth material used in the snail's farm: different portions of 6x7cm each were made, previously disinfected with ethanol (96%).
- Material used in the poultry farm: the channeling tube was cut into 8 cm portions, and then sterilized at 121°C for 20 minutes.

For all the tests carried out, an overnight culture of the microorganism was started in rich medium BHI (Brain heart infusion). The process was carried out at all times under sterile conditions to avoid contamination of the samples. As a diluent for the preparation of the different samples, a sterile solution of 50 g/L glucose and 9 g/L NaCl was used to simulate the conditions of the poultry farm, to which 3 g/L of a supplement of amino acids and vitamins for birds after sterilization. All tests were made at the same concentration of wheat extract (0.29mg/100mL of solution).

Determination of the Extract's Ability to Prevent the Formation of *S. Aureus* Biofilm

To determine the inhibitory capacity of biofilm formation, 100mL of each of the following sterile dilutions were prepared:

- Positive control: 50mL of the glucose saline solution to which 0.3g of the amino acid and vitamin supplement was added with 50mL of the overnight culture of the microorganism.
- Negative control: 100mL of the glucose saline solution to which 0.3g of the amino acid and vitamin supplement with 0.29mg of wheat extract were added.
- Sample: 50mL of the glucose saline solution to which 0.3g of the amino acid and vitamin supplement was added with 50mL of the overnight culture of the microorganism and with 0.29mg of wheat extract.

Surfaces Supplied by the Snails Farm: The surface was divided into 15 portions of 6 x 7cm, in which 50µL of the following dilutions were inoculated:

- “Positive control” in 3 portions
- “Negative control” in 3 portions
- “Sample” in 9 portions

The 50µL of each dilution was spread homogeneously by each portion using a sterile Digrafsky loop.

A sample (see section 2.3.) was taken from the portions identified as positive control, negative control and 3 replicates of

the “sample” solution at 0, 24 and 48h after the initial inoculation. During the periods between the sampling, the surface was incubated at 37°C under aerobic conditions.

Pipes from Poultry Farm: Twelve portions of pipes (8cm/portion) were inoculated with the following solutions:

- 3 portions with the “Positive Control” solution
- 3 portions with the “Negative Control” solution
- 6 portions with the “Sample” solution

A sample of the liquid and the surface (see section 2.3.) were taken from the positive and negative controls and 3 replicates of the “sample” solution at 0, 24 and 48h after the initial inoculation. During the periods between sampling, the tubes were incubated (with the corresponding solution inside) at 37°C under aerobic conditions.

Determination of the Ability of Wheat Extract to Lyse an Already Formed *S. Aureus* Biofilm

For the determination of the ability of the wheat extract to lyse an already formed biofilm of *S. aureus*, 100mL of each of the following sterile dilutions were prepared:

- Initial inoculum: 50mL of glucose saline to which 0.3g of the amino acid and vitamin supplement was added with 50mL of the microorganism overnight culture.
- Positive control: 100mL of glucose saline solution to which 0.3g of the amino acid and vitamin supplement was added.
- Negative control: 100mL of glucose saline solution to which 0.3g of the amino acid and vitamin supplement with 0.29mg of wheat extract were added.
- Sample: 100mL of the glucose saline solution to which 0.3g of the amino acid and vitamin supplement with 0.29mg of wheat extract were added.

Surfaces Supplied by the Snail's Farm: The surfaces were divided into 10 equal portions of 6 x 7cm, in each of which 50µL of the following dilutions were inoculated:

- “Initial inoculum” in all portions at initial time except those corresponding to the negative control.
- “Positive control” in 2 portions on time 24h.
- “Negative control” in 2 portions at initial time.
- “Sample” in 6 portions and the two portions inoculated with “Negative control”, on time 24h.

The 50µL of each solution was spread evenly over each portion with a sterile Digrafsky handle.

A sample (see section 2.3.) was taken from the positive control, negative control, and 3 replicates of the “sample” solution at the initial time and at 24h after inoculation on the biofilm previously formed from the “initial inoculum” solution for 24h. During the periods between inoculations and sampling, the surface was incubated at 37°C under aerobic conditions.

Pipes from Poultry Farm: Eight 8cm tubes were inoculated with the following solutions:

- “Initial inoculum” in all portions at initial time except those corresponding to the negative control.
- “Positive control” in 2 tubes on time 24h.
- “Negative control” in 2 tubes on time 24h
- “Sample” in 4 tubes on time 24h.

A sample of the liquid and the surface (see section 2.3.) Of the positive control, negative control, and 2 replicates of the “sample” solution were taken at the initial time and 24h, after inoculation of the controls and samples. During the periods between inoculations and sample collection, the tubes were incubated at 37 °C under aerobic conditions. For the formation of the biofilm with the “initial inoculum” solution during the first 24h, the tubes were emptied and incubated dry, while for the following 24h they were incubated with the corresponding solutions inside.

Sampling

On Surfaces Supplied by the Snail Farm: Sampling on these surfaces was carried out using sterile swabs moistened with 100µL of sterile Ringer's solution in order to collect the maximum number of viables present in each portion. Subsequently, the swab was suspended in a tube with 9 mL of Ringer's and 100µL were seeded in 3 plates of Mannitol-Salt Agar (3 replicates per time and portion). The plates were incubated at 37°C for 48h under aerobic conditions before proceeding to the colony count.

In tubes Supplied by The Poultry Farm

A. From the liquid Inside the Tubes: The liquid was decanted into a sterile tube so as not to affect the number of viables on the surface during handling, it was homogenized and 100µL was used to make the corresponding dilutions with sterile Ringer. The two most suitable dilutions were chosen to enable a representative and reliable CFU count. From both dilutions, 100µL were seeded in two plates of Mannitol-Salt Agar for each dilution. The plates were incubated at 37°C for 48h under aerobic conditions before proceeding to the colony count.

B. Of the Surfaces of The Interior of the Tubes: Subsequent to the vacuum of the liquid inside the tubes, a sterile swab was humidified with 100µL of sterile Ringer, and it was passed over the

entire surface of the inside of the tubes in order to take the maximum number of viable. Subsequently, the swab was suspended in a tube with 9mL of sterile Ringer, from which a serial dilution bank with sterile Ringer was performed. The two most suitable dilutions were chosen to enable a representative and reliable CFU count of both dilutions, 100 μ L were seeded in two plates of Mannitol-Salt Agar for each dilution. The plates were incubated at 37°C for 48h under aerobic conditions before proceeding to the colony count.

Results

PVC Surface Supplied by Snails Farm

The results of the PVC surface used on the snail's farm are

Table 1: Counts corresponding to the PVC surface used in the snail farm. The results are expressed in CFU/total area sampled, and the percentage variation of the average of the samples inoculated with the plant extract versus the positive control.

	Sample time (h)	Positive Control	Sample 1	Sample 2	Sample 3	Average of 3 samples	Percentage variation respect to the positive control
Concentration of microorganism with the extract	0	1.67x10 ⁴	1.71x10 ⁴	6.99x10 ⁴	3.33x10 ⁴	4.01x10 ⁴	140.12%
	24	4.97x10 ³	2.37x10 ³	1.67x10 ³	1.53x10 ³	1.86x10 ³	-62.57%
	48	7.33x10 ²	7.67x10 ²	6.33x10 ²	5.67x10 ²	6.56x10 ²	-10.50%
Addition of extract on biofilm formed	0	7.07x10 ³	6.70x10 ³	8.73x10 ³	8.47x10 ³	7.97x10 ³	12.72%
	24	1.33x10 ²	6.67x10 ¹	3.33x10 ¹	6.67x10 ¹	5.56x10 ¹	-58.19%

Pipes from the Poultry Farm

The results of the material supplied by the poultry farm for the biofilm inhibition capacity determination test are shown in (Tables 2 & 3). The results are expressed in CFU/mL of the liquid contained in each portion and in CFU/unit of sampled area. When performing the CFU counts per mL of the liquid contained within each portion, select a reduction of the percentage of viable microorganisms compared to the positive control of 10.75% at the initial moment, 74.36% at the 24h time and 85,21% at the time 48h after the inoculation of the extract. When evaluating the capacity of the

shown in (Table 1). The results are expressed in CFU/unit of surface sampled. The test of the capacity of the plant extract to inhibit the formation of the biofilm on the PVC surface provided by the snail farm (Table 1), shows an increase in the number of viables of 140.12% in the samples with respect to the positive control at initial time, while, after 24 and 48h of incubation at 37°C, they present decreases of 62.57% and 10.50% respectively with respect to the positive control. Regarding the test in which the capacity of the plant extract to destroy a biofilm on the same surface was evaluated, the counts showed an increase in the number of viable of 12.72% of the samples over the positive control at initial time, while on time 24h they showed a reduction of 58.19%.

extract to destroy a modified biofilm, the affected counts reduced the number of viable of 41.49% of the samples on the positive control at the initial moment, while at 24 h it reduced a reduction of 59.62%. When performing the CFU counts of the interior surface of each portion, a percentage reduction of viable microorganisms was observed compared to the positive control of 36.22% at initial time, 53.69% at 24h time and 91.00% at time. 48h after inoculation of the extract. When evaluating the extract's ability to destroy a formed biofilm, the counts showed a reduction in the number of viable of 41.04% of the samples over the positive control at initial time, while at 24h time they showed a reduction of 95.53%.

Table 2: Counts corresponding to the liquid inside the tubes used in the poultry farm. The results are expressed in CFU/mL and the percentage variation of the average of the samples inoculated with the plant extract versus the positive control.

	Sample time (h)	Positive Control	Sample 1	Sample 2	Average of 2 samples	Percentage variation respect to the positive control
Concentration of microorganism with the extract	0	3.35x10 ⁸	2.78x10 ⁸	3.20x10 ⁸	2.99x10 ⁸	-10,75%
	24	1.17x10 ⁸	2.58x10 ⁷	3.41x10 ⁷	3.00x10 ⁷	-74.36%
	48	1.44x10 ⁷	2.38x10 ⁶	1.88x10 ⁶	2.13x10 ⁶	-85,21%
Addition of extract on biofilm formed	0	1.88x10 ⁷	1.16x10 ⁷	1.04x10 ⁷	1.10x10 ⁷	-41,49%
	24	1.08x10 ⁸	4.20x10 ⁷	4.43x10 ⁷	4.36x10 ⁷	-59,62%

Table 3: Counts corresponding to the interior surface of the tubes used in the poultry farm. The results are expressed in CFU/area sampled, and the percentage variation of the average of the samples inoculated with the plant extract versus the positive control.

	Sample time (h)	Positive Control	Sample 1	Sample 2	Average of 2 samples	Percentage variation respect to the positive control
Concentration of microorganism with the extract	0	8.20x10 ⁶	4.25x10 ⁶	6.20x10 ⁶	5.23x10 ⁶	-36,22%
	24	1.68x10 ⁸	8.80x10 ⁷	6.76x10 ⁷	7.78x10 ⁷	-53,69%
	48	1.80x10 ⁷	1.40x10 ⁶	1.84x10 ⁶	1.62x10 ⁶	-91,00%

Addition of extract on biofilm formed	0	8.65x10 ⁷	5.50x10 ⁷	4.70x10 ⁷	5.10x10 ⁷	-41,04%
	24	8.05x10 ⁸	4.45x10 ⁷	2.75x10 ⁷	3.60x10 ⁷	-95,53%

Metallic Material Supplied by the Snail Farm

The results of the PVC surface used in the snail farm are shown in (Table 4). The results are expressed in CFU/unit of total area and percentage of variation of the samples with respect to the positive control. Viable counts on the metal surface provided by the snail farm for the test for determining the inhibitory capacity of biofilm formation, showed very high results at time 0h, which were

expressed as counts greater than 105 per unit area. In these counts it was impossible to determine the percentage reduction of the samples with respect to the positive control. At 24h and 48h time, the counts showed decreases of 52.21% and 44.44% respectively. The test carried out to determine the ability of the extract to destroy biofilm formed on the same surface, showed a decrease in the number of viables of 62.45% at initial time, and an increase of 133.63% at time 24h.

Table 4: Counts corresponding to the metal surface used in the snails farm. The results are expressed in CFU/total area sampled, and the percentage variation of the average of the samples inoculated with the plant extract versus the positive control.

	Sample time (h)	Positive Control	Sample 1	Sample 2	Sample 3	Average of 3 samples	Percentage variation respect to the positive control
Concentration of microorganism with the extract	0	>1x10 ⁵	N.A.				
	24	2.72x10 ⁴	1.17x10 ⁴	1.39x10 ⁴	1.34x10 ⁴	1.30x10 ⁴	-52,21%
	48	9.00x10 ²	2.33x10 ²	8.00x10 ²	5.00x10 ²	5.11x10 ²	-44,44%
Addition of extract on biofilm formed	0	5.30x10 ³	1.23x10 ³	2.40x10 ³	2.33x10 ³	1.99x10 ³	-62,45%
	24	3.33x10 ¹	1.00x10 ²	6.67x10 ¹	6.67x10 ¹	7.78x10 ¹	133,63%

Cloth Material Supplied by the Snail Farm

The results of the fabric area used in the snail farm are shown in (Table 5). The results are expressed in CFU/unit of total area and percentage of variation of the samples with respect to the positive control. The results obtained in the test for determining the inhibitory capacity of biofilm formation, show counts above the detection limit of the technique at initial time, which were expressed as > 10⁵ CFU/surface sampled. During this time, it was

impossible to determine a percentage reduction of the samples with respect to the positive control. At 24h and 48h time, the counts showed increases of 11.89% and 60.86% respectively. The test carried out to determine the capacity of the extract to destroy the biofilm formed showed a decrease in the number of viables of 22.78% at the initial time, and an increase of 45.88% at the 24h time. Considerable microorganism counts were not obtained in any of the portions of materials tested as negative controls.

Table 5: Counts corresponding to the surface of fabric used in the snail farm. The results are expressed in CFU/total area sampled, and the percentage variation of the average of the samples inoculated with the plant extract versus the positive control.

	Sample time (h)	Positive Control	Sample 1	Sample 2	Sample 3	Average of 3 samples	Percentage variation respect to the positive control
Concentration of microorganism with the extract	0	>1x10 ⁵	N.A.				
	24	1.43x10 ³	1.63x10 ³	1.20x10 ³	1.97x10 ³	1.60x10 ³	11,89%
	48	2.00x10 ²	4.67x10 ²	6.00x10 ²	4.67x10 ²	5.11x10 ²	60,86%
Addition of extract on biofilm formed	0	1.80x10 ³	1.38x10 ³	1.11x10 ³	1.67x10 ³	1.39x10 ³	-22,78%
	24	1.38x10 ²	6.67x10 ²	3.33x10 ¹	6.67x10 ¹	2.55x10 ²	45,88%

Discussion

The way of life of some bacteria, in which the structured biofilm structure is being formed, together with the recent increase in the last years of resistance to antibiotics, made the treatment of diseases caused by bacteria is very difficult [15]. On many occasions, biofilm bacterial growth gives bacteria greater resistance to physical or chemical agents such as antimicrobials [16-17]. Because of this, several studies have recently been conducted to demonstrate the

potential antibiotic effect of certain molecules, including various quorum detection inhibitors in *S. aureus* and other microorganisms [15,18] or of certain probiotic strains [19]. Many of the tested quorum-inhibiting molecules tested have been found to be modified to prevent biofilm formation. Even so, It has been seen that after its application on formed biofilm, the results are not as effective [15,18]. Certain probiotic strains have also been found to be determined to inhibit the biofilm formation of *S. aureus* [19]. In this study, the potential of wheat extract to inhibit the formation of

a biofilm or destroy an already formed biofilm of *S. aureus*, which is known for its biofilm-forming capacity, has been evaluated [3]. Other studies have highlighted the possible biotechnological applications of wheat extract [14] and its inhibitory effect on microbial films [1]. Different surface materials (PVC surface, metallic surface, cloth surface and drinking water channelling material) used in snail and poultry production farms have been evaluated, in which biofilm of *S. aureus* has been formed *in vitro* and, subsequently wheat extract has been added to evaluate its function.

According to the results obtained in this study, a better adherence and biofilm formation of *Staphylococcus aureus* can be considered in the water channelling material supplied by the poultry farm compared to any other surface supplied by the snail farm. In the PVC material provided by the snail farm, the microorganism has a moderate adherence and biofilm formation capacity, since it starts from values of the order of 10⁴ at the initial time for the test for determining the inhibitory capacity of biofilm formation, and of the order of 10³ at initial time for the test of the lytic capacity on biofilm formed. These values were reduced to orders of 10² CFU to 10¹ CFU after 24 or 48 hours of incubation. (Table 1) shows the percentage reduction in the number of viables in each test after 24 and 48 hours of incubation, suggesting an extract efficiency of around 10% for the inhibition test and 60% for the lysis test of the extract on said surface. Previously, high percentages of reduction by wheat extract were also reported [1]. In the material from the poultry farm, the results indicate a greater effectiveness of the plant extract at high concentrations of microorganism in both tests, presenting for the inhibition test a viable decrease of more than 85% in both liquid and surface after 48h after of inoculation.

In the test to test the ability to destroy the biofilm formed, the percentage reduction of viables was greater than 95% after 24 hours after inoculation of the extract on the biofilm formed on the inner surface (Table 3). Therefore, it can be indicated that the extract has a high efficiency on this surface in both tests tested. Both in the metallic material provided by the snail farm and in the cloth material, the microorganism has a low adherence capacity, since it has counts at initial time outside the detection range with the technique used, which decrease to residual values, in the order of 10¹ CFU to 10² CFU per unit area at 24 and 48h, which are also not indicators of biofilm formation. Therefore, it was impossible to determine the efficacy of the plant extract on these surfaces.

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