Staphylococcus epidermidis and *Staphylococcus capitis* quorum sensing as a strategy to control atopic dermatites

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Abstract

The human skin presents a diversified microbiota which influences the body homeostasis and its protection. The unbalance of this microbiota leads to skin diseases, like atopic dermatitis (AD). AD is characterized mostly by increased number of *Staphylococcus aureus*, decrease in microbial diversity and intense inflammatory response. The most common bacteria skin species found in in AD are *Staphylococcus epidermidis* and *Staphylococcus capitis* and they produce molecules that could inhibit growing of *S. aureus* by *quorum sensing* mechanisms. The *quorum sensing* is a bacterial intercellular communication system, in which specific molecules, that can be a peptide or a polysaccharide, provides communication and respond at the same way to an external stimulus. Thus, this project aims to study which substances are released by *S. epidermidis* and *S. capitis* through *quorum sensing* and its impacts on *S. aureus* and in the skin microbiota, assessing a possible treatment for AD using pre and post biotics. Liquid chromatography (HPLC - DAD) and mass spectrometry (MALDI-TOF/TOF) were used to identify PSMs among the *S. epidermidis* and *S. capitis* proteins differentially regulated by quorum sensing during growth in BHI, monitored by absorbance at 650 nm comparing with the growth pattern. The results showed that a peptide was found at the butanolic extract from *S.capitis* that promises to be a strategy to control the agr systems of *Staphylococcus* sp. The next step is challenging the bacteria with pre and post biotics, in an appropriate formulation, increasing the *S. epidermidis* and *S. capitis* to inhibits *S. aureus* activity.

Keywords: Quorum sensing; Mass spectroscopy; HPLC-DAD; skin microbiota; atopic dermatitis.

Introduction

Atopic dermatitis is a common inflammatory skin disease with a complex etiology that affects about 15 to 20% of children and 1 to 3% of adults worldwide [1]. The disease has as main symptoms intense itching, recurrent eczema, dry skin and roughness, especially in sensitive skin regions [1] [2]. AD has a great impact on the quality of life of its patients, influencing their lifestyle, since they are restricted to the clothes they can use, along with cosmetic products and their food, impairing their sleep, and the quality of their mental health [2]. The causes of AD are complex and multifactorial, there is a strong genetic component, with evidence of multiple mechanisms of genetic risks. Loss of function mutations in the gene encoding filaggrin (FLG) are the most important genetic variants, since filaggrin is an important structural protein in the epidermis [2]. The epidermis plays a crucial role as a physical barrier factor, defects in epidermis are the most significant pathological findings in AD skin [10]. The treatment of AD is aimed at improving symptoms and establishing long-term disease control, treatment planning should be patient-centered [2]. The choice of treatment is usually based on anti-inflammatory drugs, and in most cases, it is usually controlled by topical treatments, however in more severe cases it may require phototherapy or immunomodulatory therapy or both [2].

Another factor that contributes to the worsening of AD symptoms is the dysbiosis that occurs in the skin microbiota of these patients, since studies show that patients with AD have excessive colonization by *Staphylococcus aureus* in their skin, which is associated with severity and exacerbation of the disease [3].

The skin is an important first line of defense against the invasion of pathogenic microorganisms, corneocytes form a barrier of peptides and lipids secreted from keratinocytes and glands, thus providing a chemical protection to our skin [3]. Additionally, the skin's natural microbiota, which is composed of microorganisms such as bacteria and fungi, is an effective protection. It is estimated that the skin has more than 1000 different species of microorganisms, being from 19 different phyla [4].

The composition of the microbiota changes throughout life, in children there are fewer species of bacteria such as *Corynebacterium* sp and *Cutibacterium* sp, and a greater diversity of fungal species than adolescents and adults [3] [4]. An analysis carried out by

gene sequencing through 16S mRNA showed that the species most found in our skin are *Corynebacterium*, *Cutibacterium*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Betaproteonacteria* and *Gammaproteobacteria*, and this richness of diversity is influenced by micro-environmental conditions of skin such as pH, moisture, content, sebum, and topography [3].

Dysbiosis of the skin microbiota is nothing more than a change in the relative composition of different normal microorganisms, compared to a disease state, which is what occurs during the AD process [5].

In AD patients, this dysbiosis occurs with *S. aureus*, a gram-positive bacterium, which is found in the skin of these patients with rates ranging from 30% to 100%. This change will depend on the type of patient, sample size and sampling and method of analysis. In a healthy microbiota the percentage of *S. aureus* found is around 20% [4].

S. aureus can also increase AD severity through the secretion of its virulence factors, the best known and most studied are superantigens (SAgs). The SAgs work mechanically by binding to the non-peptide groove of greater histocompatibility to the class II complex, in cells that are antigen-presenting (APCs), including keratinocytes [5]. SAgs that are produced by *S. aureus* include all staphylococcal enterotoxins (Ses), and toxic shock syndrome toxin 1 (TSST-1), showing that *S. aureus* strains that produce Sags are related to AD severity [5].

Quorum sensing is a genetic, density-dependent, and environment-dependent mechanism for regulating the population of bacteria that occurs through cell-cell communication. In the genus *Staphylococcus* we find an accessory regulatory system called AGR, which is also found in other gram-positive bacteria [6]. The AGR system is composed of two transcribed and divergent units RNA II and RNA III, whose transcription is driven by the P2 and P3 promoters, respectively. RNA II contains 4 genes, namely agrA, agrB, agrC and agrD [7]. However, in clinical isolates of *S.aureus* from patients with AD, 4 different types of agr were found, type I agr, type II agr, type III agr and type IV agr [8], the most prominent in AD is the agr of the type I [9].

It is believed that the rebalancing of the microbiota of patients with AD leads to a decrease in the main symptoms of the disease, so the objective of the work is to look for ways

to reestablish the quorum-sensing of the other gram-positive bacteria that are in this microbiota, such as *Staphylococcus capitis* and *Staphylococcus epidermidis*, evaluating their activity on inhibiting the growth of *Staphylococcus aureus*.

Materials and Methods

A microbial suspension of 10^8 CFU/mL of *Staphylococcus capitis* was added to 250 mL of BHI culture medium (Difco), growth for 72 h in a shaker (Lab. Companion – model SI 300R) at a temperature of 37° and 165 RPM. The microbial suspension of *Staphylococcus aureus* and *Staphylococcus epidermidis* were also obtained by the same procedure however at 24 h growth instead of 72 h. BHI medium (50mL) without any microorganism was used as a blank or negative control.

A centrifuged (Hermle, model Z366K), at 4500 RPM, for 5 min and 4°C was used to separate the cells and the supernatant. The pellets were resuspended in 1 mL of 0.9% (w/v) saline solution and centrifuged at 4500 RPM for 5 min at 4°C, twice, and stored frozen for 48h until the performance at the mass spectrometry test (MALDI-TOF, Bruker Daltonik model GT 0264). The supernatant was filtered through a 0.45 µm x 4mm filter (Merk S/A, model S-PAK) and stored at 4°C for 24 h, until extractions were performed.

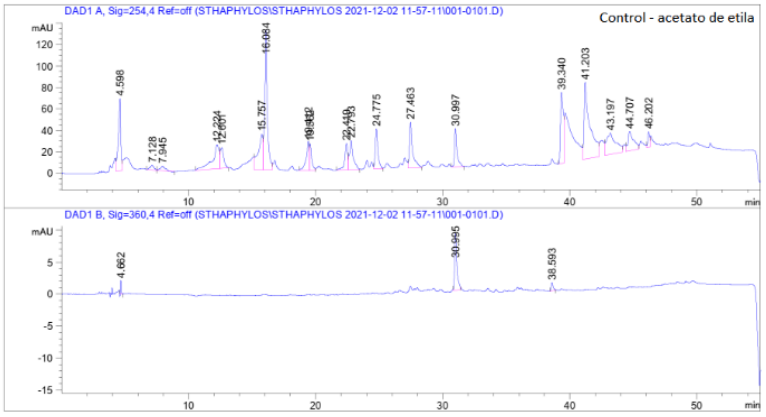
The extraction of the supernatant for the HPLC method started with ethyl acetate (Synth) and end with butanol (Synth). The proportionality was 1/3 of solvent in relation to the amount of sample to be extracted. To the negative control or blank were used 45 mL of sample and 15 mL of ethyl acetate and for the *S. capitis*, *S.epidermidis* and *S.aureus* the amount was 240 ml of sample and 72 ml of ethyl acetate. The extraction was carried out 3 times and the process were being carried out with butanol in the same way. After the extractions, the samples were passed through a speed dryer (Hudolph), the solvent was removed, and the remaining material was diluted in 2 mL of methanol (Synth). At the end of this process the following samples were obtained: Control ac. ethyl, Control butanol, *S. capitis* ac. ethyl, *S. capitis* butanol, *S. epidermidis* ac. ethyl, *S. epidermidis* butanol, *S. aureus* ac. ethyl, *S. aureus* butanol.

A volume of 1.5 mL was evaporated at a speed vacuum (Christ, model RVC 2-18) and resuspended with 1.5 mL of standard HPLC methanol, filtered at 0.45 µm, allowing the removal of any residue remaining in the sample and placed it in a specific vial for HPLC. The HPLC (Agilent, model Infinity 1260 with DAD detector, automatic injector, oven for column temperature control) with a C18 reversed phase column with silica. Solvents used were water and methanol, starting 95%/ 5%, a run of 50 min and a flow of 0.8 mL/min. The scanning methodology was used to search for particles size with wave pattern of analysis 254 and 360 nm.

Mass spectrometry analyzed the same samples analyzed in HPLC, using a MALDI-TOF (Bruker Daltonik, GT 0264, with the flexControl Version 3.4 software and the flexAnalysis Version 3.4 software for sample analysis) with MBT identification method, which has a length from 1,800 to 20,000 m/z, matrix suppression of 700 Da, and the calibrant used was the BTS (Bruker). The samples were placed on the appropriate plate and analyzed in triplicate, after drying and addition of 0.1 µL of matrix.

Results

A chromatographic analysis was carried out to survey the microbial extracts looking for peptides, carbohydrates and lipids that could be used to control *S. aureus* growth at an AD skin. The samples started with the detection of polar substances, and throughout the run, as the solvent changed, it was possible to find more nonpolar species, especially in samples that were extracted with ethyl acetate, at 360 nm, as shown in the figure 1.



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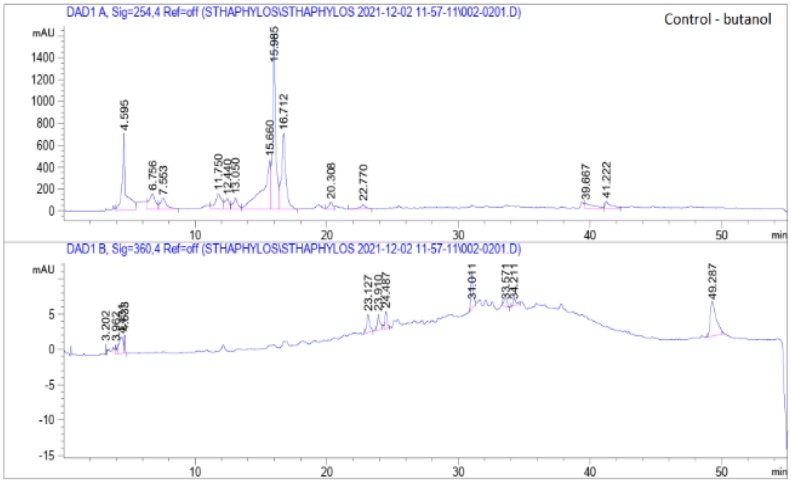

B

Figure 1. Chromatogram of the Acetate Control (A) and Butanol Control (B). In the upper part we can observe the wavelength of 254 nm, and in the lower part we can observe the wavelength of 360 nm.

The chromatograms at figure 2 represent the peaks obtained with the *S. capitis* acetate and *S. capitis* butanol samples. When compared with the Acetate Control (figure 1A), it is possible to note that at a wavelength of 360 nm after 30 min of run the sample showed peaks, indicating the presence of non-polar substances in the sample. In the *S. capitis* butanol extraction (figure 2B) when comparing with the butanol Control (figure 1B), at a wavelength of 254 nm, it is possible to observe a differentiated peak around 10 min of running, which indicates the presence of a polar substance in the sample.

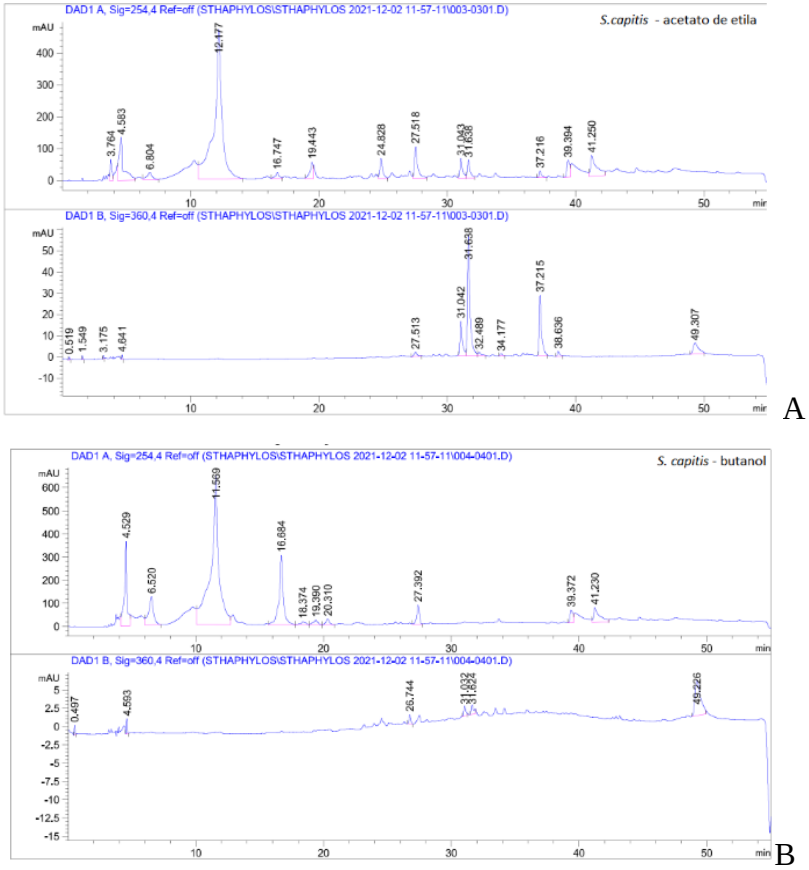

Figure 2. Chromatogram of the *S capitis* Acetate (A) and *S. capitis* Butanol (B). In the upper part we can observe the wavelength of 254 nm, and in the lower part we can observe the wavelength of 360 nm.

At figure 3 it is possible to observe chromatograms related to *S. epidermidis* acetate and *S. epidermidis* butanol samples when compared with the Acetate Control (fig.1A) at a

wavelength of 360 nm. After 25 min of run the sample showed peaks indicating the presence of non-polar substances in the sample. In the *S. epidermidis* butanol sample (figure 3B), comparing with the butanol Control (figure 1B), at a wavelength of 254 nm, a differentiated peak around 6 min of running can be observed, which indicates the presence of a polar substance in the sample.

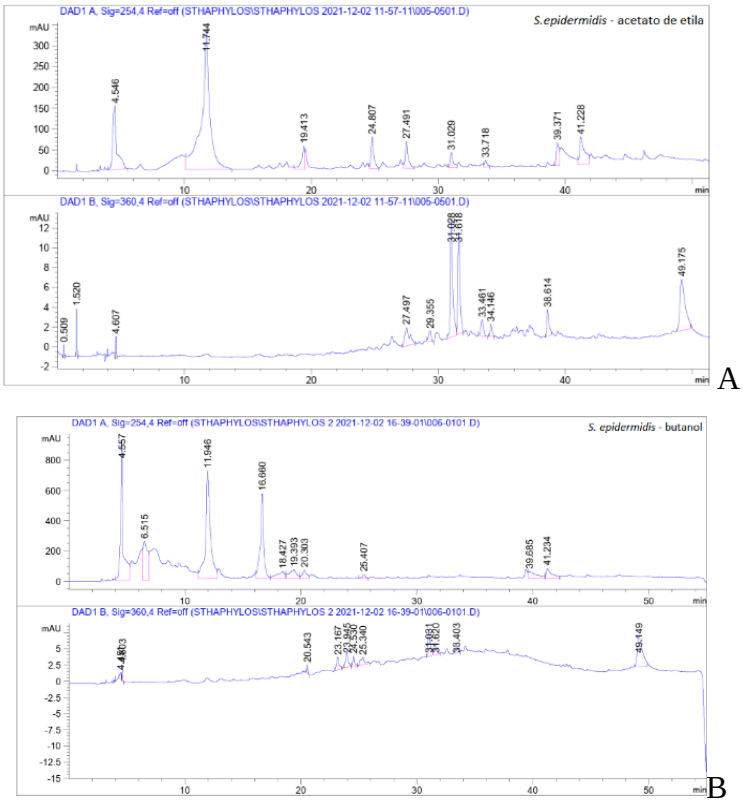

Figure 3. Chromatogram of the *S epidermidis* Acetate (A) and *S. epidermidis* Butanol (B). In the upper part we can observe the wavelength of 254 nm, and in the lower part we can observe the wavelength of 360 nm.

Finally, figure 4 showed the chromatogram of *S. aureus* acetate and *S. aureus* butanol samples. When compared with the Acetate Control (fig.1A), it is possible to observe at a wavelength of 360 nm that after 30 min of run the sample showed peaks indicating the presence of non-polar substances in the sample. In the *S. aureus* butanol sample (fig.4B) when compared with the butanol Control (fig.1B), at a wavelength of 254 nm, ot is possible to note a differentiated peak around 4 and 14 min of running which indicates the presence of a polar substance in the sample.

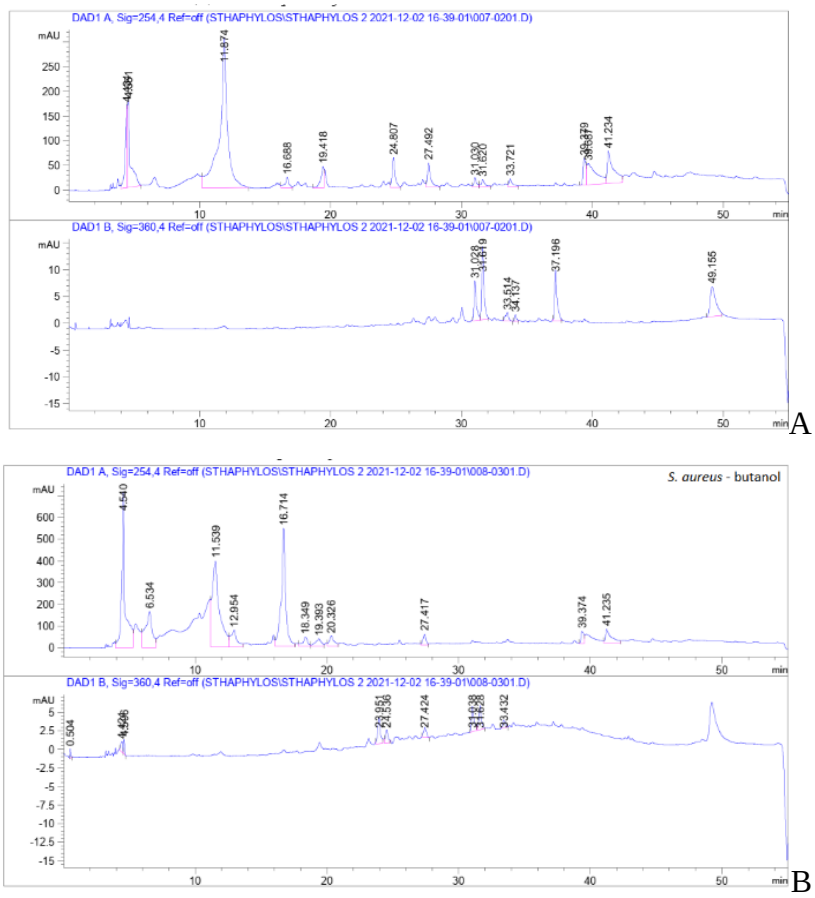

Figure 4. Chromatogram of the *S. aureus* Acetate (A) and *S. aureus* Butanol (B). In the upper part we can observe the wavelength of 254 nm, and in the lower part we can observe the wavelength of 360 nm.

The mass spectrometry analysis showed proteins that was identified by the MALDI-TOF imaging spectrometry method (figure 5). No significant peaks were found in samples with ethyl acetate (data not showed). All samples were compared to the control (in this case Butanol Control) to make sure that this peak was from the sample.

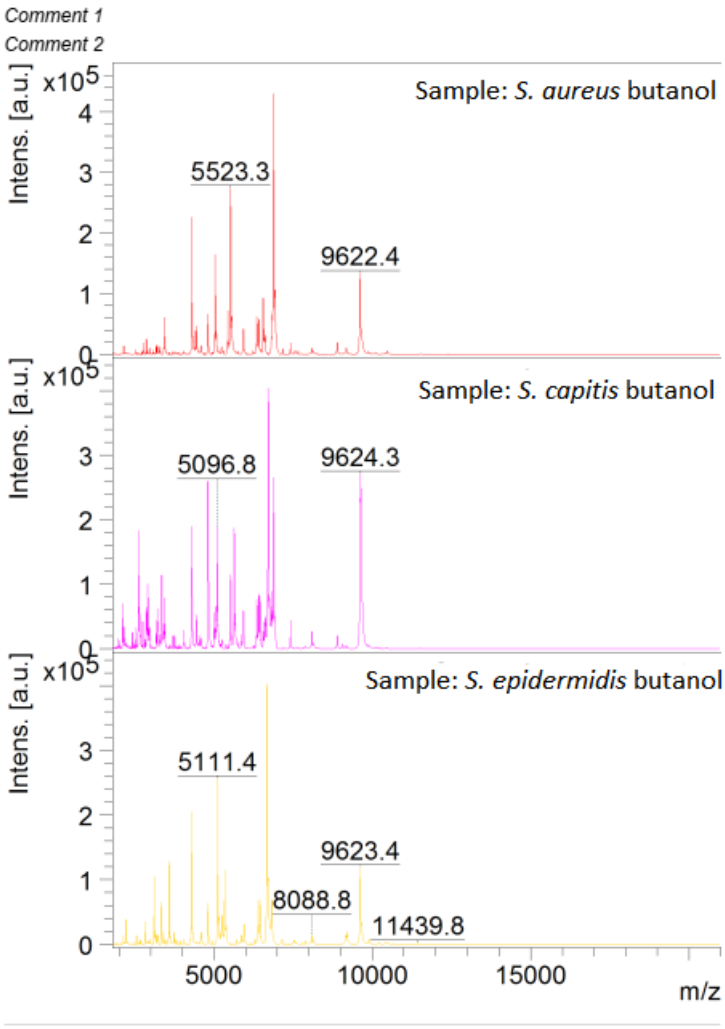

Figure 5. Peak and mass/charge results from the analysis of the three replicates of sample obtained from *S.aureus* butanol extraction (A), *S.capitis* butanol extraction (B) and *S. epidermidis* butanol extraction (C)

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Discussion

The skin is an active organ in which the keratinocytes plays an important role including the immune system and the microbiota. Bacterial stimuli cause the production of antimicrobial peptides (AMPs) and proinflammatory cytokines which interact with memory

T-cells resident in the skin itself. The interaction with the cutaneous bacterial microbiota is essential for atopic dermatitis [14].

There is a strong association between worsening disease severity and lower skin bacterial diversity. AD flares are characterized by low bacterial diversity in the absence of recent treatment. In contrast, intermittent or active treatment is associated with higher bacterial diversity. *S. aureus* is observed during disease flares; the use of AD treatment modifies microbial diversity and proportions of *Staphylococcus sp* [14].

The results showed that both microorganisms chosen, *S. capitis* and *S. epidermidis*, have proteins, which can be potential quorum-sensing molecules. Analyzing the results obtained by HPLC of the samples extracted with ethyl acetate, due to its more nonpolar property [11][12], it was possible to find peaks at the end of the run, at a wavelength of 360 nm, where the largest amount of solvent present in the equipment was methanol, presenting lipid structures, which may indicate a molecule not yet investigated.

The samples obtained through extracts from butanol, due to its more polar property [11], showed peaks at the beginning of the run, with a wavelength of 254 nm, where the largest amount of solvent present in the equipment was water. The peaks found for being of polar character, match the results that we obtained in the MALDI-TOF image spectrometry method, thus confirming that the molecules found in the extracts from butanol are peptides and proteins, and a probable indication of the same being the molecules that we seek from quorum-sensing.

There are already some tests showing the inhibition of the growth of *S. epidermidis* against *S. aureus*, where the type I agr of *S. epidermidis* against the type I, II III and IV agr of *S. aureus* was used where *S. epidermidis* proved to be effective in inhibiting the activity of the type I agr of *S. aureus*, but with no effect on the other agr [9].

It was observed that so far it has not been possible to inhibit the growth of *S. aureus* through bactericidal action, however, other studies have shown that by inhibiting the type I agr system it is possible to inhibit its growth through a bacteriostatic action, being used in this study a molecule of *S. hominis* [9]. With *S. capitis* so far, there is not much information in the literature, however, because it is one of the bacteria that is part of the microbiota of our skin, and because it has a slightly slower growth, we believe that it can have promising results ahead to *S. aureus*.

It is important to understand how the growth of *S. aureus* works, compared to the other microorganisms of our skin microbiota, since *S. aureus* has a sequence variation in agrB, agrD and agrC, which leads to a self-inducing peptide produced only by the *S. aureus*, which can inhibit agr expression in other groups [13].

Conclusion

So far, the research has shown to be promising in identify potential proteins that can be quorum-sensing molecules, through both the MALDI-TOF imaging spectrometry method, with a secondary analysis of HPLC and the presence of lipids through the HPLC method, however our research is ongoing, and more tests will be carried out until your research is completed.

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Conflict of Interest Statement. NONE

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