Effects of marine exopolysaccharides on bacterial adhesion to human skin cells and on biofilm production, applications for cosmetics

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Abstract

Some marine bacteria have the capability to produce exopolysaccharides (EPS) to protect themselves, especially against dehydration during prolonged period out of water, but also to attach themselves to natural supports (rock, wood, algae, …). These EPS have very variable structures. Some EPS contain acid groups (GlcA, GalA), sulfate or acetate groups, or even amino acids (Ala, Ser). These structures give them interesting biological activities, but also effects on microbial adhesion. We evaluated the ability of some marine EPS to inhibit the adhesion of bacteria on human skin cells (corneocytes) using an original adhesion test. Some of the tested EPSs reduce the adhesion of bacteria such as *Cutibacterium acnes* or *Staphylococcus aureus* to the surface of corneocytes by up to 50%. To better understand potential interactions between bacteria on the skin surface (corneocytes), we realised glycoprofiling. Additional experiments were made on *C. acnes* biofilm formation. The presence of EPS modified the production of *C. acnes* biofilm. It was interesting to compare for one EPS to another, its sugar-composition, its action on bacterial adhesion (or inhibition) and/or on metabolism (production of biofilm). EPS and our results are really relevant for cosmetics: for skin that presents unbalanced microbiota (*dysbiosis*), to maintain healthy conditions or to prevent excessive pathogenic strains invasion.

Key words: marine exopolysaccharides, bacteria, adhesion, lectins, biofilm.

Introduction

In natural conditions, some marine bacteria have the capability to produce biofilms composed by exopolysaccharides (EPS). These bacterial structures provide self-protection against other

bacteria and changes of environmental conditions. In addition, they ensure constant humidity around the cells but their role is also to attach themselves to natural supports (rock, wood, algae…) facilitating adhesion and development.

Polysaccharides are the most abundant and most diverse biopolymers on land and in the ocean. Polysaccharide are very complex and diverse macromolecules attested by the very high number of structures - 19.773 entries to date - listed in the "Carbohydrate Structure Databank" (http://csdb.glycoscience.ru/database/index.html) [1, 2, 3]. The very wide structural diversity is explained by the stereochemistry of carbohydrate and the numerous possibilities of linkages between residues.

In addition to the complexity of the carbohydrate backbone, polysaccharides are often decorated by organic (e.g. lactate, acetate, amino acids) and inorganic (e.g. sulfate, phosphate) derivatives, therefore increasing the number of possible structures. Many microorganisms, including marine bacteria, secrete extracellular polysaccharides, called EPS**.** The structural diversity of EPS, which is largely underestimated, constitutes an immense portfolio of novel molecules. Marine EPS have aroused considerable interest, and in vitro experiments highlight their biological activity, including anti-tumor activity, immunostimulatory activity, and anticomplementary activity, as well as the involvement in bone and tissue regeneration [5]. For cosmetic application, literature related already biological effects on epidermal renewal or inflammatory responses and more physical benefits on skin surface such as tensing or mattifying agents [6,7]. The potential biological activity of marine EPS combined with their interesting rheological properties make them very attractive for niche applications in the biomedical and cosmetic sectors.

The production of marine EPSs also present several technical advantages. Large-scale production can be easily controlled, and, in contrast to that of plant and algal EPS, is independent of seasonal variation.

Due to their very high molecular weight, they can be separated from other molecules and lend themselves to high-degree purification.

Codif Technologie Naturelle owns a collection of marine bacteria harvested under natural conditions, in places of varying salinity (open sea, Britany abers, salt marshes, etc.). It produces bacterial EPS by fermentation in a saline environment, and at normal temperatures and atmospheric pressures. The EPS produced have been purified, analysed [8, 9, 10] and tested for their physical and biological effects for cosmetic, pharmaceutical and other applications. For the study, EPS provided by Codif Technologie Naturelle are encoded EPS1, EPS3, EPS4, EPS5 and EPS15.

To mimic bacteria and its support crosstalk due to EPS in a cosmetic application, we focused on microbiome. We wondered if and how marine EPS could influence the skin microbiota comportment in the skin surface.

Materials and methods

Production, Isolation and Purification Exopolysaccharide

Marine bacterial strains were isolated from natural sampling, identified and deposited in the CNCM (Collection Nationale de Cultures de Microorganismes / *National Collection for Microorganisms Culture*).

Exopolysaccharide were produced by fermentation of marine microorganism in a fermenter containing marine broth medium supplemented with sugar at 25°C. The culture medium was inoculated at 10% (v/v) with the bacterial suspension in the exponential growth phase. The pH was adjusted to the optimum and maintained by the automatic addition of 1 M NaOH or 1 M H2SO4. The medium was oxygenated and agitated. After 72 h of fermentation, bacterial cells were removed from the culture medium by centrifugation. Then, the supernatant, containing the excreted EPS, was purified by filtration through a 1 µm filter sheet, by ultrafiltration (300 kDa) and by precipitation with isopropanol. The obtained EPS were dried to remove isopropanol and crushed.

Chemical Composition and origin

EPS code	Genus and species of the producer	Osidic composition	Other groups linked to the EPS
EPS1	*Vibrio alginolyticus*	Galactose, Lactate-Glucuronic acid or Nosturonic acid, N-Acetyl glucosamine, Galacturonic acid [8]	Lactate on Glucuronic acid Alanine on Galacturonic acid
EPS3	*Alteromonas macleodii*	Glucuronic acid, Glucose Mannose, Galactose, Galacturonic acid, N-acetylglucosamine	Alanine
EPS4	*Cobetia marina*	Glucose, Galacturonic acid, Rhamnose, Galactose,Acetyl glucosamine	Sulfate, Serine, threonine
EPS5	*Vibrio alginolyticus*	Galacturonic acid, N-Acetyl, glucosamine [9]	Alanine and Serine
EPS15	*Vibrio alginolyticus*	Galactose, N-Acetyl glucosamine, N-acetylguluronic acid/3-Acetylated N-acetylguluronic acid [10]	

Figure 1: composition of the different EPS

Size-exclusion chromatography (SEC), confirmed that the purified EPS is likely composed of one species of molecule having a molecular weight (MW) superior to of 1.4 MDa in Dextran equivalent

Composition analysis using gas chromatography (GC), after the complete hydrolysis of the polysaccharide and derivatisation of the products, revealed the osidic relative composition (see figure 1).

Corneocytes adhesion assay

Corneocytes used in this study were sampled (according to standardised procedure of patch pressure) on the day of the study from healthy volunteers, using D-Squams® disks purchased from Monaderm (Monaco).

All microorganisms used for the adhesion studies on corneocytes were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA): *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 6538), *Cutibacterium acnes* (ATCC 11827). Trypton soya broth (TSB) was purchased from Oxoïd (Basingstoke, UK) and used for the culture of *Staphyloccus* strains. Brucella broth was purchased from Condalab (Madrid, Spain) and was used for the culture of *Cutibacterium acnes*. Each microbial strain was grown according to the ATCC recommendation.

The labelling of microorganism is performed with carboxyfluorescein diacetate succinimidylester (CFDA-SE) purchased from Sigma-Aldrich (St. Louis, MO, USA) according to the supplier's technical note.

The assessment of interactions of microorganism on cell surfaces was achieved according GLYcoDiag's protocol. Briefly, the fresh corneocytes were harvested from the skin with D-Squams disks and were plated in 24 well black microplate, then incubated with a bovine serum albumine solution (BSA) in order to saturate the non-specific interactions at the surface of the cells. Afterwards, corneocytes were incubated for 1 h at room temperature with EPS at three concentrations (0.01%, 0.001% and 0.0001%) in triplicate. The cells were washed three times with 500 µL of PBS, then the labelled microorganisms were added in each well at 1.109 CFU/mL and incubated at room temperature for 2 h. After incubation, the wells containing the D-squams disks were washed three times gently with PBS. Finally, the fluorescence intensities were measured using a microplate reader (λex = 485 nm, λem = 530 nm, Fluostar OPTIMA, BMG LABTECH, France). In parallel, a calibration curve was achieved

with the labelled microbial strains solution to determine the number of microorganism that stayed in interactions with the cells.

Additionally, three test controls are prepared and used in the same way as EPS in this study: (1) a positive control corresponding to the adhesion of microorganism in absence of EPS; (2) a negative cell / microorganism adhesion control consisting of 0.5 M sucrose and (3) a medium control on the cells to determine their auto-fluorescence.

Biofilm production assay

The BioFilm Ring Test® (BRT) method was performed to assess the activity of the EPS in preventing biofilm formation. This test was realised by BioFilm Control according to its knowledge. Two types of strains were experimented, both C. acnes, but batches isolated from T zone on healthy skin and other batches isolated from forehead on acneic skin. All strains were subcultured from -20°C in M20 medium and *C. acnes* was incubated at 37°C under anaerobic conditions. Three concentrations were tested to study the preventive activity. EPS were diluted in sterile demineralised water (ED) and were concentrated 10X. Then, 20 µL of each compound were added into a 96 well-microplate. Each condition was repeated 3 times on the microplate (3 wells) and 3 biological replicates were performed (3 different bacterial suspensions).

The mobility of microbeads in presence of EPS is experimented first to verify that the BRT can be used. For that, 20 µL of each concentration of EPS 10 X were filled in the wells in triplicate and 180 µL of M20 medium with the magnetic beads (TON004) at 10 µL/mL were added in the wells. Then, microplates were incubated under anaerobic condition at 37°C during 32 h. Next, the microplate was magnetized for 1 min, scanned and analyzed with the BFC Elements 3.0 software. Biofilm Formation Index (BFI) was determined for each well to quantify the biofilm formation. BFI reveals the microbeads aggregation score: when the BFI is higher than 16, the microbeads are free to move and no interaction is observed between the EPS and the microbeads.

According to BFC knowledge, the 2 strains of *C. acnes* of the study present an adhesion at 32 h of incubation with BRT. So, the BRT was performed at 32 h in order to measure the activity of EPS at the bacterial adhesion time, and a second time (48 h) was chosen to discriminate an inhibition activity from a delayed activity.

The initial bacterial suspension was prepared in M20 medium, concentrated at 10^6 CFU/mL by measure of absorbance (OD 600 nm) and filled in each well with the magnetic beads (TON004) at 10 µL/mL. In parallel, several controls were prepared. Then, microplates were incubated under anaerobic condition at 37°C. After 32 h, the microplate was magnetized for 1 min,

scanned and analysed with the BFC Elements 3.0 software. Biofilm Formation Index (BFI) was determined for each well to quantify the biofilm formation.

BFI reveals the microbeads aggregation score: when the BFI is higher than 16, the microbeads are free to move and no biofilm is formed. When the BFI is lower than 2, more than 90% of the microbeads are trapped in the biofilm. Between BFI 2 and 16, the adhesion is partial.

In parallel with BRT, a bactericidal effect of the EPS was evaluated by spreading one well per condition on M20 agar plates and incubated at 37°C in anaerobic condition.

Doxycyclin (DOX), the active ingredient from Doxylis, was used as positive activity control. This widely used treatment for acne was tested here at 64 µg/mL.

Results

Corneocytes adhesion assay

Graphs on the figure 2 let us to compare the different EPS profiles for corneocytes adhesion, same scale, same concentration of use (0.0001%, 0.001% and 0.01%), for 3 strains of interest *C. acnes*, *S. aureus* and *S. epidermidis*.

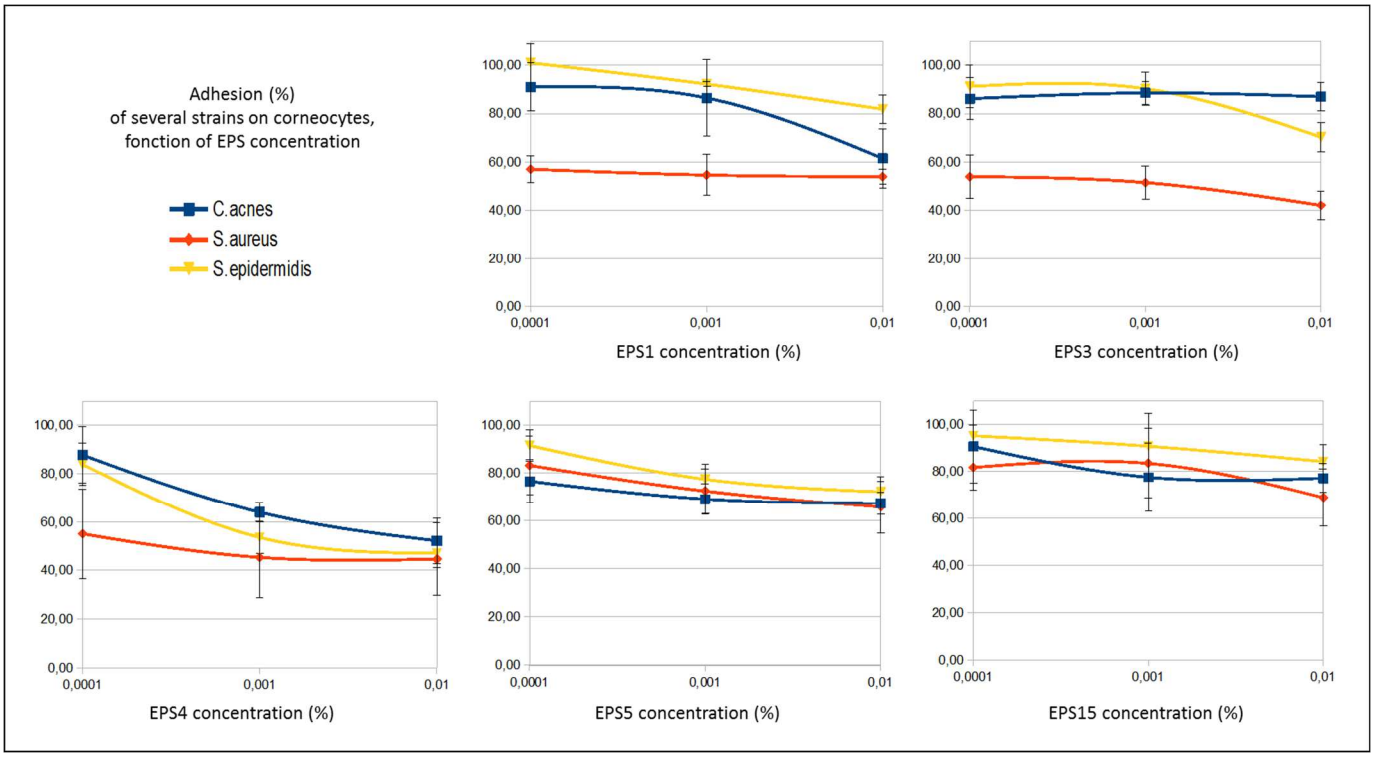


Figure 2: Adhesion of several strains in percentage against the control condition (without EPS).

EPS1 decreased adhesion of *S. aureus* on corneocytes, while it presented no effect (less than 20%) for *S. epidermidis.* Its structure may explain that distinction between *Staphylococcus*

strains, promoting adhesion of non-pathogenic strain (*S. epidermidis*), and inhibiting *S. aureus* that induce pathogenicity. EPS1 remained without any effect *C. acnes*, except for the higher dose (reduction of 38% for 0.01%). EPS3 present almost the same different profile on *Staphylococcus* but no effect any more on *C. acnes*. EPS4 is very close to two first examples with the distinction but the effect on *C. acnes* seems to exhibit a dose-response manner and seems to be higher. EPS5 and EPS15 showed similar profiles with very low and stable inhibition of all strains, without real distinction.

To go further with the biofilm production, we decided to focus on EPS1, EPS3 and EPS4, as they present adhesion profiles that are more complementary.

Biofilm production assay

First, the mobility of the microbeads in presence of the compounds was evaluated (data not shown). With a higher BFI than 16, the microbeads are free to move in all the conditions, meaning that BRT can be used to evaluated the activity of the compounds on the biofilm inhibition.

The biofilm formation was monitored over two time periods: the adhesion time and a second, later time period which confirms the inhibition (when observed) or indicates a delayed effect of biofilm formation.

Results are presented per EPS at 32h and 48h. The mean values of the tests are shown with their associated confidence intervals. In comparison with the “strain control” values, trials with significantly different results are indicated by a star.

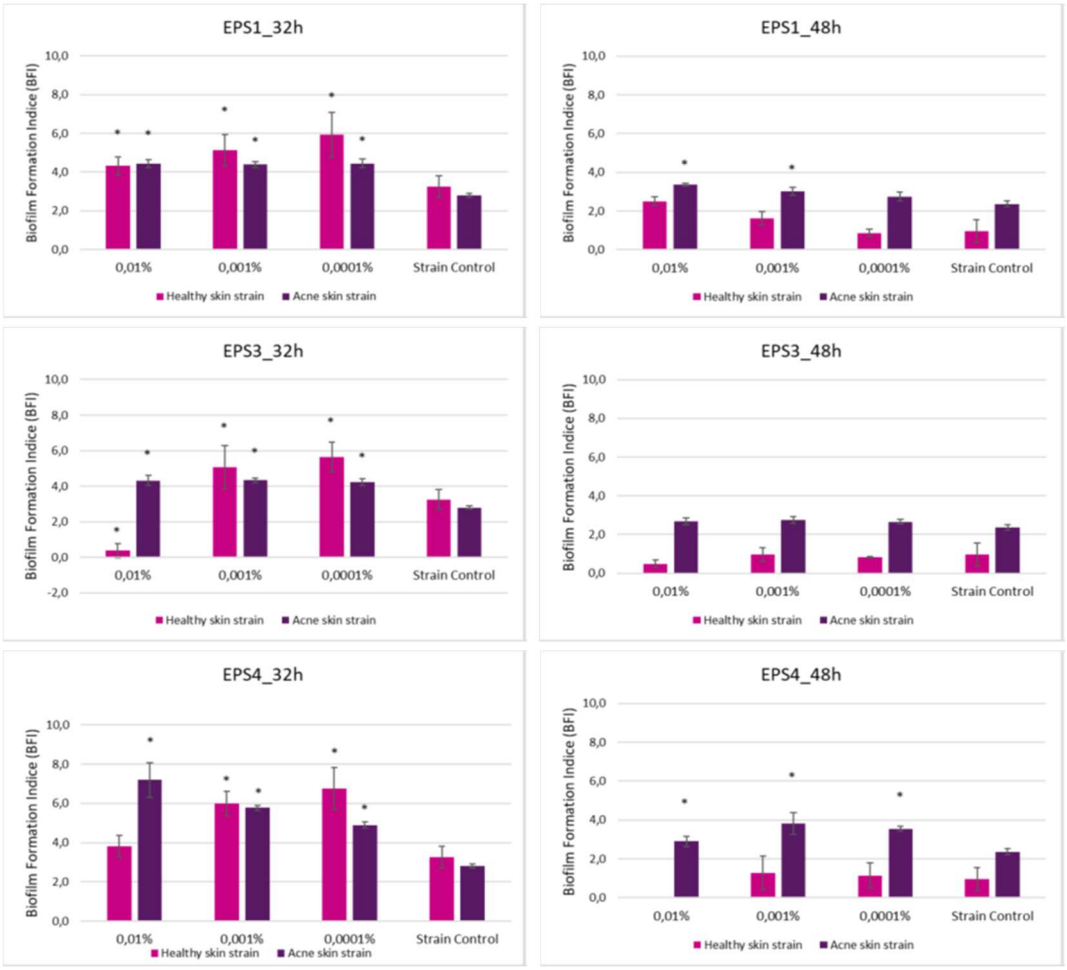
The higher the BFI index, the more the beads are mobilized, reflecting a limiting effect on the biofilm formation. When BFI>16, the biofilm formation is inhibited, under this value the inhibition is partially observed and < 4, no inhibition is measured.

No growth inhibition was observed on the control agar-plates (data not shown), the EPS could partially inhibit biofilm formation without killing the bacteria.

As shown in figure 3, for the 3 products, partial biofilm formation inhibitions were measured at 32h mainly. This activity decreased along the time. No total inhibition of biofilm formation was visible. Depending on the product and its concentration, significant differences were measured between the activity on a strain from healthy skin and a strain from acneic skin. EPS4 at 0.1% could be of interest to go further with statistical complementary analysis.

Among the panel of 6 new strains, three were isolated from healthy skins and the three others were collected on acneic skins from patients followed in a dermatology department. The

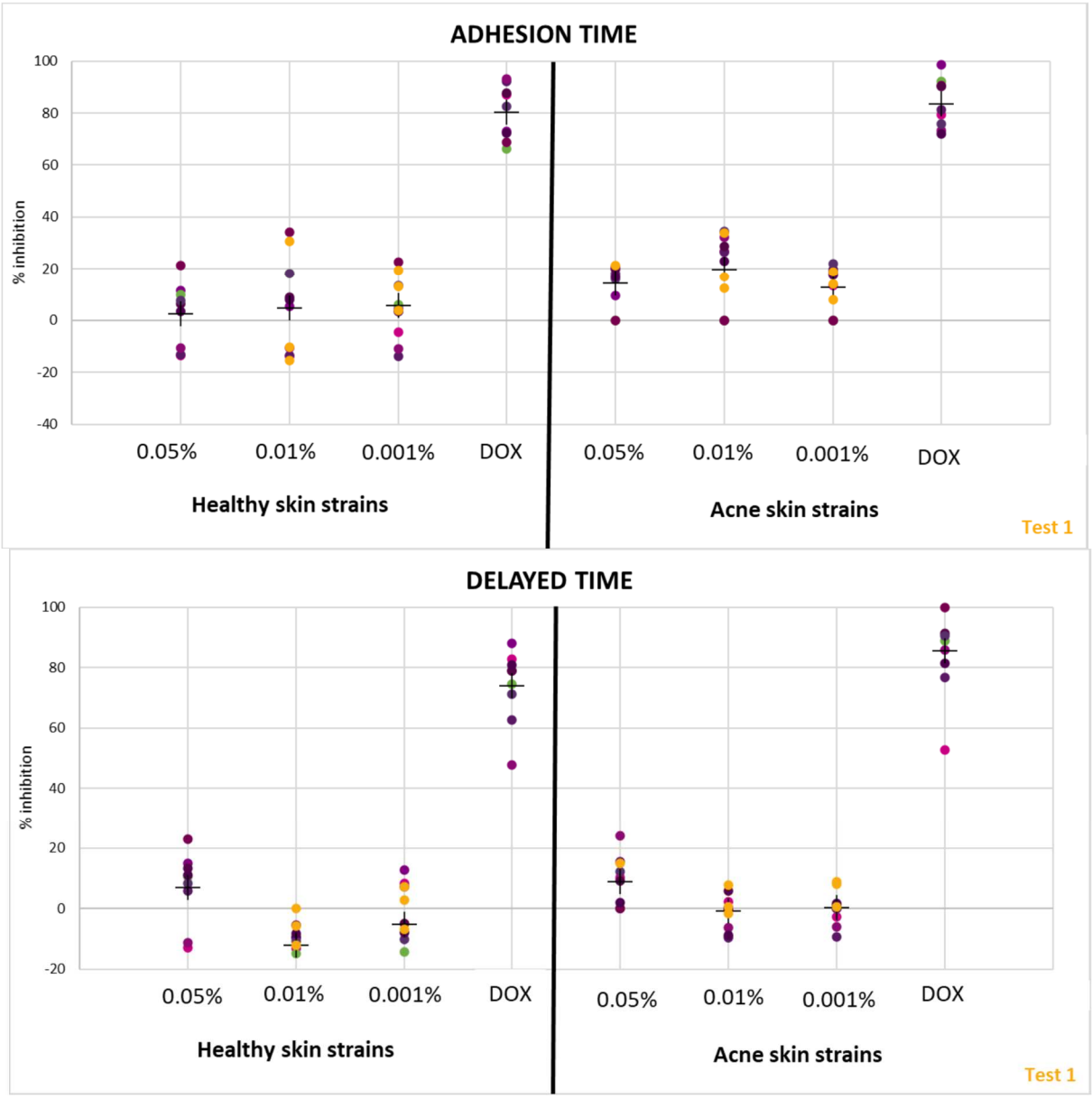
BioFilm Ring Test® (BRT) method was performed once again to assess the activity of the EPS in preventing biofilm formation.



<u>Figure 3:</u> Biofilm Formation Index, for 3 different EPS, 2 strains of *C. acnes* and 2 times of kinetics (32 and 48h).

Results from each strain and condition are summarised in figure 4 at the adhesion time and delayed time (8 hours after the adhesion time). The conditions that were already tested in the previous study are added in yellow as “Test 1”. The average value is represented by a cross.

The presence of aberrant points was checked with a Grubbs Test. On all data, no point was identified as aberrant.



<u>Figure 4</u>: Biofilm Formation Inhibition expressed in percentage, for EPS4, 3*2 strains of *C. acnes* and 2 times of kinetics (adhesion time an delayed time, 8h later).

The DOX control indicated a strong inhibition in the biofilm formation for all the strains and this lasts until the delayed time. The EPS 4 showed a partial inhibition (between 5 and 35%) without a dose effect. This partial activity is maintaining in the time only at the 0.05% concentration.

At each time, a t-test was realised to compare the global activity of the EPS on the 2 independent samples: % inhibition from healthy skin strains vs % inhibition from acne skin strains.

We realised statistical analysis as the number of results was 33 for healthy skin strains and 34 for acneic skin strains.

The conditions that were already tested in the previous study were also added. On figure 5, the average value is represented by a cross and the median by a dotted line.

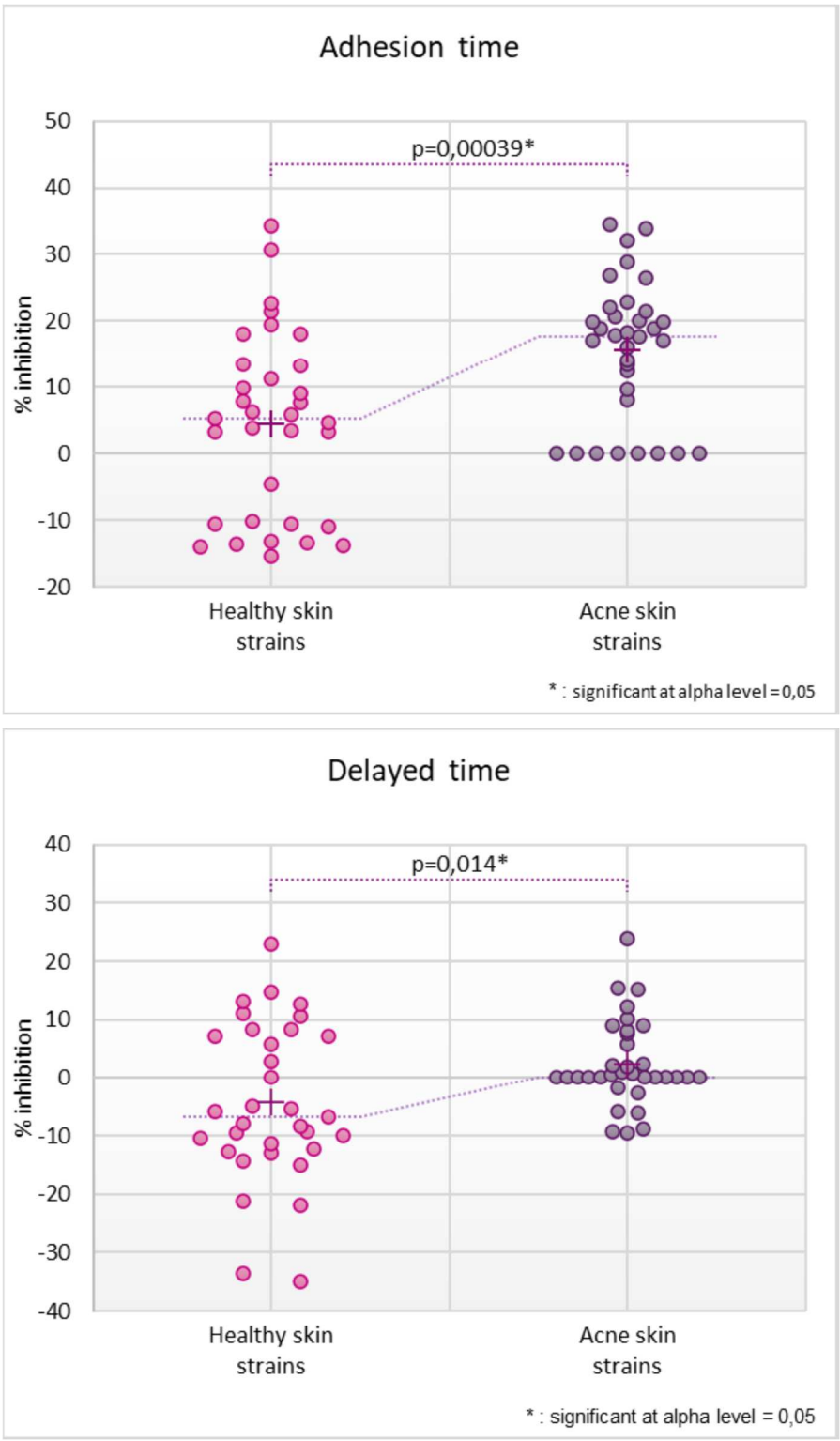


Figure 5: statistical analysis and repartition of all points for all the experiments

The p-value is equal to 0.00039 at the adhesion time, and 0.014 at the delayed time. As these p-values are less than alpha risk (0.05), averages between the two groups are significantly different. The EPS4 is more efficient on the acne skin strains that on healthy skin strains at the both times (adhesion time and delayed time after 8h).

Some pictures illustrating the different observed activities are shown in Figure 6.

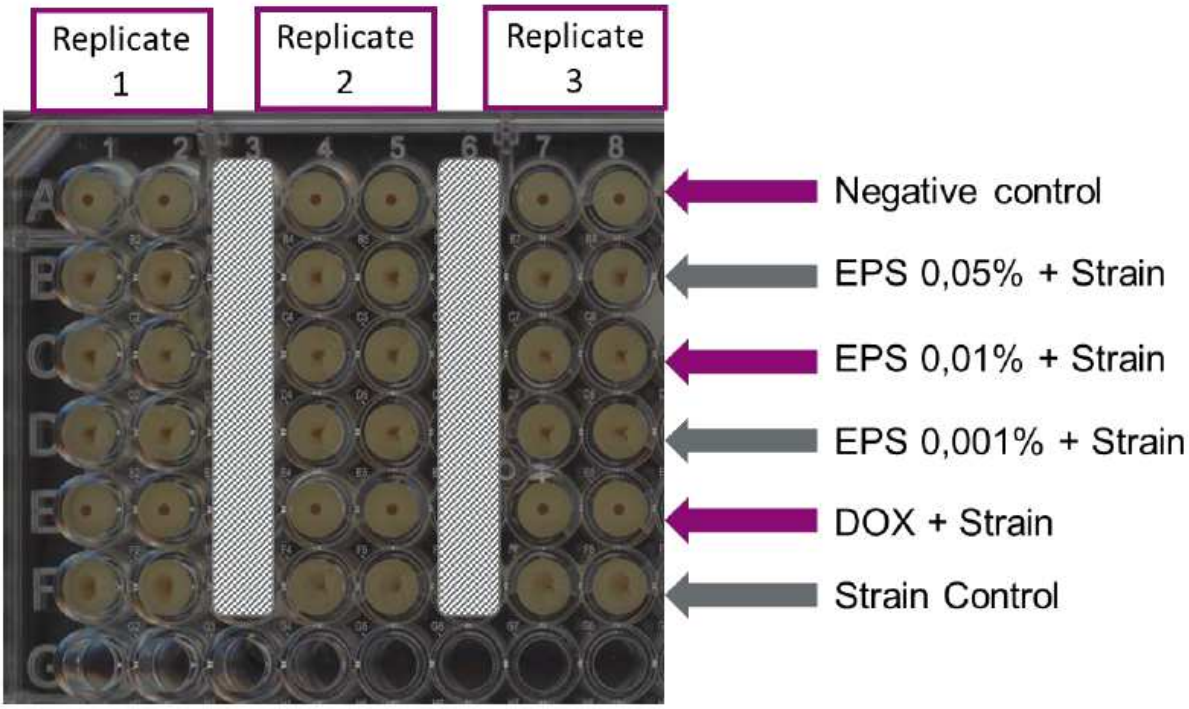


Figure 6: Observation of a culture plate containing produced biofilm and bacterial strains, without or with DOX or an EPS at 3 concentrations

Finally, no growth inhibition was observed on the agar-plates, like in our previous experiment (data not shown). The EPS4 could partially inhibit biofilm formation without killing the bacteria.

Although the activity of EPS4 is partial and concentration independent on the inhibition of biofilm formation, its activity is significantly higher on strains from acne than on strains from healthy volunteers.

Discussion

Many studies have previously showed the effects of some products on the cutaneous bacteria using growth inhibition tests. In this study, we wanted to explore the crosstalk between bacteria

and skin using a test of adhesion. We wondered if and how marine EPS could influence the skin microbiota comportment in the skin surface.

First, we wanted to define more precisely the structure and the glycans pattern of our EPS, to imagine also relations structure-efficacy. To better understand the potential interactions between bacteria on the skin surface (corneocytes), we realised glycoprofiling. Bacteria are well known to interact with their support towards lectins or glycan binding proteins (GBP), as membranes of human cells wear these molecules. Lectins and GBP are glycoproteins enabled to present specific (and releasable) affinity with glycans or oligosides. They are involve in cell-cell recognition / communication for different biological process. We performed glycoprofiling of each EPS according to an indirect method that demonstrated the inhibition of lectin/glycans interaction, developed by GLYcoDiag. Thus, thanks to these complementary informations about EPS composition and interactions, we imagined a competition in lectin/GBP recognition on the skin surface and/or on the bacteria surface. This competition could explain inhibition of bacteria adhesion on any support. In addition, we wanted to experience the results on skin cells support. We selected three common strains of skin microbiota and tested them on the skin cells that are in contact with this microbiota: corneocytes, the most superficial epidermal keratinocytes layer, also known as the *stratum corneum*.

In this step, we measured the adhesion capabilities of different common bacteria in presence of EPS. (1) *Staphylococcus epidermidis* (S. epidermidis) is a commensal bacterium, the more common of the skin surface. (2) *Staphylococcus aureus* (S. aureus) is a pathogenic bacterium responsible for numerous skin affections. (3) *Cutibacterium acnes* (C. acnes), is a well-known anaerobic bacterium, implicated in acne lesions and acidification of skin surface due to its metabolic release of propionic acid. Even if important inter-individual variations exist, the three bacteria and their ratio are considered as major indicators for healthy skin surface. To quantify bacteria adhesion, the strains, previously labelled with carboxyfluorescein diacetate N-succinimidyl ester (CFSE), were applied on corneocytes (collected with adhesive disposal) during two hours, in presence of different concentrations of different EPS (5 EPS that present different structures and glycoprofiles). After incubation then washes, adhesion of strains was calculated versus the control condition (EPS presented no effect on bacterial growth).

Adhesion profiles showed very different results from one EPS to another. For example, some of them (EPS1 and EPS3) inhibited *S. aureus* adhesion in the same manner but EPS1 only decreased *C. acnes* adhesion (EPS3 remained without any effect on *C. acnes*), while EPS3 inhibited *S. epidermidis* adhesion (EPS1 presented no effect on *S. epidermidis*). Another one (EPS5) inhibited the adhesion of all the strains in a dose dependant manner.

In a second aspect of the study, we examined with additional experiments the effect of presence of EPS on biofilm production. As for marine bacteria and numerous bacteria, bacteria of the skin surface are able to create specific biofilm that correspond to a normal step of development with the production of an adhesive and protective matrix. We used the BioFilm Ring Test® technology developed by BioFilm Control. It is based on superparamagnetical microbeads mobility measurement under magnetic field. The biofilm influences the mobility and decreases the capacity of beads movements. The substance that modifies the signal, modifies also the production of biofilm.

For this stage, we focused on two strains of *C. acnes* (one of healthy skin and one sampled on acneic lesions) and three EPS only (those presenting the main three different profiles of adhesion). On the three selected EPS, only EPS4 exhibited a trend to decrease biofilm production. After complementary experiments with different batches of each type of *C. acnes*, we realised statistical analyses that proved the significant inhibition of biofilm production due to the presence of EPS4, in both strains (healthy and pathological). Although the activity of EPS4 is partial and concentration independent on the inhibition of biofilm formation, its activity is significantly higher on strains from acne than on strains from healthy volunteers.

Taking together all the results, we could associate structures of the EPS, their effects on bacteria adhesion and biofilm production. It is interesting to note that EPS presenting the quite same adhesion profiles (EPS5 and 15 for all strains or EPS1 and 3 for *Staphylococcus* genius) are so different in structure. If we focused on *Staphylococcus aureus*, EPS 5 presented no effect while EPS1 and EPS4 inhibited adhesion on corneocytes. The structure of EPS5 appeared simpler than both others. If avoiding simple sugars, presence of glucuronic acid or lactate-glucuronic acid may be an explanation of those differences. It might be confirmed with control experience. In another hand, one EPS (1 or 3) can decrease adhesion of *Staphylococcus aureus* without any effect on *Staphylococcus epidermidis.* That suggests different systems of adhesion for bacteria even if they belong to the same biological genius.

Combining adhesion and biofilm data, we saw that on 3 EPS decreasing *Cutibacterium acnes* adhesion, only EPS4 decreased production of biofilm as well; and none EPS effected bacteria growth.

Our results exhibited and reinforced the interest of EPS in skin biology and skin microbiome. They also initiated the discussion on skin microbiota. Influencing its growth is not the only solution, when it is possible to modulate adhesion in a selective manner or to modify the behaviour of cells (biofilm production for example).

Conclusion

Results of our study show that marine EPS are not only physical fil-formers. They also play an important role in glycobiological interaction processes. They are recognised by some specific receptors for carbohydrates. Thus, they can interfere with human corneocytes and lock competitively bacterial survey (adhesion on skin surface and biofilm production). That are really interesting for cosmetic applications, and especially for skin that presents unbalanced microbiota (*dysbiosis*), in order to maintain healthy conditions or to prevent excessive pathogenic strains invasion.

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Conflict of interest: none

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