Saline nasal spray removal of bacterial biofilms, using a novel *in vitro* method

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ABSTRACT *— OBJECTIVE: About 30% of the human population are nasal carriers of* Staphylococcus aureus*. Although often asymptotic, nasal carriers have an increased risk of infections. Decolonization of nasal* S. aureus*, by biofilm dispersal has been suggested as an important treatment strategy for reducing infection. Improvement of nasal inflammatory symptoms by saline irrigation is thought to be achieved by biofilm reduction, however the contribution by the dynamic force of the irrigation devices has to our knowledge not been investigated. Preclinical testing of treatments targeting nasal biofilm needs novel* in vitro *test methodology. This study aimed to develop an* in vitro *model for nasal bacterial biofilms and, using this model, evaluate the efficacy of saline nasal sprays in removing biofilm by physical force.*

MATERIALS AND METHODS: S. aureus *biofilm was allowed to establish in the presence of mucin on a synthetic membrane placed on nutrient agar. The biofilm-membrane assembly was then placed in the insert of a two-compartment transwell system and treated with nasal spray. The removed biofilm, dislocated to the lower compartment, and biofilm remaining on the membrane, were quantitatively analyzed. Other possible effects on the biofilm were analyzed using a microtiter plate biofilm assay.*

RESULTS: Treatment with the tested nasal sprays removed the main part of the S. aureus biofilm from the membranes as demonstrated in the in vitro *nasal biofilm model. Data from the microtiter plate biofilm assay showed that the nasal spray solution containing plant extracts had higher reducing effect also at static conditions.*

CONCLUSIONS: This novel method, the Colony biofilm transwell assay, was proven useful for in vitro evaluations of nasal sprays when the effect of physical removal of biofilm needs to be simulated. With this method, we could demonstrate that nasal sprays may have a substantial reducing effect on S. aureus *biofilms on a simulated nasal mucosa.*

KEYWORDS
Bacterial biofilm, Nasal carrier, Staphylococcus aureus, *Nasal spray, Biofilm dispersal, Dynamic force.*

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INTRODUCTION

Almost one third of the human population is chronically colonized with nasal *Staphylococcus aureus* (*S. aureus*). Although often without any symptoms of infection, *S. aureus* nasal carriers have an increased risk of infections with this pathogen. Furthermore, *S. aureus* is an important cause of health care associated infections whereby nasal transport of *S. aureus* from a carrier can be the cause for infections in other patients[1-3]. Effective measures to prevent *S. aureus* infections are urgently needed since this pathogen is exhibiting increased resistance to several antibiotics[4].

S. aureus is one of the most common bacteria forming biofilm in the nasal cavity[5-9]. Biofilms are aggregates of bacteria embedded in an extracellular polymeric matrix, enabling bacterial colonization in the host tissue without detection by the host immune system. Tolerance to antibiotics and other antimicrobials is also more pronounced in biofilm bacteria. The role of bacterial biofilms in persistent infections and chronic inflammatory diseases is increasingly recognized. Biofilm dispersal has been suggested as a potential means of treating persistent *S. aureus* infections thereby exposing the bacteria and render them vulnerable to host immune defense[10]. Also, increased susceptibility to antibiotics has been observed upon treatment with biofilm dispersal agents[11]. In addition, dispersal methods could potentially be used to prevent biofilm infections. It has been shown that active surveillance for *S. aureus* nasal carriage, in combination with decolonization treatment is associated with a decreased incidence of *S. aureus* associated hospital-acquired infection[12].

Nasal saline irrigation is commonly used to reduce symptoms of inflammatory conditions in the nasal cavity. The amount of saline reaching the inflamed or infected area depends on the irrigation volume and method used, such as nasal lavage or rinse, or a spraying device that adds a dynamic force to the irrigation. Several plausible mechanisms have been suggested for the action of saline on the symptoms, such as moisturizing, reduction of inflammation and the reduction of bacterial load and biofilms[13].

Published data on the effect of nasal irrigation solutions on bacterial biofilm are from clinical investigations or animal test models. Such *in vitro* experiments found are often performed using clinical isolates from nasal biofilms but without further experimental design to simulate the nasal environment. For the investigation of the dynamics of nasal sprays there is, to our knowledge, no model published. Therefore, testing of potentially effective treatments addressing nasal biofilms requires development of new test methodology, preferably *in vitro*, mimicking the "real situation". *In vitro* screening of product concepts is necessary as a first step to avoid large test series *in vivo* and for the design of subsequent animal models.

The objectives of the present study were to design a relevant *in vitro* model for *S. aureus* nasal biofilms and, using this model, evaluate the potential efficacy of four different saline nasal spray products.

MATERIALS AND METHODS

Rationale for the Colony biofilm transwell assay

The Colony biofilm transwell assay (CBTA) was developed to study the effect of nasal sprays, *in vitro*, on established bacterial biofilms in a nasal-like environment. The overall goal was to mimic the *in vivo* situation and thus increase the predictive value of the method. Three parameters were of particular interest in the model: the characteristics of the nasal biofilm; the application of the treatment; and the possibility to recover dislocated biofilm, following application of physical force. To further elucidate other possible effects on the biofilm, the Microtiter plate biofilm assay with crystal violet staining was used[14].

To mimic the nasal cavity, the assay is based on the establishment of a *S. aureus* biofilm, in the presence of mucin, on a synthetic membrane placed on nutrient agar. Prior to the treatment, the biofilm-membrane assembly is transferred to the porous surface of the inner well of a two-compartment system. Any part of the biofilm that is dislocated from the membrane, as a result of the treatment, ends up in the lower compartment of the system and can be retrieved for analysis. After treatment, the membrane assembly with the treated biofilm may be analyzed directly for the physical effect of the spray or transferred to a new fresh agar plate. Since the biofilm is preserved by transferring the biofilm-membrane assembly, this method also allows repetitive treatments of the biofilm. The three principal steps in the CBTA are described in Figure 1.

Nasal sprays tested in the study

Four saline based nasal spray products contained in 100 ml canisters (regular size) were tested. The canisters are provided with bag-on-valve technology with different pressure applied in the spray canister, allowing for adjustment of spray force for a certain product and volume of sprayed solution for the user (Table 1).

Preparation of bacterial inoculum

S. aureus ATCC 29213 (same strain as CCUG 15915) (CCUG, Gothenburg, Sweden) was chosen for all biofilm tests. Streak plates were prepared on tryptic soy agar (TSA) and incubated for 24 h at 36 ± 1°C. Culture purity and characterization of *S. aureus* were analyzed macroscopically and microscopically by Gram staining. 1 - 3 colonies were inoculated to 5 ml tryptic soy broth

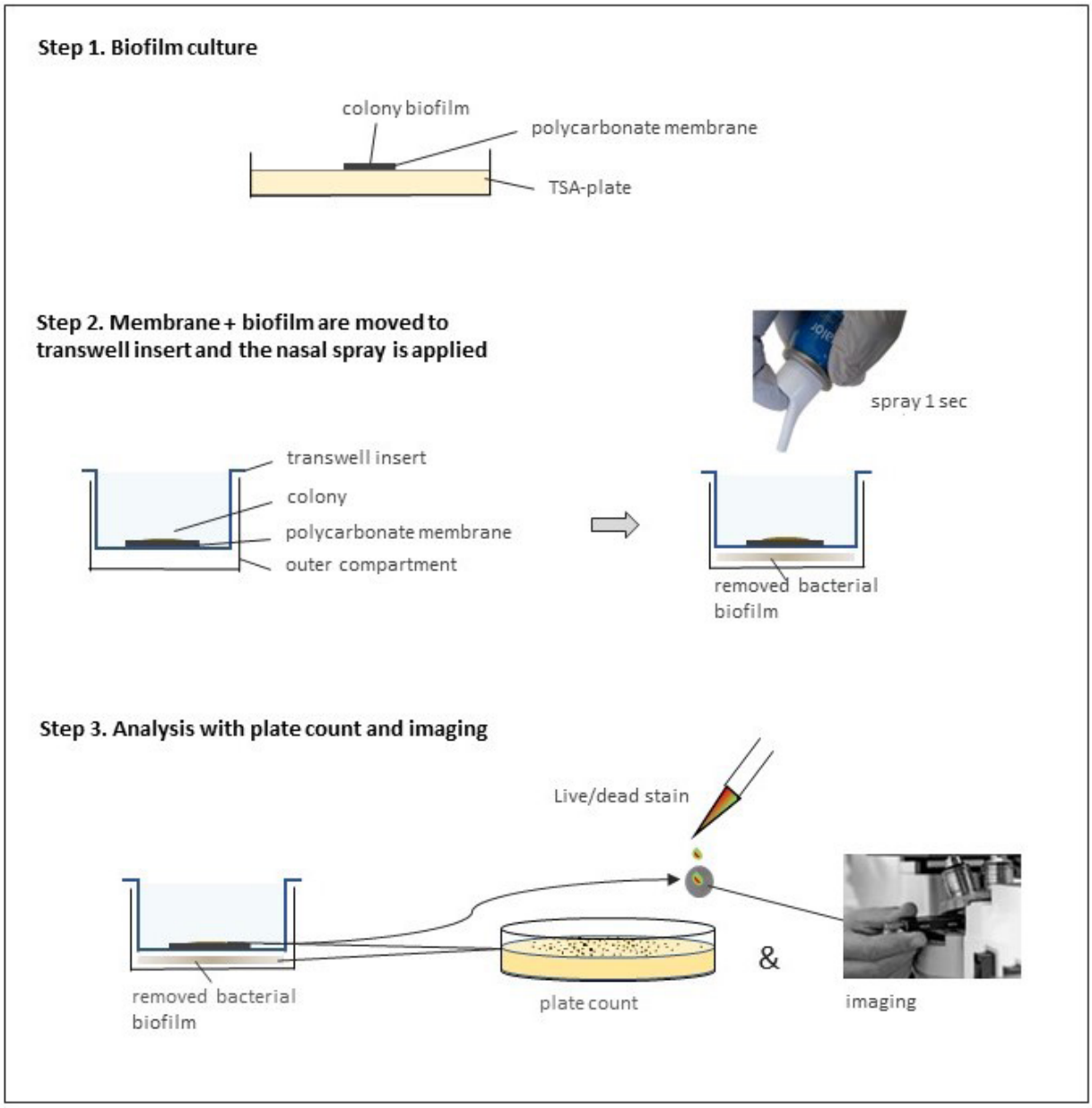

Figure 1. *The three principal steps in the Colony Transwell Biofilm Assay.*
Step 1: Colony biofilm is formed on a polycarbonate membrane placed on a nutrient agar plate (TSA) and incubated for 24 h in 36 ± 1°C. ***Step 2:*** The membrane with the established biofilm is transferred to the transwell insert whereupon the biofilm is treated with a nasal spray.
Step 3: The remaining biofilm on the membrane, as well as the removed biofilm that has ended up in the lower compartment (via large pores allowing passage of bacterial clusters), are analyzed by plate count. In parallel, additional biofilm-membrane assemblies are analyzed by fluorescence microscopy.

(TSB) in culture tubes and incubated at 36 ± 1°C for 24 h. The cell density was measured spectrophotometrically at 475 nm and adjusted with TSB to OD 0.28 at 475 nm. This equals a concentration of 1.5-3.0 $\times 10^8$ CFU/ml for most bacteria and confirmed by a calibration curve.

Colony biofilm transwell assay

Loopfuls of *S. aureus* colonies were resuspended in 5 ml TSB and mixed with mucin (Mucin type II, Sigma-Aldrich, St. Louis, MO, USA), to obtain a final OD at 475 nm of 0.28 and a mucin concentration of 2%. Punched circular UV-irradiated pieces, 5 mm Ø, of polycarbonate membranes (original size 25 mm Ø and pore size 0.2 µm (DHI Lab Products, Hørsholm, Denmark) were placed on TSA plates. One µl of the bacteria/mucin mixture was applied onto the center of each membrane and the plates were incubated at 36 ± 1°C for 24 h. Each membrane with formed biofilm was then transferred onto the porous bottom of a cell strainer, pore size 70 µm (Fisher Scientific, Article no. 11597522). Prior to the spray treatment, the strainer was placed in a well in a 6-well culture dish (Nunc, Article no. 150239).

Table I. Nasal sprays included in the study. The four nasal sprays tested in the study are from the product portfolio of Aurena Laboratories AB. Here the different spray products are given abbreviations according to the content in the spray solution. All spray products are contained in bag-on-valve spray canisters with a certain pressure applied (5.8 or 7.2 bar), which besides different physical force also adjust the sprayed volume per second. The manufacturers recommend spraying in each nostril for 1-2 seconds.

Product names in study	Product description	Volume spray/sec (µl)
ANS-Xyl	Nasal spray, 0.9% sea salt solution with 2% xylitol; 5.8 bar, fine mist spray actuator	589
ANS-AC	Nasal spray, 2.1% sea salt solution with Aloe vera (0.025%, *Aloe barbadensis mill.*, freeze dried extract of the inner leaf gel (200:1]) and Roman Chamomile (0.025%, *Chamaemelum nobile,* ethanol extraction (1:1)); 5.8 bar, fine mist spray actuator	464
ASNS-hyp	Saline nasal spray, hypertonic (2.1%); 7.2 bar, fine mist spray actuator	502
ASNS-iso	Saline nasal spray, isotonic; 7.2 bar, Jet stream actuator (flow 19.7)	818

During treatment, the spray is applied directly from the respective canister at 5 cm distance above the colony biofilm on the membrane, simulating a real treatment situation. A spray time of one second was chosen following manufacturers' recommendation. Untreated biofilm control was also included. The repeated daily treatment of the colony biofilm was performed in the same way as one single treatment. Directly after each of the first three treatments the colony-biofilm membrane assembly was transferred to a fresh TSA-plate, followed by incubation of the plate at 36 ± 1°C for 24 h.

The biofilm load remaining on the membrane, as well as the biofilm dislocated from the membrane into the lower compartment, after treatment, were then analyzed quantitatively, see procedures below. In test series with one single treatment the biofilm loads were analyzed directly after spray treatment. In test series with daily treatments for four days, analysis was performed directly after the fourth treatment, thus comprising the biofilm load on the membrane after four treatments and the biofilm dislocated to the lower compartment after the fourth treatment.

The biofilm load remaining on the membrane after one treatment was also qualitatively analyzed, macroscopically and microscopically, by fluorescence microscopy.

Quantitative assessment of biofilms

The membrane was transferred to an Eppendorf tube with 1 ml PBS, sonicated (Bransonic/B-2510 MTH, Branson Ultrasonics Corporation, Danbury, CT), vortexed vigorously, serially diluted and spread in duplicate on TSA plates. Sprayed volumes collected in the wells were weighed, serially diluted, and spread in duplicate on TSA plates. The plates were incubated at 36 ± 1°C for 24 h and analyzed in a colony counter (Scan 1200, Interscience, Saint-Nom-la-Bretèche, France). Data were presented as mean OD-values +/- SD, n=3 (in duplicates).

Qualitative macro- and microscopic assessment of biofilms after treatment

Biofilm growth on the membranes was macroscopically observed by the naked eye and photo-documented before and after treatment with the different spray products. For microscopic assessment, the colony-biofilm-membrane assembly was placed on a cover slip with the biofilm facing upwards. 100 µl staining solution, LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA), was added on top of the biofilm and incubated in darkness for 25 min. The staining solution was gently removed by rinsing with filter-sterilized water, and a cover glass was placed on top of the membranes. Stained biofilm was immediately analyzed in a fluorescence microscope (Axio Vert. A1 inverted microscope, Carl Zeiss AG, Oberkochen, Germany).

Microtiter plate biofilm assay – Crystal violet staining

The Microtiter plate biofilm assay follows the method described by Djordjevic et al[14] with minor modifications. Aliquots of 100 µl bacterial solution at 1.5-3.0 x 10^8 CFU/ml in tryptic soy broth were added to the wells of a 96-well plate and incubated for 24 h at 36 ± 1°C under static conditions, to allow the formation of mature biofilms on the inner surfaces of the wells. After incubation, the culture medium, including loosely associated bacteria, was gently removed from the wells using a pipette. For the microtiter plate biofilm assay a relevant volume of the nasal sprays was estimated, using the spray volume per second and the size of the nasal surface area. The human nasal surface area is approximately 160-180 cm^2 [15]. The volume to surface ratio for a single spray treatment was calculated using the volume dispensed during one second for the spray to be tested, resulting in test volumes of about 4 µl/well. The nasal spray solution was applied directly to

the "still wet" surface-attached biofilm. The microtiter plate was again incubated for 30 or 60 min at 36 ± 1°C under static conditions. Without any subsequent rinsing step, the treated wells as well as the untreated biofilm control wells (from which the culture medium was carefully removed by pipette), were then analyzed with crystal violet staining.

Microtiter plate biofilm assay – Daily treatments

Biofilm cultures were prepared in microtiter plates as described above under "Microtiter plate biofilm assay – Crystal violet staining". After the first treatment with the nasal spray, 100 µl TSB was added to each well without disturbing the biofilm, and the plates were incubated for another 24 h at 36 ± 1°C. These treatment and incubation steps were repeated four times and after the fourth treatment, without any rinsing step, the treated wells as well as the untreated biofilm control wells, (from which the culture medium was carefully removed by pipette), were then analyzed with crystal violet staining.

Crystal violet staining procedure

The staining procedure was performed under ambient conditions. To each well 50 µl of 0.4% (w/v) crystal violet in water was added, followed by incubation in for 45 min, and gentle rinsing 3 times with 100 µl distilled water/well using a pipette. Thereafter, the stain was immediately extracted from each well with 250 µl 95% ethanol for 45 min. 100 µl of the extraction solution, from each well in duplicates, was transferred to flat-bottomed 96-well plates for absorbance measurement at 595 nm in a microtiter plate spectrophotometer (Epoch). Data were presented as average OD-values +/- SD, n=3 (in duplicates).

For comparison of one and daily treatments the OD-values from the different experimental series were normalized relative to biofilm control and presented as mean values +/- SD, n=3 (in duplicates and n=6 (in duplicates), respectively.

Ethic approval was not necessary for this study

No clinical isolates of bacteria were included in the experiments, and no human- or animal material was used.

RESULTS

Development of a novel *in vitro* nasal biofilm assay

This *in vitro* biofilm assay was designed to simulate a nasal mucosal surface with an established bacterial biofilm formed by *S. aureus*, a common nasal colonizing pathogen. The objective of the model was to test the ability of nasal sprays to reduce the bacterial burden by dislocation of the biofilm from the surface by physical force. Macroscopic assessment of the *S. aureus*-inoculated polycarbonate membrane (the simulated nasal mucus layer) clearly showed that a distinct colony biofilm had been formed after 24 h (Figure 2). It was also confirmed that one treatment with either of the nasal sprays removed the main part of the biofilm from the membrane. Analysis of the biofilms by fluorescence microscopy revealed dense cell aggregates, surrounded by more diffuse material which is in accordance with the definition of a mature biofilm (Figure 3).

Evaluation of nasal sprays using the Colony biofilm transwell assay

The nasal spray products differ with respect to spray solution composition and pressure in the spray canister (Table 1). The results from one treatment and treatments repeated daily for four days (Figure 4 A, B and Table 2), reveal that all four nasal spray products dissolved and removed, to various extents, the biofilm from the membrane mimicking the nasal surface. The value of the bacterial load in the untreated colony biofilm was about log 10 CFU/ml, both after 24 hours (biofilm control in Figure 4A) and after 4 days incubations (biofilm control in Figure 4B). Biofilms treated once had a more

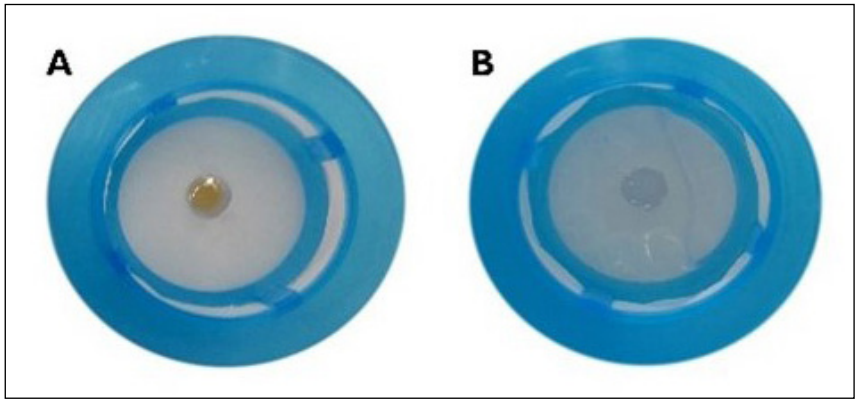

Figure 2. Example of an *S. aureus* colony biofilm grown on a polycarbonate membrane (colony biofilm-membrane assembly) which was transferred, after 24 h incubation on a TSA-plate at 36 ± 1°C, to a cell strainer or transwell insert with a porous bottom surface. (A) The colony biofilm is clearly visible on the membrane prior to treatment. (B) The main part of the biofilm on the membrane was dissolved and rinsed off upon spray treatment for one second.

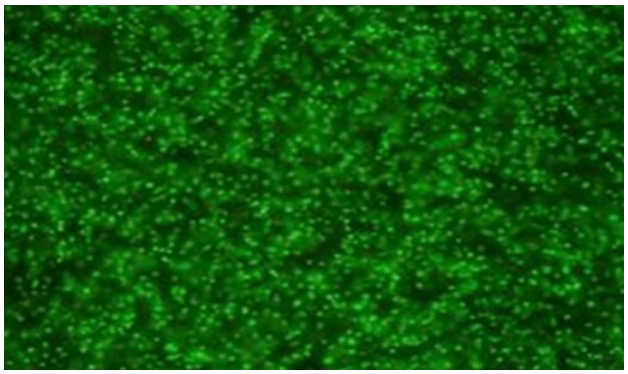

Figure 3. Fluorescence microscope image of 24 h *S. aureus* grown on a polycarbonate membrane for 24 h, stained with LIVE/DEAD™ BacLight™ Bacterial Viability Kit, and detected with fluorescence microscopy. As can be seen dense aggregates of bacterial cells has been formed, embedded in extracellular polymeric substance, which appear as diffuse "clouds". Viable bacterial cells appear in green (SYTO® 9 green) and dead cells, not detected here, would have appeared in red (by propidium iodine).

than 93% reduced bacterial load (CFU/ml) whereas daily treatment for four days had reduced the bacterial load by more than 99% (Table 2). The two spray products ANS-AC and ASNS-iso had the highest reducing effect, log 2.8 and log 2.3 CFU/ml, respectively, after one single treatment and log 2.4 CFU/ml after four treatments. For all spray treatments, major parts of the biofilms had thus been dislocated from the biofilm membrane assemblies, placed in the transwell inserts, and could be recovered in the outer compartments. The two products ASNS-hyp and ASNS-iso are both sprayed at higher pressure, especially ASNS-iso which in addition is provided with a jet actuator (Table 1). The higher value for biofilm reduction by ASNS-iso after one treatment may therefore be explained by its increased force or by larger spray volume. The higher spray pressure in ASNS-hyp, however, is not resulting in any notable increased reduction of the biofilm. After daily treatments, the different effect of the two ASNS-sprays could not be observed. In summary, all nasal spray products tested appear capable, by physical force, of dissolving and removing a mature nasal-like biofilm *in vitro*.

Evaluation of nasal sprays using the Microtiter plate CV-staining assay

In case the spray solution contains any other anti-biofilm substances, this might also affect the number of CFU/ml recovered from the biofilm membrane assembly after treatment in the CBTA. To test for possible non-dynamic/non-mechanical effects, the Microtiter

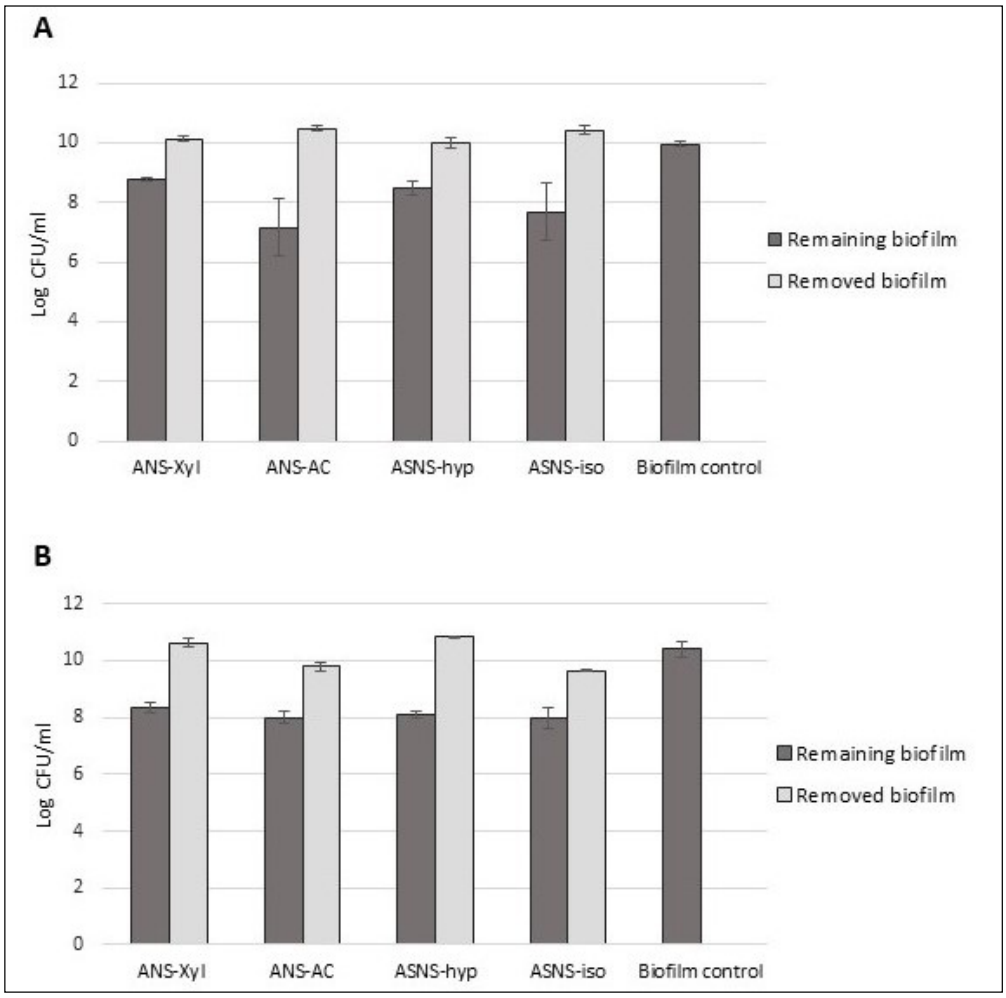

Figure 4. Effect of nasal spray treatment on simulated nasal *S. aureus* biofilms. Data from CBTA after nasal spray treatment of 24 h biofilms. **A**. one treatment, **B.** four daily treatments (total time 96 h). The bacterial load remaining on the membranes (mimicking the nasal mucus layer), and the bacterial load dislocated into the wells surrounding the transwell insert, were thoroughly dissolved, and analyzed by viable plate count (CFU/ml), (n=3 in duplicates). Data presented as mean value +/- SD.

Table II. Reduction of the bacterial load (*S. aureus* biofilm) on the membranes after one treatment and after the last of four daily treatments with nasal sprays, presented as logarithmic reductions of CFU/ml, and as percent reduction, in relation to untreated control. The untreated biofilm on membranes contained about log 10 CFU/ml both after 24 h and after 4 days incubations. (n=3 in duplicates).

Product names	One treatment		Daily treatments: 4 days	
	Reduction (log CFU/ml)	Reduction (%)	Reduction (log CFU/ml)	Reduction (%)
ANS-Xyl	1,2	93.6	2,1	99,3
ANS-AC	2,8	99.3	2,4	99,7
ASNS-hyp	1,5	96.4	2,3	99,5
ASNS-iso	2,3	98.7	2,4	99,6

plate biofilm assay with crystal violet staining was used. *S. aureus* biofilms were established in 96-well microtiter plates and treated for 30 and 60 min with the four nasal spray solutions under static conditions (Figure 5A). After 30 min treatment, it was found that all nasal spray solutions except for ANS-Xyl showed a reducing effect on the biofilm formed on the surface of the wells. After 60 min treatment the biofilm load was reduced for all four spray solutions. Among the different sprays, ANS-AC showed the highest antibiofilm effect under these static conditions. This spray solution contains two different plant extracts, gel from Aloe leaves (*Aloe barbadensis*) and extracts from Chamomille flowers (*Chamaemelum nobile).* These additives might contribute to the reduced bacterial load (CFU/ml) measured in the CBTA, where this product shows a more pronounced effect than the three other nasal sprays after one treatment (Figure 4A and Table 2). The Microtiter plate assay also reveals different effects of the two spray products ASNS-iso and ASNS-hyp, which only differ in their salt concentration, with a noticeably better effect of the isotonic ASNS-iso (Figure

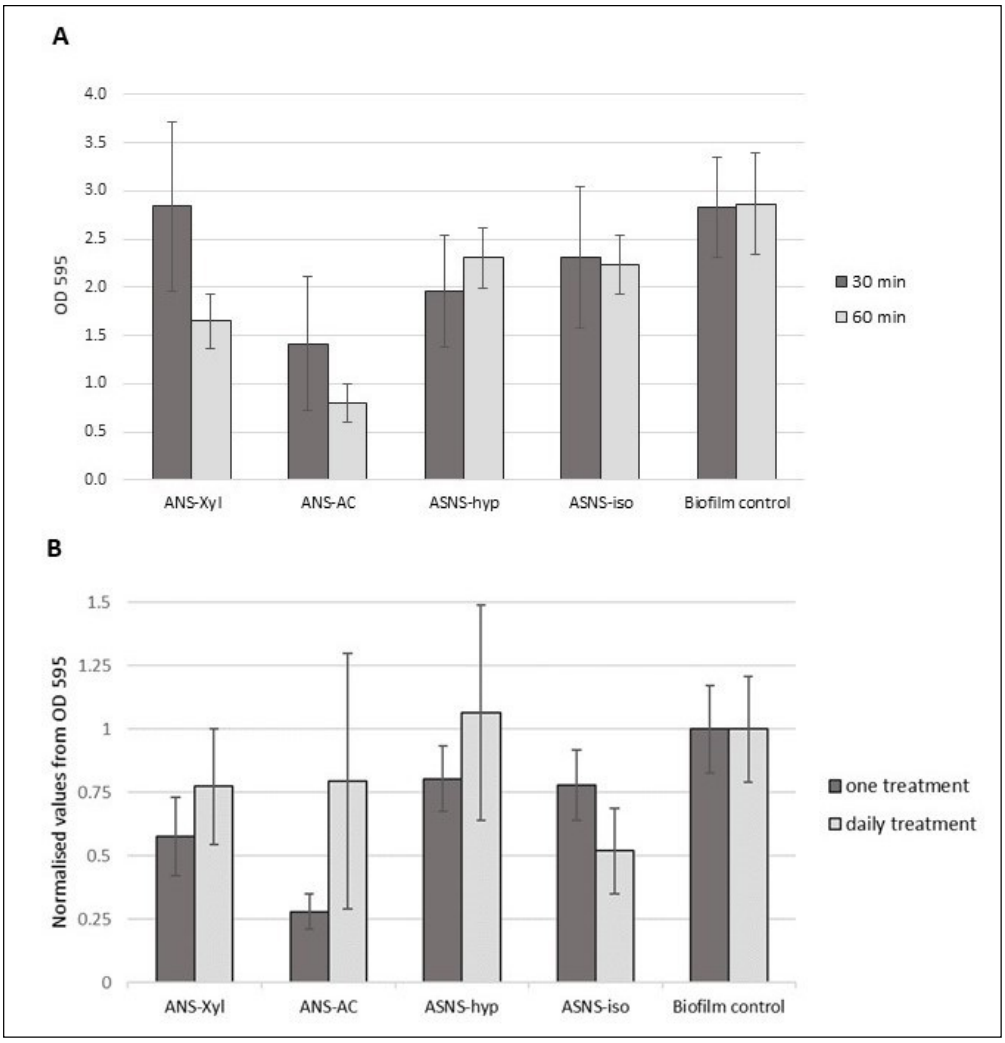

Figure 5. Effect of nasal spray solutions on *S. aureus* biofilms under static conditions. Data from microtiter plate biofilm assay with crystal violet staining. **A.** 24 h *S. aureus* biofilms treated once for 30 and 60 min with four different nasal sprays. Data presented as mean OD 595 value +/- SD, n=3 (in duplicates). **B.** *S. aureus* biofilms treated with four different nasal sprays, either once for 60 min or 60 min daily for four days. Data measured as OD 595 (one treatment n=3 (in duplicates), daily treatments n=6 (in duplicates)). For comparison of one and daily treatments the OD-values from the different experimental series were normalized relative to biofilm control (given the value 1) and presented as mean values +/- SD.

5B). However, this difference was only observed after daily treatments for four days. It thus appears that both mechanical and biochemical anti-biofilm properties of the sprays may influence the outcome of the treatments.

DISCUSSION

A novel *in vitro* nasal biofilm test model is presented. It was designed for testing the capability of the dynamic force of a sprayed saline solution to remove a bacterial biofilm from a simulated nasal mucosal surface. The two-compartment device (transwell co-culture dish) used in the model offers the possibility to retrieve both remaining (still adhered) and removed bacterial load after treatment. Calculations of the sum of these values showed similar values to the biofilm control values, indicating a proper design and experimental procedure of the test. The combination of the CBTA with the commonly used Microtiter plate biofilm assay[14] may distinguish loss of bacteria due to mechanical force from bactericidal effects due to other properties of the nasal spray. For example, the higher bactericidal effect of the nasal spray ANS-AC on *S. aureus* suggested by the CBTA could be confirmed by comparison with the results from the microtiter plate assay.

Nasal *S. aureus* biofilms have been demonstrated in human nasal mucosa by several groups[6-8]. For the CBTA, a well-known biofilm forming strain of *S. aureus* was chosen[16,17]. It has been shown that nasal colonization by *S. aureus* involves adhesion to nasal mucin[18]. The nasal cavity is lined with mucus, consisting of 2-5% mucin by weight[19]. Thus, a colony biofilm[20] formed in the presence of 2% mucin may reflect the characteristics of a nasal biofilm. Fluorescence microscopy analysis confirmed that dense cell aggregates had been formed on the membrane. These aggregates were surrounded by more diffuse material, which most probably constitute the exo-polymeric matrix surrounding the bacterial aggregates in a mature biofilm (Figure 3). These observations support that a simulated nasal biofilm was formed by a common biofilm producing nasal pathogen.

This nasal biofilm model has naturally reduced clinical complexity, however, in comparison to other *in vitro* nasal biofilm models its novelty lies in the introduced nasal environment as well as an experimental setup allowing for studies of both dynamic/physical forces and biochemical effects of nasal irrigations on bacterial biofilms. CBTA is a method easy to perform which is a great advantage, e.g., for studies of nasal irrigation devices during product development.

Our test results suggest that nasal sprays in canisters with higher pressure lead to stronger biofilm reduction. This could be explained by stronger physical force or by larger volume of the nasal spray solution during treatment (1 sec). This assumption may especially account for the effect of ASNS-iso which is a saline solution without any additives with possible antimicrobial effects (Figure 4A). However, according to the literature, also saline itself may be regarded as a quite powerful anti-biofilm solution when targeting *S. aureus* biofilm[21-23]. In addition, isotonic saline is preferable over hypertonic saline due to stronger *S. aureus* biofilm formation at higher salt concentrations[22,24]. In the present study, differences in effect between isotonic and hypertonic saline, the two spray products ASNS-iso and ASNS-hyp, would therefore be expected to be observable after treatment for four days, where new biofilm is produced between treatments. However, no such differences were observed in data from the CBTA measurements. Data from the Microtiter plate biofilm assay, on the other hand, show that daily treatments for four days noticeably improved the effect of the isotonic spray (ASNS-iso), whereas the effects were similar for the two spray solutions after one single treatment. A plausible explanation for this difference is that a more robust *S. aureus* biofilm was produced after treatment with the hypertonic solution.

Standard deviations are high for all spray products in the Microtiter plate biofilm assay. We speculate that this is an intrinsic factor of the assay due to the several rinsing steps during the biofilm staining procedure, where loosening of some of the biofilm material is difficult to avoid.

The spray solution in ANS-AC contains extracts from Aloe vera and Chamomille and these additives likely contributed to the reduction of the *S. aureus* biofilm as demonstrated in the Microtiter plate assay. Growth inhibition of *S. aureus* has been demonstrated for Chamomille[25,26], and Aloe vera has been shown to inhibit both growth and biofilm formation in several bacterial species, including *S. aureus*[27,28]. Xylitol is sometimes added to nasal irrigation solutions to increase osmolality and thereby improve inflammatory symptoms[29]. Xylitol might also contribute to inhibition of biofilm production in *S. aureus*[30,31]. Although xylitol was present in the spray product ANS-Xyl, in a concentration comparable to those found in the literature, no reducing effect was observed that could be related to this additive, not even after multiple treatments.

CONCLUSIONS

This study demonstrates, in a novel *in vitro* test model, that the dynamic force of a nasal saline spray may have a substantial reducing effect on *S. aureus* biofilms. In addition, a nasal spray with stronger dynamic force, as well as a spray with added extracts of Aloe vera and Chamomille, were found to have the highest reducing effects.

Considering the obtained data from the CBTA, this test method has proven robust and useful for *in vitro* evaluations when the effect of mechanical force needs to be realistically simulated. In combination with a test method for more direct antimicrobial activity, it may also be a useful screening tool for anti-biofilm agents with multiple mechanisms of action.

Conflict of Interest:
Anders Bared is an employee of Aurena Laboratories AB and the sprays involved in the research study are commercialized from Aurena Laboratories. Besides, the authors declare no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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