Skin Microbiota Dynamics Following *B. subtilis* formulation Challenge

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Abstract

Background

Modulating the microbiota is a leading-edge strategy for the restoration and maintenance of a healthy, balanced environment. The use of health-promoting bacteria is emerging as an alternative for skin microbiota intervention, showing promising results. *Bacillus subtilis*, with its non-pathogenic and antibacterial properties, has great potential as a microbiota-manipulating agent. Here, we investigate the experimental manipulation of the skin microbiota using *B. subtilis* incorporated into a supportive Pluronic F-127 hydrogel formulation. Indeed, the formulation is of prime importance for dermal bacterial delivery, determining the ability of live bacteria to become established at the desired action site.

Results

The *B. subtilis* challenge induced a shift in the composition and abundance of the skin microbiota. Containment of *B. subtilis* in the Pluronic F-127 hydrogel accelerated bacterial modulation compared with free *B. subtilis*. The abundance of both *Staphylococcus* and *Corynebacterium spp.* was altered as a result of the live bacterial intervention: the abundance of *Corynebacterium* increased while that of *Staphylococcus* decreased. Four days after last application of the *B. subtilis* formulation, *B. subtilis* counts returned to its initial level.

Conclusions

B. subtilis intervention can induce a shift in the skin microbiota, with an increased presence of commensal, benefit-associated bacteria and a reduced abundance of pathogen-containing bacterial genera. Containment of *B. subtilis* in Pluronic hydrogel accelerates the microbial alteration, probably by facilitating bacterial attachment and supporting continuous growth. Our results reveal the ability of *B. subtilis* in Pluronic to modulate the skin microbiota composition, suggesting that the formulation holds therapeutic potential for skin disease treatment.

Background

The skin, our largest organ, serves as the principal mechanical and biological protective barrier for the human body. Being the organ most exposed to the environment, the skin is colonized by a diverse collection of microorganisms that constitute the microbiota [1]. By communicating with epithelial cells and the immune system, the microbiota plays an important role in protecting the skin from potential damages posed by pathogenic microorganisms [2, 3]. Moreover, findings of the Human Microbiome Project have revealed that many of the resident microorganisms are harmless (commensals) or have a positive influence on human skin health and well-being (mutualists) [4, 5]. However, when homeostasis is disrupted, the microbiota enters into a state of microbial imbalance, termed dysbiosis, which can lead to

dermal immune dysregulation [6, 7]. Skin microbiota dysbiosis has been associated with skin diseases including atopic dermatitis [8], acne [9], and vitiligo [10].

Several strategies for the manipulation of the skin microbiota have been suggested, including hygiene products [11], antibiotics [12, 13], and prebiotics [14]. These methods, however, often suffer from poor efficacy and lack of selectivity towards the pathogenic bacteria [15]. Antibiotics, for example, have been associated with long-term bacterial imbalances, lasting up to several years and leading to an increased incidence of skin lesions [16, 17]. Antibiotic treatment has been showed to enhance depigmentation in vitiligo-affected skin [13] and to delay wound healing [18]. These drawbacks are driving the exploration of new microbiota intervention alternatives.

The use of health-promoting bacteria has shown promising results in the restoration of a healthy microbiota, for example by promoting the growth of beneficial microbes [19–22]. *Bacillus subtilis* has great potential as a microbiota-modulating agent since it is naturally found in human skin and displays a non-pathogenic profile [23–25]. Moreover, *B. subtilis* can efficiently outcompete important human pathogens such as *E. coli* and *S. aureus* [24, 26], probably through the production and secretion of potent antimicrobial agents [26]. However, before this knowledge can be translated into therapeutic applications, additional groundwork is required and well-controlled *in vivo* studies to assess microbiota dynamics as well as safety and efficacy aspects of living bacteria interventions must be performed. The aim of this study is, therefore, to explore the experimental manipulation of the skin microbiota using a *B. subtilis* formulation incorporated into a supportive Pluronic F-127 hydrogel delivery matrix. The microbiota of healthy skin was mapped before, during, and after the administration of a living *B. subtilis* formulation to monitor its dynamics. We hypothesized that a *B. subtilis* microbiota intervention will result in a microbial shift that will be limited to the treatment course, since human skin microbiota is relatively stable in terms of its microbial population [27, 28].

Methods

Animal husbandry

Twenty-four 8-week old C57BL/6 female mice were purchased from Envigo, Israel. The animals were caged randomly in four groups of six mice each. All animals were maintained in sterilized cages on a 12-h light/12-h dark cycle with food and water provided ad libitum. Bedding was changed once a week, and mice were given an autoclaved chow diet and sterilized water. At the end of the study, mice were sacrificed by CO_2 asphyxiation following protocols approved by the corresponding authority.

Formulations and administration

Each independently housed group of six mice received a different treatment formulation twice daily, every 12 hours, for 7 days. The treatment formulations consisted of (a) 10% v/v *B. subtillis* in lysogeny broth (LB) with 18% w/v Pluronic (“*B. subtilis* formulation”), (b) 18% w/v Pluronic, (c) 10% v/v *B. subtillis* in LB, and (d) untreated control. A stock of Pluronic F-127 was prepared by dissolving the appropriate amount

of polymer in distilled water to obtain a final concentration of 20% w/v. A stock solution of *B. subtilis* was cultured in LB agar and incubated at 37 °C overnight after which a bacterial colony was transferred to a falcon tube containing fresh liquid LB, incubated at 37 °C and allowed to reach an optical density (OD) of 0.6 at 600 nm. The *B. subtilis* formulation was prepared by adding 0.5 mL of fresh bacterial culture (OD_{600}: 0.6) to 4.5 mL of 20% w/v Pluronic, obtaining a final solution of 18% w/v Pluronic with 10% v/v *B. subtilis* in LB. 18% w/v Pluronic was obtained by adding 0.5 mL of liquid LB to 4.5 mL of Pluronic 20% w/v. To prepare 10% v/v *B. subtillis* in LB, 0.5 mL of fresh bacterial culture (OD_{600}: 0.6) were added to 4.5 mL of fresh LB media. The different formulations (100 µL) were administered to both left and right ears of the animals of the corresponding groups. Pluronic-containing formulations were allowed to harden on the skin for 1 minute until a viscous gel was obtained.

Sample collection

To sample the skin microbiota, both ears were thoroughly swabbed with a sterile FLOQSwab presoaked in buffer solution (0.15 M NaCl and 0.1% Tween 20). Sampling was effectuated every other day from day 0, before formulation administration, to day 14, a week after last application. Samples were stored at -80 °C until processing.

Bacterial DNA extraction

DNA extraction and purification were performed using the PureLink Microbiome Kit (Invitrogen, Thermo Fisher Scientific) according to manufacturer's protocol and supplementary instructions for low bio-burden samples. Concentration of purified DNA was determined using the QuBit High Sensitivity DNA quantification system (Invitrogen) and stored at 20 °C until further use.

16S rRNA gene amplification and sequencing

16S rRNA gene amplification and sequencing were carried out at the Technion's Genome Center. Sequencing libraries were prepared using the 16S rRNA Metagenomic Sequencing Library Preparation protocol by Illumina with minor adjustments. Sample input was 0.625 ng of genomic DNA and the first PCR amplification consisted of 30 cycles. V3 and V4 hypervariable regions of bacterial 16S rRNA were the amplification targets. Primers used to target the V3 and V4 regions of the 16S rRNA gene were 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG (Forward) and 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC (Reverse). The respective Illumina overhang adapter sequences were included in the primer design. All 96 libraries were sequenced on an Illumina MiSeq instrument with 250 paired-ends reads. Sequencing data was input into the 16S rRNA gene Metagenomics app on the Illumina BaseSpace sequence hub. Classification was performed using the Illumina 16S rRNA gene Metagenomics workflow, which includes demultiplexing of indexed reads, FASTQ files generation, and read classification. Operational Taxonomic Unit (OTU) clustering and classification were performed at genus and species level. An Illumina-curated version of the Greengenes database was used as the taxonomy database for the metagenomics workflow. The

algorithm used is a high-performance implementation of the Ribosomal Database Project Classifier described in Wang Q. *et al*. [29].

Bioinformatics analyses

The R statistical software package was used for all statistical tests. The Remove Unwanted Variation (RUV) normalization strategy described by Risso *et al.* [30] was employed to remove noise from unknown sources. Factors of unwanted variation using replicate samples were estimated using the RUVseq package. Parameters were set to K = 15 at the species level and K = 40 at the genus level, and corrected counts were calculated. For the calculations, the no-treatment control treatment was defined as all no-treatment samples and all day-0 samples (before treatment). Principal components analysis (PCoA) was applied on the results with EDAseq [31]. A design matrix including both the covariates of interest and the factors of unwanted variation was supplied to DESeq2 for differential analysis [32]. To test the effect of treatments, each treatment was compared with the no treatment control using Wald test as implemented in DESeq2, as previously described by Love *et al.* [32]. Only bacteria with at least 50 counts in two or more samples were included in the analysis. A bacterial genus or species was considered to differ significantly between treatments if the absolute value fold change was at least 2, with an adjusted p-value (padj) smaller than 0.05.

Results

The effects of challenging the healthy skin microbiota with a *B. subtilis* formulation were studied using the inner ear skin of laboratory mice. This experimental model highly resembles human skin sites in terms of morphology and microbiota and has been successfully used in the assessment of host-microbe interactions [33, 34]. Twenty-four 8-week old C57BL/6 female mice were randomly assigned to one of four groups: Pluronic hydrogel containing *B. subtillis*, *B. subtillis* (in LB medium), plain Pluronic hydrogel, and a no-treatment control group (Fig. 1A). Each group was administered with the corresponding formulation twice a day for seven days. The effects of the various treatments were analyzed by determining the bacterial composition of the skin before the first application (day 0) and on days 2, 4, 8, 11, and 14 (Fig. 1B).

After skin sampling, genomic DNA was extracted and the V3-V4 hypervariable regions of 16S rRNA gene were amplified and sequenced using the Illumina technology. A total of 29.5 million high-quality 16S rRNA gene sequences were obtained, each containing between 0.36% and 1.5% of the data. Noise was removed according to the Remove Unwanted Variation (RUV) strategy [30], using the untreated control group and day 0 samples (before treatment) for normalization (**Supplementary Table 1**).

The dynamics of the abundance of *B. subtilis*, in particular, and of the *Bacillus* genus, in general, before, during, and after administration of *B. subtilis* formulations was assessed (Fig. 2A **and B**) and compared with the average abundances of *B. subtilis* and *Bacillus* (dashed lines), respectively. It was found that plain Pluronic hydrogel did not impact the abundance of *B. subtilis*, whose counts remained similar to those recorded for the untreated control group (Fig. 2A). *B. subtilis* in Pluronic, conversely, had the highest

influence on *B. subtilis* counts on days 2 and 4, which were considerably higher than for the *B. subtilis* treatment. Nevertheless, four days after the last administration, all groups presented counts that were similar to that of the untreated group. For the *Bacillus* genus (Fig. 2B), counts were significantly higher for all groups compared with the untreated control group: The group that received *B. subtilis* showed a 2-fold increase while the two groups that received Pluronic (with and without *B. subtilis*) exhibited a 6-fold increase. This trend changed on day 4, when all groups presented a two-fold increase compared with the untreated group. Post-challenge, on day 8, *Bacillus* counts for the two groups that received *B. subtilis* increased 4-fold, while counts for the group that received plain Pluronic decreased to the control group level. From day 11, i.e. 4 days after the last administration, control levels were attained for all treatment groups with insignificant differences compared with the untreated group.

Principal components analysis (PCoA) was then used to assess skin microbiota dynamics on both the genus (Fig. 3A) and the species (Fig. 3B and **Supplementary Fig. 1**) levels. Our data indicated a clear clustering according to treatment day and nature as explained by PC1 and PC2, respectively (i.e., data organization according to treatment nature along PC2 axis can be observed in **Fig. 3B**). Treatment nature and time point influenced the observed clustering to the same extent, as evidenced by the similarity in effect size between PC1 and PC2 (6.97%-6.96% and 5.57%-5.86%, respectively). The *B. subtilis* in Pluronic group exhibited an enhanced microbial shift compared with the pure *B. subtilis* group, which showed a very similar pattern but with a slight delay, prominent on day 8 (Fig. 3A). One week after ceasing treatment administration, however, all groups presented a microbiota composition similar to that of the untreated control group.

We further investigated the microbiota shift upon *B. subtilis* intervention and after its cessation by analyzing the intervention's effects on the relative abundance of the most represented skin bacterial genera (Fig. 4A and **Supplementary Table 2**). Consistent with the PCoA results, altered bacterial abundance was observed for all treatment groups along the experiment. For *Corynebacterium*, the most abundant genera in the inner ear skin microbiota, application of *B. subtilis* in Pluronic resulted in a sharp increase from day 2 to 4, followed by a plateau for the remainder of the experiment. Application of pure *B. subtilis* caused an abundance increase only on day 8, which remained until day 11 and then decreased to initial values. No significant variations in abundance were observed following the application of pure Pluronic. Interestingly, the relative abundance of *Staphylococcus* exhibited an inverse trend compared with *Bacillus* for both *B. subtilis*-containing formulations: when *Bacillus* abundance increased, *Staphylococcus* counts decreased, and vice versa. To obtain a broader view of these bacterial changes, we mapped the differential representation of bacterial genera along the treatment period (Fig. 4B). Only statistically significant differences in bacterial abundance ($-1 < \log2FC > 1$; $padj < 0.05$, Wald-test) were considered for the analysis (**Supplementary Table 3**). Following the administration of *B. subtilis* in Pluronic, several highly related genera were observed to cluster, being either underrepresented or overrepresented compared with the untreated control group (Fig. 4B). For instance, the *Lentibacillus*, *Gemella*, *Marinococcus*, and *Virgibacillus* genera, of the *Bacilliales* order, were overrepresented on day 4. The *Bacillus* genus, on the other hand, was overrepresented on days 2 and 8, while no statistically significant difference to the control was presented on day 4, consistent with the trend observed in the

PCoA (Fig. 2B). *Staphylococcus* abundance decreased during the application of *B. subtilis* in Pluronic formulation (days 2 and 4) and increased on day 11, three days after the last administration.

Discussion

The concept of skin microbiota manipulation, either by promoting bacterial balance or by pathogen inhibition, is well established [35, 36]. However, despite significant progress in the field, transplantation of bacteria or bacterial ingredients that selectively stimulate or inhibit the growth and activity of one or a limited number of bacterial species is still at a very experimental stage in skin therapy [4]. Local delivery of live bacterial therapies is often challenging. Live bacterial therapy is often challenging as a local delivery system since the bacteria must reach the site of action alive and establish themselves there [15], hence the importance of proper formulation design.

In this study, administration of *B. subtilis* to the inner ear skin of mice resulted in the modulation of the skin microbial composition. Our results indicate that a suitable dermal delivery system is of prime importance for successful administration of live bacteria and, consequently, for microbiota modulation. Administration of *B. subtilis* in Pluronic hydrogel resulted in a significant increase in *B. subtilis* counts compared with a more moderate increase in bacterial levels in the absence of Pluronic. The effect of Pluronic hydrogel on the *Bacillus* genera was significant during the first two days of application: the two Pluronic groups (pure Pluronic and *B. subtilis* in Pluronic) showed a fast increase in *Bacillus* abundance that surpassed the effect exhibited by the pure *B. subtilis* group. The enhanced performance of the *B. subtilis* in Pluronic formulation can be explained by the contribution of various factors. One is the ability of Pluronic F-127 to selectively reduce the attachment and biofilm formation of several bacteria [37] probably owing to its surfactant properties [38]. Another explanation is that Pluronic F-127 gel serves as a protective layer between the bacteria and the skin, aiding the establishment of *B. subtilis* and its natural ability to produce and secrete a wide range of potent antimicrobial peptides. Pluronic F-127 was found to be very effective in reducing the degradation of such peptides while sustaining their delivery from the hydrogel to its surroundings [39, 40]. Finally, the ability of Pluronic gel to enhance the immune response, probably by stimulating the expression of vascular epithelial growth factors, may be related to the selective shift in the skin microbial composition [41]. The present study is, to our knowledge, among the first to evaluate the relationship between the use of biomaterials in live bacterial skin delivery systems for microbiota modulation.

The application of *B. subtilis* altered the abundance of common skin bacteria, including *Staphylococcus* and *Corynebacterium* spp. Some of these bacterial genera have been reported to be the most abundant organisms colonizing moist areas of the skin, including the antecubital fossa in humans, which the inner ear skin of mice resembles [42, 43]. Common skin commensals belonging to the *Corynebacterium* genus have been shown to exhibit antimicrobial activity against human pathogens and to stimulate healthy host-bacterial interactions [44]. For example, *C. striatum* can influence *S. aureus* gene expression by downregulating virulence-related genes and upregulating genes associated with the establishment of a commensal relationship [45]. Thus, the increase in *Corynebacterium* abundance induced by *B. subtilis* in

Pluronic formulation may have beneficial outcomes for skin health. As for *Staphylococcus* spp., the decrease in its abundance following the application of *B. subtilis* in Pluronic formulation may account for a decreased chance of infection development.

Conclusions

In this study, we investigated the effect of challenging the skin microbiota with *B. subtilis*, a bacterium with therapeutic potential [24–26]. The carrier, a Pluronic F-127 hydrogel, was found to facilitate *B. subtilis* administration and enhance its activity, possibly by supporting continuous bacterial growth and providing better conditions for skin attachment. The interaction between the polymer, *B. subtilis*, and epithelial cells should be further investigated to provide more insight into the mechanism of action. We demonstrated that *B. subtilis* induces a shift in the skin microbiota composition that is facilitated by the presence of the live bacteria in Pluronic hydrogel. This alteration was characterized by an increased abundance of the commensal, benefit-associated *Corynebacterium* and a reduced presence of bacterial genera containing common human pathogens such as *Staphylococcus.* Given the great potential of the live bacterial delivery approach, its clinical value for the treatment of conditions associated with skin microbiota dysbiosis (i.e. atopic dermatitis or acne) should be investigated.

Abbreviations

LB
lysogeny broth
OD
optical density
OTU
operational taxonomic unit
PCoA
principal component analysis
PCR
polymerase chain reaction
RUV
remove unwanted variation

Declarations

Ethics approval and consent to participate

All animals were cared for in compliance with protocol approved by the council for animal experiments (IL-025-02-2019), state of Israel ministry of health, in conformity with the provision of the Animal Welfare (Animal Protection) Law with all handling of live mice undertaken by an authorized person.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the BioProject database repository (BioProject ID PRJNA640303):

https://www.ncbi.nlm.nih.gov/bioproject/640303

Original R scripts are available in GitHub under request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

VM and BM conceived the study. VM carried out laboratory work with help from RB. VM carried out the *in-vivo* work with help from animal facility staff. VM analyzed the data with help from RB and GH. VM wrote the paper with the help from all co-authors. All authors read and approved the final manuscript.

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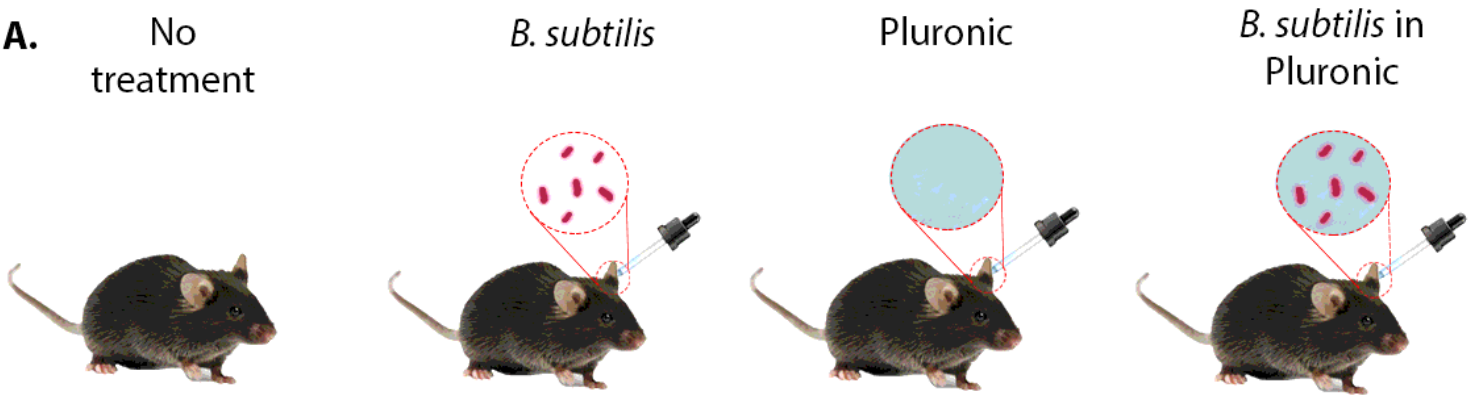
Figures

Figure 1

A.



B. **Experimental design**

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Formula application		✔	✔	✔	✔	✔	✔	✔							
Microbiome sampling	✔		✔		✔				✔			✔			✔

Figure 1

Experimental design. (A) Four groups of six C57BL/6 female mice each received different treatments: no treatment (control), B. subtilis, plain Pluronic hydrogel, and B. subtilis in Pluronic hydrogel. (B) Formulas were applied twice daily for seven days. Skin samples were collected for microbiota analysis on days 0, 2, 4, 8, 11, and 14.

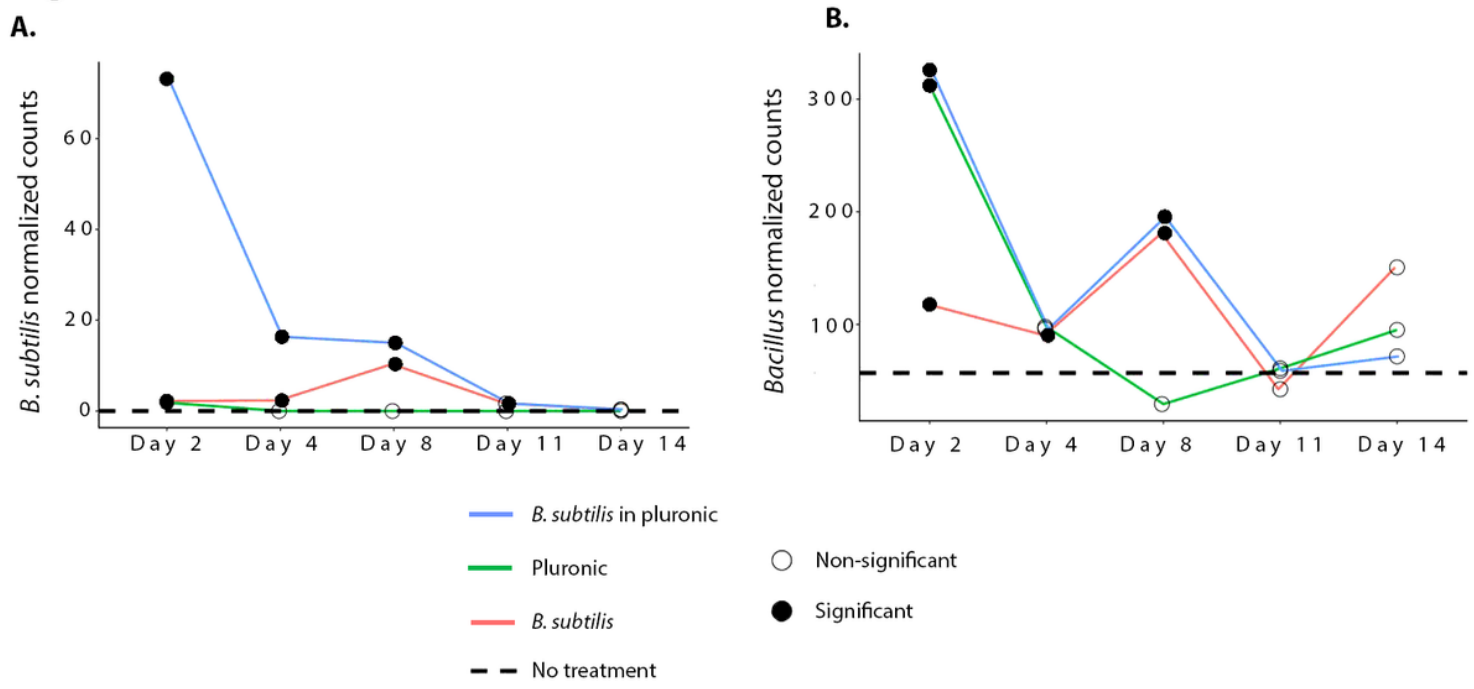


Figure 2

Temporal and treatment-dependent alteration of Bacillus subtilis species (A) and Bacillus genus (B). Dashed line represents average counts of control samples. Bacillus and B. subtilis counts that differ statistically significantly from control (padj<0.05, Wald-test) are denoted by solid circles, while empty circles represent lack of significant difference.

Figure 3

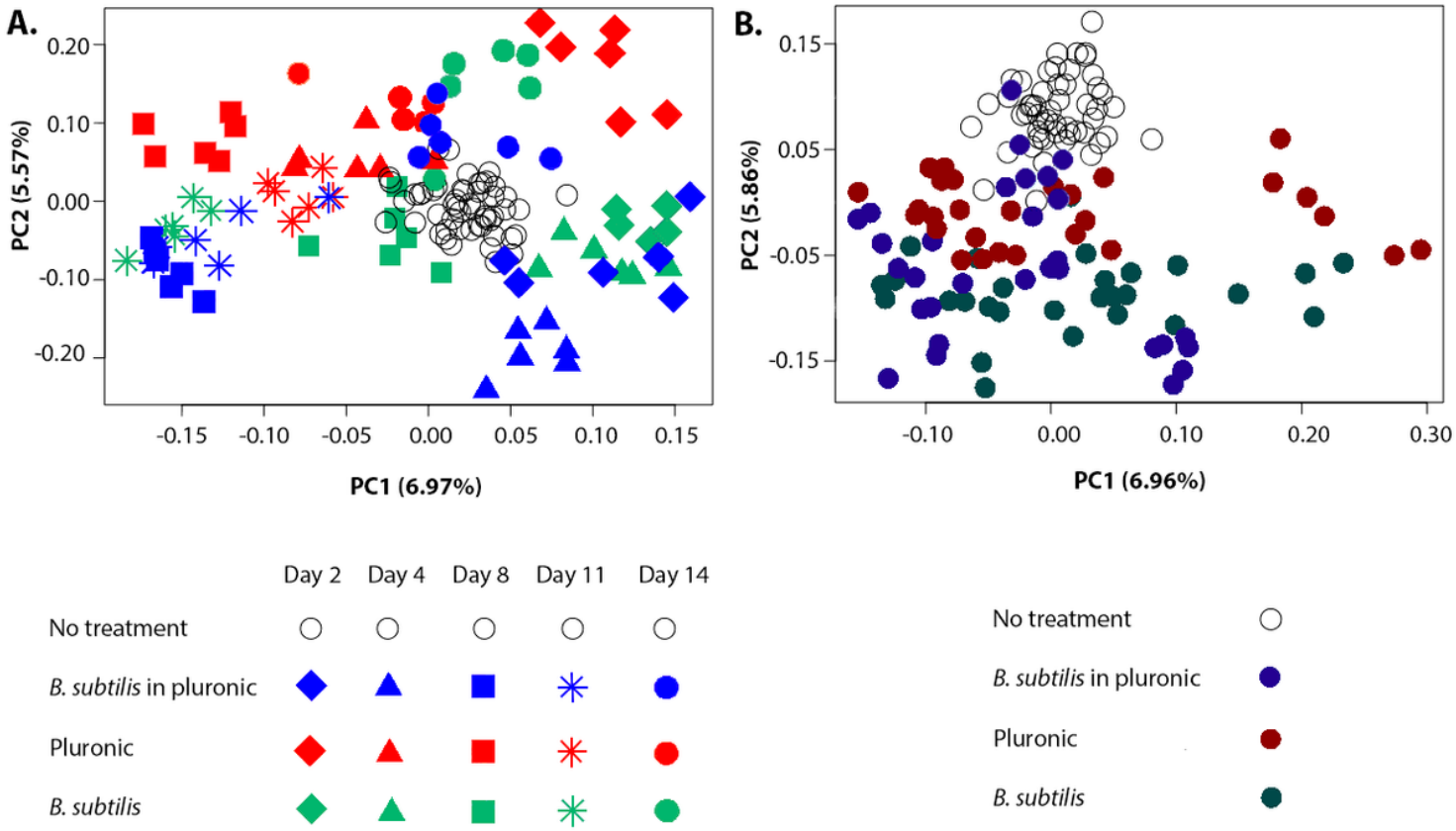


Figure 3

Principal components analysis (PCoA) of skin bacterial communities at the genus (A) and species (B) levels. Axes explain the effect of timing (PC1) and treatment (PC2) on the observed changes.

Figure 4

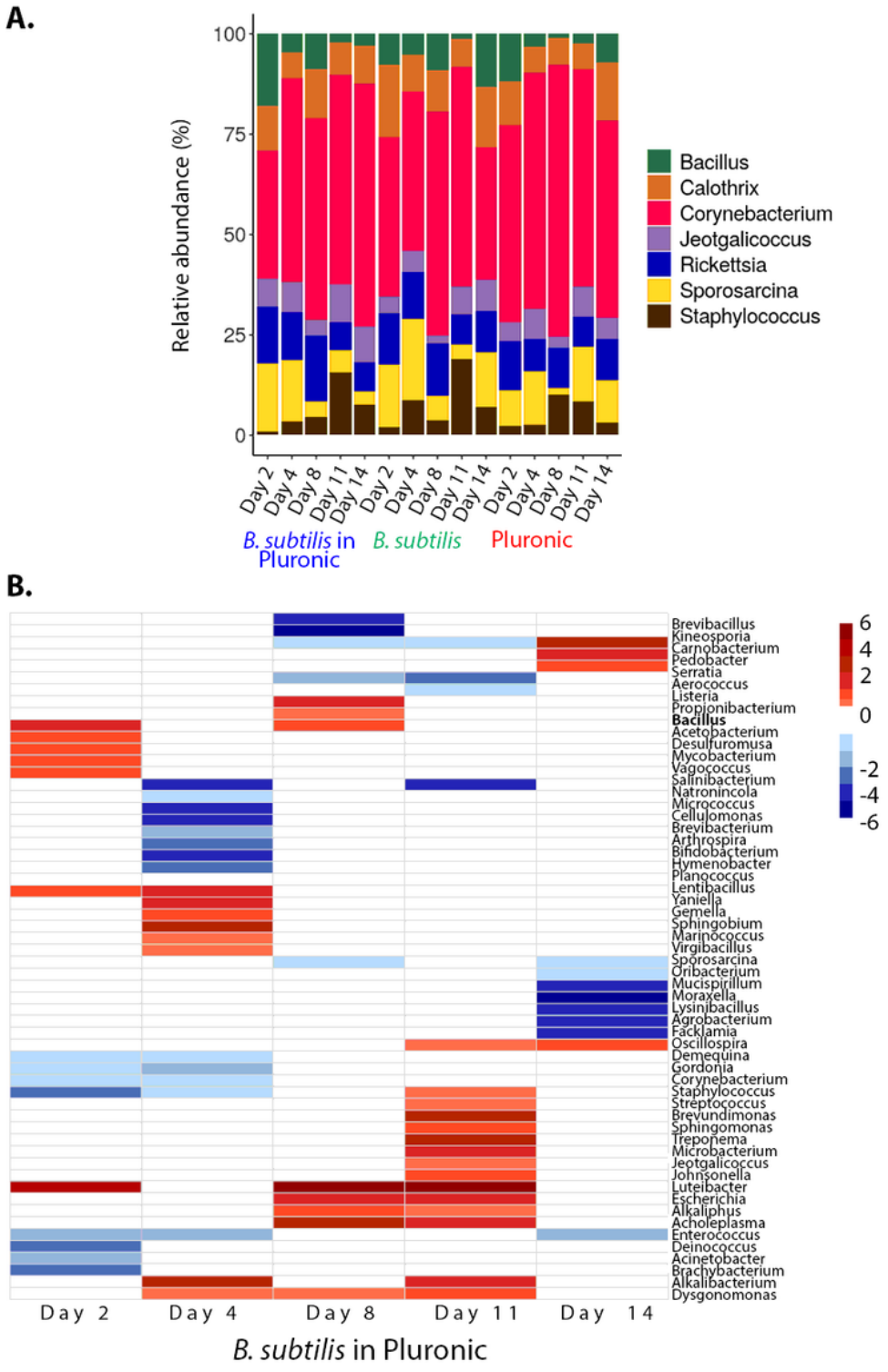


Figure 4

Effect of B. subtilis challenge on the microbial composition of the skin. (A) Relative abundance plot for the most represented bacterial genera in the inner ear skin microbiota. (B) Differential representation of bacterial genera along treatment with B. subtilis in Pluronic hydrogel. Statistically significant differences in bacterial abundance were considered (-1<log2FC>1 ;padj<0.05, Wald-test).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NC3RsARRIVEGuidelinesChecklistfillable.pdf
- Additionalfile4.xlsx
- Additionalfile3.xlsx
- Additionalfile2.xlsx
- Additionalfile1.docx