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Article

Alteration of Bacterial Communities in Anterior Nares and Skin Sites of Patients Undergoing Arthroplasty Surgery: Analysis by 16S rRNA and Staphylococcal-Specific *tuf* Gene Sequencing

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Abstract: The aim was to study alterations of bacterial communities in patients undergoing hip or knee arthroplasty to assess the impact of chlorhexidine gluconate soap decolonisation and systemic antibiotic prophylaxis. A Swedish multicentre, prospective collection of samples obtained from elective arthroplasty patients (n=83) by swabbing anterior nares, skin sites in the groin and the site of planned surgery, *before* and *after* arthroplasty surgery, was analysed by 16S rRNA (V3-V4) gene sequencing and a complementary targeted *tuf* gene sequencing approach to comprehensively characterise alterations in staphylococcal communities. Significant reductions in alpha diversity was detected for both bacterial (p=0.04) and staphylococcal (p=0.03) groin communities *after* arthroplasty surgery with significant reductions in relative *Corynebacterium* (p=0.001) abundance and *S. hominis* (p=0.01) relative staphylococcal abundance. In nares, significant reductions occurred for *S. hominis* (p=0.02), *S. haemolyticus* (p=0.02), and *S. pasteuri* (p=0.003) relative to other staphylococci. *S. aureus* colonised 35% of anterior nares *before* and 26% *after* arthroplasty surgery. *S. epidermidis* was the most abundant staphylococcal species at all sampling sites. No bacterial genus or staphylococcal species increased significantly *after* arthroplasty surgery. Application of a targeted *tuf* gene sequencing approach provided auxiliary staphylococcal community profiles and allowed species-level characterisation directly from low biomass clinical samples.

Keywords: Arthroplasty; prosthetic joint replacement; prosthetic joint infection; ; systemic antibiotic prophylaxis; Antibiotics; chlorhexidine gluconate; coagulase-negative staphylococci; *tuf* gene sequencing; staphylome, microbiome.

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1. Introduction

Arthroplasties are common surgical procedures performed to alleviate pain and to restore function in damaged or worn-out joints. Colonizing opportunistic pathogens (COPs) constitute part of the normal human microbiota [1] and comprise a risk for establishment of a prosthetic joint infection (PJI) [2]. Surgical procedures that entail breaching the epidermal protective layer increase the risk of invasive infections and an estimated 70-95% of all surgical site infections (SSIs) arise from a COP residing in the microbiota of a patient [3]. For arthroplasty, PJI incidence rates vary from low in hips, knees and shoulders (<2%), to higher risk in elbows (2-11%) and ankles (2-20%) [4]. *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) collectively account for more than half of all causative organisms derived from PJIs, with *S. aureus* being overrepresented in early postinterventional and in late acute hematogenous PJIs, and CoNS in chronic PJIs and more often as part of polymicrobial infections [2,5–7]. Recent methodological advances using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) have established *Staphylococcus epidermidis* as the most frequently isolated CoNS in PJIs (60%) followed by *Staphylococcus capitis* (11%), *Staphylococcus lugdunensis* (10%), *Staphylococcus warneri* (4%), *Staphylococcus hominis* (3%), and *Staphylococcus haemolyticus* (3%) [8,9]. In hip and knee arthroplasty, non-staphylococcal PJIs are most commonly caused by aerobic Gram-negative rods (9%), particularly *Escherichia coli* and *Pseudomonas aeruginosa,* followed by streptococci (8%), enterococci (3%), and anaerobic Gram-positive rods (4%) - typically *Cutibacterium acne*s [2].

Microbial communities vary widely across different body sites and specific bacterial genera are associated with physiological differences of the skin [10,11]. The genera *Corynebacterium* and *Staphylococcus* are integral members of the healthy human microbiota and are commonly present on moist skin including the groin (inguinal crease) and the anterior nasal cavities [12–14]. In the latter, seven distinct nasal community state types (CSTs) have been described [15], where each CST is characterised by a predominant bacterial genus or species, e.g. *S. aureus* and *S. epidermidis*. The *Staphylococcus* genus has undergone extensive evolutionary adaptation to human and animal hosts and exhibits a predominantly asymptomatic and commensal lifestyle [16]. However, the ubiquity of staphylococci, combined with their efficient acquisition of antimicrobial resistance genes and ability to form biofilm, makes staphylococcal COPs a primary concern from a clinical perspective, especially with the documented spread of multidrug-resistant (MDR) *S. epidermidis* lineages [12,17]. To reduce the risk of developing a PJI, patients scheduled for arthroplasty routinely undergo mandatory pre-operative decolonisation procedures that, according to Swedish guidelines (https://lof.se/patientsakerhet/vara-projekt/rekommendationer/), include pre-operative showers with chlorhexidine gluconate (CHG) soap along with pre-and peri-operative prophylaxis with systemic antibiotics to lower the total bacterial burden [18]. However, the impact of these treatments on the human microbiota are complex and needs better understanding.

In this study, we analysed swabs from anterior nares as well as skin swabs from groin and the site planned for surgery of patients undergoing hip or knee arthroplasty, to study bacterial communities *before* and *after* arthroplasty surgery and pre-operative decolonisation treatment with CHG soap showers and systemic antibiotic prophylaxis. To characterise the effects on *Staphylococcus* communities in detail, a novel *Staphylococcus*-centric "staphylome" approach that targets the elongation factor Tu (*tuf*) gene within the *Staphylococcus* genus was developed and applied. This approach provides staphylococcal species differentiation, with direct applicability to low abundant and polymicrobial clinical samples.

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2. Materials and Methods

2.1. Sample Collection

Materials and Methods A prospective study was performed at the Departments of Orthopaedics at the University Hospitals of Örebro and Linköping (Sweden), and the County hospitals of Karlstad and Västmanland (Sweden) from 2013 to 2015. Patients (n=83) scheduled for arthroplasty surgery were included in the study. Swabbing (Eswab, Copan Italia S.p.A., Brescia, Italy) was performed in a standardised manner i) in anterior nares ii) on the skin of the ipsilateral groin, and iii) on the skin area planned for surgery (hip or knee, hereafter referred to as operation site). Sampling was performed pre-operatively at the outpatient clinic visit 2-6 weeks scheduled arthroplasty "*before*" and post-operatively just before discharge from the hospital "*after*". The Eswab transport medium was stored at –80°C pending further analysis. All patients underwent two pre-operative showers using CHG-containing soap and the site of surgery was disinfected with a 0.5% chlorhexidine solution in 70% ethanol in the operating theatre by the scrub nurse. Antimicrobial prophylaxis was administered (i.e. for non-penicillin allergic patients, 2g cloxacillin 30-45 minutes before skin incision followed by two additional doses after 2h and 6h, respectively) as per clinical routine in Sweden.

2.2. 16S rRNA Gene Sequencing and Sequence Pre-processing

Extraction of DNA was done using FastDNA SPIN kit for Soil kits (MP Biomedicals, LLC, Solon, OH, USA) according to the manufacturer's protocol. Bacterial community profiling was performed using universal 16S rRNA V3-V4 gene primers as previously described [19] and sequences were classified using BION-meta [20] performing sequence cleaning, de-replication, chimera filtering and calculating similarities between sequences before matching against the Ribosomal Protein Database (RDP) [21]. The configuration of the software was adjusted to match the primers used and run with default parameters [20]. We initially detected high levels of contaminating *Burkholderiaceae*, in particular *Ralstonia solcenarium* and *Burkholderia spp.* However, these sequences were also detected in negative extraction controls and have been described as kit contaminants in the extraction kits that were used for DNA extraction [22]. Hence, we applied the prevalence method (threshold 0.5) of the R package decontam (version 1.4.0) to filter out contaminant sequences associated with these and other taxa [23]. Furthermore, we manually removed additional contaminants prior to downstream analysis (see details in https://github.com/ssi-dk/staphylome/tree/master/scripts).

2.3. Development of a new Tuf Gene Sequencing "Staphylome" Approach

We retrieved whole genome data from all publicly available staphylococci (>9,000 genomes accessed July 24th 2018) from RefSeq (https://www.ncbi.nlm.nih.gov/refseq/) to generate a staphylococcal reference database. From this database, *tuf* gene sequences were extracted and aligned to select suitable primer-binding sites. The staphylococcal *tuf* gene database is available at (https://github.com/ssi-dk/staphylome/tree/master/database). Amplification of a 491 bp region in the *tuf* gene was performed using primers with heterogeneity spacers (Table S3) in PCR reactions using KAPA HiFi HotStart ReadyMix (2x) Polymerase in 25 µL reaction volumes [12.5 µL 2xKAPA, 0.2 µm forward and reverse primer, 10 µL DNA template] with the PCR program (3 min at 95°C, 25 cycles: 98°C for 20 sec, 60°C for 15 sec, 72°C for 45 sec, and final extension 72°C for 5 min). Indexing of amplicons was done using Illumina's Nextera XT indexing kits (Illumina Inc., San Diego, CA, USA) in 25 µL reaction volumes [12.5 µL 2xKAPA HiFi, 2.5 µL forward and reverse index primers, and 2 µL DNA template] with the PCR program (3 min at 95°C, 20 cycles of: 98°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, and a final extension at 72°C for 5 min). After Agencourt AMPure XP (Beckman Coulter, Inc. Indianapolis, IN, USA) bead purification (x0.8 ratio), normalization and pooling, libraries were sequenced on Illumina MiSeq (Illumina Inc., San Diego, CA, USA) with a 600-cycle V3 kit (Illumina Inc., San Diego, CA, USA) with 10% PhiX spiked in.

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Demultiplexing of raw *tuf* gene reads was done using the bcl2fastq Conversion Software (Illumina) to obtain one forward and one reverse FASTQ file for each sample. The cutadapt software (version 2.3) was used to trim off heterogeneity spacers and primers at a tolerated maximum error rate of 8% corresponding to one mismatch [24]. Both read pairs were discarded if a primer was not found in one of the reads. The R package DADA2 (version 1.12.1) was used to infer amplicon sequence variants (ASVs) at single-nucleotide resolution [25]. We truncated forward reads at 270 bp and reverse reads at 241 bp to obtain reads with 20 bp overlap for merging and to retain sufficient read quality. The DADA2 pipeline (version 1.12.1) was executed with default settings (except truncation lengths) per sequencing run. The resulting ASV tables were then combined and chimeras were identified per sample, but removed from all samples globally (*removeBimeraDenovo*() function, method "consensus"). We formatted our staphylococcal *tuf* gene database to be compatible with DADA2's *assignTaxonomy()* function and used it to classify the staphylococcal ASVs with the RDP naive Bayesian classifier method. Unclassified reads were excluded from downstream analysis.

2.4. Statistical Analysis

All statistical analyses were performed in R (version 3.6.0, R Foundation for Statistical Computing, Vienna, Austria) [26]. Amplicon sequence variant (ASV) count tables, taxonomy tables and related metadata (data sets available at https://github.com/ssi-dk/staphylome/tree/master/data) were integrated with the R package phyloseq and its dependencies [27]. Packages used for the generation of plots include ggplot2 (version 3.2.1) and gplots [28,29]. Based on rarefaction curves we determined a lower cut-off at 2,000 reads per sample for both 16S rRNA (V3-V4) and *tuf* samples and excluded sample pairs from downstream analysis if one sample was below this cutoff (Supplementary Table S1). Ordination analysis by principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity was performed on Hellinger-transformed count data for both 16S rRNA (V3-V4) and *tuf* samples. We used the *betadisper()* function to test whether the variances of the groups we compared were homogenous and tested for differences in community structure between body sites and time points with permutational multivariate analysis of variance (PERMANOVA) using distance matrices with the *adonis()* function from the vegan package (version 2.5.5) [30] Bacterial alpha diversity by means of the Shannon index was calculated on untransformed 16S rRNA (V3-V4) read counts and staphylococcal *tuf* gene ASV counts, respectively, and alpha diversity *before* and *after* antimicrobial prophylactic treatment and arthroplasty was compared by paired Wilcoxon signed-rank tests. The 16S rRNA (V3-V4) read counts and staphylococcal *tuf* gene ASV counts were agglomerated at genus and species level, respectively, and transformed to relative abundance and visualized as bar plots with the top 10 most abundant bacterial genera (Figure 1B, 1C) and staphylococcal species (Figure 3A, 3B, 3C) displayed for each body site. We calculated the patient-wise change in percentage points from *before* to *after* arthroplasty for the most abundant bacterial genera (Figure 1D, 1E) and staphylococcal species (Figure 3D, 3E, 3F) and used paired Wilcoxon signed-rank tests with subsequent Benjamini-Hochberg correction for multiple testing to determine which bacterial genera and staphylococcal species changed significantly after decolonization treatment and arthroplasty. Scripts for these analysis steps are available at (https://github.com/ssi-dk/staphylome/tree/master/scripts).

3. Results

3.1. Bacterial Community Alterations Assessed by 16S rRNA (V3-V4) Gene Sequencing

Alteration of bacterial communities in anterior nares, groin, and operation sites of hip or knee arthroplasty patients, sampled *before* pre-operative decolonisation treatments and *after* arthroplasty surgery, was first examined by use of 16S rRNA (V3-V4) gene sequencing. A large proportion of the bacterial communities were not included in the analysis as too few reads (<2,000) remained after contaminant filtering, especially for samples obtained from groin and operation sites following decolonisation treatment and arthroplasty surgery (Table S1 and Figure S1, S2).

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PCoA analysis showed that bacterial communities clustered according to body site (Figure 1A) and PERMANOVA revealed no significant difference (p=0.2) in nasal bacterial community structure *before* compared to *after* decolonisation treatment and arthroplasty surgery (Figure 1B). Antibiotic prophylaxis and CHG decolonization were associated with small non-significant reductions in alpha diversity in anterior nares and significant reductions in alpha diversity at groin sites (p=0.04) (Figure S3). Among the top 10 bacterial genera in nares, only *Dolosigranulum* displayed a significant decrease across the patient cohort (p=0.002) (Figure 1D). In the groin, most patients were colonised by *Staphylococcus*, *Corynebacterium*, and *Anaerococcus* prior to decolonisation treatments and arthroplasty (Figure 1C), and a significant reduction was detected for *Corynebacterium* (p=0.0014) after arthroplasty (Figure 1E). Significantly different within-group variances between bacterial groin communities *before* compared to *after* arthroplasty (*betadisper*, p=0.01) likely resulted in significantly altered groin communities after arthroplasty (PERMANOVA, p=0.006). The *Staphylococcus* genus showed small, non-significant reductions in nares and groin, with individual patients experiencing either relative increase or decrease (Figure 1D, 1E). In groin sites, we also noted a trend with increased colonisation by Gram-negative bacteria such as *Escherichia* and *Klebsiella* (Figure 1D).

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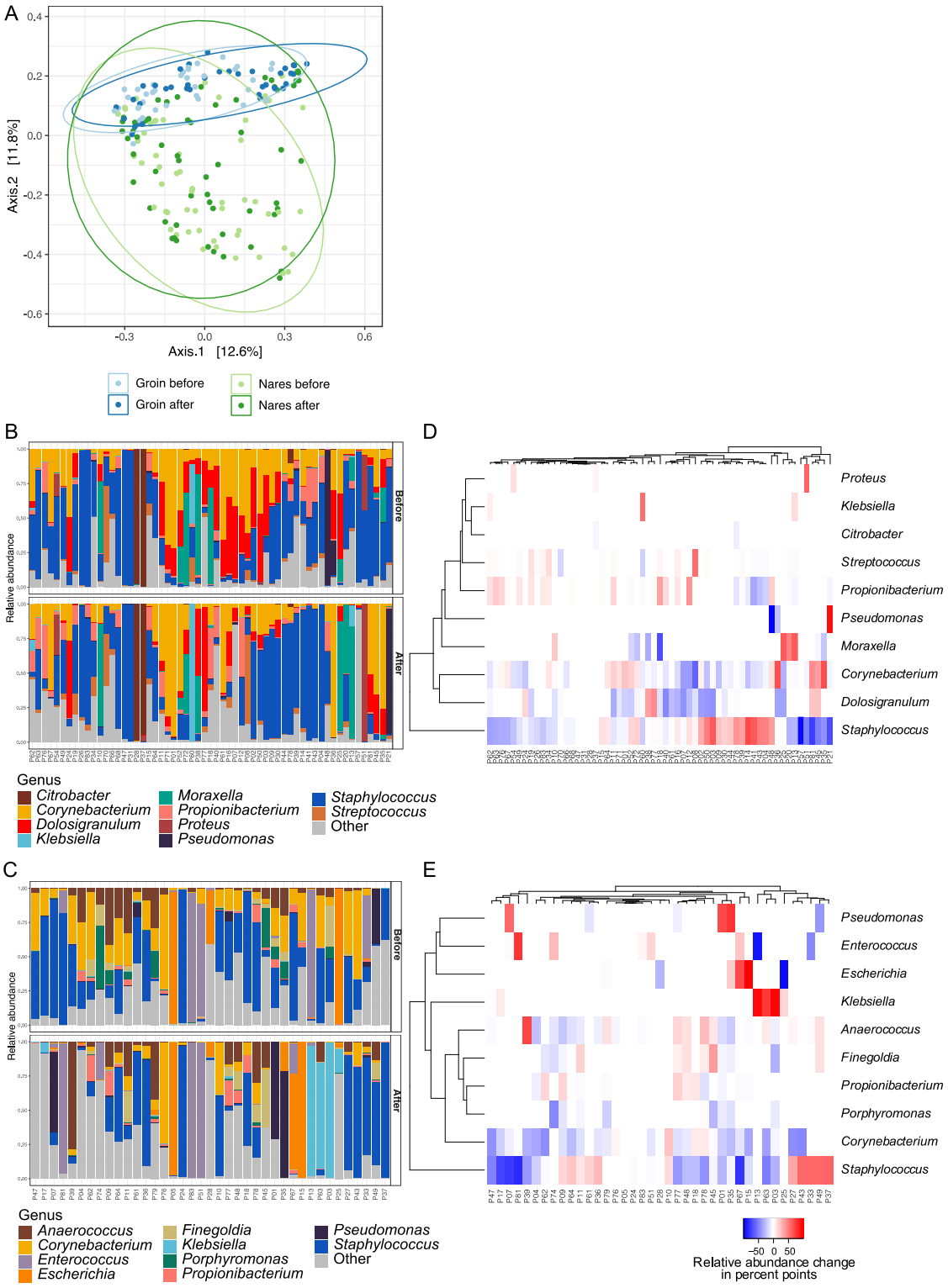

Figure 1. Bacterial community alterations *before* and *after* arthroplasty surgery assessed by 16S rRNA (V3-V4) gene sequencing. **(A)** PCoA plot of Hellinger transformed count data based on Bray-Curtis dissimilarity measures. **(B)** Bar plots of 59 bacterial communities from nares and **(C)** 39 groin communities *before* and *after* arthroplasty. Heatmaps showing patient-wise relative alterations in percentage points of the 10 most abundant bacterial genera from **(D)** nares and **(E)** groin *before* compared to *after* arthroplasty. Patients are clustered according to their similarity in relative abundance alteration for the top 10 most abundant genera.

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3.2. Development and Validation of the Staphylome Approach

To avoid kit contamination issues and to explore low-abundant *Staphylococcus* communities in greater detail, we developed and validated a *tuf* gene sequencing approach capable of detecting and differentiating individual *Staphylococcus* species (Figure 2A). First, we designed a new set of *tuf* gene primers and validated these primers on 27 different staphylococcal species and on common skin and nasal isolates (Figure S4). Next, we generated staphylococcal community profiles in triplicates from a mock community comprised of equal amounts of genomic DNA from six different *Staphylococcus* species. A dilution series revealed that all six *Staphylococcus* species could be detected down to ~10 copies/organism with decreasing technical reproducibility in the low-abundant mock communities (Figure 2B). Dilution down to ~1 copy/organism resulted in detection of between two and four of the six species in each triplicate.

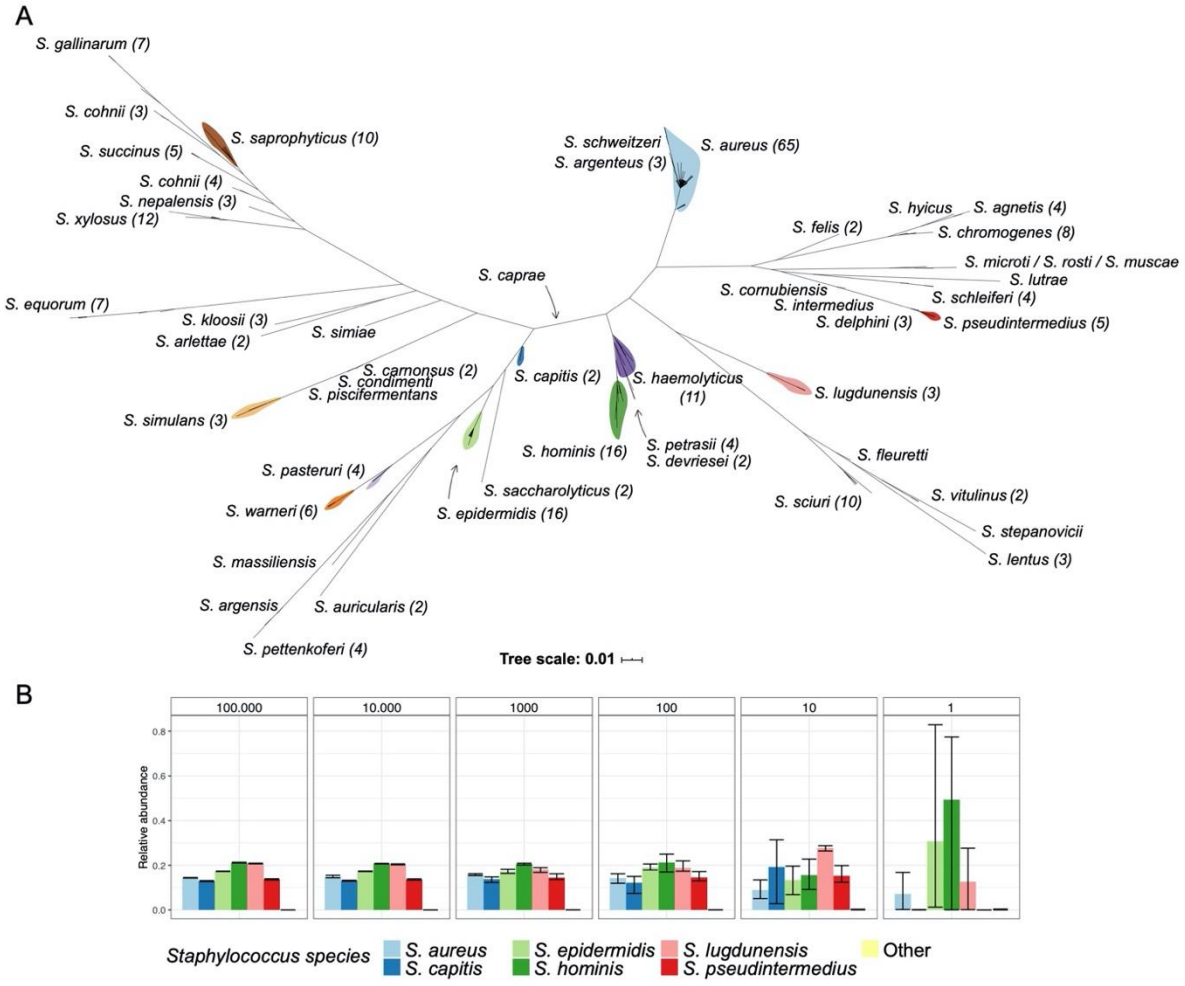

Figure 2. Phylogeny of the *tuf* gene region used in this study. **(A)** Phylogeny based on *tuf* genes from >9,000 staphylococcal genomes with *tuf* gene ASVs in each staphylococcal species shown in parenthesis. **(B)** Amplicon sequencing of *tuf* genes from a staphylococcal mock community comprised of equal amounts of genomic DNA from *S. aureus, S. capitis, S. epidermidis, S. hominis, S. lugdunensis,* and *S. pseudintermedius* in ~100,000 copies/organism (left) diluted down to ~1 copy/organism (right). Error bars indicate highest and lowest observation in each technical triplicate experiment.

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3.2. Staphylococcal Community Alterations Assessed by the Staphylome Approach

We applied the staphylome approach to study subtle alterations in staphylococcal community composition in anterior nares, groin, and operation site samples *before* and *after* arthroplasty surgery. Most patients colonized with *S. aureus* in the nares (23 out of 65) *before* prophylactic treatments (here, colonisation signifies *S. aureus* ASVs ≥1% of total *tuf* sequencing reads) retained *S. aureus* colonisation *after* arthroplasty (17 out of 23) and one patient gained *S. aureus* without being colonised *before* arthroplasty surgery.

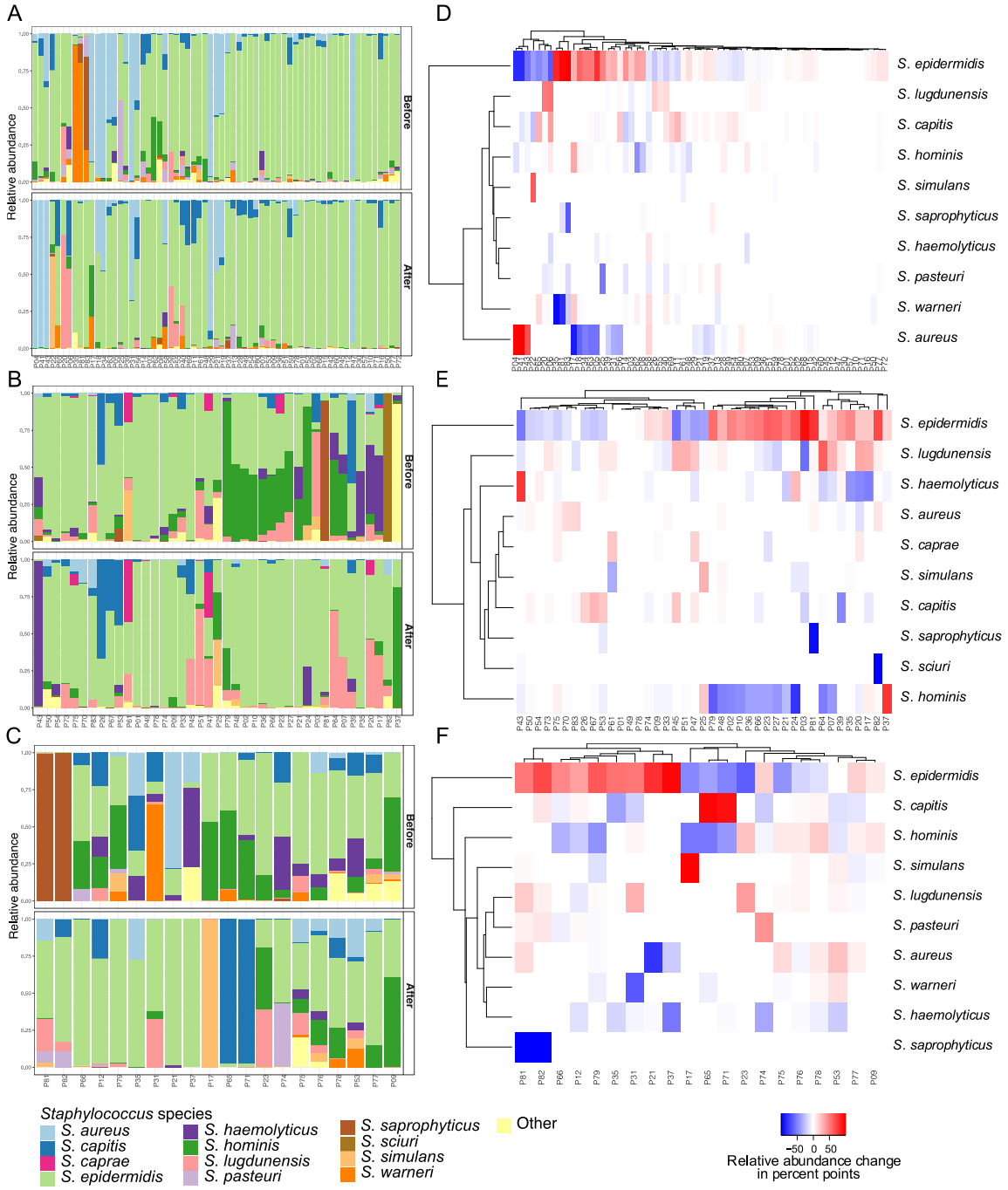

Figure 3. Staphylococcal communities from arthroplasty patients sampled *before* and *after* decolonisation treatment and arthroplasty surgery. Bar plots showing alterations in the 10 most abundant Staphylococcus species present in **(A)** nares of 65 patients, **(B)** groin of 41 patients, and **(C)** operation site of 20 patients, assessed by *tuf* gene sequencing. Heatmaps show patient-wise relative alterations in percentage points of the 10 most abundant *Staphylococcus* species from **(D)** nares, **(E)**

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groin, and **(F)** operation site from *before* compared to *after* decolonisation treatment and arthroplasty surgery. Patients are clustered according to similarities in relative abundance alterations.

We found a positive correlation (p=0.04, ρ=0.36) between *S. aureus* and total staphylococcal relative abundance in the nares, where increased presence of *S. aureus* for three patients - P4, P49, and P51 – coincided with increased total staphylococcal relative abundance (Figure S5). Three staphylococcal species, *S. hominis* (p=0.02), *S. haemolyticus* (p=0.02), and *S. pasteuri* (p=0.003), showed a significant relative decrease in the nares after arthroplasty surgery across the patient cohort (Figure 3, Table S2). The most abundant staphylococcal species, both *before* and *after* arthroplasty, at all sampling sites was *S. epidermidis*, that showed small non-significant increases in relative staphylococcal abundance at all sampling sites after arthroplasty surgery, together with *S. lugdunensis* and *S. capitis*. However, the increase in these CoNS did not correlate with increased total staphylococcal relative abundance (Table S2). Staphylococcal community alterations in groin and operation sites clustered into two distinct groups characterised by alterations in *S. epidermidis* relative abundance (Figure 3E, F). In groin samples, alpha diversity among *Staphylococcus* species decreased significantly (p=0.03) together with a significant reduction in *S. hominis* (p=0.01) (Figure 3B, 3E, Figure S3). In operation sites we detected a significant reduction in *S. haemolyticus* (p=0.039) after decolonisation treatment and arthroplasty (Figure 3C, 3F, Figure S3).

4. Discussion

The data presented here suggest that systemic antibiotic prophylaxis and decolonization treatment with CHG soap in association with prosthetic join replacement surgery and hospitalisation can alter bacterial communities with variations across different body sites. Similar alterations were observed for sub-sets of patients, while other alterations were patient specific. Overall, bacterial and staphylococcal communities remained stable in the nares after decolonization and arthroplasty, albeit with small non-significant reductions in alpha diversity and significant decreases in *Dolosigranulum* relative abundance and significant decrease in *S. hominis*, *S. haemolyticus*, and *S. pasteuri* relative staphylococcal abundance. Groin communities had significant reductions in alpha diversity in both bacterial and staphylococcal communities after arthroplasty with significantly less *Corynebacterium* and *S. hominis*. The differences observed between sampling sites likely reflect the distinctive microbiomes present at each site and the differential exposure to CHG decolonisation treatments. The nares, receiving no topical intranasal decolonisation, was predominately affected by systemic antibiotic prophylaxis, whereas skin sites were affected by both systemic antibiotic prophylaxis, preoperative CHG soap showers, and perioperative skin disinfection in operation sites. Our results are in agreement with previous culture-based findings stating that CHG showers do not eradicate bacteria, but instead decrease bacterial diversity [31]. An earlier *tuf* gene-based study also reported that nasal staphylococcal communities were not substantially altered after antibiotic treatment [32]. In this study, a lower success-rate in analysing groin and operation site samples following decolonisation treatments and arthroplasty for both 16S rRNA (V3-V4) gene sequencing and the staphylome approach points to an intended efficacy of the decolonisation treatments (Table S1 and Figure S6). Future studies incorporating absolute abundance data on the studied microbial communities as well as non-treated control subjects could help to clarify the nature of the observed alterations and assist in determining if the changes arise from preoperative decolonisation treatment, antibiotic prophylaxis, changes in environment due to hospitalisation, common fluctuations in the bacterial composition, or a combination thereof. The sensitivity of amplicon-based methods allows for analysis of low abundant bacterial communities, but simultaneously it increases the risk of contamination from the laboratory environment [33]. Contamination issues also impacted this study, including DNA extraction kit contamination issues previously reported by Salter et al. 2014 [22]. Application of the staphylome approach was not impacted by systematic contamination as staphylococci are not common kit contaminants in the extraction kits that were used, but sporadic staphylococcal contamination did occur in negative controls, likely due to cross-contamination

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between samples. However, since these contaminating *tuf* gene ASVs overlap with the most abundant staphylococcal ASVs present in the samples, a decontamination approach was not pursued.

5. Conclusions

Our data support the use of amplicon-based sequencing approaches for studying alterations in low abundant bacterial communities and highlight the use of a *Staphylococcus* genus-centric approach, by targeting the *tuf* gene as it contains sufficient sequence diversity to distinguish between closely related species (Figure 2, Figure S7). This staphylome approach could also be applied when examining subtle dynamics of the skin microbiota in other study settings, e.g. patients with atopic dermatitis or haematological malignancies, patients undergoing chemotherapy or in neonates. It remains an open question how current standard preoperative prophylactic procedures can be improved to mitigate the risk of infection with COPs or MDR nosocomial strains. We found increased presence of Gram-negative bacteria following decolonisation treatment, particularly on the skin of individual patients, which could be indicative of a reduced colonization resistance (Figure 1E). This should be considered a potential risk factor as antibiotic usage at health-care facilities favours and selects for MDR bacterial lineages which complicates treatment considerably [34,35]. High-resolution amplicon-based sequencing approaches and culture-based methods can be used in combination to provide information on how COPs respond to different treatments, along with information on MDR profiles of colonising strains, which can help guide the use of more suitable decolonization strategies and hopefully reduce the rate of postoperative infections in the future.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, **Figure S1:** Read counts in 16S rRNA (V3-V4) gene sequencing *before* and *after* arthroplasty surgery, **Figure S2:** Sample availability for each sampling in 16S rRNA (V3-V4) gene sequencing analysis, **Figure S3:** Alpha diversity plots for 16S rRNA gene sequencing and *tuf* gene sequencing, **Figure S4:** Agarose gel with *tuf* PCR products from staphylococcal and non-staphylococcal bacteria, **Figure S5:** Correlation between alterations in *S. aureus* ASVs and total staphylococcal abundance in nares *before* compared to *after* arthroplasty surgery, **Figure S6:** Sample availability for 16S rRNA (V3-V4) and *tuf* gene sequencing analysis, **Figure S7:** Single nucleotide polymorphism (SNP) differences between staphylococci in 16S rRNA (V3-V4) *tuf* gene region, **Table S1:** Overview of samples and read counts for 16S rRNA (V3-V4) and *tuf* gene sequencing, **Table S2:** Correlation between staphylococcal *tuf* gene ASVs and total staphylococcal abundance, **Table S3:** Primer table of *tuf* gene sequencing primers.

Data Availability: All sequences are available through the European Nucleotide Archive (ENA) at the European Bioinformatics Institute (EBI) (PRJEB39833). Scripts, phyloseq objects, and the database are available at https://github.com/ssi-dk/staphylome/.

Ethical Approval: The collection of pre- and postoperative samples swabs was approved by the Regional Ethical Review Board of Uppsala (reference: 2012/092 and amendment 2020-00991).

Author Contributions: Conceptualization, Åsa Nilsdotter-Augustinsson, Bo Söderquist, Marc Stegger and Paal Skytt Andersen; Data curation, Thor Bech Johannesen and Anna Cäcilia Ingham; Formal analysis, Søren Iversen, Thor Bech Johannesen, Anna Cäcilia Ingham, Emeli Månsson, Staffan Tevell, Bo Söderquist, Marc Stegger and Paal Skytt Andersen; Funding acquisition, Bo Söderquist; Investigation, Søren Iversen, Thor Bech Johannesen and Anna Cäcilia Ingham; Methodology, Søren Iversen, Thor Bech Johannesen, Anna Cäcilia Ingham, Sofie Marie Edslev, Marc Stegger and Paal Skytt Andersen; Project administration, Bo Söderquist, Marc Stegger and Paal Skytt Andersen; Software, Thor Bech Johannesen and Anna Cäcilia Ingham; Supervision, Bo Söderquist, Marc Stegger and Paal Skytt Andersen; Validation, Søren Iversen, Thor Bech Johannesen and Anna Cäcilia Ingham; Visualization, Søren Iversen and Anna Cäcilia Ingham; Writing – original draft, Søren Iversen; Writing – review & editing, Søren Iversen, Thor Bech Johannesen, Anna Cäcilia Ingham, Sofie Marie Edslev, Emeli Månsson, Staffan Tevell, Åsa Nilsdotter-Augustinsson, Bo Söderquist, Marc Stegger and Paal Skytt Andersen.

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Conflicts of Interest: The authors declare no conflicts of interest.

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