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Research Article

In Vitro Bacterial Adhesion and Biofilm Formation on Fully Absorbable Poly-4-hydroxybutyrate and Nonabsorbable Polypropylene Pelvic Floor Implants

Kim W. J. Verhorstert, Zeliha Guler, Leonie de Boer, Martijn Riool, Jan-Paul W. R. Roovers, and Sebastian A. J. Zaat*

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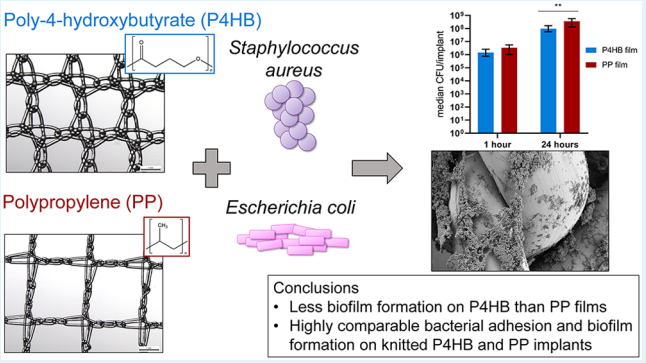
ABSTRACT: Knitted polypropylene (PP) implants for the correction of pelvic organ prolapse have been associated with complications such as vaginal exposure, infection, and pain. Since certain complications may be linked to bacterial contamination and persistent inflammation, there is a rationale to develop a biocompatible implant that is less prone to bacterial adhesion and biofilm formation. Delayed absorbable materials could meet these requirements and poly-4-hydroxybutyrate (P4HB) might be such a new material for future pelvic floor implants. We studied in vitro bacterial adhesion and biofilm formation on P4HB in comparison to PP. We investigated the influence of both polymers using flat films and compared P4HB and PP implants with different knitting designs. P4HB flat films were demonstrated to be hydrophilic with significantly less *Staphylococcus aureus* and *Escherichia coli* cultured from P4HB films than from hydrophobic PP films after 24 h of incubation. On the implants, a higher number of *E. coli* were cultured after 1 h of incubation from the knitted P4HB implant with the highest density and smallest pore size, compared to other P4HB and PP implants. No differences were observed between the implants for *E. coli* at later time points or for *S. aureus* incubation. These results show that in flat films, the polymer influences biofilm formation, demonstrated by a reduced biofilm formation on P4HB compared with PP flat films. In addition, the knitting design may affect bacterial adhesion. Despite certain design and material characteristics that give the knitted P4HB implants a higher surface area, this did not result in more bacterial adhesion and biofilm formation overall. Collectively, these results warrant further (pre)clinical investigations of P4HB pelvic floor implants.



KEYWORDS: *pelvic organ prolapse (POP), implant, mesh, absorbable, poly-4-hydroxybutyrate (P4HB), polypropylene (PP), biofilm, infection*

INTRODUCTION

Pelvic organ prolapse (POP) is a connective tissue disorder that results in the descent of one or more pelvic organs into the vagina. It can cause an impaired pelvic floor function, leading to problems with micturition and defecation and to sexual dysfunction. Between 11 and 19% of women will undergo at least one operation for POP or urinary incontinence in her lifetime.[1,2] Unfortunately, long-term results of native tissue repair are unsatisfactory, and as a result one out of four women will require multiple prolapse surgeries.[3] Pelvic floor implants, both biologic and synthetic, were introduced to reduce the risk of recurrent POP by providing mechanical support. Although biological grafts can be nonimmunogenic, they have the disadvantage of undergoing fast degradation.[4] Permanent implants, such as polypropylene (PP), have shown to be superior to native tissue repair with respect to anatomical outcomes and preventing recurrent symptomatic prolapse.[5] Unfortunately, previous heavyweight PP implants have been associated with serious and sometimes irreversible, adverse events such as vaginal exposure, pelvic pain, and infection. While these implants might have evoked minimal adverse reaction for other applications, such as in abdominal hernia repair,[6] they can provoke a sustained inflammatory response in the vagina.[7] As a result, PP implants for POP surgery have evolved to lightweight, macroporous, monofilament implants, which induce a milder host response.[8] One of the most used, latest generation PP implants is Restorelle, a monofilament,

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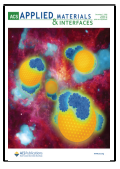

ultralightweight (18.9 g/m^2) and macroporous (3.1 mm^2) implant (Table 1). Unlike other permanent implants,

Table 1. Implant Characteristics

implant	fiber diameter (μm)	thickness (mm)	pore size (mm^2)	areal density (g/m^2)
Diamond	100	0.28	2.22	49.70
Delaware	100	0.41	0.40	94.80
Marquisette	100	0.29	3.38	60.55
Marquisette+	100	0.29	1.67 major, 0.81 minor	70.35
Restorelle	79	0.34	3.10	18.90

Restorelle has been shown to be well tolerated in animal experimental studies.[9−11] Also, clinical studies have shown some improvements in morbidity on the short term, but long-term results are yet to be reported.[12] Meanwhile, the damage caused by the earlier PP implants has led to restricted indications and limited use of synthetic permanent implants for POP surgery and caused a challenging situation for women requiring recurrent prolapse surgery.[13]

After implantation, the implant causes an initial moderate inflammatory response as part of the so called "foreign body response," leading to constructive remodeling with functional integration of the biomaterial and durable support of the native tissue.[4] One hypothesis for the occurrence of adverse events is a persistent inflammatory response, which may be related to bacterial presence on the implant.[4,14] This has been associated with complications such as exposure and pain because of contraction.[7,15,16] Bacterial contamination was seen in explanted meshes from 77 to 100% of patients with complications.[17,18] Although it is as yet uncertain whether bacterial colonization is directly related to the development of complications besides infection, there is a rationale for a biomaterial that is less prone to bacterial adhesion and subsequent biofilm formation.[19]

Biodegradable materials are considered less susceptible to infection than nondegradable materials.[20−22] However, when materials degrade too fast, that is, before sufficient new connective tissue has formed, this may result in poor surgical outcomes.[19] Materials with delayed degradation such as poly-4-hydroxybutyrate (P4HB), which retain strength in implant form for at least 12 months,[23] may therefore be better candidate materials for future POP implants. The time to complete degradation varies based on the implant's physical properties, and for P4HB lies between 18 and 24 months.[24] The P4HB Phasix mesh for abdominal hernia repair has been used for some years and demonstrated biocompatibility, few adverse event rates, and similar hernia recurrence rates compared to the PP implant.[25] However, this mesh is heavier and thicker than the currently used lightweight pelvic floor implants (i.e., density 182 g/m^2).[26] Because of the associated aforementioned adverse events, novel P4HB implants were designed to meet the requirements for pelvic floor implants. Our previous and ongoing research on knitted P4HB implants for POP surgery showed increased fibroblast proliferation and collagen formation compared with PP implants in vitro[48] and a mild host immune response in ewes (manuscript in preparation).

In the present study we evaluated in vitro bacterial adhesion and biofilm formation of *Staphylococcus aureus* and *Escherichia coli*, two common vaginal species[27,28] on different P4HB and PP implants. We investigated the influence of the P4HB and PP polymers using flat films, compared implants of both polymers with similar knitting patterns, and compared the influence of knitting patterns with four different P4HB knitting designs.

MATERIALS AND METHODS

Polymer Flat Film Development and Surface Wettability. With flat films, we studied the affinity for bacterial adhesion and biofilm formation based on the physicochemical properties of the polymers alone, thereby excluding the effect of the different implant characteristics. Flat films of P4HB and PP were produced by

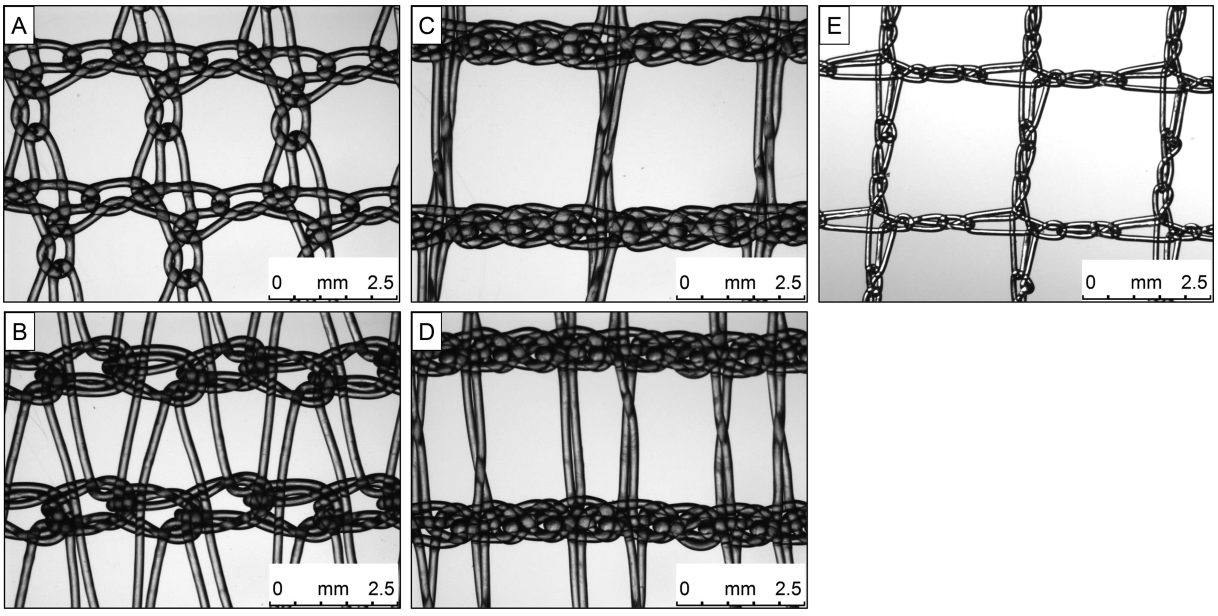

Figure 1. Knitting designs of the P4HB implants (A) "Diamond"; (B) "Delaware"; (C) "Marquisette"; (D) "Marquisette+" and PP (E) "Restorelle". Images were taken using a light microscope at random locations.

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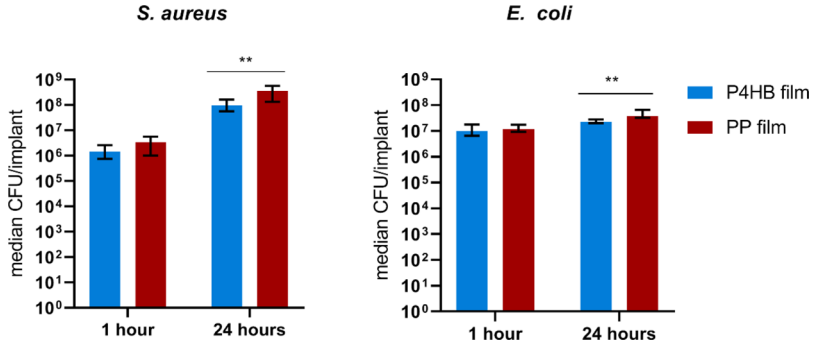

Figure 2. Bacterial adhesion of *S. aureus* and *E. coli* after 1 h and biofilm formation after 24 h on P4HB and PP flat films. Results are on a logarithmic scale and expressed as median CFU/implant with IQR. Biofilm formation at 24 h is significantly less on P4HB compared to PP films.** = $p < 0.01$. Additional analysis: P4HB *S. aureus* 1 h versus 24 h $p < 0.01$, PP *S. aureus* 1 h versus 24 h $p < 0.01$, P4HB *E. coli* 1 h versus 24 h $p < 0.01$, and PP *E. coli* 1 h versus 24 h $p < 0.01$.

compression molding. P4HB (Tepha, Inc. Lexington, MA, USA) and PP granules (SABIC, The Netherlands) were melted at 135 °C for 5 min, and at 230 °C for 10 min, respectively, while a force of 100 kN was applied. Flat films were cooled between cold plates until solidified. Advancing type water contact angles were measured with a fixed camera setup to determine the wettability of the P4HB and PP flat films as described previously.[29] In short, a droplet of 2.5 μL of ultrapure water was placed on the films with a microsyringe and after 3 s an image was recorded. Droplet contours were automatically detected and contact angles were calculated with customized MATLAB software. The reported contact angles per material are the mean of a total of nine droplets on three individual pieces of flat film. A surface with a water contact angle >90° is considered to be hydrophobic.[30]

Implants. P4HB knitted implants in four different knitting designs—"Diamond," "Delaware," "Marquisette," and "Marquisette +"—were manufactured and provided by Tepha, Inc. The PP Restorelle knitted implants were manufactured and provided by Coloplast (Minneapolis, MN, USA) (Figure 1). Table 1 provides an overview of the implant characteristics, and the data were obtained from Tepha Inc.

Inoculum Preparation. We studied *S. aureus* ATCC 49230[31,32] and *E. coli* ATCC 25922,[33,34] both clinical isolates known for their biofilm forming capacity. Bacteria from frozen stocks were cultured on sheep blood agar plates and incubated overnight at 37 °C. Single colonies were suspended in 5 mL of tryptic soy broth (TSB; BD Difco) and incubated at 37 °C at 120 rpm to the logarithmic growth phase. After incubation, the suspension was diluted with fresh TSB to 1×10^8 colony forming unit (CFU)/ml, based on the optical density at 620 nm.

Bacterial Adhesion and Biofilm Formation on Flat Films and Knitted Implants. Flat films and knitted implants of P4HB and PP were cut into 11 × 11 mm samples, sterilized for 10 min in 70% ethanol, dried in a laminar flow chamber, and transferred to individual wells of a 24-well plate. We studied bacterial adhesion after 1 and 4 h and biofilm formation after 24 and 48 h of incubation. To most sensitively assess differences in the susceptibility of the materials for biofilm formation, experiments were performed under optimal conditions for biofilm formation for the bacterial test trains. For this reason, 1% (v/v) pooled human plasma (Sanquin Blood Supply, The Netherlands) was added to the TSB incubation medium for *S. aureus* whereas experiments with *E. coli* were performed in nonsupplemented TSB (Supporting Information Figure S1). The samples of P4HB and PP films were incubated in 2 mL, and the knitted implants in 0.5 mL of 1×10^8 CFU/mL *S. aureus* or *E. coli* suspension, sufficient to ensure full covering of the respective materials. Six samples from the P4HB and PP flat films were incubated for 1 and 24 h at 37 °C under static conditions. Two independent replicate experiments were performed. Knitted implants were studied as described above but incubated for 1, 4, 24, and 48 h.

After incubation, the samples were gently washed three times in phosphate-buffered saline (PBS) to remove nonadherent bacteria. For quantitative culture, bacteria were subsequently detached from the implant in a sterile tube with 3 and 1 mL of PBS for the flat films and implants, respectively, by 30 s of vortexing followed by 15 min of sonication (Elma Transsonic T460, 35 kHz; Elma Schmidbauer GmbH, Singen, Germany). The volume of PBS was adjusted to ensure covering of the material. This detachment method was highly efficient, resulted in the removal of the vast majority of bacteria attached, and seemed comparable between P4HB and PP (Supporting Information Figure S2). Ten-fold serial dilutions of each sonicate were made in PBS and triplicate 10 μL aliquots of the sample and the dilutions were spotted on blood agar plates. The plates were incubated overnight at 37 °C and the number of CFU/implant were determined.

Visualization of Biofilm Formation by Scanning Electron Microscopy. Biofilm formation on knitted implants after 24 h of incubation was imaged with scanning electron microscopy (SEM). After incubation, the samples were fixed in 4% paraformaldehyde and 1% glutaraldehyde for 4 h at room temperature followed by a washing step with PBS and dehydration using a graded ethanol series. In order to reduce sample surface tension, samples were immersed in hexamethyldisilizane (Sigma-Aldrich) for 30 min and air dried before mounting on aluminum SEM stubs and sputter-coating with a 6 nm platinum-palladium layer, using a Leica EM ACE600 sputter coater (Leica Microsystems). Pictures were taken at random locations with a magnification of 100 × and 3000 × using a Zeiss Sigma 300 SEM (Zeiss).

Statistical Analysis. All statistical analyses were performed with IBM SPSS statistics version 25.0. Since the data were not normally distributed, medians and interquartile ranges (IQRs) are reported. The median number of CFUs recultured from flat films of P4HB and PP were compared with a Mann−Whitney-U test. The median number of CFUs recultured from different implant groups were compared using a Kruskal-Wallis test, in the case of a significant difference followed by post hoc analysis with a Mann−Whitney-U test for all possible combinations of groups. A p-value of <0.05 was considered to be statistically significant. Graphs were created with GraphPad Prism 8.3.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Flat Films: Wettability and Bacterial Quantification. P4HB flat films demonstrated lower water contact angles (77 ± 3°) than PP films (115 ± 3°), indicating that P4HB had a hydrophilic and PP a hydrophobic surface.

For both bacterial species, a significant increase of the median number of CFUs per implant over time was observed on P4HB and PP flat films (Figure 2). After 1 h of incubation, there were no significant differences in bacterial adhesion between P4HB and PP films for either *S. aureus* ($p = 0.08$) or

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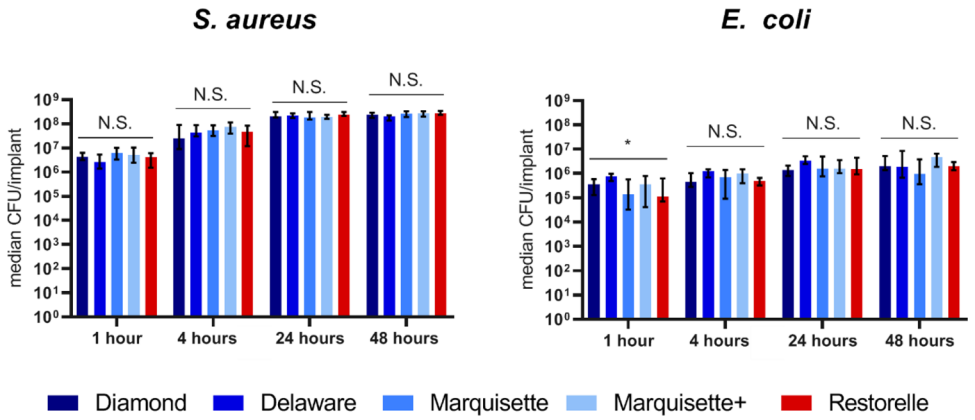

Figure 3. Bacterial adhesion of *S. aureus* and *E. coli* after 1 and 4 h and biofilm formation after 24 and 48 h on knitted implants of P4HB and PP. "Diamond," "Delaware," "Marquisette," and "Marquisette+" are made of P4HB and Restorelle is made of PP. Results are on a logarithmic scale and expressed as median CFU/implant with the IQR. For *E. coli* after 1 h there appeared to be a significant difference ($p = 0.02$) and post hoc analyses showed a significant difference between P4HB "Delaware" (7.5×10^5 CFU/implant) as compared to P4HB "Diamond" (3.7×10^5 CFU/implant ($p < 0.01$)), P4HB "Marquisette" (1.4×10^5 CFU/implant ($p < 0.01$)), and PP Restorelle (1.1×10^5 CFU/implant ($p = 0.01$)). Other time points did not show significant differences. N.S. = not significant, * = $p < 0.05$

E. coli ($p = 0.75$). However, P4HB films were demonstrated to be significantly less prone to *S. aureus* ($p < 0.01$) and *E. coli* ($p < 0.01$) biofilm formation at 24 h compared with PP films.

Material characteristics, such as surface chemistry and charge, the microarchitecture, and wettability are decisive factors for bacterial adhesion and subsequent biofilm formation.[35] In general, hydrophobic materials tend to favor bacterial adhesion and biofilm formation over hydrophilic materials.[36−39] The more hydrophobic nature of PP might be an explanation for the higher level of biofilm formation on PP compared to P4HB flat films. Furthermore, bacteria with hydrophobic properties favor hydrophobic materials and bacteria with hydrophilic properties favor hydrophilic materials for adherence and biofilm formation.[35] Since both *S. aureus*[40] and *E. coli*[36] are hydrophobic, they might favor biofilm formation on the hydrophobic PP films.

Knitted Implants: Bacterial Quantification. No significant differences were observed for *S. aureus* adhesion and biofilm formation on the different P4HB and PP implants at any of the four time points (Figure 3). For *E. coli*, a significantly higher initial adhesion (after 1 h) was observed on P4HB "Delaware" (7.5×10^5 CFU/implant) as compared to P4HB "Diamond" (3.7×10^5 CFU/implant ($p < 0.01$)), P4HB "Marquisette" (1.4×10^5 CFU/implant ($p < 0.01$)), and PP Restorelle (1.1×10^5 CFU/implant ($p = 0.01$)). Other time points did not show significant differences.

Polymer type and structural characteristics such as surface area and porosity can influence bacterial adhesion and biofilm formation.[41,42] In previous studies, comparisons were often made using various brands of implants, which differ in polymer type as well as knitting design. Since these factors both can influence adhesion and biofilm formation, it is difficult to conclude their respective influence. For this reason, we analyzed implants of different polymers with most resembling knitting designs (P4HB "Marquisette" and PP Restorelle) and four slightly different knitting designs of the same polymer, P4HB. Despite the differences in biofilm formation on the flat films, only minor differences in adhesion and biofilm formation were observed between all knitted implants, independent of the polymer used. This suggests that for the implants tested the knitting pattern may be more decisive for the adhesion and biofilm formation than the polymer used. All four knitted P4HB implants as well as the PP Restorelle are monofilament, lightweight, and macroporous implants (Table 1) and thereby of clinical relevance nowadays. These comparable characteristics may have been the reason that no, or only small differences in bacterial adhesion and biofilm formation were observed between the implants. However, these differences are not likely to be clinically relevant.

Interestingly, P4HB "Delaware," the implant with the highest density and smallest pore size, (Table 1, Figure 1), and thus the highest surface area, showed significantly more initial *E. coli* adhesion than the other P4HB implants ("Diamond" and "Marquisette"). So, *E. coli* bacterial adhesion may be related to the surface area available for adhesion. In general, the P4HB implants had smaller pore sizes, larger fiber diameters, and higher areal densities compared to the Restorelle PP implant (Table 1). The larger surface of the P4HB implants is intended to induce a more robust initial host response to compensate for the ultimate resorption of P4HB, which can only result in favorable surgical outcomes if new load-bearing tissues had developed before this time.[43] The P4HB implants thus provided the bacteria with more surface to adhere to and form a biofilm than PP Restorelle. Despite this, no differences in adhesion and biofilm formation were observed between PP Restorelle and "Diamond" or "Marquisette," the P4HB implants most resembling this PP implant (Figure 1, Table 1). Although the knitting patterns and fiber diameters of the P4HB implants and of PP are not identical, these data suggest a lower level of biofilm formation per surface area on P4HB, which is in line with the lower levels of biofilm formation on the P4HB flat films (Figure 2).

Detection of Biofilm by SEM. *S. aureus* biofilms were detected on all implants (Figure 4 and Supporting Information Figure S3; upper panels), and biofilm formation was highly similar for the different knitting designs, and in line with the number of CFUs cultured (Figure 3). However, hardly any *E. coli* biofilms were observed. On all P4HB knitted implants we observed only individual *E. coli* bacteria on the fibers, but on PP some clusters of bacteria were detected (Figure 4 and Supporting Information Figure S3; lower panels). The very low *E.coli* biofilm formation observed with SEM is not in line with the number of CFUs cultured. *E. coli* biofilms may have been less tightly attached and therefore washed off during the

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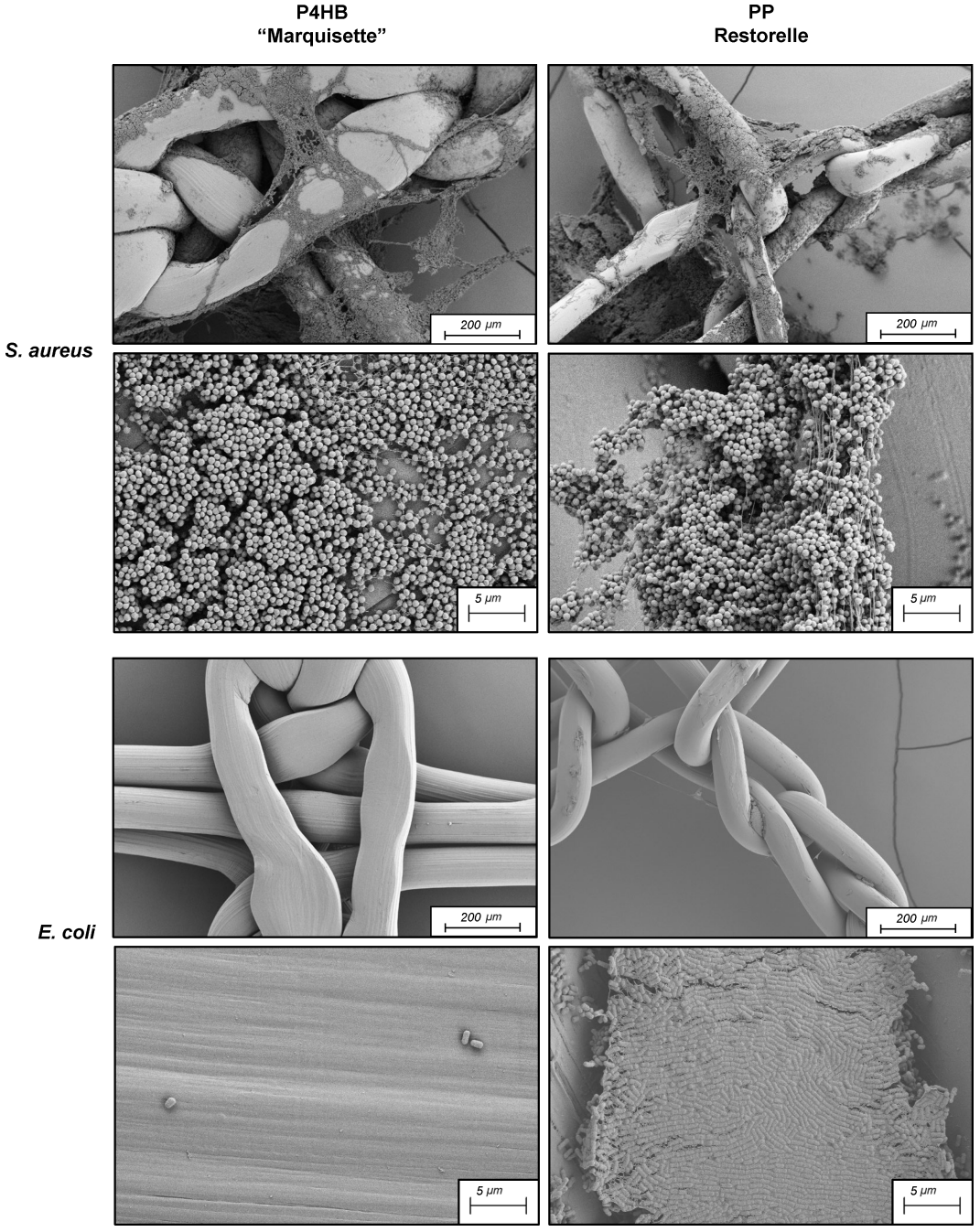

Figure 4. SEM images of knitted implants, with biofilm formation of *S. aureus* and *E. coli* after 24 h of incubation. Images were taken at 100 × and 3000 × magnification. P4HB "Marquisette" and PP Restorelle were chosen as representative implants since the knitting design of these implants is most comparable (Figure 1, Table 1). SEM images of other implant types can be found in Supporting Information Figure S3.

sample preparation. The detachment of bacteria during SEM sample processing – thereby causing a discrepancy between the quantitative culture results and SEM – has been described previously.[44] The strong biofilm formation of *S. aureus*, resisting removal during the washings, may be due to the fact that plasma was added to the growth medium of *S. aureus* since this was the optimal condition as selected for *S. aureus* biofilm formation (Supporting Information Figure S1). Plasma causes an increased expression of *S. aureus* adhesive matrix molecules, which can bind to plasma proteins bound to the implant surfaces, thereby enhancing stable biofilm formation.[45]

Both *S. aureus* and *E. coli* are part of the patient's own commensal microflora and may reside in the vagina prior to or during surgery, where they can subsequently contaminate the implants. However, there are still not many studies investigating pelvic floor implant-associated infections, and to the best of our knowledge, ours is the first study to evaluate bacterial adhesion and biofilm formation on P4HB implants. A limitation of our study is that we only investigated one strain of *S. aureus* and *E. coli*, whereas differences between strains regarding adherence and biofilm formation do exist within species. Moreover, the vaginal microflora is multimicrobial[27,28] and interspecies microbial interactions may influence biofilm formation. Our test strains for these defined in vitro studies were however selected to be strong biofilm formers allowing

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proper evaluation of bacterial adhesion and biofilm formation on the tested implants.[31−34]

In addition to its lower level of biofilm formation, P4HB may indirectly have an antimicrobial effect, since P4HB causes an upregulation of endogenous antimicrobial peptides in vitro.[46] Our in vitro setting, without the incorporation of any host factors, might therefore underestimate the in vivo (indirect) antimicrobial potential of P4HB. Indeed, P4HB implants have shown an increased resistance to bacterial contamination in vivo.[46] Moreover, bacteria likely will have difficulty to remain attached on an eroding surface and will be cleared when the implant degrades.[47] Because of the delayed absorbable nature of P4HB, the influence of degradation on bacterial adhesion and biofilm formation could not be taken into account in the present in vitro studies. It is promising that the P4HB polymer films show less biofilm formation and the higher surface area of the knitted implants does not lead to more bacterial adhesion and biofilm formation. Although the observed differences are not necessarily of direct clinical significance, this warrants in vivo studies, as small differences in vitro might have a clinically relevant outcome when combined with the local immune response. Our results and the possible beneficial effects of an absorbable material support the potential of P4HB as a new material for future pelvic floor implants.

CONCLUSIONS

In this in vitro study we compared the effects of delayed absorbable P4HB and nonabsorbable PP on bacterial adhesion and biofilm formation. No differences in bacterial adhesion on polymer flat films were observed after 1 h, but the hydrophilic P4HB films demonstrated significantly less *S. aureus* and *E. coli* biofilm formation after 24 h of incubation compared to the hydrophobic PP films. The knitted P4HB implant with the highest density and smallest pore size showed increased *E. coli* adhesion after 1 h compared to implants with lower densities and larger pores. For other time points and for *S. aureus*, we did not observe any significant differences. These in vitro data show that the P4HB polymer in films is less prone to biofilm formation and that the knitted P4HB implants do not show higher biofilm formation, despite their larger surface area in comparison to PP. These results support the potential of knitted P4HB for future POP implants encouraging further (pre)clinical research.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.0c14668.

Effect of supplements to the growth medium on biofilm formation of *S. aureus* and *E. coli*; SEM images after detachment of bacteria; SEM images of all knitted implants (PDF)

AUTHOR INFORMATION

Corresponding Author

Sebastian A. J. Zaat − *Department of Medical Microbiology and Infection Prevention, Amsterdam Institute for Infection and Immunity, Amsterdam UMC, University of Amsterdam, Amsterdam 1105 AZ, The Netherlands;* orcid.org/0000-0001-9589-186X; Email: s.a.zaat@amsterdamumc.nl

Authors

Kim W. J. Verhorstert − *Department of Obstetrics and Gynecology, Amsterdam Reproduction and Development, Amsterdam UMC, University of Amsterdam, Amsterdam 1105 AZ, The Netherlands;* orcid.org/0000-0002-9583-6657

Zeliha Guler − *Department of Obstetrics and Gynecology, Amsterdam Reproduction and Development, Amsterdam UMC, University of Amsterdam, Amsterdam 1105 AZ, The Netherlands*

Leonie de Boer − *Department of Medical Microbiology and Infection Prevention, Amsterdam Institute for Infection and Immunity, Amsterdam UMC, University of Amsterdam, Amsterdam 1105 AZ, The Netherlands*

Martijn Riool − *Department of Medical Microbiology and Infection Prevention, Amsterdam Institute for Infection and Immunity, Amsterdam UMC, University of Amsterdam, Amsterdam 1105 AZ, The Netherlands;* orcid.org/0000-0002-5444-3179

Jan-Paul W. R. Roovers − *Department of Obstetrics and Gynecology, Amsterdam Reproduction and Development, Amsterdam UMC, University of Amsterdam, Amsterdam 1105 AZ, The Netherlands*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsami.0c14668

Author Contributions

J.P.W.R.R., S.A.J.Z., and Z.G. conceived the idea and gave scientific input for the study design. J.P.W.R.R., K.W.J.V., S.A.J.Z., and Z.G. designed the experimental methodology. K.W.J.V. carried out the experiments with technical assistance of L.d.B. and M.R , supervised by S.A.J.Z. and Z.G. K.W.J.V. wrote the manuscript and the other authors gave their feedback and have given approval to the final version of the manuscript.

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Notes

The authors declare the following competing financial interest(s): J.P.W.R.R. declares that he received unrestricted research grants of Coloplast, Tepha, and Urogyn.

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ABBREVIATIONS

CFU, colony forming unit;
IQR, interquartile range
P4HB, poly-4-hydroxybutyrate
PBS, phosphate-buffered saline
POP, pelvic organ prolapse
PP, polypropylene
SEM, scanning electron microscopy
TSB, tryptic soy broth.

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