Journal of Hazardous Materials 422 (2022) 126836

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Enhanced survival of multi-species biofilms under stress is promoted by low-abundant but antimicrobial-resistant keystone species

Wisnu Adi Wicaksono [a,*], Sabine Erschen [a], Robert Krause [b,c], Henry Müller [a], Tomislav Cernava [a,c], Gabriele Berg [a,c,d,e]

[a] Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria
[b] Division of Infectious Diseases, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[c] BioTechMed Graz, Inter-university Cooperation Platform, Graz, Austria
[d] Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Potsdam, Germany
[e] Institute for Biochemistry and Biology, University of Postdam, Postdam, Germany

ARTICLE INFO

Editor: Dr. G. Jianhua

Keywords:
Biofilm
Opportunistic pathogen
Synergism
Multi-species
Metatranscriptomic

ABSTRACT

Multi-species biofilms are more resistant against stress compared to single-species biofilms. However, the mechanisms underlying this common observation remain elusive. Therefore, we studied biofilm formation of well-known opportunistic pathogens (*Acinetobacter baumanii*, *Enterococcus faecium*, *Escherichia coli*, *Staphylococcus haemolyticus* and *Stenotrophomonas maltophilia*) in various approaches. Synergistic effects in their multi-species biofilms were observed. Using metatranscriptomics, changes in the gene expression of the involved members became evident, and provided explanations for the improved survivability under nutrient limitation and exposure to disinfectants. Genes encoding proteins for vitamin B6 synthesis and iron uptake were linked to synergism in the multi-species biofilm under nutrient-limited conditions. Our study indicates that sub-lethal concentrations of an alcohol-based disinfectant enhance biofilm yields in multi-species assemblages. A reduction of the dominant taxa in the multi-species biofilm under disinfectant pressure allowed minor taxa to bloom. The findings underline the importance of minor but antimicrobial-resistant species that serve as "protectors" for the whole assemblage due to upregulation of genes involved in defence mechanisms and biofilm formation. This ultimately results in an increase in the total yield of the multi-species biofilm. We conclude that inter-species interactions may be crucial for the survival of opportunistic pathogens; especially under conditions that are typically found under hospital settings.

1. Introduction

In nature, bacteria commonly protect themselves by producing multicellular surface-associated communities embedded in an extracellular polymeric substances (EPS) known as biofilms (Flemming and Wingender, 2010). Compared to planktonic forms, biofilms are more resistant against abiotic and biotic stress factors, such as desiccation, grazing, and antimicrobial agents (Esbelin et al., 2018; O'Toole and Kolter, 1998; Raghupathi et al., 2018). Bacteria can also engage in inter-kingdom interactions with other organisms such as viruses, archaea, protozoa, and fungi in certain biofilms (Flemming et al., 2016). Multi-species biofilms are commonly found in natural habitats such as sediment surfaces in rivers, soils, and hypersaline lakes, where they fulfill important ecosystem functions (Andrews and Harris, 2000; Brislawn et al., 2019; Røder et al., 2016) as well as many artificial interfaces constructed by humans, such as ship hulls and medical devices in hospitals (Khatoon et al., 2018; Salta et al., 2013). From the perspective of humans, biofilm formation can pose a serious health problem; many opportunistic human pathogens can form biofilms, which allows them to thrive in the built environment, and to survive under clinical settings (Kelly et al., 2012; Khatoon et al., 2018).

The majority of bacterial infections in humans is associated with biofilms (Paredes et al., 2014). Hospital-acquired infections (HAIs) caused by opportunistic pathogens are suggested to further increase and to continue to pose a high healthcare burden in both developed and developing countries (Allegranzi et al., 2011; Khan et al., 2017). HAIs

* Corresponding author.
E-mail addresses: wisnu.wicaksono@tugraz.at (W.A. Wicaksono), sabine.erschen@alumni.tugraz.at (S. Erschen), robert.krause@medunigraz.at (R. Krause), henry.mueller@tugraz.at (H. Müller), tomislav.cernava@tugraz.at (T. Cernava), gabriele.berg@tugraz.at (G. Berg).

https://doi.org/10.1016/j.jhazmat.2021.126836
Received 24 March 2021; Received in revised form 11 July 2021; Accepted 4 August 2021
Available online 8 August 2021

cause costs of approximately $9.8 billion each year (Monegro et al., 2020). More than 2.5 million HAI episodes occur each year in Europe with approximately 90,000 deaths (Cassini et al., 2016). Due to their high resistance against a broad range of antimicrobial agents and common cleaning regimes, biofilms contribute to the persistence of opportunistic pathogens in hospital settings (Guyot et al., 2013; Hu et al., 2015; Khatoon et al., 2018; O'Neill et al., 2007) and are therefore suggested as a risk factor for HAIs. Biofilms are also potential hotspots for emerging multidrug-resistant bacteria due to increased antibiotic resistance gene transfer among members inside such structures (Parsek and Singh, 2003; Schwartz et al., 2003). Various opportunistic pathogens such as *Acinetobacter baumanii*, *Stenotrophomonas maltophilia*, *Escherichia coli*, *Enterococcus*, and coagulase-negative staphylococci were previously shown to form multi-species biofilms (Hu et al., 2015; Schwering et al., 2013). As bacteria generally coexist in multi-species communities, the response of single-cell cultures of a pathogen is unlikely to be sufficient to provide an indication of the degree of pathogen tolerance to antibiotics (Bottery et al., 2020). Hence, such studies should also focus on the multi-species communities in which the pathogens reside. However, detailed mechanisms underlying this common observation are still scarce. It can be assumed that a better understanding of microbial interactions and co-existence in multi-species biofilms could provide a basis for better treatment options to eradicate opportunistic pathogens.

Here we report the results of a mechanistic biofilm interaction analysis with defined single and multi-species constructs. We exposed them to two stress factors, i.e. antimicrobial exposure and nutrient-poor environments; both of them are known to commonly occur under hospital settings (Mora et al., 2016). We combined quantitative assessments of biofilm formation with whole-genome sequencing analyses and RNA sequencing in order to obtain detailed insights into the opportunistic pathogens' responses to the selected stress factors. By combining these approaches, we addressed the following questions: (i) how is biofilm formation induced in multi-species biofilms formed by opportunistic pathogens?, (ii) what are the ecological roles of each bacterial strain in multi-species biofilms?, (iii) which molecular mechanisms underpin multi-species interactions in biofilms? Our findings provide further evidence for synergistic effects in multi-species biofilms under stress pressure. Interestingly, certain species with relatively low abundance but antimicrobial resistance were shown to be important during biofilm formation; they facilitate growth of multi-species biofilm communities under antimicrobial exposure by upregulating genes involved in defence mechanism and biofilm formation which lead to the overall synergism in the multi-species biofilm.

2. Material and methods

2.1. Origin of the bacterial isolates

We used five different opportunistic pathogens namely *Acinetobacter baumanii* strain 6340276, *Enterococcus faecium* strain 6428631, *Escherichia coli* strain 6402087, *Staphylococcus haemolyticus* strain 48/6 and *Stenotrophomonas maltophilia* strain EA23. The first three isolates were obtained from the culture collection of the Department of Internal Medicine, Medical University of Graz. The *S. haemolyticus* strain 48/6 was previously isolated from a hospital setting (Oberauner et al., 2013) while *S. maltophilia* strain EA23 was previously isolated from eyecare solution and shown to be closely related to a clinical strain, *S. maltophilia* K279a (Berg et al., 1999; Lira et al., 2017). These two isolates are part of the microbial culture collection of the Institute of Environmental Biotechnology, Graz University of Technology.

2.2. DNA extraction, bacterial identification using 16S rRNA gene fragments, and whole genome sequencing

All bacterial isolates were obtained from − 80 °C stocks by inoculation of tryptic soy agar (TSA; Sigma-Aldrich, USA) and overnight incubation at 25 °C. Bacterial genomic DNA was extracted from cultures using the MasterPure™ Complete DNA and RNA Purification Kit (Lucigen, Epicentre; USA) with a modification of the manufacturer's instructions by adding an additional 15 µL lysozyme (15 µg/µL, Sigma-Aldrich; USA) during cell lysis steps.

The 16S rRNA gene fragments were amplified using the primers 8F and R1492. Polymerase chain reactions (PCR) were performed with as Whatman Biometra® Tpersonal thermocycler (Biometra 141 GmbH; Göttingen, Germany) in a total volume of 25 µL. The reaction mixture contained 1 µL DNA template, 5 µL Taq&Go (MP Biomedicals, Heidelberg, German), 1 µL of each primer that was adjusted to 10 µM, and 18 µL ultrapure water. The PCR-amplified 16S rRNA gene fragments were sequenced with Sanger sequencing at LGC Genomics GmbH (Berlin, Germany). The sequences were manually trimmed using BioEdit (Hall, 1999) to remove ambiguous sequences and compared against those of known origin using the Basic Local Alignment Search Tool (BLAST) (Boratyn et al., 2013) and the GenBank database (http://www.ncbi.nlm.nih.gov). Genomic DNA, except for *S. maltophilia* EA23 (its genome was already available at Genbank with accession no. NEQT00000000, (Lira et al., 2017)), was obtained using the Nextera XT DNA Library Preparation Kit (Illumina Inc.; Cambridge, UK) according to the manufacturer's instructions and sequenced on the NextSeq platform using the paired-end 150-bp protocol at LGC Genomics GmbH (Berlin, Germany).

2.3. Biofilm formation assay under exposure to sub-lethal disinfectant concentrations

A commercial disinfectant (Bacillol, BODE Chemie GmbH, Germany) with the active ingredients ethanol, propan-2-ol, propan-1-ol, and N-alkyl-aminopropylglycine in an approximate ratio of 10:6:1 was selected as a common alcohol-based surface disinfectant for the subsequent experiments. It is commonly used under clinical settings for surface disinfection (Brühwasser et al., 2017; Kenters et al., 2017; Reichel et al., 2014). Biofilm formation assays were performed in transparent flat-bottom polystyrene 96-well microtiter plates (Sarstedt, Germany). The method was adapted on the basis of a previous protocol by Stiefel et al. (2016). Cultures of the opportunistic pathogens that were grown overnight in TSB at 25 °C and 100 rpm, were adjusted to an optical density (OD_{600}) of 0.05 with (concentration range between 0.312% and 20%) or without Bacillol for the control treatments. Each well was inoculated with 200 µL bacterial suspension and incubated at 25 °C for 24 h under static conditions. Negative controls were included defined as columns with sterile TSB. Following the 24 h incubation, planktonic cells were removed, and the biofilms were washed twice with 0.9% NaCl to remove nonadherent bacteria. Negative controls were treated with the same procedure. Subsequently, a total of 200 µL 0.5% crystal violet (CV) solution was added to each well. After a 30 min incubation at room temperature, the CV solution was removed, and the wells were rinsed twice with 0.9% NaCl to remove excess CV. Finally, the stained biofilm was solubilized in 200 µL of absolute ethanol and quantifications were performed by measuring the OD_{595} of each well using the Infinite® 200 PRO microplate reader (Tecan; Mannedorf, Switzerland). To calculate the net amount of biofilm formed, the absorbance value of the control (wells without any bacterial inoculation) was subtracted.

2.4. Biofilm formation assays with different growth media and isolate combinations

Biofilm formation assays were performed in 96-well flat bottom microtiter plates as described above. A rich medium (tryptic soy broth, TSB; Sigma-Aldrich; USA), a minimal medium (M9; Sigma-Aldrich; USA) supplemented with 0.8% glucose (Sigma-Aldrich; USA), and TSB supplemented with a sub-lethal concentration of Bacillol (2.5%), herein referred as TSB-BAC, were used for the cultivation of the analyzed isolates. All possible combinations that consisted of either single or combined isolates (2, 3, 4, and 5 species) were implemented for the detailed

biofilm formation analyses (Supplementary Table 1). Observed biofilm interactions were defined according to Ren et al. (2015). Synergism in multi-species biofilms occurs when the total biomass of the biofilm is greater than then total biomass of the best single-strain biofilm producer that is present in the relevant combination. If this was not the case, then it was classified as non-synergy.

2.5. Metatranscriptomic analysis of single and multi-species biofilms

To assess intra-biofilm interactions on a functional level, a setup consisting of *E. faecium*, *S. haemolyticus*, and *S. maltophilia* was selected due to strong synergistic interaction in multi-species biofilms. Comparative gene expression analyses were performed with single and the abovementioned multi-species biofilm. Briefly, biofilm formation was induced as described above, except the biofilms were grown in six-well plates with a total of 4 mL bacterial suspension per well with equal inoculum densities. After removing planktonic cells, the biofilms were mixed with 500 µL TRIzol™ reagent (ThermoFisher Scientific; USA) and transferred to 2 mL reaction tubes filled with glass beads (0.25 mL–0.1 mm size). The samples were homogenized at room temperature using a FastPrep®-24 Instrument (MP Biomedicals; Germany) for 2 × 30 s at 6.0 m/s with 1 min in-between cooling on ice and subsequently incubated for 5 min at room temperature. The RNA extraction was performed according to the manufacturer's protocol. Genomic DNA was removed implementing DNAase (Lucigen, Epicentre; USA) according to the manufacturer's protocol. The RNA quality and quantity were assessed with a NanoDrop™ 2000/2000c Spectrophotometer (ThermoFischer Scientific; USA) and Qubit RNA HS Assay Kit (ThermoFischer Scientific; USA), respectively. In addition, the RNA integrity was analyzed using the Qubit RNA IQ Assay (ThermoFischer Scientific; USA) to assure sufficient quality before further processing. Library preparation (including rRNA depletion) and RNA sequencing on the Illumina NextSeq platform using the single-end 75-bp protocol was performed by the sequencing provider LGC Genomics GmbH (Berlin, Germany).

2.6. Bioinformatic analysis

The read quality of genomic and transcriptomic reads was checked using FASTQC (S. Andrews, 2010). Illumina adaptor removal and quality filtering was performed using Trimmomatic and VSEARCH (Bolger et al., 2014; Rognes et al., 2016). For whole genome sequencing analysis, high quality paired-end reads were assembled with SPAdes with the "careful option" (Bankevich et al., 2012). Contigs with a size less than 1000 bp were removed using BBTools (Bushnell, 2014). The completeness and the percentage of contaminants in the draft genomes was estimated by using CheckM (Parks et al., 2015). Open reading frames of each draft genome were predicted using GeneMark (Borodovsky et al., 2003) and annotated using the blastp algorithm in DIAMOND (Buchfink et al., 2015) against the eggNOG database 5.0 (Huerta-Cepas et al., 2019) for ecological function profiling and with the deepARG database (Arango-Argoty et al., 2018) for antibiotic resistance profiling. To minimize the risk of false-positives, reads were defined as ARG-like reads at the cutoff of *E* value of 10^{-10} and a similarity of 90%. To compare relative proportions of bacteria in the multi-species biofilm, we used Kraken2 and Bracken (Lu et al., 2017; Wood et al., 2019) to assign metatranscriptomic reads to *E. faecium*, *S. haemolyticus,* and *S. maltophilia* in the multi-species biofilm dataset. Quantification of expressed genes was performed using the Bowtie2 aligner and FeatureCounts (Langmead and Salzberg, 2012; Liao et al., 2014) as described above. The whole genome and metatranscriptomic sequencing datasets were deposited in the European Nucleotide Archive public repository (BioProject no: PRJEB43664).

2.7. Statistical analysis

All statistical analyses and graph visualizations were performed in R v3.3.1 (R Core Team, 2017) via the Rstudio IDE (available on www.rstudio.com). Analysis of variances (ANOVA) was used to determine the significance of disinfectant use on the biofilm yield. Nested ANOVA was implemented to determine the significance of the number of species in a biofilm and the species composition (nested within number of species) on the biofilm yield. The data were log (n + 1) or rank transformed whenever required to fulfil the ANOVA assumption. Linear regression analysis was used to describe correlations between the observed biofilm yield and species richness in single and multi-species biofilms. Linear regression analysis is generally more powerful than ANOVA to detect statistical significance when the response variable varies monotonically with the treatment factor (Cottingham et al., 2005). For comparative transcriptomic analysis, the read count tables from FeatureCounts (Liao et al., 2014) were used as input for DESeq2 to conduct data normalization and differential expression analysis (Love et al., 2014). To analyze the significant effects of treatment on expressed bacterial genes, normalized transcriptomic abundance profiles which were corrected using a regularized logarithm transformation were constructed using the DESeq2 R package (Love et al., 2014). For the Bray-Curtis distance matrix, the negative values in the normalized transcriptomic abundance profiles were replaced with a zero under the assumption that negative values represent very low or zero abundance and therefore are insignificant for the analysis. The permutational analysis of variance (PERMANOVA, 999 permutations) was then used to test for significant effects of the culture media (TSB vs. M9 vs. TSB-BAC) and the biofilm type (single vs. multi-species). DESeq2 was implemented to obtain a list of differentially expressed genes in pairwise comparisons between each isolate that grew in single and mixed cultures in different media. Volcano plots were used to visualize the genes that were differentially expressed under two specified conditions (Blighe et al., 2019). Gene ontology (GO) terms of the target genes (genes that were upregulated/downregulated in each pairwise comparison) were extracted from the eggNOG-mapper results and the analysis of the enrichment of GO terms was further performed using the topGO package that is implemented in R (Fisher test; p-value < 0.05; (Alexa and Rahnenfuhrer, 2010)).

3. Results

3.1. Genomic features of the opportunistic pathogens

To provide insights into the genetic background of the opportunistic pathogens used in this study, whole-genome sequencing was conducted for four bacteria (*A. baumannii* strain 6340276, *E. faecium* strain 6428631, *E. coli* strain 6402087 and *S. haemolyticus* strain 48/6) and retrieved for *S. maltophilia* strain EA23 from a public database. The estimated completeness of the genomes was more than 99% with contamination levels lower than 0.6% which can be classified as a high-quality draft genome (Supplementary Table 2). The draft genome sizes varied from 2.47 to 4.75 Mb, with the GC content in a range of 32.5–66.4%. A total of 3685, 2720, 3073, 2301, and 4010 genes from the *A. baumannii*, *E. faecium*, *E. coli* and *S. haemolyticus,* and *S. maltophilia* genomes were annotated using eggNOG database, respectively. By annotating the draft genomes with the eggNOG and deepARG databases, biofilm-associated genes such as *pgaA* (*A. baumanii*), *veg* and *divIVA* (*E. faecium*), *tomB*, *matB*, *tabA,* and fimA (*E. coli*), *clp*, *sarA*, and *agrA* (*S. haemolyticus*), *yqcC, rpfF, and rmlA* (*S. maltophilia*) were recovered from the draft genomes (Supplementary Table 3). The molecular chaperones *groL* and *grpE* (heat shock proteins) were predominantly found within the draft genomes. In addition, *hslO* a molecular chaperone that is involved in the bacterial defense system toward oxidative stress was also prevalent. The genes *sodA* and *sodC* that encode superoxide dismutase which protects against ROS-mediated host defense were also prevalent. A total of 17 antibiotic resistance gene classes were detected within the draft genomes. *E. coli* harbored 14 antibiotic resistance genes in its genome. Aminoglycoside, bacitracin, betalactam, macrolide-

lincosamide-streptogramin and multidrug resistance genes were predominantly found in the five genomes. Genes to confer resistance to glycopeptide were exclusively detected in the *E. faecium* genome, whereas genes to confer resistance against fosmidomycin, kasugamycin, as well as peptide, polymyxin, and sulfonamide antibiotics were exclusively detected in the *E. coli* genome.

3.2. Biofilm formation assays with single species

To study the effect of sub-lethal disinfectant concentration on biofilm formation of the opportunistic human pathogens, an initial screening was performed to find the optimal concentration range. All of the tested isolates were able to form biofilms; however, different sensitivities against Bacillol were observed. The highest biofilm yield was observed with *S. maltophilia* (OD_{595} of 2.86 ± 0.04) in TSB whereas the lowest yield was observed with *E. coli* (OD_{595} of 0.37 ± 0.01) (Fig. 1). *Acinetobacter baumannii, E. faecium* and *S. haemolyticus* produced biofilms with an OD_{595} of 1.54 ± 0.03, 0.67 ± 0.03, and 1.25 ± 0.02, respectively. When the isolates were grown in TSB with 5–20% Bacillol (v/v), biofilm formation was not detected (OD_{595} of < 0.05) for *A. baumannii*, *E. coli,* and *S. maltophilia* (Fig. 1). However, *E. faecium* and *S. haemolyticus* were able to form a biofilm when grown in TSB with 10% Bacillol (v/v) indicating that these isolates were more resistant to Bacillol. Interestingly, *E. faecium* showed a tendency to produce more biofilm when grown in TSB with 0.31–2.5% Bacillol (v/v) in comparison to TSB without disinfectant. The observations led to the selection of 2.5% Bacillol as the sub-lethal disinfectant concentration for mixed culture experiments, because this concentration inhibited biofilm formation (43–77% inhibition) for most of isolates except for *E. faecium* (Fig. 1).

3.3. Single and multi-species biofilm formation assays with different growth media

A relatively higher prevalence of synergy in multi-species biofilms was observed when the bacteria were grown in M9 (minimal medium) and TSB-BAC (medium with disinfectant) compared to TSB. From all possible combinations (n = 31; Supplementary Table 1), TSB-BAC led to the highest synergistic effects (53.8%), followed by M9 (42.3%), and TSB (26.9%) (Fig. 2a). The prevalence of synergistic effects in M9 and TSB-BAC showed a tendency to increase with the number of species. Within the mixed culture experiments, the combination of *E. faecium*, *S. haemolyticus,* and *S. maltophilia* yielded more biofilm in the mixed culture compared to the sum up the single cultures indicated a strong synergistic interaction (Supplementary Fig. 1).

Species richness and composition (nested under species richness) had a statistically significant effect on the biofilm yield (Table 1). In detail, the species richness significantly affected biofilm yield when the isolates were co-cultured in M9 and TSB-BAC media, but there was no significant difference when they were grown in TSB. Pairwise comparisons indicated that treatments containing 3, 4, and 5 species had a significantly higher biofilm yield compared to treatments containing 1 and 2 species ($P < 0.05$) when the isolates were cultured in M9 medium. Interestingly, in TSB-BAC, the biofilm yields significantly increased along with the increase of species richness (1 species < 2 species < 3 and 4 species < 5 species; $P < 0.05$). This strengthens the observation that enhanced biofilm formation was caused by synergistic interactions in multi-species biofilms under nutrient-poor conditions and presence of disinfectant in the media.

Based on linear regression analysis, we found that species richness had a positive and significant linear effect on the biofilm yield (Fig. 2b–d). Biofilm yields showed a tendency to increase with an increasing number of bacterial species when the isolates were grown in TSB, although the correlation was not statistically different (Fig. 2; $P = 0.056$; r = 0.112). However, a positive correlation between biofilm

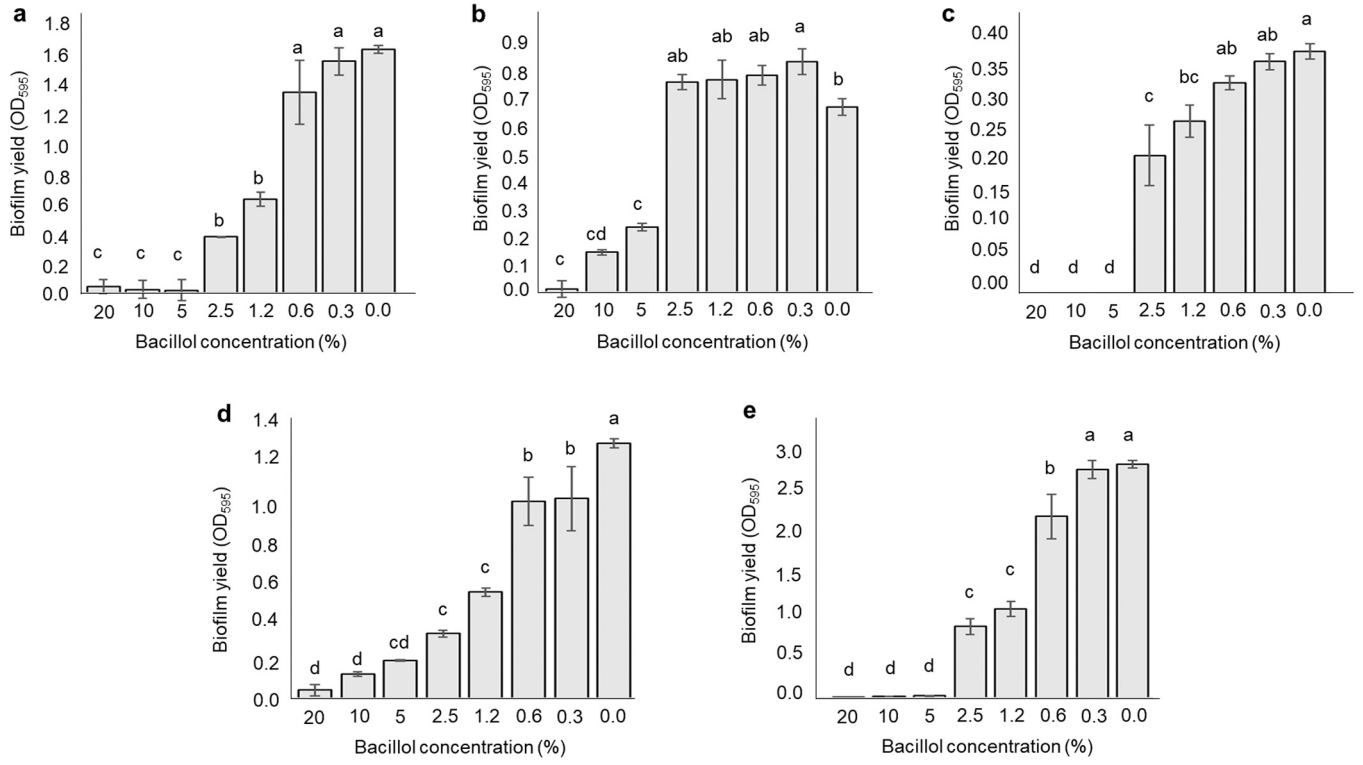

Fig. 1. Effect of the commercial disinfectant Bacillol on biofilm formation. Biofilm yields of (a) *Acinetobacter baumannii*, (b) *Enterococcus faecium*, (c) *Escherichia coli*, (d) *Staphylococcus haemolyticus*, (e) *Stenotrophomonas maltophilia* that were grown in TSB with a gradient of Bacillol (0–20%) at 25 °C for 24 h. Data are presented as mean ± SD for each treatment. The different letters above each bar indicate statistical significance at $P < 0.05$ based on the Tukey post hoc test.

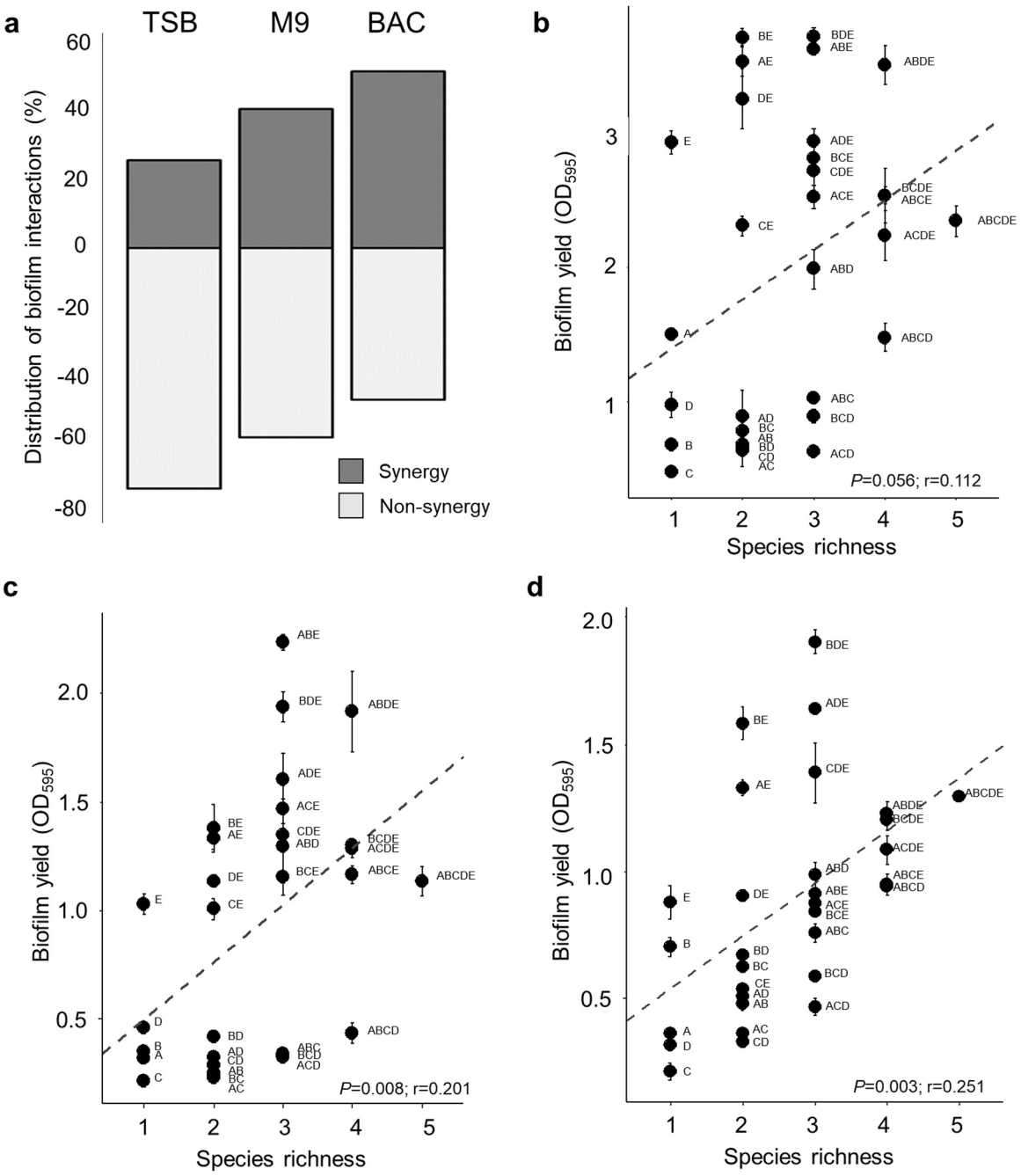

Fig. 2. Evaluation of synergism and biofilm yields with different multi-species assemblies that were grown in three different media. Synergism within multi-species biofilms was defined according to Ren et al. (2015). Single and multi-bacterial assemblies were grown in TSB (b), M9 (c), and TSB supplemented with 2.5% of the disinfectant (d). The observed data points (black dots) in panel b-d were collected by quantifying total biomass of single and multi-species biofilms of all possible cocultures (up to 5 bacterial species) using the CV assay as described in Supplementary Table 1.

yield and the number of species was observed when the isolates were grown in M9 and TSB-BAC media (Fig. 3; $P < 0.05$). Different types of growth media affected biofilm yield together with the number of species; this was supported by the higher r-values that were observed when the isolates were grown in TSB-BAC (r = 0.253), followed by M9 (r = 0.201), and TSB (r = 0.112).

3.4. Metatranscriptomic analysis of single and multi-species biofilms

In order to explore the underlying molecular mechanisms of synergy in multi-species biofilms cultivated in different media, we selected a consortium consisting of *E. faecium* strain 6428631, *S. haemolyticus* strain 48/6, and *S. maltophilia* strain EA23 due to their strong synergistic interaction in multi-species biofilms (Supplementary Fig. 1). A total of 404 million raw reads were obtained and the number of reads per sample ranged from 27,905 to 10,731,819 after quality filtering, alignment with the reference genomes and annotation using eggNOG database (Supplementary Table 4). The growth medium type substantially affected the bacterial composition in the multi-species biofilm (Fig. 3a). Multi-species biofilms obtained in TSB and M9 media were dominated by *S. maltophilia* with a relative abundance of 51.1% and 66.8%, respectively. Interestingly the proportion differed in the medium with disinfectant, TSB-BAC. Under these conditions, *E. faecium* dominated the multi-species biofilm (62.1%) which was followed by *S. maltophilia*

Table 1
Summary of the nested analysis of variance (ANOVA) with the parameter species richness and species composition on biofilm yield.

Media type	Effect	df	MS	F	*P*
TSB	Species number	4	2.83	0.99	0.429
	Species composition (species number)	26	2.86	29.00	< 0.001
M9	Species number	4	6.64	2.86	0.043
	Species composition (species number)	26	2.32	30.21	< 0.001
TSB-BAC	Species number	4	7.53	3.27	0.027
	Species composition (species number)	26	2.30	78.74	< 0.001

(33.9%). The relative abundance of *Staphylococcus haemolyticus* increased to 4% in the multi-species biofilm obtained with TSB-BAC. Here, an increased abundance of *E. faecium* indicated that this taxon plays an important role in the multi-species biofilm when disinfectant is added and hence it was defined as a keystone species.

Functional profiles were affected by the biofilm type (single vs. multi-species) as well as the utilized medium. Clustering analyses based on bacterial functions (eggNOG) showed that samples from single species biofilms and multi-species biofilms grouped separately (Fig. 3b–d). The effect was more pronounced in *E. faecium* and *S. haemoltyticus* samples (Fig. 3b and c) when compared with *S. maltophilia* (Fig. 3d). A distinct cluster was also formed in single and multi-species biofilms that were grown in M9 minimal medium compared to other cultivation media (Fig. 3b–d). PERMANOVA analysis indicated that the biofilm type (single vs. multi-species) was the major factor influencing gene expressions of *E. faecium* and *S. haemolyticus* ($R^2 = 0.303$ and 0.359, respectively; Table 2) whereas the gene expression in *S. maltophilia* was mainly affected by the medium type ($R^2 = 0.522$). Overall, the biofilm type and the cultivation medium affected the global transcriptome of all isolates ($P < 0.05$; Table 2); it explained between 60.0% and 64.1% of the variations in gene expression (Table 2).

3.5. Bacterial functions in single and multi-species biofilms

To analyze the impact of the biofilm type (single vs. multi-species)

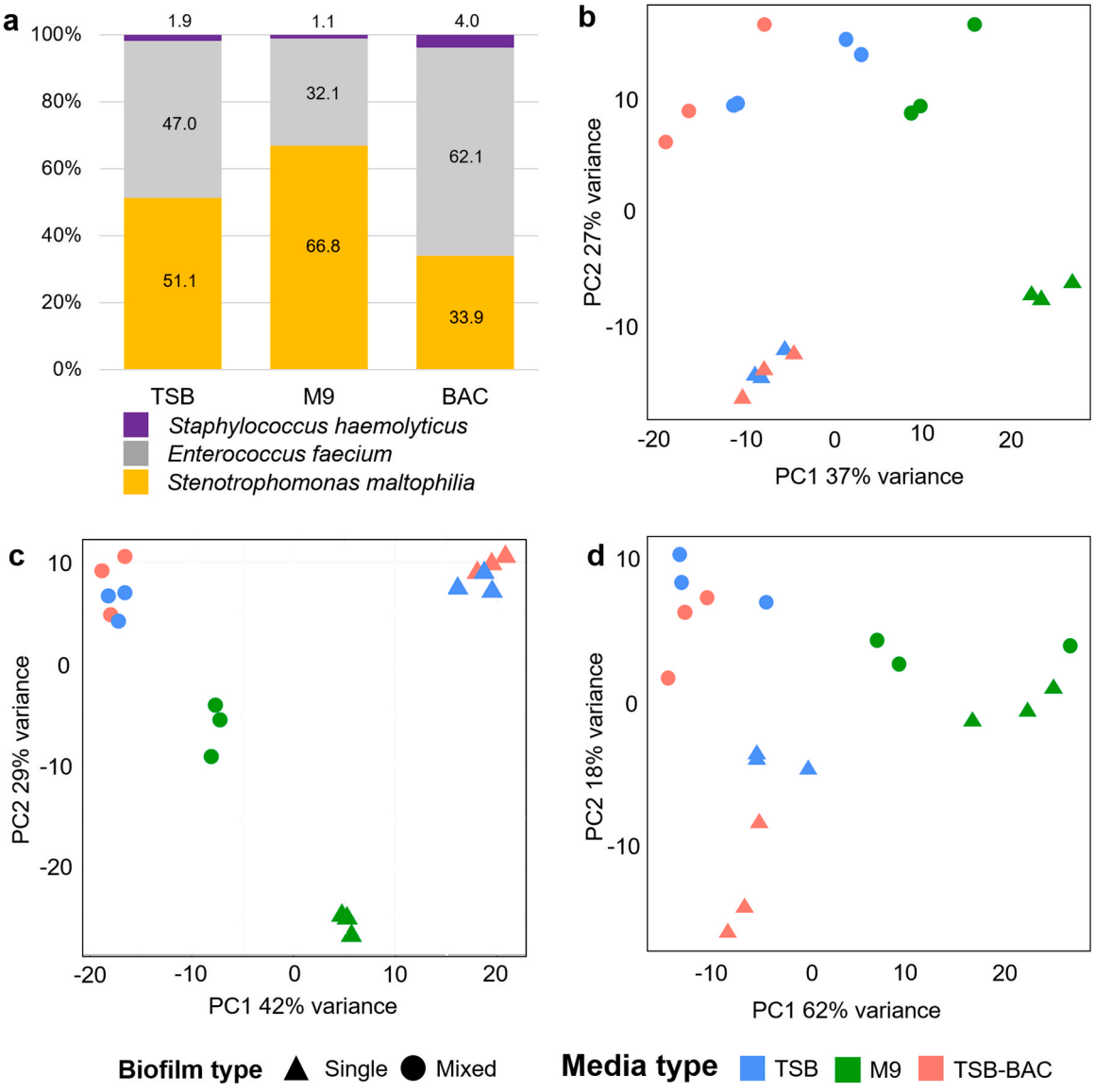

Fig. 3. Relative abundances and species composition in different multi-species biofilms. The bar plot (a) shows the relative abundance of *Enterococcus faecium*, *Staphylococcus haemolyticus,* and *Stenotrophomonas maltophilia* in different biofilms. The principal component analysis (PCA) plots (b-d) show the distribution of replicate samples. b: *Enterococcus faecium*, c: *Staphylococcus haemolyticus,* and d: *Stenotrophomonas maltophilia*.

Table 2
Effects of the biofilm type (single vs. multi-species) and the cultivation medium on bacterial gene expression.

Factor	*E. faecium* strain 6428631		*S. haemolyticus* Strain 48/6		*S. maltophilia* strain EA23	
	R^2 value	*P* value	R^2 value	*P* value	R^2 value	*P* value
Biofilm type	0.303	0.001	0.359	0.001	0.119	0.009
Medium	0.297	0.001	0.258	0.001	0.522	0.001
Biofilm type × Medium	0.098	0.071	0.201	0.001	0.066	0.200

and the cultivation medium (TSB vs. M9 vs. TSB-BAC) on the detailed bacterial transcription activity, a differential gene expression analysis was performed using DESeq2. We focused on pairwise comparisons between single species biofilms from TSB medium and such that were obtained with different media (n = 2). In addition, pairwise comparisons between multi-species biofilms from TSB medium and the other cultivation media (n = 2) were compared. This was complemented by pairwise comparison between single and multi-species biofilms that were obtained with the same medium (n = 3). Differential gene expression was visualized using volcano plots (Supplementary Figs. 2–4). The range of differentially expressed genes ($P_{adjusted} < 0.05$, log_2fold change > 1, Supplementary Figs. 2–4) varied for each species; *E. faecium* (4.2–21.6%), *S. haemolyticus* (3.7–25.4%), and *S. maltophilia* (0.5–16.4%). The preliminary results indicated that the *S. maltophilia* transcriptome was relatively less affected by the factors tested in this study in comparison to the other bacteria.

To further characterize the differentially expressed genes in the multi-species biofilm obtained with different media, a GO enrichment analysis was conducted. Peptide biosynthetic and metabolic processes were downregulated whereas the cellular response to iron was upregulated when *E. faecium* was grown in the mixed culture using M9 medium in comparison to TSB. A similar response was observed in the transcriptome of *S. maltophilia,* where biosynthetic and metabolic processes related to enterobactin were upregulated under this condition. Moreover, in the transcriptome of *S. haemolyticus*, vitamin B6 biosynthesis and related metabolic processes were upregulated under this condition. Interestingly, *S. haemolyticus* showed an increased expression of genes associated with iron ion transport in the mixed culture using M9 medium in comparison to the single culture under the same condition. Polysaccharide transport was upregulated when *E. faecium* was grown in the mixed culture using TSB-BAC medium in comparison to TSB. Moreover, trehalose biosynthesis was also upregulated. In *S. haemolyticus*, we observed upregulation of arginine biosynthesis and metabolic processes under the same conditions. Genes associated to cellular respiration and catabolic process were also overrepresented.

In the deepening analyses, we focused on differentially expressed genes that might play an important role in each bacterium's response towards limited nutrient and disinfectant pressure. *Stenotrophomonas maltophilia* was the predominant species in the multi-species biofilm obtained in nutrient-limited medium (M9). Genes encoding the putative siderophore enterobactin to scavenge iron *entE* and *entF* were upregulated when *S. maltophilia* was grown in single or mixed cultures in M9 medium in comparison to TSB. A gene that encodes a hemin import protein showed a similar pattern. These genes likely facilitate the growth of this bacterium in the mixed culture under limited nutrient availability. Two genes associated with biofilm formation in *S. haemolyticus*, *clpB* and *clpP,* were respectively upregulated and downregulated, when *S. haemolyticus* was grown in TSB-BAC. We identified a gene that encodes the antiholin-like protein lrgA; it was consistently enriched in single cultures of S. *haemolyticus* in TSB-BAC when compared to TSB. Moreover, this gene was also upregulated in the mixed culture when grown in TSB-BAC, which highlighted the importance of this gene under disinfectant pressure. Expression of *hrtA* that encodes an ABC transporter with efflux transmembrane activity was also induced by the presence of the disinfectant.

Specific genes associated with biofilm formation and defense mechanisms in *E. faecium* were induced by exposure to the disinfectant. Among the genes that are involved in cell wall formation, *murA* and *murE* were downregulated when *E. faecium* was grown in the mixed culture in comparison to the single culture regardless of the medium. In addition, *murF* and *murG* were shown to be simultaneously upregulated in the mixed culture when TSB-BAC was used in comparison to TSB. A gene (*mprF*) that is responsible for the production of lysylphosphatidylglycerol (LPG) was upregulated when *E. faecium* was grown in the single culture with TSB-BAC in comparison to TSB. Interestingly, the gene expression was also increased when compared to the mixed culture using TSB-BAC. The same gene was also upregulated in the mixed culture and the same growth media (TSB-BAC vs. TSB). It was induced by the presence of disinfectant irrespective of the biofilm type and thus likely responsible for the resilience of the keystone species, *E. faecium* under disinfectant pressure. In addition, the glycopeptide resistance gene *vanA* was found to be enriched when *E. faecium* was grown in the mixed culture with TSB-BAC in comparison to TSB (Supplementary Table 5). The same gene was enriched in the mixed culture with TSB-BAC when compared with the single culture that was grown in the same medium. This observation indicated that *vanA* was specifically induced by the disinfectant and presence of other bacterial members in the multi-species biofilm. In contrast, disinfectant exposure did not induce higher expression of any antibiotic resistance genes of the dominant member, *S. maltophilia* (Supplementary Table 5) under the same conditions. Overall, we showed that, the keystone species, *E. faecium* expressed genes that are needed to enhance survival of multi-species biofilms under antimicrobial stress.

4. Discussion

By combining various mechanistic approaches we showed that specific stress factors, namely limited nutrient availability and disinfectant exposure, induced synergistic interactions in multi-species biofilms. Moreover, we observed that in such biofilms species that are more resistant played a key role for the survival of the whole assemblages. The genomes of the implemented clinical strains *Acinetobacter baumanii* strain 6340276, *Enterococcus faecium* strain 6428631, *Escherichia coli* strain 6402087, *Staphylococcus haemolyticus* strain 48/6 and *Stenotrophomonas maltophilia* strain EA23 were shown to harbor various genes that may play a crucial role in their survival in hostile environments. For example, *grpE* (a heat-shock stress responsive chaperone) and *sodA* (superoxide dismutase) play essential roles in the survival of bacteria that cope with osmotic and oxidative stress, respectively and that they are also important for their pathogenesis (Heindorf et al., 2014; Kim et al., 2018; Salze et al., 2020; Soto-Giron et al., 2016). Several known genes that encode proteins that are responsible for biofilm formation such as *pgaA*, *divIVA*, *fimA*, *clp, sarA, agrA, rpfF* and *rmlA* (Choi et al., 2009; Fattahi et al., 2015; Frees et al., 2004; Huang et al., 2006; McCarthy et al., 2015; Sharma and Khan, 2018; Zhuo et al., 2014) were detected in the current study, indicating that the selected isolates harbored a strong potential for biofilm formation.

Multi-species biofilms reproducibly showed synergistic effects in biofilm formation under stress conditions (nutrient-poor medium and disinfectants), when specific assays were conducted. Our study showed that sub-lethal concentrations of a common disinfectant enhanced biofilm yields of pathogenic bacteria when they occur in multi-species assemblages. Low concentrations of disinfectants are known to induce biofilm production in *E. faecium* and various staphylococci (Luther et al., 2018; Pidot et al., 2018). Moreover, a foregoing study reported that increased biofilm formation by *S. aureus* grown in 2.5% ethanol (Slany et al., 2017), which is comparable with the alcohol concentration in the disinfectant that was used in this study. In multi-species biofilms, a higher prevalence of synergism was observed when the bacteria were

grown under stress, i.e. nutrient-poor medium and medium with disinfectant. Similar cooperative interactions in multi-species biofilms under selective antimicrobial have been reported previously (Parijs and Steenackers, 2018). Furthermore, we found that the overall biofilm formation increased together with the number of bacterial species in the multi-species biofilm. This cooperation could be due to changes in the global gene expression of the bacteria as a response to the environmental changes and also to the presence of other members in the multi-species biofilm. Our results are supported by previous findings (Burmølle et al., 2006; Raghupathi et al., 2018), which demonstrated that inter-species compatibility is crucial in order to ensure better survival under hostile conditions. Hence it can be argued that compatible multi-species biofilms are crucial for the survival of certain opportunistic pathogens; especially under conditions that are typically found under hospital settings. In this study, we allowed biofilms to form for only 24 h. In the built environment, bacteria are commonly exposed to long-term nutrient-poor conditions and continuous disinfection regimes. Therefore, further studies implementing combined exposure over extended periods of time will be needed to further refine our understanding of biofilm development and biological interactions therein.

S. maltophilia is known to be an efficient biofilm producer (Maes et al., 2019; Pompilio et al., 2011). It was found that biofilm formation is an important feature of *S. maltophilia,* when it has to compete with other microorganisms as well as in its natural environment, which is the plant root (Ryan et al., 2009). This is also reflected in our study, where *S. maltophilia* dominated the multispecies biofilms (> 50%) under nutrient-poor as well as under nutrient-rich conditions. We hypothesize the implemented isolates competed under ideal growth conditions (TSB medium). Competition for resources and niches, for instance, by producing biofilm inhibitory compounds leading to higher competitiveness and lower prevalence of synergism in biofilm formation is well known (Guillonneau et al., 2018; Rao et al., 2005). However, this changes under stress conditions as it was also evidenced in the present study where inter-species communication was observed in multi-species biofilm. Iron depleted conditions can inhibit bacterial growth as well as biofilm development (Banin et al., 2017; Lin et al., 2012; Oglesby-Sherrouse et al., 2014). Hence, upregulated genes that were assigned to the siderophore enterobactin and iron uptake likely facilitated thriving of *S. maltophilia* under limited nutrient availability. Interestingly, *E. faecium* upregulated cellular response to iron ion suggesting a co-habitant response in the multi-species biofilm. Simultaneously, vitamin B6 biosynthesis in *S. haemolyticus* was upregulated, which indicated increase utilization of it. Vitamin B6 is an essential cofactor for enzymes that are involved in amino acid metabolism (Rosenberg et al., 2018). Overall, we hypothesize that each bacterium in the multi-species biofilm played a specific role by producing compounds that were limited under the experimental conditions, which ultimately lead to an overall higher biofilm formation than for the individual bacteria. Further studies with knockout mutants targeting iron uptake and vitamin B6 pathways could provide deeper understanding of the molecular mechanisms behind the observed synergistic biofilm production.

Our findings also indicate that low-abundant but antimicrobial-resistant species, defined as keystone species, can play an important role in multi-species biofilms when they are exposed to disinfectants. Despite the predominance of *S. maltophilia* in the multi-species biofilm, the relative abundance of the keystone species, *E. faecium* and *S. haemolyticus* increased in multi-species biofilms under disinfectant pressure when compared to the reference growth condition. This observation indicates that bacterial community shifts are induced by disinfectants, which lead to an increase of the keystone species and simultaneously can have positive effects on the whole assemblage. The bacterial community shifts allowed low abundant taxa to bloom due to a higher resistance towards the disinfectant. They served as “protectors” for the dominant taxa. By implementing a metatranscriptomic approach, expression of *vanA* was shown to be upregulated. It is known for its potential to confer cross-resistance to both disinfectants and antibiotics (Mc Carlie et al., 2020). Numerous studies demonstrated that the application of disinfectants can induce cross-resistance to antibiotics including multidrug efflux pumps (Camarena et al., 2010; Chen et al., 2021; Merchel Piovesan Pereira et al., 2021). Our study provides further insights into the risks associated with the use of chemical disinfectants in terms of activation of cross-resistance to antibiotics in emerging opportunistic pathogens as a part of their defense strategy. Moreover, considering that biofilms are more difficult to be treated than planktonic bacteria, upregulation of certain antibiotic resistance genes suggests the potential for exacerbating bacterial virulence.

The low-abundant species also showed an enriched expression of certain genes i.e. *lrg* and *mprF* (Peschel et al., 2001; Ranjit et al., 2011; Sohlenkamp and Geiger, 2016) and pathways i.e. polysaccharide transport (Flemming and Wingender, 2010) that are involved in biofilm formation. These upregulated genes in low-abundant but antimicrobial-resistant keystone species of the assessed multi-species biofilm might be a key factor for the observed resilience towards the disinfectant and determinative for the overall synergism in the multi-species biofilm. From a previous study, diffusible signal molecules that were released by *S. maltophilia* could modulate biofilm formation and tolerance toward antibiotics in *Pseudomonas aeruginosa* indicating that interspecies signalling can influence the behavior of organisms growing in multi-species communities (Ryan et al., 2008). The overall findings of the study provide novel insights into survival mechanisms in multi-species biofilms under sub-lethal concentrations of disinfectants, where bacteria likely rely to each other. It was shown that limited nutrients and disinfectants influence the bacterial composition and gene expression in multi-species biofilms, which collectively leads to synergism (Fig. 4). Hence, our study underlines the importance of a better understanding of inter-species interactions in order to improve the control of human pathogens.

5. Conclusions

This study provides novel insights into interactions between opportunistic human pathogens in multi-species biofilms. Synergism in the multi-species biofilm was prevalently observed when the pathogens were exposed to different stress factors. Our findings shed light on potential key genes for commonly observed synergism in multi-species biofilms and highlight the so far mostly overlooked importance of low-abundant species in such structures. We highlight that improper application of can result in an unwanted outcome, i.e. higher synergism and resilience of biofilms due to changing in community composition and global gene expression. Deeper understanding of the underlying inter-species interactions might help us to effectively control the persistence and spreading of opportunistic pathogens in the built environment.

CRediT authorship contribution statement

Gabriele Berg: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Wisnu Adi Wicaksono**: Conceptualization, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Sabine Erschen:** Formal analysis, Writing – review & editing. **Robert Krause:** Writing – review & editing. **Henry Müller:** Writing – review & editing **Tomislav Cernava:** Writing – review & editing.

Funding

This study was supported by a grant from the FWF (Austrian Science Fund) and the federal state government of Styria to G.B. (P29285-BBL).

Declaration of Competing Interest

The authors declare that they have no known competing financial

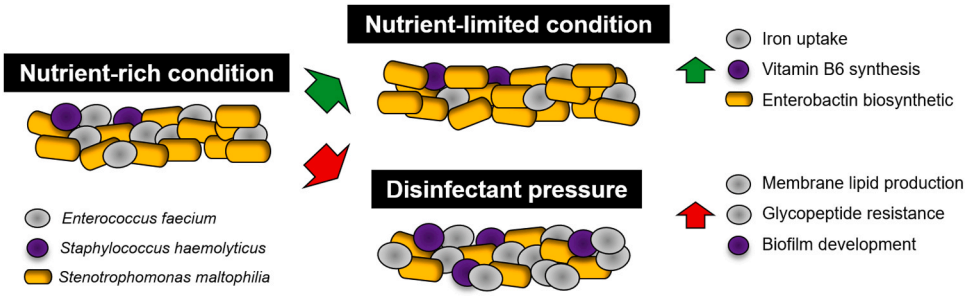

Fig. 4. Schematic illustration of multi-species biofilms that were grown under different conditions. Key genes that were differentially expressed within the mixed cultures under the experimental conditions are highlighted. Each cultivation medium, namely TSB (nutrient-rich), M9 (nutrient-limited) and TSB-BAC (nutrient-rich with disinfectant) resulted in a distinct bacterial community structure. Under nutrient-rich and nutrient-limited conditions, *Stenotrophomonas maltophilia* dominated the multi-species biofilm. Under the latter condition, all strains simultaneously increased iron uptake and vitamin B6 synthesis pathways. Under disinfectant pressure, the relative abundance of Enterococcus faecium and *Staphylococcus haemolyticus* increased and was followed by upregulation of expression of genes involved in defence mechanisms and biofilm formation. These regulations ultimately lead to an overall higher biofilm formation than for the individual bacteria under nutrient-limited and disinfectant pressure conditions. Gray: Enterococcus faecium, purple: *Staphylococcus haemolyticus,* yellow: *Stenotrophomonas maltophilia*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully thank Tamara Reisenhofer-Graber (Graz) for her support during the preparation of biofilm assays.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.126836.

References

Alexa, A., Rahnenfuhrer, J., 2010. TopGO: enrichment analysis for gene ontology, R Package Version, 2(0), 2010.
Allegranzi, B., Nejad, S.B., Combescure, C., Graafmans, W., Attar, H., Donaldson, L., Pittet, D., 2011. Burden of endemic health-care-associated infection in developing countries: systematic review and meta-analysis. Lancet 377 (9761), 228–241.
Andrews, J.H., Harris, R.F., 2000. The ecology and biogeography of microorganisms on plant surfaces. Annu. Rev. Phytopathol. 38 (1), 145–180.
Andrews, S. , 2010. FastQC: a quality control tool for high throughput sequence data.
Arango-Argoty, G., Garner, E., Pruden, A., Heath, L.S., Vikesland, P., Zhang, L., 2018. DeepARG: a deep learning approach for predicting antibiotic resistance genes from metagenomic data. Microbiome 6 (1), 1–15.
Banin, E., Hughes, D., Kuipers, O.P., 2017. Bacterial pathogens, antibiotics and antibiotic resistance. FEMS Microbiol. Rev. 41 (3), 450–452.
Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V. M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19 (5), 455–477.
Berg, G., Roskot, N., Smalla, K., 1999. Genotypic and phenotypic relationships between clinical and environmental isolates of *Stenotrophomonas maltophilia*. J. Clin. Microbiol. 37 (11), 3594–3600.
Blighe, K., Rana, S., Lewis, M., 2019. EnhancedVolcano: publication-ready volcano plots with enhanced colouring and labeling, R Package Version, 1(0).
Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120.
Boratyn, G.M., Camacho, C., Cooper, P.S., Coulouris, G., Fong, A., Ma, N., Madden, T.L., Matten, W.T., McGinnis, S.D., Merezhuk, Y., 2013. BLAST: a more efficient report with usability improvements. Nucleic Acids Res. 41 (W1), W29–W33.
Borodovsky, M., Mills, R., Besemer, J., Lomsadze, A., 2003. Prokaryotic gene prediction using GeneMark and GeneMark. Hmm. Curr. Protoc. Bioinform. 1 (1), 4–5.
Bottery, M.J., Pitchford, J.W., Friman, V.-P., 2021. Ecology and evolution of antimicrobial resistance in bacterial communities. ISME J. 15, 939–948.
Brislawn, C.J., Graham, E.B., Dana, K., Ihardt, P., Fansler, S.J., Chrisler, W.B., Cliff, J.B., Stegen, J.C., Moran, J.J., Bernstein, H.C., 2019. Forfeiting the priority effect: turnover defines biofilm community succession. ISME J. 13 (7), 1865–1877.
Brühwasser, C., Heinrich, H., Lass-Flörl, C., Mayr, A., 2017. Self-disinfecting surfaces and activity against *Staphyloccocus aureus* ATCC 6538 under real-life conditions. J. Hosp. Infect. 97 (2), 196–199.
Buchfink, B., Xie, C., Huson, D.H., 2015. Fast and sensitive protein alignment using DIAMOND. Nat. Methods 12 (1), 59–60.
Burmølle, M., Webb, J.S., Rao, D., Hansen, L.H., Sørensen, S.J., Kjelleberg, S., 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Appl. Environ. Microbiol. 72 (6), 3916–3923.
Bushnell, B. , 2014. BBTools software package. URL ⟨Http://Sourceforge.Net/Projects/Bbmap⟩.
Camarena, L., Bruno, V., Euskirchen, G., Poggio, S., Snyder, M., 2010. Molecular mechanisms of ethanol-induced pathogenesis revealed by RNA-sequencing. PLoS Pathog. 6 (4), 1000834.
Cassini, A., Plachouras, D., Eckmanns, T., Abu Sin, M., Blank, H.-P., Ducomble, T., Haller, S., Harder, T., Klingeberg, A., Sixtensson, M., 2016. Burden of six healthcare-associated infections on European population health: estimating incidence-based disability-adjusted life years through a population prevalence-based modelling study. PLoS Med. 13 (10), 1002150.
Chen, B., Han, J., Dai, H., Jia, P., 2021. Biocide-tolerance and antibiotic-resistance in community environments and risk of direct transfers to humans: unintended consequences of community-wide surface disinfecting during COVID-19? Environ. Pollut. 283, 117074.
Choi, A.H., Slamti, L., Avci, F.Y., Pier, G.B., Maira-Litrán, T., 2009. The pgaABCD locus of *Acinetobacter baumannii* encodes the production of poly-β-1-6-N-acetylglucosamine, which is critical for biofilm formation. J. Bacteriol. 191 (19), 5953–5963.
Cottingham, K.L., Lennon, J.T., Brown, B.L., 2005. Knowing when to draw the line: designing more informative ecological experiments. Front. Ecol. Environ. 3 (3), 145–152.
Esbelin, J., Santos, T., Hébraud, M., 2018. Desiccation: an environmental and food industry stress that bacteria commonly face. Food Microbiol. 69, 82–88.
Fattahi, S., Kafil, H.S., Nahai, M.R., Asgharzadeh, M., Nori, R., Aghazadeh, M., 2015. Relationship of biofilm formation and different virulence genes in uropathogenic *Escherichia coli* isolates from Northwest Iran. GMS Hyg. Infect. Control 10, 11.
Flemming, H.-C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8 (9), 623. –633.
Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. Nat. Rev. Microbiol. 14 (9), 563–575.
Frees, D., Chastanet, A., Qazi, S., Sørensen, K., Hill, P., Msadek, T., Ingmer, H., 2004. Clp ATPases are required for stress tolerance, intracellular replication and biofilm formation in *Staphylococcus aureus*. Mol. Microbiol. 54 (5), 1445–1462.
Guillonneau, R., Baraquet, C., Bazire, A., Molmeret, M., 2018. Multispecies biofilm development of marine bacteria implies complex relationships through competition and synergy and modification of matrix components. Front. Microbiol. 9, 1960.
Guyot, A., Turton, J., Garner, D., 2013. Outbreak of *Stenotrophomonas maltophilia* on an intensive care unit. J. Hosp. Infect. 85 (4), 303–307.
Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, 41(41), 95–98.
Heindorf, M., Kadari, M., Heider, C., Skiebe, E., Wilharm, G., 2014. Impact of *Acinetobacter baumannii* superoxide dismutase on motility, virulence, oxidative stress resistance and susceptibility to antibiotics. PLoS One 9 (7), 101033.
Hu, H., Johani, K., Gosbell, I.B., Jacombs, A., Almatroudi, A., Whiteley, G.S., Deva, A.K., Jensen, S., Vickery, K., 2015. Intensive care unit environmental surfaces are contaminated by multidrug-resistant bacteria in biofilms: Combined results of conventional culture, pyrosequencing, scanning electron microscopy, and confocal laser microscopy. J. Hosp. Infect. 91 (1), 35–44.
Huang, T.-P., Somers, E.B., Wong, A.C.L., 2006. Differential biofilm formation and motility associated with lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes in *Stenotrophomonas maltophilia*. J. Bacteriol. 188 (8), 3116–3120.
Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S.K., Cook, H., Mende, D.R., Letunic, I., Rattei, T., Jensen, L.J., 2019. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Res. 47 (D1), D309–D314.
Kelly, D., McAuliffe, O., Ross, R., Coffey, A., 2012. Prevention of *Staphylococcus aureus* biofilm formation and reduction in established biofilm density using a combination of phage K and modified derivatives. Lett. Appl. Microbiol. 54 (4), 286–291.
Kenters, N., Huijskens, E.G., de Wit, S.C., van Rosmalen, J., Voss, A., 2017. Effectiveness of cleaning-disinfection wipes and sprays against multidrug-resistant outbreak strains. Am. J. Infect. Control 45 (8), e69–e73.
Khan, H.A., Baig, F.K., Mehboob, R., 2017. Nosocomial infections: epidemiology, prevention, control and surveillance. Asian Pac. J. Trop. Biomed. 7 (5), 478–482.

Khatoon, Z., McTiernan, C.D., Suuronen, E.J., Mah, T.-F., Alarcon, E.I., 2018. Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. Heliyon 4 (12), 01067.
Kim, W.S., Jung, I.D., Kim, J.-S., Kim, H.M., Kwon, K.W., Park, Y.-M., Shin, S.J., 2018. *Mycobacterium tuberculosis GrpE*, a heat-shock stress responsive chaperone, promotes Th1-biased T cell immune response via TLR4-mediated activation of dendritic cells. Front. Cell. Infect. Microbiol. 8, 95.
Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9 (4), 357–359.
Liao, Y., Smyth, G.K., Shi, W., 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30 (7), 923–930.
Lin, M.-H., Shu, J.-C., Huang, H.-Y., Cheng, Y.-C., 2012. Involvement of iron in biofilm formation by *Staphylococcus aureus*. PLoS One 7 (3), 34388.
Lira, F., Berg, G., Martínez, J.L., 2017. Double-face meets the bacterial world: the opportunistic pathogen *Stenotrophomonas maltophilia*. Front. Microbiol. 8, 2190.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15 (12), 550.
Lu, J., Breitwieser, F.P., Thielen, P., Salzberg, S.L., 2017. Bracken: estimating species abundance in metagenomics data. PeerJ Comput. Sci. 3, e104.
Luther, M.K., Parente, D.M., Caffrey, A.R., Daffinee, K.E., Lopes, V.V., Martin, E.T., LaPlante, K.L., 2018. Clinical and genetic risk factors for biofilm-forming Staphylococcus aureus. Antimicrob. Agents Chemother. 62 (5).
Maes, S., Vackier, T., Huu, S.N., Heyndrickx, M., Steenackers, H., Sampers, I., Raes, K., Verplaetse, A., De Reu, K., 2019. Occurrence and characterisation of biofilms in drinking water systems of broiler houses. BMC Microbiol. 19 (1), 1–15.
Mc Carlie, S., Boucher, C.E., Bragg, R.R., 2020. Molecular basis of bacterial disinfectant resistance. Drug Resist. Update.: Rev. Comment. Antimicrob. Anticancer Chemother. 48, 100672.
McCarthy, H., Rudkin, J.K., Black, N.S., Gallagher, L., O'Neill, E., O'Gara, J.P., 2015. Methicillin resistance and the biofilm phenotype in *Staphylococcus aureus*. Front. Cell. Infect. Microbiol. 5, 1.
Merchel Piovesan Pereira, B., Wang, X., Tagkopoulos, I., 2021. Biocide-induced emergence of antibiotic resistance in *Escherichia coli*. Front. Microbiol. 12, 640923.
Monegro, A.F., Muppidi, V., Regunath, H. , 2020. Hospital acquired infections. StatPearls [Internet].
Mora, M., Mahnert, A., Koskinen, K., Pausan, M.R., Oberauner-Wappis, L., Krause, R., Perras, A.K., Gorkiewicz, G., Berg, G., Moissl-Eichinger, C., 2016. Microorganisms in confined habitats: microbial monitoring and control of intensive care units, operating rooms, cleanrooms and the International Space Station. Front. Microbiol. 7, 1573.
Oberauner, L., Zachow, C., Lackner, S., Högenauer, C., Smolle, K.-H., Berg, G., 2013. The ignored diversity: complex bacterial communities in intensive care units revealed by 16S pyrosequencing. Sci. Rep. 3, 1413.
Oglesby-Sherrouse, A.G., Djapgne, L., Nguyen, A.T., Vasil, A.I., Vasil, M.L., 2014. The complex interplay of iron, biofilm formation, and mucoidy affecting antimicrobial resistance of *Pseudomonas aeruginosa*. Pathog. Dis. 70 (3), 307–320.
O'Neill, E., Pozzi, C., Houston, P., Smyth, D., Humphreys, H., Robinson, D.A., O'Gara, J. P., 2007. Association between methicillin susceptibility and biofilm regulation in *Staphylococcus aureus* isolates from device-related infections. J. Clin. Microbiol. 45 (5), 1379–1388.
O'Toole, G.A., Kolter, R., 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. 28 (3), 449–461.
Paredes, J., Alonso-Arce, M., Schmidt, C., Valderas, D., Sedano, B., Legarda, J., Arizti, F., Gómez, E., Aguinaga, A., Del Pozo, J., 2014. Smart central venous port for early detection of bacterial biofilm related infections. Biomed. Microdevices 16 (3), 365–374.
Parijs, I., Steenackers, H.P., 2018. Competitive inter-species interactions underlie the increased antimicrobial tolerance in multispecies brewery biofilms. ISME J. 12 (8), 2061–2075.
Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 25 (7), 1043–1055.
Parsek, M.R., Singh, P.K., 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu. Rev. Microbiol. 57 (1), 677–701.
Peschel, A., Jack, R.W., Otto, M., Collins, L.V., Staubitz, P., Nicholson, G., Kalbacher, H., Nieuwenhuizen, W.F., Jung, G., Tarkowski, A., 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. J. Exp. Med. 193 (9), 1067–1076.
Pidot, S.J., Gao, W., Buultjens, A.H., Monk, I.R., Guerillot, R., Carter, G.P., Lee, J.Y., Lam, M.M., Grayson, M.L., Ballard, S.A., 2018. Increasing tolerance of hospital *Enterococcus faecium* to handwash alcohols. Sci. Transl. Med. 10 (452).
Pompilio, A., Pomponio, S., Crocetta, V., Gherardi, G., Verginelli, F., Fiscarelli, E., Dicuonzo, G., Savini, V., D'Antonio, D., Di Bonaventura, G., 2011. Phenotypic and genotypic characterization of *Stenotrophomonas maltophilia* isolates from patients with cystic fibrosis: genome diversity, biofilm formation, and virulence. BMC Microbiol. 11 (1), 1–17.
R Core Team, 2017. R Core TeamR: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.
Raghupathi, P.K., Liu, W., Sabbe, K., Houf, K., Burmølle, M., Sørensen, S.J., 2018. Synergistic interactions within a multispecies biofilm enhance individual species protection against grazing by a pelagic protozoan. Front. Microbiol. 8, 2649.
Ranjit, D.K., Endres, J.L., Bayles, K.W., 2011. *Staphylococcus aureus* CidA and LrgA proteins exhibit holin-like properties. J. Bacteriol. 193 (10), 2468–2476.
Rao, D., Webb, J.S., Kjelleberg, S., 2005. Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicata*. Appl. Environ. Microbiol. 71 (4), 1729–1736.
Reichel, M., Schlicht, A., Ostermeyer, C., Kampf, G., 2014. Efficacy of surface disinfectant cleaners against emerging highly resistant gram-negative bacteria. BMC Infect. Dis. 14 (1), 292.
Ren, D., Madsen, J.S., Sørensen, S.J., Burmølle, M., 2015. High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. ISME J. 9 (1), 81–89.
Røder, H.L., Sørensen, S.J., Burmølle, M., 2016. Studying bacterial multispecies biofilms: Where to start? Trends Microbiol. 24 (6), 503–513.
Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4, 2584.
Rosenberg, J., Yeak, K.C., Commichau, F.M., 2018. A two-step evolutionary process establishes a non-native vitamin B6 pathway in Bacillus subtilis. Environ. Microbiol. 20 (1), 156–168.
Ryan, R.P., Fouhy, Y., Garcia, B.F., Watt, S.A., Niehaus, K., Yang, L., Tolker-Nielsen, T., Dow, J.M., 2008. Interspecies signalling via the *Stenotrophomonas maltophilia* diffusible signal factor influences biofilm formation and polymyxin tolerance in *Pseudomonas aeruginosa*. Mol. Microbiol. 68 (1), 75–86.
Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., Van Der Lelie, D., Dow, J.M., 2009. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. Nat. Rev. Microbiol. 7 (7), 514–525.
Salta, M., Wharton, J.A., Blache, Y., Stokes, K.R., Briand, J., 2013. Marine biofilms on artificial surfaces: structure and dynamics. Environ. Microbiol. 15 (11), 2879–2893.
Salze, M., Giard, J.-C., Riboulet-Bisson, E., Hain, T., Rincé, A., Muller, C., 2020. Identification of the general stress stimulon related to colonization in *Enterococcus faecalis*. Arch. Microbiol. 202 (2), 233–246.
Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol. Ecol. 43 (3), 325–335.
Schwering, M., Song, J., Louie, M., Turner, R.J., Ceri, H., 2013. Multi-species biofilms defined from drinking water microorganisms provide increased protection against chlorine disinfection. Biofouling 29 (8), 917–928.
Sharma, D., Khan, A.U., 2018. Role of cell division protein divIVA in *Enterococcus faecalis* pathogenesis, biofilm and drug resistance: a future perspective by in silico approaches. Microb. Pathog. 125, 361–365.
Slany, M., Oppelt, J., Cincarova, L., 2017. Formation of Staphylococcus aureus biofilm in the presence of sublethal concentrations of disinfectants studied via a transcriptomic analysis using transcriptome sequencing (RNA-seq). Appl. Environ. Microbiol. 83 (24).
Sohlenkamp, C., Geiger, O., 2016. Bacterial membrane lipids: diversity in structures and pathways. FEMS Microbiol. Rev. 40 (1), 133–159.
Soto-Giron, M.J., Rodriguez-R, L.M., Luo, C., Elk, M., Ryu, H., Hoelle, J., Santo Domingo, J.W., Konstantinidis, K.T., 2016. Characterization of biofilms developing on hospital shower hoses and implications for nosocomial infections. Appl. Environ. Microbiol.
Stiefel, P., Rosenberg, U., Schneider, J., Mauerhofer, S., Maniura-Weber, K., Ren, Q., 2016. Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. Appl. Microbiol. Biotechnol. 100 (9), 4135–4145.
Wood, D.E., Lu, J., Langmead, B., 2019. Improved metagenomic analysis with Kraken 2. Genome Biol. 20 (1), 257.
Zhuo, C., Zhao, Q., Xiao, S., 2014. The impact of spgM, rpfF, rmlA gene distribution on biofilm formation in *Stenotrophomonas maltophilia*. PLoS One 9 (10), 108409.