Department of Agricultural Sciences, Nutritional Sciences and

Environmental Management

at Justus-Liebig-University of Giessen

and

Institute of Precision Medicine,

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Investigations on the structure and function of the microbiota in household washing machines, kitchen sponges, and on laundered textiles

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Table of Contents

List of abbreviations

AOB	Activated oxygen bleach
BUSCO	Benchmarking Universal Single-Copy Orthologs
CDC	Centres for Disease Control and Prevention
cDNA	Complementary DNA
CFU	Colony forming unit
contigs	Contiguous sequence
CsTFA	Caesiumtrifluoroacetat
DNA	Deoxyribonucleic acid
ExN50	Expression-dependant N50
FISH	Fluorescence in situ hybridization
GO	Gene ontology
HPLC	High performance liquid chromatography
ITS	Internal transcribed spacer
LogFC	Log fold change
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
mRNA	Messenger ribonucleic acid
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
pH	Potential of hydrogen (potentia hydrogenii)
RNA	Ribonucleic acid
RNASeq	Ribonucleic acid sequencing
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SIMS	Secondary ion mass spectrometry
SIP	Stable isotope probing
spp.	Multiple species

List of figures and tables

Declaration

I declare that the dissertation submitted here is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "Statutes of the Justus-Liebig-University Giessen for the Safeguarding of Good Scientific Practice"**.**

Giessen, **Date: 23.01.2023** **Susanne Jacksch**

Summary

In industrialized countries, people spend up to 90 % of their lifetime indoors, rendering domestic hygiene an important issue for human health and well-being. Due to favourable growth conditions, such as moisture, warmth, and sufficient supply of nutrients, many domestic environments harbour dense and diverse microbial communities. However, it also has been hypothesized that typical domestic cleaning and sanitation measures might shape these communities in a way that is unfavourable for human health and well-being. In order to get a deeper understanding of how environmental factors shape microbial communities in domestic environments, the structure (community composition) and function (physiology) of the microbiota in washing machines, kitchen sponges, and on washed laundry were investigated and associated with environmental factors using a polyphasic approach of cultivation-dependent and independent methods.

Washing machines are widely used tools for laundry cleaning, known to offer favourable growth conditions for microorganisms. Colonisation is further promoted by current washing trends such as short and water-saving programmes, low washing temperatures, and the use of bleach-free liquid detergent. Using 16S rRNA gene amplicon pyrosequencing and MALDI-TOF-based identification of isolated bacteria, a diverse microbial community was detected at three sampling sites of typical household washing machines (sump, rubber door seal, and detergent drawer) comprising more than 200 bacterial species. The composition of this microbiota was strongly site-dependent, with the highest bacterial diversity found inside the detergent drawer. No correlations between selected user data and bacterial community composition were found, except the fact that bacterial diversity was significantly higher in the detergent drawer of machines that are frequently used with washing temperatures of 60 °C and higher. Cell counts based on swab samples of detergent drawer and door seal showed a bacterial load of 21,000 colony-forming units (CFU) per cm^2, when averaged over all sampling sites. The lowest bacterial counts were found in the upper area of the rubber door seal, probably due to lack of water. The other sampling sites (detergent drawer, detergent chamber, bottom part of the rubber door seal) revealed similar bacterial counts of approximately 10^4 CFU per cm^2. These findings, together with the fact that about half of the most common bacterial species were classified as potentially pathogenic, show that washing machines are a domestic source of potential pathogens, malodour producers, and cross-contamination.

In order to learn more about the metabolic activities (e.g., substrate use) of bacteria on laundered textiles, a metatranscriptomic analysis pipeline was established and applied for the first time to compare bacterial gene expression on laundered cotton and polyester fabrics. The analysis revealed that 17 genes differed significantly in their expression between the two tissue types, which are involved in several different biochemical pathways, e.g. amino acid transport and metabolism or bacterial carbohydrate metabolism. The data allow careful speculation that bacteria might feed on carbohydrates released from the cotton textiles. Knowledge about substrate utilization on washed laundry might help to identify novel strategies against microbial malodour production on washed textiles.

Sponges are very popular cleaning tools in domestic kitchens. They pick up and spread microorganisms on a massive scale when cleaning dishes and other kitchen surfaces. Microwave treatment represents an effective and widely used technique to quickly reduce the microbial load of kitchen sponges. However, the long-term effects of such a treatment on the microbial community were largely unknown. When comparing the metagenome of 10 regularly microwaved and 10 untreated used kitchen sponges, microwave treatment showed a trend towards lower structural microbial diversity, while functional diversity increased. This finding clearly indicates that microwave treatment alters microbial diversity and genetic potential of resident communities in household kitchen sponges. However, further work is needed to clarify in more detail whether these changes are rather beneficial or adverse in terms of human health and well-being.

In the future, the establishment and application of methods focusing on a more functional characterisation of the microbiota in domestic environments, such as stable isotope probing or metabolomic studies in addition to metagenomics or metatranscriptomics, will provide new and important insight into the genetic potential and metabolism of the domestic microbiota and its interference with domestic cleaning measures. Such knowledge might be useful to develop novel strategies to tackle hygienic problems such as malodour formation or the selection and enrichment of (potentially) pathogenic and antibiotic-resistant species in the domestic environment.

Zusammenfassung

In Industrieländern verbringen Menschen bis zu 90 % ihrer Lebenszeit in Innenräumen, was die häusliche Hygiene zu einem wichtigen Thema für menschliche Gesundheit und Wohlbefinden macht. Aufgrund günstiger Wachstumsbedingungen wie Feuchtigkeit, Wärme und einer ausreichenden Versorgung mit Nährstoffen beherbergen viele häusliche Umgebungen individuenreiche und vielfältige mikrobielle Gemeinschaften. Es wurde jedoch auch die Hypothese aufgestellt, dass typische häusliche Reinigungs- und Hygienemaßnahmen diese Gemeinschaften in einer Weise beeinflussen könnten, die für die menschliche Gesundheit und Wohlbefinden ungünstig sind. Um ein tieferes Verständnis dafür zu bekommen, wie Umweltfaktoren mikrobielle Gemeinschaften in häuslichen Umgebungen formen, wurden Struktur (Gemeinschaftszusammensetzung) und Funktion (Physiologie) der Mikrobiota in Waschmaschinen, Küchenschwämmen und auf gewaschener Wäsche untersucht und mit Hilfe eines polyphasischen Ansatzes aus kultivierungsabhängigen und -unabhängigen Methoden mit Umweltfaktoren in Verbindung gebracht.

Waschmaschinen sind weit verbreitete Geräte zur Wäschereinigung, die bekanntermaßen günstige Wachstumsbedingungen für Mikroorganismen bieten. Die Besiedlung wird durch aktuelle Waschtrends wie kurze und wassersparende Programme, niedrige Waschtemperaturen und die Verwendung von bleichmittelfreiem Flüssigwaschmittel weiter gefördert. Mittels 16S rRNA-Gen-Amplikon-Pyrosequenzierung und MALDI-TOF-basierter Identifizierung isolierter Bakterien wurde an drei Probenahmestellen typischer Haushaltswaschmaschinen (Sumpf, Bullaugendichtung und Waschmittelschublade) eine vielfältige Mikrobengemeinschaft mit mehr als 200 Bakterienarten nachgewiesen. Die Zusammensetzung dieser Mikrobiota war stark standortabhängig, wobei die höchste bakterielle Vielfalt in der Waschmittelschublade gefunden wurde. Des Weiteren wurden keine Korrelationen zwischen ausgewählten Nutzerdaten und der Zusammensetzung der Bakteriengemeinschaft festgestellt, mit Ausnahme der Tatsache, dass die bakterielle Vielfalt in der Waschmittelschublade von Maschinen, die häufig bei Waschtemperaturen von 60 °C und höher benutzt werden, deutlich höher war. Zellzahlen auf Grundlage von Abstrichproben der Waschmittelschublade und der Bullaugendichtung ergaben eine Bakterienbelastung von 21.000 koloniebildenden Einheiten pro cm^2, wenn man den Durchschnitt über alle Probenahmestellen bildet. Die niedrigsten Keimzahlen wurden im oberen Bereich der Bullaugendichtung festgestellt, was wahrscheinlich auf Wassermangel zurückzuführen ist. An den anderen Probenahmestellen (Waschmittelschublade, Waschmittelkammer, unterer Teil der Türgummidichtung) wurden

ähnliche Keimzahlen von etwa 10^4 koloniebildenden Einheiten pro cm^2 festgestellt. Diese Ergebnisse und die Tatsache, dass etwa die Hälfte der häufigsten Bakterienarten als potenziell pathogen klassifiziert sind, zeigen, dass Waschmaschinen eine häusliche Quelle potenzieller Krankheitserreger, Geruchsverursacher und Kreuzkontaminationen sind.

Um mehr über die Stoffwechselaktivitäten (z. B. Substratnutzung) von Bakterien auf gewaschenen Textilien zu erfahren, wurde eine metatranskriptomische Analysepipeline aufgebaut und zum ersten Mal angewendet, um die bakterielle Genexpression auf gewaschenen Baumwoll- und Polyestergeweben zu vergleichen. Die Analyse ergab, dass sich 17 Gene in ihrer Expression zwischen den beiden Gewebearten signifikant unterschieden, die an verschiedenen biochemischen Stoffwechselwegen beteiligt sind, z. B. am Aminosäuretransport und -stoffwechsel oder am bakteriellen Kohlenhydratstoffwechsel. Die Daten lassen die vorsichtige Vermutung zu, dass sich die Bakterien von den aus den Baumwolltextilien freigesetzten Kohlenhydraten ernähren könnten. Das Wissen über die Substratnutzung in gewaschener Wäsche könnte dazu beitragen, neue Strategien gegen die mikrobielle Geruchsbildung von gewaschenen Textilien zu entwickeln.

Schwämme sind sehr beliebte Reinigungswerkzeuge in der Küche. Beim Reinigen von Geschirr und anderen Küchenoberflächen nehmen sie in großem Umfang Mikroorganismen auf und verbreiten sie. Die Behandlung mit Mikrowellen ist eine wirksame und weit verbreitete Technik zur schnellen Verringerung der mikrobiellen Belastung von Küchenschwämmen. Die langfristigen Auswirkungen einer solchen Behandlung auf die mikrobielle Gemeinschaft waren jedoch weitgehend unbekannt. Beim Vergleich des Metagenoms von 10 regelmäßig mikrowellenbehandelten und 10 unbehandelten gebrauchten Küchenschwämmen zeigte sich bei der Mikrowellenbehandlung ein Trend zu einer geringeren strukturellen mikrobiellen Vielfalt, während die funktionelle Vielfalt zunahm. Dieses Ergebnis deutet darauf hin, dass die Mikrowellenbehandlung die mikrobielle Vielfalt und das genetische Potenzial der ansässigen Gemeinschaften in Haushalts-Küchenschwämmen verändert. Es sind jedoch weitere Arbeiten erforderlich, um genauer zu klären, ob diese Veränderungen im Hinblick auf menschliche Gesundheit und Wohlbefinden eher von Vorteil oder Nachteil sind.

In Zukunft wird die Einführung und Anwendung von Methoden, die sich auf eine funktionellere Charakterisierung der Mikrobiota im häuslichen Umfeld konzentrieren, wie z. B. die Sondierung mit stabilen Isotopen oder metabolomische Studien, zusätzlich zu Metagenomik oder Metatranskriptomik neue und wichtige Einblicke in das genetische Potenzial und den Stoffwechsel der häuslichen Mikrobiota und ihre Beeinflussung durch häusliche

Reinigungsmaßnahmen liefern. Dieses Wissen könnte für die Entwicklung neuer Hygienestrategien nützlich sein, um Probleme wie Geruchsbildung oder die Selektion und Anreicherung von (potenziell) pathogenen und antibiotikaresistenten Arten im häuslichen Umfeld zu verhindern.

List of publications

1. Scientific publications in international peer reviewed journals as first author

Jacksch, S.†, Kaiser, D.†, Weis, S., Weide, M., Ratering, S., Schnell, S., Egert, M. Influence of Sampling Site and other Environmental Factors on the Bacterial Community Composition of Domestic Washing Machines. *Microorganisms*. 2020; 8(1):30. https://doi.org/10.3390/microorganisms8010030.
† Both authors contributed equally to this work.
Own contribution: Bioinformatics and statistical analyses, visualisation, data interpretation, data curation, manuscript writing, and reviewing.

Jacksch S., Thota J., Shetty S., Smidt H., Schnell S., Egert M. Metagenomic Analysis of Regularly Microwave-Treated and Untreated Domestic Kitchen Sponges. *Microorganisms*. 2020; 8(5):736. https://doi.org/10.3390/microorganisms8050736.
Own contribution: Supervision and assistance with wet-lab work, bioinformatics and statistical analyses, visualisation, data interpretation, data curation, manuscript writing, and reviewing.

Jacksch S., Zohra, H., Weide M., Schnell S., Egert M. Cultivation-based Quantification and Identification of Bacteria at Two Hygienic Key Sides of Domestic Washing Machines. *Microorganisms*. 2021; 9(5):905. https://doi.org/10.3390/microorganisms9050905.
Own contribution: Supervision and assistance with wet lab work, MALDI-TOF analysis, species identification, bioinformatics and statistical analyses, visualisation, data interpretation, data curation, manuscript writing, and reviewing.

Jacksch S., König C., Kaiser D., Weide M., Ratering S., Schnell S., Egert M. Metatranscriptomic Analysis of Bacterial Communities on Laundered Textiles: A Pilot Case Study. *Microorganisms*. 2021; 9(8):1591. https://doi.org/10.3390/microorganisms9081591
Own contribution: Bioinformatics and statistical analyses, visualisation, data interpretation, data curation, manuscript writing, and reviewing.

2. Further publications in international peer reviewed journals

Desiderato, C. K., Sachsenmaier, S., Ovchinnikov, K. V., Stohr, J., **Jacksch, S.**, Desef, D. N., Crauwels, P., Egert, M., Diep, D. B., Goldbeck, O., Riedel, C. U. Identification of Potential Probiotics Producing Bacteriocins Active against *Listeria monocytogenes* by a Combination of Screening Tools. *International Journal of Molecular Sciences.* 2021, *22*(16), 8615. https://doi.org/10.3390/ijms22168615
Own contribution: Sample preparation and MALDI-TOF analysis, species identification, writing, and review of the respective parts in the manuscript.

Borgolte M., Riester O., Kacerova T., Rentschler S., Schmidt M.S., **Jacksch S.**, Egert M., Laufer S., Csuk R., Deigner H-P. Methacryloyl-GlcNAc Derivatives Copolymerized with Dimethacrylamide as a Novel Antibacterial and Biocompatible Coating. *Pharmaceutics*. 2021; 13(10):1647. https://doi.org/10.3390/pharmaceutics13101647

Own contribution: Antimicrobial efficacy testing by means of contact method, review of the respective parts in the manuscript.

Weis S., **Jacksch S.**, Welkerling M., Schmidt M. S., Egert M. Do it yourself! – Initial experiences with self-synthesized CsTFA for RNA-SIP analyses. *Journal of Microbiological Methods*. 2022, 194 https://doi.org/10.1016/j.mimet.2022.106432
Own contribution: Supervision and assistance of wet lab work with Staphylococcus*, review of the respective parts in the manuscript.*

Brandau L., **Jacksch S**., Weis S., Schnell S., Egert M. Minority report: small-scale metagenomic analysis of the non-bacterial kitchen sponge microbiota. *Archives of Microbiology*. 2022; 204 (7), pp. 363. https://doi.org/10.1007/s00203-022-02969-9
Own contribution: Assistance with bioinformatics analysis, manuscript writing, and reviewing.

3. Non peer-reviewed publications

Egert M., **Jacksch S.** Wiedereröffnung der Pandora Büchse: Molekularbiologische Forschung an Küchenschwämmen. *Ernährung aktuell: der Lebensmittelbrief.* 2020, 31 (September/Oktober), pp. 10-11
Own contribution: Manuscript writing and reviewing.

Jacksch S., Egert M. Welcome to the jungle: Das Küchenschwamm-Mikrobiom. *Lebensmittelchemie.* 2020; 74 (6), 181-184. https://doi.org/10.1002/lemi.202000606
Own contribution: Manuscript writing and reviewing.

Jacksch S., Weide M., Schnell S., Egert, M. Das Waschmaschinen-Mikrobiom-Projekt. *BIOspektrum.* 2021, 27 (7), 784, https://doi.org/10.1007/s12268-021-1663-8
Own contribution: Manuscript writing and reviewing.

4. Poster publications on scientific symposia

Title: Influence of environmental factors on the bacterial microbiota of domestic washing machines (MBP409)
Authors: **Jacksch S.**, Kaiser D., Weis S., Weide M., and Egert M. Resources: Annual Conference of the Association for General and Applied Microbiology (VAAM) 2018, 15-18 April 2018, Wolfsburg (Germany), p. 45, 2018
Abstract book available under: https://www.dghm-vaam.de/fileadmin/congress/media/dghmvaam2020/druckelemente/VAAM2018_Abstractband.pdf

Title: Preliminary analysis of the microbiota of regularly microwave-sanitized and non-sanitized kitchen sponges using metagenome shotgun sequencing (EMP170)
Authors: **Jacksch S.**, Thota J., and Egert M.
Resources: Annual Conference of the Association for General and Applied Microbiology (VAAM) 2019, 17-20 March 2019, Mainz (Germany), p. 129, 2019

Abstract book available under: https://www.dghm-vaam.de/fileadmin/congress/media/dghmvaam2020/druckelemente/VAAM2019_Abstractband.pdf

Title: Influence of microwave sanitation on the metagenome of used kitchen sponges (PT177)
Authors: **Jacksch S.**, Thota J., and Egert M.
Resources: Federation of European Microbiological Societies (FEMS) 2019, 8th Congress of European Microbiologists, 7-11 July 2019, Glasgow (Scotland), p. 890, 2019
Abstract book available under:
https://fems2019.org/fileadmin/user_upload/FEMS/fems2019_abstractbook.pdf

5. Oral presentations on scientific symposia:

Repeated microwave sanitization adversely alters the genetic potential of the kitchen sponge microbiome (241-FMV)
Authors: **Jacksch S.**, Thota J., and Egert M
Resources: 6th Joint Conference of DGHM & VAAM: 72nd Annual Meeting of DGHM, Annual Conference of the Association for General and Applied Microbiology (VAAM) 2020, 08–11 March 2020, Leipzig (Germany), p. 91, 2020
Abstract book available under: https://www.dghm-vaam.de/fileadmin/congress/media/dghmvaam2020/druckelemente/DGHMVAAM2020_Abstractband.pdf

Using OMICS-technologies to unravel the mystery of malodour formation in washing machines and on washed laundry (ST204)
Authors: **Jacksch S**, König C., Kaiser D., Kaiser L., Weide M., Schnell S., Deigner H-P., and Egert M.
Resources: Annual Conference of the Association for General and Applied Microbiology (VAAM) 2022, 21–23 February 2022, online, p. 83, 2022
Abstract book available under: https://vaam-digital.de/fileadmin/documents/VAAM2022_abstract_booklet.pdf

1. The Microbiome of the Built Environment

1.1 Definitions

With respect to human health and disease, the built environment, which is defined as the human-made surroundings, represents an important factor that can promote or harm human well-being (Davison and Lawson 2006; Deschenes et al. 2020; Gilbert and Stephens 2018; Kelley and Gilbert 2013). Due to an increasing urbanization, more and more people are living in highly organized built environments and their activities and lifestyles have changed in such a way that a large proportion of people spend a majority of their time (up to 90 % in developed countries) indoors (Gilbert and Stephens 2018; Kelley and Gilbert 2013; Lam et al. 2021; Northridge et al. 2003; Prussin et al. 2020). More importantly, various studies have shown that these interiors are characterized by different microhabitats that are inhabited by a microbial community, which is composed of bacteria, fungi, archaea, algae, viruses, and small protists (Hofbauer 2021; Pakpour et al. 2016; Prussin et al. 2020; Rasli et al. 2021; Tsao et al. 2019). Of these microorganisms, bacteria and fungi are the best analysed ones, due to their potential impact on human health and well-being (Prussin et al. 2020; Rasli et al. 2021). Studies identified, among others, *Micrococcus*, *Staphylococcus*, *Pseudomonas*, and *Acinetobacter* to be the most common bacterial genera and *Cladosporidium*, *Aspergillus*, and *Penicillium* to be the most common fungal genera in a variety of buildings and on their surfaces (Li et al. 2021; Martin et al. 2015; Rasli et al. 2021). The study of indoor viruses has recently become increasingly important, especially since transmissions of viruses causing respiratory infections, such as the COVID-19 triggering *Betacoronavirus* SARS-CoV-2, were increasing (Dietz et al. 2020; Fendrick et al. 2003; Hu and Hartmann 2021).

The microbial communities in different habitats can be referred to by the term microbiota, which describes the totality of all living microorganisms present in a studied environment (Berg et al. 2020). In contrast, the term microbiome, as used by Whipps and colleagues in 1988 (Whipps et al. 1988), refers not only to the microorganisms involved but also to their "theatre of activity", which includes their specific characteristics and functions as well as their interactions with the environment, leading to the formation of specific ecological niches (Berg et al. 2020; Whipps et al. 1988). In recent years, the term became more and more associated with multi-omics approaches such as metagenomic, metatranscriptomic, metaproteomic, and metabolomic techniques that aim to characterize changes in community composition,

functionality, and host reactions under different conditions (Berg et al. 2020; Marchesi and Ravel 2015; Solbiati and Frias-Lopez 2018).

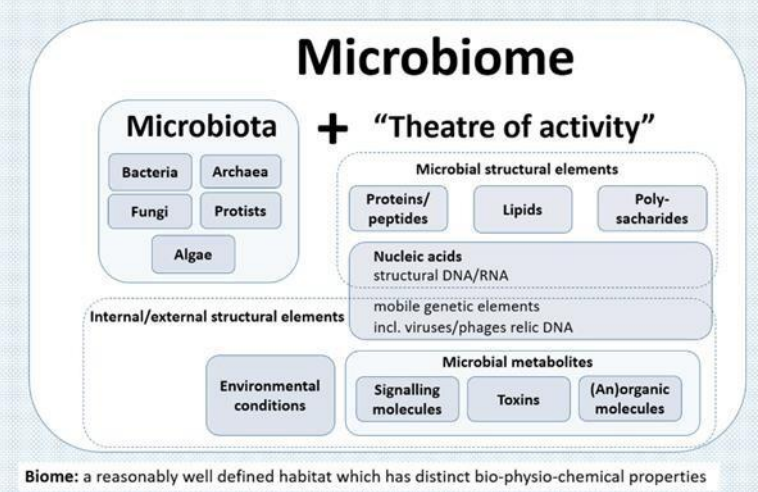

Figure 1. A schematic illustration of the composition of the term microbiome. The term microbiome comprises a living community of microorganisms (microbiota) and its "theatre of activity", as well as structural elements, metabolites, signalling molecules, and surrounding environmental conditions (Berg et al. 2020).

1.2 Methods to analyse a microbiome

To better understand how microbial communities are influenced by environmental factors, a number of studies have been conducted to characterise microbial communities in different types of buildings and on their surfaces (National Academies of Sciences, Engineering, and Medicine et al. 2017). To accomplish this, researchers used a variety of methods, both culture-dependent and culture-independent techniques, the most important of which are briefly explained below.

Culture-dependent methods

To get an overview of the quantity and composition of living microorganisms in a studied habitat, it is possible to describe and analyse specific microbial populations with conventional bacterial cultivation methods under laboratory conditions (Sarangi et al. 2019; Vartoukian 2016). However, in culture-dependent methods, the growth of microorganisms is highly dependent on the culture medium used, the particular cultivation conditions, and the incubation time, which makes it very time consuming and challenging (Zengler 2009). Cultivation of microbially highly diverse samples in particular can distort the ratios of different taxa inside a community, due to different growth rates of the respective organisms (Prakash et al. 2013; Vartoukian et al. 2010; Zengler 2009). Nevertheless, cultivation of microorganisms is still a

valuable basis today, e.g. for in-depth studies on the morphology, physiology, genetics, and pathogenicity of microorganisms, or for susceptibility testing under real life conditions (Patenge et al. 2012; Prakash et al. 2013).

One modern method to rapidly identify isolated microorganisms after cultivation is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Figure 2) (Eigner et al. 2009).

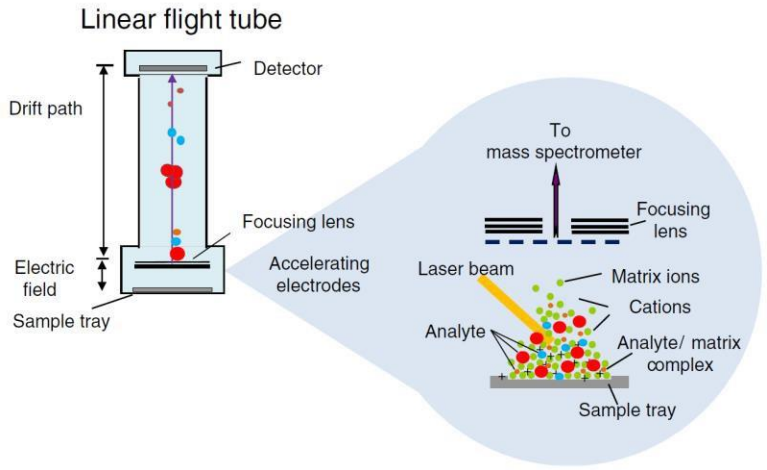

Figure 2. Schematic representation of the principle of MALDI-TOF MS identification of bacteria. The (ribosomal) proteins of bacteria (red and light blue spheres) embedded in matrix material (green spheres) are ionized by a laser. In the electric field present, the ions are accelerated according to their mass and electric charge and further separated in a vacuum tube. The measurable differences in the flight time of the desorbed particles are detected at the top of the vacuum tube. From the time of flight, the exact mass of the polypeptides can be calculated. Using the calculations, a protein spectral profile of the isolate is generated and compared with a reference database for identification (Wieser et al. 2012).

The advantage of this technique is that it allows rapid identification of bacterial and fungal species by determining the molecular masses of proteins, most of which are likely to be ribosomal proteins (Saffert et al. 2011). The basis for the analysis is sample material obtained from a pure culture, e.g. whole cells or crude extracts, which are co-crystallised with a special matrix and ionised by short laser pulses. The ionized molecules are then accelerated in an electric field and their time of flight is measured in a vacuum flight tube that separates the molecules by molecular weight. When the ions reach the detector on top of the vacuum tube, their time of flight is used to calculate the exact mass of the polypeptides (Sauget et al. 2017; Wieser et al. 2012). Based on the calculations, a protein spectral profile of an isolate is generated and compared with a reference database for identification (Alatoom et al. 2011).

Cultivation-independent methods

Many microorganisms in natural communities are difficult to culture and many methods that do not rely on cultivation have been developed over the last decades (National Academies of Sciences, Engineering, and Medicine et al. 2017). These so-called "omics" techniques have helped a lot to reveal a great phylogenetic and metabolic diversity within the built environment by analysing particular molecules such as DNA, RNA, proteins, or metabolites (Kuczynski et al. 2011; Vanwonterghem et al. 2014). However, to obtain comprehensive information on a microbial community, it is advisable to conduct polyphasic studies, as information on functional interactions and characteristics occurring in a given ecosystem cannot be obtained by studying isolated components (Siggins et al. 2012; Vanwonterghem et al. 2014).

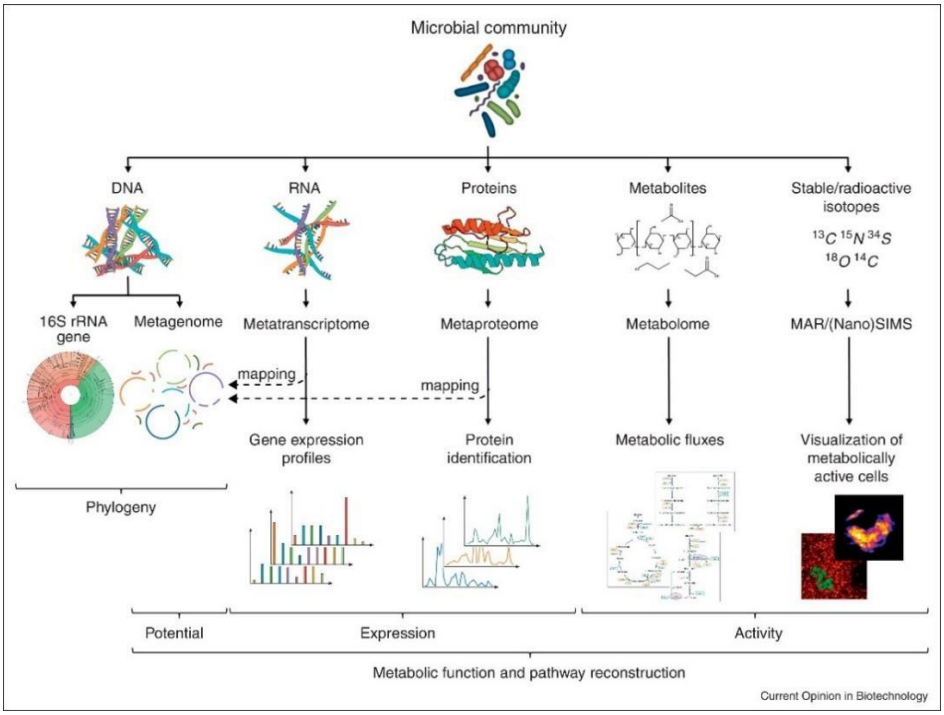

Figure 3. Overview of culture-independent methods for characterizing a microbiome. DNA-based approaches can be divided into sequencing of 16S rRNA gene amplicons, which allows determination of community composition, while metagenomics also provides information on the functional potential of a community. Metatranscriptomics can identify active microbial communities and determine important metabolic pathways and gene expression. Metaproteomics and metabolomics better reflect functional protein expression and metabolic activity. Visualisation methods based on isotope and elemental imaging can be used to enumerate metabolically active cells and to calculate substrate uptake rates and nutrient fluxes (Vanwonterghem et al. 2014).

The use of environmental DNA sequencing has revealed a large biodiversity of microorganisms in different habitats (Barberán et al. 2015; Flores et al. 2013; Fritz et al. 2018; Leung et al. 2021; Mahnert et al. 2015; Perkins et al. 2009; Sitarik et al. 2018; Turner et al. 2013). Basically, DNA-based analyses can be divided into two groups: environmental single-gene surveys or random shotgun studies of all environmental genes (Gilbert and Dupont 2011; Hamady and Knight 2009). Single-gene studies typically use stable phylogenetic marker genes that are highly conserved but exhibit sufficient variation to distinguish between taxa (Khayalethu 2013; Sharpton 2014). Such genes may be the 16S ribosomal RNA gene for bacteria or archaea, the 18S ribosomal RNA gene for eukaryotes, or the internal transcribed spacer (ITS) region for fungi (Galloway-Peña and Hanson 2020; Hamady and Knight 2009; Khayalethu 2013). These marker genes are specifically amplified using polymerase chain reaction (PCR), and the resulting amplicon products are subsequently sequenced (Galloway-Peña and Hanson 2020; Gilbert and Dupont 2011; Kuczynski et al. 2011). Random shotgun sequencing, on the other hand, uses the entire repertoire of genetic information in a sample to be sequenced, allowing simultaneous profiling of the presence as well as functional characteristics of bacteria, fungi, DNA-viruses, and other microorganisms (Galloway-Peña and Hanson 2020; Gilbert and Dupont 2011). Shotgun sequencing data can be analysed in different bioinformatic ways (Sharpton 2014). On the one hand, a simple read-based analysis can be performed, where the obtained reads are compared against different reference databases (Galloway-Peña and Hanson 2020; Quince et al. 2017). On the other hand, an assembly can be created on the basis of the reads, whereby a distinction is made here between a *de novo* assembly and a *reference-based* assembly (Wajid and Serpedin 2016). The advantage of assembly over read-based analysis is that longer sequence segments, so-called *contigs*, are formed during assembly (Ghurye et al. 2016). Thus, extended genome regions can be analysed or, even better, whole genomes can be obtained (Lapidus and Korobeynikov 2021). Sequences with low frequency, on the other hand, are lost during assembling (Nayfach and Pollard 2016).

Metatranscriptomic methods analyse the total RNA content of the microbiome sample, which has been previously extracted, enriched, and transcribed into cDNA for sequencing (Kukurba and Montgomery 2015; Kulski 2016; Moran et al. 2013; Santana et al. 2016; Shakya et al. 2019). This method can be used to determine not only the taxonomy of transcriptionally active organisms, but also the functional activity by measuring expressed transcripts within a microbiome at a specific time and under the respective environmental conditions (Galloway-Peña and Hanson 2020; Lavelle and Sokol 2018; Shakya et al. 2019; Solbiati and Frias-Lopez 2018). In addition, comparisons between different environmental conditions help to determine

which pathways might be up- or down-regulated (Galloway-Peña and Hanson 2020; Oshlack et al. 2010).

In addition to the methods mainly used in this thesis, further methods are available. Metaproteomics is used to characterize the protein expression of a microbial community under a given set of environmental conditions at a specific time point (Bastida et al. 2009; Sajulga et al. 2020; Vanwonterghem et al. 2014). It is important to note that, although the end product of mRNA are proteins, it has been shown that the transcriptome is not linearly proportional to the proteome, likely due to additional levels of cellular localisation and regulation at the protein level that are not captured by RNA measurements, such as post-translational modifications or controlled proteolysis (Hettich et al. 2013; Langley et al. 2013). However, in a metaproteome analysis, proteins are extracted from a microbial community, fractionated, separated by chromatography, and then detected by mass spectrometry to constitute most functional aspects of cellular metabolism (Hettich et al. 2013; Langley et al. 2013; Vanwonterghem et al. 2014). Metabolomics is another approach to study metabolic profiles in biological systems in response to environmental stimuli (Lankadurai et al. 2013; Lin et al. 2006). To do so, naturally occurring, low-molecular organic metabolites within a cell are usually analysed by either nuclear magnetic resonance spectroscopy or mass spectrometry (Bundy et al. 2009; Spratlin et al. 2009). All in all, metabolomics can provide insights into the cellular response of microorganisms to specific environmental stressors at the metabolic level, such as metal toxicity, or provide information about the state or conditions of the environment under study (Bundy et al. 2009; García-Sevillano et al. 2015; Lankadurai et al. 2013; Viant 2009; Viant and Sommer 2013; Zhang et al. 2021). Another way to characterise the metabolic capabilities of a microbial community are methods based on stable isotopes or radioactively labelled substances (Vanwonterghem et al. 2014). For stable isotope-based methods, selected substrates are labelled with heavy stable isotopes (mainly ^{13}C, ^{15}N), which then are subsequently assimilated by the microorganisms (Berry and Loy 2018; Hungate et al. 2015; Uhlik et al. 2013). The assimilation of the substrate used can be analysed via various techniques. On the one hand, the uptake of the labelled substrate can be visualised using imaging techniques such as Secondary ion mass spectrometry (SIMS) techniques, e.g. nanoSIMS, that enables visualisation with fine spatial resolution of single cells (Musat et al. 2012; Watrous and Dorrestein 2011). On the other hand, organisms of a microbial community that have incorporated the heavy isotope of the labelled compound into biomarkers such as DNA or RNA during microbial growth can be identified by nucleic acid sequencing after being separated by a density gradient formed during isopycnic centrifugation (Haichar et al. 2016; Hungate et al. 2015; Radajewski et al. 2000).

2. Microbiome and hygienic relevance of selected objects in the Built Environment

Within private households, there are several inanimate surfaces or appliances with which humans interact directly or indirectly almost every day, and each of these objects harbours complex microbial communities (Stephens et al. 2019). In the domestic environment, dishwashers, refrigerators, washing machines, laundry, and kitchen sponges can act as microbial hotspots (Novak Babič et al. 2020). Objects such as these are constantly inoculated with new microorganisms through their use, e.g. through handling food or direct physical contact, and microbial growth is promoted due to the environmental conditions prevailing there, such as moisture or high nutrient availability (Cardinale et al. 2017; Donofrio et al. 2012). However, because of this, such objects can represent a domestic source of (potentially) pathogenic microorganisms and cross-contaminations but are mostly not perceived as such by consumers (Donofrio et al. 2012; Marotta et al. 2018). The following section of this dissertation takes a closer look at washing machines, laundry, and kitchen sponges.

2.1. The washing machine and laundry items

Doing laundry is one of the most common household activities, and the number of washing machines in households in developed countries is generally high (Pakula and Stamminger 2010). Recently, it was estimated that up to 96 % of German households own a washing machine and up to 4 kg of laundry is washed per person per week in German households (Ellmer et al. 2017).

The main goal of doing laundry is to obtain clean and fresh clothes, free from dirt, soil, pathogens, and odours. The efficiency of laundry processes is controlled by four factors which form the so-called Sinner's Circle for Laundry and Cleaning: chemistry, mechanics, temperature, and time, with water as the connecting factor (Abeliotis et al. 2015; Ellmer et al. 2017; van Herreweghen et al. 2020). In case of the washing machine, these factors are represented by: washing temperature, type and quantity of the detergents used, mechanical action of the washing drum, duration of the washing cycles, and the water source (Alborzi et al. 2017). In any case, as shown in Figure 4, the reduction of one factor has to be compensated by the other factors in order to achieve the same cleaning effect (Basso et al. 2017).

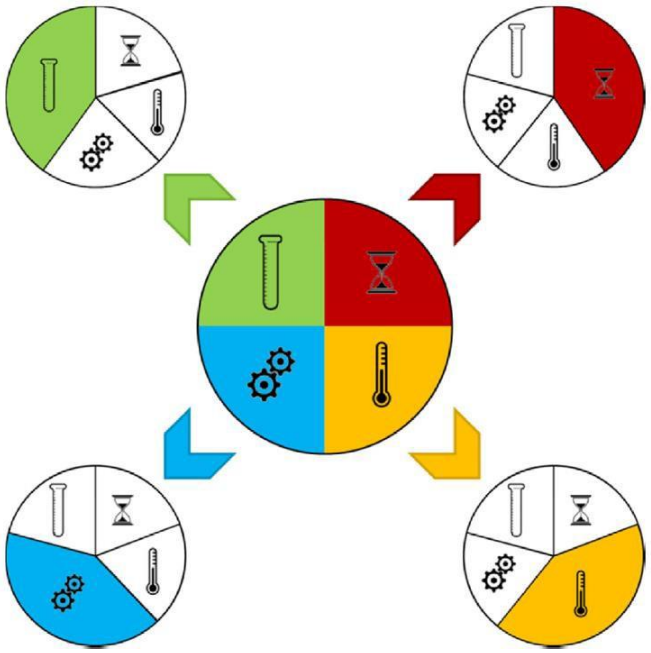

Figure 4. General representation of Sinner's circle. Factors that make up the Sinner Circle are mechanics (blue), temperature (orange), chemistry (green), and time (red). The picture also visualises how individual factors of the Sinner circle must change when one factor is increased (Basso et al. 2017).

Washing processes have a significant impact on the environment, consuming over 5.5 billion kWh of electricity and 380 million m^3 of water as well as 600,000 t of detergents in Germany annually (Ellmer et al. 2017). Due to this significant consumption of energy, water, and chemicals, modern washing trends try to reduce the energy demand and make washing processes more environmentally friendly and sustainable (Alborzi et al. 2017; Bao et al. 2017). Adaptations to the washing process include, for example, a reduced energy consumption by using low-temperature washing programmes, a reduced water consumption by eliminating one or two rinse cycles, as well as changes in detergent chemistry to avoid water pollution, such as the replacement of tripolyphosphates, addition of bleach activators or an increased use of biodegradable surfactants (Ivanković and Hrenović 2010; Reynolds et al. 2021; Terpstra 2001). Nevertheless, such adaptations can negatively affect laundry hygiene (Egert 2017). For instance, lowering the washing temperature can result in a higher number of surviving microorganisms due to lower thermal inactivation and insufficient activation of the added bleaching agents, whose antimicrobial efficacy has its optimum at 60 °C (Bloomfield et al. 2015; Bockmühl et al. 2019). In addition, reducing the number of rinses with lower amounts of water can lead to an insufficient draining of microorganisms, which are released from the fabric during the wash (Aiello et al. 2008; Terpstra 2001).

2.1.1 The microbiome of (domestic) washing machines

Microorganisms enter a washing machine mainly through worn and soiled textiles and clothing, water, and the ambient air (Hanson et al. 2016; Leung et al. 2014; Nix et al. 2015; Ren et al. 2015). On the one hand, washing machines offer favourable conditions for microbial growth, such as a warmth, humidity, and nutrients. On the other hand, microorganisms have to adapt to rapidly changing conditions, such as humidity/desiccation, high/low temperatures, neutral/alkaline pH values and the presence/absence of tensides, bleach, and other aggressive chemicals (Novak Babič et al. 2020; Savage et al. 2016). To survive under such conditions, flexible gene expression is vital for survival and enables adaptation, for example to sudden shifts in nutrient availability or pH (Donlan and Costerton 2002; Filippis et al. 2021; Jefferson 2004). One particularly important example of adaptation through systematic gene expression is the formation of biofilms (Gupta et al. 2016; O'Toole et al. 2000). Here, microbial cells live in homogeneous or heterogeneous populations, which are embedded in extracellular polymeric substances that are secreted by the constituents of the biofilm (Cortés et al. 2011; Gupta et al. 2016; O'Toole et al. 2000). More importantly, the formation of biofilms ensures that nutrients and water are bound to the specific location and help microorganisms to withstand, for example, alternating wet and dry periods, frequent temperature changes during wash cycles, or oxidative cleaning agents (Raghupathi et al. 2018). One reason for the resilience of biofilms is a top-down nutrient and oxygen gradient associated with reduced bacterial metabolic activity and thus increased bacterial cell doubling time or even dormancy, which can lead to reduced susceptibility to fluctuating conditions and antimicrobials (Gostinčar et al. 2022; Høiby et al. 2010). Most importantly, it has been shown that the growth of biofilms is associated with an increased frequency of mutations, e.g. through horizontal gene transfer, which could increase infectivity or cause resistance to certain antibiotics (Foster 2007; Galhardo et al. 2007; Høiby et al. 2010; Mangalappalli-Illathu and Korber 2006).

Inside a washing machine, biofilms are mainly formed at the water-surface interface on plastic, metal, or rubber components (Raghupathi et al. 2018). As a result, microorganisms accumulate on these machine components, such as door seal, detergent drawer, or sump, which are predominantly components with stagnant water or components which are neglected or difficult to reach and are therefore not cleaned regularly (Donlan and Costerton 2002; O'Toole et al. 2000; van Herreweghen et al. 2020). The formation of biofilms leads to unaesthetic plaques, e.g. in the detergent drawer or the door seal, and fosters a constant recontamination of laundry and the regularly cleaned machine parts, as well as an impairment of the machine's service life

by promoting, for example, the corrosion of metal parts (Callewaert et al. 2015; Egert 2017; Raghupathi et al. 2018; Ren et al. 2015; Zalar et al. 2011). Furthermore, microbial colonisation might cause machine and laundry malodour (van Herreweghen et al. 2020). In addition, the biofilms in washing machines could serve as a reservoir for pathogens (Egert 2017).

Several studies, so far, analysed the composition of microbial biofilms inside domestic washing machines (Altenbaher et al. 2011; Babič et al. 2015; Callewaert et al. 2015; Honisch et al. 2014; Nix et al. 2015; Ossowski and Duchmann 1997; Wiksell et al. 1973; Teufel et al. 2010; Stapleton et al. 2013). Nix and co-workers, for instance, demonstrated the prevalence of prokaryotic and eukaryotic microorganisms inside biofilms from washing machines using single-gene studies based on the 16S ribosomal RNA gene for bacteria and the ITS region for fungi (Nix et al. 2015). They showed that *Proteobacteria* are the dominant bacterial colonizer, while *Basidomycota* and *Ascomycota* are the main fungal representatives (Nix et al. 2015). In additional studies, *Acinetobacter*, *Bacillus*, *Brevundimonas*, *Clostridium*, *Corynebacterium*, *Escherichia*, *Micrococcus*, *Pseudomonas*, and *Staphylococcus* were identified as typical bacterial colonisers of washing machines, and *Candida*, *Fusarium, Aspergillus,* and *Exophiala* species as typical fungal representatives (Babič et al. 2015; Novak Babič et al. 2020; van Herreweghen et al. 2020). The microorganisms in a machine also show specific spatial distribution patterns that probably depend on the particular microenvironments at a given site (Babič et al. 2015; Nix et al. 2015). For instance, it has been proposed that fungi primarily prevail inside the door seal, while bacteria dominate the detergent chamber (Nix et al. 2015). In addition, it was shown that the microbial community composition is variable and influenced by various factors. Callewaert and colleagues identified the skin microbiome of the user, as well as the water source, as important influencing factors (Callewaert et al. 2015).

The extent to which consumer behaviour, including chosen washing temperature, detergent type, or regular cleaning, might additionally influence the structure and functionality of washing machine microbial communities has not yet been adequately elucidated (van Herreweghen et al. 2020).

2.1.2 The microbiome of laundry

When clothes are worn, the fabric comes into direct contact with the skin. It was shown that microorganisms associated with textiles are broadly similar to those of the microbiome of human skin (Danko et al. 2021). Various studies so far have identified *Staphylococcus* sp.,

Micrococcus sp. as well as *Streptococcus* sp., *Bacillus* sp., *Enterobacteriaceae,* and *Acinetobacter* sp. or corynebacteria and cutibacteria as common members of the textile microbiome (Callewaert et al. 2014; Callewaert et al. 2015; Danko et al. 2021; McQueen et al. 2007; Teufel et al. 2010). In addition to skin microorganisms, environmental microorganisms, e.g. from dust, soil, and food, can also be found on the fabrics, depending on the soiling or the use of the textile (Bockmühl et al. 2019; Licina and Nazaroff 2018; Nkiwane 2014; Pace-Asciak et al. 2018).

Adherence of microorganisms to the fabric fibres depends mainly on the microbial species itself but also on the fibre compositions and the bacteria-fabric contact condition, such as time, surface morphology, or hydrophobicity (An and Friedman 1998; Hsieh and Merry 1986; Hsieh et al. 1987; Takashima et al. 2004; Teufel et al. 2010). For example, Hsieh and colleagues showed that staphylococci adhere much better to cotton, polyester, and their blends than *Escherichia coli* (Hsieh and Merry 1986). Additionally, it was also found that extending the bacteria-fabric contact time gives bacteria more opportunities to attach to the fabric (Bajpai et al. 2011; Hsieh and Merry 1986).

Interestingly, a study by Rayner (2004) has shown that adherent microorganisms can form biofilms in and on the textile after wearing or using it and use transferred substances such as sweat, body fluids, sebum, or bacterial metabolic products as nutrients, from which they can also produce odour-forming substances, as it has been demonstrated for *Staphylococcus epidermidis* (Chung and Seok 2012; Rayner et al. 2004; van Herreweghen et al. 2020). In addition, further studies have shown that some microorganisms, such as *Staphylococcus aureus* or vancomycin-resistant enterococci, survived for up to 90 days on five common hospital materials, however depending on the initial amount of inoculation (Neely and Maley 2000). Furthermore, the survivability of microorganisms on textiles is also influenced by temperature, relative humidity, organic content, and deposition method (Neely 2000; Pandekar and Gurav 2019; Yeargin et al. 2016). For example, it is assumed that the higher survival rate of microorganisms on cotton compared to polyester or silk is partly due to the ability of the different fabrics to absorb and retain moisture (Colclasure et al. 2015; Riley et al. 2017). Silk, for example, as a densely woven fabric with very few pores, absorbs less liquid than cotton, which has many pores (Colclasure et al. 2015).

2.1.3 Hygienic relevance of laundry and washing machines in domestic environments

In general, laundering of clothes is meant to remove visible and invisible soiling and stains after the textile was used to ensure aesthetic fitness for reuse of the textile (Fijan et al. 2005; Terpstra 1998). Additionally, it is also performed to remove odorous substances and microorganisms from clothing to prevent transmission and reexposure to pathogens (Abney et al. 2021).

Altogether, the laundering process does not only consist of the washing process itself but is made up of several steps, starting with collection of the laundry and extending to the washing of the laundry, removal of the laundry from the machine, drying, and storage of the freshly washed laundry (Abney et al. 2021; Reynolds et al. 2021). As shown in Figure 5, each step is influenced by a number of factors that affect the potential removal of microorganisms from textiles during the washing process (Abney et al. 2021).

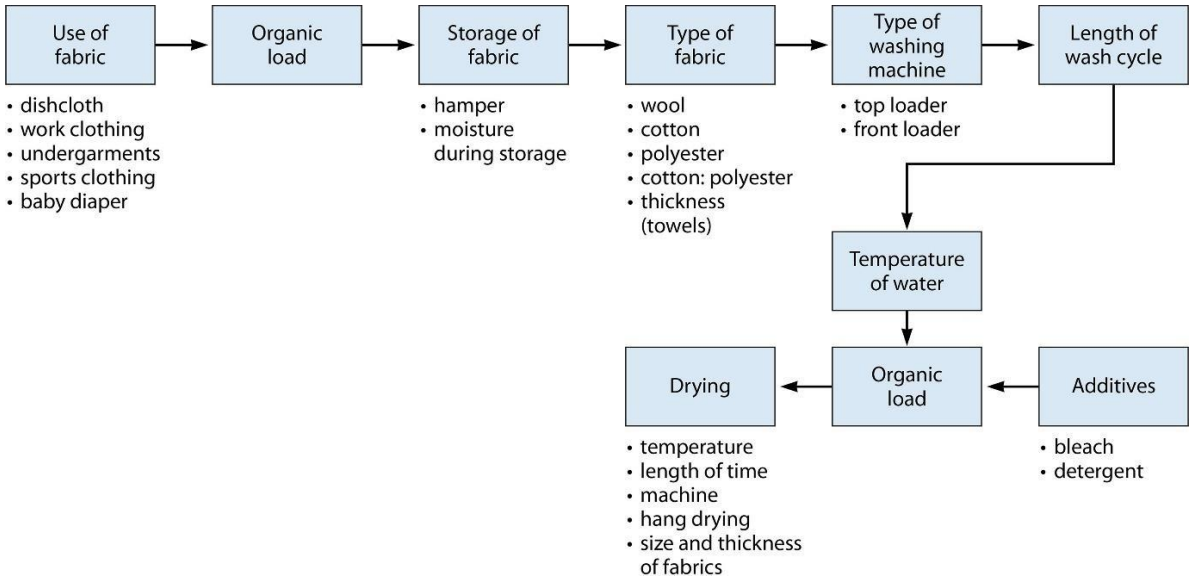

Figure 5. Influencing variables in hygienic washing. Displayed are factors that can affect the removal of microorganisms throughout the washing process (Abney et al. 2021).

For example, it has been shown that bacteria can be transferred from laundry, especially wet laundry, to hands or other surfaces during loading or unloading of the machine, folding, or final storage of the texiles (Fijan et al. 2005; Sattar et al. 2001; Scott and Bloomfield 1990). The release of microorganisms via the air when handling soiled clothing can contribute to airborne exposure of, for example, *Staphylococcus* spp., cutibacteria, corynebacteria, *Lactobacillus* spp., and *Streptococcus* spp. (Handorean et al. 2015). As a result, the handling of clothing might

contribute to cross-contamination and the transfer of microorganisms to other members or areas of the household (Handorean et al. 2015; Heudorf et al. 2017; Owen and Laird 2020).

Besides that, washing clothes in the washing machine can further contribute to the spread of microorganisms (Callewaert et al. 2015). In general, during the washing process a microbial reduction occurs through mechanical, chemical, and physical processes (Terpstra 2001). For example, it is estimated that compared to plain washing the use of a detergent can reduce bacterial contaminations by 88.9 % (Gibson et al. 1999). The further use of detergents containing activated oxygen bleach (AOB) can increase the reduction by up to one or more log levels. Furthermore, it was also determined that rinse cycles probably contribute to about one log reduction, each (Bloomfield et al. 2013). All in all, a machine wash can reduce the microbial load by three to six log levels, depending strongly on the interaction of other factors such as temperature, detergent formulation, wash cycle duration, or number of rinse cycles (Bloomfield et al. 2017).

However, if the washing conditions are changed towards more sustainability, e.g. by using less water and lower temperatures, the cleaning performance is in any case lower than with washing programmes that use higher temperatures (Cunliffe et al. 1988; Shin et al. 2020; Terpstra 2001). For instance, it has been suggested that the release of microorganisms from fabrics is more effective at higher temperatures (50 °C versus 30 °C) due to stronger convection currents (Ainsworth and Fletcher 1993). In addition to the physical effects, the washing temperature also influences microbial viability (Honisch et al. 2014; Savage et al. 2016). For example, Shin and co-workers demonstrated a higher bacterial viability of *Acinetobacter baumannii* and *Staphylococcus aureus* on cotton samples after washing at low temperature without AOB compared to high temperature (> 60 °C), which achieved a reduction rate of 99.9 % when washed without AOB (Shin et al. 2020).

Consequently, sustainable washing trends hamper a reduction of microorganisms but facilitate the survival of microorganisms in the washing machine, which in turn can accumulate on various surfaces and form biofilms (Nix et al. 2015). This may have a negative impact on hygiene, as it is assumed that biofilms can act as a reservoir for pathogens (Gibson et al. 1999). In addition, biofilms can also lead to constant re-contamination of laundry and regularly cleaned machine parts as well as an unpleasant smell of the machine and laundry or contribute to the corrosion of metal parts (Callewaert et al. 2015; Egert 2017).

In addition to biofilm formation, several studies demonstrated a microbial exchange and a mixing of microorganisms stemming from different kinds of sources, e.g. the incoming water,

skin, soiled clothing, and biofilms from inside the washing machine, which presumably contaminate all textiles of a washing load (Callewaert et al. 2015; Hammer et al. 2011). Such processes can contribute to the transmission of microorganisms through the textiles themselves in direct contact with skin or through handling after washing (Hammer et al. 2011). This can be a serious problem in case of people with a compromised immune system, such as young children or elderly people (Oosterom 1998; Shin et al. 2020). For example, Schmithausen and colleagues identified an (irregularly used) household washing machine as the reservoir and the woollen laundry washed in it as the vector of a multidrug-resistant *Klebsiella oxytoca* strain infecting newborns in a paediatric ward (Schmithausen et al. 2019). Earlier, Kundsin demonstrated the risk of cross-contamination from household laundry by describing an outbreak of *Staphylococcus aureus* skin infections among families sharing the same laundry facilities (Kundsin 1966). Pathogens that have further been proven to be transmitted via laundry comprise fungal dermatophytes (yeasts and moulds) or bacteria like *Salmonella* (Bloomfield et al. 2011; Ossowski and Duchmann 1997).

All in all, in a "healthy" household, the risk of infection through washed laundry is low (Bloomfield et al. 2011). However, if there are susceptible people in the household, special antimicrobial strategies should be applied (Egert 2017). Reynolds and co-workers attempted to quantify the risk of infection from washed laundry and summarized special laundry handling strategies in case of illness or special situations, displayed in Table 1 (Reynolds et al. 2021).

Table 1. Overview of the procedure recommended by Reynolds and co-workers for washing at different hygienic conditions. Special laundry treatment for the best possible hygienic cleaning ordered according to possible special situations that may occur in a household. (Reynolds et al. 2021)**.** (*Continued on the next page*).

Situation	Healthy households with light staining and bodily soiling	Healthy households with heavy staining and bodily soiling	Households with suspected or confirmed respiratory infections including COVID-19, influenza or the common cold	Households with confirmed or suspected enteric infections (‘stomach bugs’) Households with persons who have a weakened immune system Households with healthcare workers and first responders; laundering work clothes at home
General guidance	Standard laundry process using quality detergents provides adequate hygiene for every-day laundering	Active households with heavier soiling can benefit from higher quality detergents (characterized by multiple types of surfactants and enzymes) to deeply clean stains and body soil residues from textiles	Special precautions should be taken when handling contaminated clothes and bedding as per the CDC guidelines, but sanitizers are not needed to remove respiratory viruses	Sanitizers and/or the sanitizing cycle on the washing machine should be used during certain illness or special situations, but they should be used in combination with higher quality detergents to first remove deeply embedded soils, and should not be used for everyday cleaning
Special handling precautions	Wash hands after handling soiled laundry and transferring wet laundry from washer to dryer; avoid contact between contaminated surfaces and soiled laundry	Wash hands after handling soiled laundry and transferring wet laundry from washer to dryer; avoid contact between contaminated surfaces and soiled laundry	Wear disposable gloves when handling dirty laundry (clothes and bed linens) from a person who is sick; Dirty laundry from a person who is sick can be washed with other people's items. Do not shake dirty laundry; Clean and disinfect	Work clothes should be removed before entering the domestic environment; Wear disposable gloves when handling dirty laundry (clothes and bed linens) from a person who is sick**;** Dirty laundry from a person who is sick

			clothes hampers and contact surfaces; Remove gloves, and wash hands immediately; Wash hands again after transferring wet laundry from washer to dryer	should not be washed with other people's items; Do not shake dirty laundry; clean and disinfect clothes hampers and contact surfaces; Remove gloves, and wash hands right away; wash hands again after transferring wet laundry from washer to dryer
Recommended products	Regular (low surfactant; no enzymes) or high quality (high surfactant; enzymes) laundry detergent	Higher quality detergents (multiple types of surfactants and enzymes)	High or higher quality detergents	High-quality laundry detergent PLUS Registered Sanitizer or Sanitizing Cycle on the washing machine
Washing frequency	Wash as needed	Wash frequently	Wash as soon as possible	Wash as soon as possible
Washer settings	Regular detergent Cold water for most clothes; use warm/hot water for socks, underwear, sheets, and towels, and cleaning cloths; high-quality detergent: wash at any temperature, including cold water	Wash at any temperature, including cold water	Use the warmest water setting allowed by the care label	Hottest water allowed by the care label; follow registered sanitizer product instructions or washing machine instructions for the sanitizing cycle
Dryer setting	Medium heat; allow clothes to dry completely before storing	Low to medium heat; allow clothes to dry completely before storing	Medium to high heat; dry completely before storing	Highest heat setting allowed by the care label; dry completely before storing

Besides the health aspect, insufficient cleaning can also have other consequences. For instance, residual organic load such as remaining biofilms in the fabric, can, even if no longer active or viable, retain malodorous compounds, such as isovaleric acid or be the cause of discolouration of textiles due to microbial pigments (Mayer et al. 2021; Rayner et al. 2004; Reynolds et al. 2021). In addition, the residual organic load can serve as an additional source of nutrients for new microorganisms that are introduced to the textile when it is worn again, which therefore can lead to an increased microbial growth and contribute to the formation of malodour as shown for example, for *Staphylococcus epidermidis* (Chung and Seok 2012; Møllebjerg et al. 2021).

Hence, the life cycle of a textile is limited by the resulting loss of quality and comfort, as repeated wear can lead to an accumulation of biomass and eventually permastink (Møllebjerg et al. 2021; van Herreweghen et al. 2020).

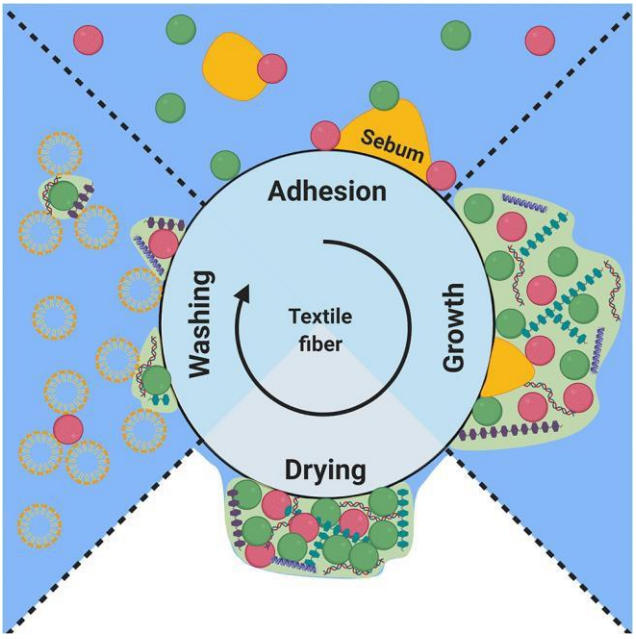

Figure 6. The life cycle of a bacterial biofilm on a textile fiber. Visualisation how bacteria repeatedly pass through constantly changing and periodic phases of attachment, growth, drying, and washing (Møllebjerg et al. 2021).

2.2 Kitchen sponges

Kitchen sponges represent the most frequently used cleaning utensil in domestic kitchens (Lagendijk et al. 2008; Mattick 2003; Turgay and Erbilir 2005). They are usually used for washing and scrubbing dishes and other kitchen utensils, such as pans and casseroles, but also for cleaning kitchen surfaces, such as sinks, refrigerators, or stove-tops (Lagendijk et al. 2008;

Møretrø et al. 2021). Today, kitchen sponges fabricated from different materials are commercially available, e.g. polyurethane or cellulose (Gerba et al. 2017; Nibedita et al. 2020). Both bind food residues or soils to the sponge during cleaning and, together with the retained moisture, create a favourable environment for microbial growth (Cardinale et al. 2017; Nibedita et al. 2020). Consequently, several studies so far have recognized kitchen sponges as a major fomite in the spread of microorganisms in domestic kitchens leading to foodborne illnesses and cross-contaminations of kitchen appliances (Donofrio et al. 2012; Turgay and Erbilir 2005).

2.2.1 Microbiome of kitchen sponges

As a cleaning tool, kitchen sponges can be contaminated by food itself, contaminated water, or by washing contaminated surfaces or dishes (Borneff et al. 1988; Mattick 2003; Møretrø et al. 2021).

Microorganisms absorbed in this way can reach up to $5.4 * 10^{10}$ cells per cm^3 sponge tissue, and remain stable thereafter (Cardinale et al. 2017; Evans and Redmond 2019; Hilton and Austin 2000; Ikawa and Rossen 1999; Møretrø et al. 2021; Rossi et al. 2013). More importantly, the varying pore size of a kitchen sponge allows for a wide distribution of microorganisms inside it, which naturally leads to a multi-level partitioning of the microbial community (Wu et al. 2022). Furthermore, these microorganisms can form biofilm-like clusters of microbial cells within the multiporous sponge tissue (Cardinale et al. 2017).

In addition to determining the microbial load, several cultural and molecular studies examined the microbial composition of kitchen sponges and found a wide diversity of mostly harmless environmental bacteria, but also opportunistic pathogenic bacteria, as well as yeasts and moulds (Cardinale et al. 2017; Hassan and El-Bagoury 2017; Turgay and Erbilir 2005; Wolde and Bacha 2016). In different studies, predominant bacteria such as *Pseudomonas*, *Bacillus*, *Micrococcus*, *Streptococcus,* and *Lactobacillus* as well as *Brevundimonas*, *Rhizobium*, *Chrysobacterium,* and *Sphingobium*, but also *Salmonella*, *Campylobacter,* and *Klebsiella* were identified (Borrusso and Quinlan 2017; Cardinale et al. 2017; Hassan and El-Bagoury 2017; Møretrø et al. 2021; Osaili et al. 2020; Wolde and Bacha 2016; Chaidez and Gerba 2000). The studies have also shown that pathogenic bacteria represent only a small fraction of all bacteria (Cardinale et al. 2017; Møretrø et al. 2021). In addition, microbial diversity was found to be influenced by the type of sponge, food handling, and food preparation habits in the household, such as cooking preferences or storage conditions (Chaidez and Gerba 2000; Møretrø et al.

2021). For example, Gerba and colleagues showed that polyurethane sponges contained 50 % fewer total bacteria and 99.9 % less *Escherichia coli* than cellulose sponges (Gerba et al. 2017).

2.2.2 Hygienic relevance of kitchen sponges in domestic environments

Since many foods are contaminated with microorganisms, kitchens are areas of particular importance for human health (Boer and Hahné 1990; Francis and O'Beirne 2001; Mattick 2003). Some studies, which analysed food poisoning outbreaks in England and Wales, estimated that 16 % of outbreaks could be linked to meals prepared in private homes (Cowden et al. 1995). Furthermore, according to the European Food Safety Authority (EFSA), one in three foodborne infectious diseases was related to the home environment (European Food Safety Authority and European Centre for Disease Prevention and Control 2018). However, due to the fact that mild private outbreaks do not have to be reported, the number of unreported outbreaks may be even higher (Redmond and Griffith 2009). Although mostly caused by improper handling of food, a considerable proportion of these diseases, however, were caused by cross-contaminations (Davies 1952; Evans et al. 1998).

Studies have shown that humid places in kitchens are environments where microorganisms are most likely to survive or grow (Flores et al. 2013). Kitchen sponges can be important vehicles for contamination transfer (Ikawa and Rossen 1999; Taché and Carpentier 2014). On the one hand, they come in contact with potentially contaminated surfaces or food during several kitchen activities, such as washing up contaminated dishes or wiping kitchen surfaces that came in contact with contaminated food (Mattick 2003). On the other hand, they can contain a relatively large amount of water even after 24 hours of drying time and therefore offer ideal growth conditions for microorganisms that may form biofilms (Cardinale et al. 2017; Møretrø et al. 2021). More importantly, sponges can become reservoirs for obligate pathogens (Cardinale et al. 2017). For example, Møretrø and co-workers could show that, once absorbed, bacteria such as *Campylobacter* can survive for one day in sponges, while *Salmonella* can even survive for more than seven days (Møretrø et al. 2021).

In any case, the use of a microbially contaminated sponge can lead to (re)contamination of kitchen utensils and surfaces (Beumer and Kusumaningrum 2003). Especially with regard to pathogens, this can pose a major health risk due to the usually low infectious dose of certain pathogens, such as the aforementioned *Campylobacter* or human noroviruses (Gibson et al. 2012; Humphrey 2002). In addition, cross-contaminations might also be a cause for food spoilage (Biranjia-Hurdoyal and Latouche 2016; Huis in't Veld 1996).

To reduce microbial contamination of kitchen sponges, several physical or chemical methods are proposed (Ikawa and Rossen 1999). These methods include, for example, the addition of hypochlorite or a dishwashing detergent to the kitchen sponge, but also cleaning by means of boiling or putting the sponge into the dishwasher, washing machine, or microwave oven (Ikawa and Rossen 1999; Park et al. 2006; Sharma et al. 2009). Several studies, e.g. by Ikawa and colleagues and Sharma and colleagues, investigated the effectiveness of the different cleaning methods for kitchen sponges and could prove that microwave radiation, emitted by a microwave oven, can effectively reduce the bacterial load of artificial contaminated kitchen sponges by five to seven log scales (Ikawa and Rossen 1999; Sharma et al. 2009).

In any case, applied in household conditions, each sanitisation method shows different efficacies in reducing microorganisms, particularly in relation to the presence of food residues that can adversely affect them and lead to a rapid recolonization of the sponge tissue by the microorganisms that have survived the sanitisation (Cardinale et al. 2017; Kusumaningrum et al. 2002; Ikawa and Rossen 1999; Sharma et al. 2009). Therefore, knowledge about the survival of pathogenic bacteria in kitchen sponges in conjunction with consumer practices is necessary to provide risk-mitigating advice to consumers (Møretrø et al. 2021).

3. Aim of the presented studies

Since washing machines, laundry and kitchen sponges are hygienically highly relevant objects in the domestic environment, that can influence human health and well-being, the aim of the presented studies was to significantly expand existing knowledge about the biodiversity and metabolism of microbial communities in these domestic microhabitats and contribute to a deeper, consumer-relevant and especially more function-oriented characterisation of their microbiomes.

To achieve this, culture-independent 16S rRNA amplicon sequencing was used to link consumer behaviour to species diversity and taxonomic composition at different sites in household washing machines, such as door seal, detergent drawer, sump, and textile fibres. The user-dependent factors selected for this analysis included the usage of liquid detergents, machine age, the perception of malodour from machine or washed textiles, the average number of washing cycles per month at ≥ 60 °C, as well as the use of fabric softener, whether mainly a powder or a liquid detergent was used for washing, and if the machine was cleaned regularly.

Additionally, culture-dependent quantification of microorganisms on different components of the washing machine, e.g. the detergent drawer and the rubber seal of the door, and the subsequent identification of several morphotypes based on MALDI-TOF should also provide more information on the actual microbial load of the different washing machine components and estimate the pathogenic potential of the isolates to allow for a better risk assessment of washing machines.

In addition to the microbial composition and the factors that control it, this work also aimed to investigate and characterize the most important metabolic activities of microorganisms of washing machines, laundry, and kitchen sponges. For this purpose, it was necessary to establish more function-oriented methods (metagenomics, metatranscriptomics, metabolomics, and SIP experiments) for these research subjects in order to gain deeper insights into the functionality of microbial communities in the domestic environment and the variables that can have an effect on them. For example, metatranscriptomic methods should be used for the first time to study the "activity" of microorganisms on washed textiles and to determine whether there are differences in microbial gene expression between washed cotton and polyester fabrics. Metagenomic shotgun sequencing, on the other hand, should be used to investigate the impact of microwave treatment on community composition and metabolic gene potential in used kitchen sponges to determine the extent to which cleaning processes such as microwave treatment may have an impact on microbial diversity and whether metabolic capabilities differ between treated and untreated kitchen sponges

Overall, new insights into important metabolic activities of microorganisms could be helpful in providing a scientific basis for the development of new and more sustainable strategies for household hygiene.

4. Influence of Sampling Site and other Environmental Factors on the Bacterial Community Composition of Domestic Washing Machines

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Influence of Sampling Site and other Environmental Factors on the Bacterial Community Composition of Domestic Washing Machines

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Abstract: Modern, mainly sustainability-driven trends, such as low-temperature washing or bleach-free liquid detergents, facilitate microbial survival of the laundry processes. Favourable growth conditions like humidity, warmth and sufficient nutrients also contribute to microbial colonization of washing machines. Such colonization might lead to negatively perceived staining, corrosion of washing machine parts and surfaces, as well as machine and laundry malodour. In this study, we characterized the bacterial community of 13 domestic washing machines at four different sampling sites (detergent drawer, door seal, sump and fibres collected from the washing solution) using 16S rRNA gene pyrosequencing and statistically analysed associations with environmental and user-dependent factors. Across 50 investigated samples, the bacterial community turned out to be significantly site-dependent with the highest alpha diversity found inside the detergent drawer, followed by sump, textile fibres isolated from the washing solution, and door seal. Surprisingly, out of all other investigated factors only the monthly number of wash cycles at temperatures ≥ 60 °C showed a significant influence on the community structure. A higher number of hot wash cycles per month increased microbial diversity, especially inside the detergent drawer. Potential reasons and the hygienic relevance of this finding need to be assessed in future studies.

Keywords: washing machines; bacterial diversity; biofilms; amplicon sequencing; hygiene

1. Introduction

Today, washing machines are one of the most common household tools targeting household hygiene. The German Federal Statistical Office determined that 96% of German households own a washing machine [1]. Due to this wide distribution, laundering clothes is one of the most widespread housework [2].

The cleaning efficiency of a washing machine is depending on the mechanical circulation of the washing drum, the flooding of the fabric with water and detergents, as well as an appropriate washing time and water temperature [3–5]. Mainly for environmental reasons, the washing process has been adapted to sustain energy in order to conserve resources and reduce costs [6]. Sustainable washing

trends include washing at lower temperatures, a reduced water consumption and an increased use of bleach-free liquid detergent [7]. However, from a hygienic point of view, these adjustments negatively affect laundry hygiene by facilitating the survival of microorganisms inside the washing machine and on the washed laundry [8].

Recent studies dealing with the antimicrobial effectiveness of modern washing processes showed that microorganism, which mainly enter the machine through worn clothing or water, were reduced, but not sufficiently killed during low temperature wash cycles [8,9]. Surviving microorganism remain inside the washing machine and either attach to different kinds of surfaces or get distributed over the wash load during the wash cycle [10]. The latter might pose a potential risk of infection to members of the household, if pathogens are involved, such as dermatophyte fungi, *Staphylococcus aureus* or *Escherichia coli* [11].

Bacteria bound to surfaces are commonly surrounded by polymeric substances, which is a main characteristic of a biofilm [12]. Biofilms can be formed on almost every surface in an aqueous environment [13]. The formation of biofilms increases microbial tolerance against mechanical, physical and chemical stress [14]. For example, it allows a protected growth, ensures a reduced diffusion rate of toxic components and strengthens the attachment and expansion capabilities of the existing community [14,15]. In addition, the occurrence of many different species at a given site might increase interspecies communication and cross-feeding and positively affect biofilm biomass [16,17]. For the consumer, however, washing machine biofilms are unpleasant, as they are causing a constant recontamination of laundry and regularly cleaned machine parts, malodour of machine and laundry, the formation of unaesthetic plaques and contribute to the corrosion of metallic components [7,9,18]. Finally, they might represent a reservoir for pathogens [19].

In order to characterize the microbial colonization of domestic washing machines, several studies analysed the microbial community of household washing machines and its potential influence on laundry [5,9,20–26]. For instance, Nix and co-workers [23] addressed the prevalence of prokaryotic and eukaryotic microorganisms at the rubber door seal and the detergent drawer using pyrosequencing of ribosomal RNA and ITS genes. The influence of environmental factors on microbial diversity, however, was not considered here. Stapelton and co-workers [24] investigated potential causes of malodour formation inside the washing machine and their effect on laundry odour. Finally, Callewaert and co-workers [9] showed that bacteria from different sources get significantly mixed during a wash cycle. Interestingly, they also suggested that laundry and washing machine microbial communities might even affect the skin microbiota of their users.

In order to better understand the microbiology of domestic washing machines, our study focused on the influence of selected environmental factors on the bacterial community composition of these widely used items. We hypothesized that factors, such as sampling site, significantly shape community composition. In order to test this hypothesis, we analysed the bacterial community at four different sampling sites using 454-pyrosequencing as a cultivation-independent technique and searched for associations of community composition with selected environmental and user-specific factors.

2. Material and Methods

2.1. Sample Collection

In the course of this study, 21 in-use domestic washing machines were examined, stemming from private households, either in the area of Villingen-Schwenningen or Waldshut-Tiengen, Germany. Sterile cotton swabs (Deltalab, Rubí, Spain), premoistened in physiological (0.9%) saline solution, were used for taking surface samples of three sampling sites, i.e., detergent drawer (drawer and chamber), door seal and sump. After sampling, the swaps were transferred into a sterile reaction tube and kept at −20 °C until further analysis. In addition to swab samples, fibres released from a wash load into the washing solution were also examined. To do so, machines were loaded with worn cotton laundry and a wash cycle was started at 30 °C with ca. 30 mL of liquid detergent (Persil Universalgel

Henkel, Düsseldorf, Germany). After half of the washing cycle (ca. 30 min), the machine was stopped, and washing solution was collected into a sterile 50 mL reaction tube and stored at −20 °C until further processing.

2.2. Factors that may Influence Bacterial Diversity in Washing Machines

A survey was issued to the machine owners to gain more information about potential environmental factors affecting the bacterial community composition. Consumers voluntarily and anonymously provided information about the age of the machine, the average number of washing cycles per month at ≥60 °C, the perception of malodour from the machine or washed textiles, as well as the use of fabric softener and the use of powder or liquid detergent. We also asked if a regular cleaning of the machine was done. To simplify the data, factors yielding a wide range of information, such as the age of the machine or the number of wash cycles per month at ≥60 °C were grouped into two categories, each.

2.3. DNA-Extraction

Textile fibres from washing solution samples were collected by centrifugation for 5 min at 121× g. Afterwards, the supernatant was discarded, and the pellet was resuspended in 500 µL of PCR-water (Sigma-Aldrich, Hamburg, Germany). DNA from the swap heads and textile fibres was isolated using the FastDNA Spin Kit for Soil and a FastPrep Instrument (both from MP Biomedicals, Eschwege, Germany) using an adjusted protocol including a reduced centrifugation force of 12,100× *g* using a MiniSpin centrifuge (Eppendorf, Hamburg, Germany), as well as an additional protein precipitation step inserted immediately after the one described in the protocol. At the end, the genomic DNA was eluted in 100 µL of DNase/ Pyrogen-free water. Extractions from blank swabs did not yield sufficient DNA for downstream analyses.

2.4. PCR and Clean Up

To determine the bacterial community composition, amplicon sequencing based on the 16S rRNA gene was applied. Barcoded amplicons were prepared using universal bacterial primers extended with the respective A or B adapters, a key sequence and a multiplex identifier (MID) sequence [27]. Pyrotag PCR was done using modified ba27f (5′-CGT ATC GCC TCC CTC GCG CCA TCA TCA-MID-Sequence-GAG TTT GAT CMT GGC TCA G-3′) and ba519r (5′-CTA TGC GCC TTG CCA GCC CGC TCA-MID-Sequence-ATT ACC GCG GCT G-3′) primers (Metabion international AG, Martinsried, Germany). Final concentrations for PCR amplification mix were 1× Dream-*Taq*-reaction buffer, 2 mM magnesium chloride, 0.2 mM PCR-nucleotide mix, 1.25 U Dream-*Taq*-polymerase (all from Thermo Fisher Scientific, Waltham, USA), 0.2 µg/µL bovine serum albumin (Roche, Penzberg, Germany), 0.5 µM of each primer and 2 µL DNA template in a final volume of 50 µL. The DNA was amplified using a T-personal thermocycler (Biometra, Göttingen, Germany) with the following thermal profile: 95 °C for 4 min for initial denaturation, followed by 28 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C and elongation for 60 s at 72 °C followed by a final elongation for 5 min at 72 °C. Correct amplicon size was verified by gel electrophoresis on 1% agarose gels and ethidium bromide staining. PCR products were purified using the NucleoSpin Gel and PCR Clean up kit (Macherey–Nagel, Düren, Germany) with the MiniSpin centrifuge (Eppendorf, Hamburg, Germany) according to the manufacturer's protocol, using Tris-HCl-buffer (5 mM, pH 8.5) for elution of DNA. Amplicon concentration and purity were measured with a NanoPhotometer P360 (Implen, München, Germany).

2.5. Pyrosequencing

16S rRNA genes amplicons from 50 samples stemming from 13 different machines were sequenced. From one machine only the door seal and detergent drawer yielded sufficient amplicons. The selected amplicon samples were delivered to Eurofins MWG Operon (Ebersberg, Germany) for

454-pyrosequencing using the GS Junior System and the Titanium sequencing kit (both from Roche, Mannheim, Germany).

2.6. Bioinformatic and Statistical Analyses

The obtained pyrosequencing data were analysed using QIIME version 1.9.1 [28]. First, the sequences were quality filtered using a quality threshold of 25. Then, the sequences were assigned to their respective samples according to their unique barcode sequence. Reads from forward and reverse primer were merged into one data set and chimeric sequences were removed using the VSEARCH method against the SILVA data base (release SILVA_128_QIIME_release) [29,30]. The remaining sequences were clustered de novo using UCLUST [31] into operational taxonomic units (OTUs) at 97% sequence similarity threshold. Representative sequences were aligned with PyNAST [32] and taxonomy was assigned using the SILVA data base. OTUs from plastids and mitochondria were subsequently removed from the data set. Further processing of the data was done using R version 3.5.3 [33] and RStudio version 1.1.463 [34] with the phyloseq package version 1.26.1 [35] and its additional packages, especially vegan (version 2.5.4) [36]. In a pre-processing step of data analysis, singletons were removed from the data set, followed by rarefaction to the minimal sequence count of all samples. In order to describe the microbial community composition, the overall relative abundance as well as the relative abundance of the OTUs at the respective sampling sites was calculated. The four most common indices (Observed, Chao1, Shannon and Simpson) were used to determine alpha diversity. A subsequent Analysis of Variance (ANOVA) was used for statistical analysis of the influence of the recorded factors on alpha diversity. Differences in beta diversity were visualised by principal component analysis (PCoA) of weighted and unweighted UniFrac measures. We used Analysis of Similarities (ANOSIM) and Permutational Multivariate Analysis of Variance (PERMANOVA) with 9999 permutations to check whether samples show statistically significant differences in community structure at the different sampling sites. Kruskal-Wallis analysis was done to analyse significant differences in bacterial community composition at the different sampling sites. Using the Wilcoxon-Mann-Whitney-U test for independent samples, we investigated the influence of the number of wash cycles per month ≥60 °C on community composition for each sampling site. p-values were adjusted for multiple testing by calculating the False Discovery Rate (FDR) using the Benjamini and Hochberg method [37]. p-values < 0.05 were regarded as statistically significant. Data were visualized using ggplot2 (version 3.1.0) [38]. To further identify the ten relatively most abundant OTUs per sampling site at species level, we performed a pairwise alignment using EzBioCloud (https://www.ezbiocloud.net/) [39] against a database of 16S rRNA gene sequences (EzBioCloud App: 16S-based ID, September 2019). Identified OTUs were classified into risk groups according to the German Technical Rules for Biological Agents (TRBA) #466 [40]. All sequence data were deposited at the European Nucleotide Archive (ENA) under the accession number PRJEB35498.

3. Results

3.1. General Bacterial Community Composition

454-pyrosequencing of the 16S rRNA gene amplicon library resulted in a total number of 110,751 raw sequences from the 50 samples, stemming from 13 domestic washing machines. After length/quality filtering, a total of 57,563 high quality forward reads and 44,564 high quality reverse reads were received. These data sets were combined and chimeric sequences (18,042) were removed. The remaining 81,206 sequences were then clustered de novo into 9211 OTUs that shared a 97% sequence similarity threshold. Further removal of mitochondrial and chloroplastic OTUs yielded 7080 bacterial OTUs, representing a total of 77,996 high quality sequences with 353–6802 sequences per sample (mean of 1560 reads per sample). After removal of singletons (4150), the whole data set was rarefied to 242 sequences per sample. Finally, 16 phyla, 36 classes, 67 orders, 124 families, 214 genera

and 229 species-like OTUs were determined as components of the bacterial community inside the investigated washing machines.

At phylum level, Proteobacteria (85.8%) was by far the dominating phylum, followed by Actinobacteria (5.3%), Firmicutes (3.0%), Bacteroidetes (2.9%) and Acidobacteria (1.1%). At class level, most sequences were affiliated with Gammaproteobacteria (57.8%), followed by Alphaproteobacteria (17.5%) and Betaproteobacteria (10.3%). Further common classes inside the washing machines were Actinobacteria (5.2%), Bacilli (2.7%), Flavobacteria (2.2%) and Blastocatellia (1.1%), whereas the main identified orders were Pseudomonadales (50.9%), Rhizobiales (9.8%) and Burkholderiales (8.3%). Within the family level, most bacteria belonged to Pseudomonadaceae (30.9%), Moraxellaceae (21.5%), and Comamonadaceae (7.0%). The predominant genera could be identified as Pseudomonas (34.3%), Acinetobacter (17.4%) and Enhydrobacter (6.5%).

3.2. Site-Dependent Bacterial Community Composition

Differences in bacterial diversity were investigated by alpha diversity using observed OTUs, Chao1, Shannon and Simpson as parameters (Table 1).

All diversity indices showed significant differences across the sampling sites (ANOVA: $p_{\text{Observed}} = 6.5 \times 10^{-4}$, $p_{\text{Chao1}} = 5.6 \times 10^{-3}$, $p_{\text{Shannon}} = 9.3 \times 10^{-4}$, $p_{\text{Simpson}} = 3.8 \times 10^{-3}$). The highest alpha diversity was found for the detergent drawer, followed by the fibres isolated from the washing solution, and the sump. The lowest alpha diversity was found inside the door seal.

In order to visualize differences in community structure between the different sampling sites, principal component analysis using weighted und unweighted Unifrac measures was done (Figure 1). Samples that originated from the detergent drawer were clearly distinct from the sump, which in turn were different from the door seal or the fibre samples. A segregation of the samples from door seal and the fibres becomes visible at the unweighted Unifrac distances, whereas the weighted analysis showed an overlay. The statistical analysis by means of PERMANOVA ($p = 1 \times 10^{-4}$ for unweighted Unifrac and weighted Unifrac) and ANOSIM (unweighted Unifrac: R = 0.4; weighted Unifrac: $R = 0.3$, $p = 1 \times 10^{-4}$ for unweighted Unifrac and weighted Unifrac) showed that the structure of the bacterial community at the sampling sites was significantly different.

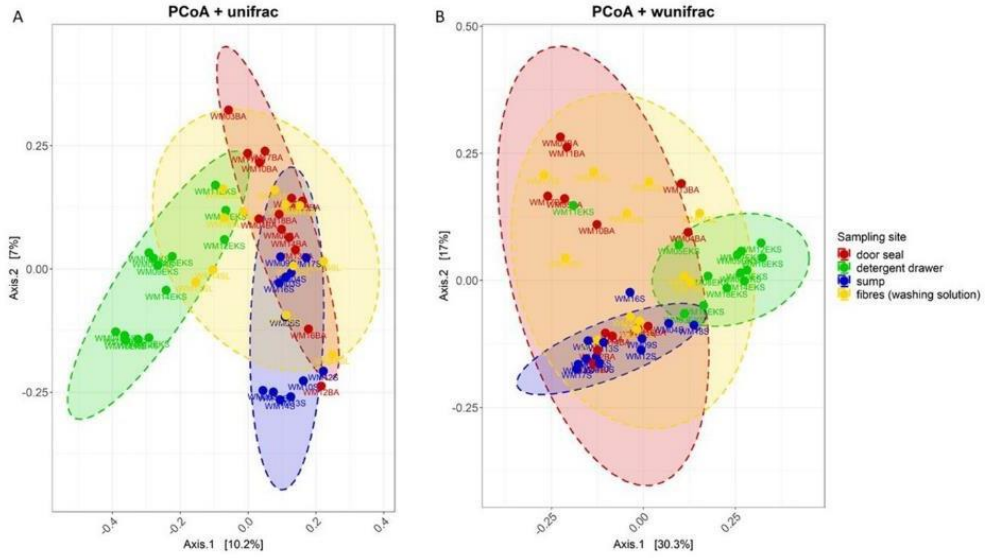

Figure 1. Principal Coordinate Analysis (PCoA) plot of unweighted (A) and weighted (B) UniFrac measures using the 16S rRNA gene sequencing data of 50 analysed washing machine samples. Colour indicates sampling site: door seal (red), detergent drawer (green), sump (blue) and fibres from washing solution (yellow). Ellipses correspond to 95% confidence intervals for each of the four sampling sites.

Table 1. Summary of the distribution of alpha diversity indices for the investigated factors potentially influencing the bacterial community of washing machines. Factors that had a significant influence are highlighted in grey. Displayed are mean values, minimum and maximum value as well as standard deviation of each diversity index.

Influencing Factor	Levels	n	Observed				Chao1				Shannon				Simpson			
			Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
Site	Door seal	13	44.54	15.50	22.00	77.00	85.44	46.88	25.00	189.80	2.57	0.63	1.88	3.59	0.83	0.09	0.70	0.96
	Detergent drawer	13	73.77	18.49	46.00	103.00	141.64	51.03	48.15	246.00	3.56	0.46	2.87	4.24	0.94	0.03	0.89	0.98
	Sump	12	53.08	16.47	25.00	74.00	90.37	33.98	34.17	154.09	2.89	0.66	1.77	3.59	0.87	0.09	0.71	0.95
	Fibres (washing solution)	12	66.08	14.08	44.00	83.00	129.83	48.74	60.62	216.00	3.22	0.50	2.08	3.81	0.90	0.07	0.73	0.96
Age	0–10 years	34	58.85	20.96	22.00	103.00	113.79	55.82	25.00	246.00	3.03	0.73	1.83	4.24	0.88	0.09	0.70	0.98
	11–20 years	16	60.44	16.74	25.00	97.00	107.85	39.38	34.17	171.88	3.14	0.53	1.77	4.07	0.90	0.06	0.71	0.97
Smell	Yes	10	62.44	20.70	28.00	97.00	123.02	54.57	32.00	216.00	3.11	0.72	1.83	4.24	0.89	0.08	0.72	0.98
	No	40	58.80	17.31	32.00	97.00	105.52	40.28	49.50	156.91	3.13	0.58	1.97	4.07	0.90	0.08	0.70	0.97
Standard wash cycles per month ≥60 °C	1–5 cycles	18	51.56	18.10	22.00	83.00	93.69	50.01	25.00	216.00	2.77	0.61	1.83	3.69	0.85	0.08	0.70	0.94
	6–10 cycles	32	63.75	19.21	25.00	103.00	122.13	49.07	34.17	246.00	3.23	0.65	1.77	4.24	0.90	0.08	0.71	0.98
Regular Cleaning	Yes	20	62.95	20.69	25.00	97.00	118.60	49.36	32.00	216.00	3.14	0.70	1.77	4.24	0.89	0.08	0.71	0.98
	No	30	56.97	18.72	22.00	103.00	107.41	52.09	25.00	246.00	3.01	0.65	1.97	4.10	0.88	0.08	0.70	0.97
Softener	Yes	16	62.44	20.70	28.00	97.00	123.02	54.57	32.00	216.00	3.11	0.72	1.83	4.24	0.89	0.08	0.72	0.98
	No	34	57.91	19.12	22.00	103.00	106.65	48.89	25.00	246.00	3.04	0.65	1.77	4.10	0.88	0.08	0.70	0.97
Detergent	Liquid	18	57.78	13.61	32.00	85.00	109.06	37.93	49.50	171.88	3.03	0.54	1.97	4.03	0.89	0.08	0.70	0.97
	Powder	32	60.25	22.36	22.00	103.00	113.48	57.30	25.00	246.00	3.08	0.74	1.77	4.24	0.88	0.09	0.71	0.98

Consequently, also the distribution of the different taxa was found to be highly site-dependent (Figure 2), in particular the phylum of Proteobacteria (Kruskal-Wallis: $p = 8.1 \times 10^{-4}$). Proteobacteria were found across all sampling sites, but in case of the door seal and the sump, this phylum accounted for 94.2% and 96.9% of all sequences, respectively, while the proportion in the detergent drawer (76.3%) and on the fibres from the washing solution (75.8%) was significantly lower. Firmicutes (Kruskal- Wallis: $p = 8.8 \times 10^{-3}$), however, were mainly found on the fibres isolated from the washing solution (9.3%) and in the door seal (2.2%). Furthermore, the relative abundance of Actinobacteria also depended strongly on the sampling site (Kruskal-Wallis: $p = 0.02$). Here, we found frequencies of around one to two percent in the sump and the door seal. The washing solution fibres and the detergent drawer on the other hand showed relative abundances of ~9%. In addition to the most common phyla, other phyla also showed significant differences between sampling sites. For instance, the phyla Planctomycetes (Kruskal-Wallis: $p = 8.1 \times 10^{-4}$), Chloroflexi, (Kruskal-Wallis: $p = 8.8 \times 10^{-3}$) and Acidobacteria (Kruskal-Wallis: $p = 3.7 \times 10^{-3}$) were found mainly in the detergent drawer but rarely at the other sampling sites.

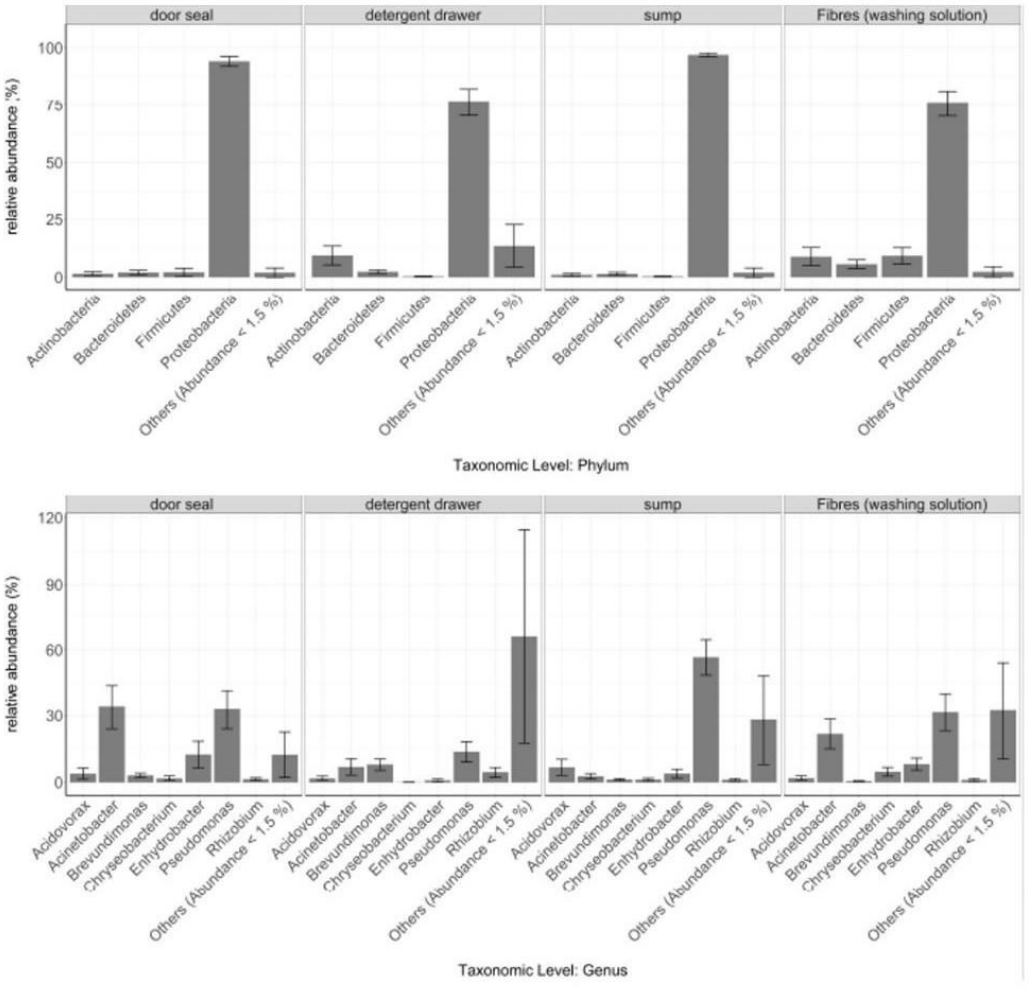

Figure 2. Relative abundances of the most abundant microbial phyla and genera at the different sampling sites. Only taxa with an overall relative abundance of ≥ 1.5% are shown Taxa with an overall relative abundance ≤ 1.5% were summed up as "Others". Data are expressed as mean ± standard error (Door seal $n = 13$, Detergent drawer $n = 13$, Sump $n = 12$, Washing solution $n = 12$).

At genus level, the genera Pseudomonas (Kruskal-Wallis: $p = 9.8 \times 10^{-3}$), Acinetobacter (Kruskal-Wallis: $p = 0.01$) and Enhydrobacter (Kruskal-Wallis: $p = 0.03$) were found at all sampling sites. However, their relative abundances varied greatly. For instance, the relative abundance of Pseudomonas in the detergent drawer (13.7%) was much lower compared to the sump (56.7 %). On the other hand, the relative abundance of this genus for door seal (32.9%) and fibres (31.4%) was similar. In contrast, Enhydrobacter occurred mostly in the door seal (12.5%) and on the textile fibres (8.2%) but only barely in the detergent drawer (0.8%). Acinetobacter, in turn, occurred more often in the door seal (34.0%), followed by the washing solution fibres (21.7%). Its relative abundance, however, was significantly lower in the detergent drawer (6.9%) and the sump (2.7%).

In order to further identify the ten most abundant OTUs per site at species level, we calculated sequence similarity against the 16S rRNA gene sequences database from EzBioCloud (Table A1). Notably, this analysis clearly revealed that the OTUs previously identified as Enhydrobacter showed a sequence similarity of 100% to the species *Moraxella osloensis*.

Significant fractions (30–60%) of the 10 relatively most abundant OTUs per sampling site could be categorized as closely related to potentially pathogenic species based on the German TRBA #466, and many of these OTUs were detected at the majority of the investigated sites. For instance, OTUs closely related to *Moraxella osloensis* were detected in up to 6 sump, 8 fibre and 9 door seal samples (Table A1).

3.3. Effect of Environmental Factors on Community Composition

In addition to the clear effects of sampling site on bacterial community composition, we investigated the effects of further parameters with a potential influence on microbial community composition. Unexpectedly, the performed ANOVA analysis revealed that only the number of wash cycles per month at ≥60 °C seemed to have an impact on the microbial diversity ($p_{\text{Observerd}} = 0.04$, $p_{\text{Chao1}} = 0.06$, $p_{\text{Shannon}} = 0.04$, $p_{\text{Simpson}} = 0.04$). Surprisingly, there was a trend towards a higher alpha diversity with an increased number of wash cycles ≥60 °C compared to a lower number of wash cycles at high temperature (Table 1). Furthermore, we also examined at which sampling site this factor had the strongest effect on microbial diversity. Figure 3 shows that there was a significantly higher alpha diversity in the detergent drawer from machines which undergo 6–10 washing cycles per month at ≥60 °C. At the other sampling sites, no clear influence of this parameter was seen.

Beta diversity revealed no clear differences between a higher and lower number of wash cycles at temperatures above ≥60 °C using PCoA or ANOSIM and PERMANOVA (data not shown). We therefore compared the relative abundances of single taxa between a high and a low number of wash cycles above 60 °C. A significant difference between a high and low numbers of wash cycles ≥60 °C was seen for the order of Xanthomonadales (Wilcoxon: $p = 7.3 \times 10^{-3}$). Its relative abundance increased with a higher number of wash cycles ≥60 °C from 0.6% to 4.8%. At the genus level, a borderline significant difference was determined for Paracoccus (Wilcoxon: $p = 0.05$). Its relative abundance increased from 0.2% at 1–5-wash cycles to 1.8% at 6–10 high-temperature wash cycles per month. Also, the minor abundant genera Kocuria, Dysgonomonas, Massilia (Wilcoxon: each $p = 0.03$) differed significantly between these two conditions.

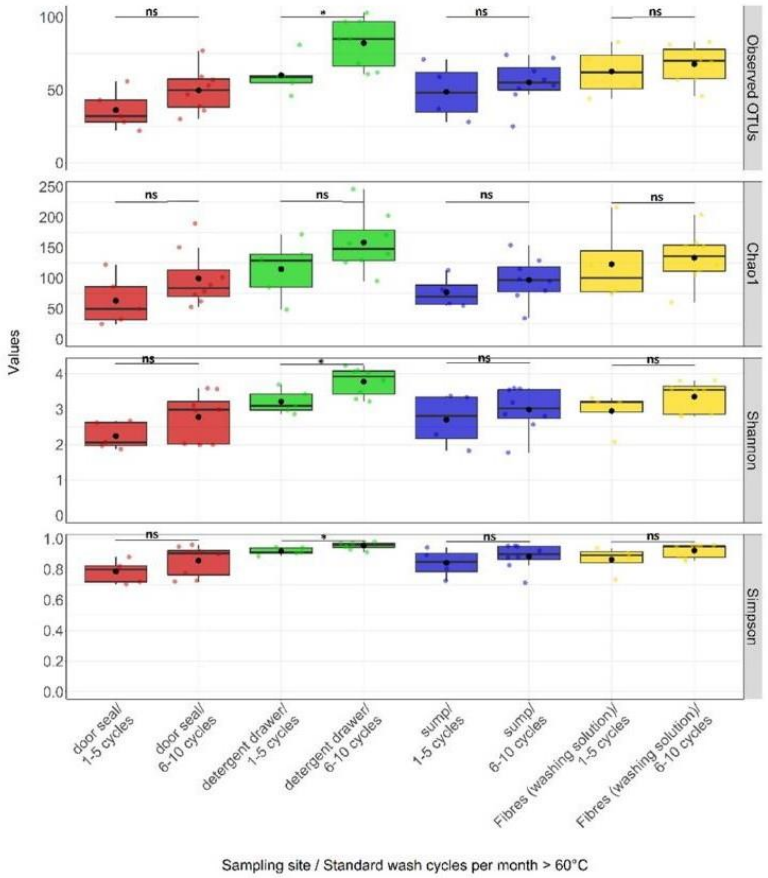

Figure 3. Box-whisker plots for the distribution of alpha diversity measures comparing sampling sites by standard wash cycle per month at temperatures ≥60 °C. Samples were categorized by sampling sites and the number of standard wash cycle per month at temperatures ≥60 °C. A box represent the 25% and 75% percentiles. The middle of the box represents the median. Mean values are displayed as black dots. The horizontal dashes above and below the boxes indicate the largest and smallest values, which were not classified as outliers. Colour indicates sampling site: door seal (red), detergent drawer (green), sump (blue) and fibres from washing solution (yellow). Coloured dots represent an individual sample. Statistical analysis was done using the Wilcoxon-Mann-Whitney-U tests for independent samples. *p*-values are indicated by asterisks, $p < 0.05$ (*) and ns = not significant.

4. Discussion

One of the main objectives of our study was to identify potential factors influencing microbial diversity in domestic washing machine. In order to achieve this, we examined 13 different household washing machines at four different sampling sites by means of 454-pyrosequencing for their bacterial community composition and statistically analysed associations with different environmental and user specific factors.

4.1. The structure of the Bacterial Community Differs between Various Sampling Sites

Using different alpha diversity parameters, we compared the specific sampling sites and were able to determine the highest alpha diversity with a high evenness for the detergent drawer and the lowest diversity for the door seal with a relatively low evenness, which corroborates findings by Nix and colleagues [23]. Similar to our study, they also identified Proteobacteria, Actinobacteria and Bacteroidetes as the main phyla in washing machines. However, we additionally examined the sump

and fibres collected from the washing solution, which extends knowledge of the core microbiome in washing machines. Firmicutes were relatively most abundant on textile fibres from the washing solution, which seems likely as Firmicutes are typical representatives of the human skin microbiota, such as staphylococci [41,42]. Diversity and species richness of sump and fibres were between the values determined for detergent drawer and door seal.

Local conditions probably play a decisive role for bacterial community composition in washing machines and may select for polyextremotolerant bacteria [43]. For example, bacteria in the detergent drawer must be particularly tolerant to the ingredients of the detergent, such as bleach, surfactants, perfumes or enzymes, and alkaline components [43,44]. In contrast, bacteria within the door seal need to handle high organic loads caused by the washed objects, alternating phases of dry and very wet conditions and changing pH values [21].

The relative abundances clearly show that some bacteria are restricted to certain sites, such as the aforementioned phylum of Firmicutes. In contrast, Proteobacteria were found in all analyzed sampling sites. Proteobacteria are known to be the most common bacteria in drinking water [45,46] and tap water serves as a means of transportation throughout all components of the machine. Accordingly, the genus Pseudomonas, also very typical for drinking water [47,48], was found at all sampling sites.

Genera such as Moraxella and Acinetobacter are members of the human skin microbiome and probably enter the machine mostly with dirty laundry [49,50]. Since the water flow in the machine is unidirectional and the washing solution does not come into contact with the detergent drawer, these bacteria were only rarely detected there. Our analyses down to species level suggest that the different sampling sites of a washing machines are not only populated by harmless environmental bacteria, but also by potentially pathogenic ones, in particular *Acinetobacter spp.* and *Moraxella osloensis*. For healthy people these bacteria are rather harmless. However, in new-borns, pregnant women, elderly persons or other immunocompromised subjects they might lead to infections [51–53].

Moraxella osloensis was also identified as a cause of malodor on laundry [8,24,54]. In particular *Moraxella osloensis's* ability to tolerate desiccation and a metabolic pathway to produce 4-methyl-3-hexenoic acid are considered key factors for survival and malodor formation in washing machines and on washed laundry [55]. Using a detergent containing disinfecting agents or bleach is recommended to control malodor formation [54]. In addition, machine parts in direct contact with laundry, that are prone to the growth of malodor producers, should be cleaned on a regular basis. In our study, an OTU closely related to *Moraxella osloensis* (sequence similarity: 100%) was relatively most abundant on the door seals. Hence regular cleaning here appears particularly suited to control this bacterium and its ability to produce malodour. Unfortunately, quantitative data on the bacterial colonization of washing machines is scarce, and in our study only relative abundances of different taxa were determined. However, in a small study Stapleton and colleagues [24] clearly showed that bacterial cell numbers of sump and rubber seal were several log scales higher than cell numbers of washing drum and drawer. Therefore, it can be carefully speculated that the prominent occurrence of *Moraxella osloensis* in the door seal samples of our study is indeed of quantitative relevance.

Finally, it is noteworthy that during the OTU identification down to species level a misclassification was revealed. OTUs classified by QIIME as Enhydrobacter were clearly identified as Moraxella, more precisely *Moraxella osloensis*, by pairwise alignment to the EzBioCloud database. The same misclassification was demonstrated before [56].

4.2. Factors Influencing Bacterial Community Composition

In addition to the influence of the sampling sites, we also investigated other, user-dependent factors. Unexpectedly, only the number of wash cycles at ≥60 °C significantly influenced the community composition, while factors such as the age of the machine or regular cleaning measures did not, at least when based on our data set.

When comparing machines with different number of washing cycles per month at temperatures ≥60 °C, an effect on alpha diversity, but not on beta diversity was seen. Different alpha diversity

parameters revealed a tendency towards an increase in bacterial diversity at 6–10 washing cycles per month at ≥60 °C compared to a number of 1–5 washing cycles. This effect was only observed for the detergent drawer, but not for sites with direct contact to the heated water. Therefore, this positive effect on bacterial diversity might be caused by heat radiation from the washing drum to the surrounding components, where it might stimulate microbial growth. However, it cannot be excluded that other factors, not recorded in this study, were responsible for this observation, such as the general number of washing cycles at both low and high levels. It might be speculated that households with a higher number of washing cycles at 60 °C or higher per month also perform more washings at lower temperatures, which in turn may influence microbial diversity.

5. Conclusions

Domestic washing machines are colonized by a diverse bacterial community probably affecting laundry hygiene. The bacterial community is dominated by taxa of water and human origin. Bacterial diversity is strongly site-dependent and shaped by the local environmental conditions. Some of the identified bacterial species here are categorized as potentially pathogenic species, that might be transmitted through laundry and cause infections in immunocompromised humans. We also demonstrated that the bacterial community composition in the detergent drawer might be influenced by the number of wash cycles per month at temperatures ≥ 60°C. Potential reasons and the hygienic relevance of this finding need to be assessed in future studies.

Clearly, such follow-up studies should not only be based on relative amplicon frequencies of 16S rRNA genes but include both quantitative (cultivation-based cell numbers, qPCR-based gene numbers) as well as more functional oriented (transcriptomic, proteomic, metabolomic) analyses in order to better understand the microbiology of domestic washing machines and its hygienic relevance.

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Conflicts of Interest: The authors declare no conflict of interests.

Appendix A

Table A1. Ten relatively most abundant OTUs per sampling site. The ten relatively most abundant OTUs per sampling site were determined and aligned against the 16S rRNA gene sequence database of EzBioCloud to calculate sequence similarities to known species. For each EzBioCloud match, sequence similarity and completness values are displayed. The identified top-hit taxa were also categorized into risk groups (RG) according to the German TRBA 466 [40]. SD = standard deviation. Positive samples = number of samples in which the OTU was detected.

Sampling Site	Relative Abundance (%)	SD	Positive Samples	OTU-ID	SILVA Genus	Top-hit taxon EzBioCloud	EzBioCloud Accession Number	Similarity (%)	Completeness (%)	RG (TRBA)
Door seal	12.1	14.8	11	denovo7218	Pseudomonas	*Pseudomonas oleovorans subsp. oleovorans*	NIUB01000072	100.00	33.3	1
	10.1	13.3	11	denovo5159	Pseudomonas	*Pseudomonas oleovorans subsp. oleovorans*	NIUB01000072	100.00	33.2	1
	7.2	14.6	8	denovo7377	Acinetobacter	JF232448_s	JF232448	98.97	33.3	-
	6.0	11.2	9	denovo6937	Enhydrobacter	*Moraxella osloensis*	APQL01000005	100.00	33.6	2
	4.1	13.5	2	denovo3157	Acinetobacter	*Acinetobacter beijerinckii*	APQL01000005	99.38	33.3	2
	3.8	6.7	7	denovo301	Enhydrobacter	*Moraxella osloensis*	CP014234	100.00	33.1	2
	3.6	8.3	7	denovo6136	Acinetobacter	*Acinetobacter parvus*	AIEB01000124	100.00	33.4	2
	3.4	8.9	5	denovo4753	Acinetobacter	*Acinetobacter parvus*	AIEB01000124	100.00	33.3	2
	3.1	7.3	5	denovo3836	Acidovorax	*Acidovorax radicis*	AFBG01000030	99.59	33.2	1
	2.7	5.9	7	denovo3896	-	*Rhizobium rosettiformans*	EU781656	98.91	32.6	1
Detergent drawer	2.5	6.6	4	denovo7377	Acinetobacter	JF232448_s	JF232448	98.97	33.3	-
	2.3	4.3	10	denovo15	Brevundimonas	*Brevundimonas vesicularis*	BCWM01000033	98.48	33.5	2
	2.1	4.5	4	denovo8752	-	*Pseudoxanthomonas mexicana*	AF273082	100.00	33.5	1
	1.9	7.0	1	denovo6661	uncultured	HQ856368_s	HQ856368	100.00	32.4	-
	1.9	5.3	3	denovo1071	Pseudomonas	*Pseudomonas aeruginosa*	BAMA01000316	99.00	34.2	2
	1.9	5.7	6	denovo5179	-	*Pseudoxanthomonas mexicana*	AF273082	99.20	34.6	1
	1.7	3.9	5	denovo7389	Rhizobium	*Rhizobium radiobacter*	AJ389904	98.47	32.8	1
	1.5	3.3	7	denovo2452	Brevundimonas	*Brevundimonas vesicularis*	BCWM01000033	100.00	31.4	2
	1.4	3.1	4	denovo8373	Aureimonas	*Aureimonas altamirensis*	BBWQ01000019	98.26	33.0	1
	1.4	4.9	1	denovo5170	Pseudomonas	*Pseudomonas avellanae*	AKBS01001374	100.00	33.4	1
Sump	20.1	14.0	12	denovo7218	Pseudomonas	*Pseudomonas oleovorans subsp. oleovorans*	NIUB01000072	100.00	33.3	1
	17.0	11.8	12	denovo5159	Pseudomonas	*Pseudomonas oleovorans subsp. oleovorans*	NIUB01000072	100.00	33.2	1
	4.7	9.0	5	denovo3836	Acidovorax	*Acidovorax radicis*	AFBG01000030	99.59	33.2	1
	2.6	5.0	5	denovo2699	Citrobacter	*Citrobacter freundii*	AJ233408	99.80	33.5	2
	2.3	3.0	8	denovo4149	-	*Diaphorobacter nitroreducens*	AB064317	99.59	33.3	1
	1.5	2.4	5	denovo7180	-	*Diaphorobacter nitroreducens*	AB064317	97.96	34.2	1
	1.5	2.9	6	denovo6937	Enhydrobacter	*Moraxella osloensis*	CP014234	100.00	33.6	2
	1.4	4.1	5	denovo213	Ochrobactrum	*Ochrobactrum anthropi*	CP000758	100.00	30.7	2
	1.2	2.6	5	denovo301	Enhydrobacter	*Moraxella osloensis*	CP014234	100.00	33.1	2
	1.2	2.0	4	denovo6665	-	*Kosakonia sacchari*	CP007215	99.80	33.4	-

Table A1. *Cont.*

Sampling Site	Relative Abundance (%)	SD	Positive Samples	OTU-ID	SILVA Genus	Top-hit taxon EzBioCloud	EzBioCloud Accession Number	Similarity (%)	Completeness (%)	RG (TRBA)
Fibres (Washing solution)	10.7	12.7	9	denovo7218	Pseudomonas	*Pseudomonas oleovorans subsp. oleovorans*	NIUB01000072	100.00	33.3	1
	10.3	12.4	10	denovo5159	Pseudomonas	*Pseudomonas oleovorans subsp. oleovorans*	NIUB01000072	100.00	33.2	1
	5.4	9.3	8	denovo7377	Acinetobacter	JF232448_s	JF232448	98.97	33.3	-
	3.0	3.9	9	denovo301	Enhydrobacter	*Moraxella osloensis*	CP014234	100.00	33.1	2
	2.9	4.9	5	denovo7076	Micrococcus	*Micrococcus aloeverae*	KF524364	100.00	32.3	-
	2.4	3.4	9	denovo2637	Acinetobacter	*Acinetobacter johnsonii*	APON01000005	98.97	33.4	2
	2.4	2.6	8	denovo6937	Enhydrobacter	*Moraxella osloensis*	CP014234	100.00	33.6	2
	2.1	3.7	6	denovo1609	Chryseobacterium	*Chryseobacterium hominis*	jgi.1096633	99.00	34.7	2
	2.0	6.9	1	denovo5400	Acinetobacter	*Acinetobacter junii*	APPX01000010	98.96	33.0	2
	1.8	2.7	7	denovo2396	Chryseobacterium	*Chryseobacterium hominis*	jgi.1096633	99.79	33.4	2

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5. Cultivation-Based Quantification and Identification of Bacteria at Two Hygienic Key Sides of Domestic Washing Machines

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Communication

Cultivation-Based Quantification and Identification of Bacteria at Two Hygienic Key Sides of Domestic Washing Machines

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Abstract: Detergent drawer and door seal represent important sites for microbial life in domestic washing machines. Interestingly, quantitative data on the microbial contamination of these sites is scarce. Here, 10 domestic washing machines were swab-sampled for subsequent bacterial cultivation at four different sampling sites: detergent drawer and detergent drawer chamber, as well as the top and bottom part of the rubber door seal. The average bacterial load over all washing machines and sites was $2.1 \pm 1.0 \times 10^4$ CFU cm^{-2} (average number of colony forming units ± standard error of the mean (SEM)). The top part of the door seal showed the lowest contamination ($11.1 \pm 9.2 \times 10^1$ CFU cm^{-2}), probably due to less humidity. Out of 212 isolates, 178 (84%) were identified on the genus level, and 118 (56%) on the species level using matrix-assisted laser desorption/ionization (MALDI) Biotyping, resulting in 29 genera and 40 identified species across all machines. The predominant bacterial genera were *Staphylococcus* and *Micrococcus*, which were found at all sites. 22 out of 40 species were classified as opportunistic pathogens, emphasizing the need for regular cleaning of the investigated sites.

Keywords: washing machine; bacteria; hygiene; MALDI biotyping

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1. Introduction

Representing wet, warm, and nutrient-rich environments, many sites of domestic washing machines offer ideal living conditions for microorganisms, such as bacteria and fungi [1,2]. Microbial contamination of washing machines might cause unaesthetic staining as well as malodor formation [3,4]. In addition, microbial biofilms might serve as reservoirs for (potentially) pathogenic microorganisms that might contaminate the laundry and thereby pose a health threat for susceptible persons [5,6].

Various studies have shown that washing machines are colonized by a considerable diversity of microbes, often capable of forming biofilms [3,7–10]. For instance, Nix and co- workers [10] investigated pro- and eukaryotic microorganisms on the rubber door seal and the detergent drawer using 16S rRNA gene and ITS1 region pyrosequencing. They identified taxa affiliated with *Proteobacteria* as the main bacterial representatives and *Basidiomycota* and *Ascomycota* representatives as the main fungal colonizers [10].

Regarding bacteria, washing machines are indeed mainly populated by the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* [7,9,10]. They largely enter the machine via soiled clothing, tap water, and maybe also air [2,4]. In a recent molecular study on the bacterial community of domestic washing machines, we identified the detergent drawer as the site with the highest bacterial diversity and the door seal as the site with highest relative abundance of malodor forming *Moraxella osloensis* species. Per site,

30–60% of the relatively most abundant sequence types were closely related to potentially pathogenic bacteria, such as *Brevundimonas vesicularis* or *Pseudomonas aeruginosa* inside the detergent drawer, and *Moraxella osloensis* or *Acinetobacter parvus* inside the door seal [9]. In a startling study, an antibiotic resistant *Klebsiella oxytoca* strain was recently isolated from biofilms of the detergent drawer and door seal of a domestic washing machine used for the woollen laundry of a paediatric hospital ward, from which it probably had colonized newborns [11].

Interestingly, quantitative data on the microbial contamination of different sites of domestic washing machines is scarce. To increase knowledge in this field, we aerobically cultivated and quantified bacteria from two sites of the detergent drawer and the door seal region of 10 domestic washing machines, each, and identified representative isolates by matrix-assisted laser desorption/ionization (MALDI) Biotyping.

2. Materials and Methods

2.1. Washing Machine Sampling

Swab samples were taken from 10 domestic (home-owned), front loading washing machines in the greater area of Villingen-Schwenningen, Germany, between April and June 2020. Each machine was sampled at the detergent drawer, the detergent drawer chamber, and the top and bottom parts of the rubber door seal. All tested machines were provided voluntarily by their owners. Similar sites of each machine were swapped with sterile cotton swaps (Deltalab, Rubí, Spain) pre-moistened in sterile physiological (0.9%) saline solution. The sampling area was ~42 cm^2 for the detergent drawer, ~28 cm^2 for the detergent chamber, and ~45 cm^2 for the upper and lower parts of the rubber door seal, respectively. After sampling, the swab heads were transferred to a sterile reaction tube containing 2 mL of sterile physiological saline solution. All samples were processed within 1 h after sampling.

2.2. Colony Counting

Colony counting was performed as previously described in König et al. [12] and Egert et al. [13] with minor modifications. Swab heads were vortexed for 1 min at maximum speed. After serial decimal dilution up to 10^{-6} with sterile physiological saline solution, 100 µL of each dilution were spread in duplicates on tryptic soy agar plates (TSA; Carl Roth Karlsruhe, Germany) and incubated under aerobic conditions for 48 h at 37 °C. Subsequently, colonies in the range of 3 to 300 colonies were counted, averaged, and used for the calculation of microbial loads per cm^2 of sample area.

One representative of each colony morphotype (differing in size, color, and/or colony morphology) per sample was picked with a sterile inoculation loop, re-streaked on TSA, and incubated aerobically at 37 °C. After control for purity, a colony from each morphotype was selected, dissolved in 300 µL of MALDI water (Honeywell, Offenbach, Germany), and stored at −80 °C for subsequent identification by MALDI Biotyping.

2.3. Identification of Isolates by MALDI Biotyping

The obtained isolates were identified with a MALDI Biotyper Microflex system (Bruker Daltonics, Bremen, Germany) according to the manufacturer's instructions. The protein extraction method was applied using ethanol/formic acid sample preparation [14]. 1 µL of the respective protein extract of each isolated colony was added to a spot on the Biotyper steel target plate. After air drying, the samples were overlayed with 1 µL MALDI-matrix solution (alpha-Cyano-4-hydroxycinnamic acid, Bruker Daltonics, Bremen, Germany). After further air drying, the samples were analyzed. The obtained mass spectra were compared against the internal MALDI Biotyper reference libraries: MBT Compass Library, revision F, v. 9, containing 8468 main spectra (MSPs); MBT Filamentous Fungi Library (revision No. 2, containing 468 MSPs); MBT Security Related Library (SR Library, revision No. 1; containing 104 MSPs). Matches with the respective spectra in the databases were displayed

as scores ranging from 0.0 to 3.0. Scores ≥ 1.7 indicated a secure genus identification and scores ≥ 2.0 a secure genus and probable species identification [15].

2.4. Statistical Analyses

The statistical analysis was performed using R (v. 3.6.1) [16] and R Studio (version 1.2.1335) [17] with the packages ggplot2 (v. 3.2.1) [18], reshape2 (v. 1.4.3) [19], and scales (v. 1.0.0) [20]. Non-parametric tests (Kruskal–Wallis rank sum test followed by Wilcoxon–Mann–Whitney post hoc tests) were used to check for statistical significance between the colony counts of the four sampling sites. p-values < 0.05 were considered as statistically significant.

3. Results and Discussion

3.1. Colony counts at the Different Sampling Sites

All investigated samples showed microbial growth. Microbial loads spanned five orders of magnitude (Figure 1). The average colony count over all samples was $2.1 \pm 1.0 \times 10^4$ colony-forming units (CFU) cm^{-2} (average ± standard error of the mean (SEM)). The sampling site with the lowest cell numbers was the top part of the rubber door seal (RDST, $11.1 \pm 9.2 \times 10^1$ CFU cm^{-2}), probably because water quickly drains off from here. Accumulation of (antimicrobial) detergent residues might be an additional reason.

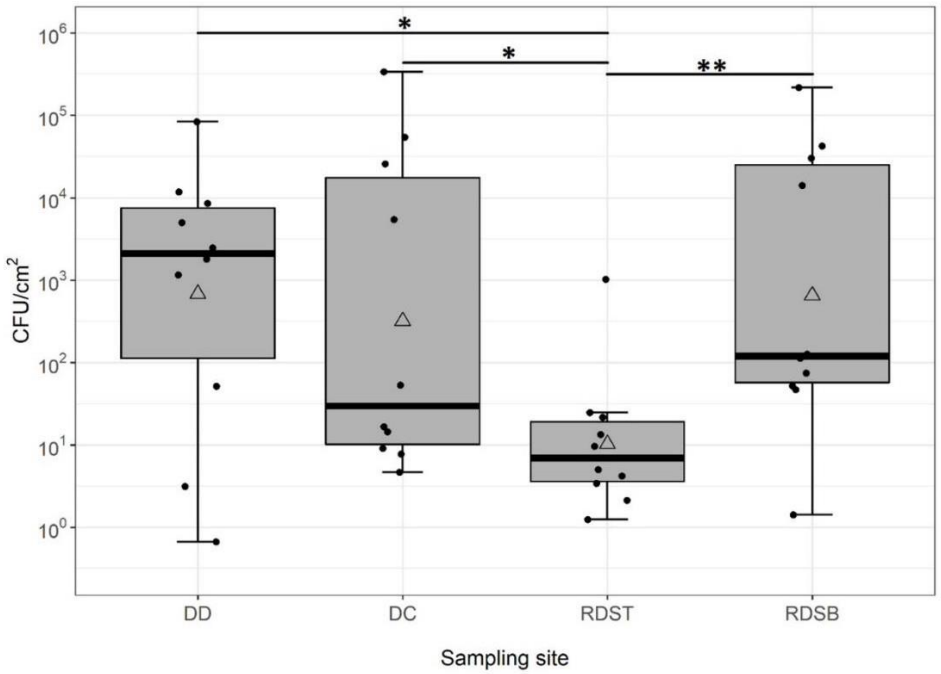

Figure 1. Box-whisker plots of aerobic colony counts per cm^2 from 4 sampling sites of 10 domestic washing machines. Each box represents the 25% and 75% percentiles. Bold horizontal lines represent medians. Mean values are displayed as triangles. Whiskers above and below the boxes indicate the lowest and highest microbial counts that were not classified as outliers. Black points represent single data points per site. The different sampling sites are detergent drawer (DD), detergent drawer chamber (DC), top part of rubber door seal (RDST), and bottom part of rubber door seal (RDSB) (n = 10 for each sampling site). Results of Wilcoxon–Mann–Whitney post hoc tests are discussed in the text; significance levels are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$).

Detergent drawer (DD), detergent drawer chamber (DC), and the bottom part of the rubber door seal (RDSB) showed similar values, with $1.1 \pm 0.74 \times 10^4$ CFU cm^{-2}, $4.2 \pm 3.0 \times 10^4$ CFU cm^{-2}, and $3.1 \pm 1.9 \times 10^4$ CFU cm^{-2}, respectively. Statistical analysis by Kruskal–Wallis rank sum test proved a significant difference when comparing the colony counts of all sampling sites ($p = 0.029$, Figure 1). Subsequent pair-wise Wilcoxon–Mann–Whitney post hoc tests indicated differences between the top part of the rubber seal and its bottom part ($p = 0.007$), as well as the detergent drawer ($p = 0.021$) and the detergent drawer chamber ($p = 0.045$). Clearly, due to the large variability of the colony counts, studies with larger sample sizes are needed to substantiate these findings.

Interestingly, little is known about the microbial load of different sites inside domestic washing machines [21]. To the best of our knowledge, only Stapelton and colleagues [21] have previously reported the microbial loads of different sampling sites, albeit only for four domestic washing machines. While our data match their results for the rubber doors seal quite well (~ 10^3 to 10^4 cm^{-2}), they also suggest the detergent drawer region as being significantly more contaminated than reported by them (~ 10^{-1} to 10^3 cm^{-2}). Clearly, also from a quantitative point for view, the detergent drawer region is an important site for washing machine hygiene and, thus, probably also laundry hygiene.

3.2. Identification of Microbial Isolates

212 microbial isolates stemming from the 40 washing machine samples were analyzed by MALDI Biotyping. Genus-level identification scores (≥1.7) were determined for 178 isolates (84%), while 34 isolates (16%) could not be identified. 118 isolates (56%) were probably identified on species level (score ≥2.0). In total, 29 genera and 40 species were found (Table 1).

Table 1. Number of microbial isolates obtained from 10 domestic washing machines and identified by matrix-assisted laser desorption/ionization (MALDI) Biotyping with identification scores ≥1.7 (genus level; *n*= 178) and ≥2.0 (species level; *n* = 118) across the four different sampling sites (DD = detergent drawer; DC = detergent drawer chamber; RDST = top part of rubber door seal; RDSB= bottom part of rubber door seal). Species categorized as risk group 2 (based on the German Rules for Biological Agents #446 [22] and #460 [23]) are marked with an asterisk. Species detected here which have been previously identified in (9) as one of the ten relatively most abundant species from door seals and detergent drawers, respectively, are written in bold.

Phylum	Class	Order	Family	Genus	Species	DD	DC	RDST	RDSB
Actinobacteria	Actinobacteria	Actinomycetales	Dermacoccaceae	*Dermacoccus*	*Dermacoccus sp.*	-	-	1	-
					Dermacoccus nishinomiyaensis	-	-	-	1
		Corynebacteriales	Corynebacteriaceae	*Corynebacterium*	*Corynebacterium sp.*	-	2	1	-
					Corynebacterium lipophiloflavum	-	-	1	-
		Micrococcales	Brevibacteriaceae	*Brevibacterium*	*Brevibacterium celere*	-	1	-	-
			Dermatophilaceae	*Arsenicicoccus*	*Arsenicicoccus bolidensis*	1	-	-	-
			Micrococcaceae	*Kocuria*	*Kocuria sp.*	-	1	1	3
					Kocuria rhizophila	-	1	2	1
				Micrococcus	*Micrococcus sp.*	-		10	3
					Micrococcus luteus	2	2	13	11

Table 1. *Cont.*

Phylum	Class	Order	Family	Genus	Species	DD	DC	RDST	RDSB
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*	*Sphingobacterium spiritivorum* *	1	-	-	-
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*Bacillus sp.*	5		2	2
					Bacillus cereus *	1	3	1	1
					Bacillus licheniformis	-	1	-	-
					Bacillus megaterium	1	-	1	1
				Lysinibacillus	*Lysinibacillus sp.*	-	-	-	1
			Paenibacillaceae	*Paenibacillus*	*Paenibacillus residui*	-	-	-	1
			Planococcaceae	*Solibacillus*	*Solibacillus sp.*	1	-	-	-
			Staphylococcaceae	*Staphylococcus*	*Staphylococcus sp.*	2	5	5	4
					Staphylococcus capitis	-	-	1	
					Staphylococcus epidermidis *	-	1	-	3
					Staphylococcus haemolyticus *	2	-	-	-
					Staphylococcus hominis *	-	1	-	1
					Staphylococcus lugdunensis *	1	-	-	-
					Staphylococcus saprophyticus	-	-	-	1
					Staphylococcus warneri	1	2	3	
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*	*Rhizobium radiobacter*	-	1	-	3
		Rhodospirillales	Acetobacteraceae	*Roseomonas*	*Roseomonas mucosa* *	-	2	-	-
		Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	*Sphingomonas sp.*	1	-	-	-
					Sphingomonas paucimobilis *	1	-	-	-
					Sphingomonas pseudosanguinis	1	-	-	-
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*	*Achromobacter sp.*	-	-	1	-
					Achromobacter mucicolens *	-	1	-	-
			Comamonadaceae	*Delftia*	*Delftia acidovorans*	2	-	-	-
	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	*Aeromonas caviae* *	-	-	-	1

Table 1. *Cont.*

Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**DD**	**DC**	**RDST**	**RDSB**
Phylum	Class	Order	Family	Genus	Species	DD	DC	RDST	RDSB
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Alishewanella*	*Alishewanella sp.*	1	-	-	-
			Shewanellaceae	*Shewanella*	*Shewanella putrefaciens* *	-	-	-	1
		Enterobacteriales	Enterobacteriaceae	*Citrobacter*	*Citrobacter freundii* *	-	-	-	1
					Citrobacter gillenii *	-	-	-	2
				Klebsiella	*Klebsiella oxytoca* *	-	1	-	2
				Pantoea	*Pantoea agglomerans* *	1	-	-	-
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	*Acinetobacter johnsonii* *	1	-	-	-
					Acinetobacter lwoffii *	1	-	-	1
					Acinetobacter parvus *	-	-	-	1
					Acinetobacter ursingii *	-	-	-	5
				Moraxella	*Moraxella sp.*	-	1	1	-
					Moraxella osloensis *	1	1	-	2
			Pseudomonadaceae	*Pseudomonas*	*Pseudomonas sp.*	4	-	-	-
					Pseudomonas alcaliphila	5	1	-	-
					Pseudomonas oleovorans	2	3	-	1
					Pseudomonas stutzeri	1	2	-	
		Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	*Stenotrophomonas maltophilia* *	1	1	1	1
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Aspergillus*	*Aspergillus sp.*	-	-	-	1
					Aspergillus fumigatus *	1	-	-	-
	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Candida*	*Candida sp.*	-	1	-	-

Standard cultivation techniques are limited, as they only detect cultivable microorganisms and thus discriminate against the vast majority of microorganisms on earth [24]. Therefore, we particularly compared the results obtained here with data from previous molecular studies, in particular, a recent one conducted by us with machines from the same region [9].

In accordance with previous molecular studies [7,9,10], *Proteobacteria* (29%), *Actinobacteria* (27%), *Firmicutes* (26%), and *Bacteroidetes* (0.5%) also represented the most abundant phyla here. In accordance with the relatively most abundant species found in our previous molecular study [9], *Pseudomonas oleovorans*, *Acinetobacter parvus*, and *Moraxella osloensis*

were also detected here by cultivation in the door seal, while *Rhizobium radiobacter* was detected in the detergent drawer (Table 1) [9].

Many of the identified species represent environmental bacteria, typically found in water habitats or the human body, such as skin-associated bacteria. In addition, some of the identified species are well-known biofilm formers, such as *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus cereus*, and *Pseudomonas sp.* [2,4,7,9,10,25–28].

To estimate their pathogenic potential, the identified bacterial species were classified into biosafety risk groups (RG) [22,23]. More than 50% (22 of 40 species) were affiliated with RG 2 organisms, i.e., representing a potential health risk, especially for immunocompromised patients, pregnant women, or elderly persons [15]. 15 out of 21 identified RG 2 bacteria were found in the detergent drawer compartment (DD and DC), and 13 out of 21 RG 2 bacteria on the entire rubber door seal.

By far, micrococci and staphylococci were the most frequently isolated genera, which is in contrast to the different molecular studies mentioned here [9,10] and might represent a cultivation bias. Micrococci and staphylococci represent ubiquitous microorganisms that are often isolated from the skin and mucous membranes of humans and animals, but also from air and water. They grow fast under a broad range of cultivation conditions [29–31]. However, they also have the ability of dormancy and might therefore well resist the dramatically changing environmental conditions inside washing machines [32,33]. The frequent detection of (non-pathogenic) micrococci on the rubber door seals might be due to the fact that these parts are more frequently touched by human hands than the other parts investigated here.

Staphylococci such as *S. epidermidis*, *S. lugdunensis*, *S. saprophyticus*, and *S. haemolyticus* possess a pathogenic potential, and may also play a role in the horizontal gene transfer of antibiotic resistance genes [34–36]. The presence and transmission of such resistance genes throughout washing machines have already been confirmed for β-lactamase [37]. β-lactamase-producing *Klebsiella oxytoca* and *Klebsiella pneumoniae* species have also been isolated from washing machines before [11,38]. It can be speculated that these bacteria can be transferred to other surfaces, e.g., via bioaerosols [34–36]. Notably, *Klebsiella oxytoca* was also found in our study; however, without knowing its resistance pattern. Clearly, the interaction between the chemistry used for cleaning and disinfection and the selection of (antibiotic) resistant microbial species is an important topic in laundry and household hygiene [39,40].

Besides bacteria, a few eukaryotic species were also isolated with the used cultivation conditions, all affiliated with *Ascomycota* (1 %). The most abundant genus was *Aspergillus*. *Aspergillus sp.* are saprophytic fungi and can recycle organic debris. *A. fumigatus* is a prevalent airborne fungal pathogen that can cause severe infections in immunocompromised people [41].

4. Conclusions

Despite its small sample size, our study clearly shows that both the detergent drawers and bottom door seals of domestic washing machines are significantly contaminated with cultivable bacteria, including significant shares of potentially pathogenic ones. Maximum loads can exceed 10^5 CFU per cm^2. For the sake of machine and laundry hygiene, both parts should be cleaned regularly. Markedly lower CFU counts from the top part of the door seal underline the importance of water for the microbial contamination of washing machines. When not in use, machines should be left open to dry out. Bacterial species identified here and in molecular studies as quantitatively important for the washing machine microbiota represent test organisms with high practical relevance for antimicrobial efficacy testing.

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6. Metatranscriptomic Analysis of Bacterial Communities on Laundered Textiles: A Pilot Case Study

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Own contribution: Bioinformatics and statistical analyses, visualisation, data interpretation, data curation, manuscript writing, and reviewing.

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Article

Metatranscriptomic Analysis of Bacterial Communities on Laundered Textiles: A Pilot Case Study

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Abstract: Microbially contaminated washing machines and mild laundering conditions facilitate the survival and growth of microorganisms on laundry, promoting undesired side effects such as malodor formation. Clearly, a deeper understanding of the functionality and hygienic relevance of the laundry microbiota necessitates the analysis of the microbial gene expression on textiles after washing, which—to the best of our knowledge—has not been performed before. In this pilot case study, we used single-end RNA sequencing to generate de novo transcriptomes of the bacterial communities remaining on polyester and cotton fabrics washed in a domestic washing machine in mild conditions and subsequently incubated under moist conditions for 72 h. Two common de novo transcriptome assemblers were used. The final assemblies included 22,321 Trinity isoforms and 12,600 Spades isoforms. A large part of these isoforms could be assigned to the SwissProt database, and was further categorized into "molecular function", "biological process" and "cellular component" using Gene Ontology (GO) terms. In addition, differential gene expression was used to show the difference in the pairwise comparison of the two tissue types. When comparing the assemblies generated with the two assemblers, the annotation results were relatively similar. However, there were clear differences between the de novo assemblies regarding differential gene expression.

Keywords: RNA sequencing; metatranscriptome; laundry hygiene; cotton; polyester

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1. Introduction

A multitude of microorganisms live in modern washing machines. The common routes of contamination are worn clothing, tap water and air [1,2]. Promoted by the warm, humid and nutrient-rich environment, microorganisms such as bacteria and fungi can settle and multiply inside the machine [2–4]. The negative effects of such a contamination are unattractive staining, malodor and biofilm formation [2,4]. In particular, the formation of resistant biofilms might pose a risk for susceptible persons, as biofilms might represent a reservoir for (potentially) pathogenic microorganisms that re-contaminate the laundry during washing [5,6].

The microbial contamination of washing machines and laundry is further promoted by largely sustainability-driven adaptations to the washing process that are common today, such as reduced water consumption, low washing temperatures and the increased use of bleach-free liquid detergents [4].

Using the molecular approach of 16S rRNA gene pyrosequencing, we recently showed that the relatively most abundant sequence types in domestic washing machines were closely related to potentially pathogenic bacteria, such as *Brevundimonas vesicularis* or *Pseudomonas aeruginosa* inside the detergent drawer, and *Moraxella osloensis* or *Acinetobacter parvus* inside the door seal [7]. While this and other structural studies have looked at the microbial community composition of washing machines and laundry items [3,8–14], studies on the metabolic activities of the laundry microbiota are often limited to distinct functionalities, such as the formation and prevention of malodor [2,15,16]. Malodor is often associated with a lack of hygiene, and can negatively affect the life cycle of a textile [17].

In contrast to metagenomics studies, metatranscriptome studies unravel the totality of the genes that are expressed in a complex microbial community [18]. The next-generation sequencing of RNA (RNASeq) can determine the metabolic potential at the time of sampling by quantifying almost all of the transcripts from the present cells, and can thus help to obtain a profound insight into the expression profiles of an entire microbiota in a single experiment, characterizing the functionality of a microbial community [19,20].

Transcriptome studies are computationally challenging and usually require several bioinformatics tools [21,22]. The major steps in a typical metatranscriptome analysis include quality trimming and the removal of contaminating sequencing reads, the reconstruction of the individual transcripts, the annotation of these transcripts and genes, and the quantification of their expression [21,23–25]. In order to reconstruct the transcriptome, de novo assemblers based on de Bruijn graphs, such as Spades (rna mode) [26], Trinity [27], Velvet/Oases [28,29], or SOAPdenovo-trans [30], focus on the relationship between substrings of a fixed length k (*k*-mers). They can be used if no reference genome is available [22,31,32]. Each of these assemblers can produce useful assemblies, but when comparing different assembler software, a considerable degree of variability becomes evident [33,34].

In this pilot case study, we aimed to analyze the expression profiles of the post-wash bacteriota on two common fabric tissue types washed in a domestic washing machine, by generating de novo assembled transcriptomes using two different assemblers. To the best of our knowledge, our study represents the first study using metatranscriptomics for the field of laundry hygiene.

2. Materials and Methods

2.1. Sample Preparation

Two common fabric types, cotton and polyester, were used for the washing experiments. Textile samples were cut from locally purchased, new, white, cotton and polyester T-shirts with an area of 8 x 15 cm (120 cm^2), and were subsequently sterilized by autoclaving. In each washing experiment (Figure 1), the cut textile samples (2 x 120 cm^2 per fabric type) were washed in a private household washing machine (an approximately five years old EcoActive W1900 appliance (Miele, Gütersloh, Germany) with approximately 4 Kg of ballast laundry consisting of worn cotton T-shirts and jean pants. A mild and short washing program for synthetics was used at 30 °C for 59 min, with a final 600 rpm spinning cycle using 30 mL of a commercial, bleach-free liquid detergent (Spee AktivGel, Henkel, Düsseldorf, Germany). The detergent was under-dosed to aid microbial survival on the washed textiles. Because many components of commercial laundry detergents are antimicrobial, we used a bleach-free liquid detergent and under-dosed it, assuming that this might increase the amount of active bacteria on the laundry after washing [35,36].

After washing, and virtually simulating "forgetting" the laundry in the washing drum, the textile samples were placed separately in a closed 38 l plastic box (Rotho Kunststoff, Würenlingen, Switzerland) and incubated at room temperature for 72 h, together with 4 pieces of washed ballast laundry (two cotton and two polyester T-shirts, with no contact with the test fabrics). This pre-incubation step was necessary to obtain sufficient

RNA for the downstream analysis. Afterwards, the textile samples were stored at −80 °C until further processing.

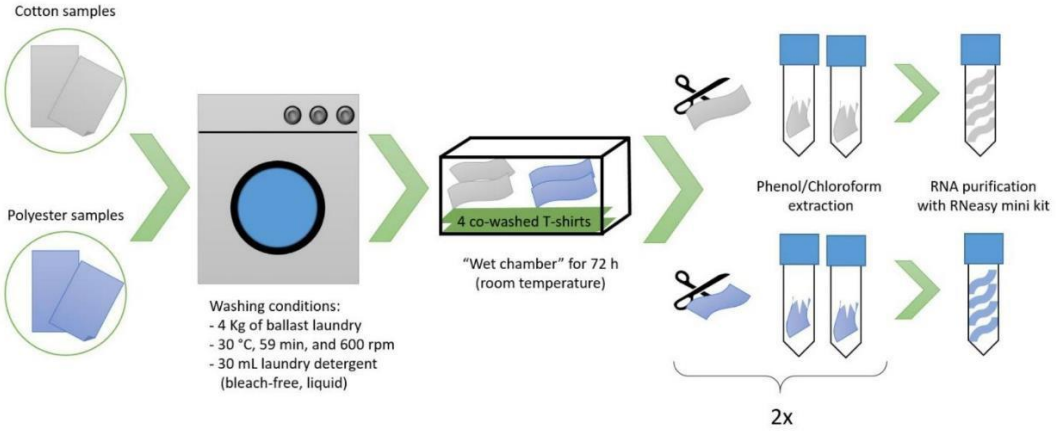

Figure 1. Schematic overview of a single washing experiment. Two sterile cotton (grey) and polyester (blue) textile samples (120 cm² each) were washed in a standard household washing machine under mild conditions. Subsequently, the textile samples were incubated in a "wet chamber" for 72 h at room temperature. Each lobe was cut into smaller pieces under sterile conditions and distributed over two reaction tubes. After the phenol/chloroform extraction, the RNA from the two cotton and polyester lobes, respectively, were combined into a single RNA extract using the RNeasy Mini Kit. This experiment was replicated once, finally yielding two independent cotton and polyester RNA extracts each (n = 2).

2.2. *RNA Extraction and Sequencing*

The total RNA was isolated using a modified phenol–chloroform extraction method from Zoetendal et al. [37]. For the cell disruption, each textile sample (120 cm²) was cut under sterile conditions into pieces of approximately 1.5 cm², and was evenly distributed into two sterile extraction tubes containing 15 sterile glass spheres (Ø 3 mm, Sigma-Aldrich, St. Louis, MO, USA) and approximately 4.3 g of a sterile ceramic silica extraction powder (Ø 0.1 mm, BioSpec Products, Bartlesville, OK, USA). Subsequently, 500 µL 10% SDS (Ambion, Carlsbad, CA, USA) and 9 mL phenol (Carl Roth, Karlsruhe, Germany) were added to each extraction tube and treated for 3 x 45 s with a FastPrep24 instrument (MP Biomedicals, Eschwege, Germany) at 5.5 m/s. Then, the extraction tubes were centrifuged for 15 min at 3220 x g and 4 °C. After the centrifugation, the upper aqueous phase of an extraction tube was transferred to two Phase Lock Gel (PLG) Heavy Tubes (5Prime, Hilden, Germany). Then, 250 µL acid phenol and 250 µL chloroform (Sigma-Aldrich, Taufkirchen, Germany) were added to each PLG tube and gently mixed. Then, the tubes were centrifuged at 13500 x g for 5 min to separate the phases. The aqueous phase was transferred into new PLG tubes, and the procedure was repeated. Afterwards, the aqueous phase was transferred to a new PLG tube, mixed with 500 µL chloroform and centrifuged again at 13500 x g for 5 min. The supernatant was transferred to a new 2 mL tube, and the RNA was purified using an RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. The RNA extracts belonging to the same textile sample were pooled during their application to the RNeasy column by the repeated transfer of 700 µl to the column, each followed by centrifugation. In order to exclude any contamination with DNA, a DNA digestion was performed two times for 20 min at room temperature. The final elution of the total RNA was performed with 30 µL TE buffer (Sigma-Aldrich, Taufkirchen, Germany). Two independent washing experiments were conducted (n = 2), yielding two RNA extracts from cotton and two from polyester samples, respectively.

The obtained RNA extracts were stored at −80 °C until the library preparation. For this, the RNA samples were reverse transcribed using the ScriptSeq Complete Kit for Bacteria (Epicentre, Madison, WI, USA) according to the manufacturer's protocol. The quality of the cDNA library was determined using an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany). Finally, the samples were sequenced on an Illumina MiSeq platform using a MiSeq Reagent Kit v2 (both Illumina, Munich, Germany).

2.3. Sequence Data Analysis

The raw sequences of each single-end library were subjected to a quality control prior to their assembly. The remaining adapters and reads with a Phred quality score < 20 were removed using Trim-galore (version 0.6.6, [38]). Furthermore, human contaminations were excluded using Bowtie2 (version 2.4.2, [39]) and the human GRCh38.p13 (Release 35, [40]). In addition, ribosomal RNA was also removed from the dataset using sortmeRNA (version 4.2.0, [41]) and its provided databases. In the next step, reads with a length of less than 50 bp were discarded, truncated at a length of 280 bp and filtered out with an average Phred quality score of 20 using Trimmomatic (version 0.36, [42]). The final quality of the preprocessed reads was visualized using FastQC (version 0.11.9, [43]) and MultiQC (version 1.9 [44]). Prior to their assembly, the preprocessed reads were error-corrected using Rcorrector (version 1.0.4, [45]), as error correction is considered the best practice for transcriptome assembly [46]. In order to assemble the reads de novo to a transcriptome, Trinity software (version 2.8.5, [27]) with default parameters and Spades software (version 3.14.1, [26,47]) with the -rna-flag and different *k*-mers (13, 15, 19, 21, 25, 29, 31, 43, 55, 67, 79, 91, 103, 115, 127) were used.

The assembly statistics were calculated by the TransRate software (version 1.0.3, [48]). As mentioned in other studies, non-redundant transcripts were removed with the CD-HIT package (version 4.8.1, [49]) with an identity threshold of 95% and a word size of 10 [50–52]. In order to reassess the quality of the clustered transcriptome, assembly statistics were generated using Quast (version 5.0.2, [53]) and rnaQuast (version 2.0.1, [54]). In order to further determine the quality of the assembly, the read representation was calculated by aligning the input reads against the transcriptome using Bowtie2.

The transcriptome completeness was evaluated using the Benchmarking Universal Single-Copy Orthologs (BUSCO) tool (version 3.0.2, [55]) against the bacteria_odb10, archaea_odb10 (both, creation date: 2020-03-06), fungi_odb10 and eukaryota_odb10 (both, creation date: 2020-09-10) databases to quantify the percentage of single-copy orthologues. Full-length transcripts were calculated using BLAST (version 2.2.31, [56,57]) against the SwissProt/UniProtKB database (version 2020_05, [58]) with the parameters max_target_seqs 1, -evalue 1.0×10^{-20}. The BLAST-results were analyzed using the "analyze_blastPlus_topHit_coverage.pl" script from the Trinity software. As recommended by the Trinity website, ExN50 statistics were calculated using RSEM software (version 1.3.3, [59]) with Bowtie2 and the "contig_ExN50_statistic.pl" script from the Trinity software.

In order to perform the annotation of the transcripts, open reading frames (ORFs) within the assemblies were determined using TransDecoder (version 5.5.0, [60]). The transcript ORFs with less than 150 bp were excluded from the dataset, and the BLAST (BLASTx and BLASTp; E-value: 1.0×10^{-3}) analysis against the SwissProt/UniProtKB database and the HMMER search (version 3.3.1, [61,62]) against the PFAM database (version 33.1 (May 2020, 18259 entries [63,64]) were performed. Finally, the annotation results were loaded into the Trinotate classification tool (version 3.2.1, [65,66]) to determine the functionality by means of Gene Ontology (GO) [67]. Non-supervised Orthologous Groups (eggNOG) [68] were used to visualize the gene expression profiles grouped according to gene genealogy. Modified trinotateR [69] functions were used to evaluate the Trinotate output with R.

For the taxonomic annotation, the different assemblies were aligned against the NCBI nucleotide database (from May 2021) using BLAST (BLASTx, E-value: 1.0×10^{-3}).

For differential expression, the transcript abundance was calculated using the "align_and_estimate_abundance.pl" and the "abundance_estimates_to_matrix.pl" scripts from the Trinity bundle using RSEM software with Bowtie2. The differential expression analysis was carried out using R (version 3.5.3, [70]), RStudio (version 1.1.463, [71]) and edgeR (version 3.24.3, [72]). The edgeR package uses negative binomial models to detect dispersion, and later determines the differential expression with the exact test, which is analogous to the Fisher exact test [72]. An overview of the bioinformatics pipeline used here is shown in Figure S1.

3. Results and Discussion

3.1. Reads and De Novo *Transcriptome Assembly*

In total, 8.3 million raw sequences with sequence lengths between 35 and 300 bp were obtained from the four samples after sequencing on the Illumina MiSeq instrument. After the various quality filtering steps, 6.8 million reads remained, with lengths of 50–280 bp. This corresponds to a loss of ~ 18%. A detailed listing of the numbers of sequences after the different quality filtering steps is given in Table S1.

In order to generate a de novo assembly, two different assemblers were used, i.e., Trinity and Spades. Both assemblers showed constant and good assemblies in a comparison of multiple assemblers with datasets of several different species, and were therefore selected [73]. The de novo assembly from the pre-processed reads generated 24,386 isoforms with lengths ranging from 201 to 64,155 bp using Trinity, and 13,147 isoforms of lengths between 365 and 112,899 bp using Spades.

The determined N50 value was 2192 bp for the Trinity assembly, and 2641 bp for the Spades assembly. The N50 value quantifies the average length of a contig, which comprises 50% of the sequence within the total assembly [23].

3.2. Evaluation of the Different de novo *Transcriptome Assemblies*

In order to investigate the quality of the assemblies, different measures were carried out (Table S2). First, the individual isoforms were assembled into clusters with 95% similarity, reducing the number of non-redundant isoforms to 22,321 for Trinity and 12,600 for Spades, respectively. For further quality control, the pre-processed input reads were aligned against the different clustered transcriptome assemblies to determine the read representation. In general, 80% of the reads mapping back to the transcriptome is considered an indication of a good assembly [74]. Our four sample reads aligned approximately 80–89% with each of the transcriptome assemblies.

In order to obtain an impression of the completeness of the generated assemblies, we applied BUSCO as a reference-based pipeline. This pipeline indicated that a large proportion of the single-copy genes were found in the bacterial domain. More precisely, of the 124 BUSCO groups examined, 69% were recovered from the Trinity assembly and 66% were recovered from the Spades assembly. Nonetheless, a large number of BUSCOs were missing or were too fragmented to be considered, more precisely 29% from the Trinity and 32% Spades assemblies. Although not complete, the values for the bacterial database showed good coverage of the transcripts to the known single copy orthologues, indicating an almost complete expected gene content [75]. The other sets of BUSCOs (archaea, eukaryotea, and fungi) revealed a completeness of less than 10%.

As suggested on the Trinity website, the number of full-length transcripts and the ExN50 values were determined for the further evaluation of the assembly. In order to determine the number of full-length transcripts, a BLAST analysis was first performed against the SwissProt database. To do so, a relatively low e-value (1.0×10^{-20}) was used to store only the single best matching proteins and to discard hits for very short sequences, as these usually do not deliver a BLAST hit [76,77]. Under these conditions, 32% of the Trinity generated isoforms and 42% of the Spades generated isoforms could be assigned to SwissProt proteins, respectively. Nevertheless, out of these, 42% (Trinity) and 51%

(Spades) of the near-full-length transcripts (> 70%) could be recovered with these assemblies, respectively. However, both assemblies seem to contain a high proportion of fragmented or incorrectly assembled transcripts [78].

The ExN50 value indicates the N50 value by using only the most highly expressed transcripts [79]. Both assemblers peak at low percentages, indicating a tendency to detect highly expressed isoforms (Figure S2). Therefore, under the experimental conditions used here, neither assembler could adequately detect low-expressed transcripts [80]. However, increasing the sequencing depth in follow-up experiments might enhance the ExN50 value to obtain a more complete representation of the transcriptome.

All in all, the above-mentioned indices show that the assemblies are of good quality, but the method clearly needs further improvement, e.g., by increasing the sequencing depth or by using multiple *k*-mers lengths to account for variable transcript expression [81,82].

3.3. Transcript Annotation

The BLAST analysis showed that bacterial sequences represented the majority (approximately 99%) of all of the sequences within the two assemblies. Eukaryotic and viral sequences were minorly abundant, probably because the ScriptSeq Complete Kit for Bacteria was used for the library preparation.

Based on the transcript counts, several bacterial genera known to be typical for washing machines and laundered textiles [7,10,13] were detected, such as *Acinetobacter* (48.5%, 51.7%), *Aeromonas* (26.1%, 21.6%), *Rhizobium* (6.0%, 6.5%), *Agrobacterium* (2.9%, 2.4%), *Moraxella* (1.8%, 2.1%) and *Pseudomonas* (0.4%, 0.4%) (the brackets show the relative abundances based on the Spades and Trinity assemblies, respectively, averaged over all of the samples). However, we also detected genera which were, to the best of our knowledge, previously not reported as being typical for washing machines or laundered textiles, such as *Sphingorhabdus* (9.9%, not detected), *Anderseniella* (2.1%, 12.1%), *Epilithonimonas* (0.9%, 1.0%), *Haematobacter* (0.5%, 0.6%) and *Escherichia* (0.04%, 0.3%). Figure S3 shows the relative abundances of the 11 most relatively abundant genera for both experiments, based on Trinity and Spades assemblies, respectively.

The identification of the functional classes was achieved by following the GO term classification using SwissProt gene symbols. These GO terms are divided in three categories, i.e., "biological process", "cellular component" and "molecular functions", which describe the attributes of a gene product [67,83]. In each of the assemblies, we detected a wide range of GO terms from all three functional categories, suggesting the active and diverse microbial gene expression of the investigated textiles (Figure 2).

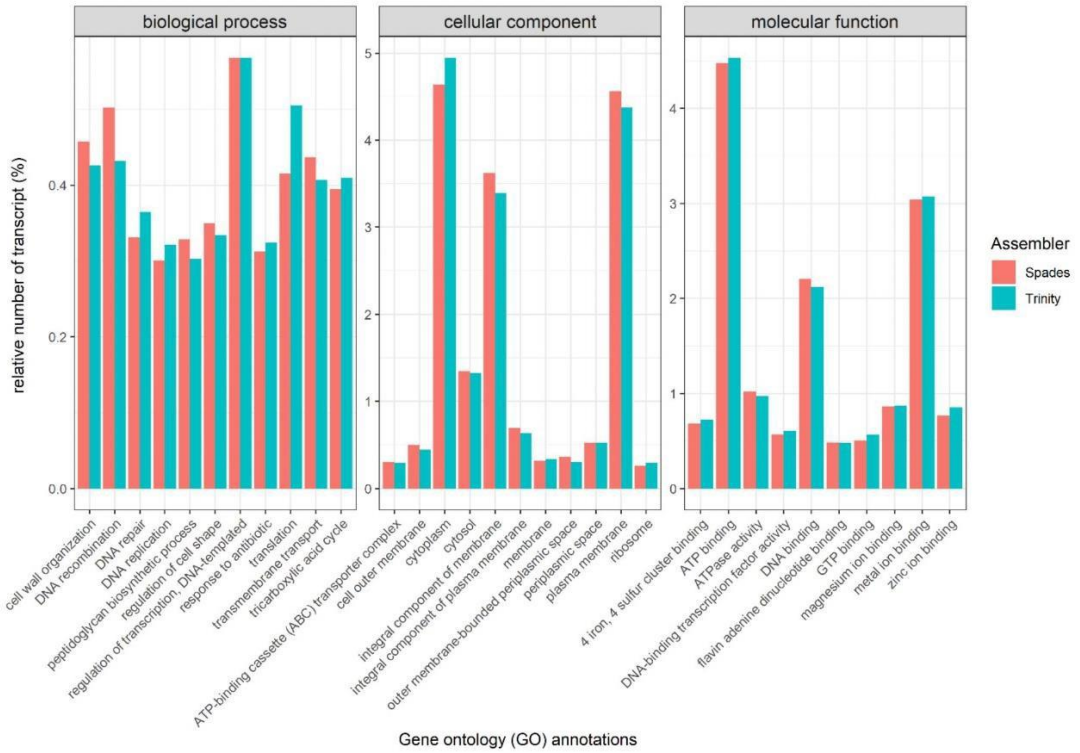

Figure 2. Bar chart of the assigned Gene Ontology (GO) terms of the annotated genes assembled with Spades (red) or Trinity (blue). The GO terms are categorized into biological process, cellular components, and molecular functions, respectively. For each category, the 10 GO terms with the highest relative number of transcripts are displayed in alphabetical order.

In total, 3881 unique GO terms were identified with the Spades assembly, whereas 4390 unique GO terms were determined with the Trinity assembly. Both assemblies shared 3686 unique GO terms. The most frequently assigned GO terms allocated to "cellular components" were related to cytoplasm (Spades: 4.6%, Trinity: 4.9%), plasma membranes (Spades: 4.6%, Trinity: 4.4%), and the integral components of the membrane (Spades: 3.6%, Trinity: 3.4%). On the other hand, most of the "molecular functions" were associated with binding: ATP binding (Spades: 4.5%, Trinity: 4.5%), metal ion binding (Spades: 3.0%, Trinity: 3.1%) and DNA binding (Spades: 2.2%, Trinity: 2.1%). Only a small proportion of the transcripts could be assigned to "biological processes". Here, the regulation of transcription (DNA-templated) (Spades: 0.6%, Trinity: 0.6%), DNA recombination (Spades: 0.5%, Trinity: 0.4%), and cell wall organization (Spades: 0.5%, Trinity: 0.4%) predominated. In summary, these data suggest that a metabolically active bacterial community was present on the investigated fabric patches.

Notably, despite using different assembly software, there was a high level of similarity between the assigned GO terms, which suggests the accuracy of the different assemblies and the assigned annotations [76]. Furthermore, previous research has shown that GO terms have high consistency across multiple species despite the intrinsic differences between the different assembly strategies and annotation pipelines, suggesting the usage of GO for comparisons with other studies [77].

3.4. Differential Expression

In order to investigate whether the tissue type had an influence on the microbial gene expression, a differential expression analysis was performed using the edgeR-R-package. After filtering and normalization using the built-in-functions of edgeR, both assemblies clearly showed a separation between the fabric types, indicating differences in their expression profiles (Figure 3). In addition, Figure 3 shows marked differences between the replicate washing experiments, strongly requiring follow-up-studies with larger sample sizes and more standardized conditions. The differences between the two experiments might originate from the different ballast laundry used. While the vertical separation of the samples seems to be textile-dependent, the horizontal separation seems to experiment-dependent.

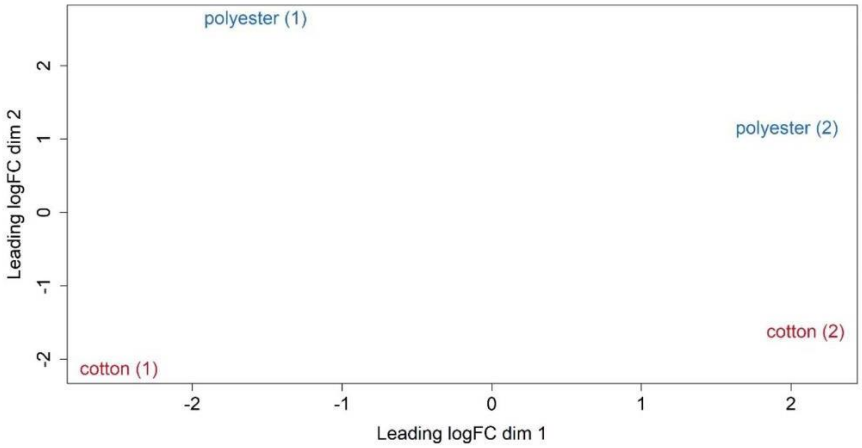

Figure 3. Multi-dimensional scaling (MDS) plot based on RNAseq expression profiles from different tissue type samples. The distances between the samples in the plot were calculated based on leading log2 fold changes (logFC) between each cotton and polyester sample, which were defined as the average log2-fold change for the 500 most differential genes between each pair of profiles [84]. The numbers in parentheses define the respective washing experiments. Here, the MDS plot for the Trinity assembly is shown. The very similar MDS plot for the Spades assembly can be found in Figure S4.

In contrast to the annotation, the differential expression analysis showed differences between the two assemblers. Overviews of the different gene expression profiles from the different assemblers, categorized into higher functional groups, are shown in Figures S5 and S6.

Performing the differential expression, we identified 8146 genes with a log2 fold change (4447 upregulated and 3699 downregulated) for the Trinity assembly and 4037 (2248 upregulated and 1789 downregulated) for the Spades assembly when comparing the cotton and polyester samples. Out of these, 16 genes were statistically significantly differentially expressed (adjusted p-value < 0.01) in the case of the Trinity assembly (Table 1). Of these, nine genes were identified as being up-regulated in the cotton samples, and seven genes were identified as being down-regulated in the cotton samples. For the Spades assembly, we identified 4037 differentially expressed genes (2248 upregulated and 1789 downregulated). However, for only one gene, the expression was significantly different. It was up-regulated in the cotton samples (Table 1).

Figures S5 and S6 suggest that the number of genes with significant differences in expression between the cotton and polyester might be higher than the 17 genes detected here. In order to prove this, more standardized studies with a bigger sample size are

needed. Interestingly, two of the differentially expressed genes were affiliated with *Moraxella* (Table 1), a genus which is well known for laundry malodor production [15,85].

The genes, which were significantly up- or down-regulated, were mainly enzymes that are predominantly involved in metabolic pathways, as well as the transport of substances across the cell membrane (Table 1). The identified genes are common genes that are found in different microorganisms and have a variety of cellular activities, such as the "AAA (ATPases Associated with diverse cellular Activities) family ATPases" or the "LysR family transcriptional transporter", which is involved in virulence, metabolism, quorum sensing and motility [86,87]. In addition, our data slightly suggest that carbohydrates might have acted as substrates on the fabric samples, and might have led to differences in gene expression, as indicated by the different expression of the genes for "Sucrose-6-phosphate hydrolase" and "PTS system sucrose-specific EIIBC component". The phosphoenolpyruvate-dependent phosphotransferase system (PTS) is found in various microorganisms, such as *Escherichia coli*, *Streptococcus mutans* and *Bacillus subtilis*, and—in addition to the transport and phosphorylation of carbohydrates—it is also involved in the movement towards carbon sources [88,89]. It is tempting to speculate that the chemical differences between cotton (made of cellulose fibers) and polyester (made of polyethylene terephthalate fibers) are responsible for these differences. Unlike natural fibers, synthetic fibers are less susceptible to bacterial degradation [90].

Table 1. Overview of the significantly differentially expressed genes. Shown here are the log fold changes (logFC), log counts per million (logCPM) and the *p*-value after the false discovery rate (FDR) correction, as calculated by edgeR for the pairwise comparison between cotton and polyester. If available, the SwissProt entry or BLAST accession number (March, 2021), as well as the organism determined by means of BLAST against the nucleotide database (May, 2021), are shown, as well as the determined up- or down-regulation of the respective gene for the cotton samples in comparison to the polyester samples. The functional orthologous groups were obtained using the EggNOG database [68]. If a protein has multiple domains, more than one functional orthologous groups is possible.

Assembly	Gene	Uniprot entry/ Blast accession #	Percent identity	E-value	Ontology	Name	Genus	log FC	log CPM	FDR	Regulation
Trinity	TRINITY_DN12211_c0_g1	WP_082183191.1	100.00	2.0×10^{-08}	Energy production and conversion	FAD-binding oxidoreductase	*Rhizobium*	−9.871	5.431	0.003	down
	TRINITY_DN12794_c0_g1	WP_126090172.1	73.08	2.5×10^{-02}	Transcription	LysR family transcriptional regulator	none available	−9.988	5.545	0.003	down
	TRINITY_DN16017_c0_g1	KIV68812.1	100.00	2.0×10^{-36}	Carbohydrate transport and metabolism	Sucrose-6-phosphate hydrolase	*Rhizobium*	−9.852	5.412	0.003	down
	TRINITY_DN16123_c0_g1	IHFA_CHRVO	41.54	1.3×10^{-10}	Transcription	Integration host factor subunit alpha	none available	−9.635	5.202	0.008	down
	TRINITY_DN19317_c0_g1	GLO22_ECOLI	51.90	4.3×10^{-15}	Inorganic ion transport and metabolism	Hydroxyacylglutathione hydrolase GloC	*Rhizobium*	−9.756	5.319	0.010	down
	TRINITY_DN6555_c0_g1	WP_164056586.1	100.00	2.0×10^{-17}	Replication, recombination and repair	AAA family ATPase	*Rhizobium*	−9.705	5.270	0.005	down
	TRINITY_DN8425_c0_g1	WP_142779495.1	100.00	1.0×10^{-18}	Cell cycle control, cell division, chromosome partitioning	ParA family protein	*Rhizobium*	−9.602	5.170	0.008	down
	TRINITY_DN12673_c0_g1	WP_042878669.1	97.53	2.0×10^{-25}	Amino acid transport and metabolism, Carbohydrate transport and metabolism	DMT family transporter	*Aeromonas*	9.611	5.184	0.008	up
	TRINITY_DN14275_c0_g1	WP_174060752.1	100.00	5.0×10^{-23}	Amino acid transport and metabolism, Inorganic ion transport and metabolism	ABC transporter permease	*Rhizobium*	9.590	5.163	0.010	up
	TRINITY_DN14310_c0_g1	WP_124801776.1	100.00	6.0×10^{-11}	Inorganic ion transport and metabolism	cation:proton antiporter	*Epilithonimonas*	9.577	5.150	0.008	up

	TRINITY_DN16876_c0_g1	YDDG_ECOLI	66.67	6.9×10^{-08}	Amino acid transport and metabolism, Carbohydrate transport and metabolism	Aromatic amino acid exporter YddG	*Acinetobacter*	9.751	5.318	0.003	up
	TRINITY_DN16965_c0_g1	STY97430.1	95.16	1.0×10^{-33}	Coenzyme transport and metabolism	Dihydroneopterin aldolase	*Moraxella*	9.920	5.481	0.003	up
	TRINITY_DN19448_c0_g1	WP_204155761.1	82.05	4.0×10^{-15}	Cell motility, Intracellular trafficking, secretion, and vesicular transport	prepilin-type N-terminal cleavage/methylation domain-containing protein	*Moraxella*	9.832	5.396	0.003	up
	TRINITY_DN19763_c0_g1	Y2604_PSEAE	69.09	6.5×10^{-21}	Cell wall/membrane/envelope biogenesis	Uncharacterized protein PA2604	*Pseudomonas*	9.985	5.544	0.004	up
	TRINITY_DN24104_c0_g1	PTSBC_SALTM	79.22	7.3×10^{-33}	Carbohydrate transport and metabolism	PTS system sucrose-specific EIIBC component	*Aeromonas*	10.140	5.695	0.005	up
	TRINITY_DN9443_c0_g1	WP_074855682.1	100.00	4.0×10^{-18}	Inorganic ion transport and metabolism	ArsC family reductase	*Pseudomonas*	9.803	5.368	0.003	up
Spades	NODE_11013_length_508_cov_2.086614_g10506	YOXD_BACSU	46.39	1.7×10^{-35}	Function unknown	Uncharacterized oxidoreductase YoxD	*Epilithonimonas*	9.511	5.043	0.000	up

The observed differences in the number of differentially expressed genes may also be due to the different ways in which the used assemblers work. As there are a number of different tools and parameters that can be used to reconstruct transcripts, it is difficult to determine a single robust method [91–94]. For future studies, merging the different assemblies created with different programs and parameters might lead to a more reliable representation of the post-wash laundry transcriptome [91].

Clearly, in this pilot case study, we only worked with small sample sizes (n = 2) for each fabric type and poorly standardized washing conditions (different ballast laundry). Small sample sizes increase the variances in gene expression, resulting in lower confidence and increasing *p*-values [95]. Therefore, the number of biological replicates should be increased in future experiments, and even technical replicates should be taken into account to reduce the technical noise [78,95].

4. Conclusions

Our study delivered the first laundry metatranscriptome, and it suggests a differential gene expression of the post-wash bacteriota on two commonly used types of fabrics. Our data provide an initial overview and characterization of the bacterial laundry transcriptome, as well as a comparison between the two de novo transcriptome assemblers used, i.e., Trinity and Spades. Clearly, the approach needs further optimization, such as a higher sequencing depth and further biological and technical replicates, ideally in combination with DNA shotgun sequencing, in order to identify the microorganisms of which the metabolic activity shapes the microbial community on laundry. Nevertheless, the assemblies created here represent a solid basis for further metatranscriptomic studies.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/microorganisms9081591/s1. Table S1: Summary of the individual quality filtering steps using individual program specifics, as well as the number of input and output reads during each step of quality filtering. Table S2: Summary of the individual assembly statistics of the generated de novo transcriptome assemblies after clustering with CD-Hit-EST. Figure S1: Schematic overview of the de novo transcriptome reconstruction workflow and analysis pipeline procedure. Figure S2: Estimated ExN50 values of assemblies using A) Trinity and B) Spades. Figure S3: Taxonomic annotation of the different samples for the two assemblers used. Figure S4: Multi-dimensional scaling plot based on RNAseq expression profiles from different tissue types samples generated with the Spades assembly. Figure S5: Mean expression versus the log2 fold change plots (MA-plots) of the Spades assembly. Figure S6: Mean expression versus the log2 fold change plots (MA-plots) of the Trinity assembly.

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7. Analysis of Regularly Microwave-Treated and Untreated Domestic Kitchen Sponges

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Article

Metagenomic Analysis of Regularly Microwave-Treated and Untreated Domestic Kitchen Sponges

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Abstract: Kitchen sponges massively absorb and spread microorganisms, leading to contamination of kitchen appliances, surfaces, and food. Microwaving as an effective and widespread technique can rapidly reduce the microbial load of kitchen sponges. However, long-term effects of such treatments are largely unknown. Notably, it has been speculated that regularly applied domestic cleaning and disinfection may select for microbial communities with a higher pathogenic potential and/or malodorous properties. In this study, we distributed newly purchased polyurethane kitchen sponges to 20 participants, with the instruction to use them under normal household conditions for four weeks. Ten of the participants sanitized their sponges regularly by a standardized microwaving protocol, while the remaining ten sponges remained untreated. Metagenomic sequence data evaluation indicated that, in addition to bacteria, viruses, eukaryotes, and archaea were also part of the kitchen sponge microbiome. Comparisons of sanitized and untreated kitchen sponges indicated a trend towards a reduced structural microbial diversity while functional diversity increased. Microwave sanitization appeared to alter composition and metabolic properties of the microbial communities. Follow-up studies will have to show whether these changes are more positive or negative in terms of domestic hygiene, human health, and well-being.

Keywords: kitchen sponge; metagenomics; shotgun sequencing; microwave; kitchen hygiene

1. Introduction

Within the domestic environment, kitchen utensils and surfaces are very frequently contaminated with microorganisms [1]. Used kitchen sponges contribute considerably to this microbial contamination [2]. The typical activity of wiping kitchen objects and surfaces following food preparation leads to the absorption of food residues and microorganisms [3]. Once contaminated, the constant nutrient-rich and humid environment offers ideal living conditions for microbial growth inside the sponge [4,5]. Several studies performed on kitchen sponges identified harmless environmental bacteria but also potential human pathogens as members of the microbial community [1,6–10]. Clearly, the use of a contaminated sponge might lead to the (re)contamination of kitchen appliances, surfaces and food, thereby increasing the risk of infections [8]. In addition, microbes might be spread to other areas of the household [11,12].

To control and reduce the microbial load in kitchen sponges, many different sanitization and cleaning procedures have been proposed [13,14], including microwaving or cleaning in a domestic

dishwasher or washing machine [15–18]. Ikawa and colleagues [15] and Sharma and colleagues [17] evaluated the efficacy of different sponge cleaning methods by measuring the reduction of the microbial load in artificially contaminated samples. Both confirmed that microwave treatment is an effective and simple method to drastically reduce the bacterial load of kitchen sponges by five to seven log-scales [15,17].

Microwave radiation, as emitted by microwave ovens, can have different effects on microbial cells including thermal and nonthermal effects [19]. Thermal effects are caused by the absorption of microwave radiation, which causes the molecules inside the cell to vibrate and thus generate heat, which in turn causes denaturation of proteins and formation of aggregations in the cytoplasm [20,21]. On the other hand, nonthermal effects include changes in cell morphology and cell wall alterations or an enhanced protein or enzyme activity [8,21,22]. Still, the understanding of how microwaving affects microorganism is limited and a field of active research [20,23].

Many of the abovementioned studies examined the efficacy of sponge sanitization methods under controlled laboratory conditions. Interestingly, Ikawa and colleagues [15] showed that consumer-used sponges were much more difficult to disinfect, presumably due to the very high number of bacteria residing in used kitchen sponges. A recent study conducted by Cardinale and colleagues [6] reported local cell densities of up to 54 billion cells per cm^3 as well as biofilm structures inside used kitchen sponges. Regularly sanitized kitchen sponges did not contain less bacteria than uncleaned ones, which is probably due to rapid recolonization of the sponge tissue by the (few) microorganisms surviving sanitization. The authors further suggested that regular cleaning might even select for higher proportions of potentially pathogenic and malodor-producing bacteria. Interestingly, there is a growing body of evidence that regular domestic cleaning and sanitization procedures might shape domestic microbial communities in a way that is non-beneficial for human health [24].

In the present study, we sought to address this hypothesis by analyzing kitchen sponges, which were either untreated or regularly sanitized by microwaving, through means of metagenomic shotgun sequencing. In addition to the structural characterization of the microbial community, functional capabilities were also examined for the influence of a regular microwave treatment. To the best of our knowledge, our study represents the first metagenomic study on used kitchen sponges, which probably represent the microbially most densely colonized inanimate objects in the domestic environment.

2. Materials and Methods

2.1. Study Design and Sample Collection

Twenty no-brand polyurethane sponges (~$7 \times 3 \times 9$ cm^3) were bought in a store for household articles in Villingen-Schwenningen, Germany. The sponges were distributed to the 20 study participants, including students and academic staff of Furtwangen University as well as private household owners in the greater area of Freiburg (Germany) and Meiningen (Germany). The participants were instructed to use the kitchen sponges "as usual" under normal household conditions for a period of about four weeks. Ten, randomly chosen participants were advised to clean their kitchen sponges regularly two to three times a week by using a standardized microwaving protocol, which was based on previous studies [16–18]. Briefly, the kitchen sponge should first be soaked with tap water containing the participants' own dishwashing detergent. Subsequently, the wet sponge should then be microwaved for 1 min at maximum wattage. After a short cooling phase, the kitchen sponge could be used again. After the study, the used (mostly wet) kitchen sponges were brought to the laboratory in a sterile bag and stored at -20 °C until further analysis.

A small survey was conducted voluntarily and anonymously to obtain information on the usage behavior by participants. Briefly, the kitchen sponges were used on average of 1 to 2 times per day for cleaning mainly dishes as well as kitchen surfaces. The household size ranged between 1 to 5 persons for the uncleaned sponge group and 1 to 7 for the cleaned ones. The maximum power of the microwaves used for sanitation was between 800 to 1200 watts.

2.2. DNA Purification

For DNA extraction, kitchen sponges were cut into halves and the corners and material from the middle part of each half were sampled with upper and lower parts (~2.5 cm^3 in total) using sterile scissors. Cuts of each sponge half were pooled as one sample. Afterwards, DNA was extracted using the ZymoBIOMICS DNA Mini Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's specifications with some modifications. Briefly, after filtration, the Zymo-spin IV spin filter was repeatedly washed with 100 µL of DNAse free water for 3 or 4 times and the flow-through of the different washing steps was collected in separate tubes. To both the flow-through and the initial washing solution, binding buffer was added, and the solutions were successively applied onto a single Zymo-spin IIIC-Z Colum. Subsequently, the extracted DNA was eluted with 50 µL DNase/RNase free water and again purified using a Zymo-spin II column and 50 µL DNase/RNase-free water. After that, DNA concentration was determined with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) using the Qubit dsDNA HS Assay Kit (Thermo Scientific, Waltham, MA, USA). To obtain a higher DNA concentration, all extracts stemming from the same sponge were combined to a single DNA extract of 50 µL by ethanol precipitation [25]. For library preparation, DNA concentration was measured again using the Qubit Fluorometer.

2.3. Library Preparation and Sequencing

The NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) was used to create the library for shotgun sequencing, according to the manufacturer's instructions. To get fragments with an estimated length of 150–300 bp, samples were incubated with the enzyme mix contained in the kit at 37 °C for 15–20 min depending on the input-DNA-concentration (if DNA concentration was <100 ng, incubation time was 15 min; if DNA concentration was >100 ng, incubation time was 20 min). The NEBNext Multiplex Oligos for Illumina (Index Primers Set 1 and 2) (New England Biolabs, Ipswich, MA, USA) were used to create the final DNA libraries. To add adapters to the fragmented DNA, the number of PCR cycles was selected depending on the amount of input DNA as specified in the manufacturer's protocol. Fragment sizes and quality of the final DNA libraries were evaluated using an Agilent Bioanalyzer with the Agilent DNA 1000 Reagent kit (both Agilent, Santa Clara, CA, USA). Finally, all samples were sequenced on an Illumina MiSeq platform, using the Illumina MiSeq v2 Reagent Kit (Illumina, San Diego, CA, USA).

2.4. Bioinformatic Analyses

The unassembled reads were uploaded to the Metagenomic Rapid Annotation using Subsys-tems Technology (MG-RAST) pipeline v.4.0.3 for downstream analyses [26]. Taxonomic and functional profiles were generated using the MD5-based, nonredundant protein database (M5nr) [27] and REfSeq [28,29] for taxonomic classification and the SEED database [30] for functional profiling. To produce annotations, which were close matches to the reference database, the "representative hits classification" with an e-value cutoff of 5, a minimum identity cutoff of 80%, and a minimum alignment length cutoff of 50 bp was used.

Further processing of the data was done using R v.3.5.3 [31] and RStudio v.1.1.463 [32]. The main packages used in the analysis were vegan (v.2.5–6) [33] and phyloseq (v.1.26.1) [34]. The generated table of frequencies was normalized by rarefication to the smallest number of reads among the samples. For statistical analyses, Analysis of Variance (ANOVA) or Wilcoxon–Mann–Whitney-U tests for independent samples were applied. The resulting p-values were adjusted by Benjamin–Hochberg's false discovery rate (FDR) [35]. Adjusted p-values < 0.05 were considered statistically significant. For alpha diversity analysis, the four most common diversity indices (Observed, Chao1, Shannon, and Simpson) were calculated and compared using ANOVA. For determination of beta diversity, taxonomic and functional profiles of samples were visualized by nonmetric multidimensional scaling (NMDS) using the Bray–Curtis distance measure. For further comparisons, Analysis of similarities

(ANOSIM) and Permutational Multivariate Analysis of Variance Using Distance Matrices (ADONIS) were applied based on Bray–Curtis distances.

All sequence data were deposited at the MG-RAST server with the project ID mgp87011 (static link: https://www.mg-rast.org/linkin.cgi?project=mgp87011).

3. Results

3.1. DNA Extraction and Sequence Analysis

Regularly microwaved sponges yielded significantly lower, nevertheless still sufficient, amounts of genomic DNA for metagenomic analyses (Supplementary Table S1). After uploading the sequences to the MG-RAST server, the platform determined a total sequence quantity of 6,486,634 total sequences, with an average sequence length of about 150 bp. After quality control, performed by MG-RAST, 6,003,330 sequences remained (Supplementary Table S1). This corresponds to a loss of approximately 8% of the total sequences. It became apparent that the regularly sanitized kitchen sponges contained a lower quantity of sequences with a smaller average sequence length compared to the untreated kitchen sponges (Supplementary Table S1). Nevertheless, of the sequences remaining after quality control, about 2,248,216 reads could be annotated using the REfSeq database and, further, 625,777 reads could be annotated using the SEED subsystem classification. For further downstream analysis, a random subsampling was performed in both the taxonomic and functional analysis. This resulted in 38,471 sequences per sample for taxonomic analysis and 12,926 sequences per sample for functional profiles. Since unicellular organisms are of higher hygienic relevance, we focused our data analysis only on microorganisms.

3.2. Taxonomic Differences in Community Composition

Using the REfSeq database, the sequences were categorized from domain down to the genus level. After random subsampling, 97.0% of the sequences were assigned to the domain *Bacteria*, and 2.7% of all sequences were affiliated with viruses. Both, *Eukaryota* and *Archaea* showed lower relative abundances (<1%). A comparison between regularly sanitized und untreated kitchen sponges showed no significant differences at this level.

According to classification of MG-RAST, 42 phyla, 80 classes, 149 orders, 286 families, and 578 genera could be determined as members of the microbial community in used kitchen sponges. The most frequently occurring sequences belonged to the phyla *Proteobacteria* (86%), *Bacteroidetes* (7%), and *Actinobacteria* (4%) followed by unclassified viruses (3%). The most frequently identified genera were *Acinetobacter* (22%), *Enhydrobacter* (8%), *Agrobacterium* (6%), *Pseudomonas* (5%), and *Chrysobacterium* (2%) (Supplementary Figure S1). Interestingly, the bacterial community composition was significantly different between regularly microwaved and non-treated sponges (Table 1).

Microwaved sponges showed reduced relative abundances of *Bacteroidetes*, while in contrast, *Proteobacteria* were relatively increased. At the class level, *Gammaproteobacteria* increased in relative abundance due to microwaving, whereas relative abundances of *Betaproteobacteria*, *Flavobacteriia*, and *Sphingobacteriia* were decreased in treated sponges. At the order level, the relative abundances of *Pseudomonadales*, *Aeromonadales*, and *Enterobacteriales* were significantly higher in the microwaved sponges whereas the proportion of *Burkholderiales*, *Sphingobacteriales*, and *Flavobacteriales* was reduced. Similar changes were found at the family level. *Moraxellaceae*, *Pseudomonadaceae*, *Enterobacteriaceae* and *Aeromonadaceae* increased, while *Flavobacteriaceae*, *Brucellaceae* and *Alcaligenaceae* showed a reduction in their relative abundance in the regularly microwaved kitchen sponges. At the genus level, the relative abundances of genera such as *Acinetobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, and *Pseudomonas* were increased while *Bordetella*, *Chryseobacterium*, and *Ochrobactrum* were relatively less abundant in the treated sponges.

Table 1. Significant differences in bacterial community composition between regularly microwaved and untreated kitchen sponges: Relative abundances of taxa with a mean relative abundance of more than one percent per treatment are shown. Wilcoxon–Mann–Whitney-U tests for independent samples were performed to identify statistically significant differences. False discovery rate (FDR)-corrected *p*-values are displayed as well. Color visualizes higher or lower relative abundances and goes from green (0%) over yellow to red (100%).

Taxonomy Level	Organism	Microwaved (%)	No Treatment (%)	*p*-Values
Phylum	*Bacteroidetes*	2.0	12.9	0.009
	Proteobacteria	92.0	79.2	0.013
Class	*Sphingobacteriia*	0.2	1.2	0.008
	Flavobacteriia	1.6	11.2	0.009
	Gammaproteobacteria	71.3	33.6	0.009
	Betaproteobacteria	2.7	7.5	0.031
Order	*Sphingobacteriales*	0.2	1.2	0.006
	Flavobacteriales	1.5	11.2	0.011
	Aeromonadales	2.0	0.2	0.006
	Enterobacteriales	19.2	3.7	0.006
	Pseudomonadales	48.1	24.1	0.035
	Burkholderiales	2.4	7.2	0.035
Family	*Sphingobacteriaceae*	0.2	1.1	0.011
	Flavobacteriaceae	1.5	11.0	0.018
	Aeromonadaceae	2.0	0.2	0.011
	Enterobacteriaceae	19.2	3.7	0.011
	Moraxellaceae	40.6	21.7	0.046
	Pseudomonadaceae	7.6	2.4	0.023
	Alcaligenaceae	0.3	3.6	0.023
	Brucellaceae	0.6	4.9	0.031
Genus	*Chryseobacterium*	0.5	3.6	0.011
	Riemerella	0.2	1.1	0.015
	unclassified *(Flavobacteriaceae)*	0.3	4.8	0.011
	Aeromonas	1.9	0.2	0.011
	Citrobacter	2.7	0.3	0.011
	Salmonella	1.4	0.3	0.011
	Escherichia	2.0	0.4	0.011
	Enterobacter	5.8	0.9	0.011
	Klebsiella	5.7	1.4	0.018
	Acinetobacter	34.6	8.4	0.011
	Pseudomonas	7.4	2.4	0.020
	Bordetella	0.1	1.0	0.011
	Achromobacter	0.2	2.6	0.024
	Brucella	0.2	2.0	0.024
	Ochrobactrum	0.5	2.9	0.033
	Caulobacter	0.4	4.4	0.043

The most frequent type of viral DNA belonged to the genus *Microvirus*, which had a total mean relative abundance of 2.7%. However, only the order *Caudovirales* was significantly different between the untreated and sanitized sponges, with higher relative abundance in the latter. Only methanogens were found within the *Archaea* with *Methanococcoides* (total mean relative abundance 0.001%), *Methanoregula* (total mean relative abundance 0.01%), and *Methanosarcina* (total mean relative abundance 0.03%) being the most frequent genera. Notably, *Methanosarcina* showed a significantly higher relative abundance in regularly sanitized kitchen sponges when compared to the untreated sponges. Further significant differences in *Eukaryota*, *Archaea*, and viruses are presented in Table 2.

Table 2. Significant differences in eukaryotic, archaeal and viral community composition (according to classification of Metagenomic Rapid Annotation using Subsystems Technology (MG-RAST)) between regularly microwaved and untreated kitchen sponges: Mean relative abundances of taxa per treatment are shown. Wilcoxon–Mann–Whitney-U tests for independent samples were performed to identify statistically significant differences. FDR-corrected *p*-values are displayed as asterisks ($p < 0.01$ (**) and $p < 0.05$ (*)).

Kingdom		***Eukaryota***				***Archaea***				**Viruses**		
Taxonomy Level	**Organism**	**Microwaved (%)**	**No Treatment (%)**	**Sig.**	**Organism**	**Microwaved (%)**	**No Treatment (%)**	**Sig.**	**Organism**	**Microwaved (%)**	**NoTreatment (%)**	**Sig.**
Class	unclassified (*Eukaryota*)	0.000	0.004	*	none	-	-	-	none	-	-	-
Order	unclassified (*Eukaryota*)	0.000	0.004	*	*Methanosarcinales*	0.008	0.000	*	*Caudovirales*	0.064	0.025	*
	unclassified (*Eukaryota*)	0.000	0.004	*	*Methanosarcinaceae*	0.008	0.000	*	*Myoviridae*	0.028	0.005	*
Family	*Metschnikowiaceae*	0.003	0.000	*					*Podoviridae*	0.010	0.001	*
									Siphoviridae	0.027	0.018	*
	Acanthamoeba	0.000	0.004	*	*Methanosarcina*	0.005	0.000	*	Lambda-like viruses	0.008	0.003	*
Genus	*Clavispora*	0.003	0.000	*					P2-like viruses	0.027	0.002	*
	Aspergillus	0.000	0.004	*					T7-like viruses	0.006	0.001	*
									unclassified (*Podoviridae*)	0.003	0.000	*

3.3. Differences in Community Structure

To determine differences in diversity and community structure of the whole data set, the most common alpha diversity (Observed, Chao1, Shannon, and Simpson) indices were used (Figure 1A). All indices revealed differences between the microwaved and untreated used kitchen sponges regarding microbial diversity. More precisely, regularly microwaved sponges tended to have lower richness and diversity. Statistical analysis by ANOVA revealed that especially the indices Observed (ANOVA: $p = 0.036$) and Chao1 (ANOVA: $p = 0.006$) were significantly different, while the other alpha diversity indices (Shannon and Simpson) showed no significant influence of microwave treatment (ANOVA: $p_{\text{Shannon}} = 0.105$, $p_{\text{Simpson}} = 0.119$).

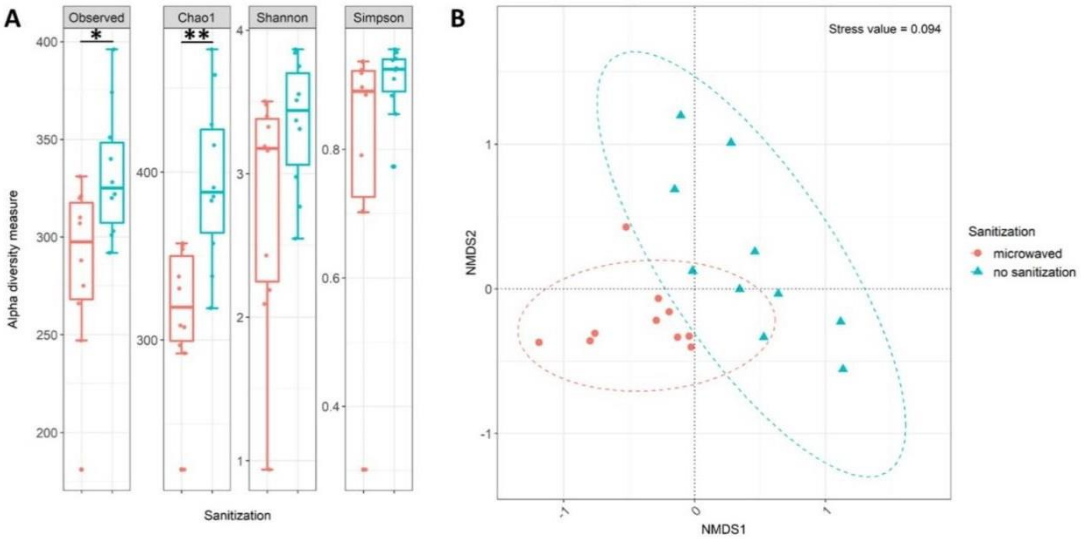

Figure 1. Taxonomic diversity analysis of used kitchen sponges calculated from the abundance table of all sequences at genus level: (**A**) Alpha diversity measures based on the four most common indices. Box plots show median as well as lower and upper quartiles. Each dot represents an individual sample. Whiskers represent minimum and maximum spread. Statistical analysis was done using ANOVA, and resulting FDR-corrected *p*-values are displayed as asterisks. The significant codes are $p < 0.01$ (**), $p < 0.05$ (*), and ( ) = not significant. (**B**) nonmetric multidimensional scaling (NMDS) plot using the Bray–Curtis distance measure of analyzed kitchen sponges. Color indicates sanitation treatment: regularly microwaved kitchen sponges (red) and untreated kitchen sponges (blue). Ellipses (dotted lines) represent the 95% confidence interval of each sanitization treatment.

The beta diversity comparison suggested significant differences in microbial community composition between microwaved and untreated sponges. For this, NMDS ordination was used for graphical representation (Figure 1B). The NMDS plot revealed that the samples clustered according to their treatment, albeit with some overlay and considerable scattering within each cluster. However, a separation by treatment was confirmed through further statistical analysis using ANOSIM (ANOSIM: $R = 0.471$, $p = 0.0001$). Similar to ANOSIM, the ADONIS ($p = 0.001$, $R^2 = 0.278$) analysis also indicated that the microbial composition of the two groups of sponges was statistically different.

3.4. Differences in Functional Annotation

Next, for investigating the metabolic potential of the sponge communities, the uploaded sequences were compared to the hierarchical SEED database. Approximately 10% of the sequences could be attributed to a potential metabolic function. After random subsampling, the metagenomic reads could be categorized into three SEED categories. The determined 28 subsystems of level 1 could be broken down further into 187 SEED level 2 categories and 940 specific functions at SEED level 3. Based on the

mean relative abundances across all samples, the most common metabolic categories were *carbohydrates* (12.2%), *clustering-based subsystems* (11.6%), *amino acids and derivatives* (11.0%), and *protein metabolism* (8.6%). Regularly microwaved and untreated sponges showed significant differences in potential metabolic functions (Figure 2).

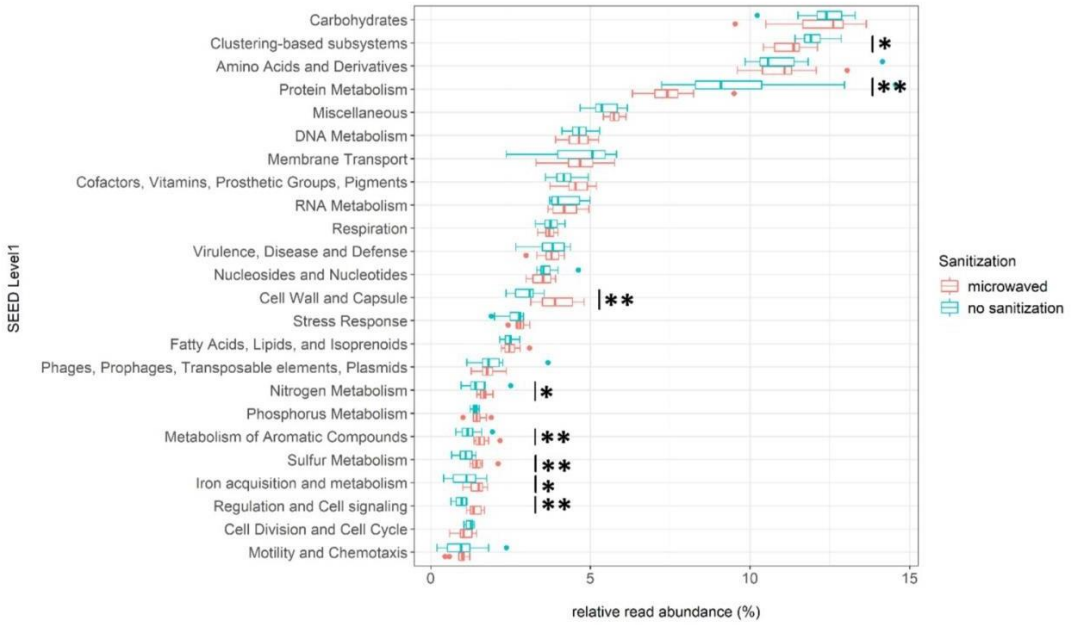

Figure 2. Functional profiles at SEED subsystem level 1 of sanitized and unsanitized used kitchen sponges: For better visualization, only the subsystems with a mean relative abundance greater than 1% are displayed. The boxes show median as well as lower and upper quartiles. Whiskers represent extremes outside upper and lower quartiles. The statistical comparison between microwaved and untreated kitchen sponges was done using Wilcoxon–Mann–Whitney-U tests for independent samples. Asterisks indicate subsystems that, in comparison, show a significant difference in relative abundance. The significant codes are $p < 0.01$ (**), $p < 0.05$ (*), and ( ) = not significant.

For example, the relative abundances of genes for *regulation and cell signaling* (Wilcoxon: $p = 0.001$) increased from 0.9% in untreated kitchen sponges to 1.4% in microwaved sponges. The same applied to the subsystems for *cell wall and capsule* (Wilcoxon: $p = 0.004$) and *sulfur metabolism* (Wilcoxon: $p = 0.006$), where the relative abundances increased in treated kitchen sponges from 3% to 4% and from 1.1% to 1.5%, respectively.

Other SEED categories, such as *metabolism of aromatic compounds* (Wilcoxon: $p = 0.006$, untreated: 1.2%, microwaved: 1.6%), *nitrogen metabolism* (Wilcoxon: $p = 0.020$, untreated: 1.5%, microwaved: 1.7%), and *iron acquisition and metabolism* (Wilcoxon: $p = 0.031$, untreated: 1.1% microwaved: 1.5%) also showed significant differences.

The relative abundance of genes belonging to *protein metabolism* (Wilcoxon: $p = 0.006$) and *clustering-based subsystems* (Wilcoxon: $p = 0.012$) decreased from 9.8% to 7.5% and from 12.0% to 11.2%, respectively, in regularly microwaved kitchen sponges.

An overview of significantly differing SEED level 2 and 3 categories for all major SEED level 1 categories that showed significant differences between treated and untreated sponges (Figure 2) is provided in Supplementary Table S2.

Comparison of alpha and beta diversity based on annotated functions revealed differences between untreated and regularly sanitized kitchen sponges. Alpha diversity, for example, showed a trend towards an increased functional diversity in regularly microwaved sponges (Figure 3A). However, statistical analysis with ANOVA revealed that these differences were not significant. A comparison of

the functional properties of SEED subsystem level 3 using NMDS displayed only minor differences between sanitized and untreated kitchen sponges with cluster overlaps and considerable scattering within each cluster (Figure 3B). A statistical analysis using ANOSIM (R = 0.21, $p = 0.0028$) confirmed the significance of the differences between the treatments. By using ADONIS ($p = 0.006$, $R^2 = 0.165$), a significant influence of sanitization was also observed.

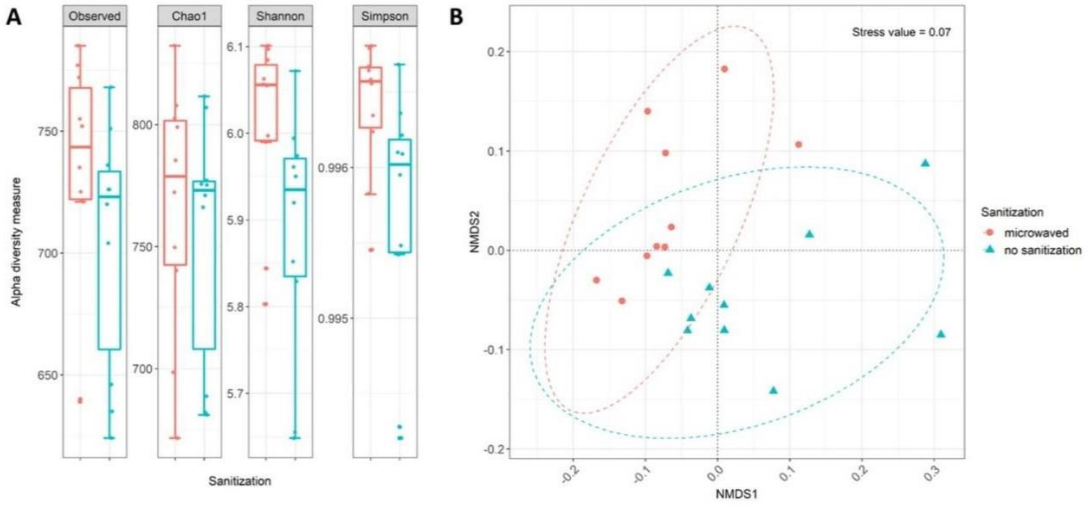

Figure 3. Diversity analysis of used kitchen sponges based on abundance table at SEED subsystem level 3: (**A**) Alpha diversity measures based one the four most common indices. Box plots show median as well as lower and upper quartiles. Each dot represents an individual sample. Whiskers represent minimum and maximum spread. (**B**) NMDS ordination for functional properties using the Bray–Curtis distance measures. Color indicates sanitation treatment: regularly microwaved sanitized kitchen sponges (red) and untreated kitchen sponges (blue). Dotted lines display ellipses, which represent the 95% confidence interval of each sanitization treatment.

4. Discussion

Several previous studies addressed the microbial colonization of domestic kitchen sponges and showed that these widespread household items harbor a high bacterial load and a diverse bacterial population [1,6,7]. Some studies have demonstrated that different sanitization methods can significantly reduce this microbial load in the short term [16,17]. However, long-term effects of such sanitization methods on microbial community composition and particularly functionality are largely unknown. In this study, we analyzed ten regularly microwaved and ten untreated kitchen sponges by means of metagenomic shotgun sequencing to explore how regular disinfection affects microbial community composition and metabolic properties of the kitchen sponge community.

Our metagenomics analysis revealed that, in addition to prokaryotes (bacteria and archaea), viruses also represent a quantitatively important part of the kitchen sponge microbial community. The viruses found most frequently in this study were mainly bacteriophages, such as the genus *Microvirus* or the order *Caudovirales* [36]. Bacteriophages are one of the most common biological entities and are found wherever bacteria can grow [37]. Therefore, in line with the high bacterial abundance found in kitchen sponges, viral relative proportions were also expected to be abundant. More importantly, bacteriophages can have a considerable influence on the structure and function of microbial communities, such as species distribution [38].

In the present study, further members of the kitchen sponge community belonged to the domains of *Archaea* and *Eukaryota*. Regarding the domain *Eukaryota*, only microbial taxa were considered here, as sequences affiliated with multicellular organisms probably represent contaminating DNA from human, food, or other environmental sources, which are of minor hygienic relevance. Typical

representatives of *Eukaryota* in the investigated kitchen sponges were different types of yeasts and molds. These have already been identified in other studies [5]. Within the archaeal domain, mainly methanogenic archaea were identified in kitchen sponges. Normally, this archaeal group needs an anoxic environment. However, it has been shown that especially the genus *Methanosarcina* contains species that can tolerate oxygen to a certain extent, which may be one reason why it represented the most abundant archaeal genus found in our study [39]. However, overall the relative abundance of *Archaea* and *Eukaryota* was low (<1%). Future studies with more detailed analyses of *Archaea* and *Eukaryota* in kitchen sponges will be crucial to identify whether these microbial groups merely represent minor important contaminations or if they are of hygienic relevance, too.

Microwave treatment of biological tissue causes thermal and nonthermal effects due to microwave radiation [23]. The data presented in this paper provides evidence that regularly microwaving also influences microbial community composition as well as functional profiles. Microbial diversity patterns of regularly microwaved and untreated sponges were clearly different. Microwaved kitchen sponges tended to have a lower alpha diversity of community composition than untreated sponges. Evaluating relative abundances at different taxonomic levels for sanitized and untreated kitchen sponges revealed that, in particular, *Gammaproteobacteria* benefited from a regular microwave treatment. Other classes, such as *Betaproteobacteria* or *Flavobacteriia*, decreased in their relative abundances. This trend implied a selection for certain bacterial populations by regular microwave sanitization, as was hypothesized by Cardinale et al. [6]. The genera that were relatively increased the most after microwave sanitization included *Acinetobacter*, *Klebsiella*, *Enterobacter*, and *Pseudomonas*. Further analyses are required to evaluate the pathogenic potential within these genera, since the use of the MG-RAST platform does not allow reliable taxa identification at species level.

Microwave radiation is also able to cause a variety of alterations within the metabolism of a cell and thereby might cause selective pressure on microorganisms [19]. The significant increase of genes affiliated with the subsystem *cell wall and capsule* observed in the regularly sanitized kitchen sponges might serve as an example. This subsystem includes genes for Gram-positive and Gram-negative cell wall components as well as capsular and extracellular polysaccharides and may be a hint for adaptive alterations in biofilm formation. It was shown that thermal stress can weaken the integrity of a microbial biofilm, which is therefore more easily sheared off from a contaminated device [40]. It should also be noted that biofilm formation is a vital characteristic for microbial survival under extreme conditions, such as heat stress [41]. The ability to form biofilms might be one cause for the positive selection of *Gammaproteobacteria*, since the genera detected at increased relative abundance in microwaved sponges here, such as *Acinetobacter* and *Pseudomonas*, are very well-known biofilm-formers [42,43].

In addition, increased relative abundances of genes belonging to the metabolism of sulfur, iron, or aromatic compounds might also be due to the higher shares of *Gammaproteobacteria*, as they possess a very versatile metabolism with respect to these substrates [44].

Since sulfur is an important component of many malodorous substances, such as H_2S, the observed relative increase in genes affiliated with sulfur metabolism might be carefully interpreted as a higher potential towards malodor formation. However, this hypothesis clearly needs to be corroborated by physiological data, including direct measurement of malodorous substances as well as environmental parameters, such as oxygen or pH, which influence microbial activities in this respect [45–47].

Despite the noticeable variability within each sanitization group, likely caused by different environmental conditions in the participants' households, there was a significant difference between them. Follow-up studies should therefore include more samples and should be more standardized. With our experimental design, it cannot be ruled out that the observed effects not only were caused by the microwave treatment but also might result from other factors differing between the two groups of participants. It is therefore important, for further studies, to collect more user and usage metadata and to define the experimental conditions (microwaving parameters, dishwashing detergent used for soaking, etc.) as precisely as possible in order to ensure a better comparability and to verify the hypotheses put forward here. Follow-up studies should particularly address the relevance of

microwave-induced changes in microbial community structure and function for domestic hygiene, human health, and well-being. In our view, pathogenic potential, biofilm formation capacity, and sulfur metabolism/malodor production appear of particular interest in this context.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/5/736/s1. Figure S1: Relative counts of regularly microwaved and un-treated used kitchen sponges summarized at phylum and genus level, Table S1: Summary statistics of the sequence data of unsanitized and regularly sanitized used kitchen sponges uploaded to MG-RAST, Table S2: Overview of the significantly differing level 3 functions from the level1 SEED subsystems that were found to be significantly different between treated and untreated sponges.

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8. Preliminary experiments and unpublished results

The processes underlying laundry odour formation are far from being fully understood, although this is a highly relevant problem in household laundry (Zinn et al. 2021). In order to deepen the knowledge of microorganisms involved in laundry odour formation and the negative odours associated with them, metabolomics and the RNA-SIP method were introduced here as new tools to study odour formation on textiles and in washing machines.

8.1. SIP experiments

The aim of the SIP experiments conducted here were to identify compounds that might serve as substrates for microorganisms on washed laundry, and subsequent malodour formation. To implement the RNA-SIP technology for laundry samples, unlabelled glucose and fully ^{13}C-labelled glucose were used as cheap and easily available model substrates.

To ensure optimal conditions for microbial growth, 120 cm^2 cotton fabric samples were washed in a mild wash cycle with worn ballast laundry. After washing, fabric samples were incubated in a "wet chamber" for 48 h or 72 h to simulate retention in a washing machine. After incubation, the fabric pieces were cut into approximately 8 x 3 cm fabric strips with sterile scissors under sterile conditions and incubated in centrifuge tubes containing M9 minimal medium with 2 % of uniformly labelled ^{13}C-glucose or unlabelled ^{12}C-glucose for 2 h, 4 h, 6 h, 8 h, and 24 h, respectively. Preliminary tests showed that only the 24 hour incubation provided a sufficient amount of RNA of about 100 ng/µl for ultracentrifugation. RNA from the 24 h incubation was extracted from the textile strips using a phenol/chloroform protocol and the RNA extraction protocol also used for the metatranscriptome analyses (Jacksch et al. 2021). Isopycnic ultracentrifugation of isolated RNA was performed in a caesium trifluoroacetate buffer without formamide (Weis et al. 2020). Initial tests showed RNA separation and thus a detectable density gradient of RNA by isopycnic ultracentrifugation, which should be further confirmed and investigated (Figure 7). Additionally, adding formamide to the gradient buffer might further enhance RNA denaturation in future experiments (Weis et al. 2020). Formamide is an effective denaturant at room temperature, which, according to spectroscopic criteria, inhibits both the base pairing and the stacking of the single strands (Pinder et al. 1974).

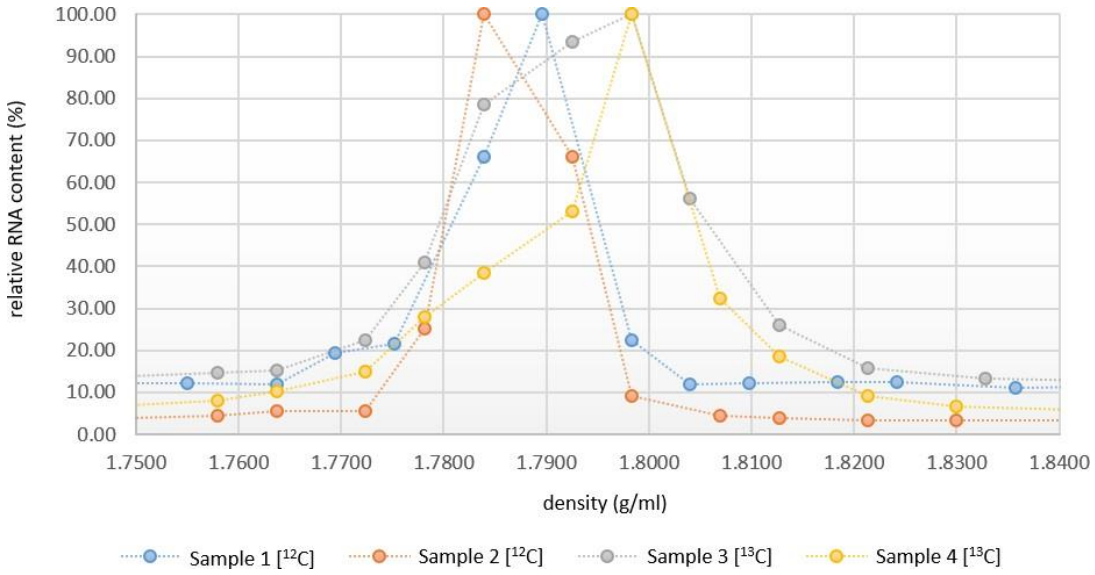

Figure 7. Density gradients and RNA contents of one of the washing experiments. RNA from textile samples was extracted after incubation in a wet chamber, incubated with unlabelled glucose and uniformly ^{13}C-labelled glucose, respectively, and separated by ultracentrifugation using CsTFA density gradients, resulting in a density dependent distribution. After re-isolation of the RNA from the different fractions, the RNA content of these fractions was quantified by a low-range Ribogreen assay and given as relative RNA content. In this figure, a clear separation is visible, the ^{13}C samples show a maximum RNA content at a density of 1.798 g/ml, whereas the RNA content of the ^{12}C samples peaks 1.783 g/ml for sample 1 and 1.789 g/ml for sample 2.

8.2. Metabolomics

In order to characterise the metabolic properties of bacteria isolated from washing machines and washed laundry, an experiment was launched in collaboration with the Metabolomics group at Furtwangen University (Lars Kaiser, Hans-Peter Deigner). More specifically, four bacterial species (*Micrococcus luteus*, *Pseudomonas oleovorans*, *Staphylococcus epidermidis*, and *Moraxella osloensis*), previously identified as abundant bacterial representatives of washing machines, were selected (Jacksch et al. 2019; Jacksch et al. 2020). Reference strains from the DSMZ as well as washing machine isolates were used. The bacteria were incubated in rich LB medium or M9 minimal medium supplemented with 37.5 % detergent solution from a domestic washing cycle and 1 g/l casamino acids. The detergent solution was obtained from a short programme at a wash temperature of 40 °C with two to three kilogram mixed ballast load (T-shirts, jeans, underwear, and socks) using 30 to 40 ml liquid detergent and was subsequently sterile filtered before use.

Depending on the growth medium used, the mixtures were incubated for either 24 h (for LB medium) or 48 h (M9 medium) at room temperature until an increase in optical density was detectable. After centrifugation, the supernatants of the media were analysed using the "AbsoluteIDQ p180" kit from Biocrates (Innsbruck, Austria) on a 4000 QTRAP mass spectrometer (AB SCIEX LLC, Framingham, MA USA), connected to a NexteraXR HPLC (Shimadzu, Kyoto, Japan) to establish a metabolic profile. Preliminary data analyses suggest an increase and decrease of several metabolites compared to the reference medium without bacteria. Differences were also observed between DSMZ strains and environmental isolates from washing machine. Interestingly, metabolites with potential importance for odour formation, e.g. putrecine, phenylethylamine, and methionine sulphoxide, were detected. Moreover, it turned out that even the reference medium (M9 medium with detergent solution and without bacteria) already contained bad odour substances like methionine sulphoxide, which likely originated from the worn textiles or were produced during the washing cycle.

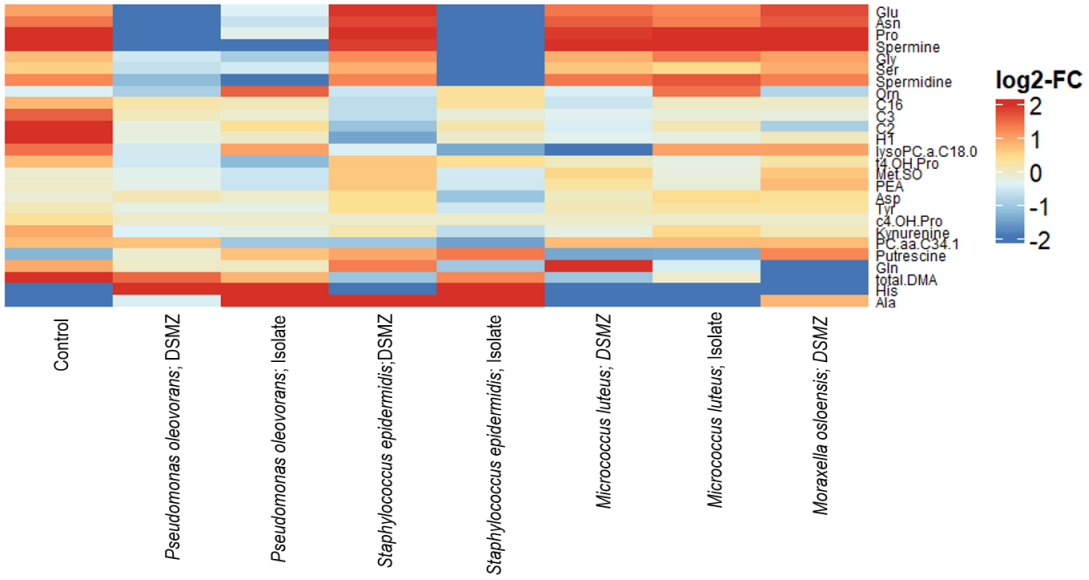

Figure 8. Preliminary statistical analysis of the differences in metabolite composition of samples incubated in LB medium. The heat map shows changes in metabolite composition of the four test bacteria incubated with LB medium when statistically comparing all washing machine isolates and DSMZ strains using an empirical Bayes approach (Casella George 1985; Gotelli and Ulrich 2010). By that, a total of 26 metabolites with an absolute log fold change (LogFC) > 1 and an adjusted p-value < 0.05 can be identified from this data set. The LogFC represents the difference between the two logarithmised mean values.

9. Synoptic discussion of published results

The aim of this thesis was to investigate changes in community composition and functionality (gene expression, metabolism) of microbial communities in different household environments under the influence of different consumer-induced variables, in order to better understand their potential role for household hygiene. The investigations were performed with various omics-methods, i.e. metataxonomics, metagenomic shotgun sequencing, and RNA sequencing using samples from objects with high hygiene relevance in typical households, namely washing machine, washed laundry, and used kitchen sponges.

9.1 Microbial colonisation of washing machines

In the first study on the microbial colonisation of washing machines (Jacksch et al. 2019), 21 household machines in use were examined at various sampling sites (detergent drawer, door seal, sump, and fibres from the washing solution) using 16S rRNA gene pyrosequencing. A comparison of the different alpha diversity parameters for determining the structural diversity of individual sampling sites showed that the detergent drawer had the highest alpha diversity with a high evenness in contrast to the rubber door seal, which had the lowest alpha diversity with a relatively low evenness. Similar observations were made by a study conducted by Nix and colleagues, which also identified *Proteobacteria*, *Actinobacteria,* and *Bacteroidetes* as the main bacterial representatives in washing machines (Nix et al. 2015). In contrast to their study, which was limited to only two sampling sites (door seal and detergent drawer), we additionally analysed laundry fibres, collected from the detergent solution, and the sump and were able to identify *Firmicutes* and *Acidobacteria* as further members of the washing machine microbial community.

Overall, with 229 species-like OTUs, the investigated washing machines showed a very diverse bacterial community that was mainly composed of typical aquatic bacteria, such as *Pseudomonas,* or skin bacteria such as *Moraxella* and *Acinetobacter,* both of which belong to the human skin microbiota (Al-Khoja and Darrell 1979; Kwon et al. 2011; McLellan et al. 2015). These microorganisms have also been identified in other studies as the main representatives in washing machines and on washed laundry alongside *Flavobacterium* sp., *Sphingomonas* sp., *Brevundimonas* sp., and *Enhydrobacter* sp. (Babič et al. 2015; Callewaert et al. 2015; Nix et al. 2015). However, their relative abundance varies in the different studies, probably due to differences in methodology, but also due to the wealth of factors influencing

microbial colonisation in washing machines and on laundry, such as the user's microbiome, the used water source, or the applied washing conditions (Callewaert et al. 2015).

Interestingly, the composition of the microbial community showed certain spatial patterns that probably depend on the prevailing conditions at each site (Babič et al. 2015; Callewaert et al. 2015; Nix et al. 2015). This applies, for example, to *Brevundimonas* sp., which was more abundant in the detergent drawer in our study, probably due to its ability to survive at extreme pH and other unfavourable conditions, but also to withstand a medium dose of sodium percarbonate, which is used as an oxidizing agent in detergent powders (Dartnell et al. 2010; Pan et al. 2018; Savage et al. 2016; Toninelli 1978). In contrast, *Moraxella* and *Acinetobacter* sp. were relatively common on door seals, maybe due to their ability to tolerate desiccation, but less common in the detergent drawer, where the bacteria must be particularly tolerant to detergent ingredients such as bleach, surfactants, or fragrances (Jawad et al. 1998; Kubota et al. 2012; Savage et al. 2016; Rojas-Herrera et al. 2015). The frequent occurrence of *Moraxella* sp. in the door seal could be particularly relevant for consumers, as *Moraxella osloensis* was linked to odour formation on laundry (Munk et al. 2001; Stapleton et al. 2013; Takeuchi et al. 2013). Hence, regular cleaning of the door seal might help preventing laundry and machine malodour formation.

Another statistically significant correlation was shown between the average number of wash cycles per month at a temperature > 60 °C and the bacterial community composition. Interestingly, a higher number of hot wash cycles seemed to increase alpha diversity in the detergent drawer, but not at the other sampling sites. The temperature in the washing machine, however, is not evenly distributed throughout the machine due to the water flow and the position of the heating element, which is usually at the bottom of the washing drum (Bertocco et al. 2020; Ortega et al. 2019; Volckmann-Kinzel 2008). Consequently, temperature probably has the greatest influence on the amount and diversity of microorganisms directly in the washing drum. In contrast, due to the limited heat transfer from the washing drum to the surrounding components (Bertocco et al. 2020), less heat reaches the detergent drawer and might even beneficially influence microbial diversity there.

Apart from the sampling location and the number of washes per month at higher temperature, no other correlations were found for factors such as machine age, odour perception from the machine or the washed textiles, use of fabric softener, powder, or liquid detergents etc. Clearly, larger studies with more homogeneous datasets are needed to verify that such factors really do not influence microbial community composition in washing machines (Lemos et al. 2011).

However, molecular studies often have limited resolution in terms of taxonomic classification (Poretsky et al. 2014). During this thesis it also turned out that quantitative data on the microbial load of washing machines is scarce. So far, only Stapleton and coworkers reported the microbial load of different sampling sites, albeit only for four household washing machines (Stapleton et al. 2013). Therefore, we used classical cultivation to perform species-level analyses and to determine the actual microbial load of 10 washing machines at the detergent drawer and the rubber door seal (Jacksch et al. 2021).

The quantitative data from this study showed that household washing machines are significantly contaminated with cultivable bacteria. Averaged over all sampling sites, microbial cell counts of ~ 21,000 colony-forming units (CFU) per cm^2 were calculated. The lowest bacterial counts were found in the upper area of the rubber door seal, probably because water drains off very quickly here and is therefore no longer available for microbial growth. If water availability is reduced, a decrease in microbial growth rate and cell yield occurs (Sperber 1983). In contrast, at sampling sites with high water availability, such as detergent drawer chamber, detergent drawer, and the bottom part of the door seal, microbial counts were around 10^4 CFU/cm^2. It is important to note, that our quantitative data regarding the door seal match those of Stapleton and co-workers quite well (~ 10^3 to 10^4 CFU/cm^2). However, microbial counts for the detergent drawer differ (~ 10^1 to 10^3 CFU/cm^2) (Stapleton et al. 2013).

A subsequent MALDI-TOF-based identification of isolated microorganisms proved that, similar to our study in 2019 (Jacksch et al. 2019), the sampled microbial community was strongly dependent on the sampling site and composed of representatives of *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* (Jacksch et al. 2019). With MALDI biotyping, a reliable identification of microorganisms on species level is possible, based on the protein fingerprints obtained from ribosomal proteins (Patel 2013; Wieser et al. 2012). However, identification of subspecies is more complex and needs a larger number of biomarkers that are not well represented in the commercially available databases (Croxatto et al. 2012). Here, the pathogenic potential of the identified microorganisms was evaluated (estimated) on species level using the classification into biosafety risk groups of the Federal Institute for Occupational Safety and Health (BAuA – German Federal Institute for Occupational Safety and Health 2015, 2016). Based on this classification, it was revealed that more than 50 % of the identified species at each site were closely associated with risk group 2 organisms. Although these microorganisms pose little risk to the general public, they could pose a potential health risk to immunocompromised individuals, pregnant women, or the elderly (Fritz et al. 2018; Montville

2012). Furthermore, the high prevalence of risk group 2 organisms also underlines the hygienic importance of the detergent drawer compartment, which should therefore be cleaned regularly in addition to the rubber door seal.

Our molecular and culture-dependent studies both consistently identified several microorganisms (*Pseudomonas oleovorans*, *Acinetobacter parvus*, *Moraxella osloensis, Rhizobium radiobacter, Micrococcus luteus*, and *Staphylococcus epidermidis)* as frequent washing machine colonizers. Due to their frequent occurrence in both studies, they can be considered as ideal model microorganisms to examine certain aspects of washing machine hygiene, e.g. to study biofilm or malodour formation in more detail. Each of the genera mentioned has the ability to form biofilms in different environments (Buswell et al. 1997; Drenkard and Ausubel 2002; Espinal et al. 2012; Fenner et al. 2019; O'Gara and Humphreys 2001; Zhu et al. 2014). Furthermore, *Moraxella osloensis, Micrococcus luteus,* and *Staphylococcus epidermidis* have been associated with the development of malodour on laundry or washing machines (Callewaert et al. 2014; Chung and Seok 2012; Kubota et al. 2012; van Herreweghen et al. 2020; Zinn et al. 2021).

9.2 Metatranscriptomic analysis of washed textiles

With the study "Metatranscriptomic analysis of microbial communities on laundered textiles – a pilot case study" from 2021 (Jacksch et al. 2021), RNASeq was established for the first time in the field of washing machine and laundry hygiene and its data used to analyse gene expression on washed cotton and polyester fabric pieces. The main objective of this study was to gain a deeper understanding of the functionality and hygienic relevance of the laundry microbiota, especially in relation to the consumer-relevant problem of odour formation.

The biggest challenge in this study was to extract sufficient mRNA for library preparation and subsequent RNA sequencing. As already mentioned, washing processes can massively reduce the bacterial concentration on textiles (three to six log levels) (Bloomfield et al. 2017). To overcome this, relatively mild washing conditions were used and the remaining microorganisms were allowed to multiply by incubation in a "wet chamber". Another challenge was that RNA is generally very sensitive to degradation which can affect RNA yield and quality (Arraiano et al. 2010). The washing machine environment with its ballast laundry, detergents, water etc. provides many resources for unwanted cellular material. Contaminants that have been shown to interfere with the successful extraction of undegraded RNA can be polysaccharides, enzymes, or secondary metabolites that mix with the RNA during cell lysis (Chen et al. 2000;

Chirgwin et al. 1979; Slater 1985). Similar problems have been observed in metatranscriptomic studies from other habitats (Carvalhais et al. 2012; Tveit et al. 2014). To avoid this, we used phenol with sodium dodecyl sulfate (SDS) in cell lysis to minimise RNA degradation, as both phenol and SDS are strong protein denaturants and inhibitors of RNases (Ghawana et al. 2011).

After overcoming these challenges, mRNA could be successfully sequenced from all incubation samples and we were able to detect a wide range of Gene ontology (GO) terms from all three functional categories, indicating an active and diverse microbial gene expression on the textiles studied. In addition, a differential gene expression analysis showed that there were differences in gene expression between the individual tissue types. In total, differences in bacterial gene expression between cotton and polyester tissues were found for 17 genes, affecting various biochemical metabolic pathways such as amino acid transport or metabolism or bacterial carbohydrate metabolism. Significantly differentially expressed genes associated with bacterial carbohydrate metabolism such as "sucrose-6-phosphate hydrolase", suggests that the textiles, especially those made from natural organic fibres, might serve as a source of nutrients for the resident bacteria. Cotton, for example, is a plant fibre consisting mainly of cellulose, which must be broken down through hydrolytic enzymes to release glucose (Bhat and Bhat 1997). In contrast, however, polyester as a synthetic fibre is less susceptible to bacterial degradation (Callewaert et al. 2014; Szostak-Kotowa 2004). In the future, information about gene expression and the affected metabolic pathways could help to find solutions for consumer-relevant problems such as malodour or biodeterioration of textiles. Possible solutions might be new detergent formulations affecting microbial gene expression or special processing to make natural fibres less susceptible to bacterial decomposition (Sanders et al. 2021).

Nevertheless, the approach described in our paper (Jacksch et al. 2021) is only a first step and several adjustments need to be made to increase the relevance to practice. For example, the mild washing conditions (mild and short programme (only 30 °C), light spin cycle, underdosed detergent) provided to promote microbial survival do not correspond to the conditions commonly used in practice when washing clothes in the machine, but rather to those of a hand wash. In addition, the protocol should be improved to further reduce the incubation time in the "wet chamber". In order to obtain meaningful statistical data, a larger number of samples, including samples from different washing machines, is required (Conesa et al. 2016; Kumar and Blaxter 2010; Martin and Wang 2011). Furthermore, the bioinformatic downstream analysis has to be adapted, since the ExN50 values show that the individual assemblies only provide an incomplete overview of bacterial gene expression, with a discrimination of the low

expressed genes (Sahraeian et al. 2017). This could possibly be remedied by merging the assemblies in order to include low expressed ones (Kumar and Blaxter 2010). Nevertheless, the assemblies produced were of good quality (good read representation, good BUSCO values) and thus definitely allow first insights into gene expression (Simão et al. 2015). In addition, despite different assembly strategies, the use of GO terminology achieved a high level of consistency that allows comparison with other metatranscriptome studies (Riesgo et al. 2012).

9.3 Effects of microwave radiation on used kitchen sponges

Kitchen sponges pick up and spread microorganisms on a massive scale, which can lead to cross-contamination of kitchen utensils, surfaces, and food (Nibedita et al. 2020). Simple sanitisation procedures should prevent this and allow the sponge to be used for a longer period of time (Ikawa and Rossen 1999; Rossi et al. 2012; Sharma et al. 2009). However, it is hypothesized that domestic cleaning and sanitisation methods might have negative side effects such as stimulation of malodour formation or the accumulation of potential pathogens (Foster 2007; Kelley and Gilbert 2013).

In our study (Jacksch et al. 2020), we used metagenomic shotgun sequencing to investigate how regular disinfection of used kitchen sponges by microwaving might affect the microbial community, as it is assumed that such methods might negatively affect microbial communities in the long term, e.g. by enriching human pathogenic microorganisms due to stress exposure (Foster 2007; Kelley and Gilbert 2013). We showed that regular sanitisation indeed exerted a selective pressure that influenced not only microbial community composition but also its genetic potential. The microwaved sponges tended to have a lower alpha diversity in community composition than untreated sponges, with a shift towards a dominance of *Gammaproteobacteria,* especially *Acinetobacter*, while *Betaproteobacteria* or *Flavobacteriia* decreased in their relative abundances. More importantly, possible pathogenic bacteria like *Citrobacter*, *Salmonella,* or *Klebsiella*, were also increasing in their relative abundance, which supports the hypothesis of accumulation of pathogenic bacteria due to stress exposure (Galhardo et al. 2007; Kelley and Gilbert 2013).

Contrary to structural diversity, the functional diversity tended to increase when treated with microwave radiation. These results seem to confirm Foster's hypothesis that there are different adaptive responses to stress, which might increase genetic variability (Foster 2007). In our study, regularly sanitised sponges showed an increase in the SEED subsystems "*regulation and*

cell signalling", "*cell wall and capsule*", "*sulphur metabolism*", "*metabolism of aromatic compounds*", "*nitrogen metabolism*", and "*iron acquisition and metabolism*". Other subsystems, however, decreased in regularly sanitised sponges, such as "*protein metabolism*" or "*clustering-based subsystems*". However, it is not clear whether these changes are triggered directly by thermal or non-thermal effects of microwave radiation, as reviewed by Jankovic and co-workers, or by changes in microbial community composition (Jankovic et al. 2014).

Further closer examination of the significantly altered SEED subsystem "*cell wall and capsule*" at deeper levels suggested that microwave treatment may induce adaptive changes in biofilm formation, whose formation can be interpreted as a "protective clothing" to various environmental factors, such as heat or radiation (Yin et al. 2019). In fact, FISH images from a recent study by Cardinale and co-workers already suggested that the bacteria in kitchen sponges indeed form biofilms (Cardinale et al. 2017). The same study also suggested that regular cleaning increased the proportion of malodour forming bacteria (Cardinale et al. 2017). This might be reflected also in our study, where genes involved in "*sulphur metabolism*" were relatively enriched.

9.4 General conclusions and outlook

The main objective of the studies presented here was to gain a deeper understanding of how environmental factors shape microbial communities in domestic environments, using commonly used items in the domestic environment, namely washing machine, laundry, and kitchen sponges.

In case of the washing machine, the studies presented show that these are highly contaminated household items that contain a diverse microbiota with more than 200 species of bacteria. Both our molecular and culture-dependent studies showed that bacterial diversity is highly site-dependent and shaped by local environmental conditions. More importantly, washing machines are significantly contaminated with cultivable bacteria, including a considerable amount of potentially pathogenic ones. Furthermore, we were able to show that an active bacterial community is present on washed cotton and polyester fabrics, showing a diverse bacterial gene expression, that differs between cotton and polyester fabrics. The differences found between the different textiles types were related to several metabolic pathways, such as carbohydrate metabolism.

In order to investigate the metabolic potential of the microbial community and to address hygienic and consumer-relevant problems such as odour formation, we started establishing additional methods such as metabolomics and RNA-SIP for even more functional characterisations. Because the formation of malodours on laundry and in the washing machine is a significant problem influenced by many factors, models close to practice are needed to study the development of odours (Zinn et al. 2021). In combination with such models, methods such as metabolomics and RNA-SIP appear as particularly important tools to provide new conclusions about possible sources of malodour precursors (Steuer et al. 2019). When used in the field of laundry and washing machine hygiene, however, these methods require further methodical adaptations.

Nevertheless, initial indications were provided by our preliminary metabolome study in which we aimed to find out which of the selected bacteria (*Micrococcus luteus*, *Pseudomonas oleovorans*, *Staphylococcus epidermidis*, and *Moraxella osloensis*) are capable of producing malodorous substances under practical conditions. In this study it became apparent that washing water already contains substances, which can be associated with bad odours. They are probably already present on the laundry and released from the clothes during washing (Braga and Varesche 2014; van Herreweghen et al. 2020). Surprisingly, when incubated with typical washing machine bacteria, these compounds might also be used as a source of nutrients rather than only being formed. Clearly, further investigation is needed to find out how this relates to bad odour on laundry and in washing machines. Notably, if precursors of malodours or malodorous substances can be identified, they may be used as substrates in future RNA-SIP experiments to determine which microorganisms in the complex microbial community on laundry particularly assimilate these substances.

To test the applicability of SIP, we incubated washed textiles with unlabelled ^{12}C and labelled ^{13}C-glucose for 24 h. First results show detectable density differences between light and heavy RNA that need to be further confirmed and investigated. Clearly, further adaptation of the protocol is needed, e.g. regarding substrate concentrations and incubation times. In addition, the use of labelled substrates which are more specific to washing machines and laundry should be considered, such as tallow, dimethyl disulphides, isovaleric acid, or sweat compounds. Then also RNA-SIP might deliver valuable data to link the structure and function of microbial communities on washed laundry (Chen and Murrell 2010; van Herreweghen et al. 2020; Zinn et al. 2021). In general, SIP experiments have already been used in several other fields and can directly link metabolic functions to distinct members of a microbial community (Radajewski et

al. 2000). Additional mRNA sequencing could further reveal active metabolic pathways of the microbial community (Lueders 2018).

With regard to the kitchen sponge metagenomic study, we were able to confirm that kitchen sponges are mostly colonised by bacteria, but sequences of viruses, archaea, and eukaryotes were also found, whose hygienic relevance still needs to be evaluated. Furthermore, it could be proven that microwave treatment has an influence on the microbial community. It was shown that kitchen sponges regularly disinfected in the microwave contained a reduced microbial alpha-diversity and higher proportions of genera that may have pathogenic potential, e.g. *Acinetobacter*, *Enterobacter*, and *Pseudomonas*. Microwave sanitization also increased the metabolic potential of the present microorganisms.

It is important to mention that metagenomics shotgun sequencing can only assess the functional potential, but not whether such genes are actually expressed under the respective conditions (Campanaro et al. 2016). Thus, metatranscriptome studies should be the next step to assess whether these changes are likely to have an impact on household hygiene or human health.

All in all, information on the metabolism of microorganisms in household appliances such as washing machines, laundry, or kitchen sponges can provide important insights into how microorganisms adapt and behave in the domestic environment. Such information will also aid the development of novel hygiene strategies with targeted efficacy and higher sustainability.

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