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POLYNUCLEOTIDE PROBE BASED, TAXON-SPECIFIC DETECTION AND ENRICHMENT OF FECAL BACTERIA

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ABSTRACT

The gut microbiome represents a key contributor to mammalian physiology, metabolism, immune function, and nutrition. The understanding of the composition and genetics of the gut microbiota under certain conditions is essential to gain insights into how microbes function individually, in a specific population, and in the complex dynamic network. Metagenomic analysis is increasingly used to study the intestinal microbiota by granting access to the genomic information of the gut microbiome that is not yet cultivable. However, for certain scientific questions it may suffice to examine in a targeted manner, only a taxon-specific sub-metagenome, *e.g. Enterococcus*. The targeted genomic characterization and in-depth analysis of certain subpopulations could open the door for progression in various fields of research and application, *e.g.* in development of therapeutics.

Here, a new variant of fluorescence *in situ* hybridization combined with flow cytometry (FISH-FACS) was established, providing access to taxon-specific sub-metagenomes of *Enterococcus* spp. belonging to the complex community of the intestinal microbiota. The key aspect of this protocol is the use of genus-specific polynucleotide probes for bacterial labeling, instead of standard oligonucleotide probes. The polynucleotide probe (polyDIII) used here, which targets domain III of the 23S rRNA, extends the resolution power in environmental samples by increasing signal intensity, and hence allows for more reliable detection of low-abundance species. Furthermore, compared to signal amplification by *e.g.* CARD-FISH, cells hybridized with this polynucleotide probe are not subjected to harsh pretreatments, and their genetic information remains intact. The protocol described here was adjusted to genus-specifically hybridize cells in fecal samples, and to purify the positively polynucleotide-labeled enterococcal cells by flow cytometric sorting. To detect and quantify enterococci in fecal samples prior to enrichment, taxon-specific PCR and qPCR detection systems have been developed.

It was successfully shown that polynucleotide probes could be used to label *Enterococcus* cells in various samples for subsequent cell sorting by flow cytometry. This was evaluated by applying fecal samples from different laboratory mouse strains, harboring diverse intestinal microbiota properties. Sorting purity was verified by microscopic examination. Significantly, this approach validated that the genomes of 10^6 genus-specifically detected and sorted bacteria were still accessible and sufficiently intact for subsequent amplification of functional genes, and the construction of gene libraries.

ZUSAMMENFASSUNG

Darmbakterien spielen bei Mensch und Tier eine bedeutende physiologische und immunologische Rolle. Um einen besseren Einblick in strukturelle und genetische Zusammenhänge des intestinalen Mikrobioms und seiner Veränderungen unter gewissen Bedingungen zu erlangen, ist es notwendig den funktionellen Anteil einzelner Mikroorganismen, bzw. Populationen, in diesem komplexen und dynamischen Ökosystem zu untersuchen. Metagenom-Analysen zur genaueren Untersuchung der intestinalen Mikrobiota werden in zunehmendem Maße eingesetzt und erlauben den Zugang zu der genetischen Ausstattung des intestinalen Mikrobioms, einschließlich der vielen bislang nicht kultivierbaren Mikroorganismen. Für gewisse wissenschaftliche Fragestellungen kann es jedoch ausreichend sein, gezielt nur eine spezifische intestinale Subpopulation zu untersuchen, z.B. einer Gattung wie *Enterococcus*. Die gezielte genomische Charakterisierung von bakteriellen intestinalen Subpopulationen könnte den Weg für weitere Fortschritte und neue Entwicklungen in verschiedenen Forschungs- und Anwendungsgebieten ebnen, wie z.B. für therapeutische Anwendungen.

In dieser Arbeit wurde ein vielversprechendes Verfahren etabliert, das die Fluoreszenz-*in-situ*-Hybridisierungsmethode mit der Durchflusszytometrie kombiniert (FISH-FACS). Die neu entwickelte Anwendung dieser Methode ermöglicht es, das taxon-spezifische Sub-Metagenom von *Enterococcus* spp. innerhalb des komplexen intestinalen Mikrobioms zugänglich zu machen. Der zentrale Aspekt liegt dabei in der Verwendung von genus-spezifischen Polynukleotid-Sonden zum Markieren der Zielbakterien, an Stelle von Oligonukleotid-Sonden. Die hierfür verwendete Polynukleotid-Sonde (‚polyDIII') hat als Zielbindungsregion die Domäne III der 23S rRNS und erhöht das Auflösungsvermögen durch gesteigerte Signalintensität. Die Signalstärke stellt bei der Verwendung von Oligonukleotid-Sonden in Umweltproben oft ein Problem dar und lässt Spezies, die im Darm unterrepräsentiert sind, möglicherweise unerkannt. Darüber hinaus werden Zellen, die mit der Polynukleotid-Sonde hybridisiert werden, nicht derart aggressiven Bedingungen ausgesetzt, wie z.B. bei der Signalverstärkung von Oligonukleotid-Sonden durch CARD-FISH, so dass ihre Erbinformation nach wie vor erhalten bleibt. Das hier entwickelte Protokoll wurde angepasst, um genus-spezifisch Zellen aus Fäzesproben durch Hybridisierung zu markieren und anschließend die polynukleotid-markierten Enterokokken unter kontrollierten Bedingungen mittels Durchflusszytometrie anzureichern. Um Enterokokken vorab in intestinalen Proben

nachzuweisen und zu quantifizieren, wurden in diesem Projekt taxon-spezifische PCR und qPCR Nachweissysteme entwickelt.

Anhand von verschiedenen Ausgangsmaterialien, u.a. Fäzes von Labormäusen mit unterschiedlicher Darmmikrobiota-Zusammensetzung, konnte erfolgreich gezeigt werden, dass Polynukleotid-Sonden zur Markierung und durchflusszytometrischen Anreicherung von Enterokokken aus komplexen Proben verwendet werden können. Die Effizienz der Anreicherung wurde durch mikroskopische Analysen verifiziert. Es wurde zudem nachgewiesen, dass 10^6 genus-spezifisch detektierte und angereicherte Bakterien ausreichend sind, um funktionelle Gene zu amplifizieren und um Genbibliotheken zu erstellen.

A. INTRODUCTION

1. GUT MICROBIOTA

Our intestinal system is exposed to many challenges such as diet, consumption of alcohol, individual stress, lifestyle, use of antibiotics, or toxic chemicals in food, water, or environment. The intestinal microbiota of an individual is composed of approximately 10^{13}–10^{14} microorganisms (ten-fold more than human body cells) [1], comprising around 1,800 genera, 15,000–36,000 species and a shared genome consisting of at least 100 times as many genes as are found in the human genome [2–5]. The microbial composition differs and the density increases along the sections of the gastrointestinal (GI) tract (stomach and duodenum: 10^{1}–10^{4} cells/mL; jejunum, ileum and cecum: 10^{4}–10^{8} cells/mL; colon and feces: 10^{10}–10^{12} cells/g). This is effected by the different physicochemical conditions in each distinct anatomical region along the GI tract which exert a selective pressure on the microbiota [6, 7]. This high biodiversity and huge genetic potential provides a wide range of metabolic functions related to human hosts, including carbohydrate metabolism, energy metabolism and storage [8, 9], as well as angiogenesis [10]. Therefore, the gastrointestinal microbiome was recently described as the "forgotten organ" due to the variety of metabolic functions for the host [4, 11]. Additionally, the microbiome is known to regulate the human immune system [12] and to prevent infections by pathogens [13]. Events that disrupt the commensal stability in the gut, *e.g.* by antibiotic treatment, may facilitate infections due to reduced biodiversity and altered composition [14]. This may lead to an increase of antibiotic-resistant organisms that normally are limited in propagation due to the healthy intestinal microbiota [15]. Furthermore, it has been suggested that microbial dysbiosis (altered balance of the intestinal microbiota) can be significant concerning obesity [16, 17], diabetes [18], inflammatory bowel disease [19], ulcerative colitis, Chron's disease [5], and also allergy symptoms [20].

Previous studies of the gastrointestinal microbiota were mostly focused on pathogenic organisms. Nevertheless, research on the influence of commensal microbiota, or their disruption effecting human health is increasingly in the center of interest. The healthy gut microbiota composition has been shown to vary greatly between individuals, each having relatively stable and unique formations of intestinal communities [21–25], dominated by only two phyla, *Firmicutes* and *Bacteroidetes*, followed by minor proportions of *Actinobacteria*, *Proteobacteria*, *Fusobacteria,* and *Verrucomicrobia* [23]. By large-scale sequencing, Arumugam *et al.* [26] recently suggested three robust "enterotypes" of human individuals, independent of origin and health

status, that are defined primarily by species and functional composition. Detailed understanding of the complex host-microbe interactions would allow improved treatment of associated diseases, *e.g.* by manipulating the microbiome with fecal bacteriotherapy [27], or by promotion of probiotics for suppression of pathogenic overgrowth [28]. Still, the microbial composition and influence on human health remains complex and its whole-scale analysis in individuals is challenging.

2. TECHNIQUES TO STUDY MICROBIOTA DIVERSITY

For studying complex and diverse environmental communities in greater depth, independent of cultivation, a variety of molecular techniques have been developed that can be categorized into two major groups depending on their genomic resolution: partial community analysis (*e.g.*, 16S rRNA profiling, qPCR, and fluorescence *in situ* hybridization) and whole community analysis, which attempt to investigate all the genetic information in the extracted DNA (*e.g.*, whole genome sequencing, conventional metagenomics, proteogenomics) [29, 30].

2.1. IMPACT OF NON-CULTURABLE MICROORGANISMS

The majority (~99%) of the microbial diversity on earth is estimated not to grow under lab conditions and therefore remain inaccessible to further analysis [31]. This huge gap is known as the 'great plate count anomaly' and was discovered based on the observation that viable counts on plates are significantly lower than corresponding microscopic counts [32]. These uncultivated bacteria were distinguished in two types of states: unknown species that have never been cultured before for lack of suitable methods (yet-to-be-cultivated cells), and known bacteria that are viable but non-culturable (VBNC), *i.e.* stagnating in a non-replicating state, with unknown cultivation conditions [31, 33]. It is suggested that many of these uncultivable bacteria are living in interspecies symbiosis based on nutrition exchange, and may require growth promoting factors (*e.g.*, siderophores) from other bacteria in their natural habitat [34, 35]. Indeed, the highest percentage of uncultivable cells was observed in nutrient-rich environments (*e.g.*, feces), decreasing in nutrient-poor environments (*e.g.*, deserts) [36]. Like the complex soil and marine ecosystems, the commensal gut microbiota ecosystem has formerly been investigated thoroughly by culture-based techniques [6, 37]. However, researchers are concerned that culture-based methods of microbiota analysis may underestimate the biodiversity of natural populations [38, 39], as it is estimated that 60% to 80% of the total human gut microbiota cannot be cultivated under standard laboratory conditions [23, 40]. This means that

microorganisms of key importance to the community and the environment may be missed and inaccessible to further analysis and understanding of complex ecosystem interactions. In this context, greater demands will be made in further development and adaption of techniques to study environmental microbiota, including the non-culturable microorganisms.

2.2. METAGENOMICS

The rise of culture-independent molecular techniques started with the polymerase chain reaction (PCR) [41] decades ago, which allowed more rapid characterization of the gastrointestinal microbiota and other environments. These culture-independent methods were based on targeted sequencing of the 16S and 23S ribosomal RNA genes used for taxonomic characterization [42–44]. Due to their ubiquitous presence in all prokaryotes, their functional constancy, slow evolutionary progression and the existence of conserved and variable domains that allow distinguishing different taxa, these ribosomal genes are presently the most comprehensively investigated phylogenetic markers for bacteria. Meanwhile, various rRNA databases for comparative sequence analysis are available [45–50]. Sequence analysis of the 16S rRNA genes of the gut microbiome was applied successfully in the 1990s by Sanger sequencing of clone libraries [22, 51, 52], and until today, it is one of the basic procedures in intestinal metagenome projects [4, 23, 26]. However, analysis of cloned DNA has largely been replaced by next-generation sequencing (NGS) of DNA extracted from environmental samples. This was accompanied by a loss of sensitivity in 16S rRNA gene profiling, but an increased depth of sampling and cost-savings enabled the publication of a wide range of data on the human microbiome [53]. Targeted single-gene studies (primarily 16S rRNA) are mainly used for comparative studies facing microbial community structures associated with individual hosts or treatment conditions [54–56]. However, targeted ribosomal gene studies suffer from a lack of functional and genetic information that could characterize host-microbe interactions, and moreover previous DNA amplification may introduce biases. Despite these drawbacks, deep sequencing is necessary to reach also less abundant species which still can have an effect on the community and the host [57].

The methodological progress of whole genome shotgun sequencing (WGS) enabled to establish a gene catalogue of a whole community and allowed for more accurate determination of the relative abundance of bacteria. The mixture of genomes that make up the metagenome is sheared randomly into small fragments and then reassembled by use of reference genomes, or by *de novo* prediction. These gene catalogues are typically matched to databases such as KEGG

(Kyoto Encyclopedia of Genes and Genomes) [58], which categorizes gene products into pathways and processes [4, 17, 25, 59–61]. However, even the application of metagenomic technology has been widely used in other environment such as soil and the sea, it is still at its beginning of studying the human gut microbiome [16, 25, 62–66]. Using the WGS approach for complex environmental samples remains difficult and poses several challenges. Beside a vast amount of metagenomics data with sequencing read length restrictions, which demands professional bioinformatic analysis, the huge bacterial diversity and genomic heterogeneity within species might complicate assembling and prediction of composite genomes. This may make classification unreliable, especially for organisms for which no reference genome is available, *i.e.* uncultivated species, or organisms that are less abundant [67–69]. Furthermore, for a meaningful understanding of microbial physiology the interpretation of genes in pathways in a full genomic context is required, but by now metagenomics data rather reflects community-level characteristics than species-specific functions [68, 70]. Another present restriction for metagenomics from the gut is the need of high quality DNA samples in sufficient quantity, currently influenced by a high risk of human contamination [53]. Some limitations were recently diminished by various advances in binning technology [71–74]. A promising metagenomic tool published by Nielsen *et al.* [72] aided assembly of microbial genomes without the need of reference sequences based on clustering of genes according their abundance in a sample. Methodological improvements like this could facilitate future *de novo* assembly in order to taxonomic and functional characterize unknown species. In combination with the rapid development of metatranscriptomics [75, 76] and with data achieved by, *e.g.* proteomics or metabolomics [30, 77, 78], the functional activity of microbial communities as well as host-microbiome interactions could be identified [79]. Although present approaches of the meta-omics still have their limitations, they were shown to have enormous potential to understand the human gut microbiome, and to provide novel clinical diagnosis and treatments [80].

2.3. TARGETED ENRICHMENT

To obtain additional information about functional couplings of uncultivable strains, various methods have been developed to physically decrease the number and diversity of bacteria within complex samples and thereby increase the resolution power. One can differentiate between two approaches: Enrichment of single cells and targeted enrichment of a specific population/microconsortia. Especially the characterization of complex microbial communities by flow cytometry (FCM) which is termed ‘cytometric fingerprinting’, is increasingly developing as it can be used to not only detect taxonomic, but also functional changes in environmental

microbial populations. However, compared to the wide utilization in eukaryotic cell analysis, microbial FCM applications are still commonly rare and an overall process standardization is yet not established [81, 82].

2.3.1. SINGLE-CELL GENOMICS

To acquire genomes of individual uncultured species, isolation and sequencing of single cells out of a mixed community is a possibility not only to expand the catalogue of reference genomes, but also to describe intra-species diversity and to characterize these uncultured bacteria by defining their core genes. As mentioned, a large pool of reference genomes is required for correctly assembling and linking metagenomic sequences assessed by NGS to taxonomic and functional classifications (A.2.2, p. 4). In 2010, the 'Human Microbiome Jumpstart Reference Strains Consortium' reported the analysis of nearly 200 microbial genomes from cultured bacteria of the human microbiome in order to establish a reference genome database [83]. Now, the Human Microbiome Project (HMP) plans to sequence 3,000 genomes from both cultured and uncultured bacteria, isolated from different body sites (http://hmpdacc.org/reference_genomes/reference_genomes.php).

The gene diversity within a species (pan-genome) is crucial to identify biological functions, *e.g.* infectivity or development of antibiotic resistance [76, 83]. After all, the approach of single-cell genomics (SCG) benefits from independency of difficulties arising by composite data from multiple cells. The workflows of single-cell whole-genome-sequencing need custom sample preparation depending on sample types and study design. Cell isolation is most commonly performed by laser capture microdissection (LCM), microfluidics, micromanipulation and limiting dilution technology, or by fluorescence-activated cell sorting (FACS) by flow cytometry (FCM) [70, 84–87]. Modern whole genome amplification (WGA) methods and genomic library preparation procedures are facing the low yield of sorted cells in targeted enrichment [85, 88]. This is usually a key step in single-cell sequencing, as a single bacterial cell typically contains only 10^{-15} g (or 1 femtogram) DNA, but most sequencing workflows require 1–500 ng of genomic input material [70]. One of the PCR-independent state-of-art methods is called Multiple Displacement Amplification (MDA), first introduced in 2002 by Dean *et al.* [89]. It allows for $>10^9$-fold amplification of the genomic input material, which is sufficient for subsequent genome sequencing.

Summarizing, the approach's advantage over conventional metagenomics is that it directly links obtained genomic data in a taxonomic context and that the data can be used to complete

reference genome databases. Furthermore, even underrepresented cells can be isolated species-specifically by labeling them fluorescently using a phylogenetic marker (*e.g.*, 16S rRNA) [88]. On the other hand, single-cell genomics is often limited by the incomplete recovery of genomes as they are frequently fragmented or unevenly amplified [90, 91]. The establishment and improvement of single-cell genomics, especially from environmental samples, is just at the beginning. Hence, by now only few studies of single-cell isolation from gut microbiota consortia have been published, *e.g.* from mice [92, 93] and honey bees [94].

2.3.2. POPULATION ENRICHMENT

Due to the high complexity of the gut microbiome, its whole-scale and in-depth analysis either via single-cell genomics, or via random metagenome sequencing remain laborious. However, for certain scientific questions it would be sufficient to examine in a targeted manner only a taxon-specific microconsortia/submetagenome, *e.g.* the intestinal species belonging to one genus of interest such as *Enterococcus*, *Bacteroides* or *Akkermansia*. This targeted physical enrichment of cells out of environmental samples can be performed by various techniques, which allow subsequent metagenomic analysis including assembly of composite genomes.

One of the first automated enrichment techniques used fluorescence-activated cell sorting (FACS) by flow cytometry (FCM) combined with molecular analysis through fluorescence *in situ* hybridization (FISH) with fluorescent oligonucleotide probes and DNA staining. In the 1990s, Wallner *et al.* [88, 95, 96] used this method for the identification of rare microorganisms from the marine and terrestrial environments. This approach is based on light scattering properties of cells (size, shape and density) and the detection of fluorescently-labeled cells. It offers parallel characterization of microorganisms according to physical and also biochemical aspects. In contrast to standard FISH analysis, a widely used technique for monitoring and identifying microorganisms in complex ecosystems, the combination of FISH and FACS (FISH-FACS) is capable of not only counting, but also of sorting specific microbial communities at high throughput [95, 97].

The combination of FCM and rRNA-targeted probes for phylogenetic analysis has been applied to various sample types, *e.g.* pure cultures [97, 98], activated sludge [96], lake water [88], marine phytoplankton [99], bacterioplankton [100, 101], as well as human feces [102–107]. The 16S rRNA gene molecule as a target was shown to provide a beneficial natural enhancement of a fluorescent signal as it is described as a multi-copy gene product containing 10^4–10^5 copies during the exponential phase of growth. Hence, it benefits the detection of even low-abundance

species [95, 108, 109]. Nevertheless, the FCM application to mixed bacterial populations is still challenging, as in contrast to eukaryotic cells, bacteria have a fairly small size and usually are not uniquely delineated by measured properties [110]. Therefore, regardless of being labeled with a fluorophore, size can limit their detection. Additionally, when applied to environmental samples, oligonucleotide probes targeting rRNA sequences often show decreased signal intensities, and hence are often undetectable above background or autofluorescence of non-target cells [97, 111, 112].

To find an inexpensive alternative to FCM, Stoffels *et al.* [113] developed a culture-independent enrichment technique, adapted and improved by Zwirglmaier *et al.* [114, 115] using biotin- and fluorescein-labeled polynucleotide probes, respectively. These taxon-specific rRNA targeted polynucleotide probes were first generated in 1985 by Schleifer *et al.* [116]. Because of its reduced specificity and complex synthesizing technologies compared with that of oligonucleotides, polynucleotide probes became less important for identification techniques. However, Trebesius *et al.* [117] discovered a special feature of polynucleotide probes targeting domain III (DIII) of the 23S rRNA, comprising *Escherichia coli* positions [118] 1366 to 1617 . These multiple-labeled ssRNA probes with a size of approx. 250–300 nucleotides were characterized by relatively high variability in contrast to the general conservation of the 23S rRNA gene, and therefore were predominantly genus-specific. Compared to mono-labeled oligonucleotide probes, these probes exhibited very strong hybridization signals even if detecting microbes with a low ribosome content. Furthermore, the polynucleotide probes were shown to detect a larger percentage of cells in environmental samples in comparison to oligonucleotide probes [112, 117]. The key feature of the polynucleotide probe ‘polyDIII’ targeting domain III of the 23S rRNA was found to be its ability to produce a halo signal surrounding the cell. This phenomenon was explained by Zwirglmaier [114] as the ‘network hypothesis’. It was suggested, considering the large number of identical probe molecules in a hybridization, that only few probes are partially bound to the target rRNA inside of the cell and serve as anchor, while simultaneously using their secondary structures to bind and build a chain of complementary probes reaching through the cell envelope. In order to enable the formation of such a network, high probe concentrations (10–100 times higher than in basic oligoFISH) and specific conditions for cell membrane permeabilization were necessary. Despite this, the concept was independent of the copy number of target molecules and also might offer enrichment possibilities for cells with low ribosome contents, *e.g.* slow growing natural populations [109, 112, 114].

Research related to polynucleotide probes and its features additionally led to the development of a FISH variant named RING-FISH. RING stands for Recognition of INdividual Genes, but

also indicates the characteristic halo appearance of the fluorescence signals at the bacterial cell periphery. The technique allowed for detection of plasmid-encoded and even chromosomal single-copy genes [119–121].

The mentioned targeted enrichment technologies can be considered as community analysis tools that can aid in gaining an extended insight into the sequence information from the collective genomes by targeting a selected submetagenome within a complex microbial consortia, *e.g.* the gut microbiome. The polynucleotide probes targeting domain III of the 23S rRNA provided the foundation of the thesis project. The techniques developed here, exploit the genus specificity and signal amplification of the inter-probe network in the cell's periphery, while in addition, the genomic DNA should remain sufficiently intact for, *e.g.* subsequent metagenomic analysis.

3. THE GENUS ENTEROCOCCUS

As a proof of principle, all protocols in this thesis were adapted to the genus *Enterococcus*. Enterococci are Gram-positive, facultative anaerobic cocci, with broad distribution in environmental microbial communities, *e.g.* soil [122], water [123], plant environments [124], food [125] and also ubiquitous in GI tracts [125, 126]. The major cause of their wide dissemination might be the ability to survive harsh conditions including a broad range of pH values and temperatures, as well as hypotonic and hypertonic conditions [127].

Although approx. 60% of fecal solids are bacteria, the physiological relevance of facultative anaerobes such as the enterococci was thought to be small in the intestinal environment, because their concentration in feces seemed to be very low (7% of total bacteria in feces) compared to dominant anaerobe microbiota (44%) [128, 129]. Nevertheless, it was found that in a densely populated part of the GI tract, the cecum (10^8 CFU/mL), facultative anaerobes like enterococci constituted the dominant microbiota (~50%) compared to anaerobes, *e.g. Bacteroides* and *Clostridium*. This suggests that enterococci might have an underestimated important physiological role in their intestinal niche such as fermentation of dietary fibers and endogenous substrates, or that they have a functional relevance for colonic diseases such as ileocecal Crohn's disease [129].

Thus, although the genus *Enterococcus* refers to the sub-dominant intestinal bacteria ($<10^9$ CFU/mL) [130, 131] and typically constitutes a relatively small proportion (<1% of total adult intestinal microbiota) [23, 132], it includes some of the most important nosocomial multidrug-resistant organisms that cause urinary tract and bloodstream infections [6, 132–134]. On

the other hand, enterococci naturally occur in raw meat and dairy products, and became an essential ingredient in fermented foods [135]. However, enterococci as probiotics were rejected because of missing safety and efficiency studies [136].

Regardless of their medical importance, only few enterococcal genomes have been sequenced until now. Presently, the HMP reference genomes database (http://hmpdacc.org/catalog) lists merely 109 draft genomes for the genus *Enterococcus*. All of which except six genomes were derived from mostly clinical *E. faecalis* and *E. faecium* isolates, which are the most frequent enterococcal species in the GI tract [134]. Importantly, enterococci possess a variety of mobile genetic elements such as plasmids, transposons, and a large pathogenicity island. Plasmids serve as a major reservoir of antibiotic resistance genes, which can be disseminated to other species. In addition, these mobile genetic elements harbor genes for bacteriocins and numerous virulence factors like aggregation substance proteins, surface exclusion proteins, extracellular surface proteins, cell wall surface anchor family proteins, cytolysin, toxins, and sex pheromones [137–140].

B. AIM OF THE THESIS

The aim of this thesis was the establishment of methods for the targeted enrichment of the metagenomes of certain taxa (*i.e.*, *Enterococcus* spp.) from complex gut consortia. Based on previous studies of our work group, the methods should be developed using nucleic acid based detection by polynucleotide probes targeting domain III of the 23S rRNA.

For this purpose, an experimental strategy with the following bullet points was formulated and adapted to the model genus *Enterococcus*:

- Selection of the polynucleotide probe and verification of its specificity
- Improvement of the method for immobilization in microplate cavities
- Development of several taxon-specific detection and quantification techniques
- Adaption of methods to fecal environment
- Development of alternative enrichment techniques
- Amplification and sequencing of functional genes from enriched cells, including uncultured gut microbiota.

Long-term aims are:

- The in-depth pangenomic characterization of the targeted taxa with respect to variation in individual hosts, including functional genomic information.
- The analysis of intra-taxa changes in the composition and functionality of microbes under different conditions (*e.g.*, in response to diet or inflammation).
- The analysis of intra-taxa changes in the composition and functionality under the influence of competing or probiotic strains.

C. MATERIAL & METHODS

Note: Enzyme solutions were prepared freshly. Buffers and other solutions were prepared with water from a Milli-Q-plus-system (Merck Millipore, Germany) and autoclaved at 121°C and 1 bar excess pressure for 20 minutes unless stated otherwise.

1. ORGANISMS

Table 1 shows organisms used in this work. Intestinal or clinical relevant enterococci are highlighted in gray color. If not stated otherwise, whenever *E. faecalis* is mentioned it refers to the *E. faecalis* type strain DSM 20478 T.

Table 1. Organisms used in this work

Organism	Strain	Growth Medium	Growth conditions	Biosafety Level
Enterococcus asini	DSM 11492 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus avium	DSM 20679 T	TSB, BHI	37°C, microaerophilic	2
Enterococcus caccae	DSM 19114 T	TSY	37°C, microaerophilic	1
Enterococcus canis	CCUG 46666 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus casseliflavus	DSM 20680 T	TSY	37°C, aerobic	2
Enterococcus cecorum	DSM 20682 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus columbae	DSM 7374 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus dispar	DSM 6630 T	TSB, BHI	37°C, microaerophilic	2
Enterococcus durans	DSM 20633 T	TSB, BHI	37°C, microaerophilic	2
Enterococcus faecalis	ATCC 29212	TSB, BHI	37°C, microaerophilic	2
Enterococcus faecalis	DSM 20478 T	TSB, BHI	37°C, microaerophilic	2
Enterococcus faecium	DSM 20477 T	TSB, BHI	37°C, microaerophilic	2
Enterococcus flavescens → *E. casseliflavus*	DSM 7370	TSB, BHI	37°C, microaerophilic	2
Enterococcus gallinarum	DSM 24841 T	TSB, BHI	37°C, microaerophilic	2
Enterococcus gilvus	DSM 15689 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus haemoperoxidus	LMG 19487 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus hirae	DSM 20160 T	TSB, BHI	37°C, microaerophilic	2
Enterococcus malodortaus	DSM 20681 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus mundtii	DSM 4838 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus pallens	CCUG 45554 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus phoeniculicola	DSM 14726 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus porcinus → *E. villorum*	CCUG 43229 T	TSY	37°C, aerobic	2
Enterococcus pseudoavium	DSM 5632 T	TSB, BHI	37°C, microaerophilic	2
Enterococcus raffinosus	DSM 5633 T	TSB, BHI	37°C, microaerophilic	2

Organism	Strain	Growth Medium	Growth conditions	Biosafety Level
Enterococcus ratti	CCUG 43228 T	TSB, BHI	37°C, microaerophilic	2
Enterococcus saccharolyticus	DSM 20726 T	TSY	37°C, aerobic	1
Enterococcus sulfreus	DSM 6905 T	TSB, BHI	37°C, microaerophilic	1
Enterococcus villorum	LMG 12287 T	TSB, BHI	37°C, microaerophilic	2
Control organisms				
Acinetobacter haemolyticus	ATCC 17906 T	M53	30°C, aerobic	2
Akkermansia muciniphila	DSM 22959 T	BHI-S	37°C, anaerobic	1
Alcaligenes faecalis	DSM 30033	LB	30°C, aerobic	2
Bacillus subtilis	Intern Collect. W23	BHI, M53	30°C, aerobic	1
Bacteroides eggerthii	DSM 20697 T	BHI-S	37°C, anaerobic	2
Corynebacterium glutamicum	DSM 20300 T	M53	30°C, aerobic	1
Enterobacter aerogenes	Intern Collect.	M53	37°C, aerobic	2
Escherichia coli	DSM 30083 T	BHI, M53	37°C, aerobic	2
Lactobacillus intestinales	DSM 6629	MRS	37°C, anaerobic	1
Lactococcus lactis ssp. lactis	DSM 20481 T	MRS	30°C, anaerobic	1
Micrococcus luteus	DSM 20030 T	M53, BHI	30°C, aerobic	1
Micrococcus sedentarius	Intern Collect. TW93	M53	30°C, aerobic	1
Moraxella catarrhalis	LMG 5128 T	BHI	37°C, aerobic	2
Morganella morganii	DSM 6675	LB	37°C, aerobic	2
Pseudomonas aeruginosa	DSM 50071T	BHI, M53	30°C, aerobic	2
Staphylococcus gallinarum	DSM 20610 T	TSY	37°C, aerobic	1
Streptococcus agalactiae	DSM 2134 T	TSB, BHI	37°C, microaerophilic	2
Streptococcus bovis	DSM 20480 T	TSB, BHI	37°C, microaerophilic	2
Streptococcus pyogenes	AF 611333	TSB, BHI	37°C, microaerophilic	2
Streptococcus salivarius	DSM 20560 T	TSB, BHI	37°C, microaerophilic	2
Tetragenococcus solitarius	DSM 5634 T	TSY	37°C, microaerophilic	1
Xanthomonas campestris	DSM 1350 T	BHI	26°C, aerobic	1

T: bacterial type strain
ATCC: American Type Culture Collection, Manassas, Virginia, USA, http://www.atcc.org
DSM: DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, http://www.dsmz.de
CCUG – Culture Collection, University of Göteborg, Sweden, http://www.ccug.se
LMG: Belgium ccordinated collections of microorgansims. Laboratorium voor Microbiologie, Universiteit Gent, http://bccm.belspo.be
AF: number of internal clinical classification of patient samples and organism isolated from clinical specimen [141].
Biosafety Level classification according to GMBI 2015 [87]

2. CULTIVATION OF ORGANISMS

2.1. CULTIVATION MEDIA

Table 2 shows all used media, their ingredients and their composition. For the preparation of agar plates, 15 g/L agar (Carl Roth, Germany) was added to the respective recipe.

Table 2. Compositions of cultivation media solutions

Growth Medium	Abbreviation	Composition per Liter	
Azide-Glucose Broth		Casein peptone	15 g
		Meat extract	4.5 g
		Glucose	7.5 g
		Sodium chloride	7.5
		Sodium azide	0.2 g
			pH 7.2 ±0.2
		Carl Roth, Germany	or 35 g/L ready-to-use medium
Brain Heart Infusion (RM)	**BHI**	Brain infusion solids	1 g
		Beef heart infusion solids	5 g
		Proteose peptone	10 g
		Glucose	2 g
		Sodium chloride	5 g
		Disodium phosphate	2.5 g
			pH 7.4±0.2
		BD, USA	or 37 g/L ready-to-use medium
BHI Supplemented	**BHI-S**	Brain Heart Infusion	37 g
		Cystein	1.4 g
		Hemin solution	10 mL
		Sodium bicarbonate (10%)	20 mL †
Corynebacterium Broth*	**M53**	Casein peptone (tryptic digest)	10 g
		Yeast extract	5 g
		Glucose	5 g
		Sodium chloride	5 g
			pH 7.3±0.1
Luria Broth	**LB**	Casein peptone (tryptic digest)	10 g
		Sodium chloride	5 g
		Yeast extract	5 g
			pH 7.0±0.2
MRS Medium*	**MRS**	see DSMZ – Medium 11	
Super Optimal Broth	**S.O.B.**	Casein peptone (tryptic digest)	20 g
		Yeast extract	5 g
		Sodium chloride	10 mM
		Potassium chloride	2,5 mM
		Magnesium chloride	10 mM †
		Magnesium sulfate	10 mM †
			pH 6.9±0.1
Super Optimal Broth with Catabolite repression	**S.O.C.**	S.O.B. medium	see above
		Glucose	20 mM

Growth Medium	Abbreviation	Composition per Liter	
Tryptic Soy Broth	**TSB**	Casein peptone (pancreatic digest)	17 g
		Soy peptone (enzymatic digest)	3 g
		Sodium chloride	5 g
		Dipotassium hydrogen phosphate	2.5 g
		Glucose	2.5 g
			pH 7.3±0.2
Tryptone Soy Yeast Extract	**TSY**	Tryptic Soy Broth	30 g
		Yeast extract	3 g
		Glucose	pH 7.0±0.2

* Recipe taken from DSMZ (https://www.dsmz.de) RM: rich medium † Addition after autoclaving

2.2. CULTIVATION

Cultivation was adapted to each organism according to its optimum growth condition (see Table 1). Liquid cultures were prepared in Erlenmeyer flasks (30 mL medium) or in anaerobic hungate tubes (5 mL medium). Prepared media was inoculated according to the used experimental setting, using small portions of glycerin stock cultures, a liquid broth culture, or single colonies from cultivated petri dishes. In contrast to microaerophilic bacteria cultures, aerobic cultures were incubated with shaking (*e.g.*, *E. coli*). Anaerobic organisms were cultured in the preferred medium (see Table 2) which was gassed with nitrogen before autoclaving in order to remove solved oxygen.

2.3. CONSERVATION

For long-term storage of pure cultures or gene libraries, 1 vol of the cell suspension (in the late exponential growth phase) was mixed with 1 vol of 50% glycerin and deposited at –80°C.

3. PRIMERS

All oligonucleotides (primers) used in this work, their characteristics and applications are listed in Appendix, Table 18, p. XI−XII.

4. POLYNUCLEOTIDE PROBES

Trebesius *et al.* [117] discovered that the part of domain III of the 23S rRNA comprising *E. coli* position [118] 1366 to 1617 is most suitable for the design of polynucleotide probes. The

ssRNA polynucleotide probe with a size of approximately 250 nucleotides was generated via *in vitro* transcription of polymerase chain reaction (PCR)-amplified rDNA based on the protocol described by Zwirglmaier *et al.* [114]. Modified universal primers (1900V, 317RT3) used for PCR amplification of the bacterial DNA target region (23S rDNA, domain III) are listed in Appendix, Table 18, p. XI. The reverse primer included the binding site for the T3 polymerase which was compulsory in subsequent *in vitro* transcription. The labelling of the probe was achieved through incorporation of fluorescently-labeled UTP and unlabeled UTP in a ratio of 0.65/0.35 during the transcription, resulting in a labelling density of approx. one labeled nucleotide every 10–20 nucleotide. Higher amounts of labeled UTP were found to decrease the yield, whereas lower amounts result in insufficient labeling [117]. The *Enterococcus*-specific rRNA targeting polynucleotide probe ('polyDIII') is characterized in Table 3. Unless stated otherwise, the 'polyDIII' probe was fluorescein-labeled by default (polyDIII-FLUOS).

Table 3. Overview of the characteristics of polynucleotide probe DIII

Probe Name	**Binding site**	***E. coli* position***	**Probe length [bp]**	**Primer set****
polyDIII	23S rDNA, domain III	1366–1617	251	1900V_mod 317RT3_mod2

* [118]
** see Appendix, Table 18, p. XI.

Reagents		
Nucleotide triphosphate (NTP) mix	100 mM	Bioline, UK
Fluorescein-12-UTP	10 mM	Roche, USA
DIG-11-UTP	1 mM	Jena Bioscience, Germany
T3 Polymerase	10 U/µL	Thermo Scientific, USA
RNase-free DNase I	1 U/µL	Promega, USA
RNase Inhibitor	40 U/µL	Thermo Scientific, USA
RNA Loading Dye	2×	Thermo Scientific, USA
Lithium chloride (LiCl)	4 M	
EDTA	0.2 M	
TE buffer, pH 7.5	1×	
$EtOH_{abs}$, $EtOH_{70\%}$		

FTP mix			**DIG mix**		
		final conc.			final conc.
3.9 µL	ATP/GTP/CTP	10.2 mM	8.6 µL	ATP/GTP/CTP	10.75 mM
1.4 µL	UTP	3.7 mM	4 µL	UTP	5 mM
25 µL	Fluorescein-12-UTP	6.6 mM	50 µL	DIG-11-UTP	5 mM

Reaction mix with label		Reaction mix without label	
2 µg	PCR product *or 18 µL of a 50 µL PCR*	≥ 1 µg	PCR product
4 µL	FTP/DIG mix	1 µL	NTP mix (100 mM)
8 µL	5× buffer	10 µL	5× buffer
2 µL	RNase Inhibitor	1.25 µL	RNase Inhibitor
8 µL	T3 polymerase	1.5 µL	T3 polymerase
ad 40 µL		*ad* 50 µL	

i. Incubate transcription mix for 3 h at 37°C.
ii. Add 1 µL/(µg template) DNase I to degrade template DNA and incubate for 15 min at 37°C.
iii. To stop the enzyme reaction, add 0.2 M EDTA to a final concentration of 8 mM.
iv. Precipitate RNA at least 2 h at –80°C, or overnight at –20°C, by addition of 0.1 volumes 4 M LiCl and 2.5 volumes $EtOH_{abs}$.
v. Centrifuge sample for 15 min (14,000 rpm, 4°C).
vi. Wash Pellet with 400 µL $EtOH_{70\%}$.
vii. Centrifuge again and carefully discard supernatant.
viii. Air-dry pellet and suspend it in 30 µL TE buffer (pH 7.5) and 1 µL RNase Inhibitor.
ix. Run standard agarose gel (2%) to check integrity of transcript probe. Use RNA loading buffer containing formamide to stabilize the transcript probe.

5. EXTRACTION OF NUCLEIC ACID

Genomic DNA was isolated using a modified protocol according to Wisotzkey *et al.* [142] based on a chloroform/isoamyl alcohol extraction. All available *Enterococcus* strains, as well as selected species used as negative controls species (see Table 1), were applied to DNA extraction.

Reagents	
Saline-EDTA (SE buffer)	0.15 M sodium chloride (NaCl) 0.01 M EDTA, pH 8
Standard saline citrate (SSC), 20x	3 M NaCl 0.3 M trisodium citrate, pH 7.0
Tris-HCl, pH 8.5	10 mM
Sodium acetate (NaAc), pH 5.5	5 M
Sodium dodecyl sulfate (SDS)	25% (w/v)
Chloroform : isoamyl alcohol (CIA)	24:1 (v/v)

Reagents		
Lysozyme from chicken egg white	20 mg/mL in 10 mM Tris-HCl	Sigma-Aldrich, USA
Proteinase K	10 mg/mL in H_2O_{MQ}	Roche, Germany
RNase A	20 mg/mL in 2× SSC	Sigma-Aldrich, USA
$EtOH_{abs}$, $EtOH_{70\%}$		

4 mL of an overnight culture (OD_{600} = ~1) were centrifuged for 5 min at 14,000 rpm and 4°C. After residue medium was discarded, the pellet was washed using 500 µL of SE buffer, and then resuspended and incubated in 500 µL of SE buffer and 20 µL lysozyme for 45 min at 37°C. This was followed by 15 min incubation at 55°C with addition of 5 µL RNase A, 60 min incubation at 37°C with addition of 10 µL proteinase K, and by 10 min incubation at 65°C applying 40 µL of 25% SDS to complete cell lysis. After addition of 755 µL CIA and 180 µL 5 M NaAc to extract proteins and cell fragments, the reaction tubes were carefully inverted for ~ 5 min, and then centrifuged for 15 min at 14,000 rpm and 4°C. The upper phase was transferred into a fresh reaction tube, while the lower phase was discarded. Two volumes $EtOH_{abs}$ were added to precipitate DNA for 1–6 h at –20°C. Precipitated DNA was centrifuged for 15 min at 14,000 rpm and 4°C, was washed with $EtOH_{70\%}$, and after drying of the DNA pellet, it was resuspended in 50–100 µL H_2O_{MQ} (on ice). The genomic DNA stock solution and a 100-fold diluted aliquot were stored at –20°C. To check extraction quality, quantity and purity, the stock was verified via gel electrophoresis (1% agarose (w/v)) and by Nanodrop measurement (see C.6).

6. PHOTOMETRIC MEASUREMENT OF NUCLEIC ACID SOLUTIONS

Due to their aromatic base moieties within their structure, nucleic acids are able to absorb short-wavelength light (260 nm). This property could be used to measure DNA concentrations in a spectral photometer. This was accomplished with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA) determining absorbance of wavelength from 230 nm to 300 nm. Various measurements could be interpreted to quantify DNA concentrations and determine DNA quality. The ratio of A260/280 shows protein contamination within the sample, while ratio of A260/230 indicates the presence of organic contaminants, such as phenol. Samples showing values between 1.8–2.1 and 2.0–2.2, respectively, were rated as pure.

7. POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR), first demonstrated by Saiki *et al.* [41], was used to specifically amplify DNA fragments. Depending on applied polymerase system different standard cycle programs were used. All primers serving as starting points for the polymerase were obtained from Eurofins Genomics, Germany and are listed in Appendix, Table 18, p. XI. The annealing temperature (T_A) for specific DNA binding was calculated using the oligonucleotide melting temperatures (T_m) given by Eurofins Genomics (see formulas), and by empirical optimization using a gradient PCR (± 6–10°C of mean T_m).

Melting temperature (>15 bases): $$T_m\ [°C] = 96.3 + \frac{41(n_G+n_C)}{s-(650/s)}$$

n = number of nucleotides of type G or C
s = number of all nucleotides per sequence

PCR annealing temperature: $$T_A\ [°C] = \frac{T_m1+T_m2}{2} - 5$$

PCR reactions mainly were performed in a Primus 96plus Thermal Cycler (MWG Biotech AG, Germany) and a Mastercycler® ep gradient S (Eppendorf AG, Germany) Thermal Cycler.

7.1. GRADIENT PCR

The optimum annealing temperature of primer sets was determined by gradient PCR using Mastercycler® ep gradient S (Eppendorf AG, Germany). This technique allows performing up to 12 different annealing temperatures in each cycler plate row during the same run. Usually a gradient of T_m ± 6°C was applied, with T_m being the mean melting temperature of used primers. To verify the optimum annealing temperature, the PCR fragments were checked by gel electrophoresis (C.8, p. 22).

7.2. TAKARA EX*TAQ*™ PCR SYSTEM

Unless stated otherwise, standard PCR applications were performed using the TaKaRa Ex*Taq*™ system (TaKaRa Bio Inc., Japan) in a volume of 50 µL. Per reaction, 5 µL of 10× Ex*Taq* buffer (20 mM Mg^{2+}), 5 µL of dNTP Mix (2.5 mM each), 0.25 µL of each primer (50 µM), 0.25 µL Ex*Taq*™ polymerase and approx. 100 ng template DNA were applied.

Table 4. Standard PCR program, specified for TaKaRa Ex*Taq*™ system.

Reaction step	Temperature	Time	Cycles
Initiating denaturation	94°C	5 min	1
Denaturation	98°C	10 s	30–35
Annealing	**x °C**	30 s	
Elongation	72°C	**y min** (1 min/kb)	
Final elongation	72°C	10 min	1

x: T_A of primer set

y: ~1 min per 1 kb fragment length

7.3. BIO*TAQ*™ PCR SYSTEM

The BIO*TAQ*™ PCR System (Bioline, UK) was used to verify insertion of DNA fragments into the vector after cloning (C.10.2, p. 23). The reaction setup was applied according to the manufacturer's protocol in a 20 µL approach using the M13 primer set (see Appendix, Table 18, p. XII).

Table 5. PCR program specified for BIO*TAQ*™ PCR system.

Reaction step	Temperature	Time	Cycles
Initiating denaturation	94°C	10 min	1
Denaturation	94°C	30 s	30–35
Annealing	50 °C *	30 s	
Elongation	72°C	**y min** (30 s/kb)	
Final elongation	72°C	10 min	1

* T_A of primer set M13V/M13R for clone check PCR

y: ~30 s per 1 kb fragment length

7.4. Q5® HOT START HIGH-FIDELITY PCR SYSTEM

The Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs, UK) is a high-fidelity, thermostable, hot start DNA polymerase with 3´→ 5´ exonuclease activity. It is recommended for crude lysates, samples that have inhibitors that were carried over, DNA that might be GC-rich and also for amplification of long fragments. This system was mainly used when applying samples considered difficult to analyze, such as fixed and hybridized cells. The reaction setup was applied according to the manufacturer's protocol.

Table 6. PCR Program specified for Q5® High-Fidelity PCR system.

Reaction step	Temperature	Time	Cycles
Initiating denaturation	98°C	10 min	1
Denaturation	98°C	10 s	
Annealing	**x °C**	30 s	25–35
Elongation	72°C	**y min** (30 s/kb)	
Final elongation	72°C	10 min	1

x: T_A of primer set

y: ~30 s per 1 kb fragment length

8. AGAROSE GEL ELECTROPHORESIS

To verify fragment size, purity and concentration of PCR products and genomic DNA, 5 µL aliquots were separated by basic agarose gel electrophoresis. A 2.5% agarose gel (w/v) was used for samples with expected fragment sizes of <300 base pairs (bp). For samples with expected fragment sizes of <800 bp and >800 bp, 2% and 1% agarose gels, respectively, were applied. The gel was prepared using particular quantity of agarose (LE agarose, Biozym Scientific, Germany) in 100 mL 1× Tris-acetate-EDTA (100× TAE stock: 4 M Tris, 2 M acetic acid, 0.2 M EDTA, pH 8.0). In order to evaluate the size and concentration of a DNA sample a standard marker was applied in given concentration (1 Kb Plus DNA Ladder, Invitrogen, USA). After electrophoresis the gel was stained for ~20 min in a ethidium bromide solution (0.15 µg/mL EtBr in H_2O_{dest}) and the fragments were visualized and documented by UV in an AlphaImager Mini (Alpha Innotec Corporation, USA).

9. PCR PURIFICATION

In order to remove residual primers, unincorporated dNTPs, enzymes, and salts from PCR products for subsequent molecular analysis, the amplification products were purified using AccuPrep® PCR Purification Kit (Bioneer, Korea) or FastGene® Gel/PCR Extraction Kit (Nippon Genetics, Japan), respectively. Both kits were applied according to the manufacturer's protocol and unless stated otherwise the purified PCR products were eluted in 30 µL H_2O_{MQ}.

10. CLONING USING TOPO TA® CLONING KIT

Cloning was performed according the manufacturer's protocol. The vector pCR® 2.1 contained in this kit has an overhang of thymidines, whereas freshly prepared PCR products show an

attached adenine overhang due to terminal transferase activity of the Ex*Taq*™ system polymerase (see C.7.2, p.20). Consequently, the TOPO TA® cloning kit uses a topoisomerase for connection of the overhangs and after heat shock transformation the fragments will be ligated inside of the bacterial cell.

Reagents		
***Escherichia coli* TOP 10 cells**		
LB Medium	see Table 2, p. 15	
Calcium chloride ($CaCl_2$)	100 mM	
Glycerin	87%	
S.O.C.	see Table 2, p. 15	
LB-Amp(X-gal)	LB medium, see Table 2, p. 15	
	100 µg/mL† ampicillin	Sigma-Aldrich, USA
	40 µg/mL† X-gal in dimethyl formamide	Sigma-Aldrich, USA

† added after autoclaving

10.1. PREPARATION OF CHEMICALLY COMPETENT *ESCHERICHIA COLI* CELLS

For the preparation of competent *E. coli* TOP 10 cells, the calcium chloride treatment described by Cohen *et al.* [143] was applied. This enabled cells to take up circular vector DNA.

A 30 mL overnight culture (37°C) was prepared by inoculation of a small portion of a –80°C (LB/25% glycerin) *E. coli* TOP 10 stock culture. Approximately 100 µL of the overnight culture was added to a fresh 30 mL LB medium and the culture was incubated at 37°C and 150 rpm until an OD_{600} of 0.5–0.6. At that time all following steps were conducted on ice. Bacterial cells were harvested by centrifugation at 4,500×g for 10 min at 4°C and were gently resuspended in 10 mL $CaCl_2$ (ice cold). After another centrifugation step and distortion of the supernatant, cells were carefully resuspended in 3 mL $CaCl_2$ and 400 µL 87% glycerin. Aliquots of 100 µL were stored at –80°C.

10.2. VECTOR LIGATION, TRANSFORMATION AND CLONE SCREENING

Purified PCR products were cloned according to the manufacturer's protocol of the TOPO TA® cloning kit #450641 (Invitrogen, USA). The ligation was performed in a 5 µL reaction using 1–2 µL of purified PCR product.

For selection of successfully cloned target cells the blue-white screening was used. The vector contains an ampicillin resistance gene and the cloning site is located in the *LacZα* reading frame. Thus, the successful ligation of a fragment into the vector will interrupt the *LacZ* gene coding for α subunit of the ß-galactosidase, and those cells will appear as white colonies if

plated on chromogen X-gal (lactose alike substrate) agar plates. Blue colonies result from *E. coli* cells containing a vector with no insert, which therefore is expressing the ß-galactosidase.

After incubation of the transformed cells in 500 µL S.O.C. medium (see Table 2, p. 14), 50 µL and 80 µL of bacterial cells were plated on LB-Amp(X-gal) and incubated at 37°C, overnight. White colonies were randomly picked from the LB-Amp(X-Gal) plates, resuspended in 100 µL LB-Amp medium and incubated at 37°C and 200 rpm for approx. 1 hour. This was followed by a PCR to determine correct insertion of the PCR fragment. The PCR was performed using 0.5 µL of the cell suspension in the BIO*TAQ*™ PCR system (see C.7.3, p.21), accomplished with primers M13F and M13R (see Appendix, Table 18, p. XII) flanking the insert region on the vector. Positive clones were detected by agarose gel electrophoresis (see C.8, p. 22) of the PCR products based on the correct fragment length.

Relevant clones were inoculated in 5 mL LB-Amp medium and incubated overnight at 37°C and 150 rpm. The cell suspension was then applied to plasmid extraction (see C.10.3, p. 24). For long-term storage of a clone library, 1 vol of the cell suspension was mixed with 1 vol of 50% glycerin and deposited at –80°C.

10.3. PLASMID EXTRACTION

Plasmid extraction was performed according to the manufacturer's protocol of the AccuPrep® Plasmid Mini Extraction Kit (Bioneer, Korea) and the EasyPrep Pro Plasmid Miniprep Kit (Biozym, Germany). The purified plasmids were eluted in 30 µL H_2O_{MQ} and deposited at – 20°C.

11. SANGER SEQUENCING

DNA sequencing according to Sanger *et al.* [144] was performed using the DNA Cycle Sequencing Kit (Jena Bioscience, Germany) and Infrared Dyes (IRD) Dy682 and Dy782 (Dyomics GmbH, Germany) as primer dyes. Sanger sequencing of PCR products or extracted plasmids as templates was applied to perform phylogenetic analysis, verification of pure cultures and cloned fragments. IRD-Primer sets targeting the 16S rRNA, 23S rRNA genes or pCR® 2.1 plasmids of cloned fragments are listed in Appendix, Table 18, p. XII. Separation and online laser detection of the fragments was carried out in a LI-COR Global IR^2 4200 DNA Sequencer (LI-COR Biosciences, USA) using 66 cm polyacrylamide electrophoresis. For analysis of the sequencing gels, *e*-Seq V 2.0 DNA Sequencing and Analysis Software (LI-COR Biosciences,

USA) was applied. For more rapid sequence investigations, not strictly dependent on detailed sequence analysis by the ARB software package [145], DNA amplicons and plasmids were sequenced by Eurofins Genomics (Germany) using cycle sequencing technique on a ABI 3730XL sequencing machine (https://www.eurofinsgenomics.eu).

11.1. COMPARATIVE SEQUENCE ANALYSIS

Genomic DNA from all project relevant enterococci and various related control strains such as streptococci (see Table 1, p. 13) was extracted according to chapter C.5, p. 18. The 16S rRNA and 23S rRNA genes were amplified (*E. coli* position 8–1511 and 115–2654, respectively) in a standard PCR. These amplicons have been applied to Sanger sequencing. The validation of the species was performed by addition of obtained sequences to the public silva databases (SSU Ref NR and LSU Ref, Version 111–119, www.arb-silva.de), and by alignment and analysis of the sequence data by the ARB software package (www.arb-home.de). Sequences were compared to existing database content and existing trees have been completed. Thus, species of the internal collection could be verified according to the respective results. Furthermore, these completed databases were used to design various specific primers and FISH probes (see Appendix, Table 18, p. XII). Besides, sequence data which were not dependent of absolute detailed analysis was applied to BLAST analysis (blastn, Basic Local Alignment Search Tool [146], http://blast.ncbi.nlm.nih.gov/Blast.cgi).

12. FECAL SAMPLE PREPARATION

Fecal samples of mice (Table 7) were kindly provided by the Chair of Nutrition and Immunology (Technische Universität München, Germany). The 'SPF' mice were raised under specific-pathogen-free (SPF) conditions, meaning that these mice were set under quarantine to ensure that diseases do not interfere with experiments. Nevertheless, these SPF mice possess a regular gut microbiota. The 'INF' feces samples were collected from germ-free mice that had been previously infected with *Enterococcus faecalis*. Consequently, this sample type should only contain *E. faecalis* if no contamination occurred.

Table 7. Different types of mouse fecal samples

Abbreviation	Mouse description	Gut microbiota status
GF	Germfree mouse	devoid of gut microbiota
SPF	'specific-pathogen-free' mouse	regular gut microbiota
INF	*E. faecalis* infected GF mouse	*Enterococcus faecalis*

After collection, mouse feces were stored at 4°C and thereafter transferred to –20°C for long-term storage. Ideally, preparation should be performed on fresh samples, followed by immediate PFA fixation (see C.13.1, p. 27). Various published protocols working with human feces were used for adjustment of the following preparation technique to remove large particles and debris [40, 147, 148]. All samples were applied to a diagnostic PCR to prove their origin and bacterial status (see C.16, p. 38 and Appendix, Table 18, p. XI), before and after the preparation. Prior to application for hybridization, fecal samples were prepared on ice as follows:

Fecal samples of mice were weighed and diluted 1:10 (assuming a sample density of 1g/mL) with ice cold sterile-filtered 1× phosphate-buffered saline (1× PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) added. For homogenization samples were rigorously vortexed for 10–15 min. Depending on feces texture, glass beads were added or a mild sonication treatment was applied (*e.g.*, 20–30 s, 20% cycles, 20% power, on ice) [149, 150]. To separate bacteria from fecal matter and debris, samples were centrifuged for 2 min at 200×g. The supernatant was transferred into a new reaction tube. PBS was added to the debris sample, followed by rigorous vortexing (5–10 min) and low-speed centrifugation (2 min, 200×g). The supernatant fractions were pooled. To store the fecal debris samples, PBS was added (10-fold volume of the fecal sample based on its weight) before freezing at –20°C. The pooled fecal supernatant was high-speed centrifuged (5–10 min, 14,000 rpm) and the cell pellet was washed three times with ice cold PBS. After the last centrifugation step, the PBS supernatant was discarded by pipetting, and the fecal cell pellet was diluted with the 10-fold volume of the fecal sample weight using PBS.

13. CELL FIXATION

Cell fixation helps to preserve cellular components and morphology and may lead to increased permeability of the cell envelope, which is necessary to allow high molecular weight molecules (*e.g.*, polynucleotide probes, labeled oligonucleotide probes) to diffuse into cells. Nevertheless, with respect to Gram-positive bacteria, probe penetration is recommended to be enhanced by enzymatic treatment. Furthermore, it was shown that cell fixation reduces autofluorescence and inhibits cellular autolysis and RNA or DNA degradation by denaturing proteins and enzymes [31, 88, 151]. Thus, even cells were not viable anymore, their morphology and genome was still intact. In respect to flow cytometry, fixation treatment shows its advantages by stabilizing the light scatter and inactivate biohazardous material. As the formation of covalent bonds by

formaldehyde works in a slow reaction, the fixation could be fine-tuned and adjusted according to the specific needs, concerning different target organisms.

For fixation of *Enterococcus* spp., a 4% paraformaldehyde (PFA) solution was applied, considering the number per milliliter should be below 10^9 cells/mL to prevent agglutination [95]. Fixed cells were stored at –20°C up to 1 year.

Reagents	
PBS, pH 7.4	1×
PFA (paraformaldehyde)	4% (w/v) in PBS
$EtOH_{abs}$	

13.1. FECAL SAMPLES

i. Split prepared fecal dilution (see C.12, p. 25) in appropriate parts and add 3 vol of 4% PFA.
ii. Incubate solutions for 5–12 h at 4°C.
iii. Centrifuge samples for 10 min at 14,000 rpm.
iv. Wash cells with appropriate amount of PBS (dependent on cell pellet volume).
v. Suspend the pellet in starting volume PBS (see C.13.1–i) and add 1.1 vol of $EtOH_{abs}$.
vi. Store samples at –20°C.

13.2. PURE CULTURES

i. Harvest 4 mL of a cell suspension during the mid-to-late exponential growth phase (OD_{600} 0.5–0.8) and centrifuge the sample at 14,000 rpm for 10 min (4°C).
ii. Remove residuals medium by washing the cell pellet with PBS.
iii. Resuspend the cell pellet in PBS (depending on cell pellet volume: 100–300 µL) and add 3 vol of PFA 4% (300–900 µL).
iv. Incubate the PFA solution for 0.5–12 h at 4°C or on ice.
v. Centrifuge the sample at 14,000 rpm for 2–10 min, and wash the cell pellet with appropriate amount of PBS (300–500 µL).
vi. Resuspend cells in a proper amount of PBS (100–500 µL) and 1.1 vol $EtOH_{abs}$.
vii. Fixed cells can be stored at –20°C up to 1 year.

14. FLUORESCENT *IN SITU* HYBRIDIZATION (FISH)

Following formulas are given to calculate optimum hybridization temperatures. As hybridizations are influenced by various parameters it is required to determine perfect conditions for each probe empirically, in order to maximize signal specificity and intensity.

DNA–DNA-hybridizations

Oligonucleotides (max. 50 nt) [152]:

$$T_D = 2 \times (\mathrm{A} + \mathrm{T}) + 4 \times (\mathrm{G} + \mathrm{C})$$

Polynucleotides [153]:

$$T_m = 81.5 + 16.6 \times logM + 0.41 \times (\%\mathrm{GC}) - 0.7 \times (\%\mathrm{FA})$$

RNA–RNA-hybridizations (max. 500 nt) [154, 155]

$$T_m = 78 + 16.6log\frac{M}{1.0 + 0.7 \times M} + 0.41 \times (\%\mathrm{GC}) - \frac{500}{D} - P - 0.35 \times (\%\mathrm{FA})$$

RNA–DNA-hybridizations

Oligonucleotides (max. 50 nt) [154, 156]:

$$T_D = 81.5 + 16.6 \times logM + 0.4 \times (\%\mathrm{GC}) - {}^{820}/_{n} - 0.5 \times (\%\mathrm{FA})$$

Polynucleotides (50 to max. 500 nt) [154, 155]:

$$T_m = 67 - 16.6 \times log10\frac{M}{1.0 + 0.7 \times M} + 0.8 \times (\%\mathrm{GC}) - \frac{500}{D} - P - 0.5 \times (\%\mathrm{FA})$$

T_m	melting temperature in °C (polynucleotides)
T_D	dissociation temperature in °C (oligonucleotides)
M	molar concentration of sodium ions in hybridization buffer
%GC	percent guanine plus cytosine
D, n	length of duplex
%FA	percent formamide in hybridization buffer
P	percent of mismatches

The method of in-solution FISH was modified according to the protocols of Wallner *et al.* [95] and Zwirglmaier *et al.* [115]. Furthermore, recommendations by Haroon *et al.* [157] were considered.

The optimal formamide concentration, pretreatment conditions, hybridization temperature and incubation time for the target bacterial taxon should be adjusted prior to the implementation by performing regular FISH experiments on slides. It is essential to permeabilize the cell walls without destroying the shape and the integrity of the bacterial cell to ensure that polynucleotide probes are able to hybridize to their target structure on 23S rRNA [114]. The optimum hybridization conditions for the Gram-positive genus *Enterococcus* are summarized in Table 8. To analyze autofluorescence and non-specific binding for each fecal sample, a negative control should be always applied using the nonEUB338 probe, unlabeled 'polyDIII' probe, or no probe (blank).

Table 8. Optimum hybridization conditions of probes targeting the taxon *Enterococcus*.

Probe	Pretreatment	Formamide concentration	Hybridization temperature	Incubation time
Polynucleotide probe (polyDIII)	10 min lysozyme on ice	80%	53°C	3–12 h
Oligonucleotide probe (Enc473)	10 min lysozyme on ice	20%	46°C	1.5–3 h

Reagents		
Lysozyme	10 mg/mL in 50 mM Tris-HCl	
Ethanol solutions	50%, 80%, 100%	
Formamide (FA)	100%	Merck Millipore, Germany
PBS, pH 7.4	1×	
Tris-HCl	1 M	
Disodium EDTA (Na_2-EDTA)	1 M	
Sodium chloride (NaCl)	5 M	
Sodium dodecyl sulfate (SDS)	25% (w/v)	
Mounting Medium with DAPI	1.5 µg/mL	Vectashield, USA
Microscope slides with 12 reaction wells		Marienfeld, Germany

Hybridization buffer:

NaCl 900 mM
Tris-HCl 20 mM pH 8.0
SDS 0.01%
Formamide **X** %

Washing buffer:

NaCl **X** mM (see Table 9)
Tris-HCl 20 mM
SDS 0.01%
(5 mM Na_2-EDTA)†

Table 9. Composition of washing buffer for oligo- and polynucleotide FISH

% formamide in hybridization buffer	Na^+ concentration [mM] in washing buffer	µL 5 M NaCl for 50 mL washing buffer	µL 1 M EDTA for 50 mL washing buffer†
0	900	9,000	–
5	636	6,360	–
10	450	4,500	–
15	318	3,180	–
20	225	2,150	250
25	159	1,490	250
30	112	1,020	250
40	80	700	250
45	56	460	250
50	40	300	250
55	28	180	250
60	20	100	250
65	14	40	250
70	10	–	250
75	–	–	250
80	–	–	250

† Only at 20% FA or more

14.1. FISH USING OLIGONUCLEOTIDE PROBES

Note: To prevent cell loss during the procedure, try to pipette and resuspend carefully. In addition, it was recommended to use low binding and low retention pipette tips (*e.g.*, SurPhob, Biozym Diagnsotik GmbH, Germany).

14.1.1. PROTOCOL FOR OLIGOFISH ON SLIDES

i. Prepare hybridization buffer (**X**% FA) and preheat it (46°C).
ii. Apply 4–8 µL of a suspension of PFA-fixed cells per microscope slide well.
iii. Dry for 5–10 min at 60°C.
iv. Dehydrate cells in increasing ethanol series (50%, 80%, 100%) for 3 min each.
v. Air dry slides and incubate each well with 10 µL lysozyme for 10 min on ice.
vi. Rinse slides with H_2O_{MQ} and dry under airflow.
vii. Dehydrate cells in a second increasing ethanol series (50%, 80%, 100%) for 3 min each and air dry slides.
viii. Put 9 µL of hybridization buffer onto each well, add 1 µL oligonucleotide probe solution (30–50 ng/µL) and mix carefully.

ix. Put slide into the hybridization tube lined with hybridization-buffer-moisturized tissue and close it.
x. Hybridize samples at 46°C for 1.5–2 h.
xi. Rinse slide with H_2O_{MQ} or preheated washing buffer (48°C).
xii. Put slide in a 50 mL tube containing preheated washing buffer, and incubate at 48°C for 15 min.
xiii. For microscopic analysis, embed wells with mounting medium (see C.14.4, p. 34). Otherwise, store slide light protected at –20°C.

14.1.2. PROTOCOL FOR IN-SOLUTION OLIGOFISH (FOR FECAL SAMPLES)

i. Prepare hybridization buffer (**X**% FA) and preheat it (46°C).
ii. Use 100 µL of a suspension of a PFA-fixed fecal sample (or 30–40 µL of fixed pure culture).
iii. Add 300 µL PBS (optional: perform a mild sonication, see p. 25) and centrifuge for 10 min at 14,000 rpm.
iv. Discard supernatant and add 200 µL of a 50% ethanol solution, try to fully resuspend the pellet by avoiding cell loss. Incubate the sample for 3 min at RT and centrifuge it for 2 min at 14,000 rpm.
v. Repeat step iv. with 80% and 100% ethanol solution.
vi. Discard supernatant by careful pipetting, and remove any residual ethanol by drying the tube in a 40°C heating block up to 15 min (depending on volume of residual ethanol left in the tube).
vii. Apply 200 µL lysozyme solution to the pellet and completely resuspend it.
viii. Incubate reaction tube on ice for 10 min.
ix. Add 300 µL PBS and centrifuge for 10 min at 14,000 rpm. Carefully discard supernatant.
x. Resuspend pellet in 100 µL preheated hybridization buffer and add 10 µL oligonucleotide probe (30–50 ng/µL).
xi. Incubate samples for 1.5–2 h at 46°C.
xii. Add ~200 µL preheated washing buffer (48°C) to the sample and wash the sample by centrifugation (2–5 min, 14,000 rpm).
xiii. Add 500 µL preheated washing buffer and incubate for 20 min at 48°C.
xiv. Wash sample with 500 µL ice-cold PBS (2–5 min, 14,000 rpm) twice, before finally resuspending the cell pellet in 500 µL ice-cold PBS.

xv. To validate the hybridization results, put ~20 µL of the hybridized sample on reaction wells of microscope slides, dry it at 50–60°C and analyze the samples embedded with mounting medium using a fluorescent microscope (see C.14.4, p. 34).

xvi. Store samples light protected at –20°C.

14.2. FISH USING POLYNUCLEOTIDE PROBE 'POLYDIII'

Note: To prevent cell loss during the procedure, try to pipette and resuspend carefully. In addition, it was recommended to use low binding and low retention pipette tips (*e.g.*, SurPhob, Biozym Diagnsotik GmbH, Germany).

14.2.1. PROTOCOL FOR POLYFISH ON SLIDES

i. Prepare hybridization buffer (80% FA) and preheat it.

ii. Apply 4–8 µL of a suspension of PFA-fixed cells per microscope slide well.

iii. Dry for 5–10 min at 60°C.

iv. Dehydrate cells in increasing ethanol series (50%, 80% and 100%) for 3 min each.

v. Air dry slides and incubate each well with 10 µL lysozyme for 10 min on ice.

vi. Rinse slides with H_2O_{MQ} and dry under airflow.

vii. (Optionally: incubate slides at 200°C for 1–3 min).

viii. Dehydrate cells in a second increasing ethanol series (50%, 80% and 100%) for 3 min each and air dry slides.

ix. Put 12 µL of hybridization buffer onto each well, add 3–4 µg RNA polynucleotide probe solution per well and mix carefully by slow pipetting.

x. Put slide into the hybridization tube lined with hybridization-buffer-moisturized tissue and close it.

xi. Denature slides with RNA probes at 80°C for 25–30 min.

xii. Hybridize samples at 53°C for 3–12 h.

xiii. Rinse slide with H_2O_{MQ}.

xiv. For microscopic analysis, embed the wells with mounting medium (see C.14.4, p. 34). Otherwise, store slide light protected at –20°C.

14.2.2. PROTOCOL FOR IN-SOLUTION POLYFISH (FOR FECAL SAMPLES)

i. Prepare hybridization buffer (80% FA) and preheat it (53°C).
ii. Use 100 µL of a suspension of a PFA-fixed fecal sample (or 30–40 µL of fixed pure cultures).
iii. Add 300 µL PBS (optional: perform a mild sonication, see p. 25) and centrifuge for 10 min at 14,000 rpm.
iv. Discard supernatant and add 200 µL of a 50% ethanol solution, try to fully resuspend the pellet by avoiding cell loss. Incubate the sample for 3 min at RT and centrifuge it for 2 min at 14,000 rpm.
v. Repeat step iv. with 80% and 100% ethanol solutions.
vi. Discard supernatant by careful pipetting and remove any residual ethanol by drying the tube in a 40°C heating block up to 15 min (depending on volume of residual ethanol left in the tube).
vii. Apply 200 µL lysozyme solution to the pellet and completely resuspend it.
viii. Incubate reaction tube on ice for 10 min.
ix. Add 300 µL PBS and centrifuge for 10 min at 14,000 rpm. Discard supernatant.
x. Resuspend pellet in 100 µL preheated hybridization buffer and add 4–5 µg RNA poly-nucleotide probe.
xi. Denature samples with RNA probes for 20 min at 80°C.
xii. Incubate for 5–12 h at 53°C.
xiii. Wash samples with 200 µL preheated washing buffer (56°C) (2–5 min, 14,000 rpm).
xiv. Add 500 µL preheated washing buffer and incubate sample for 20 min at 56°C.
xv. Wash sample with 500 µL ice-cold PBS (2–5 min, 14,000 rpm) twice, before finally resuspending the cell pellet in 500 µL ice-cold PBS.
xvi. To validate the hybridization results put ~20 µL of the hybridized sample on reaction wells of microscope slides, dry it at 50–60°C and analyze the samples embedded with mounting medium, with a fluorescent microscope (see C.14.4, p. 34).
xvii. Store samples at –20°C.

14.3. LINKING OLIGOFISH WITH POLYFISH

It is possible to combine oligo- and polynucleotide based FISH. Because of the denaturing step and the higher hybridization temperature, hybridization with the halo forming polynucleotide probes has to be completed prior to application with oligonucleotide probes. Thus, after rinsing

off the hybridization buffer containing the polynucleotide probe, the hybridization buffer with the oligonucleotide probe could be directly applied without repeated pretreatment.

14.4. MICROSCOPIC ANALYSIS

FISH approaches or enriched samples (concentrated on filters, see C.19, p. 43) were checked by microscopic analysis. Slides and filters have been embedded in DAPI-containing mounting medium (Vector Laboratories, USA) and analyzed using an epifluorescence microscope.

Fluorescence microscopic examination was performed with a Zeiss Axio Imager.M1 epifluorescence microscope. It was equipped with a 100 × EC Plan NEOFLUAR oil objective and HBO100 lamp using the Zeiss filter set 17 for the fluorescein dye (excitation 485/20, emission 515–565), 45 for the Cy3 dye (excitation 560/40, emission 630/75) and 49 for the DAPI stain (excitation G365, emission 445/50). Images were captured using AxioVision Rel. 4.8 software (Zeiss, Germany).

15. TAXON-SPECIFIC CELL ENRICHMENT IN DNA-COATED MICROPLATES

An adjusted protocol according to Zwirglmaier *et al.* [115] and Fichtl [120] was developed to enrich enterococci cells in microplate cavities. Figure 1 shows the schematic overview of the technique of the taxon-specific immobilization in microplates.

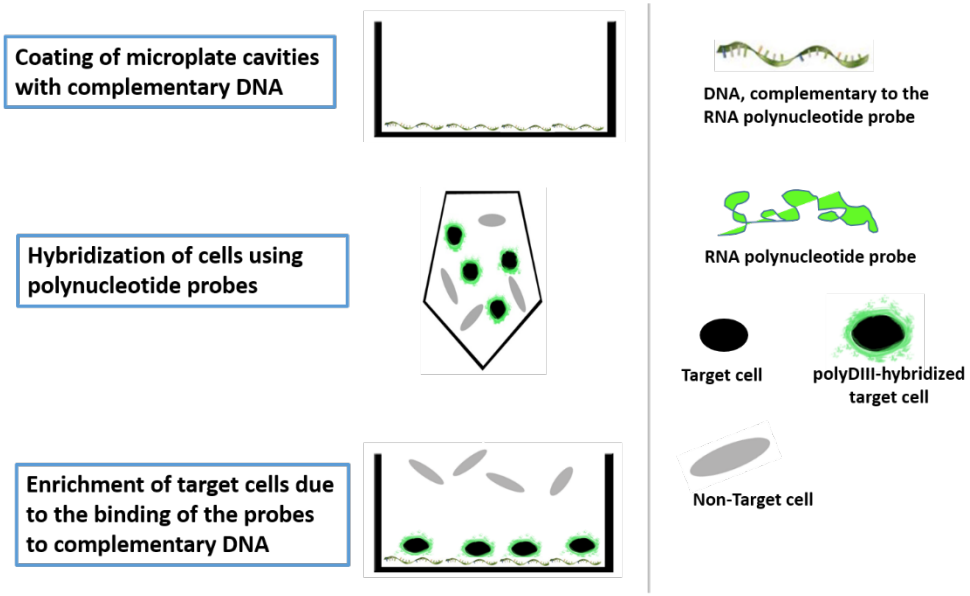


Figure 1. Schematic overview of immobilization on microplates (according to Zwirglmaier, 2003).

15.1. COATING OF MICROPLATES

In order to amplify DNA polynucleotides (sense DNA) complementary to the RNA polynucleotide probes (antisense RNA), modified PCR primers are used for amplification as used for generating the template for *in vitro* transcription (see C.4, p. 16). The reverse primer (317R) did not hold the T3 polymerase promoter sequence and the forward primer (1900V) optionally was modified by amination (by C12-amino linker) or phosphorylation at the 5'end. In addition, a linker of at least 10 thymidines was added between active primer sequences and the 5'end group of the phosphorylated primer to improve covalent binding of the fragments to the microplate surface. Information about primers are listed in Appendix, Table 18, p. XI. For covalent binding of nucleic acids to NucleoLink plates, following protocol was used.

Reagents		
PBS, pH 7.4	1×	
PBS/$MgCl_2$	0.1 M magnesium chloride ($MgCl_2$) in 1× PBS	
EDC solution	10 mM 1-Ethyl-3-(3-Dimethylaminopropyl)-Carbodiimide (EDC) in PBS/$MgCl_2$	Sigma-Aldrich, Germany
Nunc™ NucleoLink™ Microplates		Thermo Scientific, USA
Adhesive PCR sealing film		Peqlab, Germany

i. Perform a standard PCR with primer set 1900V/317R (see C.7.2, p. 20 and Table 18).
ii. In order to achieve a high yield of PCR fragments, accomplish purification of multiple PCR reactions by precipitation (using 0.1 vol 5 M sodium acetate and 2.5 vol $EtOH_{abs}$).
iii. Prepare coating-mastermix: 1 µg PCR product in 100 µL EDC-solution per each microplate cavity and apply 100 µL of coating solution/cavity.
iv. Seal microplate by application of PCR film.
v. Incubate microplate at 94°C for 20 min.
vi. Overnight incubation at 37°C.
vii. Discard plate supernatant and dry cavities for 1–2 h at 60°C.
viii. Microplates can be stored for several weeks at 4°C.
ix. Before using, wash microplates with 1× PBS twice.

Validation of correct coating was performed by DIG-labeled amplicons generated using a DIG-labeled reverse primer (317R-DIG, see Appendix, Table 18, p. XI) for standard PCR and subsequent DIG detection (see C.15.3, p. 37).

15.2. IMMOBILIZATION ON MICROPLATES

After in-solution FISH of target cells by using fluorescein-, or unlabeled 'polyDIII' probes (see C.14.1.2 and C.14.2.2, pp. 31–33), the cell suspension was subsequently hybridized to microplate cavities coated with DNA, complementary to the RNA polynucleotide probe (see C.15.1, p. 35). The protocol refers to the application of one NucleoLink™ Strip (eight cavities) (Thermo Scientific, USA).

Reagents		
PBS, pH 7.4	1×	
Maleic acid buffer (MAB)	0.15 M sodium chloride (NaCl) 0.1 M maleic acid, pH 7.5	
Blocking solution (BS)	10% (w/v) in MAB buffer	Roche, Germany
MP buffer	5× standard saline citrate (SSC) 0.02% sodium dodecyl sulfate (SDS) 2% blocking solution 0.1% N-lauroylsarcosine 33% formamide	
Coated microplates	see C.15.1, p. 35	
Adhesive PCR sealing film		Peqlab, Germany

i. Wash coated microplate with 100 µL PBS to remove unbound DNA.
ii. Toss down PBS and tap microplate on a tissue to dry.
iii. Pre-hybridize microplate cavities with 60 µL MP buffer for at least 5 min at room temperature.
iv. Centrifuge hybridized cell suspension (C.14.2.2, p. 33) at 14,000 rpm for 5 min, and resuspend the pellet in 40–42 µL/well preheated MP buffer (53°C).
v. Apply 40 µL of hybridized cell suspension in MP buffer per coated cavity (in total 100 µL).
vi. Cover microplate with adhesive PCR sealing film and incubate it for 90 min at 53°C.
vii. Carefully remove the solution without touching the walls of the microplate cavities.
viii. Pool related cavity supernatants, centrifuge them and resuspend the pellet in 10–20 µL PBS; the supernatant was used for determination of cell enrichment efficiency (see C.16.2, p.39).
ix. Carefully wash microplate cavities with PBS.
x. Microplate cavities were directly used for PCR analysis (see C.15.3, p. 37)

or

xi. Cells were recovered for microscopic analysis or qPCR (see chapter see C.19 and C.16). Therefore, add 100 µL H_2O_{MQ} per microplate cavity, incubate plate at 94°C for 5 min, and rinse cavities thoroughly to wash cells off of the cavity walls.

15.3. DETECTION OF CELLS AFTER ENRICHMENT

15.3.1. DIGOXIGENIN DETECTION

If DIG-labeled polynucleotide probes were used for hybridization, enriched target cells and enrichment efficiency was detected and measured, respectively, by following protocol. The same protocol was used for detection of coating efficiency using DIG-labeled DNA coating fragments.

Reagents		
PBS, pH 7.4	1×	
Anti-DIG-POD	Anti-digoxigenin horseradish peroxidase, fab fragments	Roche, USA
POD substrate	BM Blue POD substrate	Roche, USA
Sulfuric acid (H_2SO_4)	1 M	

i. Carefully wash microplate cavities with 100 µL (two times) and 200 µL PBS (one time).
ii. Apply 100 µL Anti-DIG-POD ($1:10^3$ in PBS/1% BS) per cavity.
iii. Incubate plate 30 min at RT.
iv. Discard supernatant by tapping down microplate on a tissue.
v. Wash microplate cavities (see step i.)
vi. Apply 100 µL BM Blue (POD substrate) per cavity.
vii. Incubate microplate 5–15 min at RT.
viii. Add 100 µL H_2SO_4 per cavity to induce color change.
ix. Absorbance values were determined using a microplate reader (450nm/650nm).

15.3.2. MICROSCOPIC DETECTION

If fluorescein-labeled polynucleotide probes were used for hybridization, enriched target cells and the enrichment efficiency were verified by microscopic examination. To concentrate samples for the analysis, 10–20 µL of the enrichment solution, or the supernatant was spotted onto polycarbonate membrane filters using a vacuum pump (see C.19, p. 43).

15.3.3. PCR DETECTION

To verify enrichment success of target cells, a PCR was directly performed in the microplate cavities. Additionally, the supernatant was used as a template. To deal with those samples considered difficult to analyze, the Q5® HS HiFi DNA PCR system (New England Biolabs, UK) was applied (C.7.4, p. 21). For parallel detection of enterococci and other bacteria, a multiplex PCR was developed which targets the 16S rRNA gene specifically for enterococci (primers Enc473V/Enc1276R) and the conserved bacterial 23S rRNA gene (primers 3010V_mod/2241R) (see Appendix, Table 18, p. XI). The optimum annealing temperature for the multiplex PCR (53.8°C) was evaluated by gradient PCR (see C.7.1, p. 20).

16. DIRECT-PCR DETECTION AND QUANTIFICATION SYSTEMS FOR *ENTEROCOCCUS* SPP.

16.1. DIRECT-PCR DETECTION SYSTEM FOR *ENTEROCOCCUS* SPP.

For a specific direct-PCR detection system which can identify the presence of the genus *Enterococcus* spp. in a sample without previous DNA extraction, a specific primer set was designed targeting the 16S rRNA of enterococci. For primer design, the 16S rRNA SILVA database SSURef NR99 119 (http://www.arb-silva.de) and the ARB software package (http://www.arb-home.de) was used [145]. The target regions were distinct including from species of the closely related genus streptococci and were validated for direct-PCR on pure cultures and fecal samples without previous extraction of genomic DNA. Table 10 shows the properties of designed oligonucleotides.

Table 10. *Enterococcus*-specific primer targeting the 16S rRNA gene, applied for genus specific detection in environmental samples.

Name	Sequence 3´–5´	*E. coli* position*	T_m
Enc473V	CRT CCC YTG ACG GTA TCT AAC	473	59.8
Enc1276R	CTG AGA GAA GCT TTA AGA GAT TWG C	1276	59.7

* [118]

Application:

A) For pretesting of fecal or environmental samples for the presence of enterococci, it was recommended to use the $1:10^2$–$1:10^3$ dilutions of washed fecal samples (see C.12, p.25) as templates.

B) Validation after cell sorting by FACS. Up to 2 µL of the lysed sort sample (see C.18, p.43) were used for a 50 µL PCR reaction. Eventually, it was useful to increase primer concentration to facilitate amplification success.

16.2. ABSOLUTE QUANTIFICATION OF *ENTEROCOCCUS* SPP. BY DIRECT QPCR

Besides qualitative identification by PCR, a taxon-specific quantitative real-time PCR (qPCR) system was developed. When applied as a pre-investigation test, the number of enterococci cells potentially available in the prepared fecal samples for sorting were assessed directly without prior DNA extraction. This may be interesting, as it was shown that a high percentage of microbial population remains attached to the debris material [158] and therefore it is recommended to check prepared fecal samples not only qualitatively, but also quantitatively before cell sorting processing of low-abundance taxa. In this project, two detection applications were evaluated, targeting the multi-copy 23S rRNA gene and the single-copy *tuf* gene, respectively.

16.2.1. PRIMER DESIGN

A 23S rRNA gene-based qPCR was already described by Ludwig and Schleifer [159], whereas the related primer set (738F and 850R) in this thesis was adjusted and modified according a validation using the latest ARB 23S rRNA database (LSU Ref 119, July 2014, http://www.arb-silva.de). The primer set targeting the enterococcal *tufA* (Ent1(*tuf*), Ent2(*tuf*)) was described earlier by Ke *et al.* [160] and was validated and slightly modified *in silico* based on a current *tuf* gene database (Wolfgang Ludwig, pers. comm.) including various enterococci sequences. Annealing temperatures for both primers range from 55°–60°C.

16.2.2. STANDARD CURVE

A standard curve for the qPCR assay was created by plotting threshold cycle (Cq) values against the number of target copies corresponding to 10-fold serial dilutions of known plasmid standard concentrations (*e.g.*, 10^8–10^2 copies/reaction) in triplicate. Plasmid standards were generated by amplifying the 23S rRNA and the *tufA* gene from the type strain *E. faecalis* (DSM-20478) with a PCR using universal primers Appendix, Table 18, p. XII. The PCR products were purified and ligated into the pCR™2.1 vector according to the manufacturer's instructions (TA Cloning® Kit, with pCR™2.1 Vector, Invitrogen), see C.10, p.22. Plasmids with correct insertion were linearized with NotI-HF (New England Biolabs, USA) according

to manufacturer's recommendation. To remove residual enzyme, linearized plasmids were purified by FastGene® Gel/PCR Extraction Kit (Nippon Genetics, Japan). If linearization was complete, a single band of the correct size was expected. Target copy numbers were estimated by a formula previously described [161].

To generate a standard curve, a 10-fold serial dilution was prepared from the stock plasmid DNA with known numbers of copies/µL (*e.g.*, 10^8–10^2 copies/reaction). The concentrations chosen for the standard curves should encompass the expected concentration range of the target in future experiments. It was necessary to mix well between the dilutions steps to avoid an inaccurate calibration of the standard curve. The stocks were stored at –20°C, but to avoid multiple freeze-thaw cycles it was recommended to store plasmid standard preparations in aliquots. The standard plasmid dilutions were applied to qPCR reaction in triplicate (see C.16.2.3, p. 40) and the results were analyzed using CFX-Manager software (Bio-Rad, USA. Various reaction parameters (including efficiency, slope, y-intercept and correlation coefficient) could be obtained by the standard curve (Table 11). They give information about the performance of the run and are further used for correct result interpretation and comparison of different qPCR approaches [162].

Table 11. Optimum values for qPCR reaction parameters [162].

Parameter	Target values
efficiency (E)	90%–110% (excellent: 95%–105%)
slope	–3.58 to –3.1
correlation coefficient (R^2)	>0.99
y-intercept (y-int)	~ 40 (Cq)

16.2.3. qPCR REACTION

Quantitative PCR was performed using a CFX96 Touch™ Real-Time PCR Detection System by Bio-Rad, USA. The qPCR reactions were perforemd in a final volume of 17 µL containing 2 µL of template with 8.5 µL of SsoAdvanced™ SYBR® Green Supermix (Bio-Rad, USA) and 250 µM of each primer. *Enterococcus*-specific qPCR primer targeting the *tufA* or 23S rRNA gene are found in Appendix, Table 18, p. XI. Photobleaching was prevented by blocking the ambient light, considering the low fluorescent signal due to the low amount of target. The PCR reaction was performed with an initial step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. To determine the specificity of the amplification, the melting curve was established by increasing the temperature from 55°C to 95°C at the end of each PCR

reaction. Fecal samples were measured in at least duplicates, including not less than three plasmid concentrations from the construction of the standard curve. Furthermore, two no-template controls per PCR run were used to check for cross-contamination. Results were analyzed using CFX-Manager software (Bio-Rad, USA), see Table 11.

17. CELL SORTING BY FLUORESCENCE-ACTIVATED FLOW CYTOMETRY

Before starting FACS analysis, it was recommended to check viability and activity of the fixed feces samples. Contamination of the FACS system had to be absolutely excluded as working with environmental material of biosafety level 2, *i.e.* material with mostly unknown organisms which could be pathogenic or infectious, might pose a safety risk or might affect subsequent experiments. In general, fixed samples do not contain any viable cells anymore and at the latest when exposure to hybridization process with 80% formamide, survival is almost impossible. Anyway, to ensure 100 µL of fixed samples were plated on full medium agar plates (*e.g.*, BHI) and incubated at 37°C anaerobically and aerobically for 1–2 days to determine any growth.

As every flow sorter model owning its individual setup procedures, sorting accuracies and laser configurations, the specific manufacturer's instruction for setup, maintenance and cleaning procedures were followed. In this study, flow cytometry was conducted with a MoFlo® (Beckman Coulter, USA) and a FACSAria™ (BD Biosciences, USA) flow sorter, both equipped with a blue laser (488 nm wavelength) for detection of fluorescein-labeled cells and optionally with an UV laser (355 nm) for DAPI detection. To set the sorting regions, target cells were initially selected by their fluorescence intensity (threshold here: $>10^3$) and side scatter characteristics (threshold SSC: 10^3–10^4), visualized on FCM scatter plots and histograms. These parameters depended on the FACS device, settings used and the hybridization success of the applied sample and had to be constantly controlled. Differences in microbial structure on FCM plots/histograms were compared to negative controls (no probe or nonEUB) and could be spotted by eye. To additionally measure these changes and to standardize the process, the analysis tool 'cytometric barcoding' (CyBar) was adopted which recommends one gate template for a single dataset (here: fecal sample) [81]. Standardized scatter plots were produced by randomly selecting 100,000 events from each acquired sample. The procedure of cell sorting recommended by Haroon *et al.* [157] was applied to the adjustment of this protocol. For regulating the voltages of the flow sorter a test sample was run at a high flow rate to calibrate. Flow sorting was conducted in two-way and purity mode. For subsequent PCR amplification at least 10^5–

10^6 cells per sample were sorted, collected in 1× PBS pH 8.5, and stored light-protected on ice before microscopic analysis. The individual settings used in this project are listed in Table 12.

Table 12. Laser settings and instrument parameters for the FACSAria™ (BD Biosciences, USA) device.

Name	**Delay**	**Area Scaling**
Blue	0.00	1.15
Parameter	**Voltage**	**Log**
FSC	220	√
SSC	500	√
eGFP	560	√

i. Homogenize hybridized samples by shaking, repeated pipetting or mild sonication (see C.12, p. 25).
ii. After confirming that hybridized cell samples are not aggregated and positively labeled by microscopic analysis (see C.14.4, p. 34), samples could be processed to FACS.
iii. First, adjust the voltages of the flow sorter using a negative sample, such that cells appear in the lower left quadrant of the FL1 vs. SSC scattergram. For that, a high flow rate (scale 11) was applied. The data were less resolved, but was acquired more quickly. A lower flow rate is generally used in applications where greater resolution is critical, i.e. while sorting.
iv. Next, run the hybridized sample of interest and determine if there is a distinct shift in the fluorescent intensity of cells.
v. For sorting, gate the regions of the scattergram that are characterizing the target population.
vi. For target cells with low-abundance it is recommended to perform multistage sorts. Hence, the first sort (at least 10^6–10^8 cells) can be accomplished using a higher sort rate (~20,000 events/s) and less stringent gating. The lower stringency on the purity are equalized by a second sort run, using a more defined gate setting and a slower sorting rate (~5,000 events/s). This may prevent increased abort counts while sorting.
vii. For subsequent PCR amplification sort at least 10^5–10^6 cells per sample and collect them via saline fluid (1× PBS, pH 8.5).

18. PROCESSING OF SORTED SAMPLES

While cell sorting, gated populations were collected in 15 mL tube due to FCM model setup. By sorting 10^6 cells for each population, the final volume of the samples was approx. 2.5 mL PBS. To facilitate further processing and concentrate the sorted products, the collected cell solution was transferred to a 2 mL reaction tube by centrifugation (14,000 rpm, 20 min, 4°C). Further, the supernatant was carefully withdrawn until 100–200 µL residue solution, to not prompt cell loss. To increase subsequent amplification success, a part of the sample was processed to single step DNA extraction using DNAreleasy Advance (FastGene, Nippon Genetics Europe GmbH), a direct PCR lysis reagent. It facilitates the release of DNA in a PCR-ready form. Therefore, it replaces the traditional DNA purification step and greatly reduces the risk of sample loss and contamination. This fact outweighs the disadvantage of releasing simply unpurified DNA. But caused by impurity, an accurate estimation of DNA yield after lysis is difficult and cannot be performed using, *e.g.* a spectrophotometer [163]. Forty microliters of sorted and concentrated cell sample were taken and mixed with 100 µL of lysis reagent (FastGene, Nippon Genetics Europe GmbH) according the manufacturer's protocol. Following lysis profile was used, recommended for samples considered difficult to analyze (Table 13). The lysate was directly used in a PCR and according to the manufacturer it can make up to 10% of most PCR mixes. The lysate was stored at –20°C for future use.

Table 13. Lysis profile used for the preparation of FCM sorted cell samples.

Step	Time	Temperature
1	15 min	65 °C
2	2 min	96 °C
3	4 min	65 °C
4	1 min	96 °C
5	1min	65 °C
6	30 s	96 °C
7	hold	20 °C

19. SORTING VALIDATION BY MICROSCOPIC ANALYSIS

Sorted samples were verified optically by microscopic analysis. To concentrate samples for the analysis and to safe material, up to 20 µL of the concentrated sample (not lysed) was spotted onto polycarbonate membrane filters (Merck Millipore, Germany, diameter 25 mm, pore size

0.22 µm) using a vacuum pump. The membrane-fixed samples were embedded in DAPI-containing mounting medium and analyzed using an epifluorescence microscope (see C.14.4, p. 34).

20. GENE LIBRARY FOR METAGENOME ANALYSIS

For gene amplification, 1–2 µL of the sorted and processed FACS sample (see C.18, p. 43) was applied to a standard 50 µL PCR reaction by using the Ex*Taq*™ or Q5® HS Hi-Fi system (see C.7.2 or C.7.4, pp. 20–21). Primer sets were targeting the 16S or 23S rDNA, or selected functional genes (see Appendix, Table 18, p. XII), whereby optionally it was useful to increase primer concentration to facilitate amplification success. Cloning was performed according the manufacturer's protocol (TA Cloning® Kit, with pCR™2.1 Vector, Invitrogen) described in C.10, p. 22. Plasmids with successful integrated amplicons were extracted and sequenced (see C.11, p. 24). Sequence data were analyzed using the ARB software package with appropriate gene databases (see C.11.1, p. 25, and W. Ludwig, pers. comm.) and the BLAST database.

D. RESULTS

1. DIRECT DETECTION SYSTEMS FOR *ENTEROCOCCUS* SPP. IN FECAL SAMPLES

In this part several diagnostic PCR and FISH approaches were developed, evaluated and optimized for the genus-specific detection and identification of enterococci in fecal samples without previous DNA extraction.

1.1. *ENTEROCOCCUS*-SPECIFIC DIAGNOSTIC PCR

For design and *in situ* validation of a genus specific primer set, for the amplification of an approx. 600–1,000 bp long DNA fragment, the ARB software package with the latest 16S rRNA database was used (see C.16.1, p. 38). All available enterococci sequences were used to identify two short oligonucleotide sequences within a conserved region for this genus.

Enc473V (*E. coli* position: 473–493) and Enc1276R (*E. coli* position: 1,276–1,300) were specified as predominantly genus-specific, corresponding to their single matching results in the 16S rRNA database using 'probe match' and were supposed to allow amplification of a 16S rRNA gene fragment with a size of ~827 bp (see Table 10, p. 38).

According to TestProbe 3.0 (http://www.arb-silva.de), the forward primer Enc473V possessed 99.8% specificity to enterococci assuming that primers with max. two mismatches can still lead to a specific amplification. Only *E. caccae* and *E. haemoperoxidus* with three mismatches were validated as not being a target of this primer. Furthermore, the primer Enc473V showed 100% specificity with zero mismatches to the members of the genus *Carnobacterium*, Gram-positive, rod-shaped bacteria, found in aquatic environments. *In situ* validation of the reverse primer Enc1276R revealed that it specifically matches all enterococcal strains (0–1 mismatch), excluding *E. columbae* with two mismatches. Further matches were found with members of the closely related genus *Tetragenococcus* (2 mismatches).

Performing TestPrime 1.0 (http://www.arb-silva.de) with both primers and max. two allowed mismatches, the primer set revealed an *Enterococcus*-specificity of 99.9%, whereas mentioned *Carnobacterium* could be excluded with zero percent specificity, as only the forward primer was specific for this genus. *E. caccae* was not found on the hitlist for the primer set.

To experimentally verify the specificity of each oligonucleotide, each primer, Enc473V and Enc1276R, was used in a PCR (Ex*Taq*™ system) by combining them with a universal bacterial 16S rDNA primer (616Valt and 100K, see Appendix, Table 18, p. XI). Genomic template DNA from available enterococcal strains and several negative control strains (Table 1) was used in the PCR reactions (Figure 2 and Figure 3). Of all samples tested with enterococcal genomic DNA gel electrophoresis of PCR application using primer set Enc473V/100K (~1,057 bp fragment) revealed positive amplification of a fragment with the length of approximately 1,000 bp (Figure 2). Noteworthy, the PCR amplification yield with *E. saccharolyticus* (Lane 12) and *E. canis* (Lane 26) DNA was lower compared to that obtained with other enterococcal strains.

The PCR approaches were also tested on negative control strains, including several intestinal bacteria and members of the genus *Streptococcus* which are phylogenetically closely related to enterococci. Samples containing the genomic DNA from the control strains *Lactococcus lactis* subsp. *lactis* (Lane A) and *Streptococcus salivarius* (Lane B), *Xanthomonas campestris* (Lane C) and *Escherichia coli* (Lane D) also resulted in amplification, but with reduced quantity despite applying equal DNA template concentrations. Samples with templates of purified genomic DNA from the other negative control strains (Lanes E–K) and the no-template controls revealed no amplification of the 16S rDNA fragment.

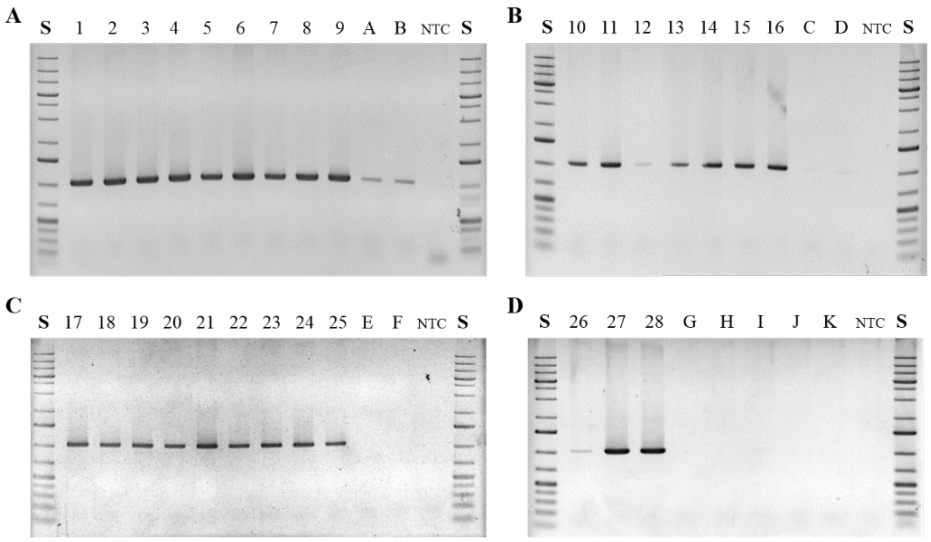


Figure 2. Validation of the *Enterococcus*-specific forward primer Enc473V, targeting the 16S rRNA gene. Gel electrophoretic analysis of primer Enc473V and 100K, applied to DNA from project relevant species. Amplification yielded in the expected product amplicon length of ~1,057 bp. Numbers refer to genomic DNA from *Enterococcus* spp., capitals refer to selected control strains. **1:** *E. avium* **2:** *E. casseliflavus* **3:** *E. cecorum* **4:** *E. columbae* **5:** *E. dispar* **6:** *E. faecium* **7:** *E. flavescens* **8:** *E. gallinarum* **9:** *E. malodoratus* **10:** *E. mundtii* **11:** *E. raffinosus* **12:** *E. saccharolyticus* **13:** *E. sulfreus* **14:** *E. faecalis* subsp. *zymogenes* **15:** *E. faecalis* subsp. *liquefaciens* **16:** *E. asini* **17:** *E. faecalis* **18:** *E. hirae* **19:** *E. durans* **20:** *E. haemoperoxidus* **21:** *E. villorum* **22:** *E. gilvus* **23:** *E. ratti* **24:** *E. porcinus* **25:** *E. pallens* **26:** *E. canis* **27:** *E. phoeniculicola* **28:** *E. faecalis* ATCC 29212 **A:** *L. lactis* subsp. *lactis* **B:** *S. salivarius* **C:** *X. campestris* **D:** *E. coli* **E:** *S. gallinarum* **F:** *M. luteus* **G:** *M. sedentarius* **H:** *C. glutamicum* **I:** *S. agalactiae* **J:** *S. pyogenes* **K:** *S. bovis*. **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder.

Primer Enc1276R was combined with universal bacterial primer 616Valt for PCR (see Appendix, Table 18, p. XI), again using DNA from several enterococci and related strains as DNA templates (Figure 3). In this approach an amplification of a fragment with the size of ~1,292 bp was detectable in all samples containing *Enterococcus* DNA, whereas amplification of *E. saccharolyticus* DNA again revealed a low yield. Beside the expected fragment, most samples showed an additional amplification band of approx. 400 bp size. However, as illustrated in Figure 3D, the PCR reactions using DNA from the negative control strains *L. lactis* (Lanes A and H), *S. salivarius* (Lanes B and I), *S. agalactiae* (Lane J), *S. pyogenes* (Lane K) and *S. bovis* (Lane L) showed an amplified fragment with the length of ~1,300 bp. The template DNA from the other control strains (Lanes C–G) and of the no-template controls (NTC) was not amplified in PCR runs.

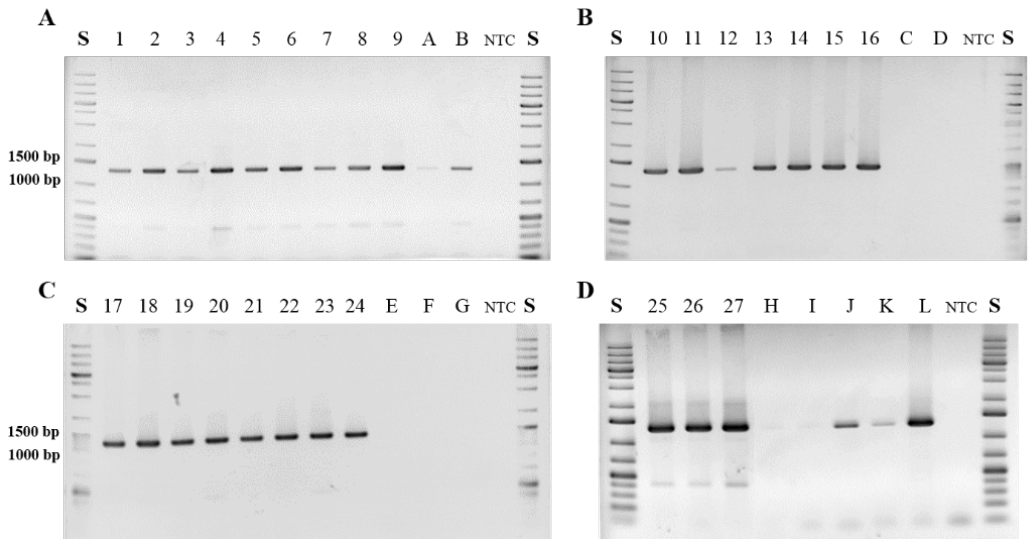


Figure 3. Validation of the *Enterococcus*-specific reverse primer Enc1276R, targeting the 16S rRNA gene. Gel electrophoretic analysis of primer 616Valt and Enc1276R, applied to DNA from project relevant species. Amplification yielded in the expected product amplicon length of ~1,292 bp. Numbers refer to genomic DNA from *Enterococcus* spp., capitals refer to selected negative strains. **1:** *E. avium* **2:** *E. casseliflavus* **3:** *E. cecorum* **4:** *E. columbae* **5:** *E. dispar* **6**: *E. faecium* **7:** *E. flavescens* **8:** *E. gallinarum* **9:** *E. malodoratus* **10:** *E. mundtii* **11:** *E. raffinosus* **12:** *E. saccharolyticus* **13:** *E. sulfreus* **14:** *E. faecalis* zymogenes **15:** *E. faecalis* liquefaciens **16:** *E. asini* **17:** *E. hirae* **18:** *E. durans* **19:** *E. haemoperoxidus* **20:** *E. villorum* **21:** *E. gilvus* **22:** *E. ratti* **23:** *E. porcinus* **24:** *E. pallens* **25:** *E. pseudoavium* **26:** *E. phoeniculicola* **27:** *E. faecalis* ATCC 29212 **A:** *L. lactis* subsp. *lactis* **B:** *S. salivarius* **C:** *X. campestris* **D:** *E. coli* **E:** *M. sedentarius* **F:** *S. gallinarum* **G:** *M. luteus* **H:** see A **I:** see B **J:** *S. agalactiae* **K:** *S. pyogenes* **L:** *S. bovis*. **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder.

Figure 4 illustrates the results for the primer set Enc473 and Enc1276R applied to DNA samples of intestinal relevant enterococci and selected negative strains. An amplified DNA-fragment with an expected length of ~800 base pairs was found for all applied *Enterococcus* DNA templates (Lanes 1–7). Although the quantity of amplified DNA differs for certain enterococci, detectable in repeated PCR reactions, all tests were positive by showing an amplified fragment.

The amplification yield of *E. caccae* was lower compared to other enterococcal strains. In the cases of the negative control strains (Lanes A–E) and the no-template control (NTC), no amplification could be detected. The same results were obtained with template DNA from the remaining available enterococcal strains (Table 1; PCR data not shown).

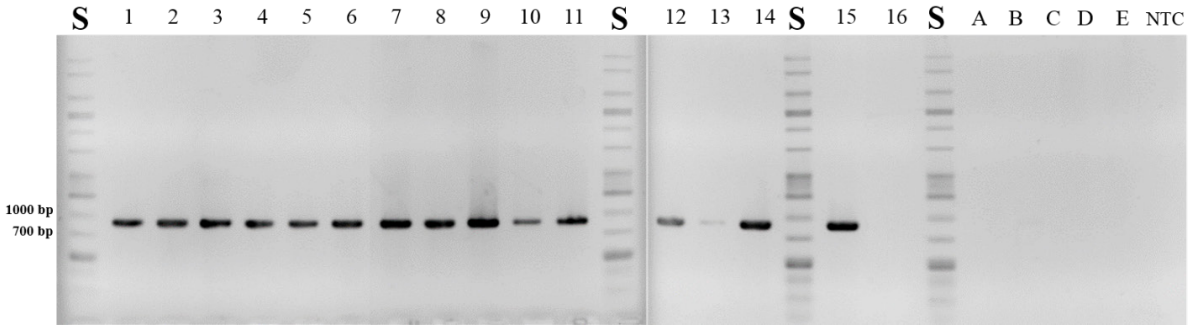
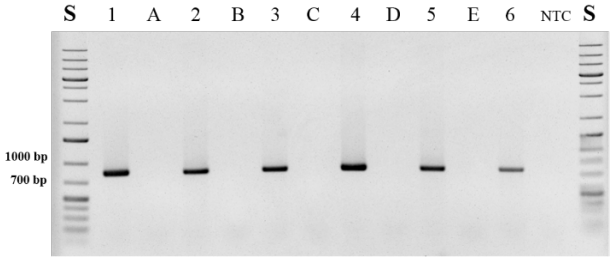


Figure 4. Validation of the *Enterococcus*-specific primer set Enc473V/Enc1276R, targeting the 16S rRNA gene. Gel electrophoretic analysis of *Enterococcus*-specific primer set, applied to DNA from relevant intestinal species. Amplification yielded in the expected product amplicon length of ~800 bp. Numbers refer to genomic DNA from *Enterococcus* spp., capitals refer to selected negative strains. **1:** *E. avium* **2:** *E. casseliflavus* **3:** *E. cecorum* **4:** *E. dispar* **5:** *E. faecium* **6:** *E. malodoratus* **7:** E. *raffinosus* **8:** *E. hirae* **9:** *E. durans* **10:** *E. gilvus* **11:** *E. pallens* **12:** *E. faecalis* **13:** *E. caccae* **14:** *E. gallinarum* **15:** *E. columbae* **16:** *Tetragenococcus solitarius* **A:** *S. bovis* **B:** *S. pyogenes* **C:** *S. salivarius* **D:** *Akkermansia muciniphila* **E:** *Bacteroides eggerthii.* **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder.

As *Enterococcus*-specific diagnostic PCR should be adapted to direct-PCR using fecal samples as template without previous DNA extraction, the amplification procedure was first adjusted to bacterial pure culture mixes. To this end, several artificial mixed samples were prepared by mixing approx. 20 µL aliquots of each pure culture (liquid, OD_{600} 0.6–0.9), and 2 µL of each mixture was used as a PCR template. The investigated bacterial mixtures and PCR amplification results are presented in Figure 5. All mixtures containing *E. faecalis*, as a representative of enterococci, resulted in the successful amplification of the primer-embraced 16S rDNA region.



Sample	Bacteria strains
1	*E. faecalis*
A	*S. pyogenes; S. bovis*
2	*E. faecalis; S. pyogenes; S. bovis*
B	*B. subtilis; S. enterica; E. coli; P. aeruginosa*
3	*E. faecalis; B. subtilis; S. enterica; E. coli; P. aeruginosa*
C	*S. salivarius; S. agalactiae*
4	*E. faecalis; S. salivarius; S. agalactiae*
D	*S. salivarius; S. agalactiae; S. pyogenes; S. bovis*
5	*E. faecalis; S. salivarius; S. agalactiae; S. pyogenes; S. bovis*
E	*B. subtilis, S. enterica; E. coli; S. salivarius; S. agalactiae; S. pyogenes; S. bovis; P. aeruginosa;*
6	*E. faecalis; B. subtilis, S. enterica; E. coli; S. salivarius; S. agalactiae; S. pyogenes; S. bovis; P. aeruginosa*

Figure 5. Evaluation of primer specificity in mixed cultures. Direct-PCR applied to bacterial pure culture mixtures using the *Enterococcus*-specific primer set Enc473V/Enc1276R, targeting the 16S rRNA gene. Gel electrophoretic analysis of the direct-PCR detection of *Enterococcus* spp. without prior DNA extraction, yielded in the expected product amplicon length of ~800 bp. Numbers refer to bacterial mixtures containing *E. faecalis* including several negative control strains, capitals refer to bacterial mixtures which only were composed of negative control strains. **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder.

To determine the detection limit of the *Enterococcus*-specific PCR system, serial 10-fold dilutions (10^{-1} to 10^{-6}) of an *E. faecalis* pure culture (~4.67×10^8 CFU/mL, see D.2.1) were prepared. These *E. faecalis* suspensions were directly applied to PCR without DNA extraction. Figure 6 shows that the detection limit was approx. at the 10^{-5} dilution which corresponded to an *E. faecalis* concentration of ~4.67 CFU/µL sample. As the goal was to apply the PCR technique directly to fecal samples, approx. 0.2 g of germ-free mice feces (GF) were prepared and diluted 10^{-1} to 10^{-4} according to initial feces volume. A 10- and 100-fold dilution of the original GF feces and the negative control (NTC) did not result in an amplification product.

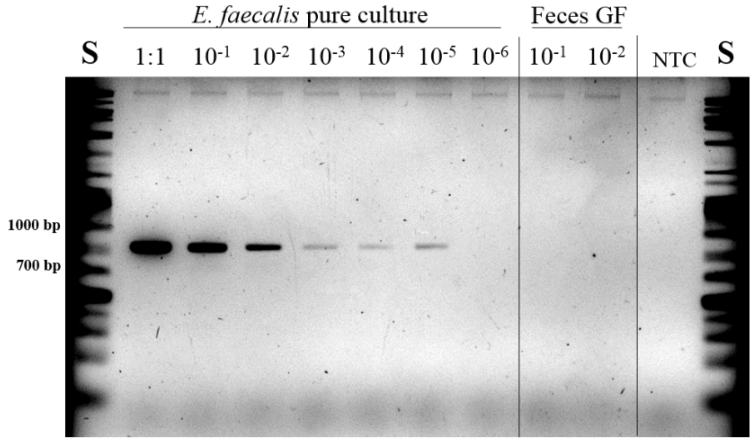


Figure 6. Detection limit of the *Enterococcus*-specific PCR system in pure cultures and application to mouse feces. Gel electrophoretic analysis of *Enterococcus*-specific primers set Enc473V and Enc1276R, applied to ten-fold dilutions of an *E. faecalis* pure culture (1:1=4.67×10^8 CFU/mL to 10^{-6}=467 CFU/mL) and ten-fold dilutions of germ-free (GF) mouse feces (~0.2 g preparation). Amplification yielded in the expected product amplicon length of ~800 bp. **NTC:** no-template control **S:** GeneRuler™ 1 kb Plus DNA ladder.

To define the detection limit in fecal samples, GF feces (10-fold and 10,000-fold diluted from original feces) were spiked with the *E. faecalis* pure culture and subsequently serially diluted (10^{-1} to 10^{-4}). Sample A (10^4-fold diluted GF feces) was inoculated with an *E. faecalis* concentration of approx. 9.34×10^6 CFU/mL and Sample B comprised half the concentration (4.67×10^6 CFU/mL). In this diluted fecal sample, *E. faecalis* was detectable until a sample concentration of approx. 47 CFU/µL. Sample C (10-fold diluted GF feces) was spiked with *E. faecalis* to a final concentration of ~1.8×10^7 CFU/mL. While the 10-fold serial dilution of this fecal suspension, detection was only possible at sample cell concentrations between ~18 and ~180 CFU/µL. No amplification products were obtained from the negative controls (NTC).

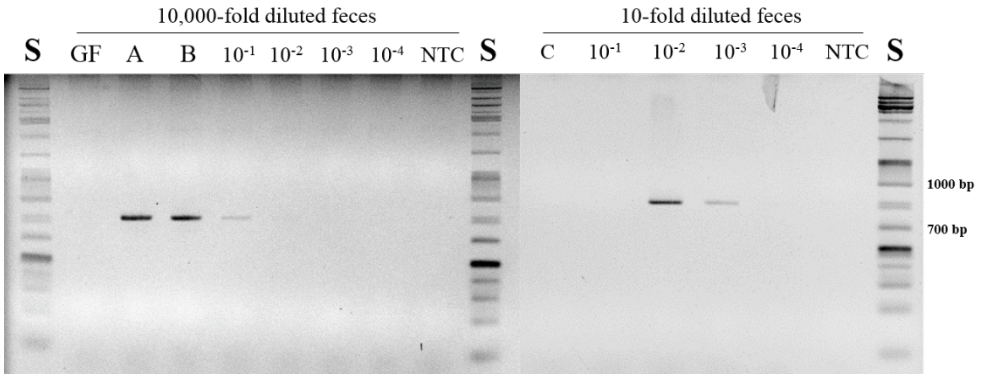


Figure 7. Detection limit of the *Enterococcus*-specific PCR system in fecal samples. Gel electrophoretic analysis of *Enterococcus*-specific primers set Enc473V and Enc1276R, applied to artificially spiked diluted germ-free mouse feces. GF: germ-free feces. **A:** GF, spiked with approx. 9.34×10^6 CFU/mL. **B:** GF, spiked with approx. 4.67×10^6 CFU/mL, followed by ten-fold dilutions. **C:** GF, spiked with approx. 1.8×10^7 CFU/mL, followed by ten-fold dilutions. Amplification yielded in the expected product amplicon length of 800 bp. **NTC:** no-template control **S:** GeneRuler™ 1 kb Plus DNA ladder.

Developed as a pretest for subsequent cell enrichment experiments, the genus-specific direct-PCR aimed to directly identify enterococci presence in fecal samples without prior DNA extraction. Figure 8 shows gel electrophoretic analysis of PCR amplification products using the primer set Enc473V/Enc1276R and 2 µL of prepared washed fecal samples (see C.12, p. 25) collected from INF and SPF mice as direct templates. Genomic DNA from *E. faecalis* was used as a positive control (PC). Gel electrophoresis of all PCR products showed positive amplification of the specific 16S rDNA region of approx. 800 bp. No amplification was detected in the no-template control (NTC).

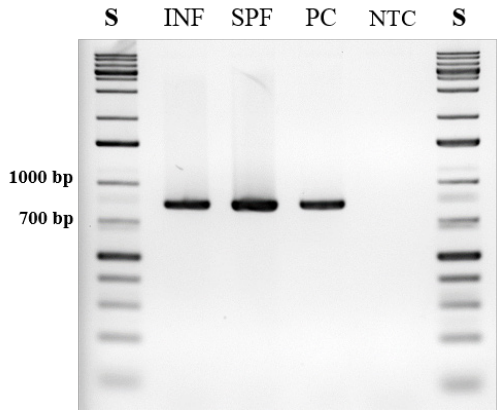


Figure 8. Direct *Enterococcus*-specific PCR applied to different mouse feces samples. Gel electrophoretic analysis of direct-PCR detection of *Enterococcus* spp. from mouse feces (diluted in PBS) using the primer set Enc473V and Enc1276R, targeting the 16S rRNA gene. Amplification yielded in the expected product amplicon length of ~800 bp. **INF:** feces of *E. faecalis* infected GF mice. **SPF:** feces of specific-pathogen-free mice. **PC:** positive control (extracted DNA from *E. faecalis*). **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder.

1.2. *ENTEROCOCCUS* IDENTIFICATION BY FISH

According to probe design recommendations by Hugenholtz *et al.* [164], the reverse complement of the target string, *i.e.* the forward primer Enc473V, was chosen as FISH probe for enterococci identification. The reverse complementary probe sequence (5′-TAG ATA CCG TCA RGG GAY G-3′) was revised with respect to melting temperature using the nearest neighbor method (≥ 57°C with 50 mM NaCl and 50 µM primer) [165]. The oligonucleotide was either 5′-labeled with yellow-absorbing fluorescent dyes Cy3 (Eurofins Scientific, Germany) or ATTO565 (ATTO-TEC GmbH, Germany), both exiting in the red spectral range (λ_{abs} = 545–575 nm, orange-red). To optimize a FISH protocol and to validate the specificity of the probe, *Enterococcus* spp. pure cultures, mixed cultures and fecal samples were applied to standard oligonucleotide FISH experiments (oligoFISH) and protocols of previous studies [120, 141, 166] were modified and adjusted according to the probe and the samples (see C.14.1, p. 30). For detection of non-specific probe binding, all samples were simultaneously hybridized with the FAM-labeled oligonucleotide probe EUB338 (λ_{abs}= 488nm, green) or stained with DAPI (λ_{abs} = 358 nm, blue) after hybridization using a DAPI-containing mounting medium (see C.14.4, p. 34). While the bacterial universal probe EUB338 is specific to most of the members of the domain Bacteria (see Appendix, Table 18, p. XII), DAPI binds to every double-stranded DNA.

According to the experiments performed in this thesis, an exposure to lysozyme (10 mg/mL) of 10–15 min on ice seemed to be appropriate for efficient uptake of the probe into the entero-

coccal cells. The formamide concentration in the hybridization buffer, which adjusts stringency, was determined to be most efficient at 20%, while a hybridization time of 3–5 h, followed by 15 min washing at 48 °C was identified as best working parameters for the *Enterococcus*-specific oligoFISH approach. Furthermore, it was observed that fixed bacteria material should not be stored longer than 1–2 months to be used in FISH studies, because thereafter the quality of the signals decreased.

Figure 9 shows the images of the microscopic analysis after FISH using *E. faecalis* pure cultures and adjusted conditions using the Enc473-ATTO565 probe. Positively hybridized enterococcal cells are shown purple using a composite overlay of the red fluorescence image (Figure 9A) and the blue DAPI channel image (Figure 9B).

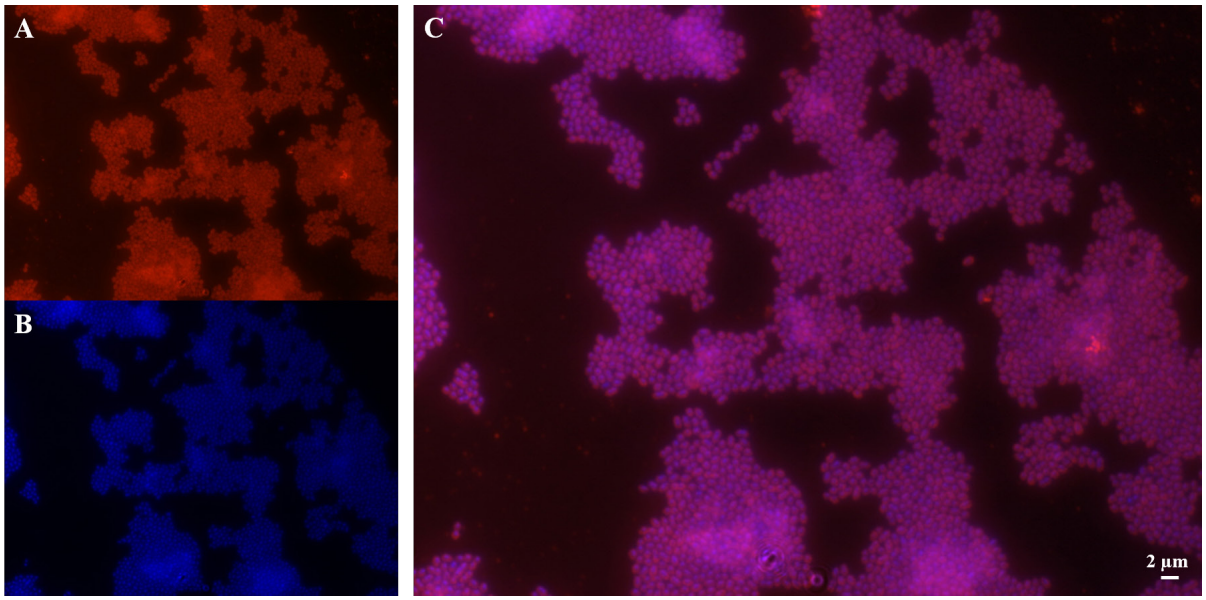


Figure 9. Specific detection of enterococci in an *E. facealis* pure culture by oligoFISH. Epifluorescence images of *in situ* hybridization with *Enterococcus* genus-specific oligonucleotide probe Enc473. **A:** Enc473-ATTO565 (red). **B:** DAPI staining (blue). **C:** composite overlay of micrographs A and B.

For verification of specificity, mixed cultures of *E. faecalis* and *Corynebacterium glutamicum* were applied to the *Enterococcus*-specific FISH approach. The composite overlay of the fluorescence image of the ATTO565 channel (Figure 10A) and FAM channel image (Figure 10B) revealed that enterococcal cells emitted the green and the red signal, leading to an orange-red signal, whereas *C. glutamicum* cells appeared green by hybridization with the EUB338-FAM probe.

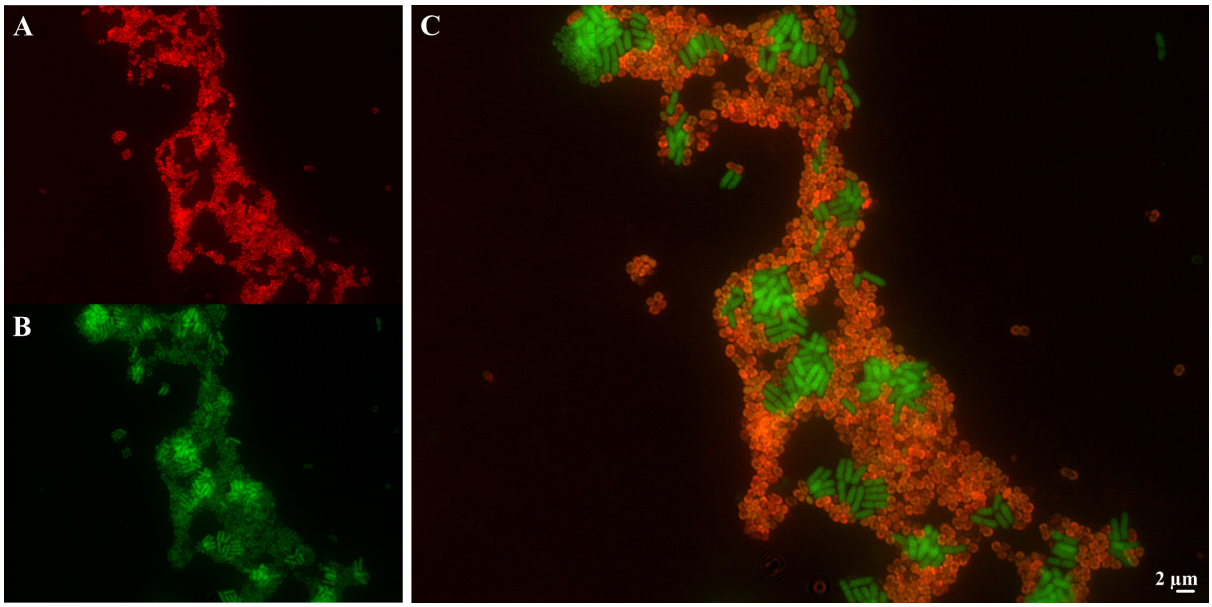


Figure 10. Specific detection of enterococci in a mixed sample of *E. facealis* and *C. glutamicum* pure cultures by oligoFISH. Epifluorescence images of *in situ* hybridization with *Enterococcus* genus-specific oligonucleotide probe Enc473 and the universal bacterial probe EUB338. **A:** Enc473-ATTO565 (red). **B:** EUB338-FAM (green). **C:** composite overlay of micrographs A and B.

The microscopic analysis shown in Figure 11 depicts a pure culture mixture of *E. faecalis* and *Bacillus subtilis*, which was hybridized with Enc473-ATTO565 and EUB338-FAM. Only enterococcal cells showed an orange-yellow signal, arising from merging the red and green channels, whereas *B. subtilis* cells only emitted the green fluorescence signal.

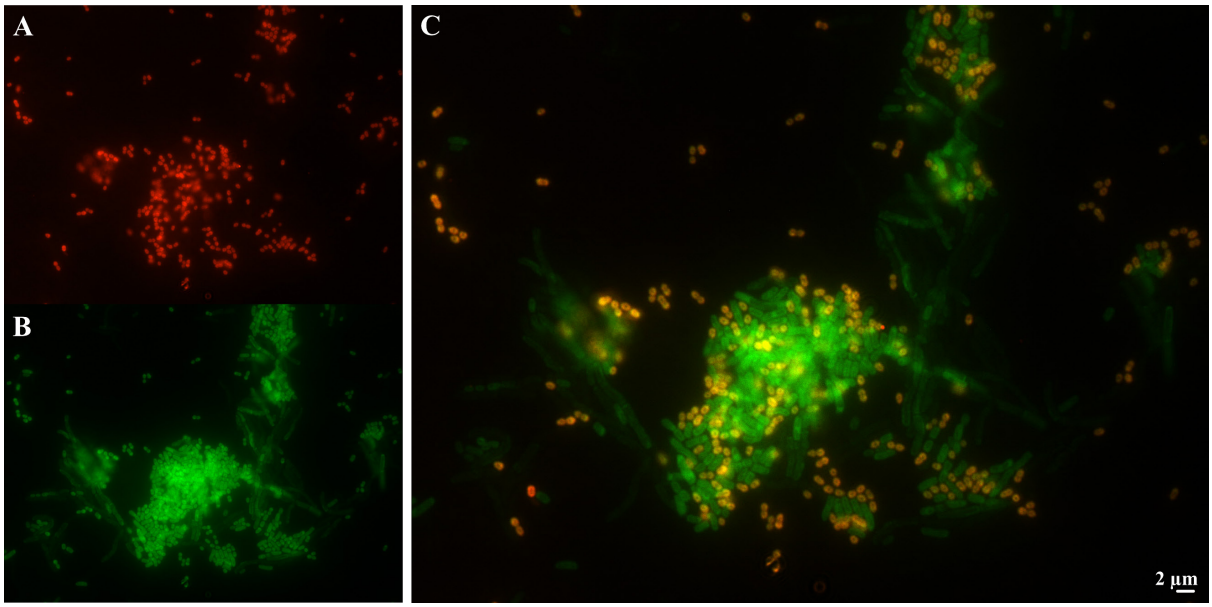


Figure 11. Specific detection of enterococci in a mixed sample of *E. facealis* and *B. subtilis* pure cultures by oligoFISH. Epifluorescence images of *in situ* hybridization with *Enterococcus* genus-specific oligonucleotide probe Enc473 and the universal bacterial probe EUB338. **A:** Enc473-ATTO565 (red). **B:** EUB338-FAM (green). **C:** composite overlay of micrographs A and B.

Figure 12 shows the microscopic analysis of FISH, which was performed using a mixture of *E. faecalis* cells and cultures of the closely related streptococci (*S. salivarius*, *S. bovis* and *S. pyogenes*). Again, signals from enterococcal cells appeared yellow, whereas streptococcal cells only emitted the green signal of the EUB338-FAM probe.

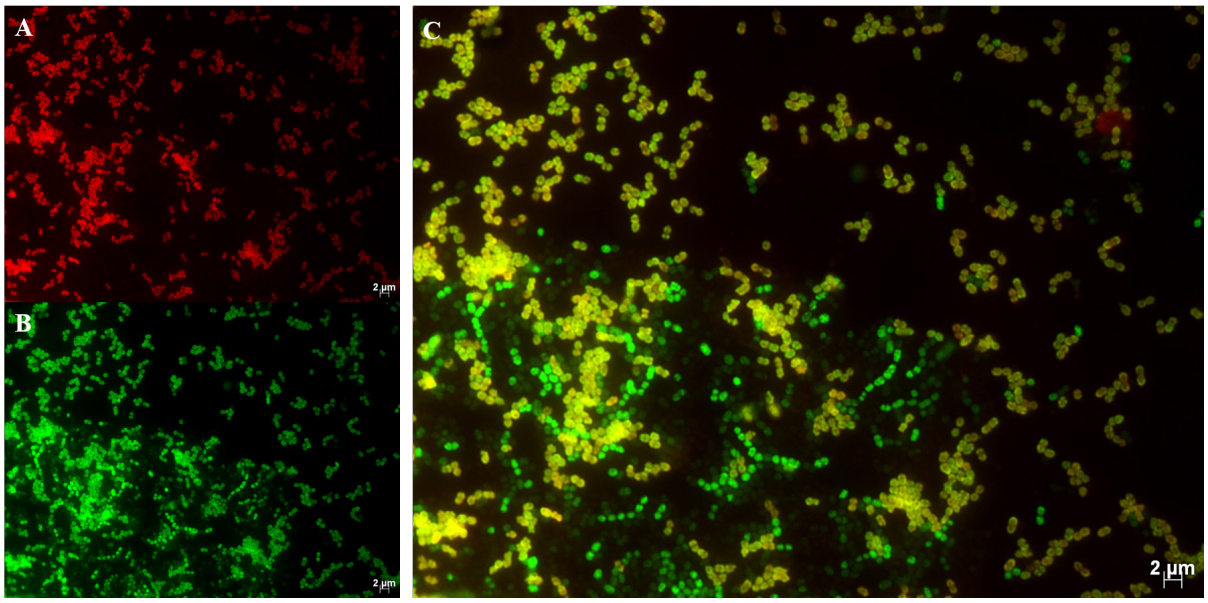


Figure 12. Specific detection of enterococci in a mixed sample of *E. facealis, S. salivarius, S. bovis* and *S. pyogenes* pure cultures by oligoFISH. Epifluorescence images of *in situ* hybridization with *Enterococcus* genus-specific oligonucleotide probe Enc473 and the universal bacterial probe EUB338. **A:** Enc473-Cy3 (red). **B:** EUB338-FAM (green). **C:** composite overlay of micrographs A and B.

More investigations were performed mainly with regard to the bacterial composition of the gastrointestinal tract. To verify the *Enterococcus*-specific oligonucleotide used for FISH, bacterial pure cultures were fixed as described in chapter C.13.2 and then used for the preparation of mixtures of relevant gastrointestinal bacteria. Figure 13 presents the FISH images resulting from hybridization with Enc473-Cy3 and the EUB338-FAM probe, using a mixture of pure cultures of *E. faecalis*, *M. luteus*, *M. sedentarius*, *E. coli*, *Moraxella catarrhalis* and *Enterobacter aerogenes*. The *Enterococcus*-specific probe Enc473 showed a high specificity for enterococci, which emitted red and green signals (= yellow). For all other relevant intestinally bacteria, only green signals were observable.

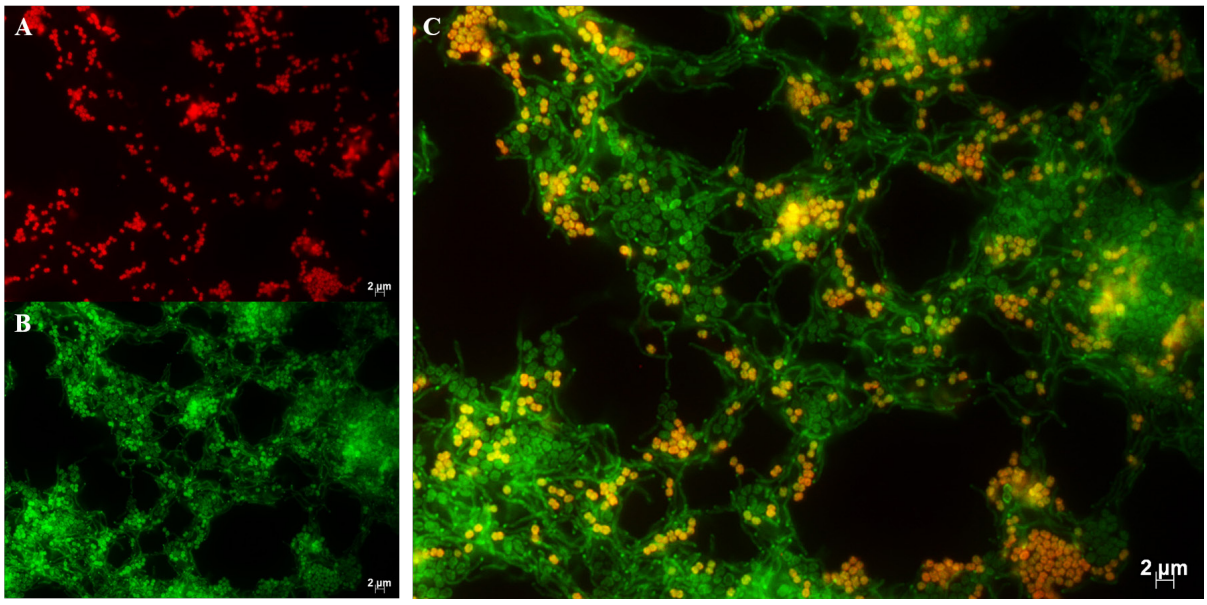


Figure 13. Specific detection of enterococci in a mixed sample of *E. facealis* and bacteria belonging to the natural gut microbiota (pure cultures) by oligoFISH. Epifluorescence images of *in situ* hybridization with *Enterococcus* genus-specific oligonucleotide probe Enc473 and the universal bacterial probe EUB338. **A:** Enc473-Cy3 (red). **B:** EUB338-FAM (green). **C:** composite overlay of micrographs A and B.

After evaluation of the *Enterococcus*-specific oligonucleotide probe Enc473 on pure cultures and cell mixtures, the probe was tested on prepared fecal samples from specific-pathogen-free (SPF) mice (see C.12, p. 25). The FISH experiments using fecal samples indicated that fecal components like organic matters absorbed a large fraction of the oligonucleotide probes. This was specifically observed for the probe labeled with a Cy3 dye which resulted in high background noise and fluorescence of the fecal compounds. According to this, the Cy3 dye was replaced by the novel ATTO565 dye that promised stronger absorption, higher fluorescence quantum yield, and higher thermal and photo-stability than Cy3.

The concentration of bacteria belonging to the genus *Enterococcus* in the real SPF samples was apparently below the detection limit of the FISH approach (only EUB338-FAM signals detectable; data not shown). To overcome this, the fecal samples were spiked with *E. faecalis* pure cultures prior to the fixation procedure. Figure 14 shows FISH of a spiked fecal SPF sample applying Enc473-ATTO565 and EUB338-FAM. In the composite overlay micrograph (Figure 14C) enterococcal cells are detectable by a yellow-orange signal, whereas non-target cells only emitted the green signal of the EUB338-FAM probe. Again however, adsorption of the probes to fecal matter and a resulting background noise was detectable.

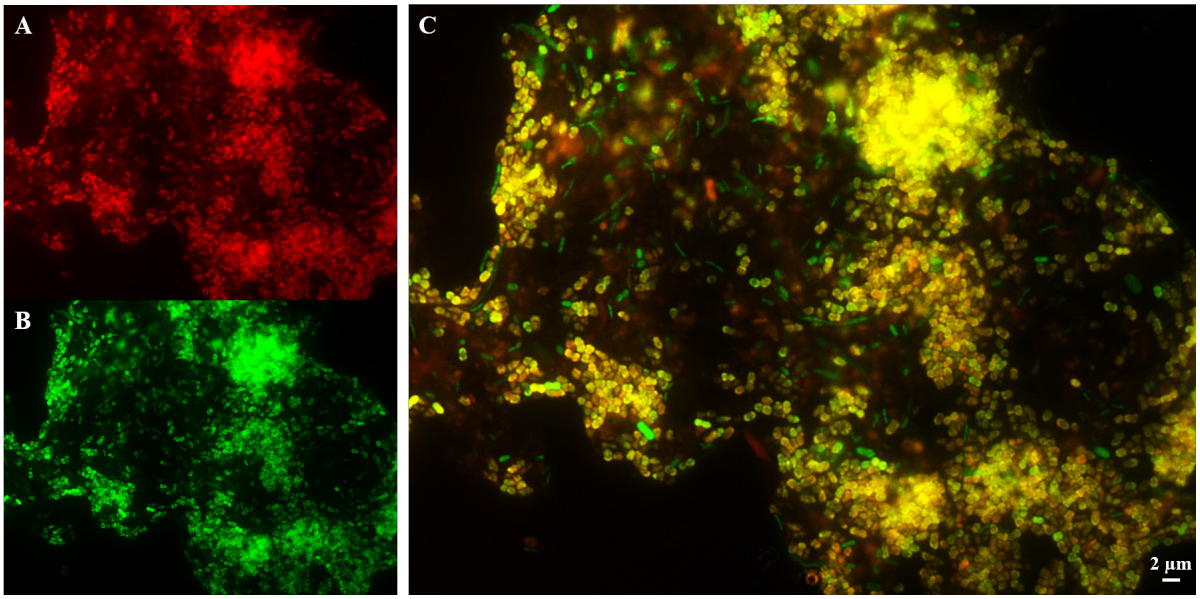


Figure 14. Specific detection of enterococci in a SPF mouse feces sample spiked with *E. faecalis* by oligoFISH. Epifluorescence images of *in situ* hybridization with *Enterococcus* genus-specific oligonucleotide probe Enc473 and the universal bacterial probe EUB338. **A:** Enc473-ATTO565 (red). **B:** EUB338-FAM (green). **C:** composite overlay of micrographs A and B.

The evaluation and verification of the *Enterococcus*-specific primers (see D.1.1, p. 45) and the oligonucleotide probe support the pretesting of fecal samples used for taxon-specific cell enrichment methods developed in this thesis. The DNA oligonucleotide probe Enc473 was also tested in combination with the *Enterococcus*-specific RNA polynucleotide 'polyDIII' (see C.14.3, p. 33). For this mutual approach, a pure culture of *E. faecalis* was used. As illustrated in Figure 15A, the DNA oligonucleotide probe showed a red fluorescence signal of the whole cell, whereas the polynucleotide probe revealed in the characteristic ring-shaped green fluorescence signal surrounding the cells (Figure 15B).

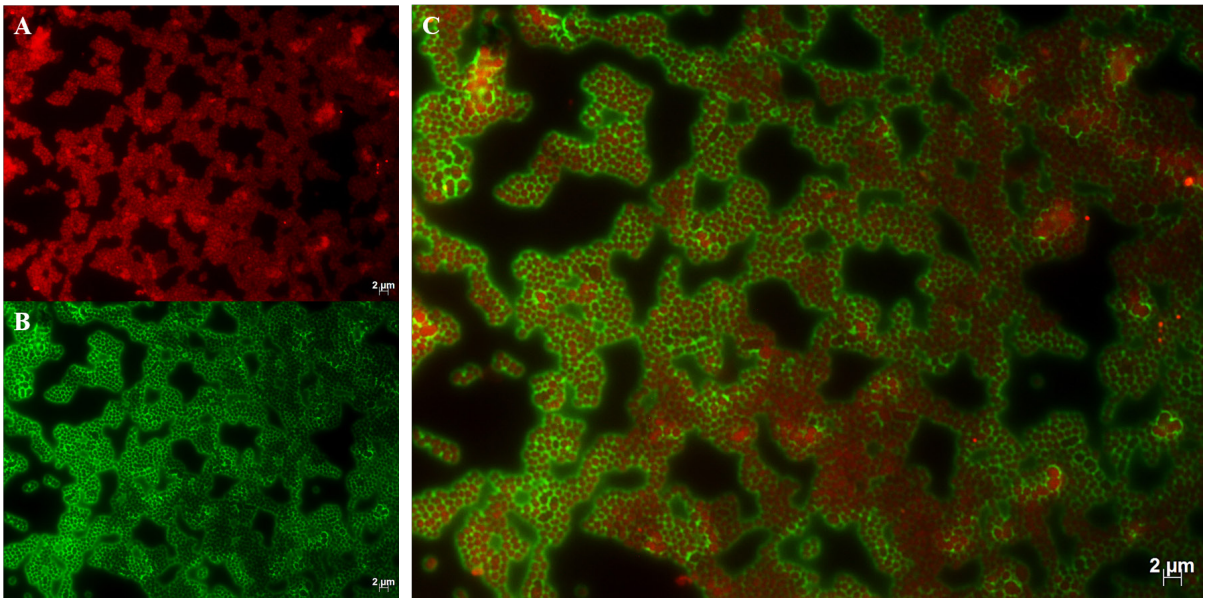


Figure 15. Specific detection of enterococci in an *E. facealis* pure culture by combined oligo- and polyFISH. Epifluorescence images of *in situ* hybridization with *Enterococcus* genus-specific probes Enc473 and polyDIII. **A:** Enc473-Cy3 (red). **B:** polyDIII-FLUOS (green). **C:** composite overlay of images A and B.

1.3. ABSOLUTE QUANTIFICATION OF ENTEROCOCCI IN FECAL SAMPLES

An absolute quantification qPCR system was developed to specifically enumerate enterococcal cells in various samples without previous DNA extraction and before sample fixation (see C.16.2, p. 39). Statistical analysis and graphs were either accomplished with the CFX-Manager software (Bio-Rad, USA) or with the statistical analysis tool OriginPro 2015 (OriginLab, USA).

1.3.1. AMPLIFICATION SPECIFICITY CONFIRMATION OF PRIMER SETS

Two systems have been developed, one targeting the 23S rRNA gene from enterococci and the other targeting *tufA* from the genus *Enterococcus*. The primer set targeting the 23S rDNA (738F/850R) (see Appendix, Table 18, p. XI) was already published by Ludwig and Schleifer [159], whereas the forward primer in this thesis was slightly modified, according to validations using the latest ARB 23S rRNA database (LSU Ref 119, July 2014, http://www.arb-silva.de). The primer set targeting the enterococcal *tufA* (Ent1(*tuf*)/Ent2(*tuf*)) (see Appendix, Table 18, p. XI) was described by Ke *et al.* [160] and was modified and validated *in silico* based on a current *tuf* gene database (Wolfgang Ludwig, pers. comm.) including various enterococcal sequences. This was accomplished in order to validate whether updated reference sequences influenced the primer sequences from Ke *et al.* in 1999. However, only one base in the forward primer had to be replaced. The best annealing temperatures of the novel enterococcal 23S

rRNA and *tufA* gene primer sets were validated by gradient PCR, revealing an optimum at 55°–60°C for both primer pairs (see C.7.1, p. 20).

Furthermore, the genus specificity of the primer sets was validated by performing a standard PCR (see C.7.2, p. 20) using DNA from relevant intestinal enterococcal strains and various negative control strain including closely related streptococci as templates. For both primer sets, gel electrophoresis confirmed the amplification of specific fragments for all enterococci templates with a size of ~100 bp. *Streptococcus* strains showed weak amplification, although *in silico* validation by ARB and TestPrime 1.0 (http://www.arb-silva.de) did not reveal matches (two allowed MM) to sequences of streptococci. The template of *Bacteroides eggerthii* did not result in any amplification (Figure 16 and Figure 17).

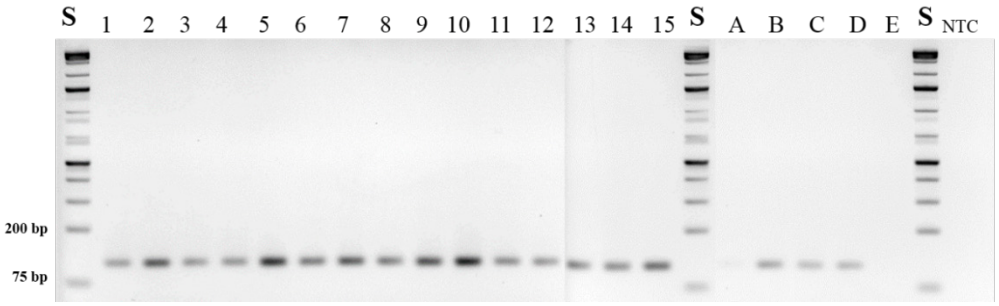


Figure 16. Validation of the *Enterococcus*-specific qPCR primer set 738F/850R, targeting the 23S rRNA gene. Gel electrophoretic analysis of 23S rDNA-targeting qPCR primer set, applied to relevant intestinal enterococcal strains and non-target strains. Amplification yielded in the expected product amplicon length of ~114 bp. Numbers refer to genomic DNA from *Enterococcus* spp., capitals refer to selected control strains. **1:** *E. avium*. **2:** *E. cassseliflavus*. **3:** *E. cecorum*. **4:** *E. dispar*. **5:** *E. faecium*. **6:** *E. malodoratus*. **7:** *E. mundtii*. **8:** *E. raffinosus*. **9:** *E. hirae*. **10:** *E. durans*. **11:** *E. gilvus*. **12:** *E. pallens*. **13:** *E. faecalis*. **14:** *E. caccae*. **15:** *E. gallinarum*. **A:** *S. salivarius*. **B:** *S. bovis*. **C:** *S. agalactiae*. **D:** *S. pyogenes*. **E:** *B. eggerthii*.

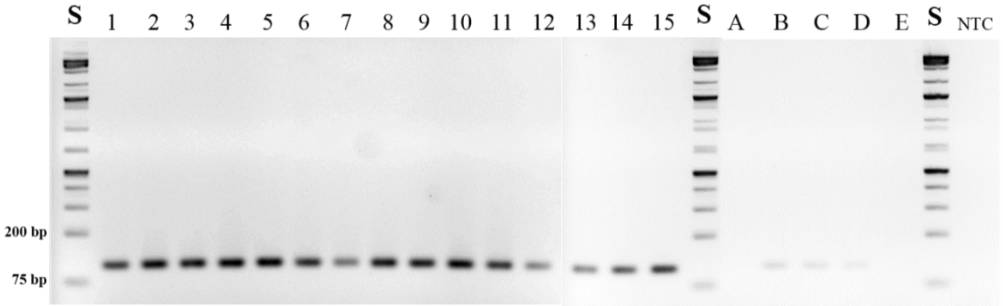


Figure 17. Validation of the *Enterococcus*-specific qPCR primer set Ent1(*tuf*)/Ent2(*tuf*), targeting *tufA*. Gel electrophoretic analysis of *tuf* gene-targeting qPCR primer set, applied to relevant intestinal enterococcal strains and non-target strains. Amplification yielded in the expected product amplicon length of ~91 bp. Numbers refer to genomic DNA from *Enterococcus* spp., capitals refer to selected control strains. **1:** *E. avium*. **2:** *E. cassseliflavus*. **3:** *E. cecorum*. **4:** *E. dispar*. **5:** *E. faecium*. **6:** *E. malodoratus*. **7:** *E. mundtii*. **8:** *E. raffinosus*. **9:** *E. hirae*. **10:** *E. durans*. **11:** *E. gilvus*. **12:** *E. pallens*. **13:** *E. faecalis*. **14:** *E. caccae*. **15:** *E. gallinarum*. **A:** *S. salivarius*. **B:** *S. bovis*. **C:** *S. agalactiae*. **D:** *S. pyogenes*. **E:** *B. eggerthii*.

Due to the non-specific amplification shown in the PCR results (streptococci in Figure 16 and Figure 17), the *tuf*-based qPCR was performed to validate the specificity and sensitivity of respective *Enterococcus*-specific *tuf* primers. Extracted DNA from *E. faecalis*, *S. bovis*, *S. agalactiae*, *S. pyogenes* and *S. salivarius* was applied to qPCR in dilutions from 10^{-2} to 10^{-5} and DNA concentrations were measured by Nanodrop (see C.6, p. 19). The mean values of the measured concentrations were compared to corresponding mean values of the qPCR results, in each case considering the dilution factors (Figure 18). Using two Y-axes, the graph reveals extremely strong qPCR sensitivity for *E. faecalis* with $1.1 \times 10^{11} \pm 4 \times 10^{9}$ detected copies/µL, by application of DNA with a concentration of 3.2 ± 0.3 µg/µL. In contrast, the maximum result obtained from streptococcal DNA was $9.4 \times 10^{7} \pm 1 \times 10^{7}$ detected copies/µL by applying DNA at a final concentration of 1.6 ± 0.4 µg/µL. For DNA from *S. bovis* it was shown that even applying almost the same concentration as from *E. faecalis* (~3 µg/µL), qPCR detected only $1.5 \times 10^{7} \pm 1 \times 10^{6}$ copies/µL, which is approx. 7,300-fold less. Finally, high sensitivity and a satisfactory specificity of the qPCR primers were shown for DNA from *S. salivarius*. By application of DNA, three-fold concentrated (~9 µg/µL) compared to *E. faecalis*, only $4.8 \times 10^{6} \pm 7 \times 10^{5}$ copies/µL were detected using the *tufA* gene-targeting primers. This corresponds to a 23,000-fold decreased detection level in contrast to *E. faecalis*.

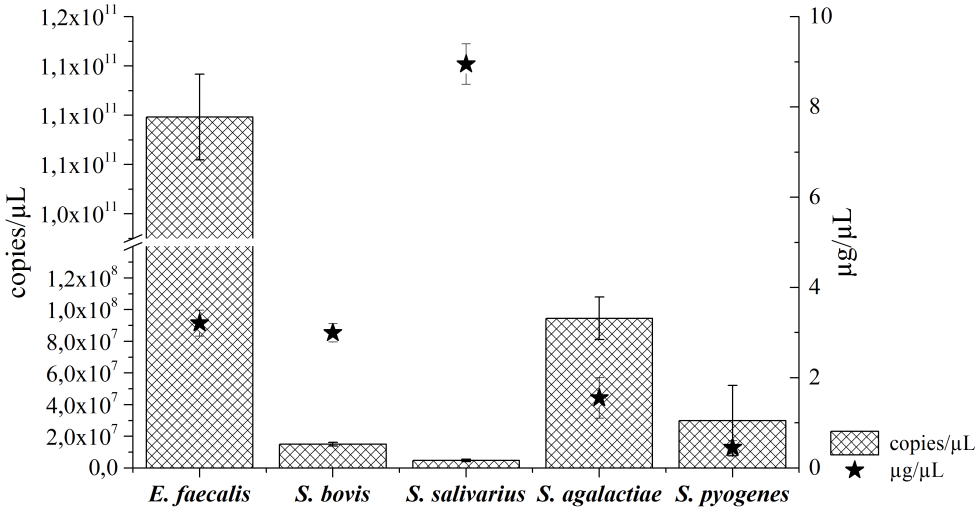


Figure 18. Evaluation of specificity and sensitivity of the *tuf*-based qPCR system. To test specificity and sensitivity of the *tuf*-based qPCR approach, different DNA concentrations (★ = µg/µL) of various negative control strains (*Streptococcus* spp.) and a positive control (*E. faecalis*) were applied and compared to qPCR results (copies/µL). Data are means ± SEM of at least 2–5 replicates. Dilution factors were considered in the analysis. The reduced specificity in respect to streptococci (Figure 16 and Figure 17) is confirmed, but however the approach revealed extremely strong sensitivity to *E. faecalis*. Particularly by comparing *E. faecalis* to *S. bovis*, the sensitivity is visualized.

1.3.2. STANDARD CURVES

The standard curves for each target were derived by applying a dilution series (from 2×10^2 to 2×10^8 copies/reaction) of known target plasmids (see C.16.2.2, p. 39) to the qPCR reaction. Plasmids contained fragments of the 23S rDNA or the *tuf* gene which were generated by including DNA extracted from the *E. faecalis* type strain as a PCR template. Relative concentrations were expressed as number of copies per µL reaction volume and the qPCR results were analyzed using the CFX-Manager software (Bio-Rad, USA). The optimal threshold was chosen automatically and was used to calculate quantification cycles (Cq) for unknown samples. Means and standard deviations were calculated for Cqs in PCR replicas. For a reliable and unequivocal interpretation of the results several standard curve parameters gave information about the performance of the reaction (see C.16.2.2, Table 11).

Standard curves, each ranging from 2×10^2 to 2×10^8 copies/reaction and their statistical analysis are shown in Figure 19. Both curves were linear in the range tested, measured by the correlation coefficient ($R^2 > 0.999$). The slopes of the log–linear phase of the amplification reaction results were –3.488 and –3.475, respectively. Thus, they were almost identical and like the following parameters displayed in Figure 19, within the target range (see C.16.2.2, Table 11). From the slopes, the amplification efficiency (E) was calculated which was 93.5% and 94.0%, respectively. The y-intercept value (y-int), useful for comparing different amplification systems and targets was 37.6 and 37.8, respectively.

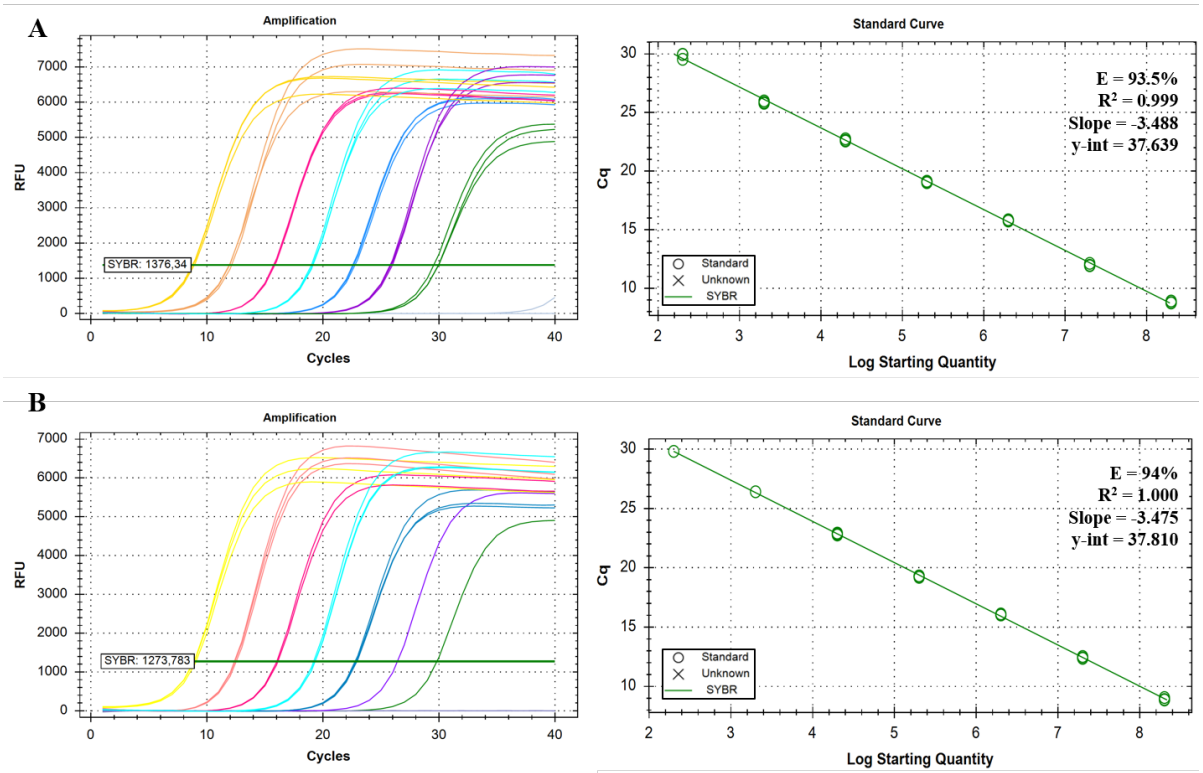


Figure 19. Amplification analysis and standard curves of *Enterococcus*-specific qPCR systems. Colors of curves were chosen randomly to discriminate different sample templates. Serial plasmid dilutions, containing the respective target gene, with 2×10^8 (yellow) to 2×10^2 (green) copies/reaction and the no-template control (grey), all in triplicate. **A:** 23S rDNA-based qPCR. **B:** *tuf* gene-based qPCR. **RFU:** relative fluorescence units. **Cq:** quantification cycle. **E:** efficiency. **R^2:** coefficient of determination. **y-int:** y-intercept.

The amplification specificity and presence of potential primer dimers was verified by melting curve analysis. During the melting reaction a plot of the negative first regression of relative fluorescence vs. temperature (–d(RFU)/dT) displays changes in fluorescence as distinct peaks. The melting curves of both primer sets occurred in single peaks (at 82.5°C and 83°C, respectively), confirming the specific amplification of the *Enterococcus* target and no presence of nonspecific secondary products or primer dimers (Figure 20).

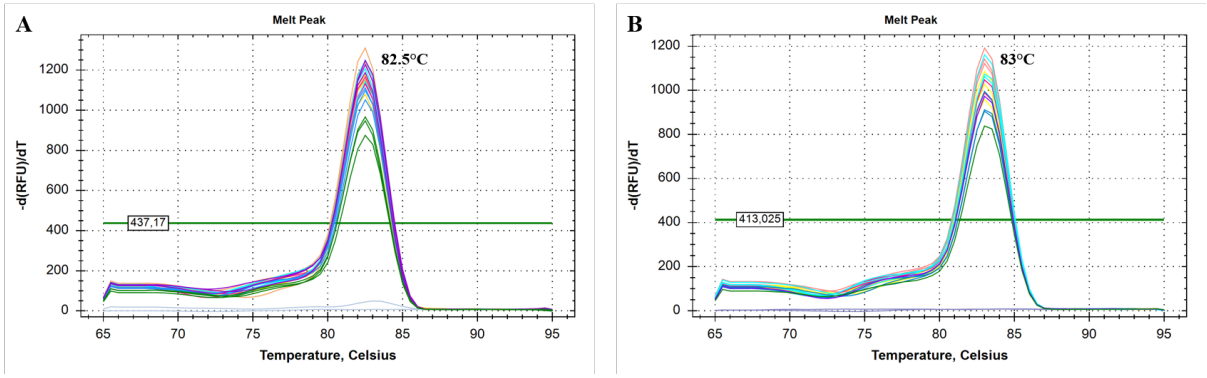


Figure 20. Melting curve analysis of the *Enterococcus*-specific qPCR assays. Colors of curves were chosen randomly to discriminate different sample templates (10-fold serial plasmid dilutions and the no-template control). **A:** 23S rDNA-based qPCR (melting peak: 82.5°C). **B:** *tuf* gene-based qPCR (melting peak: 83°C). **–d(RFU)/dT:** negative first regression of relative fluorescence vs. temperature.

1.3.3. LIMIT OF QUANTIFICATION

By determining the limit of quantification and efficiency, differences between these two qPCR systems were evaluated. The limit of quantification (LoQ) concerning the qPCR approach was defined as the minimum concentration (target gene copy number per reaction) that remained within the linear region of target concentration response. For this purpose, a freshly prepared pure culture of *E. faecalis* was prepared and a 10-fold dilution series (using PBS) was directly applied to both systems. Both approaches were performed in the same microplate to exclude inter-run variances. The determination of the starting quantity of the pure culture was performed by spectrophotometry and the culture was then diluted to specific cell concentrations (5×10^4, 5×10^3, 5×10^2, 50, 5, and 0.5 cells/µL). Amplification analysis of both qPCR approaches revealed reaction parameters almost identical and within the target range (see C.16.2.2, Table 11). Hence, they were comparable and the results could be used for interpretation.

Figure 21 shows the bar chart created from qPCR results obtained after application of the decreasing dilution series to both quantification systems. Black bars represent the *tuf* gene-based qPCR approach, while the striped bars illustrate the 23S rDNA-based quantification system. It was visualized that for both systems the gradient was detectable, whereby the quantification using the 23S rDNA-based qPCR system (copies/µL) corresponded most exactly to the concentration of the cell suspensions, calculated from the optical density (1 OD_{600} = 5×10^8 CFU/mL). The quantification by the *tuf*-based qPCR system resulted in nearly 2.1 times less detected copies/µL, but assimilated at a cell concentration of 5 cells/µL, which also was the quantification limit for both systems.

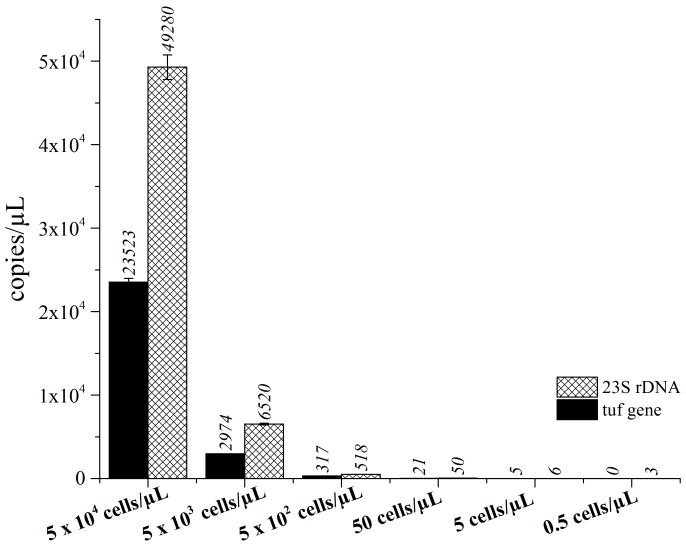


Figure 21. Limit of quantification and comparison of the efficiency of the *Enterococcus*-specific direct qPCR systems. Results were obtained by application of a gradient dilution series of a fresh *E. faecalis* pure culture (5 × 10⁴–0.5 cells/µL) to the *tuf* gene and 23S rDNA-based qPCR systems without prior DNA extraction. The indicated values are mean values from duplicate reactions.

1.3.4. REPRODUCIBILITY OF DIRECT QPCR SYSTEMS

For more accurate quantification further qPCR approaches were conducted with the *tuf* gene-based qPCR system. It was tested on different bacterial cell material to validate applicability on samples considered difficult to analyze. To exclude run-to-run differences and to ensure reproducibility, standard runs from distinct approaches were aligned to the original standard curve in a Cq/log graph including standard errors (Figure 22). Standard errors of Cq values were measured between 0.04 and 0.15.

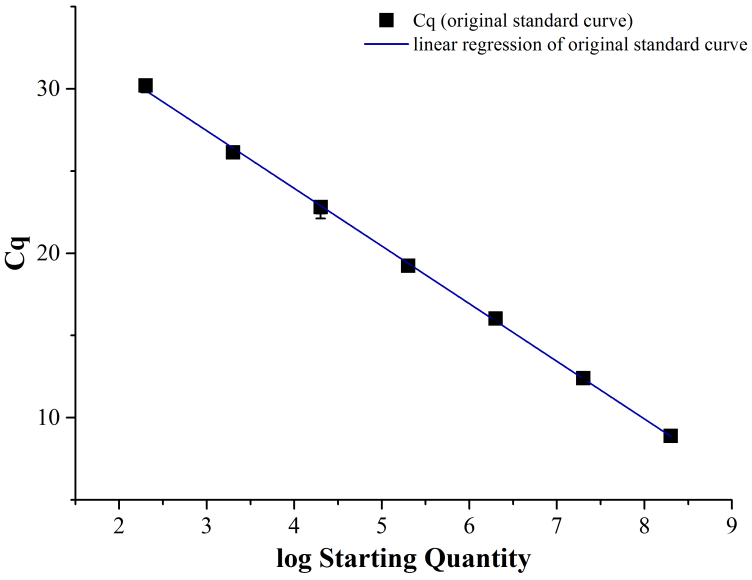


Figure 22. Reproducibility of enterococci specific direct qPCR system targeting the *tufA* gene. Original standard curve of the *tuf*-based qPCR system including inter-run standard error of Cq values. Data are Cq of standard curve ± SEM from Cq of runs, with n = 4–6.

1.3.5. FECAL SAMPLES

The study was conducted by challenging the assays with fecal samples from diverse mouse hosts. To this end, fecal samples of INF mice and SPF mice were prepared as described in chapter C.12 (p. 25) and parts of the prepared fecal suspensions were aerobically incubated in BHI at 37°C overnight without shaking. Aliquots of several preparation points were then taken as qPCR templates (INF/SPF ori: original fecal samples; INF/SPF w: washed supernatant; INF/SPF inc: cultivated supernatant). Furthermore, these aliquots were diluted 10- to 10^3-fold, to avoid reaching an upper detection limit. Amplification and melting curve analysis showed positive and specific results (83–83.5°C). Reaction parameters were within the target range (see C.16.2.2, Table 11), including efficiency with 91.1%, $R^2 = 0.996$, Slope = –3.555 and the y-int = 38.0. All results were analyzed in copies/µL, whereby dilutions were considered. In original fecal samples without preparation hardly any cell could be quantified (Figure 23A). In prepared and washed feces supernatant at least a 10^{-3} dilution was necessary to obtain a significant result (INF: $1.9 \times 10^6 \pm 2.2 \times 10^3$ copies/µL; SPF: $2.3 \times 10^4 \pm 7.5 \times 10^3$ copies/µL). The qPCR results were analyzed in copies/µL, referring to the prepared fecal suspension (see C.12, p. 25). Comparing these samples according to their origins, higher amounts of enterococci were detected in INF fecal samples (Figure 23A). Nevertheless, it has to be considered that the copy abundance is not necessarily equal to cell abundance (see Figure 21 or see E.1, p. 124). Incubated feces supernatants (inc) showed an increased quantification rate (Figure 23B). However, 10^2-fold and 10^3-fold diluted suspensions showed similar results. In summary, precultivated fecal samples showed an increased concentration of enterococci (INF: $3.1 \times 10^7 \pm 1.6 \times 10^6$ copies/µL; SPF: $9.9 \times 10^7 \pm 2.6 \times 10^6$ copies/µL) compared to the real fecal samples, corresponding to an enhancement of detected cells of approx. 16- and 4300-fold, respectively.

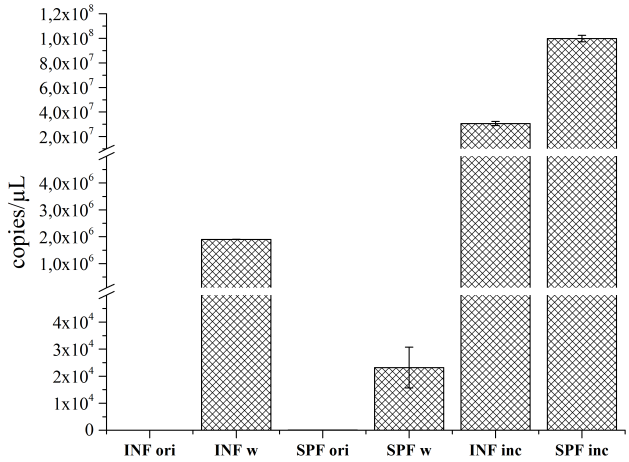


Figure 23. Enterococci quantification in fecal samples of different mouse hosts. Bar charts with two Y-axes to visualize qPCR results (*tuf*-based) of two feces types (SPF and INF). **INF:** feces of *E. faecalis* infected GF mice. **SPF:** feces of specific-pathogen-free mice. **ori:** original fecal samples (diluted in PBS). **w:** washed bacterial supernatant. **inc:** washed supernatant after aerobic BHI incubation. Data are means ± SEM with n = 2–4. Dilution factors were considered in the analysis.

1.3.6. OTHER SAMPLES

To verify whether hybridized and sorted cells were available for various molecular analysis, the qPCR system was applied to these samples as well.

First, detection and quantification of enterococci was tested on 'polyDIII'-hybridized samples. For this, prepared fixed SPF feces were spiked with fixed *E. faecalis* pure culture at increasing ratios referring to the feces volume (1:200, 1:100, 1:40, 1:20, 1:10). Samples were hybridized with 'polyDIII' according to chapter C.14.2.2 (p. 33) and resuspended in PBS. FISH images of a similar gradient experiment are shown in Figure 41, p. 85. A *tuf* gene-based qPCR was performed directly using aliquots of these samples as templates. Analysis of amplification and melting curve revealed positive and specific amplification of all samples. The qPCR results were analyzed in copies/µL referring to the prepared hybridization suspension and revealed a specific melting peak at 83−83.5°C. Statistical analysis indicated an increase of quantified copies per µL. As the hybridized samples with ratios of 1:20 and 1:10 showed an upper detection limit, these two samples were further diluted (10^{-1} or 10^{-2}) and quantified again. The results are visualized in a bar chart which shows the increased ratios of *E. faecalis* (Figure 24). The corresponding FACS dot plots are illustrated in Figure 68, p. 109. Large standard errors in samples with increased target cells (1:20 and 1:10) could indicate, that further dilution might be required to obtain more precise results.

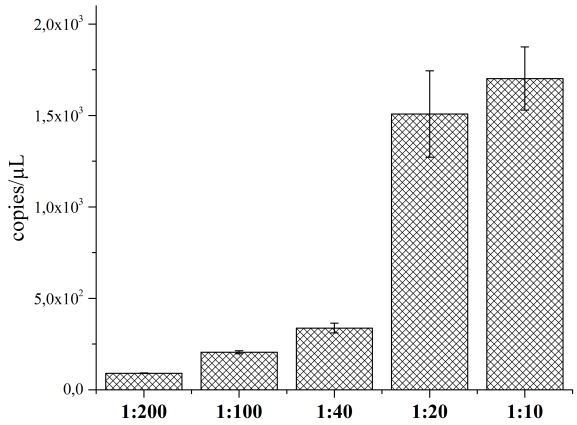


Figure 24. Detection and quantification of *in situ* hybridized spiked feces samples. Bar chart of qPCR-quantified enterococci cells (*tuf*-based) in a fecal sample that were gradiently spiked with *E. faecalis* (from ratio 1:200 to 1:10) and *in situ* hybridized with 'polyDIII' (see C.14.2.2). Data are means ± SEM with n = 2–4.

The *tuf*-based qPCR system developed to detect and quantify enterococci in original fecal samples was also tested on FACS-sorted samples to check the DNA quality of these cells. Sorted samples were concentrated and lysed before application to qPCR (see C.18, p. 43). Amplification curve and melting peak analysis revealed positive and specific amplification of the target with performance parameter values in an interpretable range (see C.16.2.2, Table 11). The qPCR results were analyzed in copies/µL referring to the prepared sorted and lysed cell suspension. Quantification of enterococcal cells revealed that sample concentrations ranged from 29–730 copies/µL (Figure 25). Differences in obtained results might be caused by irregular concentration of sorted samples by centrifugation (see C.18, p. 43), and did not influence the aim of this approach which showed that sorted cells were still applicable to qPCR.

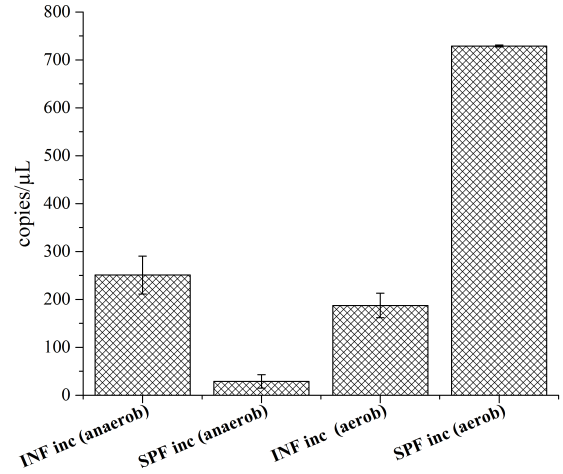


Figure 25. Detection and quantification of *in situ* hybridized fecal samples, sorted by FCM. qPCR quantification (*tuf*-based) of enterococci, present in different sorted fecal samples (INF, SPF) which were precultivated anaerobically or aerobically before *in situ* hybridization (in-solution) using 'polyDIII' (see C.14.2.2). Samples subsequently were sorted by FCM (see C.17) and applied to qPCR. **INF:** feces of *E. faecalis* infected GF mice. **SPF:** feces of specific-pathogen-free mice. The indicated values are mean values from duplicate reactions. Data are means ± SEM with n = 2.

2. FISH – IMPROVEMENT & ADAPTION FOR THE TAXON-SPECIFIC 'POLYDIII' PROBE

The FISH experiments including samples presented here as figures were conducted and validated repeatedly (n= 3–20), and results were comparable within their sample type.

2.1. FIXATION

The optimum fixation process was validated on pure cultures (see C.13.2, p. 27). Prior to fixation, liquid cultures usually are harvested at mid-to-late exponential growth phase to obtain high ribosome content. By performing a growth curve analysis of randomly chosen *Enterococcus* strains (*E. pseudoavium*, *E. gallinarum*, *E. faecalis*), together with negative control strains of closely related genera (*S. salivarius, T. solitarius*), the optimum absorbance for harvesting cells, measured at a wavelength of 600 nm (OD_{600}), was confirmed. Figure 26 represents the growth curve of *E. faecalis* (data of other species not shown, as they were similar). The exponential growth phase correlated to a mean OD_{600} from 0.2 to 0.9 (OD_{600} 1 = 5×10^8 CFU/mL in BHI). According to this, optimum values for harvesting pure cultures for fixation at the mid- to late-exponential phase were defined at OD_{600} 0.6–0.9.

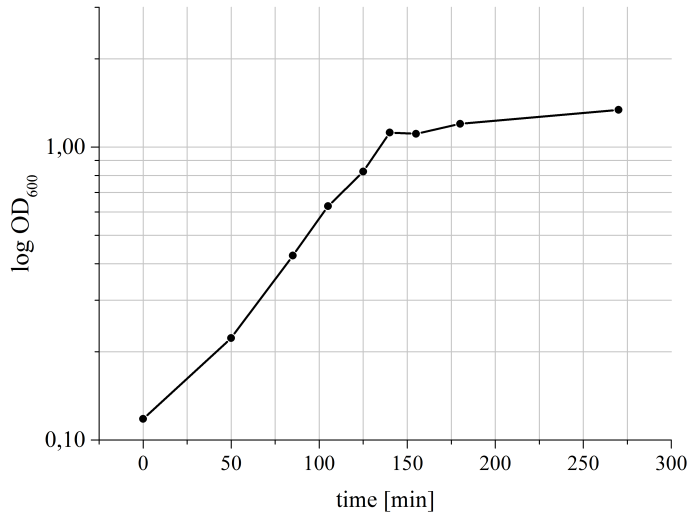


Figure 26. Growth curves of *E. faecalis*. The organism was cultivated in appropriate liquid media (BHI) under optimum conditions (see Table 1). The late-exponential phase was defined at an OD_{600} of 0.6–0.9.

The adaptation of the method for organisms with more rigid cell envelopes (*i.e.*, Gram-positive bacteria) requires modifications of the cell fixation procedures to permeabilize the cell envelope. Thus, according to previous studies, cells were fixed by a 4% PFA solution, followed by additional pre-hybridization treatments (see C.14, p. 28), further discussed in D.2.2.1 [120, 141, 166]. To check if genomic information was still available after fixation with 4% PFA

solution fixed cells were applied to extraction of genomic DNA (see C.5, p. 18), which then was applied to PCR universally targeting the 23S rDNA (primer: 118V/985R) and the *tuf* gene (primer: eftu_v_0904/ eftu_r_0904) of bacteria (see Appendix, Table 18, p. XII). Figure 27A shows successful extraction of genomic DNA from fixed *E. faecalis* pure culture. Figure 27B and Figure 27C visualize positive amplification of the 23S rRNA gene (~1,500 bp length) and the *tuf* gene (~1,000 bp length), respectively, after PCR reaction with the extracted DNA. The PCR reaction of the no-template controls (NTC) did not show any amplification products after gel electrophoresis.

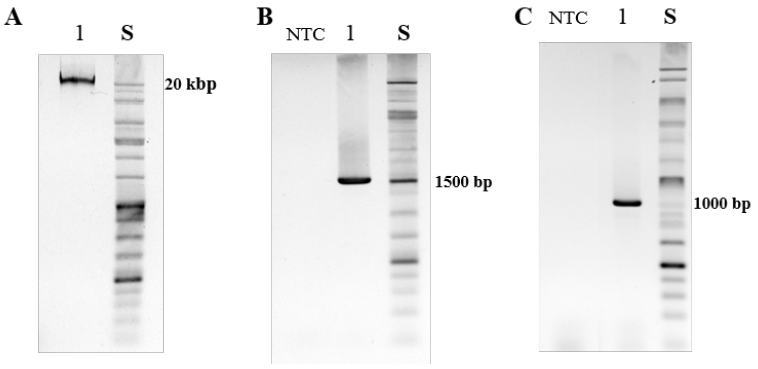


Figure 27. Genome accessibility of PFA-fixed cells after DNA extraction. Gel electrophoretic analysis of genomic DNA extracted from PFA-fixed enterococcal pure cultures and of applied PCR approaches. **A-1:** extracted genomic DNA from *E. faecalis*. **B-1:** amplified 23S rRNA gene from *E. faecalis*. **C-1:** amplified *tuf* gene from *E. faecalis.* **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder. Primer set 23S rDNA: 118V/985R; primer set *tuf* gene: eftu_v_0904/eftu_r_0904 (see Appendix, Table 18, p. XII).

Furthermore, it was examined whether PFA-fixed cells can directly be applied to standard PCR. Figure 28 shows successfully amplified PCR products after gel electrophoresis. Two universal bacterial primer sets targeting the 16S rRNA gene (616Valt/100K) and the *tuf* gene were used for PCR with 2 µL of template (PFA-fixed samples of *E. faecium*, *E. hirae* and *E. faecalis*) in a 50 µL PCR reaction. Gel electrophoretic analysis confirmed PCR amplification of both target gene fragments for all samples, resulting in specific fragments of 1,500 bp for the 16S rRNA gene and of ~1,100 bp for the *tuf* gene. Negative controls expectedly did not yield any amplification products.

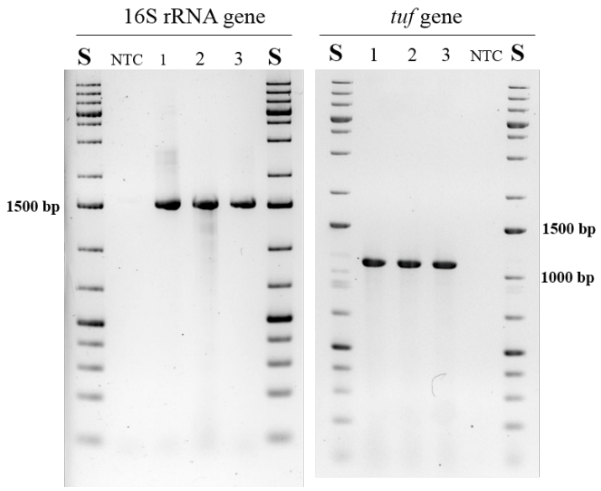


Figure 28. Direct-PCR approach with PFA-fixed cells. Gel electrophoretic analysis of PCR products obtained from 16S rRNA gene and *tuf* gene amplification. Both protocols used direct application of PFA-fixed cells without prior DNA extraction. **1:** *E. faecium.* **2:** E*. hirae.* **3:** *E. faecalis.* **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder. Primer set 16S rDNA: 616Valt/100K; primer set *tuf* gene: eftu_v_0904/eftu_r_0904 (see Appendix, Table 18, p. XII).

2.2. PRETREATMENT & HYBRIDIZATION CONDITIONS

The adequate pretreatment and hybridization conditions (lysozyme step, heat step, formamide concentration, hybridization temperature and time) had to be evaluated individually for representatives of the genus *Enterococcus*.

2.2.1. PRETREATMENT OF ENTEROCOCCI CELLS

The cell wall of PFA-fixed bacteria, especially of Gram-positives, needs to be permeabilized, specifically when using large labeled probes [167]. The rigidity of the thick peptidoglycan layer of Gram-positive bacteria can be decreased by treatment with lysozyme, which subsequently facilitates the entry of nucleic acid probes to its target region through the cell envelope. The evaluation of individual permeabilization conditions for the genus *Enterococcus* is important to not completely damage the cell structure and give rise to cell content loss.

Fichtl [120] published a pretreatment protocol for enterococci, incubating cells with lysozyme for 20 min on ice. In this project, it was shown that two ethanol series and a lysozyme incubation of 10 min on ice was sufficient for the permeabilization of the cell envelope of *Enterococcus* spp. before hybridization and the partial uptake of 'polyDIII' (see C.14, Table 8). FISH micrographs in Figure 29 revealed that when the heat pretreatment duration was increased up to 5 min, the signals obtained from 'polyDIII' were decreased, but the halo signals appeared more precisely. Cells applied to FISH with up to 1 min heat incubation showed thicker halos, more appearing like whole-cell fluorescence with strong signal intensity.

Overall, for enterococci, the combination of the enzymatic step followed by a 1–3 min heat pretreatment at 200°C was proven to be the most efficient permeabilization method for FISH on microscopic slides. The microscopic analysis revealed that the cells retained their integrity, without losing shape or complete destruction. For in-solution hybridization the heat step was not applied due to difficult implementation in plastic labware.

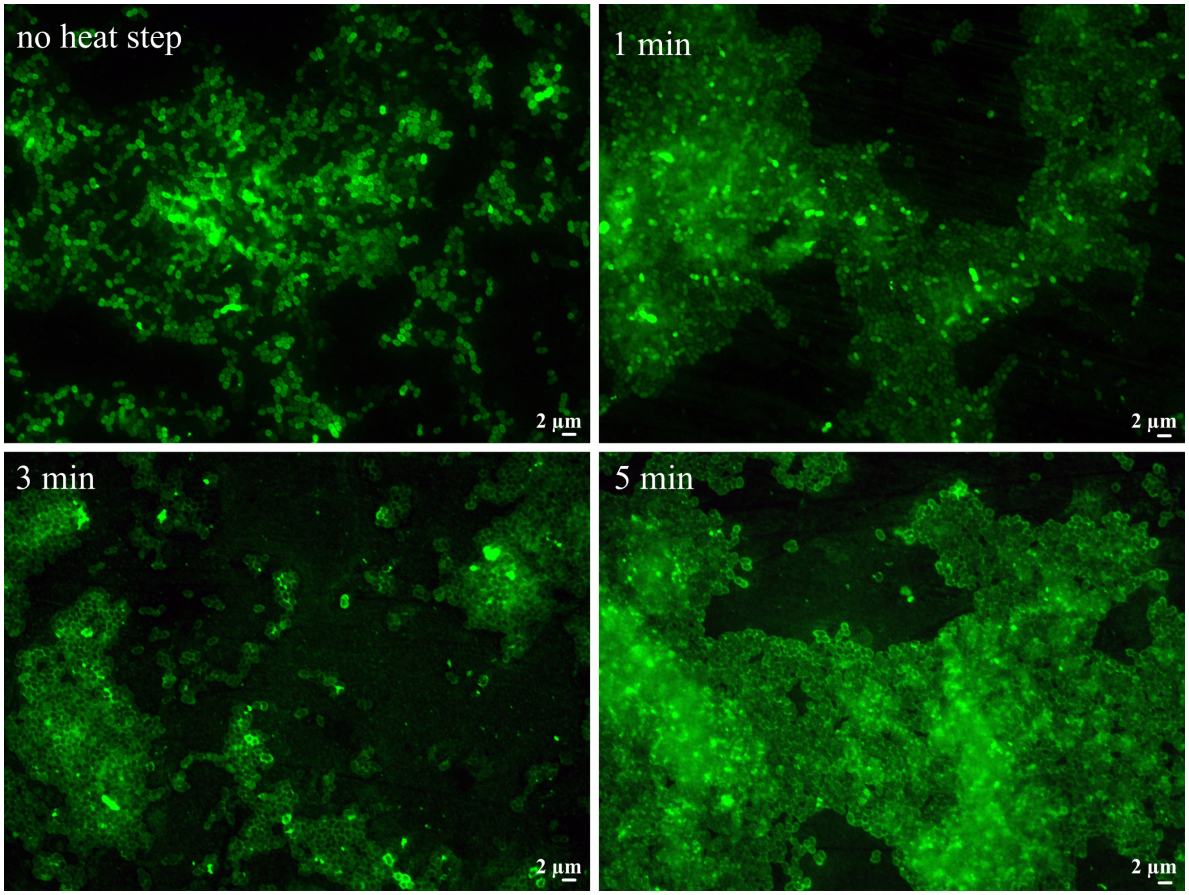


Figure 29. Pretreatment validation of the 200°C heat step. FISH micrographs acquired after *in situ* hybridization of an *E. faecalis* pure culture with 'polyDIII' on slides (see C.14.2.1). Pretreatment was applied with lysozyme and various durations of the 200°C heat step (0–5 min). Increased heat incubation led to decreased halo width, thus to decreased signal intensity.

2.2.2. HYBRIDIZATION CONDITIONS

FISH micrographs of *Enterococcus* spp. cells hybridized with 'polyDIII' under different formamide concentrations in the hybridization buffer revealed an adequate formamide concentration of 80%. The negative control strain *C. glutamicum* never gave rise to a signal after hybridization with 'polyDIII', regardless of the formamide concentration (Figure 30).

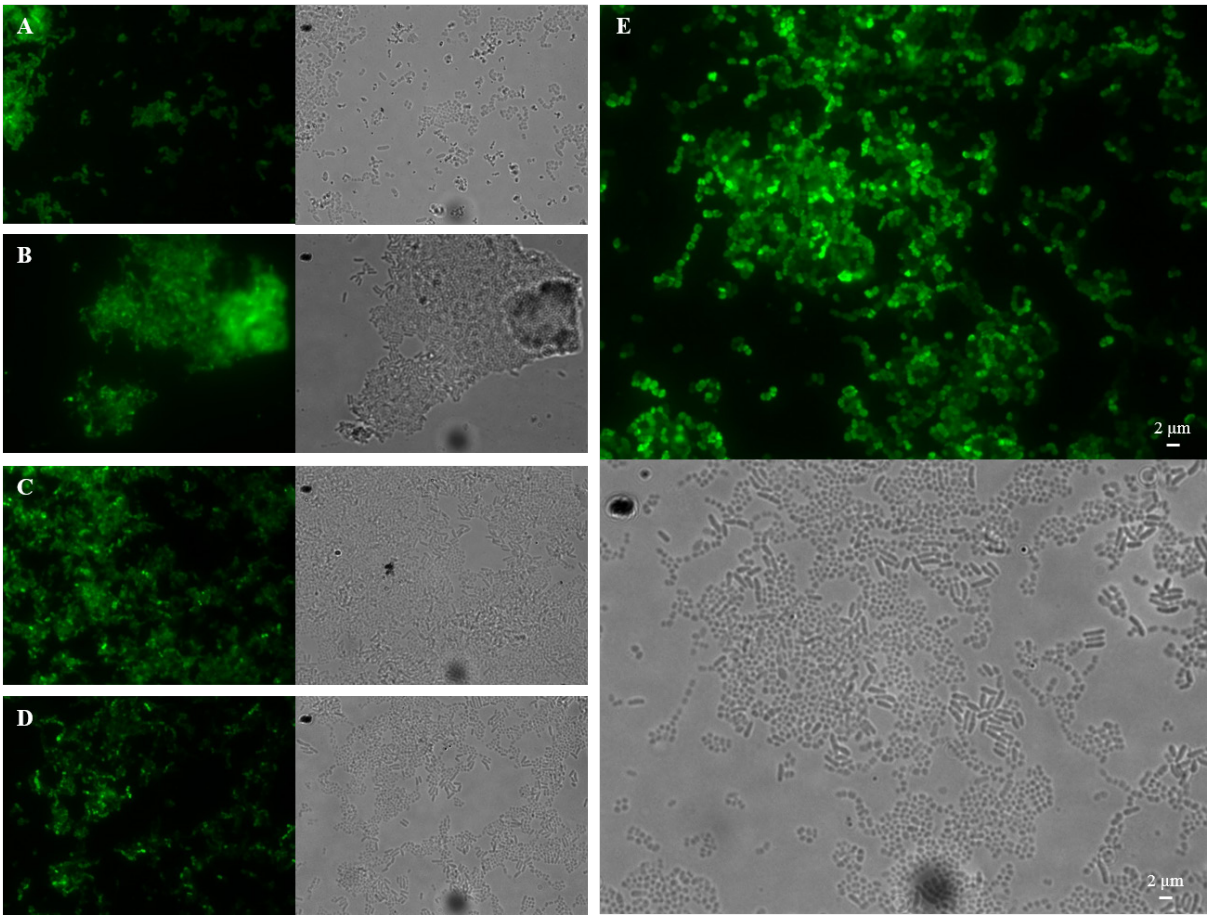


Figure 30. Validation of the most efficient formamide concentration for 'polyDIII'. FISH and phase contrast images of a mixture of *E. faecalis* ACCT 29212 and *C. glutamicum*, *in situ* hybridized with 'polyDIII' under different formamide (FA) concentrations in the hybridization buffer (see C.14.2.1). Differing conditions: 3 min at 200°C; washing 15 min at 55°C (no NaCl). **A:** 0% FA **B:** 20% FA **C:** 40% FA **D:** 60% FA **E:** 80% FA.

2.2.3. WASHING CONDITIONS

Stringency in FISH is mainly influenced by salt concentration in the buffers or by hybridization and washing temperatures. To determine the influence of washing conditions, various parameters were modified and signal intensity or signal loss was analyzed microscopically. For all tests, an approx. equal mixture of *E. faecalis* and *C. glutamicum* (as a negative control) was hybridized with the 'polyDIII' probe on slides, after being treated with lysozyme for 10 min on ice and exposed to 200°C for 2–3 min. Hybridization was performed for 5 h at 53°C using a formamide concentration of 80% (see C.14, Table 8).

In washing buffers without formamide a high stringency can be achieved by lowering the NaCl concentration and increasing the temperature [156]. Figure 31 shows samples which were washed for 15 min at 55°C, by applying washing buffer that contained increasing concentrations of sodium chloride (40–900 nM NaCl). By this means, it should be evaluated whether signal intensities were influenced by the NaCl concentration in the washing buffer. No signal

improvement or loss was detectable in this range. Therefore, the washing buffer composition was kept without NaCl as proposed in standard FISH protocols (see C.14, Table 9).

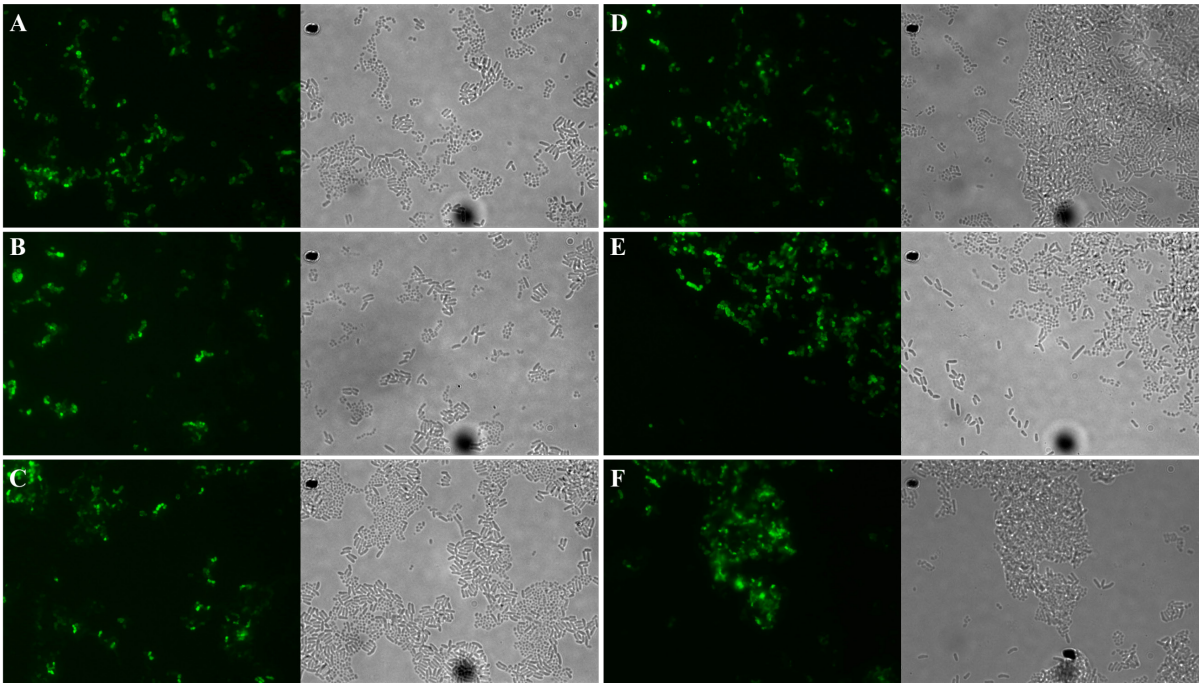


Figure 31. Verification of the influence of the NaCl concentration in the washing buffer. FISH and phase contrast images of a mixture of *E. faecalis* ACCT 29212 and *C. glutamicum*, *in situ* hybridized with 'polyDIII' (see C.14.2.1). Differing conditions: 3 min at 200°C; Washing for 15 min at 55°C using increasing NaCl concentrations. **A:** 40 mM **B:** 80 mM **C:** 160 mM **D:** 320 mM **E:** 640 mM **F:** 900 mM.

Concerning different washing temperatures, Figure 32 shows that higher signal intensities could be obtained at 48–55°C (Figure 32A and Figure 32B). Total signal loss was observed at 75°C. For subsequent FISH protocols a washing temperature of 55°C was set as adequate.

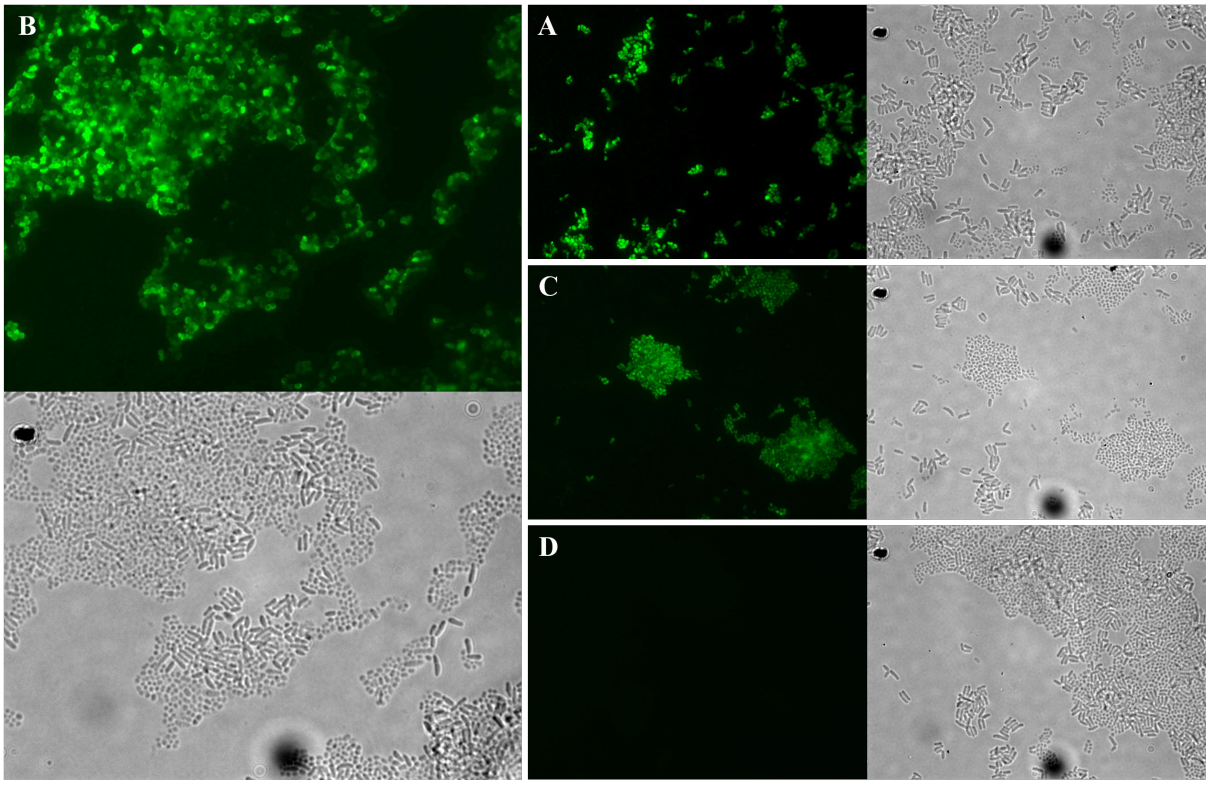


Figure 32. Validation of washing temperature for 'polyDIII' *in situ* hybridizations. FISH and phase contrast images of a mixture of *E. faecalis* ACCT 29212 and *C. glutamicum*, *in situ* hybridized with 'polyDIII' (see C.14.2.1). Differing conditions: 3 min at 200°C; 15 min washing (no NaCl) using increasing temperatures. **A:** 48°C **B:** 55°C **C:** 65°C **D:** 75°C.

Furthermore, the optimum time of the washing procedure was evaluated by varying the washing time range from 25 to 65 min. Figure 33 reveals that the washing period did not influence signal intensity. Adequate results could be established with 25 min as well as with 65 min washing incubation. Considering the time management, the protocol was kept at 25 min washing time.

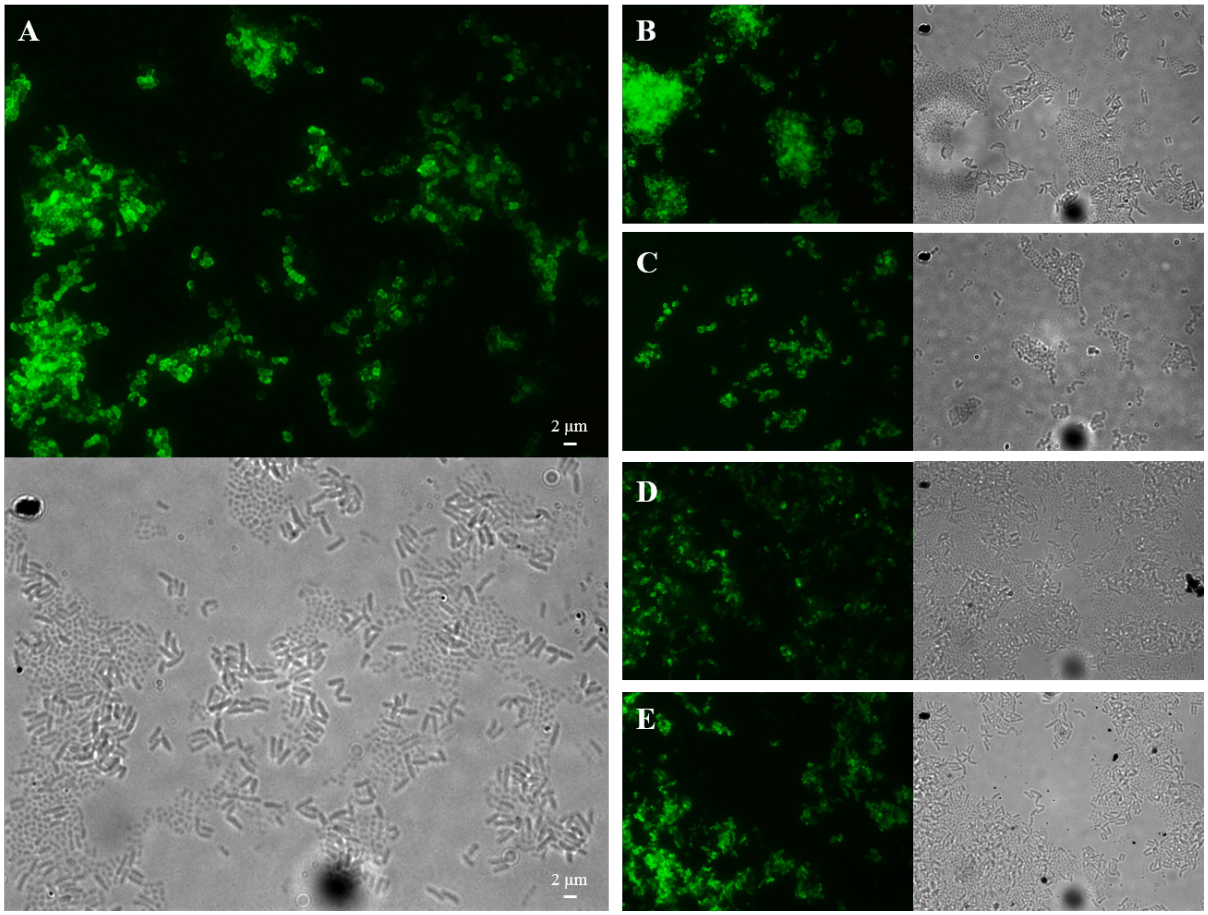


Figure 33. Verification of the washing duration for 'polyDIII' *in situ* hybridizations. FISH and phase contrast images of a mixture of *E. faecalis* ACCT 29212 and *C. glutamicum*, *in situ* hybridized with 'polyDIII' (see C.14.2.1). Other conditions: 3 min at 200°C; Washing at 55°C with different incubation durations. **A:** 25 min **B:** 35 min **C:** 45 min **D:** 55 min **E:** 65 min.

Finally, by applying all evaluated pretreatment and hybridization conditions to pure cultures of *Enterococcus* and of the genus *Streptococcus*, it could be assessed that the stringency sufficed to discriminate between these two closely related genera (Figure 34). An overview of recommended pretreatment and hybridization conditions for the *Enterococcus*-specific 'polyDIII' probe, is presented in Table 14.

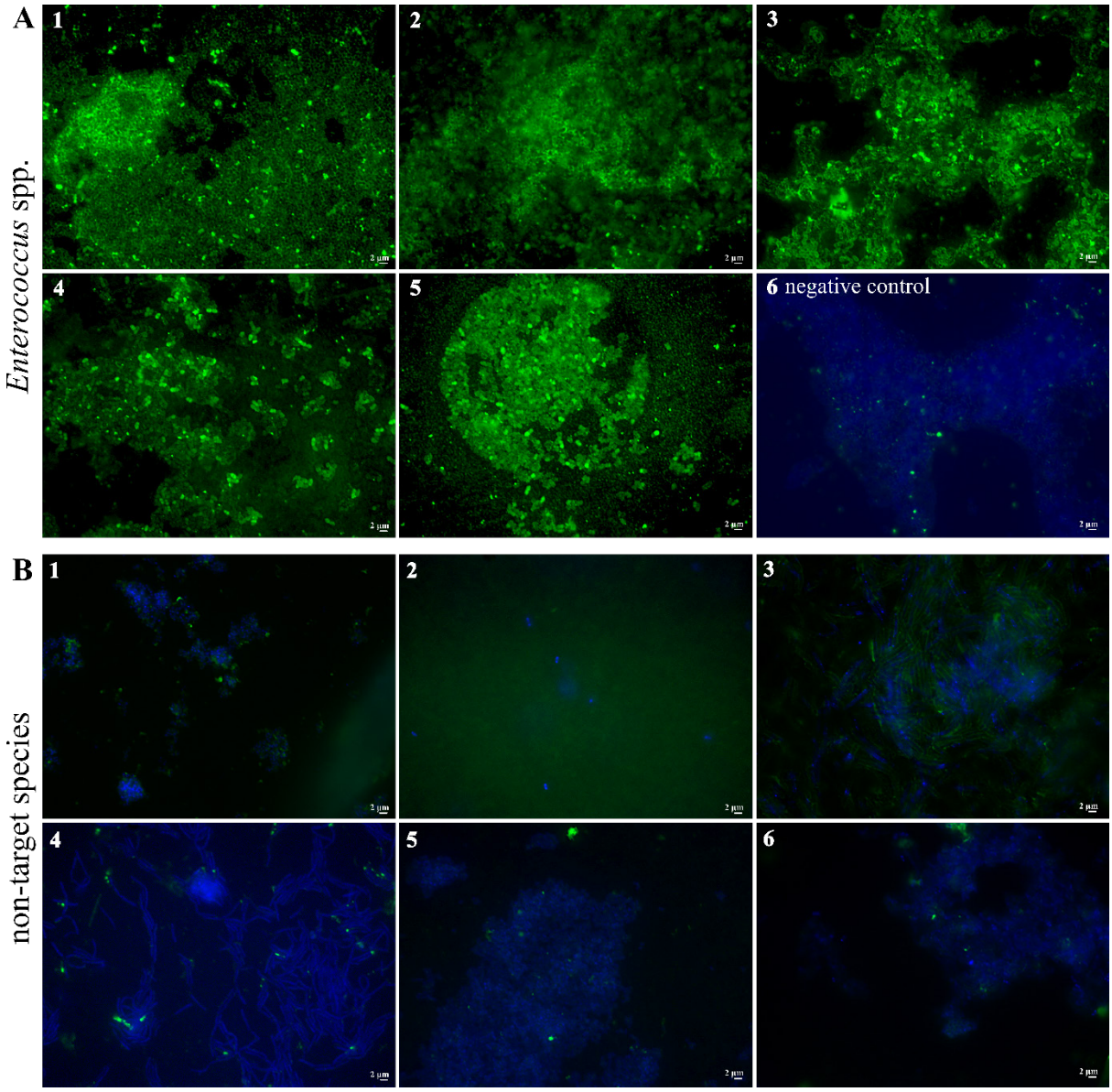
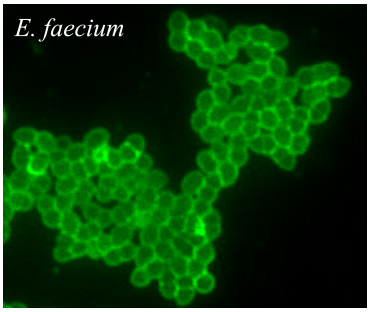


Figure 34. Verification of the genus-specificity of 'polyDIII' for *Enterococcus* spp. by FISH. Evaluated FISH conditions for 'polyDIII' were tested on various members (pure cultures) of *Enterococcus* (A) and on relevant intestinal non-target control strains, including closely related genus *Streptococcus* (B). FISH conditions: see chapter C.14.2.1; 2 min at 200°C. **A1:** *E. avium*. **A2:** *E. casseliflavus*. **A3:** *E. cecorum*. **A4:** *E. faecium*. **A5:** *E. durans*. **A6:** *E. faecalis* without 'polyDIII' (negative control). **B1:** *S. bovis*. **B2:** *Acinetobacter haemolyticus*. **B3:** *Lactobacillus intestinales.* **B4:** *L. lactis* subsp. *lactis*. **B5:** *S. agalactiae*. **B6:** *S. salivarius*. Micrographs with no detectable fluorescein signal (green) were merged with DAPI staining micrographs (blue).

Table 14. Summary for adjusted halo-forming FISH protocol using the 'polyDIII' probe for *Enterococcus* labeling.

Pretreatment	10 min lysozyme on ice (2–3 min at 200°C, on slides)
Formamide concentration	80%
Hybridization temperature	53 °C
Hybridization time	3–12 h
Washing buffer (NaCl concentration)	0 nM
Washing	25 min at 55 °C



2.3. SPECTRUM OF SPECIFICITY

To evaluate the particular specificity of the 'polyDIII' probes generated from the *Enterococcus* species relevant to the intestine, two different approaches were performed. First rDNA *in silico* sequence analysis by distance matrix methods using the 16S rRNA and 23S rRNA gene ARB databases Ver. 115 (http://www.arb-silva.de) was accomplished. These results were then compared with results of *in situ* hybridization experiments in order to evaluate the intestinally significant *Enterococcus* species with the generated enterococcal probes. The objective was to find any correlation between probe sequence similarities and hybridization success, and to find one probe or a probe combination to target all enterococci cells, regardless of species.

2.3.1. DISTANCE MATRICES OF 16S RRNA AND 23S RRNA OTUS

The validation of all available *Enterococcus* spp. type strains (see Table 1) was performed by sequencing (see C.11, p. 24) of the nearly complete 16S rRNA and the 23S rRNA genes, followed by completion and analysis of the sequence data by the ARB software package (www.arb-home.de). The ARB databases were assembled with this sequence data, which were further used to perform species-level OTU analysis. Thus, sequence similarities to other genera were detected and evaluation of a target probe covering all *Enterococcus* strains simultaneously was possible. Ludwig *et al.* [168] formerly set a sequence similarity in domain III (DIII) of the 23S rRNA gene of 78%–85%, for positive/negative signals in FISH. Another study suggested 70%–76% sequence similarity as the cut-off to discriminate non-target cells [120].

Figure 35 shows a heat map of 16S rRNA gene sequences of enterococci and closely related streptococci, lactococci and tetragenococci. It displays the sequence similarities between members of these genera. The sequence similarity of enterococci and streptococci and the three other genera, mostly are <95%, while intra-genus sequence similarity mostly is in the range of 95% [46] to 98.7% [169].

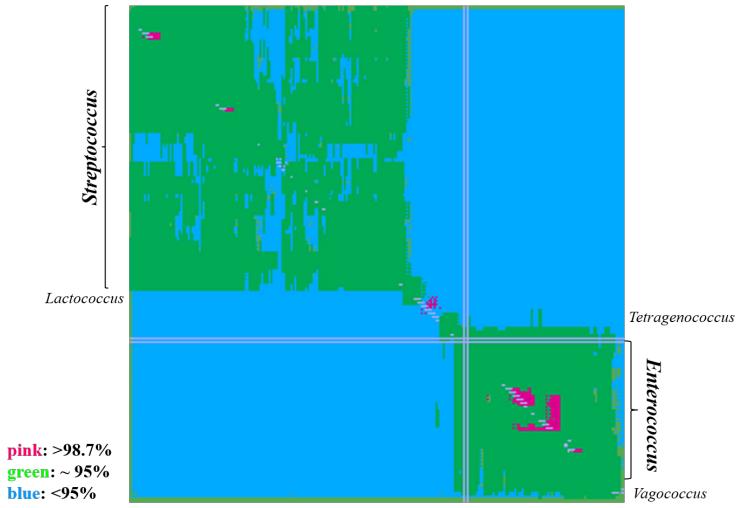


Figure 35. Sequence similarities in the 16S rRNA gene. Heat map showing sequence similarities of the complete 16S rRNA gene between the genera *Streptococcus*, *Enterococcus* and members of *Tetragenococcus* and *Lactococcus*. Blue fields indicate a sequence similarity of <95%. Sequence similarity of around 95% are illustrated in green color, while a sequence similarity of <98.7% are displayed in pink color.

Figure 36 represents the heat map built by sequences of domain III of the 23S rDNA from enterococci and streptococci. Domain III of the 23S rRNA gene constitutes the target of the 'polyDIII' probe. However, most of the compared sequences of enterococci and streptococci were lying in a span of 70%–76% similarity (green). Only a few strains showed a sequence similarity above 76% (pink) and below 70% (blue).

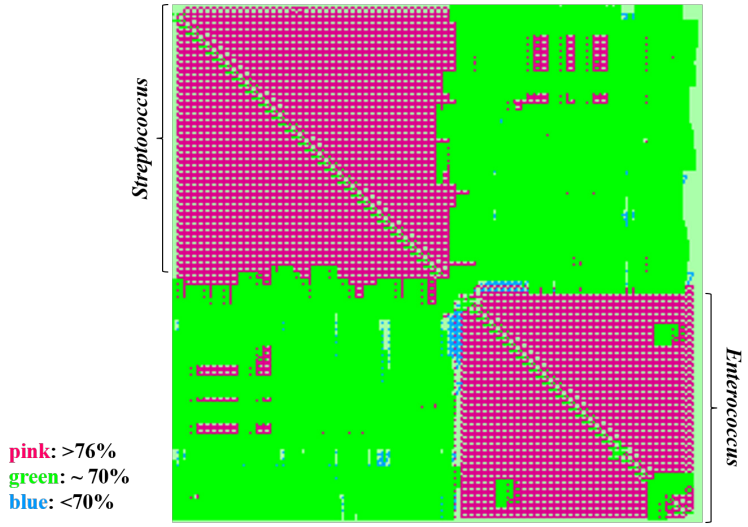


Figure 36. Sequence similarities of the 23S rRNA gene. Heat map showing sequence similarities of the whole DIII target region (within the 23S rRNA gene) between the genera *Streptococcus* and *Enterococcus*. Most of the compared sequences lie in a span of 70%–76% similarity (green). Pink color represents strains, showing a similarity above 76%, whereas blue color is indicating inter-species sequence similarity below 70%.

2.3.2. SIMILARITY MATRIX ANALYSIS OF THE WHOLE TARGET SEQUENCE OF 'POLYDIII'

To target all enterococci while excluding the streptococci depends on a proper probe validation. The target sequences of 'polyDIII' probes (*E. coli* position 1366–1601) generated for relevant intestinal enterococci were analyzed by calculating similarity matrices in the region of domain III of the 23S rRNA gene from all examined species using an existing ARB database alignment. Calculation was performed by using filters generated by ARB implemented tools to cover the whole binding region of 'polyDIII'.

In Figure 37 distance matrices of 'polyDIII' are illustrated graphically and tabularly. All genus members show sequence similarities between 81.3% (*E. faecalis* vs. *E. cecorum*) and 99% (*E. hirae* vs. *E. durans*). As proved by ARB these sequence differences were solely caused by single nucleotide varieties. Of all analyzed relevant intestinal enterococci, *E. cecorum* showed the highest sequence divergence to the other members (mean 85.5%). By counting all sequence similarities, *E. raffinosus* had the highest overall similarity to the other members (mean value 93.4%).

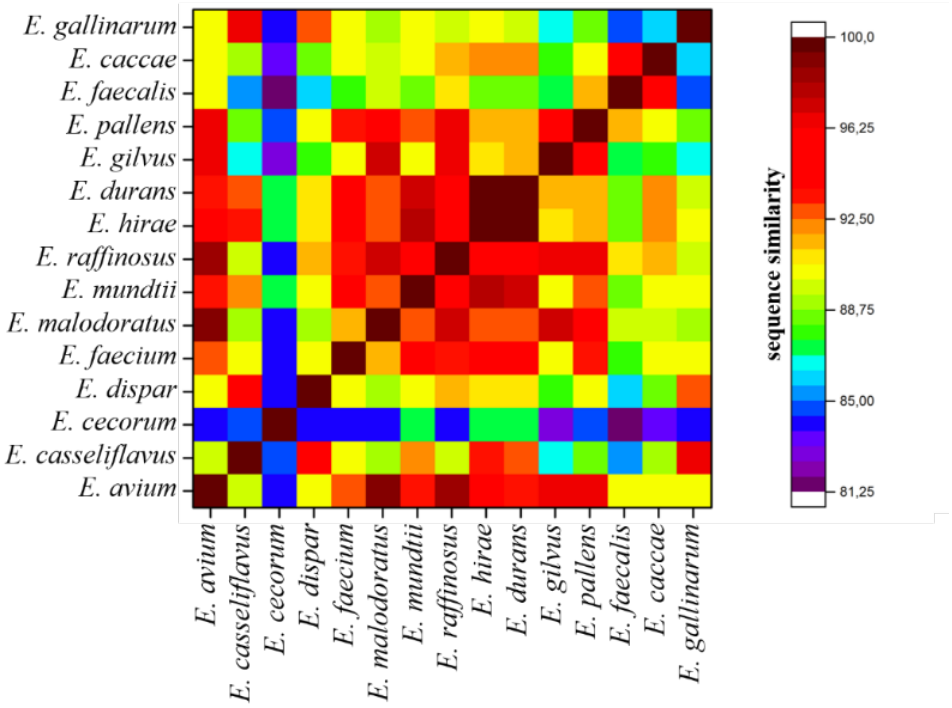


Figure 37. Sequence similarities of domain III of the 23S rRNA gene. Heat map of domain III (23S rRNA gene) sequences similarities among project relevant enterococcal strains. The individual sequences similarities are represented as colors. The color-gradient is ranging from ~81.3% similarity (violet) to 100% sequence similarity (brown).

2.3.3. DOMAIN III SEQUENCE IN FECAL SAMPLES

To investigate whether the sequence of domain III of the 23S rRNA gene offers enough phylogenetic information to perform intra-taxon discrimination, enterococci were isolated from SPF feces by plating dilution samples on enterococci-enrichment medium (2–3 × concentrated azide-glucose medium) [170]. Single colonies were analyzed by phase contrast microscopy and further plated, if coccoid cells were detected. All colonies were applied to the *Enterococcus*-specific PCR detection system (see C.16.1, p. 38), which identified three samples belonging to *Enterococcus* spp. These colonies were inoculated and grown in liquid BHI medium, and DNA was extracted (see C.5, p. 18) serving as template for PCR amplification of domain III of the 23S rRNA gene. The amplicons were further inserted into the pCR® 2.1 vector (see C.10.2, p. 23) and 12 randomly chosen clones of each colony sample were selected and checked for successful clone insertion by clone check PCR. Plasmid DNA was extracted from positive clones and plasmids were subsequently sequenced (see C.11, p. 24). The sequences were aligned using ARB and a neighbor joining tree was calculated by addition of sequences in an existing 23S rRNA database (see C.11.1, p. 25). The analyses revealed strong similarity of the sequences to the species *E. faecalis*, indicating that the short sequence of domain III of the 23S rRNA gene was sufficient to classify species from unknown samples. Nevertheless, the fecal samples showed only a small species-variance among the isolated colonies.

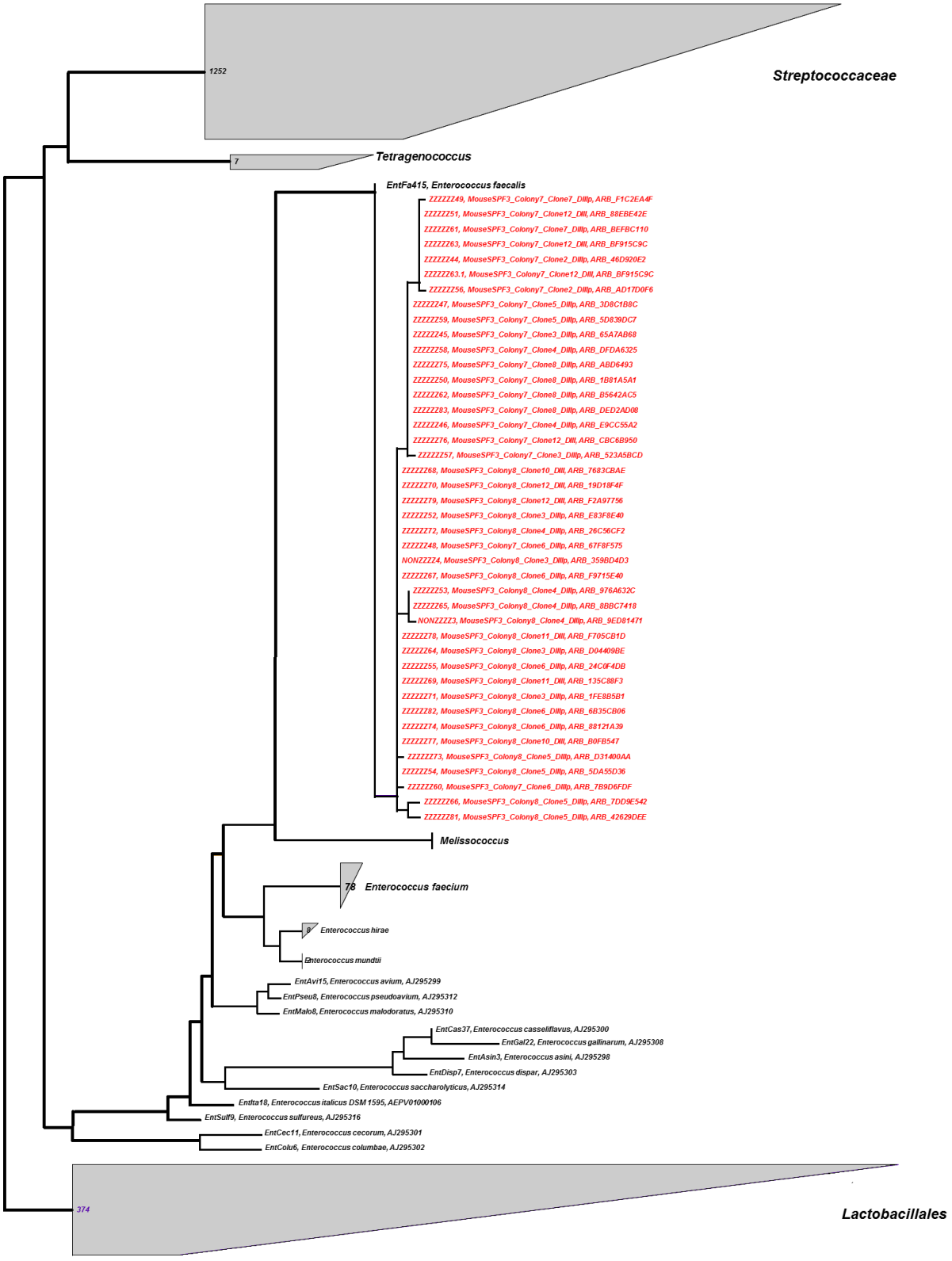


Figure 38. Phylogenetic classification of domain III sequences generated from fecal enterococci samples. The neighbor joining tree was calculated based on the sequence of domain III of the 23S rRNA gene.

2.3.4. VALIDATION OF 'POLYDIII' PROBE SPECIFICITY BY FISH

To further confirm the distance matrix results with *in situ* hybridization approaches and to examine the specificity of rRNA gene targeting probes *in situ*, all relevant intestinal enterococcal species were hybridized with the 'polyDIII' probes generated from the genomic DNA of each organism (see C.4, p. 16). All species were hybridized on slides under the same conditions with pretreatment of lysozyme and heat and experiments were performed in multiple approaches (see C.14.2.1, p. 32). In every hybridization experiment a negative control with a *C. glutamicum* pure culture was included to exclude false positive signals.

Table 15 illustrates that not all probes led to positive halo-shaped hybridization signals. In some cases, not all cells in the sample showed halos, but also very strong whole-cell fluorescence (symbol: WCF). In other cases, hybridized cells did not show any signal or only weak whole-cell fluorescence (symbols: †). Probes which worked most efficiently were generated from genomic DNA of *E. gilvus* (93% positive signals), *E. hirae* (87%), *E. durans*, and *E. faecium* (both 80%). Less efficient probes were the ones generated using DNA from *E. avium* (33%), *E. pallens* and *E. raffinosus* (both 53%) as a template. It was noticed that some fixed cells showed worse hybridization signals than others. While *E. faecium* (100% pos. signals), *E. faecalis* (100%), *E. hirae* (93%), *E. durans* (93%), and *E. mundtii* (93%) showed very good halo signals with all applied probes, *E. caccae* (20%), *E. malodoratus* (27%), and *E. avium* (33%) resulted in weak hybridization signals for all tested probes. Additionally, *E. avium*, *E. dispar*, *E. pallens*, and *E. caccae* did not show any result with the probe generated from their own genomic DNA, but with probes originating from other species. In total, 70.53% of these FISH experiments resulted in positive signals.

To ensure coverage of all enterococcal strains, further *in situ* experiments were accomplished by application of a probe mixture generated by using genomic DNA from *E. gilvus*, *E. hirae*, and *E. faecium*.

Table 15. Validation summary of 'polyDIII' *in situ* specificity presenting FISH results of project relevant organisms treated with the respective probes; symbol/color index: **√(green)**: distinct halos; **WCF(yellow)**: whole-cell fluorescence or occasional halos; **†(red)**: weak WCF or no signal; in repetitive polynucleotide probe FISH experiments.

cells \ probes		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*E. avium*	†	†	√	†	WCF	†	†	†	WCF	WCF	√	†	†	†	†
2	*E. casseliflavus*	√	√	√	√	†	†	√	√	√	√	√	WCF	√	√	√
3	*E. cecorum*	†	†	√	√	WCF	†	†	†	√	√	√	†	√	†	†
4	*E. dispar*	†	√	√	†	†	√	√	WCF	√	√	√	√	√	√	√
5	*E. faecium*	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
6	*E. malodoratus*	†	†	†	†	†	WCF	†	†	√	†	WCF	†	WCF	†	†
7	*E. mundtii*	†	√	√	√	√	√	√	√	√	√	√	√	√	√	√
8	*E. raffinosus*	†	√	†	√	√	WCF	√	√	√	WCF	WCF	√	√	√	√
9	*E. hirae*	†	√	√	√	√	√	√	√	√	√	√	√	√	√	√
10	*E. durans*	†	√	√	√	√	√	√	√	√	√	√	√	√	√	√
11	*E. gilvus*	WCF	†	†	√	√	√	†	†	WCF	WCF	√	†	†	√	†
12	*E. pallens*	†	√	√	√	√	√	WCF	†	√	√	√	†	√	√	√
13	*E. faecalis*	√	√	WCF	√	√	√	√	√	√	WCF	√	√	√	√	√
14	*E. caccae*	†	†	†	†	√	√	†	†	†	†	†	†	†	†	n.a.
15	*E. gallinarum*	√	√	√	†	√	WCF	†	†	WCF	√	√	†	†	√	WCF

2.3.5. COMBINED EVALUATION OF DISTANCE MATRIX AND FISH ANALYSIS

By combining the similarity (see Figure 37) with the *in situ* results (see Table 15) in one table (Table 16) and by analysis of the bacteria tested and the probes used, it was objected to identify correlations between used probe sequences (*in silico*) and hybridization success (*in situ*).

Table 16 and Table 17 revealed no relationship between sequence similarities and FISH results was detectable. Furthermore, no cut-off sequence similarity could be defined which could distinguish between positive and negative *in situ* results (Table 17). The minimum sequence similarity of experiments showing positive signals started at 81.3%, which is also the lowest distance similarity of two analyzed enterococcal strains (see D.2.3.2). The minimum sequence similarity for experiments with negative signals thus was illogically higher (82.6%). Both for the negative and positive FISH signal groups, the maximum sequence similarity of tested species-probe combination was 100%.

By analyzing the mean percentage of min. and max. sequence similarities of positive and negative signals, also no correlation was detected. The span for positive signals was from 85.5% / 85.8% to 98.4% / 98.7%, whereas the span for negative results ranged from 87.3% / 85.9% to 95.5%, for fixed cells and probes, respectively (Table 17). Like mentioned in D.2.3.4, *E. avium*, *E. dispar*, *E. pallens* and *E. caccae* did not show any *in situ* result with the probe generated from their own genomic DNA, despite 100% sequence similarity.

Table 16. Similarity matrix of domain III 23S rDNA (DIII) sequences from project relevant organisms in combination with polyFISH results of hybridizations using the respective probes; color index: **green**: distinct halos; **yellow**: whole-cell fluorescence (WCF) or occasional halos; **red**: weak WCF or no signal; in repetitive polynucleotide probe FISH experiments.

cells \ probes		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*E. avium*	100	89.7	84.2	90.5	92.6	98.8	93.4	98.3	93.8	93.4	96.7	96.7	90.5	90.5	90.1
2	*E. casseliflavus*	89.7	100	84.6	95.1	90.1	88.8	92.2	89.7	93.4	93.0	86.8	88.4	85.2	88.9	96.7
3	*E. cecorum*	84.2	84.6	100	84.2	84.2	83.8	87.1	84.2	87.1	87.1	82.6	85.0	81.3	83.4	83.8
4	*E. dispar*	90.5	95.1	84.2	100	90.5	89.3	90.5	91.3	90.9	90.9	88.1	90.1	86.0	88.5	93.0
5	*E. faecium*	92.6	90.1	84.2	90.5	100	91.7	95.1	93.4	94.2	94.2	90.1	93.4	88.1	90.1	90.1
6	*E. malodoratus*	98.8	88.8	83.8	89.3	91.7	100	92.6	97.1	93.0	92.6	97.1	95.5	89.7	89.7	89.3
7	*E. mundtii*	93.4	92.2	87.1	90.5	95.1	92.6	100	93.8	97.5	97.1	90.5	92.6	88.5	90.5	90.5
8	*E. raffinosus*	98.3	89.7	84.2	91.3	93.4	97.1	93.8	100	93.8	94.2	96.7	96.3	90.9	91.3	89.7
9	*E. hirae*	93.8	93.4	87.1	90.9	94.2	93.0	97.5	93.8	100	99.6	90.9	91.7	88.5	92.2	90.1
10	*E. durans*	93.4	93.0	87.1	90.9	94.2	92.6	97.1	94.2	99.6	100	91.4	91.7	88.5	92.2	89.7
11	*E. gilvus*	96.7	86.8	82.6	88.1	90.1	97.1	90.5	96.7	90.9	91.4	100	94.2	87.2	88.1	86.4
12	*E. pallens*	96.7	88.4	85.0	90.1	93.4	95.5	92.6	96.3	91.7	91.7	94.2	100	91.7	90.5	88.4
13	*E. faecalis*	90.5	85.2	81.3	86.0	88.1	89.7	88.5	90.9	88.5	88.5	87.2	91.7	100	95.5	84.8
14	*E. caccae*	90.5	88.9	83.4	88.5	90.1	89.7	90.5	91.3	92.2	92.2	88.1	90.5	95.5	100	86.0
15	*E. gallinarum*	90.1	96.7	83.8	93.0	90.1	89.3	90.5	89.7	90.1	89.7	86.4	88.4	84.8	86.0	100

Table 17. Similarities of the enterococcal DIII sequence considering positive and negative FISH signals either comparing applied cells or probes. Statistical analysis was performed based on results of Table 16. Sequence similarities are displayed in percentage (%).

		fixed cells sequence similarity				**probes** sequence similarity			
		total positive signals		total negative signals		total positive signals		total negative signals	
		% min	% max	% min	% max	% min	% max	% min	% max
1	*E. avium*	84,2	93,8	89,7	100	90,1	96,7	84,2	100
2	*E. casseliflavus*	84,6	100	88,8	90	85,2	100	84,6	93
3	*E. cecorum*	81,3	100	82,6	87,1	81,3	100	83,4	87,1
4	*E. dispar*	84,2	95	90,5	100	84,2	95	88,5	100
5	*E. faecium*	84,2	100	-	-	84,2	100	90,1	91,7
6	*E. malodoratus*	89,7	100	83,8	98,8	84,2	100	83,8	98,8
7	*E. mundtii*	87,1	100	-	93,4	88,5	100	87,1	97,1
8	*E. raffinosus*	89,7	100	84,2	98,3	89,7	100	84,2	98,3
9	*E. hirae*	87,1	100	-	93,8	87,1	100	92,2	93
10	*E. durans*	88,5	100	87,1	97,1	87,1	100	-	92,2
11	*E. gilvus*	82,6	97,1	96,7	100	86,8	96,7	82,6	100
12	*E. pallens*	85	100	-	-	85	100	88,4	96,7
13	*E. faecalis*	81,3	100	-	90,5	81,3	100	-	95
14	*E. caccae*	89,7	90,1	83,4	100	88,1	95,5	83,4	100
15	*E. gallinarum*	83,8	100	86,4	93	84,8	96,7	83,8	90,1
	min/max [%]	**81,3**	**100**	**82,6**	**100**	**81,3**	**100**	**82,6**	**100**
	Ø mean [%]	**85,5**	**98,4**	**87,3**	**95,5**	**85,8**	**98,7**	**85,9**	**95,5**

2.4. ADAPTATION OF THE IN-SOLUTION 'POLYDIII'-FISH PROTOCOL TO FECAL MATTER

To apply FISH samples to cell enrichment (see C.15, p. 34) or FACS (see C.17, p. 41), several in-solution FISH protocols [95, 115, 157] were adjusted for the use of *Enterococcus*-specific

'polyDIII' and the oligonucleotide probe Enc473 for the labeling of enterococci in fecal material (SPF and INF) (see C.14.1.2 and C.14.2.2). No heat step was necessary for the pretreatment of samples, regardless of whether they were pure cultures or prepared fecal samples.

By applying 'polyDIII' to SPF and INF samples using the same conditions, the differences between those sample types or mouse types are apparent (Figure 39). INF feces collected from germ-free mice infected with *E. faecalis* showed much more, but smaller signals (INF, Figure 39A) than SPF samples collected from mice with common gut microbiota (SPF, Figure 39B). Anyway, based on the DAPI staining, it was shown that not all cells in the INF sample were positively hybridized with *Enterococcus*-specific 'polyDIII'. In contrast, signals in the SPF samples were very rare. Furthermore, it was found that cells in the samples tend to aggregate, which can influence probe binding sensitivity. By addition of a sonication step to the protocol (see C.12, p. 25), clustering of cells could be reduced (Figure 40).

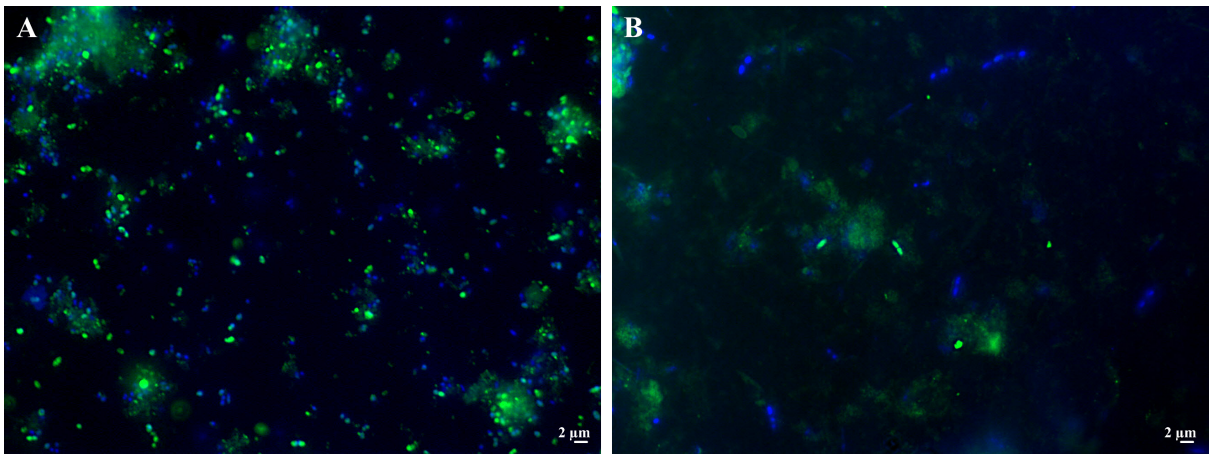


Figure 39. Comparison of 'polyDIII' *in situ* hybridized feces from different mice. The figure shows composite pictures of feces collected from **A:** an INF mouse and **B:** a SPF mouse (compare Table 7, p. 25), *in situ* hybridized with 'polyDIII' (FICT: green) in solution (see C.14.2.2) and stained with DAPI (blue). In the INF sample more 'polyDIII' signals could be obtained, compared to the SPF sample.

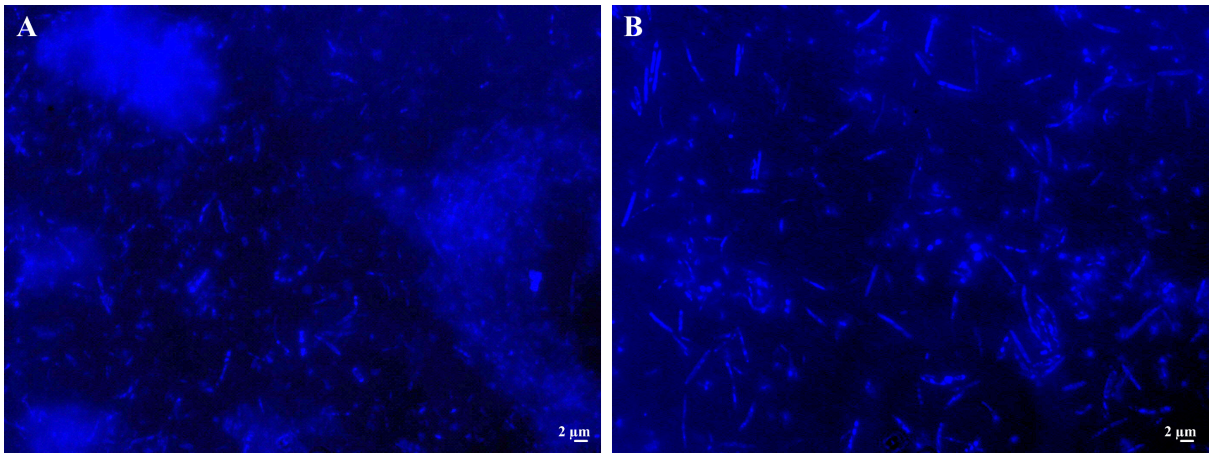


Figure 40. Aggregation status of cells in fecal samples. DAPI staining micrographs of SPF samples before applying probes to *in situ* hybridization. **A:** the sample was resuspended by vortexing and pipetting. **B:** the sample was subjected to mild sonication.

To generate positive controls for in-solution FISH cell enrichment and FACS the SPF feces samples were additionally spiked with a PFA-fixed pure culture of *E. faecalis* before hybridization with 'polyDIII'.

As a reference, samples of *E. faecalis*-spiked SPF samples in known ratios concerning the feces volume (1:100, 1:20, 1:10, 1:5, 1:2.5) were applied to hybridization. FISH analysis by epifluorescence microscopy demonstrated the increase in number of labeled target cells (Figure 41). However, it was assumed that not all *E. faecalis* cells were detected with 'polyDIII' (green) or showed lower signal intensities based on DAPI staining (blue). The increase was also visualized by qPCR (Figure 24, p. 66) and FCM analysis using dot plots (Figure 68, p. 109) when applying similar prepared samples.

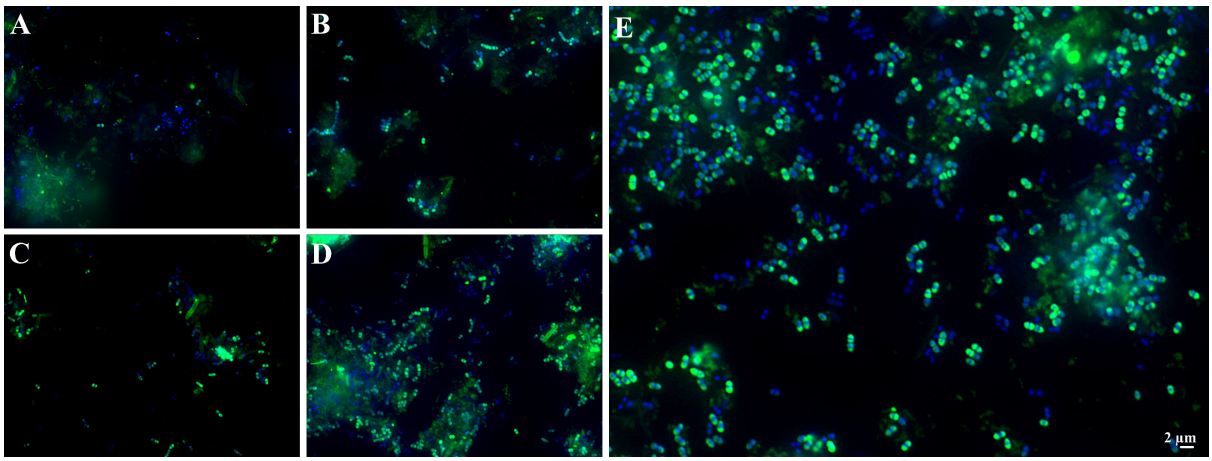


Figure 41. Detection of spiked *E. faecalis* cells in fecal samples. FISH analysis of SPF feces samples spiked with *E. faecalis* cells in gradient ratios concerning the feces volume (**A=**1:100, **B=**1:20, **C=**1:10, **D=**1:5, **E=**1:2.5) before in-solution hybridization with 'polyDIII' (FICT: green). Samples were DAPI stained (blue) before microscopic analysis.

The methods developed in this thesis aim at analyzing samples with more or less unknown content. For this, an additional FISH-based detection system was developed by designing the *Enterococcus* genus-specific oligonucleotide probe Enc473, whose evaluation is described in chapter D.1.2. To verify that this oligonucleotide probe also functions in in-solution FISH, *i.e.* as a second marker, the sample (SPF-*E. faecalis,* 1:2.5) first hybridized with 'polyDIII', was additionally hybridized with the oligonucleotide Enc473 (see C.14.3, p. 33). Figure 42 shows captured section of the spiked sample hybridized with polyDIII-FLUOS (A) and Enc473-ATTO565 (B) and the DAPI staining (C). The fact that few cells simply appear positive to one probe may arise from different signal intensities of 'polyDIII', Enc473 or the DAPI stain, resulting in separate colors in the composite image (D).

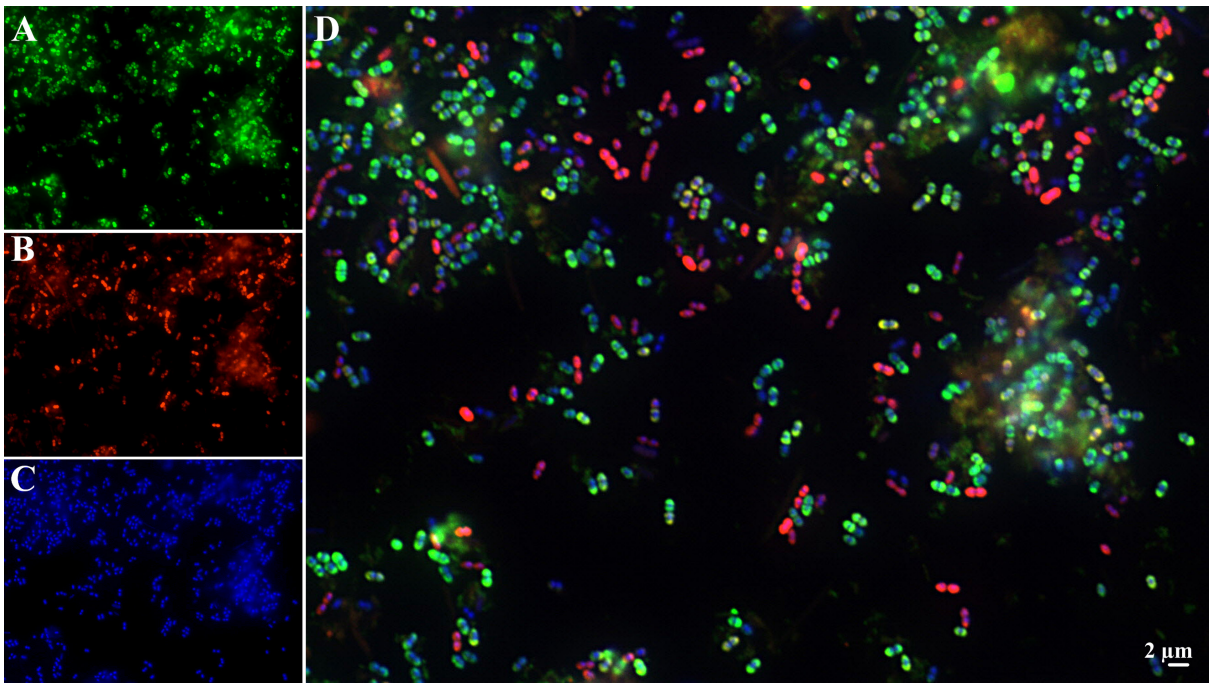


Figure 42. Fluorescent *in situ* hybridization for detection of *E. faecalis* in a fecal sample, combining an oligo- and the polynucleotide probe. FISH analysis of SPF feces sample spiked with *E. faecalis* cells (ratio 1:2.5). In-solution hybridization was performed using *Enterococcus* genus-specific probes 'polyDIII' (**A:** FLUOS, green) and Enc473 (**B:** ATTO565; red). **C:** DAPI stained (blue) sample **D:** composite micrographs A, B, and C.

As seen in Figure 39A, feces samples that were fixed with PFA after collection and storage at –20°C, showed weak signal intensities. This might be caused by long transfer passages and long-term storage of the samples. Specifically, in SPF samples the detection of enterococci is difficult, also probably due to their natural low-abundance. To increase detection rates in these samples and to compare samples to artificially spiked fecal samples, fecal matter of INF and

SPF mice were incubated aerobically (without shaking) and anaerobically in rich medium overnight at 37°C. Fixed fecal samples were directly verified by phase contrast microscopy and then applied to in-solution FISH using the 'polyDIII' probe (Figure 43, Figure 44).

Compared to feces directly fixed after long-term storage in –20°C (Figure 43A), phase contrast revealed that the precultured INF samples showed an increased occurrence of coccoid single, diplococci and chained cells, both after aerobic (Figure 43B) and anaerobic incubation (Figure 43C). FISH images of the aerobically incubated INF feces did not result in any specific probe binding, probably caused by defective probe implementation, so no cells could be detected. Additionally, anaerobically incubated INF samples showed a number of cells with different morphology, appearing as elongated diplococci. Fluorescence microscopy after in-solution FISH revealed that in these anaerobically incubated samples the rate of detection signals was increased, compared to the original fecal matter (Figure 43A) and that some detected cells showed the same changed morphology, seen in phase contrast images (Figure 43C).

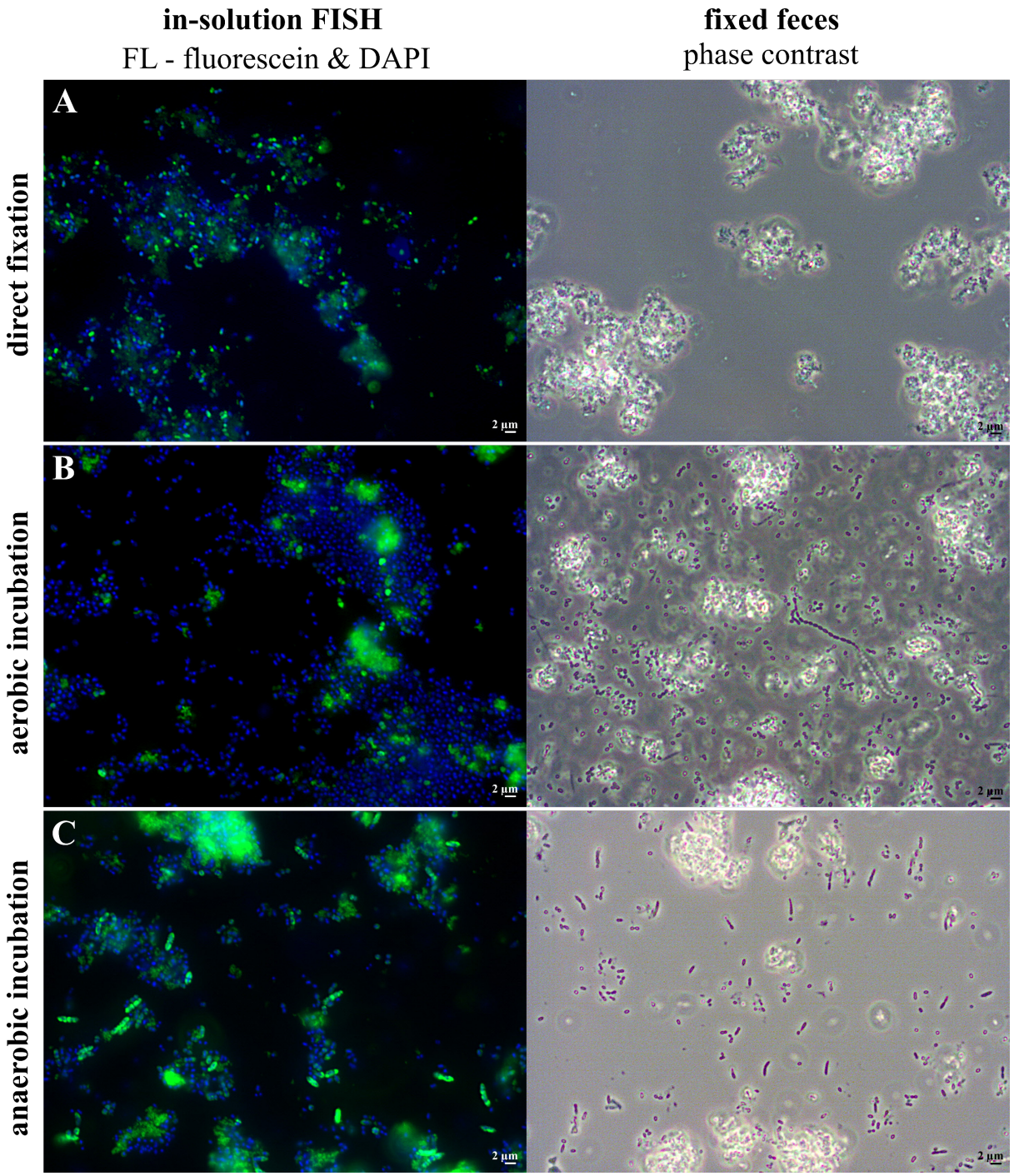


Figure 43. Comparison of INF feces samples based on precultivation procedure. Verification of samples by phase contrast microscopy and by epifluorescence microscopy of samples in-solution *in situ* hybridized with 'polyDIII' (see C.14.2.2). FISH images are displayed as composite pictures (FLUOS: green, DAPI: blue) **A:** feces directly applied to fixation after long-term storage at –20°C. **B:** feces aerobically precultivated in BHI prior to fixation. **C:** feces anaerobically precultivated in BHI prior to fixation.

Concerning SPF feces samples, phase contrast microscopy revealed occurrence of bacteria with different morphologies, *i.e.* coccoid and rod-shaped, independent from the mode of incubation (Figure 44). Fluorescence microscopy after in-solution FISH with 'polyDIII' for enterococci detection revealed that the number of signals detected in anaerobically incubated samples (Figure 44C) was significantly higher than in aerobically incubated fecal samples (Figure 44B),

and that both revealed higher numbers compared to the original fecal matter (Figure 44A). However, like for fecal matter of INF mice (Figure 43), it could also be seen for SPF samples that some of the positively 'polyDIII'-detected cells showed a slightly elongated diplococci cell shape. Due to enhanced results of anaerobically precultured samples, and considering results of the INF samples, anaerobic conditions were preferred in further analysis.

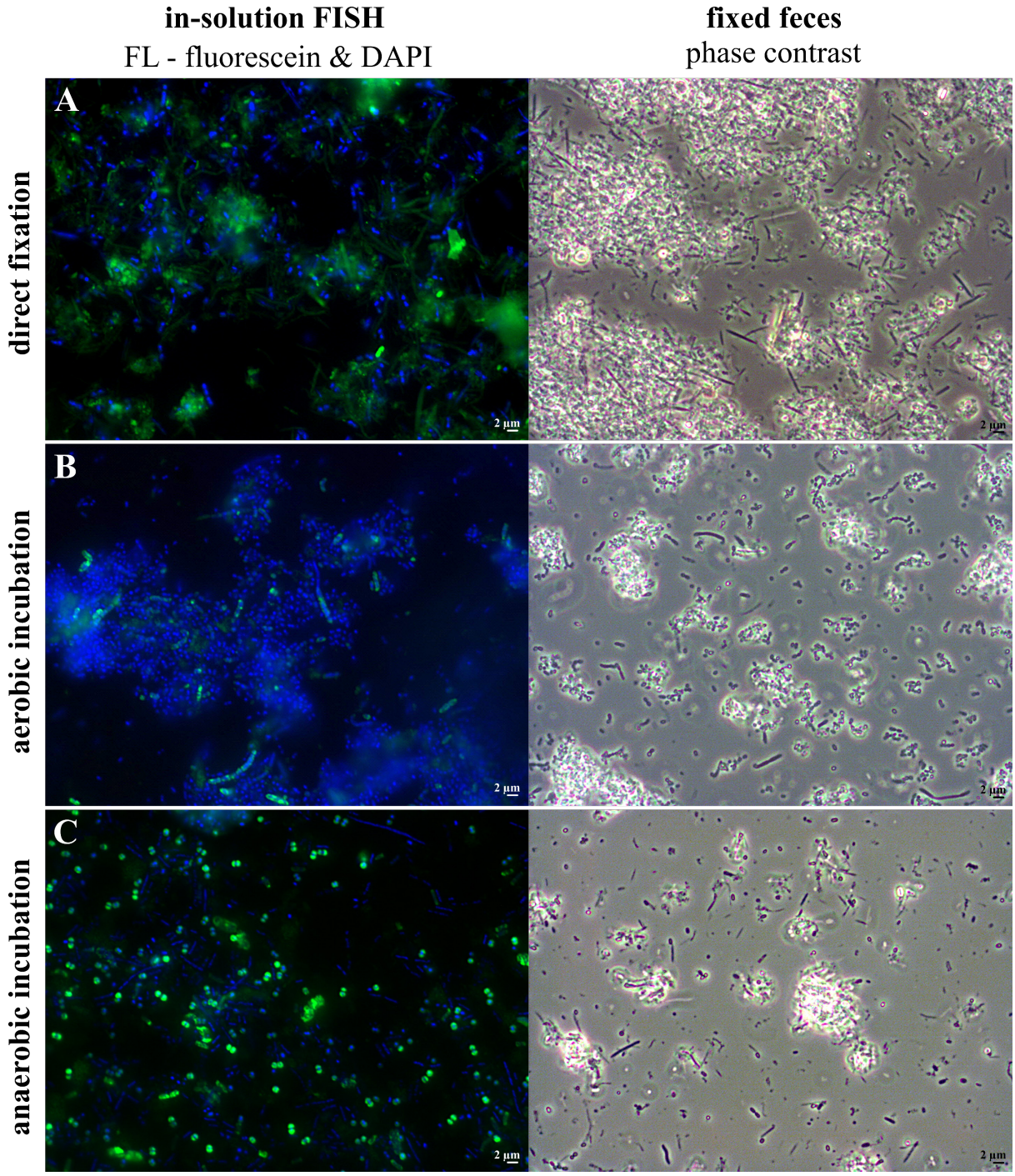


Figure 44. Comparison of SPF feces samples based on precultivation procedure. Verification of samples by phase contrast microscopy and by epifluorescence microscopy of samples in-solution hybridized with 'polyDIII'. FISH images are displayed as composite pictures (FLUOS: green, DAPI: blue). **A:** feces directly applied to fixation after long-term storage at –20°C. **B:** feces aerobically precultivated in BHI prior to fixation. **C:** feces anaerobically precultivated in BHI prior to fixation.

3. IMMOBILIZATION ON MICROPLATES

The technique of cultivation-independent cell enrichment using polynucleotide probes was first described by Stoffels *et al.* (MACS system) [113] and was further developed for enrichment in microtiter plates in other studies [115, 120, 141, 166]. These techniques are based on the phenomenon that due to the probe length, under certain conditions parts of the probes remain outside the cell's periphery and form inter-probe networks (see A.2.3.2, p. 7) [114]. These external networks are subsequently used to capture cells by immobilizing them during a second hybridization step in microplates that were coated with DNA complementary to the probe sequence (see C.15, p. 34).

3.1. COATING

To reduce the time needed for execution of the protocol, it was tested whether the incubation time for coating of the strips influenced the coating efficiency. The DIII-DNA fragments for coating were generated by including a DIG-labeled reverse primer (317R_mod-DIG) in the PCR reaction (see Appendix, Table 18, p. XI). After coating, DIII-DNA fragments could be visualized (Figure 45A) by a colorimetric detection system based on an anti-digoxigenin horseradish peroxidase conjugate in the present of a chromogenic substrate solution (see C.15.3, p. 37). After addition of 1M sulfuric acid (H_2SO_4), the absorbance was measured at 450 nm and 650 nm for wavelength correction, respectively. Figure 45B shows that strips incubated at 37°C for 45 min revealed a slightly higher absorbance than the strips incubated overnight. The negative control wells which had not been coated (NW) or were incubated with unlabeled DIII-DNA fragments, did not show any color change.

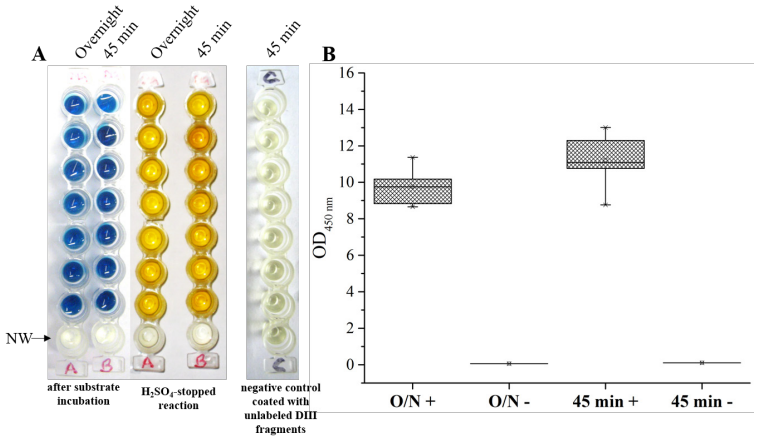


Figure 45. Comparison of strips coated for 45 min or overnight. Strips were coated with DIG-DIII-DNA fragments for 45 min or overnight (see C.15.1), including a strip incubated with unlabeled DIII-DNA fragments used as a second negative control (NW). **A:** strips after detection with BM Blue substrate (blue) and subsequent reaction with H_2SO_4 (yellow). **B:** strip absorbance measurements at 450 nm. **O/N:** overnight incubation. **+:** coated. **–:** uncoated cavity. Data shown as boxplots: horizontal line, median; boxes, 25th to 75th percentiles; whiskers, 5th to 95th percentiles.

3.2. DETECTION OF IMMOBILIZED CELLS

To determine the immobilization success of target cells in the coated cavities, various detection systems were implemented.

3.2.1. DETECTION USING THE DIG SYSTEM

Target-specific in-solution hybridization was performed using DIG-labeled 'polyDIII' RNA probes (see C.4, p. 16) on pure cultures of *E. faecalis*. After adequate washing of cells, they were subjected to immobilization on microtiter plates and detected by the anti-DIG-POD/substrate reaction (see C.15.3, p. 37), measuring the absorbance at 450nm/650nm. In Figure 46 the effects of several in-solution hybridization protocol changes are summarized, which were compared to assess the most time-saving and efficient hybridization performance. Uncoated cavities served as negative control wells (NW). Starting with comparison of different lysozyme pretreatments (Figure 46A), the application of an ethanol series (Figure 46B), a heat step (Figure 46C), duration of hybridization (Figure 46D), increasing probe concentration (Figure 46E) and evaluation of a saturation effects (Figure 46F), the optimum results were each taken as the basis for further protocol improvement. The negative controls were clearly distinct from other data. However, most of the protocol modifications merely resulted in small or no distinct changes. After the various improvement trials, the following conditions were established: 30–40 µL of a fixed pure culture (see C.13.2, p. 27) were applied to in-solution hybridization.

Pretreatment was performed using lysozyme for 10 min on ice and a gradient ethanol series. The hybridization was performed with 4–5 µg 'polyDIII' probe for 3 h.

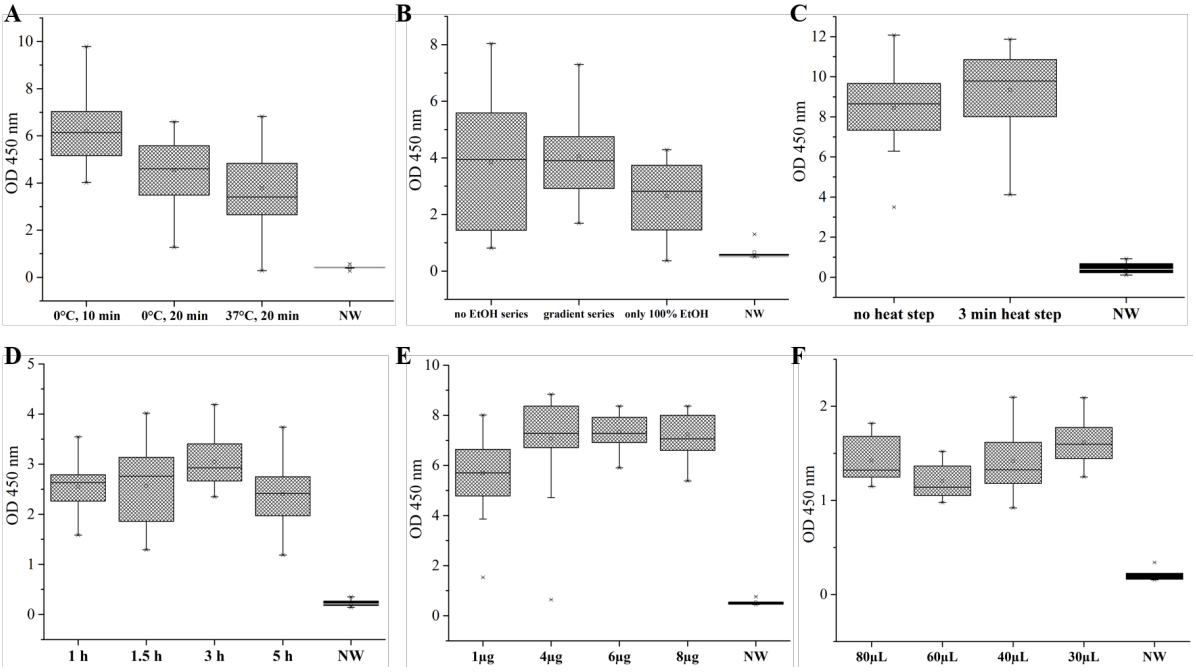


Figure 46. Immobilization efficiency evaluated after various protocol modifications. Box diagrams displaying OD_{450} ranges of DIG detection after protocol modifications (see C.15.3). **A:** lysozyme pretreatment. **B:** application of ethanol series. **C:** 200°C heat step pretreatment. **D:** hybridization time. **E:** increasing 'polyDIII' probe concentration. **F:** increasing amount of applied cells, to detect saturation effects. **NW:** negative wells (uncoated). Data shown as boxplots: horizontal line, median; boxes, 25th to 75th percentiles; whiskers, 5th to 95th percentiles.

To further challenge the usability of the adapted protocol, the detection system was also tested on non-target cells such as *E. coli*, *E. aerogenes*, or *C. glutamicum*. Figure 47A displays that also non-target strains revealed a positive colorimetric result upon anti-DIG-POD detection. Negative controls (NW, uncoated wells) were distinctly lower compared to other samples.

To determine the cause of false-positive results, several different samples were applied to the DIG detection system (Figure 47B): *E. faecalis* pure culture samples hybridized with a DIG-labeled 'polyDIII' and an unlabeled 'polyDIII' probe (see C.4, p. 16), different volumes (5 µL, 50 µL, 200 µL) of the hybridization supernatant (polyDIII-DIG hybridization) and increasing pure polyDIII-DIG probe concentrations, directly diluted in MP buffer. The sample containing cells hybridized with the unlabeled 'polyDIII' probe reacted similar to the negative controls (NW) with an absorbance around zero (OD_{450} ~0.3) (Figure 47B, unlabeled 'polyDIII'). The absorbance of the samples with extending volumes of the hybridization supernatant (5–200 µL supernatant suspension) rose, while the increase of applied pure probe concentrations (0.29 µg, 0.5µg, 1.66 µg and 5 µg) did not show any influence on the absorbance. The samples revealed

almost identical values regarding the polyDIII-DIG hybridized sample (Figure 47B, polyDIII-DIG). This indicates that sensitivity of the DIG detection system was so high that even little residual free polyDIII-DIG probes in a sample may lead to a positive signal. Thus, this detection system was considered not to be useful for this approach.

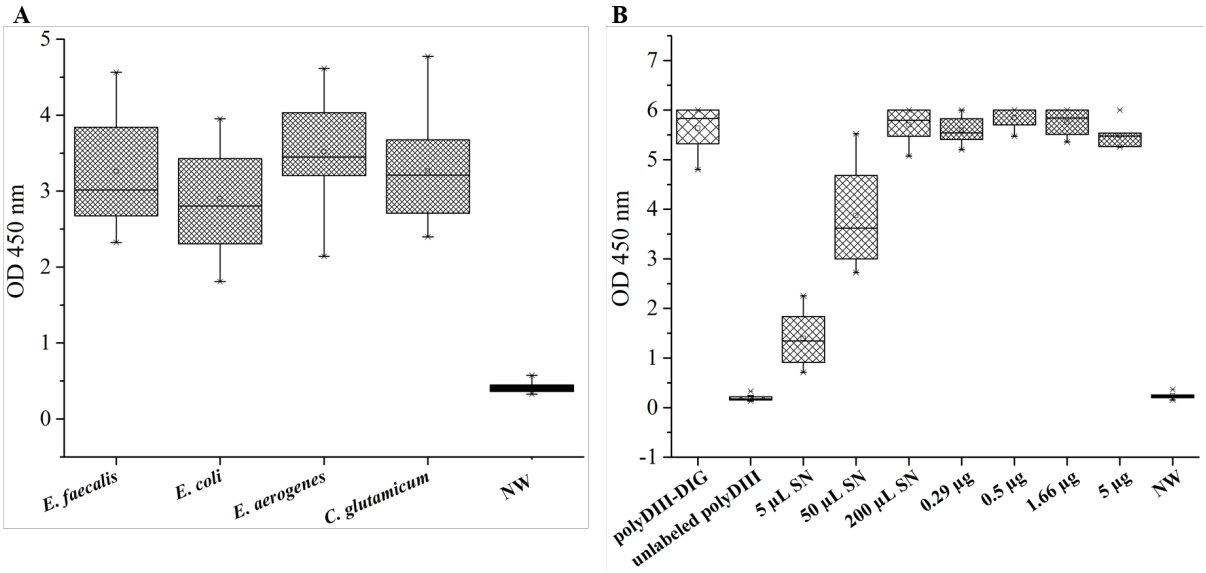


Figure 47. Immobilization efficiency evaluated after application of various sample types. Box diagrams displaying OD_{450} ranges of different samples applied to the DIG detection system after immobilization on microplates (see C.15.3). **A:** DIG detection results of *E. faecalis* pure culture samples and several non-target species. **B:** DIG detection measurements on different samples types (immobilized *E faecalis* cells hybridized with un-, or DIG-labeled 'polyDIII', increasing volumes of hybridization supernatant (polyDIII-DIG) and increasing pure polyDIII-DIG probe concentrations). **NW:** negative well (uncoated). **SN:** supernatants. Data shown as boxplots: horizontal line, median; boxes, 25th to 75th percentiles; whiskers, 5th to 95th percentiles.

3.2.2. PCR DETECTION

Samples were either in-solution hybridized with unlabeled 'polyDIII' or fluorescein-labeled 'polyDIII' (polyDIII-FLUOS). Enriched cells were directly detected by *Enterococcus*-specific PCR in the cavities (see C.15.3, p. 37). The PCR was applied to coated and uncoated cavities, as well as to the supernatants. Ideally, positive signals should be detected in the coated cavities and in the supernatant of the uncoated wells. The uncoated cavities and the supernatant of the coated wells should contain no or few target cells. Therefore, no or less efficient amplification compared to coated cavities should be detected.

Here, three different in-solution hybridization and immobilization protocols on *E. faecalis* pure cultures were compared [120, 141, 166] to detect the most efficient procedure. Gel electrophoretic analysis revealed that the protocol applied according to Fichtl [120] yielded in the most efficient PCR amplification in cavities, while the supernatant and the sample of the uncoated

well mostly did not show an amplification product (Figure 48). Hence, further modifications were tested based on this protocol. The no-template control of the PCR did not show an amplification (data not shown).

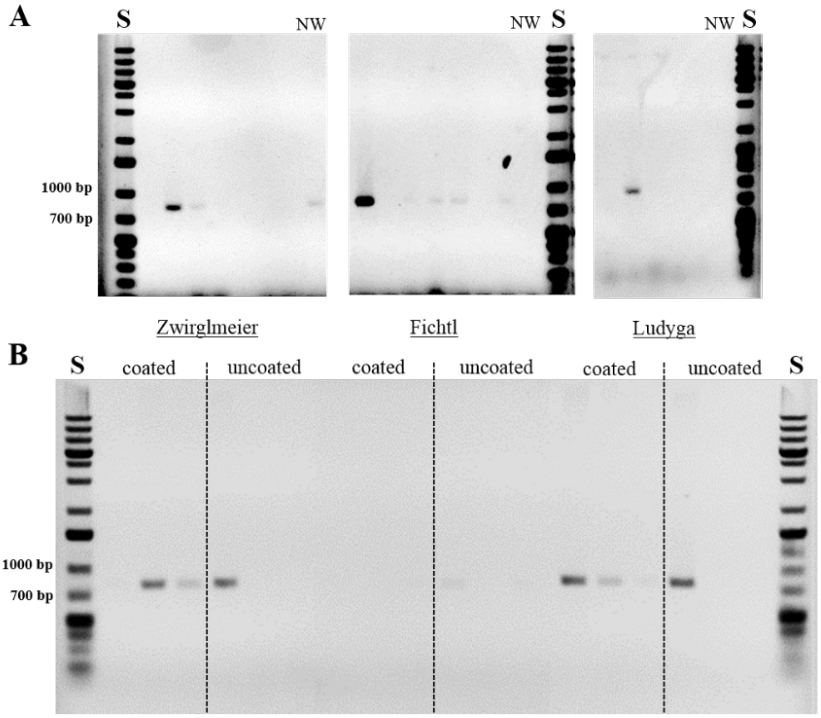


Figure 48. Immobilization efficiency evaluated by PCR applying different protocols. Comparison of different in-solution hybridization and immobilization protocols [120, 141, 166] by *Enterococcus*-specific PCR amplification (primer: Enc473V/Enc1276R). **A:** agarose gel of PCR directly performed in the cavities. **B:** agarose gel of direct-PCR applied to supernatants. **NW:** negative well (uncoated). **S:** GeneRuler™ 1 kb Plus DNA ladder.

To see whether the immobilization or the amplification result can be improved by increasing the amount of hybridization starting volume, strips immobilized with 10 µL and 20 µL of fixed pure culture sample were subjected to PCR detection using universal bacterial 23S rRNA gene primers (see Appendix, Table 18, p. XI). Figure 49 shows that the strip immobilized with in-solution hybridized pure culture samples using 20 µL of fixed cells (C.13.2, p. 27), led to better amplification results than the strip immobilized with 10 µL. Nevertheless, both uncoated cavities (NW) also showed 23S rDNA amplification products. The no-template control (NTC) did not result in PCR amplification of a product.

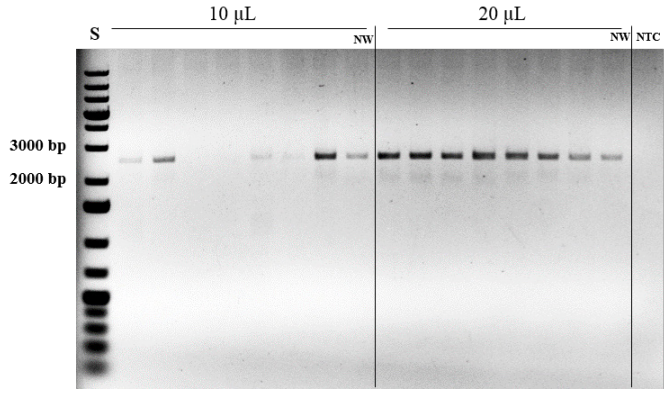


Figure 49. Impact of hybridization volume on immobilization efficiency. Gel electrophoretic analysis of bacteria-specific 23S rDNA amplification (primer: 118V/985R) directly performed in cavities. Pure culture samples were either 'polyDIII'-hybridized using 10 µL or 20 µL fixed cell suspension (see C.13.2). **NW:** negative wells (uncoated). **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder.

For parallel detection of enterococci and other bacteria, a multiplex PCR was evaluated which targets the 16S rRNA gene specifically for enterococci as well as the conserved 23S rRNA gene. The aim was to specifically obtain two fragments in samples containing enterococci (~800 bp and ~2,500 bp) and one fragment in samples containing bacteria other than enterococci (~2,500 bp). Thus, false-negative results can be excluded and furthermore, the 23S rDNA fragment can be used for phylogenetic analysis by sequencing. Figure 50 shows the gel electrophoretic analysis of a multiplex PCR experiment using primers Enc473V/Enc1276R and 3010V_mod/2241R (see C.15.3, p. 37) on several genomic DNA samples containing (Lanes A, B and PC), or not containing genomic DNA from enterococci (Lanes C–E). No amplification could be detected in the no-template control (NTC).

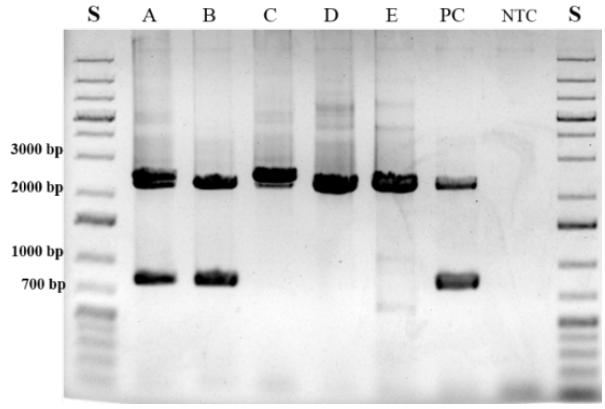


Figure 50. Development of a multiplex PCR for parallel detection of *Enterococcus*-specific 16S rDNA and bacterial 23S rDNA. Gel electrophoretic analysis of the multiplex PCR (primer sets: Enc473V/Enc1276R and 3010V_mod/2241R), to discriminate samples containing enterococci (A, B and PC) and samples only containing bacteria other than enterococci (C–E). **A:** *E. faecalis* and *C. glutamicum* **B:** *E. faecium* **C:** *C. glutamicum* **D:** *S. pygogenes* **E:** *E. coli* **PC:** positive control (extracted DNA from *E. faecalis*). **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder.

Amplification efficiency of rDNA indicating enriched cells, should further be enhanced by application of a Hot Start PCR. Therefore, two high fidelity hot start PCR systems, Q5® Hot Start High-Fidelity PCR (NEB, USA) and KAPA HiFi HotStart PCR (Kapa Biosystems, USA), both specialized on complex samples considered difficult to analyze, were compared (Figure 51). They are promoted to amplify low copy and complex DNA templates, while also tolerating the presence of PCR inhibitors (see C.7.4, p. 21).

Strips immobilized with a 'polyDIII'-hybridized *E. faecalis* pure culture were applied to direct multiplex PCR in the cavities (see C.15.3, p. 37). As controls, supernatants of the in-solution hybridization removed after the two washing steps (A, B) (see C.14.2.2 xv., p. 33), and supernatants of the cavities (C: coated and D: uncoated) were additionally used as templates for the PCR. Analysis of the gel electrophoresis revealed that by using the same PCR parameters, the Q5® Hot Start High-Fidelity PCR system led to better results, and both fragments were detectable in the strip except in cavity 5 (Figure 51A). Also control samples in lanes A–D showed positive amplification. It was noticeable that the 16S rDNA fragment of few samples could not be amplified, probably caused by minor DNA concentrations. No-template controls (NTC) were negative for both systems.

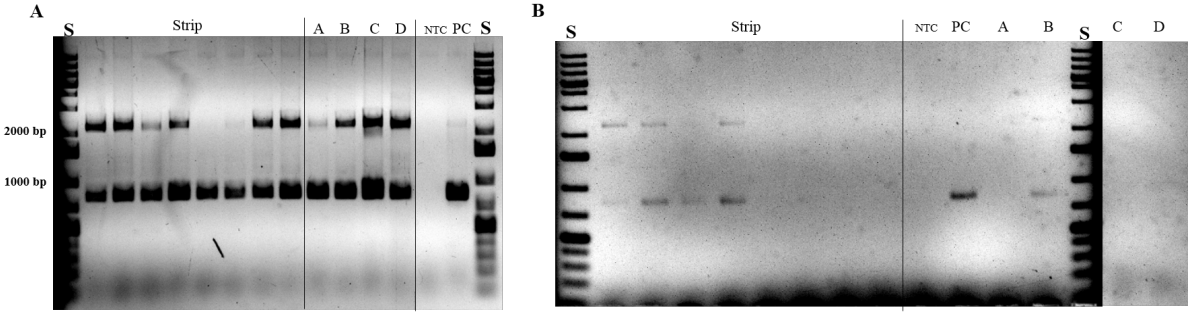


Figure 51. Immobilization efficiency compared by different hot start PCR systems. Gel electrophoretic analysis of multiplex PCR directly performed in cavities and applied on supernatants. The PCR product of the second cavity (red circle) in Figure A was forwarded to sequencing by Eurofins Genomics (see C.11). **A:** Q5® HS HiFi polymerase. **B:** KAPA Hifi HS polymerase (Kapa Biosystems, USA). **S:** GeneRuler™ 1 kb Plus DNA ladder.

After immobilization of enterococci from pure cultures, PCR detection was further tested on samples containing different non-target species (*E. coli*, *S. bovis*, and *L. intestinalis*). Gel electrophoresis revealed that in coated cavities loaded either with samples containing equal amounts of each species, or with mixtures containing a 3× vol of *E. faecalis*, amplification of *Enterococcus*-specific 16S rDNA could be detected (Figure 52). Strips containing samples with increased enterococci amount showed slightly better amplification results, whereas strips containing the samples without *E. faecalis* addition did not show any PCR amplification after gel

electrophoresis of their PCR products. Furthermore, no PCR products could be detected in samples from uncoated cavities (NW), from coated cavities only incubated with MP buffer without cells, and of the no-template control (data not shown).

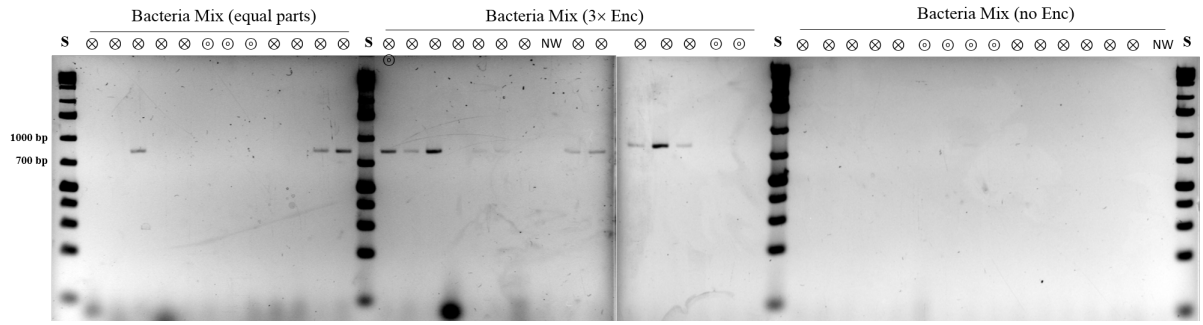


Figure 52. PCR detection in cavities tested with samples containing non-/target species. Gel electrophoretic analysis of *Enterococcus*-specific 16S rDNA amplification (primer: Enc473V/Enc1276R) directly performed in immobilization cavities. For immobilization, bacterial pure culture mixes (*E. coli*, *S. bovis*, *L.intestinalis* and eventually *E. faecalis*) were applied. In the first bacterial mix, cell concentrations of all species were equally distributed, whereas in the second mix a 3× vol of *E. faecalis* was added. In the last mix *E. faecalis* cells were excluded, and only non-target species were mixed and loaded to the cavities. **⊗:** coated cavities. **⊙:** coated cavities, incubated with MP buffer without cells. **NW:** negative well (uncoated). **S:** GeneRuler™ 1 kb Plus DNA ladder.

To exclude false-negatives or false-positives in the gel electrophoretic analysis, each fraction of the mixed bacterial samples obtained during microplate immobilization (see C.15.2, p. 36) was spotted onto filter membranes (see C.19, p. 43) and analyzed using an epifluorescence microscope (Figure 53). Regarding in-solution FISH, sample 3 (no enterococci) only showed autofluorescence signals of bacteria, while sample 1 and 2 clearly showed *Enterococcus*-specific signals derived from 'polyDIII'. For all samples it was shown that no cells were obtained from the recovered cavity samples, whereas FISH images of the supernatants (SN 1) showed roughly the same cell amount as the original in-solution hybridization sample.

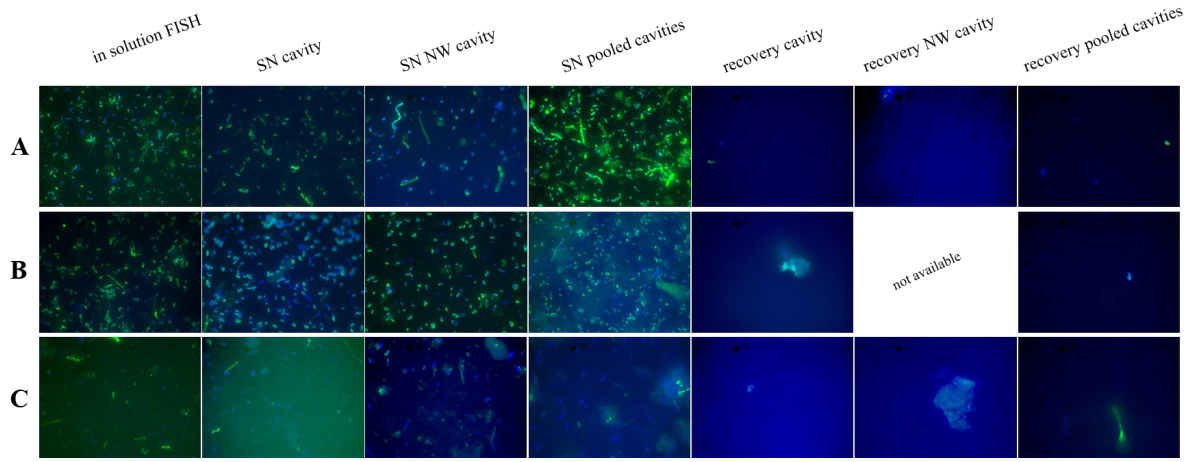


Figure 53. Immobilization efficiency evaluated by epifluorescence microscopy. Microscopic analysis of different fractions obtained during immobilization in microplates (see A.15.2). No cells could be detected in the recovered cell samples, but in the supernatants. **1:** bacterial mix containing equal amounts of non-/target species. **2:** bacterial mix containing various non-target species and an *E. faecalis* overdose. **3:** bacterial mix only containing bacteria other than enterococci. **SN:** supernatant. **NW:** negative well (uncoated). Images are displayed as composite pictures (FLUOS: green, DAPI: blue)

3.2.3. Detection and quantification by qPCR

As an alternative to direct-PCR (see C.16.1, p. 38), an *Enterococcus*-specific real-time PCR system (qPCR) was developed to directly quantify the immobilization efficiency without prior DNA extraction. The absolute quantification strategy relates the PCR signal to a known copy number using a standard curve (see D.1.3, p. 57). As the immobilization strips cannot be directly applied to the real-time PCR instrument, cells first had to be recovered (see C.15.2, xi.).

Figure 54 shows qPCR results using different immobilization fractions as templates. The strips were either immobilized with PFA-fixed or with ethanol-fixed SPF feces samples, both additionally spiked with *E. faecalis* pure cultures. The graph indicates that for both sample types most of the enterococci cells could be detected in the supernatant (SN) and only few were recovered in samples from coated cavities (PFA, EtOH, Pool). Large standard errors in SN PFA samples could indicate, that further dilution might be required to obtain more precise results.

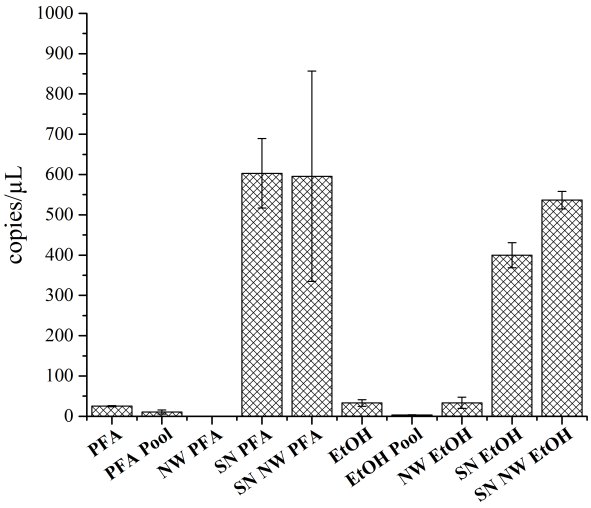


Figure 54. Immobilization efficiency of spiked fecal samples evaluated by qPCR. *Enterococcus*-specific quantification analysis by qPCR (targeting the 23S rRNA gene) of SPF feces (PFA- or EtOH-fixed) additionally spiked with *E. faecalis*. Data are means ± SEM with n = 2–16, by reference to copies/µL in 1 cavity. **PFA:** Recovered cells from a cavity immobilized with a PFA-fixed feces sample. **EtOH:** Recovered cells from a cavity immobilized with ethanol-fixed feces sample. **Pool:** Pool of recovered cells from one strip (7–8 cavities). **NW:** negative well (uncoated). **SN:** supernatant (7–8 cavities).

By comparing fecal samples additionally spiked with either *E. faecalis* or *C. glutamicum* as a non-target species it was shown that samples with *C. glutamicum* (C) could not be quantified by the *Enterococcus*-specific qPCR system (Figure 55). Samples spiked with *E. faecalis* (E) were solely detected in the supernatants of both, coated and uncoated cavities.

By applying the same templates to *Enterococcus*-specific standard PCR and by spotting the fractions on filter membranes for microscopic analysis, identical results could be obtained. Only supernatant samples (SN) yielded DNA amplification products (Figure 56) and microscopic cell signals (Figure 57).

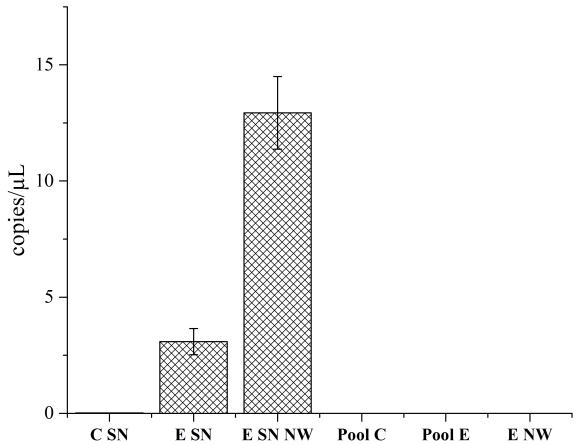


Figure 55. QPCR specificity for the assessment of immobilization efficiency in spiked fecal samples. *Enterococcus* specific quantification analysis by qPCR (23S rRNA gene) of SPF feces additionally spiked with *E. faecalis* (**E**) or *C. glutamicum* (**C**). Several immobilization fractions were used as templates. Data are means ± SEM with n = 2, by reference to copies/µL in 1 cavity. **Pool:** Recovered cells from one strip (7–8 cavities). **NW:** negative well (uncoated). **SN:** supernatant (1 cavity).

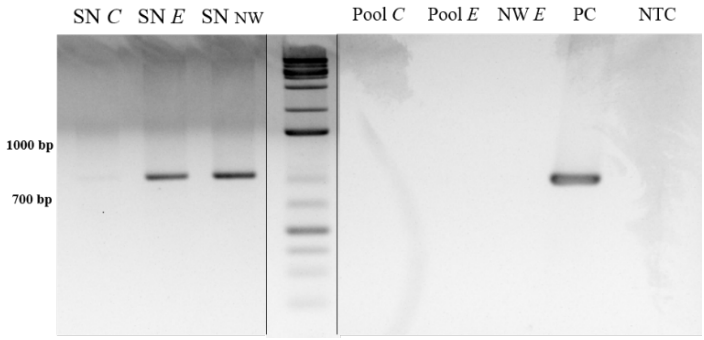


Figure 56. Direct-PCR specificity for the assessment of immobilization efficiency in spiked fecal samples. Gel electrophoretic analysis of *Enterococcus*-specific PCR amplification targeting the 16S rRNA gene (primer: Enc473V/Enc1276R). Several immobilization fractions were used as templates. **SN:** supernatant (1 cavity). **E:** SPF feces, spiked with *E. faecalis*. **C:** SPF feces, spiked with *C. glutamicum*. **Pool:** Recovered cells from one strip (7–8 cavities). **PC:** positive control (extracted DNA from *E. faecalis*). **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder.

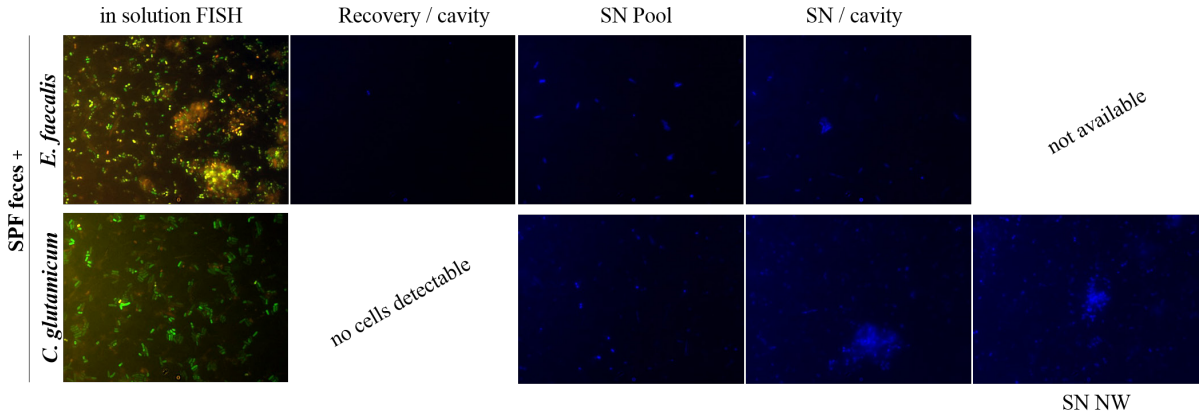


Figure 57. Immobilization efficiency evaluated by epifluorescence microscopy. Microscopic analysis of different fractions obtained during immobilization of SPF feces samples in microplates (see C.15.2). Fecal matter was additionally spiked with either *E. faecalis* (**E**) or *C. glutamicum* (**C**). No cells could be detected in the recovered cell samples, but in the supernatants. **SN:** supernatant. **Pool:** Cells from one strip (7–8 cavities). **NW:** negative well (uncoated). FISH images are displayed as composite pictures (FLUOS: green, DAPI: blue).

Figure 58 shows the quantification results of SPF feces samples increasingly spiked with *E. faecalis* pure culture (5%, 10%, 23% and 50% of hybridization volume). The graph indicates that cells could only be detected in the supernatants (increasingly, according to the spiking percentage). In contrast, in the samples of recovered cells enterococci were hardly detectable. When applying the samples to multiplex PCR (see C.15.3, p. 37), gel electrophoretic analysis also indicated positive amplification in the supernatants, whereby however the 5%–23% spiked supernatant samples did not show the 23S rDNA amplicon, possibly caused by low DNA concentrations (Figure 59). Weak amplification was visible for recovered samples, which most likely was caused by contaminated master mix (red circle) and was not further interpreted.

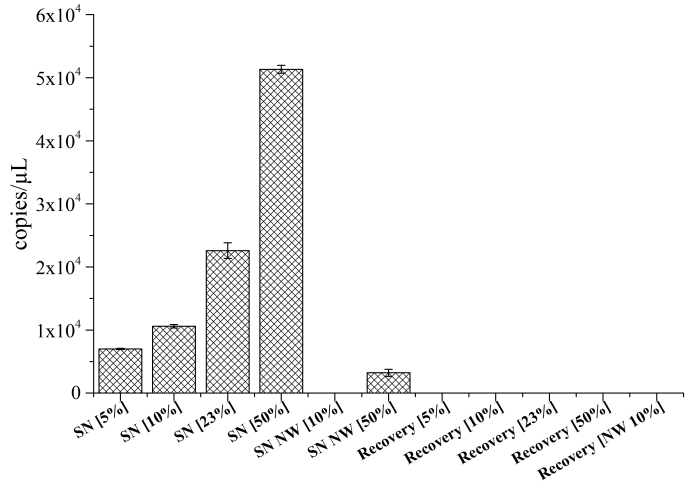


Figure 58. Immobilization efficiency of spiked fecal samples evaluated by qPCR. *Enterococcus* specific quantification analysis by qPCR (23S rRNA gene) of fractions obtained by immobilization on microplates. The SPF feces samples were increasingly spiked with *E. faecalis* (5%, 10%, 23% and 50% of hybridization volume) before hybridization and immobilization (see C.15.2). Data are means ± SEM with n = 2. **NW:** negative well, uncoated. **SN:** supernatant of one cavity. Values represent the median of duplicates.

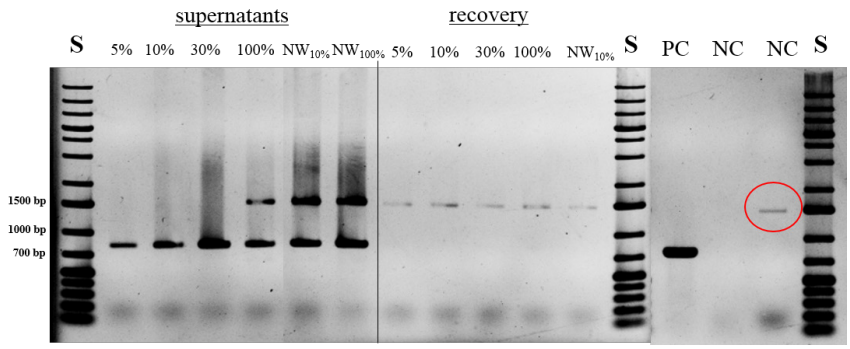


Figure 59. Immobilization efficiency of spiked fecal samples evaluated by direct-PCR. Gel electrophoretic analysis of multiplex PCR applied to supernatants and recovered cell samples of microplate immobilization. The SPF feces samples were increasingly spiked with *E. faecalis* (5%, 10%, 30% and 100% of feces volume) before hybridization and immobilization (see C.15.2). Primer sets were targeting the universal bacterial (616Valt/100K) and enterococcal (Enc473V/Enc1276R) 16S rDNA, respectively (see Appendix, p. XI) **NW:** negative wells, uncoated. **PC:** positive control (extracted DNA from *E. faecalis*). **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder.

4. CELL SORTING BY FLOW CYTOMETRY

4.1. DEVELOPMENT AND EVALUATION OF THE TAXON-SPECIFIC FACS METHOD

Evaluation started with the comparison of standard mono-labeled oligonucleotides and the polynucleotide probe 'polyDIII' concerning their application to flow cytometry, which has decreased sensitivity compared to epifluorescence microscopy.

When fecal samples were hybridized with the genus-specific DNA oligonucleotide probe (Enc473), lower signals and increased background noise was observed compared to RNA

polynucleotide probe-hybridized samples. The latter was therefore expected to enable efficient genus-specific cell sorting results through stronger signal intensities (Figure 60).

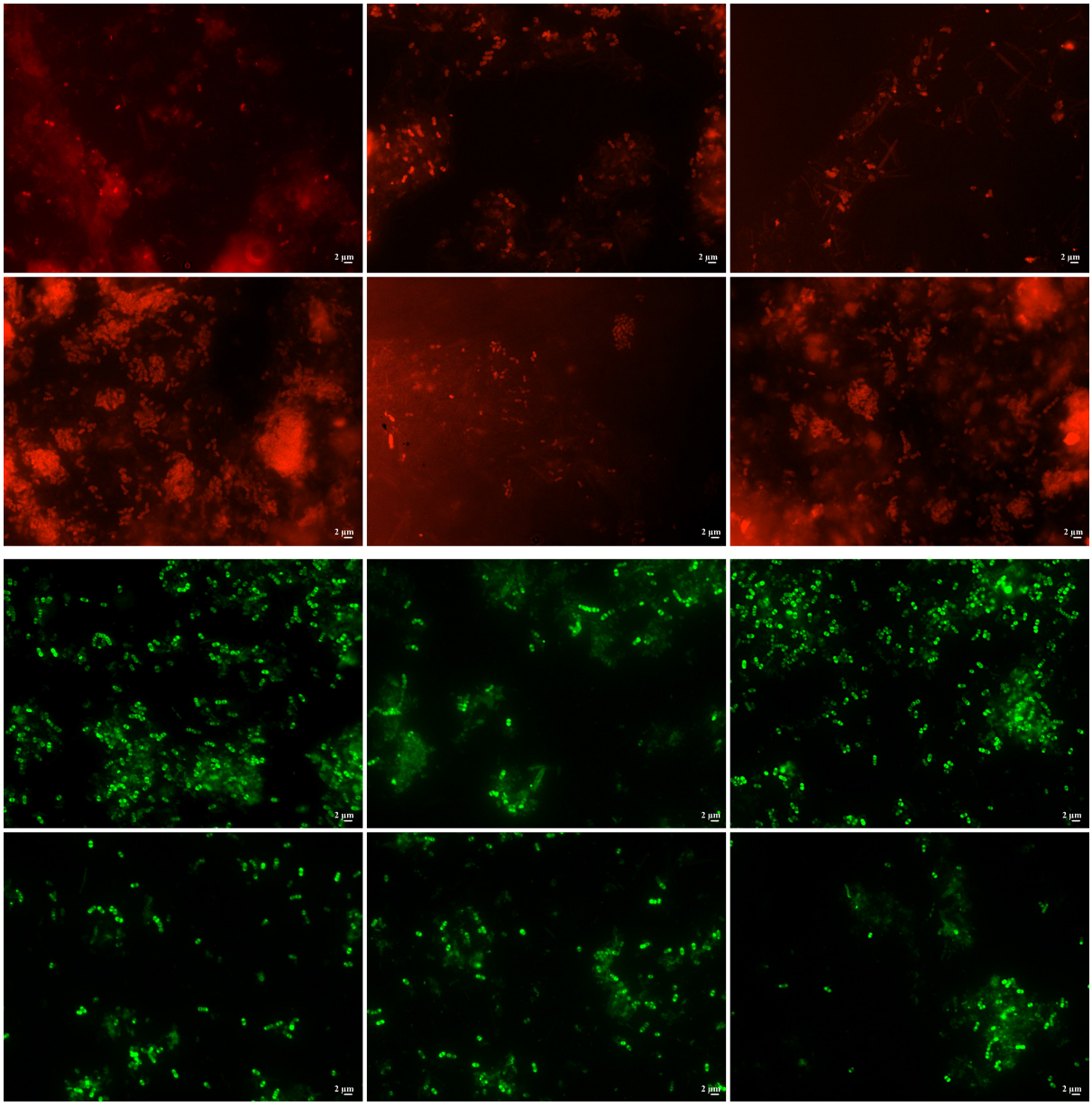


Figure 60. Application of genus-specific oligonucleotide or polynucleotide probe to mouse feces. Epifluorescence microscope image of various SPF feces samples spiked with *E. faecalis* pure culture. In-solution hybridization (see C.14.1.2 or C.14.2.2) was performed with the *Enterococcus*-specific Enc473 oligonucleotide probe (Cy3 or ATTO565: red) or with 'polyDIII' (FLUOS: green). Samples hybridized with 'polyDIII' showed increased signal intensity and minor background noise than samples hybridized with Enc473.

To directly correlate signal intensity differences between the mono-labeled oligonucleotide probe Enc473 and the 12–25× labeled polynucleotide probe 'polyDIII' (see C.4, p. 16), required exposure times for FISH image capturing were compared. Therefore, various sample types (pure culture, artificial mix and *E. faecalis*-spiked feces) were used, each under similar hybridization, as well as microscopy conditions. Figure 61 illustrates that the polynucleotide

induced a 16-, 36- or 19-fold signal amplification for the three samples, with a comparable low standard deviation, in contrast to the oligonucleotide probe. The high variance of the oligonucleotide-derived exposure times may be explained by high background fluorescence or unspecific binding to fecal matter.

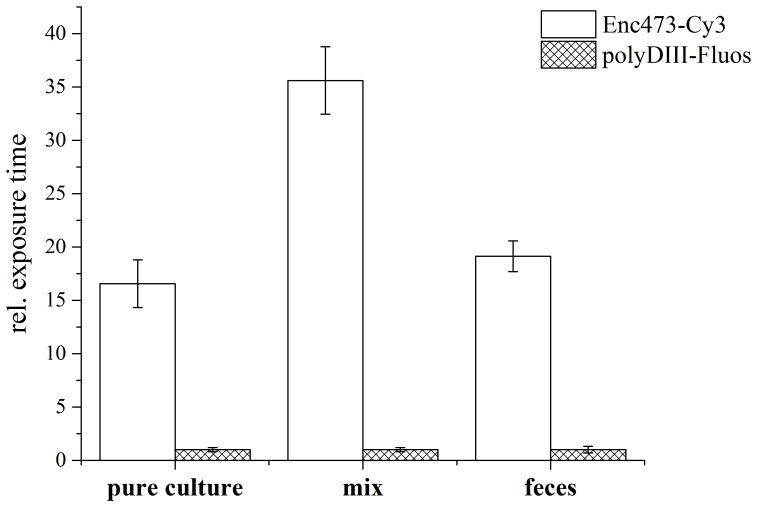


Figure 61. Signal amplification induced by polynucleotide probe polyDIII. The signal increase was measured based on the exposure time [ms] while FISH image capturing of different hybridized sample types. Each sample type (pure culture, artificial mix, *E. faecalis*-spiked feces) was hybridized with the *Enterococcus*-specific oligonucleotide probe Enc473-Cy3 (see C.14.1, p. 30) and with 'polyDIII' (see C.14.2, p. 32). The bacterial mix was composed of intestinal relevant species (*E. faecalis*, *S. bovis*, *E. coli*, *B. subtilis*, *Alcaligenes faecalis*, *Morganella morganii*), with a 3× increased density of *E. faecalis*. Data shows fold-change expressed as mean ± SEM with n=3–8.

The flow cytometric data of all samples and sample compositions presented here were repeatedly collected (n= 3–20), and results were comparable within the sample types, assuming successful *in situ* hybridization. Additionally, a negative control sample (no probe or nonEUB) was accompanied with each sample.

First trials were performed at the Max Planck Institute for Marine Microbiology (Bremen, Germany) under the instruction of Dr. Bernhard Fuchs using the MoFlo (Beckman Coulter, USA) system and the Kaluza® Flow Analysis Software (Beckman Coulter, USA). FACS data are presented as two-dimensional plots (density or dot plots) or one-dimensional histograms, both with logarithmic axes. Unless stated otherwise, plots display 100,000 events.

First *E. faecalis* pure cultures and fecal samples (see C.12, Table 7), additionally spiked (before fixation) with enterococci pure cultures in various ratios, were applied to *in situ* hybridization (see C.14.2.2, p. 33) and thereafter to FCM (see C.17, p. 41). In this way, the cell enrichment of enterococci using FACS could be developed and optimized.

Side scatter versus fluorescence intensity plots of pure cultures hybridized with 'polyDIII' revealed two populations (Figure 62B, PopA and PopB). One of them (PopA) was a distinct

population of *E. faecalis* cells not present in the negative controls hybridized with the nonEUB probe (Figure 62A). The mouse feces sample (SPF) spiked with cells from a pure culture of *E. faecalis* (Figure 63A) revealed the same characteristic subpopulation (PopA), whereas fecal samples spiked with a 10^5-fold increased volume of *E. faecalis* pure culture (Figure 63B) showed an additional third subpopulation (PopC) with identical fluorescence intensity, but an increased side scatter value (SSC). By microscopic analysis, PopA and PopC were identified as *E. faecalis* cells, while PopB probably represented target cells insufficiently hybridized with 'polyDIII' or non-target cells (Figure 64).

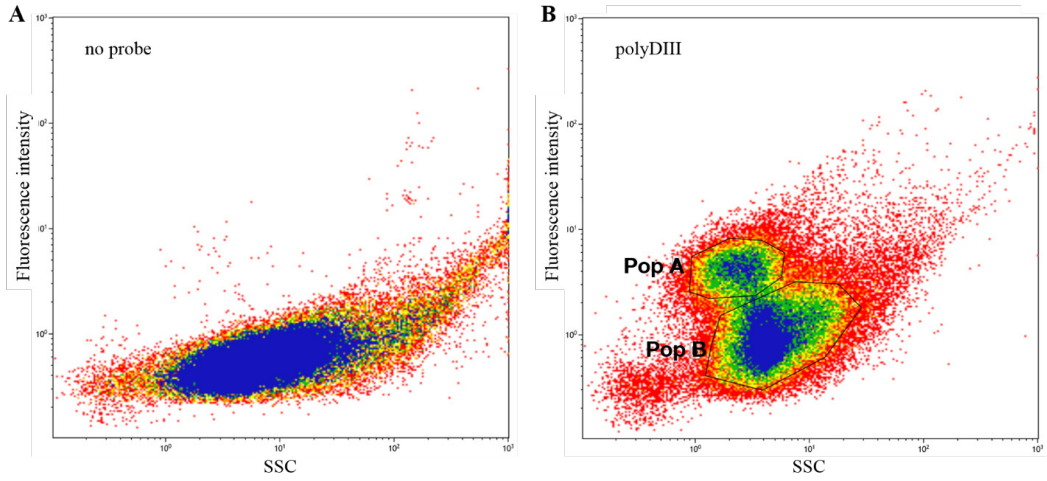


Figure 62. Characteristic population pattern of enterococci in FCM. Flow cytometric analysis (density plots: side scatter vs. fluorescence intensity) of *E. faecalis* pure cultures. Both pure culture samples were in-solution hybridized according to chapter C.14.2.2, p. 33. **A:** no probe. **B:** RNA polynucleotide probe 'polyDIII'. PopA represented positively 'polyDIII'-labeled *E. faecalis* cells, while PopB plots target cells that were insufficiently hybridized.

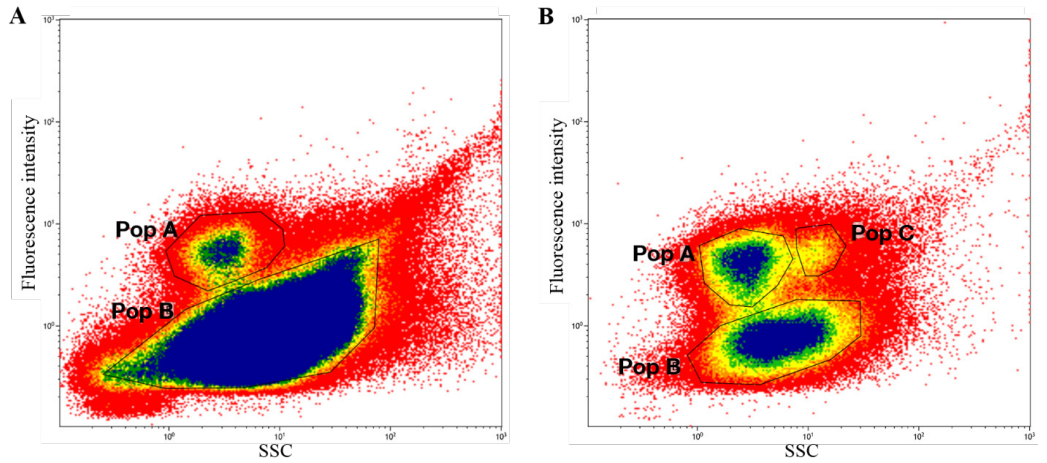


Figure 63. Characteristic flow cytometric community pattern of enterococci in feces. Flow cytometric analysis (density plots: side scatter vs. fluorescence intensity) of mouse feces samples which were in-solution hybridized according to chapter C.14.2.2, p. 33. **A:** SPF feces sample spiked with *E. faecalis* pure culture. **B:** spiked with an 10^5-fold increased amount of *E. faecalis* pure culture. PopA represented positively 'polyDIII'-labeled enterococci cells, while PopB plots target cells that were insufficiently hybridized or non-target cells.

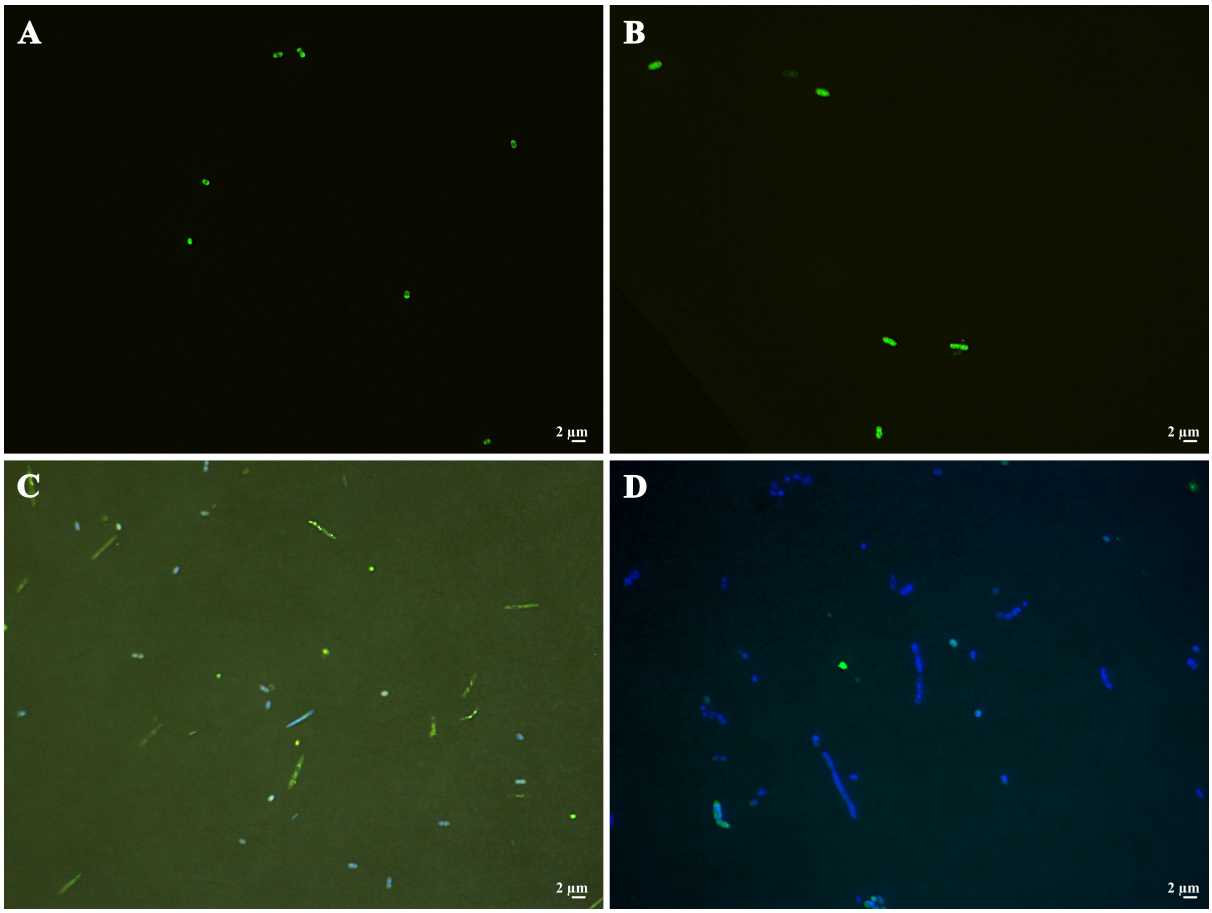


Figure 64. Microscopic analysis of fecal populations. Micrographs A–C display cells that were sorted from defined populations shown in Figure 63B (PopA, PopB, PopC). PopA and PopB displayed 'polyDIII'-labeled target cells, whereas cells in PopC appeared much larger or elongated. PopB (non-target or non(PopA) population comprised target cells that were insufficiently hybridized or non-target cells. **A:** PopA **B:** PopC **C:** PopB of spiked SPF feces sample **D:** PopB of SPF feces anaerobe precultured. Images are displayed as composite micrographs (FLUOS: green, DAPI: blue).

Extended FACS experiments were conducted at the Department of Biological Chemistry (Prof. Dr. Skerra, TU München, Germany) using the FACSAria II (BD Biosciences, USA) flow cytometer and the BD FACSDiva™ Software. Parts of the plot analyses were performed using the Flowing Software 2.5 (http://flowingsoftware.com).

To obtain quantitative information about any cell population the correct cluster first had to be defined. Based on negative (no probe or nonEUB probe) and positive controls (pure cultures or spiked samples), changes in the microbial structure were observed, and gate templates served as masks for respective sample types [81]. In this respect, the challenge was, that a defenition of a single gate template for any kind of sample type was not possible due to differences in sample matrix or hybridization success. In a scatter (dot) plot of fluorescence intensity vs. side scatter characteristic (SSC), it was possible to determine the distribution of cells based upon granularity and fluorescence intensity. On the other hand one-dimensional histograms viewing only a single parameter, *e.g.* counts of fluorescence intensity, were used to specify and count

the total number of cells that hold the selected property. Counts over a particular fluorescence intensity (section P2, threshold: 10^3) were assessed as positive events. The instrument parameters were modulated by measuring a negative sample (hybridized with no probe or a nonEUB probe, see Appendix, p. XII) against a positive sample (Figure 65), followed by application of different sample types for adjustment of the measurements (Figure 66–Figure 68).

In contrast to a sample hybridized under same conditions without any probe or nonEUB probe, application of a 'polyDIII'-hybridized *E. faecalis* pure culture led to a clear shift in fluorescence intensity in the dot plots and in the histograms (Figure 65). By defining a sorting region of events that fitted characteristic thresholds (see C.17, p. 41) and probably covered most of the target cells (PopA), the analysis of the positive control revealed almost 68% events of total counts as positive (P2) and 60% of counted events were positively gated in the region PopA (Figure 65B). In contrast, the negative control (Figure 65A) revealed only 0.3% total positive signal events and no events in the identical gate PopA. As opposed to the sample in Figure 62B, no clear distinct population (PopA), but an accumulation in this region and an almost linear shift of the cells with increased fluorescence intensity was observed in the 'polyDIII'-hybridized *E. faecalis* pure culture.

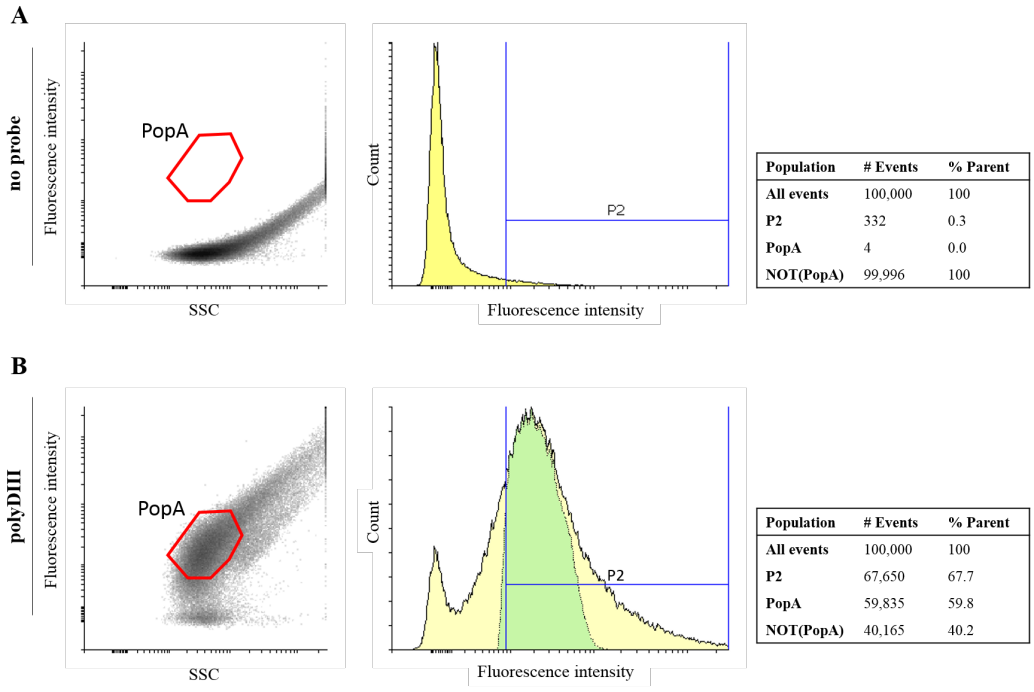
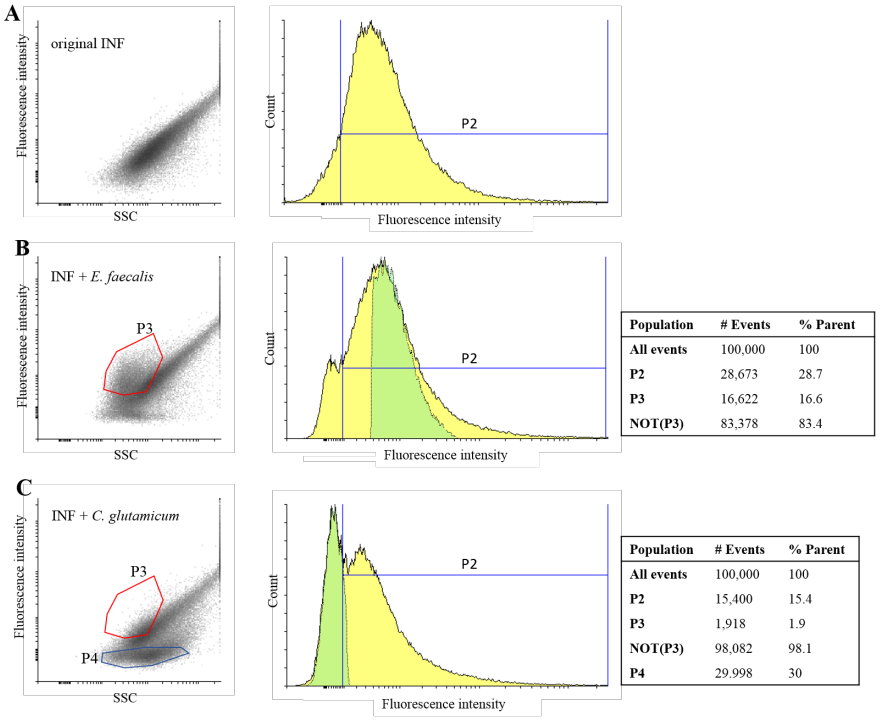


Figure 65. Detection of increased fluorescence signal intensities by 'polyDIII'. Flow cytometric analysis of *E. faecalis* pure cultures which were in-solution hybridized according to chapter C.14.2.2, p. 33. Measurements are illustrated as dot plots (fluorescence intensity vs. side scatter) and histograms (counts vs. fluorescence intensity). Selected cells (PopA) were defined according to fluorescence intensity and SSC as described in the Materials and Methods section (C.17, p. 41). The P2 section comprises all events that detected above the cut-off fluorescence intensity, thus counted as positive signals. **A:** no probe **B:** 'polyDIII'.

Figure 66 shows flow cytometric analysis of a fecal sample collected of an *E. faecalis* infected germ-free mouse (INF). Whereas Figure 66A displays the results of the original fecal sample, Figure 66B and C display INF samples additionally spiked with *E. faecalis* and *C. glutamicum* pure cultures, respectively. All samples were hybridized with 'polyDIII' under the same conditions. The original fecal INF sample did not show any clear distinct second populations (Figure 66A) as it only contains *E. faecalis* cells. It had a closer similarity to the pure culture sample in Figure 65B.

In contrast, the plot in Figure 66B indicated a small second population (P3) for the sample artificially spiked with *E. faecalis*. On the other hand, the INF sample artificially spiked with *Corynebacterium glutamicum* did not show this *Enterococcus*-characteristic population, but an accumulation of cells (P4) in the lower fluorescence intensity scale (Figure 66C). By comparing statistics of the chosen gate (P3) in Sample B (spiked with *E. faecalis*) and Sample C (spiked with *C. glutamicum*), the characteristic *Enterococcus* population could be directly measured. Gated events in Sample B are over 16% of the total count, whereas only around 1.9% in Sample C, which corresponds to the percentages of Sample A (original INF), when applying the identical sort region (data not shown).



Population	# Events	% Parent
All events	100,000	100
P2	28,673	28.7
P3	16,622	16.6
NOT(P3)	83,378	83.4

Population	# Events	% Parent
All events	100,000	100
P2	15,400	15.4
P3	1,918	1.9
NOT(P3)	98,082	98.1
P4	29.998	30

Figure 66. Specificity of 'polyDIII' in FCM. Flow cytometric analysis illustrated by dot plots (fluorescence intensity vs. side scatter) and histograms (counts vs. fluorescence intensity) of INF feces samples (*E. faecalis* infected GF mouse) *in situ* hybridized with 'polyDIII'. **A:** original INF sample. **B:** INF sample additionally spiked with *E. faecalis* pure culture. **C:** INF sample additionally spiked with *C. glutamicum*. Selected target cells (**P3**) were defined according to fluorescence intensity and SSC as described in the Materials and Methods section (C.17, p. 41). **P4:** probably accumulation of spiked non-target cells (*C. glutamicum*). **P2:** events showing signal intensities above the threshold value of 10^3.

For subsequent sorting of fecal samples, defined regions (P3) surrounding the population of interest were determined separately for each sample by using the negative controls as a reference (Figure 67). In the respective histograms the gated cells appeared as green peaks, which basically should be defined within section P2 (total positive signals) in positive samples.

Figure 67 displays the FACS analysis of fecal samples collected from SPF mice hybridized with 'polyDIII'. Sample B was additionally spiked with an *E. faecalis* pure culture before hybridization to define the *Enterococcus*-specific gate. Both samples were investigated with respective negative control samples (hybridized with the nonEUB probe or no probe) and analyses were performed with identical gating (P3). The gating region was selected on the basis of the spiked fecal sample (Figure 67B). When hybridized with 'polyDIII', it showed the characteristic Enterococcus population. While this population appeared very small in the dot plot of the original SPF sample (Figure 67A) however the amount of gated cells was quite similar (3.1%–4.2% of total counted events). Dot plots and related histograms showed similar results with a peak indicating the negative signals (red) and the positive events (green). In the negative references only 0.1% cells were counted in the applied sort region for both samples.

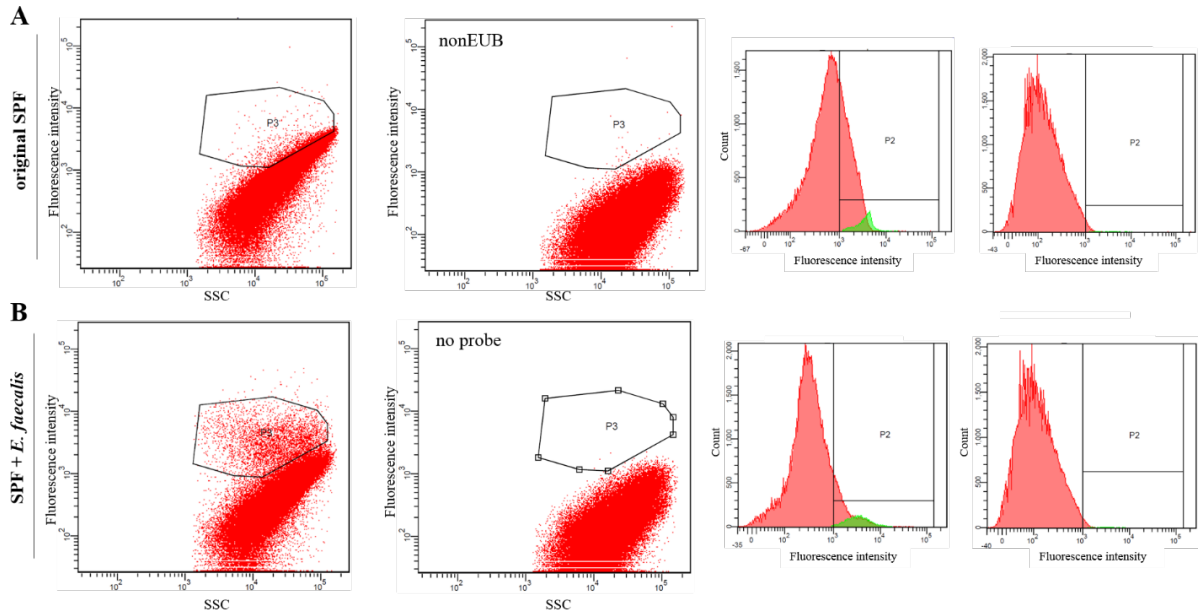


Figure 67. Selection of sort region for enterococci by analysis of spiked fecal samples. Flow cytometric analysis of fecal samples of a SPF mouse using dot plots (fluorescence intensity vs. side scatter) and histograms (counts vs. fluorescence intensity). *In situ* hybridizations with 'polyDIII' each were performed according to chapter C.14.2.2, p. 33. **A:** original SPF sample. **B:** fecal sample additionally spiked with fixed *E. faecalis* cells. Samples were analyzed using associated negative references, hybridized with the nonEUB probe or no probe. The gating region (P3) was selected on the basis of the spiked fecal sample (**B**).

An indication that population 'PopA' was the one comprising the targeted enterococci, was given by a gradient study. Increasing volumes of fixed mid exponential (OD_{600} 0.6–0.9) *E. faecalis* cells (from ratio 1:200 to 1:10) were added to fixed fecal samples (prepared as described in C.13.1, p. 27), right before hybridization. The increase of target cells was detectable on dot plots (fluorescence intensity vs. SSC) as the percentage of events in gate P3 rose from 0.4%–5.4%. In addition, the increase of added target cells was also quantifiable by qPCR (Figure 24, p. 66) and visible by epifluorescence microscopic analysis of the hybridized samples (Figure 41, p. 85).

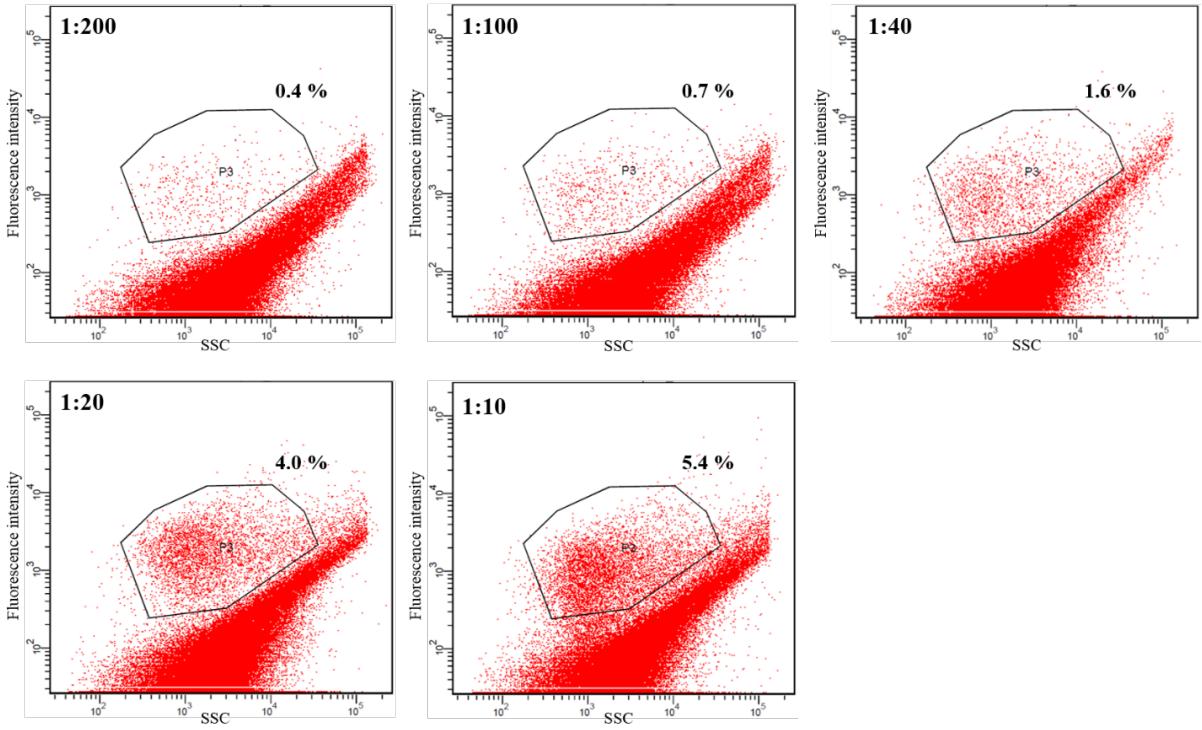


Figure 68. Specificity of enterococci characteristic subpopulation in fecal samples. Flow cytometric analysis of SPF feces samples spiked with an increasing amount of *E. faecalis* cells (1:200–1:10) before hybridization with 'polyDIII'. Analysis is illustrated by dot blots (side scatter vs. fluorescence intensity). Region P3 captures cells that will be sorted. Percentages indicate the rates of sorted cells if using the identical gate for all samples.

Figure 69 shows a complete analysis of a taxon-specific cell enrichment by FACS applied to an *E. faecalis* pure culture. It displays the FCM analysis (dot plot, A and histogram, B) and epifluorescence microscope images that were captured after hybridization with 'polyDIII' (C) and after cell sorting of the target population (D).

Dot plot, histogram (see Figure 65B) and microscope image correspond to each other, as they all confirm that most of the cells in the sample were 'polyDIII'-hybridized (Figure 69C, green cells). Target cells with signal intensities $>10^3$ (Figure 69B, section P2) were gated in the region P3 (71% of total counted) for subsequent sorting. No specific population could be identified

because cells of the pure culture showed variable cell sizes and morphology (single or diplococci). On the other hand, some cells in the sample probably were insufficiently hybridized with 'polyDIII', and therefore their signal intensity was lower (blue cells, $<10^3$). The epifluorescence image for verification of the cell sorting revealed that hybridized coccoid cells with strong signal intensity could be separated (Figure 69D).

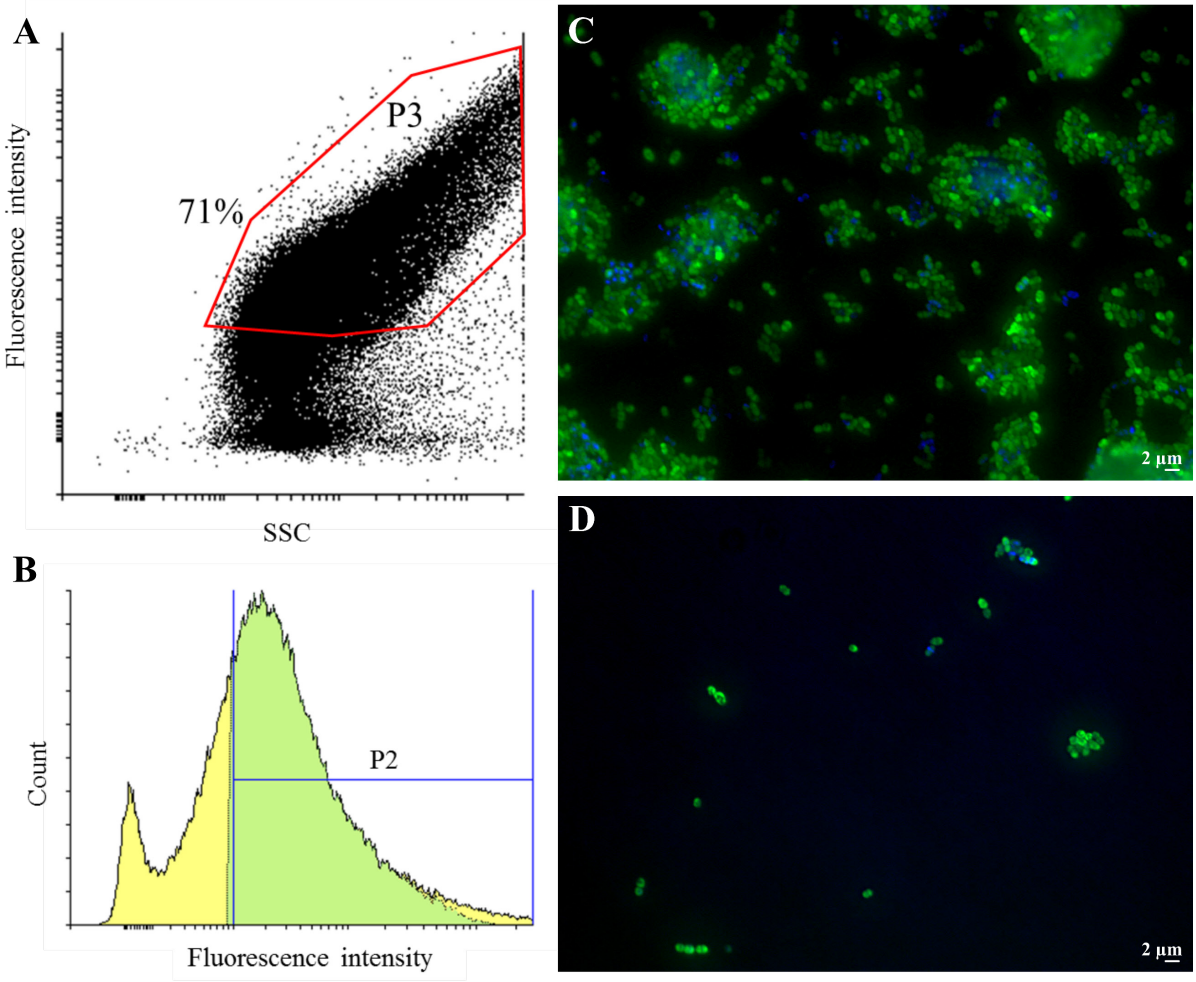


Figure 69. Complete analysis of a taxon-specific cell enrichment by FACS, applied to pure culture. Overview for the taxon-specific cell sorting procedure, applying an *E. faecalis* pure culture hybridized with 'polyDIII' (see C.14.2.2). **A:** FACS analysis (dot plot). **B:** FACS analysis (histogram). **C:** in-solution FISH imaging. **D:** cell sorting verification by microscopy (FLUOS: green, DAPI: blue). Section P2 represent the total of positively 'polyDIII'-labeled cells, whereby gated cells appeared as green peaks. As the sample was a pure culture, P3, the gate defined for sorting, was defined by the fluorescence intensity threshold of 10^3 without considering the SSC values.

Figure 70 displays the whole hybridization and sorting process for a fecal SPF sample, artificially spiked with a pure culture of *E. faecalis*. Even though the total signal intensity for this sample was not high (Figure 70A and B), the characteristic population (P3, 5.2% of total counted) indicated the presence of positively hybridized enterococci cells in the sample (Figure 70C), which successfully could be sorted by FCM (Figure 70D).

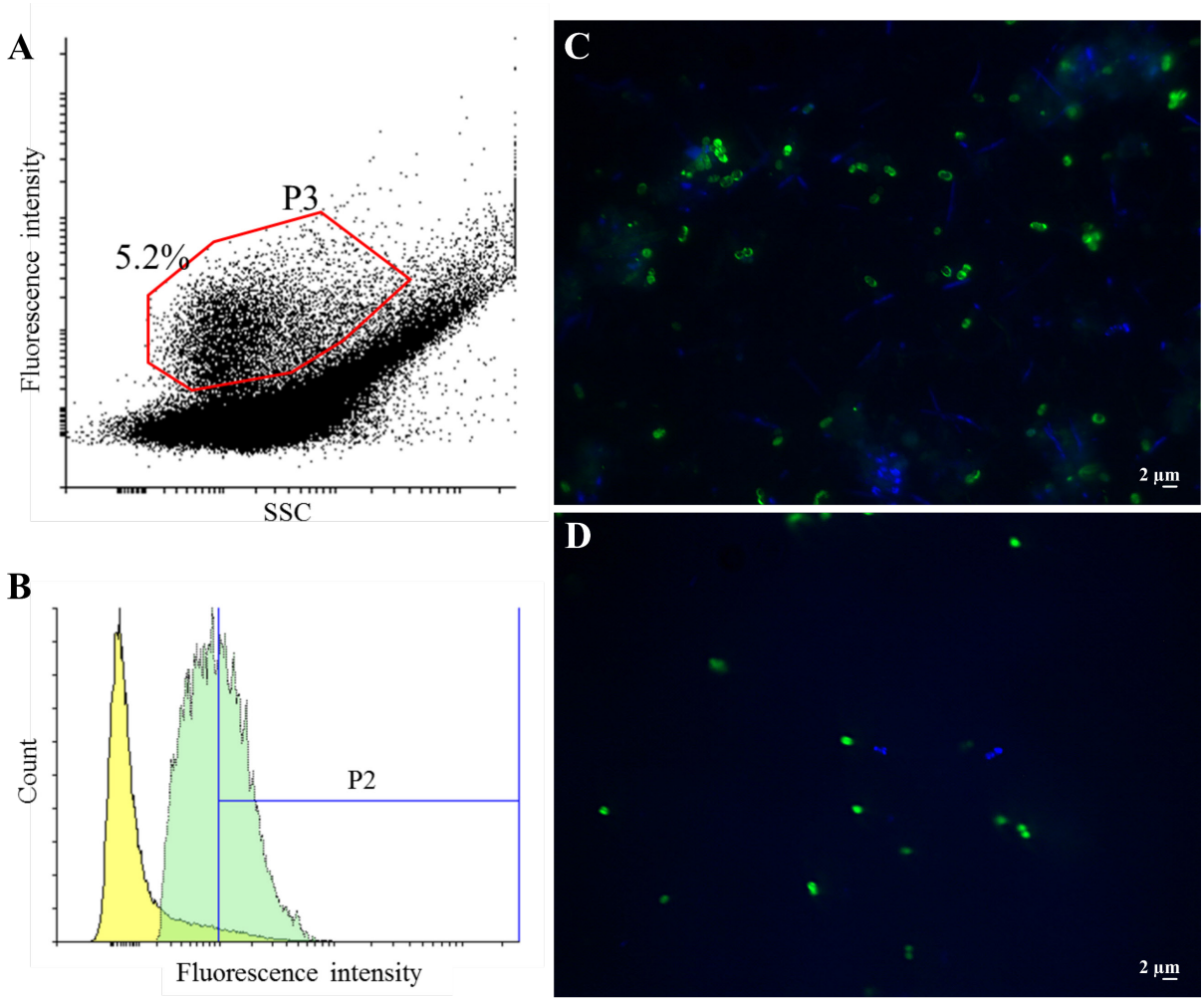


Figure 70. Complete analysis of a taxon-specific cell enrichment by FACS, applied to fecal samples. Overview for the taxon-specific cell sorting procedure, applying a SPF feces sample additionally spiked with an *E. faecalis* pure culture and hybridized with 'polyDIII'. **A:** FACS analysis (dot plot). **B:** FACS analysis (histogram). **C:** in-solution FISH imaging. **D:** cell sorting verification by microscopy (FLUOS: green, DAPI: blue). Section P2 represent the total of positively 'polyDIII'-labeled cells, whereby gated cells appeared as green peaks. P3 was the gate defined for sorting.

4.2. TAXON-SPECIFIC CELL SORTING OF FECAL SAMPLES BY FLOW CYTOMETRY

The developed genus-specific cell sorting protocol after 'polyDIII' hybridization should be tested on two mouse feces sample types, INF and SPF (see C.12, Table 7), including prior *Enterococcus* detection or quantification by PCR approaches and subsequent analysis of molecular accessibility.

While the samples in the previous chapter partly were spiked artificially with *E. faecalis* pure cultures here the ambition was to enhance the vitality of naturally occurring fecal bacteria and strengthen the discrimination of low-abundance target population for FACS validation. Thus, in some cases the fecal samples were incubated (anaerobically or aerobically, without shaking) in BHI medium at 37°C overnight after cleaning off the debris (see C.12, p. 25).

The presence and quantity of enterococci occurring in each fecal sample were evaluated by a direct-PCR specific for *Enterococcus* spp. (see C.16.1, p. 38) and a direct-qPCR targeting *tufA* from *Enterococcus* spp. (see C.16.2, p. 39). Evaluation of both systems is described in chapter D.1.1 and D.1.3. If PCR/qPCR results were positive for *Enterococcus* (in a sufficient amount), fecal samples were applied to in-solution FISH using the fluorescein-labeled RNA polynucleotide probe 'polyDIII' and subsequently were verified by microscopic examination (Figure 72). Positive 'polyDIII'-hybridized fecal samples showing coccoid bacteria in the FISH images were consequently used for cell sorting by FCM.

Figure 71 displays dot plot analysis of an original INF sample (*E. faecalis* infected GF mouse) and a precultured SPF feces samples (see Table 7, p. 25). Compliant with previous validations (see D.4.1, p. 101), target-populations of interest that were hybridized with the 'polyDIII' were defined and gated (region PopA) by following the threshold of signal intensity ($>10^3$) and size/granularity of target cells (SSC: 10^3–10^4), previously described in chapter C.17. A distinct subpopulation was detectable in the dot plots of the anaerobically incubated SPF sample (Figure 71A). On the other hand, the INF sample (Figure 71B) which was not precultivated and should only comprise *E. faecalis* cells showed similar dot plot results as the analysis of a *E. faecalis* pure culture with a roughly visible subpopulation (Figure 65, p.106). The precultured sample also displayed an increase of cell density, compared to an original SPF sample (see Figure 67A). From the INF feces, 40.2% of the counted 'polyDIII' positive cells were gated and sorted. In contrast, only 11.4% of the total counted cells from sample SPF were sorted, considering different sort regions. Approximately 10^6 cells of each defined gate (PopA) were collected in tubes. Initial and sorted samples were both checked by microscopic analysis (Figure 72).

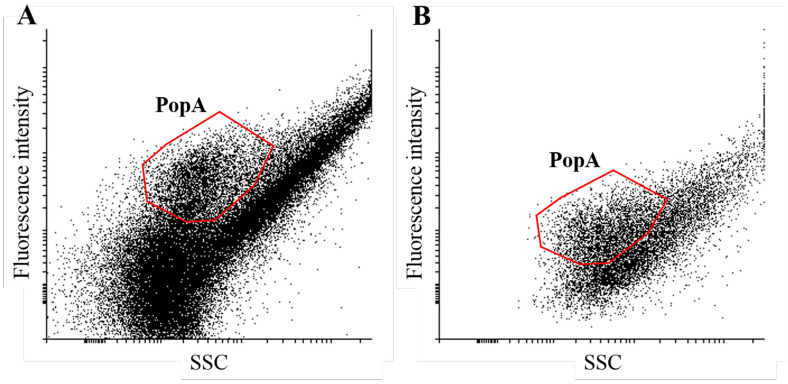


Figure 71. Enhancing of the target population for flow cytometric analysis by precultivation of feces samples. Analysis is illustrated by dot plots (fluorescence intensity vs. SSC). *In situ* hybridization with 'polyDIII' was performed according to chapter C.14.2.2, p. 33. **A:** anaerobically precultured SPF sample. **B:** original INF feces sample. The inner polygon (PopA) indicates gating used for separation of the target population and was defined as described in the Materials and Methods section (see C.17, p. 41).

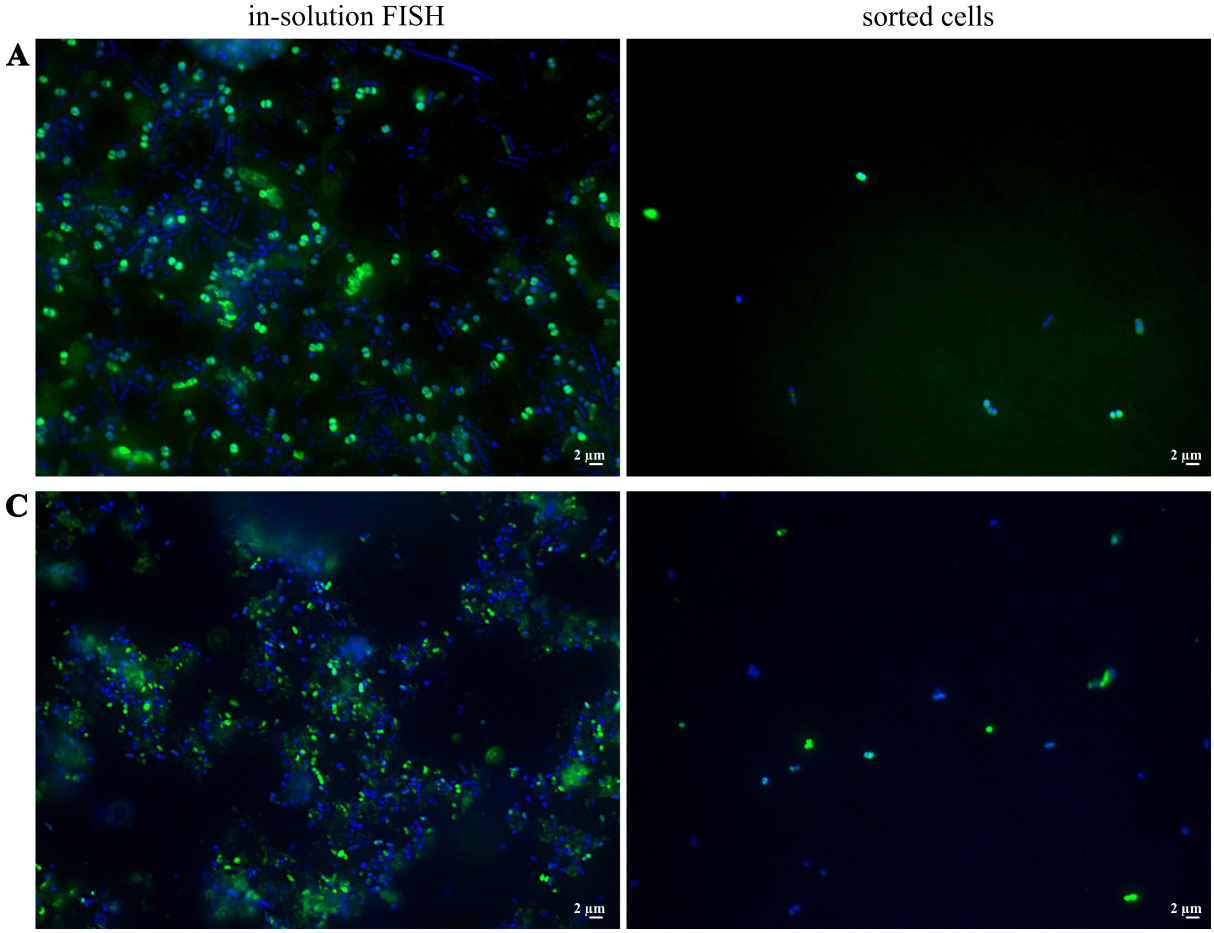


Figure 72. Microscopic analysis of precultured feces samples before and after sorting. Images on the left side show mouse feces samples after in-solution FISH with 'polyDIII' (see C.14.2.2, p. 33). Images on the right side represent target cells (region PopA, see Figure 71) after flow cytometric cell sorting of these samples. FISH images are displayed as composite micrographs (FLUOS: green, DAPI: blue). **A:** anaerobically precultivated SPF feces. **B:** INF mouse feces.

The dot plot analysis of precultivated INF (anaerobically) and SPF (aerobically) samples showed the same characteristic subpopulation (Figure 73) for both samples that was already seen with pure culture of *E. faecalis* (see Figure 65). Based on the negative reference controls and the increase of signal intensity, the sorting region PopA was separatly selected for each sample. Corresponding histograms expressed that cells gated in region PopA were counted as positive 'polyDIII'-labeled as their peak (green) was defined within section P2. The red peak in section P2 illustrated cells with high fluorescence (ideally target cells) not being gated.

Statistical analysis of the samples revealed that concering sample INF (Figure 73A), 59.8% of the cells counted for analysis were positivly 'polyDIII'-labeled, whereby 27.8% of the events were finally sorted. Almost the same percentage (around 57.6%) of the SPF sample counts showed signal intensities higher than the threshold 10^3 and hence were stated as positive, while 48% of displayed events finally were captured by the sorting region (PopA) seen in Figure 73B.

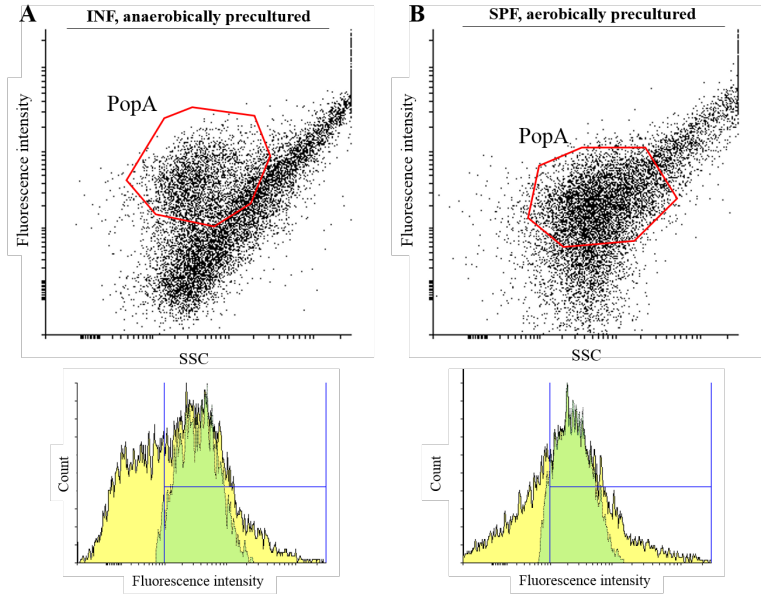


Figure 73. Detection of enterococci specific subpopulation in precultured fecal samples. Flow cytometric analysis using dot plots (side scatter vs. fluorescence intensity) and histograms (fluorescence intensity vs. cell counts) of fecal samples hybridized with the fluorescent *Enterococcus*-specific polynucleotide probe 'polyDIII'. **A:** anaerobically precultivated INF sample **B:** aerobically precultivated SPF sample. Section P2 represent the total of positively 'polyDIII'-labeled cells, whereby gated cells appeared as green peaks. PopA was the gate defined for sorting.

Concerning fecal samples which both were previously anaerobically incubated, two examples for an INF and a SPF sample are illustrated in Figure 74 and Figure 75. The figures combine several stages of the cell sorting process of the different fecal samples, starting from in-solution FISH with 'polyDIII', over flow cytometric analysis and the final sorting of 10^6 cells out of the gated target population (P3).

Epifluorescence microscopy of in-solution FISH applying the anaerobically precultivated INF sample (see Table 7, p. 25) revealed that there was a change of morphology of the coccoid samples (Figure 74A), already assessed in Figure 43C. To exclude contamination during cultivation, FCM analysis and cell sorting of two visible populations was adduced (Pop1, Pop2). Cells in region Pop2 (Figure 74B) showed almost the same signal intensities, but according to their SSC level ($>10^4$) the population differed from Pop1 and corresponded to the detection of enlarged-appearing cells in the FISH image (Figure 74A). The peaks in the P2 section of the histogram referred to the populations Pop1 and Pop2 (Figure 74C). Epifluorescence microscopy for purity analysis after sorting (see C.19, p. 43) revealed that two morphologically differing cell shapes were separated that were both 'polyDIII'-labeled. As the sort region Pop2 was chosen spontaneously by monitoring the analysis, smaller cells were also noticeable in the Pop2 micrograph (E).

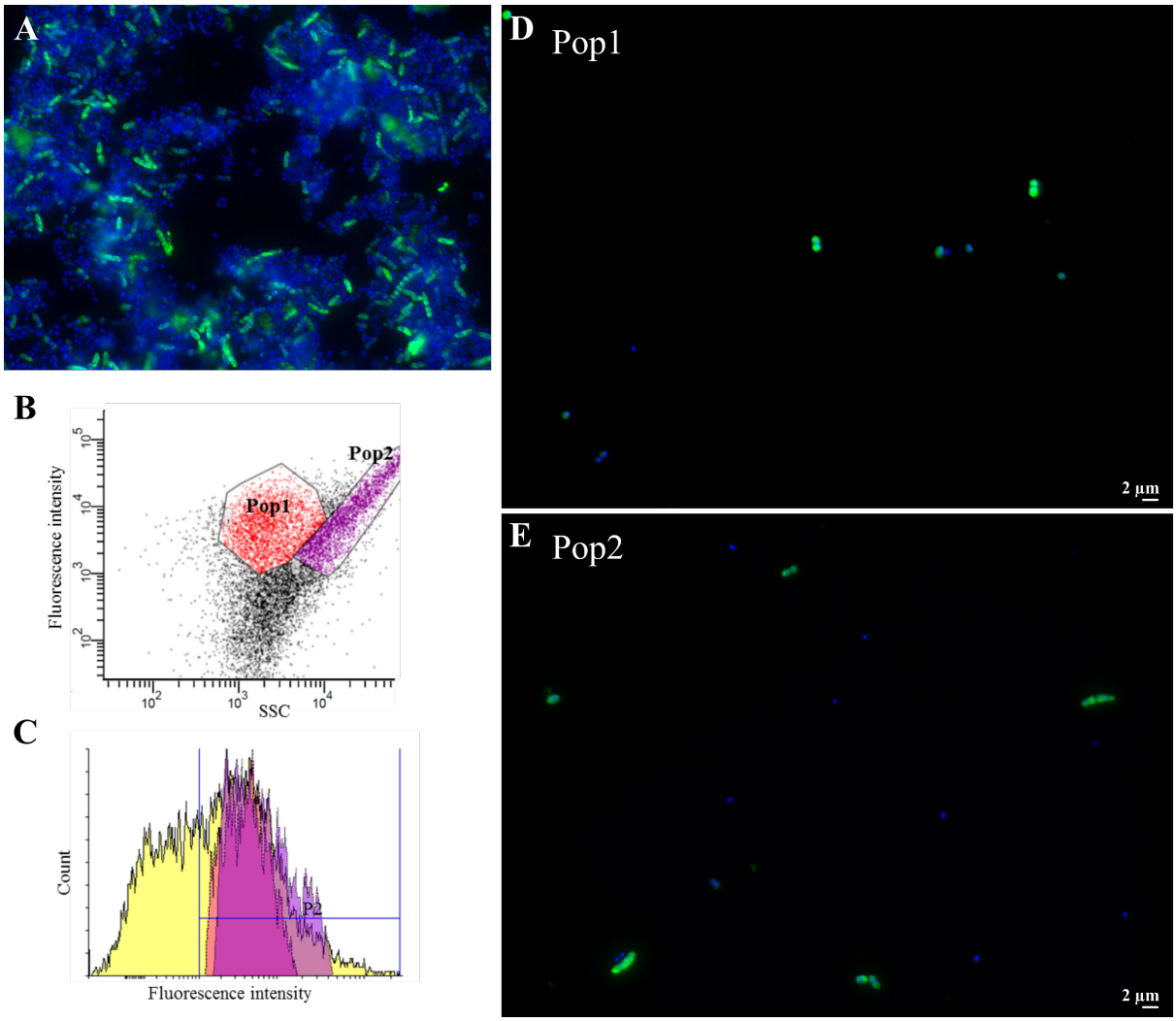


Figure 74. Complete analysis of a taxon-specific cell enrichment by FACS, applied to anaerobically precultivated fecal INF samples. Overview for the taxon-specific cell sorting procedure, applying an anaerobically precultivated fecal INF sample *in situ* hybridized with 'polyDIII' (see C.14.2.2). Pop1 and Pop2 are the gates defined for sorting. **A:** in-solution FISH image of fecal matter (precultured INF). **B:** FACS analysis (dot plot) with two sort regions (Pop1, Pop2). **C:** FACS histogram (orange: Pop1, purple: Pop2). **D:** cell sorting verification of Pop1 by microscopy. **E:** cell sorting verification of Pop2 by microscopy (FLUOS: green, DAPI: blue). Section P2 represent the total of positively 'polyDIII'-labeled cells, whereby gated cells appeared as green peaks.

Figure 75 shows the analysis overview of an anaerobically precultivated SPF feces sample applied to the developed cell sorting process. The dot plot analysis and the histogram (Figure 75A and B) approved the FISH image (Figure 75C). It shows positively 'polyDIII'-hybridized cells with different morphologies, ranging from single coccoid cells to diplococci and slightly elongated-appearing cocci. The dot plot revealed the enterococci-characteristic population (P3), but also populations with stronger signal intensities (>10^3). The histogram shows two peaks, with one referred to negative or non-target cells (left). The second peak (right) ranging from signal intensities of 10^2–10^4, indicated same level counts of events showing increasing signal intensities, whereas only events over 10^3 were counted as positive. Sorting of the region

P3 yielded in detection of single cells and diplococci, positively hybridized with ‘polyDIII’ (Figure 75D).

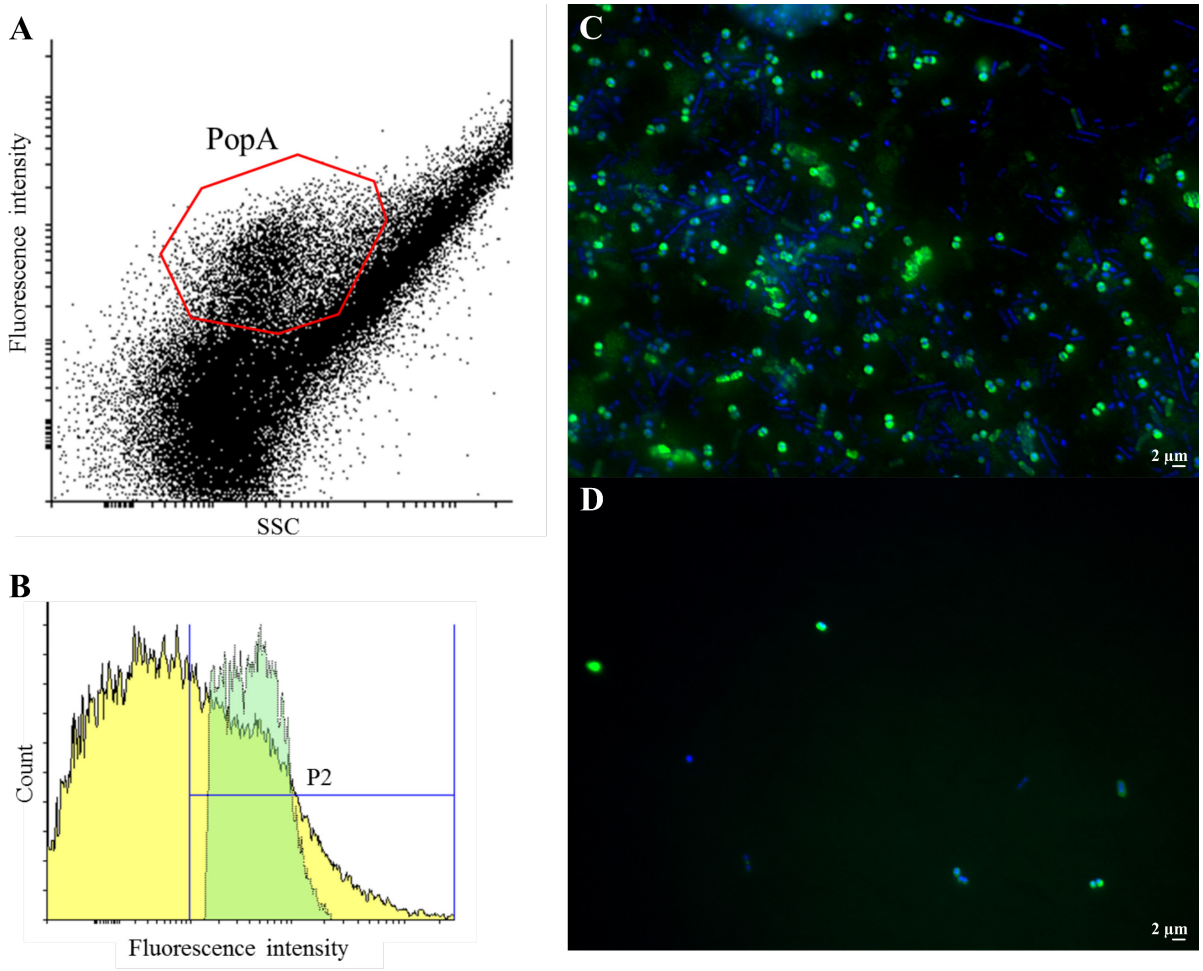


Figure 75. Complete analysis of a taxon-specific cell enrichment by FACS, applied to anaerobically precultivated fecal SPF samples. Overview for the taxon-specific cell sorting procedure, applying an anaerobically precultivated fecal SPF sample hybridized with ‘polyDIII’. **A:** FACS analysis (dot plot). **B:** FACS analysis (histogram). **C:** in-solution FISH imaging. **D:** cell sorting verification of P3 by microscopy (FLUOS: green, DAPI: blue). Section P2 represent the total of positively ‘polyDIII’-labeled cells, whereby gated cells appeared as green peaks. P3 was the gate defined for sorting.

4.3. PROCESSING OF SORTED CELLS AND GENE AMPLIFICATION

By PCR amplification of DNA from sorted cells, it was shown that they were still accessible to molecular analysis. Sorted bacterial cell samples (approx. 10^6 cells) were first analyzed for purity by microscopy (see Figure 74D–E and Figure 75D). After lysing of the sorted cells using a direct lysis reagent (see C.18, p. 43), the samples were applied to bacterial 16S rRNA and *tuf* gene amplification by PCR (for primer sets see Appendix, Table 18, p. XI−XII). Figure 76 shows the positive amplification results after gel electrophoresis for both gene fragments from all sorted target populations. PCR samples shown here corresponded to fecal samples in Figure 73. The DNA fragments showed the same size detected with the positive control (PC), whereas

no amplification was detectable in the no-template control (NTC). To verify that cells were sorted *Enterococcus*-specifically and that PCR amplification products were derived from target cells, the amplicons have to be applied to sequencing analysis (*e.g.*, by construction of a gene library).

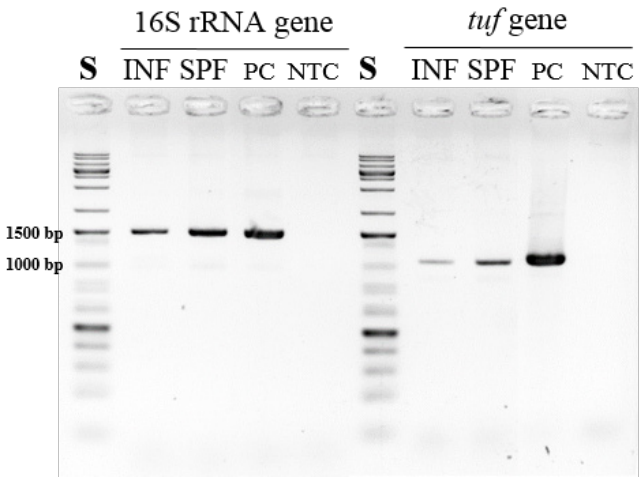


Figure 76. Accessibility to genetic information from sorted cells by FCM. Gel electrophoretic analysis of the 16S rRNA gene (primer: 616Valt/100K) and the *tuf* gene amplification (primer: eftu_v_0904/ eftu_r_0904). PCR after fluorescence-activated cell sorting and sample preparation (see C.18) verified the size of correct amplified fragments at 1,500 bp (16S rRNA gene) and 1,100 bp (*tuf* gene). **INF:** flow cytometric sorted cells of anaerobically precultivated INF feces. **SPF:** flow cytometric sorted cells of aerobically precultivated SPF feces. **PC:** positive control (extracted DNA from *E. faecalis*). **NTC:** no-template control. **S:** GeneRuler™ 1 kb Plus DNA ladder.

4.4. GENE LIBRARY

To validate the sorting specificity, the 16S rRNA gene (*E. coli* position 8–1511 and 473–1276) of the defined populations was PCR-amplified using bacterial primers and then inserted into vectors to construct a gene library (see C.20, p. 44). Beside this, clone libraries for several functional genes coding for, *e.g.* the elongation factor Tu (*tuf*), recombinase A (*recA*), heat shock protein 60kD (*hsp60*), RNA polymerase (*rpoA*) or ATP synthase (*atpB*), were created. Randomly chosen plasmids with correct fragment insertion were sequenced and the readout was performed by the e-seq software (LI-COR Biosciences, USA). As the available time for this experimental setting was limited and Sanger sequencing including sequence analysis is quite laborious, only a selection of successfully analyzed sequences is shown here. The obtained sequences were further analyzed using the BLAST database ([146], http://blast.ncbi.nlm.nih.gov/Blast.cgi). As a proof of principle, results of two sorted fecal samples are shown in Figure 77 and Figure 78.

Figure 77A shows the dot plot of an anaerobically precultivated INF feces sample from which only one region (Pop1) was sorted and used for gene amplification. According to the *rpoA* sequence 100% of the matches belonged to the species *E. faecalis*. The 16S rDNA sequences additionally matched to sequences of uncultivated bacteria, phylogenetically related to enterococci.

Figure 78A shows the analysis of an aerobically precultivated SPF feces sample, from which three different regions were sorted (Pop1, Pop2 and Pop3). Regions were chosen according their signal intensities ($>10^3$) and side scatter measurement (SSC) (see Figure 74, p. 115), whereby Pop2 corresponded to the region of Pop1 with a narrower selection. From these sorted regions, mentioned clone libraries were constructed. BLAST results were visualized in a column chart. Using this, it was possible to compare the percentage that each match was contributing to a total. Analysis of population Pop1 (Figure 77B) revealed that all obtained sequences of amplified genes indicated classification to the species *E. faecalis*, with one exception received by using the short fragment of the *Enterococcus*-specific 16S rDNA (16S-Enc). Additionally, it revealed a match to a sequence of an uncultured bacterium, phylogenetically related to enterococci. Further analysis of population Pop2 (Figure 77C) showed matches to the species *E. faecium* and also to enterococci other than *E. faecalis* and *E. faecium*. The chart of population Pop3 (Figure 77D) showed that no *E. faecium* matches could be detected, but matches for *E. faecalis*, uncultivated bacteria or other enterococcal species. In summary, the short sequence obtained by amplification of the *Enterococcus*-specific fragment (16S-Enc) showed the greatest variance in results.

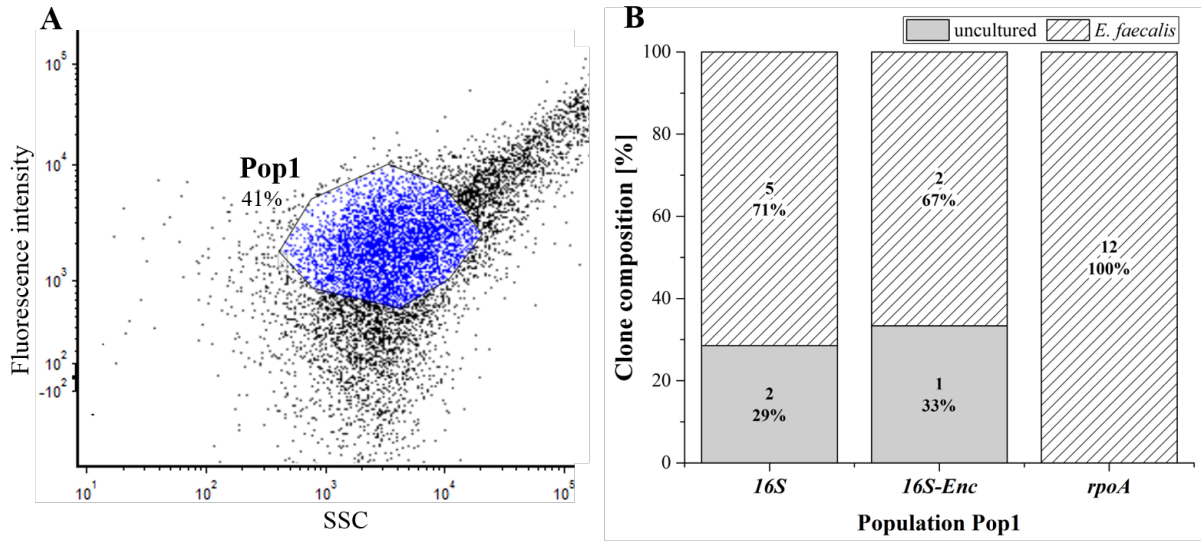


Figure 77. Validation of sorting specificity from INF feces sample. Analysis of FCM and constructed clone library of an anaerobically precultivated INF feces sample. **A:** FACS dot plot analysis illustrating the sorting region (Pop1) created by FCS Express 5 software (De Novo Software, USA). **B:** BLAST analysis of Pop1 using gene amplification sequences of complete 16S rDNA (16S), *Enterococcus*-specific 16S rDNA (16S-Enc) and of RNA polymerase α (*rpoA*) fragments. Primers are listed in Appendix, p. XI–XII.

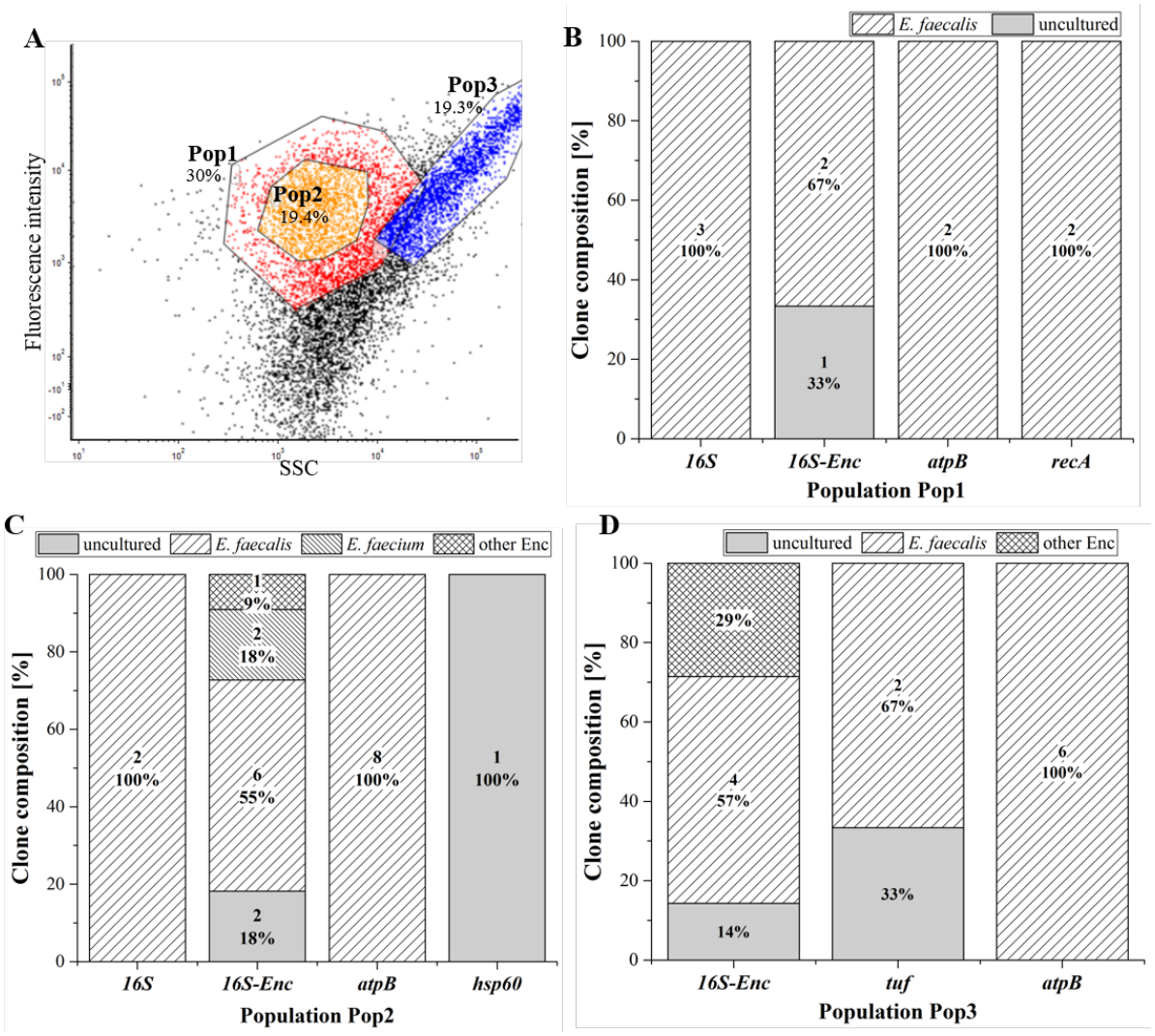


Figure 78. Validation of sorting specificity from SPF feces sample. Analysis of FCM and constructed clone library of an aerobically precultivated SPF feces sample. **A:** FACS dot plot analysis illustrating three different sorting regions (Pop1, Pop2 and Pop3) created by FCS Express 5 software (De Novo Software, USA). **B:** BLAST analysis of Pop1 using gene amplification sequences of complete 16S rDNA (16S), *Enterococcus*-specific 16S rDNA (16S-Enc), ATP synthase (*atpB*) and of recombinase A (*recA*) fragments. **C:** BLAST analysis of Pop2 using gene amplification sequences of complete 16S rDNA, *Enterococcus*-specific 16S rDNA (16S-Enc), ATP synthase (*atpB*) and of heat shock protein 60kD (*hsp60*) fragments. **D:** BLAST analysis of Pop3 using gene amplification sequences of *Enterococcus*-specific 16S rDNA (16S-Enc), *tuf* gene, and of ATP synthase (*atpB*) fragments. Primers are listed in Appendix, p. XI–XII.

E. DISCUSSION

The gut microbiota play an important role for human physiology, metabolism, immune function and nutrition. Elucidating the composition and genetics of the gut microbiota under certain conditions is essential to understand how these microbes function individually or in a specific community. For certain scientific questions it would suffice to examine in a targeted manner only a taxon-specific sub-metagenome, *e.g.* the genus *Enterococcus*. Also, in-depth analysis of certain subpopulations could offer new progressions in, *e.g.*, therapeutic developments.

The present study sought to improve and establish nucleic acid based methods for targeted enrichment of the metagenomes of the taxon *Enterococcus* from complex gut consortia. This should provide access to the genetic information of microorganisms without the need of their cultivation. The development of the method focused the application of polynucleotide probes targeting domain III of the 23S rRNA gene instead of commonly used oligonucleotide probes [96, 97, 171, 172], and their adaption to fecal environment. In this context, and as a proof of the principle various mouse feces samples were used for adaption.

1. DIAGNOSTIC SYSTEMS FOR *ENTEROCOCCUS* SPP. IN FECAL SAMPLES

As enterococci constitute a rather small portion of the microbes in human and mouse feces (10^5–10^7 CFU/g) [131], it is preferable to consider the adequate starting volume before the specific enrichment. Besides, determining the presence and quantity of enterococci occurring in each fecal sample was also important to exclude useless samples. These aspects were addressed by development of a standard PCR technique specifically targeting the 16S rRNA gene from the genus *Enterococcus* and a qPCR system targeting the 23S rRNA gene or *tufA* from *Enterococcus* spp. These target regions are commonly utilized in standard PCR or qPCR methods for the species-specific [173, 174] or genus-specific [159, 160, 175] detection of *Enterococcus*. Until now, almost all existing approaches are dependent on the previous extraction of template DNA. Their implementation is laborious, time-consuming and bears a risk of contamination [176]. Therefore, all diagnostic tools established in this study were developed as direct-PCR for detection of *Enterococcus* spp. in fecal samples without previous DNA extraction. Cell recovery or DNA extraction steps were eliminated and the prepared fecal samples could be directly used as templates in the PCR.

As the optimization of the conditions was critical for direct-PCR, the standard PCR detection system based on the 16S rRNA was first applied to pure cultures to adjust the protocol, maximize the sensitivity and determine the specificity of the primers (Figure 2–Figure 5, p. 46–49). The method was proven to be highly specific to all available *Enterococcus* strains. Even closely related species, including streptococci and tetragenococci were shown to be excluded by using the designed primers. The presence of various substances such as complex polysaccharides [177, 178], and the difficulty of lysing some bacteria [21] usually make fecal samples a challenging starting material. The detection limit of the *Enterococcus*-specific PCR as determined with the primers designed in this thesis was approximately 5 CFU/µL for the pure culture and approx. 18–47 CFU/µL for the spiked mouse feces samples (Figure 6 and Figure 7, p. 49). The fecal sample that was only 10-fold diluted before spiking showed a decreased detection range, which might occur from inhibitory effects of fecal material. Summarizing, these results agree to prior investigations that direct-PCR can be applied to fecal samples while several dilutions should be performed to avoid false-negative results. Indeed, a sufficiently high concentration of the target taxon is obligatory considering the detection limit [176, 179, 180]. While it has been shown that washing fecal samples with PBS can minimize the inhibitory effect of remaining contaminants on PCR [181], in this study this step was optional. Applied to mouse feces samples (see C.12, Table 7) the *Enterococcus*-specific PCR approach was proven to be directly applicable to environmental samples (germ-free: Figure 6; SPF and INF: Figure 8). Consequently, this DNA extraction free method was suggested as a possible application for a rapid check for contamination of germ-free laboratory mice, and as stated by Fode-Vaughan *et al.* [176], this technique might also be used to obtain PCR products for cloning, sequencing, or other analytical procedures to allow qualitative assessment of microbial community diversity.

Initially intended for quantification of microplate-enriched cells (see D.3.2.3, p. 98), a quantitative PCR system for *Enterococcus* spp. was established in this work. As the immobilization technique did not yield the desired success, the qPCR system was further improved to examine whether the amount of target cells in selected original fecal samples was sufficient for the application to flow cytometry. The quantitative PCR system for *Enterococcus* spp. established in this work (see D.1.3, p. 57) was first developed targeting the 23S rRNA gene from enterococci based on already published primers [159], slightly modified according to the latest ARB database (see C.16.2.1, p. 39) [145]. Because the number of copies of rRNA genes per genome varies among enterococci from 4 to 6, additionally another target was evaluated. The *tuf* gene from enterococci is a single- or two-copy gene (*tufA* and *tufB*, whereas all enterococci own at least *tufA*), and was already found to be more suitable for precise bacterial quantification by

qPCR than targeting of the 23S rRNA gene [182–184]. It codes for elongation factor Tu which is involved in protein biosynthesis [185]. Among multiple housekeeping genes, including *gyrA*, *gyrB*, *recA*, *rpoB*, *dnaK* and others, the *tuf* gene is highly conserved and ubiquitously distributed in bacteria, but was recently described to have a faster rate of evolutionary change than 16S rRNA genes [186, 187]. Consequently, this led to the evolution of certain regions in the *tuf* genes of bacterial species belonging to the same genus that can be used for diagnostic purpose. In this context, housekeeping genes such as the *tuf* gene were already shown to be effective bacterial phylogenetic markers [160, 188–190, 183].

Like the established *Enterococcus*-specific direct-PCR approach mentioned above (p. 122), also both quantitative pretests developed for this project (23S rRNA- and *tuf*-based) could be directly applied to pure cultures, fecal samples and even to 'polyDIII'-hybridized/sorted cells (Figure 23–Figure 25, pp. 65–66). Thereby, these qPCR approaches were useful to reduce the operational time for future cell sorting experiments by excluding samples that contained too little or no levels of target species. A copynumber of approx. 10^5 per g unprepared feces was shown to be the lower limit for successful sorting, whereby it should be considered that in this study only a small fraction (approx. 0.007 g feces per hybridization approach) was transferred to in-solution hybridization. As *tuf* from *E. faecalis* is a single-copy gene (*tufA*), and its number of copies of rRNA genes is four, quantification was previously found to be more suitable (see E.1, p. 122) [183, 184]. Nevertheless, by targeting the 23S rDNA more precise quantification for a known cell suspension could be achieved (see Figure 21, p. 63). In this context, it was suggested that for both systems an accurate quantification could not be obtained. Still, the *tuf*-based system was the application of choice as values achieved from the 23S rDNA-based systems were suggested to be over-estimated. By calculating the detected copies per gram feces, SPF mice revealed 2.3×10^9 copies/g, while INF mice had 1.6×10^{11} copies/g in a *tuf*-based qPCR approach (see Figure 23, p. 65). However, published data showed that mice with a normal gut flora approx. own 10^5 enterococci/g feces [191], and cell concentration of about 10^8–10^9 CFU/g were measured in feces of *E. faecalis* infected mice, which in turn might be depended on the experimental setting and were not precisely known for the mice in this project [192]. The loss of accuracy in quantification values might have had various reasons including failures in measurement of standard aliquot concentrations, or in calculated cell concentrations based on optical densities (OD_{600}). Nevertheless, even taking this into account, the qPCR system could be successfully used for relative quantification among different samples (*e.g.*, Figure 24, p. 66).

Although the qPCR systems mostly complied with published MIQE guidelines ('Minimum information for the publication of real-time quantitative PCR experiments') [193], the chosen primer sets targeting the 23S rRNA gene or *tufA* were not exclusively specific for the genus *Enterococcus*. A weak amplification of respective genes of the closely related genus *Streptococcus* was visible in gel electrophoresis of PCR products (Figure 16 and Figure 17, p. 58), although *in silico* validation of the primers by ARB and TestPrime 1.0 (http://www.arb-silva.de) did not reveal matches (with two allowed MM) to sequences of streptococci, which could indicate a contamination of streptococcal DNA. In the contrary case, by comparative *tuf*-based qPCR approaches using target and non-target DNA, it could be shown that *tufA* targeting primers had a very strong sensitivity for enterococcal DNA (Figure 18, p. 59). Low streptococcal DNA concentrations were only barely detected, unlike the same concentrations of *E. faecalis* DNA. Therefore, and because in this study a highly precise quantification of the developed qPCR systems was neither obtained nor required, the high sensitivity and slightly reduced specificity (signal caused by streptococci) are suggested to be sufficient for the specific quantification of *Enterococcus* spp. in fecal samples.

Both standard curves were linear in the range tested (Figure 19, p. 61, correlation coefficient R^2 >0.999). In addition, various reaction parameters (*e.g.*, slope, efficiency, y-intercept, melting curve) could be derived (see C.16.2.2, Table 11) which enable reliable and comparable interpretation of the results [162]. The reproducibility was excellent across all inter-run measurements (Figure 22, p. 63) and the limit of detection was 10–100 cells/µL for both systems. The Cq values obtained by the 23S rRNA gene targeting system were approx. 2.1 times higher than the values obtained from the *tufA* targeting system (Figure 21, p. 63). This could be explained by the higher copy number of rRNA genes in cells (up to 4–6 copies in enterococci [184]) and their potential to increase the detection sensitivity compared to single or two-copy genomic sequences, as described for the *tuf* gene [188, 194, 195]. Hence, rRNA gene targeting detection systems are useful to also quantify species with low-abundance, but when interpreting quantification results it has to be considered that the rDNA abundance does not equal cell abundance due to the multi-copy nature of the rRNA genes [195]. Samples with a high concentration of target bacteria had to be diluted to avoid reaching an upper detection limit, which was proposed to be at a concentration of approx. 1×10^8 copies/µL (Figure 23, p. 65).

In addition to the mentioned PCR based detection systems, the sequence of the enterococcal 16S rRNA gene targeting forward primer Enc473 was used to develop a genus-specific oligoFISH technique (see D.1.2, p. 51). This oligonucleotide probe was primarily developed as an alternative probe to the polynucleotide probe 'polyDIII' (see Figure 15, p. 57), discussed in

the following chapter E.2. It was proven that the Enc473-based oligonucleotide probe was highly specific for enterococci in various sample types, but when applied to fecal samples, it produces strong background noise probably by adsorption to fecal matter (Figure 60, p. 102). This problem could be reduced by using a newly developed fluorescent dye (ATTO565). This problem was already known for oligonucleotides applied to environmental samples [112], therefore for the application in future cell sorting experiments by FCM, the probe was only conceivable as an additional probe besides the polynucleotide probe 'polyDIII' to further increase its detection sensitivity (Figure 42, p. 86).

2. POLYNUCLEOTIDE PROBE 'POLYDIII'

Polynucleotide probes targeting domain III of the 23S rRNA gene were already investigated in detail in various previous studies [115, 120, 141, 166]. The probe's specificity can be triggered by usage of target organism DNA for generation via *in vitro* transcription and by changing the stringency of subsequent *in situ* hybridizations. In this project, the objective was to adjust its specificity to target the intestinal subpopulation of the genus *Enterococcus*.

2.1. TARGET ORGANISM

The genus *Enterococcus* was chosen as the organism of choice in this study because enterococci are ubiquitous in the gastrointestinal tracts of humans, animals, and insects, and the genus includes some of the most important nosocomial multidrug-resistant organisms that cause urinary tract infections and bloodstream infections [6, 132–134]. The colonization mechanism in healthy or hospitalized individuals used by this Gram-positive bacterium is not well examined. However, the rise of antibiotic resistant enterococcal strains is suggested to be influenced by the exposure of patients to antibiotics and the resulting change in the gut microbiota composition, which facilitates the colonization by drug-resistant enterococci [196, 197]. Interestingly, they only constitute a sub-dominant portion of the gut microbiota with less than 1% of the adult gut consortium, and approx. 10^5–10^7 CFU/g feces [23, 130–132]. Among the 49 published species of enterococci (1984–2015, *List of Prokaryotic names with Standing in Nomenclature*, http://www.bacterio.net/enterococcus.html), *E. faecalis*, *E. faecium, E. hirae,* and *E. durans* are the most commonly encountered enterococcal species in the human gastrointestinal tract. Additionally, *E. caccae*, *E. avium*, and *E. gallinarum* have been isolated from human feces, but their distribution appears to be low. Nevertheless, infection-associated species (*E. faecalis,*

E. faecium, E. *avium, E. casseliflavus, E. cecorum, E. dispar, E. durans, E. gallinarum, E. gilvus, E. hirae, E. mundtii, E. pallens,* and *E. raffinosus*) are rarely part of the human intestine but occur in the gut of animals or have plant origin [6, 139, 198, 199].

In this study, the focus on enterococci included the infection-associated species with intestinal origin. But if applied to real human feces samples, it might be sufficient to limit probe specificity to *E. faecalis*, *E. faecium, E. hirae*, *E. durans, E. caccae*, *E. avium,* and *E. gallinarum*.

2.2. TARGET MOLECULE

Domain III of the 23S rRNA gene as the target of the polynucleotide probe was chosen according to previous studies of our group [115, 120, 141, 166]. Even if the 23S rRNA gene is suggested to be a more informative phylogenetic marker than the 16S rRNA gene [45, 200], its drawback is that it is still not as widely used and corresponding sequence databases are underrepresented [201]. Concerning the 16S rRNA gene, Stackebrandt and Ebers [169] proposed that a sequence similarity below 98.7% of two bacterial strains is sufficient to consider them belonging to different species. Strains with similarities above 95% were suggested to refer to the same genus [46]. Although it was recently suggested not to generalize these values to all genera [202], the percentages could be confirmed for the comparative analysis of streptococci and enterococci (Figure 35, p. 77). According to the domain III of the 23S rRNA gene as a target, especially the discrimination of the closely related species *Enterococcus* and *Streptococcus* was shown to be challenging. The cut-off benchmark of 70% to 76% sequence similarities, previously defined by Fichtl [120], could be confirmed for enterococci and streptococci, and visualized in a heat map using the latest ARB databases (Figure 36, p. 77).

Indeed, in this study it could be demonstrated that even a species-level discrimination was possible with the short 'DIII' sequence, although when obtained from environmental samples (Figure 38, p. 80). Moreover, referring to the heatmaps of the complete 16S rRNA gene and the DIII sequences (23S rDNA) of enterococci and streptococci (Figure 35 and Figure 36, p. 77), probe specificity did not rely on total gene sequence similarity, but rather on sequence differences in a part of a gene, here domain III (DIII) of the 23S rRNA gene [166, 203]. Consequently, to target all enterococci while excluding the streptococci depended on a proper probe validation.

Trebesius *et al.* [117] already found that probe sensitivity and specificity for the discrimination at genus- or species level are both dependent on the stringency of the *in situ* hybridization conditions. To overcome challenges in this project caused by occurring sequence similarities

between different genera, stringency of hybridization conditions was completely adjusted to the genus *Enterococcus,* allowing differentiation from the streptococci (Figure 34, p. 75). In this context, the need of individual pretreatment and hybridization for each target organism was previously described, as between different genera of Gram-positive bacteria the conditions of accessibility of target structures varied considerably [120].

Another aim of probe triggering was to obtain the characteristic signal amplification, previously explained by Zwirglmaier *et al.* [114] as the 'network hypothesis' (see A.2.3.2, p. 7). Under certain conditions during FISH, the 'polyDIII' probes partially remain outside the cell's periphery and form inter-probe networks that can be visualized as strongly fluorescent "halos" by fluorescence microscopy (see A.2.3.2, p. 7). In comparison to applications using oligonucleotide probes which often cause strong background noise specifically in environmental samples [204, 205], the increased signal intensity of polynucleotide probes targeting the DIII region of the 23S rRNA gene allowed for a clear differentiation of target cells above background (Figure 60, p. 102).

Minor or greater modifications in the duration of the particular pretreatment or hybridization procedure achieved the optimal FISH signal results for enterococci (see Table 14, p. 75). This required a precise evaluation, as for example overpermeabilized cells might lead to increased influx of probes, and formation of an interior network which leads to a whole-cell fluorescence signal [114]. Optimal pretreatment and hybridization conditions were first assessed using pure culture of *Enterococcus* spp. including various control species, harvesting them at the mid-exponential growth phase for maximum ribosome content, and applying them to fixation with 4% paraformaldehyde (PFA). Fixation by PFA stabilizes the cell morphology, structure and surface characteristics of the cell [206], but it was shown that it often causes cell agglutination because of slow action of the fixatives, or that it led to increased autofluorescence [172, 207]. Nevertheless, in this study PFA fixation was preferred to, *e.g.* ethanol fixation, as it was shown to reveal the best FISH results in fecal samples, and that cells still could be processed for *in situ* PCR (Figure 27, p. 68). DeLong *et al.* [109] proposed a lysozyme pretreatment of the cell samples because probes that are a few hundred nucleotides in length cannot efficiently enter fixed cells. For the genus *Enterococcus*, a pretreatment of 10 min on ice was sufficient for probe uptake (see D.2.2.1, p. 69). Former studies showed that an additional heat step at 200°C further improved uptake of the polynucleotide probe [120, 141]. In this study this step was kept optional and was only applied for experiments on slides, while it was not suitable for in-solution hybridization.

Factors that mainly influence the stringency of *in situ* hybridization are the temperature and the salt concentration in the buffer. Sodium ions interact electrostatically with nucleic acids so that higher salt concentrations increase the stability of the hybrid [208]. DNA hybridization only succeeds by prolonged incubation at temperatures just below their melting point which can range from 65°C up to 75°C. As this may seriously compromise the cells' morphology or content, the organic solvent formamide is used to reduce the thermal stability (T_m) of DNA (-0.72 °C per percent formamide). The formamide concentration is dependent on the used probe as it influences the stringency, meaning the complementarity between the probe and the DNA strand [209]. When applying the 'polyDIII' polynucleotide probe, specific for *Enterococcus* spp., a concentration of 80% formamide was required to elicit the halo network effect (see D.2.2.2, p. 70).

Besides halo formation, another challenge was to adapt the hybridization conditions to capture all members of the genus *Enterococcus*, or more precisely the relevant intestinal strains, with only one probe. Therefore, 'polyDIII' probes were generated using varied DNA templates from relevant enterococcal species. By comparing all probe origins and applied enterococci target cells in a FISH result matrix (see Table 15, p. 82), the repetitive hybridization results varied. This means, that no species or its associated probe captured all target cells even though sequence similarities of all tested strains were in a range of 85.5%–93.4% (Figure 37, p. 78). The construction of an ARB tree was used as an alternative to the DIII sequence matrix (Figure 79). Using the sequences of domain III of the 23S rRNA genes from all enterococci type strains allowed the analysis of probe-strain relationships reflected by the branch lengths. Theoretically, the distance between strains of corresponding probes which most frequently provoke positive halo signals should be short, as their sequences ought to be similar. Following this hypothesis, the branch length (distance) should get greater, the less frequently a probe of an associated strain resulted in halo formation. This assumption could not be verified here as, *e.g.* probes generated from *E. gilvus* and *E. raffinosus* DNA shared higher sequence similarity (96.7%), but still the respective hybridization results differed from each other in the formation of halo signals (Table 16, p. 83). In contrast, the probes originating from the strains *E. dispar*, *E. hirae*, *E. gilvus* and *E. caccae* often revealed halo signals, but their sequence distances between each other are comparatively high (<90.9% sequence similarity).

Figure 79. ARB tree, built from sequences of domain III of the 23S rRNA genes from all enterococcal type strains. The tree was calculated by the neighbor-joining method, where the sum of the branch lengths defines the phylogenetic distance between the strains (see C.11.1, p. 25).

The FISH results combined with the distance matrix, were used to identify a cutoff benchmark distinguishing between positive or negative FISH results, but they didn't reveal any correlation between sequence similarities and the yield of positive halo signals (Table 16, p. 83). Regardless of whether FISH approaches resulted positive halo signals or not, the maximum sequence similarity of tested species-probe combination was 100% (Table 17, p. 83). This means, it was conspicuous that the spans of sequence similarities associated with positive or negative FISH results were overlapping. Furthermore, *E. avium, E. dispar, E. pallens*, and *E. caccae* didn't show any positive *in situ* result with the probe generated using their extracted DNA, although it possessed 100% sequence similarity. In contrast, positive halo results for these species could yet be obtained using probes with less sequence similarities. Consequently and because sequence differences of the enterococcal domain III are solely caused by single nucleotide polymorphisms, the ambiguities in FISH results may be explained by physical aspects (*e.g.*, tem-

perature of hybridization ovens), small changes in the hybridization procedure (*e.g.*, pretreatment, salt/buffer concentration) [120], the physical status of fixed cells (*e.g.*, growth status, fixation damage), or probe differences caused by *in vitro* transcription.

Finally, to overcome possible capture gaps, a mixture of three enterococcal 'polyDIII' probes was chosen for taxon-specific *in situ* hybridization experiments. These probes were generated by using the DNA from the most common FISH-positive *Enterococcus* species (*E. faecium*, *E. hirae* and *E. gilvus*, see Table 15, p. 82) as templates for the *in vitro* transcription (see C.4, p. 16). Consequently, this ensured the coverage of all relevant intestinal genus members. Interestingly, two of the species belonged to the most abundant enterococcal species in GI tract (see E.2.1, p. 125).

2.3. ENVIRONMENTAL SAMPLES

In general, polynucleotide probes have suitable properties for environmental samples where the detection sensitivity is limited and specificity level is not too high. Here, the optimization of the FISH protocol was performed to achieve increased signal intensities of halo-forming polynucleotide probe 'polyDIII' specifically for the genus *Enterococcus* in complex samples such as feces. For the envisaged cell enrichment application, the FISH protocol was optimized for in-solution hybridization. Consequently, neither did this protocol require the solid surface (*e.g.*, slide or filter), nor was it required to resuspend cells which are removed from a solid surface, which was in contrast necessary in other published methods [101, 210] (see C.14.2.2, p. 33). To examine the applicability of 'polyDIII' probes and respective enrichment techniques, *E. faecalis*-spiked mouse feces were used first. With respect to the most important probe parameter, the characteristic halo signal, the evaluated results obtained from spiked fecal samples established the basis for hybridizations with real mouse feces samples. This protocol was also intended to be used for human fecal samples in future.

Feces, like all environmental inhomogeneous samples, constitute a challenging material for hybridization analysis caused by difficulties related to autofluorescent organic debris and compound aggregation [207, 211]. Thus, a critical step beside sampling was the sample preparation (see C.12, p. 25). This step had to be suitable for subsequent hybridization and enrichment or FCM approaches. Particles that might cause clogging of the cytometer had to be completely removed (Figure 39, p. 84). As sample preparation came along with cell loss, all samples were tested qualitatively and quantitatively (see E.1, p. 121) to verify applicability in enrichment techniques.

While evaluating enrichment methods, the fact that the genus *Enterococcus* belongs to the part of the intestinal microbiota which is underrepresented turned out to be a limitation (see E.2.1, p. 125). Target cells in original SPF samples ('specific-pathogen-free' mouse, see C.12, Table 7) were poorly detected (Figure 40B, p. 85 and Figure 67, p. 108). In original INF mouse feces samples ('*E. faecalis* infected mouse', see C.12, Table 7) it was shown that many cells did not reveal any FISH signal, even though it could be assumed that the population only consisted of *E. faecalis* (Figure 40A, p. 85). This disadvantage was probably adversely affected by application of not well-suitable fecal sample material. Due to collection time (while cage cleaning) and long transfer passages with storage between 4°C and –20°C, the DNA of such bacterial composition was assumed to be degraded, or microbial composition might be affected by the age of the sample (nutritional limitation) [212]. Cell conditions or inhomogeneous probe uptake, a problem which is observed in complex environmental samples [111], could also play a role in incomplete hybridization. Furthermore, a reduction of cell size was found in fecal material compared to cells cultured in rich media (Figure 39 and Figure 41, pp. 84–85). This change is commonly found in VBNC cells [213], probably as a strategy to minimize energy requirements [214]. Nevertheless, a detection increase of the low-abundant enterococci might also be encouraged by larger sample volumes, while ~0.2 g might not be sufficient for that purpose.

According to the high concentration of *E. faecalis* cells in INF fecal matter, a reason for inhomogeneous signaling could also be that cells compete for a limited amount of probe. The incomplete network of probes around the target cells hence might also contribute this effect. Additionally, insufficient homogenization of the fecal matter and therefore aggregation of cells could inhibit probe uptake [207]. Consequently, for this type of sample higher probe concentrations might have been an improved effect. Additionally, to preserve sample diversity, integrity and morphology, and to allow good probe penetration, it is recommended to use fresh samples and directly transfer them to fixation immediately after collection. It was shown that the composition of the fecal microflora still is usable for FISH analysis, even after 8 months [212].

Given that the access to a fresh and a greater amount of fecal material was limited and batching was difficult, fecal samples were precultured in rich medium to enhance the underrepresented target population in fecal samples under nutritionally well-supplied conditions (Figure 43 and Figure 44, pp. 88–89). The results gained by precultivation of fecal matter from INF (*E. faecalis* infected GF mice) and SPF ('specific-pathogen-free' mice) mice differed depending on mode of incubation. Both aerobically precultivated samples showed cell growth. As it was expected

from the sample's origin, phase contrast microscopy of INF samples only showed coccoid cells (Figure 43B, phase contrast), while SPF feces samples revealed the occurrence of bacteria with different morphology, *i.e.* coccoid and rod-shaped (Figure 44B, phase contrast). However, the FISH results of the aerobically incubated samples for both feces types were more or less negative (Figure 43B and Figure 44B) indicating that no 'polyDIII' probe could be hybridized. As adaptive difficulties of the intestinal enterococci concerning aerobe conditions were excluded (growth could be observed in liquid INF culture samples), this can only be explained by problems in the hybridization performance or probe quality.

Concerning the anaerobically precultivated samples, the appearance of cells that looked like elongated diplococci could be observed, whereby INF samples were more affected (Figure 43C and Figure 44C, FISH image). Similar pleomorphisms have been already reported by Zimmermann *et al.* [215], who explained the phenomenon by nutritional situations in the environment. Changes in morphology for enterococci, *i.e.* coccobacillary shape, under certain conditions were also described by Facklam *et al.* [216] and Signoretto *et al.* [217] and could be classified in 'phenotypic heterogeneity' [218, 219]. Alternatively, phenomena like this were explained by fixation procedure incidents [207]. Contamination could be excluded as cells were positively 'polyDIII'-labeled (see Figure 74 and Figure 75, p. 115–116) and the sorted populations were verified by diagnostic PCR and sequencing (see chapter D.4.4, p. 117).

Independent of the cell shape differences (confirmed by phase contrast microscopy), varying signal intensities of the target cells generally might have occurred by diverse formation of the characteristic halo ranging from a thinner to thicker network structure (Figure 29, p. 70). It has to be emphasized that precultivation was not performed to increase the amount of ribosomes, but to increase the amount of target cells for gate definition in a complex environmental sample (without artificial spiking of pure cultures). Here, original INF samples were taken as positive controls for uncultured samples (Figure 39A and chapter E.3.2, p. 134). In this samples 'polyDIII' still produced specific signal and thus confirmed that probes were not depending on a high number of ribosomes [114]. However, upon this treatment it could be partly challenging to define the correct population in FCM. In future this procedure is only recommended for evaluation experiments as only cultivable species are enhanced while the uncultivable part might remain unidentified.

3. ENRICHMENT STRATEGIES USING 'POLYDIII'

3.1. MICROPLATES

The technique of cultivation-independent cell enrichment using polynucleotide probes was first described by Stoffels *et al.* (MACS system) [113] and was further developed for enrichment in microplates [115, 120, 141, 166]. The technique was based on the effect of the protruding parts of the polynucleotide probes in the cell's periphery which was already described previously (see A.2.3.2, p. 7 and E.2.2, p. 126). These protruding parts were subsequently used to capture cells by immobilizing them during a second hybridization step in microplates that were coated with DNA complementary to the probe sequence. The enrichment success was confirmed by target-specific PCR and epifluorescence microscopy [113].

Based on the insights gained from these previous studies, the aim in this work was to modify and improve the method for taxon-specific cell enrichment targeting *Enterococcus* spp. in complex intestinal samples. *E. faecalis*-spiked fecal samples were used to evaluate the applicability of 'poly-DIII' probes and the microplate enrichment technique. Although the application of polynucleotide probes for enrichment of bacteria out of environmental samples (*i.a.*, activated sludge, clinical samples) was already successfully conducted [120, 166, 220], it was always accompanied by various challenges. Generally, the application to Gram-negative strains where the halo formation was relatively stable, was much easier. In contrast, the application to Gram-positive cells was accompanied with cell loss due to additional washing steps. Additionally, the growth state of the organisms or naturally occurring components were thought to hamper or completely prevent the effectiveness of pretreatment. Besides, more challenges emerged when analyzing unspiked environmental samples. The amount of sample material, the number and growth state of the target organism, and naturally occurring components (inhibiting the contact of cells to the microplate, *e.g.* biofilms) in the sample material, constitute more or less unknown factors which may have an impact on successful hybridization with 'polyDIII' [120].

In contrast to this project, the studies working on 'polyDIII'-based enrichment in cavities mentioned above developed probes that were species-, but not genus-specific (*e.g.*, *E. coli*, *Acinetobacter calcoaceticus*, *Klebsiella pneumoniae*, or *Pseudomonas aeruginosa*) [115, 120, 166]. Here, as already mentioned (see chapter E.2), the application of 'polyDIII' to various bacterial and fecal samples could be accomplished, and additionally novel detection systems (*Enterococcus*-specific PCR and qPCR) for evaluation of the enrichment efficiency were developed

(see D.3.2.2 and D.3.2.3, p. 93–98). However, positive results were only obtained by application of pure cultures. But according to experiments which were done repeatedly, it could not be proven that PCR amplification products were really brought about by immobilization, or if they were just caused by cell residues due to insufficient washing of the cavities (Figure 52, p. 97). For artificial mixtures and spiked fecal samples both quantification methods (qPCR and microscopy) reproducibly failed to reveal cell enrichment in the cavities, while high concentrations of 'polyDIII'-hybridized target cells were found in the supernatants (see D.3.2.3, p. 98 and Figure 53, p. 98).

Regardless of stringent modifications applied to the method, failure might have had many reasons such as reduced sensitivity in the second hybridization, inappropriate handling while washing and recovering of cells in cavities, or inadequate working materials. Despite recent effective application to other target organisms [115, 120, 166] and elaborate modifications performed here, an adequate enrichment success for the genus *Enterococcus* could not be obtained. Furthermore, this technique requires excessive and precise hands-on-time, although only standard equipment was required. If one has access to FCM technology, it was predicted that it might be more efficient and cheaper to adapt 'polyDIII'-hybridized samples to this technique.

3.2. FACS

As the detection of rare events is extremely difficult, FCM combined with FISH constitutes a rapid and appropriate technology for environmental samples. In the project described here, the flow cytometric strategy was adapted to genus-specifically sort the microbial subpopulation *Enterococcus* based on polynucleotide probes. The technique allowed for a cultivation-free, targeted enrichment and recovery of the DNA from key gut microorganisms such as *Enterococcus* spp. (chapter D.4.4, p. 117). The most characteristic difference from other specific cell sorting techniques using FACS [101, 103, 106, 221, 222] was the application of polynucleotide probes for the identification of target cells, and the application to fecal samples. This novel methodology exploited the genus specificity and the signal amplification caused by the suggested inter-probe network in the cell's periphery [114], both previously evaluated here (see E.2, p. 125). The signal amplification, in contrast to standard mono-labeled oligonucleotides, was measured in fold change (16–36-fold increased), and corresponded to the values presented by Trebesius *et al.* (22–26-fold) [117], and to signal amplification achieved by CARD-FISH (10–41-fold) [167, 204] (Figure 60, p. 102 and Figure 61, p. 103).

One of the main challenges of flow cytometry in microbiology is the limit of detection caused by small cell sizes. This might create difficulties in distinguishing between small cells and cellular debris. Aggregation and clustering of bacterial cells or adhesion to fecal matter and other materials are additional difficulties for flow cytometry, *i.a.* due to causation of clogs in the system [207]. Therefore, thorough homogenization by mixing or sonication of fecal samples before applying to in-solution *in situ* hybridization and flow cytometric analysis was recognized as compulsory for fecal samples in this study (see E.2.3 or Figure 40, p. 85) [149, 150].

To ensure the correct gating of the target population, *E. faecalis* pure culture and artificially spiked fecal samples were first analyzed by FCM in the presence of a negative control. A characteristic population ('PopA') was detectable on respective density plots and was verified by microscopy of sorted cells from spiked fecal samples as a 'polyDIII'-hybridized (enterococcal) subpopulation (Figure 62–Figure 64, p. 104–105). Cell sizes of bacteria belonging to the same genus can vary due to differences in their physiological status which might cause difficulties in gating the correct population [223]. This might explain the third subpopulation ('PopC') in fecal samples with 10^3-fold increased spiking of pure cultured *E. faecalis* (Figure 63B and Figure 64B, p. 104–105). This subpopulation seemed to have the same fluorescence intensity range, but larger or more complex cells were identified. In microscopic analysis, 'PopC' comprised coccoid cells which appeared to be larger or of irregular shape, even rod shaped. An explanation for this phenomenon could be provided by fixation incidents, or very likely by cultivation effects (see E.2.3, p. 132) due to irregular growth states of additionally spiked *E. faecalis* cells (Figure 63B, p. 104). A similar, but not so distinct third population was also visible in original INF samples, and further analyzed in samples that were previously cultured in BHI medium (*e.g.*, Figure 74, p. 115 and Figure 78, p. 120). The corresponding microscopic results revealed that naturally occurring enterococcal cells as well showed diverse cell shapes of positively 'polyDIII'-labeled cells. This strengthened the assumption of influences by variable growth states, fixation, and inhomogeneous probe uptake including network formation.

Specificity of flow cytometric detection was further confirmed by, *e.g.*, application of artificially mixed bacterial samples (Figure 66, p. 107). However, the limitation for specific sorting was shown to be a successful hybridization with 'polyDIII' including sample preparation and homogenization. This might exclude high fluorescence signals by non-cellular debris and support a more homogeneous probe distribution (E.2.2, p. 126). Beside this, a limitation was observed by false-negative results due to cells that were not successfully hybridized with 'polyDIII'. Pure culture samples showed that not all target cells could be stained by polynucleotide probe hybridization (Figure 62, p. 104).

This ‘unstained’ population (‘PopB’) was mainly composed of autofluorescent cells, or as mentioned insufficiently labeled target cells. Moreover, non-target cells were included to ‘PopB’ in environmental samples (Figure 64C–D, p. 105). Again, probe binding was a limiting factor here, influenced by various factors. Especially in environmental samples, not all target bacteria could be accessed, probably because of irregular cell physiologic states, *e.g.* affected cell size while under nutrient-limited conditions in aged samples (see E.2.3, p. 130) [108, 223]. It is likely that the used probe concentration might also play a role in hybridization efficiency. Several members of the target-population were not fully hybridized because cells might compete for a limited amount of probe resulting in low signal intensity [166]. Incomplete network formation may lead to weaker or missing signals. According to this, in future the probe concentration should be adjusted to each sample.

When analyzing low-abundance fecal subpopulations such as enterococci, it might be difficult to set the correct gate for sorting. Specifically, this might be complicated when identification was hampered by the occurrence of not only a single distinct histogram peak in the FACS analysis (Figure 65–Figure 67, p. 106–108). Thus, the inclusion of negative controls (using no probe, nonEUB probe or no-template control species) in complex environmental samples was obligatory to correctly identify the positively hybridized cells. Additionally, the correct location of the target population could also be achieved by application of samples with increasing target cell ratios (Figure 68, p. 109). Because fresh fecal samples could not be obtained for this study, for an improved probe and method validation without artificial spiking of pure cultures it made sense to previously culture fecal SPF samples anaerobically before fixation (see E.2.3, p. 130). Thereby, it was possible to enhance the culturable part of a target population and to define the gate for the expected enterococcal population based on successful enrichment (Figure 71 and Figure 72, pp. 112–113). As discussed earlier (see E.2.3, p. 130), some of these precultivated samples led to morphological changes of the enterococcal cells, which further challenged the correct interpretation of the target populations (Figure 73A, p. 114). Nevertheless, sorted populations could be ensured by microscopic (see Figure 74, p. 115) and molecular analysis of multiple sort regions (see Figure 78, p. 120). Alternatively, prospective problems of time and gating are recommended to be minimized by multiple sort cycles. That is first sort a wide gate of the populations using less stringent purity properties, and then purify the target population using increasingly narrower ranges [88, 157]. This probably might be also important when analyzing fresh mouse or human feces samples in future.

However, as already mentioned in chapter E.2.3, precultivation had its limits for targeted enrichment as only the cultivable part of a community was enhanced. Hence, incubation in medium before hybridization and sorting might introduce an unwanted bias and uncultivable species might remain unidentified. Therefore, to avoid precultivation, in future it is recommended to use absolutely fresh feces in an appropriate starting volume (see E.2.3, p. 131) which is fixed directly after collection [212]. This may also prevent enhanced requirement of time that was necessary to sort a low-abundance population.

To subsequently perform molecular analysis an appropriate quantity of target cells had to be sorted. The references differ concerning the minimum amount of cells required for molecular analysis. Wallner *et al.* [88], Guillebault *et al.* [224], and Kleinsteuber *et al.* [225] described that approx. 10^4–10^6 cells are needed for a successful gene amplification by PCR, while Kalyuzhnaya *et al.* [226] suggested that 10^3 sorted cells are sufficient for a whole-genome amplification. Because capture of 10^6 cells from all samples except the original SPF samples was possible within a moderate time-frame, and whole-genome amplification should be avoided, sorting here was based on this quantity.

4. ACCESS TO MOLECULAR ANALYSIS

In this thesis, the taxon-specific cell enrichment by FCM using polynucleotide probes demonstrated that sorted enterococcal cells were accessible to molecular analysis of their DNA. This gives an significant advantage over harsher signal amplification techniques like CARD-FISH (catalyzed reporter deposition-FISH) which may compromise the access to DNA because of harsh enzymatic pretreatments and the application of hydrogen peroxide [101, 227]. Besides, there are only few published approaches which successfully amplified DNA from flow cytometric sorted populations [88, 210, 222, 224, 225, 228] and even less approaches targeting populations obtained from the intestinal environment [106, 229, 230]. On the other hand, next generation sequencing (NGS) benefits from its high throughput and more and more decreasing costs, but unfortunately to date this approach is computationally intense for complex populations and is restricted by its high error rate [67, 231, 232]. Various methodological improvements such as the metagenomic tool, just recently developed by Nielsen *et al.* [72], could facilitate future *de novo* assembly without the need of reference genomes. However, another present restriction for metagenomics from the gut is the need of high quality DNA samples in sufficient quantity that is still currently influenced by a high risk of human contamination [53].

In contrast to conventional metagenomics, recently single cell genomics via genomic DNA amplification using multiple-strand displacement amplification (MDA) is gaining importance in metagenomic analysis. It quite clearly benefits from the reduced amount of data and the possibility to stabilize the sequences in clone libraries. The methodology is strongly developing, but still requires substantial consumption of expensive reagents and post-amplification screenings [90, 233]. Therefore, this approach also remains difficult, specifically when complex environmental samples are used. To overcome these problems and to increase the resolution power, analyzing only a taxon-specific sub-metagenome might be sufficient for certain scientific questions, and generally justified the development of a method for targeted enrichment, which combined the advantages of NGS metagenomics and single cell genomics (Figure 80).

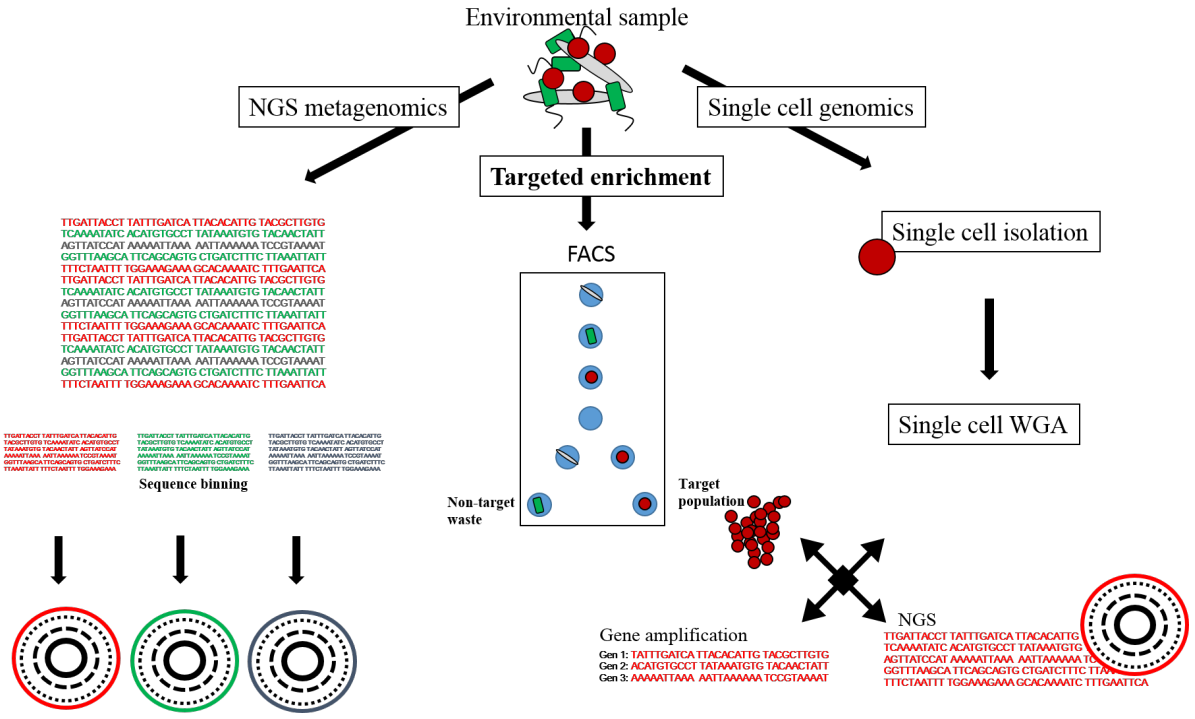


Figure 80. Overview of techniques used to investigate genomics of uncultivable bacteria in complex samples. Targeted enrichment combines the advantages of NGS metagenomics and single cell genomics (also see A.2.3.2, p. 6). The graphic was illustrated based on Blainey *et al.* [70].

Here, it was shown that 10^6 cells were sufficient to gain access to the cells' genomic information (see D.4.3, p. 116). The same amount of cells worked well in a flow cytometric approach by Koch *et al.* [234] after sorting of enrichment cultures. Cell fixation, pretreatment, low target DNA concentrations, and sodium chloride present in the cell suspension buffer may all inhibit downstream procedures such as PCR or construction of a clone library [235]. These problems were overcome by application of cells to a direct-PCR lysis reagent.

As a proof of the principle, it was successfully shown that genomic information of fixed, 'poly-DIII'-hybridized and sorted cells was still accessible for amplification of not only 16S or 23S rRNA genes, but also of functional genes (Figure 76, p. 117). These amplicons could successfully be used for the generation of gene libraries in order to proof specificity of sorted cells. Further these libraries were used to analyze and compare the genomic information of enterococci that were sorted out of different hosts feces (Figure 77 and Figure 78, pp. 119–120). Due to limited project time, sequencing and analysis of the gene clone libraries could not be fully completed (stored at –80°C) and only a small selection could be presented here. By PCR amplification of various conserved functional genes (*tuf*, *hsp60*, *atpB* and *recA and rpoA*) and by sequencing the plasmid inserts of respective clones, it was proven that all successfully obtained sequences could be assigned to the genus *Enterococcus* and mostly could be classified to the species *E. faecalis* or uncultured enterococcal species (referring to both, precultured SPF and original INF samples). As *E. faecalis* is commonly the most abundant *Enterococcus* species in feces, this is hardly surprising (also see Figure 38, p. 80) [134]. In case of the precultured SPF sample, additionally *E. faecium* and a few other enterococcal species could be detected which, as expected, could not be identified from the original (not precultured) INF mouse samples (germ-free mouse infected with *E. faecalis*).

Although the enrichment technique developed here showed that DNA of paraformaldehyde-fixed, 'polyDIII'-hybridized and sorted cell could be amplified, unfortunately it was not possible to perform a complete metagenome characterization of a sorted sample due to lack of accessibility to fresh fecal samples. The feces used here was collected while cleaning of the mouse cages and was stored at 4°C before we received it. Thus, samples were already a few days old and it was assumed that cells or their genomic DNA could be partly degraded.

Furthermore, preservation chemicals such as formaldehyde are known to induce DNA damage while fixation [236], this was obviously not influencing the sequences of the obtained functional genes. As a proof of principle, it could be demonstrated that the access to genus-specific genetic information of specific fecal samples, obtained from clone libraries of various functional genes was successful. Nevertheless, in case of metagenomic analyses it could not be verified here that the genomes are of significantly high quality for this purpose. As conventional metagenomics and single-gene studies are not sufficient yet to precisely describe the functional networks in natural microbial communities due to difficulties in interpretation, *e.g.* of gene catalogues [70], the metagenomic validation of the technique described here has to be one of the future directions for this project.

5. CONCLUSION & PERSPECTIVE

The aim of this project was the establishment of polynucleotide-based cultivation-free methods for the targeted enrichment of the metagenomes of certain genera such as *Enterococcus* from the complex gut consortia. In contrast to microplate-based enrichment, which could not be adopted properly (see E.3.1, p.133), the application of 'polyDIII'-hybridized cells from fecal samples as input for flow cytometry could be successfully implemented (see E.3.2, p.125). The advantages, disadvantages, and future perspectives of the latter were discussed in-depth along other comparable and most recently published methods.

While the design of specific oligonucleotides which are accessible for *in situ* FISH can be quite laborious, especially on genus-level, the domain III of the 23S rRNA as a target is ubiquitous in all bacteria [237–240]. Consequently, finding taxon-specific bacterial target regions for oligonucleotide probe design was not necessary anymore as the preparation of the here described polynucleotide probe could be performed by universal primers. These primers were already used to amplify corresponding fragments from rDNA from various phyla, which may allow the rapid preparation of group-specific probes also from (enriched) uncultivated, known or unknown bacteria [168]. The technique's advantage that probe specificities can be triggered by changing the stringency of hybridization is also its bottleneck. Depending on the genus of interest, the evaluation of the probe specificity, and further the adjustment of the pretreatment and hybridization conditions, might be more work-intensive than *in situ* evaluation of an oligonucleotide probe due to individual cell properties and growth conditions of genus-members. Oligonucleotide probes usually are not influenced by the permeability of the fixed cells [117]. In addition, evaluation and adjustment practically are optimized using multiple cultivable reference strains. However, once the polynucleotide's specificity and its hybridization stringency are evaluated, the detection of the target organism in environmental samples was simple and could be completed in a few hours.

Generally, a major limitation of rRNA-targeting FISH detection techniques is their limited application to environmental samples in which bacteria usually contain low ribosomal content due to starving conditions [97, 100, 111, 204, 241, 242]. Coming along that the standard oligonucleotides are commonly mono-labeled, the signal intensity of the detected cells is directly related to the number of target molecules in the cell. Therefore, naturally slow growing or starving bacteria might be below the detection limit [204, 205], particularly in flow cytometric measurements, as they have a decreased sensitivity compared to epifluorescence microscopy [221]. In contrast, the polynucleotide probe described here was inherently multi-labeled with

approx. 12–25 fluorophores (see C.2.3, p. 16). Thus, signal amplification was already included by one single probe and additionally induced by the so called network effect (see Figure 61, p. 103), but on the other hand, disadvantageously depended on a notably high probe concentration [114]. Despite this, 'polyDIII' was already shown to be independent of cellular ribosomal content, thus meets the condition for sorting cells with low ribosome contents, *e.g.* slow growing natural populations [112, 114, 115, 117, 119].

In the last decades, a number of novel signal amplification methodologies for targeted enrichment by FCM have been developed to increase the sensitivity of rRNA-targeted oligonucleotides. Many of them were based on laborious enzymatic amplification steps [101, 210, 227, 243], development of multi-label/-color probes [95, 221, 244] or multiple probes of the same specificity, binding to different sites [97, 205]. Besides, in latest techniques the ability to simultaneously detect multiple bacteria in a single sample was focused [244, 245].

Nevertheless, not all of these technologies are appropriate to flow cytometric application, sorting and subsequent molecular analysis. In addition, most of them were simply developed and evaluated to detect bacteria on "species"-level. While MiL-FISH (multilabeled FISH), a novel method recently published by Schimak *et al.* [221], might increase the signal intensity by 4-times-labeled oligonucleotide probes, and could apply unfixed environmental samples to flow cytometry, it was more focused on the simultaneous detection of various targets. Furthermore, the signal amplification, in contrast to mono-labeled oligonucleotides, could only be increased 2.9-fold ('polyDIII': 16–36-fold increase, see Figure 61, p. 103), and it was yet not shown that cells reliably could be applied to subsequent molecular analysis.

Another recently published technique was developed by Neuenschwander *et al.* [210], that was based on a fairly time-consuming 2-step enzymatic signal amplification. The scientists focused to separate ultramicrobacteria that are very small in size, and therefore very challenging for *in situ* and flow cytometric detection. Nevertheless, these bacteria were numerically dominating the examined water samples and the technique was shown to provide additional advantage only for small bacteria which were not detectable by other FISH techniques (*e.g.*, by similar CARD-FISH). However, the researchers showed a successful amplification of 16S rDNA from a PFA- or ethanol-fixed and sorted pure culture, but did not show any results for environmental samples. Furthermore, and in contrast to the polynucleotide-based technique presented in this thesis, the sorted cells (5×10^5) of Neuenschwander *et al.* were priorly conducted to multiple displacement amplification (MDA) which was not necessary for the polynucleotide-sorted cells (1×10^6) (see Figure 76, p. 117).

Nevertheless, for all methods, the possibility of a low yield of target cells (depending on sample densities) that would likely demanding for whole genome amplification techniques, might be a limitation, especially when complete population metagenomes are intended to be studied.

A general limitation for all FISH-FACS approaches is the effect of sample preservation on genome recovery by certain reagents (*e.g.*, formaldehyde or ethanol) which were already shown to induce DNA damage [150, 236]. Thus, presently the average estimated genome completeness of published single-cell genomes is only of 41% [236]. Although, PCR amplification and sequencing of genes could be successfully implemented from precultured paraformaldehyde-fixed sorted cells in this work (1 h fixation, 4% PFA) and from pure cultures in the publication of Neuenschwander *et al.* (15 h fixation, 1.7% PFA) [210], Clingenpeel *et al.* [236] recommended cryopreserved specimens or fixation-free FISH, in order to prevent from production of genomes of reduced quality. However, for fixation-free FISH, fresh samples have to be used and both fixation types are bearing hazards in exposure or contamination from unknown or clinical samples in flow cytometric systems. Besides, it was shown that samples from different origins require different fixation procedures [172, 207, 222]. While the standard procedure for feces fixation was based on paraformaldehyde as yet [212, 246], this project relied on this type of fixation. Nevertheless, ethanol fixation was also shown to be applicable for *in situ* hybridization of feces, with small loss in signal intensity (data not shown).

Another crucial step for the successful probe binding and flow cytometry was the homogeneity of the fecal matrix. Cells have to be separated and detached from fecal components, in order that dye moieties do not adhere to unspecific matter (see Figure 40 and Figure 60), especially in PFA fixed samples [207]. Furthermore, a single cell suspension is obligatory for precise detection and sorting by FACS. Usually this procedure was performed by simple mechanical homogenization of feces [103, 104, 106, 107] or with an additional centrifugation step [102, 105, 147], as well described in this project (see C.12, p. 25). Just recently, Hevia *et al.* [247] published a new potential method to separate stool microorganisms from the rest of the fecal material which might be an alternative procedure.

In the end, all present probe-based methods have in common that markers yet do not provide reliable quantitative information in environmental samples since target organism may display various structural and physiological characteristics. In this way it is not secured that every target cell in the natural sample will be detected [222]. However, the application of the polynucleotide probe, targeting domain III of the 23S rRNA, could be introduced in the concept of other FISH-FACS technologies.

In every technique, advantages and disadvantages are inherent depending on the experimental question and sample type. Besides, an overall standardization of microbial cytometric concerning sample manipulation, measurement, and data interpretation is still not fully established, and all methods were shown to have adequate room for improvement [82].

The polynucleotide probe based method described here, can be incorporated in a variety of techniques that are continuously developed, *e.g.* to gain a greater knowledge about the role of indigenous microbial populations of the GI tract and their genomes. As it is already known that the intestinal microbiota has an important role in human health and disease (*e.g.*, metabolic disorders [248]), scientists are increasingly working on strategies to profit from modulations of intestinal microbiota regarding human health. Gut microbiota is suggested to be the next generation in preventive and therapeutic medicine. In this context, one main disadvantage of the study presented here has to be mentioned. The choice and availability of fresh sample material neither allowed for sort-sufficient specific detection (*i.a.*, original SPF) nor for a complete metagenomic characterization. The application of SPF feces first appeared reasonable, as these mice owned a regular intestinal microbiota. However, it was recently presented that mice which were housed in SPF facility have a reduced cecal microbiota than wild mice, and that consequently low-abundance genera, such as *Enterococcus*, might be overlooked. Overall, discrepancies were observed at the level of specific genus/species abundance between the murine and human gut microbiota [249]. This, and the fact that the amount of fecal material was restricted and the fecal texture was more complex than from human samples, might have influenced the experimental implementation. However, the aim of the thesis to adapt the method to fecal sample material could be achieved, but in future experiments the application of more appropriate, in particular human samples is recommended.

While seeking new opportunities for therapeutic interventions based on manipulation of the gut microbiota, detailed analysis of shared/diverse genes, sequences, and data mining from a selected pan-genome might give knowledge about genetic potential in specific communities influenced and enriched under different parameters, and from different sources. The comparative functional analysis of inter-individual pan-genomes probably provides in-sights to functional differences of microbiota between individuals that cannot be explained by species composition, but are related to selective advantages including niche adaption, antibiotic resistance, and the ability to colonize different hosts [250, 251]. Particularly, regarding the gut microbiome, metagenomics using published NGS data sets and reference genomes showed that each individual might have a unique metagenomic genotype which is temporally stable [26, 252]. The technique developed in this project might pose a possibility to increase the resolution

power by physically decrease the number and diversity of bacteria within complex samples to one genus of interest. Thereby, it might be determined if a taxon-specific sub-metagenome shows response to parameters such as diet, inflammation, or to competition with other microorganisms, *e.g.* probiotics.

The polynucleotide probe based method moreover is not limited in future progression and may also be combined with physiological indicator dyes, microarrays [253], or forwarded to single-cell analysis only by using a capable flow cytometric device. It was already shown that the detection level in complex environmental samples could be further enhanced by additional combination with genus-specific 16S rRNA targeting oligonucleotide probes (Figure 42, p. 86). In addition, it benefits from the fact that it theoretically might be extended to any bacterial genus of interest, mainly limited of laborious and fairly time consuming probe evaluation by FISH, since the target of the probe is ubiquitous in bacteria. Future strategies for deeper understanding of variations in gut microbiota might be the application of this novel methodology to members of the genus *Bacteroides* or *Akkermansia*, both still being studied for their effects on human metabolism [254, 255]. The genus *Bacteroides* comprises intestinal commensal and abundant Gram-negative anaerobic bacteria, which are known to have the most antibiotic resistance mechanisms and highest resistance rates of all anaerobic pathogens [255]. To find alternative targeted therapeutic interventions it is necessary to understand its role in the gut environment. In addition, *Akkermansia muciniphila* is supposed to be rather associated to "lean" people. This was found based on studies of the intestinal microbiota composition of obese humans and mice, and those with type 2 diabetes, which both showed highly decreased levels of this bacterium [254]. Still, the detailed link between the *A. muciniphila* and the host metabolism is not known.

As a further approach, the association of taxon-specific enrichment and RING-FISH [119] might be conceivable. RING-FISH allows for *in situ* detection of any genetic material of bacterial cells (*e.g.*, functional genes or antibiotic resistance genes), regardless of copy number and metabolic activity. Consequently, in combination with novel metagenomic tools and other microbiome approaches, the method described here might further enhance the resolution power for population enrichment by FACS [72, 80, 256, 257]. Especially, the combination with metaomics for the characterization of relevant subpopulations (see A.2.2, p. 4) might make this approach to a powerful tool to understand the role which targeted microorganisms play in the gut community, as well as to understand their influence on health and disease [30]. Considering that, the quality of genomes obtained here has to be evaluated yet for conventional metagenomics.

In summary, application of the novel approach on human samples in the future may lead to highly interesting insights for the study of the human gut microbiota. The technique developed and evaluated here can be used to (i) study certain taxonomic groups in greater depth than possible with conventional metagenomics, (ii) study the diversity in-depth, including possible rare species and strains, (iii) study functional gene catalogues of taxa under different conditions (*e.g.*, in response to diet, inflammation, or competing strains), and (iv) compare selected intestinal taxa-metagenomes between different individuals or investigating intra-taxa enterotypes.

Finally, success and perspective of gut metagenome research are likely defined by the combination of novel practical and computational methods. They will provide in-depth knowledge of the 'forgotten organ' which apparently plays such a major role in human health. In this context, the statement of Hippocrates (460–370 BC): "All disease begins in the gut", has never been more relevance as today [258].

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Table 1. Primers used in this thesis, sorted by categories or applications

Name	Orientation	Target	*E. coli* position*	GC [%]	T_m [°C]	Sequence (5´ - 3´ direction)	Reference
23S rDNA domain III primer							
1900V_mod	forward	23S rRNA gene, most bacteria	1366	62.2	51.5	HAG GCG TAG GCG ATG	[168], modified
pT-1900V	forward	23S rRNA gene, most bacteria, phosphorylation & thymidine linker	1366	37.3	58.6	pho-T TTT TTT TTT HAG GCG TAG GCG ATG	[168], modified
aC12-1900V	forward	23S rRNA gene, most bacteria, amino-C12 linker	1366	62.2	51.5	aC12-HAG GCG TAG GCG ATG	[168], modified
317RT3_mod2	reverse	23S rRNA gene, most bacteria, T3-tail +GAA linker	1602	44	74.1	ATA GGA ATT AAC CCT CAC TAA AGG GAG AAC CTG TGT CGG TTT RCG GTA	[168], modified
317R_mod	reverse	23S rRNA gene, most bacteria	1602	52.5	58.3	ACC TGT GTC GGT TTR CGG TA	[168], modified
317R_mod-DIG	reverse	23S rRNA gene, most bacteria, DIG-labeled	1602	52.5	58.3	ACC TGT GTC GGT TTR CGG TA	[168], modified
Other rDNA primer							
Enc473V	forward	16S rRNA gene, *Enterococcus*	473	52.4	59.8	CRT CCC YTG ACG GTA TCT AAC	This project
Enc1276R	reverse		1276	40	59.7	CTG AGA GAA GCT TTA AGA GAT TWG C	
616Valt	forward	16S rRNA gene, most bacteria	8	45	55.2	AGA GTT TGA TYM TGG CTC AG	[260], modified
100K	reverse		1511	42.1	52.4	GGT TAC CTT CTT ACG ACT T	
118V	forward	23S rRNA gene, most bacteria	115	65.6	55.6	CCG AAT GGG GRA ACC C	[261]
985R	reverse		2654	62.5	54.3	CCG GTC CTC TCG TAC T	
3010V_mod	forward	23S rRNA gene, most bacteria	13	58	60.4	AAG SGT ACA YGG TGG ATG CC	This work group
2241R	reverse		2241	55.1	53.6	ACC GCC CCA GTH AAA CT	Lane, 1992
qPCR primers							
ENC738F	forward	23S rRNA gene *Enterococcus*	738	50	57.3	CAC GTA CGT TGA AAA GTG CG	This project
ENC850R	reverse		832	50	57.3	TTC TCA ATT CCG AGG CTA GC	
Ent1(*tuf*)	forward	*tufA* gene of *Enterococcus*	618	38.6	55.6	YAC TGA CAA ACC ATT CAT GAT G	[160], modified
Ent2(*tuf*)	Reverse		709	52.4	59.8	AAC TTC GTC ACC AAC GCG AAC	[160]

APPENDIX

Name	Orientation	Target	*E. coli* position*	GC [%]	T_m [°C]	Sequence (5´ - 3´ direction)	Reference
Plasmid primer							
M13F(–20)	forward	5' end of the *lacZ* gene in pCR2.1 plasmid	388*	56	51.7	GTA AAA CGA CGG CCA G	Invitrogen, USA
M13R(–26)	reverse		205*	47	50.4	CAG GAA ACA GCT ATG AC	
Functional genes primer							
eftu_r_0904	reverse	elongation factor Tu, most *Bacteria*	1136	55.3	57.7	ACA GTA CGT CCR CCT TCW C	[120]
eftu_v_0904	forward		53	63.9	59.4	CGG TCA CGT WGA CCA YGG	
ATPsubB_2015_F	forward	ATP synthase ß subunit, most *Firmicutes*	n.a.	51	56.3	GTT CAA GTH ATY GGT CCC G	This project
ATPsubB_2015_R	reverse		n.a.	54	53.6	CGG AAD GCT TCT TCW GG	
Enc-recA-F	forward	recombinase A, most *Firmicutes*	n.a.	50	55.6	TYA TYG ATG CNG AGC ATG C	This project
Enc-recA-R	reverse		n.a.	34	53.7	TGG TTR ATG AAA ATM GCR ATT G	
Enc-rpoA_F	forward	RNA polymerase α subunit, most *Firmicutes*	n.a.	30	49,1	ATG ATT GAR TTY GAA AAA CC	This project
Enc-rpoA_R	reverse		n.a.	41	47,9	ACA GTA TTR ATK CCT GC	
Hsp60_2015_F	forward	60-KDa heat shock protein, most *Firmicutes*	n.a.	64	57,6	GCA GAA GAY GCA CGY GC	This project
Hsp60_2015_R	reverse		n.a.	40	53,2	ATT TGA CGR AYT GGT TCT TC	
Sequencing primer							
97KV	forward	16S rRNA gene, most bacteria	342	66.7	53.3	TAC GGG AGG CAG CAG	[261]
609V	forward	16S rRNA gene, most bacteria	787	35.3	45.5	TTA GAT ACC ATR GTA GT	This work group
614R	reverse	16S rRNA gene, most bacteria	1057	68.8	56.9	ACG AGC TGA CGA CRG C	This work group
699RIII	reverse	16S rRNA gene, most bacteria	1101	65.4	43	GGG TTG HGC TCG T	This work group
1019V	forward	23S rRNA gene, most bacteria	803	50	53.7	TAG CTG GTT CTCYBC GAA	[261]
1025IRvy97	reverse	23S rRNA gene, most bacteria	1186	60	50.6	GGT AGG RGA GCD TTC	This work group
1037Rm97	reverse	23S rRNA gene, most bacteria	1954	47.6	57.9	CTT ACC CGA CAA GGA ATT TCG	[261]
***In situ* probes**							
Enc473(-ATTO565)		16S rRNA gene, *Enterococcus*	473	52.6	56.7	TAG ATA CCG TCA RGG GAY G	This project
Eub338		16S rRNA gene, most bacteria	338			GCT GCC TCC CGT AGG AGT	[97]
nonEUB		nonsense negative control				ACT CCT ACG GGA GGC AGC	[95]

*position in pCR2.1 Topo vector
T_m calculated using Eurofins Genomics (www.eurofinsgenomics.eu)
Following nucleotide ambiguity codes were used [262]: R=A or G; Y=C or T; S=G or C; W=A or T; K=G or T; M=A or C; B=C or G or T; D=A or G or T; H=A or C or T; V=A or C or G; N=any base
* [118]

LIST OF PUBLICATIONS AND FUNDING

Bruder Lena, Dörkes Marcel, Fuchs Bernhard, Ludwig Wolfgang, Liebl Wolfgang (2016). Flow cytometric sorting of fecal bacteria after *in situ* hybridization with polynucleotide probes. *Systematic and Applied Microbiology*. doi:10.1016/j.syapm.2016.08.005

Poster presentations

Bruder Lena, Ludwig Wolfgang, Liebl Wolfgang (2014). Reducing the metagenomic sequence space for analysis of the gut microflora by taxon-specific cell enrichment. Poster presentation, 4th Joint Conference of the Annual Conference of the Association for General and Applied Microbiology (VAAM) & 66th Annual Meeting of the German Society for Hygiene and Microbiology (DGHM), Dresden, Germany, 05/-08/10/ 2014.

Bruder Lena, Ludwig Wolfgang, Liebl Wolfgang (2012). Reducing the metagenomics sequence space by taxon-specific cell enrichment. Science Camp of German Research Foundation (DFG) research training group GRK 1482, Seeon, Germany, 10/-12/05/ 2012.

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°C	degree centigrade
A	adenosine
A260, A280	absorbance at 260 nm and 280 nm
abs.	absolute
Amp	ampicillin
approx.	approximately
APS	ammonium persulfate
ATP	adenosine triphosphate
atpB	gene coding for the F_1F_0 ATP synthase ß-subunit protein
BLAST	basic local alignment search tool
bp	base pairs
C	cytosine
CFU	colony forming units
CIA	chloroform : isoamylalcohol = 24:1 (v/v)
CTP	cytidine triphosphate
Cy	cyanine dye
DAPI	4', 6'-diamino-2-phenylindol-dihydrochloride
DIG	digoxigenin
DIII	domain III of the 23S rRNA gene
DNA	deoxyribonucleic acid
dnaK	gene coding for the heat shock protein (70kD) protein
DNase	deoxyribonuclease
dNTP	2'deoxy-nucleoside-5'-triphosphate
ds	double stranded
E	efficiency

e.g.	*exemplum gratiae* (lat.)
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	ethylenediamintetraacetate
Enc	*Enterococcus*
et al.	*et alteri* (lat.)
EtBr	ethidiumbromide
$EtOH_{x\%}$	x % ethanol
FA	formamide
FACS	fluorescence-activated cell sorting
FAM	fluorescein amidite
FCM	flow cytometry
FISH	fluorescence *in situ* hybridization
FLUOS	fluorescein
FSC	forward scatter
FTP	ribonucleoside-5'-tri-phosphates including fluorescein-12-UTP
g	gram
G	guanine
GC	mol % guanine + cytosine
GF	germ free
GI	gastrointestinal
GTP	guanosine triphosphate
gyrA or *B*	gene coding for the DNA gyrase (α or ß subunit) protein
h	hour(s)
H_2O_{dest}	distilled water
H_2O_{MQ}	ultra-pure water (millipore-system)
HCl	hydrochloride

hsp60	gene coding for the heat shock protein (60 kDa)
i.a.	inter alia (lat.)
i.e.	id est (lat.)
INF	*E. faecalis* infected germ free mouse
IRD	infrared dye
kb	kilo bases
kDa	kilodalton
L	liter
lacZ	gene coding for β-galactosidase
LSU	large subunit
M	molar
m	meter; milli(10^{-3})
mg	milligram
Mg^{2+}	magnesium ion
min	minutes
mL	milliliter
mM	millimolar
n	nano (10^{-9})
n.a.	not available
NaCl	sodium chloride
NC	negative control
ng	nanogram
nt	nucleotide(s)
NTC	no-template control
NTP	ribonucleoside-5'-tri-phosphates
NW	negative well (uncoated)

o/n	overnight
OD_{600}	optical density at 600 nm wavelength
oligoFISH	fluorescence *in situ* hybridization using oligonucleotide probes
p.	page
PBS	phosphate buffered saline
PC	positive control
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	negative decadic logarithm of the proton concentration
POD	peroxidase (from horseradish)
polyDIII	polynucleotide probe targeting domain III of the 23S rRNA gene from the genus *Enterococcus*
polyFISH	fluorescence *in situ* hybridization using polynucleotide probes
R^2	correlation coeffcient
rDNA	ribosomal DNA
recA	gene coding for the recombinase A protein
RM	rich medium
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	revolutions per minute
rpoA or *B*	gene coding for the RNA polymerase (α or ß subunit) protein
rRNA	ribosomal RNA
RT	room temperature
s	second(s)
SEM	standard error of the mean
SN	supernatant
SPF	specific-pathogen-free mouse

spp	species (plural)
ss	single strand
SSC	side scatter characteristics or standard saline citrate
ssp	subspecies
SSU	small subunit
T	thymine or type strain
T_A	annealing temperature
Taq	*Thermus aquaticus*
T_D	dissociation temperature
TE	Tris-EDTA
T_m	melting temperature of nucleic acids
Tris	tris-(hydroxmethyl-)aminomethane
tuf	gene coding for the elongation factor tu protein
U	unit or uracil
UTP	uridine triphosphate
UV	ultra violet
V	volt
v/v	volume / volume
vol	volume(s)
W	watt
w/v	weight / volume
X-Gal	5-chlor-4-brom-3-indolyl-β-d-galactoside
y-int	y-intercept
µ	micro (10^{-6})
µg	microgram
µL	microliter

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