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Cultivation and conservation of the human intestinal microbiota

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Contents

Abbreviations

BDM	bacterial dry matter [%]
CDI	*Clostridium difficile* infection
CFF	Continuous Flow Fermentation Medium
CFU	Colony forming units
dNTP	Deoxynukleosid triphosphat
dATP	Desoxyadenosin triphosphat
dCTP	Deoxycytidin triphosphat
dTTP	Deoxythymidin triphosphat
dGTP	Deoxyguanosin triphosphat
FMT	Fecal microbiota transplantation/ transfer
HIV	Human Immunodeficiency Virus
HPLC	High performance liquid chromatography
IMNGS	Integrated Microbial Next Generation Sequencing
NGS	Next generation sequencing
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction
Rel. cum. abundance	Relative cumulative abundance [%]
rRNA	Ribosomal ribonucleic acid
SCFAs	Short-chain fatty acids
SR	Survival rate [%]

Summary & Zusammenfassung

Summary

In this work, the establishment of a preservation process of the human intestinal microbiota, consisting of cultivation, freeze-drying and re-cultivation, was investigated. The technical realization of the developed concept was studied by comparing different characteristics of the system: cell count, metabolism, and microbial profile.

In chapters 3 and 4, it was demonstrated that a culturable, stable community with a high diversity and richness of representative gut bacteria can be produced. The produced *in vitro* microbiota can be influenced by choosing system parameters, which provide some degrees of freedom in producing a certain target microbiome. Stool characteristics had an influence as well, but the cultivation parameters had a higher impact. Being a cultivation parameter with great influence on the established system, the cultivation pH value was investigated in chapter 4. During cultivation, all investigated pH values created stable systems, with the factors of the ratio of SCFAs and the phylogenic distribution considered as being indicative of a healthy microbiota. Regarding a physiological cultivation pH value between 6.0 and 7.0, the choice of pH results in systems with certain differences. A pH of 7.0 results in a high cell count, whereas higher concentrations of butyrate were present at pH 6.0. If a system close to the original system regarding the distribution of phyla is required, the cultivation should be conducted at pH 6.0. Here, a higher ratio of Firmicutes to Bacteroidetes as well as a higher abundance of health-promoting bacteria, including *Roseburia*, *Bifidobacteria*, and *Faecalibacterium*, was detected at pH 6.0.

Further, a drying protocol was developed (chapter 5), consulting *Bifidobacterium longum* ssp. *longum* as a test strain from the human intestinal microbiota. Even if the survival was up to 40% for the single cell culture, the *in vitro* microbiota only showed a survival of 2-6% facultative aerobic and 0.1-0.9% of anaerobic cells.

When regarding the entire preservation process (chapter 6), the cultivation itself was found to have a major influence. Before drying the microbiota gets shaped and altered significantly by the cultivation system parameters. The drying step was also found to alter the microbiota significantly but the changes being reversible during re-cultivation. After 70 h, the re-established system was able to restore completely and reached a state that was comparable with the constitution of the system prior to drying. Thereby only slight, but natural changes in metabolism occurred. We detected different influences of the cultivation pH value prior to drying on the characteristics of the re-established system after drying. For cell count as well as for diversity (richness and Shannon effective index), no pH dependent influence was observed. To sum it up, all investigated pH values resulted in cells with a healthy and working metabolism after drying. Consequently, for the entire preservation process, a physiological cultivation pH value between 6.0 and 7.0 is applicable. Thereby, values

at the lower end are preferable as they create a more similar system compared with the donor stool characteristics.

Nevertheless, the clinical applicability and effectiveness should be validated by further studies including more stool samples, scale-up of the fermentation systems and additional *in vivo* studies.

Zusammenfassung

Die vorliegende Arbeit behandelt die Entwicklung, Etablierung und Validierung eines Gesamtprozesses zur Konservierung der humanen intestinalen Mikrobiota. Dieser umfasst Kultivierung, Gefriertrocknung und Re-Kultivierung zur Erfassung des Trocknungserfolges und der Wiederanzucht. Dabei wurde die rein technische Umsetzbarkeit des entwickelten Konzepts untersucht, indem die verschiedenen Charakteristika Zellzahl, Metabolismus und das mikrobielle Profil der Systeme untersucht wurden.

Kapitel 3 und 4 beschreiben die Etablierung des Kultivierungsprozesses. Hierbei konnte eine stabile mikrobielle Gemeinschaft mit hoher Diversität und Fülle an repräsentativen Darmbakterien etabliert werden. Zudem wurde ein Gehalt und Zusammensatzung an kurzkettigen Fettsäuren, den Metaboliten der Mikroben, detektiert, welcher einem normalen gesunden Erwachsenen entspricht. Die Auswahl des Stuhldonoren hat dabei ebenso einen, wenn auch deutlich kleineren, untergeordneten Einfluss verglichen mit dem Einfluss der Kultivierungsbedingungen. Die angewandten Kultivierungsparameter haben dagegen einen großen Einfluss auf die resultierende, künstliche Mikrobiota. Diese kann durch die Wahl geeigneter biotechnologischer Parameter beeinflusst werden, um bis zu einem gewissen Grad die gewünschte *in vitro* Mikrobiota zu erzeugen.

Der Einfluss des Kultivierungs-pH-Wertes in einem physiologischen Bereich zwischen 6.0 und 7.0 auf das ausgebildete System wurde in Kapitel 4 untersucht und beschrieben. Die Auswahl des pH-Wertes beeinflusst dabei direkt das ausgebildete System. Insgesamt führen alle untersuchten Kultivierungs-pH-Werte zu stabilen Systemen, wobei die ermittelten Kenngrößen, wie das Verhältnis der kurzkettigen Fettsäuren und die phylogenetische Zusammensetzung, ein System widerspiegeln, das als gesund angesehen werden kann. Während ein Wert von 7.0 in einer höheren Zellzahl resultiert, werden bei pH 6.0 höhere Konzentrationen an Butyrat gemessen. Wird ein künstliches System gewünscht, welche die Ausgangsstuhlprobe am besten repräsentiert, so sollte ein pH-Wert von 6.0 gewählt werden. Neben der Ähnlichkeit ist auch die Zusammensetzung der Phyla, gemessen unter anderem am Verhältnis von Firmicutes und Bacteroidetes, sowie die Anwesenheit von gesundheitsförderlichen Genera wie *Roseburia*, *Bifidobacteria* und *Faecalibacterium* höher bei niedrigen pH-Werten.

Im Weiteren (Kapitel 5), wurde *Bifidobacterium longum* ssp. *longum* als Testmikroorganismus zur Entwicklung eines Trocknungsprotokolls herangezogen. Während bei dieser einzelnen Zellkultur Überlebensraten von bis zu 40% erreicht werden konnten, resultierte die Trocknung der *in vitro* Mikrobiota nur in ein Überleben von 2-6% der fakultativ aeroben und 0.1-0.9% der strikt anaeroben Zellen.

Betrachtet man den Konservierungsprozess als Ganzes (Kapitel 6), so wird klar, dass der Kultivierungsschritt vor der Trocknung den größten Einfluss hat. Der Trocknungsschritt verändert die Mikrobiota ebenfalls signifikant, allerdings sind diese Veränderungen während der Re-Kultivierung reversibel. Durch eine Wiederanzucht über 70 h konnte gezeigt werden, dass sich das System komplett erholt und einen Status vergleichbar mit dem Zustand vor der Trocknung erreicht. Geringfügige Änderungen und Verschiebungen im Metabolismus werden dabei auf natürliche Schwankungen zurückgeführt. Auch der Einfluss des während der Kultivierung vor der Trocknung angewandte pH-Werts auf die Charakteristika des re-etablierten Systems wurde untersucht. Für die Zellzahl als auch Diversität (richness und Shannon effective Index) konnte kein Unterschied aufgrund des Kultivierung-pHs festgestellt werden. Zudem führen alle pH-Werte zur Ausbildung eines gesunden und funktionierenden Metabolismus. Nichtsdestotrotz gab es hier auch pH-bedingte Unterschiede. Verglichen mit der mikrobiellen Zusammensetzung von Phyla und Genera in der Stuhlprobe konnte durch den Kultivierungs-pH von 6.0 ein System erzeugt werden, was in der mikrobiellen Zusammensetzung am meisten der Ausgangsstuhlprobe ähnelt. Grundsätzlich und zusammenfassend betrachtet, ist für den gesamten Konservierungsprozess ein physiologischer Kultivierungs-pH-Wert zwischen 6.0 und 7.0 anwendbar. Basierend auf den obigen Ergebnissen scheinen aber niedrigere Werte eher empfehlenswert.

Dessen ungeachtet ist es aber zwingend notwendig, die medizinische Anwendbarkeit und Effektivität der entstehenden getrockneten Mikrobiota zu testen. Dafür sind Studien, welche eine Vielzahl weiterer Stuhlproben als auch einen Scale-up des Kultivierungssystems inkludieren, sowie *in vivo* Studien im Tierversuch als auch am Menschen unabdingbar.

1 General introduction

During the last years, the microbiota became of more and more interest in fields of science, research, and medicine. The human intestinal microbiota is a large ecosystem that hosts up to 10^{11} CFU mL^{-1} and 400-1,000 different species (Eckburg *et al.*, 2005; Gill *et al.*, 2006). The microbial distribution, variety and diversity have a major influence on human health. Disbalances can lead to several gastric diseases as an infection with *Clostridium difficile*. The standard therapy for the treatment of a *Clostridium difficile* infections is the administration of antibiotics. Unfortunately, antibiotics lead to a further disbalance and reduction of health-promoting microorganisms in the microbiota and hence in some cases to a relapse of the disease. An alternative treatment method is Fecal Microbiota transplantation, where purified stool from a healthy donor is transferred into the patient's gut to restore the microbial balance and diversity. Even if this method leads to high recovery rates, it has some disadvantages as an extensive donor screening, low storability of stool and the lack of a standardized procedure.

In the following chapter, the human intestinal microbiota with its composition, development and functions within the body is described and discussed. The occurrence of disease as *Clostridium difficile* infections is explained. As an alternative treatment method, the chances, opportunities and possibilities of Fecal Microbiota transplantations as well as the disadvantages are described. A possibility to overcome these constraints offers the production of a controlled and stable artificial colonic microbiota, which is the objective of this work.

1.1 **Human intestinal microbiota**

The human body hosts several ecosystems composed with different microorganisms, e.g. the microbiota of the skin (Sanford and Gallo, 2013), vaginal (Godha *et al.*, 2018) or oral microbiota (Lu *et al.*, 2019). The largest microbiota, regarding cell count, variety and diversity is located in the human gastrointestinal system with the large intestine hosting the highest number of cells. Therefore, the human intestinal microbiota describes mostly the community in the large intestine. This community has a high impact on health and disease of the human individual (Haller, 2018).

1.1.1 *Composition of the microbiota*

The composition of the human intestinal microbiota is dependent on the conditions in the gastrointestinal system. The gastrointestinal system is composed of the stomach, followed by the small intestine with duodenum, jejunum, and ileum. The cecum is located where the small meets the large intestine. Proximal, transverse, and distal colon form the large intestine, which ends in the rectum and anal canal. As Figure 1.1

shows, the pH value in the gastrointestinal system increases continuously from stomach (pH 2), to colon (pH 5-6.9) up to the rectum with a pH of 6 to 7 (Evans *et al.*, 1988; Payne *et al.*, 2012; McDonald, 2013). The colon is divided in three parts, with an increasing pH from proximal (pH 5.4-5.9), transverse (pH 6.1-6.4) to the distal colon (pH 6.1-6.9). Nevertheless, these values are individual and can differ from person to person within the mentioned ranges. Combined with the pH value, the number of microbial cells increases in the different parts of the gastrointestinal system (Figure 1.1). The high number of microorganisms in the distal colon results from a high retention time of food components and therefore a high amount of nutrients. When passing through the gastrointestinal tract, the food remains 20 to 37 h in the large intestine which promotes growth and abundance of the microbial cells (Metcalf *et al.*, 1987; Kim and Rhee, 2012). This high number, abundance and further diversity is important when stool samples are used for Fecal Microbiota transplantation as it will be described subsequent (Chapter 1.3).

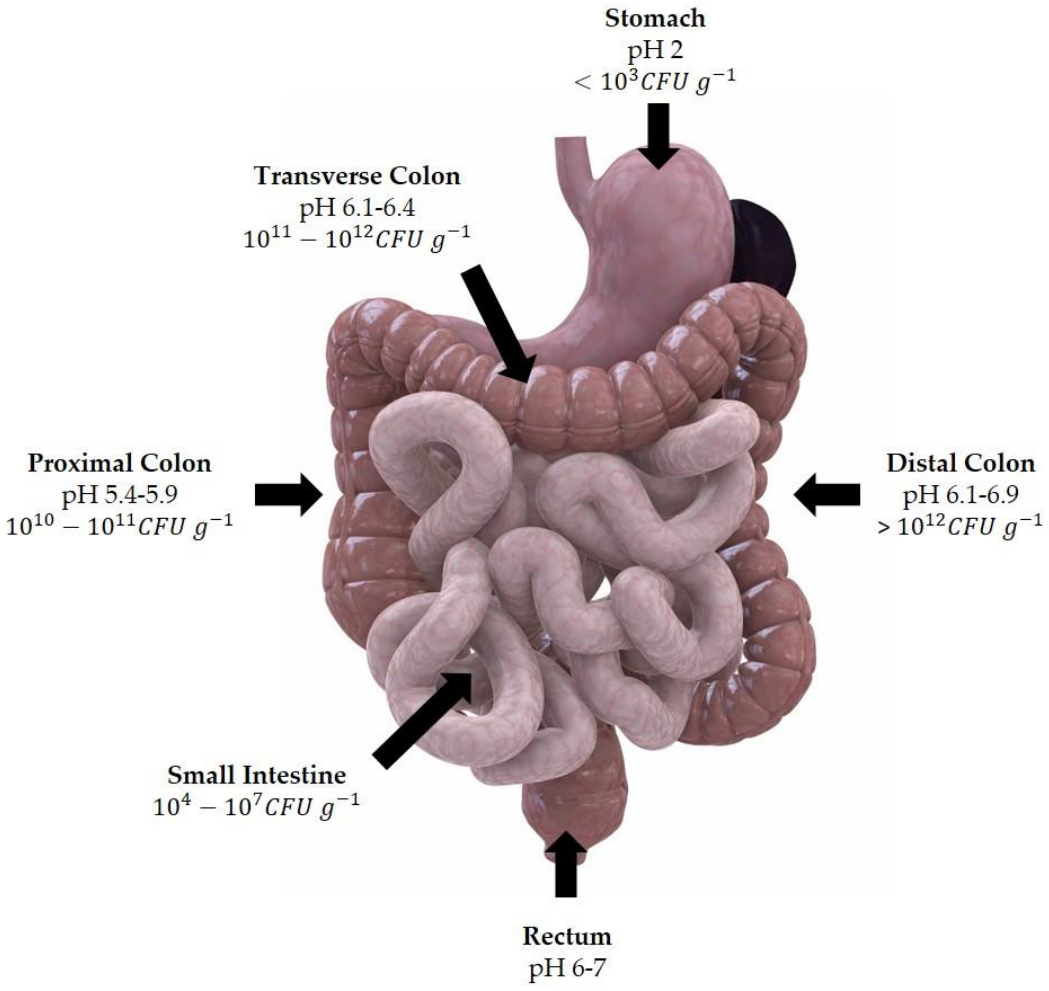

Figure 1.1: Human digestive system with stomach, small and large intestine and rectum showing the pH value and cell counts in the different segments, modified after Payne *et al.* (2012); Image from http://www.loveyourgut.com/wp-content/uploads/digestive-system.jpg

In total, the intestinal microbiota hosts 10-100 trillion of microbial cells of 400-1.000 different species, which exceeds the number of body cells in multiple cases (Eckburg *et al.*, 2005; Ursell *et al.*, 2012). These microbial cells are mainly part of the phyla of Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia. Firmicutes and Bacteroidetes represent the most abundant phyla, each constituting 40 to 50% of total bacteria. Proteobacteria (0.1 – 1%), Actinobacteria (2.5%) and Verrucomicrobia (0.1%) are the minor phyla and less abundant (Eckburg *et al.*, 2005). The ratio of Firmicutes and Bacteroidetes in the microbiota is an important factor for a healthy or diseased composition (Rajilić-Stojanović *et al.*, 2013). Koliada *et al.* (2017) detected a ratio of 0.6 to 1.0 for people with a normal, healthy body weight, which is amongst others age dependent (Mariat *et al.* 2009).

Figure 1.2 shows exemplary the distribution of phyla and genera of a healthy microbiota based on the stool of a female person. In the sample, the five known phyla as well as a small percentage of unknown phyla (1.39%) are abundant. In this stool, the phyla Firmicutes (45.28%) and Bacteroidetes (48.79%) are the major abundant phyla, indicating a healthy contribution with a ratio of 0.9.

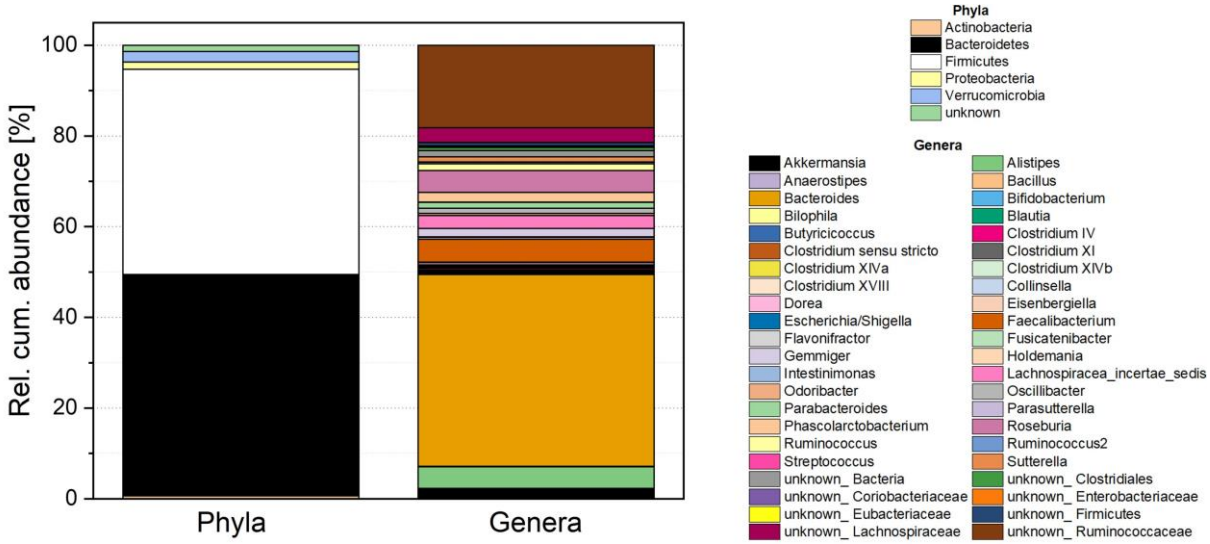

Figure 1.2: Relative cumulative abundance of phyla and genera in a stool sample of a healthy, female donor

The major phylum Bacteroidetes consists of *Bacteroides*, *Alistipes*, *Parabacteroides* and *Prevotella*. The phylum Firmicutes is represented by the class of *Bacilli* and *Clostridia* (Haro *et al.*, 2016). Typical genera are *Lactobacillus* and *Streptococcus*, as well as *Faecalibacterium*, *Ruminococcus, Blautia* and *Veillonella*. The *Clostridium* Cluster XIVa is resprestend by *Dorea* and *Lachnospira* and others (Rajilić-Stojanović *et al.*, 2013). Here, the minor phyla Proteobacteria and Verrucomicrobia are represented with 1.59% and 2.30% of relative cumulative abundance (rel. cum. abundance). The minor phylum Actinobacteria (0.65%) is mainly represented by *Bifidobacteria*, whereas *Escherichia* and *Shigella* are abundant within the phylum of Proteobacteria and *Akkermansia* within the

phylum of Verrucomicrobia (Eckburg *et al.*, 2005; Voreades *et al.*, 2014). Even though the classification and characterization of microorganisms got simplified with new techniques as Next Generation Sequencing (see chapter 1.6), parts of the genera are still un-identified (Li *et al.*, 2012).

Nevertheless, some health indicating genera were already identified. Microorganisms of the genera *Akkermansia* (van Herreweghen *et al.*, 2017), *Bacteroides* (Waidmann *et al.*, 2003), *Bifidobacterium* (Saez-Lara *et al.*, 2015), *Blautia* (Shin *et al.*, 2018), *Faecalibacterium* (Miquel *et al.*, 2013; Cheema, 2019) and *Roseburia* (Siezen and Kleerebezem, 2011) are known to have a positive effect on human health. *Escherichia Coli* Nissle is the only accredited drug on the german market, with a microorganism being the active component (Schultz, 2008). Further probiotics as *Bifidobacteria* or *Lactobacilli* also have a positive effect on health, but are only approved as dietary supplements so far (Qin, 2005; Turroni *et al.*, 2008).

For the function of the microbiota and therefore the health of the individuum it is important to host a high variety and diversity of microorganisms. Nevertheless, the composition of a healthy microbiota is never fixed and unchanging, but undergoes several changes e.g. over daytime (Rajilić-Stojanović *et al.*, 2013; Reitmeier *et al.*, 2020b; Reitmeier *et al.*, 2020a). The composition of phyla and genera are further varied by time, diet, birth, age, but also genetics, ancestry and diseases. Next to the microbial composition, the richness and diversity are of scientific interest and can be calculated using the relative cumulative abundances (see chapter 1.6.2). Hence, their absolute value is dependent on the abundant phyla or genera and varied by different factors.

1.1.2 *Development of the microbiota during life*

The human intestinal microbiota starts to develop at birth and undergoes changes through the whole life. The major, natural, and not disease-based changes in diversity and composition occur in the first three years of human life. It is influenced by diet (breast or formula feeding), start of solids, drug administration, microbiota of the parents and especially birth itself. The mode of delivery – vaginal birth or cesarean section – has a major impact on the development of the microbiota (Mitsou *et al.*, 2008; Ravel *et al.*, 2011). Due to a low pH in the birth canal (pH 4-5.5) the microbiota of vaginal delivered babies shows higher abundances of microorganisms and is dominated by facultative aerobic microorganisms as *Bifidobacteria* and *Lactobacilli*.

As the child gets older, a more and more complex microbiota develops with higher cell count, richness, stability and mainly anaerobic representatives as several *Clostridium* genera (Mariat *et al.*, 2009). During that time of life, the abundance of Bacteroidetes and Firmicutes further increases, while Actinobacteria and Proteobacteria decrease as it can be seen in Figure 1.3. When the composition of phyla changes in early childhood, also diversity, stability, and variety change. During adulthood, the stability and diversity of the microbial composition reaches the highest

value in life. While in early childhood, the microbiota is mostly influenced by birth mode and diet (formula or breast milk), the microbiota of adults is altered by diseases, drug intake, diet, hormones, or lifestyle. Figure 1.3 shows that with proceeding life, stability, diversity and variety in the abundant microorganisms alter significantly (Spor *et al.*, 2011).

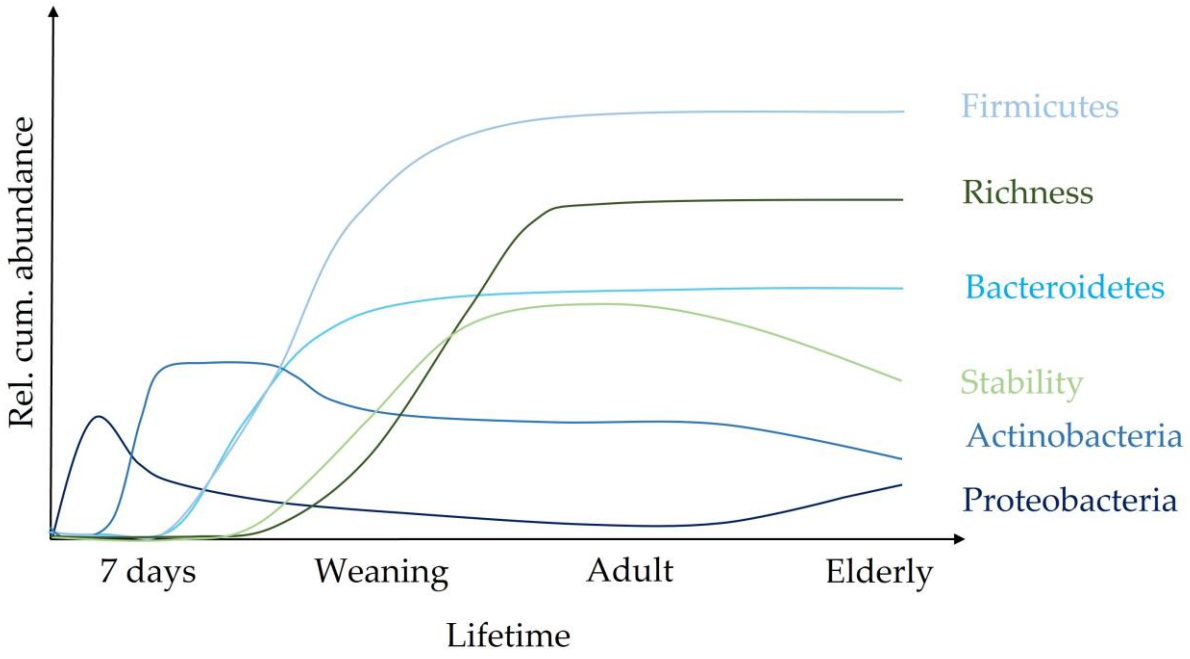

Figure 1.3: Alterations in abundance of the major phyla as well as richness and stability over lifetime; modified after Spor *et al.* 2011) and Haller (2018)

With an age of about 60 years, the digestion of elderly is altered, resulting in a longer residual time of food in the intestine as well as a reduced production of metabolites in the microbiota. This results in an increase of facultative aerobic microorganisms, the ratio of Firmicutes and Bacteroidetes alters and the abundance of Actinobacteria decreases. Consequently, more Proteobacteria as *Enterobacteria* and *Escherichia Coli* are abundant (Mueller *et al.*, 2006; Mariat *et al.*, 2009). Further, the stability of the ecosystem decreases resulting in a less active immune system and the occurrence of more diseases (Nicholson *et al.*, 2012).

1.1.3 *Influences on the microbiota*

During life, the microbiota alters constantly and is dependent on environment, sex, genetics, physical activity, cultural heritage or age of the individuum (Mueller *et al.*, 2006; Palmer *et al.*, 2007). Also, the family structure, if a person lives with elderly, siblings or pets has an influence. The microbiota even changes on a daily timescale (David *et al.*, 2014a). For instance, a high physical activity is associated with a high diversity (Monda *et al.*, 2017). Consequently, the composition of the microbiota as well as its metabolic behavior is individual and dependent on several factors, which in turn

have an influence on human health and the occurrence of several diseases. The biggest influence is done by diet and diseases connected with drug intake, especially antibiotics. Figure 1.4 sums up the influences that shape the human intestinal microbiota.

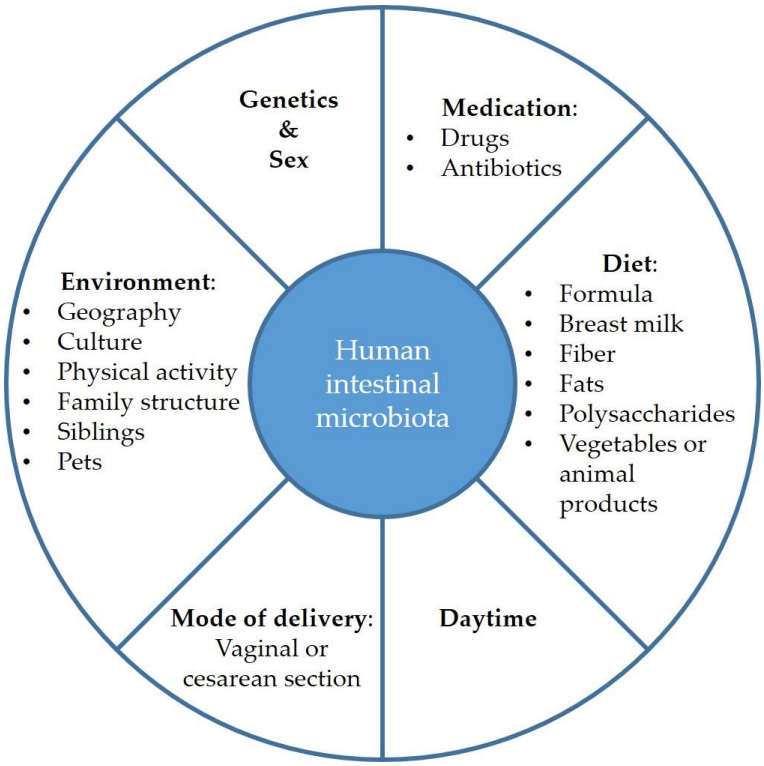

Figure 1.4: The main influencing factors that shape the human intestinal microbiota; modified after Haller (2018)

Regarding diet, a diet with a high intake of fruits, vegetables and fiber leads to a higher number and diversity of microbial cells. A western diet, which is common in industrial countries, is rich in animal protein, sugar, and starch and lacks fiber. This results into a decrease of cells compared to a diet rich in fibers (Becker *et al.*, 2011). On the other hand, persons who consume a diet high in resistant starch and fiber, often have an increased abundance of microorganisms of the phylum Firmicutes as *Ruminococcus bromii*, *Eubacterium rectale* or *Prevotella* (Walker *et al.*, 2011; Wu *et al.*, 2011). On the contrary, a diet high in animal protein and saturated acids results in a decrease of Firmicutes that metabolize dietary plant polysaccharides as *Roseburia*, *Eubacterium rectale* and *Ruminococcus bromii* and an increase of bile acid tolerant representatives of Bacteroidetes as *Alistipes* and *Bacteroides*, as well as *Clostridium ramosum* or Proteobacteria like *Bilophila* (David *et al.*, 2014b). These genera may cause the inclusion of adipose tissue and hence, the development of overweight and adiposity (Becker *et al.*, 2011; Woting *et al.*, 2014). Geographical differences in the microbiota are mainly based on locally different eating habits resulting in a different microbial composition, as well as concentration of short-chain fatty acids (Filippo *et al.*, 2010; Nam *et al.*, 2011).

However, the biggest changes in the human intestinal microbiota are induced by antibiotics. Although antibiotics aim to reduce pathogens, also microorganisms from the microbiota get influenced to a high content. The correlation between antibiotic administration, reduction of cells in the microbiota and long- and short-term aftereffects has already been proven in several studies (Jernberg *et al.*, 2007; Dethlefsen *et al.*, 2008; Jakobsson *et al.*, 2010; Panda *et al.*, 2014). Even the treatment of diseases that are not connected with the gastric system change the microbiota significantly and result in severe changes. A shift in composition and a reduction in quantity and diversity may then lead to secondary diseases (Dethlefsen *et al.*, 2008; Panda *et al.*, 2014). A further threat that comes with antibiotic treatment is the development of multi-resistant and pathogenic genes.

After all, the microbiota can be altered by several factors that result in permanent or reverse changes. Diet and antibiotics have the highest influence and may lead to severe reductions in variety and diversity. On the other hand, some changes occur naturally and do not cause automatically diseases (David *et al.*, 2014a). The connection between the function of the microbiota, human health, and alterations in the bacterial ecosystem of the microbiota will be further discussed in the following chapters 1.1.4 and 1.2.

1.1.4 *Functions of the microbiota in the human body*

The intestinal microbiota is responsible for a multiplicity of tasks in the body and is considered as an important "organ" of humans. It is involved and functions as a gut barrier system and forms and is part of the immune system. Further, microorganisms in the microbiota are involved in the depletion and admission of nutritive substances from different food components. Through nutrition, especially fermented food, a high number of living microorganisms gets applied and partly integrated in the ecosystem. It takes 20-37 h for the food and its components to pass through the large intestine (Metcalf *et al.*, 1987; Kim and Rhee, 2012). This long durance makes it possible to metabolize 10-20 % of carbohydrates from the food, which were not digested in the small intestine. These are mainly resistant starch and other carbohydrates as pectin and cellulose (Ramakrishna, 2013). They are the main source of energy for the bacteria in the microbiota and get metabolized to short-chain fatty acids (SCFAs), lactate and succinate or gases like CO_2, H_2 and methane (Flint *et al.*, 2012). The SCFAs with the major representatives acetate, propionate, and butyrate, lower the pH in the intestine and prevent the abundance of pathogens. In the same time, the microbiota has a huge influence on glucose homeostasis and lipid metabolism (Rios-Covian *et al.*, 2017). The lipid metabolism is influenced mainly by *Lactobacilli* and *Bifidobacteria*, that produce bile salt hydrolases that reduce the level of cholesterol (Devillard *et al.*, 2009; Kumar *et al.*, 2011). Consequently, the microbiota is directly connected with insulin resistance, diabetes type 2 and several diseases of the liver (Rios-Covian *et al.*, 2017). Further, *Lactobacilli* are involved in the admission of mineral nutrients and a connection

between a lack of this genus and iron-deficiency anemia is assumed (Balamurugan *et al.*, 2010). A further task of the microbiota is the vitamin synthesis including vitamin K, and B group vitamins as biotin, folates, and thiamine (Hill, 1997). Vitamins, as well as short-chain fatty acids, regulate the function of epithelium cells. The intestinal epithelium cells form antimicrobial peptides that influence the microbiota in variety and diversity (Schreiber *et al.*, 2014).

As already mentioned above, a central task of the microbiota is the production of short-chain fatty acids, that are responsible for a variety of functions in the entire body. SCFAs include the educts as acetate, propionate, butyrate, iso-butyrate, iso-valerate, valerate and intermediates as pyruvate, lactate, and succinate. Figure 1.5 shows the metabolic pathways for the formation of the main SCFAs acetate, butyrate and propionate and the involved microorganisms. Acetate and propionate are mainly produced by the phylum Bacteroidetes, whereas Firmicutes metabolize preferentially butyrate (Ramakrishna, 2013).

In detail, SCFAs are mainly formed from non-starch polysaccharides and resistant starch, that are metabolized to pyruvate in the beginning. Afterwards, pyruvate is digested in three different ways depending on the microorganism. Lactic-acid bacteria form lactate from pyruvate, which is further degraded to propionate. Another degradation path metabolizes pyruvate to oxalo-actate and finally succinate. Succinate is mainly a substrate for *Bacteroides* and *Ruminococci* that produce propionate out of it. Further, *Veillonella* and *Clostridia* are producers of propionate. The main degradation path of pyruvate is through the formation of Acetyl CoA, which is metabolized to Aceto-Acetyl-CoA and further butyrate by *Clostridia*, *Ruminococci* and *Eubacteria*. Additionally, acetate is formed by several other genera as for instance *Bacteroides*, *Bifidobacteria*, *Clostridia*, *Veillonella* or *Ruminococci*.

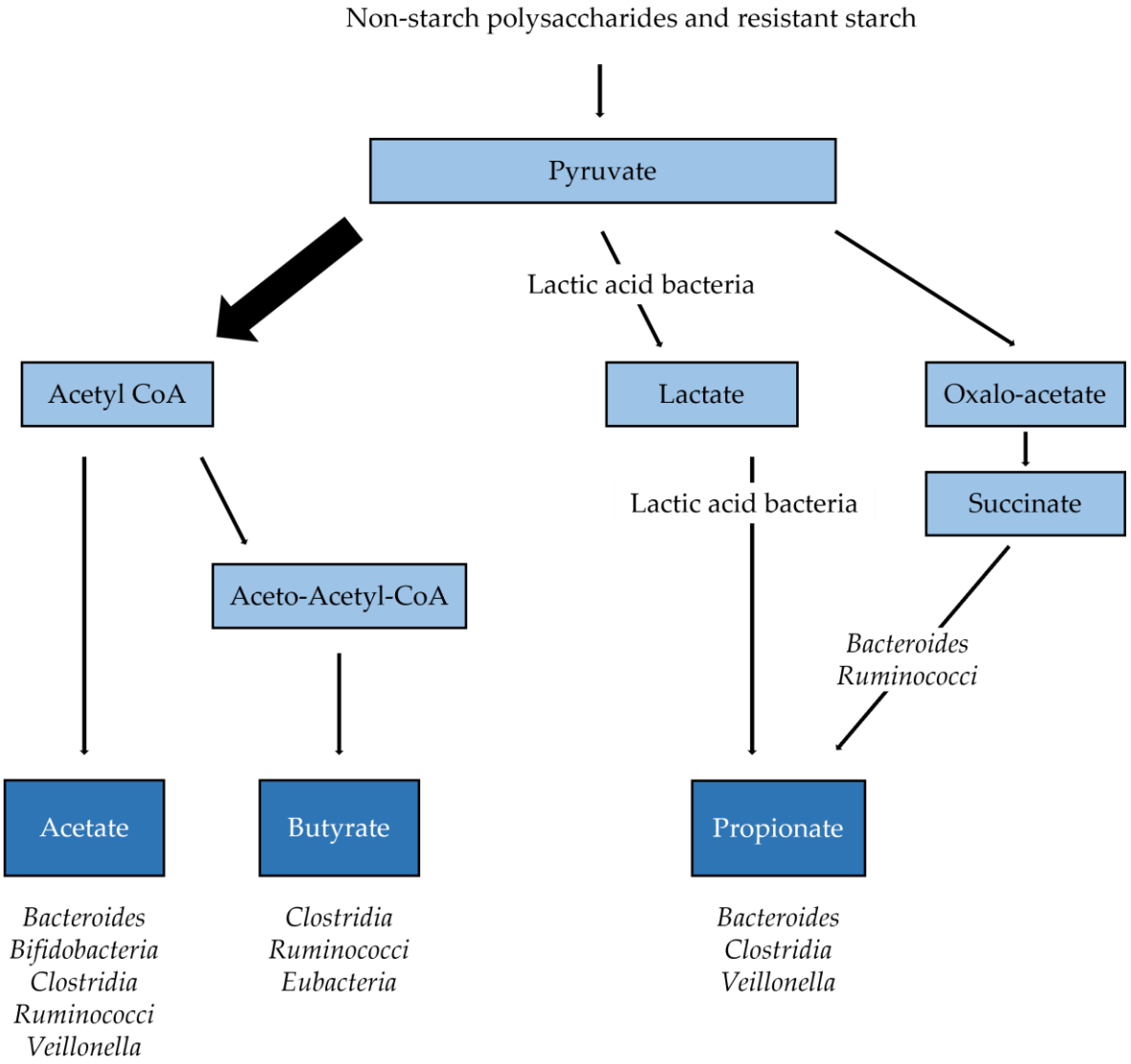

Figure 1.5: Metabolic pathways with intermediates for the formation of the main short-chain fatty acids acetate, butyrate, and propionate along with the involved microorganisms; adapted after Ramakrishna (2013)

The SCFAs have several tasks in the body and serve as substrate for different body cells, especially intestinal epithelium cells. For instance, they lower the pH and prevent the settlement of pathogens. Propionate and butyrate also influence the lever and glucose homeostasis and an increased concentration of propionate may result in a high body weight or even adiposity. Butyrate is able to improve insulin sensitivity and glucose tolerance (Vadder *et al.*, 2014). Further, butyrate is the main source of energy for the intestinal epithelium cells and can lower the risk for intestinal cancer (Ramakrishna and Roediger, 1990).

The concentration of the three main metabolites acetate, propionate and butyrate differs individually from a ratio of 3:1:1 to 10:2:1 (Macfarlane *et al.*, 1992; Rowland *et al.*, 2018). The ratio of SCFAs therefore has a direct impact on health and especially the occurrence of diseases as obesity and adiposity. Schwiertz *et al.* (2010) detected the

metabolites acetate, propionate, butyrate and iso-valerate dependent on the body mass index. Persons with a normal, healthy body weight had an average concentration of 3.03 mg mL^{-1} of acetate, 1.01 mg mL^{-1} of propionate and 1.24 mg mL^{-1} of butyrate in their stool (**Fehler! Verweisquelle konnte nicht gefunden werden.**). The c oncentrations, especially of propionate and butyrate, increased with the body mass index. For example, the concentration of propionate in obese (1.36 mg mL^{-1}) and adipose (1.43 mg mL^{-1}) is significantly higher compared to the healthy control group.

Table 1.1: Comparison of the concentrations of the short-chain fatty acids acetate, propionate, butyrate and iso-valerate of normal, overweight and adipose persons; modified after Schwiertz *et al.* (2010)

Body mass index	Acetate [mg mL^{-1}]	Propionate [mg mL^{-1}]	Butyrate [mg mL^{-1}]	Iso-valerate [mg mL^{-1}]
Normal	3.03 ± 0.76	1.01 ± 0.39	1.24 ± 0.67	0.28 ± 0.21
Overweight	3.36 ± 1.09	1.36 ± 0.59	1.63 ± 0.89	0.23 ± 0.17
Adipose	3.59 ± 1.10	1.43 ± 0.64	1.59 ± 0.88	0.29 ± 0.20

1.2 *Clostridium difficile* infections

Human health and the microbiota are strongly connected. As already discussed in chapter 1.1, health and influence on the immune system depend strongly on vitality, diversity, and the balance or disbalance of the microorganisms as well as their metabolites as SCFAs in the microbiota. Disorders of the gastric system like *Clostridium difficile* infections, inflammatory bowel disease like Colitis ulcerosa or Crohn's disease come along with an alteration of bacterial composition and disbalance and a reduced diversity and quantity of microorganisms. A disease connected with the gastric system and a threat especially for elderly and patients in hospitals are *Clostridium difficile* infections. The development and changes in the microbiota will be discussed in the following chapter.

Clostridium difficile is a Gram-positive, spore-forming, and anaerobic microorganism from the phylum Firmicutes. *Clostridium difficile* infections (CDI) cause severe diarrhea connected with an inflammation of the large intestine. These bacteria are often abundant in a healthy microbiota without causing any infection. A healthy distribution of primary and secondary bile acids prevents sporulation and growth. Mostly, the trigger for the infection is an extensive treatment (of a different disease) with antibiotics like ampicillin, amoxicillin, cephalosporin, clindamycin or metronidazole (Thomson *et al.*, 1981; Kelly and LaMont, 1998). As already described in chapter 1.1.3, the microbiota gets altered by an antibiotic treatment. For instance, clindamycin causes a reduction of strict anaerobic bacteria as Actinobacteria, Bacteroidetes and Firmicutes. Further, the abundance of Fusobacteria, Proteobacteria and Verrucomicrobia increases

(Table 1.2). Several representatives of the genera *Bifidobacterium*, *Clostridium* and *Bacteroides* decrease sharply or even disappear completely (Lagier *et al.*, 2012). In total, the microbiota of a patient with CDI is in dysbiosis with a sharp or even complete reduction of Bacteroidetes and Firmicutes. On the other side, the abundance of Proteobacteria is massively increased (Weingarden *et al.*, 2015).

Table 1.2: Alterations in the microbiota of a person with *Clostridium difficile* infection on phyla level compared to the microbiota of a healthy, normal weight person; modified after Weingarden *et al.* (2015)

	Microbiota of a patient with CDI compared to a healthy person
Firmicutes	↓
Bacteroidetes	↓
Actinobacteria	↓
Verrucomicrobia	↑
Proteobacteria	↑
Fusobacteria	↑

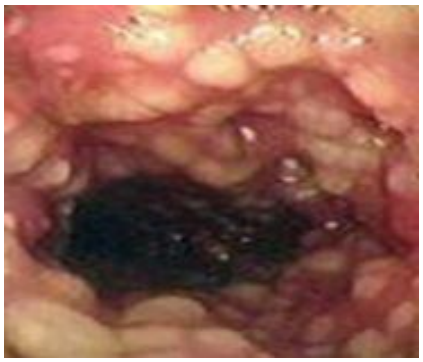

Figure 1.6: Surface of the colon with several white, pus-filled spots of a person with *Clostridium difficile* infection; image from Lagier (2016)

Additionally, the concentrations of bile acids in total as well as of primary bile acids, e.g. taurocholic acid increases (Weingarden *et al.*, 2015). Through the extensive administration of antibiotics to the patient, bacteria which can reduce these acids are decreased. This further promotes the growth and sporulation of *Clostridium difficile*. The vegetative bacterium forms the toxins A (enterotoxin) and B (cytotoxin) which lead to the outbreak of the disease (Pothoulakis, 1996). They connect to the intestinal epithelium cells and cause severe inflammation in intestinal wall (Borriello, 1990). Figure 1.6 shows these withely, yellowish running sores, that grow up to 2-4 mm of size (Riegler *et al.*, 1995). CDI is mainly a threat to elderly (> 65 years), with a weak immune system due to previous antibiotic administration as well as long hospital stays. In Germany in 2019, a number of 2,139 severe causes have been reported, whereas nearly half of them ended deadly (Robert Koch-Institut, 2020). Due to demographic alterations, the overall mortality rate doubled from 1999 to 2004 in Europe (Shears *et al.*, 2010; Wiegand *et al.*, 2012). Patients, who suffered already a

relapse of CDI, have an increased risk of 65% to sicken again on the infection (McFarland *et al.*, 1994).

The standard treatment method of CDI is the administration of antibiotics as metronidazole or vancomycin (Kelly and LaMont, 1998) resulting in recovery rates between 76% (metronidazole, severe CDI) and 98% (vancomycin, mild CDI) depending on the severity of the infection (Zar *et al.*, 2007). Relapse of disease occurred for 15% of the patients within both treatment methods. Due to an older society as well as more antibiotic treatments, an increase in cases of primary infections and relapses is expected.

This is a threat for especially older patients and further a massive burden for the health system. Consequently, scientific, and medical research is done about the investigation of the human intestinal microbiota and related diseases as well as the development of alternative treatment methods for *Clostridium difficile* infections. Next to the standard therapy of antibiotic administration, Fecal microbiota transplantation and further enhancements of this concept offer an alternative for a lasting cure of CDI.

1.3 Fecal Microbiota transplantation

Fecal microbiota transplantation (FMT) is an alternative treatment method for infections with *Clostridium difficile* (*C. diff*) but was also administered for other gastric diseases. It aims to restore the microbial community, diversity, and balance in the large intestine by transferring stool from a healthy donor into the patient's gut (Figure 1.7).

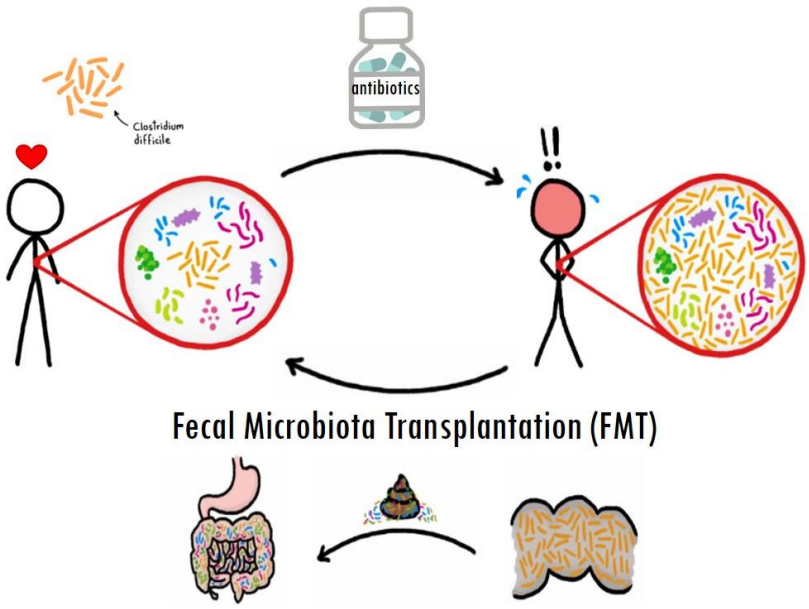

Figure 1.7: Basic principle of fecal microbiota transplantation; image modified after openbiome.org

Therefore, stool from a healthy donor gets purified, mixed with nutrient solutions as sodium chloride, filtrated, and applied in the large intestine of the patient. In advance,

the donor stool has to run through a cost and time extensive screening to prevent the transfer of diseases or bacterial, viral, and eucaryotic pathogens and fulfill a variety of different physiological and psychological criteria (Terveer *et al.*, 2017). Amongst others, the donor must not have taken antibiotics, immunosuppressives or chemotherapeutic drugs over a specific period. He or she must be healthy, not suffering from chronical diseases (e.g., infection with HIV or hepatitis B/ C), obesity or allergies. The stool must be free of parasites like giardia or microsporidia as well as pathogenic microorganisms (e.g., *Escherichia Coli*, *Salmonella*, *Helicobacter pylori*). Additionally, the potential donor stool is tested for antibiotic-resistant genes. Also, the social life of donor is of importance. Donors are excluded if they participated in high-risk sexual behaviors, travelled to areas with endemic diarrhea, used illicit drugs or have been to jail (Aroniadis and Brandt, 2013). For the treatment with FMT, stool from a close relative of the patient or a member of the same household achieved the highest effect (Gough *et al.*, 2011). After the choice of an appropriate donor and the purification of the stool, the fecal infusion material gets transferred into the patient's large intestine by enema, naso-gastric tube or coloscopy (Brandt and Reddy, 2011). Patients undergoing a treatment with FMT experience a fast decline of symptoms within a few hours (Seekatz *et al*., 2014).

Nevertheless, the mechanistic success behind FMT is still not completely revealed, although different researchers state that multiple factors contribute to the elimination of the infection with *Clostridium difficile* (Khoruts and Sadowsky, 2016; Baktash *et al.*, 2018). Khoruts and Sadowsky (2016) state, that the transferred microbial community competes with *C. diff* for nutrition and colonization resources. In a patient's gut, the pathogenic microorganism is abundant in a high number. As the fecal slurry is transferred, these bacteria can crowd over *C. diff* and replace them. Further, the offer of nutrients for the pathogens is lowered as more and other bacteria metabolize faster. Consequently, the abundance of *C. diff* drops significantly. Additionally, some bacteria are even able to kill the pathogens by interfering with its virulence factors. Transferred antimicrobials or phages are able to penetrate biofilms and pseudomembranes and consequently directly affect the viability of *C. diff* (Ott *et al.*, 2017). The fast improvement of patients' health is also caused by a direct suppression of inflammation through the action of defensins on toxin activity (Giesemann *et al.*, 2008). Further, still undefined compounds affect host pathways that underly inflammatory response (Chandrasekaran and Lacy, 2017). When transferring the donor's fecal infusion, multiple host immune defenses get activated and constrain the pathogens by producing secondary bile acids that are able to inhibit the germination and vegetative growth of *Clostridium difficile* (Khoruts and Sadowsky, 2016). Next to the microorganisms, SCFAs play a role in the mechanistic success of FMT. They lower the pH value in the intestine. Then, the re-established microbial community produces more SCFAs that are correlated with a colonization resistance against *C. diff.* A high level of SCFAs leads to an expansion of anaerobic bacteria that inhibit the growth of the pathogens. Summing up, not only the number and vitality of the transferred

microorganisms is of great importance when talking about the success of FMT. Furthermore, the whole slurry with its components, as phages, antimicrobials, anti-inflammatory compounds and SCFAs is needed to reduce the pathogen and restore a healthy environment again.

After FMT, the microbial community of the patient recovers within a short period of time, often within 24 h. After the treatment, the composition of the microbiota resembles the composition in the donor stool (Seekatz *et al.*, 2014). Recovery rates for patients treated with FMT are, depending on the severity of the disease, up to 90%. Further, the number of relapses and deaths is indeed very low (Gough *et al.*, 2011; Aroniadis and Brandt, 2013). The therapy succeeds by restoring the microbial community and diversity in the gut with a lasting effect. In the past, mainly infections with *Clostridium difficile* were treated. Characteristic for CDI is, as described in chapter 1.2, a high abundance of Proteobacteria, especially *Enterobacteriaceae*, and further a low diversity. After a treatment with FMT, the abundance of this phylum decreases, and diversity increases rapidly. Post-FMT an increase of Bacteroidetes and Firmicutes can be observed, and a healthy balance re-establishes. The composition after the therapy is very similar to the microbial distribution in the donor stool. Van Nood *et al.* (2013) investigated the microbial composition in the intestine pre- and post- treatment. Before therapy, the Simpsons reciprocal index, expressing diversity in the microbiota, was 57. After a successful FMT it rose to 179, which is similar as in the donor stool with 172. Weingarden *et al.* (2015) detected that the abundance of Proteobacteria decreased from 44-82% post-FMT to a non-detectable level after the treatment. FMT is mainly used as a treatment method for *Clostridium difficile* infections, but its potential is also applicable for other diseases (Zhang *et al.*, 2018). It was already applied successfully for Crohn's and Parkinson's disease (Borody *et al.*, 1989; Ananthaswamy, 2011), ulcerative colitis (Angelberger *et al.*, 2013) or multiple sclerosis (Borody *et al.*, 2011).

Despite the high recovery rates, FMT is not yet a standardized treatment method. The amount of fecal infusion material as well as the pre-FMT treatment of the patient differ in several studies (Gough *et al.*, 2011). The volume of slurry applied differs between 200 and 500 mL, with larger volumes yielding a better outcome (Aroniadis and Brandt, 2013). Further, the donor screening protocol is high in cost and effort. Often different donors must be screened before an appropriate one is found which may take some time. As FMT is not a standardized treatment method, the acceptability among patients can be lowered by them having safety concerns.

Therefore, several studies investigated the possibilities of an enhancement of the FMT method: establishment of minimal consortia (Becker *et al.*, 2011) or an *in vitro* microbiota (Garborg, 2015; Bircher *et al.*, 2018) as well as purified stool that gets preserved through freezing or lyophilization (Hamilton *et al.*, 2012; Vigvári *et al.*, 2019). Vigvári *et al.* (2019) were able to cure severe CDI when applying lyophilized and rehydrated stool through a nasogastric tube. With this procedure, 83% experienced a

resolution of their symptoms shortly after the treatment. After a second treatment 94% of the patients recovered. Hamilton *et al.* (2012) used purified, frozen (-80 °C) and thawed donor stool which was applied through colonoscopy. After a single treatment 86% of the patients recovered and after an additional FMT the cure rate increased to 95%.

This work aims to create a technically feasible concept where several ideas of these studies are combined. It was proven that the application of *in vitro* microbiota slurry (Bircher *et al.*, 2018) as well as of a lyophilizate (Vigvári *et al.*, 2019) has already been successful. By the generation of transferrable material *in vitro*, the concerns and challenges related to traditional FMT can be overcome. Mimicking gut conditions produce a controlled and stable artificial colonic microbiota with a high diversity and number of different gut bacteria as well as their important metabolites and compounds. A high vessel volume can create larger amounts of infusion material under controlled conditions. A following drying process leads to a product with a higher shelf life and availability. A preservation process consisting of stool sampling, *in vitro* cultivation and lyophilization may be a possible enhancement of traditional FMT and a possibility to overcome its disadvantages and constraints. Open questions in this concept are still in which way the cultivation system, as well as individual donor stool characteristics and the entire preservation chain affect the microbiota outcome and characteristics on a technical level.

1.4 *In vitro* cultivation of the intestinal microbiota

Next to applying processed stool, several researchers investigated the *in vitro* cultivation of the human intestinal microbiota to use it as infusion material. This method offers more independence from the donor stool, less extensive screening effort and a continuous accessibility of infusion material. Recently, *in vitro* microbiotas were used to investigate and modulate admission and depletion of several single as well as complex food residues and the resulting shifts in community (Boever *et al.*, 2000). Also, the evaluation of different probiotic strains and drugs can be realized *in vitro* as a preliminary stage to *in vivo* animal and human studies (Hemarajata and Versalovic, 2013). Further, the mechanistic correlation between pathogens and diseases was investigated (Le Blay *et al.*, 2009; Maccaferri *et al.*, 2010). Advantages of *in vitro* systems are an easy set-up and operation as well as the ability to simply adapt and variate it. Shifts, alterations, and manipulations can get tracked immediately and operators can react and adapt easily. Further, less ethical considerations appear. Regarding the enhancement of the FMT concept, this offers great advantages and chances. Even though, the main constraint of *in vitro* models is the lack of interactions between epithelium cells and the formation of biofilms are hardly reproducible (Macfarlane and Macfarlane, 2007)

1.4.1 *Excisting models in literature*

Existing *in vitro* models can be divided into batch systems, multistage continuous systems and systems with immobilized feces (Payne *et al.*, 2012). The complexity increases depending on how many regions of the gastric system are modelled. While some systems aim to reproduce the entire gastric system, others simulate only specific regions, mostly the large intestine.

A model consisting of several components is the so called "TIM-2" model, which simulates the entire intestine with its entire sections. For instance, this model was used to prove the positive effect of inulin on the microbiota of patients with inflammatory bowel diseases (Minekus *et al.*, 1999; van Nuenen *et al.*, 2003). Another multistage component model system is the so called "Simulated Human Intestinal Microbial Ecosystem" (SHIME), which reproduces duodenum, jejunum, ileum, caecum as well all sections of the large intestine and therefore is able to simulate occurring processes after the food left the stomach (Molly *et al.*, 1994; Boever *et al.*, 2000). Multistage component systems represent a very detailed model of the entire gastric system, but are very complex and hence harder to set-up, administer and operate than one-component systems.

Table 1.3 shows several one-component models that were already applied in literature, which were either operated as batch or continuous systems. The aim of the batch models was mainly to establish a simplified system where the effect of different food fractions as well as pro- and prebiotics could be tested. Takagi *et al.* (2016) established a model that reliably simulated the composition of the microbiota *in vitro* and determined a high impact of prebiotics on the abundance of *Bifidobacteria*. Other studies with batch models identified the dependence of metabolism and SCFAs production on the content of fibre, resistant starch, carbohydrates or other indigestible fractions from the intaken nutrition (Minekus *et al.*, 1999; Campos-Vega *et al.*, 2009; Zamora-Gasga *et al.*, 2018).

Models that are operated in a continuous mode are more complex compared to batch systems and more similar compared with the original *in vivo* system. These studies investigated the whole communities and their influence on the complex metabolism instead of the behavior of single strains.

Duncan *et al.* (2009) investigated the effect of carbohydrates on the microbial community and screened for new probiotic strains. They further tested the influence of a varying pH value from 5.5 to 6.7 on the microbial composition (Duncan *et al.*, 2003). Therefore, they used a simulated human colonic fermenter based on a bioreactor with 500 mL working volume, continuously stirred and the temperature set to +37 °C. Medium was exchanged semi-continuous with a dilution rate of 2.4 d^{-1} and experiments were performed after 4 days of stabilization time (Hillman *et al.*, 1994). In the system, the abundance of *Bacteroides* increased whereas important butyrate-producing representatives as *Faecalibacterium*, *Ruminococcus* and *Roseburia* decreased, especially at higher pH values. In total, it was shown that the pH had a major impact

on the bacterial competition for soluble polysaccharides and therefore resulted in an altered microbial abundance. Comparable results were obtained when the cultivation pH was shifted from 5.5 to 6.5 within one cultivation batch. *Bacteroides* dominated the community at the higher pH value in the beginning with approximately 80% of total bacteria, while their abundance decreased to only 20% at pH 5.5, indicating an altered metabolism. Contrary, the abundance of *Roseburia* was higher at pH 5.5, whereas it was very low at pH 6.5. In total, the production of butyrate was increased after the shift to the lower pH, opposing a lower production of acetate (Walker *et al.*, 2005).

Bircher *eta al.* (2018) investigated the cultivation of an *in vitro* microbiota at pH 5.7 and detected the abundance of three main butyrate-producing bacteria. In their system, a physiological level of SCFAs was obtained and during the cultivation of donor 1 the abundance of Firmicutes increased while Bacteroidetes decreased. For donor 2 the behavior was contrary. In accordance with both donors, the abundance of butyrate producing *Faecalibacterium* (3-11%), *Roseburia* (0-0.7%) and *Eubacterium* (0%) was decreased rapidly compared to the donor stool. Further, overall diversity was decreased in their *in vitro* system compared to *in vivo*.

Similar results were obtained when the microbiota was cultivated *in vitro* to model the human distal gut and show the effect of antibiotics as clindamycin (McDonald, 2013). In this study a steady and reproducible culture was established in a single state chemostat model of the human distal gut. The broth volume in this study was held constant at 400 mL with a retention media time of 24 h. The vessel was maintained at a temperature of +37 °C and a pH of 6.9-7.0. McDonald (2013) investigated the microbial changes as well as well as the influence of different antibiotics. Compared with the stool samples, an increase in Bacteroidetes and a reduced abundance of Firmicutes was detected after the systems stabilized. Further, the abundance of Actinobacteria and the richness in the overall community dropped, while Proteobacteria showed an enlarged abundance.

1.4.2 *Cultivation conditions in existing models*

In total, the conditions were adapted to the conditions *in vivo* by choosing appropriate values for temperature, aeration, media composition and flow. Table 1.3 shows a comparison of selected studies working with *in vitro* systems and their administered cultivation conditions.

Table 1.3: Comparison of batch, fed-batch and continuous fermentation system of the human intestinal microbiota considering fermentation volume, aeration, pH value and retention time

Study	Volume	Aeration	pH value	Operation mode	Retention Time
(Takagi *et al.*, 2016)	100 mL	N_2:CO_2 (8:2)	6.5	Batch	-
(Zamora-Gasga *et al.*, 2018)	9 mL	Anaerob	7.0	Batch	-
(Campos-Vega *et al.*, 2009)	9 mL	H_2:CO_2:N_2 (1:1:8)	-	Batch	-
(Minekus *et al.*, 1999)	1600 mL	N_2	5.8	Fed-Batch	48 h (800 mL · 24 h^{-1})
(McDonald, 2013)	400 mL	N_2	6.9–7.0	Continuous	24 h (400 mL · 24 h^{-1})
(Bircher *et al.*, 2018)	200 mL	CO_2	5.7	Continuous	8 h (600 mL · 24 h^{-1})
(Walker *et al.*, 2005)	250 mL	CO_2	5.5/ 6.5	Continuous	24 h (250 mL · 24 h^{-1})
(Duncan *et al.*, 2003; Duncan *et al.*, 2009)	250 mL	CO_2	5.5/ 6.5	Continuous	24 h (250 mL · 24 h^{-1})

The systems all have in common a cultivation at a physiological temperature of +37 °C. In total, the pH gets adapted with 0.5-2.5 M NaOH and 0.5 M HCl to values between 5.5 and 7.0. The media used in the different studies are very similar in their composition. They consist mainly of components that are also existent in the large intestine and were developed in further studies (Gibson *et al.*, 1988; Macfarlane and Gibson, 1998; Michel *et al.*, 1998; Lesmes *et al.*, 2008). Ingredients are resistant starch as amylopectin, as well as polysaccharides like xylan and arabinogalactan. Peptone serves as a main source of nutrients. Further, different salts as natrium chloride or

phosphates (K_2HPO_4 and KH_2PO_4) are mixed with cysteine, which has the function to lower the redox potential in the broth and prepare the conditions for an anaerobic milieu. Hemin provides iron for the microorganisms. The media also contain primary bile salts and vitamin K, which are essential for the growth of anaerobic intestinal bacteria. Further, mucin is included due to its protective properties. In the continuous cultivation systems, these media are exchanged completely every 8-48 h, whereas the majority of the systems chose a retention time of 24 h representing the physiological retention time of approximately 20-30 h (Kim and Rhee, 2012). In the systems, anaerobic conditions were provided by sparging the broth with either CO_2 (Duncan *et al.*, 2003; Walker *et al.*, 2005; Duncan *et al.*, 2009; Bircher *et al.*, 2018), N_2 (Minekus *et al.*, 1999; McDonald, 2013) or gas mixtures containing CO_2, N_2 and H_2 (Campos-Vega *et al.*, 2009; Takagi *et al.*, 2016). The volumes varied from 9-1,600 mL in the batch and between 200-400 mL in the continuous systems. The broth in the vessels was exchanged regularly by adding new media on the one and withdrawing broth on the other side.

1.4.3 *Challenges of in vitro cultivation models*

Up until now, only models with a vessel volume between 200 and 400 mL were prosecuted in the continuous mode. Only models with volumes too low to continuously harvest higher amounts of infusion material for FMT or complex multi-stage systems with high maintenance efforts and complicated set-ups have been applied. When performing FMT, higher infusion volumes between 200 and 500 mL provided a better clinical outcome (Gough *et al.*, 2011; Aroniadis and Brandt, 2013). Consequently, when this *in vitro* microbiota shall be used as infusion material, high volumes are required. Therefore, part of this work was to establish a less complex, easy to use system by combining different parts of existing cultivation models. For this purpose, a single-stage cultivation bioreactor without cell retention was chosen (chapter 3). The greatest discrepancy between the here compared systems next to the cultivation volume is the cultivation pH value. In total, the pH gets adapted with 0.5-2.5 M NaOH and 0.5 M HCl to values between 5.5 and 7.0. The influence of these variations on the system's behavior is great, as the abundance of microbial cells, as well as cell count and production of metabolites are dependent on the cultivation pH. With setting the pH value to different physiological values, different systems will be established. Therefore, a part of this work (chapters 3 and 4) was conducted to investigate the composition, diversity, and variety of microbes of an *in vitro* microbiota dependent on the established cultivation system and set cultivation pH value.

Next to cultivation, the viability of the microbiota should be maintained during all processing steps. Therefore, the preservation process of bacteria must be chosen wisely to guarantee a high cell survival, vitality, and ability to re-grow.

1.5 Preservation of microorganisms

Microorganisms can be preserved through freezing or different drying techniques and processes. Drying is defined as a mechanical and thermic moisture removal, where the water content is reduced to a minimum of around 1 to 10% related to the dry matter. The main objective is to extend the culture's storability.

In the industry, most commonly applied for bacteria, especially probiotic cultures, is freeze-drying although it is the most cost and time extensive drying process (Broeckx *et al.*, 2016). Freeze-drying is performed with low temperatures and pressures, which prevent chemical decomposition, are less likely to be injurious to labile bioproducts, and allow good rehydration properties (Franks, 1998). The process itself consist of three phases: freezing, primary drying and secondary drying. Freezing is mostly done by lowering the temperature of the liquid culture through freezers (e.g., -80 °C) or liquid nitrogen. Here, the choice of the freezing rate is important as it influences the survival of cells after drying (Santivarangkna *et al.*, 2008b). The main drying occurs in the frozen state below the triple point of water (6.12 mbar, 0.01 °C), where all free and frozen water is removed through sublimation. At the end of the sublimation phase, no ice crystals remain, whereas non-frozen water is still bound in product. The secondary drying phase therefore aims to remove unfrozen water molecules by desorption.

During freeze-drying, microorganisms can be harmed through freezing stress, oxidative injury, and dehydration. The main reasons for cell death are alterations in the cell membrane. The cell membrane is a lipid double layer consisting of phospholipids with a polar phosphate head and a hydrophobic tail. They form a bilayer due to their amphiphilic character with the fatty acids inside and the headgroups at the surface. It is stabilized by non-covalent bindings, especially van-der-Waals forces, or hydrogen bonds. In the native and hydrated state, the membrane is in a liquid crystalline phase, with water molecules between the lipid heads (Figure 1.8).

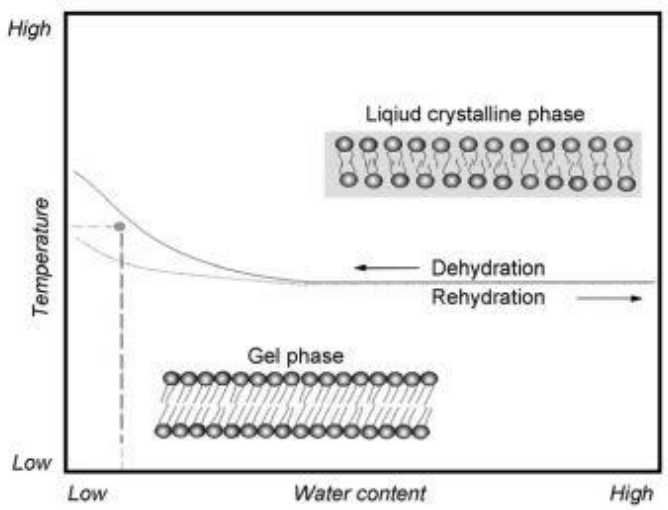

Figure 1.8: A simplified phase diagram of a phospholipid cell membrane during dehydration and rehydration and the transformation between liquid crystalline and gel phase; image from Santivarangkna *et al.* (2008)

During freezing, the cell membrane can get damaged mechanically by the formation of intracellular ice crystals (high freezing rate) was well as osmotic stress and frozen water outside the cells by a low freezing rate (Santivarangkna *et al.*, 2008b). During drying, these water molecules get removed and the cell membrane undergoes a phase change to the gel phase as Van-der-Waals forces are increasing. Thus, the phospholipids approach closer to each other. The phase transition does not occur at the same time at each part of the membrane, which leads to leakage in the cell membrane. In turn, cells with leakages lose their vitality and viability and die. These damages can be decreased by choosing appropriate process parameters.

1.5.1 *Drying of probiotics*

Nevertheless, the survival of single cell cultures is highly dependent on the cultivation and process parameters as well as the bacterial strain. For instance, for *Lactobacillus coryniformis* survival rates between 20-75% depending on the cultivation conditions were reached (Schoug *et al.*, 2008), whereas 40% of *Lactobacillus paracasei* survived a comparable freeze-drying process (Ambros *et al.*, 2018b). These microorganisms are abundant in the human intestinal microbiota as well as a variety of further different phyla and bacterial strains. This poses a nearly insolvable challenge to identify a drying process that fits all representatives in the microbiota. Part of this work was also to develop a drying protocol for a probiotic test strain of the human intestinal microbiota that can be used for the drying of the entire microbial ecosystem as well (chapter 5). As probiotic test strain *Bifidobacterium longum* was chosen, which is part of the human intestinal microbiome and additionally known for its probiotic properties (Kailasapathy and Chin, 2000; Sugahara *et al.*, 2015). The optimal pH value during cultivation for this strain is pH 6.0, which may further promote a higher survival after drying (Kiviharju *et al.*, 2005; Bauer *et al.*, 2012). During storage, different studies determined low storage temperature and a low relative humidity as key factors to obtain high survival rates (Hsiao *et al.*, 2004; Abe *et al.*, 2009; Min *et al.*, 2017).

1.5.2 *Preservation of the human intestinal microbiota*

In contrast to single cell cultures, the preservation of the human intestinal microbiota as an ecological system with several different microorganisms sets more challenges. Up until now, the microbiota has already been preserved in different ways: freezing or drying of stool samples as well as freezing of *in vitro* cultivated microbiotas. The main aim for preserving the human intestinal microbiota is to gain a product that may enhance or replace the traditional fecal infusion material for fecal microbiota transplantation. The application of a purified, frozen and afterwards thawed donor stool suspension has already been tested and resulted in recovery rates of up to 95% (Hamilton *et al.*, 2012; Smirnova *et al.*, 2019; Nicco *et al.*, 2020). Further to that, different researchers investigated the treatment success with lyophilized feces.

Vigvári *et al.* (2019) purified donor stool by suspending 60 g of sample in 200 mL of saline (0.9%). After filtration, 100 mL of the filtrate were frozen at -20 °C. The lyophilization took place at -40 °C under vacuum. After application in patients via a nasogastric tube, they determined recovery rates of up to 83%. Others prepared a fecal slurry that was lyophilized for 36 h at a shelf temperature of -20 °C, followed by a 6 h process step at +30 °C (Staley *et al.*, 2017). When powder was applied through hypromellose capsules, the treatment succeeded for 88-100% of the patients (Staley *et al.*, 2017; Staley *et al.*, 2019). After the treatment with this powder, the microbial diversity increased, as well as the relative abundances of Firmicutes and Bacteroidetes. Contrary, the relative abundance of Proteobacteria decreased and 43 of 49 patients achieved clinical success with no recurrence of CDI over two months (Staley *et al.*, 2017). Next to lyophilization as preservation method, others assessed the technical feasibility of the cryopreservation of an *in vitro* microbiota for FMT (Bircher *et al.*, 2018). Therefore, purified stool was cultivated in a continuous system for 10 days at pH 5.7, harvested, centrifuged and the pellet stored at -80 °C. The microbiota was then thawed and re-cultivated for 24 h in a batch cultivation system. In the re-cultivated system, the metabolism was lowered and the concentration of all major SCFAs decreased compared to the initial system. Further, when preserving without protectants, the abundance of butyrate producing microorganisms *Faecalibacterium* and *Roseburia* showed a strongly impaired growth.

1.6 **Microbiota profiling by 16S rRNA gene amplicon sequencing**

To identify microbial changes in different systems the determination of a molecule's nucleotide sequence, the so-called sequencing, is used. Therefore, the whole genome or only specific parts of it are sequenced. The principle is based on the sanger sequencing method, which was refined by several researchers (Labeit *et al.*, 1986; Godon *et al.*, 1997). Next generation sequencing (NGS) as a step further is referred to a high-throughput method, which enables the analysis of multiple samples in parallel (Behjati and Tarpey, 2013). The analysis of microbial communities is mostly based on the sequencing of the 16S subunit of ribosomal ribonucleic acid (16S rRNA). These genes are present in almost all bacteria and are used in reconstructing phylogenies as they only show slow rates of evolution of this region of the gene. Further, the sequence with 1,500 base pairs is large enough for bioinformatic processing purposes (Patel, 2001). In this work, not the complete 16S rRNA, but 444 nucleotides of the V3/V4 region, which contains highly conserved and variable regions, were sequenced (Figure 1.9). This allows the identification and differentiation of bacteria until the genera level.

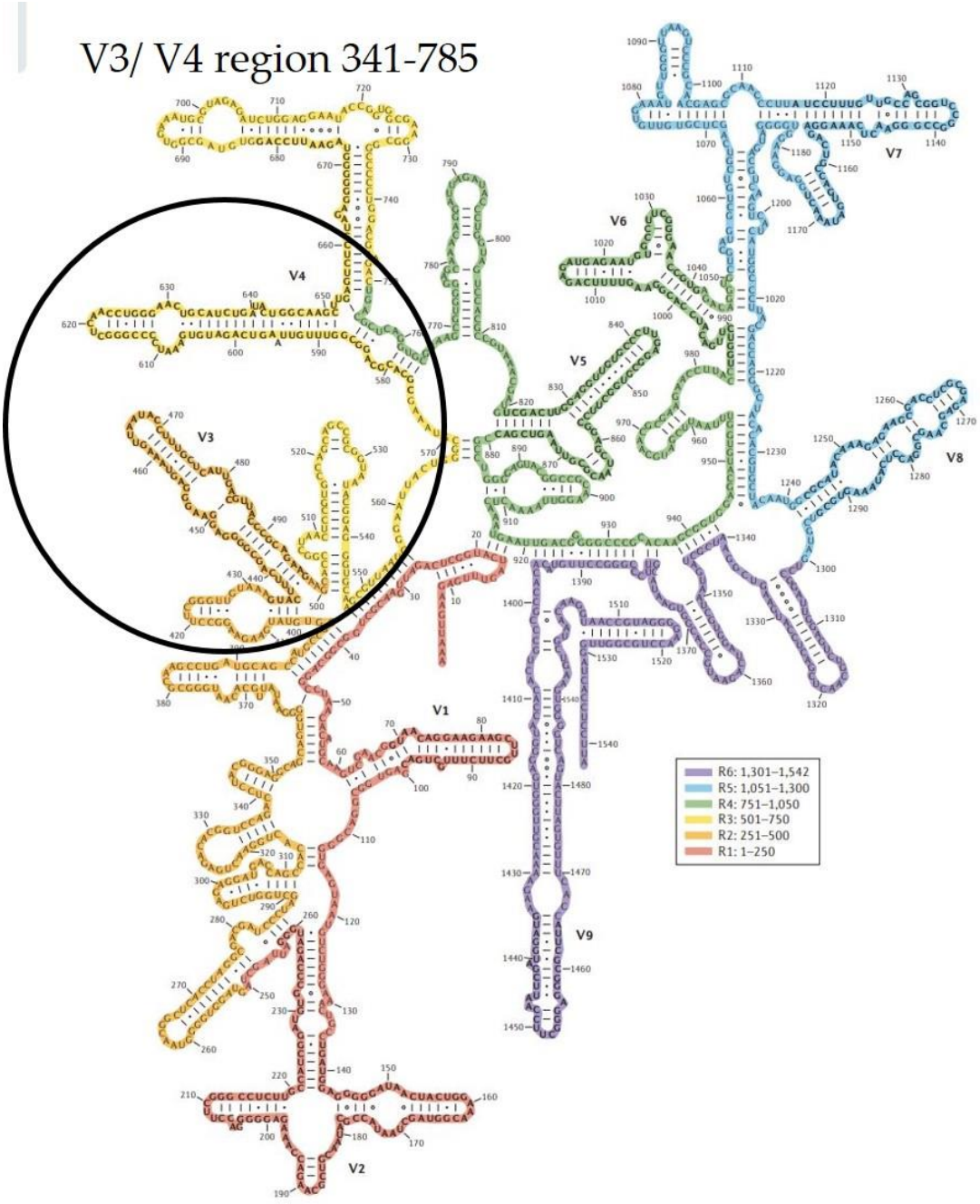

Figure 1.9: Scheme of 16S rRNA with the marked V3/ V4 region (base pair 341-785); modified from Yarza *et al.*, (2014)

1.6.1 *Principle procedure of 16S rRNA gene amplicon sequencing*

The samples get collected from the microbial environment and mostly stored with DNA stabilizer before the DNA is extracted, library constructed and the samples sequenced (Reitmeier *et al.*, 2020c). In general, the principle procedure of 16S rRNA gene amplicon sequencing is based on three steps, which are shown in detail in Figure 1.10 (Illumina, 2010):

- Library preparation
- Amplification
- Sequencing

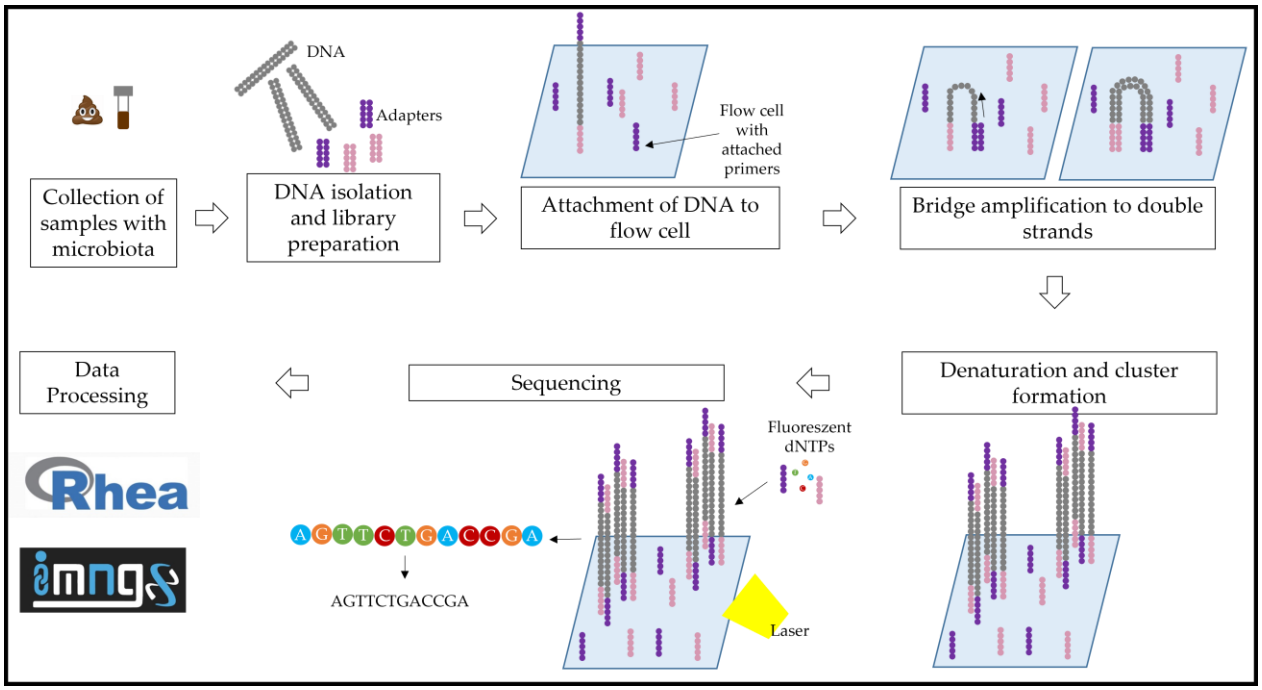

Figure 1.10: Basic process steps of 16S rRNA gene amplicon sequencing (Illumina, 2010; Lagkouvardos *et al.*, 2016; Lagkouvardos *et al.*, 2017)

Library preparation

Libraries are created by using random fragments of the collected DNA, followed by a ligation with custom linkers. Therefore, the DNA is fragmented by enzymes or by sonication to create smaller strands. The DNA ligase then ligates these fragments with short, double-stranded pieces of synthetic DNA, the so-called adapters. These adapters have a "sticky" and non-cohesive, "blunt" end with the view to join their blunt end to the blunt end of the DNA. To prevent the potential problem of base pairing between molecules and therefore dimer formation, the chemical structure of DNA is utilized. Normally, ligation takes place between 3'-OH and 5'-P ends. By removing the phosphate end, the ligase is unable to form a bridge between the two ends.

Amplification

Amplification is used to create up to 1,000 identical copies of each single template molecule to receive signals in the further process that are strong enough to be detected accurately. Therefore, the surface of the measurement flow cell is densely coated with primers that are complementary to the primers attached to the DNA library fragments. The DNA binds randomly to the surface of the flow cell channels where it is exposed to reagents for polymerase-based extension. When adding unlabeled nucleotides and enzymes, solid-phase bridge amplification is initiated, attaching the free ends of the single strands of DNA to the surface of the cell and consequently creating bridged structures. The added enzymes then incorporate nucleotides to build double-stranded bridges. After denaturation occurs, two single-stranded DNA fragments are anchored to the flow cell surface. Amplification is completed as repetition of the process leads to dense clusters of double-stranded DNA.

Sequencing

Squencing can be done by different methods as pyrosequencing, ion torrent semiconductor sequencing or reversible terminator sequencing (the Illumina principle used in this work). Each sequencing cycle starts when four terminators, primers and DNA polymerase are added to the prepared flow cell. These terminators are fluorescent labeled deoxynucleoside triphosphates (dNTP), including deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxycytidine triphosphate (dCTP) and deoxyguanosine triphosphate (dGTP). They serve as terminators for polymerization. During each sequencing cycle, a single labeled dNTP is added to the nucleic acid chain and identified by measuring the emitted fluorescence. The next sequencing cycle repeats the procedure, and the cycles are repeated to determine the entire sequence base by base in the entire fragment.

Finally, all data are aligned and compared in the further data processing with references to identify differences between the clustered samples.

1.6.2 *Data processing and characteristic values*

Data is further processed and provided into an Operational Taxonomic Units (OTU) table. In an OTU, sequences that share at least 97% of similarity comparing their 16S rRNA, are clustered. This allows the reliable differentiation of cells until the genera level. To distinguish the sequences on species level a larger part of the genome must be sequenced. Nevertheless, to obtain a reliable insight in the here used ecosystems and to track changes and alterations, the identification on genera level is considered as sufficient. The further data processing depends on the experimental approach. The sequences can be aligned with taxonomic information at different bacterial level (kingdom, phyla, classes, orders, families, and genera) or diversity within or between different groups (alpha-diversity, beta-diversity, and serial-group-comparisons) can be identified. In this work, the OTU table was obtained by the software Integrated

Microbial Next Generation Sequencing (IMNGS) and the Rhea pipline was used as a tool for post-OTU output processing (Lagkouvardos *et al.*, 2016; Lagkouvardos *et al.*, 2017). The Rhea script is based on the statistical computing software R, allowing to normalize OTU input tables, calculate alpha diversity, beta diversity, taxonomic binning, serial group comparisons, and correlations between the groups. In the following, these characteristic indicators, especially normalization, richness, Shannon index and taxonomic binning, which were used in this study, will be described in detail.

Normalization

After the sequencing process, the single reads with a similarity of at least 97% cluster in OTUs. Consequently, in different samples, a different number of OTU reads appears. Exemplary, Table 1.4 shows the number of OTUs in three different samples. For sample A a sum of 2198 of total reads, for sample B 3712 reads and for sample C 4879 reads were detected. To be able to compare these different samples, the reads get normalized. As a result, the percentage of abundance of an OTU within one sample before and after normalization is the same and the reference value of all samples is set to the same value. In the example given in Table 1.4, the reference is set to sample A and 2198 reads. Consequently, after normalization the absolute number of reads in all samples is set to 2198, maintaining the relative composition for samples B and C.

Table 1.4: Exemplary contribution of OTUs within three samples before and after normalization

	Before normalization			**After normalization**		
	Sample A	*Sample B*	*Sample C*	*Sample A*	*Sample B*	*Sample C*
OTU_1	22	51	321	22	30.20	144.61
OTU_2	289	1369	4012	289	810.63	1807.41
OTU_3	1206	58	31	1206	34.34	13.97
OTU_4	557	2146	98	557	1270.72	44.15
OTU_5	124	88	417	124	52.11	187.86
sum	2198	3712	4879	2198	2198	2198

Alpha Diversity

Alpha Diversity represents the diversity of OTUs within one sample. There are several characteristic values of diversity: richness, Simpson index, Simpson effective index, Shannon index and Shannon effective index. Calculating diversity, the cut-off level when processing data with IMNGS and Rhea is essential. The cut-off defines a limit of abundance the single OTU has to reach at least to be considered for the calculation of alpha diversity. Therefore, typical cut-offs are between 0.1-1%. If the cut-

off is set to low, the data set increases greatly, which can hinder data processing. Further, OTUs with an abundance above the limit are unlikely to have a biologically important function in the investigated system. Also, very low limits can lead to the detection of polymerase chain reaction (PCR) and sequencing artifacts or contamination. In Rhea, the normalized OTUs have a 0.5 abundance limit, which however may be adjusted.

As an important characteristic value of alpha diversity, richness is measured by the total sum of different OTUs present in one sample. Figure 1.11 shows a simplified community consisting of two different species.

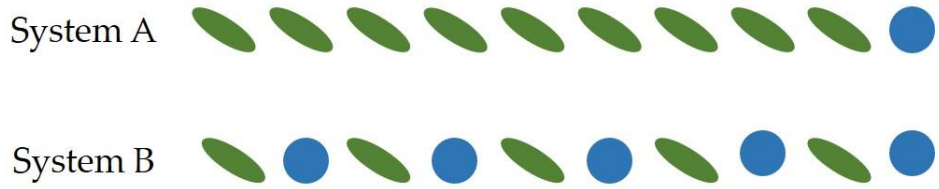

Figure 1.11: Exemplary systems with different distribution of two abundant species

Consequently, richness is 2 and equal for system A and B (Table 1.5). However, richness does not give any information about the relative abundance and distribution of different OTUs. To determine community structures, the two indices Simpson (Simpson, 1949) and Shannon (Shannon, 2001) and the effective measures, Simpson effective and Shannon effective, were developed (Jost, 2006). All contain information on the number of species, their abundance, and the overall uniformity in the sample. The Simpson index asses more the highly abundant species within one sample compared to the Shannon index. For a more direct comparison, Shannon effective and Simpson effective were invented, which give more information about the diversity itself in the community. For the given example, Simpson, Simpson effective and Shannon result in different values as Table 1.5 shows. In this work, the Shannon effective was used to compare the systems. Here, the Shannon effective for system A is 1.38, meaning there are 1.38 abundant occurring species, whereas it is 2 for sample B. Consequently, a higher Shannon effective represents a higher diversity in the systems.

Table 1.5: Calculation of Alpha diversity for the two different systems A and B shown in Figure 1.11

	Richness	**Simpson**	**Simpson effective**	**Shannon**	**Shannon effective**
System A	2	0.82	1.22	0.33	1.38
System B	2	0.5	2	0.69	2

Beta Diversity

While Alpha Diversity defines the diversity within OTUs per sample, beta diversity is defined as the diversity of OTUs between different sample groups. It statistically determines the distance in a multidimensional space above taxonomic variance. After calculation, a distance matrix is the output, which shows a pairwise comparison across all samples based on the phylogenetic distance. It is generated using a method called UniFrac, which compares the distance of different species (OTUs) in one sample to the distance of the OTUs of another other sample. Hereby, zero means the samples show an equal similarity regarding the different OTUs (Lozupone *et al.*, 2007). In this work, beta diversity was not calculated to compare different sample and experiment groups. Here, a statistical analysis based on a one-way ANOVA was consulted as this was considered sufficient to explain the behavior of the continuous flow fermentations in detail on a biotechnological level.

Taxonomic Binning

As explained above, alpha and beta diversity explain the diversity within or between samples but give no information about the present microorganisms. Therefore, taxonomic binning aims to connect each OTUs sequence read with their taxonomies. Therefore, the reads are compared with already existing 16S rRNA reads in databases as EzTaxon, Silva or rdp. The different taxonomic levels from general to detail are kingdom, phylum, class, order, family, and genus. As the sequencing is based on the 16S rRNA, a differentiation of the OTUs is only possible until the genera level. Consequently, the microbial abundances in an ecosystem based on phyla level give a first insight in the system. Nevertheless, for a better understanding of the ongoing mechanisms, a more detailed observation, based on genera level is recommendable.

2 Motivation and objectives

As the chapters above describe, disbalances in the human intestinal microbiota can lead to several gastric diseases as an infection with *Clostridium difficile*. Next to the standard therapy with antibiotics, an alternative treatment method is Fecal Microbiota transplantation, where purified stool from a healthy donor is transferred into the patient's gut to restore the microbial balance and diversity. Fecal Microbiota transplantation leads to high recovery rates. However, constraints are an extensive donor screening, low storability of stool and the lack of a standardized procedure. A possibility to overcome these constraints offers the production of a controlled and stable artificial colonic microbiota. It has already been proven that the application of *in vitro* microbiota slurry (Bircher *et al.*, 2018) as well as of a lyophilizate (Vigvári *et al.*, 2019) was already successful.

This work combines existing models and approaches and is aiming to establish a preservation concept for the generation of transferrable material *in vitro* and further conserving the artificial microbiota with an appropriate process (Figure **2.1**). Therefore, the work is divided in several parts: the establishment and validation of an *in vitro* cultivation system for the human intestinal microbiota, the development of a drying protocol and further the re-cultivation to review and validate the drying outcome and ability to re-establish.

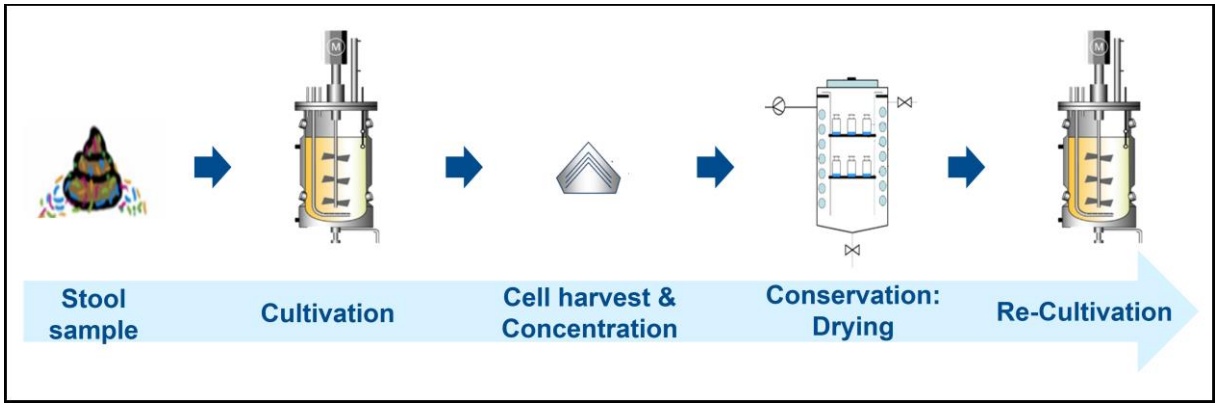

Figure 2.1: Entire preservation process applied in this work including initial donor stool, cultivation, cell harvest and concentration, conservation by freeze-drying and re-cultivation

For FMT larg infusion volumes of up to 500 mL trend to have a better outcome (Gough *et al.*, 2011; Aroniadis and Brandt, 2013). Existing models so far only provide vessel volumes up to 400 mL. Hence, a higher vessel volume can create larger amounts of infusion material under controlled conditions. Chapter 3 examines the combination of different parts of existing cultivation models to establish a less complex, easy to use system by choosing a single-stage cultivation bioreactor without cell retention with a higher vessel volume. This enables the generation of larger amounts of infusion material for FMT. Next to the cultivation system, the influence of the donor stool was investigated. The open question was if the preprocessed stool is able to establish a diverse and stable community with gut representatives suitable for a FMT treatment.

Further it was investigated in which way the cultivation system itself, but also the individual donor stool characteristics affect the microbiota characteristics in the continuously harvested cell suspension.

Existing models have a huge range of applied values from 5.5 to 7.0 (Table 1.3). However, its influence on the system's behavior is great as it affects the composition of community in the formed system. Next to the abundance of microbial cells, the number of cells and production of metabolites are dependent on the cultivation pH value. Setting the pH value to different physiological values, different systems with different microbial and metabolic composition will be established. Therefore, chapter 4 identifies the influence of the cultivation pH on the microbiota characteristics cell count, microbial diversity, and composition as well as on metabolized compounds.

Drying processes lead to products with a higher shelf life and availability. To establish a lyophilization protocol for the established *in vitro* microbiota, *Bifidobacterium longum* ssp. *longum*, a representative of the intestinal microbiota, was used as a test strain in chapter 5. Therefore, the influence of cultivation strategy, freeze-drying process (shelf temperature, addition of protectants, protectant concentration) as well as storage conditions were investigated considering survival, membrane integrity and inactivation kinetics of the test strain. It was considered and investigated whether the experiences and results of a drying process of a single strain cell culture are transferrable for the drying of the processed *in vitro* microbiota.

The here established preservation process consisting of stool sampling, *in vitro* cultivation and lyophilization may be a possible enhancement of traditional FMT and a possibility to overcome its disadvantages and constraints (chapter 6). Open questions in this concept are still in which way the individual process steps, i.e., cultivation system, cultivation pH value as well as individual donor stool characteristics and the freeze-drying process affect the microbiota outcome and characteristics on a technical level. Identifying and adapting these critical processing steps, allow to generate a re-established *in vitro* system with characteristics considered healthy and diverse.

The overall aim of this study was to demonstrate the technical feasibility of the production of a culturable, stable community with a high diversity and richness of representative gut bacteria as well as a high abundance of health-promoting microorganisms and metabolites with the perspective of applying this as an extension of the current FMT concept.

3 Establishment of an *In Vitro* System of the Human Intestinal Microbiota: Effect of Cultivation Conditions and Influence of Three Donor Stool Samples

Summary and contribution of the doctoral candidate

This study developed and investigated the establishment of an *in vitro* continuous flow fermentation system of the human intestinal microbiota (Figure 3.1). Of interest here was the influence of the cultivation system itself as well as the characteristics of three donor stool samples.

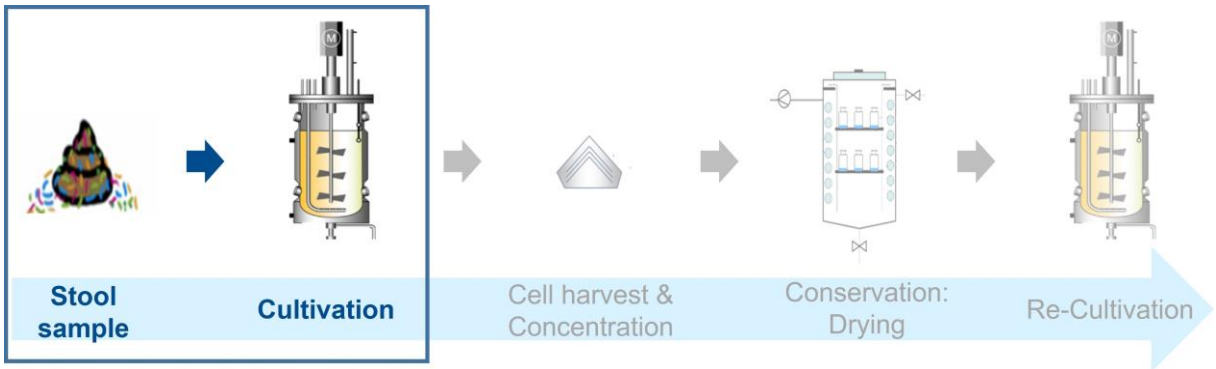

Figure 3.1: Investigated steps in the entire preservation process: influence of initial donor stool material and effect of cultivation conditions

The cultivation of a human intestinal microbiota in a continuous flow fermentation bioreactor without cell retention was inoculated with feces from three different donors each at a cultivation pH of 6.5 until a stable state was established. The influence of the fermentation conditions and donor stool characteristics regarding cell count, metabolic activity (short-chain fatty acid profile) and microbiota composition as well as richness and diversity was determined. Cultivation conditions were found to affect the microbial system regarding the number of cells and the production of short-chain fatty acids, which both showed increased values. In the composition of microorganisms, the abundance of Actinobacteria and Firmicutes decreased and Bacteroidetes increased. The abundance of the minor phyla Proteobacteria and Verrucomicrobia was unchanged. While the richness in the system was unaffected, diversity (measured by the Shannon effective index) decreased. The cultivation of stool from different donors revealed that the behavior of the created *in vitro* systems was similar and comparable, but unique characteristics of the composition of the original stool remained.

The doctoral candidate substantially contributed to this manuscript by designing the cultivation set-up and the corresponding experiments. She further critically reviewed the literature and performed all data analysis including the statistical evaluation. She additionally wrote and revised the manuscript. Co-authors

contributed to the experimental part and to the discussion of the results and provided input to the drafted publication prior to submission.

Adapted original manuscript[1]

Establishment of an *In Vitro* System of the Human Intestinal Microbiota: Effect of Cultivation Conditions and Influence of Three Donor Stool Samples[2]

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Abstract

Fecal microbiota transplantation (FMT) is an alternative method for the treatment of gastrointestinal diseases with a high recovery rate. Disadvantages are ethical concerns, high donor requirements and the low storability of stool samples. The cultivation of an *in vitro* microbiota in a continuous bioreactor was established as an alternative to FMT to overcome these problems. In this study, the influence of the system parameters and donor stool characteristics was investigated. Each continuous colonic fermentation system was inoculated with feces from three different donors until a stable state was established. The influence of the fermentation conditions on the system's behavior regarding cell count, metabolic activity, short-chain fatty acid profile and microbiota composition as well as richness and diversity was assessed. Cultivation conditions were found to affect the microbial system: the number of cells and the production of short-chain fatty acids increased. The abundance of Actinobacteria and Firmicutes decreased, Bacteroidetes increased, while Proteobacteria and Verrucomicrobia remained largely unaffected. Diversity in the *in vitro* system decreased, but richness was unaffected. The cultivation of stool from different donors revealed that the performance of the created *in vitro* system was similar and comparable, but unique characteristics of the composition of the original stool remained.

3.1 Introduction

The human gut hosts a complex, very diverse and large ecosystem called the intestinal microbiota. Due to the high retention time of food and, therefore, the high amount

[1] Adaptions refer to formatting issues: e.g., abbreviations, figure, table, equation and section numbering, citation style, notation of units, spelling, axis labeling.

[2] Originally published in *Microorganisms* (2021), Vol 9 (5), pp. 1049-1066. Permission for reuse of this article was granted.

of nutrients, the colon has the highest abundance of microbial cells with up to 10^{11} CFU mL^{-1} and 400–1,000 different species (Eckburg *et al.*, 2005; Gill *et al.*, 2006; Sender *et al.*, 2016). One function of the gut microbiota is the generation of a wide range of bioactive compounds that result from the bacterial transformation of otherwise indigestible food components. Bacterial metabolites, such as short-chain fatty acids (SCFAs), play a role in the defense against pathogens and regulate glucose homeostasis as well as lipid metabolism (Kumar *et al.*, 2011; Vadder *et al.*, 2014; Rios-Covian *et al.*, 2017).

The microorganisms in the human intestinal microbiota belong to the phyla Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia. Firmicutes and Bacteroidetes represent the most abundant phyla, each constituting 40 to 50% of total bacteria. Actinobacteria (around 2.5%), Proteobacteria (0.1–1%) and Verrucomicrobia (~0.1%) are less abundant (Eckburg *et al.*, 2005; Dethlefsen *et al.*, 2007). Variety, diversity, and cell count have a major impact on the human health and immune system. Alterations in microbiota composition, e.g., a decrease in diversity and compositional changes, the so called disbalance, are related to several diseases (Holmes *et al.*, 2011). These diseases are linked with the digestive system (Ley *et al.*, 2005; Manichanh *et al.*, 2006; Larsen *et al.*, 2010; Lagier *et al.*, 2012; Schreiber *et al.*, 2014; Koliada *et al.*, 2017). Furthermore, the microbiota has been reported to have an impact on non-digestive diseases, such as Parkin-son's disease (Hill-Burns *et al.*, 2017) or cancer (Helmink *et al.*, 2019). For example, a decrease in microbial diversity and cell count is linked to *Clostridium difficile* infections (Lagier *et al.*, 2012; Heidebrecht *et al.*, 2019).

The standard therapy for *Clostridium difficile* infections (CDI) is treatment with antibiotics, such as vancomycin or metronidazole (Zar *et al.*, 2007), but this can result in the occurrence of relapses and multi-resistant strains. Fecal microbiota transplantation (FMT) offers an alternative therapy. Here, stool from a healthy donor is transferred into the patient to restore the diversity and balance of the microbial community in the gut. The donor must be healthy, must not have taken antibiotics before FMT and must run through extensive screening in advance. For the transplantation, the stool is mixed with saline or medium, homogenized, and filtrated, and the supernatant is transferred into the patient's colon by means of a colonoscopy, nasogastric tube, or enema. To increase the chance of recovery, the transfer of microorganisms as well as other molecules, e.g., short-chain fatty acids, is re-quired (Baktash *et al.*, 2018; Xiao *et al.*, 2020). Then, the success of FMT has been shown to result in recovery rates of up to 90% (Gough *et al.*, 2011; Aroniadis and Brandt, 2013; van Nood *et al.*, 2013). Nevertheless, the extensive screening, safety, and ethical concerns, acceptability and the lack of a standardized treatment procedure are the main constraints of the method. To date, different approaches for the enhancement of the FMT treatment method include applications of frozen or freeze-dried stool samples (Hamilton *et al.*, 2012; Bircher *et al.*, 2018; Vigvári *et al.*, 2019). Further, the cryopreservation of artificial microbiota was studied (Bircher *et al.*, 2018).

This study is part of a concept aiming at the generation of transferrable material *in vitro* to overcome concerns and challenges related to traditional FMT. The expectation is that by mimicking gut conditions *in vitro*, the production of controlled and stable artificial colonic microbiota would be possible. *In vitro* models have already been applied success-fully to reveal the mechanistic effects of probiotics, drug absorption and transport (Meunier *et al.*, 1995; Mennigen and Bruewer, 2009; Hemarajata and Versalovic, 2013; Bein *et al.*, 2018; Jalili-Firoozinezhad *et al.*, 2018; Li *et al.*, 2019). Advantages of *in vitro* models are uncomplicated set-ups, operation and possibilities for variation and adaption of the system and, furthermore, the absence of ethical considerations. Several systems aiming to mimic cultivation conditions similar to the human colon have already been studied (Molly *et al.*, 1994; Minekus *et al.*, 1999; Duncan *et al.*, 2003; Walker *et al.*, 2005; Campos-Vega *et al.*, 2009; Duncan *et al.*, 2009; McDonald, 2013; Takagi *et al.*, 2016; Bircher *et al.*, 2018; Zamora-Gasga *et al.*, 2018). They commonly have a gas sparging system supplying CO_2, N_2 or forming gas to ensure anaerobic conditions. The temperature is normally set to +37 °C. Nutrients are supplied by a complex growth medium adapted in accordance with Macfarlane and Gibson (1998). To simulate the passage of food, continuous flow systems were applied with retention times of mainly 24 h (Duncan *et al.*, 2003; Walker *et al.*, 2005; McDonald, 2013), similar to the mean retention time in the human colon (Kim and Rhee, 2012). The current models worked with a vessel volume of 9 (Campos-Vega *et al.*, 2009) to 400 mL (McDonald, 2013). For FMT, the volume of the used stool suspensions varied between 200 and 500 mL, with larger volumes yielding a better outcome (Gough *et al.*, 2011; Aroniadis and Brandt, 2013). To date, only models with a vessel volume too low to continuously harvest higher amounts of infusion material for FMT or complex multi-stage systems requiring high maintenance efforts and complicated set-ups have been tested.

In this study, we combined different parts of existing cultivation models to establish a less complex, easy to use system by choosing a single-stage cultivation bioreactor without cell retention with a higher vessel volume of 850 mL. This should enable the generation of larger amounts of infusion material for FMT by the continuous removal of cell suspension in a flow-through mode according to the feed volume flow at the system's inlet to keep the volume constant over time. Nevertheless, it is unknown whether an amplification of the vessel volume and a simplification to a single-stage system without cell retention still generates a culturable and stable community with a similar richness and diversity. The approach was as follows: the impact of the cultivation conditions themselves as well as the individual characteristics of the donor stool were observed until the formation of a stable state in composition and number of cells was achieved. The open question was in which way the cultivation system itself, but also the individual donor stool characteristics, affects the microbiota characteristics in the continuously harvested cell suspension. The aim was to demonstrate the technical feasibility of the production of a culturable, stable community with a high diversity and richness of representative gut bacteria. Furthermore, the differences that

may occur by the cultivation of three various stool samples were identified. The bioreactor system established in this study may be preferentially used for the cultivation of artificial colonic microbiota instead of direct FMT in later studies.

3.2 **Materials and methods**

3.2.1 *Donor Stool*

For this study, stools from three different donors of one defecation each were used. The donors were chosen and tested regarding the criteria of Terveer *et al.* (2017). To minimize external influences, the donors were the same age group (donor A: 28 years old; donor B: 25 years old; donor C: 27 years old), ethnic group (Caucasian) and social environment. All donors had a normal body mass index (BMI) of 23 (donor A) and 21 (donors B and C) and ate a Western diet. Their last antibiotic treatment was at least 12 months ago. The stool was obtained in house and stored immediately at −80 °C prior to the experiments. To prevent a potential transfer of diseases, the donors' stool was tested for bacterial, viral and eucaryotic pathogens that are relevant for *in vitro* cultivation by the Institute for Medical Microbiology and Hygiene, University of Regensburg.

3.2.2 *Cultivation Medium*

For the preparation of inoculum and the cultivation itself, a medium mimicking the chyme in the human colon was used. It was adapted according to Macfarlane and Gibson (1998) and called Continuous Flow Fermentation Medium (CFF) in the following. As shown in Table 3.1, the medium was prepared from six different stock solutions, where stock solution 1 was mixed with 800 mL double distilled H_2O. Stock solution 2 and 3 were each mixed with 50 mL, and stock solution 4 was diluted in 100 mL. Solution 2 was subsequently filtrated with a 0.22 µm filter, whereas solution 1 and 4 were each autoclaved. For the preparation of solution 5, hemin (0.05 g) was dissolved in 1 mL 1 M NaOH and filled up to 100 mL with ultrapure H_2O. Solution 6 was produced by mixing 0.1 g Vitamin K_1 with 20 mL 95% ethanol. Solution 5 and 6 were stored in the refrigerator until use. Solution 3 was mixed, stirred, and centrifuged using an Allegra X15R centrifuge (Beckmann Coulter Inc., Brea, CA, USA; 20 min, 6000 g, +4 °C). The supernatant was subsequently filtrated (0.22 µm) and joined with solutions 1, 2, 4, 5 and 6 under sterile, anaerobic conditions. The final medium was stored at +4 °C and used within 72 h.

Table 3.1: Ingredients for preparation of 1,000 mL Continuous Flow Fermentation Medium adapted according to Macfarlane and Gibson (1998)

Stock Solution	Reagent	Manufacturer	Weight [g]	Dissolution in Double Distilled H_2O [mL]
1	Casein peptone	Gerbu Biotechnik, Heidelberg, Germany	1.3	800
	Yeast extract	Gerbu Biotechnik, Heidelberg, Germany	2	
	$NaHCO_3$	Bernd Kraft, Duisburg, Germany	2	
	$CaCl_2$	Sigma-Aldrich, Saint-Louis, MO, USA	0.01	
	Pectin from citrus	Sigma-Aldrich, Saint-Louis, MO, USA	2	
	Xylan from beechwood	Iris Biotech, Marktredwitz, Germany	2	
	Arabinogalactan	Sigma-Aldrich, Saint-Louis, MO, USA	2	
	Starch	Merck KGaA, Darmstadt, Germany	5	
	Casein	Sigma-Aldrich, Saint-Louis, MO, USA	3	
	Inulin from Dahlia tubers	Sigma-Aldrich, Saint-Louis, MO, USA	1	
	NaCl	Carl Roth GmbH, Karlsruhe, Germany	0.75	
2	K_2HPO_4	Sigma-Aldrich, Saint-Louis, MO, USA	0.04	50
	KH_2PO_4	Sigma-Aldrich, Saint-Louis, MO, USA	0.04	
	$MgSO_4$	Sigma-Aldrich, Saint-Louis, MO, USA	0.01	
3	Bile salts	Sigma-Aldrich, Saint-Louis, MO, USA	0.5	50
	L-Cysteine	Gerbu Biotechnik, Heidelberg, Germany	0.5	
4	Porcine gastric mucin (type II)	Sigma-Aldrich, Saint-Louis, MO, USA	4	100
5	Hemin solution	Sigma-Aldrich, Saint-Louis, MO, USA	10	-
6	Vitamin K1 solution	Alfa Aesar, Karlsruhe, Germany	0.2	-

3.2.3 *Fecal Inoculum*

For all experiments, the stool from one defecation of each donor was used. The preparation of inoculum was adapted in accordance with McDonald (2013) and is similar to the preparation of infusion suspensions for FMT (Aroniadis and Brandt, 2013). Therefore, a 10% (*w*/*v*) fecal slurry was prepared by mixing 30 g of stool with 300 mL CFF medium and homogenizing it for 15 s (Melissa, Adexi, Skødstrup, Denmark). To remove large food residues, the mixture was centrifuged (Allegra X15R, Beckmann Coulter Inc., Brea, CA, USA; 180 g, +4 °C, 10 min), and 120 mL of the resulting supernatant was used as the inoculum for each experiment of this study.

3.2.4 *In vitro cultivation system*

The set-up of the cultivation system was adapted in accordance with already existing systems with the focus on an easy set-up and handling as well as a sufficient generation of transferable material (Duncan *et al.*, 2003; Walker *et al.*, 2005; McDonald, 2013). Therefore, a continuous system without cell retention was chosen to constantly generate an output of a transferable slurry without a high need for constant surveillance and service. Further, a vessel volume of 850 mL was chosen. All cultivation experiments were performed in a *BioStat B* bioreactor (Sartorius AG, Göttingen, Germany). Cultivation was started by transferring 120 mL of inoculum sterile into the glass vessel containing 730 mL of anaerobic CFF medium. Anaerobic conditions in the bioreactor were maintained by sparging continuously with 8 ccm forming gas. The process conditions were adapted to the human colon by setting the temperature to +37 °C. Furthermore, the pH was adjusted to 6.5 (Payne *et al.*, 2012) and regulated with 1.25 M NaOH and 0.5 M HCl, and the broth was stirred at 200 rpm. The turbidity was measured by an internal sensor to assess the cell density. Following a batch start-up phase of 24 h to allow the low number of cells in the inoculum to grow, the system was set to a continuous mode after 24 h of processing time by adding fresh CFF medium (automatic inflow of 0.5 mL min^{-1}) and harvesting cell broth in the same amount. As a result, the mean retention time was 28.3 h, which is comparable to the human colonic transit time (Kim and Rhee, 2012). All cultivations were run for 120 h without interruption. At various time points during cultivation, cell broth was pumped anaerobically into prepared sample tubes (Greiner Bio-One, Kremsmünster, Austria). The cell broth was either used immediately (analysis of cell count) or stored at −80 °C for further analysis (analysis of SCFAs and sequencing). Storage under these conditions did not affect the analytical results.

3.2.5 *Analysis of Facultative Aerobic and Anaerobic Cell Count*

The numbers of anaerobic and facultative aerobic colony forming units (CFUs) were analyzed separately. Therefore, samples were collected and diluted with 0.25 strength Ringer's solution and either plated on Wilkins–Chalgren Anaerobe agar plates (anaerobic cell count) or Plate Count agar plates (facultative aerobic cell count). For each point of time, up to three dilution factors and at least four plates per dilution were plated. The plates were incubated either aerobically or anaerobically for 48 h at +37 °C, and only plates with 30 to 300 colonies were included in the analysis. The number of CFUs N per mL of sample was calculated according to the (3.1. Here, c is the sum of colonies of the subsequent dilutions with n_1 the number of colonies in the less diluted solution, and n_2 is the number of colonies in the more diluted solution.

$$N = \frac{c}{n_1 + (0.1 \cdot n_2)} \tag{3.1}$$

3.2.6 *Analysis of Short-Chain Fatty Acids by Means of High-Performance Liquid Chromatography*

The concentration of sugars (glucose and galactose), metabolic intermediates (succinate and lactate), as well as the main short-chain fatty acids (SCFAs) acetate, propionate, butyrate and isovalerate, were identified and measured by means of high-performance liquid chromatography (HPLC). The HPLC unit (Agilent Technologies Inc., Santa Clara, CA, USA) was equipped with an *Aminex HPXH-87H* ion exclusion column (Bio-Rad Laboratories, Hercules, CA, USA) and a refractive index detector *G1362A* (Agilent Technologies Inc., Santa Clara, CA, USA). Separation was performed with 0.0005 mol L^{-1} H_2SO_4, 0.45 mL min^{-1} flow rate and 20 to 100 µL injection volume. Before injection, the samples were centrifuged (Hermel-Z233 M-2, Hermle Labortechnik GmbH, Wehingen, Germany; 6000 g, +20 °C, 30 min), and the supernatant was used after an additional filtration (0.22 µm). The metabolites were identified and quantified using external standards (Sigma Aldrich, Saint Louis, MO, USA) and the *Agilent ChemStation Instrument 1 offline* software (Agilent Technologies Inc., Santa Clara, CA, USA).

3.2.7 *Microbial Profiling by Means of 16S rRNA Sequencing*

The composition of the microbial community, richness and diversity were characterized in the stool samples and at several points of time during cultivation by sequencing the V3/ V4 region of 16S rRNA. High-throughput 16S rRNA gene amplicon sequencing was performed by an external lab (Microbiome Core Facility, ZIEL-Institute for Food and Health, TU Munich) according to the protocol described by Reitmeier *et al.*, (2020c). After receiving the raw data, they were preprocessed using the *IMNGS* pipeline (Lagkouvardos *et al.*, 2016). Before analysis, operational taxonomic units (OTUs) with a relative abundance <0.25% across all samples were removed to prevent the detection of spurious OTUs (Reitmeier *et al.*, 2020a). Furthermore, five nucleotides were trimmed on the 5′ and 3′ ends for the R1/ R2 read, and the expected error rate was set to 3 (trim score 3). Only nucleotides with a read length between 300 and 600 were considered. Analysis of alpha diversity and taxonomy was performed by the provided R script *Rhea* (Lagkouvardos *et al.*, 2017; Reitmeier *et al.*, 2020b). The evaluation of alpha diversity richness, representing the total number of OTUs in the community and the Shannon effective index, which accounts for the evenness and abundance of species in the community, was analyzed automatically by the software.

3.2.8 *Statistical Analysis*

In this study, all analyses were repeated at least in triplicate. The arithmetic mean $\bar{x}$ represents the mean value of the number n of all samples x_i. The distribution of the values was calculated from the standard deviation s due to the random error. All data,

graphs and tables in the following show arithmetic means ± standard deviations. Statistical significance was tested using a one-way ANOVA ($p \leq 0.05$) followed by a Tukey post hoc analysis conducted with the OriginPro 2019 software (OriginLab Corporation, Northampton, MA, USA).

3.3 **Results and discussion**

In this study, an *in vitro* system representing the human intestinal system was developed to cultivate the microbiota of three different donors until a stable state was formed. Therefore, each continuous colonic fermentation system was inoculated with feces from one of the three different donors that originated from one defecation each. After 24 h of processing time, the fermentation was switched to continuous mode by adding fresh medium on the one side and removing broth in the same amount. The whole system was run until a stable state according to the criteria described below was established. The influence of the cultivation parameters on the system's behavior was investigated regarding cell count, metabolic activity and SCFA production. Additionally, microbiota composition, as well as the richness and diversity of the microbial community, was assessed.

Further, the cultivation of all three donor samples was compared to reveal whether the obtained system is influenced by the process parameters, the donor characteristics or both.

3.3.1 *Characterization of Donor Stools*

Three donors were selected for the study. They shared a common social and ethnical background as well as a comparable diet. Each donor stool originated from one defecation. The stool was tested for cell count, metabolic profile and microbial diversity. There were no significant differences ($p \leq 0.05$) in anaerobic or aerobic cell count among the three donors (Table 3.2).

Table 3.2: Characterization of the three donors by cell count, metabolites as well as richness, Shannon effective index and the ratio between Firmicutes and Bacteroidetes.

		Donor A	**Donor B**	**Donor C**
Cell count	Aerobic [10^5 CFU mL^{-1}]	0.7 ± 0.4	10 ± 9	9 ± 1
	Anaerobic [10^8 CFU mL^{-1}]	4 ± 3	2 ± 0.02	4 ± 0.6
Metabolic profile	Acetate [mg mL^{-1}]	3.17 ± 0.21	2.74 ± 0.07	2.59 ± 0.54
	Propionate [mg mL^{-1}]	2.00 ± 0.16	1.15 ± 0.04	1.02 ± 0.16
	Butyrate [mg mL^{-1}]	1.48 ± 0.08	0.99 ± 0.03	2.14 ± 0.26
	Isovalerate [mg mL^{-1}]	0.15 ± 0.02	0.24 ± 0.00	0.34 ± 0.04
	Σ SCFAs [mg mL^{-1}]	6.80 ± 0.47	5.12 ± 0.14	6.09 ± 1.00
Microbial profile	Richness [-]	100	123	122
	Shannon effective index [-]	21.42	38.43	46.34
	Ratio F:B	1.03	1.13	2.60

The cell count of facultative aerobes was between 0.7 ± 0.4 10^5 (donor A) and 10 ± 9 10^5 CFU mL^{-1} (donor B). The high variation, especially in the stool of donor B, may be due the individual availability of aerobic growth of the microorganisms. The anaerobic cell count was more homogenous and between 2 ± 0.02 10^8 (donor B) and 4 ± 3 10^8 CFU mL^{-1} (donor A). Furthermore, the concentration of SCFAs in the stool was from 5.12 ± 0.14 (donor B) to 6.80 ± 0.47 mg mL^{-1} (donor A). The ratio for acetate: propionate: butyrate was comparable as well, with 3:1:1 for donor A, 3:2:1 for donor B and 3:1:2 for donor C. The concentration of propionate (2.00 ± 0.16 mg mL^{-1}) in the stool of donor A was higher than for donors B and C. Higher values for propionate may indicate an overweight person, nonetheless, the BMI of donor A was normal (Schwiertz *et al.*, 2010). Nevertheless, the overall metabolic profile represents three healthy donors with a normal contribution of SCFAs. Richness, Shannon effective index as well as the relative abundance of phyla were determined for the microbial profile. No difference among the donors was observed for richness between 100 (donor A) and 123 (donor B). Regarding diversity, represented by the Shannon effective index, a significantly higher value of 46.34 was identified for donor C. This coincides with an increase in Firmicutes and a decrease in Bacteroidetes and Verrucomicrobia for donor C. As Figure 1 shows, donor C hosted 70.88% Firmicutes and 27.22% Bacteroidetes. The abundance of Firmicutes for donor A (48.52%) and donor B (50.06%) was lower, while the abundance of Bacteroidetes was higher (44.41% for donor B; 46.89% for donor A). For Proteobacteria, donor B had a higher relative abundance of 1.75%. The abundance of Actinobacteria varied among all donors from

0.61 (donor B) and 1.40 (donor C) to 2.02% (donor A). The phylum of Verrucomicrobia was only present in low abundances between 0.08% (donor C) and 1.98% (donor B).

The different phyla were represented by several genera. Within the phylum of Actinobacteria, only *Bifidobacteria* were abundant. *Akkermansia* represented Verrucomicrobia, whereas *Escherichia* and *Shigella* can be found within the phylum of Proteobacteria. Bacteroidetes were represented by *Bacteroides*, *Parabacteroides*, *Prevotella* and *Alistipes*. The phylum Firmicutes were comprised of several genera of *Clostridium* as well as other genera, such as *Blautia*, *Dorea*, *Dialister*, *Faecalibacterium*, *Roseburia* or *Ruminococcus*. Not all detected genera were abundant in all stool samples. Among the genera with an abundance >1%, differences were detected regarding *Dialister*, *Eisenbergiella*, *Prevotella* and *Oscillibacter*. *Eisenbergiella* and *Oscillibacter* were not abundant in donor A, but in donors B and C. Donor B instead lacked *Dialister*. Donor C showed a significantly higher abundance in *Prevotella* than donors A and B. How these differences affect the formation of a stable system and the fermentation outcome is described below. Since all donors represent a healthy intestinal microbiome that is typical for a Western diet (Dethlefsen *et al.*, 2007; Filippis and Ercolini, 2018) and effects of cultural background, body mass or age were excluded, the main influencing factors on the established in vitro cultured microbiomes may be the system parameters themselves as well as the individual compositions.

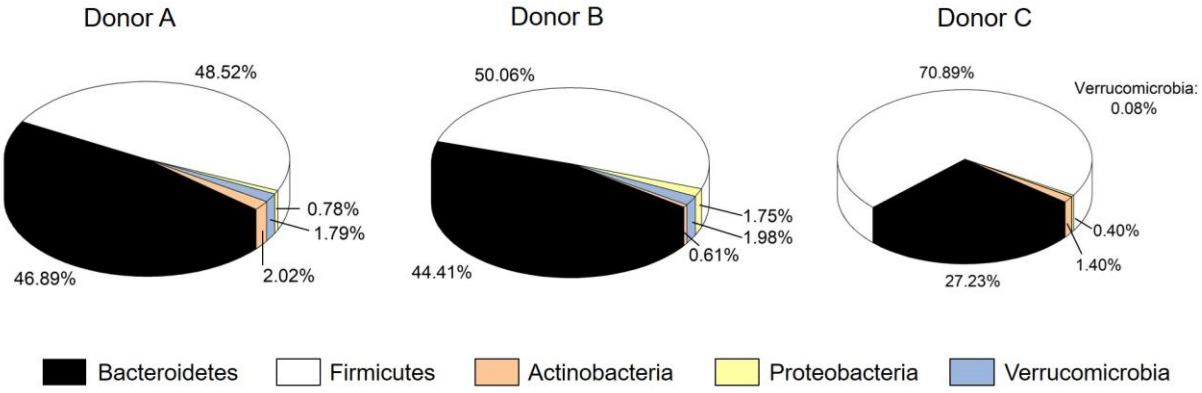

Figure 3.2: Relative cumulative abundance of the five major phyla in each stool sample.

3.3.2 *Establishment of an in Vitro Microbiota*

In the following, the establishment of the in vitro system is described by the cultivation of stool A. The same experiments were also performed for donors B and C but are not described in detail for the sake of clarity, since their behavior was comparable.

Cell Count

The inoculum cell count level was $3 \pm 0.7\ 10^6$ CFU mL^{-1} anaerobes and $3 \pm 3\ 10^4$ CFU mL^{-1} facultative aerobes. After addition to the bioreactor and dilution

with the medium, the cultivation cell broth contained a starting cell count level of $1 \pm 0.6\ 10^6$ CFU mL^{-1} anaerobes and $3 \pm 3\ 10^3$ CFU mL^{-1} facultative aerobes. Figure 3.3 shows the evolution of cell count over a processing time of 120 h.

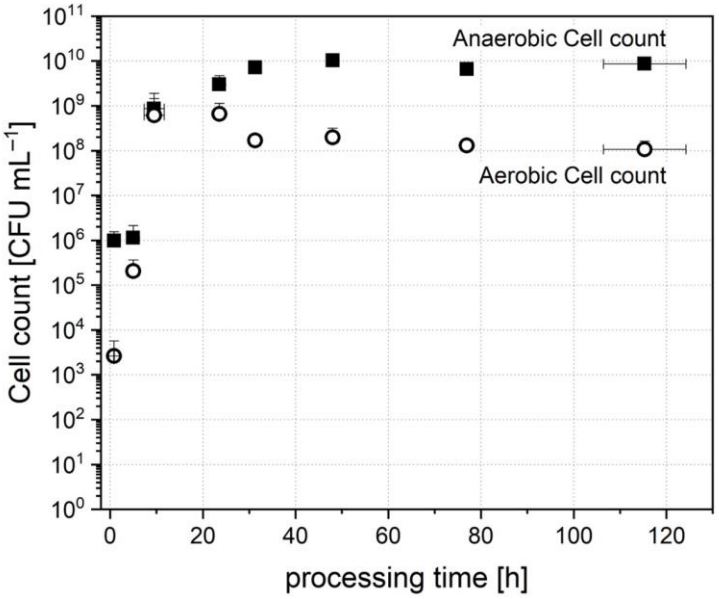

Figure 3.3: Cell count of anaerobic and aerobic cell count over processing time during cultivation of donor stool A.

The number of aerobic cells started to increase immediately after inoculation, and a peak was reached after 23.6 ± 1.3 h with a number of $6 \pm 5\ 10^8$ CFU mL^{-1} The cell count then dropped slightly and stabilized subsequently. After 115.3 ± 8.9 h of cultivation, a stable number of $1 \pm 0.5\ 10^8$ CFU mL^{-1} was reached.

In comparison, the number of anaerobic CFUs had a lag time of 5.0 ± 1.3 h in the beginning. After 9.5 ± 2.2 h, the cell count increased rapidly and reached $3 \pm 2\ 10^9$ CFU mL^{-1} after 23.6 ± 1.3 h of processing time. At that point of time, it seemed that anaerobic cells had adapted to the conditions and then established a stable amount of $8 \pm 2\ 10^9$ CFU mL^{-1} after 115.3 ± 8.9 h of processing time. The evolution of cell count and beginning of growth is in accordance with the detected turbidity (data not shown). The internal optical sensor detected a measurable total microbial growth after 5 ± 1.7 h of inoculation. Although the number of anaerobes in the inoculum was higher than that of facultative aerobes, the ratio of both was balanced after the first 9.5 ± 2.2 h. This dominance of facultative aerobes in the beginning may be explained by the fact that traces of oxygen were transferred into the reactor by the inoculation. Compared to the inoculation of gut at birth, this behavior seems similar. *In vivo*, the microbiota is also dominated by facultative aerobes, such as *Bifidobacteria* and *Lactobacilli*, after birth (Mitsou *et al.*, 2008). Regarding only cell count, the establishment *in vitro* is similar to the establishment of cells in the gut in early life. When growing up, the cell count increases further to an average human microbiota content of about 10^8 to 10^{11} anaerobic CFU mL^{-1} (Sender *et al.*, 2016). Consequently, a comparable cell count was reached in this study and could be maintained during the processing time.

Metabolic Profile and SCFA Production

In addition to cell count, the concentration of metabolic intermediates and products was determined throughout the processing time to provide further data on the molecular composition affecting microbial growth and the behavior of individual groups. When starting the cultivation, only trace amounts of SCFAs and intermediates were found in the cell broth originating from the inoculum prepared from stool. In accordance with cell growth, metabolic activity started to grow after 8 to 22 h. The development of concentration of the major SCFAs, acetate, butyrate, propionate and isovalerate, is described in the following. As Figure 3.4 shows, a low concentration of 0.17 ± 0.08 mg mL^{-1} of acetate was contained in the broth after inoculation. Growth was detected after 10 ± 1.8 h of processing time, when the SCFA concentration had increased to 1.02 ± 0.38 mg mL^{-1}. The highest concentration of acetate in the broth was detected after a processing time of 24 to 36 h. Here, a concentration of 4.14 ± 0.59 mg mL^{-1} at the peak of acetate production occurred. The amount then dropped slowly and stabilized after 76 ± 0.8 h to a stable concentration of 3.50 ± 0.22 mg mL^{-1}.

In comparison, the concentration of butyrate (Figure 3.4) after inoculation was lower (0.06 ± 0.05 mg mL^{-1}), and growth was detectable later, after 22 h (0.47 ± 0.00 mg mL^{-1}). In contrast, the maximum was reached earlier compared to the concentration of acetate. Here, a concentration of 1.64 ± 0.18 mg mL^{-1} was established after 28 ± 0.6 h of processing time. The concentration of propionate at the start of cultivation was 0.10 ± 0.02 mg mL^{-1}. It increased continuously until a stable concentration of 2.94 ± 0.13 mg mL^{-1} was reached after 78.0 ± 1.7 h. For isovalerate, the concentration after inoculation was not detectable (<0.01 mg mL^{-1}), but increased after 24.3 ± 0.3 h and reached a stable concentration of 0.25 ± 0.04 mg mL^{-1} after 77.3 ± 1.7 h. To evaluate the in vitro system, the concentration of SCFAs was compared to studies from the literature. Schwiertz *et al.* (2010) detected the concentration of SCFAs for healthy, overweight, and adipose persons. Compared to these data, acetate, butyrate and isovalerate are in the range of a healthy person with a normal weight. Only the concentration of propionate was typical for an obese person. Bircher *et al.* (2018) cultivated human microbiota in a 24 h batch system. They obtained similar concentrations for acetate and butyrate. The concentration for propionate was lower compared to the value of system A in this study. The values for systems B and C are in accordance with the findings of Bircher *et al.* (2018). This proves that the characteristics of the donor stool, e.g., a high propionate production by donor A, can also be found in the in vitro microbiome. Overall, the in vitro system represents a functional microbiome with a regularly working metabolism.

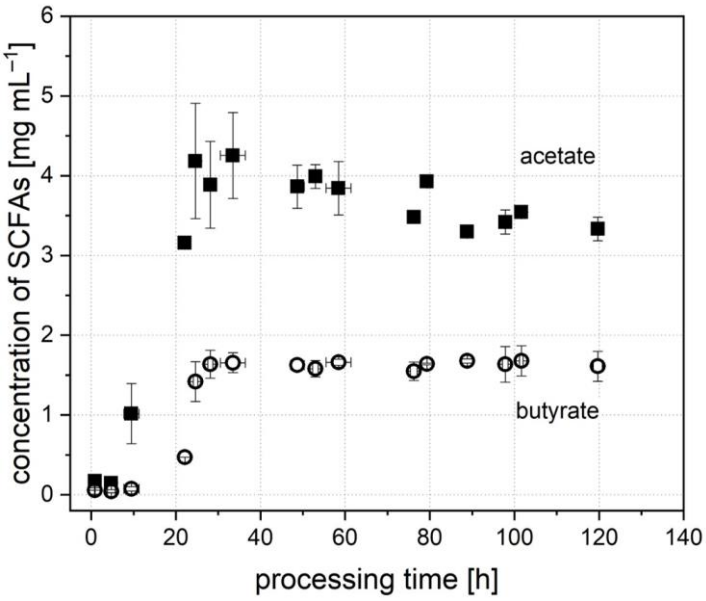

Figure 3.4: Concentration of the SCFAs, acetate and butyrate, throughout the processing time during the cultivation of donor A.

In the human intestinal microbiota, the ratio of acetate, propionate and butyrate is a marker for human health. Rowland *et al.* (2018) and Macfarlane *et al.* (1992) claim that a ratio from 3:1:1 (acetate: propionate: butyrate) to 10:2:1 as typically healthy. The *in vitro* microbiome developed in this study shows a ratio of 4:3:2. For the success of the *in vitro* alternative to FMT, not only the effective transfer of microorganisms is required. The transferred SCFAs also play a major role in the recovery of the patient (Baktash *et al.*, 2018). Consequently, the *in vitro* system obtained here represents a healthy human microbiota regarding the SCFA ratio that may be further used for FMT.

In addition to the main SCFAs, the metabolic intermediates succinate and lactate were analyzed. These substances are metabolized to acetate or butyrate , e.g., by *Bifidobacteria* (Flint *et al.*, 2012). The production of succinate started after 23.6 ± 1.2 h up to a maximum of 0.98 ± 0.02 mg mL^{-1}. The concentration was subsequently lowered to below 0.04 mg mL^{-1} after 77.4 ± 2.7 h. The progress was similar for lactate. First, a constant concentration of 0.12 ± 0.05 mg mL^{-1} was in the broth for the first 24 h until it dropped below a concentration of 0.02 mg mL^{-1}. Facultative aerobic microorganisms, such as *Lactobacilli* and *Bifidobacteria*, are the main producers of succinate and lactate in the microbiome. Consequently, the production of metabolic intermediates is in accordance with the development of aerobic CFUs. The main growth occurs in the first 80 h. Afterwards, a stable system with a constant metabolic activity and lower levels intermediates is established.

Definition of the Stable System

To compare and evaluate the *in vitro* system, a control value had to be defined. During cultivation, the value for SCFAs changed in the beginning but then plateaued, indicating stable state conditions. In this study, the stability of fermentation performance was defined as the point in time when the values for acetate, butyrate as

well as propionate did not vary by more than 1% per h of processing time. For donor A, the stable system was reached after 29.2 ± 1.9 (acetate), 76.2 ± 1.0 (propionate) and 31.5 ± 0.3 h (butyrate). Overall, a stable state was reached after a maximum of 77 h. To verify this, a cultivation was run for 180 h of processing time. Here, as well, no shift in the factors described above was detected after 77 h. For further comparison and evaluation, averaged values were used in the stable system of 77 to 120 h.

Microbial Composition and Diversitiy

The development of microbial composition, richness and diversity was determined by sequencing 16S rRNA gene amplicons. Stool A was from a healthy donor with a normal microbial distribution and diversity. In this stool, the major phyla were represented by Bacteroidetes and Firmicutes with a relative abundance of 46.89% and 48.52%, respectively. Actinobacteria (2.02%), Proteobacteria (0.78%) and Verrucomicrobia (1.79%) were present in lower relative abundance numbers. This human microbiome also hosted a richness of 100 and a diversity with a Shannon effective index of 21.42 (Table 3.2). When preparing the fecal inoculum, richness (103.40 ± 5.68) and diversity (23.35 ± 2.76) stayed constant. In contrast, the distribution of the phyla changed. As Table 3.3 shows, the relative abundance of Actinobacteria in the inoculum increased, while Proteobacteria and Verrucomicrobia decreased. The relative abundance of Bacteroidetes dropped to 1.59 ± 1.09%, while Firmicutes increased to 94.82 ± 0.75%.

Table 3.3: Evolution of microbial composition, richness, and diversity of inoculum and during cultivation of donor A.

	Processing Time					
	Inoculum	**After Inoculation (<1.0 h)**	**8.3 ± 2.6 h**	**24.6 ± 0.6 h**	**48.0 ± 1.3 h**	**Stable System (>77.0 h)**
Actinobacteria [%]	3.44 ± 0.83	3.16 ± 0.32	2.58 ± 1.01	2.27 ± 1.33	0.23 ± 0.02	0.09 ± 0.05
Bacteroidetes [%]	1.59 ± 1.09	0.90 ± 0.12	0.63 ± 0.67	37.91 ± 1.65	73.20 ± 1.46	75.99 ± 1.78
Firmicutes [%]	94.82 ± 0.75	95.78 ± 0.11	85.41 ± 7.62	49.20 ± 2.12	21.64 ± 1.69	16.92 ± 1.96
Proteobacteria [%]	0.12 ± 0.06	0.12 ± 0.14	11.37 ± 7.69	10.60 ± 3.81	3.83 ± 0.94	4.38 ± 2.42
Verrucomicrobia [%]	0.03 ± 0.01	0.04 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	1.10 ± 0.76	2.22 ± 0.72
Richness [-]	103.40 ± 5.68	94.33 ± 10.02	11.00 ± 4.36	96.33 ± 12.66	95.00 ± 11.14	105.17 ± 5.71
Shannon effective index [-]	23.35 ± 2.76	18.69 ± 0.86	8.32 ± 4.23	24.21 ± 1.35	19.28 ± 1.65	17.28 ± 1.14

This is probably due to the oxygen and shear stress sensitivity of the different phyla. During the preparation of the inoculum, the exposure to oxygen was kept as low as possible but could not be fully avoided. Further to that, by mixing and centrifugation, shear stress occurred that may have had an impact on the abundance

of various groups of cells. Consequently, the abundance of Bacteroidetes as anaerobic microorganisms decreased, but could be fully restored during cultivation. Although there was a drop after inoculation, the abundance increased after 24.6 ± 0.6 h to 37.91 ± 1.65% and finally established with a value of 75.99 ± 1.78% in the stable system. To identify the ongoing microbial changes at the genera level, we defined clusters of sequences representing single microbial entities, known as operational taxonomic units (OTUs) that share at least 97% of genetic similarity based on the 16S rRNA V3/ V4 region. In the stable system, the most represented genera with >1% relative abundance within the Bacteroidetes phylum are *Alistipes* and *Bacteroides*. They occur with a relative abundance of 6.41 ± 4.97% (*Alistipes*) and 68.17 ± 5.05% (*Bacteroides*).

In comparison, the relative abundance of the phylum Firmicutes in the inoculum is way higher (94.82 ± 0.75%). After inoculation, the abundance started to decrease to a value of 49.20 ± 2.12% after 24.6 ± 0.6 h. The final relative abundance in the stable system was 16.92 ± 1.96%. In the inoculum, the main representing genera within the Firmicutes phylum were *Blautia*, *Clostridium XIVa*, *Dialister*, *Faecalibacterium*, *Gemmiger*, *Lachnospiracea*, *Roseburia* and *Ruminococcus*. During cultivation, the abundance of *Blautia*, *Gemmiger*, *Lachnospiracea*, *Roseburia* and *Ruminococcus* lowered <1%. *Clostridium XIVa*, *Dialister* and *Faecalibacterium* were able to establish in higher abundances in the *in vitro* system. The abundance of *Clostridium XIVa*, represented with 1.95 ± 0.74% in the inoculum, was stable during cultivation (2.91 ± 0.42% in the stable system). The genera *Dialister* decreased in its abundance from 3.63 ± 0.15% in the inoculum to 1.06 ± 0.38% in the stable system. *Faecalibacterium*, which was reported to have a major influence on human health, was preserved (Miquel *et al.*, 2013). This genus was abundant in the inoculum (7.53 ± 1.07%) as well as in the stable system (3.91 ± 0.67%).

The phylum Actinobacteria decreased after inoculation and was established with an abundance of 0.09 ± 0.05% after 77 h. This phylum is represented by the genus *Bifidobacterium*, which was present with an abundance of 2.24 ± 0.55% in the inoculum but decreased to a value of 0.05 ± 0.02% in the stable system. The phylum Verrucomicrobia showed a contrary behavior. While only a low abundance of <0.05% was observed in the inoculum, it increased after 48.0 ± 1.3 h to 1.10 ± 0.76% and finally stabilized at a value of 2.22 ± 0.72%. This phylum is represented mainly by the genus *Akkermansia*. The phylum Proteobacteria showed a varying abundance during cultivation. After inoculation, the abundance increased from 0.12 ± 0.14 to 11.37 ± 7.69% after 8.3 ± 2.6 h. Then, a decrease and afterwards stabilization in the stable system at an abundance of 4.38 ± 2.42% was observed. This peak in abundance can be explained since Proteobacteria are mainly facultative aerobe microorganisms. The trend in abundance is similar to the development of aerobic CFUs. Further, the production of acetate followed a comparable development, from which it can be concluded that most of the Proteobacteria in the system produced acetate. Here, the main producers were the genera *Escherichia* and *Shigella*, which were represented in the stable system at 2.34 ± 0.59%. The progress of the different phyla shown in this study is comparable to the formation of the microbiota after birth (Adlerberth and

Wold, 2009). Here, the system was also dominated by facultative and aerotolerant bacteria, as Proteobacteria, Firmicutes and *Bifidobacterium*, in the beginning. Consequently, the formation of acetate was highest during the first 48 h. Afterwards, strict anaerobic, butyrate producing bacteria as representatives from the phylum Bacteroidetes started to grow and establish in the gut as well as in the *in vitro* system. Overall, in the *in vitro* cultivation system, the relative abundance of the phyla differed from the abundance in the donor stool. The value of Actinobacteria and Firmicutes was lower since Bacteroidetes, Proteobacteria and Verrucomicrobia have a higher abundance in vitro than in vivo in contrast. The decrease in Firmicutes may have been caused by the higher redox potential exceeding normal physiological levels in the in vitro system, which was also observed by Zihler Berner *et al.* (2013). The change from an *in vivo* to an *in vitro* environment may possibly inactivate sensitive populations of bacteria or may also convert stressed microorganisms into an abundant, but unculturable state and will, therefore, be washed out from the system. Nevertheless, the aim was not to produce an exact copy of the *in vivo* system, but rather a culturable, stable community with a high diversity and richness of representative gut bacteria. After all, this aim was reached by generating a stable system comparable to the donor stool. On the other hand, the human intestinal microbiome is also not totally stable. *In vivo* variations even on a daily timescale level were observed (David *et al.*, 2014a). Considering this, certain changes in the *in vitro* system, probably due to the cultivation parameters, would be acceptable. Our results are in accordance with the findings of McDonald (2013), who also detected a comparable distribution of phyla when cultivating microbiota at pH 7.0. Similar observations were also made for a polyfermentor intestinal model (Zihler Berner *et al.*, 2013). In these works, both obtained an *in vitro* system with a reduced abundance of Firmicutes and an increase in Bacteroidetes as well as Proteobacteria. In addition to the microbial composition of the in vitro microbiota, richness and diversity were observed. For richness, a decrease to 11.00 ± 4.36 after 8.3 ± 2.6 h was distinguished. Afterwards, an increase after 24.6 ± 0.6 h to 96.33 ± 12.66 was noticed. In the stable system, a richness of 105.17 ± 5.71, comparable to the donor stool, was established. Diversity, characterized by the Shannon effective index, showed a development similar to richness. A decrease after 8.3 ± 2.6 h to 8.32 ± 4.22 was observed as well as an increase and further stabilization to a value of 17.28 ± 1.14. In comparison to the inoculum as well as the original stool, richness and Shannon effective index showed similar values. Consequently, richness as well as diversity can be restored with the cultivation method and parameters in this study.

3.3.3 *Influence of Donor Sample on in Vitro Microbiota in the Stable System*

The influence of the donor sample was tested by comparing all values in the stable system. For all samples, the stable system was defined as the change over cultivation time in concentrations of acetate, butyrate as well as propionate <1% per h of processing time. For donor A, the stable system was reached no later than 77 h. For

donor B, the stable state was reached after 76 h, and for donor C after 74 h. Consequently, the averaged values of each factor of 77–120 h processing time were considered for a comparison.

Table 3.4 shows the value of all investigated factors in the stable system.

Table 3.4: Values of cell count, metabolic profile, and microbial profile in the stable system (>77 h processing time) for the cultivation of the three donor stools.

		Donor A	Donor B	Donor C
Cell count	Aerobic [10^8 CFU mL^{-1}]	1 ± 0.6	2 ± 1	0.7 ± 0.07
	Anaerobic [10^{10} CFU mL^{-1}]	0.9 ± 0.2	1 ± 0.3	1 ± 0.004
Metabolic profile	Acetate [mg mL^{-1}]	3.46 ± 0.22	3.82 ± 0.14	3.52 ± 0.05
	Propionate [mg mL^{-1}]	2.94 ± 0.13	2.94 ± 0.13	3.00 ± 0.07
	Butyrate [mg mL^{-1}]	1.64 ± 0.16	1.57 ± 0.16	2.39 ± 0.15
	Isovalerate [mg mL^{-1}]	0.24 ± 0.04	0.36 ± 0.08	0.31 ± 0.00
	Σ SCFAs [mg mL^{-1}]	8.29 ± 0.55	8.68 ± 0.51	9.22 ± 0.27
Microbial profile	Richness [-]	105.17 ± 5.71	96.00 ± 7.07	115.00 ± 2.83
	Shannon effective index [-]	17.28 ± 1.14	24.40 ± 1.75	21.56 ± 1.25

For cell counts, no significant differences were observed. All donor samples resulted in between 0.9 ± 0.2 10^{10} (donor A) and 1 ± 0.3 10^{10} CFU mL^{-1} (donor B) of anaerobic cells. For the concentration of SCFAs, an increase in the total amount from 8.29 ± 0.55 mg mL^{-1} for donor A to 9.22 ± 0.27 mg mL^{-1} for donor C was detected. In detail, no significant difference was observed for propionate. In contrast, the concentration of acetate, butyrate and isovalerate varied. For acetate, donor B showed a significantly higher value of 3.82 ± 0.14 mg mL^{-1}. For butyrate, the concentration of 2.39 ± 0.15 mg mL^{-1} increased for donor C. In the *in vitro* microbiota of donor A, the concentration of isovalerate decreased significantly to 0.24 ± 0.04 mg mL^{-1}. Under these conditions, the ratio of acetate: propionate: butyrate was 3:3:2 for donor A and 4:3:2 for donors B and C.

Diversity, represented by the Shannon effective index, was significantly higher in the system of donor B with a value of 24.40 ± 1.75. In terms of richness, all donors differed. Richness increased from 96.00 ± 7.07 (donor B) to 105.17 ± 5.71 (donor A) and 115.00 ± 2.83 (donor C). Figure 3.5 shows the contribution of phyla in the created stable systems, where the abundance of Actinobacteria, Proteobacteria and Verrucomicrobia did not significantly differ among the three donors based on a *p*-value of 0.05 among the three donors. Contrary to this, the abundance of Firmicutes and Bacteroidetes was different. Donor C did not show different abundances compared to donors A and B. When comparing donors A and B, A showed a decrease in Firmicutes and an increase in Bacteroidetes.

At the genus level, the genera *Bifidobacteria*, *Blautia*, *Faecalibacterium*, *Gemmiger*, *Lachnospiraceae*, *Parabacteroides*, *Roseburia* and *Ruminococcus* showed no significant

differences among the three donors. Some differences were due to the lack of genera in the donor stool. *Dialister* was neither abundant in the stool nor in the stable system of donor B. Donor A lacked *Eisenbergiella*, *Oscillibacter* and *Prevotella*. In contrast, donor C showed a high abundance of *Prevotella*, while donor B only had a low abundance of this genera. For donor C, *Prevotella* was also abundant in a high number in the stable system. In the stable system of donor B, the abundance of *Akkermansia* was reduced, while *Clostridium XlVa* increased. The abundance of *Alistipes* was significantly different between donors A and B, with A showing higher values. For *Bacteroides* as well as for *Escherichia* and *Shigella*, donor C showed a reduced abundance compared to donor B.

In general, different donor stools led to different *in vitro* microbiomes regarding several factors. For cell count, propionate, and the phyla of Actinobacteria, Verrucomicrobia and Proteobacteria no differences were observed. The abundance of Firmicutes and Bacteroidetes was indeed divergent among the donors. Therefore, the metabolization of acetate, butyrate and isovalerate was different as well as diversity and richness. Nevertheless, the produced systems were comparable to *in vitro* growth, as shown by the formation of the stable system. As Figure 3.5 shows, the shift in the system towards a higher abundance of Bacteroidetes and a lower abundance of Firmicutes was common in all three donors. In general, the cultivation system has a huge impact on which system is formed, but the *in vitro* microbiome still has some characteristics originating from the donor stool.

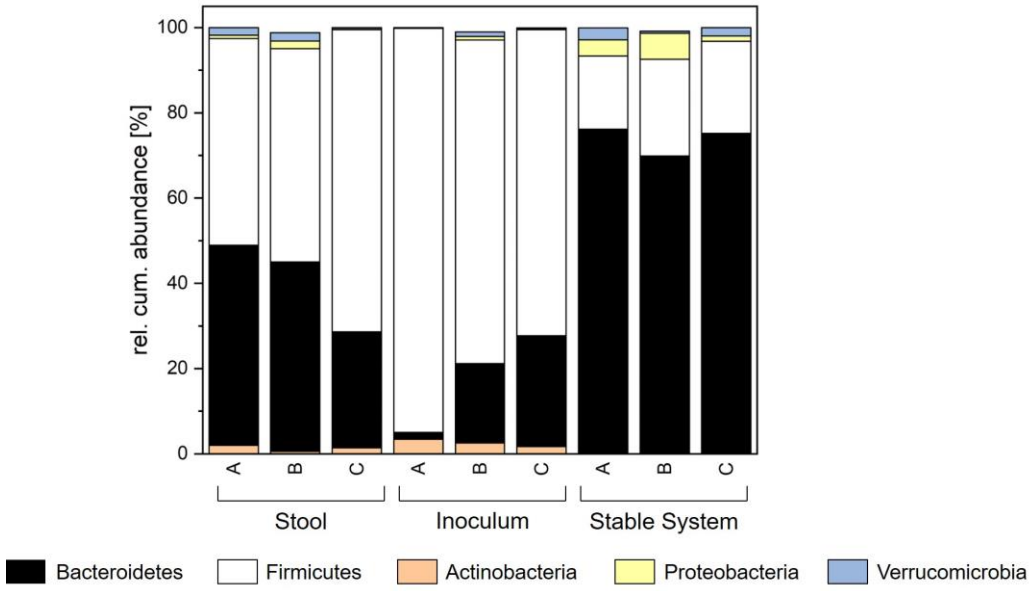

Figure 3.5: Comparison of relative cumulative abundance of the major phyla for stool, inoculum, and stable system for each donor.

3.3.4 *Comparison of Different Donor Stools*

When cultivating the microbiota *in vitro,* a high similarity with the original stool is the aim. For donor A, the cell count in the cultivated stable system differed: it increased significantly to $1 \pm 0.6\ 10^8$ (facultative aerobe) and $0.9 \pm 0.2\ 10^{10}$ CFU mL^{-1} (anaerobe). Regarding the metabolic profile, an increase was also observed. The amount of SCFAs in the broth was significantly higher with a value of 8.29 ± 0.55 mg mL^{-1} compared to the donor stool (6.80 ± 0.47 mg mL^{-1}). These increases in cell count and metabolites are probably linked since more cells produce more metabolites. Regarding the composition of these cells, the abundance of Proteobacteria and Verrucomicrobia showed no significant difference between stool and cultivated broth. On the other hand, the phyla Actinobacteria, Bacteroidetes and Firmicutes differed significantly based on a *p*-value of 0.05 from the original sample. As can be seen in Figure 3.5, the abundance of Actinobacteria in stool was 2.02%, whereas it was lower in the cultivated system (0.09 ± 0.06%). Furthermore, the abundance of Firmicutes was decreased from the original value of 48.52% in the stool to an abundance of 17.13 ± 2.12% in the stable system. In contrast, the abundance of Bacteroidetes increased during cultivation from 46.89% (stool) to 76.10 ± 1.96% in the stable system. For donor B and C, the composition of the *in vitro* microbiota was similar and comparable to donor A. All three artificial systems showed significant increases in cell count and SCFAs with slight differences in the extent of increase. The microbial composition in the stable systems was defined by an increase in Bacteroidetes and Proteobacteria and a decrease in Firmicutes and Actinobacteria. Differences can only be observed for the phylum of Verrucomicrobia. For donors A and C, an increase in abundance was detected in the stable system. However, these observations represent only a trend but no significant differences. The microbial diversity in the cultivated system of donor A showed a significantly lower value (16.99 ± 1.01) than in the stool (21.42). However, the richness in the system was not affected. Here, the richness of 107 ± 3.94 in the stable state was comparable to that in the donor stool (100). The behavior of the cultivations of donors B and C was similar. Consequently, the established system is more influenced by the system parameters themselves. The behavior of the three donor stools during cultivation was comparable and similar. Nevertheless, individual donor characteristics remained.

In this study, differences among three donor stool samples were investigated. Three samples are considered as the scientific standard and commonly accepted in this field of research, as comparable studies used similar sample numbers. In other publications, the value ranges from four (McDonald, 2013) and three (Minekus *et al.*, 1999) to even only two donor samples (Bircher *et al.*, 2018). Nevertheless, it should be considered that the donor stools used in this study shared a common social and environmental background. We cannot exclude that this fact might lead to a similar *in vitro* microbiota system and prevented the detection of differences based on the donor stool. Additionally, the sample volume of three was sufficient to validate the concept, but more samples will be needed to confirm the findings presented and conclusions drawn here.

3.3.5 *Comparison with the Inoculum*

When comparing the *in vitro* microbiota with the fecal inoculum (Figure 3.5), all phyla for all donors showed significantly different abundances, except for the abundance of Verrucomicrobia. Additionally, diversity and richness showed similar values with no significant differences. Nevertheless, inocula similar to those prepared in this study, are used in current fecal microbiota transplantation concepts. Disregarding the high differences between stool and inoculum, the method showed a high recovery rate (Brandt *et al.*, 2012; Weingarden *et al.*, 2015). This indicates that *in vitro* microbiota, irrespective of the donor, could serve as a potential enhancement of FMT, since they show a higher similarity with the stool compared to the inoculum. However, it remains unknown how in vitro infusion material adapts to the individual human gut. Therefore, further experiments and testing will be required in order to close the gap of knowledge regarding the effectiveness of *in vitro* microbiota applied in FMT.

3.4 **Conclusions**

In the current study, a human *in vitro* intestinal microbiome of three different donors was developed, established, and assessed. From the results obtained, we can conclude that the *in vitro* cultivation of a human intestinal microbiome is a valid alternative, but results in a slightly different system compared to *in vivo*. The number of anaerobic as well as facultative aerobic cells increased, as did the production of SCFAs. Regarding the microbial profile, the cultivation led to a decrease in the abundance of Actinobacteria. The abundance of Bacteroidetes increased, while Firmicutes decreased. Proteobacteria and Verrucomicrobia showed no significant changes. Diversity in the *in vitro* system decreased, but richness was not affected by cultivation. When cultivating stool from three different donors, the behavior of the created *in vitro* system was similar and comparable but showed unique characteristics originating from the composition of the stool. The aim, however, was not to produce an exact copy of the *in vivo* system, but rather to demonstrate that a culturable, stable community with a high diversity and richness of representative gut bacteria can be produced. Further, the concentration and ratio of SCFAs were required to be comparable to the *in vivo* system to increase the chance of recovery when using the broth as an infusion material. As shown by this study, this can be reached, but the cultivation system and cultivation conditions have a major impact on the resulting microbiota profile. The produced *in vitro* microbiota can be influenced by choosing system parameters, which provide some degrees of freedom in producing a certain target microbiome, as well as the stool characteristics, with the cultivation parameters having a higher impact. Nevertheless, it should be considered that the donor stools used in this study shared a common social and environmental background. We cannot exclude that this fact might lead to a similar *in vitro* microbiota system and prevented the detection of differences based on the donor stool. Additionally, the sample volume

of three was sufficient to validate the concept, but more samples will be needed to confirm the findings presented and conclusions drawn here. Consequently, further parameters, such as pH or stirring rate, as well as more and especially different stool samples from donors with different backgrounds, need to be cultivated and investigated to improve and enhance the system established here.

Comparing stool and the created system in regard to the fecal microbiota transplantation concept, we can conclude that the *in vitro* microbiome shows a higher similarity with the donor stool than the inoculum that is normally used for therapy. Nevertheless, future studies *in vivo* need to confirm the results, since it is not clear whether cultivated bacteria have the same impact on patients' recovery as FMT itself.

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References

The references were combined into a joint list of all publications, see at the end of the thesis.

4 Influence of Cultivation pH on Composition, Diversity, and Metabolic Production in an *In Vitro* Human Intestinal Microbiota

Summary and contribution of the doctoral candidate

Existing *in vitro* systems mainly differ in the cultivation pH value. Therefore, the influence of the pH on composition, diversity, and metabolic production in the continuous flow fermentation system was assessed (Figure 4.1).

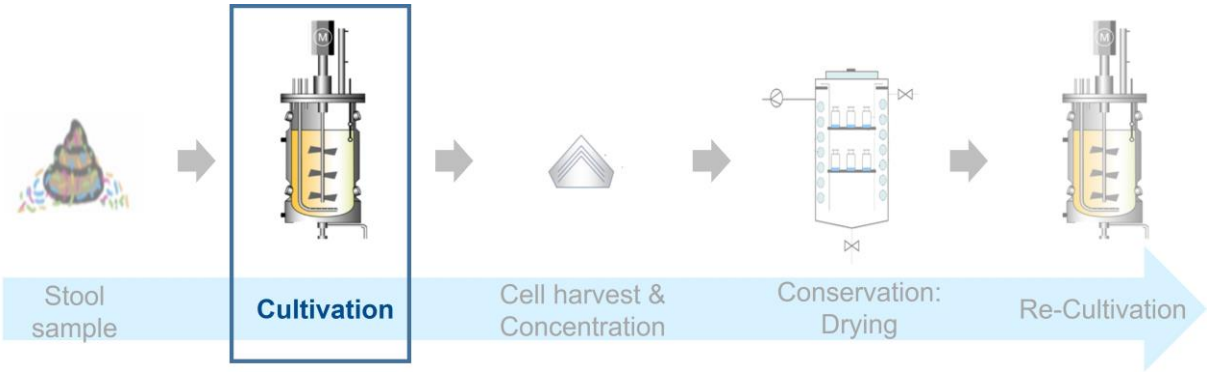

Figure 4.1: Investigated steps in the entire preservation process: influence of the cultivation pH value

As cultivation pH value a physiological range between 6.0 and 7.0 was chosen to investigate the influence on behavior and characteristics, including cell count, metabolism, and microbial composition. With an increasing cultivation pH, an increase in cell count, total amount of SCFAs, acetate, propionate, and the abundance of Bacteroidetes and Verrucomicrobia were observed. Contrary, the concentration of butyrate and the abundance of the phyla Actinobacteria and Firmicutes decreased with an increasing pH value. The cultivation pH was found to have no effect on the concentration of isovalerate, the abundance of Proteobacteria and diversity, measured by richness and Shannon diversity index. Genera known for being health-promoting, were more abundant at lower pH values. In total, all investigated values created a diverse and stable system. Ultimately, the choice of pH creates significant differences in the established *in vitro* microbiota, but no clear recommendations for a special value can be made.

The substantial contribution of the doctoral candidate was the conception, design, and execution of the experiments. She acquired the data for the manuscript and interpreted the data set. She further critically reviewed the existing literature and wrote the manuscript. Co-authors contributed to the experimental part and to the discussion of the results and provided input to the drafted publication prior to submission.

Adapted original manuscript[3]

Influence of Cultivation pH on Composition, Diversity, and Metabolic Production in an In Vitro Human Intestinal Microbiota[4]

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Abstract

Fecal microbiota transplantation, an alternative treatment method for gastrointestinal diseases, has a high recovery rate, but comes with disadvantages, such as high donor requirements and the low storability of stool. A solution to overcome these problems is the cultivation of an *in vitro* microbiota. However, the influence of cultivation conditions on the pH are yet unknown. In this study, the influence of the cultivation pH (6.0–7.0) on the system's behavior and characteristics, including cell count, metabolism, and microbial composition, was investigated. With an increasing cultivation pH, an increase in cell count, total amount of SCFAs, acetate, propionate, and the abundance of Bacteroidetes and Verrucomicrobia were observed. For the concentration of butyrate and the abundance of Actinobacteria and Firmicutes, a decrease with increasing pH was determined. For the concentration of isovalerate, the abundance of Proteobacteria and diversity (richness and Shannon effective), no effect of the pH was observed. Health-promoting genera were more abundant at lower pH levels. When cultivating an *in vitro* microbiota, all investigated pH values created a diverse and stable system. Ultimately, therefore, the choice of pH creates significant differences in the established *in vitro* microbiota, but no clear recommendations for a special value can be made.

4.1 Introduction

The human gut hosts a large and complex ecosystem, known as the intestinal microbiota. Due to the high retention time of food and the resulting high level of nutrients, a high abundance of microbial cells (up to 10^{14} CFU mL^{-1}) and 400–1,000 different species can be found in the human colon (Eckburg *et al.*, 2005; Gill *et al.*, 2006). These microorganisms belong to different phyla: 40 to 50% of gut microorganisms belong to

[3] Adaptions refer to formatting issues: e.g., abbreviations, figure, table, equation and section numbering, citation style, notation of units, spelling, axis labeling.

[4] Originally published in *Fermentation* (2021), Vol 7 (3), pp. 156-174. Permission of reuse of this article was granted.

the phylum Firmicutes, while approximately 40% belong to the phylum Bacteroidetes. The minor phylum Actinobacteria makes up around 2.5% of the gut microbiota, while the phyla Proteobacteria and Verrucomicrobia make up 0.1–1% and 0.1%, respectively (Eckburg *et al.*, 2005; Dethlefsen *et al.*, 2007). The intestinal microbiota has several functions, including transforming indigestible food components into bioactive compounds. These metabolites include short-chain fatty acids (SCFAs), which provide defense against pathogens, regulate glucose homeostasis, and metabolize lipids (Kumar *et al.*, 2011; Vadder *et al.*, 2014; Rios-Covian *et al.*, 2017). Alterations or decreases in variety, diversity, and composition of the intestinal microbiota (dysbalance) can cause various diseases (Holmes *et al.*, 2011). These diseases are often associated with the gastric system, such as inflammatory bowel (Schreiber *et al.*, 2014), Chron's (Manichanh *et al.*, 2006), and celiac disease (Collado *et al.*, 2007), but altered microbiotas are also observed in patients with obesity and type 2 diabetes (Ley *et al.*, 2005; Larsen *et al.*, 2010; Koliada *et al.*, 2017). Furthermore, neurological diseases, such as Parkinson's disease and cancer, were also reported to be associated with changes in the intestinal microbiota (Hill-Burns *et al.*, 2017; Tremlett *et al.*, 2017; Helmink *et al.*, 2019). A decrease in the microbial diversity and cell count is also linked to *Clostridium difficile* infections (Lagier *et al.*, 2012). These infections are traditionally treated with antibiotics, such as vancomycin or metronidazole (Zar *et al.*, 2007), but the occurrence of relapses and multi-resistant strains are a threat. Fecal microbiota transplantation (FMT) offers an alternative treatment method to antibiotics, with recovery rates up to 90% (Gough *et al.*, 2011; Aroniadis and Brandt, 2013). In FMT, purified stool from a healthy donor is transferred into the patient's gut via colonoscopy, nasogastric tube, or enema. In advance, the donor must undergo extensive screening, must be healthy, and must not have taken any antibiotics before FMT (Terveer *et al.*, 2017). The extensive screening protocol, as well as safety concerns, acceptability, and the lack of a standardized treatment procedure, are the main constraints of the FMT method.

By mimicking gut conditions *in vitro*, the production of a controlled and stable artificial colonic microbiota would be possible and could replace traditional FMT. *In vitro* models have already been applied successfully to reveal the mechanistic effects of probiotics, drug absorption and transport (Meunier *et al.*, 1995; Le Blay *et al.*, 2009; Mennigen and Bruewer, 2009; Hemarajata and Versalovic, 2013). These models offer advantages including easy set-up and operation, and possibilities for variation and adaption of the system and further the lack of ethical considerations. Compared to multi-component systems (Molly *et al.*, 1994; Minekus *et al.*, 1999), single component systems are easier to set up and handle, and have been studied and applied in previous reports (Duncan *et al.*, 2003; Walker *et al.*, 2005; Campos-Vega *et al.*, 2009; Duncan *et al.*, 2009; McDonald, 2013; Takagi *et al.*, 2016; Bircher *et al.*, 2018; Zamora-Gasga *et al.*, 2018). To provide conditions to mimic those *in vivo*, the cultivation systems are supplied with CO_2, N_2, or forming gas, and the temperature is set to +37 °C. To simulate the passage of food, nutrients are supplied in a continuous flow cultivation with a mean retention time of 24 h in the form of a complex growth medium (Macfarlane and Gibson, 1998;

Duncan *et al.*, 2003; Walker *et al.*, 2005; McDonald, 2013). These conditions reflect the mean retention time in the human colon (Kim and Rhee, 2012). In a previous study, the establishment of a simplified system as well as the influence of different donor stool characteristics were investigated (Haindl *et al.*, 2021a). For gas supply conditions and cultivation temperature, all existing systems suggest similar parameters. Within the accessible range of physiological pH conditions, huge differences can be observed when it comes to the cultivation pH value between existing cultivation systems, where the applied pH varies from 5.5 (Walker *et al.*, 2005) or 5.7 (Bircher *et al.*, 2018) up to 7.0 (McDonald, 2013). Therefore, a comparison of the results from these studies is hardly possible. The cultivation pH is known to have a major impact on the composition of the microbiota as well as single-cell cultivations (Kiviharju *et al.*, 2005; Duncan *et al.*, 2009). The optimal pH values for the growth of *Bifidobacteria* (Kiviharju *et al.*, 2005) and *Bacteroides* (Dalland and Hofstad, 1974) have been investigated previously. Nevertheless, results regarding pH for single-cell cultures cannot be transferred to multi-strain cultivations of the whole microbiota. However, its influence on the system's behavior is great, as the abundance of microbial cells, as well as cell count and production of metabolites are dependent on the cultivation pH. With setting the pH value to different physiological values, different systems may be established. This study was conducted to investigate the composition, diversity, and variety of microbes of an *in vitro* microbiota dependent on the set cultivation pH value. For the success of FMT, the transfer of a high amount of SCFAs is also required (Baktash *et al.*, 2018). The aim was to find a cultivation pH where a high number of overall as well as health-promoting microorganisms are abundant, and to establish a microbial composition considered healthy with a high amount of metabolites. For a successful FMT therapy, stool after defecation, representing the region of the last part of the intestine is used. Consequently, for this study as a range of pH, the physiological value of the distal colon is investigated (Evans *et al.*, 1988; Payne *et al.*, 2012). This study was conducted to identify a pH value in the physiological range of 6.0 to 7.0, which can be preferentially used for the cultivation of an artificial colonic microbiota for FMT.

4.2 **Materials and methods**

4.2.1 *Donor Stool*

For this study, stools from three different donors (one defecation per donor) were used. The donors were chosen according to the criteria, as described previously (Terveer *et al.*, 2017). To prevent the transfer of disease, the donor stool was tested for bacterial, viral, and eucaryotic pathogens that are relevant for *in vitro* cultivation by the Institute for Medical Microbiology and Hygiene, University of Regensburg. The stool was obtained in-house and stored immediately at −80 °C until use.

All donors were in the same age group, ethnic group (Caucasian), and social environment; they all ate a Western diet and had a normal body mass index (BMI).

Their last antibiotic treatment was at least 12 months ago. Before performing the experiments, the stool was characterized (Table 4.1).

Table 4.1: Characterization of donor stools (Haindl *et al.*, 2021a)

		Donor A	**Donor B**	**Donor C**
Age	years	28	25	27
BMI	-	23	21	21
Cell count	Aerobic (10^5 CFU mL^{-1})	0.7 ± 0.4	10 ± 9	9 ± 1
	Anaerobic (10^8 CFU mL^{-1})	4 ± 3	2 ± 0.02	4 ± 0.6
Metabolic profile	Acetate (mg mL^{-1})	3.17 ± 0.21	2.74 ± 0.07	2.59 ± 0.54
	Propionate (mg mL^{-1})	2.00 ± 0.16	1.15 ± 0.04	1.02 ± 0.16
	Butyrate (mg mL^{-1})	1.48 ± 0.08	0.99 ± 0.03	2.14 ± 0.26
	Isovalerate (mg mL^{-1})	0.15 ± 0.02	0.24 ± 0.00	0.34 ± 0.04
	Σ SCFAs (mg mL^{-1})	6.80 ± 0.47	5.12 ± 0.14	6.09 ± 1.00
Microbial profile	Richness (-)	100	123	122
	Shannon effective (-)	21.42	38.43	46.34
	Ratio Firmicutes:Bacteroidetes	1.03	1.13	2.60

4.2.2 *In Vitro Cultivation System*

Continuous flow fermentation medium (CFF) was used for the preparation of the inoculum as well as in the cultivation system itself. The composition and production of CFF medium were already described in previous studies (Haindl *et al.*, 2021a). After preparation, the final medium was stored at +4 °C and used within 72 h for either preparation of the inoculum or the medium for the cultivation system. For the cultivation experiments, a *BioStat B* bioreactor (Sartorius AG, Göttingen, Germany) was used. To start the cultivation, 120 mL of inoculum was transferred aseptically into a glass vessel which contained 730 mL of anaerobic CFF medium. The cultivation conditions were set to +37 °C with a stirring rate of 200 rpm (Table 4.2). Anaerobic conditions were provided by sparging the medium with 8 ccm forming gas. After 24 h of processing time, the system became continuous by supplying fresh medium with an inflow of 0.5 mL min^{-1} and removing the same amount of broth to keep the volume constantly at 850 mL.

Table 4.2: Cultivation conditions for continuous flow fermentation.

Cultivation Parameter	Set Condition
Temperature	+37 °C
Stirring rate	200 rpm
Aeration	8 ccm forming gas (95% N_2, 5% H_2)
pH value	6.0/ 6.5/ 7.0 regulated with 1.25 M NaOH and 0.5 M HCl
Medium inflow	0.5 mL min^{-1}
Volume	850 mL

Adding fresh CFF medium generated a mean retention time of 28.3 h, comparable to the human colon transit time (Kim and Rhee, 2012). During processing, samples were collected by pumping broth anaerobically into prepared sample tubes (Greiner Bio-One, Sigma Aldrich, St. Louis, USA). The broth was either used immediately for further analysis (cell count, SCFAs) or stored at −80 °C (16S rRNA sequencing).

4.2.3 *Analysis of Cell Count*

To determine the cell count in the broth, samples were collected and diluted with 0.25-strength Ringer's solution. To analyze the cell count, the solution was either plated on Wilkins–Chalgren anaerobe agar plates (anaerobic cell count) or plate count agar plates (facultative aerobic cell count). The plates were incubated either aerobically or anaerobically for 48 h at +37 °C. Only plates with 30–300 colonies were included in the analysis. The number of colony-forming units *N* per mL of sample (CFU mL^{-1}) was calculated according to the following (4.1:

$$N = \frac{c}{n_1 + (0.1 \cdot n_2)} \tag{4.1}$$

where c is the sum of colonies of the subsequent dilutions; n_1 is the number of colonies in the less diluted cell suspensions; and n_2 is the number of colonies in the more diluted solution.

4.2.4 *Analysis of Short-Chain Fatty Acids by High Performance Liquid Chromatography*

To reveal the metabolic behavior, nutritional sugars (glucose, galactose) as well as metabolic intermediates (succinate and lactate) and the end products were analyzed. The main short-chain fatty acids (SCFAs) acetate, propionate, butyrate, and iso-valeric acid were identified and measured by a high-performance liquid chromatography (HPLC; Agilent Technologies Inc., Santa Clara, CA, USA) system equipped with an

Aminex HPXH-87H ion exclusion column (Bio-Rad Laboratories, Hercules, CA, USA) and a *G1362A* refractive index detector (Agilent Technologies Inc., Santa Clara, CA, USA). Separation was performed with 0.0005 mol L^{-1} H_2SO_4 at a flow rate of 0.45 mL min^{-1} and a 20–100 µL injection volume. After centrifugation (*Hermle-Z233 M-2*, Hermle Labortechnik GmbH, Wehingen, Germany) at 6000 *g* at +20 °C for 30 min, the supernatant was filtrated (0.22 µm) and used for measurements. SCFAs were identified and quantified using external standards (Sigma Aldrich, Saint Louis, CA, USA) and the software Agilent ChemStation Instrument 1 Offline (Agilent Technologies Inc., Santa Clara, CA, USA).

4.2.5 *Microbiota Profiling with 16S rRNA Sequencing*

The microbial community, richness, and diversity were analyzed at several time points during cultivation by sequencing the V3/V4 region of 16S rRNA. High-throughput 16S rRNA gene amplicon sequencing was performed by the Microbiome Core Facility, ZIEL, TU Munich, according to the protocol previously described (Reitmeier *et al.*, 2020b). The raw data were preprocessed using the *IMNGS* pipeline (Lagkouvardos *et al.*, 2016). Operative taxonomic units (OTUs) with a relative abundance of less than 0.25% across all samples were removed to prevent the analysis of spurious OTUs (Reitmeier *et al.*, 2020a). Five nucleotides were trimmed on the 5' and 3' ends for the R1/R2 read, with an expected error rate of 3 (trim score 3). The read length only considered nucleotides between 300 and 600 base pairs. Taxonomy and alpha-diversity were analyzed by running the provided R script, Rhea (Lagkouvardos *et al.*, 2017; Reitmeier *et al.*, 2020b). The alpha-diversity richness, representing the total number of OTUs in the community and the Shannon effective index, which accounts for evenness and abundance of species in the community, were calculated automatically by the software.

4.2.6 *Statistical Analysis*

All analyses were repeated at least in triplicate. The mean values are shown as the arithmetic mean $\bar{x}$ of the number n of all samples x_i. The distribution of the values was calculated from the standard deviation s due to the random error. All graphs show the arithmetic means ± standard deviations. Statistical significance was tested using a one-way ANOVA ($p \leq 0.05$) followed by a Tukey post-hoc analysis with the software OriginPro 2019 (OriginLab Corporation, Northampton, MA, USA).

4.3 **Results and discussion**

For the cultivation of the human intestinal microbiota, a stable *in vitro* system was used (Haindl *et al.*, 2021a). The aim of this study was to reveal the influence of the pH value on the cultivation outcome in the stable system. The pH was adapted to 6.0, 6.5, or 7.0 in three separate cultivations and the behavior of the system was observed. The

impact of cultivation pH on cell count, metabolic profile, and SCFAs production as well as microbial community composition, richness, and diversity was examined.

4.3.1 *Influence of Cultivation pH*

Standardization of the Stable State

To compare and evaluate the *in vitro system*, standardized values for the above parameters were required. In this study, the stable system was defined as the point in time when the investigated value did not change by more than 1% per h of processing time. Consequently, these values obtained during the stable state were used for comparison and tested with a one-way ANOVA followed by a Tukey post-hoc test to reveal statistically significant differences. The establishment of the stable state was detected for cell count as well as acetate, propionate, butyrate, and isovalerate for all three systems. For donor A, the stable system was reached between 31.3 ± 0.0 h (values for butyrate and anaerobic cell count at pH 6.5) and 76.2 ± 0.1 h (values for propionate and aerobic cell count at pH 6.5). For donor B, the stable system was reached after 75.9 ± 1.0 h (pH 6.0), and after 74.1 ± 0.2 h (pH 6.5) for donor C. Consequently, a stable system can be reached for each pH value and system after 77 h the latest. These results are in accordance with former studies, where the system used here was established (Haindl *et al.*, 2021a). When comparing systems, only values in the stable system after 77-120 h of processing time were considered. The influence of the cultivation pH on the stable system was described by the cultivation of stool A. The same experiments were also performed for donors B and C but will not be described in detail since their behavior was comparable.

Cell Count

To analyze the number of colony-forming units, the anaerobic as well as the facultative anaerobic cell counts were determined. After inoculation (<1 h processing time), the anaerobic cell count for system A was between $4 \pm 3 \times 10^5$ CFU mL^{-1} (pH 6.0) and $10 \pm 6 \times 10^5$ CFU mL^{-1} (pH 6.5). The anaerobic cell count increased in the following processing time until a stable system was formed. As it can be seen in Figure 4.2, in the stable state, a cell count between $6 \pm 3 \times 10^9$ CFU mL^{-1} at cultivation with pH 6.0 and $9 \pm 4 \times 10^9$ CFU mL^{-1} at cultivation at pH 7.0 was reached.

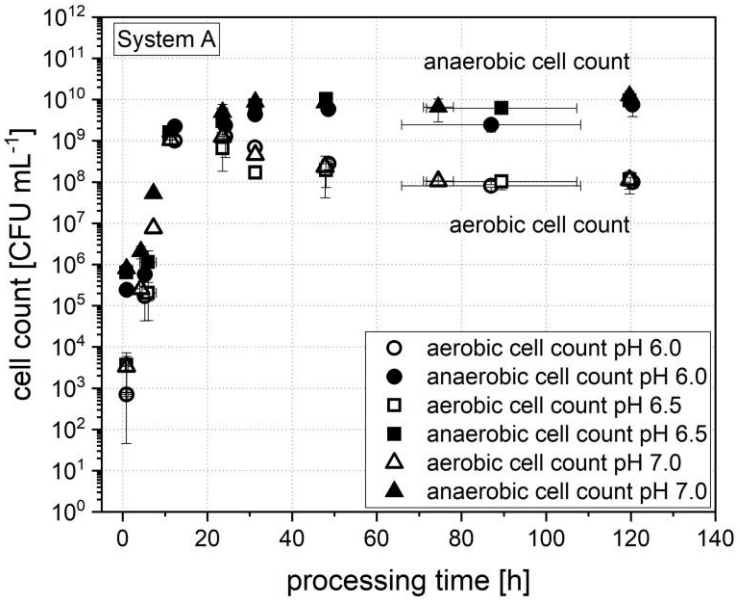

Figure 4.2: Number of aerobic and anaerobic cell counts over processing time for all examined cultivation pH values (system A).

The count of aerobic cells was higher at the beginning of cultivation, with values between $3 \pm 3 \times 10^3$ CFU mL^{-1} (pH 6.5) and $4 \pm 5 \times 10^3$ CFU mL^{-1} (pH 6.0), but as the process continued, the aerobic cell count reached lower values compared to the anaerobic cell count. In the stable system, the cell count was between $1 \pm 0 \times 10^8$ CFU mL^{-1} (pH 6.0) and $1 \pm 1 \times 10^8$ CFU mL^{-1} (pH 6.5). The influence of the cultivation pH on the overall cell count was tested by a one-way ANOVA with a *p*-value of 0.05. For system A, neither for the anaerobic nor the aerobic CFUs, a significant difference in cell counts was detected. For system B, an increase in anaerobic cells in line with pH value was detected, but the aerobic cell count did not differ with the change in pH. For system C, an increase in both the aerobic and anaerobic cell counts was observed. The cell count tended to increase with the pH value, although this trend was not obvious for all systems. In total, all cell counts determined in the *in vitro* system were comparable with the donor stools (Table 4.1) as well as with an average human intestine (Sender *et al.*, 2016).

Short-Chain Fatty Acid Production and Metabolic Profile

When performing FMT, not only is the transfer of a high number of cells important, but also the transfer of metabolic products is too (Baktash *et al.*, 2018). We tested the influence of the cultivation pH on the production of short-chain fatty acids via HPLC. In system A, the overall production of SCFAs increased from a concentration of 7.85 ± 0.94 mg mL^{-1} at pH 6.0 to 8.29 ± 0.55 mg mL^{-1} at pH 6.5, with a further slight increase to 8.28 ± 0.52 mg mL^{-1} (pH 7.0). However, these differences were only statistically significant for cultivation pH 6.0. In system B, the overall sum of SCFAs increased with the cultivation pH from 8.16 ± 0.20 mg mL^{-1} at pH 6.0 to 9.03 ± 0.36 mg mL^{-1} at pH 7.0. Here, a cultivation at pH 6.0 resulted in a significant lower concentration of SCFAs. System C showed the same results. A cultivation at the

pH value of 6.0 had the lowest concentration of SCFAs (7.00 ± 1.15 mg mL^{-1}), which increased to 8.12 ± 0.42 mg mL^{-1} at a cultivation at pH 7.0. Regarding the major SCFAs, acetate, propionate, butyrate, and isovalerate, differences in concentration at different pH values were observed. In system A, the concentration of acetate increased along with the pH value: from 2.93 ± 0.49 mg mL^{-1} (pH 6.0) to 3.90 ± 0.16 mg mL^{-1} (pH 7.0), and the concentration of acetate was significantly different between all pH values (Figure 4.3). The acetate concentration in the broth tended to increase with an increasing pH value for all three systems. Nevertheless, the difference was only statistically significant for systems A and B. System C showed an overall decreased concentration, but with the same trend. Nevertheless, only a significant difference of pH 7.0 and the other two values was observed.

In contrast, the concentration of butyrate, as shown in Figure 4.4, decreased with an increasing pH value for all three systems. In the pH 6.0 cultivation of system A, a butyrate concentration of 1.93 ± 0.24 mg mL^{-1} was detected, which decreased to 1.32 ± 0.17 mg mL^{-1} at pH 7.0. This behavior is probably due to more abundant gram-positive bacteria, which promote the production of butyrate (Duncan *et al.*, 2009). The difference in concentration was significant for system A, whereas in system B only the concentration in the pH 6.0 cultivation differed from the two other values. System C showed no significant differences, but nevertheless the same trend as systems A and B. The concentration of butyrate in all three systems was between 1.32 ± 0.17 mg mL^{-1} (system A, pH 7.0) and 1.91 ± 0.59 mg mL^{-1} (system C, pH 6.0).

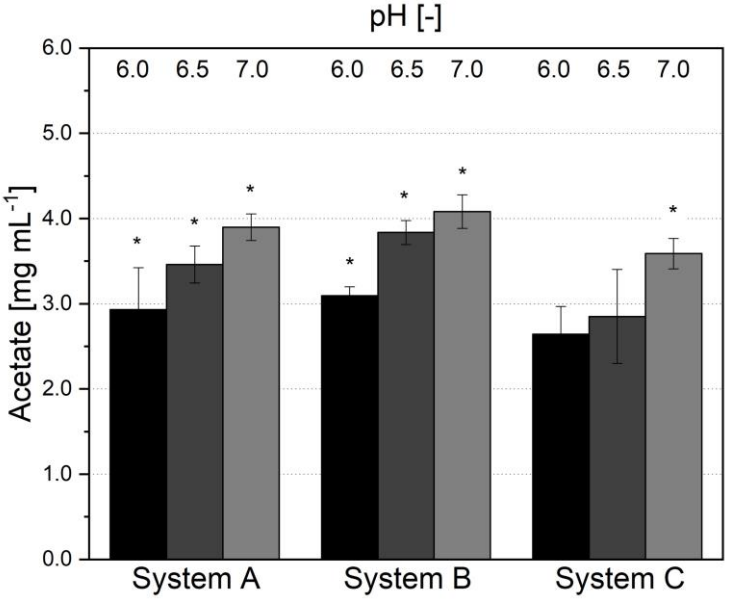

Figure 4.3: Concentration of acetate in the stable system of all three donors depending on the cultivation pH (black column: pH 6.0; dark grey column: pH 6.5; light grey column: pH 7.0); * marks statistical difference between marked pH and the other values.

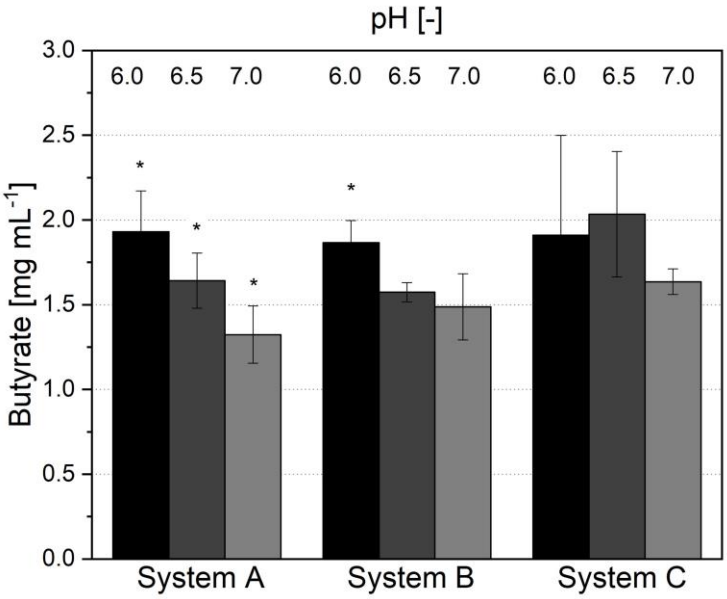

Figure 4.4: Concentration of butyrate in the stable system of all three donors depending on the cultivation pH (black column: pH 6.0; dark grey column: pH 6.5; light grey column: pH 7.0); * marks statistical difference between marked pH and the other values.

The concentration of propionate is shown in Figure 4.5. In system A, the concentration of propionate was between 2.73 ± 0.15 mg mL^{-1} (pH 6.0) and 2.94 ± 0.13 mg mL^{-1} (pH 6.5). Consequently, only the cultivations at pH 6.0 and 6.5 showed a significant difference tested on a *p*-value of 0.05. In system B, the concentration ranged from 2.77 ± 0.02 mg mL^{-1} (pH 6.0) to 3.08 ± 0.34 mg mL^{-1} (pH 7.0), and therefore showed no difference in the concentration between the three pH values. For system C, only the concentration during cultivation at pH 6.0 was significantly lowered (1.76 ± 0.30 mg mL^{-1}) compared to pH 6.5 (2.60 ± 0.42 mg mL^{-1}) and pH 7.0 (2.70 ± 0.19 mg mL^{-1}).

As Figure 4.6 shows, the concentration of isovalerate in system A ranged from 0.23 ± 0.02 mg mL^{-1} at pH 7.0 to 0.26 ± 0.06 mg mL^{-1} at pH 6.0, with no significant differences found between all tested pH values. The production of isovalerate in system B also showed no significant difference. Nevertheless, higher concentrations were reached (0.43 ± 0.03 mg mL^{-1} at pH 6.0 to 0.45 ± 0.06 mg mL^{-1} at pH 7.0). In system C, the concentration was comparable to system A. Here, only the concentration at pH 7.0 was significantly lowered (2.70 ± 0.19 mg mL^{-1}).

The results show that cultivation at different pH values creates different metabolic profiles, and the pH value affects the production of SCFAs. While acetate increases with the cultivation pH value, butyrate decreased. Propionate and isovalerate, in contrast, showed only a slight dependence on the cultivation pH value. Overall, the concentration of acetate and butyrate in each cultivation system, as well as isovalerate in systems A and C, were in the range of a healthy human with normal body weight (Schwiertz *et al.*, 2010). The concentration of propionate was increased compared to the concentration detected by Schwiertz *et al.* (2010). Nevertheless, these results are comparable with former studies (Haindl *et al.*, 2021a) as well as other *in vitro* studies

(Bircher *et al.*, 2018). Bircher *et al.* (2018) detected similar concentrations for acetate and butyrate in their *in vitro* microbiome.

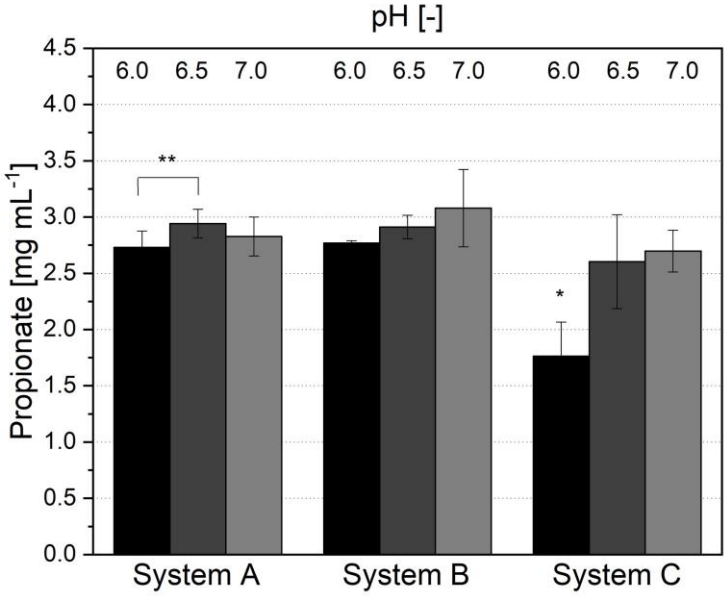

Figure 4.5: Concentration of propionate in the stable system of all three donors depending on the cultivation pH (black column: pH 6.0; dark grey column: pH 6.5; light grey column: pH 7.0); * marks statistical difference between marked pH and the other values; ** marks statistical difference between the two marked values.

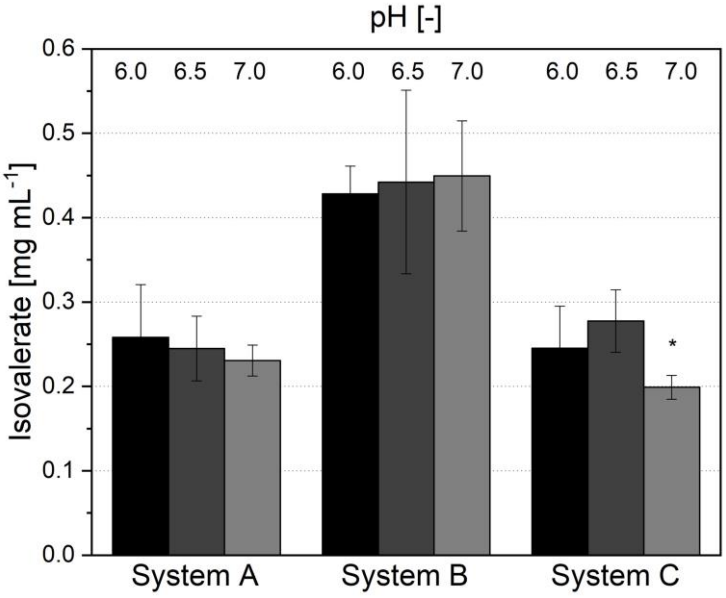

Figure 4.6: Concentration of isovalerate in the stable system of all three donors depending on the cultivation pH (black column: pH 6.0; dark grey column: pH 6.5; light grey column: pH 7.0); * marks statistical difference between marked pH and the other values.

Next to the absolute concentration, the ratio between acetate, propionate, and butyrate in the intestine is a marker for human health. We found that the ratio in system A was between 3:3:2 (pH 6.0), 4:3:2 (pH 6.5), and 4:3:1 (pH 7.0). In System B, the ratio was found to be 3:3:2 (pH 6.0) and 4:3:1 (pH 6.5 and 7.0), whereas at system C

it differed from 4:2:2 (pH 6.0) to 4:3:2 (pH 6.5 and 7.0). Consequently, despite differences in the concentration of the single SCFAs, the overall profile, measured by the ratio, is comparable for all systems and cultivation pH values. Previous researchers have stated that a ratio between 3:1:1 and 10:2:1 is healthy (Macfarlane *et al.*, 1992; Rowland *et al.*, 2018). Therefore, each cultivation pH value supports the growth of a healthy *in vitro* microbiota regarding the acetate–propionate–butyrate ratio as well as the single and overall concentration of metabolic products.

Microbial Composition

In addition to the metabolic profile, the microbial abundance and compositional changes in the systems were investigated. Clear differences for several single phyla were observed at different pH values in all systems. For Actinobacteria in system A (Figure 4.7), a decreasing trend in abundance with increasing pH was observed from 0.33 ± 0.09% at pH 6.0 to 0.04 ± 0.04% at pH 7.0.

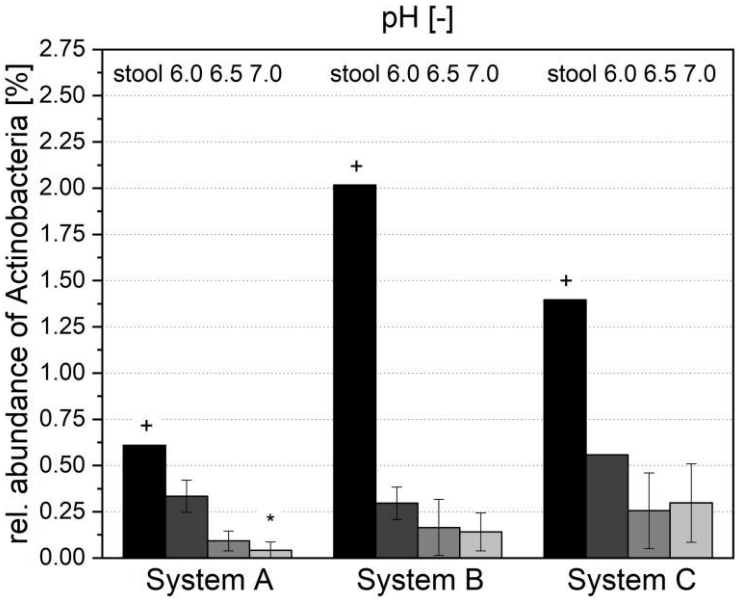

Figure 4.7: Rel. abundance of Actinobacteria in the donor stool and of all three systems depending on the cultivation pH (black column: stool; dark grey column: pH 6.0; grey column: pH 6.5; light grey column: pH 7.0); * marks statistical difference between marked pH and the other values; + marks statistical difference of donor stool and all cultivation pH values.

The same trend was observed in systems B and C; however, the differences were not statistically significant. Actinobacteria were mainly represented by the genus *Bifidobacterium* in all systems. Typical strains, such as *Bifidobacterium longum*, prefer a lower pH value (Kiviharju *et al.*, 2005). One-way ANOVA revealed statistically significant higher abundances of *Bifidobacterium* at pH 6.0 for system A. Figure 4.12 shows the rel. cum. abundance of genera in the stable system A dependent on the cultivation pH. For a better readability, only genera discussed in this study are shown; the remaining genera are summed up as "others". For systems B and C, the same trend in the abundance of *Bifidobacterium* was observed, but the differences were not statistically significant.

For Proteobacteria, no clear trend of abundance in connection with pH was observed (Figure 4.8). The abundance of Proteobacteria in system A ranged from 4.38 ± 2.42% at pH 6.5 to 3.51 ± 1.12% at pH 7.0. Systems B and C behaved comparably. In system B, the abundance in the cultivation pH 6.5 was significantly higher than in cultivation 7.0. Proteobacterial genera such as *Escherichia* and *Shigella* are able to grow at a wide range of pH (Small *et al.*, 1994; Schwan *et al.*, 2002). The Tukey post-hoc test revealed a significant influence on the abundance of *Escherichia* and *Shigella* in the low pH range of 6.0–6.5 for system A (Figure 4.12). For system B, a significant difference between all three pH values was observed, whereas no differences were detected for system C. These differences may have originated from different abundances in the donor stool. In donor stools, *Escherichia* and *Shigella* were more abundant in the stools from donors A (0.02%) and B (0.24%) compared to donor C (0.004%). These higher abundances may explain the significant differences in the stable *in vitro* systems. Verrucomicrobia, a minor phylum of the microbiota, increased with a rising pH (Figure 4.9). In system A, an abundance of 0.02 ± 0.03% at pH 6.0 was detected, which rose to 4.23 ± 1.74% at a pH value of 7.0 (Figure 4.9). Here, the differences were significant within all three systems, based on a *p*-value of 0.05. In system B, only a significant lower abundance at pH 6.0 was detected. Verrucomicrobia were represented by the genus *Akkermansia*, which show pH dependency in all three systems. The abundance of *Akkermansia* increased with the cultivation pH, which was already proven by other researchers (van Herreweghen *et al.*, 2017). For system B, the same trend in *Akkermansia* was observed, but was not statistically significant.

The two major phyla in the *in vitro* microbiome were Firmicutes and Bacteroidetes. The abundance of Firmicutes decreased with increasing pH in all systems (Figure **4.10**). For system A, at pH 6.0, an abundance of 26.04 ± 1.76% was reached, compared to 16.92 ± 1.96% at pH 6.5 and 13.35 ± 0.36% at pH 7.0. The differences between all pH values in system A were significant based on a *p*-value of 0.05. In systems B and C, only cultivation at pH 6.0 was significantly different from the others but followed the overall trend of a decrease. Abundant genera within the phylum Firmicutes were *Clostridium Cluster XIVa*, *Dialister*, *Faecalibacterium*, *Roseburia*, *Blautia*, and *Veillonella* (Figure 11). *Clostridium Cluster XIVa* and *Roseburia* showed no pH-dependent abundance for all systems. The abundance of *Faecalibacterium* decreased with an increasing pH and showed a significant dependence on pH for all three systems. *Faecalibacterium* is an important marker of gastrointestinal health (Cheema, 2019). This genus prefers a pH range of 5.7-6.7 for growth (Licht *et al.*, 2010), which is in accordance with this study. *Blautia* and *Veillonella* showed similar behavior as *Faecalibacterium*: both genera decreased in abundance with an increasing cultivation pH. This trend was statistically significant for both genera for systems A and B, but not for system C. Nevertheless, system C showed a similar trend in the abundance of *Blautia* and *Veillonella*. Representatives of the phylum Firmicutes, such as *Roseburia*, *Clostridium Cluster XIVa*, and *Faecalibacterium*, are the main butyrate-producing bacteria in the human intestinal microbiota (Barcenilla *et al.*, 2000; Louis and Flint,

2009). In this study, the highest concentration of butyrate was reached at a cultivation pH of 6.0. This relates to the high abundance of Firmicutes at pH 6.0 in all systems.

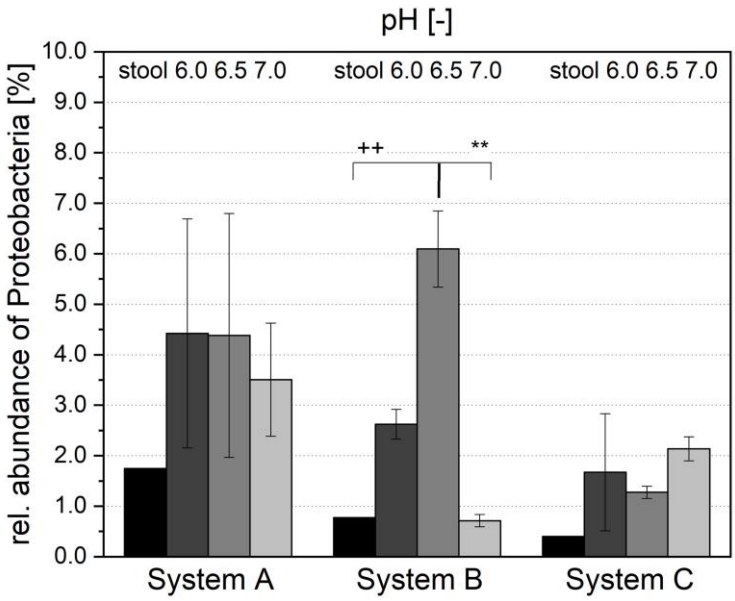

Figure 4.8: Rel. abundance of Proteobacteria in the donor stool and of all three systems depending on the cultivation pH (black column: stool; dark grey column: pH 6.0; grey column: pH 6.5; light grey column: pH 7.0); ** marks statistical difference between the two marked values; ++ marks statistical difference between the donor stool and the other marked values.

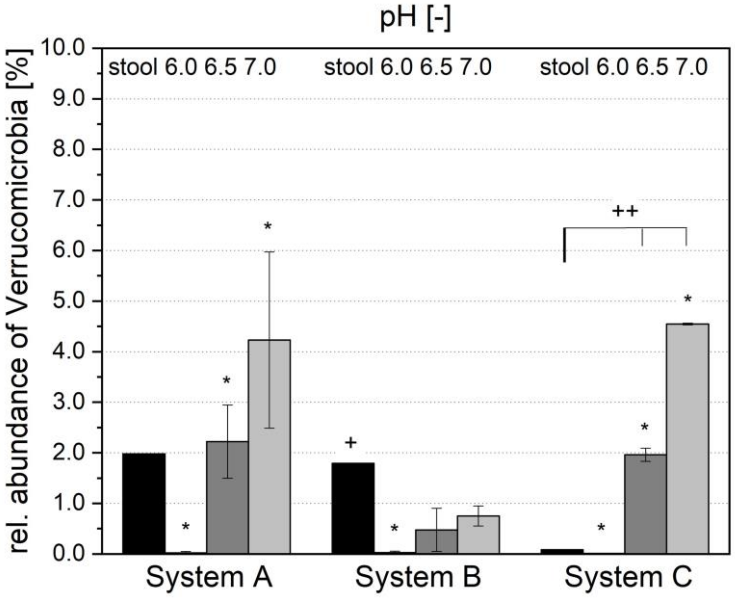

Figure 4.9: Rel. abundance of Verrucomicrobia in the donor stool and of all three systems depending on the cultivation pH (black column: stool; dark grey column: pH 6.0; grey column: pH 6.5; light grey column: pH 7.0); * marks statistical difference between marked pH and the other values; + marks statistical difference of donor stool and all cultivation pH values; ++ marks statistical difference between the donor stool and the other marked values.

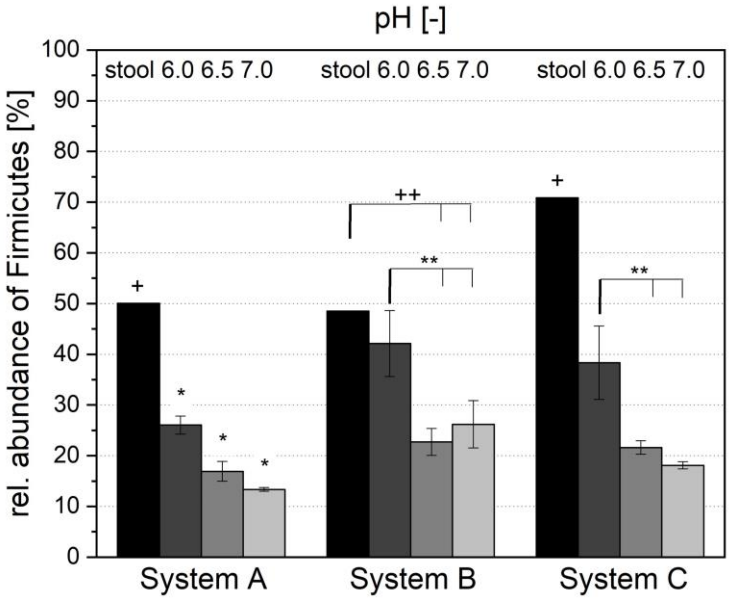

Figure 4.10: Rel. abundance of Firmicutes in the donor stool and of all three systems depending on the cultivation pH (black column: stool; dark grey column: pH 6.0; grey column: pH 6.5; light grey column: pH 7.0); * marks statistical difference between marked pH and the other values; ** marks statistical difference between the two marked values; + marks statistical difference of donor stool and all cultivation pH values; ++ marks statistical difference between the donor stool and the other marked values.

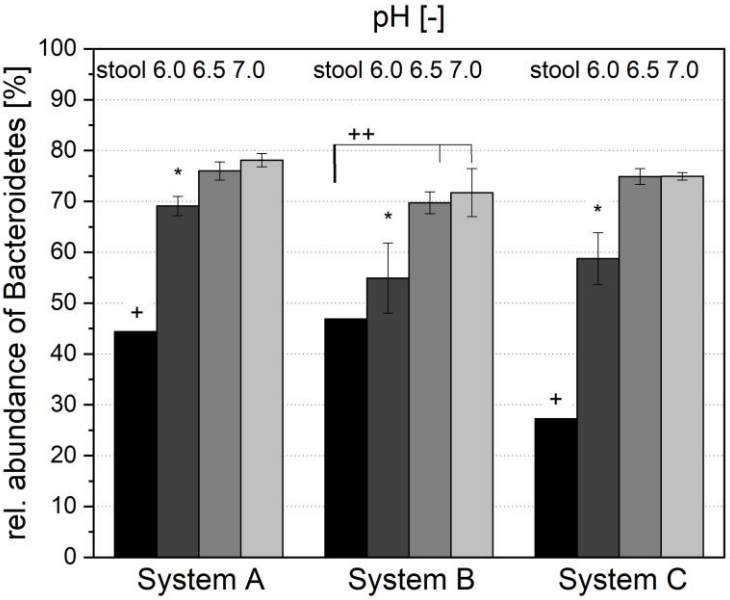

Figure 4.11: Rel. abundance of Bacteroidetes in the donor stool and of all three systems depending on the cultivation pH (black column: stool; dark grey column: pH 6.0; grey column: pH 6.5; light grey column: pH 7.0); * marks statistical difference between marked pH and the other values; + marks statistical difference of donor stool and all cultivation pH values; ++ marks statistical difference between the donor stool and the other marked values.

Conversely, the abundance of Bacteroidetes was found to increase with an increasing cultivation pH for all three systems (Figure 4.11). For system A, the relative abundance at pH 6.0 was 69.08 ± 1.88% and increased to 75.99 ± 1.78% at pH 6.5 and to

78.11 ± 1.28% at pH 7.0. This trend has also been observed by previous researchers (Duncan *et al.*, 2009; Zihler Berner *et al.*, 2013). In the current study, the influence of cultivation pH on the abundance of Bacteroidetes was found to be significant. Nevertheless, for all three systems, only the abundance at pH 6.0 showed a significant difference by the subsequent Tukey post-hoc test.

The main genera within the phylum Bacteroidetes were found to be *Alistipes*, *Parabacteroides*, *Bacteroides*, and *Prevotella* (Figure 4.12). *Alistipes* and *Parabacteroides* showed no dependance on pH for all three systems. Conversely, the abundance of *Bacteroides* was not dependent on pH in system A (61.12 ± 5.62% at pH 7.0; 68.17 ± 5.05% at pH 6.5; 67.35 ± 2.10% at pH 6.0), but was found to be statistically significant between pH values in system B and C. However, in all systems, the abundance of *Bacteroides* increased with the cultivation pH value.

An outlier occurred for the genus *Prevotella*. While this genus was not abundant in the stable systems A and B, system C showed high abundances of *Prevotella* from 1.58 ± 1.34% at pH 7.0 up to 31.28 ± 11.05% at pH 6.0. In system C, the abundance was statistically dependent on the cultivation pH. The abundance of *Prevotella* itself originated from the donor stool, as this genus was also only abundant in the stool from donor C.

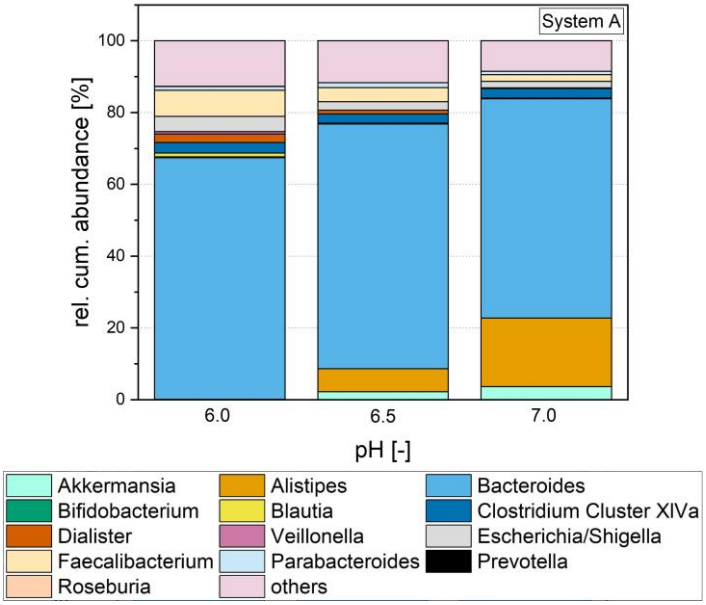

Figure 4.12: Rel. cum. abundance of genera in dependence on the cultivation pH value in system A; for a better readability only genera discussed in this study are depicted, other genera are summed up as others.

The ratio between Firmicutes and Bacteroidetes is an important measurement for human health. In general, a lower ratio is considered healthy. In a previous study, a ratio of around 0.5 was identified for a healthy control group (Lopetuso *et al.*, 2018). All *in vitro* microbiotas in the current study showed low, i.e., healthy, ratios. The ratio in system A was 0.38 at pH 6.0, 0.22 at pH 6.5, and 0.17 at pH 7.0. The same trend was observed for system B (0.77 at pH 6.0, 0.33 at pH 6.5, 0.32 at pH 7.0) and system C (0.65 at pH 6.0, 0.29 at pH 6.5, 0.24 at pH 7.0). Overall, a significant increase in the ratio at pH 6.0 was detected.

Roseburia, Bifidobacterium, and *Faecalibacterium* are genera of the intestinal microbiota that are known as health markers (Miquel *et al.*, 2013; Tojo *et al.*, 2014; Tamaki *et al.*, 2016; Tamanai-Shacoori *et al.*, 2017). In the *in vitro* microbiota of system A, the genus *Roseburia* was present in low abundance (0.00 to 0.14%) and showed no statistical dependence on the pH value during cultivation. In contrast, the abundance of *Faecalibacterium* decreased significantly with an increasing cultivation pH. As described above, the highest abundance (7.26 ± 0.28%) in system A was reached at a pH value of 6.0. In accordance, the genus *Bifidobacterium* was most abundant at pH 6.0 (0.21 ± 0.10%).

Duncan *et al.* (2009) investigated the influence of the *in vitro* cultivation pH on 33 representative human colonic bacteria. At a mildly acid pH value, less *Bacteroides* grew compared to a neutral pH value. At low pH values, more gram-positive bacteria were detected, which promote the production of butyrate. Nevertheless, gram-positive bacteria had a wildly varying tolerance for pH. For several representatives of *Clostridium Cluster XIVa*, a reduced growth at lower pH values was observed, whereas *Bifidobacterium* grew well at low values. Gram-negative bacteria, especially Bacteroidetes, were inhibited at reduced pH values. Consequently, *Roseburia* were more abundant at a lower pH value, whereas they were out-competed, especially by *Bacteroides*, at a neutral pH value. Even though these findings were investigated through in vitro cultivation of a defined microbiota, they are in accordance with the results in our study, where a whole complex microbiota was cultivated.

Microbial Richness and Diversity

The richness and diversity of microorganisms in the systems were measured by the Shannon effective index. Microbial richness and diversity are important for a functional, healthy microbiota. The richness of the in vitro microbiota of system A differed from 96.00 ± 3.46 at pH 7.0 to 105.17 ± 5.71 at pH 6.5 (Figure 4.13). At a cultivation pH of 6.0, a richness of 100.50 ± 5.96 was reached. No significant difference between the three systems was observed.

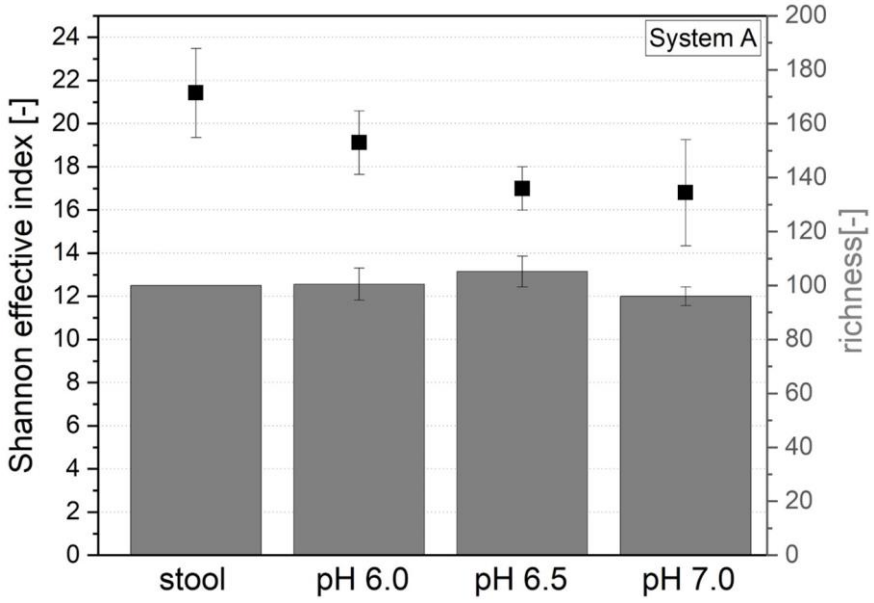

Figure 4.13: Comparison of Shannon effective index (black) and richness (grey) between the stool and cultivated system of donor A.

For systems B and C, no significantly different richness based on the cultivation pH was observed. However, the Shannon effective index in system A decreased from 19.12 ± 1.47 at pH 6.0 to 15.94 ± 1.43 at pH 7.0. The one-way ANOVA followed by a Tukey post-hoc test revealed a significant difference between the two pH values. The index of 17.28 ± 1.14 at pH 6.5 was not significantly different from the other values. The systems of donors B and C showed a similar trend in diversity, but the differences were not statistically significant. Consequently, the highest diversity was reached at lower pH values. This may be explained by the fact that *Bacteroides* dominate communities at higher pH values. Walker *et al.* (2005) detected an abundance of approximately 80% Bacteroides of total bacteria at an increased pH value, while only 20% were detected at lower pH values. These results may explain the reduced diversity at higher pH values in this study. Further, other researchers detected a higher diversity of abundant species at lower pH values when cultivating a microbiota of a defined composition of several representative bacteria (Duncan *et al.*, 2009).

4.3.2 *Comparison with Original Donor Stool*

When cultivating an *in vitro* microbiota, as well as the functionality, a high similarity with the original system is desirable. Regarding the abundance of Proteobacteria and Verrucomicrobia in system A, no significant differences between the donor stool and the cultivated broth were observed.

However, the phyla Actinobacteria in all three conditions (pH 6.0, 6.5, and 7.0) in all three systems differed significantly from the original sample (Figure 4.7). In system A, the abundance of Actinobacteria in the stool was 2.02%, whereas it was lower in the cultivated system (e.g., 0.33 ± 0.09% at pH 6.0). Systems B and C showed a similar behavior. The abundance of Proteobacteria showed no dependency on the pH for

systems A and C, and further no significant difference from the abundance in the donor stool (Figure 4.8). System B showed slight differences, as the abundance at the pH 6.5 cultivation was increased compared to pH 7.0 and the donor stool. Overall, the *in vitro* cultivation at different pH values only has a slight influence on the abundance of Proteobacteria. The abundance of Verrucomicrobia showed an increase with the cultivation pH for systems A and C. For system B, the same trend was observed, but only the abundance at pH 6.0 was significantly lowered. When comparing the abundance of Verrucomicrobia with the donor stool, different observations for the systems are made (Figure 4.9). For system A, no significant difference was observable. The abundance of Verrucomicrobia in donor stool B was significantly higher compared to the cultivated microbiotas. At system C, however, the abundance in the donor stool was significantly lower compared to cultivations at pH 6.5 and 7.0. It seems that Verrucomicrobia is abundant in higher numbers at a higher pH value, but compared to the donor stool, the behavior is individual.

As Figure 4.10 shows, the abundance of Firmicutes was significantly lower in the cultivated system compared to the original value in the stool (26.04 ± 1.76% at pH 6.0 vs. 48.52%) in system A. System C showed the same effect, whereas the abundance of Firmicutes in the donor stool B was only significantly higher than systems 6.5 and 7.0. Conversely, the abundance of Bacteroidetes in system A at pH 6.0 was 69.08 ± 1.88% compared to 46.89% in the donor stool (Figure 4.11). Systems A and C showed significantly higher abundances of Bacteroidetes than in the donor stool. For system B, only the abundance in the cultivations 6.5 and 7.0 was significantly higher, although the abundance in all three systems tended to be increased.

Consequently, all systems experienced a decrease in Actinobacteria and Firmicutes due to cultivation. Contrary, the abundance of Bacteroidetes and Proteobacteria increased during the cultivation process. The behavior of Verrucomicrobia was individual and dependent on the donor stool.

As Table 4.1 shows, the ratio of Firmicutes and Bacteroidetes in the donor stool was 1.13 (donor A), 1.03 (donor B), and 2.60 (donor C). During cultivation, the ratios dropped. In system A, ratios of 0.38 (pH 6.0), 0.22 (pH 6.5), and 0.17 (pH 7.0) were calculated. In system B, a cultivation at pH 6.0 led to a ratio of 0.77, whereas it was lower during cultivation at pH 6.5 (0.33) and 7.0 (0.32). For system C, ratios of 0.65 (pH 6.0), 0.29 (pH 6.5), and 0.24 (pH 7.0) were reached. As a ratio around 0.5 is claimed as healthy (Lopetuso *et al.*, 2018), a lower cultivation pH is recommendable to reach a healthy *in vitro* contribution of Firmicutes and Bacteroidetes.

Regarding α-diversity, an *in vitro* cultivation had no influence on the richness in systems A and B. For system A, the richness in the donor stool (100) was comparable to the richness of 101 (pH 6.0), 105 (pH 6.5), and 96 (pH 7.0) in the cultivated systems (Figure 4.13). For systems B and C, no difference was observed. Further, the Shannon effective index in system A was decreased for the cultivation at pH 6.5 (17.00) and 7.0 (16.80) compared to the donor stool (21.42). A cultivation at pH 6.0 showed no significant difference (19.12). For system B, no difference in the Shannon effective

index was detected, whereas the diversity was lowered for all cultivations in system C. Here, it seems that the richness can be preserved *in vitro* in big parts, whereas the diversity (Shannon effective) is decreased, especially at higher cultivation pH values. We concluded from these results that the original microbiota from the donor stool cannot be replicated precisely. However, all systems represented functioning *in vitro* microbiotas. Regarding the distribution of phyla (Figure 4.7 to Figure 4.11), the pH 6.0 cultivation showed the highest similarity with the original stool. At low cultivation pH values, the growth of Bacteroidetes is reduced, whereas more gram-positive bacteria as Firmicutes are abundant (Duncan *et al.*, 2009), resulting in a healthy ratio of Firmicutes to Bacteroidetes. Further, health-promoting representatives were found to be abundant at pH 6.0 compared to a value of 7.0. Regarding richness and diversity, a cultivation at a lower pH is also recommendable. The physiological pH value in the intestine increases from a value of 5.4-5.9 in the proximal colon up to 6.1-6.9 in the distal colon (Payne *et al.*, 2012). It seemed that a pH value of 7.0 is already too high to create a system similar to the *in vivo* characteristics. A cultivation at pH 6.0 provides conditions that support the growth of the phylum Firmicutes and consequently results in a system more similar to the donor stool.

4.4 Conclusions

In the current study, the influence of the cultivation pH on the *in vitro* microbiota systems created with the stool of three different donors was assessed. From the results obtained, we concluded that the microbiota differs dependent on the set pH value.

With an increasing cultivation pH, an increase in cell count; the total amount of SCFAs, acetate, and propionate; and the abundance of Bacteroidetes and Verrucomicrobia was observed. For the concentration of butyrate and the abundance of Actinobacteria and Firmicutes, a decrease with a higher pH was determined. For the concentration of isovalerate, the abundance of Proteobacteria and diversity, measured by richness and Shannon effective, no effect of the cultivation pH was observed.

The aim of this study was to detect the cultivation pH where a system with a high functionality as well as comparability with the donor stool forms. This question cannot be answered completely, as the system, and therefore the choice of pH, is dependent on the individual requirements. If a high cell count is desired, a pH of 7.0 is desirable, as this pH level promotes the growth of a higher number of cells. A higher concentration of SCFAs, including acetate and propionate, were also reached at pH 7.0. However, higher concentrations of butyrate were present at pH 6.0. When comparing the distribution of phyla in the stable systems, the composition was closer to the original system when cultivation was conducted at pH 6.0. Regarding the ratio of Firmicutes to Bacteroidetes, a cultivation at lower pH values is recommendable. Furthermore, a higher abundance of health-promoting bacteria, including *Roseburia*, *Bifidobacteria*, and *Faecalibacterium*, was detected at pH 6.0. In general, all investigated pH values created stable systems, with the factors of the ratio of SCFAs and the

phylogenic distribution considered as being indicative of a healthy microbiota. If a microbiota with a high cell count and a high amount of total SCFAs is required, the cultivation pH should be set to 7.0. On the other hand, if high concentrations of butyrate and a higher similarity with the donor stool are targeted, a cultivation pH of 6.0 would lead to the desired results. Ultimately, therefore, the choice of pH creates significant differences in the established *in vitro* microbiota, but no clear recommendations for a special value can be made.

Acknowledgements

We gratefully acknowledge the donors as well as all members of the ZIEL Core Facility Microbiome for their technical and analytical support during the experiments. The Institute for Medical Microbiology and Hygiene, University of Regensburg is thanked for the testing of the donor stools.

References

The references were combined into a joint list of all publications, see at the end of the thesis.

5 Impact of cultivation strategy, freeze-drying process, and storage conditions on survival, membrane integrity, and inactivation kinetics of *Bifidobacterium longum*

Summary and contribution of the doctoral candidate

In this publication, a freeze-drying process was developed with the aim of gentle conservation of the used model strain *Bifidobacterium longum* ssp. *longum* Reuter 1963 (Figure 5.1).

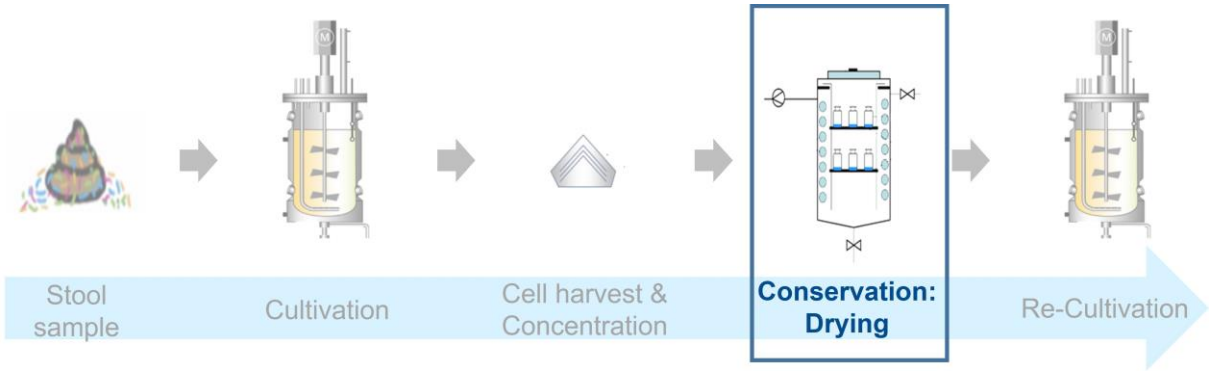

Figure 5.1: Investigated steps in the entire preservation process: development and investigation of a drying protocol based on the selected test strain

Bifidobacterium longum is one of the health-promoting microorganisms in the human gut, belonging to the phylum of Actinobacteria. In the dairy industry, it is further used as an adjunct to lactic acid starter cultures or sold as a probiotic product. Therefore, the cell suspensions get freeze-dried with protective additives to prevent activity losses. Up to date, investigations lack information covering growth and inactivation kinetics over the whole process chain consisting of cultivation, drying and storage. Therefore, the effect of cultivation conditions before and the shelf temperature during drying as well as the influence of protectants (maltodextrin, glucitol, trehalose) at various concentrations on cell survival during freeze-drying was assessed. Drying was followed by a storage at + 4 °C and + 20 °C for 70 days to evaluate inactivation kinetics. The impact and effects of the different factors were measured by the determination of survival rate, cell membrane integrity, residual moisture content and glass transition at various process points to reveal inactivation mechanisms.

A cultivation pH value of 6.0 (growth optimum of the strain) provided better conditions regarding cell survival after drying compared to non-regulated pH conditions (free acidification). The main reason for inactivation during drying is membrane leakage due to the removal of water. In total, the highest survival of 49% was obtained with cells dried at + 35 °C shelf temperature with an addition of maltodextrin (75% bacterial dry matter, w/w). The results show that Bifidobacterium longum cells are mostly inactivated during drying. Storage conditions at + 4 °C with

an addition of 75% bacterial dry matter maltodextrin relative to bacterial dry mass were able to prevent cell loss completely.

The doctoral candidate developed the concept of the experiments and methods used in this study. She critically reviewed the existing literature. Moreover, the doctoral candidate analyzed the data and interpreted the results. She further wrote the manuscript and revised it. Co-authors contributed to the experimental part and to the discussion of the results and provided input to the drafted publication prior to submission.

Adapted original manuscript[5]

Impact of cultivation strategy, freeze drying process and storage conditions on survival, membrane integrity and inactivation kinetics of *Bifidobacterium longum*[6]

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Abstract

Bifidobacterium longum, one of the main microorganisms in the human gut, is used as an adjunct to lactic acid starter cultures or sold as a probiotic product. Therefore, *Bifidobacterium longum* cell suspensions get freeze-dried with protective additives to prevent activity losses. To date, investigations covering growth and inactivation kinetics of *Bifidobacterium longum* during the whole process (cultivation, drying, and storage) have been lacking. In this study, the effect of cultivation conditions and shelf temperature as well as the influence of protectants (maltodextrin, glucitol, trehalose) at various concentrations on cell survival during freeze-drying were assessed. Drying was followed by a storage at +4 °C and +20 °C for 70 d to evaluate inactivation kinetics. The impact of the different factors was assessed by measuring survival rate and residual moisture content at various points of time over the whole process. In parallel cell membrane integrity and glass transition were determined to reveal inactivation effects (Bruno and Shah, 2003).

Cultivation strategy had a strong influence on survival with a huge potential for process improvement. A pH of 6.0 at the growth optimum of the strain provides better conditions regarding cell survival after drying than free acidification (non-regulated pH conditions). During the drying step, membrane leakage due to the removal of water is the main reason for the inactivation in this process step. In this study, the highest survival of 49 % was obtained with cells dried at +35 °C shelf temperature with an addition of maltodextrin (75 % bacterial dry matter, w/ w). The results show that *Bifidobacterium longum* cells are mostly inactivated during drying, whereas storage

[5] Adaptions refer to formatting issues: e.g., abbreviations, figure, table, equation and section numbering, citation style, notation of units, spelling, axis labeling.

[6] Originally published in *Folia Microbiologica* (2020), Vol 65 (6), pp. 1039–1050. Permission for reuse of this article was granted.

conditions at +4 °C with an addition of 75 % BDM maltodextrin relative to bacterial dry mass prevent cell loss completely.

5.1 Introduction

Bifidobacteria, attributed to the phylum Actinobacteria, are one of the core microorganisms in the human intestinal microbiome. Numerous studies have demonstrated their probiotic activities, their impact on the microbiome and their effect on human health (Quigley, 2017). *Bifidobacteria* produce organic substances such as lactic and acetic acid. These acids lower the pH in the colon and promote the growth of other beneficial intestinal bacteria (Sugahara *et al.*, 2015), as well as prevent the colonization of the gut by pathogenic microorganisms (Gibson and Wang, 1994). *Bifidobacteria* can further improve lactose utilization in case of malabsorption by lactose intolerant individuals (Kailasapathy and Chin, 2000). Typical species in the large intestine include *Bifidobacterium adolescentis*, *Bifidobacterium breve* and *Bifidobacterium longum*. The latter is abundant in the human gut and found at a concentration of 10^8 CFU/g (Matsuki *et al.*, 2004).

In industry, *Bifidobacterium longum* is used as an adjunct culture in fermented dairy products. It is also distributed as a liquid or dried probiotic nutritive supplement. Such nutritive supplement cultures are produced as starter cultures in large-scale cultivation systems. In order to be able to store and flexibly use these cultures, the obtained cells are freeze-dried. The technical production process of microorganisms, therefore, includes cultivation, drying and storage. Each step has different effects on the final number of active cells in the finished product. To prevent major losses of active cells and to minimize inactivation, processing conditions along the entire chain of production must be carefully chosen. Up until now research often concentrates on one single step of the production process. To work efficiently, it is necessary to regard the entire chain to develop a process obtaining a high cell count and survival at the end. Various works report on the effect of cultivation conditions on *Bifidobacteria* survival during the drying step. A higher survival rate was achieved when the pH during cultivation was held at the optimal value of 6.0 for *Bifidobacterium lactis* (Kiviharju *et al.*, 2005; Bauer *et al.*, 2012). Also, recommendations so far suggest that cell harvesting should be carried out during the late-logarithmic or early stationary phase as the cells here are the most resistant to drying (Saarela *et al.*, 2005). However, methods to produce and preserve microorganisms, differ. As a result, survival rates reported in previous literature, are not readily comparable. The commonly used method during industrial probiotic production of cells is freeze-drying (Champagne *et al.*, 1991; Wang *et al.*, 2004; Saarela *et al.*, 2005; Saarela *et al.*, 2006; Shamekhi *et al.*, 2013). During drying, the water surrounding the cell as well inside the bacterial cell membrane is removed. Consequently, membrane leakage occurs, and the bacterial cell is not able to recover and restart growth. This is the main reason for cells getting inactivated. During drying and storage, protectants can be added to prevent

inactivation. Protectants like maltodextrin, glucitol or trehalose, have already been used for different lactobacilli strains (Oldenhof *et al.*, 2005; Sohail *et al.*, 2013; Ambros *et al.*, 2018a). These molecules can act as cryoprotectants (Dianawati *et al.*, 2013) or through different protection mechanisms as water replacement or the glassy state (Santivarangkna *et al.*, 2008a). Studies on *Bifidobacteria* strains have demonstrated that both, the choice of protective agent and the processing conditions influence cell survival in a strain-dependent manner (Yeung *et al.*, 2016). Consequently, the individual strains' properties must be considered when selecting the conditions for the drying process.

During storage, amongst others, the environmental temperature on freeze-dried *Bifidobacterium longum* has an influence on the survival rate as examined by several researchers (Reilly and Gilliland, 1999; Bruno and Shah, 2003). Furthermore, a low storage temperature seemed to be appropriate for microencapsulated cells of *Bifidobacterium longum* (Hsiao *et al.*, 2004). At storage, low relative humidity is required to obtain high survival rates (Abe *et al.*, 2009; Min *et al.*, 2017).

However, the behavior during storage of non-encapsulated cells in combination with the addition of protectants, as well as the influence across all process unit operations including cultivation, drying, storage and the overall rate of inactivation has not yet been studied further. An investigation of how these factors in combination affect the survival of *Bifidobacterium longum* is still missing. Therefore, the purpose of this study is to investigate key factors in survival and to characterize and to improve a *Bifidobacterium longum* Reuter 1963 production process with regard to raise the level of survival. We set out to combine the factors involved in inactivation and determine how cell count evolves over the whole process including storage of the freeze-dried culture. Furthermore, the interaction among protectant and temperature during storage was investigated. A storage time of 70 d was chosen, because this was considered long enough to identify storage related effects on inactivation. Our hypothesis therefore is that by assessing the impact of factors, the rate of survival could be raised, and these effects can be better understood by measuring glass transition and cell membrane integrity in parallel.

5.2 **Materials and methods**

5.2.1 *Bacterial culture and fermentation process*

Bifidobacterium longum ssp. *longum* Reuter 1963 from the DSMZ (German Collection of Microorganism and Cell Cultures, Braunschweig, Germany), hereafter referred to as *B. longum* Reuter 1963, was chosen as the test strain.

One batch of *B. longum* Reuter 1963 was used as the inoculum for all experiments. A bioreactor *BioStat C* (Sartorius Stedim Biotech GmbH, Göttingen, Germany) containing 8 L of broth was inoculated to obtain a starting cell concentration of $A_{600} = 0.3$ ($3 \cdot 10^8$ CFU/mL). The cells were grown in MRSc medium (MRS medium with 1 g/L cystein) at +37 °C, with a mixing speed of 80 rpm and

sparged with 0.1 L/min N_2. The initial pH of the medium was adapted to pH 6.0. In the cultivation process the pH was either not regulated (free acidification) or adjusted by adding 2 mol/L NaOH or 0.5 mol/L HCl to maintain pH 6.0. These conditions were found to be of major importance by previous works (Bauer *et al.*, 2012).

5.2.2 *Sample preparation and protectants*

Following cultivation, the cell suspension was centrifuged using a HeraeusTM BiofugeTM StratosTM Centrifuge (Thermo Fisher Scientific, Waltham, USA; 15.000 rpm, 0.3 L/min, 30 min) to enable cell harvesting. Then the cells were washed by adding peptone salt solution (1 g/L peptone from casein; 8.5 g/L NaCl; 0.3 g/L KH_2PO_4; 0.6 g/L $Na_2HPO_4 \cdot 2\ H_2O$) and centrifuged again under the same conditions for another 15 min. After homogenizing the cell pellet, the protectants maltodextrin (dextrose equivalent 4.0-7.0; Sigma-Aldrich, Steinheim, Ger-many), D(+) trehalose (Sigma-Aldrich, Steinheim, Germany) or glucitol (D(-) sorbitol, Merck KGaA, Darmstadt, Germany) were added in several concentrations (0, 10, 25, 50, 75 and 100 %) related to the bacterial dry matter (BDM, w/ w). Afterwards, 1 mL aliquots of the suspensions were pipetted into freeze-drying glass vials and frozen in a -80 °C freezer BF-U538 (Buchner Labortechnik, Pfaffenhofen an der Ilm, Germany). The process scheme is illustrated in Figure 5.2.

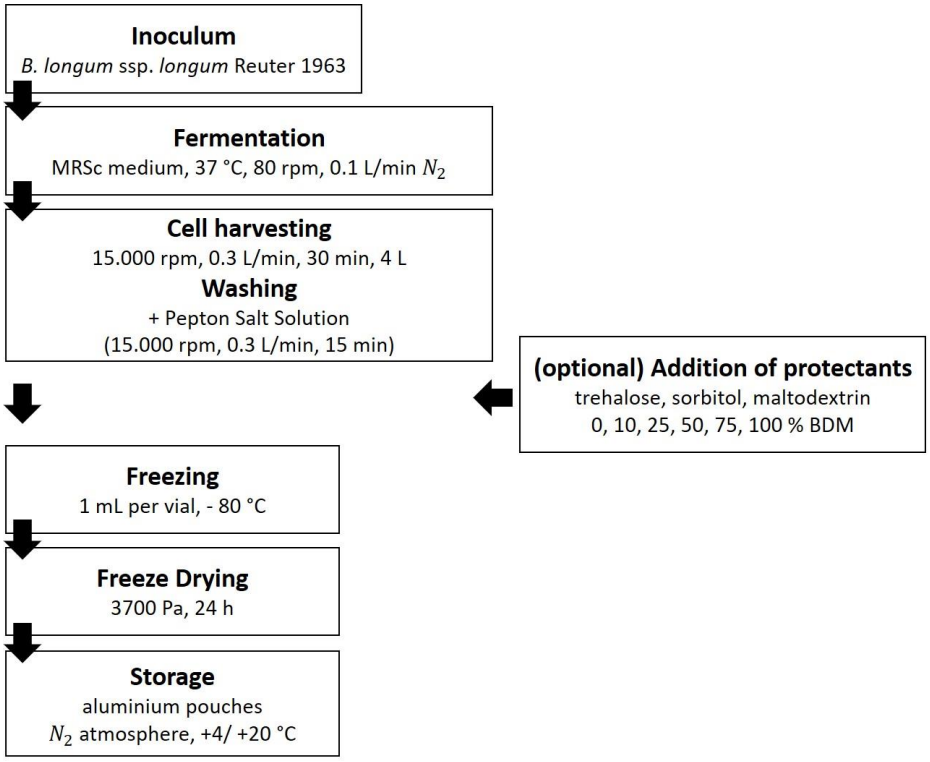

Figure 5.2: Process scheme applied in this study

5.2.3 *Freeze-drying*

Freeze-drying was conducted in three sucessive steps using a *Delta 1-24 LSC* pilot plant dryer (Christ GmbH, Osterode, Germany). The chamber pressure was kept at a constant 3700 Pa, while the shelf temperature rose in a stepwise manner (Table 5.1).

Table 5.1: Drying protocol for preservation of B. longum Reuter 1963 with the temperature in process step 3 adjusted to +25, +35, or +40 °C

Process step	Chamber pressure (Pa)	Shelf temperature (°C)	Duration (h)
1	3700	-10	12
2	3700	+10	6
3	3700	+25/ +35/ +40	6

The temperatures of the shelf and inside the product were controlled with internal sensors to avoid exceeding the set temperature limits. During freeze-drying at a constant chamber pressure, the amount of heat brought into the product is the driving force. The heat transfer rate dQ/dt is dependent on several factors as shown in equation ((5.1):

$$\frac{dQ}{dt} = A_v \cdot K_v \cdot \Delta\vartheta \quad (5.1)$$

A_v, the contact surface of the used vial and K_v, the heat-transfer coefficient of the glass vial are geometry and material dependent and constant, respectively. Consequently, the amount of heat Q that is transferred to the product in a certain time interval is only dependent on $\Delta\vartheta$, the difference of temperature between the product and the shelf. The product temperature cannot be regulated separately, but is dependent on the shelf temperature, chamber pressure and consequently moisture and heat flow rate. The shelf temperature was adjusted stepwise to remove surface and structural water molecules. Removing structural water required additional energy and, therefore, the shelf temperature during the third step of the process was adjusted to +25, +35 or +40 °C. Details regarding the choice of these conditions will be discussed later.

5.2.4 *Packaging and storage conditions*

After drying, approximately 0.5 g per sample were packed in light-proof aluminum bags (PET/ ALU/ PE - 12/ 12/ 75 µm; oxygen permeability <0.0001 cm^3/m^2 d hPa) under nitrogen atmosphere (< 5 Vol. ppm H_2O). They were stored at +4 °C or +20 °C for 7, 14, 21, 28, 42, 56 and 70 d until analysis. At each time point at least two bags were chosen for analysis.

5.2.5 *Analysis of residual moisture content and water activity*

Both after drying and at each point of time residual moisture content was determined. Water activity was analyzed with an *A_w Sprint TH-500* (Novasina, Lachen, Switzerland). The residual moisture content was measured using a *CEM Smart TurboTM 5* (CEM Corporation, Kamp-Lintfort, Germany) at a maximum sample temperature of +80 °C and 45 % power input. The results obtained with the *CEM* device were double-checked regularly by Karl Fischer titration using *TitroLine KF* (Mettler-Toledo GmbH, Schwerzenbach, Germany).

5.2.6 *Analysis of survival and inactivation rate constant*

The survival rate was determined by rehydrating the dried cell powder with sterile bi-distilled water to its initial biological dry matter (after cell harvest and concentration, before drying). The obtained suspension was then diluted with 0.25 strength Ringer's solution and plated on MRSc agar plates (MRSc broth with 15 g/L agar-agar). These plates were incubated anaerobically for 48 h at +37 °C. Plates with 30 to 300 colonies were included in counting colonies. The number of colony forming unites *N* per mL of sample (CFU/mL) was calculated according to (5.2.

$$N = \frac{c}{(n_1 + (0.1 \cdot n_2)} \tag{5.2}$$

Where *c* is the sum of colonies of the subsequent dilutions with n_1 the number of colonies in the less diluted solution and n_2 the number of colonies in the more diluted solution.

The survival rate (*SR*, %) was then calculated by the ratio of CFU as shown in the following (5.3. Hereby, $N_{0,i}$ refers to the individual plate count before each processing step (CFU before drying or storage), N_t refers to the plate count as a function of processing time and processing step.

$$SR = \frac{N_t}{N_{0,i}} \cdot 100 \tag{5.3}$$

The survival rate was determined at different points of time during the drying process and storage.

The inactivation rate during storage represents the rate of active of cells loss in a specific period of time (per d). It was determined as the logarithmic value of the ratio of the cell count at the beginning of the storage period (after drying, N_0) and after particular process time points (N_t). The residual cell count (N_t/N_0) was plotted logarithmically over time (t, d) for each storage temperature and protectant addition. The inactivation rate constant k (per d) was calculated from the slope of the linear regression of the obtained data, as shown in (5.4:

$$log \frac{N_t}{N_0} = -\frac{k \cdot t}{-2{,}303} \tag{5.4}$$

5.2.7 *Assessment of membrane preservation*

To analyze cell membrane preservation, the cells were stained with fluorochromes. The fluorochrome propidium iodide (PI) is able to pass through the damaged membrane of dead cells and can thus interact with DNA within the cell. The resulting fluorescence was detected at a filter wave length of 670 nm. On the other hand, the dye thiazole orange (TO), which is able to diffuse through the intact cell membrane and interacting with both DNA and RNA, induces fluorescence of intact cells. The absorbance of TO was measured at an excitation wave length of 530 nm. Cells with a damaged cell membrane were stained by both fluorochromes.

PI (Sigma-Aldrich Chemie, Darmstadt, Germany) was dissolved to 21 µmol/L, TO (Sigma-Aldrich Chemie, Darmstadt, Germany) was used as a 1 µmol/L solution. 100 µL of cell suspension (10^{-3} dilution) were mixed with 290 µL PBS buffer (0.2 g/L KCl; 8.0 g/L NaCl; 0.27 g/L KH_2PO_4; 1.42 g/L $Na_2HPO_4 \cdot 2\ H_2O$), 100 µL PI and 10 µL TO solutions, homogenized and incubated for 10 min at room temperature. After incubation the samples were measured in a *BD AccuriTM C6* flow cytometer (Accuri Cytometers, Ann Arbor, USA).

Membrane integrity (*MI*, %) was calculated as the ratio of the three differentiable groups in (5.5:

$$MI = \frac{N_{intact}}{N_{intact} + N_{damaged} + N_{dead}} \cdot 100 \tag{5.5}$$

Membrane preservation (*MP*, %) was calculated as the ratio of MI before drying (MI_0) and after different time points in the process (MI_t) as (5.6 shows:

$$MP = \frac{MI_t}{MI_0} \tag{5.6}$$

5.2.8 *Analysis of glass transition temperature*

The glass transition temperature of the samples after drying was measured with modulated differential scanning calorimetry *Q1000* (TA Instruments, New Castle, USA). Two hermetically closed aluminum pans were used for each measurement. An empty pan was used as reference, whereas the second pan contained 10-15 µg of the dried sample. Before measurements, the system was calibrated with indium and the measuring chamber was flooded with liquid nitrogen. During the measurement the heating rate was set to 2 K/min over a temperature range from -60 to +150 °C with a modulation time of 60 s and a temperature amplitude of ± 1 °C. Analysis was performed with the software *TA Universal Analysis 2000* (TA Instruments, New Castle, DE, USA). Therefore, the reversing heat flow (W/g), heat flow (W/g) and non-reversing heat flow (W/g) were plotted over temperature. Onset and offset glass transition temperatures were marked by applying tangents to the break points of the plotted graph of the reversing heat flow. The inflection point was calculated, and mid glass transition temperature was considered for further evaluation.

5.2.9 *Statistical analysis*

All analyses were repeated at least in triplicate. The mean values are shown as the arithmetic mean $\bar{x}$ of the number n of all samples x_i. The distribution of the values was calculated from the standard deviation s due to the random error. All graphs in the following show arithmetic means ± standard deviations. The results were tested for significance using a one-way ANOVA ($p \leq 0.05$), followed by a Tukey post-hoc analysis to reveal significant influencing parameters. The tests were conducted with the software OriginPro 2019 (OriginLab Corporation, Northampton, USA).

5.3 **Results and discussion**

The hypothesis of this work is that an investigation of several relevant process settings and inactivation during all steps allows an optimization of survival. In parallel, measurements of glass transition temperature and membrane integrity will help to explain effects causing losses in viability. The most harmful steps and conditions for *B. longum* Reuter 1963 during the production process and storage will get identified. Furthermore, the interaction among protectant and temperature during storage will be investigated. High survival rates, low residual moisture contents and a long shelf life are selected as desired variables.

5.3.1 *Effect of cultivation conditions on cell growth and survival after drying*

The production process of probiotics starts with the cultivation step. In this study the influence of the cultivation pH on cell count and survival rate after a freeze-drying process was examined. Figure **5.3** shows the absorbance and pH value obtained at two fermentation modes of *B. longum* Reuter 1963 cell suspensions: free acidification (non-regulated pH) and pH 6.0 (pH for optimal growth (Kiviharju *et al.*, 2005)).

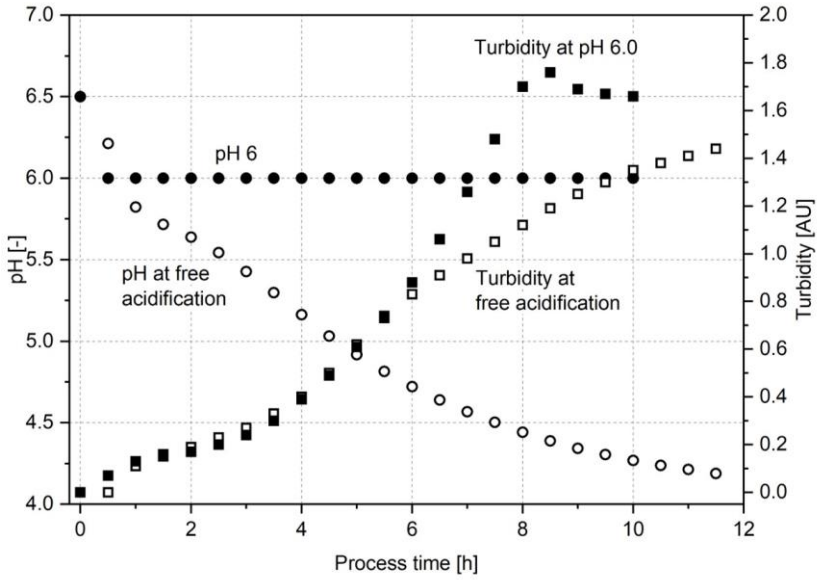

Figure 5.3: Exemplary turbidity and pH value at pH-controlled (pH 6) and non-regulated (free acidification) cultivaiton

Each cultivation was stopped in the early stationary phase. Harvesting cells in the early stationary phase achieves the best survival rate after drying as shown by Corcoran *et al.* (2004). The harvesting point was reached after 10 h at pH 6.0 and 11.5 h under non-regulated pH conditions. This is similar to times reported by Lian (2002), who also detected the stationary phase after 12-15 h when cultivating *B. longum* Reuter 1963 cells with MRS medium supplemented with 0.05 % cysteine under non-regulated pH conditions. A non-regulated pH results in free acidification, induced by the production of short chain fatty acids such as acetic or lactic acid. In the beginning, the turbidity measurements under both cultivation models were equal. After 5.5 h, however, the culture grown under free acidification conditions began to show a smaller increase in turbidity as compared to the culture kept at pH 6.0. At this point, the pH dropped below 4.8, which seems to be a critical pH for the strain since growth was restricted from that point on. This resulted in a lower turbidity value than that of the culture held at pH 6.0. After 10 h, the broth cultivated at pH 6.0 reached a turbidity of 1.66 AU. The cells cultivated without pH regulation resulted in 1.44 AU after 11.5 h. Following cell harvest by centrifugation, a comparable number of cells was established in both cases: $2 \cdot 10^{10}$ CFU/mL for non-regulated cultivation and a cell count of $5 \cdot 10^{10}$ CFU/mL after cultivation at pH 6.0. Then, the cells were freeze-dried (3700 Pa, 24 h, -10/ +10/ +35 °C, no protectant). The resulting survival rates are shown in Table **5.2**.

Table 5.2: Survival rates for the tested influencing factors during cultivation, drying, and storage; statistical analysis was done with a one-way ANOVA ($p \leq 0.05$), followed by a Tukey post hoc test

Influencing factor		Tested parameter	Survival rate [%]
Cultivation	pH control[1]	pH 6.0	40 ± 15
		Free acidification	0.5 ± 0.4
Drying	Shelf temperature [°C][2]	+25	17 ± 2
		+35	40 ± 15
		+40	6 ± 0.7
	Type of protectant[3]	Without	40 ± 15
		Glucitol	34 ± 3
		Trehalose	17 ± 7
		Maltodextrin	43 ± 3
	Concentration of MD addition [% BDM][3]	10	28 ± 3
		25	26 ± 15
		50	31 ± 9
		75	49 ± 13
		100	43 ± 3
Storage (70 d)[3]	Addition of protectant ambient temperature [°C]	4 °C without protectant	87 ± 6
		4 °C with 75 % BDM MD	100 ± 15
		20 °C without protectant	28 ± 1
		20 °C with 75 % BDM MD	57 ± 7

[1] Drying process: 3700 Pa, 24 h, -10/ +10/ +35 °C, no protectant

[2] Drying process: 3700 Pa, 24 h, -10/ +10/ *adapted due to information in table*, no protectant

[3] Drying process: 3700 Pa, 24 h, -10/ +10/ +35 °C

A high number of cells cultivated at the optimum pH of 6.0 survived the drying process (40 %). In comparison, cells cultivated under free acidification conditions were less likely to survive (0.5 %). Hence, the pH control during cultivation has a significant statistical influence based on a p-value of 0.05 on the drying outcome. These findings are in accordance with Bauer et al. (2012). They also reported the positive correlation between a cultivation pH at the optimum and a resulting higher drying resistance for *Bifidobacterium lactis* due to a different structure and composition of the cell membrane. The composition of fatty acids in the phospholipid bilayer resulted in differently stable membranes during drying. To validate those results for the strain used in this study, the membrane preservation was measured. For *B. longum* Reuter 1963, free acidification led to a preservation of only 1.0 % of membranes after drying, whereas cultivation at pH 6.0 induced more stable cell membranes, resulting in a membrane preservation of 43 %. Survival rate and residual cell membrane integrity under different cultivation conditions here also correlate directly.

5.3.2 *Effect of the drying process*

Influence of the shelf temperature during the third drying step on survival

The freeze-drying step followed cultivation, harvesting and concentration. Cells cultivated at the optimal pH of 6.0 were used for all the following experiments since they showed a significant higher rate of survival. A constant chamber pressure of 3700 Pa was used for drying. The shelf temperature T_{Shelf} was held at -10 °C for 12 h and then raised to +10 °C for 6 h. During this period, the water was mainly removed due to sublimation. Following this drying period, the residual water content was 12.9 % corresponding to an a_w-value of 0.274. At this time, most of the surface water had been removed (Higl *et al.*, 2008). To ensure storage stability, a third drying step, at a higher shelf temperature of +25,+35, or +40 °C for another 6 h, followed to remove water within the cell and cell membrane. In total, the time allowed for each drying process was 24 h. The aim of this step was to determine a temperature that yielded a stable dry product. To prevent protein denaturation and loss of activity, the temperature must not exceed the physiologically optimal temperature of the strain of +40 °C (Kiviharju *et al.*, 2005). The lower limit of +25 °C was chosen according to previous freeze-drying studies involving different starter cultures (Ambros *et al.*, 2018b). During the drying process, the temperatures of the shelf and inside the product were controlled with internal sensors to avoid exceeding the set temperature limits. At the end of each drying, the temperature difference $\Delta\vartheta$, according to (5.1, was zero. Hence, the drying came to an end as no energy was brought into the product anymore by the shelf.

Heat and dehydration are obviously the main damaging factors for microbial cells when dried. Both can cause the permanent loss of the cells' viability. Table **5.2** shows the survival rate at different temperatures of the final drying step: at +35 °C a maximum is reached.

At this temperature, a low residual moisture content of 5.5 % and a higher survival rate of 40 % compared to +25 (6 % SR) and +40 °C (17 % SR) were achieved at the end of the process. Here, an a_w-value of 0.097 was reached, indicating a stable product. Measured by $\Delta\vartheta$, no more energy was added by heat at the end of any of the three drying setups. Nevertheless, the a_w-value at +25 °C of 0.198 indicates an unstable product. Microorganisms are most sensitive in an a_w region of 0.3 to 0.5, as there is not enough water anymore to keep them in an agile state, but still too much water to bring them into a preserved state (Higl *et al.*, 2008). Here, the a_w value is lower, but somehow not low enough to keep the cells in a stable state. Consequently, at +25 °C the drying progress seems to be not efficient enough. This means that the cells were kept for longer times at higher or medium water content levels, where their sensitivity against processing stress is high. In total, the influence of a shelf temperature of +35 °C on the survival rate is significant based on a p-value of 0.05. It seems to be efficient to prevent major heat damage compared to +40 °C. It is also high enough to minimize damage through insufficient drying compared to a shelf temperature of +25 °C.

Influence of the addition of protectants on survival after freeze-drying

Another option to raise the cells' survival rate during probiotic manufacture is to use protectants. Based on results obtained with *Lactobacilli* and *Bifidobacteria*, maltodextrin (MD), glucitol (S), and trehalose (T) were chosen as candidates for *B. longum* Reuter 1963 (Oldenhof *et al.*, 2005; Sohail *et al.*, 2013; Ambros *et al.*, 2018a). Each protectant was added before drying at a concentration of 100 % related to the bacterial biological dry matter (BDM; w/ w) content.

The results in Table **5.2** indicate little, if any, protective effect of the added protectants in terms of cell survival. No significant statistical difference was detected. Adding maltodextrin (MD) resulted in a survival rate of 43 %, comparable to survival without protectant (40 %). Adding glucitol (34 %) and trehalose (17 %) resulted in reduced survival rates. Considering only survival rate, no positive protective effect could be demonstrated through the addition of protectants. However, the added protectants were found to significantly influence the residual moisture content after drying (Figure **5.4**).

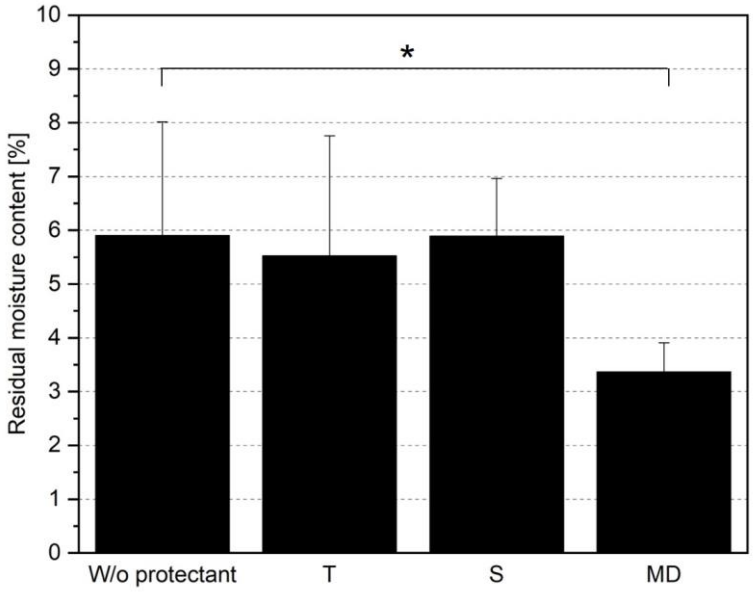

Figure 5.4: Residual moisture content for cell suspensions with an addition of 100 % BDM maltodextrin, glucitol, or trehalose as well as without protectant; significant differences between data based on a one-way ANOVA ($p \leq 0.05$), followed by a Tukey post hoc analysis are marked with an asterisk

A moisture content of 3.4 % was obtained for samples containing MD compared to samples without added protectant (5.5 %), glucitol (5.9 %) or trehalose (5.5 %). Based on a one-way ANOVA followed by a Tukey post-hoc test, there was a significant difference in the moisture content between samples without protectant and maltodextrin. Regarding the measured a_w-values of 0.109 (no protectant), 0.101 (MD), 0.194 (S) and 0.224 (T) a similar trend can be observed. This may be since trehalose and glucitol are hygroscopic. As a result, the moisture will be higher so that inactivation is also consistently higher leading to the pronounced difference in the survival rate. MD may be able to protect through a transition of the dried suspension into the glassy

state, which is important to achieve a high storability (Santivarangkna *et al.*, 2008a). Measurements with differential scanning calorimetry showed that the final product had a glass transition temperature of +40 °C. Therefore, it remained in a glassy state after drying, resulting in higher survival rates due to the decreasing rate of chemical reactions. Consequently, glucitol and trehalose do not seem to be appropriate protectants for *B. longum* Reuter 1963 in a concentration of 100 % BDM. Concerning survival rate, adding MD has no effect, but resulted in a lower residual moisture content.

To further study this effect, MD was tested as a protectant at concentrations of 10, 25, 50, 75, and 100 % BDM to assess at what concentration this effect was occurring. Measured by $\Delta\vartheta$, the 24 h drying program was known to be sufficient for all concentrations. Table **5.2** shows that with increasing MD concentration, the survival rate rises from 28 % (10 % BDM MD) to 49 % (75 % BDM MD). The residual moisture content decreased with increasing MD concentration: 7.3 % (10 % BDM MD), 5.6 % (25 % BDM MD), 4.8 % (50 % BDM MD) to 3.0 % (75 % BDM MD). Consequently, with an addition of MD no significant difference in SR, but in moisture content was detected. In this study, the addition of 75 % BDM MD was found to be sufficient. As shown here, MD prevents inactivation, resulting in a survival rate as high as without MD. Additionally, the moisture content is significantly lower, which should result in a higher SR during storage. These results appear surprising, as the survival rate normally decreases with a diminishing moisture content (Higl *et al.*, 2008). The reason for the here obtained results may be the protective effect of MD. The MD used in this study has a dextrose equivalent of 4.0 to 7.0, which leads to a degree of polymerization around 22 and an average molecular mass of about 2900 to 3500 Da (Dokic *et al.*, 2004; Rong *et al.*, 2009). MD molecules with this physical properties lead to high viscosities in the surrounding of the cells (Dokic *et al.*, 2004). This may support the transition of the product in the glassy state and should therefore produce a more stable cell membrane and a higher survival rate. Furthermore, a protection with an effect similar to the water replacement theory could be considered. Some researchers stated that the MD molecule is too big to interact with the cell membrane of *Lactobacilli* (Santivarangkna *et al.*, 2008a). On the other hand, others showed the interaction with phosphatidylcholin membranes depending on the size of the MD molecule (Koster *et al.*, 2003). A molecular mass of 1000-5000 Da and a degree of polymerization of 6-27 are the upper size limits for MD to be integrated in phosphatidylcholine membranes on the course of drying. In this study, MD may offer protection since *Bifidobacteria* have a different composition of phospholipids in the cell membrane compared to *Lactobacilli*. *Lactobacilli* membranes contain mainly phosphatidylglyceroles and lack nitrogen-containing phospholipids as phosphatidylcholin (Exterkate *et al.*, 1971; Drucker *et al.*, 1995; Novik *et al.*, 2006), whereas *Bifidobacteria* have a high content of phosphatidylcholin in their membranes (Novik *et al.*, 2006). This may result in a different interaction of MD with the cell membrane compared to *Lactobacilli* strains, explaining the divergent results in this study. Additionally, the transition into the

glassy state of products dried with MD will also have a protective effect due to the decreased rate of chemical reactions. Trehalose is known to interact with the carbonyl and phosphate groups in the cell membrane and is able to replace water molecules from the lipid headgroup (Villarreal *et al.*, 2004). Nevertheless, in this study a protection was not observed. A possible reason for that effect could be the different composition of cell membranes of *Lactobacilli* and *Bifidobacteria*, as described above. Further, glucitol is not able to protect *Lactobacilli* during drying, although the reason is not clear yet (Carvalho *et al.*, 2003). For *Bifidobacteria* also no protective effect was observed. However, the exact interaction between cells, cell membranes of *B. longum* Reuter 1963 and protectants is not fully understood to date. It needs further investigation to confirm the exact molecular mechanisms behind its protective or non-protective effects.

5.3.3 *Correlation of membrane preservation and cell survival*

Inactivation during probiotic production can occur in different ways. During the drying step, dehydration and denaturation of proteins have the largest influence on cell survival. Dehydration leads to an exponential decrease of survival rate and membrane damage (Higl *et al.*, 2008). As shown previously, the cell membrane is the component that is subject to the most damage during preservation. To verify this effect for the data obtained in this study, the membrane integrity of the dried cells was measured by flow cytometry. Table **5.3** shows the correlation of survival rate and membrane preservation for all drying set-ups. With a R^2 of 0.85, membrane preservation had an exponential impact on the survival of cells. Taking into consideration that the main part of damage occurs during drying supports the membrane integrity hypothesis. Accordingly, an intact cell membrane, shown by a high membrane preservation, after drying will result in a high survival rate. Additionally, further effects like denaturation may also have a smaller impact.

Table 5.3: Fitted correlation of survival rate over membrane preservation; fitting was done with OriginPro 2019 according to the shown statistical parameters

Process step	Correlation	R^2
Drying	exponential: $y = y_0 + A_1 \cdot e^{\frac{x}{t_1}}$ $y_0 = 0.26 \pm 1.19$ $A_1 = 0.05 \pm 0.05$ $t_1 = 7.40 \pm 1.30$	0.85
Storage	linear: $y = mx$ $m = 1.07 \pm 0.03$	0.97

5.3.4 *Effect of storage on cell survival*

Cultures are dried to increase their storage stability. In this study, a short-term storage of 70 d was investigated to reveal storage-related effects on inactivation. The impact and correlation of storage temperature and addition of maltodextrin were investigated. In parallel, the influence of membrane integrity on the survival rate was assessed to allow an explanation for inactivation. The dried samples were stored for 70 d at +4 °C (refrigerated conditions) and +20 °C (ambient conditions), with and without an addition of 75 % BDM MD. Thus, four different conditions were evaluated.

To analyze only the influence of storage, cell count and residual moisture content refer to the values after drying. Since the storage bags were flooded with nitrogen prior to sealing, there was no humidity or oxygen inside the containers. Hence, the moisture content was constant over the 70-d process (data not shown). After storage, the moisture content was between 3.3 % (+20 °C, with MD) and 6.5 % (+4 °C, without MD), which is similar to the values after drying. The storage temperature itself has no influence on the moisture content as it stays constant over the whole process. Regarding survival rate, another trend was observed, as shown in Table **5.2**. After 70 d of storage, the survival rate at +4 °C was 87 % (without MD) and 100 % (with MD) related to the cell count after drying. At +20 °C the survival rate was significantly lower with values of 28 % (without MD) and 57 % (with MD). For *B. longum* Reuter 1963, the survival rate is higher at lower temperatures. These findings are in consistence with the studies of (Abe *et al.*, 2009).

Comparing the addition of MD within one storage temperature, no significant influence was detected ($p > 0.05$). Thus, as Table **5.2** shows, the storage temperature was even more critical than the addition of MD and had a significant influence based on a p-value of 0.05. Additionally, the effect of MD is more pronounced with increasing temperature. At +4 °C the SR can be increased by 13 %, whereas at +20 °C the SR is increased by 29 % when adding the protectant. The higher SR caused by an addition of MD is probably due to the lower moisture content over storage time. No cells were lost over 70 d of storage when the freeze-dried suspensions were stored at +4 °C with added MD. Furthermore, MD was able to compensate the effects of non-ideal conditions, such as a higher storage temperature of +20 °C compared to +4 °C.

To monitor inactivation of *B. longum* Reuter 1963 over the whole storage process, the inactivation rate constant was calculated according to (5.4 The logarithmic progression of cell count over storage time is shown in Figure **5.5**. In general, a low inactivation rate constant predicts fewer inactivated cells during storage. It was visible that the inactivation rate constant was higher when samples were stored at +20 °C (Figure **5.6**). The influence of an addition of MD (0.29 per d) or absence of MD (0.46 per d) at +20 °C was far smaller. Here, the influence of the higher storage temperature caused a higher damage than the lack of MD. The lowest level of inactivation was achieved at +4 °C with an addition of 75 % BDM MD. In this case, the

constant was 0.10 per d. Hence, no significant difference could be seen for the inactivation rate constant at +4 °C, but at +20 °C.

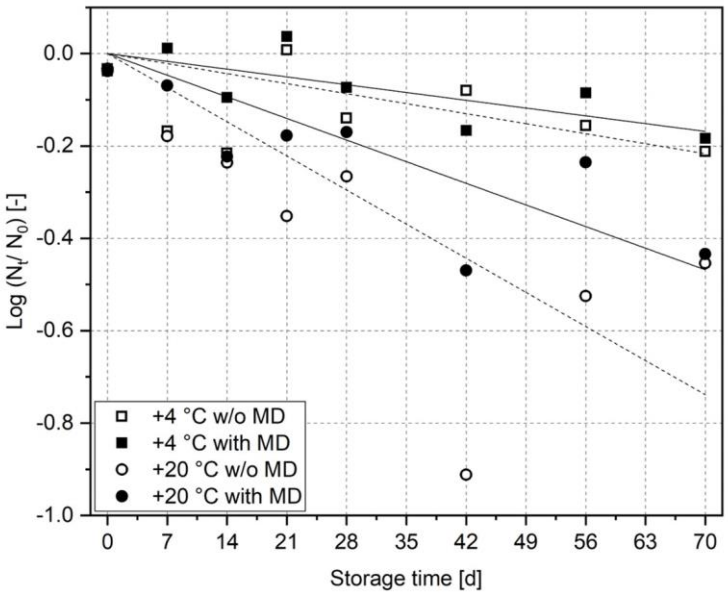

Figure 5.5: Relative viabilities of *B. longum* Reuter 1963 after freeze-drying as a function of storage time; standard deviations are not plotted for a clearer graph, but taken into consideration for calculation

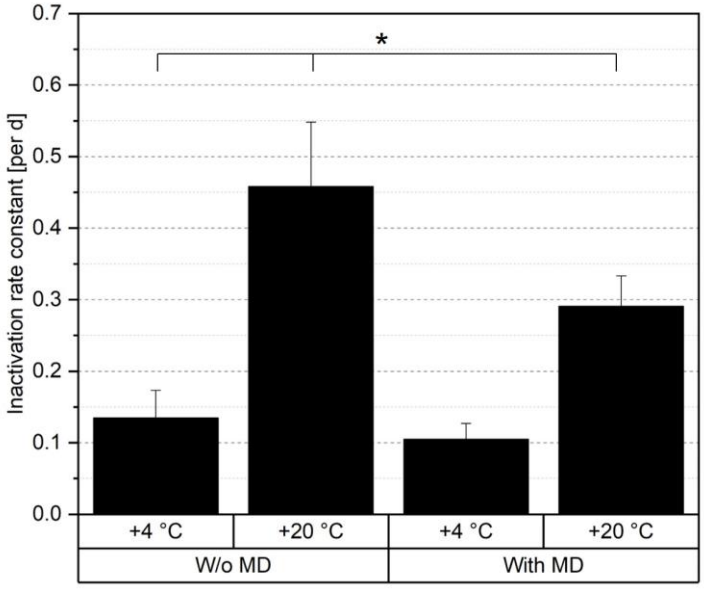

Figure 5.6: Inactivation rate constants of the four storage conditions, significant differences between data based on a one-way ANOVA ($p \leq 0.05$), followed by a Tukey post hoc analysis are marked with an asterisk

Also, during storage, the influence of membrane integrity on cell survival was investigated. For each point of time during storage, survival rate and membrane preservation were determined. Here, the survival rate is linearly affected by the membrane preservation ($R^2 = 0.97$). Consequently, cell loss during storage is probably due to leakage in the cell membranes. Storing cells with a high membrane integrity result in a high survival rate during the storage process.

Compared to results reported in literature, the data obtained in this study show a lower inactivation level. Shamekhi et al. (2013) stored freeze-dried *B. longum* Reuter 1963 cells without protectant at +4 °C and +25 °C. In their study, the cell count decreased between 1 (+4 °C) and 3 (+25 °C) log units after 70 d. The higher survival of the cells in the present study may result from storage in air-tight aluminum pouches and therefore the absence of oxygen and a low water activity in the environmental air. A low water activity in the environmental air in the pouches during storage is linked to a low inactivation rate and, consequently, a high survival rate (Higl *et al.*, 2008).

5.3.5 *Development of cell count over the whole production process*

The aim of this study was to observe and to increase *B. longum* Reuter 1963 survival rates over the whole production process, including cultivation, cell harvest, drying and storage. By regarding the whole process, we are able to detect harmful process steps and determine how cell count evolves over the whole process. It is recommended to apply probiotics in a concentration of 10^{10} CFU/mL to see a permanent abundance of the strain in the microbiome and have a positive effect on consumers' health (Saxelin, 1997; Knorr, 1998). Hence, we set out this number as desired cell count.

Figure **5.7** shows the cell count across the whole process, from cultivation to drying and storage, with all tested influencing factors. All cultivations got started with an initial cell count of $6 \cdot 10^7$ CFU/mL. Figure **5.7** shows the increase of cell count during cultivation to $8 \cdot 10^8$ CFU/mL (pH 6) and $7 \cdot 10^8$ CFU/mL (free acidification), followed by concentration, which leads to a final cell count of $2 \cdot 10^{10}$ CFU/mL (free acidification) and $6 \cdot 10^{10}$ CFU/mL (pH 6.0). As described above, when cultivating without pH regulation, the survival rate was only 0.5 % ($1 \cdot 10^8$ CFU/mL) after drying, whereas 40 % of the cells cultivated at pH 6 survived with a cell count of $2.4 \cdot 10^{10}$ CFU/mL, respectively. As the survival rate for cells cultivated at free acidification dropped by more than 2 log units after drying and less than 10^{10} CFU/mL survived, there are too less cells for application. Consequently, no further experiments were performed for drying and storage. For drying and storage all experiments were performed with cells cultivated at pH 6.0. During drying, the shelf temperatures were varied between +25, +35 and +40 °C. For these experiments no protectants were added.

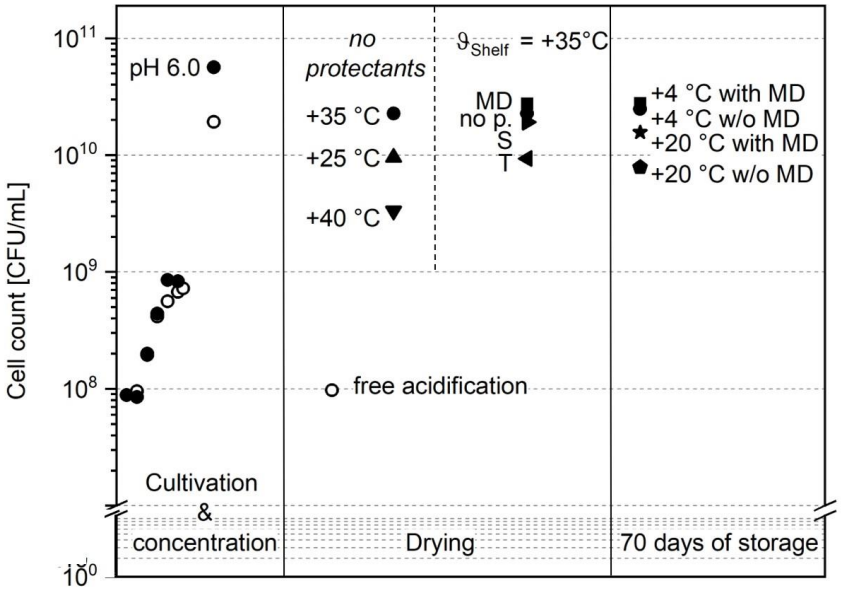

Figure 5.7: Cell count over the whole process: inoculation and cultivation, concentration, after drying and 70 days of storage; full symbols refer to experiments that were carried out with cells cultivated at pH 6.0, whereas hollow circles stand for free acidification

Afterall, a cell count of 10^{10} CfU/mL, required for a stable abundance in the microbiome, can only be reached when a shelf temperature of +35 °C is applied. Higher or lower temperatures result in a lower cell count. In comparison to that another set of drying experiments was conducted at ϑ_{Shelf} = +35 °C, while different protectants were added. As explained above, a shelf temperature of +35 °C and an addition of 75 % BDM MD led to the highest survival (49 %) with a cell count after drying of $3 \cdot 10^{10}$ CFU/mL. These settings were used for the storage experiments. During 70 d of storage, only a small decrease in cell count was observed. During storage at +20 °C without MD less than 10^{10} CFU/mL survived. On the other hand, no drop in cell count was observed for cells stored at +4 °C with an addition of MD. Consequently, a selection of appropriate storage conditions together with an adapted and improved drying process can prevent complete losses in cell count. Given that the cell count after concentration was referred to as 100 %, after drying between 50 % (pH 6, +35 °C shelf temperature, 75 % BDM MD) and 94 % (pH 6.0, +40 °C shelf temperature, no protectant) of cells were lost. At the end of the process, after drying and storage, total cell loss was between 50 % (+4 °C, with MD) and 86 % (+20 °C, without MD). The most appropriate conditions were found to include cultivation at pH 6.0, a shelf temperature of +35 °C, the addition of 75 % BDM MD and storage at +4 °C. Under these conditions, the entire inactivation through drying was 50 % (50 % SR, respectively) and 0 % through storage. Consequently, drying leads to an inactivation of a higher number of cells than storage.

5.4 Conclusions

In the current study, different factors influencing *B. longum* Reuter 1963*'s* survival were assessed. From the results obtained, we can conclude that the here investigated steps (cultivation, drying and storage) strongly influence the process. The choice of cultivation conditions here provided the biggest potential of increasing the survival after drying. The choice of appropriate cultivation conditions is important as cell membrane stability during drying correlates directly with drying resistance and survival rate. Supplementing other studies, where the effects of cultivation, drying, and storage were assessed separately, we here show how the factors involved in inactivation influence the particular process step and how some factors interact during storage. Furthermore, with choosing appropriate settings during drying, an inactivation of cells during storage can be prevented completely in the here investigated period of time. Membrane integrity has a high impact on survival both during drying and storage, as cells with leakage in the membrane fail to survive the process. Regarding the practical implication of the here shown results, cultivation at pH 6.0, a drying process of 3700 Pa, 24 h, at -10/ +10/ +35 °C shelf temperature with an addition of 75 % BDM maltodextrin and a storage at +4 °C result in the here investigated highest survival of cells. In future studies, these conclusions should be confirmed involving investigations of further probiotic strains from the human intestinal microbiome. Additionally, the interaction of maltodextrin with cell membranes needs to be the interest of following works to assume and verify the here obtained results.

References

The references were combined into a joint list of all publications, see at the end of the thesis.

6 Preservation by lyophilization of a human intestinal microbiota: Influence of the cultivation pH on the drying outcome and re-establishment ability

Summary and contribution of the doctoral candidate

The preservation by lyophilization of the human intestinal microbiota could be an enhancement of the FMT concept. This study examines the entire process chain from cultivation to cell harvest and concentration, conservation by freeze-drying and re-cultivation (Figure 6.1).

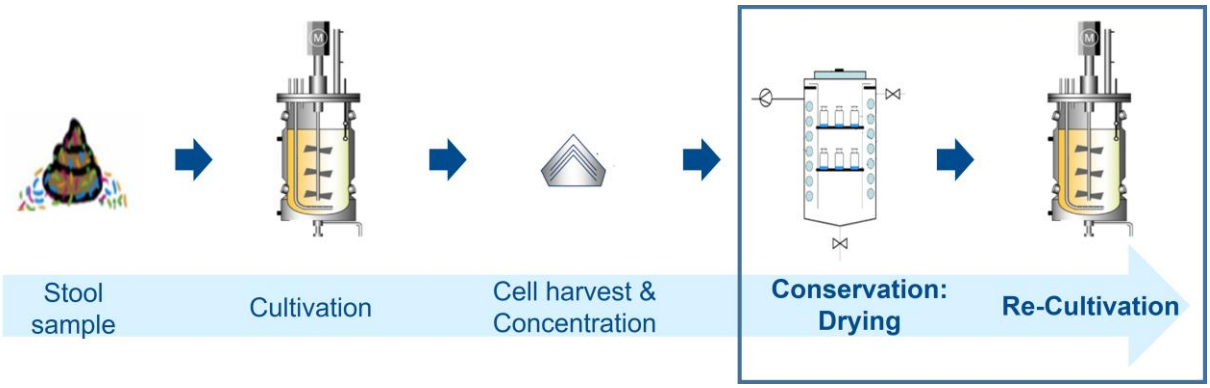

Figure 6.1: Investigated steps in the entire preservation process: influence of the entire process chain on the drying outcome and the ability to re-establish a stable system

Up to date, the influence of the entire preservation process, including cultivation and lyophilization, has not been assessed so far. In this study, the influences were measured by the determination of cell count, concentration and production of metabolites, microbial composition, and diversity in the system as evaluation criteria. All cultivation pH conditions resulted in stable, culturable communities after 70 h of re-cultivation. Cell count, richness, diversity and microbial composition were significantly affected by the drying step, but these effects were reversible and vanished during re-cultivation. As a result, comparing the re-cultivated system with the system before drying, no differences were observed. The metabolism with short-chain fatty acid concentrations as indicators showed slight changes induced by natural dynamics. Consequently, the cultivation step prior to drying was found to be the process point with the greatest influence on the entire preservation process. Hence, it offers the biggest potential for optimization. Comparing the re-established systems with the initial donor stool, the highest similarity was obtained with pH 6.0 – 6.5 during cultivation.

The substantial contribution of the doctoral candidate was the conception and the design of the experiments. She was moreover substantially involved in the performance of experiments. She acquired the data for the manuscript and interpreted the data set. She further critically reviewed the existing literature and

wrote the manuscript. Co-authors contributed to the experimental part and to the discussion of the results and provided input to the drafted publication prior to submission.

Adapted original manuscript[7]

Preservation by lyophilization of a human intestinal microbiota: Influence of the cultivation pH on the drying outcome and re-establishment ability[8]

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Abstract

Fecal microbiota transplantation is an emerging medical concept for the treatment of gastrointestinal diseases. This concept, however, has disadvantages as low storability of stool and intensive donor screening. A solution to overcome these problems would be the preservation of an in vitro microbiota through freeze–drying. However, the influence of the entire preservation process, including cultivation and lyophilization, has not been assessed so far. In this study, the influences of the process steps cultivation, drying and re-cultivation were determined with cell count, production of metabolites, microbial composition, and diversity in the system as evaluation criteria. All pH conditions resulted in stable, culturable communities after re-cultivation. Cell count, richness, diversity, and microbial composition were affected by freeze‐drying, but these effects were reversible and vanished during re-cultivation. Hence, the re-cultivated system did not differ from the system before drying. The metabolism, measured by short-chain fatty acids as indicators, showed slight changes due to natural dynamics. Consequently, the cultivation prior to drying was identified to have more influence than the drying itself on the preservation process and therefore the biggest potential

[7] Adaptions refer to formatting issues: e.g., abbreviations, figure, table, equation and section numbering, citation style, notation of units, spelling, axis labeling.

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for optimization. Hence, the highest similarity with the initial stool sample was obtained with pH 6.0-6.5 during cultivation.

6.1 **Introduction**

The human intestinal microbiota hosts a large and complex ecosystem with a high abundance of cells of up to 10^{14} CFU mL^{-1} and 400–1,000 different species (Eckburg *et al.*, 2005; Gill *et al.*, 2006). The microorganisms in the human gut belong to the phyla Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia. The two major phyla Firmicutes and Bacteroidetes represent 40 to 50% each of total bacteria. Actinobacteria (~2.5%), Proteobacteria (0.1–1%) and Verrucomicrobia (~0.1%) are less abundant and represent the minor phyla in the microbiota.

The complexity of this system is of great importance as alterations, compositional imbalances and a reduced diversity are often linked with several diseases (Holmes *et al.*, 2011). One threatening disease linked with a decreased microbial diversity is the infection with *Clostridium difficile*, a spore-forming microorganism that causes severe diarrhea, nausea and fever (Abt *et al.*, 2016). *Clostridium difficile* Infections (CDI) often occur after extensive antibiotic treatment when the balance, richness and diversity in the gut microbiota are altered. Patients suffering from CDI have a disordered gut microbiota characterized by a decrease of *Bacteroides*, *Alistipes* and *Lachnospira* and an increase of opportunistic pathogens (Milani *et al.*, 2016). Despite of the already disordered microbiota, the standard therapy is the treatment with antibiotics like metronidazole, vancomycin or clindamycin (Kelly and LaMont, 1998; Lagier *et al.*, 2012). However, a threat of repetitive application of antibiotics is the occurrence of multi-resistant strains and relapses of the disease. 15 to 20% of patients with CDI relapse after the first antibiotic treatment and patients who suffered two or more previous relapses have a risk of 65% of further occurrences of severe CDI (McFarland, 2005; Sartelli *et al.*, 2015).

Fecal microbiota transplantation (FMT) has been tested as an alternative treatment method to antibiotics, with recovery rates up to 90% (Gough *et al.*, 2011; Aroniadis and Brandt, 2013). In this therapeutic concept, stool from a healthy donor is purified and transferred into the patient's gut via colonoscopy, nasogastric tube, or enema (Brandt and Aroniadis, 2013). The aim of the transfer is to re-equilibrate the composition, diversity and thus to restore the function of a healthy microbiota in the patient's gut. The restored microbial community inhibits *Clostridium difficile* through competition for nutrients, suppression by antimicrobial peptides and bile-acid-mediated inhibition of spore germination and growth (Khoruts and Sadowsky, 2016). Further, the transferred metabolic products of the microbiota, short-chain-fatty acids (SCFAs), contribute by their anti-inflammatory properties (Tedelind *et al.*, 2007). In addition to the treatment of CDI, successful FMT applications including

diseases like ulcerative colitis (Angelberger *et al.*, 2013; Waller *et al.*, 2021), Parkinson's disease (Ananthaswamy, 2011; Xue *et al.*, 2020) or multiple sclerosis (Borody *et al.*, 2011; Li *et al.*, 2020) have been reported. Nevertheless, the method has some limitations: ethical and safety concerns, a work and cost intensive donor screening prior to FMT and mainly the availability of suitable fecal material (Terveer *et al.*, 2017; Zhang *et al.*, 2018).

So far, different approaches to enhance the FMT concept were tested for the treatment of *Clostridium difficile* infections (Chiu *et al.*, 2021). The application of a processed, frozen and thawed donor stool resulted in recovery rates for CDI of up to 95% (Hamilton *et al.*, 2012; Smirnova *et al.*, 2019; Nicco *et al.*, 2020). Further to that, different researchers investigated the treatment success with lyophilized feces. A dried microbiota offered the additional advantages of less dependence on healthy stool donors, easy storability was well as high availability and flexibility for the administration in impaired patients. Vigvári *et al.* (2019) determined recovery rates for CDI of up to 83% through the application of lyophilized and resolved feces via a nasogastric tube. When the powder was applied through capsules, the treatment succeeded for 84 – 100% of the patients suffering from CDI (Jiang *et al.*, 2018; Staley *et al.*, 2019). In our former study, a drying protocol for probiotics of the human intestinal microbiota was developed, where survival rates of up to 49% for the test strain *Bifidobacterium longum* were reached (Haindl *et al.*, 2020). In this study, this drying protocol will be now used to preserve the entire microbiota. The drying outcome and ability to re-grow and re-establish can be tested by re-cultivating the dried powder *in vitro*, which was the main purpose of this study. Next to the application of dried feces, the rectal transfer of liquid *in vitro* infusion material has also already been investigated for the treatment of CDI (Garborg, 2015). Compared to the FMT concept, the application of an *in vitro* microbiota offers advantages like a technically easy to handle set-up and operation, possibilities for variation and adaption of the system and further the lack of ethical considerations. In our previous studies the establishment of an *in vitro* system, as well as the influence of the donor stool and the cultivation pH were investigated. An applicable system was established, creating a microbiota with slight differences in the distribution of phyla compared to the donor stool (Haindl *et al.*, 2021a). Further, the influence of the donor itself was found to be small, with only some factors from the stool as e.g. high abundances of single genera and metabolic characteristics sustain in the individual cultivated system (Haindl *et al.*, 2021a). The choice of the cultivation pH had a major impact on the established *in vitro* system (Haindl *et al.*, 2021b). The choice of a physiological cultivation pH during growth in a bioreactor system was found to depend on different criteria: a pH of 7.0 created a higher number of cells, whereas a lower pH of 6.0 resulted in a system with a phyla distribution more comparable to the donor stool and an higher abundance of health indicating microorganisms (Haindl *et al.*, 2021b). The purpose in this study here compared to previous studies was to determine whether the pH value may also have an influence not only during

cultivation, but also on the resistance of the *in vitro* cultured microbiota during freeze-drying and its ability to re-establish. Bauer *et al.* (2012) showed that the fermentation conditions of single strains of probiotic cultures had an influence on the resistance against processing and thus on the survival after vacuum drying of the tested cultures. Next to freeze-drying as preservation method, Bircher *et al.* (2018) assessed the technical feasibility of the cryopreservation of an *in vitro* microbiota for FMT. Therefore, they cultivated purified feces for 10 days in a continuous system at pH 5.7. After cell harvest and centrifugation, the cell pellet was frozen and stored at -80 °C. After thawing, the microbiota was re-cultivated for 24 h in a batch fermentation system. Consequently, the used preservation (freezing at -80 °C) as well as re-cultivation (batch) process and further the used media differed from the study here. Bircher *et al.* (2018) detected a lowered metabolism in the re-cultivated system with the concentration of all SCFAs being decreased. Further, when preserving without protectants, the abundance of butyrate producing microorganisms *Faecalibacterium* and *Roseburia* showed a strongly impaired growth.

The aim of this study was to build on these reports by applying the state of knowledge regarding preserved microbiota cultivated in bioreactors for FMT instead of isolation from stool donors. As the technical feasibility of the creation of an *in vitro* cultured microbiota was already demonstrated before (Haindl *et al.*, 2021a), we aimed here to demonstrate the technical feasibility of the preservation by lyophilization of an *in vitro* microbiome. This process might promote a high survival of health-indicating microorganisms next to other advantages of a dried product as storability at ambient conditions and less weight. This study was conducted to investigate the whole processing chain including preparation of inoculum from stool, bioreactor cultivation, freeze-drying and testing survival and re-cultivation of the dried culture in a bioreactor, which has not been investigated in this complete sequence by other researchers so far. Especially the ability of the dried product to re-establish a stable system was of interest. The study investigates the influences of single process steps on survival and the ability of the technically produced cells to re-grow and re-establish a stable, cultivable community with a microbial composition comparable to the system prior to preservation as well as the initial feces. The technical feasibility of maintaining high microbial abundance, diversity and richness was determined. Other studies mainly investigated the abundance of phyla or single genera after preservation (Bircher *et al.*, 2018; Jiang *et al.*, 2018). Here, the re-establishment of several representative gut bacteria and health-markers like *Clostridium* Cluster XIVa, *Bacteroides, Faecalibacterium*, *Bifidobacterium* and *Roseburia* after drying were determined. SCFAs as the metabolic products of a healthy microbiota were analyzed because of their importance for the success and efficiency of treatment and crucial in restoring the metabolic balance of a disturbed intestinal microbiota (Tedelind *et al.*, 2007). The purpose of the study was to determine the impact of the individual processing steps, i.e., cultivation with

varied pH, freeze- drying and re-cultivation of the dried microbiota, to possibly assess the most critical processing steps along the whole preservation process chain with a major influence on the outcome of the dried and re-cultivated microbiota. By identification and adaption of these critical processing steps, a re-established *in vitro* system with characteristics similar to the donor stool was intended to achieve with the perspective of applying this as an extension of the current FMT concept.

6.2 **Materials and methods**

6.2.1 *Process Flow*

Stool from a healthy donor was purified and used as the inoculum for an in vitro cultivation system (Figure **6.2**). The microbiota was cultivated at either pH 6.0, 6.5 or 7.0 with the conditions described in chapter 2.3. After 120 h of cultivation, the broth was harvested, concentrated by centrifugation (Allegra X15R, Beckmann Coulter Inc., Brea, CA, USA; 4.000 x g, +4 °C, 10 min) and afterwards 1 mL of the sediment each was dosed into freeze-drying glass vials and stored at -80 °C. After freeze-drying, the powder was rehydrated to its initial biological dry matter (BDM, %) and used as an inoculum for the re-cultivation process. Re-cultivation was performed to assess viability, vitality, and ability of the dried microbiota to regrow in a controlled in vitro environment. For re-cultivation, the same pH value as during cultivation was used. Each cultivation run and freeze-drying was done in triplicate. Further, all analyses were executed at least three times for each experiment.

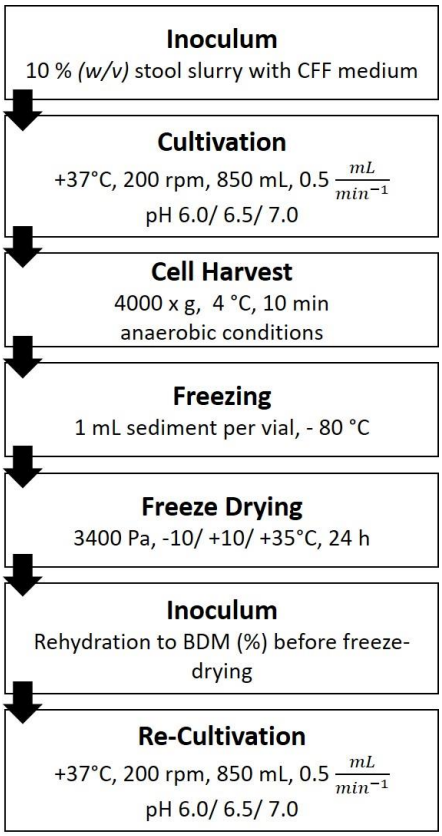

Figure 6.2: Process scheme applied in this study

6.2.2 *Donor Stool*

Former studies had already investigated the influence of different stool samples and revealed only a minor influence. Consequently, this study was conducted with stool from one healthy, female donor and one defecation (Haindl *et al.*, 2021a). The donor was chosen according to the criteria important for FMT (Terveer *et al.*, 2017) and tested prior to the experiments for bacterial, viral, and eucaryotic pathogens by the Institute for Medical Microbiology and Hygiene, University of Regensburg to prevent the transfer of diseases. The stool was obtained in-house and stored immediately at -80 °C until use. The donor was Caucasian, ate a Western diet, had a body mass index of 21 and was 25 years old. The last antibiotic treatment was over 12 months ago. The abundance of phyla in the stool was as follows: 0.65% Actinobacteria, 48.79% Bacteroidetes, 45.28% Firmicutes, 1.59% Proteobacteria, 2.30% Verrucomicrobia and 1.39% of unknown phyla. The richness was 99 and the Shannon effective index 29. Consequently, this donor hosted a microbiota considered as healthy regarding microbial composition, richness and diversity (Rinninella *et al.*, 2019).

6.2.3 *In vitro cultivation system*

Prior to and after the freeze-drying step, an *in vitro* cultivation of the microbiota was performed as already established in former studies (Haindl *et al.*, 2021a). The cultivation system was used either to obtain samples for the drying experiments or to test viability, vitality and the ability to re-establish a stable system after drying. As medium for the cultivation as well as for the preparation of inoculum, a so-called Continuous flow fermentation (CFF) medium was used, which was already described in previous studies (Haindl *et al.*, 2021a).The preparation of inoculum for the cultivation step prior to drying was already described previously (Haindl *et al.*, 2021a).

After drying, the powder was rehydrated with sterile bi-distilled water to its initial biological dry matter (after cell harvest and concentration, before drying). The obtained solution was then joint with 120 mL fresh CFF medium and served as the inoculum.

During processing, samples were collected by pumping broth anaerobically into prepared sample tubes (Greiner Bio-One, Sigma Aldrich, St. Louis, USA). The broth was either used immediately for further analysis (cell count, SCFAs) or stored at −80 °C until analysis (16S rRNA gene amplicon sequencing).

6.2.4 *Freeze-drying process*

Prior to the drying process, the samples were frozen at -80 °C for at least 24 h. The freeze-drying process used in this study was already established in a previous work based on the drying of a probiotic strain from the human intestinal microbiota (Haindl *et al.*, 2020). The process was conducted in a Delta 1-24 LSC pilot plant dryer (Christ GmbH, Osterode, Germany). The chamber pressure was set at a constant 3700 Pa through the whole processing time, while the shelf temperature rose in a stepwise manner: -10 °C for 12 h, +10 °C for 6 h and +35 °C for 6 h. Consequently, the whole drying process took 24 h.

6.2.5 *Analysis for residual moisture content and water activity*

The residual moisture content and water activity were determined after each drying process. The residual moisture content was measured by using a CEM Smart Turbo™ 5 (CEM Corporation, Kamp-Lintfort, Germany) at a maximum sample temperature of +80 °C and 45% power input. The results were double-checked regularly with Karl Fischer titration using TitroLine KF (Mettler-Toledo GmbH, Schwerzenbach, Germany).

Water activity a_w was measured using an Aw Sprint TH-500 (Novasina, Lachen, Switzerland).

6.2.6 *Analysis of cell count*

Cell counts were analyzed for anaerobic and aerobic cells separately. After collection, the samples were diluted with 0.25 strength Ringer's solution and then plated either on Wilkins-Chalgren Anaerobe agar plates (anaerobic cell count) or Plate Count agar plates (facultative aerobic cell count). The plates were incubated for 48 h at +37 °C either aerobically or anaerobically in an anaerobic chamber heated to +37 °C. For data evaluation, only plates with 30-300 colonies were considered for evaluation and the number of colony forming units N per mL of sample (CFU mL^{-1}) was calculated according to the following (6.1:

$$N = \frac{c}{n_1 + (0.1 \cdot n_2)} \quad (6.1)$$

Here, c is the sum of colonies of the subsequent dilutions; n_1 is the number of colonies in the less diluted cell suspensions; and n_2 is the number of colonies in the more diluted solution.

6.2.7 *Analysis of short-chain fatty acids by High Performance Liquid Chromatography*

The metabolic behavior of the *in vitro* microbiota was revealed by analyzing the main short-chain fatty acids (SCFAs) acetate, propionate, butyrate, and iso-valerate by a high-performance liquid chromatography (HPLC; Agilent Technologies Inc., Santa Clara, USA) system equipped with an Aminex HPXH-87H ion exclusion column (Bio-Rad Laboratories, Hercules, USA) and a G1362A refractive index detector (Agilent Technologies Inc., Santa Clara, USA). The injection volume was set to 20-100 µL and separation was performed with 0.0005 mol L^{-1} H_2SO_4 at a flow rate of 0.45 mL min^{-1}. Prior to the measurement, the samples got centrifuged (Hermel-Z233 M-2, Hermle Labortechnik GmbH, Wehingen, Germany; 6,000 x g, +20 °C, 30 min) and the supernatant filtrated with a 0.22 µm sterile filter. SCFAs were identified and quantified using external standards (Sigma Aldrich, Saint Louis, USA) and the software Agilent ChemStation Instrument 1 Offline (Agilent Technologies Inc., Santa Clara, USA).

6.2.8 *Microbiota profiling with 16S rRNA gene amplicon sequencing*

The microbial community, richness, and diversity were analyzed at several time points during cultivation. For this purpose, the V3/V4 region of 16S rRNA was sequenced by High-throughput 16S rRNA Gene Amplicon Sequencing, which was performed by the Microbiome Core Facility, ZIEL, TU Munich, according to a protocol previously described and already used in earlier studies (Reitmeier *et al.*, 2020c; Haindl *et al.*, 2021a; Haindl *et al.*, 2021b).

6.2.9 *Statistical analysis*

All cultivations, dryings and further analyses were repeated at least in triplicate. The mean values are shown as the arithmetic mean $\bar{x}$ of the number n of all samples x_i. The distribution of the values was calculated from the standard deviation s due to the random error. All graphs show the arithmetic means ± standard deviations. Statistical significance was tested using a one-way ANOVA ($p \leq 0.05$) followed by a Tukey post-hoc analysis with the software *OriginPro 2019* (OriginLab Corporation, Northampton, USA).

6.3 **Results and discussion**

The approach was to assess the effects of single processing steps, i.e., cultivation, drying and re-cultivation following rehydration and interactions between these steps. Therefore, samples were investigated throughout the whole process: after 120 h of cultivation (=before drying) and as well as during several time points in the re-cultivation (=after drying) step.

6.3.1 *Effect of cultivation pH on drying outcome*

At first, the influence and outcome of the drying process itself was identified by measuring residual moisture as well as survival of the cells by comparing the system after cultivation/ centrifugation and the powder immediately after drying. It was aimed to create a stable product with a low moisture to ensure storability.

After cultivation, the broth was harvested, concentrated and the sediment frozen at -80 °C. Afterwards, the drying process took place. After drying, residual moisture content, water activity and cell survival were determined (table 1). For the residual moisture content, values between 5.0 ± 2.4% (pH 7.0) and 8.4 ± 0.7% (pH 6.5) were measured. Water activity was 0.14 ± 0.05 (pH 7.0), 0.15 ± 0.02 (pH 6.5) and 0.14 ± 0.02 (pH 6.0). All these results indicate a stable product. Microorganisms are more sensitive in an a_w-region of 0.3 to 0.5, where there is still too much water to bring them to a preserved state, but not enough water left to keep them in an agile state. Therefore, freeze-dried microorganisms should always have a water activity below 0.3 to create a stable product (Higl *et al.*, 2008). As it can be seen from the data presented in Table **6.1**, all tested cultivations resulted in lower a_w-values, i.e., all conditions applied led to stable and storable products.

Table 6.1: Residual moisture content, water activity and survival rate after cultivation at pH 7.0, 6.5 and 6.0

	Cultivation pH value [-]		
	7.0	6.5	6.0
Residual moisture content [%]	5.0 ± 2.4	8.4 ± 0.7	7.6 ± 1.9
Water activity [-]	0.14 ± 0.05	0.15 ± 0.02	0.14 ± 0.02
Aerobic cell survival [%]	2.3 ± 0.6	5.5 ± 2.9	6.4 ± 3.3
Anaerobic cell survival [%]	0.1 ± 0.1	0.1 ± 0.1	0.9 ± 0.8

Figure **6.3** shows the aerobic and anaerobic cell count before (after 120 h of cultivation) and directly after drying for all investigated set-ups. After cultivation and before the drying process, the aerobic cell count was between $9 \cdot 10^7$ CFU mL^{-1} (pH 7.0) and $1 \cdot 10^9$ CFU mL^{-1} (pH 6.0). After drying the cell count dropped steeply in the range of 10^6 - 10^7 CFU mL^{-1}, resulting in aerobic survival rates between 2.3 ± 0.6 % (pH 7.0) and 6.4 ± 3.3 % (pH 6.0). For the anaerobic cell count, the cell counts even decreased from numbers between $7 \cdot 10^9$ CFU mL^{-1} (pH 6.0) and $3 \cdot 10^{10}$ CFU mL^{-1} (pH 6.5) before drying down to $2 \pm 2\ 10^7$ CFU mL^{-1} (pH 7.0 and pH 6.5) and $7 \pm 6\ 10^7$ CFU mL^{-1} (pH 6.0) after drying.

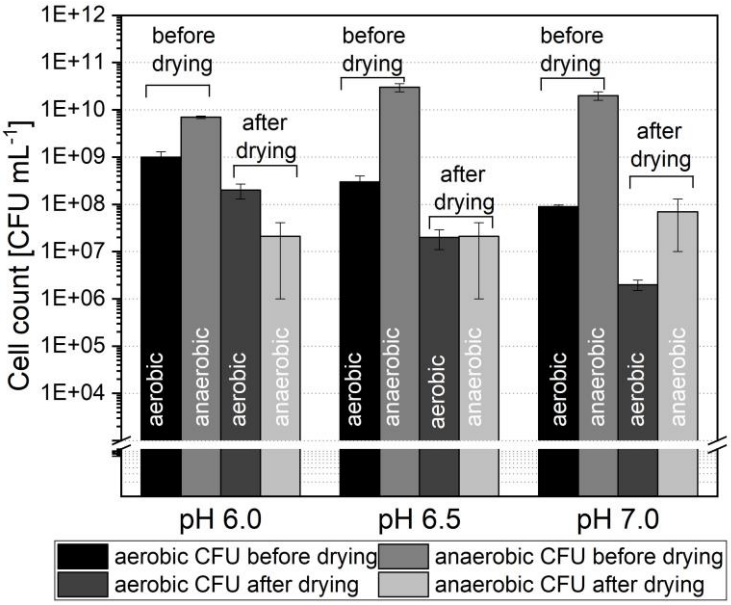

Figure 6.3: Comparison of aerobe and anaerobe cell count before and after drying for each cultivation pH value

The anaerobic survival rate therefore was only 0.9 ± 0.8% (pH 6.0) and 0.1 ± 0.1% (pH 6.5 and 7.0), indicating a significant decrease in anaerobic cell count. Compared to previous studies on the drying of single cell cultures, these survival rates are comparably low. For *Lactobacillus paracasei* and *Bifidobacterium longum*, which are also abundant in the human intestinal microbiota, higher survival rates

of e.g. 40% (Ambros *et al.*, 2018b) and 9% (Yang *et al.*, 2012) after freeze-drying were reached. For these lower survival rates, several mechanisms may be in charge. The drying of a multi-cell suspension, where the drying protocol was not optimized for every microorganism in the complex mixture, may explain the significantly lower survival rates reached in this study. Further to that, single microorganisms in the microbiota are strictly anaerobic, whereas *Lactobacilli* and *Bifidobacteria* are more tolerant to short exposures to oxygen. Despite great efforts, the contact with oxygen could not be prevented entirely through the harvesting process, which may be another reason for the lower survival rates. Additionally, not all microorganisms of the human intestinal microbiota may be culturable after drying. It may be that these species survived the cultivation before drying, but due to their bad condition, could not survive the concentration, freezing and drying step and hence died after drying what consequently lowered the survival rate. Freeze-drying consists of the freezing and the drying step. Hence, some microorganisms may be sensitive to freezing and get even inactivated before the drying step itself. Bircher *et al.* (2018) have shown, that *Roseburia* are not able to survive freezing and thawing. In this study, *Roseburia* were abundant in the cultivation step for all pH values but were below 0.01% abundance after re-cultivation. Hence, a reason for the impaired growth may be the freezing step, but this needs further investigation. The water removal during freeze-drying is due to sublimation, which may also cause damages in the cells, especially in the cell membrane, that may lead to further inactivation. Consequently, several mechanisms like exposure to oxygen, freezing sensitivity and sensitivity because of water removal may be reasons that lower the survival rates here. As these mechanisms also may interact with each other, no clear statement can be made which mechanism leads to inactivation to what extent.

Further, the influence of the cultivation pH value on the drying outcome was tested by a one-way ANOVA ($p \leq 0.05$) followed by a Tukey post-hoc analysis. It revealed no significant differences between the results for the residual moisture content as well as water activity and survival rate. Overall, it seems like the cultivation pH had no influence on the outcome directly after drying. In total, it has been shown that lyophilization has a severe impact on the survival directly after drying. Nevertheless, the influence on the ability to re-grow and re-establish a stable system must be considered and will be investigated in the following.

6.3.2 *Re-establishment of an in vitro system after drying*

After drying, a low survival rate of the cells was measured (Table **6.1**). Nevertheless, these results only inform about the drying stress, but the survival rate directly after drying does not provide evidence for the ability of the cells to re-establish and re-grow during a re-cultivation comparable to the initial bioreactor cultivation step before lyophilization. Further, the open question was how and what kind of microbiota was able to re-establish. Therefore, the dried product was

rehydrated and re-cultivated for at least 70 h. For re-cultivation the same pH value as prior to drying was used. During re-cultivation, cell count, metabolic production, microbial composition as well as diversity were measured for each experiment. In the following chapter 6.3.2, the re-establishment of the system cultivated and re-cultivated at pH 6.5 will be described in detail.

Cell Count

The initial number of colony forming units prior to drying was $3 \pm 0.3\ 10^{8}$ aerobic CFU mL^{-1} and $1 \pm 0.7\ 10^{10}$ anaerobic CFU mL^{-1} (pH 6.5). Directly after drying, the cell count dropped and was determined with $2 \pm 0.9\ 10^{7}$ aerobic CFU mL^{-1} and $2 \pm 2\ 10^{7}$ anaerobic CFU mL^{-1}. Due to the dilution by the cultivation media upon re-inoculation, the cell count dropped further and reached $5 \pm 1\ 10^{4}$ aerobic CFU mL^{-1} and $7 \pm 6\ 10^{4}$ anaerobic CFU mL^{-1} after 1 h of processing time (Figure **6.4**). Nevertheless, the cell count was able to recover and regrow to a number of $3 \pm 2\ 10^{8}$ aerobic CFU mL^{-1} and $1 \pm 0.7\ 10^{10}$ anaerobic CFU mL^{-1} after 48 h and then remained in a stable state towards the end at 71 h. Compared to the microbiota prior to drying the cell count was able to recover completely and reached similar numbers ($2 \pm 1\ 10^{8}$ aerobic CFU mL^{-1} and $1 \pm 0.3\ 10^{10}$ anaerobic CFU mL^{-1} in the stable system after 120 h of cultivation at pH 6.5 before concentration and drying).

Compared to other studies, when stool samples were preserved by freezing, the cell count irreversibly dropped about one log CFU mL^{-1} and could not reach the same level as before preservation (Carvalho *et al.*, 2021). In this study, the aerobic as well as anaerobic cell count reached the same level as before the freeze-drying preservation step. This may be due to different cultivation conditions in the study of Carvalho *et al.* (2021) and this study that resulted in lower cell counts. Further, during freezing, intracellular ice can be formed and the increase in solute concentration imposes osmotic stress to the cell (Santivarangkna *et al.*, 2008a). These mechanisms resulted in irreversible damages in the followed thawing process while they seemed not to occur during freeze- drying and sublimation.

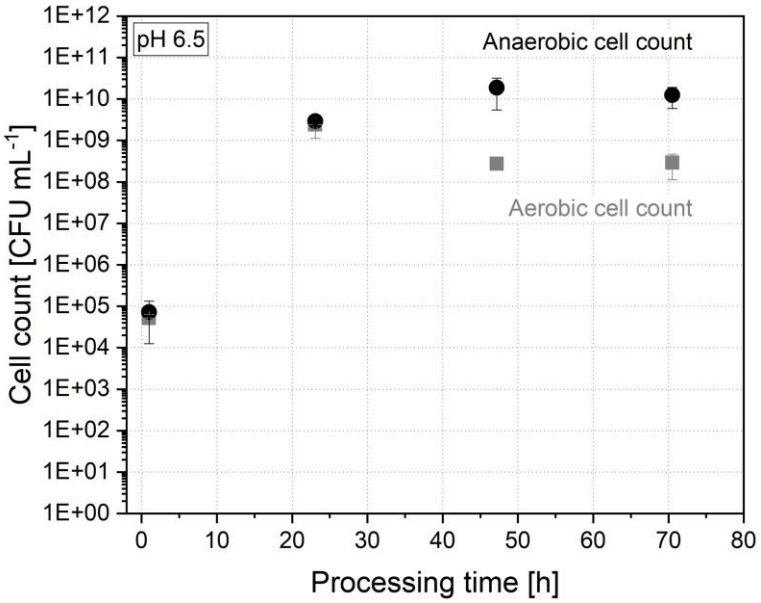

Figure 6.4: Evolution of the aerobic (■) and anaerobic (●) cell count during the re-cultivation after drying at pH 6.5

Metabolic Production

To investigate the ability of the dried microbiota to re-establish with a working metabolism, the powder was rehydrated and re-cultivated in the *in vitro* system again. The metabolic production was measured by the concentration of overall short-chain fatty acids (SCFAs) in the broth as well as the main metabolites acetate, propionate, butyrate and iso-valerate.

In total, the sum of SCFAs started to increase after 6 h when the cells started to grow and metabolize. They increased continuously until a plateau and stable state was formed after 47 h at a total concentration of all SCFAs at 8.25 ± 0.60 mg mL^{-1}. After re-inoculation, the concentration of acetate in the broth was constant for the first 6 h at 0.20 ± 0.11 mg mL^{-1}. As seen in Figure **6.5**, the concentration then steadily increased to 4.65 ± 0.19 mg mL^{-1} (47 h) and finally plateaued at a level of approximately 4.5 mg mL^{-1}.

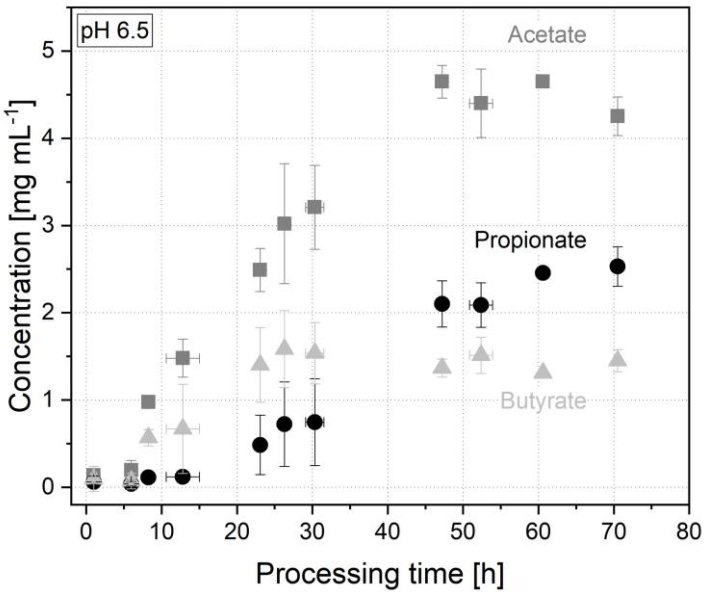

Figure 6.5: Concentration of acetate (▪), propionate (●) and butyrate (▲) during re-cultivation at a cultivation pH of 6.5

The concentration of propionate was low in the beginning (0.06 ± 0.07 mg mL^{-1}, 1 h) and started to increase after 23 h (0.49 ± 0.34 mg mL^{-1}) until a stable state was reached after 61 h at a concentration of 2.46 ± 0.02 mg mL^{-1}. The production of butyrate started earlier compared to propionate, after 6 h with a concentration of 0.07 ± 0.09 mg mL^{-1}, increased and plateaued after 26 h at a concentration of 1.58 ± 0.44 mg mL^{-1}. The concentration of iso-valerate in the broth behaved similarly to the production of butyrate (Figure **6.6**). It stayed constant at a low level for the first 6 h (0.02 ± 0.02 mg mL^{-1}) and then started to increase until a stable state was reached after 47 h at a concentration of 0.14 ± 0.05 mg mL^{-1}. In total, the re-cultivated microbiota showed a working metabolism after 6 h. First, acetate, butyrate and iso-valerate were produced, whereas the production of propionate started later after 23 h of re-cultivation. This gives the hint that a variety of different microorganisms started to grow at different points of re-cultivation metabolizing different acids.

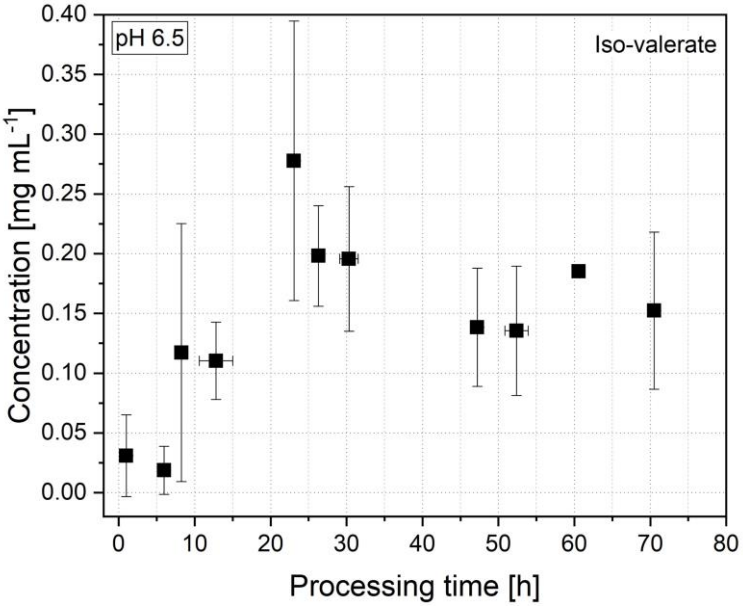

Figure 6.6: Concentration of iso-valerate during re-cultivation at a cultivation pH of 6.5

All values in the metabolic production plateaued after a certain time, indicating that a stable system was formed under steady state conditions. Along with the total amount of SCFAs, the ratio between acetate, propionate and butyrate is a marker for human health. Ratios from 3:1:1 (acetate: propionate: butyrate) to 10:2:1 are considered as typically healthy (Macfarlane *et al.*, 1992; Rowland *et al.*, 2018). Here, in the stable system (>70 h), a ratio of 4:3:1 was reached indicating a healthy contribution. Other researchers reached ratios, depending on the cryoprotectant, in a similar range of 10:2:8, 10:4:13 and 10:5:4 (Bircher *et al.*, 2018). Consequently, the concentration of SCFAs were in a comparable range with other studies and indicate that the metabolic production and behavior could be restored after the drying process. Overall, the *in vitro* system after drying still represents a functional microbiome with a regularly working metabolism.

Microbial composition

Next to the metabolic behavior of the system after drying, the microbial distribution was of interest. Therefore, the microbial composition was determined by sequencing 16S rRNA gene amplicons. When the microbiota cultivated at pH 6.5 was dried and re-cultivated, significant changes in the abundance of the phyla during re-cultivation can be observed and will be discussed in the following. The behavior in the systems cultivated at pH 6.0 and 7.0 was comparable (data shown in Table **6.2** and Table **6.3**). For one of the major phyla, i.e., Bacteroidetes, a sharp decrease after drying to 0.46 ± 0.26% relative cumulative (rel. cum.) abundance was determined. Nevertheless, the abundance of the phylum could be restored and increased to 75.01 ± 0.79% after 71 h (Figure **6.7**). Obviously, Bacteroidetes are harmed through the cell harvesting and the drying process but can be restored and re-established.

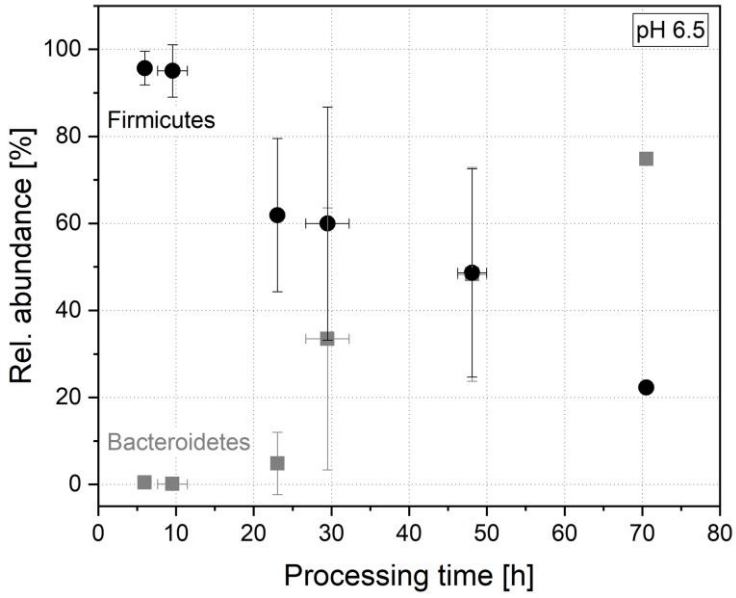

Figure 6.7: Rel. cum. abundance of the two major phyla Bacteroidetes (■) and Firmicutes (●) during re-cultivation at pH 6.5

In contrast, Firmicutes were not as sensitive after freeze-drying as Bacteroidetes. Their abundance was 95.69 ± 3.88% after 6 h (Figure **6.7**). During the further processing, the balance between Bacteroidetes and Firmicutes was re-established and the abundance of Firmicutes settled to 22.27 ± 0.33 % (71 h). Especially in the time range between 20 to 50 h of processing time, the ratio of Firmicutes and Bacteroidetes changed and resulted in a reversed ratio. The minor phylum Actinobacteria showed a similar behavior as during cultivation as the phylum Firmicutes: After adaption, the abundance increased to 0.33 ± 0.13% (30 h), then decreased and plateaued to 0.04 ± 0.03% after 71 h (Figure **6.8**). The abundance of Proteobacteria progressed in a comparable way, but the peak in abundance occurred earlier. First, the abundance increased to 33.09 ± 10.35% (23 h), then decreased, and plateaued to an abundance of 3.96 ± 2.02% (Figure **6.8**).

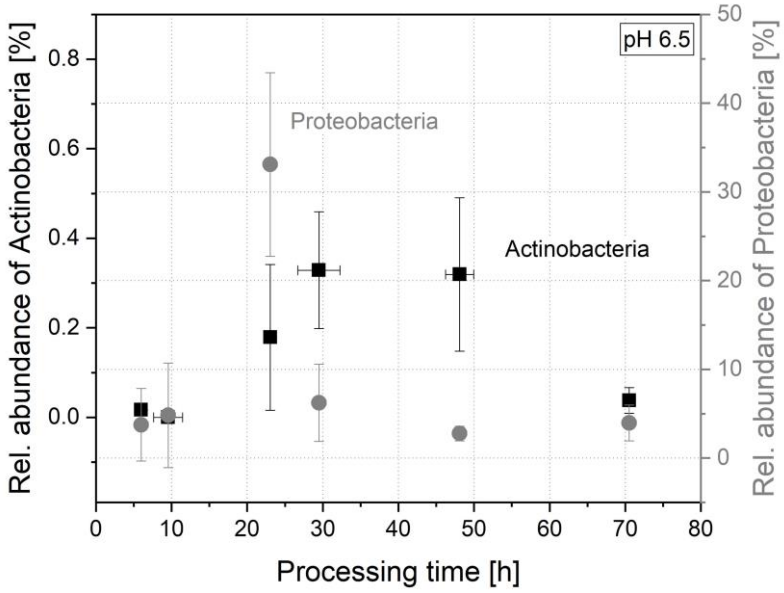

Figure 6.8: Rel. cum. abundance of Actinobacteria (■) and Proteobacteria (●) during re-cultivation at pH 6.5

In contrast, the phylum of Verrucomicrobia could not be re-established after preservation within the set limits (<0.01 % rel. cum. Abundance).

The ratio between Firmicutes and Bacteroidetes is a marker for human health. Here, during re-cultivation at pH 6.5 a ratio of 0.30 was reached, indicating a healthy microbiota (Magne *et al.*, 2020). Bircher *et al.* (2018) reached higher ratios for the re-cultivation of donor 1 with 8.73 and 3.62, whereas donor 2 was in a comparable range of the Firmicutes to Bacteroidetes ratio (0.70) obtained here, indicating that the re-established microbiota had a health indicating microbial composition.

In the human intestinal microbiome, several other genera are known as health markers, probiotics, or other members important for diversity and composition. In Figure **6.9**, the abundance of *Akkermansia* (van Herreweghen *et al.*, 2017), *Bacteroides* (Waidmann *et al.*, 2003), *Bifidobacterium* (Saez-Lara *et al.*, 2015), *Blautia* (Shin *et al.*, 2018), *Faecalibacterium* (Miquel *et al.*, 2013; Cheema, 2019) and *Roseburia* (Siezen and Kleerebezem, 2011) as markers of intestinal health, as well as the abundance of the major genera *Clostridium* Cluster XIVa, *Escherichia* and *Shigella* (Siezen and Kleerebezem, 2011) is shown and will be discussed in the following. All 30 further genera were summed up as "others" to make the graph more readable. The diversity, measured by the Shannon effective index was also comparable at 29 (donor stool), 24 ± 1 before and 24 ± 6 after drying. *Akkermansia* and *Roseburia* were able to establish in the cultivation step prior to drying in small abundances (Haindl *et al.*, 2021b). In the re-cultivation step after drying, they were hardly detectable (< 0.01 % abundance). Hence, it seemed as they are very sensitive to freeze-drying and not able to re-grow in comparable numbers as before the drying step. The abundance of *Escherichia* and *Shigella* increased after re-inoculation to 32.27 ± 9.70% (23 h) and decreased afterwards to 2.57 ± 1.42% after 71 h of

processing time. Representatives of the genus *Escherichia* and *Shigella* are able to survive and even grow in an aerobic environment (Farmer and Jones, 1976). As contact with traces of oxygen during cell harvest and concentration prior to freeze-drying and re-cultivation cannot be fully prevented, this genus was able to survive and re-grow as the abundances of *Escherichia* and *Shigella* above show. The further genera *Bacteroides*, *Bifidobacterium*, *Blautia*, *Clostridium* Cluster XIVa and *Faecalibacterium* showed a similar behavior after drying. All genera were present in low percentages after preservation but could be re-established. After 71 h of re-cultivation an abundance of 63.83 ± 15.87% for *Bacteroides*, 0.03 ± 0.02% for *Bifidobacterium*, 1.00 ± 0.73% for *Blautia*, 5.05 ± 1.21% for *Clostridium* Cluster XIVa, and 1.03 ± 0.35% for *Faecalibacterium* was detected. For these genera, the preservation process led to a decrease, but they were able to recover.

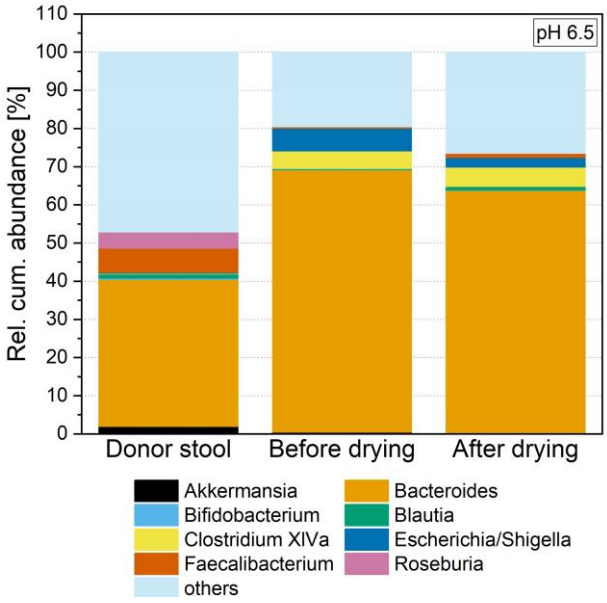

Figure 6.9: Rel. cum. abundance of genera in the donor stool and the stable system before and after drying at a cultivation pH of 6.5; for a better readability only genera discussed in this study are depicted, other genera are summed up as others

Other researches also investigated the abundance of butyrate-producing bacteria *Faecalibacterium* and *Roseburia* after the preservation of *in vitro* microbiota through freezing (Bircher *et al.*, 2018). They experienced a drop in the abundance of *Roseburia* after preservation. It seemed as *Roseburia* are sensitive to a preservation process of freezing and thawing as well as to freeze-drying. Possibly, not the removal of water is critical to this genus, but the freezing to low temperatures as it was done here as a part of freeze-drying as well as during cryopreservation performed by Bircher *et al.* (2018). Further, Bircher *et al.* (2018) measured a strongly impaired abundance of *Faecalibacterium* after cryopreservation. In this study here, it was able to re-establish. This may be due to different factors. On the one hand, the cultivation process prior to drying/ freezing differs between the two studies. *Faecalibacterium* showed impaired growth after freezing and thawing after the cultivation process applied by Bircher *et al.* (2018). Here, this genus was able to re-establish after a continuous

cultivation process and freeze-drying. Though the survival of *Faecalibacterium* may be due to the different cultivation processes (continuous vs. batch in the re-cultivation step). Further, the cells in this study did experience water removal through drying instead of thawing, which may have different effects on their ability to re-establish. As the process conditions differs significantly (continuous vs. batch cultivation; different media; different preservation method) from the study of Bircher *et al.* (2018), we cannot conclude undoubtedly that freeze-drying itself is more appropriate for the genus of *Faecalibacterium* compared to freezing.

Diversity

Next to the microbial composition, the diversity of the microbiota is of importance when it comes to human health. In this study, diversity was measured with the criteria richness, representing the total number of OTUs in the community, and the Shannon effective index, which accounts for the evenness and abundance of species in the community. In the following, the data for re-cultivation at pH 6.5 will be shown and discussed, further data (pH 6.0 and 7.0) can be found in Table **6.2** and Table **6.3**. After drying and re-inoculation, the richness in the system cultivated at pH 6.5 decreased to 47 ± 12 but increased afterwards to 104 ± 11 (71 h processing time). Compared to the system prior to freeze-drying, the richness is comparable (99 ± 7). Further, the Shannon effective index was calculated. After inoculation, the evenness and abundance of different species in the system re-cultivated at pH 6.5 was very low at a value of 3 ± 1 (6 h). As the contribution of phyla and genera already showed, the species were able to re-establish and consequently, the Shannon effective index increased to 8 ± 3 (23 h) and finally to 24 ± 6 (71 h). Compared to the stable system before preservation, the Shannon effective index was in the same range (24 ± 1 prior to freeze-drying). As a result, neither richness, nor Shannon effective index are influenced by the drying process applied in this study and therefore indicate a healthy and diverse system. Contrary results were reached by Bircher *et al.* (2018), who detected a lower diversity in the re-cultivated system. This gives the hint, as if freeze-drying is able to restore the diversity, whereas a preservation through freezing might lead to a decrease. Nevertheless, here also the different cultivation techniques have to be considered. Consequently, no clear statement can be made regarding the question of which preservation method being more appropriate.

6.3.3 *Ability to re-establish after the drying step: comparison of the pre- and post-drying system*

To investigate the influence of the ability to re-establish after the drying process, the values of the stable system prior to freeze-drying were compared with the characteristics of the re-established system (data in Table **6.4**). The open question was whether different or comparable systems were generated after drying to reveal

the impact of the drying process itself and the effect of drying stress on the individual phyla and genera.

For the *in vitro* microbiota cultivated at pH 6.5, only few significant differences based on a p-value of 0.05 were detected. For a number of aerobic and anaerobic CFUs no differences after re-cultivation were detectable. Further, the microbial composition showed no difference in the abundance of the five phyla. Also, on genera level no differences between the stable systems before and after drying occurred. The α-diversity, richness and Shannon effective index were similar as well. As chapter 6.3.1 shows, the drying step led to a significant decrease of the cell count. Nevertheless, the system was able to re-establish. Hence, number and composition of microorganisms were influenced by freeze-drying in a reversible way and the system was able to re-establish completely regarding these factors. Contrary, differences in the production of metabolites were detected. After cultivation at pH 6.5, the concentration of acetate increased after drying from 3.82 ± 0.14 mg mL^{-1} (stable system prior to drying) to 4.35 ± 0.26 mg mL^{-1} after 71 h of re-cultivation. By contrast, the concentration of propionate and iso-valerate was decreased in the broth after re-cultivation compared to the stable system before drying. Propionate decreased from a concentration of 2.94 ± 0.13 mg mL^{-1} of about 14% to 2.51 ± 0.19 mg mL^{-1} (71 h of re-cultivation). Iso-valerate showed a decrease of about 58% from 0.36 ± 0.08 mg mL^{-1} (stable system prior to drying) to 0.16 ± 0.06 mg mL^{-1} in the re-cultivated broth. The concentrations of butyrate showed no significant differences at pH 6.5. In total, the metabolism of the re-established microbiota, measured by the total concentration of SCFAs was not affected irreversible by the drying step. The systems cultivated at pH 6.0 and 7.0 showed comparable behavior with only significant changes regarding the metabolism in the established systems pre and post drying. For pH 6.0 all investigated metabolites as well as the overall concentration of SCFAs differed: As already observed at pH 6.5, the concentration of acetate was increased after drying. Here, also propionate and iso-valerate were decreased, as well as butyrate and the overall concentration (Table **6.4**). For a re-cultivation at pH 7.0, only the concentrations of butyrate and iso-valerate were decreased. The changes in metabolism after drying observed in this study are partly significant, but do not automatically indicate an altered microbial composition as the distribution of phyla before and after freeze-drying showed. Hence, the alterations are probably due to the natural dynamics of the microbiota and do not indicate a change induced completely by the drying process (Gerber, 2014). In contrast, when preserving the microbiota through freezing, a lowered metabolism was observed (Bircher *et al.*, 2018). Further, the concentration of acetate, propionate and butyrate decreased after cryopreservation. Nevertheless, this trend has to be proven urgently with more experiments and further studies, before a clear recommendation for the preservation process can be made.

6.3.4 *Influence of the cultivation pH: impact on the re-established system*

For the drying of probiotic single cell cultures, the influence of the cultivation pH was already investigated and shown to have a major impact (Bauer *et al.*, 2012). Further, the impact during the establishment of an *in vitro* microbiota was shown in former studies (Haindl *et al.*, 2021b). In this study here, the *in vitro* microbiota was cultivated prior to and after the drying process at three different physiological pH values (pH 6.0, 6.5, 7.0). The open question was whether one of the pH values has a positive effect on drying resistance and the re-established system and creates a system after lyophilization that is most similar to the donor stool. In the following, the influence of the cultivation pH on the re-established system will be evaluated. Therefore, we compared the values of all characteristics in the stable system after a cultivation time of at least 70 h based on a one-way ANOVA ($p \leq 0.05$) followed by a Tukey post-hoc analysis. The data can be seen in Table **6.4**.

For cell count, no significant difference occurred for either aerobic or anaerobic microorganisms. All of the three investigated pH values created an *in vitro* system with a comparable number of cells. Otherwise, their metabolism, measured by the SCFAs showed some pH dependent differences. In total, the sum of SCFAs showed a peak in concentration at pH 6.5. It seemed as at this pH value a more active metabolism was able to recover. This peak in SCFAs was due to the composition of the major acids. The peak at pH 6.5 was also determined for the concentration of butyrate. The concentration of acetate was not influenced by the cultivation pH. For propionate, a higher concentration was metabolized at pH 7.0, whereas iso-valerate showed a contrary behavior with the lowest concentration at pH 7.0. Consequently, the peak in metabolism, measured by the sum of all SCFAs is probably due to a high abundance of butyrate-producing bacteria in the experiment conducted at pH 6.5. Regarding the microbial composition based on phyla level in the three systems after drying, only Bacteroidetes and Firmicutes showed a pH-dependency in their abundance. While the abundance of Bacteroidetes increased with the pH value, the abundance of Firmicutes, in contrast, decreased. This effect was already shown during cultivation before drying (Haindl *et al.*, 2021b), but did not explain the peak in butyrate production after drying during re-cultivation. On genera level, *Faecalibacterium*, *Clostridium* Cluster XIVa, *Blautia* and *Roseburia* are the main butyrate-producing bacteria in the microbiota (Bircher *et al.*, 2018). Based on genera level, only *Faecalibacterium* showed a pH-dependent abundance. As already described in chapter 6.3.2, the abundance of *Roseburia* after 70 h of re-cultivation was not detectable anymore within the set limits. Nevertheless, the abundance of *Faecalibacterium* after drying was between $0.01 \pm 0.005\%$ (pH 6.0), $0.01 \pm 0.01\%$ (pH 7.0) and $1.03 \pm 0.35\%$ (pH 6.5) and therefore explained the peak in butyrate-production at pH 6.5. *Clostridium* Cluster XIVa, *Blautia and* Roseburia show no dependency in their abundance based on the cultivation pH value. *Blautia* shows a slight trend to higher abundances at higher cultivation pH values, but this trend is

not significant. Consequently, this cannot explain the peak in butyrate production, but may support the higher concentrations at pH 6.5

Overall, the variation of cultivation pH value led to different systems. Their behavior was, as discussed in chapter 6.3.3 and further studies similar to the results before drying (Haindl *et al.*, 2021b). When comparing the re-established systems after 70 h, a peak in metabolism due to a higher abundance of butyrate producing *Faecalibacterium* was observed. In total, the re-cultivation pH does only affect the metabolism as well as the abundance of Firmicutes, Bacteroidetes and *Faecalibacterium*.

6.3.5 *Influence of the entire process chain: comparison of the initial feces, pre- and post-drying system*

Former chapters above have already shown that the drying step itself does only have a small influence on the metabolism when comparing the pre- and post-drying system. The pH value during re-cultivation was found to influence the metabolism, especially butyrate production, as well as the abundance of the major phyla Bacteroidetes and Firmicutes. When preserving the microbiota, not only single process steps, but the overall process chain is of importance. In the following, the microbial composition and diversity of the initial feces will be compared with the pre- as well as the post-drying system. This data can be seen in Table **6.4**.

Comparing the microbial diversity of the feces with the post-drying system, no significant differences can be observed for either richness, or the Shannon effective index. The richness in the post-drying, re-cultivated system (e.g., 104 ± 11 for pH 6.5) was comparable to the donor stool with a richness of 99. The Shannon effective index, which was 29 in the stool was in the same range as in the re-cultivated system (e.g., 24 ± 6 for pH 6.5) and did not differ for any of the investigated pH values based on a p-value of 0.05. These results are different from other studies where the *in vitro* microbiota was preserved through freezing (Bircher *et al.*, 2018). Next to diversity, the microbial composition is important and was investigated and feces compared with the post-drying system as it can be seen in Figure **6.10**, Figure **6.11** and Figure **6.12**. Here, no differences for the minor phyla Actinobacteria, Proteobacteria and Verrucomicrobia were detected when comparing feces with all three post-drying systems. For Firmicutes, the abundance in the stool was significantly higher. In contrast, the abundance of Bacteroidetes in the stool was lower compared to pH 6.5 and 7.0, whereas the abundances at pH 6.0 (49.11 ± 14.45%) and the donor stool (48.79%) did not differ significantly. These results originate and proceed from the cultivation step. There, also the abundances of Firmicutes were decreased and Bacteroidetes increased compared to the donor stool (Haindl *et al.*, 2020).

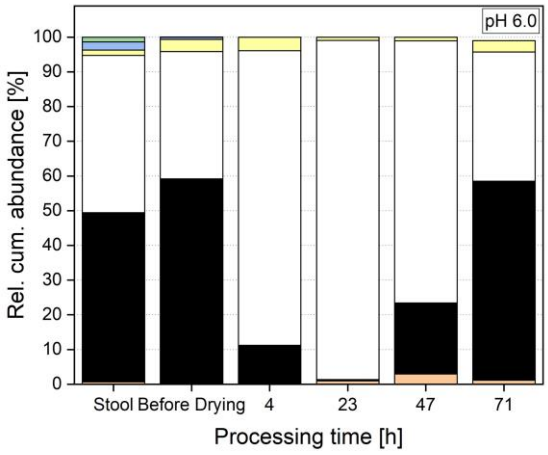

Figure 6.10: Relative cumulative abundance of phyla in the donor stool, stable system before drying and at several processing points during re-cultivation at a cultivation pH of 6.0

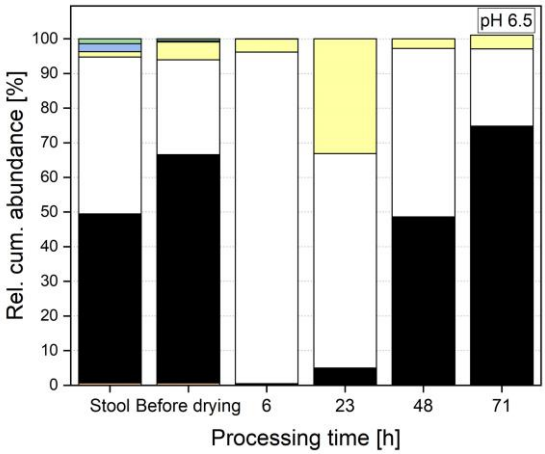

Figure 6.11: Relative cumulative abundance of phyla in the donor stool, stable system before drying and at several processing points during re-cultivation at a cultivation pH of 6.5

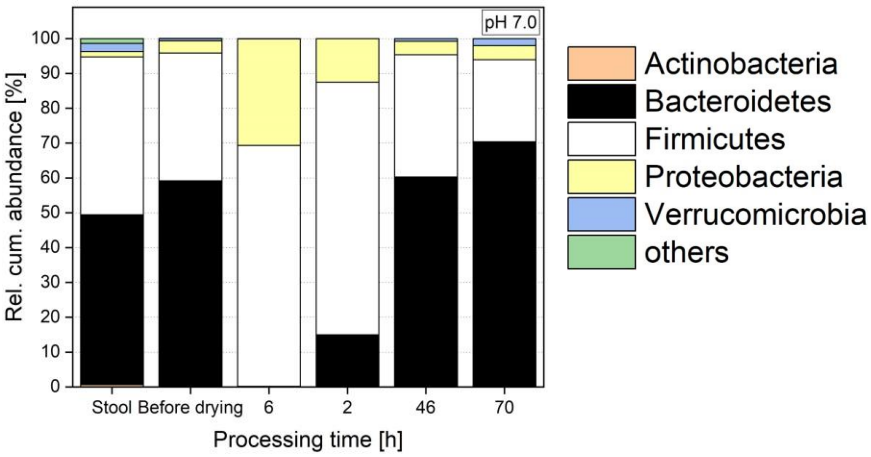

Figure 6.12: Relative cumulative abundance of phyla in the donor stool, stable system before drying and at several processing points during re-cultivation at a cultivation pH of 7.0

Next to the abundance, the ratio of Firmicutes to Bacteroidetes is of interest. In the initial donor stool, the ratio was 0.93. Regarding the distribution of phyla, a cultivation pH of 6.0 creates a system most similar to the donor sample during re-cultivation. At pH 6.0, the ratio of Firmicutes to Bacteroidetes is 0.75, compared to 0.30 (pH 6.5) and 0.34 (pH 7.0). When looking at the microbial composition at genera level, different observation for the selected genera can be made. For *Bifidobacterium*, *Blautia*, *Escherichia* and *Shigella* no significant differences in abundances between the donor stool and the re-cultivated microbiota can be seen. These genera seemed to be not sensitive to the entire preservation process and can be conserved in their initial abundances. *Bacteroides* and *Clostridium* Cluster XIVa showed increased abundances in the stable system after drying compared to the

donor stool for the cultivation at pH 7.0 and 6.5. For a pH value of 6.0 no significant differences in the abundances of these two genera compared to the donor stool were detected. These two genera had higher tolerances regarding the preservation process when cultivated at higher pH values (6.5 and 7.0) and therefore were abundant in higher values. Indeed, a cultivation at pH 6.0 led to a process resistance that created an abundance after the whole process comparable to the initial feces. However, these altered abundances did not occur during re-cultivation, but appeared already during cultivation and therefore before drying (Haindl *et al.*, 2021b). The drying process itself did not change the composition additionally. As already discussed in chapter 6.3.2, *Akkermansia* and *Roseburia* were very sensitive to the preservation process and only abundant in hardly detectable abundances in the re-cultivated system (e.g., *Akkermansia* showed an abundance of 0.001 ± 0.01% at system pH 6.0 compared to 1.98% in the donor stool). Also, the abundance of *Faecalibacterium* was lowered (e.g., 1.03 ± 0.5% after re-cultivation at pH 6.5) compared to the donor stool (6.39%). Consequently, these compositional changes resulted from the cultivation process that took place before drying.

As already investigated in previous studies, the cultivation itself and further the applied conditions during cultivation had a major impact on the composition of the *in vitro* microbiota (Haindl *et al.*, 2020; Haindl *et al.*, 2021b). The results discussed in this chapter proceed over the whole preservation process as they show the same differences between the re-cultivated microbiota and the initial donor stool system. As only slight differences in the metabolism between the cultivation systems prior and post drying were detected in chapter 6.3.3, the changes in the microbial composition in the preserved *in vitro* microbiota are probably due to the cultivation step before drying instead of the drying step itself. Concluding from that and summing up the results from chapter 6.3.3 and 6.3.4, the cultivation step had a higher impact on the entire process chain than the drying step. Further, the cultivation pH before freeze-drying was the value that may be adjusted to reach a high abundance, diversity, and richness comparable to the donor stool. Consequently, major alterations in the *in vitro* microbiota result from the cultivation step prior to drying. The drying itself did not cause any significant changes anymore.

6.4 Conclusion

In the current study, the influence of an entire preservation process on the survival, vitality, viability and ability to re-grow of a human intestinal microbiota was investigated. Therefore, the impact of the cultivation pre- and post-drying, the drying step itself on cell count, metabolism, microbial composition, diversity, as well as the re-formation of a stable system was determined.

After drying, the moisture in the received powder indicated a stable and storable product but showed a low survival rate of the cells. Nevertheless, it was proven that the system was able to re-establish and re-grow a healthy system within 24-70 h. Cell count, microbial composition as well as diversity did not differ significantly for all investigated pH values compared to the cultivation system prior to drying. Metabolism after drying showed slight changes in the concentration of SCFAs, but these alterations are more due to natural dynamics of the microbiota and do not indicate a change induced by the drying-process.

The aim of this study was to identify process steps that lead to major alterations of the *in vitro* system compared to the initial donor stool. When comparing the *in vitro* systems prior and post drying, it is clearly visible that hardly any changes occur during freeze-drying and therefore prior to drying, already during cultivation. The drying step influenced cell count and microbial composition significantly. Nevertheless, these effects were reversible and vanished during re-cultivation. The cultivation step, especially the cultivation pH value, had a major impact and offered potential for optimization of the entire preservation process. We detected different influences of the cultivation pH on characteristics of the re-established system. For cell count as well as for diversity, measured by richness and the Shannon effective index, no pH dependent influence was observed. Additionally, all cultivation pH values resulted in cells with a healthy and working metabolism. Nevertheless, a cultivation pH of 6.5 provided the most comparable, healthy characteristics in metabolism. Regarding the distribution of phyla and genera, a cultivation pH of 6.0 led to a composition that is closer to the initial system in the donor stool than at higher pH values. Consequently, for the preservation of an *in vitro* microbiota, we recommend lower physiological pH values between 6.0 and 6.5.

The application of *in vitro* infusion material instead of a traditional FMT had already been studied (Garborg, 2015). As we had proven that a dried microbiota does technically not differ significantly from that, it gives the hint that a freeze-dried *in vitro* microbiota might also be a possibility of a successful treatment. Nevertheless, it urgently needs the investigation of further stool samples as well as *in vivo* studies, since it is not clear whether the impact on patients' recovery of cultivated bacteria is the same as FMT itself.

Acknowledgements

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References

The references were combined into a joint list of all publications, see at the end of the thesis.

6.5 Supplementary Data

Table 6.2: Evolvement of all investigated values during re-cultivation at pH 6.0

	Processing time [h]					
Investigated value	*4*	*12*	*23*	*34*	*47*	*71*
CFU aerob [10^9 CFU mL^{-1}]	-	-	2 ± 0.3	-	2 ± 1	0.2 ± 0.1
CFU anaerob [10^9 CFU mL^{-1}]	-	-	3 ± 1	-	9 ± 7	7 ± 1
Σ SCFA [mg mL^{-1}]	0.19 ± 0.12	1.69 ± 0.47	2.64 ± 0.46	3.44 ± 0.50	5.51 ± 1.61	7.73 ± 1.09
Acetate [mg mL^{-1}]	0.13 ± 0.08	1.13 ± 0.13	1.93 ± 0.12	2.38 ± 0.33	3.29 ± 0.63	4.39 ± 0.34
Butyrate [mg mL^{-1}]	0.01 ± 0.02	0.30 ± 0.21	0.38 ± 0.20	0.71 ± 0.12	1.05 ± 0.12	0.97 ± 0.05
Propionate [mg mL^{-1}]	0.02 ± 0.01	0.05 ± 0.02	0.13 ± 0.07	0.17 ± 0.03	0.95 ± 0.80	2.13 ± 0.62
Iso-valerate [mg mL^{-1}]	0.02 ± 0.01	0.20 ± 0.10	0.20 ± 0.06	0.18 ± 0.01	0.22 ± 0.06	0.24 ± 0.08
Richness [-]	73 ± 28	30 ± 18	35 ± 16	40 ± 14	60 ± 21	75 ± 23
Shannon effective [-]	7 ± 4	5 ± 1	4 ± 2	8 ± 1	15 ± 7	25 ± 8
Actinobacteria [%]	0.02 ± 0.02	0.36 ± 0.62	0.96 ± 1.07	2.52 ± 0.63	2.92 ± 0.79	1.18 ± 0.92
Bacteroidetes [%]	11.17 ± 8.46	0.52 ± 0.79	0.38 ± 0.25	1.46 ± 0.81	20.46 ± 18.56	57.32 ± 3.70
Firmicutes [%]	84.90 ± 11.18	98.22 ± 1.07	97.70 ± 1.41	95.20 ± 2.03	75.54 ± 18.60	37.23 ± 1.30
Proteobacteria [%]	3.88 ± 2.81	0.89 ± 0.89	0.95 ± 0.53	0.82 ± 0.73	1.08 ± 0.83	3.27 ± 2.95
Verrucomicrobia [%]	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Akkermansia [%]	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Bacteroides [%]	8.86 ± 6.77	0.50 ± 0.80	0.35 ± 0.19	1.45 ± 0.81	20.42 ± 18.49	48.70 ± 14.23
Bifidobacteria [%]	0.02 ± 0.02	0.36 ± 0.62	0.96 ± 1.07	2.52 ± 0.63	2.78 ± 0.72	1.16 ± 0.93
Blautia [%]	0.28 ± 0.20	< 0.01	< 0.01	0.08 ± 0.08	2.29 ± 1.86	4.45 ± 3.88
Faecalibacterium [%]	0.95 ± 0.73	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Roseburia [%]	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Clostridium Cluster XIVa [%]	0.38 ± 0.30	< 0.01	< 0.01	< 0.01	0.14 ± 0.22	2.62 ± 1.80
Escherichia/ Shigella [%]	3.80 ± 2.79	0.85 ± 0.92	0.92 ± 0.50	0.78 ± 0.68	0.80 ± 0.58	2.40 ± 2.36

Table 6.3: Evolvement of all investigated values during re-cultivation at pH 7.0

	Processing time [h]					
Investigated value	*6*	*10*	*22*	*32*	*46*	*70*
CFU aerob [10^9 CFU mL^{-1}]	-	-	9 ± 0	-	2 ± 2	0.5 ± 0.2
CFU anaerob [10^9 CFU mL^{-1}]	-	-	6 ± 6	-	8 ± 9	6 ± 2
Σ SCFA [mg mL^{-1}]	0.31 ± 0.07	1.31 ± 0.99	3.88 ± 0.48	5.84 ± 0.99	7.39 ± 1.31	8.35 ± 1.11
Acetate [mg mL^{-1}]	0.21 ± 0.06	0.85 ± 0.45	2.40 ± 0.22	3.83 ± 0.19	4.77 ± 0.48	4.64 ± 0.48
Butyrate [mg mL^{-1}]	0.01 ± 0.00	0.23 ± 0.44	1.08 ± 0.15	1.05 ± 0.25	0.94 ± 0.12	0.90 ± 0.06
Propionate [mg mL^{-1}]	0.06 ± 0.00	0.10 ± 0.08	0.23 ± 0.09	0.77 ± 0.42	1.55 ± 0.68	2.67 ± 0.53
Iso-valerate [mg mL^{-1}]	0.03 ± 0.01	0.12 ± 0.02	0.17 ± 0.02	0.19 ± 0.13	0.12 ± 0.04	0.13 ± 0.04
Richness [-]	34 ± 21	32 ± 18	39 ± 18	58 ± 13	64 ± 18	68 ± 25
Shannon effective [-]	3 ± 2	4 ± 2	9 ± 4	14 ± 2	17 ± 4	18 ± 6
Actinobacteria [%]	< 0.01	< 0.01	< 0.01	0.18 ± 0.35	0.11 ± 0.19	0.02 ± 0.02
Bacteroidetes [%]	0.18 ± 0.12	0.13 ± 0.08	14.95 ± 21.12	53.77 ± 22.31	60.19 ± 13.13	70.35 ± 5.79
Firmicutes [%]	69.14 ± 35.95	77.89 ± 7.96	72.52 ± 16.01	40.29 ± 20.55	35.06 ± 11.21	23.61 ± 6.16
Proteobacteria [%]	30.63 ± 35.82	21.93 ± 7.97	12.53 ± 5.75	5.73 ± 1.63	3.89 ± 2.08	4.05 ± 1.84
Verrucomicrobia [%]	0.03 ± 0.01	< 0.01	< 0.01	< 0.01	0.75 ± 1.26	1.98 ± 3.36
Akkermansia [%]	0.03 ± 0.01	< 0.01	< 0.01	< 0.01	0.81 ± 1.22	2.00 ± 3.34
Bacteroides [%]	0.16 ± 0.11	0.12 ± 0.08	11.93 ± 16.12	43.29 ± 18.45	60.12 ± 18.14	70.79 ± 9.97
Bifidobacteria [%]	< 0.01	< 0.01	< 0.01	0.18 ± 0.45	0.11 ± 0.19	0.02 ± 0.02
Blautia [%]	< 0.01	< 0.01	< 0.01	0.66 ± 1.01	0.80 ± 0.95	0.26 ± 0.27
Faecalibacterium [%]	0.01 ± 0.00	0.02 ± 0.03	0.19 ± 0.33	< 0.01	< 0.01	< 0.01
Roseburia [%]	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Clostridium Cluster XIVa [%]	0.04 ± 0.01	0.02 ± 0.02	1.05 ± 1.76	2.78 ± 3.80	4.87 ± 2.66	4.21 ± 1.78
Escherichia/ Shigella [%]	29.64 ± 34.42	21.61 ± 7.52	10.58 ± 8.11	5.67 ± 2.36	2.70 ± 2.52	3.24 ± 2.47

Table 6.4: Summary and comparison of all investigated values of the stool sample, in the stable system before and after drying depending on the cultivation pH value

Investigated value	**Stool**	**Before drying, after cultivation (120 h) and concentration**			**After drying in the re-established system (>70 h cultivation time)**		
		pH 6.0	*pH 6.5*	*pH 7.0*	*pH 6.0*	*pH 6.5*	*pH 7.0*
CFU aerob [10^8 CFU mL^{-1}]	0.02 ± 0.01	0.9 ± 0.2	3 ± 0.3	1 ± 1	3 ± 3	3 ± 2	50 ± 20
CFU anaerob [10^9 CFU mL^{-1}]	0.1 ± 0.03	8 ± 2	12 ± 7	6 ± 2	8 ± 2	10 ± 7	6 ± 2
Σ SCFA [mg mL^{-1}]	5.12 ± 0.14	8.52 ± 0.48	8.68 ± 0.24	8.90 ± 0.36	7.92 ± 0.40	8.43 ± 0.39	8.14 ± 0.36
Acetate [mg mL^{-1}]	2.74 ± 0.07	3.14 ± 0.17	3.82 ± 0.14	4.07 ± 0.22	4.37 ± 0.30	4.35 ± 0.26	4.16 ± 0.59
Butyrate [mg mL^{-1}]	0.99 ± 0.03	1.90 ± 0.09	1.57 ± 0.16	1.38 ± 0.17	0.94 ± 0.06	1.42 ± 0.13	0.87 ± 0.07
Propionate [mg mL^{-1}]	1.15 ± 0.04	3.03 ± 0.20	2.94 ± 0.13	3.05 ± 0.21	2.39 ± 0.52	2.51 ± 0.19	2.96 ± 0.43
Iso-valerate [mg mL^{-1}]	0.24 ± 0.00	0.45 ± 0.13	0.36 ± 0.08	0.40 ± 0.18	0.21 ± 0.06	0.16 ± 0.06	0.15 ± 0.03
Richness [-]	99	100 ± 6	99 ± 7	126 ± 36	75 ± 23	104 ± 11	81 ± 14
Shannon effective [-]	29	22 ± 4	24 ± 1	24 ± 5	25 ± 8	24 ± 6	18 ± 6
Actinobacteria [%]	0.65	0.33 ± 0.09	0.68 ± 0.09	0.17 ± 0.10	1.18 ± 0.92	0.04 ± 0.03	0.02 ± 0.02
Bacteroidetes [%]	48.79	58.81 ± 8.32	65.88 ± 6.82	71.90 ± 3.62	57.32 ± 3.70	75.01 ± 0.79	70.35 ± 5.79
Firmicutes [%]	45.28	36.74 ± 10.39	27.40 ± 8.32	22.92 ± 5.58	37.23 ± 1.30	22.27 ± 0.23	23.61 ± 6.16
Proteobacteria [%]	1.59	3.50 ± 1.54	5.14 ± 1.73	8.65 ± 3.12	3.27 ± 2.95	3.96 ± 2.02	4.05 ± 1.84
Verrucomicrobia [%]	2.30	0.59 ± 0.97	0.34 ± 0.38	0.48 ± 0.40	< 0.01	< 0.01	< 0.01
Akkermansia [%]	1.98	0.03 ± 0.03	0.34 ± 0.38	0.48 ± 0.40	< 0.01	< 0.01	< 0.01
Bacteroides [%]	38.51	54.33 ± 5.50	64.83 ± 6.82	70.00 ± 3.40	48.70 ± 14.23	63.83 ± 15.87	70.79 ± 9.97
Bifidobacteria [%]	0.35	0.27 ± 0.07	0.62 ± 0.80	0.17 ± 0.09	1.16 ± 0.93	0.03 ± 0.02	0.02 ± 0.02
Blautia [%]	1.05	0.59 ± 0.05	0.36 ± 0.22	0.11 ± 0.07	0.97 ± 0.60	1.00 ± 0.73	0.26 ± 0.27
Faecalibacterium [%]	6.39	10.04 ± 1.01	0.56 ± 0.36	0.44 ± 0.43	0.01 ± 0.005	1.03 ± 0.35	0.01 ± 0.01
Roseburia [%]	4.18	0.02 ± 0.01	0.01 ± 0.02	0.01±0.01	< 0.01	< 0.01	< 0.01
Clostridium Cluster XIVa [%]	0.19	4.70 ± 0.60	3.94 ± 1.09	2.74 ± 1.79	2.62 ± 1.80	5.05 ± 1.21	4.21 ± 1.78
Escherichia/ Shigella [%]	0.24	2.43 ± 0.16	4.94 ± 1.94	0.52 ± 0.16	2.40 ± 2.36	2.57 ± 1.42	3.24 ± 2.47

7 Overall discussion and main findings

The complete preservation process of a human intestinal microbiota has not been investigated so far. Therefore, in this work, the process was split in the establishment of a drying protocol and an *in vitro* cultivation system as well as the investigation of a drying process. During cultivation, the influence of the donor and the cultivation pH value was investigated. The aim was to develop a preservation process which creates a product that has comparable biotechnological properties as the infusion material that is commonly used when applying FMT, meaning a culturable, stable community with a high diversity and richness of representative gut bacteria.

7.1 Establishment of an *in vitro* cultivation system

To create infusion material for probiotic investigations as well as for FMT, several studies already used *in vitro* cultivation systems (Hemarajata and Versalovic, 2013; McDonald, 2013; Takagi *et al.*, 2016; Bircher *et al.*, 2018). These systems offer advantages as an easy set-up, handling, and maintenance and further the lack of ethical considerations.

In chapters 3 and 4 the establishment and behavior of the developed *in vitro* system was described and evaluated. Further, the influence of three different donor stools at three different physiological cultivation pH values from 6.0 to 7.0 was determined. In the present chapter, the influence of both factors will be discussed: individual donor stool characteristics and cultivation pH value (Figure 7.1).

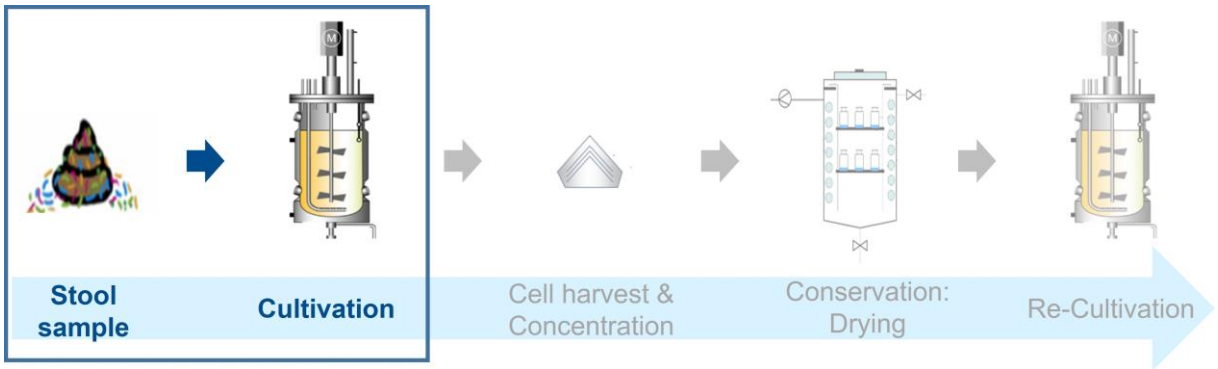


Figure 7.1: Investigated steps in the entire preservation process: influence of initial donor stool material and cultivation pH value

To minimize external influences and only regard the influence of the stool composition itself, the donors were the same age (donor A: 28 years old; donor B: 25 years old; donor C: 27 years old) and ethnic group (Caucasian) and shared the same social environment. Their body mass index (BMI) was between 23 (donor A) and 21 (donors B and C) and all of them ate a Western diet. As described above, a stable system can be reached for each donor, cultivation pH value and system after 77 h the

latest. Consequently, all donors adapted to the cultivation system and were able to establish in a comparable time span. Further, the influence of the cultivation of each donor stool on cell count, metabolism and microbial composition and diversity was investigated. The overall trends caused by cultivation overall as well as by the cultivation pH value are shown in Table 7.1. The table shows on the one hand the trend of each value marked by arrows as well as the statistical significances marked by colour. The influence of the donor stool was investigated by comparing each cultivation pH set-up of each donor with the corresponding stool sample using a one-way ANOVA followed by a post-hoc Tukey test. The same statistical analysis method was used to reveal the influence of the cultivation pH value. Here, the three different set-ups within one system were compared with each other.

Table 7.1: Comparison of the investigated values of three different system originating from three donors; arrows indicate the changes compared with the individual donor stool (↑: the value increased; ↓ the value decreased; →: value was comparable) as well as the influence of the cultivation pH in the individual system (↗: the value increased with an increasing pH; ↘: the value decreased with an increasing pH; →: value did not differ with pH); the color indicates the significant difference with the donor stool/ influence of the cultivation pH value (no color: no difference from the donor stool/ no influence of the pH; light blue: two pH values differ significantly from the donor stool/ each other; dark blue: all three pH values differ significantly from the donor stool/ each other)

Investigated value		**Influence of cultivation** Comparison of each cultivation set-up (pH 6.0, 6.5, 7.0) with the corresponding donor stool			**Influence of cultivation pH** Behavior with increasing pH value from 6.0 to 7.0		
		A	B	C	A	B	C
Cell count	*Facultative aerobic*	↑	↑	↑	→	→	↗
	Anaerobic	↑	↑	↑	↗	↗	↗
Metabolism	*Acetate*	↑	↑	↑	↗	↗	↗
	Propionate	↑	↑	↑	↗	↗	↗
	Butyrate	↑	↑	→	↘	↘	↘
	Iso-Valerate	↑	↑	↓	→	→	↘
	∑SCFAs	↑	↑	pH-dep.	↗	↗	↗
Microbial Community	*Actinobacteria*	↓	↓	↓	↘	↘	↘
	Bacteroidetes	↑	↑	↑	↗	↗	↗
	Firmicutes	↓	↓	↓	↘	↘	↘
	Proteobacteria	↑	↑	↑	↘	↘	↘
	Verrucomicrobia	pH-dep.	↓	pH-dep.	↗	↗	↗
	Richness	→	→	→	→	→	→
	Shannon eff.	↓	↓	↓	↘	↘	↘
	Ratio F:B	↓	↓	↓	↘	↘	↘

7.1.1 *Cell count*

Regarding cell count, it increased in each system compared to the donor stool. In the stable systems, the anaerobic cell count was between $3 \pm 0.0\ 10^{8}$ (system B, pH 6.0) and $1 \pm 0.02\ 10^{10}$ CFU mL^{-1} (system C, pH 7.0). These values all indicate healthy systems comparable to physiological values of 10^{10}-10^{12} CFU mL^{-1} in the large intestine (Payne *et al.*, 2012) as described above (chapter 1.1.1). The aerobic cell count accounts to facultative aerobic microorganisms that are also capable of growing with contact to or traces of oxygen as for example *Lactobacilli* or *Bifidobacteria*. Here, the values were lower and established between $4 \pm 0.4\ 10^{7}$ CFU mL^{-1} (system C, pH 6.0) and $3 \pm 0.2\ 10^{8}$ CFU mL^{-1} (system B, pH 6.5). In total, all facultative aerobic cells are also part of the anaerobic cell count. Hence, the percentage of aerobic microorganisms is between 0.6 - 2.7% depending on the donor and cultivation pH value. Marteau *et al.* (2001) detected approximately 1% of aerobic bacteria in feces. Hence, the proportion obtained *in vitro* is in the similar range. In total, compared to the initial cell count in the donor stool (chapter 4.2.1) the cell count in every *in vitro* system is erased. Further, as Figure **7.2** shows, the anaerobic cell count increases significantly with the cultivation pH value.

Consequently, an *in vitro* cultivation leads to an enrichment of cells compared to the donor stool. Further, a high cultivation pH value (here pH 7.0) creates higher anaerobic *in vitro* cell counts compared to a low value (here pH 6.0). For the facultative aerobic cells, also an increase of cells compared to the donor stool was observed, whereas it only showed a slight increase with the cultivation pH.

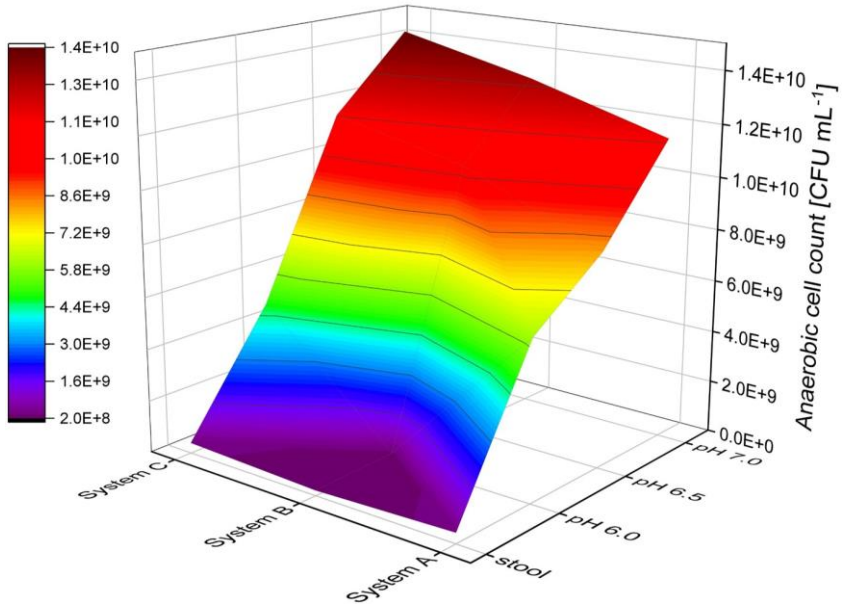


Figure 7.2: Comparison of the anaerobic cell count for the three systems in the donor stool as well as at the three cultivation pH values

7.1.2 *Metabolic profile*

Regarding the metabolic profile, an increase in most concentrations compared with the stool was also observed for all three donors (Table 7.1). This may be a consequence due to the higher cell count: more cells produce more metabolites. For the total concentration of SCFAs an increase was measured for all set-ups within systems A and B, whereas in system C only a cultivation pH of 6.5 and 7.0 led to statistically significant higher metabolic production. The metabolic production in system C at pH 6.0 was comparable with the donor stool (5.61 ± 1.56 mg mL^{-1} during cultivation vs. 6.10 ± 0.82 mg mL^{-1} in the donor stool). The concentration of single investigated SCFAs was either increased in all three systems or followed an individual behavior (Table 7.1). For acetate, propionate and butyrate in systems A and B the concentrations were increased compared to the donor stool. In system C a concentration of butyrate comparable to the content in the donor stool was measured. Iso-valerate was increased in systems A and B, whereas less iso-valerate was produced in the stable system C.

When the influence of the pH is investigated, the concentration of all metabolites in total, shows an increase with increasing pH value from 6.0 to 7.0 (Figure **7.3**). This increase in total and for each tested system, may be due to the increase of acetate (Figure **7.4**) and propionate (Figure **7.5**), the major metabolites in the broth. For instance, in system B, the highest total concentration of SCFAs at pH 7.0 of 9.03 ± 0.36 mg mL^{-1} was reached, with 4.08 ± 0.20 mg mL^{-1} of acetate and 3.08 ± 0.34 mg mL^{-1} of propionate. Butyrate was metabolized with a concentration of 1.49 ± 0.20 mg mL^{-1}, whereas only 0.45 ± 0.07 mg mL^{-1} of iso-valerate were found in the broth. For systems A and C, also acetate and propionate were the major metabolites.

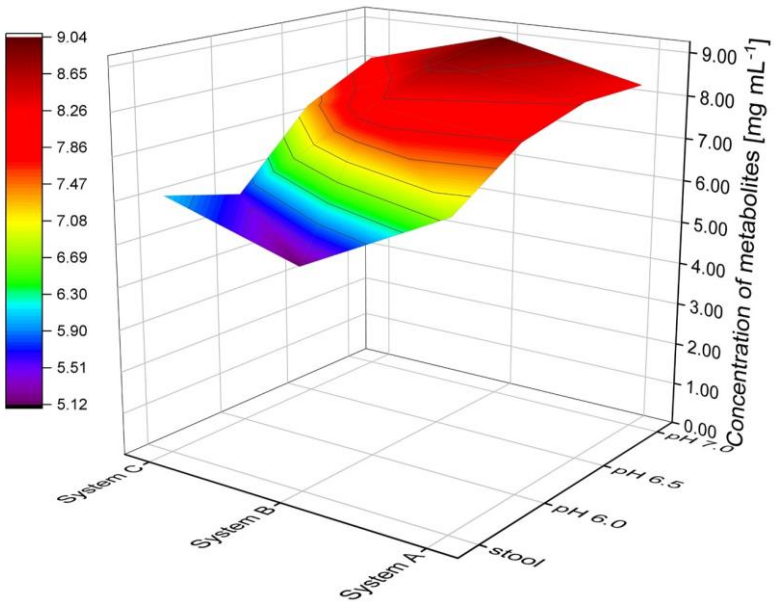


Figure 7.3: Influence of the donor system and cultivation pH value on the concentration of the total amount of metabolites in the broth

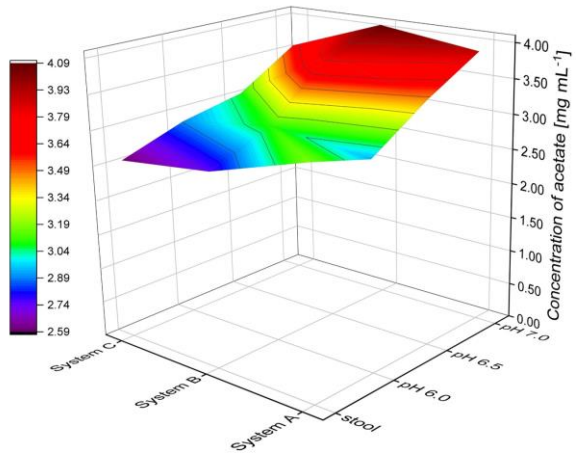


Figure 7.4: Influence of the donor system and cultivation pH value on the concentration of acetate in the broth

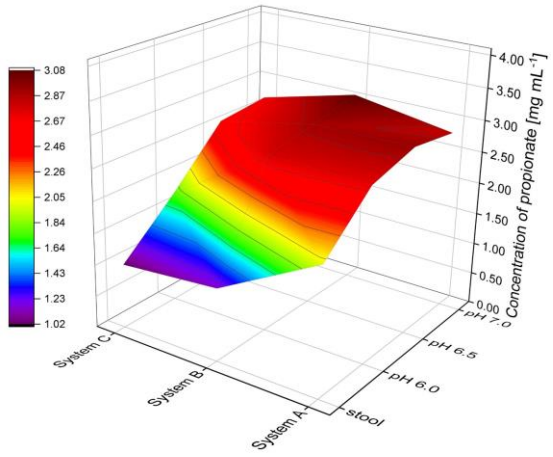


Figure 7.5: Influence of the donor system and cultivation pH value on the concentration of propionate in the broth

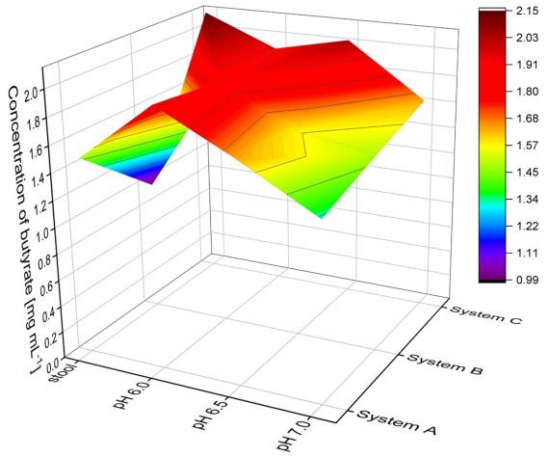


Figure 7.6: Influence of the donor system and cultivation pH value on the concentration of butyrate in the broth

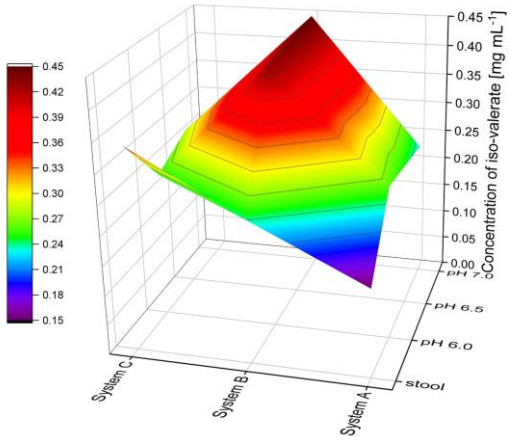


Figure 7.7: Influence of the donor system and cultivation pH value on the concentration of iso-valerate in the broth

The concentration of butyrate (Figure **7.6**) decreased with an increasing cultivation pH value for all three systems. Iso-valerate followed a more individual behavior: a continuous or slightly decreasing concentration was detected in the broth depending on the cultivation pH (Figure **7.7**).

In total, all three donors at all investigated pH values created systems with the characteristics of a working and healthy metabolism even though differences were visible. Next to the number of cells, compounds as the SCFAs are of great importance for the success of FMT. Here, all *in vitro* systems represented systems with a sufficient and even higher amounts of metabolites that would fulfill these criteria. Part of this work was to study differences among the systems created by the three chosen donor stool samples (chapter 3). When comparing the stable states of the cultivated systems, all donors showed a comparable adaption to the artificial conditions. Further, the

cultivation pH value showed a similar overall influence in every investigated system. Nevertheless, slight characteristic differences maintained. This concludes that the cultivation system itself, especially the pH value, has a higher influence than the donor stool characteristics. Even though some characteristic properties (see chapter 3) remain, all three systems behaved in a comparable way. Consequently, for an improvement of the *in vitro* cultivation system, the system parameters are of great interest and should be part of further studies.

7.1.3 *Microbial community*

The cell count and production of metabolites give hints, but do not reveal the composition of the microbial community itself. Therefore, the influence of the establishment of the system as well as the influence of the pH value on the microbial composition was investigated by 16S rRNA gene amplicon sequencing (chapter 3 and 4).

Regarding the phyla in the systems during cultivation, a trend could be observed: The abundance of Actinobacteria and Firmicutes decreased in total compared to the donor stool. Also, a decrease with an increasing pH value from 6.0 to 7.0 was observed (Table 7.1). Contrary, Bacteroidetes and Proteobacteria were increased after at least 77 h of cultivation. Here, their abundance increased with an increasing pH value. For the phylum of Verrucomicrobia an individual and pH-dependent behavior was observed. In systems A and C, the total abundance increased with the increasing pH value and was therefore only in the set-up at pH 7.0 increased compared to the abundance in the donor stool. System B also showed an increased abundance of Verrucomicrobia at pH 7.0, but these values in total were lower compared to the donor stool.

In total, an *in vitro* cultivation leads to an increase in Bacteroidetes and a decrease in Firmicutes. This can also be seen in the Firmicutes: Bacteroidetes ratio. It is lowered compared to the respective donor stool. For instance, the ratio for system C was 2.60 in the donor stool, but due to the changes in the stable *in vitro* system the ratio lowered to 0.65 (pH 6.0), 0.29 (pH 6.5) and 0.24 (pH 7.0). In other studies comparable alterations were observed (McDonald, 2013; Bircher *et al.*, 2018). Hence, it seems like the phylum of Bacteroidetes is more adaptive to the *in vitro* system than representatives of the phylum Firmicutes. Further, Bacteroidetes are more abundant at higher cultivation pH values then Firmicutes.

On genera level, some genera were more adaptive to the conditions than others. In chapter 3 and 4 several genera known as health markers, probiotics or other members important for diversity and composition were identified. In the following, the behavior of *Akkermansia* (van Herreweghen *et al.*, 2017), *Bacteroides* (Waidmann *et al.*, 2003), *Bifidobacterium* (Saez-Lara *et al.*, 2015), *Blautia* (Shin *et al.*, 2018), *Faecalibacterium (Miquel et al., 2013; Cheema, 2019), Roseburia, Clostridium* Cluster *XIVa, Escherichia* and *Shigella* (Siezen and Kleerebezem, 2011) will be discussed. Table 7.2 shows the

individual behavior of the selected genera compared to the donor stools. In total, *Bacteroides*, which are part of the phylum Bacteroidetes can adapt easily to the *in vitro* conditions as their abundance is increased significantly for all systems and pH values. Contrary, *Blautia* and *Roseburia* (both of the phylum of Firmicutes) are less capable of adapting and are decreased in their abundances. *Roseburia* even experience a total decrease down to abundances below 0.1% and seem not to be able to grow in detectable abundances *in vitro* with the conditions applied in this study. *Akkermansia*, *Bifidobacterium*, *Clostridium* Cluster *XIVa*, *Escherichia*, *Shigella* and *Faecalibacterium* behave more donor and pH dependent. Here, *Clostridium* Cluster *XIVa*, *Escherichia*, *Shigella* and *Faecalibacterium* trend to be increased in at least two set-ups, whereas *Bifidobacterium* trends to have an equal (system B and C) or decreased (system A) abundance. Overall, the abundances of the investigated genera follow the abundance of the corresponding phyla.

Table 7.2: Behavior of selected genera in the stable systems (> 77 h cultivation time) in comparison with the donor stools

Genera showing an abundance that is…				
Highly increased	**Highly decreased**	**Trending to increase**	**Trending to decrease**	**Dependent on the pH value and donor**
Bacteroides	*Blautia*	*Clostridium* Cluster *XIVa*	*Bifidobacterium*	*Akkermansia*
	Roseburia	*Escherichia* and *Shigella*		
		Faecalibacterium		

Regarding richness and diversity in the stable systems, different observations can be made (Table **7.3**). In this work, a constant richness with no significant differences for all three donor systems was detected. Consequently, the number of different OTUs within stool and cultivated systems stays constant and is not changed by the cultivation. However, the diversity and evenness, measured by the Shannon effective index is altered. During cultivation, all systems experience a drop in the index. This decrease may be mainly due to the shift towards higher abundances of Bacteroidetes, especially *Bacteroides*, which are abundant in significant higher numbers.

Table 7.3: Comparison of richness and Shannon effective index for stool and stable systems depending on the cultivation pH value for all three donor systems

		Richness [-]	**Shannon effective index [-]**
System A	*Stool*	99	21
	Cultivation pH 6.0	101 ± 6	19 ± 1
	Cultivation pH 6.5	105 ± 6	17 ± 1
	Cultivation pH 7.0	96 ± 3	16 ± 1
System B	*Stool*	123	38
	Cultivation pH 6.0	99 ± 8	20 ± 2
	Cultivation pH 6.5	96 ± 7	24 ± 2
	Cultivation pH 7.0	115 ± 9	23 ± 5
System C	*Stool*	122	46
	Cultivation pH 6.0	119 ± 1	22 ± 6
	Cultivation pH 6.5	115 ± 3	22 ± 1
	Cultivation pH 7.0	115 ± 3	18 ± 1

In total, the microbial composition in the different experimental set-ups is altered by the cultivation conditions as described above and in the chapters 3 and 4. These alterations are probably due to different reasons. During processing before cultivation and analysis, the contact with oxygen was avoided but could not prevented completely. Hence, the cells were exposed to traces of oxygen for a short period of time. Genera as *Bacteroides* are tolerant towards oxygen exposure of up to one hour (Corthier *et al.*, 1996). Others as *Blautia* are strictly anaerobic (Park *et al.*, 2013; Shin *et al.*, 2018). This may be a possible explanation for the increase in abundance of *Bacteroides* and the decrease of *Blautia*. A further reason for the alterations compared with the *in vivo* systems can be shear stress induced by the stirring in the bioreactor with 200 rpm. Also, *in vitro* models are never capable of representing identical conditions as *in vivo*. Available nutrients are changed due to different media compositions. Even the viscosity of media has an influence on the abundance as anaerobic cells favor a higher media viscosity (Tamargo *et al.*, 2018). Additionally, the formation of a biofilm in the bioreactor is hindered by the stirring and the vessel geometry. Further, interactions between microbes and microbes as well as between microbial and human cells are reduced or lack completely. The combination of all these factors eventually lead to an altered *in vitro* microbiota and a shift in the phylum ratio towards a higher abundance of Bacteroidetes and the other described effects.

The aim of this work was not to create an exact copy of the gut system, but a system with a high number and diversity of health indicating microorganisms. Here, no set-up failed to establish a system with a high richness, diversity and diverse microbial community. Even if the composition *in vitro* is altered and two investigated genera decreased rapidly, the overall richness is conserved. When it comes to diversity, a slight drop is observed. Regarding the influence of the cultivation pH value, no clear recommendation for a higher or lower value can be made. The obtained results trend to favor lower values as here a higher diversity as well a Firmicutes: Bacteroidetes ratio closer to the donor stool was observed.

7.2 Establishment of a drying protocol

The human intestinal microbiota is a very complex ecosystem hosting several different species. When preserving such a microbial consortium, the variety and diversity of the different abundant microorganisms is a huge challenge. In the past, mainly the behavior of single cell cultures during drying was investigated. Consequently, in this work, a strain with well-known properties was chosen to develop a drying protocol that might also suit a microbial community as the human intestinal microbiota. In the microbiota of the three donors the genus *Bifidobacterium* existed in significant and comparable abundances of 1.01% (donor A), 0.30% (donor B) and 0.98% (donor C). As test strain *Bifidobacterium longum* ssp. *longum* Reuter 1963 was chosen as it is a well described probiotic strain. Hence, a drying protocol for the test strain as well as the entire human intestinal microbiota was developed (Figure **7.8**).

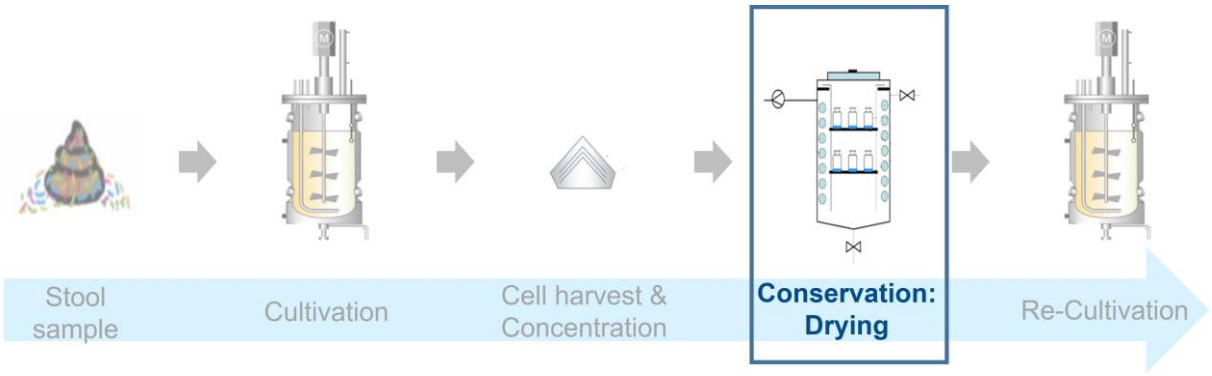


Figure 7.8: Investigated steps in the entire preservation process: development and influence of the conservation done by freeze-drying

In chapter 5 the influence of different drying process parameters as cultivation pH, shelf temperature or addition of protectants on the survival of *Bifidobacterium longum* was tested. As a result, a freeze-drying process with a pressure of 3,700 Pa, a shelf temperature increased in a stepwise manner (-10 °C for 12 h, +10 °C for 6 h, +35 °C for 6 h) and a total process time of 24 h was found to be sufficient. For *Bifidobacterium longum* a survival rate of 40 ± 15 % was reached after the process when the strain was cultivated at pH 6.0 (Table **7.4**). For a cultivation with free acidification (no pH control) a way lower survival of only 0.5 ± 0.4 % was determined.

In the past, comparable behavior was observed for other strains of the microbiota (Bauer *et al.*, 2012). A possible reason might be, as described above (chapter 5), the differently composed cell membranes due to different cultivation parameters. Additionally, the cells produce stress proteins that might cope the damages of sub-lethally injured cells and lead to higher survival rates (Morgan *et al.*, 2006). When the entire microbiota was dried, survival rates between 6.4 ± 3.3 % (aerobic cells, cultivation at pH 6.0) and 0.01 ± 0.01 % (anaerobic cells, cultivation at pH 6.5/ 7.0) were reached (Table **7.4**).

Table 7.4: Comparison of aerobic and anaerobic survival rates obtained when drying the *in vitro* microbiota and the single cell culture depending on the cultivation conditions

	***In vitro* microbiota** (Haindl *et al.*, 2022)			***Test strain*** (Haindl *et al.*, 2020)	
Cultivation conditions [pH value; -]	6.0	6.5	7.0	free acidific.	6.0
Aerobic cell survival [%]	2.3 ± 0.6	5.5 ± 2.9	6.4 ± 3.3	-	-
Anaerobic cell survival [%]	0.1 ± 0.1	0.1 ± 0.1	0.9 ± 0.8	0.5 ± 0.4	40 ± 15

These lowered survival rates compared to the single cell culture might be due to different ongoing mechanisms. Inactivation of the microbiota's cell might be due to:

- Cultivation process
- Shear stress
- Oxygen exposure
- Freezing
- Water removal
- Heat
- Membrane damage
- Unsuitable drying process

Not all microorganism in the microbiota are cultivable. Hence, the cultivation process might lead to a pre-damage of some cells and strains. That condition might be the reason why they survive the cultivation process but are then inactivated during the drying step. For the *in vitro* microbiota, only a non-significant trend regarding the influence of the cultivation conditions on the cell survival was obtained, whereas for the single cell culture significant differences in the survival rates depending on the cultivation conditions were identified. Some researchers state that the cultivation conditions in advance have an enormous influence as they promote the induction of stress response proteins that might be able to help sub-lethally injured cells to recover (Morgan *et al.*, 2006). This might be a further reason for the higher survival of

Bifidobacterium longum. It is probable, that also in the dried microbiota stress proteins are produced but due to the very complex system, their effect is not as obvious as in the single cell culture. Before drying, the broth gets harvested and concentrated by centrifugation. The occurring shear stress can lead to inactivation of shear sensitive strains. Additionally, when the broth is prepared for drying, contact with oxygen is tried to be avoided, but could not be prevented completely due to the analysis and process procedures. *Bifidobacterium longum* is, compared to other strains of the intestinal microbiota, less sensitive to the exposure to oxygen. This might have resulted in higher survival rates for the single cell culture. Also, during freeze-drying several damaging mechanisms occur. Cells can get inactivated by the freezing step, the water removal or due to heat that especially occurs in the later drying phase (Santivarangkna *et al.*, 2008b). In total, during drying the cell membrane is the part of the cell where the most damage occurs. When the water is removed, the phospholipid bilayer in the cell membrane moves from the liquid crystalline to the gel phase causing membrane leakage that might then lead to inactivation (see chapter 1.4.1). As discussed in chapter 5, different microbial strains have differently composed cell membranes. The content of phospho-lipids differs, for instance in the content of phosphatidylcholine, diphosphatidylglycerol or glucosamine-containing phospholipids (Exterkate *et al.*, 1971; Drucker *et al.*, 1995; Novik *et al.*, 2006). Hence, as the membrane is the part of the cell where the most damage occurs during drying, different microorganism have a varying sensitivity towards the drying process. The drying protocol was adapted especially for the drying of *Bifidobacterium longum*. Contrary, a broad variety of strains is abundant in the microbiota. Hence, the developed drying protocol might not fit to all of them. This may lead to lowered survival rates in the microbiota compared with the single cell culture.

Comparing the abundance of the genus *Bifidobacterium* before and after drying in the stable system of the *in vitro* microbiota to identify the survival does not make any sense. The results given by NGS are not reliable in this special case. In the here used sequencing method, cells can only get differentiated until genera level and not strain level. Hence, no information is provided about the abundance of *Bifidobacterium longum* ssp. *longum* Reuter 1963 strain within the *Bifidobacterium* genus cluster. Further, when the cells get injured irreversible during drying, the DNA and hence their 16S rRNA are still present in the broth and cannot be differentiated from the one of living cells by this sequencing method. Consequently, the abundance obtained would not give a realistic picture of the real abundance of living cells capable of a working metabolism. Further, cell survival and abundance do not correlate with each other as described in chapter 1.6. To identify the abundance of *Bifidobacterium* in the dried microbiota, it is inevitable to re-culture the cells as it will be further discussed in chapter 6. The results here only inform about the survival and stress right after the drying process but do not provide evidence for the ability of re-establishment and re-growth of the systems.

In total, the here developed drying protocol is, as described in chapter 5, suitable for the single cell culture of *Bifidobacterium longum*. Regarding only the value of cell survival for the microbiota, the impression might arise that the drying protocol does not fit the complex ecosystem. Nevertheless, this value does not give an overall impression - the ability to re-grow and re-establish is of huge importance and was tested in further studies (chapters 6 and 7.3).

7.3 Ongoing alterations in the entire preservation process

In the chapters 3, 4 and 7.1 the establishment of the *in vitro* system of a human intestinal microbiota was described. The created material was used to investigate the entire preservation process. Therefore, the donor stool was cultivated *in vitro* after a stable system was formed. The broth got harvested after 120 h of processing time, concentrated by centrifugation, and then freeze-dried by the established drying protocol (chapter 5). To validate the ability to re-form and re-establish a running *in vitro* system the rehydrated powder was cultivated again (re-cultivation) as described in chapter 6. The aim here was to demonstrate the technical feasibility of the production of a culturable, stable community with a high cell number, diversity and richness of representative gut bacteria as well as a high abundance of health-promoting microorganisms (Figure **7.9**).

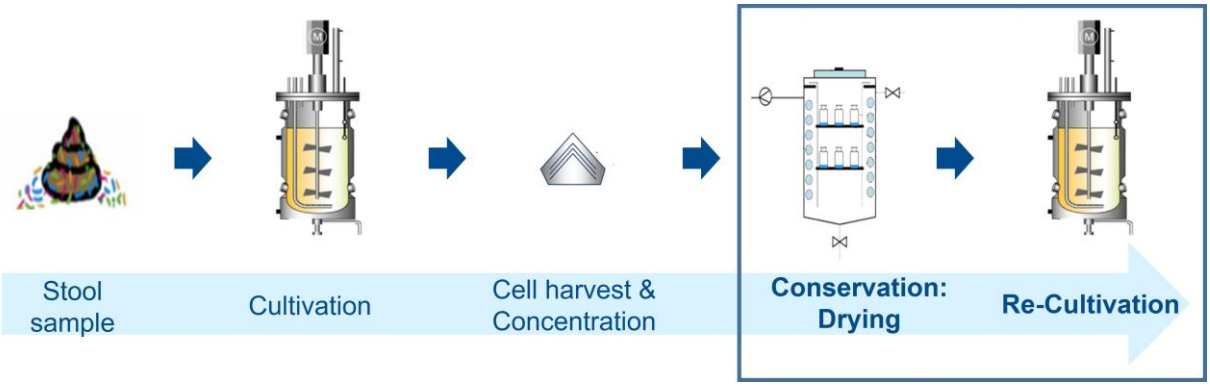


Figure 7.9: Investigation of the entire preservation process, especially the influence on the drying outcome and ability to re-establish during re-cultivation

After freeze-drying took place, the system showed huge changes and alterations. The cell count was significantly decreased. Nevertheless, after 4-6 h of re-cultivation, the cells were able to re-grow, re-start their metabolism and form a re-established stable system after 70 h. Comparing the cultivated system before drying (after 120 h) and the re-established system (after 70 h) no significant differences for any of the pH value set-ups in cell count, phyla composition or alpha diversity (richness and Shannon effective index) were detected. Also, on genera level the selected genera showed no significant changes in the systems pre and post drying. An exception was the abundance of *Faecalibacterium* in the system with cultivation pH 6.0. Whereas the abundance was 6.39% in the donor stool B, the value was increased to 10.04 ± 1.24%

before drying but dropped to a level of only 0.01 ± 0.01% after re-cultivation. Here, a significant decrease was measured. In the other set-ups, the abundance changed from 0.35 ± 0.12% (pH 6.5) and 0.28 ± 0.10 % (pH 7.0) before drying to 1.03 ± 0.43% (pH 6.5) and 0.01 ± 0.01% (pH 7.0) after 70 h of re-cultivation. It seems as *Faecalibacterium* can be preserved through lyophilization, but only in decreased abundances.

A further change in the re-established systems was observed in metabolism. Here, slight changes in the concentration of several metabolites were observed within the different pH set-ups. These changes were found to be part of natural dynamic changes in the metabolism as they could not be correlated with the abundances of single phyla or genera. Even *in vivo*, slight changes occur in the microbiota of healthy adults. Hence, these changes in the metabolism were considered natural as well and did not have an impact on the re-established system (Gerber, 2014). The lack of alterations in cell count or microbial composition pre and post drying may be attributed to the cultivation conditions. The microbial cells already adapted to the conditions *in vitro* during cultivation before drying. Consequently, they were able to recover and adapt easily after drying and re-form a similar system as before drying. In total, the cultivation pH value after drying was found to only have a minor influence. When comparing the systems after re-cultivation, differences were detected that originated already from the cultivation process. Overall, the Figure **7.10**, Figure **7.11**, andFigure **7.12** show the composition of the donor stool, the system before drying and after re-cultivation depending on the cultivation and re-cultivation pH value. As already shown during cultivation (chapters 3, 4 and 7.1.3), the distribution of genera shifts towards an increase of *Bacteroides* and a decrease of *Blautia*. *Akkermansia* and *Roseburia* were really sensitive towards the drying process and their abundances dropped to a level below 0.01%. Nevertheless, the decrease of some genera, all three investigated physiological cultivation pH values resulted in stable systems with most characteristics considered as healthy. In total, comparing all re-established systems with the donor stool, a slight recommendation towards lower pH values can be made. Here, the composition in the re-established system after 70 h of cultivation is more similar regarding the original system (donor stool) compared to a higher cultivation pH value.

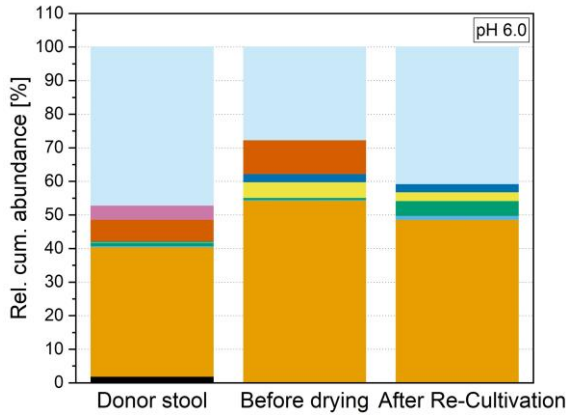


Figure 7.10: Relative cumulative abundance of the selected genera in the donor stool, before drying and after 70 h of re-cultivation at a pH value of 6.0

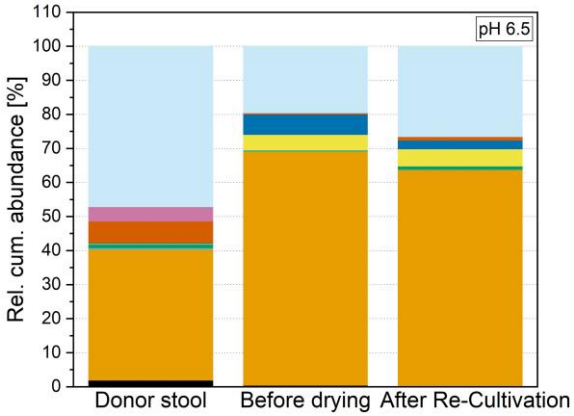
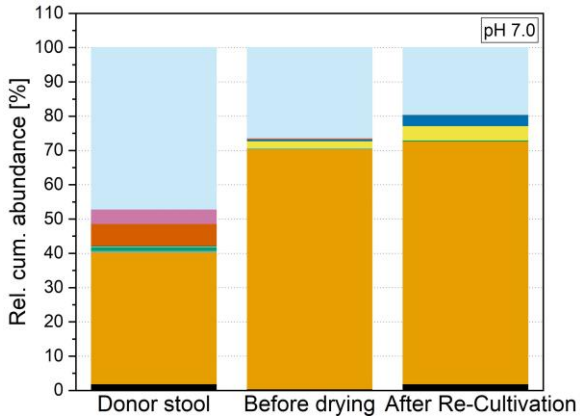


Figure 7.11: Relative cumulative abundance of the selected genera in the donor stool, before drying and after 70 h of re-cultivation at a pH value of 6.5



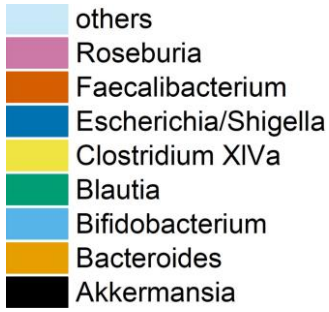


Figure 7.12: Relative cumulative abundance of the selected genera in the donor stool, before drying and after 70 h of re-cultivation at a pH value of 7.0

7.4 Comparison of the preserved *in vitro* microbiota with conventional infusion material

When treating a patient suffering from CDI with traditional Fecal Microbiota Transplantation, the procedure in different studies is not standardized yet. After an extensive donor screening, the stool gets sampled and diluted. For dilution, different solutions as saline, milk, water or media are used (McDonald, 2013). Hereby, the solution has no effect on efficiency (Brandt and Aroniadis, 2013). Afterwards, the mixture is suspended by hand shaking or the use of a blender. Large food particles are removed by centrifugation or filtration. The infusion material used as inoculum in chapters 3 and 4 was prepared in a similar way. In this work, no *in vivo* studies were performed, but the technical feasibility of the preservation and re-establishment of an *in vitro* microbiota was investigated. Figure **7.13** to Figure **7.17** show the distribution

and relative abundance of phyla in the donor stool, inoculum (prepared as described in chapters 3 and 4; before starting the cultivation process), in the stable system after 120 h of cultivation at pH 6.0, after the freeze-drying process and after 70 h of re-cultivation at a pH value of 6.0. Comparing the inoculum and state after drying, a higher abundance of Firmicutes and a decreased value for Bacteroidetes compared to the donor stool is observed. Nevertheless, the abundance of Proteobacteria, Verrucomicrobia and Actinobacteria was not significantly different as in the donor stool. The system after drying contrary experienced a loss in abundance of Actinobacteria and Verrucomicrobia, while the abundance of Proteobacteria was increased. Nevertheless, the abundance of Actinobacteria was able to increase after re-cultivation and the ratio between Firmicutes and Bacteroidetes decreased. In the stool, the ratio between Firmicutes and Bacteroidetes was 1.13, while it increased to 4.06 in the inoculum. The highest ratio was detected in the system after drying with 7.60, while it was lowered to 0.65 in the re-established system. Hence, regarding the ratio of the major phyla, the re-established system showed the highest similarity with the donor stool. Even so, the composition of the inoculum differs from the donor stool, high recovery rates after a treatment with FMT were observed (Gough *et al.*, 2011; Aroniadis and Brandt, 2013). Here, the administered fecal slurry can re-establish in the patient's gut and re-form a healthy system and cure the disease. In this work, the establishment of the inoculum in an *in vitro* system was conducted successfully. Further, it was shown, that *in vitro* the microbiota preserved by lyophilization is also able to re-establish and re-form a system considered healthy. As described in chapter 6, the systems before and after drying do not differ in the distribution of phyla. This gives a slight hint, that an *in vitro* microbiota as well as the preserved microbiota, may also have a positive effect in the treatment of CDI. Here, the technical realization was feasible, but these results give no information about the medical efficiency of such a dried product. Consequently, it is unavoidable to test this hypothesis in an animal system or *in vivo*.

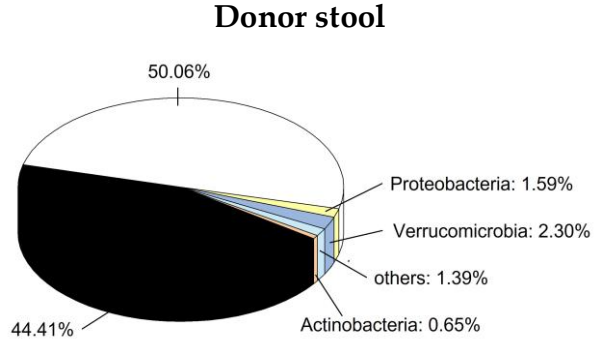


Figure 7.13: Relative abundance of phyla in the stool of donor B

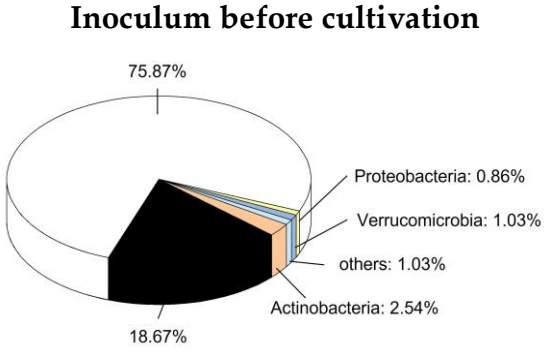


Figure 7.14: Relative abundance of phyla in the inoculum before starting the cultivation process

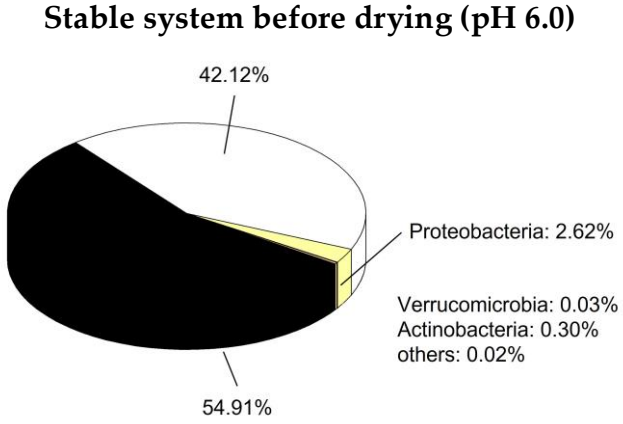


Figure 7.15: Relative abundance of phyla in the established system after 120 h of cultivation at pH 6.0

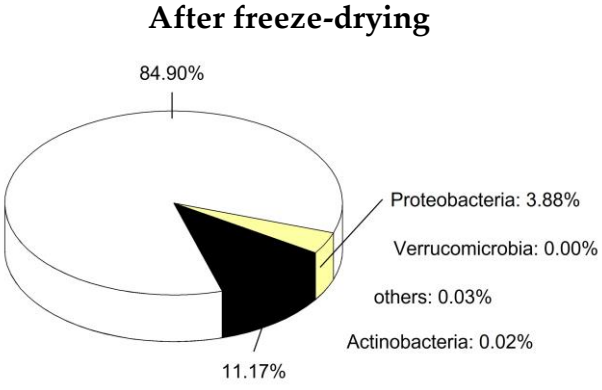


Figure 7.16: Relative abundance of phyla in the rehydrated powder after freeze-drying

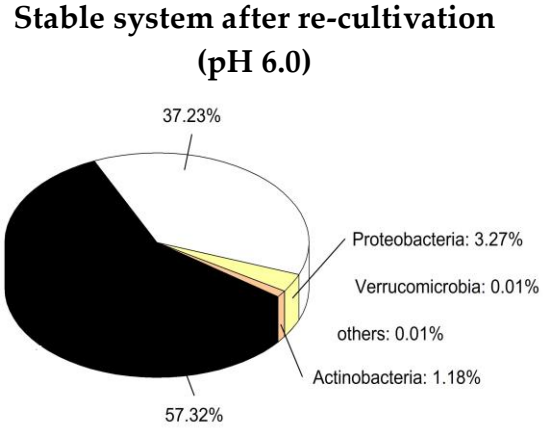


Figure 7.17: Relative abundance of phyla in the re-established system after 70 h of re-cultivation at pH 6.0

8 Overall conclusion and perspective

The aim of this work was to develop, establish and investigate a preservation process for the human intestinal microbiota with the focus on the technical feasibility of the entire process chain. The cultivation *in vitro* was successfully executed with the artificial microbiota experiencing several changes regarding the metabolic and microbial profile. The donor was found to have a minor influence, whereas the cultivation parameters had a major impact. The freeze-drying was found to have led to significant, but reversible alterations as the re-cultivated system is very similar to the system before drying. As a result, the preservation of the microbiota is possible without any major damages regarded on a technical level. Comparing the inoculum and the system after drying and re-cultivation, both form systems that are technically similar with the initial donor stool. Hence, a similar effect on the cure of CDI might be possible, even though further *in vivo* studies are inevitable. Nevertheless, this work also includes some unanswered questions. Cultivation was identified as the process step with the highest influence, but only the impact of donor stool and pH value were investigated. Hence, more parameters as stirring rate, cultivation temperature or media composition would be of interest. Additionally, the concentration step before drying can be evaluated. For the survival after drying, small survival rates of 6% and below were detected. The addition and influence of cryo- and other protectants can be of interest to further increase the survival. In this study, the drying step was seen as one process consisting of the combination of freezing, sublimation, and drying process. By separating the single steps of freeze-drying, a better understanding of the process itself and further, the occurring mechanisms and cell damage can be reached.

A further lack of this work can also be seen in the sample volume. Three samples, as regarded in chapters 3 and 4, are considered as the scientific standard and commonly accepted in this field of research. The influence of a high donor age or BMI would also be of scientific interest. Even though, it would not play a role for the enhancement of the FMT concept, as elderly or obese persons would not be chosen as donors for fecal material, it could give a better understanding of the preservation process. Despite the finding that the donor stool only had a minor influence on the established system, only one sample was used for the investigation of drying and re-cultivation. Additionally, a sample volume of three during cultivation was sufficient to validate the concept, but more samples will be needed to confirm the findings and drawn conclusions. Here, more, and different samples should be measured and investigated to substantiate, acknowledge, and further explain the results and mechanisms detected in this study.

In total, this work can be seen as a first step in the development of an enhanced FMT treatment concept, even though it concentrates on the technical feasibility and parameters. Here, it was shown that the establishment of a stable *in vitro* microbiota with characteristics regarded as healthy, was possible. Further, a drying protocol was

established that only has shown to have a reversible impact on the preserved cells. Overall, the preservation of a human intestinal microbiota was shown to be successful within the investigated technical parameters. Nevertheless, further biotechnological, microbial, molecular, and medical studies will be of high interest and inevitable.

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10 Appendix

10.1 **Peer-reviewed publications included in this thesis**

Haindl, R., Neumayr, A., Frey, A., and Kulozik, U. (2020) Impact of cultivation strategy, freeze-drying process, and storage conditions on survival, membrane integrity, and inactivation kinetics of Bifidobacterium longum. *Folia Microbiol (Praha)* **65** (6): 1039–1050.

Haindl, R., Engel, J., and Kulozik, U. (2021a) Establishment of an In Vitro System of the Human Intestinal Microbiota: Effect of Cultivation Conditions and Influence of Three Donor Stool Samples. *Microorganisms* **9** (5): 1049–1066.

Haindl, R., Schick, S., and Kulozik, U. (2021b) Influence of Cultivation pH on Composition, Diversity, and Metabolic Production in an In Vitro Human Intestinal Microbiota. *Fermentation* **7** (3): 156–174.

Haindl, R., Totzauer, L., and Kulozik, U. (2022) Preservation by lyophilization of a human intestinal microbiota: influence of the cultivation pH on the drying outcome and re-establishment ability. *Microb Biotechnol* **15** (3): 886–900.

10.2 **Other publications not included in this thesis**

Haindl, R. (2016). Bestimmung des Erntezeitpunkts des anaeroben Mikroorganismus *Bifidobacterium longum* ssp. *longum* Reuter 1963, Jahresbericht 2016 der milchwissenschaftlichen Forschungseinheiten am ZIEL, Technische Universität München.

Haindl, R., Kremer, A. (2017). Wachstumsverhalten des anaeroben Mikroorganismuses *Bifidobacterium longum* ssp. *longum* Reuter 1963, Jahresbericht 2017 der milchwissenschaftlichen Forschungseinheiten am ZIEL, Technische Universität München.

Haindl, R. (2018). Etablierung eines *in vitro* Systems zur Kultivierung der humanen intestinalen Mikrobiota, Jahresbericht 2018 der milchwissenschaftlichen Forschungseinheiten am ZIEL, Technische Universität München.

Haindl, R. (2019). Einfluss verschiedener Medienbestandteile auf das Wachstum von *Bacteroides vulgatus*, Jahresbericht 2019 der milchwissenschaftlichen Forschungseinheiten am ZIEL, Technische Universität München.

10.3 **Oral presentations**

Haindl, R., Kulozik, U. (2017). Conservation of a microbial minimal consortium of the human intestinal microbiota, ZIEL Advisory Board Meeting, Freising/ Germany, 13.3.2017

Haindl, R., Kulozik, U. (2018). Probiotika 2.0: Konservierung der humanen intestinalen Mikrobiota, Jahrestreffen der ProcessNet-Fachgruppe Lebensmittelverfahrenstechnik, Berlin/ Germany, 5.-6.03.2018

Haindl, R., Kulozik, U. (2018). Probiotika 2.0: Culturability and Preservation of the human intestinal microbiota, ZIEL Microbiome Seminar, Hohenkammer/ Germany, 20.-21.7.2018

Haindl, R., Kulozik, U. (2019). Gefriertrocknung von Mikroorganismen: Erfahrungen aus der Praxis, Seminar „Gefriertrocknung mit System“, Osterode am Harz/ Germany, 20.-21.2.2019