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DEVELOPMENT AND VALIDATION OF ANAEROBIC STABILIZATION TECHNOLOGIES FOR PRESERVATION OF POTENTIAL MICROBIAL-BASED THERAPEUTICS

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Abbreviations

ANOVA	Analysis of variance
BCFA	Branched chain fatty acids
BP	Base pair
cAMP	Cyclic adenosine monophosphate
CAZyme	Carbohydrate-active enzyme
CDI	*Clostridium difficile* infection
CD	Crohn's disease
CoA	Coenzyme A
CRC	Colorectal cancer
Cryo	Cryopreservation
DMSO	Dimethyl sulfoxide
FDA	US Food and drug administration
FMT	Fecal microbiota transplantation
GDC	Genetic diversity center
GSI	Glycerol, sucrose and inulin
HPLC	High-performance liquid chromatography
HPLC-RI	High-performance liquid chromatography with refractive index detection
IBD	Inflammatory bowel disease
IFT	Intestinal fermentation technology
Lyo	Lyophilization
MAM	Microbial anti-inflammatory molecule
MPN	Most probable number method
OD	Optical density
OTU	Operational taxonomic units
PB	Phosphate buffer
PBS	Phosphate buffered saline
PC1	Principal component 1
PC2	Principal component 2
PCA	Principal component analysis
PCoA	Principal coordinate analysis
pM	Planktonic microbiota
PSA	Polysaccharide A

qPCR	Quantitative polymerase chain reaction
rCDI	Recurrent *Clostridium difficile* infection
SCFA	Short-chain fatty acid
SI	Sucrose and inulin
sM	Sessile microbiota
t_{lag}	Lag time
UC	Ulcerative colitis
VBCN	Viable but nonculturable
μ_{max}	Maximal growth rate

Summary

Fecal microbiota transplantation (FMT) is highly efficient in resolving recurrent *Clostridium difficile* infection (rCDI) and holds potential as an alternative therapy for other gastrointestinal diseases linked to chronic inflammation. However, a treatment involving fecal matter raises several issues such as a potential risk of infection, transfer of undesired phenotypes and aesthetic concerns. These restrictions led to the ongoing development of synthetic bacteria mixtures to bypass the administration of fecal material. Intestinal fermentation technology (IFT) can readily deliver controlled and stable “artificial” colonic microbiota at high cell density. However, widespread availability of such microbial-based therapeutics can only be guaranteed if the product can be stored without losing efficiency. Consequently, there is a strong demand for anaerobic preservation technologies that maintain microbial composition and functionality. Readily available preservation techniques were mainly developed for pure, aerotolerant cultures while strict anaerobic and community level preservation has been widely neglected. Therefore, the overall aim of this thesis was to investigate, develop and validate anaerobic stabilization technologies for preservation of composition and functionality of complex colonic microbiota produced with IFT and beneficial colonic microbes in pure culture.

The aim of the first study (Chapter 2) was to investigate the effect of a penetrating (glycerol) and non-penetrating cryoprotectant (inulin) on metabolic activity of cryopreserved artificial colonic microbiota produced with IFT using the PolyFermS system. Moreover, we specifically focused on the impact of frozen storage on the stability of the highly oxygen-sensitive, butyrate-producing *Faecalibacterium prausnitzii, Eubacterium hallii* and the *Roseburia* spp./*Eubacterium rectale* group within the complex colonic microbiota as markers for preservation efficiency. The microbiota material was cryopreserved for 3 months at -80°C in a protective formulation containing either glycerol or inulin or a combination thereof. Artificial colonic microbiota was reactivated in anaerobic batch incubations before and after frozen storage. Metabolite formation, serving as activity marker, was monitored using high performance liquid chromatography (HPLC) while the re-establishment of the selected butyrate producers was determined by molecular methods (qPCR). We demonstrated that combining glycerol and inulin in the protective formulation provided a higher level of protection of cryopreserved artificial colonic microbiota than a single component formulation. We also observed a taxa-specific effect of the applied protectants. Glycerol supported cryopreservation of the *Roseburia* spp./*E. rectale* group, while inulin improved the recovery of *F. prausnitzii*. In contrast, *E. hallii* growth was affected minimally by cryopreservation. Moreover, glycerol addition maintained butyrate and propionate production and when combined with inulin resulted in faster formation of butyrate and acetate.

For a closer observation of the cryopreservation response on the previously investigated butyrate producers within a complex microbiota, we performed preservation trials with pure *F. prausnitzii*, *R. intestinalis* and *E. hallii* cultures, excluding the potential impact of a competitive microbiota (Chapter 3). Moreover, we included the gut microbes *Anaerostipes caccae* as additional butyrate producer and two propionate-producing representatives, *Bacteroides thetaiotaomicron* and *Blautia*

obeum, in the preservation trial. The selected strict anaerobic gut microbes underwent cryopreservation following storage for 3 months at -80°C and lyophilization with subsequent storage at 4°C. The protectants sucrose and inulin were added to the lyoprotective formulation and were also combined with glycerol for cryopreservation. Viability and bacterial fitness were evaluated using a combination of cultivation-dependent and cultivation-independent techniques. We identified processing by drying as generally more detrimental to bacterial viability and fitness than freezing. Lyophilization led to better storage stability. Differences in viable cell recovery between the tested bacteria pointed towards a strong species-dependent resistance to freezing and drying. We suggest membrane composition as determining factor for preservation success as the addition of membrane-interacting protectants sucrose and inulin improved viability of all lyophilized strain and of freezing-sensitive strains after cryopreservation. As glycerol also differently affected strain viability and membrane integrity prior and post cryopreservation, our results suggests that efficiency of protectants is process- and species-specific. We observed a positive impact of glycerol on viability of *R. intestinalis* what was in accordance with the previous findings of its protective effect on the reestablishment of *Roseburia sp./E. rectale* group when cryopreserved as part of a complex artificial gut microbiota. The current data also suggest that an enhanced growth rate of *E. hallii* after cryopreservation could explain its competitiveness in batch fermentation, as we reported before that *E. hallii* was little impacted by freezing within a complex artificial gut microbiota.

Lastely, we explored the impact of bacterial life style on the response of complex microbiota to preservation-induced stressors. The PolyFermS system delivers next to planktonic microbiota in reactor effluent also microbiota of sessile life style embedded in polymer beads, mimicking a biofilm-associated state. Microbes in biofilm generally exhibit a higher environmental stress tolerance than planktonic cells. We therefore hypothesized that PolyFermS derived sessile microbiota is more resistant to preservation-induced stress than the corresponding planktonic microbiota (Chapter 4). First, we characterized composition of planktonic and sessile microbiota and then exposed them to cryopreservation at -80°C and lyophilization and subsequent storage for 9 months at 4°C. Community structure and metabolic activity in batch culture were assessed prior and post preservation by using HPLC, molecular and high-throughput sequencing methods, respectively. We observed that bacterial life style is a determining factor in the establishment of microbial composition and metabolic capacity of artificial colonic microbiota. Accordingly, sessile microbiota employed a higher metabolic activity and harbored a unique bacterial and archaeal community differing from planktonic microbiota. Surprisingly, we also demonstrated that community structure and metabolic activity were better maintained by microbiota of planktonic than sessile life style during preservation for 9 months. Our data suggest that cooperating microbes might be a characteristic component of sessile microbiota possibly affecting compositional stability towards freezing and freeze-drying stressors. Preservation-induced cell death of a key taxon might provoke a concomitant decrease of depending taxa.

Independent from life style, we identified highly preservation-sensitive taxa, including several *Bacteroides* sp. but also taxa that exhibited great robustness towards preservation such as *Enterococcaceae* and *Peptostreptococcaceae*. We suggest functional redundancy, as observed for the butyrate-producing community of planktonic and sessile microbiota, as a trait that might preserve functional stability during exposure to freezing, drying and storage stress. In contrast, if a specific property, such as propionate production, is limited to a small number of taxa, the loss of key taxa can mediate loss of function. Our data demonstrated the feasibility of generating complex artificial microbiota in planktonic and sessile life-style with the continuous PolyFermS platform inoculated with fecal microbiota immobilized in gel beads, and subsequent preservation by freezing and lyophilization.

In conclusion, this doctoral thesis has allowed expanding the knowledge on the impact of different preservation methods on community structure and metabolic activity of colonic microbiota and provides important insights into the potential of community level preservation of artificially produced colonic microbiota. We successfully maintained metabolic activity of complex colonic microbiota produced with IFT during cryopreservation at -80°C for at least 3 months and preserved growth of major members of the butyrate-producing community. We demonstrated that efficiency of the applied protectants is process- and species-dependent and that cryopreservation induced effects on pure cultures, as observed with *R. intestinalis* and *E. hallii*, can partly be extrapolated on their behavior in complex microbiota. We further showed that a loss in propionate-producing activity after cryopreservation and lyophilization of sessile and planktonic microbiota can be attributed to impaired reestablishment of *Bacteroidaceae* and that maintenance of the butyrate-producing activity might come from functional redundancy in the microbial communities. Moreover, we showed that sessile microbiota is stronger impacted by cryopreservation and lyophilization than the planktonic counterpart possibly explained by a higher dependency between sessile microbes.

Zusammenfassung

Stuhltransplantation ist eine höchst effiziente Behandlungsmethode für Patienten die an einer wiederkehrenden Darminfektion mit *Clostridium difficile* leiden und birgt auch therapeutisches Potenzial als alternative Behandlung für andere chronische Magen-Darm-Erkrankungen. Eine stuhlbasierte Therapiestrategie wirft jedoch einige Bedenken auf, wie etwa ein potenzielles Infektionsrisiko, die Übertragung unerwünschter Phänotypen, begrenzte Standardisierungs-möglichkeiten und eine geringe Akzeptanz bei Patienten. Die genannten Restriktionen führten zur fortwährenden Entwicklung synthetischer Bakteriengemische, um die Verabreichung von Fäkalien zu vermeiden. Darmfermentationstechnologien (IFT) ermöglichen eine kontrollierte und stabile Produktion von «künstlicher» Darmmikrobiota in hoher Zelldichte und grossen Mengen. Eine uneingeschränkte Verfügbarkeit von Bakterien-basierenden Therapeutika kann jedoch nur gewährleistet werden, wenn sie ohne Wirksamkeitsverlust gelagert werden können. Folglich besteht eine starke Nachfrage nach anaeroben Konservierungstechnologien, die die bakterielle Zusammensetzung und Funktionalität gewährleisten. Bestehende Protokolle wurden hauptsächlich für sauerstofftolerante Reinkulturen entwickelt, während die Konservierung von strikt Anaerobe und komplexen Kulturen weitgehend vernachlässigt wurde. Das übergeordnete Ziel dieser Arbeit war daher die Entwicklung und Validierung von anaeroben Konservierungstechniken zur Bewahrung der Zusammensetzung und Funktionalität von komplexer anaerober Mikrobiota, die mit Darmfermentationstechnologie produziert wurde.

Die erste Studie (Kapitel 2) zielte darauf ab, die Auswirkung eines penetrierenden (Glycerol) und nicht penetrierenden Kryoprotektors (Inulin) auf die metabolische Aktivität einer mit IFT produzierten und kryokonservierten künstlichen Darmmikrobiota zu untersuchen. Darüber hinaus konzentrierten wir uns auf die Auswirkungen von Kryokonservierung auf das Wachstum von den Markerbakterien *Faecalibacterium prausnitzii*, *Eubacterium hallii* und der *Roseburi*a spp./*Eubacterium rectale* Gruppe innerhalb der komplexen Mikrobiota. Künstliche Darmmikrobiota wurde für 3 Monate bei -80°C in Glycerin, Inulin und einer Kombination davon kryokonserviert. Um die Metabolitenbildung mittels Hochleistungsflüssigkeitschromatographie (HPLC) zu messen und das Wachstum der ausgewählten Bakteriengruppen mittels molekularen Methoden (qPCR) zu bestimmen, wurde frische und kryokonservierte Darmmikrobiota in anaeroben Batchkulturen reaktiviert. Wir konnten aufzeigen, dass die Kombination von Glycerol und Inulin einen höheren Schutz der Darmmikrobiota bietet als wenn die Kryoproteprotektoren alleine angewendet wurden. Zudem konnte eine taxaspezifische Wirkung der Kryoprotektoren beobachten werden. Glycerol unterstützt während der Kryokonservierung die *Roseburi*a spp./ *Eubacterium rectale* Gruppe und Inulin das Bakterium *F. prausnitzii*. Das Wachstum von *E. hallii* wurde durch die Kryokonservierung nur geringfügig beeinträchtigt. Zusätzlich hielt die Glycerolzugabe die Butyrat- und Propionatproduktion aufrecht und führte in Kombination mit Inulin zu einer schnelleren Butyrat- und Acetatbildung.

Um die Wirkung von Kryokonservierung auf die ausgewählen Butyratproduzenten in einer komplexen Mikrobiota besser zu verstehen, haben wir einr Konservierungsstudie mit reinen *F. prausnitzii*, *R. intestinalis* und *E. hallii* Kulturen durchgeführt (Kapitel 3). Die Studie wurde mit dem butyratproduzierenden Darmbakterium *Anaerostipes caccae* und den zwei Propionatproduzenten *Bacteroides thetaiotaomicron* und *Blautia obeum* ergänzt. Die strikt anaeroben Darmbakterien wurden kryokonserviert und für 3 Monate bei -80°C gelagert und auch lyophlisiert mit anschliessender Lagerung bei 4°C. Die Protektoren Saccharose und Inulin wurden im Lyophilisierungsprozess verwended und zur Kryokonservierung mit Glycerol kombiniert. Fitness, Viabilität und Membranintegrität dienten als Marker für Konservierungsbeständigkeit und wurden in standardisierten Wachstumstests und mittels Durchflusszytometrie ermittelt. Gefriertrocknen war im Allgemeinen schädlicher für Viabilität und Fitness als Einfrieren, führte jedoch zu einer besseren Stabilität während der Lagerung. Grosse Viabilitätsunterschiede zwischen den getesteten Bakterien deuteten auf eine starke speziesabhängige Toleranz gegenüber Einfrieren und Gefriertrocknen hin. Die Zusammensetzung der Membran scheint ein ausschlaggebender Faktor für den Konservierungserfolg zu sein, da die Zugabe von den membraninteragierenden Protektoren Saccharose und Inulin zu einer verbesserten Viabilität aller lyophilisierten und von den gefrierempfindlichen Spezies führte. Der positiven Einfluss von Glycerol auf die Viabilität von *R. intestinalis* stimmte mit der unterstützenden Wirkung auf die *Roseburia* spp./*E. rectale* Gruppe überein, wenn kryokonserviert als Teil einer komplexen Darmmikrobiota. Die aktuellen Daten deuten auch darauf hin, dass eine erhöhte Wachstumsrate von *E. hallii* nach Kryokonservierung seine Wettbewerbsfähigkeit in der Batch-Fermentation erklären könnte, da wir zuvor berichtet haben, dass *E. hallii* durch das Einfrieren innerhalb einer komplexen künstlichen Darmmikrobiota kaum beeinträchtigt wurde.

In einem letzten Schritt untersuchten wir den mikrobiellen Lebensstil als Einflussfaktor auf die Stresstoleranz komplexer Mikrobiota gegenüber Konservierung. Das PolyFermS-System liefert neben planktonischer Mikrobiota auch Mikrobiota von sessilem Lebensstil, eingebettet in Polymerkügelchen, die einen biofilm-assoziierten Zustand imitieren. Bakterien im Biofilm weisen in der Regel eine höhere Stresstoleranz auf als planktonische Zellen. Wir haben daher angenommen, dass die von PolyFermS gewonnene sessile Mikrobiota resistenter gegen konservierungsbedingte Stress ist als die planktonische Mikrobiota (Kapitel 4). In einem ersten Schritt charakterisierten wir die Zusammensetzung der planktonischen und sessilen Mikrobiota bevor wir sie bei -80°C kryokonservierten oder lyophilisierte mit anschliessenden Lagerung für 9 Monate bei 4°C. Die mikrobielle Zusammensetzung und die metabolische Aktivität in Batchkulturen wurden vor und nach der Konservierung mit Hilfe von HPLC-, Molekular- und Hochdurchsatz-Sequenzierungsmethoden untersucht. Wir konnten aufzeigen, dass der Lebensstil ein entscheidender Faktor in der Etablierung der mikrobiellen Zusammensetzung und der metabolischen Aktivität der künstlichen Darmmikrobiota

ist. Die sessile Mikrobiota besass eine höhere metabolische Aktivität und beherbergten eine einzigartige Gemeinschaft von Bakterien und Archeen, die sich von der planktonischen Mikrobiota unterschied. Überraschenderweise haben wir auch gezeigt, dass die planktonische Microbiota die mikrobielle Zusammensetzung und die metabolische Aktivität während der Lagerung für 9 Monate besser aufrechterhalten konnte als die sessile Mikrobiota. Unsere Daten deuten darauf hin, dass kooperierende Mikroben eine charakteristische Eigenschaft der sessilen Mikrobiota sein könnten, die die strukturelle Stabilität gegenüber Gefrier- und Gefriertrocknungsstressoren beeinträchtigen könnte. Der konservierungsbedingte Zelltod eines Schlüsseltaxons kann folgedessen zum Verlust der abhängigen Taxa führen. Unabhängig vom Lebensstil identifizierten wir Taxa mit hoher Konservierungempfindlichkeit, darunter mehrere *Bacteroides* sp., aber auch Taxa, wie *Enterococcaceae* und *Peptostreptococcaceae,* die eine grosse Robustheit gegenüber Konservierung aufweisen. Wir ziehen funktionelle Redundanz, beobachtet in den Butyrat-produzierende Gruppen der planktonischen und sessilen Mikrobiota, als eine Eigenschaft in Betracht, die die funktionelle Stabilität während der Einfrierung, Trocknung und Lagerung erhalten könnte. Ist hingegen eine bestimmte Eigenschaft, wie z.B. die Propionatproduktion, auf eine geringe Anzahl von Taxa beschränkt, kann der Verlust von Schlüsseltaxa den Funktionsverlust zur Auswirkung haben. Unsere Daten zeigen die Machbarkeit der Herstellung von komplexer künstlicher Mikrobiota von planktonischen und sessilen Lebensstil mit der kontinuierlichen PolyFermS-Plattform, die mit in Polymerkügelchen immobilisierter Stuhlmikrobiota inokuliert wurde und anschließend durch Einfrieren und Lyophilisieren konserviert wurde.

Zusammenfassend lässt sich sagen, dass diese Dissertation das Wissen über den Einfluss verschiedener Konservierungsmethoden auf die Zusammensetzung und die metabolische Aktivität von künstlicher Darmmikrobiota erweitert hat.

Chapter 1

General introduction

1 Gut microbiota in health and disease

The human gastrointestinal tract features one of the body's largest interfaces between host and external environmental (Farhadi*, et al.*, 2003) and harbors a highly diverse and active microbial community that has coevolved with the host, forming a complex and mutually beneficial relationship (Backhed*, et al.*, 2005). The gut microbiota plays a central role in the human body and extensively interacts with host immune and epithelial cells. It has been long recognized that the large and dynamic microbial community actively contributes to the maintenance of a healthy state and may significantly influence the progression of diseases (Selber-Hnatiw*, et al.*, 2017).

1.1 Composition of the human gut microbiome

The human gut microbiome comprise 10^{14} microorganisms whereof the vast majority is part of the bacterial domain that harbors more than 1000 different species, mainly strict anaerobes, with an average of 160 species per individual (Eckburg*, et al.*, 2005, Gill*, et al.*, 2006, Qin*, et al.*, 2010). The remaining non-bacterial inhabitants of the gastrointestinal tract are methanogenic archaea, such as *Methanosphaera stadtmanae*, *Methanobrevibacter smithii* and *Methanomassiliicoccus luminyensis*, fungi from the phyla Ascomycota and Basidiomycota; and viruses (Dridi*, et al.*, 2012, Lozupone*, et al.*, 2012, Hoffmann*, et al.*, 2013, Kroninger*, et al.*, 2017). Several extensive phylogenetic studies of human fecal, rectal and mucosal samples, predominantly deriving from subjects of the western population, have demonstrated that gut microbiota composition greatly varies between individuals (Claesson*, et al.*, 2009, Human Microbiome Project, 2012, Falony*, et al.*, 2016, Jones*, et al.*, 2018). Claesson*, et al.* (2009) reported that most of the microbial diversity in the human gut occurs at species or strain level. General commonalities among the gut microbiota of healthy adults have mainly been found at higher taxonomic levels, where the high species diversity is restricted to only five representative phyla, namely Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, and Verrucomicrobia. Bacteroidetes and Firmicutes account for more than 90% of the bacterial species while Actinobacteria, Proteobacteria and Verrucomicrobia are less represented (Qin*, et al.*, 2010, Lozupone*, et al.*, 2012, Chassard and Lacroix, 2013).

1.1.1 Classification of the gut microbiota

Each human being harbors a unique intestinal microbiota that can be described according to the number of species (richness) and their relative abundances (evenness) (Gerritsen*, et al.*, 2011). Different attempts have been made to classify the large diversity of the human gut microbiota in distinctive groups, with the intention to define a "healthy" microbiome and identify potential markers associated with a diseased state (Backhed*, et al.*, 2012). Arumugam et al. (2011) reported that the core microbiota is shared between subjects of different countries and discriminated three discrete microbial clusters referred as 'enterotypes'. Each enterotype was characterized by a relatively high representation of *Bacteroides*, *Prevotella* or *Ruminococcus* and by distinct metabolic pathways

(Arumugam, et al., 2011). Later studies found a link between dietary habits and the proposed enterotypes, suggesting that differences in diet is a driving discriminative factor. Accordingly, enhanced intake of animal protein and saturated fat were linked with the *Bacteroides* enterotype, whereas a diet rich in carbohydrates and simple sugars correlated with a *Prevotella* enterotype (Wu*, et al.*, 2011). However, the large inter-individual variation in microbial composition cannot be attributed to a single determinant only. Falony*, et al.* (2016) identified 69 factors that significantly correlated with variation in the overall fecal microbiota composition in Western European population, including medication, health-status, age, gender, host-genetic, lifestyle and diet. Using the same data set but adding samples collected from non-European subjects (Papua New Guinea, Peru and Tanzania), a core microbiota, composed of 14 widely shared genera, including *Bacteroides*, *Blautia*, *Roseburia*, *Faecalibacterium*, *Prevotella*, unclassified *Lachnospiraceae*, *Coprococcus*, *Ruminococcus*, unclassified *Ruminococcaceae* and *Alistipes*, was established. It was shown that inter-individual variation in microbiota composition primarily derived from differences in relative abundance of these core taxa. *Ruminococcaceae*, *Bacteroides*, and *Prevotella* exhibited the largest variation in abundance, confirming their use as identifiers of the previously proposed enterotypes (Arumugam*, et al.*, 2011, Falony*, et al.*, 2016).

1.1.2 Definition of a 'healthy' gut microbiome

The gut microbiome provides a multitude of beneficial physiological functions such as supply of vitamins and nutrients from indigestible food components, protection from colonization by pathogens and regulatory effects on metabolism and immune system that are linked to gut homeostasis (Flint*, et al.*, 2012). However, the high inter-individual variability in intestinal microbiota composition makes it difficult to precisely define a microbiome distinctive for intestinal homeostasis. Microbial richness and species diversity in the human gut have been widely considered as an important indicator for health (Walsh*, et al.*, 2014). Backhed*, et al.* (2012) suggested that a 'healthy' microbiome can be described as ecologically stable and therefore resistant to structural changes after stress exposure but also by a distinctive functional profile (Backhed*, et al.*, 2012). Functional activities of the gut microbiota are generally better conserved among healthy adults than the taxonomic composition (Qin*, et al.*, 2010, Human Microbiome Project, 2012). Turnbaugh*, et al.* (2009) proposed a core microbiome at functional rather than compositional level that is shared between individuals, and stated that deviation from the core might result in an adverse physiological state. Metabolism of nutrients and other food components is one of the key functions of the human gut microbiota that greatly affects host health. The connection between metabolic function and intestinal homeostasis has led to the ongoing research to identify microorganism that are involved in metabolic processes and the underlying metabolic pathways (Rowland*, et al.*, 2018)

1.2 Metabolic activity of the gut microbiota

The gut microbiota is involved in many metabolic processes that include the degradation of food components, mainly carbohydrates and proteins that escape digestion in the digestive tract or are not digestible for the host. Humans lack the enzymes to breakdown dietary fibers such as resistant starch or polysaccharides present in plant cell walls. Therefore, these non-digestible carbohydrates withstand the passage through stomach and duodenum but undergo anaerobic fermentation in the cecum and the large intestine by the resident microbiota (Flint*, et al.*, 2012, Portune*, et al.*, 2016).

1.2.1 Bacterial carbohydrate metabolism

It is estimated that a daily amount 20-60 g of dietary carbohydrates does not readily undergo host digestion and therefore reach the proximal colon, where it serves as substrate for bacterial metabolism (Cummings and Macfarlane, 1991, Silvester*, et al.*, 1995). Diet-derived carbohydrates mainly comprise of non-digestible oligosaccharides, resistant starch and plant cell wall polysaccharides, including soluble dietary fibers, such as pectin, arabinoxylan and glucans, but also insoluble fibers like cellulose and lignin. Fermentation of such complex carbohydrates depends on the cooperative activity of different functional groups within the gut microbiota (Flint*, et al.*, 2012, Williams*, et al.*, 2017).

Primary degraders. The microbial degradation of structurally complex oligo- and polysaccharides into smaller sub-units demands for a repertoire of carbohydrate-active enzymes (CAZyme), restricting the initial breakdown to specific primary degraders that possess the required enzymatic 'equipment' (Martens*, et al.*, 2011, El Kaoutari*, et al.*, 2013). Several Firmicutes, such as *Clostridium* spp., *Ruminococcus bromii, Eubacterium rectale* and *Roseburia* spp., can act as primary degraders of resistant starch, mucin and other complex carbohydrates (Leitch*, et al.*, 2007, Ze*, et al.*, 2012). However, members of the phylum Bacteroidetes generally express a much larger repertoire of CAZymes than Firmicutes or members of other gut bacterial phyla and are therefore considered as key functional group in the primary degradation of many complex plant cell wall polysaccharides (El Kaoutari*, et al.*, 2013).

SCFA formation. In a process called cross-feeding, products formed by primary polysaccharide degraders are further converted to secondary metabolites, mainly short-chain fatty acids (SCFA), by bacteria whose degradation capabilities are restricted to mono-, di- or oligosaccharides (Belenguer*, et al.*, 2006, Cockburn and Koropatkin, 2016) (Fig. 1.1). Acetate, propionate and butyrate are the most abundant SCFAs in the colon and feces, and are estimated to be present at a molar ratio of 3:1:1 (Macfarlane, et al., 1992).The formation of SCFAs often involves the production and consumption of intermediate metabolites lactate, succinate and formate (Blaut, 2013). Approximately 95 % of the produced SCFAs are absorbed by the colonic epithelial cells (colonocytes) in the proximal and distal colon, covering 60-70 % of the epithelial energy requirement (Brahe*, et al.*, 2013, den Besten*, et al.*, 2013, Rowland*, et al.*, 2018). In addition to energy provision, SCFAs extensively interact with host cells and are regarded as beneficial for human health (Belcheva*, et al.*, 2015).

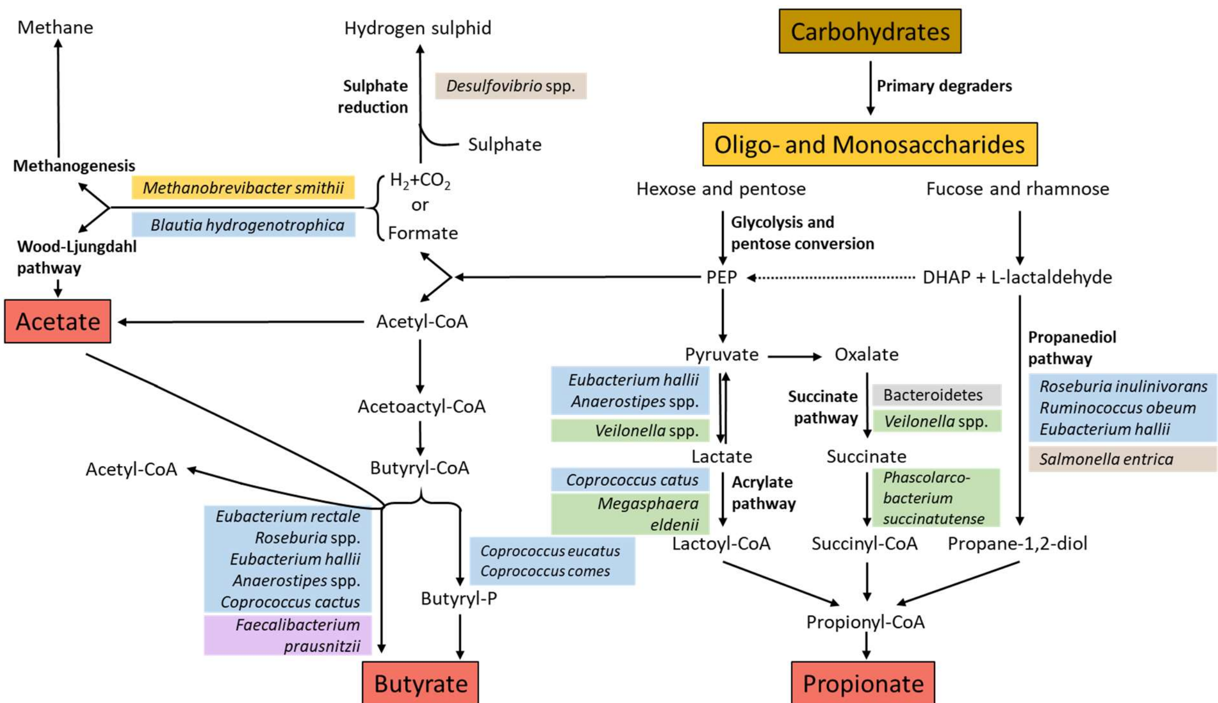

Fig. 1.1 The pathway of carbohydrate metabolism in the gut. Adapted from Flint, et al. (2012) and Rowland, et al. (2018)

1.2.1.1 Acetate formation

The ability to produce acetate is widely distributed among intestinal microbes (Morrison and Preston, 2016). Moreover, several gut bacteria require acetate for growth. *Faecalibacterium prausnitzii*, for example, only grows in pure culture if acetate is present (Duncan, *et al.*, 2004), while degradation of oligofructose by *Roseburia intesitinalis* was only detected in coculture with the acetate-producing *Bifidobacterium longum* (Falony, *et al.*, 2006). Such bacterial cross-feeding greatly impacts final SCFA balance in the gut but also determines the efficiency of substrate breakdown by human colonic microbiota (Rios-Covian, *et al.*, 2016). Acetate derived from microbial fermentation also serves as substrate for de novo lipogenesis in colonocytes. Much of the fatty acids produced this way are integrated in phospholipids and are therefore an important component in the synthesis of new cellular membranes (Zambell, *et al.*, 2003). Furthermore, acetate directly affects host lipid metabolism by stimulating leptin secretion by adipocytes that regulates the energy balance through hunger inhibition (Zaibi, *et al.*, 2010)

1.2.1.2 Butyrate formation

Butyrate can be synthesized by two different pathways involving either the enzyme butyryl-CoA: acetate CoA-transferase or the enzyme butyrate kinase. Thereof the butyryl-CoA: acetate pathway is predominant in the human gut microbiota and requires net consumption of acetate for butyrate production (Vital, *et al.*, 2013, Louis and Flint, 2017). Butyrate-producing species belong to the two predominant families of human colonic Firmicutes, *Ruminococcaceae* and *Lachnospiraceae*, but are

also found in other Firmicutes families, including *Erysipelotrichaceae* and *Clostridiaceae* (Barcenilla*, et al.*, 2000, Louis and Flint, 2009). *F. prausnitzii* (*Clostridium* cluster IV) and *Eubacterium rectale/Roseburia* spp. (*Clostridium* cluster XIVa) are considered as the most important butyrate-producers in the human intestine (Mokhtari*, et al.*, 2017). Being the key energy source for the colonocytes, the primary site of butyrate secretion in the human body is the gut epithelium (van der Beek*, et al.*, 2015). Butyrate elicits several health-promoting effects. It is involved in anti-inflammatory processes (Saemann*, et al.*, 2000, Segain*, et al.*, 2000, Mowat and Agace, 2014) and as a histone deacetylase inhibitor it is associated with anti-cancer activity by inducing inhibition of cell growth and differentiation into colonic cancer (Blouin*, et al.*, 2011, Goncalves and Martel, 2013, Foglietta*, et al.*, 2014). Additionally, there is evidence that butyrate can activate intestinal gluconeogenesis through a cAMP-dependent mechanism resulting in beneficial effects on glucose and energy homeostasis (De Vadder*, et al.*, 2014).

1.2.1.3 Propionate formation

Three different pathways are used by gut microbes to produce propionate, namely the succinate, acrylate and propanodiol pathway (Reichardt*, et al.*, 2014). Thereof two pathways depend on cross-feeding from either succinate or lactate. In the succinate route, present in Bacteroidetes and some Firmicutes of the Negativicutes class, succinate is converted to propionate (Reichardt*, et al.*, 2014). Correlation of propionate concentrations in fecal samples with relative abundance of Bacteroidetes suggests that the succinate pathway is the dominant route within the gut microbiota (Salonen*, et al.*, 2014). In the acrylate pathway lactate is used as a substrate to form propionate and is restricted to a few species of the families *Lachnospiracea* and *Veillonellaceae* (Louis and Flint, 2017). The conversion of deoxy-sugars to propionate characterizes the propanodiol pathway, present in Proteobacteria and species of the *Lachnospiraceae* family, such as *Eubacterium hallii* that can produce both butyrate and propionate as fermentation end metabolites (Reichardt*, et al.*, 2014, Engels*, et al.*, 2016, Louis and Flint, 2017). Similar to butyrate, propionate serves as fuel for epithelial cells and can activate intestinal gluconeogenesis through a mechanism complementary to butyrate. It converts into glucose in the intestinal gluconeogenesis, that directly promotes energy homeostasis by controlling the hepatic glucose production, and consequently reduces adiposity (De Vadder*, et al.*, 2014).

1.2.2 Bacterial protein metabolism

In contrast to the predominantly saccharolytic nature of the proximal colon, microbial fermentation of proteins primarily occurs in the distal part as carbohydrates are mostly depleted after transition through the proximal and transverse colon (Macfarlane*, et al.*, 1992, Macfarlane and Macfarlane, 1993). In addition to the already present endogenous protein, it is estimated that the colon receives approximately 3-12 g dietary proteins and peptides per day that can be used by the distal colon microbiota (Smith and Macfarlane, 1998, Blaut, 2013). However, only 1% of the intestinal microbiota account for amino acid-degrading bacteria (Smith and Macfarlane, 1998, Dai*, et al.*, 2010). In a first

degradation step, proteins are hydrolyzed by extracellular bacterial proteases and peptidases into oligopeptides and amino acids (Macfarlane*, et al.*, 1986, Portune*, et al.*, 2016). Proteases are produced by many common gut microbes, including *Clostridium*, *Bacteroides* and *Lactobacillus* spp. (Scott*, et al.*, 2011, Pessione, 2012). Amino acids, obtained through proteolytic activity, can be further metabolized into SCFAs, branched chain fatty acids (BCFAs), ammonia, amines, polyamines, hydrogen sulfide, thiols, phenols, indoles and gases (Blaut, 2013) (Fig. 1.2).

SCFAs. Butyrate and propionate do not only arise from carbohydrate degradation but also result from amino acid metabolism (Louis and Flint, 2017). *In vitro* incubations of human fecal microbiota with single amino acids demonstrated that propionate was preferably formed from aspartate, alanine, threonine and methionine, whereas butyrate was a major fermentation product of glutamate, lysine, histidine, cysteine, serine and methionine (Smith and Macfarlane, 1997). Thereof glutamate fermentation routes are well understood while molecular pathways involved in the production of SCFAs from other amino acids are less defined. Glutamate metabolization can be found in some butyrate-producing Firmicutes, such as *Acidaminococcus fermentans, Clostridium symbiosum*, *Fusobacterium* spp. and *Peptostreptococcus asaccharolyticus*, but also in the *Acidaminococcaceae* family with propionate rather than butyrate as metabolic endproduct (Louis and Flint, 2017). Proteolytic activity has also been observed in some Bacteroidetes members that form propionate out of peptides (Smith and Macfarlane, 1998).

BCFAs. Branched-chain fatty acids such as iso-butyrate and iso-valerate are produced by microbial fermentation of the branched-chain amino acids valine, leucine and isoleucine and are far less abundant in the luminal content than SCFAs (Liu*, et al.*, 2016). BCFA production does not involve degradation by human host enzymes and is therefore a unique feature of the microbial metabolism. Williams*, et al.* (2005) suggested BCFAs as marker for *in vivo* and *in vitro* proteolytic activity by colonic microorganisms.

Toxic by-products. *In vitro* studies with human cells and *in vivo* observations have shown that some products of bacterial protein fermentation, such as H_2S, aromatic compounds, polyamines and ammonia are potentially toxic for the host (Portune*, et al.*, 2016).

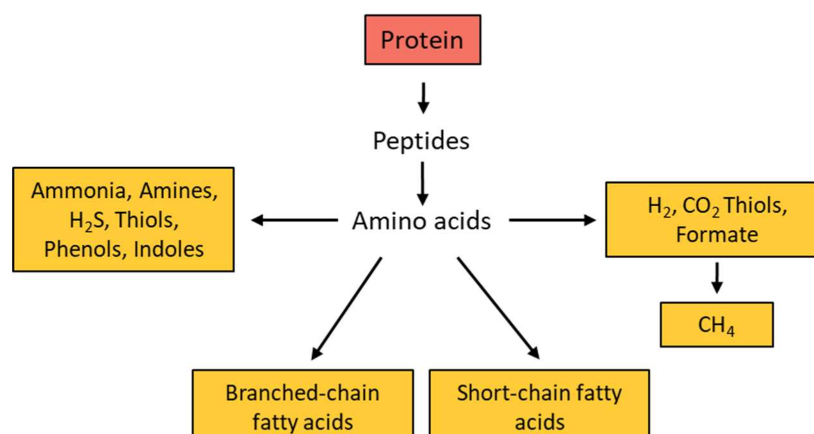

Fig. 1.2 Bacterial breakdown of proteins. Adapted from Blaut *et al*. (2013)

1.3 Gut microbiota in intestinal dysbiosis

Intestinal dysbiosis is termed as an ''imbalance in a microbial ecosystem characterized by a shift in the composition or function of microbes, which can result in pathogenesis'' (Carlucci*, et al.*, 2016). Various environmental factors, including diet, toxins, drugs, antibiotics, pathogens and diseases can trigger changes in the gut microbiota possibly leading to dysbiosis (Carding*, et al.*, 2015). Reduced bacterial diversity has been linked to a number of conditions related to metabolic, immune- and inflammatory disorders (Claesson*, et al.*, 2012, Le Chatelier*, et al.*, 2013).

1.3.1 Intestinal dysbiosis in *Clostridium difficile* infection

Clostridium difficile is an anaerobic, spore-forming and toxin-producing, opportunistic pathogen that can provoke nosocomial, antibiotic-associated diarrhea mediated by the secretion of multiple toxins (Abt*, et al.*, 2016, Schaffler and Breitruck, 2018). In healthy subjects, the indigenous gut microbiota exerts colonization resistance against *C. difficile* and therefore prevents its pathogenic activity in the gastrointestinal tract (Theriot*, et al.*, 2014). However, application of broad-spectrum antibiotics can result in disruption of the host-microbiota homeostasis by decreasing microbiota diversity, abundance of predominating taxa and thus opening niches for the establishment of *C. difficile* (Antonopoulos*, et al.*, 2009, Theriot*, et al.*, 2014). *C. difficile* colonization is further facilitated by antibiotic-induced alteration in metabolic functions of the gut microbiota, in particular the bile acid metabolism. Primary bile acids enhance while secondary bile acids inhibit growth of *C. difficile*. Consequently, an antibiotic-induced colitis that hampers secondary bile acid formation can promote *C. difficile* infection (CDI) (Theriot*, et al.*, 2016). The standard treatment of CDI involves the antibiotics metronidazole, fidaxomicin or vancomycin, which kill vegetative *C. difficile* cells, providing remission from symptoms and ideally restoring initial intestinal homeostasis (McDonald, et al., 2018). However, presence of *C. difficile* endospores or the infection with a new strain can lead to recurrence of CDI in 25–30% of the patients (Cornely*, et al.*, 2012, Abt*, et al.*, 2016, Guery*, et al.*, 2018). Recurrent CDI is a major clinical challenge, demanding for alternative therapies in cases where patients do not respond to the standard antibiotic treatment (Carlucci*, et al.*, 2016). Several studies observed taxonomic changes in the gut microbiota of recurrent CDI subjects, characterized by a decrease in overall phylogenetic richness and reduced fecal Bacteroidetes and Firmicutes along with an increase in Proteobacteria and an over-representation of opportunistic pathogens compared to healthy subjects (Chang*, et al.*, 2008, Antharam*, et al.*, 2013, Milani*, et al.*, 2016). Compositional alterations on lower taxonomic levels markedly differ between distinct patient cohorts likely due to the inter-individual variability in the intestinal microbiota (Carlucci*, et al.*, 2016).

1.3.2 Intestinal dysbiosis in inflammatory bowel disease

The term inflammatory bowel disease (IBD) summarizes a group of chronic immune-mediated inflammatory conditions that affect the gastrointestinal tract, including the two principal types of IBD, Crohn's disease (CD) and ulcerative colitis (UC). CD is characterized by a patchy and transmural

inflammation that can affect any part of the gastrointestinal tract. In contrast, the mucosal inflammation process distinctive for UC is normally continuous, starting in the rectum and extends to the colon (Lane*, et al.*, 2017). Recent studies suggest that IBD is initiated by a complex combination of several causative factors such as host genetics, dysregulation of the immune system and environmental triggers that may alter the composition and function of the intestinal microbiota, further reinforcing IBD progression (Ni*, et al.*, 2017). Coherently, IBD is clearly associated with intestinal dysbiosis but no specific microbe or microbial pattern has been proven to be causal. However, individuals suffering from IBD show reduced bacterial diversity and decreased abundances in certain taxa, including Firmicutes and Bacteroidetes compared to healthy subjects (Morgan*, et al.*, 2012, Gevers*, et al.*, 2014). Additionally, specific taxonomic shifts have been associated with IBD, including a relative increase in the abundance of *Enterobacteriaceae*, such as *Escherichia coli* and *Fusobacterium* (Ohkusa*, et al.*, 2002, Darfeuille-Michaud*, et al.*, 2004, Lupp*, et al.*, 2007). The association between intestinal dysbiosis and UC or CD leads to the increasing interest in the development of microbiome-based therapeutics for such inflammatory conditions (Carlucci*, et al.*, 2016).

1.3.3 Intestinal dysbiosis in metabolic syndrome

Metabolic syndrome is a combination of several medical conditions including abdominal obesity, atherogenic dyslipidemia, high blood pressure, insulin resistance with or without glucose intolerance, proinflammatory and prothrombotic states that my result in cardiovascular disease and type 2 diabetes as primary clinical outcome (Grundy*, et al.*, 2004). Individuals that meet three of these criteria are diagnosed with metabolic syndrome and are very likely to exhibit excessive fat accumulation (Boulange*, et al.*, 2016). Obesity is a severe public health problem that expanded to epidemic level in several industrialized countries (Engin, 2017). High caloric food intake is considered as major factor in the development of obesity, however, intestinal microbes have also been shown to influence host nutrient uptake and energy regulation and should not be neglected as contributors in the development and progression of metabolic syndrome and obesity-related disorders (Boulange*, et al.*, 2016). Several studies with obese animal and human subjects found differences in composition and activity of the gut microbiota compared to the lean control groups, characterized by reduced diversity and altered colonic fermentation profiles (Vrieze*, et al.*, 2012, Le Chatelier*, et al.*, 2013, Murri*, et al.*, 2013, Ridaura*, et al.*, 2013, Fernandes*, et al.*, 2014). In a metagenomics study, Le Chatelier*, et al.* (2013) classified the gut microbiota into two groups according to gene richness. High gene count microbiota, mainly observed in lean subjects, were associated with the butyrate-producing bacterium *F. prausnitzii* and therefore enhanced butyrate levels while low gene counts microbiota were mostly found in obese subjects, correlating with decreased butyrate formation and increased *Bacteroides* spp. and *Ruminococcus gnavus* levels (Le Chatelier*, et al.*, 2013). Diet-induced weight loss in the low gene count group could partially reverse these metabolic alterations, demonstrating the highly dynamic response of the intestinal microbiota to dietary changes (Cotillard*, et al.*, 2013).

1.3.4 Intestinal dysbiosis and colorectal cancer

Colorectal cancer (CRC) is the third most diagnosed malignancy and one of the leading cause of cancer mortality in the world (Arnold*, et al.*, 2017). Case–control and cohort studies implicated a link between diet, physical activity and the risk of cancer development. Accordingly, enhanced consumption of red and processed meat increased the risk while a diet rich in dietary fiber reduced the probability to develop CRC (Roncucci and Mariani, 2015). However, several studies also pointed towards a causal relationship between dysbiosis of intestinal microbiota and CRC (Gagniere*, et al.*, 2016). It is generally assumed that gut microbes are directly involved in colorectal carcinogenesis by inducing inflammatory responses or producing genotoxic compounds and superoxide radicals that provoke gene mutations in colonic epithelial cells (Huycke and Gaskins, 2004, Arthur*, et al.*, 2014). Several bacteria with potential carcinogenic effects were found to be overrepresented in CRC patients compared to healthy subjects, including *Fusobacterium nucleatum, Peptostreptococcus stomatis, E. coli, Bacteroides fragilis* and *Enterococcus faecalis* (Balamurugan*, et al.*, 2008, Sobhani*, et al.*, 2011, Yu*, et al.*, 2017). Simultaneously, CRC is associated with a microbial composition that is reduced in or completely lacks beneficial taxa such as *Clostridiales*, *Faecalibacterium*, *Blautia* and *Bifidobacterium* (Baxter*, et al.*, 2014, Zou*, et al.*, 2018). Raskov*, et al.* (2017) suggested therapeutic manipulation of the dysbiotic intestinal microbiota by probiotics, prebiotics or antibiotics as promising tool in the treatment and prevention of CRC.

2 Microbial-based therapeutics for modulating the gut microbiome

The tremendous research effort of the last few years in the field of the human gut microbiome has led to a more comprehensive understanding of its function in several biological processes, such as digestion, immune response and metabolism. The numbers of gastrointestinal conditions that have been connected to alterations in the gut microbiota are increasing. Microbial interventions in the human microbiome have therefore emerged as novel treatments for a wide range of gastrointestinal and non-gastrointestinal diseases (Sun*, et al.*, 2016). The main strategies to modulate the microbiome go beyond the administration of the commercially available probiotics and range from single strain approaches to defined consortium administration, to ecosystem-level intervention with fecal microbiota transplants (Olle, 2013).

2.1 From probiotics to biotherapeutics

2.1.1 Classical probiotics

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill*, et al.*, 2014). Health benefits have mainly be demonstrated for specific probiotic strains from the genera *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Streptococcus*, *Pediococcus, Leuconostoc*, *Bacillus* and *E. coli*. Thereof *Bifidobacterium* and *Lactobacillus* strains are the most extensively commercialized probiotics with shown health effects in preventing antibiotic-associated diarrhea and constipation (Fijan, 2014). In traditional probiotic treatment, bacteria are incorporated into food matrices and delivered to the gastrointestinal tract upon ingestion by the consumer. The most evaluated food carriers for probiotic administration include fermented milk and yogurts, cheese, sausages, ice-creams, fruit and vegetable juices, oats and cereals (Flach*, et al.*, 2017). Lyophilized probiotic bacteria with enhanced shelf life are increasingly used to supplement infant formula to improve general “well-being” of infants (Braegger*, et al.*, 2011).

2.1.2 Next-generation probiotics

Recently, the focus in probiotic research moved from the classical probiotics, administered via food delivery route or as supplement, to a targeted manipulation of the host gut microbiota with “personalized probiotic therapies” using functional important gut microbes (O'Toole*, et al.*, 2017). Based on the health promoting effect of butyrate as well as on the observed decline of the butyrate-producing *Clostridium* clusters IV and XIVa in IBD patients, Van Immerseel*, et al.* (2010) proposed butyrate-producing anaerobic gut bacteria as novel probiotic approach in the treatment of IBD. The increasing knowledge of the composition and functional properties of the human intestinal microbiota further extended the numbers of microorganisms with potential beneficial health effects (O'Toole*, et al.*, 2017). The idea to administer specific bacterial strains to address distinct differences in colonic microbiota profiles associated with intestinal diseases goes beyond the traditional probiotic term (Vieira, et al., 2016). Next-generation-probiotics however fit well in the FDA definition of live

biotherapeutics (O'Toole, *et al.*, 2017), termed as " biological product that: (1) contains live organisms, such as bacteria; (2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and (3) is not a vaccine" (FDA, 2016). Several strictly anaerobic gut microbes are currently under investigation as candidates for next-generation probiotics, including members of the *Clostridium* cluster XIV, such as *F. prausnitzii*, *Eubacterium hallii* and species from the genera *Roseburia* and *Bacteroides* sp. (El Hage, *et al.*, 2017, Tamanai-Shacoori, *et al.*, 2017). The probiotic potential and distinct beneficial properties of these four promising targets will be described in the following sections.

2.1.2.1 *Faecalibacterium prausnitzii*

F. prausnitzii is a highly abundant butyrate-producing gut microbe belonging to the *Clostridium* cluster XIV. It is a gram-positive, non-spore-forming, non-motile, strict anaerobe (Duncan, *et al.*, 2002, Martin, *et al.*, 2017), present in fecal microbiota of healthy human adults at levels of 2-15% (Louis and Flint, 2009). *F. prausnitzii* is considered as key butyrate-producer in the human colon, playing a crucial role in the energy supply to the colonocytes but also exerts anti-inflammatory properties that are important for intestinal health (Ferreira-Halder, *et al.*, 2017). Consequently, decreased abundance of *F. prausnitzii* has been associated with several pathologies involving gut inflammation. In terms of IBD, fecal and mucosa-associated microbiota in CD patients contain reduced proportions of *F. prausnitzii* compared to healthy subjects while individuals suffering from UC feature lower amounts thereof during active course of the disease than during periods of no symptoms (Machiels, *et al.*, 2014, Quevrain, *et al.*, 2016). Additionally, depletion of *F. prausnitzii* was also observed in CRC (Balamurugan, *et al.*, 2008, Konstantinov, *et al.*, 2013) and in type 2 diabetics (Karlsson, *et al.*, 2013). Until now, two mechanisms have been described by which *F. prausnitzii* exerts its anti-inflammatory activity. First, it possesses the ability to directly interact with the host immune system by inducing the *Clostridium*-specific interleukin-10-secreting regulatory T-cell subset that is present in different colonic cells (Quevrain, *et al.*, 2016). The second mechanism involves the secretion of the Microbial-Anti-Inflammatory Molecule (MAM) that is able to block NF-κB activation and reduces production of the proinflammatory cytokines Th1 and Th17, ultimately depressing intestinal inflammation (Sokol, *et al.*, 2008, Quevrain, *et al.*, 2016, Breyner, *et al.*, 2017). The employed anti-inflammatory properties in combination with its butyrate-producing activity highly underscore the therapeutic potential of *F. prausnitzii* in the treatment of disorders associated with chronic gut inflammation (Sokol, *et al.*, 2008).

2.1.2.2 *Eubacterium hallii*

E. hallii is a highly prevalent member of the *Clostridium* cluster XIV that is capable of metabolizing a broad range of substrates (Duncan, *et al.*, 2004). It produces butyrate as major fermentation product but also contributes to the intestinal propionate formation (Duncan, *et al.*, 2004, Engels, *et al.*, 2016). *E. hallii* might be an important player in the maintenance of the host-gut microbiota homeostasis due to its flexibility in substrate utilization and its versatility in SCFA production (Engels, *et al.*, 2016).

E. hallii has been considered for improving the metabolic status in patients diagnosed with metabolic syndrom. Vrieze*, et al.* (2012) observed enhanced *E. hallii* levels accompanied by increased fecal butyrate in subjects with metabolic syndrom after fecal microbiota transplantation from lean donors that resulted in increased insulin sensitivity. These observation were confirmed *in vivo* by direct oral administration of *E. hallii* in an obese and type 2 diabetic mouse model (db/db) that also resulted in increased energy metabolism and improved insulin sensitivity (Udayappan*, et al.*, 2016). The exact underlying protective mechanism employed by *E. hallii* is still unclear. However, Udayappan*, et al.* (2016) suggested that *E. hallii* administration modulates the function of the intestinal microbiome and that microbial metabolites, such as butyrate, contribute to the improved metabolic conditions. Accordingly, daily oral supplementation with butyrate has previously been found to beneficially affect insulin resistance and dyslipidemia in diet-induced obese mice (Gao*, et al.*, 2009). Reduced levels of butyrate-producing bacteria, characteristic for insulin resistant subjects highlight the therapeutic potential of replenishing underrepresented intestinal bacterial strains in the treatment of human insulin resistance (Karlsson*, et al.*, 2013, Le Chatelier*, et al.*, 2013).

2.1.2.3 *Roseburia* spp.

The genus *Roseburia* includes the five species *R. intestinalis*, *R. hominis*, *R. inulinivorans*, *R. faecis* and *R. ceciola* that are highly abundant in the gut of healthy subjects, accounting for 3-15% of the intestinal microbiota (Duncan*, et al.*, 2006, Tamanai-Shacoori*, et al.*, 2017). *Roseburia* spp. are considered key members of the colonic butyrate-producing community and employ anti-inflammatory properties (Mokhtari*, et al.*, 2017, Zhu*, et al.*, 2018). Reduced abundances of *Roseburia* spp. have been linked to several intestinal disorders, including the two types of IBD, metabolic syndrome and CRC (Tamanai-Shacoori*, et al.*, 2017). Accordingly, decreases in fecal butyrate in combination with a reduction in *R. hominis* and *R. intestinalis* defined dysbiosis in patients suffering from UC and was related to disease activity (Kumari*, et al.*, 2013, Machiels*, et al.*, 2014). In patients with ileal CD, reduction or disappearance of *Roseburia* spp. has been observed as a key change in the intestinal microbiota (Willing*, et al.*, 2010, Chen*, et al.*, 2014). Reduced abundances in *Roseburia* has also been reported for the gut microbiota of CRC (Wang*, et al.*, 2012) and in type 2 diabetic patients compared to normal glucose tolerant subjects (Qin*, et al.*, 2012, Zhang*, et al.*, 2013). Until now, little is known about the protective properties exerted by *Roseburia*. Butyrate, in particular, has been suggested as key factor in disease prevention by *Roseburia* (Tamanai-Shacoori*, et al.*, 2017). A recent study investigated the anti-inflammatory effects of *R. intestinalis* in colorectal colitis *in vitro* and *in vivo* and found an inhibition of the interleukin-17 secretion and an induction in the differentiation of T-regulatory cells (Zhu*, et al.*, 2018). Still, the detailed mechanisms through which *R. intestinalis* mediates cytokine secretion and T-cell differentiation need to be investigated. However, the study demonstrated the potential of *R. intestinalis* as probiotic candidate for the treatment or prevention of IBD.

2.1.2.4 *Bacteroides* sp.

Representatives of the genus *Bacteroides* are commensal gut bacteria that represent 20-40% of the intestinal bacterial population (Hong, *et al.*, 2008). Thereof, *Bacteroides fragilis, Bacteroides uniformis* and *Bacteroides acidifaciens* have shown to employ immunomodulatory properties and exhibited *in vivo* activity in preventing detrimental metabolic conditions either by direct host–bacteria interaction, or by the production of effector molecules (El Hage, *et al.*, 2017). *B. fragilis* secretes an immunomodulatory component called polysaccharide A (PSA) that can activate the T-cell dependent immune responses, involved in the establishment and homeostasis of the host immune system (Troy and Kasper, 2010). *B. fragilis* also uses PSA signaling to regulatory T-cells for activation of Toll-like receptor pathways that enhances immunologic tolerance (Round, *et al.*, 2011). PSA is therefore an important component in the maintenance of the balance between different T-cell types.

B. uniformis CECT 7771 was originally isolated from stool samples of healthy breastfed infants (Sanchez, *et al.*, 2011). Oral administration of *B. uniformis* CECT 7771 in high fat diet-fed mice improved lipid profile, decreased insulin and leptin levels, enhanced tumor necrosis factor α production by dendritic cells in response to the stimulation by lipopolysaccharide, and increased phagocytosis (Cano, *et al.*, 2012). Similarly, *B. acidifaciens* has been found to prevent obesity and improve insulin sensitivity in mice (Yang, *et al.*, 2017). Both species may therefore have probiotic potential in the treatment of metabolic diseases such as diabetes and obesity. However, selected strains are still at the very early stage of mechanistic investigation. An initial safety assessment has been performed with *B. uniformis* CECT 7771 in mice, demonstrating that ingestion does not adversely affect general health or food intake nor biochemical indicators of liver, kidney and pancreatic function or gut mucosal histology. Bacterial cells also did not translocate to blood, liver or mesenteric lymph nodes (Fernandez-Murga and Sanz, 2016). Nevertheless, further studies are required to confirm tolerability and safety in longer studies in rodents and ultimately in humans.

2.2 From fecal microbiota transplantation towards defined microbial-based products

2.2.1 Fecal microbiota transplantation (FMT)

Fecal microbiota transplantation (FMT) aims to replace or reinforce the gut microbiota of a patient with the stool microbiota from a healthy donor. Nowadays, it is nearly indisputable that FMT is a highly effective therapy for recurrent CDI, demonstrated in the resolution thereof (van Nood, *et al.*, 2013, Kelly, *et al.*, 2016, Youngster, *et al.*, 2016). The therapeutic effect of FMT is not limited to CDI but might also have a positive impact in the treatment of other gastrointestinal disorders such as inflammatory bowel disease, obesity and metabolic syndrome (Gupta, *et al.*, 2016). However, FMT has several limitations such as an extensive donor screening for pathogens and infectious diseases, the acceptance of patients towards a treatment based on stool and the potential risk of transferring an undesired

phenotype (Petrof and Khoruts, 2014, de Groot*, et al.*, 2017). These restrictions led to the ongoing development of synthetic mixtures of stool-derived microbes and defined microbial consortia to bypass the administration of fecal material.

2.2.2 Defined microbial-based therapeutics

Petrof and Khoruts (2014) defined an ideal biotherapeutic agent as "licensed by regulatory agencies, easily accessible, aesthetically unchallenged, simple to administer, and cost effective." In one of the first bottom-up approaches, dating back to 1989, a bacterial mix of 10 facultative aerobes and anaerobes resolved recurrent CDI in six patients. Strains were isolated from human feces, cultivated individually and diluted in saline prior administration (Tvede and Rask-Madsen, 1989). A recently conducted proof-of-principle study demonstrated the same outcome of curing antibiotic-resistant *C. difficile* colitis by the application of a "stool substitute" containing 33 different bacterial stool isolates such as *F. prausnitzii* and *Roseburia* spp. Again, strains were individually cultured and formulated in predetermined ratios (Petrof*, et al.*, 2013). To date, this approach has only been applied for the treatment of recurrent CDI. However, several biotechnology companies are currently developing microbial-based products for different therapeutic purposes. The most advanced ones with ongoing clinical trials are 4D Pharma, Rebiotix and Seres Therapeutics with products ranging from single bacteria strains to address specific indication, to pre-screening for desired bacterial diversity in fecal samples and bacterial spore cocktails to repopulate the gastrointestinal tract for the treatment of recurrent CDI (Sun, et al., 2016).

2.3 Intestinal fermentation technology for the production of "artificial" colonic microbiota

Intestinal fermentation models are designed for *in vitro* cultivation of complex colonic microbiota that allow investigating the function, composition and microbial interactions of intestinal communities, uncoupled from the host. Moreover, they are also a rich source of microbial material for potential therapeutic application. The top-down approach of using 'artificial' colonic microbiota for therapeutic purposes involves cultivation of complex fecal microbiota in bioreactors prior to in-depth characterization for application. This technique is in contrast to the bottom-up approaches of defined bacterial mixes or consortia where bacteria species are isolated from feces, characterized and reassembled prior application (Tvede and Rask-Madsen, 1989, Petrof*, et al.*, 2013). The setup of intestinal models range from simple batch for short-term fermentations to single or multistage continuous models that enable operation for an extended period of time due to stable substrate supply and washout of toxic byproducts (Payne*, et al.*, 2012). One of the main restrictions of intestinal fermentation models is that they only reproduce free-cell microbes and neglect the biofilm-associated bacterial life style also found in the human gastrointestinal tract (De Weirdt and Van de Wiele, 2015, Li*, et al.*, 2015).

2.3.1 PolyFermS

PolyFermS is an intestinal fermentation technology, mimicking both the planktonic and a particle associated biofilm lifestyle of complex colonic microbiota (Payne, et al., 2012). Fecal microbiota immobilization in mixed polysaccharide gel beads enables growth of sessile bacteria within the porous bead structure, while detached cells continuously released in the surrounding medium, contribute to the seeding and growth of the planktonic community (Cinquin, et al., 2004, Zihler Berner, et al., 2013). This system prevents washout of the less competitive bacteria and produces controlled and stable "artificial" colonic microbiota at high cell density and at large quantity (Fehlbaum*, et al.*, 2015, Lacroix*, et al.*, 2015). Operation conditions can be adjusted to mimick a proximal or distal colon environment, through selection of several parameters: (1) the retention time, a result of the vessel size and the continuous inflow of nutritive medium that simulates adult chyme entering the colon (Macfarlane*, et al.*, 1998); (2) the pH that is controlled by the addition of base and (3) through stirring speed. The PolyFermS system demonstrated stable operation for extended time of several months, maintaining bacterial diversity at high cell densities in continuous intestinal reactors (Cinquin*, et al.*, 2006, Fehlbaum*, et al.*, 2015). The extensive operation time and stability of the system allows an in-depth characterization of the microbial composition and function.

3 Preservation of microbial-based products

Successful usage of microorganisms for therapeutic purposes requires that the cells are in an inactive state prior to their application to ensure long-term stability of the product. Only stable microbial products guarantee widespread availability. Therefore, therapeutic application of human feces-derived microbes or complex microbiota as a whole demands for preservation technologies that warrant composition and functionality of microbial-based products.

3.1 Major long-term preservation methods and their effect on bacterial cells

Freezing (cryopreservation) and freeze-drying (lyophilization) are the two standard techniques for long-term preservation of microorganisms applied by culture collections as well as in industry (Prakash*, et al.*, 2013). However, preservation of bacteria also means exposure to various stresses and neither cryopreservation nor lyophilization guarantees complete recovery of the preserved cells (Kirsop, 1985). The degree of preservation success greatly varies between bacterial species, applied technique, storage temperature and time. Thereof, target microbes and required storage time are the major factors defining the preservation method of choice (Heylen*, et al.*, 2012).

3.1.1 Cryopreservation

The term cryopreservation describes the application of very low temperatures to maintain cell viability over an extended period of time (Pegg, 2015). Biological cell activity declines with decreasing temperature until at -196 °C all biochemical reactions cease due to lack of thermal energy what consequently also blocks diffusion activity and osmotic pressure. Long-term stability of cells can only be guaranteed in absence of liquid water at temperatures below -130 °C where cells are trapped in a glassy matrix (vitrification). Biochemical reactions are substantially reduced in cells stored above -80°C but can still occur in fractions of unfrozen water leading to a gradual loss of viable cells over time (Mazur, 1984). Accordingly, the lower the storage temperature and the exposure to temperature fluctuation, the higher the stability and recovery of viable cells from cryopreserved bacterial cultures (Heylen*, et al.*, 2012).

3.1.1.1 Cellular stresses induced by cryopreservation

Prior storage, bacterial cells need to undergo initial freezing and subsequently return to room temperature for reactivation. Both processes, cooling and thawing, are lethal to cells when unprotected. It is generally presumed that two independent deleterious mechanisms provoke cryoinjuries. Intra- and extracellular ice crystals formed during the cooling process can either directly rupture cellular membranes or indirectly affect cells due to enhanced osmotic pressure generated by solute concentration in the remaining unfrozen fractions (solute effect). Ice crystal formation can be avoided by vitrification, a process where water reaches a glassy state with non-crystalline, solid-like characteristics (Malik, 1991, Pegg, 2015).

Cellular damage caused by enhanced osmotic stress is a typical side effect of the slow-freezing process (Fowler and Toner, 2005). Fonseca*, et al.* (2016) described the physical events occurring during slow-freezing of glycerol protected *Lactobacillus bulgaricus* CLF1 cells. In a first chilling step, membranes undergo lipid phase transition, changing from fluid and disordered phospholipid bilayers to solid and ordered. Further cooling gradually leads to formation of extracellular ice crystals along with concentration of the extracellular matrix, forcing osmotic shrinkage of the supercooled cell. Further dehydration occurs until the intracellular compartment forms a glassy state inhibiting osmotic response to further increase in external hypertonicity (Fig. 1.3). Since all living microorganisms exert a limited tolerance to hyperosmotic pressure (Mille*, et al.*, 2005), slow freezing can ultimately lead to cell death if the osmotic tolerance of the bacteria culture is exceeded.

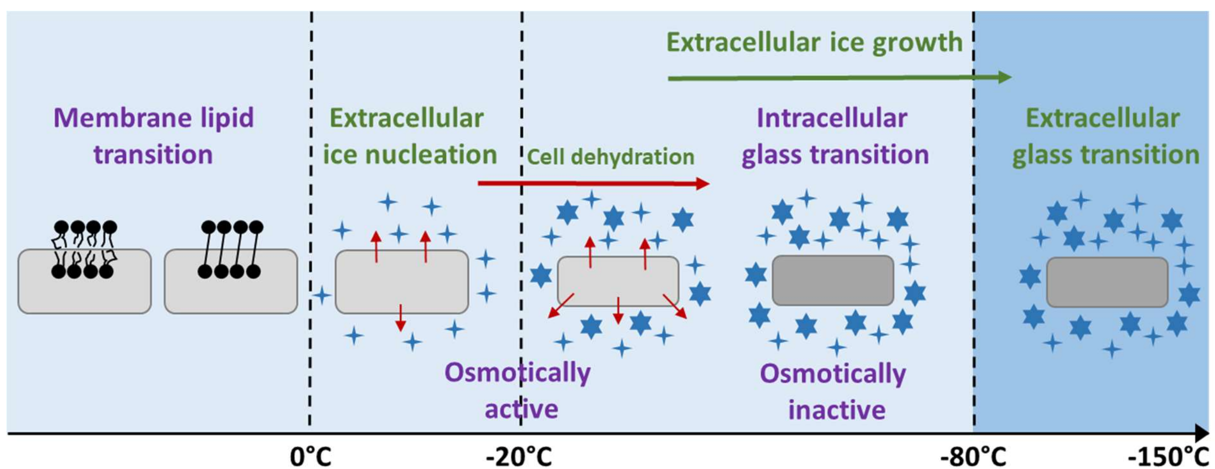

Fig. 1.3 Schematic of the transitions, which occur during cryopreservation of bacterial cells protected with glycerol. Adapted from Fonseca*, et al.* (2016)

An increased freezing rate can prevent a solute effect and concomitant excessive cellular shrinkage. However, cells frozen more quickly are prone to intracellular ice crystal formation (Fowler and Toner, 2005). In the early sixties, Mazur (1963) discovered that freezing rate is an important factor in determining the extend of intracellular freezing, because it also controls the water transport across the membrane. Too fast cooling can result in insufficient dehydration of the cell to avoid intracellular ice crystal formation. Most observations of intracellular ice formation come from investigation of eukaryotes (Fuller, 2004), nevertheless, electron microscopy also provides evidence of intracellular freezing in liquid nitrogen immersed bacterial species *Lactobacillus casei, Leuconostoc mesentroides* and *Lactobacillus delbrueckii* subs. *bulgaricus* suspended in phosphate buffer or distilled water (Albrecht*, et al.*, 1973, Fonseca*, et al.*, 2006). The exact deleterious mechanisms of intracellular ice crystal formation remain unclear, but may derive from mechanical disruption of biological membranes and organelles (Fuller, 2004). Each microorganism exhibits maximal survival at a distinctive freezing rate deriving from a combination of the solute effect and intracellular ice crystal formation (Fig. 1.4). As an example, enhanced freezing rate might reduce exposure to osmotic pressure but in return increases intracellular freezing and therefore diminish survival of the preserved cells (Pegg, 2015).

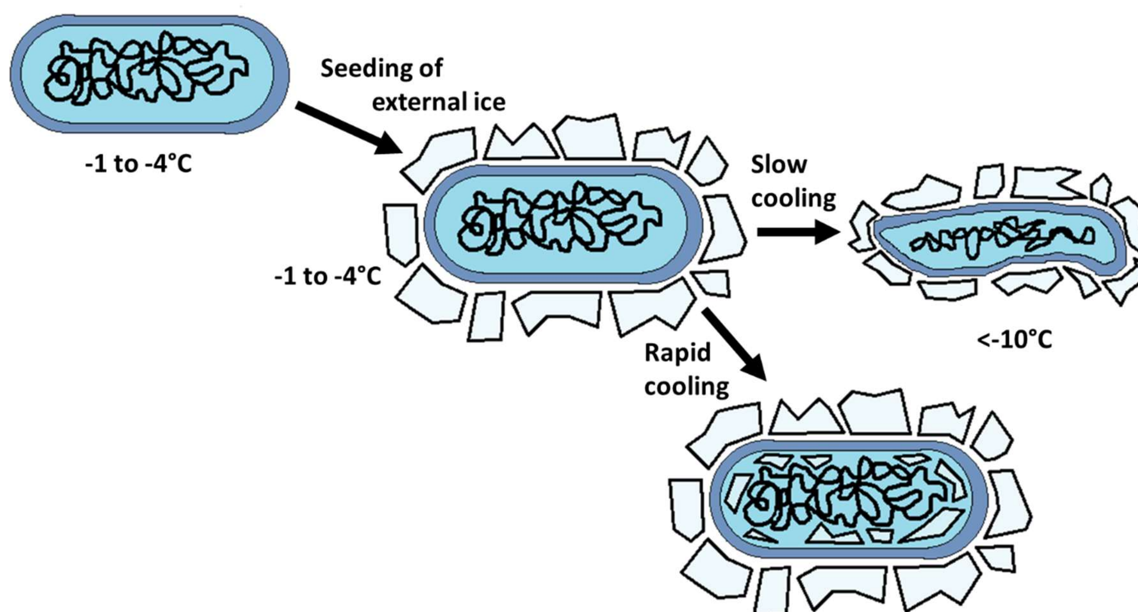

Fig. 1.4 Schema of the key events during freezing of bacteria cells. Typically, the extracellular ice is seeded between -1 and -4°C, and then the cooling process starts (Fowler and Toner, 2005).

3.1.2 Lyophilization

Lyophilization, also referred to as freeze-drying, describes the process of dehydration of a frozen product by sublimation of ice under high vacuum. Sublimation is a phase transition from solid to gas, that occurs at temperatures and pressures below the triple point of water. Water is essential for all biological processes and its removal from bacterial cells ultimately leads to inhibition of biochemical reactions that transfers the cells in a dormant state until reactivated by rehydration (Adams*, et al.*, 2015).

3.1.2.1 Cellular stresses induced by lyophilization

Bacterial preservation by lyophilization demands an initial freezing step, typically executed at -196°C in liquid nitrogen, that is considered one of the main reasons for loss of cell viability during processing (Morgan*, et al.*, 2006). Lyophilized bacterial cells are primarily exposed to the same osmotic and mechanical stresses as bacteria undergoing cryopreservation (Fig. 1.5). It has been reported that 60–70% of the cells that survive initial freezing will also survive dehydration and will remain stable over extended storage periods of 35 years and longer (To and Etzel, 1997, Heylen*, et al.*, 2012). Stresses induced by dehydration substantially differ from freezing stresses (Crowe*, et al.*, 1990). During lyophilization, the gradual removal of water further increases osmotic pressure and can cause severe drying-related injuries (Broeckx, et al., 2016). Water removal from bacterial cells is deleterious to cell wall and cellular components, especially to cytoplasmic membrane and surface proteins (Castro*, et al.*, 1997, Schwab*, et al.*, 2007). Extensive dehydration can cause phase changes in the lipid part of membranes which lead to increased membrane permeability and loss of cellular function (Crowe*, et al.*, 1992).

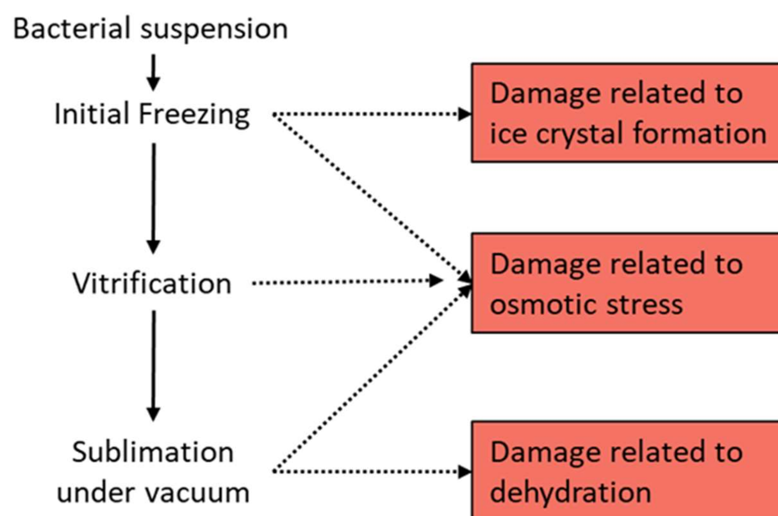

Fig. 1.5 Simplified overview of the process steps of lyophilization. The most important stress factors are indicated in red boxes (Broeckx, et al., 2016).

3.2 Protective agents and its mechanism

The composition of the protective medium is commonly considered as one of the most important factors in bacterial survival of freezing and drying processes (Morgan*, et al.*, 2006, Heylen*, et al.*, 2012). The different deleterious mechanisms involved in cryo- and lyo-injuries require protective agents capable of providing multifunctional protection during cell freezing and drying. The complexity of the preservation induced damages explains why certain organic compounds exhibit greater protective effects than others, and that structural similarity of molecules within the same functional class does not guarantee same protective activity (Fuller, 2004).

Most protectants used in cryopreservation are also protective during lyophilization due to the exposure to freezing-related stresses induced by the required initial freezing step.

3.2.1 Classification of protective agents

Protective compounds are traditionally classified according to their ability to pass through cell walls and cytoplasmic membranes. Some protective agents such as dimethylsufloxid (DMSO) and glycerol penetrate both, cell wall and membrane, whereas mono-and disaccharides, amino acids and low-molecular weight polymers selectively permeate the cell wall but not the membrane, in contrast to high-molecular weight polymers, proteins and polysaccharides which are exclusively non-penetrating (Tao and Li, 1986, Hubalek, 2003). Neglecting the ability to penetrate cell walls and only using diffusion through biological membranes as a discriminant, protective agents can be broadly divided in two groups of either membrane penetrating or non-penetrating compounds (Prakash*, et al.*, 2013).

3.2.2 Penetrating compounds with a focus on glycerol

Penetrating compounds, such as methanol, ethanol, ethylene glycol, dimethylformamide and methylacetamide, diffuse through the plasma membrane and are essential to prevent intracellular damage. Of the mentioned protectants, DMSO and glycerol are the ones most frequently added to

bacterial cell cultures to enhance viability after cryopreservation (Hubalek, 2003). Hubalek (2003) indicated that in a pairwise comparison based on published preservation experiments, glycerol is generally less effective in preserving viability of microorganisms than DMSO. However, in an application point of view, the use of DMSO is not recommended for preservation of microbial-based products with therapeutic purpose since It was shown to be toxic towards eukaryotic mouse cells and neuronal cell lines at unexpected low doses of 1-8% (v/v) (Galvao, *et al.*, 2014). In contrast, glycerol is non-toxic at concentrations required for effective cryopreservation (Meryman, 2007).

3.2.2.1 Protective mechanism of glycerol

Glycerol can prevent slow- and fast-freezing damages to cells by counterbalancing the solute effect (Lovelock, 1953) and restricting the establishment of intracellular ice (Meryman, 2007). The interaction of glycerol with water by hydrogen-bonds prevents ice crystal formation at low temperature so that the mixture remains in a glassy state (Dashnau, *et al.*, 2006). Fonseca, *et al.* (2006) reported the absence of intracellular ice in the rapidly cooled probiotic strain *L. delbrueckii* subsp. *bulgaricus* when pretreated with glycerol at a concentration of 10% (w/w) in contrast to cells suspended in distilled water. Increased viscosity with either decreasing temperature or enhanced molality is another property of the glycerol-water binary system (Weng, *et al.*, 2011). As previously demonstrated, viscosity of the unfrozen solution to which cells are exposed during freezing raises quickly in presence of glycerol what limits the diffusion process and consequently inhibits a potentially damaging osmotic response (Morris, *et al.*, 2006). It is required that glycerol enters the cell in order to exert its full protective effect. Permeable protectants have specific penetration times depending on the permeability of the cell membrane (Ryan and Smith, 2007). When compared to DMSO that penetrates within 5 min, the cell penetration rate of glycerol is with 30 min more slowly and has to be taken into account for effective cryopreservation (Hubalek, 2003, Jiang, *et al.*, 2008).

Although glycerol is very effective in preventing freezing-induced damages, its use is less suitable when bacterial cells are preserved by lyophilization. Glycerol does not readily undergo sublimation during the freeze-drying process and can therefore result in oily or sticky product rather than a dry powder when present in the protective medium (Abadias, *et al.*, 2001, Matejtschuk, 2007).

3.2.3 Non-penetrating compounds

Non-penetrating compounds, such as mono-, di- and polysaccharides, amino acids and proteins, cannot readily enter the cytoplasm of a cell. The protective effect is therefore exerted extracellularly on the cell wall, cytoplasmic membrane and surface proteins (Hubalek, 2003). However, non-penetrating agents are also capable of reducing intracellular ice formation by osmosis-derived shrinkage of the cells prior freezing (Fowler and Toner, 2005). Thus, penetrating protectants are not necessarily superior to no-penetrating protectants in preventing freezing and drying injuries.

3.2.3.1 Protective mechanism of disaccharides

Considering non-penetrating compounds, disaccharides are the far most used protectants in cryopreservation and lyophilization of bacteria. Disaccharides prevent bacterial cells from freezing- and dehydration-related lethal damages.

Cryoprotection. A general cryprotective effect of sugars derives from their osmotic activity. Enhanced sugar concentrations around the cells induce an osmotic response prior to freezing, causing an efflux of water from the cells. Reduced intracellular water consequently limits ice formation during freezing (Fowler and Toner, 2005). Stabilization of cytoplasmic membranes is another mechanism by which disaccharides protect cells from freezing-injuries. As demonstrated previously, trehalose, a disaccharide formed by a 1,1-glycosidic bond between two α-glucose units, stabilizes phospholipid bilayers by inhibiting membrane mixing during freezing and preventing aggregation of intramembranous particles what stabilizes structure and function. It was suggested that trehalose better maintains membrane integrity than the penetrating protectants glycerol or DMSO during rapid freezing in liquid N_2 followed by thawing at room temperature (Rudolph and Crowe, 1985). Due to the underlying differences in the nature of freezing and drying induced stresses, different protective mechanisms come into play when cells are preserved by cryopreservation or lyophilization (Crowe*, et al.*, 1990).

Lyoprotection. Protective mechanisms of disaccharides during drying are far better understood than during freezing and are manifold. The capacity of sugars to replace water by hydrogen bonding with cellular components, prevents denaturation of surface proteins and lyotropic phase transition of lipid membranes during dry state (Crowe*, et al.*, 1992, Leslie*, et al.*, 1995). Trehalose, maltose and glucose directly interact with the phospholipids via hydrogen bonds, substituting water by occupying 20 – 25% of the hydrogen-bonding sites conferred by the membrane (Pereira and Hunenberger, 2006). This effect is of importance since the integrity of lipid membranes may be lost during phase transition (Crowe, 2015). Similarly, structural preservation of surface proteins provided by sucrose and trehalose correlates with the amount of hydrogen bonding between sugar molecules and proteins (Allison*, et al.*, 1999). Thus, the addition of extracellular sucrose and trehalose enhanced tolerance of *E. coli*, *Bacillus thuringiensis* and *Lactobacillus paracasei* to drying (Leslie*, et al.*, 1995, Teng*, et al.*, 2017). Last, glass formation is another important mechanism of disaccharides to prevent drying-related damages. During freeze-drying, vitrified extracellular matrix compounds enclose and physically separate the cells from each other (Conrad*, et al.*, 2000).

3.2.3.2 Protective mechanism of polysaccharides with special reference to inulin

Studies based on phospholipid bilayers found that starch and dextran, two glass-forming polysaccharides, are too large for interacting with lipid head groups similar as observed for disaccharides (Crowe*, et al.*, 1996, Jain*, et al.*, 2009). Consequently, both polysaccharides were not able to influence phase transition of lipid bilayers and are therefore considered as less effective

stabilizers than low molecular weight sugars under dry and cold conditions. In contrast, inulin and levan-type fructans directly interact with phospholipids of biological membranes, exceeding the strength of interaction obtained by hydrogen bonding with trehalose, sucrose and glucose under comparable conditions (Demel*, et al.*, 1998, Vereyken*, et al.*, 2003). The polysaccharide structure and its flexibility could be central for a significant membrane interaction. Starch and dextrans are branched polymers with rigid pyranose rings, whereas levan and inulin are mainly linear molecules that contain more flexible furanose rings, what might facilitate membrane interaction (Vereyken*, et al.*, 2003). In addition, inulin is able to vitrify, which is unique for polysaccharides, and therefore combines both, water replacement properties and vitrification (Mensink*, et al.*, 2015). Inulin-type fructans are not commonly used as preservation additives despite being known as water-soluble natural protective agents synthesized by many plants, fungi and bacteria (Hubalek, 2003). In a rare study demonstrating their underestimated potential, fructo-oligosaccharides and fructans increased viability of freeze-dried *Lactobacillus reuteri* by direct interaction with the cell membrane resulting in improved integrity in dry state (Schwab*, et al.*, 2007).

3.3 Technical challenges in preservation of pure bacterial cultures and complex communities

3.3.1 Oxidative stress

The commercially available probiotic *Bifidobacterium* and *Lactobacillus* species are anaerobes or microaerophiles, but exhibit a much lower oxygen sensitivity than the strict anaerobic gut microbes that are currently proposed as next-generation probiotics (O'Toole*, et al.*, 2017). Obligate anaerobic bacteria cannot tolerate oxygen because their metabolism involves enzymes that react with oxidative agents. The exposure to ambient air consequently leads to the production of superoxide and hydrogen peroxide that can lethally damage anaerobic cells due to the lack detoxifying enzymes (Imlay, 2002). This high oxygen-sensitivity is a major challenge for the development of microbial-based therapeutics that include strict anaerobic gut microbes and has to be properly addressed to guarantee maximal survival of the oxygen sensitive cells during processing as well as during storage.

3.3.1.1 Oxygen sensitivity of different gut microbes

Flint*, et al.* (2012) previously showed that oxygen sensitivity differed between four *Clostridium*-related butyrate producers from the human gut. Thereof, *R. intestinalis* survived less than two minutes when exposed to 20% oxygen while some *Anaerostipes caccae* cells could withstand up to 60 minutes at ambient air (Fig. 1.6). Moreover, *F. prausnitzii* was able to grow under low oxygen conditions if antioxidants such as flavins, cysteine or gluthation are present, by using them as “extracellular electron shuttle” to reduce oxygen (Khan, et al., 2012). *B. thetaiotaomicron*, anotherobligate anaerobic gut microbe, also employs defense mechanisms against oxygen relying on enzymes that prevent reactive oxygen species formation and facilitates the recovery from short-term oxygen exposure (Pan and

Imlay, 2001, Mishra and Imlay, 2013). Similarly, most *Lactobacillus* strains and some bifidobacteria harbor enzymes responsible for the reduction of reactive oxygen species. This ability could be linked to their robustness to oxidative stress and their survival after spray drying (Simpson*, et al.*, 2005, Dijkstra*, et al.*, 2014).

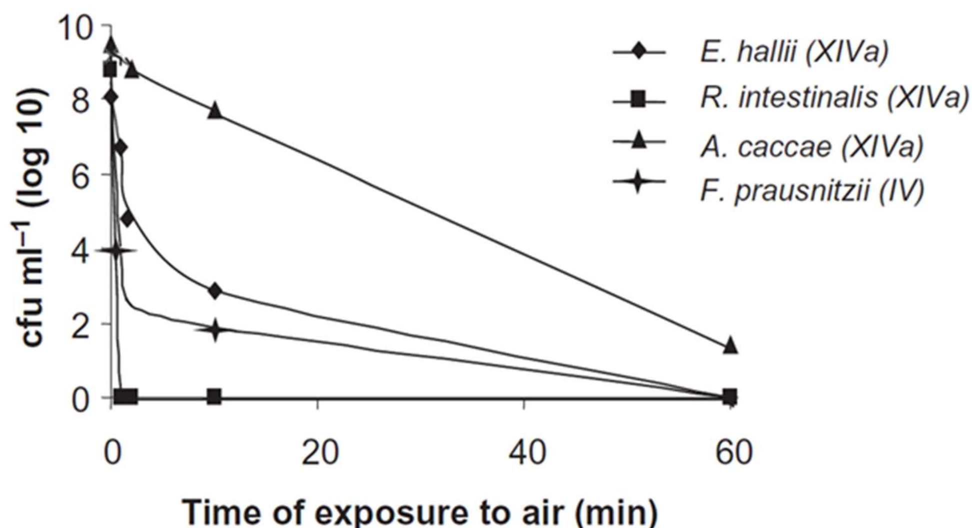

Fig. 1.6 Survival of four strains of butyrate-producing human gut bacteria following exposure to air. Strains tested were *E. hallii* L2-7, *Roseburia intestinalis* L1-82, *Anaerostipes caccae* L1-92 and *F. prausnitzii* A2-165. Dilutions were spread onto M2 agar plates in an anaerobic cabinet, exposed to air for varying time intervals, and then returned to the cabinet for incubation at 37°C (Flint*, et al.*, 2007).

3.3.1.2 Oxygen protection

Introduction of oxygen-scavenger. As mentioned above, the addition of the antioxidant riboflavin to the lyoprotective media improved survival of freeze-dried *F. prausnitzii* during exposure to ambient air by using the antioxidant as redox mediator that transfers electrons to oxygen (Khan, et al., 2014). This strategy might also work for anaerobic gut microbes not able to use antioxidants as "extracellular electron shuttle" by generating a reducing environment that protects from potential oxygen exposure during processing and storage. Supplementing the drying medium with ascorbic acid, a radical scavenger that can counterbalance oxidative stress, enhanced viability of spray-dried *Lactobacillus bulgaricus* during storage (Teixeira*, et al.*, 1995). Supplementing the protective medium with enzymes that exert oxygen-scavenging properties is another way to control oxygen exposure during processing and storage. Oxyrase® is a commercially available enzyme system, obtained from the cytoplasmic membrane fragments of *E. coli*, that produces anaerobic condition in a wide variety of environments (Copeland, 2017).

Oxygen exclusion. Controlling on oxygen during all processing steps and during storage is of major importance to prevent impairment of viability and stability of preserved strict anaerobic gut microbes. It is strongly recommended to perform processing of the anaerobic strains in anaerobic facilities and to store the preserved cultures in oxygen-impermeable containers (Malik, 1991).

3.3.2 Preservation of mixed cultures

The majority of the existing preservation protocols are designed for pure bacteria cultures and it remains unclear whether the applied techniques and conditions are conferrable to community level preservation (Prakash*, et al.*, 2013).

3.3.2.1 Strain specific response to preservation stress

Each biological cell has an optimal cooling rate that minimizes detrimental freezing effects and guarantees highest cell survival. The parameter that most strongly affects the optimal cooling rate is the membrane permeability of the cell, as a measure of the water transfer efficiency through the membrane (Pegg, 2015). This explains why two *L. bulgaricus* strains, distinguishable in their membrane fatty acid composition, reacted differently to the same stresses induced by freezing. Depending on the flexibility of the membrane, osmotic derived cell volume reduction could easily occur in the freezing resistant strain while it resulted in membrane leakage of the sensitive strain (Meneghel*, et al.*, 2017). Discordant responses to freezing and freeze-drying already observed on strain level demonstrate the complexity of preserving natural microbial communities harboring different bacterial species and strains (Modesto*, et al.*, 2004). A previous attempt to cryopreserve complex fecal microbiota at -80°C for 3 months resulted in a loss of 29% of the OTUs and a 50% reduced SCFA producing activity when incubated in basal medium over 36 h (Kerckhof*, et al.*, 2014). The applied protective formulation contained DMSO at concentration of 5% (v/v) either in distilled water or tryptic soy broth (0.3% w/v) supplemented with 1% trehalose (w/v) and was compared to distilled water as a control. The addition of protectants neither improved preservation of microbial community structure nor SCFA producing capacity. The loss of almost one third of the taxa during the course of cryopreservation implies that the proposed method does not adequately preserve community structure and demands for optimization (Kerckhof*, et al.*, 2014). The selection of protectants that did not sufficiently supported protection of freezing sensitive bacteria but also the lack of precaution to avoid oxygen exposure during processing and storage might have been the two major factors that strongly limited the success of the proposed method.

3.3.2.2 Inhomogeneous cell concentration between taxa of a complex microbiota

Different abundances among members of complex microbial communities is another important factor that might complicate community-level preservation. In pure culture, only one bacteria cell needs to survive processing and subsequent storage to guarantee the continuation of the culture after reactivation. However, the more bacteria cells are preserved the higher the probability that at least one survives. It is therefore recommended to preserve pure bacteria cultures at sufficient high cell concentrations (>10^8 cells/ml) (Morgan*, et al.*, 2006). In natural microbial communities, such as the gut microbiome, abundances greatly vary between taxa (Human Microbiome Project, 2012). Consequently, low abundant species of a complex microbiota might bear a greater risk to be irreversibly lost during long-term preservation than high abundant species.

3.4 Formulation of protective media

A supporting matrix can greatly enhance viability of microbes during processing by freezing, dehydration and during subsequent storage. Nowadays, formulation of such protective matrices are normally done on a trial and error basis and there are only few general strategies available on how to conserve a satisfying composition that supports the required high survival rates (Wessman*, et al.*, 2011). **Biocompatibility.** An optimal formulation contains protective compounds that are biocompatible and in accordance with the criteria for regulatory approval. The protectant DMSO has been previously proposed for cryopreservation of fecal microbiota and stool-based amplified microbial communities with the prospective usage as FMT without considering its potential cytotoxic effect (Kerckhof*, et al.*, 2014, Gaci*, et al.*, 2017). However, protective agents that are toxic to human cells, such as DMSO, cannot be used as component of the protective medium for bacterial products intended for therapeutic administration (Galvao*, et al.*, 2014). It is therefore recommended to restrict protectant selection to compounds that exhibit a high biocompatibility with human cells such as glycerol and several sugars. Besides cellular toxicity, protective agents can also evoke negative side-effects when ingested within the supporting formulation. Trehalose, for example, was recently shown to enhance virulence of epidemic *C. difficile* (Collins*, et al.*, 2018) and is therefore not suitable in the protective formulation of biotherapeutics intended for the treatment of rCDI.

Mixes of protective compounds. Combining protectants with different mode and site of action can potentially lead to additive or synergic protective effects (Hubalek, 2003). This was demonstrated previously with the combination of sucrose and glycerol in cryopreservation of *Helicobacter pylori* at - 80°C showing higher survival than glycerol alone (Oskouei*, et al.*, 2010), likely due to the extracellular protective effect of sucrose and prevention of intracellular ice formation and solute effect by glycerol.

Synbiotic formulation. Certain agents exert next to protective effects against preservation-induced damage also prebiotic properties. Combining probiotics and prebiotics that have a beneficial impact on the host is known as 'synbiotics'. Inulin, for example, acts as synbiotic when combined with a probiotic strain, such as *L. plantarum* (Dhewa*, et al.*, 2014). Inulin, when reaching the colon, can impact bacterial metabolite production that is associated with reduction of colorectal tumors (Pool-Zobel, 2005, Verma and Shukla, 2013) and reduced severity of symptoms in IBS (Vogt*, et al.*, 2013). Adding it to the protective formulation would therefore at first prevent preservation induced damage of the probiotic cell and secondly positively influence host health when transferred to the gut as part of the formula.

3.4.1.1 Use of glycerol in cryopreservation of gut microbes

General protocols of aerobic and anaerobic bacterial preservation advise to use glycerol as protectant for long-term frozen storage (Malik, 1991, Koh, 2013). More specifically, Bacic and Smith (2008) recommended using glycerol for cryopreservation of *Bacteroides* species that are among the predominant members of the human colonic microbiota. The universal practice of glycerol addition to

a wide range of microbes before cryopreservation and the high biocompatibility might have vindicated its use for frozen stool preparations intended for FMT in the treatment of rCDI (Satokari*, et al.*, 2015, Lee*, et al.*, 2016). The development of stool banks is essential to improve accessibility to FMT. At the European consensus conference on FMT, addition of a final concentration of 10% glycerol to a fecal suspension containing 30 g stool in 150 ml saline solution immediately before freezing at -80°C was recommended for standardization of frozen stool preparation (Satokari*, et al.*, 2015, Cammarota*, et al.*, 2017). Despite the existence of numerous bacterial cryopreservation protocols with glycerol as cryoprotectant, its application for frozen storage of anaerobic gut microbes is rather empirical and published data of its effectiveness are lacking. On single strain level, cryopreservation of *Akkermansia muciniphila* using glycerol (25% v/v) led to the survival of 6-25% of the preserved cells after one year storage at -80°C (Ouwerkerk*, et al.*, 2017). However, no comparison has been made with cells stored in a protectant-free control medium, therefore protective effect of glycerol on viability was not demonstrated. In addition, cells were immediately frozen after mixing with protective medium without taking the required 30 min equilibration time of glycerol into account. In general, neglecting the penetration time is a drawback of several other protocols; however, presence of glycerol around the cells might still block external ice crystal formation and therefore mechanical damage deriving from outside of the cells (Bacic and Smith, 2008, Satokari*, et al.*, 2015).

3.4.1.2 Use of non-penetrating compounds for cryopreservation and lyophilization of gut microbes

Recent literature provides little knowledge about the usage of non-penetrating protectants in preservation of strict anaerobic gut bacteria and the existing protocols exclusively address lyophilization. Dating back more than 40 years, lyophilization was investigated as preservation method for rumen bacteria, also including the two human gut microbes *B. fragilis* and *B. thetaiotaomicron*. Pure bacterial cultures were lyophilized in a protective medium supplemented with 7.5% glucose (v/w) under strict anaerobic condition. After 5 years of storage at 4-6°C, both *Bacteroides* sp. maintained viable cell numbers similar to fresh cultures before drying (Phillips*, et al.*, 1975). In contrast to these early preservation experiments, di- rather than monosaccharides are generally considered nowadays as the protectants of choice when preserving microorganisms by drying (Wessman*, et al.*, 2011). Comparing different disaccharides, sucrose was found to be superior over double-strength skim milk containing lactose in preservation of the strict anaerobic gut microbes *B. fragilis*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidium*, *Eubacterium lentum*, *Eubacterium limosum* and several *Fusobacterium* sp. (Staab and Ely, 1987). In a more recent study, Khan*, et al.* (2014) used a protective formulation containing inulin for lyophilization of *F. prausnitzii,* while sucrose at a concentration of 5% (w/v) was selected as protectant during lyophilization of the same bacteria in another recent study, intended for the production of probiotic tablets for therapeutic application (Allouche*, et al.*, 2018). However, the two studies did not provide any information about the effectiveness of the used protectants sucrose and inulin in preserving *F. prausnitzii*.

4 Techniques for the analysis of bacterial viability and activity

Knowing if bacteria cells are alive or dead is of major importance in the validation of anaerobic preservation methods. Several parameters of bacterial viability and activity can be used as measurements to evaluate the efficiency of different preservation techniques, to identify detrimental effects during processing and storage but also to determine the shelf-life of microbial-based product (Hammes, *et al.*, 2011). Techniques for the analysis of bacterial viability and activity can either be cultivation-dependent or cultivation-independent and should be reliable and preferably high throughput to facilitate the screening of different processing steps and protective formulations (Berninger, *et al.*, 2018).

4.1 Cultivation-dependent viability and activity assessment

Cultivation-dependent methods to detect viability are more laborious and time intensive than cultivation-independent viability assessments. Nevertheless, culturability remains the most solid evidence of bacterial viability since only live cells are able to reproduce (Kell, *et al.*, 1998).

4.1.1 Techniques for pure cultures

Determination of live bacteria in pure cultures is routinely done by plating of serially diluted bacteria samples on agar plates, followed by counting of the total number of colony-forming units (Davis, 2014). In terms of anaerobic bacteria cultures, the most probable number method (MPN), executed in oxygen impermeable Hungate tubes, is the preferred technique over conventional plating. Therefore, the concentration of viable microorganisms in a sample is estimated by means of replicate liquid broth growth in ten-fold dilutions to distinction in MPN tubes (Sutton, 2010). This method can be adapted for high throughput screening in microtiter plates that allows to greatly enhance sample number by using multichannel pipettors or robots (Kuai, *et al.*, 2001). However, sample preparation as well as execution of the analysis needs to be conducted in an anaerobic environment. This can either be achieved by running the analysis in a plate reader that has been placed in an anaerobic chamber or by creating an anaerobic environment within the microtiter plates through introduction of a reducing agent and air tight sealing (Geirnaert, *et al.*, 2014). The drawbacks of viability assessment by growth is that it only detects cells that are culturable under the given experimental conditions (Davis, 2014). Therefore not only dead or irreparable damaged bacteria are excluded but also live microbes that are dormant, a state referring to a low metabolic activity and unable to divide. Bacteria in this state are termed “viable but not cultivable” (the VBNC state) (Lahtinen, *et al.*, 2008).

4.1.2 Techniques for complex microbiota

Direct enumeration of specific groups within a complex microbial community with molecular methods does not provide information on the viability and activity status of the microbiota. However, growth of specific groups of the complex microbiota can be used as indicator for viability (Kell, *et al.*, 1998).

Cultivation of a complex colonic microbiota can be performed in anaerobic batch fermentation using a carefully selected medium that allows growth of the majority of the community members. An adequate buffering of the system is required to counterbalance a rapid acidification caused by the production and accumulation of SCFA and un-dissociated organic acids that can lead to an inhibition of microbial activity and growth (Payne*, et al.*, 2012). To assess viability by growth, cultivation-dependent approaches have to be combined with molecular methods, such as quantitative real-time PCR (qPCR) or 16S rRNA amplicon sequencing to monitor growth over time.

4.2 Cultivation-independent viability assessment

Although viability is traditionally determined by cultivation, cultivation-independent techniques are increasingly applied to understand why cells might not reproduce under certain condition (Nocker*, et al.*, 2012). Several cellular processes, essential for bacterial survival, can serve as measurable bacterial viability markers (Fig. 7). These indicators include presence of nucleic acid, membrane integrity and potential, cellular energy, efflux pump activity, respiratory and enzymatic activity (Hammes*, et al.*, 2011). Measurement of membrane integrity and membrane potential are by far the most used methods to assess bacterial viability after preservation of bacteria. The two methods, both dealing with fluorescent dye in combination with flow cytometry, spectrofluorometry or fluorescence microscopy will be therefore explained more in detail below.

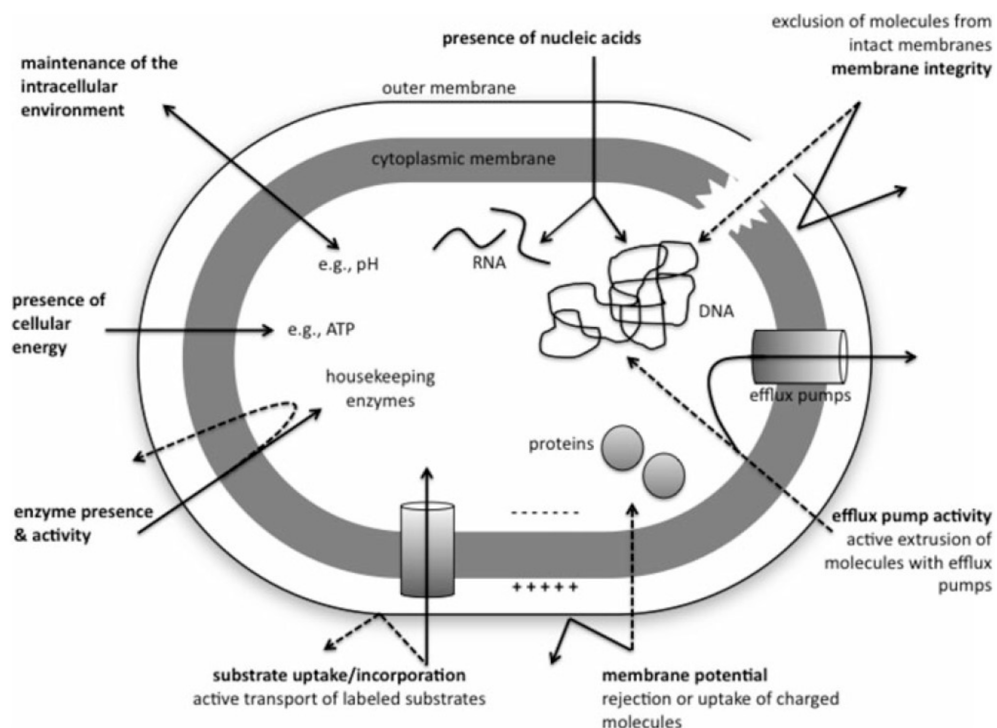

Fig. 1 The concept of evaluating various cellular processes in order to assess the viability state of the organism (Hammes*, et al.*, 2011).

4.2.1 Assessment of membrane integrity

Membrane integrity staining, also referred as LIVE/DEAD staining, is often used for bacterial viability assessment after treatments causing excessive physical or chemical cellular damage, such as chlorine

disinfection or heat treatment (Berney*, et al.*, 2008, Nocker*, et al.*, 2017). Severe structural impairment of the cytoplasmic membrane of bacteria is generally irreversible, ultimately leading to cell death (Joux and Lebaron, 2000). LIVE/DEAD staining bases on two different fluorescence nucleic acid stains that vary in their ability to penetrate bacterial cells. Propidium iodide red fluorescence dye can only access cells with permeabilized cytoplasmic membranes while a green fluorescence dye, such as SYBR green I or SYTO 9, labels all bacteria within a population (Hammes*, et al.*, 2011). The degree of cellular damage within a bacterial culture can be quantitatively analyzed based on the obtained fluorescence patterns using a flow cytometer (Van Nevel*, et al.*, 2013). As a rapid, precise and sensitive determination of viable cells, membrane integrity staining can be useful to investigate the effect of preservation-induced stresses on viability of bacteria. Bensch*, et al.* (2014) used membrane integrity staining in combination with plate counting to compare viability and cultivability of *L. plantarum* starter cultures, respectively after fluidized bed drying and found differences between viable and cultivable numbers. It was suggested that the occurrence of viable-but-non-cultivable cells might have led to the observed discrepancy. Cells with severely damaged membranes are generally considered as “dead”. In contrast, cells with integer membranes are not necessarily cultivable and may therefore not be recovered by culture (Hammes*, et al.*, 2011). In addition to viability assessment, membrane integrity is a valid indicator for detrimental processing effects on membranes (Schwab*, et al.*, 2007, Bravo-Ferrada*, et al.*, 2015).

4.2.2 Assessment of membrane potential

Membrane potential is, in contrast to membrane integrity, a more sensitive indicator of the bacterial viability status. Depolarized cells can still display intact membranes. However, only bacterial cells that are alive are able to maintain a membrane potential since its break-down exposes the cell to lethal environmental stressors, such as high salt concentration, pH gradients and oxygen exposure in case of strict anaerobes (Hammes*, et al.*, 2011). Only polarized cells can control the passage of negatively and positively charged lipophilic molecules through the cytoplasmic membrane. Cells lacking membrane potential can be selectively detected with the anionic fluorescent dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) which binds to lipid-containing intracellular components. $DiBAC_4(3)$ cannot pass through polarized cells that therefore remain nonfluorescent (Haidinger*, et al.*, 2003). $DiBAC_4(3)$ has previously been used to assess viability of the anaerobic *Bifidobacterium lactis* and *Bifidobacterium adolescentis*, two representative of the human colonic Bifidobacteria, during exposure to bile salt stress (Amor*, et al.*, 2002). To avoid a potential bias through oxygen exposure, the whole staining procedure has been conducted in an anaerobic chamber. The assessment of membrane potential appeared to overestimate the viability of *B. lactis* and *B. adolescentis* when compared to conventional plating, which was explained by a noncultivable fraction of sublethal damaged cells caused by bile salt stress. Combining different methods of viability assessment not only allows to distinguish between live and dead cells but also to discriminate between different physiological states.

Nocker*, et al.* (2012) performed a multiparameter viability assay to investigate the effect of air drying on bacterial viability of *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus hirae* and *Staphylococcus aureus*. It was concluded that cultivation demonstrated the overall impact of air drying on the ability to grow whereas the assessment of cultivation-independent viability markers, such as membrane potential and membrane integrity, provided additional insights into the stress level of the different species at given time point.

5 Background and objectives of the thesis

FMT is highly efficient in resolving rCDI and holds therapeutic potential as treatment for other gastrointestinal diseases associated with intestinal dysbiosis. However, a stool-based treatment raises several issues such as a potential infection risk, transfer of undesired phenotypes, limited standardization and low patient acceptance. These restrictions led to the ongoing research efforts in identifying beneficial gut microbes and the development of synthetic bacteria mixtures to bypass the administration of fecal material. Intestinal fermentation technology (IFT) can deliver controlled and stable "artificial" colonic microbiota at high cell density and large quantity that might act as a safe alternative for FMT. However, widespread availability of such microbial-based therapeutics can only be guaranteed if the product can be stored without losing efficiency. Consequently, there is a strong demand for anaerobic preservation technologies that warrant microbial composition and functionality. Existing preservation protocols were mainly developed for pure, aerotolerant cultures while strict anaerobic and community level preservation has been widely neglected.

For this project, we hypothesized that IFT derived complex gut microbiota can be recovered and optimally processed for long term storage, while preserving biodiversity and functionality for potential therapeutic administration.

General objective

The central objective of this thesis was to investigate, develop and validate anaerobic preservation techniques for maintaining microbial composition and metabolic activity of complex colonic microbiota produced with IFT as well as viability of health-promoting gut microbes in pure culture that are considered as next-generation probiotics.

Specific objectives

1. To test the protective effect of biocompatible cryoprotectants on the reestablishment of key butyrate producing species and metabolic activity of cryopreserved and stored artificial colonic microbiota produced with the PolyFermS platform inoculated with immobilized fecal microbiota from healthy adult donors (Chapter 2).
2. To evaluate the impact of protectants, processing (freezing and drying) and storage on viability and fitness of strict anaerobic gut microbes that are proposed as next-generation probiotics (Chapter 3).
3. To investigate the impact of microbial lifestyle (planktonic and sessile) on recovery of metabolic activity and community structure of cryopreserved and lyophilized artificial colonic microbiota produced with the PolyFermS platform inoculated with immobilized fecal microbiota from healthy adult donors (Chapter 4).

Chapter 2

Cryopreservation of artificial gut microbiota produced with *in vitro* fermentation technology

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Abstract

Interest in fecal microbiota transplantation (FMT) has increased as therapy for intestinal diseases, but safety issues limit its widespread use. Intestinal fermentation technology (IFT) can produce controlled, diverse, and metabolically active 'artificial' colonic microbiota as potential alternative to common FMT. However, suitable processing technology to store this artificial microbiota is lacking. In this study, we evaluated the impact of the two cryoprotectives, glycerol (15% v/v) and inulin (5% w/v) alone and in combination, in preserving short chain fatty acid formation and recovery of major butyrate-producing bacteria in three artificial microbiota during cryopreservation for 3 months at -80°C.

After 24 h anaerobic fermentation of the preserved microbiota, butyrate and propionate production was maintained when glycerol was used as cryoprotectant, while acetate and butyrate were formed more rapidly with glycerol in combination with inulin. Glycerol supported cryopreservation of the *Roseburia* spp./*Eubacterium rectale* group, while inulin improved the recovery of *Faecalibacterium prausnitzii*. *Eubacterium hallii* growth was affected minimally by cryopreservation. Our data indicate that butyrate producers, which are key organisms for gut health, can be well preserved with glycerol and inulin during frozen storage. This is of high importance if artificially produced colonic microbiota is considered for therapeutic purposes.

Introduction

Pathogenesis of several gastrointestinal diseases has been linked to functional alterations and compositional imbalances of the intestinal microbiota, also referred as dysbiosis. For example, decreased microbial diversity has been associated with recurrent *Clostridium difficile* infection (RCDI) (Milani*, et al.*, 2016). Changes in the abundance of the butyrate-producing bacterial community along with reduced butyrate formation were observed in inflammatory bowel disease (IBD) (Sokol*, et al.*, 2009, Kumari*, et al.*, 2013, Fuentes*, et al.*, 2017). To restore the microbial balance, transfer of fecal microbiota from a healthy donor (FMT) to diseased patients has been suggested as a therapeutic strategy. The success of FMT in treating RCDI has been demonstrated in several studies, with cure rates exceeding 90% (van Nood*, et al.*, 2013, Kelly*, et al.*, 2016, Lee*, et al.*, 2016). Using FMT as a therapy for other gastrointestinal disorders such as IBD has been proposed (Vermeire*, et al.*, 2016). To date, safety concerns and acceptability are main constraints of therapeutic uses of FMT. Fresh fecal matter is preferably obtained from relatives of the patients immediately before transplantation. A careful donor screening regarding fecal microbiota composition, pathogen status and undesirable antigens and "phenotypes" must be done preventively (Petrof and Khoruts, 2014, Alang and Kelly, 2015). Despite the increasing demand for FMT, rigorous exclusion criteria for donors strongly limit the widespread availability of suitable fecal material (Konig*, et al.*, 2017). The approach of transplanting "artificially" produced microbiota, which has been extensively characterized, might alleviate these limitations. Continuous *in vitro* intestinal fermentation technology (IFT) with immobilized fecal microbiota, mimicking both the planktonic and sessile growth, can be used to produce controlled and stable "artificial" colonic microbiota at high cell density and at large quantity (Cinquin*, et al.*, 2006, Payne*, et al.*, 2012, Zihler Berner*, et al.*, 2013, Fehlbaum*, et al.*, 2015, Lacroix*, et al.*, 2015). Nevertheless, processing for long-term preservation is required to guarantee availability of artificial fecal microbiota for transplantation.

Cryopreservation at temperatures ranging from -80°C in electrical freezers to -196°C in liquid nitrogen is a widely-used method for storing bacteria (Prakash*, et al.*, 2013). However, ice formation during freezing can cause lethal damage to bacterial cells; thus, cryoprotectants must be added to prevent cryoinjuries. The positive effects of different protective matrices composed out of polysaccharides, amino acids, peptides or more complex compounds on microbial cell physiology during freezing, storage and thawing has been studied for pure cultures and is well approved (Hubalek, 2003). Non-penetrating cryoprotectants, such as many saccharides, reduce ice formation within cells by osmosis-derived dehydration before freezing. Cryoprotective sugars can also bind to the cell surface and inhibit extracellular ice crystal formation. Penetrating cryoprotectants, such as glycerol and dimethyl sulfoxide (DMSO), change fluid properties and increase membrane glass-phase transition temperature, which results in reduced intracellular ice formation (Hubalek, 2003, Fowler and Toner, 2005). Nevertheless, DMSO is not recommended for *in vivo* administrations as it was found to be toxic to cells

at low concentrations of 2-4% (Galvao*, et al.*, 2014), whereas glycerol is a common additive for frozen fecal samples also in stool banks (Aguirre*, et al.*, 2015). The clinical efficacy of cryopreserved fecal slurries for resolving RCDI have been investigated before. It was shown that preserved slurries with or without glycerol exhibited remission rates similar to fresh FMT. Furthermore, only minor viability drops in the tested cultivable anaerobes over 6 months of frozen storage were reported (van Nood*, et al.*, 2013, Youngster*, et al.*, 2014, Costello*, et al.*, 2015, Satokari*, et al.*, 2015). However, artificially produced microbiota may lack the protective effect of a matrix naturally present in stool. Therefore, composition of the protective matrix for cryopreservation of artificially produced microbiota should be investigated.

In this work, we observed the effect of two cryoprotective agents on maintaining metabolic activity of artificial colonic microbiota produced with IFT. Glycerol and inulin were chosen as penetrating and non-penetrating cryoprotective agents, respectively, since combining compounds with different mechanisms can result in additive or synergic protective effects (Hubalek, 2003). Glycerol was selected due to its low toxicity to microbial and human cells and its widespread use for frozen stool samples intended for FMT (Hamilton*, et al.*, 2012). Inulin acts extracellularly by stabilizing membranes via interplay with membrane lipids and providing mechanical protection from enhanced surface pressure (Demel*, et al.*, 1998) and might substitute for a protective matrix.

Moreover, we also investigated the impact of cryopreservation on the re-establishment of the major butyrate producers *Faecalibacterium prausnitzii* (*Clostridium* cluster IV), *Eubacterium hallii* and the *Roseburia* spp./*Eubacterium rectale* (*Clostridium* cluster XIVa). Butyrate is an important short chain fatty acid (SCFA) that provides several benefits to the host (Tan*, et al.*, 2014). Butyrate producers of the *Clostridium* clusters IV and XIVa have been associated with a “sustained response” to FMT in IBD and could therefore be crucial in restoring the metabolic balance of a disturbed intestinal microbiota (Fuentes*, et al.*, 2017). In this study, three different colonic microbiota, originating from two *in vitro* gut fermentations inoculated with immobilized fecal microbiota of two healthy adult donors, were preserved in buffers containing either glycerol or inulin or a combination thereof. Metabolite formation, as marker of metabolic activity of the processed artificial microbiota, and re-establishment of selected butyrate producers were determined before and after storage at -80°C to identify the specific effect of cryopreservation on SCFA production and on growth of selected butyrate producers as well as to observe the protective potential of the added cryoprotectants.

Results

Bacterial composition of artificial gut microbiota

Two continuous colonic fermentation systems (F1 and F2) consisting of single reactors mimicking conditions of the proximal colon were inoculated with immobilized fecal microbiota from two healthy adult male donors and used to produce artificial gut microbiota. Two freezing trials were carried out using two effluents of F1 obtained with standard fermentation conditions (effluent 1.1) after reaching steady-state operation; or after pH stress application (effluent 1.2), which shifted microbiota composition and metabolic activity. One freezing trial was conducted with effluents of F2 obtained after reaching steady-state operation (effluent 2). Microbial composition of both fermenter effluents and donor feces were determined by sequencing the V4 region of the 16S rRNA gene amplicons (Fig. 2.1). In addition, quantitative real time PCR (qPCR) was performed to investigate the relative abundance of butyrate-producing bacteria, *F. prausnitzii*, *E. hallii* and the *Roseburia* spp./*E. rectale* group in the fermentation effluents (Table 2.1).

The microbiota in effluent 1.1 was mainly Firmicutes (79.5% of the reads). Bacteroidetes was the second most abundant phylum, accounting for 9.1% of the reads. *Ruminococcaceae* (29.5%) and *Lachnospiraceae* (36.9%) were the most prevalent families. In effluent 1.2, the abundance of Firmicutes was lower, at 67.0% of the total reads, and was represented by the families *Ruminococcaceae* (23.2%) and *Lachnospiraceae* (32.5%). Bacteroidetes were higher in effluent 1.2 (18.5%) compared to effluent 1.1. In contrast to F1, effluent from F2 was predominated by Bacteroidetes (48.7%), and contained 33.9% Firmicutes. At the family level, *Prevotellaceae* (41.7%) were predominant, followed by *Ruminococcaceae* and *Lachnospiraceae*, which represented 15.3 and 9.5% of the reads, respectively (Table S2.1 and S2.2).

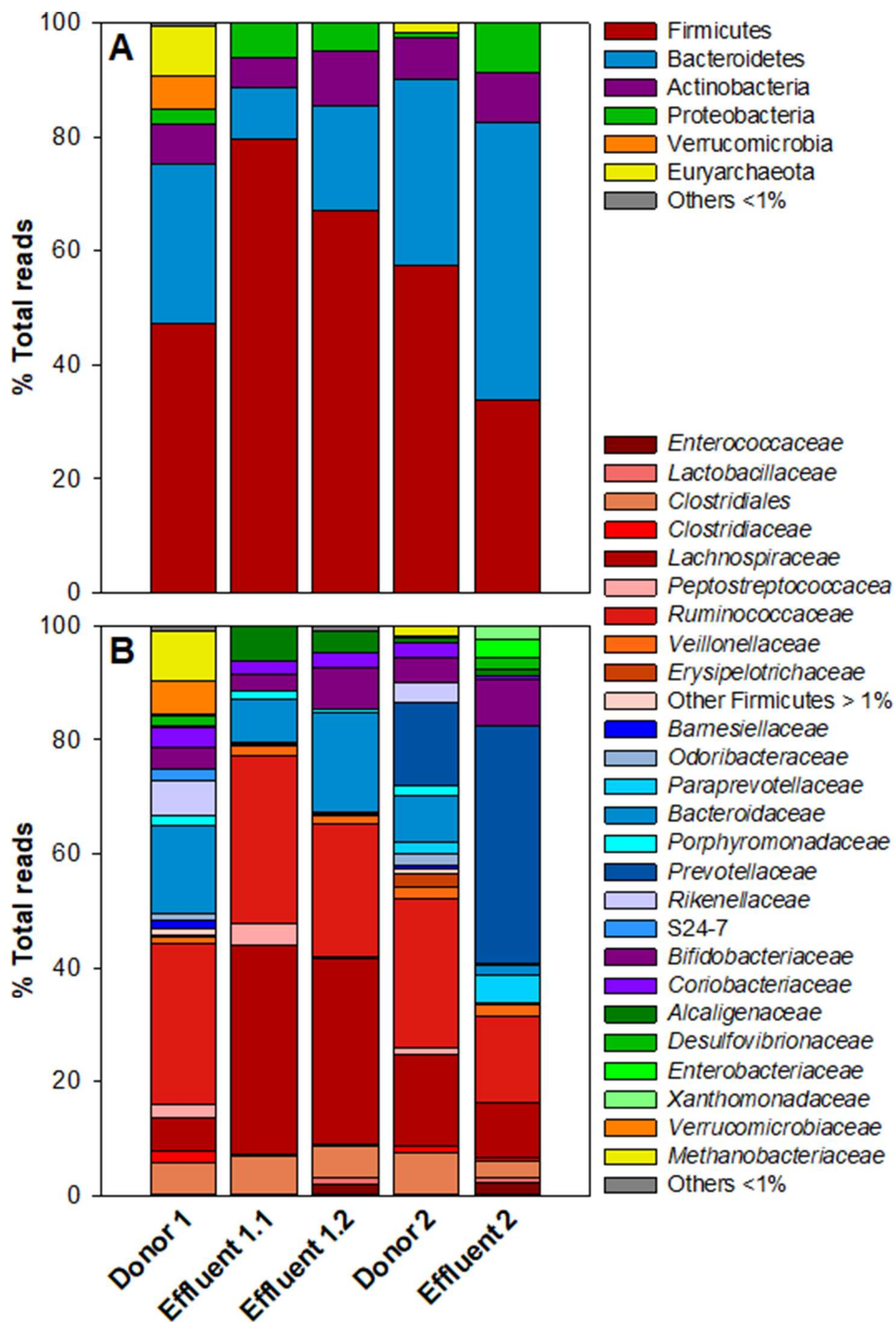

Fig. 2.1 Microbial composition of donor faeces and fermentation effluents. Relative abundance of microbial phyla (A) and families (B) in the fermentation effluents and corresponding donors was analysed by V4 region 16S rRNA gene sequencing.

F. prausnitzii represented 11.0, 5.6 and 3.1% of the bacterial community in effluent 1.1, 1.2 and 2, respectively (Table 2.1). Relative abundance of the *Roseburia* spp./*E. rectale* group ranged from 0.8% to below 0.1%, while relative abundance of *E. hallii* was between 0.002 and 0.005%.

Effluents 1.1 and 1.2 exhibited a lower diversity (Shannon index of 3.2 and 3.1, respectively) compared to the corresponding fecal sample 1 (Shannon index 4.2). Transferring fecal microbiota from donor sample to reactor decreased the Bacteroidetes:Firmicutes ratio, from 0.6 in the fecal inoculum to 0.1 in effluent 1.1. In F2, the Bacteroidetes:Firmicutes ratio shifted from 0.6 in feces to 1.4 in effluent 2,

mainly due to a decrease of *Ruminococcaceae* and an increase in *Prevotellaceae.* Overall diversity decreased in effluent 2 compared to feces, with respective Shannon indices of 2.7 and 4.2.

Table 2.1 Relative abundance of Roseburia *spp./*E.rectale group, *F. prausnitzii* and *E. hallii* in fermentation effluents.

Targeted butyrate-producing bacteria	Relative abundance[a]		
	Effluent 1.1	Effluent 1.2	Effluent 2
Roseburia spp. /*E. rectale*	0.7	0.1	0.0
F. prausnitzii	11.0	5.6	3.1
E. hallii	0.0	0.0	0.0

But genes of *Roseburia* spp./*E.rectale* and *F. prausnitzii,* and 16S rRNA of *E. hallii* were targeted by qPCR. Relative abundance was calculated relative to total 16S rRNA gene copies. Results for *but* genes were multiplied by five to account for multiple 16S rRNA gene copies.
a. Percentage of total bacteria numbers from single measurements

Metabolic profile of artificial gut microbiota

The metabolite profiles in fermentation effluents were analyzed by high-performance liquid chromatography with refractive index detection (HPLC-RI) (Fig. 2.2). Concentrations of main SCFAs, acetate, butyrate and propionate, differed between effluents (Table S3). Intermediate metabolites lactate and formate were present at low concentrations (≤1.0 mM) or were not detected. SCFAs of effluent 1.1 were dominated by acetate (62.3 mM), and were characterized by a high concentration of butyrate (46.8 mM) and a low level of propionate (12.5 mM). The predominant SCFAs of effluent 1.2 were butyrate (56.6 mM), acetate (43.1 mM), and propionate (19.0 mM), resulting in acetate:propionate:butyrate ratios of 1:0.2:0.8 for effluent 1.1 and 1:0.4:1.3 for effluent 1.2. Effluent contained 36.6 mM propionate, 29.3 mM butyrate and a high concentration of acetate (71.1 mM), giving an acetate:propionate:butyrate ratio of 1:0.5:0.4.

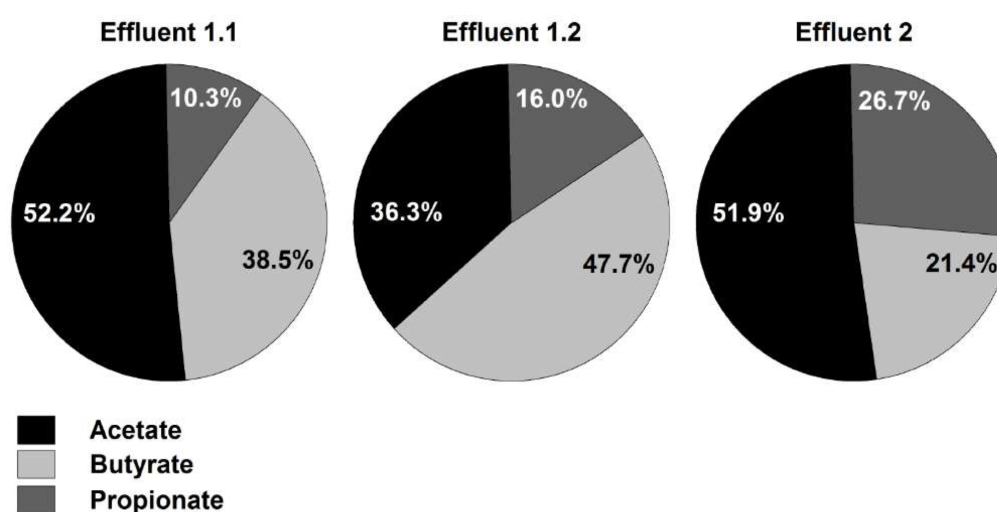

Fig. 2.2 Percentage of main metabolites in fermentation effluent samples. Ratios of major metabolites acetate, propionate and butyrate were calculated from the concentrations measured by HPLC-RI giving an acetate:propionate:butyrate ratio of 1:0.2:0.8 for effluent 1.1, 1:0.4:1.3 for effluent 1.2 and 1:0.5:0.4 for effluent 2.

SCFA formation of fermentation effluents prior to and post freezing

The ability of cryoprotectants to preserve metabolic activity of effluent microbiota was determined after cryopreservation for 3 months at -80°C, using protective buffers containing inulin (5% v/w), glycerol (15% v/v), or a combination thereof. Fresh (t_0) and preserved microbiota (3 months storage at -80°C), in phosphate buffer with or without (control) cryoprotectants, were used to inoculate adapted Macfarlane medium. Inoculated media were incubated for 24 h under anaerobic conditions to investigate the metabolic activity and re-establishment of selected butyrate-producing bacteria in batch fermentation using HPLC-RI and qPCR, respectively. Additionally, SCFA production by microbiota in preserved and reactivated effluents was determined after 3, 5 and 7 h of batch fermentation (Fig. 2.3). All fermentations were carried out in triplicate.

Final amounts of each SCFA produced by fresh microbiota differed among effluents but were impacted minimally by adding cryoprotectants (Table 2.2). SCFA amounts between the control and the treatment of microbiota 1.1 and 1.2 were similar, indicating no effect of added glycerol, inulin or combination thereof on metabolic activity. The metabolite production of microbiota 1.1 was dominated by acetate (77.9±1.1 – 80.2±4.0 mM), followed by butyrate (16.3±2.7 – 20.7±0.7 mM) and propionate (9.8±3.2 – 13.8±0.3 mM), giving a total of 107.1±6.0 – 112.7±0.87 mM SCFAs. Microbiota 1.2 produced comparable amounts of total SCFAs (107.4±1.3 – 111.1±2.2 mM), but slightly higher amounts of propionate (16.4±1.4 – 1.9±1.2 mM) at the expense of acetate (70.3±1.9 – 74.1±1.5 mM). In contrast, microbiota 2 processing with glycerol or glycerol and inulin changed final amounts of main SCFAs produced. Propionate production was significantly reduced ($P<0.05$) compared to the control (17.7±0.4 and 14.2±0.2 versus 21.6±0.3 mM), while butyrate production was enhanced with glycerol (16.4±0.2 mM) relative to levels in the control treatment (12.9±0.3 mM). After 24 h of batch fermentation lactate and formate were not detected with fresh microbiota 1.1 and 1.2; however, formate was present with microbiota 2 (9.4±1.3 mM).

After preservation, metabolic activity of effluent microbiota was generally lower compared to fresh microbiota (Table 2.2). The lowest recovery of total SCFA levels compared to fresh microbiota was measured in control samples preserved without added cryoprotectants (72.1, 82.3 and 79.9%, respectively). All three main SCFAs were significantly decreased ($P<0.05$). Glycerol alone and in combination with inulin maintained best the overall metabolic activity of the stored microbiota. The observed decrease of SCFA concentrations in microbiota samples preserved in glycerol was associated with lower acetate formation (85.5, 85.4 and 94.1% recovery), whereas the amounts of propionate and butyrate did not significantly differ from the concentrations produced by fresh microbiota. When microbiota were preserved with glycerol combined with inulin, a decrease in acetate production was also observed (88.4, 87.8 and 96.7% recovery). Microbiota 1.1 and 1.2 with glycerol and inulin formed similar amounts of propionate and butyrate compared to fresh microbiota, while microbiota in stored effluent 2 produced significantly higher concentrations of propionate and butyrate ($P<0.05$) (125.3 and

125.6%, respectively). Independent of the treatment, lactate and formate were detected after 7 h incubation (Fig. S2.1) but were not detectable anymore after 24 h batch fermentation, with the exception of cryopreserved microbiota 2, which had formate concentrations of 3.1±2.1 – 3.9±0.4 mM.

Table 2.2 Production of major SCFA after 24 h batch fermentation of effluent samples prior and post storage at -80°C.

	Effluent 1.1			**Effluent 1.2**			**Effluent 2**		
Protective buffer	Prior to Freezing (mM)	Post-freezing (mM)	Recovery (%)	Prior to Freezing (mM)	Post-freezing (mM)	Recovery (%)	Prior to Freezing (mM)	Post-freezing (mM)	Recovery (%)
Glycerol and Inulin									
Acetate	79.9±0.2	70.6±1.1[ab]	88.4	70.3±1.9	61.7±0.4[b]	87.8	54.5±0.5[a]	52.7±0.6[ab]	96.7
Propionate	12.2±0.3	13.0±0.3[a]	106.6	17.2±0.6	16.8±0.5	98.0	14.2±0.2[a]	17.8±0.2[a b]	125.3
Butyrate	17.1±0.2	18.2±0.7[a]	106.4	19.9±1.2	19.4±1.0[a]	97.5	12.9±0.6	16.2±0.1[b]	125.6
Total SCFA	109.1±0.7	101.9±0.8[ab]	93.4	107.4±1.3	98.0±0.3[a b]	91.1	81.6±0.6[a]	86.8±0.9[a b]	106.4
Glycerol									
Acetate	80.2±4.0	68.6±0.5[b]	85.5	70.6±2.0	60.3±0.5[b]	85.4	57.2±1.1[a]	53.8±1.3[ab]	94.1
Propionate	9.8±3.2	12.7±1.1[a]	129.6	16.4±1.4	16.7±0.9	102.1	17.7±0.4[a]	15.9±0.8[a]	90.0
Butyrate	17.1±1.2	16.2±0.6[a]	94.7	20.9±1.5	17.4±0.1[a]	83.5	16.4±0.4[a]	16.9±0.1[a]	103.3
Total SCFA	107.1±6.0	97.5±1.3[a]	91.0	107.9±1.9	94.5±1.2[b]	87.6	91.2±1.0[a]	86.6±1.6[ab]	95.0
Inulin									
Acetate	77.9±1.1	67.6±1.4[b]	86.8	71.0±1.2	65.6±3.1	92.4	59.4±1.0	49.0±0.9[b]	82.5
Propionate	13.8±0.3	9.3±0.4[ab]	67.4	17.7±0.1	17.4±1.1[a]	98.4	23.3±0.3[a]	16.2±0.5[a b]	69.6
Butyrate	20.7±0.7	9.4±0.2[b]	45.4	19.1±0.5	16.0±0.1[b]	83.8	14.1±0.0	14.4±1.2	102.0
Total SCFA	112.7±0.7	86.3±1.6[a b]	76.6	107.7±0.9	99.0±3.9[a]	91.9	96.8±1.3[a]	79.6±2.0[ab]	82.2
Control									
Acetate	79.7±1.5	63.5±3.3[b]	79.7	74.1±1.5	60.5±2.1[b]	81.6	59.4±0.2	47.9±0.2[b]	80.7
Propionate	12.6±0.5	4.9±0.5[b]	38.9	18.1±0.5	15.4±0.3[b]	85.2	21.6±0.3	13.3±0.4[b]	61.4
Butyrate	16.3±2.7	9.9±0.5[b]	60.7	18.9±0.2	15.6±0.8[b]	82.3	12.9±0.3	13.8±0.7	107.2
Total SCFA	108.6±1.7	78.3±3.6[b]	72.1	111.1±2.2	91.5±2.5[b]	82.3	93.9±0.3	75.0±0.9[b]	79.9

Main metabolites acetate, propionate and butyrate formed by effluents immediately after processing (t=0) and after 3 months storage at -80°C were analyzed by HPLC-RI (means and standard deviations of independent fermentation triplicates).

a. Indicates that metabolite formed in treatment is significantly different from the control within the same effluent microbiota ($p<0.05$)

b. Indicates that metabolite formed is significantly different prior and post freezing within the same treatment and effluent microbiota($p<0.05$)

Concerning the SCFA production kinetics after storage, acetate and butyrate were detectable after 7 h incubation, whereas propionate was not detected. Microbiota 1.1, 1.2 and 2 produced more acetate in batch fermentation after 7 h incubation when preserved in inulin (28.7±0.3, 38.4±1.0 and 29.8±0.5 mM) or glycerol and inulin (28.3±0.1, 35.9±0.8 and 31.6±0.4 mM) compared to the control treatment (20.7±1.7 mM, 30.9±2.0 and 24.8±0.1 mM). Glycerol in combination with inulin also increased butyrate formation (4.1±0.6, 3.1±0.2 and 3.1±0.3 mM) compared to controls (1.7±0.0, 2.1±0.2 and 2.0±0.0 mM) during 7 h incubation.

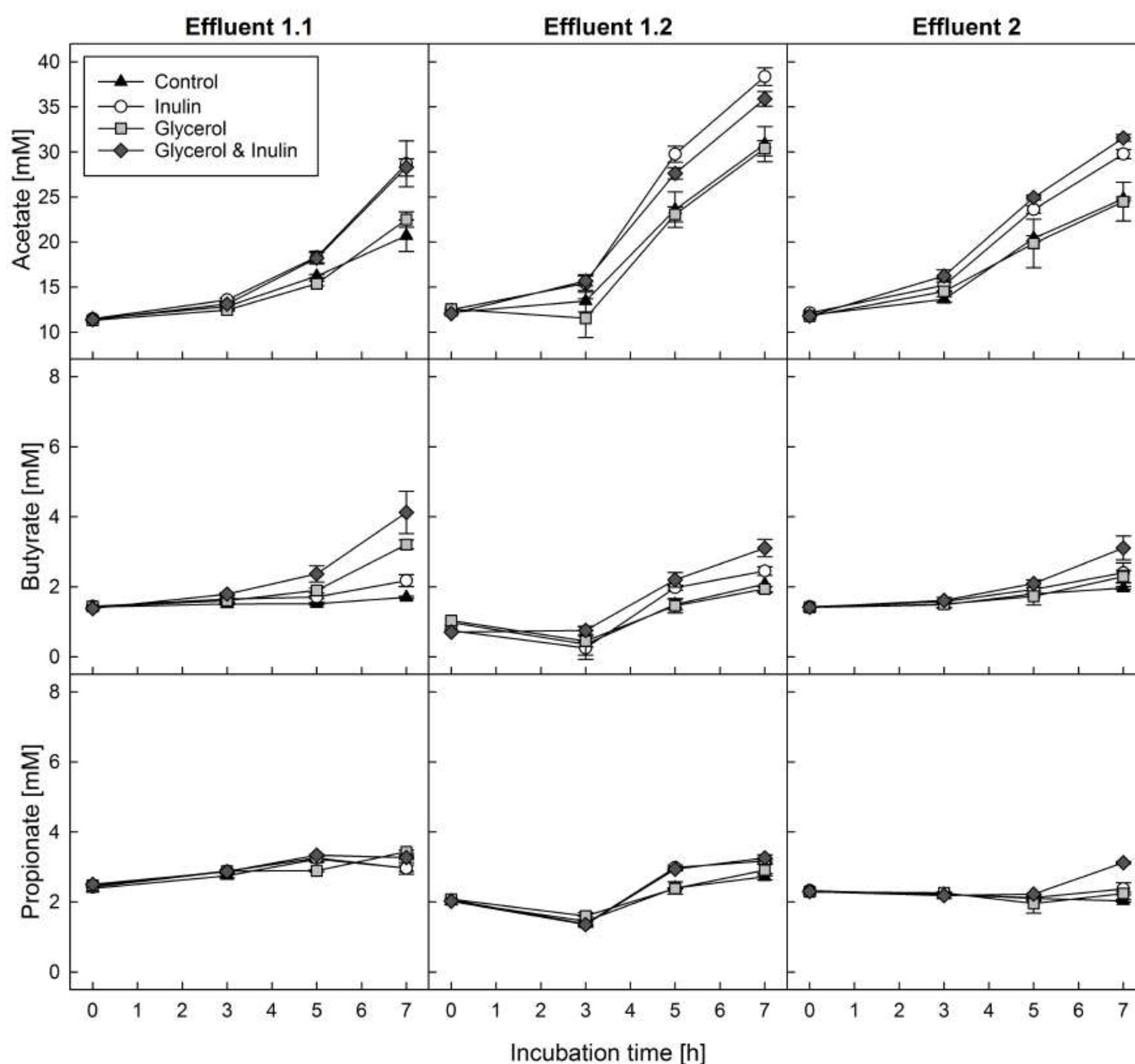

Fig. 2.3 Kinetics of main metabolites production after reactivation of effluent microbiota stored for 3 months. Main metabolites acetate, propionate and butyrate were analysed by HPLC-RI after reactivation in batch fermentations. Each point represents the average of three replicates with standard deviation.

Impact of cryopreservation on re-establishment of selected butyrate-producing bacteria

We also investigated the effect of cryoprotectants on growth and relative abundance of butyrate-producing bacteria, *Roseburia* spp./*E. rectale* group, *F. prausnitzii* and *E. hallii*, during a 24 h batch fermentation using qPCR (Table 2.3).

No significant difference in growth or relative abundance of targeted butyrate-producing bacteria was observed between control and treatments containing cryoprotectants in microbiota 1.1. After 24 h of batch fermentation, average increases of 0.9±0.2, 0.5±0.1 and 2.1±0.3 logs were observed for the *Roseburia* spp./*E. rectale* group, *F. prausnitzii*, and *E. hallii*, giving final relative abundances of 6.1±3.2, 4.2±0.9 and 0.5±0.3%, respectively. The tested treatments had only a minor impact on growth of selected butyrate-producing bacteria in microbiota 1.2. The *Roseburia* spp./*E. rectale* group showed significantly less growth ($P<0.05$) and reached a lower relative abundance when treated with glycerol and inulin (1.0±0.1 log and 4.7±0.5%, respectively) than was observed in the control (1.2±0.0 log and 7.4±0.9%, respectively). Inulin treatment resulted in slightly decreased growth ($P<0.05$) of *F. prausnitzii*

(0.5±0.0 log) compared to the control (0.8±0.1 log). Growth and relative abundance of *E. hallii* in microbiota 1.2 were not affected by treatments and control (2.0±0.1 log and 0.5±0.1%, respectively). In contrast, cryoprotective treatments strongly impacted the growth of butyrate-producing bacteria in fresh microbiota 2. Growth of *Roseburia* spp./*E. rectale* group, which was present at low relative abundance (0.1±0.0-0.2±0.1%), was significantly enhanced ($P<0.05$) by treating with protective buffers containing glycerol or glycerol and inulin (1.3±0.1 and 1.4±0.2 logs, respectively) compared to the control (0.7±0.2 log). In contrast, glycerol and inulin decreased the growth and relative abundance of *F. prausnitzii* (0.4±0.1 log and 0.3±0.0%) compared to the control (0.8±0.1 log and 1.0±0.1%). Growth of *E. hallii* (2.0±0.2 logs) was not different between treatments and control.

After cryopreservation, growth of the *Roseburia* spp./*E. rectale* group in the control and microbiota 1.1 and 1.2 that had been treated with inulin was strongly impaired compared to fresh microbiota, as shown by a decrease in log gene copies after 24 h incubation (-0.9±0.2 – -0.2±0.1 log). In contrast, glycerol alone and in combination with inulin maintained growth and relative abundance of the *Roseburia* spp./*E. rectale* group in microbiota 1.1 to levels similar to fresh samples (1.0±0.0 and 1.0±0.1 log, respectively). For microbiota 1.2, growth after cryopreservation was also not recovered when glycerol alone or in combination with inulin was added. In preserved microbiota 2, the *Roseburia* spp./*E. rectale* group grew after all treatments and in the control. Growth was significantly enhanced ($P<0.05$) in the treatments containing glycerol (1.3±0.0 and 1.7±0.1 logs) compared to the control (0.8±0.1 log), as was observed with the fresh microbiota.

Growth of *F. prausnitzii* after effluent preservation strongly depended on treatment. Control and glycerol treatment resulted in significantly impaired growth ($P<0.05$) of *F. prausnitzii* in microbiota 1.1 and 1.2 (-0.4±0.0 – 0.1±0.1 log). In contrast, treatments containing inulin in microbiota 1.2 showed growth of *F. prausnitzii* (0.7±0.0 log) that was equal to or greater than growth in fresh microbiota fermentation. In effluent 2, *F. prausnitzii* treated with inulin alone or in combination with glycerol (1.3±0.1 and 1.4±0.1 logs, respectively) grew significantly better ($P<0.05$) than in fresh microbiota. In contrast, growth of *F. prausnitzii* in the control was significantly reduced ($P<0.05$) compared to fresh samples (0.4±0.1 log).

Cryopreservation and the presence or absence of cryoprotectants had no impact on growth of *E. hallii* in microbiota 2 compared to fresh microbiota of the same condition. With microbiota 1.1, the relative abundance of *E. hallii* increased up to 5-fold (1.1±0.1 – 1.8±0.4 %) after incubation of preserved compared to fresh microbiota. In contrast, preserved microbiota 1.2 showed significantly decreased growth of *E. hallii* ($P<0.05$) compared to fresh microbiota (1.7±0.1 – 2.0±0.1 logs).

Table 2.3 Growth and relative abundance of selected butyrate-producing bacteria after 24 h batch fermentation of effluent samples prior and post storage at -80°C.

	Effluent 1.1				**Effluent 1.2**				**Effluent 2**			
	Prior to freezing		Postfreezing		Prior to freezing		Postfreezing		Prior to freezing		Postfreezing	
Protective buffer	Δlog	Rel. ab.	Δlog	Rel. ab.	Δlog	Rel. ab.	Δlog	Rel. ab.	Δlog	Rel. ab.	Δlog	Rel. ab.
Roseburia* spp. /*E. rectale												
Glycerol & Inulin	1.0±0.0	6.3±0.6	1.0±0.1[a]	6.9±0.7[a]	1.0±0.1[a]	4.7±0.5[a]	0.1±0.1[ab]	1.4±0.3[ab]	1.3±0.0[a]	0.2±0.0	1.3±0.0[a]	0.1±0.0
Glycerol	0.9±0.2	3.3±2.6	1.0±0.0[a]	6.4±0.5[a]	1.2±0.1	6.5±0.6	-0.4±0.0[ab]	0.5±0.1[b]	1.4±0.2[a]	0.2±0.1[a]	1.7±0.1[a]	0.2±0.1[a]
Inulin	1.1±0.1	9.9±1.1	-0.2±0.1[b]	0.4±0.0[b]	1.2±0.0	6.5±0.6	-0.5±0.0[ab]	0.4±0.0[b]	0.7±0.1	0.1±0.0	0.8±0.1	0.0±0.0
Control	0.8±0.2	4.8±3.6	-0.6±0.3[b]	0.2±0.1	1.2±0.0	7.4±0.9	-0.9±0.2[b]	0.2±0.1[b]	0.7±0.2	0.1±0.0	0.8±0.1	0.0±0.0
F. prausnitzii												
Glycerol & Inulin	0.4±0.0	3.6±0.5	0.5±0.1[a]	1.1±0.1[b]	0.7±0.1	0.4±0.2[a]	0.6±0.1[a]	1.7±0.4[ab]	0.4±0.1[a]	0.3±0.0[a]	1.4±0.1[ab]	1.2±0.2[ab]
Glycerol	0.4±0.1	3.8±0.7	-0.4±0.0[ab]	0.1±0.0[b]	0.8±0.0	0.4±0.0 [a]	0.0±0.1[b]	0.5±0.1	0.8±0.0	0.8±0.1	1.1±0.0[b]	0.7±0.1[b]
Inulin	0.6±0.0	5.5±0.2[a]	0.8±0.2[a]	2.0±0.3[ab]	0.5±0.0[a]	0.5±0.0	0.7±0.0[ab]	2.5±0.1[ab]	1.0±0.0[a]	1.6±0.1[a]	1.3±0.1[ab]	1.2±0.3[a]
Control	0.5±0.0	4.1±0.7	0.1±0.0[b]	0.4±0.1[b]	0.8±0.1	0.5±0.0	0.1±0.1	0.6±0.1[b]	0.8±0.1	1.0±0.1	0.4±0.1[b]	0.1±0.0[b]
E. hallii												
Glycerol & Inulin	2.6±0.1	1.0±0.1	2.5±0.2	1.8±0.4[a]	2.1±0.0	0.5±0.1	2.0±0.1[a]	2.5±0.3b[ab]	2.2±0.0	0.6±0.1	2.2±0.1[a]	0.7±0.1[a]
Glycerol	2.0±0.4	0.3±0.2	2.3±0.0	1.2±0.0[b]	2.0±0.0	0.5±0.1	1.8±0.1[b]	1.8±0.1	1.8±0.2	0.3±0.1[a]	2.2±0.1[a]	0.7±0.1[ab]
Inulin	2.0±0.1	0.3±0.0	2.3±0.0[b]	1.2±0.2[b]	2.1±0.1	0.5±0.0	1.8±0.1[b]	1.7±0.1[b]	2.1±0.1	0.6±0.1	2.0±0.1	0.5±0.1
Control	1.9±0.0	0.2±0.0	2.2±0.0[b]	1.1±0.1[b]	1.9±0.2	0.4±0.1	1.7±0.1[b]	1.5±0.1[b]	2.1±0.1	0.6±0.2	1.9±0.1	0.3±0.1

Growth (Δlog) was determined as log increase during 24 h batch fermentation of effluents immediately after processing (t=0) and after 3 months storage at -80°C analyzed by qPCR. Relative abundance (rel. ab.) was determined as ratio of target gene of respective bacterial group/total bacteria (means and standard deviations of independent fermentation triplicates).

a. Indicates that growth respectively relative abundance in treatment is significantly different from the control within the same effluent microbiota ($p<0.05$)

b. Indicates that growth respectively relative abundance is significantly different prior and post freezing within the same treatment and effluent microbiota ($p<0.05$)

Discussion

Restoring the compositional balance of the intestinal microbiota by FMT has been proposed to treat a broad range of chronic intestinal diseases associated with microbial dysbiosis (Khoruts and Sadowsky, 2016). Artificial colonic microbiota transplants derived from intestinal fermentation technology could enhance availability, acceptability and safety, associated with fecal material. For the first time, we have described enhanced storage conditions for artificial colonic microbiota by cryopreservation with glycerol and inulin with focus on maintenance of selected butyrate-producing bacteria, which are associated with "sustained remission" of gastrointestinal diseases after FMT (Fuentes, et al., 2017).

Metabolite analyses of effluents derived from F1 and F2 identified two distinct SCFA profiles. The microbiota exhibited butyrogenic (F1) or propiogenic (F2) characteristics. The high fraction of butyrate of F1 effluents was associated with a microbial community dominated by Firmicutes harboring highly abundant populations of *Ruminococcaceae* and *Lachnospiraceae* including *F. prausnitzii* and the Roseburia spp./E. rectale group, respectively, next to other unknown butyrate producers. In F2, butyrate producers were also present but Bacteroidetes (mainly *Prevotellaceae*) were more abundant than Firmicutes. Bacteroidetes form propionate via succinate pathway, which is the predominant propionate pathway in adults (Reichardt, et al., 2014). These outcomes demonstrate that IFT can reproduce, at least to a certain extent, the initial microbiota profile of the fecal donor's microbiota. Furthermore, effluent microbiota composition could be modified by short-term pH increase. Many Firmicutes are more tolerant to low pH whereas Bacteroides spp. have growth advantages at higher pH (Flint, et al., 2012). The observed shift from Firmicutes to Bacteroidetes in F1.2 compared to F1.1 was coherent with an increase in propionate production.

Since direct testing of the preserved samples with molecular methods does not provide information on the activity status of the microbiota, we used adapted Macfarlane medium to investigate growth and metabolic activity of fresh and preserved microbiota during a 24 h batch fermentation. Batch fermentations are limited by restricted substrate supply and buffer capacity (Payne, et al., 2012). However, such fermentations allow investigation of bacterial viability and activity of the inoculum in a controlled and reproducible way. Modified Macfarlane medium contains a mix of SCFA to initiate growth of butyrate-producing bacteria such as *F. prausnitzii* and *R. intestinalis* (Duncan, et al., 2002), as well as complex glycans, which provides fuel for, and can be degraded by, trophic interactions of the gut microbiota. All three investigated butyrate-producers grew in batch cultures, as indicated by qPCR data. However, changes in relative abundances from effluent microbiota to batch fermentation indicate that the applied batch conditions are more favorable to *E. hallii* and the *Roseburia* spp./*E. rectale* group than to *F. prausnitzii*.

DMSO and glycerol are the most common protectants added to bacterial cells to enhance cryopreservation (Hubalek, 2003). However, low concentrations of DMSO might result in cellular

toxicity (Galvao, et al., 2014), which limits its use for FMT unless a removal step is used before transplantation. Additionally, DMSO alone showed no significant improvement on SCFA production by fecal microbiota after frozen storage and reactivation compared to the control without cryoprotectant (Kerckhof, et al., 2014). Glycerol prevents hydrogen bonding between water molecules and thus prevents formation of intracellular ice crystals during freezing (Koh, 2013). Glycerol is commonly added before freezing and storage of fecal microbiota for FMT at -80°C (Hamilton, et al., 2012). Here, we observed only minor losses (less than 10% of total SCFA production) after 24 h incubation when effluent microbiota was frozen in 15% glycerol, which is the recommended amount for freeze-storage of bacteria cultures (Koh, 2013). In our study, the addition of glycerol selectively enhanced survival of members of the *Roseburia* spp./*E. rectale* group. The diverse *Roseburia* spp./*E. rectale* group, encompassing species such as *Roseburia inulinivorans* or *Roseburia intestinalis*, is estimated to represent between 5-10% of the fecal microbiota (Aminov, et al., 2006, Louis and Flint, 2009). In addition to its cryoprotective action, glycerol may also serve as substrate for a broad range of microbes during reactivation (Engels, et al., 2016). Nevertheless, the ability to grow on glycerol is not widely shared among members of the *Roseburia* spp./*E. rectale* group. Out of 12 tested strains, only two strains of *Roseburia hominis* and one strain of *Roseburia ceciola* were able to utilize glycerol (Duncan, et al., 2006). The final glycerol concentration (2 mmol l^{-1}), transferred with the inoculum in batch culture, was also likely too low to induce a substrate effect. Furthermore, the positive effect of glycerol addition on SCFA formation was only observed after microbiota freezing, suggesting that glycerol acted as cryoprotectant of the *Roseburia* spp./*E. rectale* group rather than as a growth substrate in our experimental set-up. Our results together with other studies point to glycerol as a suitable cryoprotectant for microbiota transplant cryopreservation.

F. prausnitzii exhibited enhanced growth in batch fermentation after freezing and storage when the cryoprotective buffer was supplemented with inulin. Inulin-type fructans are not commonly used as cryoprotectants, despite being known as water-soluble natural cryoprotective agents synthesized by many plant, fungi and bacteria (Hubalek, 2003). Fructans directly interact with lipids of biological membranes and stabilize them under cold as well as dry conditions (Demel, et al., 1998, Vereyken, et al., 2003). Inulin can also serve as a fermentation substrate, as several *F. prausnitzii* strains can use inulin (Duncan, et al., 2002). In vivo, an increased inulin intake by 10 healthy volunteers led to significantly increased numbers of *F. prausnitzii* in fecal microbiota, which indicates that *F. prausnitzii* benefits from inulin addition even in the presence of a competitive microbiota (Ramirez-Farias, et al., 2009). Here, the presence of inulin alone, or glycerol and inulin in combination, also decreased the lag time of acetate and butyrate formation. However, the amount of inulin supplied with the inoculum was likely too little (25 mg l^{-1}) to impact final SCFA formation during fermentation. Nevertheless, inulin bound to the cell membranes during freezing could possibly act as an easily accessible nutrient source

to fulfill the increased nutritional demands of stressed bacterial cells immediately after reactivation (Ray and Speck, 1973).

E. hallii, with a high prevalence in adults (Engels, et al., 2016), showed the lowest initial abundance of all three targeted butyrate-producing bacteria in effluent microbiota from both fermentation systems. Nevertheless, growth was strongly induced during batch fermentation, resulting in a more than 100-fold increase. We reported similar increases for batch fermentation of effluents derived from IFT mimicking healthy elderly colonic microbiota (Fekry, et al., 2016). The competitiveness of *E. hallii* in batch fermentation could be due to its ability to feed on lactate and acetate (Duncan, et al., 2004, Belenguer, et al., 2006). Because growth was not impacted after cryopreservation, our results also suggest that E. hallii is highly resistant against damage caused by freezing.

Conclusion

This study demonstrated that combining glycerol and inulin in protective buffers provided a higher level of protection during cryopreservation of compositionally different, artificial colonic microbiota compared to single component application. Our data indicate that the functional group of butyrate-producing bacteria, which are important for gut health but also reported to be very sensitive to environmental conditions, can be well preserved with cryoprotective agents during storage at -80°C for at least three months. The metabolic activity of frozen effluent microbiota derived from IFT and intended for FMT can be adequately maintained. The methods and preservation conditions developed in this study will be useful for further research on storage of anaerobic gut microbes and for developing new microbial-based treatments of gastrointestinal disorders. Ultimately, preserved artificial microbiota need to be transplanted in vivo to test its effectiveness in a complex system involving the host and the presence of a competitive microbiota.

Experimental procedures

Experimental design

The freezing experiments were conducted with microbiota produced with two independent fermentation systems inoculated with immobilized fecal microbiota from different male adult donors (F1 and F2), mimicking conditions of the proximal colon. F1 effluents were used to conduct two freezing trials. The first trial was carried out with microbiota produced under standard fermentation condition (effluent 1.1) for adult proximal colon, at 37°C, pH 5.7 and a mean retention time of 8 h (Payne, et al. 2012). Before the second trial, the microbiota composition of F1 was modulated by applying short-term high pH stress (pH 9) to induce a lasting shift, leading to altered metabolic activity (effluent 1.2). A stabilization period at standard fermentation condition for one week was used after the pH shock before collecting effluent sample 2. With F2 one freezing trial was carried out (effluent 2). At time point 0 h of each trial, the collected microbiota material was divided in two portions and aliquots were mixed with peptone buffer with or without protective agents. The aliquots of the first portion served as a fresh control, and were used immediately to inoculate batch fermentation medium in a prior-freezing activity test (t_0). Immediately after inoculation and after 24 h of fermentation, samples were taken for DNA extraction and metabolite quantification. The aliquots of the other portion were subjected to cryopreservation for 3 months at -80°C (t_1), after which reactivation in batch fermentation medium for 24 h in a post-freezing activity test was performed. An additional activity test was performed to more closely monitor the kinetics of metabolite production after 3, 5 and 7 h incubation.

Fecal inoculum and immobilization

For each fermentation system, fresh feces from two different adult male donors (age 30-40 years), who had not been treated with antibiotics for the last 3 months, were assigned to immobilization procedure. After defecating, approximately 5 g fecal sample was transferred to a pre-weighted Falcon tube containing 5 ml sterile, pre-reduced peptone water (0.1%, pH 7; Thermo Fisher Diagnostics AG, Pratteln, Switzerland), and placed in an anaerobic jar (Anaerojar, Oxoid, Hampshire, England) to maintain anaerobic condition during transport. Before immobilization in the anaerobic chamber, peptone water was added to the fecal sample to obtain a final v/w ratio of 20%. Fecal microbiota was immobilized in 1-2 mm gellan-xanthan gel beads as described previously (Zihler Berner*, et al.*, 2013).

Production of complex colonic microbiota with intestinal fermentation technology

The gut microbiota used for cryopreservation tests was produced in two independent continuous colonic fermentation systems inoculated with immobilized adult gut microbiota. Sixty ml of freshly-prepared fecal beads were transferred to a glass bioreactor (Sixfors, Ismatec, Switzerland) containing 140 ml sterile nutritive medium, mimicking the chyme entering the colon (Macfarlane, et al., 1998),

and supplemented with a filter-sterilized vitamin solution (Michel, et al., 1998). In the first step, batch fermentations were carried out to colonize the beads (Fehlbaum, et al., 2015). The bioreactors were continuously flushed with CO_2 to maintain anaerobiosis in the fermentation system, while the temperature was kept at 37°C and the pH maintained at 5.7 by the addition of 2.5 M NaOH (Fehlbaum, et al., 2015). Fermented medium was replaced twice by fresh medium for batch fermentations until the base consumption started to decrease. The system was set to continuous mode after the third batch fermentation by generating a constant inflow of 25 ml h^{-1} medium, targeting a mean retention time of 8 h. This rapid turnover is distinctive for the proximal colon region, where high supply of nutrients promote bacterial growth leading to a dense and highly active microbiota for production of artificial gut microbiota and preservation tests (Payne, et al., 2012). After operating in continuous mode for 10 days, microbial composition in the system was stable for collection of effluent for cryopreservation, as indicated by stable base consumption, metabolites (HPLC-RI) and population composition (qPCR).

Harvesting, processing and cryopreservation of microbiota

Effluent microbiota were directly collected from the bioreactors through a septum using a 120 mm needle (VWR International AG, Dietikon, Switzerland) connected to a 20 ml syringe flushed with CO_2. Samples were transferred to a sterile pre-reduced 50 ml Falcon tube and transported to an anaerobic chamber (10% CO_2, 5% H_2 and 85% N_2; Coy Laboratories, Grass Lake, Michigan, USA) where all further steps were performed. Effluent biomass was harvested by centrifuging 1 ml aliquots at 10’000 x g for 4 min. The supernatant was decanted and the bacterial pellet was re-suspended in 50 µl protective medium. The mixture was then incubated for 30 min at room temperature, taking into account the penetration time of glycerol, before snap-freezing in liquid nitrogen and storage at -80 °C. Three microbiota samples of each protective medium were not frozen and instead immediately used for reactivation tests.

Preparation of cryoprotective buffers

Solutions of glycerol (15% w/v; VWR International AG, Switzerland), inulin (5% w/v; Orafti®, Switzerland) and a combination thereof were prepared in phosphate buffered peptone water (PB) (0.1% v/w; Thermo Fisher Diagnostics AG) adjusted to pH 6.8 and supplemented with the reducing agents cysteine-HCl and riboflavin (both Sigma-Aldrich, Switzerland) to protect the microbiota from potential oxygen exposure during processing and storage (Khan*, et al.*, 2014). All components of the protective solutions were placed in the anaerobic chambers overnight to remove traces of oxygen. PB was prepared in oxygen-free distilled water, previously boiled and bubbled with N_2 gas. Cryoprotective agents were mixed with PB, and cysteine-HCl and riboflavin were added to final concentrations of 1 g l^{-1} cysteine-HCl and 0.3 g l^{-1} riboflavin. PB supplemented with only cysteine and riboflavin served as control to the buffers containing cryoprotective agents. The mixtures were filter-sterilized, wrapped in aluminum foil to protect from light and kept in the anaerobic chamber until usage.

Preparation of batch fermentation medium

The medium used for activity tests was a nutritive medium designed to mimic the chyme entering the colon (Macfarlane*, et al.*, 1998), and adjusted to conditions in batch fermentation. Thus, the medium was supplemented with a SCFA mix (Duncan*, et al.*, 2002) and its buffer capacity was enhanced twofold by increasing the amount of KH_2PO_4 and $NaHCO_3$. The medium composition (g l^{-1}) was as follows: 1.0 cellobiose, 1.0 xylan, 1.0 arabinogalactan, 0.5 inulin, 1.0 soluble potato starch, 3.0 casein acid hydrolysate, 5.0 bacto^{TM} tryptone, 1.5 meat extract, 4.5 yeast extract, 4.0 mucine, 0.4 bile salt, 0.05 hemin, 0.61 $MgSO_4$, 0.1 $CaCl_2{\cdot}2H_2O$, 0.2 $MnCl_2{\cdot}4H_2O$, 0.005 $FeSO_4{\cdot}7H_2O$, 0.1 $ZnSO_4{\cdot}7H_2O$, 2.0 KH_2PO_4, 6.0 $NaHCO_3$, 4.5 NaCl, and 4.5 KCl. One ml of each of Tween 80 and vitamin solution (Michel*, et al.*, 1998) were also added. Short-chain fatty acids were added to supply initial nutrients (Duncan*, et al.*, 2002) to give the following final concentrations: acetate (33 mM); propionate (9 mM); isobutyrate, isovalerate, and valerate (1 mM each). All components of the nutritive medium were purchased from Sigma-Aldrich, except for inulin, bile salts (Thermo Fisher Diagnostics AG), tryptone (Becton Dickinson, Allschwil, Switzerland) and KH_2PO_4 (VWR International AG, Switzerland). The pH of the medium was adjusted to 6.8 with 2.5 M NaOH. Cysteine was added to the medium to a final concentration of 1.0 g l^{-1} after boiling and gassing with CO_2. The medium was dispensed in 20-ml portions under flowing CO_2 into 50 ml serum flasks containing magnetic stirrers, and closed with butyl septum stoppers and aluminum caps before autoclaving.

Reactivation in batch fermentation

Three aliquots of each protective medium were excluded from snap-freezing and immediately underwent reactivation in a batch fermentation to serve as fresh reference in a prior-freezing activity test (t_0). Therefore, bacterial pellets mixed with either control or protective buffers were resuspended in 950 µl of batch fermentation medium to regain the initial concentration of the fermentation effluents. Each aliquot (0.2 ml) was inoculated into 20 ml of anaerobic batch fermentation medium in 50 ml serum flasks. The flasks were incubated at 37°C for 24 h under continuous stirring at 40 rpm. Portions (1.5 ml) of fresh (0 h) or fermented medium (24 h) were removed, and centrifuged at 10’000 x g for 5 min at 4°C. The supernatant was stored at -20°C for HPLC-IR analysis, while the microbial pellet was stored at -80°C for DNA extraction. After storing for 3 months at -80°C, three aliquots of each protective buffer were transferred to the anaerobic chamber and thawed on ice, followed by the same reactivation procedure as described above. A similar procedure was used for testing the metabolite kinetics of preserved effluents; samples were taken for HPLC-IR analysis after 3, 5 and 7 h of incubation.

Quantification of butyrate-producing bacteria

To investigate re-establishment of the major butyrate-producing bacteria, total genomic DNA from the stored pellets was extracted with the FastDNA SPIN kit for soil (MP Biomedicals, Illkirch Cedex, France).

Butyrate-producing bacteria were enumerated by quantitative PCR using primers targeting *butCoA* of *F. prausnitzii* or the *Roseburia* spp./*E. rectale* group, or the 16S rRNA gene of *E. hallii* (Table S2.4). The qPCR master mix contained 2x SYBR Green Mastermix (Life Technologies, Labgene scientific instruments, Zug, Switzerland), 0.2 µM of each forward and backward primer, and 1 µl of template genomic DNA in a total volume of 25 µl. The amplification started with a denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Melting curve analysis was performed to verify the specificity of amplification. The samples were analysed in duplicate. Standard curves were generated from ten-fold dilution series (10^2 to 10^8 copies) of linearized plasmids containing the target genes. Relative abundance was calculated as the ratio of target gene relative to total bacteria 16S rRNA gene copies. *butCoA* gene copies were normalized to five 16S rRNA gene copies to account for several 16S rRNA gene copies in the genomes (Vital*, et al.*, 2013).

Short chain fatty acid analysis by HPLC-RI

The main SCFAs acetate, propionate, butyrate and two intermediate products, lactate and formate, were measured in fermentation effluents as well as in batch fermentation samples using a high-performance liquid chromatograph (Hitachi LaChrome, Merck, Dietikon, Schwitzerland) equipped with an Aminex HPX-87H column (300 x 7.8 mm; BioRad, Reinach, Basel-Land, Switzerland) and a refractive index detector as described previously (Fehlbaum, et al., 2015). Samples were centrifuged at 13 000 x g for 5 min at 4°C. Undiluted supernatants were filtered through a 0.45 µm nylon membrane filter into HPLC vials and closed with crimp-caps. Supernatants (40 µl injection volume) were eluted with 10 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} at 40°C. SCFAs, lactate and formate were quantified using external standards.

Microbiota profiling with 16S rRNA sequencing

The microbial community was analyzed in fecal samples and fermentation effluents. The V4 hypervariable 16S region was amplified using specific primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sequencing was carried out on an Illumina MiSeq (StarSEQ, Mainz, Germany) using V4 chemistry for 2 x 250 bp read length. Raw sequencing reads were processed by merging the paired reads using USEARCH (v8.1.1756) with a minimum length of the merged read of 100 bp, an expected error threshold of 1 and minimum overlapping of 15 bp. Raw sequencing reads were filtered using PRINSEQ-lite (v0.20.4) based on selected quality criteria such as: (1) no ambiguous bases; (2) read lengths between 247 and 257 base pairs (bp); (3) the average quality score at 10 bp and a complexity threshold of 10. Sequences that passed quality filtering were clustered into OTUs at 97% identity level using UPARSE (usearch v8.0.1623). Representative sequences (the most abundant) for each OTU were aligned using PyNAST (QIIME-1.8.0) and taxonomically assigned using UTAX (usearch v8.1.1756).

Statistics

Statistical analysis of the HPLC and qPCR data (log_{10}-transformed) was done using IBM SPSS Statistics 23.0 (IBM SPSS Inc, Armonk, New York, USA). Concerning the HPLC data, the initial acetate and propionate concentration present in the medium were subtracted from the concentrations measured after 3, 5, 7 and 24 h batch fermentation. Data are expressed as means ± SD of three different batch fermentations inoculated with three aliquots from the same treatment or control. Data were tested for normal distribution using the Shapiro-Wilk test and equality of variance was assessed with the Levene test. ANOVA tests were performed on every treatment to compare qPCR and HPLC data. Treatments were compared to control with a Dunnett (two-sided) test. A non-parametric Kruskal-Wallis test was performed when data were not normally distributed or the assumption of equality of variance was violated. A paired sample t-test was performed to compare data prior to freezing with post-freezing data within one treatment. Differences were considered significant for the risk $\alpha \leq 0.05$.

Acknowledgements

16S rRNA gene amplicon libraries were analysed in collaboration with the Genetic Diversity Centre (GDC), ETH Zurich.

Supporting information

Table S2.1. Relative abundance in percentage of microbial phyla in the fermentation effluents used for cryopreservation and corresponding donors.

	Donor 1	Effluent 1.1	Effluent 1.2	Donor 2	Effluent 2
Firmicutes	47.2	79.5	67.0	57.3	33.9
Bacteroidetes	27.9	9.1	18.5	32.7	48.7
Actinobacteria	7.2	5.2	9.7	7.2	8.6
Proteobacteria	2.5	6.2	4.8	1.1	8.8
Verrucomicrobia	5.9	0.0	0.0	0.0	0.0
Euryarchaeota	8.6	0.0	0.0	1.6	0.1
Others <1%	0.7	0.0	0.0	0.0	0.0

Table S2.2 Relative abundance (in percentage) of microbial families in the fermentation effluents used for cryopreservation and corresponding donors.

	Donor 1	Effluent 1.1	Effluent 1.2	Donor 2	Effluent 2
Enterococcaceae	0.0	0.0	2.1	0.0	2.3
Lactobacillaceae	0.0	0.0	1.2	0.1	0.9
Clostridiumes	5.8	6.8	5.3	7.5	2.9
Clostridiaceae	2.0	0.3	0.4	1.1	0.5
Lachnospiraceae	6.0	36.9	32.5	15.9	9.5
Peptostreptococcacea	2.2	3.5	0.3	1.3	0.0
Ruminococcaceae	28.4	29.5	23.2	26.2	15.3
Veillonellaceae	1.1	2.0	1.5	2.1	1.9
Erysipelotrichaceae	0.1	0.2	0.1	2.4	0.1
Other Firmicutes > 1%	1.4	0.3	0.2	0.6	0.3
Barnesiellaceae	1.4	0.0	0.2	0.8	0.0
Odoribacteraceae	1.1	0.0	0.0	1.8	0.0
Paraprevotellaceae	0.0	0.0	0.0	2.0	4.9
Bacteroidaceae	15.6	7.5	17.6	8.4	1.8
Porphyromonadaceae	1.6	1.5	0.5	1.7	0.2
Prevotellaceae	0.1	0.0	0.1	14.6	41.7
Rikenellaceae	5.9	0.0	0.1	3.4	0.0
S24-7	2.2	0.0	0.0	0.0	0.0
Bifidobacteriaceae	3.8	2.8	7.1	4.6	8.1
Coriobacteriaceae	3.4	2.4	2.5	2.6	0.4
Alcaligenaceae	0.3	6.1	4.0	0.8	1.1
Desulfovibrionaceae	1.9	0.0	0.0	0.1	2.1
Enterobacteriaceae	0.2	0.0	0.0	0.1	3.1
Xanthomonadaceae	0.0	0.0	0.0	0.0	2.4
Verrucomicrobiaceae	5.9	0.0	0.0	0.0	0.0
Methanobacteriaceae	8.6	0.0	0.0	1.6	0.1
Others <1%	1.0	0.1	0.8	0.2	0.1

Table S2.3 Concentration of major SCFAs in fermentation effluents used for cryopreservation.

Metabolite concentration [mM][a]	Effluent 1.1	Effluent 1.2	Effluent 2
Acetate	62.3	43.1	71.1
Propionate	12.5	19.0	36.6
Butyrate	46.8	56.6	29.3
Total SCFA	121.6	118.7	136.9

a. Metabolite concentration of main SCFA from single measurement

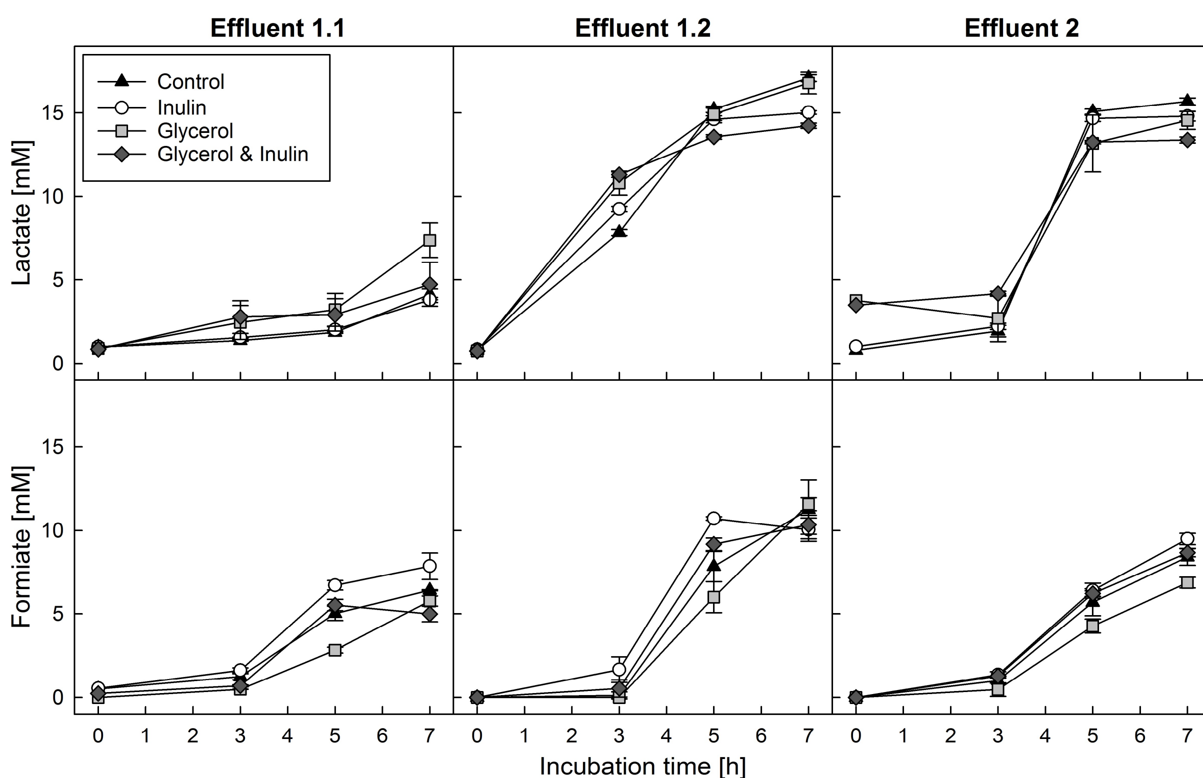

Fig. S2.1 Kinetics of production of intermediate metabolites after reactivation of long-term storage of microbiota. Formate and lactate were analyzed by HPLC-RI after reactivation of stored microbiota in batch fermentation. Each point represents the average of three replicates with standard deviation

Table S2.4 Primers used to quantify butyrate-producing bacteria within the complex microbiota

Primers	Sequence	Target gene	Reference
G_Fprsn_F	gacaagggccgtcaggtcta	*Faecalibacterium praunitzii but*	Vital *et al.* (2013)
G_Fprsn_R	ggacaggcagatRaagctcttgc		
G_RosEub_F	tcaaatcMggIgactgggtWga	*Roseburia* spp./*E.rectale* group *but*	Vital *et al.* (2013)
G_Ros_R	tcgataccggacatatgccaKgag		
G_Eub_R	tcataaccgcccatatgccatgag		
EhalF	gcgtaggtggcagtgcaa	*Eubacterium hallii* 16S rRNA gene	Ramirez-Farias *et al.* (2008)
EhalR	gcaccgragcctatacgg		
Eub 338F	actcctacgggaggcag	Total Bacteria 16S rRNA	Guo *et al.* (2008)
Eub 518R	attaccgcggctgctgg		

Chapter 3

Effect of cryopreservation and lyophilization on viability and growth of strict anaerobic human gut microbes

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Abstract

Strict anaerobic gut microbes have been suggested as "next-generation probiotics" for treating several intestinal disorders. The development of preservation techniques is of major importance for therapeutic application. The present study investigated cryopreservation (-80°C) and lyophilization survival and storage stability (4°C for three months) of the strict anaerobic gut microbes *Bacteroides thetaiotaomicron*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Anaerostipes caccae*, *Eubacterium hallii* and *Blautia obeum*. To improve preservation survival, protectants sucrose and inulin (both 5% w/v) were added for lyophilization, and were also combined with glycerol (15% v/v) for cryopreservation. Bacterial fitness, evaluated by maximum growth rate and lag phase, viability and membrane integrity were determined using a standardized growth assay and by flow cytometry as markers for preservation resistance.

Lyophilization was more detrimental to viability and fitness than cryopreservation, but led to better storage stability. Adding sucrose and inulin enhanced viability and the proportion of intact cells during lyophilization of all strains. Viability of protectant-free *B. thetaiotaomicron*, *A. caccae* and *F. prausnitzii* was above 50% after cryopreservation and storage and increased to above 80% if protectants were present. The addition of glycerol, sucrose and inulin strongly enhanced the viability of *B. obeum*, *E. hallii* and *R. intestinalis* from 0.03 – 2% in protectant-free cultures to 11 – 37%.

This is the first study that quantitatively compared the effect of cryopreservation and lyophilization and the addition of selected protectants on viability and fitness of six strict anaerobic gut microbes. Our results suggests that efficiency of protectants is process- and species-specific.

Introduction

According to the WHO/FAO, probiotics are defined as "live microorganisms, that when administered in adequate amounts confer a health benefit on the host". This definition implies viability as an important characteristic of probiotics for efficiency. Processing and preservation procedures that guarantee a high yield of viable cells are therefore essential for probiotic application. Recently, interest in probiotic research expanded from the classical probiotic *Lactobacillus* and *Bifidobacteria* species to a more targeted manipulation of the host gut microbiota with "personalized probiotic therapies" using functional important strict anaerobic gut microbes. The administration of specific bacterial strains could address distinct differences in colonic microbiota profiles associated with intestinal diseases (Vieira*, et al.*, 2016). Strict anaerobic butyrate-producing bacteria have been proposed as "next generation probiotics" in the treatment of intestinal disorders (Van Immerseel*, et al.*, 2010). Butyrate, produced during bacterial fermentation, is an important short-chain fatty acid (SCFA) providing several benefits to the host (Tan*, et al.*, 2014). *Clostridia cluster* IV and XIVa, including the highly abundant butyrate-producing *Faecalibacterium prausnitzii and Eubacterium rectale/Roseburia* spp*.,* harbor promising candidates for future probiotics (Louis and Flint, 2009, Hsiao*, et al.*, 2014, Miquel*, et al.*, 2015, Udayappan*, et al.*, 2016, Tamanai-Shacoori*, et al.*, 2017). The selection of "next-generation probiotics" however, is not limited to butyrate producers. Propionate-producers such as *Bacteroides*, can beneficially effect the host by interacting with the immune system and by maintaining host-microbiota homeostasis and might therefore contain species for future therapeutic administration (Wrzosek*, et al.*, 2013, El Hage*, et al.*, 2017).

The two main long-term preservation methods for microbes are cryopreservation and lyophilization (Prakash*, et al.*, 2013). Both techniques are well established for many aerobes or facultative anaerobes (Hubalek, 2003). However, data on preservation of strict anaerobic gut microbes are limited (Staab and Ely, 1987, Khan*, et al.*, 2014). Current preservation procedures were mainly designed for culture collections when survival of only a minor proportion of cells is required (Malik, 1992). To improve survival of preserved cells, protective agents are commonly added to minimize freezing and drying injuries. In cryopreservation, glycerol is one of the most common used penetrating protectant; it is nontoxic even at high concentrations (Meryman, 2007). Intracellular glycerol can stabilize cells during slow freezing by minimizing, or delaying osmotic derived shrinkage off the cells to a lower temperature (Fowler and Toner, 2005). It has been suggested that glycerol can prevent damage due to increased osmotic pressure, since the presence of glycerol can reduce the excessive increase in salt concentration in fractions of unfrozen water during freezing (Lovelock, 1953). In contrast, in lyophilization glycerol is less suitable as protectants, because it can lead to an insufficiently dried and sticky product at high concentrations (Abadias*, et al.*, 2001). Sugars are another classical group of protectants used in cryopreservation and lyophilization. Disaccharides, such as maltose, sucrose and trehalose are able to induce shrinkage of the cells by osmosis-derived dehydration before freezing thereby reducing

intracellular ice formation (Fowler and Toner, 2005). Sucrose has been frequently used for cryopreservation of microorganism (Hubalek, 2003) and improved tolerance to drying by protecting proteins from denaturation in the absence of water (Leslie*, et al.*, 1995). Inulin-type fructans are non-penetrating, water-soluble protective agents that are applied in lyophilization (Hubalek, 2003). The protective action is exerted extracellularly by direct interaction and stabilization of membrane lipids under dry and cold conditions (Demel*, et al.*, 1998, Vereyken*, et al.*, 2003, Schwab*, et al.*, 2007). Fructans can serve as bulking agent and protective matrix during lyophilization (Khan*, et al.*, 2014). Combining compounds with different protective mechanisms can result in greater protection of microorganisms during freezing and drying than single-component application, due to additive or synergic protective effects (Hubalek, 2003). We recently showed, that a combination of glycerol (15% v/v) and inulin (5% v/w) maintained viability and activity of the strict anaerobic, butyrate-producing microbes, *F. prausnitzii*, *Roseburia* sp./*E. rectale* group and *Eubacterium hallii* in complex “artificial” gut microbial communities during 3 months storage at -80°C (Bircher*, et al.*, 2017).

In this work, we investigated preservation of strict anaerobic gut microbes after freezing at -80°C and after lyophilization, and subsequent storage at 4°C for 3 months. Six strains were selected for preservation trials: *Roseburia intestinalis*, *F. prausnitzii*, *E. hallii, Anaerostipes caccae, Blautia obeum* and *Bacteroides thetaiotaomicron* are highly abundant representatives of human gut microbial butyrate, and propionate producers. Bacterial fitness, evaluated by maximum growth rate and lag phase, and viability were tested during processing under strict anaerobic conditions and storage using different buffers containing nontoxic protectants glycerol, sucrose and inulin to improve freezing and freeze-drying resistance.

Results

Impact of protectants on fresh cultures

The effect of the protectants inulin and sucrose alone (SI, bot 5% w/v) or in combination with glycerol (GSI, 15% v/v) on viability (MPNs, percentage of intact cells) and fitness (μ_{max} and t_{lag}) of fresh *B. thetaiotaomicron*, *B. obeum*, *R. intestinalis*, *E. hallii*, *F. prausnitzii* and *A. caccae* cultures (t_0) was evaluated after 30 min anaerobic incubation in the corresponding protective medium, and compared to a control lacking protectants.

For all tested strains, SI treated cultures did not differ from that of control cultures (Table 3.1, 3.2 and Fig. 3.1), except *A. caccae* with higher percentage of intact cells after incubation in SI than in the control (83±6% and 63±8%, respectively). In contrast, significant differences in viability and fitness of fresh cultures were observed when SI was combined with glycerol (GSI) for some strains. The MPN for *R. intestinalis* cultures was approximately 10-fold lower with GSI than in the control (7.9±0.2 and 9.0±0.3 log cells ml^{-1}, respectively), the percentage of intact cells was reduced (57±5% and 104±6%, respectively) and t_{lag} was significantly increased (3.3±0.1 and 1.3±0.1 h, respectively). Similarly, GSI treated *F. prausnitzii* exhibited a three times longer t_{lag} (3.6±0.1 and 1.3±0.1 h, respectively), a lower fraction of intact cells (65±2% and 83±7%, respectively), and a significantly reduced MPN (7.4±0.3 and 8.0±0.2 log cells ml^{-1}, respectively) compared to the control. For *B. thetaiotaomicron* treated with GIS t_{lag} was significantly increased (1.3±0.6 and 0.7±0.1 h, respectively) and the percentage of intact cells was reduced (60±5% and 87±11 %, respectively) compared to the control, but MNP was not different. GIS treatment also decreased μ_{max} of *E. hallii* compared to the control (0.23±0.02 and 0.36±0.02 OD unit h^{-1}, respectively).

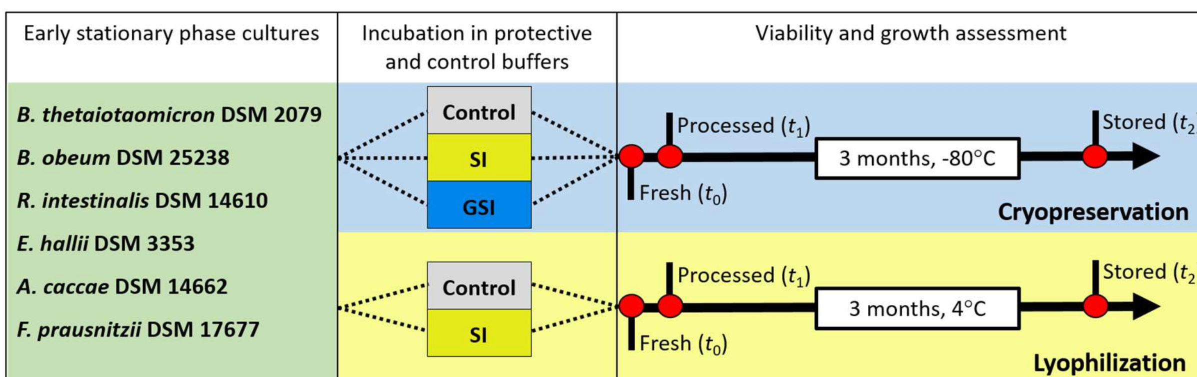

Fig. 3.1 Set-up of preservation experiments. Early stationary phase cultures were incubated for 30 min in buffers containing sucrose and inulin (SI), sucrose, inulin and glycerol (GSI) and in control buffer lacking protectants (control) before processing for preservation. Viability and growth were assessed at three different time points indicated by red dots. The first assessment was performed with fresh cultures after incubation in the protective and control buffers (t_0). The second assessment was conducted with the processed cultures immediately after freezing, respectively, lyophilization (t_1) and the third assessment with cryopreserved and lyophilized samples stored for 3 months at -80°Cor4°C, respectively (t_2).

Bacterial response to cryopreservation in the absence of protectants

The effect of cryopreservation and frozen storage at -80°C for 3 months on viability and fitness of the investigated strains was evaluated by comparing processed (t_1) and stored (t_2) control cultures lacking protectants with the fresh control culture (t_0).

R. intestinalis, *E. hallii* and *B. obeum* were strongly impacted by cryopreservation, indicated by significantly lower viable cell counts and increased t_{lag} in the processed and stored samples compared to fresh control cultures (Table 3.1). *R. intestinalis* and *E. hallii* exhibited a 100-fold lower MPN after storage (7.1±0.2 and 6.8±0.6 log ml^{-1}, respectively) compared to the fresh control (9.0±0.3 and 8.4±0.2 log ml^{-1}, respectively), along with a 5 to 6 fold increased t_{lag} (Table 3.2). The proportion of intact *E. hallii* cells declined from 71±9% in fresh to 3±0% in stored control culture. *B. obeum* was the most sensitive strain towards freezing. Its MPN was strongly reduced from 7.9±0.4 log ml^{-1} in fresh to 5.7±0.7 log ml^{-1} after processing (Table 3.1), and a further decline after storage (4.5±0.6 log ml^{-1}). Consistently, t_{lag} significantly increased from 6.4±0.2 h in the fresh to 19.7±0.8 h in the stored *B. obeum* culture. The effect of cryopreservation on μ_{max} was species dependent. *B. obeum* and *E. hallii* exhibited an increase and *R. intestinalis* a decrease of μ_{max} after processing and storage (0.26±0.03, 0.47±0.03 and 0.22±0.02 OD unit h^{-1}, respectively) compared to fresh (0.14±0.03, 0.36±0.04 and 0.29±0.02 OD unit h^{-1}, respectively) (Table 3.2).

B. thetaiotaomicron, *F. prausnitzii* and *A. caccae* were less impacted by freezing and storage as indicated by stable or little changed MPN of fresh, processed and stored cultures (Table 3.1). *B. thetaiotaomicron* was least sensitive, since the percentage of intact cells was not affected during storage (Fig. 3.2) although t_{lag} was slightly but significantly increased after freezing (1.1±0.1 h) compared to fresh culture (0.7±0.1 h). In contrast, cryopreservation and storage reduced the fraction of intact *F. prausnitzii* and *A. caccae* cells from 83±7% and 63±8% in the fresh to 27±4% and 10±3 %, respectively, in the stored samples, along with a significantly increase of t_{lag} (Table 2). A decrease of μ_{max} from fresh to processed and stored cultures was measured for all three strains (Table 2).

Impact of protectants on cryopreserved cultures

The protective effect of the non-penetrating agents inulin and sucrose alone (SI) or in combination with the penetrating glycerol (GSI) on viability and fitness of cryopreserved strains was evaluated during freezing and storage at -80°C. Processed (t_1) and stored (t_2) SI and GSI treated cultures were compared to fresh cultures (t_0) as well as processed (t_1) and stored (t_2) control samples without protectants.

SI improved viability and fitness of the stored freezing-sensitive strains, while glycerol in the protective medium (GSI) further enhanced the protective effect (Supplementary Fig. S3.1, Table 3.1 and 3.2). The viable cell counts of *R. intestinalis* and *B. obeum* stored in SI (7.7±0.3 and 6.1±0.4 log ml^{-1}) and GSI (8.3±0.2 and 7.0±0.4 log ml^{-1}) were significantly higher than without protectants (7.1±0.2 and 4.5±0.6 log ml^{-1}). Both strains exhibited similar t_{lag} with SI and GSI which was significantly lower than in the

stored control (Table 3.2). Viable cell counts and t_{lag} of stored *E. hallii* were also increased and decreased, respectively, with GSI (7.9±0.4 log ml^{-1} and 5.3±0.8 h) compared to the control (6.8±0.6 log ml^{-1} and 8.1±2.5 h), no effect was shown with SI. The highest fraction of intact cells was obtained with GSI (73±3%), compare to SI (47±5 %) and the stored control (3±0%). Unexpectedly, μ_{max} of GSI treated *E. hallii* after storage (0.30±0.02 OD unit h^{-1}) was significantly lower than for the control (0.47±0.03 OD unit h^{-1}).

The positive impact of protectants was less distinct during processing than during storage. Only small differences in viability and t_{lag} of the freezing-sensitive strains were observed between the processed control, SI and GSI treated samples, except for *B. obeum* with higher MPN in SI (7.1±0.5 log ml^{-1}) and GSI (7.7±0.04 log ml^{-1}) than the control (5.7±0.7 log ml^{-1}).

Viability and fitness of *B. thetaiotaomicron*, *F. prausnitzii* and *A. caccae*, which were less sensitive towards cryopreservation, were little impacted by the addition of SI and GSI. MPNs of *B. thetaiotaomicron* and *A. caccae* did not differ between fresh, processed and stored cultures, independent from the addition of protectants. MPN of *F. prausnitzii* remained stable after cryopreservation and storage in the GSI and control samples, but were significantly lower in SI treated culture after storage (7.6±0.3 log ml^{-1}) compared to the fresh SI culture (8.3±0.3 log ml^{-1}).

Table 3.1 Impact of protectants and cryopreservation on cell viability of fresh (t_0), processed (t_1) and stored (t_2) bacteria. Log viable cell counts ml^{-1} in the fresh culture, after freezing in liquid nitrogen and cryopreservation for 3 months at -80°C, assessed with the most probable number method (MPN). Recovery rate of viable cells (in %) was calculated relative to the average viable cell counts in the fresh control (vs. control t_0) and fresh treatment culture (vs. t_0).

	Control		SI		GSI	
Culture condition	MPN (ml^{-1})	Recovery vs. t_0	MPN (ml^{-1})	Recover vs. control t_0/t_0	MPN (ml^{-1})	Recovery vs. control t_0/t_0
B. thetaiotaomicron						
Fresh (t_0)	9.3 ± 0.2		9.4 ± 0.2	135	8.9 ± 0.3	45
Processed (t_1)	9.2 ± 0.3	85	9.3 ± 0.2	114/84	8.8 ± 0.6	31/70
Stored (t_2)	9.2 ± 0.2	93	9.3 ± 0.2	100/91	9.0 ± 0.2	60/134
B. obeum						
Fresh (t_0)	7.9 ± 0.4		7.8 ± 0.4	79	8.1 ± 0.2	139
Processed (t_1)	5.7 ± 0.7 [b]	1	7.1 ± 0.5 [a]	15/19	7.7 ± 0.4 [a]	57/41
Stored (t_2)	4.5 ± 0.6 [b]	0.03	6.1 ± 0.4 [ab]	2/2	7.0 ± 0.4 [a]	11/8
R. intestinalis						
Fresh (t_0)	9.0 ± 0.3		8.9 ± 0.4	82	7.9 ± 0.2 [a]	9
Processed (t_1)	8.4 ± 0.4 [b]	27	8.4 ± 0.3	27/33	8.3 ± 0.3	23/267
Stored (t_2)	7.1 ± 0.2 [b]	1	7.7 ± 0.3 [ab]	6/7	8.3 ± 0.2 [a]	21/247
E. hallii						
Fresh (t_0)	8.4 ± 0.2		8.4 ± 0.2	100	8.3 ± 0.2	81
Processed (t_1)	7.6 ± 0.1 [b]	16	7.8 ± 0.2 [b]	25/25	8.1 ± 0.3 [a]	52/64
Stored (t_2)	6.8 ± 0.6 [b]	2	7.1 ± 0.4 [b]	6/6	7.9 ± 0.4 [a]	37/45
A. caccae						
Fresh (t_0)	8.8 ± 0.3		8.9 ± 0.2	122	8.6 ± 0.4	63
Processed (t_1)	8.4 ± 0.2 [b]	39	8.9 ± 0.2 [a]	104/85	8.7 ± 0.2	69/109
Stored (t_2)	8.5 ± 0.2	49	8.8 ± 0.3	96/78	8.8 ± 0.3	95/152
F. prausnitzii						
Fresh (t_0)	8.0 ± 0.2		8.3 ± 0.3	181	7.4 ± 0.3 [a]	27
Processed (t_1)	7.9 ± 0.4	76	7.8 ± 0.3 [b]	64/36	7.1 ± 0.3 [a]	12/46
Stored (t_2)	7.9 ± 0.2	80	7.6 ± 0.3 [b]	45/25	7.2 ± 0.2 [a]	16/60

[a] Viable cell counts in samples with cryoprotectants are significantly different from the control samples within the same condition ($p<0.05$)

[b] Viable cell counts after processing and after storage are significantly different from the viable cell counts of the fresh culture within the same treatment ($p<0.05$)

Table 3.2 Impact of protectants and cryopreservation on fitness of fresh (t_0), processed (t_1) and stored (t_2) bacteria. Lag phase (t_{lag}) and maximum growth rate (μ_{max}) of gut microbes after freezing in liquid nitrogen and cryopreservation for 3 months at –80°C were calculated from optical density growth curves based on Baranyi's equation.

		Control		SI		GSI	
Organism	Culture condition	t_{lag} (h)	μ_{max} (OD*h^{-1})	t_{lag} (h)	μ_{max} (OD*h^{-1})	t_{lag} (h)	μ_{max} (OD*h^{-1})
B. thetaiotaomicron	Fresh (t_0)	0.7 ± 0.1	0.23 ± 0.01	0.7 ± 0.1	0.22 ± 0.01 [a]	1.3 ± 0.6 [a]	0.22 ± 0.01 [a]
	Processed (t_1)	1.1 ± 0.1 [b]	0.22 ± 0.01	0.8 ± 0.1 [b]	0.20 ± 0.01 [a]	1.6 ± 0.2	0.20 ± 0.01 [a]
	Stored (t_2)	0.8 ± 0.3	0.18 ± 0.04 [b]	0.5 ± 0.3	0.16 ± 0.04 [b]	1.2 ± 0.7	0.17 ± 0.04 [b]
B. obeum	Fresh (t_0)	6.4 ± 2.2	0.14 ± 0.02	7.0 ± 2.1	0.14 ± 0.02	7.3 ± 1.1	0.13 ± 0.03
	Processed (t_1)	15.1 ± 1.0 [b]	0.23 ± 0.01 [b]	13.7 ± 2.5 [b]	0.17 ± 0.05	13.0 ± 2.5 [b]	0.11 ± 0.02 [a]
	Stored (t_2)	19.7 ± 0.8 [b]	0.26 ± 0.03 [b]	13.5 ± 0.7 [ab]	0.21 ± 0.03 [ab]	16.1 ± 2.1 [ab]	0.21 ± 0.03 [ab]
R. intestinalis	Fresh (t_0)	1.6 ± 0.1	0.29 ± 0.02	1.6 ± 0.1	0.28 ± 0.01	3.3 ± 0.1 [a]	0.26 ± 0.01
	Processed (t_1)	3.2 ± 0.3 [b]	0.25 ± 0.01 [b]	3.1 ± 0.1	0.25 ± 0.01 [b]	3.1 ± 0.1	0.27 ± 0.00 [a]
	Stored (t_2)	9.2 ± 0.4 [b]	0.22 ± 0.02 [b]	4.7 ± 0.4 [ab]	0.18 ± 0.02 [ab]	4.4 ± 0.5 [a]	0.22 ± 0.01 [b]
E. hallii	Fresh (t_0)	1.7 ± 0.8	0.36 ± 0.04	1.5 ± 0.0	0.34 ± 0.02	1.3 ± 0.2	0.23 ± 0.02 [a]
	Processed (t_1)	4.1 ± 0.2	0.43 ± 0.09	3.4 ± 0.6 [a]	0.41 ± 0.11	1.9 ± 0.6 [a]	0.21 ± 0.04 [a]
	Stored (t_2)	8.1 ± 2.5 [b]	0.47 ± 0.03 [b]	7.3 ± 1.3 [ab]	0.43 ± 0.03 [ab]	5.3 ± 0.8 [ab]	0.30 ± 0.02 [ab]
A. caccae	Fresh (t_0)	1.5 ± 0.1	0.20 ± 0.03	1.3 ± 0.2	0.19 ± 0.03	2.0 ± 0.3	0.21 ± 0.02
	Processed (t_1)	2.4 ± 0.2 [b]	0.15 ± 0.03 [b]	1.8 ± 0.5 [b]	0.17 ± 0.02	2.2 ± 0.1 [a]	0.13 ± 0.02 [b]
	Stored (t_2)	3.3 ± 0.6 [b]	0.16 ± 0.02	2.2 ± 0.2 [ab]	0.16 ± 0.00 [b]	2.1 ± 0.3 [a]	0.14 ± 0.01 [b]
F. prausnitzii	Fresh (t_0)	1.3 ± 0.1	0.05 ± 0.00	1.4 ± 0.1	0.05 ± 0.00	3.6 ± 0.1 [a]	0.05 ± 0.00 [a]
	Processed (t_1)	2.5 ± 0.2	0.05 ± 0.01	3.3 ± 0.2 [a]	0.06 ± 0.00	4.5 ± 0.3 [a]	0.06 ± 0.00 [a]
	Stored (t_2)	5.0 ± 1.1 [b]	0.02 ± 0.00 [b]	5.4 ± 1.4 [b]	0.03 ± 0.00	5.5 ± 1.2 [b]	0.03 ± 0.00 [a]

[a] Lag phase or growth rate in samples with cryoprotectants are significantly different from the control samples within the same condition ($p<0.05$)
[b] Lag phase or growth rate after processing and after storage are significantly different (B) from the fresh culture within the same treatment ($p<0.05$)

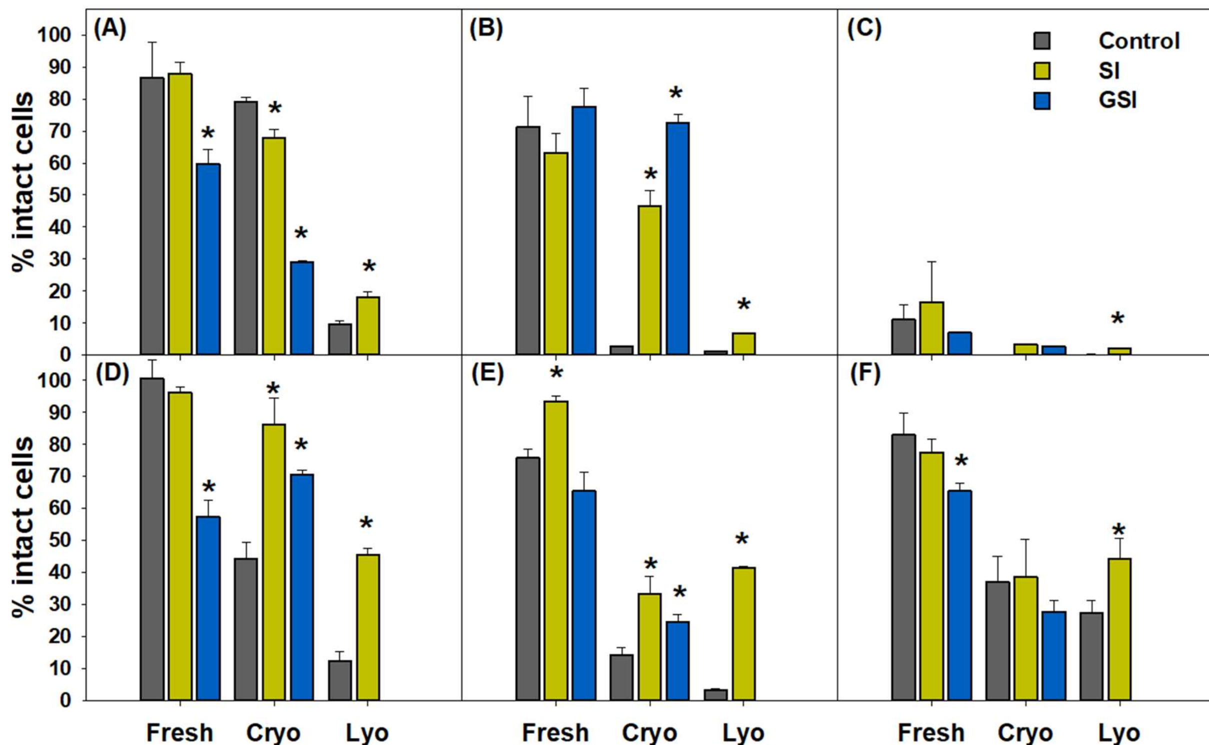

Fig. 3.2 Impact of cryoprotectants on membrane integrity of cryopreserved and lyophilized strict anaerobes. Percentage of intact cells in fresh (t_0), and cryopreserved (Cryo) and lyophilized (Lyo) *B. thetaiotaomicron* (A), *E. hallii* (B), *B. obeum* (C), *R. intestinalis* (D), *A. caccae* (E) and *F. prausnitzii* (F) after 3 months storage in control (no protectant) and treated cultures (t_2) (SI and GSI).

Bacterial response to lyophilization in the absence of protectant

The effect of lyophilization and storage at 4°C for 3 months on viability and fitness of the tested strains was evaluated by comparing processed (t_1) and stored (t_2) with fresh cultures (t_0) in the absence of protectant (control).

Lyophilization more severely affected viability and growth than freezing only. Viable cell counts decreased 100-fold, and t_{lag} increased up to 14-folf after processing but remained stable during storage of all strains except *F. prausnitzii* and *B. obeum* (Table 3.3 and 3.4). *F. prausnitzii* was the least sensitive strain towards lyophilization as indicated by a recovery of 14 % of initial viable cells (7.1±0.4 log cells ml^{-1}) and a relative high fraction of intact cells (27±4 %) in the lyophilized stored control (Fig. 3.2). In contrast, *B. obeum* was most sensitive, with a large drop of viability during processing (4.3±1.2 log ml^{-1}) and during storage (2.3±2.1) compared to the fresh control cultures (7.9±0.4 log ml^{-1}). For all strains except *B. obeum*, μ_{max} decreased between 14% and 60% after lyophilization and storage. A significant increase of μ_{max} from 0.14±0.02 in fresh to 0.27±0.00 OD unit h^{-1} in stored culture was measured for *B. obeum*.

Impact of protectants on lyophilized cultures

The protective effect of SI was evaluated on viability and fitness of lyophilized strains after processing (t_1) and storage at 4°C (t_2), and compared to the fresh cultures (t_0) as well as to processed and stored control samples with not added protectant.

The addition of SI significantly improved viability and fitness of all lyophilized strains (Supplementary Fig. S3.1, Table 3.3 and 3.4), with most effect on viability recorded for *A. caccae* and *F. prausnitzii.* Viable cell counts of *A. caccae* remained unchanged after processing (8.8±0.3 log ml^{-1}) and after 3 months storage (8.6±0.3 log ml^{-1}, 34.3% intact cells) compared to the fresh SI samples (8.9±0.2 log ml^{-1}, 83±6% intact cells). t_{lag} of SI treated *A. caccae* and *F. prausnitzii* increased approximately two-fold after processing (2.7±0.1 and 2.6±0.4 h, respectively) but was significantly lower than for the lyophilized and stored control (8.9±0.9 and 7.9±0.3 h, respectively). Viable cell counts of *F. prausnitzii* slightly but significantly decreased from 8.2±0.2 log cells ml^{-1} in the fresh SI samples to 7.8±0.4 log cells ml^{-1} after lyophilization, but remained stable during storage. t_{lag} was two-fold longer after storage (2.8±0.7 h) than in the fresh SI samples, however significantly shorter than for the lyophilized control (5.7±2.0 h).

B. thetaiotaomicron, *R. intestinalis* and *E. hallii* treated with SI had similar viable cell counts after storage (7.7±0.4, 7.6±0.2 and 6.8±0.2 log cells ml^{-1}, respectively) and improved recovery rates of 3 – 4 % viable cells compared to the control (0.01–0.2%). SI addition also significantly shortened t_{lag} by 33 – 59% after lyophilization and storage compared to the protectant free control (Table 3.4). Viability remained generally stable during storage of all lyophilized cultures with a maximal loss of 0.3 log in MPN, except for *B. obeum* that showed a 1 log decrease in MPN and an increased μ_{max} after processing and storage of SI treated samples.

Table 3.3 Impact of protectants and lyophilization on viable cell counts of fresh (t_0), processed (t_1) and stored (t_2) bacteria. Log viable cell counts ml^{-1} in the fresh culture, after lyophilization and storage for 3 months at 4°C, assessed with the most probable number method (MPN). Recovery rate of viable cells (in %) was calculated relative to the average viable cell counts in the fresh control (vs. control t_0) and to fresh treatment culture (vs. t_0).

		Control		**SI**	
Organism	Culture condition	MPN (ml^{-1})	Recovery vs. t_0	MPN (ml^{-1})	Recovery vs. control t_0/t_0
B. thetaiotaomicron	Fresh (t_0)	9.3 ± 0.2		9.4 ± 0.2	135
	Processed (t_1)	7.0 ± 0.5	1	8.0 ± 0.7 [b]	6/4
	Stored (t_2)	5.4 ± 2.0 [b]	0.01	7.7 ± 0.4 [ab]	3/2
B. obeum	Fresh (t_0)	7.9 ± 0.4		7.8 ± 0.4	79
	Processed (t_1)	4.3 ± 1.2	0.02	6.6 ± 0.2 [ab]	4/5
	Stored (t_2)	2.3 ± 2.1 [b]	0.0003	5.5 ± 0.3 [ab]	0.3/0.4
R. intestinalis	Fresh (t_0)	8.9 ± 0.4		8.9 ± 0.4	82
	Processed (t_1)	6.3 ± 0.3 [b]	0.2	7.8 ± 0.4 [ab]	7/8
	Stored (t_2)	6.2 ± 0.2 [b]	0.2	7.6 ± 0.2 [ab]	4/5
E. hallii	Fresh (t_0)	8.4 ± 0.2		8.4 ± 0.2	100
	Processed (t_1)	4.9 ± 0.4 [b]	0.04	7.1 ± 0.1 [ab]	6/6
	Stored (t_2)	5.6 ± 0.2 [b]	0.2	6.8 ± 0.2 [ab]	3/3
A. caccae	Fresh (t_0)	8.8 ± 0.3		8.9 ± 0.2	111
	Processed (t_1)	6.4 ± 0.2 [b]	0.4	8.8 ± 0.3 [a]	87/71
	Stored (t_2)	6.7 ± 0.5 [b]	1	8.6 ± 0.3 [a]	60/49
F. prausnitzii	Fresh (t_0)	8.0 ± 0.2		8.2 ± 0.2	181
	Processed (t_1)	6.1 ± 0.3 [b]	1	7.8 ± 0.4 [ab]	58/32
	Stored (t_2)	7.1 ± 0.4 [b]	14	7.8 ± 0.1 [ab]	59/33

[a] Viable cell counts in samples with lyoprotectants are significantly different from the control samples within the same condition ($p<0.05$)

[b] Viable cell counts after processing and after storage are significantly different from the viable cell counts of the fresh culture within the same treatment ($p<0.05$)

Table 3.4 Impact of protectants and lyophilization on fitness of fresh (t_0) processed (t_1) and stored (t_1) bacteria. Lag phase (t_{lag}) and maximum growth rate (μ_{max}) were calculated from optical density growth curves based on Baranyi's equation.

		Control		SI	
Organism	Culture condition	t_{lag} (h)	μ_{max} ($OD*h^{-1}$)	t_{lag} (h)	μ_{max} ($OD*h^{-1}$)
B. thetaiotaomicron	Fresh (t_0)	0.7 ± 0.1	0.23 ± 0.01	0.7 ± 0.1	0.22 ± 0.01 [a]
	Processed (t_1)	7.5 ± 0.9 [b]	0.19 ± 0.01 [b]	3.4 ± 1.2 [ab]	0.18 ± 0.01
	Stored (t_2)	10.1 ± 3.5 [b]	0.16 ± 0.04 [b]	4.1 ± 1.9 [ab]	0.14 ± 0.05 [a]
B. obeum	Fresh (t_0)	6.4 ± 2.2	0.14 ± 0.02	7.0 ± 2.1	0.14 ± 0.02
	Processed (t_1)	25.8 ± 0.3 [b]	0.25 ± 0.00 [b]	19.3 ± 2.1 [ab]	0.21 ± 0.01 [ab]
	Stored (t_2)	25.5 ± 1.6 [b]	0.27 ± 0.00 [b]	21.5 ± 2.0 [ab]	0.22 ± 0.03 [ab]
R. intestinalis	Fresh (t_0)	1.6 ± 0.1	0.29 ± 0.02	1.6 ± 0.1	0.28 ± 0.01
	Processed (t_1)	9.8 ± 0.4 [b]	0.30 ± 0.01	5.6 ± 1.1 [a]	0.26 ± 0.01 [a]
	Stored (t_2)	11.0 ± 0.5 [b]	0.25 ± 0.01 [b]	8.5 ± 0.4 [ab]	0.21 ± 0.01 [ab]
E. hallii	Fresh (t_0)	1.7 ± 0.7	0.35 ± 0.04	1.3 ± 0.2	0.30 ± 0.07
	Processed (t_1)	14.8 ± 0.2 [b]	0.27 ± 0.05 [b]	8.5 ± 0.7 [ab]	0.37 ± 0.02a
	Stored (t_2)	13.0 ± 1.3 [b]	0.21 ± 0.12 [b]	8.6 ± 0.2 [ab]	0.32 ± 0.04
A. caccae	Fresh (t_0)	1.5 ± 0.1	0.20 ± 0.03	1.3 ± 0.2	0.19 ± 0.03
	Processed (t_1)	8.9 ± 0.9 [b]	0.25 ± 0.02	2.7 ± 0.1 [ab]	0.16 ± 0.01 [ab]
	Stored (t_2)	7.9 ± 0.3 [b]	0.21 ± 0.01	2.6 ± 0.4 [ab]	0.15 ± 0.02 [a]
F. prausnitzii	Fresh (t_0)	1.3 ± 0.1	0.05 ± 0.00	1.4 ± 0.1	0.05 ± 0.00
	Processed (t_1)	6.6 ± 0.4 [b]	0.04 ± 0.01 [b]	3.3 ± 0.4 [ab]	0.06 ± 0.01 [a]
	Stored (t_2)	5.7 ± 2.0	0.02 ± 0.01 [b]	2.8 ± 0.7 [ab]	0.03 ± 0.01 [a]

[a] Lag phase or growth rate in samples with lyoprotectants are significantly different from the control samples within the same condition ($p<0.05$)

[b] Lag phase or growth rate after processing and after storage are significantly different from the fresh culture within the same treatment($p<0.05$)

Discussion

A major challenge in the production and formulation of strict anaerobic probiotics of gut origin is maintaining viability and fitness during processing and storage. In this study, we assessed the impact of the two main preservation methods, cryopreservation and lyophilization, and of subsequent storage, respectively, on viability and fitness of six strict anaerobic gut microbes.

Impact of cryopreservation, lyophilization and storage on viability

Cryopreservation was confirmed less detrimental to sensitive bacteria than lyophilization (Heylen*, et al.*, 2012), which combines freezing and drying steps, as indicated by higher viability and shorter t_{lag} of cryopreserved compared to lyophilized samples directly after processing in the absence of cryoprotectant. During freezing, bacterial cells are exposed to two main stresses. Mechanical stress due to intra- and extracellular ice crystal formation and increased osmotic pressure caused by solutes concentration in the remaining unfrozen fraction, which potentially leads to the disruption of bacterial membranes and ultimately to lethal damage (Malik, 1991, Meryman, 2007). During lyophilization, the removal of water by sublimation further increases osmotic pressure and can cause severe damage to membranes and surface proteins (Broeckx*, et al.*, 2016). However, despite lower viable cell counts in the lyophilized cultures without protectant, viability was maintained during storage at 4°C while viability of the corresponding cryopreserved cultures declined at -80°C, especially for the freezing-sensitive *B. obeum*, *R. intestinalis* and *E. hallii*. Biochemical reactions occurring when free water is present in cells stored above -80°C can cause viable cell loss over time (Mazur, 1970, Mazur, 1984). As a consequence, storage in electrical freezers will not guarantee indefinite viability of cryopreserved cells (Heylen*, et al.*, 2012)

Another important factor for stability of strict anaerobes is oxidative stress. The ability to tolerate oxygen differed between the tested microbes. An aerotolerance test identified *R. intestinalis*, *F. prausnitzii* and *E. hallii* as highly oxygen-sensitive and *A. caccae* as most oxygen-tolerant strain withstanding exposure to ambient air up to 60 minutes (Flint*, et al.*, 2007). *F. prausnitzii* can survive in the presence of low oxygen concentrations by using an “extracellular electron shuttle” over antioxidants that reduce oxygen (Khan*, et al.*, 2012). *B. thetaiotaomicron* also expresses defense mechanisms against oxygen by scavenging enzymes that prevent rapid formation of reactive oxygen species and facilitates recovery from oxygen exposure (Pan and Imlay, 2001, Mishra and Imlay, 2013). By adding riboflavin and cysteine-HCl in the buffer formulation we induced a reducing environment protecting bacteria from oxygen exposure during storage, nevertheless highly sensitive strains might require complete anaerobiosis (Khan*, et al.*, 2014). Higher oxygen tolerance of *A. caccae, F. prausnitzii* and *B. thetaiotaomicron* may explain their enhanced stability during storage at -80°C in partly oxygen-permeable screw-cap polypropylene cryo tubes. Improved stability of the lyophilized compared to cryopreserved samples might also be partly due to the absence of oxygen during storage through

vacuum-sealed glass ampules. As suggested by Malik (1991), storage stability of strict anaerobes during cryopreservation can be improved by using glass vials with oxygen-impermeable butyl rubber septa.

Impact of protectants on viability and growth of fresh cultures

Prior preservation, fresh cultures were incubated for 30 min in protective media containing 2.0 M glycerol, 150 mM sucrose and 40 mM inulin (calculated as fructose equivalents). Solutes used as protectants can cause growth inhibition due to osmotic pressure when present in growth medium at concentrations of > 1.0 M sucrose and ≥ 1.5 M glycerol (Cebrian*, et al.*, 2014). Membranes, destabilized after exposure to osmotic pressure, are suggested to cause cell death by phase transition of membrane phospholipids in interaction with volume changes of the cells (Mille*, et al.*, 2005). Glycerol in the protective buffer already decreased survival and the proportion of intact cells of fresh *B. thetaiotaomicron*, *R. intestinalis* and *F. prausnitzii* cultures after 30 min incubation. The minimal inhibitory concentration of glycerol in growth medium (1.5-2.4 M) was found to be generally lower in Gram-negative than Gram-positive bacteria. It was also suggested that osmotolerance might be strain specific (Saegeman*, et al.*, 2008, Cebrian*, et al.*, 2014).

The presence of glycerol in the protective solution reduced μ_{max} of *E. hallii* compared to the SI treatment and control without glycerol. These data may be explained by the ability of *E. hallii* to convert glycerol to reuterin (Engels*, et al.*, 2016). Reuterin is a broad spectrum antimicrobial system, which, at physiological conditions, mainly consists of 3-hydroxypropionaldehyde (3-HPA), its hydrate and dimer, and acrolein (Engels*, et al.*, 2016). Reuterin can inhibit growth of the producer strain. In the growth assessment tests, 10% (v/v) inoculation with GSI treated cultures transferred 20 mM glycerol to the YCFA medium. Hence, formation of reuterin could be responsible for reduced μ_{max} of *E. hallii*, which is supported by the absence of an effect on μ_{max} when inoculation with the glycerol containing culture was done at only 1% v/v (data not shown).

Fresh cultures incubated in protective media containing sucrose (150 mM) and inulin (40 mM) were not negatively affected in terms of viability or growth. The concentration of sucrose was likely too low to induce a significant osmotic stress. Moderate osmotic stress was shown to occur at much higher concentration of sucrose (730 mM) for *Lactobacillus delbrueckii* (Meneghel*, et al.*, 2017). In another study, growth inhibition of *Staphylococcus aureus*, *Listeria monocytogenes*, *Cronobacter sakazakii*, *Enterococcus faecium*, *Escherichia coli* and *Salmonella* Typhimurium was observed in growth medium supplemented with sucrose concentrations ranging from 1.1 to 1.8 M (Cebrian*, et al.*, 2014).

Impact of protectants on viability and growth of preserved cultures

The addition of protectants positively influenced viability and membrane integrity of the freezing sensitive *R. intestinalis*, *E. hallii* and *B. obeum*. It has been proposed, that glycerol prevents intracellular ice crystal formation at high freezing rates when bacterial cells are immersed in liquid nitrogen (Fonseca*, et al.*, 2006). The protective action of glycerol during freezing and storage also outweighed

its detrimental osmotic effect observed in fresh *R. intestinalis* cultures, and the potential growth inhibition of reuterin produced by *E. hallii*. Glycerol in the protective media exhibited greater protection than sucrose and inulin alone. However, the two protectants might have acted synergistically in combination with glycerol leading to better recovery of viable cells. The observed positive impact of glycerol on *R. intestinalis* on viability and growth performance is in accordance with earlier findings of its protective effect on the reestablishment of *Roseburia sp./E. rectale* group when cryopreserved as part of a complex artificial gut microbiota (Bircher*, et al.*, 2017)

B. thetaiotaomicron, *F. prausnitzii* and *A. caccae* were little impacted by freezing and storage. Processing conditions used in this study, characterized by a high freezing rate (immersion in liquid nitrogen) and storage temperature of -80°C, contributed to the stability of these strains, aspreviously reported for lactic acid-producing starter cultures (Fonseca*, et al.*, 2001). In agreement, only limited effects were observed on viability and the proportion of intact cells when protectants were added, and the detrimental effect of glycerol on viability of *F. prausnitzii* was still observed after freezing and storage.

The addition of SI protected cell viability during lyophilization of all strains but had little impact on storage stability. This implies that sucrose and inulin mainly protected viability during the lyophilization process than during storage. Sucrose and inulin both interact with biological membranes and stabilize during freezing and drying (Demel*, et al.*, 1998, Vereyken*, et al.*, 2003, Schwab*, et al.*, 2007), and in agreement, the proportion of cells with integer membranes was higher if SI was present during lyophilization compared to controls.

Impact of preservation on bacterial fitness

Fitness is another important marker for preservation of bacteria, which can be evaluated by μ_{max} and t_{lag} as a measure of reproductive potential (Sandegren*, et al.*, 2008, Pope*, et al.*, 2010, Adkar*, et al.*, 2017). For all tested strains, t_{lag} negatively correlated with viable cell numbers. Alteration of generation times, a known reaction to stress exposure, has been reported before for frozen and stored bacterial cells (Squires and Hartsell, 1955, Lipson, 2015, Adkar*, et al.*, 2017). Indeed, μ_{max} of *B. thetaiotaomicron*, *R. intestinalis*, *A. caccae* and *F. prausnitzii* generally decreased after preservation with only small differences between treatments and control. In contrast, cryopreserved *E. hallii,* and lyophilized and cryopreserved *B. obeum* exhibited increased μ_{max}, particularly for the treatments characterized by low viability. Stress exposure during lyophilization and cryopreservation, which was lethal to the majority of the cells, might have selected for drying and/or freezing resistant subpopulations (Patra and Klumpp, 2013, Wang*, et al.*, 2014).

Enhanced stress resistance of bacteria can be an advantage for probiotic applications. In contrast, reduced bacterial fitness leading to slower growth may impair the ability of preserved bacteria to multiply and be metabolically active in the gastrointestinal tract. Still, further evaluation is needed to better understand the interplay between t_{lag}, μ_{max} and viability in terms of probiotic application

success. As an example, higher viability of cryopreserved *E. hallii* due to the presence of glycerol might compensate for the lower μ_{max}. We recently reported that *E. hallii* was little impacted by freezing within a complex artificial gut microbiota, as indicated by comparable growth of fresh and preserved microbiota in a standardized growth assay (Bircher*, et al.*, 2017). Our current data suggest that the enhanced μ_{max} of *E. hallii* after cryopreservation could explain its competitiveness in batch fermentation. Furthermore, process-impacted bacterial fitness can be a determinant for the selection of a preservation method that offers best conditions for probiotic re-establishment *in vivo*. *F. prausnitzii* for example, exhibited a shorter t_{lag} but similar viable cell counts after lyophilization than after cryopreservation when SI was used as protectant, suggesting lyophilization as favorable preservation method for this strain.

Conclusion

To our best knowledge, this is the first study to date that quantitatively compared the effect of cryopreservation and lyophilization and the addition of selected protectants on viability and fitness of six strict anaerobic gut microbes. Viable cell recovery ranged from 11% – 100% after frozen and from 0.3% – 60% after dried storage, pointing towards a strong species-dependent resistance to freezing and freeze-drying. Membrane composition might be a determining factor as the addition of membrane-interacting protectants sucrose and inulin improved viability of all lyophilized strain and of freezing-sensitive strains after cryopreservation. As glycerol also differently affected strain viability and membrane integrity prior and post cryopreservation, our results suggests that selection of protectants has to be process- and species-specific. Based on our results, we recommend using cryopreservation with GSI for *B. obeum*, *R. intestinalis*, *E. hallii*, *A. caccae* and SI for *B. thetaiotaomicron*. *F. prausnitzii* should be preferably preserved by lyophilization with SI.

Experimental procedures

Bacterial strains and culture conditions

B. thetaiotaomicron DSM 2079, *B. obeum* DSM 25238, *R. intestinalis* DSM 14610, *E. hallii* DSM 3353, *A. caccae* DSM 14662 and *F. prausnitzii* DMS 17677 were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany). Bacterial pellets from 1 ml overnight growing cultures were snap-frozen in 100 µl phosphate buffer (pH 6.8, 0.1 M) (PB, Supplementary Table S3.1,) supplemented with glycerol (15% v/w) (VWR International AG, Dietikon, Switzerland) and stored at -80°C (stock cultures). For each experiment, a fresh stock culture was thawed in an anaerobic chamber (10% CO_2, 5% H_2 and 85% N_2) (Coy Laboratories, Grass Lake, Michigan, USA) and re-suspended with 900 µl phosphate buffered saline (0.8% v/w, pH 6.8) (PBS, Supplementary Table S3.2) to a final volume of 1 ml. Half a milliliter of reactivated culture was transferred to 10 ml yeast extract, casitone and fatty acid medium (YCFA) in a Hungate tube and incubated anaerobically for 10 h at 37°C to obtain a working culture. YCFA medium (Supplementary Table S3.3) was prepared as described previously (Duncan*, et al.*, 2003) with slight modifications. Glucose (6 g l^{-1}, Sigma-Aldrich, Buchs, Switzerland) was added as sole carbon source. All components except cystein-HCl (0.01% v/w, Sigma-Aldrich) were dissolved in deionized water and pH was adjusted to 7.4 with 2.5 N NaOH to obtain a pH of 6.8 after autoclaving. The medium was boiled while flushing with CO_2 until a color change from blue to pink occured, caused by the addition of the indicator resazurin (0.1% of 1 mg ml^{-1} stock solution). Cystein-HCl was then added and the medium was dispensed in Hungate tubes flushed with CO_2 before autoclaving.

Preparation of protective buffers

All components of the PB (0.1 M, prepared in oxygen-free distilled water) were placed in an anaerobic chamber overnight to remove traces of oxygen. Reducing agents cysteine-HCl and riboflavin (Sigma-Aldrich) were added at final concentrations of 1 g l^{-1} and 0.3 g l^{-1} respectively, to protect the bacteria from potential oxygen exposure during processing and storage (Khan*, et al.*, 2014). The pH was adjusted to 6.8 and buffers were filter-sterilized, covered in aluminum foil as light protection and stored in an anaerobic chamber until usage.
Two protective buffers were prepared by dissolving in PB sucrose (VWR International AG) and inulin (RPN Foodtechnology AG, Sursee, Switzerland) (both 5% w/v) (SI), and adding glycerol (15% v/w) to SI. PB that only contained cystein-HCl and riboflavin served as protectant-free control.

Cryopreservation, lyophilization, and storage

Two independent preservation experiments were conducted for each bacterium. Within an experiment, fresh, processed and stored samples of each treatment were analyzed in triplicates. All processing steps were either executed in an anaerobic chamber or in Hungate tubes to guarantee

anoxic conditions. A working culture was generated by incubating a reactivated glycerol stock culture for 10 h at 37°C in YCFA medium. This working culture was once sub-cultured under the same conditions to obtain a viable and active culture for preservation trials (experimental culture). For the production of experimental cultures, 10 ml portions of YCFA medium were inoculated at 0.2% (v/v) with a working culture of *B. thetaiotaomicron*, *R. intestinalis*, *E. hallii*, and *A. caccae* or at 2% (v/v) for the slow-growing strains *F. prausnitzii,* and *B. obeum*. Cultures were incubated at 37°C for 13 to 15 h, depending on the strain, until early stationary growth phase was reached. Incubation times were assessed in preliminary growth tests in Hungate tubes at 37°C. Optical density (OD) at 600 nm was monitored during incubation (data not shown). Cells were harvested by centrifugation at 4°C for 10 min at 3’000 g. The pellet was washed in 5 ml PB, centrifuged and re-suspended in either 1 ml control, SI or GSI buffer (10-fold concentration of the initial experimental culture). After an incubation time of 30 min at room temperature to allow penetration of glycerol, aliquots (100 µl) were snap-frozen in liquid nitrogen and either stored at -80°C in screw-cap polypropylene cryotubes (Bioswisstec AG, Schaffhausen, Switzerland) (control, SI and GSI cultures, Fig. 3.1) or dried with a manifold freeze-dryer (VirTis BenchTop 2K, MultiTemp Scientific AG, Kloten, Switzerland). Lyophilization of the control and SI treated cultures was carried out in long stem Vacule cryogenic ampules (Sigma-Aldrich) that were pre-reduced in an anaerobic chamber, plugged with sterile cotton wool, and contained blue silica gel with moisture indicator (Sigma-Aldrich). Frozen samples in cryogenic ampules were placed on dry-ice prior lyophilization to prevent thawing of the culture until vacuum was started. Freeze-drying was conducted at a condenser temperature of -80°C at 80 mTorr for 6 h after which ampules were flame sealed under vacuum and stored at 4°C.

Viability assessment and growth tests were conducted under anaerobic conditions at three different time points (Fig. 3.1): first with the fresh culture after incubation in control and protective buffers (t_0), then with the processed culture immediately after freezing, respectively lyophilization (t_1), and finally with the stored culture after 3 months at -80°C and 4°C for croypreservation and lyophilization, respectively (t_2). Each experimental condition was duplicated, and triplicate samples of each treatment were analyzed. Prior to the viability and growth tests, fresh cultures (t_0) were re-suspended in 900 µl PBS immediately after incubation in the buffers. Cryopreserved cultures (t_1 and t_2) were transferred from -80°C freezer to an anaerobic chamber and thawed at room temperature before re-suspending in 900 µl PBS buffer. Lyophilized cultures (t_1 and t_2) were rehydrated in 1 ml PBS for 1 h in an anaerobic chamber.

Measurement of viability

Viable cell counts of were assessed by the most probable number (MPN) method with a five-replicate design (Sutton, 2010) that was adapted to 96 well microtiter plates (Kuai*, et al.*, 2001). Before use, plates (Bioswisstec AG) were stored overnight in the anaerobic chamber to remove traces of oxygen. Samples were serially diluted ten-fold, and 20 µl of each dilution was used to inoculate 5 wells, each

containing 180 µl YCFA medium. Plates were incubated at 37°C for 48 h in an anaerobic chamber. Wells with visible turbidity were scored as growth positive.

Determination of membrane integrity

Membrane integrity of fresh and stored samples was determined with two fluorescence stains and subsequent flow cytometric analysis (Van Nevel*, et al.*, 2013). All dilution, staining and incubation steps were performed in an anaerobic chamber while flow cytometric analysis was conducted at ambient air. Fluorescence working solutions were prepared as follows: 10 µl SYBR Green I (SG; 10'000 x concentrated) (Life Technologies, Zug, Switzerland) was diluted in 990 µl filtered dimethyl sulfoxid (DMSO) (Sigma Aldrich). Twenty µl propidium iodide (PI; 20 mM) (Life Technologies) and 10 µl SG were diluted in 970 µl DMSO. Solutions were stored at −20 °C until use. Each bacterial sample was diluted to approximately 10^7 cells ml^{-1} with PBS and stained twice with SG to assess total cell counts, or with SG combined with PI to determine intact cell counts. Therefore, 30 µl diluted sample, 3 µl stain working solution and 237 µl PBS was incubated for 22 min at 37°C in the dark. PBS stained with SG and PI was used to determine background fluorescence. Prior to flow cytometric analysis, 30 µL of Flow-Count fluorospheres (Beckman Coulter International SA, Nyon, Switzerland) were added at known concentrations to determine bacterial cell counts. Samples were analyzed with a Cytomics FC 500 (Beckman Coulter International SA) equipped with an air-cooled argon ion laser emitting 20 mW at 488 nm and a red solid state diode laser emitting 25 mW at 633 nm. Gates on green against red fluorescence plots were used to assess total and intact cell counts. The percentage of intact cells were calculated by dividing the intact cell number in the green gate of the SG and PI by the total cell number in the SG stained sample.

Assessment of growth performance

Growth was assessed in 96 well microtiter plates in anaerobic conditions, as described previously (Eini*, et al.*, 2013, Geirnaert*, et al.*, 2014). Inner wells of a 96-well plate, stored overnight in an anaerobic chamber, were filled with 180 µl YCFA medium. Wells were inoculated with 20 µl (10% v/v) of the tested culture samples. Empty wells in outer rows and columns were filled with the reducing agent of an AnaeroGen bag (Thermo Fisher Diagnostics AG, Pratteln, Switzerland) to maintain an oxygen-free atmosphere when plates were moved outside of the anaerobic chamber. Plates were covered with a ClearSeal film (Labgene Scientific Instruments, Châtel-Saint-Denis, Switzerland) and the lid, which was sealed with petroleum jelly. Growth was monitored at 37°C by measuring OD at 600 nm every 30 minutes in a microplate reader (Powerwave XS). The addition of the indicator resazurin in the YCFA medium confirmed anaerobiosis in the 96-well plates during measurements. The average OD value of six wells containing sterile YCFA medium served as blank and was subtracted from OD values of inoculated wells. To calculate maximum growth rate (μ_{max}) and lag phase (t_{lag}), growth curves were

fitted using the DMFit 3.5 program (Institute of Food Research, Norwich, UK) based on Baranyi's equation (Baranyi and Roberts, 1994)

Statistics

Statistical analysis of viable cell counts (log_{10}-transformed), percentage of intact cells by flow cytometry, μ_{max} and t_{lag} was done using R studio version 3.4.1 (Boston, Massachusetts, USA). Data are expressed as mean±SD of six replicates obtained from two independent preservation experiments conducted on two different days (with three replicates each).

ANOVA tests were performed with viable cell counts, μ_{max} and t_{lag} of fresh, cryopreserved and lyophilized samples as dependant variables and either treatment or time point within a treatment as independent variables. Data were tested for normal distribution using the Shapiro-Wilk test and equality of variance was assessed with the Levene test. Tukey HSD test (multiple pairwise comparison) was used to compare treatments to control (no added protectants), and preserved and stored to fresh samples. A non-parametric Kruskal-Wallis test was performed when data were not normally distributed or the assumption of equality of variance was violated. Students t-test was performed to compare means of viable cell counts, μ_{max} and t_{lag} of fresh and lyophilized control with SI treated samples. Data were tested for homogeneity of variance with the F-test. Differences were considered significant for $\alpha \leq 0.05$.

Acknowledgements

We thank Nicole Viti for experimental assistance.

Supporting information

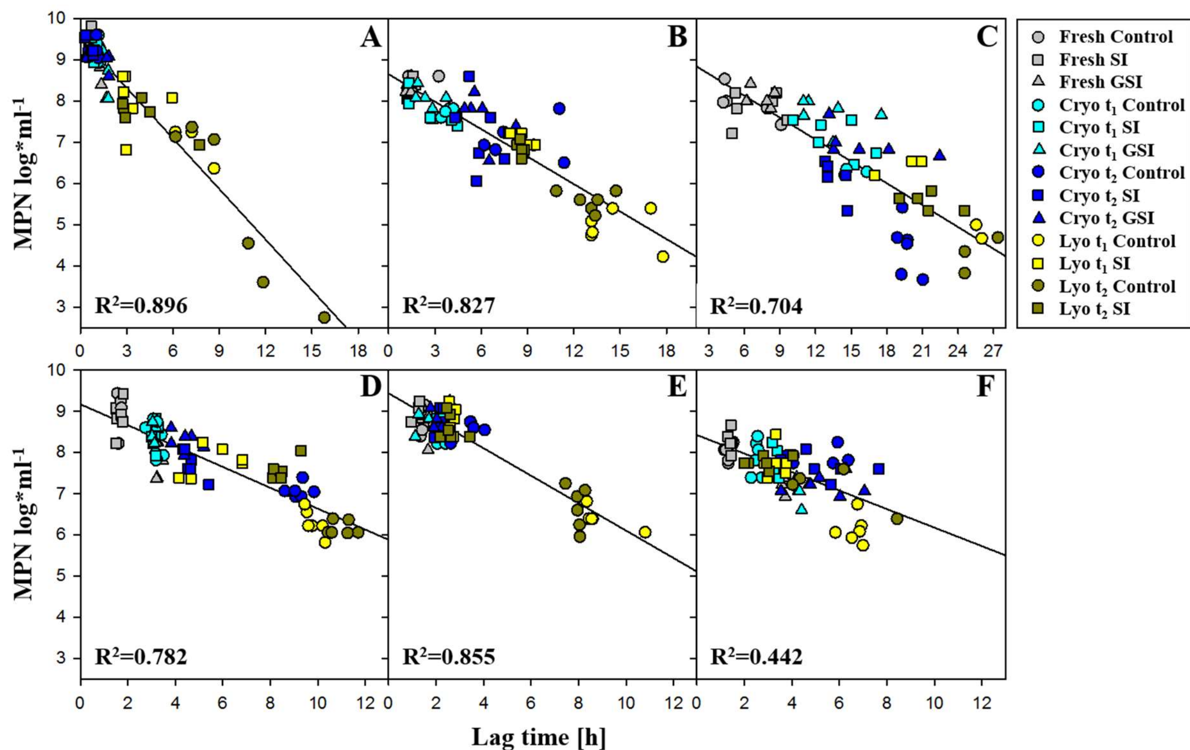

Fig S3.1 Correlation plots between viable cell counts and lag times (t_{lag}). Log viable cell counts and t_{lag} of fresh, preserved and stored *B. thetaiotaomicron* (A), *E. hallii* (B), *B. obeum* (C), *R. intestinalis* (D), *A. caccae* (E) and *F. prausnitzii* (F), assessed with the most probable number method (MPN) and optical density measurements, respectively.

Table S3.1 Composition of phosphate buffer.

Component	**g L^{-1}**
Sodium dihydrogen phosphate	6.00
Sodium hydrogen phosphate	7.10
Resazurin (1mg ml^{-1})	1.00 ml
L-cysteine hydrochloride monohydrate	1.00

Table S3.2 Composition of phosphate buffered saline.

Component	**g L^{-1}**
Potassium dihydrogen phosphate	0.24
Sodium hydrogen phosphate	1.44
Sodium chloride	8.00
Potassium cloride	0.20
Resazurin (1mg ml^{-1})	1.00 ml
L-cysteine hydrochloride monohydrate	1.00

Table S3.3 Composition of YCFA medium.

Component	g L^{-1}
Casein acid hydrolysate, from bovine milk	10.00
Yeast extract	2.50
Sodium bicarbonate	4.00
Glucose	6.00
Potassium dihydrogen phosphate	0.45
Dipotassium hydrogen phosphate	0.45
Sodium chloride	0.90
Ammonium sulfate	0.90
Magnesium sulfate	0.09
Calcium chloride	0.09
Vitamin solution (10 mg l^{-1} biotin, 10 mg l^{-1} cobalamin, 30 mg l^{-1} p-aminobenzoic acid, 50 mg l^{-1} folic acid, 150 mg l^{-1} pyridoxamine)	1.00 ml
Volatile fatty acid mix (32.9% (v/v) acetic acid, 11.7% (v/v) propionic acid, 1.6% (v/v) isobutyric acid, 1.9% (v/v) isovaleric acid, 1.9% (v/v) valeric acid, 50% (v/v) 2.5 M NaOH)	5.75 ml
Hemin (0.5 mg ml^{-1})	0.20 ml
Resazurin (1 mg ml^{-1})	1.00 ml
L-cysteine hydrochloride monohydrate	1.00

Planktonic and sessile artificial colonic microbiota reestablish differently upon frozen and freeze-dried long-term storage

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Abstract

Biofilm-associated, sessile communities are the major bacterial lifestyle, whereas planktonic cells mainly appear during initial colonization of new surfaces. Previous research, mainly performed with pathogens, demonstrated increased environmental stress tolerance of biofilm-growing compared to planktonic bacteria. Lifestyle-specific stress response of colonic microbiota, both natural and fermentation-produced, has not been addressed before. We therefore characterized planktonic and sessile microbiota produced in two PolyFermS continuous colonic fermentation models inoculated with immobilized fecal microbiota, and comparatively tested their tolerance to frozen (-80°C) and freeze-dried storage (4°C) for 9 months.

Sessile microbiota harbored next to shared taxa a unique community distinguishable from planktonic microbiota. Synergistetes and Proteobacteria were highly represented in sessile, while Firmicutes were more abundant in planktonic microbiota. Community structure and metabolic activity of both microbiota, monitored during standardized reactivation batch fermentations, were better preserved after frozen than dried storage, indicated by higher Bray-Curtis similarity and enhanced recovery of metabolite production. For both lifestyles, re-establishment of *Bacteroidaceae* was impaired after frozen and dried storage along with reduced propionate formation. In contrast, butyrate production was maintained after reactivation despite compositional rearrangements within the butyrate-producing community. Unexpectedly, recovery of metabolite production and compositional similarity to fresh microbiota was lower after preservation of sessile than planktonic microbiota. We speculate that higher functional dependencies between microbes might have led to the lower stress tolerance of sessile compared to planktonic microbiota.

Importance. Fecal microbiota transplantation has been successfully applied in the treatment of recurrent *Clostridium difficile* infection and has been suggested as an alternative therapy for other intestinal disorders such as inflammatory bowel disease or metabolic syndrome. ‘Artificial’ colonic microbiota delivered by PolyFermS continuous fermentation models can provide a controllable and reproducible alternative to fecal transplantation but effective preservation strategies must be developed. In this study, we systematically investigated the response of sessile and planktonic artificial colonic microbiota to cryopreservation and lyophilization. We suggest functional redundancy as an important factor in providing functional stability during exposure to environmental stress during processing and storage. Functional redundancy in compositionally-reduced microbial systems may be considered when designing microbial products for therapy.

Introduction

Biofilm-associated or sessile communities are the predominating lifestyle for bacteria in natural environments. In contrast, planktonic or free-cell life phases mainly prevail during colonization of new surfaces (Watnick and Kolter, 2000), and also in biotechnological processes using suspended cell reactors. Converting from sessile to planktonic lifestyle and vice versa involves changes in gene expression and physiological modifications of the alternating cells (Berlanga and Guerrero, 2016). For example, genes linked to stress response, cell envelope function and iron-sulfur metabolism are upregulated in biofilms compared to planktonic communities (Nakamura*, et al.*, 2016). Increased proportions of saturated fatty acids in membranes of sessile microbes and the production of an exopolymeric matrix are two of the biofilm-associated features that provide protection from the external environment by enhancing structural rigidity (Hobley*, et al.*, 2015, Dubois-Brissonnet*, et al.*, 2016). However, functions of biofilm matrices extend beyond physical stability. Sessile bacteria may have an advantage over planktonic bacteria because of higher environmental stress tolerance, nutrient capture that is enhanced by the spongy structure of the exopolymeric matrix, and increased enzyme retention that provides more efficient substrate conversion (Flemming*, et al.*, 2016).

Several studies have reported the occurrence of biofilms in the colonic habitat, especially on food particles and attached to the mucus layer (Sonnenburg*, et al.*, 2004, De Weirdt and Van de Wiele, 2015, Welch*, et al.*, 2017). The human gastrointestinal tract is a highly populated ecosystem that harbors more than 10^{14} microorganisms composed of more than 1000 different species, with a high cell concentration in excess of 10^{10} bacterial cells per gram (Backhed*, et al.*, 2005, Gill*, et al.*, 2006). Microscopic observations demonstrated the existence of inter-species microcolonies and bacterial biofilms on the colonic mucosa in the large intestine, and on plant particles originating from fecal matter (Macfarlane and Dillon, 2007). Microbial composition of particle-associated communities extracted from fecal samples differed from the suspended fraction. Firmicutes were more abundant in the bacterial community associated with insoluble particles, while Bacteroidetes dominated the liquid phase (Walker*, et al.*, 2008). Likewise, biofilm composition on mucin surfaces was different *in vitro* (Van den Abbeele*, et al.*, 2012) and *in vivo* (Li*, et al.*, 2015) compared to luminal or planktonic counterparts, indicating that different microbial lifestyles co-exist in the gastrointestinal tract.

The PolyFermS continuous *in vitro* fermentation model was developed to mimic both the planktonic and biofilm lifestyles of complex human colonic microbiota (Payne*, et al.*, 2012, Lacroix*, et al.*, 2015). Immobilizing fecal microbiota in porous gel beads enables diffusion of nutrients and products and growth of sessile bacteria within the bead structure and ultimately to seeding by cells released from beads in the bulk medium. Inoculation of fecal beads was shown to stabilize the modelled planktonic community, preventing washout of less competitive bacteria and enabling stable operation of the continuous model for several months (Cinquin*, et al.*, 2004, Zihler Berner*, et al.*, 2013, Fehlbaum*, et al.*, 2015). Bacterial immobilization in polymer beads was suggested as a model for ‘artificial’ biofilms,

based on specific expression patterns in immobilized microbiota, cell and physiochemical gradient formation in the beads and active detachment of cells in the surrounding liquid part, as observed in 'authentic' biofilms (Berlanga and Guerrero, 2016).

Various studies reported increased environmental stress tolerance of biofilms formed by single or mixed bacterial species compared to planktonic cultures (Doleyres*, et al.*, 2004, Türetgen*, et al.*, 2006, Kubota*, et al.*, 2009, Reimann*, et al.*, 2011, Knudsen*, et al.*, 2012, Lee*, et al.*, 2014). Investigations included species from both gram-positive and gram-negative taxa and a variety of stressors such as disinfectants, antibiotics, bile salts, acids and solvents. To our knowledge, lifestyle-dependent stress resistance of complex intestinal microbial communities has never been investigated. Therefore, based on the enhanced stress tolerance of biofilm-associated bacteria, we hypothesized that PolyFermS-derived sessile microbiota (sM) is more resistant to stressors of environmental origin than planktonic microbiota (pM). We chose cryopreservation and lyophilization as two major preservation techniques to comparatively investigate freezing, freeze-drying and storage resistance of pM and sM. Artificial colonic microbiota has been increasingly suggested to treat several gastrointestinal disorders as an alternative to fecal microbiota transplantation (Petrof*, et al.*, 2013, Gupta*, et al.*, 2016, Youngster*, et al.*, 2016, Bircher*, et al.*, 2017). Applying human feces-derived microbiota in therapy demands for preservation technologies that warrant composition and functionality of microbial-based products (Bircher*, et al.*, 2017).

In this work, we first characterized the composition of sM and pM derived from the PolyFermS models mimicking the adult proximal colon by 16S rRNA amplicon sequencing and quantitative real-time PCR (qPCR). We then challenged sM and pM with cryopreservation at -80°C or lyophilization and storage at 4°C for 9 months under strict anaerobiosis to determine the impact on metabolic activity and community structure of these communities. To improve preservation survival, the protectants sucrose and inulin (both 5% w/v), and sucrose, inulin (both 5% w/v) and glycerol (15% v/v), previously developed for stabilizing strict anaerobic gut microbes, were added for lyophilization and cryopreservation, respectively (Bircher*, et al.*, 2018). Composition and metabolic activity of preserved pM and sM were evaluated in strict anaerobic batch cultures over 24 h and compared to fresh microbiota.

Results

Life-style of PolyFermS artificial colonic microbiota impacts bacterial composition and metabolic activity

Artificial colonic microbiota were produced with two independent continuous *in vitro* fermentation systems mimicking the conditions of the adult proximal colon, inoculated with immobilized fecal microbiota (30% v/v) from two healthy donors (F1 and F2). Both fermentations maintained stable metabolic profiles of main short-chain fatty acids (SCFA), acetate, propionate and butyrate during the overall test periods of 48 and 19 days continuous fermentation, respectively (Supplementary Fig. S4.1). Each fermentation system cultivated colonic microbiota of two different lifestyles: free cell state, hereafter referred to as planktonic microbiota (pM) or entrapped in 1-2 mm gellan-xanthan beads, hereafter referred to as sessile microbiota (sM) (Fig. 4.1). The composition of initial pM and sM of both fermentations in conditions of the proximal colon was analyzed by 16S rRNA amplicon sequencing of the V4 variable region (for sampling scheme see supplementary Fig. S4.1). and compared semi-quantitatively with the fecal microbiota used to inoculate the fermentation models. pM1.1 and 2.1 were harvested for cryopreservation before sampling of pM1.2 and 2.2 for lyophilization. Main metabolites from carbohydrate and protein fermentation by pM and sM in the PolyFermS were determined by HPLC-RI.

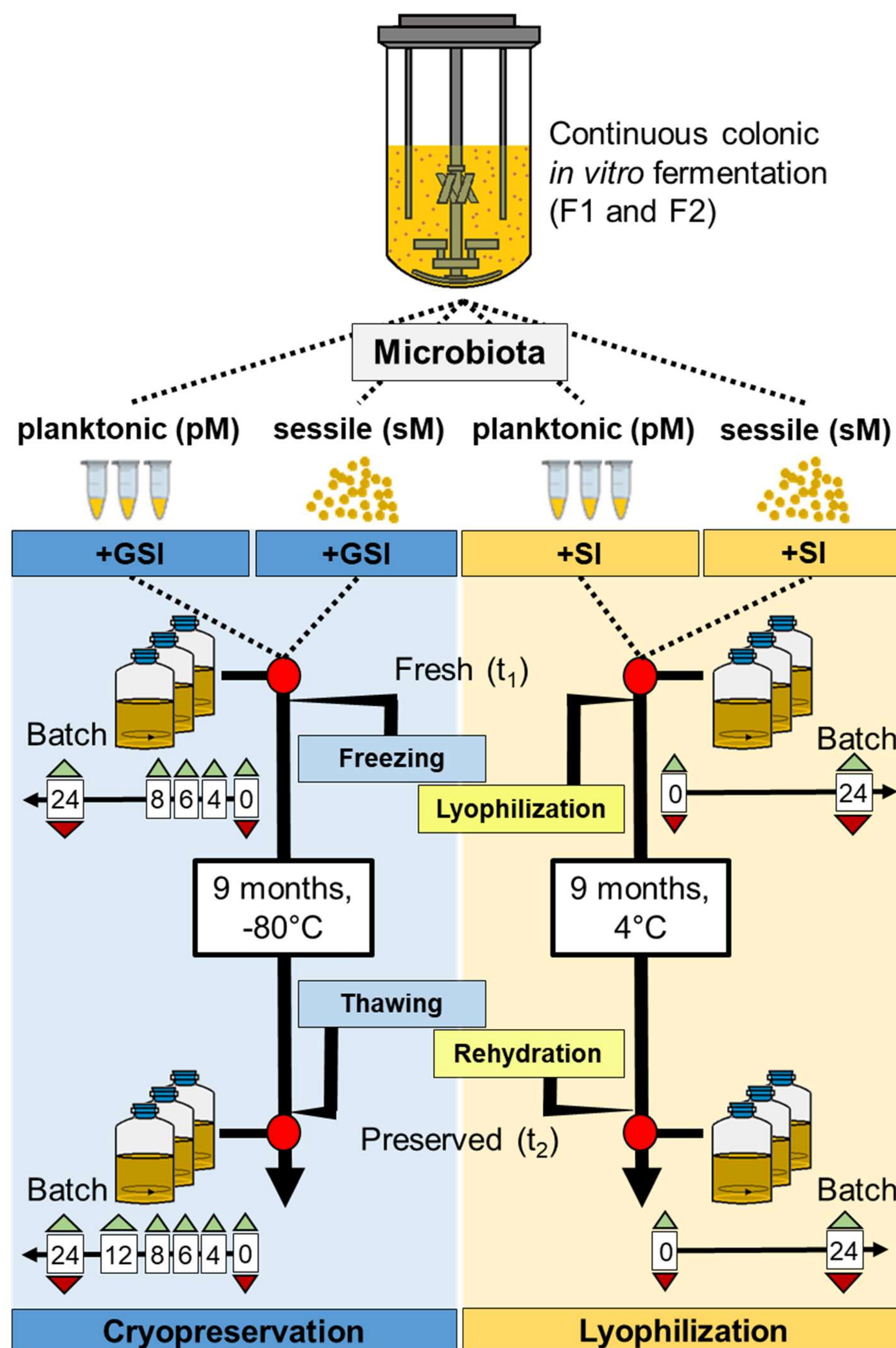

Fig. 4.1 Preservation experiments. Planktonic (pM) and sessile artificial colonic microbiota (sM) were obtained from fermentation system 1 and 2 (F1 and F2). Microbiota were reactivated in batch fermentation at two different time points (red dots). The first batch fermentation was performed with fresh microbiota after incubation with protective buffers containing sucrose and inulin (SI) or glycerol, sucrose and inulin (GSI) (t_1). The second batch fermentation was conducted with the preserved microbiota after freezing, lyophilization and storage for 9 months at -80°C or 4°C, respectively (t_2). Samples were collected from batch fermentations at different incubation time points for microbiota profiling (red triangles) and metabolite analysis green triangles.

Microbiota composition and metabolites formation

Using principle coordinate analysis (PCoA), pM and sM sequencing data of F1 and F2 were decomposed into two factors, namely principal component 1 (PC1) and principal component 2 (PC2), explaining 69% and 19% of the variance, respectively (Fig. 4.2A). The microbiota samples harvested from F2 clustered closely together, separated from F1 samples in PC1 and PC2 direction, indicating that the initial fecal

inoculum of the PolyFermS systems primarily explained the variation between F1 and F2 samples. The PolyFermS closer maintained qualitative composition of an individual fecal microbiota in bead structure than in the planktonic part as indicated by an unweighted UniFrac analysis (Supplementary Fig. S4.2). Shorter distances were observed between pM2 and sM2 than between pM1 and sM1, suggesting a higher similarity between bacterial lifestyles in F2 than in F1.

The metabolic profiles of SCFA, branched-chain fatty acids (BCFA) and fermentation intermediates differed in effluent samples of F1 and F2 inoculated with different donor microbiota (Fig. 4.2B and C). The effluent harvested from F1 contained major proportions of the SCFA acetate (41%) and butyrate (28%), followed by propionate (19%). Acetate (56%) was the major SCFA in effluents collected from F2, with lower proportions of butyrate (17%) and propionate (19%). BCFA isobutyrate and isovalerate were detected in both fermentation effluents at ratios of 1-4%. The fermentation intermediates formate, lactate and succinate were below the detection limits of the HPLC method, which were 1.5, 3.4 and 1.1 mM, respectively, indicating a complete fermentation (Supplementary Fig. S4.1).

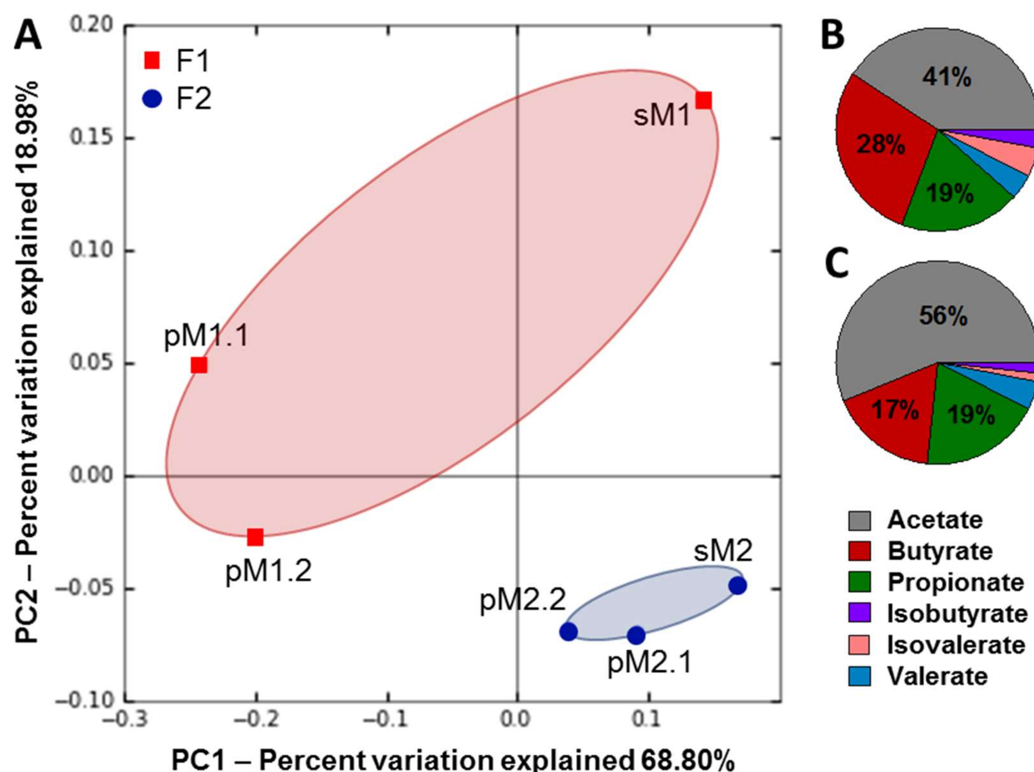

Fig. 4.2 Principal coordinate analysis (PCoA) and metabolite profile of planktonic and sessile artificial colonic microbiota. (A) PCoA plot based on weighted UniFrac distance matrices of planktonic (pM) and sessile microbiota (sM) of fermentation 1 (F1) and 2 (F2). For better visualization, F1 samples were marked with a red and F2 samples with a blue circle. Sampling of pM 1.2 for lyophilization was conducted 21 days after sampling of pM1.1 for cryopreservation, while sampling of pM2.1 and pM2.2 for cryopreservation and lyophilization, respectively, was conducted on following days (Supplementary Fig. S4.1). (B, C) Metabolite ratios of main SCFA acetate, propionate and butyrate and BCFA isobutyrate, isovalerate and valerate of F1 (B) and F2 (C) were calculated from absolute average values of three consecutive sampling days.

Sessile microbiota harbored a unique bacterial and archaeal community distinguishable from planktonic microbiota

At the phylum level, pM and sM of both fermentation systems were mainly represented by Bacteroidetes and Firmicutes (Fig. 4.3C). sM1 and sM2 were dominated by Bacteroidetes, mainly *Bacteroidaceae* (53% and 59%, respectively) with comparable levels in pM2.1 (54%) but lower abundance in pM1.1 (12%) (Fig. 3D). Firmicutes were 1.4 to 2.7 times more abundant in pM compared to the corresponding sM, and were mainly represented by the families *Lachnospiraceae*, *Ruminococcaceae* and *Veillonellaceae*. *Methanobacteriaceae* was the only representative family of the archaeal community and only detected in sM1 at a relative abundance of 2% (Fig. 4.3D).

A Venn diagram was generated to compare operational taxonomic units (OTUs) of pM and sM produced in the same fermentation system (Fig. 4.3A & B). A majority of the detected species were ubiquitous in both pM and sM. However, 13 species were unique to sM1, and 9 species were found in sM2 but not in the corresponding pM. In contrast, only one species was distinctive for pM1.1 (*Erysipelotrichaceae* sp.) and two species for pM2.1 (*Clostridium* sp. and *Eubacterium biforme*) compared to sM1 and sM2, respectively. Out of the pool of sessile-specific bacterial species, *Egerthella lenta*, *Parabacteroides distasonis*, *Rikenellaceae* sp., *Finegoldia* sp., *Bilophila* sp. and *Pyramiobacter piscolens* were present in both sM1 and sM2. Relative abundances of lifestyle-specific species were generally below 1%, except for *P. piscolens* (Synergistetes) which contributed 10% of all 16S rRNA gene reads to sM1, and *Bilophila* sp. (Proteobacteria) with a relative abundance of 12% in sM2.

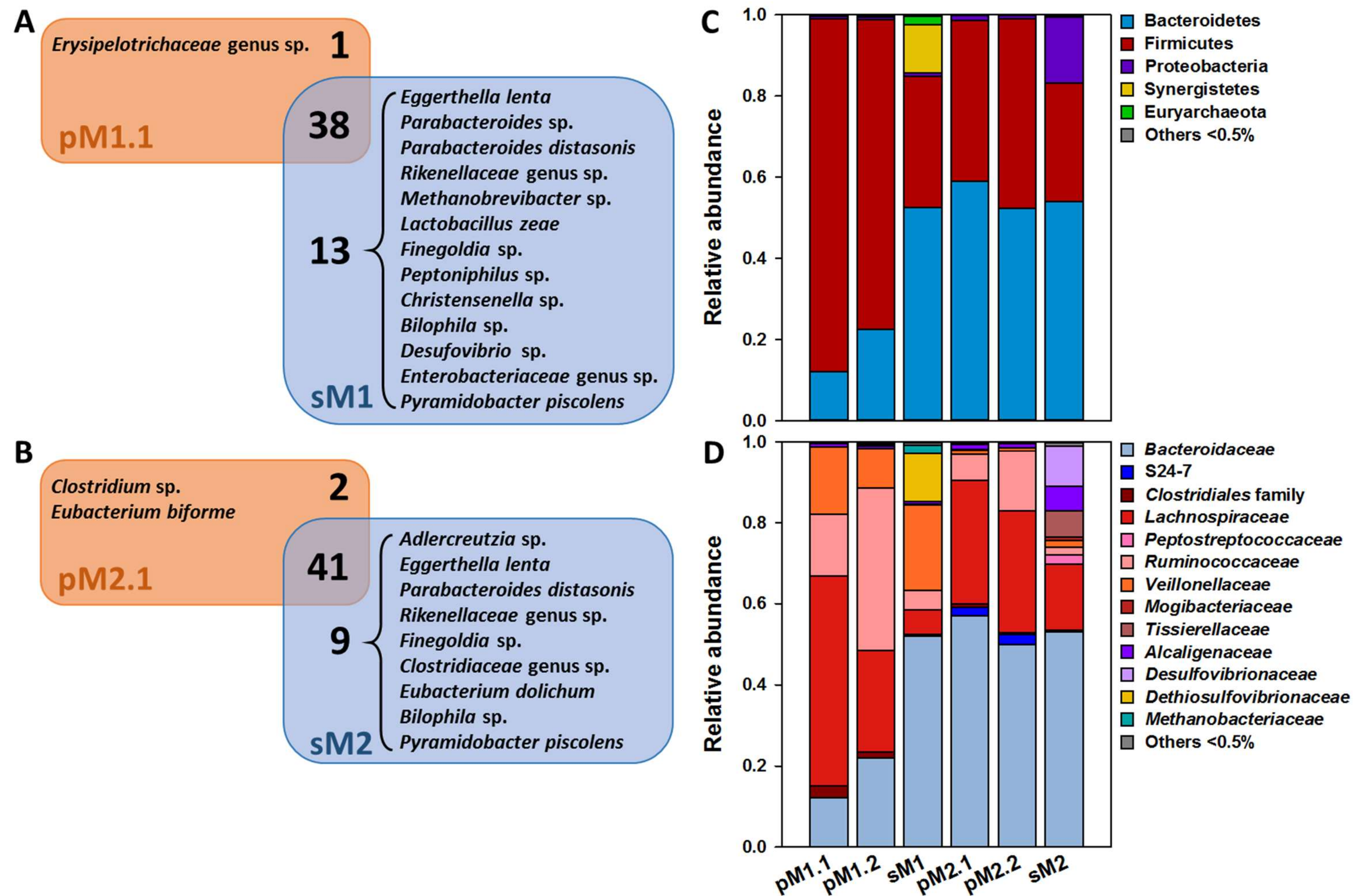

Fig. 4.3 Microbial composition of planktonic and sessile artificial colonic microbiota. (A, B) Shared and unique taxa in sessile and planktonic microbiota of F1 (A, pM1.1 and sM1) and F2 (B, pM2.1 and sM2). The number inside each square represents the amounts of OTUs observed. (C,D) Relative abundance of microbial phyla (C) and families (D) of planktonic microbiota (pM) in F1 and F2 effluents and corresponding sessile microbiota (sM) immobilized in beads was analyzed by V4 region of 16S rRNA gene sequencing. Sampling of pM for cryopreservation (pM1.1 and 2.1) was conducted 21 days before lyophilization of pM1.2 and one day before lyophilization of pM2.2 (Supplementary Fig.4.1)

Effect of cryopreservation and lyophilization on bacterial composition and metabolic activity of pM and sM after batch reactivation

To investigate the effect of cryopreservation and lyophilization, and subsequent storage on the activity of the microbiota, pM and sM samples from F1 and F2 were either snap-frozen in liquid N_2 and stored at -80°C for 9 months, or lyophilized and stored at 4°C for 9 months before reactivation in 24 h batch fermentations (t_2). Immediately before reactivation, pM in polymer beads underwent an additional redispersion step for inoculation. Fresh unfrozen microbiota processed with cryoprotectants (sucrose, inulin and glycerol for cryopreservation; sucrose and inulin for lyophilization) were used as controls (‘fresh pM or sM’, (t_1)) (Fig. 4.1). Metabolite formation and composition of pM and sM were measured after batch reactivation in modified MacFarlane medium and strict anaerobic conditions, in control samples before freezing, and after storing frozen and lyophilized pM and sM for 9 months. Microbiota composition was analyzed using 16S rRNA gene amplicon sequencing, and qPCR was performed to determine total bacteria 16S rRNA gene copy numbers. Main metabolite production, including SCFA, BCFA and fermentation intermediats, was analyzed using HPLC-RI.

Overall community structure of stored pM and sM was better preserved after cryopreservation than after lyophilization

The maximum concentrations for total bacteria after 24 h fresh pM and sM batch fermentations measured by qPCR were similar (log 9.3±0.0 to log 9.5±0.0 16S rRNA gene copies ml^{-1}) (Table 4.1). Furthermore, processing and storage did not affect total bacteria numbers in the inocula and after 24 h batch reactivation of fresh, cryopreserved and lyophilized microbiota.

Table 4.1 Bacterial growth of fresh and stored microbiota. Total log 16S rRNA gene copies ml-1 after inoculation (0 h) and after 24 h batch fermentation of planktonic and sessile microbiota (pM and sM, respectively). Shown are mean and standard deviation from three replicates obtained from fermentation 1 (F1) and fermentation 2 (F2).

		Log 16S rRNA gene copies ml^{-1}							
		pM1		sM1		pM2		sM2	
Preservation method	Condition	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
Cryopreservation	Fresh	7.5	9.3±0.1	7.0	9.5±0.0	8.0	9.3±0.1	7.3	9.3±0.0
	9 months	7.5	9.5±0.0	7.2	9.7±0.0	7.9	9.3±0.1	7.5	9.4±0.0
Lyophilization	Fresh	7.5	9.3±0.1	7.1	9.5±0.1	7.8	9.1±0.0	7.2	9.3±0.1
	9 months	7.4	9.3±0.1	6.9	9.5±0.1	7.7	9.5±0.1	7.0	9.2±0.1

Using principal component analysis (PCA) of 16S rRNA gene sequencing data, genus level composition data of fresh and preserved microbiota after 24 h batch reactivation within the same lifestyle was decomposed into two factors (PC1 and PC2), explaining in total between 87% and 92% of the total variance (Fig. 4.4). PCA analysis showed a stronger effect of lyophilization compared to cryopreservation, indicated by reactivated cryopreserved samples clustering closer to fresh samples than lyophilized samples. Independent from lifestyle, fresh samples were located in a direction

associated with *Bacteroides*. Both cryopreserved and lyophilized pM separated from fresh samples in a direction positively loaded with *Enterococcus* (Fig. 4.4A and B). No clear separation between fresh and preserved sample was observed with sM1 (Fig. 4.4C). Similarly, cryopreserved sM2 closely clustered with fresh samples while lyophilized sM2 moved in a direction associated with *Clostridiaceae* (Fig. 4.4D).

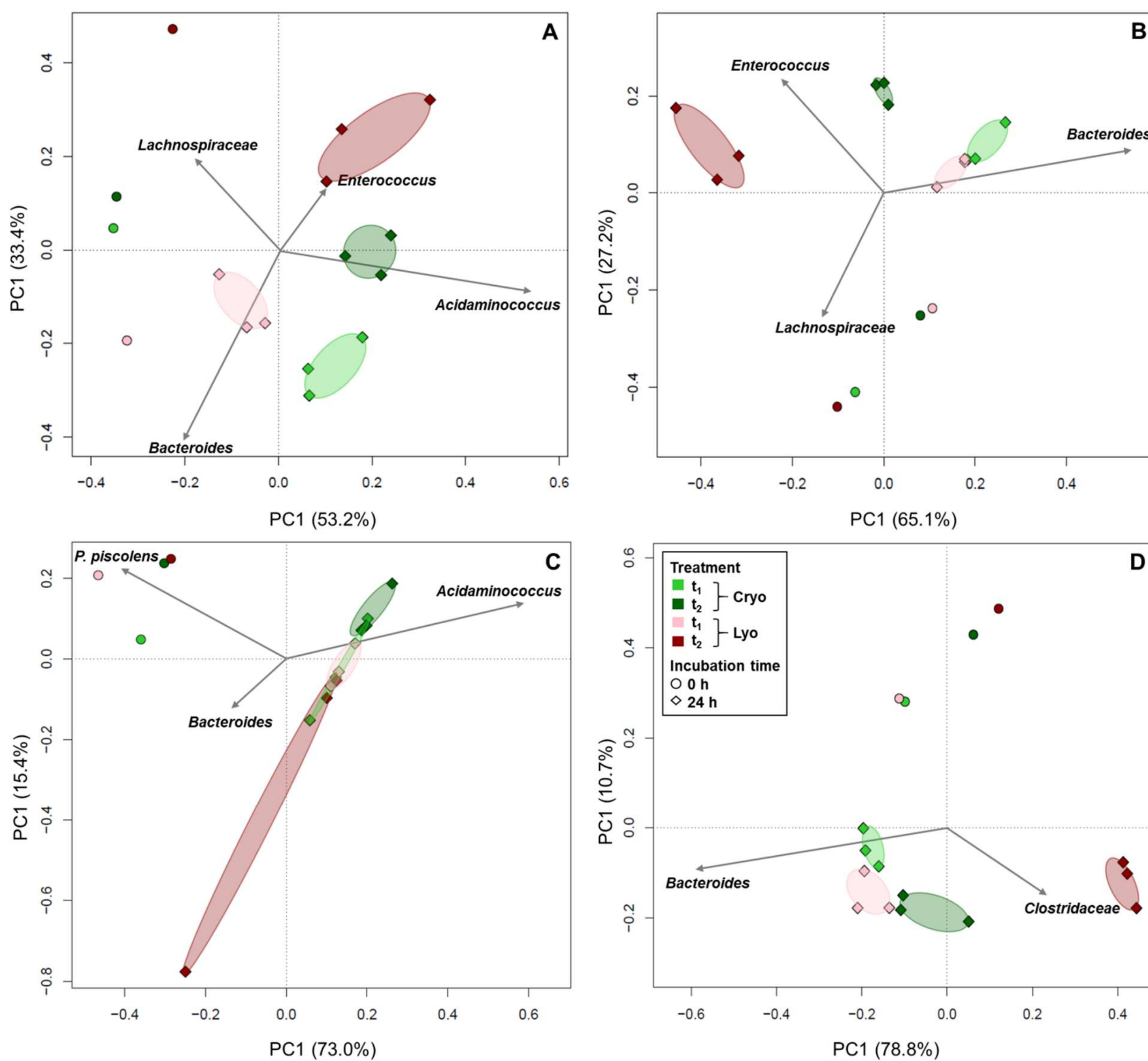

Fig. 4.4 Impact of cryopreservation and lyophilization on microbial community composition. Principal component analysis (PCA) based on weighted Unifrac distance matrices of fresh (t_1), cryopreserved and lyophilized planktonic microbiota (pM) stored for 9 months (t_2) and reactivated in batch fermentation. Each color represents a different treatment, symbol indicate batch fermentation incubation time (0, 24 h) of pM 1 (A), pM 2 (B), sM 1 (C) and sM 2 (D). Cryo, cryopreservation; Lyo, lyophilization.

Average Bray-Curtis similarity was calculated to determine the degree of compositional similarity between fresh and corresponding cryopreserved and lyophilized microbiota of the same lifestyle after 24 h batch reactivation. Based on Bray-Curtis similarity data, processed microbiota from F1 was better preserved than processed microbiota from F2, especially for lyophilized pM and sM samples. Cryopreserved microbiota from F1 and F2 were generally more similar to corresponding fresh samples than lyophilized microbiota (Table 4.2). Bray-Curtis index was highest for cryopreserved pM1 (0.84±0.03) and lowest for lyophilized sM2 samples (0.33±0.04). Preserved sM tended to be compositionally less similar to fresh microbiota than pM.

Table 4.2 Similarity of fresh and preserved microbiota. Bray-Curtis similarity between fresh and preserved planktonic (pM) and sessile (mM) microbiota after 24 h batch fermentations. Shown are mean and standard deviation from three replicates obtained from fermentation 1 (F1) and fermentation 2 (F2).

		Bray-Curtis similarity	
Preservation method	Life style	F1	F2
Cryopreservation	pM	0.84±0.03	0.78±0.03
	sM	0.77±0.05 [B]	0.73±0.06
Lyophilization	pM	0.75±0.06 [A]	0.54±0.06 [A]
	sM	0.73±0.14	0.33±0.04 [AB]

[A] Bray-Curtis similarity of lyophilized microbiota is significantly different from cryopreserved microbiota (p<0.05).
[B] Bray-Curtis similarity of preserved sM is significantly different from preserved pM (p<0.05).

Preservation impaired re-establishment of *Bacteroidaceae* in planktonic and sessile microbiota

The re-establishment of *Bacteroidaceae*, mainly represented by different *Bacteroides* species (Supplementary tables S4.1-8), was impacted by both cryopreservation and lyophilization in pM and sM (Fig. 4.5). The relative abundance of *Bacteroidaceae* decreased in reactivated cryopreserved treatments, significantly (p<0.05) for pM1, sM1 and pM2 (16±2%, 8±1% and 48±1%, respectively), compared to fresh microbiota (30±4%, 14±3% and 63±5%, respectively). The decline in relative abundance of *Bacteroidaceae* after lyophilization compared to fresh samples was most pronounced in sM2 (from 55±3% to 0.4±0.2%, P<0.05) and least impacted in sM1 (from 23±1% to 16%).

Enterococcaceae and *Peptostreptococcaceae* benefited from compositional rearrangements after preservation

Both *Enterococcaceae* and *Peptostreptococcaceae* families re-established at a higher relative abundance when reactivated with preserved rather than fresh microbiota, independent from fermentation system and lifestyle (Fig. 4.5). Relative abundance of *Enterococcaceae* significantly (P<0.05) increased from 6±1%, 4±0% and 5±2% in fresh to 12±0%, 17±1% and 8±2% in cryopreserved sM1, pM2 and sM2 respectively, but remained at levels below 0.1% in cryopreserved pM1. In

lyophilized pM1, pM2, sM1 and sM2, relative abundance of *Enterococcaceae* (14±0%, 18±5%, 8% and 5±1%,) was significantly (*P*<0.05) higher compared to fresh microbiota (3±1%, 6±0%, 5±1% and 3±0%). Lyophilization enhanced relative abundance of *Peptostreptococcaceae* in sM1, pM2 and sM2 from 0.5±0.1%, 1±0% and 8±5% in fresh to 4%, 5±2% and 18%±5% in stored samples, respectively. An increase in relative abundance of *Peptostreptococcaceae* after cryopreservation was only observed with sM1, from 1±0% in fresh to 4±1% in lyophilized samples (*P*<0.05).

Preservation induced compositional shifts within the order *Clostridiales* depending on microbiota composition and life style

Cryopreservation and lyophilization induced major rearrangements within the order *Clostridiales*. Unlike planktonic lifestyle, a sessile lifestyle favored re-establishment of *Clostridiaceae* after preservation. *Clostridiaceae* were generally present in low abundance (<1%) in all reactivated fresh microbiota and remained at comparable levels in cryopreserved pM1 and pM2, but increased to 2±1% in sM1 and 8±3% in sM2. Moreover, lyophilization enhanced abundance of *Clostridiaceae* in sM (0.1±0.1% to 5% and 8±4% to 19±3%, in fresh and lyophilized sM1 and sM2, respectively).

Relative abundance shifts of members of *Veillonellaceae* and *Tissierellaceae* were microbiota- but not lifestyle-specific. Members of *Veillonellaceae,* mainly represented by the genus *Acidaminococcus* (Supplementary table S4.1, S4.2, S4.5, and S4.6), were present in pM1 and sM1, while *Tissierellaceae*, mainly *Peptoniphilus,* was distinctive for pM2 and sM2 (Supplementary table S4.3, S4.4, S4.7, and S4.8). *Veillonellaceae* dominated batch fermentations inoculated with fresh pM1 and sM1 (58±3% and 47±7%) and remained at similar levels after cryopreservation (62±2% and 55±4%). Lyophilization significantly increased abundance of *Veillonellaceae* in reactivated pM1, from 47±3% in fresh to 57±6% (*P*<0.05). Relative abundance of *Tissierellaceae* increased from 2±0% and 3±0% in fresh to 3±0% and 10±1% in cryopreserved pM2 and sM2, respectively (*P*<0.05). Lyophilization similarly impacted *Tissierellaceae* in pM2 and sM2 with three times higher relative abundance observed in preserved (9±1 and 18±1%) than in fresh microbiota (3±1% and 6±0%, *P*<0.05). Lyophilization also specifically enhanced *Lachnospiraceae* species within pM2 and sM2, while such an effect was not observed after cryopreservation. Relative abundance of *Lachnospiraceae*, already present at 25±3% and 17±2% in fresh pM2 and sM2, almost doubled after reactivation of lyophilized pM2 and sM2 (46±2% and 35±4%, respectively; *P*<0.05).

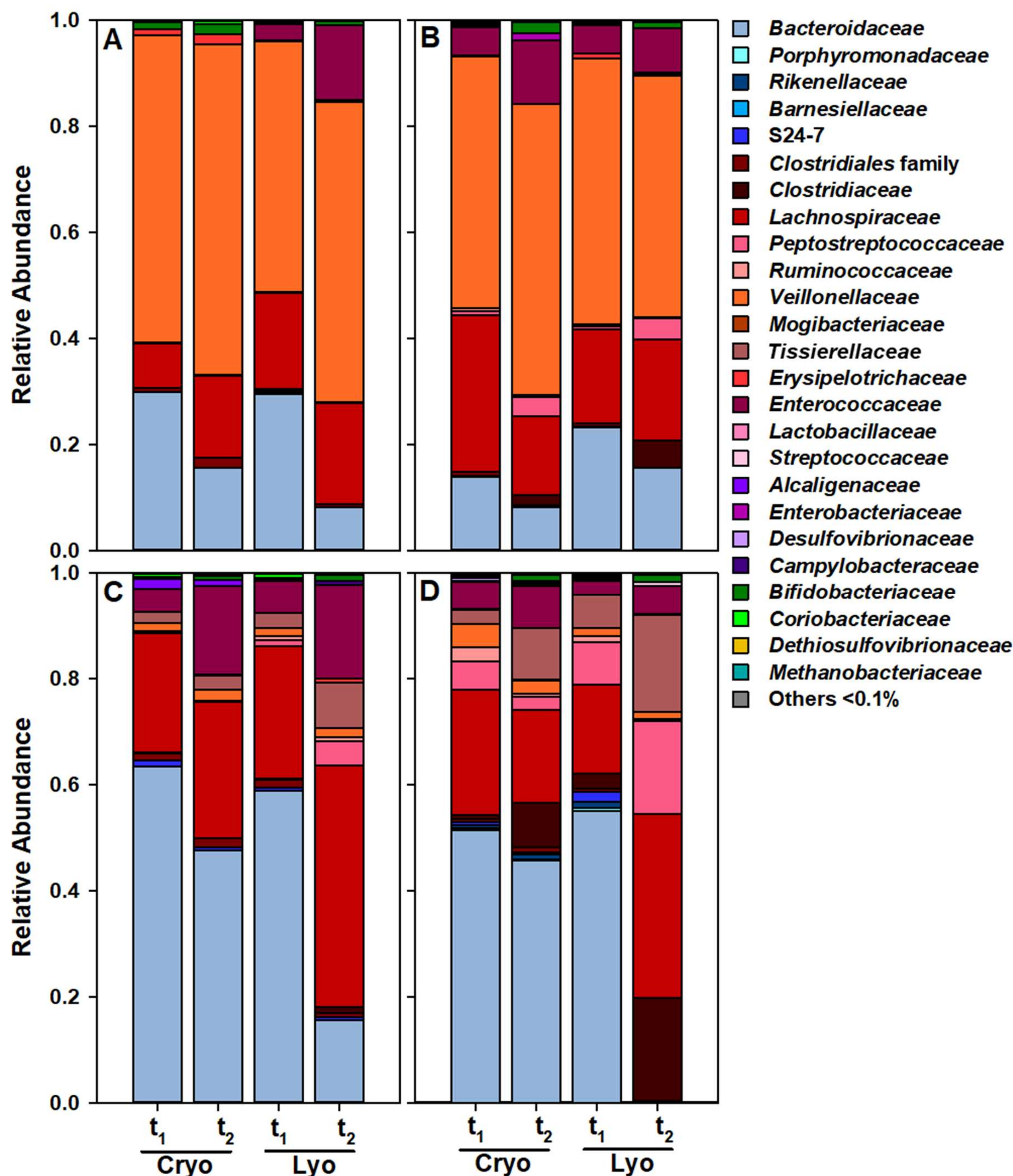

Fig. 4.5 Microbial composition of fresh and preserved planktonic and sessile microbiota reactivated in batch fermentation. Relative abundance of bacterial and archaeal families of fresh and preserved planktonic (pM) and sessile (sM) microbiota of fresh (t1), cryopreserved and lyophilized (t2) pM 1 (A), sM1 (B), pM2 (C) and sM 2 (D) was analyzed by V4 region of 16S rRNA gene amplicon sequencing after 24 h batch fermentation. Relative abundance presented are average values of three independent batch fermentations. Cryo, cryopreservation, Lyo.

Preservation induced impairment of metabolic activity was more severe in sM than pM

To evaluate the effect of the environmental stressors of cryopreservation and lyophilization following subsequent storage on microbial activity, we monitored SCFA and BCFA production during 24 h batch fermentations of fresh and preserved pM and sM. Metabolic activity of fresh sM differed from fresh pM. SCFA and BCFA productions were higher in sM compared to pM (Fig. 4.6A, B, C and D). In the cryopreservation trial, fresh pM1 and pM2 formed 81.7±2.2 and 85.5±1.0 mM total SCFAs,

respectively, and <1.5 mM BCFA, while sM1 and sM2 produced 86.6±2.0 and 95.2±2.6 mM SCFAs in addition to 5.0±1.4 and 11.6±0.8 mM isobutyrate and isovalerate, respectively. Metabolic activity of fresh pM and sM in the lyophilization trial confirmed the observed differences (Fig. 4.7A and B).

Fresh sM exhibited a more acetogenic character and lower portion of butyrate compared to pM (Fig. 4.6 and 7 (inserts)). Shifts in the metabolic profile after frozen storage of cryopreserved microbiota were distinctive for sM and pM, indicating a lifestyle-specific effect on SCFA- and BCFA-producing activity (Fig. 4.6). Compared to fresh conditions, the proportion of acetate produced by cryopreserved sM1 and sM2 was enhanced (from 55% and 61% to 62% and 68%, respectively) at the expense of propionate and butyrate in sM1 and only propionate in sM2. Butyrate-producing activity was fully recovered by cryopreserved pM (105% and 109% of corresponding fresh pM1 and pM2) after 24 h incubation along with slightly lower acetate formation (86% and 91%) (Table 4.3), shifting the metabolic profile from acetate towards butyrate (Fig. 4.6E and F). Accumulation of 6.0±2.0 mM formate in batch fermentation inoculated with cryopreserved pM1 was accompanied by a decrease in the proportion of propionate, from a concentration of 16% in the metabolic profile of fresh microbiota to 12% after frozen storage (Fig 4.6E). Besides affecting final SCFA concentrations, cryopreservation also impacted the re-initiation of metabolic activity. Onset of main SCFA formation by cryopreserved pM and sM were significantly delayed compared to fresh microbiota, while concentrations of the fermentation intermediates lactate and formate were higher after 6 h batch fermentation (Fig. 4.6).

Table 4.3 Percentage of recovery of metabolic activity after cryopreservation and lyophilization of planktonic (pM) and sessile microbiota (sM) measured by HPLC-RI.

Preservation method	Life style		Metabolite recovery (%)[a]		
			Acetate	Propionate	Butyrate
Cryopreservation	pM	1	86%	73%	109%
		2	91%	118%	105%
	sM	1	126%	67%	80%
		2	91%	59%	97%
Lyophilization	pM	1	84%	51%	87%
		2	81%	35%	112%
	sM	1	110%	18%	46%
		2	99%	16%	72%

[a] Metabolite recovery (in %) of preserved microbiota was calculated relative to the average metabolite production by fresh microbiota.

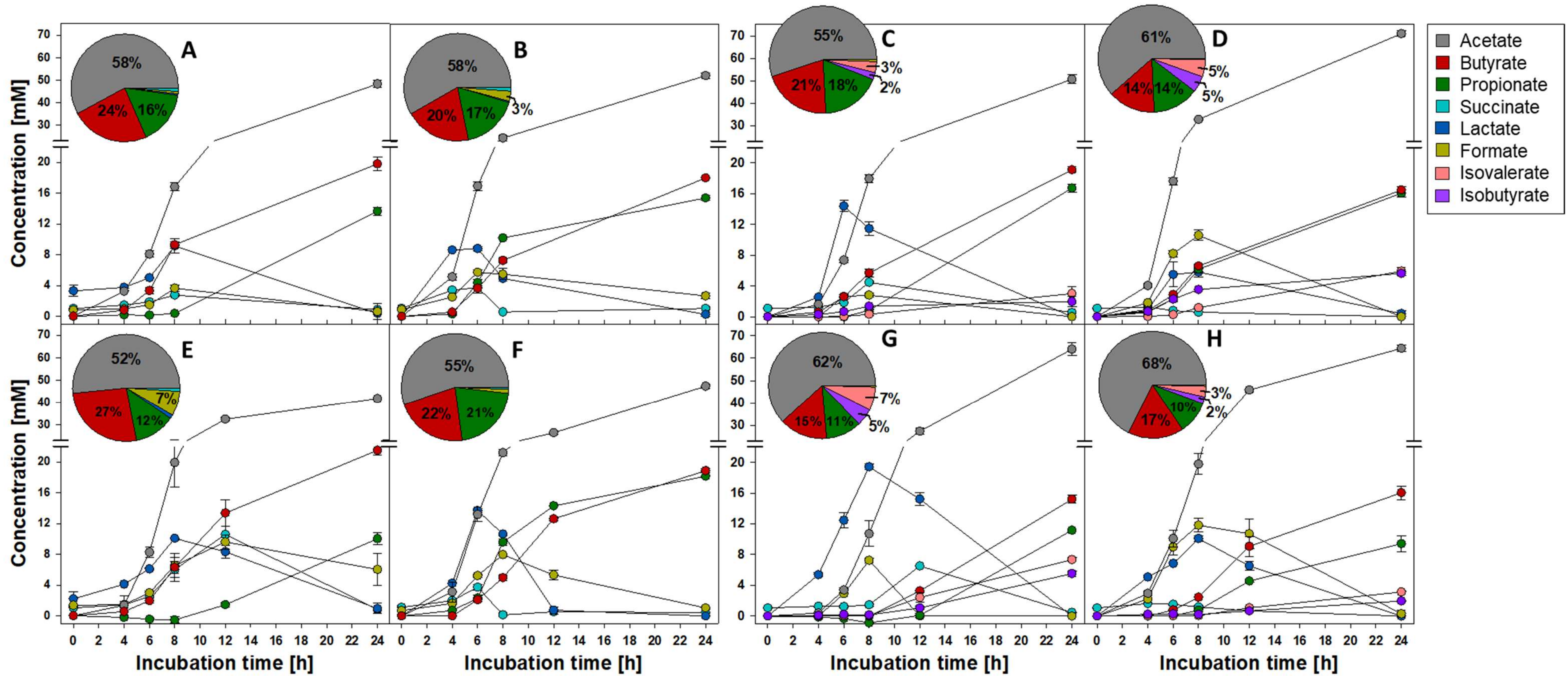

Fig. 4.6 Metabolite production by fresh and cryopreserved planktonic and sessile microbiota during batch fermentation. Shown are SCFA, BCFA and fermentation intermediates produced during batch fermentation of fresh (A - D) and cryopreserved (E - H) planktonic (A, B, E, F) and sessile (C, D, G, H) microbiota. Metabolite ratios (insets) were calculated from absolute average values of triplicate 24 h batch fermentations.

Lyophilization more severely affected metabolic activity than cryopreservation, with stronger detrimental effects on sM than pM (Table 4.3). Final butyrate concentrations did not significantly differ between fresh and lyophilized pM, while acetate formation was only partly recovered (84% and 81%, respectively). Propionate production was severely reduced, as shown by recoveries of 51% and 35% by lyophilized pM1 and pM2, respectively. As a result, metabolic profiles of lyophilized pM shifted from acetate and propionate towards butyrate. Butyrate and propionate production by sM were impacted to a greater extent by lyophilization than pM, as shown by reduced recovery rates of 54% and 72% butyrate and 19% and 16% propionate by lyophilized sM1 and sM2, respectively. In contrast, no difference in acetate formation was detected. Similar to cryopreserved pM1, formate also accumulated in lyophilized pM1 (9.4±0.4 mM) and lactate was still detected after 24 h incubation of lyophilized pM2 (4.0±2.6 mM) (Fig. 4.7C). In contrast, BCFA-producing activity was maintained in both sM1 and sM2 lyophilized compared to control fresh samples (Fig. 4.7B and D).

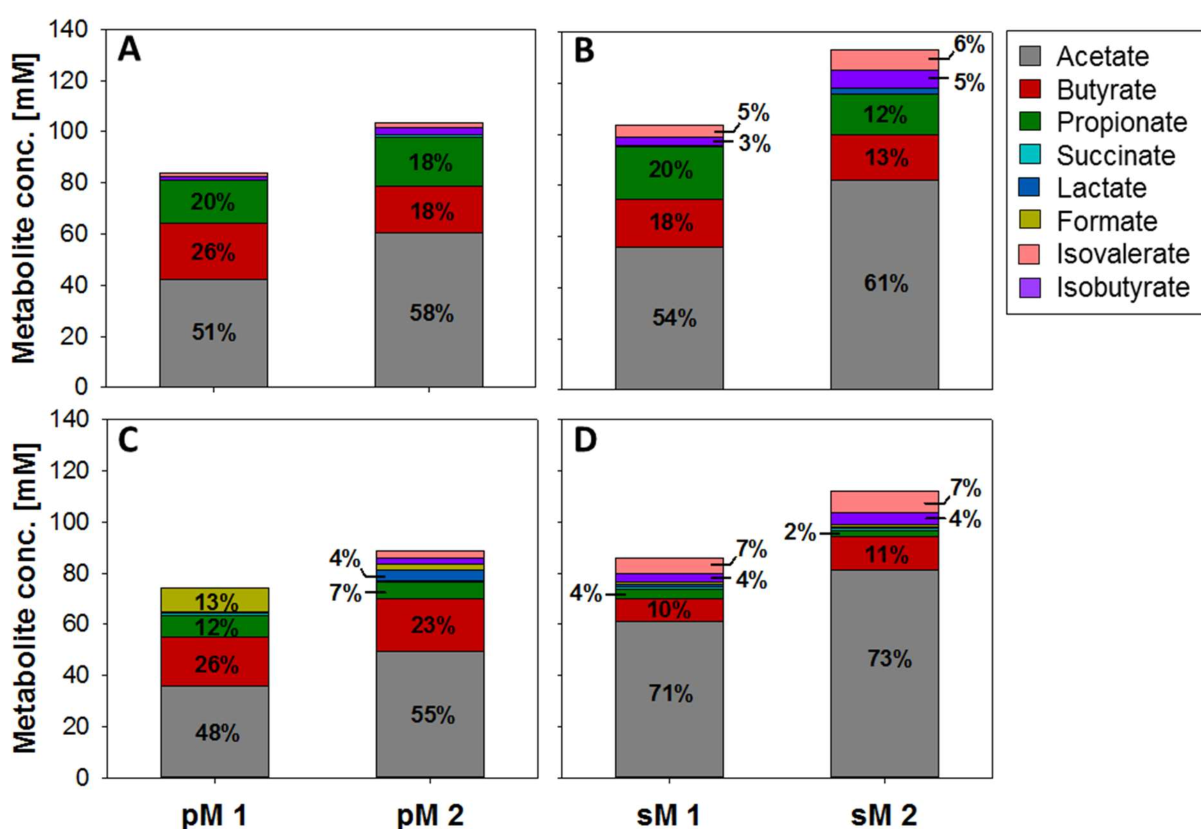

Fig. 4.7 Metabolites produced by fresh and lyophilized planktonic and sessile microbiota during batch fermentation. Shown are absolute values and ratios of main SCFA, BCFA and fermentation intermediates produced by fresh pM (A), fresh sM (B), lyophilized pM (C) and sM (D) after 24 h batch fermentation. Metabolite ratios and absolute concentrations are average values of three independent batch fermentations..

Discussion

The mammalian gastrointestinal tract harbors both planktonic and sessile mucosa- or particle-associated microbiota, which differ in community composition (Walker*, et al.*, 2008, Li*, et al.*, 2015, Liu*, et al.*, 2016). In this study, we demonstrated that two different lifestyles (planktonic and sessile) of artificially-produced colonic microbiota also differ in composition. We provide evidence that these compositional differences lead to alterations in metabolic activity, and that microbiota of the different lifestyles responded differently when exposed to technological processes applied for preservation.

Planktonic and sessile PolyFermS microbiota have different composition, metabolic activity, and trophic interactions

Both the gut and PolyFermS *in vitro* fermentation systems provide particles for microbes to adhere and form sessile mixed-species communities. In agreement with other studies from human and rumen samples (McAllister*, et al.*, 1994, Walker*, et al.*, 2008, Li*, et al.*, 2015, Liu*, et al.*, 2016, Welch*, et al.*, 2017), bacterial lifestyle impacted microbiota composition. A lower proportion of Firmicutes in sessile compared to planktonic microbiota was a lifestyle-associated discriminant of the PolyFermS system. Polymer gel used to entrap fecal microbiota demonstrated high physical stability during extended fermentation periods exceeding 40 weeks, indicating resilience to degradation by growing fecal microbes, and to mechanical and chemical stresses occurring in the continuous stirred reactors (Cinquin*, et al.*, 2004, Fehlbaum*, et al.*, 2015). This stability was corroborated in several long-term mixed fermentation processes after careful selection of entrapment gel composition (Lamboley*, et al.*, 1997, Lamboley*, et al.*, 1999, Lamboley*, et al.*, 2001). Providing a polymer-based matrix is one of the main criteria that need to be fulfilled for a structure in the intestinal ecosystem to be considered a biofilm (Sonnenburg*, et al.*, 2004). Some of the detected sessile specific species *Eggerthella lenta*, *Parabacteroides distasonis*, *Rikenellaceae* sp., *Finegoldia* sp., *Bilophila* sp. and *P. piscolens* in the PolyFermS system have previously been associated with colonic biofilms (Donelli*, et al.*, 2012). Thereof, biofilm-growing bacteria from the genera *Bacteroides*, *Parabacteroides* and *Finegoldia*, isolated from intestinal biliary stents, were able to adhere to abiotic surfaces (Donelli*, et al.*, 2012). Compositional differences between the two lifestyles might be driving the physiochemical conditions that dominate the bead and liquid phases of the fermentation systems. In the well-mixed bioreactors, continuous stirring allows more uniform spatial conditions for the growth of planktonic cells. In contrast, sharp nutrient, including oxygen, metabolic product and pH gradients are common properties of single- and multispecies biofilms, which provides a variety of microhabitats, such as in gel beads (Doleyres*, et al.*, 2002, Doleyres*, et al.*, 2004, Schepers*, et al.*, 2006, Payne*, et al.*, 2012, Flemming*, et al.*, 2016). Gradients of nutrient availability, product concentration and pH may develop within ca. 100-200 micrometers of the highly-colonized peripheral layer of polymer beads due to limited diffusion of the substrate and metabolites formed. For example microbial production of acids, such as lactate and

acetate, sharply decrease growth and activity of sessile bacteria towards the bead center (Arnaud*, et al.*, 1992, Doleyres*, et al.*, 2002, Junter*, et al.*, 2002, Cinquin*, et al.*, 2004, Schepers*, et al.*, 2006).

Our results indicate a differentiation in the use of metabolic pathways between sessile and planktonic microbiota. In general, fresh sessile microbiota encompassed a higher degradation capacity than the planktonic counterpart, as indicated by the higher levels of total metabolites formed from degradation of carbohydrates and proteins supplied by the batch medium. Higher species diversity, which would provide a greater pool of genes encoding for carbohydrate-active enzymes and breakdown of polysaccharides in bacterial cooperation, might be responsible for the increased degradation capacity of sessile microbiota in comparison to the corresponding microbiota in free-cell state (Macfarlane and Macfarlane, 2006, Flint*, et al.*, 2012, El Kaoutari*, et al.*, 2013). It was previously shown that biofilm communities populating food residues in fecal material produced higher amounts of acetate but lower proportions of butyrate from carbohydrate fermentation than their non-adherent counterpart (Macfarlane and Macfarlane, 2006). We observed similar lifestyle-associated metabolic characteristics, with sessile microbiota producing higher proportions of acetate and planktonic microbiota enhancing proportions of butyrate.

Isovalerate and isobutyrate were mainly detected in batch cultures inoculated with sessile microbiota. BCFA are specific end-products of protein fermentation by human intestinal microbiota (Macfarlane*, et al.*, 1986, Yao*, et al.*, 2016). The detection of BCFA either indicates increased proteolytic activity of sessile than planktonic microbiota, or a faster depletion of available carbohydrate sources due to enhanced metabolic activity that forces the microbes to switch to protein and amino acid metabolism when the carbohydrate energy source is exhausted. It can be speculated from our data on sM batch culture reactivation that microbiota embedded in beads might create a microenvironment more akin to nutrient limited conditions of the distal colon, including: (I) limited supply of carbohydrates which are readily accessible and rapidly fermented by the planktonic microbiota combined; (II) diffusion of substrates not preferentially fermented by the planktonic microbes, including amino-acids and proteins ; (III) protein fermentation activity by sessile microbes; (IV) a high local pH within beads resulting from the balance of low carbohydrate and high protein fermentations with production of ammonia.

Cooperation between microbes involves exchange of metabolites, genetic material and signaling molecules and is an emerged property of natural biofilms that might also apply to actively immobilized bead communities, allowing growth in microcolonies and promoting cell-cell contacts (Flemming*, et al.*, 2016). Species occurrence in the sessile microbiota also points at trophic interactions. *Pyramidobacter* sp. and *Bilophila* sp., which were abundant in sM1 and sM2, respectively, but not in planktonic microbiota, are phylogenetically different but exhibit a similar physiology. Both genera are asaccharolytic amino acid degraders that mainly produce acetate, and low amounts of isovalerate, isobuyrate and hydrogen sulfide (Baron, 1997, Downes*, et al.*, 2009). *P. piscolens*, rarely cultivated *in*

vitro, was successfully isolated from dental plaque communities in co-culture with *Methanobrevibacter massiliens*, suggesting a symbiotic relationship where *P. piscolens* uses methane produced by *M. massiliens* to form hydrogen sulfide (Marchandin*, et al.*, 2010, Huynh*, et al.*, 2017). Indeed, *Methanobrevibacter* sp. was exclusively found in sM1 where *P. piscolens* was also present in high abundance. Similarly, *Bilophila wadsworthia* was previously recovered in mixed cultures, where growth completely depended on a "helper strain" producing growth-promoting factors (quinones) (Baron, 1997, Fenn*, et al.*, 2017). Several bacteria can act as "helpers" supplying quinones, including *Eggerthella lenta*, *Eubacterium rectale* and different *Bacteroides* species. Thereof here *E. lenta* was uniquely present in sM. Both *P. piscolens* and *Bilophila* sp. did not establish in batch culture, after dissolution of the beads that forced microbiota to change from a sessile to a planktonic lifestyle. Destroying the bead structure can ultimately lead to destabilization of the symbiotic network and therefore loss of microbes that can only coexist with another species (Sachs and Hollowell, 2012). Consequently, batch fermentation favors the fraction of resuspended sessile microbiota that can grow in planktonic state and is therefore an imperfect activity read out of the preserved microbiota.

Taxa specific response to preservation stresses

Lyophilization and cryopreservation can impair growth and can lead to lethal damage of a certain fraction of preserved bacteria cells, clearing niches for more resistant taxa that are less competitive in fresh microbiota (Messer*, et al.*, 2017). Tolerance to freezing and drying varies greatly between different anaerobic gut microbes (Bircher*, et al.*, 2018). We generally observed a shift in relative abundance from gram-negative Bacteroidetes towards gram-positive Firmicutes, mainly due to a reduction in *Bacteroidaceae* that was more pronounced in lyophilized than cryopreserved microbiota. Previous preservation studies demonstrated that gram-positive bacteria have an advantage over gram-negative bacteria in surviving lyophilization (Miyamoto-Shinohara*, et al.*, 2000, Miyamoto-Shinohara*, et al.*, 2008). It has been suggested that the high portion of cross-linked peptidoglycans in the gram-positive cell walls provides structural strength during the lyophilization process, while gram-negative microbes are more prone to disruption because of their significantly thinner peptidoglycan layer (Pembrey*, et al.*, 1999). Frozen storage at -80°C similarly affected microbial composition of fecal samples by decreasing the Bacteroidetes:Firmicutes ratio (Bahl*, et al.*, 2012).

Enterococcaceae competed more effectively in the complex microbiota after cryopreservation and lyophilization than *Bacteroidaceae*. The probiotic *Enterococcus faecium* (IFA No.045) previously showed good process resistance to lyophilization in the presence of the non-reducing glucose, trehalose or sucrose, with survival rates of 80% (Strasser*, et al.*, 2009). High viability after preservation guarantees a competitive advantage for re-establishment when challenged with a complex microbiota. *Enterococcus* species are major lactate producers (Sung*, et al.*, 2017), and might have contributed to the enhanced lactate formation by cryopreserved microbiota that was observed. In return, increased lactate production can boost cross-feeding and stimulate growth of lactate-utilizing and butyrate-

producing bacteria such as *Eubacterium hallii* and *Anaerostipes caccae* and therefore indirectly also butyrate formation (Duncan*, et al.*, 2004, Belenguer*, et al.*, 2006). Indeed, relative abundance of *Anaerostipes* sp. tended to increase after both lyophilization and cryopreservation.

Functional redundancy ensures butyrate formation after processing

The composition of butyrate-producing bacteria differed between microbiota. The main contributors to butyrate formation during batch fermentations inoculated with fresh pM1 and sM1 were members of the *Lachnospiraceae* family as well as the highly abundant *Acidaminococcus*. Saccharolytic *Lachnospiraceae* mainly use the butyryl-CoA:acetate CoA-transferase pathway for butyrate production, with a net consumption of acetate (Louis and Flint, 2017). In contrast, the asaccharolytic *Acidaminococcus fermentas* and *Acidaminococcus intestine* use amino acids to produce acetate and butyrate (Jumas-Bilak*, et al.*, 2007, Chang*, et al.*, 2010, D'Auria*, et al.*, 2011). For pM2 and sM2, butyrate may be derived mainly from *Lachnospiraceae* members and *Peptoniphilus* species. *Peptoniphilus* is part of the commensal gut microbiota and includes butyrate-producing, non-saccharolytic species that use peptone and amino acids as major energy sources (Ezaki*, et al.*, 2001, Song*, et al.*, 2007). Preservation-induced stresses slowed down but did not impair butyrate production. Functional redundancy among the butyrate-producing taxa, the variability in butyrate-producing pathways and a broad substrate spectrum of pM and sM might have maintained butyrate formation after preservation (Louis and Flint, 2017, Reichardt*, et al.*, 2018). Lyophilization of sessile microbiota was an exception in terms of recovery of butyrate formation. Reduced butyrate production co-occurred with acetate accumulation, likely due to the lower net consumption that is indicative of an incomplete fermentation (Duncan*, et al.*, 2002). In contrast to butyrate, propionate formation was diminished after cryopreservation and, to a greater extent, after lyophilization. Three different bacterial pathways exist that lead to propionate in the human colon; of these, the succinate pathway contributes most to propionate formation (Reichardt*, et al.*, 2014). Impaired re-establishment of *Bacteroidaceae* after preservation, which employs the succinate pathway, might have led to the reduced propionate formation in pM and sM.

Impact of lifestyle on compositional and metabolic response to preservation stresses

We exposed planktonic and sessile microbiota to freezing and freeze-drying and hypothesized that PolyFermS-derived sM are more resistant to environmental stressors than pM. Unexpectedly, pM was less affected by preservation than sM, as shown by higher compositional similarity to fresh microbiota, and higher recovery of major SCFA butyrate and propionate. Bacteria in biofilm communities are embedded in a self-produced exopolymeric matrix that provides protection from the external environment (Hobley*, et al.*, 2015). Enhanced stress tolerance of biofilms formed by single or mixed bacterial species against acid, solvents and antimicrobials is derived partly from the limited diffusion of active substances into the biofilm matrix (Türetgen*, et al.*, 2006, Kubota*, et al.*, 2009, Knudsen*, et*

al., 2012, Lee*, et al.*, 2014). However, this protective matrix that confers a survival advantage under specific environmental conditions can turn into a burden, as diffusion of protective components into deeper layers of the polymer beads might have been limited. In contrast, planktonic cells of pM were evenly exposed to the protective medium, which allowed consistent protection of the complete microbiota.

Complex interactions between bacterial species and inter-individual dependency could establish only within the sM microbiota during extended fermentation periods. Preservation induces cell death of key microbial species and can ultimately lead to impairment of the dependent microbes not impacted by preservation (Sachs and Hollowell, 2012). This could explain the observed reduced fermentation activity of sessile compared to planktonic microbiota especially after lyophilization. *Clostridiaceae* were the only sM-specific taxon that increased after preservation, likely due to concomitant decreases of competing populations providing a new niche.

Conclusion

This study found that bacterial lifestyle is a determining factor in shaping microbial composition and metabolic capacity of artificially-produced colonic microbiota. Sessile microbiota was metabolically more active than planktonic microbiota, but was also more sensitive towards preservation-induced stressors. Our data indicate that cooperating microbes might be a characteristic component of sessile microbiota possibly affecting compositional stability towards freezing and freeze-drying stressors. Accordingly, preservation-induced cell death of a key taxon might provoke a concomitant decrease of dependent taxa. In general, taxon-dependent individual sensitivities to preservation-induced stressors might lead to a loss of initial community structure but not necessarily to a change in functionality. Functional redundancy, as observed for the butyrate-producing community of both microbiota, might be one trait that guarantees functional stability during exposure to environmental stress. In contrast, if a certain function, such as propionate production, is limited to a small number of taxa, the loss of key taxa can ultimately lead to loss of function. Our data show the feasibility of producing complex artificial microbiota in sessile and planktonic lifestyle with the continuous PolyFermS platform inoculated with fecal microbiota immobilized in gel beads, and subsequent preservation by freezing and lyophilization. Even though sessile microbiota exhibited reduced resistance towards preservation in our experimental conditions, it may be worth to investigate further the activity of entrapped microbiota directly in bead structure because the resuspension step may have led to a deorganization of metabolic networks developed during reactor cultivation.

Material and Methods

Preparation of protective media

The applied protective media was 0.1 M phosphate buffer (PB), added with the protective agents sucrose (VWR International AG, Switzerland), inulin (RPN Foodtechnology AG, Switzerland) and glycerol (VWR International AG). Prior to preparation, components of PB as well as the used protectants were stored in an anaerobic chamber (10% CO_2, 5% H_2 and 85% N_2) (Coy Laboratories, USA) overnight to remove residual oxygen. To prepare PB, sodium dihydrogen phosphate (6.0 g l^{-1}) and sodium hydrogen phosphate (7.1 g l^{-1}, both Sigma-Aldrich Chemie GmbH, Switzerland) were dissolved in oxygen-free distilled water. The pH was adjusted to 6.8 after the addition of the reducing agents cysteine-HCl and riboflavin (Sigma-Aldrich Chemie GmbH) at final concentrations of 1 g l^{-1} and 0.3 g l^{-1}, respectively, to counter potential oxygen exposure during processing and storage (Khan*, et al.*, 2014, Bircher*, et al.*, 2017). All three protectants [glycerol (15% v/w), sucrose and inulin (both at 5% w/v)] were dissolved in PB for cryopreservation (GSI), whereas a protective medium containing only sucrose and inulin (both 5% w/v) was used in the lyophilization trials (SI). Protective media were filter-sterilized, covered in aluminum foil to protect from light and stored in an anaerobic chamber before use.

Preparation of batch fermentation medium

A nutritive medium for human colonic microbiota was used to investigate metabolic activity and compositional reestablishment of fresh and preserved pM and sM in batch fermentation. The medium used for continuous intestinal fermentations was designed to imitate adult ileal chyme entering the colon (Macfarlane*, et al.*, 1998), and enriched with a filter-sterilized vitamin solution (Michel*, et al.*, 1998). This medium was adapted to conditions in batch fermentation (Bircher*, et al.*, 2017) by increasing buffering capacity, reducing carbohydrate content and adding a SCFA mix (Duncan*, et al.*, 2002). Ingredients and preparation were described previously (Bircher*, et al.*, 2017).

Production of artificial colonic microbiota with intestinal fermentation technology

For each fermentation system, fresh fecal microbiota from a different donor underwent an immobilization procedure. Feces were sampled from two healthy men, aged between 30 and 40 years, and who had not been treated with antibiotics for the last three months. The Ethics Committee of ETH Zurich exempted this study from review because sample collection was not in terms of intervention. An informed written consent was, however, obtained from the fecal donors. Immediately after defecating, 5 g fecal material was collected in a pre-weighted Falcon tube containing 5 ml sterile, pre-reduced peptone water (0.1%, pH 7) (Oxoid AG, Switzerland). Fecal samples were transported in an anaerobic jar (Anaerojar, Oxoid, England) to maintain anaerobic conditions. All further immobilization steps, involving encapsulation of fecal microbiota into 1-2 mm polymer gel beads containing gellan (2.5% v/w), xanthan (0.25% v/w) and sodium citrate (0.2% v/w), were performed in an anaerobic

chamber as described previously (Zihler Berner*, et al.*, 2013). Sixty milliliters of gellan-xanthan beads were transferred to a glass bioreactor (Sixfors, Ismatec, Switzerland) filled with 140 ml of nutritive medium. Initially, consecutive batch fermentations were carried out to colonize the beads (Bircher*, et al.*, 2017). The system was changed to continuous mode thereafter, with a constant flow rate of 25 ml h^{-1} medium, generating a mean retention time of 8 h. The pH was set to 5.7, controlled by the addition of 2.5 M NaOH, temperature was maintained at 37°C and a stable CO_2-flow produced anaerobic conditions in the bioreactors (Poeker*, et al.*, 2018). Stability in microbial composition was reached after operating in continuous mode for 10 days, as indicated by stable base consumption and metabolite formation monitored daily. On the day of the preservation experiments, 1 ml of effluent (pM) were taken for microbial metabolite analysis by HPLC-RI and microbiota composition analysis by 16S rRNA gene amplicon sequencing. Analysis of sessile microbiota was done on 0.5 g of beads (sM).

Harvesting, processing and preservation of pM and sM

Twenty-five milliliters of liquid-phase pM were directly collected from the bioreactors and transported to an anaerobic chamber where all further steps were conducted (Bircher*, et al.*, 2017). Liquid phase was divided in 12-ml portions in Hungate tubes, and biomass was harvested by centrifugation at 4°C for 10 min at 3’000 x g. The pellet was washed in 5 ml PB, centrifuged and re-suspended in either 1.2 ml SI or GSI medium (10-fold concentration) and was kept for 30 min at room temperature to allow penetration of glycerol. Aliquots of 100 µl were either used immediately for reactivation of fresh pM and sM in batch fermentation (see below) or were immersed in liquid nitrogen and either stored at -80°C for 9 months in screw-cap polypropylene cryo tubes (Bioswisstec AG, Switzerland) or filled in sterile, pre-reduced, long stem Vacule cryogenic ampules (Sigma-Aldrich) (Figure 1) for lyophilization.
For collection of sM, bioreactors were transferred to an anaerobic chamber and opened to harvest colonized gellan-xanthan beads with a metal sieve after 48 and 19 days of operation of F1 and F2, respectively. Collected beads were washed in PB and aliquots of 0.5 g were weighed into screw-cap polypropylene cryo tubes for cryopreservation, or in Vacuole cryogenic ampules for lyophilization (0.1 g). One hundred microliters of GSI medium was added to the beads in the cryo tubes, and 50 µl SI medium to the beads in the Vacule cryogenic ampules to completely cover the beads in protective medium, and samples were kept for 30 min at room temperature. Samples were either used immediately for reactivation test of fresh condition, or immersed in liquid nitrogen and stored at 80°C for 9 months, or lyophilized.
Samples were lyophilized with a manifold freeze-dryer (VirTis BenchTop 2K) (SI). Frozen effluent samples in Vacule cryogenic ampules were kept on dry ice before lyophilization to prevent sample melting before vacuum was reached. Vacule cryogenic ampules were plugged with sterile cotton wool, and with blue silica gel as a moisture indicator (Sigma-Aldrich). Samples were freeze-dried for 6 h at 80 mTorr, with a condenser temperature of -80°C. Ampules were flame sealed under vacuum and stored at 4°C for 9 months.

Reactivation of pM and sM in batch fermentation

To investigate metabolic activity and compositional reestablishment of fresh pM, three aliquots of GSI-, respectively SI-treated pM were not frozen in liquid nitrogen and instead were immediately reactivated in a batch fermentation (t_1). Aliquots were re-suspended in 900 µl of anaerobic phosphate buffered saline (PBS, supplementary table 1) to the initial volume of the liquid phase.

As with fresh pM, three aliquots of GSI-, respectively SI-treated sM were immediately reactivated in batch fermentation without undergoing liquid nitrogen freezing (t_1). Prior to inoculation, 0.5 g (cryopreservation trial) and 0.1 g portions of fresh beads (lyophilization trial) were mechanically homogenized in 400 and 850 µL PBS containing 0.4% sodium citrate, respectively, by using the tip of a sterile plastic spatula until beads were dissolved.

Serum flasks (50 ml) containing 20 ml anaerobic batch fermentation medium and CO_2 as headspace gas were inoculated at a concentration of 1% (v/v) with each aliquot of pM and 0.5% (v/w) with each aliquot of sM. Flasks were incubated for 24 h at 37°C under continuous stirring at 40 rpm. After 0, 4, 6, 8 and 24 h batch fermentation, 1-ml samples were removed, and centrifuged at 13’000 x g for 5 min at 4°C. Samples of supernatant for HPLC-IR analysis and microbial pellets for DNA extraction were separately stored at -20°C and -80°C, respectively.

After storage, three aliquots of cryopreserved sM or pM were transferred to an anaerobic chamber and quick-thawed at room temperature as described above. In addition to the five sampling time points, sampling was also performed after 12 h batch fermentation. Stored lyophilized aliquots (n=3) of pM and sM were rehydrated for 1 h in 1 ml and 900 µL PBS, respectively in an anaerobic chamber. Beads were dissolved as described above. Sampling of batch fermentations was conducted after 0 and 24 h incubation.

Metabolite analysis by HPLC-RI

Metabolic composition of main SCFAs (acetate, propionate and butyrate), intermediate fermentation metabolites (lactate, succinate, valerate and formate) and BCFAs (isobutyrate and isovalerate) were analyzed in fermentation effluents and batch fermentation samples using HPLC-RI. Supernatants were filtered through a 0.45 µm nylon membrane into HPLC vials and closed with crimp-caps. Samples were analyzed using a HPLC (Merck-Hitachi, Germany) equipped with an Aminex HPX-87H column (300 x 7.8 mm; BioRad) and a refractive index detector (Thermo Fisher Scientific AG). Supernatants (40 µl injection volume) were eluted with 10 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} at 40°C. SCFAs, BCFAs, lactate, succinate, valerate and formate were quantified using external standards.

DNA extraction

DNA was extracted from 1 ml of fermentation effluent, 0.5 g of beads and 1 ml of batch fermentation samples using the FastDNA Spin Kit for Soil (MP Biomedicals, France) including mechanical lysis of the cells with a FastPrep (MP Biomedicals, France). DNA was eluted in a final volume of 100 µL. DNA

concentration and quality was determined with a Nanodrop ND-1000 Spectrometer (Witec AG, Switzerland).

Quantification of total bacteria numbers

Total bacteria gene copy number was enumerated by qPCR using the primers Eub_339F (ACTCCTACGGGAGGCAG) and Eub_518R (ATTACCGCGGCTGCTGG) targeting the 16S rRNA gene (Guo*, et al.*, 2008). The qPCR master mix contained 12.5 µl 2x SYBR Green Mastermix (Life Technologies, Labgene Scientific Instruments, Switzerland), 0.2 µl of each forward and backward primer (5 µM), and 1 µl of genomic DNA in a total volume of 25 µl. The amplification started with a denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Melting curve analysis was performed to verify the specificity of amplification. The samples were analyzed in duplicate. Standard curves were generated from 10-fold dilution series (10^2–10^8 copies) of linearized plasmids containing the target gene.

Microbiota profiling with 16S rRNA gene amplicon sequencing

The bacterial composition was determined using the Illumina platform Miseq (Illumina, CA, USA) for tag-encoded 16S rRNA high throughput sequencing. DNA samples after 0 and 24 h incubation of fresh and preserved sM and pM in batch fermentation were selected for assessing the re-establishment of bacterial composition. The variable V4 region of the 16S rRNA gene was amplified with the primers nxt_515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and nxt_806_R (5′-GGACTACHVGGGTWTCTAAT-3′). Library preparation and sequencing was conducted in collaboration with the Genetic Diversity Center (GDC, ETH-Zürich, Switzerland). Sequencing was performed using an Illumina MiSeq flow cell with V2 2 x 250 bp paired end chemistry supplemented with 10 % of PhiX.

The raw data set containing pair-ended reads with corresponding quality scores were merged using settings as previously mentioned (Krych*, et al.*, 2018). The minimum length of merged reads was 150 bp. Quantitative Insight Into Microbial Ecology (QIIME) open source software package (1.8.0 and 1.9.0) was used for subsequent analysis steps (Caporaso*, et al.*, 2010). Purging the dataset from chimeric reads and constructing de novo Operational Taxonomic Units (OTU) was conducted using the UPARSE pipeline (Edgar, 2013). The Green genes database was used as a reference database (DeSantis*, et al.*, 2006, McDonald*, et al.*, 2012). Alpha and beta diversity analysis was performed using iterative subsampling (18’000 reads/sample) as previously described (Krych*, et al.*, 2013).

Statistics

Statistical analysis of metabolite concentration and bacterial abundance data of batch fermentation samples was done using R studio version 3.4.1 (Boston, MA, USA). Data were expressed as mean ± SD of triplicates, except for lyophilized sM1 data where only duplicates were available. One lyophilized sM1 data point had to be omitted due to an incomplete fermentation of one replicate. A students t-

test was performed to compare means of cryopreserved and lyophilized with fresh samples, excluding lyophilized sM1 data due to the limited sample size. Data were tested for normal distribution using the Shapiro-Wilk test and homogeneity of variance with the F-test. Differences were considered significant for $\alpha \leq 0.05$.

Acknowledgment

We thank Alfonso Die and Barbara Albrecht for experimental assistance.

Supporting information

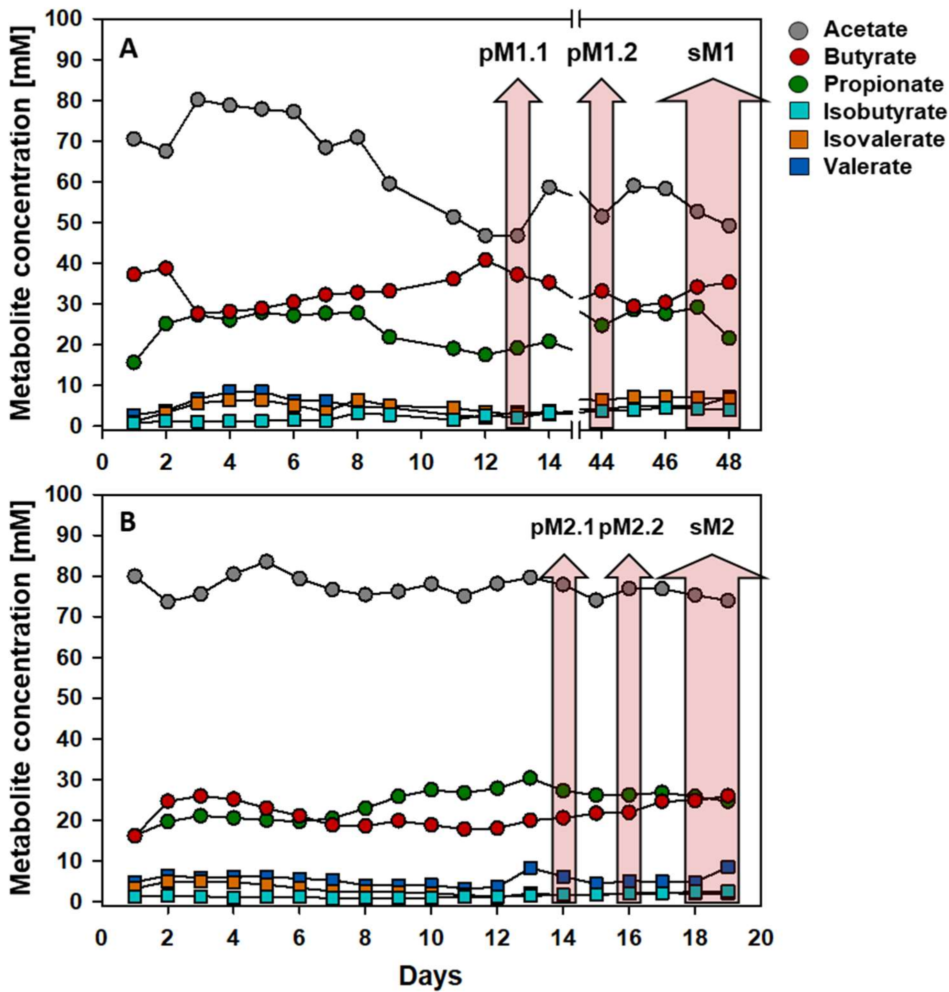

Fig. S4.1 Metabolic activity of fermentation systems. Absolute metabolite concentrations in fermentation 1 (A) and 2 (B) were measured by HPLC-RI. Sampling days of sM and pM for preservation trials are marked with red arrows. pM1.1 and pM2.1 were sampled for the cryopreservation and pM1.2 and pM2.2 for the lyophilization trial.

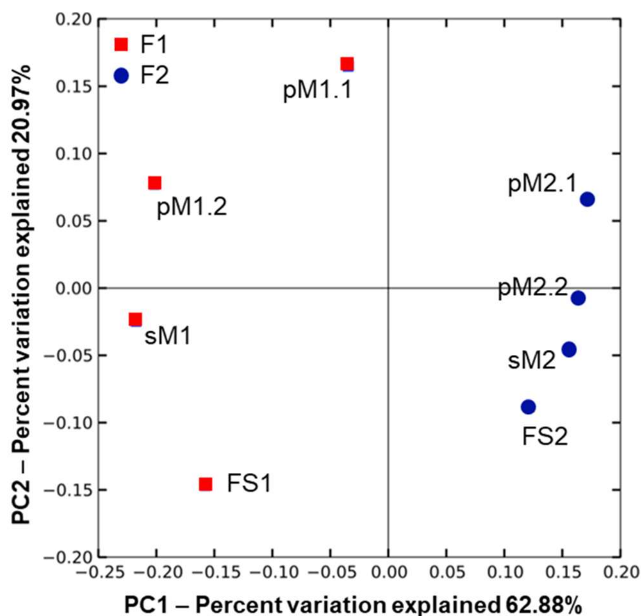

Fig. S4. 2 Beta diversity of fecal sample, planktonic and sessile microbiota. Principal coordinates analysis (PCoA) based on unweighted UniFrac distance matrices of fecal, planktonic and sessile microbiota in F1 and F2.

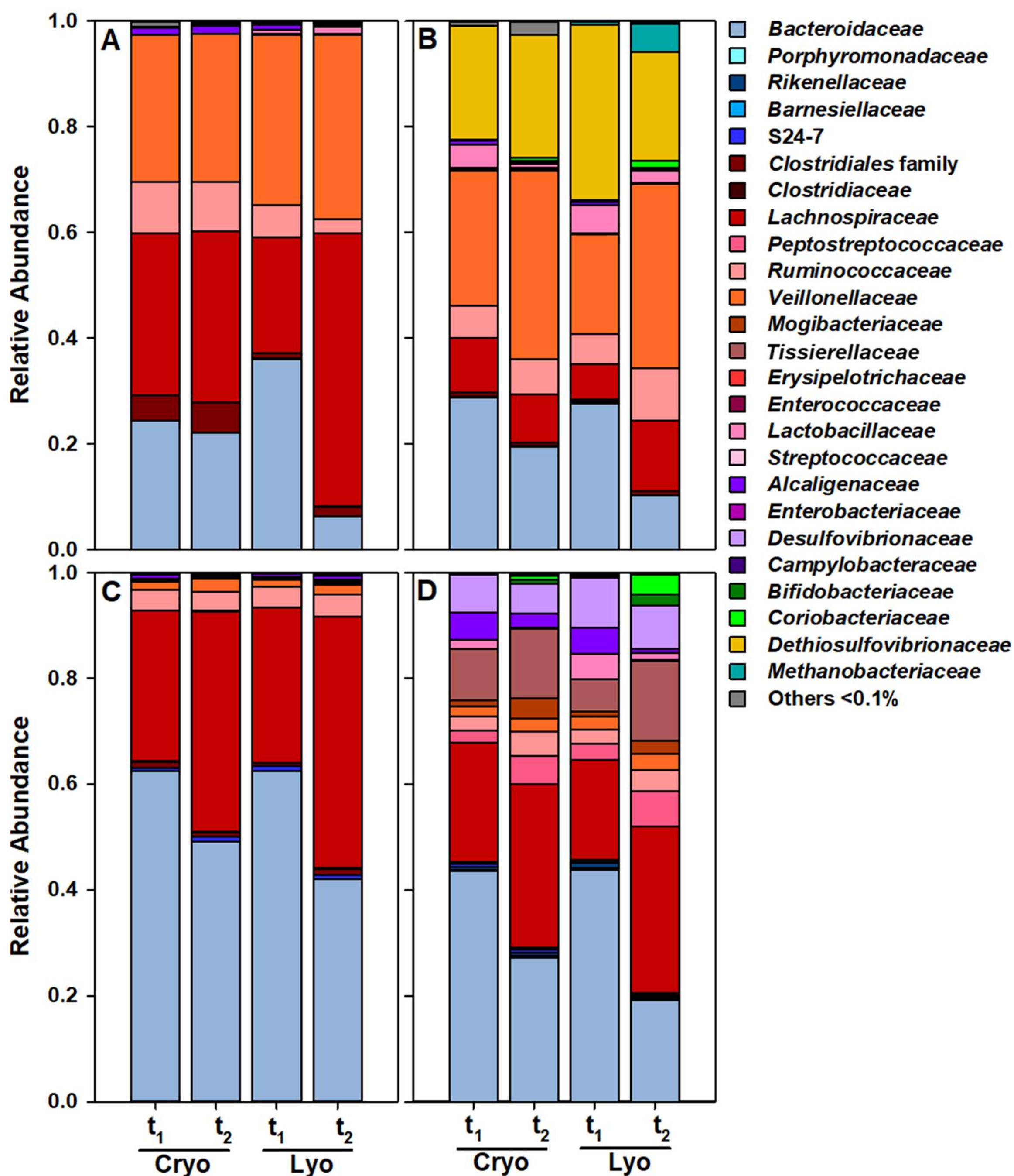

Fig. S4.3 Microbial composition of fresh and preserved planktonic and sessile microbiota. Relative abundance at family level of fresh (t_1), cryopreserved and lyophilized (t_2) pM 1 (A), sM1 (B), pM2 (C) and sM 2 (D) was analyzed by V4 region of 16S rRNA gene sequencing after inoculation in batch fermentation but before incubation (t=0 h). Samples are single measurements. Cryo refers to cryopreservation trail while Lyo refers to lyophilization trial.

Table S4.1 Microbial composition of fresh and cryopreserved pM1 in batch fermentation. Relative abundance of microbial genera was analyzed by V4 region of 16S rRNA gene amplicon sequencing. Sampling was conducted immediately after inoculation (0h) and after 24 h batch fermentation. Operational taxonomic unit (OTU).

	pM 1 Fresh		pM 1 Cryo	
OTU	0h	24h	0h	24h
Bifidobacteriaceae; Bifidobacterium adolescentis	0.1%	1.1± 1.1%	0.1%	1.7± 0.1%
Bacteroidaceae; Bacteroides species	22.9%	28.6± 3.9%	20.9%	15.1± 2.2%
Bacteroidaceae; Bacteroides caccae	0.9%	0.8± 0.2%	0.9%	0.2± 0.1%
Lactobacillaceae; Lactobacillus mucosae	0.7%	0.0± 0.0%	0.2%	0.0± 0.0%
Clostridiumes; species	4.8%	0.7± 0.3%	5.6%	1.8± 0.5%
Lachnospiraceae;Other;Other	3.1%	1.9± 1.0%	5.7%	3.0± 1.9%
Lachnospiraceae species	11.0%	3.2± 1.1%	14.2%	5.0± 1.7%
Lachnospiraceae; Anaerostipes species	0.3%	0.5± 0.4%	0.6%	4.0± 1.3%
Lachnospiraceae; Blautia species	1.5%	0.3± 0.1%	1.7%	0.5± 0.2%
Lachnospiraceae; Clostridium hathewayi	0.0%	0.9± 0.3%	0.0%	0.4± 0.1%
Lachnospiraceae; Coprococcus species	0.7%	0.7± 0.3%	0.6%	1.1± 0.2%
Lachnospiraceae; Dorea species	0.1%	0.4± 0.2%	0.1%	0.7± 0.2%
Lachnospiraceae; Lachnobacterium species	1.1%	0.0± 0.0%	1.2%	0.0± 0.0%
Lachnospiraceae; Lachnospira species	12.1%	0.2± 0.2%	7.4%	0.1± 0.0%
Ruminococcaceae; Faecalibacterium prausnitzii	2.1%	0.0± 0.0%	2.0%	0.0± 0.0%
Ruminococcaceae; Ruminococcus species	4.9%	0.0± 0.0%	4.1%	0.0± 0.0%
Ruminococcaceae; Ruminococcus bromii	2.4%	0.0± 0.0%	2.8%	0.1± 0.0%
Veillonellaceae; Acidaminococcus species	27.5%	56.3± 3.7%	27.6%	58.7± 2.8%
Veillonellaceae; Dialister species	0.2%	1.1± 0.1%	0.4%	1.4± 0.2%
Veillonellaceae; Mitsuokella species	0.0%	0.7± 0.3%	0.0%	2.1± 0.2%
Erysipelotrichaceae species	0.1%	1.1± 0.5%	0.1%	2.1± 0.9%
Alcaligenaceae; Sutterella species	1.2%	0.0± 0.0%	1.4%	0.0± 0.0%

Table S4.2 Microbial composition of fresh and cryopreserved sM1 in batch fermentation. Relative abundance of microbial genera was analyzed by V4 region of 16S rRNA gene sequencing. Sampling was conducted immediately after inoculation (0 h) and after 24 h batch fermentation.

	sM1 Fresh		sM1 Cryo	
OTU	0h	24h	0h	24h
Methanobacteriaceae; Methanobrevibacter species	0.8%	0.0± 0.0%	2.3%	0.0± 0.0%
Bifidobacteriaceae; Bifidobacterium adolescentis	0.2%	0.3± 0.0%	0.3%	2.0± 0.6%
Bacteroidaceae; Bacteroides;Other	2.0%	0.2± 0.0%	1.3%	0.2± 0.1%
Bacteroidaceae; Bacteroides species	22.6%	13.3± 3.0%	15.5%	7.5± 0.5%
Bacteroidaceae; Bacteroides uniformis	3.7%	0.2± 0.0%	2.6%	0.5± 0.1%
Enterococcaceae; Enterococcus species	0.2%	5.5± 0.6%	0.3%	12.0± 0.1%
Lactobacillaceae; Lactobacillus mucosae	4.1%	0.0± 0.0%	0.6%	0.0± 0.0%
Clostridiumes; species	0.7%	0.6± 0.0%	0.6%	0.2± 0.1%
Clostridiaceae; species	0.0%	0.0± 0.1%	0.0%	2.0± 0.8%
Lachnospiraceae;Other;Other	1.4%	8.1± 1.4%	1.1%	1.5± 0.4%
Lachnospiraceae; species	4.5%	11.7± 2.1%	3.8%	5.1± 1.1%
Lachnospiraceae; Anaerostipes species	0.0%	1.1± 0.4%	0.1%	1.2± 0.1%
Lachnospiraceae; Blautia species	0.3%	0.8± 0.1%	0.3%	0.8± 0.2%
Lachnospiraceae; Clostridium hathewayi	1.0%	1.1± 0.0%	0.8%	1.3± 0.2%
Lachnospiraceae; Coprococcus species	1.8%	5.2± 0.4%	2.0%	3.6± 0.6%
Lachnospiraceae; Lachnospira species	0.6%	0.1± 0.0%	0.3%	0.0± 0.0%
Peptostreptococcaceae; species	0.0%	0.8± 0.4%	0.0%	3.8± 0.9%
Ruminococcaceae; species	2.0%	0.2± 0.0%	3.3%	0.2± 0.1%
Ruminococcaceae; Faecalibacterium prausnitzii	2.3%	0.0± 0.0%	2.0%	0.0± 0.0%
Ruminococcaceae; Ruminococcus bromii	0.8%	0.1± 0.0%	0.5%	0.0± 0.0%
Veillonellaceae; Acidaminococcus species	16.4%	45.7± 6.7%	22.4%	52.3± 4.1%
Veillonellaceae; Dialister species	6.4%	1.0± 0.0%	10.1%	1.5± 0.2%
Veillonellaceae; Mitsuokella species	0.2%	0.7± 0.2%	0.0%	1.0± 0.0%
Veillonellaceae; Phascolarctobacterium species	2.6%	0.0± 0.0%	3.2%	0.0± 0.0%
Alcaligenaceae; Sutterella species	0.7%	0.0± 0.0%	0.4%	0.0± 0.0%
Enterobacteriaceae; species	0.0%	0.2± 0.1%	0.0%	1.3± 0.1%
Dethiosulfovibrionaceae; Pyramidobacte piscolens	21.4%	0.5± 0.1%	23.3%	0.0± 0.0%

Table S4.3 Microbial composition of fresh and cryopreserved pM2 in batch fermentation. Relative abundance of microbial genera was analyzed by V4 region of 16S rRNA gene sequencing. Sampling was conducted immediately after inoculation (0 h) and after 24 h batch fermentation.

	pM 2 Fresh			pM 2 Cryo		
out	0h	24h		0h	24h	
Bacteroidaceae; Bacteroides;Other	10.9%	8.5±	0.9%	14.3%	6.6±	0.3%
Bacteroidaceae; Bacteroides species	21.9%	43.2±	2.3%	29.5%	32.0±	0.1%
Bacteroidaceae; Bacteroides caccae	3.8%	1.1±	0.1%	3.2%	0.4±	0.1%
Bacteroidaceae; Bacteroides uniformis	12.5%	10.5±	1.4%	15.4%	9.2±	0.0%
S24-7; species	1.0%	1.3±	0.2%	0.7%	0.7±	0.0%
Enterococcaceae; Enterococcus species	0.1%	4.3±	0.3%	0.1%	16.8±	1.5%
Clostridiumes; species	0.8%	1.3±	0.0%	1.0%	1.6±	0.0%
Lachnospiraceae;Other;Other	0.6%	2.8±	0.4%	0.3%	0.2±	0.0%
Lachnospiraceae; species	29.8%	11.2±	2.8%	20.0%	13.1±	0.8%
Lachnospiraceae; Anaerostipes species	0.4%	0.8±	0.3%	0.4%	3.9±	0.6%
Lachnospiraceae; Blautia species	5.6%	3.9±	1.2%	4.8%	3.0±	0.2%
Lachnospiraceae; Dorea species	0.1%	2.1±	0.3%	0.1%	2.5±	0.0%
Lachnospiraceae; Dorea formicigenerans	1.1%	0.8±	0.2%	0.9%	1.6±	0.1%
Lachnospiraceae; Lachnospira species	1.9%	0.1±	0.1%	0.6%	0.1±	0.0%
Ruminococcaceae; Faecalibacterium prausnitzii	2.3%	0.0±	0.0%	2.4%	0.0±	0.0%
Ruminococcaceae; Ruminococcus bromii	1.1%	0.1±	0.1%	1.4%	0.1±	0.0%
Veillonellaceae; Phascolarctobacterium species	2.1%	0.6±	0.1%	1.5%	1.0±	0.0%
[Tissierellaceae]; Peptoniphilus species	0.1%	2.1±	0.2%	0.2%	2.6±	0.3%
Erysipelotrichaceae; species	0.1%	0.0±	0.0%	0.2%	0.2±	0.0%
Alcaligenaceae; Sutterella species	0.3%	2.0±	0.1%	0.8%	1.1±	0.2%

Table S4.4 Microbial composition of fresh and cryopreserved sM2 in batch fermentation. Relative abundance of microbial genera was analyzed by V4 region of 16S rRNA gene sequencing. Sampling was conducted immediately after inoculation (0 h) and after 24 h batch fermentation.

	sM 2 Fresh		sM 2 Cryo	
OTU	0h	24h	0h	24h
Bifidobacteriaceae; Bifidobacterium adolescentis	0.2%	0.4± 0.1%	0.8%	1.2± 0.1%
Bacteroidaceae; Bacteroides;Other	3.6%	4.1± 0.3%	2.9%	4.7± 1.5%
Bacteroidaceae; Bacteroides species	31.3%	37.3± 1.2%	17.7%	31.2± 6.4%
Bacteroidaceae; Bacteroides caccae	3.5%	3.9± 0.1%	3.0%	3.0± 1.3%
Bacteroidaceae; Bacteroides uniformis	5.0%	6.2± 0.6%	3.5%	6.8± 1.9%
Enterococcaceae; Enterococcus species	0.1%	5.2± 2.2%	0.0%	7.9± 1.6%
Lactobacillaceae; Lactobacillus mucosae	1.6%	0.0± 0.0%	0.2%	0.0± 0.0%
Clostridiaceae; species	0.1%	0.9± 0.4%	0.1%	8.3± 3.5%
Lachnospiraceae;Other;Other	1.0%	0.7± 0.2%	1.7%	1.0± 0.1%
Lachnospiraceae; species	11.8%	16.6± 1.6%	15.2%	8.7± 1.5%
Lachnospiraceae; Anaerostipes species	0.1%	0.2± 0.0%	0.1%	3.0± 3.8%
Lachnospiraceae; Blautia species	0.9%	3.2± 0.1%	1.9%	1.1± 0.3%
Lachnospiraceae; Coprococcus species	3.1%	0.6± 0.1%	4.0%	1.6± 0.3%
Lachnospiraceae; Dorea species	0.5%	0.3± 0.0%	1.0%	0.3± 0.2%
Lachnospiraceae; Dorea formicigenerans	3.0%	0.6± 0.1%	4.7%	0.6± 0.2%
Peptostreptococcaceae; species	2.3%	5.4± 1.8%	5.4%	2.5± 0.9%
Ruminococcaceae; Faecalibacterium prausnitzii	0.8%	0.3± 0.0%	1.0%	0.0± 0.0%
Ruminococcaceae; Oscillospira species	0.8%	1.8± 0.1%	1.2%	0.5± 0.1%
Ruminococcaceae; Ruminococcus bromii	0.8%	0.4± 0.1%	1.7%	0.0± 0.0%
Veillonellaceae; Acidaminococcus species	0.0%	2.1± 0.2%	0.1%	0.1± 0.1%
Veillonellaceae; Dialister species	1.0%	0.7± 0.1%	1.2%	0.9± 0.1%
Veillonellaceae; Phascolarctobacterium species	0.8%	1.5± 0.4%	1.3%	1.2± 0.3%
[Mogibacteriaceae]; species	1.1%	0.1± 0.0%	3.8%	0.1± 0.0%
[Tissierellaceae]; Peptoniphilus species	9.7%	2.7± 0.3%	13.1%	9.8± 0.7%
Alcaligenaceae; Sutterella species	5.2%	0.0± 0.0%	2.7%	0.0± 0.0%
Desulfovibrionaceae; Bilophila species	7.2%	0.5± 0.1%	5.6%	0.3± 0.2%

Table S4.5 Microbial composition of fresh and lyophilized pM1 in batch fermentation. Relative abundance of microbial genera was analyzed by V4 region of 16S rRNA gene sequencing. Sampling was conducted immediately after inoculation (0h) and after 24 h batch fermentation.

	pM 1 Fresh		pM 1 Lyo	
out	0h	24h	0h	24h
Bacteroidaceae; Bacteroides; species	35.5%	28.8± 1.7%	6.3%	8.1± 6.5%
Enterococcaceae; Enterococcus; species	0.1%	3.1± 1.1%	0.2%	14.2± 0.3%
Lactobacillaceae; Lactobacillus; zeae	0.5%	0.1± 0.0%	1.1%	0.1± 0.0%
Clostridiumes; species	0.9%	0.6± 0.1%	1.7%	0.5± 0.4%
Lachnospiraceae;Other;Other	2.0%	6.5± 1.7%	11.4%	7.0± 0.9%
Lachnospiraceae; species	11.5%	8.9± 2.2%	28.9%	8.1± 1.1%
Lachnospiraceae; Blautia; species	1.2%	0.7± 0.1%	3.2%	0.3± 0.1%
Lachnospiraceae; Clostridium; hathewayi	0.8%	0.8± 0.3%	0.6%	1.1± 0.3%
Lachnospiraceae; Coprococcus; species	2.9%	0.3± 0.1%	2.4%	0.5± 0.3%
Lachnospiraceae; Dorea; formicigenerans	0.5%	0.1± 0.0%	1.3%	0.0± 0.0%
Lachnospiraceae; Lachnospira; species	1.5%	0.0± 0.0%	0.6%	0.0± 0.0%
Lachnospiraceae; [Ruminococcus];Other	0.7%	0.1± 0.0%	1.6%	0.5± 0.6%
Ruminococcaceae; Faecalibacterium; prausnitzii	1.9%	0.0± 0.0%	0.4%	0.0± 0.0%
Ruminococcaceae; Ruminococcus; species	3.0%	0.0± 0.0%	0.8%	0.0± 0.0%
Veillonellaceae; Acidaminococcus; species	31.8%	45.1± 3.2%	33.9%	53.9± 6.2%
Veillonellaceae; Dialister; species	0.4%	1.4± 0.2%	1.0%	1.2± 0.1%
Veillonellaceae; Mitsuokella; species	0.1%	0.5± 0.1%	0.1%	1.6± 0.4%

Table S4.6 Microbial composition of fresh and lyophilized pM2 in batch fermentation. Relative abundance of microbial genera was analyzed by V4 region of 16S rRNA gene sequencing. Sampling was conducted immediately after inoculation (0h) and after 24 h batch fermentation.

	pM 2 Fresh		pM 2 Lyo	
OTU	0h	24h	0h	24h
Bifidobacteriaceae; Bifidobacterium; adolescentis	0.0%	0.3± 0.1%	0.0%	1.3± 0.2%
Bacteroidaceae; Bacteroides;Other	13.2%	7.0± 1.7%	8.6%	1.9± 0.6%
Bacteroidaceae; Bacteroides; species	32.7%	41.8± 1.2%	21.1%	8.4± 2.4%
Bacteroidaceae; Bacteroides; caccae	2.8%	1.5± 0.4%	1.8%	0.2± 0.1%
Bacteroidaceae; Bacteroides; uniformis	13.6%	10.2± 1.7%	10.4%	2.5± 0.7%
S24-7; species	1.0%	0.8± 0.1%	0.9%	0.6± 0.0%
Enterococcaceae; Enterococcus; species	0.1%	6.2± 0.1%	0.2%	19.0± 7.3%
Clostridiumes; species	0.6%	1.5± 0.2%	1.0%	0.7± 0.4%
Clostridiaceae; species	0.0%	0.1± 0.0%	0.0%	1.2± 0.2%
Lachnospiraceae;Other;Other	0.7%	1.1± 0.3%	1.4%	10.4± 0.0%
Lachnospiraceae; species	21.3%	14.5± 1.4%	33.7%	20.2± 0.9%
Lachnospiraceae; Anaerostipes; species	0.4%	0.8± 0.0%	0.9%	3.1± 0.6%
Lachnospiraceae; Blautia; species	3.0%	4.3± 0.2%	7.4%	4.5± 0.2%
Lachnospiraceae; Coprococcus; species	1.3%	0.4± 0.0%	1.2%	0.2± 0.2%
Lachnospiraceae; Dorea; species	0.0%	1.0± 0.2%	0.1%	1.6± 0.0%
Lachnospiraceae; Dorea; formicigenerans	0.6%	0.3± 0.1%	1.3%	4.5± 0.1%
Lachnospiraceae; Lachnospira; species	1.4%	0.2± 0.0%	0.4%	0.1± 0.0%
Peptostreptococcaceae; species	0.0%	1.2± 0.2%	0.0%	6.1± 0.7%
Ruminococcaceae; Faecalibacterium; prausnitzii	2.2%	0.0± 0.0%	2.0%	0.0± 0.0%
Ruminococcaceae; Ruminococcus; bromii	1.2%	0.3± 0.0%	1.8%	0.5± 0.0%
Veillonellaceae; Phascolarctobacterium; species	1.3%	0.9± 0.3%	1.7%	0.8± 0.1%
[Tissierellaceae]; Peptoniphilus; species	0.1%	2.2± 0.1%	0.4%	8.1± 0.5%

Table S4.7 Microbial composition of fresh and lyophilized sM1 in batch fermentation. Relative abundance of microbial genera was analyzed by V4 region of 16S rRNA gene sequencing. Sampling was conducted immediately after inoculation (0 h) and after 24 h batch fermentation.

	sM 1 Fresh			sM 1 Lyo		
out	0h	24h		0h	24h	
Methanobacteriaceae; Methanobrevibacter; species	0.6%	0.0±	0.0%	5.4%	0.0±	0.0%
Bifidobacteriaceae; Bifidobacterium; adolescentis	0.1%	0.3±	0.0%	0.3%	1.3±	0.3%
Bacteroidaceae; Bacteroides;Other	1.6%	0.5±	0.0%	1.1%	0.0±	0.0%
Bacteroidaceae; Bacteroides; species	22.5%	21.4±	0.7%	7.8%	18.8±	5.6%
Bacteroidaceae; Bacteroides; uniformis	3.2%	0.7±	0.1%	1.5%	0.0±	0.0%
Enterococcaceae; Enterococcus; species	0.0%	5.3±	0.8%	0.1%	9.9±	2.7%
Lactobacillaceae; Lactobacillus; mucosae	5.0%	0.0±	0.0%	2.1%	0.0±	0.0%
Clostridiaceae; species	0.0%	0.1±	0.1%	0.0%	7.5±	4.3%
Lachnospiraceae;Other;Other	0.8%	3.8±	1.1%	1.5%	2.3±	1.4%
Lachnospiraceae; species	3.0%	7.1±	1.3%	5.6%	9.0±	5.7%
Lachnospiraceae; Anaerostipes; species	0.0%	1.2±	0.0%	0.0%	2.3±	0.4%
Lachnospiraceae; Blautia; species	0.2%	1.2±	0.0%	0.6%	0.8±	0.3%
Lachnospiraceae; Clostridium; hathewayi	0.4%	2.2±	0.1%	1.0%	3.7±	0.5%
Lachnospiraceae; Coprococcus; species	1.6%	1.2±	0.1%	2.9%	2.8±	1.9%
Lachnospiraceae; Dorea; formicigenerans	0.1%	0.3±	0.0%	0.5%	1.7±	0.4%
Peptostreptococcaceae; species	0.0%	0.5±	0.1%	0.0%	3.3±	1.3%
Ruminococcaceae; species	2.5%	0.1±	0.0%	4.8%	0.1±	0.0%
Ruminococcaceae; Faecalibacterium; prausnitzii	2.0%	0.0±	0.0%	3.0%	0.0±	0.0%
Veillonellaceae; Acidaminococcus; species	13.2%	48.5±	3.1%	21.2%	32.5±	20.4%
Veillonellaceae; Dialister; species	3.9%	1.1±	0.1%	9.7%	1.4±	0.2%
Veillonellaceae; Phascolarctobacterium; species	1.7%	0.0±	0.0%	3.9%	0.0±	0.0%
Erysipelotrichaceae; species	0.0%	1.1±	0.3%	0.0%	0.2±	0.1%
Pyramidobacter; piscolens	33.2%	0.3±	0.1%	20.6%	0.0±	0.0%

Table S4.8 Microbial composition of fresh and lyophilized sM2 in batch fermentation. Relative abundance of microbial genera was analyzed by V4 region of 16S rRNA gene sequencing. Sampling was conducted immediately after inoculation (0 h) and after 24 h batch fermentation.

	sM 2 Fresh		sM 2 Lyo	
OTU	0h	24h	0h	24h
Bifidobacteriaceae; Bifidobacterium; adolescentis	0.3%	0.2± 0.1%	2.1%	1.4± 0.3%
Coriobacteriaceae; species	0.2%	0.0± 0.0%	1.2%	0.0± 0.0%
Coriobacteriaceae; Adlercreutzia; species	0.1%	0.0± 0.0%	1.4%	0.1± 0.1%
Bacteroidaceae; Bacteroides;Other	3.1%	4.0± 0.5%	1.1%	0.0± 0.0%
Bacteroidaceae; Bacteroides; species	31.9%	40.8± 2.9%	14.3%	0.3± 0.2%
Bacteroidaceae; Bacteroides; caccae	4.0%	4.3± 0.3%	2.0%	0.0± 0.0%
Bacteroidaceae; Bacteroides; uniformis	4.8%	6.0± 0.7%	1.8%	0.0± 0.0%
S24-7; species	0.4%	2.0± 0.2%	0.4%	0.0± 0.0%
Enterococcaceae; Enterococcus; species	0.0%	2.7± 0.4%	0.1%	5.4± 1.4%
Lactobacillaceae; Lactobacillus; mucosae	4.5%	0.0± 0.0%	1.4%	0.0± 0.0%
Clostridiaceae; species	0.0%	2.7± 0.2%	0.1%	19.3± 2.7%
Lachnospiraceae;Other;Other	1.1%	1.3± 0.4%	3.0%	1.2± 0.0%
Lachnospiraceae; species	9.7%	9.9± 1.8%	12.3%	5.8± 1.2%
Lachnospiraceae; Anaerostipes; species	0.1%	0.4± 0.1%	0.2%	7.0± 4.2%
Lachnospiraceae; Blautia; species	0.6%	2.0± 0.9%	1.3%	2.3± 0.3%
Lachnospiraceae; Coprococcus; species	2.7%	0.8± 0.4%	4.8%	7.6± 1.5%
Lachnospiraceae; Dorea; species	0.4%	0.3± 0.1%	1.2%	1.8± 0.6%
Lachnospiraceae; Dorea; formicigenerans	3.1%	0.7± 0.3%	6.3%	2.9± 1.0%
Lachnospiraceae; [Ruminococcus];Other	0.0%	0.0± 0.0%	0.1%	3.7± 1.7%
Lachnospiraceae; [Ruminococcus]; species	0.1%	0.1 0.0%	0.1%	1.4± 0.7%
Peptostreptococcaceae; species	3.0%	8.0± 4.8%	6.7%	17.5± 5.1%
Ruminococcaceae; Oscillospira; species	1.0%	0.7± 0.1%	1.2%	0.0± 0.0%
Ruminococcaceae; Ruminococcus; bromii	0.5%	0.3± 0.1%	1.4%	0.0± 0.0%
Veillonellaceae; Dialister; species	1.5%	0.8± 0.3%	1.6%	1.2± 0.3%
Veillonellaceae; Phascolarctobacterium; species	1.1%	0.7± 0.1%	1.5%	0.1± 0.1%
[Mogibacteriaceae]; species	0.9%	0.2± 0.0%	2.5%	0.0± 0.0%
[Tissierellaceae]; Peptoniphilus; species	6.0%	6.1± 0.3%	15.0%	18.3± 0.9%
Alcaligenaceae; Sutterella; species	4.9%	0.0± 0.0%	0.8%	0.0± 0.0%
Desulfovibrionaceae; Bilophila; species	9.7%	0.4± 0.0%	8.1%	0.0± 0.0%

Chapter 5

General conclusion and perspectives

General conclusion

FMT has previously demonstrated high efficiency in the resolution of rCDI and holds therapeutic potential as an alternative treatment for IBD and other gastrointestinal diseases associated with chronic inflammation. However, a stool-based treatment raises a number of concerns, including a potential infection risk, transfer of undesired phenotypes, limited standardization and low patient acceptance, that limit its availability. Alternative strategies to modulate the gut microbiome involve the application of 'artificially' produced colonic microbiota as stool-substitute or the administration of specific health promoting gut microbes. Both approaches would eliminate some of the raised issues of FMT. Intestinal fermentation technology can deliver controlled and stable "artificial" colonic microbiota at high cell density and large quantity. However, therapeutic application of microorganisms requires that the cells are in an inactive state prior administration to ensure long-term stability of the microbial-based product and thus guaranteeing widespread availability. Consequently, there is a strong demand for anaerobic preservation technologies that warrant composition and functionality. Existing preservation protocols were mainly developed for pure, aerotolerant cultures while strict anaerobic and community level preservation has been widely neglected. Therefore, the aim of this thesis was to develop and validate anaerobic preservation techniques for maintaining microbial composition and functionality of complex colonic microbiota produced with IFT. To reach our objectives we used a culture-dependent *in vitro* approach for viability and activity assessment of fresh and preserved complex artificial colonic microbiota with emphasis on metabolic activity as functionality readout. In addition, we performed a pure culture preservation study with selected members of the gut microbiota to characterize the species-specific preservation tolerance and explain their behavior towards preservation in the complex microbiota.

Therefore, **Chapter 2** aimed to investigate the effect of a penetrating and non-penetrating cryoprotectant on metabolic activity of cryopreserved artificial colonic microbiota produced with IFT by using the continuous *in vitro* colonic PolyFermS platform. Moreover, we specifically focused on the impact of cryopreservation on the re-establishment of the butyrate-producing *Faecalibacterium prausnitzii, Eubacterium hallii* and the *Roseburia* spp./*Eubacterium rectale* group within the colonic microbiota. The selected bacterial targets are considered as most important butyrate producer in the human intestine and demonstrated high sensitivity towards oxygen exposure, making them to good markers for preservation efficiency.

Complex microbiota material, harvested from reactor effluents that was generated in two independent colonic *in vitro* fermentation models mimicking proximal colon condition and inoculated with immobilized fecal microbiota from healthy male donors, was cryopreserved in buffers containing either glycerol or inulin or a combination thereof and stored for 3 months at -80°C. The cryoprotective agents were selected due to their biocompatibility, complementary protective mechanisms and proven record of effectivity in bacterial preservation. Artificial colonic microbiota was reactivated in

anaerobic batch cultures before and after frozen storage. Metabolite formation, as marker of metabolic activity, was measured using high performance liquid chromatography (HPLC) while re-establishment of selected butyrate producers was determined by molecular methods (qPCR). We demonstrated that combining membrane penetrating glycerol and non-penetrating inulin in protective buffers provided a higher level of protection during cryopreservation of compositionally different, artificial colonic microbiota compared to single component application. We also showed that the selected butyrate producers were differently affected by cryopreservation and that the effect of protectants were taxa-specific. Accordingly, glycerol supported cryopreservation of the *Roseburia* spp./*E. rectale* group, while inulin improved the recovery of *F. prausnitzii*. In contrast, *E. hallii* growth was affected minimally by cryopreservation. Moreover, glycerol addition maintained butyrate and propionate production and when combined with inulin resulted in faster formation of butyrate and acetate. Altogether, our data indicate that an adequate formulation of cryoprotective agents can preserve the functional group of butyrate-producing bacteria and maintain metabolic activity of frozen effluent microbiota derived from IFT and intended for FMT during frozen storage at -80°C for at least three months. The methods and preservation conditions developed in this study provided first insights in the storage behavior of complex anaerobic gut microbiota with special reference the butyrate-producing community.

Literature provide little comparative data on preservation of anaerobic gut microbes that might explain the distinctive behavior of the selected butyrate-producers that we observed in the previous preservation study on complex microbiota (Chapter 2). Thus, we conducted preservation trials with pure *F. prausnitzii*, *R. intestinalis* and *E. hallii* cultures for in-depth characterization of the species-specific tolerance to processing and storage by cryopreservation and lyophilization, excluding the potential impact of a competitive microbiota (**Chapter 3**). Moreover, we included the gut bacteria *Anaerostipes caccae* as additional butyrate producer and two propionate-producing representatives, *Bacteroides thetaiotaomicron* and *Blautia obeum,* in the preservation trials. The selected strict anaerobic gut microbes were lyophilized and stored at 4°C for 3 months in buffers containing sucrose and inulin in comparison to a control medium lacking protectants. Both protectants previously demonstrated high efficiency in preventing drying-related damages and where therefore included in the lyoprotective formulation. An additional protective buffer was used for preserving microbes by freezing at -80°C containing glycerol in combination with the two already mentioned protectants. Bacterial fitness, evaluated by maximum growth rate and lag phase, viability and membrane integrity were determined in fresh cultures, after processing and after storage using a standardized growth assay and by flow cytometry. Using both, viability and growth assessment we identified processing by drying as generally more detrimental to bacterial viability and fitness than by freezing. In contrast, lyophilization led to better storage stability compared to cryopreservation. Viable cell recovery ranging from 11% – 100% after frozen and from 0.3% – 60% after dried storage, pointing towards a strong

species-dependent resistance to freezing and freeze-drying. We suggest membrane composition as determining factor for preservation success as the addition of membrane-interacting protectants sucrose and inulin improved viability of all lyophilized strain and of freezing-sensitive strains after cryopreservation. As glycerol also differently affected strain viability and membrane integrity prior and post cryopreservation, our results suggests that efficiency of protectants is process- and species-specific. We observed a positive impact of glycerol on viability of *R. intestinalis* what was in accordance with the previous findings of its protective effect on the reestablishment of *Roseburia sp./E. rectale* group when cryopreserved as part of a complex artificial gut microbiota. The current data also suggest that an enhanced growth rate of *E. hallii* after cryopreservation could explain its competitiveness in batch fermentation, as we reported before that *E. hallii* was little impacted by freezing within a complex artificial gut microbiota. Altogether, our data revealed for the first time that strict anaerobic gut microbes survived well both during freezing and lyophilization and subsequent storage when carefully designed processes are applied, with strict protection from oxygen. In contrast to previous data and empirical belief, strict anaerobic microbes can be stabilized and stably stored under carefully selected conditions.

The PolyFermS system delivers next to planktonic microbiota in reactors effluent also microbiota of sessile life style embedded in polymer beads, mimicking a biofilm-associated state. Microbes in biofilm are generally considered as more robust to environmental stressors than the planktonic counterpart. We therefore hypothesized that sessile microbiota of the PolyFermS system is more resistant to preservation-induced stress than the corresponding planktonic microbiota (**Chapter 4**). In a first step, we characterized composition of planktonic and sessile microbiota and then exposed them to cryopreservation at -80°C and lyophilization and subsequent storage for 9 months at 4°C, respectively to investigate a possible life style dependent response to preservation stress. Community structure and metabolic activity in batch fermentations were assessed prior and post preservation by using high performance liquid chromatography (HPLC), molecular and high-throughput sequencing methods. We observed that bacterial life style is a determining factor in the establishment of microbial composition and metabolic capacity of PolyFermS derived artificial colonic microbiota. Accordingly, we could show that sessile microbiota was metabolically more active and harbored next to shared taxa, a unique bacterial and archaeal community distinguishable from planktonic microbiota. Surprisingly, we also demonstrated that community structure in accordance with metabolic activity were better maintained by microbiota of planktonic than sessile life style during preservation for 9 months. Our data suggest that cooperating microbes might be a characteristic component of sessile microbiota possibly affecting compositional stability towards freezing and freeze-drying stressors. Accordingly, preservation-induced cell death of a key taxon might provoke a concomitant decrease of depending taxa.
Independent from life style, we identified taxa that exhibited high sensitivity towards preservation, including several *Bacteroides* sp., but also taxa that showed great robustness such as *Enterococcaceae*

and *Peptostreptococcaceae*. We suggest functional redundancy, as observed for the butyrate-producing community of planktonic and sessile microbiota, as a trait that might preserve functional stability during exposure to freezing, drying and storage stress. In contrast, if a specific property, such as propionate production, is limited to a small number of taxa, the loss of key taxa can mediate loss of function. Our data demonstrated the feasibility of generating complex artificial microbiota in planktonic and sessile life-style with the continuous PolyFermS platform inoculated with fecal microbiota immobilized in gel beads, and subsequent preservation by freezing and lyophilization.

In conclusion, this doctoral thesis has allowed expanding the knowledge on the impact of different preservation methods on community structure and metabolic activity of colonic microbiota. We successfully maintained metabolic activity of complex colonic microbiota produced with IFT during cryopreservation at -80°C for at least 3 months and preserved growth of major members of the butyrate-producing community. We demonstrated that efficiency of the applied protectants is process- and species-dependent and that cryopreservation induced effects on pure cultures, as observed with *R. intestinalis* and *E. hallii*, can partly be extrapolated on their behavior in complex microbiota. We further showed that a loss in propionate-producing activity after cryopreservation and lyophilization of sessile and planktonic microbiota can be attributed to impaired reestablishment of *Bacteroidaceae* and that maintenance of the butyrate-producing activity might come from functional redundancy in the microbial communities. Moreover, we showed that sessile microbiota is stronger impacted by cryopreservation and lyophilization than the planktonic counterpart possibly explained by a higher dependency between sessile microbes and therefore concurrent losses of affected and dependent taxa. This thesis provides important insights into the potential of community level preservation of artificially produced colonic microbiota.

Perspectives

Monitoring functional stability during preservation can be challenging due to the high variety of beneficial physiological functions the gut microbiota provides to the host. We selected metabolic activity in *in vitro* batch incubations as marker for functional stability of artificial colonic microbiota and concluded that SCFA and BCFA production can be well conserved both during freezing or lyophilization and subsequent storage when carefully designed preservation processes are applied. Metabolite production is indisputable an important and easy applicable activity marker for optimizing preservation processes but does only partly cover the broad spectrum of beneficial functions exerted by the human colonic microbiota. In addition, metatranscriptomics could be used for further evaluations of the functional activity of both fresh and preserved microbiota by exploring the community-wide gene expression with next-generation sequencing (RNA-seq) (Bashiardes*, et al.*, 2016). This analysis will provide a more global estimation of the functional stability that goes beyond metabolic activity and might help to identify functional changes deriving from preservation and storage processes.

In vitro batch cultures, we used in this thesis for determination of the viability and activity status of the artificial colonic microbiota, are a good test environment for optimizing preservation techniques. However, an *in vivo* system is required in a next step to validate the effectiveness of the preserved colonic microbiota in a complex environment involving the host and the presence of a competitive microbiota. Experimental mice models that have been established for a broad range of gastrointestinal disorders such as CDI, IBD, colorectal cancer and metabolic syndrome, could be considered in future research for the evaluation of *in vivo* efficacy of preserved colonic microbiota (Erikstrup*, et al.*, 2015, Kiesler*, et al.*, 2015, McIntyre*, et al.*, 2015, Udayappan*, et al.*, 2016).

Furthermore, it is unknown how long 'artificial' colonic microbiota can be stored until it lose its therapeutic effect. Clinical efficiency of frozen stool suspensions (FMT) in the treatment of recurrent CDI is reported until 5-6 months at -80°C, while public stool banks such as OpenBiome or NDFB store the fecal material up to 2 years (Terveer*, et al.*, 2017). We observed maintenance of metabolic activity over storage periods of 3 – 9 months but it may be significantly longer. The exact shelf life of 'artificial' colonic microbiota might differ between preservation method and remains to be elucidated.

Our data also revealed reduced preservation resistance of sessile compared to the planktonic microbiota. However, the given experimental conditions involved destruction of the bead structure for inoculation of sessile microbiota in batch culture. It may be therefore worth to investigate further the activity of entrapped microbiota directly in bead structure because the resuspension step may have led to a deorganization of metabolic networks developed during reactor cultivation.

Regarding the pure cultures preservation study, we used bacterial survival and growth performance as marker for preservation resistance. Besides cell viability, preservation processes can also affect

important probiotic functionalities such as immunomodulation and adhesion properties attributed to the detrimental effects on bacterial cell walls (Iaconelli*, et al.*, 2015). Therefore, it is necessary to point out that viability and fitness are not the only determining parameters for the selection of an optimal preservation method or protocol. A more holistic strategy would combine viability with functionality assessments. Consequently, more research is required in identification, stabilization and evaluation of species-specific key functional properties that are associated with disease attenuation or prevention. As an example, *F. prausnitzii* employs beneficial immunomodulatory functions, attributed to the production of the Microbial-Anti-Inflammatory Molecule (MAM) and to the induction of the IL-10-secreting regulatory T cell subset (Quevrain*, et al.*, 2016). Cellular assays can be used to determine if these anti-inflammatory activities are maintained during preservation by exposing intestinal epithelial cell lines, such as Caco-2 or HT29 to supernatant of fresh and preserved *F. prausnitzii* cultures and evaluating its immune response (Quevrain*, et al.*, 2016, Breyner*, et al.*, 2017).

In general, the addition of protectants and the adaptation of processing parameters are the most applied strategies to enhance bacterial survival during preservation. We obtained good results with most of the preserved species, except with lyophilized *B. obeum* that exhibited great viability loss even in presence of protectants and therefore needs further investigation. Alternatively to a protective formulation, application of sublethal stress is a different approach to enhance preservation survival that deserves further attention. Certain adverse conditions such as unfavorable pH, starvation or osmotic pressure employed during bacterial growth can induce tolerance responses exploited in a change in cell membrane fatty acid composition, the secretion of exopolysaccharides or the production of shock proteins that might indirectly enhance tolerance to preservation induced stress (Morgan*, et al.*, 2006).

Bibliography

Abadias, M., Benabarre, A., Teixido, N., Usall, J., and Vinas, I. (2001) Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake*. *Int J Food Microbiol* **65**: 173-182.

Abt, M.C., McKenney, P.T., and Pamer, E.G. (2016) *Clostridium difficile* colitis: pathogenesis and host defence. *Nat Rev Microbiol* **14**: 609-620.

Adams, G.D., Cook, I., and Ward, K.R. (2015) The principles of freeze-drying. *Methods Mol Biol* **1257**: 121-143.

Adkar, B.V., Manhart, M., Bhattacharyya, S., Tian, J., Musharbash, M., and Shakhnovich, E.I. (2017) Optimization of lag phase shapes the evolution of a bacterial enzyme. *Nat Ecol Evol* **1**: 149.

Aguirre, M., Eck, A., Koenen, M.E., Savelkoul, P.H., Budding, A.E., and Venema, K. (2015) Evaluation of an optimal preparation of human standardized fecal inocula for in vitro fermentation studies. *J Microbiol Methods* **117**: 78-84.

Alang, N., and Kelly, C.R. (2015) Weight gain after fecal microbiota transplantation. *Open Forum Infect Dis* **2**: ofv004.

Albrecht, R.M., Orndorff, G.R., and Mackenzie, A.P. (1973) Survival of Certain Microorganisms Subjected to Rapid and Very Rapid Freezing on Membrane Filters. *Cryobiology* **10**: 233-239.

Allison, S.D., Chang, B., Randolph, T.W., and Carpenter, J.F. (1999) Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. *Arch Biochem Biophys* **365**: 289-298.

Allouche, R., Dupont, S., Charriau, A., Gervais, P., Beney, L., and Chambin, O. (2018) Optimized tableting for extremely oxygen-sensitive probiotics using direct compression. *Int J Pharm* **538**: 14-20.

Aminov, R.I., Walker, A.W., Duncan, S.H., Harmsen, H.J., Welling, G.W., and Flint, H.J. (2006) Molecular diversity, cultivation, and improved detection by fluorescent in situ hybridization of a dominant group of human gut bacteria related to Roseburia spp. or Eubacterium rectale. *Appl Environ Microbiol* **72**: 6371-6376.

Amor, K.B., Breeuwer, P., Verbaarschot, P., Rombouts, F.M., Akkermans, A.D., De Vos, W.M., and Abee, T. (2002) Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead bifidobacterium cells during bile salt stress. *Appl Environ Microbiol* **68**: 5209-5216.

Antharam, V.C., Li, E.C., Ishmael, A., Sharma, A., Mai, V., Rand, K.H., and Wang, G.P. (2013) Intestinal dysbiosis and depletion of butyrogenic bacteria in *Clostridium difficile* infection and nosocomial diarrhea. *J Clin Microbiol* **51**: 2884-2892.

Antonopoulos, D.A., Huse, S.M., Morrison, H.G., Schmidt, T.M., Sogin, M.L., and Young, V.B. (2009) Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infect Immun* **77**: 2367-2375.

Arnaud, J.P., Lacroix, C., and Castaigne, F. (1992) Counter-diffusion of lactose and lactic acid in kappa-carrageenan/locust bean gum gel beads with or without entrapped lactic acid bacteria. *Enzyme Microb Technol* **14**: 715-724.

Arnold, M., Sierra, M.S., Laversanne, M., Soerjomataram, I., Jemal, A., and Bray, F. (2017) Global patterns and trends in colorectal cancer incidence and mortality. *Gut* **66**: 683-691.

Arthur, J.C., Gharaibeh, R.Z., Muhlbauer, M., Perez-Chanona, E., Uronis, J.M., McCafferty, J.*, et al.* (2014) Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer. *Nat Commun* **5**: 4724.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R.*, et al.* (2011) Enterotypes of the human gut microbiome. *Nature* **473**: 174-180.

Bacic, M.K., and Smith, C.J. (2008) Laboratory maintenance and cultivation of *Bacteroides* species. *Curr Protoc Microbiol* **Chapter 13**: Unit 13C 11.

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., and Gordon, J.I. (2005) Host-bacterial mutualism in the human intestine. *Science* **307**: 1915-1920.

Backhed, F., Fraser, C.M., Ringel, Y., Sanders, M.E., Sartor, R.B., Sherman, P.M.*, et al.* (2012) Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe* **12**: 611-622.

Bahl, M.I., Bergstrom, A., and Licht, T.R. (2012) Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. *FEMS Microbiol Lett* **329**: 193-197.

Balamurugan, R., Rajendiran, E., George, S., Samuel, G.V., and Ramakrishna, B.S. (2008) Real-time polymerase chain reaction quantification of specific butyrate-producing bacteria, *Desulfovibrio* and *Enterococcus faecalis* in the feces of patients with colorectal cancer. *J Gastroenterol Hepatol* **23**: 1298-1303.

Baranyi, J., and Roberts, T.A. (1994) A dynamic approach to predicting bacterial growth in food. *Int J Food Microbiol* **23**: 277-294.

Barcenilla, A., Pryde, S.E., Martin, J.C., Duncan, S.H., Stewart, C.S., Henderson, C., and Flint, H.J. (2000) Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl Environ Microbiol* **66**: 1654-1661.

Baron, E.J. (1997) *Bilophila wadsworthia*: a unique Gram-negative anaerobic rod. *Anaerobe* **3**: 83-86.

Bashiardes, S., Zilberman-Schapira, G., and Elinav, E. (2016) Use of Metatranscriptomics in Microbiome Research. *Bioinform Biol Insights* **10**: 19-25.

Baxter, N.T., Zackular, J.P., Chen, G.Y., and Schloss, P.D. (2014) Structure of the gut microbiome following colonization with human feces determines colonic tumor burden. *Microbiome* **2**: 20.

Belcheva, A., Irrazabal, T., and Martin, A. (2015) Gut microbial metabolism and colon cancer: Can manipulations of the microbiota be useful in the management of gastrointestinal health? *Bioessays* **37**: 403-412.

Belenguer, A., Duncan, S.H., Calder, A.G., Holtrop, G., Louis, P., Lobley, G.E., and Flint, H.J. (2006) Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. *Appl Environ Microbiol* **72**: 3593-3599.

Bensch, G., Ruger, M., Wassermann, M., Weinholz, S., Reichl, U., and Cordes, C. (2014) Flow cytometric viability assessment of lactic acid bacteria starter cultures produced by fluidized bed drying. *Appl Microbiol Biotechnol* **98**: 4897-4909.

Berlanga, M., and Guerrero, R. (2016) Living together in biofilms: the microbial cell factory and its biotechnological implications. *Microb Cell Fact* **15**: 165.

Berney, M., Vital, M., Hulshoff, I., Weilenmann, H.U., Egli, T., and Hammes, F. (2008) Rapid, cultivation-independent assessment of microbial viability in drinking water. *Water Res* **42**: 4010-4018.

Berninger, T., Gonzalez Lopez, O., Bejarano, A., Preininger, C., and Sessitsch, A. (2018) Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. *Microb Biotechnol* **11**: 277-301.

Bircher, L., Schwab, C., Geirnaert, A., and Lacroix, C. (2017) Cryopreservation of artificial gut microbiota produced with in vitro fermentation technology. *Microb Biotechnol* 10.1111/1751-7915.12844.

Bircher, L., Geirnaert, A., Hammes, F., Lacroix, C., and Schwab, C. (2018) Effect of cryopreservation and lyophilization on viability and growth of strict anaerobic human gut microbes. *Microb Biotechnol* 10.1111/1751-7915.13265.

Blaut, M. (2013) Ecology and physiology of the intestinal tract. *Curr Top Microbiol Immunol* **358**: 247-272.

Blouin, J.M., Penot, G., Collinet, M., Nacfer, M., Forest, C., Laurent-Puig, P.*, et al.* (2011) Butyrate elicits a metabolic switch in human colon cancer cells by targeting the pyruvate dehydrogenase complex. *Int J Cancer* **128**: 2591-2601.

Boulange, C.L., Neves, A.L., Chilloux, J., Nicholson, J.K., and Dumas, M.E. (2016) Impact of the gut microbiota on inflammation, obesity, and metabolic disease. *Genome Med* **8**: 42.

Braegger, C., Chmielewska, A., Decsi, T., Kolacek, S., Mihatsch, W., Moreno, L.*, et al.* (2011) Supplementation of infant formula with probiotics and/or prebiotics: a systematic review and comment by the ESPGHAN committee on nutrition. *J Pediatr Gastroenterol Nutr* **52**: 238-250.

Brahe, L.K., Astrup, A., and Larsen, L.H. (2013) Is butyrate the link between diet, intestinal microbiota and obesity-related metabolic diseases? *Obes Rev* **14**: 950-959.

Bravo-Ferrada, B.M., Goncalves, S., Semorile, L., Santos, N.C., Tymczyszyn, E.E., and Hollmann, A. (2015) Study of surface damage on cell envelope assessed by AFM and flow cytometry of *Lactobacillus plantarum* exposed to ethanol and dehydration. *J Appl Microbiol* **118**: 1409-1417.

Breyner, N.M., Michon, C., de Sousa, C.S., Vilas Boas, P.B., Chain, F., Azevedo, V.A.*, et al.* (2017) Microbial Anti-Inflammatory Molecule (MAM) from *Faecalibacterium prausnitzii* Shows a Protective Effect on DNBS and DSS-Induced Colitis Model in Mice through Inhibition of NF-kappaB Pathway. *Front Microbiol* **8**: 114.

Broeckx, G., Vandenheuvel, D., Claes, I.J., Lebeer, S., and Kiekens, F. (2016) Drying techniques of probiotic bacteria as an important step towards the development of novel pharmabiotics. *Int J Pharm* **505**: 303-318.

Cammarota, G., Ianiro, G., Tilg, H., Rajilic-Stojanovic, M., Kump, P., Satokari, R.*, et al.* (2017) European consensus conference on faecal microbiota transplantation in clinical practice. *Gut* **66**: 569-580.

Cano, P.G., Santacruz, A., Moya, A., and Sanz, Y. (2012) *Bacteroides uniformis* CECT 7771 ameliorates metabolic and immunological dysfunction in mice with high-fat-diet induced obesity. *PLoS One* **7**: e41079.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K.*, et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335-336.

Carding, S., Verbeke, K., Vipond, D.T., Corfe, B.M., and Owen, L.J. (2015) Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis* **26**: 26191.

Carlucci, C., Petrof, E.O., and Allen-Vercoe, E. (2016) Fecal microbiota-based therapeutics for recurrent *Clostridium difficile* infection, ulcerative colitis and obesity. *EBioMedicine* **13**: 37-45.

Castro, H.P., Teixeira, P.M., and Kirby, R. (1997) Evidence of membrane damage in Lactobacillus bulgaricus following freeze drying. *J Appl Microbiol* **82**: 87-94.

Cebrian, G., Arroyo, C., Manas, P., and Condon, S. (2014) Bacterial maximum non-inhibitory and minimum inhibitory concentrations of different water activity depressing solutes. *Int J Food Microbiol* **188**: 67-74.

Chang, J.Y., Antonopoulos, D.A., Kalra, A., Tonelli, A., Khalife, W.T., Schmidt, T.M., and Young, V.B. (2008) Decreased diversity of the fecal microbiome in recurrent *Clostridium difficile*-associated diarrhea. *J Infect Dis* **197**: 435-438.

Chang, Y.J., Pukall, R., Saunders, E., Lapidus, A., Copeland, A., Nolan, M.*, et al.* (2010) Complete genome sequence of *Acidaminococcus fermentans* type strain (VR4). *Stand Genomic Sci* **3**: 1-14.

Chassard, C., and Lacroix, C. (2013) Carbohydrates and the human gut microbiota. *Curr Opin Clin Nutr Metab Care* **16**: 453-460.

Chen, L., Wang, W., Zhou, R., Ng, S.C., Li, J., Huang, M.*, et al.* (2014) Characteristics of fecal and mucosa-associated microbiota in Chinese patients with inflammatory bowel disease. *Medicine (Baltimore)* **93**: e51.

Cinquin, C., Le Blay, G., Fliss, I., and Lacroix, C. (2004) Immobilization of infant fecal microbiota and utilization in an in vitro colonic fermentation model. *Microb Ecol* **48**: 128-138.

Cinquin, C., Le Blay, G., Fliss, I., and Lacroix, C. (2006) New three-stage in vitro model for infant colonic fermentation with immobilized fecal microbiota. *FEMS Microbiol Ecol* **57**: 324-336.

Claesson, M.J., O'Sullivan, O., Wang, Q., Nikkila, J., Marchesi, J.R., Smidt, H.*, et al.* (2009) Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. *PLoS One* **4**: e6669.

Claesson, M.J., Jeffery, I.B., Conde, S., Power, S.E., O'Connor, E.M., Cusack, S.*, et al.* (2012) Gut microbiota composition correlates with diet and health in the elderly. *Nature* **488**: 178-184.

Cockburn, D.W., and Koropatkin, N.M. (2016) Polysaccharide Degradation by the Intestinal Microbiota and Its Influence on Human Health and Disease. *J Mol Biol* **428**: 3230-3252.

Collins, J., Robinson, C., Danhof, H., Knetsch, C.W., van Leeuwen, H.C., Lawley, T.D.*, et al.* (2018) Dietary trehalose enhances virulence of epidemic *Clostridium difficile*. *Nature* **553**: 291-294.

Conrad, P.B., Miller, D.P., Cielenski, P.R., and de Pablo, J.J. (2000) Stabilization and preservation of Lactobacillus acidophilus in saccharide matrices. *Cryobiology* **41**: 17-24.

Copeland, J.C., Oxyrase, Inc. (Mansfield, OH, US) (2017) Preservation and storage of biological specimens. US Patent 20170218325.

Cornely, O.A., Crook, D.W., Esposito, R., Poirier, A., Somero, M.S., Weiss, K.*, et al.* (2012) Fidaxomicin versus vancomycin for infection with *Clostridium difficile* in Europe, Canada, and the USA: a double-blind, non-inferiority, randomised controlled trial. *Lancet Infect Dis* **12**: 281-289.

Costello, S.P., Conlon, M.A., Vuaran, M.S., Roberts-Thomson, I.C., and Andrews, J.M. (2015) Faecal microbiota transplant for recurrent *Clostridium difficile* infection using long-term frozen stool is effective: clinical efficacy and bacterial viability data. *Aliment Pharmacol Ther* **42**: 1011-1018.

Cotillard, A., Kennedy, S.P., Kong, L.C., Prifti, E., Pons, N., Le Chatelier, E.*, et al.* (2013) Dietary intervention impact on gut microbial gene richness. *Nature* **500**: 585-588.

Crowe, J.H., Carpenter, J.F., Crowe, L.M., and Anchordoguy, T.J. (1990) Are freezing and dehydration similar stress vectors - a comparison of modes of interaction of stabilizing solutes with biomolecules. *Cryobiology* **27**: 219-231.

Crowe, J.H., Hoekstra, F.A., and Crowe, L.M. (1992) Anhydrobiosis. *Annu Rev Physiol* **54**: 579-599.

Crowe, J.H., Hoekstra, F.A., Nguyen, K.H.N., and Crowe, L.M. (1996) Is vitrification involved in depression of the phase transition temperature in dry phospholipids? *Biochimica Et Biophysica Acta-Biomembranes* **1280**: 187-196.

Crowe, J.H. (2015) Anhydrobiosis: An unsolved problem with applications in human welfare. *Subcell Biochem* **71**: 263-280.

Cummings, J.H., and Macfarlane, G.T. (1991) The control and consequences of bacterial fermentation in the human colon. *J Appl Bacteriol* **70**: 443-459.

D'Auria, G., Galan, J.C., Rodriguez-Alcayna, M., Moya, A., Baquero, F., and Latorre, A. (2011) Complete genome sequence of *Acidaminococcus intestini* RYC-MR95, a Gram-negative bacterium from the phylum Firmicutes. *J Bacteriol* **193**: 7008-7009.

Dai, Z.L., Zhang, J., Wu, G.Y., and Zhu, W.Y. (2010) Utilization of amino acids by bacteria from the pig small intestine. *Amino Acids* **39**: 1201-1215.

Darfeuille-Michaud, A., Boudeau, J., Bulois, P., Neut, C., Glasser, A.L., Barnich, N.*, et al.* (2004) High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. *Gastroenterology* **127**: 412-421.

Dashnau, J.L., Nucci, N.V., Sharp, K.A., and Vanderkooi, J.M. (2006) Hydrogen bonding and the cryoprotective properties of glycerol/water mixtures. *J Phys Chem B* **110**: 13670-13677.

Davis, C. (2014) Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. *J Microbiol Methods* **103**: 9-17.

de Groot, P.F., Frissen, M.N., de Clercq, N.C., and Nieuwdorp, M. (2017) Fecal microbiota transplantation in metabolic syndrome: History, present and future. *Gut Microbes* **8**: 253-267.

De Vadder, F., Kovatcheva-Datchary, P., Goncalves, D., Vinera, J., Zitoun, C., Duchampt, A.*, et al.* (2014) Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell* **156**: 84-96.

De Weirdt, R., and Van de Wiele, T. (2015) Micromanagement in the gut: microenvironmental factors govern colon mucosal biofilm structure and functionality. *NPJ Biofilms Microbiomes* **1**: 15026.

Demel, R.A., Dorrepaal, E., Ebskamp, M.J., Smeekens, J.C., and de Kruijff, B. (1998) Fructans interact strongly with model membranes. *Biochim Biophys Acta* **1375**: 36-42.

den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.J., and Bakker, B.M. (2013) The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* **54**: 2325-2340.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K.*, et al.* (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**: 5069-5072.

Dhewa, T., Pant, S., and Mishra, V. (2014) Development of freeze dried synbiotic formulation using a probiotic strain of Lactobacillus plantarum. *J Food Sci Technol* **51**: 83-89.

Dijkstra, A.R., Setyawati, M.C., Bayjanov, J.R., Alkema, W., van Hijum, S.A., Bron, P.A., and Hugenholtz, J. (2014) Diversity in robustness of *Lactococcus lactis* strains during heat stress, oxidative stress, and spray drying stress. *Appl Environ Microbiol* **80**: 603-611.

Doleyres, Y., Fliss, I., and Lacroix, C. (2002) Quantitative determination of the spatial distribution of pure- and mixed-strain immobilized cells in gel beads by immunofluorescence. *Appl Microbiol Biotechnol* **59**: 297-302.

Doleyres, Y., Fliss, I., and Lacroix, C. (2004) Continuous production of mixed lactic starters containing probiotics using immobilized cell technology. *Biotechnol Prog* **20**: 145-150.

Doleyres, Y., Fliss, I., and Lacroix, C. (2004) Increased stress tolerance of *Bifidobacterium longum* and *Lactococcus lactis* produced during continuous mixed-strain immobilized-cell fermentation. *J Appl Microbiol* **97**: 527-539.

Donelli, G., Vuotto, C., Cardines, R., and Mastrantonio, P. (2012) Biofilm-growing intestinal anaerobic bacteria. *FEMS Immunol Med Microbiol* **65**: 318-325.

Downes, J., Vartoukian, S.R., Dewhirst, F.E., Izard, J., Chen, T., Yu, W.H., *et al.* (2009) Pyramidobacter piscolens gen. nov., sp. nov., a member of the phylum 'Synergistetes' isolated from the human oral cavity. *Int J Syst Evol Microbiol* **59**: 972-980.

Dridi, B., Henry, M., Richet, H., Raoult, D., and Drancourt, M. (2012) Age-related prevalence of *Methanomassiliicoccus luminyensis* in the human gut microbiome. *APMIS* **120**: 773-777.

Dubois-Brissonnet, F., Trotier, E., and Briandet, R. (2016) The Biofilm Lifestyle Involves an Increase in Bacterial Membrane Saturated Fatty Acids. *Front Microbiol* **7**: 1673.

Duncan, S.H., Barcenilla, A., Stewart, C.S., Pryde, S.E., and Flint, H.J. (2002) Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. *Appl Environ Microbiol* **68**: 5186-5190.

Duncan, S.H., Hold, G.L., Harmsen, H.J., Stewart, C.S., and Flint, H.J. (2002) Growth requirements and fermentation products of *Fusobacterium prausnitzii*, and a proposal to reclassify it as *Faecalibacterium prausnitzii* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **52**: 2141-2146.

Duncan, S.H., Scott, K.P., Ramsay, A.G., Harmsen, H.J., Welling, G.W., Stewart, C.S., and Flint, H.J. (2003) Effects of alternative dietary substrates on competition between human colonic bacteria in an anaerobic fermentor system. *Appl Environ Microbiol* **69**: 1136-1142.

Duncan, S.H., Holtrop, G., Lobley, G.E., Calder, A.G., Stewart, C.S., and Flint, H.J. (2004) Contribution of acetate to butyrate formation by human faecal bacteria. *Br J Nutr* **91**: 915-923.

Duncan, S.H., Louis, P., and Flint, H.J. (2004) Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Appl Environ Microbiol* **70**: 5810-5817.

Duncan, S.H., Aminov, R.I., Scott, K.P., Louis, P., Stanton, T.B., and Flint, H.J. (2006) Proposal of *Roseburia faecis* sp. nov., *Roseburia hominis* sp. nov. and *Roseburia inulinivorans* sp. nov., based on isolates from human faeces. *Int J Syst Evol Microbiol* **56**: 2437-2441.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., *et al.* (2005) Diversity of the human intestinal microbial flora. *Science* **308**: 1635-1638.

Edgar, R.C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* **10**: 996-998.

Eini, A., Sol, A., Coppenhagen-Glazer, S., Skvirsky, Y., Zini, A., and Bachrach, G. (2013) Oxygen deprivation affects the antimicrobial action of LL-37 as determined by microplate real-time kinetic measurements under anaerobic conditions. *Anaerobe* **22**: 20-24.

El Hage, R., Hernandez-Sanabria, E., and Van de Wiele, T. (2017) Emerging trends in "Smart Probiotics": Functional consideration for the development of novel health and industrial applications. *Front Microbiol* **8**: 1889.

El Kaoutari, A., Armougom, F., Gordon, J.I., Raoult, D., and Henrissat, B. (2013) The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nat Rev Microbiol* **11**: 497-504.

Engels, C., Ruscheweyh, H.J., Beerenwinkel, N., Lacroix, C., and Schwab, C. (2016) The common gut microbe *Eubacterium hallii* also contributes to intestinal propionate formation. *Front Microbiol* **7**: 713.

Engin, A. (2017) The definition and prevalence of obesity and metabolic syndrome. *Adv Exp Med Biol* **960**: 1-17.

Erikstrup, L.T., Aarup, M., Hagemann-Madsen, R., Dagnaes-Hansen, F., Kristensen, B., Olsen, K.E., and Fuursted, K. (2015) Treatment of *Clostridium difficile* infection in mice with vancomycin alone is as effective as treatment with vancomycin and metronidazole in combination. *BMJ Open Gastroenterol* **2**: e000038.

Ezaki, T., Kawamura, Y., Li, N., Li, Z.Y., Zhao, L., and Shu, S. (2001) Proposal of the genera *Anaerococcus* gen. nov., *Peptoniphilus* gen. nov. and *Gallicola* gen. nov. for members of the genus *Peptostreptococcus*. *Int J Syst Evol Microbiol* **51**: 1521-1528.

Falony, G., Vlachou, A., Verbrugghe, K., and De Vuyst, L. (2006) Cross-feeding between *Bifidobacterium longum* BB536 and acetate-converting, butyrate-producing colon bacteria during growth on oligofructose. *Appl Environ Microbiol* **72**: 7835-7841.

Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K.*, et al.* (2016) Population-level analysis of gut microbiome variation. *Science* **352**: 560-564.

Farhadi, A., Banan, A., Fields, J., and Keshavarzian, A. (2003) Intestinal barrier: An interface between health and disease. *J Gastroenterol Hepatol* **18**: 479-497.

FDA (2016) *Early clinical trials with live biotherapeutic products: Chemistry, manufacturing, and control information; Guidance for industry.*

Fehlbaum, S., Chassard, C., Haug, M.C., Fourmestraux, C., Derrien, M., and Lacroix, C. (2015) Design and investigation of PolyFermS in vitro continuous fermentation models inoculated with immobilized fecal microbiota mimicking the elderly colon. *PLoS One* **10**: e0142793.

Fekry, M.I., Engels, C., Zhang, J., Schwab, C., Lacroix, C., Sturla, S.J., and Chassard, C. (2016) The strict anaerobic gut microbe *Eubacterium hallii* transforms the carcinogenic dietary heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Environ Microbiol Rep* **8**: 201-209.

Fenn, K., Strandwitz, P., Stewart, E.J., Dimise, E., Rubin, S., Gurubacharya, S.*, et al.* (2017) Quinones are growth factors for the human gut microbiota. *Microbiome* **5**: 161.

Fernandes, J., Su, W., Rahat-Rozenbloom, S., Wolever, T.M., and Comelli, E.M. (2014) Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. *Nutr Diabetes* **4**: e121.

Fernandez-Murga, M.L., and Sanz, Y. (2016) Safety Assessment of *Bacteroides uniformis* CECT 7771 Isolated from Stools of Healthy Breast-Fed Infants. *PLoS One* **11**: e0145503.

Ferreira-Halder, C.V., Faria, A.V.S., and Andrade, S.S. (2017) Action and function of *Faecalibacterium prausnitzii* in health and disease. *Best Pract Res Clin Gastroenterol* **31**: 643-648.

Fijan, S. (2014) Microorganisms with claimed probiotic properties: an overview of recent literature. *Int J Environ Res Public Health* **11**: 4745-4767.

Flach, J., van der Waal, M.B., van den Nieuwboer, M., Claassen, E., and Larsen, O.F.A. (2017) The underexposed role of food matrices in probiotic products: Reviewing the relationship between carrier matrices and product parameters. *Crit Rev Food Sci Nutr* 10.1080/10408398.2017.1334624: 1-15.

Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., and Kjelleberg, S. (2016) Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* **14**: 563-575.

Flint, H.J., Duncan, S.H., Scott, K.P., and Louis, P. (2007) Interactions and competition within the microbial community of the human colon: links between diet and health. *Environ Microbiol* **9**: 1101-1111.

Flint, H.J., Scott, K.P., Duncan, S.H., Louis, P., and Forano, E. (2012) Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* **3**: 289-306.

Flint, H.J., Scott, K.P., Louis, P., and Duncan, S.H. (2012) The role of the gut microbiota in nutrition and health. *Nat Rev Gastroenterol Hepatol* **9**: 577-589.

Foglietta, F., Serpe, L., Canaparo, R., Vivenza, N., Riccio, G., Imbalzano, E.*, et al.* (2014) Modulation of butyrate anticancer activity by solid lipid nanoparticle delivery: an in vitro investigation on human breast cancer and leukemia cell lines. *J Pharm Pharm Sci* **17**: 231-247.

Fonseca, F., Beal, C., and Corrieu, G. (2001) Operating conditions that affect the resistance of lactic acid bacteria to freezing and frozen storage. *Cryobiology* **43**: 189-198.

Fonseca, F., Marin, M., and Morris, G.J. (2006) Stabilization of frozen *Lactobacillus delbrueckii* subsp. *bulgaricus* in glycerol suspensions: Freezing kinetics and storage temperature effects. *Appl Environ Microbiol* **72**: 6474-6482.

Fonseca, F., Meneghel, J., Cenard, S., Passot, S., and Morris, G.J. (2016) Determination of iIntracellular vitrification temperatures for unicellular microorganisms under conditions relevant for cryopreservation. *PLoS One* **11**: e0152939.

Fowler, A., and Toner, M. (2005) Cryo-injury and biopreservation. *Ann N Y Acad Sci* **1066**: 119-135.

Fuentes, S., Rossen, N.G., van der Spek, M.J., Hartman, J.H., Huuskonen, L., Korpela, K.*, et al.* (2017) Microbial shifts and signatures of long-term remission in ulcerative colitis after faecal microbiota transplantation. *ISME J*10.1038/ismej.2017.44.

Fuller, B.J. (2004) Cryoprotectants: the essential antifreezes to protect life in the frozen state. *Cryo Letters* **25**: 375-388.

Gaci, N., Chaudhary, P.P., Tottey, W., Alric, M., and Brugere, J.F. (2017) Functional amplification and preservation of human gut microbiota. *Microb Ecol Health Dis* **28**: 1308070.

Gagniere, J., Raisch, J., Veziant, J., Barnich, N., Bonnet, R., Buc, E.*, et al.* (2016) Gut microbiota imbalance and colorectal cancer. *World J Gastroenterol* **22**: 501-518.

Galvao, J., Davis, B., Tilley, M., Normando, E., Duchen, M.R., and Cordeiro, M.F. (2014) Unexpected low-dose toxicity of the universal solvent DMSO. *FASEB J* **28**: 1317-1330.

Gao, Z., Yin, J., Zhang, J., Ward, R.E., Martin, R.J., Lefevre, M., *et al.* (2009) Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes* **58**: 1509-1517.

Geirnaert, A., Steyaert, A., Eeckhaut, V., Debruyne, B., Arends, J.B., Van Immerseel, F., *et al.* (2014) *Butyricicoccus pullicaecorum*, a butyrate producer with probiotic potential, is intrinsically tolerant to stomach and small intestine conditions. *Anaerobe* **30**: 70-74.

Gerritsen, J., Smidt, H., Rijkers, G.T., and de Vos, W.M. (2011) Intestinal microbiota in human health and disease: the impact of probiotics. *Genes Nutr* **6**: 209-240.

Gevers, D., Kugathasan, S., Denson, L.A., Vazquez-Baeza, Y., Van Treuren, W., Ren, B., *et al.* (2014) The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe* **15**: 382-392.

Gill, S.R., Pop, M., Deboy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., *et al.* (2006) Metagenomic analysis of the human distal gut microbiome. *Science* **312**: 1355-1359.

Goncalves, P., and Martel, F. (2013) Butyrate and colorectal cancer: the role of butyrate transport. *Curr Drug Metab* **14**: 994-1008.

Grundy, S.M., Brewer, H.B., Jr., Cleeman, J.I., Smith, S.C., Jr., Lenfant, C., National Heart, L., *et al.* (2004) Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Arterioscler Thromb Vasc Biol* **24**: e13-18.

Guery, B., Menichetti, F., Anttila, V.J., Adomakoh, N., Aguado, J.M., Bisnauthsing, K., *et al.* (2018) Extended-pulsed fidaxomicin versus vancomycin for *Clostridium difficile* infection in patients 60 years and older (EXTEND): a randomised, controlled, open-label, phase 3b/4 trial. *Lancet Infect Dis* **18**: 296-307.

Guo, X., Xia, X., Tang, R., Zhou, J., Zhao, H., and Wang, K. (2008) Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett Appl Microbiol* **47**: 367-373.

Gupta, S., Allen-Vercoe, E., and Petrof, E.O. (2016) Fecal microbiota transplantation: in perspective. *Therap Adv Gastroenterol* **9**: 229-239.

Haidinger, W., Szostak, M.P., Jechlinger, W., and Lubitz, W. (2003) Online monitoring of *Escherichia coli* ghost production. *Appl Environ Microbiol* **69**: 468-474.

Hamilton, M.J., Weingarden, A.R., Sadowsky, M.J., and Khoruts, A. (2012) Standardized frozen preparation for transplantation of fecal microbiota for recurrent *Clostridium difficile* infection. *Am J Gastroenterol* **107**: 761-767.

Hammes, F., Berney, M., and Egli, T. (2011) Cultivation-independent assessment of bacterial viability. *Adv Biochem Eng Biotechnol* **124**: 123-150.

Heylen, K., Hoefman, S., Vekeman, B., Peiren, J., and De Vos, P. (2012) Safeguarding bacterial resources promotes biotechnological innovation. *Appl Microbiol Biotechnol* **94**: 565-574.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., *et al.* (2014) Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* **11**: 506-514.

Hobley, L., Harkins, C., MacPhee, C.E., and Stanley-Wall, N.R. (2015) Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiol Rev* **39**: 649-669.

Hoffmann, C., Dollive, S., Grunberg, S., Chen, J., Li, H., Wu, G.D.*, et al.* (2013) Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. *PLoS One* **8**: e66019.

Hong, P.Y., Wu, J.H., and Liu, W.T. (2008) Relative abundance of Bacteroides spp. in stools and wastewaters as determined by hierarchical oligonucleotide primer extension. *Appl Environ Microbiol* **74**: 2882-2893.

Hsiao, A., Ahmed, A.M., Subramanian, S., Griffin, N.W., Drewry, L.L., Petri, W.A., Jr.*, et al.* (2014) Members of the human gut microbiota involved in recovery from *Vibrio cholerae* infection. *Nature* **515**: 423-426.

Hubalek, Z. (2003) Protectants used in the cryopreservation of microorganisms. *Cryobiology* **46**: 205-229.

Human Microbiome Project, C. (2012) Structure, function and diversity of the healthy human microbiome. *Nature* **486**: 207-214.

Huycke, M.M., and Gaskins, H.R. (2004) Commensal bacteria, redox stress, and colorectal cancer: mechanisms and models. *Exp Biol Med (Maywood)* **229**: 586-597.

Huynh, H.T.T., Pignoly, M., Drancourt, M., and Aboudharam, G. (2017) A new methanogen "*Methanobrevibacter massiliense*" isolated in a case of severe periodontitis. *BMC Res Notes* **10**: 657.

Iaconelli, C., Lemetais, G., Kechaou, N., Chain, F., Bermudez-Humaran, L.G., Langella, P.*, et al.* (2015) Drying process strongly affects probiotics viability and functionalities. *J Biotechnol* **214**: 17-26.

Imlay, J.A. (2002) How oxygen damages microbes: Oxygen tolerance and obligate anaerobiosis. *Advances in Microbial Physiology, Vol 46* **46**: 111-153.

Jain, P., Sen, S., and Risbud, S.H. (2009) Effect of glass-forming biopreservatives on head group rotational dynamics in freeze-dried phospholipid bilayers: a 31P NMR study. *J Chem Phys* **131**: 025102.

Jiang, J., Boese, M., Turner, P., and Wang, R.K. (2008) Penetration kinetics of dimethyl sulphoxide and glycerol in dynamic optical clearing of porcine skin tissue in vitro studied by Fourier transform infrared spectroscopic imaging. *J Biomed Opt* **13**: 021105.

Jones, R.B., Zhu, X., Moan, E., Murff, H.J., Ness, R.M., Seidner, D.L.*, et al.* (2018) Inter-niche and inter-individual variation in gut microbial community assessment using stool, rectal swab, and mucosal samples. *Sci Rep* **8**: 4139.

Joux, F., and Lebaron, P. (2000) Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes and Infection* **2**: 1523-1535.

Jumas-Bilak, E., Carlier, J.P., Jean-Pierre, H., Mory, F., Teyssier, C., Gay, B.*, et al.* (2007) *Acidaminococcus intestini* sp. nov., isolated from human clinical samples. *Int J Syst Evol Microbiol* **57**: 2314-2319.

Junter, G.A., Coquet, L., Vilain, S., and Jouenne, T. (2002) Immobilized-cell physiology: current data and the potentialities of proteomics. *Enzyme Microb Technol* **31**: 201-212.

Karlsson, F.H., Tremaroli, V., Nookaew, I., Bergstrom, G., Behre, C.J., Fagerberg, B.*, et al.* (2013) Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* **498**: 99-103.

Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R., and Barer, M.R. (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie Van Leeuwenhoek* **73**: 169-187.

Kelly, C.R., Khoruts, A., Staley, C., Sadowsky, M.J., Abd, M., Alani, M.*, et al.* (2016) Effect of fecal microbiota transplantation on recurrence in multiply recurrent *Clostridium difficile* Infection: A randomized trial. *Ann Intern Med* **165**: 609-616.

Kerckhof, F.M., Courtens, E.N., Geirnaert, A., Hoefman, S., Ho, A., Vilchez-Vargas, R.*, et al.* (2014) Optimized cryopreservation of mixed microbial communities for conserved functionality and diversity. *PLoS One* **9**: e99517.

Khan, M.T., Duncan, S.H., Stams, A.J., van Dijl, J.M., Flint, H.J., and Harmsen, H.J. (2012) The gut anaerobe *Faecalibacterium prausnitzii* uses an extracellular electron shuttle to grow at oxic-anoxic interphases. *ISME J* **6**: 1578-1585.

Khan, M.T., van Dijl, J.M., and Harmsen, H.J. (2014) Antioxidants keep the potentially probiotic but highly oxygen-sensitive human gut bacterium *Faecalibacterium prausnitzii* alive at ambient air. *PLoS One* **9**: e96097.

Khoruts, A., and Sadowsky, M.J. (2016) Understanding the mechanisms of faecal microbiota transplantation. *Nat Rev Gastroenterol Hepatol* **13**: 508-516.

Kiesler, P., Fuss, I.J., and Strober, W. (2015) Experimental models of inflammatory bowel diseases. *Cell Mol Gastroenterol Hepatol* **1**: 154-170.

Kirsop, B. (1985) The current status of culture collections and their contribution to biotechnology. *Crc Critical Reviews in Biotechnology* **2**: 287-314.

Knudsen, G.M., Nielsen, M.B., Grassby, T., Danino-Appleton, V., Thomsen, L.E., Colquhoun, I.J.*, et al.* (2012) A third mode of surface-associated growth: immobilization of *Salmonella enterica* serovar *Typhimurium* modulates the RpoS-directed transcriptional programme. *Environ Microbiol* **14**: 1855-1875.

Koh, C.M. (2013) Storage of bacteria and yeast. *Methods Enzymol* **533**: 15-21.

Konig, J., Siebenhaar, A., Hogenauer, C., Arkkila, P., Nieuwdorp, M., Noren, T.*, et al.* (2017) Consensus report: faecal microbiota transfer - clinical applications and procedures. *Aliment Pharmacol Ther* **45**: 222-239.

Konstantinov, S.R., Kuipers, E.J., and Peppelenbosch, M.P. (2013) Functional genomic analyses of the gut microbiota for CRC screening. *Nat Rev Gastroenterol Hepatol* **10**: 741-745.

Kroninger, L., Gottschling, J., and Deppenmeier, U. (2017) Growth Characteristics of *Methanomassiliicoccus luminyensis* and Expression of Methyltransferase Encoding Genes. *Archaea-an International Microbiological Journal*Artn 2756573

10.1155/2017/2756573.

Krych, L., Hansen, C.H., Hansen, A.K., van den Berg, F.W., and Nielsen, D.S. (2013) Quantitatively different, yet qualitatively alike: a meta-analysis of the mouse core gut microbiome with a view towards the human gut microbiome. *PLoS One* **8**: e62578.

Krych, L., Kot, W., Bendtsen, K.M.S., Hansen, A.K., Vogensen, F.K., and Nielsen, D.S. (2018) Have you tried spermine? A rapid and cost-effective method to eliminate dextran sodium sulfate inhibition of PCR and RT-PCR. *J Microbiol Methods* **144**: 1-7.

Kuai, L., Nair, A.A., and Polz, M.F. (2001) Rapid and simple method for the most-probable-number estimation of arsenic-reducing bacteria. *Appl Environ Microbiol* **67**: 3168-3173.

Kubota, H., Senda, S., Tokuda, H., Uchiyama, H., and Nomura, N. (2009) Stress resistance of biofilm and planktonic *Lactobacillus plantarum* subsp *plantarum* JCM 1149. *Food Microbiol* **26**: 592-597.

Kumari, R., Ahuja, V., and Paul, J. (2013) Fluctuations in butyrate-producing bacteria in ulcerative colitis patients of North India. *World J Gastroenterol* **19**: 3404-3414.

Lacroix, C., de Wouters, T., and Chassard, C. (2015) Integrated multi-scale strategies to investigate nutritional compounds and their effect on the gut microbiota. *Curr Opin Biotechnol* **32**: 149-155.

Lahtinen, S.J., Ahokoski, H., Reinikainen, J.P., Gueimonde, M., Nurmi, J., Ouwehand, A.C., and Salminen, S.J. (2008) Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria. *Lett Appl Microbiol* **46**: 693-698.

Lamboley, L., Lacroix, C., Champagne, C.P., and Vuillemard, J.C. (1997) Continuous mixed strain mesophilic lactic starter production in supplemented whey permeate medium using immobilized cell technology. *Biotechnol Bioeng* **56**: 502-516.

Lamboley, L., Lacroix, C., Artignan, J.M., Champagne, C.P., and Vuillemard, J.C. (1999) Long-term mechanical and biological stability of an immobilized cell reactor for continuous mixed-strain mesophilic lactic starter production in whey permeate. *Biotechnol Prog* **15**: 646-654.

Lamboley, L., Lacroix, C., Sodini, I., Lemay, M.J., and Champagne, C.P. (2001) Effect of inoculum composition and low KCl supplementation on the biological and rheological stability of an immobilized-cell system for mixed mesophilic lactic starter production. *Biotechnol Prog* **17**: 1071-1078.

Lane, E.R., Zisman, T.L., and Suskind, D.L. (2017) The microbiota in inflammatory bowel disease: current and therapeutic insights. *J Inflamm Res* **10**: 63-73.

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G.*, et al.* (2013) Richness of human gut microbiome correlates with metabolic markers. *Nature* **500**: 541-546.

Lee, C.H., Steiner, T., Petrof, E.O., Smieja, M., Roscoe, D., Nematallah, A.*, et al.* (2016) Frozen vs fresh fecal microbiota transplantation and clinical resolution of diarrhea in patients with recurrent Clostridium difficile infection: A randomized clinical trial. *JAMA* **315**: 142-149.

Lee, K.W., Periasamy, S., Mukherjee, M., Xie, C., Kjelleberg, S., and Rice, S.A. (2014) Biofilm development and enhanced stress resistance of a model, mixed-species community biofilm. *ISME J* **8**: 894-907.

Leitch, E.C., Walker, A.W., Duncan, S.H., Holtrop, G., and Flint, H.J. (2007) Selective colonization of insoluble substrates by human faecal bacteria. *Environ Microbiol* **9**: 667-679.

Leslie, S.B., Israeli, E., Lighthart, B., Crowe, J.H., and Crowe, L.M. (1995) Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Appl Environ Microbiol* **61**: 3592-3597.

Li, G., Yang, M., Zhou, K., Zhang, L., Tian, L., Lv, S.*, et al.* (2015) diversity of duodenal and rectal microbiota in biopsy tissues and luminal contents in healthy volunteers. *J Microbiol Biotechnol* **25**: 1136-1145.

Lipson, D.A. (2015) The complex relationship between microbial growth rate and yield and its implications for ecosystem processes. *Front Microbiol* **6**: 615.

Liu, J.H., Zhang, M.L., Zhang, R.Y., Zhu, W.Y., and Mao, S.Y. (2016) Comparative studies of the composition of bacterial microbiota associated with the ruminal content, ruminal epithelium and in the faeces of lactating dairy cows. *Microb Biotechnol* **9**: 257-268.

Louis, P., and Flint, H.J. (2009) Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol Lett* **294**: 1-8.

Louis, P., and Flint, H.J. (2017) Formation of propionate and butyrate by the human colonic microbiota. *Environ Microbiol* **19**: 29-41.

Lovelock, J.E. (1953) The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. *Biochim Biophys Acta* **11**: 28-36.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., and Knight, R. (2012) Diversity, stability and resilience of the human gut microbiota. *Nature* **489**: 220-230.

Lupp, C., Robertson, M.L., Wickham, M.E., Sekirov, I., Champion, O.L., Gaynor, E.C., and Finlay, B.B. (2007) Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of *Enterobacteriaceae*. *Cell Host Microbe* **2**: 204.

Macfarlane, G.T., Cummings, J.H., and Allison, C. (1986) Protein degradation by human intestinal bacteria. *J Gen Microbiol* **132**: 1647-1656.

Macfarlane, G.T., Gibson, G.R., and Cummings, J.H. (1992) Comparison of fermentation reactions in different regions of the human colon. *J Appl Bacteriol* **72**: 57-64.

Macfarlane, G.T., and Macfarlane, S. (1993) Factors affecting fermentation reactions in the large bowel. *Proc Nutr Soc* **52**: 367-373.

Macfarlane, G.T., Macfarlane, S., and Gibson, G.R. (1998) Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microb Ecol* **35**: 180-187.

Macfarlane, S., and Macfarlane, G.T. (2006) Composition and metabolic activities of bacterial biofilms colonizing food residues in the human gut. *Appl Environ Microbiol* **72**: 6204-6211.

Macfarlane, S., and Dillon, J.F. (2007) Microbial biofilms in the human gastrointestinal tract. *J Appl Microbiol* **102**: 1187-1196.

Machiels, K., Joossens, M., Sabino, J., De Preter, V., Arijs, I., Eeckhaut, V.*, et al.* (2014) A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* **63**: 1275-1283.

Malik, K.A. (1991) Cryopreservation of bacteria with special reference to anaerobes. *World J Microbiol Biotechnol* **7**: 629-632.

Malik, K.A. (1992) Freeze-drying of microorganisms using a simple apparatus. *World J Microbiol Biotechnol* **8**: 76-79.

Marchandin, H., Damay, A., Roudiere, L., Teyssier, C., Zorgniotti, I., Dechaud, H.*, et al.* (2010) Phylogeny, diversity and host specialization in the phylum Synergistetes with emphasis on strains and clones of human origin. *Res Microbiol* **161**: 91-100.

Martens, E.C., Lowe, E.C., Chiang, H., Pudlo, N.A., Wu, M., McNulty, N.P.*, et al.* (2011) Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts. *PLoS Biol* **9**: e1001221.

Martin, R., Miquel, S., Benevides, L., Bridonneau, C., Robert, V., Hudault, S.*, et al.* (2017) Functional characterization of novel *Faecalibacterium prausnitzii* strains isolated from healthy volunteers: A step forward in the use of *F. prausnitzii* as a next-generation probiotic. *Front Microbiol* **8**: 1226.

Matejtschuk, P. (2007) Lyophilization of proteins. *Methods Mol Biol* **368**: 59-72.

Mazur, P. (1963) Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J Gen Physiol* **47**: 347-369.

Mazur, P. (1970) Cryobiology: the freezing of biological systems. *Science* **168**: 939-949.

Mazur, P. (1984) Freezing of living cells: mechanisms and implications. *Am J Physiol* **247**: C125-142.

McAllister, T.A., Bae, H.D., Jones, G.A., and Cheng, K.J. (1994) Microbial attachment and feed digestion in the rumen. *J Anim Sci* **72**: 3004-3018.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A.*, et al.* (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* **6**: 610-618.

McDonald, L.C., Gerding, D.N., Johnson, S., Bakken, J.S., Carroll, K.C., Coffin, S.E.*, et al.* (2018) Clinical practice guidelines for *Clostridium difficile* infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin Infect Dis* **66**: 987-994.

McIntyre, R.E., Buczacki, S.J.A., Arends, M.J., and Adams, D.J. (2015) Mouse models of colorectal cancer as preclinical models. *Bioessays* **37**: 909-920.

Meneghel, J., Passot, S., Dupont, S., and Fonseca, F. (2017) Biophysical characterization of the *Lactobacillus delbrueckii* subsp. *bulgaricus* membrane during cold and osmotic stress and its relevance for cryopreservation. *Appl Microbiol Biotechnol* **101**: 1427-1441.

Mensink, M.A., Frijlink, H.W., van der Voort Maarschalk, K., and Hinrichs, W.L. (2015) Inulin, a flexible oligosaccharide. II: Review of its pharmaceutical applications. *Carbohydr Polym* **134**: 418-428.

Meryman, H.T. (2007) Cryopreservation of living cells: principles and practice. *Transfusion (Paris)* **47**: 935-945.

Messer, J.S., Liechty, E.R., Vogel, O.A., and Chang, E.B. (2017) Evolutionary and ecological forces that shape the bacterial communities of the human gut. *Mucosal Immunol* **10**: 567-579.

Michel, C., Kravtchenko, T.P., David, A., Gueneau, S., Kozlowski, F., and Cherbut, C. (1998) In vitro prebiotic effects of Acacia gums onto the human intestinal microbiota depends on both botanical origin and environmental pH. *Anaerobe* **4**: 257-266.

Milani, C., Ticinesi, A., Gerritsen, J., Nouvenne, A., Lugli, G.A., Mancabelli, L.*, et al.* (2016) Gut microbiota composition and Clostridium difficile infection in hospitalized elderly individuals: a metagenomic study. *Sci Rep* **6**: 25945.

Mille, Y., Beney, L., and Gervais, P. (2005) Compared tolerance to osmotic stress in various microorganisms: towards a survival prediction test. *Biotechnol Bioeng* **92**: 479-484.

Miquel, S., Leclerc, M., Martin, R., Chain, F., Lenoir, M., Raguideau, S.*, et al.* (2015) Identification of metabolic signatures linked to anti-inflammatory effects of *Faecalibacterium prausnitzii*. *MBio* **6**.

Mishra, S., and Imlay, J.A. (2013) An anaerobic bacterium, *Bacteroides thetaiotaomicron*, uses a consortium of enzymes to scavenge hydrogen peroxide. *Mol Microbiol* **90**: 1356-1371.

Miyamoto-Shinohara, Y., Imaizumi, T., Sukenobe, J., Murakami, Y., Kawamura, S., and Komatsu, Y. (2000) Survival rate of microbes after freeze-drying and long-term storage. *Cryobiology* **41**: 251-255.

Miyamoto-Shinohara, Y., Sukenobe, J., Imaizumi, T., and Nakahara, T. (2008) Survival of freeze-dried bacteria. *J Gen Appl Microbiol* **54**: 9-24.

Modesto, M., Mattarelli, P., and Biavati, B. (2004) Resistance to freezing and freeze-drying storage processes of potential probiotic *Bifidobacteria*. *Ann Microbiol* **54**: 43-48.

Mokhtari, Z., Gibson, D.L., and Hekmatdoost, A. (2017) Nonalcoholic fatty liver disease, the gut microbiome, and diet. *Adv Nutr* **8**: 240-252.

Morgan, C.A., Herman, N., White, P.A., and Vesey, G. (2006) Preservation of micro-organisms by drying; a review. *J Microbiol Methods* **66**: 183-193.

Morgan, X.C., Tickle, T.L., Sokol, H., Gevers, D., Devaney, K.L., Ward, D.V*., et al.* (2012) Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol* **13**: R79.

Morris, G.J., Goodrich, M., Acton, E., and Fonseca, F. (2006) The high viscosity encountered during freezing in glycerol solutions: effects on cryopreservation. *Cryobiology* **52**: 323-334.

Morrison, D.J., and Preston, T. (2016) Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* **7**: 189-200.

Mowat, A.M., and Agace, W.W. (2014) Regional specialization within the intestinal immune system. *Nat Rev Immunol* **14**: 667-685.

Murri, M., Leiva, I., Gomez-Zumaquero, J.M., Tinahones, F.J., Cardona, F., Soriguer, F., and Queipo-Ortuno, M.I. (2013) Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. *BMC Med* **11**: 46.

Nakamura, Y., Yamamoto, N., Kino, Y., Yamamoto, N., Kamei, S., Mori, H*., et al.* (2016) Establishment of a multi-species biofilm model and metatranscriptomic analysis of biofilm and planktonic cell communities. *Appl Microbiol Biotechnol* **100**: 7263-7279.

Ni, J., Wu, G.D., Albenberg, L., and Tomov, V.T. (2017) Gut microbiota and IBD: causation or correlation? *Nat Rev Gastroenterol Hepatol* **14**: 573-584.

Nocker, A., Fernandez, P.S., Montijn, R., and Schuren, F. (2012) Effect of air drying on bacterial viability: A multiparameter viability assessment. *J Microbiol Methods* **90**: 86-95.

Nocker, A., Cheswick, R., de la Rochere, P.M.D., Denis, M., Leziart, T., and Jarvis, P. (2017) When are bacteria dead? A step towards interpreting flow cytometry profiles after chlorine disinfection and membrane integrity staining. *Environ Technol* **38**: 891-900.

O'Toole, P.W., Marchesi, J.R., and Hill, C. (2017) Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. *Nat Microbiol* **2**: 17057.

Ohkusa, T., Sato, N., Ogihara, T., Morita, K., Ogawa, M., and Okayasu, I. (2002) Fusobacterium varium localized in the colonic mucosa of patients with ulcerative colitis stimulates species-specific antibody. *J Gastroenterol Hepatol* **17**: 849-853.

Olle, B. (2013) Medicines from microbiota. *Nat Biotechnol* **31**: 309-315.

Oskouei, D.D., Bekmen, N., Ellidokuz, H., and Yilmaz, O. (2010) Evaluation of different cryoprotective agents in maintenance of viability of *Helicobacter pylori* in stock culture media. *Braz J Microbiol* **41**: 1038-1046.

Ouwerkerk, J.P., Aalvink, S., Belzer, C., and De Vos, W.M. (2017) Preparation and preservation of viable *Akkermansia muciniphila* cells for therapeutic interventions. *Benef Microbes* **8**: 163-169.

Pan, N., and Imlay, J.A. (2001) How does oxygen inhibit central metabolism in the obligate anaerobe *Bacteroides thetaiotaomicron*. *Mol Microbiol* **39**: 1562-1571.

Patra, P., and Klumpp, S. (2013) Population dynamics of bacterial persistence. *PLoS One* **8**: e62814.

Payne, A.N., Zihler, A., Chassard, C., and Lacroix, C. (2012) Advances and perspectives in in vitro human gut fermentation modeling. *Trends Biotechnol* **30**: 17-25.

Pegg, D.E. (2015) Principles of cryopreservation. *Methods Mol Biol* **1257**: 3-19.

Pembrey, R.S., Marshall, K.C., and Schneider, R.P. (1999) Cell surface analysis techniques: What do cell preparation protocols do to cell surface properties? *Appl Environ Microbiol* **65**: 2877-2894.

Pereira, C.S., and Hunenberger, P.H. (2006) Interaction of the sugars trehalose, maltose and glucose with a phospholipid bilayer: a comparative molecular dynamics study. *J Phys Chem B* **110**: 15572-15581.

Pessione, E. (2012) Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. *Front Cell Infect Microbiol* **2**: 86.

Petrof, E.O., Gloor, G.B., Vanner, S.J., Weese, S.J., Carter, D., Daigneault, M.C*., et al.* (2013) Stool substitute transplant therapy for the eradication of *Clostridium difficile* infection: 'RePOOPulating' the gut. *Microbiome* **1**: 3.

Petrof, E.O., and Khoruts, A. (2014) From stool transplants to next-generation microbiota therapeutics. *Gastroenterology* **146**: 1573-1582.

Phillips, B.A., Latham, M.J., and Sharpe, M.E. (1975) A method for freeze drying rumen bacteria and other strict anaerobes. *J Appl Bacteriol* **38**: 319-322.

Poeker, S.A., Geirnaert, A., Berchtold, L., Greppi, A., Krych, L., Steinert, R.E*., et al.* (2018) Understanding the prebiotic potential of different dietary fibers using an in vitro continuous adult fermentation model (PolyFermS). *Sci Rep* **8**: 4318.

Pool-Zobel, B.L. (2005) Inulin-type fructans and reduction in colon cancer risk: review of experimental and human data. *Br J Nutr* **93 Suppl 1**: S73-90.

Pope, C.F., McHugh, T.D., and Gillespie, S.H. (2010) Methods to determine fitness in bacteria. *Methods Mol Biol* **642**: 113-121.

Portune, K.J., Beaumont, M., Davila, A.M., Tome, D., Blachier, F., and Sanz, Y. (2016) Gut microbiota role in dietary protein metabolism and health-related outcomes: The two sides of the coin. *Trends Food Sci Technol* **57**: 213-232.

Prakash, O., Nimonkar, Y., and Shouche, Y.S. (2013) Practice and prospects of microbial preservation. *FEMS Microbiol Lett* **339**: 1-9.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C*., et al.* (2010) A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**: 59-65.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F*., et al.* (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* **490**: 55-60.

Quevrain, E., Maubert, M.A., Michon, C., Chain, F., Marquant, R., Tailhades, J.*, et al.* (2016) Identification of an anti-inflammatory protein from *Faecalibacterium prausnitzii*, a commensal bacterium deficient in Crohn's disease. *Gut* **65**: 415-425.

Ramirez-Farias, C., Slezak, K., Fuller, Z., Duncan, A., Holtrop, G., and Louis, P. (2009) Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii. *Br J Nutr* **101**: 541-550.

Raskov, H., Burcharth, J., and Pommergaard, H.C. (2017) Linking gut microbiota to colorectal cancer. *J Cancer* **8**: 3378-3395.

Ray, B., and Speck, M.L. (1973) Freeze-injury in bacteria. *CRC Crit Rev Clin Lab Sci* **4**: 161-213.

Reichardt, N., Duncan, S.H., Young, P., Belenguer, A., McWilliam Leitch, C., Scott, K.P.*, et al.* (2014) Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. *ISME J* **8**: 1323-1335.

Reichardt, N., Vollmer, M., Holtrop, G., Farquharson, F.M., Wefers, D., Bunzel, M.*, et al.* (2018) Specific substrate-driven changes in human faecal microbiota composition contrast with functional redundancy in short-chain fatty acid production. *ISME J* **12**: 610-622.

Reimann, S., Grattepanche, F., Benz, R., Mozzetti, V., Rezzonico, E., Berger, B., and Lacroix, C. (2011) Improved tolerance to bile salts of aggregated *Bifidobacterium longum* produced during continuous culture with immobilized cells. *Bioresour Technol* **102**: 4559-4567.

Ridaura, V.K., Faith, J.J., Rey, F.E., Cheng, J., Duncan, A.E., Kau, A.L.*, et al.* (2013) Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* **341**: 1241214.

Rios-Covian, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de Los Reyes-Gavilan, C.G., and Salazar, N. (2016) Intestinal short chain fatty acids and their link with diet and human health. *Front Microbiol* **7**: 185.

Roncucci, L., and Mariani, F. (2015) Prevention of colorectal cancer: How many tools do we have in our basket? *Eur J Intern Med* **26**: 752-756.

Round, J.L., Lee, S.M., Li, J., Tran, G., Jabri, B., Chatila, T.A., and Mazmanian, S.K. (2011) The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* **332**: 974-977.

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., and Tuohy, K. (2018) Gut microbiota functions: metabolism of nutrients and other food components. *Eur J Nutr* **57**: 1-24.

Rudolph, A.S., and Crowe, J.H. (1985) Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. *Cryobiology* **22**: 367-377.

Ryan, M.J., and Smith, D. (2007) Cryopreservation and freeze-drying of fungi employing centrifugal and shelf freeze-drying. *Methods Mol Biol* **368**: 127-140.

Sachs, J.L., and Hollowell, A.C. (2012) The origins of cooperative bacterial communities. *MBio* **3**.

Saegeman, V.S., Ectors, N.L., Lismont, D., Verduyckt, B., and Verhaegen, J. (2008) Short- and long-term bacterial inhibiting effect of high concentrations of glycerol used in the preservation of skin allografts. *Burns* **34**: 205-211.

Saemann, M.D., Bohmig, G.A., Osterreicher, C.H., Burtscher, H., Parolini, O., Diakos, C., *et al.* (2000) Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. *FASEB J* **14**: 2380-2382.

Salonen, A., Lahti, L., Salojarvi, J., Holtrop, G., Korpela, K., Duncan, S.H., *et al.* (2014) Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. *ISME J* **8**: 2218-2230.

Sanchez, E., De Palma, G., Capilla, A., Nova, E., Pozo, T., Castillejo, G., *et al.* (2011) Influence of environmental and genetic factors linked to celiac disease risk on infant gut colonization by Bacteroides species. *Appl Environ Microbiol* **77**: 5316-5323.

Sandegren, L., Lindqvist, A., Kahlmeter, G., and Andersson, D.I. (2008) Nitrofurantoin resistance mechanism and fitness cost in *Escherichia coli*. *J Antimicrob Chemother* **62**: 495-503.

Satokari, R., Mattila, E., Kainulainen, V., and Arkkila, P.E. (2015) Simple faecal preparation and efficacy of frozen inoculum in faecal microbiota transplantation for recurrent *Clostridium difficile* infection--an observational cohort study. *Aliment Pharmacol Ther* **41**: 46-53.

Satokari, R., Mattila, E., Kainulainen, V., and Arkkila, P.E. (2015) Simple faecal preparation and efficacy of frozen inoculum in faecal microbiota transplantation for recurrent *Clostridium difficile* infection - an observational cohort study. *Aliment Pharmacol Ther* **41**: 46-53.

Schaffler, H., and Breitruck, A. (2018) *Clostridium difficile* - From Colonization to Infection. *Front Microbiol* **9**: 646.

Schepers, A.W., Thibault, J., and Lacroix, C. (2006) Continuous lactic acid production in whey permeate/yeast extract medium with immobilized *Lactobacillus helveticus* in a two-stage process: Model and experiments. *Enzyme Microb Technol* **38**: 324-337.

Schwab, C., Vogel, R., and Ganzle, M.G. (2007) Influence of oligosaccharides on the viability and membrane properties of *Lactobacillus reuteri* TMW1.106 during freeze-drying. *Cryobiology* **55**: 108-114.

Scott, K.P., Duncan, S.H., Louis, P., and Flint, H.J. (2011) Nutritional influences on the gut microbiota and the consequences for gastrointestinal health. *Biochem Soc Trans* **39**: 1073-1078.

Segain, J.P., Raingeard de la Bletiere, D., Bourreille, A., Leray, V., Gervois, N., Rosales, C., *et al.* (2000) Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut* **47**: 397-403.

Selber-Hnatiw, S., Rukundo, B., Ahmadi, M., Akoubi, H., Al-Bizri, H., Aliu, A.F., *et al.* (2017) Human gut microbiota: toward an ecology of disease. *Front Microbiol* **8**: 1265.

Silvester, K.R., Englyst, H.N., and Cummings, J.H. (1995) Ileal recovery of starch from whole diets containing resistant starch measured in vitro and fermentation of ileal effluent. *Am J Clin Nutr* **62**: 403-411.

Simpson, P.J., Stanton, C., Fitzgerald, G.F., and Ross, R.P. (2005) Intrinsic tolerance of Bifidobacterium species to heat and oxygen and survival following spray drying and storage. *J Appl Microbiol* **99**: 493-501.

Smith, E.A., and Macfarlane, G.T. (1997) Dissimilatory amino acid metabolism in human colonic bacteria. *Anaerobe* **3**: 327-337.

Smith, E.A., and Macfarlane, G.T. (1998) Enumeration of amino acid fermenting bacteria in the human large intestine: effects of pH and starch on peptide metabolism and dissimilation of amino acids. *FEMS Microbiol Ecol* **25**: 355-368.

Sobhani, I., Tap, J., Roudot-Thoraval, F., Roperch, J.P., Letulle, S., Langella, P.*, et al.* (2011) Microbial dysbiosis in colorectal cancer (CRC) patients. *PLoS One* **6**: e16393.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L.G., Gratadoux, J.J.*, et al.* (2008) *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* **105**: 16731-16736.

Sokol, H., Seksik, P., Furet, J.P., Firmesse, O., Nion-Larmurier, I., Beaugerie, L.*, et al.* (2009) Low counts of Faecalibacterium prausnitzii in colitis microbiota. *Inflamm Bowel Dis* **15**: 1183-1189.

Song, Y., Liu, C., and Finegold, S.M. (2007) *Peptoniphilus gorbachii* sp. nov., *Peptoniphilus olsenii* sp. nov., and *Anaerococcus murdochii* sp. nov. isolated from clinical specimens of human origin. *J Clin Microbiol* **45**: 1746-1752.

Sonnenburg, J.L., Angenent, L.T., and Gordon, J.I. (2004) Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? *Nat Immunol* **5**: 569-573.

Squires, R.W., and Hartsell, S.E. (1955) Survival and growth initiation of defrosted *Escherichia coli* as affected by frozen storage menstrua. *Appl Microbiol* **3**: 40-45.

Staab, J.A., and Ely, J.K. (1987) Viability of lyophilized anaerobes in two media. *Cryobiology* **24**: 174-178.

Strasser, S., Neureiter, M., Geppl, M., Braun, R., and Danner, H. (2009) Influence of lyophilization, fluidized bed drying, addition of protectants, and storage on the viability of lactic acid bacteria. *J Appl Microbiol* **107**: 167-177.

Sun, X., Fiala, J.L., and Lowery, D. (2016) Patent watch: Modulating the human microbiome with live biotherapeutic products: intellectual property landscape. *Nat Rev Drug Discov* **15**: 224-225.

Sung, J., Kim, S., Cabatbat, J.J.T., Jang, S., Jin, Y.S., Jung, G.Y.*, et al.* (2017) Global metabolic interaction network of the human gut microbiota for context-specific community-scale analysis. *Nat Commun* **8**: 15393.

Sutton, S. (2010) The most probable number method and its uses in enumeration, qualification, and validation. *J Valid Technol* **16**: 35-38.

Tamanai-Shacoori, Z., Smida, I., Bousarghin, L., Loreal, O., Meuric, V., Fong, S.B.*, et al.* (2017) *Roseburia* spp.: a marker of health? *Future Microbiol* **12**: 157-170.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A.N., Mackay, C.R., and Macia, L. (2014) The role of short-chain fatty acids in health and disease. *Adv Immunol* **121**: 91-119.

Tao, D.L., and Li, P.H. (1986) Classification of plant-cell cryoprotectants. *J Theor Biol* **123**: 305-310.

Teixeira, P.C., Castro, M.H., Malcata, F.X., and Kirby, R.M. (1995) Survival of *Lactobacillus delbrueckii* ssp *bulgaricus* following spray-Drying. *J Dairy Sci* **78**: 1025-1031.

Teng, D., Kawai, K., Mikajiri, S., and Hagura, Y. (2017) Stabilization of freeze-dried *Lactobacillus paracasei* subsp. *paracasei* JCM 8130T with the addition of disaccharides, polymers, and their mixtures. *Biosci Biotechnol Biochem* **81**: 768-773.

Terveer, E.M., van Beurden, Y.H., Goorhuis, A., Seegers, J., Bauer, M.P., van Nood, E.*, et al.* (2017) How to: Establish and run a stool bank. *Clin Microbiol Infect* **23**: 924-930.

Theriot, C.M., Koenigsknecht, M.J., Carlson, P.E., Jr., Hatton, G.E., Nelson, A.M., Li, B.*, et al.* (2014) Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to *Clostridium difficile* infection. *Nat Commun* **5**: 3114.

Theriot, C.M., Bowman, A.A., and Young, V.B. (2016) Antibiotic-Induced alterations of the gut microbiota alter secondary bile acid production and allow for *Clostridium difficile* spore germination and outgrowth in the large intestine. *Msphere* **1**.

To, B.C.S., and Etzel, M.R. (1997) Spray drying, freeze drying, or freezing of three different lactic acid bacteria species. *J Food Sci* **62**: 576-&.

Troy, E.B., and Kasper, D.L. (2010) Beneficial effects of *Bacteroides fragilis* polysaccharides on the immune system. *Front Biosci (Landmark Ed)* **15**: 25-34.

Türetgen, I., Ilhan-Sungur, E., and Cotuk, A. (2006) Effects of short term drying on biofilm associated bacteria. *Ann Microbiol* **57**: 277-280.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E.*, et al.* (2009) A core gut microbiome in obese and lean twins. *Nature* **457**: 480-U487.

Tvede, M., and Rask-Madsen, J. (1989) Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. *Lancet* **1**: 1156-1160.

Udayappan, S., Manneras-Holm, L., Chaplin-Scott, A., Belzer, C., Herrema, H., Dallinga-Thie, G.M.*, et al.* (2016) Oral treatment with *Eubacterium hallii* improves insulin sensitivity in db/db mice. *NPJ Biofilms Microbiomes* **2**: 16009.

Van den Abbeele, P., Roos, S., Eeckhaut, V., MacKenzie, D.A., Derde, M., Verstraete, W.*, et al.* (2012) Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. *Microb Biotechnol* **5**: 106-115.

van der Beek, C.M., Bloemen, J.G., van den Broek, M.A., Lenaerts, K., Venema, K., Buurman, W.A., and Dejong, C.H. (2015) Hepatic Uptake of Rectally Administered Butyrate Prevents an Increase in Systemic Butyrate Concentrations in Humans. *J Nutr* **145**: 2019-2024.

Van Immerseel, F., Ducatelle, R., De Vos, M., Boon, N., Van De Wiele, T., Verbeke, K.*, et al.* (2010) Butyric acid-producing anaerobic bacteria as a novel probiotic treatment approach for inflammatory bowel disease. *J Med Microbiol* **59**: 141-143.

Van Nevel, S., Koetzsch, S., Weilenmann, H.U., Boon, N., and Hammes, F. (2013) Routine bacterial analysis with automated flow cytometry. *J Microbiol Methods* **94**: 73-76.

van Nood, E., Vrieze, A., Nieuwdorp, M., Fuentes, S., Zoetendal, E.G., de Vos, W.M.*, et al.* (2013) Duodenal infusion of donor feces for recurrent Clostridium difficile. *N Engl J Med* **368**: 407-415.

Vereyken, I.J., van Kuik, J.A., Evers, T.H., Rijken, P.J., and de Kruijff, B. (2003) Structural requirements of the fructan-lipid interaction. *Biophys J* **84**: 3147-3154.

Verma, A., and Shukla, G. (2013) Administration of prebiotic inulin suppresses 1,2 dimethylhydrazine dihydrochloride induced procarcinogenic biomarkers fecal enzymes and preneoplastic lesions in early colon carcinogenesis in Sprague Dawley rats. *J Funct Foods* **5**: 991-996.

Vermeire, S., Joossens, M., Verbeke, K., Wang, J., Machiels, K., Sabino, J.*, et al.* (2016) Donor species richness determines faecal microbiota transplantation success in inflammatory bowel disease. *J Crohns Colitis* **10**: 387-394.

Vieira, A.T., Fukumori, C., and Ferreira, C.M. (2016) New insights into therapeutic strategies for gut microbiota modulation in inflammatory diseases. *Clin Transl Immunology* **5**: e87.

Vital, M., Penton, C.R., Wang, Q., Young, V.B., Antonopoulos, D.A., Sogin, M.L.*, et al.* (2013) A gene-targeted approach to investigate the intestinal butyrate-producing bacterial community. *Microbiome* **1**: 8.

Vogt, L., Ramasamy, U., Meyer, D., Pullens, G., Venema, K., Faas, M.M.*, et al.* (2013) Immune modulation by different types of beta 2 -> 1-fructans Is Toll-like receptor dependent. *PLoS One* **8**.

Vrieze, A., Van Nood, E., Holleman, F., Salojarvi, J., Kootte, R.S., Bartelsman, J.F.*, et al.* (2012) Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* **143**: 913-916 e917.

Walker, A.W., Duncan, S.H., Harmsen, H.J., Holtrop, G., Welling, G.W., and Flint, H.J. (2008) The species composition of the human intestinal microbiota differs between particle-associated and liquid phase communities. *Environ Microbiol* **10**: 3275-3283.

Walsh, C.J., Guinane, C.M., O'Toole, P.W., and Cotter, P.D. (2014) Beneficial modulation of the gut microbiota. *FEBS Lett* **588**: 4120-4130.

Wang, T.T., Cai, G.X., Qiu, Y.P., Fei, N., Zhang, M.H., Pang, X.Y.*, et al.* (2012) Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J* **6**: 320-329.

Wang, X., Kang, Y., Luo, C., Zhao, T., Liu, L., Jiang, X.*, et al.* (2014) Heteroresistance at the single-cell level: adapting to antibiotic stress through a population-based strategy and growth-controlled interphenotypic coordination. *MBio* **5**: e00942-00913.

Watnick, P., and Kolter, R. (2000) Biofilm, city of microbes. *J Bacteriol* **182**: 2675-2679.

Welch, J.L.M., Hasegawa, Y., McNulty, N.P., Gordon, J.I., and Borisy, G.G. (2017) Spatial organization of a model 15-member human gut microbiota established in gnotobiotic mice. *Proc Natl Acad Sci U S A* **114**: E9105-E9114.

Weng, L., Chen, C., Zuo, J., and Li, W. (2011) Molecular dynamics study of effects of temperature and concentration on hydrogen-bond abilities of ethylene glycol and glycerol: implications for cryopreservation. *J Phys Chem A* **115**: 4729-4737.

Wessman, P., Mahlin, D., Akhtar, S., Rubino, S., Leifer, K., Kessler, V., and Hakansson, S. (2011) Impact of matrix properties on the survival of freeze-dried bacteria. *J Sci Food Agric* **91**: 2518-2528.

Williams, B.A., Bosch, M.W., Awati, A., Konstantinov, S.R., Smidt, H., Akkermans, A.D.L.*, et al.* (2005) In vitro assessment of gastrointestinal tract (GIT) fermentation in pigs: Fermentable substrates and microbial activity. *Animal Research* **54**: 191-201.

Williams, B.A., Grant, L.J., Gidley, M.J., and Mikkelsen, D. (2017) Gut Fermentation of dietary fibres: Physico-chemistry of plant cell walls and implications for health. *Int J Mol Sci* **18**.

Willing, B.P., Dicksved, J., Halfvarson, J., Andersson, A.F., Lucio, M., Zheng, Z.*, et al.* (2010) A Pyrosequencing Study in Twins Shows That Gastrointestinal Microbial Profiles Vary With Inflammatory Bowel Disease Phenotypes. *Gastroenterology* **139**: 1844-U1105.

Wrzosek, L., Miquel, S., Noordine, M.L., Bouet, S., Joncquel Chevalier-Curt, M., Robert, V., *et al.* (2013) *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii* influence the production of mucus glycans and the development of goblet cells in the colonic epithelium of a gnotobiotic model rodent. *BMC Biol* **11**: 61.

Wu, G.D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.Y., Keilbaugh, S.A., *et al.* (2011) Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**: 105-108.

Yang, J.Y., Lee, Y.S., Kim, Y., Lee, S.H., Ryu, S., Fukuda, S., *et al.* (2017) Gut commensal *Bacteroides acidifaciens* prevents obesity and improves insulin sensitivity in mice. *Mucosal Immunol* **10**: 104-116.

Yao, C.K., Muir, J.G., and Gibson, P.R. (2016) Review article: insights into colonic protein fermentation, its modulation and potential health implications. *Aliment Pharmacol Ther* **43**: 181-196.

Youngster, I., Sauk, J., Pindar, C., Wilson, R.G., Kaplan, J.L., Smith, M.B., *et al.* (2014) Fecal microbiota transplant for relapsing Clostridium difficile infection using a frozen inoculum from unrelated donors: a randomized, open-label, controlled pilot study. *Clin Infect Dis* **58**: 1515-1522.

Youngster, I., Mahabamunuge, J., Systrom, H.K., Sauk, J., Khalili, H., Levin, J., *et al.* (2016) Oral, frozen fecal microbiota transplant (FMT) capsules for recurrent *Clostridium difficile* infection. *BMC Med* **14**: 134.

Yu, J., Feng, Q., Wong, S.H., Zhang, D., Liang, Q.Y., Qin, Y.W., *et al.* (2017) Metagenomic analysis of faecal microbiome as a tool towards targeted non-invasive biomarkers for colorectal cancer. *Gut* **66**: 70-+.

Zaibi, M.S., Stocker, C.J., O'Dowd, J., Davies, A., Bellahcene, M., Cawthorne, M.A., *et al.* (2010) Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. *FEBS Lett* **584**: 2381-2386.

Zambell, K.L., Fitch, M.D., and Fleming, S.E. (2003) Acetate and butyrate are the major substrates for de novo lipogenesis in rat colonic epithelial cells. *J Nutr* **133**: 3509-3515.

Ze, X., Duncan, S.H., Louis, P., and Flint, H.J. (2012) *Ruminococcus bromii* is a keystone species for the degradation of resistant starch in the human colon. *ISME J* **6**: 1535-1543.

Zhang, X., Shen, D., Fang, Z., Jie, Z., Qiu, X., Zhang, C., *et al.* (2013) Human gut microbiota changes reveal the progression of glucose intolerance. *PLoS One* **8**: e71108.

Zhu, C., Song, K., Shen, Z., Quan, Y., Tan, B., Luo, W., *et al.* (2018) *Roseburia intestinalis* inhibits interleukin17 excretion and promotes regulatory T cells differentiation in colitis. *Mol Med Rep*10.3892/mmr.2018.8833.

Zihler Berner, A., Fuentes, S., Dostal, A., Payne, A.N., Vazquez Gutierrez, P., Chassard, C., *et al.* (2013) Novel Polyfermentor intestinal model (PolyFermS) for controlled ecological studies: Validation and effect of pH. *PLoS One* **8**: e77772.

Zou, S., Fang, L., and Lee, M.H. (2018) Dysbiosis of gut microbiota in promoting the development of colorectal cancer. *Gastroenterol Rep (Oxf)* **6**: 1-12.

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