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Cryopreservation of the human gut microbiota: Current state and perspectives

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ABSTRACT

The human intestinal microbiota is a complex ecosystem that consists of thousands of bacterial species that are responsible for human health and disease. The intestinal microbiota is a natural resource for production of therapeutic and preventive medicals, such as probiotics and fecal transplants. Modern lifestyles have resulted in the extinction of evolutionally selected microbial populations upon exposure to environmental factors. Therefore, it is very important to preserve the human gut microbiota to have the opportunity for timely restoration with minimal safety risks. Cryopreservation techniques that are suitable for the preservation of viable, mixed microbial communities and a biobanking approach are currently under development in different countries. However, the number of studies in this area is very limited. The variety of morphological and physiological characteristics of microbes in the microbiota, the different cryopreservation goals, and the criteria for the evaluation of cryopreservation effectiveness are the main challenges in the creation of a universal and standardized cryopreservation protocol. In this review, we summarized the current progress of the main cryopreservation techniques for gut microbiota communities and the methods for the assessment of the effectiveness of these techniques in the context of practical application.

1. Introduction

For many years, the microbiota of the human gastrointestinal tract, especially of the large intestine, is of prime importance because of its great impact on the homeostasis of the human body (Jandhyala et al., 2015; Luna and Foster, 2015; O'Hara and Shanahan, 2006). There are two main scientific directions related to the human gut microbiota: 1) the development of precise and highly effective molecular genetic methods for identifying the composition of microbial communities in fecal samples (polymerase chain reaction (PCR) and metagenomic studies) (Vandeputte et al., 2017) and its functional analysis (proteomic and metabolic studies) (Chaplin et al., 2015), and 2) the findings of direct correlations between changes in the balance of the intestinal microbiota and human diseases (Kerckhof et al., 2014; Poluektova et al., 2014; Possemiers et al., 2009; Read et al., 2011; Van den Abbeele et al., 2013). In addition, many studies have investigated the communication mechanisms between the intestinal microbiota and the host physiology and have explored the effects of various factors on the microbiome composition.

The modern lifestyle results in a reduction of the core microbiome diversity (Blaser and Falkow, 2009) in response to environmental factors (Il'in et al., 2013), urbanization, industrialization processes, changes in dietary habits, excessive and/or irrational antibiotic use (Jakobsson et al., 2010), daily stresses (Bailey et al., 2011), formula feeding (Benson et al., 2010; Spor et al., 2011), the necessity to work in "artificial" habitats (submarines, space stations) (Il'in et al., 2013; Saei and Barzegari, 2012), etc. In addition, these factors can cause changes in the type and functionality of bacteria and can "average" the microbiome from different enterotypes, which affects the resilience of the microbiome (Kau et al., 2011; Levine and D'Antonio, 1999; Vieira et al., 2013). Thus, humans can lose evolutionarily selected microbial populations that conferred benefits to them and those that maintained the fitness of both the individual host and the population as a whole (Blaser and Falkow, 2009; Van den Abbeele et al., 2011).

Abbreviations: CDI, *Clostridium difficile* infection; CPA, cryoprotectant additive; DMSO, dimethyl sulfoxide; ECSIM, environmental control system for intestinal microbiota; FMT, fecal microbiota transplantation; NS, sterile normal saline OTUs, operational taxonomic units; PEG, polyethylene glycol; PCR, polymerase chain reaction; SCFA, short-chain fatty acids

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Therefore, it is very important to preserve microbial communities, in particular, the human gut microbiota, to use these communities as sources of restoration materials. Thus, it is necessary to develop biopreservation techniques suitable for complicated microbial communities and a biobanking approach, which is currently in progress in different countries (Barzegari et al., 2014b; Prakash et al., 2013; Terveer et al., 2017). In this review, we provide an overview of the current status quo of the main cryopreservation techniques for gut microbiota communities and methods for the assessment of the effectiveness of these techniques in the context of practical application.

2. Main objectives for the gut microbiota cryopreservation for practical needs

The intestinal microbiota is a valuable biological material that can be used for a wide range of research, biomedical, ecological and technological objectives. In this case, the intestinal microbiota could be a natural source of therapeutic and preventive resources.

2.1. Preservation of biodiversity

The development of microbial identification and cultivation methods (Forster et al., 2019; Zou et al., 2019) and the progress in understanding the mechanisms of microbiota or its separate components and the host communications make it possible to reveal: 1) new species (for example, some recently isolated microorganisms are prospective candidates for use as probiotics or food ingredients according to European Union regulations include *Bacteroides xylanisolvens, Akkermansia muciniphila*, fructophilic lactic acid bacteria, *Faecalibacterium prausnitzii*, etc.) (Brodmann et al., 2017; Martin et al., 2017); and 2) whole communities with probiotic properties. For example, strict anaerobic butyrate-producing bacteria (Tamanai-Shacoori et al., 2017; Udayappan et al., 2016; Van Immerseel et al., 2010) and propionate producing bacteria (El Hage et al., 2017) are considered to become the next generation of probiotics since the beneficial effects of short-chain fatty acids (SCFA) on human health have been confirmed in different studies (Tan et al., 2014). Moreover, new strains of already used probiotic cultures with higher therapeutic potentials could be identified without the use of genetic engineering techniques (Ermolenko et al., 2010; Suvorov et al., 2003). For example, different beneficial properties could be enhanced such as, antipathogenic activity, adhesion and colonization abilities, acid and bile tolerance, fermentation ability or the production of large amounts of valuable metabolites, such as SCFA, etc. This is extremely important for countries where the use of genetic engineering products is limited due to legislation. In this case, cryobanks that preserve the diversity of natural microbiocenosis can be considered a depot of unique, natural biotechnological and therapeutic resources, which should be preserved while they have not disappeared, and are able to be used to restore the specific and functional homeostasis of the human gut (Shenderov et al., 2014). The maximal variety of all microbiota components in a viable state is a strict requirement for the cryopreservation protocols of such cryobanks.

2.2. Therapeutic issues

There are two main techniques for gut microbiota applications in the therapies of different diseases that are associated with changes in the microbiota composition: 1) the transplantation of communities as a whole or their parts that are capable of restoring the disturbed homeostasis of a damaged biotope and 2) the administration of individual probiotic strains and/or their combinations with enhancing additives (Daliri et al., 2018). Due to the progress in understanding the key mechanisms of the human-microbiota interactions, it becomes possible to develop strategies for targeted manipulation of the microbiota for the patient's benefit and to carry out this process with minimal risks. The main features of the cryopreservation protocols for therapeutic applications are strict requirements for standardization and quality control for each manipulation.

2.2.1. Fecal microbiota transplantation

Since the pathogenesis of some inflammatory and immune diseases, diabetes, obesity, and certain cancers (Kamada et al., 2013; Matsuki and Tanaka, 2014; Sekirov et al., 2010; Young et al., 2011) is associated with functional and compositional changes in the intestinal microbiota, for example, a reduction in microbial diversity (Fuentes et al., 2017; Kumari et al., 2013; Sokol et al., 2009), then a fecal microbiota transplantation (FMT) from a healthy donor to a patient is one of the most effective treatment strategies to restore the balance of microorganisms.

The high therapeutic potential of microbiota transplantation has been demonstrated in the treatment of clostridial infections (Kelly et al., 2016; Lee et al., 2016; van Nood et al., 2013), inflammatory intestinal diseases (Vermeire et al., 2016), diabetes and obesity (de Groot et al., 2017). Recently, in a very small cohort, the high potential of FMT was demonstrated for the treatment of refractory immune checkpoint inhibitor-associated colitis (Wang et al., 2018).

Despite the high therapeutic efficiency of FMT ($>$ 90%) in antibiotic-associated diarrhea treatment, safety issues, high costs, problems with the timely availability of appropriate fecal transplants and issues with sanitation and aesthetics limit its use (Bojanova and Bordenstein, 2016; König et al., 2017; Mattner et al., 2016). Strict inclusion/exclusion criteria for donor selection (Alang and Kelly, 2015; Petrof and Khoruts, 2014), donor universalization (replacement related by nonrelated stool donors who produce multiple stool samples) (Hamilton et al., 2012; Orenstein et al., 2016; Ramai et al., 2019; Satokari et al., 2015; Youngster et al., 2014b), and the development of biobanking are currently used to make this procedure more accessible. The last approach has been of particular interest since the year 2012 (Barzegari et al., 2014b; Terveer et al., 2017). Prolonged storage allows for less frequent donor recruitment and screening and reduces the processing time and potential delay period before FMT. In addition, this may allow for the preservation of samples from large clinical studies and their shared use among laboratories by reference collection centers (Gaci et al., 2017). The ability to store stool in frozen stool banks makes their ability to function less resource intensive, more economical and, in the future, may provide a personalized approach: the opportunity for the choice of a suitable fecal transplant according to medical recommendations. All of the abovementioned studies show the necessity for efficient, standardized and validated methods for fecal transplant storage.

2.2.2. In vitro gut microbiota production for FMT

Although FMT has been an effective treatment for recurrent *C. difficile* infections, the long-term effects of FMT are still unknown (Bojanova and Bordenstein, 2016; Daliri et al., 2018). To minimize the potential risks associated with the transfer of bacterial communities from the donor to the recipient, various modifications of fecal transplantation based on the use of the feces fractions are under development (Bojanova and Bordenstein, 2016). For instance, sterile fecal filtrate (Ott et al., 2017) or, in contrast, bacterial suspensions can be used (Dubberke et al., 2016; Jones et al., 2016; Orenstein et al., 2016). Thus, transplants that consist of 33 bacterial strains isolated from the fecal material of the donor (Petrof et al., 2013) or from a mixture of 17 butyrate-producing strains of the genus Clostridium are offered as fecal-transplant stool substitutions (Mathewson et al., 2016). The other examples of “artificial” gut microbiota are the variants obtained under *in vitro* conditions using an inoculum of the donor material and various fermentation technologies, such as the intestinal fermentation technology (IFT) (Bircher et al., 2018b), TNO Intestinal Model (TIM-2) (Aguirre et al., 2015), continuous fermentation system or gut-simulating Environmental Control System for Intestinal Microbiota (P-ECSIM) (Gaci et al., 2017). According to the published data, although such technologies enable some control in the production of a diverse and metabolically active intestinal microbiota, the composition of the final product can differ both in diversity and in the microorganism proportion in comparison with fresh, natural human feces (Bircher et al., 2018b). Publications in this field are limited to the description of studies involving single donors and single samples and are related to the optimization of the production of standardized inocula (Gaci et al., 2017; Kerckhof et al., 2014) as well as long-term storage protocols for these inocula.

2.2.3. Personalized probiotics approach

In fact, the individual response of the patient to the probiotic or FMT treatment remains a key point in the efficiency of microbiota restoration, and it often remains unpredictable. The use of heterologous bacteria, even those that meet the necessary requirements (for example, generally regarded as safe status), may cause infectious processes or lead to side effects, such as metabolic disorders; excessive immunostimulation of the intestinal lymphatic system; and the formation of new bacterial strains with new pathogenic determinants through horizontal gene transfer (Barzegari et al., 2014a; Daliri et al., 2018; Ermolenko et al., 2010).

Taking into account all of the abovementioned issues, the relatively new therapeutic approach of microbiota applications can be outlined. It refers to the expansion of personalized medical capacity. The main idea is based on the preservation of indigenous microbiota that are collected from a young age, the period of maximum diversity. Such samples could be used as a source of personalized probiotics (autoprobiotics) and, if necessary, as a safe and highly effective material for autologous FMT (Ermolenko et al., 2010; Suez et al., 2018). According to some authors, the main advantage of autoprobiotics is the higher affinity for the biofilm and mucous membrane of the large intestine and the lower immune response of the patient (Ermolenko et al., 2018, 2016). In this case, the goal of cryopreservation is the same as that of the biodiversity approach: the preservation of maximal species and strain diversity of microbiota in its initial proportions. The last requirement is an important point in contrast with axenic culture preservation, where the quality of preservation is not limited by the concentration of viable cells due to its further amplification via cultivation in the selective media.

The storage time of cryopreservation is also an important parameter. If a time range of several weeks to several years is acceptable for therapeutic use, than for the cryopreservation of biodiversity and personalized resources, it is necessary to focus on years and decades.

The tasks listed have a different level of complexity and require different approaches to their implementation. The main progress is achieved in the preservation techniques of microbiota for FMT, and we are focused on these works in our review.

3. Cryopreservation of microbial communities

The main challenge in microbiota cryopreservation is its composition heterogeneity. The development of this approach is based on the systematization of knowledge and the adaptation of protocols, which are optimized for the microbiota components - pure microbial cultures.

3.1. Lessons from pure culture cryopreservation techniques

The critical factors in the cryopreservation of bacterial cells are as follows: (1) the rate of freezing and thawing, (2) the composition of the cryoprotective medium, (3) the type and concentration of the cryoprotectant, (4) the morphology and species-specific cell properties, and (5) their physiological state at the time of freezing (Fonseca et al., 2003, 2006; Hubalek, 2003; Smith and Ryan, 2012).

For pure microbial cultures, slow programmable freezing is mainly used. Various protocols are developed that take into account the following differences in bacterial morphofunctional characteristics: shape, size, density, water and lipid content, and the permeability of cell membranes and cell walls to water and other chemicals (Dumont et al., 2004; Fowler and Toner, 2005; Heckly, 1978; Hubalek, 2003; Kirsop and Doyle, 1991; Mazur, 2004). However, in some works, the authors use flash freezing (> 100 °C/min), which is achieved by sample immersion into liquid nitrogen (Fonseca et al., 2001, 2003; Fonseca et al., 2006; Novik et al., 2009). The last mentioned approach could be suitable for different types of cells with the appropriate addition of cryoprotective additives to minimize cell injuries. Some authors suggest that the intermediate cooling rates are harmful to the cell cultures (Dumont et al., 2004; Smith et al., 2008). According to various data, it can be concluded that the storage temperature should be below −80 °C to avoid water recrystallization and other processes that are not stopped under higher temperatures (Prakash et al., 2013). Thawing is usually carried out in a water bath at +37 °C.

For the cryopreservation of bacterial microorganisms, glycerol and dimethyl sulfoxide (DMSO) are the most frequently used penetrating cryoprotectant additive (CPA). Their addition at concentrations of 5–15% w/w reduces the negative freezing effects (Hubalek, 2003).

Nonpenetrating cryoprotectants, such as different saccharides (trehalose (Kerckhof et al., 2014), sucrose (Bircher et al., 2018a), inulin (Bircher et al., 2018b)), gelatin, mucin, levan (Shenderov et al., 1998), and polyethylene glycol (PEG) (Bircher et al., 2018a) are used for pure cultures to reduce ice formation, stabilize the membrane lipids and, in addition, provide nutritional support at the resuscitation stage.

Some antioxidants, such as cysteine and riboflavin, can be used to facilitate the survival of strict anaerobes (Bircher et al., 2018a; Khan et al., 2012, 2014). Combinations of these additives with inulin-type fructans were applied to protect the butyrate-producing *Bacteroides thetaiotaomicron, Faecalibacterium prausnitzii, Roseburia intestinalis, Anaerostipes caccae, Eubacterium hallii* and *Blautia obeum* from oxidative stress upon exposure to air and the cryopreservation process (Bircher et al., 2018a). Moreover, the prebiotic properties of riboflavin (Steinert et al., 2016) could be beneficial at the resuscitation stage. In addition, to support anaerobes, some authors recommend removing oxygen from the cryoprotective medium by boiling while flushing with CO_2 (Bircher et al., 2018a).

3.2. Cryopreservation of feces samples

To date, cryopreservation at −80 °C with the addition of glycerol as a cryoprotective additive is the main way to preserve the microbiota in fecal samples. Various studies have been conducted in which the efficacy (cure rate) of fresh and frozen (samples obtained after cryopreservation and restoration procedures, Table 1.) fecal transplants were compared (Costello et al., 2015; Hamilton et al., 2012; Satokari et al., 2015; Youngster et al., 2014b). Therefore, Hamilton et al established a standard protocol for the collection and preservation of fecal microbiota in a frozen state. The standard protocol included the following several steps: homogenization of 50 g of fecal material in 250 ml of sterile normal saline, filtration through sieves, centrifugation, resuspension in nonbacteriostatic normal saline with the addition of glycerol to a final concentration of 10%, storage at −80 °C for 1–8 weeks in the frozen state and thawing the samples at 0 °C for 2–4 h. All manipulations were performed under N_2 atmosphere to maintain anaerobic communities (Hamilton et al., 2012). They showed that the clinical efficacy of frozen preparation (cure rate, 90%) was similar to fresh fecal samples for *Clostridium difficile* infection (CDI) treatment. The same protocol was successfully used by Youngster, I. et al. They observed a high cure rate (90%) using different methods of administration of frozen preparation: nasogastric tube and colonoscopy (Youngster et al., 2014b). A similar protocol was used by Satokari et al. (Satokari et al., 2015), who compared CDI treatment via colonoscopic administration of frozen samples from individual and universal donors (cure rate 96%). In these works, the cure rate was detected as a release from CDI syndromes (diarrhea, etc.) and the *Clostridium difficile* toxin. In more recent studies, the cure rate was confirmed by the microbial composition analysis of the donor material and patient feces before and

Table 1
Main cryopreservation methods of fecal transplants.

Atmosphere for manipulation	homogenization	filtration through sieves/ centrifu-gation, resuspension	Cryoprotectant additive	$T_{corservation}/T_{thawing}$, thawing duration	Storage period	Number of patients, Type of fecal transplant	Compositional microbiota analysis, markers for cure rate identification	Reference
N_2	Feces: NS (1:5)	+/6000 g	10% glycerol	−80 °C/0 °C 2-4h	1-8 weeks	21 frozen/21 fresh	No, *C. difficile* toxin	(Hamilton et al., 2012)
Ambient air	Feces: NS (1:5)	+/6000 g	10% glycerol	−80 °C/37 °C	1-3 months	20 frozen	No, *C. difficile* toxin	(Youngster et al., 2014b)
Ambient air	Feces: NS (n/d)	+/+	10% glycerol	−80 °C/−20 °C 1-2 h	1-8,5 months	20 frozen capsules	No, *C. difficile* toxin	(Youngster et al., 2014a)
N_2 (85%) + H_2 (10%) + CO_2 (5%)	Feces: glycerol: NS (2:1:7)	-/-	10% glycerol	−80 °C/RT, 3h	2-10 months	16 frozen	Cultivation analysis	(Costello et al., 2015)
n/d	Feces: NS: glycerol solution (3:15:2)	filtration if necessary/-	10% glycerol	−80 °C /RT or 37 °C 4-5h	16 weeks	23frozen/26 fresh	No, *C. difficile* toxin	(Satokari et al., 2015)
n/d	Feces: NS (n/d)	-/200 g and 4600 g.	15% glycerol	−80 °C- > −20 °C up to 6 weeks/n/d	1-3 weeks - > 6 weeks	19 frozen capsules	No, *C. difficile* toxin	(Hirsch et al., 2015)
n/d	Feces: water (1:3)	n/d	No	−20 °C/25 °C overnight	< 1 month	108 frozen/111 fresh	No, *C. difficile* toxin	(Lee et al., 2016)
n/d	Feces: NS + PEG (1:3)	n/d	PEG 3350	−80 °C/n/d	n/d	34 frozen	No, *C. difficile* toxin	(Orenstein et al., 2016)
Ambient air	Feces: NS (n/d)	+/+	10% glycerol	−80 °C/37 °C	6 months	18 frozen capsules	No, *C. difficile* toxin	(Youngster et al., 2016)
n/d	Feces: NS (1:3)	+/- +/+	10% glycerol ˜17% glycerol before centrifugation	−70 °C/4 °C overnight **Note:** material for capsules were flash frozen at -55 °C	2 months	59 frozen 57 frozen capsules	No, *C. difficile* toxin	(Kao et al., 2017)
n/d	Feces: NS (1:10)	+/-	No	−80 °C/37 °C	6 months	24 frozen/25 fresh	Bacterial taxonomy (16S rRNA sequencing), *C. difficile* toxin	(Jiang et al., 2017)
n/d	Feces: NS (1:5)	+/+	Yes, non-specified	−80 °C/4 °C	n/d	34 frozen	Bacterial taxonomy (16S rRNA sequencing), *C. difficile* toxin	(Jiang et al., 2018)
n/d	Feces: NS (1:10)	+/-	No	−80 °C/n/d	6 months	2 frozen	Bacterial taxonomy (16S rRNA sequencing)	(Wang et al., 2018)

after FMT at different time points (Jiang et al., 2017, 2018; Wang et al., 2018). This type of microbiome analysis allows us to compare the changes in microbiota in response to the administered FMT product. However, it is important to note that despite the confirmation of the high efficacy of the treatment, there were no data on the viability of stool bacteria before and after the cryopreservation procedure, which are main characteristics of successful fecal transplants preparation.

Investigation of the effect of prolonged freezing storage both on the efficacy of treatment (cure rate for recurrent CDI was 88%) and on the viability of microorganisms was performed by Costello (Costello et al., 2015). The authors demonstrated the high viability of six cultivable bacterial groups (Bifidobacteria, *E. coli*, total coliforms, Lactobacilli, total anaerobic bacteria, and total aerobes) after 2 and 6 months of storage at −80 °C in 10% glycerol in comparison with the viability of microorganisms in samples stored in normal saline (NS) without cryoprotectants. The viability of microorganisms stored in glycerol was varied from 44% to 132% for total aerobes and for Bifidobacteria respectively, while cryopreservation in sterile normal saline (NS) showed a significant reduction of total coliforms (4%), *E. coli* (11%) and Lactobacilli (10%) after 6 months of storage. Costello et al. chose anaerobes and aerobes as they all cover the breadth of the bacteria present in stool. *E. coli* and coliforms were analyzed as commonly cultured bacteria and bacteria that are relevant to human health (Luo et al., 2011). Lactobacilli and Bifidobacteria were assessed because they are often used and promoted as probiotics. In addition, increases in their numbers in the stool are generally regarded as beneficial (Magill et al., 2014; Mattila et al., 2012). A similar choice was made in the works of C. Guerin-Danan et al. (Guerin-Danan, 1999) and Shenderov B. A. et al. (Shenderov et al., 1998) (Table 2). C. Guerin-Danan et al. stored fecal samples in glycerol at −80 °C for four months. They found that the facultative anaerobes, enterobacteria, enterococci, and lactobacilli were not significantly affected by freezing, while the concentration of bifidobacteria decreased significantly. Nevertheless, the authors note that this decrease did not exceed interindividual variation. Shenderov B. A. et al. stored a microbial suspension of a fecal sample using 10% mucin as a cryoprotectant at −196 °C for one year, and no significant changes in the concentration of enterobacteria, staphylococci, streptococci and anaerobe communities (lactobacilli, Bacteroides, clostridia) were observed.

The developed and standardized protocols for frozen fecal preparations can simplify clinical work by facilitating the banking of feces from carefully selected and screened donors (Satokari et al., 2015). However, the number of publications related to the investigation of freezing and storage conditions on the microbial community is limited, and further research is needed in this area.

3.3. Cryopreservation of microbial communities for in vitro gut microbiota production

In the case of cryopreservation of *in vitro* growth microbial consortia, the task is complicated by the lack of the protective effect of a matrix that is naturally present in stool. Therefore, the composition of the protective media for cryopreservation of this microbiota type should be investigated on par with freezing and thawing conditions (Bircher et al., 2018b). The main cryopreservation methods are summarized in Table 2.

In fact, combinations of different types of CPAs have been tested to preserve the different types of microorganisms, mainly based on their gram-positive and gram-negative classifications. Bircher et al showed that CPAs with different cryopreservation mechanisms of actions were able to protect different groups of microorganisms against the negative effects of the freezing process (Bircher et al., 2018a, b). Moreover, this research group tested and recommended the addition of reducing agents, such as cysteine and riboflavin, to support anaerobic butyrate producing communities (Bircher et al., 2018a). The addition of 15% glycerol (penetrating CPA) supported the cryopreservation of the *Roseburia* spp./*Eubacterium rectale* group, while 5% inulin (nonpenetrating CPA) improved the recovery of *Faecalibacterium prausnitzii* in “artificial” gut microbial communities during 3 months of storage at −80 °C. However, the effect of CPA was dramatically dependent on the initial microbial composition. For two donors, the authors obtained ambiguous results, which require further experiments and confirmation.

In the study performed by Kerckhof et al., no effect of CPA addition on the metabolic activity (SCFA production) of the fecal community or its composition before and after 3 months of cryopreservation was observed. However, even in the presence of CPA, penetrating (10% DMSO) and the combination of penetrating and nonpenetrating cryoprotectants (5% DMSO + 0.3% trehalose and tryptic soy broth (TT)), not all operational taxonomic units (OTUs) were preserved according to 16S RNA sequencing (Kerckhof et al., 2014). Despite the differences in the overall community structure, they were insignificant in comparison with the drastic community changes that occurred during the first cultivation (precultivation) step.

Gaci et al also tested penetrating (glycerol 10% and DMSO 10%) and nonpenetrating (PEG-4000 10%) CPA and their combinations for microbiota cryopreservation for a longer storage period (3 and 6 months). They used a special freezing protocol, which included the two following steps: sample storage at −20 °C for 4 h followed by transfer to −80 °C (Gaci et al., 2017). Gaci et al focused on the cryopreservation of both bacterial microorganisms and methanogenic archaea spp., which play an important role in trophic chain and human physiology (Gaci et al., 2014). A functional microbiota analysis showed that the total SCFA level and its proportions were dependent on the CPA and the cryopreservation storage time. For DMSO, alone or mixed with other CPAs, the authors observed the best efficiency for functional preservation, and the duration of preservation had little effect. Therefore, two bacterial families, the Streptococcaceae and uncultured Clostridiales of cluster II, completely disappeared during preservation. Thus, some additional CPAs and freezing processes should be tested specifically on isolated members of these families and then on the whole microbiota to improve the overall efficiency of the already tested CPAs.

Aguirre et al compared different inoculum preparations and their cryopreservation potentials at −80 °C freezing and storage with the following conditions: 1) fresh feces resuspended in dialysate solution with glycerol; 2) fecal samples frozen with 1.5 g glycerol; and 3) fecal samples frozen without CPA. Based on metabolic activity measurements and the determination of the microbiota composition, the authors concluded that the first preparation showed a similar level of viability after cryopreservation as fresh feces. This preparation protocol was proposed as the optimal way to freeze fecal material as an alternative to fresh feces for *in vitro* fermentation studies. The diversity of the *Actinobacteria, Firmicutes, Fusobacteria, Verrucomicrobia* and *Proteobacteria* groups seemed to be well preserved, whereas it declined in the *Bacteroidetes* group (Aguirre et al., 2015). In this case, dialysate (2.5 g $K_2HPO_4{\cdot}3H_2O$, 4.5 g NaCl, 0.005 g $FeSO_4{\cdot}7H_2O$, 0.5 g $MgSO_4{\cdot}H_2O$, 0.45 g $CaCl_2{\cdot}2H_2O$, 0.05 g ox bile, and 0.4 g cysteine hydrochloride) (Maathuis et al., 2009) was selected to prevent osmotic stress on the cells. The dialysate preparation included bile salts to partly reproduce the environment that the gut microbiota is usually exposed to in the colon (Ridlon et al., 2006), and cysteine hydrochloride was added as a reducing agent to improve the viability of anaerobes.

For the preservation of mixed communities, a rapid freezing technique (direct sample transfer to liquid nitrogen or dry ice and direct sample transfer to the freezer at −80 °C) with the support of CPAs is mainly used.

It should be mentioned that in most studies (Tables 1, 2), the authors included a feces dilution step in the cryopreservation protocol before freezing to produce a suspension. There are several reasons why the addition of this step may have a positive effect on the cryopreservation: (1) the maintenance of the optimum cell concentration

Table 2
Main cryopreservation methods of microbial communities for *in vitro* cultivation and personalized probiotic production.

Sample	T, °C	Freezing technique	Cryopreservation media	Cryoprotectant	Storage time	Methods for characterization of cryopreservation efficiency	Results	Aim for investigation	Reference
Fecal sample (7 donors, 4 h after defecation)	−80	Snap-freezing (4 min dry ice, transfer to −80 °C) Transfer to −80 °C	–	–	7 days	Cultivation analysis (aerobes, anaerobes, bifidobacterium), 16S rRNA sequencing	Significant difference in *Fecalibacterium* and *Leuconostoc* (fresh samples –control). Non-significant difference (different donors – control).	Investigation of the effects of freezing on fecal microbiota. **Note:** very strong difference between healthy donors.	(Fouhy et al., 2015)
Fecal sample (1 donor, 2 h after defecation)	−80	Transfer to −80 °C	Growth media	10% DMSO 5% DMSO + 0,3% trehalose and tryptic soy broth –	106 days	SCFA production (acetate, propionate, butyrate) (De Weirdt et al., 2010), 16S rRNA sequencing	No difference between cryoprotectants. Preservation of metabolic activity: 40% acetate and butyrate, 70% propionate, 50% of total SCFA. Preservation of main OTUs.	Preservation of mixed microbial communities, focus on their functionality and diversity.	(Kerckhof et al., 2014)
Fecal inocula (2 donors).	−80	Snap-freezing in LN_2 (−196 °C)	Phosphate-buffered peptone water (0.1 v/w)	15% Glycerol + cysteine 1 g/L + riboflavin 0.3 g/L	3 months	SCFA production (acetate, propionate, butyrate), 16S rRNA sequencing, qPCR for butyrate producing bacteria (after 24 h cultivation)	↑ Propionate, butyrate. ↑ *Roseburia* spp./*Eubacterium rectale* group, ~ *Eubacterium hallii*, ~ *Fecalibacterium prausnitzii*.	Cryopreservation of “artificial” gut microbiota, focus on preservation of butyrate producing bacterium: firmicutes *Fecalibacterium prausnitzii* (Clostridium cluster IV), *Eubacterium hallii and the Roseburia spp./Eubacterium rectale* (Clostridium cluster XIVa). **Note:** very strong influence of initial composition on recovery and effect of CPA.	(Bircher et al., 2018b)
Microbial pellet in protective medium after fermentation	−80			5% Inulin + cysteine 1 g/L + riboflavin 0.3 g/L	3 months		~ *Roseburia* spp./*Eubacterium rectale* group, ~ *Eubacterium hallii*, ↑ *Fecalibacterium prausnitzii*.		(Bircher et al., 2018b)
	−80			15% Glycerol +5% inulin + cysteine 1 g/L + riboflavin 0.3 g/L	3 months		↑ Acetate, butyrate. ~ *Roseburia* spp./*Eubacterium rectale* group, ~ *Eubacterium hallii*, ~ *Fecalibacterium prausnitzii*.		(Bircher et al., 2018b)
Fecal inocula (4 donors).	−80	Snap-freezing in LN_2 (−196 °C)	Dialysate solution	~10 % Glycerol	1 week	SCFA and BCFA production, 16S rRNA sequencing	~ Acetate, ~ butyrate, ~ propionate, ↓ i-butyrate, ↓ i-valerate. ~ Diversity in *Actinobacteria, Firmicutes, Fusobacteria, Verrucomicrobia, Proteobacteria,* ↓ *Bacteroidetes.*	Standardization of fecal inocula for TIM-2 fermentation system.	(Aguirre et al., 2015)
Fecal sample (4 donors)	−80	Snap-freezing in LN_2 (−196 °C)	–	~10-15 % Glycerol	1 week	SCFA production, 16S rRNA sequencing	↑ Acetate, ~ butyrate, ~ propionate, ↓↓ i-butyrate, ↓↓ i-valerate. ~ Diversity in *Actinobacteria, Firmicutes, Fusobacteria, Verrucomicrobia, Proteobacteria,* ↓↓ *Bacteroidetes.*	Standardization of fecal inocula for TIM-2 fermentation system.	(Aguirre et al., 2015)
Fecal sample (4 donors)	−80	Snap-freezing in LN_2 (−196 °C)	–	–	1 week	SCFA production, 16S rRNA sequencing	↑ Acetate, ~ butyrate, ~ propionate, ↓↓ i-butyrate, ↓↓ i-valerate. ~ Diversity in *Actinobacteria, Firmicutes, Fusobacteria, Verrucomicrobia, Proteobacteria,* ↓↓ *Bacteroidetes.*	Standardization and preservation of fecal inocula for TIM-2 fermentation system.	(Aguirre et al., 2015)

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Table 2 (*continued*)

Sample	T, °C	Freezing technique	Cryopreservation media	Cryoprotectant	Storage time	Methods for characterization of cryopreservation efficiency	Results	Aim for investigation	Reference
Feces inocula after fermentation (1 donor)	−80	Transfer to −20 °C, 4 h incubation, transfer to −80 °C.	Growth media	10% Glycerol 10% Glycerol + PEG	3 months	Gas analysis, SCFA production, HUGChip composition analysis	↑↑↑ acetate, ↑↑ propionate, changes in proportion. Disappearance of *Streptococcaceae* and uncultured *Clostridiales* of cluster II.	Development of strategies for amplification and long-term gut microbiota (especially SCFA producing anaerobe communities) preservation from fecal. Improvement facilities for sample sharing, comparisons and reproducibility over time and between laboratories. Improvement the safety and ethical issues surrounding fecal microbiota transplantations.	(Gaci et al., 2017)
				10% Glycerol 10% Glycerol + PEG	6 months		↓ SCFA production, ~ proportion. Disappearance of *Streptococcaceae* and uncultured *Clostridiales* of cluster II.		
				PEG-4000	3 months		↓ SCFA production, changes in proportion.		
					6 months		*Streptococcaceae* and uncultured *Clostridiales* of cluster II.		
				PEG-4000 + DMSO	3 months		~ SCFA production, small changes in proportion.		
					6 months		Disappearance of *Streptococcaceae* and uncultured *Clostridiales* of cluster II.		
				DMSO 10% or 10% DMSO + glycerol	3 months		~ SCFA production, ~ proportion.		
					6 months		Disappearance of *Streptococcaceae* and uncultured *Clostridiales* of cluster II.		
				10% DMSO + glycerol + PEG	3 months 6 months				
Fecal sample (20 donors)	−80	Transfer to −80 °C	pre-reduced Liquid Casein Yeast medium (LCY)	50% Glycerol	4 months	Counting plate analysis after cultivation in the selective media	~ Proportion in bacterial populations, ~ facultative anaerobes: *Enterobacteriaceae, Enterococcus, Lactobacillus,* ↓ total anaerobes, ↓ *Bifidobacterium.* **Note:** decrease did not exceed interindividual variation.	Development of fecal samples freezing technique that allows bacteriological analyses.	(Guerin-Danan, 1999)
Bacterial suspension from fecal samples	−196	Snap-freezing in LN_2 (−196 °C)	NS	10% Mucin	1 year	Counting plate analysis after cultivation in the selective media	~ *Enterobacteriaceae,* ~ *Staphylococcus,* ~ *Streptococcus,* ~ *Lactobacillus,* ~ *Bacteroides,* ~ *Clostridia.*	Preservation of symbiotic association of microorganisms.	(Shenderov et al., 1998)
				10% Levan			↓ *Enterobacteriaceae, Staphylococcus, Streptococcus,* ~ *Lactobacillus,* ~ *bacteroides,* ↓ *clostridia.*		
				10% Agarose			↓ *Enterobacteriaceae, Staphylococcus, Streptococcus,* ~ *Lactobacillus,* ~ *Bacteroides,* ~ *Clostridia.*		
				10% Gelatin			~ *Enterobacteriaceae, Staphylococcus, Streptococcus,* ~ *Lactobacillus,* ↑ *Bacteroides,* ↓ *Clostridia.*		

(*continued on next page*)

Table 2 (*continued*)

Sample	T, °C	Freezing technique	Cryopreservation media	Cryoprotectant	Storage time	Methods for characterization of cryopreservation efficiency	Results	Aim for investigation	Reference
Mixture of 17 butyrate-producing Clostridium strains	−80	Transfer to −80 °C	EG media	20% Glycerol	1 year	SCFA production, specific analysis of therapeutic effect	Local and specific alteration of microbial metabolites has direct salutary effects on GVHD target tissues and can mitigate disease severity.	Identification of alterations in gastrointestinal microbiota–derived SCFAs after allogeneic bone marrow transplant.	(Mathewson et al., 2016)

ensures their maximum survival upon freezing; (2) a uniform distribution of the cryoprotectant in a biological sample; and (3) a reduction of the matrix influence on the freezing process. In addition, the use of a combination of nonpenetrating and penetrating cryoprotectants seems to be better due to the differences in the cell wall structure of the intestine microbiome bacteria.

In studies on the conservation of mixed communities, the authors generally use combinations of DMSO or glycerin at standard concentrations with nonpenetrating cryoprotectants, for example DMSO-trehalose (Kerckhof et al., 2014), DMSO-PEG-4000 (Gaci et al., 2017), glycerol-inulin (Bircher et al., 2018a), and glycerol-sucrose. The choice of cryoprotectant remains process- and species-specific. None of the tested solutions provides the same compositional and metabolic pattern as “fresh” microbiota (Kerckhof et al., 2014).

Overall, the supportive effects of CPA limit not only the species-specific properties but also the initial species proportions, microbial diversity and precultivation conditions. In these works, the authors confine to suggestions on the maintenance of individual species in the context of their specific tasks. Based on these empirical data, it is not possible to make general recommendations.

4. Methods for evaluating mixed community viability

The comprehensive and accurate functional and compositional characterization of microbial communities before and after cryopreservation is crucial for the development of an effective cryopreservation protocol.

Currently, molecular methods based on 16S rRNA amplicon sequencing are the most commonly used and effective tools for the identification of intestinal microbiota species. 16S rRNA amplicon gene sequencing is performed on various sensitive platforms, such as Illumina’s (Kerckhof et al., 2014) MiSeq (Bircher et al., 2018b; Fouhy et al., 2015; Vandeputte et al., 2017) and HiSeq and PacBio, which are the most popular, and some works were performed using Roche 454-pyrosequencing (Carroll et al., 2012; Lauber et al., 2010). Other methods for taxonomic classification that are based on 16S rRNA detection include a hybridization assay using a HuGChip DNA microarray containing (3x) 4454 immobilized unique probes (Gaci et al., 2017; Tottey et al., 2013) and IS profiling (Aguirre et al., 2015). IS profiling differentiates the bacterial species by the length of the 16S–23S rDNA intergenic spacer (IS) region with taxonomic classification by phylum-specific fluorescently labeled PCR primers (Aguirre et al., 2015). Quantitative PCR is also used to provide functional bacterial identification and can be combined with 16S rRNA analysis; the primers used for this purpose target genes for strain or groups differentiation (Bahl et al., 2012; Gaci et al., 2017) or the presence of a specific function (for example, butCoA gene for butyrate-producing bacteria (Bircher et al., 2018b)). All these systems make it possible to determine a large diversity of microorganisms and to identify low-abundance populations that could easily be lost when cultivated in standard selection media. The main limitations of such methods are the inability to differentiate cells at the species and strain levels and to distinguish the live cells from the dead cells (Bircher et al., 2018b; Carroll et al., 2012; Fouhy et al., 2015; Gaci et al., 2017; Lauber et al., 2010).

Membrane integrity tests that are based on fluorescence microscopy (Moussa et al., 2008) or flow cytometry (Bircher et al., 2018a) can be applied to distinguish the live and dead bacterial cells.

In some works, for a relatively rough estimation of microbiota survival status, the authors used the cultivation methods in selective media followed by determining the colony forming units by taking into account the typical morphological characteristics (Costello et al., 2015; Guerin-Danan, 1999). However, it takes a rather long period of time (1–5 days of incubation), and according to the data in the literature, identification of less than 10–20% of microorganisms is feasible (Browne et al., 2016; Eckburg et al., 2005). For a more accurate quantitative detection of viable cells, the most probable number method (MPN) can be used (Bircher et al., 2018a; Kuai et al., 2001; Sutton, 2010). A detailed investigation of bacterial viability and fitness can be evaluated by the maximum growth rate and lag phase (growth test, 3-time points) (Bircher et al., 2018b).

A functional characterization of the preserved microbiota is based on the metabolic activity measurement after cultivation in the selective media. The main markers for the evaluation of metabolic activity are SCFA (acetate, propionate, and butyrate), BCFA (isobutyrate and isovalerate) (Aguirre et al., 2015) or their sum (Kerckhof et al., 2014) using HPLC-RI (Bircher et al., 2018b) and gas chromatography (Gaci et al., 2017) for their detection. In some works, the authors perform more specific analyses, for example, gas production analysis (Gaci et al., 2017) or the detection of other target substances.

The variety of therapeutic applications, microorganism restoration protocols, and individual choice of control points for measuring functional parameters (Bircher et al., 2018a; Kerckhof et al., 2014) makes it difficult to compare the cryopreservation results from different publications that were obtained in different laboratories, and sometimes in the same laboratory. Similar problem arises when interpreting the results. The strict criteria for the evaluation of the significance of characterized parameter deviations are absent. For example, in some works, the selection of cultivation conditions before and after freezing, inter-individual variation of the “healthy” microbiome is more crucial for the final microbiota composition than are the changes induced by the cryopreservation process, and the conclusions that are based on these data can lead to bias (Table 2) (Aguirre et al., 2015; Fouhy et al., 2015; Guerin-Danan, 1999).

It can be concluded that there is no complex, standardized approach that is suitable for the evaluation of microbiota preservation efficiency to date. Complex solutions that combine genetic, microbiological, proteomic and metabolomic methods of analyses are needed and should be included in the general protocols of cryopreservation.

5. Conclusions

Current cryopreservation methods are well established for pure cultures, but there are no standardized protocols for preserving ecosystems at the complex microbial community level. The cryobiological studies related to the conservation of mixed and enriched cultures, natural microbial communities and fecal transplants are at the early stages of development (Orenstein et al., 2016; Terveer et al., 2017).

The vast majority of works on cryopreservation of the human intestinal microbiota have been carried out using fast freezing techniques with a limited storage time that ranges from a week to 12 months at −80 °C (Terveer et al., 2017). Only in individual studies storage at −196 °C is recommended to prolong the cryopreservation time (Gaci et al., 2017; Shenderov et al., 2014, 1998).

The special cryobiological studies are focused mainly on the search for cryoprotectant combinations and pre/post-freezing cultivation conditions to 1) preserve the species diversity of the intestinal microbiota, 2) enhance the metabolic activity of microorganisms after thawing, and 3) standardize the starter inocula for creating *in vitro* cultivated microbiota variants. The development of cryopreservation methods is regarded as a part of the production processes of microbiota-based products. In fact, differences in research objectives and, consequently, the methods of monitoring cryopreservation efficiency make it impossible to compare results between different research groups. None of the described methods of cryopreservation are able to ensure the survival of the entire initial diversity of microbial communities to date.

Currently, the main trends in the development of the human microbiota cryopreservation methods include 1) the standardization and unification of complex microbiota state assessment protocols, 2) the development of optimized low-toxic, universal cryoprotectant compositions, and 3) the application of fast freezing techniques in creation and validation of gut microbiota preservation protocols. The realization of these tasks will help to develop a strategy for the long-term storage of the human intestinal microbiota with the possibility of its further application for medical and biotechnological purposes.

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