

IMPORTANCE OF HUMAN FAECAL BIOBANKING: FROM COLLECTION TO STORAGE

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Abstract. The freezing and storage conditions of faecal samples in biobanks influence the preservation of the integrity and stability of genetic material and play a crucial role in scientific and clinical research quality. In recent years, there has been a particular increase in the number of studies related to the gut microbiome and the importance of its impact on the functioning human body. The review includes research on faecal microbiota transplantation (FMT), microbiome analysis, ‘-omics’ research, cancer and parasites. The primary topic addressed in this research is the impact of storage conditions and freezing methods for faecal samples on the stability and diversity of the gut microbiome. Standardizing procedures for storing and analyzing faecal samples is essential to carry out this task. This standardization is not a goal but a necessity for the quality control of the storage of faecal samples to reach comparative results and to develop new diagnostic methods. Collected data presented here highlight a crucial step in microbiota research concerning optimizing biobanking conditions for faecal samples.

1. Introduction. 2. Collection of faecal samples for testing and their transport. 3. Sample processing for biobanking. 4. Securing samples for storage. 5. Storage. 6. Stability studies of faecal samples during storage in the temperature range between 15–25°C. 7. Stability testing of faecal samples during storage at 4°C. 8. Studies on the stability of faecal samples during storage in the temperature range –20 ÷ –30°C. 9. Stability testing of faecal samples during storage at –80°C. 10. Stability studies of faecal samples during storage in liquid nitrogen. 11. Applicable standards for the freezing and storage of faecal samples. 12. Clinical applications of faecal biobanking. 13. Summary.

Keywords: accreditation, biobank, cryopreservatives, faecal samples, storage conditions

1. Introduction

The storage of biological material is an essential part of the study that influences the final result. Depending on the aim of the study, several factors of the research stage should be previously optimized and validated, including essential factors, such as temperature and period of storage, while considering the application of stabilizing components. The stool is a specific biological material when it is impossible to carry out tests within a short period from its collection or in clinical studies involving many subjects and biological samples collected for harmonization/searching for new diagnostic methods or therapies. The most common approach to increase the availability of such material is to lower the storage temperature, add stabilizing compounds and standardize this pre-laboratory stage.

The type of biological material, the storage period, and the study’s purpose influence the biobanking process in faecal biobanking; CTQ (critical to quality) focuses on controlling shifts in microbial composition caused by stabilization agents, period of storage and extraction methods while ensuring optimal DNA yield. Repeatability and reproducibility are essential for consistent, high-quality data across experiments (Doukhaine *et al.* 2021). This optimization is crucial in ensuring the high quality of the biological material obtained after thawing, making the research successful. Due to the complexity, banking faecal samples is challenging. Optimization of biobanking conditions enables multidirectional research and epidemiological studies. Freezing samples should safeguard their composition for future analyses to ensure consistency of results over a more extended unit of time. Accurate biobanking allows the integrity

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of the genetic material intended for preservation and performing optimal molecular analyses, ‘-omics’, or epidemiological studies. For therapies related to intestinal microbiota transplantation, the choice of faecal freezing is crucial for storing samples from microbiota donors to ensure the quality and safety of the material intended for transplantation (Coppola *et al.* 2019).

Different freezing methods are used in biobanks storing faecal samples, depending on their purpose, the research scope, and the application. However, the insufficient standardization of biobanking methods can result in a lower level of stability of stored samples and a lack of reproducibility of test results. Implementing international requirements for standardization of biobanking methods can significantly improve the quality of stored biological materials and facilitate data sharing between resources, especially in rare diseases (Coppola *et al.* 2019).

This work aimed to analyze published biobanking modalities, the selection of compounds that prevent damage to the cell and tissue (known as cryoprotectants) before freezing faecal samples, and also review recommendations for the collection, freezing and storage of faeces to identify the impact of freezing modalities in microbiological studies, what are the challenges and limitations of faeces biobanking and future directions and emerging trends. This work also highlights the need for further research in this area to urgently address the identified challenges and explore the potential of emerging trends, offering a hopeful outlook for the future of biobanking.

2. Collection of faecal samples for testing and their transport

Sampling is the first stage of the study, which must consider several processes occurring immediately after the faecal passage. Oxidation, hydrolysis, or enzymatic degradation are just some of the reactions that can contribute variably to changes in the sought-after parameters of the test sample before analysis (O’Sullivan *et al.* 2018). The optimization of collection, transport, and storage methods is crucial as it directly impacts the quality of the research and the accuracy of the results, reducing the impact of degradation to negligible levels. Given the intricate interplay of factors and the diverse array of tests performed on faecal samples, it is challenging to establish a single protocol to optimize the steps mentioned above. Immediately after passing stool, the sample is affected by higher oxygen concentrations than those prevailing in the intestines. By conducting experiments under *in vivo* conditions ignoring the difference in atmospheric oxygen concentrations and the gut microenvironment, the final result may deviate

critically from the initial content (Thomas *et al.* 2015; Widjaja *et al.* 2023). When testing anaerobic bacteria, the time between donation and testing is crucial and should be as short as possible. Widjaja *et al.* (2023) pointed to several media for faecal sample protection collected under anaerobic conditions until transported to the anaerobic chambers for the experiment. Of further interest is a 2021 patented self-sampling device that maintains anaerobic conditions and limits donor contact with the sample (Widjaja *et al.* 2023). In the absence of access to such devices, when a donor collects a sample outside the laboratory, it is recommended to use substrates that protect the integrity of the material from destabilizing agents. These substrates have a transport and a protective function against various temperatures. One of the most frequently mentioned transport media in recent papers was the OMNIgene-GUT commercial kit. Doukhaine *et al.* (2021) designated this medium as effective in maintaining the neutrality of the microbiome. This underscores the critical importance of selecting the appropriate stabilization medium to ensure sample integrity, which is essential in sample processing for biobanking.

3. Sample processing for biobanking

While not mandatory, validation of faecal sample processing is recommended to recognize and mitigate potential impacts on sample quality, ensuring adherence to biobanking standards as outlined by the ISO, CEN standards, and BioMolecular Resources Research Infrastructure (BBMRI) guidelines. Based on the published data, several factors, such as different collection tubes and storage conditions, affect the sample stability and method validation, emphasizing the importance of precisely documenting these factors by biobanks (Neuberger-Castillo *et al.* 2020). The most important is to maintain experimental conditions as close to *in vivo* conditions as possible and process the sample in anaerobic chambers to eliminate the influence of oxygen. Bellali *et al.* (2019) developed a protocol that limited sample exposure to oxygen to 2 minutes (by collecting samples into anaerobic jars and processing steps quickly), resulting in an 87% recovery of viable anaerobic bacteria. The protocol was compared with a modified protocol using antioxidants, in which the sample was exposed to oxygen for 120 minutes. For this protocol, the recovery of viable bacteria was up to 67%. In the absence of an anaerobic atmosphere, antioxidant enrichment of the biological samples is a reasonable alternative for this experiment, providing reassurance about the validity of the research. During processing, aliquoting of samples is required. This process eliminates the risk of thawing and freezing, which degrades

most of the faecal parameters. According to the Good Laboratory Practice recommendation, homogenizing samples before aliquoting should be performed (OECD 2004). It was found that the faecal sample as a whole is differentiated in terms of aerobic bacteria in the outer layers and anaerobic bacteria in the deeper layers, which can be further subdivided into a central fermentative and a reservoir (Swidsinski *et al.* 2010; Santiago *et al.* 2014). In his experiment, Santiago *et al.* (2014) compared the results of pyrosequencing the 16S rRNA gene analysis of samples from the outer layers, inner layers, and homogenized samples, indicating a similar abundance of measurements obtained. Performing this step in the laboratory according to the procedure minimizes the risk of inappropriate homogenization due to too much hardness of the sample (Bristol scale 1.2) or unpleasant sensations during the process.

4. Securing samples for storage

Preservation of biological material plays an essential role in biobanking. Comparisons of faecal freezing without and with any cryoprotectants were concerned with the composition of the gut microbiota of fresh and frozen faeces for faecal microbiota transplantation. Some papers in this area have studied the stability and survival of viruses causing gastrointestinal infections (Alghamdi *et al.* 2022, Yang *et al.* 2022). Available results from direct freezing of faeces at -80°C and storage for up to 48 h without cryoprotectants indicate this method as the 'gold standard' for preserving the qualitative and quantitative composition of the intestinal microbiota, compared with preservation with 10% glycerol (Deschamps *et al.* 2020). Yang's (2022) metagenomics study showed that the faecal sample can be successfully stored as a supernatant at -80°C . However, freezing without a cryoprotectant will not be advisable in every experiment. Preservatives can stabilize the number of faecal microorganism species, but storing samples without that type of compounds can lead to an increase in the number of specific taxa (e.g. operational taxonomic units- OTU *Enterobacteriaceae*) (Li *et al.* 2023). In a study where the use of fresh and frozen faeces for FMT was compared, Bilinski *et al.* (2022) showed that freezing whole faeces without any cryoprotectants has an impact on the biodiversity and survival of the bacterial intestinal microbiota – the number of viable cells decreased more than fourfold, from about 70% to 15%. Among the set of commercials facilitating the preservation of faecal samples, e.g. OMNIgene-GUT mentioned earlier, was independently recognized as the best alternative to the 'gold standard', established based on the outcomes from qualitative-quantitative composition analysis. Similar results were achieved in

a protocol study for faecal samples detecting bile acids using OMNIgene-GUT, demonstrating its efficacy for metabolomics studies (Neuberger-Castillo *et al.* 2021). Of interest may be the preliminary results of an experiment by Young *et al.* (2020) on using a substrate based on guanidinium thiocyanate – eNAT when stored at room temperature for about 30 days and then transferred into -80°C for longer time of the period storage. The most often self-prepared cryoprotectants applied before sample freezing included, among others, ethanol, DMSO-EDTA salt solution (DESS) and guanidine thiocyanate. In a Japanese study, the effects of DESS or guanidine thiocyanate at room temperature or 4°C showed the α - and β -diversities with no significant differences between *Bacteroides* and *Bifidobacterium* spp. profiles (Kawada *et al.* 2019). On the other hand, Hale *et al.* (2015) found no significant effect on the microbial community of the samples studied while storing faecal samples from spider monkeys in 100% ethanol at room temperature for eight weeks. Song *et al.* (2016) came to similar conclusions, where they demonstrated that 95% ethanol effectively preserves the diversity and composition of the gut microbiome stored at room temperature for at least eight weeks. However, this solution is highly flammable and expensive to transport. The effect of 10% glycerol concentrations (through its ability to permeate cell membranes) on metabolic and biological parameters has been described. As one of the more commonly chosen protectants due to its protection of bacterial viability after freezing, it also had another characteristic – after thawing, it offers an excellent environment for microbes to thrive, thereby causing changes in the microbiome community (Widjaja 2023). Deschamps (2020) also demonstrated the effect of glycerol in reducing metabolic activity, which may be related to cell damage. According to Biclot *et al.* (2022), glycerol contributes to the formation of ice crystals inside cells, ultimately leading to cell lysis. These disparate data point to the need for further research to better understand under which conditions the integrity of samples can best be maintained for different types of analysis. Other publications analyzed the effect of stabilizing agents used in microbiome studies, i.e. Tris-EDTA buffer and 70% ethanol. Unfortunately, buffers containing EDTA may not optimally preserve the microbiological composition of faecal samples. In Young *et al.* (2020) study, samples stored in Tris-EDTA buffer at ambient temperature had lower abundances of *Bifidobacterium* and *Anaerostipes* spp. and higher abundances of *Bacteroides* and *Proteobacteria* spp. than samples stored in Tris-EDTA buffer and immediately frozen to -80°C . In the case of 70% ethanol, using it as a protectant for faecal samples at room temperature yielded results similar to those without preservatives. Hence, 70% ethanol as a protectant for this type of storage is strongly discouraged (Song *et al.* 2016). Wu *et al.*

(2021) verified a self-prepared preservation buffer (PB) that could stabilize human saliva samples, thus demonstrating the suitability of this buffer for stabilizing faecal samples and intended for sequencing without freezing facilities or logistical constraints. The study results indicate that using a cryoprotectant has a beneficial effect on the microorganisms in the faeces, the stability of the sample, and the reproducibility of the results of various diagnostic methods. In addition, commercial media can be used in molecular biology studies due to their properties, i.e., the stabilization of microbial DNA in eNAT® medium or the lack of effect on DNA extraction efficiency in OMNIgene-GUT medium (Young *et al.* 2020; Doukhaine *et al.* 2021). Understanding that choosing preservation methods is not a one-size-fits-all solution is crucial. The efficacy of these methods varies across different groups of microorganisms, making it a significant and relevant area of research. The storage temperature and duration of storage also play a pivotal role in influencing microbial DNA yield (Wu *et al.* 2021).

5. Storage

When creating a sample storage protocol with specific assumptions for the experiment, storage time and temperature should be analyzed. The bacterial composition of faeces changes after 15 minutes of storage at room temperature, which can be an obstacle for, among other things, epidemiological studies; therefore, it is desirable to freeze samples immediately (Tamada *et al.* 2022). For metabolomic studies, temperature determines microbiological and enzymatic activity and, thus, the final results. O'Sullivan *et al.* (2018) highlighted the testing of previously frozen faecal samples for glutamate and branched-chain amino acid (BCAA) levels, where higher concentrations were found relative to measurements on fresh samples. For this type of study, storing samples at temperatures below 0 can significantly affect the final results. A thorough analysis of the modes and conditions of storage on the quality and quantity of the microbiota of stored human faeces is crucial in selecting optimal conditions depending on the intended use of the collected samples (Hickl *et al.* 2019). Preserving the integrity of the genetic material of the samples after collection and storage determines the correct interpretation of the data (Hickl *et al.* 2019; Tamada *et al.* 2022). Optimizing a faecal sample's freezing and storage conditions includes the choice of container, cryoprotectant, freezing, and storage method. Many containers suitable for deep-freezing are available, e.g. Eppendorf tubes, cryo-tubes without code and with 2D code and SBS (Society for Biomolecular Screening) tubes adapted for automatic biological material handling. Before biobanking, it is necessary to analyze the

amount of frozen material, the available equipment, the number of personnel, and the choice of container. When selecting tubes with a stabilizing/transport medium, you must consider the stabilizer's possible influence on the parameter under study, e.g., the change in bacterial abundance in the buffer medium through chemical lysis or interference in metabolite studies (Chen *et al.* 2020; Neuberger-Castillo *et al.* 2021). Regardless of the container used, each Biobank needs to check the required quality parameters (depending on the purpose of the study) to ensure that the selected materials and equipment will meet the storage criteria under the prevailing conditions. Another critical factor in the correct storage process is the accurate temperature for the research. The literature data analyzed for his article of a study of the effect of the length of storage on the stability of stored faecal samples highlighted the dependence of quality parameters on different storage temperature ranges.

6. Stability studies of faecal samples during storage in the temperature range between 15–25°C

In routine diagnostic laboratories, faecal samples are usually stored at room temperature during the laboratory examination. After treatment, the remaining residue, until the result is stored at 4°C. For many research units without access to freezing equipment wishing to store faecal samples, the storage method at room temperature seems interesting. Due to several metabolic processes occurring in faecal samples at ambient temperature or fungal overgrowth (Thomas *et al.* 2015), using preservatives is crucial to maintain the sample's integrity. Their use eliminates the need for research to analyze the effect of humidity on fresh samples. In a study by Park *et al.* (2020), after evaluating the microbial profile, the authors confirmed the usefulness of transport media, i.e. NBgene-Gut and OMNIgene-GUT, for storing faecal samples at room temperature over two months. Thanks to the stabilizing media, transported faecal samples, e.g. with RNAlater® or OMNIgene-GUT, can be stored for 14–60 days (depending on the kit specification) (DNA Genotek 2010; Life Technologies 2011; Song *et al.* 2016; Wu *et al.* 2021).

In contrast, Wu *et al.* (2021) confirmed the efficacy of the 'Self-made PB buffer', which protected the microbiome in faecal samples for up to 4 weeks at room temperature. The authors suggest PB buffer as a cheaper alternative for storage at room temperature compared to its commercial counterparts. However, the limitations of this method over the long term indicate the need for further research into the long-term stability of samples. All the substrates mentioned are stored at lower temperatures (–20°C and –80°C, respectively) (DNA Genotek 2010; Life Technologies 2011; Wu *et al.*

2021). Other studies have noted the effect of room temperature on changing the ratio of Gram-negative and Gram-positive bacteria, probably due to the higher stability of bacterial DNA in Gram-negative bacteria relative to that of Gram-positive bacteria at room temperature (Hickl *et al.* 2019; Li *et al.* 2023).

7. Stability testing of faecal samples during storage at 4°C

In biobanks, temperatures of 4°C are not helpful for the long-term storage of biological samples. Amar *et al.* (2005) showed the advantage of molecular methods used for archived frozen fecal samples against conventional method immediately used for detection with the lowest percentage reconfirmation of target for *Giardia* spp. and *C. perfringens*. Results obtained by PCR indicated a 96% concordance for *Cryptosporidium* spp. after ten years of storage and a 68% concordance for *Giardia* after two years of storage, with positive results from a direct preparation. In most scientific studies on metabolites or the microbiome, a temperature of 4°C is not recommended for storage of media samples beyond 12 h because of the continuous proliferation and metabolism of some microorganisms, as well as the change in oxygen concentration in the samples, which significantly affects the anaerobic community (Thomas *et al.* 2015; Cunningham *et al.* 2020). In their study, Nogata *et al.* (2019) proved that using a medium such as Carry Blair during storage at 4°C can prevent changes in the microbiome community. For metabolomic testing of faecal water, according to O'Sullivan *et al.* (2018), storing faecal samples at this temperature for up to 24 hours shows a good alternative compared to testing on fresh samples. Wu *et al.* (2021), who studied the microbiome's composition in faecal samples subjected to different temperatures, including 4°C for up to 4h, proved the lack of effect of short-term storage on microbiome composition. Also, Cunningham *et al.* (2020) demonstrated that stool storage without cryoprotectants at room temperature and 4°C for up to 48 h has no significant effect on the microbiome in samples. The discrepancies in the period may be due to differences in the analytical methods used in the two studies, highlighting the need for standardization of procedures in this area. However, it is crucial to note that Cunningham's method should not be recommended in studies on determining the composition of faecal anaerobic microbiome by culture methods. Instead, the focus should be on pre-laboratory procedures that may cause errors affecting the microbiome's composition. If not carefully avoided, these errors can significantly alter it, underscoring the weight of a decision in maintaining its integrity.

8. Studies on the stability of faecal samples during storage in the temperature range -20 ÷ -30°C

The freezing method at -20°C has been repeatedly described as one of the most commonly used for long-term storage of samples (Biclot *et al.* 2022). Ice crystals may appear at this temperature, but the manufacturer of RNeasy [®] assures RNA stability in samples indefinitely (Life Technologies 2011). Despite using this medium in many experiments, the manufacturer's instructions do not provide information about applying it to faecal samples. An epidemiological study by Souza *et al.* (2021), conducted on archival (1998–2005) faecal samples frozen at -20°C, found genetic material in 6.8% of the samples analyzed for Adenovirus. Due to the lack of comparative results in fresh faecal samples, it is impossible to address the stability of faecal samples in the context of the presence of Adenovirus. Bilinski *et al.* (2022), in a study of faecal samples frozen at -30°C, found evidence in favour of a significant effect of low temperature on the composition of the microbiome, even though it is this way of freezing stool has more than 90% effectiveness in transplanting the microbiota in patients with *Clostridioides difficile* infection. The review did not analyze studies on the impact of the transplanted microbiota on the recipient's organism, as the main aim of this procedure is to eradicate *Clostridioides*. However, such studies may contribute to a better understanding of the transplant preparation process.

9. Stability testing of faecal samples during storage at -80°C

Biobanking using the rapid freezing method at -80°C has been recognized as the gold standard in microbiome research (Hickl *et al.* 2019; Guan *et al.* 2021; Wu *et al.* 2021; Li *et al.* 2023). This method seems more effective, especially for short-term storage of samples (up to 48 h) (Deschamps *et al.* 2020). However, some studies allow it to be used as a long-term storage method, especially in colorectal studies (Wirth *et al.* 2020). Compared to regular freezing at -80°C, the rapid drop in temperature reduces the ice crystal formation phenomenon while maintaining the integrity of the cells, further improving the isolation of Gram-positive bacterial DNA (Thomas *et al.* 2015; Li *et al.* 2023). In a study by Coryell *et al.* (2021) on the detectability of SARS-CoV-2 in faecal samples, a higher detection rate of viral RNA was demonstrated in samples stored in DNA/RNA shield stabilizing buffer, compared to samples without the addition of a stabilized stored at the same temperature. In the previously mentioned RNeasy [®] study results, confirmation was obtained for long-term storage of faecal samples without significant changes in microbiome

composition (Flores *et al.* 2015, Tap *et al.* 2019). In the case of the study by Liang *et al.* (2020) on OTU-level abundance in frozen samples, the RNAlater® substrate is not recommended due to its high diversity. Biclot *et al.* (2022) showed the effect of freezing faecal samples at –80°C on the composition of anaerobic bacterial species. They found that storing samples at this low temperature without additives, the so-called “dry condition”, significantly showed the broader species richness. In addition to using –80°C for storage, this temperature is also used to transport faecal samples for essential analyses (Williams *et al.* 2019).

10. Stability studies of faecal samples during storage in liquid nitrogen

Due to relatively high storage costs, using liquid nitrogen for freezing faecal samples is not the first chosen method. Wu *et al.* (2021) compared the economic aspects of freezing using selected techniques in their

publication. They found a financial alternative to the liquid nitrogen method (using low-cost buffers allowing samples to be kept for up to 4 weeks, based on the storage temperature. It is worth noting that liquid nitrogen, especially in combination with 10% glycerol, is an effective and reliable method for the long-term storing of faecal samples (Li *et al.* 2023). Relating to storing samples in liquid nitrogen, researchers also use nitrogen jet cooling to store samples at –80°C (Hickl *et al.* 2019; Guan *et al.* 2021).

This work’s temperature division indicates various methods for preserving faecal samples, highlighting their effectiveness based on research. The purpose, planned costs, and future application of the results should all be considered.

Table I. presents various faecal microbiome preservation methods tested across different studies, providing an overview of their effectiveness based on storage duration and temperature. The best long-term preservation method remains freezing at –80°C. In a 2020 study, Young *et al.* demonstrated that the eNAT medium can

Table I
Summary of selected storage conditions and preserving faecal samples for a microbiome study.

Stabilization compounds/ solutions	Storage temperature	Storage period	Influence on stability of microbiota	Source
Stratec DNA stabilizer	Room temperature	7 days	Stable microbiota profiles, minimal changes in diversity.	(Chen <i>et al.</i> , 2020)
OMNigene-GUT DNA stabilizer	Room temperature	7 days	Microbiota minimal changes, high DNA stability.	(Chen <i>et al.</i> , 2020)
DESS (DMSO-EDTA-salt)	Room temperature	1–3 weeks	Microbiome stability without significant changes in diversity (using targeted extraction methods).	(Kawada <i>et al.</i> , 2019)
Homemade preservation buffer (EDTA, DSD*, SCTD*, ammonium sulfate)	Room temperature	4 weeks	Stability in room temperature (or in high temperatures up to 5 days), ideal when freezing isn’t available.	(Wu & Chen <i>et al.</i> , 2021)
OMNigene-GUT or NBgene-GUT	Room temperature	65 days	The best method for room temperature storage is stable microbiome profiles comparable to frozen samples	(Park <i>et al.</i> , 2020)
95% Ethanol, OMNigene-GUT	Room temperature	8 weeks	Both methods effectively maintain microbiome stability	(Song <i>et al.</i> , 2016)
Ethanol (100%)	Room temperature	8 weeks	Stable microbiome, alternative to freezing, suitable for long-term storage (study on spider monkey’s stool)	(Hale <i>et al.</i> , 2015)
No stabilizer	–20°C	24 h	Minimal changes in taxonomic diversity, good DNA quality.	(Cardona <i>et al.</i> , 2012)
No stabilizer	–20°C (after 48h at 4°C)	48–96 h	Minimal changes in microbiome and SCFA composition, indicating that 24h refrigeration preserves sample integrity.	(Cunningham <i>et al.</i> , 2020)
No stabilizer	–80°C	48 h	Stable microbiota diversity, high DNA yield, minimal changes in bacterial composition	(Biclot <i>et al.</i> , 2022)
eNAT® medium	–80°C	30 days at room temperature, then freezing	Stability of microbiome maintained after 30 days at room temperature, no significant changes in diversity and composition	(Young <i>et al.</i> , 2020)
10% Glycerol	–80°C/liquid nitrogen	12 months	Microbiome stability maintained for 12 months, better diversity than ethanol-preserved samples at room temperature	(Li <i>et al.</i> , 2023)

* DSD- disodium salt dihydrate, SCTD- sodium citrate trisodium salt dihydrate

store samples at room temperature for up to 30 days without compromising microbial diversity or composition. Similar results can be noticed in Park et al.'s study (2020) using OMNIgene-Gut and NBgene-Gut, where the microbiome was stable for up to 65 days. The choice of the optimal storage temperature and economic considerations is necessary to analyze the impact of additional factors affecting the sample during storage.

Considering all the beneficial and adverse factors in the biobanking of faecal samples mentioned above, it is evident that the crucial factors determine the specific aim of the study. Beneficial Factors play a key role in maintaining the quality, stability, and integrity of faecal samples, thereby supporting the accuracy of research outcomes. On the other hand, adverse factors can result in sample degradation, alterations in composition, or difficulties in maintaining the necessary biobanking conditions. Microbial growth in stored faecal samples below -20°C seems unlikely, however, and there are bacteria in the environment, i.e. *Planococcus halocryophilus*, which can grow at -15°C and retain metabolic activity at -20°C (Mykytczuk *et al.* 2013). Below -20°C metabolic processes leading to cell lysis occurring reduce the number of viable bacteria and expose genetic material, facilitating the extraction step (Hale *et al.* 2015; Ahrabi *et al.* 2016).

11. Applicable standards for the freezing and storage of faecal samples

The increased awareness of the need for high-quality samples and the desire to standardize collection and transport criteria has created an international document – ISO 20387:2018 Biotechnology – Biobanking – General Requirements for Biobanking (Dagher 2022). The document systematizes the quality control and management system requirements of Biobanking entities wishing to confirm the reliability of the high-quality activities performed. To describe the different analytical steps, the technical committee has successively published standards describing pre-analytical laboratory processes for pretreatment in molecular biology (e.g. for tissue, plasma, serum, urine), microbiology, and parasitology. No guidelines that relate only to faecal samples in a broad context have been produced. Looking through ISO resources, you can find documents that generally define Biobank's handling of biological material. One such guideline is ISO 21899- “Biotechnology – Biobanking – General requirements for the validation and verification of processing methods for biological material in biobanks” This standard covers a wide range of biological samples, so it could potentially apply to faecal material if the biobank is involved in such work, as long as the methods used meet the validation requirements

outlined in the document. Another important: ISO/AWI TS 18701- “Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for human specimens – Isolated microbiome DNA”, is a forthcoming technical standard that will provide guidelines for the biobanking of faecal samples, including methods for their processing and storage. Its goal is to ensure consistency and quality in these procedures, enabling faecal samples to be effectively used in scientific and medical research. ISO/DIS 20070 “Biotechnology – Biobanking – Requirements for sample containers for storing biological materials in biobanks” focuses on the requirements for the management and quality of human biobanks, including the collection, processing, and storage of biological materials. In the context of faecal biobanking, this standard would ensure that proper protocols are followed to maintain the integrity and usability of faecal samples for future research, guaranteeing consistency in sample handling and data management.

At the same time, European technical specifications were being developed at the European Committee for Standardisation (CEN) to respond to the reproducibility crisis that emerged due to the lack of repeatability of experiments or errors in medical analysis. The main objective of the specifications was to define the requirements for the pre-analytical procedure with its complete documentation (Stumtner *et al.* 2022). In 2021, a standard describing the requirements for microbiome DNA isolation (CEN/TS 17626- Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for human specimen – Isolated microbiome DNA) was developed, which, to date, is the only document directly referring to the handling of faecal samples. Access to CEN/TS 17626 is the start to achieving standardization in handling and treating faeces in all research fields (Stumtner *et al.* 2022), from assessing sample composition to clinical trials with biobanked biological samples. Researchers are only required to use standardized methods for faecal testing if the work leads to diagnostic procedures to which the IVDR (In Vitro Diagnostic Regulation) is applied. In many cases, using these standards benefits the accuracy and reliability of the assays obtained. In addition to being reproducible, the results of such studies can be used for accreditation and the creation or updating of standards (Stumtner *et al.* 2022).

12. Clinical applications of faecal biobanking

According to the comprehensive approach to the patient and a greater understanding of the gut-brain microbiome axis, scientists have increased the number of tests performed to find solutions to many diseases. As previously mentioned, the importance of biobanking

in scientific research and clinical and laboratory diagnostics has increased. From the publications analyzed over the last five years, eight main research directions emerge, i.e. FMT, microbiome studies, -omics (genomic, transcriptomic, proteomic, metabolomic), cancer, bacteria, viruses and parasites and others. These publications analyzed or studied faecal samples stored under different conditions and periods. Sample storage is just one of the many uses of a biobank. Transparent procedures for handling banked samples, broadly written informed consents, and precise quality valuation of samples determine the success of storing material in research and clinical experiments (Coppola *et al.* 2019), such as research on cancer, metabolic diseases, infectious diseases, research transplantation and also epidemiological and population studies.

13. Summary

Analysis of the available literature on the storage of faecal samples has shown the importance of the appropriate storage method and cryoprotectants for maintaining the integrity of genetic material and the stability of the biological composition. In microbiome studies, choosing a proper cryoprotectant and storage temperature is crucial, mainly if the microbiome composition is being analyzed.

Further research into optimal storage conditions for faecal samples and developing more precise guidelines may improve the quality and reliability of microbiological results. The publications analyzed were based on in-house protocols, often impossible to reproduce under other conditions. The following International Standards Operations (ISO) underline several aspects important in biobanking, i.e., ISO 21899 with the processing of biological materials using validated and/or verified methods fit for the purpose, both ISO 18701 and similar CEN/TS 17626 with requirements and recommendations for the pre-examination phase of several human specimens, such as stool, saliva, skin and urogenital specimens intended for microbiome DNA examination, and ISO 20070 which focuses on information security management systems in an organization. The choice of freezing method depends on the goal and period of storage, as well as the resources of the Biobank. Faecal samples can be helpful in screening, cancer diagnosis, and research into gastrointestinal diseases, as well as bacterial and viral infections. Potential directions for further research should include continued research into optimal storage conditions for faecal samples, including the best cryoprotectant and storage temperature. It will undoubtedly contribute to increased efficacy in FMT therapy and the impact of microbiome composition on recipient health.

Research with faecal samples used in diagnostics of various diseases and developing more comprehensive standards for storing and analyzing faecal samples that enable reliable and reproducible results will interest future researchers.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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