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Abstract

Freeze-drying technology has been widely considered for decades as a suitable technique to preserve microorganisms. However, protective agents must be added prior to freeze drying to improve the survival and storage stability of the bacteria.

The objective of our study was to evaluate the effect of a new protectant medium containing sucrose (10%), trehalose (10%), skimmed milk (10%) and antioxidants on the viability of gut bacteria under different storage conditions. Two strains were tested, *Escherichia coli* and *Akkermansia muciniphila,* as examples of facultative aerobic and anaerobic bacteria, respectively. We studied the cell viability and bacterial morphology in 5 fecal samples in the presence and absence of this protectant medium using plating technique, flow cytometry and scanning electron microscopy.

The results of bacterial viability assessed by plating method showed that the protectant medium yielded higher survival rates for both strains whatever the storage conditions (85–93%) compared to normal saline solution (0.36-37.50%). It also showed its effectiveness on fecal samples, where bacterial viability after freeze-drying was 89.47±7.63% and 84.01±7.44%, as evidenced by flow cytometry analysis and plating method. However unprotected samples showed the lowest cell viability at 19.01±12.88% and 13.23±9.56%, as measured by flow cytometry and plating method. In addition, bacterial size and shape were conserved in the protectant medium. In contrast, storage without protectant medium severely damaged bacterial morphology.

In conclusion, our study is the first to use morphological features as well as culture-dependant and culture-independent tests to evaluate the effectiveness of a new protectant medium.

Introduction

Freeze-drying has been the most commonly used technique to enhance the storage stability of probiotics for decades. Probiotics are defined as living microorganisms that, once administered in adequate amounts, confer a health benefit for the host (WHO, 2001). A variety of probiotics such as yeast, *Lactobacillus*, and *Bifidobacterium* species have been successfully preserved by freeze-drying (Biavati et al., 2000; Gomes and Malcata, 1999). Recently, a novel group of obligate anaerobic bacteria was considered as the next generation of probiotics in the treatment of inflammatory bowel disease (Everard et al., 2013; Van Immerseel et al., 2010). Among them, *Faecalibacterium prausnitzii* is a strict anaerobic bacterium that constitutes 3–5% of all fecal bacteria (Miquel et al., 2013) and is considered a highly abundant butyrate producer (Breyner et al., 2017). *Akkermansia muciniphila*, a strict anaerobic mucin-degrading bacterium, is also considered an abundant candidate of the healthy human microbiota (1–5%) (Derrien et al., 2004). To our knowledge, only one recent study reported the preservation of *Akkermansia muciniphila* by freeze-drying (Marcial-Coba et al., 2018).

In addition, the human microbiota is known to play an important role in health and disease (Wang et al., 2017). Several studies have already reported that fecal microbiota transplantation (FMT) provided highly effective treatment of *Clostridium difficile* infection (Brandt et al., 2012; Hocquart et al., 2018; Kassam et al., 2013; Surawicz et al., 2013; van Nood et al., 2013). In recent years, FMT has emerged, evolving from the use of fresh fecal microbiota to the cryopreservation of fecal microbiota (Hamilton et al., 2012). However, colonoscopic administration remains invasive to patients and is therefore also complicated for the healthcare units at the technical and organizational level. Other FMT modalities are reported to be easier and less complicated. In fact, the orally-administered FMT via encapsulated fresh microbiota (Louie et al., 2013) and the frozen/thawed capsules seem to be

71 the best choice (Youngster et al., 2014). In addition, oral FMT showed the same clinical
72 efficiencies as fresh FMT in the treatment of recurrent *Clostridium difficile* infection (Lee et
73 al., 2016). However, these FMT administrations are limited by storage and transport
74 conditions.

75 In order to overcome these complications and simplify these procedures, we suggested oral
76 freeze-dried FMT, a far gentler and esthetically pleasing format that could be self-
77 administrated (Staley et al., 2017). It would also be less expensive and time-consuming. To
78 date, few studies have addressed the issue of freeze-dried fecal samples ready to be used for
79 fecal transplantation (Hecker et al., 2016; Hirsch et al., 2015; Jiang et al., 2017; Staley et al.,
80 2017).

81 In fact, freeze-drying combines freezing and drying stresses that were known to be more
82 detrimental to sensitive bacteria than cryopreservation (Heylen et al., 2012). Before the
83 freeze-drying or lyophilization process, bacterial suspensions need to be frozen first and
84 subsequently dried under vacuum. However, without suitable protectant medium, freeze-
85 drying severely damages cell membranes and proteins (Carpenter et al., 1987; Carpenter and
86 Crowe, 1988; Crowe et al., 1990; Leslie et al., 1995), causing a decrease in viability (Panoff
87 et al., 1998; Wolfe and Bryant, 1999). Furthermore, the importance of adding protectants such
88 as disaccharides (e.g. trehalose, sucrose) (Leslie et al., 1995), polyols (e.g. mannitol, sorbitol)
89 (Ana S. Carvalho et al., 2003; Efiuvwevwere et al., 1999) and proteins (e.g. skimmed milk)
90 (Castro et al., 1997) prior to freezing or drying, played a major role in preserving cells and
91 improving their storage viability. Meanwhile, their protective mechanisms remained unclear
92 and not fully understood. Three major protective mechanisms were reported: (i) preventing
93 the intra and extracellular ice formation (Baumann, 1964; Fowler and Toner, 2006) (ii) water
94 replacement hypothesis by hydrogen bonds formation (Leslie et al., 1995) (ii) or glassy matrix
95 formation (Crowe et al., 1998).

Each protectant medium impacts differently microorganisms and no universal protectant medium has yet been developed (Champagne et al., 1991; Sanders et al., 1999). The glycerol is one of the most commonly cryoprotectant used in preparation of frozen liquid suspensions of microbiota (Costello et al., 2016; Hamilton et al., 2012; Kaito et al., 2018; Satokari et al., 2015). However, this cryoprotectant is not recommended for freeze drying and is not useful due to its viscosity which can lead to a sticky and an insufficient dried product not amenable to encapsulation. For this, having a suitable protectant medium capable of preserving anaerobic and aerobic bacteria during freeze drying process can be challenging (Font de Valdez et al., 1983). Here, we investigate the effect of a protectant medium containing sucrose (10%), trehalose (10%), skimmed milk (10%) and antioxidants to preserve *Akkermansia muciniphila* and *Escherichia coli* during freezing, freeze-drying, and subsequent storage for 30 days at different conditions. This protectant medium was also used in a specific process to preserve fecal microbiota used in FMT. Thus, we evaluated the effectiveness and preservation rate of this protectant medium by studying cell viability and bacterial morphology in samples. To this end, we used plating technique, flow cytometry and scanning electron microscopy.

Materials & methods

1. Preservation of individual bacteria

1.1.Protectant medium composition

In this work, we used a protectant medium selected on the basis of our previous study patented in 2017 under the following number (N° WO/2018/234645). This medium contains per g/L in phosphate buffered saline (PBS) (Life Technologies, Paiseley, United Kingdom) the following elements: sucrose (10g); skimmed milk (10g); trehalose (5g); $CaCl_2$ (0.1g); $MgCl_2$ (0.1g); KOH (0.3/0.6g) and tree antioxidants being: Ascorbic acid (1g); Uric acid (0.4g) and Glutathione (0.1g). The pH of the medium was about 7.3 ± 0.2. Skimmed milk was

sterilized at 121°C for 15 min and the rest of the solution (antioxidants and sugar) was sterilized by filtration through 0.22 µm filters.

1.2.Bacterial strains and growth conditions

In order to prove the effectiveness of the protectant medium, facultative aerobic and strict anaerobic strains were tested. *Escherichia coli* (CSUR P1966) was grown in Columbia sheep blood agar plates (BioMérieux, Marcy l'Etoile, France) at 37°C for 24 hours under aerobic conditions. *Akkermansia muciniphila* strain (CSUR P6566) was grown in Columbia sheep blood agar plates at 37°C for 48 hours under anaerobic conditions using a GasPak generator (Becton Dickinson Microbiology Systems, Sparks, MD, USA). Both strains were isolated from fecal materials of two healthy donors in our laboratory.

1.3.Freeze-drying procedures

Firstly, we chose the best time to harvest bacterial colonies with higher viable rates and less dead bacteria based on flow cytometry measurements and microscopic observations at different incubation times (12, 24 and 75 hours). Fresh *Escherichia coli* and *Akkermansia muciniphila* were harvested directly from agar plates after 24 hours and 48 hours, respectively, and mixed with two solutions: (**a**) normal saline solution (NaCl 0.9%) (Fresenius Kabi, Sevres, France) as control, and (**b**) the protectant medium (milk 10% + sucrose 10% + trehalose 5%+ antioxidants), with a final concentration of 2-3x10^{10} CFU/mL and 5-4x10^{10} CFU/mL, respectively, for *Akkermansia muciniphila* and *Escherichia coli.*

Bacterial suspensions in (**a**) normal saline solution and in (**b**) the protectant medium were placed into three 2 mL, type I (Wheaton, Millville, NJ, USA) serum vials and frozen at -80°C for 5 hours. Suspensions were desiccated in a DELTA 1-24 LSC-CHRIST freeze-dryer at a condenser temperature of -80°C and at a chamber pressure of 0.63 mbar for 12 hours at 0°C, followed by 3 hours at + 30°C. After freeze-drying, vials were sealed manually, and stored at +4°C (Figure 1).

1.4.Storage conditions

Bacterial suspensions in presence of protectant medium or saline solution were stored for 48 hours and for 30 days under different conditions (Figure 1):

(i) Frozen at -80°C,

(ii) Frozen at -196°C by dipping the vials into liquid nitrogen (LN_2),

(iii) Frozen at 80°C for 5 hours then freeze-dried,

(iiii) At +4°C.

After freeze-drying, samples were rehydrated and homogenized with phosphate buffered saline (PBS) to return to their original volume (500 µL) at 25°C and incubated at room temperature.

2. Preservation of fecal samples

2.1.Stool sample collection and preparation

Fresh fecal samples were obtained from 5 healthy donors (2 women, 3 men) from France, with a normal body mass index. These volunteers were not subjected to any feeding trial, specific diet, or antibiotic treatment for the last six months prior to sampling. Stool samples were collected in a sterile stool container under anaerobic conditions by using a GasPak generator (Becton, Dickinson and Sparks, USA) and were then immediately transported to the laboratory. Donors were informed of the study and signed informed consents. The project received the IHU Méditerranée Infection ethics committee agreement under number 2016-011.

2.2.Stool sample preparation

Sample preparations were carried out under a sterile hood. Briefly, 50g of fecal matter was blenderized (BOSCH Ultracompact 400W device) with 250 mL of normal saline solution (NaCl 0.9%) (Fresenius Kabi, Sevres, France) for 5 min, then sieved using coffee strainers to remove food debris. The slurry was centrifuged for 15 min at 6,000 x g. The pellet was

suspended in one half of the initial volume in 2 media: (a) the normal saline solution used as control, and (b) the protectant medium (skimmed milk 10%+sucrose10% + trehalose 5% + antioxidants). Then, bacterial suspensions were frozen at -80°C for 5 hours and directly freeze-dried under the same conditions as described above, and finally stored at +4°C (Figure 1).

3. Cell viability determination

Bacterial viability of each storage condition, (a) in normal saline solution and (b) in protectant medium was calculated before and after freeze-drying using viable counts and flow cytometry method.

3.1.1. Enumeration of bacteria by plate count

Decimal dilutions were prepared with PBS. One hundred µL of the sample suspension was mixed with 900 µL of PBS, vortexed for 10 seconds and serially diluted with PBS. The Columbia agar plates were divided up into three or four lines into which 10µL of each dilution were speared onto it. Each dilution was plated in triplicates. The plates were incubated at 37°C for 48 h under aerobic and anaerobic (GasPak generator) conditions.

The number of colony-forming units per milliliter (CFU/mL) was determined and the viability was defined as the percentage ratio of viable cells after freeze-drying and viable cells before freeze-drying using the following equation:

$$\text{Survival } \% = \frac{Livecellsbeforefreeze-drying(CFU/ml)}{Livecellsafterfreeze-drying(CFU/ml)} \times 100$$

3.1.2. LIVE/DEAD enumeration

The membrane integrity was determined using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen, USA) as described previously (Bellali et al., 2019).

4. Scanning electron microscopy for bacterial morphology evolution

Fresh, frozen and freeze-dried samples were either directly smeared onto microscopy slides or cyto-centrifuged on cytospin slides. Slides were then processed to image acquisition, after staining with PTA (phosphotungstic acid 1 %) in order to check morphological appearance changes and cell integrity.

We used a table top scanning electron microscope SEM (Hitachi TM4000 Plus) to evaluate bacterial structures. The SEM has a capability of observing specimen in low vacuum pressure (100 Pa to 101 Pa) to reduce charge-up on the specimen's surface by the irradiated electrons. Evacuation time after the loading of specimens into the SEM Chamber is shorter than 2 minutes, which is much quicker than conventional SEMs with high vacuum condition. The imaging process for all samples in the presence and absence of protectant medium was acquired at the same acquisition settings regarding magnification, intensity and voltage mode. All settings are displayed on micrographs.

5. Statistical analysis

All experiments were carried out in triplicate. All viability data are expressed as the means standard deviations (SDs). The difference between two means before and after freeze-drying was calculated using the Mann-Whitney t-test (Prism v5.0, GraphPad). Means were considered significantly different when *P-value* was less than 0.05. Differences between fresh viable cells and all storage conditions were analyzed by one-way analysis of variance followed by a Bonferroni's multiple comparisons test (Prism v5.0, GraphPad).

Results

Evolution of the protectant medium efficacy by reporting the viability of individual bacteria after freezing and freeze-drying

1. Viability of *Escherichia coli* and *Akkermansia muciniphila* after freezing and freeze drying

The effect of protectant medium and saline water solution on the viability of *Escherichia coli* and *Akkermansia muciniphila* under the different storage conditions (for 48 hours and 30 days of storage) are summarized in Figure 2 and compared to fresh cultures suspended in protectant medium and saline water solution.

(a) Freezing at -80°C

The viability of *E. coli* with protectant medium (85.00%) did not vary after 48 hours of freezing and remained stable after 30 days of storage (84.00%). Similarly, the viability of *A. muciniphila* did not greatly decreased after 48 hours of freezing (86.67%) and even after 30 days of storage (75.00%). However, the freezing in presence of normal saline had significantly reduced the viability of *E. coli* and *A. muciniphila* to 25% and 0.67% respectively. Thus, after 30 days of storage the viability remained reduced to 24.17% and 0.04% respectively.

(b) Freezing in liquid nitrogen (-196°C)

Similar results were obtained after fast freezing in liquid nitrogen, where frozen *E. coli* and *A. muciniphila* with protectant medium exhibited high survival rates of 93.00% and 90.00% respectively. The viability of both strains remained unchanged, even after 30 days of storage (90.00% and 86.67%, respectively). However, unprotected *E. coli* and *A. muciniphila* showed low viability of 37.50% and 25.56% respectively, compared to that observed in the presence of a protectant medium. After 30 days of storage, the viability of *E. coli* and *A. muciniphila* was 25.00% and 17.00% respectively.

(c) Freeze-drying

Results showed that freeze-dried *E. coli* and *A. muciniphila* in the presence of protectant medium had the highest survival rates of 85.00% and 91.67% respectively after 48 hours. It remained stable after 30 days of storage, 80.00% and 83.33% respectively. In the presence of normal saline solution, the freeze-drying process severely damaged cell viability of *E. coli*

(2.67%) and *A. muciniphila* (0.36%) compared to freezing. The viability of *E. coli* and *A. muciniphila* significantly decreased to 0.29% and $1x10^8$ CFU/mL; 0.02%, respectively.

(d) Storage at +4°C

Bacterial suspensions of *E. coli* and *A. muciniphila* in the presence of protectant medium, exhibited high survival rates of 80.00% and 90.00% respectively after 48 hours of storage at 4°C. Meanwhile, the viability of *A. muciniphila* and *E. coli* was declined to 60.00% and 41.65% respectively after 30 days of storage. However, both *A. muciniphila* and *E. coli,* suspended in normal saline water, were affected within the 48 hours of storage, with a large drop of viability (29.17% and 33.33%, respectively) after 48 hours. After 30 days of storage, cell viability of *E. coli* and *A. muciniphila* (25.00%) and (16.67%), respectively were higher than freezing or freeze drying.

2. Evaluation of bacterial morphology and shape integrity by scanning electron microscopy after freezing and freeze-drying

Bacterial morphology and integrity of both *Escherichia coli* and *Akkermansia muciniphila* treated with (**a**) normal saline solution or with (**b**) protectant medium in fresh, frozen and freeze-dried state were evaluated for each conservation condition.

Micrographs of samples in the presence of normal saline solution showed damaged *Escherichia coli* cells under all storage conditions tested (freezing or freeze-drying). The degenerative aspect of the bacteria was clearly observed (Figure $3b_1$, $3b_2$, $3c_1$, $3c_2$, $3d_1$, $3d_2$). Similar results were obtained in *Akkermansia muciniphila* with an irregularity in bacterial shapes and an increase in cell size due to osmotic stress (2.59±1.45µm versus 0.78±0.24µm) (Figure $4b_1$, $4b_2$, $4b_3$, $4b_4$). Nevertheless, morphology of both bacteria was preserved in the presence of the protectant medium, which shape and size seemed to be better conserved. Frozen or freeze-dried *Akkermansia muciniphila* were predominantly spherical, although some were found to be irregular or elongated in the same way as fresh cells (Figure $4c_1$, $4c_3$,

$4d_1$, $4d_3$). Interestingly, we detected small shapes of *Escherichia coli* that were osmotically-dehydrated due to sucrose and trehalose contained in the protectant medium (Figures $3e_1$, $3e_2$, $3f_1$, $3f_2$, $3g_1$, $3h_1$).

Validation of the proof of concept by evaluating the effect of the protectant medium on the viability of freeze-dried fecal samples

1. Enumeration of fecal material by plate method

Total bacterial counts (anaerobic and aerobic counts), and bacterial viability were presented in Table 1. Total bacterial counts ranged from $3.42x10^9$ CFU/mL to $6.92x10^9$ CFU/mL, and from $4.10x10^9$ CFU/mL to $8.75x10^9$ CFU/mL, respectively, for samples resuspended in normal saline solution and in the protectant medium.

Before freeze-drying, we noticed differences between total cell counts in all fresh fecal samples, whatever protected or not, so that the number of anaerobic bacteria was ten times higher than that of aerobic bacteria in five samples. Moreover, anaerobic bacteria were much more numerous for samples suspended in protectant medium ($5.90x10^9 \pm 1.92\ x10^9$) than in normal saline solution ($4.25\ x10^9 \pm 1.47\ x10^9$) (Table 1).

After freeze-drying, all five freeze-dried fecal samples with normal saline solution decreased in viability and had a lower average survival rate of 13.23±9.56%, ranging from 1.69% to 26.27%, compared to samples dried in the presence of protectant medium, producing an average survival rate of 84.01±7.44% ranging from 75.60% to 94.12% (Table 1).

As shown in Figure 5A, we found significant differences before and after freeze-drying when the fecal sample was dried with normal saline solution (P=0.01). On the other hand, no significant differences were found for samples freeze-dried in the presence of protectant medium as compared to fresh fecal samples (Figure 5B).

2.1.Enumeration of fecal samples by flow cytometry

In addition to plate count methods, we used the flow cytometry method to assess the viability of freeze-dried fecal samples. IP and SYTO9 were used simultaneously for viability assessment before and after freeze-drying. Three bacterial populations were observed; live (SYTO9-stained), dead (IP-stained) and injured, which were double stained (IP/SYTO9-stained).

The relative percentages of live, dead and injured bacterial populations obtained before and after freeze-drying for the five fecal samples are presented in Figure 6. Before freeze-drying, the relative percentage of viable fecal bacteria in the presence of normal saline solution were: 84.01% (sample 1), 66.65% (sample 2), 61.93% (sample 3), 68.90% (sample 4), and 65.98%(sample 5).Therefore, after freeze-drying it decreased to 16.67% (sample 1), 23.48% (sample 2), 5.85% (sample 3), 18.67% (sample 4) and 2.27% (sample 5) (Figure 6). Simultaneously, dead and injured bacterial populations increased, whereas, in the presence of the protectant medium, we did not observe any significant changes before (79.87%, 64.52%, 68.38%, 73.13%, and 70.73%) and after (73.10%, 56.77%, 55.54%, 74.12%, 60.31%) freeze-drying (Figure 6). Overall, the presence of protectant medium during freeze-drying showed a higher protection of bacteria (89.47±7.63%), while normal saline solution damaged most of them (19.01±12.88%). These results were similar to those found by plate counting method.

Furthermore, using statistical analysis, we found a significant difference (P= 0.0079) between live bacterial counts before and after freeze-drying of samples dried with saline solution (Figure 5C). Meanwhile, viable count of samples dried with protectant medium were not statistically significant (Figure 5D).

3. Electron microscopy of fecal material before and after freeze drying

The processed fecal samples showed significant differences of their bacterial components at the level of shape and size. Before freeze-drying, stool samples in the presence of normal saline solution showed a bigger bacterial size (1.06±0.15µm) (Figure 7a_1-a_2) compared to those suspended in protectant medium (0.70±0.14µm) (Figure 7b_1-b_2). Furthermore, after freeze-drying, the protectant medium conferred a more stable state to the bacteria, where size and shape remained conserved and smaller (0.61±0.17µm) (Figure 7d_1-d_2) in contrast to the normal saline solution (1.05±0.16µm) (Figure 7c_1-c_2).

Discussion

Freeze-drying in the presence of a protective medium to ensure optimal bacterial viability plays a key role in microbiology nowadays. This quality of preservation is extremely needed in FMT and probiotics production.

In this study, we evaluated the impact of freeze drying on the bacterial viability using the normal saline solution as a control typically used in the majority (62%) of clinical studies of FMT (Gough et al., 2011; Van Nood et al., 2013), and a new protectant medium containing sucrose (10%), trehalose (10%), skimmed milk (10%) and three antioxidants (uric acid, ascorbic acid and glutathione). Those compounds were found in several studies, in combination or individually, to be effective in protecting bacteria against the injuries during freezing or freeze drying and improved storage stability. Several investigators have reported the positive effect of trehalose on bacterial survival during freeze drying (Jain and Roy, 2010; Mensink et al., 2017). Trehalose is also known as an antioxidant protecting membranes against oxidative stress (Herdeiro et al., 2006). In addition, trehalose and sucrose were the most disaccharides commonly used as protectants during the freezing-drying process (Broeckx et al., 2016; Carpenter and Crowe, 1988; A. S. Carvalho et al., 2003, 2002; Crowe et al., 1998, 1988; Leslie et al., 1995; Linders et al., 1997; Paiva and Panek, 1996; Zayed and Roos, 2004). These two sugars were capable of protecting cell membranes by water

339 replacement involved hydrogen bonding between sugars and polar-head groups of the
340 phospholipids (Crowe et al., 1990; Leslie et al., 1995; Rudolph and Crowe, 1985). They also
341 were capable of reducing the ice formation by increasing the shrinkage of cells before
342 freezing (Fowler and Toner, 2006). Remarkably, this dehydration mechanism was observed in
343 our results carried out by scanning electron microscopy, where cells of *E. coli* were found
344 dehydrated before and after freezing and drying due to the presence of sugars that induced
345 osmosis-derived dehydration. In contrast, in the absence of sugars, cells of *E. coli* and
346 *Akkermansia muciniphila* were found injured and severally damaged after freezing and drying
347 due to ice crystal formation, as described previously in several studies (Champagne et al.,
348 1991; Sanders et al., 1999). Such a mechanism still remains unclear.

349 Proteins also provided an additional protective effect by covering the cells and balancing the
350 cell membranes during freeze-drying and storage (Buitink et al., 2000). Indeed, skimmed milk
351 was selected as an efficient drying medium (Bevilacqua et al., 2012; Hubálek, 2003), it
352 contains proteins providing an additional protective layer for the cells (Abadias et al., 2001;
353 Carvalho et al., 2004) and stabilizing membrane components (Castro et al., 1996; Selmer-
354 Olsen et al., 1999). Another feature of skimmed milk is its ability to dry easier and provide a
355 higher yield of dry matter. According to the study reported by Zayed and Roos (Zayed and
356 Roos, 2004), the addition of skimmed milk to the mixture of trehalose and sucrose gave a
357 higher survival rate during subsequent storage (Abadias et al., 2001).
358 Furthermore, previous studies reported that the mixture of many components of protectant
359 medium (e.g. sucrose + trehalose, sucrose + trehalose + skimmed milk…etc.) could result in a
360 better protection of microorganisms than single component due to additive or synergic
361 protective effects (Berner and Viernstein, 2006; Celik and O'Sullivan, 2013; Hubálek, 2003;
362 Jalali et al., 2012; Keivani Nahr et al., 2015; Ming et al., 2009; Sharma et al., 2014; Yang et
363 al., 2007; Yu et al., 2017; Zhang et al., 2014).

Regarding total anaerobic bacteria in fecal samples prior to freeze drying, we found that samples suspended with our protectant medium had more anaerobes than samples without protectant. The higher number of anaerobes was explained in our previous study (N° WO/2018/234645) by the presence of antioxidants in our protectant medium preserving sensitive anaerobic bacteria from oxygen. In addition to *Akkermansia muciniphila*, our protectant medium showed superior efficacy to preserve other fastidious anaerobes such as *Treponema denticola* and *Treponema pectinovorum* (Data unpublished) and also extremely oxygen-sensitive (EOS) bacteria such as *Methanobrevibacter smithii* and *Faecalibacterium prausnitzii* (Data unpublished).

Creating a medium protecting the majority of the gut bacteria was challenging. We chose *Escherichia coli* as an example of aerobic bacteria and *Akkermansia muciniphila* as an example of anaerobic bacteria in addition to its beneficial effects. We recommend the freeze drying of anaerobic and aerobic bacteria using the protectant medium comprising sucrose (10%), trehalose (10%), skimmed milk (10%) and antioxidants. We believe that this protectant medium holds great promises for therapeutic purposes such as fecal microbiota transplant and probiotics.

The idea of creating this protectant medium comes from many conservation strategies and for the first time we used morphological, culture-dependent and culture-independent tests to evaluate its effectiveness. Further studies will be conducted to test freeze-dried microbiota transplantation in oral capsules using this protectant medium.

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397 **Competing Interests**

398 The authors declare no competing interests.

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Tables and Figures

Table 1: Total bacterial count (CFU/mL) before and after freeze drying, in the presence of protectant medium and normal saline solution and survival rates (%) after freeze drying.

Samples	Medium of conservation	Culture conditions	Before freeze- drying (CFU/mL)	After freeze-drying (CFU/mL)	% of viability
Sample 1	Normal saline	Anaerobe	3,00E+09	3,10E+08	10,33
		Aerobe	4,22E+08	7,82E+07	18,53
		Total cells	3,42E+09	3,88E+08	11,34
	Protectant medium	Anaerobe	4,60E+09	4,10E+09	67,39
		Aerobe	2,22E+08	1,82E+08	81,98
		Total cells	4,82E+09	4,28E+09	88,80
Sample 2	Normal saline	Anaerobe	6,33E+09	1,74E+09	27,49
		Aerobe	5,92E+08	7,82E+07	13,21
		Total cells	6,92E+09	1,82E+09	26,27
	Protectant medium	Anaerobe	7,60E+09	6,00E+09	78,95
		Aerobe	2,00E+08	1,82E+08	91,00
		Total cells	7,80E+09	6,18E+09	79,26
Sample 3	Normal saline	Anaerobe	3,33E+09	2,44E+08	7,33
		Aerobe	5,70E+08	6,82E+07	11,96
		Total cells	3,90E+09	3,12E+08	8,01
	Protectant medium	Anaerobe	4,00E+09	3,00E+09	75,00
		Aerobe	1,00E+08	1,00E+08	100,00
		Total cells	4,10E+09	3,10E+09	75,61
Sample 4	Normal saline	Anaerobe	3,30E+09	6,44E+08	19,52
		Aerobe	5,70E+08	8,60E+07	15,09

		Total cells	3,87E+09	7,30E+08	18,86
	Protectant medium	Anaerobe	5,00E+09	4,70E+09	94,00
		Aerobe	1,00E+08	1,00E+08	100,00
		Total cells	5,10E+09	4,80E+09	94,12
Sample 5	Normal saline	Anaerobe	5,28E+09	1,00E+08	1,89
		Aerobe	8,00E+08	3,00E+06	0,38
		Total cells	6,08E+09	1,03E+08	1,69
	Protectant medium	Anaerobe	8,30E+09	7,00E+09	84,34
		Aerobe	4,50E+08	2,00E+08	44,44
		Total cells	8,75E+09	7,20E+09	82,29
Mean	Normal saline	Anaerobe	$(4,25\pm1.47)\times10^{9}$	$(6,08\pm6.64)\times10^{8}$	13,31 (±10.18)
		Aerobe	$(5,91\pm1.35)\times10^{8}$	$(6,27\pm3.40)\times10^{7}$	11,83 (±6.87)
		Total cells	**$(4,84\pm1.56)\times10^{9}$**	**$(6,70\pm6.80)\times10^{8}$**	**13,23 (±9.56)**
	Protectant medium	Anaerobe	$(5,90\pm1.92)\times10^{9}$	$(4,96\pm1.76)\times10^{9}$	84,28 (±7.63)
		Aerobe	$(2,14\pm1.43)\times10^{8}$	$(1,53\pm4.88)\times10^{8}$	83,49 (±23.07)
		Total cells	**$(6,11\pm2.03)\times10^{9}$**	**$(5,11\pm1.79)\times10^{9}$**	**84,01 (±7.44)**

Figure 1: Schematic figure illustrating the design of the protocols established to evaluate the effect of (**a**) saline water and (**b**) the protectant medium on the viability of bacteria after freezing and freeze-drying. (**I**) On individual bacteria, *Akkermansia muciniphila* and

689 *Escherichia coli*. (**II**) On fecal samples. Bacterial viability and cell integrity were evaluated
690 using the plating method, flow cytometry and scanning electron microscopy.

691 **Figure 2:** Effect of protectant medium and saline water solution on the viability of **(A)**
692 *Akkermansia muciniphila* expressed in log CFU/mL, **(B)** *Escherichia coli* expressed in log
693 CFU/mL **(C)** *A. muciniphila* expressed the % of viability and **(D)** *E. coli* expressed the % of
694 viability, during freezing (at 80°C and in liquid nitrogen), freeze drying, and at 4°C after 48
695 hours and 30 days of storage. Freeze-dried cells were stored at 4°C. Viability was measured
696 before and after all storage conditions using the plate counting method. Statistically
697 significant differences between fresh cells and all storage conditions were analyzed by one-
698 way analysis of variance followed by Bonferroni's multiple comparisons test. $*P < 0.01$ $**P$
699 < 0.001 $***P < 0.0001$, **ns**: not significant.

700 **Figure 3: Scanning electron micrographs** of *Escherichia coli* conserved in normal saline
701 solution and with protectant medium in fresh state (**a_1- a_2**, **e_1- e_2**, respectively), after freeze
702 drying (**b_1- b_2**, **f_1- f_2**, respectively), freezing at -80°C (**c_1- c_2**, **g_1- g_2**, respectively) and in liquid
703 nitrogen (**d1- d2, h1- h2**, respectively). **Blue asterisk**: dehydrated bacteria, **yellow asterisk:**
704 non-dehydrated bacteria, **yellow arrows:** skimmed milk particles.

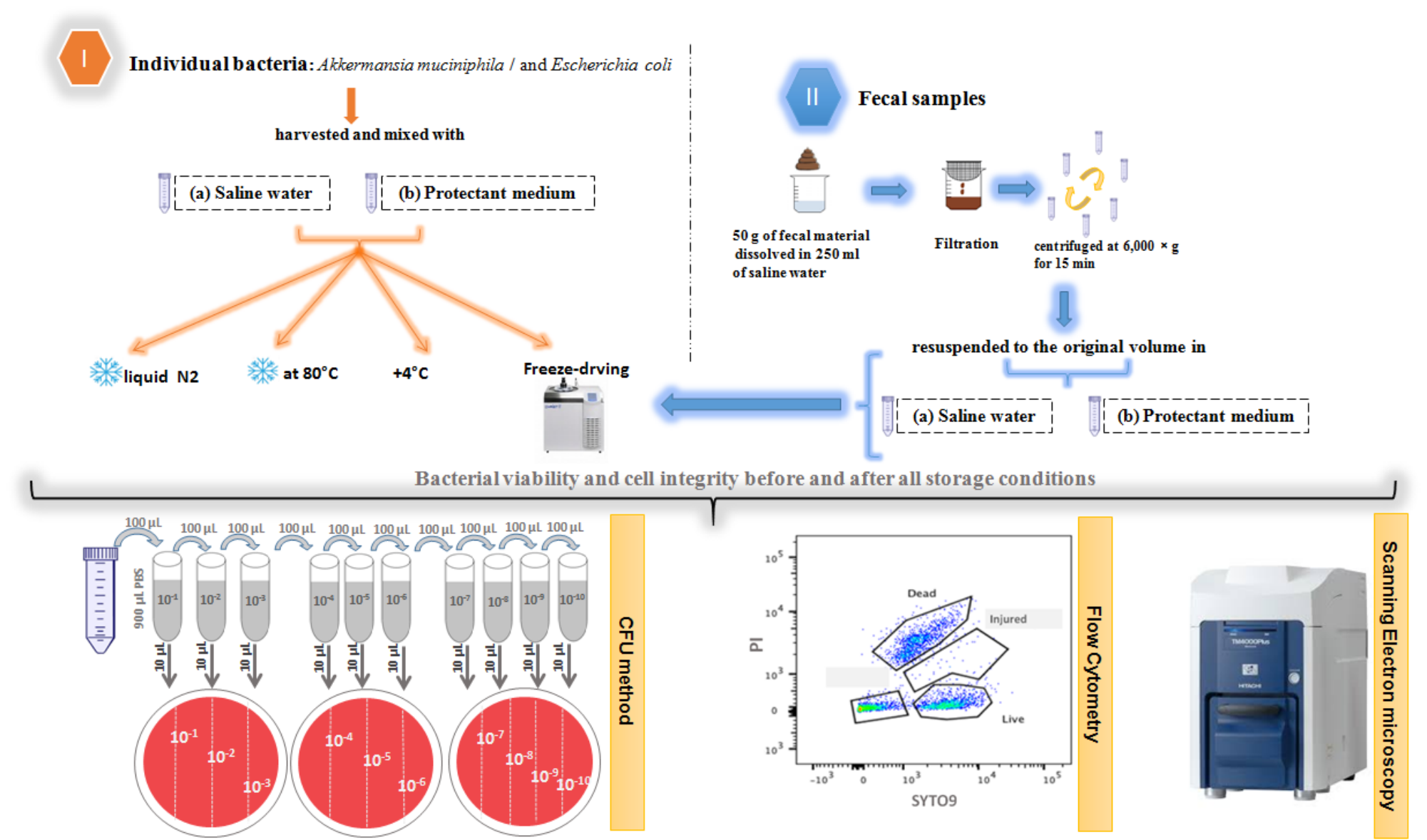
705 **Figure 4: Scanning electron micrographs** of *Akkermansia muciniphila* conserved in normal
706 saline solution and with protectant medium in fresh state (**a_1- a_4**, **c_1- c_4**, respectively) and after
707 freeze-drying (**b_1- b_4**, **d_1- d_2**, respectively). **Yellow asterisk:** *Akkermansia muciniphila*
708 bacterium, **yellow arrows:** skimmed milk particles.

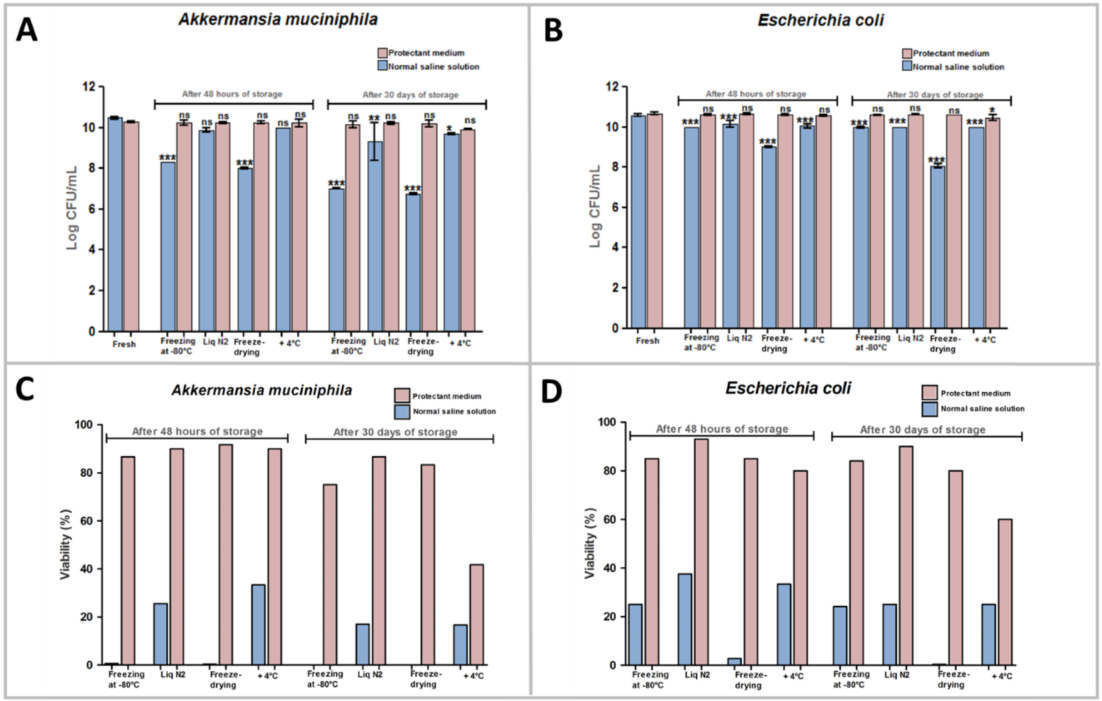
709 **Figure 5:** Statistical differences of viable count of 5 fecal samples before and after freeze
710 drying in presence of protectant medium and normal saline solution. Viable counts were
711 performed by plating count methods (log CFU/mL) (**A, B**) and flow cytometry technique (log
712 cells/ mL) (**C, D**). $*P < 0.01$, **ns**: not significant.

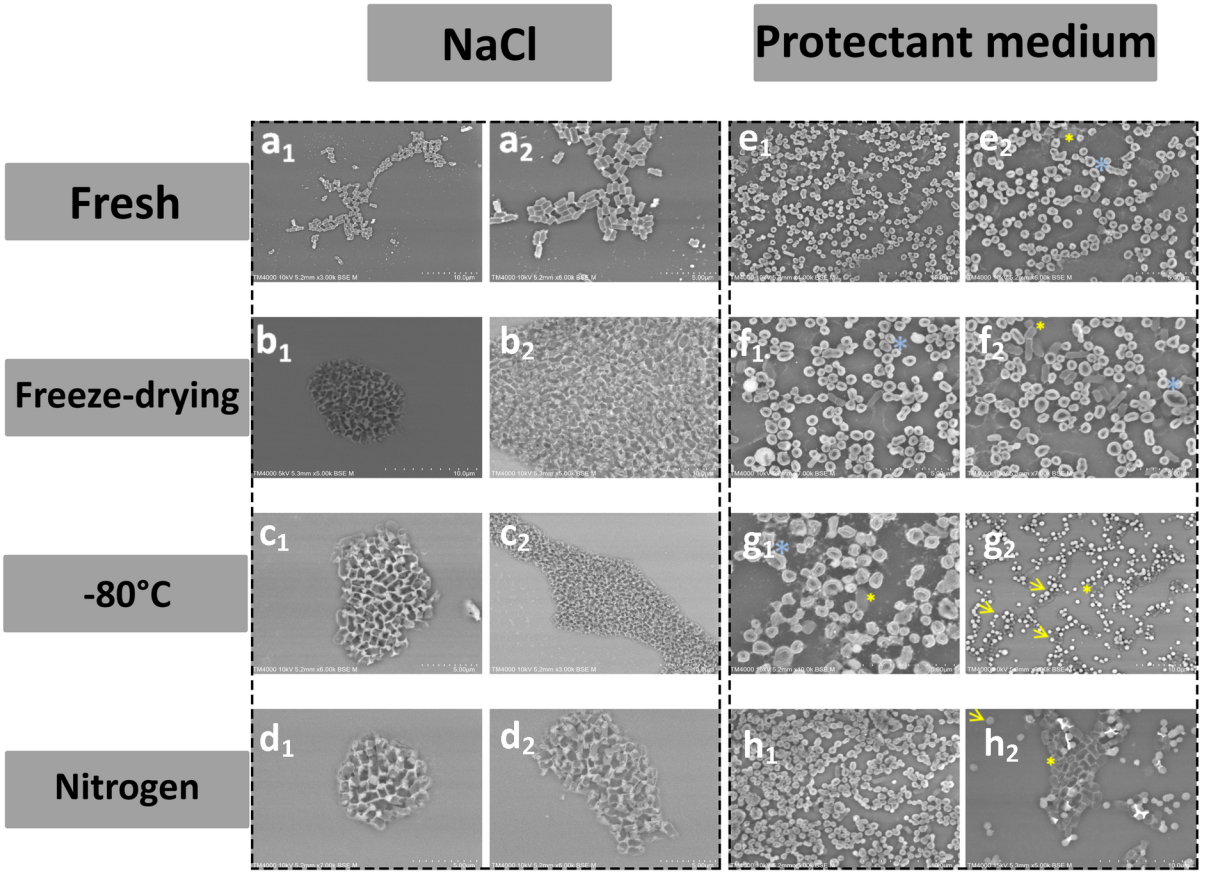
713 **Figure 6**: Relative abundance and flow cytometry measurements (Cells/mL) of Live/Dead
714 and Injured bacterial population of 5 fecal samples before and after freezing drying using
715 normal saline solution and protectant medium.

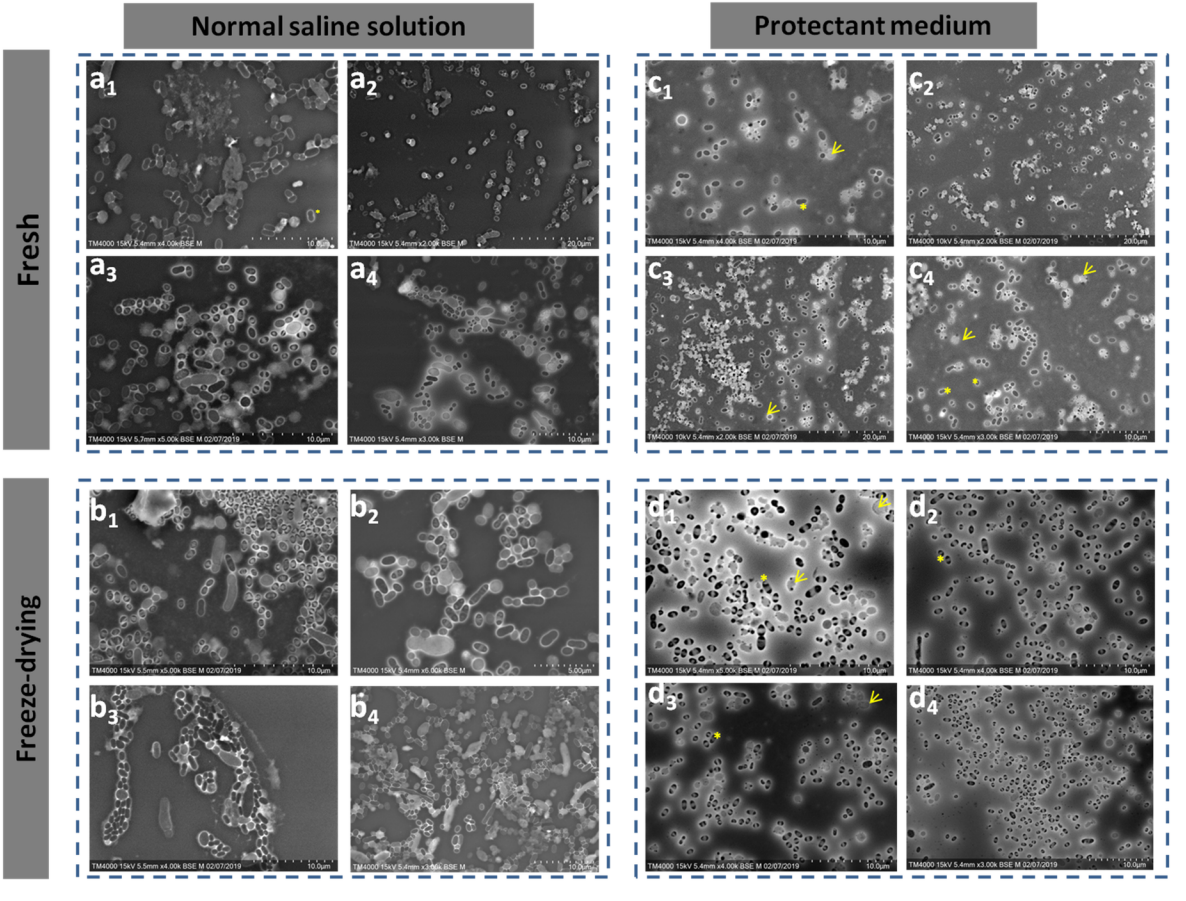
716 **Figure 7: Scanning electron micrographs** of stool sample conserved in normal saline
717 solution and with protectant medium before (**a_1- a_2**, **b_1- b_2**, respectively) and after freeze
718 drying (**c_1- c_2**, **d_1- d_2**, respectively).

719

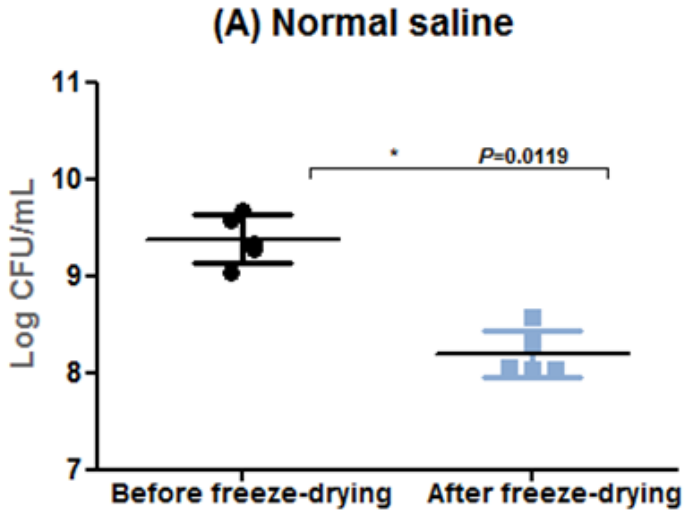

I
Individual bacteria: Akkermansia muciniphila / and Escherichia coli
harvested and mixed with
(a) Saline water
(b) Protectant medium
liquid N2
at 80°C
+4°C
Freeze-drving
II
Fecal samples
50 g of fecal material dissolved in 250 ml of saline water
Filtration
centrifuged at 6,000 × g for 15 min
resuspended to the original volume in
(a) Saline water
(b) Protectant medium
Bacterial viability and cell integrity before and after all storage conditions
100 µL
900 µL PBS
10 µL
CFU method
Dead
Injured
Live
PI
SYTO9
Flow Cytometry
Scanning Electron microscopy

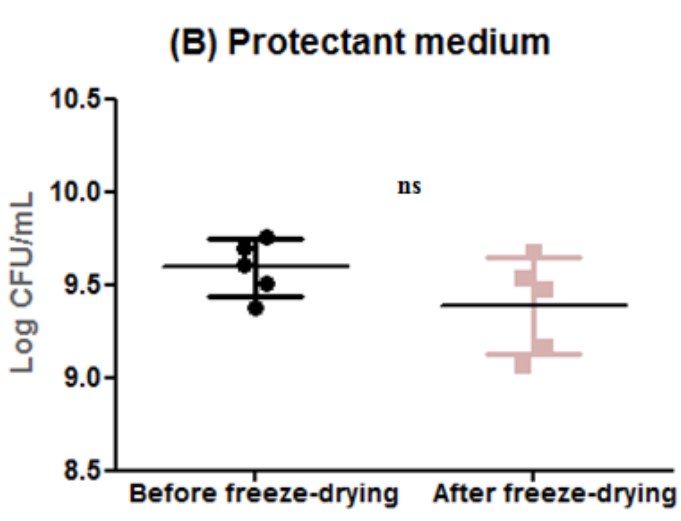

A
Akkermansia muciniphila
Protectant medium
Normal saline solution
Log CFU/mL
After 48 hours of storage
After 30 days of storage
Fresh
Freezing at -80°C
Liq N2
Freeze-drying
+ 4°C
B
Escherichia coli
C
Akkermansia muciniphila
Viability (%)
D
Escherichia coli


NaCl
Protectant medium
Fresh
Freeze-drying
-80°C
Nitrogen
a1
a2
e1
e2
b1
b2
f1
f2
c1
c2
g1
g2
d1
d2
h1
h2

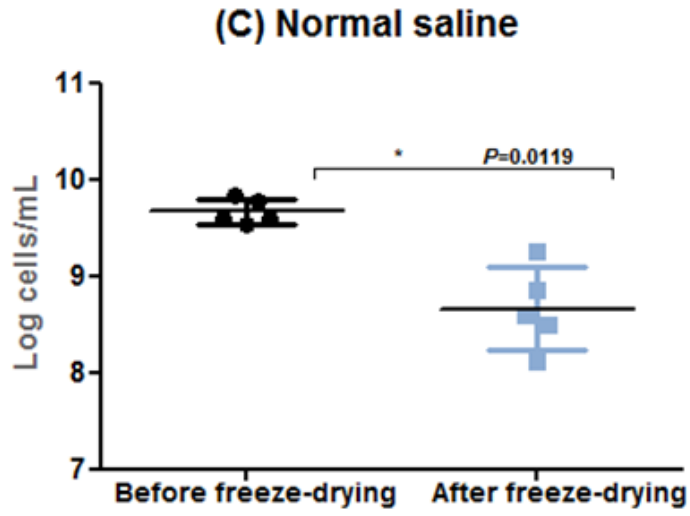

Normal saline solution
Protectant medium
Fresh
Freeze-drying
a1
a2
a3
a4
b1
b2
b3
b4
c1
c2
c3
c4
d1
d2
d3
d4

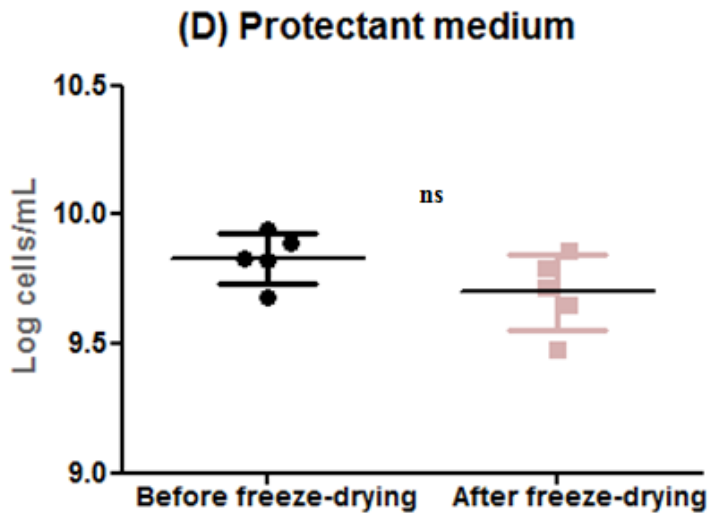
Viable counts

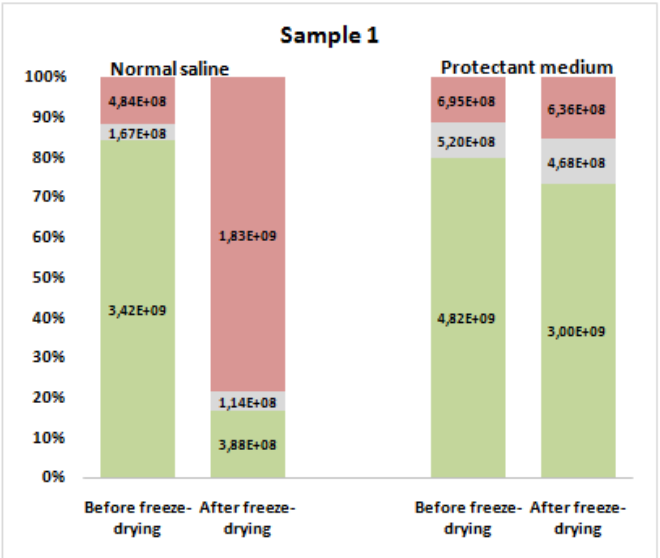


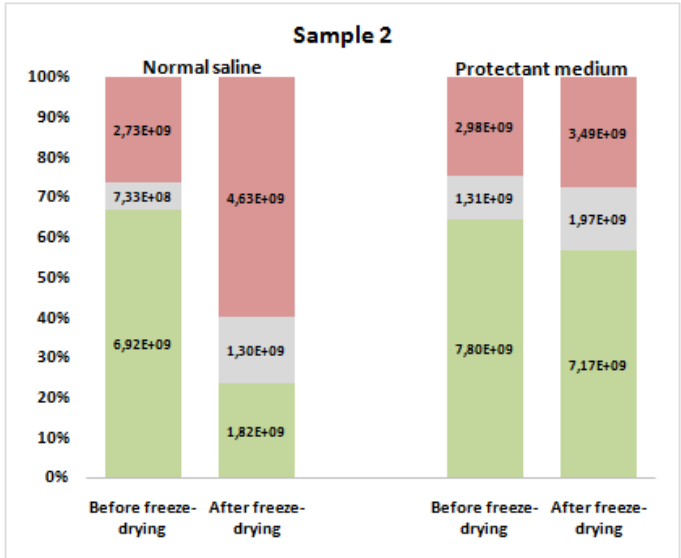


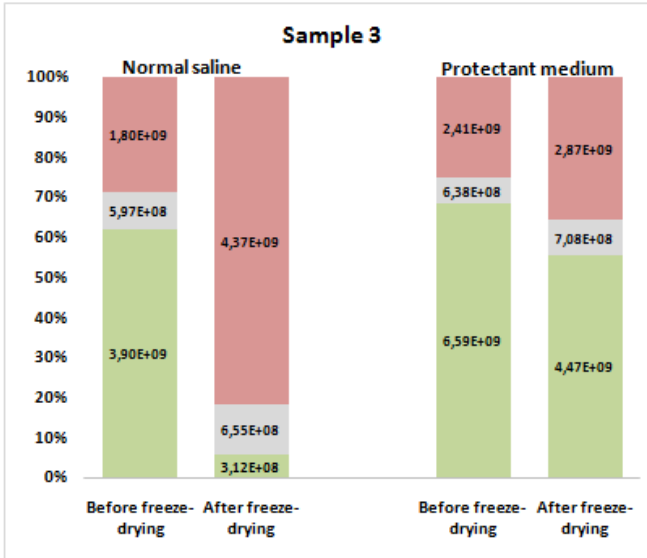
Flow cytometry counts

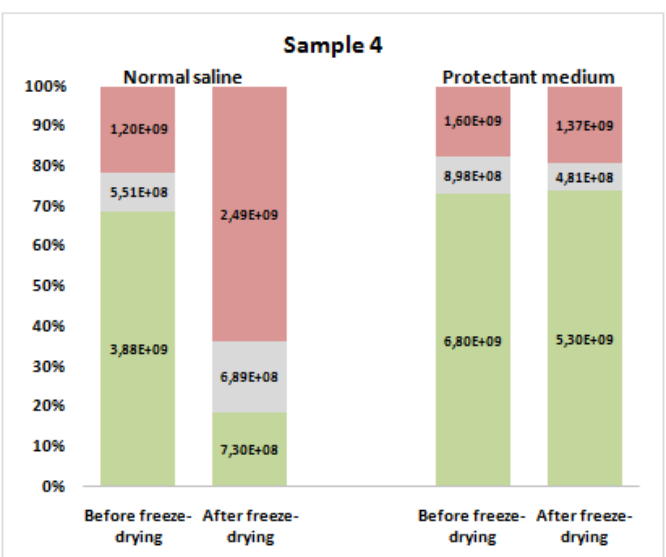


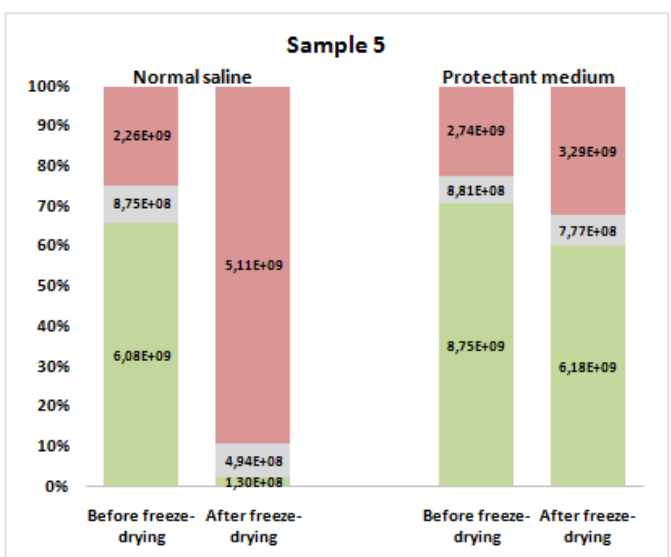


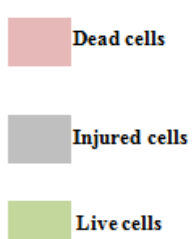

Sample 1
Normal saline
Protectant medium
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
4,84E+08
1,67E+08
3,42E+09
1,83E+09
1,14E+08
3,88E+08
6,95E+08
5,20E+08
4,82E+09
6,36E+08
4,68E+08
3,00E+09
Before freeze-drying
After freeze-drying
Before freeze-drying
After freeze-drying

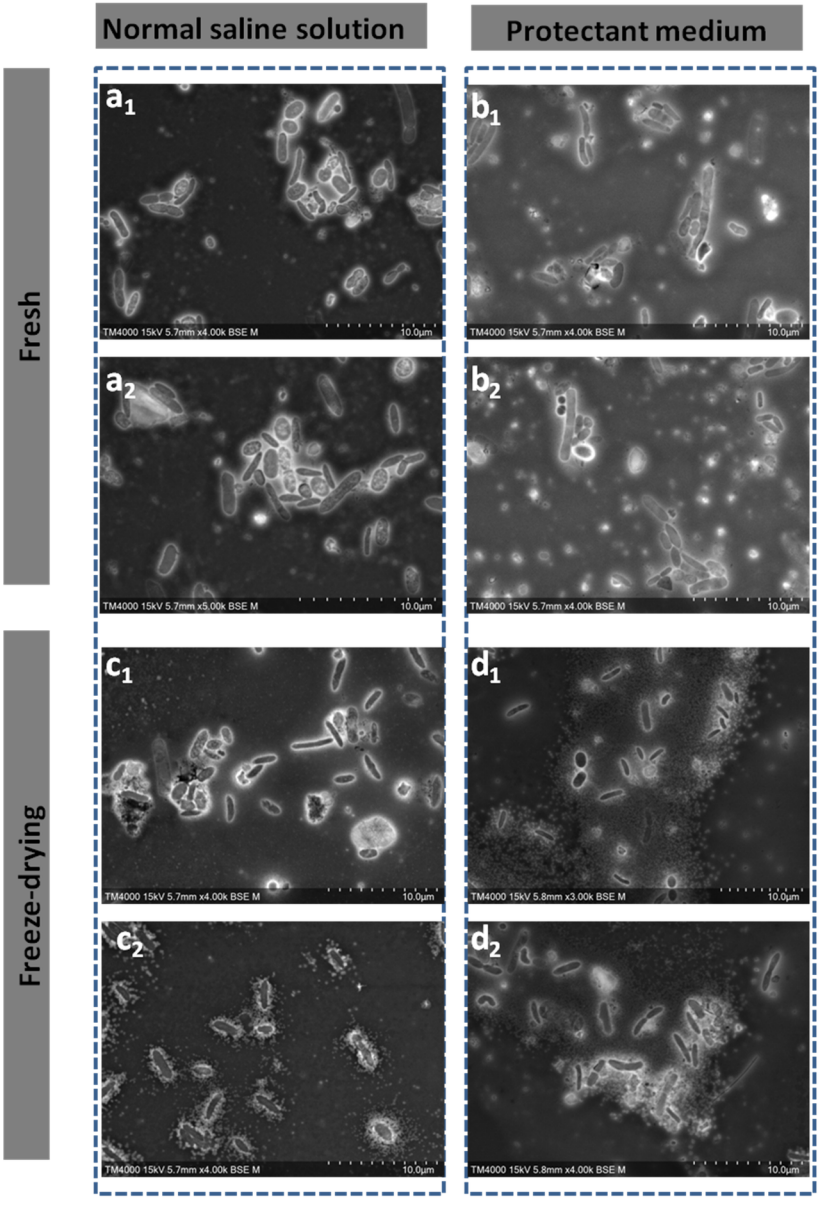

Sample 2
Normal saline
Protectant medium
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
2,73E+09
7,33E+08
6,92E+08
4,63E+09
1,30E+09
1,82E+09
2,98E+09
1,31E+09
7,80E+09
3,49E+09
1,97E+09
7,17E+09
Before freeze-drying
After freeze-drying
Before freeze-drying
After freeze-drying


Sample 3
Normal saline
Protectant medium
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1,80E+09
5,97E+08
3,90E+09
4,37E+09
6,55E+08
3,12E+08
2,41E+09
6,38E+08
6,59E+09
2,87E+09
7,08E+08
4,47E+09
Before freeze-drying
After freeze-drying
Before freeze-drying
After freeze-drying


Sample 4
Normal saline
Protectant medium
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1,20E+09
5,51E+08
3,88E+09
2,49E+09
6,89E+08
7,30E+08
1,60E+09
8,98E+08
6,80E+09
1,37E+09
4,81E+08
5,30E+09
Before freeze-drying
After freeze-drying
Before freeze-drying
After freeze-drying


Sample 5
Normal saline
Protectant medium
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
2,26E+09
8,75E+08
6,08E+09
5,11E+09
4,94E+08
1,30E+08
2,74E+09
8,81E+08
8,75E+09
3,29E+09
7,77E+08
6,18E+09
Before freeze-drying
After freeze-drying
Before freeze-drying
After freeze-drying


Dead cells
Injured cells
Live cells


Normal saline solution
Protectant medium
Fresh
Freeze-drying
a1
b1
a2
b2
c1
d1
c2
d2
TM4000 15kV 5.7mm x4.00k BSE M
10.0μm
TM4000 15kV 5.7mm x5.00k BSE M
TM4000 15kV 5.8mm x3.00k BSE M
TM4000 15kV 5.8mm x4.00k BSE M