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The Ability of *Streptococcus thermophilus* BT01 to Modulate Urease Activity in Healthy Subjects' Fecal Samples Depends on the Biomass Production Process

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Scope: This study evaluates how manufacturing conditions of probiotic biomass production, using two different cryoprotectants, Cryo-A and Cryo-B, can affect *Streptococcus thermophilus* BT01 in vivo gastrointestinal tract survival and its ability to modulate the level of urease activity in fecal samples of healthy subjects.

Methods and results: A randomized controlled cross-over study is carried out on 20 adult healthy subjects to evaluate total and viable loads, persistence of *S. thermophilus* BT01, and urease activity in fecal samples. Strain-specific quantification by using developed culture-based method and molecular qPCR tool allows to quantify viable *S. thermophilus* BT01 strain in 90% of the subjects. The quantification of both total DNA and recovered viable *S. thermophilus* BT01 in fecal samples does not reveal significant differences between Cryo-A or Cryo-B treated biomass. However, the administration of *S. thermophilus* BT01 produced with Cryo-A results in a decreased urease activity in fecal samples compared to Cryo-B protected cells.

Conclusion: This study i) highlights how the manufacturing conditions can play a role in influencing the probiotic functionality in vivo and ii) represents the first evidence that links *S. thermophilus* to a specific probiotic mechanism, the reduction of urease activity in fecal samples.

1. Introduction

The current definition of probiotics implies that the probiotic health benefits (effectiveness) on the host depend on viability (*live microorganisms*) and dose administrated (*administrated in adequate amounts*).[1] Both viability and dose are linked to the technological procedures of probiotic biomasses production.[2] During the production process probiotic viability, a key prerequisite for providing health benefits, may be affected by various factors such as growth medium composition, mechanical stress, pH and temperature conditions, oxygen stress, and cell dehydration.[2,3] Additionally, the viability of probiotics during gastrointestinal transit depends on host parameters. Major determinants of probiotic effectiveness are bound to its ability to survive the host gastrointestinal tract (GIT) conditions, such as acidic gastric environment, the disturbing activity of digestive enzymes and bile, microbicidal action of defense molecules produced by host immune response, and bound to competition with indigenous microbials, and to dietary factors.[3,4]

Producing robust and stable probiotics typically entails adding cryoprotectants such as sucrose, trehalose, sugar alcohols, polysaccharides, amino acids, protein, or complex mixtures (e.g., skimmed milk).[5–7] Cryoprotectants preserve probiotics viability, cell functions, and membrane integrity during lyophilization.[8,9] Various cryoprotectants have been explored to examine their in vitro ability to protect probiotic cells from stress factors such as low pH, simulated gastric juice, or bile salts.[10–13] However, not enough is known how those cryoprotectants influence the faith of probiotics in human GIT after ingestion. One way of evaluating the ability of probiotic strains to survive human GIT are interventional recovery studies that rely on probiotic quantification in human feces after administration. Furthermore, manufacturing conditions could play a role not only in determining the survival and viability of probiotics, but also in influencing their molecular composition and functionality in vivo.[14] However, at present, probiotic manufacturing processes remain largely unexplored as a source of variation in the results of clinical trials on probiotics.

It remains to be further clarified to which extent widely used *S. thermophilus* strains survive the gastrointestinal transit.[15] This issue is related to the lack of reliable molecular tools for the correct identification and recovery of *S. thermophilus* from fecal samples that we addressed in the previous work.[15] To accurately determine the survival rate of specific strains of *S. thermophilus*,

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development of strain-specific molecular tools might be a solution. Although PCR protocols will not allow assessment of the probiotic cells' viability, the culture-based methods could be combined with molecular tools to obtain adequate specificity and accuracy.[16,17]

Among the human GIT stressors, the most influential factor affecting the *S. thermophilus* viability is probably acidic stressor. It was reported that resistance strategies of *S. thermophilus* to acidity are attributed to the large combination of numerous different mechanisms including the over-expression of H^+-ATPase, and the release of ammonia by urea hydrolysis through the up-regulation of urease genes. Whereas the urease response to acid stress depends on urea availability in the environment, the activity of the H^+-ATPase depends on the intracellular ATP pool, thereby on the availability of metabolizable sugars.[18,19] However, in *S. thermophilus* urease activity does not play a leading role against the acid stress because urease biosynthesis is induced before the environmental pH values could be considered critical for its growth (e.g., pH close to 6). Urease rather has a key role in streamlining the energetic metabolism of *S. thermophilus* and specifically by acting on glycolysis regulation.[20–22]

In human microbiota, since the discovery of *Helicobacter pylori*, microbial ureases have been found to play a key role in the pathogenetic traits of several bacteria.[23] Several studies showed through a metagenomic approach that microbial ureases led to dysbiosis of the gut microbiota and that urease-genes are enriched in patients with irritable bowel syndrome (IBS) and depression. Additionally, urease activity is associated with autoantibodies, IBD, rheumatoid arthritis, and atherosclerosis,[24] and urease may be a potential therapeutic target for patients with inflammatory bowel diseases (IBD).[25] Brigidi et al.[26] reported that consumption of a complex probiotic supplement containing *Bifidobacterium longum, B. infantis, B. breve, Lactobacillus acidophilus, L. delbrueckii* subsp. *bulgaricus, L. casei, Lactiplantibacillus plantarum*, and *Streptococcus thermophilus* led to a decrease in fecal urease activity in IBDs patients. These patients are usually characterized by higher urease activity associated with harmful gut bacteria.[25–27]

In this study, we compared the role of two cryoprotectants on *S. thermophilus* BT01 GIT survival using a newly developed strain-specific assay that combines culture-based method and molecular tool for the detection and quantification of viable BT01 cells in fecal samples. Moreover, we evaluated the effect of *S. thermophilus* BT01 administration on the level of urease activity in fecal samples.

2. Experimental Section

2.1. Bacterial Strains, Growth Conditions, and BT01 Biomass Preparation

S. thermophilus BT01 and the other 22 *S. thermophilus* strains used for testing primer specificity were provided by Sacco Srl company. The bacterial strains *S. thermophilus* AF242, *S. salivarius*, and *S. vestibularis*, previously isolated from saliva and fecal samples, belong to Department of Food Environmental and Nutritional Sciences (DeFENS), University of Milan. *S. thermophilus* strains were cultured in M17 broth (BD Difco, Italy) supplemented with 2% w/v of lactose (Merck, Italy), whereas the *S. salivarius* and *S. vestibularis* strains were cultured in M17 broth supplemented with 2% w/v of sucrose (Merck, Italy). All strains were incubated at 37 °C for 24 h in anaerobic conditions.

Freeze-dried *S. thermophilus* BT01 biomass was prepared by Sacco Srl using a single fermentation batch. At the end of the fermentation process, *S. thermophilus* BT01 biomass was collected, concentrated 10 times by centrifugation and split in two aliquots. One aliquot was supplemented with the Cryo-A (4 wt%/v), a carbohydrate metabolizable by *S. thermophilus* BT01, and the second aliquot was supplemented with the Cryo-B (4% wt/v), a polysaccharide non-metabolizable by *S. thermophilus* BT01. Then, aliquots were freeze-dried and packaged in 1 g sachets. The viability of *S. thermophilus* BT01 Cryo-A and Cryo-B was quantified by flow cytometry (FCM) according to the protocol described by Mora et al.,[28] before and at the end of the study to confirm the biomass stability. Briefly, cell counting was performed for *S. thermophilus* BT01 Cryo-A and Cryo-B sachets using an Accuri C6 Plus FCM (BD Biosciences, Milan, Italy). A total of 1 g of each sachet was suspended in up to 10 mL of PBS (NaCl 0.15 M, KH2PO4 1 mM, Na2HPO4 3 mM, pH 7.4) and homogenized in a stomacher for 3 min at room temperature. The obtained cell suspensions were analyzed by FCM (threshold settings FSC 5000, acquisition volume 50 µL) without and by labeling with SYTO 24 (SYTO 24 is a trademark of the company Thermo Fisher Scientific) and propidium iodide (PI) (Thermo Fisher Scientific, Italy). All the parameters were collected as logarithmic signals. The 488 nm laser was used to measure the FSC values. The rate of events in the flow was generally lower than 2000 events. The SYTO 24 fluorescence intensity of stained cells was recovered in the FL1 channel (excitation, 488 nm; emission filter, 530/30), whereas PI fluorescence was recovered in the FL3 (excitation, 488 nm; emission filter, 610/20). Density plots of SYTO 24 versus PI allowed for optimal distinction between cells double stained with SYTO 24 and PI, and instrument noise or sample background. Electronic gates on the SYTO 24 versus PI density plot were used to select and measured the total bacterial cell density expressed as fluorescent unit per g (FU g^{-1}), and to selectively count live, damaged, and dead cells as described in ISO 19344 IDF 232.[29]

2.2. Development of Strain-Specific Primer for Detection and Quantification of *S. thermophilus* BT01

From draft genome of the *S. thermophilus* BT01,[30] a gene sequence encoding clustered regularly interspaced short palindromic repeats associated protein (CRISP) was identified as potential target for strain-specific primers. A DNA region downstream the gene coding for a CRISPR-associated protein Csn2 was used for the design of qPCR strain-specific primer. CRISPR-associated proteins constituted the adaptive immune system in bacteria, and they were usually hypervariable among the strains of the same species.[31] qPCR primers were designed using SnapGene (GSL Biotech LLC) and NCBI Primer-BLAST (ncbi.nlm.nih.gov/tools/primer-blast) tools. BLAST analysis also revealed that the selected primers did not show any

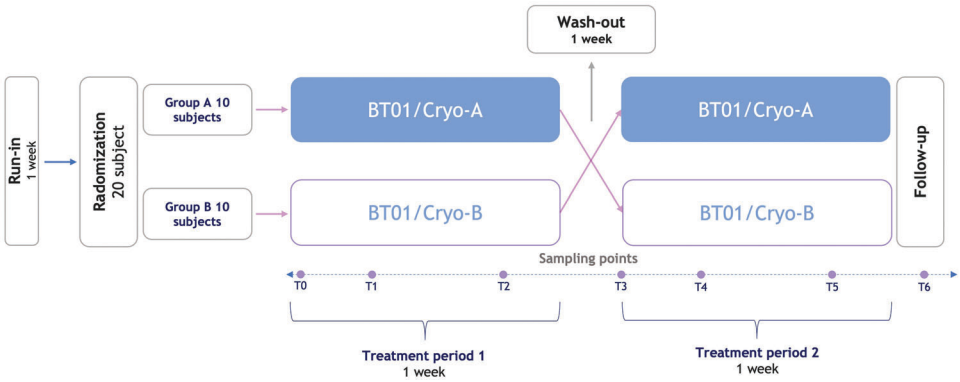

Figure 1. Study design. After 1-week run-in phase, subjects were randomly (1:1) assigned to either *S. thermophilus* BT01 Cryo-A or Cryo-B once daily for 1 week. This was followed by a washout period of 1 week before crossing over to the alternate treatment (one daily for 1 week). After 3 weeks, subjects entered in a final follow-up phase. The total duration of the study was 4 weeks. Fecal samples were obtained after run-in period (T0); visits 2 (T1) and 3 (T2) (first treatment period); wash-out (T3) visits 4 (T4) and 5 (T5) (second treatment period), and during follow up (T6).

matches with closely related *Streptococcus* spp. or other lactic acid bacteria species. In specific, the target region of BT01-F and BT01-R was absent in public available sequences belonging to the closest neighbors *S. salivarius* and *S. vestibularis* and oral streptococci. The primer sequences were the following: BT01-F 5'-CACAACCAGCAAAGAGAGCG-3', BT01-R 5'-CCGCCATCAACTTTTACCGC-3'. The size of the expected amplicon was of 165 bp.

Primer set specificity was tested on DNA of BT01, other 22 *S. thermophilus* strains and two closely phylogenetically related species, *S. salivarius* (three strains) and *S. vestibularis* (one strain) (Table S1, Supporting Information). The DNA extraction from pure cultures was performed using DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. DNA extracted was quantified in Take3 Micro-volume plate analyzed in a microplate reader with Gen5 Software (BioTek Instruments, Inc., CA, USA) at 260 nm wavelength. qPCR amplification was carried out in a final volume of 15 µL, containing 7.5 µL of EvaGreen Supermix (Bio-Rad Laboratories, Segrate, Italy), 0.5 µM of each primer, and 50 ng template DNA. The same mixture, with sterile water, and without DNA was used as a negative control. The amplification was carried out with the following thermal program: initial hold at 95 °C for 3 min, followed by 37 cycles of 95 °C for 20 s, 62 °C for 25 s, and 72 °C for 5 s. Melting curve of each amplification was analyzed with Bio-Rad CFX Manager 3.1 (Bio-Rad, CA, USA) to confirm the specificity of the amplification products.

For the total quantification of BT01 strain, the strain was grown overnight in 10 mL of M17 broth with 2% w/v lactose at 37 °C. Non inoculated M17 broth, 2% w/v lactose was used as a negative control. Standard calibration curve was prepared by spiking BT01 cells to a pool of fecal samples, previously checked for the absence of the probiotic strain. The quantification and standardization of the cell number were done by FCM as previously described.[28] Cells of *S. thermophilus* BT01 were standardized in PowerSoil Bead Solution of DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany), serially diluted in the same PowerSoil Bead Solution, and used for the spiking procedure. Specifically, 250 mg of fecal sample was spiked with known amount of BT01 ranging from 1 Log to 8 Log (cell) (250 mg feces)$^{-1}$. DNA from spiked fecal samples was extracted using Dneasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer. The reaction mixture and qPCR conditions were the same as described for primer design. The standard curve was derived by plotting the Ct values against corresponding Log cells number added to each feces-based standard dilution.

2.3. In Vivo BT01 Human Interventional Study

2.3.1. Study Title

Effect of cryoprotectants on *S. thermophilus* vital recovery in healthy adults (PROVIRTUS).

2.3.2. Study Design and Population

Randomized, controlled cross-over study (**Figure 1**), 20 healthy (non-diseased) adult volunteers of both sexes (60% females, 40% males), aged from 23 to 57 years, (average age 33 ± 11). Exclusion criteria were abnormality of the GIT (i.e., IBD such as Crohn's disease, ulcerative colitis), pregnancy, metabolic diseases, primary or secondary immunodeficiency, antibiotics intake within 1 month before the screening visit, hypersensitivity, or allergy to any ingredient of the study product, as well as participation in other clinical trial in the past 3 months.

2.3.3. Dosage Information

S. thermophilus BT01 in sachets, containing 1 g of formulation powder and corn starch excipient providing 1×10^{11} aFU of BT01 strain. The sachet product was reconstituted in drinkable water just before intake, under fasting conditions, in the morning before breakfast, or alternatively in the evening at least 2 h after the last meal of the day, before going to bed.

2.3.4. Study Protocol

During the first visit (T0) each volunteer signed a consent document and received the general information about the entire procedure. The study consisted of a *run-in* phase (1 week) during which the volunteers followed their conventional diet with a ban of probiotic-fermented milks, traditional yogurt, fermented mozzarella, fresh cheeses (e.g., stracchino and crescenza), probiotic, prebiotic, and synbiotic foods, and supplements. During this period volunteers were instructed on how to take the products in the treatment phase. Volunteers received the questionnaire to evaluate: i) the intensity and frequency of symptoms including meteorism, abdominal pain, and bloating, using a scale from 0 (no complaints) to 5 (maximal intensity), and ii) stool frequency and consistency by using Bristol stool scale.

Prior to starting with the treatments subjects were randomized (1:1) by a computer-generated random code system. Afterwards, in the first treatment phase (sampling points T1 and T2) which involved the consumption of one sachet per day (1 g) containing the lyophilized probiotic strain *S. thermophilus* BT01 in the presence of Cryo-A (arm-A) or Cryo-B (arm-B) (Figure 1). Following the 1 week of treatment, volunteers followed 1 week of a wash-out, identical to the run-in period. After the wash-out, the volunteers began the second treatment phase (sampling points T4 and T5) lasting 1 week, which involved taking one sachet per day in the presence of Cryo-B (arm-B) (for those who had taken the probiotic in the presence of Cryo-A during the first treatment phase), or Cryo-A (arm-A) (for those who had taken the probiotic in the presence of Cryo-B during the first treatment phase) (Figure 1). Volunteers delivered the completed questionnaire on the Bristol stool form scale and gastrointestinal symptoms at the end of the follow-up period.

2.3.5. Sample Collection and Analysis

Each fecal specimen (at least 2 g) was collected in sterile containers, stored at 4 °C, and delivered to the laboratory within 24 h. To verify the ability of the *S. thermophilus* BT01 strain to survive passage through the GIT, the collected fecal samples were immediately subjected to analysis. One gram of each delivered fecal samples, was diluted in up to 10 mL of Maximum Recovery Diluent (Scharlau, Italy), homogenized in a sterile Stomacher bag for 3 min, at room temperature, serially diluted, and plated on milk-based medium[32] without sucrose, and incubated anaerobically at 37 °C for 48 h. After incubation, whole biomass grown on the plate of the first serial dilution was collected and resuspended in the first solution of QIAsymphony kit. From this suspension DNA extraction was performed with QIAsymphony SP (Qiagen, Hilden, Germany) automated system by using QIAsymphony kit according to the manufacturer instructions.

For total BT01 quantification, 0.25 g of the fecal sample was weighted, resuspended in the first solution of QIAsymphony kit, and extracted by the same QIAsymphony SP (Qiagen, Hilden, Germany) automated system. Detection of viable BT01 cells and total quantification of the probiotic were carried out by qPCR according to the protocol described above.

Urease activity in the fecal sample was determined using a phenol red assay described by Lanyi[32] with some modifications. Briefly, the assay was based on pH increase due to the urea hydrolysis and ammonia release. The medium alkalization was followed at 555 nm by monitoring the phenol red color change. Frozen fecal samples were thawed at 4 °C, then 0.5 g was weighed and diluted in 10 mM potassium phosphate buffer, pH 7. The specimens were homogenized using Precellys 24 Beadblaster and centrifuged at 14 000 rpm for 10 min. The protein content of the supernatant was determined by Bradford assay.[34] Then, protein concentration was standardized and a reaction mixture for assessing urease activity was prepared. The reaction mixture contained 50 µL of the of protein extract (400 µg total protein extract/reaction), 60 µL of the solution A (2 g of CH_4N_2O, 2 mL of 95% C_2H_5OH, 4 mL of dH_2O), 940 µL of solution B (0.1 g KH_2PO_4, 0.1 g K_2HPO_4, 0.5 g NaCl, 1 mL 0.2% phenol red, 100 mL H_2O). Two hundred µL of the reaction mixture was loaded in the 96 well plate, incubated at 37 °C and kinetics was monitored every 15 min using a Biotek EON spectrophotometer (BioTek Instruments, Inc., CA, USA). The results were expressed as maximum velocity (maxV) as mOD_{555} nm min^{-1}. Analogously, starting from 0.5 g of freeze-dried biomass the urease activity was measured in Cryo-A and Cryo-B sachets.

2.3.6. Ethical Statement

The study protocol was approved by the Research Ethics Committee of the Università degli Studi di Milano (opinion no. 52/21, May 2021). Before start of interventional study all the subjects signed the informed consent form.

2.4. Statistical Analysis

Statistical analyses were performed by Prism-GraphPad software, version 8.4.3. All data were checked for normality and homoscedasticity and then parametric or non-parametric statistics was applied. The following statistical elaborations were performed to identify significant differences between treatments: parametric test including the analysis of variance (ANOVA) with repeated measures and Student's *t*-test; non-parametric tests including Wilcoxon paired data test, Freidman test. When appropriate, post-hoc tests were performed. Significance was set at $p < 0.05$.

3. Results

3.1. Development of Strain-Specific *S. thermophilus* BT01 qPCR Protocol

The specificity test of the designed primer set was carried out on 22 *S. thermophilus* strains, three *S. salivarius* strains, and one *S. vestibularis* strain. The results obtained showed amplification only from DNA obtained from *S. thermophilus* BT01 (Table S1, Supporting Information), thus confirming the strain-specificity of BT01-F and BT01-R primer set. The calibration curve showed a linear increase in the range of DNA concentration between 8 and 4 Log aFU g^{-1} with a correlation coefficient (R^2) of 0.9977 (Figure S1A, Supporting Information), and a limit of quantification (LOQ) of 4 Log cell g^{-1} feces (Figure S1B, Supporting Information).

Table 1. Quantification (FU g^{-1}) of live (live cells[a], live cells[b]), damaged cells, and dead cells in *S. thermophilus* BT01 Cryo-A and Cryo-B sachets at the beginning (T0) and at the end (T6) of the interventional study.

BT01 biomass	Live cells[a]	Live cells[b]	Total live cells	Damaged cells	Dead cells	Total
	(FU $g^{-1a)}$)					
Cryo-A (T0)	$6.9 \times 10^{10} \pm 1 \times 10^{9}$	$3.7 \times 10^{10} \pm 1 \times 10^{9}$	$1.10 \times 10^{11} \pm 2 \times 10^{9}$	$3.5 \times 10^{9} \pm 2 \times 10^{8}$	$7.5 \times 10^{9} \pm 1 \times 10^{8}$	$1.21 \times 10^{11} \pm 2 \times 10^{9}$
Cryo-A (T6)	$6.9 \times 10^{10} \pm 2 \times 10^{9}$	$3.6 \times 10^{10} \pm 1 \times 10^{9}$	$1.10 \times 10^{11} \pm 4 \times 10^{9}$	$2.6 \times 10^{9} \pm 4 \times 10^{8}$	$1.4 \times 10^{10} \pm 2 \times 10^{9}$	$1.27 \times 10^{11} \pm 6 \times 10^{9}$
Cryo-B (T0)	$1.0 \times 10^{11} \pm 2 \times 10^{9}$	$1.6 \times 10^{9} \pm 1 \times 10^{8}$	$1.03 \times 10^{11} \pm 3 \times 10^{9}$	$1.4 \times 10^{10} \pm 2 \times 10^{9}$	$3.8 \times 10^{10} \pm 1 \times 10^{9}$	$1.52 \times 10^{11} \pm 2 \times 10^{9}$
Cryo-B (T6)	$9.5 \times 10^{10} \pm 1 \times 10^{9}$	$1.1 \times 10^{9} \pm 2 \times 10^{8}$	$9.6 \times 10^{10} \pm 3 \times 10^{9}$	$1.1 \times 10^{10} \pm 2 \times 10^{9}$	$5.9 \times 10^{10} \pm 1 \times 10^{9}$	$1.66 \times 10^{11} \pm 6 \times 10^{9}$

[a)] FU g^{-1}, fluorescence unit per gram of freeze-dried biomass. Live cells[a], live cells exhibiting a higher level of SYTO 24 fluorescence; Live cells[b], live cells exhibiting a lower level of SYTO 24 fluorescence.

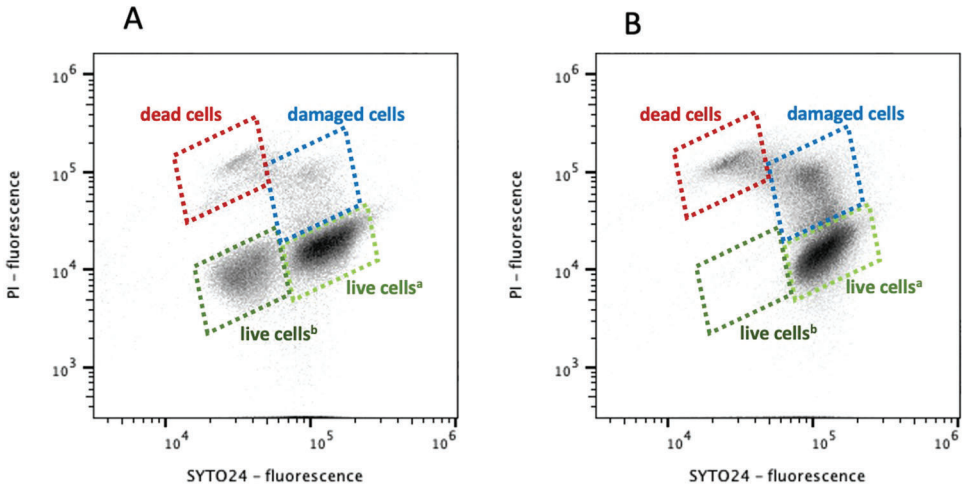

Figure 2. Dot-plots of *S. thermophilus* BT01 cell suspensions stained with SYTO 24 and Propidium iodide for the quantification of live and dead cells according to ISO 19344 IDF 232[29] and further modifications.[28] A) Dot-plots and histogram of *S. thermophilus* BT01 prepared with Cryo-A. B) Dot-plots and histogram of *S. thermophilus* BT01 prepared with Cryo-B. Green live cells[a] and live cells[b] electronic gates identify live cells; Blu gate identifies damaged cells; Red gate identifies dead cells.

3.2. Quantification of *S. thermophilus* BT01 Cell Viability and Urease Activity in Cryo-A and Cryo-B Sachets

All 20 subjects completed the study according to the protocol. Quantification of cell viability in *S. thermophilus* BT01 Cryo-A and Cryo-B sachets was determined by FCM as described in ISO 19344 IDF 232[28] and by Ni et al.[27] Cryo-A and Cryo-B biomasses showed equivalent number of viable cells, 1.10×10^{11} FU g^{-1} (Cryo-A) and 1.03×10^{11} FU g^{-1} (Cryo-B) but significantly different damaged and dead cells (**Table 1**, **Figure 2**). Interestingly, the biomass lyophilization with Cryo-A or Cryo-B had different effect on the SYTO 24 green fluorescence of the viable population (Figure 2). Indeed, using Cryo-B only one population of live cells (live cells[a]) was detectable (Figure 2B), whereas the use of Cryo-A determined the formation of an additional population of live cells (live cells[b]), exhibiting a lower level of SYTO 24 fluorescence (Figure 2A). Furthermore, both Cryo-A and Cryo-B showed to be stable, as viable cells, till the end of the study (T6) (Table 1).

Since urease activity is associated with the *S. thermophilus* species, and not commonly present in other probiotic species, the level of urease activity in *S. thermophilus* BT01 Cryo-A and Cryo-B sachets was also measured. Despite the biomass had been prepared using a single fermentation batch, and then divided in two aliquots for the freeze-drying step, the urease activity was significantly different between Cryo-A (8.10 ± 0.06 $mOD_{555}nm\ min^{-1}$) and Cryo-B sachets (5.48 ± 0.05 $mOD_{555}nm\ min^{-1}$) (**Figure 3**).

3.3. Quantification of *S. thermophilus* BT01 DNA in Fecal Samples

Fecal samples collected during the cross-over study were used for the quantification of *S. thermophilus* BT01 DNA counts by using the strain-specific qPCR assay. In five subjects out of 20 *S. thermophilus* BT01 DNA was detected after 1 week of run-in period (at T0), with counts ranging from 4.82 to 6.45 Log_{10} cells g^{-1} feces. *S. thermophilus* BT01 counts ranged from 4.76 to 8.15 (mean 7.33 Log_{10} cells g^{-1} feces), and from 4.57 to 8.23 Log_{10} cells g^{-1} feces (mean 7.44 Log_{10} cells g^{-1} feces) in subjects that consumed *S. thermophilus* BT01 Cryo-A and Cryo-B, respectively (**Table 2**, **Figure 4**A). The higher percentage of *S. thermophilus* BT01 detection was measured for those subjects that consumed *S. thermophilus* BT01 Cryo-A (Table 2) during the first (T1 and T4) as well as second (T2 and T5) dose of consumption. In details, for Cryo-A *S. thermophilus* BT01 was detected in 90%

Table 2. Quantification of *S. thermophilus* BT01 DNA in fecal samples (Log cells per g fecal sample-wet weight).

Subject	T0	Cryo-A		Wash-out	Cryo-B		Averages ± SD	
		T1/T4	T2/T5	T3	T1/T4	T2/T5	Cryo-A	Cryo-B
1[a)]	5.82 ± 0.04	5.68 ± 0.01	7.27 ± 0.03	6.96 ± 0.00	7.14 ± 0.04	6.96 ± 0.10	6.98 ± 1.12	7.06 ± 0.13
2[a)]	nd	7.41 ± 0.02	6.88 ± 0.10	nd	7.17 ± 0.02	7.40 ± 0.03	7.22 ± 0.38	7.30 ± 0.16
3[a)]	4.82 ± 0.03	6.05 ± 0.03	5.86 ± 0.01	5.95 ± 0.01	7.20 ± 0.05	5.54 ± 0.08	5.96 ± 0.14	6.91 ± 1.17
4[a)]	nd	5.58 ± 0.05	7.78 ± 0.01	nd	7.74 ± 0.01	8.23 ± 0.08	7.48 ± 1.56	8.05 ± 0.34
5[a)]	nd	nd	7.77 ± 0.09	5.31 ± 0.04	4.57 ± 0.08	7.54 ± 0.03	na	7.24 ± 2.95
6[a)]	nd	6.86 ± 0.02	7.65 ± 0.14	nd	7.14 ± 0.03	7.97 ± 0.01	7.42 ± 1.68	7.73 ± 0.58
7[a)]	nd	6.07 ± 0.04	6.88 ± 0.01	6.14 ± 0.04	6.20 ± 0.05	6.75 ± 0.04	6.64 ± 0.56	6.56 ± 0.39
8[a)]	5.45 ± 0.08	8.15 ± 0.01	8.01 ± 0.01	5.00 ± 0.08	6.51 ± 0.02	7.02 ± 0.02	8.09 ± 0.14	6.83 ± 0.36
9[a)]	5.99 ± 0.08	6.50 ± 0.00	6.93 ± 0.03	nd	nd	7.37 ± 0.03	6.76 ± 0.30	7.07 ± 0.21
10[a)]	nd	4.76 ± 0.10	6.01 ± 0.01	nd	nd	5.19 ± 0.06	5.73 ± 0.89	na
11[b)]	nd	5.30 ± 0.01	nd	5.71 ± 0.04	nd	nd	na	na
12[b)]	nd	6.61 ± 0.01	6.74 ± 0.03	nd	nd	nd	6.68 ± 0.09	na
13[b)]	nd	6.30 ± 0.01	6.65 ± 0.02	nd	5.27 ± 0.04	5.64 ± 0.04	6.51 ± 0.25	5.49 ± 0.26
14[b)]	nd	7.05 ± 0.02	7.39 ± 0.03	5.31 ± 0.03	7.98 ± 0.01	7.85 ± 0.11	7.25 ± 0.24	7.92 ± 0.09
15[b)]	nd	7.56 ± 0.04	7.49 ± 0.01	nd	6.72 ± 0.01	7.72 ± 0.04	7.52 ± 0.05	7.46 ± 0.70
16[b)]	6.45 ± 0.05	nd	7.56 ± 0.01	nd	nd	7.96 ± 0.12	na	na
17[b)]	nd	5.32 ± 0.04	6.49 ± 0.07	nd	6.82 ± 0.03	7.22 ± 0.04	6.22 ± 0.83	7.07 ± 0.28
18[b)]	nd	7.34 ± 0.00	7.75 ± 0.01	nd	nd	6.51 ± 0.04	7.59 ± 0.29	6.51 ± 0.34
19[b)]	nd	6.86 ± 0.08	7.65 ± 0.02	nd	nd	7.28 ± 0.01	7.20 ± 0.33	na
20[b)]	nd	6.07 ± 0.04	6.88 ± 0.00	nd	6.45 ± 0.06	6.92 ± 0.05	7.27 ± 0.60	6.75 ± 0.33
		Positive amplifications [%]					Total average	
	25	90	95	35	65	90	7.31	7.45

[a)] Subjects first consuming *S. thermophilus* with Cryo-A; [b)] Subjects first consuming *S. thermophilus* with Cryo-B. Cryo-A T1/T4: first sampling point of Cryo-A, T1 for subjects 1–10, T4 for subjects 11–20; Cryo-A T2/T5: second sampling point of Cryo-A, T2 for subjects 1–10, T5 for subjects 11–20; Cryo-B T1/T4 first sampling point of Cryo-B, T1 for subjects 11–20, T4 for subjects 1–10; Cryo-A T2/T5: second sampling point of Cryo-B, T2 for subjects 11–20, T5 for subjects 1–10; na, non-applicable; nd, non-detected; SD, standard deviation.

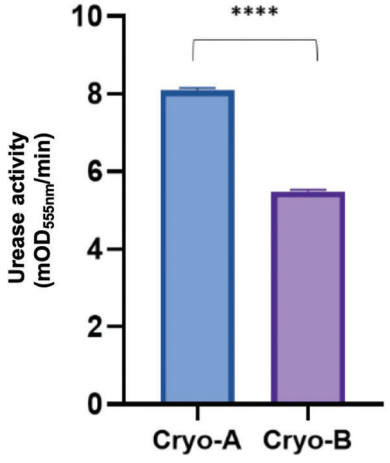

Figure 3. Quantification of urease activity in *S. thermophilus* BT01 Cryo-A and Cryo-B sachets using colorimetric assay. Student's *t*-test, $p < 0.0001$ (significance level $p < 0.05$).

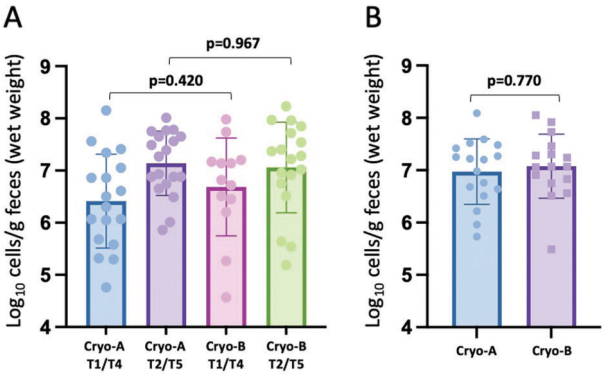

Figure 4. Quantification of *S. thermophilus* BT01 DNA in fecal samples, reported as Log_{10} cells per $g_{feces\ (wet\ weight)}$, A) with individual subject values per sampling points (T1 and T4, T2 and T5), and B) *S. thermophilus* BT01 DNA counts averages of Cryo-A and Cryo-B treatments. Wilcoxon test A) $p = 0.420$ (Cryo-A vs Cryo-B T1 and T4); $p = 0.967$ (Cryo-A vs Cryo-B T2 and T5). Wilcoxon test B) $p = 0.770$ (Cryo-A vs Cryo-B), significance level $p < 0.05$. Dots represent individual subjects' DNA counts. Due to the possible bias, subjects negative at T0 (after run-in) and positive at T3 (washout) were excluded from statistical analysis, as well as subjects that were negative for BT01 at one of time sampling points.

after the first dose of consumption (T1 and T4), and in 95% of the subjects after the second dose of consumption (T2 and T5), whereas for Cryo-B, BT01 was detected in 65% and 90% of the subjects after the first (T1 and T4) and second (T2 and

Table 3. Recovery of viable *S. thermophilus* BT01 reported as Ct values with individual subject values per sampling point.

Subject	Run-in (T0)	Cryo-A		Wash-out (T3)	Cryo-B		Follow-up (T6)	Average Ct ± SD	
		T1/T4	T2/T5		T1/T4	T2/T5		Cryo-A	Cryo-B
1[a]	nd	nd	31.2	nd	34.4	nd	33.5	31.2	34.4
2[a]		31.4	35.1		31.2	31.1	32	33.3 ± 2.6	31.2 ± 0.1
3[a]		24.4	29.4		29.5	33	33.5	26.9 ± 3.5	31.3 ± 2.5
4[a]		nd	nd		nd	nd	nd	na	na
5[a]		nd	31.9		nd	31.6	32.6	31.9	31.6
6[a]		nd	32.9		34.2	31.3	33.6	32.8	32.8 ± 2.1
7[a]		28.3	33.1		33.9	33.8	nd	30.7 ± 3.4	33.9 ± 0.1
8[a]		23.18	29.1		32	34.5	nd	26.1 ± 4.2	33.3 ± 1.8
9[a]		nd	34.3		nd	28.7	33.1	34.3	28.7
10[a]		20.5	22.2		nd	32.2	32.4	21.4 ± 1.2	32.2
11[b]		nd	nd		nd	nd	32.2	na	na
12[b]		32.5	34.7		nd	32.7	nd	33.6 ± 1.6	32.7
13[b]		nd	23.8		18.9	33.1	nd	23.8	26.0 ± 10
14[b]		33.2	nd		nd	34.5	nd	33.2	34.5
15[b]		30.6	nd		30.7	27.6	31.6	30.6	29.2 ± 2.2
16[b]		nd	nd		nd	29.6	nd	na	29.6
17[b]		23.9	30.6		21.6	32.1	nd	27.3 ± 4.7	26.9 ± 7.4
18[b]		30.2	31.2		nd	31.9	nd	30.7 ± 0.7	31.9
19[b]		33.2	21.3		nd	26.1	nd	27.3 ± 8.4	26.1
20[b]		nd	30.6		NS	32.2	30.7	30.6	32.2
Positive amplifications %	/	55	75	/	45	75	50	**Total average**	
								29.7	31.2

[a] Subjects first consuming *S. thermophilus* with Cryo-A; [b] Subjects first consuming *S. thermophilus* with Cryo-B. Cryo-A T1/T4: first sampling point of Cryo-A, T1 for subjects 1–10, T4 for subjects 11–20; Cryo-A T2/T5: second sampling point of Cryo-A, T2 for subjects 1–10, T5 for subjects 11–20; Cryo-B T1/T4 first sampling point of Cryo-B, T1 for subjects 11–20, T4 for subjects 1–10; Cryo-A T2/T5: second sampling point of Cryo-B, T2 for subjects 11–20, T5 for subjects 1–10; na, non-applicable; nd, non-detected; SD, standard deviation.

T5) dose of consumption, respectively (Table 2). However, no significant differences were found comparing the total amount of *S. thermophilus* BT01 between the first (T1 and T4), and second (T2 and T5) sampling points of Cryo-A and Cryo-B treatments ($p = 0.420$ and $p = 0.967$, respectively) (Figure 4A). Furthermore, statistics showed no significant difference ($p = 0.770$) between the total detected amount of *S. thermophilus* BT01 Cryo-A and Cryo-B (Figure 4B). At the end of the wash-out period (T3) BT01 was found in seven subjects (35%) out of 20 (Table 2).

3.4. Recovery and Persistence of Viable BT01 in the Fecal Samples

We combined the viable recovery on milk-based medium with the developed strain-specific qPCR BT01 protocol for evaluating GIT survival of *S. thermophilus* BT01 Cryo-A or Cryo-B. At baseline after 1 week of run-in (T0), no viable BT01 cells were present in the fecal samples. After the administration of the first dose (T1 and T4), *S. thermophilus* BT01 was recovered in 55% and 45% of the subjects for Cryo-A and Cryo-B, respectively. The recovery of live *S. thermophilus* BT01 increased up to 75% of the subjects for both treatments after the second administrated dose (T2 and T5) (**Table 3**). Intra-individually, in 10 subjects the recovery was higher when they consumed the Cryo-A biomass, in seven subjects the recovery was higher when the Cryo-B biomass was consumed. Only one subject (n° 4) remained negative for the whole period of the study, including the follow up, whereas viable cells of strain BT01 were detected only during the follow up (2–3 days after the end of the second treatment) in one volunteer (n° 11); one subject showed low recovery at the end of the study period (n° 16). On average, there were no significant statistical differences ($p = 0.889$; $p = 0.133$) after the first (Cryo-A vs Cryo-B, T1 and T4) and second dose (Cryo-A vs Cryo-B, T2 and T5) of *S. thermophilus* Cryo-A and Cryo-B consumption (**Figure 5**A). Moreover, when average of two sampling points (average of T1 and T4; T2 and T5) was calculated (where subject showed to be negative in one of two sampling points, only the value of the positive point was used in calculation) no statistical difference between the averages of Cryo-A and Cryo-B treatments were observed ($p = 0.170$) (Figure 5B).

The recovery of viable BT01 from fecal samples collected during follow-up (T6, 2–3 days after the end of the second treatment, Figure 1) allowed to detect *S. thermophilus* BT01 in 50% of the subjects (Figure 1, Table 3), thus highlighting the persistence of strain BT01 in human gut for at least of 2–3 days after the last product intake. On the contrary, at the end of the wash-out

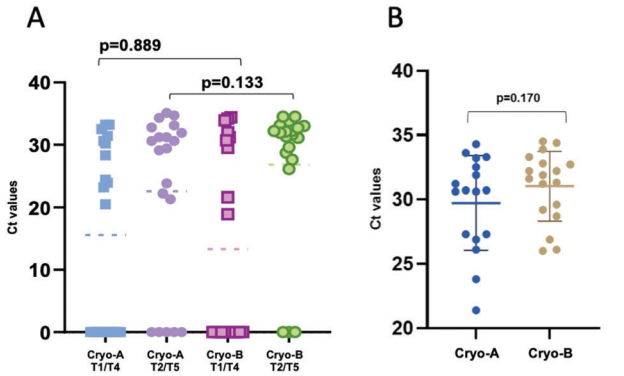

Figure 5. Recovery of viable *S. thermophilus* BT01 reported as Ct values with individual subject values per sampling point A). Recovery of viable *S. thermophilus* BT01 reported as averages of Ct values of Cryo-A and Cryo-B treatments B). Dots represent individual subjects' Ct values. Wilcoxon test A) $p = 0.889$ (Cryo-A vs Cryo-B T1 and T4); $p = 0.133$ (Cryo-A vs Cryo-B T2 and T5). Wilcoxon test B) $p = 0.170$ (Cryo-A vs Cryo-B), significance level $p < 0.05$. Due to the possible bias, subjects that were negative for BT01 at one of time sampling points were excluded from statistical analysis.

period (T3), i.e., 4–5 days from the last product intake, no one subject was positive for the recovery of strain BT01 (Table 3).

3.5. Defecation Frequency and Stool Consistency

Weekly average daily defecations were consistent throughout the whole study period (Figure S2, Supporting Information). On average there was no significant change in the number of evacuations ($p = 0.333$), as well as stool consistency ($p = 0.124$). The mean stool consistency score values were 3.7 ± 0.5 at T0 (baseline), 3.7 ± 0.5 for Cryo-A, and 3.9 ± 0.6 for Cryo-B. The percentage of subjects reporting 0, 1, 2, or 3 evacuations during the day did not change considerably from the run-in to the treatment periods, with most subjects reporting one evacuation/day (average amount of evacuation/day for each of baseline, Cryo-A, Cryo-B treatments was 1 ± 0.6). The most frequent stool consistency score was 4 (Figure S3, Supporting Information). Scores 1 and 6 were never recorded. Score 2 slightly increased in the case of Cryo-A biomass and totally decreased for Cryo-B biomass consumption (Figure S3, Supporting Information).

3.6. Quantification of Urease Activity in the Fecal Sample

Urease activity was measured in samples collected at T0 (Baseline, B), and after the Cryo-A and Cryo-B (T2 and T5) treatments. In 14 subjects, a decrease in urease activity was observed after administration of *S. thermophilus* BT01/Cryo-A compering to the Cryo-B, and in six subjects a decrease after consumption of *S. thermophilus* BT01/Cryo-B compering to Cryo-A (**Table 4**). The differences showed to be statistically significant in 13 subjects (Table 4, Cryo-A vs Cryo-B), out of which urease activity significantly decreased in 10 subjects in the case of *S. thermophilus* BT01 with Cryo-A compering to *S. thermophilus* BT01 with Cryo-B. Relative to the baseline, a decrease in the urease activity was observed in 16 subjects when *S. thermophilus* BT01 cryoprotected with Cryo-A was administered, and in 10 subjects when *S. thermophilus* BT01 protected with Cryo-B was used. Finally, *S. thermophilus* BT01 protected with Cryo-A showed to be effective to decrease the urease activity in fecal samples ($p = 0.022$) (**Figure 6**).

Table 4. Quantification of urease activity in the fecal samples.

	max V (mOD_{555nm} min^{-1})			p-value
Subject	B	Cryo-A T2/T5	Cryo-B T2/T5	Cryo-A vs Cryo-B
1[a)]	6.57 ± 0.12	1.63 ± 0.13	2.51 ± 0.52	0.016
2[a)]	3.45 ± 0.06	2.83 ± 0.03	9.08 ± 0.15	<0.001
3[a)]	5.10 ± 0.05	5.04 ± 0.07	6.75 ± 0.06	<0.001
4[a)]	1.67 ± 0.12	1.63 ± 0.05	2.99 ± 0.10	<0.001
5[a)]	1.22 ± 0.11	0.85 ± 0.05	0.75 ± 0.16	0.245
6[a)]	0.35 ± 0.37	2.06 ± 0.10	2.19 ± 0.08	0.090
7[a)]	4.20 ± 0.07	2.72 ± 0.02	3.32 ± 0.05	<0.001
8[a)]	1.44 ± 0.28	1.19 ± 0.00	1.51 ± 0.34	0.359
9[a)]	0.24 ± 0.03	0.16 ± 0.02	0.25 ± 0.09	0.094
10[a)]	4.00 ± 0.55	2.47 ± 0.19	5.02 ± 0.17	<0.001
11[b)]	4.24 ± 0.02	2.23 ± 0.02	4.56 ±0.03	<0.001
12[b)]	1.38 ± 0.23	3.59 ± 0.04	2.77 ± 0.11	<0.001
13[b)]	3.39 ± 0.15	2.58 ± 0.35	2.16 ± 0.17	0.070
14[b)]	1.61 ± 0.08	0.99 ± 0.15	0.63 ± 0.32	0.232
15[b)]	1.91 ± 0.16	2.36 ± 0.10	2.74 ± 0.03	<0.001
16[b)]	5.98 ± 0.07	5.50 ± 0.08	2.52 ± 0.03	<0.001
17[b)]	5.99 ± 0.04	2.86 ± 0.04	4.16 ±0.11	<0.001
18[b)]	1.72 ± 0.09	1.89 ± 0.40	0.94 ± 0.08	0.002
19[b)]	4.84 ± 0.07	1.75 ± 0.19	2.95 ± 0.04	<0.001
20[b)]	4.40 ± 0.06	3.63 ± 0.20	3.87 ± 0.16	0.320
Average	3.09 ± 1.96	2.33 ± 1.32	2.99 ± 2.12	Cryo-A 14 vs Cryo-B 6 U.A↓

[a)] Subjects consuming first *S. thermophilus* with Cryo-A; [b)] Subjects consuming first *S. thermophilus* with Cryo-B. Cryo-A T1/T4: first sampling point of Cryo-A, T1 for subjects 1–10, T4 for subjects 11–20; Cryo-A T2/T5: second sampling point of Cryo-A, T2 for subjects 1–10, T5 for subjects 11–20; Cryo-B T1/T4 first sampling point of Cryo-B, T1 for subjects 11–20, T4 for subjects 1–10; Cryo-A T2/T5: second sampling point of Cryo-B, T2 for subjects 11–20, T5 for subjects 1–10;B, baseline urease-activity; U.A, urease activity, ↓ decrease of urease activity. Data are the means of four replicates ± standard deviation. Cryo-A versus Cryo-B statistically elaborated using *t*-test ($p < 0.05$).

4. Discussion

Survival along human GIT is a key aspect to characterize a bacterial strain as a probiotic.[1] According to the FAO/WHO guidelines in vitro ability of probiotic strains should be validated by performing in vivo clinical trials-interventional studies.[35] *S. thermophilus*, even though used widely in probiotic supplements or probiotic food, its role as probiotic is still discussed due to the contradictory results about its capability to successfully survive human GIT. Some of these contradictory results are most likely the result of the use of ineffective culture-based methods and/or the lack of species-specific molecular tools for the selective enumeration of *S. thermophilus* strains in fecal samples.[15] The main concerns of culture-based methods and/or not species-specific molecular tools are related to the inability to discriminate *S. thermophilus* strains from intestinal streptococci, enterococci, and/or

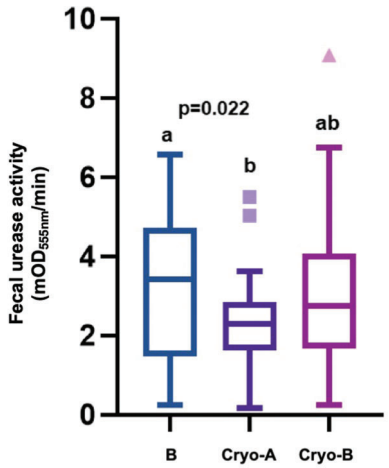

Figure 6. Quantification of urease activity in the fecal samples. B, baseline urease activity. Cryo-A, urease activity measured in fecal samples of subjects that consumed *S. thermophilus* BT01 prepared with Cryo-A. Cryo-B, urease activity measured in fecal samples of subjects that consumed *S. thermophilus* BT01 prepared with Cryo-B. Freidman test showed significant difference between baseline and treatment periods ($p = 0.019$), with Dunn's post hock test showing significant difference between B and Cryo-A treatment ($p = 0.022$).

closely related human commensals, *S. salivarius* and *S. vestibularis* species. To overcome this issue and with certainty detect and enumerate the *S. themophilus* BT01 strain, in this study we designed an effective and reliable strain-specific qPCR molecular tool targeting CRISP-*Csn2* gene sequence combined with a recently developed culture-based medium specifically set-up for the growth of *S. thermophilus*.[32]

Furthermore, being aware of the potential effect of the industrial production process on the in vivo performance of probiotics,[14] we tested the effect of two different cryoprotectants, Cryo-A and Cryo-B, on the faith of *S. thermophilus* BT01 in human GIT after ingestion. Cryoprotectants are commonly added to the probiotic biomass at the end of the fermentation process, and after its recovery by centrifugation.[6,8,19] The cryoprotectants are mixed with the probiotic biomass in a process that lasted about an hour at a low temperature (about 4 °C). Nevertheless, it could have the effect not only on the survival rate of the further freeze-drying process, but also on the physiology of the collected biomass by modulating gene expression, metabolic pathways, or single enzymatic activities.[6,36,37] In this study, the use of two different cryoprotectants, Cryo-A and Cryo-B, had different effects on the freeze-dried biomass. While Cryo-A and Cryo-B allowed to obtain a comparable number of live cells per g, the use of Cryo-A resulted in a higher number of damaged and dead cells compared to those present in Cryo-B treated cells (Table 1, Figure 2). Moreover, the measurement of the urease activity in freeze-dried biomasses showed significantly higher levels in Cryo-A than Cryo-B treated biomass (Figure 2). FCM analysis of Cryo-A and Cryo-B treated cells revealed a further difference between the two biomasses. Cells treated with Cryo-A showed an additional population less permeable to the SYTO 24 dye (Figure 2, live cells[b]). The reason of the population heterogeneity in Cryo-A treated cells needs to be clarified. However, cell heterogeneity within a homogeneous genetic background, revealed by single cell analysis by FCM, has been previously observed for other phenotypes in *S. thermophilus* DSM 20617^T and *Lactococcus lactis* M1.[38,39]

During the interventional study, at baseline (T0), no viable BT01 cells were present in the fecal samples analyzed. This was expected considering that the subjects were instructed not to consume any food or probiotic supplements possibly containing *S. thermophilus* strains during the run-in period (1 week). On the other hand, five out of 20 subjects showed the presence of BT01 DNA, thus suggesting that this strain is most likely present in marketed dairy products, and that its DNA can persist for a week after the last consumption.

The intervention study, and specifically the measurement of total *S. thermophilus* BT01 DNA in fecal samples, did not reveal significant differences between subjects that received Cryo-A or Cryo-B treated biomass. Likewise, the recovery of live *S. thermophilus* BT01 was comparable between subjects that received Cryo-A or Cryo-B treated biomass. Nevertheless, we observed that *S. thermophilus* BT01 is able of surviving the human GIT in 75% of the subjects after the second administrated dose for both Cryo-A and Cryo-B. Additionally, BT01 strain persisted up to 2–3 days (T6) after 1 week of daily consumption in 50% of the subjects. Viable counts in fecal samples of other probiotic strains ingested with a dose of 10^{11} CFU were reported to be in average 5.5 Log_{10} CFU g^{-1} fecal sample for *L. paracasei* DG,[40] and 7.3 Log_{10} CFU g^{-1} fecal sample for *Bifidobacterium animalis* subsp. *lactis* BB-12.[41] These differences in recovery demonstrate that survival of probiotics is a strain specific trait, and depends on methodology, especially choice of culture medium.

In our study, the data about viable BT01 recovery were reported as Ct values and not as for total DNA counts in cells g^{-1} feces. This is because in the fecal sample the known number of BT01 cells and not colonies were spiked. Therefore, by making a calibration curve using spiked fecal sample and plating it on modified milk-based medium (where colonies and not cells are counted) we could not find the correlation between Ct values and cell per g of fecal sample. However, considering that positive qPCR detection of BT01 means that at least one colony was detected on the plate inoculated with 0.1 mL of the 10^{-1} dilution. Therefore, in case of positive qPCR detection of BT01, its recovery was considered at least equal or higher than 2 Log_{10} CFU g^{-1} fecal sample.

Intra-individually, out of 18 (subjects whose recovery was positive), in 10 subjects' recovery was higher when Cryo-B compering to Cryo-A was used. However, due to the high inter-subject variability and total absence of BT01 recovery in two subjects we did not observe significant differences in recovery based on the use of a specific cryoprotectant (Figure 5A,B). The inter-individual variability in recovering the BT01 cells may be associated with the subjects' differences in genetics, as well as dietary patterns, which could affect gastric conditions, and thus the survival of a probiotic strain.[40–42] Furthermore, the effect of different cryoprotectants on probiotic strain survival has always been explained on the basis of in vitro conditions mimicking the gastric environment,[12,19,43] and up to date, there is no clear in vivo evidence demonstrating differences in probiotic survival through human GIT lyophilized with different cryoprotectants.

Upon probiotic treatment, no relevant changes were observed in defecation frequency and consistency.

Most subjects reported one fecal evacuation per day during the entire study duration, with inconsiderable changes in

defecation frequency when the BT01 was lyophilized by using Cryo-A or Cryo-B. Fecal consistency did not significantly change during the study period, with a score 4 (like a smooth soft sausage or snake). Already satisfactory parameters of digestive parameters and relatively short probiotic administration period could be explanation for not considerable changes.

While the recovery of *S. thermophilus* BT01 was not affected by the cryoprotectants used, the modulation of urease activity was indeed observed. Comparing to baseline (T0), a decrease in the urease activity was observed in 16 versus 10 subjects for Cryo-A and Cryo-B, respectively. On average Cryo-A protected cells showed to be significantly effective in decreasing the urease activity in fecal samples (Figure 6). In the GIT, urease (EC 3.5.1.5) is produced by numerous, mainly anaerobic species of bacteria.[44] Urease hydrolyzes urea, a major nitrogenous waste product of mammals, to yield ammonia and carbon dioxide.[23] Patients with altered gut microbiota such as juvenile chronic arthritis and IBD patients are characterized by the increased levels of fecal urease activity.[27,45] On the contrary, the consumption of the lyophilized *L. rhamnosus* GG powder [44] or commercial probiotic supplement containing *B. longum, B. infantis, B. breve, L. acidophilus, L. delbrueckii* subsp. *bulgaricus, Lactocaseibacillus casei, L. plantarum*, and *S. thermophilus*[26] decreased the fecal urease activity in these patients. Furthermore, in IBD patients, it was reported that intestinal inflammation leads to the growth suppression of indigenous beneficial urease-positive bacteria such as *Blautia* and *Ruminococcus* spp., and niche replacement by urase-positive harmful bacteria.[25,27] Modulation of urea hydrolysis by beneficial urease-positive *Lactobacillus reuteri* 100–23 strain was reported in the murine stomach.[46] Moreover, inoculation of a conventional murine host with commensal *Escherichia coli* engineered to express urease led to dysbiosis of the gut microbiota, resulting in a predominance of *Proteobacteria* species and it was associated with a worsening of immune-mediated colitis in these animals.[27] Based on our data and the available literature,[25–27,45,46] we could hypothesize that the administration of beneficial urease positive bacteria, such as *S. thermophilus*, could lead to higher urea consumption in the upper part of the gut, thus determining a decreasing of urea availability in the large intestine. This, consequently, could lead to decrease of harmful fecal urease-positive bacteria, such as *Proteus mirabilis, Klebsiella pneumoniae*, thereby determining a reduction of the overall urease activity in fecal samples. In this study, the reduction of fecal-urease was likely linked to the higher level of urease activity present in Cryo-A comparing to the Cryo-B protected BT01 cells. However, it cannot be excluded that cells lyophilized with Cryo-A could have had the modulation of other physiological traits suitable to increase their fitness in gut microbiota, and specifically against urease-positive species. Whatever the nature of the physiological variables involved, our results highlight the influence of the industrial production process on the probiotic traits of *S. thermophilus* BT01. In conclusion, our study showed the significance of reliable molecular tools design to demonstrate ability of *S. thermophilus* strains to survive harsh human GIT environment. Lyophilization of the probiotic *S. thermophilus* BT01 using two different cryoprotectants did not significantly change the strain fecal recovery. However, future investigations are needed to take into consideration the larger group of subjects involved, as well as the standardization of some inter-subject variables. Furthermore, the use of *S. thermophilus* BT01 lyophilized with Cryo-A may have serve as potential therapeutic target for patients with altered gut microbiota (e.g., IBD patients). However, clinical studies on those subjects are necessary to support our hypothesis. More in general, this study represents the first evidence that links *S. thermophilus* BT01 Cryo-A to a specific probiotic mechanism, i.e., the reduction of urease activity in fecal samples. This study highlights, how the probiotic biomass manufacturing conditions could play a role in influencing probiotic molecular composition and functionality in vivo as recently underlined for other probiotics.[14]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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This study is dedicated to Prof. Pier Luigi Manachini (1938–2022), my mentor, who first teach me the microbiology, and pushed me to study the physiology of *S. thermophilus*.

Conflict of Interest

The authors declare no conflict of interest. D.M. is in the Scientific Advisory Board of Actial Farmaceutica SRL.

Author Contributions

M.A.: experiments conduction, data analyzing and elaboration, original and final draft writing; C.M.: experiments conduction; A.S.: experiments conceptualization, methodology, supervision, writing - review and editing; M.D.: experiments conceptualization, methodology, resources, supervision, writing - review and editing. All authors have read and agreed to the submitted version of the manuscript.

Data Availability Statement

All data related to the PROVIRTUS study are available to research scientists.

Keywords

cryoprotectant, interventional study, probiotic, *Streptococcus thermophilus*, urease

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