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Sugar as a vehicle for the probiotic spore-forming bacteria delivery

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ABSTRACT

Probiotic spores have been proposed to be carried by a variety of unconventional probiotic foods, but sugar has never been considered before. In this study, five probiotic commercial bacilli spores (*Shouchella clausii* from Enterogermina, and UBBC 07; *Heyndrickxia coagulans* Unique IS2, BC30, and BC513) were added to granulated table sugar, which was then used to sweeten six different beverages, including hot and cold tisane, instant coffee, and espresso. For instant coffee and tisane brewing, two heat settings - 80 and 100 °C - were evaluated. Spores' survival and germination in beverages were evaluated over time, as well as after passing a simulated gastrointestinal passage. Significant variances among strains could be highlighted, especially concerning the spores' ability to germinate on a minimal medium. The best behaviour for all strains was recorded when probiotic sugar was used to sweeten espresso; furthermore, no statistical differences emerged when the coffee was analysed again after a ten-minute rest at room temperature. In instant coffee and tisane, strains BC30 and SA kept the initial population level of 8 Log CFU/g before and after GIT, regardless of the water temperature during brewing. Strain BC30 retained the highest bacterial loads for total counts on Luria Bertani and spores on the same medium after thermal treatment. Furthermore, even when water was brought to a boil, spores retained their capacity to germinate.

The spore content was unaffected by a two-month storage at room temperature. For the daily supply of probiotics, probiotic sugar seems to be a suitable alternative.

1. Introduction

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a beneficial effect on host health" (Hill et al., 2014). The global market for probiotics is expected to reach USD 76.7 billion by 2027, driven by rising consumer awareness of the associated health benefits (Oliveira et al., 2021).

To influence both the gastrointestinal microflora and the immune system, probiotics need to arrive alive and in sufficient numbers in the colon. In this perspective, the Italian Health Ministry (Ministero della Salute, 2018) recommended a minimum daily intake of at least 10^9 CFU per serving. This amount of alive cells has to be guaranteed up to the product's expiration date. In other words, probiotics need to retain their viability along the food processing chain and during passage through the gastrointestinal tract (Soares et al., 2023).

Despite their wide application in probiotic foods manufacturing, lactic acid bacteria (LAB) strains can be harmed or lose viability due to unfavourable or suboptimal conditions that arise during the food chain's production and storage (i.e. temperatures exceeding 60 °C), or just because of intrinsic food characteristics such as low pH or water activity (Lavrentev et al., 2021). A promising alternative to overcome such limitations is the use of spore-forming probiotic cultures (Almada-Érix et al., 2022). The high resistance of microbial spores to high temperatures, low pH, and high pressures, coupled with features such as good stability and rapid germination, make their use highly appealing in the development of unconventional probiotic foods (Foligné, Daniel & Pot, 2013; Permpoonpattana, Hong, Khaneja & Cutting, 2012; Soares et al., 2023; Zhao Zhou, Huang, Nan & Liu, 2021).

Although the use of spore-forming bacteria as probiotics is still limited, the innate ability to withstand environmental stresses is a valuable and marketable characteristic, especially in the food and feed industry (Soares et al., 2023). Among the most common spore-forming

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probiotic microorganisms, species belonging to the *Bacillus* genus stand out, since able to satisfy the most common criteria for characterizing a probiotic strain, including viability during the gastrointestinal tract (resistance to bile, stomach acid, and peristaltic movements) and the ability to synthesize several human-useful compounds (Elshaghabee, Rokana, Gulhane, Sharma & Panwar, 2017).

Anyhow, the successful delivery hinges on a thorough understanding of the food matrix and its impact on spore fate and functionality. As a matter of fact, from a probiotic perspective, spores and vegetative cells have separate but complementary functions (Bernardeau, Lehtinen, Forssten & Nurminen, 2017). Both spores and vegetative cells can promote some features, such as immunomodulation and adsorption, while others, such as the antimicrobial activity, are distinctive traits of vegetative cells (Bernardeau et al., 2017). So, in order to be beneficial, bacilli must regain their metabolic function once they have entered the host. Knowing the number of spores that could germinate during food intake and digestion is therefore essential (Blaiotta, De Sena, De Girolamo, Aponte & Romano, 2023).

Probiotic bacilli have been used to supplement many confectionery products such as chocolate, chewing gum, and gummy candy (Bartkiene et al., 2018; Erdem et al., 2014; Kahraman et al., 2023; Khullar, Nayem, Kanyakam, Thanyasrisung & Prakitchaiwattanaal, 2024), and, on the other hand, bacillus spores have been incorporated in tea and coffee before brewing (Majeed et al., 2019), but as far as we know, the incorporation in sugar for hot beverage sweetening has never been investigated yet. In the present study, the spores of five probiotic commercial bacilli were incorporated into common granulated table sugar that was then used to sweeten six distinct beverages. The adoption of more than one thermal level for the preparation allowed for a better understanding of spores' resilience to wet heating.

2. Material and methods

2.1. Probiotic strains

Bacilli used in this study were the polyantibiotic-resistant*: Shouchella* (*S.*) *clausii* (formerly *Bacillus clausii*) strain commercially available in a spore suspension marketed as Enterogermina® (Sanofi Aventis) and here designated as *S. clausii* SA. Each vial (5 mL) contains 2×10^9 spores of a mixture of four bacterial strains known as O/C, SIN, N/R, and T, characterized by an extended pattern of resistance to many antibiotics (Abbrescia et al., 2014). *S. clausii* UBBC 07 and *Heyndrickxia* (*H.*) *coagulans* (formerly *Bacillus coagulans*) Unique IS2 were both kindly provided by Unique Biotech Limited (Genome Valley, Hyderabad, India), whereas *H. coagulans* BC30™ GBI-30 6086 and BC513 were isolated from commercially available probiotic products.

2.2. Spores' production

Cultures in the exponential phase of growth of the five strains were streaked on Luria Bertani (LB - Oxoid, Basingstoke, UK) agar plates and incubated aerobically at 37 °C. Sporulation was monitored by Schaeffer-Fulton staining with Malachite Green. Plates were sealed with parafilm to avoid evaporation.

Colonies were collected using quarter-strength Ringer's solution (Oxoid). After centrifugation twice at 6500 × *g* for 30 min, the supernatant was re-centrifuged using the same settings. The pellet was then resuspended in a minimal volume of 10 % (w/vol) sterile Skim Milk (Oxoid). Suspensions were placed in sterile metal trays in layers of approximately 1 cm thickness, frozen at −18 °C, and subsequently lyophilized in a freeze-drier (Edwards® Freeze Dryer Modulyo RC.8D 9602,021, Italy) at 2–3 mbar (−1,5/−1,3 Atm).

Spores' content was checked by plate counting onto the LB (Lennox) medium (Tryptone 10.0 g L^{-1}, Yeast extract 5.0 g L^{-1}, NaCl 5.0 g L^{-1}) as well as on the same medium after treatment at 75 °C in a shaking water bath for 30 min at 50 rpm, followed by immediate cooling below 45 °C (LB75 °C). Since LB is a complex medium likely containing nutrients that may act as spore germinants, counts were also carried out on a minimal medium, the Spizizen medium (SM) with glutamic acid (1 %) as a nitrogen source (Blaiotta et al., 2023).

2.3. Probiotic sugar preparation

Spores were added to commercially available granulated white Grade 1 sugar up to 10^8 CFU/g. Specifically, in a sterile environment, lyophilized spores were added to increasing sugar amounts to achieve an even distribution. The content of spores in sugar was checked by plating onto LB, LB75 °C, and SM as described in section 2.2; then 5-gram aliquots - the amount typically found in a single-serve sugar packet - were packaged and kept at 25±1 °C in a glass vacuum desiccator (StonyLab, US) with indicated silica gel, until use. The spores' content was also assessed after two months of storage by counting on LB after thermal treatment (LB75 °C).

2.4. Beverage preparation

Probiotic sugar was used to sweeten various beverages. Specifically, the spores' behaviour was tested in six different conditions. One sachet (5 g) was used to sweeten espresso and instant coffee, as well as tisane in commercially available infusion bags. For espresso coffee, compostable pods were brewed in a dedicated machine set at 70 °C. The beverage was collected up to 25 mL in a 50 mL sterile beaker. Instant coffee was prepared following the producer's instructions: 2.5 g (roughly 2 teaspoons) were reconstituted in 150 mL of deionized water heated to 80 or 100 °C in a 250 mL sterile beaker. Similarly, the tisane (red hibiscus flowers, apple, orange zest, natural flavours 8 %, licorice root, cherry 0.5 %, blueberry 0.5 %) was prepared in sterile deionized water at both 80 and 100 °C as well as in cold water (4 °C). The volume of water for the tisane was set at 200 mL in a 250 mL beaker. Temperature and pH were monitored during the beverage cooling (pH meter HI6221–02, Hanna Instruments, Italy). In all cases, sugar was placed into the beaker before the beverage was poured. Each experiment was carried out in duplicate.

2.5. Simulated gastrointestinal transit

Except for espresso, which was tested immediately, all beverages were subjected to passage through a model mimicking the actions of the upper gastrointestinal tract (GIT) ten minutes after preparation (T0). Espresso was sampled again after a ten-minute rest at room temperature, whilst in the five other cases (instant coffee at 80 and 100 °C, tisane at 80, 100, and 4 °C), the beverages were subject to further simulated GIT after 40 min by the brewing (T1). The protocol was the one described by Ricciardi, Blaiotta, Di Cerbo, Succi and Aponte (2014) with some modifications. Samples were treated for 10 min in simulated saliva (SS-16.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$, and 1.2 g/L $NaHCO_3$, lysozyme 100 ppm, pH 6.90). The bolus was diluted 1/1 (vol/vol) with simulated gastric juice (SGJ - the SS electrolyte solution plus 0.3 % porcine gastric mucosa pepsin) and then 1:4 with simulated duodenum secretion (SDS - 6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl, 0.5 % bile salts Oxgall, and 0.1 % pancreatin). The length of treatments, 10 min for SS and 120 min for SDGJ and SDS transit, was as reported by Ricciardi et al. (2014). pH was adjusted to 2 in SGJ and 7.2 in SDS every 30 min using HCl (1 M) and NaOH (1 M), respectively. In all steps, stirring was used to simulate the peristaltic movements. Samples were analysed at time 0, as well as after SS, SGJ, and SDS. After each step, bacilli were counted on three different media (LB, LB75 °C, and SM) as previously described. Each analysis was performed in duplicate.

2.6. Probiotic espresso acceptance test

A total of 28 consumers (32 % female and 68 % male), aged 21 to 63, were asked to assume a cup of espresso sweetened with one sachet of

probiotic (S1) and standard sugar (S2) for an affective acceptance test. The strain incorporated in sugar for testing was *H. coaugulans* BC30. Each consumer evaluated the two coffee samples in one session, with a 15-minute break between samples. Consumers evaluated the products by using a 9-cm nonstructured hedonic scale anchored in the terms "dislike extremely" on the left and "like extremely" on the right (Suppl. Material 1 – Sensory Evaluation Form), for the following attributes: aroma, flavour, texture, and overall impression (Stone & Sidel, 2004).

2.7. Statistical analysis

Significant differences among data were computed by using ANOVA and Tukey *t*-test ($p < 0.05$) (XLStat 2012.6.02 statistical pocket, Addinsoft Corp., Paris, France).

3. Results and discussion

3.1. Probiotic sugar

The spores' level obtained after freeze-drying was in all cases higher than 10^9 spores/g, ranging from 9.82±0.64 Log CFU/g for strain UBBC 07 to 11.12±0.07 and 11.12±0.71 Log CFU/g for strains BC30 and BC513, respectively (Fig. 1). Spores were added to sugar up to reach a concentration of about 8 Log CFU/g. The results for counting on the three media are reported in Fig. 1 as well. The proper spore concentration, as revealed by counting on LB, was obtained only for strains BC30 and BC513. Indeed, the spores' concentration in sugar proved to be extremely uneven, as inferable by the high values of the standard deviation in more than one case. Spore counts on LB75 °C fully reflected those reported for the same medium without thermal treatment. Actually, only strain BC513 showed statistically significant differences. On the other hand, in both strains provided by Unique Biotech, a loss of germination ability on minimal medium occurred: counts on SM for strains UBBC 07 and IS2 were over 3 Logs CFU/g lower than those recorded on LB agar (Fig. 1). Anyhow, since each strain has its specific requirements in terms of nutrient, pH, temperature, and oxygen tension (Elisashvili, Kachlishvili & Chikindas, 2019), it cannot be ruled out that the five strains' varying behaviour is to be linked to the standard protocol adopted for sporulation. A two-month storage at room temperature did not affect the spore content. The number of decimal reductions (γ) calculated based on the difference between the initial (T0) and final LB75 °C count (T1) was not significant in all cases (Data not shown).

When dried date paste was used as a carrier for *H. coagulans* BC4 spores, total viable cells and spores were unaffected by water activities between 0.48 and 0.59 (Marcial-Coba, Pjaca, Andersen, Knøchel & Nielsen, 2019), suggesting that this aw range is unable to induce germination events. More recently, three probiotic bacilli, including the strain BC30, were used to assess the impact of water activity in baked goods. In no case, the water activity exerted a discernible effect. Spores of *Bacillus* usually germinate at 0.80 a_w or above (Payne, Bellmer & Jadeja, 2025). In such a perspective, the use of sugar as a spore vehicle raises no problems. According to Commission Regulation (EC) No 952/2006 of 29 June 2006, Grade 1 white sugar must have a maximum moisture content of 0.06 % and, consequently, this may limit viability losses of the embedded probiotic.

3.2. Spores' behaviour in beverages subject to simulated GIT

The probiotic sugar was tested for in potential applications, namely as a sweetener for espresso, instant coffee, and hot or cold tisane. Two thermal levels, 80 and 100 °C, were evaluated to mimic the supposable variability in the temperature of the water used by customers when preparing tisanes or instant coffee. *Bacillus* spore survival and germination during beverages' simulated GIT revealed interesting variations among strains, especially concerning the spores' ability to germinate on the minimal medium SM. When probiotic sugar was used to sweeten espresso, the strain *H. coagulans* BC513 lost about 2 Logs on LB as well as on the same medium after the thermal treatment (Fig. 2). Given that the drop occurred immediately following the brief passage in the simulated saliva, it makes sense to assume that exposure to hot water may have contributed to the spores' decreased capacity to germinate (Fig. 2). For strains SA, BC30 and BC513, counts on SM revealed that the number of spores triggered to germination by the exposure to the warm beverage was at least of around one Log lower than the number of spores counted on LB75 °C. However, the worst results were those logged for both strains supplied by Unique Biotech: counts for strain UBBC 07 fell to roughly 5 Logs, and losses for strain IS2 were even greater. Anyhow, both strains did not experience any further loss due to the GIT (Fig. 2).

When sugar was used for instant coffee prepared with water heated at 80 °C, counts on LB were satisfactory for nearly all strains with counts in the range of 7–8 Log CFU/g of sugar, but with some noteworthy differences: strains BC513 and UBBC 07 lost more than one Log. Counts on LB75 °C matched those on LB for both strains. None of the examined strains experienced any further losses as a result of the GIT.

On the other hand, when the coffee was prepared with boiling water some differences could be observed: strains BC30 and SA still kept the

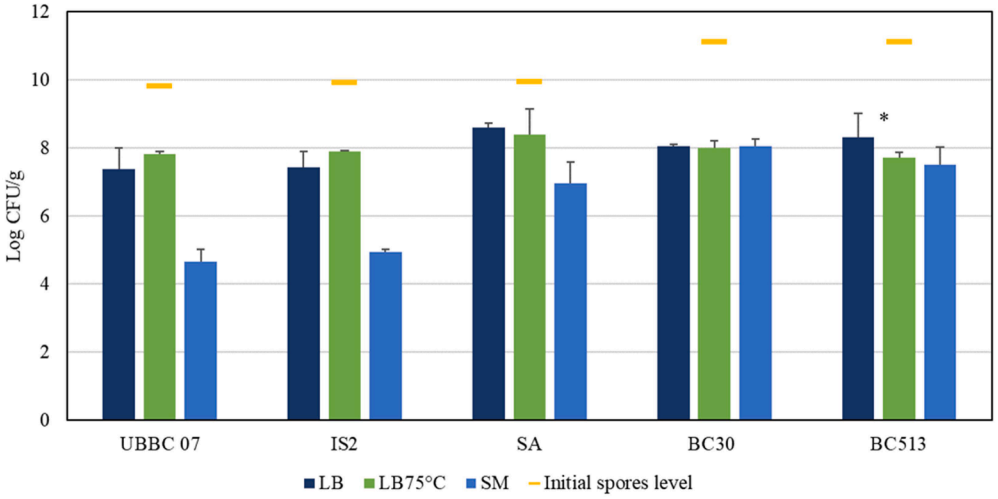

Fig. 1. Counts of probiotic spores (yellow bars) on Luria Bertani (LB) after treatment at 75 °C (LB75 °C). In the columns, results of counts (mean ±sd) on LB, LB after treatment at 75 °C (LB75 °C), and Spizizen medium (SM) of the sugar enriched with five probiotic bacilli. Asterisks indicate significant differences between media by Tukey test t ($p < 0.05$).

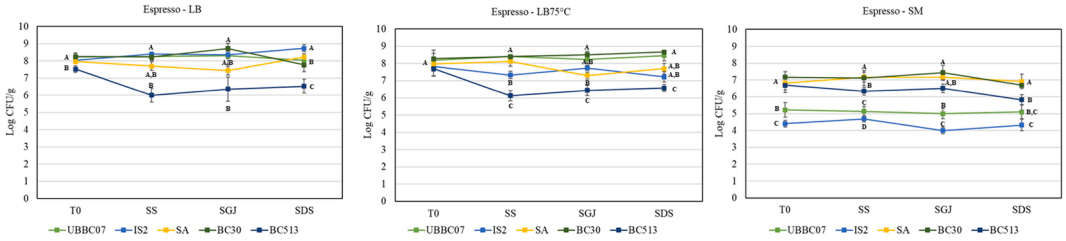

Fig. 2. Evaluation of probiotic spores incorporated in sugar used to sweeten espresso during simulated GIT. For data with the same letter, the differences between strains are not statistically significant ($p < 0.05$).
SS: Simulated Saliva; SGJ: Simulated Gastric Juice; SDS: Simulated Duodenal Secretion.

initial population level of 8 Log CFU/g before and after GIT, whereas counts on LB for strains UBBC 07 and IS2 were consistently nearly 2 Logs lower, regardless of the GIT step (Fig. 3). Results obtained for spores counted on LB after the thermal treatment were perfectly overlapping.

The strain IS2 was also used by Adibpour, Hosseininezhad and Pahlevanlo (2019a) for the coating of rock candies, known as Nabat, often used as a sweetener for warm beverages. The authors checked the spore behaviour in the temperature range of 85–50 °C, thus excluding the common use of boiling water to prepare hot beverages. After thermal treatment at 90–95 °C for 10 min, the authors counted spores on skim milk agar and found no loss. In this perspective, their data perfectly match those obtained in this study for counts on LB75 °C in the case of instant coffee made with water heated at 80 °C (Fig. 3).

Similar to espresso, spores able to germinate on the minimal medium were at most two Logs lower for the two best-performing strains (BC30 and SA). By contrast, the ability to produce colonies on SM appeared to be jeopardized for strains IS2 and UBBC 07. Above, when the water temperature was raised to 100 °C, neither strain showed any development on SM (Fig. 3).

In the case of tisane, results on LB and LB75 °C were in good agreement with those obtained for instant coffee, despite variations due to the different volumes or the different amount of solutes (Fig. 4). As a matter of fact, the dynamics of cooling were rather different in the various kinds of beverages (Fig. S1). Also in this case, a loss of germination ability could be observed as counts' lowering on the SM medium. Above all, when boiling water was used to prepare the tisane, even for strains BC30 and SA, the loss was around 3 Logs (Fig. 4).

The use of sugar in cold tisane provided controversial results. Outcomes from LB and LB75 °C counts confirmed the poor resistance of strain IS2, but unexpectedly, both UBBC 07 and IS2 were no longer detectable on SM during the simulated GIT (Fig. 4). This behaviour was not recorded when sugar enriched with probiotics was used for the preparation of the espresso.

Espresso was subject to simulated GIT again after ten minutes at room temperature, while the five other beverages were held at room temperature for an additional half-hour before testing once more. For espresso, no statistical difference occurred after 10 min, even in terms of spores triggered to germination (Fig. 5). The high pH (6.10 ± 0.1) coupled with the lower brewing temperature makes sweetened espresso an optimal option for delivering probiotic spores.

For the other hot beverages, strains UBBC 07 and IS2 showed a remarkable decrease on both LB and LB75 °C media. On the other hand, the counts after the GIT did not experience further losses. On SM, both bacilli were no longer detectable (Fig. 5). In addition to temperature, such a decline may be related to the acidic environment. The pH of instant coffee was 4.98 ± 0.3, and that of the tisane was even lower (4.88 ± 0.1). Despite this, strain BC30 demonstrated high tolerance across all media and beverages. However, strain BC30 was found to have worse resistance when added to orange juice subject to high-temperature short-time (HTST) pasteurisation by Almada-Érix et al. (2021). In this instance, the acidic matrix was linked to the decreased spore resistance. Despite observed variations (da Silveira, Tavares & Glória, 2007), instant coffee varieties typically have a pH of approximately 5, as recorded in the current investigation, which is higher than that of fruit juice.

Furthermore, for both values obtained at time zero (T0) and those obtained after the simulated digestion (SDS-10), differences between counts on LB and those for spores on LB75 °C after the beverage rest were consistently not significant (Data not shown). In other words, neither the rest nor the extended GIT period induced the spores'

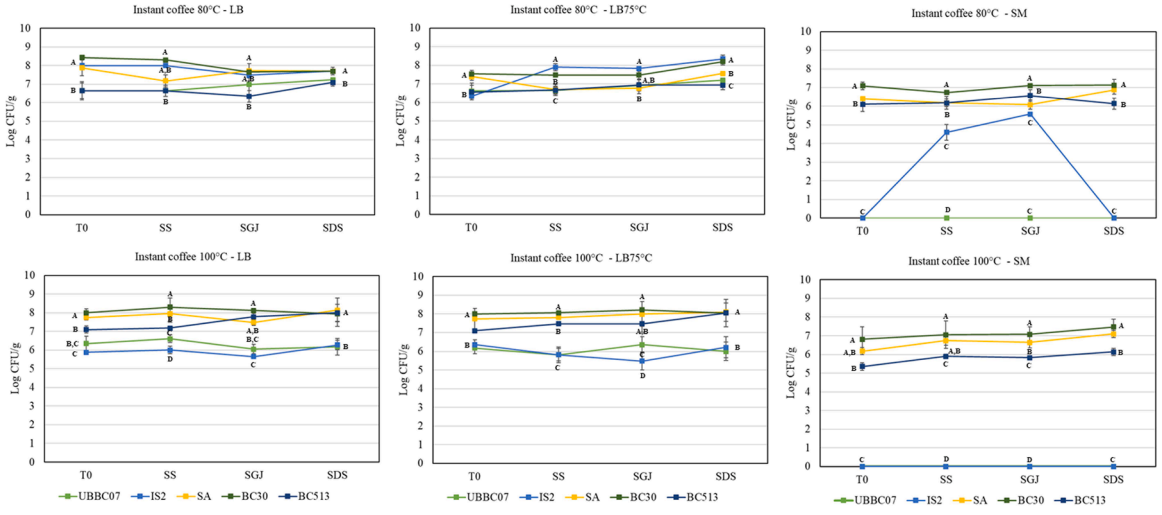

Fig. 3. Evaluation of probiotic spores incorporated in sugar used to sweeten instant coffee brewed at 80 and 100 °C during GIT. For data with the same letter, the differences between strains are not statistically significant ($p < 0.05$).

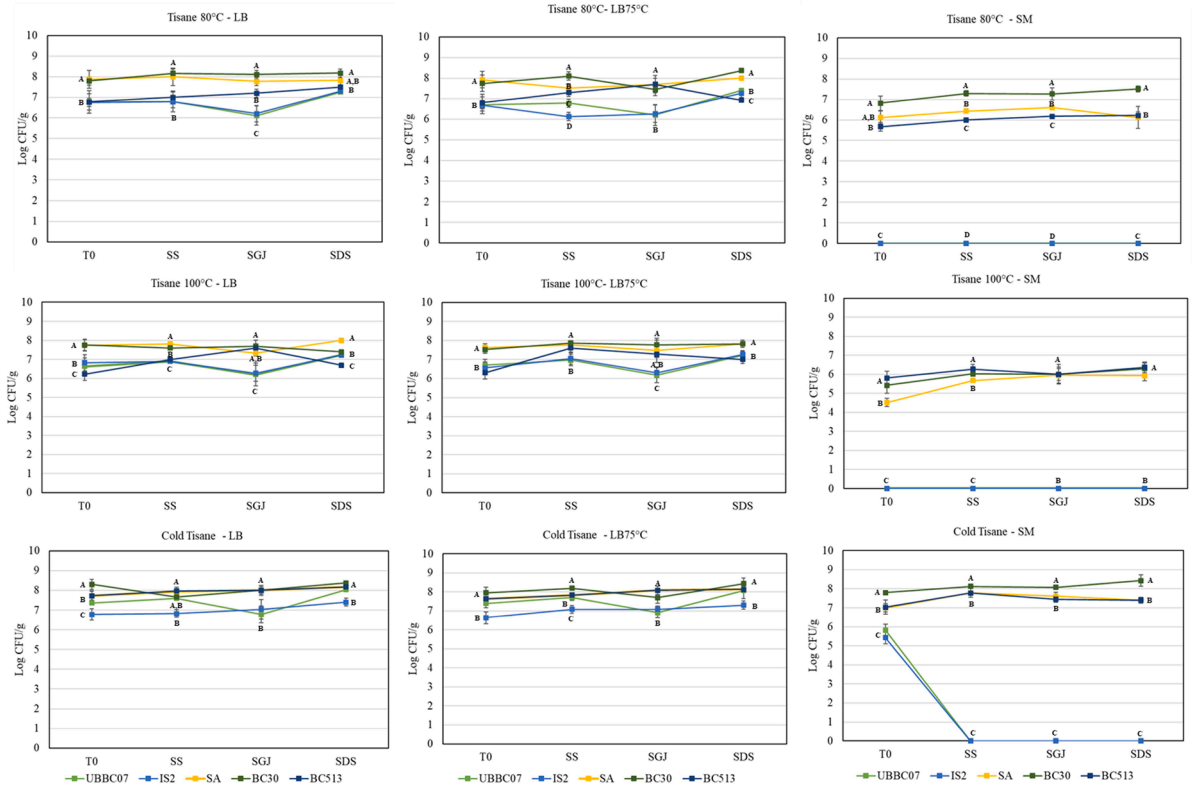

Fig. 4. Evaluation of probiotic spores incorporated in sugar used to sweeten cold tisane as well as tisanes brewed at 80 and 100 °C during simulated GIT. For data with the same letter, the differences between strains are not statistically significant ($p < 0.05$).

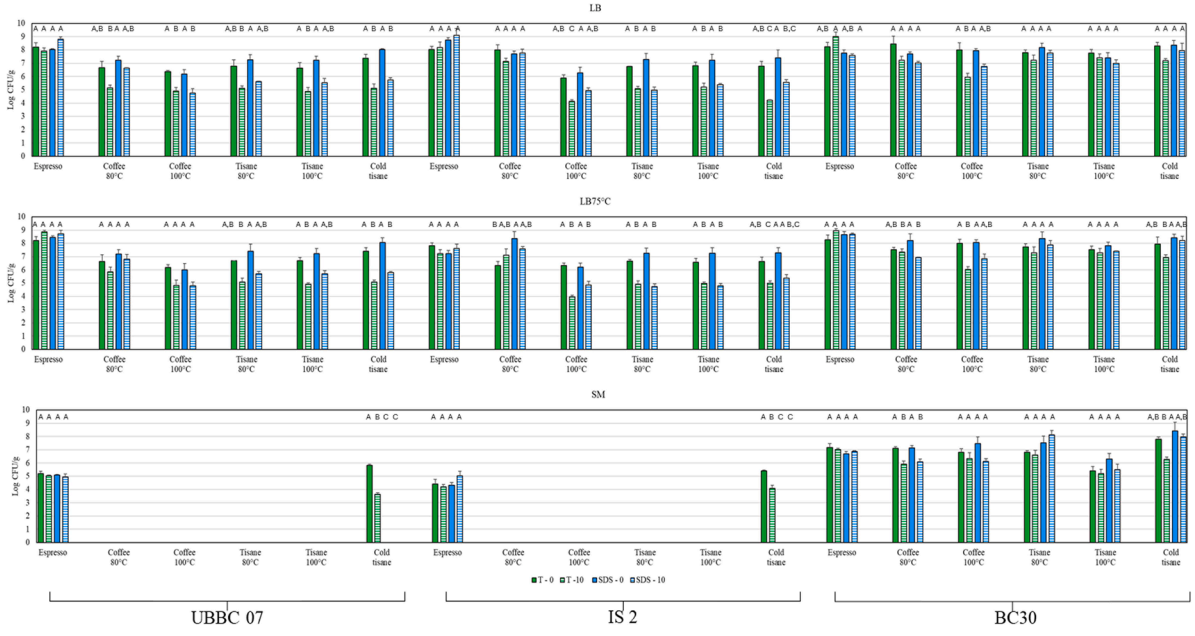

Fig. 5. Counts on LB, LB75 °C, and SM at time 0 before (T0) and after GIT (GIT T0) were compared with counts on the same media after a 10-min rest for espresso and a 30-min rest for the other five beverages before (T1) and after GIT (GIT T1). For data with the same letter, the differences are not statistically significant ($p < 0.05$).

germination. Heat treatments at sublethal temperatures are well known to be able to increase and synchronise the spore germination of Bacillales and Clostridiales (Wen et al., 2022).

There is a likelihood of spore germination in liquid coffee matrices, since the presence of nutrient germinants cannot be excluded. Germinants for *Bacillus* spp. spores include amino acids (e.g., L-alanine, L-valine, L-leucine, L-cysteine), ribosides (e.g., adenosine, inosine), and sugars (e.g., glucose, fructose) (Cho & Chung, 2020). Once germinated, metabolically active bacilli behave like any other conventional vegetative probiotics (Bernardeau et al., 2017), potentially invoking sensorial

changes related to acidity, aroma, and mouthfeels in coffee brews (Chan & Liu, 2022).

Strain BC30 showed the best performance in almost all beverages, with the highest bacterial loads for total counts on LB and spores on LB75 °C, with levels of bacilli even higher than 8 Logs. Moreover, counts on LB and LB75 °C stayed constant during the GIT thus proving the probiotic features of this strain. Furthermore, the germination capacity of strain BC30 was lowered only in the worst case: tisane at 100 °C (Fig. 4).

Actually, the resistance of this strain to wet heat has already been reported: when added to milk subject to slow pasteurization (Almada-Érix et al., 2021) or in milk to be pasteurized for the manufacture of "requeijaõ cremoso" (Soares al., 2019).

The spores of *B. coagulans* strain BC30 possess a resilient integument primarily composed of proteins, which enables them to withstand gastric acid and bile salts for effective delivery to the intestines (Maathuis, Keller & Farmer, 2010).

The impact of coffee brewing on spores has already been evaluated by Majeed et al. (2016). In this instance, the strain *H. coagulans* MTCC 5856 was added to roasted coffee, which was brewed by using water preheated at 90 °C. After 2 min at this temperature, the beverage was cooled to 77 °C and kept for 4 h at this temperature. The reduction in spores was 34 % at the end of monitoring, whereas the loss was negligible after half an hour. Results are in good agreement with those obtained for strains SA and BC30 in the current study.

Anyhow, in the present survey, it was demonstrated that BC30 spores did not lose their ability to germinate even when water was heated up to boiling and that spores are triggered to germination in a percentage, calculated as a ratio between SM and LB75 °C, of 92.9 %. On the other hand, results do not match those reported for the strain BC30 by Polo et al. (2022). According to the authors, this strain, when added to tea bags, experienced a one-Log reduction after a three-minute-long infusion in water at 100 °C, with cell densities measured as spores after thermal treatment. In any case, other than the temperature at which they are brewed, the components of tea and coffee (catechins, tannins, and caffeine) did not appear to have an impact on the survivability of probiotic bacilli (Majeed et al., 2019).

Probiotic features of strain *B. coagulans* strain BC30 have been in-depth underlined. Intestinal gas, rheumatoid arthritis, and irritable bowel syndrome (IBS) are among the GI disorders whose symptoms may be successfully reduced by BC30 spores' administration (Fares et al., 2015). Additionally, *B. coagulans* can promote the growth of beneficial bacteria and competitively exclude pathogens (Adibpour, Hosseini-nezhad, Pahlevanlo & Hussain, 2019b) by producing a bacteriocin-like substance named coagulin Honda, Gibson, Farmer, Keller and McCartney (2011). In fact, this strain is widely considered for the development of unconventional probiotic foods, such as cereal bars (de Paula Antunes et al., 2025), noodles (Wang et al., 2025), and Minas Frescal cheese (Silva et al., 2024).

3.3. Sugar acceptability

Espresso was chosen for the test due to the large acceptance of this beverage in Italy, for the fast consumption, and, in this instance, the number of spores in each serving is the highest when using the entire sachet as a sweetener. Results referring to the acceptance test (Table 1) demonstrated that all descriptors obtained an average score above 6.32 ±1.96 in probiotic sugar (S1), which corresponds to the hedonic scale "I liked" and can be considered accepted by the participants (da Silva, Tagliapietra & dos Santos Richards, 2021). Anyhow, texture obtained the worst scores for both kinds of sugar, and this can only be related to the coffee quality. However, aroma, flavour, and texture showed no differences between the samples, maybe because the presence of the spores did not interfere with the sensory profile of the espresso. Regarding the overall impression, probiotic espresso obtained a better rating (Table 1). Similar results were obtained when the strain IS2 was incorporated into a coating for Nabat: probiotic rock candies obtained a better overall acceptability (Adibpour et al., 2019a).

Table 1
Sensory acceptance of espresso sweetened with one sachet of probiotic (S1) or standard sugar (S2) by the affective acceptance test. Results are reported as mean values ±SD.

Sensory attribute	S1	S2
Aroma	$7.54 \pm 1.10_a$	$7.71 \pm 1.70_a$
Flavour	$7.64 \pm 0.99_a$	$7.43 \pm 1.29_a$
Texture	$6.32 \pm 1.96_a$	$6.36 \pm 1.52_a$
Overall impression	$7.36 \pm 1.25_a$	$6.46 \pm 1.71_b$

[a] Means followed by the same letter on the line do not differ statistically from each other by the Tukey test at 5 % significance. $N = 28$.

4. Conclusions

Despite its potential for application as probiotics, the spores of bacilli are challenging to manage in food processing. Indeed, spore-forming probiotics should not germinate in the food matrix as they may spoil the food under favourable conditions.

Since probiotics must be taken regularly to promote the expected effects, the incorporation should preferably be carried out in food largely consumed. From this perspective, going with the straightforward choice of a probiotic sugar sachet appears a promising option. Even in the worst scenery, instant coffee and tisane prepared with boiling water, strains BC30 and SA kept the initial population level of 8 Log CFU/g before. Furthermore, even when water was brought to a boil, BC30 spores retained their capacity to germinate on minimal medium SM.

The sugar was monitored for two months, and no change in the spore content was noticed. Anyhow, due to the low water activity, it is likely that longer storage would not induce any loss. On the other hand, since beverage consumption habits may be highly variable with even long rest times, the spores' germination in the matrix, especially in coffee, cannot be ruled out. In this perspective, to reap the benefits, the label should clearly state the requirements for using probiotic sugar in hot beverages.

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Ethical statement

Sugar and spores used in this study are approved and regulated for human consumption. They do not represent new chemicals, substances, biomasses, or pharmaceuticals that would require specific ethical review. All the suppliers of the ingredients used are certified producers for food sale, in compliance with current regulations. The study was conducted in agreement with the guidelines of the Declaration of Helsinki and the rules of "Personal Data Protection Code", Reg. EU 2016/679 of the European Parliament and of the Council of 27 April 2016. Informed consent was obtained from all subjects involved in the study, ensuring the respect of privacy, participants' rights, and any information related to allergy or intolerance of participants to the ingredients of our formulations. The written informed consent was developed according to the principles of the Declaration of Helsinki (1964 and its later amendments), Italian ethical requirements on research activities and personal data protection (D.L. 30.6.03 n. 196), and the ethical standards of the University of Naples Federico II (Suppl. Material 2 -Informed consent).

CRediT authorship contribution statement

Maria De Sena: Investigation, Data curation. **Raffaele Romano:**

Writing – original draft, Data curation. **Maria Aponte:** Writing – review & editing, Conceptualization. **Vittorio Valletta:** Supervision, Resources. **Giuseppe Blaiotta:** Writing – review & editing, Visualization, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2025.101586.

Data availability

Data will be made available on request.

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