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Human *in vivo* assessment of the survival and germination of *Heyndrickxia coagulans* SNZ1969 spores delivered via gummy candies

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ABSTRACT

Confectionary products hold promise as unconventional food carriers for probiotic microorganisms. This study explored the delivery of *Heyndrickxia coagulans* SNZ1969, a spore-forming probiotic, using gummy candies. In this study, we prepared gummy candies containing bacterial spores with a viable count that remained stable during a 24-month shelf-life period, meeting the label claim of at least one billion CFUs per serving (24 g). Then, we carried out an intervention trial involving 24 healthy adults who consumed one serving per day for two weeks followed by an additional two weeks of follow-up. Fecal samples were collected and analyzed with a protocol that allowed the viable counts of SNZ1969, both in spore and vegetative forms. The obtained results revealed that bacterial spores germinated in all volunteers. SNZ1969 persistence in the gut was monitored for two weeks after the end of gummy candy consumption, indicating its potential for prolonged colonization. These findings highlight the potential of unconventional food carriers for probiotic delivery and suggest that spore-forming probiotics can be metabolically active in the human intestine. These findings provide information for the development of food products containing spore-forming probiotics and their potential benefits in promoting gastrointestinal health.

1. Introduction

The ingestion of live microorganisms through food is of particular interest in the context of the “microbial depletion hypothesis” (also known as the "old-friends hypothesis"), which suggests that reduced exposure to microorganisms from the environment and food may have contributed to an increase in the incidence of immune disorders (Scudellari, 2017). In this context, enriching food with live cells of probiotic microorganisms (i.e., “*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*” (Hill et al., 2014),) can represent a valuable strategy. Probiotic microorganisms, in fact, are reported to exert their health-promoting properties primarily impacting the gut microbiome and preserving immune homeostasis (Lee et al., 2023; X. Wang et al., 2021).

Traditionally, probiotic microbes are delivered through dairy or dairy-like products derived from the fermentation of cow’s milk or plant-based infusions (primarily soy (Kumari et al., 2022),). However, there is a growing interest in using other types of food products as carriers for probiotic microorganisms, such as chocolate (Faccinetto-Beltran et al., 2021) or bakery products (Almada-Erix et al., 2022). Furthermore, the possibility of including probiotic bacteria in confectionery products such as candies appears promising, allowing a broader spectrum of consumers, including younger individuals, to access this dietary component through an enjoyable form of food. Nonetheless, studies aimed at assessing the suitability of products such as candies and chewing gum for successfully delivering live microorganisms into the human intestine are very limited.

Particularly for non-dairy alternatives, to address the challenge of probiotic cell survival during industrial manufacturing processes, the food industry is increasingly turning to spore-forming bacteria like members of the species *Heyndrickxia coagulans* (formerly *Bacillus coagulans*), whose biomass is conventionally included in food formulations as endospores, which are highly resistant structures formed by certain bacteria in response to adverse environmental conditions (Cho and

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Chung, 2020). In bacterial endospores, metabolic processes are significantly slowed down or halted, approaching a state of dormancy or cryptobiosis (Paul et al., 2019).

However, ingested microorganisms must survive gastrointestinal transit and be metabolically active in the intestine to provide benefits to the host. For example, the Italian Ministry of Health defines probiotic products as "*those foods that contain a sufficiently high number of live and active probiotic microorganisms capable of reaching the intestine, multiplying, and exerting a balancing action on the intestinal microflora through direct colonization*" (Ministero della Salute Italiano, 2018). Therefore, the survival and active metabolism of probiotic cells in the human gut are considered essential to exert their beneficial properties and should be assessed for each specific probiotic microbial strain. In the case of spore-forming probiotics, while their ability to withstand the stresses associated with food manufacturing processes and gastrointestinal transit is evident, there is also a need to establish their capacity to germinate in the intestine. Several studies have demonstrated the ability of exogenously administered spores of bacilli to germinate in the intestines of various animals, including rabbits, chickens, pigs, and mice (Popov et al., 2021). However, the germination capacity of *Bacillus* spores has been only marginally studied in the human intestine (Ghelardi et al., 2015; Latorre et al., 2014) and, to the best of our knowledge, there are no studies on spore germination in humans for *H. coagulans*, despite members of this species being widely employed as probiotics in various food matrices.

The ability of a probiotic microbe to survive gastrointestinal transit is assessed through "recovery studies." These are human intervention trials in which the microbial strain of interest is selectively quantified in feces after ingestion, using molecular and, preferably, culture-dependent methods when selective (strain-specific) cultivation conditions are available (Arioli et al., 2018; Radicioni et al., 2019; Taverniti et al., 2019).

In the context described above, we conducted a recovery study quantifying the cells of the probiotic strain *H. coagulans* SNZ1969 in feces after ingestion via gummy candies by healthy adults. Notably, this study employed an experimental strategy capable of quantifying selectively the colony forming units derived from the bacterium in the form of spores and the ones derived from the bacterium in form of vegetative cells.

2. Materials and methods

2.1. Design of the intervention trial

This study was a single-arm, open-label microbiological trial. The study design is illustrated in Fig. 1. The primary objective of the study was to quantify the viable cells of a spore-forming probiotic bacterial strain administered through gummy candies in fecal samples from healthy adult volunteers and compare the number of viable spores with that of viable vegetative forms.

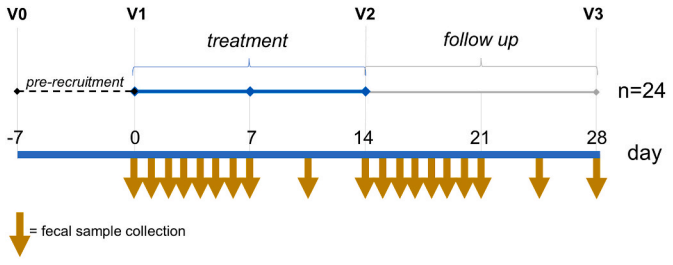

Fig. 1. Study design. Each brown arrow corresponds to a fecal sample collection point. V0–V3, the four visits attended by each study participant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Ethical statement

The study protocol received approval from the Research Ethics Committee of the Università degli Studi di Milano, with opinion nr. 89/22 expressed on October 28, 2022. Written informed consent was obtained from all the subjects before recruitment.

2.2. Study population

The number of subjects recruited was based on previous studies with a similar microbiological endpoint, where 20 subjects were recruited to assess the persistence of live probiotics in the gastrointestinal tract (Arioli et al., 2018; Radicioni et al., 2019). Therefore, estimating a 20% dropout rate, we enrolled in the project 24 adult volunteers. The target population comprised university employees, students, and their relatives. The inclusion criteria were as follows:

- Volunteers of both sexes aged between 18 and 65 years.
- Written informed consent from the volunteer.

The exclusion criteria were:

- Use of antibiotics in the month before the study
- Use of anti-acids or gastrointestinal prokinetics
- Chronic inflammatory bowel diseases
- Infectious intestinal diseases
- Diagnosis of active irritable bowel syndrome (IBS)
- Episodes of viral or bacterial enteritis in the 2 months before the study
- Episodes of gastric or duodenal ulcers in the previous 5 years
- Pregnant or breastfeeding women
- Recent history or suspicion of alcohol or drug abuse
- Any severe pathology that may interfere with treatment
- Inadequate reliability or presence of conditions that may result in non-conformity/adherence of the patient to the protocol
- Previous participation in this study
- Allergy or intolerance to the food product under study.

2.3. Product under study

A typical formulation of the gelatin-based gummies, representative of the candies used in this study, is summarized in Table 1. The gummies utilized in this recovery trial were manufactured as illustrated in Fig. 2. In specific, probiotic biomass was added to the cooked gummy candy syrup along with colors, flavors, and acids when the candy syrup reached approximately 75–85 °C. No further candy mass heating took place after the addition of probiotic biomass. The probiotic biomass consisted of an industrial lyophilized powder preparation of the bacterium *Heyndrickxia coagulans* strain SNZ1969 provided by Centro Sperimentale del Latte S.r.l. (Sacco System, Zelo Buon Persico, Italy). Viable count analyses showed that the industrial biomass used consisted exclusively of spores (no significant amounts of vegetative forms were

Table 1
Formulation of the strawberry flavored gelatin-based gummies used in this study. Percents refers to weight/weight.

Ingredient	
Glucose syrup	35–52 %
Sucrose	20–30 %
High bloom animal gelatin	6–8 %
Water	18–20 %
Strawberry juice	2.5–4 %
Acids (lactic, malic, citric)	0.6–1.2 %
Natural flavor	0.2–0.5 %
Heyndrickxia coagulans SNZ1969	at least 4.2×10^7 CFU/g

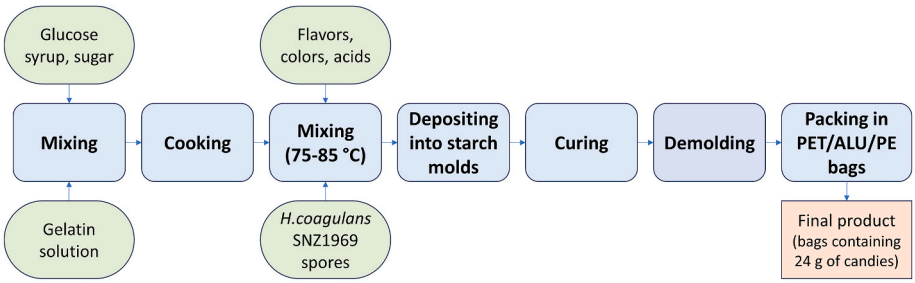

Fig. 2. Schematic of the processing steps for the gummy candies used in the intervention trial. PET/ALU/PE, polyethylene terephthalate/aluminium/polyethylene metallized pack material.

found). Subsequently, the liquid candy syrup was deposited into molds made from dried powdered starch and placed in curing rooms at controlled temperature and humidity conditions for at least 24 h. Once solidified, the gummy candy pieces were demolded and then cleaned to remove any remaining starch using an air jet. A total of 24 g of finished gummies (with a moisture content of about 20% and water activity of 0.7), each weighing approximately 4 g, were packaged in laminated plastic film [polyethylene terephthalate/aluminium/polyethylene (PET/ALU/PE) - metallized pack material]. According to label specification, gummy candies contained no less than 4.2×10^7 CFU/g corresponding to one billion CFU per serving (24 g) of the probiotic bacterium *H. coagulans* SNZ1969.

2.4. Description of the intervention trial

The study lasted for a total of 35 days. During the first visit (recruitment visit, V0; Fig. 1), we collected informed consent and personal data from the volunteers. We also provided them with general information about the study protocol and instructed them on the dietary changes they needed to follow during the pre-recruitment phase. The pre-recruitment (run-in) phase lasted for one week, during which the volunteers followed their regular diet except for the prohibition of probiotic fermented milks, probiotic food supplements, and prebiotic food supplements. At the end of the pre-recruitment period, volunteers attended a second visit (V1), during which we instructed them on how to take the product during the treatment phase. We also provided volunteers with the Bristol Stool Chart questionnaire for monitoring their bowel movements and instructed them on how to complete it. The treatment phase began with each volunteer consuming one serving (24 g, corresponding to the intake of no less than a billion CFUs) of the product once a day in mid-morning or mid-afternoon (at least an hour after or an hour before a meal). After two weeks of treatment, the volunteers underwent two weeks of follow-up, which was identical to the pre-recruitment period. The volunteers delivered the completed Bristol stool charts at the end of the follow-up period (visit V3). During the study, the volunteers provided fecal samples according to the schedule shown in Fig. 1, depending on the frequency of their bowel movements. If there were more than one bowel movement per day, we only collected the first one. We asked the volunteers to freeze the fecal sample in a domestic freezer immediately after collection, and to deliver the specimen to the laboratory within 72 h. At the beginning of the study, we provided them with a small thermal bag containing a eutectic plate to keep specimens frozen during delivery to the laboratory. Once in the laboratory, we immediately stored the samples at −20 °C and processed them within three days from collection. We conducted preliminary experiments to demonstrate that the viable counts of both spores and vegetative cells of the *H. coagulans* strain were not significantly affected by refrigeration in a domestic freezer for three days.

2.5. Viable count of H. coagulans in feces

To determine the total and spore viable counts in fecal samples, the following protocol was followed. One gram of fecal sample was diluted 1:10 in Maximum Recovery Diluent [MRD, Scharlab, containing peptone (1 g/l) and sodium chloride (8.5 g/l) at pH 7.0] within a stomacher bag. The contents of the bag were hand manipulated for 1 min and homogenized for an additional minute in the stomacher. The resulting mixture was transferred to a sterile glass tube and serially diluted in MRD before being plated on GYEA agar medium (composition per liter: glycerol 5 g, yeast extract 2 g, K_2HPO_4 1 g, bromocresol green 0.05 g, agar 15 g, pH 5.5). The plates were then incubated at 55 °C for 72 h and the colonies were enumerated. To determine spore counts, the same protocol was followed with one modification: the fecal sample was resuspended in a stomacher bag with MRD prewarmed at 90 °C, followed by incubation at 90 °C for 10 min in a laboratory water bath. To determine the number of vegetative forms, the count obtained after pasteurization (corresponding to the number of spores) was subtracted from the count obtained without pasteurization (total number of colony formant units, i.e., spores + vegetative forms).

2.6. Viable count analyses of jelly candies

The shelf life of gummy candies was determined over a period of 24 months of storage at uncontrolled room temperature, at six-month intervals, using the following protocol. Ten grams of the product were diluted ten times in MRD. After 25 min at 37 °C, the suspension was homogenized in a stomacher for 2 min. Next, 5 ml of the sample were serially diluted, plated on Bromocresol purple (BCP) agar medium, and incubated for 24 h at 37 °C under aerobic conditions. The BCP medium had the following composition per liter: casein peptone (5 g), yeast extract (2.5 g), dextrose (1 g), malt extract (2.5 g), L-cysteine-HCl monohydrate (0.1 g), bromocresol purple (0.05 g), agar (15 g), and a pH of 7.0.

We also analyzed the gummy candies used in the intervention trial with the protocol adopted for the analysis of feces (as described in the previous paragraph). In brief, 4 g of food product was resuspended in 36 ml of prewarmed MRD (37 °C) by hand manipulation until completely melted, followed by 1 min of homogenization using a stomacher. For the quantification of spores, a different sample (4 g) of jelly candies was homogenized in prewarmed MRD at 90 °C, followed by a 10-min incubation at 90 °C before serial dilution. Subsequently, dilutions were plated on GYEA agar and then incubated at 55 °C for 72 h.

2.7. Design of strain-specific primers and PCR protocol

We generated the draft genome of *H. coagulans* SNZ1969 using shotgun sequencing with an Illumina NovaSeq system. After sequence assembling, we conducted comparative genomic analysis with the available *H. coagulans* sequences in GenBank to identify genomic regions unique to strain SNZ1969. We designed four pairs of primers and verified them using endpoint PCR with metagenomic DNA and DNA from different *H. coagulans* strains. We selected a primer set (Wc-1F, 5′-TTGTCTTTGGATCAGTTACAG-3’; Wc-1R, 5′-GCATAGGAA-TACCTTGTGCA-3′) targeting a putative gene coding for a PimA-like

glycosyltransferase (phosphatidyl-myo-inositol mannosyltransferase), which produced an amplicon of 192 pb under the following PCR conditions: 2 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C, and a final elongation step of 72 °C for 10 min. We used this PCR protocol to identify colonies isolated from fecal samples on GYEA plates. To do this, we collected colonies from agar plates with a loop, resuspended them in MRD and, after centrifugation, we discarded the supernatant and froze the cell pellet for at least 1 h at −20 °C. Then, we resuspended the cell pellet in sterile milliQ water and heat-treated it at 99 °C for 15 min in a thermoblock. Finally, we cooled the samples in ice for 10 min and centrifuged them. The resulting supernatant was used as the template in PCR reactions. The experimental procedure described above is illustrated in Supplementary Fig. S1.

2.8. Statistical analysis

To assess the normal distribution of the data, we employed the Shapiro-Francia test. As the microbial viable counts data did not follow a normal distribution, we conducted statistical analyses using nonparametric methods, specifically the Wilcoxon-Mann-Whitney test, to assess for statistically significant differences between groups. We considered $P < 0.05$ as the threshold for significance and $0.05<P < 0.1$ as indicating a trend.

3. Results

3.1. Microbiological characterization of the food product

The gummy candies containing the probiotic strain *H. coagulans* SNZ1969 were found to be stable during the shelf-life experiment. In fact, the overall reduction in the viable count was only 0.12 log over 24 months of incubation at room temperature, decreasing from 1.1×10^8 to 8.3×10^7 CFU/g (corresponding to 2.6×10^9 and $2{,}0 \times 10^9$ FCU per 24g serving, see Supplementary Fig. S2). Therefore, the gummy candies prepared for this study were suitable for maintaining the number of viable probiotic cells in the product above the minimum viable number claimed on the label, i.e., 1 billion CFUs per 24 g of food serving.

In addition, we quantified the amount of viable *H. coagulans* SNZ1969 cells in gummy candies sampled from the lot administered to the volunteers of the intervention trial, adopting the same protocol used for quantifying the bacterium in fecal samples. This protocol enabled us to obtain the overall viable count and, after heat treatment at 90 °C, the viable count of the bacteria in the form of endospores. This experiment showed that the two quantifications did not differ significantly ($n = 4$, mean ± standard deviation: 7.93 ± 0.18 Log_{10} CFU/g) suggesting that *H. coagulans* SNZ1969 was present in the food product ingested by volunteers exclusively as endospores, providing a possible explanation for the marked stability of the viable counts determined over the 24-months shelf-life period.

3.2. Outcome of the intervention study

Twenty-four healthy adults (10 females and 14 males; mean age: 34 ± 4 years) provided informed consent to participate in the study, and all completed the 2-week intervention. One participant (S07) withdrew from the study on day 15 due to gastrointestinal flu (Fig. 3A). No volunteer reported any adverse events, and the product was generally well-liked.

In total, we analyzed 412 fecal samples. At the initial time point (day 0), colonies were detected on plates for 12 subjects. However, these colonies were observed only at the lowest dilution tested (1:10), and their morphology differed significantly from that expected for strain SNZ1969. We isolated 31 of these colonies, which, following 16S rRNA gene sequencing, were taxonomically classified into the following species: *Calidifontibacillus erzurumensis* (14 isolates from 5 subjects), *Bacillus stercoris* (8 isolates from 5 subjects), *Bacillus velezensis* (4 isolates from 2 subjects), *Heyndrickxia coagulans* (2 isolates from 2 subjects), *Bacillus rugosus* (1 isolate), *Paenibacillus glycanilyticus* (1 isolate), and *Enterococcus faecium* (1 isolate; the only non-spore-forming species in this list). As a result, it was discovered that two volunteers already harbored the species *H. coagulans* in their gut prior to the study, albeit at concentrations below 100 CFUs per gram of feces. This implies that the cultivation experiments performed before probiotic ingestion confirmed that the growth medium and culture conditions utilized in the study are effective in preventing interference from resident fecal microbes when quantifying the probiotic strain of interest in fecal samples.

The analysis of all fecal samples collected over the intervention trial revealed the presence of viable *H. coagulans* SNZ1969 in both spore and vegetative cell forms among all participants. However, in a limited number of fecal samples (39 out of 412, approximately 9%), the vegetative form was not detected, even though spores were present (e.g., day 6 for volunteer S01; Fig. 3A). It remains unclear whether these results are attributable to technical issues, such as inadequate pasteurization or improper fecal sample storage, or if they indicate a genuine absence of spore germination within the intestine. Nevertheless, the latter scenario appears unlikely, as vegetative forms were often detected on the day before and/or after (Fig. 3A). On the other hand, in 13 samples (approximately 3%), no colonies were detected on plates after pasteurization indicating the presence of vegetative cell forms only.

The vegetative forms of *H. coagulans* in feces accounted for a range of 18%–75% of the total CFUs detected (Fig. 4). Notably, a significant difference in counts between spores and vegetative forms was observed at only three time points: day 1 ($P = 0.040$; median counts of vegetative forms vs. median counts of spores: 5.6 vs. 5.3 Log_{10} CFU/g), day 2 ($P = 0.084$; 5.9 vs. 5.7 Log_{10} CFU/g), and day 20 ($P = 0.004$; 2.7 vs. 3.4 Log_{10} CFU/g) (Fig. 3B).

Remarkably, the study revealed that most volunteers retained viable *H. coagulans* SNZ1969 in their gut throughout the trial. Specifically, the probiotic strain of interest was isolated from the feces of 12 subjects on day 24 (i.e., 10 days after the conclusion of candy consumption) and from the feces of another 12 subjects at the study's last time point (day 28, i.e., 2 weeks after the end of candy consumption) (Fig. 3A).

Additionally, bowel movement data was systematically recorded for each subject during the study using the Bristol stool chart. A modest increase in the median fecal type was observed during week two (Fig. S3); nonetheless, the statistical analyses did not reveal any significant changes.

4. Discussion

This study investigated the delivery of probiotics via unconventional food carriers, specifically gummy candies, to enhance the intake of live microorganisms for potential health benefits. Utilizing non-dairy alternatives for probiotic delivery presents challenges, particularly in preserving probiotic cell viability during industrial manufacturing processes. To overcome this, the food industry has increasingly turned to spore-forming bacteria (Soares et al., 2023), such as *Heyndrickxia coagulans*, which holds a long history of safe use in food and has been included in the Qualified Presumption of Safety (QPS) list by the European Food Safety Authority (EFSA) (EFSA BIOHAZ Panel EFSA Panel on Biological Hazards, 2024).

Notably, several potential health-promoting activities attributed to *H. coagulans*, such as lactic acid production, secretion of antibacterial peptides, and synthesis of active enzymes enhancing gut nutrient absorption (Cao et al., 2020; Honda et al., 2011; Maathuis et al., 2010). Accordingly, various strains of *H. coagulans* were demonstrated to modulate the composition of the intestinal microbiota in older individuals (Nyangale et al., 2015), improve symptom relief in irritable bowel syndrome (IBS) (Abhari et al., 2016; Zhang et al., 2022), facilitate recovery from high-intensity endurance exercise (Jager et al., 2016), reduce incidence and duration of acute rotavirus diarrhea in infants (Chandra, 2002), and enhance protein absorption (Jager et al., 2018).

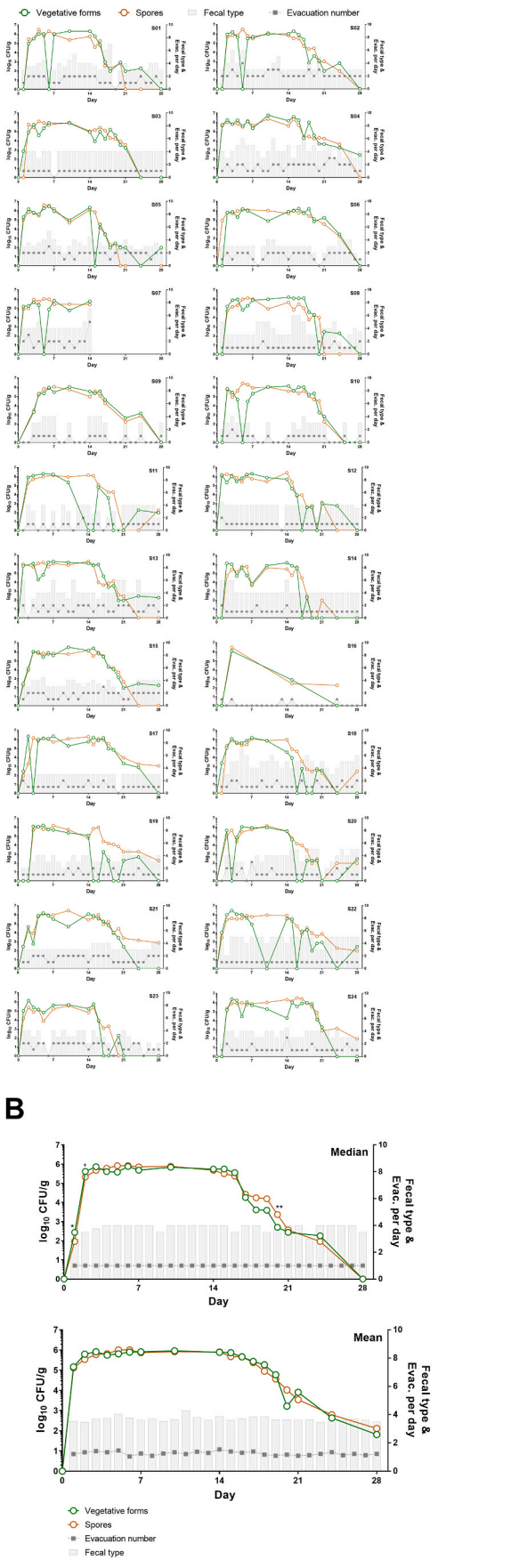

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Fig. 3. Kinetics of viable *H. coagulans* SNZ1969 in fecal samples during the trial. The orange lines (Spores) represent the viable counts determined after pasteurization of the fecal sample, while the green lines (Vegetative forms) represent the difference between the viable counts without pasteurization (total count) and the viable counts after pasteurization (spore count). **A**. Viable counts quantified from fecal samples for each volunteer at specific time points, according to the study scheme illustrated in Fig. 1. Each graph also reports the fecal type and the number of evacuations per day as determined by means of the Bristol stool chart. **B**. Graphs for median and mean data of all volunteers. Due to the non-parametric distribution of data, statistical analysis was conducted with median data using the Wilcoxon test to identify significant differences between the viable counts of vegetative forms and spores at each time point. **, $P < 0.01$; *, $P < 0.05$; +, $0.05<P < 0.10$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Based on this literature, we decided to use a *H. coagulans* strain (namely SNZ1969) as probiotic in the gummy candies under investigation.

The gummy candy production protocol used in this study, despite involving steps at high temperatures, effectively preserved the probiotic's viability. In fact, the final product contained a sufficiently high probiotic count to support daily intake of at least one billion CFU per serving, aligning with the daily consumption recommended by the Italian Ministry of Health (Ministero della Salute Italiano, 2018). In addition, shelf-life tests conducted confirmed that the live bacteria in the gummy candies remained stable at room temperature for up to 24 months after production, indicating compatibility with the industrial and commercial requirements for such products.

H. coagulans is a bacterium with minimal nutritional requirements and thrives in a broad temperature range (15–60 °C). These characteristics enabled the development of a cultivation protocol with high specificity and selectivity for the probiotic strain under study, which was based on the use of glycerol as carbon source and an incubation temperature of 55 °C. Experiments conducted at baseline (i.e., before the intake of gummy candies) using this protocol revealed the presence of thermophilic members of the Bacillaceae family in about half of the volunteers' feces. However, these were in relatively low quantities (less than 100 CFU per gram of feces) and exhibited distinct colony morphology compared to the target strain, confirming the specificity of the isolation and cultivation protocol developed for *H. coagulans* SNZ1969.

To demonstrate that *H. coagulans* endospores can germinate *in vivo*, we developed a protocol for the enumeration of viable *H. coagulans* SNZ1969 that included a step involving the pasteurization of the fecal sample in order to distinguish between the bacterium in the form of spores and vegetative cells. This differentiation provided insights into the fate of this probiotic strain within the gastrointestinal tract. Only a limited number of studies have explored the ability of probiotic endospores to germinate *in vivo*. For instance, the germination of *B. subtilis* was confirmed in the gastrointestinal tract of mice (Casula and Cutting, 2002), with experimental data suggesting that bacterial spores may complete their entire life cycle within this environment (Tam et al., 2006). Other studies demonstrated the capability of endospores from members of the Bacillaceae family to germinate and reproduce in the guts of insects (Margulis et al., 1998), poultry and pigs (Jadamus et al., 2002; Jadamus et al., 2001). Notably, Latorre et al. (2014) observed in chickens that the spore germination capability of *Bacillus* species can reach a high level (approximately 90%) within an hour of ingestion. Only a few studies were conducted in humans. For instance, Ghelardi et al. isolated *Shouchella clausii* colonies from the feces of 20 healthy adults for 12 days after ingesting spores of this bacterium, calculating that in some volunteers, the total quantity exceeded the number of spores ingested, suggesting germination and proliferation of this bacterium had occurred (Ghelardi et al., 2015). Furthermore, Colom et al.'s research revealed that orally ingested *Bacillus subtilis* DE111® spores could germinate in the small intestine of humans within 3 h after

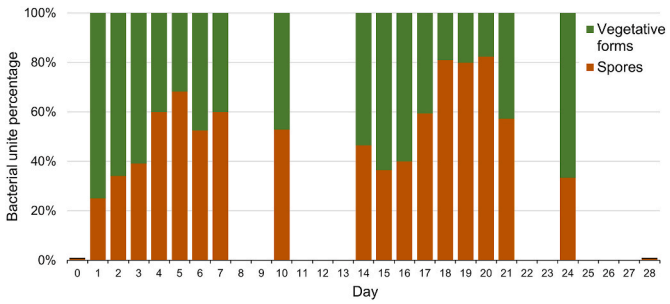

Fig. 4. Percentage ratio between the median viable counts of vegetative forms and spores at each time point during the trial.

ingestion (Colom et al., 2021). In our current study, even though the bacterium in the administered gummy candies was entirely in the form of endospores, we observed a comparable amount of this bacterium in both the vegetative and spore forms in feces, suggesting that the ingested spores germinated during their passage through the human gastrointestinal tract. Importantly, this conclusion held true for all 24 healthy adult volunteers participating in the trial.

Although we reported the percentage of vegetative forms relative to spores, the data from this study do not allow us to determine how many of the ingested spores were actually able to germinate. Indeed, two possible scenarios with potentially opposite effects could not be explored with our experiments: (i) only a minority of the ingested spores germinated, and the resulting vegetative forms reproduced (leading to an increase in the vegetative forms identified in the feces); (ii) some of the vegetative forms originating from the ingested spores completed their full life cycle and sporulated again (leading to an underestimation of the number of ingested spores that actually germinated).

The gummy candies used in this study contain noticeable amounts of sugars (sucrose, glucose, maltose, and higher oligomers of glucose). Reportedly, certain sugars have been demonstrated to trigger germination of *B. subtilis* when combined with L-asparagine, and KCl (Atluri et al., 2006). In addition, glucose has also been recognized as a germinant for *B. megaterium* spores (Gupta et al., 2013; Hyatt and Levinson, 1964). Therefore, we can hypothesize that the sugar in the candies may have contributed to the probiotic spores' germination in the gut of the volunteers in our study.

Furthermore, the results of this study indicate that the investigated probiotic strain has a strong ability to persist in the gastrointestinal tract. In fact, viable cells of *H. coagulans* SNZ1969 were isolated from the feces of most volunteers more than 10 days after discontinuation of jelly candy consumption. The available literature contains conflicting reports concerning the ability of probiotic microorganisms to survive gastrointestinal transit. Nevertheless, the viable fecal recovery observed for *H. coagulans* SNZ1969 in the gummy candies developed for this study is, in terms of both the percentage of volunteers and the duration after cessation of intake, comparable to or even superior to that of well-established probiotic strains such as *Lacticaseibacillus paracasei* Shirota, *Lacticaseibacillus rhamnosus* GG, and *Bifidobacterium animalis* subsp. *lactis* BB12 (Arioli et al., 2018; Granata et al., 2013; Poutsiaka et al., 2017; R. Wang et al., 2015).

Interestingly, some studies have identified engraftment of probiotics in the human gut even for extended periods. For instance, *Bifidobacterium longum* AH1206 was shown to stably persist in the gut of 30% of individuals for at least 6 months (Maldonado-Gomez et al., 2016). Furthermore, *Lactiplantibacillus plantarum* ATCC 202195 and *B. longum* subsp. *infantis* EVC001 were shown to persist in infants for approximately 6 months and one year, respectively, after discontinuing probiotic administration (O'Brien et al., 2022; Panigrahi et al., 2017). In our study, the presence of *H. coagulans* SNZ1969 in feces was monitored for only two weeks after the cessation of probiotic intake, so we cannot establish the exact duration of its intestinal persistence. However, despite a relatively low abundance (ranging from 10^2 to 10^3 CFU per g), viable SNZ1969 was still detectable in the feces of approximately half of the subjects at the end of the follow-up phase, suggesting a capacity for prolonged colonization.

5. Conclusion

The study described herein provides insights into the delivery of probiotic microorganisms within the human intestine using unconventional food carriers, such as gummy candies. The information provided by this study is not readily available for most commercial probiotic food products, as human intervention trials assessing viable fecal recovery are most conducted using probiotic supplements in the form of capsules, powders, or sachets. Moreover, studies that differentiate between vegetative and spore forms of spore-forming probiotic strains are exceptionally rare.

In specific, this trial demonstrated that the gummy candies developed in this study can effectively deliver the strain *Heyndrickxia coagulans* SNZ1969 into the human intestine, allowing for its germination *in vivo*. Therefore, the findings of this study highlight the potential of unconventional food carriers such as gummy candies in delivering and maintaining probiotic microorganisms within the human intestine. These findings also suggest that spore-forming probiotic bacteria of the species *H. coagulans* can be metabolically active in the human gut and, consequently, exert their health-promoting properties. However, further controlled trials are needed to establish any potential health benefits for the consumer from ingesting this probiotic product.

CRediT authorship contribution statement

Susanna Perotti: recruited and trained volunteers and collected questionnaire data, developed laboratory protocols for the strain-specific quantification of the probiotic in feces and performed the preliminary experiments, processed fecal samples and performed viable count, Formal analysis, also performed PCR experiments, Data analysis and statistics were carried out, Writing – original draft, with the contribution. **Giacomo Mantegazza:** developed laboratory protocols for the strain-specific quantification of the probiotic in feces and performed the preliminary experiments. **Elena Pierallini:** processed fecal samples and performed viable count, Formal analysis, also performed PCR experiments. **Natalja Kirika:** conceived the study, gave support in the realization, storage and, Formal analysis, of the gummy candies used in the intervention trial at Perfetti Van Melle laboratory, The manuscript was revised. **Robin Duncan:** processed fecal samples and performed viable count, Formal analysis, All authors read and approved the final version of the manuscript for publication. **Nicolò Telesca:** processed fecal samples and performed viable count, Formal analysis. **Andrea Sarrica:** conceived the study, gave support in the realization, storage and, Formal analysis, of the gummy candies used in the intervention trial at Perfetti Van Melle laboratory, The manuscript was revised. **Simone Guglielmetti:** conceived the study, Supervision, the study, Data analysis and statistics were carried out, Writing – original draft, with the contribution.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used the AI-powered language model ChatGPT-3.5 (https://chat.openai.com/) in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The following authors have affiliations with organizations with

direct or indirect financial interest in the subject matter discussed in the manuscript:

- Natalja Kirika and Andrea Sarrica are employed by Perfetti Van Melle S.p.A., the company that provided financial support for this study.
- Simone Guglielmetti served as a consultant for Perfetti Van Melle S.p.A. during the period when this study was conducted.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100793.

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