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ORIGINAL ARTICLE

Survival and persistence of *Bacillus clausii* in the human gastrointestinal tract following oral administration as spore-based probiotic formulation

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Abstract

Aims: This study aimed to investigate the fate of *Bacillus clausii* spores orally administered as lyophilized or liquid formulation to healthy volunteers.
Methods and Results: The study was a randomized, open-label, cross-over trial in which two commercial probiotic formulations containing spores of four antibiotic-resistant *B. clausii* strains (OC, NR, SIN, T) were given as a single dose administration. Faecal *B. clausii* units of each strain were counted on selective media and extrapolated for the total weight of evacuated faeces. RAPD-PCR typing was used to confirm *B. clausii* identification. *Bacillus clausii* was found alive in faeces for up to 12 days. In some volunteers, the recovered amount of OC, NR or SIN was higher than the number of administered spores. Bioequivalence among the two formulations was demonstrated.
Conclusions: *Bacillus clausii* spores survive transit through the human gastrointestinal tract. They can undergo germination, outgrowth and multiplication as vegetative forms. *Bacillus clausii* strains can have different ability to survive in the intestinal environment. *Bacillus clausii* spores administered as liquid suspension or lyophilized form behave similarly *in vivo*.
Significance and impact of the study: This work contributes towards a better understanding of the behaviour of *B. clausii* spores as probiotics.

Introduction

Manipulating the gut bacterial community by using probiotic bacteria is a therapeutic choice for treating intestinal microbial imbalance (Iannitti and Palmieri 2010; Rijkers *et al.* 2010). Probiotics are live microbial feed supplements that beneficially impact on host health by exerting a plethora of biological actions having the effect of restoring the microflora symbiosis in the intestinal tract (Sherman *et al.* 2009)]. Most probiotics are bacteria similar to those naturally found in the human gut. The most commonly used probiotic formulations contain lactic acid bacteria (e.g. lactobacilli, enterococci, streptococci and bifidobacteria) (Fuller 1991; Atlas 1999). Despite long considered soil micro-organisms, *Bacillus* spp. have been used for more than 50 years in the form of fermentation products or spore-based supplements (reviewed in Cutting 2011). During the recent years, it has become apparent that these bacteria are more prevalent in the faeces of animals than previously recognized (Fakhry *et al.* 2008; Hong *et al.* 2009a; Hoyles *et al.* 2012) and should be considered gut commensals rather than solely soil micro-organisms (Hong *et al.* 2009b).

Over other probiotics based on nonspore formers, probiotics based on *Bacillus* species have some advantages due to the fact that they are delivered as spores. Being extremely stable and resistant, the spore is capable of surviving the low pH of the gastric barrier and reaching the intestine intact (Cutting 2011). In addition, spore-based products can be indefinitely stored without refrigeration or in a desiccated form without any deleterious effect on viability. However, administration of dormant bacterial

forms raises the question of how they can exert a beneficial effect on the gastrointestinal flora. In fact, if spores can directly compete for adhesion sites and have an immunomodulatory effect, enzymes, antimicrobials or other substances with probiotic activity can only be produced by growing vegetative cells derived from spore germination (Tam *et al.* 2006). Despite studies in animal models showed that *Bacillus* spores can germinate in the small intestine, grow and proliferate and then re-sporulate (Mazza 1994; Hoa *et al.* 2001; Tam *et al.* 2006; Wilks *et al.* 2006; Leser *et al.* 2008; Jung *et al.* 2012), human studies focused at evaluating these aspects are still lacking. This limits the comprehension of the mechanisms responsible for the claimed probiotic activity of formulations containing bacterial spores.

Enterogermina (Sanofi-Aventis S.p.A.) is a spore-based probiotic registered as a pharmaceutical preparation in 1958 and having an over-the-counter medicinal status since 1999. Enterogermina contains spores of four antibiotic-resistant *Bacillus clausii* strains (OC, NR, SIN, T) (Senesi *et al.* 2001) and it is recommended for restoring intestinal microbial balance particularly during combined antibiotic treatment (Courvalin 2006). Nowadays, two different formulations, i.e. lyophilized capsules and liquid vials, are commercialized in 55 Countries around the world for the treatment of intestinal dysbiosis and the prevention of infectious gastrointestinal diseases (Nista *et al.* 2004; Gabrielli *et al.* 2009).

This study aimed to investigate the gastrointestinal fate of Enterogermina *B. clausii* spores in humans. The pharmacokinetics of the four *B. clausii* strains was evaluated in terms of faecal bacterial recovery following oral administration of Enterogermina capsules or vials to healthy volunteers. The equivalence between the two formulations was also evaluated.

Material and methods

Subjects' recruitment

Twenty healthy adult volunteers of European descent were recruited for the study. Inclusion criteria were healthy persons, aged 18–40 years, weight within 10% of the ideal weight reported in the Metropolitan Height and Weight, and acceptance of the study protocol. Exclusion criteria were the following: history of metabolic or gastrointestinal diseases, blood parameters outside the normal range and considered clinically significant, food allergies, medications associated with intestinal diseases, alcohol, cocoa, coffee or caffeine containing drinks abuse, drug use, participating in another clinical study and unwillingness to follow the study protocol. Antibiotics and probiotics were not allowed from 1 month before and during the intervention. Background information was collected from the volunteers through screening interviews and included questions regarding general health, medications and manner of living. The general health of the volunteers was confirmed by blood and urine analyses during the run-in period (Fig. 1). Inclusion criteria were hepatic and renal function, complete blood count, uric acid and standard urine analysis within normal limits. All samples were analysed by a certified clinical laboratory at the University Hospital of Pisa, Italy. Volunteers were asked to continue to follow their usual diet regimen during the

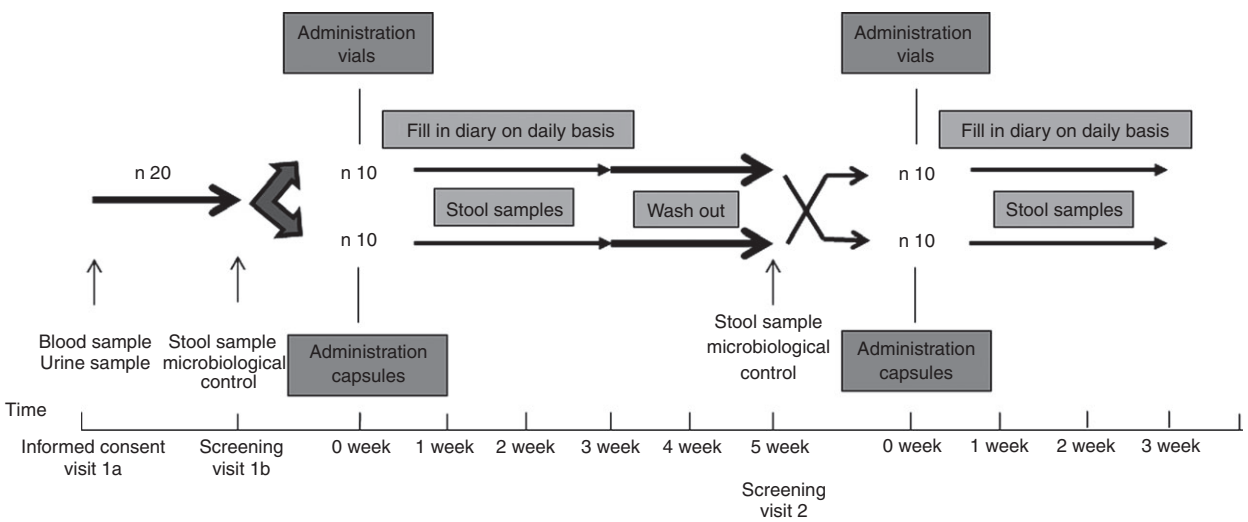

Figure 1 Study design. Subjects were screened for eligibility into the study. Subjects eligible for participation (*n*: number of subjects) were randomly allocated to receive either the capsules or the vials. Intervention period was 3 weeks, followed by a wash-out period of another 2 weeks after which volunteers received the other treatment.

study. At the end of the run-in and wash-out periods, stool cultures were performed to exclude the presence of enteric pathogens and spores/vegetative forms of antibiotic-resistant *Bacillus clausii* (see below). This human-intervention study was approved by the University Hospital Pisa Ethical Committee and conducted in full accordance with the principles of the Declaration of Helsinki. All subjects gave their written informed consent before their inclusion in the study. Analysis of the study samples was blinded. After having signed the informed consent, subjects were screened for eligibility into the study.

Probiotic products

The probiotic preparations (Enterogermina, Sanofi-Aventis SpA, Milan, Italy) consisted of a mixture of spores of four antibiotic-resistant *B. clausii* strains named OC, NR, SIN and T. Each Enterogermina capsule or vial is stated to contain 2×10^9 CFU of *B. clausii* spores. The CFU of each *B. clausii* strain contained in the preparations (capsules: batch 811; vials: batch 22088) were analysed as follows. Capsules were opened and the powder content dissolved in 5 ml sterile distilled water. Vials contained a 5 ml suspension of *B. clausii* spores in water. As preliminary studies indicated good correlation between microscopic counts of spore suspensions and number of CFU obtained by seeding nonheat-treated spore suspensions on MHa, serial dilutions of capsule-derived and vial suspensions were plated (100 μl per plate) onto Mueller Hinton agar (MHa) supplemented with 50 μg ml^{-1} chloramphenicol (CLF; Merck, Darmstadt, Germany), 25 μg ml^{-1} tetracycline (TTC; Sigma, St. Louis, MO, USA), 50 μg ml^{-1} rifampicin (RMP; Sigma) or 200 μg ml^{-1} streptomycin (STM; Merck). The number of CFU was determined after incubation at 37°C for 24 h.

Analysis of stool samples

Subjects were instructed to collect and weight faeces every day, to annotate the total weight of faeces in the study diary of the volunteer, and to bring a stool sample in a sterile container to the laboratory within one h or, if not possible, store it in the fridge before bringing it to the laboratory within 12 h. As soon as the faecal samples arrived to the laboratory, they were weighted, suspended by vigorous shaking in phosphate buffered saline (PBS) at 0·5 g ml^{-1}, and divided into two aliquots. One aliquot (2 ml) was thermally treated by exposing stool suspensions to 80°C for 15 min prior to plating. Thermally treated and untreated suspensions were serially diluted in PBS and seeded (100 μl per plate) on MHa plates containing CLF, TTC, RMP or STM for selection of the Enterogermina *B. clausii* strains. Plating was performed in triplicate and the plates were incubated at 37°C for 24 h. The detection limit of this method was estimated to be 10 bacteria for 50 mg of stool sample. As the four *B. clausii* strains produce identical colonies on MH supplemented with the above mentioned antibiotics, the counts of all the colonies with typical *B. clausii* morphology were determined. Gram staining of selected colonies was used to confirm the presence of Gram-positive bacilli and molecular typing was applied for the identification of the Enterogermina *B. clausii* strains. The mean of bacterial counts was extrapolated for the total weight of faeces collected.

Molecular typing

Three colonies grown on selective media were collected at day one, five, and seven from stool cultures of each patient. Bacteria were stored at −70°C. At the end of each intervention period, randomly selected strains were subjected to molecular typing. Genomic DNA was extracted and purified as previously described (Celandroni *et al.* 2000). Randomly amplified polymorphic DNA (RAPD) fingerprinting of bacterial genomes was performed with the primers RPO_2 and M13 as described previously for the *B. clausii* strains contained in Enterogermina (Senesi *et al.* 2001). *Bacillus clausii* OC, NR, SIN and T were used as controls. PCR products were visualized after electrophoresis on 1% agarose gel stained with ethidium bromide (0·5 μg ml^{-1}). Amplification patterns were analysed by the IMAGE MASTER 1D ELITE software (Pharmacia Biotech, Uppsala, Sweden), and dendrograms based on S_{AB} values (similarity between the patterns for every pair of strains) were generated with the IMAGE MASTER ID DATABASE software based on the unweighted pair group method with arithmetic means. An S_{AB} value of 1·00 indicated identity between the patterns generated by the two strains.

Statistics

Bacterial counts were first expressed as the mean of values obtained from three countable sample dilutions each seeded in triplicate. A log-transformation of the mean values was applied, outliers were identified by the Grubb's test for outliers and each outlier was subjected to winsorization (Wilcox 1998) (STATGRAPHICS software, StatPoint Techonologies, Warrenton, VA, USA) (Table S1). Vial and capsule formulations were compared by means of a bioequivalence approach using daily total viable counts from each volunteer and statistical analysis was performed by the nonparametric Wilcoxon test (STATGRAPHICS software). The 95% Confidence Interval for the differences of the means was calculated by the unpaired *t* test with Welch's correction.

Results

Study design

The study was a randomized, open-label, cross-over trial in which two Enterogermina formulations (vials and capsules) were compared with each other. Before trial initiation, CFU counts of each *B. clausii* strain contained in vial and capsule batches were performed. Due to the typical antibiotic resistance of each strain (Courvalin 2006), OC, NR, SIN and T were selected on plates containing CLF, TTC, RMP and STM respectively. Similar total spore counts (~$2{\cdot}1 \times 10^9$) were obtained for the two formulations. Strain NR was the predominant, with counts of $2{\cdot}1 \pm 0{\cdot}2 \times 10^9$ for vials and $2{\cdot}1 \pm 0{\cdot}7 \times 10^9$ for capsules. The amount of the other strains was $3{\cdot}8 \pm 0{\cdot}6 \times 10^7$ (OC), $2{\cdot}1 \pm 0{\cdot}6 \times 10^6$ (SIN), and $6{\cdot}5 \pm 0{\cdot}3 \times 10^6$ (T) for vials and $4{\cdot}1 \pm 0{\cdot}2 \times 10^7$ (OC), $7{\cdot}0 \pm 0{\cdot}5 \times 10^7$ (SIN), and $6{\cdot}6 \pm 0{\cdot}3 \times 10^6$ (T) for capsules.

Twenty healthy adult volunteers (10 males and 10 females) were recruited for the study and randomly allocated to receive a single administration of three capsules or three vials at day zero of each intervention period (Fig. 1). The intervention period was 3 weeks, followed by a washout period of another 2 weeks after which volunteers received the other treatment. All the volunteers were free of enteric pathogens and antibiotic-resistant *B. clausii* strains at the screening visit 1b and 2 (Fig. 1). Nineteen volunteers (Table S2) completed the trial. One volunteer dropped out because of antibiotic treatment for upper respiratory tract infection. No adverse reactions were observed with both formulations.

Detection of *Bacillus clausii* in faecal samples

During each intervention period, stool samples were analysed for the presence of the Enterogermina *B. clausii* strains. The seeding of nonthermally treated samples occasionally resulted in the isolation of other antibiotic-resistant bacteria. However, *B. clausii* could be easily identified by colony morphology and Gram staining. RAPD-PCR amplification was used to confirm the identity of the collected bacteria with the Enterogermina strains. As previously reported (Senesi *et al.* 2001), this technique can be successfully used to differentiate OC, NR, SIN and T from other *B. clausii* strains. All the collected strains generated identical patterns ($S_{AB} = 1{\cdot}00$) that are typical of the Enterogermina strains (Fig. 2).

The *B. clausii* CFUs obtained seeding nonthermally treated samples (count of germinated and heat-resistant spores plus vegetative cells) were higher or almost equal

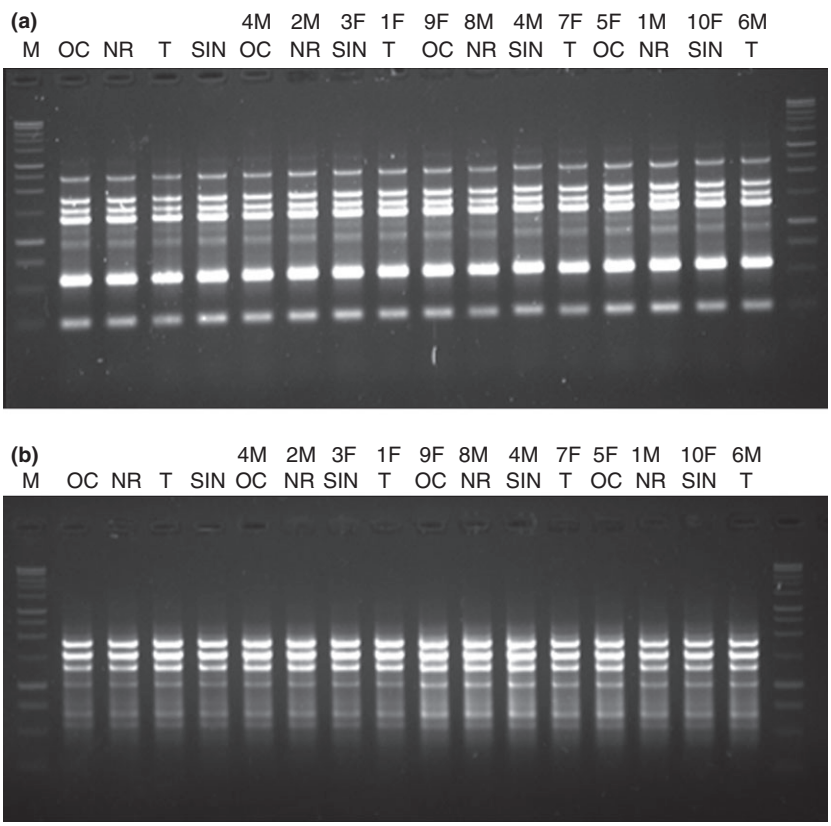

Figure 2 Representative electrophoretic profiles of RAPD-PCR amplicons obtained with the primers M13 (a) or RPO2 (b). OC, NR, T, SIN: RAPD-PCR profiles of the Enterogermina strains OC, NR, T and SIN respectively. 4M OC: strain isolated from volunteer 4M on MHa containing CLF; 2M NR: strain isolated from volunteer 2M on MHa containing RMP; 3F SIN: strain isolated from volunteer 3F on MHa containing STM; 1F T strain isolated from volunteer 1F on MHa containing TTC; 9F OC: strain isolated from volunteer 9F on MHa CLF; 8M NR: strain isolated from volunteer 8M on MHa containing RMP; 4M SIN: strain isolated from volunteer 4M on MHa containing STM; 7F T: strain isolated from volunteer 7F on MHa containing TTC; 5F OC: strain isolated from volunteer 5F on MHa containing CLF; 1M NR: strain isolated from volunteer 1M on MHa containing RMP; 10F SIN: strain isolated from volunteer 10F on MHa containing STM; 6M T: strain isolated from volunteer 6M on MHa containing TTC.

than those obtained seeding heat-treated samples (count of heat-resistant spores). This result suggested that *B. clausii* spores were able to undergo germination in the human gut. Figure 3 shows the total number of *B. clausii* viable units excreted by each volunteer every day after the administration of vials or capsules, independently from the intervention period (for daily counts of each strain see Table S1). Although variability was observed among volunteers, *B. clausii* strains were already detectable at day one after administration and they were present for more than 10 days in the faeces of some volunteers. No significant difference was emerged by the Wilcoxon analysis in the trend of excretion (Fig. 3) following administration of vials or capsules ($P > 0{\cdot}05$). In addition, no effect of the intervention period was observed. In fact, the mean values of the total viable counts recovered from volunteers dosed with vials ($4{\cdot}4 \pm 3{\cdot}2 \times 10^9$) or capsules ($9{\cdot}4 \pm 18{\cdot}7 \times 10^9$) in the first intervention period were not statistically different from those obtained in the second period with the same formulation (vials: $7{\cdot}0 \pm 13{\cdot}0 \times 10^9$; capsules: $2{\cdot}7 \pm 18{\cdot}0 \times 10^{10}$) ($P > 0{\cdot}1$). No statistical difference also emerged from the comparison of the mean recovery values obtained after administration of the two formulations in the same intervention period ($P > 0{\cdot}1$).

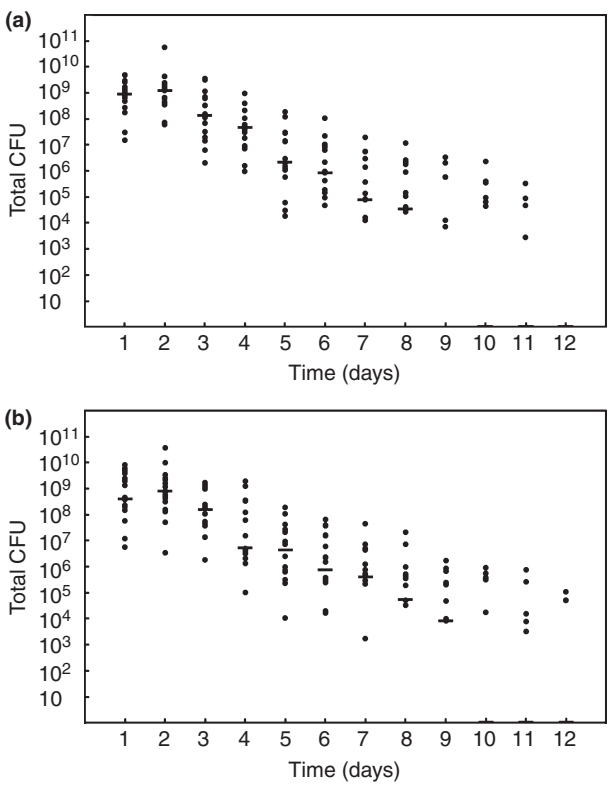

Figure 3 Total number of *Bacillus clausii* viable units (CFU) excreted by each volunteer every day after administration of vials (a) or capsules (b), independently from the intervention period. Bars represent median values.

Recovery of the single Enterogermina *Bacillus clausii* strains

To investigate on the gastrointestinal fate of the single Enterogermina strains, we calculated the total amount of OC, NR, SIN and T excreted by each volunteer after administration of vials or capsules. The volunteers showing a higher, almost equal or smaller amount of recovered bacteria compared to the administered spores were grouped (Table 1).

Differences in the behaviour of the four strains were observed. Strain OC appeared the best adapted to multiplication in the gut, being five and five the number of volunteers dosed with vials or capsules in which recovered OC units were higher than the number of administered spores (Table 1). A lower number of volunteers showed a higher recovery of NR (four and one) or SIN (three and one) compared to the number of spores administered as vials or capsules. None of the volunteers showed an increase in the number of recovered T units compared to the number of administered T spores. In addition, in 19 and 16 volunteers, the total recovery of this strain was from 0 to 1-log lower than the number of spores administered by vials or capsules.

NR, the predominant strain in both formulations, displayed the longest persistence in the intestine, being the last strain excreted by almost all volunteers before complete *B. clausii* clearance (Table S1).

Discussion

The use of pharmaceutical preparations containing *Bacillus* spores in the treatment or prophylaxis of intestinal dysbiosis is widespread and supported by encouraging results. As regards *B. clausii*, its purported beneficial effect has been attributed to antimicrobial and immunomodulatory activity (Urdaci *et al.* 2004; Ciprandi *et al.* 2005), effect on the expression of genes involved in immune responses, cell growth and differentiation, cell-cell signalling, cell adhesion, signal transcription and transduction (Di Caro *et al.* 2005), secretion of vitamins, such as riboflavin (Salvetti *et al.* 2003) and activity in reducing gastrointestinal risk originating from genotoxic agents (Cenci *et al.* 2008). However, most of these positive effects can only be the result of spore survival, germination and outgrowth in the gut.

A strain persisting in the gut must resist stomach acidity, proteases, bile acids, and lipases. In addition, it must develop some basic metabolic activity and resist the selective pressure of immunoglobulin A (IgA), which is

Table 1 Total recovery of *Bacillus clausii* strains after administration of vials or capsules*

		Group 1		Group 2		Group 3	
Bacterial strains	Total administered (log CFU)	Total recovery range (log CFU†)	Total no. of volunteers	Total recovery range (log CFU†)	Total no. of volunteers	Total recovery range (log CFU†)	Total no. of volunteers
OC vials	8·06	6·21–7·05	6	7·06–8·06	8	8·07–8·40	5
OC capsules	8·08	6·61–7·07	1	7·08–8·08	13	8·09–8·16	5
NR vials	9·80	6·92–8·79	6	8·80–9·80	9	9·81–10·64	4
NR capsules	9·80	8·35–8·79	1	8·80–9·80	17	9·82–10·79	1
SIN vials	6·80	0–5·79	4	5·80–6·80	12	6·81–9·95	3
SIN capsules	8·32	5·80–7·31	6	7·32–8·32	12	8·33–8·55	1
T vials	7·29	0–6·28	19	7·29–8·29	0	>8·29	0
T capsules	7·30	0–6·29	16	6·30–7·30	3	>7·30	0

*Volunteers were clustered into three groups based on the amount *B. clausii* recovered from faeces in the whole study period.
†Count of spores and vegetative bacteria × mass of stool of each day × no. of days.

abundantly secreted into the intestine. This study demonstrates that *B. clausii* spores are able to survive during transit in the human gut and persist in the intestine, being recovered in faeces for up to 12 days after a single administration.

Total *B. clausii* recovery in faeces showed a gradual reduction in the excreted viable units during time (Fig. 3). Maximal elimination was recorded in the first 2 days after administration. Taking into consideration that the colonic mean transit-time of a radioactive marker in humans is 2 days (Graff *et al.* 2001), this result suggests that part of the administered *B. clausii* spores transits intact through the gut during this time period. However, the longer persistence of *B. clausii* in faeces and the increased number of faecal *B. clausii* units compared to the number of administered spores in some volunteers indicates that another part of the administered spores germinates and undergoes multiplication in the intestinal environment (Table 1). This hypothesis is supported by data showing that the Enterogermina *B. clausii* spores are able to germinate after an acid challenge and growth as vegetative cells both in the presence of bile and under limited oxygen availability *in vitro* (Cenci *et al.* 2006).

The progressive decline in *B. clausii* excretion to a complete wash-out in less than 2 weeks shown in all volunteers indicates that this bacterium does not give rise to a permanent population competing with, or even substituting, the resident flora.

The Enterogermina *B. clausii* strains show low level of intraspecific diversity and high degree of genomic conservation (Senesi *et al.* 2001). Nevertheless, diversities in their growth rate in various environmental conditions and in their cellular proteomic profiles were demonstrated (Cenci *et al.* 2006; Lippolis *et al.* 2011). Our comparative analysis of the recovery of single *B. clausii* strains from faeces highlights a different ability of these strains to survive and multiply in the human gut (Table 1 and Table S1). Indeed, while the amount of OC, NR and SIN recovered in the whole period was equal or even greater than the administered dose in many volunteers, strain T disappeared quickly or was never detected in faeces. In addition, strain NR was isolated from all volunteers for the longest time. This finding could be the result of the predominance of this strain in both formulations, but best propensity of NR for adhesion and persistence in the gut cannot be excluded. This hypothesis is supported by data demonstrating that *Bacillus* spores of different strains display a diverse adhesion propensity to Caco-2 cells and persistence in the mouse gut (Tam *et al.* 2006). However, the finding that OC, NR, SIN and T behave differently in the gut of healthy volunteers does not necessarily imply that they maintain the same behaviour in patients undergoing antibiotic treatment or suffering from gastrointestinal diseases.

Randomized cross-over trials are designed to test whether different formulations of the same drug are equivalent in rate and extent to which the active ingredient is adsorbed from a drug and becomes available in the system (Zheng *et al.* 2012). Probiotics are feed supplements containing microbes that can transit intact, die or multiply in the host. Therefore, equivalence of probiotic formulations can only be evaluated in terms of microbial recovery in faeces and trend of excretion. A cross-over design, in which each subject receives two formulations in different orders, appears a powerful tool also for comparing survival, persistence and potential efficacy of different probiotic preparations. In this randomized, open-label, cross-over study, through the analysis of *B. clausii* recovery and trend of excretion in faeces, we demonstrate the bioequivalence of Enterogermina capsules and vials. Therefore, the administration of *Bacillus* spores as aqueous suspensions or lyophilized forms does not appear to influence bacterial stability *in vivo*. Statistical analysis for our cross-over design underlined no significant sequence, period and formulation effects.

In conclusion, our work highlights new information regarding the gastrointestinal fate of *B. clausii* spores, which are used as probiotics in many Countries around the world. *B. clausii* survives transit in the gut and maintains a considerable intestinal titre for up to 12 days after a single oral administration. *B. clausii* strains show different ability to survive and persist, suggesting a strain-dependent adaptation to this environment. During transit in the gastrointestinal tract of healthy volunteers, germination, outgrowth and multiplication of *B. clausii* can occur. Therefore, even if assessment of the functionality of probiotics should ideally be performed directly in the target population (Rijkers *et al.* 2010), our findings show that, in healthy subjects, *B. clausii* spores possess basic properties that can allow them to behave as probiotics.

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Conflict of Interest

The authors declare that they do not have conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 *Bacillus clausii* CFUs (log values) daily recovered from each volunteer and extrapolated for the total weight of faeces collected. Values in bold represent winsorized data [33] of the outliers identified by the Grubb's test for outliers.

Table S2 Sex, age and weight distribution of the 19 volunteers that completed the trial.