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Overall Assessment of a Model Probiotic Bacterium: from Gut Colonization to Clinical Efficacy

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ABSTRACT

Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”. This definition is inclusive of a broad range of microbes and applications, whilst capturing the essence of probiotics (microbial, viable and beneficial to health). Specific guidelines describe the minimal requirement for the probiotic status. In particolar, the conventional process of selecting novel potential probiotic strains includes the assessment of: 1) the ability of the strains to survive during the gastrointestinal transit and reach alive the intestine, 2) the actual impact of the probiotic bacterium on the intestinal microbial ecosystem (IME).

To evaluate these two aspects, in my PhD project we first carried out two recovery studies (in children and adults) with a selected probiotic bacterial strain, named *Lactobacillus paracasei* DG. Then, to evaluate the impact of the strain DG on IME we partecipated in a multicenter, randomized, double-blind, cross-over, placebo-controlled, pilot trial in irritable bowel syndrome (IBS) and determined the overall structure of the intestinal microbial communities by 16S rRNA gene profiling.

Specifically, to demonstrate the capability of the selected bacterial strain to survive the gastrointestinal transit when consumed by healthy subjects, we developed and adopted in the recovery studies a strategy that combined culture-based methods and molecular methods for strain specific enumeration of viable cells in fecal samples. The results showed that the *L. paracasei* DG was re-isolated from at least one fecal sample of all the volunteers, survived the gastrointestinal transit and proliferated in the intestine, and persisted after the interruption of the probiotic intake up to 5 days in adults and 3 days in children.

The results of the pilot study in IBS showed that *Lactobacillus paracasei* DG is able to modulate gut microbiota structure/function and reduce immune activation in IBS. Specifically, the strain induced a significant

reduction in genus *Ruminococcus*, a significant increase in the short chain fatty acids (SCFAs) acetate and butyrate, and a significant reduction in the pro-inflammatory cytokine interleukin-15.

Finally, we also investigated on mice the site of colonization of the probiotic bacterium in the intestine (animal study). The obtained results demostrated that *L. paracasei* DG colonized preferentially caecum and colon compared to ileum, suggesting a specific use of this probiotic in case of pathological situations with a localization at colonic level, such as diverticular disease and IBD, which are conditions including dysbiosis in their etiopathogenesis.

At the end of my PhD, we focused on another very important point in the probiotic world, i.e. the “neglected” bacterial components of commercial probiotic formulations. In fact, it is quite clear that not only live, but also dead cells are present in probiotic products and they can generate beneficial biological responses. This can have several implications for the production and application of probiotics, influencing the potential health promoting effects since the relative proportions of live and dead cells in a probiotic formulation is usually unkwnon. This aspect can be very important, even while conducting clinical trials aiming at studying the efficacy of a probiotic product.

RIASSUNTO

I probiotici sono "microrganismi vivi che, se somministrati in quantità adeguate, conferiscono un beneficio alla salute dell'ospite". Questa definizione comprende una vasta gamma di microbi e applicazioni, mentre cattura l'essenza dei probiotici stessi (microbici, vitali e benefici per la salute). Linee guida specifiche descrivono il requisito minimo per lo stato di probiotico. In particolare, il processo convenzionale di selezione di nuovi potenziali ceppi probiotici comprende la valutazione di: 1) la capacità dei ceppi di sopravvivere durante il transito gastrointestinale e raggiungere vivi l'intestino, 2) l'impatto effettivo del batterio probiotico sull'ecosistema microbico intestinale (IME).

Per valutare questi due aspetti, nel mio progetto di dottorato abbiamo prima di tutto condotto due studi di “recovery” (in bambini e adulti) con un ceppo batterico probiotico selezionato, chiamato *Lactobacillus paracasei* DG. Quindi, per valutare l'impatto del ceppo DG sull'IME abbiamo partecipato a uno studio clinico pilota multicentrico, randomizzato, in doppio cieco, cross-over, controllato con placebo, nella sindrome dell'intestino irritabile (IBS) ed abbiamo determinato la struttura complessiva delle comunità microbiche intestinali mediante 16S rRNA *gene profiling*.

In particolare, per dimostrare la capacità del ceppo batterico selezionato di sopravvivere al transito gastrointestinale quando consumato da soggetti sani, abbiamo sviluppato e adottato negli studi di recovery una strategia che combinava metodi basati su coltura e metodi molecolari per l'enumerazione specifica di cellule vitali del ceppo nei campioni fecali. I risultati hanno mostrato che *L. paracasei* DG è stato nuovamente isolato da almeno un campione fecale di tutti i volontari, è sopravvissuto al transito gastrointestinale ed ha proliferato nell'intestino e persisteva dopo l'interruzione dell'assunzione di probiotici fino a 5 giorni negli adulti e 3 giorni nei bambini.

I risultati dello studio pilota nell'IBS hanno mostrato che *Lactobacillus paracasei* DG è in grado di modulare la struttura/funzione del microbiota

intestinale e ridurre l'attivazione immunitaria nell'IBS. In particolare, il ceppo ha indotto una significativa riduzione del genere *Ruminococcus*, un aumento significativo degli acidi grassi a catena corta (SCFA) acetato e butirrato ed una riduzione significativa della citochina pro-infiammatoria interleuchina-15.

Infine, abbiamo anche studiato nei topi il sito di colonizzazione del batterio probiotico nell'intestino (studio *in vivo* su animali). I risultati ottenuti hanno dimostrato che *L. paracasei* DG colonizzava preferibilmente il cieco e il colon rispetto all'ileo, suggerendo un uso specifico di questo probiotico in caso di situazioni patologiche con una localizzazione a livello del colon, come la malattia diverticolare e l'IBD (Inflammatory Bowel Disease), che sono condizioni che includono la disbiosi nella loro etiopatogenesi.

Alla fine del mio dottorato di ricerca, ci siamo concentrati su un altro punto molto importante nel mondo dei probiotici, ovvero i componenti batterici "trascurati" delle formulazioni probiotiche commerciali. In effetti, è abbastanza chiaro che non solo le cellule vive, ma anche quelle morte sono presenti nei prodotti probiotici e possono generare risposte biologiche benefiche. Ciò può avere diverse implicazioni per la produzione e l'applicazione di probiotici, influenzando i potenziali effetti di promozione della salute poiché le proporzioni relative di cellule vive e morte in una formulazione probiotica sono generalmente sconosciute. Questo aspetto può essere molto importante, anche quando si conducono studi clinici volti a studiare l'efficacia di un prodotto probiotico.

Index

INTRODUCTION and LITERATURE REVIEW 9

AIMS and OBJECTIVES 15

CHAPTER 1 17

Quantitative Recovery of Viable *Lactobacillus paracasei* CNCM I-1572 (L. casei DG®) After Gastrointestinal Passage in Healthy Adults

Abstract 19

Introduction 20

Materials and methods 22

Results 27

Discussion 33

References 39

CHAPTER 2 46

Survival of L. casei DG® (*Lactobacillus paracasei* CNCMI1572) in the gastrointestinal tract of a healthy paediatric population

Abstract 49

Introduction 50

Methods 52

Results 56

Discussion 59

References 64

CHAPTER 3 76

***L. paracasei* DG® (*L. paracasei* CNCM I-1572) affects bacterial localization along the intestinal tract and modulates immune system responses and gut serotonergic metabolism in mouse**

Abstract 77

Introduction 77

Materials and methods 79

Results and Discussion 82

References 91

CHAPTER 4 **97**

Effect of *Lactobacillus paracasei* CNCM I-1572 on symptoms, gut microbiota, short chain fatty acids, and immune activation in patients with irritable bowel syndrome: A pilot randomized clinical trial

Abstract 98

Introduction 100

Materials and methods 101

Results 107

Discussion 114

References 119

CHAPTER 5 **124**

The neglected microbial components of commercial probiotic formulations

Abstract 126

Introduction 127

Overdosing of viable microbial cells in probiotic products 128

Viable cells in nongrowing states (the "hidden" viable biomass) 129

Non-viable microbial cells in probiotic formulations 130

Extracellular microbial products in probiotic formulations 131

Methodologies for the microbiological characterization of probiotic products 132

Conclusions 135

References 136

GENERAL CONCLUSION **145**

IMPLICATIONS and FUTURE DIRECTIONS **147**

APPENDICES **148**

INTRODUCTION and LITERATURE REVIEW

The gut is inhabited by microbial communities in so massive way that the prokaryotic cells probably overpass of one order of magnitude the eukaryotic cells present in the human organism forming an intimate beneficial association with the host (the concept of "holobiont") (Simon et al. 2019; Postler TS & Ghosh S 2017). For this reason, the intestinal microbiota has been proposed as an additional organ of the human body that is deputed to numerous functions ranging from vitamin production and immunomodulation to nutrient bioavailability and competitive exclusion against potential detrimental microorganisms (Bäckhed et al. 2015; Postler TS & Ghosh S 2017). Consequently, the modification of the gut microbiota composition plausibly induces functional changes affecting the host physiology (Bäckhed et al. 2015; Morris. 2018; Postler TS & Ghosh S 2017). In this context, the ability to modulate the intestinal microbial ecosystem is clearly an important element in supporting a healthy life. Notably, it appears possible to modify the relative abundance of specific bacterial groups by means of dietary interventions and, consequently, modulate the concentrations of health-affecting microbial metabolites in the gut such as butyrate, a bacterial fermentation product that plays a regulatory role on intestinal motility, epithelial barrier, and mucosal inflammation (Liu et al. 2018; Ferrario et al. 2014). Among these interventions, probiotics play a dominant role.

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al. 2014). This definition is inclusive of a broad range of microbes and applications, whilst capturing the essence of probiotics (microbial, viable and beneficial to health; Hill et al. 2014).

The Italian Ministry of Health has regulated the use of probiotic bacteria in the food sector over the past 12 years and, in 2013, confirmed the use of the word probiotic for food and food supplements under certain conditions, including a minimum number of viable cells (1×10^9 CFU)

administered per day, a full genetic characterization of the probiotic strain and a demonstrable history of safe use in the Italian market (Hill et al. 2014).

The survival of gastrointestinal transit has been listed among the criteria that a microorganism should satisfy to qualify as a probiotic (Borchers et al. 2009; Verna and Lucak 2010; Millette et al. 2013; Terpou et al. 2019). In the literature studies of probiotic viability in humans after oral intake are mainly based on quantification in feces and are also referred to as “persistence” or “recovery” studies. The recovery of viable microrganisms from feces is technically challenging because feces host thousands of different microbial species, which are not always easily distinguishable from one another. Furthermore, probiotics mostly belong to the genera *Lactobacillus* and *Bifidobacterium*, natural inhabitants of the human gut, that can be co-isolated with the probiotic strain of interest.

The conventional process of selecting new potential probiotic strains includes first of all the *in vitro* evaluation of the ability of the strains to survive at low pH, in simulated gastric juice or in the presence of bile salts (Millette et al. 2013; FAO/WHO, 2001). However, *in vivo* evaluation of probiotic viability is a more challenging task, probably because of the difficulties associated with carrying out intervention studies involving human volunteers and because of technical limitations. The use of human biopsies is an impractical option (it is not admitted for ethical issues), and therefore, the ability of probiotic microorganisms to survive in the gastrointestinal tract (GIT) is evaluated by analyzing fecal samples. However, conventional selective and/or discriminative growth media can hardly distinguish a specific probiotic strain from other ones belonging to closely related taxa, which are naturally present in the sample. The development of molecular approaches based on the design of strain-specific primer may solve the problem of selectivity, although the PCR protocols may lack sensitivity and, above all, do not allow the evaluation of the viability of the probiotic cells. For these reasons, culture-based methods have been combined with molecular approaches to achieve adequate

sensitivity and specificity (Dommels et al. 2009; Poutsiaka et al. 2017; Arioli et al. 2018; Radicioni et al. 2018). Nevertheless, the *in vivo* assessment of the ability to survive gastrointestinal transit has been conducted so far only for a few well-known commercial probiotics (Verna and Lucak 2010; Derrien and van Hylckama Vlieg 2015).

The identification of the actual impact on IME of a probiotic, prebiotic, pharmacologic or dietary intervention in general is surely of importance, but it is a difficult task, since the effects may be hindered by the complexity and the profound variability of the microbiota compositions among subjects. For this reason, the IME can be studied only by adopting proper trial design associated to next-generation analysis supported by solid statistical approach (Veiga et al. 2014; Ferrario et al. 2014; Ticinesi et al. 2018).

It is commonly accepted that the ability of a probiotic to influence health moves first from its ability to influence the microbial ecology of the gut. In line with this notion, in Italy the Ministry of Health allows the only health claim for probiotics "*promote the balance of intestinal flora*" (Italian Ministry of Health 2018) and, in Canada, the Food Inspection agency identified as acceptable the non-strain specific probiotic claim "*provides live microorganisms that contribute to healthy gut flora*". Several studies have been reported in the literature demonstrating for some strains their ability to rebalance the gut microbiota and thus bring a benefit to the health of the host.

However, few studies have gone into assessing specifically the ability of the probiotics to colonize specific districts of the intestine. This aspect is fundamental for targeted therapies. In fact, the awareness that a microorganism is able to specifically colonize the ileum or colon could open the way for "sartorial therapies": specific strains for specific diseases, that have a well-defined intestinal localization.

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AIMS and OBJECTIVES

This Ph.D. thesis research project is aimed to study the impact of a specific probiotic bacterium on the intestinal microbial ecosystem (IME) focusing on some aspects related to its intestinal colonization, such as the evaluation of the bacterial survival during intestinal transit and the specific site of colonization. In addition, the Ph.D. project is also aimed to study the efficacy of this probiotic bacterium in a population of adults with diagnosed irritable bowel syndrome (IBS). To reach this goal, sensitive analytical tools, such as microbiomics, and appropriate designs of intervention and clinical trials have been adopted. Specifically, in this project, we selected a specific bacterial strain, L. casei DG® (*Lactobacillus paracasei* CNCMI1572), that is contained within the probiotic product Enterolactis®, commercially available for more than 10 years. This strain has already demonstrated its efficacy in modulating the gut microbiota and the butyrate levels in healthy subjects and in exerting health-promoting properties in several conditions, such as diverticular disease, *Helicobacter pylori* infection, small intestinal bacterial overgrowth (SIBO), ulcerative colitis and IBS. L. casei DG® is well characterized and both the genome and plasmidome are known. During the characterization process, it was also found that a specific exopolysaccharide (EPS), with chemical structure never identified before, is secreted by and covers the bacterium. This EPS has been demonstrated to have immunostimulatory properties and therefore may contribute to the ability of the probiotic *L. paracasei* DG not only to interact with the immune system, but also to exert the beneficial effects in the conditions reported above.

Within the overall objective mentioned above this Ph.D thesis project is subdivided into the following activities:

A1) Recovery trial: this study aimed at the evaluation of the ability of the probiotic strain to cross alive the gastrointestinal tract and colonize it. The

activity started with a microbiology bench work and the design of the study, followed by evaluation of the number of live probiotic cells in the fecal samples.

A2) Animal Study: this study aimed to investigate on mice the preferential site of colonization of the probiotic bacterium. The study has been carried out in collaboration with the division of Agricultural Zootechnics at the University of Milan. The activity began with a bench work based on the design of the study, followed by evaluation of the preferential intestinal site of colonization of bacterium probiotic by means of quantitative PCR experiments.

A3) Irritable bowel syndrome (IBS) crossover multicentric intervention trial: the activity started with a microbiology bench work, based on the design of the study, the definition of endpoints and the centers to involve. Then, a microbiological part followed, based on 16S rRNA gene profiling (by MiSeq Illumina sequencing) of the metagenomic DNA isolated from fecal samples.

A4) Review on the "neglected" bacterial components of commercial probiotic formulations: the aim of this review is to focus on the "probiotic paradox" that both live and dead cells are present in probiotic products and they can generate beneficial biological responses; particularly, the factors influencing the *live:dead* cells ratio in a probiotic product are discussed.

CHAPTER 1

Arioli, S., Koirala, R., Taverniti, V., Fiore, W., Guglielmetti, S. 2018. Quantitative Recovery of Viable *Lactobacillus paracasei* CNCM I-1572 (L. casei DG®) After Gastrointestinal Passage in Healthy Adults. Frontiers in Microbiology, 9:1720

Quantitative Recovery of Viable *Lactobacillus paracasei* CNCM I-1572 (L. casei DG®) After Gastrointestinal Passage in Healthy Adults

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Probiotics are live microorganisms, and viability after transit through the gastrointestinal tract (GIT) is considered an inherent property of the health benefits of probiotics. The aim of the present study was to quantify the viable and total loads of *Lactobacillus paracasei* DG cells after passage through the GIT following the consumption of the probiotic product Enterolactis (L. casei DG® = *L. paracasei* CNCM I-1572 = *L. paracasei* DG) from drinkable vials by healthy adults. We developed a novel method for discriminating and enumerating culturable *L. paracasei* DG cells based on the unique sticky, filamentous phenotype of this strain on MRS agar containing vancomycin and kanamycin. The identity of DG was also confirmed with strain-specific primers by colony PCR. This method was used for a recovery study of the DG strain to quantify viable cells in the fecal samples of 20 volunteers during a 1-week probiotic consumption period and a 1-week follow-up. We isolated *L. paracasei* DG from at least one fecal sample from all the volunteers. The highest concentration of viable DG cells [ranging from 3.6 to 6.7 $\log_{10}$ colony-forming unit (CFU) per gram of feces] in the feces was observed between 4 and 8 days from the beginning of Enterolactis intake and for up to 5 days after cessation of intake. As expected, the total DG count determined by real-time quantitative PCR (qPCR) was mostly higher than the viable DG cells recovered. Viable count experiments, carried out by combining ad hoc culture-based discriminative conditions and strain-specific molecular biological protocols, unambiguously demonstrated that *L. paracasei* DG can survive gastrointestinal transit in healthy adults when ingested as Enterolactis in drinkable vials containing no less than one billion CFU at the end of shelf life.

Keywords: probiotic, Enterolactis, EPS, qPCR, isolation

INTRODUCTION

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al. 2014). Therefore, by definition, the term probiotic is restricted to live microbial cells. According to the regulations of numerous countries, the actual number of microbial colony-forming units (CFUs) in a probiotic product cannot be lower than the value indicated on the label until the end of the shelf life of the product. Consequently, both producers and competent public authorities constantly assess the viable counts of commercial probiotic products to ensure compliance with label specifications. Simultaneously, many industrial efforts are being made to identify strategies to keep bacterial cells viable during the various production steps and in the final product until the end of the shelf life; these strategies include the selection of appropriate culture media, the use of protective agents during the freeze-drying process, microencapsulation, and improvements in packaging systems (da Cruz et al. 2007; Savini et al. 2010; Goderska 2012; Mai et al. 2017).

Although microbial cell viability is constantly monitored for each marketed probiotic product, only limited data are available regarding the capacity of a particular microbial strain in a specific probiotic formulation to survive in the gastrointestinal tract (GIT) upon ingestion. Nonetheless, viability is conventionally considered a prerequisite for the health benefit of a probiotic, and accordingly, viable probiotics have been demonstrated to be more effective than non-viable probiotics for certain health-promoting activities (Lahtinen 2012). In this context, the first "FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics" stated that the ability to remain viable at the target site should be verified for each potential strain (FAO/WHO 2001).

The conventional process of selecting novel potential probiotic strains includes in vitro assessment of the ability of the strains to survive at low pH, in simulated gastric juice or in the presence of bile salts. However, in vivo assessment of probiotic viability is a more challenging task, possibly due to the difficulties associated with setting up intervention trials with human volunteers and because of technical limitations. The use of human biopsies is an impractical option, and therefore, the ability of probiotic microorganisms to survive in the GIT is assessed by analyzing fecal samples. However, conventional selective and/or discriminative growth media can barely distinguish a specific probiotic strain from other members of closely related taxa that are naturally present in the sample. The development of molecular approaches based on strain-specific primer design may solve the problem of selectivity, although PCR protocols may lack sensitivity and, most importantly, will not permit assessment of the viability of the probiotic cells. For these reasons, culture-based methods have been combined with molecular approaches to obtain adequate sensitivity and specificity (Dommels et al. 2009; Poutsiaka et al. 2017). Nevertheless, in vivo assessment of the ability to survive gastrointestinal transit has been carried out so far for only a limited number of well-known commercial probiotics (Verna and Lucak 2010; Derrien and van Hylckama Vlieg 2015).

Lactobacillus paracasei CNCM I-1572 (commercially known as L. casei DG®; *L. paracasei* DG) is a bacterial strain commercially available as part of the Enterolactis® product line. Enterolactis® is currently the best-selling probiotic food supplement in Italy, which is the country with the largest probiotic market in the world. *L. paracasei* DG has been demonstrated to possess the ability to modulate the intestinal microbial ecosystems of healthy adults (Ferrario et al. 2014) and to influence host immune response (Balzaretti et al. 2015; Cremon et al. 2017) via its unique polysaccharide capsule (Balzaretti et al. 2017). *L. paracasei* DG has also been demonstrated to

possess therapeutic potential for several dysfunctions and pathological conditions such as ulcerative colitis (D'Incà et al. 2011), diverticular disease (Tursi et al. 2013; Turco et al. 2017), small intestinal bacterial overgrowth (Rosania et al. 2013), and irritable bowel syndrome (Compare et al. 2017; Cremon et al. 2017).

In this study, we present the development of a strategy that combines culture-based methods and molecular methods for strain-specific selective enumeration of viable *L. paracasei* DG cells in fecal samples. Subsequently, we adopted this protocol to demonstrate the ability of *L. paracasei* DG to survive gastrointestinal transit when consumed by healthy adults via a probiotic formulation consisting of at least one billion bacterial CFU in a 10-ml suspension.

MATERIALS AND METHODS

Bacterial Strain and Selective Medium

Lactobacillus paracasei DG (CNCM I-1572) was routinely cultivated anaerobically at 37°C for 24 h in MRS broth or in vk-MRS agar (Difco Laboratories Inc., Detroit, MI, United States) supplemented with 1 µg/ml vancomycin and 10 µg/ml kanamycin (Sigma-Aldrich, Steinheim, Germany). The culturable bacterial content per vial used for the study was determined by resuspending at least 5 g of freeze-dried *L. paracasei* DG biomass in maximum recovery diluent (MRD) (Scharlab, Milan, Italy); then, this initial cell suspension was homogenized in a sterile Stomacher bag by using a Colworth Stomacher 400 instrument (Seward, West Sussex, United Kingdom) for 3 min. Serial 10-fold dilutions were prepared in MRD, and total microorganismal content was determined by the spread plate technique on vk-MRS agar.

Human Intervention Study Methods

Study title: recovery study with L. casei DG® (Enterolactis®) in drinkable vials in healthy adult volunteers (REVENANT-DG). Study design: open-label pilot microbiological study (Figure 1).

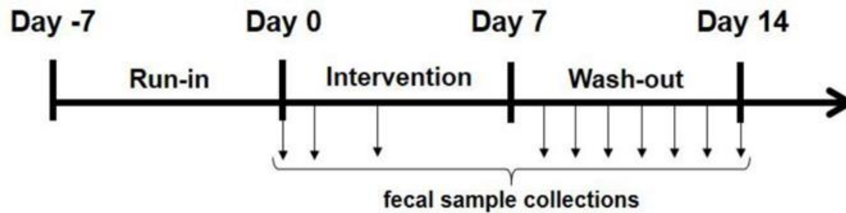


FIGURE 1: *Design of the study. Vertical arrows indicate the day of collection of the fecal sample (when available) from the volunteer*

Number of participants: 20 volunteers (Table 1). Study population: healthy (non-diseased) adult volunteers of both sexes, aged 18–55 years, who provided signed informed consent of their participation in the study. Exclusion criteria were as follows: (i) antibiotic consumption in the month preceding the start of the trial; (ii) consumption of antacids or prokinetic gastrointestinal drugs; (iii) chronic inflammatory bowel diseases; (iv) intestinal diseases of infectious origin; (v) episodes of viral or bacterial enteritis in the 2 months prior to the study; (vi) episodes of gastric or duodenal ulcers in the previous 5 years; (vii) pregnancy or breast-feeding; (viii) recent history of alcohol abuse or suspected drug use; and (ix) any severe disease that may interfere with treatment. Probiotic formulation under study: Enterolactis® (Sofar, Trezzano Rosa, Italy) in drinkable vials, which consisted of a plastic vial containing 10 ml of 2% fructose solution (additives: citric acid as an acidity controller, and sodium benzoate and potassium benzoate as preservatives) and a plastic/aluminum push-button cap (DryCap technology) containing at least one billion CFU/vial of freeze-dried *L. paracasei* DG biomass. Study protocol: during the initial visit, each volunteer provided signed informed consent and was trained on the entire procedure; then, the study consisted of a pre-recruitment phase (run-in, 1 week), during which the volunteers followed their

conventional diet with a ban on probiotic-fermented milks (traditional yogurt was allowed during this phase) and probiotic, prebiotic and symbiotic foods and supplements. At the end of this period, the volunteers were invited to consume one drinkable vial of Enterolactis per day for 1 week. The product was consumed on an empty stomach in the morning, at least 10 min before breakfast, or, if forgotten, in the evening, before bedtime and at least 2 h after the last meal. Following the 7 days of administration, the volunteers underwent a 1-week follow-up, which was identical to the period of pre-recruitment. Sample collection: at the beginning of the study, the volunteers were trained to collect and deliver the fecal samples as follows: each stool specimen (at least 2 g) was collected in special sterile containers, stored at room temperature, and delivered to the laboratory within 24 h. Preliminary experiments demonstrated that strain DG can survive in human feces at room temperature and 37°C at least 48 h without significant decrease of the viable count (not shown). To verify the ability of the DG strain to survive passage through the GIT, the fecal samples collected were immediately subjected to viable bacterial counts. To obtain fecal bacterial counts, 1-g fecal samples were diluted in MRD, homogenized in a sterile Stomacher bag, plated on vk-MRS and incubated anaerobically at 37°C for 48 h. Throughout the study period, the frequency and consistency of the stools were evaluated according to a validated fecal scoring system (Bristol stool scale). Ethical statement: the study protocol was approved by the Research Ethics Committee of the Università degli Studi di Milano (opinion no. 37/16, 15th December 2016). Written informed consent was obtained from all the subjects before recruitment. Volunteer compliance: volunteer compliance, as determined by verbal assessment, was almost 100%. All programmed fecal samples were delivered by volunteers, with the only exception of subject S1, we voluntarily interrupted fecal sample collections at day 12.

Subject (n = 20)	Sex (8F/12M)	Age (22–53 years)
S01	M	53
S02	F	46
S03	F	47
S04	F	27
S05	F	27
S06	F	28
S07	M	25
S08	F	23
S09	M	23
S10	F	27
S11	M	24
S12	M	28
S13	F	31
S14	M	31
S15	M	31
S16	F	26
S17	M	26
S18	F	26
S19	F	42
S20	M	22

TABLE 1. *Basic characteristics of study participants*.

DNA Extraction

After microbiological analysis, the fecal samples were stored at −80°C until DNA extraction. Samples collected from each subject on different days (n = 9; T0–T8) were thawed on ice and mixed vigorously for 2–3 min with a sterile spatula; then, 250 mg of each sample was weighed and processed with a DNeasy® PowerLyzer® PowerSoil® Kit (Qiagen, Hilden, Germany) with the following modifications: tubes containing samples were incubated at 65°C for 10 min after addition of solution C1. Before extraction, mechanical lysis of the cells was carried out using a Precellys 24 bead homogenizer (Bertin Technologies, Montigny le Bretonneux, France). Then, the extraction was conducted according to the manufacturer's specifications. The DNA extracted from fecal samples was quantified by using a NanoDrop (BioTek Instruments,

Inc., CA, United States). Finally, the DNA was stored at −80°C until molecular analysis.

L. paracasei DG Quantification by qPCR

Real-time quantitative PCR (qPCR) protocols were adopted for the quantification of *L. paracasei* DG in fecal metagenomic DNA, targeting the glycosyl transferase gene welF with the primers rtWELFf (5′-TACTAAAGAAATTAGCTTTTGT-3′) and rtWELFr (5′-AGTAATGTCTGCATCCTCCA-3′) (Ferrario et al. 2014) in a final volume of 15 µl containing 7.5 µl of EvaGreen Supermix (Bio-Rad Laboratories, Segrate, Italy) and 0.5 µM of each primer; 50 ng of template DNA samples was used in each reaction. The amplification was carried out using the following thermal program: initial hold at 95°C for 3 min followed by 39 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. A standard calibration curve for the absolute quantification of the total number of L. casei DG® was prepared by mixing five different fecal samples (of varying consistency) that were collected before the consumption of probiotics. Different numbers of L. casei DG® cells (n = 10; 1–1 × 109) were added to 250-mg fecal samples; one fecal sample was used as a control (without the addition of bacterial cells). All the samples were subjected to DNA extraction as mentioned above. The standard curve was obtained by plotting the average Cq values versus log10 of the number of cells added to each fecal sample. Melting curves were analyzed with Bio-Rad CFX Manager 3.1 software to confirm the specificity of the amplification products.

Colony PCR for Identification of *L. paracasei* DG Colonies

To confirm the identities of the DG colonies, we carried out end-point colony PCR by randomly selecting colonies with sticky, filamentous phenotypes. Colonies with different phenotypes were always included as negative controls. PCRs were performed in 25-µl reaction mixtures, each

containing one colony (picked with a sterile wooden stick), 2.5 µl of 10× reaction buffer, 200 µmol/l of each dNTP, 0.5 mmol/l MgCl2, 0.5 µmol/l each primer (rtWELFf and rtWELFr), and 0.5 U DreamTaqTM DNA polymerase (Thermo Fisher Scientific Inc., Monza, Italy). Amplifications were carried out using a Mastercycler 96 (Eppendorf, Milan, Italy). The PCR mixtures were subjected to the following thermal cycling conditions: initial hold at 95°C for 3 min followed by 39 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Amplification products were resolved by electrophoresis on a 2% (w/v) agarose gel (with 0.2 µg/ml ethidium bromide) in 1× TAE buffer (40 mmol/l Tris-acetate, 1 mmol/l EDTA, pH 8.0) and photographed. A 1-kb GeneRuler DNA Ladder Mix was used as a size marker.

RESULTS

Development of a Method for the Enumeration of Live *L. paracasei* DG Cells

To develop culture conditions for selective and discriminative growth of *L. paracasei* DG, we implemented the cultivation protocol suggested by the Italian Higher Institute of Health (ISS) for the enumeration of heterofermentative lactobacilli in probiotic products (ISSN 1123-3117 ISTISAN 08/36; available at http://www.iss.it/binary/publ/cont/08-36_web.1229959899.pdf as accessed on February 10th, 2018). The ISSN protocol suggested the use of 1 µg/ml vancomycin for the selective counting of heterofermentative lactobacilli; however, during the preliminary experiment, the use of such a medium allowed the growth of many non-DG colonies, which hampered the identification and counting of the probiotic strain under investigation. For this reason, based on the antibiotic resistance profile of *L. paracasei* DG (Table 2), we also added 10 µg/ml kanamycin to the medium (vk-MRS medium), which resulted in an evident decrease in the background without affecting the growth of the DG strain compared to the growth of this

strain in normal MRS medium or MRS supplemented with the only vancomycin. The use of higher concentrations of the antibiotics resulted in reduced viable count of the DG strain.

Antibiotic molecule	ISO10932 (μg/ml)*	MIC (μg/ml) LMG 12586	MIC (μg/ml) *L. paracasei* DG
Ampicillin	0.5–2	1	1
Vancomycin	Not required	>16	>16
Gentamycin	1–4	4	4
Kanamycin	16–64	32	256
Streptomycin	8–32	16	32
Erythromycin	0.062–0.25	0.125	0.125
Clindamycin	0.062–0.25	0.125	0.125
Tetracycline	1–4	2	8
Chloramphenicol	4–8	4	4

The results are compared with cutoff values defined by EFSA (*) for the purpose of distinguishing resistant strains from susceptible strains within the taxonomic group Lactobacillus casei/paracasei (European Food Safety Authority [EFSA], 2012).

TABLE 2. *Minimum inhibitory concentrations (MICs) of* Lactobacillus paracasei *DG and the EFSA reference strain* L. paracasei *LMG 12586 determined via a microdilution assay*.

In addition, we observed that the DG colonies on vk-MRS agar had a peculiar sticky, filamentous phenotype, allowing the discrimination of this strain from the colonies of closely related lactobacilli, which typically have a creamy consistency (Figure 2 and Supplementary File S1).

FIGURE 2. *Sticky, filamentous consistency of the colonies of* Lactobacillus paracasei *DG grown on vk-MRS agar plates*

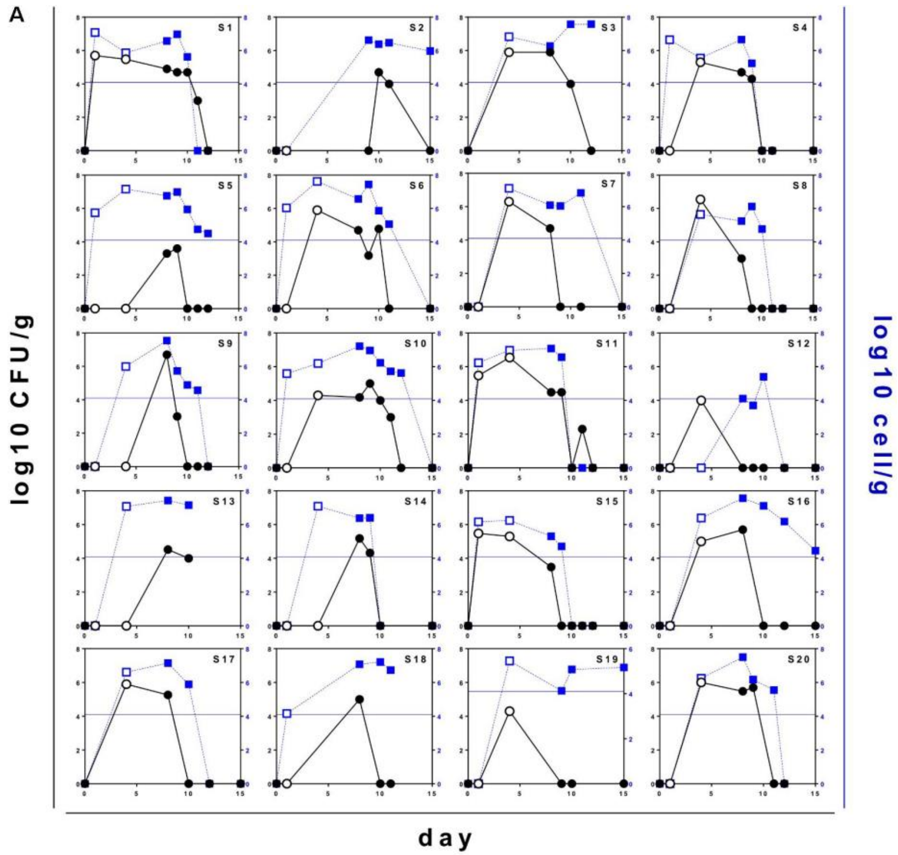
Finally, to unambiguously confirm that the colonies isolated on the vk-MRS plates belonged to the DG strain, we performed end-point colony PCR analysis with strain-specific primers on randomly selected colonies. The results confirmed that only those colonies with the sticky, filamentous phenotype belonged to *L. paracasei* DG. This method allowed us to precisely distinguish the DG colonies from the other fecal microorganisms and to selectively count colonies of the probiotic strain under study.

Viable Counts of *L. paracasei* DG in the Fecal Samples of Healthy Adults

We used the protocol based on vk-MRS medium combined with strain-specific PCR of the isolated colonies to determine viable counts of *L. paracasei* DG in the fecal samples of 20 adult volunteers over 1 week of probiotic intake and during a 1-week follow-up. Subject compliance was excellent, and all 20 subjects completed the study. Moreover, no adverse events were recorded for

the entire duration of the probiotic treatment. Based on the results of strain-specific PCR (Supplementary Figure S1), 100% of the analyzed colonies with sticky, filamentous phenotypes belonged to *L. paracasei* DG, confirming that vk-MRS is a suitable medium for the selective counting of this strain. Notably, although we found wide inter-individual variability, we isolated *L. paracasei* DG from at least one fecal sample from all 20 volunteers, demonstrating that this probiotic bacterium can survive gastrointestinal transit when consumed via the formulation Enterolactis in drinkable vials. Overall, we found the highest concentration of viable *L. paracasei* DG in the fecal samples obtained between 4 and 8 days after the beginning of Enterolactis intake. The highest concentration of DG isolated from a single subject ranged between 3.6 and 6.7 $\log_{10}$ CFU per gram of feces (mean of 6.1 $\log_{10}$ CFU/g). In particular, we observed profound inter-subject variability in terms of kinetics of persistence. In fact, while from some subjects (e.g., S1) DG cells were retrieved from the first evacuation after intake of the probiotic product, from others (e.g., S2 and S9), viable DG cells were isolated only after the end of the 1-week probiotic intake period (Figure 3). In general, however, viable DG cells were isolated from the feces of the volunteers until 5 days after the cessation of Enterolactis intake (Figure 3).

A



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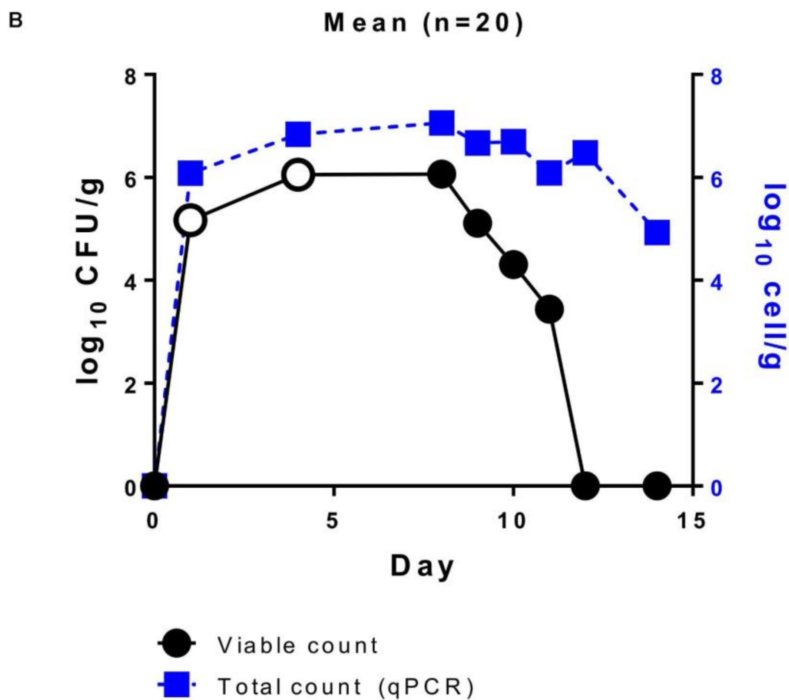

FIGURE 3. *Viable (black lines and circles) and total (blue lines and squares) counts of* Lactobacillus paracasei *DG in the fecal samples of healthy adult volunteers who ingested Enterolactis in drinkable vials once daily. (A) Data per subject; (B) mean data (n = 20). Open symbols refer to fecal samples collected during the week of probiotic intervention.*

Total *L. paracasei* DG Counts in the Fecal Samples of Healthy Adults

The fecal samples collected during the 2-week trial were also used for the quantification of total *L. paracasei* DG cells by means of qPCR with strain-specific primers. As expected, the total DG counts determined via qPCR were mostly higher than the viable DG counts (Figure 3). Accordingly, the highest concentration of DG cells isolated from each subject, as calculated by qPCR, ranged between 5.4 and 7.6 log_{10} cells per gram of feces (mean of 7.1 log_{10} cells/g). However, while some subjects exhibited very similar total and viable counts (e.g., S1, S15, S17, and S20), others exhibited total counts that far exceeded viable counts in terms of both cell concentration and persistence (e.g., S2, S5, and S19; Figure 3). Overall, while the recovery of culturable DG cells was possible for up to 5 days after the intake period, the detection of DG cells by qPCR was possible for up to 7 days on average.

Bowel Habits and DG Recovery

The Bristol Stool Chart did not reveal any significant alteration in bowel habits, and no gastrointestinal abnormalities were reported by the volunteers during the entire study. Although intestinal transit time and bowel habits could plausibly determine the differences observed among the different volunteers, the data regarding fecal types and evacuations per day did not correlate with the results of viable or total DG recovery (Supplementary Figure S2).

DISCUSSION

Survival of gastrointestinal transit has been listed among the criteria that a microorganism should fulfill to qualify as a probiotic (Borchers et al. 2009; Verna and Lucak 2010). Studies of probiotic viability in humans after ingestion mostly rely on quantification in feces and are also referred to as "persistence" or "recovery" studies. The viable recovery of probiotics from feces is technically challenging because feces are microbiologically very complex, hosting thousands of different microbial species. Furthermore, probiotics mostly belong to the genera *Lactobacillus* and *Bifidobacterium*, which naturally inhabit the human gut and can, therefore, be co-isolated with the probiotic strain of interest. Reportedly, antibiotics and colony morphology have been used to address this challenge and facilitate the selective identification of colonies belonging to specific strains under study (Larsen et al. 2006; Mai et al. 2017; Poutsiaka et al. 2017). In addition, more reliable results have been obtained when molecular approaches have been combined with conventional isolation on agar plates. For instance, Tuohy et al. (2007) enumerated the *L. paracasei* strain Shirota using lactitol-LBS-vancomycin agar combined with pulsed-field gel electrophoresis to confirm colony identity. In another study, the Shirota strain was selectively quantified in feces using lactitol-lactobacillus selection-vancomycin agar plates with ELISA for confirmation of colony identity (Wang et al., 2015). Furthermore, fluorescent whole-cell hybridization was used to identify colonies of *Bifidobacterium animalis* subsp. *lactis* BB-12-like colonies grown on MRS agar supplemented with cysteine-HCl and tetracycline (Larsen et al. 2006). Molecular fingerprinting (rep-PCR, RAPD-PCR, or AP-PCR) of colonies isolated from feces has also been used to confirm strain identity (Songisepp et al. 2005; Prilassnig et al. 2007; Verdenelli et al. 2009; Pino et al. 2017).

Here, we designed an effective and reliable protocol for the selective enumeration of viable cells of *L. paracasei* DG in human feces via the

exploitation of the exopolysaccharide (EPS) capsule of this bacterium (DG EPS) (Balzaretti et al. 2017). According to the analysis of the complete genome (chromosome and plasmids), *L. paracasei* DG does not have any antibiotic resistance genes (Balzaretti et al. 2015), and therefore, the modestly increased ability of the DG strain to resist certain antibiotics can be reasonably explained by the existence of the EPS capsule, which may partially impede antibiotic penetration into the cell. In addition, many strains of lactobacilli have been reported to have high natural resistance to aminoglycosides (e.g., gentamicin and kanamycin). We exploited the observed modest resistance of strain DG to certain antibiotics, which is intrinsic and is not associated with horizontally transmissible genetic elements, by adding the antibiotics vancomycin and kanamycin to the medium developed in this study to enumerate the DG strain. In addition, the DG EPS imparts a sticky, filamentous texture to the colonies, allowing easy discrimination of DG from other lactobacilli. Finally, the genetic region encoding the DG EPS has a unique DNA sequence (Balzaretti et al. 2017), therefore, permitting the design of strain-specific primers (Balzaretti et al. 2015). All the colonies with the sticky, filamentous phenotype detected on the plates were demonstrated by colony PCR with strain-specific primers as belonging to the DG strain, demonstrating that the developed protocol is suitable for the selective enumeration of strain DG.

Apparently, the literature contains contradictory reports regarding the ability of probiotic microorganisms to survive gastrointestinal transit. Previous studies, in fact, have demonstrated that the recovery of live cells of probiotic microorganisms after gastrointestinal transit in humans is poor (Hamilton-Miller et al. 1999; Temmerman et al. 2003). In a subsequent study, out of six different commercially available products, only *Escherichia coli* Nissle 1917 and *Enterococcus faecium* SF 68 were consistently detected in human feces, whereas ingested bifidobacteria and lactobacilli (including a *L. paracasei*

strain) were not recovered from stool (Prilassnig et al. 2007). A higher number of studies, however, have reported the successful recovery of different probiotics from human feces after ingestion (Verdenelli et al. 2009; Derrien and van Hylckama Vlieg. 2015). Overall, the results are inconclusive, primarily because the recovery of probiotics from human feces depends on several pivotal factors: (i) the dose of the ingested live microbial cells; (ii) the intrinsic ability of the microorganism to resist chemical and physical stresses in the stomach and gut (e.g., acidity and bile salts); and (iii) the product composition in terms of excipients and/or ingredients. Accordingly, for instance, *Lactobacillus fermentum* ME-3 was retrieved from the feces of all volunteers (n = 16) who received the probiotic as fermented goat milk but not from volunteers who ingested the probiotic cells as gelatin-coated capsules (n = 12) (Songisepp et al. 2005). The results of another study showed that, compared to capsules and yogurt, cheese negatively influenced the fecal quantity of *Propionibacterium freudenreichii* subsp. *shermanii* JS and *B. animalis* subsp. *lactis* BB12 in human feces, whereas *Lactobacillus rhamnosus* GG and LC705 were not affected by the matrix (Saxelin et al. 2010). Therefore, intestinal recovery should be investigated for specific probiotic strains in precise product formulations. Nonetheless, reliable studies have been carried out only for a few well-known commercially available probiotics, such as *L. paracasei* Shirota, *L. rhamnosus* GG, and *B. animalis* subsp. *lactis* BB12. For instance, Wang et al. isolated viable cells of the Shirota strain from the feces of all volunteers (n = 25) after 7- and 14-day periods of consumption of a milk-based probiotic beverage (corresponding to a daily intake of approximately 10 billion CFU); however, the Shirota strain was detected in the feces of only three subjects (out of 25) 7 days after cessation of product ingestion (Wang et al., 2015). In another study, the Shirota strain was retrieved from the feces collected from 9 healthy adult volunteers after 7, 14, and 21 days of daily consumption of a fermented milk drink, corresponding to a total intake of approximately 50 billion CFU per day; 7 days after cessation of fermented milk intake, the Shirota strain was still

isolated from the feces of six subjects, albeit at a much lower concentration (Tuohy et al. 2007). The longer persistence of the probiotic in a subgroup of volunteers observed in the study by Tuohy et al. (2007) than that in the REVENANT-DG trial was possibly due to the longer treatment (3 weeks) and much higher total daily intake of probiotic cells (50 billions) in the study by Tuohy et al. (2007).

More recently, the well-known probiotic strains *B. animalis* subsp. *lactis* BB12 and *L. rhamnosus* GG were successfully recovered alive from the stools of 16 out of 19 healthy volunteers who each ingested both strains together at a quantity of one billion CFU per day for 3 weeks as a powder in a sachet; the quantitative culture-based experiment, however, failed to isolate strain BB12 or GG 28 days after the end of the supplementation period (Poutsiaka et al. 2017). *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB-12, in particular, have been retrieved from the feces of healthy subjects and patients when administered with a variety of formulations, including different pharmaceutical forms and foods (Larsen et al. 2006; Ahlroos and Tynkkynen. 2009; Dommels et al. 2009; Saxelin et al. 2010; Granata et al. 2013). Nonetheless, this information is not available for most commercially available probiotics.

In our study, we examined the recovery of live *L. paracasei* DG from the GITs of healthy individuals after oral ingestion of Enterolactis, a probiotic supplement consisting of at least one billion CFU per dose of bacterial cells, suspended in a 10-ml fructose solution in drinkable vials. The intervention lasted only 1 week and was based on the intake of a single one-billion-CFU dose of probiotic cells per day; nonetheless, the intervention was effective enough to lead to the recovery of viable *L. paracasei* DG from all 20 volunteers enrolled in the study, clearly demonstrating that the Enterolactis in drinkable

vials, containing at least one billion CFU, is suitable for successful delivery of probiotic cells to the human intestine.

The abovementioned studies demonstrated that the colonization of the human intestinal tract by an ingested probiotic microorganism is transient, and after the cessation of ingestion, the probiotic rapidly approaches the detection limit via kinetic mechanisms that possibly depend on the dose of the administered microbial cells. In particular, the results of our study on *L. paracasei* DG are consistent with the literature regarding the *L. paracasei* Shirota strain, the persistence of which in the guts of healthy adults was demonstrated to disappear within 1 week after cessation of probiotic intake (Tuohy et al. 2007; Wang et al. 2015).

In this study, we invited volunteers to ingest the probiotic on an empty stomach, at least 15 min before breakfast. There is no convincing information in the scientific literature to answer the question of whether probiotics should be taken with food or on an empty stomach. However, this factor could affect microbial survival in the stomach and intestine upon ingestion, and future investigations on the topic are warranted.

In conclusion, in this report, we presented the results of a comprehensive recovery study of the probiotic strain *L. paracasei* DG. Viable count experiments carried out by combining ad hoc culture-selective/discriminative conditions and strain-specific molecular biological protocols unambiguously demonstrated that *L. paracasei* DG can survive gastrointestinal transit in healthy adults when ingested as Enterolactis in drinkable vials, a formulation consisting of a 10-ml drinkable suspension containing no less than one billion CFU. Recovery studies to assess microbial viability after gastrointestinal transit should be a mandatory step in the characterization process of any

probiotic product. Our study shows that reliable verification of microbial survival in feces can be performed in a rigorously strain-specific manner by developing enumeration protocols for viable cells based on the specific genetic and phenotypic characteristics of probiotic microorganisms of interest.

Author Contributions

SG and WF conceived and planned the intervention trial. SG and SA developed the protocol for the strain-specific isolation of *L. paracasei* DG. SA, VT, and RK carried out the experiments. SG took the lead in writing the manuscript. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest Statement

The author WF is an employee of Sofar S.p.A., which is the company that financially supported the study. The probiotic product used in the study is commercialized by the company that financially supported the study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01720/full#supplementary-material

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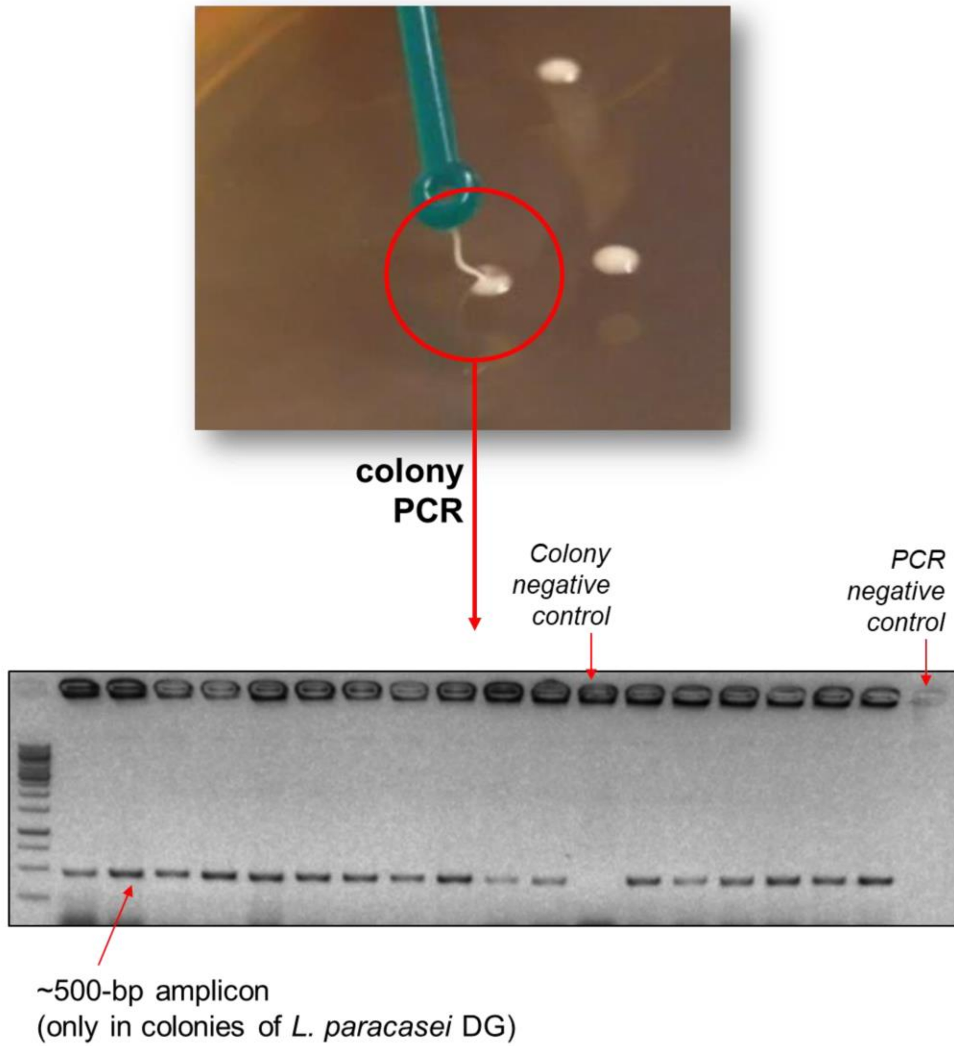
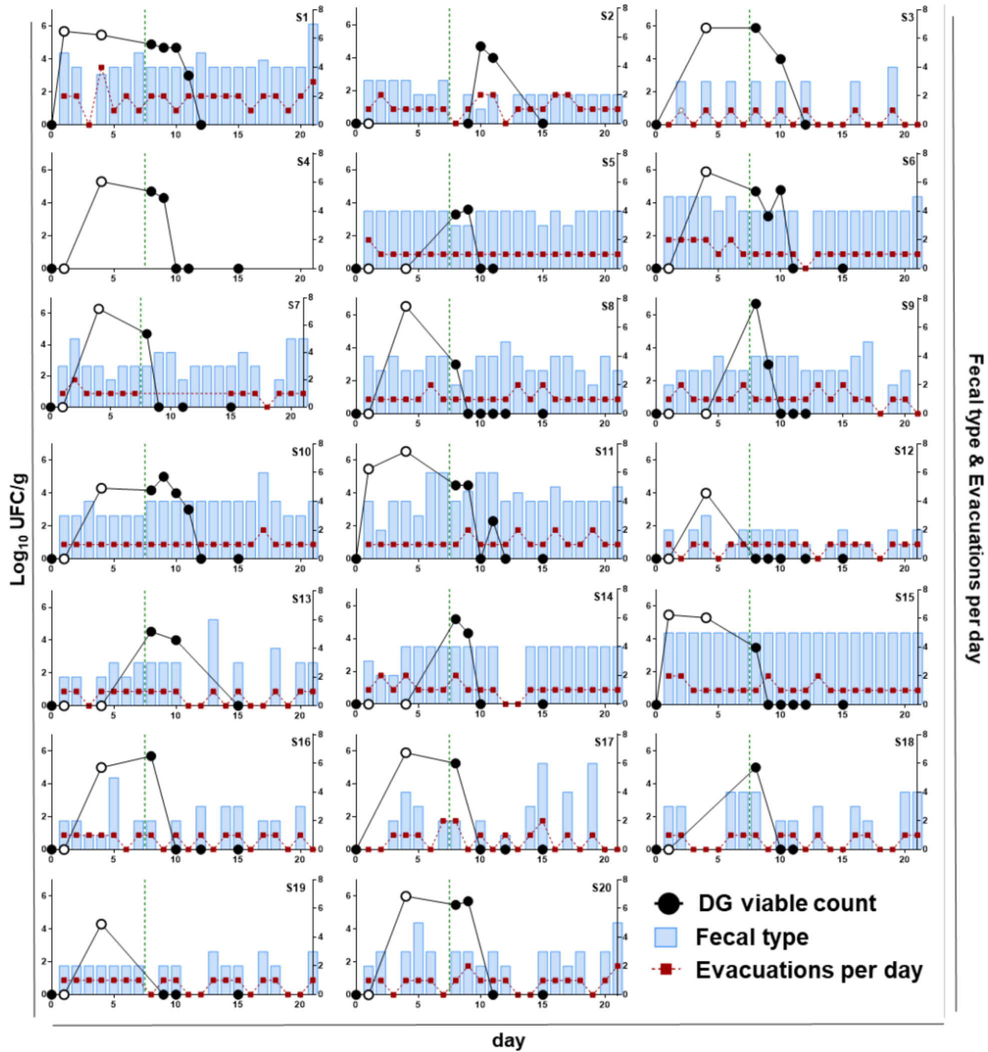
SUPPLEMENTARY MATERIAL

Supplementary Figure 1. *Confirmation of the identity of colonies by colony PCR with DG-specific primers*



Supplementary Figure 2. *Viable counts (black lines and circles) of Lactobacillus paracasei DG in the fecal samples, fecal type (blue columns) and evacuation per day (red squares and line) of healthy adult volunteers who ingested Enterolactis in drinkable vials (n=20). White circles refer to the viable counts of the DG strain in fecal samples collected during the week of probiotic intervention.*



CHAPTER 2

Radicioni, M., Koirala, R., Fiore, W., Leuratti, C., Guglielmetti, S., Arioli, S. 2018. Survival of L. casei DG® (*Lactobacillus paracasei* CNCMI1572) in the gastrointestinal tract of a healthy paediatric population. European Journal of Nutrition, pp. 1-10

Survival of L. casei DG® (*Lactobacillus paracasei* CNCM I1572) in the gastrointestinal tract of a healthy paediatric population

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Keywords: Healthy children, probiotics, L. casei DG® recovery, *Lactobacillus paracasei* CNCM-I1572

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Abbreviations

CFU: Colony Forming Unit, GCP: Good Clinical Practice; GI: gastrointestinal; GRAS: Generally Recognized As Safe; ICH: International Conference on Harmonisation; LCDG: L. casei DG®

ABSTRACT

Purpose: Ability to survive the digestive process is a major factor in determining the effectiveness of a probiotic. In this study, the ability of the probiotic L. casei DG® (*Lactobacillus paracasei* CNCM I-1572) to survive gastrointestinal transit in healthy children was investigated for the first time.

Methods: Twenty children aged 3-12 years received L. casei DG® as drinkable solution of 1 $x10^9$ colony forming units (CFU), once daily for 7 consecutive days. Recovery in faecal samples was evaluated at baseline and at different time-points during and after administration. Defecation frequency, faeces consistency, digestive function and product safety were also assessed.

Results: Nineteen (95%) of the 20 enrolled children presented viable L. casei DG® cells in their faeces at least once during the study, with a maximum count (mean: 4.3 log_{10} CFU/g ±2.3) reached between day 4 and 6 from the beginning of consumption. Notably, for 11 (57.9%) of the 19 children with viable cells, L. casei DG® survived in faecal samples up to 3 days after treatment end. Defecation frequency, faeces consistency and digestive function did not change considerably during or after study treatment. Safety of the study product was very good.

Conclusions: This study showed for the first time that L. casei DG® survives the gastrointestinal transit when ingested by children with a paediatric probiotic drinkable solution containing 1 $x10^9$ CFU, and persists in the gut up to 3 days after the end of product intake, demonstrating resistance to gastric juices, hydrolytic enzymes and bile acids.

INTRODUCTION

A first assessment of probiotics efficacy was made in 2001 by an International Expert Consultation group, working for the Food and Agricultural Organization (FAO) of the United Nations and the World Health Organization (WHO), resulting in the Guideline for the Evaluation of Probiotics in Food, published in 2002 (FAO/OMS 2001). One output was a reworking of the definition of probiotics, which was accepted in 2014 by the International Scientific Association for Probiotics and Prebiotics (Hill et al. 2014), with only a minimal grammatical change, as follows: "*Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*".

The health promoting effects of probiotic bacteria, mostly lactobacilli and bifidobacteria, are being increasingly reported, in particular in patients affected by pathological conditions (FAO/OMS. 2001; Hill et al. 2014; Saxelin et al. 2005; Derrien & van Hylcklama Vlieg, 2015; Ljungh & Wadström, 2006; Guglielmetti et al., 2011; Shen et al., 2014). In a very recent review on the role of probiotics, Khalesi *et al.* (2018) confirmed that probiotic supplementation generates a transient improvement in gut microbiota and has a role in improving immune system responses, stool consistency, bowel movement and vaginal lactobacilli concentration also in healthy subjects. In addition, the authors confirmed that in healthy adults probiotic consumption can have a beneficial effect on the immune, gastrointestinal and female reproductive health systems.

An effective probiotic should be preferably of human origin, remain viable during storage and use, be generally recognized as safe (GRAS), confer health benefits on the host, modulate host immunity, prevent or treat a specific pathogen infection by antimicrobial production, adhere to human intestinal cells, contain a large number of viable cells and be capable of surviving in the gut (Ljungh & Wadström. 2006). It follows that a major factor in determining the effectiveness of a probiotic is its ability to survive the

digestive process and thrive in the gastrointestinal tract (Perdigon et al. 1995; Perdigon et al. 2001; Dommels et al. 2009; Saxelin et al. 2010; Hütt et al. 2011). In the gut, in fact, ingested bacteria are confronted with many physicochemical effects that may adversely influence bacteria viability. These include gastric acid, bile acid and digestive enzymes, along with the highly diverse and competitive environment presented by the gut microflora (Tuohy et al. 2007; Larsen et al. 2006).

Interestingly, survival of different *lactobacilli* strains in the gastrointestinal tract after oral ingestion has been demonstrated in several faecal recovery studies conducted in healthy volunteers (Saxelin et al. 1995; Oozer et al. 2006; Wang et al. 2015).

Lactobacillus paracasei is a normal component of healthy individuals' intestinal microflora, commonly used in probiotics products. L. casei DG® (*Lactobacillus paracasei* CNCM I1572; LCDG) is a probiotic strain isolated from human faeces and developed by SOFAR S.p.A. in the Enterolactis® line products. LCDG was deposited at the Pasteur Institute, Paris (deposit N. CNCM I1572).

Characteristics of LCDG are its ability to adhere to the small intestine mucosae, to produce lactic acid, to survive under pH 3.0 conditions and in the presence of bile acids, and not to induce antibiotics resistance (De Vecchi et al. 2008; Ferrario et al. 2014; Balzaretti et al. 2017; Cremon et al. 2017; Balzaretti et al. 2015).

Consistently with these peculiarities a number of *in vitro/in vivo* studies support its therapeutic use: in healthy adults LCDG was shown to have the ability to modulate the intestinal microbial ecosystem (Ferrario et al. 2014) and to influence host's immune responses (Balzaretti et al. 2017; Cremon et al. 2017) through its unique exopolysaccharide capsule (Balzaretti e al. 2015). In addition, LCDG is endowed with therapeutic potential for several dysfunctional and pathological conditions such as ulcerative colitis (D'Incà et al. 2011),

diverticular disease (Turco et al. 2017; Tursi et al. 2013), small intestinal bacterial overgrowth (Rosania et al. 2013) and irritable bowel syndrome (Compare et al. 2017; Cremon et al. 2017).

A previous study in healthy adult volunteers, administered an adult LCDG formulation containing $8.5x10^9$ CFU, once a day for 7 days, demonstrated the presence of live LCDG cells in the collected faeces up to 7 days after the end of treatment (Drago et al. 2002). In the study by Ferrario *et al.* (2014), LCDG cells in faecal samples of healthy adults were significantly increased as compared to baseline after 4-week once daily administration of capsules (Enterolactis® Plus) containing at least $24x10^9$ viable cells. The same study also demonstrated that the intake of LCDG modulated gut microbiota, in particular by increasing the Costridiales geni *Coprococcus*:*Blautia* ratio, which, according to the literature, could potentially confer a health benefit on the host. More recently, LCDG was found to be able to survive after passage through the gastrointestinal tract in healthy adults (Arioli et al. 2018).

The aim of the present open-label, one-week treatment study was to confirm the ability of an LCDG paediatric formulation, containing $1 x10^9$ live bacteria, to transit alive through the gastrointestinal tract in children during and after the administration period. Product safety, defecation frequency, faeces consistency and digestive function were also evaluated.

METHODS

Study design and participants

This was a single centre, open-label, one-arm, recovery study, which included a screening visit, a one-week run-in, a one-week administration period, a two-week follow-up period and a final visit. After the screening visit (V1), subjects attended the clinical centre on the day before the first administration (day -1, V2), on day 8 (V3) and for the final visit (day 22/23) (Figure 1).

The study protocol (No. PSC-DS RECENT-BS 16) was approved by the Ethics Committee of Canton Ticino, Switzerland. All the subjects were given a detailed description of the study and all of them gave written informed consent before enrolment. The study was performed from August to October 2017, in accordance with the Declaration of Helsinki, harmonised European standards for Good Clinical Practice (ICH E6 1.24) and the applicable local laws.

Healthy male and female children, aged 3-12 years and classified as not overweight based on the body mass index chart for sex and age (SSP SGP, 2012), were enrolled in the study. All children were in good physical health, as assessed through a full physical examination at screening. No subjects were on abnormal diets or vegetarians. Children with a defecation frequency above 3 stools per day or less than 3 stools per week were not enrolled. Exclusion criteria also included the following: history or presence of significant diseases, in particular inflammatory/infective intestinal diseases, viral or bacterial enteritis, gastric or duodenal ulcer, metabolic diseases, primary or secondary immunodeficiency; antibiotics intake within 1 month before the screening visit; any other medication, including over the counter drugs, for 2 weeks before the study. Subjects were not enrolled if they were hypersensitive or allergic to any study product's ingredient or food components and if they had participated in other clinical trials in the past 3 months.

Investigational product

Enterolactis® is a probiotic formulation based on L. casei DG® (= *Lactobacillus paracasei* CNCMI1572 = LCDG viable cells). The product was supplied as vials containing 1 x10^9 CFU as powder in the cap (SOFAR SpA, Italy) and a 2 % fructose solution (additives: citric acid as acidity controller, and sodium benzoate and potassium benzoate as preservatives).

All children enrolled in the study received one vial of the investigational product, once daily from day 1 to day 7.

The product was reconstituted just before intake. Upon opening of the vial, the powder in the cap directly mixed with the drinkable solution. For the intake, after the vial was shaken, the children drank the content of the vial directly, under fasting conditions, in the morning at least 10 min before breakfast, or alternatively in the evening before going to bed, at least 2 h after the last meal of the day. Administrations date and time were recorded on a daily diary. Product accountability and diary check were performed to check treatment compliance.

During the entire study, the subjects continued their normal diet except for fermented milk, probiotics food supplements or any other probiotic-containing products and prebiotics food supplements, which were forbidden from the start of the run-in phase until study end. Traditional yoghurts were allowed. The intake of any medication was reported as a protocol deviation.

Faecal sample collection and analysis

Faecal samples were collected at baseline (day -2), during the one-week treatment (day 1 and 4) and at follow-up (day 8, 11, 14, 17 and 20). Collection times could vary of +1 day at baseline or +2 days at all the other time-points. Samples were collected in sterile containers, stored at home at approximately 2-8°C, picked up by a courier as soon as possible after defecation and delivered at 2-8°C to the Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Italy.

Each fresh faecal sample was processed immediately after the delivery to the laboratory, that is within 24 h after defecation, in order not to affect the viability of the probiotic strain. The protocol for the analysis is described in Arioli *et al.*, 2018. Specifically, after homogenization of the sample, 1 g of faeces was resuspended in 9 mL Maximum Recovery Diluent

(MRS; Scharlau) and mixed with a Stomacher. Then, the faecal suspension was serially 1:10 diluted and inoculated by spreading on agar plates containing MRS medium (Difco) supplemented with 1 mg/L vancomycin and 10 mg/L kanamycin (vkMRS). Finally, plates were incubated anaerobically at 37°C for up to 48 h. The identification of the colonies as LCDG strain was carried out by assessing the sticky/filamentous texture of the colony and through an end point-colony PCR with strain specific primers (rtWELFf and rtWELFr) Ferrario et al., 2014). PCRs were performed in 25-μL reaction mixtures, each containing 1 colony (picked with a sterile wooden stick), 2.5 μL of 10× reaction buffer, 200 μmol/L of each dNTP, 0.5 mmol/L $MgCl_2$, 0.5 μmol/L each primer, and 0.5 U DreamTaqTM DNA polymerase (Thermo Fisher Scientific Inc., Monza, Italy). Amplifications were carried out using a Mastercycler 96 (Eppendorf, Milan, Italy). The PCR mixtures were subjected to the following thermal cycling conditions: initial hold at 95 °C for 3 min followed by 39 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Amplification products were resolved by electrophoresis on a 2% (w/v) agarose gel (with 0.2 μg/mL ethidium bromide) in 1×TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA, pH 8.0) and photographed. A 1-kb GeneRuler DNA Ladder Mix was used as a size marker. The method has a detection limit of 100 cells LCDG/g of wet faeces. Result values are presented as $\log_{10}$ CFU/g of wet faeces.

Defecation frequency, stool consistency, digestive function and safety assessments

Besides investigational product administration date/time, study subjects or their parent(s) reported in a daily diary: defecation date/time, stool consistency, adverse events occurrence and concomitant medication intake. Stool consistency was assessed according to the illustrations associated with the 1-7 score system of the Bristol stool scale (Lewis & Heaton, 1997). Scores were as follows: 1. separate hard lumps like nuts; 2. sausage-shaped but

lumpy, 3. like a sausage but with cracks on the surface, 4. like a sausage or snake, smooth and soft; 5. soft blobs with clear-cut edges; 6. fluffy pieces with ragged edges, a mushy stool; 7. watery, no solid pieces, entirely liquid.

In addition, digestive function was evaluated daily in the diary as bad (score 1), normal (score 2), good (score 3) or optimal (score 4) from the day before first administration until day 8. Product intake global evaluation was assessed by the investigator on day 8.

Safety and general tolerability of the investigational product were based on treatment-emergent adverse events occurrence, daily diary check and physical examinations performed at screening and final visit.

Sample size and data analysis

Study sample size was not based on any formal calculation but was deemed appropriate for the descriptive and pilot nature of the study.

The data documented in this trial and the parameters measured were described using classic statistics, i.e. mean, SD, CV (%), minimum and maximum values, for quantitative variables and frequencies for qualitative variables. Data not available were evaluated as "missing values". The analysis was performed using SAS® version 9.3 (TS1M1).

Adverse events were coded using the Medical Dictionary for Regulatory Activities version 20.1.

RESULTS

Demography and disposition of the study participants

Twenty (20) healthy children, 10 males and 10 females, satisfying the study inclusion/exclusion criteria, were enrolled, received all planned doses of

the investigational product and were included in the data analyses. Demographic characteristics of the study subjects are presented in Table 1.

Nineteen (19) children completed the study per protocol, while one (subject 12) discontinued the study during the follow-up phase, after completing the one-week treatment period, due to an antibiotic therapy to cure a tooth abscess (i.e. azithromycin 180 mg suspension) not allowed according to the study requirements.

L. casei DG® (LCDG) faecal recovery

At baseline, no viable LCDG cells were present in the analysed faecal samples. This was expected considering that the children were instructed not to consume any probiotic/prebiotic food components or supplements.

During the administration period most subjects showed variable counts of live LCDG CFU in their faeces. In particular, viable cells of LCDG were isolated from at least one faecal sample in 19 (95%) of the 20 treated children, with the only exception of one child for whom no viable cells were detected (Table 2 and Table 3).

In general, most of the viable LCDG cells were isolated during the week of probiotic treatment, with a maximum count (mean log_{10} CFU/g of 4.3±2.3 [range 3.7 - 6.3]; Table 3) reached between day 4 and 6 after the beginning of the intake.

For 3 of the 19 children with viable cells (15.8%), LCDG was already detected on day 3 (assessment time: day 1 [+2]) at counts of 4 - 4.8 log_{10} CFU/g, whereas for the other 17 children no viable LCDG was detectable at this time point.

Notably, for 11 (57.9%) of the 19 children with detectable live cells, LCDG survived in faecal samples up to 3 days after treatment end (day 10, i.e. assessment time: day 8 [+2]; Table 2 and 3). At this time-point, viable

LCDG counts ranged from 3.7 to 5.5 log_{10} CFU/g, with a mean log_{10} of 2.8±2.2 CFU/g.

Defecation frequency and stool consistency

Weekly average daily defecation numbers are consistent throughout the study periods (Figure 2). Percentage of subjects reporting 0, 1, 2 or 3 evacuations during the day did not change considerably from the run-in to the administration period and from the administration period to the follow-up, with most subjects reporting one defecation / day throughout the study.

The most frequent stool consistency score was 3 during most study days (Figure 3). Scores 1 and 6 were seldom recorded (frequency < 5%) and score 7 was never recorded. Score 2 slightly increased and score 5 slightly decreased with time, during and after treatment.

Consistent with the overall evidence on defecation frequency and stool consistency, the children scored their digestive function most frequently as "Optimal" both at baseline (50%) and at the end of the administration period (55%), with the majority of the children who had an "Optimal" digestion at baseline maintaining the same digestive function during all study periods. Digestion was "Good" for 30% of the children at baseline and for 25% at study end. Notably, for one child who had a "Good" digestion at baseline digestion improved to "Optimal" starting from day 4 up to the last assessment (day 8). For the remaining children, digestive function was graded as "Normal", with one child improving from "Normal" at baseline to "Good" at study end. No children scored their digestive function as "Bad" at any evaluation.

Global evaluation and safety assessments

The individual global evaluation of the product intake was very good for 15 out of the 20 (75%) children. Of the other children, 3 (15%) judged product intake as good and 2 (10%) as normal.

The investigational product, administered to the study children once daily for 7 days, showed a very good safety profile. Only 4 subjects (20%) reported mild to moderate treatment-emergent adverse events either at the end of the treatment period or during the follow-up phase. The most common event was headache experienced by 2 (10%) children. All other adverse events (i.e. oropharyngeal pain, abdominal discomfort, pyrexia chills and tooth abscess) were reported by 1 (5%) subject each. The reported adverse events were judged as not related to study product intake, the majority of them were flu symptoms, and all resolved before study end. No clinically relevant findings were observed at the physical examination performed at the final visit.

DISCUSSION

In the present study, we have demonstrated for the first time that LCDG is capable of surviving the transit through the gastrointestinal tract of 3-12 years old children during and after a one-week consumption of a drinkable paediatric formulation, administered at the daily dose of $1x10^9$ CFU.

Nineteen (19) of the 20 treated children (95) had LCDG CFU in their faecal samples during the administration period, 3 of them already after 1-3 days of treatment. Maximum viable LCDG counts were found at day 4-6 (mean 4.3 log_{10} CFU/g ±2.3 [range 3.7 - 6.3 log_{10} CFU/g]).

These results confirm the ability of LCDG strain to pass the gastrointestinal barrier, i.e. to survive the untoward actions of gastric acid, bile acids and hydrolytic enzymes, also in children. According to these findings, *in vitro* results have previously shown that LCDG can resist at extreme pH (as low as pH 3) and bile acids conditions (De Vecchi et al. 2008; Balzaretti et al. 2015).

Although no previous studies evaluated the survival of LCDG in children, a few studies were performed in infants who were administered other lactobacilli strains with different formulations. In a study performed in 2 months-6 years old children suffering from acute diarrhoea and administered for 5 days *L. rhamnosus* 573L/1, 573L/2, 573L/3 strains as milk/glucose solution (1.2x10 CFU; strain 1:1:1 proportion), viable bacterial cells were detected on the last treatment day in faeces samples of 37 out of the 46 (80.4%) treated children (Szymanski et al. 2006).

In another study, Marzotto *et al.* (2006) observed that 92% of 26 (12-24 months old) infants retained viable *L. paracasei* A cells, at counts ranging from 4.3 to 8.2 $\log_{10}$ CFU/g after the first week of consumption of 100 g fermented milk containing 8.2 $\log_{10}$ CFU/g of this *Lactobacillus* strain. As also previously reported, in fact, in most cases, ingested strains are still detected after a few days (Firmesse et al. 2008; Fujimoto et al. 2008). In the above cited study (Marzotto et al. 2006), the percentage of children with positive samples decreased to 16% during the wash-out that followed the overall 4-week treatment. Notably, in the present study, live LCDG in faeces was present up to day 10, i.e. 3 days following the last product intake, in 57.9% of the study children at counts ranging from 3.7 to 5.5 $\log_{10}$ CFU/g, indicating a rather sustained persistence.

For comparison, in a study conducted in healthy adult volunteers (Ferrario et al. 2014) continuing their usual diet throughout the investigation, administration of a probiotic capsule containing at least 24 $\times 10^9$ viable LCDG, every day for 4 weeks, resulted in a significant increase ($p<0.001$) in bacterial cells, detected in faecal samples of all subjects at the end of the probiotic intervention at a mean count of 7.5±0.7 $\log_{10}$ CFU/g (range 6.2 - 8.3 $\log_{10}$ CFU/g), as compared to baseline (7/12 subjects; mean 5.1±0.3 $\log_{10}$ CFU/g; range 4.7 - 5.6 $\log_{10}$ CFU/g). Interestingly, after a 4-week washout period, the LCDG cell number decreased to the amount before probiotic intake. More recently, the ability of LCDG to survive gastrointestinal transit in healthy adults

after one-week consumption of 1 x 10^9 CFU per dose was evaluated (Arioli et al., 2018). The main finding of the study was that all 20 enrolled subjects were positive at least once for LCDG alive cells in the faecal sample, with the highest concentration between 4 and 8 days from the beginning of probiotic consumption. Alive probiotic cells were countable up to 5 days after the end of the Enterolactis® formulation intake.

In the study by Drago *et al.* (2002), after administration of 8.5x10^9 CFU LCDG to 12 healthy adult volunteers once daily for 7 days, viable cells were detected in all samples during consumption, with mean counts ranging from 1.2x10^5 on day 3 to 2.3x10^6 CFU/g on day 7, and one week after treatment cessation (mean 1.1x10^6 CFU/g).

The results of the present study are also consistent with previously published data obtained with various lactobacilli strains where bacteria were found in numbers ranging from < 2 $\log_{10}$ CFU/g to 8 $\log_{10}$ CFU/g [see e.g. Larsen et al. 2006; Saxelin et al. 1995; Oozer et al. 2006; Wang et al. 2015; De Vecchi et al. 2008; Drago et al. 2002; Marzotto et al. 2006; Fujimoto et al. 2008; Ahlroos & Tynkkynen. 2009).

In this study, 19 of the 20 enrolled and treated children were positive for viable LCDG cells at least once. Children 3 and 5 were found positive only during the follow up phase, likely because recovery of bacteria in faecal samples is consistently variable between individuals (Derrien & van Hylcklama Vlieg. 2015). Unexpectedly, for subject 10 a higher number of viable LCDG cells in faeces were found during the follow-up rather than during the week of treatment. As in the other referenced studies, a high variability in recovered live cells in faecal samples was observed. It is known that the diet can indirectly affect the survival of ingested probiotics (Salonen & de Vos. 2014). The different amount of recovered LCDG cells in different subjects may thus be associated with the food consumed, which could affect the gastric emptying rate and thus the survival of the probiotics (Russo et al. 2011), although other factors could have contributed to the variability observed. Faecal presence of

ingested strains, also referred to as persistence, reflects not only the dose of the ingested strain, but also the extent of cell death (mainly in the upper gastrointestinal tract), and the subsequent replication of surviving cells.

In the present study, digestive function was also evaluated, in order to assess whether LCDG intake for a short time period and in a healthy paediatric population could already exert a beneficial effect. Results showed that digestive function was reported as "Optimal" or "Good" for the majority of subjects already before the consumption of the investigational product. The digestive function either did not change (for 18/20 children) or improved only very slightly and only for 2 children at the end of the one-week administration period as compared to baseline.

In addition, the majority of subjects reported one stool evacuation each day during the whole study duration, with negligible changes in defecation frequency between the study periods. Stool consistency did not significantly change during the study, with score 3 (*like a sausage but with cracks on the surface*) being the most frequent at all assessment times. To note that score 3 is an indicator of a satisfactory stool consistency. Upon treatment, score 2 (*sausage-shaped but lumpy*) slightly increased and score 5 (*soft blobs*) slightly decreased, suggesting a very modest digestion improvement, although not clinically relevant, during and after treatment. Based on currently available evidence, *L. rhamnosus* GG strain has proven to be efficacious in the treatment of children acute gastroenteritis, prevention of antibiotic-associated diarrhoea and prevention of nosocomial diarrhoea (Rosania et al. 2013; Hojsak. 2017; Allen et al. 2010; Szajewska et al. 2007). In addition, similar to the findings of the present investigation, a previous study in healthy adults showed that a 2-week administration of fermented milk containing a strain of *L. casei* (i.e. *L. casei* Shirota) did not change bowel movements frequency or stool consistency (Wang et al. 2015).

In the present study, general digestive conditions of the enrolled healthy children, including defecation frequency, stool consistency and

digestive function, were already satisfactory at study entry, due to the restrictions imposed by the study inclusion criteria. It is likely that this, together with the short administration period, could be the reason why no relevant changes were observed upon probiotic treatment.

In the present study, the good safety profile and palatability of LCDG drinkable paediatric formulation were also confirmed.

In conclusion, the present preliminary study, carried out in healthy children, aged 3-12 years, demonstrated for the first time that *L. casei* DG® survives the gastrointestinal transit when ingested with the paediatric probiotic drinkable formulation containing 1×10^9 CFU, and persists in the gut up to 3 days after the end of probiotic consumption, demonstrating resistance to gastric juices, hydrolytic enzymes and bile acids.

Ethical statements

The study protocol (No. PSC-DS RECENT-BS 16) was approved by the Ethics Committee of Canton Ticino, Switzerland.

All the subjects were given a detailed description of the study and all of them gave written informed consent before enrolment.

The study was performed from August to October 2017, in accordance with the Declaration of Helsinki, harmonised European standards for Good Clinical Practice (ICH E6 1.24) and the applicable local laws.

Conflict of interest

W.F. is an employee of SOFAR S.p.A., Italy; M.R. and C.L. are employees of CROSS Research S.A.; SA, RK and S.G. are employees of DeFENS, Milan University. CROSS Research S.A. and DeFENS, Milan University, were contracted by SOFAR S.p.A. and received financial support

for their services. The authors declare that they have no other relationships or activities that could appear to have influenced the submitted work.

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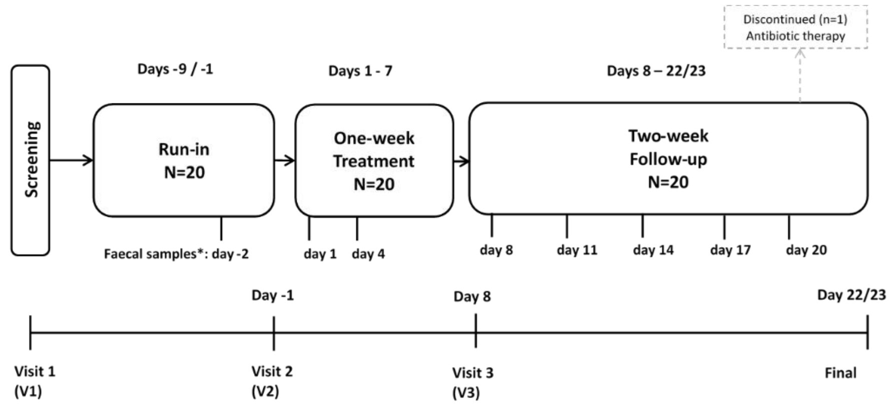
Figure captions

Fig. 1 *Graphic representation of the study design*



**+ 1 day collection window for day 1 and day 4 samples; + 2 days collection window for follow-up (days 8, 11, 14, 17 and 20) samples*

Fig. 2 *Average percentage of children reporting 0, 1, 2 or 3 defecations/day during the run-in, treatment and follow-up (days 8-14 and 15-22) study phases. N=20*

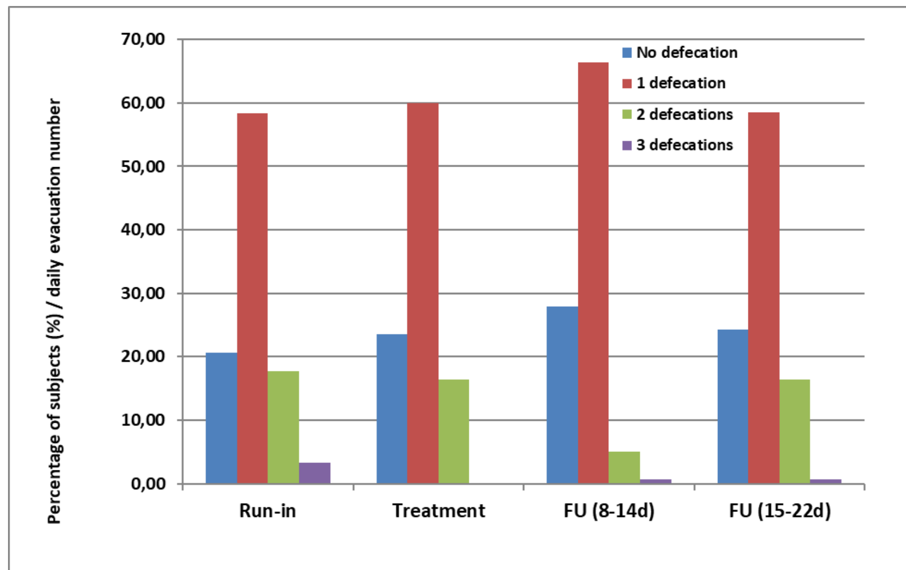


Fig. 3 *Average children percentage data for each stool consistency score, assessed daily using the Bristol 1-6 score scale*, during the run-in, treatment and follow-up (days 8-14 and 15-22) study phases. N=20. *Score 1: separate hard lumps like nuts; score 2: sausage-shaped but lumpy; score 3: like a sausage but with cracks on the surface; score 4: like a sausage or snake, smooth and soft; score 5: soft blobs with clear-cut edges; score 6: fluffy pieces with ragged edges, a mushy stool; score 7: watery, no solid pieces, entirely liquid.*

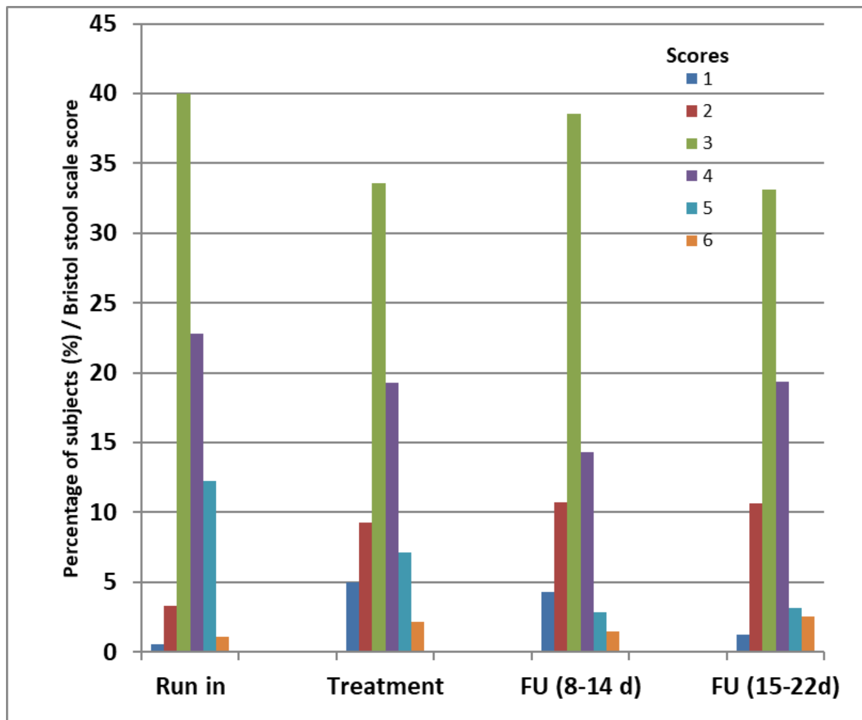


Table 1. *Demography of the study children*

Parameter	Analysed subjects N = 20
Sex	
Male – n (%)	10 (50%)
Female – n (%)	10 (50%)
Race	
White	20 (100.0%)
Age (Years)	
Mean ± SD	7.0±2.8
Median (Range)	6.5 (3-12)
Body weight (kg)	
Mean ± SD	27.07±11.64
(Range)	25.05 (13.4 – 59.5)
Height (cm)	
Mean ± SD	125.1±19.0
(Range)	125.0 (94 – 170)
Body mass index (kg/m^2)	
Mean ± SD	16.49±1.89
(Range)	15.75 (14.2 – 20.9)

Table 2. *Percentage of children with viable L. casei DG® cells in faecal samples collected at baseline (day - [+1]), during treatment (Day 1 [+2]), Day 4 [+ 2]) and at follow-up (Day 8 [+ 2] and days 11, 14, 17 and 20 [+2])*

Assessments	Subjects Number	Subjects, n(%) with viable L. casei DG® in faecal sample				
		Baseline	One-week treatment		Follow-up	
		Day -2 (+1)	Day 1 (+2)	Day 4 (+2)	Day 8 (+2)	Day 11, 14, 17, 20 (+2)
Daily assessment	20	0 (0.0%)	3 (15.0%)	16 (80.0%)	11 (55.0%)	0 (0.0%)
Overall	***20***	***0 (0.0%)***	***19 (95.0%)***			***0 (0.0%)***

Table 3. *Individual and mean (±SD) counts of viable L. casei DG® in faecal samples of the study children (N=20) at baseline, during the probiotic administration period and at follow-up*

Subject	Viable L. casei DG® counts (log_{10} CFU/ g faeces)				
	Baseline	One-week administration period		Follow-up	
	Day -2 (+1)	Day 1 (+2)	Day 4 (+2)	Day 8 (+2)	Days 11 (+2), 14 (+2), 17 (+2), 20 (+2)
1	BDL	BDL	5.7	3.7	BDL
2	BDL	BDL	4.5	BDL	BDL
3	BDL	BDL	BDL	5.5	BDL
4	BDL	BDL	5.7	BDL	BDL
5	BDL	BDL	BDL	4.7	BDL

6	BDL	BDL	4.7	BDL	BDL
7	BDL	BDL	5.9	BDL	BDL
8	BDL	BDL	5.3	4.7	BDL
9	BDL	4	6.3	4	BDL
10	BDL	BDL	3.7	4.7	BDL
11	BDL	BDL	5	3.95	BDL
12*	BDL	BDL	5.3	BDL	BDL**
13	BDL	BDL	5.9	3.3	BDL
14	BDL	BDL	5	4.7	BDL
15	BDL	4.8	5.3	4	BDL
16	BDL	BDL	5.9	4.3	BDL
17	BDL	BDL	5.5	4.5	BDL
18	BDL	4.5	BDL	BDL	BDL
19	BDL	BDL	BDL	BDL	BDL
20	BDL	BDL	5.3	4.5	BDL
Mean±SD	**BDL**	**0.5±1.6**	**4.3±2.3**	**2.8±2.2**	**BDL**

BDL: Below detection limit. BDL values on days 1 (+1), 4 (+2), 8 (+2) were considered as "0" in the calculation of the mean±SD values

** Subject 12 discontinued the study on day 20. This subject completed study treatment (days 1-7), whereas assessments at days 14(+2), 17(+2) and 20(+2) were not performed.*

*** Day 11(+2) only.*

CHAPTER 3

ANIMAL STUDY

L. paracasei DG® (*L. paracasei* CNCM I-1572) affects bacterial localization along the intestinal tract and modulates immune system responses and gut serotonergic metabolism in mouse.

ABSTRACT

Lactobacillus paracasei species have been largely employed as probiotics. However, the exact mechanism by which lactobacilli may exert beneficial effects remains unknown. In order to better understand the mechanism of action of probiotics in the host's intestine we have selected a specific strain, *Lactobacillus paracasei* DG, and, in a mouse model we have evaluated the *in vivo* impact of the strain on the microbiota and host's gene expression in different intestinal sites. The results have shown that *L. paracasei* DG preferentially colonized the cecum and the colon. Furthermore *L. paracasei* DG affected the expression of several genes involved in serotonin pathway, mainly in the colon, and in immune responses, particularly, in the ileum, suggesting that this bacterium may play a potential anti-inflammatory/regulatory activity in the gut.

INTRODUCTION

Lactobacilli are non-spore-forming, Gram-positive rods that are an important part of the normal human bacterial flora commonly found in the mouth, gastrointestinal (GI) tract and female genitourinary tract (Slover et al. 2008). Specific strains of the species *Lactobacillus paracasei* are found naturally in a number of fermented food products, and they have traditionally been used in the production of fermented milks and cheeses. Nowadays, they have been largely employed as probiotics, i.e. "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*" (Hill et al. 2014). Due to its attitude to resist stresses during the industrial production, largely most of the microbial biomasses of probiotic products on the market consists of lactobacilli species and some bifidobacteria.

Various species of lactobacilli may provide benefit in certain infectious diarrheas or other illnesses. Several studies have reported success in treating irritable bowel syndrome (IBS) in school age children, decreasing colic

symptoms in infants, preventing diarrhea secondary to antibiotic treatment for respiratory infections, and decreasing the duration of acute diarrhea in young children (Pant et al. 1996; Arvola et al. 1999; Gawronska et al. 2007; Savino et al. 2007). As typical part of the microbial flora in the GI and urogenital tracts, lactobacilli have been shown to prevent pathogenic bacteria from causing infection. These bacteria exhibit several properties that make them useful for preventing infectious disease.

Several mechanisms have been proposed to explain how probiotics are able to exert their effects. The acids produced by lactobacilli, such as acetic, propionic and lactic acids, may reduce intestinal pH, thereby potentially inhibiting the growth of pathogenic bacteria. However, the exact mechanism by which lactobacilli may exert such beneficial effects remains unknown. In recent years, the efficacy of *Lactobacillus* spp. probiotics has been extensively studied. Many clinical trials aimed at the comparison between *Lactobacillus* probiotics and placebo or standard treatment options have been done for many situations such as for example gastrointestinal disorders, cholesterol management, and bacterial vaginoses, and even to attempt immunomodulation (Slover et al. 2008).

Recently, specific strains of *L. paracasei* have been used in probiotic dietary supplements, including the strain *L. paracasei* DG (commercially known as L. casei DG® [Enterolactis®]). A range of health-promoting properties have been assigned to *L. paracasei* DG, including the improvement of ulcerative colitis, a slight improvement in eradication rate and a reduction in the side effects associated with therapies for the eradication of *Helicobacter pylori*, and the treatment of small intestinal bacterial overgrowth (Balzaretti et al. 2017). *L. paracasei* DG has proved to be also able to modulate the levels of fecal *Clostridiales* bacteria and butyrate levels in healthy adults (Ferrario et al. 2014). Despite the significant clinical evidence for the above health benefits

associated with the intake of *L. paracasei* DG, the molecular mechanisms underlying these health effects are still unknown.

Several mechanisms have been proposed to explain how the probiotic strain exert their effect. One of the most studied mechanisms concerns the ability of probiotic bacteria to antagonize pathogenic organisms through the excretion of antimicrobial agents (Spinler et al. 2008) or the displacement of pathogenic organisms through the competitive occupancy of adhesion sites (Guglielmetti et al. 2010). In addition, several reports suggest that health benefits result from stimulation of the immune system by components presented at the surfaces of probiotic strains (Taverniti et al. 2013; Guglielmetti et al. 2014). Many experiments conducted both *in vivo* and *in vitro* have demonstrated that the polysaccharides present at the surfaces of the bacteria, such as for example exopolysaccharides (EPSs), can play roles not only in the displacement of pathogenic organisms but also in the stimulation of the immune system (Balzaretti et al. 2017; Hidalgo-Cantabrana et al. 2010; Ruas-Madiedo et al. 2006).

In order to contribute to the greater comprehension of the mechanisms of actions of *L. paracasei* DG in the host's intestine, mainly in case of functional disorders such as SIBO and IBS, here we set out to define in a mouse model the *in vivo* impact of the intake of *L. paracasei* DG on the microbiota and host's gene expression in different intestinal sites.

MATERIALS AND METHODS

Bacterial strains, preparation, and growth conditions. *L. paracasei* DG was grown in the Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories Inc., Detroit, 63 MI, USA). Bacterial strain was inoculated from frozen glycerol stocks and sub-cultured twice in MRS using a 1:100 inoculum. Incubation temperature was 37°C in aerobic conditions. To prepare fresh cultures to be used in *in vivo* experiments, bacterial cells from an overnight culture were

collected, washed twice with sterile PBS and then resuspended in PBS at the concentration of 1 × 10^9 cells ml^{-1}, by using a Neubauer Improved counting chamber. Bacterial cells have been prepared fresh each day of mice treatment.

Mice treatment with probiotic strains. Two-month old female C57BL/6 mice were housed in a traditional, specific pathogen-free, nonsterile environment in the animal facility of the Department of Agricultural and Environmental Sciences of Università degli Studi di Milano. After one-week adaptation, mice were separated in cages in 2 groups of 5 mice each. Mice were treated with bacterial suspension or the vehicle (as a 200 µl suspension) *via* oral gavage once a day for 5 days. Finally, mice were euthanized 4 h after the last gavage. Following sacrifice, biopsies of distal ileum, caecum and proximal colon portions were collected from each mouse and DNA was then extracted.

Nucleic acid isolation from intestinal biopsies. DNA was obtained from mouse biopsies by means of a PowerFecal® DNA Isolation Kit (MO BIO Laboratories) and it was employed to quantify bacterial cell number through qPCR. The homogenization of mouse biopsies was performed by using a Precellys bead beater (3 x 30 s at 6800 *rpm*; Advanced Biotech Italia s.r.l., Seveso, Italy). After that, DNA isolation was conducted following manufacturer's instructions. For RNA isolation, flushed biopsies stored at -80°C have been thawed on ice, and RNA later was removed. Biopsies were then immediately resuspended in Qiazol (Qiagen) and homogenized by using an IKA T10 basic Ultraturrax (30.000 *rpm* for 30 s). Following steps of RNA extraction have been performed by using RNeasy Lipid Tissue Mini Kit (Qiagen), in accordance with manufacturer's instructions. Concentration and purity of nucleic acids were determined with the Take3 94 Micro-Volume (BioTek Instrument).

The total number of bacteria was quantified by using 16S rRNA-targeting primers 357F-907R. Bacterial DNA isolation was carried out with the same kit employed to extract DNA from biopsies.

Preparation of RNA and reverse transcription. After extraction, RNA integrity was checked by loading 100 ng of RNA on a 1% agarose gel in non-denaturing conditions. Afterwards, removal of DNA was performed by using DNase I (Sigma-Aldrich) by following manufacturer's protocol. Briefly, 8 µl of RNA were incubated with 1 µl of DNase I for 30 min at room temperature; afterwards inactivation of DNase was obtained by adding 1 µl of Stop solution (Sigma-Aldrich) and incubating at 70°C for 10 min. RNA was re-quantified after DNA removal. One microgram of total RNA was then reverse transcribed with the iScript Select cDNA Synthesis Kit (Bio-Rad Italia, Segrate, Italy) using the following thermal cycle: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. The expression levels of the genes of interest were determined through reverse transcription quantitative PCR (RT-qPCR) with SYBR Green technology using SsoFast EvaGreen Supermix (Bio-Rad Italia, Segrate, Italy) on a Bio-Rad CFX96 system according to manufacturer's instructions. Primers, where not taken and adapted from literature, were designed by using Primer3 Tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and checked with OligoAnalyzer 3.1 Tool (http://eu.idtdna.com/calc/analyzer), and for specificity with Nucleotide BLAST. Gradient and efficiency analyses were performed to select the most suitable primer's annealing temperatures and concentrations. Afterwards, the amplifications of selected target genes were carried out in a total volume of 15 µl containing 2×SsoFast EvaGreen Supermix, forward and reverse primers (concentration of 300 µM for ZONU, 5HTR3 and 5HTR4 oligos; concentration of 500 µM for the other primer pairs), ultrapure sterile water, and cDNA (15 ng in reaction). The cycling parameters were initiated by 3 min at 95°C, followed by 44 cycles of 10 s at 95° C, 30 s at 58° and 5 s at 72°C using the Bio-Rad CFX96 system. An annealing temperature of 55.5°C was used for THP1 and ZONU primers. Amplification

reactions were performed in duplicates, and DNA contamination controls were included. The amplifications were normalized to the expression of the glyceraldehyde 3-phosphate dehydrogenase encoding gene, which resulted the most stable reference gene in preliminary comparison experiments compared with 18S and beta-actin (Data not shown) Relative transcript levels were calculated applying the 2(-ΔΔCT) method. Specific amplification was checked by melting curves analysis, confirmed by run of amplification products on agarose gel.

Statistical analysis. Statistical calculations were performed using the software program GraphPad Prism 5. The significance of the results was analyzed by unpaired Mann-Whitney test with two-tailed distribution. $P < 0.05$ was considered to be significant.

Ethics statement. All experiments have been accepted by the Ethic Committee of the University of Milan (protocol n. 3/2013).

RESULTS AND DISCUSSION

***L. paracasei* DG modifies the bacterial load in different mouse intestinal sites**. Quantitative PCR (qPCR) with strain-specific primers was used to quantify *L. paracasei* DG in the ileum, cecum and colon of mice gavaged once daily for 5 days with the bacterial cells or PBS. As expected, qPCR resulted negative in samples from PBS-gavaged mice. Conversely, we quantified 6.47±0.83, 9.25±0.08 and 9.38±0.39 $\log_{10}$ cells/g respectively in the ileum, cecum, and colon of DG-gavaged mice (Figure 1).

Subsequently, we performed qPCR with panbacterial primers targeting the 16S rRNA gene to quantify the total bacterial cells in the same mouse intestinal samples used for the quantification of DG. We found a significantly higher concentration of bacterial cells in the cecum and colon (11.43±0.13 and 10.99±0.34 $\log_{10}$ cells/g, respectively) compared to the ileum (9.22±0.61 $\log_{10}$

cells/g); the difference between the bacterial concentration in the cecum and the colon resulted statistically significant too (Figure 1).

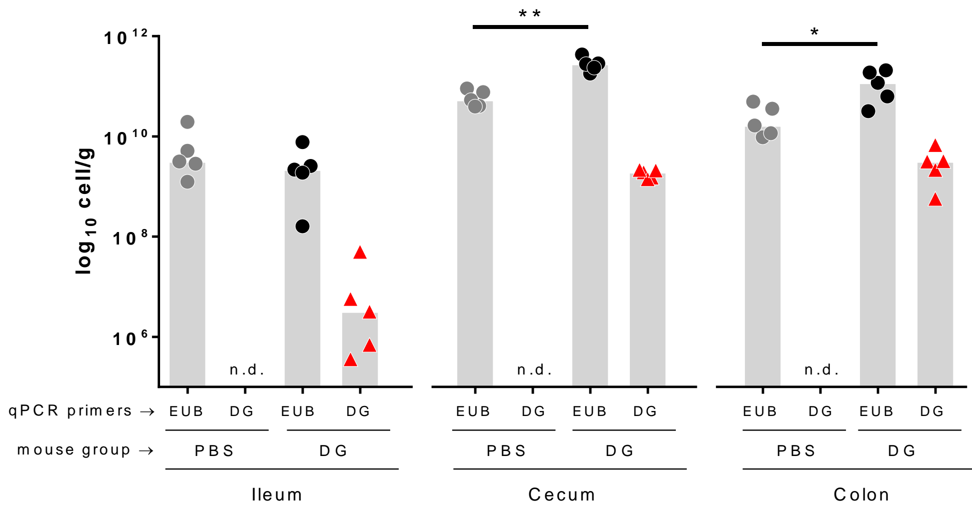


Figure 1 *Quantification of* L. paracasei *DG cells expressed as* log_{10} *mean total bacterial cell. *=P < 0.05; **=P < 0.01. Significant differences are according to Mann-Whitney test.*

Overall, these data show that *L. paracasei* DG preferentially colonized the cecum and the colon.

These results may help us to identify a specific use of probiotic in case of pathological situations with a localization at colon level, such as diverticular disease and IBD, which are conditions that include the dysbiosis in their etiopathogenesis.

***L. paracasei* DG affects the gene expression of the serotonergic pathway in the mouse intestine.** The intestinal serotonergic signalling

system seems to play a very important role in many gastrointestinal disorders, such as IBS and IBDs (Inflammatory Bowel Diseases). In fact, serotonin (or 5-hydroxytryptamine, 5-HT) is a neurotransmitter and neuromodulator released by enterochromaffin cells (EC), that is involved in the regulation of both motor and sensory functions of the GI tract (Camilleri. 2002; Yan et al. 2012). Over 95% of serotonin in the body is found right in the gut. Some alterations in the serotonergic signaling, in terms of 5-HT biosynthesis, content, release, and/or reuptake, have been reported in the gut of IBS patients. It was observed that mucosal release of 5-HT stimulates both intrinsic sensory neurons (most likely via 5-HT_4 receptors) affecting peristalsis, secretion and vasodilation, and extrinsic sensory neurons (via 5-HT_3 receptors), affecting gastric emptying, pancreatic secretion, satiation, pain, discomfort and nausea (Gershon. 1999). Therefore, the availability of 5-HT and some serotonergic receptors, such as 5-HT_3R, 5-HT_4R, could influence the risk of developing problematic symptoms common in the IBDs and IBS, including visceral hypersensitivity and changes in bowel habits (Coates et al. 2017; Yan et al. 2012).

In this work we hypothesized that a change in gut motility may be related to the modifications in bacterial load that affect the serotoninergic pathway in the different intestinal districts. To test this hypothesis, we studied the expression of genes involved in the intestinal serotonin metabolism. Specifically, with the aim to see potential modulation of serotonergic pathways by bacteria, we decided to evaluate the expression of tryptophan hydroxylase-1 (TPH1) gene, which represents the rate-limiting step of serotonin biosynthesis and of the gene coding the serotonin reuptake transporter SERT, which is widespread in all intestinal epithelial cells of intestinal mucosa (Chen et al. 1998). In this work, we also evaluated the potential modulation of two of the functional receptors for serotonin 5-HTR_3 and 5-HTR_4 by probiotics.

RT-qPCR experiments evidenced that the strain affected the transcription of the genes involved in serotonin metabolism. Particularly, it

downregulated the expression of the serotonin receptor $5HT_3R$ in the colon, whose activation is more likely to worsen the inflammatory process (Fig. 2).

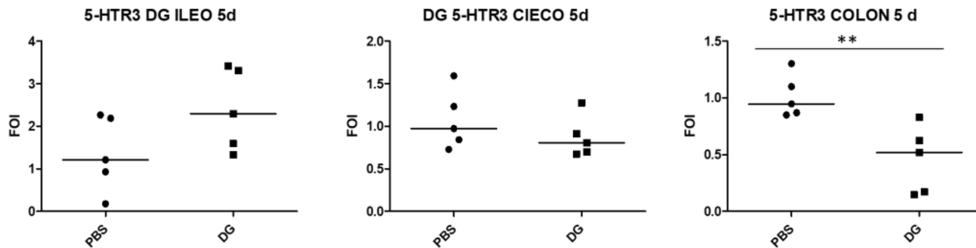


Figure 2: *Serotonergic gene expression modulated by* L. paracasei *DG at three different districts (ileum, caecum and colon), reported as fold of induction (FOI) (* = P value < 0.05, ** = P value < 0.01)*

In addition, we observed a significant downregulation in the ileum coding for the genes TPH1 and $5HT_4R$.

As reported above, TPH1 represents the rate-limiting enzyme involved in the majority of serotonin synthesis within the periphery, including the gastrointestinal tract. Recent evidences suggest that 5-HT can affect the development and severity of inflammation within the gut. Minderhoud *et al.* (2007) have demonstrated an inverse relationship between TPH-1 RNA levels and IBS-like symptoms. Specifically, they have observed that increased TPH-1 levels in colonic biopsies from Crohn disease patients were linked to increased risk of IBS symtoms (Coates et al. 2017).

As regards $5HT_4R$, a serotoninergic receptor located in the gastrointestinal tract, its downregulation can positively affect the likelihood and severity of intestinal inflammation and/or its complicating symptoms. To date, only few clinical trials have been conducted in order to study these relationships in a targeted way (Coates et al. 2017).

L. paracasei **DG affects the intestinal immune system and permeability of mice.** Since the gut microbiota and the serotonergic pathway are associated to the overall gut homeostasis, we also investigated the intestinal immune response and permeability by performing RT-qPCR targeting the genes of several cytokines, such as IL-1β, IL-6, IL-10, TGF-β, and TNF-α, the cyclooxygenase 2 (COX-2), the iNOS (inducibile Nitric Oxide Synthase) and the zonulin protein. The latter is the protein responsible for the disengagement of the protein zonula occludens of the tight junction complex, the major component of epithelial barrier function (Fasano. 2001). An uncontrolled zonulin activity might be a leading factor in several chronic inflammatory disorders (CID), as increased permeability allows the entrance of several environmental stimuli, which have been shown to be the crucial event in the onset of CID as IBD, necrotizing enterocolitis, multiple sclerosis, type-1-diabetes, rheumatoid arthritis (Sturgeon & Fasano. 2016). As regard iNOS enzyme, it is involved in the synthesis of nitric oxide, whose production has both beneficial and detrimental consequences, depending on the physiologic environment and magnitude of expression (Lind et al. 2017). The constitutive presence of iNOS in normal ileal epithelium indicates a role for this enzyme in maintaining intestinal homeostasis (Hoffman et al. 1997) and it was demonstrated that iNOS is involved in villous reepithelialization of mucosa upon injuries. In a previous *ex vivo in vitro* experience the strain *L. paracasei* DG has been already demonstrated to be able to impact on the gene expression of the iNOS in the ileum in an *ex vivo in vitro* study on colonic biopsies (Turco et al. 2017).

In this work, similarly to the gene expression analysis of the serotonergic metabolism, we observed that, out of eight investigated genes in the three intestinal sites, the strain only increased the expression of TGF-ß (fold of induction, FOI=1.96; $p<0.01$) and iNOS (FOI=1.55; $p<0.01$) in the ileum (Fig. 3). The gene coding for zonulin was modulated exclusively in the

colon after 5 days, where its expression was reduced in DG-treated mice (FOI=0.32; p<0.01).

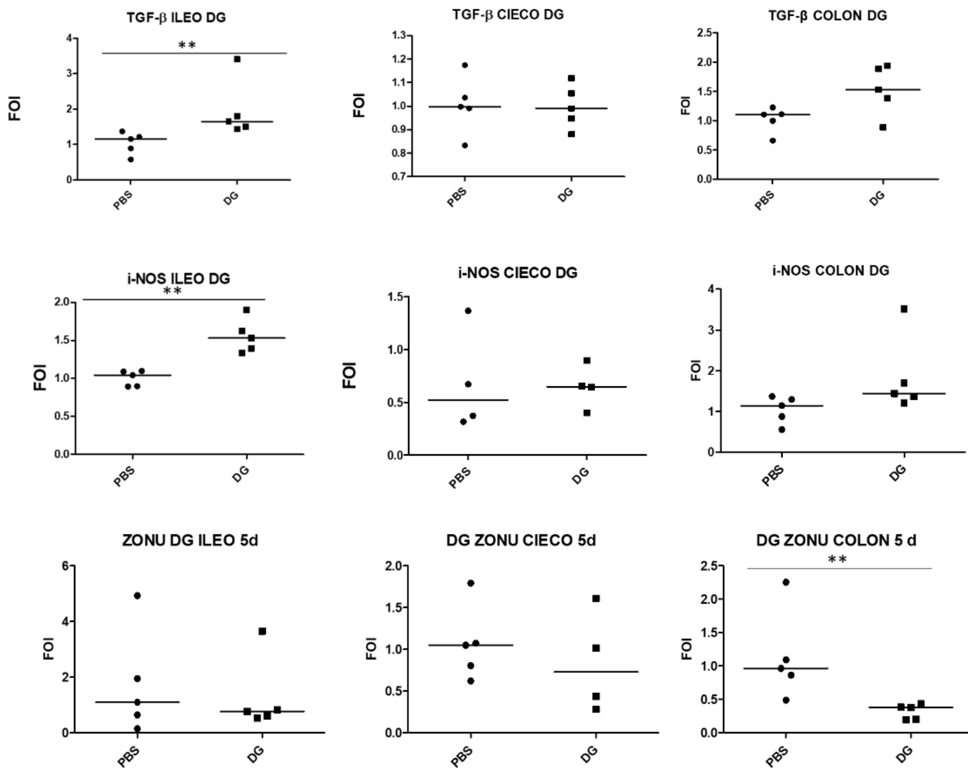


Figure 3: *immune system and zonulin gene expression modulated by L. paracasei DG at three different district (ileum, caecum and colon), reported as fold of induction (FOI) (* = P value < 0.05, ** = P value < 0.01)*

Overall, these results show that the administration of *L. paracasei* DG affected the expression of several genes involved in immune responses in the mouse intestine and, particularly, in the ileum, suggesting that this bacterium may play a potential anti-inflammatory/regulatory activity in the gut.

A summary of all the results of gene expression obtained in the study is reported below (Figure 4).

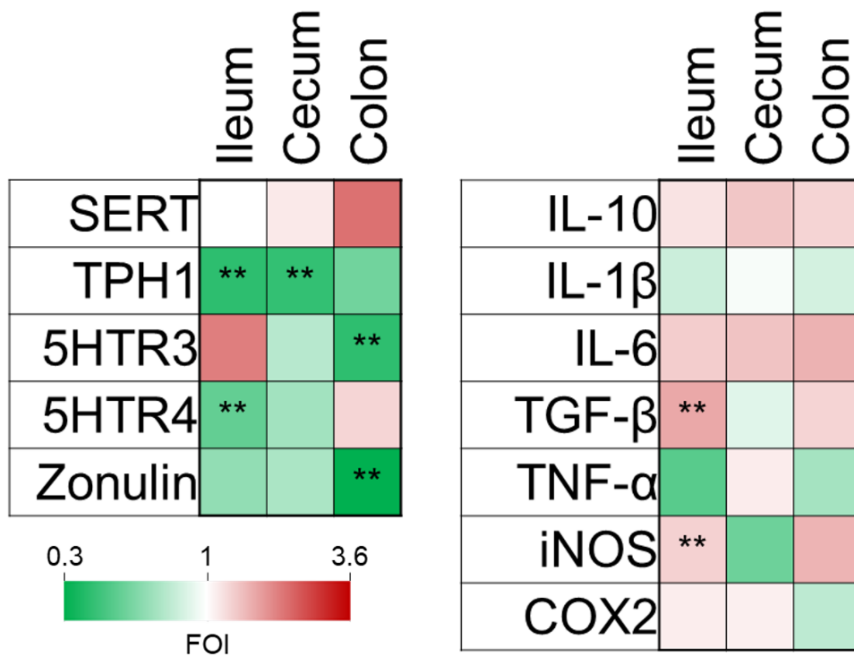


***Figure 4**: summary of the results of gene expression modulated by* L. paracasei *DG at three different district (ileum, caecum and colon), reported as fold of induction (FOI) (* = P value < 0.05, ** = P value < 0.01)*

This study aimed to contribute to the understanding of the mechanisms that support the health promoting properties of the probiotic human intestinal microorganism *Lactobacillus paracasei* DG. The strain may affect host's health through diverse mechanisms that can be classified into two main classes: (i) interaction with the intestinal microbial ecology (Singh et al. 2013; Gargari et al. 2016) and (ii) modulation of host's mucosal metabolism in the gut, mainly in terms of immunomodulation (Guglielmetti et al. 2014a; Kawahara et al. 2017; Taibi et al. 2017; Tanaka et al. 2017). Here we investigated both aspects by means of a murine model based on conventionally reared wild-type adult mice; we did not use a murine model for a specific disease or dysfunction, to meet the meaning of probiotics that,

according to the European Food Safety Authority (EFSA), are foods or food supplements intended for the healthy population.

It is commonly accepted that the ability of a probiotic to influence health first moves from its ability to influence the microbial ecology of the gut. In line with this notion, in Italy the Ministry of Health allows the only health claim for probiotics "*promote the balance of intestinal flora*" (Italian Ministry of Health. 2013) and, in Canada, the Food Inspection agency identified as acceptable the non-strain specific probiotic claim "*provides live microorganisms that contribute to healthy gut flora*". Accordingly, the initial step of this *in vivo* study consisted of studying the preferential site of intestinal colonization of the *L. paracasei* DG. The obtained results showed that the strain is able to preferentially colonize the cecum and the colon. The colon is a very important organ as it is responsible for performing many functions in the intestine: to absorbe water and electrolytes, to produce mucus and immunoglobulins (antibodies), and, above all, to advance its content to the rectum, through two types of contractions: the segmental and the propulsive (peristaltic) ones. In this last function, a fundamental role is played by serotonin. Serotonin is a neurotransmitter that regulates motility and intestinal secretions, where the presence of enterochromaffin cells containing serotonin is conspicuous; it determines diarrhea if present in excess and constipation if present in defect. In this *in vivo* study it was observed that *L. paracasei* DG was able to modulate the gene expression of some compounds related to the serotonergic pathway, with a potential positive effect on the intestinal peristalsis. At the same time, the daily administration for five days of *L. paracasei* induced (i) the downregulation of zonulin, that is involved in the leaky-gut and, therefore, the gut permeability, and (ii) the stimulation of expression of the antinflammatory cytokine TGFβ in the ileum.

The above effects are very important in some disorder that affects the large intestine, such as the IBS.

IBS is one of the most common gastrointestinal disorders, with a prevalence of 11.2% in the United States and Europe (Lacy et al. 2016); it is characterized by abdominal pain and changes in bowel habits. In recent years, alterations in the luminal factors, the epithelial barrier, and the immune, endocrine, and nervous systems have been found in a large proportion of IBS patients (Barbara et al. 2016). Furthermore, it has been observed that the microbiota alteration can represent another factor of fundamental importance in the etiopathogenesis of this disorder. In fact, many studies have reported changes in the composition and stability of the intestinal microbiota in IBS patients over time (Rajilić-Stojanović et al. 2011; Jalanka-Tuovinen et al. 2014; Simrén et al. 2013). We still don't know if this altered microbiota is the cause or effect of IBS, but in any case the improvement of symptoms observed after probiotics (Moayyedi et al. 2010; Ford et al. 2014) or non-absorbable antibiotics (Pimentel et al. 2011) supports the fact that there are intestinal bacteria-host interactions in the pathophysiology and symptomatology of IBS.

Clear alterations within the gastrointestinal mucosa or lumen at tissue, cellular and molecular level, although reported in literature, are variable and have not been irrefutably identified in IBS (Gazouli et al. 2016). Improper immune responses seem to be involved (Sinagra et al. 2016), but they cannot fully explain the symptomatology. Similarly, intestinal 5-HT and its receptors also appear to play an important role in the modulation of the development and intensity of inflammation. (Coates et al. 2017).

L. paracasei DG, which was demonstrated to be able to modulate gut microbiota structure/function in IBS, possesses noticeable immunomodulatory activities, and can positively affect the human intestinal microbiota (Cremon et al. 2017; Compare et al. 2017). However, the precise mechanism of action supporting the efficacy of *L. paracasei* DG in the management of IBS remains, at least partly, unexplained. The possible missing piece of the puzzle may be the enteric nervous system (ENS), that is described inside the Gershon' book "The second Brain" as an independent neuroendocrine organ governed by the

neurotransmitter and hormone serotonin. Gershon's book brought to speculate that, besides immune system, microbiota, and mucosal barrier, a wider understanding of the mechanisms of interaction between the probiotic strains and the host could be gained considering also the ENS and, particularly, the serotoninergic metabolism. In this study, we have also collected data related to the serotoninergic pathway, showing positive results that can help us add another small piece in understanding of mechanism of action of the strain and in its potential efficacy in the treatment of functional bowel disorders such as IBS.

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CHAPTER 4

Cremon, C., Guglielmetti, S., Gargari, G., Taverniti, V., Castellazzi, A.M., Valsecchi, C., Tagliacarne, C., Fiore, W., Bellini, M., Bertani, L., Gambaccini, D., Cicala, M., Germanà, B., Vecchi, M., Pagano, I., Barbaro, M.R., Bellacosa, L., Stanghellini, V., Barbara, G. 2018. Effect of *Lactobacillus paracasei* CNCM I-1572 on symptoms, gut microbiota, short chain fatty acids, and immune activation in patients with irritable bowel syndrome: A pilot randomized clinical trial. United European Gastroenterol J. 6(4):604-613.

Effect of *Lactobacillus paracasei* CNCM I-1572 on symptoms, gut microbiota, short chain fatty acids, and immune activation in patients with irritable bowel syndrome: A pilot randomized clinical trial

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Abstract

Background: Evidence suggests a role of intestinal microbiota-host interactions in the pathophysiology and symptoms of irritable bowel syndrome (IBS).

Objective: The objective of this article is to assess the effects of *Lactobacillus paracasei* CNCM I-1572 on clinical and gut microbiota-related factors in IBS.

Methods: We conducted a multicenter, randomized, double-blind, cross-over, 18-week, placebo-controlled, pilot trial assessing the effect of *Lactobacillus paracasei* CNCM I-1572 on symptoms, gut microbiota composition, fecal short chain fatty acid (SCFA), immunoglobulin A, and cytokines in IBS. The intestinal microbial ecosystem was characterized by 16S rRNA gene profiling.

Results: Forty IBS patients were enrolled from five Italian centers. *Lactobacillus paracasei* CNCM I-1572 did not significantly improve IBS symptoms, including primary efficacy variables worst abdominal pain/discomfort and IBS degree of relief. Interestingly, *Lactobacillus paracasei* CNCM I-1572 induced a significant reduction in genus *Ruminococcus*, dominated by taxa related to *Ruminococcus bromii* and *Ruminococcus*

callidus, a significant increase in the SCFAs acetate and butyrate, and a significant reduction in the pro-inflammatory cytokine interleukin-15.

Conclusions: This pilot study shows that *Lactobacillus paracasei* CNCM I-1572 is able to modulate gut microbiota structure/function and reduce immune activation in IBS. As no statistically significant effect on IBS-symptoms was found, further studies are necessary to determine the role of this probiotic in IBS. The study was registered at ClinicalTrials.gov registry under identifier NCT02371499.

Keywords Irritable bowel syndrome, dietary compounds, probiotics, microbiota

Key summary

Although probiotics, as a class, have a small but significant therapeutic effect on irritable bowel syndrome (IBS) symptoms, the optimal probiotic strategy in IBS and the mechanism of action by which these compounds exert their beneficial actions in humans are virtually unknown.

Lactobacillus paracasei CNCM I-1572 induces a significant reduction in genus *Ruminococcus*, a significant increase in the fecal short chain fatty acids acetate and butyrate, and a significant reduction in the pro-inflammatory cytokine interleukin-15 in patients with IBS.

We identify plausible biological mechanisms by which this probiotic may exert its effects in patients with IBS.

Introduction

Irritable bowel syndrome (IBS) is characterized by abdominal pain and changes in bowel habits. IBS is one of the most common gastrointestinal disorders, affecting 11.2% of the population in the United States and Europe (Lacy et al. 2016). Recently, advanced microscopic and molecular techniques have revealed alterations in the luminal factors, the epithelial barrier, and the immune, endocrine, and nervous systems in a large proportion of patients with IBS (Barbara et al. 2016).

Several lines of evidence suggest a pathogenetic contribution of the intestinal microbiota in IBS. Prospective studies have shown that 3% to 36% of enteric infections disrupting the intestinal ecosystem lead to de novo onset of so-called post-infection IBS (Barbara et al. 2016; Spiller et al. 2009). A number of studies have reported changes in the composition and stability of the intestinal microbiota in patients with IBS over time (Rajilić-Stojanović et al. 2011; Jalanka-Tuovinen et al. 2014; Simrén et al. 2013). Although these data do not allow us to determine if the abnormal microbiota is the cause or effect of IBS, the improvement of symptoms described in studies using probiotics (Moayyedi et al. 2010; Ford et al. 2014) or non-absorbable antibiotics (Pimentel et al. 2011) implicates intestinal bacteria-host interactions in the pathophysiology and symptoms of this common disorder. However, current data are inconsistent because of the lack of control of diet, concomitant use of antibiotics, different bowel habit subtypes and gut transit.

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al. 2014). Systematic reviews of the literature and meta-analyses indicate that probiotics, as a class, have a small but significant therapeutic effect on IBS symptoms (Moayyedi et al. 2010; Ford et al. 2014). However, the quality

of probiotic trials in IBS and their sample sizes remain suboptimal. The great variety of species, strains, and doses of probiotics tested in clinical trials makes it difficult to provide generalizable advice about the optimal probiotic strategy in IBS (Irvine et al. 2016). For all these reasons, it is questionable if meta-analyses are really applicable to trials of probiotics. Understanding of the mechanism of action by which probiotics exert their beneficial actions in humans is limited because these aspects were evaluated mainly in pre-clinical studies or a small number of clinical trials (Hill et al. 2014; Irvine et al. 2016). In one clinical study (O'Mahony et al. 2005), probiotics were shown to have potent anti-inflammatory properties. In particular, *Bifidobacterium longum* subsp. *infantis* 35624 was capable of normalizing the interleukin (IL) 10/IL12 ratio, indicative, although not validated, of a pro-inflammatory T helper (Th)-1 type immune response, in patients with IBS (O'Mahony et al. 2005). In a recent study of healthy volunteers (Ferrario et al. 2014), the intake of *Lactobacillus paracasei* CNCM I-1572 significantly modulated fecal *Clostridiales* bacteria and butyrate levels, potentially conferring a health benefit to the host. In addition, *Lactobacillus paracasei* CNCM I-1572 was able to modulate colonic microbiota in intestinal chronic inflammation, partly modifying Toll-like receptor expression when rectally administered (Tursi et al. 2013; D'Incà et al. 2011).

In this context, we designed a randomized, double-blind, placebo-controlled, cross-over pilot study to assess the efficacy, safety, and mechanism of action of *Lactobacillus paracasei* CNCM I-1572 in patients with IBS.

Materials and methods

Study design

This was a multicenter, randomized, double-blind, cross-over, placebo-controlled, pilot trial designed to study the effect of *Lactobacillus*

paracasei CNCM I-1572 (L. casei DG®, (LCDG), Enterolactis® plus, Sofar S.p.A., Trezzano Rosa, Milan, Italy, deposited at Institute Pasteur of Paris with number I1572) on the symptoms, fecal microbiota composition, and short chain fatty acid (SCFA), immunoglobulin (Ig) A, and cytokine levels in patients with IBS. The probiotic preparation consisted of a gelatin capsule containing at least 24 billion viable cells of the bacterial strain LCDG. Placebo and probiotic capsules, identical in color, texture, and taste, were delivered in aluminum boxes sealed with a plastic cap containing desiccant salts. Eligible patients entered a two-week run-in phase and were randomly assigned to either LCDG twice daily for four weeks or the equivalent product without bacteria (placebo), followed by a washout period of four weeks before crossing over to the alternate treatment (twice daily for four weeks). After 14 weeks, patients entered a four-week follow-up phase (Figure 1). Study visits occurred every four weeks during the treatment period and follow-up. The randomization schedule was determined by a computer-generated random code system. Intervention sequence assignments were not revealed until the study was completed. Patients, study investigators, and sponsor staff were blinded to the randomization codes. All participants underwent a formal clinical assessment and were further phenotyped using validated questionnaires as described below. In all cases, fecal samples were obtained at the start and end of the first (visits 2 and 3) and the second (visits 4 and 5) treatment period, and at the end of the follow-up.

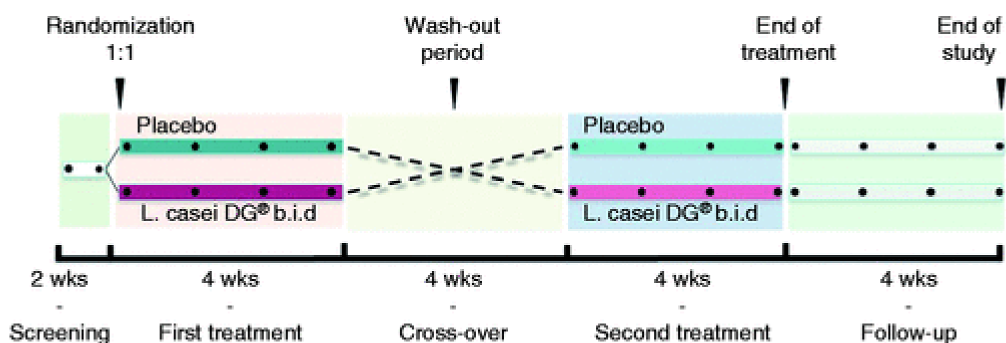

Figure 1. *Study design. After a two-week run-in phase, patients were randomly (1:1) assigned to either* Lactobacillus paracasei *CNCM I-1572 twice daily for four weeks or placebo. This was followed by a washout period of four weeks before crossing over to the alternate treatment (twice daily for four weeks). After 14 weeks, patients entered a four-week follow-up phase. The total duration of the study was 18 weeks. Fecal samples were obtained at visits 2 and 3 (first period), visits 4 and 5 (second period), and at the end of follow-up.*

The protocol was designed by the coordinating center. Data were collected by investigators and monitored by the sponsor with the supervision of OPIS, a contract research organization. OPIS personnel, in collaboration with the coordinating center, analyzed the trial data. A statistical analysis plan (SAP) was released and approved by the sponsor prior to the database lock and unblinding of the treatment sequence. The protocol was approved by an independent ethics committee at each center (in particular, it was approved by the Ethics Committee of St. Orsola-Malpighi Hospital of Bologna on October 7, 2014, approval identification no: 145/2014/O/Sper) and carried out according to the Declaration of Helsinki and the principles of good clinical practice. All patients provided written informed consent. All authors have access to the study data and reviewed and approved the final manuscript. The trial was registered in a public registry (ClinicalTrial.gov No. NCT02371499).

Patients

Eligible patients with symptoms meeting Rome III criteria for IBS (Longstreth et al., 2006), irrespective of bowel habit, were recruited from five Italian centers.

Study assessment

Data collection was carried out using an electronic clinical case report form (eCRF). Patients recorded all symptoms daily in a paper patient diary. The patients' lifestyle and eating habits were controlled during the study and were the same throughout all the study periods. Compliance with the suggested lifestyle and eating habits was checked weekly and noted in the patient diary. Use of concomitant medication and adverse events were recorded at each visit.

Primary efficacy variables were: (1) worst abdominal pain/discomfort in the last 24 hours (responders were defined as patients with ≥ 30% reduction in the weekly mean worst abdominal pain and/or discomfort score, versus mean value of the run-in period, in at least two of the four weeks of the treatment period) using a daily 11-point numeric rating scale (NRS); (2) IBS degree of relief in the past seven days compared to before the trial started (responders were defined as patients reporting being "completely relieved" or "considerably relieved" in at least two of the four weeks of the treatment period) using a weekly seven-point balanced ordinal scale; (3) daily stool frequency and consistency as assessed by the Bristol Stool Scale Form (BSSF); (4) gut microbiota composition, fecal SCFAs, immunoglobulin A (IgA), and cytokines assessed every four weeks during the treatment periods and at the end of follow-up.

Secondary efficacy variables included: (1) overall satisfaction with treatment at the end of both the treatment periods as assessed by a 10-point visual analog scale (VAS); (2) Hospital Anxiety and Depression Scale (HADS) (Zigmond & Snaith, 1983); (3) quality of life assessment using the validated Short-Form 12 Items Health Survey (SF-12) (Ware et al. 1996) and consumption of rescue medications.

Analysis of the bacterial composition of fecal samples

The bacterial community structure of the fecal microbiota was analyzed as described elsewhere (Ferrario et al. 2014; Gargari et al. 2016; Duranti et al. 2016).

Quantification of fecal SCFAs

SCFAs were quantified in the fecal samples as previously described.

Fecal IgA and cytokine analysis

Fecal IgA and cytokines (including interleukin (IL)6, IL8, IL10, IL12, IL15, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β) were detected by an enzyme-linked immunosorbent assay (ELISA) test as previously described (Avazini et al. 1992).

Statistical analysis

This was a pilot study; thus, no sample size was calculated. Forty patients were included in the study based on feasibility criteria and previously published studies (Halmos et al. 2014). Nevertheless, when the sample size in each sequence group is 20 (a total sample size of 40) a 2 × 2 cross-over design has 80% power to detect a difference between treatments, assuming a medium effect size, using a two group t test (cross-over analysis of variance (ANOVA)) with a 0.05 two-sided significance level (Cohen. 1988).

Continuous data were summarized by mean, standard deviation (SD), median, first and third quartile, minimum, and maximum. Categorical data were presented by absolute and relative frequencies or contingency tables. Patients were included in each analysis based on available assessments. The

prevalence approach was applied unless otherwise indicated; therefore, missing data were not replaced.

The full analysis set (FAS) included all randomized patients. The safety set included all randomized patients who received at least one dose of the study treatment and had at least the post-baseline safety assessment. The intent-to-treat (ITT) set included all randomized patients who received at least one dose of the study treatment and had at least one efficacy assessment in each cross-over period. The per protocol (PP) set included all randomized patients who completed the study without any significant protocol violation. Primary efficacy analyses were performed on the ITT set and PP set provided supportive data.

For the binary efficacy variables, Prescott's test for a direct treatment effect was applied after verifying the absence of a treatment-by-period interaction using the test proposed by Armitage and Hills (Armitage & Hills, 1992). When a treatment-by-period interaction was evident, the analysis was based on the data from the first period only, using chi-square or Fisher's exact test to determine the treatment effect. In addition, for primary variables, a generalized estimating equations model for repeated measures (i.e. subject within sequence) was applied considering sequence, period, and treatment as fixed effects. For the continuous efficacy variables, a mixed-effects model with repeated measures was applied after verifying the absence of a carryover effect.

All statistical tables, figures, listings, and analyses were produced using SAS for Windows release 9.4 (64-bit) (SAS Institute Inc, Cary, NC, USA). Unless otherwise specified, each statistical test used a two-tailed α-level of 0.05.

Results

Study patients

Study enrollment and randomization are shown in Figure 2. The study was conducted from January to November 2015. Forty-two patients (95.5%) were randomized (22 assigned to the LCDG-placebo sequence and 20 assigned to the placebo-LCDG sequence) and included in the FAS (all performed at visits 1 and 2). A total of 40 patients (90.9%) were seen at visits 3 and 4 and included in both the ITT set and safety set, whereas 39 patients remained for visit 5 and the follow-up phase. The primary reasons for study withdrawal were withdrawn consent, non-compliance, and adverse events. Almost all patients had a normal compliance (between 80% and 120%). The demographic and baseline characteristics of the subjects are reported in Table 1.

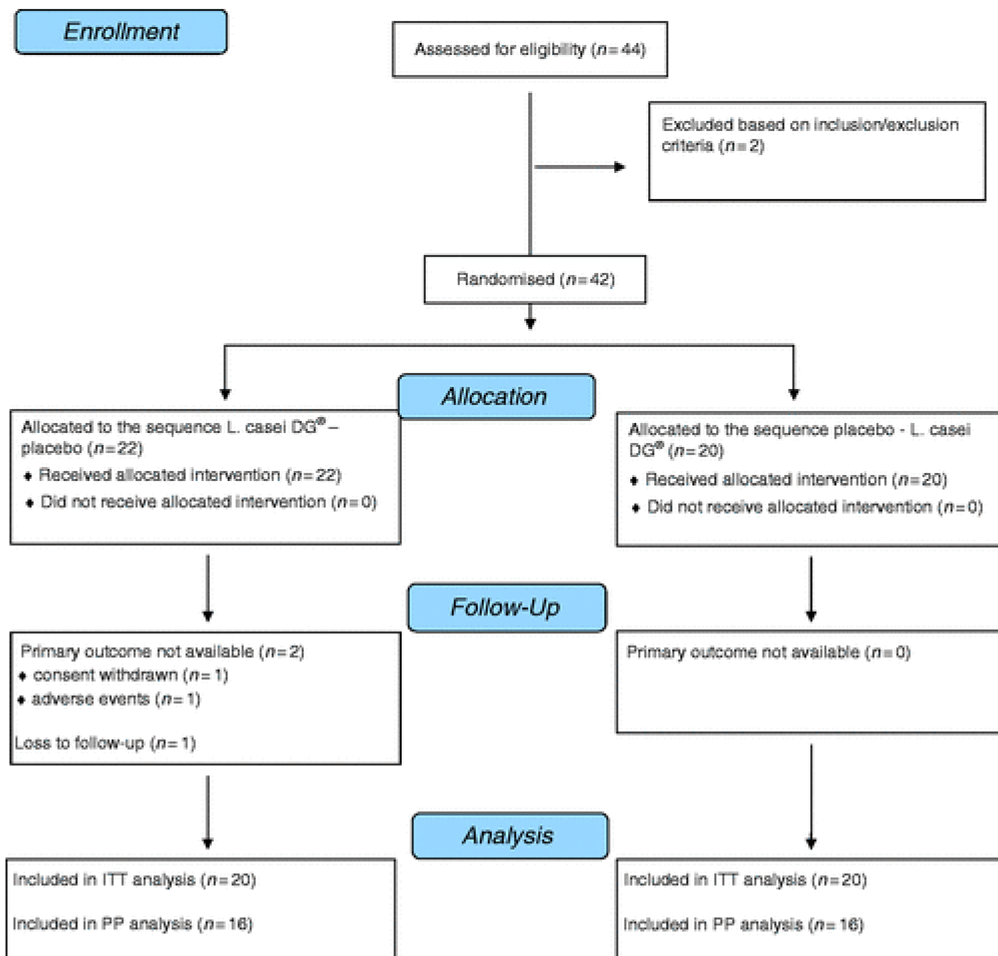

Figure 2. *DG® Flowchart of enrollment CNCM I-1572 and randomization of the study.* L. casei*:* Lactobacillus paracasei*; ITT: intent-to-treat; PP: per protocol.*

Characteristics	Placebo/*Lactobacillus paracasei* CNCM I-1572 (*n* = 20)	*Lactobacillus paracasei* CNCM I-1572/placebo (*n* = 20)
Age, years	44.55 ± 12.98	37.35 ± 11.25
Female gender	15 (75%)	11 (55%)
Ethnic origin		
Caucasian	20 (100%)	20 (100%)
Other	0 (%)	0 (0%)
IBS subtype (4)		
IBS-D	6 (30%)	8 (40%)
IBS-C	7 (35%)	5 (25%)
IBS-M	1 (5%)	2 (10%)
IBS-U	6 (30%)	5 (25%)
Abdominal pain score[a]	2.70 ± 1.24	3.28 ± 1.95

Table 1. *Baseline characteristics of study participants*.

Effect of treatment on digestive symptoms

Abdominal pain/discomfort

Considering both treatment periods together, the proportion of responders was higher in patients who took LCDG (15/40, 37.5%) than placebo (12/40, 30%), but these differences were not significant in the model ($p = 0.336$). Analyzing the overall results by treatment in the PP set, the proportion of responders (overall) was the same in both groups of patients (11/32, 34.4%).

IBS degree of relief

Considering both treatment periods together, the proportion of responders was higher in patients who took LCDG (9/40, 22.5%) than placebo (6/39, 15.4%), but these differences were not significant in the model ($p = 0.195$). Similar results were obtained for the PP set.

Daily stool frequency and form

Stool frequency was collected daily and stool consistency was assessed using the BSSF. For both the features, no significant differences

were found in either the ITT set or PP set. Although better results (i.e. bowel function normalization) were obtained in patients with IBS with diarrhea (IBS-D) and mixed IBS (IBS-M) treated with LCDG, there was no significant difference.

For all the investigated digestive symptoms, no carryover effect resulted statistically significant, indicating that values at the beginning of the second period are statistically equal to baseline values.

Effect of treatment on the gut microbiota

The within-sample biodiversity was analyzed in terms of bacterial richness and evenness (α-diversity) using the Chao1, Shannon, and InvSimpson indexes, while the inter-sample relationships (β-diversity) was measured by principal coordinate analysis (PCoA) based on weighted and unweighted UniFrac distances. The differences between LCDG and placebo in modulating α and β diversity were not significant. Next, we assessed the effect of treatment on the modulation of specific bacterial taxa. We showed a significant increase in genus *Lactobacillus* (a plausible effect of the ingested probiotic cells) and *Oscillospira*, and reduction in genus *Ruminococcus* (Table 2(a)). In addition, only LCDG induced a significant change in the level of bacterial taxa; specifically, we observed an expansion of genera *Parabacteroides*, *Lactobacillus*, and an unidentified member of the family Barnesiellaceae (Table 2(b)).

(a)

	p value	Median relative abundance (%)			
		L. paracasei CNCM I-1572		Placebo	
		Baseline	Post-treatment	Baseline	Post-treatment
Family					
p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Lactobacillaceae	0.022	0.01	0.34	0.01	0.02
Genus					
p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Ruminococcus	0.042	4.44	3.94	5.25	5.62
p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Oscillospira	0.042	0.37	0.42	0.38	0.41
p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Lactobacillaceae.g_Lactobacillus	0.011	0.01	0.34	0.01	0.02

(b)

	p value	Median relative abundance (%)	
Lactobacillus paracasei CNCM I-1572 treatment		Baseline	Post-treatment
Order			
p_Firmicutes.c_Bacilli.o_Lactobacillales	0.025	0.56	1.66
Family			
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Porphyromonadaceae	<0.001	0.17	0.36
p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Lactobacillaceae	<0.001	0.01	0.34
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Barnesiellaceae	0.022	0.05	0.11
Genus			
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Porphyromonadaceae.g_Parabacteroides	0.013	0.17	0.36
p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Lactobacillaceae.g_Lactobacillus	<0.001	0.01	0.34
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Barnesiellaceae.g_unidentified	0.049	0.05	0.11
Placebo treatment			
none_			

Table 2. *Bacterial taxa that were significantly modified by probiotic (*Lactobacillus paracasei *CNCM I-1572) or placebo treatments. Median relative abundance before (baseline) and after treatment is shown*.

Because of the reported association between IBS and members of the genus *Ruminococcus* (Taverniti & Guglielmetti. 2014; Rajilić-Stojanović et al.. 2015), we further investigated the data concerning this taxon. Using Basic Local Alignment Search Tool (BLASTn) and ClustalW global alignment algorithms, we assigned three of the most represented Ruminococcus-associated de novo sequences to the species *R. bromii* (67.7% of the *Ruminococcus* reads), *R. bicirculans* (7.7%), and *R. callidus* (4.3%).

Effect of treatment on SCFAs

We demonstrated that SCFAs acetate and butyrate increased significantly with LCDG treatment, but no significant differences were found after placebo (Table 3). The median levels of acetate and, particularly, butyrate before the placebo were higher than before the probiotic treatment. Although this difference was not statistically significant, a carryover effect of the probiotic on SCFA levels (i.e. an insufficient washout period) cannot be excluded.

L. paracasei CNCM I-1572 treatment	*p* value	Median relative abundance (mmol/kg)	
		Before	After
Acetate	**0.021**	**36.63** (±22.62)	**47.83** (±26.14)
Propionate	0.289	15.18 (±10.35)	16.37 (±11.97)
Butyrate	**0.047**	**5.99** (±8.30)	**10.52** (±8.51)
Isobutyrate	0.133	1.11 (±0.98)	1.55 (±1.13)
Isovalerate	0.428	1.14 (±0.81)	1.04 (±1.03)
Valerate	0.080	1.82 (±1.43)	2.45 (±1.34)
Placebo treatment			
Acetate	0.388	43.06 (±26.65)	33.08 (±26.70)
Propionate	0.622	16.73 (±10.51)	17.13 (±8.89)
Butyrate	0.746	10.73 (±7.68)	8.47 (±9.06)
Isobutyrate	0.387	1.22 (±1.22)	1.64 (±1.13)
Isovalerate	0.36	0.95 (±1.12)	1.28 (±1.22)
Valerate	0.572	2.14 (±1.92)	1.9 (±2.00)

Table 3. *Fecal levels of short chain fatty acids (SCFAs) throughout treatment. Median values (±standard deviation) from before (baseline) and after treatment are given.*

Significant differences appear in bold and were determined by the Wilcoxon-Mann-Whitney test.

Effect of treatment on fecal IgA and cytokines

The mean fecal IgA level, expressed as ng/g, decreased during LCDG treatment (mean change −5.4), and increased during treatment with placebo (mean change 14.1), with a borderline difference ($p = 0.068$) (Table S1). The mean IL6 level, expressed as pg/g, decreased during LCDG treatment (mean change −0.2), and increased during treatment with placebo (mean change 0.7), with a borderline difference ($p = 0.056$). The mean IL15 level, expressed as pg/g, decreased during LCDG treatment (mean change −173.4), and increased during treatment with placebo (mean change 35.4), with a significant difference ($p = 0.042$). For the other fecal cytokines, no significant differences were found.

Correlations between microbiomic, clinical, and immunological features

The correlations between biological and clinical features are reported in Table 4.

Genus	Family	Stool frequency	Stool form (BSSF)	Abdominal pain	IgA	IFNγ	TGFβ	TNFα	Acetate	Butyrate	Propionate	Lactate	Isobutyrate	Isovalerate	Valerate
Lactobacillus	Lactobacillaceae											++	+		
Parabacteroides	Porphyromonadaceae				+										
Oscillospira	Ruminococcaceae	++	++				+		++	++	++				
Ruminococcus	Ruminococcaceae					+	++		++	+	++				

Color scale: +0.5 to -0.5

Table 4. *Correlation analyses performed using the relative abundances of the bacterial taxa modified by the* Lactobacillus paracasei *CNCM I-1572 treatment (predictors) and clinical parameters, immunological factors, and fecal SCFA levels (dependent variables).*

Safety

Treatment-emergent adverse events during the study are reported in Table 5. Although no significant difference was found between the patients with at least one treatment-emergent adverse event in the two treatment groups ($p = 0.742$), one participant allocated to the sequence LCDG-placebo dropped out because of worsening of abdominal pain. No patient experienced a serious, severe, or related adverse event during the treatment period. All reported adverse events were unrelated to the experimental products.

Event	Placebo ($n = 39$)	*L. paracasei* CNCM I-1572 ($n = 40$)
Adverse events		
Headache	7 (17.9%)	10 (25.0%)
Upper respiratory tract infection	5 (12.8%)	4 (10.0%)
Diarrhea	3 (7.7%)	3 (7.5%)
Abdominal pain	2 (5.1%)	3 (7.5%)
Asthenia	1 (2.6%)	3 (7.5%)
Nausea	2 (5.1%)	1 (2.5%)
Dyspepsia	2 (5.1%)	0 (0%)
Serious adverse events		
	0 (0%)	0 (0%)

Table 5. *Treatment-emergent adverse events during the study.*

Discussion

LCDG significantly reduces the genus *Ruminococcus*, induces a significant increase in the fecal levels of SCFA butyrate, and significantly reduces the pro-inflammatory cytokine IL15. LCDG improves IBS symptoms, though the

differences over placebo did not reach a statistical significance. Despite this, we identify plausible biological mechanisms by which this probiotic may exert its effects in patients with IBS.

Given the growing evidence of the role of dysbiosis in the pathophysiology of IBS (Barbara et al. 2016; Spiller et al. 2009), probiotics have been evaluated as a potential therapeutic option in these patients. Probiotics may reduce abdominal symptoms and benefit patients with IBS (Moayyedi et al. 2010; Ford et al. 2014). A recent meta-analysis of 43 clinical trials of different products showed that probiotics improve global IBS symptoms, pain, bloating, and flatulence (Ford et al. 2014). Although probiotics may act through multiple mechanisms, whether they modify abdominal symptoms through direct modulation of the microbiota or indirect action via the gut immune system, or other ways, is unclear (Hill et al. 2014; Irvine et al. 2016). In our study, LCDG was not statistically superior to placebo in any of the clinical efficacy variables evaluated. However, this was a pilot study not full powered for clinical endpoints aimed at investigating underlying mechanisms of action by which this probiotic induces its effect.

We showed that LCDG significantly reduces *Ruminococcus*. Members of the intestinal microbiota ascribed to the genus *Ruminococcus* have been found to be increased in IBS patients (Jalanka-Tuovinen et al. 2014; Taverniti & Guglielmetti. 2014; Rajilić-Stojanović et al.. 2015; Rigsbee et al.. 2012). Therefore, the observed ability of LCDG to reduce the relative abundance of this taxon can be considered beneficial in IBS. In particular, we ascribed most of the *Ruminococcus*-associated reads (~72%) to the species *R. bromii* and *R. callidus*, which were recently proposed as potential microbial biomarkers for diagnosing IBS (patent WO/2011/043654). Correlation analyses supported the proposed dominant involvement of bacteria from the genus *Ruminococcus*

in IBS. We found that *Ruminococcus* negatively correlates with fecal levels of the main SCFAs in the human gut (i.e. acetate, butyrate, and propionate), which play important roles in maintaining intestinal homeostasis (Correa-Oliveira et al. 2016; Rios-Covián et al. 2016). Accordingly, an ecological link could exist between the significant reduction in *Ruminococcus*, which is a dominant genus of the microbiota (overall median relative abundance ~5%), and the increase in butyrate and acetate observed over the course of the LCDG intervention. The data on intestinal microbial ecology presented in this study agree with the results of a previous intervention study that demonstrated the ability of LCDG to modulate SCFAs and Clostridiales bacteria in healthy adults (Ferrario et al., 2013). In addition, the inverse correlation between the *Clostridiales* genus *Oscillospira*, which was modulated by LCDG but not placebo, and stool frequency and form suggests that the active treatment may regulate gut physiology.

We assessed the fecal levels of IL6, IL8, IL12, TNF-α, and IFN-γ, which are typical Th-1 pro-inflammatory cytokines, and TGF-β and IL10, regulatory cytokines capable of suppressing inflammatory responses (Abraham & Cho. 2009). In addition to its well-known pro-inflammatory role, IL6 also possesses anti-inflammatory properties exerted through its ability to stimulate IgA secretion (Fagarasan & Honjo. 2003; Goodrich & McDee 1999). This evidence may explain why, in our study, the significant decrease in IL6 levels is also accompanied by a decrease in fecal IgA levels after treatment with LCDG, but not placebo (Fagarasan & Honjo. 2003; Goodrich & McDee. 1999). IL15 is produced by intestinal epithelial cells and able to stimulate intraepithelial lymphocytes and their interactions with enterocytes. IL15 plays a primary role in the development of several inflammatory diseases, including celiac disease and IBD, affecting the integrity of the mucosal barrier (van Veel. 2006). The significant decrease in IL15 levels observed in our study after treatment with LCDG, but not placebo, suggests that this product may play an important role

in the restoration of intestinal regulation and mucosal integrity (van Veel. 2006; Pagliari et al.. 2013). The role of IL15 in IBS should be clarified in ad hoc studies.

The strength of this study is that we used the same rigorous criteria, design, and endpoints as classical pharmacological efficacy studies. In addition, as suggested by recent guidelines (Irvine et al. 2016), we previously demonstrated that the test organism was present in the stools of exposed individuals (Ferrario et al. 2014); here, we clarified the mechanisms by which it may benefit patients with IBS. However, we acknowledge the limitations of the present study. Clearly, we recognize the downsides of the cross-over design, particularly in studies of patients with functional bowel disorders; however, we opted for this design because it seemed most applicable in pathophysiological studies in which endpoints are measured objectively. Furthermore, because of the pilot and mechanistic nature of the study, the sample size was limited and clearly not powered for clinical endpoints. We did not show any significant differences between the active treatment and placebo, though better results were obtained with LCDG. Whether this absence of significant differences reflects a true treatment ineffectiveness or a type 2 error should be clarified in ad hoc studies. Finally, for all these reasons, the generalizability of our results requires caution and further confirmation.

In conclusion, although causality is not proven and only an association can be reported, we showed that LCDG improves IBS symptoms, though not in a statistically significant manner, through modulation of the gut microbiota, its metabolic pathways, and pro-inflammatory cytokines. As in this study no statistically significant effect on IBS symptoms was found, further studies are necessary to determine the role of LCGD in the management of IBS.

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Author contributions: Giovanni Barbara, Vincenzo Stanghellini and Cesare Cremon planned the study, designed the protocol, contributed to the writing of the manuscript, and were involved in the screening and periodic visits of the patients. Simone Guglielmetti contributed to the writing of the text concerning the analysis of the intestinal microbial ecosystem (IME). Simone Guglielmetti, Giorgio Gargari, and Valentina Taverniti carried out the IME analyses, bioinformatic and statistical analysis of IME data, and the preparation of fecal waters. Anna Maria Castellazzi, Chiara Valsecchi, and Carlotta Tagliacarne contributed to the experimental design, to the writing of the manuscript, and carried out the analyses on IgA and cytokines. Walter Fiore contributed to the writing of the protocol and manuscript. Massimo Bellini, Lorenzo Bertani, Dario Gambaccini, Michele Cicala, Bastianello Germanà, Maurizio Vecchi, Isabella Pagano, Maria Raffaella Barbaro, and Lara Bellacosa contributed to the experimental design, and were involved in the screening and periodic visits of the patients.

Declaration of conflicting interests

None declared.

Ethics approval

The protocol was approved by an independent ethics committee at each center (in particular, it was approved by the Ethics Committee of St. Orsola-Malpighi Hospital of Bologna on October 7, 2014, approval identification no: 145/2014/O/Sper) and carried out according to the Declaration of Helsinki and the principles of good clinical practice.

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Informed consent

All patients provided written informed consent.

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CHAPTER 5

Fiore W, Arioli S, Guglielmetti S. 2020. The neglected microbial components of commercial probiotic formulations.

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The neglected microbial components of commercial probiotic formulations

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Abstract

BACKGROUND: Probiotics are live microorganisms, and viability is conventionally considered essential to their health benefits. To date the label of these products indicates only the minimum numbers of viable microorganisms at end of shelf-life expressed as colony-forming units (CFUs). Label specifications, however, describe only a fraction of the actual microbiological content of a probiotic formulation.

SCOPE AND APPROACH: This paper describes the microbiological components of a probiotic product that are not mentioned on the label, such as the actual number of CFUs, the presence of viable cells that cannot generate colonies on agar plates, and the abundance of dead cells. To this aim we analyzed a few reference commercial probiotic products by flow cytometry (FC).

KEY FINDINGS AND CONCLUSIONS: Industry adopts cell overdosing of cells in probiotic formulations, which results in a fraction of CFUs that is not declared on the label. FC analyses confirmed that viable unculturable and dead microbial cells are virtually always variably present in probiotic formulations, but not specified in the label. All these hidden microbial fractions in probiotic products can promote biological responses in the host and, therefore, they should not be ignored because they may influence the efficacy and can be relevant for immunocompromised or fragile consumers. The use of flow cytometry can provide a relevant contribution for a more comprehensive microbiological quantification of probiotic formulations. In conclusion, we propose the minimum requirements for the microbiological characterization of a probiotic product to adopt for the label specifications and clinical studies.

1. Introduction

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al. 2014). Therefore, by definition, the term probiotic is restricted to live microbial cells. The number of live microbes in a probiotic formulation is generally recognized as relevant for the effectiveness of the product. Accordingly, review of literature led national public authorities in Canada and Italy to suggest in their guidelines that the minimum number of live probiotic microorganisms to impart general health benefits should be at least 10^9 colony-forming units (CFUs) per day (Italian Ministry of Health) or per serving (Health Canada) (Hill et al. 2014). In addition, reportedly, for specific health conditions, benefits can only be observed above a certain concentration of live cells introduced with the probiotic; for instance, *Lactobacillus rhamnosus* GG was shown to be most likely effective in treating infectious diarrhea in children when administered at a dose of at least 10^{10} CFUs per day (Caffarelli et al. 2015; Szajewska et al. 2013). In another study, authors observed that probiotics may significantly reduce systolic and diastolic blood pressure only when daily doses $\geq 10^{11}$ CFUs were used (Khalesi et al. 2014).

Since the amount of live microbial cells administered is profoundly relevant for the health benefit that probiotics can provide to host, the regulations of several countries specify that the minimum numbers of viable microorganisms at the end of shelf-life must be indicated on the label of the probiotic product. Nonetheless, label specifications describe only a fraction of the actual microbiological content of a probiotic formulation. Within this framework, in this commentary paper, we will describe the microbiological components constituting a probiotic product that are not mentioned on the label, discussing their relevance in the context of clinical research aiming at elucidating the health-promoting properties of probiotics. In addition, we will make methodological considerations in support of the use of flow cytometry for the characterization of microbial viability in a probiotic formulation,

presenting the results of the analysis of a few reference commercial probiotic products.

2. Overdosing of viable microbial cells in probiotic products

Producers constantly evaluate the viable microbial content of commercial probiotic products in order to ensure compliance with the information reported on the label. Specifically, the amount of microbial CFUs per single dose indicated on the label refers to the minimum concentration of live cells that must be present at the end of the shelf-life. During the shelf-life of a probiotic product, which is typically longer than 12 months, inevitably part of the microbial cells die. To manage this issue, the primary solution adopted by producers consists in overdosing the live microbial cells in the probiotic product. In fact, commonly, more CFUs of probiotic microorganisms are added to the product in order to guarantee that the viable concentration does not decrease below the limit declared on the label before the end of the shelf-life. Nonetheless, overdosing leads to a significant increase in production costs. For this reason, probiotic producers try to contain it as much as possible by adopting and optimizing strategies aimed at preserving microbial survival, which concern the cultivation media used for the production of the biomass, the freeze-drying protocols, the use of protective agents during freeze-drying, microencapsulation, and the packaging systems (Arioli et al. 2018; da Cruz et al. 2007; Goderska. 2012; Mai et al. 2017; Savini et al. 2010). In brief, the overdosing of viable microbial cells in probiotic formulations is practically always adopted, resulting in a fraction of CFUs that is not declared on the label but can surely contribute to the health effects of the product. Therefore, overdosing can significantly alter the actual load of a dose and, plausibly, the outcomes of an intervention trial.

3. Viable cells in nongrowing states (the “hidden” viable biomass)

By a convention that dates to the time of Koch, a microbial cell is considered “viable” if it reproduces to form a colony on an agar plate that supplies key nutrients for its replication. More recent advances, however, revealed that microorganisms may exist in a variety of metabolic states (García-Cayuela et al. 2009; Volkert et al. 2008), most of which do not involve active replication (Davis. 2014). In fact, besides “culturable” microbial cells, which may multiplicate and form a colony on agar plate, other non-colony forming physiological states have been described such as the “**non-replicating**” state, characterized by an active physiology and intact cytoplasmic membrane, the “**starving**” state, characterized by a dramatic decrease in metabolism, the “**dormant**” state, possessing low metabolic activity and inability to divide without a preceding resuscitation phase (also defined “**viable but not culturable**”; VBNC), and the “**irreparably damaged**” state, characterized by progressively declining metabolism that irreversibly leads to death (Davis. 2014). The relevance of non-culturable states in the interaction with host health has been demonstrated for several pathogens, which were observed to retain their pathogenicity after entering the VBNC or dormancy states (Gengenbacher & Kaufmann. 2012; Li et al. 2014; Wesche et al. 2009). Although more rarely investigated, it has also been shown in probiotics that microbial cells failing to grow on agar media can have several typical properties of viable cells, such as enzymatic activities (e.g. esterases and reductases) and an intact cytoplasmic membrane maintaining the electrochemical gradient (Lahtinen et al. 2008; Lahtinen et al. 2005; Lahtinen et al. 2006). Notably, viable unculturable microbial cells can still maintain some metabolic activity that could contribute to the promotion of health benefits (de Almada et al. 2016). In addition, plausibly, as also observed for intestinal pathogenic bacteria (Senoh et al. 2012), probiotic microbial cells in nongrowing states may resuscitate (i.e., reacquire the ability to reproduce)

once in the favorable environmental conditions of the gut, therefore starting to interact with host and resident microbiota similarly to cultivable viable bacteria.

4. Non-viable microbial cells in probiotic formulations

Death of a bacterial cell is generally defined as “the point where the extent of injury is beyond the ability of a cell to resume growth” (Bogosian & Bourneuf. 2001). From a structural and functional point of view, non-viable (dead) microbial cells are characterized by inability to reproduce, irremediably damaged plasma membrane, dissipated proton gradient, and absence of any metabolic activity (Davey. 2011).

Dead cells are virtually always present in a probiotic formulation. Part of them are generated by stresses of industrial manufacturing, including biomass production and concentration, cryopreservation, and lyophilization. In addition, a progressive increase of dead cells occurs during shelf-life, according to a cell death kinetics that depends on taxon/strain-associated properties, product formulation and packaging (Fig. 1).

By definition, viability is an essential prerequisite to qualify a microorganism as probiotic and it is conventionally considered essential to exert health benefits. In this regard, the first report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food in 2001 stated that “the ability to remain viable at the target site should be verified for each potential strain” (FAO/WHO, 2001). Nonetheless, although viable probiotic cells may be more effective than the same non-viable microorganisms (Lahtinen. 2012), increasing literature is demonstrating that also inactivated (dead) probiotic cells may exert beneficial effects on human health (Nakata et al. 2019; Nishida et al. 2017; Pique et al. 2019; Taverniti & Guglielmetti. 2011). Accordingly, several products intentionally constituted by non-viable microbial cells are increasingly present on the market (Adams. 2010; Ananta & Knorr. 2009; Barros et al. 2020; de Almada et al. 2016; Pique

et al. 2019; Shigwedha et al. 2014). For such products the term "**paraprobiotics**" was proposed with the specific definition of "*non-viable microbial cells (intact or broken) or crude cell extracts (i.e. with complex chemical composition), which, when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal consumer*" (Taverniti & Guglielmetti. 2011). The non-vital microbial material that fall within the definition of paraprobiotic is able to interact with host health primarily through mechanisms of immunomodulation (Taverniti & Guglielmetti. 2011), mediated at intestinal level (principally in the ileum (Derrien & van Hylckama Vlieg, 2015)) by the microbe-associated molecular patters (MAMPs) that constitute the microbial cell, such as outer surface molecules [(lipo)teichoic acids (Smelt et al. 2013), S-layer proteins (Taverniti et al. 2013), polysaccharidic capsules (Balzaretti et al. 2017), outer surface proteins (Guglielmetti et al. 2008; Guglielmetti et al. 2014)] and other cellular components (e.g. genomic DNA and unmethylated cytosine-phosphate-guanine-containing oligodeoxynucleotides (Qi et al. 2020)).

5. Extracellular microbial products in probiotic formulations

Usually, in industrial manufacturing, immediately after fermentation, the broth culture is concentrated (often from 1:5 to 1:10) by continuous centrifugation before freeze drying. Therefore, the lyophilized microbial biomass used to prepare a probiotic product contains about 10% of residual growth medium, which includes microbial metabolites produced and secreted/released by probiotic cells during growth. Such microbial products may include primary metabolites (e.g., lactate, acetate, propionate), bacteriocins, reuterin, or other secreted molecules (e.g., immunomodulatory secreted peptides; (Bauerl et al. 2019)), which can be defined as "**postbiotics**" (Tsilingiri & Rescigno. 2013). The actual contribution of these molecules to the interaction of probiotic formulations on host health is not

known but, considering data originating by in vitro experiments, cannot be excluded (Aguilar-Toala et al. 2019; Gao et al. 2019; Tsilingiri et al. 2012).

6. Methodologies for the microbiological characterization of probiotic products

The analysis of a probiotic formulation is conventionally carried out by counting CFUs through serial dilution method to measure the viable cell number. This method only allows the identification of microbial units (single cells or aggregates) that may create visible colonies on an agar medium, while being unable to provide any information on cells that are present in unculturable physiological states. In order to obtain a more comprehensive microbiological characterization of probiotic products flow cytometry (FC) has been proposed (Chiron et al. 2018). In fact, FC can overpass some disadvantages of the cell viability measurement based on plating, such as the impossibility to provide results in real time, the lack of information regarding cell integrity and metabolic activity, and, most importantly, the inability to quantify both dead cells and viable microbial cells that cannot produce colonies on agar plates. Specifically, FC associated to the use of fluorescent dyes can simultaneously generate data concerning viability, structural integrity, and physiological state in individual cells (Jackson et al. 2019). In addition, FC can analyze up to thousands of cells/events per second. For these reasons, FC is becoming increasingly popular as a rapid alternative method for microbial detection, enumeration, and population profiling of microorganisms (Van Nevel et al. 2017), including probiotics (Lahtinen et al. 2006). Accordingly, in 2015, the International Organization for Standardization and the International Dairy Federation (ISO and IDF) published a standard method for the quantification of active and/or total lactic acid bacteria and probiotics in dairy products and fermented milk products by FC (ISO19344:2015/IDF232:2015). Here, we adopted the ISO19344:2015/IDF232:2015 standard (protocol B) to analyze 14 commercial

probiotic products purchased at a local pharmacy, in order to assess their bacterial count. Specifically, the utilized protocol consisted of a dual staining targeting nucleic acids with the non-permeant red-fluorescent dye propidium iodide (PI) and the cell-permeant green fluorescent dye, SYTO™ 24 (S24). S24 permeates and green-labels all bacterial cells, whereas PI can permeate and stain only bacterial cells with damaged plasma membrane, causing the reduction of S24 fluorescence and subsequent substitution of the green with a red fluorescence emission. Subsequently, stained samples were analyzed with a BD Accuri™ C6 Plus flow cytometer combining light scattering and the detected emitted fluorescence. Such analysis permits the detection of three sub-populations of cell events (Mora et al. 2019): Active Fluorescent unit (AFU, or viable cells with green fluorescence); non-Active Fluorescent Unit (nonAFU, or dead cells with only red fluorescence); and the so-called "damaged cells" possessing contemporaneously the green and the red staining (considered as a transient step between active and membrane-compromised or irreversibly membrane-damaged states (Díaz et al. 2010)).

Tested products included formulations made of a single strain or blends of strains belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Bacillus* and *Enterococcus* (Fig. 2). In addition, different packaging types have been included: cellulose capsules in aluminium (Alu) vial tube with desiccant, capsules in Alu/Alu blister or polyvinyl chloride (PVC)/Alu blister, lyophilized powder in Alu pouch located within the cap of 10-ml pastic bottles, orosoluble lyophilized powder in Alu foil stick and lyophilized powder in flexible multi-layered (plastic and aluminium) sachet. Furthermore, probiotic products were differently distant from the expiration date indicated on the label (from 5 to 17 months). The obtained results indicate that, independently from formulation, packaging and distance from the end of the shelf-life, all products contained dead cells (from 4.6 to 36.8 % of all cells; mean ± standard deviation: 12.3 ± 8.9 %) and possessed more viable cells than what declared on the label [on average, approximately 5-fold overdosing,

calculated comparing CFUs specified on the label vs CF determined active fluorescent units (AFUs)]. The only exception was found for product I, for which the number of CFUs on the label coincided with the measured AFUs. In addition, product I was characterized by the highest abundance of dead cells (about 37 %) and by the second highest concentration of damaged cells (about 43 %). In general, damaged cells were quantified in the range 9.3-44.6 % (mean 22.1 %).

We also analyzed five products formulated with bacterial biomasses constituted by a single *Lactobacillus* strain and industrially produced by the same manufacturer (products H1-5). The results obtained from the FC analyses of these products showed a similar ratio between viable, damaged and dead cells, independently from dose and packaging. Nonetheless, we found packaging-dependent differences concerning the deduced overdosing. In fact, while the two formulations in sachet and stick showed approximately 2:1 ratio between AFUs and CFUs on the label, the two formulations in capsule had approximately a 3-fold overdosing and the formulation in bottle had more than 10-fold overdosing (Fig. 2).

In these analyses, we compared the viable microbial cells calculated with the FC (active fluorescent units; AFU) with the CFUs indicated on the label. We are aware that this is an approximation because, reportedly, the ability of probiotics to form colonies (CFUs) may decrease during shelf-life more rapidly than their ability to be counted as AFUs in FC (Jackson et al. 2019). Nonetheless, we believe that this approximative calculation did not prevent from achieving to the actual goal of our experiments, which was the demonstration that a dominant fraction of commercial probiotic products is constituted by microbial cells in different physiological states that are not mentioned on the label, including an actual amount of viable cells higher than the declared concentration.

7. Conclusions

In this commentary paper, we discussed the microbiological components constituting a probiotic product, which are very often more abundant than the number of live cells (expressed as CFUs) indicated on the label, and include (i) additional CFUs, (ii) viable cells that cannot generate colonies on agar plates, and (iii) dead cells. Determining the complete composition in terms of physiological states of microbial cells in a probiotic product is difficult, also in consideration of the fact that the abundance of the different microbiological components changes over time (Fig. 1). Nonetheless, the use of flow cytometry can provide a relevant contribution for a more comprehensive microbiological quantification of a probiotic formulation.

In clinical trials aimed at studying the efficacy of a probiotic product, reference is most often given to the quantity of CFUs indicated on the label. However, ignoring the other microbial components of a probiotic product, since all of them (including dead cells) can promote biological responses in the host, can greatly prevent the possibility of making an adequate comparison between different studies and different probiotic formulations. In addition, limiting the information on the label of a probiotic product to the only minimum concentration of CFUs guaranteed at the end of the shelf-life significantly hampers the possibility of health professionals to make a fully aware prescription of probiotic products. In fact, reportedly, the non-cultivable microbiological components (both viable and dead) influences the immune system. Therefore, overlooking these microbial fractions, which can also be 10 times greater than the limit indicated on the label (Fig. 2), can represent a potential risk for immunocompromised or fragile subjects such as infants or older people.

In light of the above considerations, we believe it is important to provide more information about the microbial viability states of a probiotic product, both on the label and while conducting a clinical study. In this context,

we propose the following **minimum requirements for the microbiological characterization of a probiotic product**:

- On the label of commercial products: in addition to the minimum number of CFUs at the end of the shelf-life, the total number of microbial cells determined by flow cytometry must be declared.
- In clinical trials: viable count by plating according to standard procedures (e.g. the methods reported in the document ISTISAN 08/36 by the Italian National Institute of Health) must be determined for each lot of the probiotic formulation under investigation immediately before the beginning of the intervention to be aware of the actual number of viable cells administered to study participants. In addition, the probiotic formulation must be analyzed by flow cytometry according to standard methods (e.g. ISO-IDF protocols) in order to determine the total number and the proportion of viable, damaged and dead microbial cells.

In our opinion, the minimum requirements of microbiological characterization proposed above can significantly contribute to a more precise understanding of the potential efficacy of the myriad of different probiotic products on the market in promoting health benefits.

Conflict of interest

WF is an employee at SOFAR S.p.A., a pharmaceutical company commercializing probiotic supplements. SA and SG act as scientific consultant for several food and pharmaceutical companies.

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Legends and figures

Fig. 1. *Exemplifying representation of the microbial viability states of a hypothetical probiotic product over time.*

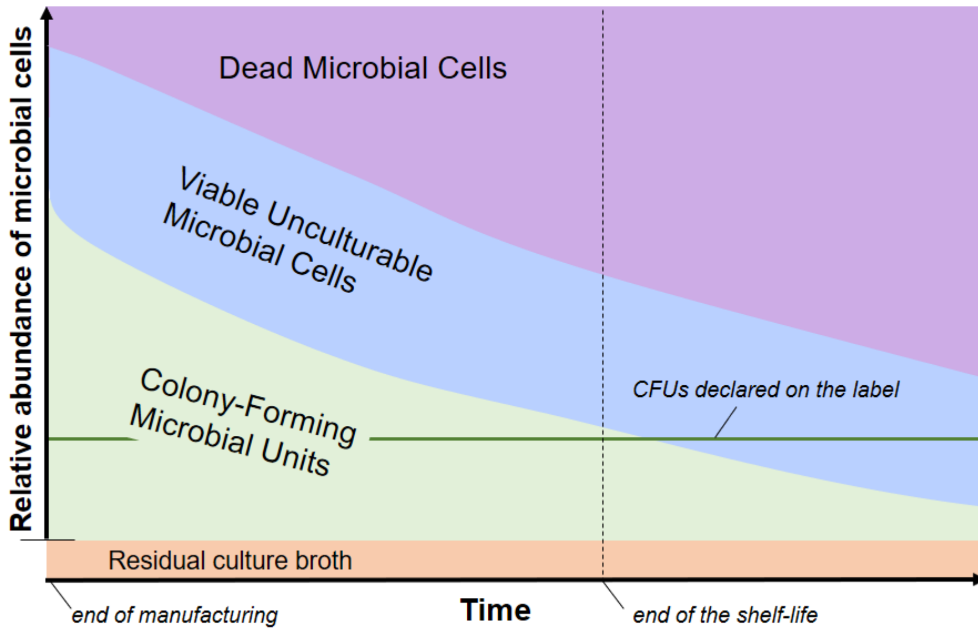
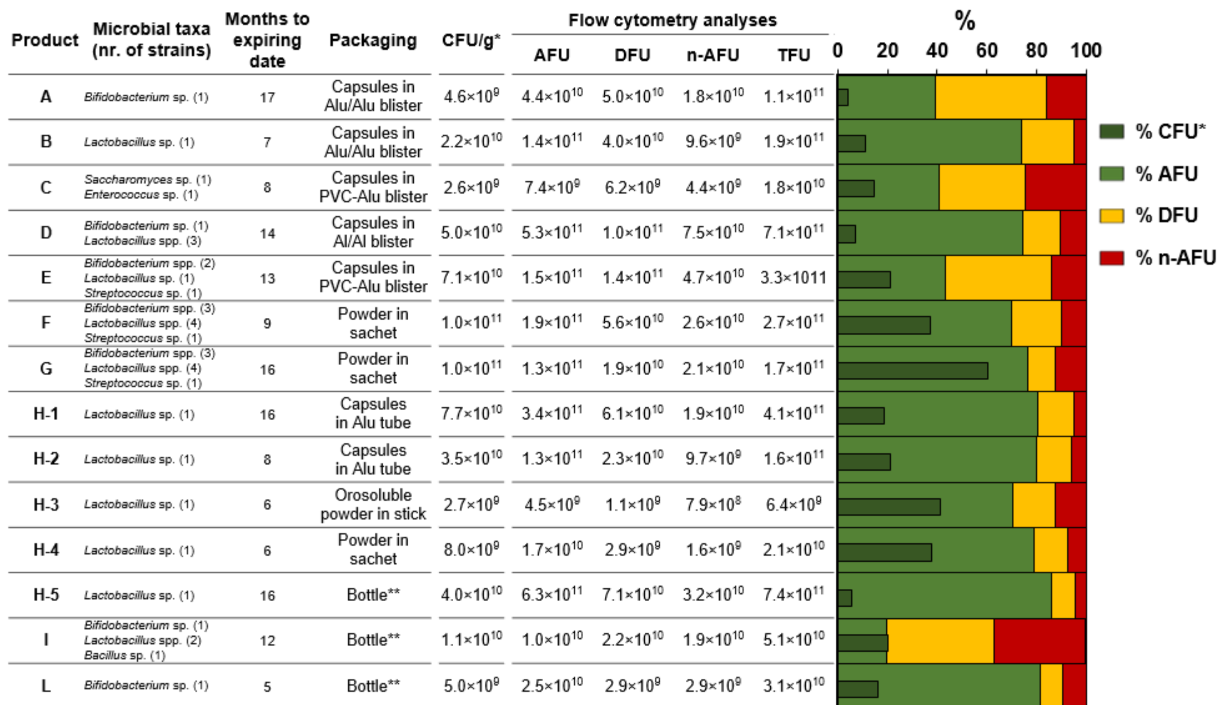


Fig. 2. *Analysis of probiotic products by flow cytometry (FC). The figure shows the data obtained from the analysis of 14 commercial products. Histogram on the right represents the percentages of the different microbial fraction in the formulation setting as 100% the total number of cells detected by FC (total fluorescent unuts, TFU). AFU = active fluorescent units, indicating viable cells as determined through FC; DFU, damaged fluorescent units; n-AFU, non-active fluorescent units (dead cells). H1-5, same probiotic strain in different commercial formulations. *, CFU/g calculated from the concentration declared on the label. **, probiotic cells were included in a small sealed aluminum pouch located within the bottle cap*.



Product	Microbial taxa (nr. of strains)	Months to expiring date	Packaging	CFU/g*	Flow cytometry analyses			
					AFU	DFU	n-AFU	TFU
A	*Bifidobacterium* sp. (1)	17	Capsules in Alu/Alu blister	4.6×10^{9}	4.4×10^{10}	5.0×10^{10}	1.8×10^{10}	1.1×10^{11}
B	*Lactobacillus* sp. (1)	7	Capsules in Alu/Alu blister	2.2×10^{10}	1.4×10^{11}	4.0×10^{10}	9.6×10^{9}	1.9×10^{11}
C	*Saccharomyces* sp. (1) *Enterococcus* sp. (1)	8	Capsules in PVC-Alu blister	2.6×10^{9}	7.4×10^{9}	6.2×10^{9}	4.4×10^{9}	1.8×10^{10}
D	*Bifidobacterium* sp. (1) *Lactobacillus* spp. (3)	14	Capsules in Al/Al blister	5.0×10^{10}	5.3×10^{11}	1.0×10^{11}	7.5×10^{10}	7.1×10^{11}
E	*Bifidobacterium* spp. (2) *Lactobacillus* sp. (1) *Streptococcus* sp. (1)	13	Capsules in PVC-Alu blister	7.1×10^{10}	1.5×10^{11}	1.4×10^{11}	4.7×10^{10}	3.3×1011
F	*Bifidobacterium* spp. (3) *Lactobacillus* spp. (4) *Streptococcus* sp. (1)	9	Powder in sachet	1.0×10^{11}	1.9×10^{11}	5.6×10^{10}	2.6×10^{10}	2.7×10^{11}
G	*Bifidobacterium* spp. (3) *Lactobacillus* spp. (4) *Streptococcus* sp. (1)	16	Powder in sachet	1.0×10^{11}	1.3×10^{11}	1.9×10^{10}	2.1×10^{10}	1.7×10^{11}
H-1	*Lactobacillus* sp. (1)	16	Capsules in Alu tube	7.7×10^{10}	3.4×10^{11}	6.1×10^{10}	1.9×10^{10}	4.1×10^{11}
H-2	*Lactobacillus* sp. (1)	8	Capsules in Alu tube	3.5×10^{10}	1.3×10^{11}	2.3×10^{10}	9.7×10^{9}	1.6×10^{11}
H-3	*Lactobacillus* sp. (1)	6	Orosoluble powder in stick	2.7×10^{9}	4.5×10^{9}	1.1×10^{9}	7.9×10^{8}	6.4×10^{9}
H-4	*Lactobacillus* sp. (1)	6	Powder in sachet	8.0×10^{9}	1.7×10^{10}	2.9×10^{9}	1.6×10^{9}	2.1×10^{10}
H-5	*Lactobacillus* sp. (1)	16	Bottle**	4.0×10^{10}	6.3×10^{11}	7.1×10^{10}	3.2×10^{10}	7.4×10^{11}
I	*Bifidobacterium* sp. (1) *Lactobacillus* spp. (2) *Bacillus* sp. (1)	12	Bottle**	1.1×10^{10}	1.0×10^{10}	2.2×10^{10}	1.9×10^{10}	5.1×10^{10}
L	*Bifidobacterium* sp. (1)	5	Bottle**	5.0×10^{9}	2.5×10^{10}	2.9×10^{9}	2.9×10^{9}	3.1×10^{10}

GENERAL CONCLUSION

Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al. 2014). Therefore, by definition, the term probiotic is restricted to live microbial cells. Among the criteria that a microorganism should fulfil to be qualified as a probiotic is its survival during gastrointestinal transit. Studies on probiotic viability in humans after ingestion are mainly based on quantification in the feces and are also referred to as "persistence" or "recovery" studies. To date, reliable studies have been conducted only for some well-known commercially available probiotics, such as *L. paracasei* Shirota, *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12. The viable recovery of probiotics from feces is technically challenging because the feces are microbiologically very complex and host thousands of different microbial species.

In my Ph.D project we have conducted two recovery studies, in both children and adults, on a selected strain, *L. paracasei* DG. Viable count experiments carried out by combining ad hoc culture-selective/discriminative conditions and strain-specific molecular biological protocols unambiguously demonstrated that *L. paracasei* DG can survive gastrointestinal transit in healthy subjects. Recovery studies to assess microbial viability after gastrointestinal transit should be a mandatory step in the characterization process of any probiotic product.

Once in the intestine, the probiotic strain is able to proliferate and colonize the gut. We have used a mouse model to evaluate the impact of the selected strain on microbiota and host’s gene expression at three different districts of the intestine: ileum, caecum and colon. The obtained results have provided a rationale to use the strain in case of pathological situations with a localization at colon level, such as diverticular disease and IBD, that include

the dysbiosis and inflammatory processes in their etiopathogenesis. In addition, the administration of *L. paracasei* DG affected the expression of several genes involved in immune responses in the mouse intestine and, particularly, in the ileum, suggesting that this bacterium may play a potential anti-inflammatory/regulatory activity in the gut.

These beneficial effects observed in the clinical study in IBS patients may be due to the above actions of the strain and mainly to its capability to positively modulate the gut microbiota structure/function and to affect the inflammatory and immune system pathway. Furthermore, as repoted by *Barbara et al.* (Barbara et al. 2016), serotonin and visceral hypersensitivity play an important role in the etiopathogenesis of the IBS. The results obtained in the mouse model could be linked directly to the mechanism of action of a potential clinical benefit of the strain in these patients.

To date we have considered only probiotic defined, as reported above, as “live microrganisms”, but, plausibly, the beneficial effects observed in the clinical practice could be exerte also with the contribution of dead microbial cells, which are unavoidably present in all probiotic products. *Taverniti & Guglielmetti* (2011) introduced the concept of “paraprobiotic” to indicate the use of inactivated microbial cells or cell fractions to confer a health benefit to the consumer. These dead cells have an effect mainly on inflammatory pathways. So, in the future we need to take into consideration that probiotic products could affect host health in manner that depends on the relative proportion between live and dead cells. Flow cytometry, which makes possible to distinguish the two types of cells, should be considered a mandatory tool for the microbiological characterization of probiotic products in the next years.

IMPLICATIONS AND FUTURE DIRECTIONS

The results observed in my Ph.D thesis may have useful implications both at industrial and clinical research level.

In fact, at industrial level, the presence of live and dead cells in probiotic products and their proportion is enormously affected by the production process. The demonstration that both cell types can have beneficial effects on the host gut could allow companies, for instance, to produce products with less live cells inside. In some cases, companies may even be able to completely eliminate living cells from their products and maintain only dead cells (paraprobiotic formulations), reducing significantly production, packaging and storing costs.

The contribution of dead cells to the potential health benefits of probiotic products is also important in clinical studies. One product may be more effective than another only due to a different ratio between live and dead cells. Flow cytometry can help to accurately determine this ratio obtaining a more comprehensive characterization of the probiotic formulation than the conventional agar plate count. For this reason, we recommend that a probiotic product be characterized microbiologically by flow cytometry immediately before its use in a clinical study.

APPENDICES (additional details, poster(s), activities list)

Publication list

Arioli S, Koirala R, Taverniti V, **Fiore W**, Guglielmetti S. 2018. Quantitative Recovery of Viable *Lactobacillus paracasei* CNCM I-1572 (L. casei DG®) After Gastrointestinal Passage in Healthy Adults. Front Microbiol. 9, 1720

Radicioni M, Koirala R, **Fiore W**, Leuratti C, Guglielmetti S, Arioli S. 2018. Survival of L. casei DG® (*Lactobacillus paracasei* CNCMI1572) in the gastrointestinal tract of a healthy paediatric population. Eur J Nutr. 2018 Nov 29 (Epub ahead of print)

Cremon C, Guglielmetti S, Gargari G, Taverniti V, Castellazzi AM, Valsecchi C, Tagliacarne C, **Fiore W**, Bellini M, Bertani L, Gambaccini D, Cicala M, Germanà B, Vecchi M, Pagano I, Barbaro MR, Bellacosa L, Stanghellini V, Barbara G. 2018. Effect of *Lactobacillus paracasei* CNCM I-1572 on symptoms, gut microbiota, short chain fatty acids, and immune activation in patients with irritable bowel syndrome: A pilot randomized clinical trial. United European Gastroenterol J. 6(4), 604-613.

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Turco F, Andreozzi P, Palumbo I, Zito FP, Cargiolli M, **Fiore W**, Gennarelli N, De Palma GD, Sarnelli G, Cuomo R. 2017. Bacterial stimuli activate nitric oxide colonic mucosal production in diverticular disease. Protective effects of L. casei DG® (*Lactobacillus paracasei* CNCM I-1572). United European Gastroenterol J. 5(5), 715-724.

Balzaretti S, Taverniti V, Guglielmetti S, **Fiore W**, Minuzzo M, Ngo HN, Ngere JB, Sadiq S, Humphreys PN, Laws AP. 2017. A Novel Rhamnose-Rich Hetero-exopolysaccharide Isolated from *Lactobacillus paracasei* DG Activates THP-1 Human Monocytic Cells. Appl Environ Microbiol. 83(3)

Activities list

Fiore W. Overall Assessment of a Model Probiotic Bacterium: from Gut Colonization to Clinical Efficacy. **Poster** presented and discussed at 22nd Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Free University of Bozen, Bozen, September 20th-22nd, **2017**

Fiore W. Overall Assessment of a Model Probiotic Bacterium: from Gut Colonization to Clinical Efficacy. **Oral communication** at 24th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Florence, September 11th-13th, **2019**