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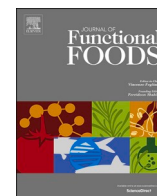
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Effect of a carotenoid-producing *Bacillus* strain on intestinal barrier integrity and systemic delivery of carotenoids: A randomised trial in animals and humans

Yala Stevens^{a,b,*}, Iris Pinheiro^c, Bouke Salden^{a,b}, Cindy Duysburgh^d, Selin Bolca^c, Jeroen Degroote^e, Maryam Majdeddin^e, Noémie Van Noten^e, Béatrice Gleize^f, Catherine Caris-Veyrat^{f,1}, Joris Michiels^e, Daisy Jonkers^a, Freddy Troost^a, Sam Possemiers^c, Ad Masclee^a

^a Department of Internal Medicine, Division of Gastroenterology-Hepatology, School of Nutrition & Translational Research in Metabolism (NUTRIM), Maastricht University, Maastricht, the Netherlands

^b BioActor BV, Maastricht, the Netherlands

^c MRM Health NV, Technologiepark 82, 9052 Ghent, Belgium

^d ProDigest BV, Technologiepark 82, 9052 Ghent, Belgium

^e Department of Animal Sciences and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

^f INRAE, Avignon Université, UMR SQPOV, F-84914 Avignon, France

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ABSTRACT

The aim of the present study was to investigate effects of the carotenoid-producing *Bacillus indicus* strain PD01 on intestinal barrier function and its ability to survive passage through the gastrointestinal tract and to assess systemic bioavailability of these carotenoids *in vivo*. As model for impaired barrier function, 16 early weaned piglets were randomly assigned to a control diet or control diet with PD01 for 23 days. In addition, 67 overweight/obese, otherwise healthy individuals were randomly assigned to groups receiving PD01 or placebo for 6 weeks. PD01 survived passage through the gastrointestinal tract in piglets and human subjects and resulted in significant accumulation of PD01 derived carotenoids (methyl-glycosyl-apo-8'-lycopenoate and glycosyl-apo-8'-lycopene) in human plasma after 3- and 6-weeks supplementation versus baseline (0.044 and 0.076 vs 0 μ M, respectively; $p < 0.001$). PD01 supplementation resulted in higher expression levels of occludin in the distal small intestine (1.38 ± 0.31 vs 0.59 ± 0.14 ; $p = 0.044$) and transepithelial electrical resistance in the mid colon (34.1 ± 3.01 vs $24.3 \pm 1.13 \Omega \cdot \text{cm}^2$; $p = 0.019$) of early weaned piglets compared to control. In overweight/obese individuals with preserved barrier integrity, PD01 did not affect sugar excretion ($p \geq 0.104$). In summary, PD01 survived transit through the gastrointestinal tract, resulted in systemic carotenoid accumulation and improved compromised barrier function outcomes.

1. Introduction

The intestinal epithelial barrier plays an important role in the maintenance of intestinal homeostasis and in overall human health. It acts as a selective barrier allowing the absorption of substances such as water and essential nutrients, while preventing the translocation of luminal content and pathogens. In general, the barrier consists of a

mucus layer, a single layer of epithelial cells sealed by junctional complexes and underlying lamina propria. In addition to these components, other factors such as the mucosa-associated lymphoid tissue and the intestinal microbiota play a key role in maintaining the integrity and function of the intestinal barrier (Konig et al., 2016; Vancamelbeke & Vermeire, 2017). Defects in intestinal barrier functioning have been associated with the development and progression of various

* Corresponding author at: Maastricht University, Department of Internal Medicine, Division of Gastroenterology-Hepatology, P.O. Box 616, 6200 MD Maastricht, the Netherlands.

E-mail address: yala.stevens@maastrichtuniversity.nl (Y. Stevens).

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gastrointestinal (GI) and metabolic diseases, including inflammatory bowel disease (IBD) and diabetes (Konig et al., 2016; Vancamelbeke & Vermeire, 2017). Therefore, treatments targeted at reducing intestinal permeability or preventing intestinal barrier dysfunction, could contribute to improve (GI) health. In this context, nutritional interventions for instance with probiotic bacteria have been proposed. Probiotics may improve intestinal barrier function by stimulating the immune system, strengthening the physical barrier and by modulating the microbiota composition in favour of lactic acid bacteria (Bron et al., 2017; Maldonado Galdeano, Cazorla, Lemme Dumit, Velez, & Perdigon, 2019). Globally, lactic acid bacteria and *Bifidobacterium* spp. are the most consumed bacteria included in probiotic formulations. However, species belonging to other genera such as *Enterococcus*, *Streptococcus*, *Bacillus*, along with *Saccharomyces* spp., have also gained interest over the past years. Although not all *Bacillus* strains are considered safe for human consumption (Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017), probiotic properties including the prevention of pathogen colonization and reduced intestinal permeability have been shown for *Bacillus* spp. (Elshaghabee et al., 2017; Lopetuso, Scaldaferrri, Franceschi, & Gasbarrini, 2016). Within this genus, the species *Bacillus indicus* has an extra attribute which is of interest to intestinal health. It has the ability to produce carotenoids which are lipophilic isoprenoid compounds with well-known antioxidant, anti-apoptotic, and anti-inflammatory properties (Rodríguez-Concepcion et al., 2018). Furthermore, positive effects on microbiota composition have also been shown for carotenoids (Dingeo et al., 2020; Wiese et al., 2019). Therefore, in addition to the strain itself, the carotenoids that are produced may also play a role in improving barrier dysfunction via their antioxidant and anti-inflammatory effects and their ability to modulate the microbiota composition. Various animal studies have indeed shown beneficial effects of carotenoids on intestinal barrier function such as an increased expression of tight junction proteins and a reduction of intestinal epithelial damage (Hwang, Koh, Lee, Kim, & Lim, 2014; Singh et al., 2016; Trivedi & Jena, 2015; Wang et al., 2019).

The *Bacillus indicus* strain PD01 is a spore-forming bacterium originally isolated from human faeces from a healthy volunteer as HU19 (Duc, Fraser, Tam, & Cutting, 2006), which fails to grow anaerobically. The bacterium naturally produces high levels of two glycosyl-apo-lycopene carotenoids (methyl-glycosyl-apo-8'-lycopenoate and glycosyl-apo-8'-lycopene) that do not degrade during passage through the stomach and are equally or even more bioaccessible and bioavailable than dietary carotenoids currently on the market (unpublished data from ProDigest, Ghent, Belgium). In addition to the potential probiotic properties of PD01 itself, the carotenoids released in the intestine may also result in local and systemic effects. Until now, data confirming these properties *in vivo* are lacking. Therefore, the aim of the present study is to investigate the effects of PD01 on intestinal barrier function *in vivo*. We chose to study this effect in early weaned piglets and in healthy yet overweight or obese subjects, as both early weaning stress and overweight have been associated with GI alterations, including a potential reduction in intestinal barrier integrity (Campbell, Crenshaw, & Polo, 2013; Fasano, 2017; Salden et al., 2018). Additionally, we evaluated whether PD01 is able to survive transit through the GI tract in both piglets and humans, and whether PD01 is able to release systemically absorbable carotenoids that can be detected in human plasma.

2. Methods

2.1. Animal study

The animal study was conducted in accordance with the ethical standards and recommendations for accommodation and care of laboratory animals covered by the European Directive 2010/63/EU on the protection of animals used for scientific purposes and the Belgian royal decree KB29.05.13 on the use of animals for experimental studies.

2.1.1. Animals and housing

Sixteen healthy male and female piglets (Topics hybrid × Piétrain) were weaned at the age of 19 days (approx. average weight 6.5 kg), origin Stefaan Debaerdemaeker, Evergem, Belgium. The piglets, originated from four litters with four piglets/litter and were assigned to the pens according to litter of origin, sex and body weight (BW). Besides these criteria, the animals were allocated randomly to either one of the two treatments: basal control diet (CD) or basal control diet supplemented with *Bacillus indicus* strain PD01 at a concentration of 2×10^9 spores/kg of diet (CD + PD01). The treatments lasted for a total of 23 days, and were replicated in two pens of four piglets per pen ($n = 8$ animals per treatment). Piglets were housed at the Laboratory for Animal Nutrition and Animal Product Quality of the Faculty of Bioscience Engineering from Ghent University (Ghent, Belgium). Four piglets were housed per pen (2.10 m²/pen) with full slatted floors, conventional ventilation scheme, starting ambient temperature at 30 °C and a 24L schedule till day (d)5 post-weaning. From d6 till d23, ambient temperature was linearly adjusted to 28 °C with 18L:6D light schedule. No medications in feed or water were used. Health was recorded on a daily and individual basis. No animals or data points were excluded during the study because of signs of disease or animal deaths.

2.1.2. Experimental diets and study product

The basal control diet was formulated to meet or exceed the piglets' requirements (adapted from Centraal Veevoeder Bureau, 1997) and was aimed to represent a human Western diet in terms of nutrients (Gunnness et al., 2016). The diets were semi-synthetic, including minerals, and vitamins, but excluding supplementary organic acids, and Cu and Zn beyond animal requirements. The ingredient composition and calculated nutrient composition of the basal control diet is provided in Table s1. Feeds were prepared as mash. One batch was made and then split up to make the two experimental diets (CD and CD + PD01). *Bacillus indicus* strain PD01 (MRM Health, Ghent, Belgium) was grown and lyophilized on a maltodextrin (Pineflow; Matsutani Chemical Industry, Hyogo, Japan) carrier with 89% spores per vegetative cells. Strain PD01, deposited at BCCM/LMG as *Bacillus indicus* PD01, was originally isolated and characterised by Duc et al. (2006) at Royal Holloway University of London (UK) from human faeces from a healthy volunteer (*Bacillus indicus* HU19 - NCIMB 41359). For the CD + PD01 diet, strain PD01 was added (2×10^9 spores/kg of diet) on top by premixing in a limited amount of basal diet, prior to complete mixing. All diets were fed *ad libitum*. No fasting period before sampling or sacrifice was included. Water was available *ad libitum* at all time.

2.1.3. Animal follow-up

The BW (kg) of the piglets was followed regularly: d0, d5, d14 and at the end of the trial (d23). For each period (d0-5, d5-14, d14-end and total period, d0-end) growth (g/d), feed intake (g/d) and feed to weight gain ratio (g/g) was recorded. All piglets were inspected two times a day for general health and presence of diarrhoea during the experimental period. The system used to score faecal consistency is provided in Table s2. To assess whether PD01 was detectable in the gastrointestinal tract of the piglets, at d14 and d23 a fresh non-contaminated faecal sample was collected from the rectum of each piglet. In addition, at d23 samples from the mid colon were also collected post-mortem for the same purpose.

2.1.4. Quantification of *Bacillus indicus* PD01

The samples collected at d14 and d23 from the rectum and mid colon were weighted, and 0.5 g of sample was dissolved in sterile PBS. Serial dilutions of this faecal slurry (10^{-1} to 10^{-6}) were plated in Luria Broth (LB) agar plates for colony counting (total cell count) upon incubation at 37 °C for 24 h. Because the PD01 strain of *Bacillus indicus* produces carotenoids, the colonies are easily identified by their bright yellow-to-orange appearance. For spore counting, the same samples were pasteurised at 65 °C for 30 min. Only spore-forming bacteria are able to

survive this treatment. Pasteurized samples were then plated in the same manner and colonies counted. Results are provided as colony forming units (CFU)/g of faecal sample.

2.1.5. Collection of samples post-mortem

All piglets were sacrificed on d23 postweaning. Just before euthanasia, the weight of the piglets was registered. Piglets were euthanized by intra-peritoneal pentobarbital (90 mg/kg BW), followed by exsanguination. After piglets were killed, the abdomen was immediately opened to collect several intestinal sections: 50% and 90% of small intestinal length and of the mid colon. Samples were either used for histomorphology, RNA extraction and quantitative (q)PCR, or for Ussing chambers.

2.1.6. Histomorphology of intestinal segments

Five cm segments of the 50% and 90% of small intestinal length were collected for histomorphology measurements (segments were flushed with saline and immersed in formalin). In brief, after fixation in neutral-buffered formalin, intestinal tissue samples were processed under standard conditions in an automatic tissue processor, embedded in paraffin wax and subsequently 5 μ m slides were stained with haematoxylin-eosin (van Nevel, Decuypere, Dierick, & Molly, 2003). Villus length (from tip to base) and crypt depth (from base to opening) of all well-oriented villi and adjacent crypts were measured using a microscope equipped with a camera and computer with appropriate software (Olympus BX61 microscope and image analysis software, analySIS Pro, Olympus, Aartseelaar, Belgium). Villus: crypt ratio was calculated as the mean value of the ratios of the obtained villus heights and adjacent crypt depths.

2.1.7. Intestinal permeability and ion transport analysis using Ussing chambers

Twenty cm segments of the 90% of small intestinal length and mid colon were collected for Ussing chamber measurements. Segments were flushed with saline, then cut along the mesenteric border, stripped of the muscle layers and mounted in modified Ussing Chambers (Andreas Mund Scientific Instruments, Simmerath, Germany) as flat sheets on a segment holder, with an exposed tissue area of 1.07 cm² (Chen et al., 2015). Immediately after mounting the tissues in the chambers, both half-chambers were filled with the Ringer's buffer solution (pH 7.4) containing (in mmol/L): 115 NaCl, 25 NaHCO₃, 0.4 NaH₂PO₄·H₂O, 2.4 Na₂HPO₄·2H₂O, 5 KCl, 1.2 CaCl₂·2H₂O, MgCl₂·6H₂O and 12 D-glucose, however at the mucosal side of the epithelium the 12 mmol/L D-glucose in buffer was replaced by an equimolar amount of mannitol. Paracellular intestinal permeability of the tissue was assessed by the apparent permeability for FITC-dextran, 4 kDa (Sigma-Aldrich, Overijse, Belgium) in two chambers (P_{app} FD-4). Parameters of intestinal ion transport and tissue integrity in another two chambers. Two sets of Ag/AgCl electrodes were connected to the half-chambers by 3 mmol/L KCl-agar bridges. One pair was used to record the potential differences between the half-chambers, while an external current ran through the other pair. After correction for solution resistance, the *trans*-epithelial potential difference was clamped to 0 mV by applying an external short-circuit current (I_{sc}). Ion transport was evaluated by measuring the baseline I_{sc} and chloride- (Cl⁻) stimulated secretion by the agonists carbachol (10 μ M serosal-side; a Ca²⁺-mediated secretagogue; Δ I_{sc}_{carbachol}) and theophylline (5 mM, bilateral; a cAMP/cGMP mediated secretagogue; Δ I_{sc}_{theophylline}) (Chen et al., 2015). Barrier function was determined by measuring the transepithelial electrical resistance (TEER).

2.1.8. RNA extraction and qRT-PCR of tight junction protein genes

Ten cm segments of the 50% and 90% of small intestinal length, and mid colon, were collected for real-time quantitative reverse-transcription (qRT)-PCR of two tight junction (TJ) protein genes: TJ protein 1 (*Tjp1*), encoding Zonula occludens 1 (ZO-1), and occludin (*Ocln*). For that, segments were flushed and mucosa was scraped,

collected and stored in RNeasy[®] (Sigma-Aldrich) at -80 °C until further RNA isolation. Total RNA was extracted by using the RNeasy Plus Mini Kit from Qiagen (Germantown, USA) and complementary (c) DNA was synthesized by using the High-Capacity cDNA Reverse Transcription kit from Thermo Fisher Scientific (Dreieich, Germany) according to the manufacturer's instruction (0.5 μ g of RNA was used for synthesis). All qPCRs were performed by using the StepOnePlus[™] Real-Time PCR System (Applied Biosystems, Foster City, USA), the SensiMix SYBR Hi-ROX Kit from Bioline (London, UK) and by using 12.5 ng of template per reaction together with 250 nM of each primer (BioLegio, Nijmegen, the Netherlands) (all samples were tested in triplicate). Primer nucleotide sequences were as follows: *Tjp1*-Fw: 5'-ATCTCG-GAAAAGTGCCAGGA-3'; *Tjp1*-Rev: 5'-CCCCTCAGAAACCCATACCA-3'; *Ocln*-Fw: 5'-CATGGCTGCCTTCTGCTTCATTGC-3'; *Ocln*-Rev: 5'-ACCATCACACCCAGGATAGCACTCA-3'. The hydroxymethylbilane synthase (*HMBS*) gene was used as internal reference for normalization (*HMBS*-Fw: 5'-AGGATGGGCAACTCTACCTG-3'; *HMBS*-Rev: 5'-GATGGTGGCTGCATAGTCT-3') (Nygard, Jorgensen, Cirera, & Fredholm, 2007). Relative quantification (RQ) was performed according to the comparative 2^{- $\Delta\Delta$ Ct} method (Livak & Schmittgen, 2001) (for that a reference sample from the control diet group was randomly chosen for normalization) using the StepOne Software v2.3 (Applied Biosystems).

2.2. Clinical study

The clinical study was part of a larger project in which the effect of *Bacillus indicus* strain PD01 on cardiovascular health and microbial environment was investigated (Salden, 2017). The study was approved by the Medical Ethics Committee of the Maastricht University Medical Centre + (MUMC+) and conducted in full accordance with the principles of the Declaration of Helsinki of 1975 as amended in 2013 and with the Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). The study was performed at the MUMC+ from August 2015 to December 2015. All participants gave written informed consent before participation. The trial has been registered in the Clinical Trials register (NCT02622425).

2.2.1. Subjects

Healthy, overweight or obese volunteers aged 18–70 years with a body mass index (BMI) between 25 and 35 kg/m² were recruited by advertisement. Participants were excluded from the study when meeting one or more of the following exclusion criteria: any medical condition that might interfere with the study and might jeopardize the health status of the participant; smoking; high intake of fruits and vegetables (>75th percentile of dietary intake of fruits and vegetables in the general Dutch population); abuse of alcohol (>20 alcoholic units / week) and drugs; absence of a stable body weight 3 months prior to the study (\pm 3 kg); plans to lose weight or to follow a hypocaloric diet during the study period; use of medication/vitamin-, mineral- or antioxidant supplements; consumption of pro-, pre- or synbiotics during study period and in the 30 days prior to start of the study; use of antibiotics 90 days prior to the study; pregnancy and lactation; history of any side effects towards the intake of pro-, pre-, synbiotic supplements or carotenoids. To assess the fruit and vegetable intake of the participants, a 3-day food record was completed prior to start of the study (during screening). During the study, the subjects were instructed to maintain their habitual diet. The sample size calculation was determined for the primary outcome of the original research protocol, *i.e.* lipid peroxidation, which is not included in this manuscript. Based on previous research (Visioli, Riso, Grande, Galli, & Porrini, 2003), it was calculated that a sample size of at least 60 subjects (*i.e.* 30 participants per intervention group) would be required.

2.2.2. Study products

Bacillus indicus strain PD01 (MRM Health, Ghent, Belgium) was grown and lyophilized on a maltodextrin (Pineflow; Matsutani Chemical

Industry, Hyogo, Japan) carrier with 89% spores per vegetative cells. The study products were provided as a powder in sachets. The PD01 group received per day one sachet containing each 5×10^9 CFU PD01 with maltodextrin (3 g) as carrier material. The amount of PD01 carotenoids present inside the spores of the clinical batch was determined after lipophilic extraction by spectrophotometry at 455 nm in dichloromethane ($\epsilon = 165\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ and $\text{MW} = 750\text{ g}\cdot\text{mol}^{-1}$). This amount was quantified as $2.81\text{ }\mu\text{g}$ per 10^9 CFU, resulting in a total of $14.05\text{ }\mu\text{g}$ PD01 carotenoids per day. The placebo group received one sachet containing 3 g maltodextrin per day. Subjects were asked to stir the content of one sachet in 150 mL whole fat milk and ingest the solution each morning, just before consuming breakfast, for a total of six weeks.

2.2.3. Design and intervention

This study was designed as a randomized, placebo-controlled, double-blind, parallel-group study. Each subject underwent three test days. Participants were randomly assigned to one of the two intervention arms: PD01 or placebo (maltodextrin). An independent and blinded person generated the randomization list, using a computerized procedure (<http://randomizer.org>). All participants and investigators remained blind to treatment until all analyses were completed. Participants were instructed to abstain from strenuous physical exercise, consumption of alcohol and carotenoid-rich food products on the day prior to each test day. Assessments were performed in a quiet, temperature-controlled ($20\text{--}24\text{ }^{\circ}\text{C}$) room. After an overnight fast, subjects handed in a faecal sample on the first test day. Then, anthropometric measurements (height, body weight, waist-to-hip circumference) were performed. Subsequently, venous blood samples were collected from an antecubital vein in the fore-arm. Then, subjects ingested a multi-sugar drink and collected full urine output for 24 h measurement. Finally, the subjects completed a questionnaire to assess the presence of GI symptoms, stool frequency and stool consistency. After completion of the baseline measurements, participants received the study product for the following six weeks. After three weeks of daily supplementation, the second test day was organized. Measurements were identical to the baseline measurements performed during the first test day, with the exception of the multi-sugar drink with 24 h urine collection, which was not performed after three weeks of intervention. After six weeks of daily administration of the study product, the third test day was organized, which was identical to the first test day. To assess compliance, participants were asked to collect the empty sachets and to return these at the last visit.

2.2.4. Quantification of *Bacillus indicus* PD01

Prior to analysis, faecal samples were thawed for 20 min and homogenized in sterile distilled water. Homogenized faecal samples were serially diluted in PBS buffer and total cell count and spore count were determined according to the methods described above.

2.2.5. Bioavailability of bacterial carotenoids in plasma

After collection, blood samples were kept in an ice-water bath and light exposure was avoided. Plasma was isolated by centrifugation (10 min , 1400 g , $4\text{ }^{\circ}\text{C}$) within 2 h following collection and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis. Carotenoids were extracted by the Bligh and Dyer method (Bligh & Dyer, 1959). Then, an enzymatic step, according to Breithaupt, was carried out to hydrolyse esterified carotenoids (Breithaupt, 2000). Carotenoids were quantified by reverse-phase HPLC as described by Gleize, Steib, Andre, and Reboul (2012) using an HP1100 Agilent system equipped with a $150 \times 4.6\text{ mm i.d.}$ C30 column (YMC, Kyoto, Japan) set at $35\text{ }^{\circ}\text{C}$. Mobile phase consisted of a gradient of methanol (A), methyl *tert*-butyl ether (B) and water (C) and the flow rate was 1 mL/min . The gradient profile of the mobile phase (A:B:C) was set at 96:2:2 and changed linearly to 18:80:2 in 27 min, and then the mobile phase was changed back to 96:2:2 from 31 to 35 min. The carotenoids were identified at 460 nm based on retention time and UV-Visible

spectra (for PD01 carotenoids spectra, see Perez-Fons et al. (2011)). The absorption spectra of each standard were measured between 300 and 550 nm in the mobile phase to cross-check the identification of sample molecules. Quantification was performed comparing peak area with standard calibration curves. Lutein, β -carotene and lycopene standards were purchased from Carotenature (Münsingen, Switzerland) and PD01 carotenoids standard was purified from PD01 spores as detailed by Sy, Dangles, Borel, and Caris-Veyrat (2015).

2.2.6. Gastrointestinal permeability

Gastrointestinal permeability was assessed by using a validated multi-sugar test (van Wijck, van Eijk, Buurman, Dejong, & Lenaerts, 2011; van Wijck et al., 2013), measuring the urinary excretion of ingested sugar probes reflecting permeability of four segments of the GI tract. The method of measurement has been described previously by Mujagic et al. (2014). Urinary sugar probes were measured by HPLC-MS as previously described (van Wijck et al., 2011; van Wijck et al., 2013).

2.2.7. Gastrointestinal tolerance

The occurrence of GI symptoms was assessed using the validated gastrointestinal symptom rating scale (GSRs), consisting of 15 items combined into five symptom clusters describing reflux, abdominal pain, indigestion, diarrhoea, and constipation (Svedlund, Sjodin, & Dotevall, 1988). In the seven-point graded Likert-type GSRs, a score of 1 represents absence of troublesome symptoms and a score of 7 represents very troublesome symptoms. Defecation frequency and stool consistency were assessed by using the Bristol Stool Form Scale Chart (Riegler & Esposito, 2001).

2.3. Statistical analyses

Normality of the data was evaluated by using the Shapiro-Wilk test and statistical tests were applied accordingly. For the animal study, statistically significant differences between the control and test diet were assessed by unpaired, two-tailed Student's *t*-tests for normally distributed data and Mann-Whitney U tests for data that were not normally distributed. Differences between two time points were assessed by two-tailed paired Student's *t*-tests.

For the clinical study, baseline differences between intervention groups were tested using unpaired, two-tailed Student's *t*-tests, Mann-Whitney U tests or Chi-square tests when appropriate. To compare the presence of PD01 in faeces and bacterial carotenoids in plasma between baseline samples and samples collected after three and six weeks of supplementation, per protocol analyses were performed based on all participants that actually consumed the study product, by using the Friedman test with post-hoc Wilcoxon Signed Rank test. For GI permeability, GI tolerance, stool consistency and stool frequency per protocol analyses were performed based on all participants that completed the study protocol. For these outcomes, differences between intervention groups (PD01 or placebo) were assessed using linear mixed models with intervention group (placebo and PD01), time (baseline, three weeks and six weeks) and intervention group*time as fixed factors, where an unstructured covariance structure was used for repeated measures. The linear mixed model accounts for the correlation between repeated measures and missing data, where a likelihood approach was used assuming data missing at random and can be used for data showing limited skewedness providing a sufficient sample size. Final conclusions were confirmed in a sensitivity analysis using log transformed data.

All statistical analyses were performed using IBM SPSS Statistics for Windows (version 25.0, Armonk, NY, USA). Data analysed by parametric tests are presented as mean \pm SEM or medians with interquartile ranges for data analysed by non-parametric tests. Two-sided *p*-values ≤ 0.05 are considered significant. Correction for multiple testing was performed by Bonferroni correction based on a correction for multiple time points.

3. Results

3.1. Animal study

3.1.1. Faecal quantification of *Bacillus indicus* PD01

Faecal samples from the rectum and mid colon of early weaned piglets were collected at day 14 (only rectum) and day 23 (rectum and mid colon). The number of total bacterial cells and spores detected in the rectum was high and did not increase significantly from day 14 to day 23 (Table 1). In the faeces collected post-mortem from the mid colon, the number of total cells and spores was also high (1.83×10^6 and 1.38×10^6 CFU/g faeces, respectively). This indicates that PD01 was present in a viable form in the gut of the animals that received PD01.

3.1.2. Intestinal barrier function

PD01 supplementation did improve barrier function in early weaned piglets (Table 2). Both in the distal small intestine and mid colon, PD01 supplementation resulted in higher TEER values ($p = 0.070$ and $p = 0.019$ at 90% of small intestinal length and mid colon, respectively), although this effect was only significant in the mid colon. To investigate whether the positive effects observed could be attributed to changes in the expression of TJ proteins, we have measured the expression of *Tjp1* and *Ocln* in the small intestine and mid colon of the piglets. A significantly higher *Ocln* gene expression in the distal small intestine was observed for the group fed PD01 when compared to the control diet (Fig. 1).

3.1.3. Gastrointestinal tolerance

Although statistical analyses could not be performed on indices per pen, the average pen data indicate that supplementation of the piglet's diet with *Bacillus indicus* PD01 did not have a major impact on animal performance in terms of total body weight, daily growth or feed intake (Table S3). The mean faecal score was also similar between the two diets. No diarrhoea was recorded for any of the groups. Also no differences were observed in histomorphology in terms of crypt depth and villus height, both at 50% and 90% small intestinal length (Table 3; Figure S1).

Table 1

PD01 total cells and spores (CFU/g) in the gastrointestinal tract of piglets at day 14 and at the end of the study in the CD + PD01 group. Total cell count was performed by plating serial dilutions of collected samples in LB agar plates. Spore count was performed in the same manner and on the same samples after pasteurization at 65 °C. Vegetative cells are calculated as the difference between total cell count and spore count. Average CFU/g of fecal sample are presented, and percentage from total cells were calculated for both spores and vegetative cells. All values are presented as mean \pm SEM.

	Faeces (rectum)		P-value	Faeces (mid-colon)
	Day 14 (n = 6)	Day 23 (n = 8)		Day 23 (n = 8)
Total cells	1.52E+06 \pm 3.08 E+05	2.62 E+06 \pm 6.46 E+05	0.218	1.83 E+06 \pm 8.29 E+05
Spores	1.45 E+06 \pm 4.24 E+05	1.86 E+06 \pm 3.64 E+05	0.541	1.38 E+06 \pm 2.36 E+05
Percentage of total	96%	71%		75%
Vegetative cells	6.33 E+04 \pm 2.85 E+05	7.63 E+05 \pm 3.90 E+05	0.331	4.48 E+05 \pm 7.08 E+05
Percentage of total	4%	29%		25%

Differences between day 14 and day 23 were tested with a paired student's *t*-test. CD: basal control diet.

Table 2

Effect of control (CD) and test diets (CD + PD01) on intestinal permeability and electrophysiological parameters as determined in the distal small intestine and mid colon sections of piglets by using Ussing chambers. All values are presented as mean \pm SEM (n = 8/group/segment).

		CD	CD + PD01	P-value
90% of small intestinal length	SI length (m)	9.88 \pm 0.35	10.5 \pm 0.37	0.244
	P _{app} FD-4 (10 ⁻⁷ cm/s)	13.4 \pm 2.62	9.89 \pm 1.91	0.303
	TEER (Ω .cm ²)	36.4 \pm 2.47	46.2 \pm 4.30	0.070
	Δ Isc _{carbachol} (μ A/cm ²)	34.8 \pm 11.11	35.8 \pm 10.0	0.951
Mid colon	Δ Isc _{theophylline} (μ A/cm ²)	30.6 \pm 7.38	17.4 \pm 2.28	0.100
	P _{app} FD-4 (10 ⁻⁷ cm/s)	23.6 \pm 3.58	16.9 \pm 3.37	0.194
	TEER (Ω .cm ²)	24.3 \pm 1.13	34.1 \pm 3.01	0.019
	Δ Isc _{carbachol} (μ A/cm ²)	63.2 \pm 9.54	71.9 \pm 9.78	0.538
	Δ Isc _{theophylline} (μ A/cm ²)	53.1 \pm 9.18	46.1 \pm 6.12	0.533

Differences between control and test diet were tested with an unpaired student's *t*-test. CD: basal control diet; SI: small intestine (90% length); P_{app} FD-4: apparent permeability for FITC-Dextran 4 kDa; TEER: transepithelial electrical resistance of tissue; Δ Isc_{carbachol} and Δ Isc_{theophylline}: changes in short-circuit current upon stimulation with chloride secretagogues carbachol and theophylline, respectively.

3.2. Clinical study

3.2.1. Study subjects

For the clinical trial, 67 healthy overweight or obese volunteers were enrolled of which 62 completed the entire study protocol (Fig. 2). One randomised participant did not start the study for a personal reason. Four participants dropped out during the intervention period: one due to the need for antibiotic treatment being unrelated to the current study, another for unspecified, private reasons, and the two remaining participants experienced mild GI complaints. These complaints were present already at the start of the study and were not associated to PD01 intake. From these participants only baseline characteristics were available. Baseline characteristics are presented in Table 4. From the total of 67 randomized subjects, 33 participants in the placebo group and 29 participants in the PD01 group completed the study protocol.

3.2.2. Quantification of *Bacillus indicus* PD01

In the group of subjects receiving placebo, PD01 was not detected in any of the faecal samples. In the PD01 group, PD01 was not detected in any of the faecal samples at baseline, but was found in faecal samples of all 29 subjects after three weeks of supplementation and was present in all of the subjects that consumed PD01 during the complete study period (n = 27) after six weeks. As shown in Table 5, PD01 total cells and spores were significantly increased both after three weeks and six weeks supplementation, compared to baseline (all $p < 0.001$). No significant differences could be observed between three and six weeks of supplementation both for total cells and spores. The total amount of viable PD01 cells in human faeces after six weeks of PD01 supplementation was quantified as 2.26×10^7 CFU/g, of which 3.43×10^6 CFU/g are PD01 spores. Thus, although administered as spores, PD01 was mainly present in the faeces as vegetative cells (1.99×10^7 CFU/g; 73% of total counts), indicating effective germination under intestinal conditions.

3.2.3. Bioavailability of PD01 carotenoids in plasma

In fasted plasma, PD01 carotenoids were detected in samples of all subjects after three weeks (0.044 μ M) and six weeks (0.076 μ M) of PD01 supplementation, but not in the baseline samples. The total plasma concentration of PD01 carotenoids were significantly increased after

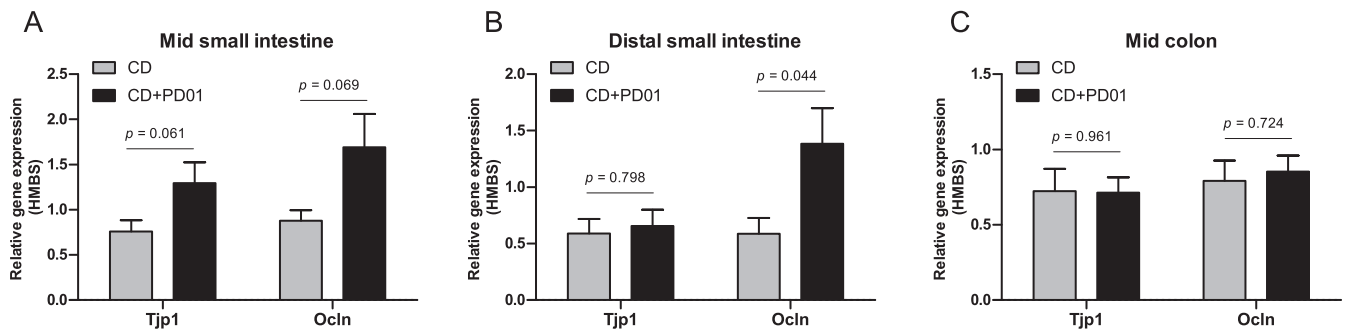


Fig. 1. Changes in tight junction protein gene expression in the (A) mid small intestine, (B) distal small intestine, and (C) mid colon of piglets upon supplementation of diet with PD01. Means and standard errors are shown ($n = 8$ animals/group/segment). Mid and distal small intestine correspond to the 50% and 90% small intestinal length tissues, respectively. The expression of tight junction protein 1 (*Tjp1*) and occludin (*Oc1n*) were normalized to hydroxymethylbilane synthase (*HMBS*). Differences between control and test diet were tested with an unpaired student's *t*-test. For 90% SI length, *TJP1* expression was tested with a Mann-Whitney *U* test.

Table 3
Effect of control (CD) and test diets (CD + PD01) on histomorphology of small intestinal mucosa. Data are presented as mean \pm SEM ($n = 8$ /group/segment).

		CD	CD + PD01	P-value
50% SI	Crypt depth (μm)	143 \pm 2.51	145 \pm 4.59	0.710
	Villus height (μm)	358 \pm 20.1	331 \pm 27.3	0.435
	Villus: crypt ratio	2.56 \pm 0.13	2.31 \pm 0.13	0.191
90% SI	Crypt depth (μm)	139 \pm 7.02	136 \pm 4.92	0.764
	Villus height (μm)	305 \pm 18.1	328 \pm 26.5	0.495
	Villus: crypt ratio	2.25 \pm 0.12	2.44 \pm 0.13	0.205

Differences between control and test diet were tested with an unpaired student's *t*-test. For 90% SI length, the villus: crypt ratio was tested with a Mann-Whitney *U* test. CD: basal control diet; SI: small intestine (50% and 90% length).

three weeks and continued to increase during the six weeks of daily supplementation (all $p \leq 0.027$, Table 6). The PD01 carotenoids detected in plasma were methyl-glycosyl-apo-8'-lycopenoate and glycosyl-apo-8'-lycopene, with methyl-glycosyl-apo-8'-lycopenoate making up 74.6% and 85.3% of the total concentration after 3 and 6 weeks, respectively. *Bacillus indicus* PD01 supplementation did not induce significant changes in the plasma content of lutein, β -carotene or lycopene during the study period (all $p \geq 0.618$, Table 6). The presence of (bacterial) carotenoids in plasma samples of subjects from the placebo group was not assessed, as bacterial carotenoids proved undetectable in the baseline sample of the subjects. Representative chromatograms of carotenoids found in plasma before and after supplementation are shown in Fig. 3.

3.2.4. Gastrointestinal permeability

The GI permeability results, as determined by the multi-sugar test, are presented in Table 7. Neither did the excretion of sugars indicative for small intestinal ($p = 0.842$) or whole gut permeability ($p = 0.266$) differ after six weeks PD01 supplementation nor did the changes in sugar excretion reflecting gastroduodenal ($p = 0.131$) or colonic ($p = 0.104$) permeability reach statistical significance after six weeks PD01 supplementation compared to placebo.

3.2.5. Gastrointestinal tolerance

GI tolerance was assessed by GSRS subdimension scores at every test day. As shown in Figure s2, the scores for indigestion decreased in the PD01 treatment group after six weeks of supplementation (uncorrected $p = 0.045$), but this reduction did not remain statistically significant after correction for multiple testing ($p = 0.135$). For the other gastrointestinal symptom scores and time points, also no significant changes were observed after PD01 supplementation compared to placebo (all $p \geq 0.183$, Figure s2), indicating that PD01 was well tolerated. Neither

PD01 nor placebo did affect stool frequency or consistency throughout the study period (data not shown).

4. Discussion

This is the first *in vivo* study evaluating the effect of a carotenoid-producing *Bacillus* strain on intestinal barrier function as well as its intestinal fate and ability to release systemically absorbable carotenoids in a combined animal and human approach. We have shown that *Bacillus indicus* strain PD01 survives transit through the GI tract in both piglets and human subjects and was able to release carotenoids *in situ*. Supplementation with PD01 resulted in a significant improvement in outcomes of intestinal barrier function in early weaned piglets, but did not significantly affect intestinal barrier permeability in healthy overweight or obese human subjects. Repeated intake of PD01 did not result in any adverse effects, not in early weaned piglets nor in human subjects.

In both piglets and humans, PD01 survived passage through the GI tract and was able to germinate into vegetative cells under intestinal conditions, although germination seems to be more efficient in humans based on the percentage of vegetative cells present in the faeces after supplementation. This difference in results between the piglets and humans may be due to interspecies differences in GI physiology, in particular gastric emptying (Hatton, Yadav, Basit, & Merchant, 2015; Suenderhauf & Parrott, 2013). Furthermore, we have shown that in humans, daily PD01 supplementation over a period of six weeks led to a significant accumulation of PD01 carotenoids in plasma. These carotenoids, methyl-glycosyl-apo-8'-lycopenoate and glycosyl-apo-8'-lycopene, are normally not present in the diet. In plasma, no changes in concentrations of the three main dietary carotenoids (*i.e.* lutein, β -carotene and lycopene) were observed, indicating that subjects did not change their dietary carotenoid intake during the study and these novel PD01 carotenoids do not interfere with the absorption and transport of other carotenoids. Plasma concentrations of the PD01 carotenoids were slightly lower in comparison with those of the dietary carotenoids. This is not surprising as in general the average intake of these carotenoids (10 mg/day) is of a much higher magnitude than the calculated daily dose of 14.05 μg PD01 carotenoids (Sluijs et al., 2015). Still, the fact that bacterial carotenoid levels reached similar order of magnitude levels in plasma upon a six-week intake, despite the much lower intake levels, further suggests that PD01 is capable of local production of high amounts of bacterial carotenoids in the intestine, in combination with high bioavailability of the released carotenoids.

In early weaned piglets, we observed an improvement in intestinal barrier function in animals fed a diet supplemented with *Bacillus indicus* strain PD01. Others did previously show with this model that the probiotic strain *Lactobacillus frumenti* was able to promote intestinal barrier function, via increased expression levels of the TJ proteins ZO-1, *Oc1n*

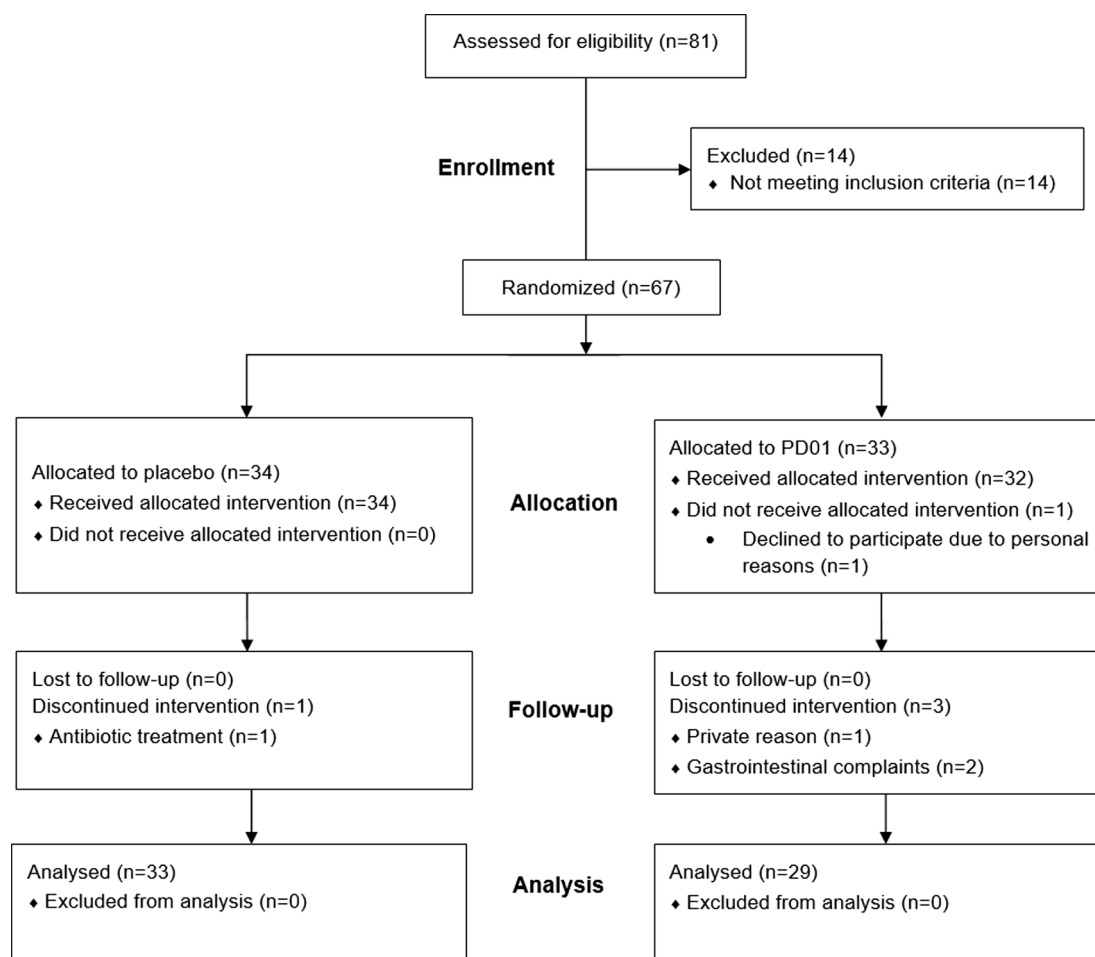


Fig. 2. CONSORT flow diagram.

Table 4

Baseline characteristics of the clinical study participants. Values are presented as medians [Q1; Q3] or numbers.

	Total population (n = 67)	Placebo (n = 34)	PD01 (n = 33)	P-value
Age, years	53.0 [46.0; 64.0]	51.0 [40.5; 64.5]	58.0 [50.0; 64.0]	0.129
Sex, M/F	29/38	12/22	17/16	0.180
WHR	0.91 [0.85; 0.98]	0.90 [0.84; 0.96]	0.94 [0.86; 1.00]	0.127
BMI, kg/m ²	29.3 [26.7; 32.3]	29.4 [27.1; 34.3]	29.3 [26.3; 31.6]	0.527

Differences in Age and BMI between placebo and PD01 were tested with a Mann-Whitney *U* test. Differences in WHR between placebo and PD01 were tested with an unpaired student's *t*-test. Differences in gender between placebo and PD01 were tested with a Chi-square test. M: male; F: female; WHR: waist-to-hip-ratio; BMI: body mass index.

and Claudin-1 (Hu et al., 2018). In addition to any potential effects of the *Bacillus indicus* strain PD01 itself, the carotenoids released in the intestine following PD01 supplementation may have been responsible or could have contributed to the observed improvement in barrier function. To date, in different mouse models associated with intestinal barrier disruption, treatment with the carotenoids β -carotene and lycopene resulted in an improvement in markers for intestinal barrier integrity and for colonic damage (Singh et al., 2016; Trivedi & Jena, 2015; Wang et al., 2019). In the current study, PD01 supplementation resulted in higher expression levels of *Ocln* in the distal small intestine of the

piglets. These increased expression levels were not accompanied by a significant change in TEER or transcellular permeability in the small intestine, although a slight improvement in both outcomes could be observed. In the mid colon, PD01 supplementation resulted in a significantly increased TEER, while TJ gene expression levels of *Tjp1* and *Ocln* were not affected. These findings do not exclude effects on protein levels, but can also be due to other tight junction or adherens junction related factors (Ulluwishewa et al., 2011). In future studies, additional analyses on the expression of junctional proteins and activity of signalling cascades will contribute to further insight in underlying mechanisms.

In our population of overweight or obese subjects, no significant effects on barrier function were observed as a result of PD01 intake. Intestinal barrier function was assessed by the use of a multi-sugar test measuring urinary recovery of sugars indicative of gastroduodenal, small intestinal, colonic and whole gut permeability. This method has been widely used and validated for non-invasive measurement of GI permeability in human subjects (van Wijck et al., 2013). Although overweight and obesity have been associated with an impaired barrier function, a recent study in morbidly obese patients that assessed intestinal permeability using the same sugar test showed that only gastroduodenal permeability was significantly increased in obese subjects, while small intestinal and colonic permeability were not increased when compared to healthy lean subjects (Wilbrink et al., 2019). These recent results indicate that in overweight/obese subjects who are otherwise healthy, in contrast to previously expected, intestinal permeability is not increased. Therefore, this group may not have been the most suitable study population to study effects on this outcome. So far, data from

Table 5

PD01 total cells and spores (CFU/g) in faecal samples of the clinical study participants at baseline, after 3 weeks and after 6 weeks supplementation with PD01 ($n = 27$). All values are presented as medians [Q1; Q3].

	Baseline	3 weeks	6 weeks	P_1	P_2	P_3	P_4
Total cells	0 [0; 0] [†]	2.50 E+07 [1.11 E+07; 3.98 E+07]	2.26 E+07 [1.06 E+07; 4.19 E+07]	<0.001	<0.001	<0.001	0.939
Spores	0 [0; 0]	5.77 E+06 [1.28 E+06; 1.56 E+07]	3.43 E+06 [1.85 E+06; 1.06 E+07]	<0.001	<0.001	<0.001	0.117
Percentage of total	0	30 [11; 53]	27 [7; 39]				
Vegetative cells	0 [0; 0]	1.70 E+07 [4.20 E+06; 3.95 E+07]	1.99 E+07 [5.65 E+06; 2.93 E+07]	<0.001	<0.001	<0.001	>0.999
Percentage of total	0	70 [47; 89]	73 [61; 93]				

Differences between baseline and 3 weeks and 6 weeks supplementation were tested with Friedman test with post hoc Wilcoxon signed rank test. Correction for multiple testing was performed by Bonferroni correction. P_1 represent the p values for the overall difference between the three time points tested with the Friedman test. P_2 represent the adjusted p values for the analysis of baseline vs. 3 weeks of intervention. P_3 represent the adjusted p values for the analysis of baseline vs. 6 weeks of intervention. P_4 represent the adjusted p values for the analysis of 3 weeks vs. 6 weeks of intervention. [†] Values that could not be detected, depicted here as 0 (all such values).

Table 6

Carotenoid concentrations (μM) in fasted plasma of the clinical study participants at baseline, after 3 weeks and after 6 weeks supplementation with PD01 ($n = 27$). All values are presented as medians [Q1; Q3].

	Baseline	3 weeks	6 weeks	P_1	P_2	P_3	P_4
PD01 carotenoids	0 [0; 0] [†]	0.044 [0.016; 0.073] ^{\$}	0.076 [0.042; 0.14] [¥]	<0.001	<0.001	<0.001	0.027
Lutein	0.13 [0.063; 0.19]	0.12 [0.084; 0.21]	0.12 [0.091; 0.22]	0.618	–	–	–
β -Carotene	0.28 [0.19; 0.54]	0.32 [0.23; 0.41]	0.32 [0.17; 0.44]	0.692	–	–	–
Lycopene	0.18 [0.091; 0.29]	0.18 [0.10; 0.26]	0.18 [0.087; 0.28]	0.936	–	–	–

Differences between baseline and 3 weeks and 6 weeks supplementation were tested with Friedman test with post hoc Wilcoxon signed rank test. Correction for multiple testing was performed by Bonferroni correction. P_1 represent the p values for the overall difference between the three time points tested with the Friedman test. P_2 represent the adjusted p values for the analysis of baseline vs. 3 weeks of intervention. P_3 represent the adjusted p values for the analysis of baseline vs. 6 weeks of intervention. P_4 represent the adjusted p values for the analysis of 3 weeks vs. 6 weeks of intervention. In case post hoc Wilcoxon signed rank test was not applicable, no p values are reported. [†] Values that could not be detected, depicted here as 0. ^{\$} Sum of methyl-1-glycosyl-3,4-dehydro-apo-8'-lycopenoate (74.6%) and 1-glycosyl-3,4-dehydro-apo-8'-lycopenoate (25.4%), non-esterified carotenoids of PD01. [¥] Sum of methyl-1-glycosyl-3,4-dehydro-apo-8'-lycopenoate (85.3%) and 1-glycosyl-3,4-dehydro-apo-8'-lycopenoate (14.7%), non-esterified carotenoids of PD01.

human intervention trials investigating the effect of probiotics on intestinal barrier function in overweight or obese subjects are scarce and show varying results (Krumbeck et al., 2018; Leber et al., 2012) while data on carotenoids are still lacking. For example, daily supplementation with *Lactobacillus casei* Shirota for three months did not have any significant effects on intestinal permeability (Leber et al., 2012), while the intestinal barrier was found to be significantly improved after three weeks of supplementation with *Bifidobacterium adolescentis* IVS-1 or *Bifidobacterium lactis* BB-12 (Krumbeck et al., 2018). Altogether, the results from the piglet and human studies show that while supplementation with PD01 was not able to induce any changes in intestinal barrier permeability in a healthy overweight/obese population, it was able to do so in an animal model of impaired barrier function. Therefore, further investigations regarding the effect of PD01 supplementation in specific patient groups with a more clearly compromised barrier integrity, particularly those in which immune-mediated barrier disruption is likely, are worthwhile. Use of a stressor that is able to induce a transient disruption of intestinal barrier function could also provide additional insight.

As this was one of the first studies performed *in vivo* with this particular *Bacillus* strain (PD01), animal growth performance and GI tolerance were assessed during the intervention periods. In animals, no changes in total body weight, daily growth, feed intake or faecal scores were observed as a result of PD01 feeding. In human subjects, gastro-intestinal symptom scores, stool frequency and stool consistency were also not significantly affected by the intake of PD01. These results show that PD01 did not result in any adverse effects and was well tolerated in

early weaned piglets and in healthy overweight/obese subjects. This is in line with data from a previous study assessing the safety profile of a similar strain (HU36) in guinea pigs and rabbits (Hong et al., 2008). Furthermore, toxicity and safety trials in mice and healthy lean and obese human subjects with supplementation periods of up to 90 days showed that repeated intake of *Bacillus indicus* strain PD01 was not associated with any adverse effects (unpublished data from ProDigest, Ghent, Belgium).

Some potential shortcomings of our study should be mentioned. First, the diet of the participants was not strictly controlled in the clinical study. We instructed participants to maintain their habitual dietary intake throughout the study period, as we aimed to assess the effects of PD01 as supplement to their habitual diet, which is in line with future applications. Based on the unchanged plasma concentrations of lutein, lycopene, and β -carotene it can be assumed that the habitual diet was indeed maintained. Second, the study population chosen for the current study was relatively healthy, because PD01 is a new product which so far has only been tested in healthy human volunteers. Third, the required sample size of the human intervention study was not calculated based on an improvement in intestinal permeability, as this was not the primary outcome of the original study protocol. Therefore, this study might have lacked sufficient power to confirm the effects on intestinal barrier function observed in animal models. Fourth, statistical analyses could not be performed on piglet performance indices, as these were based on data per pen. Last, the barrier function results in the animal study were in some cases borderline significant or a trend toward significance, indicating that the results have to be interpreted with caution.

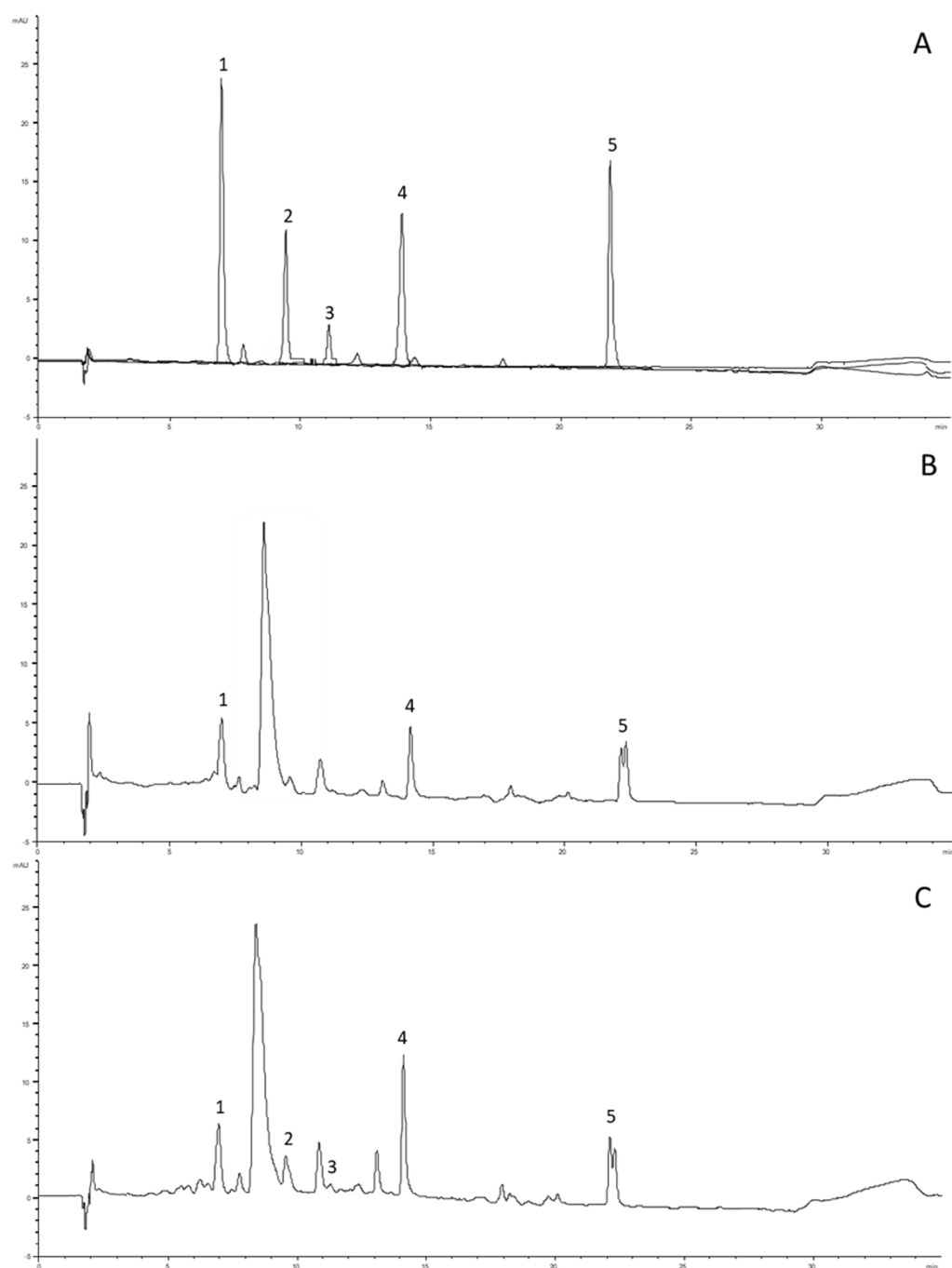


Fig. 3. Representative chromatograms of carotenoid standards (A) and carotenoid profiles in fasted plasma at baseline (B) and after 6 weeks supplementation with PD01 (C). Carotenoids were analyzed by reverse-phase HPLC on a HP1100 Agilent system using a YMC C30 column. Free forms of carotenoid were detected at 460 nm and identified by retention time compared with pure standards. The absorption spectra of each standard were measured between 300 and 550 nm in the mobile phase to cross-check the identification of sample molecules. 1: Lutein, 2: Methyl-glycosyl-apo-8'-lycopenoate (free form of orange bacterial carotenoid produced by PD01 strain), 3: Glycosyl-apo-8'-lycopene (free form of yellow bacterial carotenoid produced by PD01 strain), 4: β -Carotene, 5: Lycopene.

Table 7

Permeability test: sugar excretion (μmol) and ratios of excreted sugars as measured in urine (0–5, 5–24 and 0–24 h fractions) of the clinical study participants at baseline and after 6 weeks supplementation ($n = 62$). All values are presented as mean \pm SEM.

	Placebo		PD01		P
	Baseline	6 weeks	Baseline	6 weeks	
0–5 h sucrose	8.92 \pm 1.76	12.0 \pm 3.22	11.4 \pm 2.14	8.46 \pm 2.04	0.131
0–5 h L/R ratio	0.042 \pm 0.010	0.032 \pm 0.003	0.049 \pm 0.010	0.042 \pm 0.008	0.842
5–24 h S/E ratio	0.013 \pm 0.001	0.018 \pm 0.004	0.051 \pm 0.025	0.017 \pm 0.002	0.104
0–24 h S/E ratio	0.014 \pm 0.001	0.014 \pm 0.002	0.031 \pm 0.014	0.016 \pm 0.002	0.266

Differences between placebo and PD01 were tested with an unstructured linear mixed model with correction for baseline values. L/R: lactulose/l-rhamnose; S/E: sucralose/erythritol.

In conclusion, this study provides the first evidence that PD01 survives transit through the GI tract, is able to germinate and is able to release bacterial carotenoids, which are absorbed and detected in human blood plasma. Furthermore, PD01 supplementation resulted in improved barrier function outcomes in an animal model of disturbed intestinal barrier in early weaned piglets. While no significant effects on barrier function were found in an overweight/obese, but otherwise healthy population, these results warrant further research in specific target populations to investigate the specific bioactivities of PD01 in the intestine and possibly also on systemic parameters.

CRedit authorship contribution statement

Yala Stevens: Investigation, Formal analysis, Writing - original draft. **Iris Pinheiro:** Investigation, Formal analysis, Writing - original draft. **Bouke Salden:** Conceptualisation, Methodology, Formal analysis, Writing - original draft. **Cindy Duysburgh:** Investigation, Project administration, Writing - review & editing. **Selin Bolca:** Conceptualisation, Methodology, Project administration, Writing - review & editing. **Jeroen Degroote:** Investigation. **Maryam Majdeeddin:** Investigation. **Noémie Van Noten:** Investigation. **Béatrice Gleize:** Investigation, Writing - review & editing. **Catherine Caris-Veyrat:** Supervision. **Joris Michiels:** Conceptualisation, Methodology, Supervision, Writing - review & editing. **Daisy Jonkers:** Writing - review & editing. **Freddy Troost:** Conceptualisation, Methodology, Supervision, Writing - review & editing. **Sam Possemiers:** Conceptualisation, Methodology, Project administration, Supervision, Writing - review & editing. **Ad Masclee:** Conceptualisation, Methodology, Supervision, Writing - review & editing. All authors approved the final version of the manuscript.

Ethics statement

Animal study

The study was conducted in accordance with the ethical standards and recommendations for accommodation and care of laboratory animals covered by the European Directive (2010/63/EU) on the protection of animals used for scientific purposes and the Belgian royal decree (KB29.05.13) on the use of animals for experimental studies. No ethical approval was required for this trial as animals were kept under farm practices without interventions causing harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice, and because animals were killed solely for the use of their organs or tissues (2010/63/EU).

Clinical study

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Centre + (MUMC+) and conducted in full accordance with the principles of the Declaration of Helsinki of 1975 as amended in 2013 and with the Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). All participants gave written informed consent before participation. The trial has been registered in the Clinical Trials register (NCT02622425).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: YS is an employee of BioActor BV. IP, SB and SP are employees of MRM Health, which markets the strain discussed in this study. DJ is involved in research projects as part of private public partnership grants (i.e. TKI/Well on Wheat project and CCC NWO Carbokinetics). AM has received a ZON MW, The Netherlands Organization for Health Research and Development, health care efficiency grant to evaluate efficacy of

peppermint oil in IBS; has received an unrestricted research grant from Will Pharma SA and received research funding from Allergan and Grünenthal on IBS topics; has given scientific advice to Bayer (topic: IBS), to Kyowa Kirin (topic: constipation) and to Takeda (topic: gastroparesis); received funding from Pentax Europe GmbH and has received funding from the Dutch Cancer Society related to endoscopy and to colorectal polyps.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2021.104445>.

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