Journal Pre-proofs

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PII: S0963-9969(20)30605-0
DOI: https://doi.org/10.1016/j.foodres.2020.109580
Reference: FRIN 109580

To appear in: *Food Research International*

Received Date: 7 May 2020
Revised Date: 20 July 2020
Accepted Date: 20 July 2020

Please cite this article as: Patrignani, F., Parolin, C., D'Alessandro, M., Siroli, L., Vitali, B., Lanciotti, R., Evaluation of the fate of *Lactobacillus crispatus* BC4, carried in Squacquerone cheese, throughout the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), *Food Research International* (2020), doi: https://doi.org/10.1016/j.foodres.2020.109580

Evaluation of the fate of *Lactobacillus crispatus* BC4, carried in Squacquerone cheese, throughout the Simulator of the Human Intestinal Microbial Ecosystem (SHIME)

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Abstract

Lactobacillus crispatus strain BC4, isolated from the human healthy vaginal environment and characterised by a strong antimicrobial activity against urogenital pathogens and foodborne microorganisms, was employed as a probiotic culture in the cheesemaking of the soft cheese Squacquerone. Such cheese is intended as a "gender food", that could be used as a hedonistic dietary strategy to reduce the incidence of woman vaginal dysbiosis and infections, given the evidence that a probiotic strain able to survive to the entire digestive process once ingested, can pass from intestine to vagina. This work was aimed to evaluate the resistance of *L. crispatus* BC4, carried in Squacquerone cheese, to different challenges of the human gastrointestinal tract, including the colon stage. The digestion process was tested using a Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). The viability and metabolic activity of *L. crispatus* BC4 during the colon simulation were monitored by qPCR and gas chromatography, respectively, also in the presence of a complex microbiota. The results showed that *L. crispatus* BC4 survival was not affected by the gastric condition, while it was significantly affected by bile salts and pancreatic juice in small intestine conditions, where it decreased of approx. 0.6 log (colony-forming units) CFU/g. Differently, during colon simulation *L. crispatus* BC4 was able to grow in sterile colon conditions and to maintain viability in the presence of a complex microbiota. Moreover, during colon simulation, *L. crispatus* BC4 was metabolically active as demonstrated by the higher production of short chain fatty acids (SCFA) and lactate. In the presence of a complex gut microbiota, a decrease of lactate was observed, due to its conversion into propionate (anti-cholesterol activity) and butyrate (anti-inflammatory activity) by cross-feeding. However, no differences in propionate and butyrate production could be observed between control cheese and cheese containing *L. crispatus* BC4. Despite this may appear as a negative outcome, it must be taken into account that, in this setup, only a single dose of the cheese was tested and the outcome of the colonization and impact of the gut microbiota might be different when daily repeated doses are tested.

Keywords

Functional cheese, *Lactobacillus crispatus* BC4, SHIME®, "gender food", dairy products, probiotic activity, vaginal strain.

1. Introduction

The impact of dairy foods, especially containing probiotic bacteria, on human health has been investigated for many years (Heller, 2001), highlighting scientific evidence for their beneficial effects (Ouwehand, Salminen, & Isolauri, 2002; Tuomola, Crittenden, Playne, Isolauri, & Salminen, 2001; Burns et al., 2015; Patrignani et al., 2019). Particularly, the search for strains gifted of good resistance to the biological barriers of the human gastrointestinal tract (GIT) and suitable technological and functional features can result into new probiotics for tailored functional food products (Ugarte, Guglielmotti, Giraffa, Reinheimer, & Hynes, 2006). However, the fate of probiotic bacteria, when carried in foods, depends on several factors, including food matrix composition, since proteins, fats and carbohydrates can significantly influence microbial growth and survival in the food and in the GIT during digestion (Dommels et al., 2009). Also, physic-chemical treatments applied during food processing (*e.g.* heat treatment, High pressure Homogenization-HPH; Pulsed Electric Fields-PEF, antimicrobials *etc*,) are reported to affect viability and functionality of probiotics during storages and simulated digestion (Muramalla & Aryana, 2010; Tabanelli et al., 2013; Patrignani, Siroli, Serrazanetti & Lanciotti, 2018). Recently, the literature data highlighted also the use of polymers such as alginate, pectin, chitosan, carrageenan, whey, gelatin and lipids for microencapsulation of bacteria with positive effects in protection of probiotic cells during storage condition and GIT environment (Inguva, Ooi, Desai, & Heng, 2015; Patrignani et al., 2017). On the other hand, the survival and functionality of a probiotic, besides being strictly strain-dependent, is reported to vary also in relation to its physiological state (Sumeri, Arike, Adamberg, & Paalme, 2008). Among dairy

products, cheese has been reported to be a good vehicle to deliver viable probiotic bacteria (Boylston, Vinderola, Ghoddusi, & Rheinheimer, 2004; Lanciotti et al., 2004; Settanni & Moschetti, 2010; Burns et al., 2008; Burns et al., 2015) since, if compared with fermented milks, it has lower acidity, high buffering capacity, protein matrix and high fat content which could also protect bacteria against environmental changes during production, ripening and ingestion (Gobbetti, Corsetti, Smacchi, Zocchetti, & de Angelis, 1998; Kailasapathy & Chin, 2000; Phillips, Kailasapathy, & Tran, 2006; Vinderola, Costa, Regenhardt, & Reinheimer, 2002). Moreover, some current exciting papers underline how foods, with their components and microbial communities and probiotics as adjuncts, can modulate the human symbiotic microbes (Patrignani et al., 2019; Prakash, Tomaro-Duchesneau, Saha, & Cantor, 2011; Sanz, 2011) in relation also to the gender differences. In fact, diet and probiotic foods are expected to differently influence the male and female health through the modulation of their symbiotic microbes. However, in order to exert a positive effect, it is mandatory that a probiotic strain arrive alive *in situ*, surviving, thus, to the digestion process. Among probiotics, *Lactobacillus* strains have been used for decades as both food ingredients and supplements, for their ability to treat human dysbiosis and restore healthy conditions. Indeed, lactobacilli are commonly resident in the human gut, and even dominate the vaginal microbiome of premenopausal healthy women, contributing to the maintenance of the ecosystems' eubiosis and preventing pathogen overgrowth, including sexually transmitted agents (Foschi et al., 2017; Heeney, Gareau, & Marco, 2018; Ceccarani et al., 2019; Ñahui Palomino et al. 2019). In the cervicovaginal niche, the presence of *L. crispatus* has been recognised as a hallmark of a healthy and stable status, such role has been related to its capability to produce antimicrobial compounds, such as hydrogen peroxide and lactate, responsible for maintaining a low vaginal pH, and to positively interact with the mucosae and modulate the immune response (Petrova et al. 2015; Anton et al., 2018; Calonghi et al 2018; van der Veer et al 2019). *L. crispatus* strain BC4 was isolated from the vagina of a healthy premenopausal woman (Parolin et al 2015) and widely characterized for its antimicrobial properties and for its metabolic and technological features. Indeed, *L. crispatus* BC4 was proved to possess antagonistic activity towards vaginal pathogens including

Candida albicans (Parolin et al 2015), *Neisseria gonorrhoeae* (Foschi et al. 2017), *Chlamydia trachomatis* (Nardini et al. 2016), Group B *Streptococcus* (Marziali, Foschi, Parolin, Vitali, & Marangoni, 2019), food-borne and gut pathogenic species including *Lysteria* spp., enterococci and coliform bacteria (Siroli et al., 2017). In addition, *L. crispatus* BC4 was characterised for antibiotic susceptibility and tested for technological properties in pasteurized whole milk, before being included in a probiotic cheese (Siroli et al., 2017, Patrignani et al., 2019). In particular, *L. crispatus* BC4 has been employed as adjunct in Squacquerone cheese production, enabling the chance for the production of high quality "gender cheese", that could be used, after proper *in-vivo* trials, as potential hedonistic dietary strategy to reduce the incidence of woman vaginal dysbiosis and infections (Patrignani et al., 2019). The rationale of including a beneficial vaginal strain in a "gender food" is based on the evidence that a probiotic strain able to survive to the entire digestive process once ingested, can pass from intestine to vagina, given to the anatomic proximity of the two apparatus (Vitali et al., 2012; Vujic, Jajac Knez, Despot Stefanovic, & Kuzmic Vrbanovic, 2013). In our recent paper (Patrignani et al. 2019), we have only partially demonstrated the survival and the maintenance of metabolic activity of *L. crispatus* BC4 during the digestion process of the probiotic cheese. In fact, only the survival of the lactobacilli group was described in relation to the digestion steps from gastric to duodenum one. Thus, the principal aim of the present research was to demonstrate the ability of *L. crispatus* BC4, used as probiotic adjunct and carried in Squacquerone cheese, to survive to different simulated biological stresses, from gastric to colon scenario. The simulation was performed by using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). During colon phase, the viability and metabolic activity of *L. crispatus* BC4 were studied also in the presence of a complex microbiota simulating human gut conditions, in order to explore the potential interactions between *L. crispatus* and the other gut microbes.

2. Materials and Methods

2.1 Lactobacillus crispatus BC4 culture conditions and Squacquerone cheesemaking

L. crispatus BC4, a vaginal strain isolated from healthy woman vagina, gifted of good technological (Siroli et al., 2017) and antimicrobial properties (Parolin et al. 2015) was used in this study for the production of Squacquerone cheese, according to the protocol described in Patrignani et al. (2019). Briefly, *L. crispatus* BC4 was grown overnight in de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, UK), at 37°C, in anaerobic jars supplemented with CO_2 gas generating kit (Oxoid). Suspensions were centrifuged at 8,000 × g for 20 min at 4˚C, the bacterial pellet was washed twice with sterile saline (0.9% NaCl in distilled water) and resuspended in commercial milk for the inoculums in the industrial environment. In the present research, two kinds of Squacquerone cheese were produced: i) one supplemented with *L. crispatus* BC4 as adjunct and ii) one without supplementation of *L. crispatus* BC4, used as control cheese. The cheesemaking was carried out in a pilot-scale plant of a local dairy farm (Mambelli, Bertinoro, Italy). A commercial freeze-dried culture of *Streptococcus thermophilus* St 0.20 (Sacco S.R.L., Como, Italy) was used as starter at 6 log CFU/ml *L. crispatus* BC4 was added in cheese at level of 7 log CFU/g (Patrignani et al., 2019). The cheeses were packed in modified atmosphere and stored at 4˚C. All the experiments were carried out on Squacquerone cheeses ripened at 4˚C for 8 days, since, after this period of time, the product is commercially ready for the consumption.

2.2 Evaluation of the fate of L. crispatus BC4 in Simulator of the Human Intestinal Microbial Ecosystem (SHIME®)

The survival of the vaginal strain *L. crispatus* BC4 was evaluated in an adapted SHIME® (ProDigest-Ghent University, Ghent, Belgium) (Van den Abbeele et al., 2012; Van Den Abbeele et al., 2013), following SHIME® patent conditions updated based on the Infogest consensus method (Minekus et al., 2014; Van de Wiele et al., 2015) . This system, composed of a reactor used in a sequential setup to simulate over time first the stomach and then the small intestine and colon, was used for the accurate *in vitro* simulation of the ability of the probiotic bacterium to merge into the intestinal environment (Govender et al., 2014). For this, appropriate retention time and pH were chosen in order

to resemble *in vivo* conditions in the different parts of the gastrointestinal tract in fed state. Control and test cheeses were incubated to simulate gastric, upper intestinal phases, and colon environments. All the experiments were performed in triplicate to account for biological variability. Along the simulation, samples were collected and used to evaluate viable contents of *Lactobacillus* and *L. crispatus* species. The applied conditions used in the simulator are reported in detail in Van de Wiele et al. (2015) and summarized below.

2.2.1 Gastric phase (fed state)

The two types of cheese (2.5 g) were incubated at 37˚C for 2 h in Simulated Gastric Fluid (SGF) added with pepsin (final concentration 1,000 U/mL, with the activity being standardized by measuring absorbance increase at 280 nm of TCA-soluble products upon digestion of hemoglobin (reference protein) while mixing via stirring, with sigmoidal decrease of the pH profile up to pH=2. Phosphatidylcholine was also added, followed by the addition of SHIME® complex nutritional medium; the salt (NaCl and KCl) levels were implemented according to Mackie and Rigby (2015).

2.2.2 Small intestinal phase (fed state)

The small intestine phase was characterized by an incubation of the cheese matrices, after gastric phase, at 37˚C for 3 h in Simulated Intestinal Fluid (SIF), while mixing via stirring. In this step, pH was increased to 7.4. Pancreatic extract (pancreatin, final concentration 100 U/mL) and bovine bile extract (final concentration 10 mM) were supplemented. Along the simulation, the samples resulting from the two different types of Squacquerone cheese were collected at time 0 (initial part of stomach), end of stomach, small intestine after 1, 2 and 3h.

2.2.3 Colon simulation

A subsequent simulation of the proximal part of the colon was then coupled to the upper GIT. After transit in the upper GIT, the test samples entered a sterile (without the inoculum of a complex gut microbiota) simulated proximal colon. According to the SHIME®, the mixing of the digestive slurry in the compartment is obtained with magnetic stir bars. The entire SHIME® system was kept

anaerobic by flushing the headspace of the respective compartments with N_2 gas or a 90/10 % N_2/CO_2 gas mixture. The incubation took place at 37°C for 48 h.

Moreover, a final test has been conducted with a colon simulation in presence of a complex microbiota (control vs. treatment) according to the SHIME® proposed by Van de Wiele et al (2015). During the colonic phase, three samples were considered: i) Vaginal strain alone: the cheese containing the vaginal strain (*L. crispatus* BC4) enters a colonic simulation under sterile conditions (without inoculum of a complex gut microbiota); ii) Control + microbiota: the blank/standard cheese enters a full colonic simulation with a complex microbiota; iii) Vaginal strain + microbiota: the cheese containing the vaginal strain enters a full colonic simulation with a complex microbiota.

Along the colon simulation, in presence or not of a complex microbiota, samples were collected after 24 and 48h. At each end point Short Chain Fatty Acids (SCFA), lactate production and colon acidification were also measured.

2.3 Short chain fatty acid production and pH measurement

The SCFA in the lumen samples of the SHIME were extracted with diethyl ether and analyzed using a gas chromatograph as described by De Weirdt et al. (2010). The sample concentration in mM of acetate, propionate, butyrate, was determined. pH measurements were carried out by a Mettler MP200 pH meter (Mettler Toledo, Columbus, OH, USA).

2.4 Determination of total Lactobacillus and L. crispatus species survival by qPCR

In order to selectively detect the alive fraction of *Lactobacillus* and *L. crispatus* cells, Propidium Monoazide (PMA) treatments, followed by bacterial DNA extraction combined with a molecular detection method (qPCR), were used. Samples were collected during SHIME® simulation for control and *L. crispatus* BC4-added cheeses.

2.4.1 Bacterial DNA extraction from viable cells of SHIME samples

Samples collected during SHIME® simulation were firstly subjected to PMA-treatment following the protocol described in Moens, Duysburgh, van den Abbeele, Morera, & Marzorati (2019). PMA is a photoreactive DNA-binding dye that enters only dead cells and binds to dsDNA. A photoreaction of the chemical is then induced to covalently bond PMA to dsDNA, thus rendering DNA of dead cells non-amplifiable by PCR. Living cells do not react with PMA as they have intact cell membranes, so their DNA can be amplificated by PCR. Briefly, the sample were diluted in 450 µl of sterile anaerobic peptone water, and adding PMA (Biotium, Hayward, CA, USA) to reach a final concentration of 50 µM. Samples were shaken at room temperature in the dark for 5 min, then placed in a LED-Active Blue system (Ingenia Biosystems, Barcelona, Spain) for 15 min followed by centrifugation for 5 min at 7,690×g. Cell pellets were stored at -20°C.

Afterwards, DNA was extracted from PMA-treated cells by using the DNeasy UltraClean Microbial Kit (QIAGEN, Venlo, The Netherlands) according to manufacturer's instructions, DNA was eluted in a volume of 100 µL.

2.4.2 Quantitative PCR for Lactobacillus and L. crispatus species

Real-time PCR was used to quantify total *Lactobacillus* and *L. crispatus* species, according to Vitali et al. (2015), by using a LightCycler instrument (Roche, Mannheim, Germany) and SYBR Green fluorophore. Primer sets targeted to 16S rRNA gene of *Lactobacillus* genus or *L. crispatus* species and thermal cycling conditions were used as described by Vitali et al. (2015). PCR was carried out in a final volume of 14 µL containing each primer at 0.5 µM, 4 µL of LightCycler FastStart DNA Master SYBR Green I (Roche) and 2 µL of template. Different quantities of genomic DNA extracted from *L. crispatus* DSM 20584 were used as standards for PCR quantification; melting curve analysis was carried out to confirm that the PCR products from SHIME® samples and standards had identical melting points.

Data were expressed as log copies of target DNA for samples obtained from stomach and small intestine simulation steps, and as log copies of target DNA per mL of colon liquid for samples obtained from colon simulation.

2.5 Statistical analysis

Three independent trials were set up in SHIME®. All the experimental data are expressed as the mean of six repetitions (2 repetitions x the 3 trials in SHIME®) ± standard deviation. For each kind of determination, the data were statistically analyzed by Statistica software (version 8.0; StatSoft, Tulsa, Oklahoma, USA) and subjected to the analysis of variance (ANOVA) and the test of mean comparison, according to Fisher's least significant difference (LSD), was applied on all obtained data. Level of significance was $p < 0.05$.

3. Results and discussion

The study of the fate of *Lactobacillus crispatus* BC4, when carried in Squacquerone cheese, was performed by SHIME®. According to regulation of FIL-IDF (International Dairy Federation), it is necessary that probiotics maintain in food at least a cell loads of 6 log CFU/g throughout food shelf-life in order to assure the optimal intake level, ranging between 8 and 11 CFU/day, in relation to the probiotic strain, and to exert positive effect on the host (FIL-IDF, 2008; Burns et al., 2015; Patrignani et al., 2019). According to our previous results performed in Squacquerone cheese at the same conditions of the present research, *L. crispatus* BC4 maintained levels of about 7 log CFU/g up to 13 days of cheese refrigerated storage, allowing the intake of 9 CFU/day, consuming a cheese portion of 100g. However, it was necessary to investigate in deep the fate of this strain when carried in Squacquerone cheese in order to prove its viability and metabolic activity during digestion process. The figures 1-2 show, respectively, the log counts of total lactobacilli and *L. crispatus* after the control and test cheese passage in SHIME® at the gastric and small intestine steps. The results were obtained by means of PMA-PCR using specific primers for *Lactobacillus* genus and *L. crispatus* species and enabling to detect viable cells.

The results related to *Lactobacillus* spp. show that control and supplemented cheeses were characterized by initial cell loads of 7.6 and 8.7 log (16S rRNA copy number), respectively. However,

comparing the data with Fig. 2, *L. crispatus* BC4 counted for 6.6 log order in the supplemented cheese, while it was absent in the control cheese. This result it is not surprising since the main *Lactobacillus* population in mature cheese is represented by *L. plantarum, L. casei, L. paracasei* which are able to survive to the initial thermal treatment and they can represent the non-starter lactic acid bacteria (NSLAB) population (Gobbetti, De Angelis, Di Cagno, Mancini, & Fox, 2015). At the end of gastric step, the *Lactobacillus* population characterizing the control cheese decreased at 6.2 log order while the *Lactobacillus* population of the supplemented cheese remained quite stable at level of 8.4 log. Regarding *L. crispatus* BC4 carried in Squacquerone cheese, the transit in the stomach led to a decrease of approx. 0.3 log units, and a further decrease of 0.5 log units was observed after the exposure to simulated small intestine conditions for 60 min. Afterwards, *L. crispatus* BC4 loads remained quite constant at the level of 5.6 log. In the present research, the data showed that the vaginal strain used is weakly affected by the low pH at the end of the gastric condition while it is significantly affected by the presence of bile salts and pancreatic juice in the first 60 min of small intestine (SI) stress condition. On the other hand, also Vamanu (2017) showed significant reductions for different LAB strains in the presence of the enzymes and bile salts related to small intestine stress when their viability was tested in GIS1 simulator. Also, Bianchi et al. (2014) adopted SHIME® to evaluate the fate of *L. casei* Lc-01 inoculated in beverage, showing a significant reduction in the viability of the strain under stomach and duodenum conditions. According to Xanthopoulos, Litopoulou-Tzanetaki, and Tzanetakis (2000), the bile salt resistance varies in significant way among different lactic acid bacteria species and among strains of the same species. According to Saarela, Mogensen, Fondén, Mättö and Mattila-Sandholm (2000), bile salts are toxic to microbial cells because they disrupt the cellular membrane structure and, therefore, bile salt tolerance is considered one of the required characteristics for the survival of lactic acid bacteria in the duodenum. The results obtained in the present research showed that *L. crispatus* BC4 viability was affected by the passage in the duodenum, and it entered the colon at level of about 5.6 log, which corresponds to about 4 log (16S rRNA copy number) per mL of colon liquid in our simulated conditions. However, *L. crispatus*

BC4 was able to multiply in the colon environment or at least to remain stable in presence of a complex microbiota. In fact, following the passage in the upper GIT of the supplemented Squacquerone cheese (containing the *L. crispatus* BC4) and of the control one, the contents of the *ileum* were used to perform a colonic inoculum under sterile conditions and in presence of a complex background microbiota resembling the human gut microbiota.

Fig. 3 shows the total viable lactobacilli detected by PMA-qPCR in the three samples corresponding to the supplemented cheese inoculated in colon without a complex microbiota, the control cheese inoculated in colon with a complex microbiota, and the supplemented cheese inoculated in colon with a complex microbiota. It is possible to observe that the concentration of viable lactobacilli in samples containing *L. crispatus* BC4 at the beginning of the colon simulation is approx. 4.5 log units/mL while the background community is below the detection limit. Along the 48h of incubation under sterile conditions, *Lactobacillus* spp. can grow and increase the concentration up to 6.7 log (16S rRNA copies/mL). The same behaviour was registered for the control sample, in which *Lactobacillus* spp. reached a final concentration of 6.3 log (16S rRNA copies/mL). On the contrary, in the sample represented by the cheese containing the vaginal strain and a background microbiota, it was possible to observe an initial increase of *Lactobacillus* spp. of 0.5 log (16S rRNA copies/mL) followed by a decrease at 4.0 log after 48 h. The data obtained by species specific qPCR confirmed that *L. crispatus* BC4 had a good survival rate and it was able to grow in colon sterile conditions (Fig. 4). In fact, at the end of the colon simulation, after 48 h, 5.1 log out of 6.75 were represented by *L. crispatus* BC4 while it was absent in the control samples without complex microbiota. Differently, when the simulation was performed in the presence of a complex microbiota, *L. crispatus* represented initially 4.1 log out of 4.4 log of lactobacilli detected, and it slightly decreased over time reaching 3.9 log out of 4.0 log of lactobacilli detected at the end of the simulation (Fig. 4). These data confirmed the ability of *L. crispatus* BC4, although with significant reduction with respect to the initial inoculum level in cheese, to survive to simulated gut conditions also in the presence of a complex microbiota, challenging the hypothesises that this strain, gifted with good anti-*Candida* and anti-foodborne

pathogen features (Parolin et al., 2015; Siroli et al., 2017), can be used to reduce vaginal dysbiosis throughout a dietary strategy. Moreover, samples collected from the colon simulation were analysed for changes in pH (Table 1), total and main SCFA (Fig. 5) and lactate production (Fig. 6), to assess the potential metabolic activity of the test product and its impact on the metabolism of the gut microbiota. Table 1 shows that a colonic acidification occurred independently on the presence of a complex microbiota, with no differences among the different conditions of the study. This drop in pH is linked to a saccharolytic activity of the gut microbiota, as shown in Fig. 5 which reports the total SCFA and main SCFA, such as acetic acid, propionic acid, and butyric acid, and Fig. 6 describing the production of lactate, in the presence of a very low proteolityc activities, as shown in Fig. 7 reporting the branched-SCFAs, usually considered marker for proteolysis. During all colonic incubations the concentration of SCFA increased, reaching the highest concentrations in the presence of a complex microbiota (Fig. 5). Moreover, a very mild increase of SCFA occurred between 24 and 48 h in the presence of a complex microbiota. The production of SCFA due to the LAB could be due to the proteolytic and peptidase or esterase activities since they are scarcely gifted of lipolytic potential (Vannini et al., 2008). All the tested cheeses led to a strong production of lactate in the first 24 h of colonic simulation and lactate increase was higher for *L. crispatus* BC4 supplemented cheeses (Fig. 6). After 48 h of colonic simulation, lactate concentration remained constant under conditions characterized by the absence of microbiota, while it decreased in the presence of a complex microbiota, for both the control cheese and the *L. crispatus* supplemented one. Interestingly, the decrease in lactate concentration was correlated to an increase in propionate and butyrate indicating that a cross-feeding reaction occurred between 24-48 h in presence of a complex microbiota (Fig. 5). This is of relevance since also bacteria metabolic activity can represent an important feature for the selection of *L. crispatus* BC4 as a probiotic candidate. In fact, according to Douillard and de Vos (2019), the criteria for probiotic selection include the presence of genes associated to the survival in the GIT, but also the ability to interact with host-bacteria. Our data demonstrated that, when entering the colon, the vaginal *L. crispatus* BC4 strain led to strong lactate production (clear indication that

the strain is still metabolically active). In presence of a complex microbiota, lactate is further converted by cross-feeding into propionate and butyrate, having potentially a significant biological role (Rios-Covian et al 2016). Propionate and butyrate metabolisms have received much attention during the last years, mainly due to their role in diseases and inflammatory processes in which they are involved. For example, a reduction in obesity and insulin resistance in experimental animals on high-fat diet after dietary supplementation with butyrate has been observed (Gao et al., 2009). Moreover, butyrate and propionate have been reported to induce the production of gut hormones, such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are key modulators of energy homeostasis and glucose metabolism, also reducing food intake (Lin et al., 2012). It has also been observed that SCFA protect against the development of colorectal cancer (CRC), with most studies focusing on butyrate (Keku et al., 2015). Butyrate promotes colon motility, reduces inflammation, increases visceral irrigation, induces apoptosis, and inhibits tumor cell progression (Louis et al., 2014). In addition, butyrate and propionate have also been reported to induce the differentiation of T-regulatory cells, assisting to control intestinal inflammation (Louis et al., 2014). As reported by Douillard and de Vos (2019), also the immunomodulation properties represent a criterion for selection of probiotic strains. However, no differences in propionate and butyrate production could be observed between control cheese and cheese containing *L. crispatus* BC4. Notably, the results obtained in the present research are related to a single dose of product, but the outcome of the colonization and impact of the gut microbiota might be different when daily repeated doses are tested. In fact, as reported by many authors (Madison & Kiecolt-Glaser, 2019; Salonen & de Vos, 2014; Sonnenburg & Bäckhed, 2016), diet is one of the most important drivers on the microbiota composition and activity that in turn may have an important systemic impact on the host.

Conclusion

The PMA-qPCR analysis, using specific primers for the target species, showed that the vaginal *L. crispatus* BC4 strain, when vehicolated in cheese carrier, could well withstand the acidic conditions of the stomach with a slight decrease in viable cells. In fact, a cell decrease was observed in the transition between stomach and duodenum indicating that bile salts are, anyway, a source of stress for *L. crispatus* BC4. When entering the colon, the vaginal *L. crispatus* BC4 demonstrated to increase its cell loads during incubation time in the absence of other microrganisms and to remain stable in presence of a complex microbiota. However, it led to strong lactate production (clear indication that the strain is still metabolically active). In presence of a complex microbiota, lactate was further converted by cross-feeding into propionate and butyrate, demonstrating a potential positive effect on the host. However, no differences in propionate and butyrate production could be observed between control cheese and cheese containing *L. crispatus*. Despite this may appear as a negative outcome, it must be taken into account that, in this setup, only a single dose of the cheese was tested and the outcome of the colonization and impact of the gut microbiota might be different when daily repeated doses are tested. Moreover, also the initial inoculum level of the target strain in cheese could be a little bit increased in order to guarantee a higher level of survive during all the GIT passage. However, even if human trials should be set-up to confirm the effective role of *L. crispatus* BC4 to positively interact with the host, these data suggest its potential use to obtain a functional cheese as potential dietary strategy to guarantee the woman well-being.

Acknowledgements

This study was carried out in the framework of the Alma Idea Project "Approcci biotecnologici per la produzione di un alimento di genere—Biotechnological approaches to produce a gender food", coordinated by Francesca Patrignani, University of Bologna, Italy.

Declarations of interest: none

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Figure caption

Fig. 1. Total viable Lactobacilli in upper GIT analyzed by PMA-qPCR data with primers specific for *Lactobacillus* genus. Results for the *L. crispatus* supplemented cheese and the control cheese are shown as average ± standard deviations. The collected samples are relative to time 0 (ST0), end of stomach (ST2), Small intestine after 60 min (SI60), 120 min (SI120) and 180 min (SI180). Column with different letters are significant different ($p < 0.05$).

Fig. 2. Viability of *L. crispatus* in upper GIT analyzed by PMA-qPCR data with primers species-specific for *L. crispatus*. Results for the *L. crispatus* supplemented cheese and the control cheese are

shown as average ± standard deviations. The collected samples are relative to time 0 (ST0), end of stomach (ST2), Small intestine after 60 min (SI60), 120 min (SI120) and 180 min (SI180). Column with different letters are significant different ($p < 0.05$).

Fig. 3. Total viable Lactobacilli in complex gut microbiota analyzed by PMA-qPCR data with primers specific for *Lactobacillus* genus. Results are shown as average ± standard deviations for the *L. crispatus* supplemented cheese in the presence or absence of complex gut microbiota , and control cheese in complex gut microbiota. 0, 24 and 48h represent the sampling points at the beginning and after 24 and 48 of incubation. Column with different letters are significant different ($p < 0.05$).

Fig. 4. Viability of *L. crispatus* in simulated colon analyzed by PMA-qPCR data with primers species-specific for *L. crispatus*. Results are shown as average ± standard deviations for the *L. crispatus* supplemented cheese in the presence or absence of complex gut microbiota, and control cheese in complex gut microbiota. 0, 24 and 48h represent the sampling points at the beginning and after 24 and 48 of incubation. Column with different letters are significant different ($p < 0.05$).

Fig. 5. Total SCFA and main SCFA, namely acetic acid, propionic acid, and butyric acid concentration (mmol/L) occurring along the 48h of colonic simulation. Results are shown as average ± standard deviations. For each detected compound column with different letters are significant different ($p < 0.05$).

Fig. 6. Total lactate concentration (mmol/L) occurring along the 48h of colonic simulation. Results are shown as average ± standard deviations. Column with different letters are significant different ($p < 0.05$).

Fig. 7. Branched SCFA concentration (mmol/L) occurring along the 48h of colonic simulation. Results are shown as average ± standard deviations. Column with different letters are significant different ($p < 0.05$).

Table 1

pH quantification during the 48h of colonic simulation for the 3 conditions of the study. Results are shown as average ± standard deviations. Means followed by different superscript letters a, b are significant different ($p < 0.05$).

	pH during colonic simulation		
	0h	24h	48h
L. crispatus supplemented cheese without complex microbiota	6.41±0.06[a]	5.68±0.06[b]	5.68±0.07[b]
control cheese in complex gut microbiota	6.35±0.05[a]	5.60±0.05[b]	5.71±0.06[b]
L. crispatus supplemented cheese in complex gut microbiota	6.36±0.07[a]	5.61±0.06[b]	5.67±0.05[b]

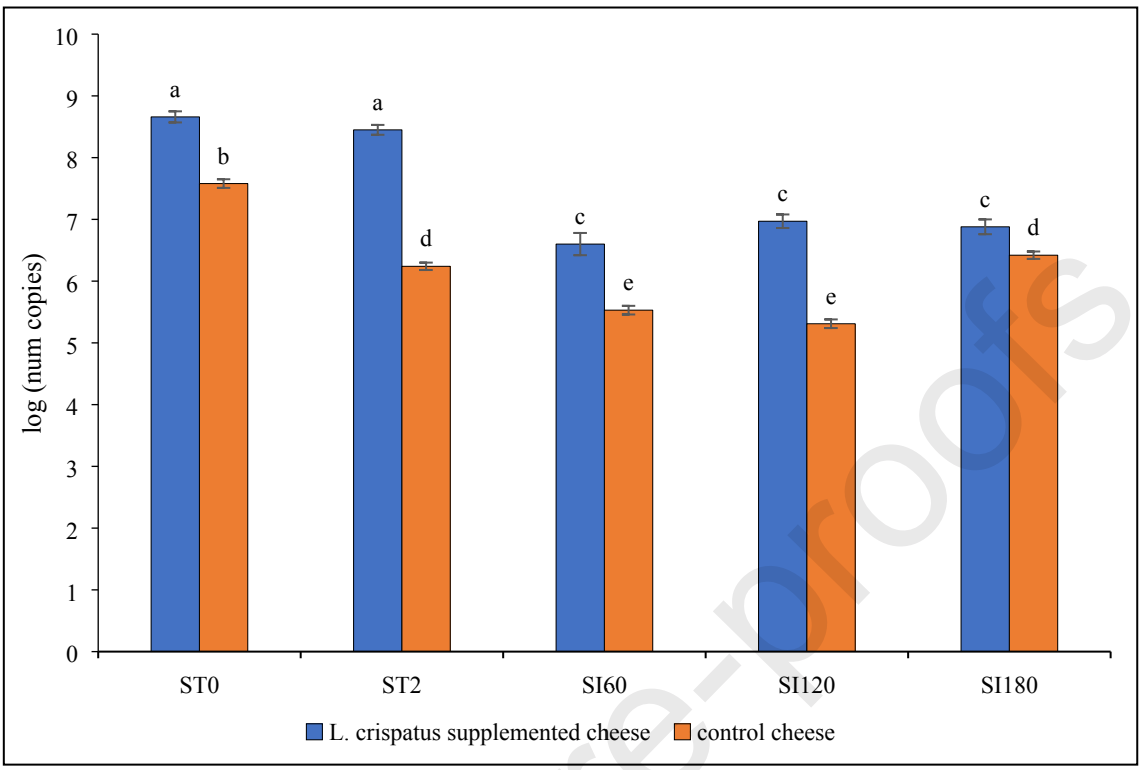


Figure 1.

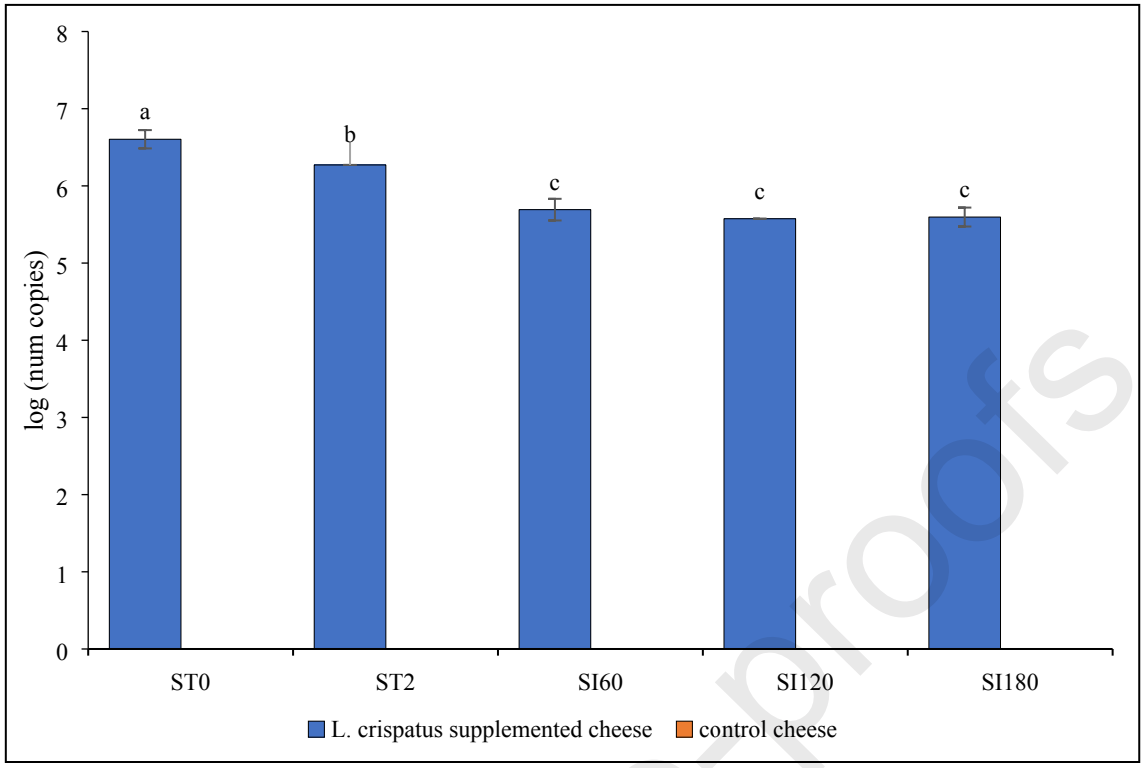


Figure 2.

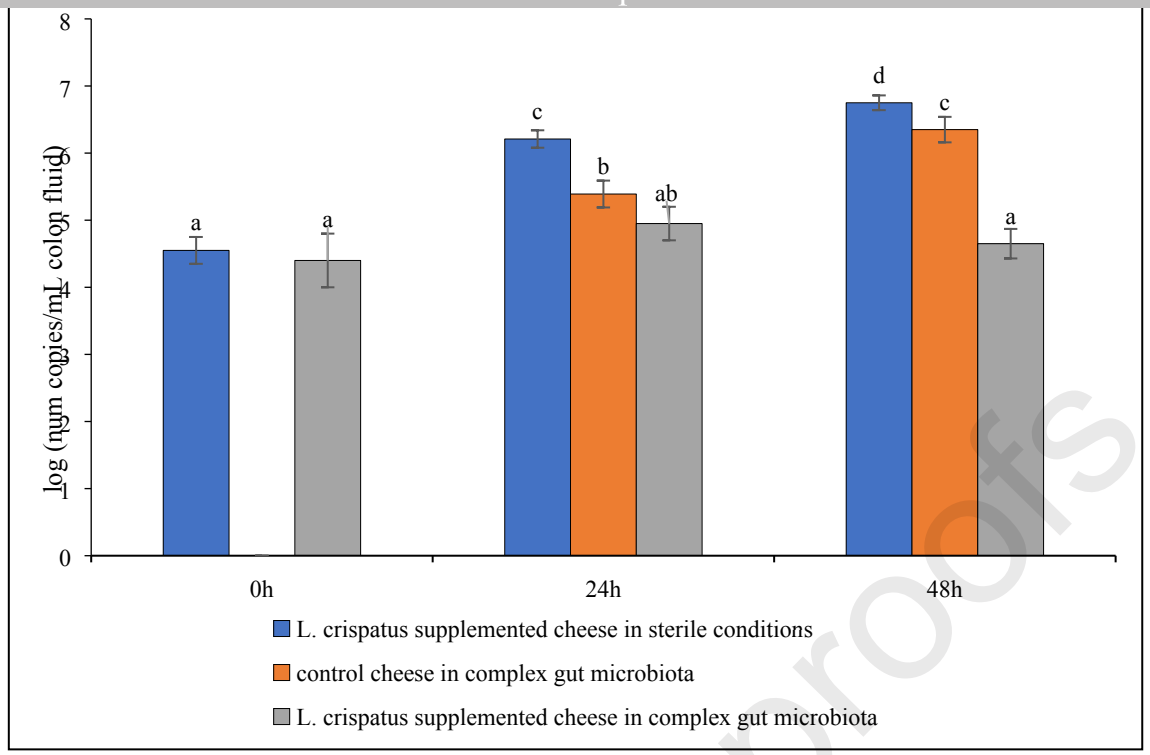
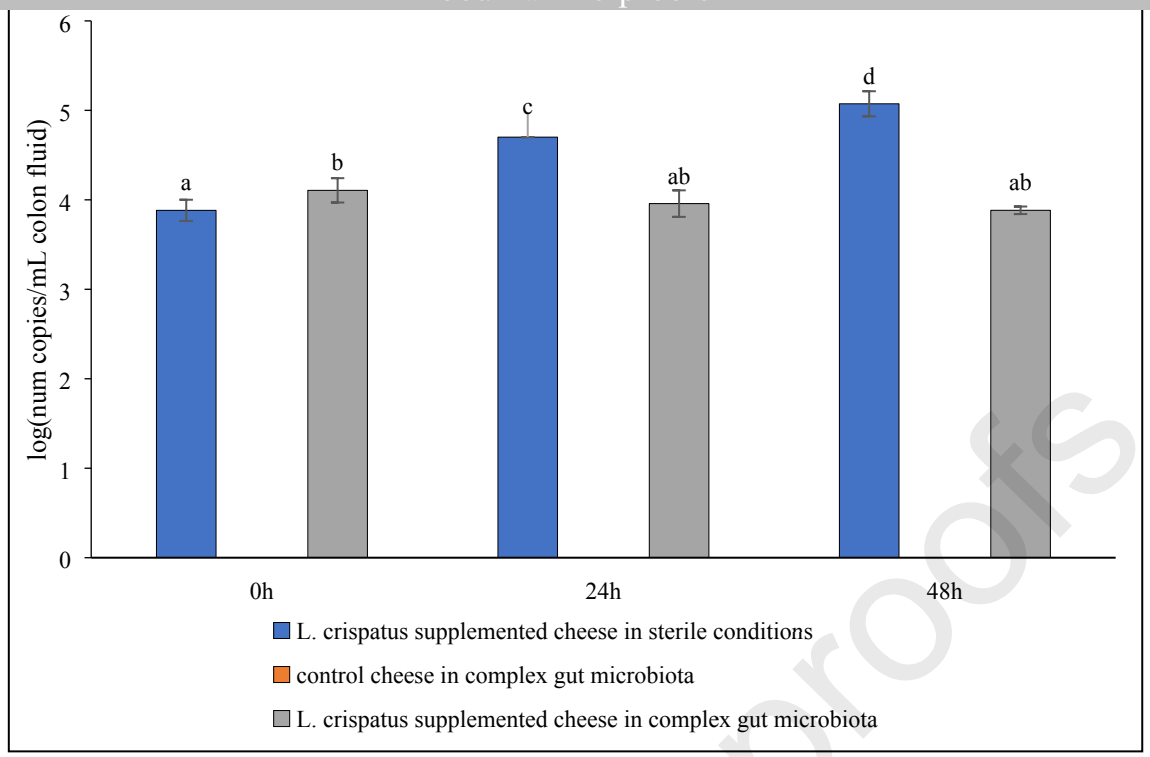


Figure 3.



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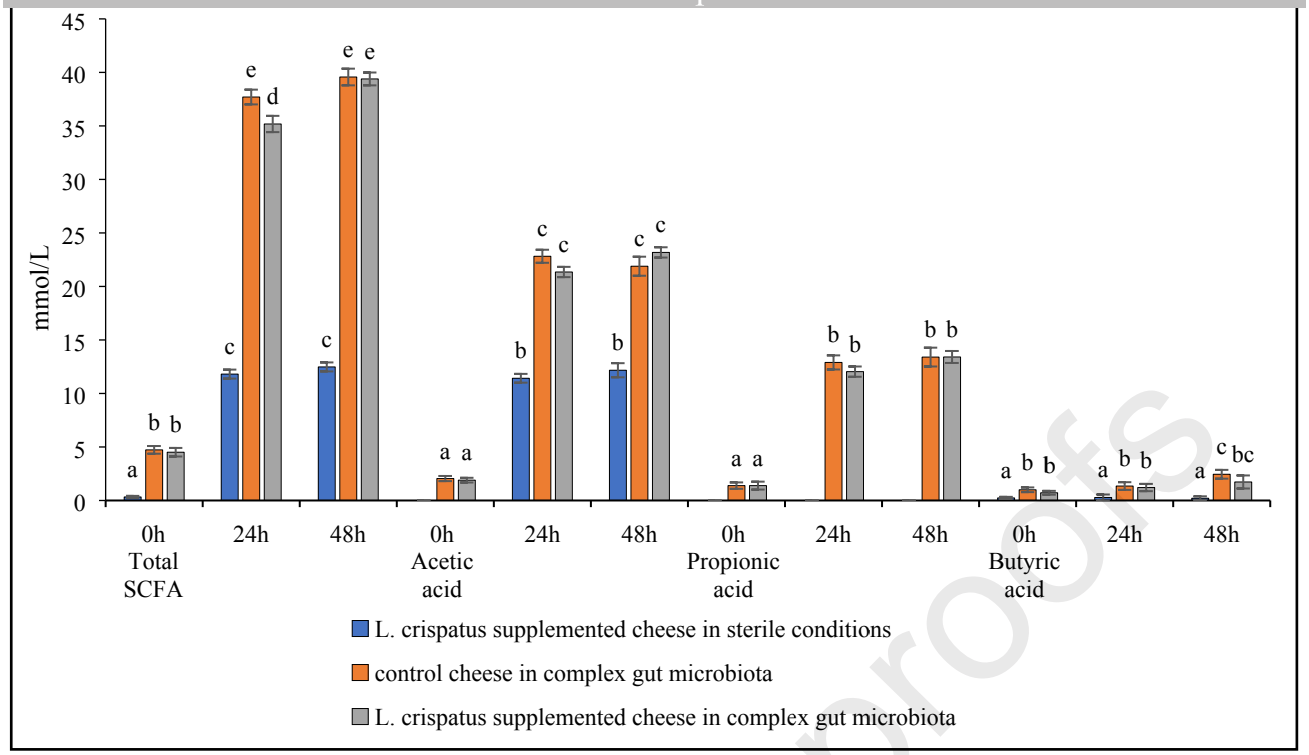


Figure 5.

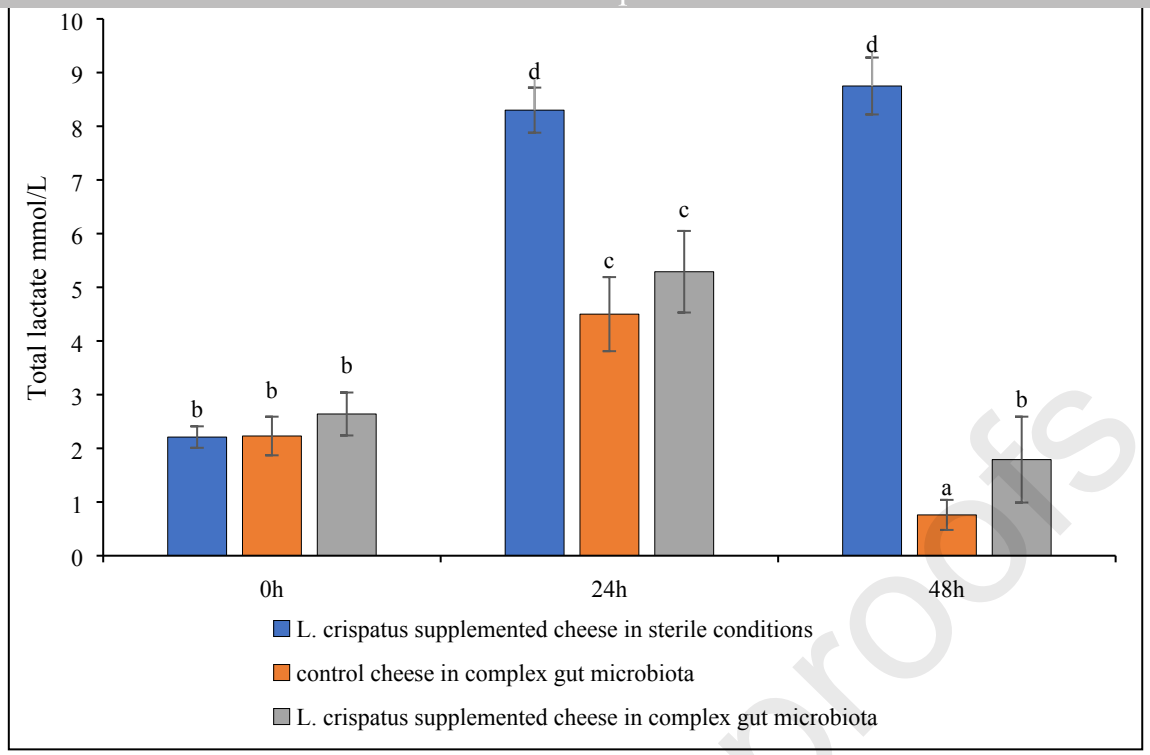


Figure 6.

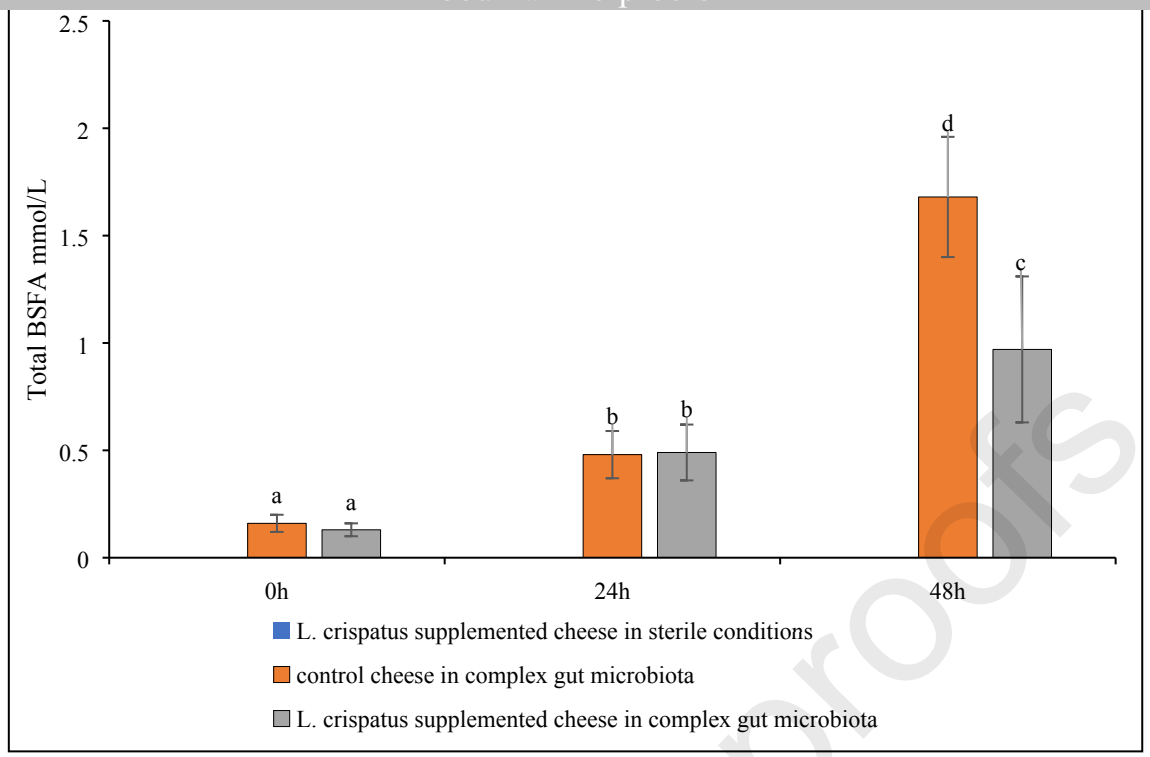
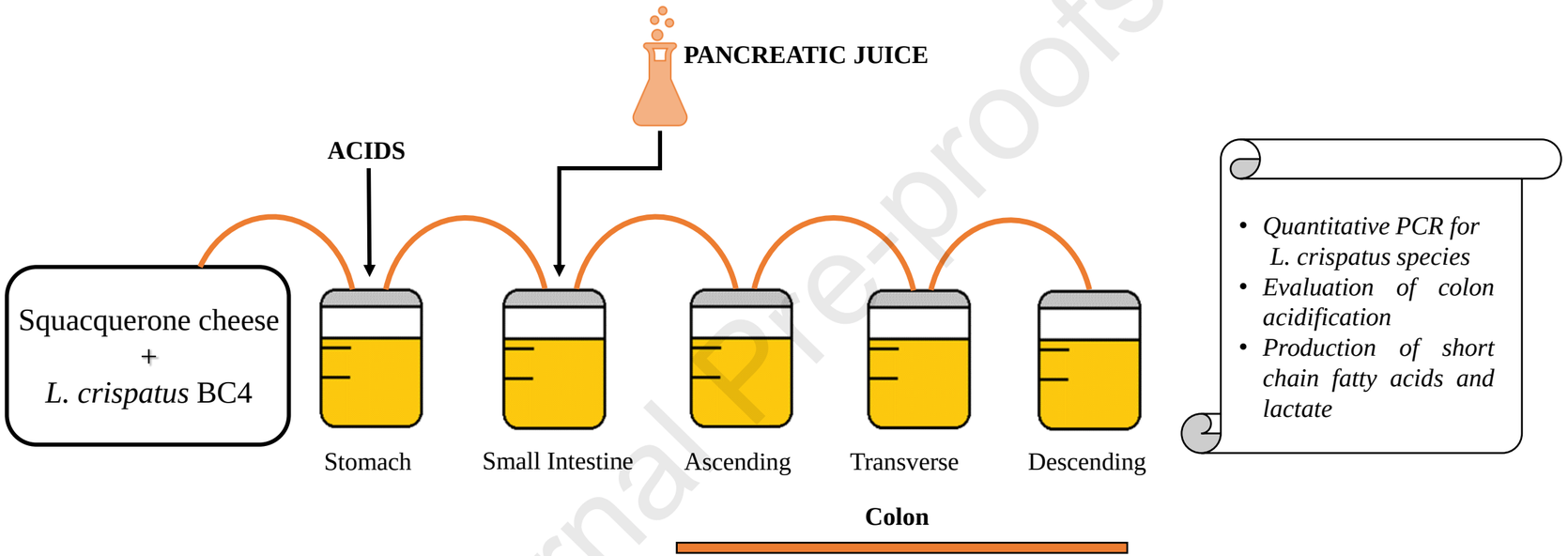


Figure 7.

Graphical Abstract



Highlights

Lactobacillus crispatus BC4 remained metabolically active during SHIME simulation

L. crispatus BC4 maintained viability in presence of a complex microbiota in colon

L. crispatus BC4 produced short chain fatty acids and lactate during colon simulation

L. crispatus BC4 could be a suitable candidate to produce "gender cheese"

Conflict of Interest
No conflict of interest declared.

Credit Author Statement

Francesca Patrignani: Conceptualization and Writing, Funding acquisition

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