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Survivability and metabolic activity of *Lactobacillus casei* 01 incorporating lychee juice plus inulin under simulated gastrointestinal environment

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Abstract

A study of survivability of *Lactobacillus casei* 01 in simulated gastrointestinal environment under three treatment conditions i.e. *L. casei* 01 plus lychee juice, *L. casei* 01 plus lychee juice plus 3% inulin and *L. casei* 01 plus lychee juice plus 5% inulin was investigated, while the control, *L. casei* 01 in the colon fluid also run simultaneously. It was found that *L. casei* 01 combined with lychee juice plus inulin had significantly increased the survival cells in the gastric-bile environment. This treatment condition also significantly enhanced the growth of colon lactobacilli and benificial bifidobacteria after fermenting for 24 h but suppressed the growth of harmful microbes including fecal coliforms, clostridia and total anaerobic bacteria. Moreover, this specific treatment also stimulated significant increase of total short chain fatty acids (SCFA) in the colon section. Among all SCFA, acetate was the predominant metabolic product followed by propionate and butyrate respectively.

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Introduction

Lactic acid bacteria, Gram-positive bacteria that were present in large numbers among normal animal and human gastrointestinal flora, are some of the most widely-used probiotics in several daily products and fruit drinks (García-Fontán *et al*., 2006; Penna *et al*., 2007; Rivera-Espinoza and Gallardo-Navarro, 2010; Chaikham *et al*., 2012, 2013; Chaikham and Apichartsrangkoon, 2012, 2013). According to the definition of Food and Agriculture Organization of the United Nations/World Health Organization (2002), probiotics are live microorganisms that confer a health-benefit on the host when administered in adequate amounts. Numerous health-promoting effects of probiotic administration are identified, for instance, maintenance of normal intestinal gut microflora, control of inflammatory bowel diseases, protection against gastrointestinal pathogens, enhancement of the immune system, reduction of lactose intolerance, reduction of serum cholesterol level and blood pressure and anti-carcinogenic activity as well as improved utilization of nutrients and nutritional values of food (Lourens-Hattingh and Viljoen, 2001; Chaikham *et al.*, 2012, 2013). Probiotics, i.e. *Lactobacillus casei* strains, have been shown to have probiotic properties and their positive effects can occur both on metabolic and protective levels. On the metabolic level, these strains are involved in the production of beneficial short chain fatty acids (SCFA) including acetate, propionate and butyrate (Gibson *et al.*, 1989; Nowak and Libudzisz, 2006). A commercial beverage containing *L. casei* strain Shirota had been shown to inhibit the growth of *Helicobacter pylori* (Cats *et al*., 2003). Some *L. casei* might be effective in alleviation of gastrointestinal pathogenic bacterial diseases (Hudault *et al.*, 1997).

Nowadays, it is interesting in developing fruit juice based functional beverages with probiotics because they have taste profiles that are appealing to all age groups and because they are perceived as healthy and refreshing foods (Sheehan *et al.*, 2007). Lychee (*Litchi chinensis* Sonn), the most popular fruits of northern Thailand, is an excellent source of 40 - 90 mg/100 g ascorbic acid (Menzel, 2002), 16.8 g/100 g total sugar content (Chan *et al*., 1975) and 0.8 mg/g total phenolic compounds (Somsang, 2007), thus to add value to its juice by fortification with probiotics is a challenging implementation for this research area.

The influences of probiotics in several foods through *in vitro* gastrointestinal environments were investigated by several researchers. For instance, Chaikham *et al*. (2012, 2013) simulated oral administration encapsulated *Lactobacillus acidophilus* LA5 or *L. casei* 01 combined with pasteurized or pressurized longan juices, through the dynamic gastrointestinal tract. They found that both microbes in processed longan juices simulated the growth of several beneficial bacteria

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including lactobacilli and bifidobacteria in the colon compartments, and enhanced a decrease of fecal coliforms and clostridia. In addition, both encapsulated probiotics led to significant formation of lactic acid and SCFA, such as propionate, butyrate and acetate. Similar results were found by Possemiers *et al.* (2010) with probiotic *Lactobacillus helveticus* CNCM I-1722 and *Bifidobacterium longum* CNCM I-3470 immobilized in chocolate.

To improve the growth of these probiotics, prebiotics such as a short chain inulin could be utilized by *Bifidobacterium* species (Biedrzycka and Bielecka, 2004; Rossi *et al.*, 2005). Van de Wiele *et al*. (2007) fed inulin into the Simulator of the Human Intestinal Microbial Ecosystem (SHIME reactor) and found that inulin exerted prebiotic effects with significantly higher butyrate and propionate formation and stimulating the growth of lactobacilli and bifidobacteria in distal colon compartments. Thus, the objective of this research was to investigate the survivability and metabolic activity of *L. casei* 01 incorporated in lychee juice plus inulin (3% and 5%) on the exposure to the simulated stomach, small intestinal and colon environments.

Materials and Methods

Probiotic strain and growth condition

L. casei 01 was purchased from Chr. Hansen (Hørsholm, Denmark). Ten grams of dried probiotic cells were rehydrated in 100 ml de Man Rogosa and Sharp (MRS) broth (HI-Media, India) for 10 min at room temperature and incubated anaerobically at 37°C for 24 h using Anaerocult® C system (Merck, Germany). Stock culture was prepared by mixing the incubated MRS-grown culture with 25% (v/v) sterile glycerol (Merck) and placing 1 ml of the culture fraction into sterile cryogenic vials, and then kept at -80°C until use. One vial of stock cultures that was thawed at room temperature for 30 min was transferred into 100 ml MRS broth and consequently incubated at 37°C for 24 h in anaerobic environment using anaerobic system for recovery the cells. Subsequently, 1% (v/v) recovered culture was inoculated into MRS broth and incubated anaerobically at 37°C for 12 h to achieve its early stationary stage. After incubation, probiotic cells were harvested and washed twice with 0.85% (w/v) sterile saline water by centrifugation at 4,000 rpm at 4°C for 15 min. The cell pallet was diluted to provide a bacterial concentration of 10^{11} CFU/ml by saline water.

Preparation of lychee juice incorporating probiotic

The lychee fruit (cv. Hong Huay) was purchased from an orchard in Chiang Mai, Thailand. Its juice was extracted using a juice extractor (MJ-68M; Panasonic, Thailand). Subsequently, 250 ml of separated lychee juice or lychee juice plus inulin (3% and 5%, w/v) was packed in a retort pouch and sterilized at 121°C for 3 min. The reason for sterilizing lychee juice in this experiment was to assure that all existent microbes were completely eliminated, so that no wild microbes left to interfere in the simulated gut system. The lychee juice has pH 3.48 ± 0.02 and total soluble solids 17.63 ± 0.22°Brix.

Simulated stomach and small intestine experiments

Gastric, duodenal and bile fluids were prepared according to the method described by Oomen *et al.* (2003) and Chaikham *et al.* (2013). One milliliter of *L. casei* 01 was inoculated into 100 ml gastric fluid (pH 1.4) or the mixture of 50 ml gastric fluid plus 50 ml sterile lychee juice which had been transferred into sterile glass bottles sealed with a rubber cap and a metal ring. The mixed solution was incubated anaerobically at 37°C with gentle shaking at 100 rpm for 30 min. After the incubation period in simulated stomach, 45 ml duodenal fluid plus 50 ml bile fluid (pH 8.1) were fed into the system (pH 6.5 - 6.9), allowed further incubation for 1 h. For the viable counts either in stomach or small intestine sections, 1 ml of incubated fluid was withdrawn and diluted with 9 ml peptone water (HI-Media). Several dilutions were made and plated on MRS agar (HI-Media). The colonies were appeared after the incubation in anaerobic condition at 37°C for 48 - 72 h.

Experiment of simulated colon section

Preparation of carbohydrate-based medium

The carbohydrate-based medium was prepared according to Van de Wiele *et al.* (2004) and Chaikham *et al.* (2012, 2013) by mixing the following substances; 4 g potato starch (O.V. Chemical, Thailand), 1 g (+)-arabinogalactan (Sigma, USA), 2 g pectin (Sigma), 1 g xylan (Sigma), 0.4 g D-(+)-glucose (Sigma), 3 g yeast extract (Oxoid, UK), 1 g special peptone water (Oxoid), 4 g mucin (Sigma) and 0.5 g *L*-cysteine (Sigma) and dissolved in 1 L deionized water. This medium was then sterilized at 121°C for 15 min and adjusted with either 0.5 M HCl or 0.5 NaOH to pH 5.5.

Preparation of fecal fluid

The fecal fluid was prepared by collecting fecal sample from a healthy adult volunteer (aged 30 years) who had no history of antibiotic treatment in the last 6 months. A 25 g of freshly fecal sample was diluted with 150 ml of 0.7 M phosphate buffer (pH 7; Merck), containing 1.5 g sodium thioglycolate (Sigma) as a

reducing agent, subsequently homogenized for 10 min using stomacher and centrifuged at 3,000 rpm for 3 min. The supernatant was separated to use as "fecal fluid" (Chaikham *et al.*, 2012).

Batch experiment in colon section

The colon fluid was prepared by mixing 50 ml fecal fluid with 50 ml carbohydrate-based medium in a sterile bottle to use as "colon fluid" for a control condition. For a treatment condition, the colon fluid was a mixture of 50 ml fecal fluid, 25 ml carbohydrate-based medium and 25 ml sterile lychee juice. Three treatment conditions defined as treatment 1, 2 and 3 were *L. casei* 01 plus lychee juice, *L. casei* 01 plus lychee juice plus 3% inulin and *L. casei* 01 plus lychee juice plus 5% inulin respectively. One milliliter of diluted cell pallet (~10^8 CFU/ml) was inoculated into the sterile bottles filled with 100 ml colon fluids, subsequently flushed with nitrogen gas to simulate an anaerobic environment. All simulated colon bottles were then incubated at 37°C for 24 h with gentle shaking at 100 rpm. During the incubation period, the colon fluids were collected at the initial state, 6, 12 and 24 h for further analysis.

Determination of short chain fatty acids

SCFA were extracted as described by Chaikham *et al.* (2012) with some modifications. Two milliliters of sample were mixed with 0.4 g NaCl and acidified by adding 0.5 ml H_2SO_4 conc. (Merck). A 0.1 ml 2-methylhexanoic acid (internal standard) (2.8 ml in 1 L deionized water; Fluka, Switzerland) and 2 ml diethylether (Merck) were added, and subsequently SCFA were extracted using a shaker for 10 min and centrifuged at 1,000 rpm for 5 min. One microlitter aliquot of the diethylether layer (top layer) was injected and measured with a Shimadzu GCMS-QP2010 gas chromatography (Shimadzu Cooperation Analytical & Measuring Instruments Division, Japan) equipped with a flame ionization detector. The gas chromatograph was equipped with a capillary free fatty acid-packed column (25 m x 0.53 mm, film thickness 1.2 µm: Superchrom, Italy). Nitrogen was used as the carrier gas at a flow rate of 20 ml/min. The column temperature was 130°C and the temperature of the injection port and detector was 195°C.

Enumeration of colon microbial communities

Decimal dilutions in physiological solution of samples from the colon section were plated on 5 types of selective media as follows; LAMVAB agar (Hartemink *et al.*, 1997) for colon lactobacilli (72 h, anaerobic), McConkey agar (Oxoid) for total coliforms (24 h, aerobic), TSC-agar (Merck) containing *Clostridium perfringens* selective supplement (Merck) for clostridia (24 h, anaerobic), RB-agar (Hartemink *et al.*, 1996) for bifidobacteria (96 h, anaerobic) and BHI-agar (Oxoid) for total anaerobes (24 h). All plated media were subsequently incubated at 37°C for colony counting.

Data analysis

All data were the means of triplicate determinations with standard deviations (means ± SD). Analysis of variance (ANOVA) was carried out using SPSS Version 15.0, and determination of significant differences among treatment means was done by Duncan's multiple range tests ($P \leq 0.05$).

Results and Discussion

Survival of probiotic during incubation in simulated gastrointestinal fluids

Table 1 illustrates the survival of *L. casei* 01 during incubation in simulated gastric and small intestinal juices. The cells of this probiotic in the control batch (colon fluid) were completely eliminated after administering for 45 min, but their survivability could be prolonged up to 60 min under the environment of lychee juice (treatment 1). This might be due to the presence of sugar (total soluble solids 17.63°Brix) in lychee juice providing more carbohydrate source to the microbes. With the addition of 3% and 5% inulin (treatments 2 and 3), the survivability of *L. casei* 01 could be substantially restored and the cells could survive up to 90 min, although significant ($P \leq 0.05$) reducing rate during the incubating period was observed. It was worth noting that higher amount of prebiotic like 5% inulin (treatment 3) gave rise to significantly ($P \leq 0.05$) increasing viable cells. Besides this fructo-oligosaccharide, other carbohydrate resources such as lactulose also has some impact on the survivability of probiotic cells. Hernandez-Hernandez *et al.* (2013) reported an increase of survival rate of *Lactobacillus bulgaricus* ATCC7517 and *Lactobacillus plantarum* WCFS1 in simulated gastric and small intestinal juices containing prebiotic lactulose after incubation at 37°C for 1 and 3 h. Similar results were obtained by Pitino *et al.* (2010) with *Lactobacillus rhamnosus* ssp. Additionally, Wichienchot *et al*. (2010) demonstrated that soluble fibers from pitaya (dragon fruit) also had a prebiotic function with *Bifidobacterium bifidum* and *Lactobacillus delbrueckii* strains.

Effect of probiotic combinded with lychee juice and inulin on microbial communities and its metabolic products in simulated colon section

Table 2 shows that at the initial state lactobacilli count in the control batch consisted of colon fluid was

Table 1. Survival cells of *L. casei* 01 in simulated gastric juice and sequential following small intestinal juice incubated at 37°C

Incubation times (min)	Treatment conditions			
	L. casei 01	Treatment 1	Treatment 2	Treatment 3
	Simulating stomach environment (CFU/ml)			
Initial state ^{ns}	$6.24\pm0.65^{a}x10^{10}$	$5.93\pm1.03^{a}x10^{10}$	$6.11\pm0.48^{a}x10^{10}$	$5.84\pm0.82^{a}x10^{10}$
15	$1.66\pm0.44^{bC}x10^{6}$	$4.21\pm1.11^{bB}x10^{6}$	$5.20\pm0.65^{bA}x10^{7}$	$5.92\pm0.60^{bA}x10^{7}$
30	$2.02\pm0.54^{cD}x10^{4}$	$6.45\pm0.42^{cC}x10^{5}$	$1.79\pm0.88^{cB}x10^{6}$	$6.25\pm0.71^{cA}x10^{6}$
	Simulating small intestinal environment (CFU/ml)			
45	$9.80\pm2.10^{dD}x10$	$4.03\pm0.55^{dC}x10^{2}$	$1.03\pm0.40^{dB}x10^{4}$	$5.88\pm0.52^{dA}x10^{4}$
60	not detected^{eD}	$9.90\pm0.30^{eC}x10$	$1.05\pm0.45^{eB}x10^{3}$	$8.12\pm1.30^{eA}x10^{3}$
90	not detected^{eC}	not detected^{fC}	$6.21\pm0.40^{fB}x10^{2}$	$9.64\pm0.82^{fA}x10^{2}$

Means in the same column or row followed by the same lowercase or capital letters indicate non-significant difference (P > 0.05). Each data point is the average of three replications. (ns = non-significant difference, treatment 1 = *L. casei* 01 plus lychee juice, treatment 2 = *L. casei* 01 plus lychee juice plus 3% inulin and treatment 3 = *L. casei* 01 plus lychee juice plus 5% inulin)

increased markedly ($P \leq 0.05$) by 3 log cycles after incorporating *L. casei* 01 in the system (treatment 1). This probiotic enhanced a significant ($P \leq 0.05$) increase of colon lactobacilli in every treatment condition up to 12 h of incubation, beyond this period a drawback situation occurred by significantly ($P \leq 0.05$) reducing cell numbers at the final states in every treatment. This presumably caused by exhausting nutrients along with an accumulation of toxic substances such as lactic acid or SCFA which could injure the colon lactobacilli. When considering treatment 3, the quantity of colon lactobacilli significantly ($P \leq 0.05$) increased in every incubating time compared with other treatments. This also affirmed the strengthening function of prebiotic inulin. The finding of Van de Wiele *et al.* (2007) and Kontula *et al.* (1998) supported that inulin combined with oat bran could raise the numbers of lactobacilli in the simulated colons. Impact of various probiotic strains on colon lactobacilli community has been reported by several researchers. For instance, Possemiers *et al.* (2010) fed probiotics *L. helveticus* CNCM I-1722 and *B. longum* CNCM I-3470 immobilized in chocolate into a gastrointestinal model and observed that colon lactobacilli were stimulated by at least 1 log cycle after fermentation in the colon compartments. Identical results were also reported by Maillard and Landuyt (2008) for encapsulated *Lactobacillus* Rosell-52 and *Bifidobacterium* Rosell-175.

SCFA are the major end-products of bacterial fermentation in the gastrointestinal tract. Figure 1 exhibits the influence of different treatments on formation of SCFA in the simulated colon batch. Every SCFA in all treatments including the control noticeably increased ($P \leq 0.05$) in comparison with its initial states. In particular, the addition of inulin gave rise to the highest rate of SCFA formation. Among all SCFA, acetate was the major metabolic product of every treatment followed by propionate and butyrate. Usually, acetic acid is the metabolic product of colon lactobacilli and/or bifidobacteria (Chaikham

Table 2. Viable cells of colon lactobacilli in simulated colon section incubated at 37°C for 24 h

Treatment conditions	Incubating times (h)			
	Initial state (CFU/ml)	6 (CFU/ml)	12 (CFU/ml)	24 (CFU/ml)
Control	$4.88\pm0.33^{bC}x10^{3}$	$6.42\pm0.84^{cB}x10^{3}$	$8.28\pm0.75^{cA}x10^{3}$	$5.22\pm1.45^{cB}x10^{3}$
Treatment 1	$2.97\pm0.82^{aD}x10^{6}$	$6.28\pm0.26^{bB}x10^{6}$	$9.11\pm0.45^{bA}x10^{6}$	$4.92\pm0.49^{bC}x10^{6}$
Treatment 2	$3.02\pm0.61^{aC}x10^{6}$	$6.14\pm1.34^{bB}x10^{6}$	$9.05\pm1.32^{bA}x10^{6}$	$6.02\pm0.52^{bB}x10^{6}$
Treatment 3	$2.71\pm0.34^{aC}x10^{6}$	$8.70\pm0.59^{aB}x10^{6}$	$1.61\pm0.41^{aA}x10^{7}$	$9.20\pm0.40^{aB}x10^{6}$

Means in the same column or row followed by the same lowercase or capital letters indicate non-significant difference (P > 0.05). Each data point is the average of three replications. (treatment 1 = *L. casei* 01 plus lychee juice, treatment 2 = *L. casei* 01 plus lychee juice plus 3% inulin and treatment 3 = *L. casei* 01 plus lychee juice plus 5% inulin)

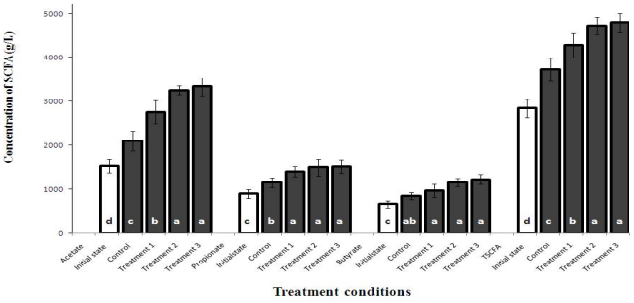

Figure 1. Formation of SCFA in colon section during fermentation at 37°C for 24 h, means in the same SCFA set followed by the same lowercase letters indicate non-significant difference (P > 0.05). Each data point is the average of three replications (solid bars = fermented 24 h, non-filled bars = initial states, treatment 1 = *L. casei* 01 plus lychee juice, treatment 2 = *L. casei* 01 plus lychee juice plus 3% inulin and treatment 3 = *L. casei* 01 plus lychee juice plus 5% inulin)

and Apichartsrangkoon, 2013), since these bacteria produce mainly acetate through pentose phosphate or bifidus shunt pathways (Zani *et al*., 1974; Fernando *et al*., 2010). For propionate and butyrate, they are normally synthesized by other bacteria in the colon including *Anaerostipes caccae*, *Eubacterium halli*, *Megasphaera elsdenii*, *Roseburia* ssp., *Veillonella* ssp. and *Selenomonas* ssp. (Chaikham *et al*., 2013). Several researchers had noticed an increase of SCFA after inoculating various probiotic strains or prebioitc types into the simulated colon systems. For instance, Van de Wiele *et al.* (2004) revealed that the formation of butyrate and propionate significantly rose after the fermentation of probiotics along with chicory inulin in the gut simulator. Marzorati *et al.* (2010) observed a significant increase of SCFA in all colon compartments during treatment with two commercially-available plant polysaccharide supplements. Additionally, Fernando *et al.* (2010) fermented fecal cultures along with various probiotic strains including *L. rhamnosus* ATCC 7469, *Lactobacillus acidophilus* ATCC 11975, *Bifidobacterium breve* ATCC 15700 and *B. longum* ATCC 15707. They found substantial formation of acetate up to 50 - 85% of the total SCFA, while the formation of butyrate and propionate was comparatively less. Van de Wiele *et al.* (2004) stated that the increase of SCFA created more acidic

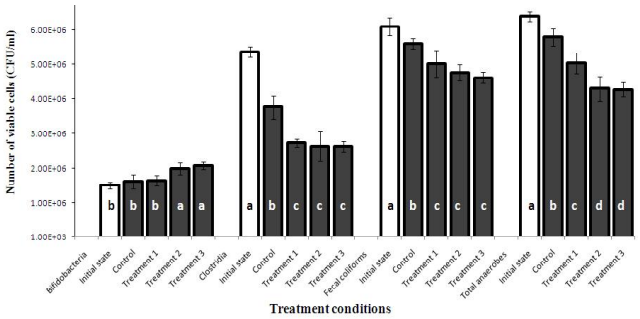

Figure 2. Other microbial communities determined in the colon section during fermentation at 37°C for 24 h, means in the same probiotic group followed by the same lowercase letters indicate non-significant difference ($P > 0.05$). Each data point is the average of three replications. (solid bars = fermented 24 h, non-filled bars = initial states, treatment 1 = *L. casei* 01 plus lychee juice, treatment 2 = *L. casei* 01 plus lychee juice plus 3% inulin and treatment 3 = *L. casei* 01 plus lychee juice plus 5% inulin)

environment to the colon which was important in terms of colonizing resistance against pathogens *in vivo*. In this study, the significant increase of each SCFA compared with its control was observed especially the treatments including inulin. Acetate serves as energy resource of liver and peripheral tissues and also acts as signaling molecules in metabolic pathways of gluconeogenesis and lipogenesis (Zambell *et al*., 2003). Propionate was believed to lower lipogenesis, serum cholesterol levels and carcinogenesis in colon tissues (Scheppach *et al*., 1995; Hosseini *et al*., 2011), while butyrate could protect colon from inflammatory bowel disease (Van Immerseel *et al.*, 2010).

When considering the impact of probiotic and lychee juice plus inulin on other bacterial communities, Figure 2 elucidates the development of beneficial and harmful bacteria in the colon section. Although bifidobacteria were the least amount in comparison with other bacterial communities, after fermentation for 24 h, significant ($P \leq 0.05$) increase of these viable cells could be observed especially in treatments 2 and 3. On the other hand, pathogenic bacteria such as fecal coliforms, total anaerobes and clostridia in all treatments after the fermentation were significantly decreased ($P \leq 0.05$) compared with their initial states. Treatments 2 and 3 were the least reduction which was an indirect impact of inulin strengthening the growth of *Lactobacillus* community to colonize the colon or suppressing the growth of other bacteria. A study of Van de Wiele *et al.* (2004) who found that chicory inulin had significant influence on the growth of lactobacilli and bifidobacteria in a human gut reactor. Gmeiner *et al.* (2000) fed *L. acidophilus* 74-2 plus prebiotic fructo-oligosaccharide in milk-based product into the gut reactor. They found bifidobacteria counts markedly increased in all colon compartments, while *Escherichia coli* and enterobacteria counts apparently decreased. Chaikham *et al.* (2012) noted that lactobacilli and bifodobacteria could produce antimicrobial low-molecular-weight substances such as lactic acid, various SCFA and hydrogen peroxide to inhibit various harmful bacteria.

Conclusions

L. casei 01 in lychee juice could be survived in the simulated gastrointestinal environment for 45 min, while the addition of inulin could prolong its survivability. For the experiment in colon section, it was found that *L. casei* 01 in lychee juice plus inulin had significantly enhanced the growth of colon lactobacilli and bifidobacteria after fermenting for 24 h but suppressed the growth of harmful microbes including fecal coliforms, total anaerobic bacteria and closteria. Colon microorganism had produced beneficial SCFA such as acetate, propionate and butyrate in which acetate was the predominant metabolic product followed by propionate and butyrate. This specific treatment also stimulated significant increase of total SCFA in the colon section.

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