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Investigation of *Saccharomyces cerevisiae* var. *boulardii*-enriched yogurt fermentation

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Abstract. *Saccharomyces cerevisiae* var. *boulardii* (also known as *Saccharomyces boulardii*) is the only probiotic yeast with the ability to tolerate the low pH condition of human stomach and the effect of bile salts in man and animal intestines. Rather than bacteria, *S. boulardii* has been reported to ameliorate antibiotic-associated disorders. Inclusions of *S. boulardii* in food system are increasingly investigated to develop health-promoting products. This research aimed to examine the effect of *S. boulardii,* as starter culture, in interaction with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yogurt fermentation. *S. boulardii* cells were prepared in YMA agar at 30°C for 48 h. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were isolated and cultivated under anaerobic condition in MRS agar at 37°C for 72 h and in M17 agar at 37°C for 48 h, respectively. Results showed that the yeast reached live cells density of $\geq 10^6$ cfu/g in yogurt after fermentation. The presence of *S. boulardii* insignificantly affected the growth of lactic acid bacteria and vice versa. In addition, the yeast showed insignificant effect on the fermentation parameters including time, pH and the content of lactic acid accumulated in the prepared yoghurt. However, at the cell density of $\geq 10^7$ cfu/g, *S. boulardii* likely cause bubble-structure and alcohol-flavour to the yogurt due to CO_2 and ethanol production. The effect of fermentation temperature showed that yeast cell density reduced 0.12 log cfu/g after 4.5 h of yogurt fermentation. Over the transit in simulated gastric fluid, the survivability of *S. boulardii* showed a reduction to 42–43% after 30 min. However, in the simulated intestinal fluid, the density of in-yogurt-yeast cells significantly increased up to 94% of the original load, whereas the survivability of the free cells continued to reduce to 34%.

Keywords: *Lactobacillus bulgaricus, Saccharomyces boulardii*, *Streptococcus thermophilus,* Survivability, Yogurt fermentation

1. Introduction

According to FAO/WHO [1], any food product containing probiotic must have at least 10^6 live cells per g or mL of the product. Only from this density, the probiotic exhibits biological effect on the host. In fact, this takes effect only when the probiotics are capable to exist in the harsh condition of the gastro-intestinal system of the host, which exposes the presences of hydrolytic enzymes, bile salt and low pH condition of the GI tract [2]. *Saccharomyces cerevisiae* var. *boulardii* (also called as *Saccharomyces boulardii*), belongs to the family of *Saccharomyces cerevisiae*, however, it differs in terms of some genetic, metabolic and physiological characteristics. Within yeasts, *S. boulardii* is called a probiotic due to its adaptability in the harsh environment of guts. Rather than bacterium, *S. boulardii* has been reported to persist before antibiotic and protease enzymes. This yeast can neutralize the toxins of *Clostridium difficile*, *Vibrio cholera* and *Escherichia coli*, improves dose tolerance for treatment of *Helicobacter pylori* infection. *S. boulardii* are capable of persisting in the mimicking pH condition of human stomach

for 4 h, resistant and capable of growing in the presence of 0.3% bile salt, has good adhesion ability to human intestinal mucosa [3-5].

S. boulardii is a facultative anaerobic microorganism. The yeast is asexually reproduced by cell budding and can grow in medium containing lactic acid [6]. Rather than other yeasts in carbohydrate fermentation, *S. boulardii* produces limited alcohol content (up to 0.5%) [7]. Lourens-Hattingh *et al* [6] reported that, the yeast is likely to use nutrients from milk to grow and maintain cell density of 10^6 cfu/mL upward. Especially, *S. boulardii* is very suitable in producing alcohol-flavour yoghurt like kefir, due to its ethanol production. In an experiment of producing kefir of Ivanova *et al* [8], *S. boulardii* was well adapted and combined with the micro-organisms from kefir grains, total yeast cells density was doubled in the sample containing *S. boulardii*.

As it renders a probiotic yeast with protection against antibiotic [4], *S. boulardii* has been increasingly utilized to develop health-promoting products in food industry. Although literature is abundant on this state of art, slight information is available on the application of *S. boulardii* in fermentation of yogurt. This research aimed to examine the inclusion of *S. boulardii*, in concomitant with other two lactic acid bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus* used as starter cultures for yogurt fermentation. The implications of *S. boulardii* on the growth of lactic acid bacteria, pH value and lactic acid content of yogurt, and the survivability of the yeast in yogurt over gastro-intestinal fluids will be investigated.

2. Materials and methods

2.1 Materials

Fresh cow milk was collected by after milking from the farm of Nong Lam University, Ho Chi Minh City (Vietnam). The milk's dry matter was approximately 12%.

Lactobacillus bulgaricus and *Streptococcus thermophilus* were anaerobic isolated from thermophillic yoghurt starter culture labelled YC-X11 (Chr Hansen–Denmark) on MRS agar (HiMedia-India) at 37°C for 72 h and on M17 agar (HiMedia-India) at 37°C for 48 h, respectively. Lyophilized *Saccharomyces boulardii* cells (Biocodex, Gentily, France) were isolated on yeast malt extract agar (YMA, Sigma Aldrich, Australia) at 30°C for 48 h.

Chemicals such as ethanol, sodium hydroxide (Cemaco, Vietnam), hydrochloric acid, sodium chloride (Xilong, China), bile salt (HiMedia, India) were used in this study. Besides, the enzyme such as pepsin and pancreatin (Sigma, Singapore) also were used in this study.

2.2 Sample preparation

2.2.1 Fresh cow milk preparation

Fresh cow milk was supplemented with refined sugar (8% w/w) and skim milk powder (2% w/w, Darigold-USA) to reach a total solid of 14-15% [9] and homogenized at 11.000 rpm/min for 15 min at 55-65°C. The suspension was heat-treated at 85°C for 30 min using an autoclave then cooled down to 40-45°C before fermentation [9, 10].

2.2.2 Skim milk suspension

Skim milk suspension was prepared to a concentration of 10-12% w/v, pasteurized at 85°C for 30 min and cooled down to 40-45°C before use. The suspension was utilized for producing stock culture.

2.2.3 Starter cultures preparation for yogurt fermentation

L. bulgaricus and *S. thermophilus* after isolation were separately inoculated in MRS broth (Himedia-India) at 37°C for 24 h to reach the late-log phase. Cells from the broths were centrifuged, washed twice

in sterile saline (0.85%) under centrifugation then further cultivated in the pasteurized skim milk suspensions at 37°C for 16 h. pH and sensorial appearances of this propagation milk were determined over 0, 4, 8, 12 and 16 h of incubation.

2.2.4 Preparation of S. boulardii cells for yogurt fermentation

Yeast cells after isolation were cultivated in YPD broth (HiMedia-India) at 37°C for 16-18 h. Cells from the culturing broth were centrifuged, washed twice in sterile saline (0.85%) under centrifugation, and re-suspended into the pasteurized skim milk, which the preparation mentioned previously, before adding into fermenting mixture at a density of 10^6 cfu/g.

2.2.5 Yogurt fermentation

Prepared fresh cow milk was added to the culture suspensions containing of *L. bulgaricus* and *S. thermophilus*. Ratio of the culture suspensions were kept at 1:1 (v/v) with total concentration in final yoghurt samples arranged from 5, 10 and 15% (v/v). Simultaneously, the *S. boulardii* cells previously prepared in skim milk was also added to the fresh milk at densities varied from 10^5, 10^6 to 10^7 cfu/g. The mixtures were finally poured into sterile glass jars and incubated at different temperatures of 30, 37 and 43°C. pH value, lactic acid content and densities of microorganisms of the samples were observed during fermentation in intervals of 2 h; and sensorial values of the samples were determined after 24 h kept at 5°C. The fermentation finished when pH value and lactic acid content of the yogurts reached 4.4-4.5 and lactic acid concentration of 0.85 - 1% (85-100°D), respectively [9]. After fermentation, the yogurt was moderately cooled down to 20°C in ice water and stored at 5°C.

2.2.6 Viability of S. boulardii in gastrointestinal fluids

This *in-vitro* experiment was developed from the method of Picot and Lacroix [11] to evaluate viability of fresh-free and in-yogurt *S. boulardii* cells through a mimicking condition of human upper gastrointestinal fluid.

Simulated gastric fluid was formulated by adding pepsin (porcine gastric mucosa, P7012, Sigma-Aldrich) to a concentration of 0.26 g/L in 0.1 N HCl and pH 1.9, adjusted by 1 N NaOH. The simulated intestinal fluid was a solution of bile salt (B3883, Sigma Aldrich, 0.3%) and porcine pancreas (P7545, Sigma Aldrich, 1.95 g/L) at pH 7.5. The bile salt solution was prepared by dissolving bile salt in distilled water followed by a filter-sterilisation. The simulated pancreatic fluid was prepared by adding porcine pancreas into sterile sodium phosphate buffer (0.22 M, pH 7.5) and adjusted to pH 7.5 by 1 N NaOH.

S. boulardii free-cells sample was prepared by inoculating *S. boulardii* cells in YPD broth at 37ºC for 16 h. Yeast cells after inoculation were collected and washed by repeated centrifugation in sterile saline of 0.85%. Two samples include the *S. boulardii* free cell and the *S. boulardii* cells contained in yogurt sample (1 g) which were then dissolved into gastric fluid. The mixtures were immediately adjusted to pH 1.9 by 1 N HCl, kept stirring for 30 min then quickly adjusted to pH 7.5 by 1 N NaOH to stop pepsin's activity. Aliquots from this transit were withdrawn to check for the viability of *S. boulardii*.

To perform simulated intestinal transit, sodium phosphate buffer (500 mM, pH 7.5) and sterile bile salt solution were quickly added into the fluids after the first passage. pH of the fluids was repeatedly adjusted to 7.5 by 1 N NaOH before pancreatin addition. At each interval of 0, 1, 2, and 3 h, aliquots were withdrawn to check for viability of *S. boulardii*.

2.3 Analysis method

2.3.1 pH

The pH of yogurt samples was figured out by using a pH meter (WTW, 7110, Germany) at the temperature to 20 – 25℃.

2.3.2 Lactic acid content

The total acidity in lactic acid is quantified by direct potentiometric titration method [8]. 10 g of yogurt sample that has been stabilized at temperature of 20 - 25℃ was transferred into a 50 mL cup together with 10 mL of distilled water and well stirred. The total acidity of the sample was titrated by 0.1N NaOH solution, with kept-stirring, until a pH of 8.3 ± 0.03 was reached. Total lactic acid content (%, w/w) of the sample was calculated as below (1):

$$\text{Lactic acid content (\%)} = \frac{V \; x \; 0.9}{m} \quad (1)$$

Where: V: volume of 0.1N NaOH used for titration (mL), m: mass of test sample (g), 0.9: conversion coefficient for lactic acid.

2.3.3 Cells enumeration

S. boulardii cells were enumerated on yeast-malt-extract agar (YMA, Sigma-Aldrich, Australia) at room temperature for 48 h. *L. bulgaricus* and *S. thermophillus* were enumerated on MRS agar (HiMedia, India) at 37°C for 72 h under anaerobic condition and on M17 agar (HiMedia, India) at 37°C for 48 h, respectively. To investigate the cell count of the sample, 1 mL of the broth or 1 g of the sample was suspended into 9 mL of sterile saline (0.85%) with constant stirring. After serial dilution, an aliquot of 0.1 mL was transferred into the medium plates to enumerate the actual growth of the probiotic. The N value was calculated as below (2):

$$N = \frac{\sum C}{((n_1+0.1n_2)x\ d} \quad (2)$$

Where,

$\sum C$: total number of colonies in the counted plates.

n_1: number of the plates of the first dilution.

n_2: number of the plates of the second dilution.

d: dilution coefficient of the first dilution.

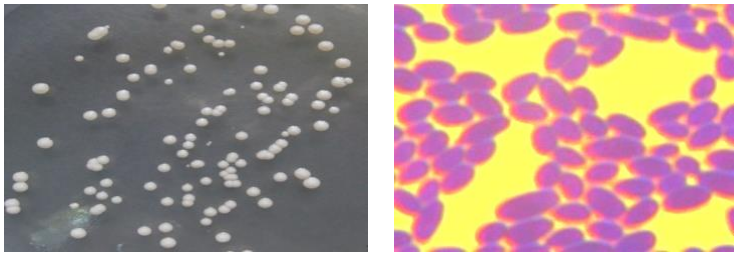
2.3.4. Statistical analysis

The One-way analysis of variance (ANOVA) and Least significant difference (LSD) tests were carried out using JMP 8.0 software at $P < 0.05$.

3. Results and discussion

3.1 Isolation of S. boulardii

Inoculating YM agar (Sigma-Aldrich, Australia) is specific for the isolation and cultivation of yeasts. In this experiment, only one colony type from the lyophilized *S. boulardii* was observed on YM agar, in accordance with the statement of the manufacturer (Biocodex-France) that the product contained only *S. boulardii*. Shapes of the colonies (Figure 1a) were consistent with the description of Larone [13] for typical yeast, and for *S. boulardii* accordingly to McCullough *et al* [12]. After 48 h of incubation at 30°C on YM agar, the colonies were in round to oval shapes, creamy white, entire edge and slightly raised centre giving “cone-like” appearances. The stained cells (Figure 1b) of *S. boulardii* sample under light microscope (Novex-Holland) with oil immersion and magnification of 1000x had round or oval shape, sometimes with buds. This morphology, according to Lesage and Bussey [14], was typical for *S. boulardii*.



a b

Figure 1. Colonies of *S. boulardii* grown on YM agar (a) and crystal violet stained cells of *S. boulardii*, with light microscope magnification of 1000x (b).

3.2 Isolation of L. bulgaricus and S. thermophilus

The lyophilized YC – X11, according to the manufacturer (Chr Hansen-Denmark), was specific for yogurt preparation. In this experiment, *L. bulgaricus* and *S. thermophilus* consisted in the product were separated in order to simplify the combination ratio and enumeration of these species for yogurt fermentation.

Results of this experiment (data not shown) showed that colony morphologies of *L. bulgaricus* and *S. thermophilus* isolated from YC - X11 in MRS and M17 agars, respectively, were in consistence with that described by Chandan [15]. The colonies had dried surfaces, round shapes and smooth edges. However, *L. bulgaricus* was slightly yellow and flat surface; while the latter was in milky-glossy-white colour and had slightly-raised centre. Under light microscope, crystal violet stained cells of *S. thermophilus* were in strings of spherical cells (Figure 2a) and that from *L. bulgaricus* were in single or strings of rod cells (Figure 2b). These two species, in addition, induced opaque media and biomass settles after 24 h of growing in MRS broth at 37°C (Figure 3a-b).

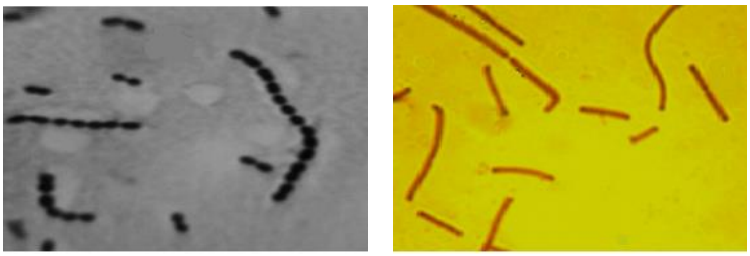


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Figure 2. Crystal violet stained cells of *S. thermophilus* (a) and *L. bulgaricus* (b).

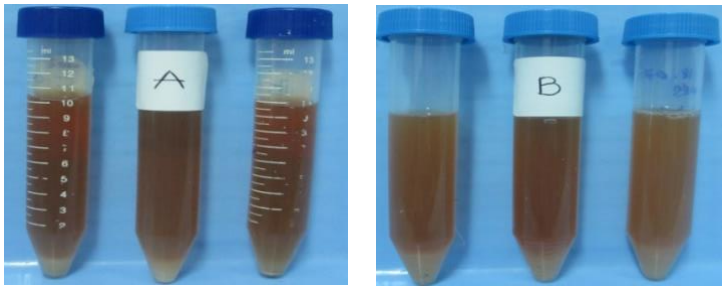

Figure 3. Biomass of two strains of lactic acid bacteria incubated in MRS broth media, at 37℃ for 24 h. (A) *S. thermophilus* strain, (B) *L. bulgaricus* strain.

3.3 Growth of L. bulgaricus and S. thermophilus in skim milk prepared as starter culture for yogurt fermentation

Starter culture prepared in skim milk suspension was for instant use in yogurt fermentation, without centrifugation and washing steps as that complicatedly prepared from micro-nutrient broth. In this experiment, pH of the propagation medium was started at 6.0 for *L. bulgaricus* and 6.5-6.0 for *S. thermophilus* (Figure 4).

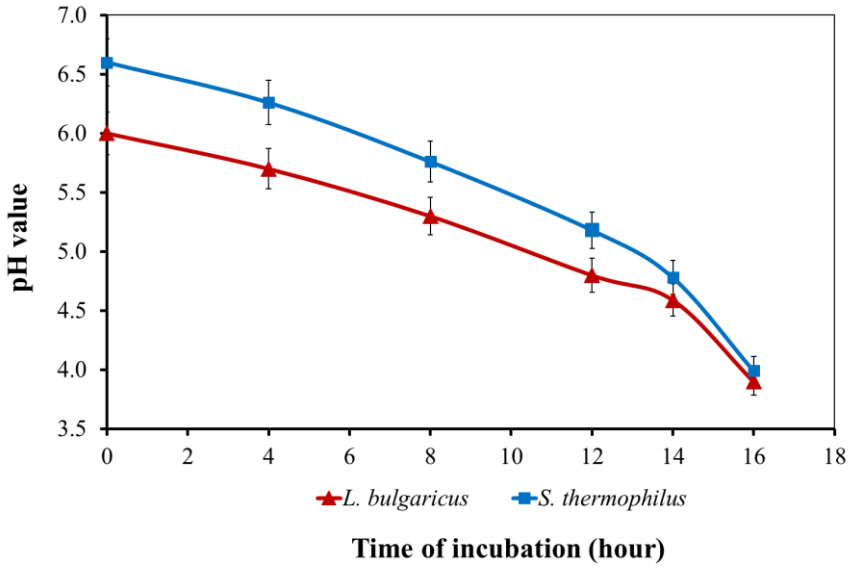

Figure 4. pH of propagation milks containing of *L. bulgaricus* and *S. thermophilus.*

pH 4 is favourable for the growth of *L. bulgaricus*, however, it is detrimental to *S. thermophilus* [9]. According to the results in Figure 4, pH of the propagation suspensions adversely reduced to 4.5-4.7 and nearly to 4.0 after 14 and 16 h of incubation, respectively. Coincidently, the densities of *L. bulgaricus* and *S. thermophilus* were found to reach the peak of growing after 14 h incubation (data not shown). Therefore, the propagation of *L. bulgaricus* and *S. thermophilus* in skim milk at 37ºC, as prepared for yogurt fermentation, would better last for 14 h. At 16 h, the pH of the yeast culture was too low (< 4.0). Consequently, when mixed into milk solution, an unfavourable condition for the growth of *S. thermophillus* strain may be induced and the coagulation of milk protein in yogurt may be under controlled thus far.

3.4 Effect of starter culture amount on quality of yogurt containing S. boulardii

During fermentation of yogurt, *L. bulgaricus* and *S. thermophilus* metabolized lactose and produced a large amount of lactic acid thus decreased pH value of the yogurt. At the same fermentation temperature, higher number of starter bacteria obviously intensifies fermentation rate and shortens fermentation time. In a different manner, *S. boulardii* used lactic acid, together with glucose and galactose from lactose hydrolysis activity of lactic acid bacteria, as carbon source for its growing [16]. The metabolism resulted in certain amounts of lactic acid, ethanol and CO_2 for the fermented product [17].

According to Fox [18], formation of lactic acid reduced yogurt's pH, leads to destabilization of casein micelles. At pH 5.0-5.1, calcium-phosphate complex is completely released from the micelle; linking-force between individual casein to form micelle is reduced. At pH 4.6-4.7, the dissociation between kappa-casein and beta-casein results in the aggregation of casein micelles, consequently special texture of yogurt is formed.

As in Figure 5 and Figure 6, fermentation time of the yogurt samples with or without the presence of *S. boulardii* (cells density of 10^6 cfu/g) reduced with the increase of starter cultures amount. Whereas, at the same amount of starter culture but different *S. boulardii* application densities, lactic acid content and pH of the samples were not much different from each other. For detail, yogurt samples with and without 15% *S. boulardii* achieved pH of 4.43 and 4.49, and lactic acid content of 0.90% and 0.87%, respectively, after fermentation at 43℃ for 5.5 h. It is likely that fermentation time, pH value and lactic acid content of the yogurt samples were affected by starter culture amount only, not the presence of *S. boulardii*.

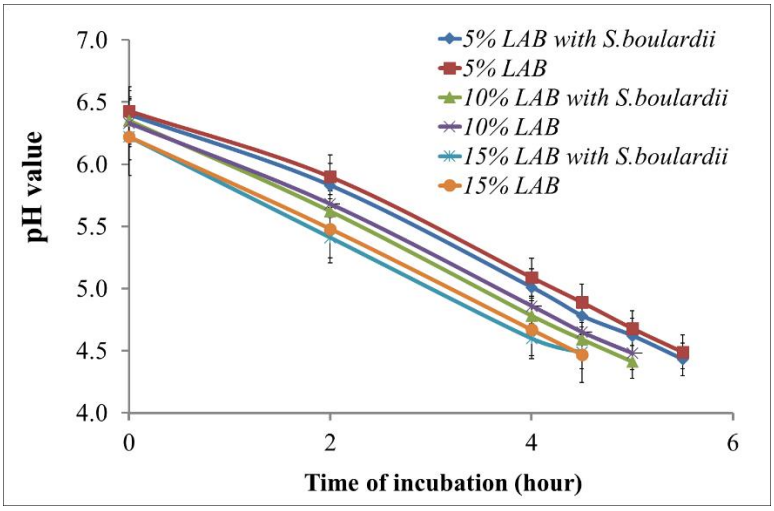

Figure 5. Change of yogurt's pH during fermentation, LAB (*L. bulgaricus* and *S. thermophilus*, 1:1 ratio) starter culture suspensions of 5-10-15% (v/v), with or without the presence of *S. boulardii*.

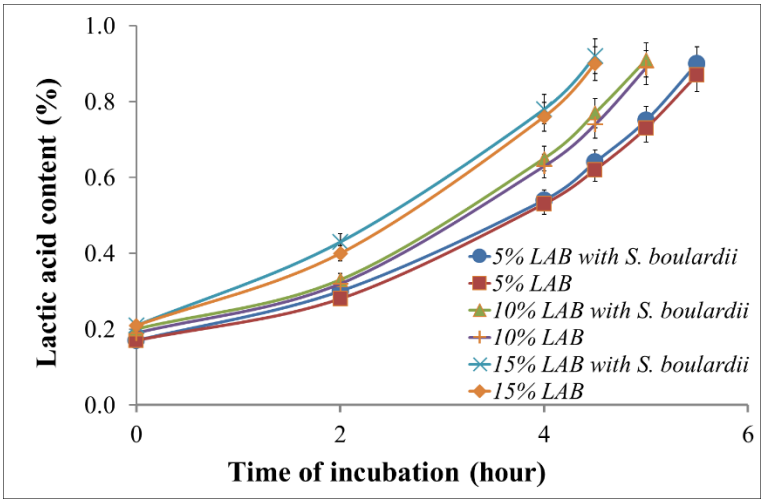

Figure 6. Accumulation of lactic acid (%) during yogurt fermentation, LAB (*L. bulgaricus* and *S. thermophilus*, 1:1 ratio) starter culture suspensions of 5-10-15% (v/v), with or without the presence of *S. boulardii*.

3.5 Effect of starter culture on the growth of S. boulardii during yogurt fermentation

In this study, the growth of lactic acid bacteria and *S. boulardii* in the yogurt samples during fermentation are expressed in Figure 7. It is likely that the growths of *L. bulgaricus* and *S. thermophilus* were insignificantly affected by the presence of *S. boulardii* in the yogurt. According to Fleet [16], *S. boulardii* is unable to assimilate lactose, but capable to assimilate glucose, galactose and lactic acid

which are produced by lactose hydrolysis activity of lactic acid bacteria during yogurt fermentation. Yeast, by its metabolism activity, provides amino acids and vitamins, especially vitamin B, that can stimulate the growth of bacteria. According to Mathara *et al* [19], no other intestinal bacteria were found in fermented dairy product containing yeast. It is likely that the reduced pH environment of the yogurt and the symbiotic relationship between lactic acid bacteria and *S. boulardii* inhibited the growth of spoilage bacteria and extended the shelf life of yogurt. In this experiment, *S. boulardii* was found to produce a small amount of ~ 0.09% ethanol. However, this is not a lethal cause to lactic acid bacteria, even till the concentration of 7% (v/v) [18].

As in Figure 7, cell density of *S. boulardii* decreased 0.25 log cfu/g after 5 h of yogurt fermentation. Fietto *et al* [2] also found a decline of 50% of free *S. boulardii* cells after 1 hour exposure at temperature of 49°C. Whereas, Lourens-Hattingh *et al* [4] found that this yeast grew from a density of 7.7 to 8.1 log cfu/g after 29 days mixed and stored in fruit-mixed yogurt at 5°C. Therefore, the decline of yeast cell in this experiment might be due to unfavorable condition of yogurt fermentation temperature at 43°C.

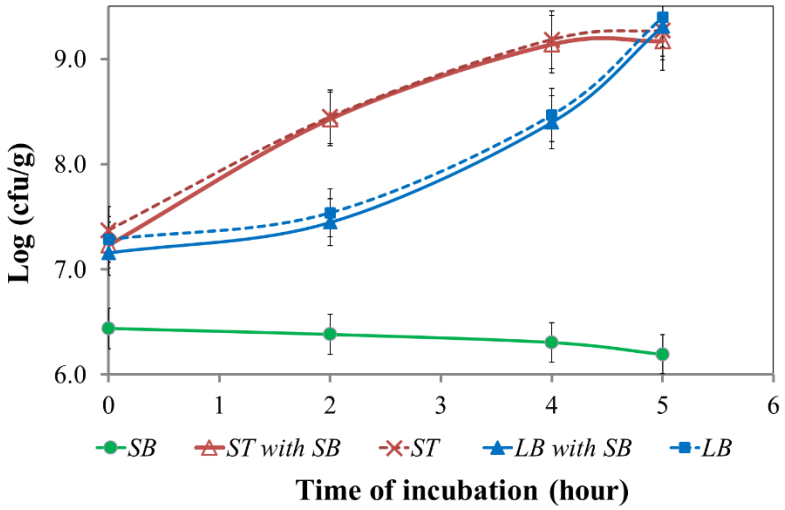

Figure 7. Growths of *L. bulgaricus (*LB), *S. thermophilus* (ST) and *S. boulardii* (SB) in yogurt.

In addition, it is noticeably that the growth curves of the two starter cultures in the yogurt containing or without containing *S. boulardii* expressed the same manners. By the density of 10^7 cfu/g, *S. thermophilus* was likely in earlier adaptation and growth, compared to that of *L. bulgaricus*. However, the growth of this bacterium was slowed down after 4 h fermentation while that of *L. bulgaricus* continued to develop. According to Lourens-Hattingh and Viljoen [6], in the initial stage of fermentation, *S. thermophilus* quickly utilized amino acid provided by *L. bulgaricus*. After that, its growth is slowed down due to the accumulation of lactic acid. Adversely, the bursting growth and development of *L. bulgaricus* at the late stage of fermentation is due to formic acid provided by *S. thermophilus* and the more optimal pH condition. However, development of the two bacteria finally reached the same point when the yogurt fermentation was finished.

3.6 Effect of S. boulardii on the formation of lactic acid and changes of pH in yogurt

In this experiment, amount of starter cultures were fixed at concentration of 15% (v/v, ratio of 1:1), *S. boulardii* densities varied from 10^5, 10^6 to 10^7 cfu/g. The fermentation was performed at temperature of 43°C until a pH of 4.4-4.5 was accomplished in the yogurt. Changes of pH and formation of lactic acid during fermentation were shown in Figure 8 and Figure 9.

According to Figure 8 and Figure 9, results showed that *S. boulardii* supplementations at 10^5, 10^6 and 10^7 cfu/g did not affect on pH, fermentation time and the formation of lactic acid in yogurt samples. At each density, yogurt samples reached pH of 4.46, 4.45, and 4.44, lactic acid content of 0.88%, 0.90%, and 0.91% within the same fermentation time of 4.5 h. In yogurt fermentation, lactic acid was produced by lactic acid bacteria, not by yeast [16]. Result in this research was in consistence with that of Lourens-Hattingh *et al* [6], which found that lactic acid concentration was unchanged in the UHT milk and UHT yogurt samples supplemented with *S. boulardii* and stored at 5°C for 29 days.

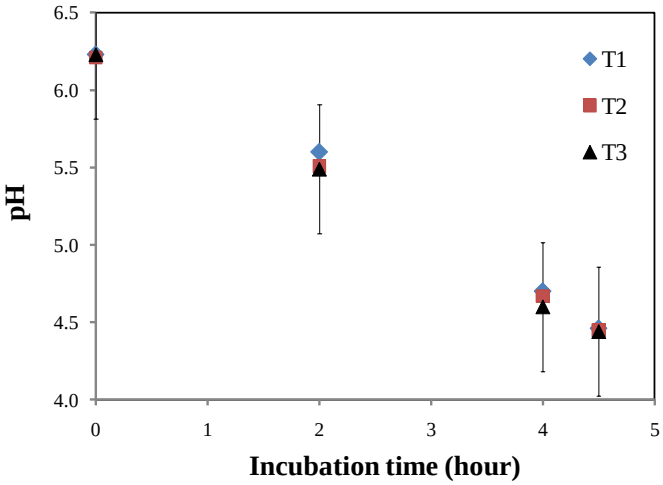

Figure 8. Change of yogurt's pH over *S. boulardii* cell densities of 10^5 (T1), 10^6 (T2) and 10^7 (T3) cfu/g.

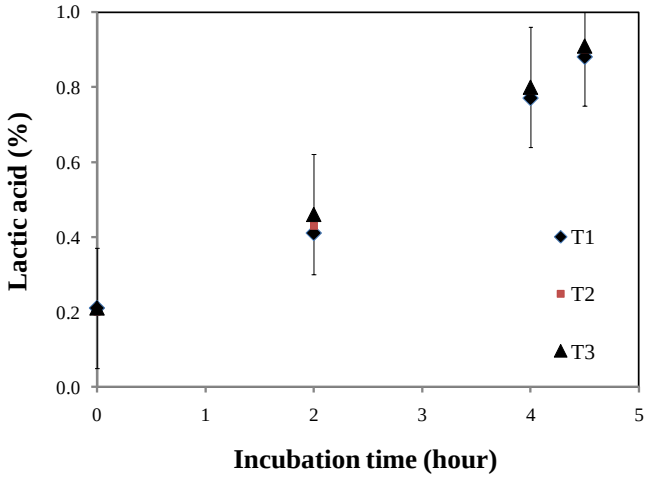

Figure 9. Formation of lactic acid in yogurt samples over *S. boulardii* supplementations of 10^5 (T1), 10^6 (T2) and 10^7 (T3) cfu/g.

Effect of starting densities on the yeast cells count during yogurt fermentation was shown in Figure 10. Results showed that all *S. boulardii* treatments decreased about 0.05-0.12 log cfu/g, compared to the initial loads, in the yogurt samples after 4.5 h of fermentation at 43°C. Surprisingly, this was consistent with the findings of Fietto *et al* [2]. The authors stated that the light reduction of yeast cells was due to unfavorable temperature condition of yogurt fermentation.

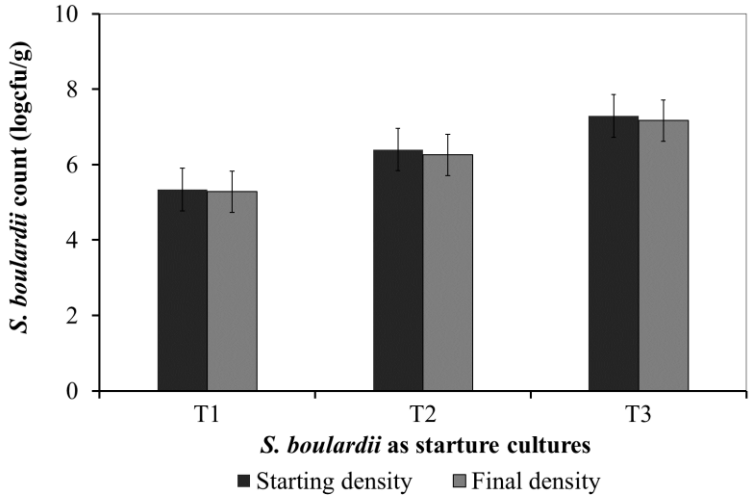

Figure 10. Cell counts of *S. boulardii* before and after fermentation, over *S. boulardii* supplementations of 10^5 (T1), 10^6 (T2) and 10^7 (T3) cfu/g.

3.7 Effect of fermentation temperature on lactic acid formation, pH, growths of lactic acid bacteria and S. boulardii

This experiment, by starter culture amount of 15% (v/v) and starting yeast cell density of 10^6 cfu/g, was run at various temperatures of 30, 37 and 43°C until the yogurt's pH reached 4.4-4.5, corresponding to acidity 85-100ºD. Figure 11 and Figure 12 show the **changes** in pH and concentration of lactic acid during fermentation. According to the results, fermentation rate of the yogurts, which started from the same densities of *L. bulgaricus*, *S. thermophilus* and *S. boulardii*, varied accordingly to the fermentation temperature. In yogurt, formation of lactic acid depends on the activity of lactic acid bacteria. Growth optimum temperatures for *L. bulgaricus* and *S. thermophilus* ranged from 43 to 46°C and 35 to 42°C, respectively [21]. At fermentation temperature of 30°C, lactic acid bacteria grow in a slow speed and thus produced a small amount of lactic acid. At fermentation temperature of 43°C, the bacteria were in favorable condition and the fermentation rate was speeded up.

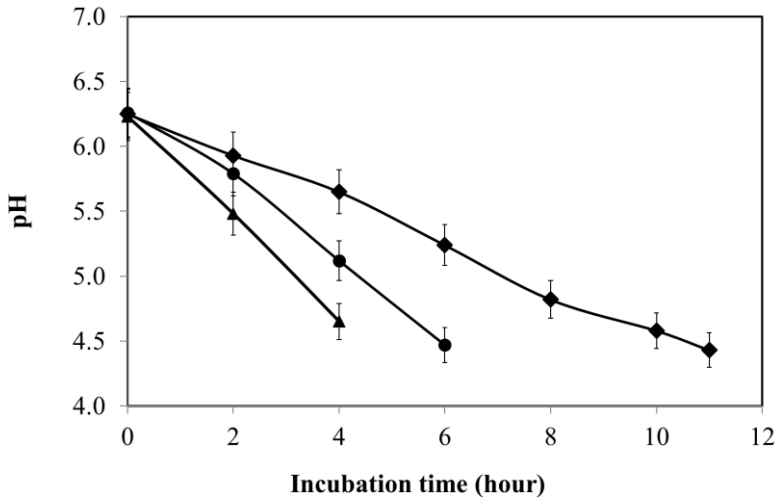

Figure 11. Change of yogurt pH over fermentation temperatures of 30°C (■), 37°C (●) and 43°C (▲).

According to Graff *et al* [22], *S. boulardii* optimally grows at temperatures between 30-37ºC. However, Fietto *et al* [2] stated that the growth rate of *S. boulardii* at 30ºC seems to be more vigorous than that at 37ºC. Results in this study showed that viability of *S. boulardii* after yogurt's fermentation at temperatures of 30ºC, 37ºC and 43ºC were significantly different ($P < 0.05$). With fermentation temperature of 30ºC, yeast cell density increased by about 0.8 log cfu/g (from 6.12 ± 0.08 to 6.92 ± 0.03) and that at 37ºC was 0.39 log cfu/g, while at 43ºC, yeast cells decreased 0.14 log cfu/g (Figure 13). Fietto *et al* [2] also stated that, at fermentation temperature of 43ºC, yeast is still growing; however, the number of new-born cells is always less than the number of death-cell. To some extent, at appropriate temperature, *S. boulardii* is capable to assimilate milk components for growing and cells number was increased.

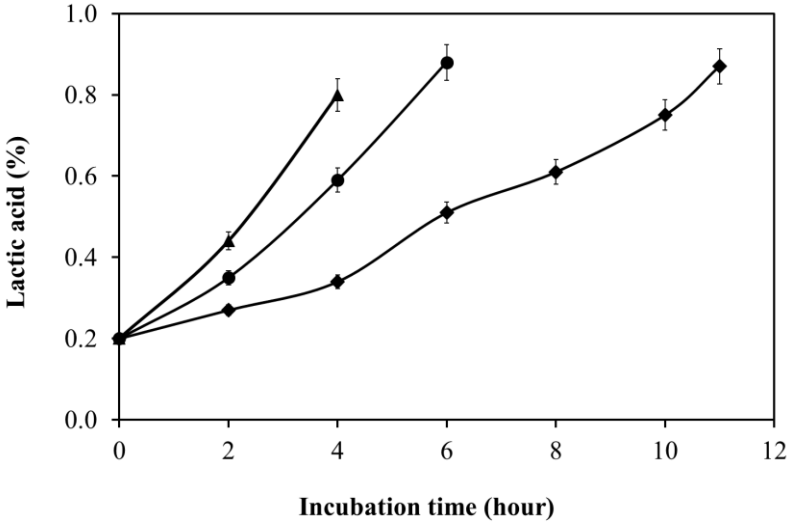

Figure 12. Accumulation of lactic acid (%) in yogurt samples fermented at 30°C (■), 37°C (●) and 43°C (▲).

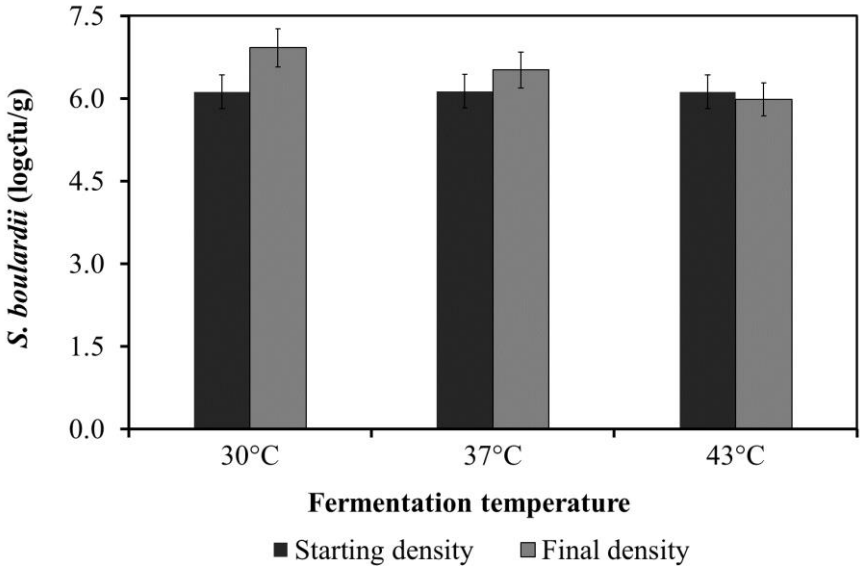

Figure 13. Cell count of *S. boulardii* before and after yogurt fermentation, with fermentation temperatures of 30, 37 and 43°C.

3.8 Viability of S. boulardii in simulated human upper gastro-intestinal fluids

One important characteristic of probiotic is that it can survive in the harsh condition of gastro-intestinal system with the presences of enzymes, organic acids, bile salts and low pH [23]. In this experiment, viability of *S. boulardii* cells over 0, 1, 2 and 4 h exposed in simulated gastro-intestinal juice was examined. According to the results expressed in Figure 14, cells count of *S. boulardii* in free-fresh and in-yogurt samples reduced 42-43% after 30 min exposed in simulated gastric juice, which were from 8.68 x 10^8 to 3.70 x 10^8 (cfu/mL) for the free-fresh cells and from 5.82 x 10^6 to 2.45.10^6 (cfu/mL) for the in-yogurt cells. In a different research, Fietto *et al* [2] found a survival of 70% of *S. boulardii* after 60 min exposure in simulated stomach fluid which was in pH 2, pepsin (3 g/L) and NaCl (5 g/L). This difference in yeast cell tolerance might be due to the pH condition in this study was more extreme.

By after the transit in simulated gastric juice, the probiotic samples were rapidly moved to simulated intestinal fluids. Undoubtedly, the temperature of 37ºC in this exposure was still optimal for the yeast growth. However, the digestive environment with presences of bile salts, pancreatin and pH 7.5 was challenging to the yeast. Figure 14 shows that the cells count of *S. boulardii* in free-fresh sample could not maintain stably during this transit, while the viability of the cells in yogurt was almost linearly increased from 72% to 85%, which was extrapolated from the changes of the yeast cell densities originally from 5.82 x 10^6 cfu/mL to 4.21 x 10^6 and 4.97 x 10^6 (cfu/mL) after 1 and 4 h transit, respectively. In fact, there were some free amino acid, lactic acid, galactose, sucrose, etc…. in yogurt which play as nutrition source for yeast's growth during the transit in the intestinal fluid.

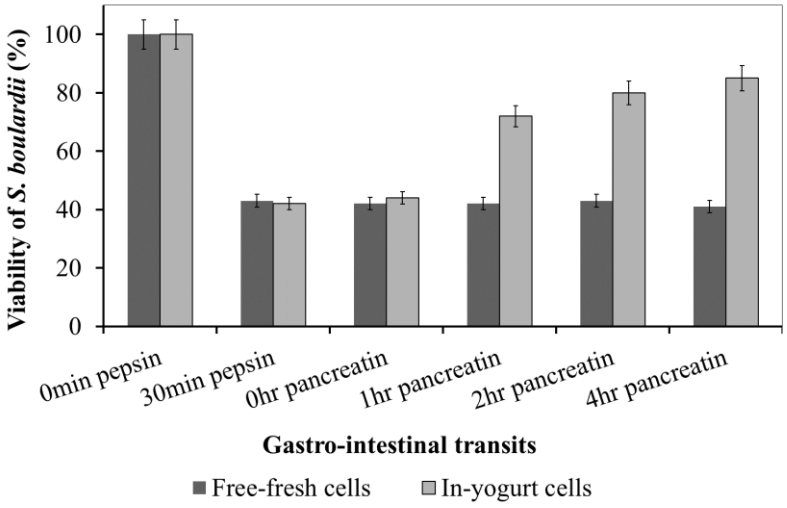

Figure 14. Changes in viable cell counts of free-fresh and in-yogurt cells of *S. boulardii* during simulated gastrointestinal digestion.

4. Conclusions

S. boulardii exhibited potential characteristics to be used as starter culture with other lactic acid bacteria in yogurt production. At the temperature condition of yogurt fermentation, *S. boulardii* was likely to grow to a cells count more than 10^6 cfu/g, which is more than required to be reached with a probiotic in probiotic containing product. With yogurt fermentation, *S. boulardii* insignificantly affected the growths of the other two starter cultures *L. bulgaricus* and *S. thermophilus*, and vice versa. The growth of this yeast exhibited insignificant effect on the fermentation parameters such as pH value, lactic acid accumulation and fermentation time of yogurt. *S. boulardii* also expressed its tolerance to the severe condition of gastro-intestinal fluids, which is required to be for a probiotic. Even being considered to

have very limited production of ethanol and CO_2, however, these should be further investigated to control the quality of product when applying *S. boulardii* in yogurt fermentation.

Conflict of interest

The author declares that there are no conflicts of interest to disclose related to this manuscript.

Ethical approval

This article does not contain any studies with human or animal subjects.

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