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Using Probiotic Bacteria for Soymilk Fermentation

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Abstract

Five probiotic bacterial strains (*Lactobacillus plantarum ATCC 14917*, *Lactobacillus casei DSM 20011*, *Lactobacillus acidophilus ATCC20552*, *Lactococcus thermophilus DSM 20259*, and *Bifidobacterium longum B41409*) were used as monoculture, and combined with them as co-cultures for the fermentation of soymilk. The total number is 20 co-cultures, separated similarly into two parts, the first half of each co-culture consisted of two strains, and the second half of each co-culture consists of three strains. The findings revealed that these cultures were capable of fermenting soymilk at only 8 h with a pH drop of between 4.42 and 4.89. Among 25 cultures, eight cultures (3 monoculture *of L. plantarum ATCC 14917 (C1), Lc. thermophilus DSM 20259 (C4),* and *B. longum B41409 (C5)* and co-cultures of two strains *L. plantarum ATCC 14917 + Lc. thermophiles DSM 20259 (C8)* and of three strains *L. plantarum ATCC 14917 + Lc. thermophilus DSM 20259 + B. longum B41409 (C18), L. plantarum ATCC 14917+ L. casei DSM 20011+ Lc. thermophilus DSM 20259 (C19), L. casei DSM 20011+ L. acidophilus ATCC20552+ Lc. thermophilus DSM 20259 (C22),* and *L. acidophilus ATCC20552+ Lc. thermophilus DSM 20259 + B. longum B41409 (C25)*) which recorded the greatest significant viability of bacterial cells, titratable acidity, total organic acids (lactic, acetic, and propionic acids) contented, and pH in fermented soymilk ranged from 9.26 to 9.98 Log CFU/mL, 3.06 to 3.32 %, 19.90 to 18.40 g/L (lactic acid), 13.30 to 12.30 g/L (acetic acid), and 16.35 to15.00 g/L (propionic acid), and 4.38 to 4.62. The soy yogurt produced from the selected fermentation of soymilk was more preferred in the chemical composition of protein, carbohydrate, and fat contents than non-fermented soymilk. So the results show that the integrative use of probiotics may achieve the nutritional value of soymilk after fermentation had been improved by probiotic strains, therefore, making it a more health-beneficial functional food.

Keywords: Beneficial food, Fermented soymilk, Functional food, Probiotic, Prebiotics, Titratable acidity

1 Introduction

The term 'probiotic' means pro-life. The Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) are currently defining probiotics as 'live microorganisms that have a healthy effect on the host when consumed in sufficient amounts by improving the intestinal balance. (Sanchez et al 2017). It can be used for the processing of fermented food and yoghurt. Lactic acid-producing, non-pathogenic bacteria such as *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Propionibacterium*, and *Enterococcus*, or non-pathogenic yeasts such as *Saccharomyces boulardii*, are the large majority of these probiotics. Probiotics are consumed by oral and live in the intestine. Probiotics affect health status directly and indirectly by altering the intestinal microflora and stimulating the immune response through the vitamins, and short-chain fatty acids production, the undigested food substances degradation (Holck et al 2017). Furthermore, protection against infections, (Mallina et al 2018) prevention of cancer (Aragon et al 2015), decrease in a gut inflammatory response, (Fábrega et al 2017), and prevention of allergies (Velez et al 2015). For our intestinal microbiome to work properly, humans need a great mix of several bacterial strains (probiotic) and compounds (prebiotics) to energize them. They are mostly administered in combination with specific prebiotics (indigestible food directly metabolized by probiotics), many food components, in particular oligosaccharides and polysaccharides (including dietary fiber) have been used as prebiotics for the formation of synbiotics mixtures (Heydari et al 2018). Through disease prevention and as alternatives to reduce the risk of disease, synbiotics, and other food supplements now play an important role in human health and nutrition. The role of synbiotics in human health has been demonstrated in studies on the relationship between human intestinal microbiota and immunology. Possible combinations of the most compatible probiotics and prebiotics may eliminate specific microbial disorders, and reduce the risk of intestinal diseases (Pandey et al 2015). Because of its extraordinary nutritional importance and health properties, soymilk has become a very impressive food substance. It contains isoflavones, which are very essential in the daily diet, and a very valuable source of high-quality beneficial proteins, soluble and insoluble dietary fibers, and unsaturated fatty acids (Božanić 2006). Soymilk, owing to its high protein and phytochemical content, is quickly becoming a promising milk alternative for consumers accused of being healthy. (Olaoye et al 2015). Besides, soybean provides all the amino acids required for human feeding, so for those that are lactose intolerant or allergic to milk proteins, it can be a healthy food base for milk replacement (Vanga et al 2018). There is evidence that soymilk intake has potential health benefits associated with cardiovascular disease, menopausal symptoms, osteoporosis, breast and prostate cancer, as it is a rich source of bioactive phenolic compounds (Rodríguez-Roque et al 2013). For soymilk product fermentation. The addition of probiotics would improve the health and commercial value of soymilk and produce more alternative soy products to the delight of consumers (Olaoye et al 2017). The fermentation may reduce the beany flavor caused by lipoxygenase activity which lowering the acceptability of soymilk (Peng and Guo 2015). The contents of oligosaccharides, especially verbascose, stachyosis, and raffinose, are also reduced by the source of flatulence of fermentation. Fermented soymilk can be readily digested and has antioxidant properties that resist cancer (Ziaei and Halaby 2017). By converting isoflavone glycosides into aglycones, these are bioactive forms known for their health benefits by enhancing the function of β-galactosidase (Liu et al 2018).

The principal goals of this research were to produce fermented soymilk by probiotic bacteria (*lactic acid bacteria* and *bifidobacteria*) and shortening the fermentation time. Then, as a supplement for fermented milk, use this highly nutritious, wellbeing and economic benefit milk to help those people who are lactose intolerant and allergic to milk proteins.

2 Materials and Methods

2.1 Samples collection

Soybean (Glycine max) seeds were collected from markets in Cairo, Egypt, and used for the preparation of soymilk.

2.2 Probiotic bacterial strains used

Five probiotic bacterial strains (*Lactobacillus plantarum* ATCC 14917, *L. casei* DSM

20011, *L. acidophilus* ATCC 20552, *Lactococcus thermophilus* DSM 20259 and *Bifidobacterium longum* B 41409) were collected from the Food Technology Research Institute, Agricultural Research center in Giza, Egypt. These strains have been used to ferment soymilk.

2.3 Media used

de Man, Rogosa, and Sharpe (MRS) broth Erdogrula and Erbilir (2006), it was used for maintenance and preservative of all probiotic bacterial strains except *Lactococcus thermophilus*. It has the following composition (g / L): protease peptone, 10.00, yeast extract, 5.00, dextrose, 20.00, polysorbate 80, 1.00, ammonium citrate, 2.00, sodium acetate, 5.00, magnesium sulphate, 0.10, manganese sulphate, 0.05, dipotassium phosphate, 2.00 and agar, 15. The final pH (at 25°C) was 6.5 ± 0.2. * MRS broth medium was the same as MRS agar medium without adding agar.

M17 agar (Strahinic et al 2007) it was used for maintenance and preservative *Lactococcus thermophilus* strain. It has the following composition (g / L): tryptone, 2.5, meat peptone, 2.5, soya peptone (papainic), 5.0, yeast extract, 2.5, meat extract, 5.0, sodium glycerophosphate, 19.0, magnesium sulfate, 0.25, ascorbic acid, 0.5, lactose, 5.0 and agar, 15, Final pH 7.0 +/- 0.2 at 25°C.

2.4 Soymilk preparation

Soybeans were washed and soaked overnight in distilled water at 5°C. After the segregation of water, the soybeans combined 1:5 w/v with distilled water. The resulting slurry was then filtered through a double-layer cheesecloth and sterilized at 121°C for 15 min (Wang et al 2002).

2.5 Maintenance of cultures

Stoke culture slants were maintained at 5ºC on a preservation media after incubation at 37°C for 48 h.

2.6 Inoculum preparation

Bacterial standard inoculum was prepared by inoculation of a conical flask (250 ml in volume) containing 50 mL of MRS or M17 broth media (1 or 2) with a stock culture loop. inoculated flasks were incubated for 48 h at 37°C under static conditions. The contents of these flasks were used as standard inoculum (1 ml contained colony-forming unit 2.3 × 10^7 (CFU)/mL) for the fermentation process study. The CFU/mL of the inoculum was measured by plating the appropriate dilutions (made in 0.9% NaCl) on MRS agar plates. Plates were incubated at 37°C for 48 h (Shah et al 2007).

2.7 Soymilk fermentation process

In 250 mL plugged Erlenmeyer flasks, each containing 100 mL of soymilk and inoculated with 5% of the standard inoculum, the fermentation process was performed. These flasks were incubated for 12 h at 37°C. Samples were collected at 2 h periodic intervals. During fermentation, for the cell growth determination, pH changes, and total acidity, aliquots from each batch of inoculated soy milk were taken at periods between 0 and 12 h.

2.8 Effect of single and consortia probiotic cultures on soymilk fermentation

Soymilk was inoculated with 5% (v/v) inoculum size of each probiotic bacterial culture separately as a single culture. Co-cultures (di and tri-cultures) as the below have also been inoculated with different inoculum sizes of 5ml. A previous propagation procedure has been used.

Single cultures being C1= *L. plantarum* ATCC 14917, C2= *L. casei* DSM 20011, C3= *L. acidophilus* ATCC 20552, C4= *Lc. thermophilus* DSM 20259, and C5= *B. longum* B 41409.

Di-cultures being C6 = *L. planturum* ATCC 14917 + *L. casei* DSM 20011, C7= *L. planturum* + *L. acidophilus* ATCC 20552, C8= *L. planturum* ATCC 14917 + *Lc. thermophilus* DSM 20259, C9= *L. planturum* ATCC

14917+ *B. longum, C10*= *L. casei* + *L. acidophilus* ATCC 20552, C11= *L. casei* DSM 20011+ *Lc. thermophilus* DSM 20259, C12= *L. casei* DSM 20011+ *B. longum* B 41409, C13= *L. acidophilus* + *Lc. thermophilus* DSM 20259, C14= *L. acidophilus* ATCC 20552+ *B. longum* B 41409, and C15= *Lc. thermophilus* DSM 20259+ *B. longum* B 41409.

Tri-cultures were C16=*L. planturum* ATCC 14917+ *L. casei* DSM 20011 + *L. acidophilus* ATCC 20552, C17= *L. planturum* ATCC 14917+ *L. acidophilus* ATCC 20552+ *Lc. thermophilus* DSM 20259, C18= *L. planturum* ATCC 14917 + *Lc. thermophilus* DSM 20259+ *B. longum* B 41409, C19= *L. planturum* ATCC 14917 + *L. casei* DSM 20011 + *Lc. thermophilus* DSM 20259, C20= *L. planturum* ATCC 14917+ *L. casei* DSM 20011 + *B. longum* B 41409, C21= *L. planturum* ATCC 14917+ *L. acidophilus* ATCC 20552+ *B. longum* B 41409, C22 = *L. casei* DSM 20011+ *L. acidophilus* ATCC 20552+ *Lc. thermophilus* DSM 20259, C23= *L. casei* DSM 20011+ *Lc. thermophilus* DSM 20259+ *B. longum* B 41409, C24= *L. casei* DSM 20011 + *L. acidophilus* + *B. longum* B 41409, and C25= *L. acidophilus* ATCC 20552+ *Lc. thermophilus* DSM 20259+ *B. longum* B 41409.

2.9 Analytical methods

2.9.1 pH value

The pH of the fermented soymilk was measured with a calibrated digital pH meter (Model Adwa 1000).

2.9.2 Determination of total acidity

The titratable acidity of the sample was measured using 10 mL of weighed sample into a conical flask and 3 drops of phenolphthalein indicator were added and titrated with 0.1 mL of sodium hydroxide (NaOH) until a pink color appeared. The titer value was recorded and was expressed as a percentage of lactic acid, acetic acid, and probionic acid (Olorunnisomo et al 2014). Organic acids productivity (g/L/ h) was calculated as the ratio of organic acids concentration (g/L) to the fermentation time (h) (Abdel-Rahman et al 2013).

2.9.3 Cell viable count (viability)

The cell viability of probiotic bacterial cultures was quantified using the pour plate method with MRS agar and M17 agar (media 1 and 2) and incubated for 48 h at 37 °C. Tenfold serial dilution of the samples was done using sterile 0.1 g/L peptone water (Oxoid Ltd., Basingstoke, Hampshire, England). On MRS agar and M17 agar plates, the diluted samples were poured (Oxoid Ltd., Basingstoke, Hampshire, England). The cell viable count (viability) was calculated after incubated for 12 h at 37°C (Shah et al 2007).

2.9.4 Chemical analysis

Total solids, fat, total nitrogen, and ash contents of the samples were determined according to AOAC (2007). The content of carbohydrates was calculated by subtracting the moisture, protein, fat, and ash content from the total mass.

2.9.5 Energy determination

The energy value was calculated by using the formula: $Energy\ Value = (Protein \times 4 + Fat \times 9 + Carbohydrate \times 4)$ kcal/100g (Abiodun et al 2019).

2.10 Statistical analysis

The data were seen as mean ± standard deviation (SD). The statistical analysis was done with SPSS (version 20.0). A one-way analysis of variance (ANOVA) technique was used, followed by several range tests for Duncan'spost-hoc.

3 Results and Discussion

3.1 Soymilk fermentation by probiotic bacterial cultures as monoculture

3.1.1 Growth of probiotic bacterial strains in soymilk

The five probiotic bacterial strains of *L. plantarum* ATCC 14917 (C1), *L. casei* DSM 20011 (C2), *L. acidophilus* ATCC 20552 (C3), *Lc. thermophilus* DSM 20259 (C4), and *B. longum* B41409 (C5) were used as a single for soymilk fermentation, for the production of soy yogurt. During the 12 h fermentation period the samples were taken every 2 hours. Results in **Table 1** show that the population of the tested strains increased significantly ($p \leq 0.05$) in fermented soymilk with an increase in fermentation time up to 8 h, after which the cell counts were constant or decreased slightly. The bacterial viability or the viability of inoculum in fermented soymilk (soy yogurt) increased from 3.55 to 9.47 Log CFU/mL, 3.20 to 8.26 Log CFU/mL, 3.41 to 8.11 Log CFU/mL, 3.81 to 9.37 Log CFU/mL, and 3.89 to 9.26 Log CFU/mL for *L. plantarum* ATCC 14917 (C1), *L. casei* DSM 20011 (C2), *L. acidophilus* ATCC 20552 (C3), *Lc. thermophilus* DSM 20259 (C4), and *B. longum* B 41409 (C5) during fermentation period between 0 and 8 h, respectively for 8 h of incubation at 37°C with over increase reached to 2.66, 2.58, 2.37, 2.45, and, 2.38-Fold, respectively.

Results also indicated that the growth of *L. plantarum* ATCC 14917 (C1), *Lc. thermophilus* DSM 20259 (C4), and *B. longum* B41409 (C5) were more significant ($p \leq 0.05$) than *L. casei* DSM 20011 (C2), and *L. acidophilus* ATCC 20552 (C3). A high positive correlation coefficient (r) between fermentation time and the growth of the tested probiotic bacterial strains =0.90.

The logs CFU / ml at 24 hours for many of these bacteria (probiotic bacteria) were greater than the 8 log CFU / ml suggested for health benefit (Champagne et al 2016). The monoculture of *L. plantarum* ATCC 14917, *L. casei* DSM 20011, *L. acidophilus* ATCC 20552, *Lc. thermophilus* DSM 20259, and *B. longum* B41409 showed good growth in soymilk, the fermentation time is 8 h has been reported by (Božanić et al 2008). The probiotic bacteria had their growth favored reaching the population of 8 log CFU/mL within 8 h of fermentation (Claudia et al 2014), and the cell viable count of probiotic cultures increased significantly during the first 8 h of fermentation (Rathore et al 2012). Also, (Kumari et al 2018) reported that the viability of fermented soymilk with a variety of viable counts ($p \leq 0.05$) improved significantly from 4.21 to 8.01 Log CFU / ml in *L. casei*, and from 4.13 to 7.83 Log CFU / ml in the comparison probiotic strain *L. casei* strain at 30°C for 24 h of incubation. In furthermore, (Niyibituronsa et al 2018) found that the maximum final population of *L. plantarum*, *L. reuteri*, and *L. brevis* in fermented soymilk was 9.03, 8.99, and 8.98 log CFU / mL after 24 h, respectively. `To achieve the desired acid content and pH decrease, which affects organoleptic properties and shelf life and avoids product contamination, high viable counts are required (Rathore et al 2012).

3.1.2 Change in pH, total acidity, and the content of organic acids (lactic and acetic acids) during soymilk fermentation by single strains

Data illustrated by **Fig 1 s**hows the changes in pH and titratable acidity (TA) of fermented soymilk during the 12 h of the fermentation period. At zero time, the initial pH value of soymilk was 7.20, the tested probiotic monoculture decreased dramatically at the first 8 h and the pH was between 4.54, and 4.89. Whereas TA was significant ($p \leq 0.05$) increased with increase fermentation period from zero time up to 8 h being from 0.030 to 3.17%, 0.045 to 3.03%, 0.015 to 3.11%, 0.059 to 3.15%, and 0.072 to 3.07% for *L. plantarum* ATCC 14917 (C1) fermented soymilk, followed by *L. casei* DSM20011 (C2), *L. acidophilus* ATCC20552 (C3), *L. thermophilus* DSM20259 (C4) and *B. longum* B41409 (C5), respectively. Results also indicated that there

Table 1. Viability of probiotic bacterial cultures as monoculture during soymilk fermentation at 37°C is affected with fermentation time

Fermentation period (h)	Probiotic cultures (Log CFU/mL)					Mean
	C1	C2	C3	C4	C5	
0	3.55±0.48	3.20±0.35	3.41±0.40	3.81±0.44	3.89±0.44	3.43^{f}±0.22
2	5.79±0.36	4.55±0.78	5.51±0.84	5.79±0.56	5.63±0.59	5.26^{e}±0.50
4	7.79±0.36	6.66±0.59	6.37±0.49	7.72±0.63	7.59±0.95	7.02^{d}±0.05
6	8.77±0.68	7.79±0.37	7.79±0.87	8.42±0.36	8.84±0.62	7.96^{c}±0.12
8	9.47±0.35	8.26±0.87	8.11±0.64	9.37±0.20	9.26±0.52	8.84^{a}±0.09
10	9.41±0.30	8.23±0.50	8.11±0.64	9.07±0.20	9.21±0.52	8.80^{ab}±0.15
12	9.37±0.30	8.20±0.50	8.08±0.64	9.04±0.20	9.16±0.52	8.77^{b}±0.10
Mean	7.73^{a}±0.03	6.90^{b}±0.15	6.76^{d}±0.25	7.61^{ab}±0.19	7.65^{ab}±0.20	
r	0.90	0.90	0.90	0.90	0.90	

- C1= *Lactobacillus plantarum* ATCC14917, C2= *L.casei* DSM20011, C3= *L.acidophilus* ATCC20552, C4= *Lactococcus thermophilus* DSM20259, C5= *Bifidobacterium longum* B41409.
- The data is provided as mean ± SD. The mean values within the same column or row with different superscript letters (^{a,b}) are significantly different at P ≤ 0.05. r= Correlation coefficient between fermentation time and viability of probiotic bacterial strains as a single culture during soymilk fermentation.

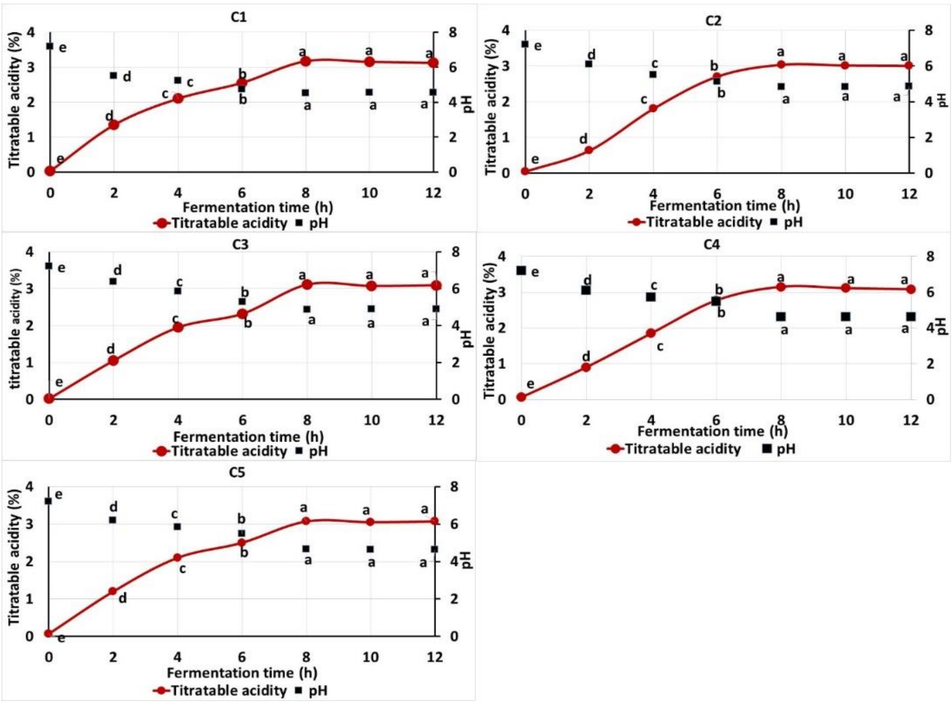

Fig 1. Changes in pH and titratable acidity (TA) during soymilk fermentation by probiotic bacterial strains as a single culture affected with fermentation time.

- C1= *Lactobacillus plantarum* ATCC14917, C2= *L.casei* DSM20011, C3= *L.acidophilus* ATCC20552, C4= *Lactococcus thermophilus* DSM20259, C5= *Bifidobacterium longum* B41409.
- The mean values above the line or points with different superscript letters (^{a,b}) are significantly different at P ≤ 0.05.

was no significant difference between the increase of the soymilk fermentation time from 8 h to 12 h in the reduction of pH and the increase in TA.

The resulted reduction in pH levels and increased probiotic bacterial population suggested that soymilk strains could adapt and survive (Rathore et al 2012). At the end of the incubation period, pH decreased from 6.79 to 4.03, and to 4.64, while TA increased from 0.01% to 0.09%, and to 0.07% for soymilk fermented with a single cultures of *L. casei* PLA5, and *L. casei* Shirota, respectively (Kumari et al 2018).

Fig 2 shown that the formation of organic acids by all the tested bacteria during soymilk fermentation was significantly improved by an increase in the fermentation period of up to 8 h. Whereas organic acid content was constant (not significant $p > 0.05$) after 10, and 12 h of the fermentation period for all tested bacteria. the production of lactic acid has been increased as the major organic acid accompanied by a small amount of acetic acid during the fermentation of soymilk. Data obtainable in Fig (2) showed the maximum lactic and acetic acid content (18.9±0.27, and 12.6±0.10 g / L) with the productivity of 2.36, and 1.57 g/L/h were recorded by *L. plantarum* ATCC14917(C1) fermented soymilk, followed by *Lc. thermophilus* DSM20259 (C4), and *B. longum* B41409 (C5) at 18.7±0.19, and 12.5±0.18 g / L with the productivity of 2.33, and 1.55 g/L/ h and 18.4 ±0.20, and 12.3±0.18 g / L with the the productivity of 2.30, and 1.53 g/L/ h, respectively. *B. longum* B41409 (C5) was only one of the tested cultures capable of producing propionic acid during fermentation and gave the highest content being 15.0±0.05 with a productivity of 1.87 g/L/ h after 8 h of fermentation time.

3.2 Soymilk fermentation with consortia probiotic bacterial cultures

3.2.1 Growth of consortia probiotic bacterial strains in soymilk

The date presented in **Table 2** clearly showed that the viability of probiotic bacterial cultures on fermented soymilk as a mixed culture (2 cultures). The viability of fermented soymilk increased significantly ($p \leq 0.05$) from zero time to 8 h with a range of viable counts from 4.36 to 8.55 Log CFU/ mL for *L. plantarum* ATCC14917+ *L. casei* DSM20011 (C6), from 4.20 to 8.49 Log CFU/ mL for *L. plantarum* ATCC14917+*L. acidophilus* ATCC20552 (C7), from 3.63 to 9.89 Log CFU/ mL for *L. plantarum* ATCC14917+ *Lc. thermophilus* DSM20259 (C8), from 4.11 to 9.33 Log CFU/ mL for *L. plantarum* ATCC14917+ *B. longum* B41409 (C9), from 3.41 to 8.83 Log CFU/ mL for *L. casei* DSM20011+ *L. acidophilus* ATCC20552 (C10), from 4.51 to 9.55 Log CFU/ mL for *L. casei* DSM20011 + *Lc. thermophilus* DSM20259 (C11), from 4.41 to 8.55 Log CFU/ mL for *L. casei* DSM20011+*B. longum* B40419, (C12), from 3.51 to 8.77 Log CFU/ mL for *L. acidophilus* ATCC20552+*Lc. thermophilus* DSM20259 (C13), from 3.55 to 8.25 Log CFU/ mL for *L. acidophilus* ATCC20552 + *B. longum* B41409 (C14), and from 4.51 to 9.27 Log CFU/ mL for *Lc. thermophilus* DSM20259+*B. longum* B41409 (C15) for 8 h incubation at 37°C with over increase reached to 1.96, 2.02, 2.72, 2.27, 2.59, 2.12, 1.93, 2.50, 2.32, and 2.05-Fold, respectively. Maximum viability (9.89 Log CFU/mL and 9.55 Log CFU/mL with r= 0.95) was obtained in a co-cultures of *L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259 (C8), and *L. casei*

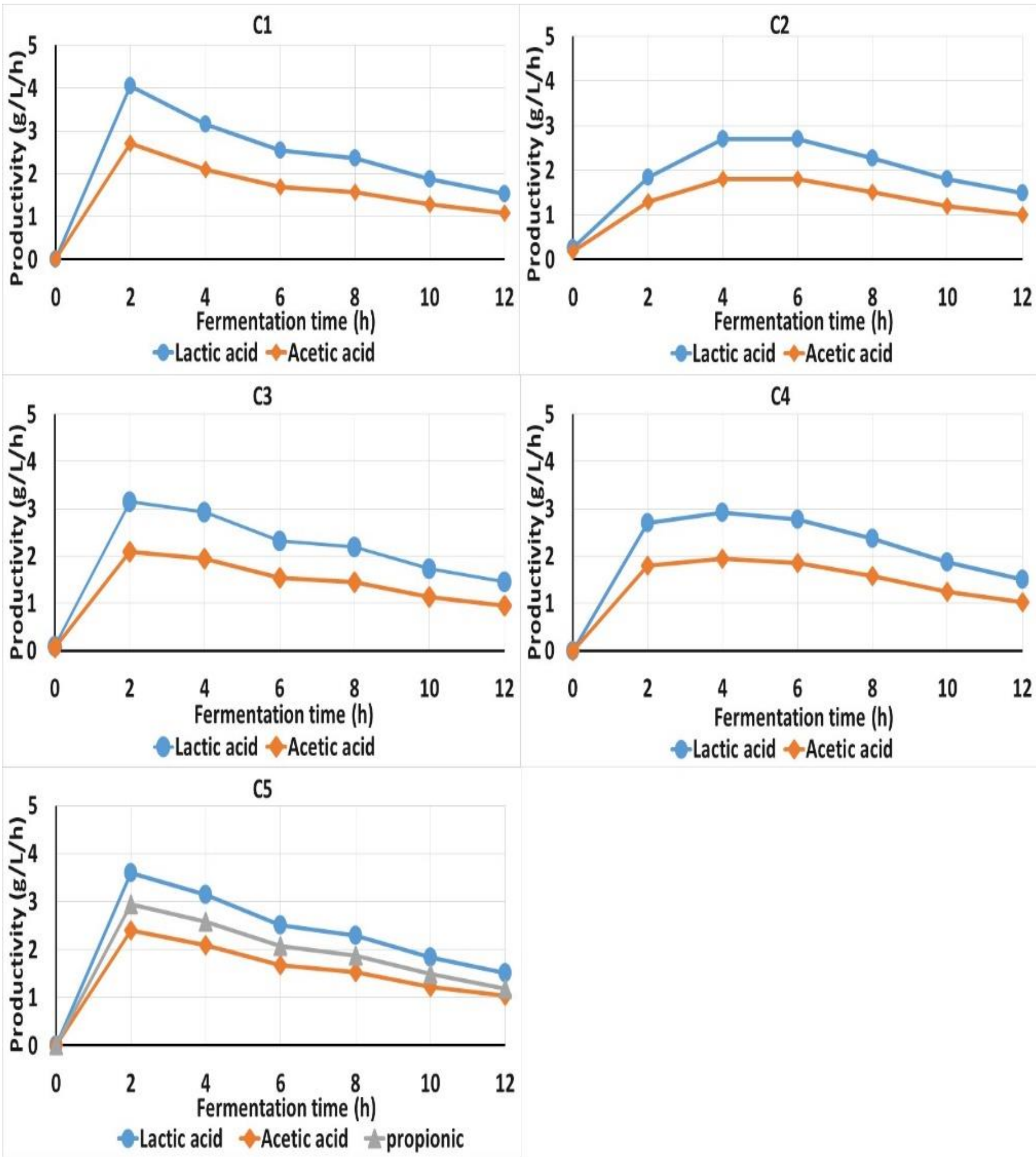

Fig 2. Organic acids (lactic and acetic or propionic acids) productivity during soymilk fermentation by probiotic bacterial strains as a single culture is affected with fermentation time

- C1= *Lactobacillus plantarum* ATCC14917, C2= *L.casei* DSM20011, C3= *L.acidophilus* ATCC20552, C4= *Lactococcus thermophilus* DSM20259, C5= *Bifidobacterium longum* B41409.

Table 2. Viability of probiotic bacterial strains as a co-culture (two and three strains) during soymilk fermentation is affected with fermentation time

Fermentation period (h)	Two probiotic cultures (Log CFU/mL)										
	C6	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**C13**	**C14**	**C15**	**Mean**
0	4.36±0.50	4.20±0.50	3.63±0.50	4.11±0.50	3.41±0.50	4.51±0.50	4.41±0.50	3.51±0.50	3.55±0.50	4.51±0.50	4.02^{e} ±0.04
2	6.20±0.55	6.26±0.50	5.57±0.50	6.41±0.50	5.11±0.50	5.36±0.50	5.77±0.50	5.26±0.50	5.11±0.50	5.20±0.50	5.61^{d} ±0.19
4	7.77±0.15	7.77±0.50	7.61±0.50	7.51±0.50	6.63±0.50	7.19±0.50	7.51±0.50	6.36±0.50	6.20±0.50	7.51±0.50	7.16^{c} ±0.21
6	8.11±0.44	8.16±0.50	8.40±0.50	8.26±0.50	7.77±0.50	8.20±0.50	8.11±0.50	7.55±0.50	8.00±0.50	8.77±0.50	8.11b ±0.42
8	8.55±0.18	8.49±0.50	9.89±0.50	9.33±0.50	8.83±0.50	9.55±0.50	8.55±0.50	8.77±0.50	8.25±0.50	9.27±0.50	8.94^{a} ±0.17
10	8.51±0.09	8.47±0.50	9.88±0.50	9.31±0.50	8.80±0.50	9.51±0.50	8.52±0.50	8.72±0.50	8.21±0.50	9.25±0.50	8.91^{ab} ±0.22
12	8.53±0.09	8.45±0.50	9.87±0.50	9.30±0.50	8.82±0.50	9.55±0.50	8.51±0.50	8.77±0.50	8.24±0.50	9.22±0.50	8.92^{ab} ±0..21
Mean	7.43^{d}±0.19	7.40^{d}±0.05	8.26^{a}±0.11	7.74^{b}±0.33	7.05^{f} ±0.09	7.34^{e} ±0.33	7.34^{e}±0.41	7.00^{f} ±0.50	6.80^{g} ±0.21	7.68^{c} ±0.15	
r	0.87	0.89	0.95	0.93	0.94	0.95	0.89	0.95	0.93	0.93	
	Three probiotic cultures (Log CFU/mL)										
	C16	**C17**	**C18**	**C19**	**C20**	**C21**	**C22**	**C23**	**C24**	**C25**	**Mean**
0	4.41±0.50	4.55±0.50	3.51±0.50	4.51±0.50	4.20±0.50	4.51±0.50	4.55±0.50	4.41±0.50	4.51±0.50	3.89±0.50	4.20^{e}±0.10
2	6.22±0.50	6.51±0.50	6.41±0.50	6.51±0.50	5.41±0.50	6.36±0.50	5.77±0.50	6.20±0.50	6.63±0.50	5.26±0.50	6.02^{d}±0.17
4	7.20±0.50	7.51±0.50	7.59±0.50	7.55±0.50	6.63±0.50	7.41±0.50	7.53±0.50	7.36±0.50	7.48±0.50	7.51±0.50	7.37^{c}±0.30
6	8.10±0.50	8.26±0.50	8.39±0.50	8.11±0.50	7.55±0.50	8.20±0.50	8.12±0.50	8.00±0.50	8.14±0.50	8.79±0.50	8.14^{b}±0.20
8	8.63±0.50	8.83±0.50	9.98±0.50	9.63±0.50	8.55±0.50	9.15±0.50	9.87±0.50	8.17±0.50	8.66±0.50	9.77±0.50	9.11^{a}±0.05
10	8.60±0.50	8.82±0.50	9.95±0.50	9.60±0.50	8.51±0.50	9.11±0.50	9.85±0.50	8.12±0.50	8.60±0.50	9.75±0.50	9.09^{ab}±0.07
12	8.61±0.50	8.80±0.50	9.93±0.50	9.63±0.50	8.50±0.50	9.12±0.50	9.82±0.50	8.10±0.50	8.63±0.50	9.77±0.50	9.08^{ab}±0.02
Mean	7.40^{d}±0.19	7.61^{b}±0.22	8.01^{a}±0.19	7.93^{ab}±0.12	6.91^{f}±0.11	7.69^{b} ±0.32	7.94^{ab}±0.10	7.19^{e} ±0.09	7.52^{c}±0.30	7.90^{ab}±0.19	
r	0.90	0.91	0.95	0.92	0.94	0.95	0.95	0.91	0.89	0.94	

- C6= *Lacobacillus plantarum* ATCC14917+ *L. casei* DSM20011, C7= *L. plantarum* ATCC14917+ *L. acidophilus* ATCC20552, C8= *L. plantarum* ATCC14917+ *Lactococcus thermophilus DSM20259*, C9= *L.plantarum* ATCC14917+ *B. longum* B41409, C10= *L.casei* DSM20011+ *L. acidophilus* ATCC20552, C11= *L. casei* DSM20011 + *Lc. thermophilus* DSM20259, C12= *L. casei* DSM20011+ *B. longum B41409*, C13= *L. acidophilus* ATCC20552+ *Lc. thermophilus DSM20259*, C14= *L. acidophilus ATCC 20552+ B. longum* B41409and C15= *Lc. thermophilus DSM20259+ B. longum B41409*. C16= *Lactobacillus plantarum* ATCC14917+ *L. casei* DSM20011+ *L. acidophilus* ATCC20552, C17= *L. plantarum + L. acidophilus* ATCC20552 + *Lactococcus thermophilus*, C18= *L. plantarum* ATCC14917 + *Lc. thermophilus DSM20259+ Bifidobacterium longum* B41409, C19= *L. plantarum* ATCC14917+ *L. casei* DSM20011+ *Lc. thermophiles DSM20259*, C20= *L. plantarum ATCC14917+L. casei* DSM20011+ *B. longum B41409*, C21= *L. plantarum* ATCC14917 + *L. acidophilus* ATCC20552+ *B. longum B41409*, C22 = *L. casei* DSM20011+ *L. acidophilus* ATCC20552+ *Lc. thermophilus* DSM20259, C23= *L, casei* DSM20011+ *Lc. thermophiles* DSM20259 + *B. longum* B41409, C24= *L. casei* DSM20011 + *L. acidophilus* ATCC20552+ *B. longum* B41409and C25= *L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259 + *B. longum* B41409.
- The data is provided as mean ± SD. The mean values within the same column or row with different superscript letters (^{a,b}) are significantly different at $P \leq 0.05$. r= Correlation coefficient between fermentation time and viability of probiotic bacterial strains.

DSM20011 + *Lc. thermophilus* DSM20259 (C11), respectively followed by both of *L. plantarum* ATCC14917 + *B. longum* B41409 (C9), and *Lc. thermophilus* DSM20259+*B. longum* B41409 (C15) at 9.33, and 9.27 Log CFU/mL with high positive correlation coefficient r= 0.93. The lowest viability was 8.25 Log CFU/ mL in the presence of mixed cultures *L. acidophilus* ATCC20552+*B. longum* B41409 (C14).

Data in **Table 3** clearly showed that the viability of probiotic bacterial cultures on fermented soymilk as a co-culture by 3 cultures for 12 of the fermentation periods at 37°C. Fermented soymilk viability increased substantially ($p< 0.05$) from zero to 8 h with a range of viable counts. from 4.41 to 8.63 Log CFU/mL for *L. plantarum* ATCC14917 + *L. casei* DSM20011 + *L. acidophilus* ATCC20552 (C16), 4.55 to 8.83 Log CFU/mL for *L. plantarum* ATCC14917+*L. acidophilus* ATCC20552+*Lc. thermophilus* DSM20259 (C17), 3.51 to 9.98 Log CFU/mL for *L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259 + *B. longum* B41409 (C18), from 4.51 to 9.63 Log CFU/mL for *L. plantarum* ATCC14917 + *L. casei* DSM20011+*Lc. thermophiles* DSM20259 (C19), from 4.20 to 8.55 Log CFU/mL for *L. plantarum* ATCC14917 + *L. casei* DSM20011 + *B. longum* B41409 (C20), 4.51 to 9.15 Log CFU/mL for *L. plantarum* ATCC14917 + *L. acidophilus* ATCC20552 + *B. longum* B41409 (C21), from 4.55 to 9.87 Log CFU/mL for *L. casei* DSM20011 + *L. acidophilus* ATCC20552+*Lc. thermophilus* DSM20259 (C22), 4.41 to 8.17 Log CFU/mL for *L. casei* DSM 20011 + *Lc. thermophiles* DSM20259 + *B. longum* B41409 (C23), 4.51 to 8.66 Log CFU/mL for *L. casei* DSM20011 + *L. acidophilus* ATCC20552 + *B. longum* B41409 (C24), 3.89 to 9.77 Log CFU/mL for *L. acidophilus* ATCC20552+*Lc. thermophilus* DSM20259+*B. longum* B41409 (C25) with overgrowth increase about 1.96, 1.93, 2.84, 2.14, 2.03, 2.02, 2.17, 1.85, 1.92, and 2.51-Fold, respectively. The highest viability (9.98 Log CFU/mL with $r = 0.95$) was recorded in fermented soymilk by mixed culture *L. plantarum* ATCC14917+*Lc. thermophilus* DSM20259+*B. longum* B41409(C18) followed by *L. casei* DSM20011+*L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259 (C22), *L. acidophilus ATCC20552+Lc. thermophilus* DSM20259 + *B. longum* B41409 (C25), and then *L. plantarum* ATCC14917 + *L. casei* DSM20011 + *Lc. thermophiles* DSM20259 (C19) being 9.87, 9.77, and 9.63 Log CFU/mL, respectively. The 3 mixed cultures of *L. casei* DSM20011+*Lc. thermophiles* DSM20259+*B. longum* B41409 (C23) were restarted as a minimum of viability (8.17 Log CFU/mL). A high positive correlation coefficient (r) between the fermentation time and growth for all tested bacteria strains, which value ranged between 0.89 and 0.95 are shown in **Fig 2.**

It was concluded from the aforementioned results that mixed cultures of two strains *L. plantarum* ATCC14917+ *Lc. thermophiles* DSM20259(C8) and three strains *L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259 + *B. longum* B41409 (C18), *L. plantarum* ATCC14917 + *L. casei* DSM20011+ *Lc. thermophiles* DSM20259 (C19), *L. casei* DSM20011 + *L. acidophilus* ATCC20552+ *Lc. thermophilus* DSM20259 (C22), and *L. acidophilus* ATCC20552+ *Lc. thermophilus* DSM20259 + *B. longum* B41409 (C25) had the greatest significant viability of probiotic bacterial cultures in fermented soymilk.

Probiotic cultures (*L. plantarum* ATCC14917, *L. casei* DSM20011, *L. acidophilus* ATCC20559, *Lc. thermophiles* DSM20259, and *B. longum* B41409) were chosen for soymilk fermentation because of the positive interaction between probiotic bacterial strains (Tamime et al 2005). Besides that, *L. plantarum* and *Lc. thermophilus* is always the dominant strain in co-cultures and shows the greatest growth regardless of the fermentable substrates, e.g. in soymilk (Božanić et al 2008, Rathore 2012).

From the above findings, it could be observed that soymilk fermentation with consortia probiotic bacterial strains of two and three mixed cultures increased viability more than single cultures fermentation.

Table 3. Organic acids formation during soymilk fermentation by probiotic bacterial strains as a co-culture (three strains) affected with fermentation time

Probiotic cultures	Organic acid content (g/L)	Fermentation time (h)							Mean
		0	2	4	6	8	10	12	
C16	Lactic acid	0.18^{e}±0.08	7.90^{d}±0.08	11.6^{c}±0.18	14.0^{b}±0.19	17.73^{a}±0.19	17.72^{a}±0.19	17.70^{a}±0.19	12.39^{c}±0.09
	Acetic acid	0.12^{e}±0.06	4.60^{d}±0.16	7.40^{c}±0.05	9.30^{b}±0.18	11.82^{a}±0.18	11.82^{a}±0.18	11.81^{a}±0.18	8.12^{cd}±0.01
	Propionic acid	-	-	-	-	-	-	-	-
C17	Lactic acid	0.135^{e}±0.05	6.5.8^{d}±0.17	10.6^{c}±0.05	15.8^{b}±0.17	18.20^{a}±0.20	18.0^{a}±0.19	18.0^{a}±0.19	12.46^{c}±0.07
	Acetic acid	0.09^{e}±0.05	4.40^{d}±0.03	6.40^{c}±0.05	10.20^{b}±0.03	12.12^{a}±0.18	12.0^{a}±0.18	12.0^{a}±0.29	8.09^{c}±0.04
	Propionic acid	-	-	-	-	-	-	-	-
C18	Lactic acid	0..31^{e}±0.03	11.2^{d}±0.05	12.6^{c}±0.05	16.91^{b}±0.20	19.73^{a}±0.27	19.7^{a}±0.25	19.93^{a}±0.50	14.28^{a}±0.12
	Acetic acid	0.21^{e}±0.03	7.50^{d}±0.10	8.40^{c}±0.10	11.27^{b}±0.18	13.19^{a}±0.10	13.17^{a}±0.15	13.37^{a}±0.17	9.51^{a}±0.10
	Propionic acid	0.28^{e}±0.03	9.17^{d}±0.10	10.3^{c}±0.10	13.09^{b}±0.18	16.35^{a}±0.10	16.21^{a}±0.19	16.13^{a}±0.10	11.75^{a}±0.17
C19	Lactic acid	0.27^{e}±0.06	9.31^{d}±0.06	11.7^{c}±0.05	15.61^{b}±0.05	18.57^{a}±0.19	18.52^{a}±0.39	18.52^{a}±0.21	12.64^{b}±0.21
	Acetic acid	0.18^{e}±0.05	6.24^{d}±0.05	7.84^{c}±0.03	10.37^{b}±0.10	12.63^{a}±0.18	12.60^{a}±0.28	12.62^{a}±0.11	8.62^{b}±0.19
	Propionic acid	-	-	-	-	-	-	-	-
C20	Lactic acid	0.22^{e}±0.02	8.62^{d}±0.06	11.7^{c}±0.50	12.43^{b}±0.20	16.93^{a}±0.22	16.81^{a}±0.44	16.72^{a}±0.11	11.60^{e}±0.11
	Acetic acid	0.15^{e}±0.07	5.80^{d}±0.05	7.80^{c}±0.09	8.30^{b}±0.10	10.73^{a}±0.38	10.69^{a}±0.19	10.71^{a}±0.25	7.63^{d}±0.15
	Propionic acid	0.18^{e}±0.08	6.90^{d}±0.08	9.90^{c}±0.11	10.57^{b}±0.12	13.91^{a}±0.40	13.9^{a}±0.32	13.9^{a}±0.50	9.71^{e}±0.11
C21	Lactic acid	0.09^{e}±0.04	7.20^{d}±0.03	12.0^{c}±0.50	14.19^{b}±0.18	17.93^{a}±0.20	17.83^{a}±0.28	17.87^{a}±0.33	12.54^{c}±0.10
	Acetic acid	0.05^{e}±0.0.01	4.80^{d}±0.13	8.00^{c}±0.09	10.0^{b}±0.05	11.90^{a}±0.18	11.87^{a}±0.10	11.82^{a}±0.29	8.35^{c}±0.09
	Propionic acid	0.07^{e}±0.09	5.90^{d}±0.08	10.0^{c}±0.11	12.20^{b}±0.05	14.72^{a}±0.05	14.81^{a}±0.22	14.80^{a}±0.19	10.35^{c}±0.10
C22	Lactic acid	0.03^{e}±0.06	8.37^{d}±0.06	12.61^{c}±0.05	15.10^{b}±0.18	18.82^{a}±0.22	18.71^{a}±0.50	18.17^{a}±0.40	12.59^{b}±0.12
	Acetic acid	0.02^{e}±0.05	5.60^{d}±0.05	8.70^{c}±0.03	10.40^{b}±0.05	12.45^{a}±0.33	12.44^{a}±0.18	12.41^{a}±0.28	8.54^{b}±0.11
	Propionic acid	-	-	-	-	-	-	-	-
C23	Lactic acid	0.02^{e}±0.02	7.40^{d}±0.06	11.81^{c}±0.05	15.0^{b}±0.18	16.72^{a}±0.44	16.61^{a}±0.30	16.61^{a}±0.41	12.47^{c}±0.15
	Acetic acid	0.01^{e}±0.05	4.90^{d}±0.05	7.90^{c}±0.03	10.11^{b}±0.05	11.19^{a}±0.19	11.0^{a}±0.22	11.0^{a}±0.19	8.03^{d}±0.19
	Propionic acid	0.01^{e}±0.02	6.00^{d}±0.08	10.13^{c}±0.05	12.21^{b}±0.05	13.81^{a}±0.32	13.82^{a}±0.35	13.28^{a}±0.30	10.01^{d}±0.10
C24	Lactic acid	0.09^{e}±0.03	8.90^{d}±0.03	12.61^{c}±0.18	13.81^{b}±0.22	16.19^{a}±0.20	16.17^{a}±0.33	16.14a±0.35	12.17^{d}±0.21
	Acetic acid	0.05^{e}±0.0.01	5.60^{d}±0.13	8.40^{c}±0.05	9.20^{b}±0.50	10.17^{a}±0.18	10.15^{a}±0.14	10.11a±0.29	7.68^{e}±0.19
	Propionic acid	0.07^{e}±0.07	6.40^{d}±0.08	10.32^{c}±0.05	11.34^{b}±0.22	12.93^{a}±0.05	12.91^{a}±0.29	12.91^{a}±0.10	9.54^{e}±0.11
C25	Lactic acid	0.02^{d}±0.03	9.90^{d}±0.03	13.92^{b}±0.22	16.93^{b}±0.22	18.35^{a}±0.20	18.32^{a}±0.22	18.30^{a}±0.25	12.62^{b}±0.12
	Acetic acid	0.01^{d}±0.13	6.60^{d}±0.13	9.41^{b}±0.50	10.61^{b}±0.50	12.23^{a}±0.18	12.21^{a}±0.10	12.20^{a}±0.16	8.57^{b}±0.17
	Propionic acid	0.02^{d}±0.08	7.40^{d}±0.08	11.47^{b}±0.22	13.84^{b}±0.22	15.03^{a}±0.05	15.01^{a}±0.19	15.00^{a}±0.29	10.50^{b}±0.10

- C16= *Lactobacillus plantarum* ATCC14917+ *L. casei* DSM20011+ *L. acidophilus* ATCC20552, C17= *L. plantarum + L. acidophilus* ATCC20552 + *Lactococcus thermophilus*, C18= *L. plantarum* ATCC14917+ *Lc. thermophilus DSM20259+ Bifidobacterium longum*, C19= *L. plantarum* ATCC14917+ *L. casei* DSM20011+ *Lc. thermophiles DSM20259*, C20= *L. plantarum ATCC14917+L. casei* DSM20011+ *B. longum B41409*, C21= *L. plantarum* ATCC14917+ *L. acidophilus* ATCC20552+ *B. longum B41409*, C22 = *L. casei* DSM20011+ *L. acidophilus* ATCC20552+ *Lc. thermophilus* DSM20259, C23= *L, casei* DSM20011+ *Lc. thermophiles* DSM20259+ *B. longum* B41409, C24= *L. casei* DSM20011+ *L. acidophilus* ATCC20552 + *B. longum* B41409and C25= *L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259 + *B. longum* B41409 and (-) = Not detected.
- The data is provided as mean ± SD. The mean values within the same column with different superscript letters (^{a,b}) are significantly different at P ≤ 0.05.

This suggested that mixed strain fermentation can make up for the deficiency of single strain fermentation, shorten the fermentation time, give full play to the advantages of multi-strain fermentation, and obtain more satisfactory fermentation products. But in the fermentation process, we still need to consider the antagonism between different strains (Ruokun et al 2020).

3.2.2 Change in pH, total acidity and the content of organic acids (lactic and acetic acids) during soymilk fermentation by consortia strains

Data in **Fig 3 s**howed that the initial pH (0 h) of soymilk was found to decrease and increase acidity after inoculation with probiotic bacterial as a mixed culture (2 cultures) for 8 h at 37°C. The pH declined from 7.20 to 4.87, 4.82, 4.51, 4.62, 4.66, 4.71, 4.82, 4.86, 4.61 and 4.64 for *L. plantarum* ATCC14917 + *L. casei* DSM20011 (C6), *L. plantarum* ATCC14917 + *L. acidophilus* ATCC20552 (C7), *L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259 (C8), *L. plantarum* ATCC14917 + *B. longum* B41409 (C9), *L. casei* DSM20011 + *L. acidophilus* ATCC20552 (C10), *L. casei* DSM20011 + *Lc thermophilus* DSM20259 (C11), *L. casei* DSM20011 + *B. longum* B41409 (C12), *L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259 (C13), *L. acidophilus* ATCC20552 + *B. longum* B41409 (C14) and *Lc. thermophilus* DSM20259 + *B. longum* B41409 (C15), respectively. Data also indicated in **Fig 4** that the mixed culture of *plantarum* ATCC14917 + *L. casei* DSM20011 (C6), *L. plantarum* ATCC14917 + *L. acidophilus* ATCC20552 (C7), *L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259 (C8), *L. casei* + *L. acidophilus* ATCC20552 (C10), *L. casei* DSM20011 + *Lc. thermophilus* DSM20259 (C11) and, *L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259 (C13), achieved hetero fermentation of soymilk and lactic acid and acetic acid, were produced whereas the mixed strains of *L. plantarum* ATCC14917 + *B. longum* B41409 (C9), *L. casei* DSM20011 + *B. longum* B41409 (C12), *L. acidophilus* ATCC20552 + *B. longum* B41409 (C14) and, *Lc. thermophilus* DSM20259 + *B. longum* B41409 (C15) generated lactic acid, acetic acid, and propionic acid. The content and productivity of organic acids (lactic acid and acetic acid or propionic acid) had been increased during the fermentation of soymilk. The highest production of lactic acid and acetic acid is being 19.8 and 13.2 g/L for *L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259 (C8). Low production of organic acids (16.97 g/L of lactic acid and 11.32 g/L of acetic acid) were recorded by *L. casei* DSM20011 + *L. acidophilus* ATCC20552 (C10).

In the case of soymilk fermentation by 3 combination strains, data obtainable in **Fig 5 s**howed that the initial pH (0 h) of soymilk has been decreased and the acidity was increased after inoculation with probiotic bacterial as a mixed culture (3 cultures) for 8 h at 37°C. The pH declined from 7.20 to 4.42, 4.49, 4.38, 4.43, 4.54, 4.51, 4.48, 4.52, 4.53, and 4.56 for *L. plantarum* ATCC14917 + *L. casei DSM20011* + *L. acidophilus* ATCC20552 (C16), *L. plantarum* ATCC14917 + *L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259 (C17), *L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259+ *Bifidobacterium Longum* B41409 (C18), *L. plantarum* ATCC14917+ *L. casei* DSM20011+ *Lc thermophiles* DSM20259 (C19), *L. plantarum* ATCC14917 + *L. casei* DSM20011 + *B. Longum* B41409 (C20), *L. Plantarum* ATCC14917 + *L. acidophilus* ATCC20552+ *B. longum* B41409 (C21), *L. casei* DSM20011 + *L. acidophilus* ATCC20552+ *Lc. thermophilus* DSM20259 (C22), *L. casei* DSM20011+ *Lc. thermophiles* DSM20259 + *B. longum* B41409 (C23), *L. casei* DSM20011+ *L. acidophilus* ATCC20552 + *B. longum* B41409 (C24) and *L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259 + *B. longum* B41409 (C25), respectively.

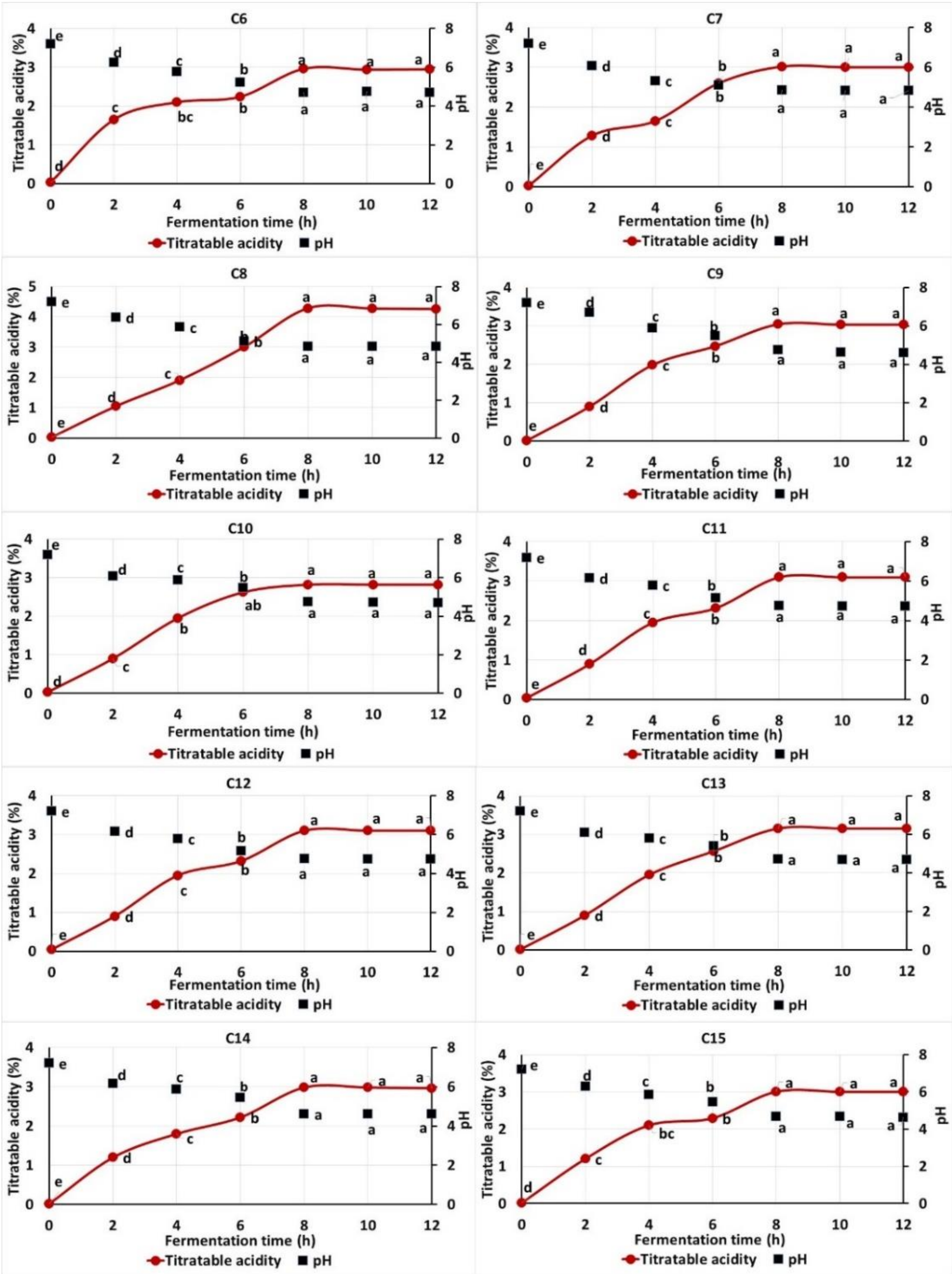

Fig 3. Changes of pH and titratable acidity (TA) during soymilk fermentation by probiotic bacterial strains as a mixed culture (two strains) affected with fermentation time

- C6= *Lactobacillus plantarum* ATCC14917 + *L. casei* DSM20011, C7= *L. plantarum* ATCC14917 + *L. acidophilus* ATCC20552, C8= *L. plantarum* ATCC14917 + *Lactococcus thermophilus DSM20259*, C9= *L. plantarum* ATCC14917 + *B. longum B41409*, C10= *L. casei* DSM20011 + *L. acidophilus* ATCC20552, C11= *L. casei* DSM20011 + *Lc. thermophilus* DSM20259, C12= *L. casei* DSM20011 + *B. longum B41409*, C13= *L. acidophilus* ATCC20552 + *Lc. thermophilus DSM20259*, C14= *L. acidophilus ATCC 20552* + *B. longum* B41409and C15= *Lc. thermophilus DSM20259* + *B. longum* B41409.
- The mean values above the line or points with different superscript letters (^{a,b}) are significantly different at $P \leq 0.05$.

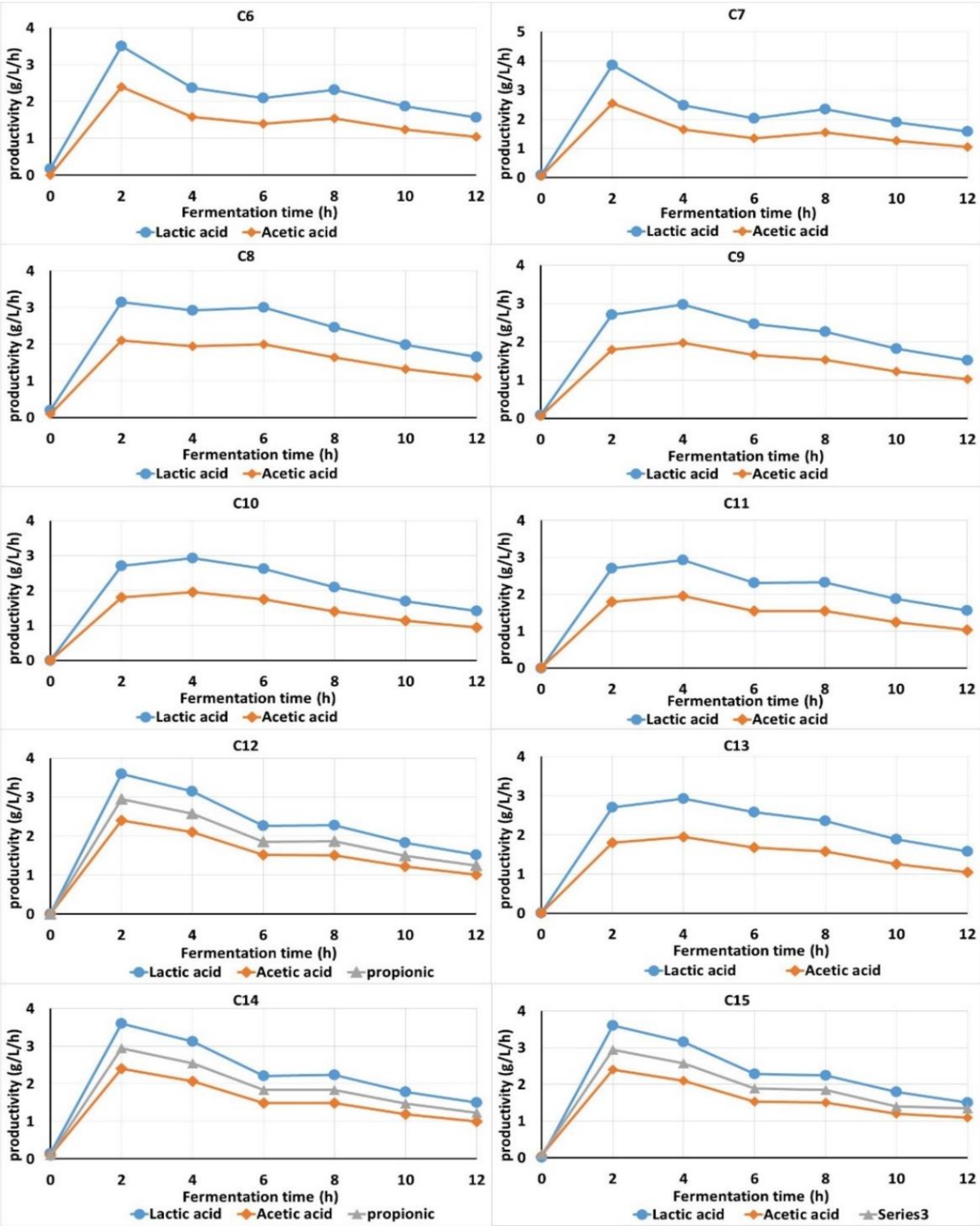

Fig 4. Organic acids (lactic and acetic or propionic acids) productivity during soymilk fermentation by probiotic bacterial strains as two mixed cultures affected with fermentation time

- C6= *Lactobacillus plantarum* ATCC14917+ *L. casei* DSM20011, C7= *L. plantarum* ATCC14917+ *L. acidophilus* ATCC20552, C8= *L. plantarum* ATCC14917+ *Lactococcus thermophilus DSM20259*, C9= *L. plantarum* ATCC14917+ *B. longum B41409*, C10= *L. casei* DSM20011+ *L. acidophilus* ATCC20552, C11= *L. casei* DSM20011 + *Lc. thermophilus* DSM20259, C12= *L. casei* DSM20011+ *B. longum B41409*, C13= *L. acidophilus* ATCC20552+ *Lc. thermophilus DSM20259*, C14= *L. acidophilus ATCC 20552*+ *B. longum* B41409and C15= *Lc. thermophilus DSM20259*+ *B. longum* B41409.

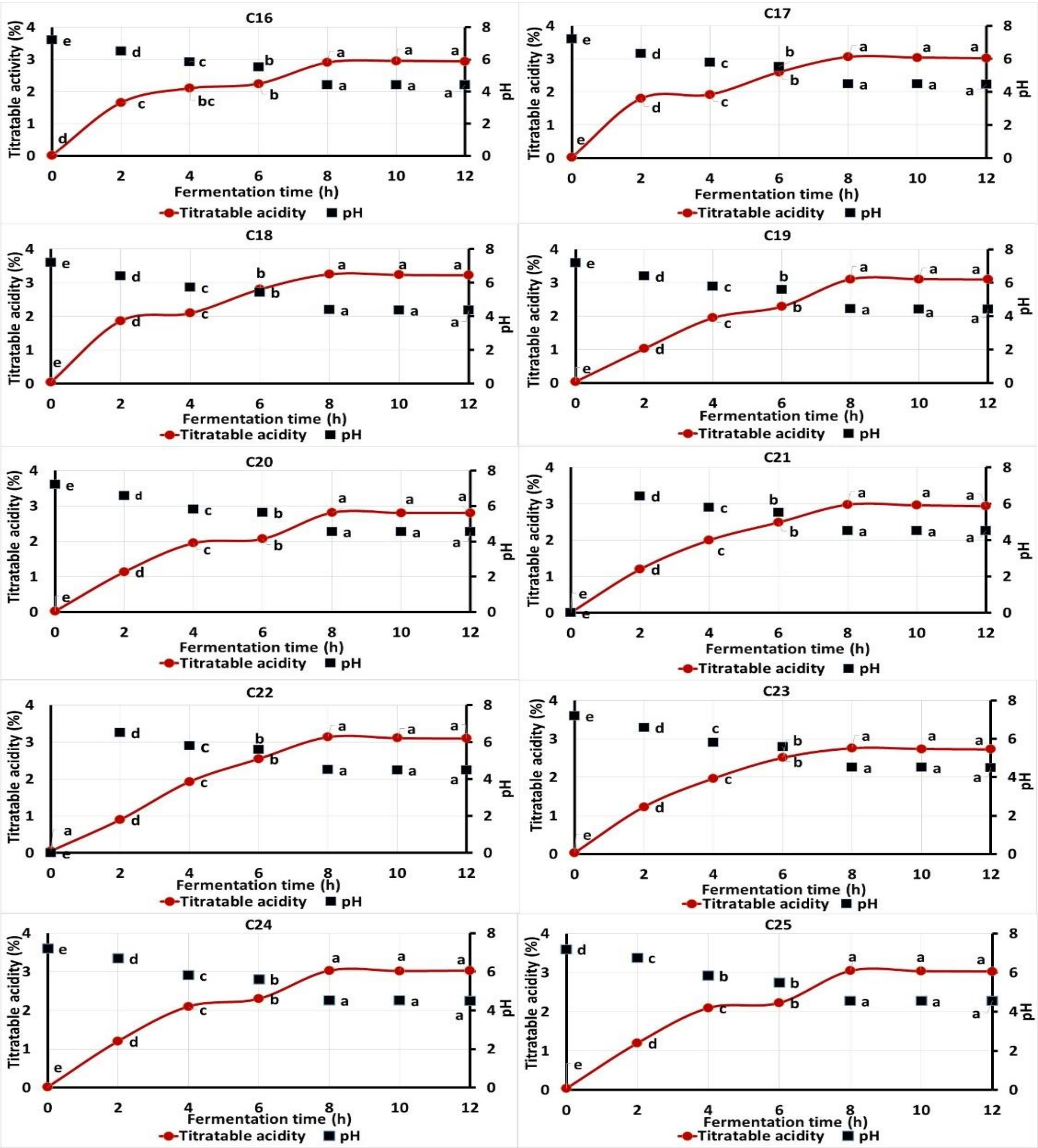

Fig 5. Changes of pH and titratable acidity (TA) during soymilk fermentation by probiotic bacterial strains as a co-culture (three strains) affected with fermentation time

- C15= *Lc. thermophilus DSM20259*+ *B. longum B41409*. C16= *Lactobacillus plantarum* ATCC14917+ *L. casei* DSM20011+ *L. acidophilus* ATCC20552, C17=*L. plantarum* + *L. acidophilus* ATCC20552 + *Lactococcus thermophilus*, C18= *L. plantarum* ATCC14917+ *Lc. thermophilus DSM20259*+ *Bifidobacterium longum*, C19= *L. plantarum* ATCC14917+ *L. casei* DSM20011+ *Lc. thermophiles DSM20259*, C20= *L. plantarum ATCC14917*+*L. casei* DSM20011+ *B. longum B41409*, C21= *L. plantarum* ATCC14917+ *L. acidophilus* ATCC20552+ *B. longum B41409*, C22 = *L. casei* DSM20011+ *L. acidophilus* ATCC20552+ *Lc. thermophilus* DSM20259, C23= *L, casei* DSM20011+ *Lc. thermophiles* DSM20259+ *B. longum* B41409, C24= *L. casei* DSM20011+ *L. acidophilus* ATCC20552+ *B. longum* B41409and C25=*L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259+ *B. longum* B41409.,
- The mean values above the line or points with different superscript letters (^{a,b}) are significantly different at $P \leq 0.05$.

Data in **Table 3** and illustrated by **Fig 6** indicated that lactic acid and acetic acid production has been increased during the fermentation of soymilk. The highest production of lactic acid and acetic acid or propionic acid is being 19.73, and 13.19 or 16.35 g/ L with productivity 2.45, and 1.65 or 2.03 g/ L/ h for *L. plantarum* ATCC14917+ *Lc. thermophilus* DSM20259 + *B. longum* B41409 (C18) followed by *L. plantarum* ATCC14917+*L. casei* DSM20011+*Lc. thermophiles* DSM20259 (C19) (18.57 and 12.63 g/ L with productivity 2.32 and 1.57 g/ L/ h), *L. casei* DSM20011+*L. acidophilus* ATC20552+*Lc. thermophilus* DSM20259 (C22) (18.82, and 12.45 g/L with productivity 2.35 and 1.55 g/ L/ h) and *L. acidophilus* ATCC20552+*Lc. thermophilus* DSM20259+*B. longum* B41409(C25) (18.35, and 12.23 or 15.03 g/ L with productivity 2.29, and 1.53 or 1.88 g/L/h), respectively. Minimum organic acid content was recorded in the case of *L. casei* DSM20011+*L. acidophilus* ATCC20552+*B. longum* B41409 (C24) being 16.19 and 10.17 or 12.93 g/L with productivity 2.02 and 1.27 or 1.62 g/L/h).

This trend is similar with (Obadina et al 2013) reported that although the pH of all soymilk yoghurt decreased with fermentation time, the observed differences in the pH reduction of the fermentation yoghurt pre-mix may reflect the ability of the yoghurt bacteria to grow and ferment the carbohydrates contained in the pre-mix.

The pH value of fermented soybean milk was lower than that of unfermented soybean milk because a certain amount of organic acid was produced in the fermentation process. However, as fermentation continued, the accumulation of other metabolites and the synthesis of nitrogen-containing substances such as ammonia and amines delayed the pH value. It is also linked to the change in the total acid content (Fang et al 2020).

Normally probiotic bacterial cultures convert lactose in milk into organic acid, mostly lactic acid (Widyastuti et al 2014). Considering lactose as the key substrate for probiotic bacteria, researchers typically add lactose from skim milk to soya milk to increase the activity of probiotic bacteria. Pure soymilk was used in this analysis with skim milk to ensure that the findings were obtained only from the fermentation of soymilk, not from other ingredients. Changes in organic acids formed during fermentation may be due to probiotic bacterial cultures which convert most of the available carbohydrates in the substrate into lactic acids with a small amount of acetic acid (Farnworth 2005).

Also, indicated that a lower pH and higher acidity could be obtained with mixed cultures of lactic acid bacteria and *Bifidobacteria* than with a single strain. The introduction of *Bifidobacteria* in the starter culture with lactic acid bacteria resulted in a higher content of acids than that with a single strain of the respective lactic acid bacteria during fermentation. soymilk inoculated with the mixed culture of *L. plantarum, L. thermophilus,* and *B. longum* contained a higher lactic and acetic acid content of 2.25 m moll^{-1} & 1.50 m moll^{-1} and *L. acidophilus, L. thermophilus* and *B. longum* 2.16 and 1.44 m moll^{-1} compared with those of 2.16 and 1.44 m moll^{-1} and 1.89 and 1.26 m moll^{-1}, in soymilk inoculated with the single culture of *L. plantarum and L. thermophilus*, respectively. This result agreement with (Wang et al 2003).

The above results clarified that fermentation of soymilk varied according to different types of probiotic bacteria whether it was a single strain or mixed strains. As shown strains number *L. planturum* ATCC14917(C1), *L. thermophilus* DSM20259(C4), and *B. longum* B41409(C5) as single culture and *L. planturum* ATCC14917 + *Lc.* thermophilus DSM20259 (C8), *L. planturum* ATCC14917 + *Lc. thermophilus* DSM20259 + *B. longum* B41409 (C18), *L. planturum* ATCC14917 + *L. casei* DSM20011 + *Lc. thermophilus* DSM20259 (C19), *L. casei* DSM20011 + *L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259 (C22), and *L. acidophilus* ATCC20552 + *Lc. thermophilus DSM20259* + *B. longum* B41409 (C25) as mixed culture gave the highest value of growth, decrease the pH and higher acidity

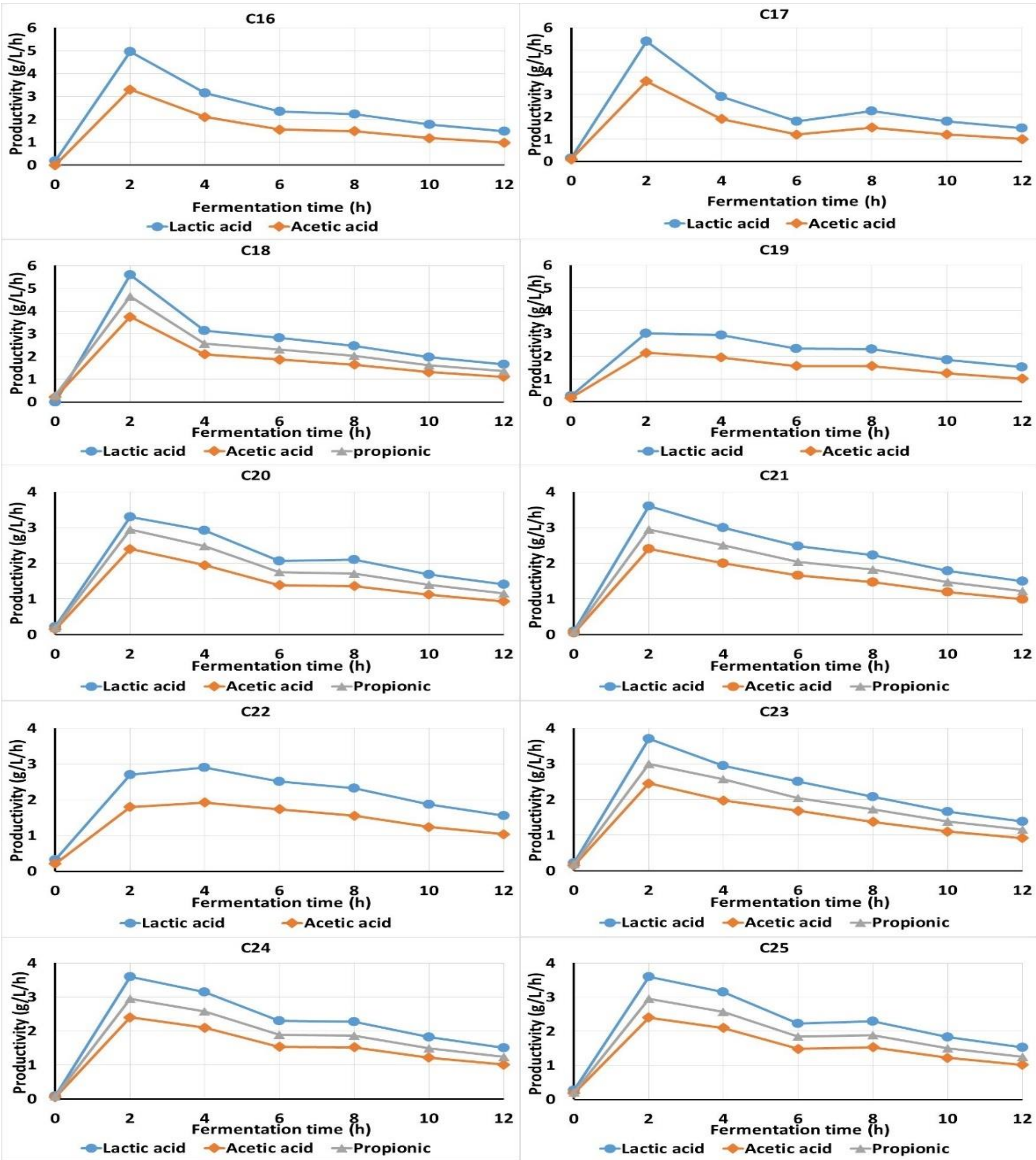

Fig 6. Organic acids (lactic and acetic or propionic acids) productivity during soymilk fermentation by probiotic bacterial strains as two mixed cultures affected with fermentation time

- C16= *Lactobacillus plantarum* ATCC14917+ *L. casei* DSM20011+ *L. acidophilus* ATCC20552, C17= *L. plantarum* + *L. acidophilus* ATCC20552 + *Lactococcus thermophilus*, C18= *L. plantarum* ATCC14917+ *Lc. thermophilus DSM20259*+ *Bifidobacterium longum*, C19= *L. plantarum* ATCC14917+ *L. casei* DSM20011+ *Lc. Thermophiles DSM20259*, C20= *L. plantarum ATCC14917+L. casei* DSM20011+ *B. longum B41409*, C21= *L. plantarum* ATCC14917+ *L. acidophilus* ATCC20552+ *B. longum B41409*, C22 = *L. casei* DSM20011+ *L. acidophilus* ATCC20552+ *Lc. thermophilus* DSM20259, C23= *L, casei* DSM20011+ *Lc. thermophiles* DSM20259+ *B. longum* B41409, C24= *L. casei* DSM20011+ *L. acidophilus* ATCC20552+ *B. longum* B41409and C25= *L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259+ *B. longum* B41409.

consequence the production of lactic acid and other acids, it means these strains had a higher activity in converting soymilk become fermented soymilk as compared with other strains. So, these strains were selected for further studies.

3.3 Nutritional values of fermented products by probiotics strains

To identify nutritional values, several experiments have been done on final products. **Table 4** tabulates the chemical composition of fermented soymilk products by probiotic bacteria. Fermented products were found to consist of varying percentages of protein, ash, fat, and carbohydrates as compared to non-fermented products. A decrease in moisture, fat, and carbohydrate contents was observed, while an increase in the total solids, protein, and ash content of fermented soymilk was noticed.

The initial moisture content falls within the range 1.01-Fold to 0.99 and 0.98-Fold and is similar to reports of Namrata and Gurmukh (2007), Tunde-Akintunde and Souley (2009), Orhevba (2011). As a consequence of microbial cell proliferation, the decrease observed in moisture content as fermentation time progressed may be attributed to increased dry matter content (Morris et al 2004, Obadina et al 2013) stated that the accumulation of nutrients was typically increased by a reduction in moisture. Moisture content decreased as fermentation time increased, while the overall solid content in soymilk fermentation increased.

The results also revealed and development in the number of fermented soymilk products in protein, which ranged from 1.5 to 1.9-Fold, as compared to non-fermented products, respectively. The increase identified in fermenting soymilk protein content compared to soymilk could be attributed to certain anabolic processes leading to polymer build-up or microbial cell proliferation (Obadina et al 2013).

Although the fat content improved slightly. This may be due to the increased lipolytic enzyme activity during fermentation that hydrolyses fat components (triacylglycerol) into fatty acid and glycerol and used as energy sources have been suggested by (Astuti et al 2000).

The content of ash was increased in fermented soymilk with ranging from 1.33 to 2.9 -Fold as compared to non-fermented. This increase in ash refer to enhance the value with minerals (such as magnesium, iron and calcium)in fermented soymilk products have been described by (Obadina et al 2013).

The carbohydrate content in this study decreased significantly from 1.8 to 1.6 -Fold at 8 h of fermentation. The decrease in the carbohydrate content of fermented soybean as the fermentation period increased could be explained by the activities of fermenting microorganisms which that used and transformed them into growth energy and other cellular activities (Osundahunsi et al 2007). The energy values ranged from 33.84 kcal/100g *Lc. thermophilus* DSM20259 (C4) to 39.59 kcal/100g *L. plantarum* ATCC14917+ *Lc. thermophilus* DSM20259 + *B. longum* B41409(C18). The decrease may be due to the energy utilization of the microbes in this drink. The higher energy value of the samples was a result of their higher protein and fat contents. The number of calories in a given food is termed the energy value of the food and is a good factor for comparing the true value of different foods (Abiodun et al 2019).

Statistics show that after 8 hours of the fermentation period, there is a significant difference between products of fermented soymilk products (soybean yogurt) in each of the content of moisture, protein, fat, carbohydrates, ash, and total solids, ranging from 93.16 to 91.73, 2.10 to 3.95, 2.13 to 1.40, 2.43 to 1.51, 0.13 to 0.52, and 5.21 to 8.70 %, respectively.

Table 4. Chemical composition of soymilk and fermented soymilk using the most significant mono and co-cultures

Treatments	Parameters (%)						
	Moisture	**Protein**	**Fat**	**Carbohydrates**	**Ash**	**Total solids**	**Energy (kcal/100)**
Control	93.16^{a}±0.5	2.10^{e}±0.24	2.13^{c}±0.05	2.43^{f}±0.34	0.18^{c}±0.19	6.84^{e}±0.09	37.29^{b} ± 0.71
C1	92.48^{b}±0.22	3.92^{a}±0.52	1.42^{cd}±0.24	1.87^{de}±0.02	0.31^{b}±0.01	8.22^{bc}±0.11	35.94^{d} ± 0.16
C4	92.88^{f}±0.19	3.40^{c}±0.65	1.40^{cd}±0.37	1.91^{d}±0.10	0.41^{ab}±0.05	7.82^{c}±0.05	33.84^{e} ± 0.32
C5	92.65^{c}±0.32	3.11^{de}±0.22	1.68^{b}±0.45	2.18^{ab}±0.17	0.38^{b}±0.03	8.18^{b}±0.09	36.28^{c} ± 0.17
C8	92.45^{de}±0.48	3.81^{ab}±0.50	1.75^{ab}±0.07	1.51^{c}±0.11	0.48^{d}±0.06	8.50^{b}±0.09	37.03^{bc} ± 0.22
C18	91.73^{e}±0.33	3.95^{a}±0.47	1.83^{ab}±0.27	1.97df±0.09	0.52^{a}±0.11	8.57^{b}±0.15	39.59^{a} ± 0.09
C19	92.58^{d}±0.61	3.66^{b}±0.34	1.69^{b}±0.05	1.83^{cd}±0.17	0.24^{bc}±0.09	8.32^{bc}±0.03	37.17^{bc} ± 0.11
C22	92.20^{ef}±0.52	3.19^{d}±0.17	1.92^{a}±0.11	2.22^{a}±0.07	0.47^{ab}±0.01	8.70^{a}±0.10	39.00^{ab} ± 0.27
C25	92.61^{bc}±0.49	3.12^{de}±0.49	1.52^{bc}±0.21	2.24^{a}±0.19	0.51^{a}±0.07	7.48^{d}±0.01	35.12^{d} ± 0.07

- C1= L. plantarum ATCC14917, C4= Lc. thermophilus DSM20259, C5= B.longum B41409, C8= *L. plantarum* ATCC14917+ *Lc thermophilus DSM20259*, C18= *L. plantarum* ATCC14917+ *Lc. thermophilus DSM20259+ Bifidobacterium longum*, C19= *L. plantarum* ATCC14917+ *L. casei* DSM20011+ *Lc. thermophiles DSM20259*, C22 = *L. casei* DSM20011+ *L. acidophilus* ATCC20552+ *Lc. thermophilus* DSM20259, C25= *L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259+ *B. longum* B41409., Control= non fermented soymilk.
- The data is provided with mean ± SD. The mean values within the same columns or rows with different superscript letters (^{a,b}) are significantly different at $P \leq 0.05$.

During food fermentation, the role micro-organism transforms the chemical constituents of raw materials, thereby improving the bioavailability of nutrients, enhancing the sensory consistency of food, imparting bio-preservative effects and improving food safety, destroying toxic components and anti-nutritional elements, generating antioxidants and antimicrobial compounds, stimulating the working of probiotics (Bourdichon et al 2012). Soymilk with positive health benefits has been recognized as a healthy source of protein. The chemical structure and mineral profile of fermented soy milk have changed relative to its unfermented equivalent. (Kumari et al 2018).

So, it could be stated that the highest contents in these products were protein and carbohydrates followed by fat were recorded in products fermented by *L. plantarum* ATCC14917 (C1), *L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259 (C8), and *L. plantarum* ATCC14917+ *Lc. thermophilus* DSM20259 + *B. longum* B41409 (C18).

4 Conclusions

Soymilk was extracted from soybean (*Glycine max*) seeds and fermented with five probiotic bacterial strains (*L. Plantarum* ATCC14917, *L. casei* DSM20011, *L.* acidophilus ATCC20552, *Lc. thermophilus* DSM20259 and *B. longum* B41409) as a single and mixed cultures for the manufacture of soybean yogurt. The soymilk was fermented by the tested strains after 8 h when the pH ranged from 4.42 to 4.89. There was a significant increase in the bacterial cell count, T.A., and total organic acids in fermented soy milk. Finally, in the chemical composition of protein, carbohydrate, fat, ash, and total solids, the soybean yogurt product (fermented soymilk) was improved as compared to the non-fermented. Therefore, in the future, the fermented soy milk (soybean yogurt) product will be used as a functional food for dairy yogurt.

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