MICROBIOLOGICAL CHARACTERIZATION OF *BUDU*, AN INDIGENOUS MALAYSIAN FISH SAUCE

Sim Kheng Yuen[1], Chye Fook Yee[2] & Ann Anton[1]

[1]Biotechnology Research Institute,
Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia
[2]School of Food Science & Nutrition,
Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia

ABSTRACT. *The study aimed to characterize the microbiological changes during fermentation of budu and hydrolytic properties of the isolated strains. The initial microbial load of the fish substrate was 5.13 ± 0.01 log CFU/g before decreased gradually to 3.20 ± 0.02 log CFU/g after 12 months of fermentation. Micrococcus sp was the predominant bacteria to initiate the fermentation before replacing by Staphylococcus sp that survived throughout the traditional process. Halophilic bacteria especially Micrococcus luteus and Staphylococcus arlettae exhibited good proteolytic and lipolytic activities compared to other isolated bacterial strains. However, most of the identified strains showed weak amylolytic and pectinolytic activity. Lactobacillus plantarum LP1, LP2, Staphylococcus arlettae SA1, Saccharomyces cerevisiae SC3 and Candida glabrata CG2 strains showed potential probiotic activities. These demonstrate that budu is a mixed cultures fermentation involving functional strains in hydrolyzing fish protein into solubilized liquid. Further study is suggested to focus on the selection of technologically important strains as starter cultures for reliable and enhanced budu fermentation.*

KEYWORDS. Fermentation, Fish sauce, Microbial isolates, Probiotic

INTRODUCTION

Fish sauce is a liquid product developed during fermentation of heavily salted fish material in a closed tank at tropical temperature. This hydrolyzed fish condiment comes with many different names such as *nampla* (Thailand), *bakasang* (Indonesia), *yu-lu* (China), *patis* (Philippines), *ngapi* (Burma), *shotshuru* (Japan), *Colombo-lumre* (India and Pakistan), *aekjeot* (Korea) and *budu* (Malaysia). The unique flavour of fish sauce attracted many consumers around the world and has great financial impact to the exporting countries. Malaysian fish sauce, *budu* is quite popular in the east coast of peninsular Malaysia (Kelantan, Terengganu and Pahang) and commonly consumed as a condiment or flavoring agent in certain dishes. The budu production is considered as backyard industry, the production starts off by adding salt to the anchovies (*Stolephorus spp*.) with a ratio of fish to salt of 2:1 or 3:1 and is allowed to ferment at ambient temperature (30-40°C) for 6-12 months in a large concrete tank. During fermentation, a water-soluble protein rich *budu* is formed due to the action of digestive and microbial enzymes. The subsequent budu processing steps include filtration, pasteurization and bottling.

The fermentation process normally takes a long period to ensure the solubilization of *budu* produced. The budu fermentation relies on adventitious microorganisms to initiate the process. It is believed that a wide variety of microorganisms may present at the initial stage of *budu's* fermentation before microbial succession occurs which confines specific microorganisms to sustain in the process. Thus, the organisms found in budu fermentation can be associated with protein degradation and flavour-aroma development to the end product. According to Lopetcharat *et al.* (2001), bacteria involved in most fish sauce can be classified into 2 major groups; the first group is bacteria that produce proteolytic enzymes, such as *Bacillus* sp., *Pseudomonas* sp., *Micrococcus* sp., *Staphylococcus* sp., *Halococcus* sp., *Halobacterium salinarum*, *Halobacterium cutirubrum* while the second group consists of bacteria that related to the flavour and aroma development of the fish sauce such as *Bacillus* sp and *Staphylococcus* sp. In addition of that, many species belonging to *Bacillus, Micrococcus, Staphylococcus, Streptococcus, Pediococcus*, and other halophilic bacteria are found in several fish sauces, including *nampla (*Tanasupawat and Komagata*,* 2001*) and bakasang (*Ijong and Ohta, 1996*)*. Until present, little information on the microbial diversity involved in budu fermentation is being reported. Therefore, this study aimed to characterize the microbiological changes during *budu* fermentation and hydrolytic properties of the isolated strains which could be further selected as starter cultures for controllable fermentation process.

MATERIALS AND METHODS

Sample Collection

The fresh anchovy's mixtures (30kg) were obtained from a *budu* processing factory located in Tumpat, Kelantan, Malaysia. The sample was transferred to sterilized porcelain pots to allow fermentation similar to that of the producer. Samples of *budu* were collected aseptically from the pots for the monthly microbiological analysis.

Microbiological Analysis

Fish mixtures (10g) were transferred aseptically to a stomacher bag containing 90 mL of 0.1 % (w/v) peptone solution having 10 % (w/v) NaCl. The mixture was mixed vigorously for 1 min using a stomacher. The total plate count was determined using plate count agar (PCA) which was incubated for 48 hours at 37°C while determination on halophilic count was done by using PCA with 10% NaCl and incubated for 2-14 days at 37°C. Lactic acid bacteria (LAB) were isolated on MRS agar supplemented with 0.01% (w/v) cyclohexamide and incubated for 48-72 hours at 30°C. Yeast and mould count were performed using yeast-malt extract agar and PDA respectively and incubated for 72 hours at 25°C. Violet Red Bile Agar (VRBA) was used to determine total enterobacteriaceae count which was incubated for 48 hours at 37°C. The determination of proteolytic count was done using nutrient agar (NA) with 10% NaCl, supplemented with 1% (w/v) casein hydrolysate and left incubated 48 hours at 37°C (Tanasupawat *et al*., 1992). Microbiological data were transformed into logarithms of the number of colony forming units (CFU/g). Identification of the isolates until species level was done by using the Biolog Micrology Database Software (Release 4.2, Biolog Inc.) which determines the species of the isolated bacteria (AN, GP2, GN2), yeasts and filamentous fungi (FF) based on their ability to utilize or oxidize the carbon sources. Additional test kits such as API (Bio-Merieux, France) were used to screen some isolates especially yeasts with standard taxonomical method (Kurtzman and Fell, 1998).

Technological Characterizations of Isolates

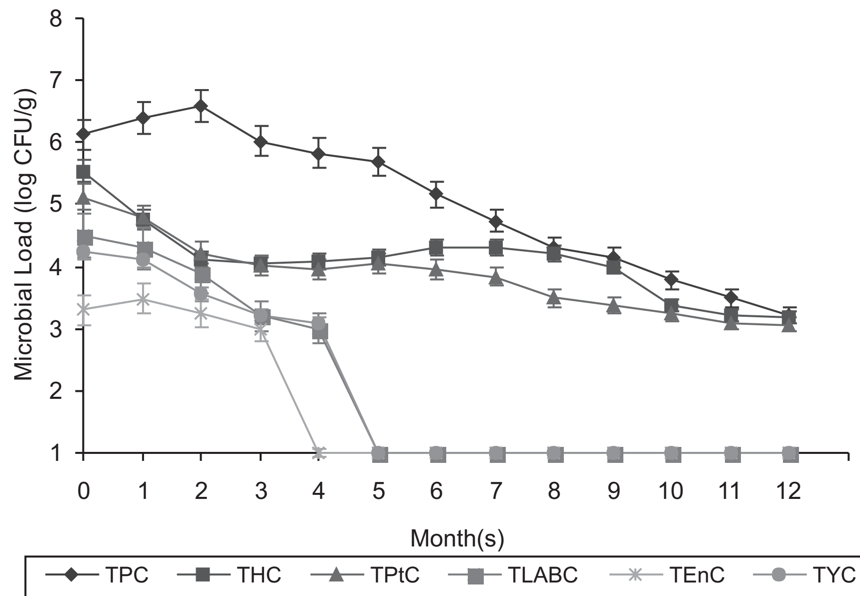
All the isolates were further screened for hydrolytic properties, namely proteolytic, lipolytic, amylolytic (Harrigan and McCance, 1976) and pectinolytic (Speck, 1984) activities. The presence of hydrolytic activities is indicated by the opalescent halo zone surrounding the colony. The estimation on hydrolytic activity of an isolates was determined according to Ionita *et al* (1997). On the other hand, the screening on bile and acid tolerance test was according to Psomas *et al*. (2001), while antimicrobial activity followed the method suggested by Uhlman *et al*. (1992).

RESULTS AND DISCUSSION

An increase of the microbial load for the total plate count (TPC) was observed from the initial 6.13±0.15 to 6.42±0.10 log CFU/g after 3 months of fermentation (Figure 1). It was then decreased significantly ($p<0.05$) to 3.20±0.02 log CFU/g after 12 months of fermentation. The increase in the TPC at the initial stage of fermentation could be due to the bacterial and autolytic spoilage that occurred once the anchovy's mixture was prepared. The result is similar to the finding of Anihouvi *et al*. (2007) who found that the aerobic mesophilic count of the *lanhouin* decreases gradually when the less halophilic bacteria is eliminated throughout the fermentation. In contrary, the halophilic count decreased at the initial process and started to increase until 6th month fermentation (4.32±0.10 log CFU/g) before it reached plateau at the latter stage of fermentation. This was because most of the halophilic bacteria were not adapt well at the initial process but they managed to grow well at the latter stage of fermentation once they were adapted to the extreme condition compared to other bacteria group. In this study, none of the spoilage microorganisms such as moulds were detected throughout the fermentation of *budu*.

This study reports for the first time on microbial population dynamics during the budu fermentation. A total of 134 strains were identified in this spontaneous fermentation with the greatest number of strains corresponded to *Micrococcus* species (32.1% of total strains), followed by *Staphylococcus* (27.6%), *Pediococcus* (10.4%) and *Candida* (8.9%) (Data not shown). Meanwhile, two species of *Micrococcus* were identified, namely *Micrococcus luteus* and *Micrococcus luteus* ATCC 9341, while four species of *Staphylococcus* were identified as *Staphylococcus arlettae*, *Staphylococcus cohnii*, *Staphylococcus carnosus* and *Staphylococcus xylosus*. The microbial composition was evenly distributed at the initial fermentation as *Micrococcus luteus* was the predominant strain at the initial fermentation (Table 1). Their presence at the initial stage was associated with microbial degradation on the fish mixture. This was because they possessed catalase and hydrolytic activities that could degrade the fish protein into smaller components once fermentation started.

The fermentation was further carried out by the *Staphylococcus arlettae*, which became the dominant strains found at the latter stage of budu fermentation. These strains tolerated well to high salt condition (20-25% NaCl). Their ability to grow in low oxygen condition enabled them to endure at the later stage of budu fermentation. The presence of *Staphylococci* species at the latter fermentation stage was vital as they might improve the color and flavor changes of budu via a continuous hydrolysis on budu mixture. This result is in accordance to the findings of Anihouvi *et al*. (2007) who reported that the dominant organisms isolated from *lanhouin* (fermented cassava fish) are from salt tolerant group such as *Bacillus* and *Staphylococcus spp*. Apart from that, Achi *et al*. (2007) even reported that *Staphylococcus sp* are part of the dominant microflora involved in the protein rich fermented crab meal (ogiri-nisiko), this shows that these species of organisms are commonly found in protein rich fermented foods.



TPC- Total plate count, THC- Total halophilic count,TPtC- Total Proteolytic count,
TLABC- Total LAB countTYC-Total Yeast Count, TMC- Total Mould Count

Figure 1. Microbial load during budu fermentation.

Yeast was not a prevalent species in this study as only 4 species of yeasts (*Saccharomyces cereviseae*, *Candida famata*, *Candida glabrata* and *Candida parasilopsis*) were found during fermentation. All the yeasts present at the initial fermentation while only *Candida famata* sustained until the middle stage (6 months) of budu fermentation. The *Candida famata* was able to sustain in such extreme condition due to the toleration of its cell wall to equal amounts of Na^+ and K^+ (Neves *et al*., 1997) and the initiation of stress protection of the metabolic systems against high intracellular sodium concentration (Gonzalez-Hernandez *et al*., 2004). In addition, this yeast species was able to survive because they were sustained by the extracellular molecules such as polysaccharides, fatty acids, vitamins and single cell proteins after the autolysis of other yeasts in the early stage of fermentation. However, this yeast species was absent at the latter stage of fermentation due to limited nutrients as well as unfavorable growing condition especially inadequate oxygen level.

Table 1. Distribution of microbial strains during budu fermentation.

Species	Fermentation Time (Months)													Number of strains
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
Micrococcus luteus	4(16.0%)[a]	3(12.0%)	4(16.0%)	5(20.0%)	3(12.0%)	2(8.0%)	2(8.0%)	1(4.0%)	1(4.0%)	0	0	0	0	25
M.luteus ATCC 9341	3(16.7%)	4(22.2%)	3(16.7%)	3(16.7%)	2(11.1%)	1(5.5%)	1(5.5%)	1(5.5%)	0	0	0	0	0	18
Staphylococcus arlettae	2(14.3%)	1(7.2%)	0	1(7.2%)	1(7.2%)	1(7.2%)	0	1(7.2%)	3(21.4%)	1(7.2%)	1(7.2%)	1(7.2%)	1(7.2%)	14
S.cohnii	4(33.3%)	2(16.7%)	1(8.3%)	1(8.3%)	1(8.3%)	1(8.3%)	1(8.3%)	0	1(8.3%)	0	0	0	0	12
S.carnosus	2(28.3%)	1(14.3%)	1(14.3%)	1(14.3%)	1(14.3%)	1(14.3%)	0	0	0	0	0	0	0	7
S.xylosus	1(25.0%)	1(25.0%)	0	1(25.0%)	1(25.0%)	0	0	0	0	0	0	0	0	4
Lactobacillus plantarum	2(33.3%)	1(16.7%)	1(16.7%)	0	2(33.3%)	0	0	0	0	0	0	0	0	6
L.delbrueckii sp delbrueckii	2(50.0%)	1(25.0%)	1(25.0%)	0	0	0	0	0	0	0	0	0	0	4
Pediococcus pentosaceus	3(42.9%)	2(28.6%)	2(28.6%)	0	0	0	0	0	0	0	0	0	0	7
P.acidilactici	2(28.6%)	3(42.3%)	2(28.6%)	0	0	0	0	0	0	0	0	0	0	7
Lactococcus lactis ssp lactis1	2(50.0%)	1(25.0%)	1(25.0%)	0	0	0	0	0	0	0	0	0	0	4
Corynebacterium	1(25.0%)	1(25.0%)	1(25.0%)	1(25.0%)	0	0	0	0	0	0	0	0	0	4
Rahnella aquatilis	1(50.0%)	1(50.0%)	0	0	0	0	0	0	0	0	0	0	0	2
Enterobacter	1(50.0%)	1(50.0%)	0	0	0	0	0	0	0	0	0	0	0	2
Saccharomyces cerevisiae	1(16.7%)	1(16.7%)	1(16.7%)	1(16.7%)	1(16.7%)	1(16.7%)	0	0	0	0	0	0	0	6
Candida famata	1(16.7%)	1(16.7%)	1(16.7%)	1(16.7%)	0	1(16.7%)	1(16.7%)	0	0	0	0	0	0	6
C.parasilopsis	1(33.3%)	1(33.3%)	1(33.3%)	0	0	0	0	0	0	0	0	0	0	3
C.glabrata	1(33.3%)	1(33.3%)	1(33.3%)	0	0	0	0	0	0	0	0	0	0	3
Total	**34**	**27**	**21**	**15**	**12**	**8**	**5**	**3**	**5**	**1**	**1**	**1**	**1**	**134**

a- Figure without bracket indicated the number of strains; figures in bracket indicated the % of prevalence of the strains on that particular period of time

A total of 114 (85.1%) out of 134 strains (except *Corynebacterium*, *Rahnella aquatilis*, *Enterobacter agglomerans bgp7* (pantoea) and *Candida glabrata*) exhibited potential hydrolytic activity (data not shown). The result revealed that all the *Micrococcus luteus*, *Staphylococcus carnosus* and *Staphylococcus xylosus* strains exhibited good proteolytic activity (R/r > 2.0) compared to other tested strains. Meanwhile, majority of *Micrococcus* and *Staphylococcus* strains also showed good lipolytic activity (R/r > 2.0) in this study. This indicated that the predominant strains played significant role in budu fermentation because they might secrete bacterial protease or peptidase that assist in the hydrolysis and solubilization process of *budu*. Furthermore, the ability of the strains to undergo proteolysis might decrease the peptide size to form more amino acids that improved the chemical attributes of the budu. However, most of the LAB identified in this study exhibited moderate proteolytic and lipolytic activity (1.0<R/r<2.0). Strains of other minority species in *budu* fermentation such as *Saccharomyces cereviseae* and *Candida sp* demonstrated good proteolytic and lipolytic activities as 50.0 and 66.7% of the strains showed good proteolytic activity respectively. Several studies have found out that yeasts with good lipolytic activity play major role in cheese ripening as they are able to degrade the complex components into fatty acids which determine the flavor or texture of the end products (Jakobsen and Narvhus, 1996; Gardini *et al*., 2006). However, information on the hydrolytic property shown by yeasts in fermented meat or fish products is still inadequate (Sorensen, 1997; Dura *et al*., 2004). On the other hand, majority of *Lactobacillus plantarum*, *Lactobacillus delbrueckii ssp delbrueckii*, *Saccharomyces cereviseae*, *Candida parasilopsis* and *Candida famata* had shown moderate and weak amylolytic activity among tested strains. The actual role and mechanisms played by the amylolytic producing strains in the fermented fish products remains unknown. In the contrary, none of the strains showed pectinolytic activity and this phenomenon could be due to the absence of possible pectinolytic enzymes especially depolymerases in the microorganisms tested.

Only *Lactobacillus plantarum* LP1 and LP2, *Staphylococcus arlettae*SA1, *Saccharomyces cereviseae* SC3 and *Candida glabrata* CG2 strains were potential probiotic candidates as they were resistant to the acid and bile salt test as well as the antimicrobial activity compared to other tested strains (Table 2). All tested strains did not grow well at acidified broth with pH 1.5 as this extreme condition might inhibit their normal metabolism. Only 3 out of 5 species (*Lactobacillus plantarum*, *Lactobacillus delbrueckii ssp.delbrueckii* and *Lactococcus lactis ssp lactis 1*) of LAB were able to survive at 0.3% bile acid liquid medium, which is normally used to screen the efficiency of the bacterial against the bile effect. However, it is surprising to note that *Lactobacillus plantarum* LP1 and LP2 strains could resist to bile acid up to 1.0% as most of the probiotic LAB strains can withstand the bile acid at the maximum level of 0.3% which is equivalent to human bile range. This might due to the presence of bile salt hydrolase enzymes (BSHs) that hydrolyzed the bile salt and thus decreased their solubility (Erkkila and Petaja, 2000). The result presented here also suggests that the *Lactobacilli* strains has a better resistant to bile acid compared to other LAB species as the BSH enzyme may weaken detergent effect of bile acids and enable *Lactobacilli* to survive under intestinal bile stress (De Smet *et al*., 1995).

Much attention is given to the potential bacteria to produce bacteriocins or related antimicrobial substances such as organic acid, diacetyl or hydrogen peroxide that can prevent food spoilage as well as to retard the growth of emerging food borne pathogens. In most lactic acid fermentation, LAB will produce lactic acid which decrease the pH and to inhibit some food borne pathogens by producing lactic acid and thus decreasing the pH and ensuring stability of food products.

This usually occurs in fermented dairy, wine and meat products as LAB actively secrete organic acid to preserve the food. In this study, most of the LAB strains tested actively inhibited the grow of selected food borne pathogens, namely *Listeria monocytogenes* L55, *Staphylococcus aureus* S-277, *Salmonella typhimurium* S1000 and *Escherichia coli* O157:H7. It is observed that 31 out of 36 tested strains fully exhibited antibacterial activity against selected food borne pathogens and the inhibition zone was fluctuating between 8 to 12 mm. On the other hand, all *Staphylococci* and yeasts strains (except *Candida fermata*) showed effective antibacterial effect against selected food borne pathogens. There was no concrete study to prove the antibacterial mechanisms against the pathogenic bacteria found in this study, however in another study done by Laukova and Marekova (1993) who found that bacteriocins-like substances produced by *Staphylococci* can penetrate into the cell's wall and exhibit their antibacterial effect. Besides, Papamanoli *et al*. (2000) also reported that bacteria like substances secreted by *Staphylococcus xylosus*, *Staphylococcus carnosus* and *Kocuria varians* strains show antagonism antimicrobial activity against 3 strains of *L. monocytogenes*, which also indicates the potential of *Staphylococci* strains to inhibit food borne pathogens. Yeasts are generally recognized as potential biocontrol agents as most of them are non-pathogenic and do not produce mycotoxins or allergenic spores (Fredlund *et al*., 2002). Gotcheva *et al*. (2002) reported that *Candida strains* can be good antimicrobial agents as it show good inhibitory effect against *S. enteridis*. However, the mode of action of yeasts as biocontrol still remains unknown.

Table 2. Screening on probiotic activity of the identified strains.

Isolates	Increase[a] in counts after incubation in the presence of different pH concentrations				Increase[a] in counts after incubation in the presence of different bile salt concentrations (%)				Antimicrobial activity (inhibition zone, mm)[b]			
	1.5	2.0	3.0	5.0	0.1	0.3	0.5	1.0	LM	SA	ST	EC
Lactobacillus plantarum												
LP1*	0.86	2.98	2.27	1.48	1.12	0.63	0.35	0.21	12	11	8	9
LP2*	1.12	3.13	3.21	1.38	1.61	1.27	0.78	0.37	11	9	10	8
LP3	-1.07	-3.35	-3.37	1.28	0.95	-1.32	-0.95	-0.43	10	9	10	9
LP4	-1.12	-2.56	-2.70	1.12	1.30	-1.17	-0.96	-0.52	11	9	11	9
Saccharomyces cerevisiae												
SC1	-0.45	-0.82	-1.14	1.48	1.48	0.83	-0.72	-0.60	14	12	11	10
SC2	-0.53	1.92	2.02	1.14	1.53	0.89	0.71	0.53	14	11	11	9
SC3*	0.47	2.01	2.13	1.56	1.61	0.76	0.99	0.57	13	10	9	8
Candida glabrata												
CG1	-0.67	-1.21	-1.98	0.82	1.20	0.63	0.48	0.26	12	10	8	9
CG2*	0.35	1.18	2.03	1.05	1.18	0.75	0.50	0.27	11	10	9	7
Staphylococcus arlettae												
SA1*	-2.42	0.32	0.85	1.17	2.15	1.93	1.21	1.10	13	10	10	8
S.xylosus												
SX1	-3.6	-3.4	-3.1	-1.9	1.59	1.43	1.30	1.13	15	14	12	11
SX2	-2.9	-3.0	-3.1	-2.0	1.42	1.40	1.28	1.20	16	13	11	10
S.cohnii												
SCH1	-3.7	-3.4	-3.9	-1.52	1.12	1.07	0.98	-1.30	12	11	9	8
SCH2	-3.8	-3.1	-2.8	-1.47	1.23	1.05	-1.68	-1.13	11	9	8	7
SCH3	-3.6	-3.2	-2.8	-1.50	1.26	1.07	1.12	-1.12	10	9	8	7
S.carnosus												
SCN1	-3.7	-3.60	-3.10	-1.53	0.98	0.74	-0.83	-1.32	17	16	10	8
SCN2	-3.6	-3.57	-2.74	-1.32	1.61	1.47	0.78	0.54	14	11	9	7
SCN3	-3.5	-3.60	-2.68	-1.36	1.72	1.42	0.68	0.45	13	12	10	9
L.delbrueckii ssp. Delbrueckii												
LD1	-1.83	-0.93	1.32	0.94	1.30	0.15	-1.10	-1.58	11	12	9	x
LD2	-1.78	-0.95	1.31	0.88	1.36	0.20	-1.18	-1.60	11	10	9	7
LD3	-1.80	-0.97	1.36	0.90	1.42	0.25	-1.09	-1.56	10	9	8	X

Lactococcus lactis ssp lactis 1												
LL1	-1.46	-0.58	1.13	-0.32	1.12	0.23	-0.62	-1.27	11	10	X	x
LL2	-1.50	-0.62	1.25	-0.42	1.25	0.45	-0.57	-1.34	10	9	X	x
LL3	-1.48	-0.53	1.32	-0.12	1.36	0.38	-0.63	-1.32	10	9	x	x
Pediococcus pentosaceus												
PP1	-1.76	-1.42	0.78	0.25	0.87	-0.93	-1.15	-1.45	12	10	X	9
PP2	-1.78	-1.38	0.80	0.30	0.87	-0.89	-1.20	-1.27	11	10	X	8
PP3	-1.69	-1.36	0.75	0.28	0.98	-0.82	-1.31	-1.31	9	8	X	8
PP4	-1.76	-1.28	0.68	0.27	0.82	-0.78	-1.18	-1.08	9	7	x	8
P.acidilactici												
PA1	-1.09	-0.93	0.27	-0.18	0.18	-1.46	-1.69	-2.01	X	x	X	x
PA2	-1.10	-0.96	0.32	-0.19	0.20	-1.27	-1.52	-2.10	X	x	X	x
PA3	-1.03	-0.89	0.28	-0.16	0.28	-1.30	-1.48	-2.05	x	x	x	x
C.parasilopsis												
CP1	-1.45	-1.01	-0.46	0.15	0.98	0.67	-0.38	-0.86	10	11	7	6
CP2	-1.37	-1.15	-0.98	0.35	0.87	0.87	-0.26	-0.65	11	9	7	6
C.famata												
CF1	-1.56	-0.97	-0.72	0.32	0.72	0.54	0.23	-0.45	X	X	X	X
CF2	-1.38	-0.83	-0.72	0.46	0.82	0.66	0.38	-0.48	x	x	x	x

a $(\text{lo g cfu/ml})_{final}$ $(\text{lo g cfu/ml})_{initial}$

LM - *Listeria monocytogenes* L55, SA - *Staphylococcus aureus* S277, ST- *Salmonella typhimurium* S1000, EC - *Escherichia coli* O157:H7

CONCLUSION

Budu fermentation was driven by dominant microorganisms as the conversion of fish mixture into liquids was observed due to the aid of microbial degradation. Therefore, further studies are necessary to evaluate the feasibility of the technologically important strains to perform as starter cultures for controllable *budu* fermentation.

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