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Survival of commercial probiotic strains to pH and bile

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Abstract: This study was performed to enumerate the total viable cell count of probiotic in five brands (A to E) of commercially cultured milk drinks that are available in the Malaysian market as well as to test their tolerance to various pH and bile concentrations by simulating the human gastrointestinal pH and bile concentration. The acid tolerance test was studied under pH 1.5 and 3.0 with 7.2 as control. The cell count for the acid tolerance test was obtained at an interval of 0, 1.5 and 3 hours respectively and was plated onto duplicate MRS agars to be incubated at 37°C for 48 hours. All cells recovered after 3 hours of pH treatment were selected for bile tolerance test in MRS broth containing bile concentrations of 0% (control), 0.3% and 2.0% and cell counts were recorded after 24 hours of incubation. The probiotic strains in products A, B, C & D met the suggested initial count of 10^6 CFU/ml with brand C recording the highest at 9.19 ± 0.14 log CFU/ml. Strains in product A, B & C showed good tolerance to pH 3.0 and 7.2 recording a count of $>10^6$ CFU/ml after 3 hours with a range of 6.60 – 9.04 log CFU/ml. The higher bile concentrations resulted in lower growth of strains in all the brands. Upon pH 1.5 treatment, only brand C recorded growth in all bile concentrations. After pH 3.0 treatment, all brands except brand E met the requirement of survival at 0.3% bile concentration. Results showed probiotics in product A, B & C met the initial count requirement, and exhibited good acid and bile tolerance therefore being a potentially good source of probiotic.

Keywords: Acid and bile tolerance, cultured milk drink, *Lactobacillus*, probiotic, total viable cell count

Introduction

Recent increase of awareness towards human nutrition over the past few decades, especially in the developed countries has seen a shift from the concept of adequate to optimal nutrition. This is evident in the rapid growth of interest in probiotics to promote better health and well-being which shows a substantial promise to expand the food industry into new fields. Strains from genera of *Lactobacillus* and *Bifidobacterium* species, both of which are indigenous to the human intestine, are predominantly selected for use although some other species have also been used (Holzapfel and Schillinger, 2002). Probiotics, also termed as functional foods, are commonly found in dairy products such as yogurt and cultured milk drinks or even in the form of health supplements.

The notion of probiotics evolved from a theory first proposed by Nobel laureate, Elie Metchnikoff, who associated longevity with the consumption of fermented milk products. He postulated that the bacillus present could positively modify the bacterial community structure of the colon, thus contributing to human health status (Vasiljevic and Shah, 2008). Probiotics were later termed as live micro-organisms which, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2006). The amount required to gain any therapeutic benefits is a minimum of 106 viable probiotic cells per millilitre during storage until the expiry date (Samona and Robinson, 1994; Lourens-Hattingh and Viljoen, 2002; FAO/WHO, 2006).

In the past two decades, there has been renewed interest in the study of the nutritional and therapeutic aspects of the mentioned products (Parvez *et al*., 2006). It is widely accepted that probiotics may exert positive influence on the host through modulation of the endogenous ecosystem and stimulation of immune system as well as maintaining a healthy intestinal microflora (Silva *et al*., 1987; Goldin *et al.*, 1992; Lee and Salminen, 1995; Lu and Walker, 2001; Marteau *et al*., 2001; MacFarlane and Cummings, 2002). However, research suggests that health benefits are strain specific and vary by amount ingested and duration administered.

Several studies have revealed that some probiotic products in the market have deficiencies in the viability of probiotic strain(s), especially in products containing bifidobacteria (Fasoli *et al*., 2003; Masco *et al.*, 2005). This may be due to storage conditions, manufacturing or food technologies setbacks such as inappropriate packaging materials that could affect probiotic stability through variations in oxygen permeability (Miller *et al*., 2002).

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The viability of these cells after consumption remains obscure as the bacteria are also subjected to unfavourable physiological conditions of the Gastrointestinal (GI) tract such as acidic environment and bile secretions (Holzapfel *et al*., 1998). These include variation in the level of acidic conditions and bile secretion at different incubation time simulating the physiological aspects of human digestive system. Viability of these bacteria upon ingestion and sufficient survival through the transit to GI tract is crucial to confer any health benefits to the host (Salminen *et al*., 1998; Hou *et al*., 2003; Krasaekoopt *et al*., 2003; Kailasapathy, 2006). Consequently, the survival of commercial probiotic strains when subjected to various pH and bile conditions similar to that of human GI conditions should be assessed and substantiated accordingly. Therefore, it is reasonable to enumerate the viable cell counts in commercial cultured milk drinks upon reaching consumers in this study. Hence five brands of commercial cultured milk drinks were selected off the shelves in a local hypermarket in Malaysia.

Most studies evaluating the resistance of potential probiotic strains to gastric and bile secretion have been conducted *in vitro* (Del Piano *et al*., 2006) although some discrepancy between *in vitro* and *in vivo* observations does exist due to the complex nature of the human system (Morelli 2000). However, in two seperate studies conducted by Marteau *et al*. (1997) and Lin *et al*. (2006); no significant differences were observed between *in vitro* and *in vivo* data, indicating the predictive value of the model for the survival of the bacteria. Thus, in this study, an *in vitro* methodology was employed to assess the transit tolerance of the probiotic samples. The effects of bile on probiotic strains are more difficult to assess by *in vitro* due to variation of bile concentration in the system at any given moment (Marteau *et al*., 1997). Hence, various concentration of bile are considered and used in this study to mimic the physiological conditions closely.

Materials and Methods

Five commercial cultured milk drinks labeled as A, B, C, D, and E were obtained from a local hypermarket at Taman Connaught for this study (Table 1). Each batch of the sample consists of five bottled cultured milk drinks of the same flavour. Therefore, the sample selected for each repeated test was of the same flavour to ensure consistency. These cultured milk drinks with respect to each brand are expected to be the same in terms of its composition, quality and quantity of probiotics. Thus, the sample was randomly selected and stored immediately in an ice box to preserve the optimal storage environment before transporting it to the laboratory. They were then stored at 4°C and were utilized two weeks before its expiry date.

Table 1. List of species and presence of prebiotics contained in each cultured milk brand

Brand	Species	Prebiotics*	Amount of prebiotics
A	*Lactobacillus acidophilus*	Inulin and polydextrose	Not Stated
B	*Lactobacillus acidophilus*	None	-
C	*Lactobacillus casei* Shirota strains	None	-
D	*Lactobacillus acidophilus, Streptococcus thermophilus, Bifidobacterium;*	None	-
E	*Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium.*	None	-

**: Prebiotics are stated in the ingredients of the cultured milk.

De man, Rogosa, Sharpe (MRS) (DIFCO™, USA) agar was selected as the growth medium for the probiotics in the samples. On the other hand, MRS broth (OXOID, UK) was used for determination of bile tolerance. The MRS broth used has the exact formulations as MRS agar, without the agar as a solidifying agent.

The samples (cultured milk drinks) were shaken to ensure the homogeneity of the contents. Serial dilutions were performed to enumerate the total number of viable cells upon consumption. Plating with duplicates was done in the laminar flow and the plates were incubated aerobically for brand A, B, and C and anaerobically for brand D and E at 37°C for 48 hours. Anaerobic condition was achieved by enclosing the plated cultures with an activated Anaerocult A gas pack (Merck, Germany) in a sealed anaerobic jar (Merck, Germany).

Acid tolerance test

The method used to evaluate the viability of the cells under acidic stress in this study was adapted from Conway *et al*. (1987), Brashears *et al*. (2003) and Tsai *et al*. (2007). Various simulated GI conditions were achieved by subjecting the samples to different pH levels at a designated incubation time. Jacobsen *et al*. (1999) and Huang and Adams (2004) had described the use of sterile PBS adjusted to different pH to study the acid tolerance of the microorganisms. Hence, the pH of PBS was adjusted to pH 1.5, 3.0 and 7.2 (control) respectively using 1M HCl in this study. In addition to that, the test was also subjected to three incubation periods of 0, 1.5 and 3.0 hour.

Initially at 0 hour, 1.0 ml of the sample was inoculated into the universal bottle containing 9 ml of PBS, pH 1.5 and was mixed thoroughly. It was then serial diluted with sterilized PBS pH 7.2 in microcentrifuge tubes. The appropriate dilution factor was determined and plating of serial dilutions

of the culture was performed on MRS agar plates. Each assay was performed in duplicates. These plates were then incubated aerobically/ anaerobically at 37°C for 48 hours. The same procedure was repeated for pH 3.0 and pH 7.2 under the same experimental conditions for the 0 hour. The aforementioned process was performed for 3 incubation periods of 0, 1.5 and 3.0 hour. Acid tolerance was estimated by comparing the growth of viable cell counts in all the MRS agar plates after 48 hours.

Bile tolerance test

The effects of bile on the growth of probiotic strains were examined using methods modified from those of Gilliland and Walker (1990) and Tsai *et al*. (2007). A series of bile concentrations were employed in this study considering the fluctuation of bile concentration at different times. Broth with 0% bile concentration serves as a control of the study.

Bile tolerance test was commenced at the end of the third hour of acid pretreatment where 5 ml of sample was each pipetted out from the universal bottles incubated earlier (pH 1.5, pH 3.0 and pH 7.2) into three pH-labeled centrifuge tubes. Centrifugation was carried out at 4000 rpm for 10 minutes at 25°C. The supernatants were discarded and the pellets were washed with PBS of pH 7.2. Centrifugation was repeated and the supernatants were once again discarded. The three remaining concentrates were then re-suspended with MRS broth.

The next procedure involves the inoculation of 1 ml of pH 1.5 suspensions into 9 ml of three MRS broths with different bile concentrations (0%, 0.3% and 2.0%). The step was repeated for pH 3.0 and pH 7.2 mixtures as well. The MRS broth containing cells were incubated aerobically/ anaerobically at 37°C for 24 hours. Subsequently, 0.1 ml was pipetted out from each of the MRS broth and serial dilutions were performed for plating (duplicates). All the plates were incubated aerobically/ anaerobically at 37°C for 48 hours. Bile tolerance was determined by comparing the viable cell counts on MRS agars with and without bile salt. All samples were analysed in duplicates, all experiments were repeated thrice. Data obtained from the study was expressed in terms of $\log_{10}$ CFU/ml and analysed as mean ± standard deviation (SD).

Results and Discussions

Initial counts of probiotics

All brands have met the minimum requirement set by FAO/WHO (1 x 10^6 CFU/ml) except for brand E. The highest number of live probiotics was recorded by Brand C with a count of 1.55 x 10^9 CFU/ mL and the lowest was recorded by Brand E with a count of 2.40 x 10^5 CFU/ mL (Table 2). Judging by this initial count, Brand A, B, C and D are considered good probiotic sources except for Brand E. Amongst the reasons for the low count in Brand E could be affected by the temperature during the fermentation process as well as during the inoculation period and most importantly during transportation (Shah and Dave, 1998). Oxygen that is dissolved in the product during manufacturing could stress the probiotics as too much oxygen will delay their growth (Klaver *et al*., 1993; Kailasapathy and Supraidi, 1996; Lankaputra *et al*., 1996; Shah and Dave, 1998; Godward *et al*., 2000; Vinderola and Reinheimer, 2000; Vinderola *et al*., 2003) especially since Brand E is strictly anaerobic. The inulin and polydextrose contained in Brand A may have caused it to increase the initial count of the probiotics as they acted as prebiotics which aid the growth of probiotics especially of *Lactobacilli* genera (Huebner *et al*., 2006).

Table 2. Total Plate Count (TPC) for five commercially cultured milk products on MRS agars under aerobic/anaerobic conditions at 37°C for 48 hours

	Product	TPC ($\log_{10}$CFU/ mL)[a]
Aerobic	A	7.92 ± 0.02
	B	7.04 ± 0.09
	C	9.19 ± 0.14
Anaerobic	D	6.84 ± 0.34
	E	5.38 ± 0.28

[a]: Results were expressed as mean ± standard deviation (SD) with each data point an average of two repeated measurements from a total of three independently replicated experiments; n=3.

Acid tolerance

Another important criterion to be a good source of probiotics is the tolerance of high acid levels, which is present in our stomachs. The lowest pH recorded has been pH 1.5 (Huang and Adams, 2004; Lin *et al*., 2006). Good probiotic sources should withstand at least pH 3.0 (Fernandez *et al*., 2003). High concentrations of acid reach pH 1.5 during fasting. Generally there is a reduction in probiotic count, as they were exposed to pH 1.5 and pH 3.0 and the count is fairly constant at pH 7.2 (control) (Table 3).

All brands recorded a count except for Brand B which had no growth during the initial phase of inoculation (zeroth hour). After 1.5 hours of incubation, all the brands showed no growth except for Brand D albeit a low count of 2.52 ± 1.97 $\log_{10}$ CFU/ mL. After 3 hours of incubation, all brands showed no growth of probiotics suggesting most probiotics were killed by this harsh pH (Table 3).

According to Zavaglia *et al*. (2002), acid such as the hydrochloric acid (HCl) that is found in the human stomach is a strong oxidizer. Thus, it can oxidize many important biomolecular compounds in the cells and

Table 3. Total plate counts for five commercially cultured milk products on MRS agars at different pH values of 1.5, 3.0 and 7.2(control) over 1.5 hour intervals

pH value	Brand	Total plate counts (log_{10} CFU/ mL) #		
		0 hour	1.5 hour	3.0 hour
1.5	A	6.09 ± 0.13 ^{3}	-	-
	B	-	-	-
	C	8.53 ± 0.64 ^{4}	-	-
	D	5.93 ± 0.15 ^{a,2}	2.52 ± 1.97 ^{b}	-
	E	2.32 ± 2.03 ^{1}	-	-
3.0	A	7.17 ± 0.05 ^{a,3}	7.08 ± 0.03 ^{b,3}	6.94 ± 0.03 ^{c,4}
	B	6.82 ± 0.04 ^{a,2}	6.73 ± 0.05 ^{ab,2}	6.60 ± 0.06 ^{b,3}
	C	9.06 ± 0.06 ^{a,4}	9.06 ± 0.03 ^{a,4}	9.04 ± 0.06 ^{a,5}
	D	6.83 ± 0.44 ^{a,2}	6.17 ± 0.23 ^{ab,2}	5.92 ± 0.25 ^{b,2}
	E	5.25 ± 0.02 ^{a,1}	4.10 ± 0.70 ^{b,1}	3.59 ± 1.12 ^{b,1}
7.2	A	7.57 ± 0.05 ^{a,4}	7.51 ± 0.04 ^{ab,4}	7.46 ± 0.04 ^{b,3}
	B	7.02 ± 0.08 ^{a,2}	6.89 ± 0.05 ^{ab,3}	6.68 ± 0.08 ^{b,2}
	C	9.12 ± 0.08 ^{a,5}	9.05 ± 0.07 ^{a,5}	9.04 ± 0.07 ^{a,4}
	D	7.24 ± 0.14 ^{a,3}	6.73 ± 0.09 ^{b,2}	6.58 ± 0.22 ^{b,2}
	E	5.16 ± 0.11 ^{a,1}	5.48 ± 0.29 ^{b,1}	5.43 ± 0.20 ^{ab,1}

: Each value in the table represents the mean value ± Standard Deviation (SD). Each data point is the average of two repeated measurements from 3 independently replicated experiments, n = 3

^{abc}: Mean value with different superscripts in the same row differs significantly (P< 0.05).

- : The hyphen symbol represents plate counts of < 1 log10CFU/ mL on the MRS agar plates.

* : The period of 0 hour is the time when the samples from milk cultures were plated immediately for assay upon being exposed to PBS with different pH values.

^{123}: Growth increases numerically. Mean value with different superscripts for each pH value in the same column differs significantly (P<0.05).

disrupt them while it will undergo reduction. Amongst the important biological compounds that acid can destroy includes fatty acids, proteins, cholesterol and the DNA (Pan *et al*., 2008). As demonstrated by Sultana *et al.* (2000) and Chan and Zhang (2005), aciduric members such as *L. acidophilus*, generally could not survive in low pH environment as these cells were proven to be vulnerable at pH 2.0 and below. Low pH environments are thought to inhibit the metabolism activity and growth of *L. acidophilus*, thus reducing the probiotics' viability. Another study conducted by Mandal *et al*. (2006) also confirmed that the viability count of the bacteria declined tremendously when exposed to simulated gastric juice of pH 1.5 after an incubation period of 3 hours. The threshold point to determine acid resistance was set at pH value of 3.0 and incubation period of 3 hours in this *in vitro* study as it simulates the residence time in the stomach (Prasad *et al*., 1998; Haddadin *et al*., 2004). This is in accordance with findings from Liong and Shah (2005) which stated that resistance at pH 3 is set as standards for acid tolerance of probiotic culture. Therefore, result in Table 3 indicates the strong inhibition on the viable bacteria numbers at pH 1.5 was well supported.

As for pH 3 (Table 3) in Brand A and B, there were strains of *L. acidophilus* that survived perhaps because the pH was not too high so as to cause complete destruction of all the cells. At any given time, there were cells that were still dying at pH 3 and there were those that survived, but after 3 hours the rate of cells dying outnumbers the rate of survival cells.

For Brand C (Table 3), the number of cell counts remained significantly unchanged during all the interval transit at pH 3 and pH 7.2. Good acid tolerance properties exhibited by the bacteria are closely related to their strains specification as they are always strains dependent (Huang and Adams, 2004; Lin *et al*., 2006).

Based on the results obtained (Table 3), the survival of probiotic differed considerably when compared at 0 and 3.0 hour for Brand D, with the former achieving higher viability than the latter. With the number of counts of log_{10} 5.92 CFU/ ml, the sample has proven to be unsuccessful to meet the minimum requirement of 10^6 viable probiotic cells per ml at pH 3 after exposure for 3 hours. Having multiple strains in this brand, has indeed affected the overall viability count. It is well established that *L. acidophilus*, is more resistant compared to *Bifidobacterium* spp. in terms of high acidity (Boylston *et al*., 2004). Comparatively, the intrinsic resistance of acid of *S. thermophilus* has been reported to be fairly poor (Conway *et al*., 1987; Vinderola and Reinheimer, 2003). However, the addition of *S. thermophilus* may also increase the survival of certain strains of *Bifidobacterium* through the reduction of oxygen pressure (Ishibashi and Shimamura, 1993; Nogueira *et al*., 1998). Nevertheless, the results obtained in this *in vitro* study may not truly reflect their performance *in situ* as many other physiological conditions that might affect the survival of the strains (Morelli, 2000).

Bile tolerance

The survival rate of bacteria supplemented with bile was similar to the trend portrayed in acid tolerance test, with higher inhibition of growth seen as the bile concentrations increased for all the brands. 0% bile acted as control for all experiments and it recorded the highest growth. Upon exposure to bile acids, cellular homeostasis disruptions causes the dissociation of lipid bilayer and integral protein of their cell membranes, resulting in leakage of bacterial content and ultimately cell death.

Data obtained from the acid tolerance study indicates that bacteria from all samples that cannot survive at high pH 1.5, also failed to grow in this subsequent bile test except Brand C (Figure 1). As for pH 3 and 7.2 (Figures 2 and 3), there is a gradual decline in viable count as the bile concentrations increased.

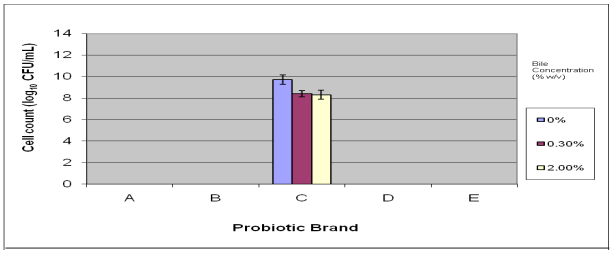

Figure 1 Cell growth in different probiotic brands at different bile concentration upon pH 1.5 treatment during 24 hours of incubation at 37°C

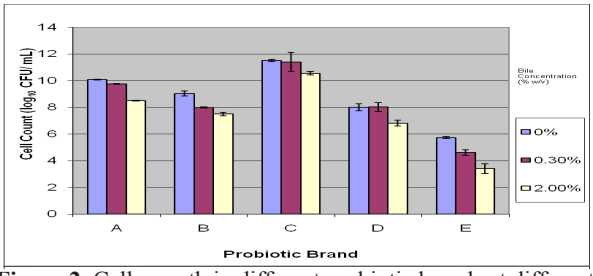

Figure 2. Cell growth in different probiotic brands at different bile concentration upon pH 3 treatment during 24 hours of incubation at 37°C

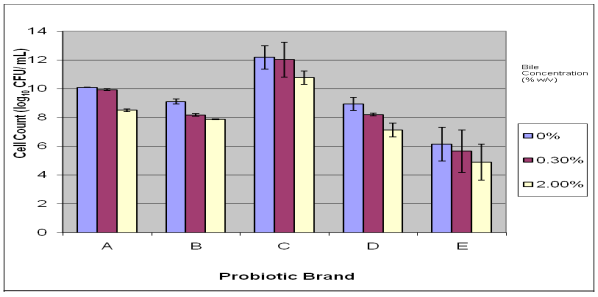

Figure 3. Cell growth in different probiotic brands at different bile concentration upon pH 7.2 treatment during 24 hours of incubation at 37°C

The findings in this study are in accordance to Chou and Weimer (1999) which also combined two selection criteria, acid and bile in their assessment. A common observation among these studies, despite major design differences, is that acid and bile have separate and combined effects on the growth of bacteria. As bile stress takes place after pH stress in the stomach, Leyer and Johnson (1993) and Lin *et al*. (2006) postulated that sub-lethally injured microorganisms may have a different and unpredictable resistance to new stress factors.

Overall in the results, bile did not inhibit the growth of the bacteria completely as even when subjected to 2% of bile, there is still a high number of bacterial count (8.51 ± 0.03 log units)(Brand A, Figure 2). The high growth of the bacteria at 2% bile could be that stress adaptation mechanism may serve as a logical rationalization to explain the increased growth with longer incubation hours after pre-exposure to acid stress. The enhanced survival capabilities appeared to be due to the acclimatization of the bacteria to the low pH environment, therefore minimising the relative toxicity to glycoconjugates in the intestine (Begley *et al*., 2005; Martoni *et al*., 2007). The protective effect of food matrix also may prevent the bacteria from bile exposure and hence, giving rise to the increased bile resistance of the strains (Begley *et al*. 2005).

Besides that, some of the cells that were not entirely killed off and upon being exposed to the MRS broth, they proliferate. Polysorbate 80, also commercially known as Tween 80, is a non ionic surfactant and emulsifier commonly found in MRS agar medium. As bile is a detergent, the addition of Tween 80 is postulated to enhance the stability of the cell membrane and thus contributing to the bile tolerance observed in some strains (Kimoto *et al*., 2002).

Apart from that, the revival of these bacteria after 24 hours of incubation with bile may also be attributed to the pH of the bile itself. The pH of the bile was expected to be in the range of 7.0 and higher. After acid pre-treatment, the revival of the bacteria might be associated with the higher pH encountered in bile tolerance test. This is postulated to be a positive factor that contributes to the increased revival of these probiotics after prolonged incubation with bile solution. However, the actual pH of the oxgall used in this study was not determined as it was not a part of the research design. Thus, it is suggested that future study may need to investigate the relationship of the bile's pH and the revival of probiotics when assessing the sensitivity of probiotic strains.

The probiotic strains proved to exhibit an excellent quality of bile tolerance. Another important factor is the bile salt hydrolase (BSH) activity which accounts for the bile salt resistance. It is observed in some strains where BSH hydrolyse conjugated bile, thus reducing its bactericidal effect (Du Toit *et al*., 1998). Later a study by Suskovic *et al*. (2001) proved that BSH was active when tested on *lactobacilli* and showed a relatively strong resistance to bile such as in Brand A, B and C. This may explain the sensitivity of some strains which lacked this BSH activity. However, sensitivity to bile may also have positive consequences as lysis of cells during passage through GI tract may result in the release of β-galactosidase (Marteau *et al*., 1997). Yazid *et al.* (1999) reported that bile acid had a greater inhibitory effect on the *lactobacilli* than *bifidobacteria* strains.

Conclusion

All brands except brand E has met the minimum initial count requirement as set by WHO/FAO 2006. The acid tolerance for all brands is the same where increasing level of acidity has a negative impact on the viability of the probiotics. This trend is noticeable for all intervals tested (0, 1.5 and 3.0 hour) as findings observed a decline in viable counts when the sample was subjected to increasing acidity levels. Only brand A, B and C can survive pH 3.0 and have met the criteria to be considered as good sources of probiotics. The bile tolerance for all the brands show the same trend where increasing levels of bile shows more inhibitory effect. Brand A, B, C and D shows good bile tolerance with growth more

than 6 log units at 0.3% of bile. Brand C shows the best tolerance towards bile, surviving with more than 6 log units even after pH 1.5 and pH 3.0 treatments with the highest count amongst all the samples.

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