

Characterising the synbiotic beverages based on barley and malt flours fermented by *Lactobacillus delbrueckii* and *paracasei* strains

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RESEARCH ARTICLE

Abstract

This research was conducted to study physicochemical characteristics of fermented cereal-based beverages. The changes in levels of microbial population, pH, total titrable acidity (TTA), sugars (fructose, maltose and glucose), and organic acids (lactic and citric acids) of these beverages were determined during 48 h fermentation of malt, barley and barley-malt media by two potentially probiotic strains of *Lactobacillus paracasei* and *Lactobacillus delbrueckii*. Results showed that the selected probiotics well grew on single and mixed substrates without any supplementation. The highest microbial growth (9.7 log cfu/ml) was found in malt medium after 15 h fermentation. A significant decrease in pH value to 4.25 and a considerable increase in TTA level to 2.96 g/100 g lactic acid were obtained by initial 6-h fermentation of *L. paracasei* on malt medium. High-performance liquid chromatography analysis showed that lactic acid and glucose, respectively, were the major produced acid and metabolised sugar in the developed beverages. Higher sugar consumption by *L. paracasei* in comparison to *L. delbrueckii* was also observed. *L. paracasei* synthesised 5.12, 11.22 and 7.64 g lactic acid/l in the media containing barley, malt and barley-malt, respectively. Moreover, malt-based beverages fermented with *L. delbrueckii* were the best sample due to the highest cell viability (1.2×10^6 cfu/ml) after 4 weeks under cold-storage.

Keywords: probiotic beverage, lactic acid bacteria, fermentation process, functional foods, malt

1. Introduction

In recent years, increasing consumer demand to healthy diets and changing dietary habits has encouraged the food industry toward the production of functional foods (Charalampopoulos *et al.*, 2002b; Gharibzahedi *et al.*, 2013). Although, most functional foods are based on dairy products, but these functional foods have disadvantages such as lactose intolerance, allergy and high cholesterol content (Prado *et al.*, 2008; Rathore *et al.*, 2012; Yoon *et al.*, 2006).

Cereal grains can be used as suitable fermentable substrates/ carriers for probiotic microorganisms (lactic acid bacteria (LAB) or bifidobacteria) to make new functional products in the food industries. Because of the presence of significant amounts of biologically active ingredients like non-digestible carbohydrates, dietary fibre oligosaccharides

and resistant starch, these grains may be served as prebiotics (Charalampopoulos *et al.*, 2002b; Gupta *et al.*, 2010; Kedia *et al.*, 2007; Krahel *et al.*, 2009). Thus, they have a positive effect on the growth and colonisation of intestinal microflora (Schrezenmeir and De Vrese, 2001). Barley among the different types of cereals is an ancient grain which is widely used as animal feed and the substrate for malt production (Rozada-Sánchez *et al.*, 2008). Recently, barley is known as a potentially substrate for production of functional beverages due to high contents of its phenolic compounds, β -glucans and tocopherols (Liu and Yao, 2007; Qingming *et al.*, 2010; Zhao *et al.*, 2006).

Barley along with oat has the highest amount of β -glucan among other grains (Angelov *et al.*, 2006). It has been demonstrated that barley consumption led to reduce total and serum-cholesterol, intestinal protection, and cardiovascular benefits (Pins and Kaur, 2006), type-2

diabetes (Wood, 2007; Yokoyama, 2006), and colon and breast cancer (Dykes and Rooney, 2007). Gamel and Abdel-Aal (2012) also reported that barley in comparison to oat was much more effective in reducing both glucose and insulin responses. Although cereals provide the considerable amounts of macronutrients (carbohydrates, proteins and fibres) and micronutrients (vitamins and minerals) required for body, they have shortage in the nutritional value especially essential amino acids and sensory characteristics (Coda *et al.*, 2011). Fermentation is the most antiquity technology used by human which could be a suitable election for cereal processing. This process not only is an economical and convenience method, but also can enhance bioavailability of many nutrients such as B-group vitamins, shelf-life, digestibility and sensory properties (Keşkekoğlu and Üren, 2013; Passos *et al.*, 1993). Fermentation also provides optimum pH for enzymatic degradation of phytate which may increase the amount of soluble iron, zinc and calcium (Blandino *et al.*, 2003; Haard *et al.*, 1999). Cereals as prebiotics can perfectly replace to extension of non-dairy probiotic beverages. Use of probiotic microorganisms in certain number ($6\text{--}7 \log \text{cfu/ml}$) could beneficially affect the host via connect to intestinal epithelial cells, colony formation, improvement immune system by the elimination of pathogenic microorganisms (Prado *et al.*, 2008), reduction of the risk of colon cancer and blood cholesterol, and anti-diarrheal properties (Saarela *et al.*, 2002; Wood, 2007).

Based on these findings, barley and malt could be suitable substrates for the production of functional beverages and could fulfil the consumer demand for non-dairy beverages (Coda *et al.*, 2011). Therefore, the aims of this study were development and characterisation of cereal-based drinks based on barley and malt medium fermented with *Lactobacillus delbrueckii* and *Lactobacillus paracasei* and investigation of the growth rate and substrate metabolism during their fermentation period.

2. Materials and methods

Bacterial strains and culture media

Probiotic LAB used in study (*L. delbrueckii* DSMZ 20006 and *L. paracasei* DSMZ 15996) were obtained by the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). All bacteria cultures as an inoculum were stored frozen at -20°C in de Man, Rogosa and Sharpe (MRS) broth medium (Merck Chemical Co., Darmstadt, Germany) containing 20% glycerol. The starter culture was reactivated on MRS broth at 37°C for 24 h. Barley and malt flours were also supplied from Beh Malt Co. (Karaj, Iran).

Preparation of fermentation media

The fermentation medium used in this study was prepared with flour of barley, malt and barley-malt (equal proportions) and dissolved in distilled water in a constant ratio of 15% (w/w). This ratio was selected according to preliminary studies. The suspensions were transferred to the volumetric flasks and thermostatically remained in a controlled water-bath at 95°C for 15 min for the partial gelatinisation of starch. The samples prior to inoculation were cooled to 37°C .

Fermentation process

LAB were cultured in the MRS broth, harvested by centrifugation at $4,000\times g$ for 10 min (4°C), washed and re-suspended in sterile distilled-water. The slurry was inoculated with 10% (v/v) starter culture ($>10^7 \text{cfu/ml}$) and incubated at 37°C for 48 h. The sampling was performed at specified intervals during the fermentation time for chemical and microbiological analysis. For chemical analysis, the samples were initially centrifuged at 4,000 rpm for 10 min and then the supernatant maintained at -20°C when was needed.

Cell growth enumeration

Determination of viable cells (cfu/ml) was performed by the method of standard plate count using MRS nutrient agar (pH=5.7) after the incubation at 37°C for 48 h (Charalampopoulos *et al.*, 2002a).

pH and total titratable acidity

A digital pH-meter (Metrohm 744; Metrohm Applikon B.V., Schiedam, the Netherlands) was used for the measurement of pH-value based on the method 945.10 of AOAC (1990). The total titratable acidity (TTA) was determined by titrating the samples (10 g beverage in 90 ml of distilled water) with 0.1 N sodium hydroxide (NaOH) solution (Merck Chemical Co.) to get a pH value of 8.3 and expressed as percentage lactic acid (AOAC, 1990).

Determination of organic acids and sugars

A high-performance liquid chromatography system (HPLC; Knauer, Berlin, Germany) was used to identify and quantify sugars (fructose, maltose and glucose) and organic acids (lactic and citric acids) present in the beverages. A K-2301 refractive index detector (Knauer, Germany, Berlin) and a K-2600 UV-visible detector (Knauer) were respectively applied for the analysis of sugars and organic acids. The carbohydrates were separated by a $10 \mu\text{m}$ column (Eurokat H 300 $\times$ 8 mm; Knauer) and a mobile phase containing 0.01 N sulphuric acid (Merck Chemical Co.). Flow rate of the mobile phase was 0.4 ml/min and the operation temperature

was maintained at 45 °C. The injection volume of 20 µl sample for each run was selected. Temperature of separation column (Ultrasep ES-FS 250×9×30 mm; Knauer) for the analysis of organic acids was room temperature. The mobile phase was 2.25 mM sulphuric acid. Volume and flow rate of the injected sample were 20 µl and 0.2 ml/min, respectively (Mousavi *et al.*, 2011).

Identification of the compounds was performed on the basis of their retention times via comparison with standards made up from pure sugars and organic acids. Concentration of these compounds in the fermented beverages can be determined by comparison with their external calibration curves.

Statistical analysis

All the determinations were carried out in triplicate, and data are represented as mean ± standard deviation. The SAS statistical computer package (version 9.2; SAS Institute Inc., Cary, NC, USA) was used to analyse the experimental data. The means were compared using the Duncan's multiple ranges test at a significant level of $P < 0.05$.

3. Results and discussion

Growth kinetic

As mentioned earlier, three types of suspensions from barley, malt and barley-malt in a ratio 15% (w/w) were prepared and then inoculated with 10% (v/v) lactic starters of *L. paracasei* and *L. delbrueckii* for obtaining an initial cell density of 10^7 cfu/ml. The higher number of viable microorganisms can help to produce more organic acids and achieve appropriate pH value in short time. This fact can lead to the improvement of sensory properties, shelf-life and reduction of the contamination risk (Angelov *et al.*, 2006; Rathore *et al.*, 2012). The evolution of cell populations for each medium in fermentation process is presented in Figure 1.

Two used LABs of *L. delbrueckii* and *L. paracasei* were found capable to rapidly utilise cereal media without pH adjustment and nutrients supplementation. For all the samples, no drop in the microbial population was observed. The initial cell populations of *L. paracasei* and *L. delbrueckii* were about 7.20–7.36 log cfu/ml. The population densities of these bacteria after 15 h fermentation at 37 °C, respectively, reached nearly 8.90 and 8.70 log cfu/ml in barley medium, 9.40 and 9.30 log cfu/ml in barley-malt medium and, 9.75 and 9.73 log cfu/ml in malt medium. The higher growth in malt and barley-malt media than barley was exhibited. The suitability of these substrates was due to the presence of monosaccharides (glucose and fructose) and disaccharides (maltose and sucrose) in malt medium which could lead to the high quantities of probiotics

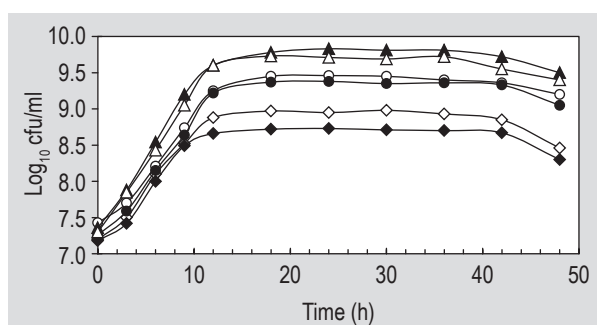


Figure 1. Microbial growth curve of used lactic acid bacteria during fermentation of cereal substrates of malt (triangle), barely-malt (circle) and barely (diamond) at 37 °C (white symbol = *Lactobacillus paracasei* and black symbol = *Lactobacillus delbrueckii*).

(Charalampopoulos *et al.*, 2002a; Rathore *et al.*, 2012). Our results show that *L. paracasei* had a higher population density than *L. delbrueckii*. This fact suggests *L. paracasei* in comparison with *L. delbrueckii* under same conditions has a more capability in the compounds exploitation of culture medium.

pH and acidity

The changes in pH and TTA values of the each sample during 48 h fermentation are shown in Figure 2. This figure indicates that the reduction in pH value and acidification rate was rapidly happened in exponential growth period, while in the stationary phase dropped slowly. Mixed and single cereal media had an initial pH-value of 6 and the strains lowered it to below 4.5 after 10 h fermentation. In this study, the more rapid drop in pH and an increase in TTA were observed by *L. paracasei* in malt medium as respectively reached to 4.25 and 2.96 g/100 g lactic acid in the first 6 h. Increasing the acidification rate reduced fermentation time and prevented the growth of undesirable microorganisms in raw material and thus caused an enhancement in the strain viability (Marklinder and Lönner, 1992). Reduction of the pH also is due to the production of organic acids with sugar consumption by used LAB (Figure 3 and 4).

Sugar consumption and acid production

HPLC analysis was conducted to investigate the amount of acid production in metabolic pathways. As illustrated in Figure 3, lactic acid at the beginning of the fermentation process was not detected, but its concentration was significantly increased during the process so that this metabolite recognised as major organic acid in the fermented beverage. *L. paracasei* produced 5.12, 7.64 and 11.22 g lactic acid/l in the media containing barley, barley-malt and malt, respectively. The concentration of lactic acid produced by *L. delbrueckii* during 15 h fermentation in barley, barley-malt

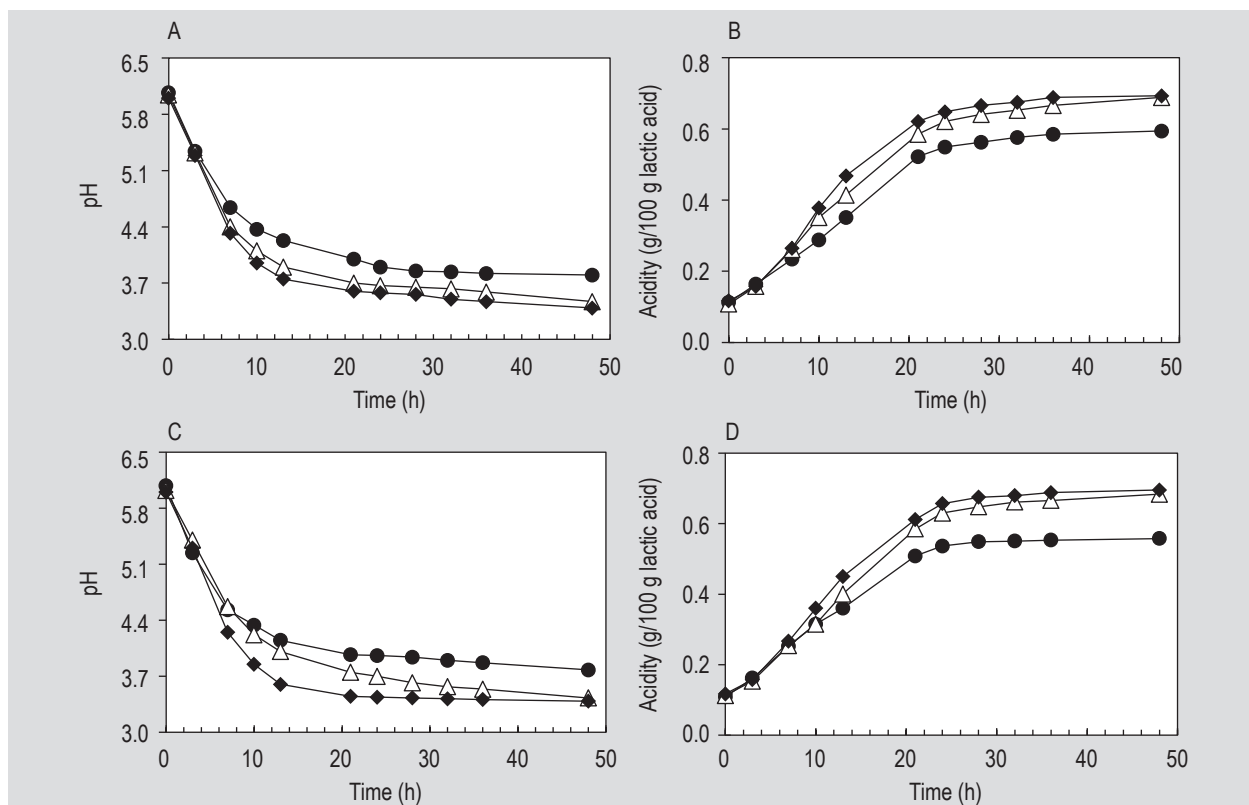


Figure 2. Changes in (A and C) pH and (B and D) TTA levels during 48 h fermentation by *Lactobacillus delbrueckii* (A and B) and *Lactobacillus paracasei* (C and D) (black diamond = malt medium; white triangle = barley-malt medium; black circle = barley medium).

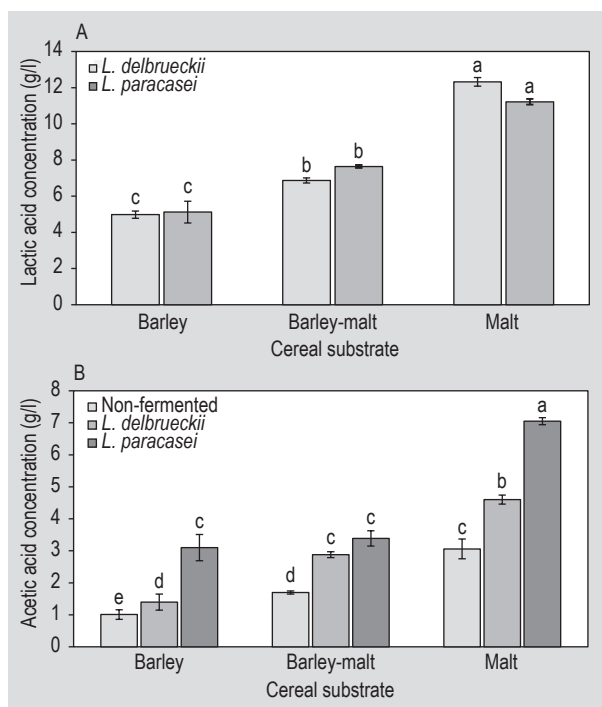


Figure 3. Production of (A) lactic and (B) acetic acids during 15 h fermentation of cereal substrates by *Lactobacillus delbrueckii* and *Lactobacillus paracasei*.

and malt media was 4.98, 6.87 and 12.32 g/l, respectively. As is evident, lactic acid was significantly produced in larger amounts by *L. delbrueckii* in malt medium. But *L. paracasei* in other media produced higher amounts of lactic acid in comparison with *L. delbrueckii*. The initial concentration of acetic acid (Figure 3) present in non-fermented cereal media respectively was 3.06, 1.70 and 1.01 for malt, barley-malt and barley. Our results indicated that acetic acid produced by *L. paracasei* in malt medium was significantly higher than other samples. Acetic acid concentration for *L. paracasei* and *L. delbrueckii* was 7.05 and 4.6 g/l in malt, 3.39 and 2.88 g/l in barley-malt, 3.1 and 1.4 g/l in barley, respectively. Production of this organic acid was previously reported by some strains of *Lactobacillus casei* as a metabolic end product (Desai *et al.*, 2004). Jahandideh *et al.* (2011) by studying the lactic fermentation of *Echium amoenum* extract found that lactic acid is the main metabolite produced by all used lactic strains. They also reported the highest concentration of acetic acid is produced by *L. paracasei* which is in accordance with our results.

The presence of lactic and acetic acids at a certain value can prevent spoilage by other microorganisms due to their antimicrobial characteristics. Passos *et al.* (1993) showed that accumulation of organic acids (lactic and acetic acids) during fermentation process in synthetic media without pH control leads to a decrease in the medium pH. These

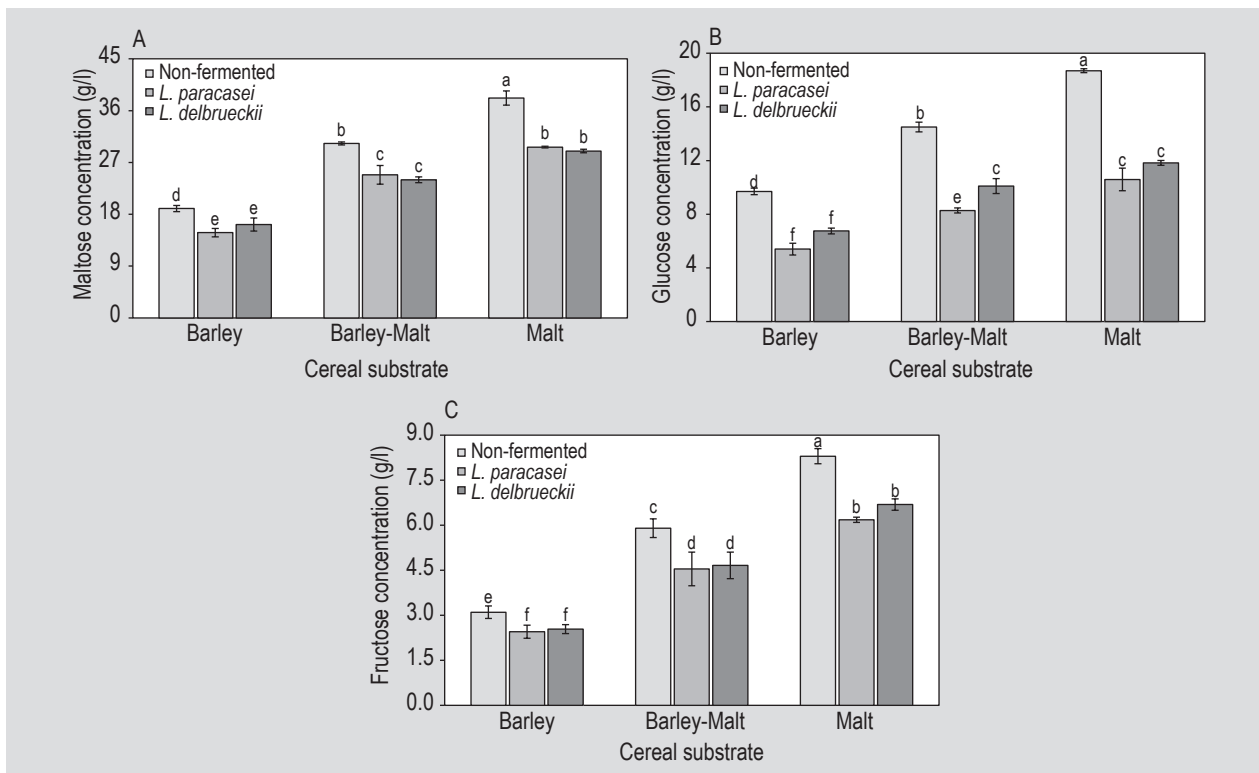


Figure 4. Consumption of (A) maltose, (B) glucose, and (C) fructose sugars during 15 h fermentation of cereal substrates by *Lactobacillus delbrueckii* and *Lactobacillus paracasei*.

compounds through undissociated and dissociated forms or indirectly prevent the microbial growth by releasing the protons (H^+).

Results also indicated that among metabolised sugars by all the strains of in the different media, glucose was the main carbon source followed by fructose and maltose (Figure 4). However, the consumption amount in various samples was varied by the different strains. Wang *et al.* (2003) also showed that glucose is the most important carbon/energy source for the *lactobacilli* and *bifidobacteria* fermentations.

The highest decrease in sugar content was observed in malt medium fermented by *L. paracasei* as respectively 43.36, 25.58 and 22.34% initial glucose, fructose and maltose were consumed. However, a minimum ability for sugar consumption by all the strains exhibited in barley medium. *L. paracasei* respectively reduced fructose and maltose concentrations from 3.10 to 2.45 g/l and from 19.00 to 14.81 g/l in barley medium. This microorganism also decreased glucose concentration from 14.50 to 8.28 g/l in malt-barley and from 9.70 to 5.40 g/l in barley medium. The glucose content reduced to 6.75, 10.1 and 11.83 g/l during *L. delbrueckii* fermentation in malt, barley-malt and barley media, respectively. Hou *et al.* (2000) demonstrated that metabolism of carbohydrates by LAB depends on several factors such as the type strain used, fermentation time and applied substrate. Similar results have also been obtained

for fermentation of *E. amoenum* extract by some strains of lactic probiotics. It was proved that LAB consumed glucose as the first carbon source and *L. paracasei* among all the strains had a more affinity to sugar consumption (Jahandideh *et al.*, 2011). The results obtained in this study are in agreement with the findings obtained by Mousavi *et al.* (2011), who found that glucose and fructose sugars were consumed by the used strains during the lactic fermentation of pomegranate juice.

Effect of cold storage on cell viability of probiotic lactic acid bacteria

In order to evaluate cell viabilities of two species of LAB, the fermented samples were stored at 4 °C for 4 weeks and the sampling was carried out at weekly intervals. The changes in cell viability are shown in Table 1. In the barley medium, the viable cells count of strains was gradually reduced as *L. paracasei* and *L. delbrueckii* lost their viability completely after 2 and 3 weeks, respectively.

The results showed that malt was an ideal medium for more surviving of LAB during the cold-storage. For this reason, survival of the strains in malt and barley-malt (mixed) media maintained even after 4 weeks from fermentation time. Charalampopoulos *et al.* (2003) reported that the cereal extracts specially malt due to the high content of sugar had a positive effect on the viability of probiotic LAB under

Table 1. Effect of cold storage on the cell viability (cfu/ml) of lactic acid bacteria cultures in fermented single and mixed cereal substrates.^{1,2}

Time (week)	Barley		Barley-Malt		Malt	
	<i>Lactobacillus paracasei</i>	<i>Lactobacillus delbrueckii</i>	<i>Lactobacillus paracasei</i>	<i>Lactobacillus delbrueckii</i>	<i>Lactobacillus paracasei</i>	<i>Lactobacillus delbrueckii</i>
0	8.2±0.24×10 ^{8a}	4.9±0.22×10 ^{8a}	2.05±0.21×10 ^{9b}	1.7±0.12×10 ^{9a}	4.47±0.31×10 ^{9a}	5.7±0.25×10 ^{9a}
1	3.3±0.21×10 ^{6b}	3.5±0.16×10 ^{7b}	3.2±0.34×10 ^{8b}	2.26±0.25×10 ^{8b}	6.5±0.26×10 ^{8b}	5.0±0.12×10 ^{8b}
2	2.0±0.12×10 ^{5b}	2.3±0.29×10 ^{6c}	4.0±0.34×10 ^{6c}	3.23±0.12×10 ^{7c}	2.3±0.22×10 ^{8c}	3.3±0.17×10 ^{7c}
3	ND ³	3.3±0.24×10 ^{5c}	1.8±0.29×10 ^{5c}	4.0±0.21×10 ^{6c}	3.3±0.21×10 ^{7c}	4.1±0.12×10 ^{6c}
4	ND	ND	3.0±0.25×10 ^{4c}	2.6±0.08×10 ^{5c}	3.0±0.16×10 ^{5c}	1.2±0.09×10 ^{6c}

¹ The experimental values are means ± standard deviation; n=3.

² Values in the same medium followed by different superscript letters are significantly different ($P<0.05$).

³ ND = not detected.

acidic conditions (Charalampopoulos *et al.*, 2003). In this study, cell concentration of *L. delbrueckii* in malt medium was at an acceptable level (10^6 - 10^7 cfu/ml) after 4 weeks of storage at 4 °C. At the same time, microbial population of *L. paracasei* reached 3.0×10^5 cfu/ml. *L. paracasei* has previously shown less resistant to the high acidic conditions during cold storage (Mousavi *et al.*, 2011).

Minimum dose of LAB in the final probiotic product for the achieving maximum health benefits and therapeutic effect should be 10^6 cfu/ml based on 100 ml daily dose (Sanders and Huis in 't Veld, 1999; Shah, 2001). The main factor for losing viability of the added probiotic bacteria was low pH and accumulation of organic acid as a result of their growth and fermentation (Hood and Zottola, 1998; Shah and Jelen, 1990). Although the other factors such as cold storage, lactic acid production, oxygen level, lack of nutrients, culture condition and fermentation time could affect the cell viability of probiotic organisms in the product (Shah, 2001).

4. Conclusions

In summary, lactic cultures reduced the pH values less than 4.5 and the viable cells count reached more than 10^8 cfu/ml after 15 h fermentation at 37 °C. The used strains had the greatest growth rate and substrate metabolism in the malt compared to other media. All three types of sugars of glucose, fructose and sucrose were consumed by bacterial strains with a higher preference of glucose. *L. paracasei* metabolised higher amount of glucose than *L. delbrueckii*. The maximum amounts of lactic and acetic acids were respectively produced by *L. delbrueckii* and *L. paracasei* in malt medium. LAB stability was enhanced in the presence of malt medium. However, *L. paracasei* in barley medium could not survive due to the low pH and high acidity as

its viability was failed after only 2 weeks of cold storage at 4 °C. It was proposed that malt and *L. delbrueckii* were the best substrate and lactic strain for producing a functional beverage with the highest cell viability (1.2×10^6 cfu/ml after 4 weeks).

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