

## Fermentation of Vegetable Substrates by Lactic Acid Bacteria as a Basis of Functional Foods

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### Abstract

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People suffering from lactose intolerance, cow's milk allergy or phenylketonuria or people on low-protein diet are restricted in the consumption of dairy products. Their consumers' basket should be variegated and enriched with probiotics. The main task was to evaluate important growth and metabolic characteristics of lactic acid bacteria in rice, natural rice, corn, chickpea and barley. Suspensions of the respective flours in water (8% w/w) were supplemented with glucose (1% w/w), pasteurised and fermented. Suitable combinations of cultures and substrates enable the growth of microorganisms minimally by 2 decimal orders. This is connected with a specific shape of the acidification curve pH should be higher than 4.5 after 4-h fermentation. The vegetable samples contained lower concentrations of organic acids than milk samples because of their lower contents of the buffering substances. Fermentation did not result in any decrease in the concentration of protein or phenylalanine. Thus, special formulas of foods for people on phenylketonuria diet or low-protein diets should be conceived. Fermentation of vegetable substrates seems to be a prospective technology in functional foods manufacturing.

**Keywords:** non-dairy probiotic foods; growth curves; acidification curves; phenylketonuria diet; low-protein diet

Dairy products like yoghurts, probiotic beverages, dairy spreads, dressings, dairy desserts, etc., represent an important part of consumers' basket in most of countries. These products contain various bioactive compounds and valuable nutrients (e.g. vitamins, bio-available minerals, bio-active peptides, antimicrobial substances, enzymes, proteins with ideal composition of essential amino-acids, essential fatty acids, etc.) (LANOU *et al.* 2011). Nevertheless, there are a number of consumers who have to restrict the dairy products consumption (due to lactose intolerance; cow's milk allergy; low-protein diet; phenylketonuria) or who dislike

dairy products (due to various superstitions; vegetarianism or personal preferences).

Alternative foods for these groups of consumers should be developed to variegate their diet and to enrich it with probiotics and other lactic acid bacteria and their metabolites (lactic and acetic acid, bacteriocines and other antimicrobial compounds, bio-active compounds liberated from fermented substrates, etc.).

The most common alternative substrate for fermentation is soy, especially in form of soymilk, which was used in many works (e.g. BEHRENS *et al.* 2004; PYO *et al.* 2005; WOO *et al.* 2009; BOŽANIĆ

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*et al.* 2011). Although probiotic soy beverages may exhibit positive effects on humans health (antihypertensive effect, reduction in serum cholesterol, modulation of post-menopausal disorders, etc.) they may be refused by consumers due to their beany-flavour caused by the contents of *n*-hexanol and pentanol (Woo *et al.* 2009). Another negative aspect of the soy consumption is soy allergy. This type of allergy occurs especially in infants – 0.4% of 1-year-old children suffer from soy allergy but at the age of 7 years the prevalence of this disease reduces in half (SAVAGE *et al.* 2010).

This work is focused on alternative substrates for the manufacturing of products other than soy fermented by lactic acid bacteria. The substrates selected were rice, natural rice, barley, corn and chickpea.

In comparison with other main cereals, rice has the lowest content of proteins (Table 1). Moreover, these proteins are hardly digestible for humans and monogastric animals. On the other hand amino-acids composition of rice proteins is more balanced than that of other cereal proteins due to a relatively high content of lysine (OSZVALD *et al.* 2008). Thus, rice seems to be a good raw material for manufacturing of foods for people on low-protein diet or suffering from phenylketonuria.

Additional benefits (or attraction for consumers) can be brought by the use of natural (brown) rice that is, as well as other whole grain cereals, generally recognised as a material with higher contents of dietary fiber and micronutrients like vitamin E, folates, zinc, iron, selenium, copper, manganese, carotenoids, phenolic acids, choline, sulphur amino-acids, etc. in comparison with that refined one (FARDET *et al.* 2008).

Rice (both refined and natural), corn or chickpea were selected as gluten-free materials that can be consumed by patients with coeliac disease (RENZETTI *et al.* 2008).

As concerns the essential amino-acids, cereals are generally deficient in lysine (SHEWRY 2007). On the contrary, legumes have typically amino-acids composition that fulfills the demands of the

human diet on the ratios of lysine, arginine, leucine and other essential amino-acids except of sulphur amino-acids (cysteine and methionine) and tryptophan (IQBAL *et al.* 2006). Chickpea was selected for testing as a representative of legumes.

Barley was selected because of its contents of  $\beta$ -glucans and arabinoxylans as major non-starch polysaccharides. Especially  $\beta$ -glucans have been associated with lowering plasma cholesterol, reducing glycaemic index, reducing the risk of colon cancer (IZYDORCZYK & DEXTER 2008) and supporting intestinal bifidobacteria due to their prebiotic properties (MITSU *et al.* 2010).

Naturally fermented foods based on vegetable materials (especially wheat, rice, chickpea, cowpea, corn, sorghum or millet) are important mainly in the developing countries where the lack of resources limits the energy and capital intensive processes for food preservation. These traditional foods are e.g. ogi, bhalla, bhatura, dhokla, dosa, idli, jalebee, kaajibhat, khaman, kulcha, modhubhat, nan, pantabhat, papadam, vadai, warri, zilapi, etc. (PARVEEN & HAFIZ 2003).

Nevertheless, as described above, there is a considerable potential in manufacturing fermented functional foods for specific groups of consumers that are based on cereals, pseudocereals or legumes. The main task of this work is to evaluate the important growth and metabolic characteristics of lactic acid bacteria in the selected vegetable substrates as a basis for the design of functional foods. Some of the possible final foods were described in the work of KEJMAROVÁ *et al.* (2011).

## MATERIAL AND METHODS

**Microorganisms.** Lactic acid bacteria in the form of liquid milk-based starter cultures were obtained from the Culture Collection of Dairy Microorganisms Laktoflora® (Prague, Czech Republic). The cultures were propagated in a particular vegetable substrate (see below) prior to the use in the experiments in or-

Table 1. Average concentration of protein in various vegetable substrates (VELÍŠEK & HAJŠLOVÁ 2009)

|        | Protein (g/100 g) |        | Protein (g/100 g) |
|--------|-------------------|--------|-------------------|
| Rice   | 7.4               | Wheat  | 11.7              |
| Corn   | 9.2               | Oat    | 12.6              |
| Barley | 10.6              | Lentil | 25.8              |
| Rye    | 11.6              | Soy    | 44.7              |

der to minimise milk protein concentration. Probiotic cultures *Lactobacillus delbrueckii* CCDM 707 and *Lb. fermentum* CCDM 154 and the mesophilic culture CCDM 17 (containing *Lactococcus lactis* subsp. *lactis*, *Lcc. lactis* subsp. *cremoris*, *Lcc. lactis* subsp. *lactis* biovar *diacetylactis*, and *Leuconostoc mesenteroides*), that are currently used in dairy products manufacturing in the Czech Republic, were tested as cultures for fermentation of vegetable substrates. Cultures with enhanced proteolytic activity *Lb. casei* subsp. *casei* CCDM 145, CCDM 198, CCDM 199, and CCDM 650, *Lb. paracasei* subsp. *paracasei* CCDM 211, CCDM 212 and CCDM 213, *Lb. helveticus* CCDM 850 and CCDM 961 and *Lb. gasseri* CCDM 215 were tested for their ability to lower the contents of protein and phenylalanine.

**Fermentation of vegetable substrates.** Rice, natural rice, barley, corn and chickpea flours were bought in the market. The suspension of the respective flour in water (8% w/w) were supplemented with glucose (1% w/w) to enhance the content of fermentable carbohydrates (BEHRENS *et al.* 2004). Afterwards, batch pasteurisation (85°C/10 min) took place under continuous stirring in order to inactivate most of the contaminating microorganisms present and to make gelatinise the starch. Sterile milk (fat 0.5%) was used as a reference medium. The samples were subsequently cooled and inoculated (1% v/v) with the starter cultures tested. Fermentation took place at 37°C (lactobacilli) or at 30°C (the mesophilic culture).

**Growth and acidification curves.** Sampling was carried out after 0, 4, 8, 12 and 16 h of fermentation. Active acidity (pH) was measured and the density of lactic acid bacteria was determined by the cultivation plate methods according to the international standard (IDF 1997).

**Fermentation profiles.** The increase in the concentration of lactic acid or acetic acid was expressed as a difference between the result obtained after 16 h of fermentation and the result obtained with non-fermented sample. Organic acid analysis was performed by capillary electrophoresis (IONOSEP 2003; RECMAN, Ostrava, Czech Republic) according to the application schedule (RECMAN 2008).

**Proteolytic activity.** Totally 10 strains, which are known for their enhanced proteolytic activity in comparison with other more common cultures, were chosen from the Culture Collection of Dairy Microorganisms Laktoflora® (Prague, Czech Republic). Rice and corn substrates were prepared

and fermented by these strains as described above. The samples before fermentation and after 16 h fermentation were analysed to test the ability of the strains to reduce the concentration of protein or phenylalanine. Protein was determined by the Kjeldahl method (Kjeltec 2200; FOSS Analytical, Hilleroed, Denmark) (Foss Tecator 1987), phenylalanine by the ion-exchange high performance liquid chromatography (Summit; Dionex Corp., Sunnyvale, USA) with post-column derivatisation (PCX 5200; Pickering Laboratories, Inc., Mountain View, USA) (MOORE & STEIN 1951).

**Statistics.** All fermentation experiments were performed in duplicate. Moreover, both parallel samples were analysed twice (totally  $n = 4$ ). The results were expressed as the means of the data obtained under particular conditions. Standard deviations of the results on organic acids, protein and phenylalanine were 5%, 3% and 3%, resp. The data were processed by MS Excel 2007.

## RESULTS AND DISCUSSION

The growth curves of the strains tested are shown in Figure 1. Microbial density after fermentation 16 h at optimal temperature reached about 7–8 log CFU/g in most of the substrates, thus such fermented products would fulfill the demands on dairy-like fermented foods and on therapeutic minimum in probiotic ones (CHARALAMPOPOULOS *et al.* 2002).

An important parameter to be observed is the increase in density during fermentation. It should be minimally about 2 decimal orders to reach the same density as is that in the inoculated culture (inoculum 1%). From this point of view, the mesophilic culture CCDM 17 grew well in all substrates except of corn, *Lb. fermentum* CCDM 154 in all substrates (in natural rice and corn even about 3 decimal orders) and *Lb. delbrueckii* CCDM 707 in all substrates expect of natural rice and corn. It seemed that some microbial strains could grow better in the particular substrate than other ones.

Less suitable combinations culture-substrate, as mentioned above, had the same shape of acidification curve (Figure 2). Active acidity in these samples was below 4.5 after 4 h fermentation. Similar results were obtained by BEHRENS *et al.* (2004) for fermentation of soymilk by a mixture of *Streptococcus thermophilus*, *Bifidobacterium lactis*, and *Lb. acidophilus*. Such fast drop in pH

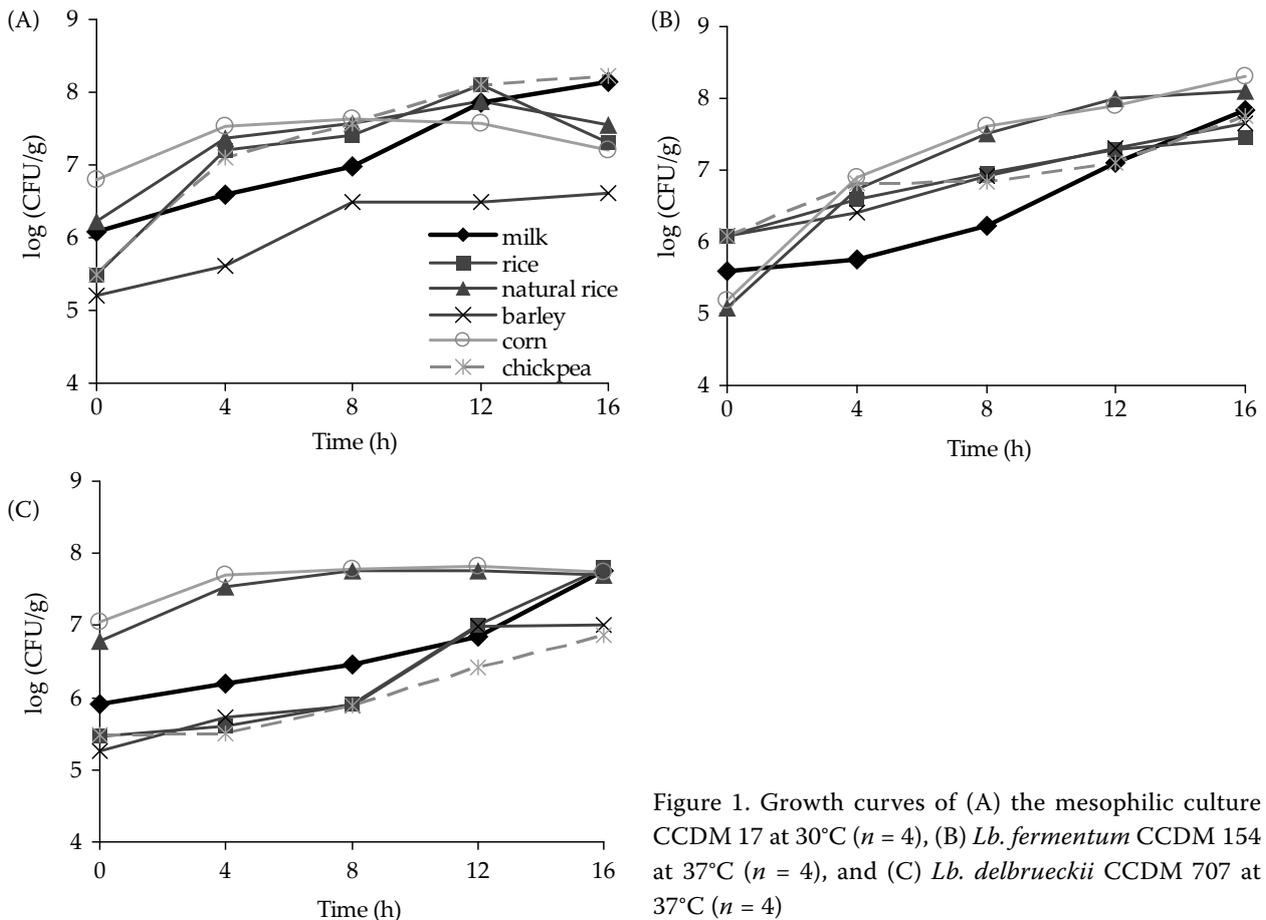


Figure 1. Growth curves of (A) the mesophilic culture CCDDM 17 at 30°C ( $n = 4$ ), (B) *Lb. fermentum* CCDDM 154 at 37°C ( $n = 4$ ), and (C) *Lb. delbrueckii* CCDDM 707 at 37°C ( $n = 4$ )

could disable the adaptation of the culture and its further growth. In these cases, the increase in bacterial density during the whole fermentation process was maximally about 1 decimal order, which could lead to a loss in culture viability after a few re-inoculations.

On the other hand, a relatively fast drop in pH could prevent contaminating microflora from growing. This is important especially in respect of spore-formers that could survive pasteurisation, and their pathogenic representatives like *Bacillus cereus* (RÖSSLAND *et al.* 2003). These findings were related to various acidification curves in milk. However, the drop in pH during fermentation of the vegetable substrates was faster than in milk, especially for the mesophilic culture CCDDM 17 that seemed to be a good tool for the safeguard of functional foods.

Final pH (after 16 h of fermentation) varied between 3.7–4.5 and was comparable with or somewhat lower than those in common fermented dairy beverages. However, lower amounts of organic acids were sufficient to reach this active acidity (Figure 3) due to the lower contents of buffering

compounds, e.g. proteins, in vegetable substrates, especially those based on cereals and rice. While milk contained about  $3.2 \pm 0.16$  g/100 g proteins, vegetable substrates flour (8% w/w) contained only between  $0.57 \pm 0.03$  and  $1.14 \pm 0.06$  g/100 g of proteins, with the highest concentration in the chickpea substrate (data not shown).

Specific profiles of organic acids were obtained with *Lb. delbrueckii* CCDDM 707. This strain was formerly identified as *Bifidobacterium* sp. due to its growth characteristics and fermentation profile in milk typical of bifidobacteria (ratio of lactic acid and acetic acid 2:3, Figure 3C). On the contrary, in the vegetable substrates the concentration of acetic acid was about ten-fold lower than the concentration of lactic acid. It is possible that glucose addition led to the preferential use of homofermentative Embden-Meyerhof-Parnas pathway with the only final product – lactic acid (CURRY & CROW 2003).

Moreover, the exploitation of lactic acid bacteria was tested for lowering protein or phenylalanine concentrations in functional foods for patient on low-protein diet or phenylketonuria diet. This

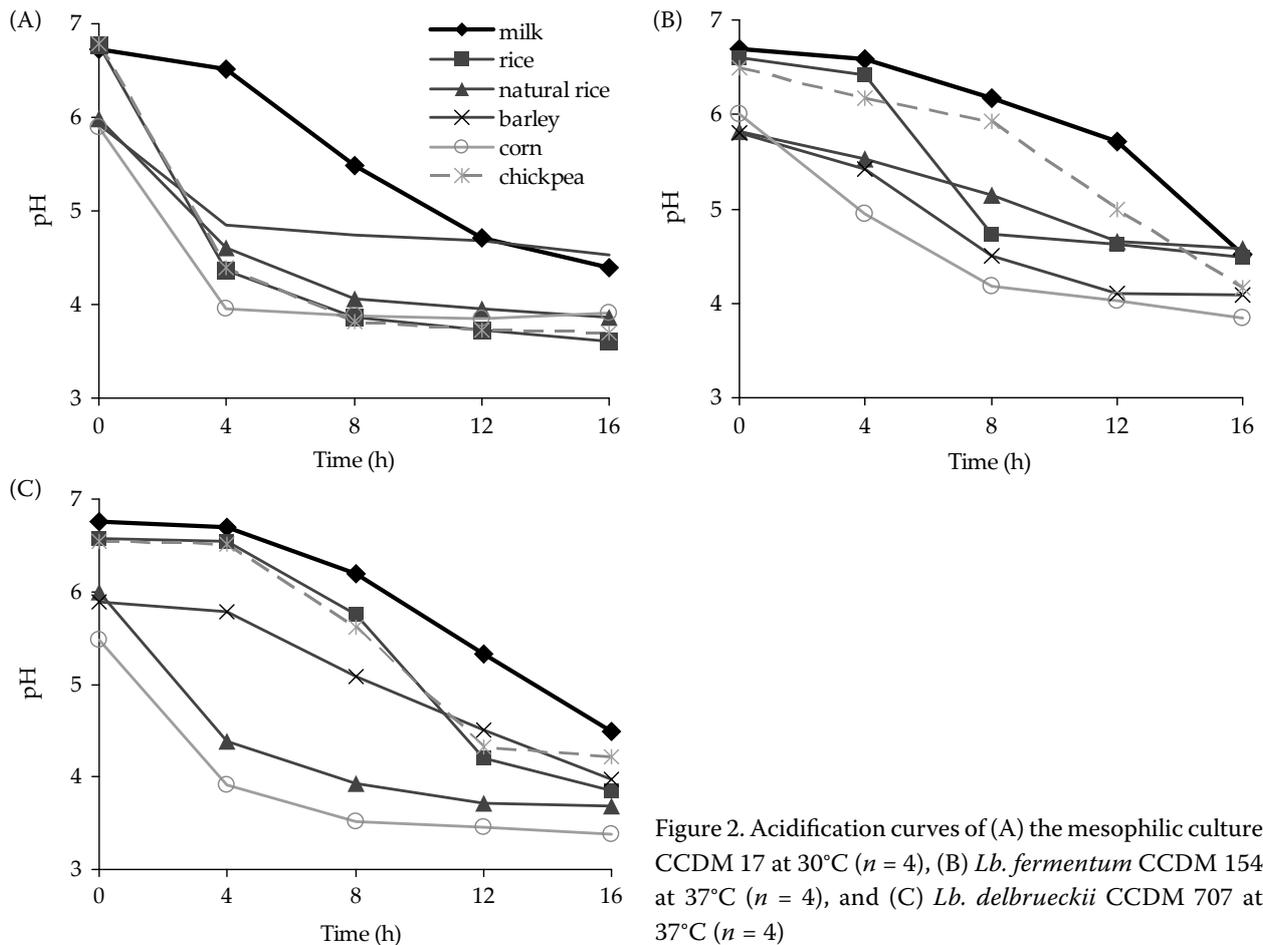


Figure 2. Acidification curves of (A) the mesophilic culture CCDM 17 at 30°C ( $n = 4$ ), (B) *Lb. fermentum* CCDM 154 at 37°C ( $n = 4$ ), and (C) *Lb. delbrueckii* CCDM 707 at 37°C ( $n = 4$ )

special use was expected to require special high-proteolytic cultures applied to the substrates with the lowest content of protein or phenylalanine that were based on corn and rice.

In non-fermented samples, protein concentrations were  $0.68 \pm 0.02$  g/100 g and  $0.59 \pm 0.02$  g/100 g, resp., phenylalanine concentrations  $0.0463 \pm$

$0.0014$  g/100 g and  $0.0316 \pm 0.0009$  g/100 g, respectively. The corn sample with the lowest concentration of these nutrients was fermented by *Lb. paracasei* subsp. *paracasei* CCDM 212 and contained  $0.67 \pm 0.02$  g/100 g protein and  $0.0447 \pm 0.0013$  g/100 g of phenylalanine. Rice sample with the lowest concentration of these nutrients was

Table 2. Concentration (g/100 g) of protein and phenylalanine after 16 h fermentation of rice and corn substrate ( $n = 4$ )

|   | Rice            |                     | Corn            |                     |
|---|-----------------|---------------------|-----------------|---------------------|
|   | protein         | phenylalanine       | protein         | phenylalanine       |
| Before fermentation                                   | $0.59 \pm 0.02$ | $0.0316 \pm 0.0009$ | $0.68 \pm 0.02$ | $0.0463 \pm 0.0014$ |
| <i>Lb. casei</i> subsp. <i>casei</i> CCDM 145         | $0.58 \pm 0.02$ | $0.0310 \pm 0.0009$ | $0.71 \pm 0.02$ | $0.0451 \pm 0.0014$ |
| <i>Lb. casei</i> subsp. <i>casei</i> CCDM 198         | $0.57 \pm 0.02$ | $0.0309 \pm 0.0009$ | $0.66 \pm 0.02$ | $0.0473 \pm 0.0014$ |
| <i>Lb. casei</i> subsp. <i>casei</i> CCDM 199         | $0.58 \pm 0.02$ | $0.0310 \pm 0.0009$ | $0.69 \pm 0.02$ | $0.0466 \pm 0.0014$ |
| <i>Lb. paracasei</i> subsp. <i>paracasei</i> CCDM 211 | $0.62 \pm 0.02$ | $0.0308 \pm 0.0009$ | $0.65 \pm 0.02$ | $0.0504 \pm 0.0015$ |
| <i>Lb. paracasei</i> subsp. <i>paracasei</i> CCDM 212 | $0.60 \pm 0.02$ | $0.0308 \pm 0.0009$ | $0.67 \pm 0.02$ | $0.0447 \pm 0.0013$ |
| <i>Lb. paracasei</i> subsp. <i>paracasei</i> CCDM 213 | $0.59 \pm 0.02$ | $0.0310 \pm 0.0009$ | $0.74 \pm 0.02$ | $0.0450 \pm 0.0014$ |
| <i>Lb. casei</i> CCDM 650                             | $0.59 \pm 0.02$ | $0.0311 \pm 0.0009$ | $0.66 \pm 0.02$ | $0.0463 \pm 0.0014$ |
| <i>Lb. helveticus</i> CCDM 850                        | $0.57 \pm 0.02$ | $0.0312 \pm 0.0009$ | $0.70 \pm 0.02$ | $0.0505 \pm 0.0015$ |
| <i>Lb. helveticus</i> CCDM 961                        | $0.57 \pm 0.02$ | $0.0307 \pm 0.0009$ | $0.71 \pm 0.02$ | $0.0494 \pm 0.0015$ |
| <i>Lb. helveticus</i> CCDM 215                        | $0.58 \pm 0.02$ | $0.0309 \pm 0.0009$ | $0.68 \pm 0.02$ | $0.0459 \pm 0.0014$ |

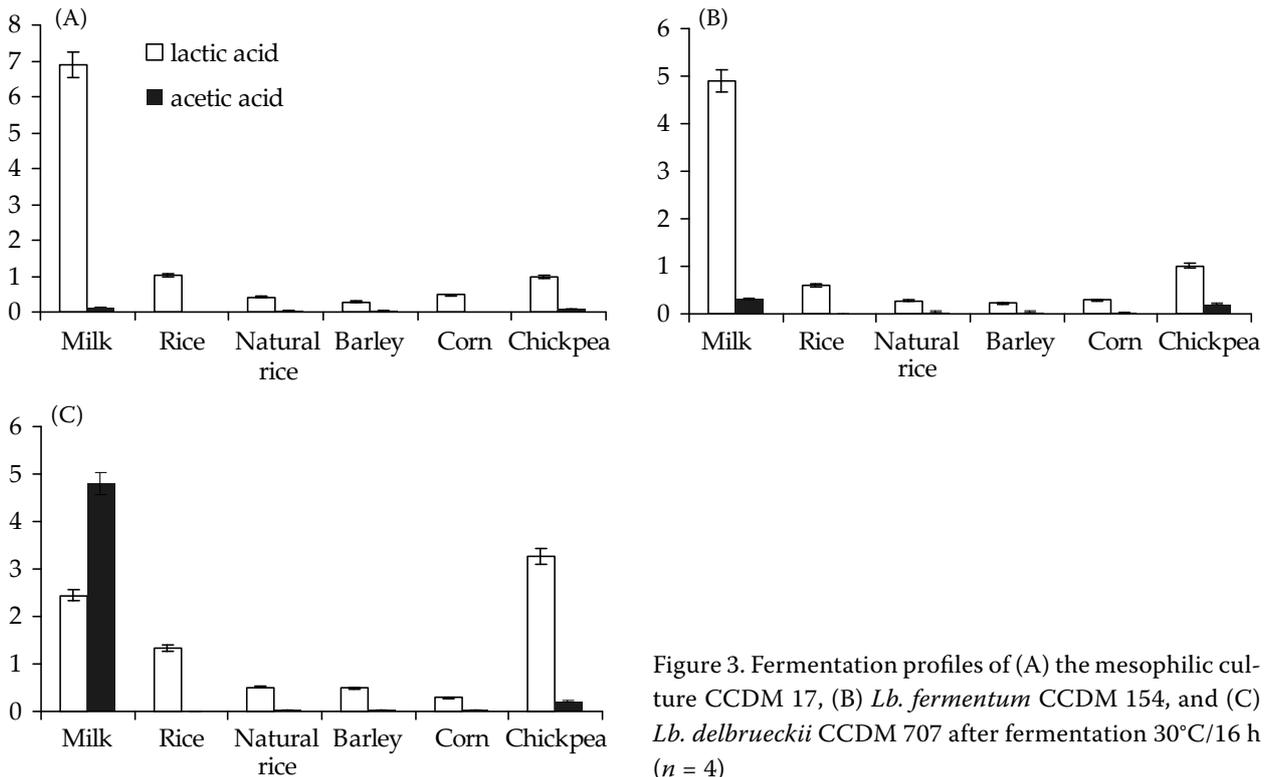


Figure 3. Fermentation profiles of (A) the mesophilic culture CCDM 17, (B) *Lb. fermentum* CCDM 154, and (C) *Lb. delbrueckii* CCDM 707 after fermentation 30°C/16 h ( $n = 4$ )

fermented by *Lb. helveticus* CCDM 961 and contained  $0.57 \pm 0.02$  g/100 g of protein and  $0.0307 \pm 0.0009$  g/100 g of phenylalanine.

Obviously, the differences in protein or phenylalanine concentration between the non-fermented and fermented samples are insignificant (Table 2). However, fermentation enhances the digestibility of cereal proteins (CHARALAMPOPOULOS *et al.* 2002) and lactic acid bacteria with proteolytic activity (e.g. *Lb. casei*) could play a positive role in the aroma formation and off-flavour reduction (BEHRENS *et al.* 2004).

As concerns the demands on foods for people on low-protein diet (2 g/1000 kJ) or for those suffering from phenylketonuria (0.02 g/100 g) (Decree No. 157/2008), special formulas should be developed containing lower concentrations of protein and/or phenylalanine sources.

## CONCLUSION

Lactic acid bacteria can be used for the manufacturing of functional foods based on vegetable substrates. Nevertheless, it is necessary to select strains suitable for particular substrates that can be characterised by the specific shapes of growth and acidification curves.

A rapid drop in pH (below 4.5 within 4 h) could inhibit the strain from reaching the same density as is that in the starter culture. On the other hand, acidification slower than that in milk could lead to outgrowth of the contaminating microflora. In general, for suitable cultures and substrates, the fermentation for minimally 12–16 h along with the final pH below 4.6 seems to be convenient for the fermentation of vegetable substrates by lactic acid bacteria. Diversions are supposed to vary from case to case rather than between particular types of the vegetable substrate.

The final pH of fermented vegetable substrates was comparable to that of fermented dairy beverages, although the concentrations of organic acids were lower in the vegetable substrates due to lower contents of buffering substances, e.g. proteins.

Functional foods for people on low-protein diet or for people suffering from phenylketonuria, which are based on vegetable substrates, could be manufactured using special formulas. In such case, the effect of proteolytic strains is insignificant.

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