Isolation and characterization of acid-sensitive \textit{Lactobacillus plantarum} with application as starter culture for Nham production

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\textbf{A R T I C L E  I N F O}

Article history:
Received 10 November 2009
Received in revised form 22 March 2010
Accepted 23 March 2010
Available online 30 March 2010

Keywords:
Acid-sensitive
H\textsuperscript{+}-ATPase activity
Internal pH
\textit{Lactobacillus plantarum}

\textbf{A B S T R A C T}

The aim of this study was to derive new starter culture variants that are unable to grow below pH 4.6, the desirable pH of the Thai fermented pork sausage, Nham, specified by Thailand Food Standard, and apply them in Nham fermentation. Several acid-sensitive mutants of one of the commercial Nham starter cultures, \textit{Lactobacillus plantarum} BCC 9546, were isolated as spontaneous neomycin-resistant mutants. The growth of three representative mutants was characterized in MRS broth, which revealed that their cell numbers and acid production were lower than that of the wild-type. The H\textsuperscript{+}-ATPase activities of the three mutants were found significantly lower than that of the wild-type under either neutral or acidic conditions. Consequently, internal pH values of the mutants appeared to be lower, especially in acidic environment (pH 5). The most acid-sensitive mutant was applied in experimental Nham production and the pH of Nham fermented with the mutant had significantly higher pH at the end of fermentation (3 days) and after an additional 4 days of storage at 30 °C. These results indicate that the use of acid-sensitive \textit{L. plantarum} as starter culture can reduce the severity of post-acidification and increase the shelf life of Nham at ambient temperature.

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1. Introduction

Nham is a Thai-style fermented pork sausage that is made of minced pork, boiled pork rinds, cooked rice, garlic, salt, sugar, pepper, chili and sodium nitrite. It is packed in banana leaves or plastic sheets and allowed to ferment for approximately 3–4 days at ambient temperature (about 30–35 °C in Thailand), after which period the pH of the product turns to 4.5–4.6 (Valyasevi and Rolle, 2002). Nham is either fermented spontaneously, or starter cultures are used to initiate fermentation for better quality control in terms of consistency and microbiological safety of the product. A hazard analysis found that the Nham fermentation’s critical limit for product safety is pH 4.6 or lower (Paukong and Kunawasen, 2001). \textit{Lactobacillus plantarum} BCC 9546 is one of the commercial Nham starter cultures available in the Thai market.

Nham producers generally do not store the product at their factories, storage usually takes place at the retailer’s facilities in refrigerated conditions. Many Nham manufacturers tend to keep the product at room temperature during product handling, transportation or storage which can result in prolonged fermentation (over-fermentation). This process is mainly caused by lactic acid bacteria that produce organic acids, mostly lactic acid, which contribute to a variety of undesired flavor and texture changes in Nham such as water dripping, discoloration and off-flavor development. Since Nham producers prefer reducing the product loss caused by over-fermentation there is a considerable interest in an economic solution to prevent over-fermentation. A mutated starter culture whose growth is sensitive to high acidity, i.e. pH 4.6, may offer a strategy to the manufacturer to overcome the above problem. The potential new acid-sensitive starter strain would possibly have reduced energy metabolism and would be unable to continue the fermentation below pH 4.6, thereby over-fermentation could be prevented.

There are several reports about acid-sensitive mutants of lactic acid bacteria such as an acid-sensitive \textit{Lactobacillus delbrueckii} subsp. \textit{bulgaricus} in yoghurt fermentation has reduced post-acidification that prolongs the viability of \textit{Bifidobacterium breve} in yoghurt during refrigerated storage (Ongol et al., 2007). An \textit{Oenococcus oeni} mutant that lacks malolactic activity (Galland et al., 2003), as well as an acid-sensitive variant strain of \textit{Lactobacillus helveticus} (Yamamoto et al., 1996) and \textit{Streptococcus bovis} mutants (Miwa et al., 2000) had reduced H\textsuperscript{+}-ATPase activity. These mutants were obtained with the use of neomycin as a selective tool for isolating H\textsuperscript{+}-ATPase deficient...
strains. In *Escherichia coli*, a correlation between resistance to neomycin *(Kanner and Gutnick, 1972)* and defects in ATPase activity has been reported. Some of the independently obtained amino-glycoside antibiotic-resistant ATPase mutants of *Escherichia coli* appeared to have membranes leaky to protons, and a decrease in proton gradient across such membranes was demonstrated *(Tsuchiya and Rosen, 1975; Humbert and Altendorf, 1989)*. It was postulated that defective H+–ATPase would lead to cellular energy shortage that would result in a reduced uptake of neomycin into the cell, thereby leading to resistance to the antibiotic *(Humbert and Altendorf, 1989)*. H+–ATPase plays an important role in cytoplasmic pH regulation in lactic acid bacteria by expelling protons out of the cell utilizing the energy released by ATP hydrolysis. Mutants defective in H+–ATPase are impaired for survival at low extracellular pH *(Hutkins and Hutkins, 1991)*, likely because many cytoplasmic enzymes have their pH optima in a neutral range *(Hutkins and Hutkins, 1993)*. Besides the H+–ATPase there are also other enzymes that may contribute to pH homeostasis of lactic acid bacteria such as glutamate decarboxylase and arginine deiminase. It was demonstrated that in *Lactobacillus* spp. glutamate decarboxylase catalyze the decarboxylation of glutamate, resulting in the stoichiometric release of the end products γ-aminobutyric acid (GABA) and CO₂ *(Hanaoka, 1967)*. The reaction product, GABA is exported from the cell via the GlüK+/CABA α-antiporter, leading to the decrease of cytoplasmic H+ ion concentration and a slight increase of the internal pH. The arginine deiminase (ADI) pathway may also contribute to pH homeostasis converting arginine to ammonia, ornithine and carbon dioxide and generating 1 mol of ATP per mol of arginine. Ammonia production contingent upon arginine availability may contribute to survival at acidic environment by neutralizing the pH. This process has been reported in oral streptococci *(Marquis et al., 1987; Casiano-Colón and Marquis, 1988; Curran et al., 1995)* as well as in *Streptococcus lactis* and *Streptococcus cremoris* *(Poolman and Konings, 1998)* and in a *L. plantarum* strain isolated from orange peel *(Arena et al., 1999)*. All the above three mechanisms reduce acidification of the internal compartment and are important in maintaining acid resistance for the survival of lactic acid bacteria.

*L. plantarum* is one of the most intensively studied *Lactobacillus* strains, however the application of its mutant as an acid-sensitive starter have not been reported to date. In this study we attempted to isolate an acid-sensitive *L. plantarum* strain with mutation in either H+–ATPase, glutamate decarboxylase or arginine deiminase and employ it in Nham production to hinder the fermentation below pH 4.6 and subsequently prolong the shelf life during storage at ambient temperature (30 °C).

2. Materials and methods

2.1. Bacterial strains: acid isolation of acid-sensitive mutants

*L. plantarum* BCC 9546 and *Pediococcus acidilactici* BCC 9545 were obtained from BIOTEC Culture Collection, Pathumthani, Thailand. *Lactobacillus brevis* LSF 8–13 was a gift from Chulalongkorn University, Thailand. Bacteria were grown in MRS medium until mid-exponential phase was reached (A₆₅₀ about 0.8–0.9). Then 0.1 ml aliquots of 10– and 100-fold dilution of the culture was spread onto half strength MRS plates. Viable counts were determined after 2 days incubation at 30 °C. Another portion of the sample was diluted 50-fold with CO₂-free distilled water and titratable acidity was measured by the method of AOAC *(2000)*. The pH of the culture broth was measured using a pH meter *(Mettler Toledo, Switzerland)*. Growth was monitored by measurement of absorbance at 660 nm (Helios-α spectrophotometer, Thermo Electron Corp., UK).

2.2. Measurement of growth characteristics

Growth parameters such as viable count, culture pH, and titratable acidity were measured during 72 h culture in MRS broth at 30 °C. Samples were taken from the culture broth at various time points and were serially diluted with sterile saline and spread onto half strength MRS plates. Viable counts were determined after 2 days of incubation at 30 °C. Another portion of the sample was diluted 50-fold with CO₂-free distilled water and titratable acidity was measured by the method of AOAC *(2000)*. The pH of the culture broth was measured using a pH meter *(Mettler Toledo, Switzerland)*. Growth was monitored by measurement of absorbance at 660 nm (Helios-α spectrophotometer, Thermo Electron Corp., UK).

2.3. Measurement of H+–ATPase activity

Activity of H+–ATPase was assayed using the method of *Matsumoto et al.* *(2004)* with minor modifications. Cells cultivated in MRS broth overnight at 30 °C were harvested by centrifugation (8000 × g for 5 min) and washed three times in sterile saline, then harvested cells were suspended in sterile saline to an A₆₅₀ around 27. Each bacterial suspension (1.0 ml) was added to 10 ml of MRS broth with different pH (4.0, 5.0, 6.0 and 7.0 adjusted with l-lactic acid) and then was incubated for 1 h at 30 °C. Cells were centrifuged at 8000 × g for 5 min, resuspended in 1 ml of 75 mM Tris–HCl buffer (pH 7) containing 10 mM MgSO₄ and permeabilized by the addition of 30 μl of toluene:acetone mixture (1:9, v:v; Lauret et al., 1996) and vigorous mixing for 5 min. Then permeabilized cells were kept at –80 °C before use. H+–ATPase activity assay was conducted as described by *Belli and Marquis* *(1991)* with minor modifications. Permeabilized cells (100 μl) were mixed with 1 ml of 50 mM Tris–maleate buffer (pH 6.0) containing 10 mM MgSO₄ and 10 μl of 0.4 M N,N′-dicyclohexyl carbodiimide (DCCD; Wako Pure Chemicals Industries Ltd., Osaka, Japan) in ethanol or 10 μl of ethanol (as control). Mixtures were incubated at 37 °C for 1 h, then a 10 μl aliquot of 0.5 M ATP (Fluka) was added to start the assay. Mixtures were incubated at 37 °C for 20 min and subsequently put on ice to stop the reaction. Liberated inorganic phosphate (Pi) in the reaction mixtures was measured using the Phosphor C test kit (Wako). Results of the assay are expressed as the amount of Pi produced per minute per mg protein of permeabilized cells. Protein content of the cells was determined by the Bradford method *(Bradford, 1976)*.

2.4. Internal pH measurement

Bacterial intracellular pH was measured as outlined by *Kurdi et al.* *(2000)*. Strains were cultured in MRS broth to mid-exponential phase (6–7 h) at 30 °C, harvested and washed twice in ice cold 150 mM KPO₄, 1 mM MgSO₄, pH 7.0 buffer then resuspended in 150 mM KPO₄, 1 mM MgSO₄, pH 7.0 buffer to A₆₅₀ ~ 10. The cell suspension was diluted to a final volume of 9 ml with prewarmed buffer (A₆₅₀ ~ 0.5),...
and then preloaded with the membrane permeable precursor probe 5 (and 6-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes Inc., Eugene, OR, USA) at 30 °C for 1 h. After that the cell suspension was centrifuged (8,000 × g for 5 min) and the cells were resuspended in 150 mM KPO4, 1 mM MgSO4 buffer, pH 7.0, then 1 M glucose was added to a final concentration of 10 mM and the mixture was incubated at 30 °C for 1 h, divided into two parts and centrifuged (8000 × g for 5 min). One part was resuspended in the same buffer (pH 7.0), while the other part was resuspended in the same buffer with pH 5.0. The two cell suspensions were incubated at 30 °C for another 30 min. After washing once, the cells were resuspended in the same buffer followed by internal pH measurements using a spectrofluorometer (FP 6500, Jasco Co., Tokyo, Japan) with excitation and emission wavelength of 490 and 520 nm, respectively. Calibration of the fluorescent signal was carried out using de-esterified cells in buffers pH 5.0 and pH 7.0. De-esterification was achieved by adding valinomycin (Fluka) and nigericin (Fluka), both from a 2 mM stock to a final concentration of 2 µM, while DCCD (0.01 mM final concentration) was added to the cells in the cuvette (30 °C) placed in the spectrofluorometer when its effect on the internal pH of the cells was investigated.

2.5. Measurement of arginine deiminase activity

Every strain was grown to stationary phase (15–24 h) in GYP–Arg broth, while L. plantarum BCC 9546 was also inoculated to GYP broth and used as Arg-free control. The GYP medium contains (g/l): 0.2, glucose; 3, yeast extract; 5, peptone; 1 ml, Tween 80; 5 ml, salt solution. The salt solution comprised of (g/l): 40, MgSO4·7H2O; 2, MnSO4·4H2O; 2, FeSO4·7H2O; 2, NaCl. Such GYP medium was supplemented with 3.484 g/l arginine (20 mM) (GYP–Arg) and both media were adjusted to pH 6.5 with 6 N HCl before sterilization at 121 °C for 15 min. All L. plantarum strains and P. acidilactici BCC 9545 as positive control were harvested by centrifugation at 8000 × g for 5 min at 25 °C suspended in the same media to AnoG around 1, then these cell suspensions were used to inoculate (1%) GYP–Arg broth and GYP broth. Fifty ml samples were taken at 0, 9, 24, 48 and 72 h. The arginine deiminase activity was detected in terms of ammonia production from arginine by using NH4 + ion-selective electrode (Metler Toledo, Switzerland), and was defined as the amount of ammonium ion produced per mg of protein. The protein concentrations of the cell suspensions were determined by Bradford method using bovine serum albumin as standard (Bradford, 1976).

2.6. Determination of glutamate decarboxylase activity

Bacterial strains were cultured in GYP–Glu medium (per liter: glucose, 5 g; proteose peptone, 5 g; yeast extract, 5 g; Tween 80, 1 ml; salt solution (as described above), 5 ml; monosodium glutamate, 9.36 g (50 mM)) for 24 h. Cells were harvested by centrifugation (8000 × g for 5 min), then incubated at AnoG ~ 10 in 50 mM sodium acetate buffer pH 4.5 containing 50 mM monosodium glutamate for 24 h. After centrifugation (8000 × g for 5 min), 10 µl supernatants were subjected to TLC analysis on Silica gel 60 F254 plates (Merck) using 1-butanol:glacial acetic acid:H2O = 3:2:1 as solvent. The TLC plate was developed with 0.5% ninhydrin in acetone. A known γ-aminobutyric acid (GABA) producer L. brevis LSF 8–13 was used as positive control. The amount of glutamate that Lactobacillus strains consumed was measured by a biochemical analyzer (YSI 7100MBS, YSI Inc., Yellow Springs, Ohio, USA) using glutamate specific membrane. Assuming that consumed glutamate was fully converted to GABA by the intracellular glutamate decarboxylase enzyme the GABA production of our strains was calculated. The protein concentrations of the cell suspensions were determined as mentioned above.

2.7. Nham fermentation

One loopful of stock cultures of L. plantarum BCC 9546 and N750-1 were cross-streaked on half strength MRS agar and half strength MRS agar plates containing 1500 µg/ml neomycin, respectively and were incubated at 30 °C for 24–48 h. A single colony of bacteria was transferred into 5 ml of MRS broth and incubated at 30 °C for 15 h. Cells were harvested by centrifugation at 25 °C at 8000 × g for 5 min and washed with 5 ml of 0.1% sterile peptone water. Nham sausages were mainly produced at Product Development Division, Department of Livestock, Pathumthani using the standard recipe (Visessanguan et al., 2004), and transported to the laboratory for further analysis. Nham was prepared by mixing ground pork (52%), cooked pork rind (35%), sucrose (0.4%), garlic (4.3%), salt (1.9%), cooked rice (4.3%), sodium erythorbate (0.2%), sodium tripolyphosphate (0.2%), monosodium glutamate (0.2%), whole bird chili (2%), and potassium nitrite (0.01%). Half of the sausages (two batches) were prepared with a modified formula which contained the same ingredients but only 3% of garlic. The ingredients were thoroughly mixed and stuffed into plastic casings (3.0 cm diameter, approximately 200 g each). A total of four batches of Nham were prepared by combining two kinds of starter cultures (the wild-type and N750-1) with two kinds of Nham formulations (normal formulation and reduced garlic (3%) formulation). Nham sausages were incubated at 30 °C for 7 days. Samples were taken at 0 h, 12 h and every 24 h up to 7 days.

Nham sample (25 g) was aseptically transferred to a sterile plastic bag and pummeled at 230 rpm for 30 s in a stomacher Model 400 (Seward, England), with 225 ml of sterile peptone water. Appropriate decimal dilutions of the samples were prepared using the same diluent and 0.1 ml of each dilution was plated in triplicates on half strength MRS agar containing 0.5% CaCO3 incubated at 30 °C for 1 day for total LAB count. After that, all colonies were replicated on half strength MRS-neomycin plates (1500 µg/ml of neomycin sulfate) containing 0.5% CaCO3 and incubated at 30 °C for 2 days for neomycin-resistant mutant count. Direct pH measurements were carried out using a pH meter.

2.8. Statistical analysis

Results are expressed as means ± standard deviations of triplicate analyses for each sample unless otherwise stated. A one-way analysis of variance and Duncan’s multiple range tests were used to establish the significance of differences among the mean values at the P < 0.05 significance level. The statistical analyses were performed using SPSS version 15.0 for Windows (2006).

3. Results and discussion

3.1. Isolation of acid-sensitive mutants of L. plantarum BCC 9546

Approximately 6,700 spontaneous neomycin-resistant mutants (600 µg/ml neomycin sulfate) were obtained with a frequency of 10-5–10-6. Only one mutant was found to have its culture broth pH near the desired pH 4.6 after 72 h of growth (strain R5 with a culture broth pH of 4.4). Whereas, among the mutants resistant to 750 µg/ml neomycin sulfate, strain N750 had a culture broth pH of 4.5 at 24 h which decreased to pH 4.19 at 72 h. To generate mutants with resistance to higher neomycin concentration, R5 and N750 were restreaked on half strength MRS plates containing 1500 µg/ml neomycin sulfate. Twenty-six colonies of R5 mutants were found not to lower the pH of the MRS broth below 4.4 during 72 h of incubation at 30 °C. In addition, four of N750 mutants resistant to 1500 µg/ml neomycin sulfate had their culture pH above 4.4 after 72 h of incubation at 30 °C.
From the above mutants with culture broth pH not lower than 4.4 after 72 h, three representative strains were selected for further studies, namely: R5 (resistant to 600 μg/ml neomycin), R5-18 (an R5-derivative resistant to 1500 μg/ml neomycin) and N750-1 (an N750-derivative resistant to 1500 μg/ml neomycin).

3.2. Growth characteristics of the representative mutants

Growth and acid production of the three acid-sensitive mutants compared to parent strain BCC 9546 are shown in Fig. 1 A and B, respectively. After 8 h of culturing, the cell number of R5-18 and N750-1 were similar to that of the wild-type strain, while the viable count of R5 was slightly lower. Afterwards, until 48 h of cultural time, the viable count of the three mutants declined by 3 to 10 fold and were lower than that of the wild-type strain. However, after 48 h the cell number of R5 and R5-18 increased again and surpassed that of the wild-type strain while the viable count of N750-1 remained stagnant (Fig. 1A). The acid production of all strains increased until 24 h culture time followed by virtually no or substantially less acid production in the next 24 h. While no more acids were produced by the parent strain and N750-1 the acidity of the culture broths of R5 and R5-18 were increased considerably between 48 and 72 h (Fig. 1B). Overall, N750-1 produced about half as much acid as the wild-type after 72 h, while the acid production of the other two mutants did not differ much from that of N750-1 until 48 h. The tendencies observed in acid production were also reflected in the pH values of the culture broths (Fig. 1B). The pH of the culture broth of all mutants differed from that of the wild-type strain by one pH unit (at 744 hours). The pH of the culture broth of all mutants did not differ much from that of N750-1 until 48 h. The acid production of all strains increased until 24 h culture time followed by virtually no or substantially less acid production in the next 24 h. While no more acids were produced by the parent strain and N750-1 the acidity of the culture broths of R5 and R5-18 were increased considerably between 48 and 72 h (Fig. 1B). Overall, N750-1 produced about half as much acid as the wild-type after 72 h, while the acid production of the other two mutants did not differ much from that of N750-1 until 48 h. The tendencies observed in acid production were also reflected in the pH values of the culture broths (Fig. 1B). The pH of the culture broth of all mutants differed from that of the wild-type strain by one pH unit at 8 h and the pH of their culture broths did not decrease between 24 and 48 h. Yet after 48 h the culture broth pH of R5 and R5-18 decreased nearly to the pH value of the wild-type strain's culture. The only culture broth pH of N750-1 (~ 4.5) was substantially higher than that of the wild-type strain at the end of the 72 h culturing period. Taken together these observations the cell number increase of R5 and R5-18 accompanied by increased acid production and culture pH reduction between 48 and 72 h suggest that the mutations that made R5 and R5-18 acid-sensitive are unstable and can be reversed after 48 h of continuous culture. This makes R5 and R5-18 not suitable for the application as Nham starter cultures. On the other hand, the acid-sensitivity of N750-1 is probably caused by a stable mutation and seem to be at the desired level, since this strain appear to reduce the pH of the culture broth no lower than about 4.5, what makes it a good candidate for a new Nham starter culture.

3.3. ATPase activity under acidic conditions

To investigate the cause of acid-sensitivity, we measured the ATPase activity of the wild-type and the three mutant strains in different pHs (pH 4–7, this pH range resembles that of in Nham between the beginning and the end of fermentation). Table 1 shows the ATPase activity of L. plantarum BCC 9546 and its acid-sensitive mutants at different acidic pHs in the presence (A) and absence (B) of DCCD, a relatively specific inhibitor of the F0 part of the F0F1-ATPase. The difference in ATPase activity with or without DCCD was also calculated and since it is the activity inhibited by DCCD this value can be considered as the activity of H+–ATPase (Table 1C). The ATPase activity of all mutant strains were significantly lower than that of wild-type strain at all conditions and at pH 4 the ATPase activity of all mutants decreased below the values at other pHs (5–7). Since the H+–ATPase activity (DCCD inhibited) of the wild-type strain was significantly higher than those of all mutant strains at the above pH interval it indicates that the mutants had reduced membrane-bound H+–ATPase activity. It is postulated that mutants with reduced H+–ATPase activity do not generate sufficient energy to concentrate neomycin, therefore they are resistant to neomycin because neomycin uptake by bacterial cells is energy dependent process (Kanner and Gutnick, 1972). Such defect in the energy metabolism in our acid-sensitive mutants might explain their growth characteristics and reduced acid production in MRS broth.

3.4. Measurement of internal pH

In order to characterize the pH homeostasis of acid-sensitive mutants, their internal pH upon energization with glucose was investigated. Internal pH (pHi) measurements revealed that the mutants had significantly lower pHi (by about 0.2 pH unit) at external pH 7.0 than that of wild-type (Table 2). At acidic condition (pH 5.0) the internal pH of the mutants were also lower than the pHi of the wild-type however only that of N750-1 did by a significantly margin. At pH 5.0 the difference between external and internal pH (ΔpH) of the parent strain was one unit, whereas N750-1 could maintain a ΔpH of only 0.43, and it is likely that acidification of the cytoplasm under acidic condition affects the physiology of the latter strain considerably. Lactic acid is a weak organic acid with increased proportion of its protonated (neutral) form at low pH that can freely pass the cell membrane, then dissociating at the more alkaline cytoplasmic pH (Kashket, 1987). This possibly results in the disturbance of the internal pH regulation in N750-1, at least, due to its reduced H+–ATPase activity, therefore it cannot maintain its cytoplasmic pH near neutral at external pH 5.0 as the wild-type strain.

In order to reveal the overall effectiveness of other internal pH maintenance processes DCCD was applied during the internal pH measurements to inhibit H+–ATPase. A representative experiment shown in Fig. 2 demonstrates that after the cells were first energized by addition of glucose (10 mM), addition of DCCD resulted in
a decline in internal pH. After energization the cytoplasm became more alkaline than the extracellular buffer, and assuming that DCCD inhibited proton translocating ATPase, decrease in the internal pH upon DCCD addition was likely the result of a net proton leak through the cell membrane. When the rate of internal pH decrease was measured following the DCCD addition, the results revealed that (Table 2) the wild-type had the smallest proton leak among the strains at both tested pHs, while the internal pH decrease rate was highest in N750-1 at acidic condition (pH 5.0). This also implies that the mechanisms involved in pH homeostasis excluding the H⁺-ATPase are probably the weakest in N750-1 among the strains at least at acidic conditions. Moreover, that is coupled with the lowest H⁺-ATPase activity in N750-1 which is probably the main reason behind that this strain has the smallest internal pH at extracellular pH 5.0. Since these parameters indicate disturbed pH homeostasis and smaller cellular energy level (smallest ΔpH), it is reasonable to assume that all these factors combined have also a profound impact on the survival of this strain in acidic environment.

### Table 2

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<th>Strain</th>
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<th>Proton leakb</th>
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<td>pHex pH 4.0</td>
<td>pH 5.0</td>
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<tr>
<td>Wild-type</td>
<td>7.46 ± 0.02</td>
<td>6.02 ± 0.16</td>
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<tr>
<td>R5</td>
<td>7.22 ± 0.04*</td>
<td>5.83 ± 0.38</td>
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<tr>
<td>R5-18</td>
<td>7.32 ± 0.03*</td>
<td>5.58 ± 0.23</td>
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<tr>
<td>N750-1</td>
<td>7.25 ± 0.01*</td>
<td>5.43 ± 0.03*</td>
</tr>
</tbody>
</table>

Results are means ± standard deviation obtained from three independent experiments.

*Statistically different (P < 0.05) from that of the wild-type strain at the same pH.

**Measured as described in Section 2.

Time (h)

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<th>Time (min)</th>
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<td>10</td>
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### 3.5. Measurement of arginine deiminase activity

The arginine deiminase activity may also contribute to the pH homeostasis in some lactic acid bacteria (Marquis et al., 1987) including *L. plantarum* strain from orange (Arena et al., 1999) therefore the ammonium ion production from arginine by *L. plantarum* strains was investigated. Fig. 3 shows that wild-type and mutant *L. plantarum* strains produced very small amounts of ammonium ion. Their ammonium ion production profiles were not significantly different from each other at every sampling point. The ammonium ion concentrations in the culture broths of *L. plantarum* strains were slightly higher after 24 h of culturing (by 30–50%) than at the beginning of the cultivation, while the culture broth pHs of these strains were dropped to 4.7 at 9 h without a change onwards (data not shown). However, this small ammonium ion concentration increase might not be the result of arginine deiminase activity, since similar ammonium ion concentration pattern was observed cultivating the wild-type strain, *L. plantarum* BCC 9546, in arginine-free GYP medium (43% increase). The virtually no ammonia production by any of our *L. plantarum* strains and their
slightly decreasing culture broth pH is a sharp contrast to the ammonium ion production by the positive control (*P. acidilactici*) which also coupled with culture broth pH increase to pH 8.3 (data not shown). Therefore, considering all these findings, we conclude that arginine cannot be metabolized by our *L. plantarum* strains.

### 3.6. Measurement of glutamate decarboxylase activity

The glutamate decarboxylase catalyzes the α-decarboxylation of glutamic acid to yield γ-aminobutyric acid and carbon dioxide. This intracellular reaction results in the decrease of cytoplasmic H⁺-ion concentration and a slight increase of the cytoplasmic pH. Fig. 4 demonstrates that all of our *L. plantarum* strains were able to produce GABA from glutamate, albeit at a seemingly lower extent than the positive control *L. brevis* LSF 8–13. To quantify the GABA production the amount of glutamate consumed by *L. plantarum* strains were measured by biochemical analyzer. It was found that N750-1 showed the lowest GABA production (0.07 ± 0.12 μmol/mg protein/24 h), while the wild-type produced nearly twice as much GABA (0.12 ± 0.04 μmol/mg protein/24 h). The other two mutants, R5 and R5–18, produced about twofold more GABA than the wild-type strain (0.26 ± 0.02 and 0.24 ± 0.05 μmol/mg protein/24 h, respectively). According to the H⁺-ATPase activity measurements (Table 1C) N750-1 showed less activity than R5 and R5–18 which probably result in less available cellular energy for solute, e.g. glutamate, transport in this strain. That would provide a possible explanation for this strain’s lowest glutamate decarboxylase activity although the likelihood of down-regulation or mutation in the glutamate decarboxylase enzyme could not be ruled out. On the other hand, R5 and R5–18 had higher glutamate decarboxylase activity than that of the wild-type, which might be due to a slight up-regulation as a compensatory response to their acid-sensitivity.

Nevertheless, these results indicate that the moderate/weak glutamate decarboxylase activity of these strains might play only a marginal role in their pH homeostasis and this effect might be realized over a longer period of time (24 h), therefore it might not provide effective protection against the much faster cytoplasmic acidification. This latter notion is also supported by the growth experiment data (Fig. 1), which showed almost no difference in cell number, total acidity and culture broth pH among the tree acid-sensitive mutants in MRS culture up to 48 h. This suggest that in spite of glutamate most likely available in MRS broth the higher glutamate decarboxylase activity of R5 and R5–18 is not able to make these mutants more acid-resistant than N750-1 with the least glutamate decarboxylase activity (until 48 h).

### 3.7. Nham fermentation

From among the mutants only N750-1 was selected for Nham fermentation trials because of its lowest H⁺-ATPase activity, mutation stability and because its MRS broth pH was the highest after 72 h (around pH 4.48, Fig. 1B). This mutant and the wild-type strain (WT, *L. plantarum* BCC 9546) were used in two formulas, (1): normal formula (using 4.3% garlic) and (2): limiting carbon source in Nham by decreasing concentration of garlic to 3%.

Table 3 shows the number of total lactic acid bacteria (LAB) and of the mutant during Nham fermentation. The mutant was counted by replica-plating on half strength MRS-neomycin plates (1500 μg/ml of neomycin sulfate) to confirm that most of the colonies that grew on these plates were N750-1. The initial inoculation level was approximately 10⁴ cfu/g Nham in all four treatments. The viable count of total LAB and N750-1 increased until 12 h then the bacterial counts of all Nham formulations were slightly decreased until the end of incubation time (7 days). However, the number of

![Fig. 4. Glutamate conversion to γ-aminobutyric acid by *Lactobacillus* strains as demonstrated with TLC. Glu: glutamate (negative control), GABA: γ-aminobutyric acid, L. b. 8–13: *Lactobacillus brevis* LSF 8–13 (positive control), WT: wild-type (*L. plantarum* BCC 9546). This TLC profile is a representative of three independent experiments that gave similar results.](image_url)
total LAB in Nham fermentation using the wild-type or N750-1 starter were about three times higher than the viable count of the N750-1 starter cell strain in Nham. This means that the number of N750-1 starter cells represents about one third of the total LAB cell number in Nham. This affirms that in Nham inoculated by the mutant starter acid-sensitive \textit{L. plantarum} cells play a major (or dominant) fermentative role. Comparing the pH of Nham (Table 4) in all trials shows that the pH values at 0 and 12 h in both wild-type and mutant fermented sausages were not significantly different but after 24 h there is a significant difference in pH of Nham fermented by different starters until 7 days. In Nham fermented by the wild-type the pH decreased lower than 4.6 and faster than Nham fermented with mutant in normal formulation. N750-1 as a new starter culture gave Nham a consistent final pH about 4.5 until 7 days (in normal formulation) while wild-type fermented Nham had a pH about 4.6 between the 1st and the 2nd days. Using 3% of garlic did not result in a product with significantly higher pH than that of produced with the traditional 4.3% garlic content neither when the acid-sensitive mutant nor when the wild-type strain was used as starter. Although, when the mutant was used as a starter at the end of 7 days of fermentation the pH of Nham formulated with 3% garlic was higher than that of Nham formulated with 4.3% garlic by about 0.2 units. This implies that garlic amount reduction in the formulation of Nham may contribute to less acid production in Nham fermented with the mutant. Concerning the safety of Nham fermented by the mutant, the sausages formulated with 3% garlic were above the critical pH limit of 4.6 (Paukatong and Kunawasen, 2001), therefore these are not considered to be safe. However the mutant fermented Nham with 4.3% garlic content had safe pH (4.6 or below) from the 4th day onwards. Therefore, the above results indicate that acid-sensitive \textit{L. plantarum} has a potential for use as starter culture for Nham production to prevent over-fermentation.

4. Conclusions

Spontaneous acid-sensitive \textit{L. plantarum} BCC 9546 mutants were isolated which have shown to have reduced H+-ATPase activity. Experimental Nham fermented by one of the acid-sensitive mutant starters (N750-1) had significantly higher pH than Nham fermented by the wild-type strain and could be kept at ambient temperature for four days without over-fermentation. \par

Acknowledgements

Authors would like to thank the National Center for Genetic Engineering and Biotechnology (BIOTEC) for the financial support for the project BT-B-02-NC-BG-5003 and this research is partially supported by the Center of Excellence on Agricultural Biotechnology, Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education, Thailand. 

References


Table 3

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<th>WT-normal</th>
<th>WT-3% G</th>
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Mean values and standard deviations obtained from three independent experiments. Different letters (a–d) in the same row indicate significant differences (P < 0.05).

Table 4

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