Isolation of Acid–Sensitive *Lactobacillus plantarum* and its application as starter in *Nham* production to prevent over–fermentation

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*Pornpan Jaichumjai[1,2], Apinya Assavanig[1], Ruud Valyasevi[3] and Peter Kurdi[3]**

Abstract

Acid-sensitive mutants of *L. plantarum* BCC 9546 were isolated from spontaneous neomycin-resistant mutants with potential to be used as starter to reduce the post-acidification in *Nham* during storage at ambient temperature. In MRS broth, their growths and acid productions (at 30 °C for 48 h) were lower as well as their culture broth pH were higher than the wild-type strain. Three representative acid-sensitive mutants had reduced H^+-ATPase activity in acidic condition (pH 5) corresponding to their lower internal pH than the wild-type strain. Besides, the arginine deiminase activities of the wild-type and mutant strains were also investigated and they were found not significantly different from each other. Finally, *Nham* fermentation trial was carried out for 3 weeks and *Nham* fermented with the wild-type strain had significantly higher amounts of released water than *Nham* fermented using one of the mutants until one week but no significant difference in color was observed, while the total biogenic amine content was higher in mutant fermented *Nham*. In conclusion, *Nham* fermented with mutant had longer shelf-life and less post-acidification with a consistent product pH about 4.6 compared to *Nham* fermented by wild-type.

Keywords: Acid-sensitive neomycin-resistant mutant, H+-ATPase, Internal pH

[1] *Department of Biotechnology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand.*

[2] *Center of Excellence for Agricultural Biotechnology: AG-BIO/PERDO-CHE.*

[3] *National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Paholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand.*

**Corresponding author, e-mail address: peter.kur@biotec.or.th*

Introduction

Nham is a traditional Thai 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 which is fermented at ambient temperature for approximately 3-4 days, after which period the pH of the product turns to 4.5-4.6 (Valyasevi and Rolle 2002). *Nham* fermentation used adventitious microflora as natural starters which are mainly come from raw materials would make the product's quality variable. Therefore, starter cultures for *Nham* production have been developed. *Lactobacillus plantarum* BCC 9546 is one of the commercial *Nham* starter cultures available in the Thai market to ensure good quality and product consistency (Valyasevi et al., 2001). While, the use of starter cultures generally result in a rapid decrease in pH prolonged exposure to the ambient temperature (handling, transportation) can lead to excessive acid production (over-fermentation) causing product deterioration such as water dripping, discoloration, and off-flavor development which are unacceptable characteristics for both manufactures and consumers (Valyasevi et al., 2001).

There are several reports about acid-sensitive mutants of lactic acid bacteria such as an acid-sensitive *Lactobacillus delbrueckii* subsp. *bulgaricus* which has shown to be able to reduce post-acidification in yoghurt and prolonged the viability of *Bifidobacterium brevis* in yoghurt during refrigerated storage (Ongol et al., 2007). An *Oenococcus oeni* mutant that lacks malolactic activity (Galland et al., 2003), as well as acid-sensitive variants of *L. helveticus* (Yamamoto et al., 1996) and *Streptococcus bovis* (Miwa et al., 2000) have also shown to be able to reduce H^+-ATPase activity. All of these mutants were obtained with the use of neomycin as a selective tool to isolate H^+-ATPase deficient strains. Additonally, these mutants with defective in H^+-ATPase, are all impaired for survival at low pH (Nannen and Hutkins, 1991), likely because many cytoplasmic enzymes have their pH optima in the neutral range (Hutkins and Nannen, 1993).

In addition, the arginine deiminase (ADI) pathway in certain lactic acid bacteria may also contribute to the pH homeostasis by converting arginine to ammonia, ornithine, and carbon dioxide. Ammonia production (contingent upon arginine availability) might play an important role in survivability at acidic environment by neutralizing the pH (Marquis et al., 1987; Casiano-Colon and Marquis, 1988; Poolman and Konings, 1988; Curran et al., 1995). All the above mechanisms are associated with the reduction in acidification of the internal compartment which are important in maintaining acid resistance for the survivability of lactic acid bacteria. As a result from considering all of these above mechanisms, our study was then aimed to obtain acid-sensitive *L. plantarum* starter strain from spontaneous neomycin resistant mutants. The new starter culture selection targeted mutants with reduced energy metabolism, which would make them unable to continue the fermentation at pH below 4.6, thereby preventing an over-fermentation.

Materials and Methods

Bacterial strains acid isolation of acid-sensitive mutants

Lactobacillus plantarum BCC 9546 and *Pediococcus acidilatici* BCC 9545 were obtained from BIOTEC Culture Collection, Pathumthani, Thailand (BIOTEC). *Lactobacillus brevis* LSF 8-13 was a gift from Chulalongkorn University.

Bacteria were grown in MRS medium until mid-exponential phase which was manifested by A_{660} value 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 containing 600 or 750 μg/ml neomycin sulfate for acid-sensitive mutant generation and incubated at 30 °C for 2 days. After 2 days of incubation neomycin plates were used as master plates for replica-plating on half strength MRS pH 4.5 or pH 4.6 and on half strength MRS plates. After incubation at 30 °C for 2 days the acid-sensitive colonies were identified as the ones unable to grow on half strength MRS pH 4.5 or pH 4.6 plates and were picked up from the half strength MRS replica plates. They were cultured in MRS broth for 15 hours and adjusted to A_{660} about 0.8 then it was used as an inoculum (2%) for the growth experiments in MRS broth. The pH of the culture broth was monitored for 24-72 hours and the isolates with the highest culture broth pH were stored in 20% glycerol at-80 °C. To obtain isolates with higher acid-sensitivity mildly acid-sensitive mutants (pH 4.2) were re-streaked onto half strength MRS containing 1500 μg/ml neomycin plates and incubated at 30 °C for 24-48 hours then each single colony was picked up and then inoculated to MRS broth for 15 hours at 30 °C. The cultures were adjusted to A_{660} around 0.8 then inoculated (2%) to MRS broth. The pH of the culture broth was monitored for 24-72 hours and the isolates with the highest culture broth pH were kept and stored in 20% glycerol at-80 °C.

Determination of growth characteristics of the mutant

Growth parameters were determined during 72 h cultivation in MRS broth at 30 °C. Samples were taken from the culture broth at various time points and were serially diluted with sterile normal saline and spread onto half strength MRS plates. Viable counts were determined after 2 days of incubation at 30 °C.

Measurement of acidity and pH

Another portion of the sample was diluted 50-fold with CO_2-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. Growth was monitored by measurement of absorbance at 660 nm (Helios α, Thermo electron corporation), and by pH measurement of the culture broth.

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 for overnight at 30 °C were harvested by centrifugation (8000xg for 5 min) and washed three times in sterile normal saline, then harvested cells were suspended in sterile normal saline to an A_{660} at approximately 27 (from cultivated in MRS broth, as above). 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). They were adjusted to the assigned pH by using L-lactic acid) and then was incubated for 1 h at 30 °C. Cells were centrifuged at 8,000xg for 5 min, resuspended in 1 ml of 75 mM Tris-HCl buffer (pH 7) containing 10 mM $MgSO_4$ 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 $MgSO_4$ 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 (P_i) in the reaction mixtures was measured using the Phosphor C test kit (Wako). Results of the assay are expressed as the amount of P_i produced per minute per mg protein of permeabilized cells. Protein content of the cells was determined by the Bradford method (Bradford, 1976).

The 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 two times in ice cold 150 mM KPO_4, 1 mM $MgSO_4$, pH 7.0 buffer then resuspended in 150 mM KPO4, 1 mM $MgSO_4$, pH 7.0 buffer to A_{660} ~ 10. The cell suspension was diluted to a final volume of 9 ml with prewarmed buffer (A_{660} ~ 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 (8,000xg for 5 min) and the cells were resuspened in 150 mM KPO_4, 1 mM $MgSO_4$, pH 7.0 buffer then 1 M glucose was added to a final concentration of 10 mM and the mixture was incubated for 1 hour at 30 °C, ??? What divided into two parts and centrifuged (8000 x g for 5 min). One part was resuspended in the same buffer at pH 7.0, while the other part was resuspended in the same buffer at pH 5.0. The two cell suspensions were incubated for another 30 min at 30°C. After washing once, the cells were resuspended in the same buffer followed by internal pH measurements using a spectro fluorometer (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-energized cells in buffers pH 5.0 and pH 7.0. De-energization was achieved by adding valinomycin (Fluka) and nigericin (Fluka), both from a 2mM 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.

Measurement of Arginine deiminase activity

Every strain was grown to stationary phase (15-24 h) in GYP-Arg broth, while *L. plantarum* BCC 9546 inoculated into GYP broth was used as an 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 and it was supplemented with 3.484 g/l, arginine (20 mM) in GYP-Arg, both medium were adjusted to pH 6.5 with 6 N HCl before sterilization in autoclave at 121°C for 15 min. The salt solution comprise of (g/l): 40, $MgSO_4x7H_2O$; 2, $MnSO_4x4H_2O$; 2, $FeSO_4x7H_2O$; 2, NaCl. All *L. plantarum* strains and *P. acidilatici* BCC 9545 as positive control were harvested by centrifugation at 8000xg for 5 min at 25 °C suspended in same media to A_{660} around 1, then 1% of cell suspension was used to inoculate GYP-Arg broth and GYP broth. Fifty ml samples were taken at 0, 12, 24, 48 and 72 h. Activity was detected in terms of ammonia production from arginine by using NH_4^+ ion-selective electrode (Mettler Toledo, Switzerland). The arginine deiminase activity was defined as the amount of ammonium ion produced per mg of protein. The protein concentration of the cell suspension was

determined by Bradford method using bovine serum albumin as standard (Bradford, 1976).

Nham fermentation

Preparation of starter culture

One loopful of a stock culture of *L. plantarum* BCC 9546 and N750-1 were cross-streaked on half strength MRS agar and half strength MRS 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 5ml of MRS broth and incubated at 30 °C for 15 h. Cells were harvested by centrifugation at 25 °C at 8000xg for 5 min and washed with 5 ml of sterile peptone water. Finally, the cell concentration was adjusted to 10^7cfu/ml with sterile peptone water.

Nham preparation

Nham sausages were produced at Product Development Division, Department of Livestock, Pathumthani using the standard recipe developed by Paukatong et al., 1999 as shown below, and transported to Food Biotechnology Laboratory, BIOTEC for 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%), erythrobate (0.2%), trisodium polyphosphate (0.2%), monosodium glutamate (0.2%), whole bird chili (2%), and potassium nitrite (0.01%). Half of the sausages 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). Four batches of Nham were prepared by using the wild-type strain and N750-1 as starter cultures in normal formulation and in reduced garlic (3%) formulation. Nham sausages were incubated at 30 °C and samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 10, 13, 16, 19 and 22 days (3 weeks).

Microbiological and chemical analyses

Nham sample (25 g) was aseptically transferred to a sterile plastic bag and pummeled for 30 sec at 230 rpm in a stomacher Model 400 (Seward, England), with 225 ml of 0.1/100 g 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 triplicate on different growth media. The following media and incubation conditions were used: half strength MRS agar containing 0.5% $CaCO_3$ incubated at 30 °C for 1 day for total LAB count and for neomycin-resistant mutant count by replica-plating on half strength MRS-neomycin plates (1500 μg/ml of neomycin sulfate) containing 0.5% $CaCO_3$ incubated at 30 °C for 2 days. Direct pH measurements were carried out using a pH meter (Mettler Toledo 320, Switzerland). Biogenic amines in Nham were extracted and analyzed by high performance liquid chromatography (HPLC) according to the method of Eerola et al. (1993).

Color evaluation and released water

The surface color of Nham samples were measured by using a tristimulus colorimeter (Minolta Color Meter CR300; Minolta Camera Ltd., Osaka, Japan). Before each measurement, the apparatus was standardized against a white tile (L = 90.7, a = -0.9 and b = -0.1). Color parameters (*L**: lightness, *a**: redness and *b**: yellowness) were measured in the CIE Lab mode. The variation of each measurement was compensated by recording the average of three readings taken on the round surface of sample. Color measurement was carried out in two replicates. The percentage of water released from samples was measured immediately upon sample collection according to the method of Nakao et al. (1991).

Sample with casing was weighed (A). After removing the sample from the casings, water released on the surface was absorbed using filter paper and sample was weighed (B). The empty casing was weighed (C). The percentage of released water will be calculated according to the following equation:

Released water (%) = 100 x {(A-B)-C}/ (A-C)

Statistical analysis

Results are expressed as mean ± 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.

SPSS for Windows, 2006 SPSS for Windows, (2006). User's manual, version 15.0. Chicago, IL: SPSS Inc.

Results and discussion

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 (R5, pH 4.4). Due to the low number of adequate acid-sensitive mutants replica plating was applied as another strategy to obtain neomycin resistant mutants. Among the mutants obtained with replica plating (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 re-streaked on haft strength MRS plates contain 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 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 we selected three representative strains (R5, as well as R5- and N750-derivatives, R5-18 and N750-1, respectively, with increased neomycin resistance) for further study.

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 Table 1. All three mutant strains grew slower than the wild-type strain at 24 h but after 48 h culture time viable counts of R5 and R5-18 increased again and surpassed that of the wild-type strain (A). In contrast, the viable count of N750-1 after 24 h was declined slightly to the end of culturing period (72 h). In general, mutant strains produced less acid than the wild-type strain and between 24 and 48 h all mutants produced about half of the amount of acid produced by the wild-type strain. However, after 48 h R5 and R5-18 produced nearly the same amount of acid produced by the wild-type strain (B). At the end of the 72 h culturing period only mutant N750-1 produced about half of the amount of acid produced by the wild-type strain. The tendencies observed in acid production were also reflected in the pH values of the culture broths (C). 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 during 24-48 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. Only the culture broth pH of N750-1 was higher than that of the wild-type at the end of the 72 h culturing period. These results suggest that the mutations that made R5 and R5-18 acid sensitive are unstable since the growth characteristics of these mutants after 48 h became similar to that of the wild-type strain.

Table 1. Growth and fermentative characteristics of *L. plantarum* BCC 9546 and its acid-sensitive mutants during 3 days of culture (A-C) in MRS broth

(A) The viable count (log CFU/ml)

Strain	0 h	8 h	24 h	48 h	72 h
Wild-type	7.07 ± 0.14	9.10 ± 0.21	9.21 ± 0.11	8.87 ± 0.16	7.62 ± 0.14
R5	7.08 ± 0.04	8.66 ± 0.08	8.49 ± 0.03	8.18 ± 0.09	8.95 ± 0.10
N750-1	7.23 ± 0.08	9.06 ± 0.03	8.38 ± 0.02	8.19 ± 0.03	8.12 ± 0.02
R5-18	7.19 ± 0.05	9.38 ± 0.01	8.75 ± 0.07	8.30 ± 0.14	8.86 ± 0.14

(B) The total acidity (%)

Strain	0 h	8 h	24 h	48 h	72 h
Wild-type	0.553 ± 0.02	1.043 ± 0.13	2.535 ± 0.11	2.549 ± 0.10	2.540 ± 0.15
R5	0.573 ± 0.01	0.723 ± 0.01	1.243 ± 0.06	1.287 ± 0.03	2.082 ± 0.09
N750-1	0.555 ± 0.00	0.798 ± 0.01	1.157 ± 0.00	1.272 ± 0.00	1.272 ± 0.00
R5-18	0.544 ± 0.01	0.723 ± 0.01	1.157 ± 0.00	1.359 ± 0.06	2.024 ± 0.20

(C) The pH of culture broth

Strain	0 h	8 h	24 h	48 h	72 h
Wild-type	6.13 ± 0.03	4.58 ± 0.24	3.84 ± 0.01	3.85 ± 0.02	3.85 ± 0.03
R5	6.09 ± 0.01	5.47 ± 0.04	4.65 ± 0.01	4.51 ± 0.08	3.98 ± 0.11
N750-1	6.14 ± 0.00	5.01 ± 0.01	4.58 ± 0.00	4.51 ± 0.00	4.48 ± 0.01
R5-18	6.17 ± 0.00	5.14 ± 0.02	4.59 ± 0.00	4.43 ± 0.05	4.05 ± 0.08

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

ATPase activity under acidic conditions

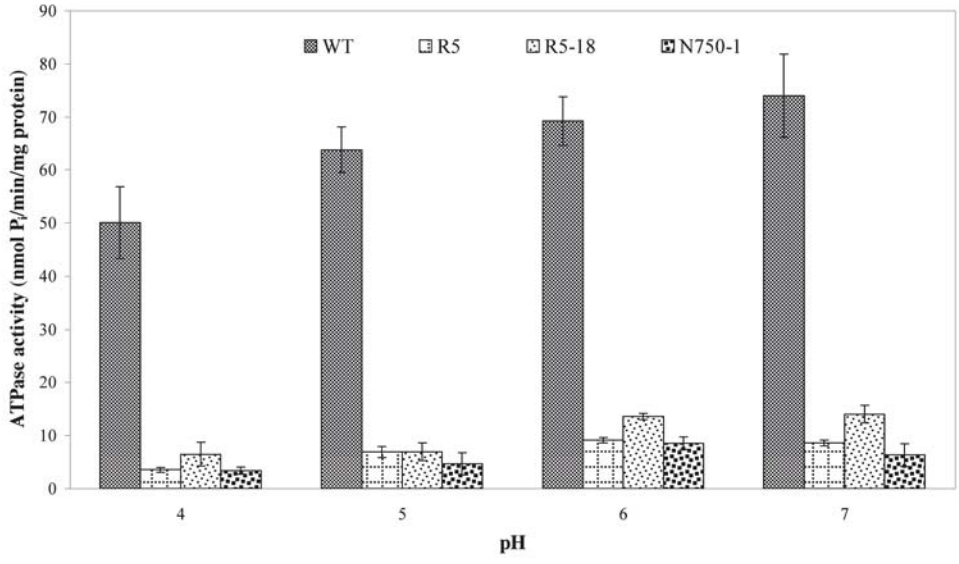

Figure 1. ATPase activity of *L. plantarum* BCC 9546 and its acid-sensitive mutants at different pHs (pH 4-7) in the absence of DCCD.

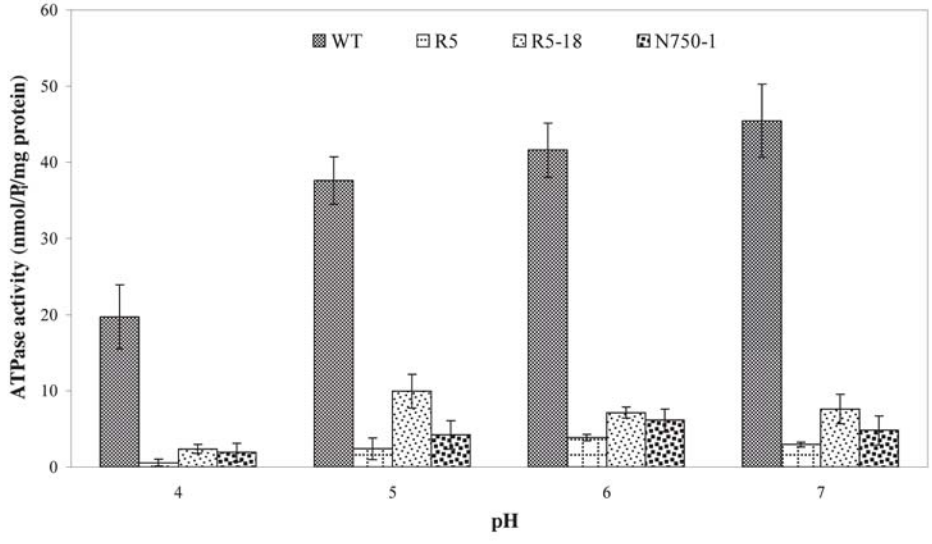

Figure 2. ATPase activity of *L. plantarum* BCC 9546 and its acid-sensitive mutants at different pHs (pH 4-7) in the presence of DCCD.

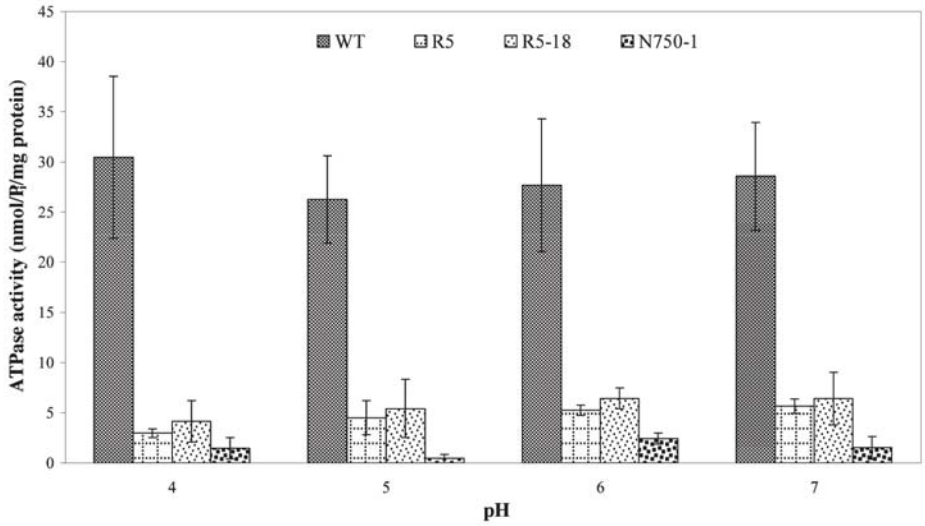

Figure 3. ATPase activity of *L. plantarum* BCC 9546 and its acid-sensitive mutants at different pHs (pH 4-7) inhibited by DCCD.

To investigate the cause of acid sensitivity, the ATPase activity of wild-type and the three mutant strains in different pHs ranging from 4 to 7 were measured. Figure 3 shows the ATPase activity of *L. plantarum* BCC 9546 and its acid-sensitive mutants at different acidic pHs in the presence and in the absence of DCCD, a relatively specific inhibitor of the F_0 part of the F_0F_1-ATPase. The difference in ATPase activity with or without DCCD was also calculated and since it's activity was inhibited by DCCD this value can be considered as the activity of H^+-ATPase. The ATPase activity of all mutant strains were significantly lower than the ATPase activity of wild-type strain at all conditions. At pH 4 the ATPase activity of all strains decreased below the values at other pHs (5-7). Since the H^+-ATPase activities (DCCD inhibited) of the wild-type strain were significantly higher than that of all mutant strains at all conditions, it is possible that the acid sensitivity of all mutants is caused by reduced membrane-bound H^+-ATPase activity. It is postulated that mutants with reduced membrane H^+-ATPase activity do not generate sufficient energy to concentrate neomycin and are resistant to neomycin because neomycin uptake by bacterial cells is energy dependent process (Kanner and Gutnick, 1971). In addition, the H^+-ATPase activity is important for acid tolerance of *L. plantarum* since acid-sensitive mutants of *L. plantarum* have reduced H^+-ATPase activities and are less acid-tolerant than the wild-type strain, which also indicates that the wild-type strain can survive in acidic environment better than the mutant strains. These results also explain why the mutant strains had lower growth ability than the wild-type strain in MRS broth.

Measurement of internal pH

In order to characterize the pH homeostasis of acid-sensitive mutants their internal pH were investigated upon energization with glucose (Figure 4). Internal pH (pH_i) measurements revealed that the mutants had somewhat lower pH_i at external pH 7.0

than that of wild type (Table 3). At acidic condition (pH 5.0) the internal pH of R5 and R5-18 were similar to that of the wild type, while the pH_i of N750-1 was considerably lower than that of the other three strains.

At pH 5.0 the difference between external and internal pH (ΔpH) of the parental strain was 1.3 units, whereas N750-1 could maintain a ΔpH of only 0.85, and it is likely that acidification of the cytoplasm under acidic conditions affect the physiology of the latter strain considerably. Lactic acid is a weak organic acid with increased proportion of its neutral form at low pH and can easily pass the cell membrane in the protonated form, dissociating then at cytoplasmic pH (Kashket, 1987). This results in the disturbance in the internal pH regulation in N750-1, at least, due to its low H^+-ATPase activity, therefore it could not maintain its cytoplasmic pH near neutral at external pH 7.0 as the wild-type strain.

Table 3. Internal pH and proton leak in *L.plantarum* BCC 9546 and its acid-sensitive mutants at extracellular pH 7.0 and 5.0.

Strain	Internal pH		Proton leak*	
	pH 7.0	pH 5.0	pH 7.0	pH 5.0
Wild type	7.39 - 7.40	6.34 - 6.35	0.013	0.018
R5	7.21 - 7.22	6.60 - 6.61	0.034	0.019
R5-18	7.20 - 7.21	6.12 - 6.13	0.009	0.016
N750-1	7.33 - 7.34	5.85 - 5.86	0.021	0.041

* Unit of internal pH decrease in energized cells after a few minutes of DCCD addition, see figure. 4.

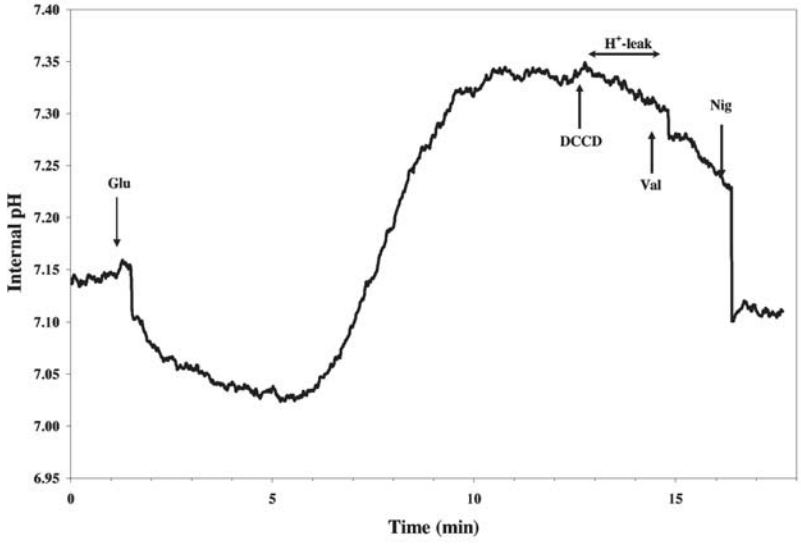

Figure 4. Proton leakage in the mutant, N750-1 at pH 7.0 upon DCCD addition. Val: valinomycin, Nig: Nigericin.

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 Figure 4 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 N750-1 had the highest net proton leak at acidic condition (pH 5.0), corresponding to its H^+-ATPase activity that was the lowest among the tested strains. Since the proton leak of this mutant can be related to a disturbed pH homeostasis and smaller cellular energy level, it is reasonable to assume that it has also a profound impact on the survival of the strain in acidic environment.

Measurement of arginine deiminase activity

The arginine deiminase activity may also contribute to the pH homeostasis so we investigated the ammonium ion production by *L. plantarum* strains.

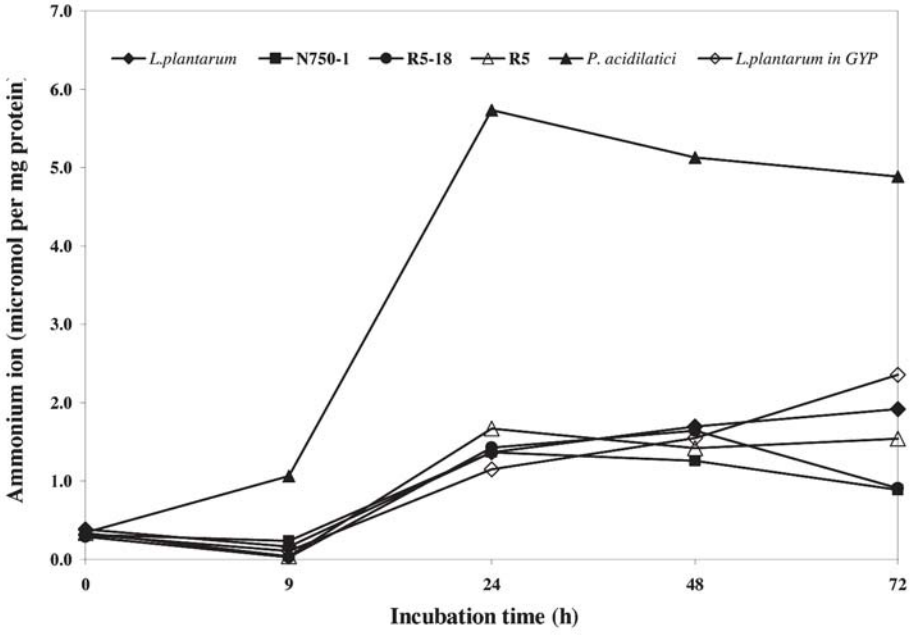

Figure 5. The ammonium ion production by *L. plantarum* and *P. acidilatici* strains in GYP-Arg medium for 72 h at 30°C.

Figure 5 shows that our *L. plantarum* strains produced very small amount of ammonium ion. Their ammonium ion production profiles were not different from that of at 0 h while the culture broth pH of all *L. plantarum* strains were decreased to 4.7 at 9 h without a change outwards until the end of incubation time (data not shown). Besides, these result indicated that cultivation of *L. plantarum* BCC 9546 in Arg-free GYP resulted in nearly the same amount of ammonium ion in the culture broth as in the cases using GYP-Arg medium. The virtually no ammonia production by *L. plantarum* strains is a sharp contrast to the ammonium ion production by the positive control (*P. acidilatici*) which also coupled with culture broth pH increase to pH 8.3 (data not shown). Therefore we conclude that there is a possibility that arginine cannot be metabolized by our *L. plantarum* strains.

Nham fermentation

In this study strain N750-1 was selected because of its lowest H^+-ATPase activity and it also gave the highest pH in MRS broth at 72 h (around pH 4.48). This mutant and the wild-type strain (WT) 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%.

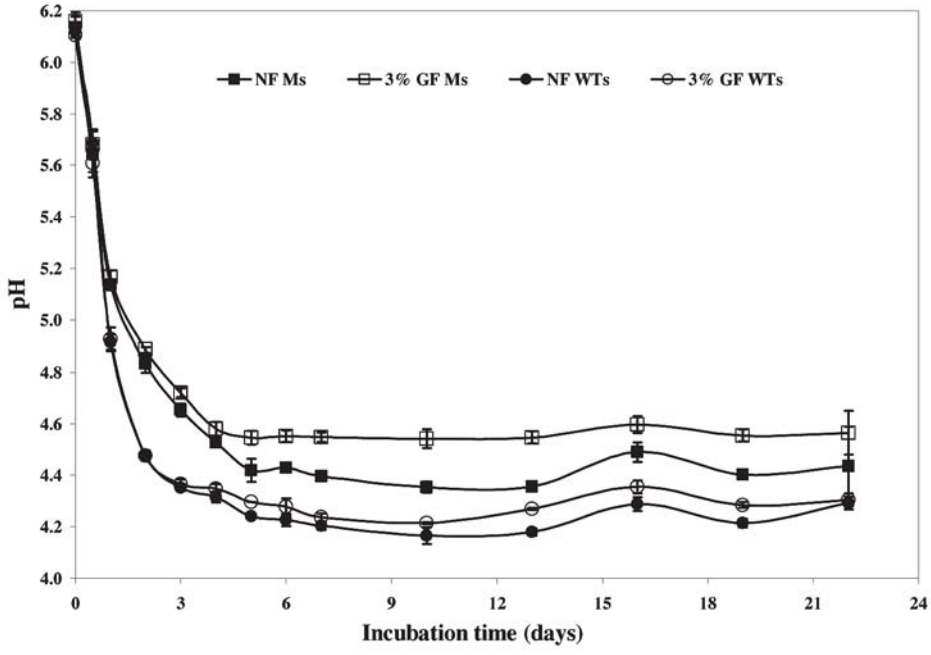

Figure 6. Change in pH of *Nham* during 3 weeks at 30 °C. 3% GF: *Nham* with 3% garlic formulation, NF: *Nham* with 4.3% garlic (normal) formulation, Ms: *Nham* fermented by mutant starter, WTs: *Nham* fermented by wild-type starter.

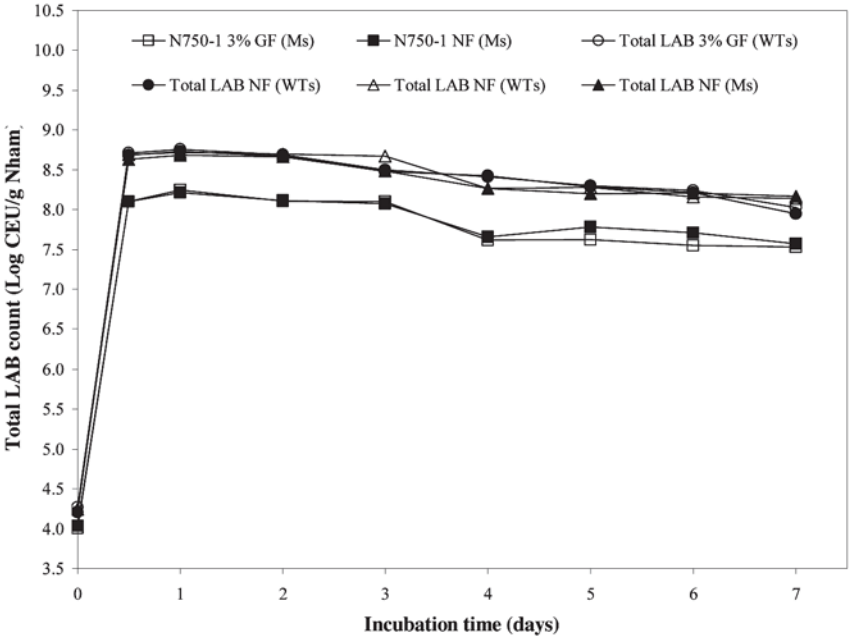

Figure 7. Change in number of total LAB (log cfu/g *Nham*) in *Nham* during 7 days at 30 °C. 3% GF: *Nham* with 3% garlic formulation, NF: *Nham* with 4.3% garlic (normal) formulation, Ms: *Nham* fermented by mutant starter, WTs: *Nham* fermented by wild-type starter.

It is considered that *Nham* with pH lower than 4.6 is safe for consumption (Paukatong & Kunawasen, 2001). *Nham* inoculated with the wild-type exhibited lower pH than that of with mutant in both formulations (Figure 6). The pH was lower than 4.6 within 2 days for both formulations using wild type. In case of the mutant starter the pH gradually decreased to 4.6 within 3 days and 4 days for normal formula and limiting carbon source formula (3% garlic), respectively. The results indicated that using the mutant as starter culture combined with limiting carbon source kept the pH 4.6, which did not change significantly from 4 days at 30 °C. Figure 7 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 10^4 cfu/g *Nham* in all four treatments. The viable count of total LAB and mutant increased until 12 h then the bacterial count of all *Nham* formulations were slightly decreased until the end of incubation time (7 days). However the total LAB count in *Nham* fermentation using the wild-type or mutant starter were a little bit higher than the viable count of the mutant starter culture strain in *Nham.* Our results also revealed that the number of mutant 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 cells play a major (or dominant) fermentative role.

Changes in Nham color

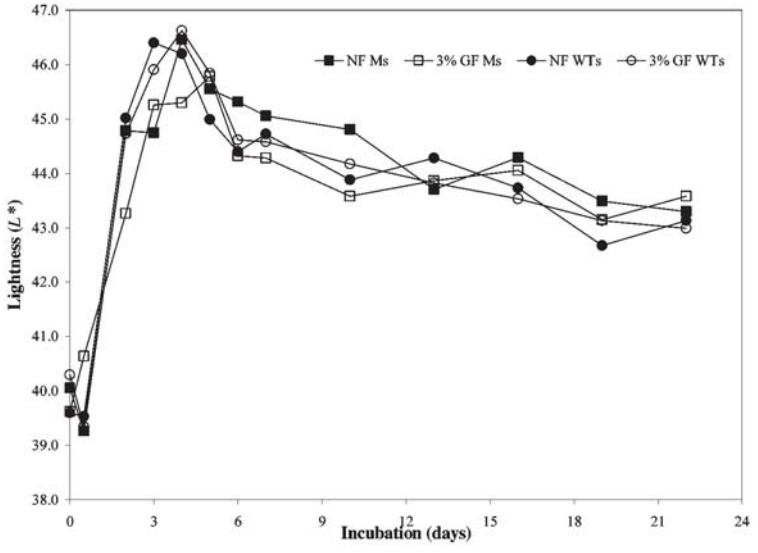

Figure 8. Changes in lightness (*L**) of *Nham* at 30 °C for 3 weeks. 3% GF: *Nham* with 3% garlic formulation, NF: *Nham* with 4.3% garlic (normal) formulation, Ms: *Nham* fermented by mutant starter, WTs: *Nham* fermented by wild-type starter.

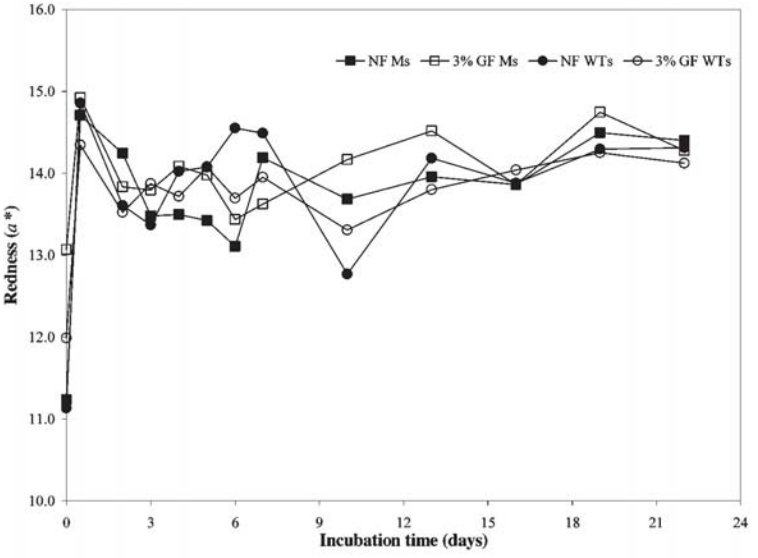

Figure 9. Changes in redness (*a**) of *Nham* at 30 °C for 3 weeks. 3% GF: *Nham* with 3% garlic formulation, NF: *Nham* with 4.3% garlic (normal) formulation, Ms: *Nham* fermented by mutant starter, WTs: Nham fermented by wild-type starter.

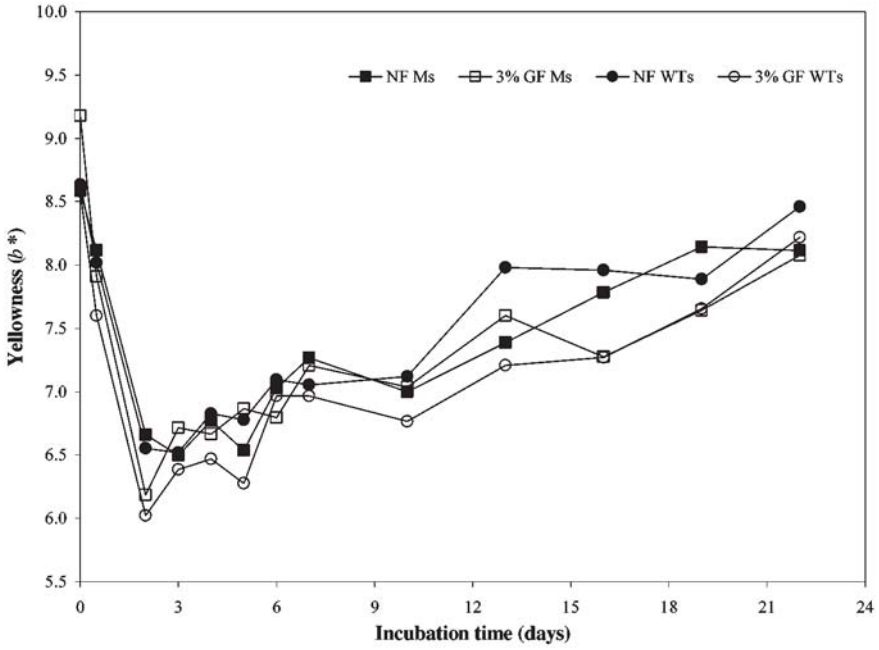

Figure 10. Changes yellowness (*b**) of *Nham* at 30 °C for 3 weeks. 3% GF: *Nham* with 3% garlic formulation, NF: *Nham* with 4.3% garlic (normal) formulation, Ms: *Nham* fermented by mutant starter, WTs: *Nham* fermented by wild-type starter.

The results show that *L** values (lightness) increased to a maximum at 24 h before remaining relatively constant thereafter and no significant differences between *Nham* fermented using wild-type and mutant starter. Redness (*a**) increased somewhat during the fermentation preriod with no significant differences between *Nham* fermented using wild-type and mutant starter. *b** values or yellowness slightly decreased during the first 24 h but at the end of fermentation time (22 days) the yellowness of all *Nham* (Figure 10) was almost the same as at 0 h ($P>0.05$).

Released water

Table 5. Released water (%) of *Nham* during fermentation at 30 °C for 3 weeks.

Time (day)	Mutant-normal	Mutant-3% G	WT-normal	WT-3% G
3	0.83 ± 0.15 a	1.01 ± 0.08 a	0.75 ± 0.10 a	1.69 ± 0.12 b
4	1.29 ± 0.03 a	1.15 ± 0.06 a	1.85 ± 0.06 b	1.85 ± 0.17 b
5	1.75 ± 0.17 b	1.38 ± 0.07 a	3.50 ± 0.07 d	3.13 ± 0.07 c
6	3.09 ± 0.35 ab	2.67 ± 0.11 a	3.68 ± 0.02 c	3.29 ± 0.05 bc
7	3.41 ± 0.43 a	2.70 ± 0.09 a	4.58 ± 0.15 b	4.47 ± 0.47 b
10	6.09 ± 0.34 a	5.62 ± 0.20 a	5.41 ± 0.15 a	5.65 ± 0.52 a
13	7.28 ± 0.08 a	6.75 ± 0.30 a	7.73 ± 0.10 a	7.63 ± 0.38 a
16	7.36 ± 0.11 a	7.16 ± 0.14 a	8.50 ± 0.58 a	7.82 ± 0.46 a
19	9.57 ± 0.11 b	8.87 ± 0.14 a	9.13 ± 0.07 a	9.90 ± 0.07 c
22	9.88 ± 0.06 b	9.47 ± 0.23 a	10.25 ± 0.07c	10.34 ± 0.16 c

Mean values and standard deviations with different letters in the same row indicate significant differences ($P < 0.05$).

Nham fermented with the wild-type strain had significantly higher amounts of released water than *Nham* fermented using the mutant from day 3 to day 7. An increase in released water was presumably caused by denaturation of proteins during fermentation. Given that *Nham* fermented with mutant as starter resulted in less acid production compared to *Nham* fermented with wild-type, it is likely the reason why mutant fermented *Nham* had significantly lower amounts of released water than the by wild type. However after 7 days *Nham* fermented with the mutant released water nearly as much as *Nham* fermented by the wild type but in *Nham* containing 3% garlic fermented with the mutant had the lowest amount of released water until the end of fermentation.

Determination of biogenic amines

Biogenic amines (BA) are compounds commonly presented in living organisms in which they are responsible for many essential functions and they can also be produced in high amounts by microorganisms through the activity of amino acid decarboxylases. High microbial counts, which characterize fermented foods, often unavoidably lead to considerable accumulation of biogenic amines. In general, histamine, putrescine, cadaverine, tyramine, tryptamine, 2-phenylethylamine, spermine and spermidine are the most important BA in foods (Shalaby, 1996). Excessive consumption of these amines could cause nervous, gastric, intestinal, and blood pressure problems (Suzzi & Gardini, 2003). Therefore the biogenic amines in *Nham* produced with different starter cultures were measured (Table 6).

Table 6. Biogenic amine concentrations in *Nham* during fermentation at 30 °C for 3 weeks.

Time (day)	Mutant-normal	Mutant-3% G	WT-normal	WT-3% G
0	18.74 ± 1.38 a	17.56 ± 2.49 a	24.09 ± 24.09 a	18.26 ± 1.54 b
2	183.16 ±16.45a	125.91 ± 13.68 b	104.41 ± 8.74 b	197.91 ±19.03 a
3	194.20 ± 1.63 b	144.76 ± 12.10 c	120.21 ± 9.79 ab	131.01 ± 6.06 a
5	326.14 ± 37.27 b	266.85 ± 45.72 b	155.26 ± 26.32 a	137.84 ± 4.46 a
7	440.57 ± 36.45 c	357.84 ± 37.36 d	141.37 ± 10.07 b	227.86 ± 10.96 a
10	947.12 ± 5.03 c	636.12 ± 52.71 d	191.99 ± 58.09 b	409.58 ± 9.75 a
13	991.05 ± 82.65 b	727.23 ± 74.73 c	170.98 ± 0.99 a	267.56 ± 2.10 a
16	721.34 ± 80.34 b	766.02 ± 3.94 b	173.63 ± 10.59 b	694.89 ± 39.85 a
19	1084.55 ± 114.72 b	754.58 ± 49.99 c	195.28 ± 8.57 a	342.24 ± 19.01 a
22	405.20 ± 39.86 a	876.91 ± 77.05 a	284.11 ± 49.23 a	403.81 ± 3.17 a

Mean values and standard deviations with different letters in the same row indicate significant differences ($P < 0.05$).

Tryptamine and 2-phenylethylamine were not detectable in all formulations (data not shown). Total biogenic amine concentrations increased during the test period until the 22nd day and *Nham* fermented with mutant starter had significantly higher total biogenic amine content than *Nham* fermented with wild-type (Table 6). Histamine was not detectable in *Nham* fermented with wild-type, while it was present in *Nham* fermented with mutant (data not shown). However, histamine content of *Nham* fermented with mutant was 10.57 mg/kg when product reached the pH of 4.6 (3 days) in normal formulation and 7.23 mg/kg in 3% garlic formulation. Nout (1994) pointed out that histamine contents could be in the range of 50-100 mg/kg in sausage processed according to ''Good Manufacturing Practice (GMP)'. Even though *Nham* fermented by mutant had some histamine content it was lower than that allowed by GMP until 7 days of fermentation (data not shown). Tyramine, putrescine and cadaverine were the most abundant BA in *Nham* (data not shown). Higher amounts of cadaverine were found in *Nham* fermented by mutant than by wild-type, while spermine and spermidine were found at constantly low levels in all the samples. That agrees with the already published data and with the hypothesis that these latter polyamines are not formed by bacterial decarboxylation of amino acids during fermentation but they are naturally present in the meat used as raw material (Bardócz, 1993; Hernández-Jover et al., 1997).

High amount of biogenic amines can be found in fermented products derived from raw materials with high protein content. The microbial growth often leads to high concentrations of biogenic amines (Silla Santos, 1996). Several reports about BA in fermented sausages from retail market showing that a wide variety of amine contents can be found (Tiecco et al., 1985; Santos-Buelga et al., 1986; Bauer et al., 1989). Formation of biogenic amines in food generally occurs due to decarboxylation of amino acids by microbial substrate-specific enzymes. The production of biogenic amines is a characteristic of several groups of microorganisms such as enterobacteriaceae, *Pseudomonas* spp., micrococcaceae, enterococci, and LAB (Halász et al., 1994). Therefore, biogenic amines are also being concerned to be related to food spoilage because they were come from decarboxylase activity of spoilage microflora during the storage of food (Vidal-Carou et al., 1990). Considering all these and our findings we can

conclude that although biogenic amine content in mutant fermented was higher than in wild-type starter fermented Nham, their concentrations are still in the acceptable limits (at 7 days of fermentation) for human consumption.

Conclusions

This study's goal to modify the starter *L. plantarum* strain to obtain a mutant, with reduced H^+-ATPase activity was successfully reached (N750-1) and the mutant was applied in *Nham* production. Limiting carbon source (3% garlic concentration) combined with the acid-sensitive mutant as starter culture has a potential for use in *Nham* production to prevent over-fermentation.

Acknowledgements

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