Relation between free Amino Acids and the Biogenic Amines Contents in Green Tomatoes inoculated with *Lactobacillus plantarum*

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Abstract

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The aim of the work presented here was the evaluation of the profile of free amino acids as the possible precursors of biogenic amines (BAs) in tomatoes and its changes during tomato ripening. Hygienically significant amounts of BAs can be formed by the action of amino acid decarboxylases provided the tomatoes contain a high enough amount of free amino acids. In view of this, the effect was studied of *Lactobacillus plantarum* strains 976H and 3626 on the BAs formation in green tomatoes. Homogenates of green tomatoes were adjusted to the model conditions of conservation by the addition of NaCl to the concentration of 1.5% (w/w) and of glucose to the concentration of 2.0% (w/w). The homogenates were subsequently inoculated with the two strains given above. The formation of BAs was monitored for 186 h and compared with that in the control non-inoculated samples. The changes in the BAs content taking place during fermentation were estimated by means of statistical methods. It was found that, during fermentation of green tomatoes with the two strains of *Lactobacillus plantarum* used, the conditions were not fulfilled that enable BAs formation, i.e. as sufficient amount of free amino acids together with a sufficient production of decarboxylases.

Keywords: tomato; biogenic amine; free amino acids; *Lactobacillus plantarum*

Dietary biogenic amines are interesting from several aspects. They rank among potential indicators of bacterial spoilage and, as such are studied in foods from the hygienic point of view. Their function in human organism is also known (protein synthesis and nucleic acids regulation, blood pressure control, membrane stabilisation). Their high concentrations can cause toxic defects whose knowledge is important from the viewpoint of the foodstuff health security (Davídek 1995; Greif *et al.* 1999).

The absolute majority of the foodstuff biogenic amines (BAs) originate from decarboxylation of free amino acids which is catalysed by bacterial decarboxylases. It is important to take into account the accessibility of free amino acids, the occurrence of bacteria containing amino acid decarboxylases and the conditions of their growth including biosynthesis of decarboxylases and their activity (Křížek & Kalač 1998; Kalač *et al.* 2000; Standara *et al.* 2001). That is why biogenic amines are supposed to occur almost in all foods which contain proteases or free amino acids and which are subject to the conditions enabling microbial and biochemical activity.

The objective of this work has been to evaluate the relationship between free amino acids and the occurrence of BAs in green tomatoes subjected to lactic acid fermentation. The lactic acid fermentation of green tomatoes is a promising method

for the steroid glycoalkaloid degradation in the technological treatment of tomatoes (Preiss *et al*. 1996; Veselá 2001). The work has been carried out within the framework of the research of the possibility of healthy food production.

MATERIALS AND METHODS

Tomatoes. The green fruits of Tornado variety were harvested together with ripe red fruits at one week intervals during September and October 2000. After the harvest the fruits were stored in a freezer at the temperature of –18°C and processed continually.

Lactic bacteria. Cultures used during the work came from the MILCOM, a. s., collection (*Lactobacillus plantarum* 976H) and from the Czech Collection of Microorganisms, Brno (*Lactobacillus plantarum* 3626). Freeze-dried cultures of lactic bacilli sealed in glass vials were inoculated into test tubes containing 9 ml of Lactobacillus MRS Broth nutritive medium (Hi Media Lab. Bombay, India). Cultivation lasted three days at the temperature of 37°C. The cultures with the initial concentration of *Lactobacillus plantarum* 976H 1.4 × 10^8 CFU/ml and of *Lactobacillus plantarum* 3626 1.8 × 10^8 CFU/ml, respectively, were used for the inoculation of green tomatoes.

Lactic fermentation. Green tomatoes (4.5 kg) without any visible physiological and/or microbial damage were homogenised. The homogenate was supplemented with D-glucose and NaCl to make the concentrations 2% (w/w) for D-glucose and 1.5% (w/w) for NaCl was subsequently divided into three portions. The first portion was the control, *Lactobacillus plantarum* 976H was added to the second one and *Lactobacillus plantarum* 3626 was added to the third portion (40 ml of the inoculate, 1.4 × 10^8 CFU/ml). The mixtures were placed into 3l Erlenmayer flasks, closed with cotton stoppers, and left to ferment at the temperature of 25°C for 186 h. The experiment was carried out three times.

Determination of free amino acids

Sample preparation. 10 g of the homogenised sample was weighed and transferred quantitatively into a 50 ml graduated flask with 30 ml of lithium buffer (pH 2.2). The flask was kept in an ultrasound bath for 15 min, filled with solvent buffer up to the mark and the sample was filtered so that the amino acids could be perfectly scattered into the solution. The pure sample prepared was drawn into a 400 μl cassette of the automatic sampler of an amino acid analyser.

Liquid chromatography. The amino acids were determined in the system of 5 lithnocitrate buffers with increasing pH values (2.8 to 4.6) at two temperature programs (38.5°C and 58°C) on a column (26.0 × 0.37 cm) of OSTION LG FA ion exchanger (SPCHV Ústí n. L.) using the automatic Mikrotechna 339 amino acid analyser (Mikrotechna, Prague). The photometric detection at 520 nm was used after the derivatisation by the ninhydrine agent, the duration of analysis was 305 min. The results were evaluated in CSW employing PC. Free amino acids were identified by the comparison of the individual peak retention times in the sample with those of the peaks of the standard amino acid solution. After the elimination of outer effects (e.g. aging of the agent), the standard in the ratio of 2:1 of the sample benefit was integrated into the series.

Biogenic amine determination

Sample preparation. 200 g of sample was homogenized in a kitchen mixer (5 min at 2nd degree). 10 g of the homogenate was weighed with the accuracy of 0.01 g. The sample was extracted in the mixer with 40 ml of 5% trichloro acetic acid 3 times for 5 min. Afterwards, it was transferred into a 100 ml volumetric flask and the mixer was rinsed twice with 20 ml of extract solution and the flask was filled up to the mark. After cooling to 3–4°C (eventual fat removing), the sample was centrifuged at 10 000 g for 10 min. The part of supernatant under the top layer (2–3 mm thick) was cleaned via membrane filter (Synpor 0.45 μm). The filtrate was ready for the chromatographic analysis either directly or after the dilution with the dosing buffer.

Liquid chromatography. Biogenic amines, such as histamine (HA), tyramine (TA), putrescine (PU), cadaverine (CD), tryptamine (TR), agmatine (AG), spermidine (SD) and spermine (SM) were determined by ion exchange liquid chromatography using OSTION LG ANB (column 0.37 × 6.0 cm, automatic amino acid analyser Mikrotechna 339 M, Prague) in the system of sodium/potassium citrate buffers after post-column ninhydrine derivatisation. The content of the total N-substances was determined using Kjel-Fos apparatus.

For the individual BAs, the limits of determinability varied from 1.0 mg/kg (e.g. PU or TA) to

3.0 mg/kg in TR. The relative standard deviations (RSD, %) in the individual analytes decreased on the monitored levels with the increasing concentration of the analyte: HA (14 up to 7%), TA (to 4%), SD (12 to 6%), CD (6 to 2%), TR (15 to 5%), SD (7 to 4%) and SM (15 to 6%). The yields varied in the samples of fermented vegetables at the levels of tens mg/kg from 78.5% in AG to 101.6% in CD.

The resultant BAs contents were tested by statistical methods. The main statistic characteristics (RSD) were calculated and the significance of the difference between two mean values of the independent complexes was tested (Student's *t*-test).

RESULTS AND DISCUSSION

Almost all twenty amino acids except proline and tryptophane were found in both green and ripe tomatoes. γ-Amino butyric acid (GABA) and an increased content of ammonia were detected (even after the comparison with ammonia from other sources). Glutamine represented the main component of the amino acid profile in green tomatoes. GABA, non-protein amino acid, represented the second most widely occurring free amino acid in green tomatoes. The relative contents of some amino acids, e.g. isoleucine or phenylalanine, dropped while the relative contents of some others, especially glutamic acid, increased. In the groups of green and of ripe tomatoes, the determined characteristic differences in free amino acid contents referred especially to glutamic acid and glutamine. Glutamic acid in ripe fruits represented the main component of the amino acid profile. Figure 1 shows the record of the chromatographic separation of free amino acids in green tomatoes and the amino acid standard. This is contrasted by the record of the free amino acid separation in green and in ripe tomatoes in Figure 2. Table 1 represents the data on free amino acid concentrations in green and in ripe tomatoes of the Tornado variety.

During the tomato development, approximately 70% of all amino acids in the pulp was represented by the glutamate family. While γ-amino butyric acid and glutamine prevailed in green tomatoes, the concentrations of glutamine, glutamate and aspartic acid increased significantly during the process of the tomato ripening. The data obtained with the Tornado cultivar ripening are in agreement with the data given in literature (Valle *et al.* 1998; Boggio *et al.* 2000). The accumulation of free glutamic acid in tomato fruits is positively the result of the ripening process. Therefore, considering the given variety, we can take its concentration in the fruits as a marker of the ripening process.

The results obtained by means of the automatic amino acid analyser were compared with the total N-substance content as detected with Kjel-Foss apparatus. It showed that, from the point of view of the distribution, more than 70% of the nitrogenous substances were contained in tomato juice. Therefore, the free amino acid profile was monitored also in this matrix. We found a profile

Table 1. The concentration of free amino acids in samples of green and ripe tomatoes

Amino acid	Abbreviation	Amino acid content (mg/kg)	
		green tomatoes	ripe tomatoes
Asparagine	Asp	31 ± 8	789 ± 57
Threonine	Thr	37 ± 12	77 ± 17
Serine	Ser	62 ± 5	118 ± 9
Glutamic acid	Glu	91 ± 17	2290 ± 64
Glutamine	Gln	806 ± 23	1540 ± 125
Alanine	Ala	93 ± 4	96 ± 6
Isoleucine	Ile	24 ± 2	9 ± 3
Leucine	Leu	35 ± 10	25 ± 5
Phenylalanine	Phe	53 ± 10	68 ± 13
γ-Amino-*n*-butyric acid	GABA	941 ± 150	648 ± 128
Ammonia		230 ± 21	142 ± 15
Lysine	Lys	26 ± 8	58 ± 17
Histidine	His	14 ± 5	82 ± 6

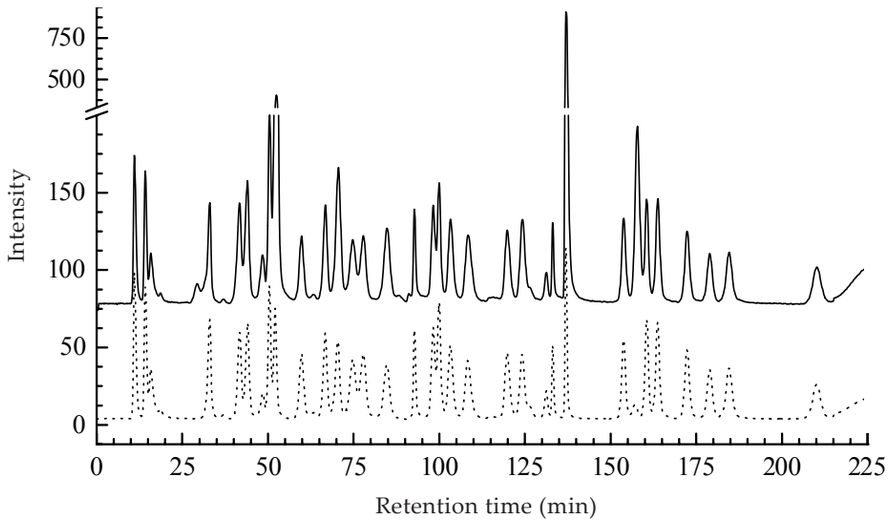


Figure 1. Chromatographic spectrum of green tomato sample (solid line) and of commercially available standard of amino acids (dotted line), retention times in minutes: 11.07 – cysteine, 14.24 – taurine (tau), 15.85 – phosphatidylethanolamine, 18.65 – urea, 33.05 – asparagine (asp), 37.13 – hydroxyproline (HO-pro), 41.79 – threonine (thr), 44.13 – serine (ser), 48.5 – asparagine (asn), 50.48 – glutamic acid (glu), 52.15 – glutamine (gln), 59.87 – α-aminoadipic acid, 63.3 – proline (pro), 66.7 – glycine (gly), 70.4 – alanine (ala), 74.7 – citrulline (cit), 77.8 – α-aminobutyric acid (α-ABA), 84.71 – valine (val), 92.7 – cysteine (cys), 98.2 – methionine (met), 100 – cystathionine, 103.2 – isoleucine (ile), 108.3 – leucine (leu), 119.8 – tyrosine (tyr), 124.2 – phenylalanine (phe), 131.2 – β-alanine (β-ala), 133.1 – β-aminobutyric acid (β-ABA), 137 – γ-aminobutyric acid (GABA), 153.8 – ethanolamine (EA), 157.1 – ammonia (NH_3), 160.5 – ornithine (orn), 163.7 – lysine (lys), 172.3 – histidine (his), 178.9 – 1-methylhistidine, 184.6 – 3-methylhistidine, 210.1 – arginine

similar to that in the ripe fruits with the highest levels of glutamic acid and glutamine. However, the free amino acid concentration was by 20% lower than that in the fresh fruits. The presence of peptides as well as of other ninhydrine-positive substances was also detected in both matrix samples. Unlike in the fresh fruits, the monitored matrix showed a significantly higher peptide concentration, probably due to the partial protein hydrolysis.

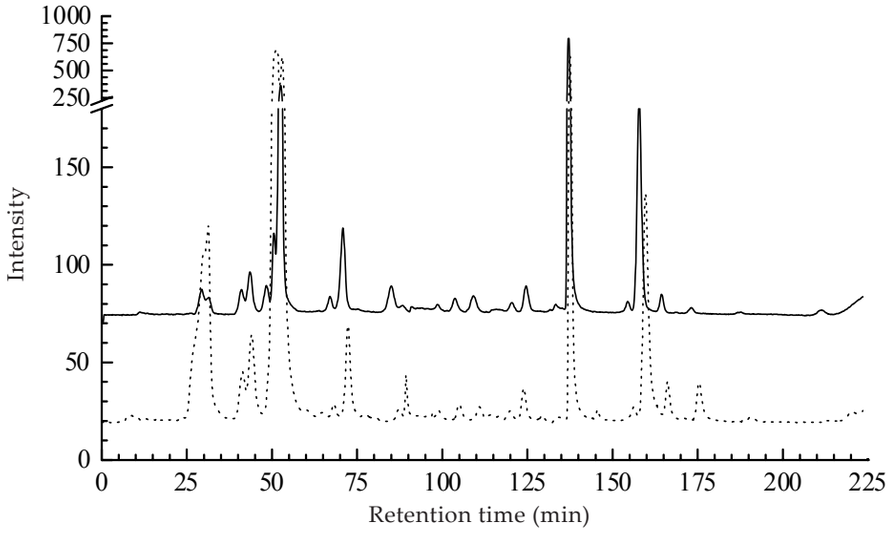


Figure 2. The chromatograms comparing green tomato sample (solid line) and ripe tomato sample (dotted line)

The aim of the work was to evaluate the profile of free amino acids as BAs precursors with respect to the occurrence of amino acid-decarboxylases in tomatoes, namely in the dependence on the production of free amino acids in the course of tomato ripening.

Considering in details the published knowledge on the amino acid biosynthesis in fruits, our work did not observe the enzyme activity catalysing N-substances and the activity of amino-decarboxylases, synthetases of amino acids, and proteases.

Biosynthesis of amino acids in fruits is an enzymic process which is catalysed mainly by synthetases (e.g. glutamine synthetase). In green tomatoes, glutamine synthetase which catalyses the synthesis of glutamine from glutamate, ATP, and ammonia was found. Other amino acid metabolising enzymes were detected during the process of fruit ripening, e.g. aspartate amino transferase and, especially, such decarboxylases as histidine decarboxylase, glutamate decarboxylase and arginine decarboxylase, the highest levels of which are found in the early stages of the fruit ripeness. These decarboxylases may cause the production of hygienically significant contents of BAs in the case of sufficiently high levels of relevant free amino acids as precursors (Picton *et al.* 1993). With respect to the presence of the given decarboxylases in ripening tomatoes, the profile of biogenic amines was monitored in the samples. We concentrated on those BAs the production of which is enabled either by the presence of the relevant decarboxylases (histidine-, arginine-, glutamine-decarboxylase) or by the microflora present during fermentation.

The evaluation of BAs production in green tomatoes during fermentation using the given strains must take into account the fact that green tomatoes contain natural levels of BAs which are not connected with microbial activity, and also the possibility of the BAs production by the activity of the contaminating microflora. Biogenic amines: histamine (HA), tyramine (TA), putrescine (PU), cadaverine (CD), tryptamine (TR), agmatine (AG), spermidine (SD) and spermine (SM) in green tomatoes were determined by liquid chromatography. The necessity of a study on the effect of *Lactobacillus plantarum* strains 976H and 3626 on the BAs production in green tomatoes emerged from the standpoint mentioned above. The production of BAs in the samples inoculated with the cultures of the strains under study was monitored during 186 h and compared with the group of the original homogenate and the group of spontaneous fermentation complemented with NaCl to 1.5% and glucose to 2.0% to create the conditions of conservation. The mean values determined in the monitored complex of samples: HA 7.1 (2.8 –10.7), TA 8.7 (ND – 17.4), PU 11.9 (6.8–18.1), CD 1.7 (ND – 10.2) mg/kg. TR, AG, SD and SM levels were below the detection limits. The determined values correspond to the literary data on the BAs contents in green tomatoes (Askar 1979; Suhaj & Kováč 1996; Velíšek 1999).

On the addition of the cultures, the production of HA was significantly decreased ($P \leq 0.05$). The comparison of the both cultures showed that HA level was more efficiently influenced by the *Lactobacillus plantarum* 976H culture. HA concentration was influenced mainly during the initial

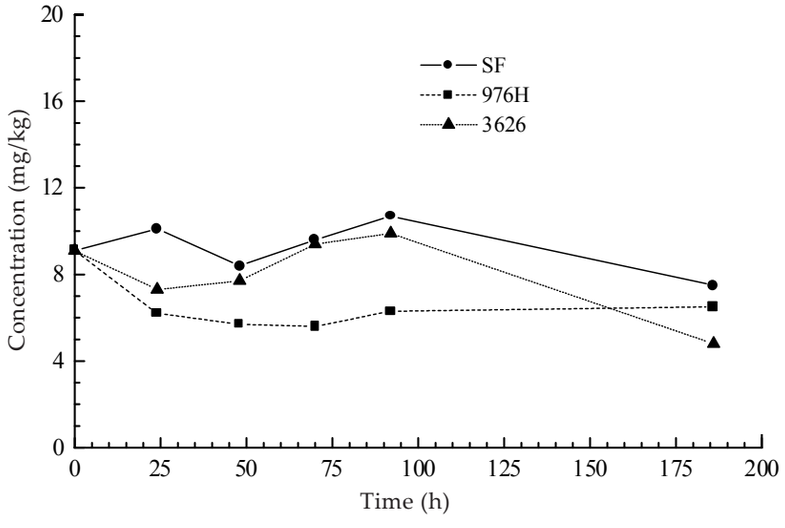
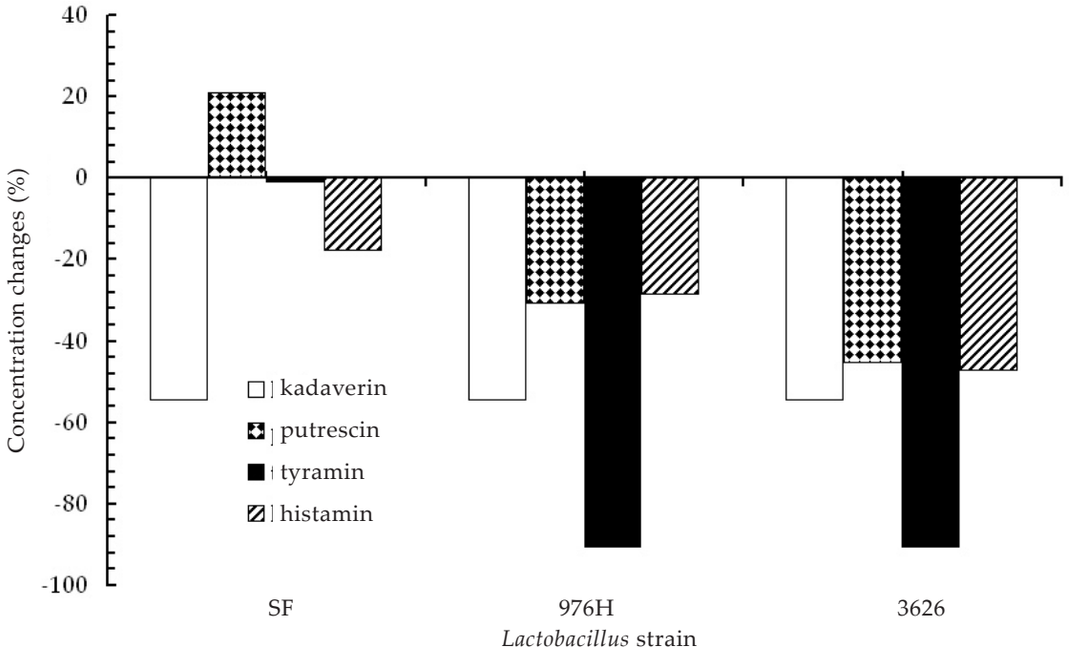


Figure 3. The concentration of histamine during fermentation – SF = spontaneous fermentation (control sample)



SF = spontaneous fermentation (control sample)

Figure 4. The concentration changes after 186 hours of fermentation for selected biogenic amines. The changes are expressed as $-(c(0) - (t))/c(0)$

24 hours of cultivation (Figure 3). HA concentration decreased in all groups during fermentation and reached its lowest values at the end of the experiment (Figure 4). In contrast to the reports of other authors (Greif *et al.* 1999), the drop of HA production was recorded even in the final phase of the spontaneous fermentation. The absolute value of the drop of HA concentration was greater than the reliability interval of the method. The drop might be explained by the growth of lactobacilli, epiphytic microflora and, consequently, by the conservation effect of lactic acid produced during the spontaneous fermentation.

The effect of the cultures on TA production was similar as in the case of HA. At the end of fermentation, a significant drop of TA concentration occurred (Figure 4).

Unlike the observed changes of HA and TA concentrations, PU level in tomato samples reached its maximum after 70 to 90 h of fermentation of the whole course of 186 h (Figure 5). The impact of the both lactobacilli cultures on the decrease of the amine levels was significant even in the case of PU production ($P \leq 0.5$). However, it was not sufficient enough to prevent the rise of the levels during fermentation. At the end of the experiment (186 hours), PU levels were lower in the samples with the inoculants than its levels in the original homogenate at the beginning of the experiment. In the spontaneously fermented samples, the concentrations of PU were statistically significantly higher ($P \leq 0.5$) during the whole experimental period. PU increase during the spontaneous fermentation of a vegetable (sauerkraut) was monitored before (Greif *et al.* 1999). Unlike in our work, PU was produced especially during the initial phase of fermentation.

Higher levels of CD were found in the control samples than in those with the inoculants. However, the differences were not significant. CD concentration dropped in all groups during the experiment and so, at the end of the experiment, traces only of CD were found or it was not detected at all.

The BAs contents detected in fermented green tomatoes are comparable with BAs contents in the ripe tomatoes (Suhaj *et al.* 1996) and they are close to the Asian fermented vegetables and soya products if compared with various kinds of fermented vegetables. During the spontaneous fermentation, the increase in TA and especially PU contents during the beginning of fermentation is contributed to the effect of the contaminating microflora which

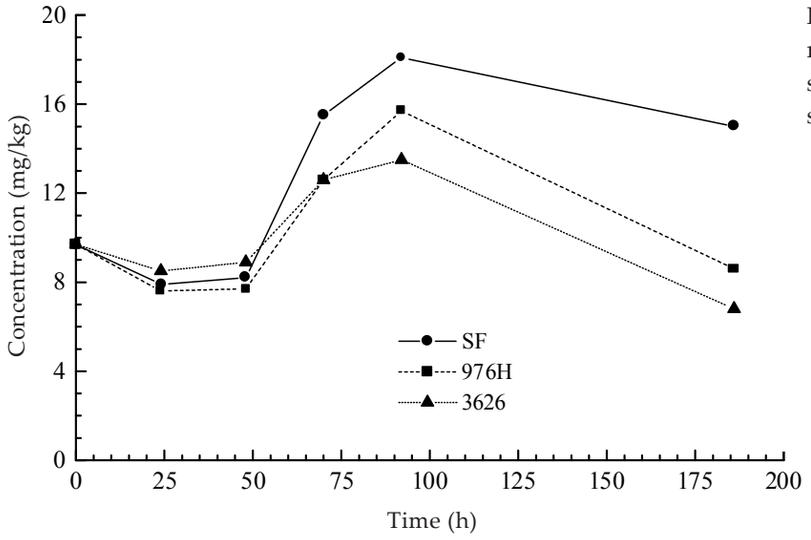


Figure 5. The concentration of putrescine during fermentation – SF = spontaneous fermentation (control sample)

is gradually suppressed during the fermentation both by the lactic bacteria present and their primary metabolite – lactic acid causing the drop of pH. The phenomenon can also be attributed to the possible production of bacteriocine (the fermentation is usually terminated by the strains of *Lactobacillus plantarum*).

In the study presented here it was found that, in the green tomato fermentation employing the selected strains of lactic bacteria, the conditions for the BAs production are not fulfilled from the point of view of the technological microflora.

Apart from the possible relatively negative effects (production of BAs), there are also positive aspects of *Lactobacillus plantarum* application. That is why it finds its commercial applications especially in starting cultures. Lactic fermentation of carbohydrates results in the lactic acid production and thus in a drop of pH which causes a conservation effect – the inhibition of acid-sensitive germs. The inhibition of pathogenic and decarboxylase producing microflora can possibly explain the decrease of the BAs production after the addition of *Lactobacillus plantarum* cultures in tomatoes under condition that the bacteria themselves are not decarboxylase positive.

In agreement with our previous publications, we can state that the industrial application of a suitable *Lactobacillus plantarum* starter culture selection can lead to the production of healthier foodstuffs (Veselá & Drdák 2000). This is caused by the reduction of BAs levels together with the levels of steroid glycoalkaloids and nitrates (Karovičová *et al.* 1999).

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Souhrn

Veselá M., Drdák M., Standara S. (2003): **Vztahy mezi obsahem volných aminokyselin a biogenních aminů v zelených rajčatech inokulovaných bakteriemi** *Lactobacillus plantarum*. Czech J. Food Sci., **21**: 51–58.

Vyhodnocovali jsme profil volných aminokyselin rajčat jako možných prekurzorů biogenních aminů zejména při změně profilu volných aminokyselin během zrání. Enzymy dekarboxylasy mohou způsobit tvorbu hygienicky významného množství biogenních aminů, pokud je v rajčatech přítomno velké množství volných aminokyselin. Z tohoto hlediska byl studován účinek mléčných bakterií *Lactobacillus plantarum* kmenů 976H a 3626 na tvorbu biogenních aminů v zelených rajčatech. Homogenizovaný vzorek zelených rajčat byl inokulován těmito kulturami. Koncentrace NaCl v homogenizovaném vzorku byla upravena na 1,5 % hm. a glukózy na 2 % hm. s cílem modelovat podmínky konzervace. Tvorba biogenních aminů byla sledována během 186 hodin fermentace také na kontrolním vzorku, který nebyl inokulován. Změny koncentrací biogenních aminů během fermentace byly statisticky posouzeny a vyhodnoceny. Zjistilo se, že při fermentaci zelených rajčat použitými mléčnými bakteriemi nebyly z technologického hlediska splněny všechny podmínky pro vznik biogenních aminů. To znamená, že ve vzorcích zelených rajčat nebyl dostatečný obsah volných aminokyselin a zároveň nebyly produkovány enzymy dekarboxylasy v takovém množství, které by mohlo způsobit vznik biogenních aminů v hygienicky významném množství.

Klíčová slova: rajčata; biogenní aminy; volné aminokyseliny; *Lactobacillus plantarum*

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