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EFFECT OF PARA-AMINOBENZOIC ACID MILK SUPPLEMENTING ON THE AMINO ACID PROFILE OF SEMI-HARD CHEESE

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Abstract. The objective of this work is to analyze the cheese amino acid profile through the ripening period. The evaluation of the effect of Para-aminobenzoic acid (PABA) on the proteolytic activity of *Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris*, and *Streptococcus salivarius subsp. thermophilus*, allows to deduce their vitality during the ripening period within 28 days. Four cheeses have been prepared and analyzed (a) control cheese, (b) cheese with 0.1 g/l PABA, (c) cheese with 0.2 g/l PABA and (d) cheese with 0.4 g/l PABA – in two stages: t_0 (beginning of ripening period) and t_1 (end of ripening period). The protein content was measured by Kjeldahl method and the amino acid profile was done by High-Performance Liquid Chromatography (HPLC). In the cheeses with 0.1 and 0.2 g/liter (PABA), significantly higher contents of most amino acids were measured compared to those for the control cheese. In the cheese samples with 0.4 g/liter PABA all amino acids were significantly higher in quantity than those in the control cheese.

***Keywords*:** Para-aminobenzoic acid (Vitamin B10), food biotechnology, semi-hard cheeses, starter culture bacteria, amino acid profile

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ВЛИЯНИЕ ДОБАВЛЕНИЯ ПАРААМИНОБЕНЗОЙНОЙ КИСЛОТЫ В МОЛОКО НА АМИНОКИСЛОТНЫЙ ПРОФИЛЬ ПОЛУТВЕРДОГО СЫРА

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Аннотация. Целью данной работы является анализ аминокислотного профиля сыра в течение периода созревания. Оценка влияния парааминобензойной кислоты (ПАБК) на протеолитическую активность *Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris и*

Streptococcus salivarius subsp. thermophilus позволяет сделать вывод об их жизнеспособности в период созревания в течение 28 дней. Были приготовлены и проанализированы четыре сыра: a) контрольный сыр; b) сыр с 0,1 г/л ПАБК; c) сыр с 0,2 г/л ПАБК и d) сыр с 0,4 г/л ПАБК – в два этапа: t_0 (начало периода созревания) и t_1 (конец периода созревания). Содержание белка в сырах было определено методом Кьельдаля, а аминокислотный профиль – методом ВЭЖХ. В сырах с 0,1 и 0,2 г/литр ПАБК было найдено более высокое содержание большинства аминокислот по сравнению с их содержанием в контрольном сыре. В образце сыра с 0,4 г/литр ПАБК содержание всех аминокислот было значительно выше, чем их содержание в контрольном сыре.

Ключевые слова: парааминобензойная кислота (витамин B10), пищевая биотехнология, полутвердые сыры, заквасочные бактерии, аминокислотный профиль

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Introduction

Para-aminobenzoic acid (PABA), referred to Vitamin B10, has been widely recognized for its involvement in human health, ranging from skin health to protein utilization. However, the increasing demand in functional foods, which are food products fortified with additional nutrients to provide benefits to health beyond basic nutrition [1], has prompted new research into potential PABA applications.

PABA was classified as a part of the Vitamin B complex some decades ago due to its activity as a coenzyme in the biosynthesis of folic acid in some bacteria [2]. PABA is being reevaluated for its potential health advantages, despite the fact that it is not considered essential for humans. Recent research has begun to highlight the possible benefits of PABA supplementation. These include antioxidant capabilities [3], and anti-inflammatory effects [4]. These health benefits suggest that PABA could be a tempting component in the functional foods industry.

Over the last two decades, the functional food enterprises have grown at an exponential rate, with an increasing demand for food that deliver positive health benefits beyond basic nutrition [5]. Vitamin, mineral, probiotic, and other bioactive ingredient enrichment is becoming a prevalent method for boosting the value of food items and addressing customer demand for health-promoting foods [6]. While plenty of foods are fortified with well-known vitamins notably B12, C, and D, the potential for implementing PABA as a food additive in functional food is still being researched. There are potential obstacles in adding PABA into foods, such as its stability, bioavailability, and sensory qualities, which must be thoroughly studied [7].

Amino acids, the fundamental components of proteins, are required for a wide range of biological functions in organisms, from the cellular to the organismal level. While humans and animals must absorb specific amino acids, known as essential amino acids, through nutrition, many bacteria can synthesis these critical compounds from scratch [8]. The mechanism for bacterial amino acid biosynthesis has piqued scientists' curiosity, notably because of its possible implications in bioengineering, antibiotics, and food production.

The involvement of PABA in bacterial amino acid biosynthesis has far-reaching ramifications for many scientific fields. There is potential in food science to grow the nutritional content of fermented foods. PABA supplementation of bacterial cultures may boost the production of critical amino acids in meals fermented with these bacteria, adding to higher nutritional value [9].

The study of PABA's involvement in bacterial amino acid biosynthesis has significant ramifications for multiple fields, including food science. The manufacture of critical amino acids in food-producing bacterial cultures might be improved by using PABA strategically. This would boost the nutritional content of some meals, particularly those fermented with these bacteria, and might contribute to the emerging field of functional foods [10]. PABA's promise in increasing bacterial amino acid production goes beyond nutritional uses. It may have consequences for bioengineering, since increasing amino acid synthesis in bacterial cultures might boost the produc-

tion of physiologically active molecules such as medicines and industrial enzymes [11].

Given the expanding body of literature supporting the leveraging and vital PABA's role in amino acid biosynthesis, health advantages, as well as the growing demand for functional foods, it appears that it is high time to investigate PABA's potential as a functional food additive. The aim of this research is to study the effect of PABA on increasing the amino acids content in semi-hard cheeses through inducing their biosynthesis by the starter culture strains used in cheese manufacturing.

Materials and methods

Production of control cheese

Raw cow's milk was used to produce semi-hard cheeses. Its nutritional value is presented in Table 1.

Table 1

Nutritional value of milk

Fat	Protein	Dry skimmed milk residues	Lactose	Urea
3.21	2.77	7.93	4.65	15.0

Five liters of raw milk were poured into cheese maker vat. Milk was heated to 65 °C for 15 minutes for pasteurization. 0.1 g/l of calcium chloride was added to the milk after cooling it down to 40 °C. After complete dissolving, the milk was once again cooled to a temperature of 32 °C. Starter culture containing *Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris*, and *Streptococcus thermophilus*, was added to the milk with continuous mixing till complete dissolving. This temperature and continuous mixing were obtained for 30 minutes. Microbial rennet enzyme was added in a ratio of 0.01 g/l. The enzyme was rapidly mixed with milk, then the mixture was left without mixing for 20 minutes for coagulation.

After clotting, the curd was continuously cut until a suitable grain size was obtained. Next, the mixture was heated to a temperature of 40 °C, and held for 20 min. After that, the whey was drained, cheese grains were placed in a mold and pressed for 3 hours. Finally, the cheese was salted using a 20 % saline solution for 2 hours, left to dry overnight at 9 °C, and coated by food-grade latex. The cheese was kept for 30 days for ripening at 9–13 °C.

Production of experimental cheeses

Three fortified cheeses were produced with different amounts of PABA. Doses of 0.1 g/l, 0.2 g/l, and 0.4 g/l were used to produce experimental fortified cheeses to be compared to control cheese. Just before initiating milk fermentation, PABA was added to the milk after cooling it down to 32 °C. The same protocol of control cheese production was applied.

Determination of physical and chemical characteristics of cheeses

Physical and chemical characteristics, mass fraction of protein, fat, moisture, and dry matter, of the four cheeses were determined. Mass fraction of protein was determined by Kjeldahl method in accordance with the requirements of GOST 25179-2014 “Milk and dairy products. Methods for determining the mass fraction of protein”. Mass fraction of fat was determined by Gravimetric method in accordance with the requirements of GOST R 51457-99 “Cheese and processed cheese. Gravimetric method for determining the mass fraction of fat”. Finally, mass fraction of moisture and dry matter were determined in accordance with the requirements of GOST 3626-73 “Milk and dairy products. Methods for determining moisture and dry matter”.

Microbiological analysis

On the 7^{th}, 14^{th}, 21^{th} , and 28^{th} days, microbiological analysis was conducted for all the cheeses to monitor the growth of the bacterial starter culture throughout the ripening period. It can be considered as a product’s quality control). Briefly, in sterile conditions, cheese samples were homogenized, serially diluted, and inoculated on M17 agar nutrient media. Petri dishes were incubated at 30 °C for 48 hours. Parallelly, determination of *E.coli* and *Coliform* bacteria was conducted by inoculation of samples in test tubes with Kessler’s liquid nutrient medium and Derham’s trumpet (Food safety control).

Determination of amino acid profile in cheese samples

Before starting measurements, the chromatograph was calibrated with a calibration standard of amino acids produced by Agilent Technologies (Item: 5061-3332).

A sample weighed with an accuracy of 0.0001 g was placed in a glass ampoule and filled with 6 M hydrochloric acid. The ampoule prepared in this way was sealed and placed in a thermos-reactor with a temperature of 110 °C for a period of 24 hours. After this, the ampoule was cooled to room temperature.

After that, the ampoule was opened, and removal of hydrochloric acid immediately began. Removal of hydrochloric acid was carried out on

a rotary evaporator at a temperature of 70 °C. To do this, the hydrolysate from the ampoule was quantitatively transferred into a pear-shaped flask, after which the ampoule was rinsed with distilled water, and the rinses are poured into the same flask. The hydrolysate in the flask was evaporated to dryness. Then 2 ml of acetonitrile-water mixture was poured into the dry residue. The sample was then filtered using a 0.45 μm syringe filter. After that, the sample was measured using HPLC. If necessary, the sample was diluted with water to the required concentration before measurement.

In this work, a High-Performance Liquid Chromatograph Agilent 1260 Infinity II (Germany) was used. The measurement method was based on the separation of a mixture of amino acids in a column filled with a C_{18} carrier grafted onto a high-purity silica gel base, using a reverse-phase mechanism.

The chromatograph was equipped with a multi-wavelength detector and equipped with an Agilent ZORBAX Eclipse AAA 4.6×150 mm 3.5-Micron reverse-phase analytical column. Gradient elution with two eluents was used. A phosphate buffer based on Na_2HPO_4 with a concentration of (0.038 M) was used as the first eluent, and the eluent used was acetonitrile : methanol : water in a ratio of 45:45:10. The elution rate was 2 ml/min, and the column temperature was maintained at 40 °C.

Results and discussion

Physico-chemical characteristics of cheeses

Yield of cheese production, mass fraction of protein, fat, moisture, and dry matter in the four cheeses are presented in Table 2.

According to the results presented in Table 2, the addition of PABA to the milk did not result in any significant differences in physical and chemical characteristics of the produced cheeses.

Viability of starter culture bacteria in cheeses throughout the ripening period

Viability of starter culture bacteria in cheeses throughout the ripening period is presented in Figure 1.

According to the results presented in Figure 1, the addition of PABA to milk affected the growth and viability of starter culture bacteria in experimental cheeses. The graph shows higher CFU per gram od cheese in all experimental cheeses on day 7 of ripening period. Specifically, cheeses C and D had a significantly higher CFU/g, compared to control cheese (Cheese A). On day 14 of ripening, cheese C and D had slightly higher CFU/g. On the 21st day, all three experimental cheeses had significantly lower CFU/g at the end of ripening period. This indicates that supplementation of PABA has led to not only a significant increase in the number of colony forming units, but also to a shorter life cycle of the starter culture bacteria colonies with shorter time in each phase. According to results on the 7th day, it can be concluded that the bacteria in experimental cheeses, especially Cheeses C and D, have passed the lag phase and already at the log phase. Moreover, it can be noticed that the bacteria in experimental cheeses have started death phase at the 21st day, earlier than in case of the control cheese.

The rate of developing starter cultures influences on the biochemistry of the cheese-ripening process. As bacteria multiply and metabolize, they create enzymes that degrade proteins, lipids, and carbohydrates in cheese. These breakdowns produce a variety of chemicals important for taste. The higher bacterial growth rate can hasten this process, resulting in faster flavor formation. The rapid growth of starter cultures can assist with acidification, possibly out-competing and limiting the growth of pathogenic or spoiling microorganisms, assuring both cheese quality and safety.

Table 2

Physico-chemical characteristics of produced cheeses

Product	Yield, kg	Protein content, %	Fat content, %	Moisture content, %	Dry matter content, %
Cheese “A”	0,427	24.89	27.46	35.45	64.55
Cheese “B”	0,433	25.63	27.33	35.21	64.79
Cheese “C”	0,431	26.83	27.22	36.11	63.89
Cheese “D”	0,434	26.57	28.80	33.74	66.26

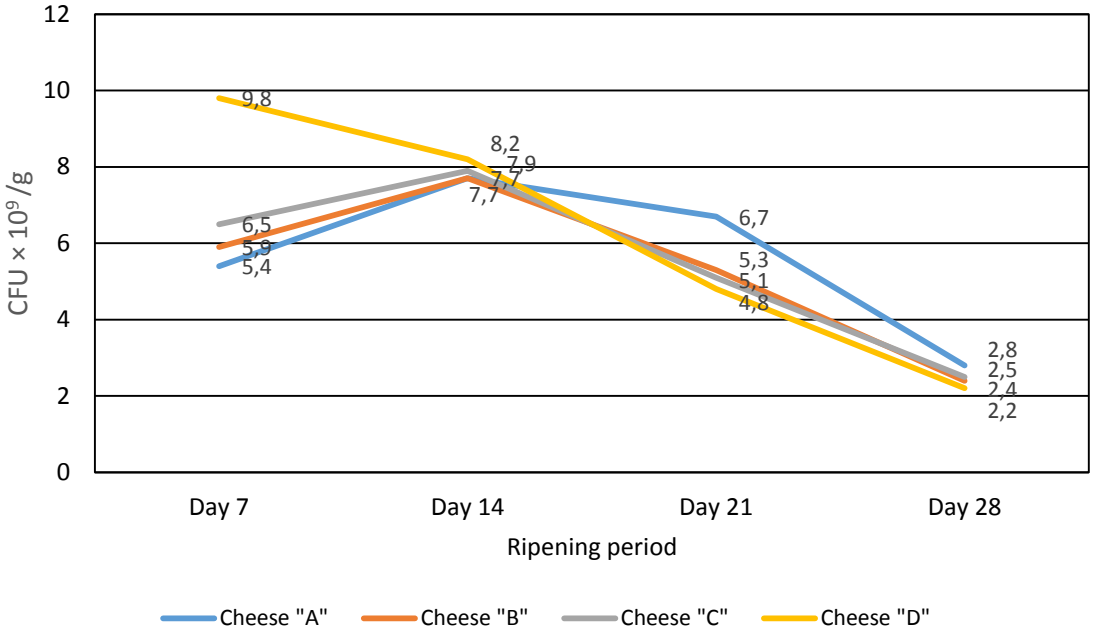

Fig. 1. Microbiological assessment of viability of starter culture bacteria in produced cheeses throughout the ripening period

In test tubes with inoculated samples in Kessler's liquid nutrient media there was no signs of coliform bacterial growth. This indicates that throughout the production procedure and incubation period, there was no contamination with pathogens.

Amino acid composition in cheeses

HPLC results of the amino acid content in the cheeses studied, presented in mg/100 g of cheese, are shown in Table 3. The experimental results were expressed as mean ± standard deviation (SD) and using Analysis of Variance (two-way ANOVA) for data. All calculations were performed using statistical analysis software (GraphPad Prism 8.0.2). The results were considered statistically significant when $P < 0.05$.

Ripening Effects on Amino Acid Profiles in Cheeses

At the culmination of the ripening period for the control cheese (fourth week), there was a significant increase in the concentrations of aspartic acid, glutamic acid, serine, glycine, threonine, and cystine compared to their initial levels at the beginning of the ripening period. Additionally, a negligible increase in the levels of histidine and tyrosine was observed. Conversely, the content of arginine, valine, methionine, phenylalanine, isoleucine, leucine, lysine, and proline was significantly higher at the outset of the ripening period, with an insignificant change in alanine content.

For the cheese with 0.1 g PABA per liter of milk, at the conclusion of the ripening period, all amino acid contents increased significantly, except for proline, which remained constant, and methionine and asparagine, which significantly decreased.

For the cheese with 0.2 g PABA per liter of milk, all amino acid contents increased significantly by the end of the ripening period, except for proline, isoleucine, phenylalanine, methionine, and glutamine, which were significantly higher at the beginning of the ripening period.

The addition of 0.4 g PABA per liter of milk resulted in a substantial increase in the content of all the amino acids by completing the ripening period.

Impact of PABA Addition to Milk on Amino Acid Profiles in Cheeses

In the cheese with 0.1 g PABA per liter of milk, there was a significantly higher concentration of glycine and proline at the beginning of the ripening period, while the levels of other amino acids were significantly higher in the control cheese. However, by the end of the ripening period, the contents of glutamic acid, glycine, arginine, alanine, cystine, valine, leucine, lysine, and proline were significantly elevated.

In the cheese with 0.2 g PABA per liter of milk, the concentrations of glycine, glutamine, and lysine were notably higher at the outset of the

Table 3

Amino acid profile of produced cheeses at the beginning and end of ripening period

Amino acid	Cheese "A"		Cheese "B"		Cheese "C"		Cheese "D"	
	Day 7	Day 28	Day 7	Day 28	Day 7	Day 28	Day 7	Day 28
Asparagine	2161 ± 2	2327 ± 3	1652 ± 3	1347 ± 2	1129 ± 2	1414 ± 2	2054 ± 3	2705 ± 2
Glutamine	5598 ± 1	6006 ± 5	4294 ± 3	7572 ± 5	7433 ± 4	7308 ± 5	5504 ± 4	8783 ± 3
Serine	1396 ± 3	1417 ± 4	997 ± 1	1221 ± 2	1109 ± 2	1203 ± 2	1528 ± 3	1617 ± 1
Histidine	955 ± 6	958 ± 3	626 ± 1	966 ± 2	881 ± 2	1022 ± 2	1141 ± 2	1499 ± 2
Glycine	303 ± 2	329 ± 2	327 ± 1	591 ± 1	384 ± 1	586 ± 1	385 ± 1	1193 ± 1
Threonine	938 ± 2	1043 ± 3	798 ± 2	981 ± 1	700 ± 1	889 ± 2	1042 ± 1	1579 ± 0
Arginine	970 ± 4	914 ± 1	614 ± 2	968 ± 2	814 ± 2	1059 ± 2	978 ± 1	1748 ± 1
Alanine	625 ± 1	617 ± 1	471 ± 1	667 ± 1	517 ± 1	957 ± 2	704 ± 2	1593 ± 2
Tyrosine	1519 ± 3	1519 ± 2	1103 ± 3	1287 ± 3	1204 ± 3	1254 ± 3	1509 ± 3	1855 ± 2
Cystine	73 ± 1	131 ± 1	26 ± 1	225 ± 1	41 ± 1	142 ± 1	149 ± 1	76 ± 0
Valine	1758 ± 4	1641 ± 2	1292 ± 2	2015 ± 3	1633 ± 3	1883 ± 3	1727 ± 2	3577 ± 3
Methionine	834 ± 2	730 ± 1	451 ± 1	401 ± 1	515 ± 1	367 ± 1	650 ± 1	707 ± 1
Phenylalanine	1424 ± 2	1250 ± 1	972 ± 2	1136 ± 3	1306 ± 2	1095 ± 2	1242 ± 3	1523 ± 2
Isoleucine	2257 ± 5	1165 ± 2	897 ± 2	984 ± 2	1010 ± 2	942 ± 1	1068 ± 3	1319 ± 2
Leucine	2599 ± 4	1538 ± 3	1410 ± 3	2535 ± 3	2241 ± 3	2481 ± 3	1058 ± 2	3637 ± 3
Lysine	2304 ± 4	1310 ± 2	1121 ± 1	2504 ± 3	2372 ± 2	2738 ± 3	1405 ± 2	2695 ± 2
Proline	4923 ± 3	3615 ± 3	6433 ± 4	5583 ± 3	4392 ± 3	3950 ± 3	5216 ± 3	6044 ± 4

ripening period, while the levels of other amino acids were significantly higher in the control cheese. However, by the end of the ripening period, the content of all amino acids had significantly increased, except for asparagine, serine, threonine, tyrosine, methionine, and phenylalanine, which remained significantly higher in the control cheese.

In the cheese with 0.4 g PABA per liter of milk, there was a significantly higher content of serine, histidine, glycine, threonine, alanine, and cystine. The increase in arginine was insignificant, while the concentrations of other amino acids were significantly higher in the control cheese. Nonetheless, by the end of the ripening period, the content of all the amino acids was significantly higher, with the exception of cystine and methionine, which remained considerably higher in the control cheese.

Discussion

Bacteria have the unique capacity to produce folic acid, yet humans must receive it from food. PABA is a significant phase in this pathway. PABA is specifically involved in the synthesis of dihydropteroate, a precursor to dihydrofolate and, later, tetrahydrofolate (THF). THF is an essential coenzyme in the synthesis of several amino acids, including glycine, and serine. It acts as a methyl donor or acceptor in a number of processes that result in the production of these amino acids [12]. According to research, PABA is involved in the biosynthesis of various amino acids, including methionine, glycine, and serine, which are produced from intermediates in the folic acid synthesis pathway [13]. Furthermore, numerous investigations have shown that PABA supplementation can increase bacterial synthesis of these and possibly other amino acids [14].

Conclusion

In conclusion, this study provides compelling evidence that the addition of PABA to cheese during production can significantly influence the amino acid profile of semi-hard cheeses over a 28-day ripening period. The data indicates that cheeses fortified with varying concentrations of PABA not only exhibited higher concentra-

tions of amino acids compared to the control but also demonstrated variations in the viability and growth dynamics of starter culture bacteria. This suggests that PABA could play a beneficial role in enhancing the nutritional value of cheese, thereby contributing to the development of functional foods that offer health benefits beyond basic nutrition. Further studies focusing on the long-term stability, sensory qualities, and consumer acceptance of PABA-fortified foods are recommended to fully realize their potential in food science and industry.

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