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ORIGINAL RESEARCH PAPER

CHARACTERIZATION OF LACTIC ACID BACTERIA POSTBIOTICS: BIOACTIVE PEPTIDE ON YOGURT WITH THERMAL INACTIVATION PROCESS

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Abstract

Postbiotics are non-living microorganisms and/or their metabolites that offer health benefits to the host. The stability of postbiotics is maintained due to the inactive state of microorganisms, making them more resistant to environmental factors like temperature and oxygen. One of the promising metabolites acting as a postbiotic is peptides. Thermal process is one of the methods for obtaining postbiotics, influencing their physicochemical properties and quality. This study investigates the impact of thermal processes on bioactive peptide compounds and evaluates their potential health benefits in yogurt fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. Peptide sequences were analyzed before and after thermal inactivation, while health benefits were assessed *in silico* using the BIOPEP-UWM application. Results showed that thermal inactivation increases the diversity of bioactive peptides, particularly those with smaller molecular weights. Based on health benefit analysis, bioactive peptides with molecular weights <3 kDa from the thermal process predominantly have potential bioactivities as Angiotensin Converting Enzyme inhibitors and dipeptidyl peptidase IV inhibitors.

Keywords: bioactivity, inactivation, *in silico*, bioactive peptides, postbiotics

Introduction

Yogurt is recognized as a fermented food product containing lactic acid bacteria (LAB) that offers health benefits. According to the food category definition by the Codex Alimentarius Commission for fermented milk standards, “yogurt is a milk

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product obtained through the fermentation of milk using lactic acid bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*" (Codex Alimentarius Commission, 2022). Yogurt benefits include immune system stimulation, enhanced nutrient absorption and digestion, minimized pathogenic bacteria growth, and improved gut health (Zolkiewicz *et al.*, 2020).

At the end of the shelf life of LAB-containing products, LABs may die, and the potential of these dead cells and their metabolites becomes an intriguing research focus. According to The International Scientific Association of Probiotics and Prebiotics (ISAPP, 2021), postbiotics are dead microorganisms and/or their metabolites produced through specific processes that offer health benefits to the host. LAB fermentation can produce metabolites such as short-chain fatty acids (SCFA), amino acids, enzymes, bioactive peptides, exopolysaccharides (EPS), and vitamins (Germani *et al.*, 2014; Zolkiewicz *et al.*, 2020; Peredo-Lovillo *et al.*, 2022). Based on the ISAPP definition of postbiotics, these metabolites qualify as postbiotic components.

According to Salminen *et al.* (2021), postbiotics have to meet criteria including: (1) genomic characterization of the microorganisms used in the fermentation process; (2) detailed description of the inactivation process; (3) confirmation of inactivation; (4) proof of health effects through controlled human clinical studies; (5) detailed description of the postbiotic composition; and (6) food safety assessment of postbiotics. Given these criteria, identifying bioactive components in fermented LAB products is essential to evaluate their potential as postbiotics. This study identifies bioactive components, particularly bioactive peptides, in yogurt fermented using *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*.

Milk proteins contain amino acid sequences that are mostly inactive within the primary protein structure, they are also known as peptides. Peptides in their inactive form lack health benefits and require additional processing to become active forms (Korhonen and Pihlanto, 2006; Rubak *et al.*, 2020; FitzGerald *et al.*, 2020). Peptides can be activated through various processes, including: (1) hydrolysis with digestive enzymes; (2) hydrolysis with proteolytic microorganisms; (3) enzymatic hydrolysis by microorganisms; and (4) physical/chemical treatments (e.g., ultrasonic, microwave, acid/base addition). During LAB fermentation of milk, protein hydrolysis occurs, releasing bioactive peptides from the primary protein structure. The health benefits of bioactive peptides depend on their amino acid sequences (Korhonen and Pihlanto, 2006) and molecular weights (Manzoor *et al.*, 2022; Peredo-Lovillo *et al.*, 2022; Guo *et al.*, 2023). Bioactive peptides can aid in digestion, cardiovascular health, immune function, and nervous system support, as well as promote overall health (Hafeez *et al.*, 2014).

The thermal inactivation of microorganisms in fermented food can affect the structure and bioactivity of metabolites, influencing their bioactive potential as postbiotics. Various inactivation methods, such as heating, high-pressure processing, sonication, irradiation, and ultraviolet treatment, can be applied (De Almada *et al.*, 2016).

This study aimed to investigate the effects of thermal inactivation on bioactive peptide components and to identify their potential health benefits using *in silico* methods. A comparative analysis of bioactive peptides was performed before and after thermal inactivation to evaluate the impact of thermal processing. The potential bioactivity of peptides generated through thermal processes was further assessed using *in silico* analysis via the BIOPEP-UWM application.

Materials and methods

Yogurt culture

The yogurt culture used in this study was a freeze-dried culture containing a mixture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* obtained from International Flavors and Fragrances (IFF) with the code YO-MIX 883 LYO 250 DCU.

Yogurt Preparation

Yogurt was prepared by fermenting pasteurized (90 ^{o}C for 10 minutes) reconstituted milk consisting of 10% full cream milk and 1% skim milk, inoculated with *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. The fermentation process was carried out at 43 °C for 7 hours. After fermentation, the yogurt was packaged in pouches, heated to 78 °C for 15 minutes, and then cooled to a temperature of 25 – 28 °C.

Bioactive peptide analysis

Peptide Fractionation

A sample of 40 g yogurt was placed into a 50 mL falcon tube and centrifuged (5789×g, 15 minutes, 4 °C). The resulting supernatant was centrifuged again using a 10 kDa filter tube (1967 × g, 30 minutes, 4 °C). The filtrate (< 10 kDa) was then centrifuged again under the same operating conditions using a 3 kDa filter tube. The final fraction (< 3 kDa) was collected for further identification.

Peptide Identification

Peptides were characterized by Thermo Scientific™ Dionex™ Ultimate 3000 RSLCnano UHPLC coupled with Thermo Scientific™ Q Exactive™ High Resolution Mass Spectrometer. The mobile phase used in this study was 0.1% formic acid in distilled water (nano pump A) and 0.1% formic acid in 80% acetonitrile (nano pump B). The analytical column was an EASY-Spray™ column (15 cm × 75 µm i.d., PepMap C18, 3 µm; Thermo Fisher Scientific). The < 3 kDa fraction obtained from the sample preparation was filtered using a 0.22 µm filter. 10 µL of the samples were injected into the system. The flow rate was 100 µL/min with a 60-minute gradient. Samples were analyzed under Full Scan MS at 70,000 FWHM (Full Width at Half Maximum) and data-dependent MS2 at 17,500 FWHM. Full Scan MS spectra were recorded in positive mode using easy nano spray ionization. The obtained data were identified using Thermo Scientific Proteome Discoverer 2.2 software.

Bioactive peptides analysis and potential health benefits in silico

The analysis was conducted by evaluating the presence of bioactive fragments in peptide sequences (A) and specific biological activity values (B) using the BIOPEP-UWM application. The method for identifying value A is based on Minkiewicz *et al.* (2008):

$$A = \frac{a}{N} \quad (1)$$

where A = frequency of bioactive fragment presence in the peptide sequence; a = number of fragments with a given activity in a protein sequence; *N* = the number of amino acid residues in a protein.

$$B = \frac{\sum_{i=1}^{k} \frac{ai}{EC50i}}{N} \quad (2)$$

where B = potential biological activity of protein; ai = the number of repetitions of *i*th bioactive fragment in protein sequence; EC50i = the concentration of *i*th bioactive peptide corresponding to its half-maximal activity [mM]; k = the number of different fragments with a given activity; N = the number of amino acid residues in protein

Data analysis

The analysis of bioactive peptides and potential health benefits was performed using Principal Component Analysis (PCA) and Agglomerative Hierarchical Clustering (AHC) methods with the XLSTAT application.

Result and discussion

Effect of thermal inactivation on peptide sequences

Bioactive peptides are organic compounds consisting of 2–20 amino acids with potential biological activity (Manzoor *et al.*, 2022). These peptides can be generated during fermentation, and their bioactivity depends on the peptide sequence. The type of bacteria used in fermentation influences the resulting peptide sequence due to their role in proteolytic reactions during the process (Peredo-Lovillo *et al.*, 2022). Using LC-HRMS, 10 peptide sequences were identified in yogurt before and after thermal inactivation, as shown in Table 1.

The research results identified in Table 1 show that the thermal inactivation process: (1) causes changes in peptide sequences in yogurt products after the thermal inactivation process; (2) generates new peptides derived from κ-casein (A0A140T8A9) and α-s1-casein (A0A4W2H1U3); (3) results in the loss of several peptides derived from κ-casein (A0A140T8A9), serine/threonine kinase (F1MSZ0), and reverse transcriptase (A0A4W2IJ09); (4) does not affect peptides derived from glycosylation (A0A4W2FPA7) and α-s2-casein (P02663); and (5) produces peptide sequences with lower molecular weight ranges (831.49 – 1436.72 Da) compared to before the thermal inactivation process (831.49 – 4160.13 Da).

The findings of Rendon-Rosales *et al.* (2022) identified 11 peptide sequences in fermented milk products processed using *Lactobacillus lactis* NB-571. The resulting peptide sequences were derived from κ-casein, β-casein, α-s1-casein, α-s2-casein, α-

lactalbumin, and serotransferrin. The molecular weight of the peptide sequences ranged from 592.3 to 1717 Da. This demonstrates that the same substrate but different bacterial strains can produce different peptide sequences.

Table 1. Peptide sequence before and after thermal inactivation

Protein	Peptide sequence	m/z (Da)	MH+ (Da)	Molecular Weight (Da)	Retention Time (Min)		Before inactivation	After inactivation
					Before inactivation	After inactivation		
κ-casein (A0A140T8A9)	SPPEINTVQ	492.75	984.50	983.49	9.51		√	
	FMAIPPKKN	349.20	1045.59	1044.58	15.53	14.15	√	√
	AVRSPAQIL	477.79	954.57	953.57	12.61	12.75	√	√
	GEPTSTPTXE	516.25	1031.49	1030.48		9.33		√
	QDKTEIPTIN	579.80	1158.60	1157.59		10.25		√
Glycosylation (A0A4W2FPA7)	DASAQFIR	454.24	907.46	906.46	9.50	9.47	√	√
	AQPTDASAQFIR	652.83	1304.66	1303.65	10.18	10.18	√	√
	ILNKPEDETHLE	479.91	1437.72	1436.72	13.70	13.36	√	√
α-S2-casein (P02663)	ALPQYLK	416.75	832.49	831.49	10.88	10.52	√	√
	ALPQYLKT	467.27	933.54	932.53	11.17	11.00	√	√
Serine/threonine kinase (F1MSZ0)	EETKTKVKRVIKQVRGRLMPLLKLQHAHISI	1825.61	3650.22	3649.21	1.14		√	
Reverse transcriptase (A0A4W2II09)	KVGLKLNIQKTKITASSPITSWEIDGET VETMSDFMF	321.02	4161.13	4160.13	16.46		√	
α-S1-casein (A0A4W2H1U3)	DVPSERYL	489.75	978.49	977.48		11.00		√

where A=Alanine; R=Arginine; N=Asparagine; D=Aspartic acid; E=Glutamic Acid; Q=Glutamine; G=Glysin; H=Histidine; I=Isoleucine; L=Leucine; K=Lysine; M=Methionine; N=Aspargine; F=Phenylalanine; P=Proline; S=Serine; T=Threonin; V=Valine; Y=Tyrosine; X=not identified or others. √ = identified

Heating processes can alter protein conformation, leading to disruption in protein structure, the generation of new peptides, and potentially increased bioactivity (Peredo-Lovilo *et al.*, 2022). Heating at 70 °C can enhance the dissociation of κ-casein but reduce the dissociation of β-casein and αs-casein (Krishna *et al.*, 2021). The research results showed that Glycosylation-related proteins and α-s2-casein retained most peptides across inactivation states, indicating their stability under these conditions. Unlike other proteins, κ-casein displayed changes in peptide detectability, with some peptides being undetectable after inactivation, indicating structural modifications or susceptibility to inactivation. Minor proteins are components of milk protein outside the main protein fractions with potential bioactivity (Wynn and Sheehy, 2013). Some minor proteins are categorized as whey proteins, which are sensitive to heat treatment. Denaturation processes can result in whey protein fractions forming complexes with κ-casein and preventing the dissociation of β-casein and αs-casein (Krishna *et al.*, 2021).

Bioactivity of peptide sequence

Several previous studies have identified bioactivities derived from fermented products as follows: (1) casein fermented with *Lactobacillus helveticus* R0389 and *Lactobacillus rhamnosus* R0011, which produce ACE inhibitors; (2) camel milk fermented with *Lactobacillus lactis* KX881782 and *Lactobacillus acidophilus* DSM9126, which exhibits anticancer, antihypertensive, antidiabetic, and antioxidant activities; and (3) cow's milk fermented with *Lactobacillus plantarum*, which demonstrates antioxidant, antimicrobial, antihaemolytic, antimutagenic, and anti-inflammatory activities (Manzoor *et al.*, 2022).

In this study, peptide sequences identified through LC-HRMS analysis were further evaluated *in silico* using the BIOPEP-UWM application (data accessed: July 14, 2024). The *in silico* analysis revealed 23 types of bioactivity in the yogurt product, both before and after the thermal inactivation process. The types of bioactivities identified are presented in Table 2.

Table 2 highlights the diverse bioactivities of the analyzed peptides before and after thermal inactivation, which can be broadly grouped based on their overall health benefits. Compounds with cardiovascular health benefits include ACE inhibitors, ACE2 inhibitors, renin inhibitors, and antithrombotic agents, which collectively contribute to blood pressure regulation and vascular protection (Manzoor *et al.*, 2022). Metabolic health is supported by alpha-amylase and alpha-glucosidase inhibitors, pancreatic lipase inhibitors (Levuok-Mena *et al.*, 2023), and hypouricemic agents, which help manage glucose and lipid metabolism and reduce uric acid levels (Scanu *et al.*, 2022). Anti-inflammatory and antioxidative properties contribute to reducing oxidative stress and immune modulation (Manzoor *et al.*, 2022). Additionally, antibacterial activity and bacterial permease ligand action indicate antimicrobial potential (Manzoor *et al.* 2022). Neurological benefits are suggested by neuropeptides and glutamate carboxypeptidase inhibitors, while several enzyme inhibitors, such as dipeptidyl peptidase inhibitors and CaMPDE inhibitors, support various physiological processes (Akbarian *et al.*, 2022). These

bioactivities underscore the multifunctional health-promoting potential of these peptides.

Table 2. Bioactivity before and after thermal inactivation.

Bioativity	Before inactivation	After inactivation
ACE Inhibitor	√	√
Alpha-amylase inhibitor	√	√
Alpha-glucosidase inhibitor	√	√
Anti inflammatory	√	√
Antibacterial		√
Antioxidative	√	√
Antithrombotic	√	√
Bacterial permease ligand	√	√
CaMPDE inhibitor	√	√
Dipeptidyl peptidase III inhibitor	√	√
Dipeptidyl peptidase IV inhibitor	√	√
Inhibitor of cytosol alanyl aminopeptidase	√	
Lactocepin inhibitor	√	√
Leucyltransferase inhibitor	√	
Neuropeptide	√	√
Pancreatic lipase inhibitor	√	
Renin inhibitor	√	√
Stimulating	√	√
Xaa-pro inhibitor	√	
ACE2 inhibitor	√	√
Glutamate carboxypeptidase II inhibitor	√	√
Glutamate carboxypeptidase inhibitor	√	√
Hypouricemic		√

where √ = identified

Table 2 demonstrates the selective effects of thermal inactivation on bioactivities. Many bioactivities, including ACE inhibition, alpha-amylase and alpha-glucosidase inhibition, anti-inflammatory effects, antibacterial activity, antithrombotic properties, bacterial permease ligand binding, CaMPDE inhibition, neuropeptide activity, renin inhibition, and glutamate carboxypeptidase inhibition, remained unaffected by thermal inactivation, showcasing their stability under heat treatment. This resilience suggests their potential application in heat-processed products where maintaining functional bioactivity is essential.

In contrast, certain bioactivities exhibited sensitivity to thermal processing. Notably, inhibitors of cytosolic alanyl aminopeptidase, leucyltransferase inhibitors, pancreatic lipase inhibitors, and xaa-pro inhibitors were not detected following thermal inactivation treatment. This indicates that thermal inactivation can degrade bioactive components that are structurally or chemically sensitive to heat.

The data in Table 2 reveal that thermal inactivation not only impacts existing bioactivities but also results in the emergence of new activities. Specifically, antibacterial and hypouricemic became detectable after thermal inactivation, despite being absent prior to the treatment. These findings show that thermal processing may induce structural changes in the bioactive compounds, leading to the release of bioactive peptides or the formation of secondary metabolites with antibacterial and hypouricemic properties. Antibacterial activity points to the generation of compounds capable of inhibiting microbial growth, potentially through mechanisms such as disruption of bacterial cell walls or interference with bacterial metabolism (Manzoor *et al.,* 2022). While, hypouricemic compounds are known to reduce uric acid levels, making them beneficial for managing conditions like hyperuricemia and gout (Scanu *et al.,* 2022).

The bioactivity of peptides is influenced by enzymatic hydrolysis, fermentation processes, and chemical or physical treatments (FitzGerald *et al.*, 2020). This study utilized fermentation processes and a physical process in the form of thermal inactivation. Thermal processes can alter protein structures, thereby affecting the peptides produced. Thermal treatments may enhance certain bioactivities, although in other cases, they may have no effect on specific bioactivities (Peredo-Lovillo *et al.*, 2022).

The evaluation of bioactive peptides was also conducted by assessing the potential presence of bioactive fragments in peptide sequences (A) using the BIOPEP-UWM application (data accessed: July 14, 2024). The total A value for specific bioactivity potentials provides an overview of the biological activity potential of these peptides (Din *et al.*, 2022).

The analysis of peptide bioactivity in yogurt was conducted using PCA and AHC methods with the XLSTAT application. This analysis grouped bioactivity potential based on the A value and the number of peptide sequences. The results, as shown in Figure 1, reveal two clusters of bioactivity potential. Cluster 1 exhibits a higher profile in terms of the frequency of bioactive fragment presence and the number of peptide sequences compared to Cluster 2. Cluster analysis indicates that Cluster 1 has more dominant bioactivity than Cluster 2. The dominant bioactivities include ACE inhibitor and DPP IV inhibitor (Cluster 1A), as well as DPP III inhibitor and antioxidative activities (Cluster 1B).

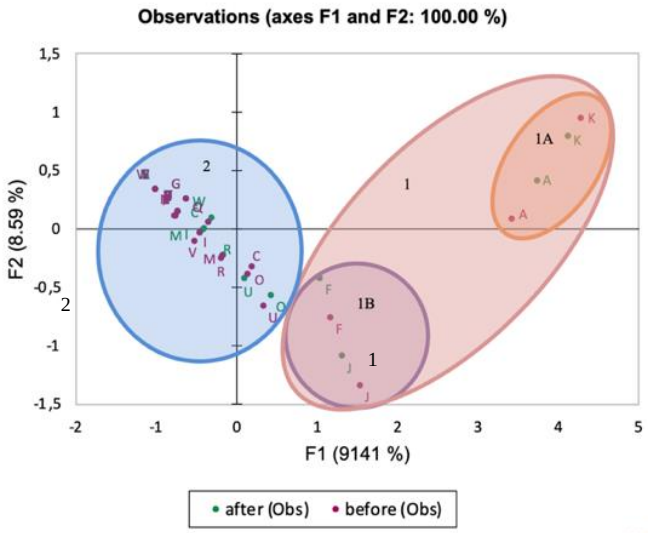

Figure 1. Bioactivity based on the frequency of bioactive fragment presence and the number of peptide sequences. Cluster 1 represents dominant peptide bioactivity. Cluster 2 represents non-dominant peptide bioactivity. A-ACE inhibitor, F-antioxidative, J-DPP III inhibitor, K-DPP IV inhibitor.

The identification of dominant bioactivity potential through clustering methods confirms findings from previous studies on the bioactivity observed in milk proteins and fermented dairy products (Barati *et al.*, 2020; FitzGerald *et al.*, 2020; Peredo-Lovillo *et al.*, 2022; Manzoor *et al.*, 2022; Guo *et al.*, 2023). The application of physical processes, such as heat treatment, can influence bioactivity by enhancing, reducing, or having no effect on it (Peredo-Lovillo *et al.*, 2022). Further analysis compared the A values before and after the thermal inactivation process for the four identified dominant bioactivities. The results of this analysis are presented in Table 3.

Table 3. Frequency of bioactive fragment presence before and after thermal inactivation.

Bioactivity	Before inactivation	After inactivation
ACE inhibitor	4.56	5.27
DPP IV inhibitor	6.43	6.10
DPP III inhibitor	0.95	0.98
Antioxidative	1.19	1.41

The results of the analysis in Table 3 indicate that the thermal inactivation process can enhance the bioactivity potential of ACE inhibitors, DPP III inhibitors, and antioxidative properties in yogurt products. The total A values for these three bioactivities were higher after thermal inactivation. However, the thermal inactivation process reduced the bioactivity potential of DPP IV inhibitors in yogurt

products, as evidenced by lower total A values for DPP IV inhibitor activity after thermal inactivation.

Potential health benefits

The calculation of biological activity potential (B) depends on the EC_{50}/IC_{50} values, which are not always available in the BIOPEP-UWM application (Minkiewicz *et al.*, 2019). Based on data accessed on July 14, 2024, the B values for DPP III inhibitor and antioxidative bioactivities could not be identified due to the absence of EC_{50}/IC_{50} values. Therefore, the analysis of potential health benefits in this study was focused on ACE inhibitor and DPP IV inhibitor bioactivities, as both A and B values were available for these activities.

Dominant bioactivity

The analysis of dominant bioactivity after the thermal inactivation process was conducted by clustering the A and B values of identified peptide sequences using PCA and AHC methods in the XLSTAT application. The results, displayed in Figure 2A, reveal three dominant bioactive peptides with higher combined A and B profiles compared to other clusters. These dominant bioactive peptides are FMAIPPKKN, GEPTSTPTXE, and DVPSERYL, all of which were identified as dominant for ACE inhibitor bioactivity. Peptides with ACE inhibitor bioactivity demonstrate higher potential health benefits compared to peptides with DPP IV inhibitor bioactivity, as evaluated *in silico*.

Dominant bioactive peptides

Further analysis was conducted to evaluate the dominant bioactive peptides for ACE inhibitor and DPP IV inhibitor bioactivities. Clustering of bioactive peptides based on their A and B values for both bioactivities was performed using PCA and AHC methods. The analysis results indicate that the dominant bioactive peptides for ACE inhibitor bioactivity are FMAIPPKKN, GEPTSTPTXE, and DVPSERYL (Figure 2B). Meanwhile, the dominant bioactive peptides for DPP IV inhibitor bioactivity are ILNKPEDETHLE, ALPQYLK, ALPQYLKT, and QDKTEIPTIN (Figure 2C). The findings reveal that after the thermal inactivation process, three newly identified peptides are GEPTSTPTXE, DVPSERYL, and QDKTEIPTIN emerged as dominant bioactive peptides *in silico*. These results align with the study by Peredo-Lovillo *et al.* (2022), which demonstrated that heat treatment can enhance the release of bioactive peptides, thereby potentially increasing their bioactivity.

The research showed that bioactive peptides serve potential bioactivities as ACE inhibitor and DPP IV inhibitor. Whereas these bioactivities are crucial in managing chronic conditions such as cardiovascular diseases and type 2 diabetes. ACE inhibitors reduce blood pressure by inhibiting the angiotensin-converting enzyme, preventing the formation of angiotensin II, a vasoconstrictor. DPP IV inhibitors, on the other hand, regulate blood glucose by inhibiting the degradation of incretin hormones, thereby enhancing insulin secretion and glycemic control (Manzoor *et al.*, 2022).

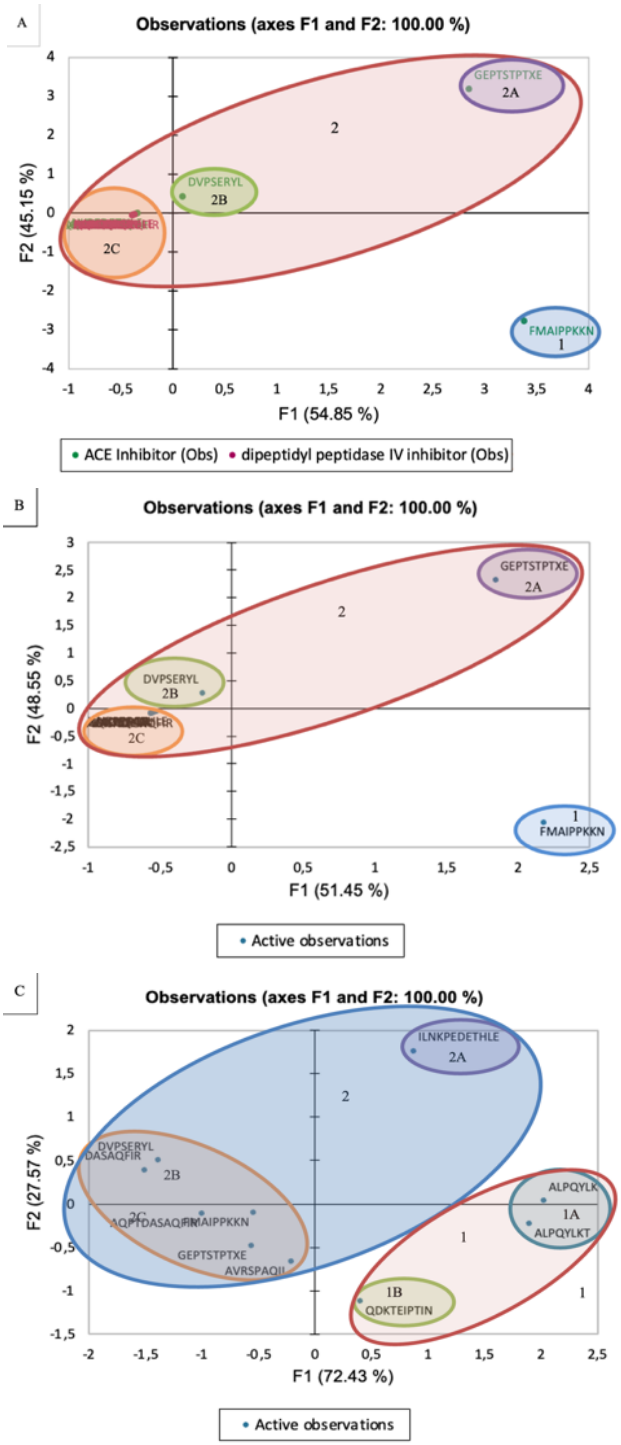

Figure 2. Bioactive peptides on dominant bioactivity (A) Bioactive peptides dominant on ACE inhibitor (B) Bioactive peptides dominant on DPP IV inhibitor (C).

Identification of bioactive peptide fragments

The identification of potential health benefits was performed by profiling bioactive fragments within peptide sequences (Minkiewicz *et al.*, 2008). The identification of bioactive fragments was conducted *in silico* using the BIOPEP-UWM application (data accessed: July 14, 2024). The results of this analysis are presented in Table 4.

Table 4. Bioactive peptide fragments as ACE inhibitor and DPP IV inhibitor.

Bioactivity	Peptide Sequences	Bioactive peptide fragments
ACE inhibitor	FMAIPPKKN	IPP, AIP, IP, AI, PPK, AIPP, PP, MAIPPK, MAIPPKK
	GEPTSTPTXE	GEP, GE, PT, TP, ST
	DVPSERYL	RY, YL, VP, VPSERYL, RYL, ER
DPP IV inhibitor	ILNKPEDETHLE	KP, HL, ET, IL, LN, TH
	ALPQYLK	LP, AL, PQ, QY, YL, LPQ
	ALPQYLKT	LP, AL, PQ, QY, YL, LPQ
	QDKTEIPTIN	IP, EI, IN, KT, PT, QD, TE, TI

where A=Alanine; R=Arginine; N=Asparagine; D=Aspartic acid; E=Glutamic Acid; Q=Glutamine; G=Glysin; H=Histidine; I=Isoleucine; L=Leucine; K=Lysine; M=Methionine; N=Aspargine; F=Phenylalanine; P=Proline; S=Serine; T=Threonin; V=Valine; Y=Tyrosine.

The identified bioactive peptide fragments can serve as a basis for confirming the bioactivity of bioactive peptides present in yogurt products subjected to thermal processes. Currently, two commercial products contain tripeptides VPP and IPP, with claims of biological activity as ACE inhibitors, marketed in Japan and Finland. These products are derived from fermentation processes using *Lactobacillus helveticus* and *Streptococcus cerevisiae* (Japan) and *Lactobacillus helveticus* LBK-16H (Finland) (Korhonen and Pihlanto, 2006).

Conclusions

Thermal inactivation during yogurt production, fermented using *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*, can enhance the diversity of bioactive peptides with lower molecular weights compared to before thermal inactivation.

In silico evaluation indicates that although thermally processed yogurt exhibits dominant health potential as an ACE inhibitor and DPP IV inhibitor, its activity is more prominent as an ACE inhibitor. The bioactive peptides identified with ACE inhibitor activity are FMAIPPKKN, DVPSERYL, and GEPTSTPTXE, while those with DPP IV inhibitor activity are ILNKPEDETHLE, ALPQYLK, ALPQYLKT, and QDKTEIPTIN.

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