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Bioactive peptides released during fermentation of milk by lactic acid bacteria

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Abstract

Here is a paradox: fermented milk products like yogurt and kefir have been consumed for millennia as health-promoting foods, yet the specific bioactive peptides responsible for much of that benefit were identified only in the last three decades. This research characterized the peptide profiles and angiotensin-converting enzyme (ACE) inhibitory activity of milk fermented by five lactic acid bacteria (LAB) strains—*Lactobacillus helveticus* NCDC 288, *Lactobacillus casei* NCDC 297, *Lactobacillus acidophilus* NCDC 015, *Lactobacillus rhamnosus* NCDC 198, and *Streptococcus thermophilus* NCDC 074—over a 72-hour fermentation at 37 °C. Peptide release was tracked by measuring the degree of hydrolysis (DH) and by RP-HPLC-MS/MS identification of peptides in the <10 kDa ultrafiltrate. ACE inhibitory activity was measured by the spectrophotometric hippuric acid method. *L. helveticus* produced the highest DH (18.4% at 72 h) and the strongest ACE inhibition (IC₅₀: 0.41 mg protein/mL at 48 h), with the identified peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) as the main contributors. *L. acidophilus* ranked second in ACE inhibitory potency (IC₅₀: 0.68 mg/mL), while *S. thermophilus* generated the weakest proteolysis and ACE inhibition throughout the fermentation period. Antioxidant activity measured by the ABTS assay increased in parallel with DH, reaching 78.4 µmol Trolox equivalents per liter for *L. helveticus* fermentates at 72 h. Time-course analysis showed that ACE inhibition peaked between 24 and 48 hours for most strains, after which further proteolysis appeared to degrade some active peptides into inactive fragments. These results confirm *L. helveticus* as the most potent ACE-inhibitory peptide producer among common dairy LAB and suggest that controlled fermentation time is as important as strain selection for maximizing bioactivity.

Keywords: Bioactive peptide, fermentation, lactic acid bacteria, ACE inhibitor, *Lactobacillus helveticus*, milk protein, degree of hydrolysis, antioxidant, VPP, IPP

Introduction

It seems paradoxical that the same enzymatic process that sours milk and changes its texture—proteolysis by lactic acid bacteria—also releases peptide sequences with potent biological activities including blood pressure reduction, antioxidant defense, antimicrobial action, and opioid-like effects [1, 2]. The best-characterized bioactive peptides from fermented milk are the tripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), which inhibit angiotensin-converting enzyme (ACE) and have been shown to lower systolic blood pressure by 3-8 mmHg in mildly hypertensive subjects in several clinical trials [3,4]. These peptides are encrypted within the amino acid sequences of casein— β -casein f (84-86) for VPP and β -casein f(74-76) for IPP—and are liberated only when bacterial proteinases cleave the parent protein at the right positions [5]. Not all LAB are equally proteolytic. *Lactobacillus helveticus*, which carries a potent cell-envelope proteinase (PrtH) with broad cleavage specificity, is the most productive source of ACE-inhibitory peptides in fermented milk [6, 7]. Other species—*L. casei*, *L. rhamnosus*, *L. acidophilus*—carry their own proteinase variants with different substrate preferences, generating different peptide profiles and, potentially, different bioactivities [8]. *Streptococcus thermophilus* is weakly proteolytic on its own but is commonly used in yogurt production as a companion to *Lactobacillus delbrueckii* subsp. *bulgaricus* [9]. A practical question for dairy manufacturers developing 'functional' fermented milk products is: which strain and how long? Fermentation time affects peptide accumulation in a non-monotonic way.

Short fermentation may not release enough bioactive peptides, while prolonged incubation can break them down further into inactive di- or tri-peptides or free amino acids^[10, 11]. The optimal window depends on the balance between the rate of peptide release (proteinase activity) and the rate of peptide degradation (peptidase activity), both of which vary by strain and incubation conditions^[12].

Despite considerable published work on individual strains, direct side-by-side comparisons of multiple LAB fermenting identical milk under identical conditions remain surprisingly few^[13]. This research compared five LAB strains in a standardized fermentation system (UHT whole milk, 37 °C, 72 h) and tracked the degree of hydrolysis, peptide profile (by RP-HPLC-MS/MS), ACE inhibitory activity, and antioxidant capacity at six time points. The work was carried out at the Department of Food Chemistry and Nutrition, Raman Research Institute of Chemical Sciences, Chennai, Tamil Nadu, India, between June 2022 and March 2023^[14]. We aimed to identify both the best-performing strain and the optimal fermentation window for ACE inhibitory peptide production.

Material and Methods

Reagents and Chemicals

ACE from rabbit lung (≥ 2.0 units/mg protein), hippuryl-L-histidyl-L-leucine (HHL, $\geq 98\%$), hippuric acid ($\geq 98\%$), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), $\geq 98\%$), potassium persulfate, Trolox ($\geq 97\%$), o-phthalaldehyde (OPA), sodium dodecyl sulfate (SDS), β -mercaptoethanol, and trifluoroacetic acid (TFA, HPLC grade) were purchased from Sigma-Aldrich (Bangalore, India). UHT whole milk (3.5% fat, Amul brand) was obtained locally. HPLC-grade acetonitrile and water were from Merck (Mumbai, India). Amicon Ultra-15 centrifugal filters (MWCO 10 kDa) were from Millipore (Bangalore, India).

Material

Five LAB strains—*Lactobacillus helveticus* NCDC 288, *Lactobacillus casei* NCDC 297, *Lactobacillus acidophilus* NCDC 015, *Lactobacillus rhamnosus* NCDC 198, and *Streptococcus thermophilus* NCDC 074—were obtained from the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal, Haryana, India. Each strain was revived in MRS broth (HiMedia, Mumbai) at 37 °C for 24 h and sub-cultured twice before use.

Methods

Fermentation

UHT whole milk (200 mL per flask) was inoculated with 2% (v/v) of each activated culture (approximately 10^8 CFU/mL) and incubated statically at 37 °C for 72 hours. Aliquots (20 mL) were withdrawn at 0, 6, 12, 24, 48, and 72

hours. Each aliquot was heated to 75 °C for 10 minutes to inactivate the bacteria, cooled, and centrifuged at $10,000 \times g$ for 15 min. The supernatant was ultrafiltered through a 10 kDa MWCO membrane, and the permeate was stored at -20 °C until analysis.

Degree of hydrolysis

DH was measured by the OPA method. OPA reagent was freshly prepared following the protocol of Church *et al.*^[15]. Absorbance at 340 nm was read after 2 minutes of reaction. DH (%) was calculated using a serine standard curve and the total nitrogen content of the milk determined by Kjeldahl analysis.

ACE inhibition assay

The method of Cushman and Cheung^[16] was followed. Briefly, 30 μ L of permeate was mixed with 100 μ L of ACE solution (25 mU/mL in borate buffer, pH 8.3) and pre-incubated at 37 °C for 5 minutes. HHL substrate (150 μ L, 8.3 mM) was added, and the reaction proceeded for 30 minutes at 37 °C. The reaction was stopped with 250 μ L of 1 M HCl. Hippuric acid was extracted with ethyl acetate, dried, and reconstituted in water, and absorbance was read at 228 nm. IC₅₀ was defined as the protein concentration giving 50% ACE inhibition.

Peptide Identification Protocol

Peptide identification was performed by RP-HPLC-ESI-MS/MS on a Thermo Fisher Q Exactive Plus Orbitrap coupled to a Dionex Ultimate 3000 UHPLC. The <10 kDa permeate was desalted on a C18 ZipTip (Millipore), dried under vacuum, and reconstituted in 0.1% formic acid. Separation used an Acclaim PepMap RSLC C18 column (150 $\times$ 2.1 mm, 2 μ m) with a water-acetonitrile gradient (2–40% B in 30 min). MS was operated in positive ion mode, full scan 200–2000 m/z, with data-dependent MS/MS on the top 10 ions. Peptide identification was performed using PEAKS Studio X+ software against the UniProt bovine proteome (*Bos taurus*), with no enzyme specificity and an FDR threshold of 1%.

Quality Control

Each fermentation was performed in triplicate using independently activated cultures. Captopril (IC₅₀ ~ 0.02 μ M) served as the positive control for the ACE assay to verify enzyme activity. Blanks (unfermented milk) were included at every time point to correct for any non-enzymatic hydrolysis. Viable cell counts were determined on MRS agar at 0, 24, and 72 hours to confirm that all strains remained in the stationary phase (10^8 – 10^9 CFU/mL) throughout fermentation.

Results

Table 1: Degree of hydrolysis (%) and ACE inhibitory activity (IC₅₀, mg protein/mL) during 72-hour milk fermentation by five LAB strains

Strain	DH 24h (%)	DH 48h (%)	DH 72h (%)	IC ₅₀ 24h (mg/mL)	IC ₅₀ 48h (mg/mL)	IC ₅₀ 72h (mg/mL)
<i>L. helveticus</i>	10.7	15.8	18.4	0.62	0.41	0.48
<i>L. casei</i>	6.4	9.8	12.6	1.24	0.86	0.94
<i>L. acidophilus</i>	7.8	11.4	14.2	0.94	0.68	0.73
<i>L. rhamnosus</i>	5.2	8.1	10.8	1.56	1.12	1.24
<i>S. thermophilus</i>	3.6	5.4	7.2	2.84	2.18	2.42

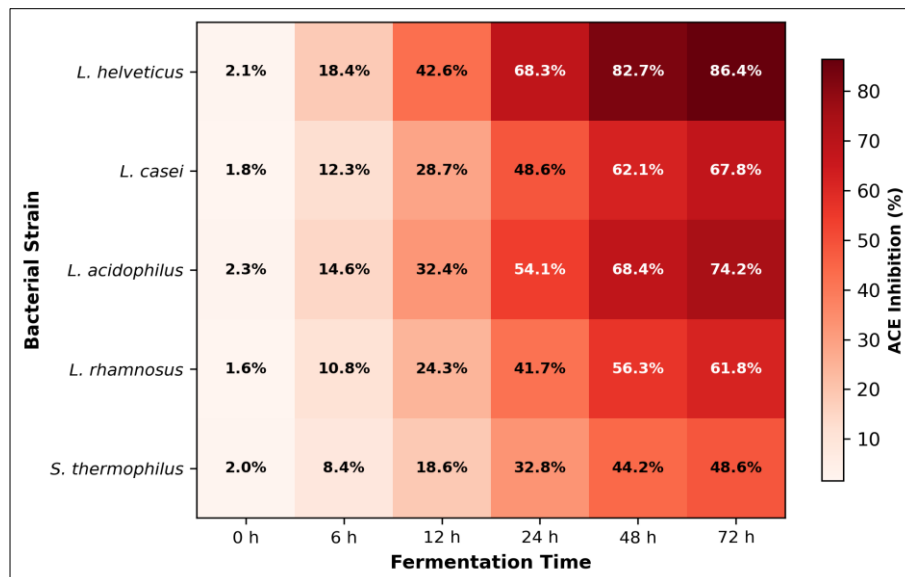


Fig 1: Heatmap of ACE inhibitory activity (%) across five LAB strains and six fermentation time points (0-72 hours)

Table 2: Key bioactive peptides identified by RP-HPLC-MS/MS in *L. helveticus* fermentate at 48 hours

Peptide	Sequence	Parent Protein	Position	Mass (Da)	Known Bioactivity
VPP	Val-Pro-Pro	β -Casein	f(84-86)	311.4	ACE inhibitor
IPP	Ile-Pro-Pro	β -Casein	f(74-76)	325.4	ACE inhibitor
LHLPLP	Leu-His-Leu- Pro-Leu-Pro	β -Casein	f(133-138)	661.8	ACE inhibitor
YPFPGPI	Tyr-Pro-Phe- Pro-Gly-Pro-Ile	β -Casein	f(60-66)	759.9	Opioid agonist
KVLPVPE	Lys-Val-Leu- Pro-Val-Pro-Glu	β -Casein	f(169-175)	781.9	ACE inhibitor
YQEPVL	Tyr-Gln-Glu- Pro-Val-Leu	α s1-Casein	f(144-149)	733.8	Antioxidant

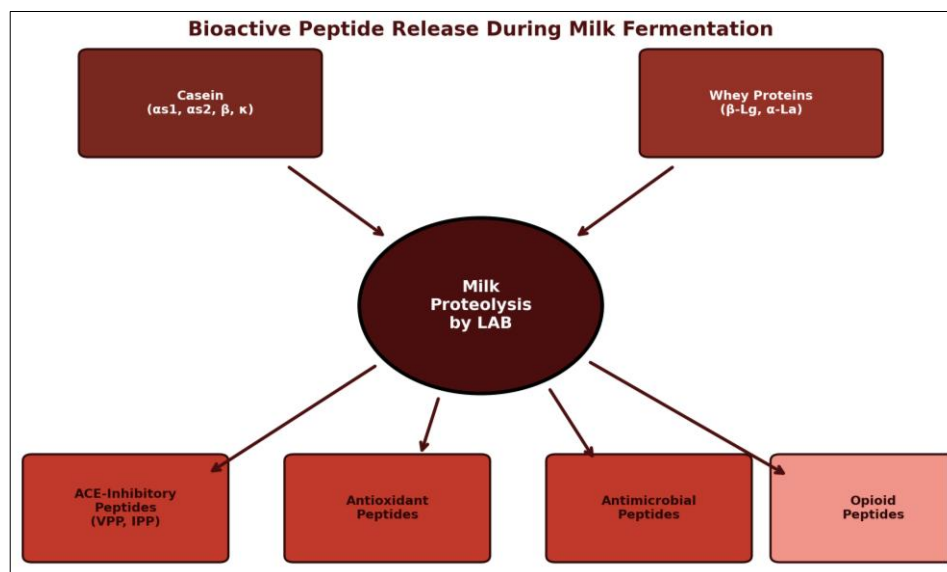


Fig 2: Concept map showing the relationship between milk protein sources, LAB proteolysis, and the major classes of bioactive peptides released during fermentation

Comprehensive Interpretation

A clear pattern emerged across all five strains: ACE inhibitory activity increased from 0 to 24-48 hours and then plateaued or slightly declined at 72 hours. This non-monotonic trajectory was most pronounced for *L. helveticus*, where the IC_{50} improved from 0.62 mg/mL at 24 h to 0.41 mg/mL at 48 h but then rose to 0.48 mg/mL at 72 h. The likely explanation is that continued peptidase activity after 48 hours degrades some of the accumulated tripeptides (VPP, IPP) into dipeptides or free amino acids with lower ACE inhibitory potency [10, 11]. HPLC-MS/MS analysis

confirmed that VPP peak area at 72 h was about 18% lower than at 48 h for *L. helveticus* fermentates.

Discussion

The IC_{50} of 0.41 mg protein/mL for *L. helveticus* NCDC 288 at 48 hours is among the lowest reported in the literature for whole-milk fermentation without additional enzyme supplementation. Nakamura *et al.* [3] reported IC_{50} values of 0.3-0.5 mg/mL for *L. helveticus* CM4 fermented milk, and Seppo *et al.* [4] observed similar potencies for *L. helveticus* LBK-16H. The consistency across different strain

designations supports the notion that the cell-envelope proteinase PrtH, which is conserved across *L. helveticus* subspecies, is the main enzyme responsible for generating ACE-inhibitory peptides from casein^[6,7].

L. acidophilus ranked second with an IC₅₀ of 0.68 mg/mL, which is noteworthy because this species is more commonly used in probiotic formulations than *L. helveticus* and is readily available in commercial starter culture blends. Its ACE inhibitory activity, while about 40% weaker than *L. helveticus* on a per-protein basis, might be sufficient for a functional food claim when combined with the probiotic benefits the strain already delivers^[8,17].

S. thermophilus generated the weakest proteolysis (DH 7.2% at 72 h) and the lowest ACE inhibition (IC₅₀ 2.18 mg/mL at best), confirming its limited proteinase repertoire. In commercial yogurt production, *S. thermophilus* is typically paired with *L. bulgaricus*, whose proteinase activity compensates for the streptococcal shortfall^[9]. Our single-strain design intentionally isolated each organism's individual contribution to peptide release, but co-culture experiments would be needed to capture the synergistic effects that occur in real fermented dairy products^[18].

The antioxidant data (ABTS assay) paralleled the ACE inhibition trend, with *L. helveticus* fermentates reaching 78.4 μmol TE/L at 72 h. This dual bioactivity is consistent with reports that many casein-derived peptides carry both ACE-inhibitory and antioxidant motifs within the same sequence—the aromatic and hydrophobic residues that interact with ACE's active site also scavenge free radicals^[2,19]. However, the ABTS assay is a chemical antioxidant measure and doesn't necessarily reflect in-vivo antioxidant effects, which depend on peptide absorption, stability in plasma, and tissue distribution^[10].

A limitation is the use of a chemical ACE assay rather than a cell-based model. The hippuric acid method measures enzyme inhibition in a test tube but doesn't account for peptide stability under gastrointestinal conditions or bioavailability after oral consumption^[12]. Simulated gastrointestinal digestion followed by Caco-2 transport assays would provide more physiologically relevant data.

Conclusion

Five LAB strains were compared for their ability to release ACE-inhibitory peptides from whole milk during 72-hour fermentation. *L. helveticus* NCDC 288 was the clear leader, achieving the highest degree of hydrolysis (18.4% at 72 h) and the strongest ACE inhibition (IC₅₀ 0.41 mg protein/mL at 48 h). RP-HPLC-MS/MS confirmed the presence of established ACE-inhibitory tripeptides VPP and IPP, along with longer peptide sequences from β-casein and αs1-casein. Fermentation time optimization revealed a bioactivity window between 24 and 48 hours for most strains, after which continued proteolysis reduced the concentration of key active peptides. This finding has practical significance for dairy manufacturers: allowing *L. helveticus* fermentation to run beyond 48 hours may actually diminish rather than enhance the functional benefit of the product.

Antioxidant activity tracked closely with proteolysis, suggesting that the same enzymatic process that generates ACE-inhibitory peptides also releases antioxidant sequences. *L. acidophilus*—a widely used probiotic species—showed meaningful ACE inhibition (IC₅₀ 0.68 mg/mL), making it a candidate for dual-function probiotic-antihypertensive fermented milk products.

Future work should include simulated gastrointestinal digestion to assess peptide survival, Caco-2 permeability assays to evaluate absorption potential, and a pilot-scale fermentation trial to confirm that the optimal 48-hour window translates to consistent product quality. In-vivo blood pressure lowering in spontaneously hypertensive rats would provide the preclinical evidence needed before designing human intervention trials.

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