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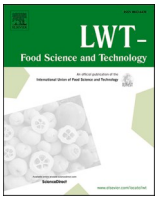

Synergistic use of fermentation and extrusion processing to design plant protein-based sausages

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ABSTRACT

The synergistic effect of lactic acid fermentation and high-moisture extrusion processing of pea protein on the microbiological and sensory properties of plant-based sausages was investigated. Plant-based sausages were formed by combining fermented pea protein concentrate (PPC) biomass with high-moisture extruded pea protein isolate (PPI). Pea protein concentrate (PPC) was fermented with *Lactococcus lactis subsp. lactis* and *Lactiplantibacillus plantarum* to improve the perceived structure, texture, and flavour (specifically via expression of glutamic acid which is connected to umami flavour) of plant-based sausages.

The sausages were prepared by stuffing the mixture of extruded PPI and fermented PPC (addition level 70:30) inside vegetarian casings followed by steam cooking. After preparation and cooking of the sausages, a trained sensory panel evaluated the intensities of ten selected attributes defining the flavour, odour, colour, and texture. In addition, dry matter content, acidification, microbial quality, and glutamate contents were analysed.

The results demonstrated that fermentation decreased the pea-like odour and improved the texture of the sausages. In addition, yeast-like odour and umami taste were observed. The study was able to demonstrate novel clean-label processing approaches by combined fermentation and extrusion to generate *in-situ* meat-like flavour and texture based on plant protein ingredients.

1. Introduction

Global demand for food production is estimated to increase 60% by 2050 to fulfil the dietary needs of the constantly growing human population (Alexandratos & Bruinsma, 2012). Traditional meat production is inefficient in protein conversion for every 1 kg of animal protein 6 kg of plant protein is required as feed (Pimentel & Pimentel, 2003). Furthermore, consumers' increased awareness of the adverse effects of meat consumption on the environment, human health, as well as ethical issues associated with animal farming has directed their interest towards plant-based foods (Y. Wang et al., 2022). To facilitate the transition towards a plant-based diet, it is essential to design novel, appealing meat alternatives based on diverse, sustainable protein sources. This transition is already visible in the market: the global plant-based meat market is predicted to reach USD 15.7 billion by 2027, with a compound annual growth rate of 14.7% (*Plant-Based Meat Market Players, Analysis Report (2027)*, 2022).

Plant protein isolates and concentrates are common ingredients in meat alternatives (Wang et al., 2022). Compared to protein concentrates, production of protein isolates requires water- and energy-intensive processing, which introduces sustainability challenges for their use (Schutyser, Pelgrom, van der Goot, & Boom, 2015). On the other hand, meat alternative products containing protein concentrates have been associated with inferior texture (e.g., softness, compactness, dryness) and sensory characteristics (e.g., bitterness, astringency, beany taste), limiting their applicability (Flores & Piornos, 2021; Y. Wang et al., 2022). Fermentation or extrusion processing of plant proteins have been reported to improve the flavour and texture characteristics of meat alternatives (Kaleda et al., 2020; Youssef et al., 2020). Plant proteins can be texturised with high-moisture extrusion processing (HMEP) to form similar fibrous structure to animal meat (Nisov, Nikinmaa, Nordlund, & Sozer, 2022). Fermentation has been demonstrated as a potential tool for flavour modification of plant proteins. Shi, Singh, Kitts, and Pratap-Singh (2021) reported successful degradation of off-flavours in pea protein isolate (PPI) by fermentation with *Lactiplantibacillus plantarum* (former name *Lactobacillus plantarum)*. In addition, lactic acid bacteria (LAB) fermentation has been shown to have a positive impact on the aroma, flavour and texture profiles of several plant proteins such as lupin and PPI (Schindler et al., 2011; Shi et al., 2021).

In general, consumers' acceptance towards plant-based meat alternatives is substantially reduced by the lack of meaty "umami" flavour

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Abbreviations

PPC	Pea protein concentrate
PPI	Pea protein isolate
HMEP	High-moisture extrusion processing
LAB	Lactic acid bacteria
TTA	total titratable acidity

(Giacalone, Clausen, & Jaeger, 2022). Free amino acids, nucleotides and peptides modify the overall taste of protein-based foods by interacting with other substances. These interactions can enhance sweet and salty taste or inhibit perception of sourness and bitterness (W. Wang, Zhou, & Liu, 2020). A diverse group of umami compounds have been identified including amino acids (glutamic acid, aspartic acid) and 5' nucleotides (inosine monophosphate (IMP) and guanosine monophosphate (GMP)) (W. Wang et al., 2020). The number of umami precursor compounds can be enriched during the fermentation process through proteolysis or by conversion of glutamine to glutamic acid with glutaminase enzyme (Diepeveen, Moerdijk-Poortvliet, & van der Leij, 2022). For instance, there is recent evidence of glutaminase activity and expression of L-glutamic acid, one of the main umami compounds, in LAB, (Lee et al., 2021).

Although it has been shown that fermentation induces development of umami flavour, this process has been underutilized in plant-based sausages lacking the umami taste. Instead, fermentation in plant-based meat alternatives has been mainly limited to filamentous fungi for biomass production (Quorn™ type of products) or as a binder for the structure (tempeh). Moreover, to the best of our knowledge, there are no previous studies focusing on synergistic use of high-moisture extrusion with fermentation in the production of plant-based sausages. The present study elucidates the effects of combined HMEP and fermentation in novel plant-based sausage models. Furthermore, the study strives to evaluate the role of fermentation and extrusion especially in structure and flavour formation.

2. Materials and methods

2.1. Raw materials

This study investigated commercially available Pea Protein Isolate (PPI) (Nutralys F85M, Roquette, Lestrem, France, 89% dm protein), Pea Protein Concentrate (PPC) (Pea protein F55X, Vestkorn, Tau, Norway, 55% dm protein), and corn starch (Maizena, Unilever, Hamburg, Germany). Corn starch was added to the mixture of PPI and PPC to improve the chewiness and elasticity of the extrudates.

2.2. Bacterial strains, cultivation, and preparation of the inoculum

Lactococcus lactis subsp. *lactis* (VTT E−991172) and *Lactiplantibacillus plantarum* (VTT E−78076) strains were obtained from the VTT Culture Collection (www.culturecollection.vtt.fi). The strains were selected based on a comprehensive screening of several LAB strains for their ability to acidify the plant protein matrix and a survey of their known characteristics. The strains were routinely cultivated in General Edible Medium (GEM) broth (Saarela et al., 2004) at +30 °C. To prepare the inoculant, the 24-h cell cultures were collected by centrifugation (4000 rpm, 15 min), washed twice, and resuspended in sterile distilled de-ionized water (DDIW) to obtain the desired cell density. Cell suspensions at a density of 6.0 log colony-forming units per gram (cfu/g) were used to inoculate the PPC matrix.

2.3. Production of fermented pea protein concentrate with selected strains

PPC was fermented with either: 1. *L. lactis* (VTT E−991172), 2. *L. plantarum* (VTT E−78076) or 3. as a co-cultivation of *L. lactis* (VTT E−991172) and *L. plantarum* (VTT E−78076). In addition, two control samples, spontaneous fermentation and chemically acidified control, were prepared. In spontaneous fermentation the raw material was incubated without applying the starter. The chemically acidified control was prepared by adjusting the pH to 4.0 with lactic acid followed by incubation. The PPC fermentations were prepared with a total weight of 450 g using glass bottles with loosely covered tops. Each batch contained 74% (w/w) sterile DDIW including the inoculum (target 6.0 log cfu/g), 23% (w/w) PPC and 3% (w/w) of sucrose. The ingredients were weighed and mixed into each bottle followed by incubation at 30 °C for 24 h in aerobic conditions.

2.4. Acidification of the fermented pea protein concentrate

The ability of the strains to acidify the protein concentrate was analysed by measuring pH and total titratable acidity (TTA). The pH value was measured before and after the fermentation with a pH meter (Basic pH meter, Denver Instrument, Denver, CO, USA) containing a double junction pH electrode (ORION 9110DJWP, Thermo Scientific, Waltham, MA, USA). TTA was analysed with an automated titrator (T50, Mettler Toledo, Columbus, OH, USA) by diluting 5 g of sample into 50 mL of DDIW and titrating against 0.1M NaOH until pH 8.5 was obtained. Results were expressed as mL NaOH consumed per g of sample.

2.5. Microbiological assessment of the fermented pea protein concentrate biomass and plant-based sausages

The cell counts of aerobic heterotrophic bacteria, aerobic spore-forming bacteria, lactic acid bacteria, and yeasts and molds were determined to assess the microbiological quality of the fermented PPC. The bacterial counts of aerobic heterotrophic bacteria and aerobic spore-forming bacteria were analysed on TSA (Tryptic Soya Agar, BD Difco™, Heidelberg, Germany) supplemented with 0.001% cycloheximide (SR0222C, Oxoid, Hampshire, UK). To enumerate the spore-forming bacteria, the sample was heated in a water bath at80 °C for 10 min to inactivate vegetative cells before cultivation. LAB, yeasts and mold cell counts were determined on MRS (De Man, Rogosa and Sharpe, CM0361, Oxoid, Hampshire, UK) supplemented with 0.001% cycloheximide and YMA (Yeast and Mold agar, 271210, BD Difco™, Heidelberg, Germany) supplemented with 0.01% chloramphenicol (C0378, Sigma-Aldrich, St. Louis, MO, USA), 0.01% chlortetracycline (430960250, Acros organics, Geel, Belgium) and 0.02% Triton™ X-100 (M143, VWR Chemicals, Radnor, PA, USA), respectively. TSA and MRS plates were incubated at 30 °C for 2 days. MRS plates were cultivated in an anaerobic atmosphere created with the Anoxomat Mart II system (Mart Microbiology B.V.®, Lichtenvoorde, Netherlands). YMA plates were incubated at 25 °C for 5 days.

In addition, cell counts of *Bacillus cereus* (Mannitol Egg Yolk Polymyxin agar, CM0929B, SR0047, SR0099, Thermo Scientific, Waltham, MA, USA) and coliforms (Chromocult® Coliform agar, VWR Chemicals, Radnor, PA, USA) were determined to ensure sufficient microbiological quality of the sausages. The plates were incubated at 30 °C for 2 d and 37 °C for 24 h, respectively.

2.6. High-moisture extrusion processing of pea protein isolate

A mixture containing 80% PPI, 10% PPC and 10% of corn starch was extruded with a twin-screw extruder (Process 11 Hygienic, screw diameter 11 mm, L/D 40:1) equipped with a cooling die. The schematic drawing of the extruder used is reported in Nisov et al., 2022 study. The drawing describes the heating zones, water feed position and the long slit cooling die in more detail. The temperature profile was set to

155-155-155-155-90-80-70-60 °C (from die to feed), flour feed rate to 300 g/h, water feed rate to 300 g/h, screw speed to 200 rpm, and cooling die temperature to 50 °C. Extrudates were collected as strips to zip lock bags and frozen (−18 °C) until further processing. Prior to processing into sausages, the extrudates were thawed and coarsely ground with a meat grinder (MG450, Kenwood, Havant, UK).

2.7. Preparation of plant-based sausages

The filling for plant-based sausages was prepared by combining the coarsely ground extrudates with fermented PPC biomass. The target dry matter content for the sausages was 40% (w/w) of which 70% (w/w) was the extruded ingredient and 30% (w/w) fermented PPC in dry matter basis. In addition, 0.5% (wet w/w) salt was supplemented into the mixture. Control samples were prepared similarly except that the fermented PPC was replaced with either native PPC (native control) or chemically acidified PPC where the pH was adjusted to 4.0 with lactic acid (acid control).

The sausages were prepared by stuffing the mixture inside the vegetarian casings (19 mm Veggie casing, Viscofan, Navarra, Spain) with a meat grinder combined with sausage stuffer equipment (MG450, Kenwood, Havant, UK). The prepared sausages were steam cooked for 5 min and stored in at −20 °C until further analysis.

2.8. Total titratable acidity, pH, dry matter determination and glutamic acid content of vegetarian sausages

Sausages pH and TTA were analysed as described in section 2.4.

Sausages dry matter content (DMC) of the was determined gravimetrically by drying the samples at 105 °C until constant weight was achieved. The results were expressed as g/100g.

For glutamic acid analysis, the samples were freeze-dried (Alpha 1–4 LSC basic, Christ®, Osterode, Germany) and ground with mortar. An aliquot of 100 mg of each sample were suspended into 0.9 mL potassium phosphate buffer (50 mM, pH 7.0) and mixed with vortex to obtain a homogenous suspension. Samples were incubated at room temperature for 10 min, centrifuged at 6000 rpm for 5 min and filtered before analysis (0.45 μm pore size). The glutamic acid content was determined spectrophotometrically using a commercially available assay kit (K-GLUT, Megazyme, Wicklow, Ireland). The assay was performed according to manufacturer's instructions except 232 μL reaction volume for the microtiter plate method. The absorbance was measured at 492 nm (Multiscan SkyHigh, Thermo Scientific, Waltham, MA, USA). The results were expressed as mg/100g dry matter.

2.9. Sensory profiling of the vegetarian sausages

The vegetarian sausages were thawed at room temperature and tempered in oven at 40 °C for 10 min before the evaluation. Samples were served in transparent containers which were randomly coded with three-digit numbers. The descriptive profiling was performed with a trained sensory panel consisting of 8 assessors following a previously reported protocol (Pöri, Aisala, Liu, Lille, & Sozer, 2023). Before the evaluation, the descriptive terms and intensity scales in addition to reference standards were defined by the assessors followed by a training session to clarify the selected terms. In total, the intensities of 10 attributes were evaluated (pea-like odour, sour odour, yeast-like odour, colour darkness, pea-like flavour, sour taste, umami taste, sweet taste, number of chewings, graininess) on a 0–10-line scale. The evaluation was performed in duplicate. The data were collected and analysed statistically with Eyequestion software version 5.0.8.5. with EyeOpenR (Logic8 BV., Elst, the Netherlands). The results were analysed with a two-way mixed model analysis of variance (ANOVA) with samples as a fixed factor and assessors as a random factor, and $p < 0.05$ as the limit for significance.

3. Results and discussion

3.1. Microbiological assessment and acidification of the fermented pea protein concentrate

Before the fermentation with selected strains, the LAB and total aerobic heterotroph cell counts were 2.1 log cfu/g and 3.6 log cfu/g, respectively (Table 1). During fermentation, the LAB cell counts increased from 2.1 log cfu/g to 9.6–10.5 log cfu/g resulting in good acidification of the PPC matrix. The fermentation with selected strains decreased the pH to 4.3 with TTA values of 2.1–2.3 mL/g resulting in good acidification of the PPC matrix. To avoid the growth of typical food spoilage microbes and food-borne pathogens, fast acidification, and pH levels under 4.5 are preferred (Pernu, Keto-Timonen, Lindström, & Korkeala, 2020; Sperber, 2009). The decrease in pH during fermentation is strongly affected by the buffering capacity of the raw material (Zare, Champagne, Simpson, Orsat, & Boye, 2012). Pea protein has been confirmed to have a high buffering capacity over a pH range from 4 to 10 indicating that a high acidifying potential of the strains is required to decrease the pH to desired levels (Reinkensmeier, Bußler, Schlüter, Rohn, & Rawel, 2015).

Spontaneous fermentation resulted in relatively high levels of total aerobic heterotrophic bacteria, spore formers and lactic acid bacteria (>5.0 log cfu/g) with final pH of 4.9 and TTA value of 1.5 mL/g. The pH of the chemically acidified control was rapidly decreased from 6.4 to 4.1 prior to the incubation step which suppressed the growth of aerobic heterotrophic bacteria and spore-forming bacteria, as well as inhibited the growth of LAB. This indicates that fast acidification of the raw material is required to avoid the growth of undesirable microbes and to achieve microbiologically safe products.

3.2. Microbiological assessment, pH, TTA and glutamate content of the sausages

The pH, TTA and dry matter content of the sausages are shown in Table 1. Sausages containing fermented PPC resulted in lower pH (5.4–5.6) while the pH of native control was 6.8. In addition, higher amounts of acids were detected in fermented PPC samples. The microbiological assessment (Table 1) revealed minimal growth of any microbial groups, which demonstrates that good hygienic processing practices were followed during the sausage making, and that the heat-treatment of the samples was successful.

The glutamic acid content of the sausages was analysed to evaluate the potential of fermentation to produce and enhance the umami taste (Table 1). The L-glutamic acid and its ionic form, glutamate, is one of the main umami taste compounds (W. Wang et al., 2020). A minor increase in the L-glutamic acid content (1.7–7.4 mg/100 g) was observed compared to the native sample during fermentation. Interestingly, the L-glutamic acid content was significantly lower (59.2 mg/100g) in the chemically acidified control compared to native control (92.2 mg/100 g dry). This might be explained by the interactions and changes in proteins or amino acids caused by the fast acidification.

3.3. Sensory profile

The results of the sensory analysis are presented in Figs. 1 and 2. Fermentation and acid incubation (chemically acidified control) decreased the pea-like odour ($p < 0.05$ vs the control sample) of the sausages. The sausage containing *L. lactis* scored the highest values in umami taste ($p < 0.05$ vs the control sample), while the sausage containing *L. plantarum* scored highest for texture attributes, including graininess ($p < 0.1$ vs the control sample, defined as a high degree of texture left after chewing) and number of chewings ($p < 0.05$ vs the control and co-cultivated samples) required before ingestion. Thus, they exhibited the closest resemblance to meaty characteristics. The chemically acidified control showed a similar outcome related to the texture

Table 1
Total titratable acidity, pH, L-glutamic acid content and Microbiological assessment results of fermented pea protein concentrate (Fer) and plant-based sausages (Sau).

Sample	Ctrl		Acid Ctrl		Spontaneous		*L. lactis*		*L. plantarum*		Co-cultivation	
Fermented PPC/sausage	Fer	Sau	Fer	Sau	Fer	Sau	Fer	Sau	Fer	Sau	Fer	Sau
pH	6.4	6.8	4.1	5.6	4.9	na	4.3	5.4	4.3	5.6	4.3	5.5
Total titratable acids (mL/g)	0.6	0.3	2.3	1.0	1.5	na	2.1	1.1	2.1	0.8	2.1	1.0
Dry matter content (g/100 g)	na	39.3 (±0.4)	na	40.4 (±0.3)	na	na	na	39.0 (±0.4)	na	38.9 (±0.1)	na	40.7 (±0.4)
l-glutamic acid content (mg/ 100g dry)	na	90.5 (±0.9)	na	59.2 (±0.4)	na	na	na	92.2 (±1.5)	na	97.9 (±2.1)	na	92.6 (±3.4)
Total aerobic heterotrophs (cfu/g)	3.6 log	nd	nd	nd	>5.0 log	na	7.2 log*	nd	>6.0 log*	nd	7.2 log*	nd
Aerobic spore-forming bacteria (cfu/g)	nd	2.2 log	nd	2.3 log	>5.0 log	na	nd	2.6 log	nd	2.5 log	nd	1.7 log
Yeasts and Molds (cfu/g)	nd	nd	nd	nd	nd	na	nd	nd	nd	nd	nd	nd
Lactic acid bacteria (cfu/g)	2.1 log	nd	2.1 log	nd	>5.0 log	na	9.7 log	7.8 log	10.5 log	<7.0 log	9.6 log	7.0 log
B. cereus (cfu/g)	na	nd	na	nd	na	na	na	nd	na	nd	na	nd
Coliforms (cfu/g)	na	nd	na	nd	na	na	na	nd	na	nd	na	nd

na = not analysed, nd = not detected, * = growth of LAB on TSA.

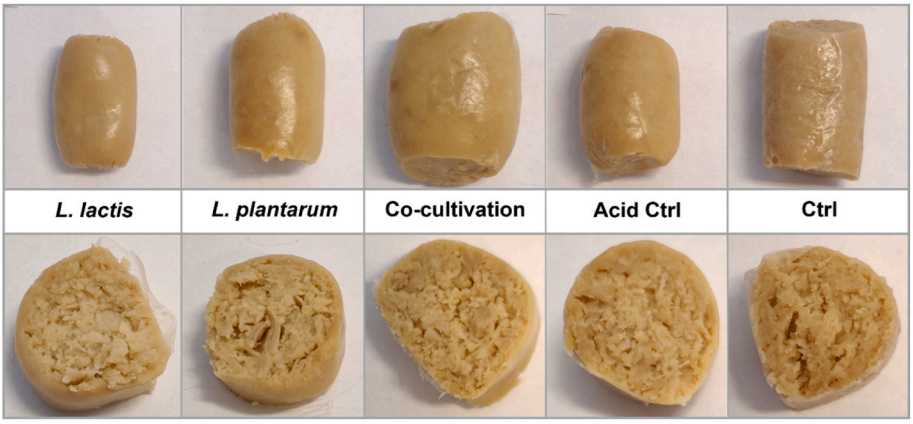

Fig. 1. Surface and cross-sectional pictures of the sausages.

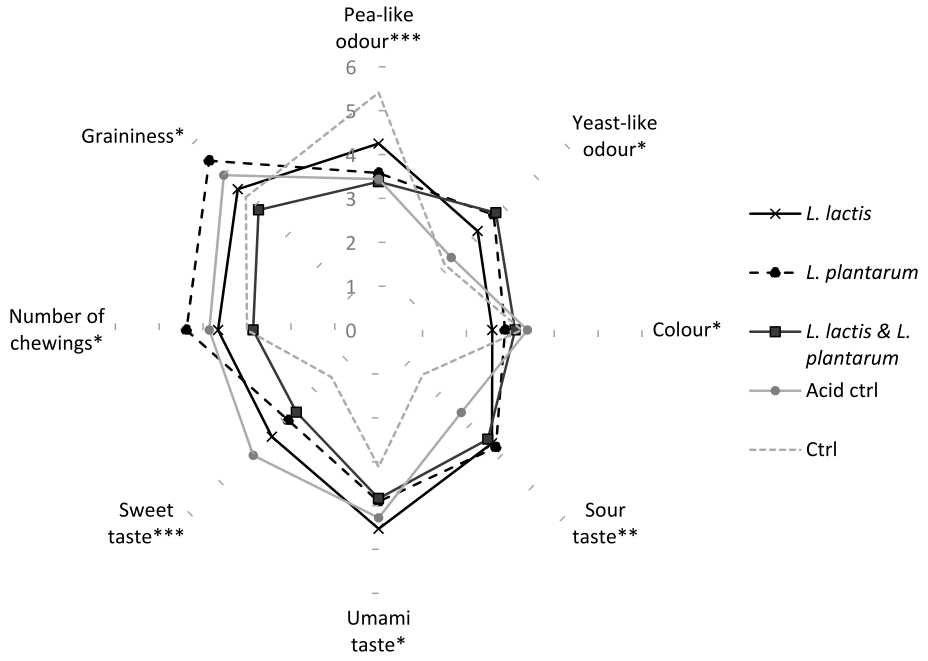

Fig. 2. Sensory profiles of the plant-based sausages. Only attributes with statistically significant differences in the two-way mixed model ANOVA (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$)are displayed.

and umami flavour attributes. These attributes were lower in intensity in the co-cultivated sample. The texture improvement in acid control was most likely caused by acid-induced gelation where the pH decreases near the isoelectric point of the protein, which reduces the protein solubility, and thus, promotes protein aggregation (Klost & Drusch, 2019; Ma et al., 2022; Yang, Su, & Li, 2020). On the other hand, the sausage containing *L. plantarum* indicated that fermentation improved the texture by gelation through beneficial proteolytic activity of the strain (Ju, Otte, Madsen, & Qvist, 1995; Ren & Li, 2022).

Yeast-like odour (defined as the odour of proofing bread) and umami taste could be associated with meat-like characteristics (Maga, 1994; Raza et al., 2020). Ferrocino et al. (2018) applied a metagenomic approach to understand the connections between the microbiota, microbiome and release of volatiles during ripening by utilising a commercial starter culture (mix of *Lactobacillus sakei* and *Staphylococcus xylosus*) in meat-based sausages. The group found that carbohydrate metabolism was mainly contributing to the generation of precursor volatiles such as acetate, acetoin, diacetyl, acetic acid and isobutyric acid. During the ripening process the volatiles were evolved into for example 3-methyl-2-buten-1-ol which is a yeasty odour descriptor. However, formation of yeast-like odour and umami taste during fermentation is rather complex where proteins and lipid hydrolysis generate aroma precursors (e.g. free fatty acids, and free amino acids) which contributes to formation ofvolatile aroma compounds (Flores & Piornos, 2021).

In our study both yeast-like odour ($p < 0.1$) and umami taste were perceived more intensely in the fermented sausages while the native control scored the lowest values. Acid control showed the second most intensive value for umami, whereas the yeast-like odour scored almost the lowest value. Umami taste compounds usually impact the overall perception of umami taste through synergistic interactions with other taste compounds (Mouritsen, Duelund, Calleja, & Frøst, 2017; W. Wang et al., 2020). Several studies have shown the effect of fermentation through proteolysis on enhancing the umami flavour in foods by both releasing high levels of free amino acids, especially glutamic acid and aspartic acid, as well as free flavour nucleotides, such as inosine monophosphate (IMP) and guanosine monophosphate (GMP) (Chen, Gao, et al., 2021; Hartley, Liem, & Keast, 2019; Mouritsen et al., 2017; Zhao et al., 2018). In addition, glutamine can be converted to L-glutamate via glutaminase activity of the selected strain during fermentation (Diepeveen et al., 2022). As for the L-glutamate analysis, no significant differences were observed indicating the detected umami taste might be caused by the protein hydrolysis and release of nucleotides rather than the glutaminase activity of the strains (Chen, Fang, et al., 2021; Chen, Gao, et al., 2021).

Fermented sausages showed increased sour taste ($p < 0.05$) compared to the native control, which correlated well with the pH and TTA results. This is explained by the organic acids and volatile compounds produced during fermentation, which can be perceived as sourness (Kaleda et al., 2020; Laaksonen et al., 2021). Interestingly, acid control was not perceived as sour as the fermented sausages although they exhibited similar TTA and pH values. The more intense sweet taste ($p < 0.01$) was perceived in the fermented samples and in the acid control. This outcome might be an indication of incomplete sugar consumption during fermentation in addition to protein hydrolysis releasing sweet peptides and amino acids, such as serine, proline, glycine, threonine, alanine (Yamamoto et al., 2014).

4. Conclusions

The present study demonstrated the feasibility of combined HMEP and lactic acid fermentation in production of novel plant-based sausages. The application of fermentation showed successful degradation of pea-like odours and exhibited the closest resemblance to meaty characteristics in terms of texture attributes. In addition, yeast-like odours and umami taste were formed and perceived in fermented samples. This study demonstrated novel pathways for further studies to develop appealing plant-based products. The authors highlight that more comprehensive studies on characterization of the sausages are required to evaluate the possible flavour, colour and textural changes during storage. For example, protein and lipid oxidation pathways and how they contribute to flavour formation could be included in future studies. Moreover, metabolic pathways of diverse categories of strains and co-cultures could be studied to gain a wider understanding of the effect on the aroma and flavour formation in plant protein-based sausages.

CRediT authorship contribution statement

Anniina Valtonen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Heikki Aisala:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, reviewing & editing. **Anni Nisov:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Markus Nikinmaa:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, writing – editing. **Kaisu Honkapää:** Conceptualization, Writing – review & editing, Supervision. **Nesli Sozer:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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