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Lactococcus cell envelope proteases enable lactococcal growth in minimal growth media supplemented with high molecular weight proteins of plant and animal origin

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Abstract

Lactic acid bacteria (LAB) have evolved into fastidious microorganisms that require amino acids from environmental sources. Some LAB have cell envelope proteases (CEPs) that drive the proteolysis of high molecular weight proteins like casein in milk. CEP activity is typically studied using casein as the predominant substrate, even though CEPs can hydrolyze other protein sources. Plant protein hydrolysis by LAB has rarely been connected to the activity of specific CEPs. This study aims to show the activity of individual CEPs using LAB growth in a minimal growth medium supplemented with high molecular weight casein or potato proteins. Using *Lactococcus cremoris* MG1363 as isogenic background to express CEPs, we demonstrate that CEP activity is directly related to growth in the protein-supplemented minimal growth media. Proteolysis is analyzed based on the amino acid release, allowing a comparison of CEP activities and analysis of amino acid utilization by *L. cremoris* MG1363. This approach provides a basis to analyze CEP activity on plant-based protein substrates as casein alternatives and to compare activity of CEP homologs.

Keywords: *Lactococcus cremoris*; cell envelope proteases; minimal growth medium; proteolysis; plant protein; amino acid secretion

Introduction

Lactic acid bacteria (LAB) play an important role in traditional fermented foods. Several LAB have highly adapted proteolytic systems that can induce protein changes in food raw materials (Christensen et al. 2022). Peptides liberated from plant proteins, like potato proteins, can entail bioactivities such as antioxidant and emulsifying activities (García-Moreno et al. 2020b). Additionally, amino acid (AA) liberation can affect pH and flavor formation (Gänzle 2015).

LAB have adapted to nutrient rich habitats, becoming fastidious microorganisms with complex nutritional requirements (Makarova et al. 2006). Several LAB are auxotrophic for a number of AAs, and only some LAB possess a proteolytic system to satisfy their AA requirements. The proteolytic system of *Lactococcus cremoris* [formerly *Lactococcus lactis* subsp. *cremoris* (Li et al. 2021)] has been characterized in dairy fermentations. Among the milk proteins, caseins have been shown to be the preferred substrates for the proteolysis (Savijoki et al. 2006). A cell-wall anchored protease, PrtP, hydrolyzes casein into oligopeptides of 4 to 30 AAs with no or minor release of di- or tripeptides or free AAs (Konings 2002, Juillard et al. 1995a). Oligopeptides of 4 to 35 AAs can be transported across the cell membrane into the bacterial cell by the oligopeptide transport system (Opp) of ABC transporters (Berntsson et al. 2011). During lactococcal growth in milk, oligopeptides are preferred as AA source over available free AAs (Juillard et al. 1995b). Following the peptide uptake, intracellular peptidases hydrolyze the oligopeptides into free AAs for protein biosynthesis. Nitrogen metabolism based on oligopeptide uptake rather than individual AAs is not likely to generate AAs in the balanced ratio required for protein biosynthesis (Konings 2002), leaving an intracellular excess of certain AAs. The excess of AAs can be precursors for other AAs, metabolic intermediates, or volatile organic compounds (VOCs) like alcohols, aldehydes, and esters (Konings 2002, Smit et al. 2005). AAs in excess can also be secreted from the cytoplasm into the environment (Hernandez-Valdes et al. 2020) via specific lactococcal symporter systems (Konings 2002).

Increasing levels of intracellular branched-chain AAs stimulate the repression by the CodY regulon in *L. cremoris* (den Hengst et al. 2005a). CodY is a repressor protein, which targets the promoter region of *prtP*, *prtM*, and genes encoding other proteins of the proteolytic system and AA metabolism (den Hengst et al. 2005b, Gajic 2003). The maturase PrtM is a prolyl cis/trans isomerase, which is required for proper activation of some, but not all PrtP homologs (Siezen 1999, Liu et al. 2010, Ikolo et al. 2015).

LAB from both dairy and plant-based habitats can possess PrtP with similar structures (Christensen et al. 2023), though dairy associated PrtP homologs dominate the literature dealing with biochemical characterization of this protease group (Christensen

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et al. 2022). The most studied PrtP homologs are from dairy associated *L. cremoris* NCDO712, SK11, and Wg2 (Gasson 1983, Kok et al. 1988, de Vos et al. 1989). PrtP homologs have, based on their selective cleavage of the casein proteins, been divided into two major types, PI and PIII (Exterkate et al. 1993, Savijoki et al. 2006). $PrtP_{Wg2}$, a PI-type, primarily hydrolyses β-caseins and to lesser extent κ-caseins, whereas $PrtP_{SK11}$, a PIII-type, degrades α_{s1}, β-, and κ-caseins equally well. $PrtP_{SK11}$ and $PrtP_{Wg2}$ differ in 45 positions of their first 1800 AAs (Christensen et al. 2023) and share high sequence identity with other less characterized PrtP homologs like $PrtP_{MS22333}$ of *L. lactis* MS22333, from which they differ in 68 and 50 AA positions, respectively (Hansen and Marcatili 2020). The substrate selectivity and specificities of these and other PrtP homologs have been studied with casein proteins and peptides as substrates. However, the underlying mechanisms for the proteolytic diversity among PrtP homologs remain to be clarified.

The specific activity of extracellular proteases on plant protein substrates has rarely been analyzed (Christensen et al. 2022). Plant proteins have different structural-physiochemical properties than casein (Sim et al. 2021), necessitating other medium conditions to prevent precipitation of the proteolytic target, besides balancing the optimal conditions for LAB growth and PrtP activity (Christensen et al. 2022). Plant-based protein suspensions with protein concentrations similar to or higher than dairy products may contain low molecular weight peptides for LAB utilization. Such peptides will activate the CodY repression of PrtP expression besides stimulating LAB acidification, blurring the impact of PrtP activity on LAB growth and metabolite production.

This study analyzes PrtP activity in a chemically defined medium, providing the minimal growth requirements of *L. cremoris* with a high molecular weight protein source. Potato protein is applied as a plant protein alternative to the known casein substrate of the studied dairy associated PrtP homologs. Extracellular proteolytic activity is directly related to LAB growth of strains carrying PrtP homologs inserted into *L. cremoris* MG1363 as isogenic background.

Methods

Strains and plasmids

Bacterial strains and plasmid constructs used in this study are listed in Table 1. The strains were propagated in M17 broth with 0.5% (w/v) glucose or on corresponding agar plates at 30°C. Additionally, 5 µg/mL erythromycin (Ery) was added for selection of Ery-resistant strains (Ery^R).

Genomic DNA of the wild-type *L. cremoris* and *L. lactis* strains was purified, using the procedure of the DNeasy UltraClean Microbial Kit (Qiagen). The Plasmid Mini AX kit (A&A Biotechnology) was used to purify recombinant plasmids of *L. cremoris* (Table 1, Fig. S1), following the protocol of the manufacturer with an extended cell lysis step. Resuspended cells in lysis buffer were mixed with 8000 U lysozyme (A&A Biotechnology) and 100 U mutanolysin (A&A Biotechnology), following incubation at 50°C for 30 min.

Recombinant plasmids were constructed following the procedure of the Gibson Assembly Master Mix (New England Biolabs), using linear DNA with designed overlapping termini of the pAK80 vector fragment (5151 bp) and the *prtM/prtP* inserts regions (6962-7325 bp) from the wild-type strains SK11, Wg2 and MS22333 (Table 1). Linear DNA was PCR amplified with specific oligonucleotide primers (TAG Copenhagen A/S) and Phusion™ High-Fidelity DNA Polymerase (Thermofisher) (Table S1). PCR amplified linear DNA was extracted from SYBR Safe (ThermoFisher) stained tris-acetate-EDTA (TAE) buffered agarose gels using the QIAEX II Gel Extraction Kit (Qiagen). Purified recombinant plasmids were validated by Sanger sequencing, using the Mix2Seq Kit (Eurofins Genomics) with the primers pAK80_seq_fw and pAK80_seq_rv (Table S1). Recombinant plasmids of the Gibson reaction mix were directly transferred into electrocompetent *L. cremoris* MG1363 by electroporation (Holo and Nes 1989).

Minimal growth medium

The chemically defined medium (CDM) was inspired by a minimal growth medium developed for *L. cremoris* (Jensen and Hammer 1993) (Table S2). Our CDM did not contain any AAs, but instead each CDM contained a high molecular weight protein source. CCDM contained casein (Casein Sodium Salt from Bovine Milk, 9005–46-3, Sigma) and PCDM contained potato protein (soluble food-grade protein extract, KMC-food, Brande, Denmark) as nitrogen source. HCDM contained casein hydrolysate (65072–00-6, OXOID) as a low molecular weight source of organic nitrogen.

Protein stock solutions (0.3% (w/v) were prepared, solubilizing protein powder in distilled water before autoclaving (121°C, 15 min). 10 x MOPS [3-(N-Morpholino)propanesulfonic acid] solution and 100 x vitamin solution were prepared (Neidhardt et al. 1974, Jensen and Hammer 1993) with 1.5 mM Na-acetate and without NaCl. To obtain the final media with a pH of 7.0 and a protein content of 0.25% (w/v), 10 x MOPS solution, 100 x vitamin solution, 0.132 M K_2HPO_4, and 1 M Glucose were added to the protein stock solutions in the specified order (Table S2).

Batch culture cultivation

The inoculum for growth experiments was prepared from overnight cultures of *L. cremoris* strains cultivated in M17 with glucose and Ery. Cells were harvested by centrifugation (4000 x g, 10 min, 4°C) and washed in 0.9% (w/v) NaCl followed by an extra centrifugation step. The washed cells were resuspended in 0.9% (w/v) NaCl and standardized to OD_{600} of 1.0. All growth experiments used 1% (v/v) of the standardized washed cells as inoculum. Sterile water was added instead of the inoculum for negative controls. Biological replicas were made using different colonies of each strain.

Homogenized and pasteurized skimmed milk (0.1% fat, Arla, Denmark), supplemented with 1.0% (w/v) glucose and 5 µg/ml Ery, was used in the milk fermentation as control experiment. The bacterial cultures were cultivated as 100 mL standing batch cultures at 30°C under aseptic conditions. Milk acidification was monitored using an iCinac Wired L.A.B Fermentation Monitor (AMS Alliance) for pH measurements every minute.

Freshly prepared CDM, HCDM, CCDM and PCDM were supplemented with 5 µg/mL Ery and cultivated as 45 mL batch cultures in 50 mL tubes with screw caps. The cultures were incubated as standing cultures at 30°C and were handled under sterile conditions during sampling for pH, colony forming units (CFUs), and metabolite measurements. CFUs were counted on Ery selective M17 agar plates, making relevant serial culture dilutions in peptone physiological salt solution.

Extracellular metabolite analyses

Cells were immediately removed from the batch culture samples by centrifugation (13 000 × g, 10 min, 4°C). The supernatant was stored at –20°C until sample preparation for high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) analyses. Glucose and lactate as well

Table 1. Bacterial strains and plasmid constructs used in this study.

Strains and plasmids	Description[a]	Source or reference
Strains		
Lactococcus cremoris subsp. *cremoris* MG1363	Recombinant, Plasmid-free *L. cremoris* NCDO712	(Gasson 1983, Liu et al. 2010)
L. cremoris subsp. *cremoris* MS22418	MG1363 derivate with pAK80, Ery^R, $PrtP^-$	This work
L. cremoris subsp. *cremoris* MS22421	MG1363 derivate with plasmid pIL18, Ery^R, $PrtP^+$ ($PrtP_{SK11}$: DQ149245.1)	This work
L. cremoris subsp. *cremoris* MS22425	MG1363 derivate with plasmid pIL22, Ery^R, $PrtP^+$ ($PrtP_{Wg2}$: P16271)	This work
L. cremoris subsp. *cremoris* MS22427	MG1363 derivate with plasmid pIL24, Ery^R, $PrtP^+$ ($PrtP_{MS22333}$: WWDI00000000)	This work
L. cremoris subsp. *cremoris* SK11	WT strain, $PrtP^+$ ($PrtP_{SK11}$: DQ149245.1)	(Siezen et al. 2005)
L. cremoris subsp. *cremoris* Wg2	WT strain, $PrtP^+$ ($PrtP_{Wg2}$: P16271)	(Otto et al. 1982, Haandrikman et al. 1990)
Lactococcus lactis subsp. *lactis* MS22333	WT strain, $PrtP^+$ ($PrtP_{MS22333}$: WWDI00000000)	(Bragason et al. 2020, Hansen and Marcatili 2020)
Plasmids		
pAK80	Contains the minimal theta replicon of pCT1138, Ery^R	(Israelsen et al. 1995)
pIL18	pAK80 derivate containing the *prtP/prtM* genic region of *L. cremoris* SK11, Ery^R	This work
pIL22	pAK80 derivate containing the *prtP/prtM* genic region of *L. cremoris* Wg2, Ery^R	This work
pIL24	pAK80 derivate containing the *prtP/prtM* genic region of *L. lactis* MS22333, Ery^R	This work

[a]Erythromycin resistance (Ery^R), Accession number of PrtP homologs are from either the UniProt database or the NCBI database

as acetoin, acetate, ethanol, formate, lactose and pyruvate were quantified by HPLC (van Rijswijck et al. 2017). To quantify AAs, UPLC was used and comprised a deproteinization and a derivatization step on undiluted samples with L-norvaline as internal standard (Scott et al. 2021).

For VOCs measurement, 2 mL of each batch culture was directly transferred to 10 mL GC vials. Samples were kept at –20°C until analysis by headspace solid phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS) (van Rijswijck et al. 2017), using a SPME fiber assembly Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Supelco).

Chromeleon 7.2 Chromatography Data System Software was used to pre-process raw HPLC and UPLC data and to identify VOCs. After relevant data normalization, concentrations were determined using the calibration curves of the applied standards for HPLC and UPLC data. For VOC analysis, peak integration was carried out with ICIS algorithm. Mass spectral profiles of VOCs were matched with known profiles in the NIST main library (van Rijswijck et al. 2017).

Statistical analysis

Statistical analyses and plots were performed in R version 4.1.1 (R Core Team 2021), as shown in supplementary material.

All analyses used a significance level of 0.05. Analysis of variance (ANOVA) was used to test group differences, with Kruskal-Wallis test as a non-parametric alternative when appropriate. Significant ANOVA and Kruskal-Wallis tests were followed by pairwise group comparisons using t-test or Wilcoxon test, respectively, with no assumption of equal variance. False discovery rate method was used to correct for multiple testing (Benjamini and Hochberg 1995).

Z-scores were calculated, using the relative AA abundances of each sample (x), the mean of all non-inoculated samples (μ_{Media}), and the column standard deviation (σ_{Col}):

$$z = \frac{x - \mu_{Media}}{\sigma_{Col}}$$

The z-scores were visualized in a heat map with samples hierarchically clustered based on Euclidean distances. Differences in distances between groups were tested using permutational analysis of variance using distance matrix (PERMANOVA) and visualized using a principal coordinate analysis (PCoA).

Results

Growth on high molecular weight proteins

A minimal growth medium for *L. cremoris* was supplemented with high molecular weight casein or potato protein to analyze the extracellular protease activity by monitoring lactococcal growth. The potato protein composition was recently analyzed (García-Moreno et al. 2020a), and it was applied as an alternative to casein that represents the standard substrate for the dairy associated PrtP homologs. CCDM and PCDM were designed to contain low protein concentrations in order to reduce the levels of oligopeptides and free AAs present in the protein preparations. Oligopeptides and free AAs would support growth of our strains independent of proteolytic activity. The AA contents of the protein-supplemented media correspond to the minimal growth requirements of *L. cremoris* (Table S3), assuming that the entire protein can be hydrolyzed and utilized for lactococcal growth. The basic CDM was given a low ionic strength and a high buffer capacity in order to keep the protein in solution during the fermentation (Table S2).

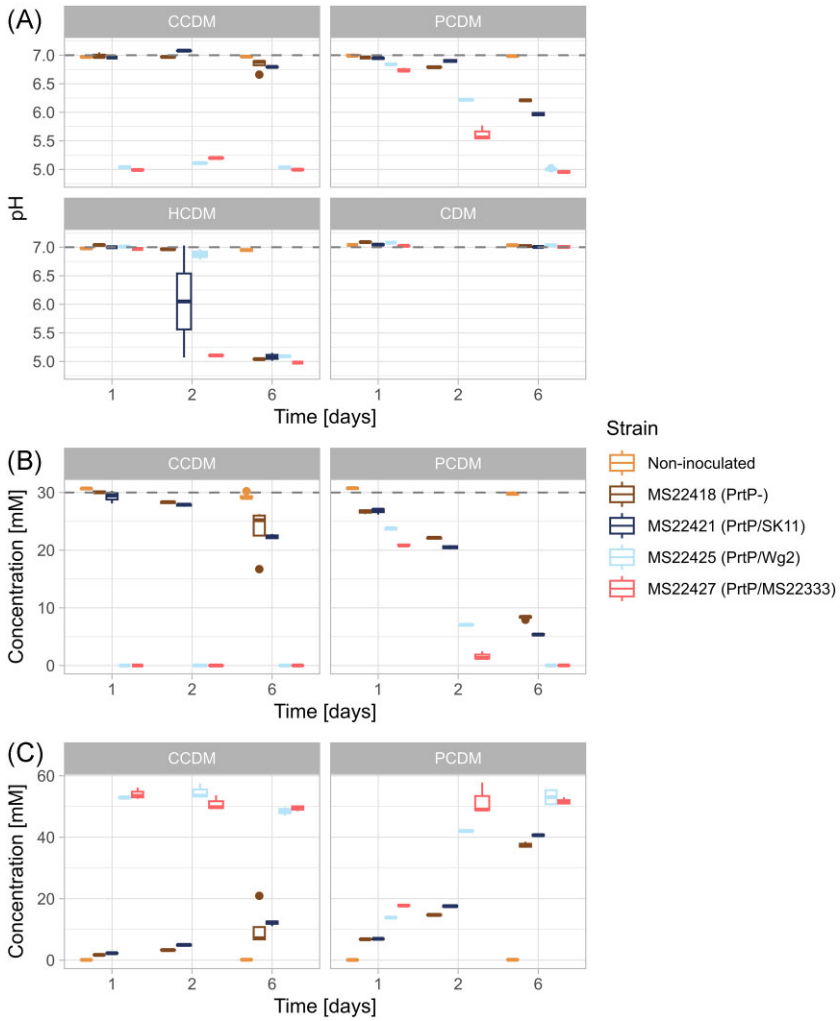

Figure 1. Growth of *Lactococcus cremoris* in protein-supplemented media. A chemically defined medium (CDM) was supplemented with casein hydrolysate (HCDM) or a high molecular weight protein as casein (CCDM) and potato protein (PCDM). Batch cultures were incubated at 30°C for 6 days (Day 0–6). Samples were taken at day 1 and 6 for non-inoculated media and at day 1, 2 and 6 for monocultures of recombinant *L. cremoris* strains. *L. cremoris* MS22418 is PrtP-negative (PrtP^{--}), while *L. cremoris* MS22421, MS22425, and MS22427 are PrtP-positive expressing $PrtP_{SK11}$, $PrtP_{Wg2}$, and $PrtP_{MS22333}$, respectively. *L. cremoris* growth was analyzed based on pH changes (A) and changes in glucose (B) and lactate (C) concentrations. Horizontal dashed lines indicate the pH and glucose concentration of the original, non-inoculated media. The boxplot includes biological replicates as follows: CCDM and PCDM at day 1 (n = 3), day 2 (n = 2–3) and day 6 (n = 3–4), CDM (n = 2), HCDM (n = 2).

$PrtP_{SK11}$, $PrtP_{Wg2}$, and $PrtP_{MS22333}$ were expressed and active in the cloned PrtP-positive *L. cremoris* strains (MS22421, MS22425, and MS22427), which all acidified bovine milk in contrast to the PrtP-negative strain MS22418 (Table 1, Figs S1 and S2). The applied vector was chosen because it has a low copy number (Israelsen et al. 1995), mimicking the natural abundance of the *prtP/prtM* regions. The *prtP/prtM* intergenic regions of the PrtP homologs contain approximately 330 bp, showing high sequence identity (91–98%) with identical promoter elements (Fig. S3). Based on the comparable promoter activity of *L. cremoris* SK11 and Wg2 (Gajic 2003), we assume that the sequence variations of the *prtP/prtM* intergenic regions will have only minor effect on the PrtP homologs' expression.

Non-inoculated media maintained a stable pH during the course of the experiment (Fig. 1). pH of CDM lacking AAs remained constant with all inoculated strains, indicating absence of growth. HCDM was used as positive control and reached a pH of 5.0 by day 6, for all strains. In CCDM and PCDM, only the two PrtP-positive strains MS22425 ($PrtP_{Wg2}$) and MS22427 ($PrtP_{MS22333}$) reached pH 5.0. The PrtP-negative strain MS22418 and the strain MS22421 expressing $PrtP_{SK11}$ did not acidify CCDM but did acidify PCDM moderately to 6.2 and 6.0, respectively. The best acidifying PrtP-positive strains MS22425 and MS22427 reached high viable plate counts, showing that the presence of either $PrtP_{Wg2}$ or $PrtP_{MS22333}$ facilitates lactococcal growth on the high molecular weight proteins (Fig. S4). However, all cultures showed a reduction of the viable plate count at day 6 compared to day 1. As expected, no colony forming units were detected in non-inoculated media. The PrtP-positive strains MS22425 and MS22427 depleted glucose in CCDM before PCDM, whereas the other strains did not reach glucose depletion of the media (Fig. 1). A glucose to lactate molar conversion ratio of 1 to 1.7 was found. These observations demonstrate that the PrtP-positive strains MS22425 and MS22427 grow well on both protein substrates, with casein providing the fastest growth.

The potato protein supplement must contain short peptides and/or free AAs that the bacteria could access, as the PrtP-negative strain MS22418 and the strain MS22421 expressing $PrtP_{SK11}$ grew slightly in PCDM but not in CCDM. However, the proteolytic-negative strains showed distinctly lower growth

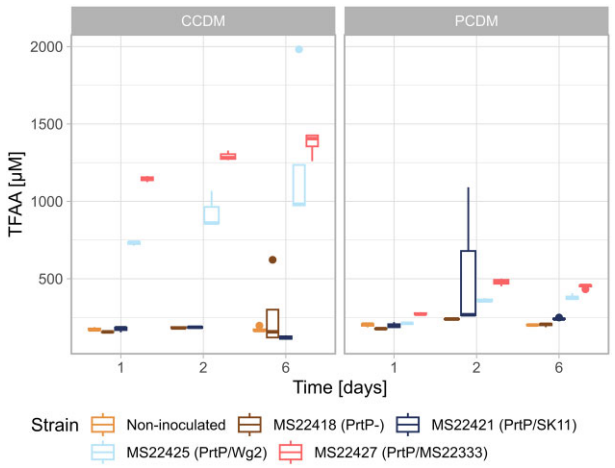

Figure 2. Total amount of free amino acids over time. Concentration of total free amino acids (TFAAs) at day 1, 2, and 6 were measured in CCDM and PCDM that were chemically defined media (CDM) supplemented with casein and potato proteins, respectively. The boxplot includes biological triplicates (n = 3) at day 1 and 2, and biological quadruplicates (n = 4) at day 6.

as compared to the proteolytic-positive strains MS22425 and MS22427 in both media. This indicates that *L. cremoris* expressing $PrtP_{Wg2}$ or $PrtP_{MS22333}$ were proteolytically active in CCDM and PCDM, whereas the *L. cremoris* expressing $PrtP_{SK11}$, like the PrtP-negative strain, was not.

Quantitative and qualitative amino acid analysis

Extracellular proteolytic activity was studied indirectly by measuring changes in the extracellular AA pool over time. Both media were low in total free AAs (TFAAs), with similar stable TFAA levels over time without *L. cremoris* growth (Fig. 2). The PrtP-negative strain MS22418 and the MS22421 strain expressing $PrtP_{SK11}$ did not significantly change the TFAA levels of CCDM, but MS22421 slightly increased the TFAA level of PCDM at day 6. Therefore, PCDM might contain small peptides for PrtP targeted hydrolysis. Cultures of strains expressing $PrtP_{Wg2}$ and $PrtP_{MS22333}$ both provided significant increase in the TFAA pool sizes when compared to cultures of strains expressing $PrtP_{SK11}$, with $PrtP_{MS22333}$ achieving in both tested media the highest increase in pool size. These observations are in agreement with the growth data, establishing *L. cremoris* MS22425 and MS22427 as proteolytic strains. These strains preferred casein over potato proteins, providing significantly higher TFAA enhancements in CCDM compared to PCDM.

The relative AA abundances of all samples were hierarchically clustered in the heat map (Fig. 3). Samples of biological replicas cluster closely. The non-inoculated medium samples cluster, showing that the relative AA abundances of CCDM and PCDM were similar. The non-proteolytic *L. cremoris* strains MS22418 and MS22421 did not induce large changes of the AA composition in CCDM. However, those strains induced changes of the AA composition of PCDM over time. Samples of the proteolytic strains cluster together, though the relative AA abundances appeared to differ in PCDM and CCDM. Additionally, the AAs were clustered into two overall groups in the heat map. Ile, Trp, Ser, Gly, Tyr, Thr, and Val clustered together, having relative low abundances with little sample variations. The cluster of the other AAs such as His, Pro, Cys, and Phe showed larger variation in their relative abundances between the samples. A PERMANOVA test of the Euclidian distances, confirmed that strain, time, and medium significantly contributed to AA composition variance. Almost 49% of the variance can be explained by the strain alone, whereas the time and the medium each explain 9% and 8% of the variance. The combination of strain and medium explains an additional 19% of the variance. Hereby, the protein substrate of the medium is the second most important contributor to the AA composition, after the strain type.

The PCoA plot based on the same distance matrix summarizes the largest differences of the AA compositions (Fig. 4). In the PCoA plot, PC1 and PC2 each explain 62% and 26% of the variation. PC1 is dominated by the variations of Trp, Ile, Gly, Tyr, and Ser, whereas PC2 captures the variations in the remaining AAs. The AA compositions of CCDM and PCDM were similar with relatively high abundances of Ile, Trp, and Gly, which showed no changes over time. PC1 values decreased over time, for all inoculated media, which was consistent with depletion of Ile and Trp pools by *L. cremoris* (Fig. S5). The AA changes appeared faster for the proteolytic strains than the non-proteolytic. On day 6, samples with non-proteolytic strains had negative PC1 values, without changes in PC2 for CCDM samples, while PC2 values for PCDM samples decreased. The enrichment of relative and absolute abundances of Ala, Asp, Glu and Gln/Arg in PCDM, compared to CCDM, might be caused by *L. cremoris* utilization of smaller peptides present in PCDM (Figs 3 and 4, and Fig. S5). PC2 values increased for CCDM samples with proteolytic strains, corresponding to higher relative and absolute abundances of His, Leu, Lys, Pro, and sulfur-containing AAs. The PC2 values were less affected, but became negative for PCDM samples with proteolytic strains. Strains carrying $PrtP_{MS22333}$ and $PrtP_{Wg2}$ delivered similar AA composition changes in both CCDM and PCDM, which appeared to occur slightly faster in cultures of *L. cremoris* expressing $PrtP_{MS22333}$ as compared to cultures of *L. cremoris* expressing $PrtP_{Wg2}$ (Fig. 4). A supplementary PERMANOVA test revealed that the proteases significantly explained 2% of the AA composition variance between the proteolytic strains, indicating that their PrtP homologs had significantly different activities.

In summary, the extracellular proteolytic activity caused quantitative and qualitative AA changes in PCDM and CCDM when compared to non-proteolytic strains. The proteolytic strains' abilities to change relative and absolute AA abundances depend on the protein substrate, with CCDM achieving the quickest changes for the two proteolytic strains. The protease activities of those strains appeared to be significantly different from each other, contributing to the overall AA changes.

Volatile organic compounds (VOCs)

The profiles of VOCs of the non-inoculated and the inoculated CCDM and PCDM were examined to determine if AA changes could be linked to potential differences in VOC formation.

The VOC levels in both the non-inoculated and inoculated media were low, with only a few VOCs identified (Fig. 5). Hexanal, a common off-flavor in legumes, was detected in non-inoculated PCDM, and the level of this compound was significantly reduced by all *L. cremoris* strains. Benzaldehyde and acetaldehyde were the only detected VOCs that might be products of AA catabolism. Benzaldehyde and acetaldehyde were found to be significantly enriched in cultures of the two proteolytic *L. cremoris* strains in CCDM. *L. cremoris* transaminase pathways generate benzaldehyde from Phe via α-keto acids, whereas *L. cremoris* lyase pathway with threonine aldolase converts threonine to glycine and acetaldehyde (Smit et al. 2005). Proteolytic *L. cremoris* produced significantly more Phe and Thr than non-proteolytic *L. cremoris* in both

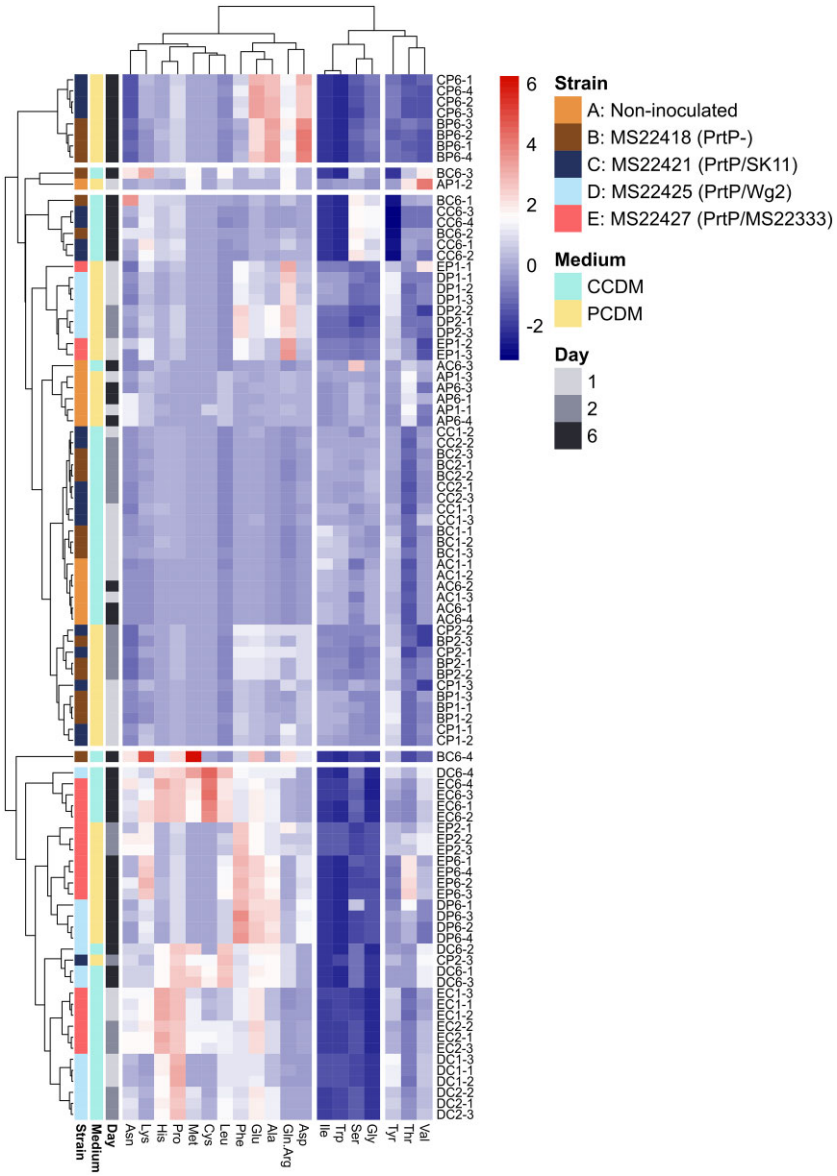

Figure 3. Hierarchical clustered heat map of the composition of the extracellular amino acid pool. The relative abundance of amino acids is compared across all samples of non-inoculated and *Lactococcus cremoris* inoculated minimal growth media supplemented with high molecular weight casein protein (CCDM) or potato protein (PCDM). The heat map shows the scaled data by row. The data is scaled according to a modified z-score, using the mean of non-inoculated CCDM and PCDM. The heat map is organized by hierarchical clustering of samples, using Euclidean distances. Data include biological replicas (n) at day 1 and 2 (n = 3) and at day 6 (n = 4). Sample names, such as DP6-1, were given as follow: D (Strain D = MS22425), P (Medium P = PCDM), 6 (Day 6)-1 (Sample number to specify among the biological replicas).

media. However, the proteolytic strains were found to produce significantly more acetaldehyde and benzaldehyde as compared to the non-proteolytic strains in CCDM, but not in PCDM. In CCDM, the proteolytic strains produced equal amounts of Thr, whereas the strain expressing $PrtP_{MS22333}$ provided significantly more Phe, explaining why this strain produced significantly more benzaldehyde than *L. cremoris* expressing $PrtP_{Wg2}$.

Discussion

This current study applies a minimal medium with high molecular weight protein sources to study the extracellular proteolytic activity of *L. cremoris* based on growth. This medium has the benefit of having a well-defined composition in comparison to milk and other raw food matrices. Additionally, the protein supplemented minimal medium has the advantage of modeling the situation in food for AA source uptake, unlike CDM supplemented with individual AAs. *L. cremoris* is known to favor uptake of oligopeptides over free AAs, importing oligopeptides with preferable 9 AAs via Opp (Juillard et al. 1995a, b). The energetics of peptide uptake is more favorable than the energetics of individual AA uptake (Konings 2002). The oligopeptide import via Opp may cost 2 ATP molecules per peptide molecule translocated, just as for other ABC importers, irrespective of the peptide length (Oldham et al. 2008, Locher 2016). The intracellular excess of AAs represents a buildup in entropy energy, which can be converted to an electrochemical potential by symporters (Konings 2002). The symport of an AA with a proton from the intracellular to extracellular environment can yield a quarter to a third of an ATP molecule (Konings 2002). In this way, the import of oligopeptides longer than six

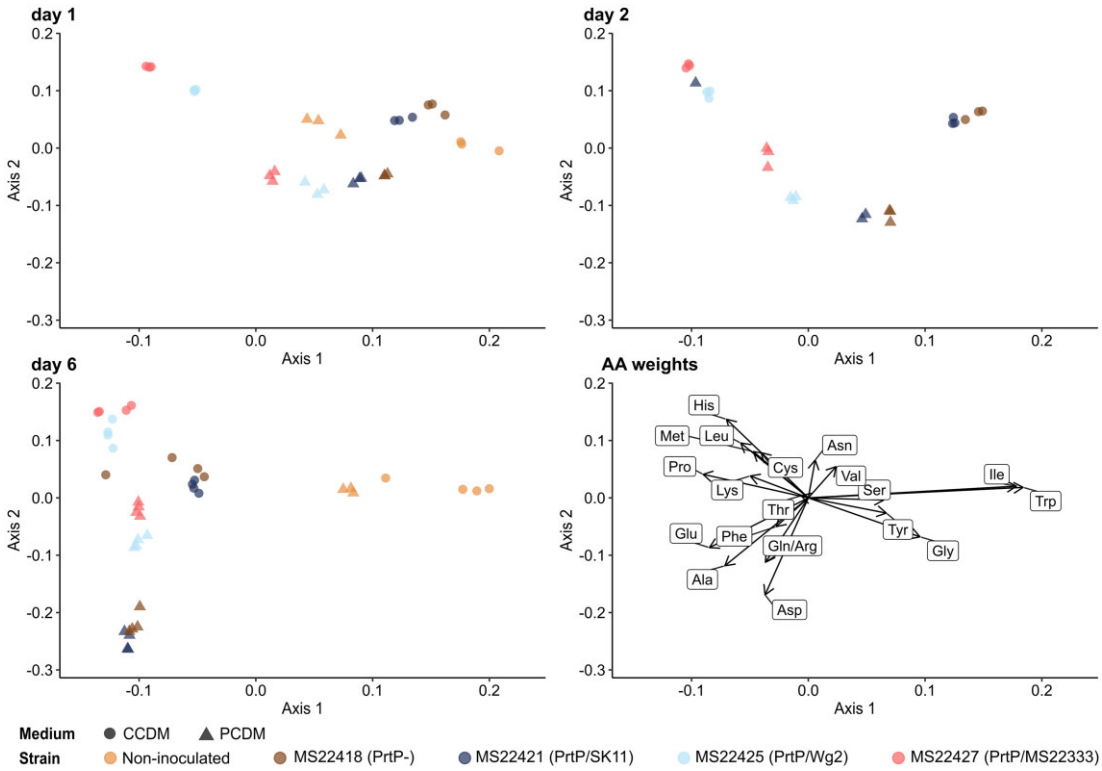

Figure 4. Principal coordinate analysis (PCoA) plot of the extracellular amino acid (AA) pool. The extracellular AA composition of *Lactococcus cremoris* strains was studied over time (Day 1, 2 and 6) in chemically defined medium supplemented with casein (CCDM) or potato protein (PCDM). The PCoA plots reveal that the first two principal coordinates on axis 1 (PC1) and 2 (PC2) explained 62% and 26% of the AA variance between the groups. The weight of each AA is represented with arrows, indicating how each AA contributed to the overall variation. The length of each arrow represents the strength with which each AA drags the variation in a specific direction.

to eight residues might generate energy by intracellular hydrolysis and subsequent secretion by appropriate symporters. *Lactococcus* has symporters for at least 14 AAs (Konings 2002). Lactococcal AA secretion will be fundamentally different in situations where the bacterium is growing on high molecular weight proteins in comparison to the situation of growth on minimal medium supplied with only essential AAs. Therefore, minimal media with high molecular weight proteins as a nitrogen source have several advantages for studying AA metabolism and flavor formation.

In CCDM and PCDM, *L. cremoris* MS22421 expressing $PrtP_{SK11}$ did not differentiate from the PrtP-negative strain, showing the inability of $PrtP_{SK11}$ to hydrolyse casein and potato proteins in CDM. Low expression is unlikely to be the cause of the PrtP-negative phenotype as the strain is PrtP-positive in milk. $PrtP_{SK11}$ may have a lower affinity for the protein substrate than $PrtP_{Wg2}$ and $PrtP_{MS22333}$, requiring higher substrate concentrations for efficient proteolysis than provided in the minimal growth media. The close PrtP homologs have critical sequence variations in their substrate binding regions (Vos et al. 1991, Børsting et al. 2015, Christensen et al. 2023), which may affect the yet-to-be-determined kinetic parameters. The PrtP homologs' activity is dependent on salt concentrations (Exterkate 1990). Calcium depleted media can release $PrtP_{SK11}$ and $PrtP_{Wg2}$ from the cell envelope, with different effects on their activity and stability (Exterkate and Alting 1999). Therefore, the PrtP homologs are likely liberated in CDM. Cell bound PrtP can facilitate high local peptide availability of the cell and consequently support lactococcal growth and fitness (Bachmann et al. 2011). In co-cultures with PrtP-positive and PrtP-negative strains, this advantage of bound PrtP is only achieved when the PrtP-positive is underrepresented. The PrtP release from the cell-envelope may lower the local peptide availability, reducing the lactococcal growth in the start of the fermentation of CDM with protein supplements. Although the low ionic strength of CDM is required to keep potato proteins in solution, salt concentrations may be optimized to support specific activities of PrtP homologs.

Different factors limit the growth of proteolytic *L. cremoris* in CCDM and PCDM, which have protein supplements with theoretically similar AA profiles (Table S3). For the two proteolytic strains, both media provide sufficient nitrogen to allow complete consumption of glucose. However, the AA availability seems to be rate-limiting in PCDM as glucose depletion and acidification in this medium occur later than in CCDM.

Proteolytic *L. cremoris* generates excess of AAs, including some branched-chain and aromatic AAs in CCDM and PCDM. These AAs, together with sulfur-containing AAs, are the main precursors of VOCs related to nitrogen metabolism in dairy (Smit et al. 2005, Flahaut et al. 2013). Nonetheless, the generally low VOC levels in the protein-supplemented media highlight that released AA levels are limited and prioritized to support proteolysis driven growth of *L. cremoris*. *L. cremoris* with $PrtP_{MS22333}$ releases significantly more Phe in CCDM than in PCDM, resulting in a higher benzaldehyde amount in CCDM. This illustrates the dependency of flavor formation on AA accessibility, though proteolysis is normally not highlighted as a rate-limiting step for flavor formation. *L. cremoris* MG1363, which is a plasmid cured strain, produces relatively low amounts of VOCs in comparison to other *L. cremoris* strains (van de Bunt et al. 2014). High flavor production may require addition of α-ketoglutarate and pyridoxal-5-phosphate, which can enhance aminotransferase activity, converting AAs into α-keto acids as the first metabolic step (van de Bunt et al. 2014).

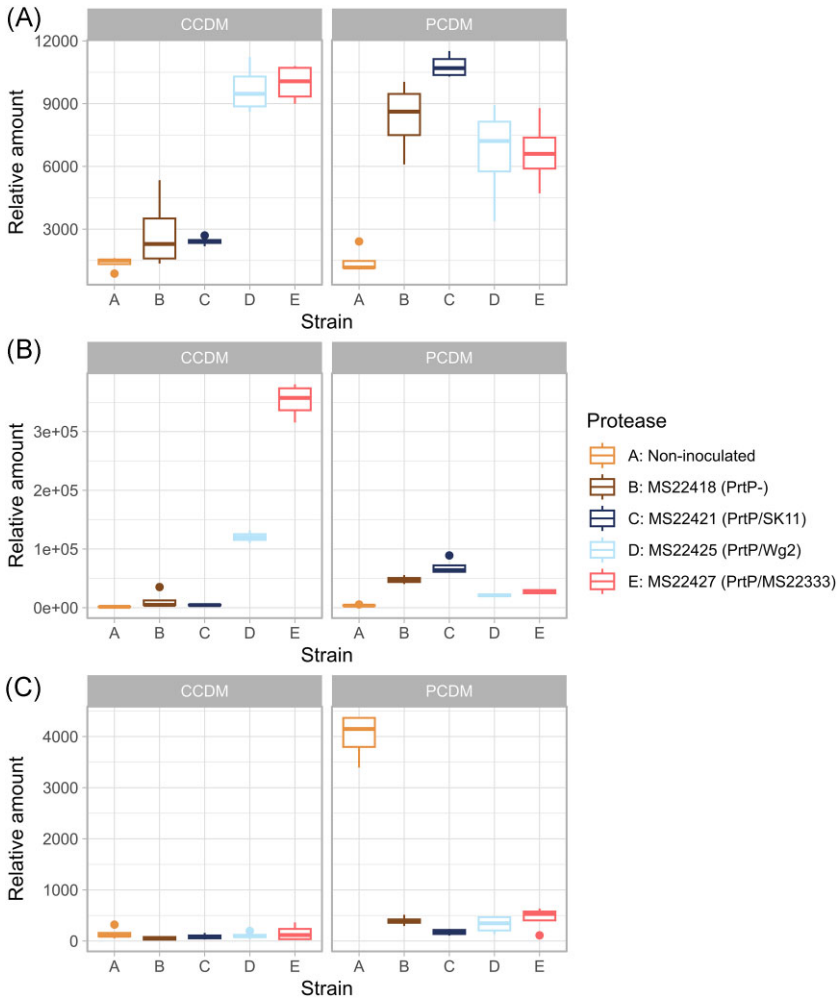

Figure 5. Relative amounts of volatile organic compounds (VOCs). Relative amounts of acetaldehyde (A), benzaldehyde (B), and hexanal (C) were measured as the peak area (counts x min) for day 6. The VOCs were analyzed for non-inoculated and *Lactococcus cremoris* inoculated minimal defined media, which were supplemented with either casein protein (CCDM) or potato protein (PCDM). The boxplot includes data for biological quadruplicates (n = 4).

The growth of proteolytic *L. cremoris* shows that the essential AA requirements are met in both media, though not all AAs are secreted in excess. *L. cremoris* can have diverse AA secretion profiles, of which some *L. cremoris* strains have relatively low secretion of essential AAs (Hernandez-Valdes et al. 2020). Different *L. lactis* strains appear to secrete more Glu than other essential AAs, though Val and Leu are also secreted in relatively high amounts (Hernandez-Valdes et al. 2020). This is consistent with the observations in CCDM and PCDM. Met, on the other hand, is secreted in relatively low amounts by proteolytic *L. cremoris* in CCDM but is absent in PCDM. All the enzymatic genes for Met biosynthesis are present in *L. cremoris* MG1363, but Cys inhibits MetC activity (Flahaut et al. 2013). Therefore, the auxotrophy to Met may depend on the presence of Cys. Due to the lack of Cys in PCDM, *L. cremoris* may be able to produce Met via biosynthesis if proteolysis is insufficient.

A striking result of the AA analysis is the complete depletion of Ile and Trp by all strains, even by the two proteolytic strains. Ile is the main regulator of the CodY repressor (den Hengst et al. 2005a, 2005b). Therefore, *L. cremoris* must avoid intracellular Ile accumulation as this would lead to repression of a large set of genes involved in nitrogen metabolism, causing starvation for other AAs (Chambellon and Yvon 2003). Excess Ile is metabolized primarily through the transaminase BcaT (Chambellon and Yvon 2003), which converts Ile to α-keto-β-methylvalerate. It would be interesting to study the fate of this α-keto acid and the connected regeneration of α-ketoglutarate to glutamate as we did not observe related VOCs. *L. cremoris* also decreases the Trp levels in CCDM and PCDM, with no apparent effect of extracellular proteolytic activity. *L. cremoris* MG1363 can synthesize Trp (Jensen and Hammer 1993, Flahaut et al. 2013). Trp is categorized as the most energetically expensive AA to synthesize (Priya et al. 2014), explaining a potential restraint of *L. cremoris* to secrete Trp. Contrary to Ile, Trp does not inhibit growth of transaminase deficient strains (Chambellon and Yvon 2003), and we do not see a metabolic need to deplete the pool of Trp.

Compared to the potato protein, casein was clearly preferred as a substrate for the *L. cremoris* strains carrying the dairy-associated PrtP homologs $PrtP_{Wg2}$ and $PrtP_{MS22333}$. This is in line with the observed modest microbial extracellular hydrolysis of plant proteins by proteases of LAB (Reale et al. 2021, Shirotani et al. 2021). The activities of $PrtP_{Wg2}$ and $PrtP_{MS22333}$ differ significantly. $PrtP_{MS22333}$ reaches higher levels of TFAAs than $PrtP_{Wg2}$, and the two proteases generate different AA compositions. These differences of their proteolytic activities may not solely be kinetically determined, but instead, related to their different cleavage patterns. However,

proteomics studies are needed to elucidate the substrate selectivity and cleavage specificity of these PrtP homologs.

In conclusion, the current study demonstrates that extracellular protease activity of *L. cremoris* can be detected in a minimal growth medium supplemented with high molecular weight proteins. Using *L. cremoris* MG1363 as isogenic background, extracellular protease activity was found to be directly related to *L. cremoris* growth. In PCDM and CCDM, proteolytic *L. cremoris* strains distinguished themselves from non-proteolytic *L. cremoris* strains by their significantly better growth and higher extracellular AA pools.

Supplementary data

Supplementary figures and tables are available at *FEMSLE Journal* online. Raw data and analyses performed in R are available at https://doi.org/10.11583/DTU.24582246

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