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Lotte Bach Larsen:

MicroLink – Forudsigelse af mikrobiologiske enzymers indflydelse på holdbarhed og kvalitet af UHT mælk vha omics-teknologier

MicroLink – Prediction via omics-technologies of influence of microbiological enzymes on quality deterioration in UHT milk

Mejeribrugets
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Final report

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1. Title of the project

Danish: MicroLink - Forudsigelse af mikrobiologiske enzymers indflydelse på holdbarhed og kvalitet af UHT mælk vha omics-teknologier

English: MicroLink – Prediction via omics-technologies of influence of microbiological enzymes on quality deterioration in UHT milk

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4. Sources of funding

Milk Levy Fund, Arla Foods amba, Aarhus University, DTU, CSC (Chinese scholarship for PhD)

5. Project period

Project period with DDRF funding: Jan 19-Dec21

Revised, if necessary: Jan 19-Dec22

6. Project summary

Danish:

Det overordnede formål er at anvende omics-teknologier og screeningsteknikker til at forudsige den mikrobiologiske kvalitet i mejeriprodukter. Et af de væsentligste kvalitetsproblemer i ultra-høj temperatur behandlet (UHT) mælk kan

føres tilbage til aktivitet fra kvalitetsforringende enzymer, der stammer fra koens celler eller fra kuldeelskende (psykrotrofe) bakterier, som kan vokse under køling. Bakterierne overlever ikke varmebehandlingen, men det gør enzymerne, som efterfølgende kan reducere produkternes holdbarhed og kvalitet, samt ultimativt føre til produktspild. Projektet leverer viden baseret på anvendelse af "omics"-teknologier til at belyse kvalitetsødelæggelse i UHT-mælk som resultat af mikrobiel kontaminering. Sigtet er at etablere værktøjer til tidlig påvisning af problem-batches, så denne mælk ikke bruges til produktion af langtidsholdbare UHT-produkter.

Det er blev undersøgt hvordan fire indkøbte Pseudomonas-stammer varierer i deres påvirkning af kvalitet af UHT-mælk opbevaret ved stuetemperatur i 45 dage, og fundet sammenhæng mellem udtryk af aprX-genet og proteinnedbrydning i UHT-mælk. Peptidomics-analyser af den opbevarede UHT-mælk viste, at selvom κ-kasein er mest hydrolyseret i begyndelsen, vil de øvrige af mælkens proteiner med tiden også hydrolyseres, især αs1-kasein. Dernæst blev den proteinnedbrydende enzymaktivitet og enzymatisk varmestabilitet analyseret hos 56 Pseudomonas-stammer fra Arla Foods amba mikrobiologiske bibliotek over indsamlede kvalitetsforringende stammer over mange år. Af disse blev 24 stammer udvalgt til helgenomsekventering og derfra opdelt i grupper, og sammenhæng mellem denne sekvensbaserede gruppering og tilhørende potentiale for proteinnedbrydning blev påvist. Desuden blev specifikke genetiske modifikationer, som kunne relatere AprX sekvensvariation inden for grupperne til potentiale for proteinnedbrydning, identificeret. Projektet viser, at det mere er forskelle mellem stammer mht AprX genudtryk end andre forskelle, som ligger til grund for den variation man hidtil har observeret mellem stammer af Pseudomonas. Denne viden er brugbar i relation til udvikling af metoder til mere nøjagtig forudsigelse af egnethed af enkelte UHT-batches til produktion af UHT-mælk. Den nye viden kan fx bruges til udvikling af en PCR-baseret metode og formindske madspild og kvalitetsforringelse af UHT mælk. Udvikling af en sådan PCR-baseret metode er fortsat i et nyt MFF projekt, som ledes af professor Lisbeth Truelstrup Hansen, DTU, og med projektdeltagelse fra Arla Foods amba.

English:

The overall aim is to use omics technologies and screening techniques to predict the microbiological quality of dairy products. One of the main quality problems in ultra-high temperature treated (UHT) milk can be traced back to the activity of quality-deteriorating enzymes originating from either the cow's cells or from cold-tolerant (psychrotrophic) bacteria that can grow during refrigeration. The bacteria do not survive the heat treatment, but the enzymes do, which can subsequently reduce shelf life and quality of dairy products, and ultimately lead to product waste.

The project provides knowledge based on the use of "omics" technologies to elucidate quality deterioration in UHT milk as a result of microbial contamination. The aim is to establish tools for early detection of problem batches, so that unsuitable milk is not used for production of long-life UHT milk.

It was in the MicroLink project initially investigated how four purchased Pseudomonas strains differed in their influence on UHT milk quality during storage (room temperature for 45 days). A correlation between expression of the aprX gene and protein degradation in UHT milk was found. Peptidomic analysis of the stored UHT milk showed that κ-casein was most hydrolysed at the beginning of the storage, and that the other milk proteins will eventually also be hydrolysed by time, especially αs1-casein. Next, the protein-degrading enzyme activity and its heat stability were analyzed for 56 Pseudomonas strains from the Arla Foods amba microbiological library of quality-deteriorating strains, collected over many years. Of these, 24 strains were selected for whole-genome sequencing, and based on spoilage potential from the determined proteolytic activities, the strains and sequences could be grouped. Specific genetic modifications that could relate AprX sequence variation within these spoilage groups to potential for protein degradation were identified. The project shows, that observed variation in spoilage potential between strains is mainly dertermined by expression levels of spoilage enzymes, like aprX is the main determinant. This knowledge is useful in relation to the development of methods for more accurate prediction of the suitability of individual UHT batches for the production of UHT milk. The new knowledge can, for example, be used to develop a PCR-based method and reduce food waste and quality deterioration of UHT milk. The development of such a PCR based method is taken on in a newly granted DDRF project, led by Professor Lisbeth Truelstrup Hansen, DTU, and with project participation from Arla Foods amba.

7. Project aim

Danish:

Det overordnede formål er at anvende omics-teknologier og screeningsteknikker til at forudsige den mikrobiologiske kvalitet i mejeriprodukter.

Et af de væsentligste kvalitetsproblemer i ultra-høj temperatur behandlet (UHT) mælk kan føres tilbage til aktivitet fra kvalitetsforringende enzymer, der stammer fra koens celler eller fra kuldeelskende (psykrotrofe) bakterier, som kan vokse under køling. Bakterierne overlever ikke varmebehandlingen, men det gør enzymerne, som efterfølgende kan reducere produkternes holdbarhed og kvalitet, samt ultimativt føre til produktspild. Projektet leverer viden baseret på anvendelse af "omics"-teknologier til at belyse kvalitetsødelæggelse i UHT-mælk som resultat af mikrobiel kontaminering. Sigtet er at etablere værktøjer til tidlig påvisning af problem-batches, så denne mælk ikke bruges til produktion af langtidsholdbare UHT-produkter.

English:

The overall aim is to use omics technologies and screening techniques to predict the microbiological quality of dairy products.

One of the main quality problems in ultra-high temperature treated (UHT) milk can be traced back to the activity of quality-deteriorating enzymes originating from the cow's cells or from cold-tolerant (psychrotrophic) bacteria that can grow during refrigeration. The bacteria do not survive the heat treatment, but the enzymes do, which can subsequently reduce the shelf life and quality of the products, and ultimately lead to product waste.

The project provides knowledge based on the use of "omics" technologies to elucidate quality degradation in UHT milk as a result of microbial contamination. The aim is to establish tools for the early detection of problem batches, so that this milk is not used for the production of long-lasting UHT products.

8. Background for the project

In UHT milk, the main cause of spoilage during storage is a result of proteolytic action of heat-stable enzymes. Attempts to assure quality of UHT milk by routinely monitoring total psychrotrophic counts are currently costly and takes time. Incidents of spoilage of a whole-batch of produced UHT milk product are still encountered, and leads to financial loss and potential brand damage, particularly for overseas markets. Assuming 1 in 500 productions has heat resistant enzymes, the chances to find the microbial cause in eg. 20 silos milk samples would be quite low, and have, in spite of extensive samplings, in many cases led to inconclusive results (information from Arla foods). A major challenge of current approaches is that only some, very specific psychrotrophic bacteria are capable to producing these heat resistant enzymes, and there is therefore no fixed association between the level of spoilage-enzyme-producing organisms and total psychrotrophic bacteria count. **Our scientific hypothesis is that survival of heat resistant enzymes secreted from re-contaminating microorganisms during the cool and/or processing chain can lead to severe quality deteriorations, while the microorganisms themselves are killed by the heat treatments.** See project rationale in Fig. 1.

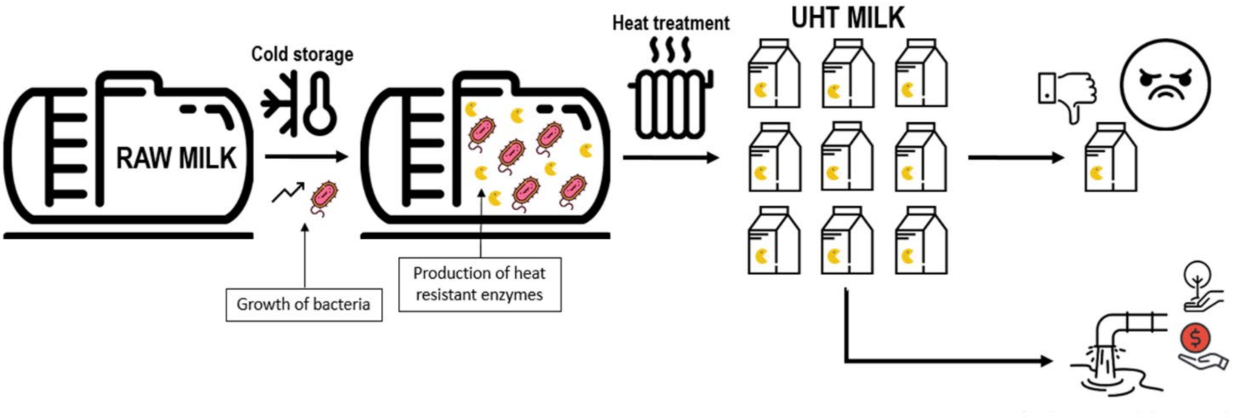


Fig. 1. Project rationale, where knowledge and potential new detection method could enable sorting of unsuitable batches of milk for UHT application and avoid downstream product spoilage and waste.

9. Sub-activities in the entire project period

The project consists of four work packages (WPs) as outlined in Fig. 2 and in the Gannt diagram, and have been performed by AU, DTU and Arla Foods amba. All milestones have been completed.

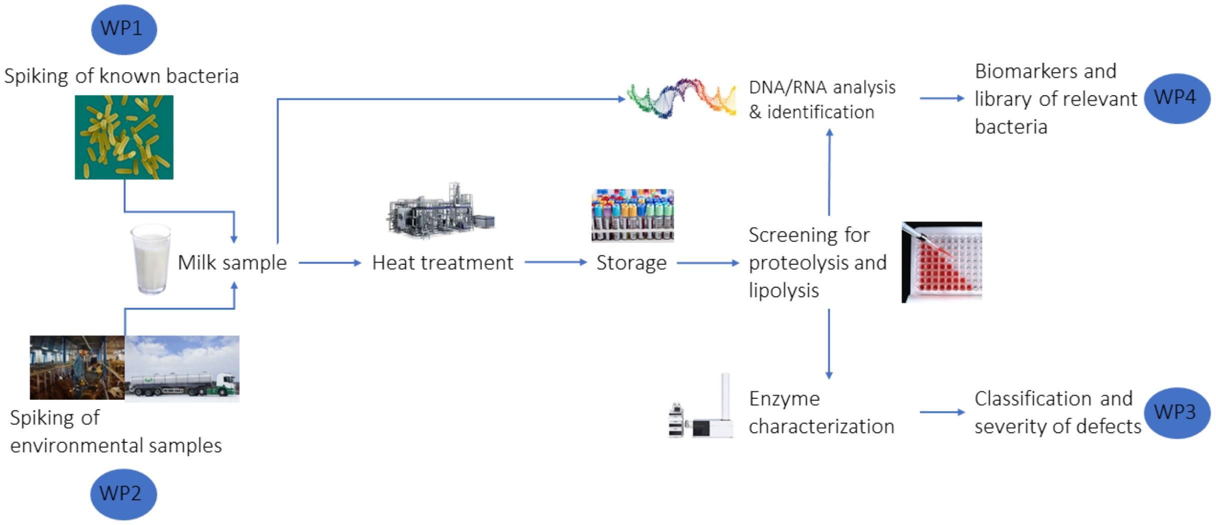


Fig. 2. Overview of WPs in the MicroLink project.

WP1: Establishment of screening methods using a positive control (WP leader AU)

Aim: Establish link between inoculation level, quality deterioration and enzyme activities through the production of a positive control of known psychrotrophic bacteria. The spiking samples are prepared by inoculating heat resistant enzyme-producing bacteria into milk, followed by test UHT heat treatment.

Pseudomonas representing two relevant strains of psychrotrophic bacteria from microbiological library at Arla Foods will be selected based on combination of knowledge from dairies and a literature study. The selected strains will be spiked into raw tank milk at 3 different levels, and incubated for 3 different time intervals.

The incubated milk will be tested for enzyme activity before and after UHT treatment by implementation of a lab based UHT system in stainless steel tube combined with a pump (Arla foods). The milk will be stored and characterized by enzymatic and microbiological analyses:

- Fast assay for free fatty acids as a result of lipolysis (NEFA).
- Extent of proteolysis by measurement of level of free amino-terminals from breakdown of proteins.
- Protein integrity by gel electrophoresis and protein mapping by liquid chromatography and mass spectrometry.
- Milk functional characteristics (processing characteristics, micelle and fat globule size characteristics, pH and calcium distribution.

Samples of the used strain will be delivered to WP4 for DNA/RNA extraction and development methods for identification of these microorganisms.

WP2: Characterization of unknown heat-resistant enzymes from microorganisms in the environment (WP leader Arla)

Aim: Evaluate the spoiling potential of a "spoilage mix" made based on samples from dairies and added to milk before and after lab scale UHT treatment.

Ten microbial samples from dairy silos will be collected from different source dairies, preferably from different countries. The samples will be inoculated alone and in mixtures into milk, before and after lab scale UHT treatment, incubated 1-2 days at 4°C and screened for heat resistant enzymes according to the protocol and methods established in WP 1.

WP3: Characterization of heat-resistant enzymes (WP leader AU)

Aim: To characterize new, heat-resistant enzymes from the from inoculation mix.

New heat-resistant enzyme activities discovered in WP2 will be characterized regarding:

- Cleavage pattern on milk proteins by peptide mapping of created breakdown products using peptidomic methods combined with bioinformatics searches using in-house databases of the milk proteins and their different variants.
- Lipolysis by measuring individual free fatty acids by gas chromatography and mass spectrometry.
- Type of spoilage by characterization of off flavor and gelation of the inoculated samples.
- Heat inactivation behavior of the enzymes added using lab-scale UHT and assessing the enzyme activities.

The expectation is that at least 3 new enzymes will be identified and characterized regarding specificity and effect.

WP4: Characterization of heat-resistant enzyme producing bacteria (WP leader DTU)

Aim: Identification of DNA/RNA biomarkers for *Pseudomonas* and *Bacillus* species with the perspective of establishing fast method for detection.

The challenge is to detect and characterize a very low level of these heat resistance-enzyme producing bacteria in a mixed microbial flora contaminated by naturally present lactic acid bacteria, *E. coli* and various numbers of other bacteria present in the milk and in the added microbiological mixtures also present, but not being the target of spoilage enzymes. To address this issue, we will develop two strategies: First, a non-biased metagenomics that combines efficient pre-analytical sample preparations methods with deep sequencing and refined bioinformatics. Second, using the so-called "PadLock" probes, which is a targeted nucleic amplification test for inclusion of up to 30 000 probes as a combinatory biomarker. DTU has through previous projects obtained experience with both technologies that will be used in connection with the bioinformatic facility MG Mapper (Petersen et al., 2017). Potential markers for establishment of fast method will be evaluated.

Time plan for the MFF project period January 2019 - December 2021, including milestones (M) (MAF part of the project was extended till end of 2022, but GSTS part of funding proceeded into 2023, also with a PhD defense in 2023):

	2019				2020				2021				2022			
M1: Strain for WP1 pilot study has been selected	▨	▨ √														
M2: Pilot study WP1 carried out and methods established		▨	▨	▨ √												
M3: Method for bacterial identification established in WP4 based on WP1 pilot experiment			▨	▨	▨ √											
M4: Samples for inoculation study in WP2 collected					▨	▨ √										
M5: Spoilage potential of dairy mix has been determined in WP3							▨	▨	▨	▨	▨	▨	▨	▨ √		
M6: Heat resistant enzymes from dairy mix has been characterized in WP3								▨	▨	▨	▨	▨	▨	▨ √		
M7: Microorganisms from inoculation study in WP2 has been characterized in WP4								▨	▨	▨	▨	▨	▨	▨ √		
M8: Knowledge of flora and heat-resistant enzymes has been combined in PhD thesis and publications													▨	▨	▨	▨ √
M9: Evaluation of potential parameters for fast methods WP2+3+4															▨	▨ √

10. Deviations

The project was extended with 1 y, partly due to exchange of anchor person at DTU during the project period, partially in order to accommodate the finalizing of the embedded PhD. The PhD was finalized in 2023.

11. Project results

Initial work centered around implementation of methods for characterizing UHT milk spoilage and mechanisms around that in non-inoculated UHT milk (paper 1), as well as performing a range of cultivation and inoculation storage experiments, to know the system and try to standardize and enable reproducibility, which is a big challenge in this area.

Initially, 4 strains of Pseudomonas were bought from DSM and tested for their different spoilage potential. The strains were cultured and induced for enzyme production. Expression analysis was carried out and then extracts were made by filtration and dialysis of culture media and used for inoculation of UHT milk in a storage experiment over 45 d at room temperature. Then proteolytic activity was measured as generation of new N-terminals in the UHT milk during storage by Flourescamine assay. This was accompanied by visual analysis of physical changes as well as gravitometric separation of sediment and liquid eventually formed during the shelf-life experiment. Also pH development was followed, as well as peptidomics was carried out.

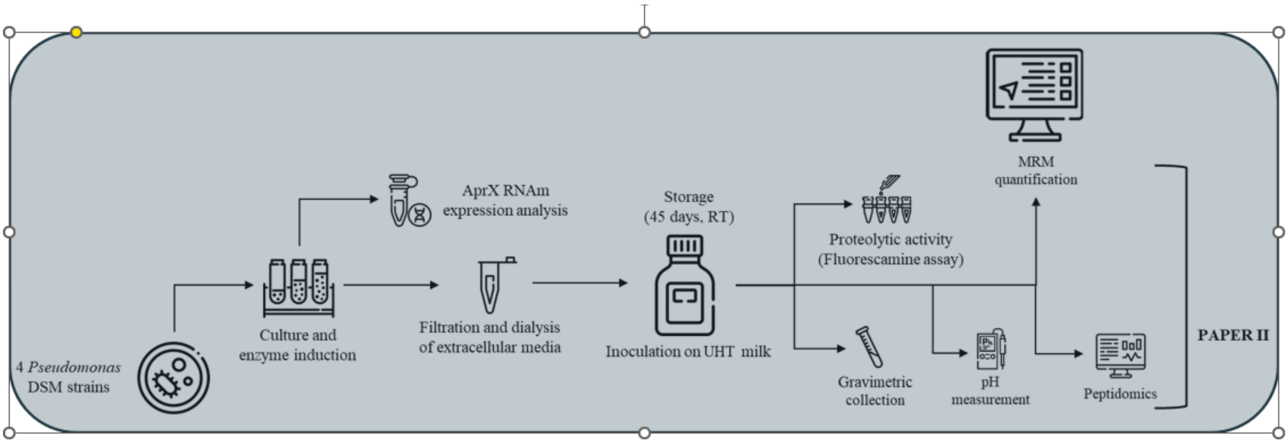
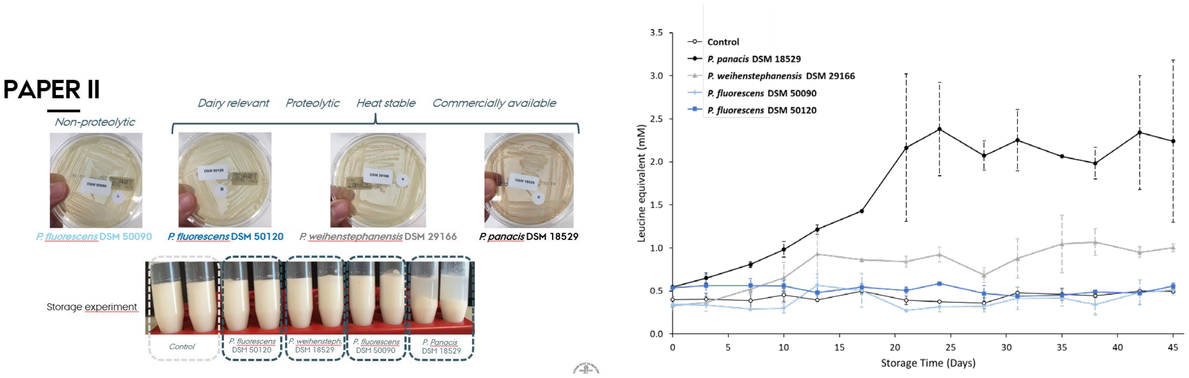


Fig. 3. Overview of experimental strategy for investigations on spoilage potential of 4 commercial strains of Pseudomonas.

Overview of used commercially available strains and effect on UHT milk appearance after inoculation and storage for 45 d at RT is shown in Fig. 4A. Result from determination of development in level of free N-terminals by Flourescamine assay of the inoculated UHT milk during storage at 45 d at RT is shown in Fig. 4B. It is seen that the development in levels of free N-terminals (expressed as Leucine equivalents) is increasing over storage period, especially for milk inoculated with *P. panacis* culture extract. Also, a small increase was observed for *P. weihenstephanensis* inoculated UHT milk. This result is in accordance with the visual appearance in Fig. 4A.


A)

B)

Fig. 4. A) Overview of used commercial strains and their effect after inoculation into UHT milk and after storage for 45 d at RT. It is seen that P. panacis resulted in visible change in quality and phase separation/sedimentation. Control was without inoculation. B) Development in level of free N-terminals in UHT milk during storage at 45 d at RT after inoculation with each of the 4 different tested commercial strains of Pseudomonas, as well as an non-inoculated control.

Furthermore, expression of mRNA for aprX gene in the media from the cultivation and enzyme induction was performed. The result is shown in Fig. 5, and it is evident that the expression level of aprX in *P. panacis* medium is almost 5000 x higher than the control strain *P. flourescens* DSM 50090, while the expression level for *P. weihenstephanensis* was approximately 100 times higher than control strain. This is in accordance with the results from the shelf life study and indicate that the reason behind differences in spoilage potential between strains is related to expression level of aprX gene and thereby expectedly also spoilage enzyme level.

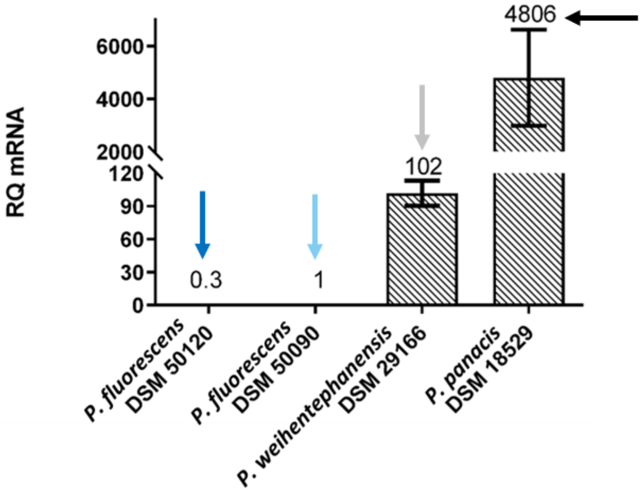


Fig. 5. Expression level of aprX gene in cultivation media of the four investigated commercial strains. Level of P. flourescens DSM 50090 was set as 1 and the others expressed relative to that.

Next peptidomics to profile peptides generated in the shelf life experiment as a result of the inoculations and storage was carried out using nanoLC timsTOF MS/MS and bioinformatics. The result of the peptide generation is shown in Table 1. It is seen that all samples, including control, had a relatively high level of generation of peptides from β-casein, which may indicate that these are formed by eg plasmin. Especially *P. panacis* had a high level of peptides generated from α_{S1}-casein, but also that the *relative* ion intensities were not very different.

Table 1. Peptide formation expressed as relative ion intensity of generated peptides from major milk proteins in each of the inoculation experiments after 45 d of storage at RT.

		Control	***P. panacis* DSM 18529**	***P. weihenstephanensis* DSM 29166**	***P. fluorescens* DSM 50090**	***P. fluorescens* DSM 50120**
Relative ion intensity distribution (%)	β-casein	65	67	67	61	62
	αs1-casein	14	26	17	18	15
	αs2-casein	8	3	9	9	9
	κ-casein	7	2	3	7	8
	β-lactoglobulin	5	3	4	5	6

The deciphered cleavage sites observed to be involved in the proteolysis of the major milk proteins are shown in Fig. 6, as well as the *absolute* intensities of the observed cleavage sites, based on peptide intensities behind these cleavages. It is observed that there is not a big difference between strains in their specificities, but instead in the intensity of the peptides generated, meaning that spoilage is linked to more generation of peptides but not really a different pattern.

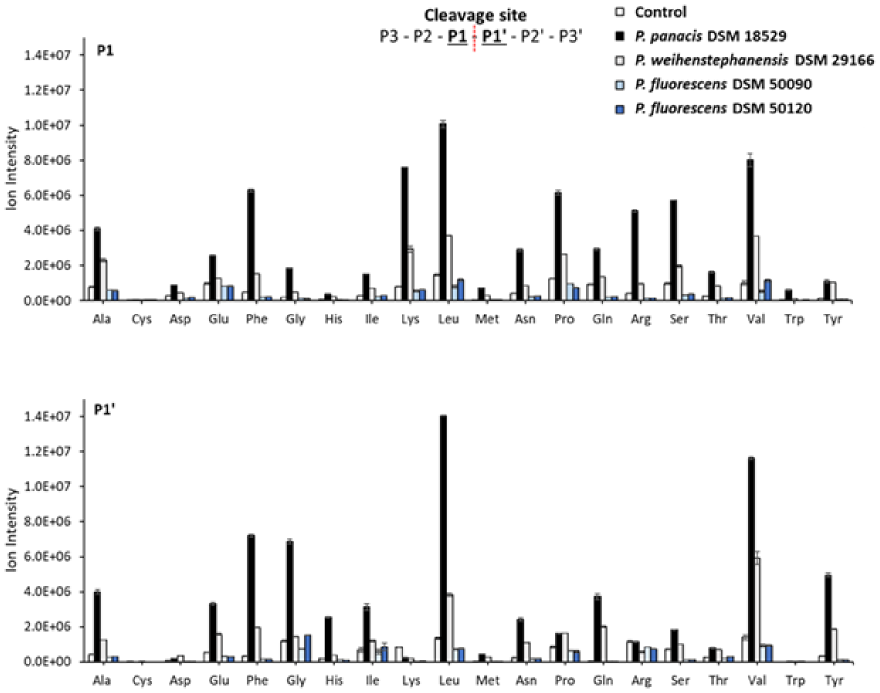


Fig. 6. Absolute ion intensities of cleavage sites observed in inoculation experiment with the four commercial strains after 45 d of storage at RT, as based on peptide quantifications and cleavage patterns behind as determined by nanoLC tim-sTOF MS/MS and PEAKS bioinformatics.

Milestones related to this part of the study:

M1: The strains for the shelf-life tests were bought from DSM and tested: A) *Pseudomonas weihenstephanensis,* DSM 29166; B) *Pseudomonas flourescens,* DSM 50120; C) *Pseudomonas flourescens,* DSM 50090; D) *Pseudomonas panacis,* DSM 18529.

M2: Pilot study 1 of inoculation of known strains in milk and identification of their proteolytic potential has been carried out, and showed marked and visual changes in the stored and inoculated UHT milk, with highest spoilage potential for strain D.

M3: DNA/RNA extracted from the DSM strains in the batches obtained from Arla foods were assessed for the presence of *apr*A using PCR primers based on sequence studies from literature and databases. AprX gene expression was found to be significantly higher in strains C and D compared with A and B. The enzymes were found only in strains C and D. Relation was found between expression level of AprX gene and proteolysis, indicating that the differences in spoilage potential between *Pseudomonas* strains are likely due to regulatory differences in their expression.

In the next study, the strain collection at Arla Foods collected over many years from dairies, products and other places was screened for spoilage potential and proteolytic activity before and after heat treatment. The experimental strategy is shown in Fig. 7.

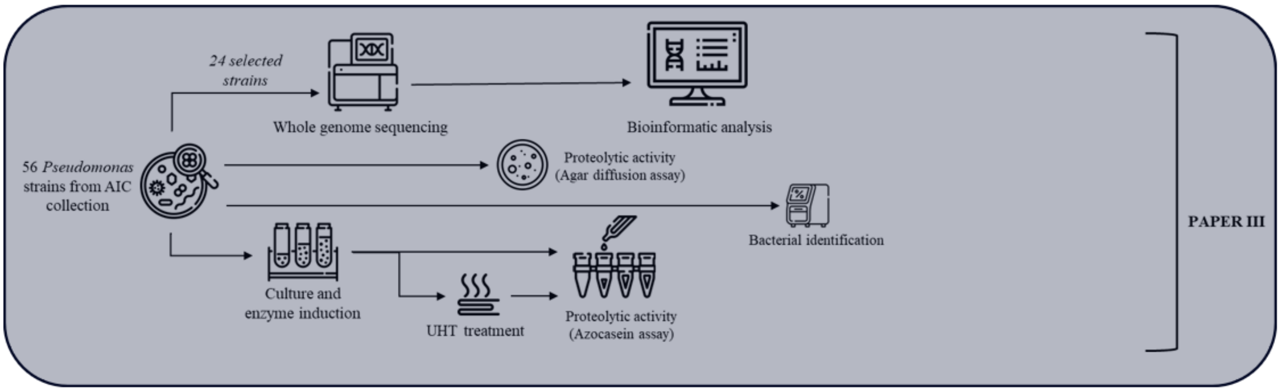
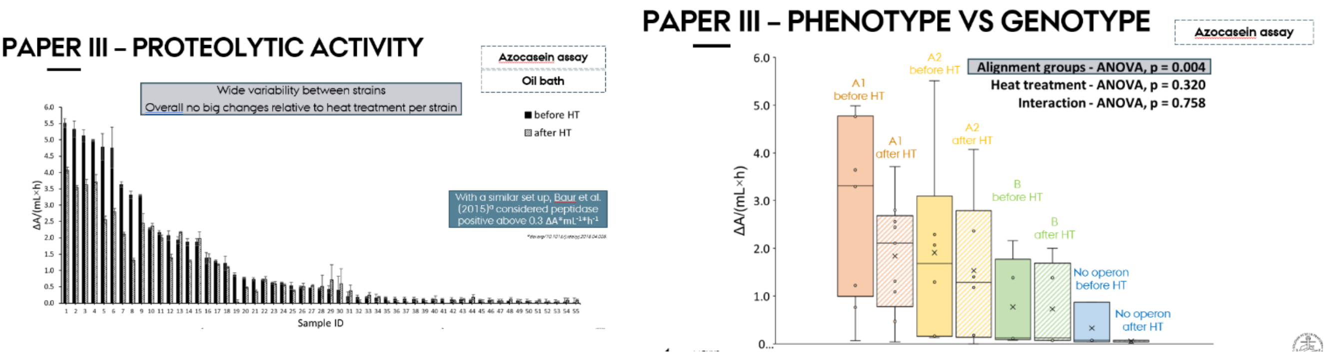


Fig. 7. *Overview of experimental strategy for investigation of level of proteolytic activity of strains from AIC collection (agar diffusion assay), combined with whole genome sequencing of 24 out of a total of 56 strains selected based on screening for proteolytic activity in culture medium before and after lab UHT treatment. s on spoilage potential of 4 commercial strains of Pseudomonas.*

56 strains from AIC collection were screened by agar diffusion assay and by azocasein assay. The 56 strains were then typed by MALDI ToF MS at AIC and showed that 55 of these contained Pseudomonas and then included in next step. Culture media after enzyme induction were subjected for assay of proteolytic activity by azocasein assay before and after lab UHT treatment (135°C, 10 s). Based on high and low values from this screening, 24 strains were selected for whole genome sequencing and bioinformatics to investigate motifs related to high spoilage potential before and after UHT treatment. Result from azocasein assay before and after lab UHT treatment of the 55 strains from AIC collection is shown in Fig. 8A. Based on this result, 25 extremes were selected and the strains behind were subjected to whole genome sequencing. The sequencing results from this were subjected to bioinformatics and alignment analyses and this enabled some grouping of the strains based on aprX gene sequences to be performed (Fig. 8B). There were found to be statistically significant levels of azocasein assay performance between alignment groups (Fig. 8B).



A)

PAPER III – PROTEOLYTIC ACTIVITY

Wide variability between strains
Overall no big changes relative to heat treatment per strain

With a similar set up, Baur et al. (2015)ª considered peptidase positive above 0.3 ΔA*mL⁻¹*h⁻¹

B)

PAPER III – PHENOTYPE VS GENOTYPE

Alignment groups - ANOVA, p = 0.004
Heat treatment - ANOVA, p = 0.320
Interaction - ANOVA, p = 0.758

Fig. 8. Level of proteolytic activity of 55 strains from AIC collection as measured by azocasein assay before and after lab UHT treatment (135°C, 10 s).

Milestones related to this part of the study:

M4: Arla´s library of ~ 50 dairy samples which were earlier identified to contain *Pseudomonas* spp. were biotyped by MALDI ToF MS and tested for enzyme activity before and after lab scale UHT heating. Based on this a subset was selected for sequencing and structure analysis.

M5: Variation in spoilage potential of Arla strains was determined measuring proteolytic potential before and after lab scale UHT treatment.

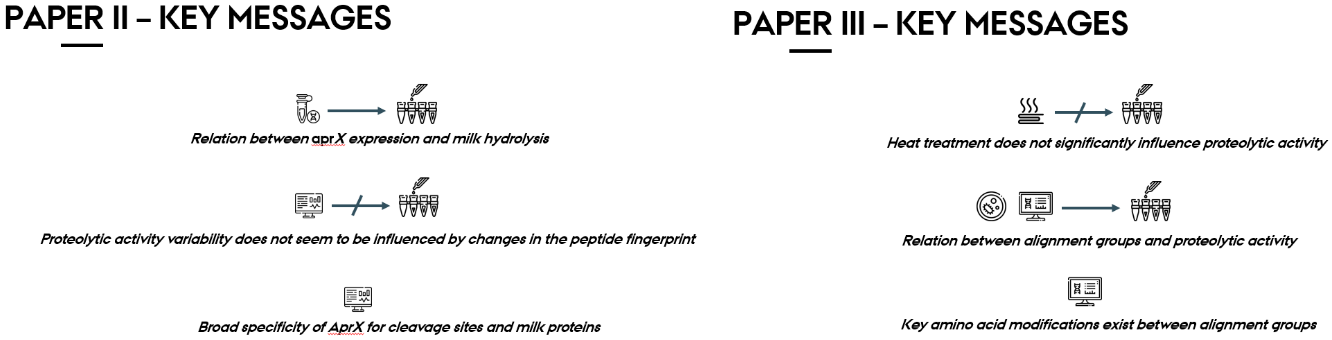
M6: The heat resistance of the enzymes from Arla's library has been determined at simulated UHT conditions using an oil bath. No significant difference in activity before and after heat treatment was found, indicating that the enzymes are very heat resistant.

M7: The subset of bacteria from the Arla collection were sent to sequencing, and based on this it was possible to group the different strains into structure groups that were relatable to spoilage potential.

M8: The results have been published into 2 scientific papers and a PhD thesis have been made and defended successfully.

M9: Using the generated knowledge on structural variation of environmental *Pseudomonas* strains relative to spoilage potential, it should be possible to develop PCR-based detection method for spoilage bacteria in subsequent projects that can be used to predict the quality of UHT milk batches at an early stage.

Conclusions:



PAPER II – KEY MESSAGES

Relation between aprX expression and milk hydrolysis

Proteolytic activity variability does not seem to be influenced by changes in the peptide fingerprint

Broad specificity of AprX for cleavage sites and milk proteins

PAPER III – KEY MESSAGES

Heat treatment does not significantly influence proteolytic activity

Relation between alignment groups and proteolytic activity

Key amino acid modifications exist between alignment groups

Fig. 9. Summary of key messages from the project.

12. The relevance of the results, including relevance for the dairy industry

The project has in short term contributed with knowledge on relationship between microbiological quality and spoilage of UHT milk, as well as on new insight into explaining why the TPC (total plate count) is not a useful tool for understanding and predicting spoilage potential of UHT milk batches. We have shown that differences in proteolytic level between strains can be related to level of expression of the spoilage enzyme, and thereby that differences in spoilage potential seem to be linked to regulatory differences of the AprX operon in the different bacteria. This knowledge is essential in relation to development of new screenings techniques for assessment of batches of milk for UHT processing suitability.

The second part of the results, showing that the bacteria can be grouped on basis of their operon sequences and that this grouping also mirrors spoilage potential point on the possibility of developing a targeted PCR based method for categorizing *Pseudomonas* flora in milk batches intended for UHT processing, and thereby enabling sorting. This will ultimately enable keeping the high quality of UHT products for expansion on both national and not the least, export markets by filtering away problem batches for UHT processing, which can then be used for other purposes.

The results of the MicroLink project can form the basis of spin-off project(s) using the obtained knowledge on spoilage enzymes and their structures to develop screening method for specific bacterial strains or specific spoilage enzymes, instead of the currently employed plate assay used as quality control of dairy batches of milk for UHT treatments. This current plate assay is carried out by Eurofins, and both dairies and Eurofins are interested in implementing a faster and more reliable method that is more directly linked with the milk quality in relation to being used in UHT products. Such a method could rely on a PCR method to be developed and that could be used for sorting milk prior to UHT treatment. The solution is illustrated in Fig. 10.

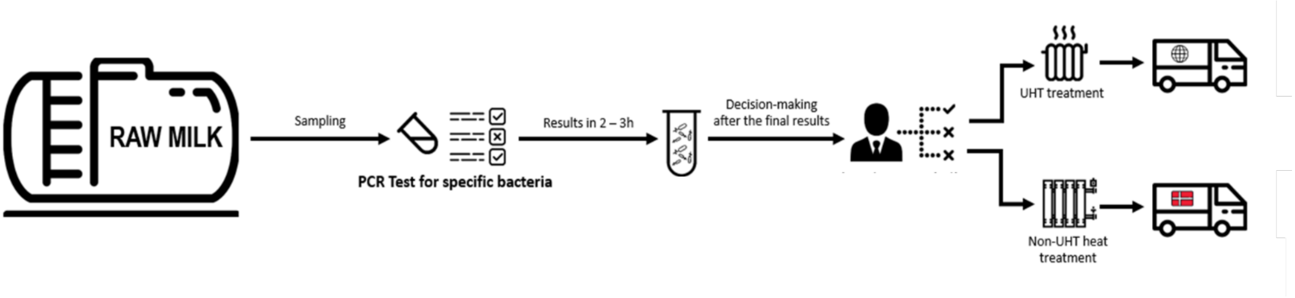


Fig. 10. Schematic representation of potential solution based on a PCR test enabling detection of problem milk batches in relation to presence of expression products from spoilage bacteria, like Pseudomonas. This could enable that only milk batches suitable for UHT milk production are directed towards that.

13. Communication and knowledge sharing about the project

Papers in international journals:

Aguilera-Toro, M., Poulsen, N. A., Akkerman, M., Rauh, V., Larsen, L. B., Nielsen, S. D. (2022). Development in Maillard reaction and dehydroalanine pathway markers during storage of UHT milk representing differences in casein micelle size and sedimentation. Foods, 11, 1525.

Miguel Aguilera-Toro, Søren Drud-Heydary Nielsen, Martin Laage Kragh, Yinghua Xiao, Lisbeth Truelstrup Hansen, Valentin Rauh, Lars Wiking, Nina Aagaard Poulsen, Lotte Bach Larsen (2022). Peptidomic fingerprints of stored UHT milk inoculated with protease extracts from different *Pseudomonas* strains relative to aprX expression and visible spoilage. *Dairy* 2023, *4*(1), 83-97; https://doi.org/10.3390/dairy4010005.

Miguel Aguilera-Toro, Martin Laage Kragh, Amalie Vestergård Thomasen, Vittoria Piccini, Valentin Rauh, Yinghua Xiao, Lars Wiking, Nina A. Poulsen, Lisbeth Truelstrup Hansen & Lotte B. Larsen (2022). Proteolytic activity and heat resistance of the protease AprX 1 from *Pseudomonas* in relation to genotypic characteristics. International Journal of Food Microbiology, 391-393, 110147.

Easily read papers:

Aguilera-Toro M, Poulsen NA, Rauh V, Truelstrup-Hansen L, Xiao Y & Larsen LB (2021). Catching the persistent bacteria. Mælkeritidende 9, p. 1-2, 2021.

Miguel Aguilera Toro, Valentin Rauh, Yinghua Xiao, Lars Wiking, Nina Aagaard Poulsen, Lisbeth Truelstrup Hansen & Lotte Bach Larsen (2024). Spoilage of UHT milk by enzymes from microorganisms**.** Mælkeritidende, submitted.

Student theses:

Louise Almer (2021). Detection of the aprX gene in UHT milk using genomic amplification. Technical University of Denmark, Kgs. Lyngby, Denmark (M.Sc. thesis).

Amalie Vestergård Thomasen (2022). Milk spoilage enzymes from psychrotrophic bacteria. June 2022. Aarhus University, Denmark (M.Sc. thesis).

Miguel Aguilera Toro (2022). Influence of protein interactions and Pseudomonas-derived proteolysis on UHT milk quality and spoilage. PhD thesis, Jan, 2023.

Oral presentations at scientific conferences, symposiums etc.:

Miguel Aguilera Toro, Valentin Rauh, Yinghua Xiao, Lisbeth Truelstrup Hansen, Nina Aagard Poulsen, Lars Wiking, Lotte Bach Larsen (2021). Spoilage of UHT milk by thermoresistant bacterial enzymes. Abstract and flash presentation for Dairy Science and Technology Symposium, June 21-25, online, Aarhus, Denmark.

Aguilera-Toro, M., Thomasen, A. V., Piccini, V., Rauh, V., Xiao, Y., Hansen, L., Kragh, M. L., Poulsen, N. A., Wiking, L., Larsen, L. B. (2022). Screening of proteolytic activity and heat resistance of Pseudomonas strains. Dairy Science and Technology Symposium 2022. Abstract and on-line presentation.

Oral presentations at meetings:

Presentations at regular project meetings in the project group, as a point of origin 2-4 times/y.

Project presentation by Wiking & Toro (2020) at DDRF steering group meeting in Technology and Microbiology group, Autumn 2020.

Project presentation by Toro and Larsen (2022) at DDRF steering group meeting in Technology and Microbiology group, May 2022.

Aguilera-Toro, M., Poulsen, N. A., Larsen, L. B. (2022). Spoilage of UHT milk by thermoresistant proteases from Pseudomonas spp. 2nd Tech Microbiome Initiative, Aarhus University. Abstract and poster.

Project end presentation by Larsen LB (2023) at DDRF steering group meeting in Technology and Microbiology group, Autumn 2023.

Other: Project homepage https://food.au.dk/microlink/

14. Contribution to master and PhD education

Teaching at AU-FOOD at MEF education and teaching at DTU.

Miguel A Toro had several external research stays at DTU-FOOD to work together with Martin and Lisbeth on expression analyses of the bought bacteria strains as well as of the strains from the Arla collection.

Miguel A Toro and Amalie V Thomasen worked in several periods at Arla Foods Innovation Centre in Agro Food Park on cultivating and testing Pseudomonas strains and different extracts from them to use in AU-FOOD laboratories. Plating and MALDI ToF MS typing of strains from Arla library collection was carried out at AIC.

Louise Almer (2021). Detection of the aprX gene in UHT milk using genomic amplification. Technical University of Denmark, Kgs. Lyngby, Denmark (M.Sc. thesis).

Amalie Vestergård Thomasen (2022). Milk spoilage enzymes from psychrotrophic bacteria. June 2022. Aarhus University, Denmark (M.Sc. thesis).

Miguel Aguilera Toro (2022). Influence of protein interactions and Pseudomonas-derived proteolysis on UHT milk quality and spoilage. PhD thesis, Jan, 2023.

15. New contacts/projects

Discussions with Eurofins Milk Testing during the project, which lead to applications to GUDP and InnovationFund Denmark, which unfortunately were not funded.

Project result discussion between AU-FOOD and Arla Foods amba, Oct 2022.

The development of a PCR based method is taken on in a newly granted DDRF project, led by Professor Lisbeth Truelstrup Hansen, and with project participation from Arla Foods amba.