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Lene Jespersen:

Improve Dairy Life – Forebyggelse af gærkontamineringer i fermenterede mejeriprodukter for forbedring af kvalitet og holdbarhed

Improve Dairy Life – Prevention of yeast spoilage of fermented dairy products for improved quality and shelf-life

Mejeribrugets
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Final report

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1. Title of the project

Danish: Forebyggelse af gærkontamineringer i fermenterede mejeriprodukter for forbedring af kvalitet og holdbarhed

English: Prevention of yeast spoilage of fermented dairy products for improved quality and shelf-life (Improve Dairy Life)

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4. Sources of funding

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5. Project period

Project period with dairy funding: January 2020 – December 2022

Revised, if necessary: January 2020 – June 2023

6. Project summary

Danish: Formålet med projektet var at karakterisere fordærvelsesgær med henblik på genetisk diversitet, vækst i mejeriprodukter, og hvordan mejerirelevante faktorer påvirker vækst og fordærvelsesevne. Projektet har fokuseret på kortlægning af gærkontamineringer gennem produktionen af salatost (white-brined cheese, WBC) på et dansk mejeri. Gær blev analyseret på 26 steder langs produktionsprocessen. De højeste gærantal og diversitet blev fundet i ostemasse, der tilbageføres i produktionen, hvor der blev identificeret 11 forskellige gærarter. Hyppigst fundne gær inkluderede *Candida intermedia*, *Kluyveromyces marxianus* og *Pichia kudriavzevii*. Successionen af gær blev efterfølgende undersøgt i WBC med urter (WBC-H) og med soltørrede tomater (WBC-T) over 52 ugers opbevaring ved lav temperatur (henholdsvis 5 °C and 10 °C). Ud af de 10 identificerede gærarter, var *Candida zeylanoides* og *Debaryomyces hansenii* de gær, der var bedst til at vokse

i WBC under opbevaring. Modelleringsforsøg af væksten af *D. hansenii* og *C. zeylanoides* på osteagar under mejerirelevante forhold viste lineær korrelation mellem de kinetiske parametre (maksimal specifik vækshastighed og lagfase) og koncentrationen af NaCl samt temperatur. Studier vedr. gær-gær interaktioner viste at *D. hansenii*, *Kluyveromyces lactis* og *Wickerhamomyces anomalus* havde stamme-specifikke inhiberende effekter mod andre gærarter isoleret fra WBC. Gærstammer af *K. marxianus*, *P. kudriavzevii* og *Torulaspora delbrueckii* blev isoleret fra skyr og vurderet med hensyn til deres fordærvelsespotentiale. Blandt disse gær, viste *K. marxianus* det højeste fordærvelsespotentiale i skyr. Dette skyldes blandt andet at *K. marxianus* i højere grad er i stand til at formere sig ved lav temperatur. *K. marxianus* er i skyr i stand til at producere flygtige organiske forbindelser (VOCs), ethanol og kuldioxid som fører til off-flavours og luftdannelse i produktet. *P. kudriavzevii* havde en moderat produktion af VOCs, mens *T. delbrueckii* havde en begrænset produktion. Dette skyldes til dels deres ringere evne til at vokse i skyr. Projektet har belyst kilder til gærkontaminering i osteproduktion samt identificeret gærarter, hvilket er afgørende for at forebygge krydskontaminering i mejeriproduktion og forhindre produktfordærvelsen. Projektet pegede på at udover kvantitative optællinger har gærdiversitet en stor betydning for produktets stabilitet, hvorfor det i en effektiv kvalitetskontrol er vigtigt at identificere de kontaminerende gær. Projektet har i særdeleshed opklaret forskelle blandt gærarter i deres fordærvelsespotentiale, og afklaret hvorledes gær metabolitter bidrager til udvikling af off-flavours og andre kvalitetsproblemer under opbevaring af mejeriprodukter.

English: The project aimed to characterize yeast spoilers in terms of genetic diversity, succession in dairy products, and spoilage capacity influenced by various factors. It focused on identification of yeast contaminants through the production of white-brined cheese (WBC) at a Danish dairy. Yeasts were analyzed at 26 locations along the production process, with the highest yeast counts and diversity found in old curd samples where 11 yeast species were identified. Most abundant spoilage yeasts included *Candida intermedia*, *Kluyveromyces marxianus*, and *Pichia kudriavzevii*. Succession of yeasts was further studied in WBCs with herbs (WBC-H) and sundried tomatoes (WBC-T) during 52 weeks of cold storage (5 °C and 10 °C). Out of 10 species identified in WBC, *Candida zeylanoides* and *Debaryomyces hansenii* were the best growing yeasts in WBC during storage. Modeling experiments of the growth of *D. hansenii* and *C. zeylanoides* under dairy-related conditions showed linear correlation between the kinetic parameters (maximum specific growth rate and lag phase) and the concentration of NaCl and temperature. Studies on yeast-yeast interactions showed that *D. hansenii*, *Kluyveromyces lactis*, and *Wickerhamomyces anomalus* had strain-specific inhibitory activity against other yeasts from WBC. Strains of *K. marxianus*, *P. kudriavzevii*, and *Torulaspora delbrueckii*, were isolated from skyr and assessed for their spoilage potential. Among these yeast, *K. marxianus* exhibited the highest spoilage potential in skyr. Compared to other yeast species, *K. marxianus* proliferated most extensively at cold storage, producing volatile organic compounds (VOCs), ethanol and carbon dioxide associated with off-flavours and package swelling. *P. kudriavzevii* had a moderate impact on VOCs, while *T. delbrueckii* had a minor effect, which could be attributed to comparatively lower viable counts of this species in skyr. The project identified yeast contamination sources and species, which is crucial for preventing cross-contamination and product spoilage in dairy production. It emphasized the importance of yeast taxonomic diversity alongside quantitative counts for quality control. Furthermore, it highlighted differences in growth and spoilage potential among yeast species, shedding light on how yeast metabolites contribute to off-flavour formation and other quality issues during cold storage of dairy products.

7. Project aim

Danish: Formålet med projektet er at identificere og karakterisere fordærvelses-gær isoleret fra danske mejeriprodukter samt at undersøge, hvorledes deres vækst og fordærv påvirkes af produktionsvilkår, mælkematrixen, interaktioner med starterkulturer og opbevaringsforhold. Baseret på dette udarbejdes prædiktive modeller til bestemmelse af holdbarheden af syrnede mejeriprodukter. Formålet bliver opnået gennem fem arbejdspakker: undersøgelse af gær kontaminering i mejeriproduktion (AP1); taksonomisk identifikation og

karakterisering af fordærvelsesgær isoleret fra danske mejerier (AP2); bestemmelse af uønskede smagsstoffer associeret med fordærvelsesgær (AP3); interaktionsstudier mellem fordærvelsesgær og relevante starterkulturer (AP4); og prædiktiv modellering af fordærvelses-gær under forhold specifikke for de enkelte mejeriprodukter (AP5). Projektresultaterne giver mejeriindustrien nye viden og redskaber til at forebygge vækst af fordærvelsesgær uden brug af fordyrende biobeskyttende kulturer samt sikre en forlænget holdbarhed af produkterne.

English: The main objectives of the project are to characterize the yeast spoilage consortium with regard to their taxonomic identity, genetic diversity, and spoilage capacity as affected by the dairy matrix, microbial interactions and storage conditions as well as to develop predictive models for yeast growth in fermented dairy products. The objectives are achieved through five work packages: monitoring yeast contamination in dairy production (WP1); identification and characterization of spoilage yeasts (WP2); influence of spoilage yeasts on flavour development in dairy products (WP3); studies on microbial interactions between spoilage yeasts and dairy starter cultures (WP4); and predictive modeling of yeast spoilage at dairy-relevant conditions (WP5). The outcome of the project provides the dairies with new knowledge and tools to prevent yeast spoilage of fermented dairy products without the use of costly bio-protective cultures and to extent the shelf-life of the products.

8. Background for the project

Microbiological spoilage is of major concern in production of fermented dairy products, compromising product quality, causing reduction of shelf-life, and, thereby, contributing to food waste and financial losses. Especially yeasts species are known to spoil fermented dairy products as e.g., cheese and skyr, before the end of the product shelf-life. However, yeasts are a very broad group of microorganisms, and their spoilage potential vary tremendously depending on their ability to grow in the dairy product, to form off-flavours and to cause discoloration[1,2]. It is, therefore, very important to be able to identify the spoilage yeast species to estimate its spoilage potential. Likewise, even within the same yeast species some variations might occur at the strain level and strain typing becomes essential in order to trace the original source of contamination[3,4]. Growth of yeast species in dairy products depends on their ability to propagate at refrigeration temperatures, to assimilate available sugars, to tolerate high concentrations of organic acids in milk such as lactic acid, and to grow at reduced water activity[5,6,7]. Other factors in the dairy production, such as cross-contamination during production, temperature fluctuations along the storage and distribution chain, will likewise affect the yeast growth. As dairy shelf-life should be viewed in a holistic perspective, taking into consideration all significant aspects affecting product quality, models predicting growth of contaminating yeasts need to be developed and a knowledge base established, linking the spoilage potential of each specific yeast species with the shelf-life of the dairy product.

References:
1.) Gori, K., Sørensen, L.M., Petersen, M.A., **Jespersen, L**., Arneborg, N. 2012. *Debaryomyces hansenii* strains differ in their production of flavour compounds in a cheese-surface model. Microbiology Open, 1, 161-168. 2.) Sørensen, L.M., Gori, K., Petersen, M.A., **Jespersen, L**., Arneborg, N. (2011) Flavour compound production by *Yarrowia lipolytica*, *Saccharomyces cerevisiae* and *Debaryomyces hansenii* in a cheese-surface model. Int. Dairy J., 21, 970-978. 3.) Ryssel M, Johansen P, Al-Soud WA, Sørensen S, Arneborg N, **Jespersen L**. 2015. Microbial diversity and dynamics throughout manufacturing and ripening of surface ripened semi-hard Danish Danbo cheeses investigated by culture-independent techniques. Int. J. Food Microbiol. 215:124–130. 4.) Krych L, Castro Mejia J, Moesby DN, Mikkelsen M, Rasmussen M, Nielsen DS. 2018. Finally, Bulk Typing of Bacterial Species down to Strain Level using ON-rep-seq (posted on bioRxiv: http://biorxiv.org/cgi/content/short/402156v1) 5.) Gori K, Ryssel M, Arneborg N, **Jespersen L**. 2013. Isolation and identification of the microbiota of Danish farmhouse and industrially produced surface-ripened cheeses. Environ. Microbiol. 65:602–615. 6.) Mortensen H, Gori K, Siegumfeldt H, Nissen P, **Jespersen L**, Arneborg N. 2006. Intracellular pH homeostasis plays a role in the NaCl tolerance of *Debaryomyces hansenii* strains. Appl. Microbiol. Biotechnol. 71:713-719. 7.) Petersen KM, Westall S, **Jespersen L**. 2002. Microbial succession of *Debaryomyces hansenii* strains during the production of Danish surfaced-ripened cheeses. J. Dairy Sci. 85:478–486.

9. Sub-activities in the entire project period

The updated five Work Packages (WPs) with tasks and deliverables and the Gantt chart with milestones are presented below.

WP1. Monitoring yeast contamination in dairy plants. ***Task 1.1. Sample collection.*** A plan for sample collection was established in cooperation between a Danish dairy and the FOOD-KU team. Samples were collected across all stages of WBC production during 2 trials conducted at a Danish dairy. ***Task 1.2. Enumeration of yeasts*****.** Samples were analyzed for the total yeasts counts to reveal the "hot-spots" of yeast contamination using standard microbiological procedures. **Deliverables:** *(i) Samples from cheese–making facilities for yeast analysis in WP2; and (ii) Identification of "hot-spots" of yeast contamination in dairy production.*

WP2. Identification and characterization of spoilage yeasts. ***Task 2.1. Taxonomic and phenotypic characterization of yeasts.*** Yeast isolates from WBC production were grouped according to their micro- and macromorphology and differentiated using rep-PCR. The representative isolates were further identified by 26S rRNA gene and the 5.8S rDNA-ITS region sequencing. Additionally, yeasts were isolated from skyr and identified to species level as described above and differentiated to a strain level by pulse-field gel electrophoresis (PFGE). Closely related species that could not be distinguished by the DNA sequencing (e.g., *K. marxianus* and *K. lactis*) were differentiated through phenotypic tests based on the utilization/fermentation profiles of carbohydrates. ***Task 2.2. Yeast diversity and succession in dairy products.*** Succession of yeasts was analyzed in two types of cheese products: WBC added herbs and WBC added tomatoes provided in modified atmosphere packaging by a Danish dairy. WBC packages were stored at 5 °C and 10 °C and withdrawn every two weeks during a shelf-life period of 52 weeks for yeast enumeration and identification, as described in Task 2.1. **Deliverables:** *(ii) A panel of yeasts with known taxonomy isolated from WBC production and from the dairy products; (iii) Insight into succession of spoilage yeasts species in dairy products throughout the shelf-life.*

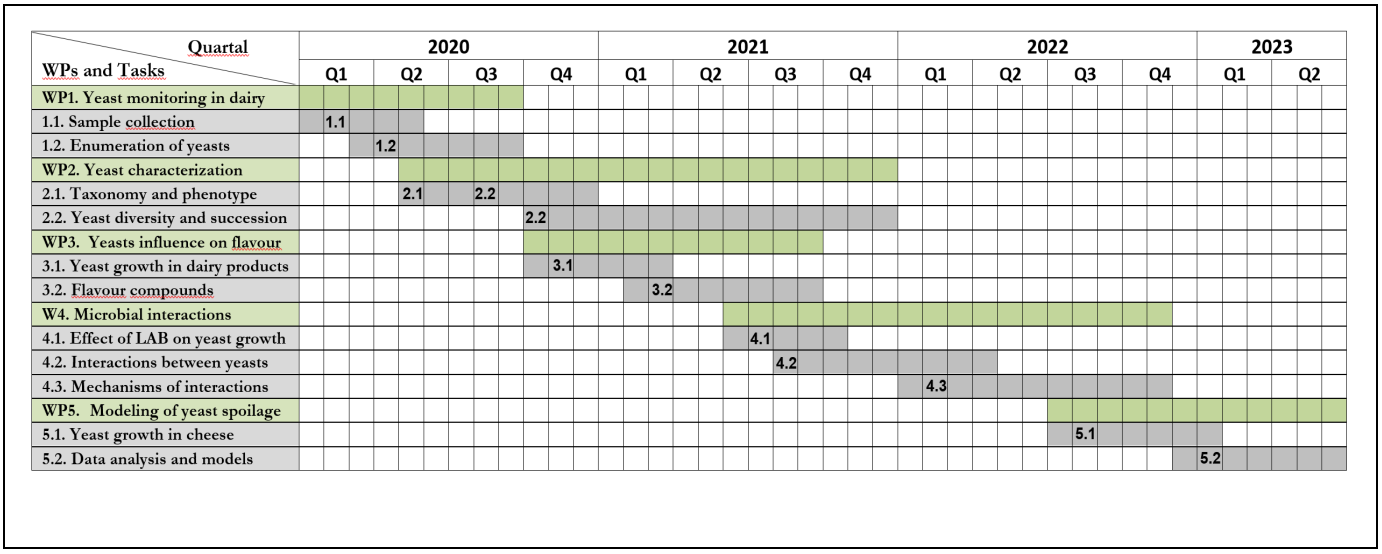
WP3. Influence of spoilage yeasts on flavour development in dairy products. ***Task 3.1. Yeast propagation in dairy products.*** Yeasts isolated from skyr in WP2 and in previous studies (5 strains of three species) were incubated in skyr under cold storage conditions (8°C). Samples were collected after every 7 days over 21-days storage period for microbial counts and biochemical analyses. ***Task 3.2. Determination of flavour compounds.*** Volatile aroma compounds, organic acids, carbohydrates, ethanol, and carbon dioxide were quantified in skyr samples collected in task 3.1 using advanced chromatography (GC-MS and HPLC) and standard biological methods. **Deliverables***: (i) List of metabolic compounds associated with the spoilage activity of yeasts in skyr; and (ii) Knowledge on the spoilage potential of specific yeast species and strains.*

WP4. Microbial interactions in dairy products. ***Task 4.1. Effect of lactic acid bacteria (LAB) on growth of spoilage yeasts.*** Dairy-related LAB cultures were assessed for their ability to either inhibit (antagonistic interactions) or enhance (synergistic interactions) the growth of yeast isolates from WBC and skyr in skim milk by measurements of viable counts. Additionally, inhibitory activity was evaluated using well-diffusion and radial agar assays. ***Task 4.2. Interactions between spoilage yeasts.*** We performed a large scale screening of 84 potential killer strains and 19 sensitive strains isolated from WBC and the production environment for their antagonistic interactions using the seeded agar-plate technique. ***Task 4.3. Mechanisms of microbial interactions.*** To characterize the inhibitory compounds and their genetic factors associated with antagonistic interactions, the yeast cultures were subjected to treatments with proteinases and heat, as well as plasmid and dsRNA curing. Subsequently, alterations in their killer activity were analyzed. Selected yeast strains were sequenced using Oxford Nanopore Technologies GridION system and the open reading frames (ORF) with sequence similarities to genetic elements from the NCBI GenBank database associated with killing activity were identified. **Deliverables:** *(i) List of yeast strains having potential to inhibit the growth of spoilage yeasts in dairy products; and (ii) Understanding of molecular mechanisms behind the yeast-yeast inhibition.*

WP5. Predictive modeling of yeast spoilage. ***Task 5.1. Yeast growth in cheese medium.*** Growth modelling experiments were performed with *D. hansenii* and *C. zeylanoides* isolated from WBC. The strains were grown on cheese agar medium containing variable content of sodium chloride at different temperatures. Growth kinetics of yeasts were monitored until stationary phase by quantifying colony-forming units (CFUs). ***Task 5.2. Explorative data analysis and growth models.*** The CFU data obtained for yeasts grown on cheese agar were analyzed using ComBase software to calculate the key kinetic parameters, including maximum specific growth rate (μ_{max}) and lag phase values. Subsequently, these data were employed to develop the mathematical models describing yeast growth dynamics as effected by sodium chloride content and storage tempera-

ture. **Deliverables:** *(i) Kinetic data of yeast growth as affected by temperatures and food matrices; (ii) predictive models for growth of spoilage yeasts in dairy products, and (iii) validated knowledge on the potential of yeast species to spoil the dairy products before the end of shelf life.*

Gantt chart (updated) showing the time schedule of WPs, tasks and milestones:



WP1: M1.1. Plan for sample collection, including specific locations within the entire WBC production developed (2th month). **M1.2**. Trials at a Danish Dairy finalized (4th month);
WP2: M2.1. Methods for strain differentiation and typing optimized (rep-PCR, PFGE) (5th month); **M2.2.** Procedures for the DNA sequencing and data analysis established (8th month); **M2.3.** Set-up and procedures for sample collection from WBC packages developed (10th month);
WP3: M3.1. A panel of spoilage yeasts isolated and identified from skyr (11th month). **M3.2**. Methods for quantification of flavour compounds from skyr (GC-MS, HPLC etc.) implemented (15th month);
WP4: M4.1. Screening agar-based methods to study microbial interactions optimized (19th month). **M4.2**. A panel of spoilage yeasts identified and deposited to the FOOD-KU culture collection (20th month) **M4.3**. Methods of DNA/RNA curing and high-throughput DNA sequencing (e.g., Nanopore) implemented (26th month);
WP5: M5.1. Design of the growth modelling experiments defined (32th month); **M5.2**. Kinetics data of yeast growth collected (37th month).

10. Deviations

Scientific deviations: WP2: Analysis of yeast microbiota in cheese samples using Illumina sequencing resulted in unsatisfactory yeast classification, which could be attributed to the potential inadequacy of databases or the poor DNA quality. Instead yeast classification was performed based on media isolation followed by 26S rRNA gene and 5.8S rDNA-ITS region sequencing. WP4: Since no growth inhibition or promotion was observed between LAB and yeasts, the studies were focused on yeast-yeast interactions. We conducted mechanistic studies employing appropriate DNA-based methodologies, such as DNA curing and whole-genome sequencing (Oxford Nanopore Technologies), instead of single-cell studies using microscopic techniques.
WP5. Challenge tests at the industry were not performed due to time constraints because of COVID-19 restrictions.

Financial deviations: No

Deviations related to the timetable: The project timeline has been extended to June 2023, primarily due to the delays in WP4 and WP5 activities caused by COVID-19 restrictions.

11. Project results

WP1. Monitoring yeast contamination in dairy plants.

Results are published in: Geronikou et al., 2022. doi.org/10.3390/microorganisms100610791079

Task 1.1. Sample collection.

WBC: Sampling was conducted at 26 sites across the production of two independent batches of WBC at a Danish Dairy, including cheese, curd, whey, ingredients, air, surface swaps, and finished products, among others.

***Task 1.2. Enumeration of yeasts*.**

WBC: Yeasts were detected at several locations, including old curd beneath the turning machine (5.44 log CFU/g), whey (1.48 log CFU/mL), and air in the draining room (1.02 log CFU/m^3), indicating "hot spots" of yeast contamination. Other samples collected from WBC production sites were tested negative for yeasts (less than 2 CFU/g).

WP2. Identification and characterization of spoilage yeasts

Results are published in: Geronikou et al., 2020. doi.org/10.3389/fmicb.2020.582778; Geronikou et al., 2023. doi.org/10.1016/j.fm.2023.104266

Task 2.1. Taxonomic and phenotypic characterization of yeasts.

WBC: In total 100 yeast isolates from WBC production were identified to species level. The predominant species in whey samples and old curd were *P. kudriavzevii* (26% of isolates), *Candida intermedia* (20% of isolates), and *Kluyveromyces marxianus* (15% of isolates). The largest abundance and diversity of yeasts were found in the old curd with *C. intermedia* prevailing as the dominant species (24% isolates from the curd). Other identified yeast species included *Candida pseudoglaebosa*, *Candida sojae*, *Cutaneotrichosporon curvatus*, *Papiliotrema flavescens*, *Rhodotorula mucilaginosa*, *Vanrija humicola*, and *Wickerhamiella sorbophila*. Air samples collected from the draining room primarily contained *K. marxiamus*, and *Candida parapsilosis*.

Skyr: Yeast isolates from skyr (10 in total) were all identified as *K. marxianus* and differentiated to strain level by PFGE. These isolates were used in WP3.

Task 2.2. Yeast diversity and succession in dairy products.

WBC: Succession of yeasts was investigated in WBC added herbs (WBC-H) and WBC added sundried tomatoes (WBC-T) during incubation at 5°C and 10°C for 52 weeks. Extensive growth was obtained within the initial 12-14 weeks, starting from undetectable levels (<2 log CFU/g) (Fig 1). Subsequently, the yeast counts stabilized, fluctuating from 4.2 to 7.1 log CFU/g depending on incubation temperature and WBC type. Notably, a higher incubation temperature resulted in lower yeast counts and a greater diversity of yeast species, particularly observed in WBC-T. Lower yeast counts were probably due to negative interactions between yeast species leading to inhibition of otherwise dominating species, like *C. zeylanoides* and *D. hansenii*.

A total of 469 yeast isolates were purified from WBCs and classified using the $(GTG)_5$-rep-PCR technique. Among these, 132 representative isolates were further identified via the 26S rRNA gene sequencing. The distribution of yeast species in WBCs over time is illustrated in Figure 2. Predominant yeasts included *C. zeylanoides* and *D. hansenii*, commonly associated with yeasty and bitter off-flavours. Additionally, *K. lactis* was consistently found in lower numbers in WBC-H stored at 10°C and in WBC-T stored at both 5°C and 10°C. Less frequently identified species comprised *C. parapsilosis*, *Kazachstania bulderi*, *K. lactis, Pichia fermentans, P. kudriavzevii, R. mucilaginosa, T. delbrueckii,* and *W. anomalus*. The diversity of yeasts in WBC-T was generally greater, suggesting a more nutrient-rich environment facilitating the growth of species such as *K. bulderi, P. kudriavzevii*, *P. fermentans*, *R. mucilaginosa*, and *T. delbrueckii*. Notably, some of the identified yeasts, as *C. zeylanoides*, *C. parapsilosis*, and *P. kudriavzevii*, are considered as opportunistic pathogens. Interestingly, species *C. parapsilosis, P. kudriavzevii,* and *R. mucilaginosa,* were also identified in the production of WBCs (WP1) indicating potential cross-contamination from the production facilities.

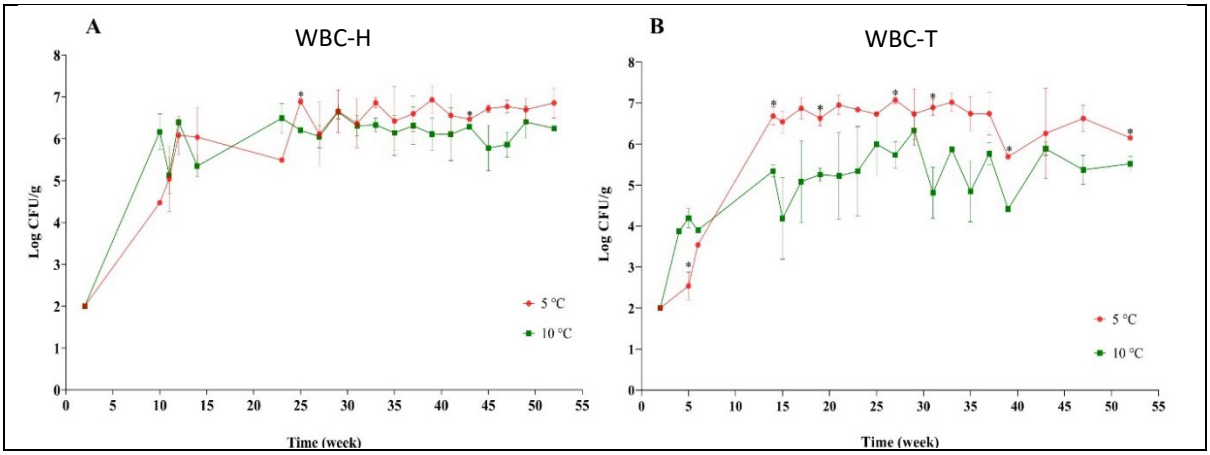


Figure 1. Yeast viable counts (log CFU/g) during incubation of white-brined cheeses added herbs WBC-H (A) and sun-dried tomatoes WBC-T (B) at 5°C and 10°C for 52 weeks. CFU counts are presented as mean values and SD from two biological and two technical repeats. Asterisks (*) denote significant differences between the temperatures at the same time-point using the unpaired Student's t-test ($P < 0.05$).

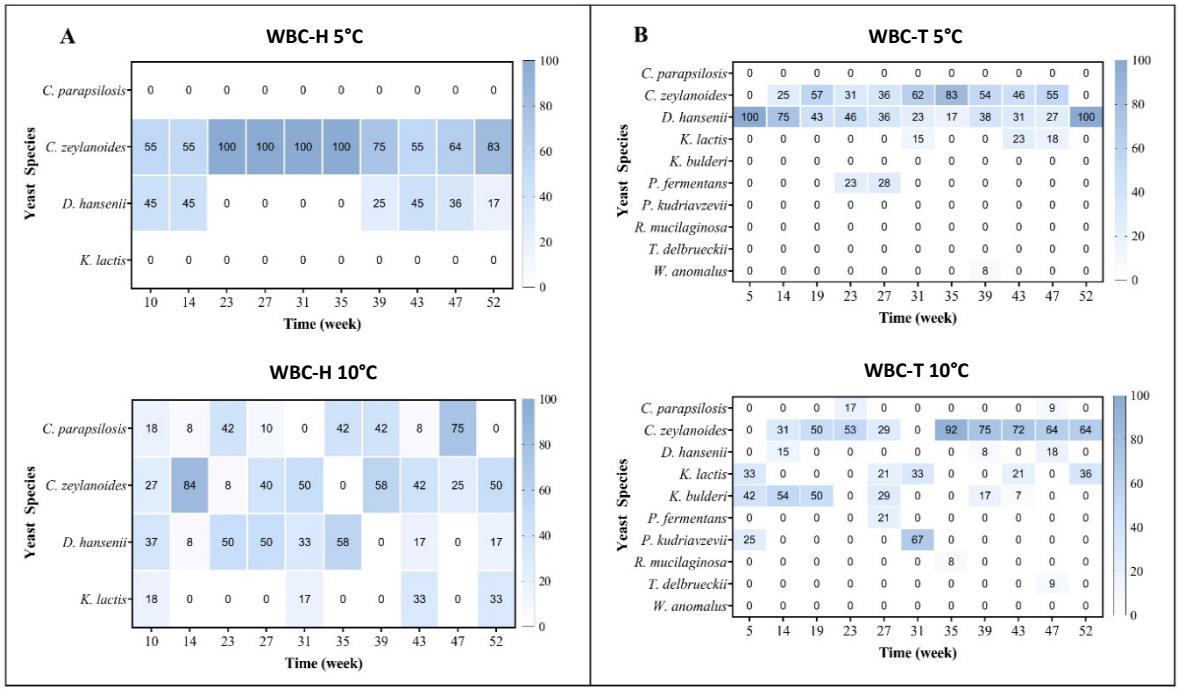


Figure 2. Heat maps showing relative distribution (%) of yeast species isolated from (A) white-brined cheese added herbs (WBC-H) and (B) white-brined cheese added sundried tomatoes (WBC-T) during 52 weeks of storage at 5°C and 10°C.

WP3. Influence of spoilage yeasts on flavour development in dairy products

Results are published in: Srimahaeak et al., 2021. doi.org/ 10.3390/foods11121776

Task 3.1. Yeast propagation in dairy products.

Skyr: Strains of *K. marxianus* (Km1, Km2 and Km3) identified in WP1 along with *P. kudriavzevii* Pk1 and *T. delbrueckii* Td1, derived from skyr and provided by Arla Foods, were used for the experiments. The strains were tested for spoilage potential in skyr during 21 days incubation at 8°C. All yeast strains were capable of growing in skyr, with *K. marxianus* attaining the highest counts (7.0 ± 0.2 Log CFU·g^{-1}), while *P. kudriavzevii* Pk1 and *T. delbrueckii* Td1 were grown to lower numbers (5.4 ± 0.2 and 5.7 ± 0.1 Log CFU·g^{-1}, respectively). The pH values and viable counts of lactic acid bacteria in skyr (9.6 ± 0.2 Log CFU·g-1) remained stable and unaffected by yeasts throughout the incubation period.

Task 3.2. Determination of flavour compounds.

Skyr: *K. marxianus* strains produced a diverse array of volatile compounds (VOCs) linked to off flavours (Fig. 3A). The gradual increase in alcohols (from 10% to 78%) during incubation indicated fermentation of lactose in skyr under oxygen limitation conditions. Among these, isoamylalcohol (whiskey, malt aroma), 2-methyl-1-propanol (alcoholic aroma), 1-pentanol (fusel) and 1-octanol (orange) were abundant alcohols possessing low sensory thresholds. Esters of formic, lactic, propionic acetic acid, etc., commonly associated with fruity notes, increased significantly in *K. marxianus* incubations (from 2% to 14% after 21 days). Specifically, ethyl acetate (solvent aroma), ethyl butyrate (pineapple), ethyl hexanoate (aniseed, apple), ethyl octanoate (sour apple) and isoamylacetate (banana, pineapple) were produced in quantities above thresholds. Aldehydes were reduced by *K. marxianus* (from 8% to 2%), which can be considered as favourable as straight-chain aldehydes (e.g. hexanal) are associated with rancidity in foods. Key aroma compounds, diacetyl and acetoin, linked to buttery, creamy notes, significantly decreased in skyr incubated with strains of *K. marxianus* and *P. kudriavzevii* Pk1 compared to controls. Additionally, the growth of *P. kudriavzevii* Pk1 resulted in modest increases in alcohols and esters (mainly 3-methyl-1-butanol and ethyl acetate).

The levels of lactose and galactose (Fig. 3B) gradually decreased during incubation of *K. marxianus* in a strain-dependent manner, indicating their capacity to assimilate lactose and galactose in skyr. During growth of *P. kudriavzevii* Pk1 and *T. delbrueckii* Td1 lactose was reduced after 7 days incubation and remained stable afterwards. In contrast to the other yeast species, lactose utilization by *K. marxianus* led to production of ethanol and carbon dioxide (Fig. 3C). In conclusion, extensive growth capacity, formation of undesired aroma and carbon dioxide indicated high spoilage potential of *K. marxianus* contaminants. *T. delbrueckii* Td1 demonstrated lowest spoilage potential, with minor changes in metabolite production compared to other tested yeasts.

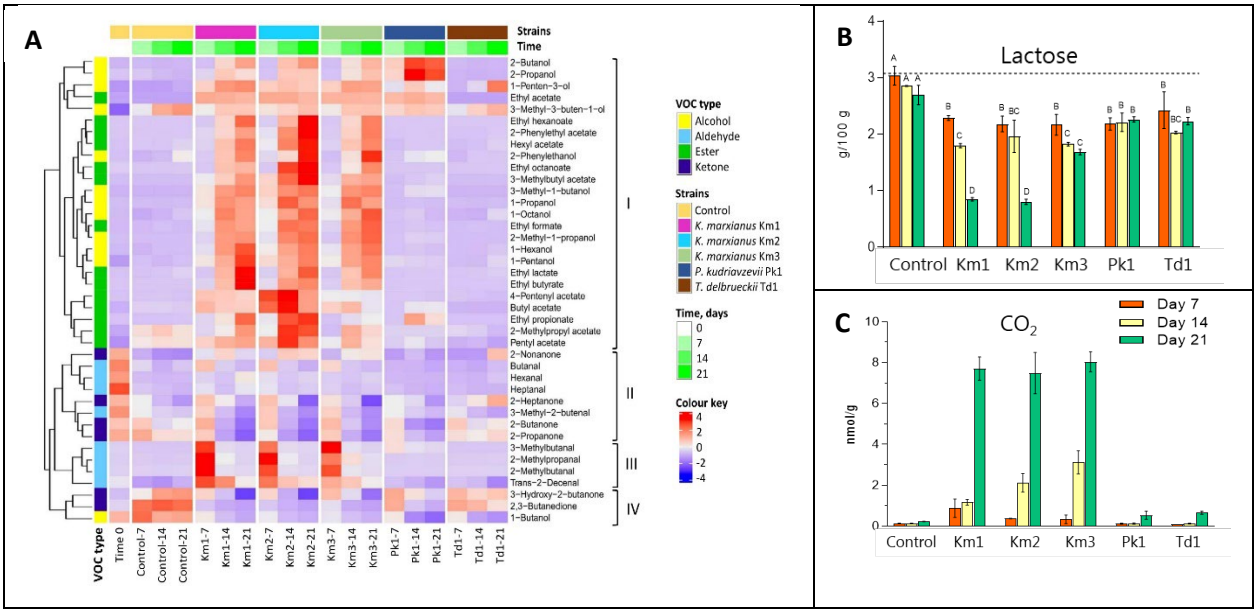


Figure 3. Volatile organic compounds (VOC), lactose and carbon dioxide (CO_2) during growth of *K. marxianus* strains (Km1, Km2 and Km3), *P. kudriavzevii* Pk1 and *T. delbrueckii* Td1 in sky after 0, 7, 14 and 21 days at 8°C. Mean values and SD from triplicate experiments are shown. (A) VOC heat map with hierarchical cluster analysis (Pearson's correlation); (B) Lactose concentration (g/100g skyr) with initial content indicated by the dashed line. Statistical differences denoted by different subscripts ($p < 0.05$) within the same incubation period (one-way ANOVA, Tukey's post-hoc analysis); and (C) Carbon dioxide (CO_2) production (nmol·g^{-1}).

WP4. Microbial interactions in dairy products.

Task 4.1. Effect of lactic acid bacteria (LAB) on growth of spoilage yeasts.

The growth of yeasts, comprising eleven strains from eight different species found in WBC and skyr, was not significantly influenced by the tested LAB, which consisted of five strains from three species obtained from the Sacco collection. Agar assays revealed no antagonistic interactions between the tested LAB and yeast strains.

Task 4.2. Interactions between spoilage yeasts.

In total, 37 strains (out of 84 tested) belonging to *D. hansenii*, *K. lactis* and *W. anomalus* exhibited inhibitory activity against at least one strain of *K. lactis, K. marxianus, W. sorbophila, T. delbrueckii*, *Candida* spp.*, Cutaneotrichosporon* spp.*,* and *Pichia* spp*.* (Fig. 4).

Task 4.3. Mechanisms of microbial interactions.

Strains of *D. hansenii*, *K. lactis* and *W. anomalus* displaying the broadest inhibitory spectrum were subjected to proteinase and heat treatments. As a result, the inhibitory activity of these strains was reduced by 40%, indicating the proteinaceous nature of the inhibitory compounds. Moreover, a decrease in inhibitory activity by 50-60% was observed in *K. lactis* and *W. anomalus* after curing of the cells for plasmids, suggesting that the killer phenotype in these strains was related to plasmids. In case of *D. hansenii,* genetic determinants of the killer activity were likely linked to dsRNA as evidenced by a significant reduction of yeast inhibition after curing for dsRNA. Genome sequencing of *D. hansenii* and *K. lactis* revealed similarities to multiple genes associated with autonomous gene replication, DNA virus transcription, killer plasmids, such as pGKL2 in *K. lactis*, and other genetic elements associated with antimicrobial functions.

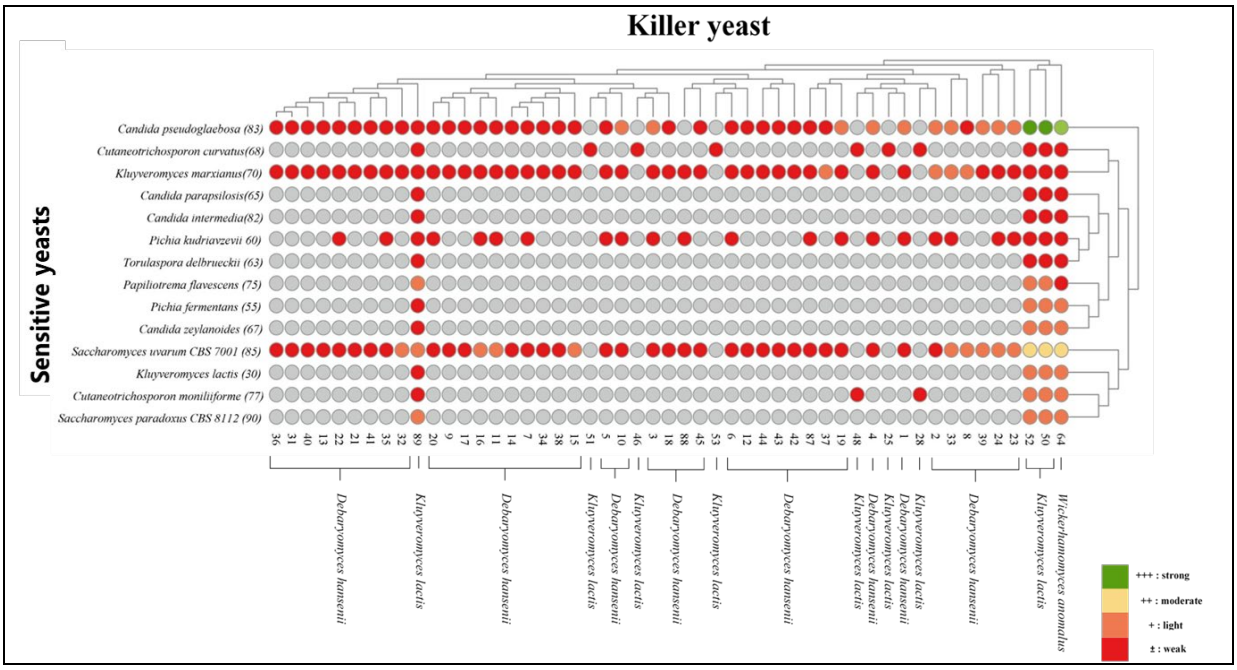


Figure 4. Inhibition profile of yeasts isolated from WBC. Inhibition assay was performed using the seeded-agar plate technique on YMA agar pH 4.5 added 2% w/v NaCl. The inhibition activity was calculated from the area of the clear zone (mm^2) after 5 days incubation and described as weak (red, < 2 mm^2), light (orange, 2 – 6 mm^2), moderate (yellow, 6 – 10 mm^2), and strong (green, > 10 mm^2).

WP5. Predictive modeling of yeast spoilage

Task 5.1. Yeast growth in cheese.

Concentration of sodium chloride (2, 5 and 8% w/v NaCl) and incubation temperatures (5, 10 and 15°C) had a significant effect on growth of *D. hansenii* and *C. zeylanoides*, cultivated on cheese agar (Fig. 5). *D. hansenii* demonstrated better growth reaching higher CFU counts (~ 8 Log CFU/g) at stationary phase compared to *C. zeylanoides* (~ 7 Log CFU/g).

Task 5.2. Explorative data analysis and growth models.

A high NaCl concentration (8% w/v), combined with low temperature (5°C), resulted in an extended lag phase and lower growth rates. This effect was particularly pronounced for *C. zeylanoides*. In contrast, *D. hansenii*

was more tolerant to low temperatures and high NaCl concentration exhibiting higher growth rate and shorter lag phase compared to *C. zeylanoides*. This suggests a higher spoilage potential of *D. hansenii*.

Based on the growth data, the maximum growth rate (μ_{max}) and lag phase for *C. zeylanoides* as well as lag phase for *D. hansenii* exhibited a linear response to the changes in NaCl C_{NaCl} and temperature (Temp). This relationship can be presented by the following model:

μ_{max} (or lag phase) = a × (C_{NaCl} - α) × (Temp - β) + γ, where "a" is a slope and "α, β and γ" are constants.

Prediction of the maximum growth rate for *D. hansenii* was uncertain due to lack of data regarding the impact of high NaCl concentrations. Overall, the effect of temperature on the lag phase was more significant than salt, emphasizing that refrigerated storage is an effective strategy for protecting cheese products against yeast spoilage.

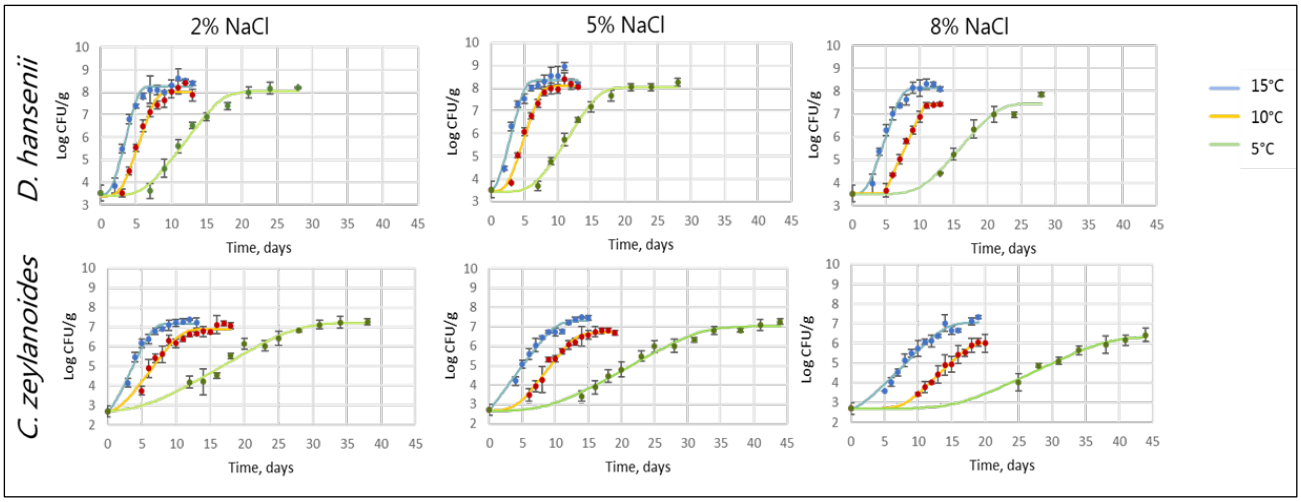


Figure 5. Viable counts of *D. hansenii* and *C. zeylanoides* (log CFU/g) grown on cheese agar as affected by NaCl concentration (2, 5 and 8% (w/v) NaCl) and incubation temperature (5, 10 and 15 °C). The points and vertical bars represent the mean values from triplicate experiments (± SD), while the solid lines represent the fitted model values using ComBase software.

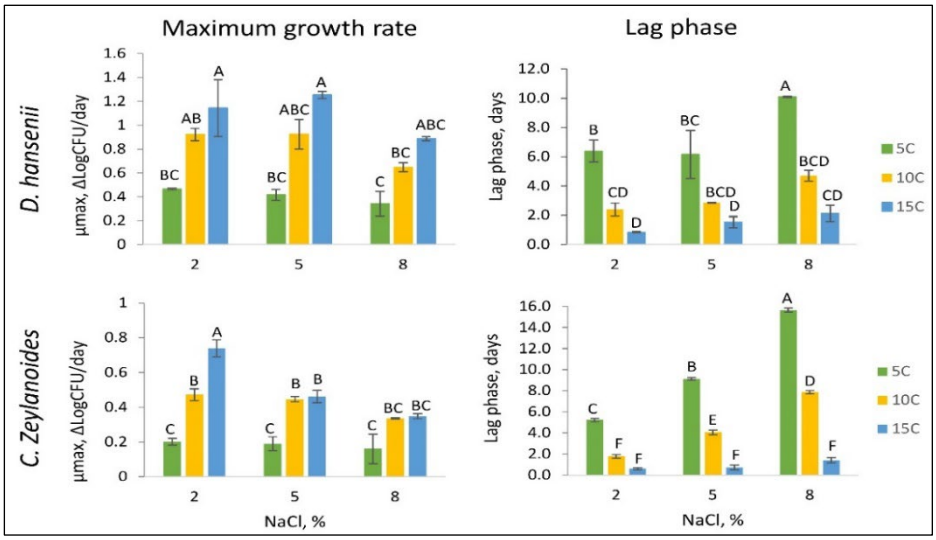


Figure 6. Maximum growth rate (μmax, ΔLogCFU/day) and Lag phase (days) of *D. hansenii* and *C. zeylanoides* grown on cheese agar with 2, 5 and 8% w/v NaCl at 5, 10 and 15°C. Mean values (± SD) from triplicate experiments are represented. Different letters denote significantly different values (Anova Tukey's post hoc test, $p<0.05$).

Overall conclusions on project results:

White-brined cheese:

Diverse yeast species in cheese production: The study identified a high diversity of yeast species (13 in total) as contaminants during the production of WBC. Some of these species (e.g. *C. intermedia*, *K. marxianus*, *P. kudriavzevii*) are common dairy spoilers, while others originate from different sources.

Yeast growth in WBC at cold storage: Yeast contaminants were capable of growing in WBC during cold storage (5 and 10°C for up to 52 weeks), reaching substantial populations of up to 7 log CFU/g.

Temperature and ingredients influence yeast diversity in WBC: The abundance and diversity of yeast species varied during storage, with more diverse communities and lower yeast counts associated with increased temperatures and the addition of sundried tomatoes to WBC.

Dominant yeast species in WBC: Throughout the 52-week shelf-life of WBC, the dominant yeast species were *C. zeylanoides* and *D. hansenii*, with several other less dominant species present.

Importance of taxonomic heterogeneity: An inverse correlation was observed between yeast viable counts and yeast heterogeneity, particularly in WBC with sundried tomatoes, highlighting the importance of considering yeast heterogeneity in quality assessment.

Salt and temperature impact on yeast growth: The growth parameters of *D. hansenii* and *C. zeylanoides* in cheese matrices showed linear correlation with NaCl concentration and incubation temperatures. High NaCl concentration (8% w/v) combined with low temperature (5°C) had a particularly detrimental effect on yeast growth, with *C. zeylanoides* being most adversely affected.

Inhibitory activity of yeasts: Specific isolates of *D. hansenii*, *K. lactis* and *W. anomalus* from WBC exhibited inhibitory activity against other yeasts, including *C. pseudoglaebosa*, *K. marxianus*, *P. kudriavzevii*, and *Saccharomyces uvarum*.

Skyr:

Species-specific growth and metabolite production in skyr: Spoilage yeasts, specifically *K. marxianus*, *P. kudriavzevii*, and *T. delbrueckii*, had the ability to proliferate in skyr during cold storage (5 °C and 10 °C), each displaying unique growth characteristics and metabolite production profiles.

High spoilage potential of *K. marxianus*: Species *K. marxianus* is of particular concern in skyr, as it exhibited the highest viable counts and released significant amounts of various aroma compounds (VOCs), carbon dioxide and ethanol, which are associated with off-flavours and product deterioration, highlighting its high spoilage potential in skyr.

General conclusion:

Importance of distinguishing between yeast species and strains: This study emphasizes the importance of distinguishing between contaminating yeast species and even strains when assessing their impact on product quality and shelf-life. It suggests a paradigm shift in quality control in dairy from solely determining CFU to more detailed studies that focus on yeast species identification, growth patterns, and metabolite profiles. Such an approach can help in better understanding and managing yeast-related spoilage in dairy products like cheese and skyr.

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

Contamination with yeasts is a common problem in dairy production recognized for decades. Even though huge variations in growth characteristics exist between yeast species, academic knowledge on the spoilage potential of specific yeast species in acidified dairy products does practically not exist. The genotypes of

spoilage yeasts as well as their growth characteristics are currently not known to Danish dairies. As a consequence, the dairies are today not able to differentiate between harmless yeast species not being able to multiple in the dairy products and yeast species with huge spoilage potential.

The knowledge acquired by monitoring yeast contamination throughout the dairy production process, identifying hot-spots and classifying yeast species, enables the dairies to implement advanced quality control measures and differentiate between contaminating yeast species that can potentially spoil dairy products. The project provides detailed knowledge on contaminating yeast species in dairy products with a particular focus on their classification, succession patterns and spoilage potential. This knowledge encompasses various aspects, including growth characteristics of yeasts during product self-life, their interactions of other microbial communities, their ability to produce undesirable flavours, organic acids, gases and other quality-altering changes. To address the issue of spoilage, the project systematically examines how yeast growth is affected by dairy-specific conditions, such as temperature fluctuations during storage, using advanced mathematical models predicting growth of spoilage yeasts based on multiple intrinsic and extrinsic factors.

By thoroughly characterizing yeast spoilage potential and developing predictive growth models, the project facilitates the dairies with innovative tools and solutions for controlling yeast-related spoilage. This not only reduces the reliance on costly bio-protective cultures but also paves the way for exploration of new innovative approaches to enhance product quality and extend shelf life.

13. Communication and knowledge sharing about the project

Papers in international journals:

- Geronikou A, Srimahaeak T, Rantsiou K, Triantafillidis G, Larsen N and Jespersen L. 2020. Occurrence of Yeasts in White-Brined Cheeses: Methodologies for Identification, Spoilage Potential and Good Manufacturing Practices. Frontiers in Microbiology. 11, 582778. https://doi.org/10.3389/fmicb.2020.582778
- Srimahaeak T, Petersen MA, Lillevang SK, Jespersen L and Larsen N. 2021. Spoilage Potential of Contaminating Yeast Species *Kluyveromyces marxianus*, *Pichia kudriavzevii* and *Torulaspora delbrueckii* during Cold Storage of Skyr. MDPI Foods, 11,1776. https://doi.org/10.3390/foods11121776
- Geronikou A, Larsen N, Lillevang SK, and Jespersen L. 2022. Occurrence and identification of yeasts in production of white-brined cheese. MDPI Microorganisms, 10, 1079. https://doi.org/10.3390/microorganisms10061079
- Geronikou A, Larsen N, Lillevang SK, and Jespersen L. 2023. Diversity and succession of contaminating yeasts in white-brined cheese during cold storage. Food Microbiology, 113. https://doi.org/10.1016/j.fm.2023.104266

Easily read papers:

- Larsen N, Geronikou A and Jespersen L. 2020. Gærkontamineringer skal være fortid i dansk mejeriindustri. Mælkeritidende. Nr. 11.
- Larsen N, Kristensen L and Jespersen L. 2024. Vigtig viden for at begribe og begrænse uønskede gær i mejeriprodukter. Mælkeritidende nr. 4, 18-19.

Student theses:

PhD thesis:

- Thanyaporn Srimahaeak. “Potential use of beneficial microbes: in food and for health”, June 2022.
- Athina Geronikou. “Characterization and diversity of spoilage yeasts in dairy production with focus on their killer effect”. Submitted October 2023.

MSc thesis and reports:

- Pedersen EM. “Identification of spoilage yeasts in white-brined cheese and investigation of contamination sources in the production line”. March 2020.

- Gröbner SR. "Functional characteristics and spoilage potential of yeasts isolated from Danish white-brined cheese". January, 2021.
- Liu Q. "Interactions between the dairy-related spoilage yeasts and lactic acid bacteria". January, 2021.
- Christos Toliopoulos and Eleni Mucka, University of Aegean. Report "Modelling yeast growth in white-brined cheese as affected by salt concentration and temperature". May 2023.

Oral and poster presentations at scientific conferences, symposiums etc.:

- Geronikou A, Larsen N, and Jespersen L. Identification and succession of spoilage yeasts in the Danish feta-type cheese. MiFFi, November 2021. Frederiksberg, Denmark
- Geronikou A, Larsen N, and Jespersen L. Occurrence of spoilage yeasts in production of the Danish white-brined cheese and their succession in the final cheese product. FoodMicro Conference, August 2022, Athens.

Oral presentations at meetings:

Consortium meetings were held quarterly including presentations of project results and activities by PhD students and Postdocs.

14. Contribution to master and PhD education

The following persons were educated during the project:

- 2 PhD students: Thanyaporn Srimahaeak and Geronikou Athina
- 3 MSc students: Pedersen Emma, Gröbner Susann and Liu Quanyin
- 2 Erasmus Mundus exchange students: Christos Toliopoulos and Eleni Mucka

Knowledge obtained in "Improve Dairy Life" has been integrated into various student courses at FOOD-KU, including the MSc courses "Microbiology of Fermented Food and Beverages", "Dairy Microbiology", and "Yeast Physiology and Application".

15. New contacts/projects

2 MSc projects dealing with inhibition of yeasts contaminants have been planned together with Chr. Hansen starting November 2023.