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Paw Dalgaard:

Cbot-PREDICTOR – *Clostridium botulinum* og sikre oste

Cbot-PREDICTOR – *Clostridium botulinum* and safe cheeses

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Final report

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1. Title of the project

DK: *Clostridium botulinum* og sikre oste (Cbot-PREDICTOR)

ENG: *Clostridium botulinum* and safe cheeses (Cbot-PREDICTOR)

2. Project manager

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3. Other project staff

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4. Sources of funding

Danish Dairy Research Foundation (Mælkeafgiftsfonden/Milk Levy Fund), National Food Institute (DTU Food), Arla Foods amba and KU Leuven.

5. Project period

Project period with DDRF funding: November 2018 – October 2021

6. Project summary

In Danish:

Cbot-Predictor projektet fokuserede på udviklingen af vækst- og vækstgrænse-modeller for proteolytiske *Clostridium botulinum*. Dette er en patogen bakterie, der producerer en række meget potente neurotoksiner (BoNT'er). På grund af denne markante giftighed af *C. botulinum* toksiner, kræves særlige laboratorier og procedurer ved forsøg med denne bakterie. Dette gør laboratoriearbejde med bakterien meget kostbart og mange typer af forsøg kan ikke udføres. Af denne grund blev ikke-giftige *C. sporogenes* stammer anvendt som surrogater for toksin-producerende *C. botulinum* gennem dette projekt. En cocktail af tre *C. sporogenes* isolater blev anvendt til udvikling, kalibrering og evaluering/validering af en omfattende vækst og vækst-grænse model for proteolytisk *C. botulinum*. Proteolytiske *C. botulinum* (Gruppe 1) er en mesofil bakterie, som vokser godt ved stuetemperatur og som ikke vokser når temperaturen er under 10°C. Sporer af proteolytisk *C. botulinum* er udbredt i naturen og det er derfor meget sandsynligt at finde disse i fødevarer. Selvom udbrud af botulisme på grund af indtagelse af smelteost er sjældne, kan *C. botulinum* vokse og producere

toksiner i smelteoste, der opbevares ved stuetemperatur. Det er derfor relevant at efterspørgsel efter smelteost på nye markeder (f.eks. Afrika og Mellemøsten) har skabt en udfordring i forhold til køledistribution og opbevaring. Endvidere giver de tilgængelige retningslinjer for håndtering af proteolytisk *C. botulinum* ikke fleksibilitet til produktudvikling af denne type produkter. Disse retningslinjer angiver minimum 10 % salt i produkters vandfase, eller en pH-værdi under 4,6, hvis der ikke anvendes et tilstrækkeligt varmebehandlingstrin (121°C i 3 min.) i løbet af forarbejdningen af fødevaren.

En ny vækst- og vækstgrænsemodel for *C. sporogenes*, som surrogat af *C. botulinum*, blev udviklet indenfor dette projekt. Modellen inkluderede effekten af elleve miljøfaktorer, herunder mejerispecifikke ingredienser, såsom organiske syrer og smeltesalte, samt effekten af interaktionseffekten mellem de elleve faktorer. Modellen blev kalibreret med smelteost og succesfuldt valideret for smelteost og kødprodukter. Den nye model har et bredere anvendelsesområde og bedre evne til at forudsige vækst og vækstgrænse sammenligning med andre tilgængelige modeller for proteolytiske *C. botulinum*. Den nye model og det udviklede prædiktionsværktøj kan anvendes af fødevarevirksomheder til produktudvikling eller omformulering af eksisterende produktopskrifter samt til dokumentation af fødevaresikkerhed. Desuden kan modellen bruges til undervisning og af myndighederne til at understøtte mere fleksible retningslinjer således unødvendig over-konservering reduceres.

In English:

To facilitate management of *Clostridium botulinum* (Proteolytic; Group 1) in dairy products at ambient temperature this Cbot-Predictor project focused on the development of a mathematical model to predict growth and growth boundary for the bacterium. *C. botulinum* is a spore-forming pathogenic bacterium that produces very potent neurotoxins (BoNTs). Due to the high toxicity of *C. botulinum* demanding requirements are requested for premises/laboratories and personnel that handle the bacterium. The use of non-toxigenic surrogates for laboratory experiments is therefore more efficient and less costly. A three strains cocktail of *C. sporogenes* was used to develop, calibrate and evaluate/validate an extensive growth and growth boundary model. This model allowed prediction of the combined effect of product characteristics and storage temperature to prevent growth and toxin formation by proteolytic *C. botulinum*.

Proteolytic *C. botulinum* is a mesophilic bacterium which grows well at ambient temperatures and does not grow below 10°C. Spores of proteolytic *C. botulinum* are widespread in nature and therefore very likely to be found in food. Although outbreaks of botulism due to consumption of processed cheeses are rare, *C. botulinum* can grow and produce toxins in processed cheeses stored at ambient temperatures. The demand for processed cheese in new markets (e.g. Africa, Middle East) has created a challenge in relation to chilled distribution and storage. Furthermore, the available guidelines for management of proteolytic *C. botulinum* do not provide flexibility for product development of this type of products. These guidelines suggest a minimum water phase salt (WPS) of 10% or a pH below 4.6, if a sufficient heat-treatment step (121°C for 3 min) is not applied during processing. A new growth and growth boundary model for *C. sporogenes*, as a surrogate of *C. botulinum* was developed. The model contained the effect of eleven environmental factors, including dairy specific ingredients such as organic acids and melting salts, and the interaction effect between the eleven factors. The model was calibrated with processed cheese and successfully validated for processed cheese and meat products. This extensive model showed a wider range of applicability and superior performance when it was compared to other available proteolytic *C. botulinum* models for processed cheese. The new model and predictive tool developed in the present project can be used by food business operators for product development or reformulation of existing recipes and for documentation of food safety. Furthermore, the model can be used for teaching and by the authorities to support more flexible guidelines so that unnecessary over-preservation is reduced.

7. Project aim

In Danish:

Projektet udvikler en matematisk model og et software til at forudsige betingelser, der forhindrer vækst af den væsentlige human-patogene bakterie *Clostridium botulinum* i smørbare oste ved distribution uden køling. Denne model og software vil gøre det lettere at udvikle sikre smørbare oste. I Danmark er der behov for at forarbejde et overskud af mælk til produkter, der kan eksporteres til vækstmarkeder udenfor Europe. Dette kræver innovation og projektet bidrager til fleksibel produkt-udvikling samt reduceret udviklingstid for nye sikre smørbare oste. Studier af *C. botulinum* i fødevarer er kostbare og tidskrævende fordi bakterien danner en meget farlig nervegift. Derfor vil vi indenfor projektet udvikle mutanter af *C. botulinum*, som ikke danner nervegift. Disse uskadelige mutanter anvendes derefter til at udvikle en matematisk vækst og vækst-grænse model for *C. botulinum*. Projektet indeholder et samarbejde mellem to forskningsgrupper med meget forskellige kompetencer og et tæt samarbejde med den danske mejerisektor.

In English:

The project aims to develop a mathematical model and a tool to reliably predict no-growth conditions for the important pathogenic microorganism *Clostridium botulinum* in spreadable cheeses during distribution at ambient temperature. This predictive tool will facilitate product formulation and innovation of safe spreadable cheeses. There is a need to increase processing of excess milk in Denmark into new value-added products for export to growing markets outside Europe. Innovation is needed and the project contributes to flexible product development and reduced time-to-market for new spreadable cheeses. Studies of *C. botulinum* in food are costly and time consuming due to its formation of a very potent neurotoxin. A new approach is applied, and non-toxigenic mutants of *C. botulinum* are constructed within the present project and then used to develop a growth and growth-boundary model. The project includes two research groups with complementary skills and close collaboration with the Danish dairy sector.

8. Background for the project

After the removal of EU quota in 2015 there has been an interest in using excess milk in Denmark to create added value dairy products for export out of Europe, as very limited growth within the EU market was expected. By producing new processed/spreadable cheeses it was estimated that more value could be created compared to the production of milk powder. Processed cheese was interesting because its consumption is expected to increase in several regions where consumption of dairy products exceeds local production e.g. in China and the Middle East. To experimentally identify conditions resulting in a desired safe recipe and its related shelf-life at ambient temperature for processed/spreadable cheeses is both costly and time-consuming. Clearly, available guidelines for safe product formulations can be used to manage *C. botulinum* at ambient temperature but then product characteristics must include pH below 4.6 or water phase salt above 10%. These conditions are not compatible with development of new processed/spreadable cheeses. Alternatively, challenge testing can be used to document safety of recipes where various product characteristics contribute to control microbial hazards including *C. botulinum* (NACMCF, 2010). However, these approaches do not convey the flexibility and speed needed for innovation e.g. of recipes with reduced salt/food additives. Predictive food microbiology models can facilitate product development by predicting combinations of product characteristics and storage or distribution conditions that result in safe products. This approach is increasingly used but sufficiently accurate models are not available for many relevant microorganism/product combinations and appropriate models are not available for *C. botulinum* in many dairy products including processed/spreadable cheese. To obtain relevant predictions, models must include the effect of both general product characteristics (temperature, NaCl, pH) and dairy specific ingredients including melting salts and organic acids. Such extensive models have been developed for the food-borne pathogen *Listeria monocytogenes* (Mejlholm and Dalgaard 2009; Martinez-Rios et al. 2019a, b; 2020) but they are lacking for other human pathogens including *Clostridium botulinum* which is the main safety hazard for spreadable cheeses distributed at ambient temperature (Doyle et al. 2015). *C. botulinum* can be present in milk, and it is particularly found in dehydrated dairy ingredients (Carlin et al. 2004) and processed cheese (Franciosa et al. 1999). In processed cheeses, control of *C. botulinum* is based on suitable product formulations including combinations of phosphates salts and to a lesser extent UHT treatment in combination with aseptic packaging. Different studies show that the pathogen can grow in processed cheeses in the absence of an inhibitory formulation (Tanaka et al. 1986; Teer Steg and Cuppers 1995) and it is important to control potential growth for this product group. In fact, outbreaks of botulism have previously resulted from the consumption of contaminated processed/spreadable cheese (Briozzo et al. 1983) and other dairy products (Doyle et al. 2015). *C. botulinum* produces a very potent neurotoxin and it therefore requires dedicated and very costly facilities and procedures to study its growth in food. The present project will use a cost-effective approach relying on construction of new non-toxigenic mutants of *C. botulinum* (Surrogates) for development and validation of the desired growth models. The overall objective of the project is to develop a predictive tool to facilitate innovation of safe spreadable cheeses.

Specific project aims include:

- Construction and selection of new non-toxigenic *C. botulinum* mutants (Surrogates; WP1).
- Development and validation of an extensive growth and growth boundary model for mesophilic *C. botulinum* group I (WP2).
- Programming and evaluation of a predictive tool for application of the developed mathematical model (WP3)

9. Sub-activities in the entire project period

Activity	Start date (yyyy-mm-dd)	End date (yyyy-mm-dd)	Half year period 1	2	3	4	5	6
Literature work- WP1	2018/11/02	2018/12/31						
Experimental work -WP 1	2019/01/01	2019/02/17						
External stay (KU Leuven) - WP 1	2019/02/18	2019/05/17						
Literature work- WP 2	2019/02/18	2019/05/17						
Experimental work- WP 2	2019/05/18	2021/04/30						
Literature data extraction- WP 2 (Corona lockdown)	2020/03/01	2020/05/31						
Programming model in MS Excel - WP 3	2021/04/01	2021/06/30						
Article writing - WP 2	2021/04/01	2021/06/30						
Article writing - WP 2	2021/06/01	2021/07/31						
Thesis writing	2021/08/01	2021/10/31						

In order to meet its objective and aims the project has been divided into three work-packages (WP). The description of these WPs are included below as taken from the original Expression of Interest for the present project:

WP1: New non-toxigenic mutants of *C. botulinum* group I for cheese studies.

New mutants will be constructed at KU Leuven by using their recently developed gene replacement strategy (Clauwers et al. 2016). Gene replacement and the occurrence of adventitious mutational events will be verified by whole genome sequencing. 4-8 new non-toxigenic mutants will be constructed to allow relevant fast-growing mutants to be selected. Toxigenic isolates from culture collections including the culture collection at DTU will be studied. WP1 includes an external research stay for PhD-student from DTU Food to the research group of Prof. Chris W. Michiels at KU Leuven to study mutant construction and analyses of whole genome sequences. At DTU Food growth of each constructed non-toxigenic mutant will be quantified in spreadable cheese and broth. Comparison with available growth data for toxigenic *C. botulinum* group I isolates will be used for selection of mutants to include in growth modelling studies (WP2). Arla provides spreadable cheeses with specified characteristics and information about the products. Available data on growth kinetics and time-to-toxin formation is collected from the literature and from challenge tests with dairy products.
Deliverable: Non-toxigenic mutants for development of growth and growth-boundary model.
Milestone: Scientific paper on construction and selection of relevant mutants (Months 12)

WP2: Growth and growth-boundary model for *C. botulinum* group I.

DTU Food quantifies growth responses for a cocktail on non-toxigenic *C. botulinum* group I mutants and develops an extensive growth and growth-boundary model including the effect of relevant spreadable cheese product characteristics and storage conditions. A specific qPCR method will be developed for enumeration of mutants in cheeses with a natural microbiota and gene-sequence information from WP1 will be used for this task. The inhibiting effect of product characteristics and storage conditions are quantified and mathematical terms for their effect are developed and included in a cardinal parameter growth and growth-boundary model. This type of predictive models has been selected as they can be expanded by addition of terms including the effect of various growth inhibiting conditions. Arla contributes with information on dairy specific ingredients, including melting salts, processing and distribution conditions. The expanded model is product calibrated and evaluated by using data from both the literature and the present study. Conditions where model evaluation is successful are used to determine the range of applicability for the final model.
Deliverable: Fitted and validated growth and growth-boundary model
Milestone: Scientific paper on model development and validation (Months 30)

WP3: *C. botulinum* growth predictor.

The developed and validated growth and growth-boundary model for mesophilic *C. botulinum* group I will be included in a predictive tool (*Cbot*-PREDICTOR) to facilitate the practical application of the models. DTU Food develops the Cbot-PREDICTOR by using MS Excel and R. Arla evaluates the new tool in relation to their development and distribution of selected spreadable cheeses. Furthermore, a workshop for the Danish dairy sector will be organized to demonstrate the new tool. Feedback from Arla and workshop participants is used to improve the predictive tool and the final version is disseminated from the homepage of the Food Spoilage and Safety Predictor (FSSP) software (http://fssp.food.dtu.dk).
Deliverable: Cbot-PREDICTOR
Milestone: Freely available predictive tool and popular article describing its development and application (Months 36).

10. Deviations

WP 1 was performed with some modifications compared to the original plan. At KU Leuven six non-toxigenic mutants were constructed from toxigenic *C. botulinum* isolates as planned. However, mutants were generated by insertionally inactivating toxin genes rather than by using a gene replacement strategy. Furthermore, growth characteristics of the developed mutants differed from their toxigenic wild-types and consequently, non-toxigenic *C. sporogenes* strains were used as an alternative surrogate for toxigenic *C. botulinum* isolates.

WP 3 developed a predictive tool using MS Excel as planned. This tool is freely available as planned but distributed via e-mail rather than from a DTU Food homepage.

11. Project results

WP 1 Construction and selection of new non-toxigenic *C. botulinum* mutants.

Five toxigenic *C. botulinum* isolates from the DTU culture collection were studied at KU Leuven. Six non-toxigenic mutants were constructed from these isolates the ClosTron system and insertional inactivation of the toxin gene. Whole genome sequencing of the five isolates (WT, wild types) and of the six mutants were carried out to verify inacitivation of the toxin genes. The effect of storage temperature (14-18°C), pH (4.6 – 5.0) and salt (4.2-4.8% NaCl) of wild types and mutants were studied and compared. Unfortunately, all the six mutants grew slower than their corresponding wild types when temperature, pH or salt were changed to less favorable conditions. In this situation the mutants were not considered suitable for development of a growth and growth boundary model. As an alternative surrogate for toxigenic *C. botulinum* isolates, three non-toxigenic *C. sporogenes* strains (NCTC 532^{T}, NCTC 8594 and NCTC 12925) from the Public

Health England culture collection were used as a cocktail for model development as mentioned above under 10. Deviations. The three strains were selected as the fastest growing of seven studied *C. sporogenes* strains.

WP 2 Growth and growth-boundary model for *C. botulinum* group I.

Growth responses for *C. sporogenes* were quantified and an extensive growth and growth-boundary model including the effect of relevant spreadable cheese product characteristics and storage conditions was developed. This model includes the inhibitory effect of 11 environmental factors. 626 maximum specific growth rates (μ_{max} values) in broth were generated to formulate model terms and determine cardinal parameter values for the growth inhibiting effect of temperature, pH, NaCl/water activity (aw), organic acids (acetic, benzoic, citric, lactic and sorbic acids) and phosphate melting salts (ortho-, di- and tri-phosphates) (Fig. 1 and 2).

A cardinal parameter model was developed, and this model took into account the effect of interaction between the 11 factors included in the model (Koukou et al., 2022). This model was able to predict both growth and growth boundary conditions. The new model predicts so called Ψ-values corresponding to specific combinations of product characteristics and storage temperature. A Ψ-value of 1.0 corresponds to the growth boundary whereas Ψ-values < 1.0 indicate that growth is predicted and Ψ-values > 1.0 corresponds to prediction of no growth. To efficiently prevent growth in processed cheese the combined effect of product characteristics and storage temperature should result in a predicted Ψ-values > 2.0.

A specific real-time PCR method was developed for enumeration of *C. sporogenes* in cheeses with a natural microbiota. Growth responses of *C. sporogenes* in well characterized processed cheeses were used to product calibrate the developed model. Thereafter, the model was evaluated by using data from both the literature and the present study. μ_{max}-values for *C. sporogenes* growing in well-characterized processed cheeses were used for product calibration (n = 10) and for product evaluation of the developed broth-model (n = 29). 112 growth/no-growth responses and including 104 μ_{max}-values from the scientific literature for 58 different isolates of proteolytic and toxigenic *C. botulinum* (Group I) were used for further model evaluation. The developed model had less bias and a higher percentage of correct predictions than available models and was acceptable for processed cheese (Fig. 3) and good for meat products (Fig. 4).

Very little data is available in the scientific literature for growth of *C. botulinum* (proteolytic and toxigenic) in processed cheese. However, some data are available for the time-to-toxin (TTT) formation by *C. botulinum* in processed cheese, other dairy products and other foods. In fact, the present project collected 509 TTT data extracted from the scientific literature. These data were used to evaluate the performance of the developed growth model. For this to be possible the growth model was expanded to become able to predict TTT as the time required for *C. sporogenes* to grow corresponding to a 3.3 log-increase in cell-concentration. Then TTT-predictions by the new models were compared with predictions from other available models for processed cheese. This comparison showed the new model to have the widest range of applicability (Fig. 5) and the lowest percentage of fail-dangerous predictions (Table 1).

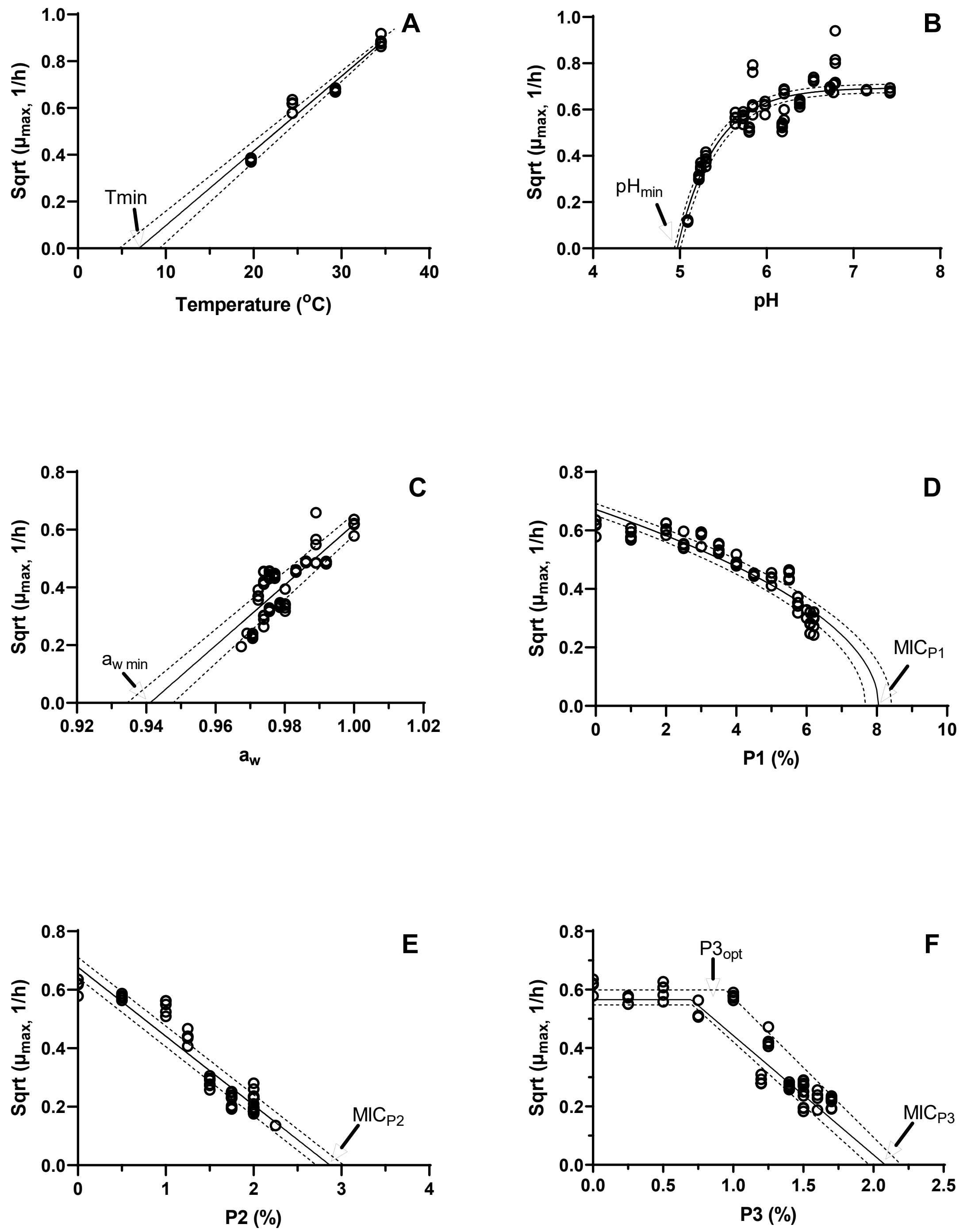

Figure 1. Effects of temperature (A), pH (B), water activity (C), ortho- (D), di- (E) and tri- phosphates (F) on square-root transformed maximum specific growth rates (μ_{max} values) of a cocktail of three *C. sporogenes* strains in a liquid laboratory broth. Studies were performed at 25°C and at pH 6.00 except of course for the two series of experiments where temperature and pH was changed. Solid lines represent the fitted model terms and dashed lines the 95% confidence intervals.

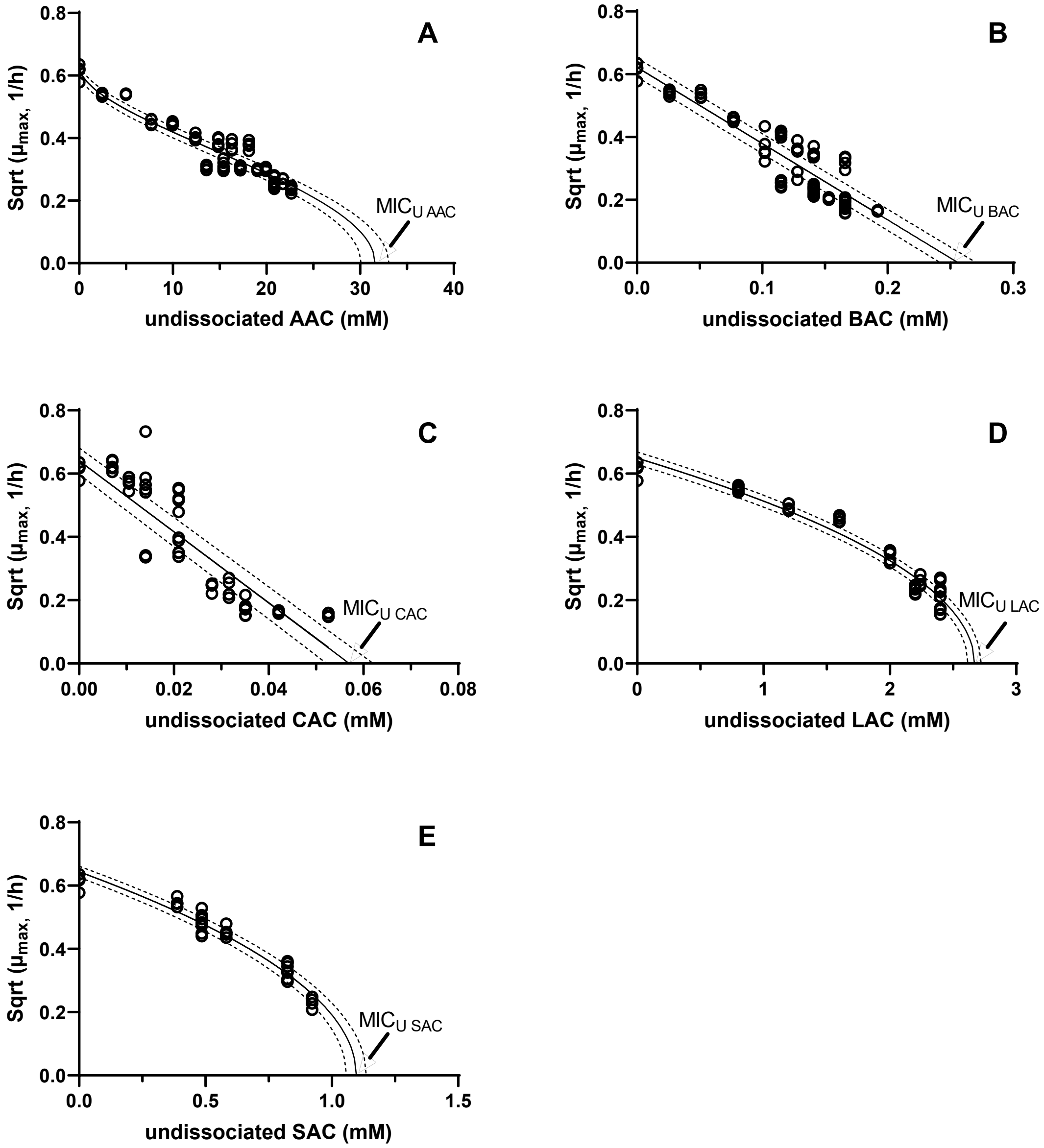

Figure 2. Effects of undissociated acetic (A), benzoic (B), citric, (C) lactic (D) and sorbic (E) acids on square-root transformed maximum specific growth rates (μ_{max}) of a cocktail of three *C. sporogenes* strains in a liquid laboratory broth at 25ºC and pH 6.00. Minimum inhibitory concentrations (MICs) were determined from fitted model terms. Solid and dashed lines represent the fitted model terms and confidence intervals (95%), respectively.

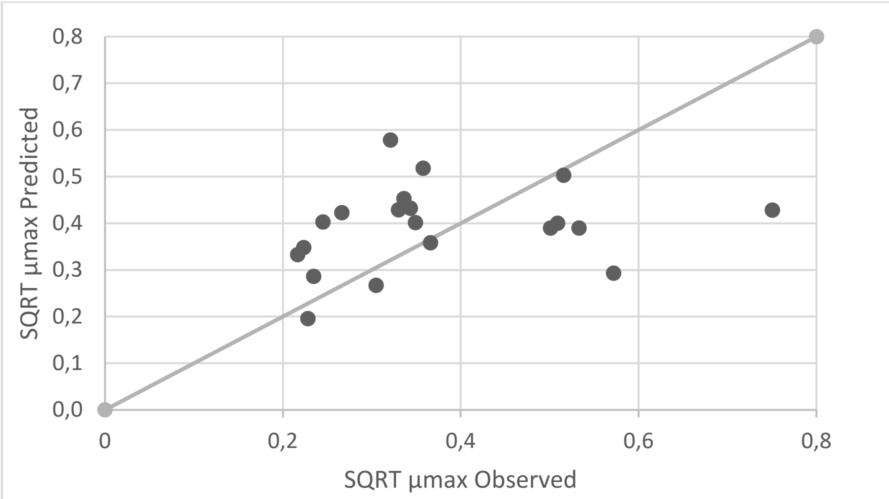

Figure 3. Square root transformed growth rates of *C. sporogenes* in processed cheeses as observed by challenge tests in the present project (Observed) and predicted by the new *C. sporogenes* model (Predicted).

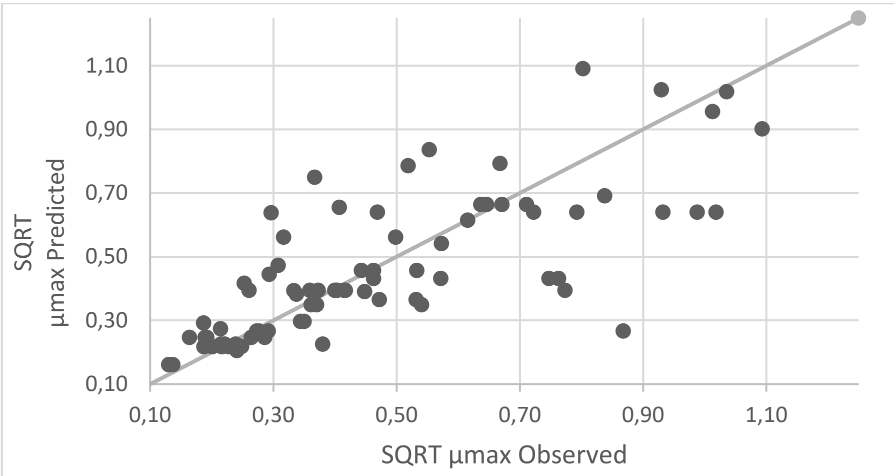

Figure 4. Square root transformed growth rates of proteolytic and toxigenic *C. botulinum* in meat products as extracted from the scientific literature (Observed) and predicted by the new *C. sporogenes* model from the present project (Predicted).

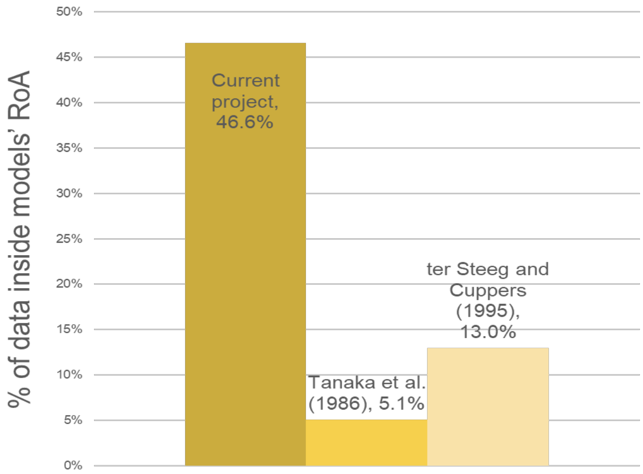

Figure 5. Percentage of data inside the range of applicability of the model from the present study (dark color), model of Tanaka et al. (1986) (medium color) and model of ter Steeg and Cuppers (1995) (light color).

Table 1. Percentage of fail-dangerous predictions by new and available models when used inside their range of applicability.

Models	**Current project**	**Tanaka et al. (1986)**	**ter Steeg and Cuppers (1995)**
Environmental factors included	Temp., pH, NaCl/a_w, acetic, benzoic, citric, lactic, sorbic acids, ortho-, di-, tri- phosphates	Temp., pH, NaCl, DSP, moisture	Temp., pH, total salts, lactic acid, citrates
Predicted responses	3.3 Log-increase	TTT	2.0 Log-increase
Fail-dangerous	1%	4%	18%

As planned this task resulted in a scientific publication (Koukou et al. (2022).

<u>**WP 3** DTU Food Predictive Tool for Proteolytic *Clostridium botulinum* (Group 1).</u>

The developed and validated growth and growth-boundary model for *C. sporogenes* have been programmed as a predictive tool named DTU Food Predictive Tool for Proteolytic *Clostridium botulinum* (Group 1). This predictive tool was programmed using MS Excel and it is freely available for the entire dairy sector and obtained by contacting DTU Food (pada@food.dtu.dk). The following example shows how this predictive tool can be used to identify combinations of product characteristics to prevent growth and toxin formation by *C. botulinum* (Fig. 6). At 25°C growth is predicted for processed cheese with 55% moisture, 1.5% NaCl, 1.5% orthophosphate, pH 6.0, 0.1% citric acid, 0.1% acetic acid and 0.5% lactic acid (Fig. 6, left). By increasing the citric acid concentration (from 0.1% to 0.2%) and reducing pH from 6.0 to 5.8 growth is predicted to be efficiently prevented (Fig. 6, right; Ψ-value = 3,1). This product (with Ψ-value of 3,1 at 25°C), when stored at 35°C, instead of at 25°C, the new model predicts a Ψ-value of 1.5 and therefore that growth is also prevented for this processed cheese at 35°C.

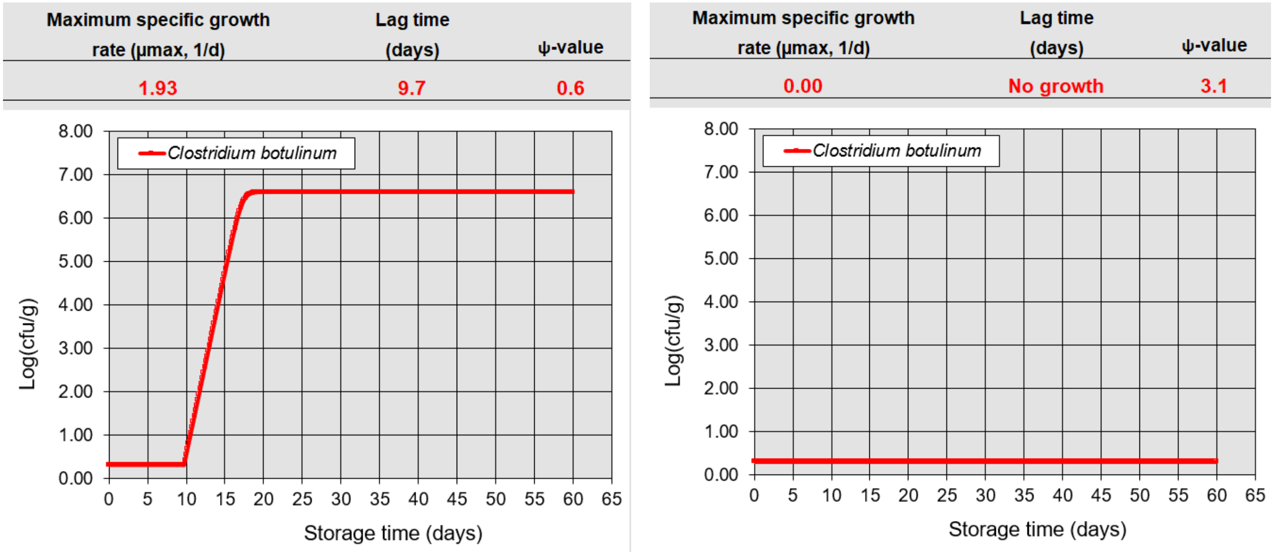

Figure 6: Example showing how the new growth and growth boundary model and predictive tool can be applied.

Concerning application of the predictive tool it should be noted that the new growth and growth boundary model was developed to predict combinations of product characteristics and storage conditions that prevent growth and toxin-formation by *C. botulinum* (Group 1). The new model was not developed to provide more precise predictions of the time required for *C. botulinum* to form toxin in processed cheese and the predictive tool MUST NOT be used for that type of predictions. Furthermore, the new growth and growth boundary model must be used in the same way as when the model was developed. Specifically, this means that the percentage of NaCl in a product is used as a model input to obtain predictions whereas the measured water activity is not used as model input. Also, the pH used as model input must be measured for cheese diluted in water and not by using a probe for directly measuring pH in chesses.

In conclusion, a cocktail of non-toxigenic *C. sporogenes* strains were used, as surrogates for *C. botulinum* (Group 1), to develop an extensive growth and growth boundary model. This model was calibrated to provide unbiased predictions for processed cheese. The calibrated model was then validated for growth and toxin formation in processed cheese, poultry/meat products and vegetables. The successfully validated model was included in the DTU Food Predictive Tool for Proteolytic *Clostridium botulinum* (Group 1). This predictive tool is freely available and facilitates the correct application of the developed model. The new model and predictive tool can support formulation of recipes for processed cheese and other foods that efficiently prevent growth and toxin formation by proteolytic *C. botulinum* (Group 1). This can be useful for flexible product development and to reduce the time-to-market e.g. for sodium reduced products.

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

Scientific relevance:

The performed studies showed non-toxigenic *C. sporogenes* strains to be a suitable surrogate for toxigenic and proteolytic *C. botulinum.* This is important as non-toxigenic strains are markedly easier to use, for example in challenge testing and for development of predictive models.

The developed growth and growth boundary model includes the effect of more dairy product characteristics than available models. This is important as the prediction of combinations of product characteristics to prevent growth and toxin formation by proteolytic *C. botulinum* can then be both more flexible and also more relevant for processed cheeses.

Relevance for the society:

The new model and predictive tool can support teaching in food safety and advice to industry and authorities e.g. by DTU Food. Furthermore, the model can be used by the authorities to support more flexible guidelines that do not rely on individual-factor-growth-limits of proteolytic *C. botulinum* that may lead to unnecessary over-preservation.

Relevance for the industry:

The new model and predictive tool can support formulation of recipes for processed cheese and other foods that efficiently prevent growth and toxin formation by proteolytic *C. botulinum* (Group 1). This can be useful for flexible product development and to reduce the time-to-market e.g. for sodium reduced products.

13. Communication and knowledge sharing about the project

Papers in international journals:

Koukou, I., Stergioti, T. la Cour, R., Gkogka, E., Dalgaard, P., 2022. *Clostridium sporogenes* as surrogate for proteolytic *C. botulinum* - Development and validation of extensive growth and growth-boundary model. Food Microbiology 107, 104060. https://doi.org/10.1016/j.fm.2022.104060.

Easily read papers:

Dalgaard, P., Koukou, I. (2022). *Clostridium botulinum* og sikre oste. Mælkeritidende, 7, side 20-21. https://maelkeritidende.dk/sites/default/files/udgivelser/Forskningsartikler/sider_fra_mt_7_2022_1908_hoej_slut.pdf.

Dalgaard, P., Koukou, I., 2019. *Clostridium botulinum* og sikre oste. Mælkeritidende, MT14, Oct., 2019. https://issuu.com/maelkeritidende/docs/mt_14_2019_h_j_opl.

Student theses:

PhD thesis by Ioulia Koukou with title 'Management of *Clostridium botulinum* in food - A predictive food microbiology approach'. November 2021, DTU National Food Institute. https://orbit.dtu.dk/en/publications/management-of-iclostridium-botulinum-iin-food-a-predictive-food-m.

MSc thesis by Thomai Stergioti with title 'Validation of cardinal parameter model for growth of *Clostridium sporogenes* in processed cheese' July 2021, DTU National Food Institute.

BSc thesis by Gustav H.B. Jørgensen with title: '*Clostridium botulinum* i ost – vurdering af matematisk model til forudsigelse af vækst som bidrag til produktudvikling'. June 2021, DTU National Food Institute.

Oral presentations at scientific conferences, symposiums etc.:

Dalgaard., P. (2025). Hvor er vi i forhold til at kunne forudsige mikrobiel vækst? Inviteret foredrag inden for sessionen ”Forudsigelse af mikrobiel vækst” ved Mejeribrugets dag 2025 ”Fra forskning til praktisk anvendelse”, 12. marts 2025, Herning, Danmark. https://mejerimedier.dk/nyhed/mejeribrugets-dag-samler-branchen-om-den-nyeste-viden/.

Dalgaard., P. (2023). Predictive food microbiology models and software – tools to facilitate safety evaluation of new products. Invited presentation at “New products from idea to supply chain”. Danmarks Mejeritekniske Selskab, 28 September 2023, Billund, Denmark.

Dalgaard, P. (2023). *Clostridium botulinum* growth boundary models for development of stabilized new food products. Invited presentation within the symposium "*Clostridium botulinum* – Opportunities and Challenges for new Testing Methods to Maintain Food Safety in Ready-to-Eat Foods. IAFP's European Symposium on Food Safety; Aberdeen, Scotland; 3-5 May 2023.

Koukou, I., Stergioti, T., Gkogka, E., Dalgaard., P., 2022*. Clostridium sporogenes* as surrogate for proteolytic *C. botulinum* - Development and validation of extensive predictive model using data for growth and time-to-toxin formation. Abstract for oral presentation at FoodMicro 2022, 27th International ICFMH Conference, 28-31 August 2022, Athens, Greece.

Oral presentations at meetings:

Koukou, I., Dalgaard., P., 2022. *Clostridium botulinum* and safe cheeses (Cbot-Predictor). Closing project presentation with Arla (online meeting) on 3rd June 2022.

Koukou, I., Dalgaard., P., 2019. *Clostridium botulinum* and safe cheeses (Cbot-Predictor). Project presentation at Arla Innovation Centre on 18th June 2019.

Presentations at DDRF steering group meetings.

14. Contribution to master and PhD education

One PhD student, Ioulia Koukou (see section 13, student theses).

One 35 ECTS MSc-project and one 20 ECTS BSc-project (see section 13, student theses).

15. New contacts/projects

Genomic-driven **R**isk **AS**sessment of **P**lant-based foods (GRASP; 2024-2027). Collaboration between Wageningen University & Research, DTU Food, Arla Foods and ISI Food Protection funded by NNF within the Plant2Food programme.

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