SEAFOOD TOMORROW

Nutritious, safe and sustainable seafood for consumers of tomorrow

Grant agreement no: 773400

Deliverable D3.4

Optimized/selected predictive food microbiology models and tools

Due date of deliverable: 30/09/2020

Actual submission date: 22/10/2020

Start date of the project: 01/11/2017 **Duration: 42 months**

Organisation name of lead contractor: DTU

Revision: v1

Project co-funded by the European Commission within the H2020 Programme	
Dissemination Level	
PU Public	X
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	

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1. Summary

The SEAFOOD[TOMORROW] consortium aims at the development of eco-innovative seafood products including sodium reduced salmon pâté and smoked salmon (Task 2.1) and new recipes, developed by professional cooking schools, for youth/children, pregnant women and elderly people/seniors (Task 2.2). The objectives of D 3.4 within task 3.4 is to evaluate safe shelf-life of these eco-innovative solutions with focus on the pathogenic microorganism *Listeria monocytogenes.*

An available mathematical model (Martinez-Rios et al. 2019) has been evaluated and used to predict the potential growth of *L. monocytogenes* depending on product characteristics and storage conditions for the suggested eco-innovative seafood products. A DTU Food Predictive Tool for *Listeria monocytogenes* has been developed to facilitate the determination of safe shelf-life determined as the time required for *L. monocytogenes* to grow 100-fold in the evaluated products. When required the DTU Food Predictive Tool for *Listeria monocytogenes* has been used to identify new recipes with longer safe shelf-life or new recipes that both had reduced sodium content and longer safe shelf-life.

For salmon pâté from task 2.1 the safe shelf-life was 6 days at 8°C and new product formulations have been suggested. These include recipes that prevented the growth of *L. monocytogenes* as well as growth and toxin formation by psychrotolerant *Clostridium botulinum.* Safe shelf-life of 3-5 weeks at 8°C was predicted for the new product formulations from task 3.4.

Smoked salmon from task 2.1 contained 5.8% salt in the water phase and had safe shelf-life at 8°C of 12-31 days. New product formulations with 3.5 % water phase salt was suggested. These new recipes included acetic acid and they prevented growth of *L. monocytogenes* and allowed the safe shelf-life to be 3-5 weeks at 8°C.

Six different dished developed for specific population groups (Winner products from task 2.2) were each divided into their major fractions. Each fraction was analyzed for water activity, pH, water content/dry matter, salt (NaCl) and organic acids including acetic, citric and lactic acids (> 400 analyses at DTU) to evaluate potential growth of *L. monocytogenes.* The products for youth/children had a safe shelf-life at 5°C of 10-12 days and the fish fraction was most sensitive with respect to potential growth of *L. monocytogenes.* Dishes for pregnant women had safe shelf-life at 5°C of 11 days. Fish balls with marinara sauce and the mussel soup for elderly people/seniors had safe shelf-life at 5°C of 6 days.

The DTU Food Predictive Tool for *Listeria monocytogenes* was developed to include a calculator for determination of water phase salt in products depending on their content of NaCl and KCl. The tool has broad range of applicability and allowed safe shelf-life of various seafoods to be determined. Importantly, this new tool allowed new safe seafood recipes to be formulated including products with reduced content of sodium and with shelf-life that allow distribution under realistic conditions in chill chains.

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2. Objective

The objectives of D 3.4 within Task 3.4 is to evaluate safe shelf-life of eco-innovative solutions including sodium reduced salmon pâté and smoked salmon from task 2.1 and new recipes, developed by professional cooking schools, for youth/children, pregnant women and elderly people/seniors (Task 2.2). Safe shelf-life was evaluated with focus on the pathogenic microorganism *Listeria monocytogenes* and the objective of D 3.4 included development of the DTU Food Predictive Tool for *Listeria monocytogenes.* This tool was used to suggest new safe seafood recipes with reduced content of sodium and sufficient shelf-life for chilled distribution.

3. Background

The pathogenic bacterium *Listeria monocytogenes* remains a major human health hazard in seafood products both world-wide and in Europe (Jami et al. 2014). *L. monocytogenes* in seafood products can cause the important food-borne disease listeriosis with a high case fatality rate (15.6% during 2018 within the EU). Importantly, a statistically significant increasing trend of confirmed listeriosis cases in the EU/EEA has been observed in 2009-2018 as well as during 2014-2018 (EFSA/ECDC, 2019). 2549 human cases of listeriosis were reported within the EU during 2018. During 2010-2017 'mixed food' and 'fish and fish products' caused most of the so-called strong-evidence food-borne outbreaks of listeriosis (EFSA/ECDC, 2019). At processing 3.18% of tested single samples for fish and fish products were positive for presence of *L. monocytogenes* (EFSA/ECDC, 2019). Consumer groups with highest risk of listeriosis include babies, pregnant women and elderly people/seniors. *L. monocytogenes* is heat sensitive and inactivated by thorough cooking, hot-smoking and equivalent heat treatments of seafood (den Besten et al. 2018). Consequently, and as recognized by the European regulation, occurrence and growth of *L. monocytogenes* is most relevant for ready-to-eat (RTE) products that are not thoroughly heated immediately prior to consumption (EC 2073/2005). The European regulation divides RTE foods into three categories depending on the consumer and on the ability of the product to support or not the growth of *L. monocytogenes* (EC 2073/2005). Category 1.1 concerns baby food and is not relevant for the present study. Category 1.2 RTE-products are those that support more than 3-fold growth of *L. monocytogenes* within their shelf-life. For these RTE-product, *L. monocytogenes* must be absent in 5 samples of 25 g at processing and the pathogen must not grow to more 100 cfu/g within the shelf-life of the product. Category 1.3 RTE-products does not support more than 3-fold growth of *L. monocytogenes* within their shelf-life. For these RTE-products, the *L. monocytogenes* concentration should be less than 100 cfu/g at processing and within the shelf-life of products (EC 2073/2005).

To document control of *L. monocytogenes* in RTE seafood products it is the responsibility of food business operators (FBO) to evaluate their products by using data from durability studies with naturally contaminated products, challenge studies with inoculated products or predictions from relevant mathematical models (EC 2073/2005).

The application of predictive food microbiology models to evaluate and document *L. monocytogenes* related safety of seafood is clearly indicated within the European regulation (EC 2073/2005). Nevertheless, the

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adaptation of this technology by national food safety authorities has been slow within Europe. For some European countries the national food safety authorities does not in practice allow the use of predictive food microbiology models to evaluate and document *L. monocytogenes* related safety of seafood. The European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm) has included the use of a simple predictive food microbiology temperature model in their guidance document on shelf-life studies (EURL *Lm*, 2014/2019). In Denmark and in the Netherlands application of more extensive predictive food microbiology models has been adapted by their national food safety authorities to evaluate and document *L. monocytogenes* related safety of seafood. Clearly, to be applied in this way the relevant models must have been successfully validated for seafood. These successfully validated predictive food microbiology models for *L. monocytogenes* have the advantage that they can:

- predict potential growth and survival of *L. monocytogenes* in a seafood depending on product characteristics and storage conditions and
- predict how product characteristics and storage conditions need to be changed to reduce the potential growth of *L. monocytogenes* to an acceptable level.

Numerous predictive food microbiology models are available to predict growth, growth boundary, growth probability and survival of *L. monocytogenes* in seafood (Ross and Dalgaard, 2004). However, the number of successfully validated models are much more limited (Mejlholm et al. 2010). *L. monocytogenes* models that allow prediction of growth and survival as well as prediction of new product characteristics that will stabilize a seafood against growth of *L. monocytogenes* must include the effect of all product characteristics and storage conditions that have an important effect on the potential growth *L. monocytogenes* (Dalgaard and Mejlholm, 2019).

Various product characteristics in seafood influence the growth response of *L. monocytogenes* including its lag time and growth rate. Clearly, potential growth of *L. monocytogenes* depends on the storage time of a seafood product. For seafood, as for other foods, some variability in product characteristics is always observed and this will influence the potential for growth of *L. monocytogenes*. Consequently, a measure that takes into account product variability is needed when safe shelf-life of a seafood product is established depending on its product characteristics and storage conditions. One such measure is the ψ-value (psi-value) that expresses how far a specific set of product characteristics and storage conditions is from the growth boundary of *L. monocytogenes* with ψ-value of 1.0. For seafoods with ψ-values below 1.0, growth of *L. monocytogenes* is observed whereas for seafoods with ψ-values above 1.0 no growth of *L. monocytogenes* is observed. The ψ-value is important as a simple measure to evaluate safe shelf-life of RTE seafood and it has been suggested that products with (i) ψ-value below 1 should have a chilled shelf-life of less than 2-3 weeks; ψ-value of 1-2 corresponds to safe shelf-life of less than 3-5 weeks and (iii) ψ-value above 2 match with safe shelf-life of more than 5 weeks (Mejlholm and Dalgaard, 2009; Dalgaard and Mejlholm, 2019).

Mejlholm and Dalgaard (2007) developed and validated a growth and growth boundary model for *L. monocytogenes* in seafood. This model was further expanded by Mejlholm and Dalgaard (2009) and it has been extensively validated for prediction of growth responses in seafood and meat products (Mejlholm et al. 2010). Subsequently, Mejlholm et al. (2015) confirmed the ability of this model to predict growth of *L. monocytogenes* in naturally contaminated lightly preserved seafood including smoked salmon. The growth

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and growth boundary model of Mejlholm and Dalgaard (2009) include the effect of temperature, atmosphere (CO_2), a_w/NaCl, pH, smoke components (phenol), nitrite and acetic/di-acetic, benzoic, citric, lactic and sorbic acids. This model has been included in version 4 of the Food Spoilage and Safety Predictor (FSSP) software from 2014 (http://fssp.food.dtu.dk). The inclusion of this extensive growth and growth boundary model for *L. monocytogenes* in a user-friendly application software markedly increased its use in practice for both seafood and meat products. In fact, the Mejlholm and Dalgaard (2009) model included in the FSSP software has been adapted by the food safety authorities in Denmark and in the Netherlands.

Although popular with more than 16,000 users in more than 120 countries the Mejlholm and Dalgaard (2009) model in the FSSP software has a range of applicability with respect to product characteristics that limits its use for some products. One limitation of this model is that is has not been validated for product with pH below 5.6 (Mejlholm et al. 2010). More recently, this limitation was studied by Martinez-Rios et al. (2019) and they introduced a new pH-term that allowed the model to be successfully validated for products with pH as low as 4.6.

Predictive food microbiology studies performed in relation to D 3.4 within Task 3.4 of SEAFOOD^{TOMORROW} will use the model of Martinez-Rios et al. (2019) and this model will be included in a MS Excel based predictive tool.

Studies of salmon pâté have been performed within Task 2.1 of SEAFOOD^{TOMORROW} and reported as part of D 2.1. Importantly, D 2.1 is marked as confidential ("CO") whereas the present D 3.4 is public ("PU") according to the DoA. With D 2.1 being confidential it is difficult to include data from Task 2.1 in D 3.4. However, data from D 2.1 has been published by Nielsen et al (2020) and that information is included in D 3.4. In the same way, studies of smoked salmon have been performed within Task 2.1 of SEAFOOD^{TOMORROW} and reported with confidentiality as part of D 2.1. In that case data from D 2.1 was published by Muñoz et al. (2020) and again information from that publication is included in D 3.4. Concerning the six new recipes for youth/children, pregnant women and elderly/seniors (Task 2.2) the related product development is described in D 2.1 and this document is also confidential ("CO"). As part of the development of D 3.4 DTU has contacted IDmer to request if non-confidential information about the winner projects is available. This was not the case and that situation clearly complicates the reporting of data related to safety of the six new recipes. This challenge was presented by DTU to the SEAFOOD^{TOMORROW} consortium during the project meeting in November 2019 in Porto, Portugal. Decisions on confidentiality of deliverables was not changed by the SEAFOOD^{TOMORROW} consortium. However, it was agreed that DTU should perform an extensive chemical characterization of the six winner products and their factions. This product characterization would allow DTU to identify the least preserved factions of each of the six products. Furthermore, the chemical characteristics determined by DTU will belong to DTU and they will not be confidential. These data have been used within D 3.4 to evaluate the safe shelf-life of the six winner products.

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4. Experimental design

4.1. Salmon pâté from task 2.1

As reported by Nielsen et al. (2020) three types of salmon pâté were studied in Task 2.1. These included salmon pâté with (A) 100% of the sodium as sodium chloride, (B) 60% of the sodium as sodium chloride and 40% as Saltwell and (C) 20% of the sodium as sodium chloride and 80% as Saltwell. Saltwell is a commercially available salt preparation including NaCl and KCl. Data for water content, water activity, pH and growth of *L. monocytogenes* were used as reported by Nielsen et al. (2020). Form the reported water activity the concentration of salt in the water phase of the salmon pâté was determined as close to 2.0%. The concentration of lactic acid in the product was assumed to be 0.04% corresponding to 625 mg/liter (ppm) in the water phase.

Nielsen et al. (2020) reported that growth of *L. monocytogenes* in the three types of salmon pâté did not differ significantly. The observed growth of *L. monocytogenes* in the studied salmon pâté at 8°C was compared with the growth rate predicted by the extensive growth and growth boundary model of Martinez-Rios et al. (2019). The observed growth rate was determined by fitting all growth data for the three studied products together. The Logistic model was used for this fitting of growth data (Dalgaard and Mejlholm, 2019).

Nielsen et al. (2020) observed a 6 log-increase (from ca. 10^2 cfu/g to ca. 10^8 cfu/g) in concentrations of *L. monocytogenes* within the first 17 days of salmon pâté storage at +8°C. The model of Martinez-Rios et al. (2019) was used to identify combinations of product characteristics and storage conditions that reduced growth of *L. monocytogenes* and resulted in safe shelf-lives that were sufficient for practical distribution of a chilled RTE salmon pâté. Recipes with differences in pH, organic acid content, modified atmosphere packaging and salt content were evaluated with respect to predicted growth of *L. monocytogenes.*

4.2. Smoked salmon from task 2.1

Smoked salmon was studied in Task 2.1 of SEAFOOD[TOMORROW] and the main results have been published by Muñoz et al. (2020). A full factorial experimental design was completed including (i) smoked salmon produced with either wood smoke (6 treatments) or liquid smoke (6 treatments), (ii) the temperature of smoking included cold smoking (6 treatments) or hot smoking (6 treatments), finally (iii) for each combination of smoke type and smoke temperature the % NaCl/% KCl were (a) 100%/0%, 75%/25% and 50%/50%. Product characteristics including water content, water activity (a_w), pH, lactic acid (LAC) and phenol was determined as reported by Muñoz et al. (2020) and shown in Table 3.

Growth of *L. monocytogenes* was predicted at the studied storage temperature of 1°C as well as at the more realistic commercial storage temperatures of 5°C and 8°C. Predictions were obtained by using the model of Martinez-Rios et al. (2019). This model has already been extensively validated for smoked salmon as Martinez-Rios et al. (2019) used the validation dataset from Mejlholm et al. (2010) to evaluate their model. This data set included 193 growth responses for seafood and these were predicted with a good model performance. In fact, the ration between predicted and observed growth rates was 1.0 (Martinez-Rios et al.,

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2019). Therefore, further evaluation of this model was not required prior to prediction of growth responses in smoked salmon.

The smoked salmon studied in Task 2.1 (Muñoz et al., 2020) included a high concentration of salt corresponding to more than 5% salt in the water phase (Table 3). The model of Martinez-Rios et al. (2019) was therefore used to suggest recipes with less salt that at the same time prevented growth of *L. monocytogenes* during storage at 5°C or 8°C.

4.3. Seafood dishes for specific population groups - Winner products from task 2.2

On the basis of recipes developed by different professional cooking schools, the SEAFOOD[TOMORROW] participant IDmer has been in charge of a semi-industrial production of six winner RTE products for consumer and market acceptance tests. Each of the six products has as target groups youth/children, pregnant women or elderly/seniors.

Each product was placed in plastic trays, skin packed and frozen. For each of the six winner products IDmer sent five frozen packaged to DTU, a total of 30 packages. At DTU the content of each of the 30 packages was separated into its major fractions as shown in Table 1. This resulted in a total of 85 fractions. Each of these factions were analyzed for water activity, pH, water content/dry matter, salt (NaCl) and organic acids including acetic, citric and lactic acids and this resulting in more than 400 analyses.

Table 1. Fractions of the six winner products.

	Fractions of dishes for analyses			
Products	1	2	3	4
Fish sausage and vegetables	Fish sausage	Carrots	Tomato/onion	Sauce
Fish balls with purée	Fish balls	Banana	Purée	
Fish roulade	Fish	Cabbage	Potato	Sauce
Fish fillet and wheat salad	Fish	Wheat salad		
Fish balls with marinara sauce	Fish balls	Vegetables	Sauce	
Mussel soup	Mussel soup			

For each fraction the potential growth of *L. monocytogenes* was predicted at 5°C by using the model of Martinez-Rios et al. (2019). The time for a 100-fold increase in cell concentration was predicted and reported together with the maximum specific growth rate (μmax) and the ψ-value.

The six winner dishes are mixed meals and the different fractions are likely to have markedly different product characteristics. When evaluating growth of *L. monocytogenes* it is therefore important to evaluate the growth potential for the separate fractions rather than for the average product characteristics of the entire dish. As one example, the dish 'Fish sausage and vegetables' include both a fish sausage with pH 6.24 and a tomato/onion fraction with pH 4.66. At 5°C *L. monocytogenes* will not grow in the tomato/onion fraction due to the low pH. However, *L. monocytogenes* will grow in the fish sausage with pH 6.24. If the entire dish had been homogenized the pH would be well below pH 6.24 as both carrots and tomato/onion

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fractions had pH below 5. Consequently, the potential growth of *L. monocytogenes* in the fish sausage (the most sensitive fraction of the dish) would then be underestimated.

4.4. DTU Food Predictive Tool for *Listeria monocytogenes*

A tool to predict growth responses of *L. monocytogenes* for various seafood products was developed by programming the extensive growth and growth boundary model of Martinez-Rios et al. (2019) in a Microsoft Excel 2016 (Microsoft Corp. Redmond, WA, USA) Excel file. The logistic model with delay was used as primary model to predict growth with storage time at constant conditions with respect to product characteristics and storage conditions including temperature and atmosphere. The secondary model of Martinez-Rios et al. (2019) was used to predict the effect of product characteristics and storage conditions on lag-time (d), maximum specific growth rate (μ_{max} , 1/d), time for 100-fold increase in cell concentration (d) and the ψ-value. This secondary model includes the effect of temperature, atmosphere (CO_2), a_w/NaCl, pH, smoke components (phenol), nitrite and acetic/di-acetic, benzoic, citric, lactic and sorbic acids as well as the effect of interaction between all these factors. As a new development the model of Martinez-Rios et al. (2019) include a pH-term that allow the model to predict the growth response of *L. monocytogenes* at pH-values at low as 4.6. This has been important for example to predict growth responses in fractions of winner products from task 2.2 as several of these had pH below 5. The model of Martinez-Rios et al. (2019) is available as an open access publication (https://doi.org/10.3389/fmicb.2019.01510).

5. Results and Discussion

5.1. Salmon pâté from task 2.1

The observed maximum specific growth rate (μ_{max}) of *L. monocytogenes* in salmon pâte was 1.00 1/d, whereas the predicted growth rate was 1.26 1/d (Fig. 1). The model of Martinez-Rios et al. (2019) thus overestimated the observed growth rate by ca. 26 % and this is considered to be an acceptable model performance (Dalgaard and Mejlholm, 2019). In fact, good model performance corresponds to predicted growth rates being 95 – 111 % of observed growth rates and acceptable model performance corresponds to predicted growth rates being from 87 – 95 % or from 111 % to 143 % of observed growth rates (Mejlholm et al. 2010).

The model of Martinez-Rios et al. (2019) acceptably predicted the observed rapid growth of *L. monocytogenes* in salmon pâté (Fig. 1). This suggests that the product characteristics with pH of 6.4 and about 2% water phase salt explained the rapid growth. When predicting growth of *L. monocytogenes* the model of Martinez-Rios et al. (2019) include a lag time as lag times are observed for *L. monocytogenes* growing in naturally contaminated seafood (Mejlholm et al. 2015). Nielsen et al. (2020) did not observe a lag time for *L. monocytogenes* when growing in the studied salmon pâté (Fig. 1). This is most likely due to inoculation of the product with unstressed laboratory cultures of *L. monocytogenes* that may not represent *L. monocytogenes* in a seafood processing environment.

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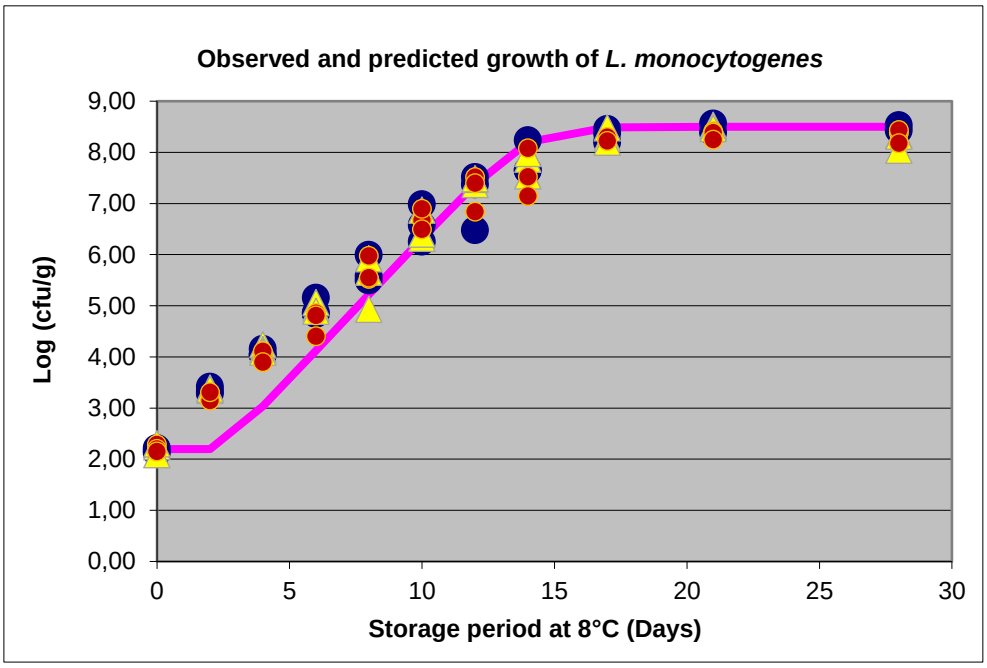

Figure 1. Observed and predicted growth *L. monocytogenes* in salmon pâté. Symbols represent growth for the three recipes with different ratios of sodium chloride and Saltwell. The line (Magenta) shows growth as predicted by the model of Martinez-Rios et al. (2019).

When seafood is naturally contaminated with *L. monocytogenes* in seafood processing environments then the concentrations of the pathogen in products are often low but concentrations as high as about 1 cfu/g has been reported (Mejlholm et al. 2015). Therefore, safe shelf-life can be predicted as the time required for *L. monocytogenes* to grow 100-fold from 1 cfu/g to 100 cfu/g. With 100 cfu/g being the maximum acceptable concentration is RTE seafood (EC, 2005).

The studied salmon pâté was baked at 125°C during 40 min. and subsequently cooled to a core temperature at 8°C whereafter individual packages were vacuum-packed (Nielsen et al. 2018). The heat treatment seems sufficient to inactivate *L. monocytogenes* (Den Besten et al. 2018). However, contamination of the cooked salmon pâté may occur during refrigeration and until the time of packaging. In this situation, the very rapid observed growth of *L. monocytogenes* in the salmon pâté at 8°C is most critical unless the commercial chilled shelf-life of the product is limited to less than 6 days (Product recipe A in Table 2 below). For longer commercial chilled shelf-life the product must be re-formulated (Table 2). The growth of *L. monocytogenes* can be delayed by lowering pH of salmon pâté from 6.4 to 5.9, adding lactic and acetic acid to the product as well as by using a modified atmosphere packaging with a CO_2 concentration of for example 25% in the head-space gas at equilibrium (Recipes B to D, Table 2). These recipes reduced the growth of *L. monocytogenes* and increased the time it takes to grow 100-fold from 6 days (Recipe A, Table 2) to 24 days (Recipe D, Table 2). However, these recipes do not prevent the growth of *L. monocytogenes* in salmon pâté as shown from the predicted ψ- and μ_{max}-values (Table 2). Furthermore, these recipes with 2.0% salt in the water phase of the product do not prevent the growth and toxin formation by *Clostridium botulinum.* To prevent growth and toxin formation by *C. botulinum* at 8°C in salmon pâté recipes E and F can be used (FSA,

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2016). For recipe E with 2.0% salt the product characteristics including lactic, acetic and benzoic acids and modified atmosphere packaging also prevented the growth of *L. monocytogenes* as shown by the ψ- value of 1.09. This ψ-value corresponds to a safe shelf-life of 3 - 5 weeks (Dalgaard and Mejlholm, 2019). For recipe F with 3.5 % salt but without benzoic acid and without modified atmosphere packaging the ψ- value is 0.68. This ψ-value corresponds to a safe shelf-life of less than 3 weeks and this match well with the predicted time of 22 days for a 100-fold increase in growth (Recipe F, Table 2).

Table 2. Predicted growth responses for *Listeria monocytogenes* in salmon pâté at 8°C depending on product characteristics.

Product recipe	% WPS[a]	pH	Organic acids in water phase, ppm[b]			% CO_2	Predicted[c] growth responses		
			Lactic	Acetic	Benzoic		ψ-value	Growth rate (μ_{max}, 1/d)	Time for 100-fold increase (d)
A	2.0	6.4	625	0	0	0	0.20	1.26	6
B	2.0	6.1	6000	1000	0	0	0.46	0.82	9
C	2.0	5.9	8000	1000	0	0	0.65	0.44	18
D	2.0	5.9	8000	1000	0	25	0.68	0.33	24
E	**2.0**	**5.9**	**8000**	**1000**	**1000**	**25**	**1.09**	**0.00**	**Not reached**
F	**3.5**	**5.9**	**8000**	**1000**	**0**	**0**	**0.68**	**0.35**	**22**

[a] Water phase salt.

[b] mg/Liter of water phase in the product (ppm).

[c] Growth responses were predicted by the model of Martinez-Rios et al. (2019) and included a prediction of lag time.

The salmon pâté developed in Task 2.1 as described by Nielsen et al. (2020) is a high-risk product with respect to potential growth of *L. monocytogenes* as well as with respect to growth and toxin formation by *Clostridium botulinum.* It is not necessary to develop such a high-risk product and the recipe of the salmon pâté from Task 2.1 must be re-formulated for example as shown for recipe E or F in Table 2. This very strong recommendation that salmon pâte developed in Task 2.1 must be re-formulated is based on (i) the results obtained in Figure 1 and Table 2; (ii) the WHO recommendation suggesting persons in risk groups to avoid consumption of pâtes (WHO https://www.who.int/news-room/fact-sheets/detail/listeriosis) and (iii) lessons learned from a major *L. monocytogenes* outbreak with spiced meat roll which is another cooked and chilled RTE product (Jensen et al. 2016). Like salmon pâté, spiced meat roll is cooked, chilled and then vacuum-packed. This product caused a major outbreak of listeriosis in 2014 with 41 persons becoming sick and 17 deaths occurred. The typical commercial shelf-life of vacuum-packed spiced meat roll is 28 days at 5°C and during this period *L. monocytogenes* can grow to high concentrations in the product if no specific anti-listerial food preservatives are added to recipes. For spiced meat roll (and many other cooked and chilled RTE foods) it is now common practice to include acetic acid/acetate in the recipes to stabilize the products against growth of *L. monocytogenes*. A similar approach is needed for the studied salmon pâté. During the SEAFOOD[TOMORROW] project the application of predictive food microbiology to determine safe shelf-life and determine safe product recipes were presented by DTU to the consortium during the SEAFOOD[TOMORROW] project meeting in Rome (3-5 May 2018) and again in Ghent (14-15 May 2019).

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5.2. Smoked salmon from task 2.1

Predicted growth of *L. monocytogenes* very much depended on product temperature, pH and a_w as the water phase concentrations of lactic acid, from endogenous origin, was similar irrespective of smoking and sodium reduction. Furthermore, the low phenol concentrations of 2.2 - 2.8 ppm in wood and cold-smoked salmon had limited inhibiting effect on the predicted growth of *L. monocytogenes* (Table 3). For *L. monocytogenes* a 2-log increase in cell concentration, corresponding to safe shelf-life, was predicted to take more than 90 days at 1 °C, from 39 to more than 90 days at 5 °C (Munoz et al., 2020) and 12-31 days at 8 °C (Table 4). If shelf-life longer than these critical times is desired then smoked salmon needs to be reformulated to become more inhibiting against the growth of *L. monocytogenes* at storage temperatures realistically occurring during the retail and consumer storage. The studied smoked salmon had a_w of 0.963-0.968 (Munoz et al., 2020) which is low in comparison with a_w above 0.97 often reported for smoked salmon (Jørgensen et al. 2000). This contributed to reduce growth of *L. monocytogenes* in the smoked salmon studied in task 2.1. However, at 8 °C, the predicted critical concentration of *L. monocytogenes* was reached after 12 to 31 days. A longer safe shelf-life can be achieved by using a formulation able to stabilize the smoked salmon against growth of *L. monocytogenes*, at 8°C and this can be obtained even for recipes where the average water phase salt (WPS) concentration is reduced from 5.7 % as reported by Munoz et al. (2020) to 3.5% WPS. A WPS concentration of 3.5% is needed in smoked salmon and other chilled RTE seafoods to prevent growth and toxin formation by *C. botulinum* (FSA, 2016). Unless, of course, other combinations of preserving parameters are included to manage *C. botulinum* and that was not the case for the studied smoked salmon. The smoked salmon studied by (Munoz et al., 2020) had an average pH of 5.82 (Table 3). At that pH and with 3.5% WPS the smoked salmon can be stabilized against growth of *L. monocytogenes* by adding acetic acid to a concentration of 2.5 g/L (2,500 ppm). Alternatively, a recipe with 1.0 g/L (1,000 ppm) of acetic acid and a total of 15.0 g/L (15,000 ppm) of lactic acid will prevent growth of *L. monocytogenes* (Table 3). The studied smoked salmon naturally included 9.0 g/L (9,000 ppm) of lactic acid in the water phase and thus 6.0 g/L (6,000 ppm) of lactic acid in the water phase should be added. The addition of organic acids to salmon fillets can be carried out together with the addition of salt for example by injection of a brine. Importantly, smoke components (phenol) have an inhibiting effect on growth of *L. monocytogenes* in cold-smoked salmon whereas this inhibiting effect is not observed for hot-smoked salmon. Therefore, in Table 3 a typical concentration of smoke components, corresponding to 10 ppm phenol, is used when predicting the growth response of *L. monocytogenes* in cold-smoked salmon whereas no phenol is included when predicting growth in hot smoked salmon (Table 3). ψ-values of 1.10 to 1.21 at 8°C were obtained for the suggested recipes that prevented growth of *L. monocytogenes*. These ψ-values correspond to safe shelf-lives of 3-5 weeks for the four suggested recipes with 3.5% water phase salt (Dalgaard and Mejlholm, 2019). The model of Martinez-Rios et al. (2019), as included in the predictive tool described below, can be used to identify relevant combinations of product characteristics and storage condition to prevent growth of *L. monocytogenes* in smoked salmon.

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Table 3. Predicted growth of *Listeria monocytogenes*[a] in vacuum-packed smoked salmon at 8°C depending on product characteristics[b]

Smoking and salting			Product characteristics[c]					Predicted growth responses for *L. monocytogenes*		
Smoke type	Smoke temp.	% NaCl; % KCl	% WPS[d]	pH	Organic acids in water phase, g/L[e]		Phenol, ppm[f]	Ψ-value	Growth rate (μ_{max}, 1/d)	Time for 100-fold increase (d)
					Lactic	Acetic				
Product characteristics as measured by Munoz et al. (2020)										
Wood	Cold	100; 0	5.95 ± 0.76	5.92 ± 0.21			2.2 ± 0.5	0.53	0.51	15
Wood	Cold	75; 25	5.65 ± 0.69	5.77 ± 0.17	9.0 ± 4.2	-	2.8 ± 0.9	0.65	0.34	23
Wood	Cold	50; 50	5.62 ± 0.63	5.87 ± 0.32			2.4 ± 0.3	0.56	0.48	16
Wood	Hot	100; 0	6.11 ± 0.92	5.77 ± 0.32			-	0.64	0.36	22
Wood	Hot	75; 25	5.43 ± 0.85	5.79 ± 0.35	8.5 ± 3.0	-	-	0.59	0.47	17
Wood	Hot	50; 50	5.96 ± 0.48	5.93 ± 0.32			-	0.51	0.59	13
Liquid	Cold	100; 0	5.66 ± 0.64	5.75 ± 0.31			-	0.64	0.38	21
Liquid	Cold	75; 25	5.25 ± 0.08	5.85 ± 0.25	8.5 ± 5.0	-	-	0.53	0.58	13
Liquid	Cold	50; 50	5.41 ± 0.36	5.90 ± 0.27			-	0.51	0.63	12
Liquid	Hot	100; 0	6.09 ± 1.40	5.75 ± 0.32			-	0.73	0.25	31
Liquid	Hot	75; 25	5.68 ± 0.31	5.73 ± 0.30	9.7 ± 5.0	-	-	0.73	0.26	30
Liquid	Hot	50; 50	5.89 ± 0.73	5.75 ± 0.44			-	0.72	0.27	29
Product characteristics of recipes stabilized against growth of *L. monocytogenes*										
Wood	Cold	-	3.5	5.82	9.0	2.5	10	1.20	0.00	Not reached
Wood	Cold	-	3.5	5.82	15.0	1.0	10	1.21	0.00	Not reached
Wood	Hot	-	3.5	5.82	9.0	2.5	-	1.10	0.00	Not reached
Wood	Hot	-	3.5	5.82	15.0	1.0	-	1.11	0.00	Not reached

[a] Growth responses was predicted by the model of Martinez-Rios et al. (2019). [b] Product characteristics were obtained from Muñoz et al. (2020).

[c] Values indicate mean ± standard deviation. [d] Percentage water phase salt (WPS) determined from measured a_w.

[e] Determined for each combination of smoke type and temperature (n = 10-12). [f] Phenol was exclusively measured for cold-smoked salmon produced with wood smoke as models to predict the effect of other types of smoking are not available.

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Table 4. Product characteristics[a] and predicted growth of *Listeria monocytogenes* at 5°C in fractions of seafood dishes (Winner products).

Products and fractions	% Dry matter	% NaCl in water phase	pH	Organic acid in water phase (g/L)			Predicted growth responses for *L. monocytogenes*		
				Acetic acid	Citric acid	Lactic acid	Ψ-value	μ_{max}, (1/d)	Time for 100-fold increase (d)
Fish sausage and vegetables									
- Fish sausage	18.1 ± 0.8	1.95 ± 0.5	6.24 ± 0.09	< LOD[b]	0.91 ± 0.17	2.05 ± 0.17	0.30	0.63	12
- Carrots	20.7 ± 0.5	0.48 ± 0.1	4.83 ± 0.06	0.37 ± 0.08	1.59 ± 0.29	0.21 ± 0.05	>10	0.00	Not reached
- Tomat/onion	9.3 ± 0.9	0.26 ± 0.1	4.66 ± 0.04	0.29 ± 0.03	4.22 ± 1.12	0.14 ± 0.10	>10	0.00	Not reached
- Sauce	18.0 ± 2.2	1.59 ± 0.1	6.04 ± 0.06	< LOD[b]	1.34 ± 0.18	1.54 ± 0.23	0.31	0.63	12
Fish balls with purée									
- Fish balls	28.9 ± 1.6	0.49 ± 0.0	6.91 ± 0.05	< LOD[b]	0.25 ± 0.07	1.03 ± 0.30	0.26	0.75	10
- Banana	51.2 ± 2.6	0.53 ± 0.1	5.60 ± 0.05	0.74 ± 0.19	2.71 ± 0.01	< LOD[b]	0.50	0.36	22
- Purée	17.7 ± 0.2	0.81 ± 0.0	5.63 ± 0.03	0.34 ± 0.00	1.18 ± 0.08	< LOD[b]	0.37	0.44	18
Fish roulade									
- Fish	22.9 ± 0.6	2.05 ± 0.1	6.69 ± 0.01	< LOD[b]	0.61 ± 0.08	0.96 ± 0.09	0.27	0.66	12
- Cabbage	11.8 ± 0.6	1.76 ± 0.1	6.39 ± 0.05	< LOD[b]	1.06 ± 0.08	0.62 ± 0.07	0.27	0.66	12
- Potato	20.9 ± 0.7	0.51 ± 0.1	6.02 ± 0.03	< LOD[b]	3.01 ± 0.16	0.13 ± 0.02	0.27	0.69	11
- Sauce	14.3 ± 1.1	0.68 ± 0.1	6.32 ± 0.05	-[c]	-[c]	-[c]	0.26	0.72	11

[a] Values indicate mean ± standard deviation.

[b] Below limit of detection (LOD).

[c] Not enough sample available for analysis.

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Table 4. Continued

Products and fractions	% Dry matter	% NaCl in water phase	pH	Organic acid in water phase (g/L)			Predicted growth responses for *L. monocytogenes*		
				Acetic acid	Citric acid	Lactic acid	Ψ-value	μ_{max}, (1/d)	Time for 100-fold increase (d)
Fish fillet with wheat salad									
- Fish fillet	22.5 ± 5.8	0.37 ± 0.1	6.70 ± 0.10	1.17 ± 0.71	0.37 ± 0.20	2.00 ± 0.56	0.32	0.64	12
- Wheat salad	27.2 ± 1.7	0.35 ± 0.0	4.44 ± 0.05	< LOD[b]	3.47 ± 0.46	1.99 ± 0.20	>10	0.00	Not reached
Fish balls with marinara sauce									
- Fish balls	28.0 ± 1.7	1.81 ± 0.3	6.62 ± 0.09	1.88 ± 0.20	1.44 ± 0.18	0.93 ± 0.11	0.26	1.03	8
- Vegetables	23.7 ± 3.2	0.35 ± 0.2	5.65 ± 0.20	< LOD[b]	0.92 ± 0.15	< LOD[b]	0.19	1.28	6
- Sauce	13.2 ± 1.1	1.42 ± 0.1	6.24 ± 0.17	0.69 ± 0.08	1.26 ± 0.07	0.75 ± 0.11	0.26	1.06	7
Mussel soup									
- Mussel soup	20.9 ± 1.1	1.15 ± 0.1	6.09 ± 0.03	< LOD[b]	1.12 ± 0.04	< LOD[b]	0.19	1.32	6

[a] Values indicate mean ± standard deviation.

[b] Below limit of detection (LOD).

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5.3. Seafood dishes for specific population groups - Winner products from task 2.2

The intended distribution of the six studied dishes as catering products, retail products or both have not been finally communicated from task 2.1. If these products are heated prior to consumption the relevance of studying the growth potential of *L. monocytogenes* becomes less important. However, heating of the six products by consumers (or even by catering facilities) prior to consumption may not be sufficient to inactivate *L. monocytogenes.* Therefore, chilled storage and distribution is relevant with respect to of *L. monocytogenes* in both catering and retail products. The growth potential of *L. monocytogenes* was evaluated for storage of these products at 5 °C. The time for a 100-fold increase in the predicted concentration of *L. monocytogenes* has been used to determine safe shelf-life or safe chilled distribution time. If heated appropriately prior to consumption *L. monocytogenes* will be inactivated and potential growth during chilled distribution will not directly be related to the listeriosis risk of these dishes. However, packs will be opened prior to heating and to limit potential cross contamination with *L. monocytogenes* in catering facilities or in kitchens of consumers, growth of the pathogen in the chilled products should be limited.

The products for youth/children were (i) fish sausage and vegetables and (ii) fish balls with purée. For these products' distribution at 5 °C must be limited to less that 10-12 days to avoid the risk of more than a 100-fold increase in concentrations *L. monocytogenes*. For both products the fish fraction was the most sensitive with respect to potential growth of *L. monocytogenes* although the sauce included together with the fish sausage and vegetables was as sensitive as the fish fraction (Table 4). It needs to be noted, however, that babies but not youth/children is a high-risk-group of consumers with respect to listeriosis. Thus, quality changes during storage at 5 °C seem more important for these products than potential growth of L. monocytogenes.

Fish roulade and fish fillet and wheat salad were developed for pregnant women. For fish roulade the different fractions were about equally sensitive to growth of *L. monocytogenes* and safe shelf-life at 5°C was below 11 days (Table 4). *L. monocytogenes* was unable to grow in the wheat salad due to a low pH of 4.44 but safe distribution time of the fish fillet at 5°C must be limited to less than 12 days (Table 4). Pregnant women are a high-risk group with respect to listeriosis and chilled distribution of the developed dishes should preferably be much shorter than 12 days.

The fish balls with marinara sauce and the mussel soup developed for elderly people/seniors both supported growth of *L. monocytogenes* and the safe shelf-life at 5°C must be limited to less than 6 days. For fish balls with marinara sauce the vegetable fraction was the most sensitive with respect to potential growth of *L. monocytogenes* and the fraction limited the safe shelf-life of the dish. Listeriosis is a major challenge for elderly people/seniors and it is most important to limit the chilled distribution time for the developed dishes.

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5.4. DTU Food Predictive Tool for *Listeria monocytogenes*

The developed DTU Food Predictive Tool for *Listeria monocytogenes* include a 'INPUT-OUTPUT' sheet and a 'Calculator–salt and acids' sheet (Fig. 2). Product characteristics and storage conditions to be evaluated are entered in fields with black background within the 'INPUT-OUTPUT' sheet. Predictions for those conditions are then provided in the right part of the screen (Fig. 2). The DTU Food Predictive Tool for *Listeria monocytogenes* has a broad range of applicability as shown in Figure 2. This is important to facilitate product development and innovation. Due to the unfortunate high occurrence of *L. monocytogenes* in RTE seafood within Europe it is often important to limit product shelf-life or to identify combination of product characteristics and storage conditions that reduce or prevent growth of this important human pathogenic bacteria. The DTU Food Predictive Tool for *Listeria monocytogenes* is developed for this task as illustrated above by the studies of salmon pâté, smoked salmon and winner products. Importantly this tool can predict both growth and growth boundary for *L. monocytogenes* and it determines the ψ-value that express how far a given set of product characteristics and storage conditions is from the growth boundary of the pathogen. The ψ-value is important to determine the safe shelf life of a specific recipe as shown by the examples in this document. It must also be noted that for products that are stabilized against growth of *L. monocytogenes* the growth rate is always zero but the ψ-value indicate if the product is close to the growth boundary (ψ-value above but close to 1.0) or if the product is well preserved so that it can have a long chilled safe shelf-life of more than five weeks (ψ-value above 2).

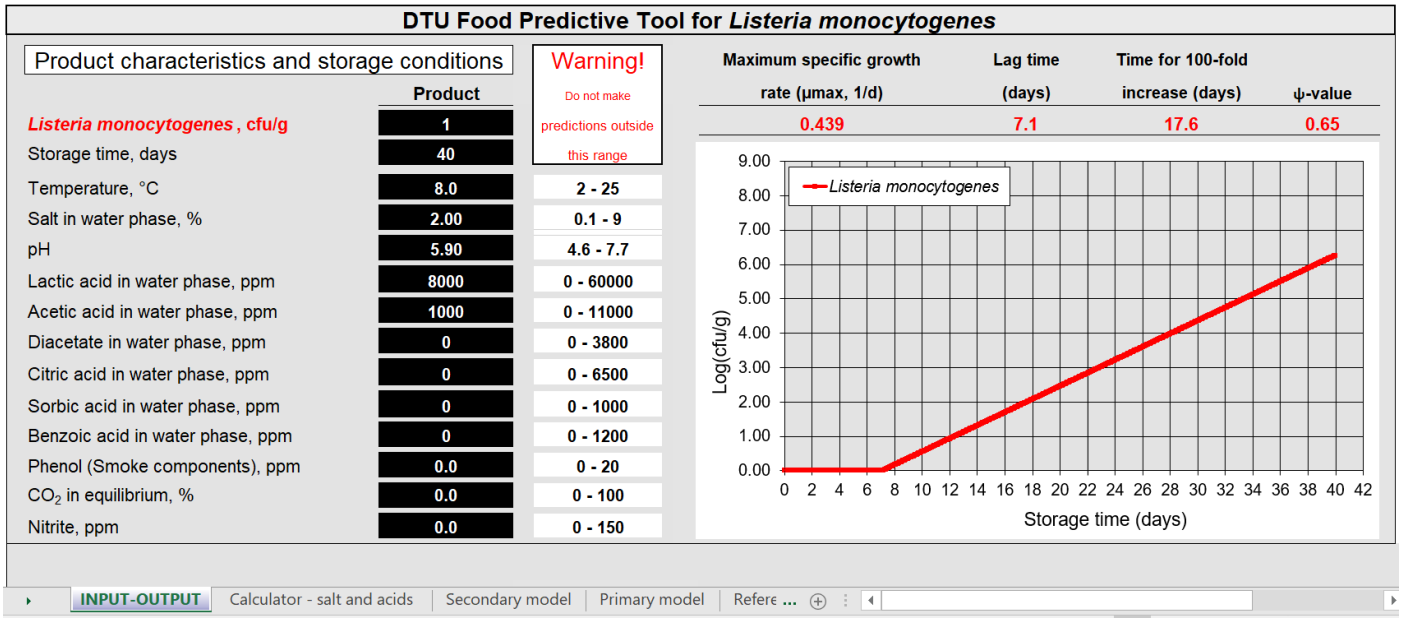

Figure 2. DTU Food Predictive Tool for *Listeria monocytogenes* including the model of Martinez-Rios et al. (2019) and used to evaluate growth potential of the pathogen in different seafood within task 3.4.

Within the sheet 'Calculator–salt and acids' the DTU Food Predictive Tool for *Listeria monocytogenes* includes formulas to facilitate the calculation of concentrations for water phase salt and organic acids in water phase of products. Within task 2.1 of the SEAFOOD^TOMORROW project studies of both salmon pâté and smoked salmon included products where sodium chloride (NaCl) was replaced the potassium chloride (KCl).

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In this situation it is important to calculate the concentrations of water phase salt in products that allow the DTU Food Predictive Tool for *Listeria monocytogenes* to accurately estimate the growth potential of the pathogen. For both NaCl and KCl the inhibiting effect on *L. monocytogenes* is due to the Cl^-ion (See references in Munoz et al., 2020). The model of Martinez-Rios et al. (2019) was developed and validated with NaCl at model input and therefore when used to make predictions concentration of the Cl^-ion in products must be expressed as an equivalent concentration of NaCl. To facilitate the required calculations, the DTU Food Predictive Tool for *Listeria monocytogenes* includes a simple calculator that expresses the percentage of both NaCl and KCl in a product as the equivalent concentration of NaCl in the water phase of the product ('% water phase salt in product') (Fig. 3).

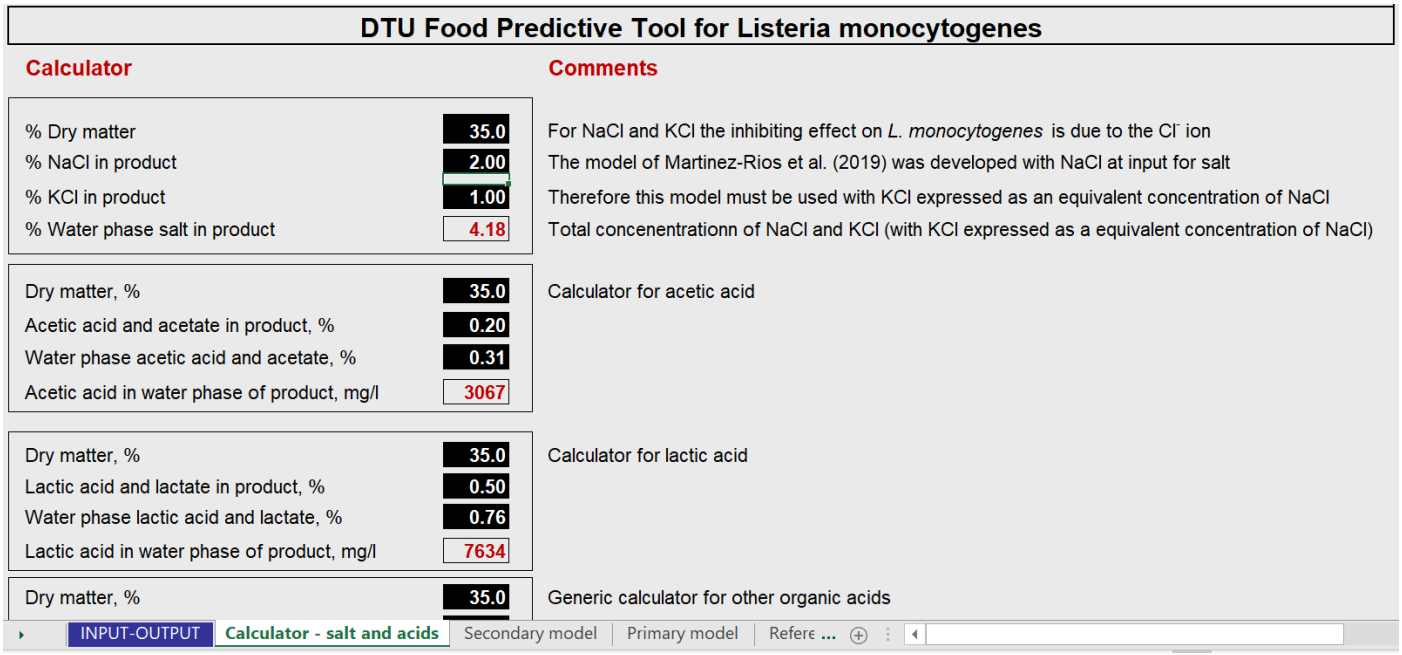

Figure 3. DTU Food Predictive Tool for *Listeria monocytogenes* including calculators to determine concentrations of salt and organic acid in the water phase of products. These calculations are important as it is the concentrations in the water phase rather than concentrations in the entire product that determine the growth inhibiting of salt and acids on the pathogen.

The DTU Food Predictive Tool for *Listeria monocytogenes* is programmed by using MS Excel. This has the advantage that the tool can be used on different types of computers irrespective of the operating system being Windows or OSX (Mac/Apple). The MS Excel based tool is not locked and users can change the way the included primary and secondary models are working. This can be an advantage as the tool can be set up to make prediction for a range of conditions and the relevant data can be easily included by copy/paste from other spreadsheets. This option has for example been beneficial when making the predictions underlying Table 2 to Table 4 above. However, this flexibility can also be a disadvantage as user may change models to provide incorrect predictions. To overcome the latter problem the model of Martinez-Rios et al. (2019) can be used as part of the Food Spoilage and Safety Predictor (FSSP) software (http://fssp.food.dtu.dk) which has been developed entirely outside the SEAFOOD^TOMORROW project and therefore are not commented on within this SEAFOOD^TOMORROW deliverable. It probably also should be noted that development of the Martinez-Rios et al. (2019) was funded by the Danish Dairy Research Foundation and developed entirely outside the SEAFOOD^TOMORROW project.

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6. Conclusions

The objective of this deliverable was to evaluate safe shelf-life of eco-innovative solutions from task 2.1 and 2.2 within the SEAFOOD[TOMORROW] project. The DTU Food Predictive Tool for *Listeria monocytogenes* was developed to facilitate determination of safe shelf-life as well as to facilitate the identification of new recipes that prevented growth of the pathogen. Salmon pâté from task 2.1 had a safe shelf-life of 6 days at 8°C and this is insufficient for practical distribution of the product. New recipes were suggested to obtain longer shelf-life of products where growth of *L. monocytogenes* as well as growth and toxin formation by psychrotolerant *Clostridium botulinum* was prevented. For smoked salmon from task 2.1 new safe recipes were suggested with reduced sodium content and safe shelf-life of 3 - 5 weeks at 8°C. For six different dished developed to specific population groups (Winner products from task 2.2) the most sensitive fractions with respect to growth of *L. monocytogenes* were identified and safe distribution time at 5°C was determined.

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