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Quantitative microbiological risk assessment of traditional food of animal origin produced in short supply chains in Poland

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Abstract

Polish raw-milk cheeses produced in short supply chains may pose a threat to consumer safety due to pathogen presence. *Listeria monocytogenes* is a bacterium of great importance for the food safety of refrigerated RTE foods due to its ability to grow at refrigeration temperatures. During the EU-FORA fellowship, a stochastic risk assessment was designed and executed to estimate the risk for consumers from *L. monocytogenes* in these products. The aim was to develop a probabilistic QMRA model that would incorporate the variability and uncertainty of the model's inputs such as prevalence, initial concentration levels, product intrinsic factors, domestic storage temperature and consumer behaviour. The project involved data collection and analysis, growth model selection, mathematical modelling and Monte Carlo analysis in R programming language. Microbiological and physicochemical testing were carried out throughout the year on two types of cheeses in combination with a domestic refrigerator temperature survey and accompanying consumption questionnaire. Collected data were fitted to probability distributions using R. The appropriate growth model for the pathogen was selected based on an inoculation study performed on one of the raw-milk cheeses and the chosen mathematical model was written into the R script developed for the QMRA. The dose–response model used the ingested dose calculated from the modelled concentration of *L. monocytogenes* at the time of consumption and the single serving size from the questionnaire to estimate the probability of illness. The final risk was expressed as probability of listeriosis for Polish consumers per serving of raw-milk cheese.

Keywords: *Listeria monocytogenes*, QMRA, raw-milk cheese, risk assessment

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1. Introduction

Polish cheese consumption is dominated by Polish white-fresh cheeses, which account for 54% of total sales volume in the country **(WORLD AND POLAND PER CAPITA CHEESE CONSUMPTION,** n.d.**)**. The most popular among these are 'twarog', an acid-set cheese, and 'ser podpuszczkowy', a rennet cheese, made from naturally acidified milk. These types of cheeses from unpasteurized cow's milk are commonly produced by small scale farms and sold at a local level by producers directly to customers (Lucey, n.d.). Consumer Interest in small-scale locally produced cheeses, such as the aforementioned, has for the past years been on the rise in Poland **(Soroka and Wojciechowska-Solis,** 2019**)**.The biological risk associated with the consumption of such products has not been thoroughly assessed in Poland. The small scale of production by farmers and the use of unpasteurised milk raises concerns about the safety of these products. Products produced locally, often using traditional methods,14 are rarely controlled in terms of safety, and the farmers/producers themselves often lack extensive knowledge related to good hygiene and production practices.

Food of animal origin can be a very good substrate for the growth of microorganisms. Specifically, for fresh cheeses, the high nutrient content, high moisture, low-temperature heat treatment and lack of additives, conduce to the growth of various microorganisms, including possibly dangerous pathogens, such as *Listeria monocytogenes*, that could pose a threat to consumers of these products. The small dose required for illness and the severity of listeriosis in humans warrant high vigilance for all RTE foods including cheeses (The European Union One Health 2019 Zoonoses Report, 2021; The European Union One Health 2020 Zoonoses Report, 2021).

Commission Regulation (EC) No 2073/2005 on microbiological criteria, distinguishes between RTE products able to support *L. monocytogenes* growth and those that inhibit growth. The physicochemical characteristics of these two products, pH and water activity (a_w), in this case place them in category 1.2, for which two criteria apply. These are absence in 25 g at the end of production and $<$ 100 CFU/g during its shelf life. These safety criteria are required to achieve the appropriate level of protection (ALOP) for EU consumers.

The Rapid Alert System for Food and Feed (RASFF) is a European system for reporting food safety issues within the Union, and is a key feature of EU food safety. The RASFF report for 2020 included 3 alerts regarding raw-milk cheeses out of a total of 25. The Polish chief sanitary inspectorate (Główny Inspektorat Sanitarny), has enforced 6 recalls of cheeses due to detection of *Listeria* from 2019 to 2021 **(Główny Inspektorat Sanitarny,** 2021**)**. These included cheeses made from raw-milk, and traditional products. Pyz-Łukasik et al studied the occurrence of *L. monocytogenes* in artisanal Polish cheeses and found the prevalence to be much higher than the European average reported by EFSA. Their findings suggest a prevalence of 6.2% for artisanal cheeses, thus warranting the assessment of listeriosis risk (Pyz-Łukasik et al., 2021).

To ascertain the listeriosis risk associated with the consumption of such products, a quantitative microbiological risk assessment (QMRA) was conducted. QMRA is a mathematical modelling method used to estimate the risk from a hazard-food combination for a specific population. The methodology involves the steps of hazard identification, hazard characterisation, exposure assessment and risk characterisation. The process aims to quantitatively describe the fate of the studied pathogen in the food from production to consumption and associate the consumers' subsequent exposure with the risk of an adverse health outcome. Considering the variability and uncertainty associated with all the model inputs (food intrinsic factors, contamination levels, consumption patterns, etc.), the stochastic approach was selected to better portray real-life scenarios. In the stochastic approach, the point estimate values of QMRA inputs are substituted by probability distributions describing variability. Subsequently the final outcome of the risk assessment (RA) is a probability distribution of risk.

2. Description of work programme

2.1. Aims

The working programme aimed to familiarise the fellow with all aspects of an QMRA by providing hand-on experience in performing a RA of a traditional RTE food of animal origin. The fellow carried out activities spanning from relevant data collection to mathematical modelling in R. By applying a stochastic approach, emphasis was placed on describing the variability and uncertainty of most of the model inputs. Throughout the fellowship's duration the fellow undertook learning to code in R, an open-source programming language, popular in the RA community. Thus, a custom R script was

written to fit probability distributions to data, estimate kinetic parameters, model bacterial growth for *L. monocytogenes* and lactic acid bacteria (LAB), perform Monte Carlo analysis and produce a final risk estimate for listeriosis arising from the consumption of raw-milk cheeses produced in short supply chains in Poland.

2.2. Activities/Methods

2.2.1. Prevalence estimation – Cyclical microbiological testing

Microbiological analyses were performed on 46 raw-milk cheese samples (26 twarog and 20 ser podpuszczkowy) from a farmer/producer based in the Silesian voivodeship. Testing for *L, monocytogenes* was carried out according to EN ISO 11290-1:2017 and EN ISO 11290-2:2017 aiming to detect the presence, as concentrations were expected to be low. In cases of detection, *L. monocytogenes* was biochemically verified (LISTERIA-ID Microgen Bioproducts, Camberley Surrey, UK). *L. monocytogenes* was detected in 3.85% of acid-set cheeses and 10% of rennet cheeses, for a total product prevalence of 6.52%. In their study of Polish artisanal cheeses, Pyz-Lukasik et al. estimated a mean prevalence of 6.2% for Listeria monocytogenes.

An important parameter affecting the fate of *Listeria* in dairy products is their indigenous lactic acid microflora which is characteristic of these cheeses, as they are produced without the addition of a starter culture. LAB act by acidifying the food matrix via lactic acid production, competing for resources and producing antimicrobial peptides such as bacteriocins. *Listeria* growth suppression due to the action of LAB is referred to in literature as the 'Jameson Effect' (Mellefont et al., 2008; Sip et al., 2012; de Niederhäusern et al., 2020). To include this important parameter in the final QMRA model, mesophilic LAB were enumerated following ISO 15214:2002.

Additionally, cyclical microbiological ISO standard-based testing was carried out on samples for presence of *Salmonella* spp., *Campylobacter* spp., *Clostridium perfringens*, coagulase-positive *Staphylococcus aureus*, *Escherichia coli* O157:H7, *E. coli*, total number of microorganisms, coliforms, Enterobacteriaceae, moulds and yeasts.

2.2.2. Product intrinsic factors

The ability of *L. monocytogenes* to grow in food and the rate at which it can proliferate is dependent on temperature, water activity, pH and organic acids such as lactic acid (Mellefont et al., 2008; Sip et al., 2012; de Niederhäusern et al., 2020). The product intrinsic factors influencing bacterial growth were measured for every sample and used to better describe the batch-to-batch variability in product characteristics. Water activity was measured with an Aqualab 4TE metre (METER Group, Pullman Washington, USA) with $\pm$ 0.003 accuracy, and pH was measured with an accuracy of $\pm$ 0.01 using a FiveEasy Benchtop pH-metre (Mettler Toledo, Columbus Ohio, USA). Additionally, cheese samples (n = 6) were sent to the institute's chemical department in Warsaw for lactic acid quantification by HPLC.

2.2.3. Domestic refrigerator temperature survey

Domestic refrigerated storage is a crucial part of the cold chain and proper storing temperatures contribute to food safety. Refrigerators can vary considerably and temperature fluctuates constantly during the cooling cycle. This variability in storage temperatures is of paramount importance in a stochastic quantitative microbial risk assessment. Since artisanal and traditional farmer products are mainly sold directly to consumers, the majority of storage time is spent in households. Due to this temperature variation, contaminated products may end up in an environment that could allow for additional growth of the pathogen, thus increasing the final dose consumed. The fact that *L. monocytogenes* is one of the few food-borne pathogens that can adapt and grow slowly under refrigeration temperatures emphasised the need for domestic storage temperature data (Taoukis et al., n.d.; Tasara and Stephan, 2006). As there was no data regarding storage temperatures in Poland in the literature, a refrigerator temperature survey was conducted in order to incorporate this source of variability into the exposure assessment. The survey included 78 inhabitants of Lodz, Poland, and measured the temperature of the middle shelf with a data logger (LogTag model TRIX-8) at 5-min intervals for 24 h. The results of this survey are under consideration for publication for use in future risk assessments.

2.2.4. Consumer questionnaire

Alongside the temperature survey, a consumption questionnaire was provided to participants. Questions were related to personal characteristics of the consumers, cheese storage habits, consumption patterns and refrigerator use. An English version of the questionnaire is provided in the Appendix A. The received questionnaires (n = 56) were used to estimate the variability in portion size (single serving consumed in one sitting), and time of consumption for consumers of these traditional products. The information collected was incorporated into the final model.

2.2.5. Inoculation study – model selection

To select an appropriate predictive microbiology model for *L. monocytogenes* in the fresh raw-milk cheeses, a 10-day inoculation study was performed on ser podpuszczkowy. A *L. monocytogenes* inoculum strain mix was prepared from two reference strains for food (ATCC 19111, WDCM 1/2a) and one strain isolated from the product. The working culture strains were prepared from reference stock following the procedures of EN ISO:11133:2014. Individual cheese samples in replicates were inoculated to achieve an initial concentration level of 4.04 logCFU/g. The aerobically packed, inoculated samples were stored at temperatures of 5°C and 15°C for 10 days. The sample's physicochemical properties were measured (pH, a_w, lactic acid) on the first and last day. Enumeration was performed for *L. monocytogenes* and LAB on each sampling day (days 0, 2, 4, 7, 8, 10) by plating out the appropriate dilution on OCLA and MRS media (Oxoid, Basingstoke Hampshire, UK) respectively (Figure 1).

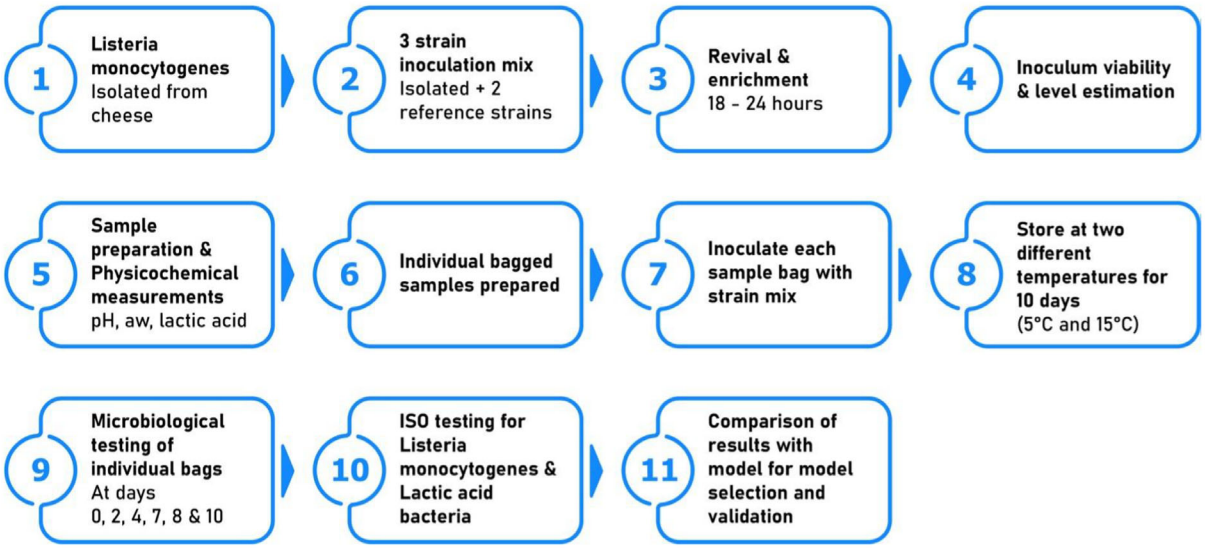

Figure 1: Inoculation study plan of *Listeria monocytogenes* on ser podpuszczkowy

Enumeration results were graphically compared for model selection, taking into account plate count uncertainty of 0.3–0.6 logCFU (Jarvis et al., 2007). The Food Spoilage and Safety Predictor (FSSP), developed by DTU, was selected as it included LAB and lactic acid concentration as an input in its *L. monocytogenes* cottage cheese model ('Growth of *Listeria monocytogenes* in cottage cheese in combination with LAB') (Østergaard et al., 2014) (Figure 2).

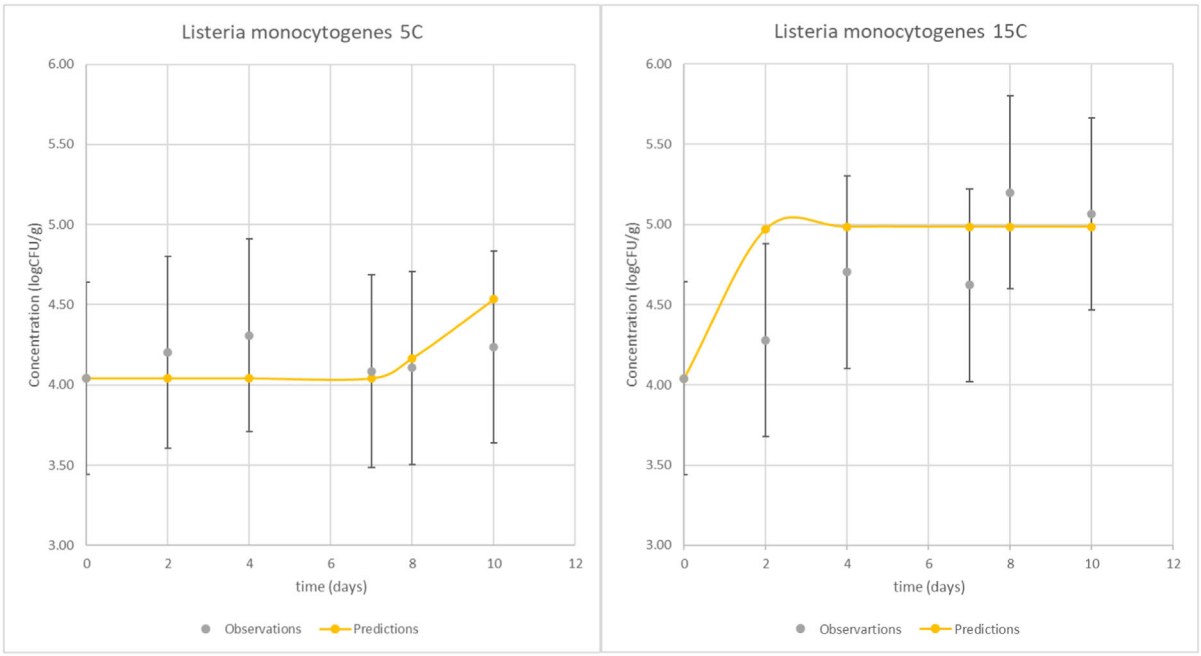

Figure 2: Graphical comparison of *L. monocytogenes* FSSP cottage cheese model prediction (line) and average observed counts (points) from the inoculation study at 5°C and 15°C

The relative error % (RE) for each set of predictions-observations was calculated according to equation (1). At 5°C, all predictions were within the ±10% RE zone while at 15°C 50% of the predictions were within the ±10% RE zone, with one outside of the ±20% RE zone.

$$Relative\ Error\%(RE) = \frac{N_{observed} - N_{predicted}}{N_{observed}} \times 100 \quad (1)$$

2.2.6. QMRA in R

R is an open-source programming language used for statistical analysis and graphics. The language has become a popular choice for risk assessors due to its flexibility and transparency, an important aspect of risk assessment. During the fellowship, the fellow learned to code in R and developed a custom R script for use in stochastic quantitative risk assessment of cheeses.

Probability distribution fitting scenarios of the collected data were statistically tested for goodness of fit. The distribution selection was made based on the chi-square test, the Kolmogorov–Smirnov test, the Cramer–Von Mises test, the Anderson–Darling Test, the Akaike information criterion and the Bayesian Information Criterion. Based on the summary of these statistics, appropriate distributions were selected to describe the model inputs where possible. In cases where the data was insufficient or fitting based on different goodness of fit tests was not conclusive, variables were assigned distributions according to scientific literature. In the case of *L. monocytogenes*, initial concentration N0 was assumed to follow a beta-general distribution as described in the EFSA opinion on *L. monocytogenes* contamination of RTE foods (Ricci et al., 2018). The variability in a_w and pH values was described by applying the beta-pert distribution based on the minimum, maximum and most likely values. The cumulative distribution function was used for the time of consumption and serving size to describe consumer behaviour. The mean lactic acid concentration was used to calculate the undissociated lactic acid (uLA) in mM, based on the product pH value according to equation (2).

$$uLA = \frac{\frac{LAC}{Mr_{lactic\ acid}}}{\left(1 + 10^{(pH - pka_{lactic\ acid})}\right)} \quad (2)$$

For the prevalence of *L. monocytogenes* in the tested products a beta distribution was chosen to incorporate the uncertainty resulting from the limited sample size (Figure 3).

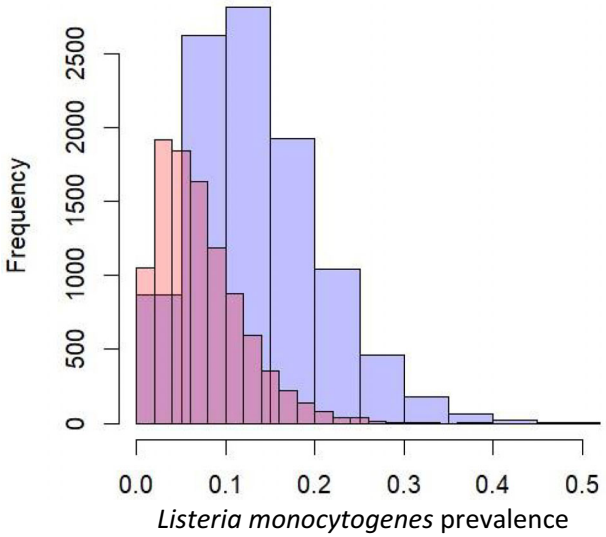

Figure 3: Estimated prevalence distribution of *Listeria monocytogenes* in raw-milk twarog (pink) and ser podpuszczkowy (purple)

To predict the fate of Listeria in the contaminated products, the growth model of Østergaard et al from FSSP was coded into the R script (Østergaard et al., 2014). The FSSP model follows the Jameson effect approach, illustrating the inhibiting influence of mesophilic LAB on the growth of *L. monocytogenes*. This model also includes the term ξ for quantifying the effects of interactions between temperature, pH and organic acids on the growth/no growth limit (le Marc et al., n.d.). The model estimated the LAG time, maximum growth rate $\{_{max}$, the growth rate of *Listeria* in the presence of LAB $\{_{lm}$ and the final concentration N for each simulated scenario. A total of 10,000 simulations were run for the complete model, sampling from the inputted distributions for the Monte Carlo analysis, calculating the final concentration of *L. monocytogenes* at the time of consumption. These results were multiplied with the distribution of the single serving size resulting from the cumulative distribution, to estimate the dose ingested by consumers (lamda) (Figure 4, Table 1).

Table 1: Overview of the R stochastic model parameters for both products

Model parameters	Description	Probability Distribution/Value		Units	Source
		Twarog	Ser podpuszczkowy		
N0	Initial concentration of *Listeria monocytogenes*	rbetagen(iterations, shape1 = 0.194, shape2 = 3.177, min = −1.69, max = 7)		logCFU/g	*EFSA Journal* 2018;16(1):5134, 173 pp. Gombas et al. (2003)
LAB	Initial concentration of lactic acid bacteria	rweibull(iterations, shape = 7.659300, scale = 7.859085)	rweibull(iterations, shape = 10.066707, scale = 7.959734)	logCFU/g	Microbiological analysis
pH	Product pH	rpert(iterations, min =3.98, max = 4.66, mode = 4.32)	rpert(iterations, min = 4.67, max = 5.95, mode = 5.84)	–	Physicochemical testing
aw	Product water activity	rpert(iterations, min = 0.9550, max = 0.9985, mode = 0.9980)	rpert(iterations, min = 0.9696, max = 0.9951, mode = 0.9832)	–	Physicochemical testing
LAC	Lactic acid concentration	Mean = 10,340	Mean = 8,395	ppm	Physicochemical testing
Temp	Domestic refrigerator Temperature	Truncated normal distribution(TBP)		°C	Temperature survey

Model parameters	Description	Probability Distribution/Value		Units	Source
		Twarog	Ser podpuszczkowy		
times	Time of consumption during product's shelf life	recdf(tofcon[(a)], iterations)		Days	Consumption questionnaire
SSS	Single Serving Size	recdf(SS[(b)], iterations)		g	Consumption questionnaire
lamda	Dose received	$=10^{N} \times$ SSS		CFU	WHO/FAO (2004)
iterations	Number of iterations for the Monte Carlo analysis	10,000		–	–
P_Contam	Prevalence of *Listeria monocytogenes* in tested products	rbeta(iterations, s + 1 = 2, n-s + 1 = 26)	rbeta(iterations, s + 1 = 3, n-s + 1 = 19)	–	Microbiological testing
P_ill	Probability of illness	$= 1 - e(-r^{(c)} \times$lamda)		–	WHO/FAO (2004)
Risk	Listeriosis risk for consumers of Polish raw-milk cheeses produced in short supply chains	$=$ P_Contam $\times$ P_ill		–	–

TBP: to be published.
(a): tofcon:Time of consumption Data from Consumption questionnaire.
(b): SS: Single Serving Data from Consumption questionnaire.
(c): $r = \frac{nb\ of\ cases\ in\ the\ subpopulation}{nb\ of\ L.monocytogenes\ ingested\ by\ the\ population}$.

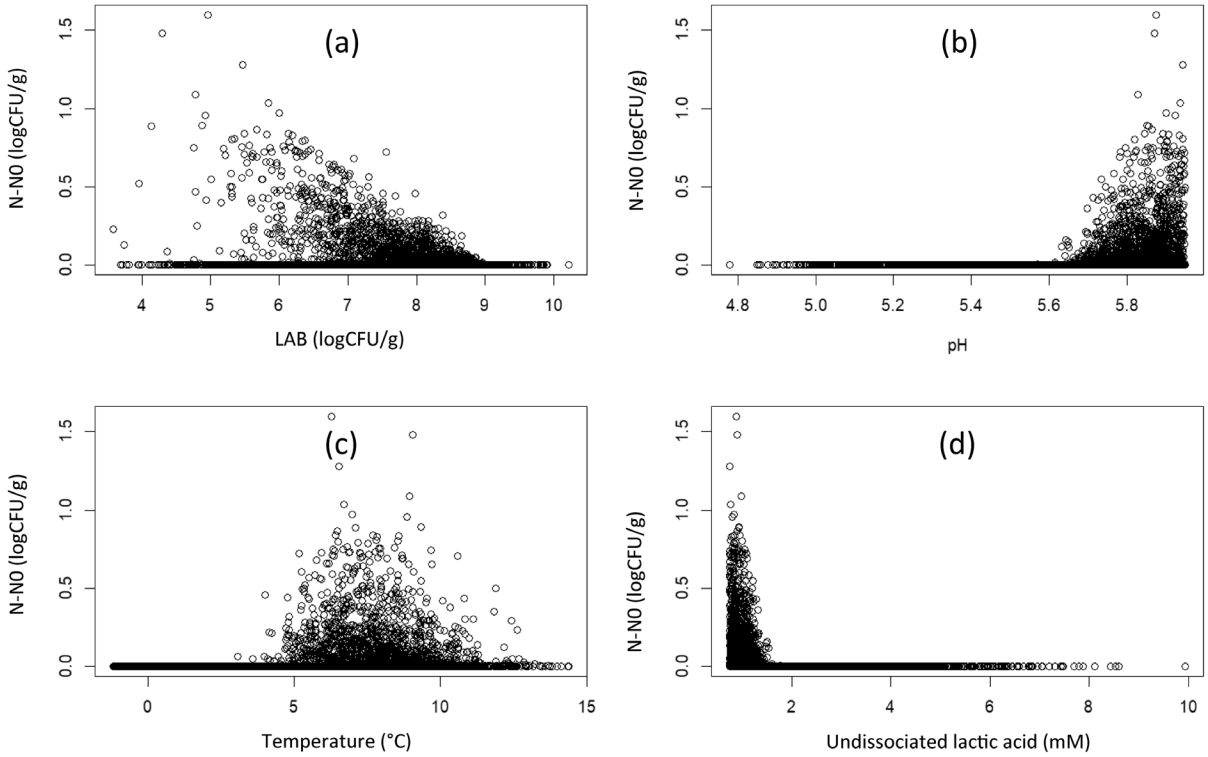

Figure 4: Ser podpuszczkowy *L. monocytogenes* growth in relation to LAB concentration (a), pH (b), Temperature (c) and undissociated lactic acid (d) for each of the 10,000 Monte Carlo simulations

The exposure assessment results were then inserted into the exponential dose response model of WHO/FAO (2004) for the hazard characterisation. Two scenarios were examined for listeriosis infection resulting from the consumption of fresh raw-milk cheeses, one for the healthy general population and one for susceptible consumers with the r parameter values being $2.37 \times 10^{(-14)}$ and $1.06 \times 10^{(-12)}$, respectively. The resulting probability of illness was a probability distribution which was used in the risk characterisation. The final risk to consumers of these products was calculated by multiplying the probability of illness by the prevalence of Listeria in each product.

The Monte Carlo analysis allowed for estimating the risk of foodborne illness per serving with the simulation model, drawing the values of input variables such as prevalence, initial *L. monocytogenes* concentration, initial LAB concentration, storage temperature, pH, a_w, time of consumption and individual serving size from the described probability distributions (Figure 5).

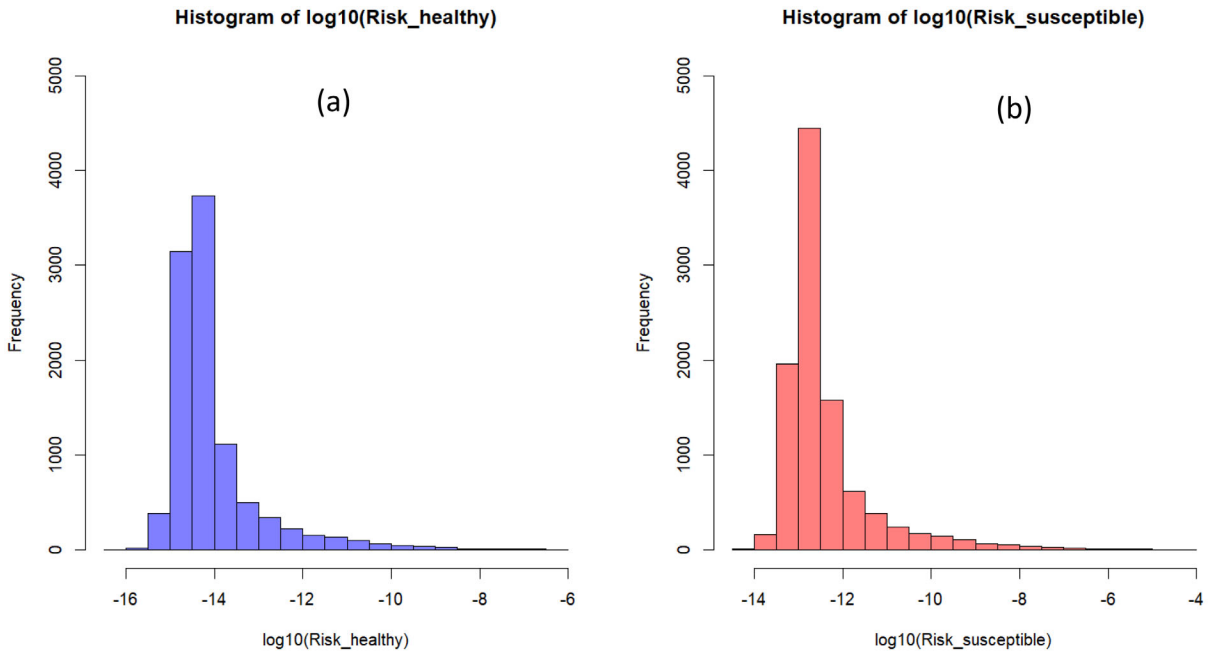

Figure 5: Probability distribution of Log10(Risk) of listeriosis associated with the consumption of one serving of raw-milk ser podpuszczkowy cheese by (a) typical healthy consumers and (b) susceptible high-risk consumers

3. Conclusions

The main aim of the project was the development and application of a stochastic quantitative microbial risk assessment model for estimating the health risk for listeriosis arising from the consumption of RTE animal origin products produced in small supply chains in Poland. The working programme enabled the fellow to gain expertise in the many components of a QMRA by applying the methodology on the specific food-hazard combination of raw-milk cheese and *L. monocytogenes*. In the course of the fellowship, the fellow became familiarised with appropriate data selection from scientific literature, collection of data to fill in identified case-specific data gaps, designing a risk assessment pathway, mathematical modelling of bacterial growth and developing a complete QMRA model for risk estimation, taking into account input and output variability and uncertainty. A particularly valuable facet of this process has been the introduction to R language programming, which has allowed for the model to be conceived and designed probabilistically with the use of Monte Carlo analysis.

Based on 10,000 simulations of the developed model, *Listeria* did now grow in twarog but displayed limited growth in 1.17% of the ser podpuszczkowy simulations. The mean log10(Risk) per serving for healthy consumers of local raw-milk twarog and ser podpuszczkowy was estimated −14.40 (min = −16.80, median = −14.66, max = −6.73, sd = 1.05) and − 14.05 (min = −16.08, median = −14.35, max-6.11, sd = 1.02), respectively. In the case of susceptible consumers, the mean log10(Risk) was increased, at −12.75 (min = −15.84, median = −13.01, max = −4.77, sd = 1.04) for twarog and − 12.40 (min = −14.49, median = −12.70, max = −4.22, sd = 1.03) for ser podpuszczkowy. These estimates are due to the short shelf life, low pH values and high lactic acid concentration due to LAB the products. Thus, the risk of Listeriosis arising from the consumption of these products can be considered as very low, although translating the risk into qualitative terms should be done with caution when communicating with risk managers as there is no consensus on translating probabilities (Scientific Opinion on Risk Assessment Terminology, 2012).

The fellowship programme has been an invaluable opportunity for the fellow to obtain new scientific knowledge and skills, and join the thriving and growing network of the EU-FORA fellows and alumni, promoting the dissemination of risk assessment expertise in Europe.

4. Additional activities during the EU-FORA fellowship

The hosting site arranged a visit to the on-farm facilities of the producer in the Silesian voivodeship participating in the project. During the visit, the facilities were inspected and samples from the production environment were taken and analysed for pathogen and spoilage microorganisms. Throughout the year the fellow was also given the opportunity to attend in person and virtually various scientific conferences and webinars to expand his knowledge in the fields of food safety and risk analysis.

- Attended virtually the Recent Advances in Food Analysis (RAFA) 2021 conference.
- Presented in person at the IAFP 2022 scientific conference in Munich on the topic of quantitative risk assessment of food-borne viruses (QVRA).
- Attended virtually the 4th BVL/MRI Course on Food Safety, Food Authenticity and Risk Management
- Followed a presentation by researchers from the Institute Of Technology Of Agricultural Products ITAP of Greece on photometric methods for microbial spoilage assessment.
- Attended virtually the EFSA ONE HEALTH conference 2022.
- Took part in a 10-h intensive QMRA in R workshop, PredMicro2022.
- Participated in the setting up of a QDA sensory profiling of Polish cheeses.
- Took Polish language classes at a Polish Language School for Foreign students.

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Abbreviations

ALOP	Appropriate Level of Protection
CFU	colony forming units
DTU	Danmarks Tekniske Universitet
FAO	Food and Agriculture Organization
FSSP	Food Safety and Spoilage Predictor
HPLC	high-performance liquid chromatography
LAB	lactic acid bacteria
MRS	de Man Rogosa and Sharpe agar
OCLA	Oxoid Chromogenic Listeria Agar
QMRA	quantitative microbiological risk assessment
RA	risk assessment
RASFF	Rapid Alert System for Food and Feed
RTE	ready to eat
uLA	undissociated lactic acid
WHO	World Health Organization

Appendix A – Consumption Questionnaire

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PAŃSTWOWY INSTYTUT BADAWCZY

Questionnaire

The information provided will be used exclusively for research purposes by IBPRS.
Thank you for participating.

Date: ...../...../.....

General Information

- Age: ○ 18 – 30 ○ 31 – 40 ○ 41 – 50 ○ 51 – 60 ○ > 60
- Gender: ○ Male ○ Female
- Number of kids (< 18 years) living at home: .....
- Number of adults living at home: .....
- Employment: ○ Full-time ○ Part-time ○ Remote work ○ Retired ○ Domestic ○ Unemployed
- Education: ○ Elementary school ○ High school ○ Higher education ○ Other (please specify .....)
- Do you or anyone in your household take antacid digestion medication: ○ Yes ○ No
- Do you or anyone in your household have a long-term disease or allergy: ○ Yes ○ No
  If yes, please mention the condition: .....
- Do you or anyone in your household take immunosuppressant medication: ○ Yes ○ No

Q1 Do you work in the food industry or food science field?
○ Yes
○ No

Q2 Who in your household does the food shopping?
○ Always me
○ Mainly me, occasionally someone else
○ Usually someone else, occasionally me
○ Always someone else

Q3 How often do you buy farmer raw milk cheese?
○ Once a month
○ Once a week
○ I don't buy artisanal cheeses
○ Other (please specify .....)

Q4 How much cheese do you usually purchase?
○ < 200 g
○ 200 – 300 g
○ 300 – 400 g
○ 400 – 500 g
○ > 500 g (please specify .....)

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Q5 How long do you store cheese for?

- 1 – 3 days
- 3 – 5 days
- A week
- Other (please specify .....)

Q6 How much cheese do you usually consume in one sitting (portion size)?

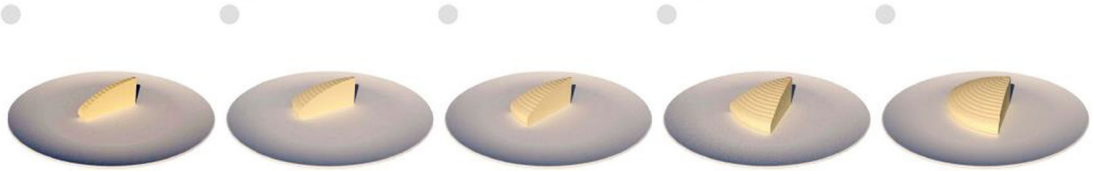

Plate diameter: 27 cm

Q7 Do you check the expiration date on food packaging?

- Always
- Never
- Sometimes

Q8 Where in the fridge do you usually store cheese?

- Door shelf
- Top shelf
- Middle shelf
- Bottom shelf

Q9 How is your refrigerator's temperature regulated?

- Automatically
- Manually

Q10 Do you personally regulate your refrigerator's temperature settings?

- Yes
- No

Q11 How long does it take you to return home from the purchasing point?

- 5 minutes
- 5 – 10 minutes
- 15 – 30 minutes
- > 30 minutes (please specify .....)

Q12 Do you use a special insulated bag when transporting refrigerated products?

- Yes
- No

Q13 Time in: ..... Time out: .....