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Project title: Innovative Bio interventions and Risk Modelling Approaches for Ensuring Microbial Safety and Quality of Mediterranean Artisanal Fermented Foods

Deliverables 6.1 and 6.2: Report on the First Predictive Dynamic Models of the Viability of Pathogens along Processing of Mediterranean Artisanal Fermented Foods and Report on the Optimised Process Variables to Enhance their Microbiological Safety (R, PU; August 2022)

Content

1. Introduction

Deliverable 5.4 outlined relevant information about the fate studies of selected foodborne pathogens conducted on the different artisanal fermented products by the ArtiSaneFood project's consortium; whereas Deliverable 5.5 compiled all raw data of the pathogens' kinetics and accompanying microbiota obtained from such fate studies. The current Deliverables (6.1 and 6.2) meet the objective of Work package 6, which was to build up predictive microbiology models that describe the viability of the selected pathogens in the target artisanal fermented foods (this is, the data obtained from the fate studies); and to provide recommendations to optimise the microbiological safety and stability of those products. To avoid being repetitive, this report does not describe the fate studies conducted nor the methodologies employed by the partners. Such information is provided in Deliverables 5.4 and 5.5.

2. Partner IPB: *alheira* sausage and goat's raw milk cheese

2.1 Description of the predictive microbiology models adjusted to the data from fate studies in the products

Table 1 compiles the data sets of fate studies where predictive microbiology models were adjusted in order to extract the kinetic parameters of pathogens in the two artisanal foods. It is worth mentioning that the inoculated matrices are prototypes produced in the laboratory that resemble the artisanal fermented foods.

Table 1. Data sets of fate studies conducted by IPB and their characteristics

Data set	Artisanal food	Pathogen inoculated	Preservation method	Stage followed
#1	*Alheira* raw sausage	*Staphylococcus aureus*	Ethanolic extract of *Bixa Orellana* L. at 0.0, 0.5%, 1.0% and 1.5%, added to the batter	Maturation for 12 days at 10ºC
#2	*Alheira* raw sausage	*Salmonella* Typhimurium	Mild heat treatment of stuffed sausages (before maturation) at temperatures	Mimicking smoking and heating

			of 63°, 60°, 57° and 54 °C.	
#3	Goat's raw milk cheese	*Staphylococcus aureus*	Ethanolic extract of spearmint at 0.0 and 1.0%, added to the raw milk	Ripening for 12-14 days at 10ºC
#4	Goat's raw milk cheese	*Staphylococcus aureus*	Ethanolic extract of lemon balm at 0.0 and 1.0%, added to the raw milk	Ripening for 12-14 days at 10ºC
#5	Goat's raw milk cheese	*Staphylococcus aureus*	Ethanolic extract of sage at 0.0 and 1.0%, added to the raw milk	Ripening for 12-14 days at 10ºC
#6	Goat's raw milk cheese	*Staphylococcus aureus*	Cocktail of four indigenous lactic acid bacteria with antimicrobial properties, added to the raw milk	Ripening for 12-14 days at 10ºC
#7	Goat's raw milk cheese	*Staphylococcus aureus*	Mild heat treatment of milk (before coagulation) at temperatures of 55 °C, 58 °C, 61 °C, 62.5 °C and 64 °C	Mimicking thermisation of raw milk

Data sets #1, 3, 4 and 5 have in common (1) that they all applied extracts which produced a decay in the foodborne pathogen inoculated during maturation/ripening; and (2) that acidification took place, which was quantified as pH. To each of these data sets (control and treatments), a dynamic model was adjusted that consisted of a log-decay function with tail in differential form as primary model (with parameter D-value), coupled to a secondary model Bigelow equation of D-value as a function of the varying pH (with parameters log D_{ref} at pH 7.0 and z_{pH}), as follows:

$$\frac{dN}{dt} = -kN\left(\frac{1}{1+C_c}\right)\left(1-\frac{N_{res}}{N}\right)$$

$$\frac{dC_c}{dt} = -kC_c$$

$$D = \frac{\ln(10)}{k}$$

$$\log D = \log D_{ref} - \left(\frac{pH - pH_{ref}}{z_{pH}}\right)^2 \quad (1)$$

Herein, N is the population density (CFU/g), k is the inactivation rate (1/day), Cc is the physiological state of the cells, N_{res} is the residual population density (CFU/g), D is the decimal reduction time (day) at the constant temperature T (10 °C) and at the pH of the food matrix, pH_{ref} is the reference pH (set to 7.0), z_{pH} is the distance of pH from pH_{ref} which leads to a ten-fold change in decimal reduction time, and D_{ref} is the decimal reduction time (day) at pH_{ref}.

To mathematically describe **data set #2** , which consisted of experimental curves resembling log-linear decay, the following procedure was used. For every survival curve, at a fixed temperature, the D value [min] was estimated by adjusting the log-linear decay equation:

$$logN(t) = logN_0 - \frac{t}{D}$$

where N(t) is the microbial concentration [CFU/g] at time t [min]; N_0 is the initial microbial concentration [CFU/g], a parameter to be estimated. The inactivation rate, k [1/min] can be estimated as the reciprocal of D. After estimating the D values at the fixed temperature T, the Bigelow secondary model was adjusted to extract the parameters log D_{ref} (i.e., D value at the reference temperature T_{ref} of 50 ºC) and z value (i.e., change in temperature that causes a 10-fold change in the D-value).

$$log\ D = log\ D_{ref} - \frac{(T - T_{ref})}{z} \quad (2)$$

Data set #6 was amenable to be characterised by the Lotka-Volterra competition model, which is described by the following system with two inhibition functions,

$$\frac{1}{STA}\frac{dSTA}{dt} = \mu_{STA}\left(1 - \frac{STA + \alpha_{STA-LAB} \times LAB}{STA_{max}}\right)$$

$$\frac{1}{LAB}\frac{dLAB}{dt} = \mu_{LAB}\left(1 - \frac{LAB}{LAB_{max}}\right) \quad (3)$$

where $\alpha_{STA-LAB}$ is the inhibition or interaction coefficient measuring the effect of lactic acid bacteria (LAB) on *S. aureus*. If $\alpha_{STA-LAB} < 1$, the effect of LAB on *S. aureus* is less than the effect of LAB on its own population.

Data set #7 involved non log-linear decay curves. The modelling approach opted for was the omnibus or global modelling one, in which primary and secondary models are fitted all at once to the data. The log CFU/mL concentration measured at time *i* when subjected to experimental condition *j* was estimated as:

$$Y_{ij} = Y_{0\,j} - \left(\frac{t}{\chi_j}\right)^{\beta_j} + \varepsilon_{ij}$$

$$Y_{0\,j} = Y_{0\,mean} + u_j$$

$$\sqrt{\chi_j} = a_1 + a_2 * Temperature + a_3 * Temperature^2 + v_j$$

$$\sqrt{\beta} = b_1 + b_2 * Temperature + b_3 * Temperature^2 \quad (4)$$

χ and β are the scale and shape parameters of the underlying Weibull distribution, respectively. The scale parameter χ indicates the time for first decimal reduction (minutes), whereas the shape parameter accounts for upward concavity of a survival curve ($\beta < 1$), a linear survival curve ($\beta = 1$), or a downward concavity ($\beta > 1$). Since the initial microbial concentration Y_0 was different between conditions, this variability was accounted for by adding a random-effects term *u*. Another random-effects term *v* was added to the mean of the intercept a_1 of the polynomial expression predicting $\sqrt{\chi}$. This was done because a fraction of the variability in the scale parameter could not be explained solely by its fixed-effect predictor. Hence, the random effects *u* and *v* were assumed to take in random shifts subject to a given condition *j* defined by the inactivation temperature. The two random effects were assumed to follow normal distributions with means zero and covariance matrix $[s_u^2, s_{uv}^2; s_{uv}^2, s_v^2]$. The residual error ε_{ij} followed a normal distribution with mean zero and variance s^2.

2.2. Summary of fitted microbial kinetic parameters

The kinetic parameters of the foodborne pathogens in the two artisanal fermented foods, obtained from the seven fate studies, are compiled in Table 2. Among the plant-based extracts, *Bixa orellana* at 1.0-1.5% presented the highest antimicrobial effect against *S. aureus* in *alheira* sausage during maturation; whereas spearmint at 1.0% was the most effective in reducing *S. aureus* populations in goat's raw milk cheese during ripening.

Table 2. Kinetic parameters of foodborne pathogens in *alheira* raw sausage and goat's raw milk cheese, as affected by biopreservation

Data set	Artisanal food	Pathogen inoculated	Preservation method	Kinetic parameters
#1	*Alheira* raw sausage	*Staphylococcus aureus*	*Bixa orellana* L. at 0.0% at 0.5% at 1.0% at 1.5%	Equation (1) Log D_{ref} =1.036 (SE=0.142) z_{pH}=2.426 (SE=0.561) Log D_{ref} =1.040 (SE=0.294) z_{pH} = 2.144 (SE=0.230) Log D_{ref} =1.040 (SE=1.033) z_{pH}=2.113 (SE=1.033) Log D_{ref} =1.079 (SE=0.303) z_{pH}=2.005 (SE=0.904)
#2	*Alheira* raw sausage	*Salmonella* Typhimurium	Mild heat (smoking and heating) between 54 to 63 ºC	Equation (2) Log D_{ref} =2.302 (SE=0.304) z=5.016 (SE=0.839) ºC.
#3	Goat's raw milk cheese	*Staphylococcus aureus*	Spearmint at 0.0% at 1.0%	Equation (1) Log D_{ref} =0.932 (SE=0.166) z_{pH} =1.727 (SE=0.392) Log D_{ref} =0.621 (SE=0.061) z_{pH} =3.172 (SE=0.660)
#4	Goat's raw milk cheese	*Staphylococcus aureus*	Lemon balm at 0.0%	Equation (1) Log D_{ref} =0.996 (SE=0.056) z_{pH} =1.851 (SE=0.007)

			at 1.0%	Log D_{ref} =1.189 (SE=0.200) z_{pH} =2.339 (SE=0.835=
#5	Goat's raw milk cheese	*Staphylococcus aureus*	Sage at 0.0% at 1.0%	Equation (1) Log D_{ref} =0.796 (SE=0.068) z_{pH} =2.054 (SE=0.131) Log D_{ref}=0.996 (SE=0.278) z_{pH}=2.006 (SE=0.677)
#6	Goat's raw milk cheese	*Staphylococcus aureus*	Selected LAB cocktail Without cocktail With cocktail	Equation (3) LAB=15.16 (SE=0.237) STA=9.538 (SE=0.197) μ_{LAB}=1.219 (SE=0.177) LAB_{max}=18.83 (SE=0.117) μ_{STA}= 0.138 (SE=0.216) STA_{max}=9.452 (SE=0.470) LAB=16.35 (SE=0.155) STA=9.935 (SE=0.163) μ_{LAB}=1.059 (SE=0.089) LAB_{max}=20.40 (SE=0.076) μ_{STA} = 0.094 (SE=0.504) STA_{max}=9.581 (SE=2.582) $\ln(\alpha_{STA-LAB})$=0.491 (SE=0.038)
#7	Goat's raw milk cheese	*Staphylococcus aureus*	Mild heat treatment of milk (between 55 and 64 °C)	Equation (4) Predictors of $\sqrt{\chi}$ a1 =325.0 (SE=32.58) a2 =-10.25 (SE=1.099) a3 =0.081 (SE=0.009) Predictors of $\sqrt{\beta}$ b1=67.09 (SE=19.91) b2=-2.183 (SE=0.671) b3=0.018 (SE=0.006)

2.3 Optimised process variables to enhance the microbiological safety of the food products

In *alheira* raw sausages, the addition of selected extracts to the batter has a beneficial effect in controlling the growth of *S. aureus* during maturation. When the *alheiras* were not formulated with extracts (i.e., control), the inoculated concentration of *S. aureus* (5.0 log CFU/g)

persisted throughout maturation despite acidification and dehydration. At the 12th day of maturation, sage extract added in *alheira* batter at a dose between 0.5-1.0% produced an average reduction of *S. aureus* counts of 1.146 log CFU/g (SE=0.065 log CFU/g; results not shown in Table 2). Extracts of *Bixa orellana* L. added to *alheira* batter in 0.50, 1.00 and 1.50% caused a comparable level of drop in *S. aureus* mean concentration, which was gradually higher at 1.05, 1.20 and 1.35 log CFU/g, respectively, on the 12th day of maturation. It is therefore expected that further inactivation is attained during shelf-life. In addition to a reformulation of the raw sausage with these extracts, it is proposed that a mild heat treatment be carried out right after smoking. It was estimated that for inactivation of Salmonella Typhimurium in one-log in the geometric centre of the sausage, a temperature of 57ºC must be kept for 7.9 minutes in the geometric centre of the sausage, or alternatively 3.7 minutes at 60 ºC. Higher temperatures would induce undesirable organoleptic changes. These outcomes are very relevant to local producers since these two pathogens are often introduced to *alheiras*: *Salmonella* spp. through raw meats, and *S. aureus* through inadequate handling during mixing and stuffing, in particular when *alheiras* are artisanally produced.

In the case of the goat's raw milk cheese, the addition of the indigenous lactic acid bacteria cocktail as a starter culture reduced only marginally the time needed for one-log decrease, and in practice corresponded to a reduction of 0.50 log CFU/g after 14 days of maturation. Among the extracts, spearmint extract was the most effective. The three extracts in doses of 1.0% significantly decreased the time to achieve one log reduction, which in practical terms corresponded to a reduction of 0.63 log CFU/g (sage), 0.67 log CFU/g (lemon balm) and 1.38 log CFU/g (spearmint) by the end of maturation, in contrast to the control groups, where a mean *S. aureus* decay of 0.33 log CFU/g was observed by the end of maturation. Depending on the initial concentration *of S. aureus* in raw milk, it is also proposed that thermisation be applied for a very short time. This milk treatment markedly reduces the number of spoilage bacteria, and, in the case of *S. aureus*, the log reduction is such that toxin formation in the cheese, which requires a microorganism count greater than 5 log CFU/g, is highly unlikely. At the same time, thermisation causes minimum collateral heat damage to milk constituents and milk renneting properties, mild effect on the raw milk flora and the functionality of milk caseins and salts, and reduced impact on the sensory profile of the final cheeses. Since the heat load is lower compared

to that used in pasteurisation, enzymes involved in cheese flavour development, such as lipoprotein lipase, are less inactivated, thus avoiding changes in ripening and in aroma and flavour improvement of the cheese. According to the model adjusted, the time to reach first decimal reduction of *S. aureus* in goat's raw milk at 58 ºC is 9.7 min; where the first decimal reduction is attained in 0.57 min at 61 ºC. The omnibus model predicting the inactivation of *S. aureus* by thermisation contributes to the body of work using predictive microbiology to describe pathogen heat-inactivation in goat's raw milk, which is scarce, particularly if pasteurisation is a treatment disregarded for this traditional food product.

3. Partner UCO: *Soft cheese*

3.1 Description of the predictive microbiology models adjusted to the data from fate studies in the product

We conducted an in-house study to develop a mathematical model that characterises the responses of *S. aureus* in cheeses. To gather data, we used a Bioscreen C equipment and employed a full factorial design (2x4) to investigate the impact of temperature (10, 17, 24, and 30 °C) and sodium chloride concentration (NaCl; 0, 1.5, 3, 4.5% w/v) on the behavior of *S. aureus* in modified M-17 broth. By constructing calibration curves, we were able to convert absorbance values (OD) into counts (log CFU/mL). Each experimental condition was replicated five times, resulting in a total of 80 growth curves. We fitted the growth curves using the model proposed by Baranyi and Roberts (1994) (Equation 5) to estimate the growth kinetic parameters of *S. aureus* under various conditions.

$$y(t) = y_0 + \mu_{max}t - \frac{1}{\mu_{max}} ln\left(e^{-vt} + e^{-h_0} - e^{-vt-h_0}\right) - \frac{1}{m} ln\left(1 + \frac{e^{m\mu_{max}t+\frac{1}{\mu_{max}}\ln\left(e^{-vt}+e^{-h_0}-e^{-vt-h_0}\right)-1}}{e^{m(y_{max}-y_0)}}\right) \quad (5)$$

where y_0 and y_{max} are the natural logarithm of the initial number of cells and maximum population density, respectively; μ_{max} is the maximum specific growth rate; m is the curvature parameter to characterize the transition from exponential to stationary phase; v is the curvature parameter to characterize the transition to the exponential phase; h_0 is a dimensionless parameter quantifying the initial physiological state of the cells. From that, the lag time can be determined as follows: $\lambda = h_0/\mu_{max}$.

The secondary model of Ratkowsky (Equation 6) was applied to relate *S. aureus* growth rates, estimated by fitting the Baranyi and Roberts model to the kinetic curves, with temperature.

$$\sqrt{\mu_{max}} = b\ x\ (T - T_{min}) \quad (6)$$

where b is the slope of the line and T_{min} is the minimum theoretical growth temperature of *S. aureus*. The developed model was further calibrated to predict the *S. aureus* in soft cheeses, using growth data extracted from the ComBase database (https://www.combase.cc/index.php/es/). By doing so, a calibration factor (F_c) was determined, so that by multiplying Equation 6 by F_c, the model became suitable to predict changes in *S. aureus* numbers in soft cheese (Burgos type) during storage at different temperatures.

On the other hand, it was aimed at developing a *L. monocytogenes* growth model in artisanal fresh goat cheeses at different storage temperatures and on its application to assess the exposure of consumers to the pathogen during the product shelf-life. The effect of storage temperature (4-25ºC) for up to 18 days on *L. monocytogenes* was evaluated in a lab-scale goat fresh cheese initially inoculated with a three-strain cocktail of the pathogen (ca. 2-3 log CFU/g). To describe the relationship between *L. monocytogenes* concentration with growth rate (μ_{max}, log CFU /d), storage temperature and time, the Baranyi and Ratkowsky models were fitted to the collected growth data following a one-step approach (Equations 5-6).

Finally, to evaluate the effect of the addition of LAB starter cultures, a Weibull inactivation model was fitted to describe the decay in *L. monocytogenes* in goat milk fresh cheese stored at room temperature (Equation 7).

$$logS(t) = -\left(\frac{t}{\delta}\right)^{p} \quad (7)$$

Where δ is the time parameter or time of first decimal reduction, i.e., the time necessary to reduce the initial population (N_0) to $N_0/10$ (δ corresponds to the time when log S(t)= −1) and p is the equivalent of n, i.e., the shape parameter.

3.2. Summary of fitted microbial kinetic parameters

The kinetic parameters of the foodborne pathogens in the artisanal fermented foods, obtained from the fate studies, are compiled in Table 3.

Table 3. Kinetic parameters of foodborne pathogens in cheeses at different conditions.

Data set	Artisanal food	Pathogen inoculated	Preservation method	Kinetic parameters
#1	Cheese simulation medium	*Staphylococcus aureus*	Refrigeration	Equation (6) b = 0.0153 ± 0.0005 log CFU/d.°C T_{min} = 2.37 ± 0.66 °C
#2	Soft cheese	*Staphylococcus aureus*	Refrigeration	Equation (6) F_c = 0.81 b = 0.0153 ± 0.0005 log CFU/d.°C T_{min} = 2.37 ± 0.66 °C
#3	Goat's milk fresh cheese	*L. monocytogenes*	Refrigeration	Equation (6) b = 0.057 ± 0.006 log CFU/d.°C T_{min} = -4.7 ± 1.1 °C
#4	Goat's milk fresh cheese	*L. monocytogenes*	Addition of LAB starter cultures	Equation (7) δ = 4.84 ± 0.77 d p = 1.52 ± 0.29 logN_0 = 4.64 ± 0.17 log CFU/g

3.3 Optimised process variables to enhance the microbiological safety of the food products

In the light of the results of the fate studies, for the cheese simulation medium, the tested NaCl concentrations (0, 1.5, 3, 4.5% w/v) did not affect *S. aureus* behaviour, which indicate that a salt reduction in this type of products would not affect its safety with regards to the presence of *S. aureus*. Since *S. aureus* growth rates are reduced at low temperatures, we recommend lowering down the storage temperatures of soft cheeses to values close to the minimum temperature for *S. aureus* growth, to delay the growth of the pathogen.

Regarding *L. monocytogenes*, the μ_{max} showed a positive correlation with temperature, with values ranging from 0.296±0.023 to 2.122±0.466 log CFU/d from 4-25ºC. The estimated secondary model parameters were b=0.057±0.006 and T_{min}= -4.7±1.1ºC and the global model showed a good fit to data (RMSE=0.849). The addition of microbial starter cultures led to a reduction of *L. monocytogenes* population of more than 2 log CFU/g during a 10-d storage, thus, showing a promising biopreservation strategy in this type of artisanal cheeses.

Nevertheless, the results confirm that the distribution chain conditions of fresh goat cheeses enable *L. monocytogenes* growth over their shelf-life. Hence, focused control measures, as reinforcing manufacturing, cleaning and disinfection practices and strict storage temperature controls, should be implemented to reduce the *L. monocytogenes* consumer's exposure.

4. Partner AUA: *Katiki* cheese and *Noumboulo* sausage

4.1 Description of the predictive microbiology models adjusted to the data from fate studies in the products

Table 4 compiles the data sets of fate studies where predictive microbiology models were adjusted in order to extract the kinetic parameters of pathogens in the two artisanal foods.

Table 4. Data sets of fate studies conducted by AUA and their characteristics

Data set	Artisanal food	Pathogen inoculated	Preservation method	Stage followed
#1	*Katiki* cheese	*3 strain cocktail of Salmonella* Typhimurium	Oregano essential oil (OEO) at 0.0, 0.44% and 0.88%, added to the final product	Mimic storage conditions at 7ºC
#2	*Katiki* cheese	3 strain cocktail of *Salmonella* Typhimurium	Oregano essential oil (OEO) encapsulated into β-cyclodextrins at 0.0 and 0.88%, added to the final product	Mimic storage conditions at 7ºC
#3	*Katiki* cheese	3 strain cocktail of *Salmonella* Typhimurium	Oregano essential oil (OEO) encapsulated into liposomes at 0.0 and 0.44% added to the final product	Mimic storage conditions at 7ºC
#4	*Katiki* cheese	3 strain cocktail of *Listeria monocytogenes*	Oregano essential oil (OEO) at 0.0 and 0.88%, added to the final product	Mimic storage conditions at 7ºC
#5	*Katiki* cheese	3 strain cocktail of *Listeria monocytogenes*	Oregano essential oil (OEO) encapsulated at two ratios (1:99 & 8:92) into β-cyclodextrins at 0.0 and 0.88%, added to the final product	Mimic storage conditions at 7ºC
#6	*Katiki* cheese	3 strain cocktail of *Listeria monocytogenes*	Oregano essential oil (OEO) encapsulated into liposomes at 0.0 and 0.88% added to the final product	Mimic storage conditions at 7ºC
#7	*Noumboulo* sausage	3 strain cocktail of *Listeria monocytogenes*	Oregano essential oil (OEO) incorporated into edible films (WPI: Whey protein isolate) at 0.0, 1.5% and 2.5% and used to the final product	Mimic post-processing contamination of slices when stored under vacuum at 10ºC

All treatments (Data set 1 to 7) were applied in the final products in order to study the behaviour of the selected pathogens as post-processing contamination, during normal storage conditions. The majority of the experimental curves presented a sigmoidal behavior, with an initial shoulder, followed by a maximum inactivation rate (k_{max}) period with or without a final tailing tendency. To those curves the Geeraerd model (Equation 1) was applied.

$$Log_{10}(N_t) = Log_{10}(N_0) + Log_{10}\left\{(1-f)\exp(-k_{sens}t)\frac{\exp(k_{sens}t_s)}{1+[\exp(k_{sens}t_s)-1]\exp(-k_{sens}t)} + f exp(-k_{res}t)\left[\frac{\exp(k_{sens}t_s)}{1+[\exp(k_{sens}t_s)-1]\exp(-k_{sens}t)}\right]^{\frac{k_{res}}{k_{sens}}}\right\} \quad (8)$$

Where k_{sens} stands for the inactivation rate of the sensitive subpopulation of microorganisms at the current environmental conditions, k_{res} stands for the inactivation rate of the more resistant subpopulation of microorganisms at the current environmental conditions, and *ts* stands for the shoulder parameter.

When the experimental curves resembled a log-linear decay, the Bigelow model was applied (Equation 9). In brief, Bigelow model assumes that the microbial inactivation for a constant temperature follows a first order kinetics reaction; and, therefore, a linear relation exists between the logarithm of the number of microorganisms (*N*) and the time (*t*) as shown in Equation 9.

$$\log_{10} N(t) = \log_{10} N_0 - \frac{1}{D_T}t \quad (9)$$

The parameter D(T) (also named D-value) equals the negative inverse of the slope of the line.

4.2. Summary of fitted microbial kinetic parameters

The kinetic parameters of the foodborne pathogens in the two artisanal fermented foods, obtained from the seven fate studies, are compiled in Table 5.

Table 5. Kinetic parameters of foodborne pathogens in *katiki cheese* and *noumboulo* sausage, as affected by biopreservation

Data set	Artisanal food	Pathogen inoculated	Preservation method	Kinetic parameters
#1	Katiki cheese	3 strain cocktail of *Salmonella* spp.	Oregano essential oil (OEO) at 0.0%	Equation 8 D = 2.48 ±0.83

				t_s = 1.08 ±1.74
			at 0.44%	D = 3.04±0.45 t_s = -0.86 ±1.03
			at 0.88%	D = 2.26±0.27 t_s = -3.41 ±1.12
#2	*Katiki* cheese	3 strain cocktail of *Salmonella* spp.	Oregano essential oil (OEO) encapsulated into β-cyclodextrins	Equation 8
			at 0.0%	D = 2.48 ±0.83 t_s = 1.08 ±1.74
			at 0.88%	D = 4.19±0.16 t_s = -5.5 ±0.51
#3	*Katiki* cheese	3 strain cocktail of Salmonella spp.	Oregano essential oil (OEO) encapsulated into liposomes	Equation 8
			at 0.0%	D = 6.27 ±1.72 t_s = -8.41 ±4.12
			at 0.44%	D = 2.11±0.38 t_s = 1.16±0.63
#4	*Katiki* cheese	3 strain cocktail of *Listeria monocytogenes*	Oregano essential oil (OEO)	Equation 8
			at 0.0	D = 6.12 ±0.99 t_s = 3.89 ±1.01
			at 0.88%	D = 4.73±1.08 t_s = 6.24±1.23
#5	*Katiki* cheese	3 strain cocktail of *Listeria monocytogenes*	Oregano essential oil (OEO) encapsulated at two ratios (1:99 & 8:92) into β-cyclodextrins	Equation 8
			at 0.0%	D = 6.12 ±0.99 t_s = 3.89 ±1.01
			bCDs (1:99) at 0.88%	Eq. 8 : D= 26.00±1.97
			bCDs (8:92) at 0.88%	Eq. 7 : D= 5.32±0.69

				t_s = 4.28 ±0.92
#6	*Katiki* cheese	3 strain cocktail of *Listeria monocytogenes*	Oregano essential oil (OEO) encapsulated into liposomes at 0.0% at 0.88%	Equation 8 D = 6.12 ±0.99 t_s = 3.89 ±1.01 D = 5.19±1.08 t_s = 4.01±1.03
#7	*Noumboulo* sausage	3 strain cocktail of *Listeria monocytogenes*	Oregano essential oil (OEO) incorporated into edible WPI films at 0.0% at 1.5% at 2.5%	Equation 8 D = 32.46 ±5.46 D= 23.16±5.57 D= 17.86±3.21

4.3 Optimised process variables to enhance the microbiological safety of the food products

In *katiki* cheese, regardless of the application method of OEO (as free OEO, or encapsulated in liposomes or β-cyclodextrins), *Salmonella* spp. appeared to be more sensitive compared to *L. monocytogenes*. Incorporation of OEO into different inclusion complexes such as β-cyclodextrins and/or liposome, exhibited different antimicrobial activity against the pathogens tested. More specifically, OEO at 0.88% (v/v) reduced *L. monocytogenes* in *katiki* cheese, in the following order of rate and magnitude of log reductions: free OEO at 0.88% > OEO at 0.88% in β-CDs at the 8:92 ratio>>OEO at 0.88% in liposomes. *Salmonella* populations were reduced by OEO in *katiki* cheese, as follows:: Free OEO at 0.88% > OEO at 0 .88% in β-CDs at the 8:92ratio > OEO at 0.88% in β-CDs at the1:99 ratio > OEO at 0.88% in liposomes. The results are indicative of the efficiency of encapsulated OEO as a means to control pathogens in *katiki* cheese.

In the case of *noumboulo* sausage, incorporation of OEO into edible WPI films was able to cause a 2 log reduction in the levels of *L. monocytogenes,* regardless of the OEO concentration. D values (Table 5) were found to depend on the OEO concentration incorporated

into the film. The higher the OEO concentration, the lower the D value. The results are indicative of the potential edible films to increase product safety during storage.

5. Partner UNIBO: *Squacquerone* cheese

5.1 Description of the predictive microbiology models adjusted to the data from fate studies in the products

Aim of the fate studies: To assess the ability of the food to support the growth of *L. monocytogenes*, knowing that the food supports the growth if a growth potential higher than 0.5 log CFU/g is observed. The parameters are given in Table 1.

Table 6. Results of the fate studies of *L. monocytogenes* in Squacquerone cheese

	Log10 cfu/g															
Hours B1–B2	**0**	**24**	**72**	**144**	**216**	**312**	**384**									**Δ**
Batch 1 (3°C)	3,38	3,03	3,63	3,63	3,11	3,55	3,22									0,25
Batch 2 (10°C)	3,26	2,70	3,66	3,66	3,21	3,63	3,21									0,40
Batch 3 (10°C)	3,07	3,55	3,25	3,44	3,07	2,82	2,59	2,90	3,01	2,97	2,84	2,84	2,90	2,95	2,85	0,48
Hours B2	**0**	**144**	**168**	**192**	**216**	**240**	**264**	**288**	**312**	**336**	**360**	**408**	**432**	**456**	**480**	
Standard deviation	0,16															

Target inoculation level: 3 log10 cfu/g.

Limit of quantification of the method used: 1 log CFU/g.

5.2 Summary of fitted microbial kinetic parameters

The standard deviation of the log counts at Time 0 was below the limit of 0.30. Therefore, all inoculations are acceptable. The estimated growth potential was 0.448; corresponding to the maximum value observed for batch 3. This is lower than the decision criterion of 0.5 log CFU/g.

5.3 Optimised process variables to enhance the microbiological safety of the food products

In the light of the results of the fate studies, the food does not support growth under the time/temperature conditions of storage assessed.

6. Partner Anses/CNIEL: *Camembert* cheese

Growth tests were carried out for the two microbial species (*L. monocytogenes* and *Salmonella*) on soft raw milk cheeses according to the PDO specifications. The behaviour of four different strains was studied during the production, maturation and microbiological life span (MLS) of the cheeses. These tests were performed in pilot at INRAE Aurillac by ACTALIA according to the ISO 20976-1:2019 standard. The pathogens were inoculated into the milk used to make the cheeses at approximately 100 CFU/mL. For each of the strains, three production batches were monitored and three tests were carried out per production at each enumeration point were performed.

6.1 Description of the predictive microbiology models adjusted to the data from fate studies in the product

Different types of primary and secondary models were used to describe the behaviour of the two pathogens during the cheese processing and storage.

For the primary growth model, the logistic growth model was used:

$$\begin{cases} \ln(x_0) & ,t \le lag \\ \ln(x_{\max}) - \ln\left(1 + \left(\frac{x_{\max}}{x_0} - 1\right) \cdot \exp\left(-\mu_{\max} \cdot (t - lag)\right)\right) & ,t > lag \end{cases} \quad (10)$$

For *Salmonella* and *Listeria monocytogenes*, the secondary cardinal model of Rosso was used for growth rate (Augustin et al., 2005). It takes into account the effect of temperature, pH, water

activity and concentration of undissociated lactic acid. Interaction between environmental factors (γ(int)) was taken into account according to Augustin et al. (2005).

$$\mu_{max} = \mu_{opt} \cdot \gamma(T) \cdot \gamma(pH) \cdot \gamma(a_w) \cdot \gamma(LAC) \cdot \gamma(int)$$

with

$$\gamma(T) = \frac{(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})\left[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})\left((2-1)T_{opt} + T_{min} - 2T\right)\right]}$$

$$\gamma(pH) = \frac{(pH - pH_{max})(pH - pH_{min})}{(pH_{opt} - pH_{min})(pH - pH_{opt}) - (pH_{opt} - pH_{max})(pH_{min} - pH)}$$

$$\frac{(aw - aw_{max})(aw - aw_{min})^2}{(aw_{opt} - aw_{min})\ \ [(aw_{opt} - aw_{min})(aw - aw_{opt}) - (aw_{opt} - aw_{max})(aw_{opt} + aw_{min} - 2aw)]}$$

$$\gamma(LAC) = 1 - \left(\frac{c}{CMI}\right)^{\alpha} \qquad (11)$$

Only μ_{opt} values were fitted to challenge-test data.

For *L. monocytogenes*, the primary model used the individual cell growth probability taken from Augustin & Czarnecka-Kwasiborski (2012). The model used to assess the growth probability during ripening once the conditions get favourable to growth. It is determined as follows:

$$p(T, \mathrm{pH}, a_w) = p(T) \cdot p(\mathrm{pH}) \cdot p(\mathrm{a_w}) \text{ with}$$

$$p(T) = \begin{cases} 0, & T \le T_{\mathrm{inf}} \\ \dfrac{\exp(T/c) - \exp(T_{\mathrm{in}}/c)}{\exp\left(T_{\mathrm{sup}}/c\right) - \exp(T_{\mathrm{inf}}/c)}, & T_{\mathrm{inf}} < T < T_{\mathrm{sup}} \\ 1, & T \ge T_{\mathrm{sup}} \end{cases}$$

$$p(\mathrm{pH}) = \begin{cases} 0, & \mathrm{pH} \le \mathrm{pH_{inf}} \\ \dfrac{\exp(-\mathrm{pH}) - \exp(-\mathrm{pH_{inf}})}{\exp(-\mathrm{pH\ sup}) - \exp(-\mathrm{pH_{inf}})}, & \mathrm{pH_{inf}} < \mathrm{pH} < \mathrm{pH_{sup}} \\ 1, & \mathrm{pH} \ge \mathrm{pH_{sup}} \end{cases}$$

$$p(a_w) = \begin{cases} 0, & a_w \leq a_{w,\,inf} \\ \dfrac{a_w - a_{w,inf}}{a_{w,sup} - a_{w,inf}}, & a_{w,\,inf} < a_w < a_{w,sup} \\ 1, & a_w \geq a_{w,sup} \end{cases} \quad (12)$$

No fitting of model parameters was carried out for growth probability (default parameters from the original paper were used).

For *Salmonella* during ripening and storage, the inactivation was modelled using a Weibull primary model:

$$log_{10}\big(N(t)\big) = log_{10}(N_0) - \left(\frac{t}{\delta}\right)^p \quad (13)$$

Delta and p values were adjusted on challenge-test data set.

6.2. Summary of fitted microbial kinetic parameters

The kinetic parameters of the foodborne pathogens in the cheese, obtained from the different fate studies, are compiled in Table 8.2.1.

Table 7. Synthesis of kinetic parameters of foodborne pathogens in Camembert de Normandy cheese

Data set	Artisanal food	Pathogen inoculated	Preservation method	Parameters
#1	Soft cheese	*Listeria monocytogenes*	Milk maturation, ripening conditions and storage	Equation (12) mu_{opt}(h-1) (1 value per batch) L_mono_AER_101: 0.69, 0.45, 0.52 L_mono_UNIR_100: 0.67, 0.46, 0.51
#2	Soft cheese	*Salmonella*	Milk maturation	Equation (11) mu_{opt}(h-1) (1 value per batch)

<table>
<tr><td></td><td></td><td></td><td></td><td>Salmonella Dublin: 1.00, 1.01, 1.04
Salmonella Mbandaka: 0.99, 1.00, 1.07</td></tr>
<tr><td>#2</td><td>Soft cheese</td><td>Salmonella</td><td>Ripening and storage</td><td>Equation (13)
<table>
<tr><td></td><td></td><td>Core</td><td></td><td></td></tr>
<tr><td>Strain</td><td>Batch</td><td>Parameters</td><td>best fit</td><td>Inf</td></tr>
<tr><td></td><td></td><td>p</td><td>0.274</td><td>0.146</td></tr>
<tr><td>S_Dublin</td><td>1</td><td>log_(10)(delta)</td><td>1.461</td><td>0.481</td></tr>
<tr><td>S_Dublin</td><td>2</td><td>log_(10)(delta)</td><td>1.481</td><td>0.478</td></tr>
<tr><td>S_Dublin</td><td>3</td><td>log_(10)(delta)</td><td>1.444</td><td>0.449</td></tr>
<tr><td>S_Mbandaka</td><td>1</td><td>log_(10)(delta)</td><td>1.456</td><td>0.324</td></tr>
<tr><td>S_Mbandaka</td><td>2</td><td>log_(10)(delta)</td><td>1.469</td><td>0.473</td></tr>
<tr><td>S_Mbandaka</td><td>3</td><td>log_(10)(delta)</td><td>1.419</td><td>0.353</td></tr>
</table>
<table>
<tr><td></td><td></td><td></td><td>Rind</td><td></td><td></td></tr>
<tr><td>Strain</td><td>Batch</td><td>Sup</td><td>best fit</td><td>Inf</td><td>Sup</td></tr>
<tr><td></td><td></td><td>0.403</td><td>2.744</td><td>0.521</td><td>4.967</td></tr>
<tr><td>S_Dublin</td><td>1</td><td>2.441</td><td>3.141</td><td>2.945</td><td>3.336</td></tr>
<tr><td>S_Dublin</td><td>2</td><td>2.485</td><td>3.15</td><td>2.982</td><td>3.319</td></tr>
<tr><td>S_Dublin</td><td>3</td><td>2.44</td><td>3.134</td><td>2.981</td><td>3.288</td></tr>
<tr><td>S_Mbandaka</td><td>1</td><td>2.588</td><td>3.19</td><td>3.061</td><td>3.319</td></tr>
<tr><td>S_Mbandaka</td><td>2</td><td>2.465</td><td>3.165</td><td>3.074</td><td>3.257</td></tr>
<tr><td>S_Mbandaka</td><td>3</td><td>2.484</td><td>3.181</td><td>3.077</td><td>3.285</td></tr>
</table></td></tr>
</table>

6.3 Optimised process variables to enhance the microbiological safety of the food products

According to PDO specifications, processing conditions are not the main levers to control the fate of the pathogens during processing. Control of the microbiological quality of raw milk as well as batch testing for the presence of the pathogen are more important to address to reduce risk.

7. Partner ISBST-UMA: Fermented cow milk *(Lben)* and Dried fermented sausage *(*Dry *Merguez)*

7.1 Description of the predictive microbiology models adjusted to the data from fate studies in the products.

Table 8 compiles the data sets of fate studies where predictive microbiology models were adjusted to the kinetic parameters of pathogens in the Tunisian dry fermented sausages (Dry *Merguez*) and fermented cow milk (*Lben*). It is noteworthy mentioning that the inoculated matrices are prototypes produced in the laboratory that resemble the artisanal fermented foods.

Table 8. Data sets of fate studies conducted and their characteristics

Data set	Artisanal food	Pathogen inoculated	Preservation method	Stage followed
#1	Dry sausages (*Merguez*)	*Listeria monocytogenes*	Ethanolic extract of Tunisian mint at 1×MIC, 3×MIC, 6×MIC, added to the batter	Maturation + drying for 16 days at 27°C
#2	Dry sausages (*Merguez*)	*Listeria monocytogenes*	Adjusting the maturation conditions	Maturation + drying for 16 days at 27°C
#3	Dry sausages (*Merguez*)	*Listeria monocytogenes*	Adjusting the maturation condition. Ethanolic extract of mint at 0.49 % added to the batter	Maturation +drying for 16 days at 27°C
#4	Fermented cow milk (*Lben*)	*Listeria monocytogenes*	Raw milk pasteurisation and *L. paracasei* isolated lactic acid bacteria used for milk fermentation	Storage for 7 days at 4°C
#5	Fermented cow milk (*Lben*)	*Listeria monocytogenes*	Raw milk pasteurisation and *L. paracasei* isolated lactic acid bacteria used for milk fermentation supplemented with ethanolic extract of lemon citrus at 2×MIC (added to fermented milk before storage)	Storage for 7 days at 4°C

Data sets #1 and 2 applied extracts + controls which produced two phases of behaviour of the foodborne pathogen during maturation/drying as the water activity decreased progressively: a growth phase and inactivation phase. The first data sets (control and treatments) were adjusted using the Huang model (Huang, 2008) which was chosen as the primary model characterising the growth of *L. monocytogenes* in sausages during drying, whereas the Weibull model was chosen for inactivation modelling. The effect of pH and a_w was considered in the secondary models but not developed in this part. Thus, the primary models are described by equations (14) and (15):

Growth phase Huang model

$$N(t) = N0 + Nmax - ln\{exp(N0) + [exp(Nmax) - exp(N0)] \times exp[-k \times exp(Nmax)B(t)]\},$$

where $B(t) = t + 1\,\alpha\, ln\, 1 + exp[-\alpha(t - \lambda)]\, 1 + exp(\alpha\,\lambda)$

(14)

Initial population N_0 is expressed in $\log_{10}$ (CFU/g), maximum population N_{max} expressed in $\log_{10}$ (CFU/g), Maximum growth rate μ_{max} expressed in $\log_{10}$ (CFU/g) h^{-1} and lag time ʎ expressed in h. The variable α is the rate constant for the exponential phase. It is not the specific growth rate, and has the unit of $(CFU/g{\cdot}h)^{-1}$.

Inactivation phase Weibull model:

$$Log(N) = Log(N0) - \left(\frac{t}{\chi}\right)^{\beta}$$

(15)

where Log(N) is the *Listeria monocytogenes* concentration at given time, Log(N_0) is the average value of the initial *Listeria monocytogenes* concentration of three replicates at time zero of maturation and drying), χ is the time (hours) required for the first log reduction of *Listeria monocytogenes*, β is a dimensionless parameter describing the shape of the inactivation curve (i.e. $\beta < 1$ concave; $\beta = 1$ linear and $\beta > 1$ convex) and t is the time (hours).

The unknown kinetic parameters were estimated by least-squares optimization, using the 'nlme' library implemented in the R software. The goodness-of-fit of the developed models was

assessed by standard error of the parameter estimates, residual sum of squares (RSS), root mean squared errors (RMSE).

Data set #3: the experimental data produced a log decay in the foodborne pathogen therefore only the Weibull model was applied (Equation 15).

For **data sets #4 and #5,** the experimental data produced a log decay during the whole storage phase of fermented cow milk (*Lben*), which was produced with a starter culture previously isolated from fermented milk sequenced and identified as *L. paracasei* and supplemented or not a citrus lemon extract (2×MIC) and then inoculated during storage phase with the pathogen *L. monocytogenes*. Two models: Weibull and Baranyi and Roberts models were used to mathematically fit the growth of the pathogen *L. monocytogenes* inoculated in fermented milk.

The first primary model "Weibull model" is previously shown in Equation (15). Three parameters (initial concentration Log(N_0) (log CFU/ml), χ time required for first tenfold reduction (hour), and curvature parameter β) were fitted.

Additionally, the complete Baranyi and Roberts model (Equation (16) was also fitted to the experimental results using DMFit version 3.5 Excel add-in (ComBase, Australia, https://www.combase.cc/index.php/en/).

$$\mathrm{Log\ N(t)} = \log \mathrm{N_0} + \frac{1}{\mu_{max}} \mathrm{x} \left[\mathrm{time} + \frac{1}{\mu_{max}} \mathrm{x\ Ln} \left(\frac{\exp(-\mu_{max} \mathrm{x\ time} + q_0)}{1 + q_0} \right) \right]$$
$$- \frac{1}{\log(10)} \mathrm{x\ Ln} \left[1 + \frac{\exp\left(\mu_{max} \mathrm{x} \left[\mathrm{time} + \frac{1}{\mu_{max}} \mathrm{x\ Ln} \left(\frac{\exp(-\mu_{max} \mathrm{x\ time}) + q_0}{1 + q_0} \right) \right] \right) - 1}{\exp\left(\log(\mathrm{N_{max}}) - \log(\mathrm{N_0}) \right)} \right] \quad (16)$$

Where N (t) is the population at time t (CFU)/mL; N_0 is the initial value of CFU/mL (scaled logarithmically), Nmax is the maximum value of CFU/mL (scaled logarithmically), µmax is the maximum specific growth rate 1/h; q_0 is a constant related to λ (lag phase), where λ=q_0/µmax.

For this model, four main parameters (initial concentration, lag/shoulder, maximum growth rate, final concentration) were determined.

7.2. Summary of fitted microbial kinetic parameters.

The kinetic parameters of the foodborne pathogens in the two artisanal fermented foods, obtained from the fifth data sets are compiled in Table 9.

Table 9. Kinetic parameters of foodborne pathogens in dry sausages (*Merguez*) and fermented cow milk (*Lben*)

Data set	Artisanal food	Pathogen inoculated	Preservation method	Kinetic parameters	
				Growth Equation (1)	**Inactivation Equation (2)**
#1	Dry sausages	*Listeria monocytogenes*	Mint extract at 0.0%	$Y0$ =12.47 (SE=0.23) $Ymax$ =17.36 (SE=0.163) μ_{max}= 13.34 (SE=0.603) λ = 2.690 (SE=0.356)	$log\ N0$ =7.80 (SE=0.422) χ =223.2 (SE=14.52) β =0.86 (SE=0.211)
			at MIC%	$Y0$ =11.87(SE=0.031) $Ymax$ =17.48(SE=0.022) μ_{max}= 3.07(SE=0.172) λ = 1.15 (SE=0.053)	$log\ N0$ =7.59 (SE=0.05) χ =221.8 (SE=17.28) β =0.88 (SE=0.25)
			at 3×MIC%	$Y0$ =11.82(SE=0.032) $Ymax$ =16.93(SE=0.022) μ_{max}= 3.80 (SE=0.171) λ = 1.248 (SE=0.05)	$log\ N0$ =7.52 (SE=0.024) χ =222.5 (SE=17.04) β =0.87(SE=0.025)
			at 6×MIC%	$Y0$ =10.96 (SE=0.292) $Ymax$ = 16.48 (SE=0.21) μ_{max}= 2.36 (SE=0.439) λ = 0.490(SE=0.34)	$log\ N0$ =7.06 (SE=0.548) χ =173.04 (SE=20.4) β =0.85 (SE=0.028)
#3	Dry sausages	*Listeria monocytogenes*	Modification at maturation condition + mint extract at 3×MIC%		$log\ N0$ =5.11(SE=0.06) χ =244.5(SE=10.78) β =1.97(SE=0.174)
#4	Fermented cow milk (Lben)	*Listeria monocytogenes*	*L. paracasei* (isolated strain) without lemon extract	**Equation (15)** χ = 173.5 h (154.5, 192.5) β= 0.4645 (0.1495, 0.7795) N_0= 5.85 (5.578, 6.123) **Equation (16)** N_0 (CFU/mL) = 5.81 Lag time (h)= 25.486	

				μmax (log CFU/mL/h) = -0.137 Final Value, N_f (CFU/mL) = 4.934
#5	Fermented milk	*Listeria monocytogenes*	*L .paracasei* (isolated strain) with 2×MIC Citrus lemon extract	**Equation (15)** χ (h)= 44.09 (20.15, 68.03) β= 0.6929 (0.4585, 0.9274) N_0 (CFU/mL) = 5.934 (5.607, 6.262) **Equation (16)** N_0 (CFU/mL) = 5.86 Lag time (h) =14.943 μmax (log CFU/mL/h)= -0.0257 Final Value, N_f (CFU/mL) = 3.702

7.3 Optimised process variables to enhance the microbiological safety of the food products.

In the framework of the design of risk minimisation strategies, the quantified listericidal effect of mint extract can be used to establish a corrective measurement as well as controlling the conditions of maturation/drying of traditional dried sausages, a practical control allowing the maintenance of the product authentic characteristics.

The sausages formulated with extracts (i.e., control) and inoculated with a concentration of *L. monocytogenes* (5.0 log CFU/g) exhibited growth that persisted throughout drying despite acidification and dehydration. Only after 8 days of drying, the effect of water activity dropping, and higher acidity prompted the pathogen's inactivation. In these fate studies, the effect of the extract on the development of the pathogen was therefore slightly noticeable. Furthermore, it was estimated that adding MIC% extract to sausages decreases the log reduction time. At the 8th day of drying, mint extract at a 6×MIC dose produced a lesser reduction of *L. monocytogenes* of 0.43 log CFU/g, in comparison to the control (without added extract).

After adjusting the maturation condition, and with a dose of 3×MIC of extract as indicated in 3rd data set (Table 9), the behaviour of the *L. monocytogenes* drastically changed, where no growth was perceived. It is therefore expected that further inactivation is attained at the end of drying and even during shelf-life. These results suggest that meat producers can use the extract to design lethality treatments in order to achieve specific reductions of *Listeria monocytogenes* while using the mathematical models developed.

In fermented cow milk (*Lben*) (data sets #4 and #5), the decay rates (log CFU/mL/h) described the decrease in *L. monocytogenes* load in the product during storage. Even in previous studies *L. monocytogenes* was described as able to grow at temperatures as low as -0.4°C; however, no growth was observed in the fermented milk. Based on the adopted models, the time required for a first tenfold reduction of the *L. monocytogenes* population (χ) at 4 °C is estimated at 173.5 h for fermented milk without extract with a lag time of 25.486 h and μmax of -0.137 log CFU/mL/h. The present results indicate that for producers, the type of starter culture used for milk fermentation prior to refrigerated storage does offer a way to improve the safety of their products. Besides, level and type of starter and incubation temperature may affect the acidification process of raw milk, and consequently, the final pH and the antimicrobial compounds produced by lactic bacteria. This may provide information on the conditions needed to achieve specific log reductions for inactivation of pathogen. In the case of fermented milk with lemon extract, the inactivation of *L monocytogenes* was more significant. The parameters χ was equal to 44.09 ± 2 hours, which is about 3.9 folds lower than the χ value of not supplemented fermented milk samples (173.5 h), and the inactivation rate was -0.0257 log CFU/mL/h. The obtained results showed that the application of citrus lemon peel extract significantly affected the growth and the survival of *L. monocytogenes*. The combined application of selected lactic acid bacteria and citrus lemon peel extract was found to be a successful combination of natural agents for pathogen reduction. The use of natural extract could be a good choice providing antimicrobial effect and special organoleptic attributes to the traditional product; however, this probably would affect the consumers' choices.

8. Partner UIZ

8.1 Description of the predictive microbiology models adjusted to the data from fate studies in the products

Based on laboratory-created prototypes that replicate the properties of these fermented foods, Table 10 provides information from studies that employed predictive microbiology models to determine pathogen kinetics in two artisanal foods.

Table 10. Data sets of fate studies conducted by UIZ and their characteristics

Data set	Artisanal food	Pathogen inoculated	Preservation method	Stage followed
#1	*Merguez* fresh sausage	*Salmonella* Typhimurium (cocktail of 3 strains)	Essential oil of *Origanum ompactum* at 0.0, 0.075%, 0.15%, 0.3%, added to the simulate sausage medium	Storage conditions at 8 ºC
#2	*Merguez* fresh sausage	*Salmonella* Typhimurium (cocktail of 3 strains)	Essential oil of *Origanum ompactum* at 0.0, 0.075%, 0.15%, 0.3%, added to the simulate sausage medium	Storage conditions at 14 ºC
#3	*Merguez* fresh sausage	*Salmonella* Typhimurium (cocktail of 3 strains)	Essential oil of *Origanum ompactum* at 0.0, 0.075%, 0.15%, 0.3%, added to the simulate sausage medium	Storage conditions at 20 ºC
#4	*Merguez* fresh sausage	*Listeria monocytogenes* (cocktail of 3 strains)	Monoculture of indigenous lactic acid bacteria with antimicrobial properties, added to the simulated sausage medium	Fermentation conditions at 25 ºC
#5	*Merguez* fresh sausage	*Listeria monocytogenes* (cocktail of 3 strains)	Monoculture of indigenous lactic acid bacteria with antimicrobial properties, added to the sausage batter	Storage conditions at 8 ºC
#6	Goat's fresh cheese	*Staphylococcus aureus*	Refrigeration	Storage conditions at 8 ºC
#7	Goat's fresh cheese	*Listeria monocytogenes* (cocktail of 3 strains)	Refrigeration	Storage conditions at 8 ºC

All treatments (Data sets 1 to 3) were applied in the simulated sausage medium in order to study the behaviour of the *Salmonella* spp. as post-processing contamination, during normal storage and extreme conditions. To gather data, an experimental design using a Doehlert matrix was performed to determine the effects of four factors on *Salmonella* inactivation. The factors evaluated were oregano essential oil (OEO), sodium chloride (NaCl), pH, and temperature. A total of 23 experiments (including three central points) were carried out within the experimental domain shown in Table 11. Weibull model was used as the primary model in order to determine the survival kinetics of *Salmonella* spp. in SSM. In order to fit the model of Weibull to

inactivation data, GInaFiT software was used. The Weibull model has two parameters: ρ (dimensionless) and Delta (δ, h). While ρ is a shape parameter (for ρ > 1, convex curves are obtained, and for ρ < 1, concave curves are described), δ refers to the time required for the first decimal reduction when ρ is fixed.

$$log_{10}\big(N(t)\big) = log_{10}(N_0) - \left(\frac{t}{\delta}\right)^p \tag{17}$$

In order to quantify the effect of the independent variables on the primary kinetic parameters, polynomial models were developed.

Datasets 4 and 5 could effectively be characterized using the Lotka-Volterra competition model, as outlined by the ensuing system of two inhibitory functions.

$$\frac{1}{STA}\frac{dSTA}{dt} = \mu_{STA}\left(1 - \frac{STA + \alpha_{STA-LAB} \times LAB}{STA_{max}}\right)$$
$$\frac{1}{LAB}\frac{dLAB}{dt} = \mu_{LAB}\left(1 - \frac{LAB}{LAB_{max}}\right) \tag{18}$$

To mathematically describe **data sets 6 and 7**, the kinetic parameters of the selected pathogens were evaluated using the Baranyi model applied to the collected data through the DMFit software.

$$y(t) = y_0 + \mu_{max}t - \frac{1}{\mu_{max}} ln\big(e^{-vt} + e^{-h_0} - e^{-vt-h_0}\big)$$
$$-\frac{1}{m} ln\left(1 + \frac{e^{m\mu_{max}t + \frac{1}{\mu_{max}}\ln\left(e^{-vt}+e^{-h_0}-e^{-vt-h_0}\right)-1}}{e^{m(y_{max}-y_0)}}\right) \tag{19}$$

Table 11. Experimental range and levels of the selected factors

Factors	**Range and levels**						
Coded variable	−1	−0.5	0	0.5	1		
Oregano (% v/w)	0	0.075	0.15	0.225	0.3		
Coded variable	−0.866	−0.577	−0.289	0	0.289	0.577	0.866
Sodium chloride (%, w/w)	0.9	1.4	1.9	2.4	2.8	3.3	3.8
Coded variable	−0.816	−0.612	−0.204	0	0.204	0.612	0.816
pH	4.7	5	5.5	5.8	6	6.5	6.8
Coded variable	–0.791	0	0.791				
Temperature (°C)	8	14	20				

8.2. Summary of fitted microbial kinetic parameters

The kinetic parameters of the foodborne pathogens in the two artisanal fermented foods, obtained from the seven fate studies are compiled in Table 12.

Table 12. Kinetic parameters of foodborne pathogens in fresh sausages (*Merguez*) and goat's fresh cheese

Data set	Artisanal food	Pathogen inoculated	Preservation method	Kinetic parameters
#1	*Merguez* fresh sausage	*Salmonella Typhimurium* (cocktail of 3 strains)	Oregano essential oil (OEO) at 0.075% at 0.15% at 0.30%	Equation 17 δ (h) = 301.07 ±3.02 ρ = 6.04 ±0.10 δ (h) = 1.86 ±0.28 ρ = 0.32 ±0.00 δ (h) = 3.91 ±0.21 ρ = 1.60 ±0.36

#2	*Merguez* fresh sausage	*Salmonella Typhimurium* (cocktail of 3 strains)	Oregano essential oil (OEO) at 0.0% at 0.075% at 0.15% at 0.30%	Equation 17 δ (h) = 156 ±12.95 ρ = 1.5 ±0.14 δ (h) = 51.12 ±4.67 ρ = 0.88 ±0.05 δ (h) = 8.84 ±0.37 ρ = 0.48 ±0.01 δ (h) = 1.99 ±0.32 ρ = 0.55 ±0.03
#3	*Merguez* fresh sausage	*Salmonella Typhimurium* (cocktail of 3 strains)	Oregano essential oil (OEO) at 0.075% at 0.15% at 0.30%	Equation 17 δ (h) = 10.28 ± 0.38 ρ = 0.4 ±0.04 δ (h) = 180 ± 12.09 ρ = 2.62 ± 0.33 δ (h) = 20 ± 7.09 ρ = 1.10 ± 0.03
#4	*Merguez* fresh sausage	*Listeria monocytogenes* (cocktail of 3 strains)	Selected LAB Without monoculture With monoculture	Equation 18 μ_{LAB}= 1.99 ± 032 μ_{LIS} = 0.59 ± 0.13 μ_{LAB}= 0.31 ± 0.04 μ_{LIS} = 0.09 ± 0.03
#5	*Merguez* fresh sausage	*Listeria monocytogenes* (cocktail of 3 strains)	Selected LAB Without cocktail With monoculture	Equation 18 μ_{LAB} = 0.08 ± 0.03 μ_{LIS} = 0.01 ± 0.00 μ_{LAB} = 0.07 ± 0.02 μ_{LIS} = -0.02 ± 0.00
#6	Goat's fresh cheese	*Staphylococcus aureus*	Refrigeration	Equation 19 N_0 (CFU/g) = 3.051 ± 0.347 µmax (log CFU/g/h) = 0.014 ± 0.003 Final Value, N_f (CFU/g) = 8.4 ± 1.25

#7	Goat's fresh cheese	*Listeria monocytogenes* (cocktail of 3 strains)	Refrigeration	Equation 19 N_0 (CFU/mL) = 3.544 ± 0.14 µmax (log CFU/mL/h) = 0.023 ± 0.002 Final Value, N_f (CFU/mL) = 7.683 ± 0.129

8.3 Optimised process variables to enhance the microbiological safety of the food products

Based on our experimental data, there are significant implications for the microbiological safety of artisanal food products (fresh sausage and cheese) with the optimal application of certain process variables. Our evidence clearly demonstrates that the incorporation of OEO has a strong effect on the inactivation of *Salmonella* spp. in a simulated sausage medium (SSM), irrespective of the concentration of OEO added. Moreover, through an evaluation of the δ Weibull parameter, the evidence indicates that a reduction in time for the first log reduction is considerably lower in a SSM that contains a higher concentration of OEO (3.91 ±0.21h at 0.30% when stored at 4°C), confirming that high OEO concentrations favour the early inactivation of *Salmonella* spp.

Further analysis reveals that an increase in storage temperature, paired with an elevated NaCl concentration, reduces the δ, which implies that a temperature increase to room temperature combined with high NaCl concentration aids in the inactivation of the pathogen. This outcome is notable despite *Salmonella* spp.'s mesophilic nature and tolerance for temperatures as low as 5°C. The tested strain demonstrated resilience against refrigerated conditions, thus posing a challenge for the storage of perishable foods.

Adding to the complexity of controlling *Salmonella* spp., our studies revealed that the application of microbial starter cultures effectively reduced *L. monocytogenes* loads in simulated fresh sausage by 3.6 to nearly 6 log CFU/g. This shows the potential of these strains as adjunct cultures to enhance the safety of finished products.

In the case of fresh cheese, both *S. aureus* and *L. monocytogenes* demonstrated accelerated growth rates over storage time, with the growth rate increasing as the temperature increased. However, these effects can be mitigated by strategic process enhancements.

Based on these experimental results, we recommend the careful regulation and monitoring of temperature and NaCl concentration in the storage and preparation of fresh sausage. Moreover, the application of OEO should be considered as a natural preservation method, especially for perishable foods kept under refrigeration. Additionally, the use of microbial starter cultures shows promise in enhancing product safety, and further investigation of these cultures is suggested for their potential broader application. However, continual monitoring of pathogens, notably *S. aureus* and *L. monocytogenes* in fresh cheese, is required to maintain microbiological safety in light of their rapid growth during storage. The safety of food products is a multifaceted challenge, but through strategic interventions and continuous monitoring, significant enhancements in food safety can be achieved.