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

Standardisation of inoculum and optimisation of the inoculation procedure in the selected matrices

Leader Partner: UCO

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1. Aim of the fate-studies

The fate-studies performed within the scope of this project aim at studying the microbial behaviour in artisanal products to evaluate biopreservation strategies and to develop mathematical models that can be further applied in a decision-support tool for Mediterranean artisanal food producers. Once the appropriate biopreservatives – starter cultures (WP3) and natural extracts (WP4)– have been selected, and any decision on the adjustment of process variables made (WP2), a series of inoculation studies will be performed per pathogen, i.e., *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus* and *Escherichia coli* (STEC), and selected artisanal food, i.e., cheeses and fermented sausages. In this document, relevant aspects to be considered for inoculation of the food matrices for the performance of these fate-studies are described. This document was elaborated based on published studies and mainly on the ISO/FDIS 20976, which provides the guidelines for conducting challenge tests with foods.

2. Selection of microbial strains to be used in fate-studies

The identification of the microbial strains used in fate studies may be known in sufficient detail, through previous biochemical, and/or serological and/or genetical characterization. It is preferred the selection of strains previously isolated from fermented products, including raw materials, ingredients, and end products. In addition, strains isolated from the production environment or from clinical, food, environmental samples in outbreaks involving fermented products, are preferred rather than strains from a culture type collection. The selected strains might be available in national or international culture collection for future testing, if required.

A mixture of 3-5 strains of the same microbial species is usually used in fate studies so that variations among strains are considered (Rosshaug et al. 2012; Leggett et al. 2012; Mataragas et al. 2015; Gunvig et al. 2016). In growth kinetics studies to estimate growth parameters in foods, e.g., growth rates, only one strain shall be used per challenge test (ISO/FDIS 20976-1).

3. Inoculum preparation

Inoculation might mimic contamination scenarios that may occur during the production or storage of the fermented products under study. The inoculum may reflect the microbial

responses that may appear due to adaptation and the microbial variability that may be encountered in food matrices, processing, and retail environments.

Culture media and reagents shall be prepared following procedures specified in ISO11133 and in the International Standard developed for the specific microorganism evaluated. For microbial detection and quantification, internationally accepted and validated protocols shall be applied (e.g., ISO 16140-2). A generic scheme of inoculum preparation is shown in Figure 1.

3.1. Maintenance of bacterial stock cultures

The selected strains may be kept in culture broth supplemented with glycerol (20-25 %) at temperatures ≤ -20 °C.

3.2. Preparation of the bacterial cultures

For the preparation of the bacterial cultures, two successive cultures of the selected strain(s) should be conducted. The first one may be performed in a culture broth that enables optimum microbial growth, while the second may be performed in a medium that allows the adaptation of cells to the natural conditions of the food to be inoculated, and therefore mimics its conditions. During incubation, bacterial cells may reach the early or the end of the stationary phase in the two successive cultures. When applying a multi-strain cocktail, the cultures of each individual strain selected may be prepared by two successive cultures, as described above. To prepare the cocktail, aliquots of grown cultures of each individual strain selected may be transferred to a sterile tube. Each strain may be present at the same concentration on the final cocktail.

It has been reported in some studies that after the second incubation (second sub-culture), microbial cultures or cocktails are submitted to successive centrifugation steps followed by the re-suspension in a non-promoting growth media, e.g., phosphate buffer solution, to avoid the interference of the nutrients available in broth on microbial behaviour (Mataragas et al. 2015; Gunvig et al. 2016).

Following, serial dilutions of the individual cultures or the cocktails shall be performed in a dilution medium that does not promote microbial growth to adjust the initial inoculum concentration to a desired level. Both individual cultures and the cocktail must be enumerated in the same medium that will be used for enumeration in the challenge test. Inoculation of the tested units may be performed immediately after preparation to avoid changes in inoculum concentration.

If inoculation is performed in end products to evaluate microbial behaviour during their storage at different conditions, it is convenient to consider the adaptation of bacterial cells to stress conditions that may take place during the production processes of the selected fermented products. To this end, strain(s) shall be submitted to treatments that mimic food production processes, i.e., injury and/or stress to induce adaptation. See ISO/FDIS 20976-1 for more detail in injury protocols. If only the maximum growth rate is estimated in challenge tests, there is no need to induce any adaptation on the selected strain(s).

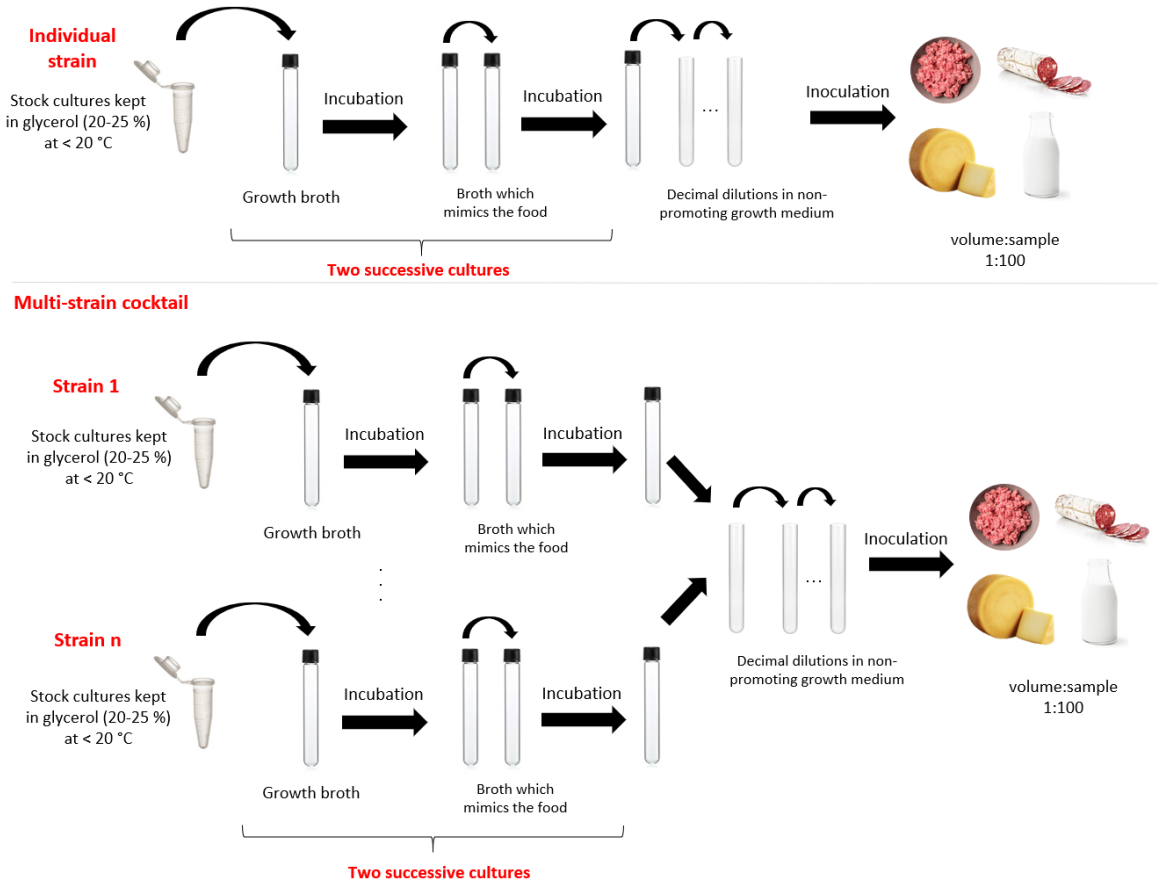

Figure 1- Generic scheme of inoculum preparation

4. Inoculation of the test units

The inoculation must be performed in such a way that all the test units receive the same level of a microorganism. Besides, the inoculum volume must not change the physicochemical characteristics of the food. Moreover, the ratio volume:sample should not exceed 1:100 (ISO/FDIS 20976-1). Before inoculation, the initial temperature of the samples shall be equilibrated to the test target temperature.

The inoculation level shall be justified and adjusted in accordance with the expected microbial behaviour in the fermented foods. In case microbial growth is expected on fermented products, inoculum levels usually range between 2-3 log cfu/g or log cfu/mL (Schvartzman et al. 2011; Tiwari et al. 2014). On the contrary, if a survival pattern or inactivation is expected on the product under study, an inoculum at 5-7 cfu/g or cfu/mL is usually applied (Lobacz, Kowalik, and Zulewska 2020; Mataragas, Stergiou, and Nychas 2008; Drosinos et al. 2006). In any case, the inoculum level selected shall be within the quantification limit of the enumeration method applied. When the inoculum level or expected counts are low, the quantification limit of the enumeration method can be lowered by adapting the volumes plated on the culture media (in accordance with ISO 7218).

The inoculation procedure shall be described in detail and justified. It might mimic contamination events of interest. For example, to simulate the contamination of the main raw materials to be used to produce cheese and fermented sausages, inoculation shall be performed in milk and on the meat batter, respectively, so the inoculum is homogeneously distributed in test units (Campagnollo et al. 2018; Schvartzman et al. 2011; Drosinos et al. 2006) (Figure 2). To simulate cross-contamination scenarios on processing environments, contamination of whole products or slices of the products is reasonable (Tiwari et al. 2014; Schvartzman et al. 2014; Gounadaki et al. 2007). Alternatively, sausages have been inoculated in depth by injecting inoculum at different points (Iannetti et al. 2017). In any case, enumeration shall be performed in the whole test unit.

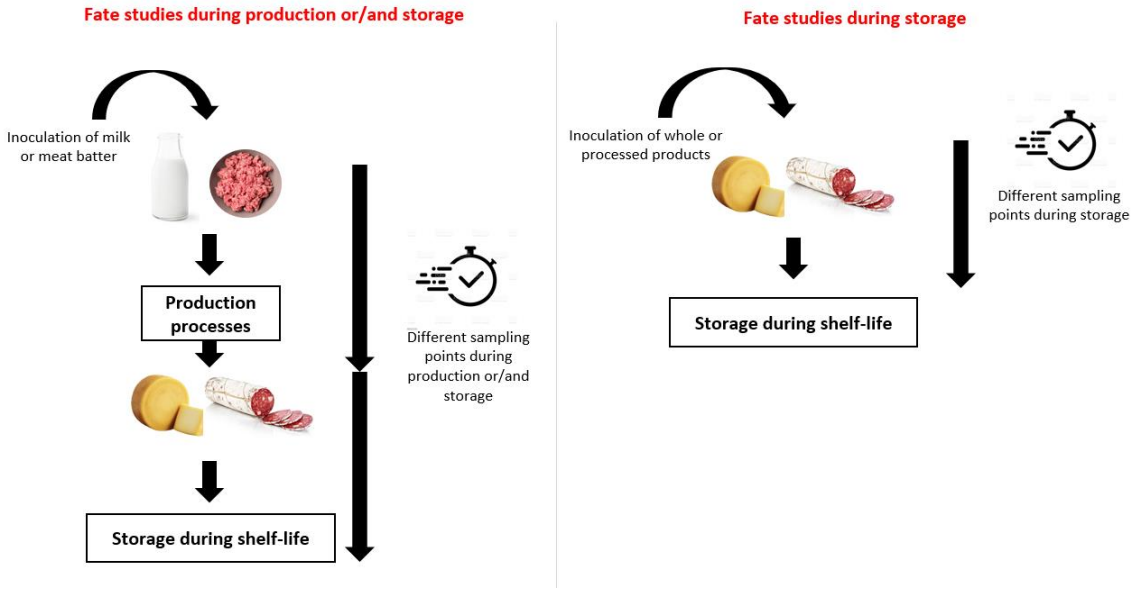

Figure 2- Generic scheme of fate studies performed during production or/and storage

5. Controls

Relevant physicochemical characteristics such as pH, water activity, lactic acid concentration, concentrations of other preservatives, background microbiota or/and added starter cultures may be determined in control test units, to assist in the proper interpretation of results and to know the influence of the involved factors on microbial evolution. For the preparation of control units, the same inoculation procedure applied for the test units may be performed using a non-growth promoting diluent, free of the pathogenic cells. It is recommended to analyse control units at least at the first (t_0) and the last (t_{end}) sampling point of the fate study, although it is better to analyse at least one control unit per sampling point.

Food controls may be also be performed and compared with the physicochemical characteristics of the test units, to assure that the processing and inoculation procedures would not change the characteristics of the food of interest.

6. Storage of the test units

The inoculated test units might be stored at the conditions mimicking either the production processes or/and the storage of fermented products, i.e., temperature, relative humidity, time. These conditions vary for the different artisanal products selected by partners and might be monitored and recorded throughout the duration of the challenge tests.

7. Analysis of inoculated test units

Microbiological and physicochemical determinations shall be performed at different sampling points according to the experimental design of the challenge-tests. A minimum of 5 sampling points is recommended in fate studies to evaluate growth potential in foods, while a minimum of 8 sampling points is recommended in studies to estimate the growth rates (ISO/FDIS 20976-1). In all cases, an initial determination shall be performed on the day of inoculation of the test-units of fermented products. All determinations should be conducted using recognized and accepted protocols (See the list of normative references below). To reduce both the uncertainty derived from the sampling and analytical methods and the variability derived from the artificial inoculation, food matrix and other sources, sampling units must be analysed at least in duplicate per sampling point and at least three different batches of the food products may be evaluated.

Normative references of interest:

ISO/FDIS 20976-1: Part 1: Microbiology of the food chain — Requirements and guidelines for conducting challenge tests of food and feed products — Challenge tests to study growth potential, lag time and maximum growth rate.

EURL Lm: Technical guidance document for conducting shelf-life studies on *L. monocytogenes* in ready-to eat foods, 2019.

ISO 11133: Microbiology of food, animal feed and water — Preparation, production, storage, and performance testing of culture media

ISO 7218: Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations

ISO/IEC 17025: General requirements for the competence of testing and calibration laboratories

ISO 16140-2: Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method

ISO 6887-1: Microbiology of the food chain — Preparation of test samples, initial suspension, and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions

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Normative references of interest:

Summary tables of inoculum preparation and procedures to be adopted for the fate studies in the artisanal cheeses and sausages

Table 1: Summary of inoculum preparation and procedures to be adopted for the fate studies in the artisanal cheeses

Question	IPB (Portugal)	UCO (Spain)	AUA (Greece)	UNIBO (Italia)	UIZ (Morocco)
Microorganisms evaluated	*Listeria monocytogenes* and *Staphylococcus aureus*	*Salmonella* and *Staphylococcus aureus*	*Listeria monocytogenes* and *Salmonella*	*Listeria monocytogenes*	*Listeria monocytogenes* and *Staphylococcus aureus*
Number of strains of each microorganism	2	3	3	5 (2 reference + 3 field strains)	1
Inoculated individually or as a cocktail?	Individually	Cocktail	Cocktail	Cocktail	Cocktail
Strain sources	Previously isolated from the evaluated food matrices and obtained from culture collection	Previously isolated from the evaluated food matrices and obtained from culture colection	Previously isolated from other food matrices, clinical isolates, obtained from culture collection	Previously isolated from other food matrices and obtained from culture colection	Strains obtained from culture collection
Why were the strain(s) selected?	One from culture collection for comparison, the other has been isolated from cheese and is recurrent.	To mimic variability that can be found in reality	The selection of strains was made in order to account variation in isolation, growth / survival characteristics and serotype.	Reference strains and field strains will be selected according to ANSES protocol for *Listeria monocytogenes c* hallenge studies in RTE foods	*L. monocytogenes* and *S. aureus* are the most related pathogens to the cheese products
Concentration to be inoculated	6 log cfu/mL	2-3 log cfu/mL (g)	5-6 log cfu/g	2-3 log cfu/g (mL)	3-4 log cfu/mL or g
Will the inoculation be performed in milk as initially planned?	Yes, in milk, and then followed throughout processing and shelf life	Inoculation will be performed according to what will be agreed with the company	The inoculation will be performed on final products to mimic post-process contamination during packaging.	According to what will be agreed in the pilots	Yes. But also a contamination by the pathogens at cheese surface, in particular via a contamination scenario during the brining of the product is possible
What steps of the production chain will be evaluated?	Production and storage of end products	Production and storage of end products	Storage of end products	Storage of end products	Production and storage of end products
The influence of which factors on microbial behaviour will be evaluated?	Temperature, presence of starter cultures and addition of natural extracts	Water activity, pH, lactic acid concentration, temperature, presence of starter cultures	Water activity, pH and addition of natural extracs	Water activity, pH, lactic acid concentration, temperature	pH, lactic acid concentration, temperature, presence of starter cultures, addition of natural extracts, relative humidity
If possible, provide some details about the experimental design or the levels of the factors to be evaluated.	For natural extracts, only addition/no addition. For starter cultures, addition/no addition. In both types of fate studies, three temperatures will be used for shelf life	We are going to compare microbial behaviour in the presence/absence of bioprotective cultures during production and shef-life	1 product (katiki) X 2 essential oils (Oregano, Rosemary) X 3 types of application (pure EE, encapsulated in liposomes, encaspulated in cyclodextrin) X 1 storage temperature X 2 microorganisms (L. monocytogenens, Salmonella spp.)	-	A multi-level factorial design will be performed using "Design Expert" Package (version 12) with two or three levels of each factor
How many batches will be evaluated in challenge-tests?	2	2	2	4	at least 4

Table 2: Summary of inoculum preparation and procedures to be adopted for the fate studies in the artisanal sausages

Question	IPB (Portugal)	UCO (Spain)	AUA (Greece)	UNIBO (Italia)	UIZ (Morocco)
Microorganisms evaluated	*Salmonella* and *Staphylococcus aureus*	*Salmonella* and *Staphylococcus aureus*	*Listeria monocytogenes* and *Salmonella*	*Staphylococcus aureus*	*Listeria monocytogenes* and *Salmonella*
Number of strains of each microorganism	2	3	3	5	1
Inoculated individually or as a cocktail?	Individually	Cocktail	Cocktail	Cocktail	Cocktail
Strain sources	Previously isolated from the evaluated food matrices and obtained from culture collection	Previously isolated from the evaluated food matrices and obtained from culture colection	Previously isolated from other food matrices, clinical isolates, obtained from culture collection	Previously isolated from the evaluated food matrices and other matrices, strain obtained from culture colection	Previously isolated from the evaluated matrices, strains obtained from culture collections
Why were the strain(s) selected?	One from culture collection for comparison, the other has been isolated from cheese and is recurrent.	To mimic variability that can be found in reality	The selection of strains was made in order to account variation in isolation, growth / survival characteristics and serotype.	Isolated from a similar matrix	Salmonella isolated from the target product
Concentration to be inoculated	6 log cfu/g	2-3 or 6-7 log cfu/g (according to microbial responses observed in previous tests)	5-6 log cfu/g	2-3 log cfu/g	4 log cfu/g
Will the inoculation be performed in the meat batter as initially planned?	It is very likely that for safety reasons, inoculating will be done by puncturing in mini sausages, with a syringe needle at different points in the sausage. Each sausage would be directly stomached when analysing. Nonethess, a preliminary experiment will be conducted in January to finally decide.	Inoculation will be performed in the meat batter	Inoculation will be performed in the final product (storage experiments) and at the marination step during the production of noumpoulo	According to the pilot study	Inoculation will be performed in the meat batter
What steps of the production chain will be evaluated?	Production and storage of end products	Production and storage of end products	Production and storage of end products	Storage of end products	Production and storage of end products
The influence of which factors on microbial behaviour will be evaluated?	Temperature, presence of starter cultures and addition of natural extracts	Water activity, pH, lactic acid concentration, temperature, presence of natural extracts	Water activity, pH, temperature and addition of natural extracs	Water activity, pH, lactic acid concentration, temperature	pH, lactic acid concentration, temperature, presence of starter cultures, addition of natural extracts, relative humidity
If possible, provide some details about the experimental design or the levels of the factors to be evaluated.	For natural extracts, only addition/no addition. For starter cultures, addition/no addition. In both types of fate studies, three temperatures will be used for shelf life	We are going to compare microbial behaviour in the presence/absence of bioprotective cultures during production and shef-life	1 product (Noumpoulo) X 2 essential oils (Oregano, Rosemary) X 3 types of application (pure EE, encapsulated in liposomes, encaspulated in cyclodextrin) X 1 storage temperature X 2 microorganisms (L. monocytogenens, Salmonella spp.)	-	A multi-level factorial design will be performed using "Design Expert" Package (version 12) with two or three levels of each factor
How many batches will be evaluated in challenge-tests?	2	2	2	4	at least 5