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Definition of Food Safety Criteria for Bacteria Food-Borne Pathogens in Ready to Eat products

Tesi presentata da: Federica Bovo

Coordinatore Dottorato

Prof. Giovanni Dinelli

Relatore

Prof. Gerardo Manfreda

Correlatrice

Dott.ssa Alessandra De Cesare

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INTRODUCTION

1. Food safety and public health protection

Food safety is a broader term, which means an assurance that food will not cause harm to the consumers when it is prepared and/or eaten according to its intended use (CAC, 1997). Food safety is a global issue that affects the health of populations in both industrialized and developing countries. It is one of the highest priorities of public health at national and international level. Food crises that have occurred over the last 20 years (Bovine Spongiform Encephalopathy (BSE), dioxins, foot and mouth disease, etc), changing nutritional habits, new food production processes, increasing international trade and emerging risks, have led consumers to be more sensitive to food safety issues and risk managers to develop and strengthen a more effective food safety system (Manfreda, De Cesare 2014). In the aftermath of the BSE crisis and several other food scandals, the EU decided to have and action plan for a pro-active new food policy, developing a "Farm to Fork" approach that covers all sectors of the food chian. Infact, safety, quality and hygiene of food products depend on the joint effort of all stakeholders during the complex chain of production, processing, transport and retailing of food. Moreover, it is also important that consumers give their attention to food hygiene, preparation and proper storage to have the guarantee to consume safe food products.

The changing process began in January 2000, when the European Commission gives off "the White Paper on Food Safety" in which is outlined a new strategy: the food safety can be assured only by using integrated systems of control in the supply chain, from

the production of raw materials to the food consumption. A new system was created, applicable in a uniform manner throughout Europe, based on solid scientific basis and on a modern legislative framework, aimed to identify, characterize and verify all the hazardous factors to the health, from the production to the consumption of the food product. This path of innovation, started defining the principles and requirements of food law, continued with the EC Regulation 178/2002 establishing the European Food Safety Authority (EFSA) and setting procedures to be implemented, in order to ensure, by the food industry, a high level of protection of public health without forget the domestic market, which still require the free movement of safe and wholesome food products to achieve a smooth operation. During 2004, the U.E. issued a set of regulations which, together with the EC Regulation 178/2002, reperesent the so-called "Hygiene package". These Regulations, in force since 1st January 2006, identify and separate the responsibilities of those involved in food safety, defining new rules for the industry as well as the control measures to carry out by the competent authorities.

These Regulations are:

- Regulation (EC) No 852/2004 on the hygiene of foodstuffs. The safety of foodstuffs is mainly ensured by a preventive approach, such as implementation of good hygiene practice and application of procedures based on hazard analysis and critical control point (HACCP) principles (Regulation EC n. 2073/2005).

- Regulation (EC) No 853/2004 laying down specific hygiene rules for food of animal origin (excluding vegetable origin foods and mixed foods) in order to guaranteee a high level of food safety and public health.

- Regulation (EC) No 854/2004 putting in place a Community framework of official controls on products of animal origin intended for human consumption.

- Regulation (EC) No 882/2004 on official controls performed to verify the compliance to the regulations in the field of feed and food, and the compliance to the rules on the health and welfare of animals.

Furthermore, in December 2005 other Community legislations were issued, among which the Regulation n. 2073/2005 on microbiological criteria for foodstuffs.

The World Trade Organization (WTO) has been a central force in stimulating the concept of harmonization of food safety control procedures, introduced in the WTO Agreement on Sanitary and Phytosanitary (SPS) Measures. In this agreement, and in case of differences, each WTO member must accept the sanitary measures of other members as equivalent to their own measures, provided they offer the same level of protection. Safe food is produced by adhering to good hygienic practices (GHP), good manufacturing practices (GMP), good agricultural practices (GAP) and implementation of food safety risk management systems such as hazard analysis critical control points (HACCP).

However, the level of safety that these food safety systems are expected to deliver has seldom been defined in quantitative terms. Therefore, in 2002, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) held a joint consultation meeting to explore the principles and to establish guidelines for incorporating microbiological risk assessment in the development of food safety standards, guidelines and related texts. In this consultation, concepts such as appropriate level of protection (ALOP) and food safety criteria were discussed in detail. In 2003, the Codex Alimentarius Commission (CAC) adopted the Guidelines for the Judgment of Equivalence of Sanitary Measures Associated with Food Inspection and Certification Systems (CAC, 2003). Afterwards, in 2004 it defined the so-called Food Safety Objective (FSO) and Performance Objective (PO).

2. The Microbiological criteria on food safety in the European legislation

At European level, in 2002, the Regulation (EC) 178 of the European Parliament and of the Council states that, in order to achieve the general objective of a high level of protection of human health and life, food law shall be based on risk analysis, except where this is not appropriate to the circumstances or the nature of the measure (EC, 2002).

European countries have traditionally attempted to improve food safety by setting microbiological criteria for raw or for finished processed products. However the frequency and extent of sampling used in traditional food testing programs may not provide a high degree of consumer protection (ICMSF, 2006).

Microbiological Criteria have been the corner stone on which food regulation regarding microbiological hazards has been sustained. By means of its application could be determined if a product is acceptable with regard to the absence/presence or concentration of the microorganism per mass unit, volume, area or lot of food (Todd, 2003; FAO/WHO, 2001a; Pérez-Rodríguez et al., 2007). A Microbiological Criterion has to refer to a microorganism of interest and affirm clearly the reason of its consideration; besides, it has to include the list of method(s) for the detection or quantification of the microorganism, indicate the number of samples, the method of the sample and the size of the analytic unit; identify the appropriate microbiological limits in each specific point in the food chain and the number of analytic units which it constitutes (Pérez-Rodríguez et al., 2007). In the Commission Regulation 2073/2005, microbiological criteria have been defined for specific biological risks in selected food products. Overall, the definition of a microbiological

criterion should be able to assess (1) the microbiological quality of a food; (2) the adherence to GHP; (3) the suitability of a food or ingredient for a particular purpose; and (4) the acceptability of a food or ingredient from another country or region where the conditions of production are unknown or uncertain. A microbiological criterion consists of a statement of the microorganisms of concern and/or their toxins/metabolites and the reason for that concern; the food to which the criteria applies; the analytical methods for their detection and/or quantification, generally represented by an ISO reference culture method; a sampling plan defining the number of field samples to be taken (i.e., n) and the size of the analytical unit (e.g., 25 g); microbiological limits (i.e., m and M) considered appropriate to a food at the specified point in the food chain (e.g., at the market); the number of analytical units that should conform to these limits (i.e., c) and the actions to be taken when the criteria is not met (Manfreda, De Cesare 2014).

However, the microbiological criteria included in the EU Regulation n. 2073/2005 for foodstuffs are not based on risk analysis. Moreover, at a governmental level, the microbiological criteria covers the range of different food chains related to a certain food product or product group, including all relevant producers, manufacturing sites and food service establishments within the country as well as those importing into the country.

In particular, different targets of hazard are included in the Regulation n. 2073/2005, such as *Salmonella spp.*, *Listeria monocytogenes*, *Verotoxigenic E. Coli*, *Staphylococcal enterotoxins*, *Enterobacter sakazakii*, Histamine. These food risk are related to specific food matrix most of them included RTE products. For instance, the microbiological criteria for *Salmonella spp*. in RTE products corrresponds to absence in 10 or 25 g of product, depending on food matrix, Staphilococci enterotoxins: absence in 25 g of milk and cheese products heat treated at lower temperatures than pasteurisation. These criteria are different from those for *L. monocytogenes*, as the criteria are not related to storage time or the last day of shelf life. For example, for healthy human population, foods where the

levels do not exceed 100 CFU/g are considered to a negligible risk. Therefore, the EU microbiological criterion for *L. monocytogenes* is set as ≤ 100 CFU/g for RTE products on the market. Specifically, the *L. monocytogenes* criteria included are related to the following condition:

• In RTE products intended for infants and for special medical purposes *L. monocytogenes* must not be present in 25 g of sample.

• *L. monocytogenes* must not be present in levels exceeding 100 CFU/g during the shelf-life of other RTE products.

• In RTE foods that are able to support the growth of the bacterium, *L. monocytogenes* may not be present in 25 g of sample at the time of leaving the production plant; however, if the producer can demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 CFU/g throughout its shelf-life, this criterion does not apply (EFSA, 2015).

No safety criteria are included in this European Regulation for different biological hazard such as *Bacillus cereus* or *Campylobacter* that normally rapresent a significant risk for human consumers.

On the other hand, the increasing international trade in food and the fact that manufacturing sites in one country may provide raw materials to other manufacturers or finished goods (products) for large numbers of consumers living in importing countries, demonstrates the need to harmonize at global level the microbiological criteria in order to improve the safety for consumer (Manfreda, De Cesare 2014).

3. Food Safety Criteria

As mentioned before, in 2003, the CAC adopted the Guidelines for the Judgment of Equivalence of Sanitary Measures Associated with Food Inspection and Certification Systems (CAC, 2003), based on so-called Food Safety Objective (FSO) and Performance Objective (PO).

The Microbiological Criterion described in EU Regulation 2073 is an element belonging to the traditional Microbiological Risk Management Systems that still has capacity in the new framework ruled by the FSO (Gorris, 2005; Pérez-Rodríguez et al., 2007). In many cases, when it is unknown if there have been applied HACCP programs and/or GMP guides, Microbiological Criteria, based on an established sampling plan, can be used as a decision-making element to accept or eject a lot (Pérez-Rodríguez et al., 2007).

This new approach is built on three-stage process as follows:

(1) risk assessment: an assessment is made of the risk to human health associated with a particular food-borne hazard;

(2) risk management: decisions are made regard- ing the acceptable level of risk and measures implemented for the control of this risk;

(3) risk communication: information about the risk and chosen methods of control are communicated amongst interested parties (Henson, Caswell 1999).

Risk analysis helps risk managers in governmental functions to decide on food safety control measures in a structured, open and transparent way. New terms and concepts have been introduced to describe public heath goals (Gorris, 2004). The World Trade Organization Agreement on the application of sanitary and phytosanitary measures

introduced the concept of appropriate level of protection (ALOP). This is a public health objective defined as: “The level of protection deemed appropriate by the Member establishing a sanitary or phytosanitary measure to protect human, animal or plant life or health within its territory.” In food microbiological risk management, an “ALOP refers to a level of protection of human health established for a foodborne pathogen”. Tipically an ALOP would be articulated as a value related to the desease burden associated with a particular hazard/food combination and its consumption in a country (Rieu et al., 2007). The ALOP is not the most adequate concept to develop and implant the necessary control measurements throughout the food chain (Pérez-Rodríguez et al., 2007): it is extremely difficult for any government body or international agency to quantify the level of risk that a society is willing to tolerate or accept, or even to specify who has the ultimate responsibility to make such a decision (Manfreda, De Cesare 2014). The terms in which the ALOP is expressed do not form the part of the “language” that the industry or the other operators of the food chain use for food safety management (Pérez-Rodríguez et al., 2007); on a global scale countries and regions have their own legislative measurements regarding food safety and quality. Even within EU there are still many differences regarding food safety legislation between countries, making trade often complicated (Trienekens , Zuurbier 2007). To bridge this gap, the concept of food safety objective (FSO) was introduced by the International Commission on Microbiological Specifications for Foods (ICMSF), adopted later by the Codex Alimentarius Food Hygiene Committee (CCFH), and defined as: “The maximum frequency and/or concentration of a hazard in a food at the time of consumption that provide(s) or contributes to the appropriate level of protection” (Rieu et al., 2007). The ICMSF (2002) porposes the creation of a new concept, the Food Safety Objective (FSO), whose function is to establish a link between the ALOP, and food safety management in the food chain (Pérez-Rodríguez et al., 2007) (Figure 1).

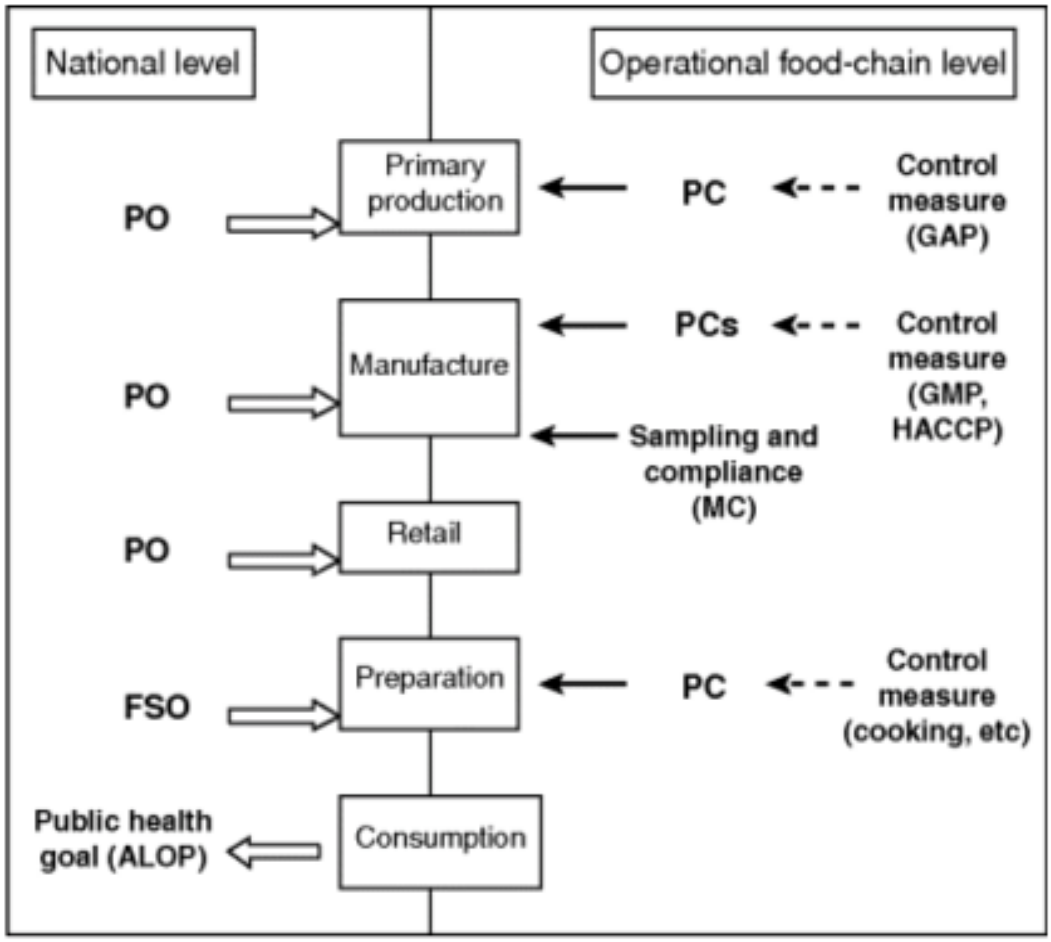

Figure 1 – Schematic diagram of the positions of the risk-based microbiological metrics at national level and operational level along an imaginary food chain. The operational level measures are embedded in the food safety management sustems operated in the chain, such as GAP, GHP, GMP, HACCP (Gonzales-Barron et al., 2014).

Because, conceptually, an FSO should be derived from the ALOP, there is a need for additional milestones that ensure the appropriate frequency and/or concentration of a hazard at specific steps along the food chain. This need has been addressed with the POs that should be based on the FSO. The codex defines a PO as “the maximum frequency and/or concentration of a hazard in a food at a specified step in the food chain before consumption that provides or contributes to an FSO or ALOP, as applicable”. A PO may

be the same as the FSO if the frequency/concentration of the hazard stays at the same level between the point of the PO and FSO. Otherwise, Codex guides the PO to be more or less stringent than the FSO according to the likeliness of the hazard to increase or decrease between the PO and FSO (CAC, 2005; Manfreda, De Cesare 2014). There is intentional similarity in the concepts of FSO and PO since both are guidance values for the hazard level at points in a food chain. Whereas FSOs by concept are only set by competent bodies/governments, POs can be set by industry or by such bodies/governments. The latter, for instance, could propose PO values when they want to define default milestones in a typical food production chain in a generic ‘‘guidance’’ fashion. Industry can choose to define PO values in the very specific case of a food production chain, for instance, to improve the integration of the overall supply chain management.

A new term has been proposed (CAC, 2004) to describe the overall effect of the control measures on the hazard level at a step, namely the Performance Criterion (PC). A PC indicates the change in hazard level required at a specific step in order to reduce the hazard level at the start of the step to a level at the end of the step that complies with the PO or the FSO. PCs are the specific operational, supply chain measures at a specific step(s) that result in meeting the objective for that step, the PO. When a PC is effective at time of consumption (e.g. a required minimum effect of a heat treatment during preparation in order to cause a specific reduction in the hazard level) it actually is the FSO that is met. Such a PC can be part of the product design, but can be relied upon only under specific conditions. PCs may concern a required reduction of the hazard, avoiding increase (limit to 0) or assuring a minimal increase. PCs in general will be decided on by food safety managers as key points in the design of the production of a food in a supply chain (Gorris, 2005). One example where such specific measures could prove to be important is in the prevention of cross-contamination at the point where food is prepared

for final consumption. In this case, the occurrence of cross-contamination is a generic issue affecting the safety of all ready-to-eat products. For instance, pathogens present on raw food products such as red meats or poultry products could transfer to processed, ready- to-eat foods through manual handling, cooking utensils or surfaces. In this case, both the meat and poultry as well as the ready-to-eat product may have FSOs associated. However, as they are generic appropriate preventative or control measures relating to cross-contamination should better not be linked to a specific food (i.e. as a PO, PC or control measure at that step) but should be part of general hygiene measures to be kept to in all cases during preparation (Gorris, 2005).

Developing meaningful microbiological criteria for a food or ingredient is a complex process that requires considerable efforts and resources. Therefore, a microbiological criterion should be provided only when there is a need and when it can be shown to be effective and practical. Governments establish microbiological standards only when they are deemed appropriate to ensure the safety of the foods for which they have regulatory responsibility (Manfreda, De Cesare 2014).

The implementation of risk-based metrics, such as POs and FSOs, in future European Regulations might assist governments in conveying health goals throughout the food chain. An ALOP/FSO/PO based policy requires more than a better understanding of risks assessment or better process management of individual businesses. It requires an integrated approach in risk assessment, in management, and above all in risk communication (Manfreda, De Cesare 2014).

4. Food-borne diseases in Europe

The microbiological aspects of food safety have been studied intensively for many decades. However, even in industrialized countries, there is still a considerable burden of foodborne illness (Haavelaar et al., 2010) and increases in the recorded incidence of food-borne illness alongside the recent history of high-profile outbreaks that have been linked to food in a number of industrialised countries, have created both political and economic demands for more effective food safety controls (Martinez et al., 2007). Illness from foodborne pathogens is a significant global health concern. Population level incidence estimates, however, are uncertain due to underreporting and difficulty in attributing illness to food consumption. For instance, in the U.S. the Centers for Disease Control estimate that contaminated foodborne pathogens cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths among a population of 273 million each year. In developing countries, where it is more difficult to separate water and foodborne illness, approximately 2.2 million people die from these causes. Such a level of illness and mortality drains productivity, imposing an in-kind of tax on human energy (Hoffmann, 2010).

The response of a human population to exposures to a foodborne pathogen is highly variable, reflecting the fact that the incidence of disease is dependent on a variety of factors such as the virulence characteristics of the pathogen, the numbers of cells ingested, the general health and immune status of the hosts, and the attributes of the food that alter microbial or host status (Buchanan et al., 2000). Any consideration of microbiological dose-response relations must take into account the various modes of pathogenicity associated with different pathogenic foodborne bacteria (Buchanan et al., 2000). Human populations are highly diverse in relation to their response to infectious

agents, reflecting the population's diversity in terms of genetic backgrounds, general health and nutrition status, age, immune status, stress levels, and prior exposure to infectious agents (Buchanan et al., 2000). The final leg of the disease triangle is the influence that the food in which the pathogen is transmitted has on dose-response relations. Previously, food was generally viewed as a neutral vehicle for the pathogen and as such had little impact on dose-response relations. However, during the past few years there has been an increasing awareness of the magnitude of the impact that food matrix effects can have on the likelihood of disease (Buchanan et al., 2000).

Food can be contaminated by many human pathogenic microbes and components. According to the last annual report from EFSA, which presents the results of the zoonoses monitoring activities carried out in 2013 (Table 1), the number of reported confirmed cases of human campylobacteriosis was 214.779, that confirmed *Campylobacter* as the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union (EU) since 2005. Anyway, considering the high number of human campylobacteriosis cases, the severity in terms of reported case fatality was low (0.05 %). Salmonellosis confirmed cases were 82,694, resulting in an EU notification rate of 20.4 cases per 100,000 population. This represented a 7.9 % decrease in the EU notification rate compared with 2012, and there was a declining trend of salmonellosis in the EU/European Economic Area (EEA) in the five-year period of 2009-2013. A total of 6,471 confirmed cases of yersiniosis were reported in 2013, making it the third most commonly reported zoonosis in the EU. The EU notification rate was 1.92 cases per 100,000 population which was a decrease of 2.8 % compared to 2012. There was a statistically significant decreasing five-year trend in the EU in 2009–2013. Although the total number is lower, the cases of *Listeria* and *Verocytotoxic E. Coli* (VTEC) are increasing with a total of 1,763 and 6043 cases respectively, with an enhancement between 2012 and 2013 equal to 8.6% for *Listeria* and to 5.9% for VTEC. There was a statistically significant increasing trend of

listeriosis in the EU/EEA over the period 2009-2013. A total of 191 deaths due to listeriosis were reported in 2013 with France reporting the highest number, 64 cases. The EU case-fatality rate was 15.6 % among the 1,228 confirmed cases with known outcome. In relation to VTEC, the EU notification rate in the two consecutive years following the large outbreak in 2011 was higher than before the outbreak, possibly an effect of increased awareness and of more laboratories testing also for other serogroups than O157 (EFSA, 2015).

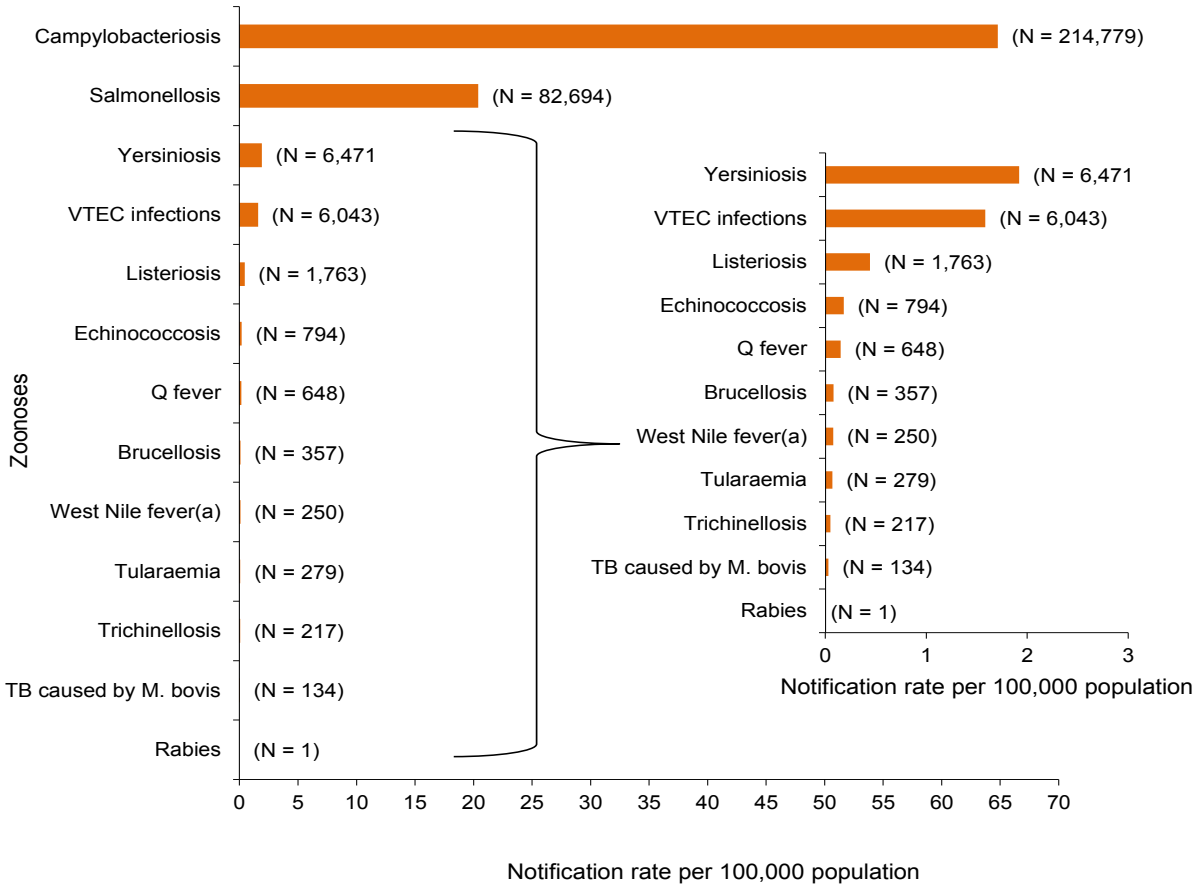

Table 1 - Reported notification rates of zoonoses in confirmed [(b,c)] human cases in the EU, 2013 (EFSA, 2015)

(a): For West Nile fever, the total number of cases was used.

(b): The ordering of the diseases is according to the notification rate.

(c): Total number of confirmed cases is indicated in parenthesis at the end of each bar.

A total of 5,196 food-borne outbreaks were reported by the 24 reporting MS in 2013, compared with 5,363 outbreaks reported in total by 25 MS for 2012. The main causative agents in these outbreaks were *Salmonella*, bacterial toxins, viruses and *Campylobacter*. However, in 2013, compared with 2012, a decrease was observed in the number of reported outbreaks caused by *Salmonella* and *Campylobacter*, whereas the number of outbreaks due to bacterial toxins and viruses increased, becoming the second and third most commonly reported causative agents in 2013. In fact, during the six-year period from 2008 to 2013 within the EU, the annual total number of *Salmonella* outbreaks has decreased markedly by 38.1 %, whereas the annual total number of outbreaks due to bacterial toxins increased by 58.9 %. Overall, the outbreaks reported by MS involved 43,183 human cases, 5,946 hospitalisations and 11 deaths. Of the nine fatalities related to strong-evidence outbreaks, three were associated with *Salmonella*, three with *Listeria*, one with *Clostridium perfringens* toxins, one with mushroom toxins and one with an unknown agent (EFSA, 2015).

Moreover in 2013, nine European Member States (MS) reported 278 outbreaks in which *Bacillus* toxins were the causative agent, representing 5.4 % of all outbreaks reported within the EU, which is more than in 2012 when 10 MS reported 259 outbreaks representing 4.8 % of all outbreaks. The overall reporting rate in the EU was 0.1 per 100,000 population. France reported the vast majority (84.9 %) of these outbreaks and reported that 2,099 human cases, 69 hospitalisations and no deaths were involved. In the 54 strong-evidence Bacillus outbreaks, ‘Mixed food’ was the most commonly implicated food vehicle (29.6 % of outbreaks), followed by ‘Vegetables and juices and other products thereof’ (11.1 % of outbreaks), and ‘Cereal products’ (9.3 %). Information on the type of outbreak was available for all the *Bacillus* strong-evidence outbreaks: 51 were general

outbreaks, and three were household/domestic kitchen outbreaks. The setting most frequently reported was ‘School or kindergarten’ (17 outbreaks), followed by ‘Restaurant, café, pub, bar, hotel’ (12 outbreaks) (EFSA, 2015).

In relation to Listeria foodborne, as previously mentioned, in 2013 27 MS reported 1,763 confirmed human cases. The EU notification rate was 0.44 cases per 100,000 population which was an 8.6 % increase compared with 2012. The vast majority of cases were reported to be domestically acquired, and 15 MS provided information on hospitalisation for all or the majority of their cases (which represented 42.1 % of all confirmed cases reported in the EU) in 2013. On average, 99.1 % of the cases were hospitalised. This is the highest proportion of hospitalised cases of all zoonoses under the EU surveillance and reflects the focus of the EU surveillance on severe, systemic listeriosis infections. Human listeriosis is a relatively rare but serious zoonotic disease, with high morbidity, hospitalisation and mortality rates in vulnerable populations. Of all the zoonotic diseases under EU surveillance, listeriosis caused the most severe human disease with 99.1 % of the cases hospitalised and 191 cases being fatal (case fatality rate 15.6 %). This also reflects the focus of EU surveillance on severe, systemic infections. In the last five years, there has been an increasing trend of listeriosis in the EU/EEA and, in 2013, the EU notification rate increased by 9.4 % compared with 2012. In 2013, seven strong-evidence food-borne outbreaks caused by *L. monocytogenes* were reported by five MS. These outbreaks resulted in 51 cases, 11 hospitalisations and three deaths, i.e. 37.5 % of all deaths due to strong-evidence food-borne outbreaks reported in 2013. Three outbreaks were related to crustaceans, shellfish and molluscs and products thereof, and other sources were: mixed salad, meat and meat products, cheese and pig meat and products thereof. In addition, one non-MS reported one strong-evidence outbreak associated with the consumption of half-fermented trout and responsible of one fatal case (EFSA, 2015).

Concerning salmonellosis cases in the EU, as in previous years the two most commonly reported *Salmonella* serovars in 2013 were *S.* Enteritidis and *S*. Typhimurium, representing 39.5 % and 20.2 %, respectively, of all reported serovars in confirmed human cases (N=73,627). Together, these two serovars accounted for 68 % of the human cases with the serotype reported. As in previous years, most of the *S.* Enteritidis outbreaks were attributed to the consumption of eggs and egg products (59.9 %), while those caused by *S.* Typhimurium were mostly attributed to pig meat and products thereof (46.7 %). In 2013, 207 outbreaks with strong evidence were caused by *S.* Enteritidis, followed by *S.* Typhimurium (66.0 % and 9.6 % of the total, respectively, excluding water-borne outbreaks). The EU case-fatality rate was 0.14 % and 59 deaths due to non-typhoidal salmonellosis were reported in the EU in 2013. The most important source of food-borne *Salmonella* outbreaks in 2013 was again eggs and egg products, followed by sweets and chocolates, although largely reported by one MS, and then pig meat and products thereof. The use of eggs and egg products is very diverse and the risk derived from egg-borne hazards such as *S.* Enteritidis is affected by the storage conditions of the eggs, such as temperature and time; however, the pooling of eggs is also important in household, food service and institutional settings. On the other hand, other foods such as broiler meat, that might also be a source of *S.* Enteritidis, are normally consumed cooked, mitigating the risk of human infection. The highest levels of non-compliance with *Salmonella* criteria generally occurred in foods of meat origin, although at low levels, and, overall, non-compliance with the *Salmonella* food safety criteria was at a level comparable to the previous years. (EFSA, 2015).

Overall *Bacillus* toxins and *Listeria* cases of food-borne diseases are increasing whithin the Europe Member States, and moreover infection diseases caused by *Listeria monocytogenes* are rare but characterized by a high mortality rate, while *Salmonella enterica* remains the second most common zoonosis in humans in the EU.

In this contest, the EU New Food Law points out the food provider is responsible for the food safety of their products, indicating that the industry has to consider the risks of these bacteri as well, as well as for the product quality. To do so, research studies relevant for their production, i.e. ready to eat products like mixed food, are needed.

5. The Ready-to-eat foods as source of food-borne pathogens

Ready-to-eat foods include various types of food products that can be categorized in many different ways. According to the definition given by the Codex Alimentarius Commission, RTE foods include any food (including beverages) that is normally consumed in its raw state or any food handled, processed, mixed, cooked, or otherwise prepared into a form in which it is normally consumed without further processing (Almualla et al., 2010). The increased consumption of these foods has led to an increase in the number of foodborne illnesses linked to them especially for fresh-cut produce, since processing steps (peeling and cutting) enhance its susceptibility to microbial growth. Minimally processed produce has been implicated in outbreaks caused by foodborne pathogens (Tian et al., 2012). Ready-to-eat and convenience food products are generally the same dishes as traditionally prepared ones, the main difference being that the industrially produced ones are stored for many days, even weeks or months, between production and consumption, while the traditional dishes are prepared shortly before they are consumed, either at home, in restaurants and in food service outlets. Not all ingredients used in the traditional dishes are suited for long term storage, and some even accelerate the quality degradation of other ingredients when mixed. Also, food safety status is challenged by mixing of ingredients. For instance, rice used in various salads may contain *Bacillus* spp. which is able to survive as spores during cooking, and regenerate during subsequent storage. Similarly, raw vegetables may contain *Salmonella, Shigella, VTEC* and other pathogens

(Jørgensen et al. 2001).

Except for *Bacillus*, the bacteria mentioned get killed if the food is sufficiently heat treated. In modern ready-to-eat products, however, raw and cooked ingredients are often mixed, and then, the bacteria present in the raw ingredients are carried into the products. Mixing of warm and cold ingredients also increases the food safety risk potential of the pathogens even more (Jørgensen et al. 2006; De Buyser et al. 2001). Food can be considered as a growth medium for bacteria, both spoilage bacteria, pathogenic bacteria, desired microbes like lactic acid bacteria and fungi needed for maturation of the products, etc. Mixing of ingredients can also increase or enhance the growth potential of the microbes. The challenges related to mixing of ingredients are often overlooked both in research and by food authorities in the development of food legislation. The industry is however well aware that raw vegetables in combination with heat treated cream, seafood and meat are typical examples of combinations which cause rapid spoilage and varying quality even if each of the ingredients are relatively stable during storage. This is because vegetables are often contaminated with non-growing bacteria, as as vegetables don't contain much organic nitrogen compounds. Pasteurised milk and cream, as well as heat treated seafood and meat, on the other hand, do usuallly not contain bacteria, but a lot of organic nitrogen compounds being essential for growth. Combination of raw vegetables and other ingredients ay therefore lead to undesired growth of bacteria, if the temperature is sufficiently high, the pH and salt content are in the range that allows growth, and there are no inhibiting compounds or process treatments in the product (Lorentzen et al., 2010).

The research activities described in this thesis aim to clarify and improve knowledge on the effect of mixing of different ingredients on safety and quality of RTE products, with reference to conventional productions. To reach this aim, spelt salad has been selected according to its composition in terms of presence of a mixture of fresh ingredients (i.e., raw vegetables and cheese) and cooked ingredients (i.e., meat, spelt and pasta).

6. *Bacillus cereus*

Bacillus cereus sensu lato is a large and diverse group of facultatively anaerobic, Gram-positive spore-forming bacteria belonging to the Firmicutes phylum. They are widely distributed in nature, being commonly found in the soil but also in the digestive tracts of vertebrate and invertebrate animals. As such, they also frequently contaminate and spoil raw and processed foodstuffs, including starchy food, vegetables, meat and dairy products. Furthermore, some members of the *B. cereus* group are food-borne pathogens, causing emetic or diarrheal disease.

The emetic syndrome is caused by cereulide, a heat-stable, acid and protease-resistant cyclic peptide toxin that is produced in the food before ingestion. Cereulide intoxication is highly associated with rice and rice products, has a short incubation time of 30 min to 6 h, and generally lasts for 6 to 24 h with symptoms of nausea and vomiting, similar to those of Staphylococcus aureus intoxication. The diarrheal syndrome is an infection with an incubation time of 8–16 h resulting from the production of heat-labile protein enterotoxins in the small intestine. Diarrheal strains can produce several enterotoxin complexes, like hemolysin BL (Hbl) and nonhemolytic enterotoxin (Nhe), or single protein enterotoxins such as cytotoxin K and enterotoxin T. The contribution of each of these toxins to the disease is not exactly known, and it remains to be investigated whether they act separately or in combination. Diarrheal disease symptoms include abdominal pain, watery diarrhea and, occasionally, nausea and emesis (Luu-Thi et al., 2014).

Six species are commonly distinguished within the *Bacillus cereus* group based on specific phenotypic properties, and the foodborne pathogens are designated as *B. cereus* sensu stricto. However, more recent DNA-based methods have revealed that these phenotypically delineated species do not form homogeneous genetic groups. An analysis

carried by Hue Luu-Thi et al. in 2014 showed that *B. cereus* sensu stricto, which groups the strains that can cause foodborne illness, is genetically and phenotypically heterogeneous, being dispersed over four phylogenetic groups. Moreover, strains from phylogenetic group VII, which have been recently assigned to the novel species Bacillus cytotoxicus, are also foodborne pathogens (Table 2).

Phylogenetic group	Growth temperature	Species
I	10-43 °C	*B. pseudomycoides*
II	7-40 °C	*B. thuringiensis II or B. cereus II*
III	15-45 °C	*B. thuringiensis III, B. cereus III or B. anthracis*
IV	10-45 °C	*B. thuringiensis IV or B. cereus IV*
V	8-40 °C	*B. thuringiensis V or B. cereus V*
VI	5-37 °C	*B. weihenstephanensis, B. mycoides or B. thuringiensis VI*
VII	20-50 °C	*B. cytotoxicus*

Table 2 - Species composition and growth temperature range of seven phylogenetic groups of *B. cereus* sensu lato (Luu-Thi et al., 2014).

A large comparative study of hundred *B. cereus* strains from various sources revealed that strains producing emetic toxin had higher heat resistance than diarrheal and environmental strains. When the same strains were later typed it was concluded that spore heat resistance was lowest for group VI, highest for groups III and VII, and intermediate for groups II, IV and V. However, even this large study leaves some questions unanswered. For example, the comparison between phylogenetic groups was based on analysis of the

heat resistance of the spores at only one single temperature (90 °C), and it is therefore not known whether spores from the different genetic groups have different values in relation to the parameter reflecting temperature sensitivity of the heat resistance.

7. Listeria monocytogenes

The bacterial genus *Listeria* currently comprises 10 species, but human cases of listeriosis are almost exclusively caused by the species *Listeria monocytogenes* (*L. monocytogenes*). *Listeria* species are ubiquitous organisms that are widely distributed in the environment, especially in plant matter and soil. The principal reservoirs of *Listeria* are soil, forage and water. Other reservoirs include infected domestic and wild animals. The main route of transmission, to both humans and animals, is through consumption of contaminated food or feed (EFSA, 2014). There are at least 13 serotypes of *L. monocytogenes;* however, only serotypes 4b, 1/2a, and 1/2b are known to commonly cause human infection. Serotype 4b has been the type most commonly responsible for invasive listeriosis, whereas serotypes 1/2a and 1/2b have been the dominant isolates in outbreaks of gastroenteritis (Ooi, Lorber 2005). In humans, severe illness mainly occurs in developing fetuses, newborn infants, the elderly and those with weakened immune systems. Symptoms vary, ranging from mild flu-like symptoms and diarrhoea, to life-threatening infections characterised by septicaemia and meningoencephalitis (EFSA, 2014). The symptoms most frequently reported are fever (in 60%–100% of patients), diarrhea (in 33%–88%), arthromyalgia (in 20%–100%), and headache (in 15%–88%). In most outbreaks, 170% of patients had at least 1 gastrointestinal symptom (e.g., diarrhea, vomiting, nausea, and/or abdominal pain). The incubation period from the time of food ingestion to the onset of symptoms is usually 24 h or less, but it has ranged from 6 h to 10

days (Ooi, Bennett 2005). Virulent strains of *L. monocytogenes* may invade the gastrointestinal epithelium and enter phagocytic host cells, where the bacteria are able to survive and multiply, hereby permitting access to the brain or the fetus in pregnant women (Nørrung, 2000). The infection can spread to the fetus, leading to severe illness at birth or death in the uterus, resulting in abortion. Illness is often severe with high hospitalisation and mortality rates. Human infections are rare yet important, given the associated high mortality rate. These organisms are among the most important causes of death from food-borne infections in industrialised countries (EFSA, 2014). The highest incidence is among individuals at increased risk due to alterations or deficiencies in the normal immune response as a result of immunosuppressive drugs, cancer, AIDS, etc. The very young and the very old may also be affected and the unborn child is particularly at risk, because listeriosis may lead to abortion, stillbirth or septicaemia and meningitis in neonates (Nørrung, 2000).

8. Salmonella enterica sub. enterica

Two major clinical syndromes caused by *Salmonella* infection in humans are enteric or typhoid fever and colitis/diarrheal disease. Enteric fever is a systemic invasive illness caused by the exclusively human pathogens *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi A and B. Clinical manifestations include fever, headache, abdominal pain, and transient diarrhea or constipation, and infection can produce fatal respiratory, hepatic, spleen, and/or neurological damage. Without treatment, the mortality is 10 to 20%, decreasing to 1% among patients treated with the appropriate antibiotics. In there are many nontyphoidal *Salmonella* (NTS) strains that cause diarrheal disease in humans and can, in addition, infect a wide range of animal hosts. According to data

obtained from the World Health Organization, *S.* Enteritidis and *S.* Typhimurium are the two serovars most commonly isolated in clinical practice. In all regions except North America and Oceania, *S.* Enteritidis is more prevalent than *S.* Typhimurium. In an immunocompetent host, NTS serovars cause self-limiting diarrhea that has an untreated case fatality rate of approximately 0.1% in developed countries (Fabrega, Vila 2013). Enteric infection with *Salmonella* cannot be reliably clinically distinguished from that caused by other enteric bacterial pathogens. Patients typically present an acute onset of fever, cramping, abdominal pain, diarrhea with or without blood associated with inflammation of the large bowel, and very often nausea and vomiting as well; there is a wide spectrum of severity of illness. Disease usually occurs after the ingestion of greater than 50,000 bacteria in contaminated food or water and after an incubation period of approximately 6 to 72 h, which depends on host susceptibility and inoculum. Approximately 5% of individuals with gastrointestinal illness caused by NTS develop bacteremia, a serious and potentially fatal problem. Bacteremia is more likely to occur in young children, immunologically compromised patients, and patients with comorbid medical conditions (e.g., HIV, malaria, or malnutrition) (Fabrega, Vila 2013). On the other hand, the mortality rate due to NTS is as high as 24% in developing countries, where *Salmonella* infections contribute to childhood diarrhea morbidity and mortality and are a common cause of hospital admission among children, being among the most frequent etiological agents causing bacteremia (20% of cases) (Fabrega, Vila 2013) .

The genus *Salmonella* consists of two species, *S. enterica* and *S. bongori*. In turn, *S. enterica* can be divided into six subspecies: *S. enterica* subsp. enterica (I), *S. enterica* subsp. salamae (II), *S. enterica* subsp. arizonae (IIIa), *S. enterica* subsp. diarizonae (IIIb), *S. enterica* subsp. houtenae (IV), and *S. enterica* subsp. indica (VI). *S. bongori* (V) was initially considered to be another subspecies but it has now been classified separately from the rest of the *S. enterica* lineages as a distinct species. *S. bongori* as well as

subspecies II, IIIa, IIIb, IV, and VI are rarely isolated from clinical specimens but rather are found principally in cold-blooded vertebrates and in the environment. Therefore, almost all *Salmonella* organisms that cause disease in humans and domestic animals belong to *S. enterica* subspecies enterica (I). Alternatively, *S. enterica* strains can also be classified on the basis of the O (lipopolysaccharide [LPS]) surface antigen into 67 serogroups and into 2,557 serotypes or serovars when strains are differentiated by both their O and H (flagellar) antigens. Among them, 1,531 serovars are recognized to belong to subspecies I. Nowadays, according to the current classification, the familiar names given to serovars, such as *S. enterica* serovar Typhimurium, Enteritidis, or Choleraesuis, are maintained and not replaced by their antigenic formulas (Fabrega, Vila 2013). The common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals, which may result in a variety of foodstuffs, of both animal and plant origin, becoming contaminated with faecal organisms either directly or indirectly. Transmission often occurs when organisms are introduced into food preparation areas and are allowed to multiply in food, e.g. due to inadequate storage temperatures, inadequate cooking or cross-contamination of RTE food. The organism may also be transmitted through direct contact with infected animals or humans or faecally contaminated environments. Infected food handlers may also act as a source of contamination for foodstuffs (Burnett, Beuchat 2001).

OBJECTIVE

Even if microbiological criteria for *Salmonella and L. monocytogenes* RTE products are included in the European Regulation, these parameters are not in any case enough in limiting or decreasing cases of both foodborne disease due to the contamination of foods, and in particular RTE products, whithin the European Union, as described in the last EFSA report that includes data collected up to 2013. Moreover current EU regulations on food safety do not include microbiological criteria for *Bacillus cereus* in RTE products.

The aims of this research study is to explore the opportunity to set up PO parameter for specific food/risk combination to propose for the future European Regulations. In particular, this approach has been developed for RTE products that rapresent a significant target for modern food habit. In fact, the ingredients to make RTE products are prepared and, occasionally, stored in conditions where they are likely to spoil rapidly and where pathogenic bacteria may have better opportunities to grow. Moreover, cutting and mixing different ingredients increases the contact between microbes being present on the surface of the ingredients, and essential micronutrients being released from the inner part to the surface, leading to increased probability of growth. Finally chemical and physical degradation processes like browning and drying are also faster in cut and mixed ingredients than in whole, single ingredients.

1. Fate of Salmonella enterica in a mixed ingredient salad containing lettuce, Cheddar cheese, and cooked chicken meat

Ready-to-eat salads containing mixtures of fresh-cut leafy vegetables and various nonproduce ingredients are provided to consumers through retail outlets and the food service trade. The formulation of mixed ingredient salads offered in specific markets is affected by cultural and socioeconomic factors. Added ingredients may consist of other minimally processed items such as vegetables or fruits, nuts, cereals, dairy products, cooked seafood, and cooked or cured meats. Mixed ingredient salads are distributed in formats ranging from bulk containers to single-serve polypropylene trays that may include packets of dressings or sauces for addition immediately prior to consumption. Although some ingredients may be subjected to treatments that ensure microbiological stability, mixed ingredient salads are highly perishable products that must be refrigerated to maintain quality and safety during distribution. Centralized processing is possible where proximity to significant markets and the availability of suitable transpor- tation systems allow timely delivery to consumers. Alternatively, products may be formulated at the point of sale using ingredients obtained from external suppliers. This approach is increasingly common in urban retail outlets where there is strong impetus to provide customers with varied and convenient food products requiring little or no additional preparation.

Fruits and vegetables may become contaminated with enteric bacterial pathogens such as Salmonella enterica during production or postharvest handling (Brandl, 2006). Although infrequent, outbreaks of salmonellosis associated with the consumption of fresh produce, including fresh-cut vegetables, have been documented in many parts of the world. Surveys of market fresh produce indicate that prevalence rates for Salmonella are

generally low, ranging from 0.1 to 2.3% in European Union countries (Berger et al., 2010), 0.15% in the United States (Achen, 2012), and 0.16% in Canada (Arthur et al., 2007). The surface of actively growing plants is a nutrient poor environment (Lindow et al., 2002). Research on the behavior of Salmonella in the phyllosphere suggests that some growth is possible within sheltered anatomical features (such as stomata) where nutrients are released by the plant, provided sufficient water is available (Brandl, 2006). However, epidemiological evidence that outbreaks of foodborne illness have resulted from the growth of enteric bacterial pathogens in whole fresh produce is scant. In contrast, wounds inflicted to plant tissues during processing of fresh-cut produce may induce leakage of cellular fluids containing nutrients that can support active bacterial proliferation (Harris et al., 2003). *S.* enterica Typhimurium can grow in cellular fluids extracted from common vegetables, including tomato, broccoli, bell pepper, jalapeño pepper, and lettuce, although both the rate and extent differed with vegetable type (Nutt et al., 2003). Direct experimental evidence has revealed that *Salmonella* populations can increase during storage of fresh- cut lettuce at growth-permissive temperatures (Manios et al., 2013; Sant'Ana et al., 2012; Tian et al., 2012). The extent of growth reported in individual studies differs, likely because of differences in experimental protocols and their influence on biotic factors (e.g., lettuce variety and level and composition of background microflora) and abiotic factors (e.g., processing treatment, packaging system, and storage temperature) known to affect the fate of enteric bacterial pathogens in fresh-cut produce.

Research on the fate of enteric bacterial pathogens in fresh-cut produce has largely been carried out in experimental systems consisting of a single commodity or mixtures of fresh-cut fruits or vegetables. Some nonproduce items used in the formulation of mixed ingredient salads are known to support rapid growth of bacterial pathogens. For example, estimates of maximum exponential growth rates for Salmonella derived from kinetic data in the Combined Database for Predictive Microbiology (ComBase; http:// www.combase.com)

were 0.50 Log units per hour at 15°C in produce and 0.13 Log units per hour in cooked chicken, which suggests that cooked chicken is a far better substrate for growth. The blending of fresh-cut leafy vegetables with ingredients that support rapid growth could therefore influence the fate of *S. enterica* during storage and distribution of mixed ingredient salads. Hence the purpose of the present work was to examine the effect of nonproduce food ingredients on the behavior of the bacterial species present on lettuce tissues.

2. Relevance of spelt salad as source of Bacillus cereus and Listeria monocytogenes foodborne disease

The study of the ecological and phenotypic properties of the different phylogenetic groups will provide a better insight into the causes of *B. cereus* group foodborne illness, and allow the development of more effective control measures. Since *B. cereus* is a sporeformer, foods that are mildly heated and subsequently refrigerated are a particular concern, not only with regard to foodborne illness but also food spoilage (Luu-Thi et al., 2014).

The probability of developing foodborne illness upon consumption of a given concentration of *B. cereus* depends on the type of strain (psychrotrophic/mesophilic), the physiological state of the microorganisms (cell/spore), the food product and the helth of the consumer (Daelman et al., 2013). Foodborne illness attributed to *B. cereus* spp. generally occurs when enterotoxigenic strains multiply to numbers ≥ 10^6 CFU/g (Samapundo et al., 2011). Nevertheless, it is generally accepted that *B. cereus* concentrations of more than 10^5 CFU/g (spores or cells) are unacceptable; infact spores are able to pass the stomach more easily than vegetative cells; subsequently they can

germinate, colonize the intestine and produce enterotoxin. For cells the situation is different. If a product contains 10^5 cells/g during any stage of production or shelf life, production of the emetic toxin is possible. And since this toxin is heat stable it will still be present after pasteurisation (Daelman et al., 2013). As a result of the ubiquitous occurrence of its spores, it is practically impossible to prevent the contamination of food products by *B. cereus* group spp. Reports can indeed be found in literature which highlight the frequent isolation of *B. cereus* group spp. from several types of products i.e. ready-to-eat (RTE) food products. RTE food products have of late gained much interest owing to their mild heat processing (typically pasteurisation) which leads to the possibility of bacterial spores surviving. These mild heat treatments serve to inactivate the vegetative flora but may also inadvertently activate the surviving spores. Depending on their psychrotolerance and the nature of the intrinsic and extrinsic environments, the activated spores may in turn germinate, grow out, spoil and/or produce emetic or diarrhoeal enterotoxins in the food in the absence of competition during refrigerated storage. Alternatively spores surviving a mild heat treatment may also be consumed in the food and due to their inherent resistance to low pH and proteases, they can pass intact through the stomach to the small intestines were they could potentially germinate, grow out and cause illness via toxin production (Samapundo et al., 2011).

Concerning *Listeria,* peculiar property that affects its food-borne transmission is the ability to multiply at low temperatures, whithin a range from 1°C to 45°C. *Listeria monocytogenes* is a ubiquitous, psychrotrophic pathogen that may contaminate different foods. Most human listeriosis case appear to be caused by consumption of refrigerated RTE foods that are contaminated with high levels of *L. monocytogenes.* While initial *L. monocytogenes* levels in contaminated foods are usually low, the ability of *Listeria monocytogenes* to survive and multiply at low temperatures allows it to reach levels high enough to cause human disease. Contamination of RTE products with *L. monocytogenes*

may occur at several stages before consumption (Skalina et al., 2010), and can be found in raw foods and in processed foods which are contaminated after processing. Cooking at temperatures higher than 65 °C destroys *Listeria*, but the bacteria are able to multiply at temperatures as low as +2/+4 °C, which makes presence of *Listeria* in RTE foods, with a relatively long shelf-life, of particular concern (EFSA, 2014).

For example, a study undertaken in the UK as part of the European Commission (EC) coordinate programme for 2005 to determine the frequency and level of *L. monocytogenes* on pre-packaged RTE mixed salad containing meat or seafood ingredients from retail premises, showed that 0.2% of the samples exceeded EC legal food safety criteria due to the presence of *L. monocytogenes* in excess of 100 CFU/g. Overall contamination of *Listeria spp.* and *L. monocytogenes* found in samples of mixed salads in the UK was 10.8% and 4.8%, respectively (Skalina et al., 2010). The infective dose of *L. monocytogenes* is not yet known but is believed to be strain and host dependent. Based on current epidemiological information from several countries, a concentration of *L. monocytogenes* not exceeding 100/g of food at the time of consumption is of low risk to the consumers. In order to establish such levels, knowledge of the shelf life and behavior of *L. monocytogenes* in the food during prevailing storage and distribution conditions is needed (Nørrung, 2000).

Hence, RTE spelt salad has been selected to investigate quality and safety changes during mixing of conventional ingredients and preparation of the final product, because this RTE product contains both fresh and cooked ingredients and the knowledge of the impact of fresh ingredients in terms of pathogenic bacteria transmission in the final product was a key goal of this reserch study. The process includes cooking and cooling of spelt and peas, and the addition of raw vegetables and fresh cheese which are not treated in any way before consumption. Before packaging the fresh and cooked ingredients are mixed with a brine containing lemon decreasing the overall product pH and supporting the

product stability. The safety aspects investigated were related to presence, count, persistence and survival of biological hazards selected according to their relevance for public health, epidemiological characteristics, and data needs. Such hazards were *Bacillus cereus* and *Listeria monocytogenes* in pasta salad. In the selected product the standard ingredients were also tested in order to assess variability in the safety of the ingredients.

The quality parameters addressed to spelt salad were pH and number of microorganisms indicative of the process hygiene, like lactic acid bacteria (LAB), total mesophilic count (TMC), enterobacteriaceae (ENT) and psycrophilic bacteria (PSI) and *Listeria* spp.

Both safety and quality parameters were determined using validates methods, mainly represented by ISO protocols, as well as molecular techniques.

3. Target of research activities

The specific targets perfomed during my PhD has been:

1. to study the behavior of *Salmonella* in RTE product.
2. to assess changes in food safety and quality parameter occurring in RTE spelt salad when different ingredients are combined, different processing procedures are applied, variables in distribution and storage occur.
3. to define performance objectives (POs) for *Bacillus cereus* and *Listeria monocytogenes* in selected igredients added to RTE mixed spelt salad packaged under air or modified atmosphere.
4. Setting of sampling plans and risk-based metrics (POs and FSOs) for *Bacillus cereus* and *Listeria Monocytogenes* in spelt salads.

MATERIALS AND METHODS

1. Study on the behavior of Salmonella in RTE product.

I. RTE product selected fo the experimental study.

A model system was developed to examine the fate of *S. enterica* applied to lettuce tissues placed in contact with Cheddar cheese or cooked poultry meat. Observations derived from experimentation with the model system were verified in a commercial salad product containing fresh-cut lettuce and both ingredients.

II. Microorganisms and inoculum preparation.

Experiments were performed with inoculum prepared by mixing five *S. enterica* serovars: *S*. Agona (alfalfa sprout outbreak, British Columbia, Canada, 1996), *S*. Typhimurium (ATCC 14028), *S.* Enteritidis, *S*. Brandenberg, and *S*. Kentucky (poultry litter isolates, Pacific Agri-Food Research Centre culture collection). Stock cultures were preserved at -80°C in glycerol, and working cultures were maintained at 4°C on tryptic soy agar (TSA; Difco, BD, Sparks, MD). Inoculum was prepared from cultures grown overnight in tryptic soy broth (TSB; Difco, BD) at 37±1°C. Equal volumes of the cultures were mixed, the cell suspension was spun at 3,000 rpm for 10 min at room temperature, and the resulting pellet was resuspended in sterile distilled water and diluted as required for the experiments.

III. Model system studies: effect of cooked chicken or cheese on the fate of S. enterica on romaine lettuce tissue.

Romaine lettuce was obtained from a local retail outlet. The outer leaves were discarded, and several inner leaves were separated from the stalks. Square pieces of tissue (4 by 4 cm) were removed from the leaves with a sterile scalpel blade and placed adaxial side up in individual sterile petri plates in a biosafety cabinet. Each piece was inoculated with 16 5-ml drops of inoculum applied in an array consisting of four columns and rows to achieve a total inoculum level of 10^4 CFU. After 1 h of drying, a scalpel blade was used to score the surface of half of the inoculated lettuce pieces to simulate tissue injury. The blade was pulled gently across the surface (to avoid cutting through the tissues) eight times on each lettuce piece.

Scored and unscored lettuce pieces were assigned to one of three treatments. Controls consisted of lettuce pieces incubated alone. For the lettuce plus cheese treatment, commercial vacuum-packaged mild Cheddar cheese bricks (approximately 4 by 4 by 20 cm) were cut into 0.2- to 0.3-cm-thick slices with a sterile sharp knife, and one slice of cheese was placed on each piece of lettuce. For the lettuce plus cooked chicken treatment, raw chicken breast was placed in boiling water until the temperature measured with a thermocouple inserted in the center of the breast reached 70°C. After cooling to 4°C the meat was sliced into pieces (4 by 4 by 0.2 to 0.3 cm). One piece of cooked chicken was placed on each piece of lettuce. The petri plates containing samples from each treatment were wrapped with Parafilm and divided into two lots, which were placed in incubators set at 6 or 14°C.

S. enterica populations in three samples from each treatment were determined at the outset and after 3 and 6 days of incubation at both temperatures. Each sample was

blended with 160 ml of buffered peptone water (BPW) in a laboratory stomacher (Seward Co., Worthington, UK) for 2 min at high speed. Decimal dilutions of the homogenates were prepared in BPW, and duplicate aliquots (0.1 ml) were spread onto xylose lysine deoxycholate (XLD; Oxoid, Nepean, Ontario, Canada) agar plates and incubated for 24 h at 37°C. The selectivity of XLD agar was confirmed by testing three to five typical colonies with the latex agglutination test for the presumptive identification of *Salmonella* spp. (Oxoid). Lactic acid bacteria populations were estimated on de Man Rogosa Sharpe (MRS; Oxoid) agar incubated at 30°C for 48 h. Populations were expressed as log CFU per square centimeter of lettuce.

IV. Model system studies: effect of cooked chicken or cheese on the fate of S. enterica on romaine lettuce washed in chlorinated water.

Four sterile glass containers (20-liter volume) were placed in a biosafety cabinet. One container was filled with a 70 mg/liter (ppm) free chlorine solution prepared from a 10.8% sodium hypochlorite solution (Javex 12, Colgate-Palmolive Inc., Toronto, Ontario, Canada). Final chlorine concentration was adjusted with a test kit (model CN-66, Hach, Loveland, CO). The remaining three containers were filled with sterile distilled water.

A grid of squares (4 by 4 cm) was drawn on the adaxial surface of several romaine lettuce leaves with a permanent marker. Sixteen 5-ml drops of inoculum were placed in each marked square (10^4 CFU of *S. enterica*) and the leaves were held for 1 h in the biosafety cabinet. Stainless steel alligator clips affixed to glass rods were then used to suspend the leaves (from the stem end) in the containers for complete immersion in the treatment solutions. Half of the inoculated leaves were submerged in the 70-mg/liter chlorine solution for 1 min followed by 1 min in sterile water. The other half of the leaves were twice immersed in sterile water for 1 min. The leaves were held in the cabinet for 1 h to remove excess water, and the marked square tissue sections were excised with a

scalpel blade. Individual pieces were placed in petri plates and overlaid with Cheddar cheese or cooked chicken as described above. The samples were divided into two lots, which were placed in incubators set at 6 or 14°C.

S. enterica populations were measured in three samples from each treatment at the start of the experiments and after 6 days of incubation at both temperatures. Each sample was pummeled with 160 ml of BPW in a laboratory stomacher for 2 min at high speed. Decimal dilutions of the homogenates were prepared in BPW, and duplicate aliquots (0.1 ml) were spread onto XLD agar plates, which were incubated for 24 h at 37°C. Lactic acid bacteria populations were estimated on MRS agar plates incubated at 30°C for 48 h.

V. Model system studies: measurement of RH.

The relative humidity (RH) above the samples of intact or scored romaine lettuce tissues overlaid with Cheddar cheese and cooked chicken was measured with a Humidat-TH2 Thermoconstanter hygrometer (Novasina AG, Lachen, Switzerland). For measurements of samples consisting of combined ingredients, lettuce and the second component were added to the test chamber in the ratio used for the experiments. Measurements were taken after 60 min at 21°C.

VI. Fate of S. enterica in a commercial mixed ingredient salad.

Eight mixed ingredient salads packed in polypropylene clamshell-type containers were obtained on the day of manufacture from a local retail outlet and were transported to the laboratory in a cooler (approximately 15 min driving distance). Each clamshell was labeled with a ‘‘best before’’ date of 3 days postmanufacture. The salads consisted of a layer of cut romaine lettuce (approximately 150 g) overlaid with shredded Cheddar cheese (approximately 50 g), cooked chicken strips (approximately 150 g), and four to six cherry tomatoes. Core temperatures were measured with a thermocouple probe upon arrival at

the laboratory. The cooked chicken and Cheddar cheese were removed from each salad, and the uppermost layer of lettuce (approximately 50 g) and tomatoes was discarded. The clamshells containing the remainder of the lettuce and retained ingredients were held at 4°C until used (within 2 h of delivery to the laboratory). Discarded lettuce was replaced with 50 g of cut romaine lettuce inoculated with *S. enterica* using methods described by Delaquis et al. in 2002. The outer leaves of whole romaine lettuce were removed, and the heads were cut into pieces (4 by 4 cm) that were placed in a large autoclave bag. All further handling was done in a biosafety cabinet. Inoculum was added in a ratio of 1 ml to 100 g of lettuce, and the contents were mixed by inverting the bag several times. After 1 h at room temperature, the lettuce was placed in a 70-mg/liter (free) chlorine solution at 4°C for 1 min, dipped in sterile distilled water for 1 min, and spun in a salad spinner to remove excess water. Fifty grams of the inoculated lettuce was then layered over the remaining lettuce in each clamshell. The shredded Cheddar cheese and cooked chicken were returned to half of the clamshells; the rest of the clamshells received no further treatment. Two clamshells with and without added Cheddar cheese and cooked chicken were stored for 3 days at 6 or 14°C.

S. enterica populations were measured in samples withdrawn at the start of the experiments and after 6 days of incubation at both temperatures. Approximately one third of the clamshell contents was removed, cut into small pieces with scissors, and mixed. Twenty-five-gram samples were pummeled with 225 ml of universal preenrichment broth (Difco, BD) in a laboratory stomacher. Suitable dilutions were spread onto XLD agar to estimate *S. enterica* populations as described above. The initial homogenate was also placed in an incubator at 37°C for 24 h for enrichment to confirm the presence of *S. enterica* when populations may have fallen below the limit of detection afforded by the plating assay (10 CFU/g). Fluids from the enrichments were spread onto XLD agar for the detection of *S. enterica*. Populations of lactic acid bacteria were estimated on MRS agar

incubated at 30°C for 48 h, and total aerobic populations were grown on standard plate count agar (Difco, BD) incubated at 30°C for 24 h.

A molecular fingerprinting technique was used to identify *S. enterica* serovars recovered from the stored salads. Five typical colonies on XLD agar plates associated with each sample were picked and transferred to TSA for purification. A single colony was then transferred to TSB and incubated for 24 h at 37°C. DNA was extracted using an UltraClean microbial DNA isolation kit using procedures described by the manufacturer (MO BIO Laboratories, Inc., Carlsbad, CA). Enterobacterial repetitive intergenic consensus (ERIC) PCR was performed with the single ERIC-2 primer (59- AAGTAAGTGACTGGGGTGAGCG-39) (Lim et al., 2005). The 50-ml PCR volume was composed of 3 ml of template DNA, 5 ml of 10x PCR buffer plus 2.0 mM (final) $MgCl_2$ (Lucigen, Middleton, WI), 4 ml of ERIC-2 primer solution, 1 ml of 10 mM deoxynucleoside triphosphate mixture (Fermentas, Thermo Scientific, Ottawa, Ontario, Canada), 0.25 ml of Econo Taq DNA polymerase (5 U/ ml; Lucigen), and 36.8 ml of nuclease-free PCR grade water (Millipore, Billerica, MA). Amplification was performed in a programmable thermocycler using the following program: preliminary denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 49°C for 1 min, and 72°C for 3 min, followed by a final extension at 72°C for 10 min. The amplicons were separated by electrophoresis on 1.5% agarose gels (0.5x Tris-borate-EDTA buffer at 60 V for 90 min) and stained with 10 mg/ml ethidium bromide for 10 min. Images were captures with a UV gel imaging system (Lim et al., 2005).

VII. Statistical analysis.

Two independent replicate experiments were performed with the model system (n = 3) using lettuce obtained on different dates and with commercial salads (n = 2) manufactured on separate days. An analysis of variance was performed using the linear model procedure of SAS (SAS Institute Inc., Cary, NC), and differences between treatments were assessed using LSMEANS ($P < 0.05$).

2. *Study on changes in food safety and quality parameter occurring in RTE spelt salad when different ingredients are combined, different processing procedures are applied, variables in distribution and storage occur.*

I. Product used as model system

The model system used in this study is spelt salad made with steam cooked spelt (47.5% w/w) and peas (10.45 % w/w), cut Edamer cheese (7.14% w/w), fresh cut celery (8.75 w/w) washed in 200 ppm of chlorine solution, canned pepper (8.57% w/w), black olives (10.45% w/w), fresh basil (0.94% w/w) and a brine containing sunflower oil (4.75 % w/w), black pepper (0.05% w/w), salt (0.8% w/w) and lemon juice (0.94% w/w). The salad sold in the market is packaged under modified atmosphere (MAP) containing 50% CO2 and 50% N2. The shelf-life of the product stored refrigerated is 12 days. The process production flow chart is illustrated schematically in Figure 2.

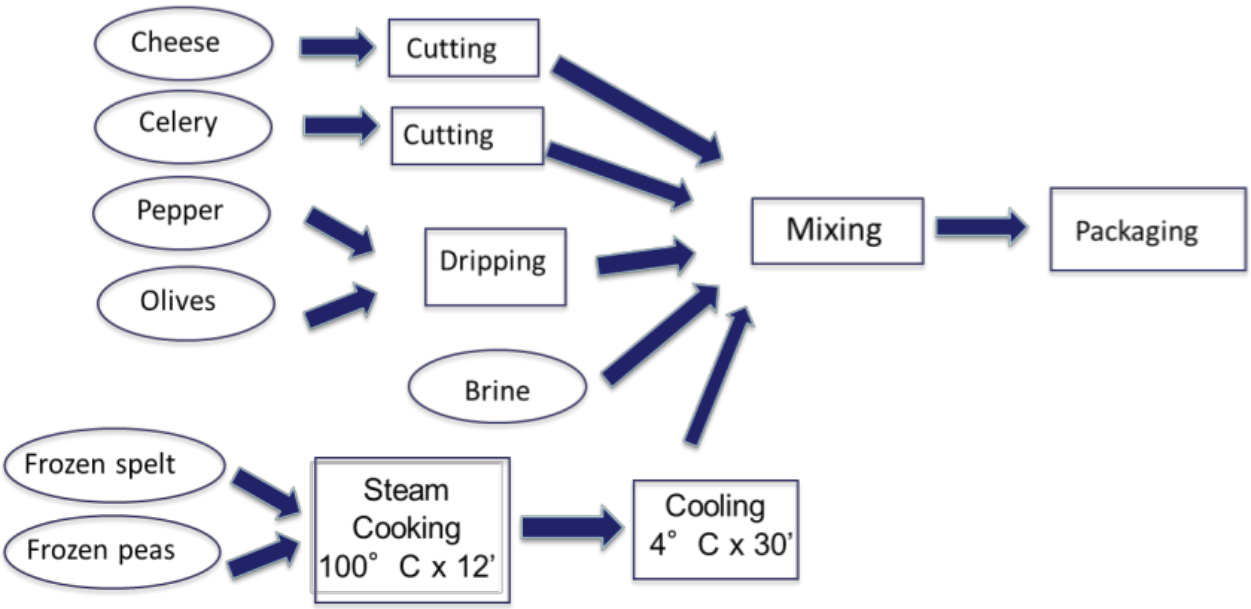

Figure. 2 Spelt salad flow chart

The steam cooking of spelt and peas is performed at 100°C for 12 minutes followed by cooling at 4°C for 30 minutes. Then, spelt and peas are mixed with all the other ingredients before packaging.

II. Microbiological characterisation of spelt salad as currently produced by the industry.

Fifteen lots of spelt salad were tested to estimate the intra-lot and inter-lot variability in pH, number of mesophilic bacteria Total Microbial count (TMC), *Lactic Acid bacteria* (LAB), *Enterobacteriaceae* (ENT) and Psychrotrophic bacteria (PSY), *Listeria* spp. (LIS) and *L. monocytogenes* (LM).

Five different lots of each product were sampled in different days in the period October 2012 – February 2014. Fourteen sample units (packs) for each lot were taken at the end of the production process and cooled at 0 - 4°C overnight before dispatching.

Transports of the lot samples were made in refrigerated trucks that were also used for distribution to customers. The temperature in the boxes that contained the samples was recorded using Dataloggers (model Escort iMiniPlus PDF, Cryopack US) (accuracy ±0.3°C). At their arrival in the laboratory two sample units (packages) per each lot were analysed, while the other packages were divided in two groups of six, which were stored up to 18 days (the end of shelf life given by the producer) at +6±0.5°C and +14±0.5°C. The temperature in the storage cabinets (Cooled Incubator VELP Scientific Model FOC 225I), which have a tolerance of ±0.5°C, was controlled using the Dataloggers described above and every day with a MIG (Mercury in glass) thermometer. Two packs per each lot and storage temperature were taken at 7, 14 and 18 days and 25 g of analytical samples were taken to represent the different components of the salads. Two analytical samples for each package were analysed. The standard methods ISO

2917:1999 (Anonymous, 1999), ISO 4833:2004 (Anonymous, 2004), ISO 15214:1998 (Anonymous 1998), ISO 21528-2:2004 (Anonymous, 2004), ISO 17410:2003 (Anonymous, 2003), ISO 11290-1:2004 (Anonymous, 2004a) and ISO 11290-2:2004 (Anonymous, 2004b) were used for the measurement of pH, TMC, LAB, ENT, PSY, LIS and LM, respectively.

For the pH measurement, 20 g of the product were homogenized in 20 ml of distilled water by stomaching for 2 minutes at normal speed. The pH-meter was calibrated using two buffer solutions with pH values of 4 and 7, and a temperature in a range of 20±2 °C. pH values were obtained from the bags with homogenized sample reading the pH directly from the instrument (Crison, pH-meter 507).

According to the ISO methods, 10 g of the product were homogenized in 90 ml of Physiological solution by stomaching for 2 minutes at normal speed. From the bags with homogenized samples, 1 ml of the initial suspension was transferred in a tube containing 9 ml of Physiological solution, to make serial dilutions of the sample. From each tube, 1 ml was taken and transferred in double in a sterile Petri dish. Approximately 15 ml of the “plate count agar” (PCA) (Oxoid, Milan, Italy) for TMC and “de Man, Rogosa and Sharpe” (MRS) (Oxoid, Milan, Italy) for LAB count, and 10 ml of the “Violet Red Bile Glucose Agar” (VRBGA) (Oxoid, Milan, Italy) for ENT count, already prepared and placed in a water-bath at 44 °C to 47 °C, were pour into each Petri dish. The inoculum was carefully mixed with the medium by rotating the Petri dishes and allowed the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface. The prepared dishes of PCA and MRS were inverted and placed in the incubator at 30°C±1°C for 72h±3h, while 15 ml of VRBGA were overlaied on the VRGBA plates already solidified, before incubation at 37°C for 24h±2h. After the specified period of time, the colonies in each dish were counted. ENT colonies were purple/pink coloured and surrounded by purple halos.

For Psychrotrophic bacteria count, according to the ISO method 17410:2003, from the serial-dilutions tubes, 100 µl of the initial suspension were spreaded in double onto plates of PCA (Oxoid, Milan, Italy). Plates were inverted and incubated at 6,5°C for 10 days. PCA was prepared pouring 20 ml portions of the complete medium into sterile Petri dishes and allow to solidify. After the specified period of time of incubation, the colonies in each dish were counted.

Qualitative changing in the product were also evaluated during the storing period of time at 6 and 14 °C.

According to the ISO 11290-2:2004, for the LM detection and enumeration, 10 g of the sample were diluted in 90 ml of Buffered Peptone Water (BPW) (Biolife, Milan, Italy) as primary enrichment broth. Then, the initial suspension was kept for 1±5 min at 20±2°C in order to resuscitate the stressed microorganisms. For the enumeration, from the primary enrichment in BPW, 0.1 ml of the suspension were transferred to each of two plates of Agar Listeria Ottaviani-Agosti (ALOA) (Biolife, Milan, Italy) and spread over the surface of the agar plates. Plates were incubated at 37°C for 24-48 h and then examined for the presence of colonies of LM. After 48 h the characteristics colonies of LM grow as green colonies surrounded by a narrow, clear, light zones of β-haemolysis. Colonies with these kind of characteristics were confirmed as LM and counted.

According to the ISO 11290-1:2004, for the LM detection, BPW dilutions were incubated at 30°C for 24h±2h. After the incubation, 0.1 ml of the culture was transferred to a tube containing 10 ml of Fraser broth (secondary enrichment medium) (Biolife, Milan, Italy), and incubated for 48h±2h at 37°C. Both primary and secondary enrichment cultures were inoculated onto the surface of the selective plating medium (Oxford agar) (Oxoid, Milan, Italy) so that well-separated colonies were obtained. Then, Oxford agar was incubated for 48h±2h at 37°C. After incubation, dishes were examined for the presence of

suspected LM colonies. The suspected LM colonies were purified and tested for the positivity of mobility, catalase and ramnosio reactions.

3. *Definition of Performance Objectives (POs) for Bacillus cereus and Listeria monocytogenes in selected ingredients added to RTE mixed spelt salad packaged under modified atmosphere*

I. Incidence of Bacillus cereus and Listeria monocytogenes in commercial spelt salad and specific risk ingredients used to formulate the final product.

Nine different samplings were conducted between July 2012 and February 2014 in a medium size industry located in Romagna (Italy) purchasing ingredients from several selected national supplier facilities. During each sampling, fifteen packs of 250 g each of spelt salad belonging to the same lot and five sample units of 100 g each of frozen spelt, cut celery, and cut cheese belonging to the lots used to make the spelt salads were collected. These ingredients were identified as potentially contaminated by the pathogens according to preliminary information obtained from the industry (data not shown). After sampling the ingredients and the final product were transported to the laboratory under refrigeration conditions and analyzed within two hours.

The lots of spelt salad and the ingredients that were used for their formulation were analysed for the detection and quantification of *Bacillus cereus*, *Listeria* spp. and *Listeria monocytogenes*.

Moreover, values on pH of the final products and the single ingredients were collected following the ISO method 2917:1999, as previously described in paragraph 2.B. (Materials and Method).

II. Bacillus cereus detection, enumeration and confirmation

Bacillus cereus detection, enumeration and confirmation were performed on de-frozen spelt, cut celery, cut cheese and the final mixed product. *B. cereus* was detected and enumerated in the sample units described above using the ISO procedures 21871:2006 and 7932:2004, respectively.

According to the method ISO 21871:2006 for *B. cereus* detection, 25 g of the product were homogenized in 50 ml of Physiological solution by stomaching for 2 minutes at normal speed. Bags with homogenized sample were incubated at 37°C for 24 h. Then, five sampling pools were made taking 10 ml of suspension from each of the fifteen bags. From each pool (30 ml/pool), 1 ml of the initial suspension was diluited in 9 ml of “Trypticase Soy Polimixin Broth Base” (Oxoid, Milan, Italy) and incubated at 30°C for 48 h. From each tube, the suspension was streaked onto “Polymixin pyruvate Egg yolk Mannitol Bromothymol Blue Agar” (PEMBA) (Biolife, Milan, Italy) in double and incubated at 37°C for 24 h. PEMBA was prepared pouring about 12,5 ml aliquots of the complete medium to sterile Petri dishes and left them to solidify. The prepared dishes were inverted and placed in the incubator at 37°C for 18 h to 24 h.

Bacillus cereus typical colonies of presumptive *B. cereus* were about 2 mm to 5 mm in size, had an irregular edge which is between ragged and root-like with ground glass surface, were turquoise to peacock blue, possibly with a greyish white colony centre against a blue background, and had a precipitation halo (egg yolk reaction) up to 5 mm wide.

For the *B. cereus* enumeration, according to the ISO method 7932:2004, 1 ml of the initial suspension was spreaded onto 3 plates of “Mannitol egg Yolk Polimixin agar” MYP (Biolife, Milan, Italy) (0.33 ml for each plate), and plates were incubated at 30°C for 24 h. MYP was prepared pouring 15 ml to 20 ml portions of the complete medium

into sterile Petri dishes and allow to solidify. The prepared dishes were inverted and placed in the incubator at 30°C for 18h to 24.

Bacillus cereus typical colonies are 2 mm to 5 mm in size and are ragged. They have a pink coloration against a crimson background and are surrounded by a precipitation halo (egg yolk reaction) up to 5 mm wide.

Confirmation tests were performed analyzing five (when available) suspected *B. cereus* colonies isolated from each presumptive positive sample using the Bacillus ID (MicrogenTM, UK). Microgen Bacillus ID is a biochemical identification system to identify those *Bacillus spp.* and related genera associated with food spoilage and poisoning. This identification system comprises 24 biochemical substrates specifically selected to provide accurate and efficient identification of *Bacillus spp.* Each kit contains suspending medium and sufficient microwell strips for 20 identifications, holding tray and reporting cards. Positive and negative results of Sugar Fermentation tests, Citrate and Urease tests were reported on the form provided after 24 and 48 h of incubation at 30°C.

III. Listeria spp. and Listeria monocytogenes detection, enumeration and confirmation

Listeria spp (LIS) and *Listeria monocytogenes* (LM) were quantified in 5 sample units each of spelt salad, cut celery and cut cheese using the ISO method 11290-1:2004 (Anonymous 2004a) and ISO 11290-2:2004 (Anonymous 2004b) for the detection of LIS and LM respectively. In fact, raw vegetables and cheese might represent a potential vector of LM. The procedure used for enumeration and cont of LIS and LM has been previously described in paragraph 2.B. (Materials and Methods).

IV. POs calculation approach

The strategy followed to find the concentration levels in specific ingredients allowing to meet an established FSO in the spelt salad place on the market at the end of the shelf life consisted of applying the standardized in-equation proposed by ICMSF (2002):

$$\mathbf{H0 - \sum R + \sum I \leq FSO} \qquad \text{(Eq. 1)}$$

Where H0 is the initial contamination of the target ingredient, ∑ R and ∑ I are the sum of all the log reduction and increase of the bacterial concentration in all the step until consumption of the final product, that concerns the contamination produced by the target ingredient. The PO can be defined, analogously the FSO as:

$$\mathbf{H0 - \sum R + \sum I \leq PO} \qquad \text{(Eq. 2)}$$

$$\mathbf{PO - \sum R' + \sum I' \leq FSO}$$

Where sum over R and I concern the reduction and increase in the production step before the point (in time and space) where performance PO is set and is needed, to set limits on H0 after PO has been defined through eq.2. Sum over R' and I' concern reduction and increase happening after the PO definition until consumption. In our case PO is rapresented by H0 directly over single ingredients. Mathematically PO is defined as:

$$\mathbf{PO = \sum R' - \sum I' + FSO}$$

$$\mathbf{H0 = \sum R - \sum I + PO = \sum Rtot - \sum Itot + FSO}$$

In this study naturally contaminated spelt salads were investigated. Their contamination with foodborne pathogens is often under the limit of quantification (LOQ) of the microbiological method used for enumeration, that corresponds to 30 CFU/g for *B. cereus* and to 10 CFU/g for *Listeria spp*. when pathogen are enumerated using the ISO methods.

- ***POs calculation approach for Bacillus cereus***

The PO calculation should include enumeration but also presence/absence results, being able to identify 'positive' samples also with very low concentration, corresponding to 1 CFU/25g. The presence/absence test gives a 'positive' when the contamination level is higher than the limit of detection (LOD). Otherwise, enumeration data are replaced by 'censored data', i.e. the number of positive belonging to a certain contamination interval (x < LOD is 'negative', LOD < x < LOQ is 'positive', x > LOQ provides an enumeration result). Therefore, the PO definition (Eq. 2), can be considered as a maximum contamination level of a biological hazard at a particular step in the food production chain or the maximum frequency of a 'positive' under a certain LOQ and over the correspondent LOD. This can be done determining the most probable distribution of the censored data set, i.e., the probability density function that maximizes the likelihood.

To achieve both variability and uncertainty of the results, Monte Carlo simulations were performed (Busschaert et al., 2010; Commeau et al., 2012). The 95^{th} percentile of the distribution was used to be compared to the requested PO. It is also possible to determine an estimation of the pathogen concentration using only presence/absence data (Andritsos et al., 2012). The population of certain pathogens in a sample can be assumed to follow a Poisson (xM) distribution, where x is the mean of the pathogen concentration in the sample (CFU/g) and M is the sample size analyzed (g), i.e., 25 g.

The probability of at least one pathogen cell being present in a sample of M=25 g is:

$1 - e^{-xM}$

since the probability of having no pathogen cells in a sample of M=25 g is given by a Poisson probability mass function:

$p(0) = e^{-xM}$.

Therefore, the probability to find a 'positive' score is:

$P_{pos} = 1 – e^{-xM}$ and the fraction of false positives or negatives is considered equal to zero. $p(x) = P_{pos}$ is used as probability of success in a binomial test B(p(x),n,s) where s is the number of 'positive' (successes) and n is the number of samples tested given x. The probability to measure more than s 'positives' given p is Cp(n,s) = 1 - cumulative(B(p,n,s)) and it grows if the concentration x increases. The most appropriate approach to be used is suggested by the characteristics of the data set; in fact when enumeration data form more than 10% of available data a censored data fit is suggested in order to produce accurate results (Busschaert et al., 2010; Commeau et al., 2012).

- ***POs calculation approach for Listeria spp. and Listeria moncytogenes***

Since no data are available directly on *Listeria Monocytogenes*, a 2D Monte Carlo simulation of the food production process has been performed using "Tools for Two-Dimensional Monte-Carlo Simulations ("http://cran.r-project.org/web/packages/mc2d/index.html") according to Pouillot et al., 2010. To define POs aside the experimental data, several initial contamination distributions, have been used as input to describe the concentrations of *L. monocytogenes* in celery, cheese and spelt salad. According to Jarvis (1989) in order to take into account impact of variability was selected the Lognormal distribution. The parameters of the distributions used (mean concentration and standard deviation) have been chosen according to the quantification measures of *Listeria* spp. of each lot product analysed, in order to reflect real values for variability.

The production chain until consumption can be divided into two step: mixing of all the ingredients and storage of the finite product. Each of these steps can be simulated separately and the output for *L. monocytogenes* concentration after mixing can be used as input to simulate the growth during storage.

Using different initial concentrations for *L. monocytogenes* in cheese and celery make us able to quantify the average effect of each step over a single ingredient, taking into account real conditions for variability. This values will be used to estimate a PO for the contamination in cheese and celery.

V. Statistical methods to derive POs values

➢ *Correlation between single ingredients and final product contamination*

Contingency tables were used to study the correlation between ingredients and final product contamination. They were based on the exact Fisher test in which a score is given to the correlation between the two variables in use and a p-value assesses that at least such a score is produced by chance from uncorrelated variables. If there are two variables A=(p1, n1) and B=(p2, n2) and p1,2 (n1,2) represents the number of positive (negative) related to a certain measure concerning A or B, four classes can be defined: pp, is the number of measure where A and B are always both positive; pn (np) the number of measures where A is positive and B negative (or vice versa); nn the number of measures where A and B are always both negative. Fisher demonstrated that the probability of obtaining any set of values from A and B, if they are equally distributed populations, is given by the hypergeometric distribution (i.e., null-hypothesis):

P=[(pp+np)!(pp+pn)!(pn+pp)!(np+nn)!]/[pp!pn!np!nn!]

Where ! is the factorial operator and P is the probability. To perform such a test the function “scipy.stats.fisher_exact” in Pyton 2.7 language was used providing a prior odd ratio and a p-value. The p-value represents the probability of obtaining a distribution at least as extreme as the one that was actually observed, assuming that the null hypothesis

is true. If the measures of A and B are coupled the values pn and np discriminate the two samples. The confidence interval of the observed difference can be defined as:

D=d±sd

d = np/tot-pn/tot

sd=√(pn+np-(d)2/tot)/tot

- ***Monte Carlo models to simulate the production process***

To simulate the production of a complex food matrix, like the spelt salad, the production chain may be divided in several steps (Figure 3).

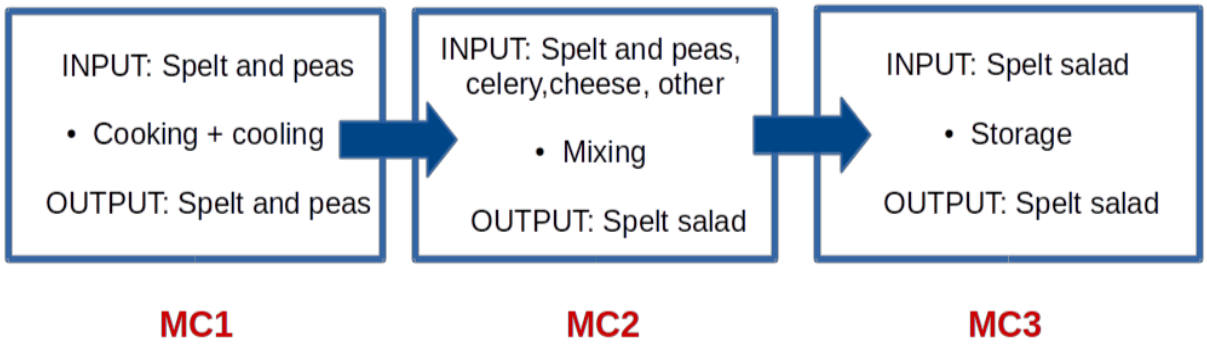

Figure 3 - Description of the main production steps: MC1 includes the treatments of spelt and peas before mixing of other ingredients; MC2 includes the process of mixing of all ingredients; MC3 includes storage of the final product. The connection between MC1, MC2 and MC3 is sequential.

Each step can be simulate separately to predict how the product contamination changes according to the treatment applied to the food in that step. To each variable included in the process is assigned a distribution taking into account its variability (first order model) and uncertainty (second order model). Variability for biological hazards is represented by a LogNormal distribution (Whiting et al., 2006). The uncertainty is linked to the number of measures. In Monte Carlo simulations, the uncertainty over first order model

parameters can be included assigning to the parameters a distribution (second order model) depending by a set of hyper-parameters instead of a numeric value.

When there are no information on such distribution for the second order model, the uninformative distribution over an appropriate interval should be considered.

The variables taken into account in the first order model were bacterial concentration (Log CFU/g), temperature, pH, time and weight of the ingredients. The mathematical description of the treatments in the flow chart follows that presented by Nauta, 2001. Inactivation during cooking (MC1 Figure 3) is described by the following expression:

Nout~Norm(E(Nout),sqrt(E(Nout)*(Nin-E(Nout/Nin))

E(Nout)=Nin*exp(-labda*time)

Where Nout is the distribution associated to the pathogen concentration after inactivation (cooking of spelt), approximated by a Normal distribution when Nin >16*E(Nout)/16-E(Nout)), otherwise it can be approximated by a Negative Binomial distribution; E(Nout) is the expected value for pathogen concentration after inactivation (CFU/g); Nin is the input of the model corresponding to the pathogen concentration in spelt and peas before cooking; lambda is the inactivation rate (1/h); time is time of cooking (h).

The effect of mixing of ingredients (MC2 Figure 3) over pathogen concentration is described as sum of all pathogen contamination from each ingredient (CFU=concentration x weight) divided by the total weight.

The growth during storage (MC3 Figure 3) of the final product is described by the following expressions:

Nout~Norm (E(Nout), sqrt(E(Nout)*(E(Nout)-Nin/Nin))

E(Nout)=Nin*exp(mumax*time)

where Nout is the distribution associated to the pathogen concentration after the end of the shelf life, approximated to a Normal distribution when E(Nout)>Nin^2/(Nin-16); otherwise it can be approximated by the initial contamination plus a negative binomial;

E(Nout) is the expected value for pathogen concentration after storage (CFU/g); Nin is the input of the model which is the pathogen concentration in the final product after mixing; mumax is the maximum growth rate (1/h); time is the time of storage (h). Mumax, lambda, weight and time are taken from a tiny interval with uninformative distribution. Mumax and lambda means were taken from ComBase including the respective experimental values of temperature during treatments and food pH.

4. Setting of sampling plans and risk-based metrics (POs and FSOs) for Bacillus cereus and Listeria Monocytogenes in spelt salads

When the natural concentration of a pathogen in a food matrix is low it is difficult to achieve a PO through numerical evaluation, at least using the microbiological methods currently available.

A sampling plan to reject the lots non complaint with the estimated PO can be defined setting an appropriate sensitivity test (Whiting et al., 2006). The main steps are: define the standard deviation of samples inside the lot, define the just acceptable lot respect to the PO, choose the appropriate sensitivity of the test. Since spelt salad is an heterogeneous product, the standard deviation can be fixed at 0.8 Log CFU/g and the pathogen contamination can be assumed as Log normally distributed (Whiting et al., 2006). According to Whiting et al., 2006 the just acceptable lot is defined as the lot that has 0.13% of the samples exceeding the PO. However, to achieve such a precision 2200 samples should be analyzed to have 95% CL that at least one sample would be positive. As an alternative, it is possible to apply analytical methods with a high sensitivity in order to reject the lot with enough precision (95% CL) testing a limited number of samples. A lot would be rejected when at least one of the samples results positive to the test. Sensitivity level of the analytical procedure of the m value and number of samples to be analyzed (n) are related by equation 3 for standardized z values between 0 and 2.2. For a given just

acceptable lot with m=0.5 Log CFU/g, mean concentration (μ) = 1.0 Log CFU/g and standard deviation (σ)=0.8 Log CFU/g, the probability that a sample would exceed the m value is represented by p:

$z = (m-\mu)/ɞ$

$p = 0.5-(z(4.4-z))/10$ (Eq. 3)

The number of samples can be then calculated from a give probability of rejecting the lot ($P_{(R)}$=0.95) as:

$n=\log_{10}(1- P_{(R)})/\log_{10}(1-p)$ (Eq. 4)

coresponmding to the follow formula:

$n=\log_{10}(1- 0,95)/\log_{10}(1-0,266)$

In this example, ~ 26.6% of the samples would exceed the m value and 10 samples would be needed to reject the lot at 95% of confidence level.

RESULTS

1. Study on the behavior of Salmonella in RTE product.

According to ComBase, *Salmonella* is capable of progressively faster growth at water activities above 0.94. Relative Humidity (RH) measurements (Table 3) indicated that all the materials and combinations examined in the model system could theoretically support the growth of *Salmonella.*

	RH (%)	
Sample	Mean	SE
Intact lettuce	95.3	0.2
Scored lettuce	98.8	3.4
Cheddar cheese	97.5	0.5
Cooked chicken	98.5	1.7
Intact lettuce + chicken	98.9	0.5
Intact lettuce + cheese	97.5	1.6

Table 3 - Relative humidity (RH) measured above samples of each ingredient and combinations of ingredients used in the model system used to examine the fate of S. *enterica* on the surface of romaine lettuce.

RH values were higher in wounded than intact lettuce samples, likely because of leakage of water from damaged tissues and wound-induced increases in respiration rates. Respiration results in the release of water from stomata through transpiration, a process that modifies the RH of the surrounding atmosphere in an enclosed system. However, transfer of water to the atmosphere may be delayed when lettuce leaves are in intimate contact with other materials, resulting in variable water activity at the leaf surface. Hence,

the actual water activity at the leaf surface cannot be deduced from the RH measurements.

Results (Tables 4-5) revealed that *S. enterica* survived but did not grow on intact or wounded tissues of lettuce leaves incubated in air for 6 days at either 6 or 14°C.

***S. enterica* (log CFU/cm^2)[a]: Unscored lettuce**				
Temp (°C)	**Time (days)**	**Lettuce**	**Lettuce + cheese**	**Lettuce + chicken**
6	0	4.32 ± 0.13 A	4.32 ± 0.13 A	4.32 ± 0.13
	3	2.09 ± 0.12 B a	3.15 ± 0.40 B b	4.07 ± 0.17 c
	6	3.46 ± 0.49 B ab	3.21 ± 0.35 B a	4.02 ± 0.17 b
14	0	4.07 ± 0.15	4.07 ± 0.15	4.07 ± 0.15 A
	3	4.40 ± 0.30 a	3.91 ± 0.05 a	8.72 ± 0.06 B b
	6	4.29 ± 0.09 a	3.70 ± 0.15 b	9.52 ± 0.17 B c

Tables 4 - *Salmonella enterica* populations on unscored romaine lettuce tissues incubated at 6 and 14°C with alone, in contact Cheddar cheese, or in contact with cooked chicken.

***S. enterica* (log CFU/cm^2)[a]: Scored lettuce**				
Temp (°C)	**Time (days)**	**Lettuce**	**Lettuce + cheese**	**Lettuce + chicken**
6	0	3.42 ± 0.17	3.42 ± 0.17	3.42 ± 0.17 A
	3	2.86 ± 0.40 a	2.79 ± 0.39 a	4.16 ± 0.19 B b
	6	3.18 ± 0.70 a	2.85 ± 0.21 a	4.00 ± 0.15 B b
14	0	3.16 ± 0.40 A	3.84 ± 0.42 A	3.84 ± 0.42 A
	3	4.28 ± 0.21 B a	4.19 ± 0.30 B a	8.55 ± 0.20 B b
	6	3.30 ± 0.30 A a	4.08 ± 0.19 B b	9.40 ± 0.11 C c

Tables 5 - *Salmonella enterica* populations on scored romaine lettuce tissues incubated at 6 and 14°C with alone, in contact Cheddar cheese, or in contact with cooked chicken.

Notes: [a] Values are means ± standard deviations (n = 6). Within a row for each treatment, means with different lowercase letters are significantly different ($P < 0.05$). Within a column for each temperature, means with different uppercase letters are significantly different ($P < 0.05$).

S. enterica populations also did not increase at 14°C on lettuce tissues placed in contact with Cheddar cheese. On the contrary, populations increased significantly ($P < 0.05$, approximately 6 log CFU/cm^2) over 6 days on lettuce tissues in contact with cooked

chicken meat in samples stored at 14°C.

Results shown in tables 6-8 revealed that washing in water or sanitizer reduced but did not eliminate *S. enterica* applied to the romaine lettuce. Residual populations remained unchanged upon subsequent incubation of lettuce tissues alone or in contact with Cheddar cheese, irrespective of treatment or temperature. However, large population increases (7 log CFU/cm^2) were again evident when lettuce was incubated in contact with cooked chicken at 14°C.

S. enterica (log CFU/g)a: Lettuce not washed				
Temp (°C)	**Time (days)**	**Lettuce**	**Lettuce + cheese**	**Lettuce + chicken**
6	0	4.44 ± 0.10 A	4.44 ± 0.10 A	4.44 ± 0.10 A
	6	3.13 ± 0.52 B a	3.16 ± 0.20 B a	5.58 ± 0.07 B b
14	0	4.11 ± 0.17	4.11 ± 0.17	4.11 ± 0.17 A
	6	3.76 ± 0.23 a	3.57 ± 0.26 a	9.85 ± 0.07 B b

Table 6 - *Salmonella enterica* populations on unwashed romaine lettuce tissues incubated at 6 and 14°C alone, in contact with Cheddar cheese, or in contact with cooked chicken.

S. enterica (log CFU/g)a: Lettuce washed in water				
Temp (°C)	**Time (days)**	**Lettuce**	**Lettuce + cheese**	**Lettuce + chicken**
6	0	2.57 ± 0.16	2.57 ± 0.16 A	2.57 ± 0.16
	6	1.85 ± 0.21 a b	1.29 ± 0.24 B a	2.29 ± 0.16 b
14	0	2.01 ± 0.22	2.01 ± 0.22 A	2.01 ± 0.22 A
	6	2.46 ± 0.33 a	3.30 ± 0.61 B b	9.26 ± 0.22 B c

Table 7 - *Salmonella enterica* populations on washed romaine lettuce tissues incubated at 6 and 14°C alone, in contact with Cheddar cheese, or in contact with cooked chicken

S. enterica (log CFU/g)a: Lettuce washed in 70 µg/ml chlorine				
Temp (°C)	**Time (days)**	**Lettuce**	**Lettuce + cheese**	**Lettuce + chicken**
14	0	2.04 ± 0.21	2.04 ± 0.21 A	2.04 ± 0.21
	6	1.00 ± 0.00	1.00 ± 0.00 B	1.85 ± 0.21
	0	1.28 ± 0.14	1.28 ± 0.14	1.28 ± 0.14 A
	6	2.66 ± 0.55	2.15 ± 0.74 a	8.45 ± 0.22 B b

Table 8 - *Salmonella enterica* populations on sanitized romaine lettuce tissues incubated at 6 and 14°C alone, in contact with Cheddar cheese, or in contact with cooked chicken.

Notes: [a] Values are means ± standard deviations (n = 6). Within a row for each treatment, means with different lowercase letters are significantly different (P < 0.05). Within a column for each temperature, means with different uppercase letters are significantly different (P < 0.05).

The salads chosen for this work were obtained from a large retail outlet, where they are assembled from outsourced fresh-cut romaine lettuce, Cheddar cheese, and cooked chicken. All were stamped with a 3-day expiration date. The arrangement of ingredients in the original package was maintained during the experiments, which were carried out over the anticipated shelf life of the product. Results (Table 9) revealed that *S. enterica* populations in control salads consisting of lettuce alone and in mixed ingredient salads formulated with shredded Cheddar cheese and cooked chicken remained essentially unchanged during 3 days at 6°C.

Storage of mixed ingredient salads at 14°C led to significant growth of *S. enterica* (P , 0.05, approximately 4.0 log CFU/g over 3 days), which occurred despite simultaneous large surges in the populations of potentially competitive lactic acid and total aerobic bacteria. An increase in *S. enterica* (P < 0.05, approximately 1.5 log CFU/g over 3 days) also was noted in the control salads, a result not in agreement with observations in the model system. Although efforts were made to completely remove nonproduce ingredients

from the control salads, carryover of small amounts sufficient to support limited growth of the test bacteria in the control salads cannot be ruled out.

		S. enterica[b]		Lactic acid bacteria		Total aerobes	
Temp °C	Time Days	Control	Treatment	Control	Treatment	Control	Treatment
6	0	2.19 ± 0.06 A	1.76 ± 0.07	1.82 ± 0.99 Aa	4.88 ± 0.12 b	5.06 ± 0.09 A	5.04 ± 0.18 A
6	3	1.53 ± 0.26 B	1.07 ± 0.08	4.81 ± 0.22 B	5.57 ± 0.34	6.76 ± 0.14 B	6.81 ± 0.24 B
14	0	2.29 ± 0.13 Aa	1.52 ± 0.19 Ab	0.97 ± 0.88 Aa	5.06 ± 0.07 Ab	5.00 ± 0.17 A	4.87 ± 0.17 A
14	3	3.93 ± 0.40 Ba	5.68 ± 0.31 Bb	6.98 ± 0.03 B	7.45 ± 0.35 B	8.51 ± 0.04 Ba	9.11 ± 0.22 Bb

Table 9 - *Salmonella enterica*, lactic acid bacteria, and total aerobic bacteria in salads made with fresh-cut romaine lettuce alone (control) or layered with shredded Cheddar cheese and cooked chicken strips (treatment) during 3 days of storage at 6 and 14°C[a]

Notes: [a] Values are the means ± standard deviations for two experiments (n = 4). Within a row, means with different lowercase letters are significantly different ($P < 0.05$). Within a column for each temperature, means with different uppercase letters are significantly different ($P < 0.05$).

[b] One third of the lettuce in all salads was inoculated with a five-strain cocktail of S. enterica.

Observations derived from experimentation with a commercial salad indicated that *S. enterica* survived storage at 6°C for 3 days and that growth of the pathogen was stimulated by nonproduce ingredients at 14°C. Isolates recovered on the selective medium were purified and differentiated by ERIC-PCR to determine whether this behavior was common to all five experimental strains used to prepare the inoculum. All strains, except serovar Kentucky (a poultry litter isolate), were detected in controls and mixed ingredient salads stored for 3 days at 6°C, and serovar Agona (alfalfa sprout isolate) was recovered at the highest frequency (Figure 4). All the experimental strains were found in both control and mixed ingredient salads after 3 days at 14°C, and serovar Agona was again isolated at higher frequency were serovars Enteritidis, Typhimurium, Kentucky, or Brandenburg.

Although *S. enterica* Agona appeared to be slightly better adapted to survival and growth in mixed ingredient salads, recovery of all five serovars after 3 days provided strong evidence that the ability to survive and grow in this environment may be common among the many serovars of this species.

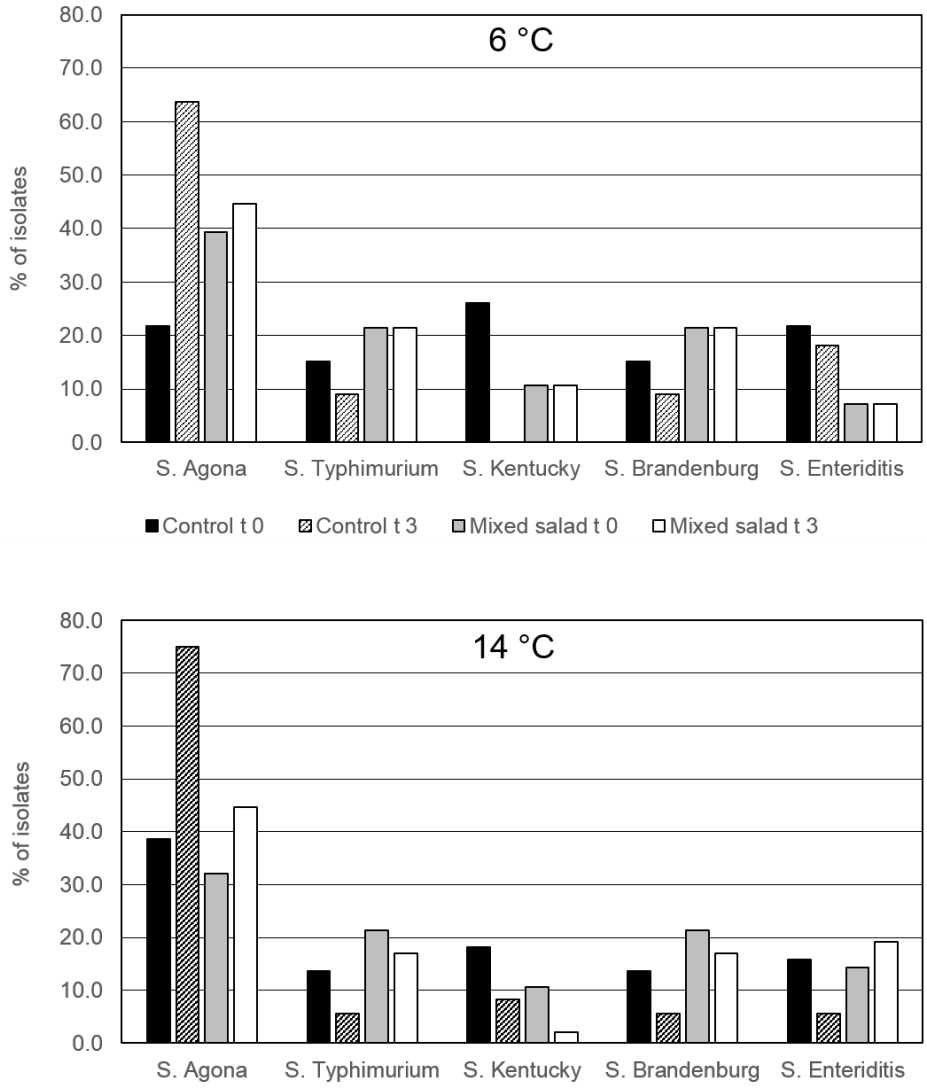

Figure 4. Proportion (% of total) of *S. enterica* serovars recovered from control and mixed ingredient salads stored for 3 days at 6 or 14 °C. Serovars were differentiated by ERIC-PCR.

2. *Study on changes in food safety and quality parameter occurring in RTE spelt salad when different ingredients are combined, different processing procedures are applied, variables in distribution and storage occur.*

I. Microbiological characterisation of spelt salad as currently produced by the industry.

The average counts of the microbial groups investigated in five lots of spelt salad from packaging up to the end of the product shelf life after storage at 6 and 14°C, showed a general increase of Lactic Acid bacteria (LAB), Psychrotrophic bacteria (PSY) and Total Mesophilic count (TMC) combined to a general decrease of pH and Enterobacteriaceae (ENT). The number of *Listeria* spp (LIS) did not change significantly and remained very low for the entire period of storage. The presence of *Listeria monocytogenes* (LM) was never detected. LAB were the most representative microorganisms of spelt salad (figure 5), and the numbers of TMC, with a mean value of 6.06 Log CFU/g, reflects the high numbers of LAB, which were in the range between 4.73 and 6.89 Log CFU/g (mean 6.17). The ENT counts were in a range between 1.15 and 4.80 Log CFU/g, and the majority of the lots (4 out of 5) showed countable numbers of LIS in a range between 0.88 and 2.05 Log CFU/g.

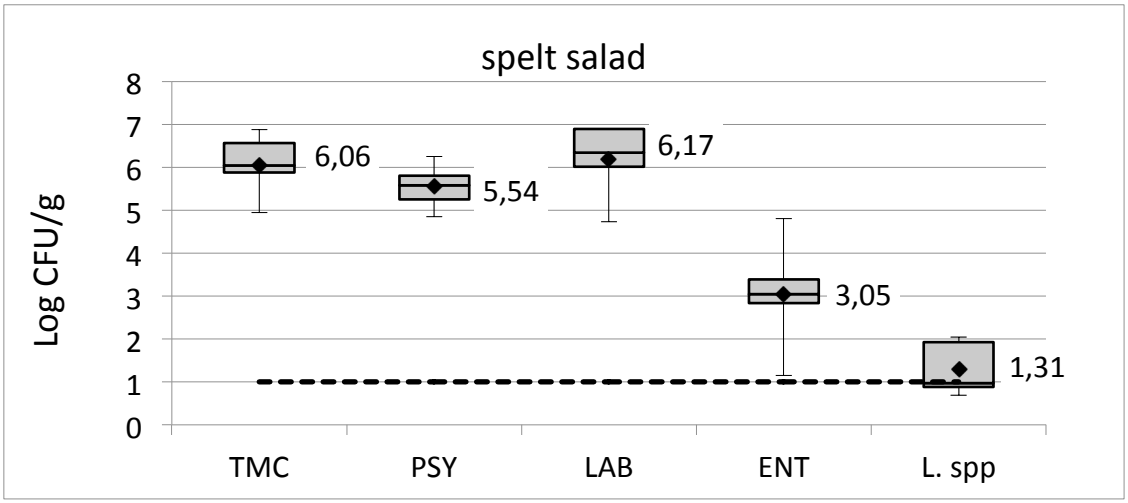

Figure 5 – Box and whisker plots of the number of indicator bacteria in ready-to-eat deli salads at day 1 of shelf life (storage 0 - +4°C)

◆ mean; dot line = limit of quantification (LOQ=1 CFU/g);

In particular, the median number of LAB increased progressively at chilling temperature of 6°C from 6.34 CFU/g (CI95% 4.99-6.89) to 8.12 CFU/g (CI95% 8.04-8.51) in 14 days and remained approximately at the same level (8.19 CFU/g) at day 18. The number of ENT showed a slight reduction from 3.05 CFU/g (CI95% 1.48-4.52) to 2.80 CFU/g (CI95% 1.23-3.50) (Figure 6). The number of LIS did not change significantly, showing a small decrement from 0.98 to the limit of quantificaton (0.69 Log CFU/g) (CI95% 0.69-0.83) (Figure 7). The pH changed from a median value of 5.12 at day 1 (CI95% 4.89-5.49) to 4.73 at day 18 (CI95% 4.44-4.94) (Figure 6-7).

Furthermore, at abuse temperature the number of LAB increased more rapidly than at 6°C (Figure 8-9). Their median number was 8.29 CFU/g at 7 days (CI95% 8.27-9.46) but further increment was slower reaching a maximum of 9.72 CFU/g at the end of the storage period (CI95% 8.39-9.72). The pH median values decreased from 5.12 at day 1 to 4.23 (CI95% 3.79-4.79) at 18 days. The median number of ENT showed a progressive decline from a median value of 3.05 (CI95% 1.48-4.52) at day 1 reaching a minimum (below the limit of quantification) at day 14 and a slightly higher number at the end of the

storage (1.08 CFU/g) (Figure 8). Finally, LIS was never detected during the storage of samples of spelt salad held at abuse temperature, remaining below the LOQ of 0.69 Log CFU/g (Figure 9).

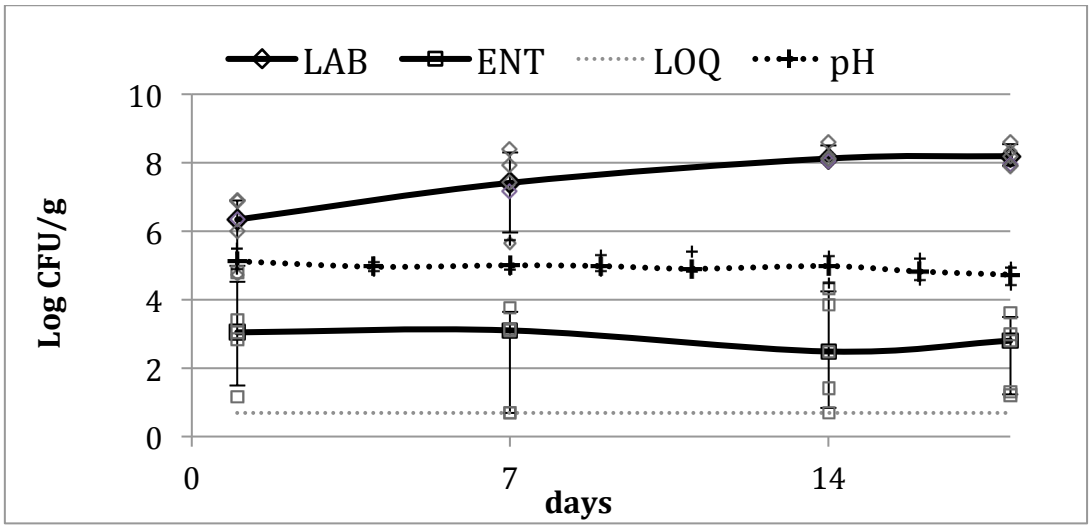

Figure 6 - Changes in the number of LAB and pH versus ENT in spelt salads stored at 6°C (home refrigerator) for 18 days.

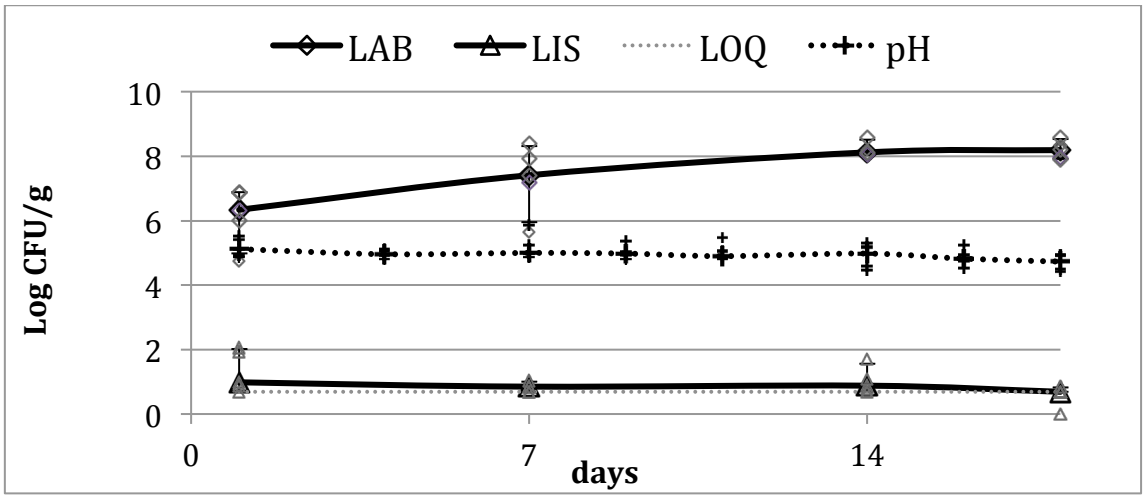

Figure 7 - Changes in the number of LAB and pH versus LIS in spelt salads stored at 6°C (home refrigerator) for 18 days.

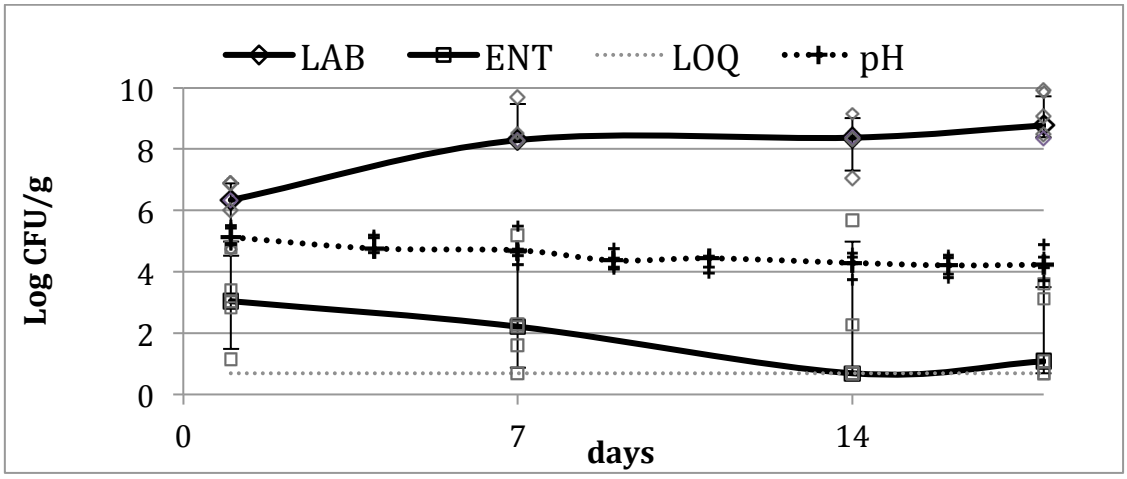

Figure 8 - Changes in the number of LAB and pH versus ENT in spelt salads stored at 14°C (temperature abuse) for 18 days.

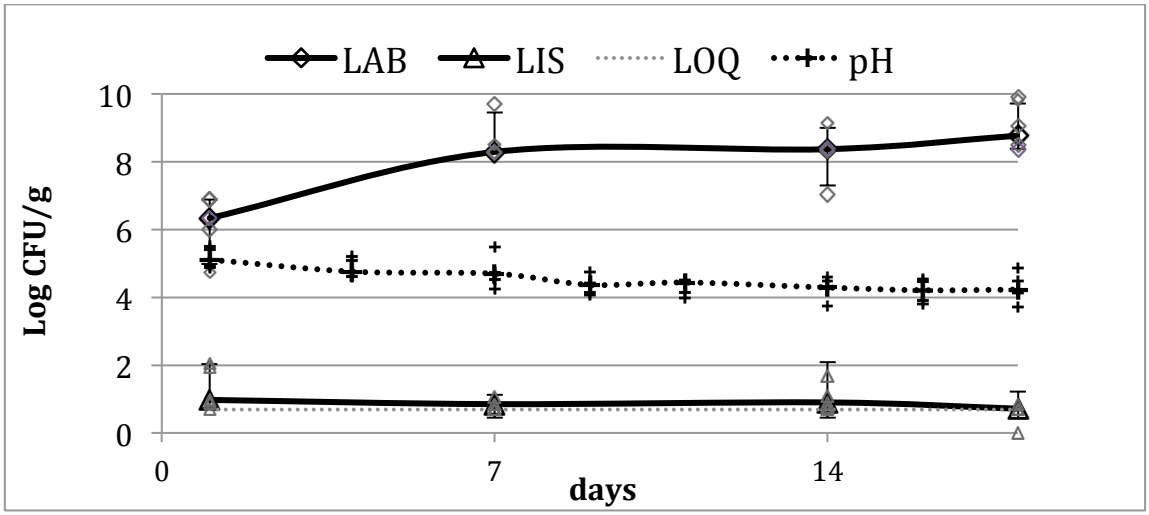

Figure 9 - Changes in the number of LAB and pH versus LIS in spelt salads stored at 14°C (temperature abuse) for 18 days.

The changes of the microbial counts and pH values of spelt salad recorded at 1, 7, 14 and 18 days of storage of intact spelt salad packages are reported in Table 10. The numbers of LAB had significant increments during the shelf life. The difference between the initial and the highest mean values reached during the shelf life was approximately 2.04 Log CFU/g in the samples stored at 6°C. The increments were similar in the samples held at 14°C (2.44 Log CFU/g), but the growth of LAB was faster at 14°C. In fact, while the

LAB reached values of 8.21±0.22 Log CFU/g after two weeks of storage at 6°C, differently after 7 days of storage at 14°C there is a significant increase of LAB to a value of 8.61±0.62 Log CFU/g, remaining at the same level up to the last day of the storage.

		1 day	7 days	14 days	18 days	difference	P(T≤t)
TMC	6°C	6.06±0.74	7.25±1.08	7.54±0.53	7.26±1.86	1.48§	NS
	14°C	6.06±0.74[a]	8.16±1.09[b]	8.28±0.41[b]	8.80±0.38[b]	2.74§	0.009
PSY	6°C	5.54±0.53[a]	5.88±1.47[b]	7.83±0.87[b]	6.63±2.84[b]	2.29§	0.019
	14°C	5.54±0.53[a]	7.95±0.47[b]	7.67±0.94[b]	6.83±2.15[ab]	2.41§	<0.001
LAB	6°C	6.17±0.89[a]	7.31±1.04[ab]	8.21±0.22[b]	8.19±0.28[b]	2.04§	0.001
	14°C	6.17±0.89[a]	8.61±0.62[b]	8.26±0.76[b]	8.94±0.67[b]	2.44§	0.001
ENT	6°C	3.05±1.31	2.28±1.47	2.56±1.55	2.39±1.07	-0.77¤	NS
	14°C	3.05±1.31	2.40±1.69	2.00±2.16	1.84±1.41	-1.21¤	NS
LIS	6°C	1.31±0.64	0.82±0.14	1.02±0.40	0.73±0.08	-0.58¤	NS
	14°C	1.31±0.64	0.81±0.29	1.10±0.73	0.78±0.38	-0.33¤	NS
pH	6°C	5.17±0.28[a]	5.19±0.41[a]	4.90±0.36[ab]	4.71±0.24[b]	-0.46¤	0.023
	14°C	5.17±0.28[a]	4.74±0.46[ab]	4.28±0.33[b]	4.28±0.43[b]	-0.86¤	0.002

Table 10 – Changes in the numbers (Log CFU/g) of TMC, PSY, LAB, ENT, LIS and pH in spelt salad during the shelf life (mean values ± standard deviation of five lots).

Notes: Different letters in superscript following values per each row indicate statistical significance differences. The value reported P(T≤t) is the probability of null hypothesis between the means with different superscripts (the value is calculated for the mean values that are closest). NS=Not Significant. §Log_{10} CFU/g increase was calculated as the difference between the highest Log_{10} concentration reached (underlined) and the initial value (1day). ¤Decrease for pH or bacteria was calculated as the difference between the initial value and the lowest value reached (underlined).

The numbers of TMC and PSY had a similar trend, starting at day 1 from values of 6.06±0.64 Log CFU/g and 5.54±0.53 Log CFU/g, respectively. During the storage, TMC and PSY manteined a linear growing trend, reaching a maximum value at 14 days in packs of spelt salad stored at both temperatures, followed by a slight decrease of the bacteria numbers after 14 days of storage, except for TMC in packs stored at 14°C, where a further increase occurred at day 18 of storage (from 8.28±0.41 to 8.80±0.38 Log CFU/g).

While LAB, TMC and PSY in general had an increasing trend, ENT, LIS and pH, on the other hand had the opposite behaviour. The numbers of ENT and LIS did not change significantly in the spelt salads. For ENT the initial value at day 1 of storage was 3.05±1.31 Log CFU/g and gradually decreased by 0.77-1.21 Log CFU/g, especially in products stored at 14°C where a strong increase of LAB was observed, and for LIS a slight decreasing from a value of 1.31±0.64 Log CFU/g to the limit of quantification was observed during the storage at both temperatures. Finally, the pH values decreased significantly from 5.17±0.28 to 4.28±0.33 after two weeks for samples stored at 14°C; differences became significant at days 18 for spelt salad stored at 6°C (4.71±0.24).

Moreover, softening of cheese were observed after two weeks in 20-40% of the spelt salad packs stored at 14 or 6°C, while blowing of packs were observed after 7 and 11 days of storage at 14 and 6°C, respectively, associated with an high count of LAB.

3. Definition of Performance Objectives (POs) for Bacillus cereus and Listeria monocytogenes in selected ingredients added to RTE mixed spelt salad packaged under modified atmosphere.

I. POs calculation for Bacillus cereus in spelt salad

- **Detection, quantification and confirmation of Bacillus cereus in spelt salad and corresponding ingredients**

According to the expected prevalence and concentration of the selected pathogens, a number of 9 lots analyzed was considered representative to calculate the pathogens distribution in the selected ready to eat products.

The direct enumeration of *B. cereus* in spelt salad and relative ingredients was always under the LOQ (i.e., 30 CFU/g). The presence of *Bacillus cereus* was detected in 3 out of 9 lots of spelt salad (final product; mean prevalence 33.3%; CI95% 12.1-64.6), but the number was always below the LOQ. Celery, cheese and frozen spelt were also positive for the presence of *Bacillus cereus*, but their number was below the level of quantification (<10 CFU/g). Frozen spelt, cheese and celery can be considered as a sources of this spore forming pathogen in the final product (Table 11). In particular, *Bacillus cereus* turned out as positive in all five sample units obtained pooling together 25 g collected from three separate packs of spelt salads. In the first lot, *B. cereus* was also detected in three over five samples of the frozen spelt before cooking. Starting from the third lot, *B. cereus* was investigated also in five sample units of cut celery and cheese. Celery and frozen spelt were both positive in one sample unit over five in the 8th sampling, while the presence of *B. cereus* was also detected in 1 out of 5 sample units of cheese cut in dices in the 3rd lot, and also in the last lot analyzed, in which were enumerated 3 UFC/g of *B. cereus*, corresponding to the limit of quantification of 0.7 Log CFU/g (Table 11). The

confirmation tests performed on *B. cereus* presumptive colonies, isolated on selective plates, ranged between good an excellent identification of *B. cereus* group. Moreover, the pH values showed a higher variability between 4.88 and 5.20 in the spelt salad, between 6.17 and 6.53 in celery, and between 5.25 and 5.48 in cheese.

Lot number (production date)	Frozen spelt n.positive/n. sample units	Cut celery* n. positive/n. sample units	Cut cheese n. positive/n. sample units	Spelt salad n. positive/n. sample units
1 (03/07/12)	3/5	Not done	Not done	5/5
2 (24/07/12)	0/5	Not done	Not done	5/5
3 (06/11/12)	0/5	0/5	1/5	0/5
4 (27/11/12)	0/5	0/5	0/5	0/5
5 (08/01/13)	0/5	0/5	0/5	0/5
6 (29/01/13)	0/5	0/5	0/5	3/5
7 (15/01/14)	0/5	0/5	0/5	0/5
8 (11/02/14)	1/5	1/5	0/5	0/5
9 (25/02/14)	0/5	0/5	1/5	0/5

* The cut celery was sampled after washing into a chlorine solution (200 ppm)

Table 11 - Presence and count of *B. cereus* in spelt salad and raw ingredients.

These results confirmed that the steam cooking process cannot guarantie the inactivation of the spores of *B. cereus* which can survive and germinate. However the number of vegetative *B. cereus* cells was below the quantitation limit.

- ***Relation between presence/absence of Bacillus cereus in single ingredients and contamination of final product***

The association between presence/absence of *B. cereus* in single ingredients (i.e., spelt,

celery and cheese) and the final spelt salads was estimated using the Fisher exact test on contingency tables (Table 12) and calculating the confidence interval of the observed differences.

	Mixed +	Mixed -
Oddratio=0 p-value =1		
Celery +	0	1
Celery -	3	31
Oddratio=0 p-value =1		
Cheese +	0	2
Cheese -	3	30
Oddratio=10.2 p-value =0.06		
Frozen spelt +	3	1
Frozen spelt -	10	34

+=presence; - = absence

Table 12 - Association between presence/absence of *Bacillus cereus* in celery, cheese and spelt and its presence/absence in the final product

The results show a positive association (i.e., p-value=0.06) between presence/absence of *B. cereus* in spelt and its presence/absence in the final mixed spelt salad. Unfortunately, cheese and celery added in the first two lots of spelt salads, which turned out as positive for *B. cereus*, were not tested for the pathogen. Therefore, the score of the Fischer test (i.e., odd-ratio) is zero. The two tailed p-values give the probability that the null hypothesis is true, which means that the samples are equally distributed and therefore the two variables completely correlated. The estimation of p-values shows that contamination in spelt is not equally distributed respect to the final spelt salad at 93%. This result is justified by the fact that raw spelt is cooked before mixing in the spelt salad. Since the tested samples belonged to the same lot, the confidence interval of the observed differences should be taken into account. Despite the absence of pp in cheese and celery, they are fresh ingredients and should be tested anyway to guarantee spelt salad safety.

- ***Estimation of Bacillus cereus concentration (censored data analysis)***

Applying the method described by Andritsos et al., 2012 to the detection results obtained in the nine lots tested, it is possible to obtain the cumulative for the *B. cereus* estimated concentration. The values associated to the 95th percentile of the cumulative distributions are shown in Table 13 and their mean values range between 6 and 7 CFU in 100 g, with a standard deviation lower than 2 CFU/g. When more than one limit (i.e., LOD or LOQ) is present in the dataset, it is possible to estimate the concentration results fitting a parametric distribution on the censored data (Pouillot et al., 2010). Following this approach it is possible to assign a parametric distribution to each quantity in the model, estimates uncertainty (non-parametric bootstrap) and performs Monte Carlo simulations.

Lot	Frozen spelt	Celery	Cut cheese	Spelt salad
1	-0.59	Not done	Not done	Not assigned
2	-1.22	Not done	Not done	Not assigned
3	-1.22	-1.22	-0.97	-1.22
4	-1.12	-1.22	-1.22	-1.22
5	-1.22	-1.22	-1.22	-1.22
6	-1.22	-1.22	-1.22	-0.59
7	-1.22	-1.22	-1.22	-1.22
8	-0.97	-0.97	-1.22	-1.22
9	-1.22	-1.22	-0.97	-1.22
Mean	-1.11	-1.19	-1.15	-1.13
SD	0.21	0.10	0.12	0.24

Table 13 - Estimated *Bacillus cereus* concentration (Log CFU/g) associated to the 95th percentile for the given prevalence and sample weight

The cumulative distribution associated to the contamination levels in spelt salad and relative ingredients through this method is shown in Figure 10, whereas the estimated *B. cereus* concentration at the 95th percentile of the cumulative distribution for the median and the corresponding 2.5 and 97.5 percentiles of the uncertainty distribution around the

median are reported in Table 14, showing that pathogen concentration ranges between 1 CFU in 10 g of final product and 3 CFU in 100 g of cut celery.

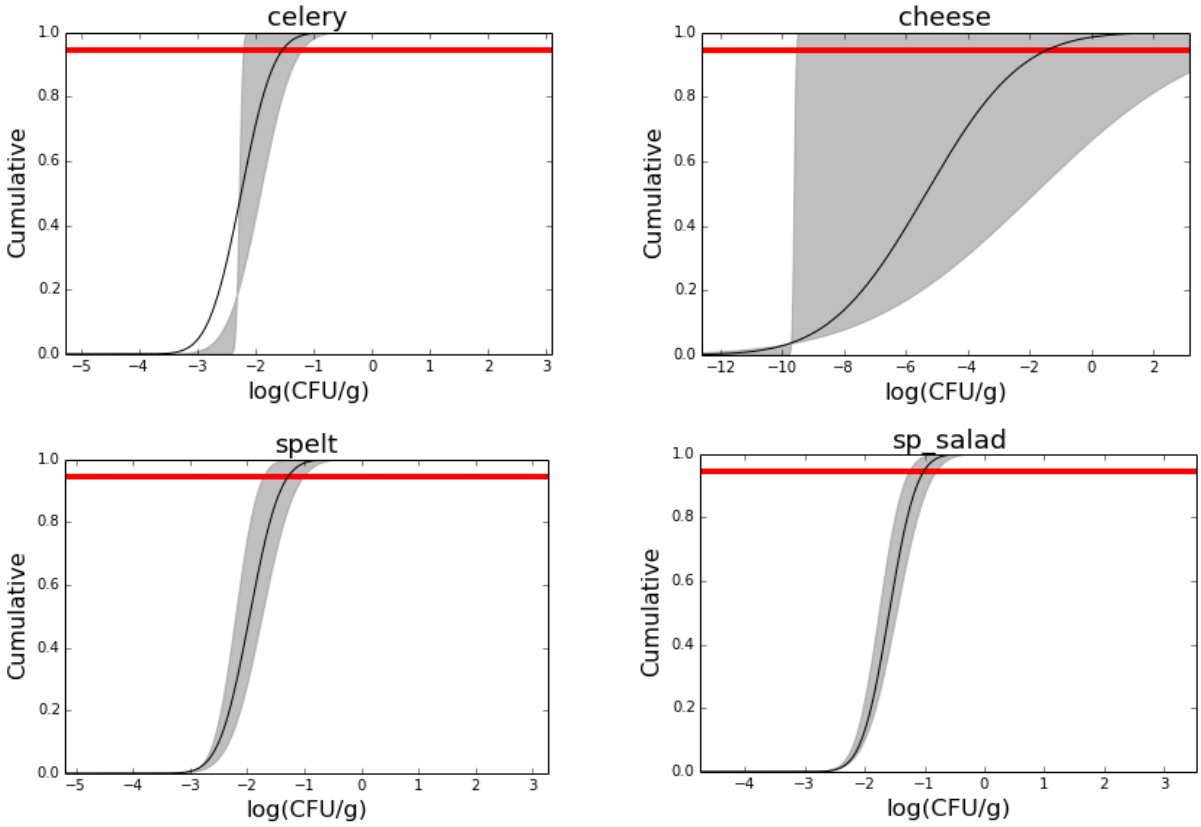

Figure 10 - Cumulative of the normal distribution of positives per lot as a function of the expected bacterial concentration (Lof CFU/g) (the shadow represent the uncertainty at 95CL).

Sample	2.5th	97.5th	Median
Celery	-2.20	-1.17	-1.51
Cheese	-9.53	5.08	-1.34
Spelt	-1.69	-0.98	-1.26
Spelt salad	-1.28	-0.77	-0.98

Table 14 - Estimated *B. cereus* concentration (Log CFU/g) at the 95th percentile of the cumulative distribution for the median and the corresponding 2.5 and 97.5 percentiles of the uncertainty distribution around the estimation (95CL) using R package fitdistrplus, taking into account LOD and LOQ.

In the simulation was assumed no cross contamination and that spelt salad contamination may arise only from the ingredients listed above (i.e., spelt and cheese). Three phases in the spelt salad production process that significantly affect the final product contamination with *B. cereus,* has been identified to perform a Monte Carlo simulation (Figure 3). Each of these sub process can be simulated separately and the output of one can be used as input of another one. Each output can be compared with experimental results. Overall, the simulation underestimates the concentration for percentiles < 0.75 and it overestimates the concentration for percentiles > 0.75. Generally the simulation increases the standard deviation of the distribution respect to the empirical one; this happens because the dataset contained less than 10% of enumeration data. To define POs aside the experimental data, several initial contamination distributions, defined theoretically, may be used to study the impact on the final product, especially if the experimental data are not enough to give accurate predictions. According to Jarvis (1989), in order to take into account impact of variability, the Lognormal distribution has been selected. The simulation has been performed according to Pouillot et al., 2010 applying the following packages: (1) Fitdistrplus: Help to Fit of a Parametric Distribution to Non-Censored orCensored Data'fitdistrplus' http://cran.r-project.org/web/packages/fitdistrplus/index.html and (2) "Tools for Two-Dimensional Monte-Carlo Simulations" http://cran.r-project.org/web/packages/mc2d/index.html. The results of the simulations based on the experimental data for each of the three phases are shown in Figures 11, 12 and 13. Moreover, the correspondent values for the distributions are summarized in Tables 15, 16, 17 and 18. Table 15 refers to a shelf life of 12 days, starting from the simulated contamination after mixing; Table 16 refers to a shelf life of 12 days, starting from the estimated contamination in the final product.

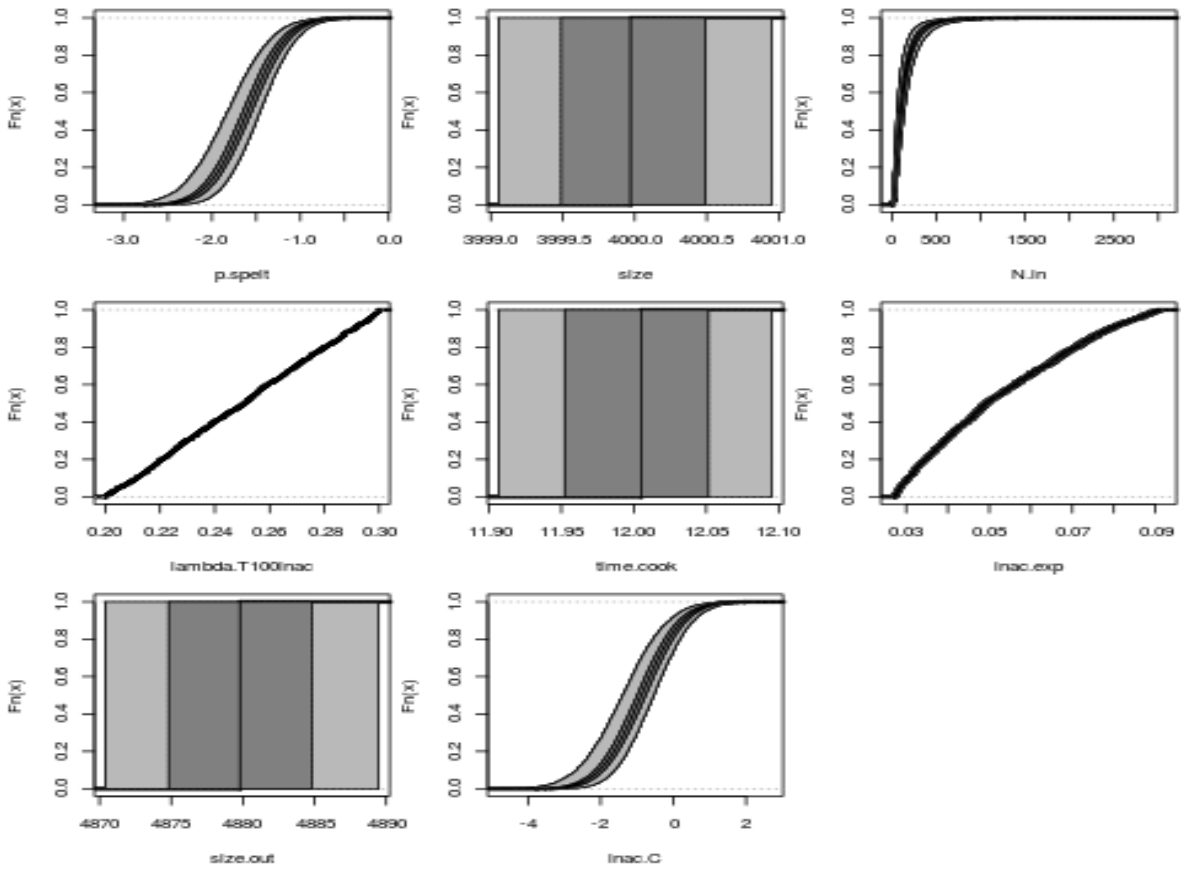

Figure 11 - Results of the Monte Carlo simulations with the R package 'mc2d' for MC1 for the cumulative distributions of the simulated variables: *B. cereus* concentration, Log CFU/g for 'p.spelt' and CFU for 'N.in', distribution in frozen spelt (Normal and LogNormal), variability in weight (uninformative) of spelt ('size') and spelt and peas ('size.ou'); variability in cooking time (uninformative), i.e.,l time.cook; B. cereus concentration after cooking in CFU (inac.exp) and Log (CFU/g) (inac.C).

Uncertainty	Mean	sd	Min	2.5th	25th	50th	75th	97.5th	Max
Median	-6.93	1.08	-12.82	-9.36	-7.52	-6.84	-6.22	-5.09	-4.01
Mean	-6.95	1.08	-12.99	-9.37	-7.54	-6.86	-6.23	-5.09	-3.98
2.5th	-7.39	0.96	-15.79	-10.15	-8.04	-7.30	-6.62	-5.40	-4.52
97.5th	-6.60	1.22	-11.02	-8.76	-7.16	-6.52	-5.94	-4.82	-3.24

Table 15 - Results of the simulation of *B. cereus* contamination in spelt and peas after cooking and cooling using the R package mc2d. For selected percentiles of the final distribution for variability (columns), median, mean and 95CL (2.5th and 97.5th percentiles) of the uncertainty distributions (rows) are shown.

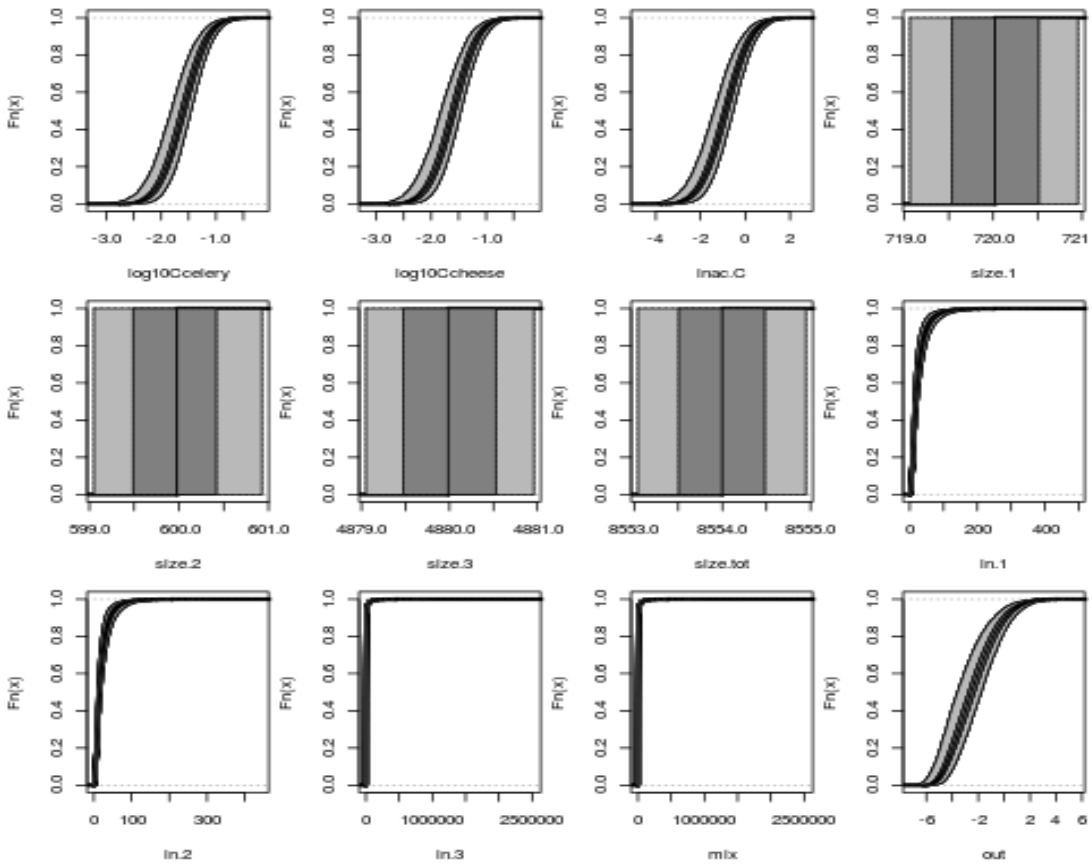

Figure 12 - Results of the Monte Carlo simulations with the R package 'mc2d' for MC2 for the cumulative distributions of the simulated variables: LogCelery, LogCheese and inac.C are *B. cereus* concentration of celery, cheese and spelt and peas after cooking in Log CFU/g; size 1, size 2, size 3 and size tot are the weight of the ingredients and the total weight of the final product; in.1, in.2, in.3 and mix are CFU in each ingredient and in the final product; out is the B. cereus concentration (Log CFU/g) in the final product.

Uncertainty	Mean	sd	Min	2.5th	25th	50th	75th	97.5th	Max
Median	-5.39	0.62	-7.33	-6.58	-5.81	-5.40	-4.99	-4.19	-3.34
Mean	-5.42	0.62	-7.37	-6.61	-5.84	-5.43	-5.01	-4.19	-3.31
2.5th	-5.87	0.53	-8.17	-7.22	-6.33	-5.89	-5.42	-4.49	-3.80
97.5th	-5.10	0.72	-6.77	-6.17	-5.48	-5.11	-4.71	-3.90	-2.68

Table 16 - Results of the simulation of *B. cereus* contamination in the final product after mixing of all ingredients using the R package mc2d . For each of the several percentiles of the final distribution for variability (columns), median, mean and 95CL (2.5th and 97.5th percentiles) of the uncertainty distributions (rows) are shown.

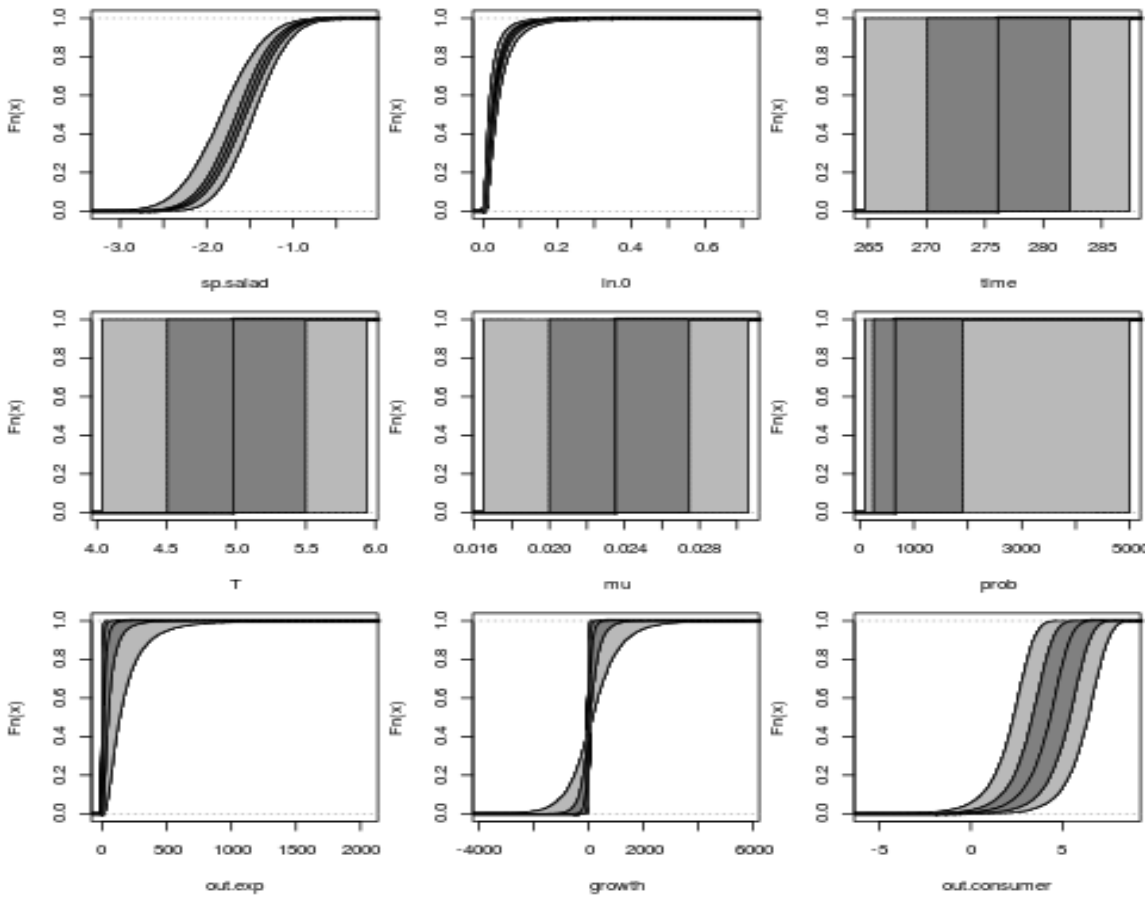

Figure 13 - Results of the Monte Carlo simulations with the R package 'mc2d' for MC3 for the cumulative distributions of the simulated variables: sp.salad and in.0 are the *B. cereus* concentrations in spelt salad after mixing in Log CFU/g and CFU/g, respectively; time, T (temperature) uninformative around the expected values; mu is the maximum growth rate estimated by ComBase; outconsumer is the B. cereus concentration at time of consumption (Log CFU/g).

Uncertainty	Mean	sd	Min	2.5th	25th	50th	75th	97.5th	Max
Median	-0.32	1.32	-6.39	-3.36	-1.05	-0.18	0.56	1.84	2.83
Mean	-0.35	1.32	-6.50	-3.38	-1.06	-0.20	0.54	1.78	2.80
2.5th	-2.47	1.19	-10.15	-5.62	-3.2	-2.32	-1.55	-0.32	0.60
97.5th	1.78	1.45	-3.44	-1.12	1.08	1.92	2.66	3.92	5.06

Table 17 - Results of the simulation of *Bacillus cereus* contamination in the final product at the end of the shelf life using the R package mc2d. For each of the several percentiles of the final distribution for variability (columns), median, mean and 95CL (2.5th and 97.5th percentiles) of the uncertainty distributions (rows) are shown.

The results of the simulations, based on the experimental data, for each of the three phases show that the mean *B. cereus* contamination in spelt and peas after cooking and cooling is around 1 cfu/10 ton (Figure 11, Table 15) and increases to 3 CFU/ton after mixing with all ingredients (Figure 12, Table 16). However, at the end of the shelf life the mean *B. cereus* contamination in the final product (Figure 13) is about 5 cfu/10g using the simulated contaminations (Table 17) and about 15000 CFU/g using the experimental results (Table 18).

Uncertainty	Mean	sd	Min	2.5^{th}	25^{th}	50^{th}	75^{th}	97.5^{th}	Max
Median	4.14	1.17	-1.86	1.27	3.56	4.35	4.94	5.87	6.58
Mean	4.17	1.18	-1.99	1.30	3.59	4.36	4.97	5.89	6.62
2.5^{th}	2.13	1.08	-5.78	-0.83	1.54	2.34	2.95	3.88	4.61
97.5^{th}	6.19	1.30	0.99	3.38	5.62	6.40	6.99	7.93	8.67

Table 18 - Results of the simulation of *B. cereus* contamination in the final product at the end of the shelf life using experimental data of spelt salad after mixing as input and simulating the shelf life using the R package mc2d. For each of the several percentiles of the final distribution for variability (columns), median, mean and 95CL (2.5th and 97.5th percentiles) of the uncertainty distributions (rows) are shown.

This difference can be explained taking into account that *B. cereus* was not enumerated in cheese and celery added in the first two lots of spelt salads which turned out as positives. Therefore, the initial contamination associated to the ingredients underestimates the contamination of the final product, also because few enumeration results were available for the analysis. Moreover, possible cross contaminations were excluded as well as contaminations coming from ingredients other than spelt, cheese and celery. To assess the impact of cooking the ComBase parameters were included and they might be different from the real ones. However, the real parameters are difficult to evaluate because the natural *B. cereus* contamination is usually very low. Beside the difference between simulated and experimental results the approach presented takes into account

the whole production process and for each production step simulations were performed (Figures 11-13).

- ***FSO estimation and derivation of Performance Objectives for Bacillus cereus***

A FSO for *B. cereus* is currently not included in the EU regulations. Therefore, in order to show the approach to derive the POs was fixed an hypothetical FSO at 4 Log CFU/g. This value was selected because, according to expert opinions, the maximum tolerable *B. cereus* concentration in raw materials has been fixed at 10^4 CFU/g (Uyttendaele et al., 2010) increased with 10^2 for variability and uncertainty (Daleman et al., 2013). However, this last increment is not incorporated in the model because the more restrictive limit was applied. According to outbreak data, the number of *B. cereus* cells able to cause disease was estimated to be eaten 10^4 and 10^8 CFU/g of food (Granum, 1997). However, this range may be even wider, most probably due to differences in (entero)toxin production and/or growth rate of the involved strains (Wijnands et al., 2006). The effect of each phase in the process chain can be quantified as increase or decrease in the distribution of the mean contamination. In MC1 (i.e., cooking and cooling) the inactivation rate of *B. cereus* during cooking was calculated using the available ComBase inactivation predictive model and including the experimental pH values of spelt and peas, as well as time and temperature of the treatment. The estimated log reduction associated to the process was 3.02 Log for 0.2 h at 100°C. Comparing the output of the simulation with our experimental data on concentration of *B. cereus* in spelt, the effect of the treatment resulted in a reduction of 5.3 Log CFU/g respect to the contamination in raw spelt, also due to the dilution related to mixing with peas. This result may, however, overestimate the reduction, since the quantification of the initial contamination cannot be defined accurately due to

data limitations. In MC2 (i.e., mixing of all ingredients of the salad), as it is shown by the differences in mean values reported in Tables 15 and 16, the effect was an increase of 1.53 Log CFU/g respect to the contamination of spelt and peas after cooking; in MC3 (i.e., storage), considering that the pH of the final product has a mean value of 4.9, the increase was about 4.9 Log CFU/g for spelt salad stored 12 days at 5°C in air (0.8% NaCl), while it was about 4.04 Log CFU/g for spelt salad stored 12 days at 5°C in modified atmosphere (50% CO2 0.8%NaCl) (ComBase predictor).

II. POs calculation for Listeria spp. and Listeria monocytogenes in spelt salad

- ***Detection, quantification and confirmation of Listeria spp. and Listeria monocytogenes in spelt salad and corresponding ingredients***

Concerning *Listeria* spp. and *Listeria monocytogenes*, the results obtained testing the lots of ingredients to make spelt salads showed that fresh vegetables are sources of *Listeria* spp. The analysis at one day of shelf life of samples of spelt salad had very low numbers of *Listeria* spp. in the range between < LOQ and 2.05 Log CFU/g (mean±sd 1.16±0.45). The analyses on ingredients showed that raw vegetables and diced cheese were often positive for *Listeria* spp., although their number was low (Table 19). The presence of *Listeria* spp. was very common in celery (78%; CI95% 45-94%) and diced cheese (89%; CI95% 56-98%), with low mean numbers (i.e. 1.54 and 1.41 Log CFU/g, respectively).

In relation to pathogenic species, the presence of *L. monocytogenes* was detected in one out of 9 batches of cut celery and one out of 9 batches of cheese (mean prevalence=11.1%; CI95% 2-43%). However, the pathogen quantification was always under the detection limit of the microbiological technique, corresponding to 1 Log CFU/g.

Enumeration of *Listeria* spp. and detection of *L. monocytogenes* have been performed over two single ingredients, celery and cheese, and over the final product before storage.

Listeria spp.		
	Cut celery	**Cut cheese**
n° lots	9	9
≥LOQ	7 (78%)	8 (89%)
UCL95%≥LOQ	94%	98%
LCL95%≥LOQ	45%	56%
Mean±sd*	1.54±0.66	1.41±0.70
Median*	1.21	1.28

Table 19 – Incidence (%) of *Listeria* spp. in celery and cheese.

From the data (Table 20) it can be noted that cheese samples are often under the limit of quantification for *Listeria* spp., while detection of *L. monocytogenes* is always negative. Instead in celery as well as in the final product (Table 19-20) there is a measurable population of *Listeria* spp. and a single positive sample of celery for *L. monocytogenes* in one of the highest *Listeria* spp. contaminated lots has been identified.

	Cheese			Celery		
Lot	**Mean Log(UFC/g) LIS**	**S. D. LIS**	**Pres./abs. LM**	**Mean Log(UFC/g) LIS**	**S. D. LIS**	**Pres./abs. LM**
1	-	-		-	-	-
2	-	-		-	-	-
3	0.76	0.13	no	1.17	0.75	no
4	0.69*	0*	no	1.14	0.38	no
5	0.69*	0*	no	1.43	0.42	no
6	0.69*	0*	no	1.16	0.59	no
7	0.82	0.16	no	2.66	0.07	no
8	0.76	0.13	no	0.82	0.16	no
9	0.82	0.27	no	2.10	0.45	yes

Table 20 – Mean and standard deviation of *Listeria* spp. (LIS) and presence/absence of *L. monocytogenes* (LM) in cheese and celery.

Lot	**Mean Log(UFC/g) Lspp**	**St. Dev. Lspp**	**Presence/absence LM**
1	-	-	-
2	-	-	-
3	2.05	0.23	no
4	0.91	0.34	no
5	1.00	0.30	no
6	1.00	0.30	no
7	1.18	0.68	no
8	0.69	0	no
9	0.79	0.39	no

Table 21 – Mean and standard deviation of *Listeria* spp. (LIS) and presence/absence of *L. monocytogenes* (LM) in the final product.

- ***Estimation of Listeria moncytogenes concentration (censored data analysis)***

Due to the negative results for *Listeria monocytogenes* in all nine lots of cheese used for the formulation of spelt salad any cumulative estimation has been performed using the method described by Andritsos et al. 2012, as previously applied for *Bacillus cereus.*

On the contrary, based on the contamination level of celery (Table 20) and final product (Table 21), has been possible to make an estimation of the distribution parameters of the bacterial population in the products, assuming that the distribution is a Log Normal distribution. The distributions obtained with such parameters are used as input concentrations to perform 2D Monte Carlo simulation of the mixing process and the storage process in AIR and MAP. A simulation is performed for each of the seven lot analyzed for *Listeria* spp. and *L. monocytogenes*. In the simulation was assumed no cross contamination, and that spelt salad contamination may arise only from the ingredients listed above (i.e., cheese and celery). Only one phase in the spelt salad production process that significantly affect the final product contamination with *L. monocytogenes,* has been identified to perform a Monte Carlo simulation. To define POs aside the experimental data, several initial contamination distributions, defined theoretically, may be used to study the impact on the final product, especially if the experimental data are not enough to give accurate predictions. According to Jarvis (1989), in order to take into account impact of variability, was selected the Lognormal distribution. The simulation has been performed according to Pouillot et al. 2010 applying the following packages: (1) Fitdistrplus: Help to Fit of a Parametric Distribution to Non-Censored orCensored Data'fitdistrplus' http://cran.r-project.org/web/packages/fitdistrplus/index.html and (2) "Tools for Two-Dimensional Monte-Carlo Simulations" http://cran.r-project.org/web/packages/mc2d/index.html. The results of the simulations for lot numer 3 based on the experimental data are shown in Figures 14 and 15. Moreover, the

correspondent values for the distributions are summarized in Tables 22 and 23. Table 22 refers to simulated contamination in the final product after mixing; Table 23 refers to a shelf life of 12 days, starting from the simulated contamination in the final product after mixing storage in Air condition. Table 24 refers to a shelf life of 12 days, starting from the simulated contamination in the final product after mixing storage in MAP condition.

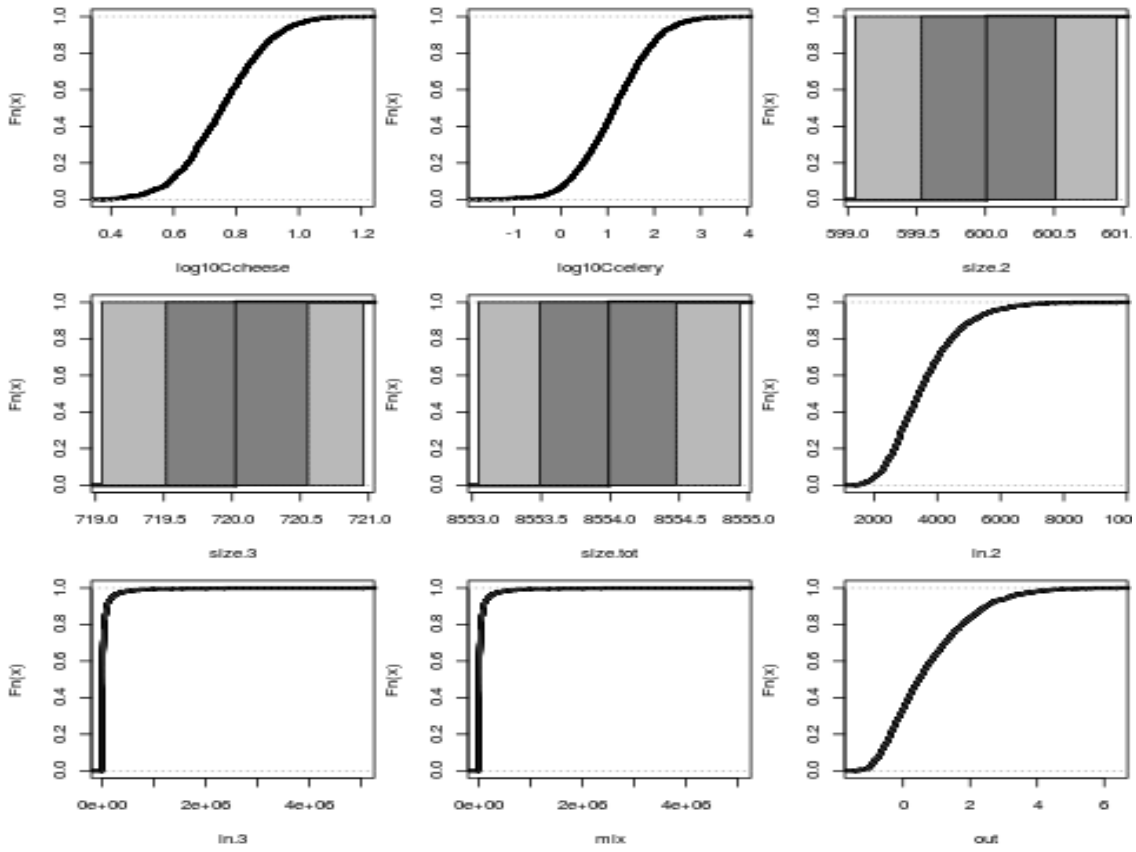

Figure 14 - Results of the Monte Carlo simulations with the R package ‘mc2d’ for the cumulative distributions of the simulated variables. The mixing treatment in lot number 3 over the concentration of *Listeria* spp. has been simulated. The variables log10cheese and log10celery (a and b) are lognormally distributed and correspond to the estimated natural contamination of the raw ingredients before mixing. The size 1,2 and tot are the weight of cheese and celery to be added respectively and of the final product spelt salad, they are taken from an uninformative distribution correspondent to a tiny interval around the value defined in the recipe. in1 and in2 are the CFU of cheese and celery that are directly added to obtain the final contamination. out is the contamination in log(CFU/g) in the final product before storage.

Uncertainty	Mean	sd	Min	2.5th	25th	50th	75th	97.5th	Max
Median	0,51	0,66	-0,81	-0,59	-0,01	0,46	0,94	1,92	3,11
Mean	0,51	0,66	-0,81	-0,59	-0,01	0,46	0,94	1,92	3,11
2.5th	0,51	0,66	-0,81	-0,59	-0,01	0,46	0,94	1,92	3,11
97.5th	0,51	0,66	-0,81	-0,59	-0,01	0,47	0,94	1,92	3,11

Table 22 - Results of the simulation of *Listeria monocytogenes* contamination in the final product after mixing of all ingredients using the R package mc2d. For each of the several percentiles of the final distribution for variability (columns), median, mean and 95CL (2.5th and 97.5th percentiles) of the uncertainty distributions (rows) are shown.

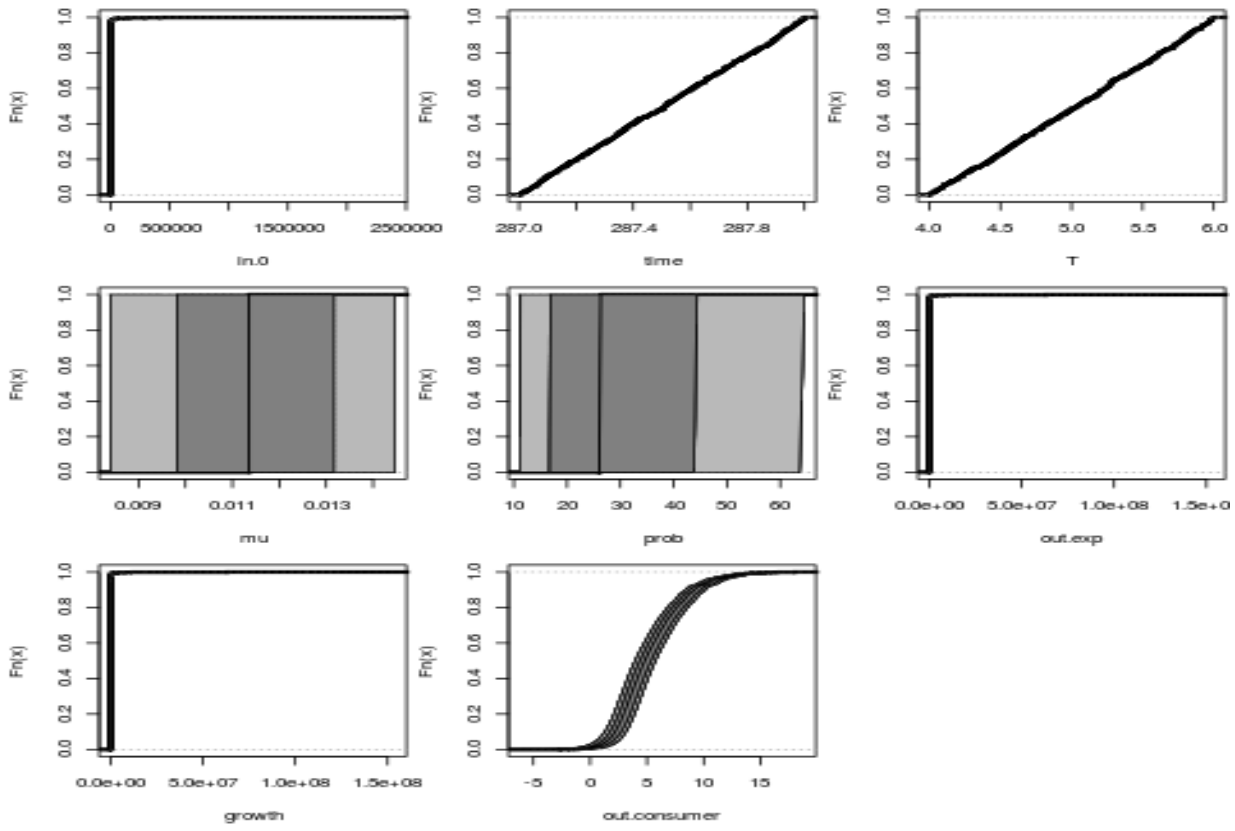

Figure 15 - Results of the Monte Carlo simulations with the R package 'mc2d' for the cumulative distributions of the simulated variables. The MAP storage treatment in lot number 3 over the concentration of *Listeria* spp. has been simulated. The variables considerated are the initial contamination in0 (lognormal), the temperature T (uninformative around the mean), the estimated maximum growth rate mu uninformative inside the CL interval considered by ComBase. out.consumer is the contamination at consumption in Log(CFU/g).

Uncertainty	Mean	sd	Min	2.5th	25th	50th	75th	97.5th	Max
Median	4,59	1,63	-2,05	1,38	3,56	4,57	5,63	7,90	10,49
Mean	4,59	1,63	-2,26	1,39	3,55	4,56	5,62	7,90	10,49
2.5th	3,74	1,57	-5,31	0,42	2,70	3,71	4,77	7,05	9,66
97.5th	5,48	1,69	-0,03	2,40	4,46	5,44	6,51	8,80	11,37

Table 23 - Results of the simulation of *Listeria monocytogenes* contamination in the final product at the end of the shelf life, in MAP storage, using the R package mc2d. For each of the several percentiles of the final distribution for variability (columns), median, mean and 95CL (2.5th and 97.5th percentiles) of the uncertainty distributions (rows) are shown.

Uncertainty	Mean	sd	Min	2.5th	25th	50th	75th	97.5th	Max
Median	0,71	1,21	-1,42	-0,95	-0,20	0,46	1,46	3,68	5,70
Mean	0,71	1,21	-1,42	-0,95	-0,20	0,46	1,46	3,68	5,70
2.5th	0,71	1,21	-1,42	-0,95	-0,21	0,46	1,46	3,68	5,69
97.5th	0,71	1,21	-1,42	-0,95	-0,20	0,46	1,46	3,68	5,70

Table 24 - Results of the simulation of *Listeria monocytogenes* contamination in the final product (lot n° 3) at the end of the shelf life, in MAP storage, using the R package mc2d. For each of the several percentiles of the final distribution for variability (columns), median, mean and 95CL (2.5th and 97.5th percentiles) of the uncertainty distributions (rows) are shown.

The same calculation has been performed with storage in AIR, where mu_max was different. Below these values are reported:

min=0.004, max=0.008 in map

min=0.00823, max=0.0147 in air

mu_max_combase_air = [0.011 +/- 0.003] (log.conc/h)

mu_max_combase_map = [0.006 +/- 0.002] (log.conc/h)

The effect of mixing and storage treatments are established making the difference between the means of the concentration distributions (assumed Log-Normal) before and after the treatment. The mean effect of the treatment and its variability between lots is calculated averaging over the effects of the seven lots and calculating the related standard deviation (Table 25).

Overall the simulation of the effect of mixing is compatible with the experimental measure in the final product, the difference between the mean of the distribution averaged over the lots is :

<(spsalad L.spp. mean conc)-(mix L.spp. mean conc)> = - 0.17 + - 1.35 log (CFU/g).

Treatment	Effect	Mean Log(UFC/g)	St. Dev.
Mixing respect to cheese	I	0.52	1.26
Mixing respect to celery	R	-0.23	0.66
Storage in AIR 12days	I	5.08	1.64
Storage in MAP 12days	I	3.61	1.66

Table 25 – Effect of reduction/increase in each step of the food chain for cheese and elery, the standard deviation of the mean effect of each treatment is due to variability between lots.

➢ FSO estimation and derivation of Performance Objectives for *Listeria monocytogenes*

A possible FSO for Listeria monocytogenes can be set up to the value for this risk including in the microbiologial criteria regulation (Regulation 2073/2005). This value correspond to 2 Log(CFU/g) and consequently the effect of each treatment is established making the difference between the means of the concentration distributions (assumed

Log-Normal) before and after the treatment. The mean effect of the treatment and its variability between lots is calculated averaging over the effects of the seven lots and calculating the related standard deviation (Table 25). In MC3 (i.e., storage), as it is shown by the differences in mean values reported in Tables 25, the effect was an increase of 0,52 Log CFU/g in cheese respect to the initial level of this ingredient. In the final product the increase was about 5,08 Log CFU/g for spelt salad stored 12 days at 5°C in air (0.8% NaCl), while it was about 3,61 Log CFU/g for spelt salad stored 12 days at 5°C in modified atmosphere (50% CO2 0.8%NaCl) (ComBase predictor).

Finally, to calculate PO with 95%CL also the standard deviation of the final distribution should be included in the calculation (Table 26), in order to take into account the difference of the concentration of the pathogen at 95th percentile respect to the mean concentration of the distribution at consumption. For this purpose the minimum values reported in table 26 has been used.

Lot	**St. Dev.** **AIR**	**St. Dev.** **MAP**
mean	1.7	1.8
min	0.35	0.35
max	2.8	2.8

Table 26 – Standard deviation of *Listeria* spp. distribution after storage.

4. Setting of sampling plans and risk-based metrics (POs) for Bacillus cereus and Listeria Monocytogenes in spelt salads

I. Sampling plans to reject lots non-compliant to the established PO for Bacillus cereus

In order to calculate sampling plans for cheese and spelt, considering a real possibility of contamination of these ingredients, the method reported in material and methods (Whiting et al., 2006) has been used. Celery can be treated analogously to cheese because both are raw ingredients and have almost the same weight proportion in the final product. Differently from cheese, celery can be controlled through washing with chlorinated water to increase safety. In the calculations, the effect of all ingredients over the total contamination after mixing is taken into account. From the simulation results it can be assumed that mixing increased the mean contamination of the final product of about 1.53 Log CFU/g respect to the mean contamination of spelt and peas after cooking due to other ingredients, while the contamination after mixing remains almost equal respect to the contamination of cheese, because in this particular case the presence of other ingredients numerically compensate the ”dilution” of cheese bacterial counts into the whole product. It should be noted that MAP packaging improves product safety during the shelf life limiting the growth of pathogens and allowing to test less samples for *B. cereus* in the single ingredients, with particular reference to cheese. MAP was included in the simulation changing the maximum growth rate parameter (based on predictions from ComBase) of *B. cereus* used in the Monte Carlo simulations. According to the simulation results, there was a significant increase of the variability in the contamination distribution, up to 3 Log CFU/g. Therefore, the selection of representative samples to test and their correct homogenization before microbiological testing is critical and crucial in order to reduce the variance. This indication must be part of the sampling procedure.

The results concerning sampling plans for spelt and cheese are shown in Tables 27, 28, 29 and 30. Several sensitivity thresholds were used in the calculation, leading to different numbers of necessary samples to test in order to reject unsafe lots with 95% CL. Tables 27-30 show how much changes the number of samples to test in order to reject a lot at 95%CL according to different values of p exceed. The lower is p exceed, the higher is the probability to accept lots at 95%CL (assuming a random sampling). However, p exceed must be defined according to a reasonable number of samples to be analyzed. Tables 27-30 should be used by food safety managers in order to fix a sampling plan according to their acceptable level of risk.

Sensitivity test (Log CFU/g)	**p exceed[a] (%)**	**n[b]**
0.53	15.71	18.0
0.33	22.37	12.0
0.13	30.29	9.0

Table 27 - Sampling plans for *B. cereus* in spelt to be added to spelt salad packaged under MAP.

Sensitivity test (Log CFU/g)	**p exceed[a] (%)**	**n[b]**
-0.35	15.45	18.0
-0.55	22.12	12.0
-0.76	30.11	9.0

Table 28 - Sampling plans for *B. cereus* in spelt to be added in spelt salads packaged under air.

Sensitivity test (Log CFU/g)	**p exceed[a] (%)**	**n[b]**
-1.06	15.30	19.0
-1.26	21.99	13.0
-1.47	30.00	9.0

Table 29 - Sampling plans for *B. cereus* in cheese to be added in spelt salads packaged under MAP

PO (Log CFU/g)	p exceed[a] (%)	n[b]
-1.97	14.26	20.0
-2.19	21.03	13.0
-2.40	29.24	9.0

Table 30 - Sampling plans for *B. cereus* in cheese to be added in spelt salad packaged under air.

Notes: [a]percentage of samples of the just acceptable lot that would exceed the sensitivity threshold; [b]number of samples to test to reject the lot at 95CL

II. Sampling plans to reject lots non-compliant to the established PO for Listeria monocytogenes

Following the method proposed by Withing sampling plans are established for cheese and celery. In the calculation the absolute numerical values for PO are used, instead of PO95%CL. Then just acceptable lot for the ingredients are estimated as the lots exceeding 0.013% the PO, taking into account the average standard deviation values for cheese and celery distributions, respectively 0.17 Log (CFU/g) and 0.40 Log (CFU/g). It should be stressed that in this case was used the st. dev. of the ingredients instead of the st.dev. of the final product because sampling is performed over the ingredients and should take into account the ingredients standard deviations. Through the EQ. 3 and 4 different sensitivity tests was set in order to complain PO with 95%CL.

The results from calculations are shown in Tables 31 to 34.

Sensitivity *m* Log(CFU/g)	**Samples *n***	***P exceeding***
-3.39	20	14.12
-3.50	13	20.9
-3.61	9	29.14

Table 31 – Sensitivity of the test, number of samples and probability to reject just unacceptable lot at 95CL for whiting sampling plans in celery to be added in spelt salad stored in air.

Sensitivity *m* Log(CFU/g)	**Samples *n***	***P exceeding***
-1.91	19	14.7
-2.02	13	21.41
-2.12	9	29.54

Table 32 – Sensitivity of the test, number of samples and probability to reject just unacceptable lot at 95CL for whiting sampling plans in celery to be added in spelt salad stored in MAP.

Sensitivity *m* Log(CFU/g)	**Samples *n***	***P exceeding***
-3.83	19	14.94
-3.88	13	21.66
-3.92	9	29.74

Table 33– Sensitivity of the test, number of samples and probability to reject just unacceptable lot at 95CL for whiting sampling plans in cheese to be added in spelt salad stored in air.

Sensitivity *m* Log(CFU/g)	**Samples *n***	***P exceeding***
-2.48	18	15.74
-2.54	11	24.89
-2.60	7	36.31

Table 34 – Sensitivity of the test, number of samples and probability to reject just unacceptable lot at 95CL for whiting sampling plans in cheese to be added in spelt salad stored in MAP.

CONCLUSIONS AND DISCUSSION

- ***Behavior of Salmonella in RTE product.***

To our knowledge, no explicit attempts have been made to determine the minimum growth temperature of *Salmonella enterica* in fresh-cut lettuce. Investigations carried out at discrete storage temperatures have led to variable observations ranging from slight population declines at 4°C (Kakiomenou et al., 1998) to survival without change in population size at 8°C (Manios et al., 2013) or very slow growth at 7°C (Sant'Ana et al., 2012). Disparities in outcomes between studies carried out at temperatures below 10°C can be ascribed to differences in the sensitivity to cold stress and/or minimum growth temperatures of experimental strains. In contrast, lack of growth at 14°C was unexpected given that *S. enterica* has been widely reported to grow above 10°C in fresh-cut material prepared from diverse lettuce cultivars, including romaine (Koseki, Isobe 2005; Sant'Ana et al., 2013; Tian et al., 2012). Lack of growth at temperatures above 10°C has been reported previously. Horev et al. (Horev et al., 2012) found that *S. enterica* Typhimurium populations remained unchanged on romaine lettuce leaves stored at 20°C in air or under modified atmospheres in experiments performed with whole leaves sanitized in 200 mg/ml free chlorine solutions, rinsed with water, and dried by centrifugation.

The first step of the experiment was to examine the growth of *S. enterica* on deliberately wounded and comparatively intact leaf tissues, in order to better understand the possible role of nutritious substances released from the wounded leaves on the growth of *S. enterica*. In fact, separation of leaves from the lettuce head and subsequent handling undoubtedly injures tissues, although the damage to whole leaves is probably slight in comparison with that inflicted by paring and slicing during further fresh-cut processing. Since *S. enterica* did not grow on romaine lettuce at both temperature of storage, irrespective of tissue damage, these observations suggest that the surface of romaine

lettuce leaves does not provide conditions conducive to active growth of this pathogen at the temperatures investigated in this study.

S. enterica populations also failed to increase at 14°C on lettuce tissues placed in contact with Cheddar cheese that yielded a pH of 5.1 an a viable lactic acid bacteria population of 10^5 CFU/g. Growth of *S. enterica* at low pH is influenced by the innate resistance of individual serovars to acidic conditions, the nature of the acidulants, medium composition, and temperature. For example, several serovars grew in a laboratory medium adjusted to pH 3.8 to 4.0 at 30°C but grew at pH 4.4 to 4.8 only at 10°C (Ferreira, Lund 1987). Reduction of pH due to the accumulation of lactic acid during fermentation of Cheddar cheese is achieved by inoculation with *Lactococcus lactis* subsp. *lactis* or *cremoris*, which can persist in the fermented product (Vedamuthuet al., 1966). Consequently, the combined effects of lactic acid and and low pH resulting from contact with Cheddar cheese may have restricted the growth of *S. enetrica* on tissue surface. In contrast, populations increased approximately 6 log CFU/g, over 6 days on lettuce tissues in contact with the comparatively pH neutral cooked chicken meat.

In this study was used the model system to examine the fate of *S. enterica* on lettuce tissues washed in water or a hypochlorite solution, the most common sanitizer used in most processing schemes for fresh cut vegetables, in accordance with regulatory requirements, which may differ among jurisdictions. Results confirmed that prior washing either in water or in a sanitizing solution did not prevent stimulation of *S. enterica* growth by contact with cooked chicken meat. However, the design of the model system ensured intimate contact between the lettuce tissue surface and the cheese or cooked meat. In commercial salads, the contact area between lettuce surfaces and added ingredients may be limited because of heterogeneous distribution of the ingredients or the geometry of the packaging system. Therefore, more realistic simulation of the mixed ingredient salad environment was accomplished by experimentation with a product formulated and

packaged in a commercial setting. The mixed ingredient salads used in the present work were offered for sale in a refrigerated retail display cabinet. Core temperatures of the salads upon arrival at the laboratory approached 10°C, which is well in excess of recommended norms for ready-to-eat foods, including prepared salads. Unfortunately, abuse temperatures may occur at several stages along cold chains, including the home. The storage temperatures selected for this study are representative of median (6°C) and maximum (14°C) values recorded in household refrigerators (James et al., 2008; Koutsoumanis et al., 2010). Hence, growth of *S. enterica* in a salad consisting of fresh-cut romaine lettuce mixed with nonproduce ingredients exceeded that in lettuce alone at temperatures known to occur in distribution systems or during subsequent handling by consumers.

Evidence derived from experiments performed with a model system and in the mixed ingredient salad indicated that contact with cooked chicken meat stimulated rapid *S. enterica* growth at the surface of contaminated lettuce leaves. These findings highlight the critical importance of strict temperature control during the manufacture, distribution, handling, and storage of salads formulated with ingredients that could stimulate the growth of pathogens such as *S. enterica*.

Further research will be necessary to verify the effect of other nonproduce ingredients on the ecology of human pathogens in mixed ingredient salads. Overall, these eveidences confirmed the need to evaluate, for each RTE product obtained with a mixing ingredients, the beahaviour of possibile microbial pathogens able to contamiante the specific food product. The data collected have to be included in the matemathical models used to estimated the changes of microbial contamination during the whole production chain.

- ***Microbiological characterisation of spelt salad as currently produced by the industry.***

Lactic acid bacteria (LAB) were the most prominent group of microorganisms of spelt salad, in association with drop of pH and often blowing of packages especially in the products held at 14°C. The carbohydrate-rich composition of these products, the microaerophilic condition (packaging in modified atmosphere containing 50%CO_2 and 50% N_2) and cold storage are factors that make them very competitive. In spelt salad their numbers was already high at 1 day of shelf life (approximately 5-7 Log CFU/g) and increased significantly (approximately 2-3.5 Log CFU/g) within 1-2 weeks, reaching high mean maximum population densities equal to 8.2-8.9 Log CFU/g. The spelt salad includes cheese as a source of LAB (i.e. Edam cheese), fresh cut vegetables and had an higher initial pH values (i.e., mean pH equal to 5.17). Some researchers reported that LAB, such as *Leuconostoc* mesenteroides and *Lactobacillus* spp. more likely contaminate the fresh cut and rinsed vegetables during processing (Barth et al. 2009, Pothakos et al., 2014), therefore the presence of LAB in steam cooked spelt, probably can also derive by contamination during processing.

The time to reach the maximum population densities of LAB was affected by the temperature of storage and was shorter at 14 than at 6°C (1 and 2 weeks, respectively). The inhibitory effect of LAB is mainly accomplished through formation of antimicrobial metabolites, such as lactic acid, which is the major end-product of LAB metabolism. Under experimental condition in artificial media, the concentration of the undissociated lactic acid [LaH] and pH are almost constant until the concentration of LAB reach approximately 6.5-7 Log CFU/ml, then the bacterial cells pass from the exponential growth phase to the stationary growth phase and variations of [LaH] (increase) and pH (reduction) are observed (Vereecken and Van Impe, 2001). Combinations of low pH, high concentration of lactic acid and low temperature can inhibit the development of *Listeria* spp. (Tienungoon et

al., 2000; Le Marc et al., 2002). The metabolic activity of LAB is affected by their number, the substrate composition (i.e. concentration of easily fermentable carbohydrates and buffering capacity), the initial pH (i.e. time to reach conditions that limit their growth) and the temperature. Mejlholm and Dalgaard (2015) modelled the simultaneous growth of *L. monocytogenes* and psychrotolerant lactic acid bacteria in processed seafood and mayonnaise-based seafood salads and observed that the onset of microbial interaction was at the time when LAB concentration is close to their maximum population densities. In the present study, the LAB maximum population density was reached within 1 week at 14°C and within 2 weeks at 6°C*. Listeria* spp. did not grow in spelt salads neither at 6° nor at 14°C. It is possible that *Listeria* spp. did not growth because the onset of the interaction with LAB preceded the end of the Lag phase duration and therefore the high numbers of LAB and the significant reduction of pH may have played a role in the inhibition of *Listeria* spp.

The presence of Enterobacteriaceae (ENT) in spelt salads was probably associated to its formulation. The presence of ENT was common in presence of fresh raw vegetables with numbers as high as 3 Log CFU/g, and are not necessarily associated with faecal contamination (Fröder et al., 2007). Moreover, many psychotropic ENT, such as Citrobacter, Enterobacter, Escherichia, Klebsiella, Proteus, Serratia, Hafnia and Erwinia, can growth at temperature above 6°C and are involved in food spoilage (Ledenbach and Marshall, 2009; Baylis et al., 2011). The fast growth of LAB and the concomitant reduction of pH may have had an impact in limiting their growth. In fact, their number showed a decline in the samples stored at 14°C at 14 and 18 days, whereas their number remained high in the samples held at 6°C. Possible risk management strategies for the fresh vegetables should include selection of suppliers (i.e. adopting controls on the quality of water and excluding the use of untreated manure) and effective cleaning and sanitation programs (Shen et al 2013; Catford et al., 2014).

- ***Incidence of Bacillus cereus and Listeria monocytogenes in commercial spelt salad and specific risk ingredients used to formulate the final product.***

The presence of *Bacillus cereus*, which was detected in 3 out of 9 lots of spelt salad at day 1 of the shelf life, can derive from different sources (i.e. frozen pre-cooked spelt, celery and cheese). Their number was always below the detection limit by the plating method (0.7 Log CFU/g). This value was relatively low compared to the estimated initial number of *B. cereus* (1.5 Log CFU/g) for ravioli filled with ricotta and spinach that received a thermal treatment equivalent to a pasteurization value (P7010) ranging between 10.5 and 200 min (Chaves Lopez et al,1998). Their growth model estimated that a maximum population density equal to 3.3 Log CFU/g can be reached after storage in MAP at 4°C for 30 days. The results of this study showed that spelt salad was characterized by the presence of an high number of LAB and that *Listeria* spp. did not growth during their shelf life. An hypothesis suggested by this work is that the onset of microbial interaction between psychotropic LAB and *Listeria* spp. was relatively fast due to the relevant metabolic activity of LAB in these products, which are rich in carbohydrates. This hypothesis should be more thoroughly investigated.

Moreover, the results obtained in spelt salads showed that fresh vegetables represent the main source of foodborne pathogens in RTE products and their appropriate washing is a key step to increase product safety. Since most fresh produce receives minimal processing and is often eaten raw, pathogen contamination can represent serious risk. Further, cutting, slicing or peeling cause tissue damage which releases nutrients and facilitates growth of microorganisms (Harris et al., 2003). *Bacillus cereus* and *Listeria monocytogenes* can occasionally contaminate the salads even if at very low concentrations. However, keeping modified atmosphere packaging (MAP) during storage and decreasing the initial product pH, with the consequent growth of lactic acid bacteria, seem efficaciously control the multiplication of those pathogens. One of the most essential

functions of MAP is to maintain integrity of packages. If the pack leaks, the optimized atmosphere within the food pack will become compromised as the protective gas mixes with normal atmosphere, consequently resulting in the loss of the beneficial effect of the modified atmosphere used (Smolander et al., 1997). In MAP applications, reduced O_2 and high CO_2 levels are used to extend product quality by controlling firmness, enzymatic browning, and decay of fresh vegetables. According to Rojas-Grau et al., 2009 in pack O_2 concentration must be sufficient to limit respiration but also prevent anaerobic respiration. Using low levels of O_2 and high concentrations of CO_2 in combination with a low storage temperature (< 7 °C) has been proposed by researchers as the optimal conditions for storing fresh-cut vegetables, maintaining sensorial and microbial quality (Jacxsens et al., 2000).

- ***POs and FSO values for Bacillus cereus and Listeria monocytogenes in spelt salad.***

The PO is a risk management concept we should became familiar with in the next future (Manfreda, De Cesare 2014). The achievement of a PO for a target microbiological hazard in a specific food product should help food industries to put on the market lots compliant to the FSO defined by food authorities for that microbiological hazard at the time of consumption. Each PO must be calculated for specific ingredients and/or intermediate products, according to the distribution of the microbiological hazard in those ingredients or intermediate products. Furthermore, the impact of each single production step and storage conditions on the hazard in the food up to consumption must be assessed.

Risk assessment models for the specific food/hazard combinations for which PO and FSO are calculated should attest that the compliance with the PO during the production process and the FSO at the time of consumption significantly affect the incidence of human foodborne diseases associated to the selected microbiological hazard and the proportion of food recalls, causing huge economic losses to food companies.

In this project is presented the approach to derive POs for *B. cereus* in spelt, cheese and celery, and POs for *Listeria monocytogenes* in spelt and cheese to be added as ingredients in a spelt mixed salad, packaged under modified atmosphere or air with a shelf life of 12 days. The results collected showed that spelt and cheese are risky ingredients for *B. cereus,* while cheese and celery for *L. monocytogenes* in the mixed RTE salad investigated. Furthermore, two steps in the process were identified as critical for the *B. cereus*: cooling after cooking of spelt and peas, that should be performed as fast as possible to inhibit spore germination, and mixing of ingredients, in which cross contamination may occur. On the other hand, the mixing of ingredients was considered the only critical step for the contamination of *L. monocytogenens* in the final product.

In order to derive POs for the selected ingredients, I assumed no cross contamination during mixing of ingredients and that contamination may arise only from the ingredients listed above. In order to estimate the contamination levels at consumption, expressed as the 95th percentile of concentration, the values of simulated initial contaminations, in which as input there was a single lognormal distribution, were used as parameters in the simulation. Moreover, the mixed salad has been considered an heterogeneous product and the standard deviation was considered equal to 0.8 Log CFU/g.

Specifically for *Bacillus cereus* the MC1 (after cooking few minutes may pass before cooling) step represents a critical phase of process production. This can be taken into account decreasing the reduction effect for *B. cereus* in that step. In my study a reduction ranging between 1.3 and 5.3 Log CFU/g during cooking has been considered since the experimental data showed that simulation underestimates about 4 Log the contamination of the final product. This is due to the fact that an higher number of positives is found in spelt salad in comparison to the single ingredients, to the lack of ”re-growth” estimation before and during cooling, and to possible cross contaminations. Therefore, a final reduction estimated by ComBase (i.e., 3.02 Log CFU/g for 0.2 h at 100°C) together with a further reduction due to the increase of the salad weight (i.e., 0.086 Log CFU/g). for addition of peas was included in the statistical model.

Specifically for *Listeria monocytogenes* the mixing phase and storage represent critical step. In fact as reported in Table 25, an increase of 0,52 Log CFU/g in cheese as well as an increase of about 5,08 Log CFU/g for spelt salad stored in Air and 3,61 Log CFU/g for spelt salad stored MAP was observed.

The POs were derived for *L. monocytogenens* and *B. cereus* by interpolating the proposed FSO in the regression obtained by representing the simulated contamination levels versus the contamination level at time of consumption, which was the output of the model (Figures 16-17).

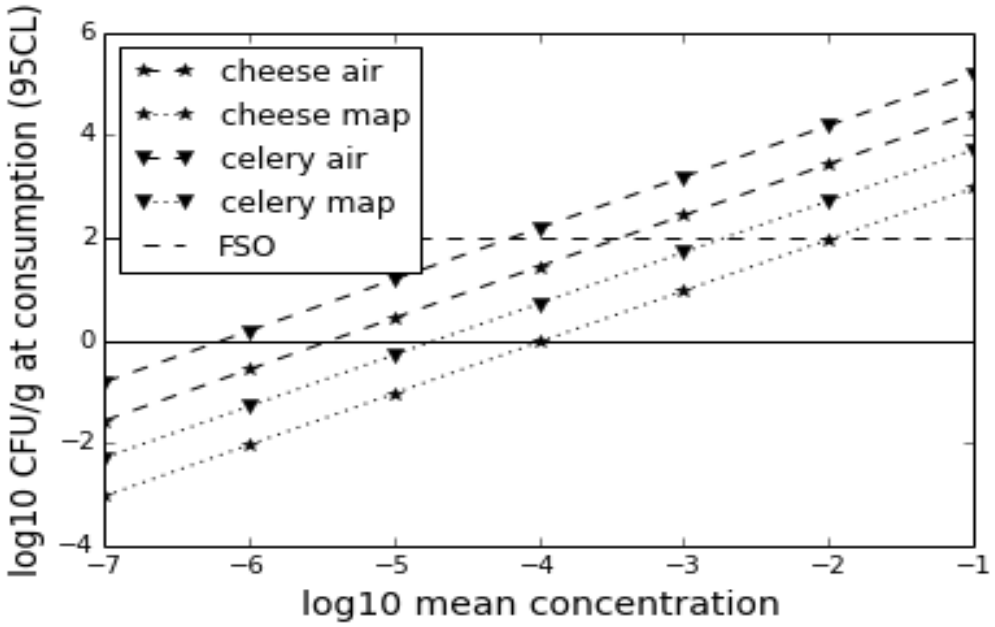

Figure 16 – POs for *Listeria monocytogens* in cheese and celery added in spelt salad packaged under air or MAP. The numerical values of the PO may be obtained interpolating the FSO line, the PO is respect to the 95 percentile of the cumulative distribution.

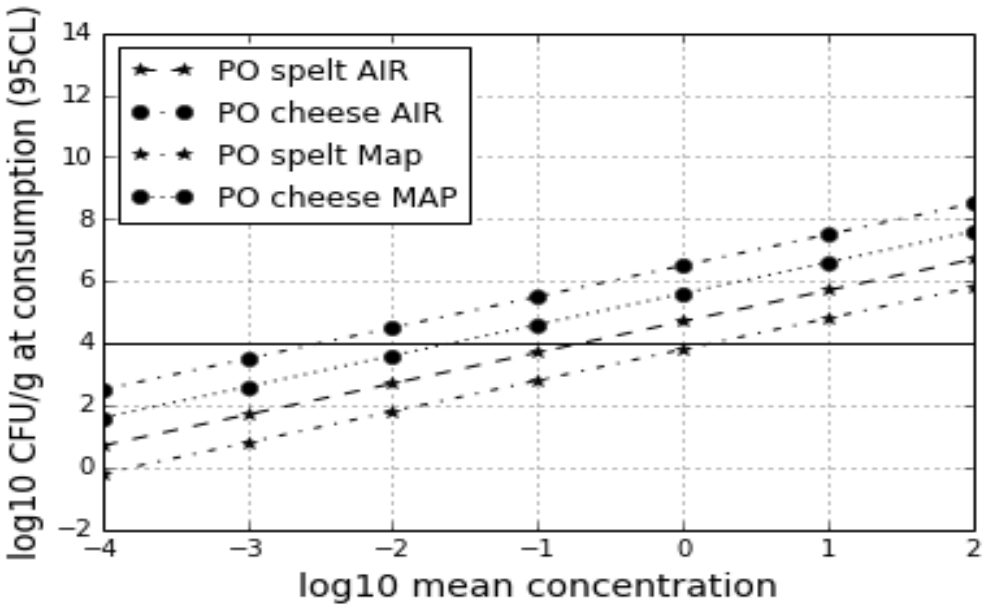

Figure 17 - POs for *Bacillus cereus* in cheese and spelt added in spelt salads packaged under air or MAP. The numerical values of the PO may be obtained interpolating the FSO line, the PO is respect to the 95 percentile of the cumulative distribution.

In order to provide to food industries clear quantitative targets for *Bacillus cereus* to have 95%CL of probability to commercialize food lots compliant, at the time of consumption, comply with proposed FSO, the PO values listed below, calculated at the 95 percentile of the cumulative distribution, must be reduced of the mean value of the distribution, corresponding to 1.32 Log CFU/g.

Therefore, the PO values to provide to the industries correspond to:

- PO1 = 4 (i.e., FSO) - 4.9 (storage in air) + 3.02 (cooking) + 0.086 (mixing with peas) – 1.53 (mixing of all ingredients) – 1.32 = - 0.64 Log CFU/g for spelt to be added in spelt salads stored under air;
- PO2 = 4 – 4.04 (storage in MAP) + 3.02 + 0.086 – 1.53 - 1.32 = 0.22 Log CFU/g for spelt to be added in spelt salads stored under MAP;
- PO3 = 4 – 4.9 (storage in air) – 1.32 = - 2.22 Log CFU/g for cheese to be added in spelt salads stored under air;

- PO4 = 4 – 4.04 (storage in MAP) – 1.32 = - 1.36 Log CFU/g for cheese to be added in spelt salads stored under MAP;

Concerning *Listeria monocytogenes* to derive POs with 95%CL of probability to commercialize food lots compliant, at the time of consumption, comply with proposed FSO, the PO values listed below, calculated at the 95 percentile of the cumulative distribution, must be reduced of the mean value of the distribution, corresponding to (– 0.58 Log CFU/g.

Therefore, the PO values to provide to the industries correspond to:

- PO1 = 2 (I.e., FSO) + 0.23 (mixing with celery) - 5.08 (storage in air) - 0.58 = - 3.43 Log CFU/g, for celery to be added in spelt salads stored under air;
- PO2 = 2 + 0.23 (mixing with celery) - 3.61 (storage in MAP) - 0.58= - 1.96 Log CFU/g, for celery to be added in spelt salads stored under MAP;
- PO3 = 2 - 0.52 (mixing with cheese) - 5.08 (storage in air) - 0.58= - 4.18 Log CFU/g, for cheese to be added in spelt salads stored under air;
- PO4 = 2 - 0.52 (mixing with cheese) - 3.61 (storage in MAP) - 0.58= - 2.71 Log CFU/g, for cheese to be added in spelt salads stored under MAP.

According to the PO calculated for *Bacillus cereus* and *Listeria monocytogenes* the only value detectable with the current microbiological methods correspond to *B. cereus* in spelt salad packaged under MAP.

- ***Sampling plans to reject lots non-compliant***

Overall, the lower is p exceed, the higher is the probability to accept lots at 95%CL (assuming a random sampling) comply with PO values.

The results concerning sampling plans for *Bacillus cereus* in spelt and cheese, reported in Tables 27-30 show a very few changes in number of samples to collect in the different ingredients as well as in different storage conditions (ranging between 18 to 20 samples) when the high sensitivity value (ranging between 14,26 to 15,71%) was applied. On the contrary no differences in terms of samples number to analysed (9) was identified using low sensitivity value (ranging between 29,24 to 30,29%).

Similarly the results concerning sampling plans for *Listeria monocytogenes* in celery and cheese, reported in Tables 31-34 show a very few changes in number of samples to collect in the different ingredients as well as in different storage conditions (ranging between 18 to 20 samples) when the high sensitivity value (ranging between 14,12 to 15,74%) was applied. When used a low sensitivity value (ranging between 29,14 to 36,31% the number ranged between 7 to 9 samples.

In conclusion the sampling plans proposed in this project can be used by food safety managers in order to fix a sampling plan according to their acceptable level of risk. Moreover the compliance of the POs should be done testing the sample number corresponding to the lower percentage of samples of the just acceptable lot that would exceed the sensitivity threshold, using a detection method including an enrichment step or molecular methods with extremely low detection and quantification levels.

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- M. Trevisani, A. De Cesare, R. Mancusi, F. Bovo, S. Vitali, G. Manfreda. 2015. Microbiological characteristics of pasta, spelt berry and rice salads and influence of storage temperature. *International Journal of Food Microbiology* (in press).

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