STANDARDIZATION OF INOCULATION PROCEDURES FOR SALMONELLAE AND SURROGATE BACTERIA FOR VALIDATING SPICE/HERB PATHOGEN REDUCTION PROCESSES

A Dissertation

by

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ABSTRACT

Suitable spice inoculation procedures using surrogates of *Salmonella* are needed to perform adequate validations of spice disinfection processes. In this study, inoculation procedures of dried oregano and onion powder (OP) were developed and standardized using *Salmonella*, and *Pediococcus faecium,* as a potential surrogate. The effectiveness of traditional liquid inoculation was compared with dry inoculation of spices using silica and talcum powder (as inert vehicles for the microorganisms). A small amount of inoculated OP was used as starter for inoculation of larger bathes of OP. The stability of the inoculated microorganisms was monitored over time at -18, 4, and 25 °C.

Salmonella strains isolated from outbreaks associated with spices (SRSS) and non-associated with spices (non-SRSS) were evaluated to determine their appropriateness to represent the most resistant pathogenic strains during spice desiccation processes.

For dry oregano, the final concentrations of SRSS, non-SRSS, and *P. faecium* were 5.5, 3.6, and 4.2 log CFU/g, respectively, when using a liquid inoculation. When removing 18% of the oregano's EO, the concentration of SRSS, non-SRSS, and *P. faecium* increased to 7.2, 5.1, and 6.4. log CFU/g, respectively; and when removing 58% of the oregano's EO, the final counts were 7.0, 5.6, and 7.2 log CFU/g, respectively. Dry inoculation of oregano using pre-inoculated inert powders yielded in silica: 6.7, 5.0, and 6.9 CFU/g, and in talcum: 6.0, < 3, and 7.8 log CFU/g of SRSS, non-SRSS, and *P. faecium,* respectively.

For OP, liquid inoculation was not successful since the consistency changed from powdery to clumpy and sticky. However, the OP consistency was maintained when

inoculating in a 1:50 v/w (inocula/OP) ratio. After desiccation, SRSS, non-SRSS, and *P. faecium* had a reduction of 0.7, 0.8, and 0.0 log CFU/g of SRSS, non-SRSS, and *P. faecium*.

The SRSS showed higher resistance to desiccation than the non-SRSS, whereas *P. faecium* showed the greatest resistance between the 3 groups of microorganisms.

In general, all microorganisms were more stable at -18 and 4 °C than at 25 °C during storage independently of the matrix tested.

DEDICATION

To all my beloved family and to Robert, the love of my life, thank you for your endless love, sacrifices, patience, prayers, support, and advices.

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NOMENCLATURE

a_w	Water activity
CFU	Colony forming units
EO	Essential oil
FDA	Food and Drug Administration
non-SRSS	Non-spice-related *Salmonella* strains
OV	Overlay
SRSS	Spice-related *Salmonella* strains
VHTO	Vacuum-heat treated oregano
WTO	Water-treated oregano

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1 INTRODUCTION

In light of the new evidence that questions the safety of spices, more attention has been paid to the mitigation and control of pathogens from farm to table to reduce public health risk associated with spice consumption. Food safety specialists have paid special attention to the development of validation procedures, supported with challenge studies, to evaluate disinfection processes of spices.

The design of validation procedures using challenge studies starts with the selection of target pathogens and their potential surrogates, inoculation procedures, stabilization of the inoculated product, and verification of the surrogate suitability when a specific control measure is used. In this study, standardized and stable methodologies were developed for the inoculation of two type of spices, dried oregano leaves and onion powder.

The use of sanitary practices during production, harvesting, and processing of spices may not be sufficient to reduce the risk of contamination since these products are commonly grown in open fields and usually subjected to drying processes in open environments. Therefore, the use of antimicrobial treatments is essential to minimize the possible presence of foodborne pathogens.

Various antimicrobial treatments have been developed to sterilize spices and herbs. The major treatment categories are 1) irradiation, such as gamma and e-beam rays, 2) fumigation, with propylene oxide or ethylene oxide, and 3) heat processes like high-pressure steam and dry heat *(55).*

Ideally, the efficacy of these treatments at reducing bacterial pathogens should be validated to provide quantifiable control assurance. Validation consists of collecting evidence that demonstrates the control measure is capable of controlling the hazard to specified levels *(34)*. This evidence can include data from scientific literature, pathogen reduction modelling, in-plant data collection, challenge studies, and/or hazard enumeration.

Although quantification of the reduction in the naturally occurring hazard is ideal in validating a control measure, pathogens are not commonly present in spices, and if present, they are usually inconsistent and in low concentrations. Validations have been conducted by quantifying naturally occurring microorganisms such as aerobic plate counts, *Enterobacteriaceae,* coliforms, and non-pathogenic *Escherichia coli (47, 94, 134, 142, 170).* The downside of using these organisms is that they are often at levels below the targeted reduction of the pathogen that they may represent, and therefore not being adequate for validating the control measure. Even if they were present at concentrations sufficiently high to precisely quantify their reduction, depending on the selected group of microorganisms, these reductions may not accurately predict the reduction of the target pathogen. Therefore, this approach may not be adequate to measure such intended reduction *(55).*

The use of challenge studies is usually preferred due to their accuracy; but artificially inoculating the pathogen of concern in spices or any other food product, is not tolerable in food processing plants due to safety concerns. A recommended solution is to conduct challenge studies using surrogates. Surrogates are defined as nonpathogenic

microorganisms that behave similarly to the pathogen under the same conditions *(25)*. In order to conduct any validation procedure, it is necessary, first, to develop and standardize inoculation methods of both, the pathogen and the surrogate at the laboratory level *(25)*. Traditionally, the liquid inoculation has been used when performing challenge studies. However, increasing the moisture content in low-a_w foods, such as spices, would likely modify their texture and a_w and perhaps, would be difficult to return it to the initial values. Modification of such properties could mislead the selection of treatment parameters required to assure control of the pathogen (*e.g. Salmonella* reduction rates are higher in products with higher a_w than with low a_w).

Therefore, standardizing inoculation procedures that do not alter moisture content of spices is one of the tasks needed to perform adequate validation of disinfection processes.

2 LITERATURE REVIEW

2.1 Safety of low-moisture foods

Low-moisture foods are considered to be those products with water activity (a_w) values lower than 0.7 *(68)*. Examples include chocolate, cereals, honey, pasta, peanut butter, powder infant formula, beef jerky, spices, and dried coconut. Although these products are microbiologically stable (*i.e.* do not sustain pathogenic bacterial growth) *(68)*, they have been shown to allow the survival of pathogenic bacteria for extended periods of time *(85, 86, 163)*. In addition, several outbreaks of foodborne illness have been linked to low moisture foods *(16, 83, 95, 132, 144, 162)*. Furthermore, bacteria exposed to low-moisture environments can increase their heat tolerance *(50)*, thus, stronger thermal inactivation treatments are usually required to reduce pathogens in low-moisture foods *(30, 102)*.

Among the pathogenic bacteria found in low a_w foods are spore-forming bacteria, for example, *Clostridium botulinum, Clostridium perfringens,* and *Bacillus cereus,* and non-sporeforming bacteria, such as *Cronobacter sakazakii, Listeria monocytogenes,* Shiga-toxigenic *E. coli, Staphylococcus aureus,* and *Salmonella (28, 55, 166)*. Microorganisms such as *Clostridium botulinum* and *S. aureus* will need to grow to produce sufficient amount of toxin to produce illness. Therefore, other microorganisms such as *Salmonella* become of greater concern since few cells can cause illness *(6, 65, 144)*.

2.1.1 Safety of spices

Among these low a_w products, spices have attracted the attention of food safety experts due to their relatively unexpected participation as vehicles of *Salmonella* causing outbreaks of foodborne illness, high probability of becoming contaminated during harvesting and drying if adequate hygiene is not applied, they are frequently ready-to-eat products, and are usually consumed by people from almost all ages. Spices are produced from diverse farm sizes and agricultural practices all around the world. In many cases the production of spices come from different small producers where harvesting is performed by hand, farm animals are used to plow, and/or production and processing of spices take place in open fields increasing the risk of contamination *(6, 55)*.

In the United States, Food and Drug Administration (FDA) regulations require that all spices intended to be imported meet the federal regulatory requirements for food safety and cleanness. In a survey, this agency reported a prevalence of *Salmonella* in imported spices to be close to 7%, almost twice as all other FDA-regulated imported foods *(55)*. This prevalence could be underestimated considering the reported challenges in the recovery of pathogenic bacteria from spices *(68)*. A combination of factors such as the natural antimicrobial compounds of spices and the desiccation processes may create a hostile environment for bacterial growth and survival. Therefore, cells that are surviving under these conditions likely will present some sort of injury. This ultimately can result in their inability to grow in selective culture media if an adequate testing method is not used *(55, 144)*. Outbreaks of foodborne illness linked to consumption of contaminated spices have been reported *(6, 168)* even if spices are usually subjected to decontamination

processes (such as ethylene oxide, propylene oxide, gramma irradiation, heat and steam) before and/or after importation *(6)*. Van Doren, *et al. (168)* conducted a comprehensive literature review related to the foodborne disease outbreaks associated with spices. In the period of 1973 - 2010, fourteen outbreaks were documented in United States, Canada, and some European countries. Remarkably, 10 of the 14 outbreaks (71%) were caused by *Salmonella.* The other four (29%) were attributed to *Bacillus* spp. The high number of outbreaks related to *Salmonella* in spices is of great concern when considering that even if they cannot grow in dry spices due to low moisture, only few cells present can be sufficient to cause infection, especially when considering that spices are consumed by people from all ages (toddlers to elderly) and all health conditions, increasing the risk of getting infected especially if contaminated spices are added to ready-to-eat foods *(55)*.

The spice production and processing from farm to table involves several steps where contamination can occur *(6, 55)*. As with other food commodities, using sampling and testing as a tool to determine the presence or absence of contamination does not seem to be an effective measure to minimize the risk of foodborne illness, and therefore the industry relies on the application of good agricultural practices and good manufacturing practices as well as the application of disinfection treatments to reduce the risk of contamination of spices. In several instances, the current spice disinfection processes for the reduction of *Salmonella* (or other pathogens) have been found not to be correctly validated *(72)*. The efficacy of these treatments has been measured by quantifying the reduction of microorganisms already present in the spices such as total aerobic plate counts, yeast and molds, coliforms, *E. coli*, and *Enterobacteriaceae (55)*. However, these

microorganisms would accurately represent the pathogens only if their sensitivity/resistance to the disinfection is similar to the sensitivity/resistance of *Salmonella* under the same conditions, and if the concentrations of the indicators are high enough to allow measurement of the reduction *(55)*. Therefore, there is a critical need for comprehensive research related to inoculation procedures for spices to perform validation of the disinfection of spices measured in terms of logarithmic reductions of *Salmonella*. For in-plant studies, using adequate surrogates is recommended to predict the inactivation kinetics of *Salmonella* in a disinfection process.

To evaluate the ability of surrogate bacteria to represent a specific pathogen, it is necessary to establish and standardize an inoculation procedure for both the surrogate and the pathogen in the food matrix. This is conducted in laboratory or pilot plant settings. The inoculation procedure for spices may be challenging *(55, 144)*. In challenge studies, replicating real conditions of contamination, handling and storage of the dry food, etc. will increase the reliability of the validation study. However, in spices, replicating natural conditions may have a negative effect on the inoculated bacteria by exposing them to stressors such as antimicrobial compounds present, osmotic shock and the drying process after inoculation *(9, 31, 124, 150)*, and therefore, care must be taken to apply laboratory methods capable of detecting cells that have been injured due to stress.

2.2 *Salmonella enterica*

The *Salmonella* genus was discovered and named after Dr. Daniel E. Salmon. This genus belongs to the family *Enterobacteriaceae. Salmonella* genus is divided in two species, *S. enterica* and *S. bongori.* The latter is more associated with cold-blooded animals, although rarely can cause disease in humans *(58)*. *S. enterica* is the most commonly associated with human salmonellosis; therefore, for the rest of this document reference will be made only to *S. enterica*. *S. enterica* is divided in the subspecies: *S. enterica* subsp. *arizonae, S. enterica* subsp. *diarizonae, S. enterica* subsp. *enterica, S. enterica* subsp. *houtenae, S. enterica* subsp. *indica,* and *S. enterica* subsp. *salamae (115). Salmonella* is a Gram negative rod-shaped bacterium. Generally, *Salmonella* spp. are motile with a peritrichous arrangement, all are facultative anaerobes and non-spore forming. The optimal growth temperature is 37 °C and can grow in a range of 5 to 40 °C. The optimal pH is neutral-alkaline from 6.6 to 8.2. One of the unique characteristics of *Salmonella* is its ability to survive under low a_w environments. *Salmonella* can grow under a_w of 0.93, although the optimal a_w is > 0.98 *(93)*. This pathogen is not a fastidious organism and does not require of special nutrients to grow. It can survive for long periods of time in different food matrices and environments, even if they are not optimal. It shows an special ability to survive desiccation processes and remain alive for long time in low a_w environments, making it of a major concern in the low-moisture food industry *(57)*.

Every year in the U.S., 1.2 million cases of salmonellosis are reported, with an annual incidence of 15.2 illnesses per 100,000 people *(29, 145)*. Infections occur when eating contaminated food or water or by not having a good hygiene after touching

contaminated animals. In average, the first symptoms appear 12 – 72 h after infection and the illness can last 4 – 7 days.

The natural habitat of *Salmonella* is the intestinal tract of humans and animals, but it can survive for long periods in hostile environments. The most common vehicles of *Salmonella* causing foodborne outbreaks are contaminated eggs, poultry, meat, unpasteurized milk and juice, cheese, contaminated raw fruits and vegetables including sprouts and melons, nuts, and spices *(161)*. Among them, spices have become of special concern since *Salmonella* has shown high resistance to desiccation and dry conditions for extended periods of time, making them a food safety priority *(100)*.

2.3 Surrogate organisms

Surrogate microorganisms are nonvirulent bacterial strains that possess well-known characteristics and can substitute pathogenic bacteria in challenge studies. The criteria of an ideal surrogate was listed by Busta, *et al. (25)* and includes the following characteristics: nonpathogenic, similar behavior to the target pathogen, stable and consistent growth characteristics, easy preparation and maintenance, the ability to yield high-density populations, easy and inexpensive enumeration and detection, genetic stability, unlikelihood of spoiling food or damaging equipment, and susceptibility to injury similar to the target pathogen.

When a food product is subjected to a control measure, surrogates are used to evaluate the efficacy of the cleaning, sanitation, and disinfection processes.

Pediococcus faecium (previously *Enterococcus faecium*) strain NRRL B-2354 has been used as a surrogate of *Salmonella* in challenge studies especially when testing foods with high a_w such as dairy products *(7)*, juice *(131)*, and meat products *(90, 99)*. However, this coccus has been studied to be used for disinfection of low a_w food subjected to thermal processes showing good correlation with *Salmonella (4, 30, 49, 82, 85)*. It has been claimed that certain *Pediococcus* strains lack suitability as surrogates of *Salmonella* when tested in low a_w products *(127)*. For example, Peña-Meléndez *(127)* evaluated the *E. faecium* OSY 31284, an isolate from pistachios, as a potential surrogate of *Salmonella* during the disinfection of pistachios using a heat-ozone treatment. In their study, it was found a much higher resistance of *E. faecium* OSY 31284 to the treatments (> 2 log cycles). According to the conclusions of these authors, the higher resistance of *E. faecium* OSY 31284 made it not a good surrogate of *Salmonella* since the recommended resistance of a surrogate has to be equal to the pathogen or slightly higher *(25)*.

Almonds were one of the first low a_w products inoculated with *Pediococcus faecium* NRRL B-2354 for the evaluation of disinfection processes. Due to the good results, *Pediococcus faecium* NRRL B-2354 has been being evaluated as a potential surrogate of *Salmonella* other products, like peanut butter and spices, using other technologies for disinfection such as irradiation and ethylene oxide *(8, 116)*.

2.4 Foodborne disease outbreaks associated with spices

The United States Food and Drug Administration (FDA) defines spices as "any aromatic vegetable substance in the whole, broken or ground form, except for those substances which have been traditionally regarded as foods, such as onions, garlic and celery; whose primary function in food is seasoning rather than nutritional; that is true to name; and from which no portion of any volatile oil or other flavoring principle has been removed" *(54)*. Spices can be obtained from different parts of the plants such as seeds, leaves, stalks, bark, fruits and rhizomes *(31)*. The particular smell and taste is given by a mixture of essential oils (EOs). These secondary metabolites are a mixture of terpenoids, phenolic compounds, alcohols, aldehydes, aliphatic hydrocarbons, acylic esters and/or lactones that are found mainly in secretory cells, cavities, canals, glandular trichomes or epidemic cells. *(113)*. The antimicrobial effect depends on the active compound, its concentration, and the interaction with the composition of the cell walls *(160)*. The antimicrobial properties of EOs have been studied extensively *(61, 140, 158, 160, 176)*. EOs such as eugenol, carvacrol, thymol, basil oil, and anise oil have been added directly or encapsulated to cultures of pathogenic bacteria *in vitro* or onto contaminated food surfaces to measure their antimicrobial effect *(9, 31, 124, 140, 150, 176)*.

Although spices in general are known to possess antimicrobial compounds and do not support bacterial growth due to the low water activity, they can harbor pathogenic bacteria. Over several years, multiple recalls and outbreaks related to different spices contaminated with pathogenic bacteria have been reported all around the world *(26, 69, 79, 83, 95, 154)*.

From a thorough literature review, Van Doren, *et al. (168)* collected information of 14 outbreaks of foodborne illness associated with spices from 1973 to 2010. The identified vehicles of the pathogenic bacteria included peppercorn, paprika, turmeric, pepper, curry powder, anise seeds, fennel seeds, spice blend, and seasoning mix, and broccoli powder.

The high proportion of the outbreaks associated with *Salmonella* is quite remarkable, accounting for 71% of the outbreaks (9/14). The remaining 29% (4/14) of the outbreaks was attributed to *Bacillus* spp. *(168)*. The country of origin was identified in 62% (9/14) of the outbreaks, and in all these cases they were imported *(168)*. The identified importers of the contaminated spices included Brazil, China, India, Malaysia, Turkey, and Vietnam *(168)*.

The occurrence of outbreaks and the prevalence of *Salmonella* reported in spices at the different points from farm-to-table reflect the significant problem behind all the farm-to-table continuum to control the safety of spices, especially when considering their production takes places principally in open fields and they are usually harvested by hand. Even though contamination of spices probably occurred before importation in the majority of the outbreaks, it is suspected that spices were possibly contaminated in an after-importation process in at least two of the outbreaks (2/14) *(26)*. By the end of 2008 and beginning of 2009, a multistate outbreak of *Salmonella* Rissen infections was associated with contaminated white pepper imported from Vietnam. Although sampling of an unopened bag tested positive for *Salmonella*, indicating that the white pepper was tainted before importation, an unsanitary post-importation process indicates that cross-

contamination could have been possible. From July 2009 to April 2010 another large multistate outbreak of salmonellosis was associated with black and red pepper in salami snacks, affecting 272 people in the U.S. *(27)*. Samples from ingredient suppliers, patients and processing facility were compared and results from the next-generation sequencing revealed a closer similarity of the *Salmonella* Montevideo strain from the outbreak and the only strain isolated from the processing plant. These results suggested a possible cross-contamination in a post-importation processing *(42, 96)*.

Salmonella has been shown to possess better survival capacity than other non-sporeforming pathogenic bacteria under low water activity environments and food, including spices *(50, 70, 85, 132, 168)*. Survival of the pathogen depends on the strain, serotype, stage of growth, route of contamination, point of contamination during the spice processing (*i.e.* before harvesting, during drying, in a post-drying process), and stressing factors that cells have been exposed to, such as temperature, addition of solutes, acidity, and EOs *(50, 66, 143)*.

2.5 General characteristics of oregano (*Oreganum vulgare* L.)

Oregano (*Oreganum vulgare* L.) is the leaf of a perennial herb widely distributed but main commercial producers are Turkey and Mexico. It is used as a condiment for its aromatic properties given by the EOs. The main EO compounds found in oregano are carvacrol and thymol which possess antimicrobial, antifungal, antiparasitic, and antioxidant properties. The total production of oregano worldwide is estimated to be 15,000 tons per year from which Turkey produces 10,000 tons. The US is the largest

importer of oregano with 6,000 tons per year *(5, 62)*. In developing countries, it is common to dry oregano under the sun for several days until reaching 10% of moisture *(40, 133)*. Mechanical drying is also used to dehydrate oregano, but is less popular in developing countries, especially if the final product is the result of the production from different small farms *(40, 133)*. This conventional drying increases the bacterial load in the oregano leaves and the probability of contamination with foodborne pathogens since the product is exposed directly to the environment. Then, feces, dust, rodents, birds, and other wild animals are the most likely route of contamination of oregano at the farm. Contamination during packaging can be possible when sanitary conditions are not ideal *(26)*.

Oregano's EO has one of the most powerful antibacterial effects due to the relative high concentration of carvacrol and thymol among other aromatic volatile compounds. In fact, the oregano's EO has been studied to reduce *Salmonella,* pathogenic *E. coli,* and other pathogens *in vitro* and has been proposed to be used for pathogen control specially in fresh vegetables *(22, 140)*. For example, Boskovic, *et al. (22)* reported a minimum inhibitory concentration (MIC) of 320 μg/ mL for *S.* Senftenberg and *S.* Give when using oregano's EO, and 640 μg/ mL when using thyme's EO. Among the individual active compounds, carvacrol and thymol had the same MIC of 320 μg/ mL to inhibit *Salmonella*, while cinnamaldehyde and eugenol required 640 and 1280 μg/ mL, respectively *(22)*. This higher antimicrobial effect of oregano's EO has been reported also for other microorganisms *(59, 61, 76, 98, 105)*. In spite of the above, the EO has to be released from the leaf to get in direct contact with the bacteria to exert the antimicrobial effect. Hence, it is not surprising finding levels of 3-4 and 2 log CFU/g of mesophiles and coliforms in

dried oregano *(104, 148)*. Some reports have found levels > 5 log CFU/g of *Enterobacteriaceae* in dried oregano leaves *(104)*.

2.6 General characteristics of onion (*Allium cepa* L.)

Onion (*Allium cepa* L.) is an herbaceous biennial belonging to the *Amaryllidaceae* family *(171)*, thought to be native to Afghanistan *(118)*. Approximately 88.5 million ton of this crop are produced every year, primarily in mainland China (17,000,000 ton), India (9,600, 000 ton), United States of America (3,200,000 ton), Turkey (2,000,000 ton), and Iran (1,600,000 ton) and about 8% is traded internationally *(52, 107)*. Onion powder is not considered a spice by the FDA for regulation purposes *(45)*, however, in the traditional classification of spices, it is considered as an aromatic vegetable *(141)*.

Onion bulbs are deemed mature when the leaves of the plant bow and fall over. When 70 to 80% of the crop has reached maturity (100 to 140 days for most plants and 35-45 days for spring onions), harvesting begins. In developing countries, the bulbs are usually loosened with a fork and extracted manually. Relative to mechanical methods used in developed countries, manual excavation is laborious and inefficient. Mechanical harvesting techniques generally proceed by first mowing existing leaves and foliage. In moist, mild climates, onion bulbs are immediately excavated using modified potato harvesters, sieved to remove debris, manually graded, collected in bins or bunches, and finally transported for further mechanical drying. This method is referred to as one-phase harvesting. Alternatively, two-phase harvesting can be implemented for crops cultivated in warm conditions with low humidity. In this technique, onions are undercut, stubbed,

and sieved, then rolled into rows for windrowing (drying) in the field. Under ideal conditions, sufficient drying is achieved within 2 weeks. At this time, bulbs are again harvested and sieved, manually graded, and transferred into trailers or crates for transport. Although two-phase harvesting eliminates the need and expenditures for mechanical drying, the labor costs for this method well exceed those of one-phase harvesting *(45, 122)*. Production of onion powder is performed by dehydrating under the sun, but drying in a cabinet at 55-60 °C for 10-15 h is more commonly used. The final moisture content of onion powder is sought to be 4% *(45)*.

Phenolic compounds, protocatechuic acid, catechol, and sulfur compounds present in onions impart an antimicrobial effect. Sulfur compounds are also responsible of the characteristic flavor of onions *(171)*. However, to exert the antimicrobial effect, it is necessary disrupting the cell to release an enzyme that converts S-(1-propenyl)-L-cysteine sulfoxide to thiopropanal-S-oxide.

In vitro studies showed that the juice and vapors released by onions inhibits the growth and/or production of toxins of *Bacillus cereus, Clostridium botulinum, Lactobacillus, Salmonella, Shigella, S. aureus, Aspergillus flavus, Candida albicans, Rhodotorula, Saccharomyces,* etc. *(15, 48, 139)*. However, the organosulfur compounds from onion showed a lower antimicrobial effect than those compounds from garlic and ginger when tested against *Salmonella, E. coli,* and *Bacillus subtilis (11)*.

2.7 Mechanisms of *Salmonella* survival to desiccation

The unusual ability of *Salmonella* to survive under low-moisture conditions is not fully elucidated but some mechanisms proposed are described below.

The level of survivability in desiccation processes depends of the *Salmonella* strain, the solids present in the food matrix before dehydration (*e.g.* sugars and fat), previous exposure to other stresses, etc. Once the bacteria have survived desiccation, the length of survival if affected by the storage temperature, oxygen presence, and final a_w *(91).*

The mechanisms for the survival of *Salmonella* in low-moisture environments are represented in Figure 1 *(50)*. When the bacteria are being exposed to low-moisture environments, the first mechanism of defense for survival is increasing the concentration of internal solutes that bind molecules of water to maintain a correct turgor pressure by balancing the osmotic pressure. The solutes can be produced by the bacteria or can be obtained from the same environment. Examples of low-molecular solutes are proline, glycine-betaine, ectoine, which are transported by the ProP and ProU proteins. These solutes are transported through two membrane porins; the OmpF and the OmpC that are expressed as a response of a low a_w *(173)*. Another solute is trehalose, produced from glucose utilizing ATP obtained from the catabolism of fatty acids.

As reported for other stresses, the sigma factors (σ) play an important role for the survival of *Salmonella* during as a response of sublethal stresses. For *Salmonella*, the σ^S is associated with survival to desiccation while the σ^E is associated with the survival during long periods of time in the desiccated product *(51, 67)*.

The production of glycocalyx during biofilm formation has been reported to protect against desiccation *(123).* A compound of the slime, colanic acid is composed by D-glucose, D-galactose, L-fructose, and D-glucuronic acid in a ratio 1:2:2:1 *(123, 156).* This exopolysaccharide has the ability of absorbing large amounts of water, protecting the bacterial cell against desiccation *(137).*

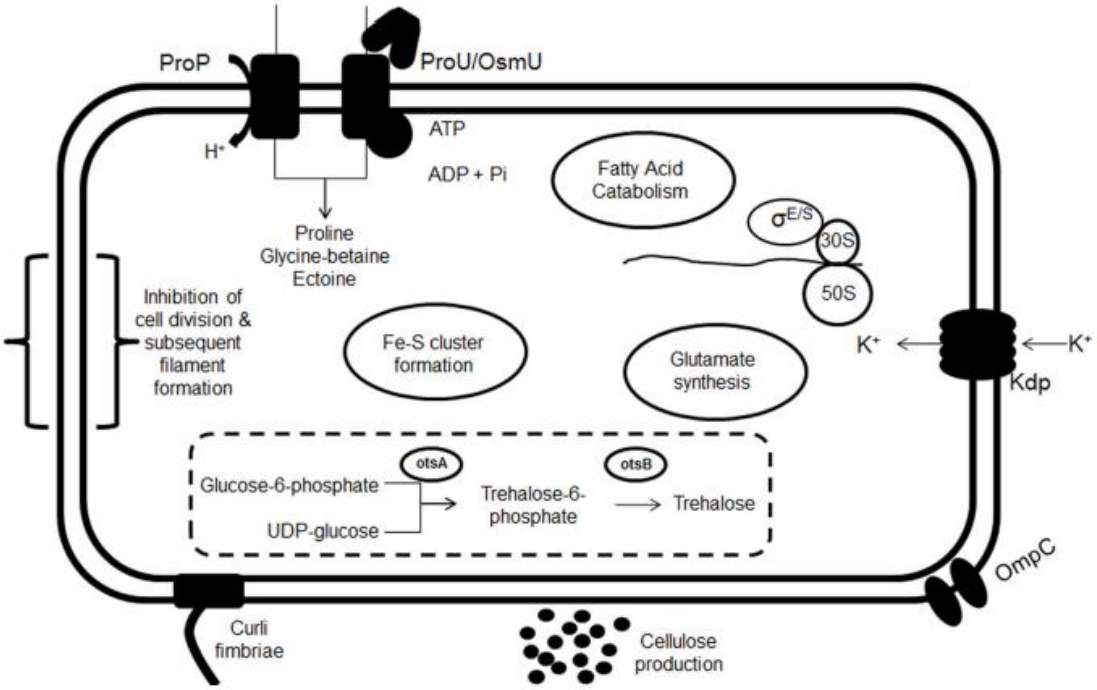

Figure 1 Mechanisms of *Salmonella* survival in low-moisture foods.
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2.8 Validation of control measures

Various antimicrobial treatments have been developed to sterilize spices and herbs. The major treatment categories are 1) irradiation, such as gamma and e-beam rays *(3, 12,*

60, 88, 94, 110, 128, 134, 142, 153, 165), 2) fumigation, with propylene oxide or ethylene oxide *(39, 60, 109, 116, 148, 165)*, and 3) heat processes like high-pressure steam and dry heat *(6, 55, 82, 134, 142, 170)*.

Ideally, the efficacy of these treatments at reducing bacterial pathogens should be validated to provide quantified assurance of control.

The effectiveness of steam treatments for the reduction of *Salmonella* in spices is *Salmonella* has become the target pathogen in the spice control measures due to the higher prevalence and higher association as an outbreak-causing pathogen in spices. The United States Food and Drug Administration (FDA), in the document entitled: "Draft Risk Profile: Pathogens and Filth in Spices" *(55)* addresses and gives an overview of the health risk associated with consumption of spices in the U.S., as well as the current control measures, data gaps, and research needs to reduce or prevent new episodes of illnesses associated with microbial contaminated spices. One of the important needs is the generation of information to conduct validation of the treatments to reduce *Salmonella* in spices. Part of this information includes the determination of the prevalence and concentration of *Salmonella* in spices and the level of post-process contamination. Knowledge of the risk of post-process contamination becomes essential since about 70% of the spices consumed in the U.S. are imported and subjected to post-importation processing. As for other food commodities, post-disinfection processes increase the likelihood of recontamination, especially if the hygienic practices are not adequate *(26, 27)*. Then, one question that arises is: what the safe reduction level should be for a disinfection treatment in the meantime of getting this missing data *(146)*. Schaffner, *et al.*

(146) concluded that where there is no enough available data, a 5-log cycle reduction may be used as reference assuming the good manufacturing practices are effective and the initial concentration of the pathogen tends to be low with a low prevalence *(146)*.

Validation of control measures has represented a challenge since the procedures to perform the validation have not been established and standardized. Some of the obstacles are the establishment of suitable inoculation methods for challenge studies, selection of the pathogens and their surrogates, and development or improvement of methodologies for isolation and quantification of pathogens. Hence, one of the only resources used are the quantification of naturally occurring microorganisms such as aerobic plate counts (APCs), *Enterobacteriaceae*, coliforms, and non-pathogenic *Escherichia coli (3, 47, 94, 134, 142, 170)*. However, validation of a control measure consists of collecting evidence that demonstrates the treatment applied is capable of controlling a specific hazard to safe levels. It is important to highlight that concentration of these pathogenic bacteria in the food product is not always high enough to accurately measure the logarithmical reduction after the treatment.

Ideally, a validation should be performed before the implementation of the control measure and whenever any parameter is modified, and the Codex Alimentarius *(34)* recognizes various approaches to validate control measures (Figure 2).

When conducting experimental studies, it is desirable to perform these measurements at the food processing plant since it would be more effective, but pathogens are not commonly present in spices *(1, 13, 56, 63, 71, 108)* and when present, they are usually inconsistent and in low concentrations *(69, 71, 80, 89, 95, 164, 167)*. Then, with

those limitations, the use of a pilot plant would be ideal, however, they are usually not available. Hence, the next more reliable option is the performance of challenge studies using surrogates (Figure 2) *(2).*

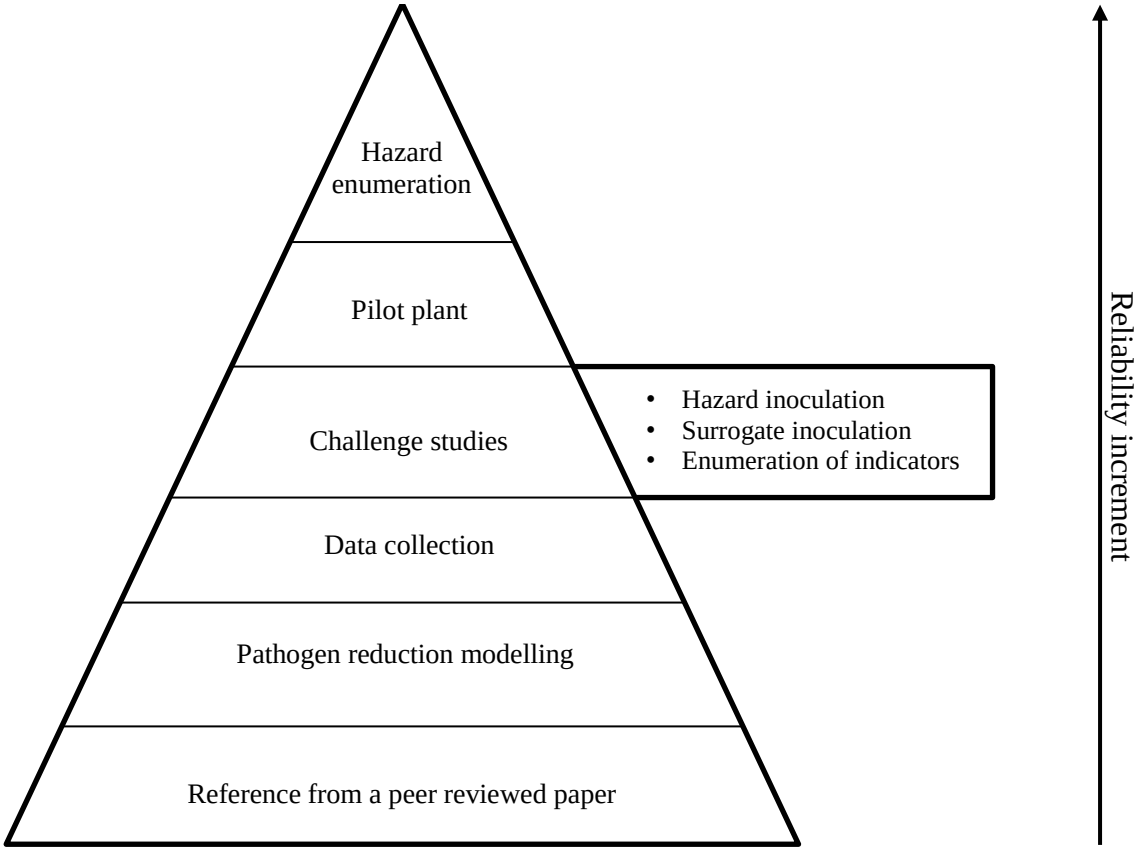


Figure 2 Increment in reliability when conducting validation of control measures. Reprinted from "Process Validation for the Control of Pathogens in the Food Industry" by Acuff *et al*., 2015. Reprinted with permission.

Selection of spice-related *Salmonella* is capital when conducting validations of control measures. As mentioned above, bacteria get resistant to different stressors when previously exposed to sublethal conditions. Therefore, strains of *Salmonella* isolated from

spices would more likely to show a higher resistance to a disinfection treatment than other strains isolated from other sources. This phenomenon has been described for *Salmonella* and other microorganisms in low-moisture environments *(50, 77)*. *Salmonella* have shown to have high resistance to desiccation and higher and more prolonged survival when stored at low temperatures *(77, 81, 162)*. In addition, the resistance to dehydration differs between various serotypes of *Salmonella* and can cause cross-tolerance to other stressors, including high temperatures, salts (*e.g.* NaCl and bile salts), disinfecting agents (ethanol, hydrogen peroxide, sodium hypochlorite, and quaternary ammonium compounds), and UV irradiation *(66, 77)*.

Different approaches aiming towards the evaluation of the control measures for low a_w products have been reported *(85)*. These approaches vary from investigator to investigator. Methodologies for the evaluation of the control measures should be established especially for the tasks prior to the validation of the control measures such as the *Salmonella* strains, the type of inoculation (*i.e.* liquid vehicle inoculum or dried vehicle inoculum), the moment of inoculation (*i.e.* fresh herbs or dried spices), dehydration procedures to reach the original a_w (*i.e.* temperature, RH, and time), and determination of the sample size to be treated. The control of these variables has as an objective to mimic as much as possible the common contamination routes, avoiding the exposure of the bacteria to other stresses that may skew the conclusions of the effectiveness of the control measure. Standardization of the inoculation procedures may differ for each type of products. For instance, inoculation of dried spices may be specific for powders, leaves, and seeds. Liquid inoculation is the most common technique used to inoculate food in

challenge studies. However, this technique may not be suitable when working with low-moisture foods. Some of the downsides of utilizing liquid inoculation are the possible release of water-soluble antimicrobials, changes of the water activity and original texture that perhaps may not be reversible *(20, 125, 176)*.

The release of antimicrobials in the medium can reduce the levels of the microorganisms being inoculated, therefore, the targeted concentration of these bacteria will be reduced.

On the other hand, changes in texture, such as stickiness and clumping, could be more noticeable if the spices are hygroscopic powders such as onion powder *(64, 78)*, making it difficult to return to the original texture. Thus, dry inoculation procedures are promising for low-moisture products. These procedures consist of using a dry culture or a dry vehicle of the microorganism to inoculate the low-moisture food *(8, 21, 49, 78, 116)*. Some of the different types of dry inoculum reported are the freeze-dried culture and the inert powders such as silica, talcum powder, and chalk *(21)*. Information available for inoculation of low aw food commodities is scarce *(21)* and it is limited for spices.

Table 1 summarizes some inoculation procedures of spices found in the literature. As can be seen, these procedures vary making it difficult to compare among them. For example, some researchers dry the inoculated matrix to a specific a_w while others do not control the a_w. Fluctuation of this variable can lead to an over or underestimation of the effect of a control measure, since the excessive removal of water from the inoculated matrix can lead to a higher resistance of the bacteria to desiccation and other stresses.

Table 1 Procedures to inoculate spices and other low a_w reported in literature

Low a_w product	Inoculation	Post inoculation	Reference
Black peppercorn and cumin seeds	50 g of spice were inoculated with 20 ml of a suspension of *Salmonella*	Dried at room temperature for 24 h to reach an a_w of 0.3	*(23)*
Black peppercorn and cumin seeds	*Salmonella* was incubated 24 h in 1-cm depth TSB containing peppercorn or cumin seeds arranged in a monolayer	Dried at room temperature for 24 h until reaching an a_w of 0.3	*(23)*
Black peppercorn and cumin seeds	Dry inoculation using inoculated silica (3 g, ~7.8 Log CFU *Salmonella*/g, a_w of 0.3) mixed with 20 g of spices, shaken 24 h and then sieved for 30 s	NA	*(23)*
Chalk	Chalk soaked in a 24 h suspension of *Salmonella*	Dried at 37 °C for ~72 h until getting the original chalk's weight	*(78)*
Meat and bone meal	*Salmonella* grown in a sterilized suspension of meat and bone meal mixed with water (1:5)	Centrifuged and the pellet was dried at 40 °C under vacuum for 12 h to an a_w 0.46	*(135)*
Quartz	*L. monocytogenes* added to white quartz	Mixture was shaken at 35 °C/24 h and vacuumed, then it was filtered and dried at 22 °C/24 h	*(41)*
Almonds	A *Salmonella* suspension added to almonds	Dried at 23 °C/ 24 h, no a_w or humidity measurements	*(162)*

3 MATERIALS AND METHODS

3.1 Dried spices

Dried oregano (*Origanum vulgare* L.) and onion (*Allium cepa* L.) powder, from Turkey, were donated by the International Life Sciences Institute of North America. These spices received no antimicrobial treatment before or after the shipment. When received, the oregano and onion powder were stored at 25 °C in polypropylene bags in *cardboard* boxes and in food grade high-density polyethylene buckets, respectively.

3.2 Bacterial cultures

3.2.1 Spice-related Salmonella strains (SRSS group)

S. enterica serotype Rissen (SAL1449) and *S. enterica* serotype Montevideo (SAL4599) were obtained from the Food and Drug Administration. *S.* Rissen is an environmental isolate from the outbreak linked to white peppercorn in 2009 *(26)*. *S.* Montevideo was an isolate from black pepper used to prepare salami linked to a nationwide outbreak in 2009-2010 *(27)*.

3.2.2 Non-spice-related Salmonella strains (non-SRSS group)

S. enterica panel ATCC® MP-15™ was purchased from the American Type Culture Collection (ATCC). This panel consists of 5 *Salmonella* strains commonly associated with contaminated food and water. The MP-15 panel included *S. enterica* serotypes Choleraeasuis (ATCC 13312), Enteritidis (ATCC 4931), Newport (ATCC

6962), Typhimurium (ATCC 700720) and Typhi (ATCC 6539). For this study, *S.* Typhi was excluded and only the remaining four strains in the ATCC panel MP-15 were used.

3.2.3 Pediococcus faecium NRRL B-2354

Pediococcus faecium (*Enterococcus faecium* NRRL B-2354) was obtained from Dr. James Dickson from Iowa State University (Ames, IA). This strain has been found to be a reliable surrogate for *Salmonella* when testing thermal processes for other low-moisture foods *(49, 82, 85),* and was tested in this study as a potential surrogate to use in further studies to validate treatments to reduce *Salmonella* in dry spices.

3.2.4 Preparation and bacterial stock maintenance

Salmonella and *Pediococcus* organisms were stored in cryocare vial beads (Key Scientific Products, Stamford, TX) at -80 °C. A bead of each frozen culture was aseptically transferred into sterile 10-ml tryptic soy broth (TSB, Becton Dickinson Co., Spark, MD) tubes and incubated at 35 °C for 18-24 h. A loopful of each culture was transferred to another sterile 10-ml TSB tube and incubated at 35 °C for 18-24 h. This allowed the total bacterial activation. A loopful of the last incubated TSB cultures of each strain was streaked onto tryptic soy agar (TSA, Becton Dickinson Co.) slants and incubated at 35 °C for 18-24 h. After incubation, the slants were sealed with parafilm and stored at 4-7 °C. Fresh working slants were prepared every 2 months.

3.3 Inoculum preparation

Salmonella and *P. faecium* strains were transferred from TSA slants into 10-ml tubes of TSB and TSB with 0.6% of yeast extract (TSBYE), respectively, using a sterile loop. The inoculated TSB and TSBYE were incubated at 35 °C for 18-24 h. The resulting suspensions were used either to prepare the inoculum for the liquid or the dry inoculation procedures as described below.

3.3.1 Inoculum preparation for oregano, silica, and talcum powder

A highly-concentrated inoculum was obtained using the bacterial lawn method *(39)*. Two ml of the TSB cultures were individually transferred onto a sterile TSA surface of 225 cm^2 in a tissue culture flask (TCF, Falcon™, Corning, NY). The inoculum was spread onto the TSA surface by adding approx. 80 sterile glass beads (5 mm-diameter, Thermo-Fisher Scientific, Waltham, MA) and rotating the beads at a rate of one rotation/ s for 10 rotations over the entire area of the agar. The inoculated TSA was incubated for 18-24 h at 35 °C to obtain the bacterial lawn. The incubated cultures were harvested by adding 15 ml of 0.1% peptone water (PW, Becton Dickinson Co.) into the TCFs and rotating the beads as described above. SRSS, non-SRSS, and *P. faecium* cocktails were prepared by transferring the suspension of the specific strains for each cocktail into sterile 160-ml dilution bottles.

3.3.2 Inoculum preparation for the onion powder starter

Forty-five ml of sterile TSB contained in a 50-ml conical centrifuge tube (VWR, Sugarland, TX) were inoculated with a loopful of the previously incubated TSB and incubated at 35 °C for 18-24 h. *Salmonella* cultures were centrifuged (Jouan B4i centrifuge, Thermo-Fisher Scientific) for 20 min at 2885 x *g* and *P. faecium* culture was centrifuged for 15 min at 2209 x *g*. The resulting pellets were resuspended in 500 µl of PW. The SRSS and the non-SRSS cocktails were made by combining the corresponding bacterial strains into 15-ml conical centrifuge tubes (VWR).

3.4 Preliminary studies for bacterial enumeration

In all cases, enumeration of *Salmonella* from inoculated oregano was performed by diluting 10-g samples in 90 ml of PW, mixing the sample with PW in a stomacher bag. The samples were pummeled during 60 s at 230 rpm using a Stomacher® model 400 Circulator, Seward Ltd. Samples were diluted decimally in tubes with 9 ml of sterile PW and spread plated onto XLT4. Plates were inverted and incubated at 35 °C for 18-24 h.

Resuscitation procedures using TSB and TSA were evaluated to determine their appropriateness when recovering injured cells. One-g samples were diluted in 99 mL of TSB or PW and homogenized as described above. Samples diluted in TSB were incubated at 25 °C for 1 and 2 h, and afterwards, decimal dilutions were plated onto XLT4. Samples diluted in PW were plated as described in *Section 3.6*

3.5 Bacterial enumeration

The inoculated spices or inert powders contained in whirl-pak bags were homogenized using a disposable polypropylene spatula (VWR). Samples were taken from different parts of the bag. One-gram samples were diluted with 99 ml of PW in a stomacher bag. Each sample was pummeled during 60 s at 230 rpm using a Stomacher® model 400 Circulator, Seward Ltd. Samples were diluted decimally in 9 ml of sterile PW and spread plated and/or poured plated according to the specifications for each experiment.

3.6 Two-step overlay procedure

To make sure that any cells injured by the drying process were also detected, samples were subjected to a resuscitation step to allow the injured cells recovering *(84)*. Recovery of injured cells was performed by spread plating decimal dilutions of the sample onto ~ 16-ml of TSA plates. TSA plates were inverted and incubated 3 h at 35 °C. After the resuscitation time, an overlay of 8-10 ml of Xylose-Lysine-Tergitol 4 agar (XLT4, Becton Dickinson Co.), for *Salmonella* samples, or KF *Streptococcus* agar (KFS, Becton Dickinson Co.), for *Pediococcus* samples, at ~50 °C were poured on the incubated TSA plates. XLT4 agar was supplemented with 4.6 ml of tergitol 4 (7-ethyl-2-methyl-4-undecanol hydrogen sulfate, sodium salt) per liter of agar. KFS agar was supplemented with 10 ml of 2,3,5-triphenyl tetrazolium chloride 1% (TTC 1%) per liter of agar. Once XLT4 and KFS solidified, the plates were inverted and incubated at 35 °C to continue incubating for a total of 18-24 h.

Salmonella colonies were completely black or black in the center with a yellow to red periphery on XLT4. Likewise, *Pediococcus* had red to pink centers on KFS plates.

3.7 Liquid inoculation of spices

Inoculating spices using a suspension of the bacteria was used to represent the scenario of contamination where contaminated high-moisture material such as wild animal's excreta get in contact with spices before desiccating in open fields.

3.7.1 Single and double liquid inoculation of oregano

The first and second liquid inoculation of oregano consisted in inoculating oregano twice using SRSS (Figure 3). As a control, oregano was pre-treated with water (to simulate the first inoculation) and dried. Then, the dried water-treated oregano was inoculated with *Salmonella* and dried (to simulate the second inoculation). The final water activity *(a_w)* of oregano was 0.45 ± 0.01.

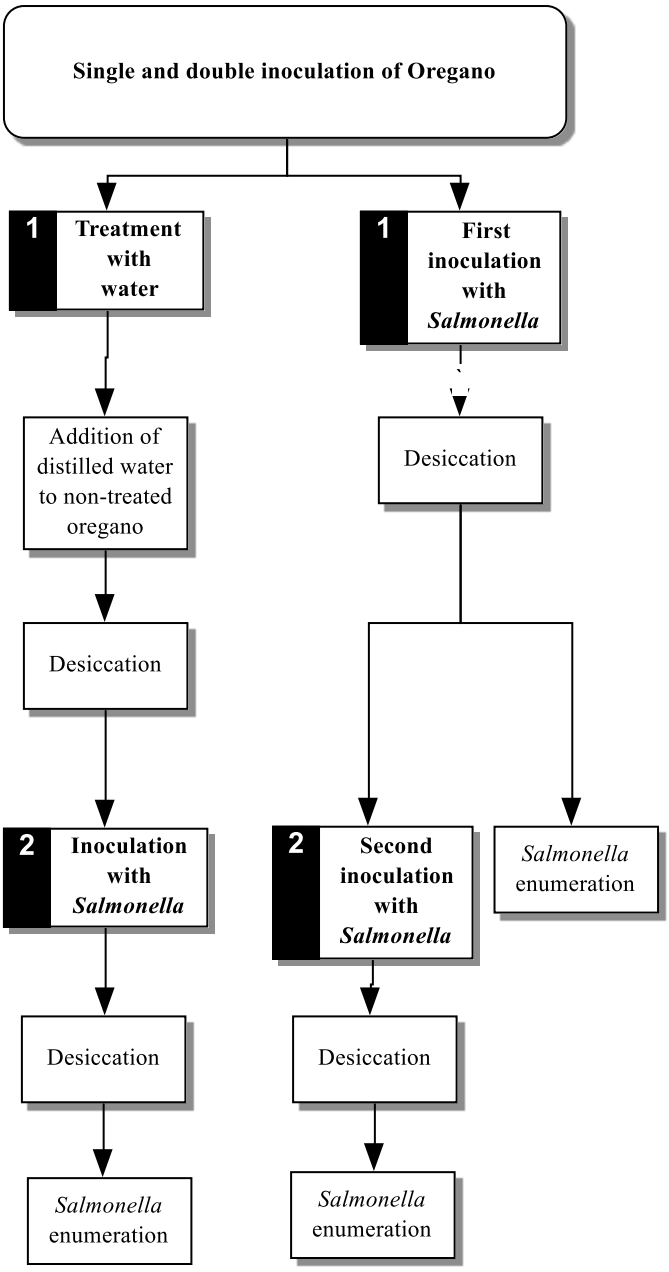


Figure 3 Single and double liquid inoculation of oregano using *Salmonella*.

3.7.1.1 Single and double inoculation of oregano using Salmonella

Single inoculation. One hundred g of non-treated oregano were placed in a polyethylene bag and mixed with 20 ml of the SRSS cocktail from the bacterial lawn procedure. The inoculated oregano was hand-massaged for 2 min to homogeneously distribute the inoculum. Afterwards, the treated oregano was dried on a monolayer of approx. 0.5 cm on a tray at 35 °C for 12 h to recover the original a_w of 0.45 ± 0.01. Three 1-g samples were sampled as described in *Section 3.5* to quantify the concentration of *Salmonella* using the overlay procedure.

Double inoculation. Fifty grams of the inoculated and dried oregano from *Section 3.7.1.1* were re-inoculated with 10 ml of the SRSS cocktail. Homogenization, desiccation, and sampling were performed as described in *Section 3.7.1.1*.

3.7.1.2 Water-treated oregano inoculated with Salmonella

Fifty grams of non-treated oregano were mixed with 10 ml of sterile distilled water. Homogenization, desiccation, and sampling were performed as described in *Section 3.7.1.1.* After drying, 10 ml of the SRSS cocktail were added to the water-treated oregano. Homogenization, desiccation, and sampling were performed as described in *Section 3.7.1.1.*.

3.7.2 Liquid inoculation of vacuum-heat-treated oregano (VHTO)

The temperature to evaporate essential oils (EOs) under vacuum conditions was calculated using the Clapeyron equation (Equation 1).

$$ln\frac{P_1}{P_2} = \frac{-\Delta H_{vap\ essential\ oil}}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$ ….…… Equation 1

Calculation of the exact temperature needed to vaporize carvacrol was performed with the Clapeyron equation (Equation 2). This equation describes the relationship of a pure substance vapor pressure change in relation to a temperature change. The Clapeyron equation is useful when calculating the enthalpy of vaporization of a pure substance, that is, the energy that must be supplied to a liquid substance to vaporize a mole of molecules.

$$ln\frac{P_1}{P_2} = \frac{-\Delta H_{vap\ essential\ oil}}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$ …….…… Equation 2

Where,

P_1, pressure in atm.

T_1, temperature of the EO vaporization in Kelvin at P_1.

$\Delta H_{vap\ essential\ oil}$, enthalpy of the essential vaporization in $J \cdot mol^{-1}$ at P_1.

P_2, fixed pressure to treat oregano in atm.

T_2, temperature for vaporization of the EO in Kelvin at P_2.

R, gas ideal constant equal to 8.314 $J\ °K^{-1}mol^{-1}$

The enthalpy of vaporization (ΔH_{vap}) is the heat needed to transform a liquid into gas and it is dependent of the pressure. Carvacrol not only is one of the most concentrated terpenes in oregano's EO, it also possesses one of the highest ΔH_{vap} (Table 2). This ΔH_{vap} is 56,980 $J/Mol\,°K$ at 1 atm *(97)*. Therefore, carvacrol's ΔH_{vap} was used as reference to calculate the temperature to vaporize the EO in oregano. Hence, all other terpenes and monoterpenes with a lower ΔH_{vap} would also vaporize.

Table 2 Vaporization enthalpies of oregano EO compounds

Compound	% of the total EO	ΔH_{vap} (kJ/mol)	Reference
Carvacrol	14.5	56.98	*(97, 157)*
ß-Fenchyl alcohol	12.8	51.61	*(33, 157)*
Thymol	12.6	42.23	*(97, 157)*
γ -Terpinene	11.6	51.4	*(119, 157)*
γ-Terpineol	7.5	54.0	*(119, 157)*
ß -Cymene	6.8	44.7	*(119, 157)*
α-Terpinene	3.7	40.11	*(32, 157)*

The temperature needed to vaporize carvacrol at 1 atm is 237.65 °C (510.8 °K) *(152)*. Treating oregano at this temperature would likely result in overheating, therefore changing the physical properties of the oregano leaves. To overcome such problem, oregano was treated under vacuum conditions to reduce the carvacrol's vaporization temperature. The maximum vacuum pressure held in the oven used in this study was 0.958 atm. Thus, the values of each variable and constants from the Clapeyron equation were:

P_1 = 1 atm

T_1 = 510.8 °K

$\Delta H_{carvacrol}$ at 1 atm = 56,980 $J \cdot mol^{-1}$

P_2 = 0.042 atm

R = 8.314 $J\ °K^{-1}mol^{-1}$

T_2 = ?

Solving the equation for T_2 gave:

$$ln\frac{P_1}{P_2} = \frac{-\Delta H_{vap\ carvacrol}}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

$$ln\frac{1\ atm}{0.042\ atm} = \frac{-56{,}980\ J/mol}{8.314\ J/mol \cdot °K}\left(\frac{1}{510.8\ °K} - \frac{1}{T_2}\right)$$

$$ln(23.81) = -6{,}853.50\ \left(1.96 \cdot 10^{-3} - \frac{1}{T_2}\right)$$

$$-\frac{1}{T_2} = -\frac{3.17}{6{,}853.50} - 1.96 \cdot 10^{-3}$$

$$\frac{1}{T_2} = \frac{3.17}{6{,}853.50} + 1.96 \cdot 10^{-3}$$

$$\frac{1}{T_2} = 2.40 \cdot 10^{-3}$$

$$T_2 = 412.79\ °K$$

$$\therefore T_2 = 140\ °C$$

Fifty-gram portions of non-treated oregano were spread on a try to form an approx. 0.5-cm monolayer. Then, oregano was dried at 70 °C for 24 h ($VHTO_{70°C}$) and at 140 °C for 2.5 h ($VHTO_{140°C}$) at 0.958 atm in a vacuum oven (Isotemp® Vacuum Oven model 285A, Fisher Scientific®, Waltham, MA).

Fifty-gram batches of non-VHTO, $VHTO_{70\ °C}$, and $VHTO_{140\ °C}$ were inoculated individually with 10 ml of the SRSS, non-SRSS, and *P. faecium* cocktails. Homogenization, desiccation, and sampling were performed as described in *Section 3.7.1.1*.

3.7.2.1 Color measurement of VHTO

To measure the color change of the oregano treated with heat under vacuum conditions, a Minolta colorimeter (CR400, Minolta Co., Osaka, Japan) was used. The values L* (lightness), a* (green-red intensity) and b* (blue-yellow intensity) obtained were compared against the control (non-VHTO).

3.7.2.2 Essential oil extraction using steam distillation

The EO from the oregano samples was extracted using steam distillation *(92)*. The steam distillation apparatus was assembled as shown in Figure 4.

Fifty-five-grams of oregano were transferred to a 2-l round-flat-bottomed flask (Pyrex®, Greencastle, PA) containing 900 ml of tap water. The cold-water flux was activated before the mixture of oregano and water was heated until boiling. The extraction

was set for 3 h after the first drop of the condensate water-oil dripped into the separatory funnel.

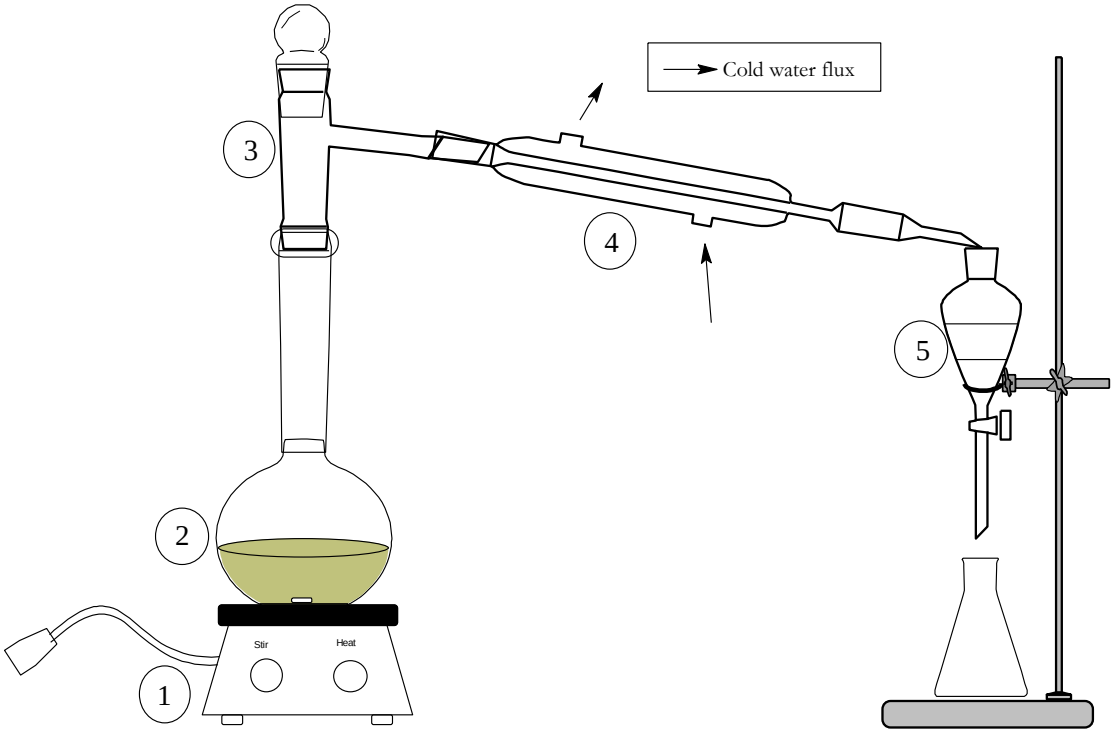


Figure 4 Steam distillation apparatus. 1) Hot plate, 2) Round-flat bottom flask with a stir bar, 3) Adapter, 4) Condenser, 5) Separatory funnel

To separate the oil from the water collected in the separatory funnel, 15 ml of hexane were mixed with the condensate by inverting the funnel 10 times and allowed to repose for 2 h. The heaviest liquid phase (*i.e.* water), contained at the bottom of the funnel, was collected in a 250-ml flask. Then, the light liquid phase, containing the EO and hexane, was transferred to test tubes. The heavy liquid phase was poured back into the funnel to separate any residues of EO from the water. Five ml of hexane were added to the

funnel and mixed by inverting 10 times as described above. After reposing for 2 h, the heavy liquid phase was discarded and the remaining light liquid phase was transferred to the test tubes.

The hexane was evaporated from the EO using a vacuum-heat-centrifuge (Centrivap concentrator, Labconco®) at 45 °C and 30 in-Hg of vacuum during 3 h. To eliminate the remaining water from the oil after centrifuging, 1 ml of hexane and a pinch of sodium sulfate anhydrous (EMD, Billerica, MA) were added to each tube and homogenized *(114)*. Then, the content from the tube was filtered by pouring the solution in a funnel with cotton to retain the hydrated sodium sulfate. The filtrated (*i.e.* essential oil-solvent) was collected in a tared test tube and subjected to vacuum-heat-evaporation as described above. The EO mass was calculated by calculating the weight difference between the tube with the EO minus the empty test tube. The amount of the EO extracted from the non-VHTO, the $VHTO_{70\ °C}$, and the $VHTO_{140°C}$ was compared.

3.7.2.3 Survival of the SRSS, non-SRSS, and P. faecium in VHTO

Fifty-gram batches of non-VHTO, the $VHTO_{70°C}$, and the $VHTO_{140°C}$ were individually inoculated with 15 ml of the SRSS, non-SRSS and *P. faecium* cocktails obtained from the bacterial lawn procedure. The inoculated oregano was homogenized and dried as described in *Section 3.7.1.1*. Enumeration of *Salmonella* and *P. faecium* for each treatment was performed using the overlay procedure.

3.7.3 Liquid inoculation of onion powder

Fifty-g of onion powder were inoculated with 5 ml of the inoculum obtained from the bacterial lawn procedure. The inoculated onion was homogenized and dried as described in *Section 3.7.1.1*.

3.8 Dry inoculation of spices

3.8.1 Inoculation of silica and talcum powder

Fifty g of silica and talcum powder were inoculated individually with 15 ml of suspensions of the SRSS, the on-SRSS, and *P. faecium*. Each bacterial suspension was obtained from the bacterial lawn procedure. The inoculated powders were massaged during 2 min to distribute the inoculum homogeneously. Then, the powders were dried on monolayer of approx. 0.5 cm on a tray at 35 °C for 12 h to recover an a_w of 0.45 ± 0.01. Three 1-g samples were drawn to quantify *Salmonella* or *Pediococcus* using the overlay procedure. After drying, each powder was pulverized with a mortar and pestle to disintegrate lumps and then sieved using a 15x15-cm plastic mesh sheet with a pore size of 0.2 cm.

3.8.2 Dry inoculation of oregano

Three grams of the inoculated silica or talcum powder described in *Section 3.8.1*. were mixed with 10 g of non-treated oregano in a 950-ml sealing glass jar (Kerr®, Waxahachie, TX). The mixture was shaken during 2 minutes doing vertical and horizontal movements. To separate the powder from the oregano, a 15x15-cm plastic mesh sheet

(pore size of 0.2 cm) was affixed to the mouth of the glass jar. A second glass jar was placed mouth-mouth with the jar that contained the mixture and affixed with masking tape and parafilm. The mixture was sieved for 3 min by making circular and vertical movements to separate the oregano from silica or talcum powder.

3.8.3 Inoculation of onion powder

Bacterial suspensions were obtained as described in *Section 3.3.2*. One ml of the SRSS, the non-SRSS cocktails, and *P. faecium* were individually added to 50-g batches of non-treated onion powder contained in a coffee grinder bowl (Cuisinart, East Windsor, NJ). The inoculum was homogenized by activating the grinder every 1 s for 30 s. After inoculation, each batch was split into 12.5-g portions and placed in weighing boats (4-in diameter, VWR) in polycarbonate waterproof containers (Dri-Loc, Plano, IL) with a moisture absorbing sachet (Stack-on®, Wauconda, IL) at 25 °C during 12 h. The final a_w reached was 0.30 ± 0.01.

The starter was mixed with untreated onion powder in a 1:10 ratio. *Salmonella* and *P. faecium* were enumerated as described in *Section 3.5* using the overlay procedure.

3.9 Resistance of SRSS and non-SRSS to desiccation

The resistance of SRSS and non-SRSS to desiccation was tested in sterile talcum powder. A 0.5-cm monolayer of talcum powder was autoclaved at 121 °C for 60 min. Fifty-g batches of sterile talcum were inoculated individually with 15 ml of suspensions of SRSS and non-SRSS. Homogenization and desiccation were performed as described in

Section 3.8.1 using sterile materials and utensils. One-gram samples of talcum inoculated with SRSS and non-SRSS were diluted with 99 ml of PW and pummeled at 230 rpm for 1 min. SRSS and non-SRSS were enumerated using the overlay procedure *(Section 3.6)* and by plating only onto TSA. All plates were incubated 18-24 h at 35 °C.

In this same experiment, the effectiveness of recovering injured *Salmonella* cells using the two-step overlay method was evaluated. Thus, samples were also enumerated using the two-step overlay method described in *Section 3.5.* and the results were compared with the counts from plating only onto TSA.

3.10 Survival of *Salmonella* and *P. faecium* in oregano, talcum and onion powder

The survival of SRSS, non-SRSS and *P. faecium* inoculated in oregano, talcum and onion powder stored at -18, 4, and 25 °C was monitored up to 24 weeks. The inoculated and dried materials were stored in watertight containers to maintain the a_w constant over the extent of this experiment. Quantification of *Salmonella* and *Pediococcus* were performed periodically as described in *Section 3.5*.

3.11 Water activity measurements

The complete batch of the inoculated oregano, onion powder, silica or talcum contained in a Whirl-pak bag was individually homogenized with a sterile plastic spatula (VWR) for 2 min. One-g samples, randomly taken from the bag with sterile spatulas, were individually weighted in a digital balance and placed individually into disposable sample

cups to measure the water activity using a water activity meter (Aqualab series 3 Decagon Devices Inc, Pullman, WA).

3.12 Statistical analysis

Bacterial counts were transformed to $\log_{10}$ before the statistical analysis. Experiments were conducted in independent triplicates. The mean concentration of *Salmonella* or *P. faecium* for each treatment was compared using one-way analysis of variance and significant mean differences ($P < 0.05$) were compared with the Turkey test. All of the statistical analysis were performed using JMP statistical software v8 (SAS, Institute, Cary, NC).

The stability curves were modeled using DMFit, an add-in for Microsoft Excel, and the survival/death rates for each group of microorganisms were obtained from the output generated by DMFit *(14)*.

4 RESULTS AND DISCUSSION

4.1 Preliminary experiments: selection of a procedure to recover injured cells

The concentration of *Salmonella* after inoculating oregano was estimated to be as high as 8.0 - 10.5 log CFU/g, however, the counts of *Salmonella* in all cases, fell below the limit of detection (LOD, < 3 log CFU/g) after direct plating on XLT4 agar. It is unlikely that a lethal effect would have resulted from the exposure of *Salmonella* to stressors such as drying and exposure to the natural antimicrobials of oregano. Instead, these factors may stress the *Salmonella* cells, resulting in a sub-lethal injury and that a resuscitation step needed to be included in the enumeration method. Diluting oregano to 1:100 instead of 1:10 for plating, may have helped reducing the toxic levels of the EO.

Results from enumeration of *Salmonella* using a resuscitation procedure indicated that cells in fact were injured after being added to oregano. It was also determined that not all resuscitation procedures were effective in fixing injured cells in dry oregano. The mean count of *Salmonella* obtained when resuscitating on TSA incubating for 3 h, followed by overlaying with XLT4 was 7.3 log CFU/g, whereas the counts obtained by resuscitating in TSB for 1 – 2 h and then plating on XLT4 were consistently not detectable (< 3 log CFU/g).

A major disadvantage of using liquid media (*i.e.* TSB) when recovering injured cells is the risk of allowing the cells to reproduce if the time of the recovery overtakes the lag phase, affecting the accuracy of the initial bacterial counts. If the time of incubation is shortened to prevent such growth, the risk is that there may not be enough contact with the TSB nutrients to ensure full recovery of injured cells. The time required for injured

cells to recover vary and non-target cells can multiply or limit the growth of the microorganism of interest *(155)*. Therefore, using non-selective solid media (*i.e.* TSA) resulted in a better recovery of the injured cells. In addition, if the cells overtake the lag phase and reproduce on the agar, the initial concentration of cells would not be affected since each colony on the plate represents an initial single cell *(84, 155)*.

4.2 Liquid inoculation of spices

4.2.1 Single and double liquid inoculation of oregano

The objective of performing a double inoculation of oregano was to increase the final concentration of the pathogen in the dried oregano. The hypothesis was that a portion of EO would be removed after subjecting oregano to desiccation once inoculated. As a consequence, the EO concentration would be reduced allowing a better recovery of *Salmonella* after a second inoculation process. Even though conventional drying methods are not set at high temperatures (*i.e.* > 100 °C), a portion of the EO is still removed *(18, 19, 121, 149)*. For instance, Hassanpouraghdam *(74)* reported that even drying basil at temperatures as low was 40 – 60 °C, the EO could be reduced by almost 1% depending the drying method. In their experiment, a 0.9, 0.8, 0.5, and 0.4% EO reductions were obtain after shade drying, oven drying at 40 °C, sun drying, and oven drying at 60 °C, respectively.

Although the concentration of *Salmonella* in the WTO was significantly higher than in the first-inoculated oregano before drying ($P < 0.05$), no significant differences ($P > 0.05$) were detected after desiccation (Table 3).

Table 3 Concentration of spice-related *Salmonella* strains in oregano after the 1st and 2nd inoculation

Sample	Treatment	After inoculation	Dried	Log CFU/g reduction
		Log CFU/g	Log CFU/g	
Oregano	1st inoculation	$7.3 \pm 0.1_{A}$[b]	$3.8 \pm 1.3_{A}$	3.5
	2nd inoculation	$8.5 \pm 0.1_{B}$	$6.6 \pm 0.2_{B}$	1.9
WTO[a]	1st inoculation	$8.0 \pm 0.7_{B}$	$3.8 \pm 1.4_{A}$	4.2

[a]WTO, water treated oregano.
[b]Data within the same column followed by different letters are significantly different ($P < 0.05$).

The pathogen concentration after the second inoculation increased 1.2 logs after inoculation (Table 3). However, after drying, the concentration of *Salmonella* was significantly higher in the double-inoculated oregano than in the other two treatments ($P < 0.05$). Pre-treating oregano with water did not increase the final concentration of *Salmonella* in the dried oregano when compared with oregano inoculated once. In both cases, the final concentration of the pathogen was 3.8 log CFU/g ($P > 0.05$) (Table 3).

Even though the double-inoculated oregano had at least 6 log CFU/g after drying, physicochemical changes in the oregano leaves are expected to occur seen at least as the reduction of the EO *(18, 19, 121, 149)*. On the other hand, it is important to highlight that the inoculated *Salmonella* is subjected to stressful processes twice, increasing the chance of developing resistance to further antibacterial treatments in challenge studies *(101, 102, 130)*. In such case, the calculation of D-values for a pathogen control measure can be overestimated.

Ideally, inoculation of food in challenge studies should simulate the common ways of contamination for the particular type of food *(111)*. For dry spices, possible ways of contamination include dry vehicles such as dust and feces, wet vehicles such as fecal material from birds, rodents, insects, and other wild animals, etc. In this study, dry vehicles were used for the inoculation of dry spices. *(17, 55)*. Dust can contaminate spices when drying under the sun in opened fields and during processing that can lead to cross-contamination *(16, 17, 38, 43, 55, 87)*.

4.2.2 *Liquid inoculation of oregano using P. faecium*

Although *Pediococcus* is has been inoculated in other dry products *(49, 85)*, it was not known if *Pediococcus* also required a resuscitation step to recover injured cells when inoculating and drying spices. Therefore, the overlay procedure was evaluated to recover possible injured cells. As control, quantification of *Pediococcus* was performed by pour plating KFS agar in the serial diluted samples.

As can be seen from Figure 5, no significant differences between techniques were identified in the enumeration of *Pediococcus* ($P < 0.05$) perhaps because of a high variability in the results (Figure 5).

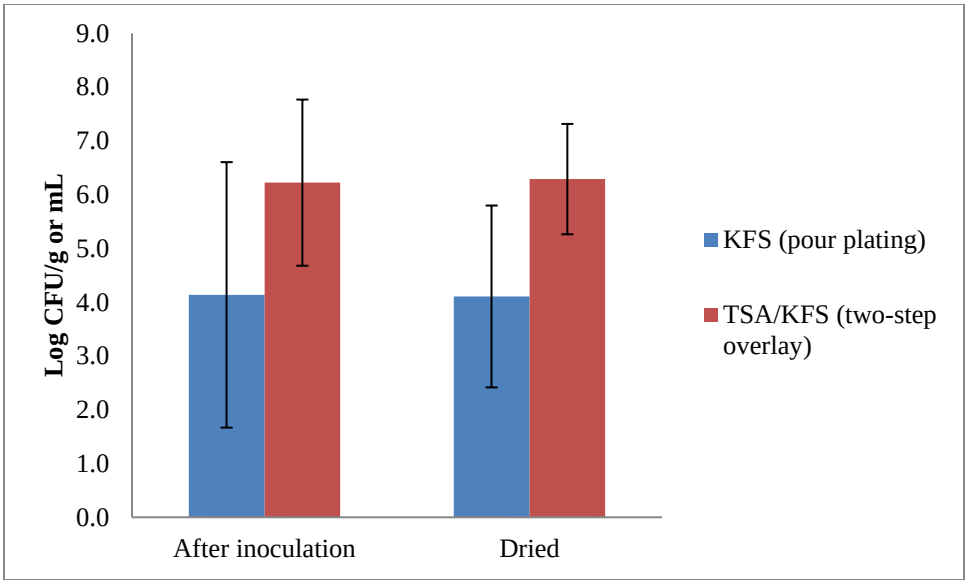


Figure 5 Liquid inoculation of oregano using *Pediococcus faecium*

The effect of desiccation on the survival of *P. faecium* was null, in other words, no significant differences of the *P. faecium* concentration were detected before and after desiccation with or without the resuscitation procedure ($P < 0.05$).

4.2.3 Liquid inoculation of vacuum-heat-treated oregano

As mentioned earlier, the EO found in oregano functions as antimicrobial, principally by the action of carvacrol and thymol *(44, 174)*. The EO in oregano is composed mainly by a mixture of monoterpenoids and monoterpenes such as *p*-cymene, ɣ-terpinene, thymol, and carvacrol *(120)*. The concentration of each compound varies according to factors such as the geographic origin and the oregano species *(159)*. However, carvacrol is one of the most concentrated components found in oregano's EO independently of the geographic origin of the species *(106, 120, 159)*.

Because of the potential antimicrobial effect of the oregano's EO, a number of *Salmonella* cells die after being added to the dry oregano leaves. Therefore, in order to increase the survival of *Salmonella* in oregano, a partial removal of the oregano's EO was performed using a vacuum-heat procedure.

The temperature needed to vaporize carvacrol at 0.042 atm was 140 °C. It was observed a change of color after drying at 140 °C due to the overheating process. Since the objective of vacuum drying the oregano was to increase the final *Salmonella* concentration after inoculation and desiccation, a lower temperature, 70 °C, was tested.

When the differences of the oregano's color were compared with the Minolta equipment (Table 4), no significant differences were observed in $VHTO_{70°C}$ final color ($P > 0.05$) (Figure 6). On the contrary, $VHTO_{140°C}$ had a significantly darker color ($P < 0.05$) due to higher intensity in the red and blue colors (higher a* and b* values) when compared with the non-VHTO and $VHTO_{70°C}$ values ($P < 0.05$).

Table 4 VHTO Minolta color measurements

Sample	L	a	b
Non-VHTO	51.40_{AB}[a]	0.33_{A}	23.42_{A}
$VHTO_{70\ °C}$	52.84_{A}	0.21_{A}	24.24_{A}
$VHTO_{140\ °C}$	51.05_{B}	2.61_{B}	25.82_{B}

[a]Data within the same column followed by different letters are significantly different ($P < 0.05$)

The analysis of the EO concentration showed a significant difference when heating oregano at 140 °C than at 70 °C, both at 0.042 atm of pressure ($P < 0.05$). The removal of EO in $VHTO_{140°C}$ was 58.4%, whereas it was only 18.3% in $VHTO_{70°C}$.

Table 5 Oregano's essential oil removal

Oregano treatment	EO content (%)	EO removed (%)
Non-treated	$1.25 \pm 0.11_{C}$[a]	N/A
70 °C for 24 h at 0.042 atm	$0.93 \pm 0.06_{B}$	18.3 ± 4.62
140 °C for 2.5 h at 0.042 atm	$0.52 \pm 0.03_{A}$	58.4 ± 4.36

[a]Data within the same column followed by different letters are significantly different ($P < 0.05$)

a) Non-treated

b) 70 °C for 24 h at 0.042 atm

c) 140 °C for 2.5 h at 0.042 atm

Figure 6 Color change of vacuum-heat-treated oregano *vs* non-vacuum-heat treated oregano.

4.2.3.1 *Inoculation of VHTO using Salmonella*

SRSS and non-SRSS concentration after desiccation was in average 1.6 logs higher, in both, $VHTO_{70°C}$ and $VHTO_{140°C}$ than in non-VHTO ($P < 0.05$) (Figure 7). No significant differences were observed in the SRSS concentration when inoculated in the $VHTO_{70°C}$ and $VHTO_{140°C}$ after dying ($P > 0.05$). Similar results were found in the non-SRSS inoculated in $VHTO_{70°C}$ and $VHTO_{140°C}$ ($P > 0.05$).

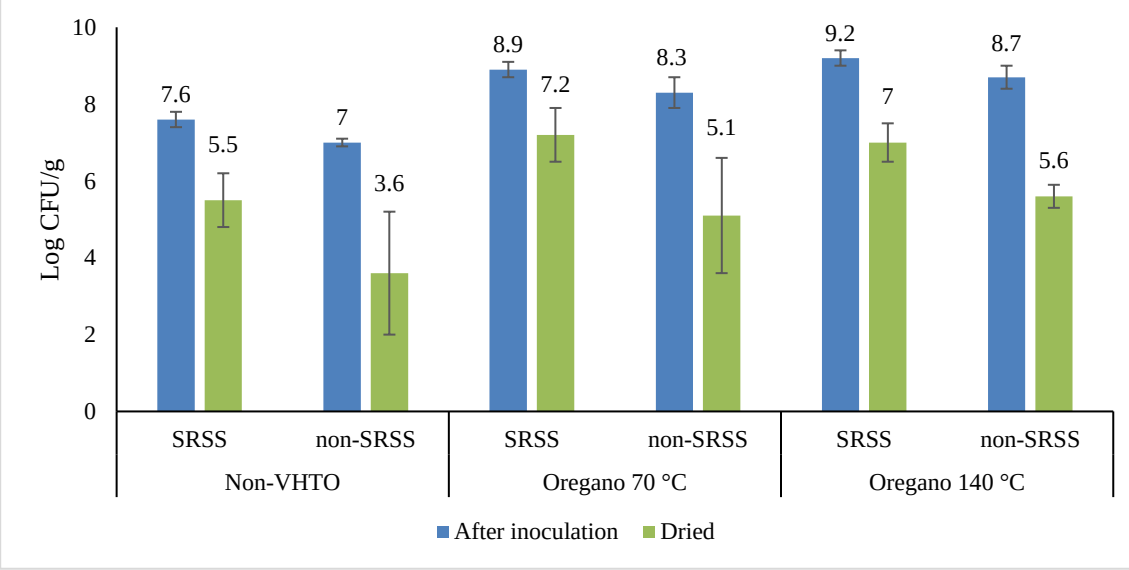


Figure 7 Survival of spice-related (SRSS) and non-spice-related *Salmonella* strains (non-SRSS) in vacuum-heat-treated oregano (VHTO)

The concentration of SRSS was at least 7 log CFU/g after desiccation in both VHTO samples. Although the removal of EO from oregano also benefited the survival of the non-SRSS, the maximum concentration reached after desiccation was 5.1 and 5.6 log CFU/g for $VHTO_{70°C}$ and $VHTO_{140°C}$, respectively.

The SRSS and non-SRSS concentration was significantly different for the non-VHTO, VHTO$_{70°C}$, and VHTO$_{140°C}$ after drying (Figure 8). Even though the SRSS and non-SRSS populations in the inocula were the same (10.2 ± 0.3 and 10.3 ± 0.2 log CFU/ml, respectively) ($P > 0.05$), the SRSS survival was higher in all three treatments before and after desiccation ($P < 0.05$).

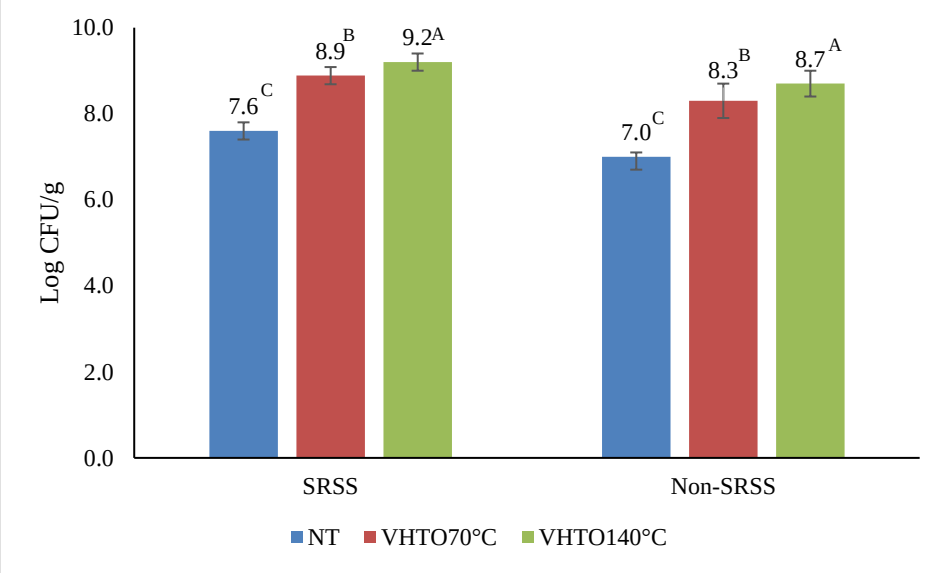


Figure 8 Concentration of spice-related (SRSS) and non-spice-related *Salmonella* strains (non-SRSS) after inoculating in vacuum-heat-treated oregano (VHTO)

The Non-SRSS survival was more affected than the SRSS after desiccation as the oregano's EO content increased (Figure 9). Thirty-three (3/9) and 11 (1/9) % of the dried non-VHTO and VHTO$_{70°C}$ samples inoculated with non-SRSS had counts that fell under the LOD (< 3 log CFU/g). All VHTO$_{140°C}$ dried samples inoculated with non-SRSS had

levels ≥ 5 log CFU/g and all of the dried samples containing SRSS fell above the LOD (Figure 9).

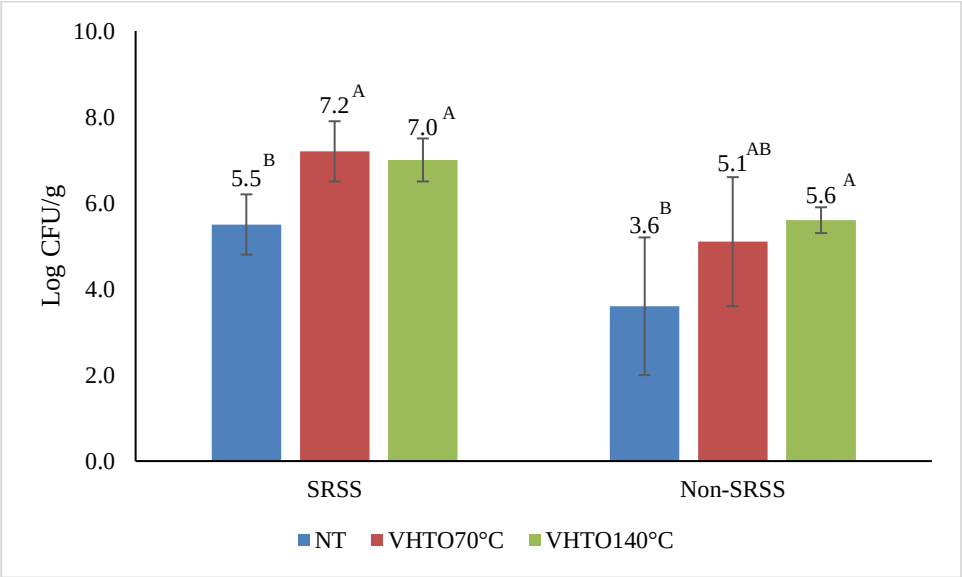


Figure 9 Concentration of spice-related (SRSS) and non-spice-related *Salmonella* strains (non-SRSS) after desiccating the inoculated vacuum-heat-treated oregano (VHTO)

Survival of *Salmonella* was directly affected by the % of EO in oregano (%EO) ($P < 0.0001$), the desiccation process ($P < 0.0001$), and the strains used (*i.e.* SRSS or non-SRSS) ($P < 0.0001$) (Table 6). Analysis of interactions between these 3 factors showed a strong effect given by the type of strains subjected to desiccation ($P < 0.001$). Although the concentration of the EO played an important role in the survival of *Salmonella*, it affected similarly to both, SRSS and non-SRSS, and no strong interactions were detected ($P > 0.05$). These results are not surprising since the non-SRSS have shown more

susceptibility to desiccation in different matrices than the SRSS (*see Section 4.3.1 and 4.3.6*).

Table 6 Effect of factors in the survival of *Salmonella* in inoculated vacuum-heat-treated oregano

Factor	Degrees of freedom	Sum of Squares	F Ratio	Prob > F
Strain[a]	1	34.84320	66.5409	<.0001*
%EO	2	60.98513	58.2324	<.0001*
Time	1	179.25791	342.3331	<.0001*
Strain*%EO	2	0.84028	0.8023	0.4513
Strain*Time	1	9.42043	17.9904	<.0001*
%EO *Time	2	0.52014	0.4967	0.6101
Strain*%EO *Time	2	0.40921	0.3907	0.6776

[a]Strain refers to the SRSS or non-SRSS, %EO is the % of EO in oregano, and Time refers to the moment in which the sample was analyzed: before or after desiccation.

4.2.3.2 Comparison of the concentration of Salmonella when inoculated in oregano that had been stored for 2 years

Results from the survival of *Salmonella* after oregano's inoculation and desiccation from *Section 4.2.1* are significantly higher than those from *Section 4.2.3.* In both instances the oregano was taken from the same batch. The only difference was the date when the inoculation was performed. The experiment in *Section 4.2.1* was executed in 2014, within 6 months of the oregano's arrival, while *Section 4.2.3* was completed in 2016, 2 years after the oregano's arrival. As can be seen in Figure 10, the survival of *Salmonella* is significantly higher in oregano stored for 2 years even after inoculation ($P < 0.05$).

The higher *Salmonella* recovery in the experiment performed in 2016 could be due to possible partial loss of the oregano's EO. Indeed, the expiration date for spices is determined according to the loss of color and aroma and the loss of these attributes is driven by factors such as humidity, temperature, and sunlight *(103)*. The aroma is attributed principally to the concentration of the EO remaining in the sample *(129)*. Then, when oregano is stored under ideal conditions (*i.e.* low light, warm-cold temperature, and a dry environment), the best-if-used-by date can be extended to 1 – 3 years after the batch was produced *(103)*. Despite the fact that the oregano used in this study was stored at 25 °C, in a dry environment and away from the sunlight, the loss of EO during these 2 years could be possible. As seen in Figure 7, it was not necessary to substantially remove all the EO from oregano to be able to significantly increase the concentration of *Salmonella* after inoculation.

Paraskevakis, *et al. (126)* reported a 5.8% EO loss after storing oregano under shade and ambient conditions. These authors analyzed individual compounds from EO that showed significant reductions of carvacrol at the end of the 6^{th} month, from an initial concentration of 91.21 to 81.28% ($P < 0.05$) *(126)*. Rosado, *et al. (138)* reported a EO reduction of 0.1% per month in basil leaves while Verma, *et al. (169)* found a 20% EO loss in rosemary leaves after a year of storage under shade at room temperature. The grade of EO reduction depends on its volatile-oil composition, which is directly associated with the specific type of spice and the storing method (*i.e.* whole, in pieces, or ground) *(169)*.

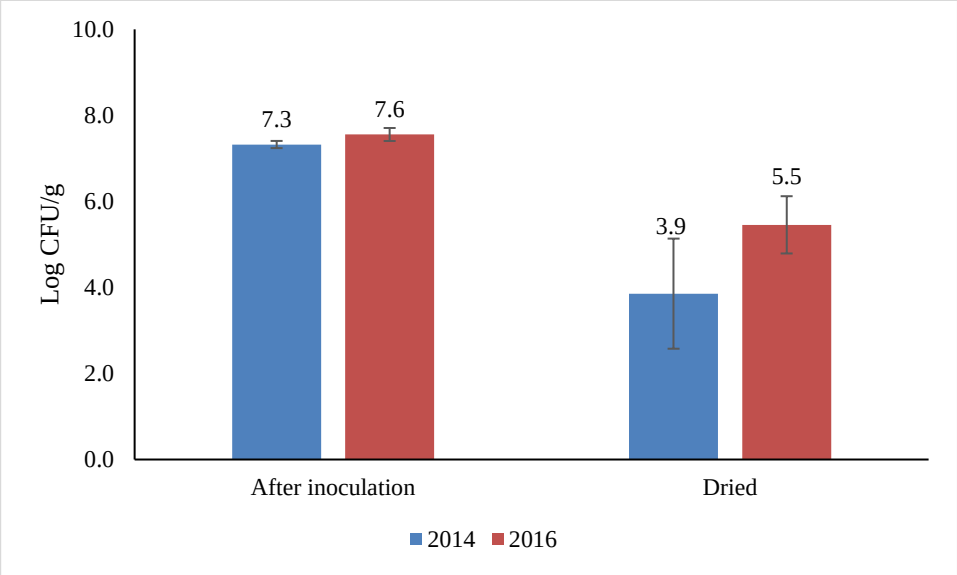


Figure 10 Recovery of *Salmonella* inoculated in oregano stored for 2 years

4.2.3.3 Inoculation of VHTO using Pediococcus faecium

Pediococcus faecium showed a similar survival pattern to the *Salmonella* in the VHTO trials where the partial removal of the oregano's EO allowed a higher survival of the microorganism ($P < 0.05$). The improvement in the recovery of *P. faecium* after inoculating in $VHTO_{70°C}$ and $VHTO_{140°C}$ was 1-log cycle higher than in non-VHTO ($P < 0.05$) (Figure 11). The differences in the bacterial load after inoculation were due to the effect of the EO concentration. A higher antimicrobial effect was shown in non-VHTO (Figure 11), where the no EO was removed ($P < 0.05$) (Table 5).

After desiccation, the concentration of *Pediococcus* decreased and this reduction was dependent on the EO percentage present in the sample (Table 7).

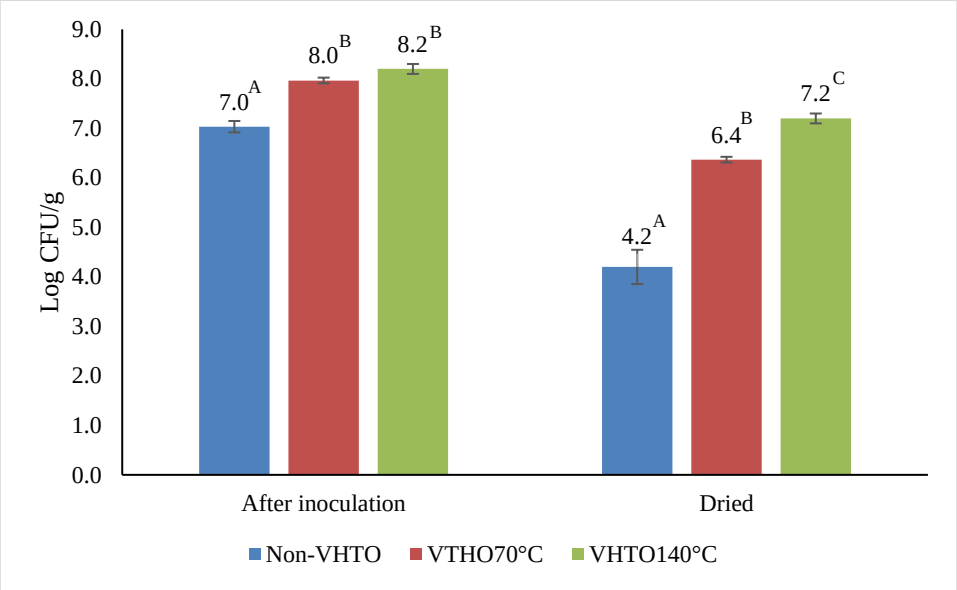


Figure 11 Vacuum dried oregano inoculated with *P. faecium*

Table 7 Effect of factors in the survival of *Pediococcus faecium* in vacuum-heat-treated oregano

Factor	Degrees of freedom	Sum of Squares	F Ratio	Prob > F
%EO[a]	2	14.054444	263.5208	<.0001*
Time	1	14.760556	553.5208	<.0001*
%EO*Time	2	2.621111	49.1458	<.0001*

[a] %EO is the % of EO in oregano and Time refers to the moment in which the sample was analyzed: before or after desiccation.

The *P. faecium* concentration was 2.2 and 3.0 log CFU/g higher in $VHTO_{70°C}$ and $VHTO_{140°C}$, respectively, than in non-VHTO (Figure 11). $VHTO_{140\ °C}$ had significantly higher concentration of *Pediococcus* after desiccation than the $VHTO_{70\ °C}$ with 7.2 and 6.4 log CFU/g, respectively ($P < 0.05$). Nevertheless, this difference is not relevant for practical purposes since the goal of the inoculation procedure was having at least 6 log CFU/g, for both, *Salmonella* and *Pediococcus.*

In summary, partial removal of the oregano's EO resulted in an increase of the survival of *Salmonella* and *P. faecium* in dry oregano. Although the effect desiccation played a major role in reducing the final bacterial load, the final concentration was high enough, > 6 log CFU/g, to be used in challenge studies.

A strong interaction of the *Salmonella* strains subjected to desiccation was found, in which the non-SRSS were more sensitive to desiccation than the SRSS independently of the VHTO sample tested.

4.2.4 Liquid inoculation of onion powder

Liquid media has been the most used vehicle for inoculation of microorganisms in food for challenge studies. Usually the inoculated food has a high a_w and the addition of a liquid inoculum does not substantially affect the moisture content, a_w, or other physicochemical characteristics of food. These changes are especially important to maintain stable when inoculating low a_w products such as spices.

For this study, when onion powder was inoculated at ratios as low as 1:10 v/w, the consistency always changed from powdery to sticky. Desiccating the onion powder did not restore the consistency to the original characteristics (Figure 12). This is a common problem when liquid inoculating highly hygroscopic materials *(78)*. This evidenced the differences between spice matrices, which may or may not be adequately restored to original conditions after drying following wet inoculation. It was concluded that for onion powder, wet inoculation was not adequate in a protocol for inoculating onion powder in

challenge studies. Thus, the following experiments involving *Salmonella* enumeration in onion powder followed the dry inoculation only.

a) Non-treated b) Treated

Figure 12 Onion powder texture a) before inoculation and b) after inoculation and homogenization

4.3 Dry inoculation of spices

In this study, silica and talcum powder were used as scenarios of foreign matter contamination of spices. As mentioned earlier, contamination of spices with dust or any other dry matter is likely to occur.

For powdery spices, such as onion powder, the use of the starter represents the scenario of cross contamination during processing and packaging of the same spice *(26)*.

4.3.1 *Inoculation of silica and talcum powder with Salmonella*

Dry inocula have been used as an alternative to inoculate low a_w food products without changing the physical properties of the food matrix being inoculated for challenge

studies. Example of these products inoculated with dry inocula are almonds, nuts, peanut butter, whole peppercorn, and cumin *(21, 23, 49, 73, 85)*.

Dry inocula can be obtained when freeze, vacuum drying bacteria, and by liquid inoculating inert powders *(21, 49, 78)*. Certainly, the election of one or another way to obtain the dry inocula depends especially on economic resources *(78)*.

In this study, two inert powders were used to make the dry inocula to inoculate oregano: silica and talcum powder.

Inoculation of silica and talcum powder was adapted from the method described by Blessington, *et al. (21)* for inoculation of almonds. A 1:3 ratio v/w of inoculum in silica did not modified substantially the final consistency of silica. Silica showed higher hydroscopic properties than talcum powder. It was evident the formation of small grumps that were not easy to dissolve with the mortar and pestle. It was observed, in preliminary experiments, that the addition of liquid to silica changed the physical properties of the powder. For example, the initial consistency of silica did not return to the original one even after drying at 35 °C for at least 24 h. These physical changes were more evident when working with small amounts of silica, for instance, 10 g.

a) b)

Figure 13 Silica powder consistency, a) non-treated and b) 2 ml of inoculum added to 10 g of silica

When inoculating and drying a larger volume of silica (*e.g.* 50 g), the final physical consistency was similar to the non-treated one (Figure 14).

The disadvantage of using silica was its high tendency to develop static charge, making it difficult to handle even though proper equipment was used.

a) Non-treated b) Inoculated silica (1:3 v/w ratio)

Figure 14 Powdery consistency of silica

Handling talcum powder was easier than working with silica powder. Although talcum powder's consistency was similar to clay after adding the liquid inocula, after

desiccating at 35 °C during 12 h returned to the powdery consistency. After desiccation, sieving and pulverization were needed to dissolve the grumps formed.

a) b)

Figure 15 Powdery consistency of talcum a) non-treated and b) inoculated, dried, and pulverized (1:3 v/w ratio)

SRSS concentration was higher than non-SRSS in both, silica and talcum ($P < 0.05$). This difference was more evident once the powders were dried (Table 8). The reductions after the desiccating talcum were 3.4 and 4.6 Log CFU/g for SRSS and non-SRSS, respectively.

Table 8 Concentration of SRSS and non-SRSS after inoculation and desiccation of silica and talcum powder

Sample	Silica powder		Talcum powder	
	SRSS	non-SRSS	SRSS	non-SRSS
After inoculation	$9.0 \pm 0.2_{aB}$[a]	$9.1 \pm 0.2_{aB}$	$9.7 \pm 0.3_{aA}$	$9.2 \pm 0.1_{bB}$
Dried	$8.4 \pm 0.2_{aA}$	$7.3 \pm 0.2_{bA}$	$6.3 \pm 0.2_{aC}$	$4.6 \pm 0.5_{bD}$

[a]Within a row, values with different lower-case letters are significantly different ($P < 0.05$). Within a column, values with different upper-case letters are significantly different ($P < 0.05$).

Reduction of *Salmonella* after a drying process has been reported in other studies *(21, 23, 49, 77)*. The magnitude of such reductions has been reported to be dependent of the temperature of desiccation, pH, composition of the media tested, if the bacteria were grown as planktonic cells or as biofilm, *Salmonella* serotype, and the growth phase at the time of desiccation among others *(67)*.

For instance, Hiramatsu, *et al. (77)* reported that the survival of *Salmonella* spp. in low-moisture foods was even 100 times higher in food with high concentration of lipids than in dried paper disks. In this study, the survival of *Salmonella* was dependent of pH and no *Salmonella* was detected after inoculation and desiccation of acidic foods with a pH < 4. The presence of non-reducing solutes such as sucrose also improves the survival of *Salmonella* after drying *(50, 102)*. Hiramatsu, *et al. (77)* reported a high survivability in products such as chocolate, peanuts, potato chips, and squid chips but not in plain dried squid nor paper disks. The difference between products were the sucrose and fat content.

In this study, survival of the non-SRSS in silica was significantly lower than the SRSS ($P < 0.05$) with 1.8 and 0.6 log reduction respectively. The lower survival of non-

SRSS was also identified when inoculating oregano (Figure 7), which strongly suggested that the non-SRSS are more sensitive to the desiccation effect.

The desiccation effect in the survival of SRSS and non-SRSS was further evaluated and is described in *Section 3.9*.

4.3.2 Inoculation of oregano using talcum and silica powder

Concentrations higher than 6 log CFU/g were obtained when inoculating oregano with SRSS using silica and talcum powder as a vehicle of the pathogen (Table 9). Although the levels of non-SRSS were higher in talcum than the levels reached with the SRSS, a more efficient transfer of SRSS was found when inoculating oregano (Table 9). Using talcum powder inoculated with 6.3 log CFU/g yielded 6 log CFU/g of SRSS in oregano. On the contrary, the non-SRSS levels in talcum were too low (4.5 log CFU/g) and non-detectable counts were reported after the inoculation of oregano (< 3 log CFU/g).

Table 9 Concentration of SRSS and non-SRSS in oregano after inoculation with silica and talcum powder

Sample	Silica powder		Talcum powder	
	SRSS	non-SRSS	SRSS	non-SRSS
Dried	8.4 ± 0.2[a]	7.3 ± 0.2	6.3 ± 0.2	4.6 ± 0.5
Inoculated oregano	6.7 ± 0.1	5.0 ± 0.3	6.0 ± 0.8	ND[b]

[a]Log CFU/g ± standard deviation
[b]No detectable levels with a limit of detection < 3 log CFU/g

A noteworthy observation during inoculation of silica was the formation of lumps when the liquid inoculum was added to the powder. The formation of lumps was more evident when inoculating small amounts of silica than when inoculating larger amounts (Figure 13b). The dried inoculated silica was sieved and the remaining lumps were pulverized. Although this procedure was repeated twice, there were still small lumps, smaller than the mesh pore size (0.2 mm), that were difficult to pulverize (Figure 13b). It is possible that clusters of bacteria were contained in the small lumps reducing the transference of *Salmonella* to oregano. This could explain that when sieving the mixture oregano-silica, the lumps would not transfer *Salmonella* cells to the oregano leaves, decreasing the efficacy of the transfer.

a) b)

Figure 16 Dry inoculated oregano leaves, a) Sieved silica and oregano residues and b) Inoculated oregano

4.3.3 Preliminary experiments: inoculation of silica, talcum powder, and oregano using P. faecium

When the inoculated plates were incubated for 24 h, plates from the overlay procedure presented colonies with a diameter of ≥ 0.05 cm whereas, colonies from the KFS pour plated plates were not easily distinguished (Table 10).

Table 10 *Pediococcus faecium*'s colony size after incubating 24 h at 35 °C

Procedure	Plating media	Diameter (mm) of colonies by dry vehicle	
		Silica	Talcum
Resuscitation[a]	TSA	0.58 ±0.01A[b]	0.50 ± 0.00A
	TSA+YE	0.59 ±0.01A	0.54 ± 0.01A
No resuscitation	KFS	ND	ND

[a]Refers to the two-step overlay procedure where samples were plated onto TSA or TSA+YE and incubating 3 h at 35 °C. Afterwards, an overlay of KFS was poured on the incubated plates and incubation was continued to 24 h
[b]Data within the same column followed by different letters are significantly different (P < 0.05)
[c]No detectable

If injured *P. faecium* cells are plated directly on selective media, *i.e.* KFS agar, it is possible that sublethally injured cells get inhibited *(84)*. Furthermore, another potential factor that can add up inhibition during the enumeration is the heat-shock received when pour-plating KFS at 45- 50 °C. An advantage of performing the two-step overlay procedure is that by the time the KFS is poured, cells are already resuscitated. In fact, the overlay procedure shows to recover the same or even more cells form the dried materials. This can be seen in the enumeration of *Pediococcus* in talcum powder were the variability in the counts is small (greatest standard deviation of ±0.2 Log CFU/g) when compared with silica powder (greatest standard deviation of ±1.0 Log CFU/g) (Table 12)

Additionally, the overlay procedure allowed reading the plates at 24 h of incubation instead of 48 h (as the pour plating procedure dictates). No significant differences were detected at 24 h of incubation between the counts of *Pediococcus* in silica and talcum powder when using TSA or TSAYE ($P > 0.05$) (Table 11).

There was a significant difference in the counts after 48 h of incubation, when using TSA and TSAYE in dried silica ($P < 0.05$), whereas no significant differences were obtained when comparing the overlay procedure (TSA and TSAYE) *vs* using only KFS ($P > 0.05$). The variability in the results when using only KFS was high, making it difficult to see a true difference when compared with TSA and TSAYE. Smaller variability in the counts was obtained in the talcum powder samples. Despite the significant differences in the final counts in dried talcum when using the resuscitation and non-resuscitation procedure (Table 12) these differences of 0.2 - 0.3 logs are not significant in practical terms.

Table 11 Recovery of *P. faecium* from silica and talcum powder before and after desiccation. Selection of the enumeration procedure when incubating plated samples for 24 h

Procedure		After inoculation		After desiccation	
		Silica	Talcum	Silica	Talcum
Resuscitation[a]	TSA	9.0 ± 0.2A[b]	9.1 ± 0.1A	7.4 ± 1.0A	9.0 ± 0.1A
	TSAYE	8.9 ± 0.2A[c]	9.1 ± 0.1A	8.0 ± 0.2A	9.0 ± 0.1A
No resuscitation	KFS	N/A	N/A	N/A	N/A

[a]Refers to the two-step overlay procedure where samples were plated onto TSA or TSA+YE and incubating 3 h at 35 °C. Afterwards, an overlay of KFS was poured on the incubated plates and incubation was continued to 24 h.
[b]Data within the same column followed by different letters are significantly different ($P < 0.05$)
[c]Log CFU/g ± standard deviation

Table 12 Recovery of *P. faecium* from silica and talcum powder before and after desiccation. Selection of the enumeration procedure when incubating plated samples for 48 h

Procedure		After inoculation		After desiccation	
		Silica	Talcum	Silica	Talcum
Resuscitation[a]	TSA	9.0 ± 0.2A[b]	9.1 ± 0.0A	7.2 ± 1.0B	9.0 ± 0.1A
	TSAYE	8.9 ± 0.2A[c]	9.1 ± 0.2A	8.0 ± 0.2A	9.1 ± 0.2A
No resuscitation	KFS	8.8 ± 0.2A	9.1 ± 0.2A	7.8 ± 0.5AB	8.8 ± 0.1B

[a]Refers to the two-step overlay procedure where samples were plated onto TSA or TSA+YE and incubating 3 h at 35 °C. Afterwards, an overlay of KFS was poured on the incubated plates and incubation was continued to 24 h
[b]Data within the same column followed by different letters are significantly different (P < 0.05).
[c]Log CFU/g *Log CFU/g ± standard deviation.

The final concentration of *P. faecium* in dried silica and talcum powder was high enough to be used for further inoculation of oregano, with > 7 log CFU/g in silica and > 8.8 log CFU/g in talcum powder. Smaller reductions of *P. faecium* were obtained after desiccation when inoculating in talcum powder than silica.

No significant differences were found when enumerating *P. faecium* from oregano inoculated with talcum powder using the resuscitation procedure or without it (P > 0.05). Also, the addition of yeast extract to TSA did not seemed to improve the recovery of *P. faecium* from oregano inoculated neither with silica nor talcum (Tables 12-14). However, counts of *P. faecium* from oregano inoculated with silica showed lower significant differences between enumerating with the two-step OV using TSA than when using TSAYE and the non-resuscitation procedure (P < 0.05).

Independently of the powder used to inoculate oregano, the final concentration of *P. faecium* reached at least the 6 log CFU/g. However, inoculation of oregano using talcum powder yielded between 7.4-7.8 log CFU/g, one logarithm higher than when using silica (Tables 12-14).

Table 13 Recovery of *P. faecium* from oregano inoculated with silica and talcum powder. Selection of the enumeration procedure when incubating plated samples for 24 h

Procedure		Dried powder		Inoculated oregano	
		Silica	Talcum	Silica	Talcum
Resuscitation[a]	TSA	7.4 ± 0.1A[b]	9.0 ± 0.1A	6.9 ± 0.8A	7.4 ± 0.6A
	TSAYE	8.0 ± 0.1A[c]	9.0 ± 0.1A	6.4 ± 0.1A	7.4 ± 0.5A
No resuscitation	KFS	N/A	N/A	N/A	N/A

[a]Refers to the two-step overlay procedure where samples were plated onto TSA or TSA+YE and incubating 3 h at 35 °C. Afterwards, an overlay of KFS was poured on the incubated plates and incubation was continued to 24 h
[b]Data within the same column followed by different letters are significantly different (P < 0.05).
[c]Log CFU/g *Log CFU/g ± standard deviation.

Table 14 Recovery of *P. faecium* from oregano inoculated with silica and talcum powder. Selection of the enumeration procedure when incubating plated samples for 48 h

Procedure		Dried powder		Inoculated oregano	
		Silica	Talcum	Silica	Talcum
Resuscitation[a]	TSA	7.2 ± 1.0B[b]	9.0 ± 0.1A	6.9 ± 0.8A	7.8 ± 0.1A
	TSAYE	8.0 ± 0.2A[c]	9.1 ± 0.2A	6.4 ± 0.1AB	7.8 ± 0.2A
No resuscitation	KFS	7.8 ± 0.5AB	8.8 ± 0.1B	5.8 ± 0.2B	7.7 ± 0.1A

[a]Refers to the two-step overlay procedure where samples were plated onto TSA or TSA+YE and incubating 3 h at 35 °C. Afterwards, an overlay of KFS was poured on the incubated plates and incubation was continued to 24 h
[b]Data within the same column followed by different letters are significantly different (P < 0.05).
[c]Log CFU/g *Log CFU/g ± standard deviation.

Despite the final concentration of *Pediococcus* in oregano was > 6 logs when using silica, this powder was not used in further experiments due to the difficulty when handling it. As previously mentioned, silica gets easily charged with statics increasing the probability of getting dispersed all over the working area. This problem represents a high concern especially when working with pathogens.

4.3.4 Mechanisms of oregano contamination using silica and talcum powders

At the beginning of the study, it was thought that the mechanism of dry inoculation of oregano consisted in transferring bacteria from the inert powder to the oregano leaves (Figure 17) due to the electrostatic interactions such as van der Waals attraction *(175)*.

However, a change of the oregano's color was evident after inoculating using silica and talcum, suggesting that a fine powder layer covered the leaves even after sieving (Figure 18-19).

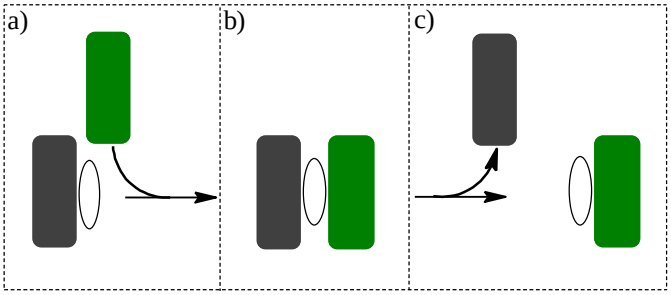


Figure 17 Mechanism of bacterial transference from inert powders to oregano originally suggested: a) inert powder with bacteria attached is mixed with oregano leaves, b) the bacterial cells get in contact with the surfaces of oregano leaves and attach to them, c) during sieving, the inert powders are separated without the bacterial cells, now attached to the oregano leaves.
Grey squares represent the inert powder surfaces, green squares represent the oregano leaves surfaces, and the white ellipses represent the bacterial cells.

Figure 18 Color of silica-inoculated oregano (left) and non-treated oregano (right)

Figure 19 Color difference of talcum-inoculated oregano (left) and non-treated oregano (right)

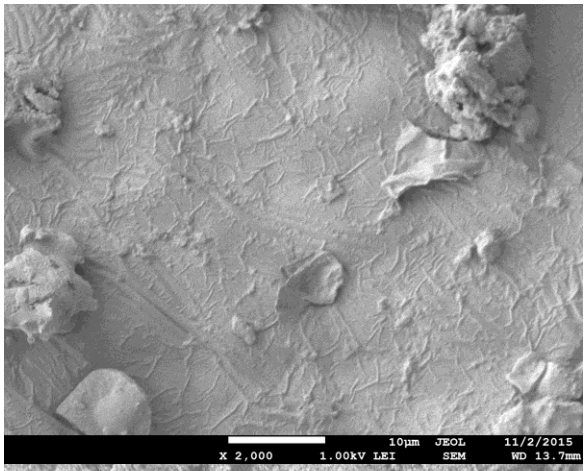
The amount of silica and talcum powder after sieving should be, in theory, the 3 grams initially added to oregano. When measured, the amount of silica and talcum powder recovered was 4 and 3.8 g, respectively. Therefore, the recovered inert powders contained ~1 g of oregano (Figure 20). Although a 2-mm pore size mesh was used during sieving, the mechanical motion when mixing the oregano with the powders could have broken the

oregano leaves that passed through the mesh during sieving. Furthermore, the inoculated oregano was analyzed using scanning electron microscopy (SEM).

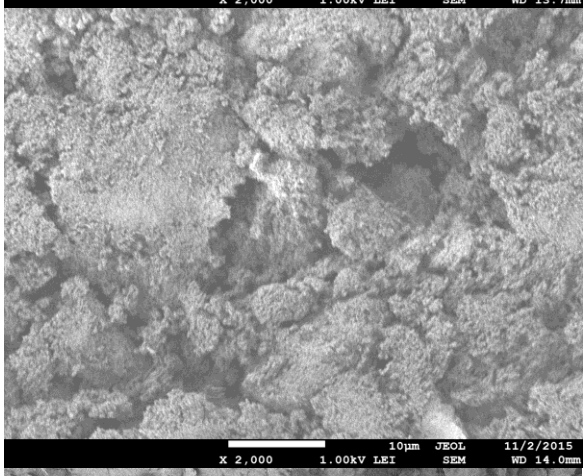
Figure 20 Sieved silica with oregano residues

The SEM showed that, effectively, oregano leaves were totally coated with silica or talcum particles after sieving as shown in Figure 21. Although a true transference of bacteria could have happened from the powders to the leaves, it seems that the principal mechanism of inoculation is a mere mixture of the inert powders, bacterial cells, and oregano leaves.

a)



b)



c)

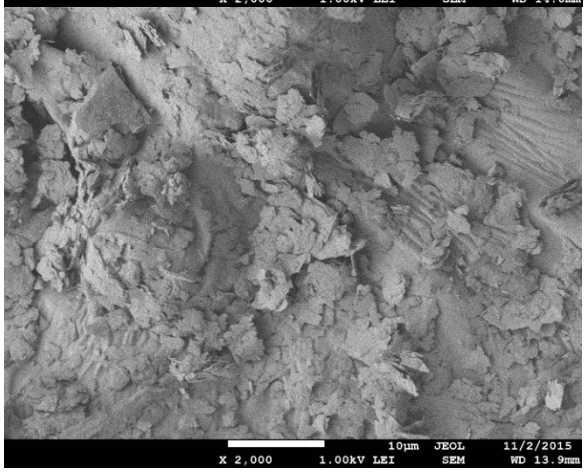
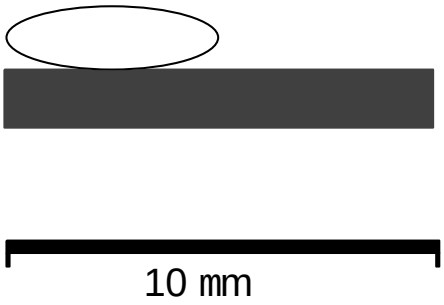


Figure 21 Scanning Electron Microscopy pictures of a) non-inoculated oregano, b) oregano inoculated with silica (sieved), and c) oregano inoculated with talcum (sieved)

Silica and talcum particle sizes are 0.2-0.3 and 10 μm, respectively. *Salmonella* cells are about 2- 5 μm in size, therefore, silica is ~10-25 times smaller than *Salmonella*. Then, it is more likely that silica binds around the cells instead of the other way around. Although the talcum particle sizes are about 2 times bigger than *Salmonella* cells, and it is possible that the cells attach to the powder as shown in Figure 22a, the SEM images revealed high heterogeneity (Figure 22b) perhaps as a result of the mechanical motion during homogenization and sieving.

a)



b)

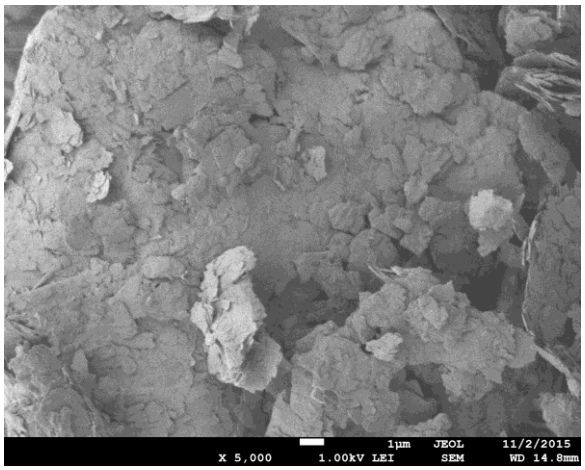


Figure 22 a) Comparison of *Salmonella* and talcum powder sizes where the grey rectangle represents talcum powder and the white ellipse represents a *Salmonella* cell. b) Scanning electron microscopy image of non-inoculated talcum powder shows heterogeneity of the particle sizes.

It cannot be discarded that bacteria can be transferred from the inert powder to the oregano leaves. The adherence of a bacterial cell to solid surfaces is given principally by the van der Waals and electrostatic forces *(136)*. The total strength of the attachment depends at least on the cell shape, the presence of flagella, and the solid material topography and polarity. Bacterial cell exterior is negatively charged as well as the inert powders *(112, 175)*. If bacterial cells approach to another negatively charged surface, they will repel from one another unless the bacterial cells pass a stable distance, which is known

as secondary minimum *(175)*. Young *(175)* described the secondary minimum as the distance between 4 and 10 nm from the surface where the electrostatic repulsion still exists but cells are close enough to initiate a reversible physical contact with the surface by using pili, secreting polymeric capsular materials, and/or using adhesins located at the cell poles. As mentioned earlier, this type of attachment is weak and reversible *(175)*. A plant leave polarity depends on the type of proteins and molecules found on the surface. The polarity of the leave depends on the surface being measured. For example, the upper surface is often waxier than the lower surface *(46)*. It seems like the total polarity of the oregano leave is strong enough to retain even the inert powders as shown in Figure 19. However, further studies are needed to measure the polarity of dry oregano leaves.

4.3.5 Dry inoculation of onion powder

During standardization of the dry inoculation of onion powder, a liquid inoculation was still needed. The lumping was decreased by adding a small volume as inoculum (1 ml of the inoculum mixed with 50 g of onion powder in a coffee grinder). To measure the effect of this approach in the moisture of the inoculated onion powder, the a_w was measured, and found to increase from 0.3 to 0.4 after dry inoculation, whereas for the liquid inoculation, the a_w substantially increased from 0.3 to almost 1.0. The inoculated and dried onion powder starter, was used then as inoculum for new and bigger batches of onion powder.

4.3.5.1 Inoculation of onion powder using Salmonella

The final concentration of the SRSS and non-SRSS inocula of 10.6 and 10.7 log CFU/ml showed no significant differences ($P > 0.05$). After inoculation, the SRSS had significantly a higher survival before and after desiccation ($P < 0.05$). However, these differences of 0.2 and 0.3 logs for the wet and dried onion powder, respectively, are not significant in practice (Table 15).

Table 15. Dry inoculation of onion powder using SRSS and non-SRSS

Sample	SRSS	Non-SRSS
	Log CFU/g or ml ± SD	Log CFU/g or ml ± SD
Cocktail	$10.6 \pm 0.3_{A}$[a]	$10.7 \pm 0.2_{A}$
Onion powder after inoculation	$8.8 \pm 0.1_{B}$	$8.6 \pm 0.2_{A}$
Onion powder after desiccation	$8.1 \pm 0.1_{B}$	$7.8 \pm 0.2_{A}$

[a]Data within the same row followed by different letters are significantly different ($P < 0.05$)

Interestingly, the reduction of SRSS and non-SRSS after desiccation was smaller in onion powder than the other 3 matrices tested previously (*i.e.* oregano, talcum powder, and silica powder) (Table 16).

The sensitivity of non-SRSS to desiccation can be easily appreciated in Table 16 for oregano, silica, and onion powder. However, the difference of the reduction between SRSS and non-SRSS is minimal (0.1 log) in desiccated onion powder. One explanation to this phenomenon is the temperature of desiccation used for each sample. Desiccation of oregano, silica and talcum powder was performed at 35 °C and for onion powder at 25 °C.

This difference in temperature could explain why the effect of desiccation is smaller and almost equal for both, SRSS and non-SRSS. Also, the total reduction for each *Salmonella* cocktail in onion powder was < 1 log cycle when in silica, talcum powder and oregano was between 1.7 – 4.6 log cycles.

A higher tolerance of *Salmonella* to desiccation has been related to factors such as the media of growth. When cells are grown in solid media, the production of biofilms is promoted and favors a better adaption to environmental stresses than planktonic bacteria.

This adaption is related to the expression of several stress-response genes in the cell *(37, 147)*. In this experiment, TSB was used to prepare the inoculum for *Salmonella* and no large reductions were obtained after desiccation. It seems that other factors such as the inoculated matrix composition (*i.e.* solutes and type of antimicrobials), the final a_w, and the temperature for desiccation had a less aggressive effect in *Salmonella*'s survival in onion powder.

The concentration of solutes, such as sugars, in onion powder is higher than in oregano, with 35 g of sugar/ 100 g of onion powder while oregano only has 4 g *(35, 36)*. This could have provided more protection to *Salmonella* cells during desiccation. For example, Hiramatsu, *et al. (77)* found a substantially higher bacterial survival rate of 10 to 79 times as the level sucrose concentration increased when inoculating in paper disks.

The high fat content has also associated with a higher protection of the bacterial cells in low a_w environments *(10, 66, 75, 151)*. Even though the total fat content in oregano is 10% and in onion powder is only 1%, this high fat content oregano is composed basically by only EO (*i.e.* antimicrobials).

Table 16. Reduction of *Salmonella* after desiccation of oregano, silica, talcum and onion powder

Sample	Reduction after desiccation (log CFU/g)		Difference of the reduction between Non-SRSS and SRSS
	SRSS	Non-SRSS	
Non-VHTO	2.1	3.4	1.3
$VHTO_{70°C}$	1.7	3.2	1.5
$VHTO_{140°C}$	2.2	3.1	0.9
Silica powder	0.6	1.8	1.2
Talcum powder	3.4	4.6	1.2
Onion powder	0.7	0.8	0.1

Another important factor that contributed to the higher *Salmonella* survival in onion powder was the final lower a_w of 0.3 compared with the a_w of 0.45 reached in oregano, silica, and talcum powder. The increase in the survival of *Salmonella* as the a_w decreases has been reported previously *(16, 24, 101).*

4.3.5.2 Inoculation of onion powder using P. faecium

Before running the inoculation of *P. faecium* experiments, it was necessary to determine if the cells became injured after desiccation and the resuscitation procedure was needed. To test this, inoculated and desiccated onion powder was sampled and plated using 1) only KFS and 2) the overlay procedure (TSA and an overlay with KFS). The addition of yeast extract and potassium sulfite to TSA was evaluated in the overlay procedure. As control, *Pediococcus* was enumerated by pour plating KFS in the serial decimal diluted samples. The addition of yeast extract to TSA intended to provide sources of vitamins and cofactors, proteins, and carbohydrates for a better *Pediococcus* growth.

Volatile disulfide compounds (*i.e.* di-*n*-propyl disulfide and *n*-propyl allyl disulfide) have been recognized as natural antimicrobials present in onion. To determine the inhibition effect by these compounds, TSA was supplemented with 0.5% K_2SO_3 *(172)*.

The calculated concentration of *Pediococcus* when adding 1 ml of the inoculum (10.2 log CFU/ml) in 50 g of onion powder was 8.5 log CFU/g. As shown in Table 17, no bacterial reductions were observed after inoculation or after desiccation. This same pattern was observed when inoculating SRSS and non-SRSS in onion powder *(Section 4.3.5.1)*.

The comparison of the different supplements added to TSA in the overlay procedure showed no significant differences for the enumeration of *Pediococcus* ($P > 0.05$). On the other hand, the enumeration of *Pediococcus* using the resuscitation procedure did not proved to improve the recovery of *Pediococcus* than when enumerating without resuscitating the cells ($P > 0.05$). This suggests that *Pediococcus faecium* does not get injured after desiccating in onion powder at 25 °C.

Table 17 Inoculation of onion powder using *P. faecium*

Procedure	Media	After inoculation	Dried
No resuscitation	KFS	$8.5 \pm 0.1_{aA}$[b]	$8.6 \pm 0.1_{aA}$
Resuscitation[a]	TSA	$8.6 \pm 0.1_{aA}$	$8.7 \pm 0.1_{aA}$
	TSA + K_2SO_3	$8.6 \pm 0.1_{aA}$	$8.7 \pm 0.2_{aA}$
	TSAYE	$8.6 \pm 0.1_{aA}$	$8.7 \pm 0.2_{aA}$
	TSAYE + K_2SO_3	$8.6 \pm 0.1_{aA}$	$8.7 \pm 0.2_{aA}$

[a]Refers to the two-step overlay procedure where samples were plated onto TSA or TSA+YE and incubating 3 h at 35 °C. Afterwards, an overlay of KFS was poured on the incubated plates and incubation was continued to 24 h

[b]Within a row, values with different lower-case letters are significantly different ($P < 0.05$). Within a column, values with different upper-case letters are significantly different ($P < 0.05$).

4.3.6 Resistance of Salmonella to desiccation

Selection of the *Salmonella* strains is important for challenge studies since the resistance among strains for a determined treatment varies. It is recommended selecting those that show higher resistance or those which have been isolated from the food of interest. However, special attention has to be taken to avoid using strains with atypical high resistance to a certain treatment *(111)*. An example of an unusual resistance is *Salmonella* Senftenberg 775W which has an extremely resistance to moist heat *(117)*.

In this study, SRSS showed to have higher resistance to desiccation in silica, talcum powder, oregano, and onion powder. However, to determine if there was a true difference in the susceptibility to desiccation, SRSS and non-SRSS were inoculated in sterile talcum powder (described in *Section 3.9*) and enumerated on TSA. No selective media was used to assure that potentially injured and well as non-injured cells were recovered. At the same time, the resuscitation procedure was evaluated to determine if the 3-h recovery time was long enough for the desiccation-injured cells to recover.

The comparison of the enumeration using only TSA versus the resuscitation procedure demonstrated that the 3-h resuscitation time was enough to allow the injured cells to recover ($P > 0.05$) (Figure 23).

The concentration of SRSS and non-SRSS inocula was 10.5 and 10.8 log CFU/ml. No significant differences were detected between the concentration of SRSS and non-SRSS with 9.4 log CFU/g each ($P > 0.05$) right after inoculation of the talcum powder.

However, the deleterious effect of desiccation on the non-SRSS was significantly higher than the SRSS with 3.5 and 2.2 log reduction, respectively ($P < 0.05$).

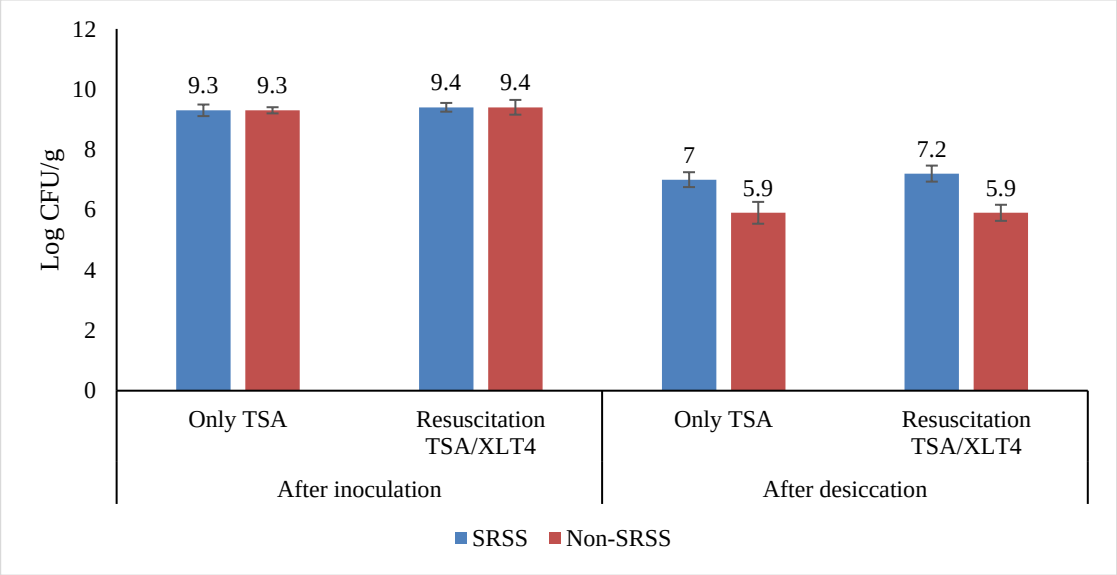


Figure 23. Resistance of *Salmonella* to desiccation

The intention of including the non-SRSS (*i.e.* the MP15 *Salmonella* strains) was to account for variability of the survival rates among the different strains *(111)*. However, based on the results of these studies, the non-SRSS was more sensitive to desiccation. Hence, using the non-SRSS panel was not considered to be adequate for further experiments studies involving an accelerated desiccation (*i.e.* 35 °C / 12 – 24 h). The higher SRSS recoverability after desiccation can be associated with the origin of the strains: dry spice *(55)*.

4.4 Survival of *Salmonella* and *P. faecium* in talcum powder, onion powder, and oregano over time

In general, for all samples, the survival rate was dependent on the temperature of storage and independent of the type of sample. This behavior is explained below.

4.4.1 Vacuum-heat treated oregano (VHTO)

4.4.1.1 Non-VHTO

Populations of SRSS significantly declined by 1.0 and 0.8 log CFU/g within a month of storage at 25 and 4 °C, respectively. These populations remained constant from the 4th to the 13th week at those temperatures (Figure 24). At -18 °C, SRSS showed no significant reductions during the 13-week storage period ($P > 0.05$).

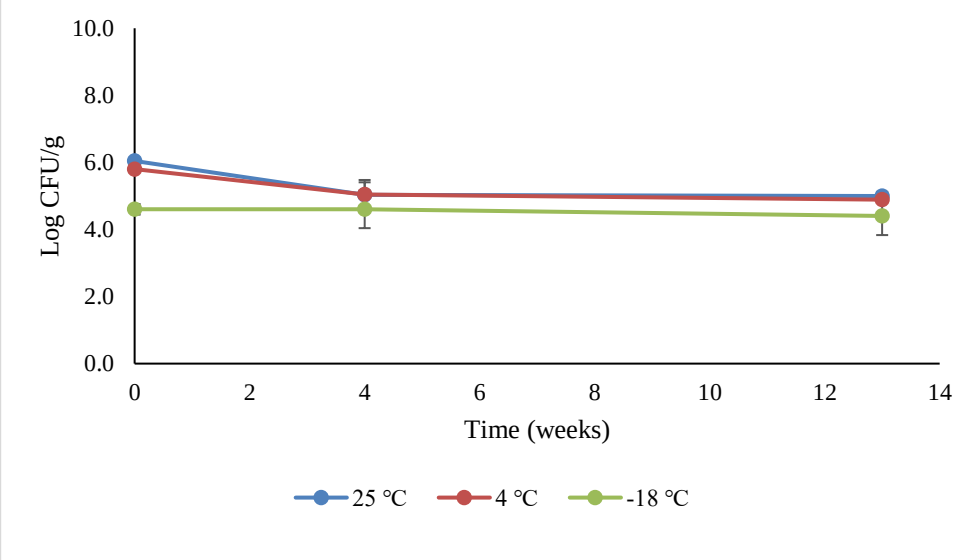


Figure 24 Survival of spice-related *Salmonella* strains in non-vacuum-heat-treated oregano stored at 25 °C, 4 °C, and -18 °C.

At 25 °C, the non-SRSS populations declined below the level of detection (LOD, of 3 log CFU/g) within a month of storage. At the 13th week, the non-SRSS concentration was 3 log CFU/g. Non-SRSS showed a better survival at 4 °C than at 25 °C. At 4 °C, the non-SRSS were reduced by 0.8 log CFU/g during the first 4 weeks and maintained its concentration during the rest of the storage period (Figure 25).

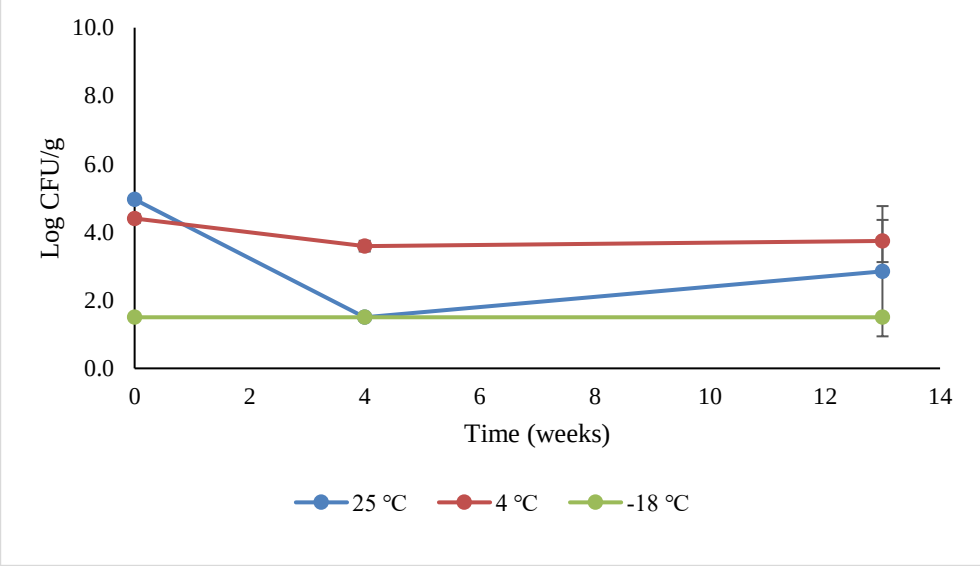


Figure 25 Survival of non-spice-related *Salmonella* strains in non-vacuum-heat-treated oregano stored at 25 °C, 4 °C, and -18 °C.

The *P. faecium* populations were more stable than the SRSS and the non-SRSS over the 13 weeks at the 3 different temperatures (Figure 26). The overall reduction at the end of the study was 0.5, 0.3, and 0.4 logs at 25, 4 and -18 °C, respectively ($P > 0.05$). Even though there was a reduction of 0.5 logs at the 4th week of storage at 25 °C, this was not significant ($P > 0.05$).

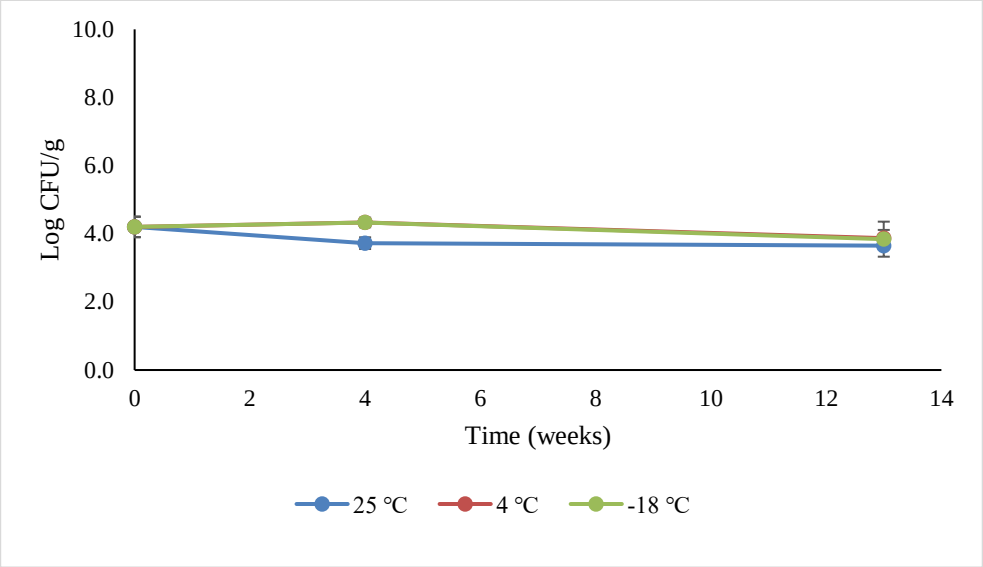


Figure 26 Survival of *P. faecium* in non-vacuum-heat-treated oregano stored at 25 °C, 4 °C, and -18 °C.

4.4.1.2 Vacuum-heat-treated oregano at 70 °C (VHTO$_{70°C}$)

The SRSS survival patterns in VHTO$_{70°C}$ at the 3 different temperatures were similar to the survival patterns in non-VHTO (Figure 27). At 25 and 4 °C, SRSS had reductions of 1.5 and 1.1 logs, respectively, and maintained those concentrations until the 13th week of storage. At -18 °C, no significant changes were detected throughout the study ($P > 0.05$).

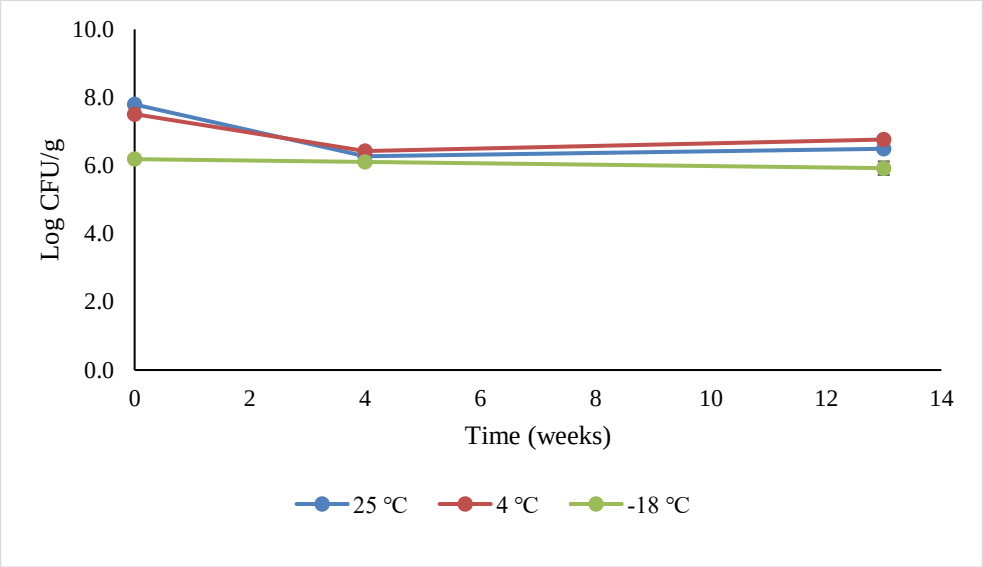


Figure 27 Survival of spice-related *Salmonella* strains in vacuum-heat-treated oregano (70 °C) stored at 25 °C, 4 °C, and -18 °C.

The non-SRSS population levels in $VHTO_{70^\circ}$ were more stable when stored at -18 °C and 4 °C than at 25 °C (Figure 28). The initial populations were maintained constant at -18 and 4 °C during the 13 weeks of storage ($P > 0.05$). However, at 25 °C, significant reductions of the non-SRSS populations were detected at the 4^{th} and 13^{th} week of storage ($P < 0.05$).

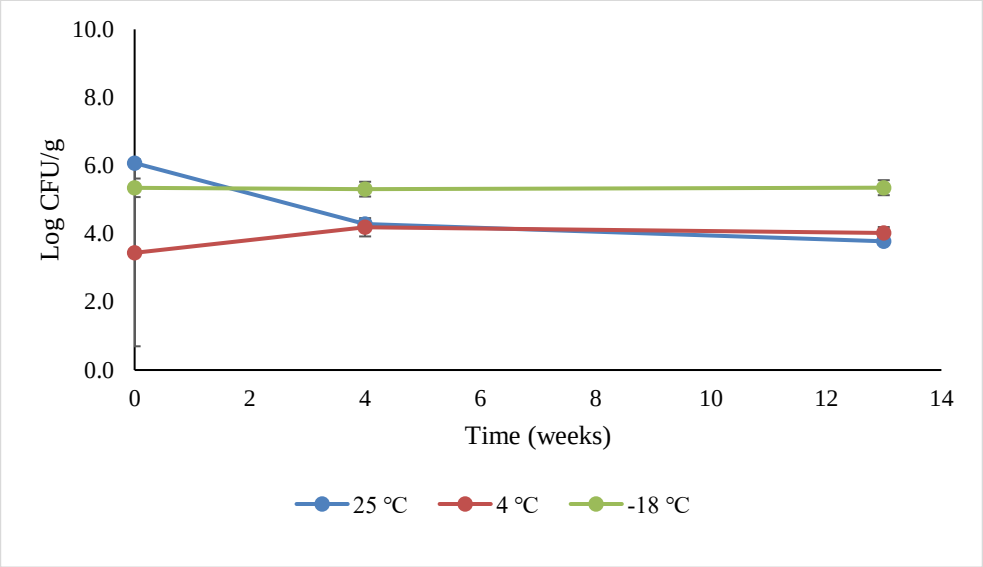


Figure 28 Survival of non-spice-related *Salmonella* strain in vacuum-heat-treated oregano (70 °C) stored at 25 °C, 4 °C, and -18 °C.

The concentration of *P. faecium* in $VHTO_{70°C}$ slowly declined by 0.4 logs by the 13th week at 25 °C ($P < 0.05$). The population of *P. faecium* at 4 and -18 °C at the end of the study was not significantly different than the initial counts ($P > 0.05$). Although there was an increment of 1 log at -18 °C at the 4th week of storage, no bacterial growth can occur at those conditions (*i.e.* -18 °C and a_w of 0.45). This apparent increment was attributed to a technical laboratory error when processing the samples.

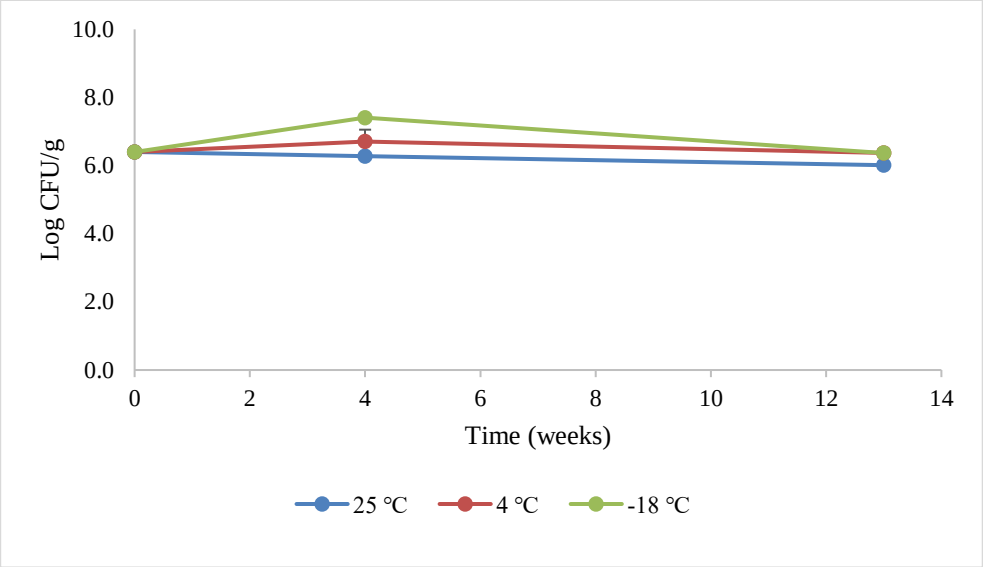


Figure 29 Survival of *P. faecium* in vacuum-heat-treated oregano (70 °C) at 25 °C, 4 °C, and -18 °C.

4.4.1.3 Vacuum-heat-treated oregano at 140 °C ($VHTO_{140°C}$)

The SRSS population levels in $VHTO_{140°C}$ were more stable when stored at cold temperatures than at room temperature (Figure 30). At 1 month of storage, SRSS populations significantly decreased by 1.8 logs ($P < 0.05$). Although there was a slightly reduction of 0.4 logs from the 4th to the 13th week, this was not significant ($P > 0.05$).

The SRSS survival at 4 °C had a small, but significant, decrement from 6.6 to 6.2 log CFU/g by the end of the 13th week ($P < 0.05$).

At -18 °C, the SRSS concentration remained constant throughout the storage period ($P > 0.05$).

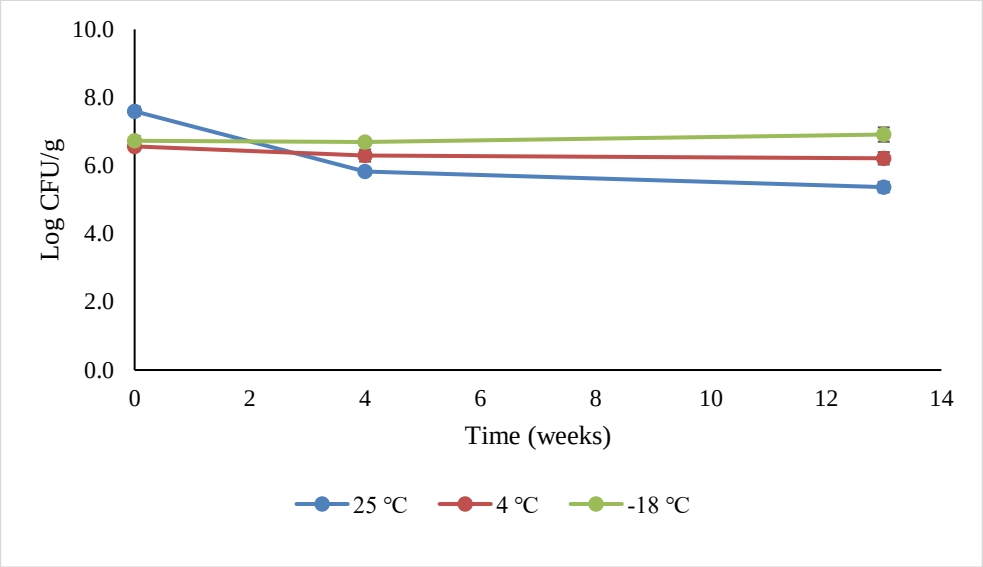


Figure 30 Survival of spice-related *Salmonella* strains in vacuum-heat-treated oregano (140 °C) stored at 25 °C, 4 °C, and -18 °C.

The non-SRSS concentration was more stable at -18 °C than at 4 and 25 °C over 13 weeks (Figure 31). In contradistinction to the non-VHTO and $VHTO_{70°C}$ survival patterns at 4 °C, the non-SRSS populations in $VHTO_{140°C}$ had a significant reduction of 2.3 logs within a month of storage ($P < 0.05$). A similar pattern occurred in $VHTO_{140°C}$ at 25 °C with a 1.7 log reduction within a month of storage ($P < 0.05$). However, the reduction level at 4 °C and 25 °C of 2.3 and 1.7 logs respectively, was not significant ($P > 0.05$). After a month of storage, the non-SRSS populations maintained constant at 4 °C in the non-VHTO, $VHTO_{70°C}$, and $VHTO_{140°C}$. On the contrary, by the 13th week at 25 °C, the non-SRSS population was below the LOD (< 3 log CFU/g).

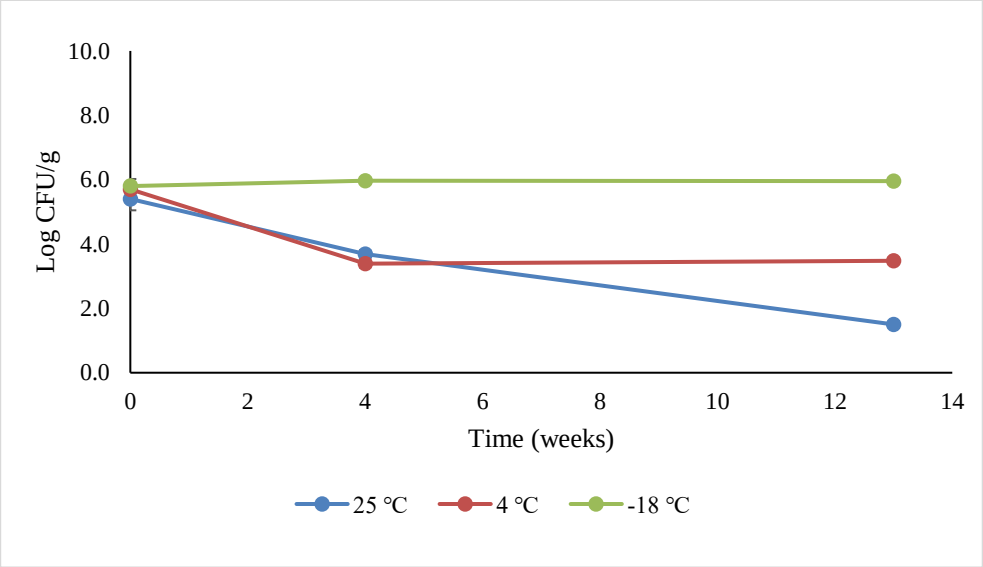


Figure 31 Survival of non-spice-related *Salmonella* strains in vacuum-heat-treated oregano (140 °C) stored at 25 °C, 4 °C, and -18 °C.

Pediococcus faecium populations at the 3 temperatures, 25, 4 and -18 °C, were constant throughout the storage period. No significant differences of the *Pediococcus* levels were detected in the counts at the 4th and 13th week for all 3 temperatures ($P > 0.05$) (Figure 32).

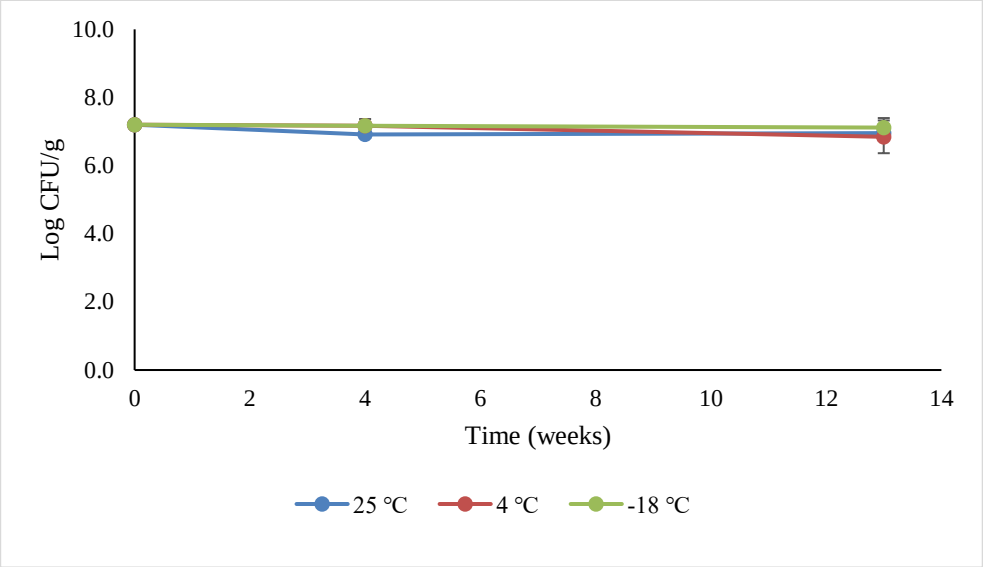


Figure 32 Survival of *P. faecium* in vacuum-heat-treated oregano (140 °C) stored at 25 °C, 4 °C, and -18 °C.

4.4.1.4 Salmonella and P. faecium survival rates in VHTO

The fitted curves obtained from DMFit are shown in Figures 25-33. The blue dots represent the experimental data and the red and continuous lines represent the fitted curve using the primary level Baranyi's model. Due to the pattern obtained from all curves, the reduction was considered a linear estimation.

In general, the survival of SRSS in non-VHTO and $VHTO_{70°C}$ presented similar patterns where the maximum reductions were reached within a month of storage at 24 and 4 °C. More stable SRSS levels were detected at -18 °C for all 3 types of oregano (non-VHTO, $VHTO_{70°C}$, and $VHTO_{140°C}$).

Analysis of the survival rates presented in Table 18 show a correlation between the storage temperature with the survivability of *Salmonella* independently of the %EO present in oregano. In general, storing oregano samples inoculated with SRSS and non-

SRSS at 25 °C resulted in higher reduction rates (Table 18). The time required to reduce 1 log cycle of *P. faecium* was calculated using the change rate. For *P. faecium,* this time was at least the 13 weeks of storage at all 3 temperatures.

Although higher reductions of *P. faecium* occurred at 25 °C, the rates changes obtained when storing for 13th weeks are small enough to be considered non-significant especially in $VHTO_{70°C}$ and in $VHTO_{140°C}$.

Table 18 Death rates of salmonellae and *P. faecium* inoculated in oregano

Treatment	Bacterial cocktail	Temperature (°C)	Time to reach 1 log reduction (Weeks)	Rate change (Log CFU/g per week)	SD[a]	
Non-VHTO	SRSS	25	> 13.0	-0.0675	0.0580	Figure 33
		4	> 13.0	-0.0612	0.0400	
		-18	> 13.0	-0.0163	0.0053	
	Non-SRSS	25	9.1	-0.1093	0.2380	Figure 34
		4	> 13.0	-0.0394	0.0515	
		-18	Error			
$VHTO_{70°C}$	SRSS	25	12.6	-0.0791	0.0948	Figure 35
		4	> 13.0	-0.0414	0.0723	
		-18	> 13.0	-0.0202	0.0002	
	Non-SRSS	25	6.4	-0.1564	0.0916	Figure 36
		4	> 13.0	0.0342	0.0483	
		-18	> 13.0	0.0010	0.0034	
$VTHO_{140°C}$	SRSS	25	6.6	-0.1509	0.0915	Figure 37
		4	> 13.0	-0.0238	0.0136	
		-18	> 13.0	0.0161	0.0076	
	Non-SRSS	25	3.5	-0.2897	0.0425	Figure 38
		4	7.1	-0.1404	0.1377	
		-18	> 13.0	0.0095	0.0101	
Non-VHTO	*P. faecium*	25	> 13.0	-0.0365	0.0260	Figure 39
		4	> 13.0	-0.0297	0.0197	
		-18	> 13.0	-0.0322	0.0203	
$VHTO_{70°C}$	*P. faecium*	25	> 13.0	-0.0297	0.0004	Figure 40
		4	> 13.0	-0.0081	0.0266	
		-18	> 13.0	-0.0216	0.0862	
$VTHO_{140°C}$	*P. faecium*	25	> 13.0	-0.0151	0.0178	Figure 41
		4	> 13.0	-0.0288	0.0067	
		-18	> 13.0	-0.0063	0.0005	

[a]SD, standard deviation of the fitted model from DMFit

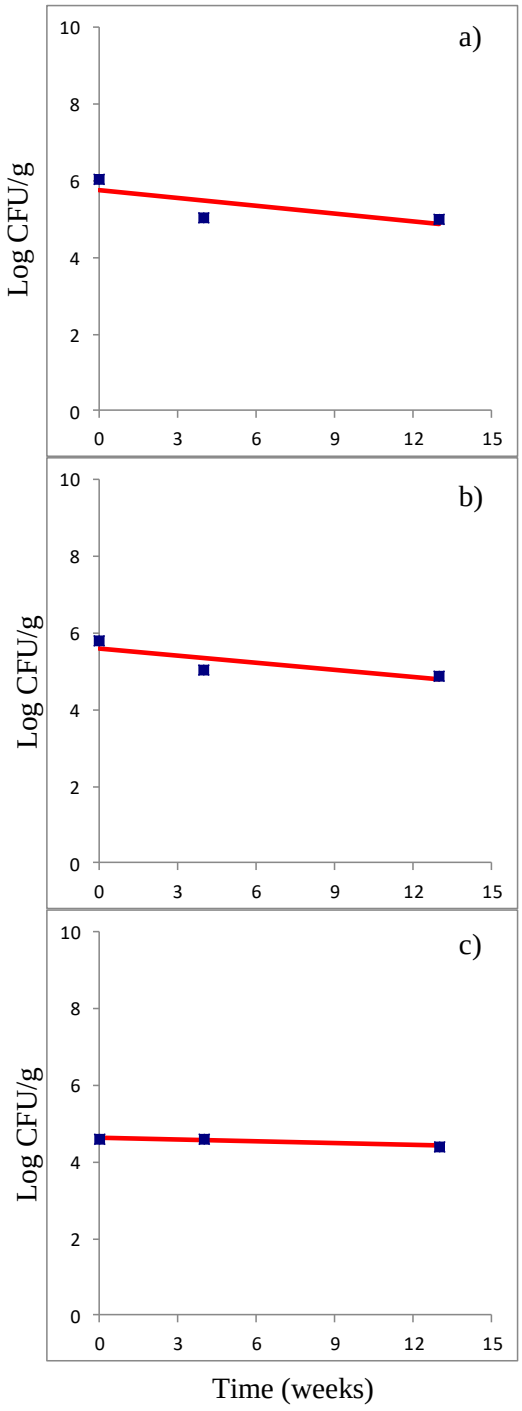


Figure 33 Survival of spice-related *Salmonella* strains in non-vacuum-heat-treated oregano stored at a) 25 °C, b) 4 °C, and c) -18 °C.

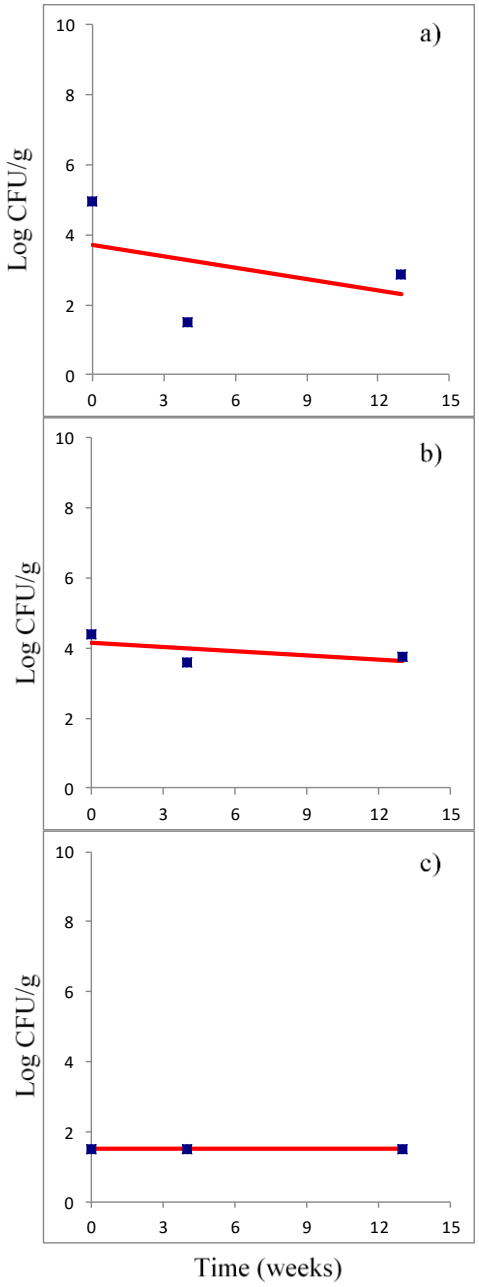


Figure 34 Survival of non-spice-related *Salmonella* strains in non-vacuum-heat-treated oregano stored at a) 25 °C, b) 4 °C, and c) -18 °C.

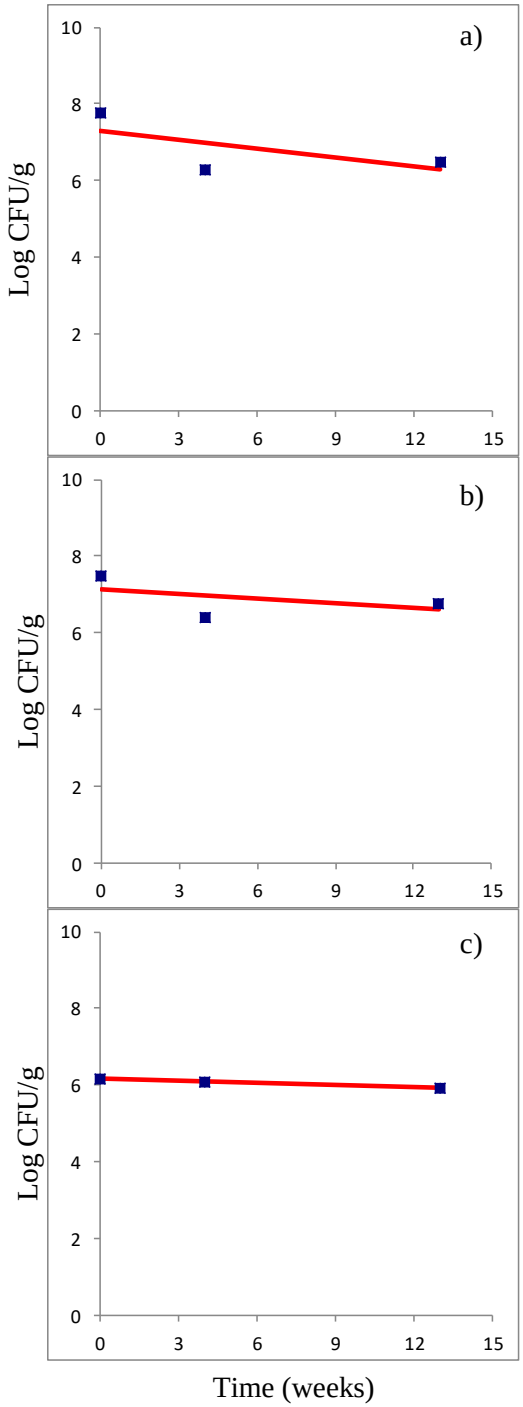


Figure 35 Survival of spice-related *Salmonella* strains in vacuum-heat-treated oregano (70 °C) stored at a) 25 °C, b) 4 °C, and c) -18 °C.

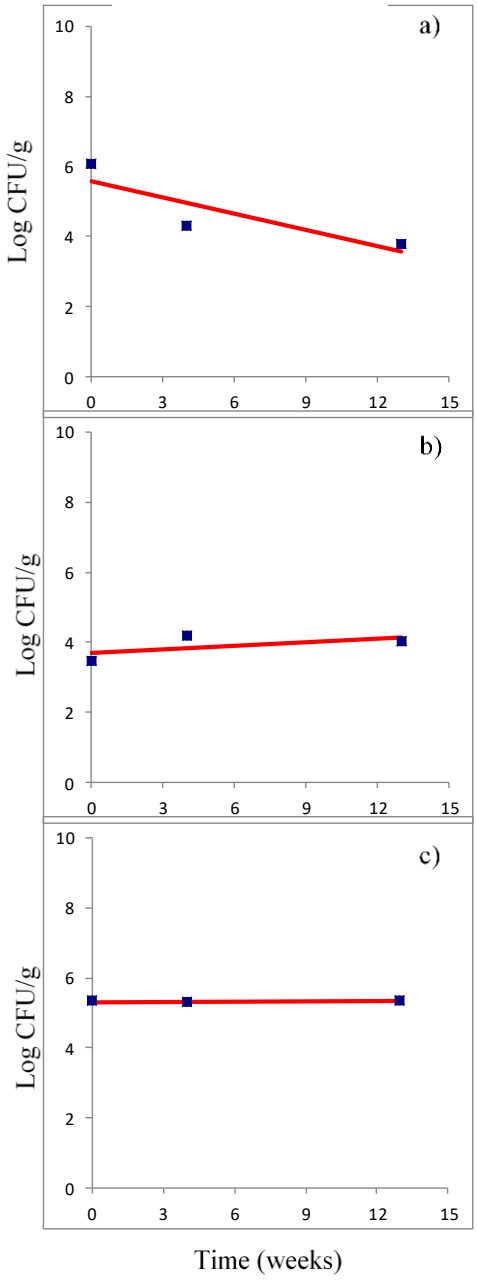


Figure 36 Survival of non-spice-related *Salmonella* strains in vacuum-heat-treated oregano (70 °C) stored at a) 25 °C, b) 4 °C, and c) -18 °C.

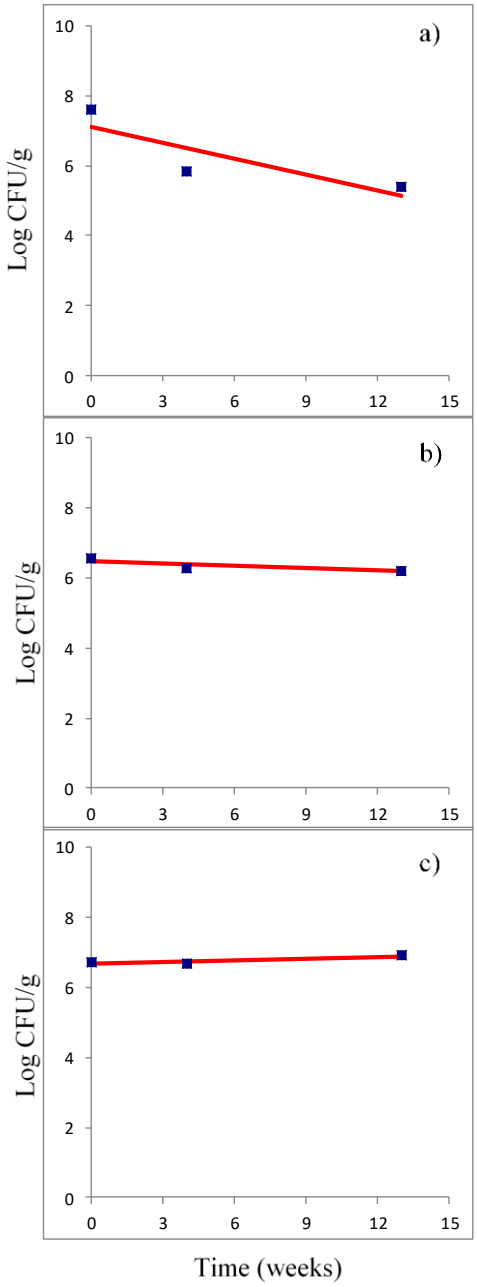


Figure 37 Survival of spice-related *Salmonella* strains in vacuum-heat-treated oregano (140 °C) stored at a) 25 °C, b) 4 °C, and c) -18 °C.

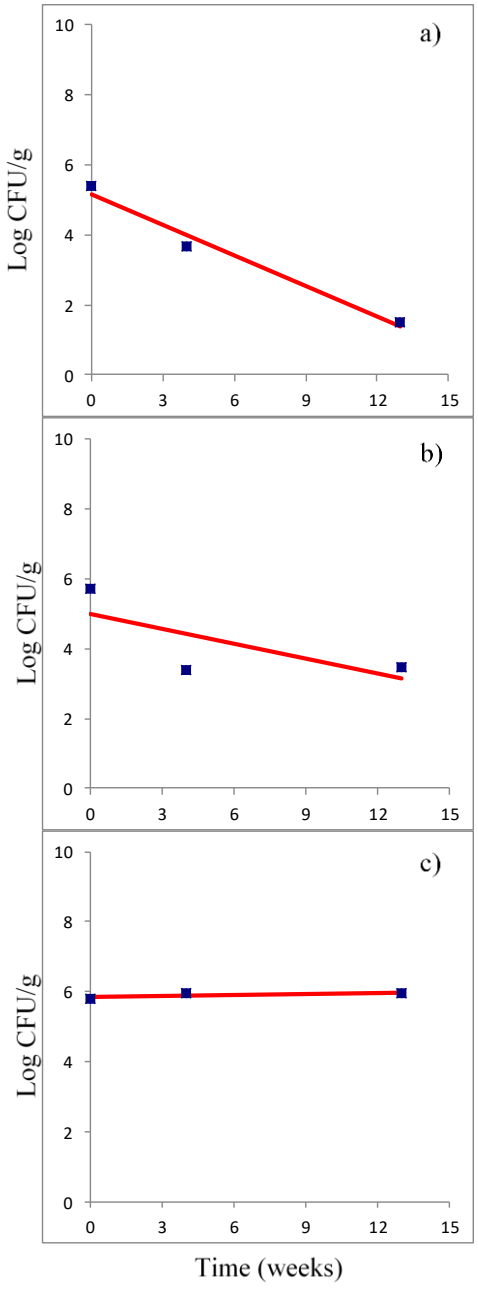


Figure 38 Survival of non-spice-related *Salmonella* strains in vacuum-heat-treated oregano (140 °C) stored at a) 25 °C, b) 4 °C, and c) -18 °C.

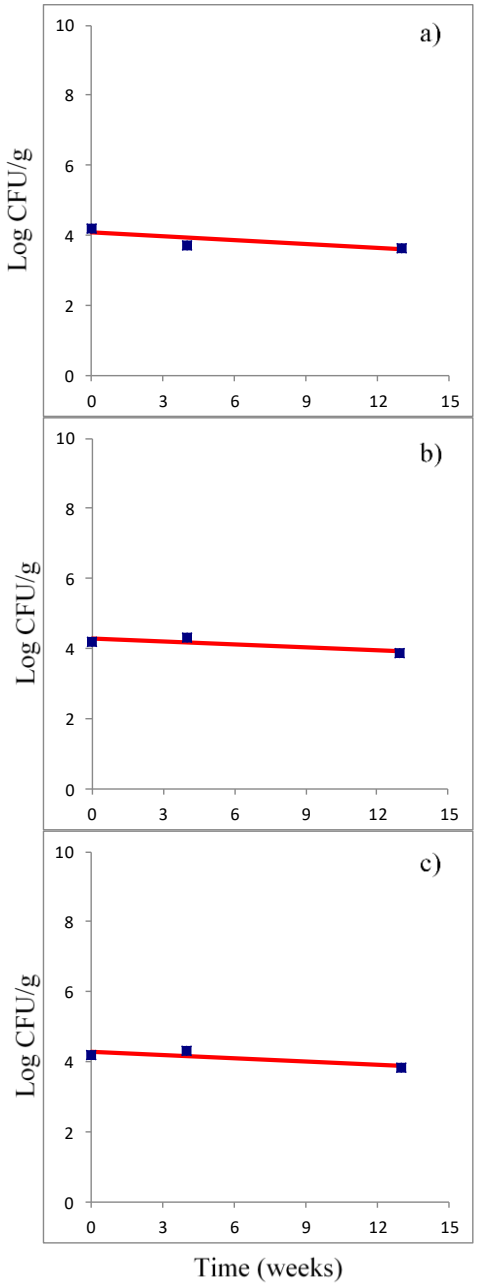


Figure 39 Survival of *P. faecium* in non-vacuum-heat-treated oregano stored at a) 25 °C, b) 4 °C, and c) -18 °C.

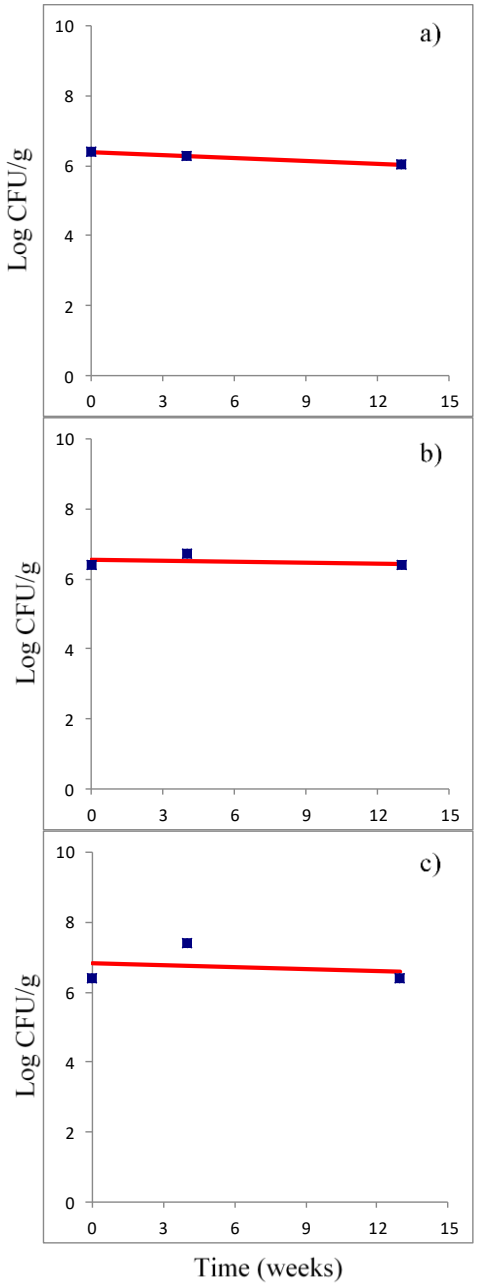


Figure 40 Survival of *P. faecium* in vacuum-heat-treated oregano (70 °C) stored at a) 25 °C, b) 4 °C, and c) -18 °C.

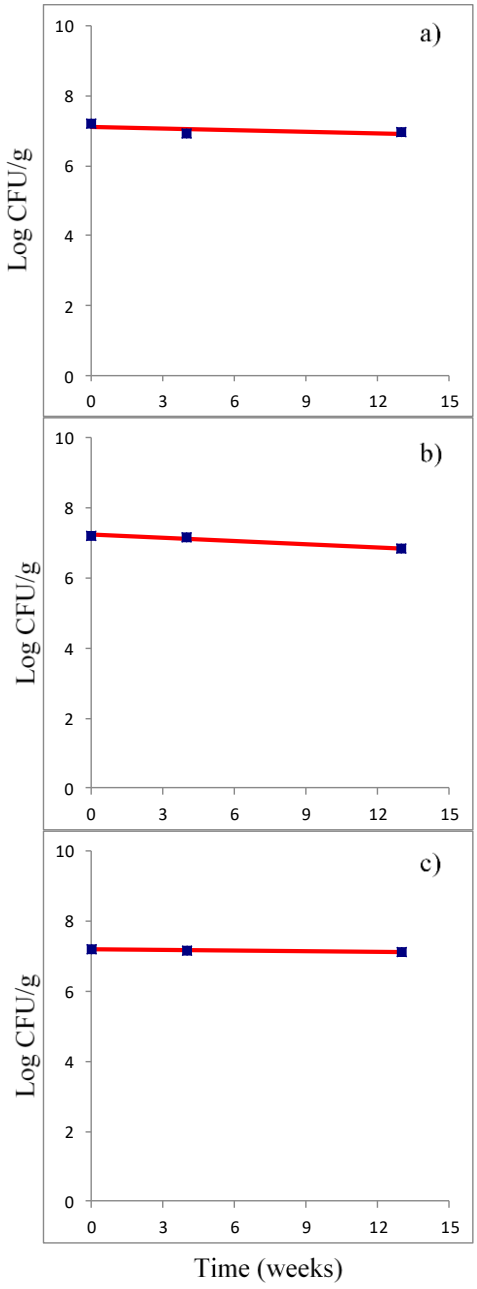


Figure 41 Survival of *P. faecium* in vacuum-heat-treated oregano (140 °C) stored at a) 25 °C, b) 4 °C, and c) -18 °C.

4.4.2 Survival of SRSS, non-SRSS, and *P. faecium* in talcum and onion powder

The survival of *Salmonella* and *P. faecium* was followed during 24 weeks of storage at -18 and 25 °C and for 20 weeks at 4 °C. It was not possible to continue the study at 4 °C until the 24th week of storage due to a lack of samples.

In general, all the strains stored at 4 and -18 °C were more stable over the 20 and 24 weeks, respectively. The differences in survival at 4 and -18 °C vs 25 °C were more evident in onion powder than talcum powder, for both, *Salmonella* and *P. faecium*. The description of each behavior is described below.

4.4.2.1 Survival in talcum powder

Populations of SRSS inoculated in talcum powder and stored at 25, 4 and -18 °C were overall constant. The SRSS concentration fluctuated during the first 11 weeks at the 3 temperatures (Figure 42). At 25 °C, SRSS concentration decreased by 0.6 logs at the 11th week to render 5.8 log CFU/g until the end of the study. Therefore, storing *Salmonella* strains at room temperature is not recommended unless the inoculated matrices are being used within 7 weeks of storage.

At 4 °C, SRSS were more stable than at 25 °C. The reduction of SRSS at 4 and 25 °C was 0.6 logs from the 1st to the 24th week. However, the greatest reduction at 4 °C was registered at the 24th week. The SRSS maintained a concentration of 6.6 – 6.8 log CFU/g during the first 11 weeks at 4 °C. The greatest reduction of 0.6 logs was observed from the 11th to the 20th week ($P > 0.05$).

In general, SRSS remained above 6.5 log CFU/g during the extent of the study at -18 °C and no significant differences on their concentration were observed between at the 1st and 24th week (P>0.05).

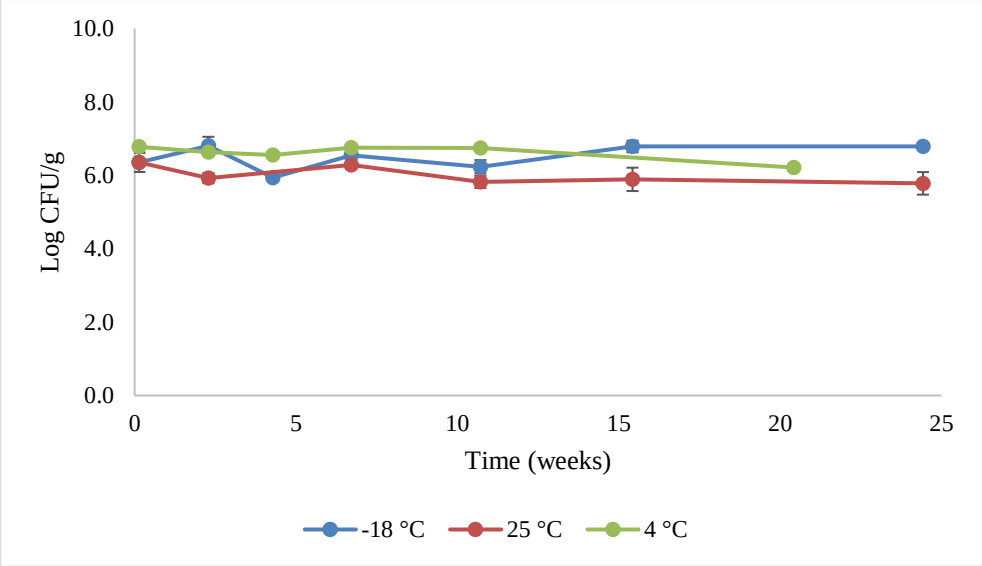


Figure 42 Survival of SRSS inoculated in talcum powder stored at 25, 4, and -18 °C

In overall, the non-SRSS remained constant when stored at 25 °C during 15 weeks. Although a reduction of 0.8 logs was registered from the 7 to the 11th week, the numbers were restored at the 15th week. A significant reduction of 1.1 logs occurred from the 15th week to the end of the study (P <0.05).

At 4 °C, no significant reductions of non-SRSS occurred (P >0.05) even though there was reduction of 0.7 logs from 1st to the 20th week. This lack of significance can be explained by the high standard deviation of the samples in the 20th week (0.5 logs).

The non-SRSS populations at -18 °C did not decreased throughout the extent of the storage period. Although the concentration of non-SRSS at -18 °C seemed to increase after the 1st week, this phenomenon is more likely to be a technical artifact. No bacterial growth can occur at these harsh conditions in which the non-SRSS were subjected (*i.e.* a_w of 0.45 and -18 °C).

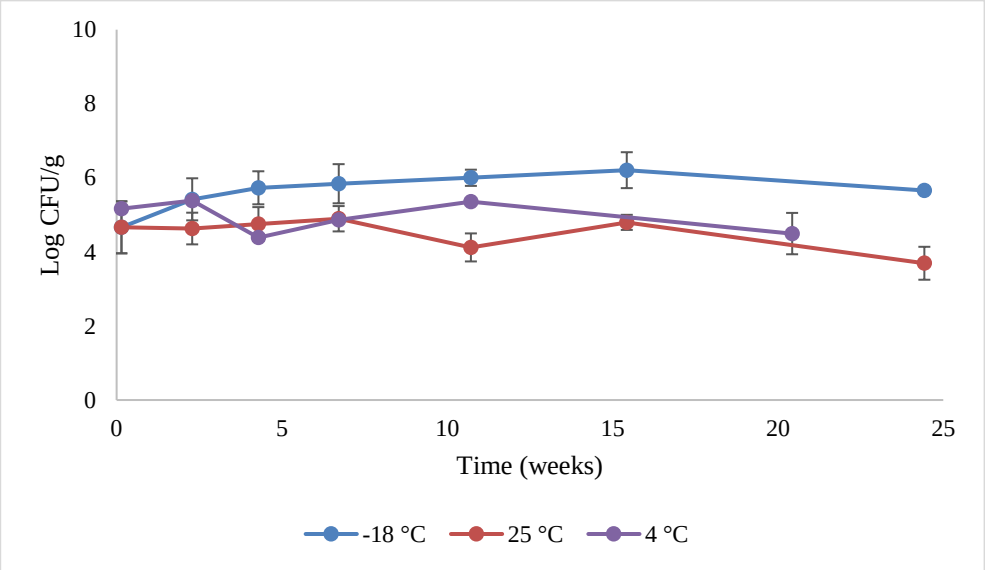


Figure 43 Survival of non-SRSS inoculated in talcum powder stored at 25, 4, and -18 °C

Pediococcus faecium demonstrated greater stability than *Salmonella* at the 3 temperatures during the whole storage period of 20 to 24 weeks ($P < 0.05$). Indeed, populations of *P. faecium* remained rather constant at the 3 temperatures ($P > 0.05$) (Figure 44). The 0.8-log reduction observed at the 5th week at 4 °C was attributed to a technical error especially because the *P. faecium* load returned and maintained in its original levels

for the rest of the study. The counts of *P. faecium* were not significantly different at the 3 temperatures over the 20 and 24 weeks of storage ($P > 0.05$).

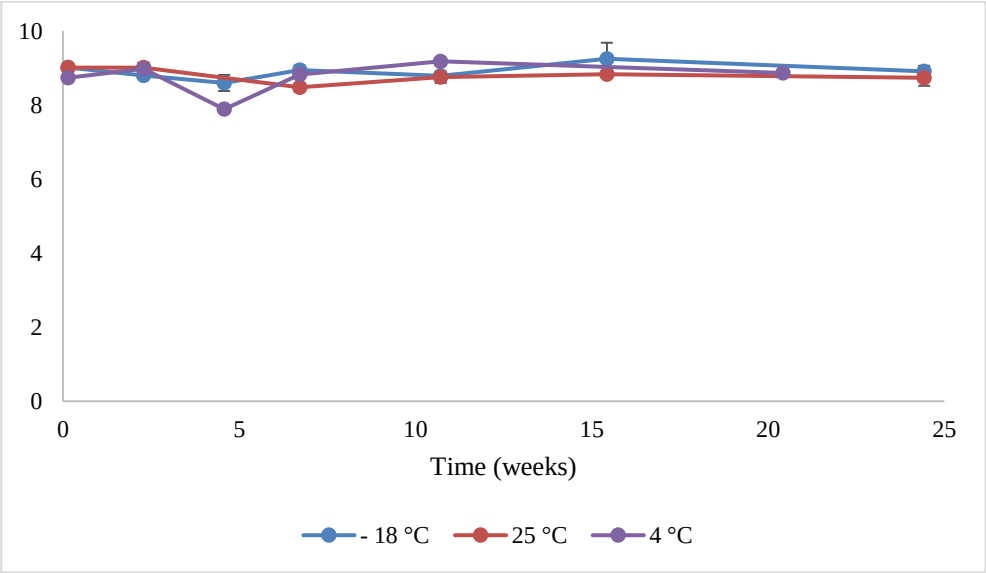


Figure 44 Survival of *P. faecium* inoculated in talcum powder stored at 25, 4 and -18 °C.

The reduction or survival rates for each curve presented above are presented as rate changes in Table 19.

This growth rates are the values obtained from the Baranyi's model using DMFit add-in for excel. This rate change parameter describes in this case, the tendency of each strain to survive or die at each temperature in talcum powder. The time needed to observe a log reduction was calculated using the change rate. Although some variability can be observed between time points, in general the patterns are considered stable over time.

Then, the time needed to reduce 1 log was > 20 weeks for all temperatures and microorganisms.

The fitted curves obtained from DMFit are shown in Figures 38-40. Blue dots represent the experimental data and the red and continuous lines represent the fitted curve using the primary level Baranyi's model. Due to the pattern obtained from almost all curves, the reduction was considered a linear estimation. The only exception of this consideration was the SRSS curve at -18 °C (Figure 45c). The estimation of the survival/death rate for Figure 45c yielded a positive and high survival value that could have been considered as an actual growth rate. As bacterial growth cannot occur at -18 °C and at 0.45 a_w, the estimation of the rate was corrected to avoid the calculation of the exponential rate in the model.

Table 19 Growth rates of SRSS, non-SRSS, and *P. faecium* in talcum powder at different temperatures

Bacterial cocktail	Temperature (°C)	Time to reach 1 log reduction (Weeks)	Rate change (log CFU/g per week)	SD[a]	
SRSS	25	> 24.0	-0.0190	0.0098	Figure 23
	4	> 20.0	-0.0230	0.0092	
	-18	> 24.0	0.0175	0.0158	
Non-SRSS	25	> 24.0	-0.0330	0.0151	Figure 22
	4	> 20.0	-0.0250	0.0266	
	-18	> 24.0	0.0322	0.0222	
P. faecium	25	> 24.0	-0.0080	0.0103	Figure 24
	4	> 20.0	0.0158	0.0295	
	-18	> 24.0	0.0072	0.0103	

[a]SD, standard deviation of the fitted model from DMFit

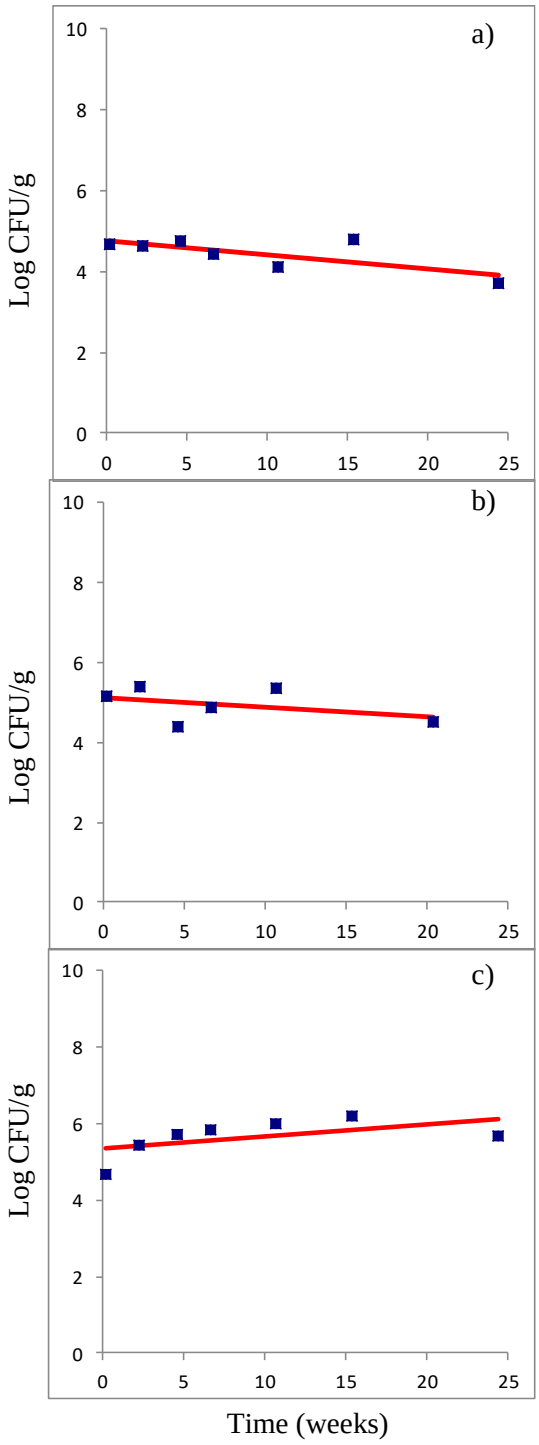


Figure 45 Survival of non-spice-related *Salmonella* strains in talcum powder stored at a) 25 °C, b) 4 °C, and c) -18 °C.

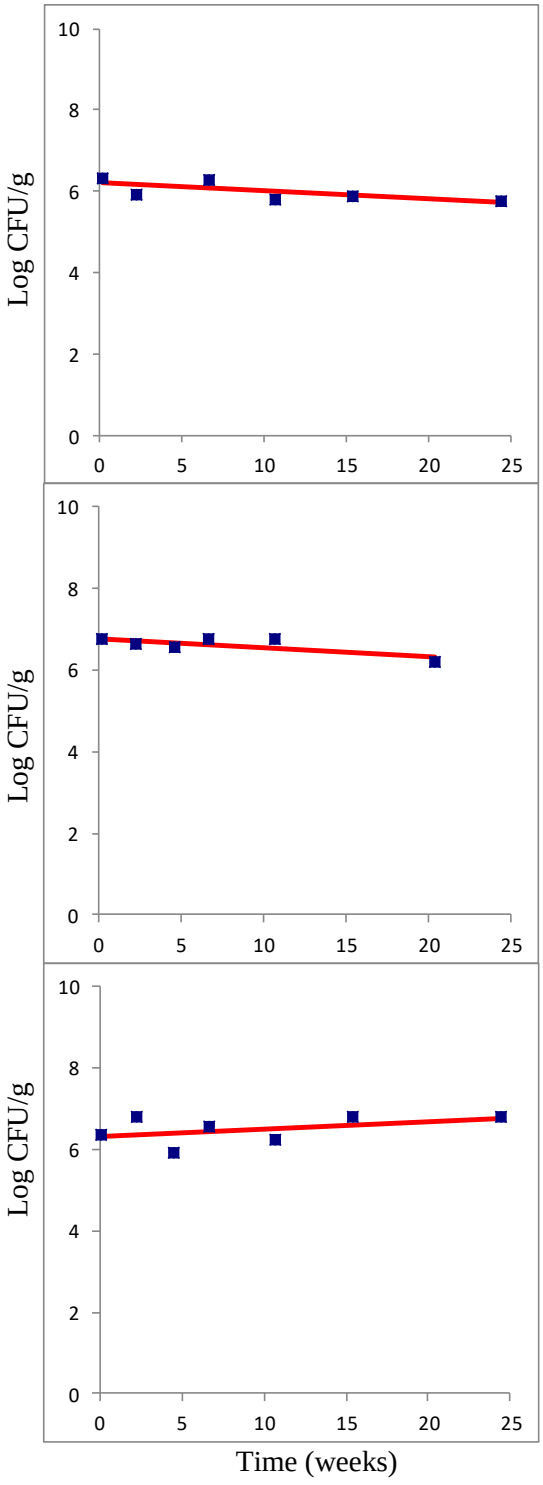


Figure 46 Survival of spice-related *Salmonella* strains in talcum powder stored at a) 25 °C, b) 4 °C, and c) -18 °C.

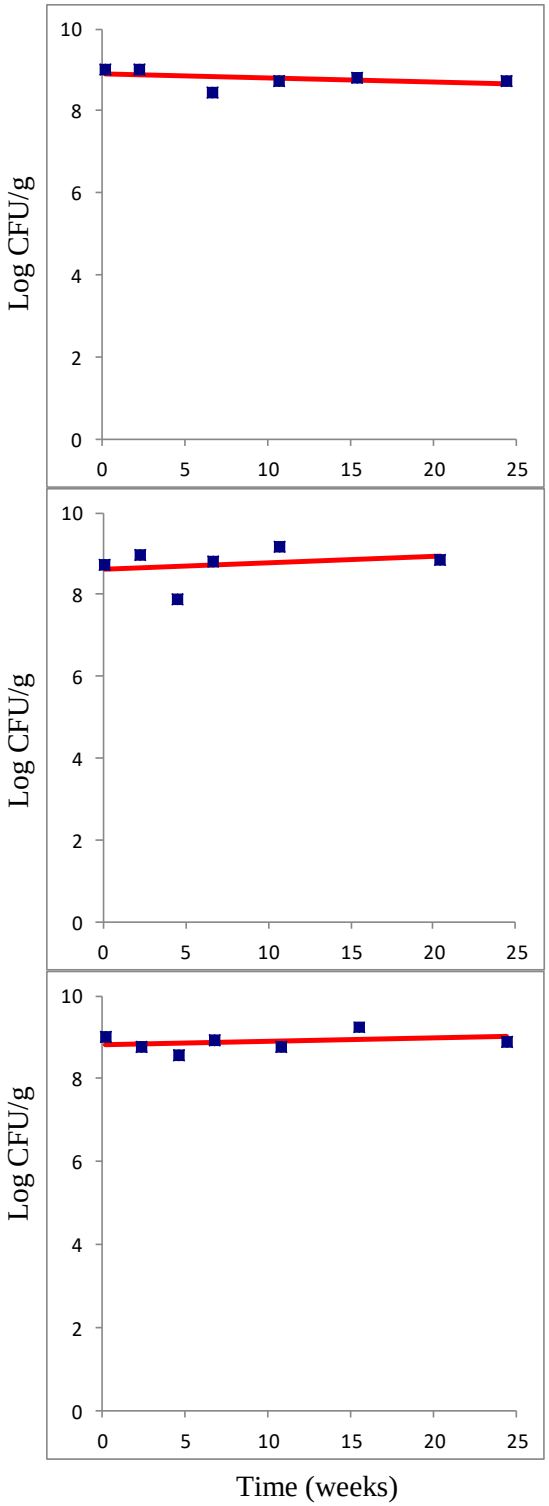


Figure 47 Survival of *P. faecium* in talcum powder stored at a) 25 °C, b) 4 °C, and c) -18 °C.

4.4.2.2 Survival in onion powder

Reductions of *Salmonella* and *P. faecium* were more prompt to occur in onion powder than in talcum powder, and at 25 °C than at cold temperatures.

As previously mentioned, the highest reduction of SRSS occurred at 25 °C. The overall reduction at 25 °C was > 4.7 logs and non-detectable numbers were reported by the end of the study (*i.e.* < 3 log CFU/g).

SRSS counts throughout the 20 weeks at 4 °C slightly decreased in 1 log over the storage period. Although the differences between the concentration in the 1st and 20th week are significant ($P < 0.05$), for practical purposes, the inoculated onion powder, or starter, can still be mixed with new batches of onion powder in a ratio 1:10 w/w and render at least 6 log CFU/g within the 20-week window at 4 °C.

Considering that the SRSS concentration varied in ± 0.1 log at -18 °C during the 24 weeks of storage and no significant reductions were observed ($P > 0.05$), the survival of SRSS was considered stable.

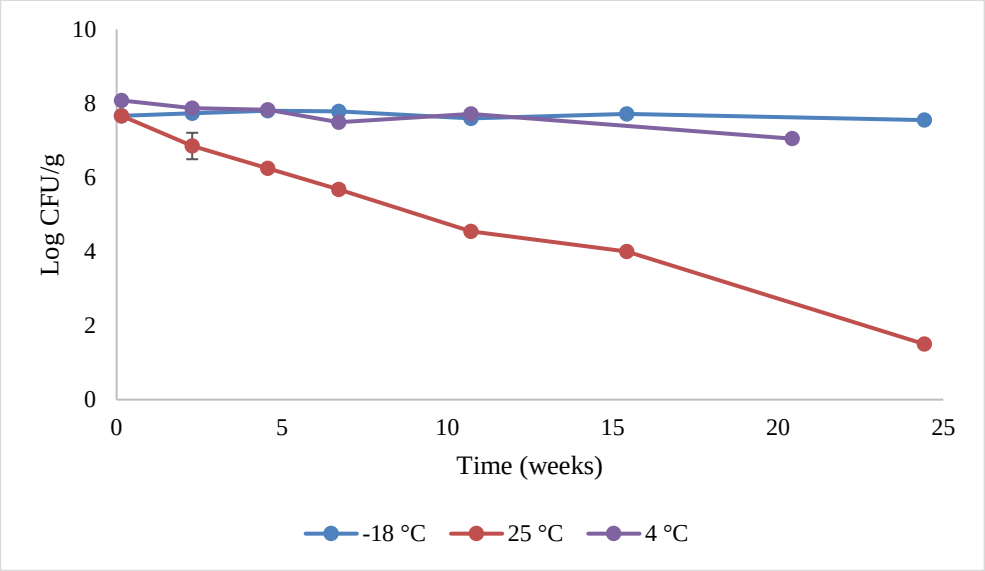


Figure 48 Survival of spice-related *Salmonella* strains inoculated in onion powder stored at 25, 4, and -18 °C

The survival of non-SRSS and SRSS presented similar survival patterns in onion powder at the 3 temperatures.

Reductions at 25 °C were more evident and non-detectable levels were reached at the 24th week of storage. The first log reduction of the non-SRSS occurred at the 2nd week and the overall reduction was > 4.6 logs.

At 4 °C, populations of non-SRSS remained constant until the 11th week and a log reduction was registered by the 20th week (Figure 49).

At -18 °C, the non-SRSS load remained constant during the length of the study with an average of 7.6 ± 0.1 log CFU/g. Although there was a reduction of 1 log at the 2nd week of storage, this was considered a technical laboratory error since the later time points showed a constant concentration.

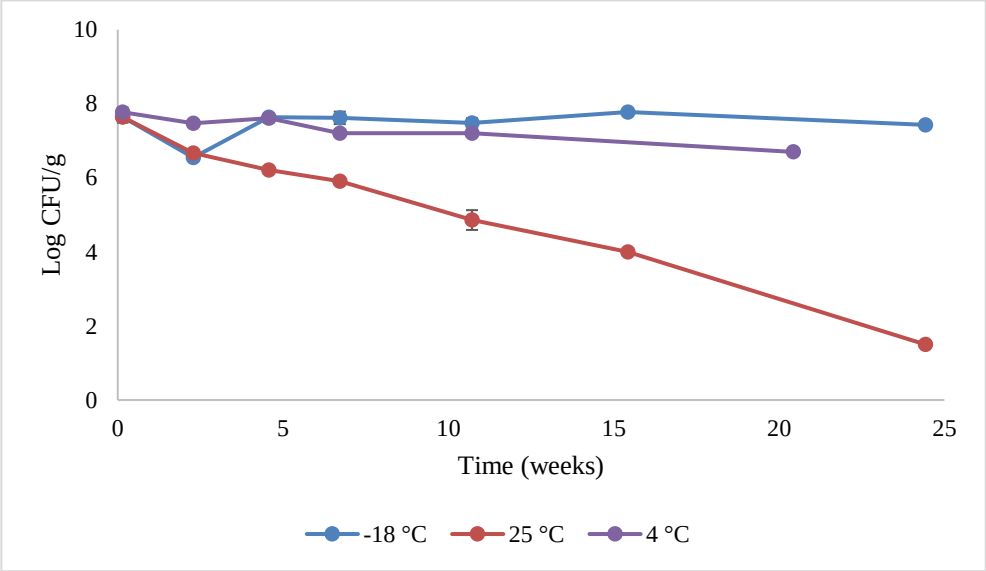


Figure 49 Survival of non- spice-related *Salmonella* strains inoculated in onion powder stored at 25, 4, and -18 °C

Pediococcus faecium counts started declining after the 2nd week at 25 °C and reached 6.5 log CFU/g at the 24th week. The total reduction was 2.4 logs by the end of the storage period at 25 °C. In contrast, *P. faecium* survival's patterns were stable at cold temperatures. In fact, no significant reductions were detected at 4 and -18 °C ($P > 0.05$) (Figure 50).

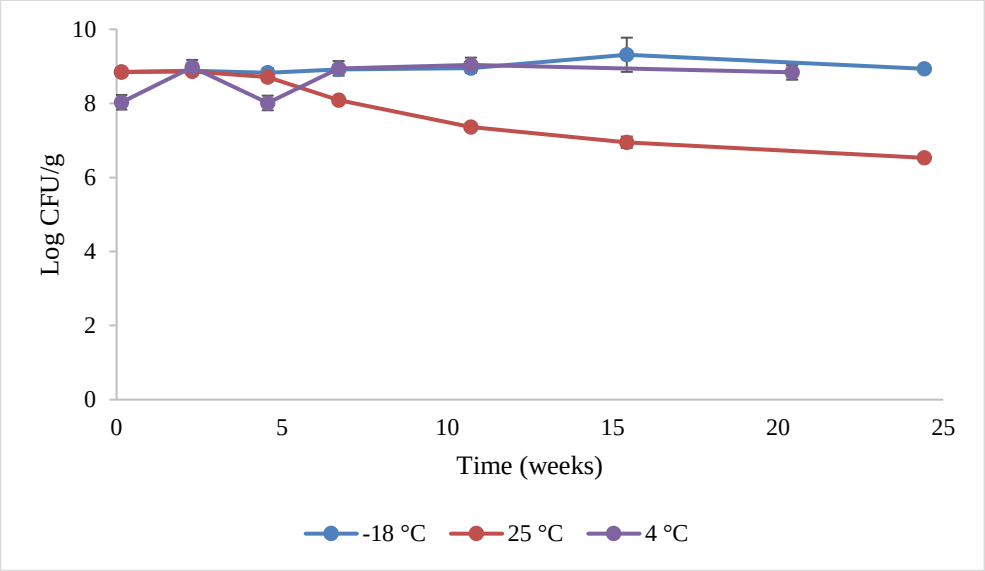


Figure 50 Survival of *P. faecium* inoculated in onion powder stored at 25, 4, and -18 °C

The parameters given by fitting the data points in DMFit are showed in Table 20. The time required to reduce 1 log were calculated using the rate changes. As described above, storing the inoculated powder at 25 °C had a higher impact in the survival of the microorganisms (Table 20). Then, the time needed to reduce 1 log of *Salmonella* was ~ 4 weeks and for *Pediococcus* was 9 weeks while storing at 4 and -18 °C maintained reductions < 1 log for at least 20 and 24 weeks for all microorganisms.

Table 20 Growth rates of *Salmonella* and *P. faecium* inoculated in onion powder and stored at 25, 4 and -18 °C

Onion powder	Temperature (°C)	Time to reach 1 log reduction (Weeks)	Growth rate (log CFU/g per week)	SD[a]	
SRSS	25	4.1	-0.2430	0.0112	Figure 25
	4	20.0	-0.0458	0.0094	
	-18	> 24.0	-0.0103	0.0044	
Non-SRSS	25	3.7	-0.2732	0.0226	Figure 26
	4	20.0	-0.0495	0.0078	
	-18	> 24.0	0.0117	0.0212	
P. faecium	25	9.1	-0.1103	0.0134	Figure 27
	4	> 20.0	0.0325	0.0315	
	-18	>24.0	0.0082	0.0079	

[a]SD, standard deviation of the fitted model from DMFit

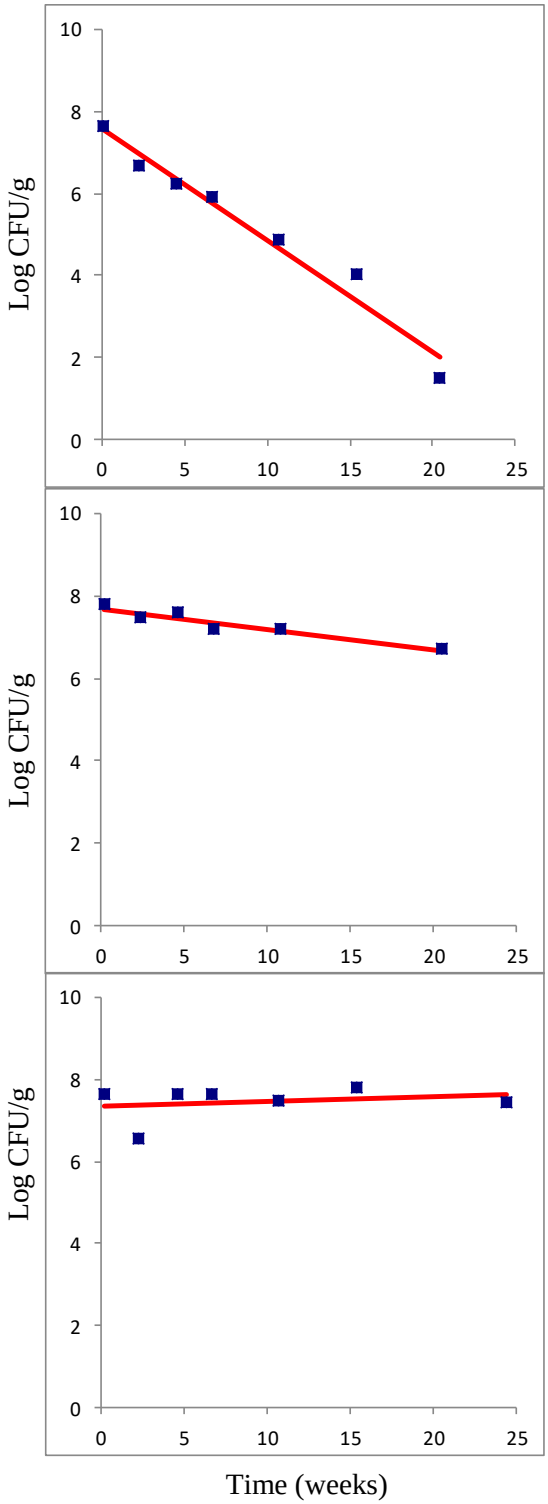


Figure 51 Survival of non- spice-related *Salmonella* strains in onion powder stored at a) 25 °C, b) 4 °C, and c) -18 °C.

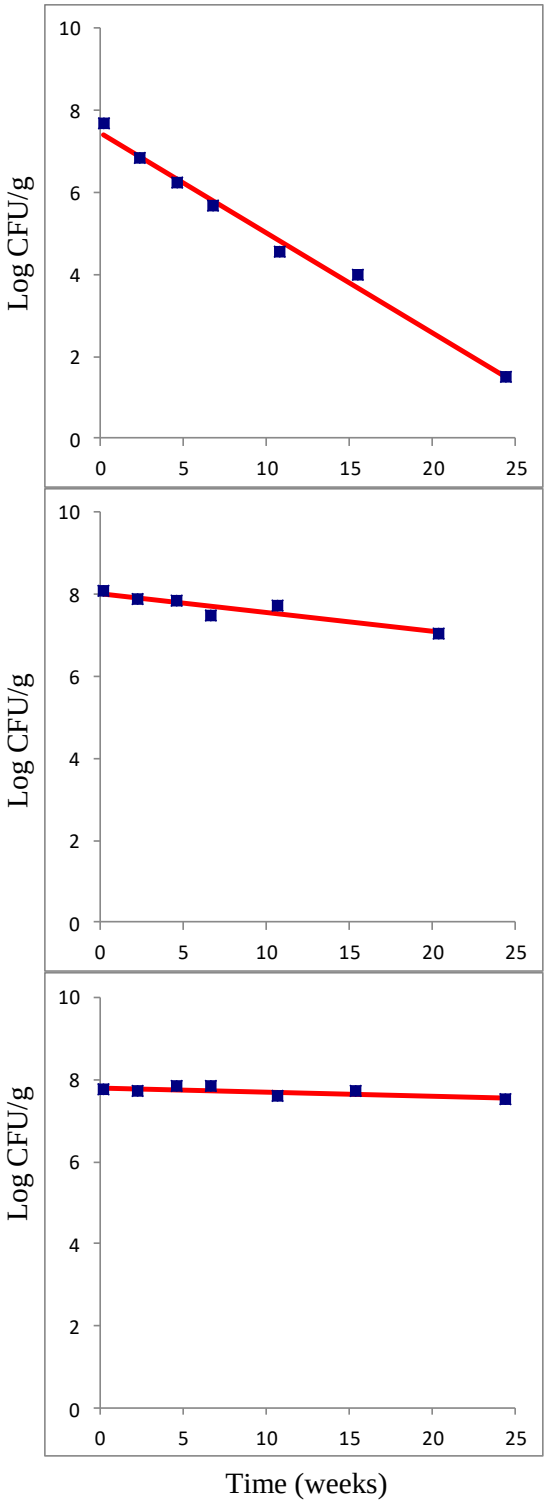


Figure 52 Survival of spice-related *Salmonella* strains in onion powder stored at a) 25 °C, b) 4 °C, and c) -18 °C.

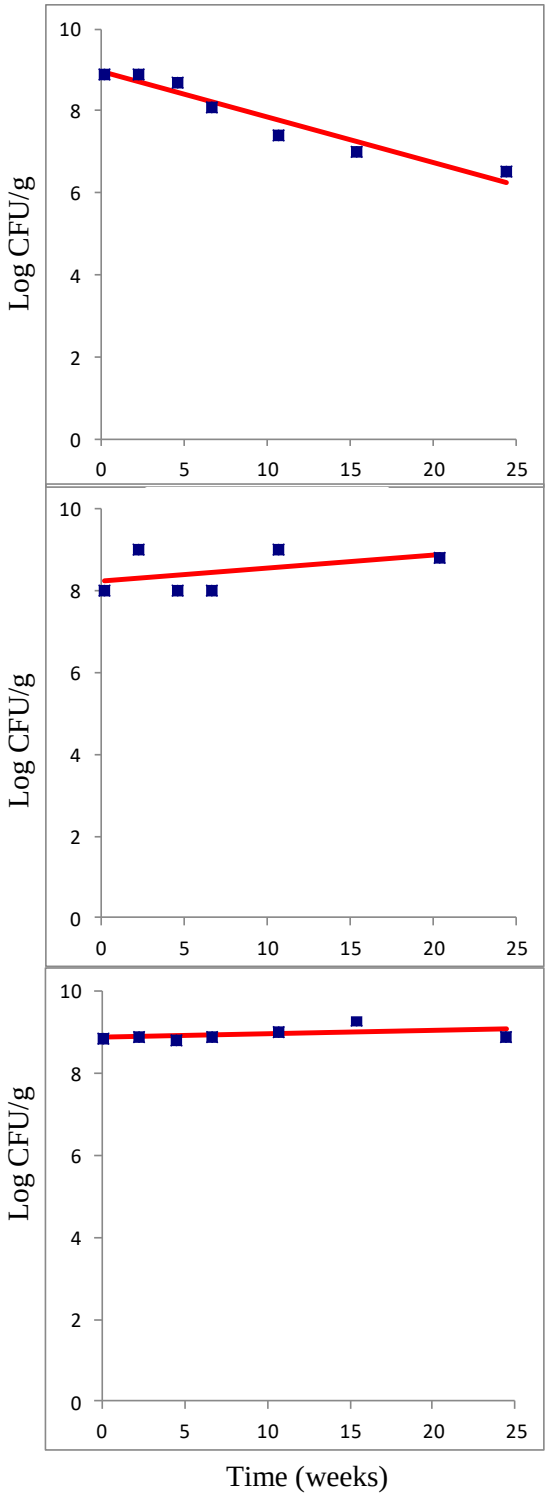


Figure 53 Survival of *P. faecium* in onion powder stored at a) 25 °C, b) 4 °C, and c) -18 °C.

As mentioned in Section 4.3.5 (Table 16) the *Salmonella* reduction was < 1 Log CFU/g when desiccating inoculated onion powder. Indeed, it is noticeable that the difference between the reductions of the non-SRSS and the SRSS was small (0.1 log) (Table 16). Higher differences in the reduction of both groups of *Salmonella* cocktails were observed in the silica, talcum, and oregano. However, the *Salmonella* reduction patterns in onion powder were greater than in the other 3 matrices (Figures 27-32, 40, 41, 46, and 47). Such low stability could have been the result of the exposure of *Salmonella* to the antimicrobial compounds found in onion (*i.e.* organosulfur compounds, phenolic compounds, protocatechuic acid, and catechol) *(11, 15, 48, 139)*. In fact, the FDA procedures for isolation of *Salmonella* from onion require the addition of K_2SO_3 to inhibit the antimicrobial compounds *(53)*. A hypothesis to explain the higher recovery of *Salmonella* at the beginning of the stability study is that as the desiccation of the inoculated onion powder was performed at 25 °C for 12 h, then, the effect of the antimicrobial compounds was not noticeable than when stored for a longer period of time. A similar pattern was observed in non-VHTO when stored over time. Oregano was dried at 35 °C and it was possible that the antimicrobial compounds vaporized faster than if drying at 25 °C as for onion powder. Although the oregano's essential oil strongly impacted the initial survivability of *Salmonella* after desiccation, the reduction of the cells over time was faster when compared with silica and talcum.

5 CONCLUSIONS

The development of inoculation procedures for oregano, onion powder, and talcum powder will serve as a base for inoculation of other spices or even other low-moisture foods. Therefore, these procedures represent a potential solution for inoculation of food products that have not been validated properly, precisely due to the lack of inoculation procedures.

As demonstrated on dry oregano, a small portion of the same spice may be pre-treated with heat under vacuum conditions to remove essential oils that have antimicrobial activity, and then using as dry vehicle for inoculating larger amounts of product. This would avoid the use of foreign materials such as silica or talcum powder as inoculum vehicles, as recommended by some authors. These procedures can be used as reference for inoculation of spices during challenge studies.

The results of this study showed the importance of selecting suitable organisms for challenge studies, including the selection of an adequate surrogate and, if the study involves inoculation of pathogens, the selection of adequate bacterial strains. In this study, *Salmonella* strains associated with spices were more resistant to the air-desiccation processes (*i.e.* at 35 °C/12 h) than *Salmonella* strains that were not associated with spices. However, the survival of these non-spice-related *Salmonella* strains was not substantially affected when inoculated and desiccated in onion powder at 25 °C (not air-drying). This highlights that the type of desiccation, the food matrix, and final a_w played a major role in the survival of pathogenic cells, even if they were not previously exposed to low a_w environments.

Pediococcus faecium showed high survivability after inoculation and desiccation of oregano, onion powder, and inert powders (silica and talcum powder). It also maintained stable populations over time when stored at different temperatures. The survival patterns of *Salmonella* and *P. faecium* during storage were similar, showing a greater reduction at room temperature, and were more stable at cold temperatures (4 °C and -18 °C). The best temperature for storage was -18 °C for both microorganisms and all the matrices tested.

There were differences in the stability between the 2 *Salmonella* groups*;* the SRSS and non-SRSS, when stored at the 3 different temperatures. However, the survivability at -18 °C was more stable for both groups of *Salmonella* strains.

Storing the inoculated matrices at low temperatures (≤ 4 °C) maintained the concentration of microorganisms stable. This stability is especially important for *P. faecium* since inoculated ready-to-use inert powders can be stored for at least 6 months. However, more studies need to be performed to test the suitability of *P. faecium* as a surrogate when stored for a long period of time at such temperatures.

The advantage of using dry inocula is that inoculation of spices can be done right before performing the challenge studies without the need of drying them afterwards, this feature becomes important for the industry. However, more research is needed to determine if using these dry inert powers shift the D-values of the microorganism obtained by specific control measures.

Finally, talcum powder showed to be a better dry vehicle of microorganisms for inoculation of oregano than silica powder. Silica powder is more difficult to handle since

it was easily electrostatically charged which resulted in a lack of control when trying to weight it and transferred it from one container to another.

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