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DETERMINATION OF THERMAL DEATH KINETIC PARAMETERS OF *NEOSARTORYA* *FISCHER* IN FRULACT PREPARATIONS BASED ON TEMPERATURE AND WATER ACTIVITY VARIATION

MATILDE MARIA DE CARVALHO LOPES
MASTER THESIS IN CHEMICAL ENGINEERING
PRESENTED TO THE FACULTY OF ENGINEERING
OF THE UNIVERSITY OF PORTO

Master in Chemical Engineering

**Determination of thermal death kinetic
parameters of *Neosartorya fischeri* in FRULACT
preparations based on temperature and water
activity variation**

Master dissertation

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Matilde Maria de Carvalho Lopes

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held in

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Supervisor at FEUP: Prof. Manuel Simões

Coordinator at Frulact: Eng. Cristina Martins Rodrigues



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Abstract

The contamination of food by heat-resistant filamentous fungi poses a significant concern for food companies. These organisms are characterized by the formation of ascospores, which are extremely heat-resistant spores capable of surviving the temperatures applied in industrial pasteurization processes, thereby compromising the food quality and safety. *Neosartorya fischeri* is one of the primary thermoresistant fungi known to survive pasteurization. Certain strains can produce mycotoxins and fumitremorgins, which are natural compounds known to be toxic and capable of causing dangerous foodborne illnesses to those who consume them. The objective of this dissertation was to assess the thermal resistance of *N. fischeri* in peach preparations, both sugared (42.8 °Brix, pH 3.85, a_w 0.94) and sweetened (6.6 °Brix, pH 3.75, a_w 0.99), under the influence of different production (30 °C/1 h, 50 °C/1 h, 30 °C/3 h, 50 °C/3 h) and pasteurization (85 °C/5 min, 90 °C/5 min, and 93 °C/10 min) time/temperature binomials. The thermal death kinetic parameters, D (min) and Z (°C), were determined while considering the nutritional characteristics of the matrix, water activity (a_w), sugar concentration, and pH of the preparation. The highest D -value obtained was 63.3 min (for the sugared preparation at 85 °C with the production binomial of 50 °C/3 h), while the lowest value recorded was 6.4 min (for the sweetened preparation at 93 °C with the production binomial of 30 °C/1 h). The results revealed a trend of decreasing D -values with increasing pasteurization temperature. Conversely, an increase in production time and temperature led to an augmentation of the D -value, with the increase in time (from 1 to 3 h) exerting a more pronounced effect on this parameter than an increase in temperature (from 30 °C to 50 °C). Approximately 75 % of the D -values obtained for the peach preparation exhibited increased thermal resistance proportional to the rise in °Brix/sugar concentration. The influence of pH and ascospore age on *N. fischeri* resistance was not determined; however, a discussion of their potential influence is presented. The Z -values obtained ranged from 13.7 to 10.8 °C for the sugared preparation and from 16.3 to 12.4 °C for the sweetened preparation. Overall, a tendency was observed for the Z -value to decrease when the D -values for an experiment were higher. The sterilization (F) values (4 log reduction) for the peach preparations ranged from 22 to 39 min. Considering that a typical thermal treatment applied in the Food Industry varies between 90-95 °C for 2-5 min, it is concluded that such treatments may not be sufficient to ensure a 4-log reduction in the preparations studied. Consequently, the effect of the addition of preservatives on sterilization time is further discussed.

Keywords:

ascospores, *Neosartorya fischeri*, thermal resistance, fruit preparations

Resumo

A contaminação de alimentos por fungos filamentosos termorresistentes representa uma grande preocupação para a indústria alimentar. Estes organismos são caracterizados pela formação de ascósporos, que são esporos extremamente resistentes ao calor, capazes de sobreviver às temperaturas aplicadas nos processos de pasteurização utilizados a nível industrial, comprometendo assim a qualidade e segurança dos alimentos. *Neosartorya fischeri* é um dos principais fungos termorresistentes, conhecido por sobreviver a tratamentos de pasteurização. Estirpes específicas podem produzir micotoxinas e fumitremorginas, que são compostos naturais conhecidos por serem tóxicos e capazes de causar doenças alimentares perigosas a quem os ingere. O objetivo desta dissertação foi avaliar a resistência térmica de *N. fischeri* em preparados de pêssago, tanto açucarados (42.8 °Brix, pH 3.85, a_w 0.94) quanto edulcorados (6.6 °Brix, pH 3.75, a_w 0.99), sob a influência de diferentes binómios tempo/temperatura de produção (30 °C/1 h, 50 °C/1 h, 30 °C/3 h, 50 °C/3 h) e pasteurização (85 °C/5 min, 90 °C/5 min, and 93 °C/10 min). Os parâmetros cinéticos de morte térmica, D (min) e Z (°C), foram determinados tendo em conta as características nutricionais da matriz, atividade de água (a_w), concentração de açúcares e pH da preparação. O valor máximo de D obtido foi de 63.3 min (para o preparado açucarado a 85 °C com o binómio de produção de 50 °C/3 h), enquanto o valor mais baixo registado foi de 6.4 min (para o preparado edulcorado a 93 °C com o binómio de produção de 30 °C/1 h). Os resultados revelaram uma tendência de diminuição dos valores de D com o aumento da temperatura de pasteurização. Pelo contrário, um aumento no tempo e temperatura de produção resultou num aumento do valor de D , sendo que o aumento do tempo (de 1 para 3 h) teve um efeito mais pronunciado neste parâmetro do que o aumento da temperatura (de 30 °C para 50 °C). Aproximadamente 75 % dos valores de D obtidos para o preparado de pêssago apresentaram um aumento da resistência térmica proporcional ao aumento do valor de °Brix/concentração de açúcares. A influência do pH e da idade dos ascósporos na resistência de *N. fischeri* não foi determinada; no entanto, é discutida a sua potencial influência. Os valores de Z obtidos variaram entre 13.7 e 10.8 °C para o preparado açucarado e entre 16.3 e 12.4 °C para o edulcorado. No geral, observou-se uma tendência de diminuição do valor de Z quando os valores de D para uma experiência eram mais elevados. Os valores (F) de esterilização (redução de 4 log) para os preparados de pêssago variaram de 22 a 39 min. Considerando que o típico tratamento térmico aplicado na Indústria Alimentar varia entre 90-95 °C durante 2-5 min, conclui-se que tais tratamentos podem não ser suficientes para garantir uma redução de 4 log nos preparados estudados. Consequentemente, é discutido o efeito da adição de conservantes no tempo de esterilização.

Palavras-chave:

ascósporos, *Neosartorya fischeri*, resistência térmica, preparados de frutas

Declaration

I hereby declare, under word of honour, that this work is original and that all non-original contributions is indicated and due reference is given to the author and source

Matilde Lopes

22/07/2023

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Notation and Glossary

a_w	Water activity	
°Brix	Degree Brix (scale for measuring the amount of sugar)	1 g/100 g H ₂ O
D	Decimal reduction time	min
F	Sterilization value	min
N_0	Initial population of microorganisms	UFC/g
N	Final population of microorganisms	UFC/g
P_0	Partial vapor pressure of pure water	Pa
P	Partial vapor pressure of water in food	Pa
T	Temperature	°C
Z	Temperature variation required to change the D -value by one log	°C

Greek Letters

γ	Number of decimal reductions
σ	Standard deviation

List of Acronyms

ASAE	Autoridade de Segurança Alimentar e Económica
BPW	Buffered Peptone Water
DEPC	Diethylpyrocarbonate
EFSA	European Food Safety Authority
FDA	Food and Drug Agency
GHPs	Good Hygiene Practices
GMPs	Good Manufacturing Practices
HACCP	Hazard Analysis and Critical Control Points
HPP	High-Pressure Processing
HPTP	High-Pressure Thermal Processing
HRMs	Heat-resistant molds
PDA	Potato Dextrose Agar
PEF	Pulsed Electric Field
RM	Raw Material
TDT	Thermal Death Time
UHT	Ultra High-Temperature
UV	Ultraviolet
WHO	World Health Organization

1 Introduction

1.1 Background and presentation of the work

Fruit preparations and their derivatives are the most susceptible foods to deterioration by heat-resistant fungi, a problem documented in many countries [1]. Most fungi have limited thermal resistance and the application of heat easily destroys them. However, heat-resistant molds are characterized by the formation of ascospores (sexual reproduction), which are known as the most stress-resistant eukaryotic cells [2].

The most frequent preservation methods of fruit-based products are pasteurization and the Ultra High-Temperature (UHT) process. However, this use of high temperatures can activate dormant ascospores which will germinate, outgrow, and, consequently, spoil the food products, resulting in an economic loss [2]. Among the thermoresistant molds known to survive pasteurization treatments is *Neosartorya fischeri* [3]. Certain strains of *N. fischeri* can produce mycotoxins and fumitremorgins, which has sparked concern about potential health implications related to the presence of these biomolecules. In this way, this mold is not only of concern to food spoilage, but also to food safety aspects [3]. It is of high importance to be able to eliminate these microorganisms from the food products, which is attempted by pasteurization at Frulact. Due to the limited availability of bibliographic data concerning the heat resistance of microorganisms in fruit, the assumption of a "worst case scenario" is made, resulting in an over-processing of Frulact preparations. The estimation of the appropriate time/temperature pasteurization binomials becomes feasible by obtaining knowledge of the thermal death kinetic parameters in Frulact preparations under real conditions. This, in turn, ensures the preservation of organoleptic characteristics and the reduction of energy costs inherent in the production process.

The main goal of this thesis was to evaluate the effectiveness of different pasteurization time/temperature binomials in the inactivation of spores of *N. fischeri* when inoculated in different fruit preparations, considering their production process. This method of predictive biology uses mathematical models to study the behavior of microorganisms in the various stages of the production process, allowing to know how much they may interfere with their shelf life [4].

The thermal death kinetics parameters, D (min) and Z ($^{\circ}\text{C}$), were determined based on the time/temperature binomials previously applied in the production process and based on the nutritional characteristics of the matrix, the a_w (water activity), the concentration of sugars and the pH of the preparation. D stands for decimal reduction time and it represents the time

required at a specific temperature to reduce the population of microorganisms by 90 %. On the other hand, the *Z*-value represents the temperature variation required to change the *D*-value by one logarithmic unit [5].

Based on the data obtained, the validation of the minimum sterilization (*F*) time/temperature binomials on an industrial scale was necessary for reducing energy costs, ensuring food safety, quality preservation, and avoiding the over-processing of fruit preparations.

1.2 Presentation of the company

Frulact is a food company that is currently positioned as a top-ranked innovative company in the supply of added-value ingredients for the food and beverage industries dedicated to fruit preparations, plant-based ingredients, and other specialty ingredients. The company is organized into three different business units that answer to different market demands: Frulact, Frusenses, and Oatvita [6].

Frulact was first founded in 1987 by Arménio Miranda. Due to its rapid success, in 1992, the Miranda family proceeded with the construction of an industrial unit in Maia, increasing the production capacity and boosting demand for new international markets. Later, the first international unit was inaugurated in Morocco to supply global and local brands linked to the food industry.

In 2006, a larger factory was opened in Tortosendo, Covilhã, and in 2009, a new unit was acquired in southeastern France. In 2010, Frulact launched the FRUTECH project - Frulact's Center of Research, Development, and Innovation in Maia, Portugal, to boost innovation. Between 2011 and 2013, there was the creation of Frulact South Africa, the implementation of a production unit in Pretoria, and another in Morocco. Also in 2013, Frusenses emerged as a reinforcement of Frulact's strategic orientation of expanding its offer, thus entering new segments such as food flavorings. By the year 2017, Frulact opened a unit in Kingston, Canada, expanding its international presence to a total of 3 continents [6].

In response to customer demand for alternatives to animal by-products, in 2018 Frulact acquired Oatvita which is a producer of vegetable-based ingredients, namely alternatives to dairy products. Finally, in 2020, Frulact was acquired by Ardian, a world leading private investment company.

Over the years, Frulact has won several international awards, being, in 2019, elected International Company of the Year at the SAPCC - ABSA Business Excellence Awards. Among its customers are renowned brands such as Nestlé, Mimosa, Danone, Becel, Olá, and McDonald's.

We can therefore say that, in a competitive market such as the food industry, Frulact is a company at the forefront of innovation reaching turnovers of over 260 million euros [6].

1.3 Contribution of the author to the work

The work described in this report was carried out by me, Matilde Lopes, supported by the supervision and guidance of prof. Manuel Simões and Eng. Cristina Rodrigues.

1.4 Organization of the dissertation

The dissertation is organized into six chapters:

- Chapter 1: provides the introduction, where the background and presentation of the work is described, including the main objectives, and the presentation of the company;
- Chapter 2: consists of the state of the art, where the theoretical foundations are addressed to properly contextualize the topic under study, providing details on the research and data obtained previously by diverse researchers;
- Chapter 3: describes the materials and methods used throughout the experimental work;
- Chapter 4: the results obtained in the project are presented and discussed;
- Chapter 5: provides a concise description of the main conclusions of the work developed;
- Chapter 6: an assessment of the work done is presented, as well as research needs.

2 Context and State of the art

2.1 Food Safety and Hygiene

Food is one of the bases of life but, if contaminated, it can cause illness and, in extreme cases, death. The primary purpose of food hygiene is then to ensure the safety of food from production to consumption, by eliminating/reducing the risk of exposure to foodborne illnesses. These illnesses are a global public health concern that can result in substantial costs to individuals, the food industry, and the economy [7]. According to the World Health Organization (WHO), it is estimated that every 1 in 10 people in the world gets ill after ingesting contaminated food, resulting in 420.000 deaths per year [8]. Food becomes contaminated when unwanted materials are found in the product, affecting its quality or the process [7]. Several factors contribute to the contamination of food, more specifically the presence of hazards. Hazard is defined as a “biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect” [7]. These contaminations can enter the food chain at any stage, from ingredient supplies by producers to transport and storage operators, by inappropriate processing or handling [9]. Briefly, biological contamination occurs when living organisms or the substances they produce contaminate food, compromising its value and safety. Six types of microorganisms can cause foodborne illness, such as bacteria, parasites, viruses, protozoa, fungi, and prions. These illnesses can range from gastroenteritis with soft symptoms such as nausea, abdominal pain, diarrhea, and fever, to illnesses caused by enteric viruses such as hepatitis A and E [9, 10]. Some examples of the most common pathogens are bacterial species of *Staphylococcus*, *Salmonella*, *Clostridium*, *Bacillus*, and *Escherichia coli* [10]. There can also happen chemical contamination when a food product is exposed to chemicals that can cause harm through toxic effects (such as food additives, heavy metals, pesticides, cleaning chemicals), or physical when the items can cause harm through direct choking or injury (such as metal, glass, plastics, stones, etc) [7]. In this work, biological contamination by fungi is the focus.

Preventing food contamination is essential to ensure food safety and prevent foodborne illnesses. Food safety embraces anything in the processing, preparation, or handling of food to ensure it is safe to eat [11]. Therefore, adequate control is needed throughout the food chain to maintain its safety, either by preventing the entry of hazards into the process, destroying them, or reducing contamination [7].

To maintain food safety, it is essential to implement good practices throughout the food chain. Good Manufacturing Practices (GMPs) and Good Hygiene Practices (GHPs) are guidelines and

procedures used to maintain a clean and safe environment during food production and processing. GMPs guidelines cover a wide range of areas, including staff qualifications and training, building and facilities, cleanliness and sanitation, quality control procedures, and valid documentation. GHPs, on the other hand, are prerequisite measures that focus more specifically on ensuring good hygiene practices in manufacturing facilities. GHPs guidelines cover areas such as staff personal hygiene and training, facility and equipment sanitation, and pest control [7]. Additionally, food businesses should also implement Hazard Analysis and Critical Control Points (HACCP) practices, which are a systematic approach to identifying, evaluating, and controlling food safety hazards [12]. HACCP is based upon the use of control practices in certain production steps where there is a greater probability of occurrence of health hazards [7]. This is composed of seven main principles that are applied to food-related services, which have been accepted by government agencies, trade associations, and the food industry around the world [12]. In addition to these measures, each country has a regulatory agency or organization that enforces food safety standards. In the USA, the Food and Drug Agency (FDA) is responsible for public health by ensuring the safety of the food supply [13]. The European Food Safety Authority (EFSA) is the primary agency responsible for food safety in the European Union and its member states, and in Portugal, this responsibility belongs to *Autoridade de Segurança Alimentar e Económica* (ASAE).

Food safety is a critical issue affecting everyone, from individuals to communities and the economy. It is essential to ensure that the food we eat is free from harmful substances and will not cause any harm to the population. Achieving food safety involves all stakeholders involved in the food chain and the implementation of good practices. By working together, foodborne illnesses can be prevented and a healthier and safer food supply for all is promoted.

2.2 Filamentous fungi

The kingdom of Fungi is one of the most diverse groups on Earth. Fungi can be either unicellular or multicellular organisms that possess a cell wall rich in glucans and chitin. They are organochemoheterotrophs, meaning that they obtain nutrients through the absorption of organic compounds as sources of energy and carbon [14, 15]. These eukaryotic organisms are present in all habitats, but most live in soil or on plant material (e.g. leaves and wood) rather than in water environments [16].

In general, fungi can grow as either yeast or filamentous fungi. Yeasts are spherical, oval, or cylindrical and reproduce by budding or fission, forming sexual states which are not enclosed in a fruiting body [17]. Filamentous fungi (or molds), however, are characterized by the formation of masses visible to the naked eye called mycelium, composed of long intertwined

filaments known as hyphae [14, 18]. For these microorganisms to multiply, the mycelium may fragment into several segments (spores) that can survive extreme conditions and grow into a new individual. Fungal spores are stabilized cells that can range from macroscopic sizes between 3 to 30 μm . They can be formed either sexually or asexually, depending on the species. Asexual spores, also known as conidia, are produced by one parent only (after mitosis without the involvement of meiosis) and are genetically identical to that parent [19]. Sexual spores, on the other hand, are formed through the fusion of two compatible nuclei of different mating types of hyphae [17]. This introduces genetic variation into a population of fungi and often occurs in response to adverse environmental conditions. These spores allow the distribution of fungi in space and time. This means that fungal cells travel by air, water, insects, animals, or other vehicles toward new possible areas for colonization and remain dormant awaiting conditions that are more appealing for growth [14, 16].

Many filamentous fungi are beneficial to ferment and ripening foods since they contribute to developing the desired characteristics for the product. However, some species of fungi growing on foods are a major constraint to food safety [20]. One main single cause of food spoilage is invasion by microorganisms such as molds, yeast, and bacteria. Most molds require less available moisture than yeast and bacteria to grow. Their optimal temperature for growth can range from 25 °C to 30 °C and in a vast range of pH values from 2 to 8.5, although the majority is favored by a low pH [18]. In case of mold spoilage, a furry growth covers the surface of the food, producing an unappealing smell. Fruit preparations are usually extremely prone to this spoilage because of their high levels of sugars and acidic pH. So, the main concern regarding foodborne fungi is their capability to produce a wide variety of secondary metabolites, including mycotoxins, that can contaminate food and are toxic and allergenic [21].

The great biodiversity of fungal species is reflected in their macroscopic characteristics, biological properties, varied enzymatic systems, and vast metabolic production. Some fungi present distinct characteristics when they are in the asexual (anamorphic fungi) and sexual stage (teleomorphic), so the identification of filamentous fungi by classical taxonomy and molecular biology remains difficult and for specialists only [14, 15].

2.2.1 Heat-resistant filamentous fungi

Most of the commonly occurring filamentous fungi present a limited resistance to temperature *i.e.* *Aspergillus*, *Penicillium*, *Fusarium*, *Mucor*, and *Rhizopus* spp. These molds are easily inactivated by the application of pasteurization processes such as heating for 10 min at 70 °C. However, some fungi are characterized as having high resistance to temperature and can survive heat treatments for longer than 30 min at 75 °C [22]. These are called heat-resistant molds (HRMs).

HRMs are characterized by the formation of ascospores which are extremely heat-resistant spores inside an ascus mother cell [2]. The most common examples of these heat-resistant fungi are *Neosartorya fischeri*, *Byssoschlamys nivea*, *Talaromyces flavus*, and *Eupenicillium* spp. [23]. The presence of the ascospores of these molds has been reported in soils and can easily contaminate fruits harvested from or close to the ground [24]. These fungi have been intensively studied due to their ability to survive temperatures normally employed in pasteurization treatments given to acidic foods such as fruit juices, purees, and juice-containing beverages [22]. The high temperatures employed in these treatments allow the ascospores to transit from a dormant state to a metabolically active state, after which they can germinate and grow [2, 23]. By surviving these treatments, these fungi can cause subsequent food spoilage at room temperature resulting in a grand economic loss. In addition to their high thermal tolerance, the ascospores of HRMs have a very high tolerance to adverse environmental conditions. They can survive under low pH, low oxygen (O₂), and high carbon dioxide (CO₂) atmospheres. Therefore, their presence can compromise the microbial stability of processed fruit products [2]. Some HRMs are known to produce various mycotoxins during their growth in fruit products [1]. These substances are poisonous to humans and other animals even if consumed in low doses, which means that quite small amounts of the compound can still represent significant health effects [25]. Toxicity commonly arises after ingestion (called mycotoxicosis), but dermal and inhalation exposure can also result in adverse effects. Some mycotoxins cause primarily acute and highly reversible effects, others, however, can cause irreversible organ damage. These natural products can also cause neoplasia (cancer) in humans and severe kidney and neurological diseases, hence are of major concern to public health [26].

2.2.2 Mycotoxins

Mycotoxins are naturally occurring compounds that can be produced by fungi as a means of defense against other organisms or to aid in their growth and survival. However, many mycotoxins are toxic [27]. Mycotoxins are present in foods such as grains, vegetables, fruits, milk, nuts, and meat, which can cause foodborne illnesses seriously harmful to human health [28]. These illnesses can range from acute poisoning to long-term effects such as immune deficiency and cancer [27]. Mycotoxins may invade the food products via many contamination pathways either before harvest, through seeds, soil, and air, or during the postharvest especially in storage [29].

Table 1 lists some of the major foodborne mycotoxins of public health concern, the producer fungi, the associated foods where they grow, and their toxic effects both in humans and animals.

Table 1. Mycotoxins produced by fungi and associated effects.

Mycotoxin	Producing fungi	Associated food	Effects/diseases	Ref.
Aflatoxins	<i>Aspergillus flavus</i> , <i>A. parasiticus</i> and <i>A. nomius</i>	Cereals, tree nuts, oilseeds, maize, dried fruits and spices	Abdominal pain, vomiting, diarrhea, carcinogenic, teratogenic, mutagenic, chronic hepatitis, nutritional disorders, death at high doses	[27] [28] [29] [30]
Ochratoxin A (OTA)	<i>Aspergillus ochraceus</i> , <i>A. carbonarius</i> , <i>A. niger</i> and <i>Penicillium verrucosum</i>	Cereals, cocoa, coffee, wine, beer, soybeans, spices, dried fruit, and meat	Kidney problems, immunosuppression, carcinogenic, neurotoxic, nephrotoxic	[29] [28] [30]
Fumonisin	<i>Fusarium verticillioides</i> , <i>F. proliferatum</i> and <i>Aspergillus niger</i>	Maize	Esophageal and liver cancer, may cause birth defects and idiopathic congestive heart disease	[28] [30]
Patulin	Some strains of <i>Penicillium</i> , <i>Aspergillus</i> and <i>Byssoschlamys nivea</i>	Fruits, seeds of plants	Nausea, lung congestion, epithelial cell degeneration, genotoxic, liver and kidney damage, toxic effect on intestinal barrier function, inhibitory effect on a variety of enzymes	[28] [29]
Citrinin	<i>Penicillium citrinum</i> , <i>P. verrucosum</i> and <i>Aspergillus ochraceus</i>	Fruits, herbs, beans, spices, and cereals	Kidney problems, yellow rice fever	[29]
Zearalenone	<i>Fusarium culmorum</i> , <i>F. graminearum</i> , <i>F. verticillioides</i> , <i>F. sporotrichioides</i> , <i>F. semitectum</i> , <i>F. equiseti</i> , and <i>F. oxysporum</i>	Cereal grains	Vomiting, nausea, diarrhea, genotoxic, strong embryological toxicity, strong estrogen activity (may lead to human high estrogen syndrome, female reproductive organ changes, cervical cancer, etc)	[28] [31]
Deoxynivalenol (DON)	<i>Fusarium culmorum</i> and <i>F. graminearum</i>	Maize, wheat, barley and oats	Acute gastroenteritis, interfere with protein synthesis	[28] [30]
Fumitremorgin (A, B, C)	<i>Neosartorya fischeri</i> , <i>Aspergillus caespitosus</i> and <i>A. fumigatus</i>		Sustained tremors, convulsions, and death in animals, tremors and headaches in humans	[4] [24] [32] [33]
Verruculogen	<i>Neosartorya fischeri</i> , some strains of <i>Penicillium</i> and <i>Aspergillus</i>		Severe tremors in animals	[32] [34]

2.2.3 Production and Activation of Ascospores

If fungi go through a long period of very low nutrient availability or with poor conditions that limit their development, they produce stabilized cells for survival without growth. Stabilization is characterized by the lowering of their metabolic activity, the absence of cellular extension, and the increase of stress resistance allowing the protection of their cell constituents and biomolecules. To overcome these adverse periods, fungi most notable survival strategy is the production of stabilized cells, commonly known as ascospores [35].

The formation of these ascospores is the main characteristic of the HRMs. The size, shape, and ornamentation of these ascospores vary within the type, species, and strain of microorganism, as well as the environmental conditions under which the spores are formed [36]. Ascospores are produced, in groups of eight, inside a bag-like structure called an ascus [37]. Once it reaches a mature state, the ascus ruptures and releases the ascospores. Asci of some of these molds are covered by a large fruiting body called the ascocarp. There are two different types of ascocarps: the cleistothecium (rigid, spherical, smooth-walled, and completely closed membrane) or gymnothecium (wall formed by a tangle of hyphae) [36]. Some species can even produce asci without the presence of an ascocarp, like the genus *Byssoschlamys* [4].

From the moment that the ascospores of heat-resistant fungi are formed, they become dormant over time, characterized by a state of low metabolic activity [4]. Dormancy allows spores to avoid germination under conditions that may not be optimal or could even be harmful. Under sub-optimal conditions, the spores may perish due to environmental stress or lack of nutrients. On the other hand, if spores stay dormant for too long, they may miss a valuable opportunity to proliferate and grow. As a result, the maintenance of dormancy is carefully regulated [4]. Eventually, these stabilized cells must regain growth after this period of dormancy. The triggers that stimulate ascospore germination from dormancy can vary depending on the strain of microorganism and environmental conditions, such as time, temperature, growth medium, or heating. For example, some ascomycetes require exposure to light or a specific temperature range to initiate germination, while others may require the presence of certain nutrients or chemical signals [36].

One of the most common ways to induce the activation of these ascospores is with a sub-lethal thermal treatment (usually a heat shock). This process is known as heat activation [23]. This activation is closely linked to the pasteurization processes used in the industry. The time/temperature binomials used in these treatments are not sufficient to eliminate these microorganisms. Instead, the heat shock will activate the ascospores present in food, which results in germination, outgrowth, and spoilage. It is necessary to find the ideal binomial that allows the elimination of the filamentous fungi in fruit products, instead of their activation.

2.2.4 *Neosartorya fischeri*

Neosartorya fischeri (anamorph *Aspergillus fischerianus*) is the only *Neosartorya* species able to cause spoilage in food [38]. It grows in damp environments, such as soil, decaying vegetation, and organic debris. The ascospores produced by this mold are frequently isolated from fruits that grow or are harvested from the ground, like apples, grapes, strawberries, and others. Ascospores of *N. fischeri* are highly thermoresistant and are often associated with the spoilage of pasteurized fruit products such as juices, purees, jellies, jams, and canned fruits [37].

N. fischeri can grow at temperatures between 10 and 52 °C, being the optimal temperature around 26-45 °C. Just like most fungi, they can resist extreme conditions, like oxygen levels as low as 0.1 % at 25 °C and a broad range of pH (3-8) [39]. The mycelium is white, dense, and has a granular appearance with a large development of cleistothecium (**Figure 1**). When this structure reaches the state of maturation, the ascus ruptures, and the ascospores are released from its interior. *N. fischeri* ascospores are ellipsoidal or oval, between 7 to 8 µm long, with two prominent longitudinal flanges. The ascospore walls may be smooth, irregularly roughened, or even spinose. This depends on the variety of *N. fischeri* specie that is present since there are three varieties: *N. fischeri* var. *fischeri*, *N. fischeri* var. *glabra*, and *N. fischeri* var. *spinosa*. Each species possesses differences in the ornamentation of its ascospores, and types of toxins/secondary metabolites produced [4, 40].

Some strains of *Neosartorya fischeri* are also capable of producing mycotoxins such as fumitremorgins, verruculogen, and terrain, which are major causes of public health concern [32]. These compounds can act on the central nervous system causing tremors, convulsions, and death in animals. Verruculogen is the most toxic of the three, producing side effects in pigs and sheep at a concentration as low as 5-10 mg/Kg of body weight when administered intravenously [4].

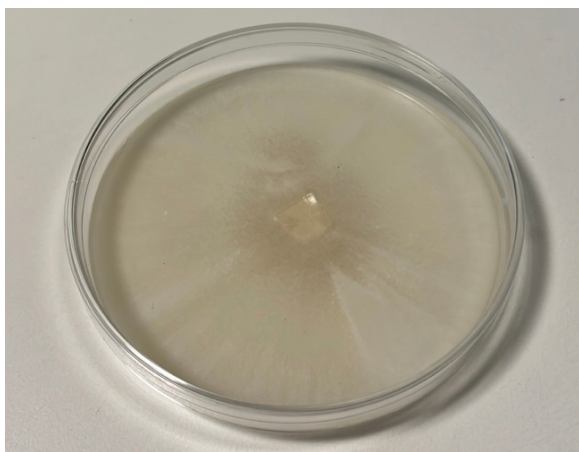


Figure 1. *Neosartorya fischeri* grown on Potato Dextrose Agar (PDA).

Neosartorya is the second most common of the *Aspergillus* teleomorphs found in foods [40]. For these reasons, it is important to study the resistance of this mold to heat processes to be able to eliminate it from food products. Ascospores of *N. fischeri*, along with those of the genus *Byssoschlamys* sp., are considered the most heat-resistant fungal spores known, as some studies have shown that thermal processes at 75 °C for 30 min did not inactivate these molds [32]. According to Jesenská et al. [41], *N. fischeri* can even survive temperatures of 70 °C for 120 min and 85 °C for 60 min.

2.3 Preservation methods

Preservation of food is a crucial process that restrains the development of microorganisms that are associated with food spoilage. The aim of these preservation methods include not only maintaining food taste, texture, flavor, quality, and nutritional value but also increasing its shelf-life and reducing the wastage of excess food [42].

2.3.1 Thermal processing

The most common method to inactivate microorganisms and enzymes for increasing the shelf life of fruit products is by thermal processing [43]. Heat is applied to food to kill or inactivate harmful microorganisms. By reducing the number of these contaminations, this thermal processing can extend the shelf life of foods, making them safer for longer periods and decreases the risk of foodborne diseases.

In the food industry, the pasteurization and sterilization processes are the most commonly employed. Sterilization is one of the most effective food preservation methods since it can provide near complete inactivation of microorganisms, including spores. This is achieved by applying high-intensity heat usually between 121 °C and 140 °C to food products. However, these high temperatures can lead to serious quality losses of the products [44]. These negative effects have motivated a great interest in the development of new technology that offer advantages of using low processing temperatures and energy consumption and retaining the nutritional and sensory properties of the food [43]. On the other hand, pasteurization is a relatively mild heat treatment, compared to heat sterilization, since food is only heated to below 100 °C. This causes smaller changes to the nutritional, functional, and sensory characteristics of most foods and it is the preferred method used by Frulact. However, even though the temperatures used are effective against *E. coli* and *Salmonella* they are not effective against ascospores of heat-resistant molds, becoming one of its biggest limitations [43]. These thermal processes are mainly based on the kinetics of the thermal death of microorganisms, being dependent on the combined action of time and temperature that is applied to the food products.

2.3.2 Examples of non-thermal processing

Non-thermal processing methods are food preservation techniques that aim to inactivate or control microorganisms without significantly raising the temperature of the food. Some examples of non-thermal processing are:

- High-pressure processing (HPP): involves subjecting food products to high hydrostatic pressure (usually at over 600 MPa) in a short period of time. HPP is capable of inactivating foodborne spoilage and pathogenic microorganisms by erupting their cell membranes but maintaining the organoleptic properties of the food products [13, 15].
- Pulsed electric field (PEF): application of short duration (1-100 μ s) and high intensity (20-80 kV/cm) pulses of electricity, which affects the cell wall of microorganisms by electroporation, inactivating them. Though PEF cannot be used solely as a sterilization technology, this process combined with heat can become a potential sterilization technology [14, 15].
- Ultraviolet (UV) light treatment: food surfaces, water, and packing materials are irradiated with UV light (from 100 to 400 nm). The microbial inactivation effect of UV light and pulsed light is achieved by damaging the microorganism's DNA. The cost of UV treatment is low, and no chemical residue or significant amount of heat is generated during processing. UV-C (200-280 nm) is considered to be the most effective against bacteria and viruses [13, 15].
- Food irradiation: inactivates microorganisms by exposing food to ionizing irradiation, such as electron beam, X-ray, and gamma-ray. Despite consumer concerns, these sources do not have sufficient energy to induce radioactivity. Precise irradiation dosing to maintain sensory and nutritional quality is crucial. Additionally, gamma rays require greater investment in protective facilities and radioactive waste treatment [44].
- Preservatives: used to preserve food by lowering the pH value and settling the redox potential of the food. These work by interfering with the metabolic processes of microorganisms or by creating an environment that is inhospitable to their growth. Food preservatives are divided into two classes: natural preservatives and artificial/chemical preservatives. Some chemical preservatives such as sodium benzoate and potassium sorbate are often used to prevent microbial spoilage of fruit juices. Natural food preservatives are usually of plant, animal, or microbial origin [13, 14].
- Cold plasma: a partially ionized gas that contains a mixture of electrons, ions, atomic species, UV photons, and charged particles is used to inactivate microorganisms. Cold plasma uses less water, has lower operating temperatures, and lower costs when compared to thermal processing. It is being explored as a method to reduce surface contamination on fruits and vegetables [44].

2.4 Factors that affect the thermal resistance of ascospores

The heat treatment to which a product is subjected must be suitable for the inactivation of most heat-resistant fungi, without affecting the quality of the processed product. It is then important to know how the thermal resistance of ascospores can vary depending on multiple factors and how to improve inactivation. Their resistance varies between different fungal species and even within the same species under different conditions. These conditions can be related to factors intrinsic to the food matrix, such as pH, a_w , presence of organic acids, soluble solids content, addition of preservatives, and type of heating media [1, 45]. There are also factors external to food matrices that also influence the thermal resistance of these microorganisms, such as incubation time and temperature. It should be noted that it is the combination of different factors that leads to greater or lesser thermo-resistance, and this influence on the ascospores will be reviewed in the following sub-section.

2.4.1 Variation of resistance among different strains of heat-resistant fungi

Different species and strains of fungi may exhibit varying responses to environmental and food preservation stresses. This variability gives rise to differences in stress resistance and plays a crucial role in food safety and food quality. Some species and strains are more resistant to heat, while others may be more susceptible [46].

Regarding the variety between different species, Scott et al. [46] compared the thermal resistance of *T. flavus* and *N. fischeri* isolated from commercial fruit juices. Ascospores of *N. fischeri* had a *D*-value of 1.4 min at 190 °F (87.8 °C) while *T. flavus* had *D*-values of 7.8 min at 190 °F, showing that the ascospores of *T. flavus* were more resistant than those of the strain of *N. fischeri* studied. On the other hand, Quintavalla et al. [47] compared the thermal resistance of *T. flavus*, *N. fischeri*, and *B. nivea* in grape juice, where ascospores of *N. fischeri* proved to be more heat resistant than the other strains, showing that besides the variety between different organisms, the growth media also affects their resistance.

The variety within the strains of the same species also affects the resistance of the ascospores. Recently, Brule et al. [48] reported that the heat resistance of various strains of the HRM *Paecilomyces variotii* was highly variable. Decimal reduction times of thermal treatments at 60 °C (D_{60}) ranged from 3.5 to 27.6 min when different strains were evaluated, all belonging to the same species. In another study, Brule et al. [49] studied 20 different strains of the fungi *Penicillium roqueforti*, stating that the most heat-resistant strain was DTO 013-F5, while the most heat-sensitive strain was the 130-C1 with D_{56} -values of 13.6 ± 3.0 and 1.6 ± 0.38 min, respectively.

2.4.2 Incubation time and temperature

Several studies suggest that the thermo-resistance of spore-forming microorganisms tends to increase with increasing incubation time and temperature.

Tournas et al. [50] studied the influence of the age of *Neosartorya fischeri* ascospores on their thermal resistance. Ascospores were incubated at 30 °C for 1, 2, 3, and 6 months. After the application of thermal treatment at 88 °C for 1 h, it was verified that the increase in the incubation time provided an increase in the thermal resistance of the fungus. In Evelyn and Silva et. al [37], spores of *Byssoschlamys nivea* and *N. fischeri* were obtained after cultivation of the stock culture for periods of 4, 8, 10, and 12 weeks at 30 °C. This period of cultivation was considered the age of the spores. That study investigated the efficacy of 600 MPa high-pressure thermal processing (HPTP) at 75 °C, and it showed that the spore resistance increased with increasing age for both species, with differences in the extent of inactivation of up to 2 log between spore age groups.

Regarding the influence of temperature, Beuchat et al. [51] studied the resistance of a *Talaromyces flavus* culture when inoculated in apple juice. The heat resistance was lower when *T. flavus* was incubated at 21 °C, compared to 25 and 30 °C. Engel and Teuber et al. [52] studied the influence of the incubation temperature on the thermal resistance of ascospores of *Byssoschlamys nivea* and verified that the ascospores produced at 30 °C, in malt extract agar for a period of 21 days, were more heat-resistant than those produced under the same conditions at temperatures of 20, 25, and 37 °C. However, the authors observed that production at 30 °C provided the highest thermal resistance, which goes against other reports that mention that the highest temperatures produce spores with the highest resistance.

2.4.3 Influence of the heating media

2.4.3.1 pH

It has been well established that most microorganisms have an optimum pH for their growth, survival, and enzymatic activity around 7.0 (6.6-7.5) and that few can grow at pH values below 4. Filamentous fungi and yeasts are highly tolerant to pH variations whereas bacteria tend to be more sensitive, growing in a very restricted pH range [53].

The minimum and maximum pH values tolerated for each microorganism are greatly influenced by the composition of the food itself. The high acidity content of fruits restricts bacterial growth but provides a suitable environment for fungi and yeast spoilage, and this is due to the capacity of these organisms to grow at pH values <3.5 [53]. **Table 2** shows the average pH of some fruit products.

Table 2. pH values of some fruit products. Adapted from Jay et al. [53].

Fruit Product	pH
Apple	2.9-3.3
Bananas	4.5-4.7
Figs	4.6
Grapes	3.4-4.5
Limes	1.8-2.0
Orange (juice)	3.6-4.3
Watermelons	5.2-5.6
Peach	3.4-3.6
Cherry	3.2-4.0

Microorganisms are most resistant to heat at their optimum pH of growth, which is generally about 7.0. But as pH is lowered, there is a consequent decrease in spores resistance. The advantage is taken of this fact in the heat processing of high-acid foods, where considerably less heat is applied to achieve sterilization compared to foods at or near neutrality. This method also helps to preserve some nutritive and sensorial properties, since the temperature used for eliminating these organisms does not need to be as high as for others [53]. King et al. [54] studied the heat resistance of *Talaromyces flavus* using synthetic heating media (glucose 16 °Brix/tartaric acid 0.033 M) adjusted to pH 3.6, 5.0, and 6.6. The *D*-values showed an increase in thermal resistance with increasing pH of the heating medium. They also commented on the effect of pH on other microorganisms. The resistance of *Neosartorya fischeri* increased in a pH range from 2.5 to 3.5; *Byssoschlamys fulva* resistance also increased in two tests, in the range of pH 1.4 to 6.0 and 3.0 to 3.5. In another study, Bayne and Michener et al. [55] verified that the ascospores of five strains of *B. fulva*, suspended in a standard medium adjusted to pH 5.0, were more resistant than in the same medium at pH 3.6. López et al. [56] evaluated the influence of the pH of heating medium on heat resistance of *Bacillus stearothermophilus* spores. The pH values tested were 4.0, 5.0, 6.0 and 7.0 at temperatures of 115, 120, 125, 130 and 135 °C. In general, there was a decrease in *D*-value when the pH was lowered, stating that at that pH the thermal resistance was reduced.

2.4.3.2 Water activity

Water is essential to life. Therefore, water plays a very important role, both in terms of food composition, but also in the way it mediates the reactions that occur in the food matrix. However, water is also primarily responsible for the deterioration of food during storage. Water causes the growth of microorganisms, is the medium for chemical and enzymatic reactions, confers certain properties in terms of texture, color, and odor, and defines the shelf life of food. Water in food is found substantially in two forms: combined and free. Combined water is bound to different molecules such as proteins and carbohydrates; free water is all water that

is not strongly bound to substrates and therefore acts as a solvent [57]. It is this free water that supports the growth of bacteria, yeasts, and molds, which can affect the safety and quality of food. The water availability in foods is most readily measured as water activity. Water activity (a_w) is a measurement that effectively quantifies the relationship between moisture in foods and the ability of microorganisms to grow on them [40]. By mathematical definition, a_w is defined by the ratio between the partial vapor pressure of water in food (P) and the partial vapor pressure of pure water (P_0) at the same temperature [58] (Equation 1).

$$a_w = \frac{P}{P_0} \quad (1)$$

In many practical situations, a_w is the dominant environmental factor concerning food stability or spoilage. Microbial growth is directly related to water activity, with certain types of microbes being unable to grow when a_w is below some critical value [59]. The degree of tolerance to low a_w determines the minimum a_w at which germination and growth can occur, and the minimum value of a_w at which microorganisms can grow is 0.60. Fungi that can grow at low a_w are termed xerophiles meaning that they can grow at a_w 0.60-0.70, but the minimum a_w for mycotoxin production by molds is 0.80, with the majority not producing mycotoxins at $a_w < 0.85$ [60]. Table 3 shows the range of a_w values favorable for the proliferation of some fungi.

Table 3. Minimal a_w for growth and toxin production by fungi. Adapted from Beuchat et. al [61] and Evelyn et al. [37].

Fungi	Minimal a_w	
	Growth	Toxin production
<i>Aspergillus parasiticus</i>	0.82	0.87
<i>Aspergillus clavatus</i>	0.85	0.99
<i>Aspergillus ochraceus</i>	0.81	0.88
	0.76	-
<i>Penicillium cyclopium</i>	0.87	0.97
	0.82	-
<i>Penicillium martensii</i>	0.83	0.99
	0.79	-
<i>Stachybotrys atra</i>	0.94	0.94
<i>Byssosclamyces nivea</i>	0.89	-
	0.84	-
<i>Neosartorya fischeri</i>	0.90	-

The range of a_w values that foods can have is very wide, from those with very little free water to almost completely free water [58]. Fresh products have values close to 1, while dried and dehydrated products are among the foods that have the lowest values, appearing very close to 0. The degree of water availability of a food product will impact how easily it can be spoiled

by microorganisms. Low a_w foods have clear advantages with respect to controlling the growth of foodborne pathogens, as the food will be less susceptible to biological contamination [60]. Representative a_w of foods are shown in Table 4.

Table 4. Representative a_w of foods. Adapted from Ross et al. [58].

Foods	Water activity (a_w)
Milk, fruit, vegetables	0.995-0.998
Fresh meat, fish	0.990-0.995
Bread	0.90-0.95
Fruit cakes, preserves, soy sauce	0.80
Dried fruit	0.75-0.6
Dried milk (8 % moisture)	0.70
Cereals, confectionary, dried fruit, peanut butter	0.70-0.80
Dried pasta, spices, milk powder	0.20-0.60
Freeze-dried food	0.10-0.25

In food microbiology, reducing a_w is essential to improve the microbiological stability of the product. The water activity of an environment can be reduced by the addition of solutes/water-binding substances, or by the removal of liquid water (*i.e.*, drying, freezing) [58]. The value of a_w is also highly influenced by the presence of dissolved sugars, other sweeteners, and salts in confections, while the increase of these elements tends to decrease the a_w [59]. Although it is ideal to decrease the water activity in food, low a_w foods can also suffer some consequences if the product is already contaminated by a heat-resistant mold. When water activity is low (*e.g.*, in dry environments), ascospores tend to remain dormant. This effect is further intensified by the impact of a_w on both the cell and its surrounding environment. Reduced a_w usually correlates to decreased chemical activity, which may help preserve microorganisms present in the foods. This is particularly true for low a_w (*e.g.*, <0.7) products, in which microbial survival may be enhanced in comparison to that at higher a_w , resulting in enhanced resistance to heat damage. Conversely, high water activity (*e.g.*, in moist environments) activates ascospores and makes them more susceptible to heat damage. Water also facilitates heat transfer within the spore, potentially leading to faster thermal damage [58].

Murrel and Scott et al. [62] reported that the thermal resistance, as explained by D -values (time required for 90 % reduction in the population of a specific pathogen), of bacterial spores of six different species equilibrated to a_w values of 0.2 to 0.4 varied from 2 to 24 h at 110 °C. Beuchat et al. [61] showed a higher D -value for *B. nivea* at an a_w of 0.89 (49 min) than with an a_w of 0.99 (39 min), at 80 °C, stating that the heat resistance of the ascospores would increase when the water activity was lower.

2.4.3.3 Organic Acids

Several studies were carried out to understand the influence that organic acids exert on the thermal resistance of fungal spores. The tolerance of ascospores depends highly on the type of acid present, their concentration, and the pH of the heating medium [4]. Rajashekhara et al. [63] studied the thermal resistance of *N. fischeri* isolated in mango juice in which was individually added different organic acids (citric, lactic, malic, and tartaric acid). The maximum destruction of ascospores, after heat treatment at 85 °C, occurred when the heating medium contained citric acid and the lowest was achieved using tartaric acid. Beuchat et al. [64] investigated the tolerance of ascospores of three strains of *T. flavus* to organic acids after exposure to high temperatures. Fumaric, sorbic and benzoic acids were more lethal than citric, malic, acetic, and tartaric acids. Conner & Beuchat et al. [65] verified an increase in the rate of thermal inactivation of *N. fischeri* ascospores when heating media were added with fumaric acid (1 and 2 %) at pH 2.5, 3.0, and 3.5. In contrast, acetic, citric, and tartaric acids, at the same concentrations and pH, resulted in only a moderate increase in inactivation rates. This effect was also studied by Splittstoesser & Splittstoesser et al. [66]. After treatment at 85 °C for 120 min, the resistance of *B. fulva* ascospores was greater when the solutions had a pH 3.0 containing malic and tartaric acid, with tartaric acid providing the best percentage of survival. However, this was not observed for *Aspergillus* sp. since the addition of organic acids had no influence on thermoresistance.

2.4.3.4 Soluble solids

Higher concentrations of soluble solids in the heating medium also exert a protective effect on the ascospores of heat-resistant fungi. The water activity of an environment can be reduced by the addition of solutes, as this reduction of a_w can lead to increased resistance of the ascospores, as previously explained in subchapter 2.4.3.2 [58]. Splittstoesser & Splittstoesser et al. [66] studied the influence of glucose concentration on the thermal resistance of *B. fulva* and *Aspergillus* sp. The concentration of soluble solids in grape juice was adjusted to 5, 26, and 54 °Brix. Great protection was verified on ascospores of both fungi as the concentration of the glucose solution increased, after thermal treatment. Beuchat & Toledo et al. [67] verified that ascospores of *B. nivea* were protected from inactivation by heat when the heating medium (grape juice) was supplemented with sucrose. This protection was greater with the increase in the amount of sucrose added to the medium. The reason for this protection is not well explained, but the osmotic pressure differential between the heating medium and the ascospores may favor thermal resistance. In another study, King & Whitehand et al. [54] also verified an increase in the thermal resistance of ascospores of *T. flavus* as the content of soluble solids in the medium increased, when suffering an inactivation at 90 °C.

2.4.3.5 Addition of preservatives

The presence of preservatives in the heating medium tends to increase the lethal effects of heat. Beuchat et al. [68] tested the effectiveness of sulfur dioxide, potassium sorbate, sodium benzoate, and diethylpyrocarbonate (DEPC) in preventing the outgrowth of *Byssosclamyces nivea* ascospores. Sulfur dioxide was the most inhibitory since complete inhibition of colony formation occurred in acidified PDA (pH 3.5) containing 50 ppm of the preservative. However, the presence of up to 100 ppm potassium sorbate in grape juice during heat activation appears to have a stimulatory effect on breaking dormancy, while the other test preservatives at this concentration decreased the heat resistance of *B. nivea* ascospores.

King et al. [69] added various concentrations of sodium benzoate and potassium sorbate to grape juice and found that fungal growth was prevented by adding 250 ppm of sodium benzoate or potassium sorbate for up to 16 days at room temperature. In the same study, the authors also evaluated the fungistatic effect of sulphur dioxide (SO₂) and concluded that this was the most efficient preservative in preventing the growth of *B. fulva* ascospores. Rajashekhara et al. [63] evaluated the thermal resistance of *N. fischeri* in grape and mango juice containing potassium sorbate (0.1 %) or sodium benzoate (0.1 %) or a combination of both (0.05 % of each) in its formulation. After thermal treatment at 85 °C, the highest thermal inactivation of ascospores was verified with the addition of potassium sorbate in grape juice. For mango juice, the lethality effect conferred by the addition of sodium benzoate (0.1 %) and by the addition of benzoate-sorbate (0.05 % of each) showed the greatest inactivation effect.

2.4.3.6 Types of heating media

Conner & Beuchat et al. [65] tested three strains of *N. fischeri* in three different heating media: apple juice (12.3 °Brix, pH 3.8), grape juice (16.6 °Brix, pH 3.4), and phosphate buffer (0.1 M, pH 7.0). The rate of thermal inactivation of ascospores was lower in apple juice than in the other media. Rajashekhara et al. [70] also verified that the ascospores of *N. fischeri* presented better survival to a thermal treatment at 85 °C when suspended in fruit juices than when they were heated in phosphate buffer (pH 7). Splittstoesser & Splittstoesser et al. [66] studied the influence of different heating media on the thermal resistance of ascospores of *B. fulva* and *Aspergillus* sp. in a thermal treatment for 1 h at 85 °C. The heating media used were: grape juice (5 °Brix, pH 3.5), apple juice (11 °Brix, pH 3.4), tomato juice (6.6 °Brix, pH 4.1), and distilled water (control). The percentages of *B. fulva* ascospores recovered in these media were 8.4 %, 5.7 %, 9.2 %, and 0.06 % and for *Aspergillus* sp. it was in the following order of 14 %, 11 %, 12 %, and 0.61 %, respectively.

2.5 Thermal death parameters for *Neosartorya fischeri*

As previously mentioned, the resistance of HRM ascospores varies based on factors like a_w , pH, matrices, solute concentrations, and temperature profiles. This resistance is quantified by using the decimal reduction time (D), which measures the time needed at a specific temperature to reduce the microorganism population by 90 %. The equations used to calculate the thermal death parameters will be soon clarified in sub-section 3.2.4. **Table 5** provides a compilation of the D -values for *N. fischeri* at different temperatures and heating media conditions.

Table 5. D -values of *Neosartorya fischeri* at different temperatures and heating media.

T (°C)	Heating medium	pH	°Brix	D (min)	Ref.
80	Apple juice	3.8	12.3	27.0	[71]
		2.5	11.3	81.3	[36]
		3.5	11.3	153.85	[36]
	Papaya juice	3.6	13	129.9	[1]
		3.9	13	146.6	[72]
		3.9	27	183.4	[72]
	Pineapple juice	3.45	12.6	19.6	[50]
		3.4	42.7	29.5	[50]
		3.7	13	225.4	[72]
85	Apple juice	3.8	12.3	13.2	[71]
		4.5	11.3	38.61	[36]
	Papaya juice	3.6	13	19.0	[1]
		3.9	10	37.3	[72]
		3.9	30	48.5	[72]
	Pineapple juice	3.4	12	13.2	[1]
		3.7	10	35.8	[72]
		3.7	30	46.5	[72]
	Mango juice	3.5	15	63.51	[63]
	Grape juice	3.4	16.6	5.8	[71]
		3.4	16.6	10.1	[71]
90	Apple juice	3.5	11.3	1.03	[36]
		4.5	11.3	2.20	[36]
	Papaya juice	3.6	13	1.9	[1]
		3.9	13	3.3	[72]
		3.9	27	5.8	[72]
	Pineapple juice	3.4	12	1.5	[1]
		3.7	13	3.9	[72]
		3.7	27	6.3	[72]
		3.4	42.7	7.6	[50]
92	Pineapple juice	3.5	12	1.06	[45]
95	Pineapple juice	3.45	12.6	1.7	[50]
		3.4	42.7	2.3	[50]

2.6 Frulact's industrial processing

The raw materials required for the preparation of fruit-based products undergo several processing stages, as illustrated in **Figure 2**, before being incorporated into the final product.

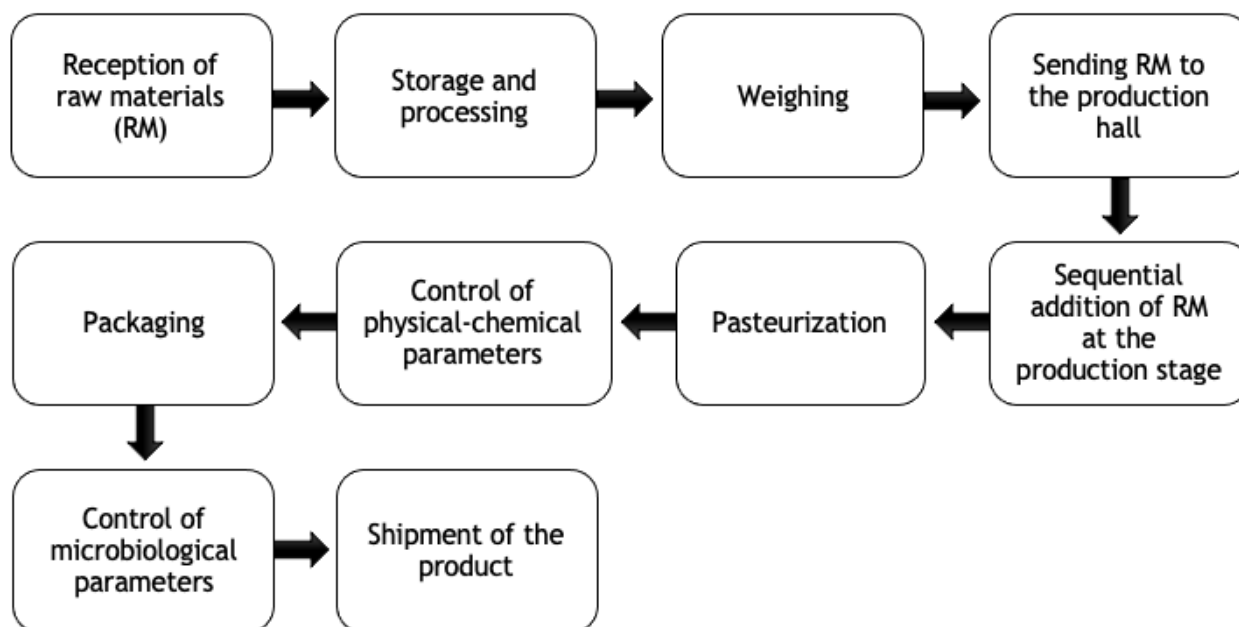


Figure 2. Diagram of the industrial processing stages. Adapted from Brito et al. [73].

Upon arrival at the factory, the raw materials are coded according to their respective batches, thus ensuring proper inventory management and traceability throughout the entire production process. Following the reception stage, these materials are stored according to their specific requirements, including freezing, refrigeration, or room temperature storage. If necessary, the raw materials may also undergo physical processes such as sorting, hydration, pulping, or cutting, depending on the formulation's specifications.

During the weighing stage, the raw materials are measured according to the formulation of the specific product. Subsequently, they are sent to the production area, where they are sequentially added under agitation, complying with the stipulated production time/temperature binomial. This binomial will be one of the factors studied to assess its impact on the behavior of the microbial load, particularly in terms of increasing or decreasing the thermal resistance of the microorganism in question [73].

Next, the product is passed through the pasteurizer, where the pasteurization time/temperature binomial is applied. In this study, the binomials will be equivalent to 85, 90, and 93 °C for 5 or 10 min. Subsequently, the physical-chemical parameters are checked to confirm whether the product is within the limits agreed with the client. Finally, the product is packaged and shipped in accordance with the compliance of microbiological parameters.

3 Materials and Methods

3.1 Preparation of the material

To carry out the experimental procedure, it was first necessary to prepare the necessary culture and enrichment media. The culture media used in the experiment was Potato Dextrose Agar (PDA) (Liofilchem, Italy). This medium was prepared according to the manufacturer's instructions in Schott glass flasks with a maximum capacity of 500 mL. These bottles were then sterilized in an autoclave (Panasonic MLS-3781L), according to a time/temperature binomial of 121 °C for 15 min. After sterilization, the medium was placed in Petri dishes to be further used. This procedure was performed either inside a laminar flow chamber or by flame, to avoid contamination. Schott glass flasks with a capacity of 100 mL were filled with 90 mL of Buffered Peptone Water (BPW) (Merck, Germany), necessary for the suspension of the samples. The dilution tubes necessary to perform successive dilution of the samples were prepared by filling each test tube with 9 mL of BPW. Finally, all the materials were also subjected to sterilization in an autoclave for 15 min at 121 °C, including the 1 mL tips used in the micropipettes and the filter paper, funnel, and tweezers used for the preparation of the inoculum.

3.2 Experimental procedure

3.2.1 Inoculum

N. fischeri was obtained from the Frulact microbial collection. The mold was cultured in a Petri dish at 30 °C for several weeks on PDA. Ascospores were harvested by flooding the medium surface with 9 mL of sterile distilled water and gently rubbing the mold with a sterile glass rod [1]. The ascospore suspension was then filtered through a filter paper with pore sizes between 10-20 µm to remove hyphal fragments with the help of a funnel [73]. The inoculum was diluted and kept in a fridge at 5 ± 2 °C until further use.

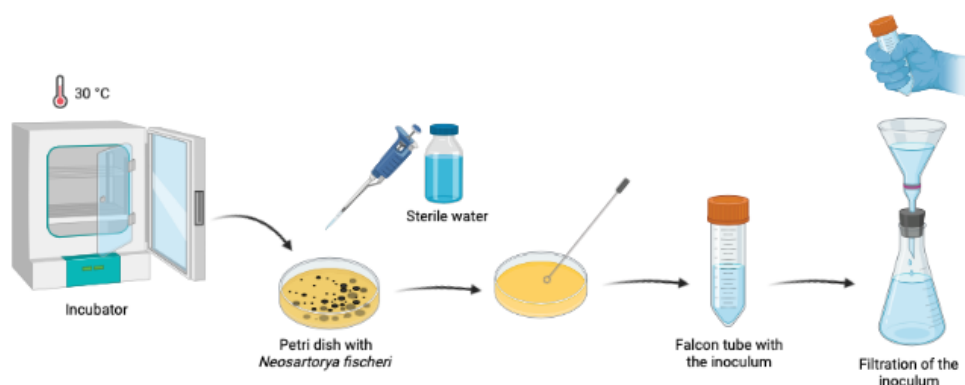


Figure 3. Representative scheme of the preparation of the inoculum.

3.2.2 Elaboration of fruit preparations and chemical analysis

The fruit preparations used were produced according to the formulations supplied by Frulact. After the initial mixer was done, it was pasteurized for 5 min at a temperature of 90 °C. The preparations were then packed in sterile containers of 1 L at a temperature of 70 °C. A small sample of each preparation was saved to be analyzed after 24 h. The values of physical and chemical parameters were obtained and presented in **Table 6**, namely pH (potentiometer, Consort C860), °Brix (refractometer, Bellingham+Stanley Ltd. RFM732), and a_w (Rotronic Hygrolab3). The samples were then stored in refrigerators at 5 ± 2 °C until the date of use.

Table 6. Physical and chemical characteristics of the fruit preparations.

Preparation	Parameters		
	pH	a_w	°Brix
Sugary peach	3.85	0.94	42.8
Sweetened peach	3.75	0.99	6.6

To investigate the impact of water availability on the thermoresistance of the microorganism, high and low °Brix preparations were chosen. The preparations all share a similar level of acidity, with pH values very closely together.

3.2.3 Heat treatment

To start the experimental procedure, a simulation of the production step was carried out on a laboratory scale, using a thermal bath. This step intends to simulate the binomial time/temperature applied to the factory preparations. The binomials studied in this project are 30 °C/1 h, 50 °C/1 h, 30 °C/3 h, and 50 °C/3 h. By using the help of a flame, 50 g of the preparation being studied were weighed into 120 mL plastic sterile cups, under aseptic conditions, and inoculated with 6 % (3 mL) of the *N. fischeri* suspension previously presented in sub-section 3.2.1. A total of 19 cups were used per experiment. These cups were placed in a thermal bath to simulate the different production time/temperature binomials and homogenized every 10 min to avoid the creation of clusters of the ascospores. To control the temperature of the sample, a chemical thermometer, with a sensitivity of 1 °C, was placed in a similar cup with the same amount of preparation, but without being inoculated or sterile.

After the production step, these cups were then subjected to three different temperatures inside a thermal bath: 85, 90, and 93 °C, for 5 or 10 min. During the pasteurization time, samples were taken from the bath corresponding to times 0, 1, 2, 3, 4, and 5 min or to times 0, 2, 4, 6, 8 and 10 min. These samples were carried out in triplicate and a control cup for the temperature was also used. After achieving the desired times, the cups were placed in an ice

bath, to cause a thermal shock. For all temperatures tested, there was a sample relating to time 0 before pasteurization, which was submitted only to a previously defined activation treatment to quantify the microbiological cargo after the production process and before the heat treatment.

To determine the number of ascospores that survived, 10 g of each cup were weighed into flasks containing 90 ml of BPW, where they were left to rest for 30 min. Working either by the flame or inside the laminar flow chamber, 1 mL of this BPW suspension was transferred into a dilution tube containing 9 mL of BPW, with the help of a volumetric micropipette. The number of dilutions made for the samples varied depending on the cargo that they might have, varying from 10^{-1} to 10^{-3} . After this, 200 μ L of the dilutions were transferred into a Petri dish containing PDA and spread using a spreader. This process was always made in triplicate. After all the plates were identified and inoculated, they were incubated for at least 5 days at 30°C before counting. The procedure employed in this study was based on prior thesis research conducted at the Frulact facilities with some added adaptations [73].

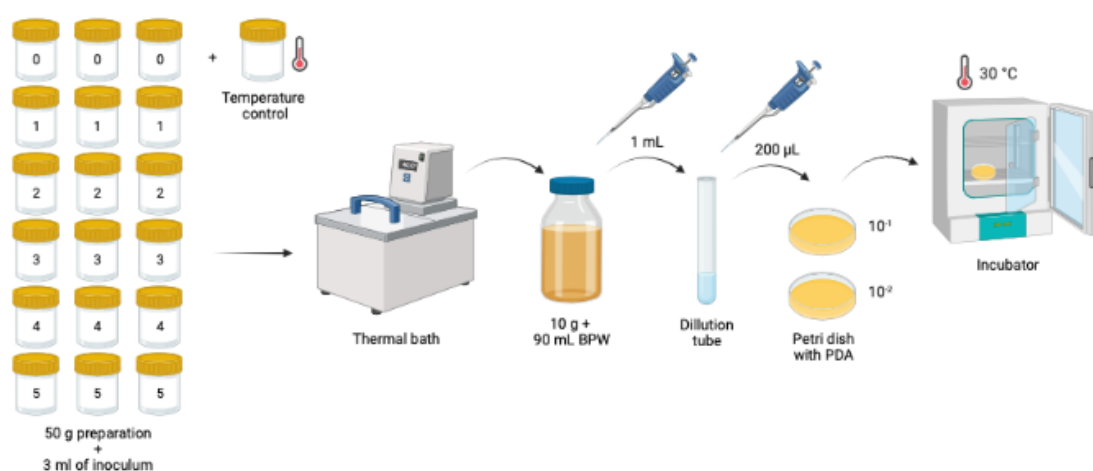


Figure 4. Representative scheme of the pasteurization step followed by the microbiological analysis.

3.2.4 Calculation of thermal death rate of ascospores

The most used mathematical measure of the death rate is the *D*-value. The decimal reduction time (*D*) represents the time required at a specific temperature to reduce the population of microorganisms by 90 % (i.e., by one order of magnitude or “one log”). The level of microbial reduction achieved is quantified by the number of decimal reductions. This is given by $\log(N_0/N)$, where N_0 is the initial population and N is the final population; and it is determined from the equation that follows [1, 5].

$$\log\left(\frac{N_0}{N}\right) = \frac{\text{Heating time}}{D} \quad (2)$$

The D -value can be obtained by plotting the log of the population versus time and finding the first-order kinetic that relates the two. This relation is shown in **Figure 5**. The temperature to which the D -value applies is indicated by a subscript, for example, D_{70} to 70 °C. If a microorganism has a D_{70} of 10, it means that subjecting it to 70 °C for 10 min will result in a 90 % reduction in the microbial population, but 20 min will achieve $2D$ (99 %), 30 min will achieve $3D$ (99,9 %) and so on. However, theoretically, a 100 % reduction is never possible.

Another parameter used to assess the thermal resistance of microorganisms is the Z -value. The Z -value represents the temperature variation required to change the D -value by one logarithmic unit. In other words, it represents how much the temperature needs to increase or decrease to have a 90 % change in the microbial population's response to the heat treatment. A higher Z -value indicates that the microorganism is more heat-resistant, as it requires a greater temperature variation to achieve the same effect. The relationship between D at two different temperatures (T_1 , T_2) and the Z -value is given by the following equations and its graphic calculation is shown in **Figure 5** [5].

$$\log \left(\frac{D_1}{D_2} \right) = \left[\frac{(T_2 - T_1)}{Z} \right] \quad (3)$$

An alternative way of using Z -values is for comparing processes. For example, if it is known that a temperature of 68 °C is effective for 10 min and the Z -value is 6 °C; then equally effective processes would also be 62 °C for 100 min or 74 °C for 1 min [5].

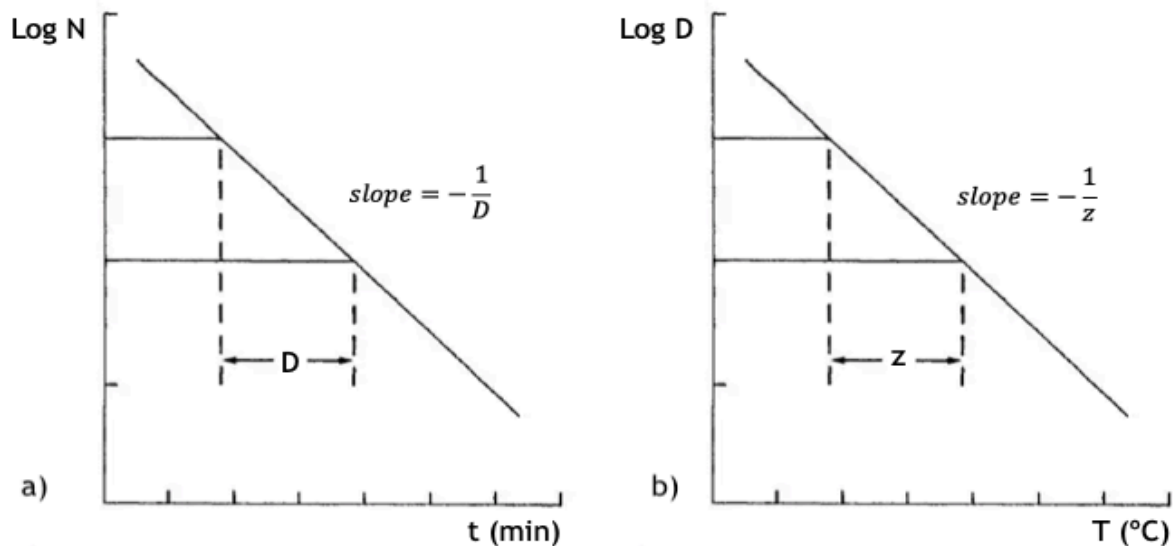


Figure 5. a) Relationship between the log of the population of microorganisms and the time at a constant heating temperature to obtain the D -value, b) relationship between the log of the decimal reduction time and the temperature to determine the Z -value. Adapted from Lewis et al. [5].

3.2.5 Calculation of sterilization (F) value

To verify whether the commercial thermal treatment applied in the fruit preparations is enough to destroy *N. fischeri* ascospores, the sterilization F value was calculated. Sterilization refers to the process of eliminating/destroying all forms of viable microorganisms from a given environment, object, or substance. Since the commercial thermal treatment commonly applied in the Food Industry employs a temperature of between 90-95 °C for 2-5 min, the temperature considered for calculating the F -value was 95 °C. As the D -values obtained in this work were only calculated for 85, 90, and 93 °C, it is necessary to obtain the D -value for 95 °C using the equation of the Thermal Death Time (TDT)-equivalent curve (**Figure B.3** in Appendix B).

The F -value was calculated through the equation that follows, where γ stands for the number of decimal reductions and D is equivalent to the decimal reduction time.

$$F = \gamma D \quad (4)$$

Salomão et al. [1] considered the probability of survival of 1 ascospore of *N. fischeri* in 1000 (10^3) infected packs. So, the number of decimal reductions required for the calculation of the F -value is 4, *i.e.* $\gamma = 4$.

3.4 Statistical analysis

In this project, all the experimental results were analyzed using statistical methods. The average of the microbial load values obtained from different tests was calculated, along with the corresponding standard deviation. To determine the relationship between the observed production time/temperature combinations, a single-factor ANOVA test was performed at a significance level of 95 %. This analysis was carried out using the Data Analysis supplement of the Microsoft Office Excel program. Additionally, the R^2 values associated with each planned linear fit were calculated to determine the kinetic parameters of thermal death (D and Z) [73]. All these values are presented in Appendix D.

4 Results and Discussion

4.1 Assessment of the impact of different time/temperature binomials for production and pasteurization on the microbial load of peach preparations

Four production binomials - 30 °C/1 h, 50 °C/1 h, 30 °C/3 h, and 50 °C/3 h - were applied to both sugared (42.8 °Brix) and sweetened (6.6 °Brix) peach preparations inoculated with 6 % (3 mL) of a *N. fischeri* suspension. One of the objectives was to examine how the production stage influenced the thermal resistance of *N. fischeri* ascospores. Subsequently, a heat treatment (pasteurization) was carried out and the thermal death parameters (*D* and *Z*) outlined in sub-section 4.2 were computed based on the analysis of the microbial load. Upon performing analyses for each individual production time/temperature binomials and applying diverse pasteurization temperatures to the preparations, the results illustrated in **Figure 6** were obtained by calculating the microbial load. These results correspond to the average microbial load obtained at each pasteurization time, as presented in Appendix A.

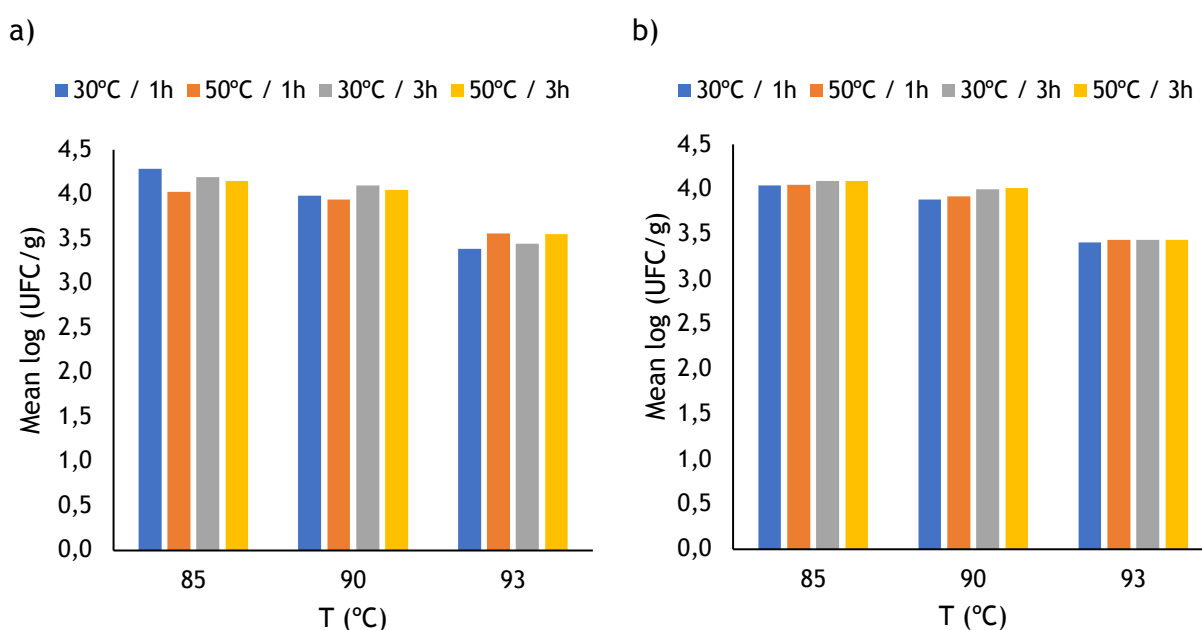


Figure 6. Average microbial load obtained in each of the time/temperature binomials as a function of the pasteurization temperature applied to: **a)** sugared; and **b)** sweetened peach preparation.

Upon examining **Figure 6**, it becomes apparent that the growth of *N. fischeri* is observable across the four distinct production time/temperature binomials that were studied. Initially, an attempt was made to apply a pasteurization binomial of 95 °C/5 min to the preparations; however, this condition resulted in a remarkably low microbial load that would prevent viable

results from being obtained. Subsequently, a more rigorous treatment involving a temperature of 93 °C for a duration of 10 min was reevaluated as the most intensive approach. When the fungus was subjected to this pasteurization binomial, complete eradication was never achieved in the peach preparations. This observation demonstrates the high resistance of *N. fischeri* to extreme temperatures. Despite subjecting it to conditions that are typically highly lethal to many microorganisms, this strain exhibited a noteworthy ability to endure and survive.

Upon analyzing **Figure 6**, a clear trend emerges in both the sugared and sweetened preparations: an increase in pasteurization temperature consistently leads to a reduction in the microbiological load, regardless of the specific production binomial used.

When exclusively examining **Figure 6b)** related to the sweetened peach preparation, it becomes evident that an increase in the microbiological load is observed across all pasteurization temperatures as both production temperature and time experienced augmentation. In general, it can be assumed that *N. fischeri* tends to exhibit a higher microbial load when subjected to the production binomial of 50 °C/3 h. Given the fungus ascospores capability to initiate growth and germinate within the temperature span ranging from 10 to 50 °C, the adoption of this production temperature emerges as an optimal scenario for its proliferation [39]. In addition, the extension of the retention time from 1 to 3 h, a period during which the preparations are subjected within the factory, allows the fungus to adapt to the heating medium. This adaptation contributes to the enhancement of the microorganism stability and increases its resistance to heat, ultimately contributing to its longer survival and persistence within the given conditions.

However, when observing **Figure 6a)**, corresponding to the sugary preparation, a different scenario emerges. At 85 °C, the 30 °C/1 h production binomial results in *N. fischeri* exhibiting a higher microbial load. Similarly, at 90 °C, this behavior is mirrored by the 30 °C/3 h binomial. Furthermore, at 93 °C, a close tie is observed with both the 50 °C/1 h and 50 °C/3 h binomials. This discrepancy in results could potentially be attributed to the fact that, although *N. fischeri* demonstrates growth in a broad temperature spectrum, its optimal temperatures vary within the range of 26-45 °C [39]. Consequently, in this situation, incubation at 30 °C may offer more favorable conditions for growth compared to the 50 °C.

Adding to the complexity, the inoculum utilized in the experiments was progressively developed over time, leading to variations in the concentration of the initial microbial load. This changing characteristic of the inoculum might have contributed to the differing outcomes observed in the results. A better explanation of this impact in the results obtained in this paper is presented in sub-section 4.2.5.

4.2 Determination of the thermal death kinetic parameters

To determine the parameters of the thermal death kinetics of *N. fischeri* in the peach preparations, the protocol outlined in subchapter 3.2.3 was implemented. Following the collection of microbial load data (present in Appendix A) over the course of the pasteurization process across three distinct temperatures (85, 90, and 93 °C), the resultant thermal death rate curves for *N. fischeri* ascospores were obtained, and displayed in **Figure B.1** within Appendix B. The linearized forms of these thermal death rate curves for *N. fischeri* ascospores at 85, 90, and 93 °C are presented in **Figure B.2**, again within Appendix B. The thermal death rate curve parameters, alongside the heat resistance (*D*) values for *N. fischeri*, were deduced from these linearized curves. These essential parameters are documented in **Tables B.1** and **B.2** (Appendix B).

Furthermore, the *Z*-values associated with the peach preparations were extracted from the TDT-equivalent curve, showcased in **Figure B.3** (Appendix B). By employing linear regression analysis, the corresponding *Z*-values were determined and meticulously documented in **Tables B.3** and **B.4** (Appendix B).

A compilation of all the obtained *D* and *Z*-values, collected from the testing of different production time/temperature binomials for the varying peach formulations, is presented below in **Table 7**.

Table 7. Values of *D* and *Z* obtained for the sugared and sweetened peach preparations.

Fruit preparation	Production binomial	<i>D</i> ₈₅ (min)	<i>D</i> ₉₀ (min)	<i>D</i> ₉₃ (min)	<i>Z</i> (°C)
Sugared peach	30°C / 1 h	27.7	14.1	7.0	13.7
	50°C / 1 h	34.4	15.9	8.0	12.8
	30°C / 3 h	55.9	39.1	8.9	10.8
	50°C / 3 h	63.3	37.5	10.4	10.8
Sweetened peach	30°C / 1 h	21.4	16.3	6.4	16.3
	50°C / 1 h	28.3	18.7	7.6	14.7
	30°C / 3 h	41.7	29.7	8.4	12.4
	50°C / 3 h	49.5	31.5	11.3	13.2

The highest determined *D*-value was 63.3 min for a pasteurization temperature of 85 °C, specifically for the 50 °C/3 h binomial within the sugared peach preparation. At the same pasteurization temperature and for the same species, thermal resistance tests were conducted by Rajashekhara et al. [63] using mango juice with a soluble solids concentration (15 °Brix, pH 3.5) lower than that employed in this study. The objective was to investigate the impact of organic acids and preservatives on the resistance of *N. fischeri*, but the *D*-value for the control experiment without additives was found to be 63.5 min, which was relatively close to the value obtained in the present study. The lowest *D*-values were observed at a pasteurization temperature of 93 °C, varying from 6.4 to 11.3 min in both preparations. Slongo et al. [45], while testing the thermal resistance of *N. fischeri* in pineapple juice at pH 3.5 and 12 °Brix, reported a *D*-value at 92 °C of 1.06 min. Since this *D*-value is lower than the ones obtained in this study for a lower temperature (92 °C < 93 °C), it seems that the ascospores examined in this project exhibit considerably greater resistance compared to those in the aforementioned study. The higher resistance observed could be related to the strain of the microorganism, the spore production process, the method of determining heat resistance, and/or the product in which the spores were subjected to heat. At this temperature (93 °C), the times required for a one-logarithmic reduction (*D*) exhibit greater similarity, indicating a weaker influence of water activity and solid concentration on this parameter.

The determined *Z*-values in this study demonstrated relatively close outcomes across all pasteurization temperatures and studied binomials for the sugared and sweetened peach preparations (*Z* = 13.7, 12.8, 10.8, 10.8 °C and *Z* = 16.3, 14.7, 12.4, 13.2 °C, respectively). These values exceeded those reported in the literature. Salomão et al. [1] reported *Z*-values ranging from 5.0 to 5.9 °C for *N. fischeri* in apple (11.3 °Brix, pH 3.5), papaya (13 °Brix, pH 3.6), and pineapple (12 °Brix, pH 3.4) juices. Tournas et al. [50] found *Z*-values of 9.2 and 8.9 °C when working with the same microorganism in pineapple juice (12.6 °Brix, pH 3.45) and pineapple juice concentrate (42.7 °Brix, pH 3.4), respectively.

4.2.1 Influence of pasteurization temperature on thermal death parameters

From Table 7, it becomes evident that, in a general sense, *D*-values decrease with the increase in pasteurization temperature. This trend holds true for both sweetened and sugared references across the studied production time/temperature binomials. In all cases examined within this study, the *D*-value at the pasteurization temperature of 93 °C is consistently found to be lower than that at 90 °C, and correspondingly, the *D*-value at 90 °C is lower than that at 85 °C.

As a result, if there is a decrease in the *D*-value as the pasteurization temperature increases, this means that the *N. fischeri* ascospores become more sensitive to heat at higher temperatures. In other terms, higher temperatures are more effective at killing the

microorganisms in a shorter period. This is a desirable outcome in food processing, as it means that the pasteurization process is becoming more efficient and faster. However, should the *D*-value become larger as the pasteurization temperature increases, it would imply that the microorganisms are becoming more heat-resistant at elevated temperatures. Such an occurrence could indicate an issue in the pasteurization process. The fundamental goal of pasteurization resides in the elimination or great reduction of the microbial load. Consequently, an increase in the *D*-value at higher temperatures would suggest an insufficiency in effectively killing the microorganisms, thereby causing concern regarding the process efficacy.

Within the context of the current study, a consistent pattern emerges wherein the *D*-value always diminishes with an increase in pasteurization temperatures. These findings align with the studies made by Salomão et al. [36], where the inactivation of *N. fischeri* in apple preparations with different pH values was investigated and a decrease in *D* was observed as the temperature increased from 80 to 90 °C. Similarly, when *N. fischeri* was subjected to hot treatment by Slongo et al. [45], it was found that the *D*-value varied from 30.3 to 1.06 min as the pasteurization temperature increased from 85 to 92 °C. This is a common conclusion since usually higher temperatures need less time to achieve the destruction of 90 % of the microbial population present in the sample.

4.2.2 Influence of production time/temperature on thermal death parameters

In general, it can be inferred from **Table 7** that an increase in production temperature and time results in higher decimal reduction (*D*) values at the same pasteurization temperature.

When considering the sugared peach preparation, a clear trend is observed in the increase of *D*-values as production time and temperature also increase. Focusing on the pasteurization temperature of 85 °C, for a 1 h production period, a noteworthy increase in *D*-value is observed from 27.7 to 34.4 min (an increase of 6.7 min) when transitioning from a production temperature of 30 °C to 50 °C. However, at the same 30 °C temperature, extending the production time from 1 to 3 h results in a more substantial surge in *D*-value from 27.7 to 55.9 min (an increase of 28.2 min). Similarly, under 50 °C conditions, extending production time from 1 to 3 h also originates a higher microbial resistance, leading to a change in *D*-values from 34.4 to 63.3 min (an increase of 28.9 min). At a pasteurization temperature of 90 °C, this propensity for bigger *D*-values relating to increased production time and temperature remains evident, with the exception of the 30 °C/3 h binomial (39.1 min). This experiment shows a lower decimal reduction value for the 50 °C/3 h binomial (37.5 min), diverging from the other results obtained. Moving on to 93 °C, the difference between the *D*-values for the varying binomials is smaller, yet the tendency for this value to increase with increasing production time and temperature persists.

The overall trend observed in the sugared preparation indicated a reduction in the Z -value with an increase in D -values, showing the presence of a negative correlation between Z and the heat resistance of ascospores. Salomão et al. [1] investigated the thermal resistance of *N. fischeri* across various juices and reported the same behavior. The Z -values for the 30 °C/3 h and 50 °C/3 h combinations are identical, which is likely attributed to the unexpected D -value relating to the 90 °C pasteurization temperature for the 30 °C/3 h binomial, as mentioned earlier.

Shifting the focus to the sweetened peach preparation, the tendency for D -values to ascend with increasing production time and temperature continues. At 85 °C, for 1 h of production, the D -values rise from 21.4 to 28.3 min (an increase of 6.9 min) when transitioning from 30 °C to 50 °C. Conversely, at the same temperature of 30 °C, extending the production time from 1 to 3 h induces a more substantial surge in D -value from 21.4 to 41.7 min (an increase of 20.3 min). This augmentation in D -values, as the time and temperature of the production binomials increase, remains constant for both 90 °C and 93 °C pasteurization temperatures. Regarding the Z -values in the context of the sweetened peach preparation, in general, augmenting the decimal reduction values corresponds to diminished Z -values. An exception is observed in the 50 °C/3 h binomial, which, despite exhibiting higher D -values than the 50 °C/3 h binomials, it also obtained a higher Z -value of 13.2 °C.

Table 8 shows the variance in D -values (min) when the production time is tripled for the same production temperature (1 h → 3 h), or when the production temperature is increased for the same production time (30 °C → 50 °C). From this Table, it becomes apparent that increasing production time generally causes a greater increase in the thermal resistance of *N. fischeri* ascospores compared to raising production temperature while maintaining a constant time.

Table 8. Variance in D -values for the sugared and sweetened peach preparation when the production time or temperature was altered, for each pasteurization temperature.

Fruit preparation	Pasteurization temperature	ΔD (min)			
		1 h	3 h	30 °C	50 °C
		30 °C → 50 °C	30 °C → 50 °C	1 h → 3 h	1 h → 3 h
Sugared peach	85	+ 6.7	+ 7.4	+ 28.2	+ 28.9
	90	+ 1.8	- 1.6	+ 25	+ 21.6
	93	+ 1	+ 1.5	+ 1.9	+ 2.4
Sweetened peach	85	+ 6.9	+ 7.8	+ 20.3	+ 21.2
	90	+ 2.4	+ 1.8	+ 13.4	+ 12.8
	93	+ 1.2	+ 2.9	+ 2	+ 3.7

In summary, this study proposes that, in general, an increase in production time and temperature results in an elevation of the thermoresistance of *N. fischeri* ascospores, regardless of the pasteurization temperature applied. However, it is worth noting that this trend has not consistently been observed in the literature. Brito et al. [73] conducted another study, also at the Frulact facilities, to assess the impact of different production time/temperature binomials (30 °C/1 h, 30 °C/3 h) on the behavior of this same fungus in sugared and sweetened preparations of blackberry, raspberry, and blueberry, considering various pasteurization temperatures (70, 80, and 90 °C).

In the case of blackberry and raspberry preparations, at a pasteurization temperature of 90 °C, there is a decrease in the thermal resistance of *N. fischeri* with increasing production time (from 1 to 3 h), which contradicts the findings of this present study. These values decrease from 7.5 to 5.1 min and 37.0 to 25.6 min in the case of sugared (29 °Brix, pH 3.8) and sweetened (11 °Brix, pH 3.7) blackberry preparations, respectively. For sugared (36 °Brix, pH 3.5) and sweetened (9 °Brix, pH 3.5) raspberry, these values vary from 45.5 to 16.9 min and 23.3 to 5.6 min, respectively. However, in the case of the blueberry preparation, at a pasteurization temperature of 90 °C, there is an increase in the thermoresistance of *N. fischeri* with increasing time in the production stage. With this increased time, the *D*-values shift from 23.3 to 35.7 min for sugared preparation (52 °Brix, pH 3.5) and from 32.2 to 36.0 min for sweetened preparation (24 °Brix, pH 3.5). This trend aligns more closely with the values obtained in the current study.

It is, however, important to emphasize that the *D*-values obtained for these three fruit preparations at 90 °C ranged from 5.1 to 45.5 min, indicating a significant disparity in results, generally with values considerably higher than those obtained in the present study. Therefore, comparison of results should be made with caution.

4.2.3 Influence of water activity and soluble solids on thermal death parameters

In this scenario, the sugared preparation exhibited higher Brix levels (42.8 °Brix) and lower water activity (a_w 0.94), leading to the observation that the obtained *D*-values are greater than those identified in the sweetened preparation, which features lower Brix levels (6.6 °Brix) and higher water activity (a_w 0.99).

The *D*-values obtained in this experience can be better understood by considering the impact of water activity and Brix levels on the heat resistance of microorganisms. A crucial role is played by the Brix levels of a solution, representing the concentration of dissolved solids, such as sugars. A high Brix level/solute concentration reduces the availability of free water molecules, resulting in lower water activity. Consequently, microorganisms within this environment face restricted access to water for growth and survival. In contrast, the sweetened

preparation with lower Brix levels contains a relatively higher proportion of free water molecules, thus leading to higher water activity. This environment provides microorganisms with more accessible water, thereby supporting their growth and survival and reducing the obstacles to their development.

Concerning the D -values, the difference in water activity and Brix levels directly influences these values. In the sugared preparation with higher Brix levels and lower water activity, microorganisms face a less favorable environment for growth due to reduced water availability. As a result, their resistance to heat treatment increases, leading to elevated D -values. Furthermore, sugar solutions provide protection against the inactivation of more resistant structures, such as fungal and bacterial spores, by acting as a protective barrier against the effects of heat [72]. On the other hand, the sweetened preparation with lower Brix levels and higher water activity offers a more conducive environment for microorganisms. This results in lower resistance to heat treatment and, subsequently, a lower time is needed for the reduction of 90% of the population.

From **Table 7**, which displays all the D -values obtained in the study, it can be observed that most of the decimal reduction values for the sugary preparation (42.8 °Brix, a_w 0.94) were higher than for the sweetened one (6.6 °Brix, a_w 0.99). For instance, for the 30 °C/3 h binomial, at a pasteurization temperature of 85 °C, the D -value decreased from 55.9 to 41.7 min when transitioning from the high-sugar content preparation to the low-sugar content preparation. Similarly, for the same binomial, at 90 °C, this value decreased from 39.1 to 29.7 min, and at 93 °C, it reduced from 8.9 to 8.4 min.

However, some values did not follow this pattern. Particularly, for the pasteurization temperature of 90 °C and the production binomials of 30 °C/1 h and 50 °C/1 h, the D -values for the sweetened preparation ($D_{90}^{30\text{ °C}/1\text{ h}}=16.3$ min, $D_{90}^{50\text{ °C}/1\text{ h}}=18.7$ min, respectively) exceeded those for the sugared one ($D_{90}^{30\text{ °C}/1\text{ h}}=14.1$ min, $D_{90}^{50\text{ °C}/1\text{ h}}=15.9$ min, respectively). This pattern also recurred at 93 °C, specifically for the 50 °C/3 h binomial, with the sweetened preparation exhibiting a D -value of 11.3 min, and the sugared one, 10.4 min.

Table 9 illustrates the disparity between the D -values resulting from the transition from sweetened to sugared preparations. Green values indicate an increase in ascospore resistance when they are present in preparations featuring a high Brix, compared to when they were in preparations with lower solute concentrations. Conversely, the red value means a reduction in resistance as Brix levels decrease and water activity increases.

Table 9. Variance in *D* and *Z*-values when comparing the sweetened and sugared peach preparation.

Sweetened (6.6 °Brix) -> Sugared (42.8 °Brix)				
Production binomial	ΔD_{85} (min)	ΔD_{90} (min)	ΔD_{93} (min)	ΔZ (°C)
30 °C/1 h	+ 6.3	- 1.9	+ 0.6	- 2.6
50 °C/1 h	+ 6.1	- 2.8	+ 0.4	- 1.9
30 °C/3 h	+ 14.2	+ 9.4	+ 0.5	- 1.6
50 °C/3 h	+ 13.8	+ 6	- 0.9	- 2.4

In conclusion, it seems evident that the concentration of soluble solids had an impact on the *D*-values. In general, an increase in the Brix level of the preparation led to an increase in the *D*-value (Table 9). It is well-established that a higher concentration of soluble solids in the heating medium provides greater protection to the ascospores of heat-resistant fungi, which significantly influences the design of thermal processing [72].

Tournas et al. [50] also documented an increased percentage of survival among *N. fischeri* ascospores with a rise in the percentage of solids in the heating medium. For instance, at a pasteurization temperature of 85 °C, the *D*-value for pineapple juice (12.6 °Brix, pH 3.45) was 19.6 min, whereas for its concentrate, with a higher sugar concentration of 42.7 °Brix, it was 29.5 min (there was a 9.9 min increase). Also, for a pasteurization temperature of 90 °C, an augmentation in the resistance of the ascospores was observed, obtaining *D*-values that increased from 4.8 to 7.6 min (2.8 min increase), when comparing the pineapple juice to its concentrated form. At 95 °C, this was also observed by an increase in the *D*-values from 1.7 min, for the juice, to 2.3 min, for the concentrate. It was observed that as the sugar concentration of the pineapple juice increased from 12.6 to 42.7 °Brix, the *D*-values increased by 9.9, 2.8, and 0.6 min at pasteurization temperatures of 80, 90, and 95 °C, respectively. Studies were conducted by Souza et al. [72] about the inactivation of *N. fischeri* in papaya and pineapple juices, considering temperature and soluble solids content as variables. The soluble solids concentration in both juices was adjusted to 10, 20, and 30 °Brix. For a pasteurization temperature of 85 °C, a significant influence of the °Brix value on the resistance of this organism was observed. In the case of papaya juice, the decimal reduction time increased from 37.3 to 48.5 min as the solute concentration increased from 10 to 30 °Brix. Likewise, for pineapple juice, this change in values increased from 35.8 (10 °Brix) to 46.5 min (30 °Brix). An increase in the *D*-value of 11.2 min for papaya juice and 10.7 min for pineapple juice was noted as the sugar concentration increased from 10 to 30 °Brix. The solids concentration values changed when the same study was carried out at a temperature of 90 °C. For papaya, *D*-values of 3.3 (13 °Brix, pH 3.9) and 5.8 min (27 °Brix, pH 3.9) were obtained; for pineapple, these values

were 3.9 (13 °Brix, pH 3.7) and 6.3 min (27 °Brix, pH 3.7). This time, as the sugar concentration of the juices increased from 13 to 27 °Brix, the *D*-value suffered an augmentation of 2.5 min for papaya juice and 2.4 min for pineapple juice.

Upon reviewing **Table 9** and the literature discussed, a protective effect on the thermal resistance of *N. fischeri* ascospores becomes evident, possibly attributed to an increased percentage of solids. The precise mechanism underlying this protective effect remains unclear; however, it appears that the osmotic pressure differential between the heating medium and ascospores may favor resistance to heat. Souza et al. [72] have previously reported that the protective effects of solids could be associated with a reduction in water activity.

To finalize, the *Z*-values were consistently higher for the sweetened preparations. As previously noted, *Z* generally increases with decreasing decimal reduction values. When the *Z*-values between the two preparations are compared, there is clearly a reduction in this value from sweetened to sugared products for any binomial studied, as shown in **Table 9** by the red values. However, usually a higher *Z*-value indicates that the microorganism is more heat-resistant, as it requires a greater temperature variation to achieve the same effect. Nevertheless, our recent conclusion, which indicated a decrease in *D* with a corresponding reduction in solids concentration, implying reduced heat resistance, appears to be contradicted by the higher *Z*-values observed in the case of this peach preparation.

4.2.4 Influence of pH on thermal death parameters

As described above, it is expected that in environments with lower pH values, a reduction in the thermal resistance of spores will be observed. In acidic conditions, cell structures and enzymatic activities can be disrupted by the presence of hydrogen ions (H⁺). As a result, microorganisms may become more susceptible to heat treatment, resulting in their easier inactivation, thereby decreasing their *D*-value. Conversely, when the pH of a solution is increased, microorganisms can become more heat-resistant, leading to higher *D*-values.

In this case study, when **Table 7** is evaluated, it can be concluded that, in general, the decimal reduction values of the sweetened preparation are lower than those of the sugared one. Correspondingly, the pH of the sweetened preparation (3.75) is lower than that of the sugared (3.85) one, so it can be inferred that, overall, the decrease in pH led to a reduction in *D*-values. Nevertheless, it appears that the difference in pH values may not be sufficiently significant to solely account for this decrease in *D*-values for the sweetened preparation (difference of 0.10). Instead, it can be deduced that this influence on the decrease in *D*-values for the sweetened preparation is attributed more to the decrease in the concentration of soluble solids (explained in subchapter 4.2.3) rather than the reduction in pH.

4.2.5 Influence of the preparation of the inoculum on thermal death parameters

The inoculum utilized in the experiments was progressively developed in accordance with the specifications presented in sub-section 3.2.1. Due to the lack of knowledge regarding the required quantity of inoculum throughout the duration of the work and the extended time needed for its production, the inoculum employed for the experiments was usually generated at the beginning of each working week. Consequently, despite attempts to sustain uniformity in the concentration and maturation of the ascospores within the inoculum, this parameter was at times difficult to control. Once the preparation of the inoculum had been perfected, it was concluded that the concentration of the inoculum generally approximated 6 log, although it could undergo some fluctuations (Appendix C). These fluctuations may correspond to the variations in the microbiological load previously mentioned in sub-section 4.1.

Associated with these variations is also the age of the ascospores. At the start of the research period, multiple PDA plates were inoculated with *N. fischeri* and incubated at 30 °C to promote growth. Approximately one month in incubation, the initial PDA plates were removed to start inoculum production. As the plates were used, subsequent replicates were generated to sustain the cultivation of the organism. Nonetheless, one minor oversight that may have been introduced into the study was the potential variation in thermal resistance associated with the age/incubation duration of the ascospores. The influence of the age of *N. fischeri* ascospores on their thermoresistance in apple juice (12.3 °Brix, pH 3.8) and grape juice (16.6 °Brix, pH 3.4) was investigated by Conner and Beuchat et al. [71]. When subjected to a pasteurization temperature of 85 °C, the *D*-values for apple juice increased from 6.9 to 13.2 min as the age of the ascospores ranged from 16 to 21 days. Similarly, under identical conditions but with grape juice, these values ranged from 5.8 to 10.1 min. The effect of ascospore age on resistance to thermal inactivation was also investigated by Tournas et al. [50]. Heating of 1, 2, 3 and 6 month old ascospores in deionized water resulted in an increase in *D*-values as the age of the ascospores also increased (**Figure A.1** - Appendix A). The notably lower heat resistance observed in one-month-old spores was likely due to incomplete maturation and a higher water content, a known factor that facilitates heat-induced destruction [50]. This finding aligns with previous studies indicating that older fungal spores exhibited higher resistance compared to the younger ones.

Therefore, it is conceivable that the variability in ascospore ages within the inoculum employed in the current study may have exerted an influence on certain obtained *D*-values. However, it should be noted that rigorous monitoring of this parameter was lacking throughout the project, making it inconclusive as to which *D*-values correspond to the highest ascospore maturation. For future research endeavors, it is recommended that measures should be taken to ensure

uniformity in the age of the ascospores within the inoculum. This can be accomplished by refrigerating the PDA plates once they have reached the desired age, inducing spore hibernation, and thereby maintaining a consistent age profile.

4.3 Determination of the sterilization (F) value

To determine the optimal commercial heat treatment to be applied to peach preparations for the eradication of *N. fischeri* ascospores, the sterilization value (F) of the product was computed using the D -values corresponding to each production binomial. The thermal treatments applied by the Food Industry usually vary between a temperature of 90-95 °C for 2-5 min. The selected sterilization temperature was 95 °C, as it is the maximum applicable temperature used. However, given that the D -values calculated in this study were exclusively available for 85, 90, and 93 °C, there is a need to derive the D -value for 95 °C. This is possible by using the equation of the TDT-equivalent curve present in **Figure B.2** (Appendix B).

In this case, as detailed in sub-section 3.2.5, the computation of the sterilization value (F) at 95 °C involves the application of a logarithmic reduction of 4. This calculation is subsequently governed by equation 5.

$$F_{95} = 4D_{95} \quad (5)$$

Table 10 is a summary of the D and F -values, at 95 °C, when the number of decimal reductions is 4.

Table 10. Values of D_{95} and F_{95} when $\gamma = 4$ for the sugared and sweetened peach preparations.

Fruit preparation	Production binomial	D_{95} (min)	F_{95} (min)
Sugared peach	30 °C/1 h	5.5	22
	50 °C/1 h	5.9	24
	30 °C/3 h	8.0	32
	50 °C/3 h	8.7	35
Sweetened peach	30 °C/1 h	5.9	24
	50 °C/1 h	6.5	26
	30 °C/3 h	7.5	30
	50 °C/3 h	9.8	39

Taking into consideration that only free *N. fischeri* ascospores are present, it can be deduced that, for the sugared preparation a pasteurization process of at least 35 min at 95 °C is necessary to sterilize the product if exposed to a production binomial below 50 °C for 3 h. In the case of the sweetened peach preparation, the minimum pasteurization time to be applied to ensure safety is 39 min. However, it must be acknowledged that factory-produced peach preparations might contain both free ascospores and asci. Given that this experiment employed a suspension containing only free ascospores, there exists a belief that the applied treatments may not be enough for product sterilization. Furthermore, it should be noted that ascospores and asci from fungi occurring in nature might exhibit greater resistance compared to those obtained through incubation, potentially necessitating an extension of pasteurization time when this heat treatment is made at a big scale [36].

Upon analyzing **Table 10**, a notable conclusion arises: within the same fruit preparation, the sterilization time increases alongside the augmentation of both production temperature and time. However, this *F* parameter seems to exhibit an inversely proportional behavior with the decimal reduction value in a way that, despite the sweetened preparation generally having lower *D*-values than the sugared one, indicating relatively lower thermal resistance, it requires longer sterilization durations. This phenomenon could be attributed to the higher *Z*-values for the sweetened preparation as opposed to the sugared one.

It is evident that the sterilization values obtained in this project exceed the levels typically employed at an industrial scale. When contemplating the most rigorous pasteurization regimen usually utilized by the Food Industry, which involves heating at 95 °C for 5 min, it becomes apparent that such heat treatment would not be adequate to attain a survival rate of fewer than 1 ascospore per 1000 packages ($\gamma = 4$). This is due to the fact that all the *F*-values derived from this study exceed 5 min. Nonetheless, it is imperative to take into consideration that industrially manufactured preparations may incorporate preservatives that were not included in the fruit preparations used in this study. The inclusion of preservatives, such as potassium sorbate, represents a technique aimed at safeguarding fruit products against microbiological degradation.

The effect of common preservatives on the thermal death rates of *N. fischeri* ascospores at 85 °C in mango and grape juices was investigated by Rajashekhara et al. [63]. In the presence of a preservative, a reduction of approximately 60 % was noted in the time required for the destruction of 1 logarithmic unit of ascospores, as compared to the control treatment with no preservative. Specifically, in the case of mango juice, the introduction of potassium sorbate resulted in a decrease in *D*-values from 63.5 to 29.4 min. In grape juice, the *D*-value for the experiment without preservatives was 69.3 min, whereas with the addition of potassium

sorbate, it reduced to 25.07 min. It can therefore be inferred that, in the context of Industrial preparations, the inclusion of this preservative may result in lower decimal reduction values than those derived from this study. As a result, the F_{95} values could decrease, aligning more closely with the pasteurization regimen employed by companies at an industrial level, which corresponds to a treatment at 95 °C for 5 min in the most stringent scenario.

In the event of a higher level of safety consideration, to a pasteurization temperature of 95 °C, the number of decimal reductions can be increased to ensure a safer product. The F -values at 95 °C for higher decimal reductions are displayed in **Table 11**.

Table 11. Values of F_{95} when $\gamma = 6, 8, 10$ for the sugared and sweetened peach preparations.

Fruit preparation	Production binomial	F_{95} (min)		
		$\gamma = 6$	$\gamma = 8$	$\gamma = 10$
Sugared peach	30 °C/1 h	33	44	55
	50 °C/1 h	36	47	59
	30 °C/3 h	48	64	80
	50 °C/3 h	52	70	87
Sweetened peach	30 °C/1 h	36	47	59
	50 °C/1 h	39	52	65
	30 °C/3 h	45	60	75
	50 °C/3 h	59	78	98

As expected, with an increase in the number of logarithmic reductions, there is a corresponding rise in the sterilization (F) value. This phenomenon occurs because, as a more comprehensive microbial reduction is desired, the related sterilization process must be more intense, resulting in a higher sterilization value.

5 Conclusions

After analyzing the data acquired, it can be concluded that the primary objectives set forth for this dissertation project have been achieved. The study investigated the response of *N. fischeri* in sugared and sweetened peach preparations, featuring both low and high a_w levels, when exposed to various production and pasteurization time/temperature binomials. Furthermore, the thermal death kinetic parameters (D and Z) were ascertained for each condition, enabling the calculation of the sterilization value (F) for each respective scenario.

Concerning the response of this microorganism to varying pasteurization time/temperature binomials, it can be deduced that the D -value diminishes with increasing temperature. This observation was consistent across all the investigated preparations and various production time/temperature pairings. It is evident that as the pasteurization temperature escalates from 85 to 90 °C and ultimately to 93 °C, the corresponding D -value consistently decreases.

Upon analyzing the impact of the production time/temperature binomials, it can be generally observed that their increase leads to higher decimal reduction values (D). Specifically, when the production time is tripled for the same production temperature (1 h to 3 h), the D -values can increase over 1 to 7.8 min; when the production temperature is heightened while maintaining the same production time (30 °C to 50 °C), the corresponding increase in D -values can be from over 1.9 to 28.9 min. It can be concluded that, in general, extending the production time results in a more significant increase in the thermal resistance of *N. fischeri* ascospores compared to raising the production temperature. An exception to this trend was observed in the case of the 30 °C/3 h binomial for the sugared peach preparation at a pasteurization temperature of 90 °C, where the D -value in such circumstances ($D_{90}^{30\text{ °C}/3\text{ h}}=39.1\text{ min}$) surpassed the D -value obtained when the production temperature was elevated to 50 °C for the same time ($D_{90}^{50\text{ °C}/3\text{ h}}=37.5\text{ min}$).

When assessing the influence of water activity and sugar concentration on the organism's resistance, it was generally observed that the sugared preparation (42.8 °Brix, a_w 0.94) yielded higher D -values compared to the sweetened preparation (6.6 °Brix, a_w 0.99). This divergence can be attributed to the lower Brix content and higher water activity in the latter. These findings show a protective effect on the thermal resistance of *N. fischeri* ascospores, which is proportionate to the percentage of solids present. However, it is worth noting that certain results did not align with this pattern. For the pasteurization temperature of 90 °C and the production binomials of 30 °C/1 h and 50 °C/1 h, the D -values for the sugared preparation

($D_{90}^{30^{\circ}\text{C}/1\text{h}}=16.3\text{ min}$, $D_{90}^{50^{\circ}\text{C}/1\text{h}}=18.7\text{ min}$) exceeded those of the sweetened preparation ($D_{90}^{30^{\circ}\text{C}/1\text{h}}=14.1\text{ min}$, $D_{90}^{50^{\circ}\text{C}/1\text{h}}=15.9\text{ min}$). Additionally, at the temperature of 93 °C, particularly for the 50 °C/3 h binomial, the preparation with the lowest sugar concentration exhibited a higher D -value (11.3 min) than the preparation with the highest °Brix value (10.4 min). Observing the Z -values, these were in general higher for the sweetened preparation. A higher Z -value indicates that the microorganism is more heat-resistant, as it requires a greater temperature variation to achieve the same effect. This conclusion seems to bring some contradiction to what was previously stated.

Regarding the influence of pH on thermal death kinetic parameters, in the present study, the preparations examined did not exhibit a sufficiently significant difference in pH values to account for their impact on the thermal resistance of *N. fischeri*. Instead, the influence on the D -values is attributed more to the variation in the concentration of soluble solids rather than the small difference in pH.

The preparation of the inoculum also emerged as an important factor in influencing the acquired D -values. Given that the inoculum employed in the experiments was cultivated throughout the research period, both the concentration and age of the ascospores exhibited some variation. Recognizing that ascospores become more resistant to heat treatment as they mature, a more rigorous monitoring of this parameter over the course of the project would have been essential. This oversight has rendered it inconclusive as to which D -values correspond to higher ascospore maturation and concentration.

Lastly, to determine the optimal commercial heat treatment for the eradication of *N. fischeri* fungal ascospores in peach preparations, the sterilization value (F) of the product was calculated utilizing the corresponding D -values for each production binomial. The Food Industry typically applies heat treatments ranging between 90-95 °C for 2-5 min. Consequently, the chosen sterilization temperature was 95 °C. Nevertheless, under these conditions, all sterilization values obtained in this project (ranging from 22 to 39 min) surpass the commonly employed levels used in the industrial-scale process. However, it is imperative to take into consideration that industrially manufactured preparations may incorporate preservatives that were not included in the fruit preparations used in this study. The inclusion of these preservatives may lead to lower decimal reduction values compared to those derived from this study, thereby resulting in F_{95} values that are also lower than those obtained.

6 Assessment of the work done

6.1 Objectives Achieved

After analyzing the data obtained, it can be concluded that the main objectives proposed for this dissertation project were achieved. The behavior of *N. fischeri* was investigated in sweetened and sugared Frulact peach preparations with low and high a_w values when exposed to various production and pasteurization time/temperature binomials. Furthermore, the thermal death kinetic parameters (D and Z) were determined for each condition, allowing the simulation of pasteurization time/temperature binomials to be employed in the factory, based on the sterilization values (F) obtained.

6.2 Future work

For future work, it is recommended that some of the tests be repeated to gain a more comprehensive understanding of the microbial behavior of *N. fischeri* in Frulact preparations. Consequently, conducting a larger number of replicates is desirable, given that fungal counts are consistently associated with significant variability and potential error. Additionally, investigating various production temperatures is suggested to better comprehend the influence of this stage on the enhancement or reduction of *N. fischeri* thermal resistance. This is especially pertinent as the production temperatures utilized in this study are favorable to the growth and proliferation of this fungus. Due to the inability to examine alternative fruit matrices, it is imperative to assess identical binomials of production and pasteurization for preparations with differing pH levels, as this parameter remained unexplored in this particular study. It is also advisable to explore the impact of the addition of preservatives on the thermal resistance of the microorganism and, separately, to investigate the influence of ascospore age on the thermal inactivation.

6.3 Final Assessment

In summary, this semester's work has deepened my understanding of the crucial importance of comprehending the inactivation values of filamentous fungi. I've developed a clear understanding of the diverse factors affecting these organisms' thermal resistance for achieving industrial-level food safety. This knowledge not only contributes to our field but also has practical implications, serving as a solid foundation for future research and safer food production processes.

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Annex A - Additional informational regarding the influence of ascospore age

The investigation of the impact of *N. fischeri* ascospore age on resistance to thermal inactivation was conducted by Tournas et al. [50]. **Figure A.1** illustrates the fluctuation in *D*-values when 1, 2, 3, and 6 months old ascospores were subjected to heating at 88 °C in deionized water. Within this experimental set, these values ranged from 7.4 to 15.5 min.

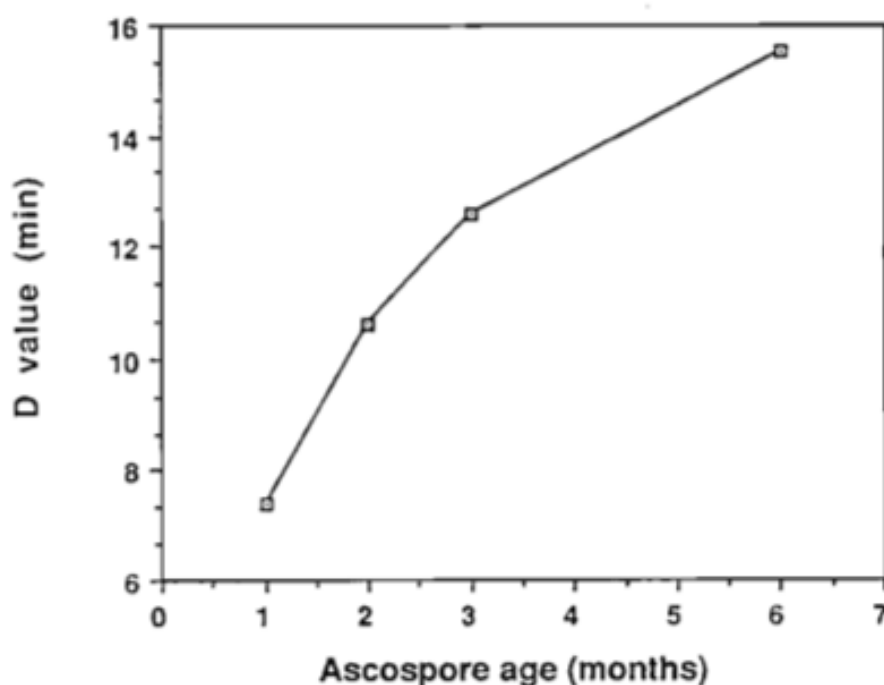


Figure A. 1. Effect of ascospore age of *N. fischeri* on heat resistance (*D*-value); heating at 88 °C in deionized water.

Appendix A - Microbial load associated with the production and pasteurization binomials

The data in the Tables present in this appendix refer to the microbial load associated with the production and pasteurization time/temperature binomial for each preparation. The data is in accordance with ISO 7218:2007, i.e. only colony numbers between 10 and 300 have been taken into account.

After the incubation period, the number of CFU on each Petri dish was calculated considering the dilution used for each pasteurization time, which could vary from -1 to -3. All the values obtained for each pasteurization time resulted from the mean of three replicates. Some sampling points were discarded to obtain the best fit of the lines, represented by the value of R^2 , while obtaining the value of D (appendix B).

Thus, in the following Tables, the logarithm of each of these values is presented, as well as the logarithm of the associated standard deviation.

Table A. 1. Microbial load of the sugared peach preparation calculated using the mean logarithm of CFU/g and respective standard deviation, for the production binomial of 30 °C/1 h and for the respective pasteurization binomials.

Temperature (°C)	Pasteurization time (min)	30 °C/1 h	
		Mean (log CFU/g)	Standard deviation
85	0	4.372	0.014
	1	4.318	0.033
	2	4.312	0.048
	3	4.232	0.010
	4	-	-
	5	4.194	0.042
90	0	4.203	0.059
	1	4.090	0.065
	2	4.012	0.063
	3	3.922	0.056
	4	3.800	0.074
	5	3.898	0.067
93	0	4.054	0.034
	2	3.890	0.074
	4	3.572	0.087
	6	3.154	0.061
	8	2.965	0.084
	10	2.713	0.024

Table A. 2. Microbial load of the sugared peach preparation calculated using the mean logarithm of CFU/g and respective standard deviation, for the production binomial of 50 °C/1 h and for the respective pasteurization binomials.

Temperature (°C)	Pasteurization time (min)	50 °C/1 h	
		Mean (log CFU/g)	Standard deviation
85	0	4.106	0.062
	1	4.083	0.045
	2	4.028	0.041
	3	3.987	0.061
	4	3.956	0.050
	5	3.986	0.059
90	0	4.152	0.028
	1	4.027	0.039
	2	3.917	0.093
	3	3.902	0.102
	4	3.853	0.058
	5	3.818	0.022
93	0	4.089	0.030
	2	3.992	0.034
	4	3.747	0.035
	6	3.476	0.166
	8	3.176	0.079
	10	2.878	0.091

Table A. 3. Microbial load of the sugared peach preparation calculated using the mean logarithm of CFU/g and respective standard deviation, for the production binomial of 30 °C/3 h and for the respective pasteurization binomials.

Temperature (°C)	Pasteurization time (min)	30 °C/3 h	
		Mean (log CFU/g)	Standard deviation
85	0	4.227	0.038
	1	4.220	0.041
	2	4.208	0.037
	3	4.181	0.020
	4	-	-
	5	4.141	0.017
90	0	4.161	0.029
	1	4.152	0.035
	2	-	-
	3	4.080	0.049
	4	4.091	0.024
	5	4.026	0.033
93	0	4.088	0.039
	2	3.732	0.007
	4	3.452	0.018
	6	3.388	0.030
	8	3.115	0.115
	10	2.903	0.107

Table A. 4. Microbial load of the sugared peach preparation calculated using the mean logarithm of CFU/g and respective standard deviation, for the production binomial of 50 °C/3 h and for the respective pasteurization binomials.

Temperature (°C)	Pasteurization time (min)	50 °C/3 h	
		Mean (log CFU/g)	Standard deviation
85	0	4.199	0.040
	1	4.188	0.032
	2	4.130	0.030
	3	4.111	0.029
	4	4.118	0.046
	5	4.134	0.020
90	0	4.105	0.041
	1	4.100	0.039
	2	-	-
	3	4.050	0.027
	4	4.034	0.031
	5	3.960	0.035
93	0	4.013	0.040
	2	-	-
	4	3.498	0.100
	6	3.489	0.078
	8	3.202	0.050
	10	-	-

Table A. 5. Microbial load of the sweetened peach preparation, calculated using the mean logarithm of CFU/g and respective standard deviation, for the production binomial of 30 °C/1 h and for the respective pasteurization binomials.

Temperature (°C)	Pasteurization time (min)	30 °C/1 h	
		Mean (log CFU/g)	Standard deviation
85	0	4.157	0.017
	1	4.131	0.028
	2	4.024	0.033
	3	-	-
	4	3.977	0.023
	5	3.925	0.064
90	0	4.112	0.042
	1	-	-
	2	3.855	0.077
	3	3.881	0.044
	4	3.806	0.046
	5	3.794	0.072
93	0	4.081	0.045
	2	4.020	0.026
	4	3.491	0.028
	6	-	-
	8	2.751	0.058
	10	2.713	0.024

Table A. 6. Microbial load of the sweetened peach preparation, calculated using the mean logarithm of CFU/g and respective standard deviation, for the production binomial of 50 °C/1 h and for the respective pasteurization binomials.

Temperature (°C)	Pasteurization time (min)	50 °C/1 h	
		Mean (log CFU/g)	Standard deviation
85	0	4.109	0.031
	1	4.071	0.042
	2	4.044	0.041
	3	-	-
	4	3.967	0.048
	5	-	-
90	0	4.036	0.042
	1	3.951	0.022
	2	3.939	0.048
	3	3.917	0.039
	4	3.786	0.064
	5	-	-
93	0	3.987	0.030
	2	4.042	0.034
	4	3.585	0.055
	6	3.155	0.039
	8	2.998	0.113
	10	2.851	0.076

Table A. 7. Microbial load of the sweetened peach preparation, calculated using the mean logarithm of CFU/g and respective standard deviation, for the production binomial of 30 °C/3 h and for the respective pasteurization binomials.

Temperature (°C)	Pasteurization time (min)	30 °C/3 h	
		Mean (log CFU/g)	Standard deviation
85	0	4.138	0.026
	1	4.132	0.033
	2	4.111	0.019
	3	4.098	0.023
	4	4.038	0.020
	5	4.029	0.008
90	0	4.082	0.029
	1	4.070	0.020
	2	3.994	0.020
	3	3.989	0.040
	4	3.980	0.031
	5	3.901	0.038
93	0	3.994	0.011
	2	3.533	0.022
	4	3.423	0.066
	6	3.341	0.035
	8	2.905	0.144
	10	-	-

Table A. 8. Microbial load of the sweetened peach preparation, calculated using the mean logarithm of CFU/g and respective standard deviation, for the production binomial of 50 °C/3 h and for the respective pasteurization binomials.

Temperature (°C)	Pasteurization time (min)	50 °C/3 h	
		Mean (log CFU/g)	Standard deviation
85	0	4.151	0.025
	1	4.108	0.031
	2	4.115	0.020
	3	4.096	0.042
	4	4.055	0.031
	5	4.045	0.049
90	0	4.075	0.026
	1	4.070	0.016
	2	-	-
	3	4.020	0.017
	4	4.004	0.018
	5	3.895	0.042
93	0	3.947	0.039
	2	3.623	0.053
	4	3.435	0.044
	6	3.563	0.036
	8	3.002	0.086
	10	3.059	0.049

Appendix B - Determination of thermal death parameters

In order to accurately determine the thermal death kinetics parameters (D and Z) of *Neosartorya fischeri*, a comprehensive analysis was conducted using the data derived from the microbiological tests outlined in Appendix A. This process involved a series of steps to derive the necessary parameters.

To start, the investigation focused on establishing the D -value for the different preparations. This was achieved by plotting a graph of the average logarithmic values of Colony Forming Units per gram (CFU/g) vs the corresponding pasteurization time, across different temperature conditions (**Figure B.1**). Each combination of pasteurization time and temperature was treated as a separate dataset. By employing a linear regression analysis, equations were derived to describe the lines that best fit the data points. The slopes of these lines (**Figure B.2**) were particularly significant, as they were indicative of the rate at which microbial inactivation occurred over time. To calculate the D -value, which represents the time it takes for a particular microbial population to reduce by one logarithmic cycle, the negative inverse of each line's slope was computed.

The resulting D -values were tabulated in **Tables B.1** and **B.2**.

With the D -values obtained for various pasteurization binomials, the focus shifted to the assessment of the Z -value for each specific production time/temperature combination. To accomplish this, a graph was generated where the logarithm of the previously calculated D -values was plotted against their corresponding pasteurization temperatures (**Figure B.3**). Employing linear regression analysis again, a new line was derived, characterized by its slope. Where its slope enables the calculation of the Z -value, which signifies the change in temperature required to alter the D -value by a factor of ten. The Z -value was determined by taking the negative inverse of the slope from the linear regression.

The calculated Z -values were then presented in **Tables B.3** and **B.4**.

Table B. 1. Calculations for the determination of D for each binomial of the sugared peach preparation, accompanied by the equations of the corresponding lines and regressions.

Production binomial	Pasteurization binomial	Line equation	R^2	D (min)
30 °C/1 h	85 °C/5 min	$y=-0.0361x+4.3651$	0.9384	27.7
	90 °C/5 min	$y=-0.071x+4.1647$	0.8401	14.1
	93 °C/10 min	$y=-0.1414x+4.0984$	0.9865	7.1
50 °C/1 h	85 °C/5 min	$y=-0.0291x+4.0973$	0.8438	34.4
	90 °C/5 min	$y=-0.063x+4.1022$	0.9060	15.9
	93 °C/10 min	$y=-0.1253x+4.186$	0.9810	8.0
30 °C/3 h	85 °C/5 min	$y=-0.0179x+4.2351$	0.9647	55.9
	90 °C/5 min	$y=-0.0256x+4.1685$	0.9105	39.1
	93 °C/10 min	$y=-0.112x+4.0063$	0.9738	8.9
50 °C/3 h	85 °C/5 min	$y=-0.0158x+4.186$	0.6287	63.3
	90 °C/5 min	$y=-0.0267x+4.1192$	0.8923	37.5
	93 °C/10 min	$y=-0.0962x+3.9833$	0.9472	10.4

Table B. 2. Calculations for the determination of D for each binomial of the sweetened peach preparation, accompanied by the equations of the corresponding lines and regressions.

Production binomial	Pasteurization binomial	Line equation	R^2	D (min)
30 °C/1 h	85 °C/5 min	$y=-0.0466x+4.1547$	0.9497	21.5
	90 °C/5 min	$y=-0.0613x+4.0612$	0.8310	16.3
	93 °C/10 min	$y=-0.156x+4.1597$	0.9563	6.4
50 °C/1 h	85 °C/5 min	$y=-0.0353x+4.1095$	0.9965	28.3
	90 °C/5 min	$y=-0.0535x+4.0327$	0.8741	18.7
	93 °C/10 min	$y=-0.132x+4.0964$	0.9343	7.6
30 °C/3 h	85 °C/5 min	$y=-0.024x+4.1509$	0.9168	41.7
	90 °C/5 min	$y=-0.0337x+4.0871$	0.9059	29.7
	93 °C/10 min	$y=-0.1185x+3.9133$	0.9178	8.4
50 °C/3 h	85 °C/5 min	$y=-0.0202x+4.1457$	0.9174	49.5
	90 °C/5 min	$y=-0.0317x+4.0954$	0.8193	31.5
	93 °C/10 min	$y=-0.0882x+3.8791$	0.8474	11.3

Table B. 3. Calculations for the determination of Z for each binomial of the sugared peach preparation, accompanied by the equations of the corresponding lines and regressions.

Production binomial	Pasteurization binomial	Log D	Line equation	R^2	Z (°C)
30 °C/1 h	85 °C/5 min	1.442	$y = -0.0725x + 7.628$	0.9781	13.8
	90 °C/5 min	1.149			
	93 °C/10 min	0.850			
50 °C/1 h	85 °C/5 min	1.536	$y = -0.078x + 8.1823$	0.9880	12.8
	90 °C/5 min	1.201			
	93 °C/10 min	0.902			
30 °C/3 h	85 °C/5 min	1.747	$y = -0.0926x + 9.6985$	0.7852	10.8
	90 °C/5 min	1.592			
	93 °C/10 min	0.951			
50 °C/3 h	85 °C/5 min	1.801	$y = -0.0927x + 9.7458$	0.8619	10.8
	90 °C/5 min	1.573			
	93 °C/10 min	1.017			

Table B. 4. Calculations for the determination of Z for each binomial of the sweetened peach preparation, accompanied by the equations of the corresponding lines and regressions.

Production binomial	Pasteurization binomial	Log D	Line equation	R^2	Z (°C)
30 °C/1 h	85 °C/5 min	1.332	$y = -0.0613x + 6.5958$	0.8117	16.3
	90 °C/5 min	1.213			
	93 °C/10 min	0.807			
50 °C/1 h	85 °C/5 min	1.452	$y = -0.068x + 7.2739$	0.8801	14.7
	90 °C/5 min	1.272			
	93 °C/10 min	0.879			
30 °C/3 h	85 °C/5 min	1.620	$y = -0.0809x + 8.5622$	0.7999	12.4
	90 °C/5 min	1.472			
	93 °C/10 min	0.926			
50 °C/3 h	85 °C/5 min	1.695	$y = -0.0758x + 0.1914$	0.8732	13.2
	90 °C/5 min	1.499			
	93 °C/10 min	1.055			

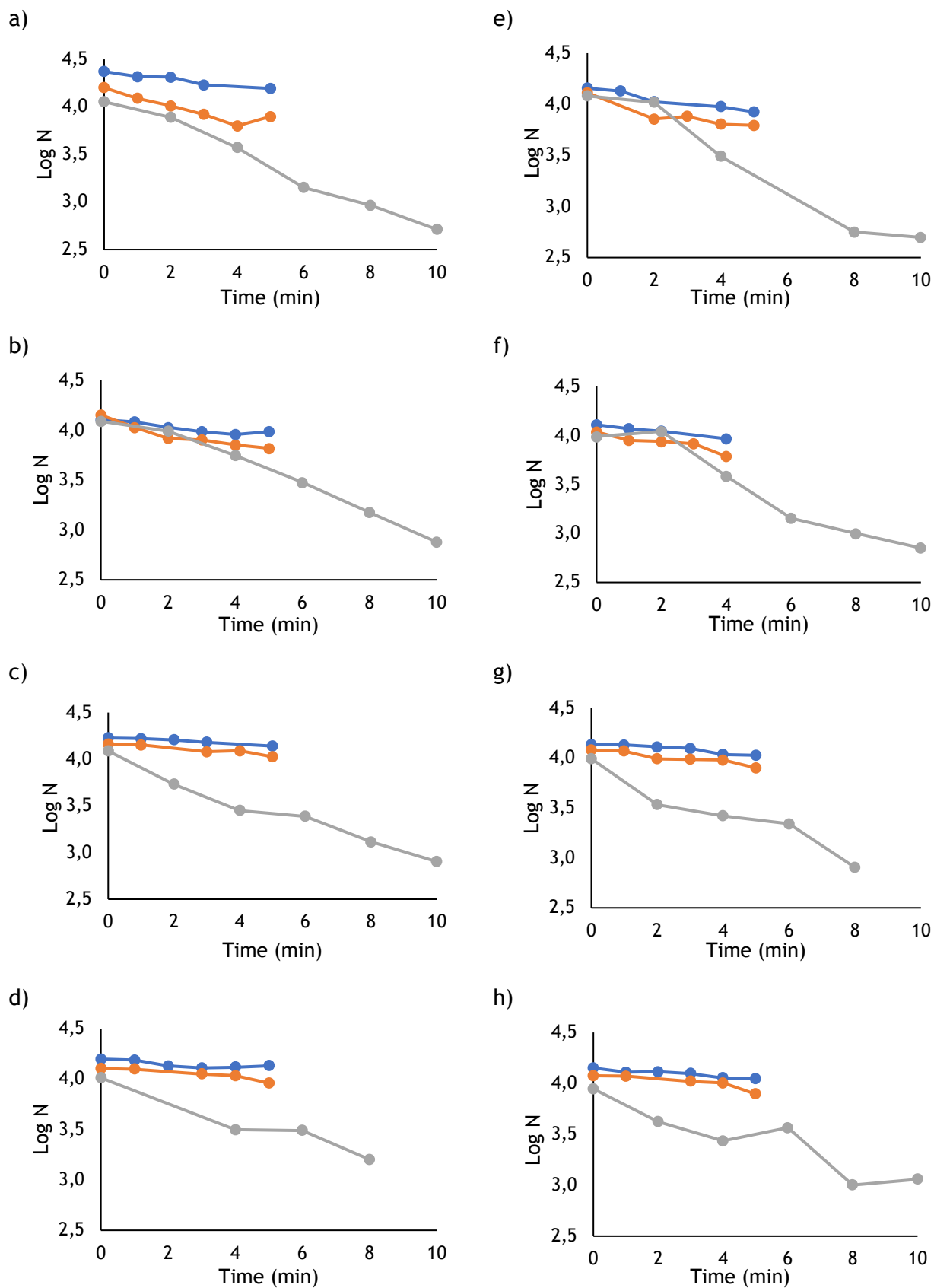


Figure B. 1. Thermal death rate curves for *N. fischeri* ascospores heated at 85 °C, 90 °C and 93 °C in sugared peach: a) 30 °C/1 h, b) 50 °C/1 h, c) 30 °C/3 h, d) 50 °C/1 h; and in sweetened peach: e) 30 °C/1 h, f) 50 °C/1 h, g) 30 °C/3 h, h) 50 °C/1 h.

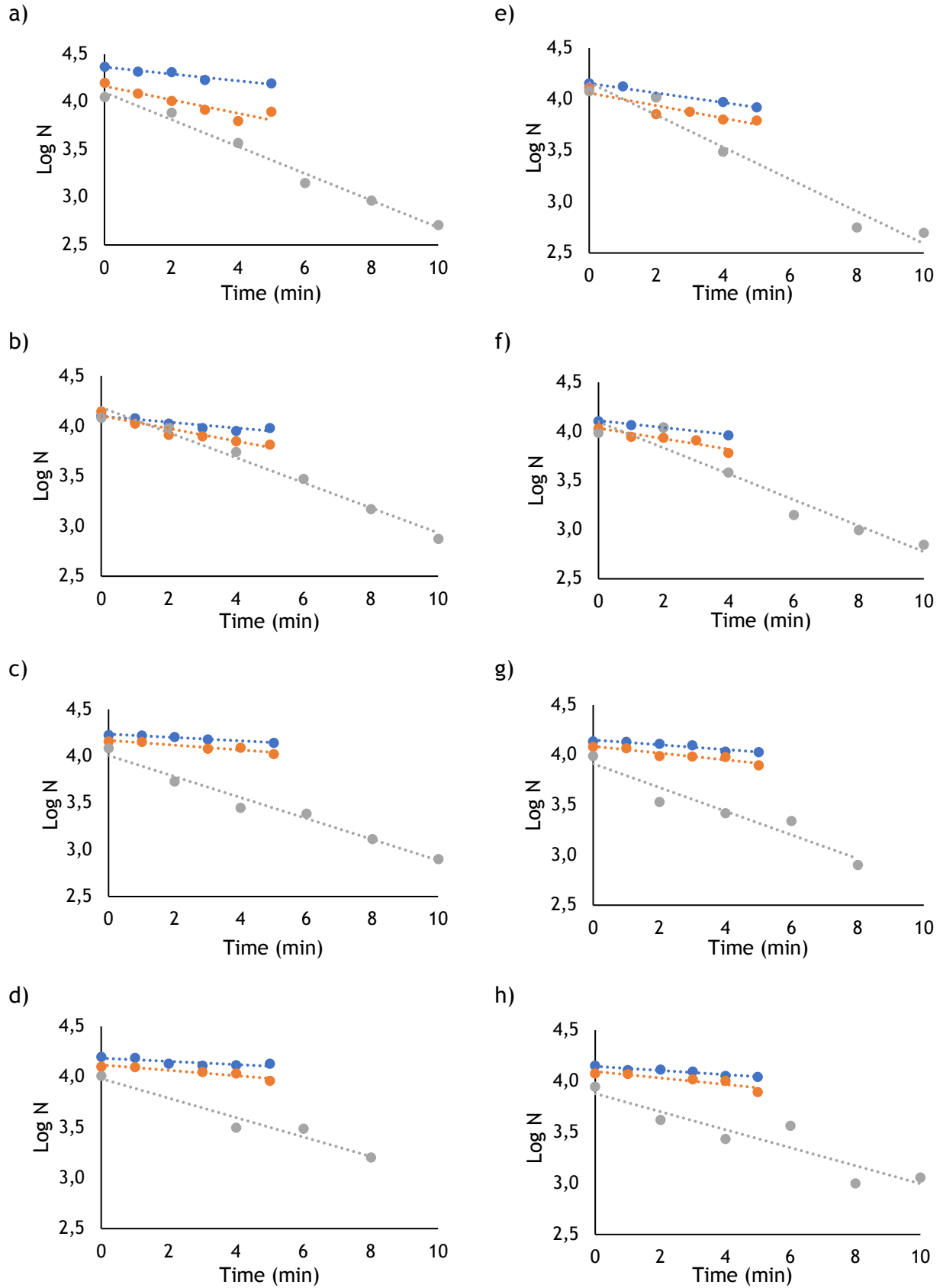


Figure B. 2. Linearized thermal death rate curves for *N. fischeri* ascospores heated at 85 °C, 90 °C and 93 °C in sugared peach: a) 30 °C/1 h, b) 50 °C/1 h, c) 30 °C/3 h, d) 50 °C/1 h; and in sweetened peach: e) 30 °C/1 h, f) 50 °C/1 h, g) 30 °C/3 h, h) 50 °C/1 h.

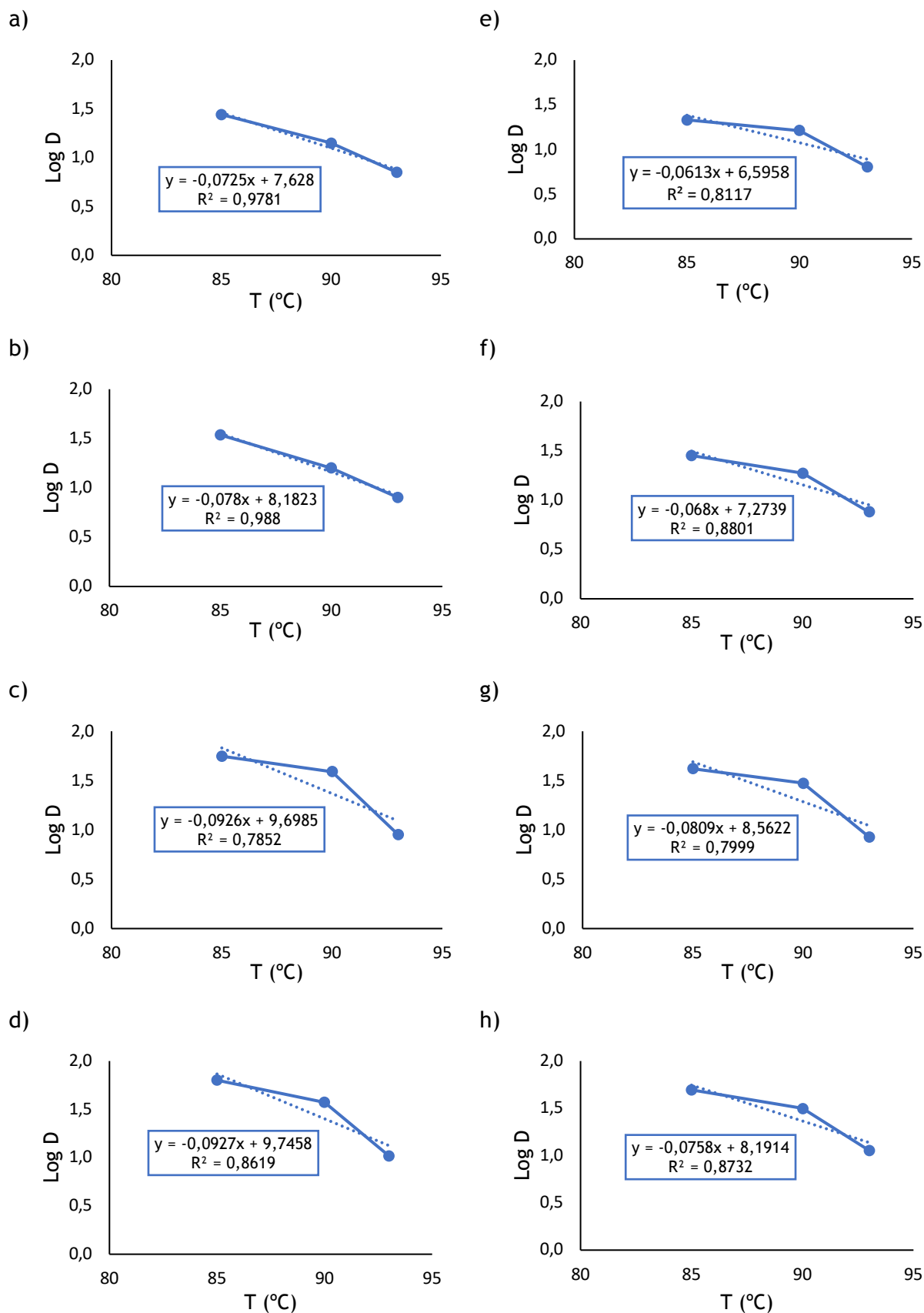


Figure B. 3. TDT curves for *N. fischeri* in sugared peach: a) 30 °C/1 h, b) 50 °C/1 h, c) 30 °C/3 h, d) 50 °C/1 h; and in sweetened peach: e) 30 °C/1 h, f) 50 °C/1 h, g) 30 °C/3 h, h) 50 °C/1 h.

Appendix D - ANOVA: Single factor test

For each preparation, statistical differences were tested between the production binomials used, for the same pasteurization binomial, using the *Single Factor ANOVA* test with a significance level of 0.05.

The only values of the pasteurization binomials that are statistically significant are the ones where $p < 0.05$ and $F > F_{crit}$.

Table D. 1. Results of the *Anova: single factor* test between the different production binomials of the sugared peach preparation.

Pasteurization binomial	P-value	F	F crit
85 °C/5 min	5.07316E-05	6.57598507	2.35380896
90 °C/5 min	5.36574E-07	9.73267724	2.35380896
93 °C/10 min	1.40879E-35	161.049614	2.35380896

Table D. 2. Results of the *Anova: single factor* test between the different production binomials of the sweetened peach preparation.

Pasteurization binomial	P-value	F	F crit
85 °C/5 min	0.003024565	4.02140847	2.35380896
90 °C/5 min	0.005455584	3.66839078	2.35380896
93 °C/10 min	1.58684E-25	72.8798912	2.35380896