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Quantification of the effect of process, product and storage conditions on the spoilage risk of pasteurized fruit based products by Heat-Resistant Moulds (HRMs)

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Bioscience Engineering

Dutch title: “Kwantificering van het effect van proces-, product- en bewaarcondities op het risico op bederf van gepasteuriseerde, op fruit gebaseerde producten door hitteresistente schimmels”.

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Juliana Lane Paixão dos Santos

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List of Abbreviations

°Brix	% Soluble solids
AIC	Akaike's information criterion
AIJN	European fruit juice association
ANOVA	Analysis of variance
AP	Apple puree
aPDA	Acidified potato dextrose agar
a_w	Water activity
BIC	Bayesian Information Criterion
CDFs	Cumulative distributions functions
Cfu	Colony forming units
Ch	Chapter
CI	Confidence Interval
CIP	Cleaning-in-place
ClO_2	Chlorine dioxide
CO_2	Carbon dioxide
COP	Cleaning-out-of-place
CYA	Czapek Yeast Autolysate agar
FAO	Food and Agricultural Organization
FDA	The U.S. Food and Drug Administration
GAP	Good Agricultural Practices
GHPs	Good Hygiene Practices
GMPs	Good Manufacturing Practices
HACCP	Hazard Analysis Critical Control Point
HHP	High hydrostatic pressure
HPTP	High-pressure processing
HRMs	Heat-resistant moulds
HRP	Heat resistant puree
HSP	Heat sensitive puree
MAP	Modified atmospheric packaging
MEA	Malt Extract Agar
Min	Minutes
Mg	Milligrams
Mm	Millimeters
Nf	*Neosartorya fischeri*

NFC	Not from concentrate
O_2	Oxygen
PDA	Potato Dextrose Agar
PET	Polyethylene terephthalate
ppm	Parts per million
QMRA	Quantitative Microbial Exposure Risk Assessment
QMSRA	Quantitative Microbial Spoilage Risk Assessment
RMSE	Root Mean Square Error
Sec	Seconds
SP_C	Strawberry puree concentrate
SP_{SS}	Single strength strawberry puree
SSO	Specific spoilage organism
SSOPs	Sanitation Standard Operating Procedures
T	Temperature (°C)
TOS	Trehalose-based oligosaccharides
TS	Ultrasonication
t_v	Time to mycelial visible growth
WHO	World Health Organization

Objectives and Outline

Fruit concentrates (juices and purees) are widely used as ingredient in a range of food and beverages products. A diversity of fruit based products are currently available on the market, including juices, ready-to-eat purees, smoothies, baby food, dairy based products and others. These products typically present high acidity (pH<4.0), relative high sugar content, low O_2 content, they are commonly pasteurized and have a shelf-life up to several months. Hence, the conditions found in pasteurized fruit based products (formulation and process) are sufficient to prevent most of the present spoilage microbiota present before pasteurization, such as heat-sensitive moulds, yeasts and bacteria. On the other hand, their spoilage caused by heat-resistant moulds (HRMs) is a topic of great concern of fruit processing industries worldwide, as it can result in economic losses, health issues due to mycotoxin production, besides causing a very negative impact on food brands (Snyder and Worobo, 2018, Snyder et al.,2018). The HRMs not only present an extreme heat resistance, but they can also tolerate various other stress conditions such as high-pressure, high sugar content, high acidity and modified atmospheres, which make them the target specific spoilage organisms for this type of food product.

The HRMs heat resistance have been investigated for more than eight decades and a vast number of data have been generated. Conversely, few data are currently available regarding the effect of a combination of relevant factors on the HRMs growth and/or on the growth inhibition of such microorganisms. Moreover, the majority of available data focused on assessing mycelial radial growth rates of HRMs. These data are difficult to translate in practical applications, more specific in mould free shelf-lives as food products are considered spoiled as soon as the mycelium is visible for the consumer. In this thesis, the microbial stability of food products is therefore assessed by estimating the time required for the present HRMs ascospores to form visible growth (t_v).

Furthermore, while an impressive number of research on “food safety” has been published in the last decades, the number of scientific publications in the area of “food quality and microbial spoilage” during the same period was much less expressive (Snyder and Worobo, 2018). However, it has been estimated that 25% of the post- harvest food supply may be wasted due to microbial food spoilage (Gram al., 2002) and that 64% of fruit processors in Europe and the United States (during a survey) have experienced spoilage of finished product due to HRMs (Snyder and Worobo, 2018). Therefore, the study (quantification) of the effects of food processing and storage conditions on the microbial spoilage of fruit based products is crucial and necessary to estimate more accurately their risk of spoilage in the food supply chain.

The primary objective of this PhD was to create insights in the effect of various aspects regarding raw material contamination, process, product and storage on the spoilage risk of pasteurized high-acid fruit based products by HRMs. To do so, various data regarding HRMs heat resistance, time to visible growth and growth/no growth conditions were collected. Ultimately a “proof of concept” of a quantitative microbial spoilage risk assessment (QMSRA) approach for HRMs was proposed for fruit purees. To be able to do this, the research was divided into 5 sub-objectives which are listed below and are reflected in 8 chapters which are schematically represented in Figure 1.

Objectives

1. To collect quantitative and qualitative data regarding the HRMs occurrence throughout the processing of pasteurized high-acid fruit products (Ch.2).

2. To assess the inter and intra-species variability of heat resistance and potential subsequent growth of HRMs (Ch.3).

3. To develop an experimental set-up (method) to attain and maintain low-oxygen-levels (<1%) in a solid medium for long incubation periods (Ch. 5).

4. To determine growth limit conditions and to assess the effect of process, product and storage conditions on the time to visible growth of HRMs (Ch.4, Ch.6 and Ch.7).

5. To develop a spoilage risk assessment approach for heat-resistant moulds (HRMs) (Ch.8).

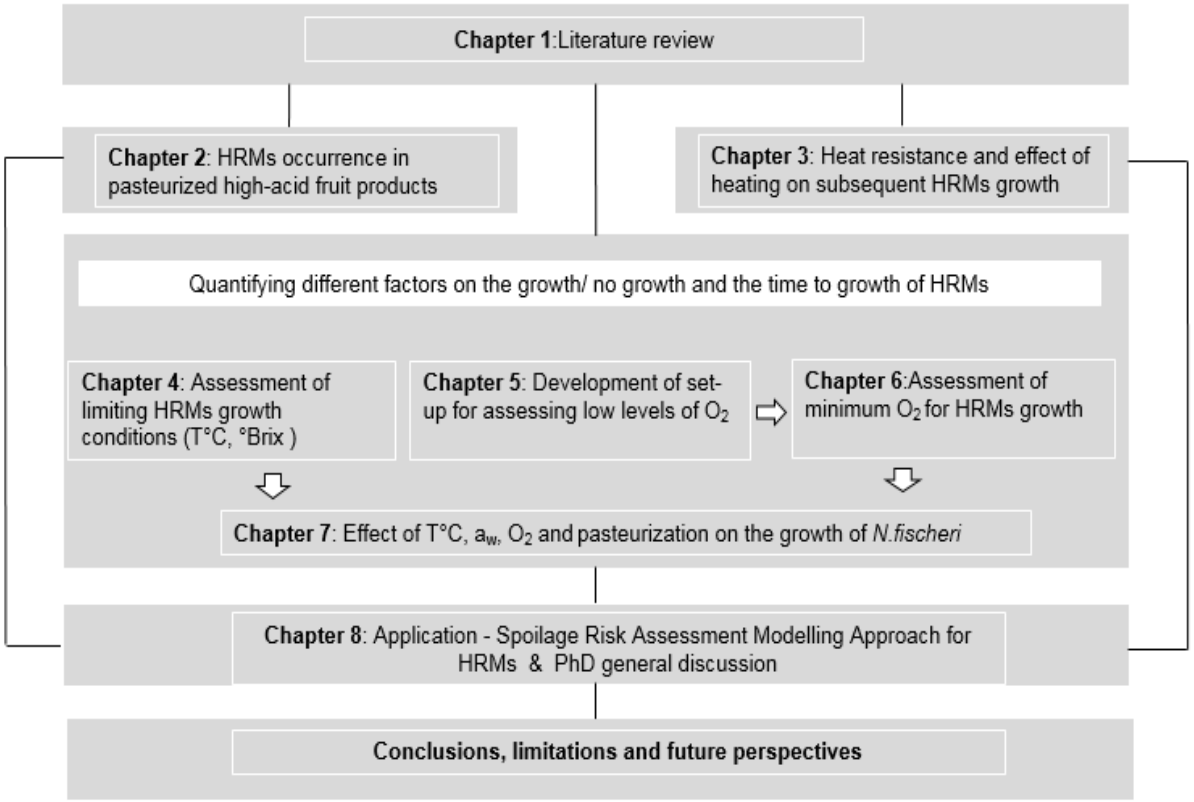

Figure 1**.** Outline of the PhD thesis entitled: “Quantification of the effect of process, product and storage conditions on the spoilage risk of pasteurized fruit based products by Heat-Resistant Moulds (HRMs)”.

Summary – Samenvatting

Summary

Chapter 1 presents a literature review. Firstly, a general introduction of high-acid fruit product characteristics and its economic importance were addressed. In order to introduce the heat-resistant moulds (HRMs) as the specific spoilage organism (SSO) of such products, an overview of the heat resistant microflora is discussed. Next, an extensive review of the different aspects of the HRMs (general characteristics, sources of contamination and physiology aspects) is given. The main strategies currently applied towards the control of HRMs, including recent non-thermal technologies, is summarized. Finally, a comparison of the terminology of quantitative microbial risk assessments (QMRA) applied in food safety and food quality is provided. Additionally, some examples of QMRA intended to determine the risk of food spoilage are given.

Chapter 2 was intended to obtain quantitative and qualitative data of HRMs throughout fruit product processes. To do so, 332 samples from 111 batches were collected and analyzed from three processing plants: strawberry puree, concentrated orange juice and apple puree. HRMs were detected in 96.4% and 59.3% of the batches and samples, respectively. However, the majority of the samples were either not contaminated or presented low levels of HRMs (<10 ascospores/100g). In addition, an assessment on the effect of processing on the contamination levels of HRMs in these products was carried out. While no significant reduction ($p > 0.05$) was observed during the strawberry puree and concentrated orange juice process, a significant decrease on the HRMs levels was found during the processing of apple puree ($p < 0.05$). Twelve HRMs species were morphologically and molecularly identified belonging to four genera - *Byssochlamys*, *Aspergillus* with *Neosartorya*-type ascospores, *Talaromyces* and *Rasamsonia*. Ultimately, the data of contamination levels of ascospores (cfu/100g) were expressed as exponential distributions to allow incorporation during the development of Quantitative Microbial Spoilage Risk Assessment (QMSRA) in Ch. 8.

Chapter 3 aimed to investigate the inter- and intra-species variability of *Byssochlamys* strains regarding (i) their heat resistance and (ii) the effect of heat treatment intensity on subsequent outgrowth. Estimated parameters were obtained after inactivation experiments: inactivation rate, curve shape and the time for first decimal reduction, and after growth experiments: lag times and growth rates. Inter- species variability were observed for both inactivation and growth estimated parameters among *B. fulva* and *B. nivea* strains. Interestingly, some injured ascospores required very long lag times (>20 days) to recover and germinate after heating. The inter-strain variability observed in this study reinforces the importance of considering the

distribution of inactivation and growth parameters in quantitative approach studies. By doing so, strains with marginal behavior will be also taken into account during predictions. The data were further incorporated in a Quantitative Microbial Spoilage Risk Assessment (QMSRA) focused on spoilage of fruit purees by HRMs (Ch. 8).

Chapter 4 intended to investigate the growth/no growth limits regarding maximum sugar concentration (44–59°Brix) and minimum storage temperature (4-14°C) of six HRMs species isolated from fruit products (Ch. 2). The biological variability of individual ascospores regarding their individual time to form visible growth (D=2mm) was quantified and described as parametric statistics distributions. Overall, the HRMs are not likely to grow at T < 10°C. Yet, visible colonies of *N. hiratsukae* were observed at 7°C. At lesser or higher extent, all the HRMs strains showed high tolerance to an increase of sugar content. The most sugar-tolerant strain, *N. udagawae*, was able to grow out at the highest °Brix evaluated (59°/a_w=0.86). Large ranges of individual times to form visible mycelia were mainly observed under conditions at the growth/no growth regions. The obtained data of growth/no growth limits of HRMs strains and the variability of individual ascospores was further used as inputs for a Quantitative Microbial Spoilage Risk Assessment (QMSRA) focusing on spoilage of fruit purees by HRMs (Ch. 8).

Chapter 5 aimed to develop an experimental set-up (method) to attain and maintain low-oxygen-levels (<1%) in a synthetic solid medium for up to two months. This set-up would enable the assessment of the effect of low O_2 levels on the growth of HRMs and was developed into two parts. Firstly, the evolution of dissolved O_2 as a function of depth (0-12 cm) in a solid medium was determined. From these experiments, it became clear that relying on the O_2 gradient created as function of depth in a solid medium was not a good strategy as any change in the O_2 concentration in one layer (at one depth) would influence the overall gradient and the concentrations of O_2 in the other layers. Thereafter, a set-up consisting of obtaining desired levels of dissolved O_2 by adjusting the headspace O_2 levels in 3-cm-deep glass jars via O_2 scavengers was developed. The set-up was effective in maintaining the set O_2 levels for up to two months. Hence, the method was used to assess the growth limiting conditions of HRMs regarding their minimum oxygen requirement (Ch. 6).

Chapter 6 aimed to assess the effect of strict anaerobic conditions and low oxygen (O_2) concentration (0.03%, 0.15%, and 0.90%) on the time to growth of six HRMs species isolated from fruit products (Ch. 2). The assessment was performed in acidified potato dextrose agar (aPDA, pH=3.5) and fruit-based medium. Strict anaerobic conditions and low O_2 levels were set and monitored inside glass jars filled with aPDA, inoculated and stored at 22°C. Gas concentrations were determined via attached Oxydot's® by means of an OxySense®.

Colonies of the HRMs were visible within a short period (3-6 days) at 0.9 and 0.15% O_2. Complete inhibition did not occur even at very low levels of dissolved O_2 (*ca*. 0.01-0.1 % O_2). However, lowering the O_2 to 0.03% did result in significant ($p<0.05$) longer times to visible growth for most of the HRMs. Growth inhibition for the majority of HRMS sp. was observed under strict anaerobic condition. Both, *B. fulva* and *N. fischeri* showed visible growth when inoculated into three fruit-based media (apple, strawberry and orange) at O_2 concentrations of 0.15% and 21%. Due to the extreme HRMs tolerance to low O_2 concentrations, strategies to inhibit their growth should therefore not be based entirely on establishing low headspace O_2 levels. Therefore, the effect of low O_2 in combination with other hurdles (sugars (a_w), storage temperature and pasteurization intensity) was investigated, in order to predict more accurately the growth inhibition of the HRMs in heat treated fruit based products (Ch.7).

Chapter 7 aimed to assess the combined effect of storage temperature (10-30°C), water activity (a_w, 0.87-0.89), oxygen (O_2) level in the headspace (0.15-0.80%) and pasteurization intensity (95°C, 100°C or 105°C/15sec) on the time to visible growth of *Neosartorya fischeri* on acidified Potato Dextrose Agar (aPDA, pH 3.6) for up to 90 days. Thereafter, 13 conditions were selected and assessed in strawberry-puree based medium. Ultimately, the effect of O_2 (0.05 and 1%) and pasteurization intensity (95°C and 105°C/15sec) were evaluated on real fruit purees and concentrates over a 60 day storage period. Storage temperature had the greatest effect on the time to growth of *N. fischeri*. At 10°C, no visible growth was observed for all the conditions, whilst it was present in 37% and 89% of the conditions at 22°C and 30°C, respectively. Pasteurization intensity had only a minor effect on the subsequent outgrowth of *N. fischeri*. Overall, the HRM growth was inhibited when a_w was reduced to 0.870 ± 0.005 in combination to very low headspace O_2 levels (0.15%± 0.10), regardless of the incubation temperature (22° or 30°C) and heat pasteurization intensity. Furthermore, longer times to growth were required when incubation was done at 22°C (range = 27 to 85 days) compared to 30°C (range = 10 to 86 days). The ability of *N. fischeri* to form visible colonies was impaired or delayed in strawberry media in comparison to outgrowth on aPDA. With regards to real fruit purees ($a_w \geq 0.980$), blueberry, raspberry, blackberry and sour cherry purees did not support the growth of *N. fischeri* when the headspace O_2 level was reduced to 0.05%, regardless of the pasteurization intensity. On the other hand, visible colonies were observed in strawberry puree in all the evaluated conditions. No growth was observed in all 14 concentrated fruit purees tested ($a_w \leq 0.860$). The data obtained (growth/no growth) and time to visible growth were further incorporated in a Quantitative Microbial Spoilage Risk Assessment (QMSRA) focusing on spoilage of fruit purees by HRMs (Ch. 8).

Chapter 8 aimed at transferring the obtained results of previous chapters into practical implications and to make some general conclusions and perspectives. Therefore, a “proof-of-concept” of a quantitative microbial spoilage risk assessment (QMSRA) approach for HRMs was proposed. This concept was applied to develop a risk spoilage model due to HRMs at the time of use under various scenarios regarding formulation, processing and storage for three types of packaged fruit purees being representative for a heat sensitive single strength puree (strawberry puree), a heat sensitive concentrated puree (strawberry concentrate) and a heat robust fruit puree (apple puree). Overall, high probabilities of spoilage (≥40%) were obtained in strawberries purees due to *N. fischeri* ascospores after milder pasteurization treatments (85 and 90°C /15sec-60sec) and when stored at ambient temperatures. For such purees the spoilage risk was only effectively reduced by increasing pasteurization intensities at T ≥95°C for 45sec or more and by lowering O_2 and a_w (for concentrates purees). On the other hand, the spoilage risk of apple purees due to *B. nivea* ascospores was predicted to be reduced twofold by increasing the holding time of pasteurizations at 85°C, 90°C and 95°C and by reducing the shelf-life (ca. 1 month). In general, zero risk of spoilage was predicted for all types of purees when they are stored at chilled conditions (<10°C), after high intensity pasteurization (i.e. 100°C /15sec), and at strict anaerobic conditions. Lastly, a general discussion of this PhD is provided followed by final conclusions and perspectives for future research.

Samenvatting

In **Hoofdstuk 1** wordt een literatuuroverzicht gegeven. In eerste instantie wordt een algemene inleiding gegeven over de eigenschappen en het economische belang van fruit met een hoge zuurtegraad. Vervolgens wordt de hitteresistente microbiologie besproken, worden hitteresistente schimmels (HRS) als specifieke bederforganismen geïntroduceerd en worden hun verschillende eigenschappen besproken (algemene eigenschappen, mogelijke besmettingsbronnen, fysiologie). De belangrijkste controlemaatregelen die momenteel worden toegepast, waaronder ook recente niet-thermische technologieën, worden daarna kort samengevat. Tenslotte wordt de terminologie van kwantitatieve risicobeoordelingen die in de voedselveiligheid en voedselkwaliteit worden gebruikt met elkaar vergeleken en worden er enkele voorbeelden gegeven van risicobeoordelingen die uitgevoerd worden om het risico op voedselbederf in te schatten.

In **Hoofdstuk 2** werden kwantitatieve en kwalitatieve gegevens verzameld over het voorkomen van HRS gedurende het productieproces van fruitgebaseerde producten. Hiervoor werden 332 stalen van 111 batchen verzameld en geanalyseerd in drie productie-eenheden: aardbeipuree, geconcentreerd appelsiensap en appelpuree. Hitteresistente micro-organismen werden gevonden in 96,4% van de batchen en 59,3% van de stalen. De meerderheid van de stalen waren ofwel niet gecontamineerd of bevatten zeer lage aantallen van hitteresistente schimmels (<10 ascosporen/100 g). Vervolgens werd nagegaan wat het effect was van het productieproces op het voorkomen van besmetting met HRS in deze producten. Voor aardbeienpuree en geconcentreerd appelsiensap werd geen significante verlaging ($p > 0,05$) gezien in besmettingsgraad met HRS terwijl er wel een significante afname werd waargenomen tijdens de verwerking van appelpuree ($p > 0,05$). Twaalf HRS soorten konden morfologisch en moleculair geïdentificeerd worden en behoorden tot vier genera: *Byssochlamys*, *Aspergillus* met *Neosartorya*-type ascospores, *Talaromyces* and *Rasamsonia*. Ten slotte werden de gegevens van de besmettingsgraad uitgedrukt als exponentiele distributies zodat deze gebruikt konden worden in een kwantitatieve microbiologische bederf risicobeoordeling (Hoofdstuk 8).

In **Hoofdstuk 3** worden de inter- en intrasoortvariatie van *Byssochlamys* stammen onderzocht op vlak van (i) hitteresistentie en (ii) het effect van hittebehandelingsintensiteit op de daaropvolgende groei. De geschatte paramaters werden enerzijds gebaseerd op inactivatie experimenten: inactievatiesnelheid, vorm van de curve en tijd voor de eerste decimale afname en anderzijds na groei-experimenten waarin de lag-tijden en groeisnelheden werden bepaald. De inter- en intrasoortvariëteit werd zowel voor de inactivatie als voor de groei gezien voor *B.*

fulva en *B. nivea* stammen. Sommige beschadigde ascosporen hadden een zeer lange lag-tijd (> 20 dagen) nodig om te herstellen en te kiemen na verhitting. De variatie tussen de verschillende stammen versterkt het belang om de inactivatie- en groeiparameterdistributies mee te nemen bij kwantitatieve studies. Hierdoor zullen stammen met een afwijkend gedrag ook in rekening genomen worden bij de voorspellingen. De data werden verder gebruikt in de kwantitatieve microbiologische bederf risicoanalyse van fruitpuree (Hoofdstuk 8).

In **Hoofdstuk 4** worden de groei/geen groei limieten van zes HRS soorten geïsoleerd van de fruitproducten (Hoofdstuk 2) onderzocht op vlak van suikerconcentratie (44–59°Brix), en minimum bewaartemperatuur (4-14°C). De biologische variatie van de individuele tijd om zichtbare groei (D=2mm) te ontwikkelen werd gemeten en beschreven als parametrische statistische verdelingen. In het algemeen is het niet waarschijnlijk dat de onderzochte HRS groeien bij een temperatuur lager dan 10°C. Maar, voor *N. hiratsukae* werden wel zichtbare kolonies gevormd bij 7°C. Alle HRS vertoonden in meer of mindere mate tolerantie naar een verhoging van de suikerconcentratie. De stam met de hoogste suikertolerantie was *N. udagawae* en kon nog groeien bij een °Brix van 59° (a_w=0.86). Er werd een grote range aan individuele tijden waargenomen om zichtbare mycelia te vormen wanneer condities tegen de groei/geen groei grens werden onderzocht. De bekomen data van de groei/geen groei limieten van de HRS en de variatie tussen individuele ascosporen werd verder gebruikt als input voor de kwantitatieve microbiologische bederf risicobeoordeling van fruitpuree.

Het doel van **Hoofdstuk 5** was om een experimentele methode te ontwikkelen om lage zuurstof niveaus (<1%) te bekomen en te behouden in een synthetisch vast medium gedurende twee maanden. Een dergelijk proefopzet maakt het mogelijk om het effect van lage zuurstofconcentraties op de groei van HRS te onderzoeken en werd ontwikkeld in twee delen. In eerste instantie werd de evolutie van opgelost zuurstofgas als functie van de diepte (0-12 cm) in synthetisch medium onderzocht. Uit deze experimenten kon afgeleid worden dat enkel en alleen vertrouwen op de zuurstof gradiënt in functie van diepte in een vast medium geen goede strategie is aangezien elke verandering in O_2-concentratie op een bepaalde diepte de algemene en de O_2-concentraties in de andere lagen beïnvloedden. Daarna werd een andere methode getest waarin de zuurstofgas concentratie werd bekomen door de kopruimte aan te passen in 3 cm diepe glazen potjes met behulp van zuurstofgasabsorbeerders. Met behulp van deze methode kon de zuurstofgasconcentratie gedurende 2 maand stabiel worden gehouden. Deze methode werd gebruikt om de groeilimiterende condities voor HRS te onderzoeken op vlak van minimum zuurstofgasconcentratievereiste (Hoofdstuk 6).

Hoofstuk 6 wil het effect evalueren van strikte anaerobe condities in combinatie met een lage zuurstof (O_2) concentratie (0.03%, 0.15% en 0.90%) op de tijd tot groei van zes HRS stammen geisoleerd uit fruitproducten (Ch. 2). Deze bepaling werd uitgevoerd in potato dextrose agar met verlaagde pH (aPDA, pH=3.5) en fruit-gebaseerd medium. Strikte anaerobe condities en lage O_2 waarden werden ingesteld en opgevolgd binnen een glazen fles met aPDA, geïnoculeerd en bewaard bij 22°C. De gas concentraties werden bepaald via Oxydot's ® door middel van een OxySense®. Kolonies van HRS waren zichtbaar binnen een korte periode (3-6 dagen) bij 0.9 en 0.15% O_2. Complete inhibitie vond niet plaats, zelfs niet bij erg lage waarden van opgeloste O_2 (ca. 0.01-0.1% O_2). Het verlagen van de O_2 tot 0.03% resulteerde in een significant ($p<0.05$) langere tijd nodig om visueel zichtbare groei te verkrijgen voor de meeste HRS. Groei werd geïnhibeerd voor het grootste deel van de HRS sp. onder strikte anaerobe condities. Zowel *B. fulva* als *N. fischeri* toonden zichtbare groei wanneer geïnoculeerd in drie fruit-gebaseerde media (appel, aardbei en sinaasappel) bij O_2 concentratie van 0.15% en 21%. Door de extreme tolerenatie van HRS aan lage O_2 concentraties, zouden strategieën om hun groei te inhiberen niet enkel gebaseerd moeten zijn op het verkrijgen van lage O_2 waarden in de kopruimte. Om dit verder te onderzoeken werd het effect van lage O_2 in combinatie met andere parameters (suiker (a_w), opslagtemperatuur en pasteurisatie intensiteit) onderzocht om zo beter de inhibitie van groei van HRS in hitte behandelde fruit-gebaseerde producten te voorspellen (Ch. 7).

Hoofdstuk 7 wil het gecombineerde effect van opslag temperatuur (10-30°C), wateractiviteit (a_w, 0.87-0.89), zuurstof (O_2) waarden in de kopruimte (0.15-0.80%) en pasteurisatie intenstiteit (95°C, 100°C of 105°C/15sec) onderzoeken op de tijd tot zichtbare groei van *Neosartorya fischeri* in aangezuurde Potato Dextrose Agar (aPDA, pH 3.6) tot 90 dagen. Hierna werden er 13 condities geselecteerd en geanalyseerd in een medium gebaseerd op aardbeienpuree. Uiteindelijk werd het effect van O_2 (0.05 en 1%) en pasteurisatie intensiteit 95°C en 105°C/15 sec) geëvalueerd op echte fruitpuree en concentraten gedurende 60 dagen bewaring. De opslagtemperatuur had het grootste effect op de tijd tot groei van *N. fischeri*. Bij 10°C werd er geen zichtbare groei gezien bij alle condities, terwijl er wel groei was bij respectievelijk 37% en 89% bij 22°C en 30°C. Pasteurisatie intensiteit had enkel een beperkt effect op groei van *N. fischeri*. In het algemeen was de HRM groei geïnhibeerd wanneer de a_w gereduceerd werd tot 0.870 ± 0.005 in combinatie met erg lage zuurstofwaarden in de kopruimte (0.15% ± 0.10), en dit onafhankelijk van de incubatietemperatuur (22°C of 30°C) en pasteurisatie intensiteit. Verder waren er langere tijden voor groei nodig wanneer de incubatie gedaan werd bij 22°C (van 27 tot 85 dagen) in vergelijking met 30°C (10 tot 86 dagen). De mogelijkheid van *N. fischeri* om zichtbare kolonies te vormen is gehinderd of vertraagd in aardbei medium in vergelijking met groei op aPDA. Bij de echte fruit puree ($a_w \geq 0.980$), blauwe

bessen, frambozen, braambessen en zure bessen werd de groei niet ondersteund voor *N. fischeri* wanneer de O_2 concentraties in de kopruimte verlaagd werd naar 0.05%, onafhankelijk van de pasteurisatie intensiteit. Aan de andere kant werden er zichtbare kolonies gezien in aardbeienpuree in alle geëvalueerde condities. Er werd geen groei gezien in alle 14 geconcentreerde fruit purees die getest werden ($a_w \leq 0.860$). De data die verkregen werd (groei/geen groei) en de tijd tot zichtbare groei werden verder ingebouwd in een kwantitatieve microbiologische bederf risicobeoordeling (QMSRA) dat focust op het bederf van fruit purees door HRMs (Ch. 8).

Hoofdstuk 8 richt zich op het toepassen van de resultaten uit de vorige hoofdstukken in praktische implicaties en maakt enkele algemene conclusies en perspectieven. Hiervoor wordt een “proof-of-concept” van het kwantitatieve microbiologische bederf risicobeoordeling (QMSRA) voor HRMS voorgesteld. Dit concept werd toegepast om een risico bederf model te ontwikkelen voor HRMs op het moment van gebruik van verschillende scenario’s omtrent de formulatie, verwerking en opslag van drie types van verpakte fruit purees die representatief zijn voor een basis, enkele sterkte hitte gevoelige puree (aardbeienpuree), een hitte gevoelige geconcentreerde puree (aardbeien concentraat) en een fruit puree die bestand is tegen de hitte (appelpuree). In het algemeen was er een grote kans op bederf (≥40%) in aardbeienpuree door *N. fischeri* ascosporen wanneer milde pasteurisatie behandelingen (85 en 90°C/15 sec-60sec) en wanneer er opslag op kamertemperatuur plaats vond. Voor deze puree was het bederf risico enkel gereduceerd door een toename in pasteurisatie intensiteit bij T ≥95°C voor 45 seconden of meer en door het verlagen van de O_2 en a_w (voor geconcentreerde purees). Aan de andere kant, werd het bederf risico van appelpurees door *B. nivea* ascosporen voorspeld om tweevoud verminderd te zijn door het toenemen van de pasteurisatietijd bij 85°C, 90°C en 95°C en door het reduceren van de houdbaarheid (ca. 1 maand). In het algemeen werd er geen risico voorspeld voor alle types van puree wanneer ze bewaard werden onder koeltemperaturen (<10°C), na een hoge intensiteit pasteurisatie (vb 100°C/15sec), en onder strikte anaerobe condities. Tot slot word de algemene discussie van deze PhD weergeven, gevolgd door de finale conclusie en perspectieven voor toekomstig onderzoek.

Chapter 1

Literature Review

1.1 Pasteurized fruit based products

1.1.1 Economic importance

The increasing demand for natural, healthy, safe, convenient and appealing food products has driven the food industry to increasingly meet such consumers expectations by incorporating fruit in a whole range of food products. The daily consumption of fruits to 400g or five portions constitutes one of the strategies of the World Health Organization (WHO, 2018) to promote a healthier diet to reduce the risk of chronic diseases such as heart disease, cancer, diabetes and obesity. The demand for products which are rich in nutrients, free of chemical preservatives and with reduced sugar content has driven fruit processors worldwide to diversify their product ranges. Thus, besides the consumption of fruit in bulk, a variety of fruit based products are currently available on the market, such as ready-to-eat fruit purees, fruit juices, smoothies, yoghurts, fruit fillings, etc. On the other hand, the same trends have affected negatively some fruit sectors. As an example, fruit juice consumption in Europe has been reported to have slightly decreased (-1.1%) in the last years (AIJN, 2018). This may be attributed to the preference of consumers for food products with low(er) sugar content and reduced consumption of juices which are prepared from concentrates and stored at ambient conditions. Conversely, juices which are chilled, not originating from concentrate fruit juices (NFC), submitted to milder processes and produced as 100% natural juice have gained popularity in Europe and recorded an annual increase of 3.6% from 2016 to 2017. Therefore, these products represent nowadays one of the main segments in this sector (AIJN, 2018).

The consumption of ready-to-eat fruit purees, primarily by infants, may also represent one of the growing trends in the processed fruit market. The global fruit puree market is expected to increase by 4.4% yielding US$14.5 million by 2023 (Market Research Future, 2019). Besides being directly consumed as a snack or dessert, fruit purees have been extensively used as ingredients in the other sectors of the food and beverage industry such in, among others, smoothies, ice creams, nectars, baby food, pastry and bakery (Silva and Silva, 1997, Hui et al., 2006).

1.1.2 General characteristics

High-acid pasteurized fruit products are characterized by their low pH values (3.0-4.0), high nutritional value, the presence of sugars and organic acids and low dissolved oxygen (O_2), protein and amino nitrogen content (Silva and Gibbs, 2004, Lawlor et al., 2009). Included within this category are, among others, juices, nectars, concentrates, purees and pulps.

Fruits can be processed in many different ways depending on the raw materials and end product specifications. The processing of fruit products often involves the use of various techniques to extend the shelf life, prevent spoilage and meet consumer expectations. Overall, the processing includes the following steps: storage of fresh fruit, washing, grading, peeling, cutting, crushing, heating, cooling, packaging and storage (Hui et al., 2006). The main strategies applied to preserve processed fruit products include thermal pasteurization, storage under chilled or frozen conditions, robust formulation by decreasing the available water [(= reducing the water activity (a_w)] (as in concentrates) or increasing the sugar content, by using aseptic and/or hot fill techniques and by reducing the oxygen (Hui et al., 2006, Sandoval et al*.,* 1994, Silva and Silva, 1997, Lawlor et al., 2009). The thermal pasteurization process may range from 85°C for 15 to 120 secs to 105°C for few seconds, depending to the end product requirements, raw materials, formulation and storage conditions. While the use of mild(er) pasteurization temperatures is intended to keep the quality and sensorial characteristics of the products as intact as possible, followed by storage at chilled conditions, shelf-stable fruit products receive more intense heat treatments, i.e. higher temperatures, which allow them to be stored at ambient temperatures for long periods.

1.2 Potential Spoilage Microorganisms

From a quality point of view, processed fruit products are expected to present characteristics such as fresh-like appearance, taste, aroma and flavor that should be preserved during storage. These primary quality attributes together with the microbial stability of such products will define their shelf-lives and acceptance by consumers (Hui et al., 2006). However, consumers demand for food products with no chemical preservatives, reduced sugar levels, natural ingredients and less processing, all contributing to an increase in the threat of microbial spoilage.

With regard to processed fruit products, both the natural microflora of the fruits and the microflora that gain access to the food product during processing may be relevant as potential spoilage microorganisms (Lawlor et al., 2009, Hernández et al., 2018, Snyder et al., 2018). Fruits are the main carriers of spoilage and pathogenic microorganisms into the processing facilities. Contact with soil can highly increase the probability of contamination by spoilage spore forming microorganisms (Ugwuanyi and Obeta, 1991, Frąc et al., 2015). Potential spoilage microorganisms may also have access to processed food products *via* other different sources (routes) including equipment such as rinsers, fillers, extractors, pipelines, cappers, coolers, pallets and packaging materials (Rico- Munoz et al., 2017, Jay and Anderson 2001, Narciso and Parish 1997, Tribst et al., 2009).

The classification of specific spoilage organisms (SSO) of fruit based products is dependent on the type of fruit, ingredients, processing applied and storage conditions (Gram et al., 2002). These products in their various forms are submitted to thermal treatments which are sufficient to destroy most of the spoilage microorganisms, such as heat sensitive moulds, yeasts and lactic acid bacteria, in addition to also inactivate pathogenic bacteria (Leff and Fierer, 2013, Iqbal et al., 2016, Hernandez et al., 2018, Tribst et al., 2009). Therefore, the spoilage risk due to heat sensitive microorganisms is highly associated with sanitization program failures, cross- and post-contamination. On the other hand, the surviving microorganisms on such products are restricted to spore forming microorganisms which are able to withstand pasteurization. These include spore forming bacteria such as *Alicyclobacillus acidoterrestris*, *Clostridium pasteurianum*, *Bacillus coagulans* and several species of heat-resistant moulds (HRMs) such as *Paecilomyces* (*Byssochlamys* morph*)* and *Aspergillus* (*Neosartorya* morph) sp. (Lawlor et al., 2009, Tribst et al., 2009, Massaguer et al., 2014). Due to the intrinsic characteristics of high-acid fruit products, such as pH values <4.0, presence of organic acids and low dissolved O_2 levels, the microorganisms which are able to germinate and grow out in the finished product are limited to the acidophilic spore forming microorganisms - HRMs and *Alicyclobacillus* sp. On the other hand, spoilage by *C. pasteurianum* and *B. coagulans* is mostly associated with fruit products with slightly low acidity such as canned tomato, pears and figs (Clark et al., 1946, Feng et al., 2010, Silva and Gibbs, 2004, Lawlor et al., 2009, ICMSF, 2005). Therefore, there is great concern among fruit processors with regards to the spoilage by both HRMs and *Alicyclobacillus* sp. (Snyder et al., 2018). Although *Alicyclobacillus* sp. are well known as potential spoilers of fruit juices, they require O_2 for growth and are not likely to grow out in products with ≥18ºBrix, which is typical of many processed fruit products other than juices (Chang and Kang, 2004, Walls and Chuyate, 1998). Thereby, HRMs were determined to be SSOs of the high-acid fruit products evaluated in this thesis. Hence, the following sections of this chapter will be focusing on this specific group of microorganisms.

1.3 The Heat Resistant Moulds (HRMs)

1.3.1 General Characteristics, Taxonomy and Nomenclature

Heat resistant moulds (HRMs) belong to the sub-kingdom Ascomycotina, commonly referred to as ´ascomycetes´. They have both, an asexual phase in which non-heat resistant spores are produced (=conidia) and a sexual phase that produces the heat resistant ascospores (see Figure 1.1). Ascospores are commonly produced in sets of eight inside a specialized cell called an ascus (plural: asci) in which they are protected by a thick cell wall (Dijksterhuis, 2007). The asci are themselves produced inside fruiting bodies which are called ascoma or ascomata.

Neosartorya spp. produce fruiting bodies known as cleistothecia while *Byssochlamys* spp. produces asci which are not enclosed (Pitt and Hocking, 2009). Both types of ascoma release ascospores by rupturing. The morphology of the ascospores, asci, and fruiting bodies have been crucial in the characterization and morphological identification of HRMs (Fig. 1.2).

Whilst spores (conidia) are genetically identical to the parental mycelium after asexual production (mitosis), ascospores are the product of meiotic division after sexual reproduction, resulting in diversity genetic which confer them competitive advantages to survive diverse conditions (Snyder et al., 2018).

Species belonging to the genera *Byssochlamys*, *Neosartorya* and *Talaromyces* are encountered frequently and are of economic relevance with regards to their ability to spoil foods (Frac et al., 2015, Pitt and Hocking, 2009). *Eupenicillium*, *Hamigera*, *Thermoascus*, *Rasamsonia* and *Monascus* sp. are less commonly encountered (Samson et al., 2010).

A new taxonomy of fungi has been proposed during the last years due to the increased availability of advanced molecular data. The principle of 'one fungus: one name' has been established in this proposal (Houbraken and Samson, 2017, Norvell et al. 2011). This nomenclature relies on the exclusion of dual nomenclature and adaptation of a single name for each fungus. Therefore, according to the new nomenclature, HRMs no longer have a specific name linked to their sexual stage, instead HRMs are named solely according to their asexual form as is presented in Table 1.1. The main changes regarding the nomenclature of HRMs are associated with *Byssochlamys* sp. which will be known as *Paecilomyces* sp., *Neosartorya* sp. which will be known as *Aspergillus* sp. and *Eupenicillium* sp. which will be known as *Penicillium* sp. (Houbraken and Samson, 2011, Samson et al., 2011). Besides, it is recommended to include the sexual morph behind the species name in order to distinguish strains that produce ascospores, e.g. *Paecilomyces niveus* (byssochlamys morph). Additionally, recent scientific publications have referred to some HRMs sp. as 'aspergilli with *Neosartorya*-type ascospores' (Berni et al., 2017, Tranquillini et al., 2017).

As transition to the new nomenclature was still occurring in the field of Food Mycology during the period of this thesis, the 'old' nomenclature was still used throughout this PhD thesis.

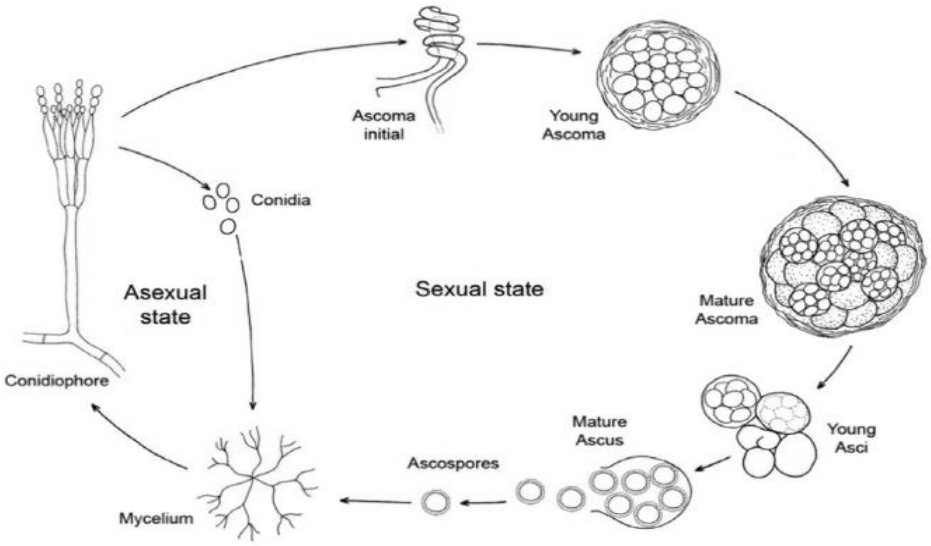

Figure 1.1 Life cycle of HRMs, showing both reproduction states: asexual state where conidia are formed and sexual state where ascospores are produced (Samson et al., 2010).

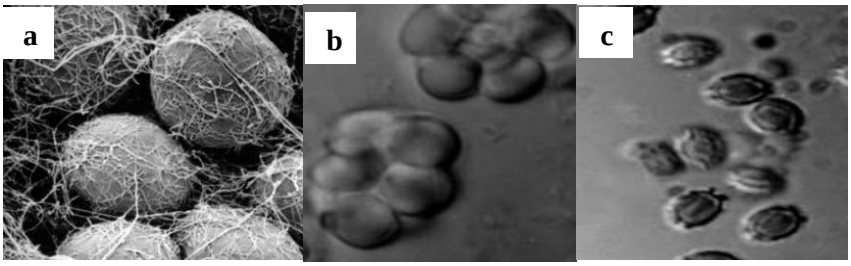

Figure 1.2 HRMs propagules. (a) cleisthotecia (b) asci and (c) ascospores (Pitt and Hocking, 2009).

Table 1. 1 New nomenclature proposed for HRMs.

Old nomenclature	New nomenclature
Byssochlamys fulva	*Paecilomyces fulvus*
Byssochlamys nivea	*Paecilomyces niveus*
Byssochlamys spectabilis	*Paecilomyces variotti*
Neosartorya fischeri	*Aspergillus fischeri*
Neosartorya glabra	*Aspergillus neoglaber*
Neosartorya pseudofischeri	*Aspergillus thermomutatus*
Eurotium herbarium	*Aspergillus glaucus*
Eupenicillium javanicum	*Penicillium javanicum*

1.3.2 Sources of contamination by HRMs

Ascospores of HRMs are soilborne and widely distributed in vineyards, orchards, and fields designated for fruit cultivars (Yates, 1974, Jesenská et al., 1993, Piecková et al., 1994, Frąc et al., 2015, Amaeze et al., 2010). Consequently, they can contaminate raw materials which come in contact with soil before delivery to processing plants. Therefore, fruits that grow in contact with soil or that come in contact with soil during harvesting e.g. strawberries, pineapples, blueberries, apples, grapes, papayas, etc., are more likely to be contaminated with heat resistant ascospores.

Even though the raw materials (raw fruits) are the main source of contamination when fruit based food products are considered, ascospores have also been recovered from several ingredients, packaging materials and the processing environment. Hence, all those sources of contamination may contribute to the introduction of ascospores into processing facilities which may result in contamination and eventual spoilage of pasteurized fruit based products (Rico-Munoz, 2017).

Table 1.2 shows an overview of the occurrence of HRMs in fruits, processed products and washing water. Spoilage incidence of thermally processed high-acid fruit products HRMs have been reported for over 80 years (Kavanagh et al., 1963, Olliver and Rendle, 1934). In this period various HRMs sp. have been isolated from several fruit and fruit-based products. These include strawberries (purees, pulps, canned and pasteurized products), apples (juices, concentrates, nectars), grape juice, pineapple (juices and concentrates), tomato pulp, blueberries and lemon cells (Table 1.2). Among the HRMs sp., *Byssochlamys* (*B. fulva* and *B. nivea*), *Neosartory fischeri* and *Talaromyces macrosporus* (= *T. flavus*) have been predominantly isolated from these types of fruits and fruit products. Even though HRMs are widely spread in nature and occur in a broad range of fruit matrices, they are not likely to occur in levels >10 cfu/100g. However, higher counts (>64 cfu/100g) may be found in strawberries and strawberry based products as recently reported by Tranquillini et al. (2017). Moreover, despite the number of available data regarding the incidence of HRMs, very few are quantitative, i.e. very few describe the levels and distribution of HRMs on the products throughout their processing.

Several ingredients (liquid and dry) have also been reported as potential sources of contamination by HRMs. These include, for instance, liquid sweeteners, pectin, root powders and other beverage ingredients (Rico-Munoz et al., 2007, Rico-Munoz, 2017). A recent study has shown that the ascospores of HRMs are widely spread within processing environments for beverages and in bottle manufacturing facilities. The incidence of HRMs was reported in packaging storage areas, the de-palletizer and the palletizer areas, on empty bottles,

ingredient storage rooms, wooden pallets, palletizers, cap boxes, airveyors, bottle conveyors rinsers, fillers, cappers, coolers, forklifts, ingredient coolers, and pallet jacks (Rico-Munoz, 2017). The same author also reported that incidence of ascospores was highest in the de-palletizer and palletizer areas, as well as on wooden pallets. Furthermore, HRMs have also been found in processing wash water such as *N. fischeri* at a tomato pulp processing plant (<1- 4 cfu/100 ml wash water) (Baglioni et al., 1999) and *Eupenicillium* sp. at an apple juice concentrate processing plant (1 cfu/100 ml wash water) (Salomão et al., 2014).

Moreover, packaging materials such as empty polyethylene terephthalate (PET) bottles and paperboard, which are used for aseptic processing, have also been reported as possible sources of ascospores (Rico-Munoz, 2017, Rico-Munoz et al., 2007, Delgado et al., 2012). PET bottles, which are considere sterile after being produced (=blown), may get contaminated after they are exposed to the processing environment. Therefore, if ascospores gain access to packaging materials and they are not eliminated during the bottle rinsening (hot water), they can potentially be activated during hot-filling of fruit products and spoil the product during storage (Rico-Munoz, 2017). Delgado et al. (2012) detected *T. macrosporus (= T. flavus*) on the surface of paperboard materials (1 cfu/100 cm^2) to be filled with tomato pulps. Such packaging materials are commonly sterilized by means of a combination of heat and hydrogen peroxide before they come into contact with ‘sterile’ food during aseptic processing. Therefore, the contamination of these materials is associated with product failures during storage or with the presence of moulds which are both heat and chemical resistant (Tournas 1994).

Table 1.2 Summary of HRMs occurrence in fruit, fruit products and fruit processing.

HRM specie	Isolated from	HRMs Count	Reference
Eupenicillium, *Talaromyces*	Strawberry pulp (refrigerated)	1 cfu/g	Aragão, 1989
Neosartorya and *Byssochlamys* spp.	Strawberry pulp (frozen)	< 1 - 10 cfu/g	
N. fischeri	Raw material (tomato pulp processing)	< 1-8 cfu/100ml	
	Wash water (tomato pulp processing)	< 1-4 cfu/100 ml	
	Non pasteurized tomato pulp	< 1 - 1 cfu/100 ml	Baglioni et al., 1999
	Pasteurized tomato pulp	< 1 - 1 cfu/100ml	
	Pasteurized tomato pulp after 3 months of shelf-life	< 1 cfu/100 ml	
Talaromyces spp.	Concentrate pineapple juice	4 cfu/kg	
Talaromyces spp., *N. fischeri*	Refrigerated pineapple juice	3 cfu/kg	
	Aseptic single-serving pack fruit juices	<1 cfu/kg	
	Apple juice concentrate	<1 cfu/kg	Enigl et al., 1933
	Cranberry juice concentrate	<1 cfu/kg	
	Tomato juice	<1 cfu/kg	
	Apple, orange and pineapple juice	<1 cfu/kg	
	Apple, pear, passion fruit and banana juice	<1 cfu/kg	
N. fischeri	Raw material (grape juice processing)	0.3-4.8 cfu/100ml	Marcolino, 2003
	Pasteurized grape juice	0.3-3.0 cfu/100 ml	
Talaromyces spp.	Apple juice concentrate	1.7 x 10^2 cfu/mL	
B. fulva	Apple juice concentrate	2 cfu/100 ml	
Eupenicillium spp.	Wash water (apple juice concentrate processing)	1 cfu/100mL	Salomão et al., 2014
N. fischeri, *Eurotium* spp.	Washed apples (apple juice concentrate processing)	3cfu/100g	
Eupenicillium spp.	Apple juice (before concentration)	1 cfu/100mL	
Several HRM spp.	Frozen blueberries	4-54 cfu/ kg	
Neosartorya, *Talaromyces*, *Monascus* spp.	Frozen lemon cells	<10-200 cfu/kg	Tranquillini et al., 2017
Various HRM spp.	Frozen strawberries	2-642 cfu/kg	

1.3.3 Heat resistance of HRMs

One of the most important characteristics of the HRMs is the extreme resistance of their ascospores to thermal processes. Ascospores of *Neosartorya*, *Byssochlamys* and *Talaromyces* sp. are often regarded as the most stress-resistant eukaryotic cells known to date. In addition to their ability to survive high temperatures and drought, they also exhibit other types of extreme stress resistance, which will be discussed below.

It is known that fungal propagules have developed some adaptations to survive stress (Snyder et al., 2018, Dijksterhuis et al., 2007, Wyatt et al., 2013). The mechanisms behind the great heat resistance of HRMs has been well investigated in the past years. The presence of a thick cell wall, high viscosity cytoplasm, low water content and high content of protective compatible solutes (such as trehalose) have been reported to strongly contribute to the stress resistance of ascopores in comparison to vegetative hyphae and conidia (Dijksterhuis, 2017, Samson et al., 2014, Dijksterhuis, 2007, Wyatt et al., 2014, Dijksterhuis et al., 2002, Wyatt et al., 2015). The above mentioned solutes are termed 'compatible' as they can accumulate intracellularly without impairing cell metabolism during ascospores maturation. The concentration of accumulated solutes decreases sharply shortly after germination, which coincides with loss of heat resistance (Wyatt et al., 2015, Dijksterhuis et al., 2007). Trehalose, trehalose-based oligosaccharides (TOS) and mannitol comprise the compatible solutes of more relevance to the heat resistance of ascospores (Wyatt et al., 2014, Dijksterjuis and de Vries, 2008). The accumulation of such solutes may differ between different species of HRMs. For instance, ascospores of *N. fischeri* have been reported to accumulate high concentrations of mannitol and TOS, while ascospores of *T. macrospores* are known for their high trehalose content (Dijksterhuis et al., 2002, Dijksterhuis et al., 2007, Dijksterhuis, 2019, Wyatt et al., 2014, Wyatt et al., 2015).

The inactivation kinetics of HRMs have been largely modelled as log-linear curves (Fig. 1.2), from which the D-value (the time required to cause one log cycle reduction in the microbial population at certain temperature) and z-values (temperature range (°C) required to cause a 10-fold variation in the D-value) are estimated (Engel and Teuber, 1991, Rajashekhara et al., 1996, Tournas, 1994, Tribst et al., 2009, Kikoku et al., 2008, Scaramuzza and Berni, 2014). Conversely, many studies have shown that the inactivation of ascospores are better described by non-linear kinetics. These are associated with the presence of a non-uniform population of ascospores, resulting in shoulders or tails in the death curves, which may approximate linearity at higher temperatures (Bayne and Michener, 1979, Stumbo, 1965). The non-linear Weibull model (Geeraerd et al., 2005, Mafart et al., 2002, Peleg and Cole, 1998) has been used to describe the inactivation kinetics of HRMs and to estimate three important parameters - (i) the

time required for the first log reduction (δ, min), (ii) the inactivation rate (b , min^{-1}) which is related to the velocity of microbial inactivation and (iii) the survival curve shape parameter (n) (Sant' Ana et al., 2009, Evelyn and Silva, 2015, Menezes et al., 2019, Souza et al., 2017).

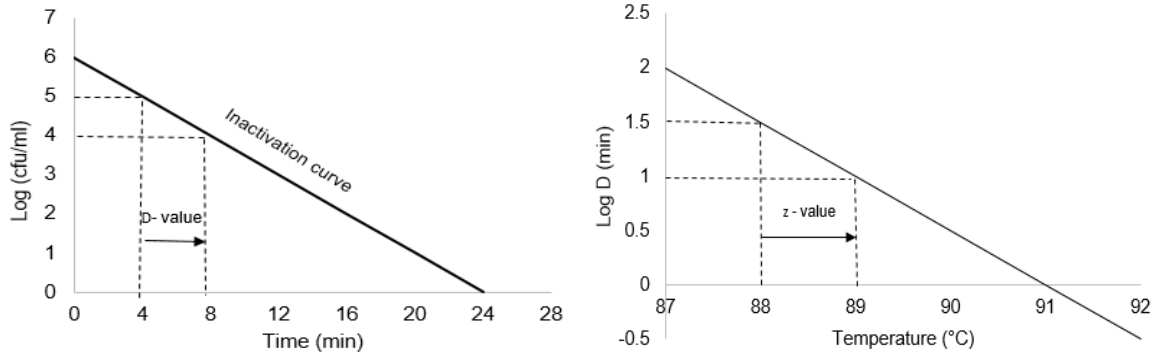

Figure 1.2. Representation of log-linear thermal death curve (left) and decimal reduction time curve (right).

The thermal resistance of HRMs and, thus, their inactivation kinetic parameters, are dependent on many factors such as temperature, properties of the heating medium, (e.g. soluble solids, pH, a_w, organic acids) and ascospores age. Table 1.3 summarizes the available studies on the heat resistance of ascospores of HRMs as a function of diverse intrinsic and extrinsic factors. Most of the heat resistance data were collected for temperatures ranging from 80°C to 95°C. The application of milder temperatures (80-85°C) enables HRMs to survive for long periods, i.e. from a few to many minutes (>300 min), depending on the species and properties of the heating medium. Higher temperatures (85-95°C) result in much shorter D-values, ranging, in general, from seconds to a few minutes (see Table 1.3). As observed in the data available in literature (Table 1.3), the ability to survive heating is highly variable among different HRM species. In a recent meta-analysis, 455 D-values of HRMs were collected and the HRMs were classified into two groups: (i) HRMs with moderate resistance, which are those able to survive several minutes at milder temperatures, i.e. 75-85°C but which are not likely to withstand temperatures above 90°C and, (ii) extremely heat-resistant HRMs, including *Neosartorya* spp. (*N. fischeri*, *N. hiratsukae*, *N. thermomutatus*, *N. neoglaber*) and *Talaromyces* spp. (i.e. *T. macrosporus* and *T. bacillisporus*) (Dijksterhuis, 2019).

Heating temperature, the content of soluble solids (°Brix), together with the type of fruit matrix and the type of organic acid have major impact on HRMs inactivation kinetics (Tournas, 1994, Tribst et al., 2009). Moreover, it has been reported that the heat resistance of HRMs may increase with the age of the ascopores (Conner et al., 1987, Evelyn and Silva, 2017, Tournas

and Texeller, 1994, Casela et al., 1990, Slongo and Aragão, 2008, Wyatt et al., 2015). The addition of sugars to natural substrates or synthetic media has been reported to significantly enhance the heat resistance of ascospores (Beuchat and Toledo, 1977, Rajashekhara et al., 1996). Tournas and Traxler (1994) showed that *N. fischeri* exhibited much higher heat resistance in concentrated pineapple juice (42.7°Brix) compared to pineapple juice (12.6°Brix) and deionized water. Thus, differences in heat-resistance within same species of HRMs may be associated with the composition of the heating media as well with differences in ascospores age and growing conditions. The effect of fruit composition on the HRMs heat resistance has been reported by several studies (Souza et al., 2017, Conner and Beuchat, 1987, Splittstoesser and Splittstoesser, 1977, Kotzekidou, 1997). For instance, *N. fisheri* ascospores were reported to exhibit higher heat resistance in apple juice compared to grape juice and cranberry juice (Conner and Beuchat, 1987, Splittstoesser and Splittstoesser, 1977).

The lower heat resistance of young ascospores (<15 days maturation) may be associated with incomplete maturation and higher water content, which facilitates the heat inactivation (Conner et al., 1987, Wyatt et al., 2015). As an example, *N. fischeri* ascospores from 11-day-old cultures did not survive 2 min at 85°C in ACES buffer, while mature ascospores from 50-day-old cultures could survive 50 min at 85°C in the same buffer (Wyatt et al., 2015).

A recent study demonstrated that pH within the range 4-6 did not significantly affect the D-values of *B. nivea* ascospores (Samapundo et al., 2018). On the other hand, this effect seems to be very pronounced in high acid media. For instance, Bayner and Michener (1979) reported that the heat resistance of *Byssochlamys* sp. diminished at pH 3.6 compared to pH 5.0 value. Additionally, the same authors observed that this effect is strain specific. Likewise, Beuchat (1988) observed an increase in lethality of *T. flavus* (=*T. macrosporus*) when the pH was decreased from pH 5.0 to 2.5. In addition, lethality of *T. flavus* was determined to be also dependent on the type of acid present in the heating medium. Therefore, the presence of benzoic, sorbic, citric and acetic acid was determined to reduce the heat resistance of HRMs compared to their resistance in neutral conditions (Beuchat, 1988, Rajashekhara et al.,1998).

Therefore, it is important to take into account these factors affecting microbial inactivation during the design of appropriate pasteurization processes, product reformulation or development of new products. Nevertheless, due to the extreme heat resistance of ascospores, increase in demand of minimally processed products and the adverse effect of high pasteurization intensities on many fruit products, it is crucial to also consider other strategies in order to ensure the microbial stability of pasteurized fruit products.

Table 1.3. Heat resistance of ascospores of HRMs at different temperatures.

HRMs species	T (°C)	D-value	z-value (°C)	Medium	Reference
Byssochlamys fulva	90	13.0 min		Buffer pH 3.6-5.0, 16°Brix	a
	87.8 - 90.0	17.7 - 47 min	6.2	Glucose solution pH 5.0 16°Brix	b
	85	8.2 - 14.6 min		0.1 M potassium phosphate pH 7	c
	80-85	12.3 - 70.6min		Saline solution	v
	86	13.0 min		Grape juice	d
	87.8 90.0	4.8 -11.3 min	6.0	Grape juice 3.4-3.5 and 10-11°Brix	b
	85-90	19.8 - 26.7 min	4.2	Tomato juice pH 4.2 16°Brix	c
	98-107	0.6-27 min	5.4	Passion fruit nectar	e
	85-95	1.81 - 42.98*	6.3	Clarified apple juice pH 3.88 11°Brix	f
	80-85	16.7 - 89.5 min		Apple juice pH 3.7, 11.2°Brix	v
Byssochlamys	88-92.0	1.4 - 8.0 sec	6.0	Ringer solution	g
nivea	85	8.8 min		0.1 M potassium phosphate pH 7	c
	85-95	1.1-16.2 min	7.1	Malt extract broth (MEB) pH 4-6	v
	75	60 min		Grape syrup pH 3.2 13.6°Brix	i
	80-85	13.8 - 1.4 min		Saline solution	v
	80-85	2.0 - 15.4 min		Apple juice pH 3.7, 11.2°Brix	v
	85-90	1.5 - 41.3 min	7.1	Tomato juice pH 4.2 16°Brix	c
	85-92	1.2 - 45.5 min	4.5	Pineapple juice pH 3.0-3.5 12-15°Brix	j
	95-104	0.9 - 55.2 min	5.5	Pineapple nectar pH 3.0-3.5 12-15°Brix	e
	85-90	1.8 - 13.7min	5.7	Strawberry puree pH 3.4 8°Brix	k
	80-93	1.7-193.1 min	6.2	Strawberry pulp pH 3.0 15°Brix	l
	78-92	1.5 - 4.54*		Pineapple juice pH 3.7 10-30 °Brix	u
	78-92	2.9 - 374.5 min*		Papaya juice pH 3.9 and 10-30 °Brix	u

Neosartorya	85	10.4 min		Buffer pH 7.0	m
fischeri	87.8	1.4 min	5.6	Apple juice pH 3.6-3.9 11.6°Brix	n
	85-93	0.4 - 15.1 min	5.3	Apple pulp pH 3.6 15.5°Brix	o
	80-90	1.0 - 312.5 min	4.6	Apple juice pH 3.5-4.5 11.3°Brix	q
	85	13.2 min		Apple juice pH 3.8 12.3°Brix	m
	80-85	78 - 141.7		Mango juice pH 4.0 10-45°Brix	p
	85	56.25 min		Mango drink pH 3.39 15°Brix	p
	85	69.75 min		Mango-pineapple drink pH 3.61 14.8°Brix	p
	80-85	34.7 - 88.5min		Grape juice pH 4.0 10-45°Brix	p
	85	10.1 min		Grape juice pH 4.0 10-45°Brix	m
	85	36.75 min		Orange juice pH 3.04 13.4°Brix	p
	85	44.25 min		Pineapple juice pH 3.4 12-13.8°Brix	p
	85-91	<2 -116 min		Blueberry filling pH 3.46 27.8°Brix	r
	85-91	<2 - 45.0 min		Strawberry filling pH 3.47 33.4°Brix	r
	85-91	<2 - 51.8 min		Cherry filling pH 3.87 25.2°Brix	r
	85-91	<2 - 41.1 min		Raspberry filling pH 3.19 30.3°Brix	r
	85-91	<2 - 43.9 min		Peach filling pH 3.31 29.8°Brix	r
	78-92	0.8 - 424.0 min*		Pineapple juice pH 3.7 10-30 °Brix	u
	78-92	0.7 - 427.0 min*		Papaya juice pH 3.9 and 10-30 °Brix	u
Talaromyces macrospores	90	2 - 8 min		Buffer glucose pH 5.0 16°Brix	t
(T. flavus)	90.6	2.2 min	5.2	Apple juice pH 3.6-3.9 11.6°Brix	n
	85	30 -100 min		ACES-buffer, 10 mM, pH 6.8	w
T. trachyspermus	75-82	2.6 - 90.9 min	4.7	Blueberry and grape juice pH 3.50 12.5°Brix	x
	75-82	1.6 - 50.0 min	4.7	Buffered glucose solution pH 3.60 12.5°Brix	x
T. bacillisporus	82-91	1.2 - 44.4 min	5.6	Blueberry and grape juice pH 3.50 12.5°Brix	x

	82-91	1.2 - 60.9 min	5.2	Buffered glucose solution pH 3.60 12.5°Brix	x
Hamigera avellanea	87 - 95	0.68 - 49.50 min	4.3	Apple juice pH 3.80 12.7 °Brix	y
Thermoascus crustaceus	90 - 95	1.8 – 24.4 min	4.4	Apple juice pH 3.80 12.7 °Brix	y

* = time for the first decimal reduction estimated using the Weibull model (Mafart et al., 2002). References: [a] Bayne and Michener, 1979, [b] King et al. (1969), [c] Kotzekidou, 1997, [d] Michener et al.(1974), [e] Ferreira et al. (2011), [f] Sant' Ana et al. (2009), [g] Engel and Teuber, 1991 [h] Samapundo et al. (2018), [i] Beuchat, 1977, [j] Salomão et al. (2007), [k] Evelyn and Silva, 2015, [l] Aragão, 1989, [m] Conner and Beuchat, 1987, [n] Scott and Bernard, 1987, [o] Gumerato, 1995, [p] Rajashekara et al. (1996), [q] Salomão et al. (2004), [r] Beuchat, 1986, [s] King, 1997, [t] King and Halbrook, 1987, [u] Souza et al. (2017), [v] Hosoya et al. (2012), [w] Dijkersterhuis and Teunissen, 2004, [x] Tranquillini et al. (2017) [y] Scaramuzza and Berni, 2014.

1.3.4 Dormancy, activation and germination of ascospores

The production of ascospores during the sexual-cycle of HRMs may not only be initiated in response to environmental stress but also by self-fertile fungi (homothallic fungi) without a sign of stress (Conner and Beuchat, 1987, Goddard et al., 2005). During dormancy, ascospores can survive long periods of time due to their low metabolic rates, and the protection of compatible solutes (Wyatt et al., 2013, Dijksterhuis, 2017). In contrast to the exogenous dormancy of asexual spores (conidia), the constitutive dormancy inherent of ascospores is not broken when ascospores are exposed to favourable growth conditions. This resilience occurs either due to a metabolic block, action of self-inhibiting compounds or a physical barrier which prevents the entry of nutrients (Dijksterhuis, 2004). External physical or chemical triggers are required to activate ascospores, which enables germination and outgrowth to occur (Wyatt et al., 2013). The use of heat (pasteurization) and high-pressure during processing has been repeatedly reported to break the dormancy of ascospores (Reyns et al., 2003, Kikoku, 2003, Dijksterhuis and Teunissen, 2004, Samapundo et al., 2018, Sant'Ana et al., 2009). Moreover, optimum activation conditions may differ among different HRM species and heating/pressure conditions, e.g. *B. nivea* (75ºC/10-20 min), *B. fulva* (75ºC/10 min), *N. fisheri*, (85ºC/10 min), *T. macrosporus*, (85ºC/7-10 min or 6000 Bar) (Paula et al., 2006, Dijksterhuis and Teunissen, 2004, Sant'Ana et al., 2009, Samapundo et al., 2018). Therefore, most of the thermal treatments applied by the fruit industry may either inactivate or activate them, the latter resulting in increased risk of spoilage.

The transition from a low to a highly metabolic active state (= activation) of ascospores is characterized by the degradation of compatible solutes (which are used as a carbon source), decrease in the viscosity of the cytoplasm, disruption of the thick cell wall, uptake of nutrients and the initiation of the germination process (Splittstoesser et al., 1972, Beuchat, 1986, Dijksterhuis et al., 2002, Dijksterhuis and Samson, 2006, Dijksterhuis, 2007). Fig.1.3 shows the schematic representation of the biological process of spoilage by HRMs including initial contamination, activation of the ascospores, germination and outgrowth into visible mycelia (visible colonies). The germination of activated (asco)spores is characterized by isotropic growth (swelling) and polarized growth (germ tube formation and elongation) followed by branching and linear mycelial growth (=spoilage) (Dantigny et al., 2005, Gougouli and Koutsoumanis, 2013). Therefore, if the ascospores are present and activated inside the food matrix, and, if the physico-chemical properties are favorable, the ascospores may germinate, grow out and spoil the product during storage.

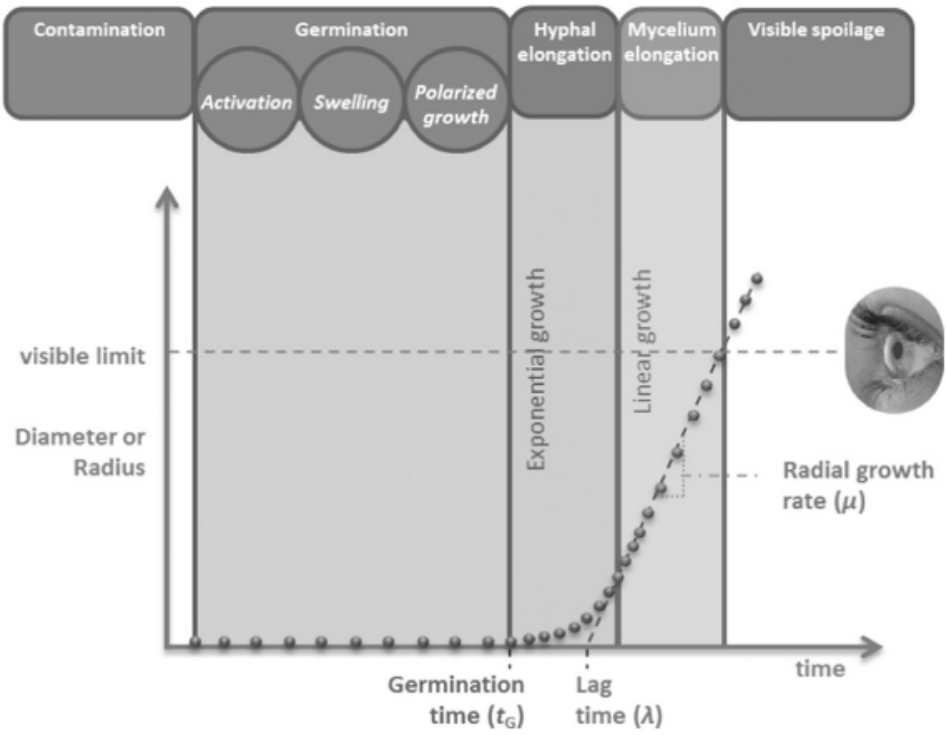

Figure 1.3. Schematic representation of the biological process of spoilage by HRMs, which include contamination by ascospores, activation of ascospores, germination, linear hyphal growth and formation of visible mycelia (= visible spoilage) (Dagnas et al., 2015).

1.3.5 Effect of physico-chemical properties of food on the growth of HRMs

A summary of the conditions required for the growth of HRMs are presented in Table 1.4. In contrast to bacteria, water activity (a_w) is the most important factor influencing fungal growth, followed by temperature. Therefore, the germination and growth of HRMs can be drastically reduced and impaired by lowering a_w (Valík and Piecková, 2001). The minimum a_w required for growth is dependent on the species, temperature, as well as composition of the food or growth medium. The large effect of temperature on the minimum a_w required for the germination and growth of fungi has been extensively reported (Tassou et al., 2009, Pitt and Hocking, 2009, Roland and Beuchat, 1984, Panagou et al., 2010). Overall, most HRMs are able to grow out at a_w-values ≥0.87 (see Table 1.4). However, some xerophilic species such as *Byssochlamys spectabilis* and *Emericella nidulans*, are able to grow at a_w values as low as 0.80 (Pitt and Hocking, 2009). According to the presented data, HRMs can be classified as thermo-tolerant fungi as the maximum temperature for growth occurs at 45-50°C and poor growth occurs at temperatures less than 20°C (6-15°C). Moreover, most HRMs grow optimally at 30–35°C (Mouchacca, 2007).

Overall, fungal species associated with food spoilage require oxygen for germination and growth. HRMs, on the other hand, have been reported to tolerate and grow under extremely low oxygen levels, i.e. under nearly anaerobic conditions. As an example, *B. fulva* and *B. nivea* were reported to present radial growth at <0.5% oxygen (Taniwaki et al., 2009). According to King et al. (1969), *B. fulva* may grow under oxygen concentrations within the range 0.27 -21% after three days. *N. fischeri* presented growth and produced mycotoxins under atmospheres containing 0.1-20.9% O_2 and pure N_2 (Nielsen et al.,1989). Hillmann et al. (2015) reviewed the main aspects of the metabolic mechanisms that enable fungal survival under low-oxygen conditions. It is known that oxygen is dissolved in the food matrix and equilibrates with the headspace during the shelf-life. Therefore, the microbial stability of food products is largely dependent on the total in-pack oxygen (Snyder et al., 2018). It is also dependent on the food matrix, solute composition, pasteurization and filling temperature (Snyder et al., 2018). Furthermore, oxygen has also been reported to have an interactive effect with other gases, such as carbon dioxide (CO_2). As an example, Taniwaki et al. (2010) reported that both, *B. nivea* and *B. fulva*, were able to grow under 60% CO_2 in the presence of residual O_2 (<0.5%). Yates et al. (1967) observed that an atmosphere containing 80% CO_2 and 20% O_2, did not significantly affect the growth of *B. nivea* and in comparison with atmospheric air (21% O_2). Hull (1939) suggested that the growth of *B. fulva* in contaminated cans of processed fruits was inhibited by the lack of oxygen rather than the production of CO_2 to inhibitory concentrations. The same author also highlighted that the headspace size and the storage temperature strongly affect the amount of mycelial growth that occurs in contaminated processed canned fruit products.

Whilst pH may strongly affect the survival and inactivation of ascospores, its effect seems to be less pronounced during fungal development as HRMs are able to grow across a broad pH range (2-9) (Pitt and Hocking, 2009, Tournas,1994), including very acid environments such as the ones present in the majority of the fruit based products. The acid tolerance of HRMs is associated with some physiological strategies such as acid-proton and anion outflow and oxidative degradation of organic acids during catabolism (Snyder et al., 2018).

Additionally, fruit compounds, such as the type and amount of organic acids and sugars may influence the ability of HRMs to grow out (Amaeze, 2013, Beuchat, 1977, Valík and Piecková, 2001, Zimmermann et al., 2013). As an example, Amaeze et al. (2013) observed that citric acid strongly delayed germination whereas glucose promoted germination and outgrowth of *N. fischeri*. Beuchat (1977) observed discrepancies in the time required for growth when *B. nivea* ascospores were inoculated in different fruit juices and nectars. According to Zimmermann et al. (2013), HRMs may grow differently depending on the type of fruit juice. For

instance, whilst the growth of *B. nivea* was observed at the center of bottled papaya juice, growth was only detected on the surface of pineapple juice which was composed of two separated phases.

Table 1.4. Conditions required for the growth of various HRMs.

HRMs species	Growth conditions	Reference
B. fulva	T = 10-45°C, min a_w = 0.89, pH = 2-9, 60% CO_2, 0.27 % O_2	1, 2
B. nivea	T = 15-40°C, min a_w = 0.89, 60% CO_2, <0.5 % O_2	1, 3
B. spectabilis	T = 5-48°C, min a_w = 0.80	4
N. fischeri	T = 11-52°C, 0.1 % O_2, min a_w = 0.89, pH= 3-8	5, 6, 7 ,8
N. hiratsukae	min a_w = 0.90	9
N. glabra	min a_w = 0.87	9
E. nidulans	T= 6-51°C, min a_w = 0.81	4
T. bacillisporus	T= 15-45°C	10

[1] Panagou et al. (2010), [2] King et al. (1969), [3] Roland and Beuchat, 1984, [4] Pitt and Hocking, 2009, [5] Nielsen et al. (1989a), [6] Nielsen et al. (1989b), [7] Samson et al. (2010), [8] Tournas, 2004, [9]Berni et al. (2017), [10] Mouchacca, 2007.

1.3.6 Negative consequences of HRMs growth

Spoilage by HRMs is primarily associated with visual defects due to appearance of fungal mycelia (colonies). Moreover, HRMs may strongly diminish some quality attributes of the product by the production of off-flavors, discoloration, swelling and degradation of the texture due to the production of pectinolytic and cellulolytic enzymes which break down complex food components such as lipids, sugars and proteins into utilizable products (Kregiel et al., 2018, Filtenborg et al., 1996, Dijksterhuis, 2007, Tournas, 1994, Tribst et al., 2009, Snyder et al., 2018). The term “puffballs” has been used to describe the dense filamentous appearance of HRMs at the bottom of food and beverage containers (Snyder and Worobo, 2018). It is worth mentioning that while the majority of cases of spoilage fungi is associated with the appearance of visible colonies at the surface of the food where O_2 is abundant, HRMs do not appear to require high amounts of O_2 to form visible mycelia. Therefore, the spoilage of food products with low oxygen contents by HRMs is likely to occur. Spoilage incidents of thermally processed high-acid fruit products, including fruit juices, purees, fruit-based beverages, sport and energy drinks and flavored mineral waters have been reported for more than eight decades (Beuchat, 1998, Filtenborg et al., 2004, Obeta and Ugwuanyi, 1995, Olliver and Rendle, 1934, Baglioni et al., 1999, Rico-Munoz et al., 2019). Nevertheless, spoilage by HRMs is still a topic of great concern among fruit processors.

Globally, nearly 25% of all food supply is wasted due to postharvest microbial spoilage (Gram et al., 2002). A recent survey of European and American fruit processors reported that 92%

and 89% of fruit juice manufacturers have experienced fungal spoilage in the finished products and ingredients, respectively (Snyder and Worobo, 2018), while the spoilage of HRMs in finished product has been reported by 64% of the participants. Fungal growth in processed fruit products and thus spoilage, not only results in waste and economic losses, but also brings a very negative impact and mistrust of food brands (Snyder et al., 2018).

Furthermore, many HRM species are known to produce toxic secondary metabolites (mycotoxins) such as patulin, byssochlamic acid, bissotoxin A, assymetrin, variotin, fumitremorgin C and A, verrucologen, fischerin, and eupenifeldin which may cause adverse effects on human health (Tournas, 1994, Frac et al., 2015). Thus, such HRMs may be not only classified as spoilage microorganisms, but they may be also a food safety issue due to the production of mycotoxins. An overview of the mycotoxins produced by different HRMs species is depicted in the Table 1.5.

Table 1.5. Summary of mycotoxins produced by HRMs species.

HRMs species	Mycotoxin	Reference
B. nivea	Patulin, mycophenolic acid, byssochlamic acid	1
B. fulva	Byssochlamyc acid, byssotoxin A	2
B. spectabilis	Viriditoxin	2
N. fischeri	Fumitremorgin A, B & C, fischerin, verruculogen	3
N. udagawae	Fumigatin, fumagillin, tryptoquivalone	4
N. fumigata	Gliotoxin, fumigaclavines, fumitoxins,fumitremorgins, verruculogen	3

[1] Kramer et al., 1976, Rice, 1977, Houbraken et al., 2006,[2] Houbraken et al., 2006, [3] Samson et al., 2010, [4] Samson et al.,2007.

1.4 Prevention and intervention strategies

Different strategies have been employed for the prevention and intervention of HRMs contamination. These include, for instance, the use of sanitation programs, fruit selection, thermal and non-thermal inactivation.

Good Manufacturing Practices (GMPs) have been widely employed as a means of primary control. GMPs, for instance, include the physical separation of ingredient and packaging areas from the food processing environment, application of good hygiene practices (GHPs) and raw material selection (Wareing and Davenport , 2007). These strategies are mostly intended to prevent fungal contamination of the finished product and spreading of spores within processing facilities (Snyder and Worobo, 2018). The selection of high-quality fruits and/or ingredients with high potential of HRMs incidence, such as liquid sweeteners, pectin, root powders, coconut water, fruit concentrates (juices and purees) should be performed prior to processing

(Rico-Munoz, 2017). Thereby, the exclusion of damaged, injured and decayed fruits is a starting point to reduce the introduction of fungal contaminants into the facilities (Tribst et al., 2009, Keller 2006). In addition, sanitation programs such as Sanitation Standard Operating Procedures (SSOPs), Hazard Analysis Critical Control Point (HACCP), cleaning-in-place (CIP) and cleaning-out-of-place (COP) regimes are employed for the purposes of food quality and safety control (Rico-Munoz, 2017, Wareing and Davenport, 2007). The antimicrobial efficacy of industrial sanitizers containing chlorine dioxide (ClO_2) and iodine (iodophors) were recently assessed towards HRMs ascospores. Treatment with 100ppm ClO_2 for at least 10 min or 75 ppm iodine (iodophor) for 16h is required to inactivate *T. macrosporus* and *B. spectabilis* ascospores (Dijksterhuis et al., 2018). However, it is worth mentioning that the use of intense sanitation programs, such as high concentrations of chlorine-based products, can have potential negative environmental impacts and safety issues. Furthermore, these industrial sanitizers are preferentially used during the disinfection of food-contact surfaces and equipment and utensils and not for washing of fruits, which are the main source of ascospores. According to many fruit processors, strategies to control ascospores which are entirely based on use of sanitizers are insufficient. 69% of fruit processors agree that additional targeted methods should be taken into account to reduce or prevent spoilage due to fungal spores (Snyder and Worobo, 2018).

The adoption of mitigation strategies such as the use of a strict cold chain and/or elevated heat processing (pasteurization) temperatures have also been proposed for the inhibition or elimination of HRMs. However, these strategies have some limitations. Applying very intense thermal pasteurization processes may result in loss of quality and sensorial aspects depending on the type of fruit. Storage of pasteurized products at chilled conditions may not be feasible to apply for low-cost products, such as fruit juices from concentrates and ready-to-eat fruit purees. Moreover, the increase in the globalization of the food supply chain has led to complexities with regards to the maintaining and tracing reduced storage temperatures.

The growing consumers demand for minimally processed food products wherein most of the original nutritional value is retained and the extreme heat tolerance of HRMs, have driven many researchers to investigate, besides the thermal pasteurization, the use of non-thermal technologies to inactivate ascospores in products during processing. These include high hydrostatic pressure (HHP), the combination of mild heat treatment and high-pressure processing (HPTP), ultrasonication/thermosonication (TS), high pressure cycles and ultraviolet (UV) light (Evelyn and Silva, 2015, Evelyn and Silva, 2017, Ferreira et al., 2009, Palou et al., 1998, Laboissiére et al., 2007, Marcellini et al., 2006, Menezes et al., 2019). These techniques are mainly intended to replace thermal pasteurization or reduce the intensity (time and

temperature) of the pasteurization and reduce its negative effects on the quality of the products. However, many of those methods still need to be combined with a mild(er) pasteurization process in order to be effective. Among them, the application of a 600 MPa HPTP-thermal technique seems to be more effective in inactivating ascospores of *B. nivea* and *N. fischeri* compared to HPTP, TS and ordinary thermal methods (Evelyn et al., 2017). It has been reported that HPTP-thermal (600 MPa, 75 °C) can steadily reduce the counts of ascospores of *B. nivea* and *N. fischeri* by 3.4 and 5.2 log, respectively, after 40 min. On the other hand, as for thermal treatment (75°C), TS treatment (0.33W/mL/75°C) caused a steady and slow increase in the ascospores count prior to inactivation, which compromises its commercial application, as opposed to HPTP technology (Evelyn et al., 2017). In addition to HPTP-thermal, the application of pressure cycles seems to be also effective in inactivating ascospores (>4 log reductions) in fruit juices and nectars (Palou et al.,1998, Ferreira et al., 2009). Furthermore, a recent study showed that the use of UV-C (at 38W/m^2 /20min) resulted in ≥4 log reductions of *N. fischeri* ascospores in apple juice adjusted to various concentrations of soluble solids (12, 25, 30, 40, 50, 60 and 70 °Brix) (Menezes et al., 2019). However, the use of such techniques is limited to transparent and liquid food products, mostly fruit juices. Nevertheless, most of the presented treatments need to be applied in combination with heating or for long time in order to completely inactivate HRMs ascospores.

Therefore, despite being promising for application to some of types of fruit products, the use of non-thermal techniques still needs to be better investigated regarding their application, economic viability, effects on quality of the products and safety standards.

1. 5 Quantitative approach towards risk assessment of microbial spoilage

Quantitative approaches have been extensively employed to assess the effect of processing, formulation and storage on the levels of pathogens and spoilage microorganisms (Membré et al., 2015, Gougouli et al., 2011, Zimmerman et al., 2013, Rigaux et al., 2014, Huchet et al., 2013, Deschuyffeleer et al., 2015). Quantification of the effect of intrinsic and extrinsic factors on microbial responses (growth, survival, or inactivation) is commonly described by predictive mathematical models which are valuable tools that can be applied, among others, during development (formulation) of new products, shelf-life determination, quality control management and defining process step settings (McMeekin et al., 1993). Predictive models can be classified into deterministic and stochastic models. As previously mentioned, deterministic models are intended to estimate a single point of output, i.e. the microbial load associated after a certain time during storage or pasteurization. The stochastic approach predicts probability functions for microbial loads, for instance, at a specific time of shelf-life or

pasteurization. The stochastic approach entails the use of probabilistic models which consider a range of values with their associated probability of occurrence resulting in outcomes with a confidence interval. Therefore, they provide more realistic risk predictions (Poschet et al., 2003, Vásquez et al., 2014).

For roughly 15 years, predictive mycology has promoted the use of methods and predictive models for fungal development (germination and linear growth), which take into account mould specificities (Dantigny et al., 2005, Dantigny and Panagou, 2013). Through deterministic primary models, kinetic growth parameters have been estimated, including the radial growth rate ($mm.day^{-1}$ or $mm.h^{-1}$), lag time (days/ h), germination time (days/h) and time to visible growth (days/h). These type of models are used to describe changes in a microbial property, such as the temporal colony diameter. In contrast to such models, secondary probability models are intended to predict the probability of microbial growth over storage time. To do so, these models take into account the presence/absence of microbial growth and/or toxin production, i.e. whether these events may occur or not under certain environmental conditions (Dantigny et al. 2005, Garcia et al., 2009).

Table 1.6 shows an overview of the available (primary and secondary) predictive models for the growth of HRMs. Many factors have been reported to strongly influence the kinetic growth parameters of fungi (lag time, germination time and time to visible growth). These include the media/food matrix composition, extrinsic factors and inoculum size (Dantigny et al., 2002, Sant'Ana et al., 2010, Judet et al. 2008, Dantigny, 2016). Nonetheless, most of the available models are kinetic models investigating the influence of temperature and a_w on the growth response of HRMs. It can be seen that the majority of the studies were performed in real fruit matrices. Moreover, the estimation of the growth rates and lag times has often been reported in these studies (see Table 1.6). On the other hand, the use of probabilistic models has to date been limited to only one study describing the combined effect of temperature and a_w on growth/no growth boundaries of *B. fulva* and *B. nivea* in malt extract agar (MEA) (Panagou et al., 2010).

Additionally, it is noted that the majority of the studies were focused on describing the effect of environmental factors on mould growth, i.e. the linear extension of mycelium. However, as recommended by Dantigny (2016), these studies have less application on determination of the mould free shelf-lives as food products are considered spoiled as soon as mycelium is visible (on the surface or inside product). On the contrary, the times required for visible growth as a function of various growth determining parameters may be preferentially estimated. These are very often defined as the time at which the diameter of the mycelium is equal to 2-3 mm, which

corresponds to the lag time and the beginning of the linear growth (Gougouli et al., 2011) (see Fig. 1.3).

Table 1.6. Available predictive models for heat resistant moulds (HRMs) growth.

HRMs species	Model output	Parameters range	Medium	Reference
B. fulva and *B. nivea*	Growth/no growth boundary	Temperature [10-45°C] a_w [0.88-0.99]	Malt Extract Agar (MEA)	1
B. nivea	Growth rate (mm.h^{-1}) and lag time (h)	a_w [0.90-0.99] and ascospores age (30 - 90 days)	Papaya juice	2
N. fischeri	Growth rate (mm.h^{-1}) and lag time (h)	a_w [0.90-0.99] and ascospores age (30 - 90 days)	Pineapple juice	3
B. fulva, *N. fischeri* and *T. avellaneus*	Growth rate (mm.day^{1}) and lag time (day)	a_w [0.85-0.99]	Sabouraud agar	4
B. fulva, *N. fischeri*	Growth rate (mm.h^{-1}) and lag time (h)	Temperature [10-30°C]	Apple juice	5
B. fulva	Time for 10% bottles spoiled $t_{10\%}$(h)	Initial load [5 and 19 ascospores/100 ml] Temperature [21 and 30°C]	Clarified apple juice	6

[1]Panagou et al. (2010), [2] Zimmermann, et al. (2013), [3] Zimmermann, et al. (2011), [4] Valık and Pieckova, 2001, [5] Tremarim et al. (2015), [6] Sant' Ana et al. (2010).

According to the European Commission the term unsafe not only refers to 'damage to health' but also to products 'not suitable for consumption'. Therefore, both safety and spoilage are acknowledged by the food authorities in Europe (European Commission, 2002). While risk analysis is widely used by food safety authorities, the application of risk within a food processing context could also be used to cover food quality issues. Risk analysis studies comprise three main steps: Risk assessment, Risk management and Risk communication (Codex Alimentarius Commission, 1999). In the first step, the probability of occurrence of a hazard or quality issue is scientifically evaluated. During the development of risk assessment models (within a food processing context), the risk (applied for both food safety and food quality in case of extension to food spoilage) is defined and thereafter quantitatively and/or qualitatively evaluated through four stages described below according to Membré and Boué (2018):

i) Hazard identification: Identification of target microorganisms which are pathogens in case of microbial food safety issues or spoilage microorganisms if a microbiologically determined quality issue is to be tackled;

ii) Hazard characterization: Characterization and clear definition of the "risk". In the case of microbial food safety issues, it is associated with the nature of adverse health effects caused by certain pathogens, while the effects associated to

spoilage, such as maximum microbial concentrations and/or food defects, need to be defined for microbiologically determined quality issues;

iii) Exposure assessment: In this step, it is intended to estimate the final likely intake of a pathogen or likely level of spoilage microorganisms in the food, taking into account changes throughout the food supply chain up to consumption (from farm to fork);

iv) Risk characterization: Consists of estimating the probability of occurrence and severity of potential adverse health effects in a specific population or, in case of spoilage, in estimating the probability of food quality alterations and spoilage.

Risk management is intended to evaluate and weigh the outputs from a risk assessment study and potentially select and implement possible control alternatives. Ultimately, risk communication aims to translate these results into practical and understandable terms to the different stakeholders (Lammerding, 1997).

The first Risk Analysis step, known as Quantitative Microbiological Exposure Risk Assessment (QMRA), is defined as a structured process intended to determine and characterize the risk associated with biological hazards in a food matrix (Codex Alimentarius Commission, 1999, FAO et al., 2001). During the development of a probabilistic QMRA model four key steps need to be considered (see Figure 1.4). The first stage refers to the definition of purpose and scope of the model as well as the population of interest, followed by the second stage where common terminology is defined. The third step comprises the model building. It starts with the definition of the model structure, i.e. the connections between different processing steps, including thermal processing or other processes aimed at microbial reduction/destruction. It also covers the microbial growth and/or post-process contamination before or during storage. Next, mathematical equations are determined and included in order to estimate changes in microbial response after each processing step. The model inputs are also collected and/or defined at this stage. These are mainly linked to management options such as process settings and product formulation (Membré and Valdramidis, 2016). In addition, the outputs may be deterministic values or probabilistic distributions provided beforehand through expert opinions, scientific publications, reports, books, databases, company data or experimentally collected data. Probabilistic inputs may indicate more accuracy than deterministic ones the predictions as they are included as probability distributions (stochastics models) which will generate and output distributions (Jacxsens et al., 2016).

Furthermore, this approach allows for the characterization of both uncertainties and variability in those variables and therefore also on the estimates (= outputs). Thus, they will indicate real

life scenarios. On the other hand, deterministic inputs may also be included as a fixed value, for instance, when they are precisely known such as a specific property of the food product under consideration. However, as these are not related to process settings they cannot be used to reduce or control the risk. In this case, the uncertainty is negligible. The last and fourth stage corresponds to the simulation of what-if-scenarios and the interpretation of model outputs. Changing settings (contamination, processing, formulation, storage) will result in diverse outcomes (probabilities distributions) which can be illustrated by means of tools such as iso-risk curves, in order to facilitate the interpretation by stakeholders (Membré and Boué, 2018).

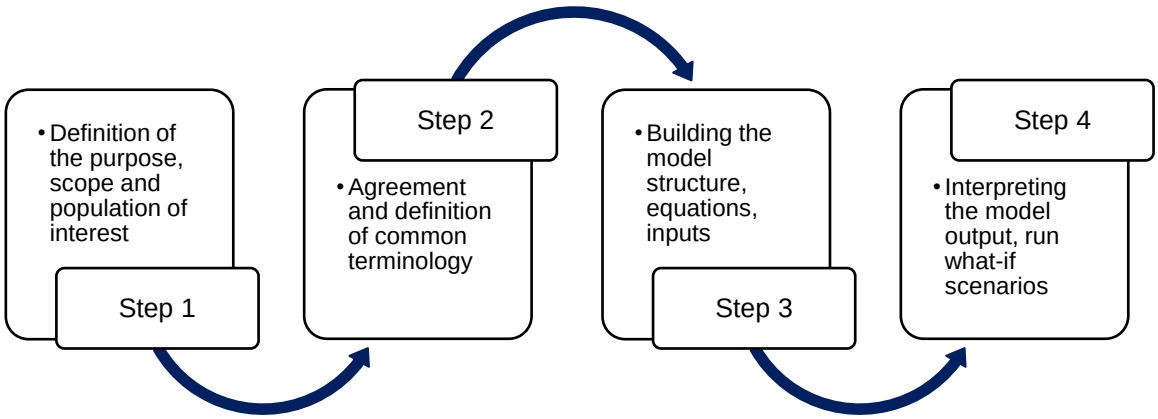

Figure 1.4. Key steps in QMRA model development. Adapted from Membré and Boué (2018).

During the QMRA model building process it is very important that both uncertainty and variability are separated and included (Nauta, 2000). Uncertainties refer to lack of knowledge about certain parameter and thus can be reduced by further study (Haas, et al., 2014). When predictive models are used as input it is important to know and include the uncertainty associated with their predictions.

On the other hand, variabilities are associated with natural randomness (true heterogeneity) and hence it is not reducible by further study. Different sources of variability may affect microbial responses during passage through the food chain such as post-processing contamination, contamination between packs, storage condition (time-temperature) and biological variability at cell and strain level (Rigaux et al., 2014, Membré and Boué, 2018, den Besten et al., 2017, Pouillot and Lubran, 2011). Therefore, the study and incorporation of these factors are crucial to better estimate the risk of spoilage/illness. A common way to measure uncertainties in QMRA is by running simulations. Monte Carlo simulation is widely used to run

different scenarios, through simulations resulting in output distributions such as the probability of occurrence of contaminated packs of food in a batch. This technique relies on building in-silico samples representative of the input distributions, and through processing some functions/modeling determine outcomes (Poschet et al., 2003, Mokhtari and Frey, 2005, Koutsoumanis and Angelidis, 2007). The process is repeated according to the number of iterations and will result in different scenarios (various outcomes). Thereby, the use of Monte Carlo simulations will generate an outcome which reflects the uncertainties and variabilities, which is more likely to occur in real-life scenarios. The number of iterations will highly influence the accuracy of the response variable distribution; in other words, the larger the number of iterations, the higher the probability of including marginal results such as data from the tails of a normal distribution. However, the number of iterations is usually limited by the available computing power.

Currently, a vast number of QMRA studies are available which have been widely applied as tools for the determination and management of risks posed by foodborne pathogens in the food supply chain (Cassin et al., 1998, Delignette-Muller et al., 2008, Ross et al., 2009). However, the QMRA approach has not been applied as extensively towards determination of risk of spoilage. A very small number of studies (three) are available whereby the QMRA approach has been applied to determine the risk of spoilage (Gougouli and Koutsoumanis, 2017, Rigaux et al., 2014). These will be further discussed.

As previously mentioned, the development of QMRA models focusing on food quality (risk of spoilage) requires some adjustments to the QMRA framework. Firstly, they differ regarding their risk definition. Whilst risk refers to a determined threshold value of a pathogen in the food at the time of consumption in the food safety approach, spoilage risk may be correlated to the microbial load in the food through food chain, but also to visual defects, such as the appearance of visible mycelia on the food product, or alterations of sensorial and quality characteristics in spoilage by fungi (Gougouli and Koutsomanis, 2017). With regards to end-points application, the QMRA food safety approach is mainly intended to estimate the probability (risk) of developing foodborne illness upon consumption of food contaminated by a pathogen/microbial toxins, usually expressed as the number of human cases in a population, while the food quality approach aims to predict the probability (risk) of having spoilage packs of food during supply chain (Membré and Boué, 2018). Whilst in the food safety approach the distribution of pathogen contamination levels is mainly focused on the food at the moment of consumption, the effect of each process step on the spoilage risk is often more intensively studied in QMRA food quality studies. On the other hand, both approaches share: (i) the same

"scope-model-simulation-communication" methodology (§*1.5.1*), and (ii) use of mathematical predictive models and characterization / integration of uncertainties and variabilities.

1.5.1 Examples of QMRA applied to food quality (food spoilage)

The use of QMRA for food quality issues may be applied through two approaches. In the first approach the microbial spoilage is taken into account during microbial risk assessment intended to assess a food safety issue. While the second approach is entirely associated with quality issues i.e. is based on the estimation of the spoilage risk of target spoilage microorganisms in a specific food matrix.

The three QMRA studies performed to date on food spoilage issues are discussed below and are summarized in Table 1.7. In addition to these examples, Membré and Boué (2018) provided an extensive scientific background and guidance towards the development of a QMRA model in an industrial context, including two case studies focusing on food process optimization and shelf-life determination.

Table 1.7 QMRA models applied to food spoilage.

Microorganisms	**Food product**	**Risk model Outputs**	**Reference**
Pseudomonads, *E. coli* O157:H7	Ground beef	Concentration of pseudomonads and *E. coli* O157:H7 at the end of retail storage; % of spoilage packages	Koutsoumanis (2009)
Geobacillus stearothermophilus	Canned green beans	Concentration of *G. stearothermophilus* after sterilization and incubation test; % of green bean cans with defect	Rigaux et al. (2014)
Aspergillus niger	Yogurt	Probability distribution (%) of the number of cups which a visible mycelium of *A. niger* at the consumption time	Gougouli and Koutsoumanis (2017)

During an exposure assessment of *Escherichia coli* O157:H7 and pseudomonads in ground beef, by Koutsoumanis (2009), both organisms were taken into consideration, followed by estimation of their growth rates under retail storage conditions and final counts at the end of retail. A positive correlation was observed between the counts of *E. coli* and pseudomonads in ground beef packages after retail as the packages with high *E. coli* counts also had high counts of pseudomonads. The results showed that hazard risk predictions without considering the quality aspects of contaminated food at the time of consumption may lead to overestimated predictions. Moreover, the initial counts of spoilage microorganisms may strongly affect the estimation of the biological hazard risk. For instance, if the product is highly contaminated with

spoilage microorganisms, the risk of biological hazards tends to be lower, as the shelf-life will be reduced as spoilage is occurring earlier. Therefore, it is of great importance to consider the quality aspects of the food at the time of consumption when hazard risk predictions are made (Koutsoumanis, 2009).

The next two examples intended to estimate the spoilage risk (utilizing the second approach). Rigaux et al. (2014) proposed a microbial risk assessment model to predict the percentage of non-stability of canned green beans due to *Geobacillus stearothermophilus*. The spoilage risk was determined by means of the non-stability rate of cans submitted to a biological stability test (55°C for 7 days), used as a quality indicator. Spoilage was determined to occur when *G. stearothermophilus* grew out after the test, i.e. positive cans reaching concentrations of 7 log CFU/g. The model takes into account the microbial counts changing along the canned green-bean processing chain, including the initial microbial contamination of fresh unprocessed green beans, cross-contamination during processing, inactivation, probability of survival and growth at different stages of processing. The model developed predicted that about 92% of cans containing at least one spore after sterilization will develop spoilage, considering the high growth ability of germinated spores (Rigaux et al., 2014).

The only available QMRA study focusing on fungi focused on the spoilage of yogurt by *Aspergillus niger* (Gougouli and Koutsoumanis, 2017). In this study, the risk of spoilage was assessed through a stochastic model by including important sources of variability inherent to fungi, such as individual spore lag times, time-temperature and variability between consumers regarding visual assessment of spoilage (product rejection). The final output, i.e. the probability (risk) of having spoiled units of yogurt presenting visible mycelium of *A. niger* at the moment of consumption was achieved by combining the true prevalence of *A. niger* spores in yogurt with the probability of the fungus forming visible colonies before the end of shelf life (Gougouli and Koutsoumanis, 2017). For instance, for a batch of 100,000 cups (= 100,000 iterations) where 1% of the cups are contaminated with *A. niger*, it was estimated that eight cups would result in visible mycelium at the time of consumption (=spoilage).

Spoilage risk predictions for fungi will be based on the combination of prevalence (probability to be contaminated), inactivation (probability of survive) if pasteurized food products are evaluated, probability of recontamination and probability of germination and formation of visible colonies (mycelia) before the end of the shelf-life or time of consumption (Membré and Boué, 2018). Moreover, the risk of spoilage will be associated to the appearance of mycelia and not to threshold counts, as for bacteria.

Ultimately, the outcomes obtained from QMRA models may lead to improvement of risk-based quality management decisions in identifying and quantifying the impact of possible interventions during product formulation, processing and storage of fruit-based products to eliminate or greatly reduce this risk of spoilage.

Chapter 2

Occurrence, distribution and contamination levels of heat-resistant moulds throughout the processing of pasteurized high-acid fruit products

Redrafted after

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Abstract

Heat-resistant moulds (HRMs) are well known for their ability to survive pasteurization and spoil high-acid food products, which is of great concern for processors of fruit-based products worldwide. Whilst the majority of the studies on HRMs over the last decades have addressed their inactivation, few data are currently available regarding their contamination levels in fruit and fruit-based products. Thus, this study aimed to quantify and identify heat-resistant fungal ascospores from samples collected throughout the processing of pasteurized high-acid fruit products. In addition, an assessment on the effect of processing on the contamination levels of HRM in these products was carried out. A total of 332 samples from 111 batches were analyzed from three processing plants (= three processing lines): strawberry puree (n=88, Belgium), concentrated orange juice (n=90, Brazil) and apple puree (n=154, the Netherlands). HRMs were detected in 96.4% (107/111) of the batches and 59.3% (197/332) of the analyzed samples. HRMs were present in 90.9% of the samples from the strawberry puree processing line (1-215 ascospores/100g), 47.0% of the samples from the orange juice processing line (1–200 ascospores/100g) and 48.7% of samples from the apple puree processing line (1-84 ascospores/100g). Despite the high occurrence, the majority (76.8%, 255/332) of the samples were either not contaminated (40.7%) or presented low levels of HRMs (36.1%), i.e., <10 ascospores/100g. For both strawberry puree and concentrated orange juice, processing had no statistically significant effect on the levels of HRMs ($p>0.05$). On the contrary, a significant reduction ($p<0.05$) in HRM levels was observed during the processing of apple puree. Twelve species were identified belonging to four genera - *Byssochlamys*, *Aspergillus* with *Neosartorya*-type ascospores, *Talaromyces*. *Rasamsonia* sp. *N. fumigata* (39.7%), *N. fischeri* (27%) and *B. nivea* (7.9%) were the predominant species in pasteurized products. The quantitative data (contamination levels of HRMs) were fitted to exponential distributions and will ultimately be included as input to spoilage risk assessment models which would allow better control of the spoilage of heat treated fruit products caused by heat-resistant moulds.

2.1 Introduction

Spoilage incidents of thermally processed high-acid fruit products by heat-resistant moulds (HRMs) have been reported for over 80 years and thus, considered as a widesproblem problem for the fruit industry, influencing both import and export markets (Beuchat, 1998, Filtenborg et al., 2004, Olliver and Rendle, 1934).

The most important species of heat-resistant fungi isolated from food products belong to the genera *Byssochlamys*, *Aspergillus*, *Talaromyces* and *Eupenicillium* (Dijksterhuis, 2007, Pitt and Hocking, 2009, Samson et al., 2010). HRMs have already been detected in various fruits and fruits products, such as strawberries, blueberries, lemon cells and apple juice (Aragão, 1989, Kikoku et al., 2008, Salomão et al., 2014, Tranquillini et al., 2017).

Besides their occurrence in raw materials, they have also been isolated from cardboard materials used in aseptic packaging (Delgado et al., 2012). The presence of HRMs in raw materials and processing environments may compromise the microbial stability of heat-treated fruit products. The spoilage risk is not only due to their great resistance to thermal processing and chemical compounds (e.g. chlorine, alcohol and hydrogen peroxide) (Dijksterhuis and Teunissen, 2004, Silva and Gibbs, 2004, Tournas, 1994), but also due to their ability to grow under low pH (pH<4.0) and limited headspace oxygen levels (Nielsen et al., 1989, Taniwaki et al., 2009, Tournas, 1994). The (asco)spores of many species are activated after a sub-lethal temperature or pressure trigger (Dijksterhuis and Teunissen, 2004, Reyns et al., 2003). Moreover, once present, even in low numbers, some HRMs can germinate during storage at room temperature and spoil the products (Sant´Ana et al., 2010a), causing large economic losses. Besides the spoilage risk, many species of heat-resistant fungi may also pose a health risk due to mycotoxin production (Frąc et al., 2015, Houbraken et al., 2006, Sant´Ana et al., 2010, Tournas, 1994).

In order to increase the microbial stability of high-acid fruit products, different tools can be used in the food process chain. These tools include the adoption of good manufacturing practices (GMPs) which are crucial to prevent entry of microbial contaminants into food processing environments and inactivation of potentially present ascospores by thermal (Kikoku et al., 2008, Sant'Ana et al., 2009, Scaramuzza and Berni, 2014, Souza et al., 2017) and non-thermal (Evelyn and Silva, 2015, Evelyn and Silva, 2017, Ferreira et al., 2009) processes. In addition, prevention of growth of HRMs is an equally important tool to increase the microbial stability of high-acid fruit products (Berni et al., 2017, Panagou et al., 2010, Taniwaki et al., 2009, Tremarin et al., 2015). However, the exact impact of these measures in reducing the probability of spoilage of high-acid fruit products by HRMs has not yet been adequately quantified.

Quantitative microbial risk assessment (QMRA), which is mostly used as a tool to determine and manage the risks of foodborne pathogens, has recently been used to assess the risk of spoilage of food by fungi (Gougouli and Koutsoumanis, 2017). This is a very valuable approach which

incorporates the variability related to the occurrence, inactivation and growth of target spoilage microorganisms along the food chain. Despite the vast amount of information regarding the inactivation of ascospores, few data (e.g. Aragão, 1989, Salomão et al., 2014, Tranquillini et al., 2017) are currently available regarding their occurrence in fruit products. Moreover, where data are available, only a low number of samples were analyzed leading to scarcity in quantitative data.

Therefore, there is a need for more data in order to determine the distribution of contamination levels of HRMs and their variability, not only in the raw material but also throughout the processing steps and in pasteurized products. Likewise, these data can be very useful to assess the spoilage risk of high-acid fruit products by heat resistant moulds. Thus, the major objectives of this study were to determine (i) the incidence and distribution of contamination levels of fungal ascospores in three fruit processing lines: strawberry puree, orange juice and apple puree; (ii) the identity at genus and species levels of detected HRMs; and (iii) the effect of processing on the contamination levels of ascospores.

2.2 Material and Methods

2.2.1 Sampling

A total of 332 samples (each *ca*. 150g) were aseptically collected from 111 batches of three processing plants: strawberry puree (Belgium), concentrated orange juice (Brazil) and apple puree (the Netherlands). The process flow diagrams are shown in Fig. 2.1. With regards to the strawberry puree processing, frozen strawberries are received and stored at -18ºC until they are used. After thawing, the strawberries are crushed and sieved and then held in a buffering tank until they are pasteurized and packaged. Samples of strawberry puree (n=29 batches, 88 samples) were collected during May to September 2016 at the follow stages: after crushing (n=29), after sieving (n=29) and after pasteurization (n=30). In the orange juice processing line, fresh oranges are received, washed and screened according their sizes/variety. The juice is then extracted and filtered before pasteurization in a heat exchanger. Orange juice concentrate samples (n=30 batches, 90 samples) were collected in August and September 2016 at the follow stages of processing: at extraction output (n=30), at evaporator inlet (n=30) and after pasteurization (n=30). With regards to the apple puree processing line, apples are received, washed and screened. The apple puree is obtained after extraction/sieving followed by pasteurization. Samples of apple puree (n=52 batches, n=154) were collected in October and

November 2016 at the following stages of processing: raw material-apples (n=52), after sieving (n=52) and after pasteurization (n=50). Pasteurization was in all cases performed for a few seconds. With regards to the relative intensity of the pasteurization applied, strawberry puree was subjected to the least intense pasteurization process, whereas apple puree was subjected to the most intense pasteurization process. All the samples were stored at -20ºC until the analysis were performed.

2.2.2 Quantification of heat-resistant fungal ascospores

Hundred grams (100 g) of sample was analyzed for heat-resistant fungal ascospores. Firstly, the samples were aseptically transferred to stomacher bags and diluted in 150ml of sterile distilled water. The diluted samples were then homogenized in a stomacher (Lab Blender 400, Seward Laboratory, London, UK) for two minutes and heat-sealed half way along their length to avoid the presence of air bubbles. After sealing, the bags were heat treated in a temperature-controlled water bath (Memmert, WB 10, Germany) at 80±1ºC for 30 min. Thereafter, the samples were aseptically transferred to Schott bottles containing 250ml of molten double strength Malt Extract Agar (MEA) (Oxoid, Basingstoke, UK) supplemented with chloramphenicol (200 mg/l, Oxoid, Basingstoke, UK) and tempered to 55°C. Subsequently, the heat-treated samples were thoroughly mixed with the agar and distributed into seven 140mm diameter Petri dishes. The dishes were placed in plastic bags and then incubated at 30°C for up to 30 days and visually checked for growth every 7 days. The results obtained after 30 days were expressed as ascospores/100g.

2.2.3 Isolation and morphological identification

All colonies recovered from analyzed samples were picked with a sterile inoculation loop, streaked onto MEA plates and incubated at 30°C for 7 days. This was repeated until pure cultures (isolates) were obtained, i.e., when identical macroscopic characteristics (e.g. color, size, colony appearance) were observed. The pure isolates were then cultured on different media and incubated at different temperatures according to the identification keys of Pitt and Hocking (2009) and Samson et al. (2010).

2.2.4 Molecular identification

Gene sequencing was used to identify the HRMs at the species level. The sequencing was performed in three stages: DNA extraction, DNA amplification and DNA sequencing. Firstly, the isolates were grown for 14 days at 25°C in 3 ml of potato dextrose broth (PDB) (Oxoid, Basingstoke, UK) in 12-well microplates. The resulting mycelial mats were harvested in Eppendorf tubes after centrifugation at 8000xg, 5 min and 4°C. The pellets obtained were then lyophilized at -80°C for 20 hours to produce a fine powder. Subsequently, the genomic DNA was obtained using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instructions. After extraction, the DNA was then amplified using the universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGC-3'). The PCR amplification reactions were performed by adding 2µl of genomic DNA to 23µl of reaction mixture (1.75µL of each primers, 5µL of PCR buffer, 0.5µL dNTPs, 0.15 µL Taq DNA polymerase and 13.85 µl ultrapure sterile water). Amplification was performed using a FlexCycler PCR thermocycler (Analytic Jena, Germany) programmed for an initial denaturation at 94°C for 10 min followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 60s, elongation at 72°C for 60 s and a final chain elongation at 72°C for 10min. Amplification products were then separated in 1.5% agarose gels in TAE-buffer at 100V for 25 min (MUPID-ONE electrophoresis system, Eurogentec, Seraing, Belgium) and visualized by ethidium bromide staining on a UV transilluminator (Gel Doc XR+ System, Bio-Rad, USA). The purification was performed using USB ExoSAP-IT PCR product cleanup reagent (Isogen, The Netherlands). Finally, the sequences were determined by LGC Genomics GmbH (Berlin, Germany) using Sanger sequencing. Consensus sequences were then created using the sequence alignment editor of BioEdit (BioEdit 7, USA). The consensus sequences were then blasted using the nucleotide blast (blastn function) of the BLAST (Basic Local Alignment Search Tool) tool of the National Center for Biotechnology Information (National Institute of Health, US), which can be found on http://blast.ncbi.nlm.nih.gov. The BLAST tool compares the consensus sequences to those in its database in order to ascertain sequence similarity.

2.2.5 Statistical analysis

Statistical analysis was performed using SPSS® version 24 (IBM, New York, USA) to assess whether the HRM contamination levels was affected by processing steps at which the sampling was performed. The contamination data (ascospores/100g) were firstly checked for normality by using the Shapiro–Wilk test ($p<0.05$), Q-Q plots and histograms. Non-normally distributed data

were then analyzed by means of the non-parametric, Kruskal-Wallis test, with ascospores/100g as the dependent variable and the processing stages of each processing as independent variables. P-values ≤0.05 were considered as indicating that the median HRM for at least one of the processing steps differs significantly from the other. The data (ascospores/100g) from the three processing lines were then fitted to statistical distributions by using @Risk 7.0 software for Excel (Palisade Corporation, NY, EUA). The goodness of fit was measured by the Chi-square test statistic, using the BestFit® data fitting module of @Risk.

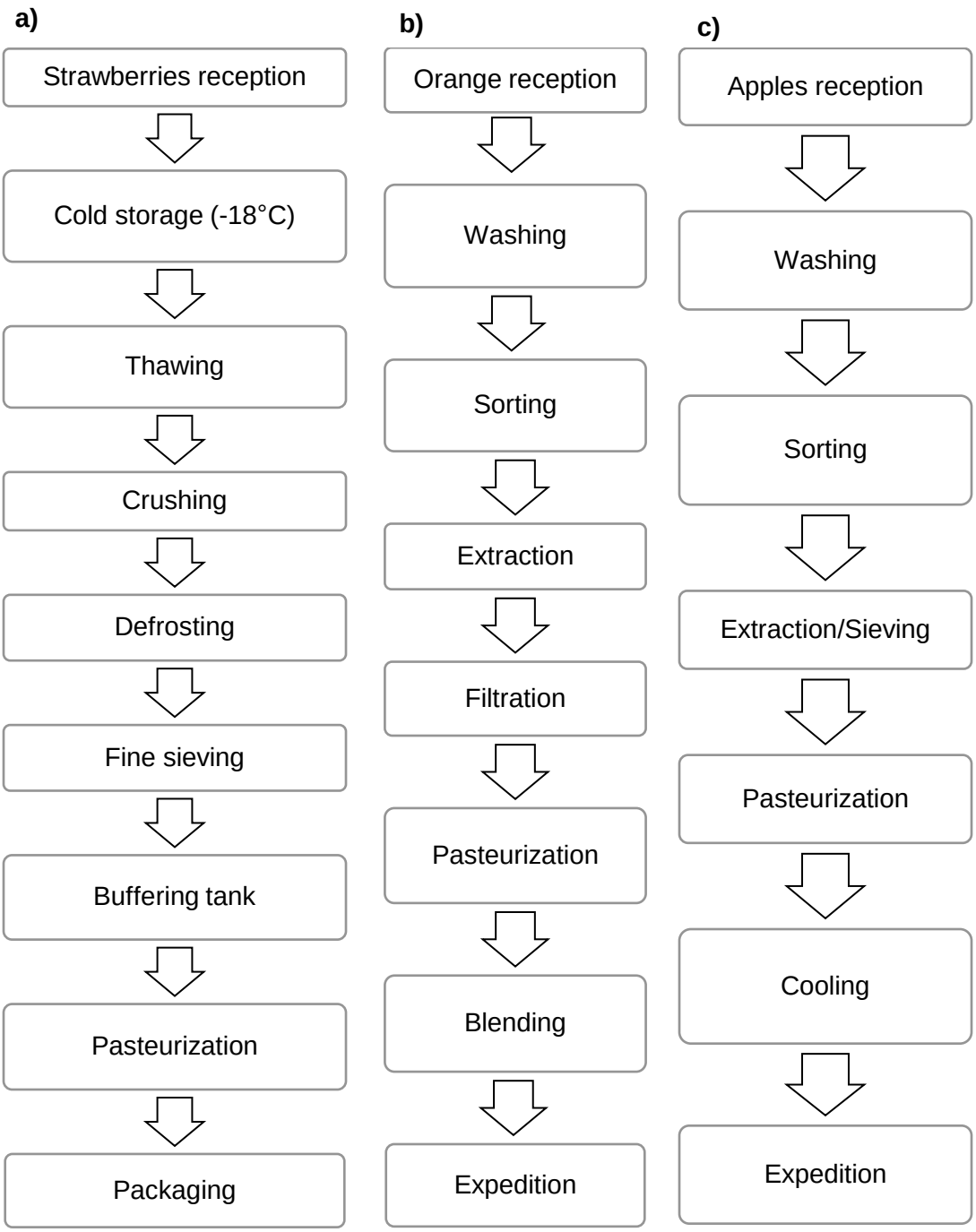

Figure 2.1 Flow charts of the a) strawberry puree, b) concentrated orange juice and c) apple puree processing lines evaluated in this study.

2.3 Results

During the months of June to November 2016, three high-acid fruit processing facilities (strawberry puree, apple puree and concentrate orange juice) were sampled. HRMs in the range of 1 to 215 ascospores/100 g fruit product were detected in 96.4% (107/111) and in 59.3% (197/332) of the analyzed batches and samples, respectively. The incidence and distribution of HRMs in the individual products are described in more detail below.

2.3.1 Occurrence and identification of HRMs in strawberry puree during processing

HRMs were detected in 90.1% (80/88) of the samples collected on the strawberry puree processing line at counts ranging from 1 to 215 ascospores/100 g (Fig. 2.2). To investigate whether processing had an effect on the HRM contamination levels, samples were collected from three different processing stages as described previously (*see* §2.2.1). HRMs were detected in 89.7% (26/29) of the samples collected immediately after crushing at levels ranging from 0 to 215 ascospores/100g, in 96.6% (28/29) of the samples collected immediately after sieving at levels ranging from 0 to 97 ascospores/100g and in 83.3% (25/30) of the strawberry puree samples collected after pasteurization at levels ranging from 0 to 157 ascospores/100g. Although the incidence of HRMs appeared to be lower in the pasteurized strawberry purees, it was determined that processing had no significant effect on the level of HRMs ($p>0.05$) (Fig. 2.3). A total of 263 isolates of HRMs (out of 1558 detected colonies) were recovered from the samples collected at the strawberry puree processing line. These isolates were identified to the genus level according the keys proposed by Pitt and Hocking (2009) and Samson et al. (2010).

The isolates belonged to two genera, *Byssochlamys* and *Aspergillus* with *Neosartorya*-type ascospores. HRMs belonging to *Aspergillus* with *Neosartorya*-type ascospores dominated the isolates which were identified, accounting for 98% of the isolates. Thereafter, 60 isolates were selected and identified at the species level by means of gene sequencing (Table 2.1). From the *Neosartorya*-type aspergilli, eight species were identified, namely *N. fumigata*, *N. fischeri, N. spinosa, N. glabra, N. hiratsukae, N. udagawae, N. laciniosa and N. coreana*. With exceptions of *N. spinosa* and *N. hiratsukae*, isolates of *Neosartorya* type aspergilli were recovered from the three processing steps evaluated. *N. fischeri* and *N. fumigata* were the most encountered species. They were isolated at all three stages of processing evaluated, being detected in 55.7 and 52.3% of the samples and in 86.2 and 82.8% of the batches analyzed, respectively. These were followed

by *N. laciniosa*, which was detected in 11% and 24.1% of samples and batches, respectively (Table 2.1).

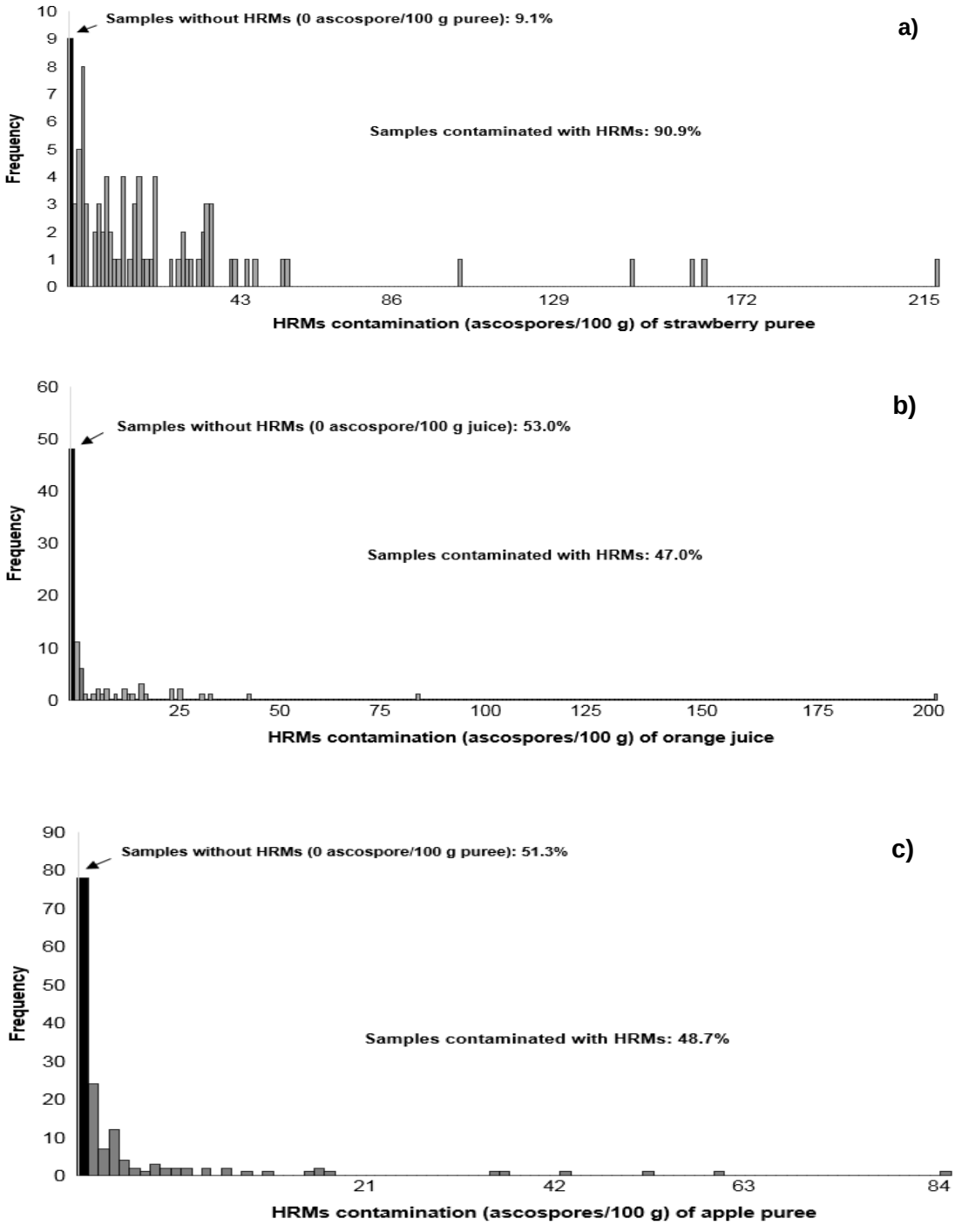

Figure 2.2 Distribution of HRM contamination levels in samples collected on a) a strawberry puree processing line, b) an orange juice processing line and c) an apple puree processing line.

The *Byssochlamys* species isolated were *B. nivea* and *B. fulva.* These were isolated from the three processing steps of strawberry puree line. *B. fulva* and *B. nivea* were present in 4.5% and 10.2% of the samples and in 13.8% and 27.6% of the batches analyzed, respectively. The relationship between species and level of contamination was also assessed. Despite the high occurrence of ascospores, absence of contamination was observed in 9.1% of samples (8/88) and 36.4% (32/88) of the strawberry samples were contaminated at low levels (1-10 ascospores/100g puree). The samples with the highest counts of HRMs (>100 ascospores/100g) were also the samples were *N. fumigata* and *N. fischeri* were recovered from as the dominating species. On the contrary, *B. fulva*, *B. nivea* and *N. coreana* were only detected in very low numbers (<10 ascospores/100g).

Table 2.1 Species of HRMs isolated from samples collected throughout the processing of strawberry puree.

Heat-resistant mould	Samples (%)	Batches (%)	After crushing	After sieving	After pasteurization
Byssochlamys fulva	4.5	13.8	X	X	X
Byssochlamys nivea	10.2	27.6	X	X	X
Neosartorya fischeri	55.7	86.2	X	X	X
Neosartorya fumigata	52.3	82.8	X	X	X
Neosartorya spinosa	2.3	6.8	X	X	
Neosartorya glabra	4.5	13.8	X	X	X
Neosartorya udagawae	7.9	24.1	X	X	X
Neosartorya laciniosa	11.0	24.1	X	X	X
Neosartorya hiratsukae	1.1	3.4	X		
Neosartorya coreana	10.0	17.2	X	X	X

% Samples: n contaminated samples/total samples. % Batches: n contaminated batches / total batches. X: indication of isolation of heat-resistant fungi from the correspondent step of processing

2.3.2 Occurrence and identification of HRMs in orange juice during processing

HRMs were detected in 46.7% (42/90) of the samples collected from the orange juice processing line at counts ranging from 1 to 202 ascospores/100g (Fig. 2.2). 56.7% (17/30) of the samples collected at extraction output and at evaporator inlet had counts ranging from 0 to 41 ascospores/100g and 0 to 200 ascospores/100g, respectively, whilst 26.7% (8/30) of the pasteurized samples had counts ranging from 0 to 80 ascospores/100g. Despite the lower incidence of HRMs in pasteurized juice, processing had no significant effect ($p>0.05$) on the level of HRMs (Fig. 2.3). Even though high levels of HRMs were sometime detected, 53% (48/90) of

samples were not contaminated and 27.8% (25/90) had low contamination levels (1-10 ascospores/100 g).

A total of 102 HRMs were isolated (out of 652 colonies) and purified after enumeration of the samples from the orange juice processing line. After morphological characterization of the isolates, four genera were identified, namely *Byssochlamys*, *Talaromyces*, *Rasamsonia* and *Aspergillus* with *Neosartorya*-type ascospores. As observed for strawberry processing, *Aspergillus* with *Neosartorya*-type ascospores were predominant, representing 86.3% (88/102) of the identified isolates. 32 isolates were selected for identification to the species level by means of gene sequencing. These were determined to belong to five species, namely *B. nivea*, *Talaromyces bacillisporus, Rasamsonia brevistipitata*, *N. fischeri* and *N. fumigata* (see Table 2.2). *N. fischeri* was predominant, being recovered from 31.1% (28/90) of the samples and 66.7% (12/30) of the batches. Moreover, it was isolated from all three stages of processing evaluated. However, *N*. *fischeri* was largely present in samples which had low counts of HRMs (<10 ascospores/100 g). The second most common species, *N. fumigata*, was also recovered at all processing steps evaluated, occurring in 16.7% (15/90) and 36.7% (11/30) of samples and batches, respectively. Moreover, it was largely isolated from the most contaminated samples (>100 ascospores/100g). HRMs with the teleomorph state of *Penicillium* sp. were represented by *B. nivea* and *Talaromyces bacillisporus. B. nivea* was isolated from all processing steps evaluated, and detected in 4.4% (4/90) of the samples and 13.3% (4/30) of the batches at relatively low levels (<10 ascospores/100 g). In contrast, *T. bacillisporus* and *Rasamsonia brevistipitata* were only isolated from 1.1% of samples (1/90) and 3.3% of batches (1/30) at the evaporator inlet (after filtration) and from pasteurized orange juice, respectively.

Table 2.2 Species of HRMs isolated from samples collected throughout the processing of concentrated orange juice.

Heat-resistant mould	Samples (%)	Batches (%)	After extraction	At evaporator	After pasteurization
Byssochlamys nivea	4.4	13.3	X	X	X
Talaromyces bacillisporus	1.1	3.3		X	
Neosartorya fischeri	31.1	66.7	X	X	X
Neosartorya fumigata	16.7	36.7	X	X	X
Rasamsonia brevistipitata	1.1	3.3			X

% Samples: n contaminated samples/total samples. % Batches: n contaminated batches / total batches. X: indication of isolation of heat-resistant fungi from the correspondent step of processing.

2.3.3 Occurrence and identification of HRMs in apple puree during processing

HRMs were detected in 48.7% (75/154) of the samples collected from the apple puree processing line at levels ranging from 1 to 84 ascospores/100g puree (Fig. 2.2). In general, the majority of the samples collected on this processing line were either not contaminated by HRMs (51.3%, 79/154) or were contaminated at low levels (1-10 ascospores/100 g) (38.3%, 59/154). HRMs were detected in 84.6% (42/52), 50% (26/52) and 10% (5/50) of the apple, sieved apple puree and pasteurized apple puree samples, respectively. In difference to the trends observed on the strawberry and orange processing lines, a significant reduction ($p<0.05$) was observed in the HRM levels during processing (Fig. 2.3), with the counts after pasteurization being significantly lower than those of apples and sieved puree.

From the 688 recovered colonies, 99 were isolated on MEA and grouped according to their phenotypic characteristics. From these, 25 were molecularly identified and determined to be *Byssochlamys*, *Talaromyces*, *Rasamsonia* and *Aspergillus* with *Neosartorya*-type sp. As can be seen in Table 2.3, a total of seven species were identified, namely *N. fumigata*, *N. fischeri*, *N. glabra*, *B. nivea*, *B. fulva*, *T. bacillisporus* and *Rasamsonia brevistipitata*. *N. fumigata* was predominant, being isolated from 30.5% (47/154) and 73.1% (38/52) of the samples and batches, respectively. *B. nivea* was the second most prevalent species after *N. fumigata*, being detected in 14.3% (22/154) of samples and 42.3% (22/52) of batches. In addition to their high incidence, *B. nivea* and *N. fumigata* were the only species isolated from all three stages of the processing evaluated in this study. *B. fulva* and *N. fischeri* were detected in only 1.3% (2/154) and 1.9% (3/154) of the samples from the apple processing line, respectively. Less occurring, *N. glabra*, *T. bacillisporus* and *R. brevistipitata* were equally isolated from 0.7% of samples (1/154). The levels of HRMs found in pasteurized apple puree were very low (1 ascospore/100g). Of the five pasteurized apple puree samples which were contaminated by HRMs, two were contaminated with *B. nivea* and three with *N. fumigata.*

Table 2.3 Species of HRMs isolated from samples collected throughout the processing of apple puree.

Heat-resistant mould	Samples (%)	Batches (%)	Apples	After sieving	After pasteurization
Neosartorya fumigata	30.5	73.1	X	X	X
Byssochlamys nivea	14.3	42.3	X	X	X
Byssochlamys fulva	1.3	3.9	X		
Neosartorya fischeri	1.9	5.8	X	X	
Neosartorya glabra	0.7	1.9	X		
Talaromyces bacillisporus	0.7	1.9		X	
Rasamsonia brevistipitata	0.7	1.9	X		

% Samples: n contaminated samples/total samples. % Batches: n contaminated batches / total batches. X: indication of isolation of heat-resistant fungi from the correspondent step of processing.

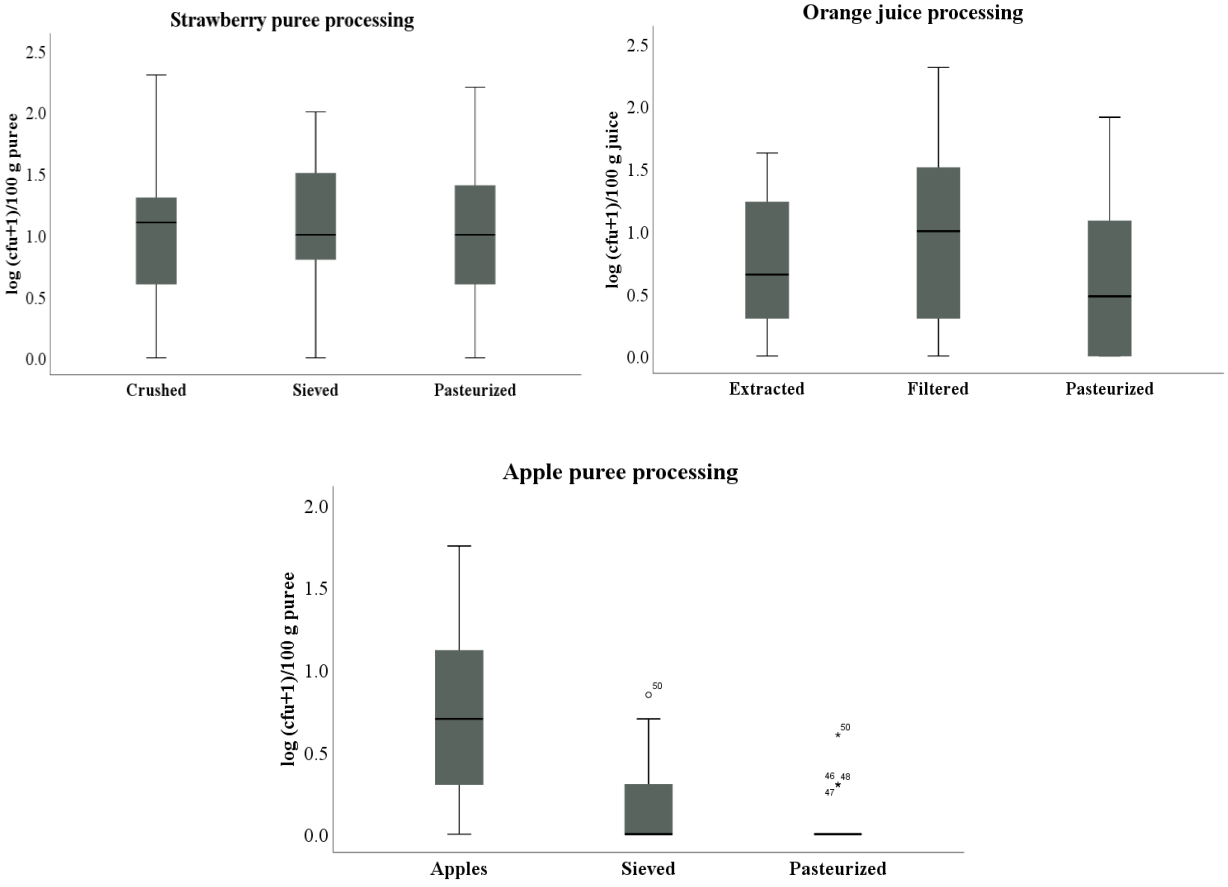

Figure 2.3 Effect of processing on the levels of HRMs during the processing of strawberry puree, orange juice and apple puree.

2.3.4 Distribution of HRMs in pasteurized high-acid fruit products

The results regarding the contamination levels (ascospores/100g) of samples from each processing line were fitted to statistical distributions in @Risk. Each distribution represents the probabilities of contamination of the product (strawberry puree, orange juice or apple puree) throughout its processing. Therefore, different scenarios of possible HRMs contamination were expressed by the best fitting statistical distribution. In order to include the scenario where no contamination was observed, the results from non-contaminated samples (0 ascospores/100g) were also considered. The best fitting statistical distribution for each process line was based on the chi-square statistic, available in @Risk. According to this statistic, exponential distributions were selected and fitted to the data. The distribution of HRM contamination levels in the three fruit products were represented by right-skewed curves lower bounded at 0 and represented by the following statistic parameters (mean of ascospores levels expressed as ascospores/100g and the shift of the curve on the x-axis): mean = 9.2, RiskShift = -0.04 for strawberry puree; mean = 7.5, RiskShift = -0.08 for orange juice and mean= 4.5, RiskShift = -0.03 for apple puree (Table 2.4).

Table 2.4 Statistical distributions fitted to HRMs occurrence in high-acid fruit products.

Fruit Processing	**Statistical distribution**[1]
Strawberry puree	RiskExpo (9.2;Risk Shift(-0.04))[2]
Orange juice	RiskExpo (7.5;Risk Shift(-0.08))
Apple puree	RiskExpo (4.5;Risk Shift(-0.03))

[1] Exponential distributions selected according to chi-square statistic.
[2] RiskExpo (mean; minimum value shifted from 0)

2.4 Discussion

The preservation of pasteurized fruit products is mainly based on a combination of their high acidity and the heat process applied. Although this is usually sufficient to eliminate and prevent the growth of most spoilage microorganisms and pathogens, it is not sufficient to eliminate the spores of some microorganisms. To assess the occurrence and variability of HRMs contamination levels during processing, three fruit processing lines were investigated. From the 332 samples analyzed, 59.3% were contaminated with ascospore-forming moulds at levels ranging from 1 to 215 ascospores/100g of product. The counts observed were predominantly low, i.e. <1 ascospore/g. However, it has been reported that the incidence of ascospores at levels as low as 5 per 100g (ml), just prior to the heat treatment, may indicate a problem (Rico-Munoz et al., 2015).

Despite this, no criteria have yet been set regarding the minimum (acceptable) level of ascospores in food products. In this regard, some factors may play important roles such as type of product and storage conditions. As HRMs are unlikely to grow at temperatures below 10°C, the acceptable levels of contamination in chill stored food products would be expected to be higher than those for shelf-stable food products stored at room temperature. The acceptable levels of contamination in ingredients such as fruit purees and concentrated fruit juices which are diluted in the final product would be expected to be proportionally higher than those determined to be acceptable in the final product. In addition, the intensity of processing (thermal and/or non-thermal pasteurization) and the composition of the final product, such as presence and level of preservatives, sugar concentration etc. (Rico-Munoz et al., 2015) will also play a role in setting robust microbial criteria for these products.

The predominant incidence of HRMs at low levels has also been observed in other studies, for instance, on strawberry pulp (1-10cfu/g) (Aragão, 1989), apples (≤3 cfu/100g) (Salomão et al., 2014), concentrated apple juice (≤2 cfu/100ml) (Salomão et al., 2014), grape juice (<5 cfu/100ml) (Marcolino, 2003), pineapple juice (≤3 cfu/kg) (Enigl et al., 1993), frozen blueberries (4-54 cfu/kg) (Tranquillini et al., 2017), frozen lemon cells (< 200cfu/kg) and frozen strawberries (2-642 cfu/kg (Tranquillini et al., 2017). However, these data were collected from a very restricted number of samples, mostly from the raw material without taking into account the distribution of contamination throughout processing, such as from pasteurized fruit products.

The incidence of HRMs was higher in the samples collected from the strawberry puree processing line (90.1%), almost double of the incidence observed on the samples collected on the orange juice processing line (46.7%) and the apple puree processing line (48.7%). These results could be expected as strawberries typically grow close to the ground with a high risk of contamination from the soil. Moreover, the differences in intensities of pasteurization could have potentially contributed to these results. The high occurrence of HRMs in strawberries has also been reported by other authors (Aragão, 1989, Tranquillini et al. 2017). Strawberry puree is largely used as an ingredient in many food products including yoghurts, juices, purees, ice cream and baked goods. Consequently, the high incidence of HRMs can be considered as a threat to the microbial stability of these products, especially those stored at room temperature. The strawberry puree was obtained from 100% of fruit, without addition of ingredients. The strawberries used as raw material at the processing line evaluated in this study were obtained from different countries and combined according to requirements just before crushing was performed. Therefore, the variability observed

with regards to the levels of HRMs in the samples collected on the strawberry processing line may partly be related to the geographical differences in their origin and transportation conditions.

A great paucity currently exists with regards to data on the occurrence of HRMs in citrus fruit and their derivatives. The only available data is limited to a recent study by Tranquillini et al. (2017) in which the incidence of HRMs in lemon cells was reported. That study determined that 50% of lemon cells were contaminated by HRMs, which agrees with the findings of this study, whereby HRMs were detected in 46.7% of the samples collected on the concentrated orange juice processing line. Concentrated orange juice is a raw material for the production of single strength orange juice (= reconstituted orange juice) and is exported as frozen concentrated orange juice. In this study, the samples collected after extraction and before evaporator (= after filtration), had similar HRM occurrence levels (56.7%), indicating that filtration did not have an effect on the contamination levels ($p>0.05$).

HRMs have been detected in apples and its derivatives, such as in concentrated apple juice processing from different steps e.g. wash water and pasteurized products (Salomão et al., 2014). The findings from our study suggest that these HRMs are introduced to the processing facilities by the apples, which had the highest contamination levels. The reduction observed from sieving may be due to the fact that a significant amount of the HRMs are located on the apple peel which is separated from the pureed flesh during sieving. Likewise, the intensity of the heat treatment applied to the apple puree may have resulted in inactivation (and/or possibly activation) of present ascospores, which could explain the drop in counts of HRMs.

It is also important to mention that the most frequently occurring species on the apples were also the ones which were encountered in samples of pasteurized apple puree. This finding shows, that the levels of HRMs in the raw material may strongly affect the microbial quality and subsequent stability of the heat-treated apple puree products.

Although the counts of HRMs in the samples collected on the apple puree processing line decreased significantly ($p<0.05$) during processing, none of the thermal processes employed on all three processing lines evaluated in this study were able to completely eliminate ascospores. HRMs were found in 34.5% (38/110) of the pasteurized fruit products evaluated in this study (Fig. 2.4). With the exception of *N. spinosa* and *N. hiratsukae*, all the other HRMs which were isolated and identified from the three processing lines were also isolated from the heat-treated fruit

products. The incidence of HRMs was higher in pasteurized strawberry puree (83.3%, 25/30), followed by concentrated orange juice (26.6%, 8/30) and apple puree (10.0%, 5/50). The levels of occurrence and the HRMs species isolated from samples of pasteurized products are presented in Fig. 2.5. *N. fumigata* (39.7%), and *B. nivea* (7.9%) were isolated from pasteurized samples of all three products. In addition to these two, *N. fischeri*, was present in 27% of the pasteurized products evaluated in this study. Rather than inactivate, many (sub-lethal) thermal process as may actually activate present ascospores. Consequently, heat activated ascospores may germinate and spoil the food under favorable conditions during storage.

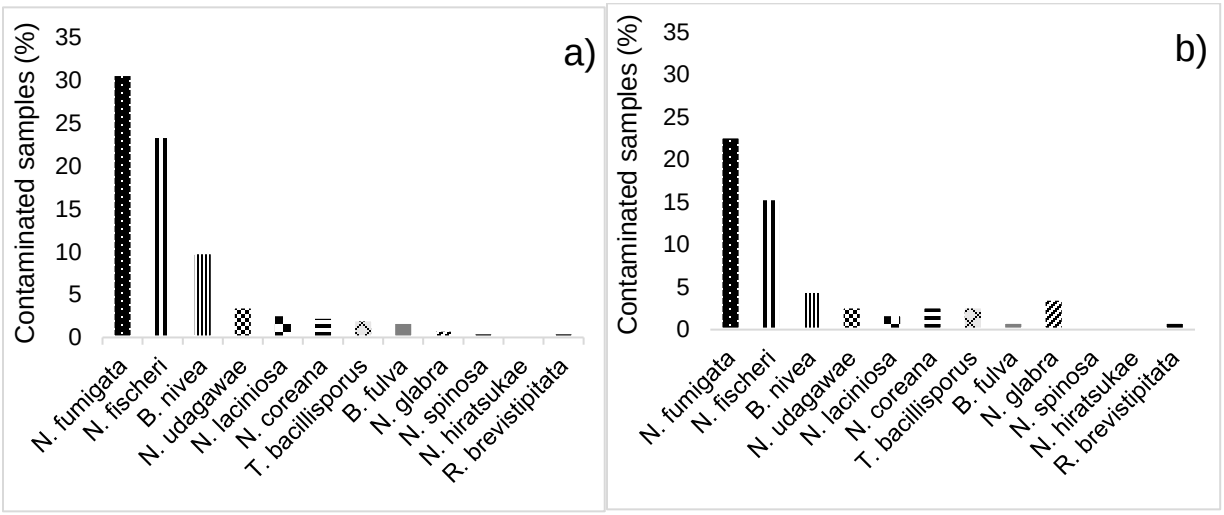

Figure 2.4 Occurrence of HRMs in a) high-acid fruit products (strawberry puree, orange juice and apple puree), N=332 and in b) pasteurized high-acid fruit products, N=110.

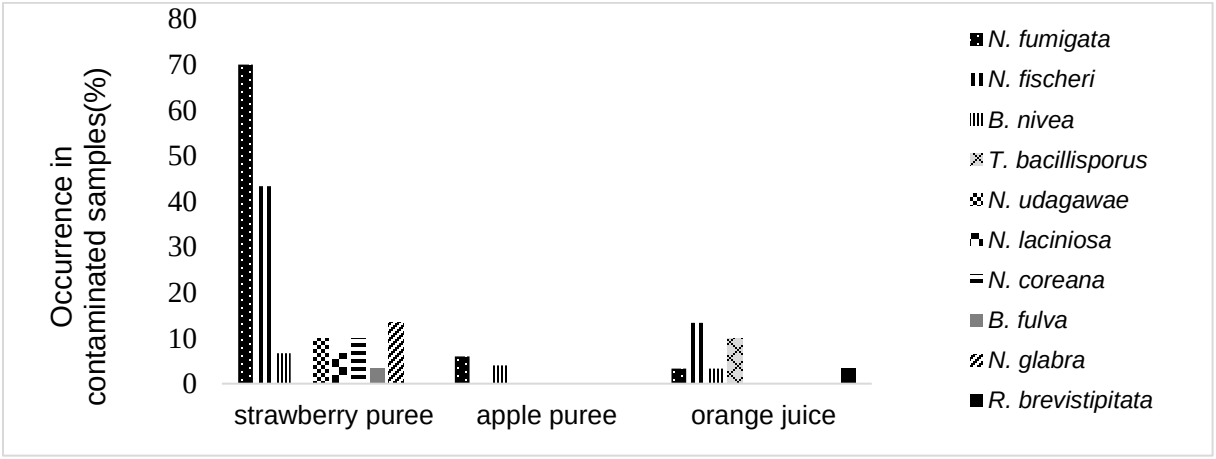

Figure 2.5 Occurrence of HRMs (% contaminated samples) in high-acid pasteurized fruit products.

The use of quantitative microbial risk assessment (QMRA) to assess the risk of food spoilage is restricted to a recent study of *A. niger* in yoghurt (Gougouli and Koutsoumanis, 2017). This probabilistic approach incorporates different sources of variability to predict the risk, e.g., environmental and biological variability. As observed in this study, the HRMs may occur at different levels in the high-acid fruit products depending on the matrix, processing stage and many other factors, such as geographical source of the fruits. However, due to the predominance of low levels, exponential right-skewed distributions were chosen to present the best fit to the data. The importance of using this stochastic approach in a spoilage risk study is the possibility of simulating different scenarios of contamination and combine these probabilities with inactivation and growth probabilities for each high-acid fruit product.

Despite the limited number of HRMs identified (*Byssochlamys* sp., *Aspergillus* with *Neosartorya*-type ascoma, *Talaromyces* sp. and *Rasamsonia* sp.) in this study, the majority are of great economic importance as they are associated with the spoilage of pasteurized high-acid fruit products. Fungi belonging to *Aspergillus* section *Fumigati,* represented by aspergilli with *Neosartorya*-type ascospores, dominated the identified isolates. *N. fumigata* is a cosmopolitan contaminant, soilborne, thermo-tolerant and mostly present in warm environments (O'Gorman et al., 2009; Samson et al., 2010). It was isolated from all three processing lines evaluated and also from samples of the pasteurized fruit products processed on these lines. Besides being isolated from food (Copetti et al., 2011, Pitt and Hocking, 2009, Wigmann et al., 2015), *A. fumigatus* is a well-known indoor air contaminant which is capable of producing several mycotoxins such as fumigaclavines, fumitoxins, fumitremorgins, verruculogen (Pitt and Hocking, 2009, Samson et al., 2007, Samson et al., 2010). *Aspergilli* with *Neosartorya*-type ascoma were isolated from all three matrices studied, with markedly higher incidence in samples collected on the strawberry processing line, followed by the samples collected on the orange processing line. Occurrence was determined to be low in samples collected on the apple processing line. These results may be attributed to the geographical origin of fruits. It is known that food spoilage by *Aspergillus* sp. are predominantly encountered in tropical zones (Pitt and Hocking, 2009). The oranges and strawberries analyzed in this study came mostly from tropical countries and from east and south Europe during summer. Within the aspergilli, *N. fischeri* was the most frequently isolated species in this study. *N. fischeri* has been commonly isolated from heat-treated food products (Pitt and Hocking, 2009, Salomão et al., 2008, Samson et al., 2010) due to the great heat resistance of its ascospores (D_{92^oC} = 0.8 min) (Evelyn and Silva, 2017, Souza et al., 2017).

Byssochlamys sp., mostly *B. fulva* and *B. nivea*, have been extensively studied, due to their well-known importance as potential spoilers of heat-treated fruit products (Baglioni et al., 1999, Olliver and Rendle, 1934, Pitt and Hocking, 2009, Salomão et al., 2008, Samson et al., 2010, Tournas, 1994). The occurrence of *Byssochlamys* sp. was confirmed in all three fruit processing lines and in the pasteurized fruit-products processed on these lines as well. Although *B. nivea* was isolated from all processing lines evaluated, it occurred predominantly in samples collected from the apple puree processing line, where it was also present at generally higher levels (up to 62 ascospores/100g puree) than at the other processing lines (<10 ascospores/100g product). In general*, B. fulva*, occurred at a lower incidence than *B. nivea* and was only isolated from samples collected on the strawberry and apple puree processing lines. It is important to mention that both, *B. fulva* and *B. nivea* are known to form asci's containing eight ascospores and that no procedure was performed in this study to release the ascospores from the asci's. As a result, the HRM contamination levels were potentially underestimated. Nevertheless the contamination levels reported represent the number of ascospores and ascis as naturally occur in the food products. However, in addition to its great heat resistance (D_{95^oC} = 1.81 min, Sant'Ana et al., 2009), *B. fulva* is also able to grow in atmospheres with high CO_2 and/or very low oxygen contents (Pitt and Hocking, 2009; Taniwaki et al., 2009). These attributes can contribute to its ability to spoil fruit products. Besides their incidence in fruit and fruit products (Salomão et al. 2008, Tournas, 1994, Tranquillini et al., 2017) some *Byssochlamys* species may also produce toxic secondary metabolites such as patulin, mycophenolic acid, byssochlamic acid and byssotoxin A (Houbraken et al., 2006).

Despite their lower occurrence, HRMs belonging to *Talaromyces* sp. have also been isolated from various pasteurized food products such as berries and lemon cells (Pitt and Hocking, 2009, Salomão et al., 2014, Samson et al., 2010, Tranquillini et al., 2017, Yaguchi et al., 2005). The main species related to food spoilage are *T. trachyspermus*, *T. flavus*, *T. macrosporus* and *T. bacillisporus*. Of these, *T. bacillisporus* was isolated from the samples collected on the orange juice and apple processing line in this study - just before pasteurization (at evaporator inlet), from pasteurized concentrated orange juice and also from sieved apple puree. *T. bacillisporus* is able to grow at up to 45°C (Mouchacca, 2007) and its ascospores are highly heat resistant (D_{91^oC} = 1.24 min, Tranquillini et al., 2017). *Rasamsonia* has only recently been recognized as a distinct genus (Houbraken et al., 2012). They have already been isolated from pineapple (Yaguchi, 2005), heat treated fruit concentrate, indoor environment, soil (Houbraken et al., 2012) and more recently, from berries (Tranquillini et al., 2017). *R. brevistipitata* is a thermo-tolerant fungus, able

to growth up to 45°C (Houbraken et al., 2012).

2.5 Conclusions

The incidence of HRMs in fruit products and their ability to overcome pasteurization is still a challenge for the fruit processing industry. This study determined the distribution of HRM contamination levels in fruit samples collected at various processing steps of three high-acid fruit processing lines. Despite their higher occurrence in the raw fruits, HRMs were also isolated from intermediate products and from pasteurized strawberry puree, orange juice and apple puree. It was observed that the heat treatment applied for apple puree may contribute to reducing the HRM levels. However, none of the heat treatments applied on these three processing lines were enough to completely inactivate the ascospores. From twelve HRM species identified, *N. fumigata* (39.7%), *N. fischeri* (27%) and *B. nivea* (7.9%) were the predominant species in pasteurized products. Besides their potential to cause spoilage, these moulds may also produce mycotoxins. Moreover, the most frequently isolated species from each processing line were also isolated from the heat-treated products. The data obtained were then fitted to exponential distributions which will be used as input for spoilage risk assessment studies of high-acid pasteurized fruit products by heat-resistant moulds. The findings from this study suggest that HRMs may potentially affect the microbial stability of fruit products and that different scenarios of contamination should be considered in studies focused on preventing spoilage by these microorganisms.

Chapter 3

Inter- and intra-species variability in heat resistance and the effect of heat treatment intensity on subsequent growth of *Byssochlamys fulva* and *Byssochlamys nivea*

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Abstract

The major aims of this study were to assess inter- and intra-species variability of heat resistant moulds (HRMs), *Byssochlamys fulva* and *Byssochlamys nivea*, with regards to (i) heat resistance and (ii) effect of heat treatment intensity on subsequent outgrowth. Four-week-old ascospores were suspended in buffered glucose solution (13° Brix, pH 3.5) and heat treated in a thermal cycler adjusted at 85°C, 90°C and 93°C. Two variants of the Weibull model were fitted to the survival data and the following inactivation parameters estimated: b (inactivation rate, min^{-1}), n (curve shape) and δ (the time taken for first decimal reduction, min). In addition to the assessment of heat resistance, outgrowth of *Byssochlamys* sp. from ascospores heated at 70°C, 75°C and 80°C for 10 minutes and at 93°C for 30 and 70 s was determined at 22°C for up to 30 days. The Baranyi and Roberts model was fitted to the growth data to estimate the radial growth rates (μ_{max}, $mm.day^{-1}$) and lag times (λ, days). Inter-species variability and significant differences ($p<0.05$) were observed for both inactivation and growth estimated parameters among *B. fulva* and *B. nivea* strains. The effect of heat treatment intensity on outgrowth of *B. fulva* strains was more apparent at the most intense heat treatment evaluated (90°C/10 min), which was also the condition in which greater dispersion of the estimated kinetic parameters was observed. On the other hand, *B. nivea* strains were more affected by heating, resulting in greater variability of growth parameters estimated at different heating intensities and in very long lag phases (up to 25 days). The results show that inter- and intra-species variability in the kinetic parameters of *Byssochlamys* sp. needs to be taken into account for more accurate spoilage prediction. Furthermore, the effect of thermal treatments on subsequent outgrowth from ascospores should be explored in combination with other relevant factors such as °Brix and oxygen concentration to develop thermal processes and storage conditions which can prevent the growth of HRMs and spoilage of heat treated food products.

3.1 Introduction

Quantitative microbial risk assessment (QMRA) studies, which are mostly used to determine the risk of foodborne pathogens in food, have more recently been applied to assess the risk of spoilage microorganisms in foods (Gougouli and Koutsoumanis, 2017). The use of QMRA to predict the spoilage risk of fruit products should consider certain variabilities (e.g. microbiological, environmental) and uncertainties (Zwietering, 2015), as is done in food safety studies. It is known that overall microbiological variability may arise from different sources such as experimental,

reproduction (intra-strain variability) and intra-species variability (or inter-strain variability). Also, growth and inactivation kinetics may be strongly affected by inter-strain variability and the variability within strain population i.e. the population heterogeneity (den Besten et al., 2017, Pouillot and Mubran, 2011). However, despite the availability of studies which have reported inactivation of HRMs by thermal (Kikoku et al., 2008, Sant'Ana et al., 2009, Scaramuzza and Berni, 2014, Souza et al., 2017) and non-thermal processes (Evelyn and Silva, 2015, Evelyn and Silva, 2017, Ferreira et al., 2009), there is no information on variability in response at the level of the strain.

Not only the high heat resistance of HRMs have challenged fruit processors and researchers but also their ability to grow in a broad range of conditions. Furthermore, there is a lack of information regarding how the heat treatments affect the time required by sub-lethally injured ascospores to recover and form visible colonies (= lag time). This is very valuable information for assessing the viability of heat treated ascospores and predicting the growth of HRMs in heat treated fruit products (= spoilage). Likewise, whilst several studies have focused on prevention of the growth of HRMs (Berni et al., 2017, Panagou et al., 2010, Taniwaki et al., 2009, Tremarin et al., 2015), the vast majority is performed using single strains and hence do not take into account potential inter-strain variation. Therefore, inactivation and growth studies performed with single strains, may fail to accurately predict the behavior of other strains. Hence, the use of kinetic parameters estimated from single strains in QMRA studies may be not adequate to robustly estimate risk of spoilage.

Therefore, the major objective of this study was to assess variability in HRMs through: (i) assessing variability in heat resistance among *Byssochlamys* strains isolated from a strawberry puree processing line, (ii) assessing the effect of heat treatment intensity on variability of germination and outgrowth (i.e. lag duration and growth rates) of *Byssochlamys* ascospores.

3.2 Material and methods

3.2.1 Isolates

Seven *Byssochlamys* sp. isolated from samples collected on a strawberry puree processing line were used in this study: *B. fulva* (*Bf*1) isolated from pasteurized strawberry puree; *B. fulva* (*Bf*2) isolated from sieved strawberry puree; *B. fulva* (*Bf*3) isolated from crushed strawberry puree; *B.*

nivea (*Bn*1) isolated from pasteurized strawberry puree; *B. nivea* (*Bn*2) isolated from sieved strawberry puree; *B. nivea* (*Bn*3) isolated from pasteurized strawberry puree and *B. nivea* (*Bn*4) isolated from crushed strawberry puree.

3.2.2 Preparation of ascospore suspensions

Each isolate was grown for 30 days at 30°C on 45 plates of Malt Extract Agar (MEA, Oxoid, UK) before the ascospores were harvested. Ascospores suspensions were obtained by flooding each Petri dish with 4ml of sterile 0.1% Tween 80 (Sigma-Aldrich, USA). The resulting suspensions were then filtered through sterile cotton and centrifuged for three times at 8.000 g for 15 min at 4°C. After each centrifugation, the supernatant was discarded and 10 ml of sterile distilled water added to the pellet and mixed. *B. nivea* strains were sonicated in an ultrasonic bath (Vibra ≈ Cell^{TM} 75186, United States) for 4 min to separate the ascospore clusters. To determine the ascospores concentration, the first dilution of each suspension was then heat treated in a water bath (Memmert, WB 10, Germany) at 80°C for 10 min. Thereafter, the suspensions were enumerated by spread plating serial decimal dilutions (in sterile 9ml-distilled-water tubes) of the suspensions on MEA. Enumeration was done after incubation of the plates for 5-7 days at 30°C. The final suspensions, with 10^6-10^7 ascospores/ml, were then transferred to 50 ml falcon tubes containing sterile glass beads and kept at 2°C for at most 30 days.

3.2.3 Evaluation of thermal resistance

Thermal resistance tests were carried out in buffered glucose solution (prepared by mixing KH_2PO_4 9.073 g/l with $NaHPO_4$ 11.867 g/L), adjusted to pH 3.6 with 0.67M tartaric acid and 13° Brix by adding sucrose. Before the trials, 1 ml of each suspension was transferred to a sterile Eppendorf tube and centrifuged at 13000 g for 15 min. The ascospores were then suspended in 1ml of the adjusted buffer-glucose solution. Thirty microliters of suspended ascospores were transferred to thin walled PCR tubes (Bioplastics, Landgraaf, Netherlands) which were heat treated in a thermal cycler (Arktik Thermal Cycler; Thermo Fisher Scientific, Pittsburgh, PA, USA) adjusted to 85°C, 90°C and 93°C. Initially the heating program was set at 80°C for 1 min to standardize the initial sample temperatures and to reduce the come-up-time (time to reach the target temperature). Time 0 was assumed after this initial heating. Triplicate tubes were then removed at each of eight set time intervals and immediately immersed in an ice-water bath. After cooling, serial decimal dilutions were prepared in 96-well plates containing 0.1% bacteriological

peptone (Oxoid, England) broth and spread plated onto MEA plates. Colonies were counted after incubation of the MEA plates at 30 °C for up to 7 days.

3.2.4 Estimation of inactivation parameters

Two variants of the Weibull model: Eq. 1 (Peleg and Cole, 1998; Weibull, 1951) and Eq.2 (Mafart et al., 2002) were fitted to each replicate (n=3) of the survival data (log of surviving ascospores as a function of the heating time) to estimate the inactivation parameters: b = scale factor (inactivation rate, min^{-1}) (from Eq. 1); δ = time (min) for the first decimal reduction (from Eq. 2) and n = survival curve shape (from Eq. 1); where $n < 1$ = survival curves with concave upwards (tails), $n > 1$ = concave downwards (shoulders) and $n = 1$ corresponds to log-linear curves.

$$log\frac{N}{N0} = -bt^n \quad \text{(Eq. 3.1)}$$

The parameters of Eq. 1 were estimated by means of the non-linear regression function of SPSS® version 25 (IBM, New York, US) whereas those of Eq. 2 were estimated by means of GInaFiT v1.6 (Geeraerd et al., 2005).

$$log\frac{N}{N0} = -(t/\delta)^{\cdot} n \quad \text{(Eq. 3.2)}$$

3.2.5 Effect of intensity of heat treatments applied to ascospores on outgrowth of HRMs

To assess the effect of heat treatment intensity on the outgrowth of (surviving) ascospores, 30 µl of ascospores resuspended in buffer glucose solution (adjusted to pH 3.6 and 13°Brix as described above) from each strain were heat treated in a thermal cycler (Arktik Thermal Cycler; Thermo Fisher Scientific, Pittsburgh, PA, USA) adjusted at 70°C, 75°C, 80°C, 85°C and 90°C for 10 minutes and at 93°C for 30 and 70s. After been cooled in a water-ice bath, 10µl (≤10^4 ascospores/ml) of the heat treated ascospores were centrally inoculated on duplicate acidified MEA plates (pH 3.6) and incubated at 22°C. Growth was assessed by measuring perpendicular colony diameters (mm) with a digital caliper at regular intervals for at most 30 days. The experiment was repeated twice. A 30-day-incubation period was used taking into account that sub-lethally injured ascospores would potentially require a longer time to recover and grow out.

3.2.6 Estimation of growth parameters

The growth parameters, maximum growth rate (μ_{max}, mm.day^{-1}) and the lag time (λ, days), were estimated by fitting the Baranyi and Roberts (1994) model to non-transformed growth data (colony diameter as a function of time) for each replicate (n=4) of each strain. The fitting was performed by means of the non-linear regression function of SPSS® version 25 (IBM, New York, US).

$$D(\mathrm{t}) = \mu_{max}\, A - \ln\left[1 + \frac{\exp(\mu_{max}\, A) - 1}{\exp(D_{max})}\right] \quad \text{(Eq. 3.3)}$$

$$A = t + \left(\frac{1}{\mu_{max}}\right) ln[exp(-\mu_{max} t) + exp(-\mu_{max}\lambda) - \exp(-\mu_{max} t - \mu_{max}\,\lambda)] \quad \text{(Eq.3.4)}$$

Where *D*(t) is the colony diameter (mm) as function of time (days), μ_{max} is the maximum growth rate (mm.day^{-1}), λ (days) is the lag time, which is the inflection point, and $D_{max}\,(\mathrm{mm})$, the maximum colony diameter attained.

3.2.7 Statistical analysis

The average of each estimated inactivation or growth parameter, their standard deviations and their 95% CI´s were calculated in SPSS® version 25 (IBM, New York, US) considering the data obtained from two repeated experiments, each one with two replicates for the growth and three replicates for the thermal resistance study per strain and treatment. Significant differences (at α = 0.05) between strains and treatments were determined by comparing their 95% CI´s for overlap or lack thereof.

3.3 Results and discussion

3.3.1 Thermal resistance

The thermal resistance of ascospores of the *Byssochlamys* strains was evaluated at 85ºC, 90ºC and 93ºC in acidified buffered glucose. The thermal death curves of *B. fulva* and *B. nivea* are shown in the Fig. 3.1. Two variants of the Weibull model were used to estimate the inactivation parameters (see Eq. 3.1 and Eq. 3.2) on the basis of the non-linearity of the survival curves. From Eq. 3.1, two parameters were estimated: inactivation rate, *b* (min^{-1}), and the shape parameter, n.

From Eq. 2, δ (min), time for the first decimal reduction was estimated. The estimated parameters are shown in the Table 3.1.

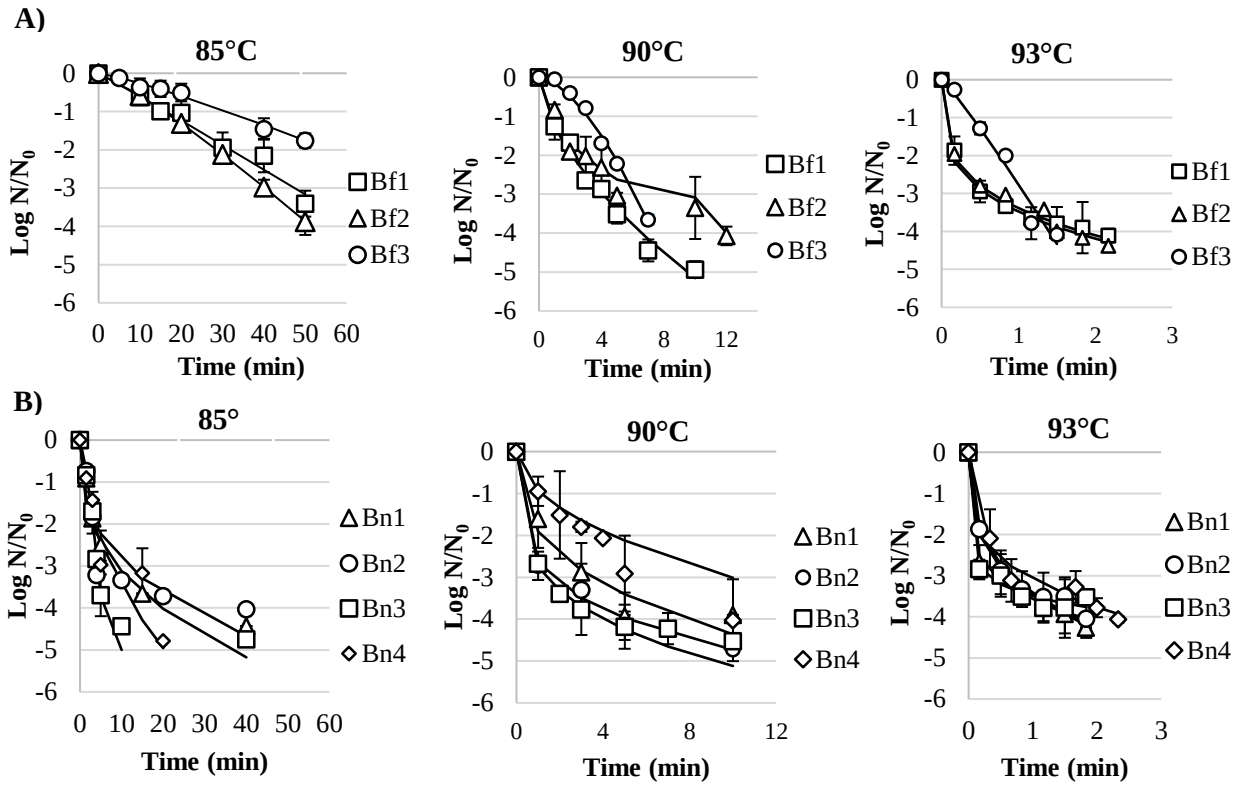

Figure 3.1 Survival curves of A) *B. fulva* strains (*Bf*1, *Bf*2 and *Bf*3) and B) *B. nivea* strains (*Bn*1, *Bn*2, *Bn*3 and *Bn*4) in acidified buffer glucose solution at 85°C 90°C and 93°C. Solid line = fitted Weibull model (Eq. 3.1).

In general, *B. fulva* strains were more heat resistant than those of *B. nivea*. At 85°C, the thermal death curves of *B. fulva* at 85°C approached log linear kinetics, characterized by slight downward concavity indicating the presence of a heat resistant sub-population (Fig. 3.1). By increasing the heating temperature, distinct survival curves were observed for the *B. fulva* strains. The thermal death curves of *Bf*1 and *Bf*2 at 90 and 93°C were characterized by tailing, indicating the presence of two sub-populations, the largest of which was heat-labile (inactivated at the beginning of the treatments) and the smaller being more heat-resistant. On the contrary, the most heat-resistant strain, *Bf*3, had a more homogeneous population as no prominent tailing or shoulders were observed at all heat treatments applied. The survival curves of *Bf*3 were characterized by slight downward concavity ($n > 1$) (see Table 3.1). The thermal death curves of all four *B. nivea* isolates were characterized by upward concavity ($n < 1$, tailing) (Table 3.1) at all heating temperatures, indicating the presence of dominant heat-labile sub-population and a minor heat resistant sub-

population. The more resistant fraction of the population was observed at the highest temperature after a short treatment time compared to lower temperatures applied for long time (Fig. 3.1-b).

Inactivation kinetics of *Byssochlamys* sp. have been extensively studied. Inactivation parameters such as D- and z-values have been estimated from log-linear survival curves (Bayne and Michener, 1979, Castella et al., 1990, Engel and Teuber, 1991, Kotzekidou, 1997) and more recently, inactivation parameters such as the time for first log reduction (δ) or inactivation rates (b) have been estimated from non-log linear survival curves (Evelyn and Silva, 2015, Evelyn and Silva, 2017, Sant'Ana et al., 2009, Souza et al., 2017). Despite the easier application of D- and z-values in thermal food processes, it has been reported that non-linear survival kinetics may be more representative for HRMs (Sant'Ana et al., 2009, Souza et al., 2017).

The inactivation rate parameter, b, is indicative for the microbial inactivation velocity. In this study, it ranged from 0.017 to 3.593 min^{-1} and as expected increased with heating temperature. As can be seen in Table 1, the *B. fulva* isolates were generally more heat resistant than those of *B. nivea* as they had lower inactivation rates at the same treatment. Greater spread (variability) of the inactivation rates (b) were observed when the ascospores of isolates of both species were heated at 90ºC for 10 min. At this heating condition, the inactivation parameter ranged from 0.16 to 1.28 min^{-1} for *B. fulva* and from 0.94 to 2.75 min^{-1} for the *B. nivea* strains (see Table 3.1). It is important to highlight that this was one of the most intense heat treatments assessed in this study, which may explain the increased variability. The inter-strain variability in heat resistance became more apparent among *B. fulva* isolates with increase in the heating temperature. For instance, at 90ºC, isolate *Bf*3 had significantly lower ($p<0.05$) inactivation rates (0.16 min^{-1}) than *Bf*1 (1.28 min^{-1}) and *Bf*2 (1.22 min^{-1}). Similarly, as can be seen in Table 3.1 variability between the *B. fulva* isolates was observed at 93ºC. Variability between the *B. nivea* isolates was more pronounced at 85ºC and 90ºC. As an example, the inactivation rate of isolate *Bn*3 at 85°C (0.42 min^{-1}) was significantly lower ($p<0.05$) than that of *Bn*1 (1.26 min^{-1}) (see Table 3.1). Whilst at 90ºC, isolate *Bn*4 was significantly ($p<0.05$) more heat resistant than *Bn*2 and *Bn*3.

In comparison to our finding, Evelyn and Silva (2015) reported a lower inactivation rate (0.18 ± 0.03 min^{-1}) for *B. nivea* at 90°C in strawberry puree (pH = 3.4, 8.1 °Brix). Inter-strain (biological) variability and differences in the methods and heating media used may account for the differences observed.

Due to their greater heat resistance, the estimated times for first log reduction, δ- values, obtained for *B. fulva* strains were much longer (11.35-28.41 min) at 85ºC compared to *B. nivea* strains (δ = 1.15-1.48 min). As the heating temperature was increased, the differences in the estimated δ values between the *Byssochlamys* sp. became smaller (see Table 3.1). Regarding to the intra-species variability, no significant difference ($p > 0.05$) was observed among *B. fulva* and among *B. nivea* strains (see Table 3.1). The δ values obtained in this study were much lower than those obtained at 85°C in pineapple juice (27.0 ± 16.90 min) of pH 3.7 and 10 °Brix by Souza et al. (2017). The same authors also obtained higher δ values of 3.6 ± 0.7 min at 90°C in pineapple juice of pH 3.7 and 13 º Brix). The greater heat resistance of *B. nivea* in fruit juice could be partly explained by the protective effect from fruit compounds. The influence of the heating matrix (fruit vs. buffered glucose solution) on the estimation of inactivation parameter have been already assessed for *Hamigera avellanea* and *Thermoascus crustaceus* isolated from pasteurized acid products (Scaramuzza and Berni, 2014). The authors observed that the differences on D-values obtained from fruit-based medium and buffer glucose solution decreased as the heating temperature increased. Moreover, no statistically significant differences ($p>0.05$) between the inactivation parameters obtained in different media was observed by Scaramuzza and Berni (2014).

Table 3.1 Weibull model parameters for the survival of *Byssochlamys* strains after heat treatment at 85°C, 90°C and 93°C in acidified buffer glucose solution (pH=3.6, 13ºBrix).

Strain	T (ºC)	*b*	95%CI	*n*	95%CI	δ	95%CI
Byssochlamys fulva							
Bf1[1]	85	0.06	0.01-0.13^{AD*}	1.03	0.69-1.36^{A}	23.22	-6.60-53.05^{AB}
Bf1	90	1.28	1.02-1.54^{B}	0.61	0.49-0.71^{B}	1.14	-3.12-5.39^{AB}
Bf1	93	3.39	3.26-3.52^{C}	0.27	0.22-0.33^{C}	0.10	-0.26-0.46^{A}
Bf2	85	0.04	-0.05-0.12^{A}	1.18	0.58-1.79^{AB}	11.35	2.19-20.51^{B}
Bf2	90	1.22	0.86-1.57^{B}	0.48	0.33-0.62^{BD}	1.90	-0.83-4.63^{AB}
Bf2	93	3.49	3.28-3.69^{C}	0.26	0.18-0.35^{CD}	0.04	0.00-0.07^{A}
Bf3	85	0.02	0.00-0.03^{A}	1.19	0.96-1.42^{A}	28.41	-64.27-121.11^{AB}
Bf3	90	0.16	0.08-0.23^{A}	1.62	1.36-1.89^{A}	3.07	2.69-3.45^{AB}
Bf3	93	2.76	2.29-3.22^{D}	1.11	0.65-1.57^{AB}	0.44	0.06-0.82^{A}
Byssochlamys nivea							
Bn1[2]	85	1.26	0.68-1.83^{A}	0.36	0.20-0.51^{A}	1.19	-0.36-2.75^{A}
Bn1	90	1.91	0.95-2.88^{AC}	0.36	0.08-0.63^{AC}	1.28	-8.76-11.32^{A}
Bn1	93	3.59	3.32-3.86^{B}	0.19	0.09-0.29^{AC}	0.38	-0.31-1.08^{A}
Bn2	85	1.34	0.18-2.50^{ACD}	0.37	0.00-0.73^{AC}	1.36	-13.13-15.85^{A}
Bn2	90	2.65	2.27-3.03^{C}	0.25	0.17-0.33^{AC}	0.59	-0.81-1.98^{A}
Bn2	93	3.44	3.21-3.68^{B}	0.32	0.22-0.42^{A}	0.07	-0.57-0.71^{A}
Bn3	85	0.42	0.22-0.63^{D}	1.35	1.02-1.69^{B}	1.48	-4.36-7.33^{A}
Bn3	90	2.75	2.54-2.97^{C}	0.27	0.20-0.34^{A}	0.22	-0.67-1.11^{A}
Bn3	93	3.49	3.29-3.69^{B}	0.12	0.05-0.20^{C}	0.03	-0.22-0.28^{A}
Bn4	85	1.09	-0.08-2.26^{AD}	0.51	0.09-0.92^{AC}	1.15	-5.84-8.14^{A}
Bn4	90	0.94	0.73-1.16^{A}	0.64	0.51-0.76^{A}	1.49	-13.43-16.43^{A}
Bn4	93	3.11	2.75-3.46^{BC}	0.28	0.12-0.44^{AC}	0.10	-1.04-1.24^{A}

[1]*Byssochlamys fulva* strains and [2]*Byssochlamys nivea* strains isolated from different steps of a strawberry processing line. b are the inactivation rates (min-1), n the shape factor and δ, the time for first log reduction (min) fitted by Weibull model. CI 95% is the confidence interval at probability 0.05 considering three replicates for each trial.

3.3.2 Effect of heat treatment intensity on the growth of Byssochlamys sp.

The growth kinetics of colonies arising from heat treated *Byssochlamys* ascospores were evaluated in this study. The heat treatments applied were set at 70, 75, 80, 85 and 90ºC for 10 min and at 93ºC at 30 and 70 s. Heat treatments at 70, 75 and 80°C are mostly applied to activate the ascospores from their dormant native state (= heat shocks) as well as to kill vegetative cells, whereas heat treatments at 90-95°C, ranging in duration from a few seconds up to two minutes, are commonly applied by processors of fruit-based products during pasteurization.

The growth curves of *B. fulva* and *B. nivea* colonies arising from native ascospores, from ascospores heated for 10 min at 70, 75, 80, 85 and 90ºC and at 93ºC for 30 and 70s are shown in the Fig. 3.2. The curves were typical of fungal (radial) growth curves with initially a lag phase followed by linear growth and an upper asymptote defined by the Petri dish diameter. As can be observed in Fig. 3.2, the lag times were in general more dependent on the intensity of the heat treatment than the radial growth rates.

In predictive mycology, the lag time is defined as the time required for germination and the time of initiation of hyphal elongation. The lag phase duration is a very important parameter with regards to fungal spoilage as food is considered spoiled as soon as mycelia are visible, which occurs very shortly after the lag time (Gougouli et al., 2011, Gougouli and Koutsoumaniss, 2013). The estimated lag phases (λ, day) of colonies arising from native and heat treated ascospores of the *Byssochlamys* isolates evaluated in this study are shown in Tables 3.2. In general, increase the heating temperature resulted in longer lag times when heating was done for 10 min. The lag times of colonies arising from native ascospores of *B. fulva* strains and *B. nivea* strains were in general significantly shorter ($p<0.05$) than those of the colonies arising from ascospores heat treated for 10 min at ≥70 ºC. The estimated lag phases of strains of both *Byssochlamys* species were significantly longer ($p<0.05$) after the ascospores had been heated at 90ºC for 10 min compared to those obtained after the other heat treatments. The longest average lag phase duration was observed for *Bf*1 (12.5 days) after 90ºC/10 min. It is important to mention that this was the most heat sensitive *B. fulva* strain*,* thus the surviving ascospores which were able to grow were part of the heat resistant fraction of population (= tails), while the ascospores of *Bf*3 which had shorter lag times are more heat labile and came from a more homogeneous population. The *B. fulva* strains evaluated in this study were previously determined to be more resistant than the *B. nivea* strains (see §3.3.1).

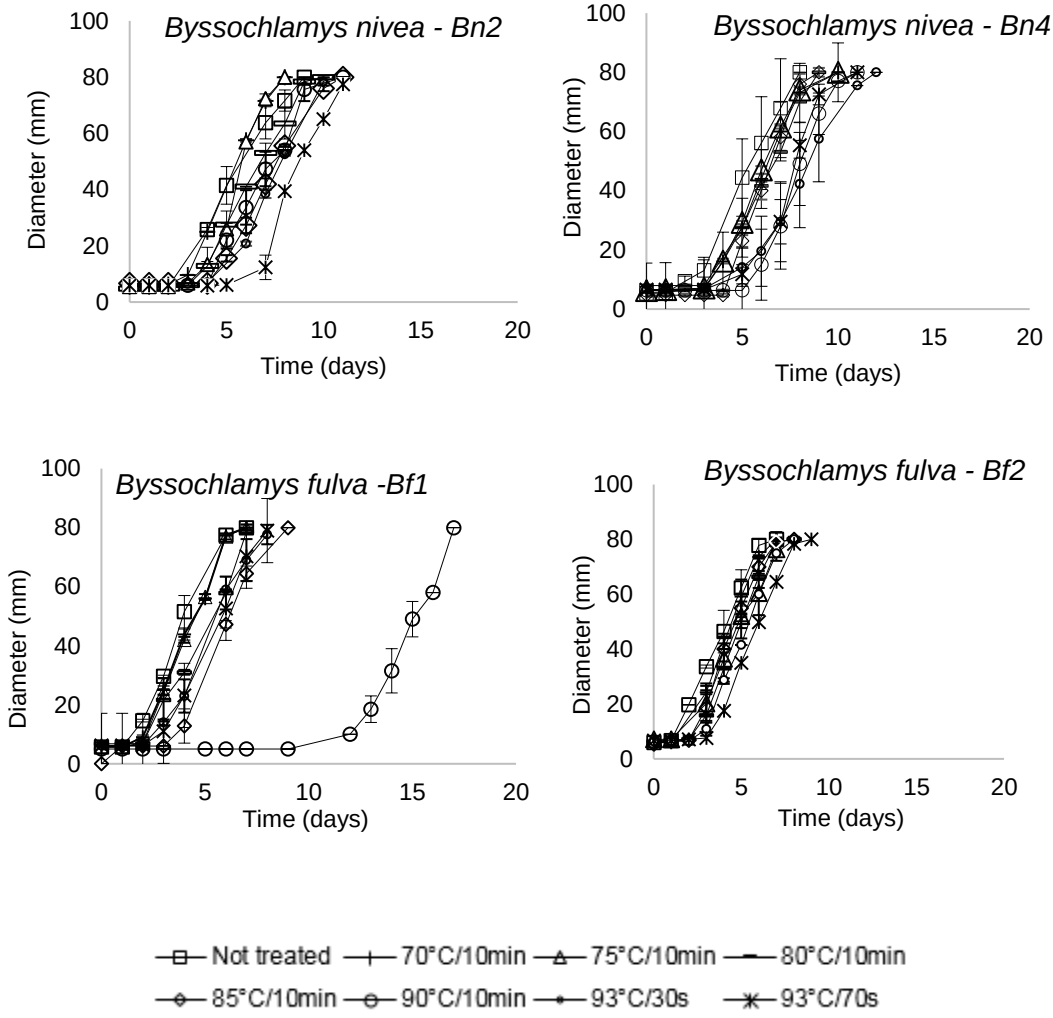

Figure 3.2 Effect of heat treatment intensity on the growth curves of *Byssochlamys* strains. No growth was observed after heat treatment at 90ºC for 10 minutes among the replicates of *B. fulva* strain Bf2.

Among *B. nivea*, the longest average lag phases were observed after heat treatments of 90ºC/10 min (12.1 days) for *Bn*1 and after heating at 93ºC for 70s for *Bn*3 (9.4 days) (Table 3.2). The long lag phases observed for the *B. nivea* strains, imply that the surviving ascospores, belonging to the most heat stable subpopulation, are potentially sub-lethally injured and hence require longer times to recover, germinate and grow out.

Table 3.2 Estimated lag phase duration (λ, days) of *Byssochlamys* sp. on acidified malt extract agar (MEA, pH 3.6) after different thermal treatments and incubation at 22°C**.**

Strain	Native ascospores λ 95% CI (d)	70°C/10 min. λ 95% CI (d)	75°C/10 min. λ 95% CI (d)	80°C/10 min. λ 95% CI (d)	85°C/10 min. λ 95% CI (d)	90°C/10 min. λ 95% CI (d)	93°C/30s λ 95% CI (d)	93°C/70 s λ 95% CI (d)
Byssochlamys fulva								
Bf1	1.41 1.37-1.46^{a*1**}	1.81 1.77-1.85^{a2}	1.89 1.74-2.05^{a2}	3.17 2.69-3.65^{b3}	3.34 3.02-3.67^{a3}	12.5 11.23-13.77^{a4}	2.77 2.40-3.14^{a3}	2.8 2.70-2.90^{a3}
Bf2	1.21 1.12-1.32^{b1}	1.89 1.76-2.02^{a2}	1.9 1.86-1.94^{a2}	2.47 2.44-2.50^{a3}	2.99 2.98-3.01^{b4}	NG -	2.51 2.47-2.55^{a3}	3.17 2.49-3.85^{a134}
Bf3	1.19 1.18-1.20^{b1}	1.94 1.74-2.14^{a2}	2.05 1.90-2.21^{a2}	3 1.49-4.51^{ab23}	3.1 2.74-3.46^{ab3}	6.47 4.59-8.35^{b4}	4 1.30-6.62^{a234}	2.69 2.26-3.55^{a3}
Byssochlamys nivea								
Bn1	2.02 1.28-2.75^{a1}	3.46 3.44-3.45^{a2}	2.57 1.78-3.35^{ab12}	3.57 3.49-3.65^{a2}	4.75 4.39-5.09***	12.06 0.00-37.07^{a4}	6.67 6.00-7.15^{a4}	NG -
Bn2	2.18 1.79-2.57^{a1}	2.74 2.31-3.17^{b12}	3.09 2.94-3.26^{a23}	3.5 3.31-3.68^{a3}	4.29 3.84-4.74^{a4}	5.03 4.62-5.44^{b4}	4.57 3.93-5.21^{b4}	5.24 4.22-6.26^{a4}
Bn3	2.53 2.39-2.67^{a1}	2.77 2.32-3.22^{b12}	2.95 2.78-3.12^{a1}	3.62 3.41-3.83^{a2}	ND -	7.75 3.17-12.32^{ab2}	4.02 3.67-4.38^{b2}	9.39 0.36-18.43^{a12}
Bn4	2.1 1.75-2.45^{a1}	3.39 3.31-3.49^{a2}	3.31 3.27-3.35^{b2}	3.53 3.32-3.74^{a23}	4.11 2.39-5.84^{a123}	4.38 1.58-7.17^{b123}	5.28 3.54-7.02^{ab3}	4.72 4.53-4.90^{a3}

*Different superscript letter indicates significant differences (p<0.05) among strains at same treatment.
**Different superscript numbers indicate significant differences (p<0.05) among the treatments for the same strain.
***Estimated from one replicate (no growth was observed for the other replicates).
NG: No Growth was observed in any of the replicates (n=4).
ND: Not detectable.

Regarding variability, broad ranges for the lag times (including inter-strain variability and intra-strain variability, i.e. within replicates) were observed after the ascospores of the *B. nivea* strains had been given heat treatments of 90ºC for 10min (4.13-17.50 days) and 93ºC for 70s (3.8-25.4 days), whilst the same was observed after ascospores of the *B. fulva* strains which had been given a heat treatment of 90ºC for 10min (6.3-12.6 days). At this condition, significant differences ($p<0.05$) were observed between the lag times of the three *B. fulva* strains, of which *Bf*1, had the longest lag phase of 12.5 days, whilst *Bf*3 had al lag phase approximately half as long as that of *Bf*1 (6.5 days) and *Bf*2 did not grow out over the 30 days incubation period (Table 3.2). In contrast, the inter-strain variability between the *B. nivea* strains was significant ($p<0.05$) and more apparent when the ascospores had been given a heat treatment of 93°C for 30s with significant longer lag times ($p<0.05$) being obtained for strain *Bn*1 (6.7 days) compared to strains *Bn*2 (4.6 days) and *Bn*3 (4 days).

The estimated growth rates (μ_{max}, $mm.day^{-1}$) of colonies arising from the heat treated ascospores of the *Byssochlamys* isolates are shown in Table 3.3. The average growth rates, which were estimated for each strain, ranged from 13.8 $mm.day^{-1}$ (90ºC/10min) to 21.9 $mm.day^{-1}$ (80ºC/10min) for *B. fulva* and from 11.9 $mm.day^{-1}$ (70ºC/10min) to 18.3 $mm.day^{-1}$ (90ºC/10min) for *B. nivea*. In general, differences between the growth rates of colonies arising from native ascospores spores and those arising from ascospores heat treated at 70-80 ºC for 10 min were not so pronounced. Among *B. fulva* the growth rates increased as the heating temperature was increased to 75-80 ºC after which further increase in the heating intensity resulted in a decrease in the growth rate (see Table 3.3). For instance, after a heat treatment of 90ºC for 10 min, the *B. fulva* strains had significantly lower growth rates ($p<0.05$) than observed for colonies arising from native ascospores and ascospores given milder heat treatments (70-85 ºC for 10 min) (Table 3.3). In contrast, higher growth rates were observed for colonies arising from heat treated *B. nivea* ascospores compared to native ascospores, but with most being determined to be not significantly different ($p>0.05$). Interestingly, it was observed that, the growth rates of *B. nivea* colonies arising from ascospores which had been subjected to an intense heat treatment, were higher than those of colonies arising from ascospores subjected to milder treatments. For instance, *Bn*1 had the highest growth rates from colonies arising from ascospores heated at 93ºC/30s (19.6 $mm.day^{-1}$) followed by 90ºC/10min (18.3 $mm.day^{-1}$). This result shows that heat-resistant (injured) ascospores, which are survive the heat treatment, once germinated, grow even more rapidly than those which are not injured or have been subjected to less thermal injury. This is in contrast to, *B. fulva*, which seems to grow faster when the heating was milder, i.e. when treatments commonly

used to activate the ascospores are applied. The inter-strain variability of *B. fulva* was more apparent when the heat treatment of 93ºC for 30s was applied, whereby *Bf*1 had statistically slower growth rates ($p<0.05$) (15.21 mm.day^{-1}) than *Bf2* (15.69 mm.day^{-1}) and *Bf*3 (16.71 mm.day^{-1}) (Table 3.3). Likewise, the inter-strain variability of *B. nivea* was pronounced at 93ºC/30s, where strain *Bn*1 had significant higher growth rates ($p<0.05$) (19.65 mm.day^{-1}) than strains *B*n3 (14.11 mm.day^{-1}) and *Bn*4 (13.93 mm.day^{-1}).

After the *Byssochlamys* strains had been subjected to the heat treatments, it was not possible to standardize the initial inoculum in the growth experiments. However, it was estimated from the Weibull models that they ranged from $<10^2$ to 10^4 spores/ml. Biological variability in the growth responses has been assessed on the basis of the germination time (Dagnas et al., 2015, Dantigny et al., 2005, Gougouli and Koutsoumanis, 2012, Kalai et al., 2014), and as lag times of individual spores (Burgain et al., 2013, Dagnas et al., 2015, Dagnas et al., 2017, Gougouli and Koutsoumanis, 2013, Samapundo et al., 2007). The germination time has been reported to vary highly between spores from the same population (Gougouli and Koutsoumanis, 2012, Judet et al., 2008) and that the variability in growth response may increase with magnitude of stress applied (Dagnas et al., 2015, Dagnas et al., 2017). The intra-species variability on the growth of non-ascospore forming fungi (*Aspergillu**s***, *Fusarium* and *Penicillium* sp.) has been previously assessed (Astoreca et al., 2010, Baert et al., 2007, Garcia et al., 2011, Samapundo et al., 2007, Tassou et al., 2009). This variability seems to be higher under conditions marginal for growth (Baert et al., 2007, Garcia et al., 2011, Samapundo et al., 2007). This is in agreement with our findings whereby at the most stressful condition i.e. at most heat intensity treatment, greater variability in the growth response of the *Byssochlamys* isolates was observed. In addition, as the inoculum size in the growth assessment study differed as a function of the heat treatment applied, it may have also contributed to the variability observed. It is known that large inoculums are mostly represented by ascospores with short germination and lag times (Burgain et al., 2013). In our study this effect was more pronounced after a heat treatment of 90ºC for 10 min for both *B. fulva* strains and *B. nivea* strains. At this condition, the differences in heat resistance (= log reductions achieved) resulted in a greater variation in the inoculum size which could partly explain the intra-species variability.

The use of inoculum consisting of a single strain versus the use of cocktails in predictive mycology has been already discussed (Garcia et al., 2010, Garcia et al., 2014). Despite the estimated parameters obtained from cocktails of strains being more representative for estimating fungal

growth in a worst-case scenario, they may not evaluate and include some strains with the marginal behavior (Garcia et al., 2014), such as observed in this study for the very long lag times required for some injured ascospores to grow. From one side, the estimation of kinetic parameters from individual strains has the advantage to include different behaviors and marginal cases which are very important information to assess more accurately the risk of spoilage. However, this approach may be time-consuming and costly.

Consumer demand for fruit products with high sensorial quality and less chemical preservatives has challenged the fruit processing industry. On one hand, the application of milder treatments, <90°C, is necessary to optimize the sensorial quality, whilst on the other hand, these milder heat treatments may increase the risk of fungal spoilage of fruit products. The assessment of mild heat treatments for long intervals such as 85˚C for 10 min and as well as 90˚C for 10 min and 93ºC for 70s may overestimate the treatments applied by industries. However, the results obtained in this study reinforce not only the ability of *Byssochlamys* ascospores to overcome these treatments, but also how the heating stress affects the viability of spores during subsequent outgrowth. As observed, some injured ascospores required very long times to recover, germinate and grow out, while others are less injured or, in some cases, inactivated after sub-lethal treatments. Moreover, the intra-strain variability in growth response (within replicates) may be the result not only of experimental variability but also from the biological variability at spore level. As spoilage by fungi can occur at low spore contamination levels, variability in growth response of colonies arising from individual spores i.e., population variability, should be take into account as well. In addition to the intra-species and intra-strain variability, differences in growth response as a function of the intensity of the heat treatment were observed between the two *Byssochlamys* species, named inter species variability. These results show the need for more studies to be performed to determine how the heating intensity affects the growth of HRMs of importance to the food industry. Lastly, it is important to mention that despite the comparison analyse of 95% CI´s (confidential Intervals) overlap or lack thereof imply statistical significance when confidential intervals non-overlapping, no real statistic conclusion can be made when the intervals overlap.

Table 3.3 Estimated growth rate (μ_{max}, mm.day^{-1}) of *Byssochlamys* sp. on acidified malt extract agar (MEA, pH 3.6) after different thermal treatments and incubation at 22°C.

Strain	Native ascospores	70°C/10 min.	75°C/10 min.	80°C/10 min.	85°C/10 min.	90°C/10 min.	93°C/30s	93°C/70 s
	μ_{max}	μ_{max}	μ_{max}	μ_{max}	μ_{max}	μ_{max}	μ_{max}	μ_{max}
	95% CI	95% CI	95% CI	95% CI	95% CI	95% CI	95% CI	95% CI
Byssochlamys fulva								
Bf1	16.3	16.7	16.8	16.9	16.3	13.8	15.21	14.77
	15.7-16.9^{ab*1**}	16.0-17.4^{a1}	15.9-17.7^{ab1}	15.3-18.4^{a1}	14.9-17.6^{a13}	13.5-14.2^{a2}	14.81-15.62^{b3}	14.10-15.43^{a23}
Bf2	15.8	16.9	15.8	17.1	14.9	NG	15.69	16.71
	14.9-16.8^{a13}	16.2-17.7^{a12}	14.8-16.9^{a123}	16.9-17.2^{a2}	13.9-15.9^{a3}	-	15.66-15.72^{a13}	14.78-18.65^{a123}
Bf3	17.3	18.3	17.6	21.9	14.9	13.9	16.71	15.8
	16.9-17.6^{b1}	16.3-20.3^{a1}	17.2-17.5^{b1}	14.9-28.9^{a12}	13.6-16.2^{a23}	13.1-14.8^{a3}	15.70-17.73^{a12}	14.58-17.02^{a123}
Byssochlamys nivea								
Bn1	13.7	13.7	14.6	13.9	13.7	18.3	19.65	NG
	11.5-15.8^{a12}	13.4-13.7^{a1}	14.4-14.7^{a1}	13.1-14.6^{a1}	12.17-15.23^{a1***}	15.4-21.2^{a2}	15.67-23.63^{a12}	-
Bn2	12.6	11.9	15.4	14.4	12.9	17.1	15.15	15.42
	11.2-14.2^{a1}	8.9-14.8^{a12}	12.6-18.1^{a12}	12.9-15.7^{a12}	11.4-14.4^{a1}	14.7-19.4^{a2}	13.91-16.39^{abc12}	13.36-17.48^{a12}
Bn3	14.1	16.1	15.8	14.5	ND	14.5	14.11	14.52
	12.7-15.5^{a1}	15.5-16.6^{b2}	14.7-16.9^{a12}	12.8-16.2^{a12}	-	13.4-15.6^{a12}	13.19-15.03^{bc1}	12.11-16.93^{a12}
Bn4	14.7	14.6	12.5	14.1	17.1	17.7	13.93	15.21
	14.1-15.3^{a1}	13.7-15.6^{ab1}	9.9-15.1^{a1}	12.6-15.5^{a1}	9.4-24.7^{a1}	9.5-26.0^{a1}	12.41-15.52^{bc1}	12.98-17.43^{a1}

*Different superscript letter indicates significant differences ($p<0.05$) among strains at same treatment.
**Different superscript numbers indicate significant differences ($p<0.05$) among the treatments for the same strain.
***Estimated from one replicate (no growth was observed for the other replicates).
NG: No Growth was observed in any of the replicates (n=4).ND: Not detectable.

3.4 Conclusions

An exploration of strain variability (heat inactivation and growth after heat treatment) of *Byssochlamys* sp. was presented in this study. Despite the limited number of strains used, significant differences ($p<0.05$) were observed between the strains (in both inactivation and growth kinetics) for *B. fulva* and *B. nivea*. This variability may strongly influence the development of predictive modeling and their accuracy on prediction of fungal spoilage, performance of heat processing operations used in the food industry and of shelf-lives. The findings obtained in this study suggest the incorporation of biological variability on estimation of kinetic parameters, which will include strains with divergent behavior. Moreover, the capacity of injured ascospores to recover and germinate after long incubation periods (of up to 25 days) after application of sub-lethal treatments represents a risk to the fruit industry as the fruit products are pasteurized and stored at mostly at ambient temperatures. Therefore, the effect of heat intensity treatments should be better understood and explored in combinations with other factors, such as stored temperature, °Brix and oxygen concentration to prevent the germination and growth of heat-resistant fungi during the shelf-life of heat treated fruit-based products.

Chapter 4

Effect of sugar concentration (°Brix) and storage temperature on the time to visible growth of individual ascospores of six heat resistant moulds isolated from fruit products

Redrafted after

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Abstract

Heat-resistant moulds (HRMs) pose great challenges to processors of fruit-based products due to their thermal resistance and ability to grow across a broad range of conditions. Therefore, the quantification of the effect of inhibitory factors (conditions) on the growth of HRMs is very important to be used to prevent spoilage during shelf-life. This study assessed the minimum temperature and maximum sugar content (°Brix) for the growth of six HRMs (*Byssochlamys* spp. and *Neosartorya* spp.) previously isolated from fruit products. In addition, the time to form a visible colony (t_v, days) was determined to assess biological variability of individual ascospores within same population. Heat activated ascospores (10 min at 80°C) were spread plated (±100 spores) on acidified Potato Dextrose Agar (aPDA, pH 3.5) plates from which the °Brix was adjusted with fructose-glucose (1:1) to levels between 44–59 °Brix followed by incubation at 30°C. To assess the effect of temperature, inoculated plates of aPDA were incubated at 4, 7, 8, 10, 12 and 14°C. Three replicates (= 3 aPDA plates) were prepared per condition evaluated. The number of visible colonies were counted daily for up to two months. Probability distribution functions were then fitted in @Risk to the cumulative t_v's. With regards to cold tolerance, *B. nivea* was the most cold sensitive as it had the least ability to germinate and form visible colonies at low temperatures (no growth when T ≤ 10°C). On the other hand, *N. hiratsukae* was the most cold tolerant, being able to form visible colonies at temperatures ≥ 7°C. Likewise, *B. nivea* was the most sensitive to increased °Brix values, whilst *N. udagawae* was able to grow out at the highest °Brix evaluated (59°/a_w=0.86). The tolerance of potential spoilage HRMs to high sugar levels and their ability to grow under chilled conditions represents a challenge for the microbial stability of high-sugar fruit products. Differences in individual t_v's were mostly observed under conditions at the growth/no growth regions. For instance, individual t_v's of *B. nivea* ascospores ranged from 24 to 46 days at 12°C and those of *N. udagawae* ranged from 20 to 45 days at 59°Brix. The t_v's data from each HRM and condition evaluated were then fitted to different statistical distributions (Exponential, Normal, Lognormal, Weibull, Logistic or Pareto) to allow the use of the obtained data in further Quantitative Microbial Spoilage Risk Assessment work for pasteurized fruit products.

4.1 Introduction

Many studies have focused on the effect of environmental factors on the growth of HRMs through the estimation of fungal growth rates and lag times (Tremarin et al., 2015, Panagou et al., 2010), often of high inocula. However, less attention has being given to these effects on the time to visible growth of HRMs, i.e., the time required for the colony to develop to a size that consumers can detect (Roland and Beuchat, 1984, Berni et al., 2017, Santos et al., 2017).

Nevertheless, this parameter is of great interest of fruit processors as it can be translated into a predicted time of rejection, i.e., the mould-free shelf-life of the product (Dantigny et al., 2016, Huchet et al., 2013, Burgain et al.,2013). Moreover, contamination of food products by HRMs is most likely to occur by only one or a few spores (Berni et al., 2017, Tranquillini et al., 2017, Pitt and Hocking 2009). Therefore, the incorporation of individual ascospore growth response (= biological variability) as a stochastic process needs to be taken into account in studies aiming to determine mould-free shelf-lives.

The biological variability inherent to single fungal spores belonging to the same population has been explored by some authors (Garcia et al., 2010, Gougouli and Koutsoumanis, 2013, Samapundo et al., 2007, Dagnas et al., 2017), which highlighted the importance of performing growth kinetic studies at the individual spore level. These studies focused on the effect of environmental factors on the distribution of individual lag times of heat sensitive spores, while to date there are no available data on the time to visible growth of individual ascospores.

Therefore, this study aimed to assess the inhibitory levels of °Brix and temperature on the growth of 6 different species of HRMs. In addition, the time to form a visible colony (t_v) by individual ascospores (= biological variability) for each HRM species and condition was assessed and further fitted to statistical distributions.

4.2 Material and Methods

4.2.1 HRMs strains

Six heat-resistant moulds (HRMs) previously isolated from raw and processed fruit products (Ch. 2) were investigated in this study: *Byssochlamys nivea* (Bysso nivea 76-1) and *Neosartorya laciniosa* (Neosart laciniosa 67-2) - isolated from pasteurized strawberry puree, *Byssochlmays fulva* (Bysso fulva 56-2) and *Neosartorya hiratsukae* (Neosar hiratsukae 77-5) - isolated from strawberries, *Neosartorya udagawae* (Neosar udagawae 54-3) - isolated from sieved strawberry puree, and *Neosartorya fischeri* (Neosar fisheri 95-1)- isolated from extracted orange juice. The isolates were maintained in the culture collection of the Laboratory of Applied Mycology (MYCOLAB; Department of Food Technology, Safety and Health, Ghent University, Belgium).

4.2.2 Preparation of ascospore suspensions

Ascospore suspensions were prepared was described in Ch. 3 (see §*3.2.2*). The final suspensions were kept at 2°C and used up to 7 days after harvesting.

4.2.3 Effect of °Brix on the time to visible growth

The effect of °Brix was assessed on acidified PDA (pH 3.5, HCl 6N) from which the °Brix was adjusted with fructose (F-0127, Sigma) and glucose (G-8270, Sigma) (1:1) to 44–59 °Brix (= a_w 0.86-0.91) (see Table 1). The values of °Brix (i.e. the value (%) of total sugar content) were checked by means of a pocket refractometer (HR25/800 Kruess, Germany). Before inoculation of the aPDA was done, the ascospores suspensions were standardized to 10^2 -10^3 spores by serial decimal dilutions in sterile 9ml-glycerol solutions tubes adjusted to the same a_w-values as the media to be inoculated. After activating the suspensions in a water bath at 80°C for 10min, aliquots of 100µL of standardized suspensions were spread plated (±100 spores) in triplicate on aPDA plates and incubated at 30°C±1°C. All Petri dishes were then sealed with Parafilm® to avoid dehydration after which plates with the same °Brix were placed together in sealed plastic boxes. Non-inoculated plates with adjusted aPDA were incubated together with inoculated plates with similar a_w-values. a_w measurements were performed as a_w is a more useful parameter in food microbiology as it objectively describes the amount of water available whilst degree Brix is universally applied in the fruit processing industry. The a_w of the media was checked monthly by means of a water activity meter Sprint TH500, Novasina Thermoconstanter, Pfapfikkon, Switzerland). The plates were checked periodically for visible growth (colony diameter= 2mm) for up to 60 days. Growth/no growth data, time to visible growth (t_v, days) and the number of visible colonies were assessed. The experiment was performed twice, with a total of three plates checked per condition and experiment (n=6).

Table 4.1. Formulation of sugar-based growth media.

Fructose (g)	Glucose (g)	PDA (g)	Distilled water (g)	°Brix	a_w ± SD
105	105	19.5	290	44	0.904 ± 0.003
110	110	19.5	280	47	0.902 ± 0.007
115	115	19.5	270	50	0.894 ± 0.007
120	120	19.5	260	53	0.883 ± 0.005
125	125	19.5	250	56	0.869 ± 0.006
130	130	19.5	240	59	0.860 ± 0.002

4.2.4 Effect of temperature on the time to visible growth

For the experiments assessing the effect of temperature, the suspension of each HRM was initially standardized to 10^2 -10^3 spores/ml in sterile acidified phosphate buffer solution prepared by dissolving 68g/L of potassium dihydrogen phosphate (Sigma Aldrich) in 1000 ml of distilled water acidified to pH 3.5 with phosphoric acid (Merck). After being heat activated (80°C for 10 min) aliquots of 100µL of standardized suspensions were spread plated (±100 spores) in triplicate on acidified Potato Dextrose Agar (aPDA, pH 3.5, HCl 6N) plates. After inoculation, the plates were sealed with Parafilm® and incubated at 4±1°C, 7±1°C, 8±1°C, 10±1°C, 12±1°C and 14±1°C. The plates were periodically checked for visible growth (colony diameter ≥ 2mm) for up to 60 days. Growth/no growth data, t_v's, and the number of visible colonies were assessed. The experiment was performed twice with a total of three plates checked per condition and experiment (n=6).

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4.2.5 Statistical analysis

Statistical distributions were fitted to the data, i.e., number of observed colonies *vs*. time, for each condition by means of @Risk 7.0 software for Excel (Palisade Corporation, NY, EUA). The probability distributions available in @Risk were visually compared (adjusted-fit plot, Probability-Probability (P-P) plots and Quantile-Quantile (Q-Q) plots) and ranked based on the Bayesian Information Criterion (BIC). The distributions with the best fits were chosen and their estimates for important parameters such as the mean, 95% confidence interval (CI), and standard deviation, were obtained. Significant differences (at $\alpha = 0.05$) between t_v's were determined by comparing their 95% CI's for overlap or lack thereof.

4.3 Results

The six °Brix levels evaluated (expressed as the total sugar content, %), were selected based on data in literature (Berni et al., 2017, Beuchat and Toledo, 1977) and our own preliminary studies (data not shown). In general, visible mycelia (i.e., fungal colony diameter ≥ 2mm) were observed within 2 to 45 days at 30°C, depending on the sugar content (°Brix) and isolate (Table 4. 2). *B. nivea* was the least tolerant to elevated sugar levels and was unable to grow at sugar concentrations ≥ 53°Brix ($a_w \leq 0.883$). The other five isolates evaluated grew out at sugar concentrations equal to 56°Brix within the two month incubation period. Of these, *N. udagawae* was the most tolerant to elevated sugar levels as it was the only isolate able to develop visible colonies at 59°Brix (a_w=0.86) within the incubation period evaluated. At the lowest sugar concentration evaluated, 44°Brix (a_w=0.904), very short t_v's (of 2-12 days) were observed for

all HRMs evaluated. Overall, increase in sugar concentration resulted in longer t_v's. With the exception of *N. fischeri*, significantly longer t_v's ($p<0.05$) were observed for the *Neosartorya* strains when the sugar concentration was increased from 44 to 56°Brix. In addition, a significant increase ($p<0.05$) in the t_v of *N. udagawae* was observed when the sugar concentration increased from 56° to 59°Brix, in which visible colonies were observed after 20-45 days. On the other hand, the growth of *N. fischeri* was less affected when the sugar concentration was increased from 44 to 56°Brix, with significantly longer ($p<0.05$) t_v's being observed only between the two lowest and highest sugar concentrations evaluated. Unlike *B. nivea*, the response of *B. fulva* towards changes in the °Brix was similar to that exhibited by the *Neosartorya* strains. Increase in sugar concentration in the range of 44 to 53°Brix did not result in the significant differences ($p>0.05$) in the t_v's of *B. fulva* and the *Neosartorya* strains.

The minimum growth temperatures (cold tolerance) of the six HRMs strains were evaluated on aPDA (a_w=0.99/ 4°Brix). The t_v's ranged from 6 to 46 days, depending on the incubation temperature and species (isolate) (Table 4.3). *B. nivea* was the most cold-sensitive as it was not able to germinate and form visible colonies at low temperatures (≤10°C) and it presented visible growth at 12°C after up to 46 days of incubation. On the other hand, *N. hiratsukae* was the most tolerant to low temperatures and was able to form visible colonies at temperatures ≥7°C after 27-40 days, while *B. fulva*, *N. fischeri*, *N. laciniosa* and *N. udagawae* were able to form visible colonies at temperatures ≥10°C. The growth of these HRMs was significantly delayed ($p<0.05$) at 10°C, with visible growth being exhibited only after 19-38 days. On the other hand, visible outgrowth at 14°C occurred just after 6-13 for all the *Neosartorya* strains and after 8-23 days and 18-35 days for *B. fulva and B. nivea*, respectively.

The results of this study show that ascopores tv's originating from the same population are highly variable. Therefore, this parameter would be better described by probability distributions describing variabilities and/or uncertainties. In this study it was assumed that each visible colony detected after a determined period originated from a single ascospore. Thus, the *ca*. 100 t_v values (from *ca*. 100 ascospores) were obtained for each isolate at each experimental condition. These t_v's were fitted to statistical distributions in @Risk. The best fitting distributions were identified (see Tables 4.2 and 4.3). Fig. 4.1 shows the cumulative t_v's of ascospores of isolates evaluated in this study on aPDA at sugar concentrations ranging from 44 to 59°Brix at 30°C. Most of the data regarding the effect of °Brix was best represented by right-skewed exponential curves bounded at the estimated parameter "Risk shift", i.e. a shift value from 0, and described by a single scale parameter. The most likely values from exponential curves are concentrated in the lower boundary, which in this study indicate the shortest t_v's observed for the majority of the ascospores in a population (= single HRM). Moreover, in some cases,

different types of distributions were selected, according to the goodness of fit (Table 4.2). For instance, the t_v data for *N. udagawae* at 56 and 59°Brix were best represented (described) by lognormal and normal distributions, respectively. Likewise, *N. laciniosa* ascospores tv's data were best represented by a Weibull distribution at 56°Brix (Fig. 4.1). These differences in distributions were also associated with the growth/no growth regions in which wider t_v ranges were observed implying greater biological variability among ascospores from the same population under these conditions.

Table 4.2. Estimated parameters of cumulative distributions fitted to individual times to growth (t_v , days) data obtained on aPDA (pH=3.5) plates adjusted to different °Brix (44-59) stored at 30°C. No growth means that no visible mycelium was observed in any of the replicates.

HRM	°Brix	a_w ± SD	Distribution	t_v (days) ± SD	5th percentile	95th percentile
N. hiratsukae	44	0.904 ± 0.003	RiskExpon(0,29641;RiskShift(5,99911))	6.3 ± 0.69	6	8
	47	0.902 ± 0.007	RiskExpon(0,65418;RiskShift(3,99811))	4.6 ± 1.07	4	7
	50	0.894 ± 0.007	RiskNormal(5,64211;0,88313)	5.6 ± 0.88	4	6
	53	0.883 ± 0.005	RiskExpon(0,47244;RiskShift(5,99814))	6.5 ± 1.01	6	8
	56	0.869 ± 0.006	RiskExpon(2,9192;RiskShift(8,9705))	11.9 ± 3.57	9	20
	59	0.860 ± 0.002	No Growth	-	-	-
N. udagawae	44	0.904 ± 0.003	RiskExpon(0,47368;RiskShift(2,99644))	3.5 ± 0.73	3	5
	47	0.902 ± 0.007	RiskExpon(1,2256;RiskShift(2,9908))	4.2 ± 1.72	3	7
	50	0.894 ± 0.007	RiskExpon(1,8684;RiskShift(2,9902))	4.8 ± 1.67	3	9
	53	0.883 ± 0.005	RiskExpon(2,8246;RiskShift(4,9835))	7.8 ± 2.87	5	16
	56	0.869 ± 0.006	RiskLognorm(10,134;3,2801;RiskShift(1,9221))	12.1 ± 3.3	9	20
	59	0.860 ± 0.002	RiskNormal(31,1053;8,0195)	31.1 ± 8.01	20	45
N. laciniosa	44	0.904 ± 0.003	RiskExpon(1,038;RiskShift(1,9934))	3.0 ± 1.26	2	7
	47	0.902 ± 0.007	RiskExpon(0,69427;RiskShift(2,99558))	3.7 ± 1.91	3	7
	50	0.894 ± 0.007	RiskExpon(0,88889;RiskShift(3,9924))	4.9 ± 1.59	4	8
	53	0.883 ± 0.005	RiskExpon(0,4661;RiskShift(5,99605))	6.5 ± 1.45	6	10
	56	0.869 ± 0.006	RiskWeibull(1,6185;9,4512;RiskShift(11,5347))	20 ± 5.36	14	31
	59	0.860 ± 0.002	No Growth	-	-	-
N. fischeri	44	0.904 ± 0.003	RiskExpon(0,21698;RiskShift(2,99795))	3.2 ± 0.5	3	4
	47	0.902 ± 0.007	RiskExpon(1,1282;RiskShift(2,9904))	4.1 ± 2.19	3	12
	50	0.894 ± 0.007	RiskExpon(1,7699;RiskShift(2,9843))	4.8 ± 3.1	3	16
	53	0.883 ± 0.005	RiskExpon(1,3883;RiskShift(3,9865))	5.4 ± 2.64	4	9
	56	0.869 ± 0.006	RiskExpon(3,4066;RiskShift(9,9626))	13.5 ± 2.78	10	19
	59	0.860 ± 0.002	No Growth	-	-	-
B. nivea	44	0.904 ± 0.003	RiskExpon(2,7045;RiskShift(4,9385))	7.7 ± 2.20	5	12
	47	0.902 ± 0.007	RiskExpon(2,8966;RiskShift(6,9001))	9.9 ± 3.38	7	16
	50	0.894 ± 0.007	RiskExpon(2,1639;RiskShift(7,9645))	10.2 ± 2.58	8	16
	53	0.883 ± 0.005	RiskExpon(6,9211;RiskShift(14,8179))	21.9 ± 5.26	15	35
	56	0.869 ± 0.006	No Growth	-	-	-
	59	0.860 ± 0.002	No Growth	-	-	-
B. fulva	44	0.904 ± 0.003	RiskExpon(1,2286;RiskShift(1,9649))	3.2 ± 1.30	2	7
	47	0.902 ± 0.007	RiskExpon(0,63636;RiskShift(2,94215))	3.6 ± 1.28	3	7
	50	0.894 ± 0.007	RiskExpon(1;RiskShift(3,9804))	5.0 ± 2.11	4	10
	53	0.883 ± 0.005	RiskExpon(1,8103;RiskShift(5,9688))	7.8 ± 4.67	6	24
	56	0.869 ± 0.006	RiskExpon(1,1455;RiskShift(9,9792))	11.1 ± 3.93	10	20
	59	0.860 ± 0.002	No Growth			

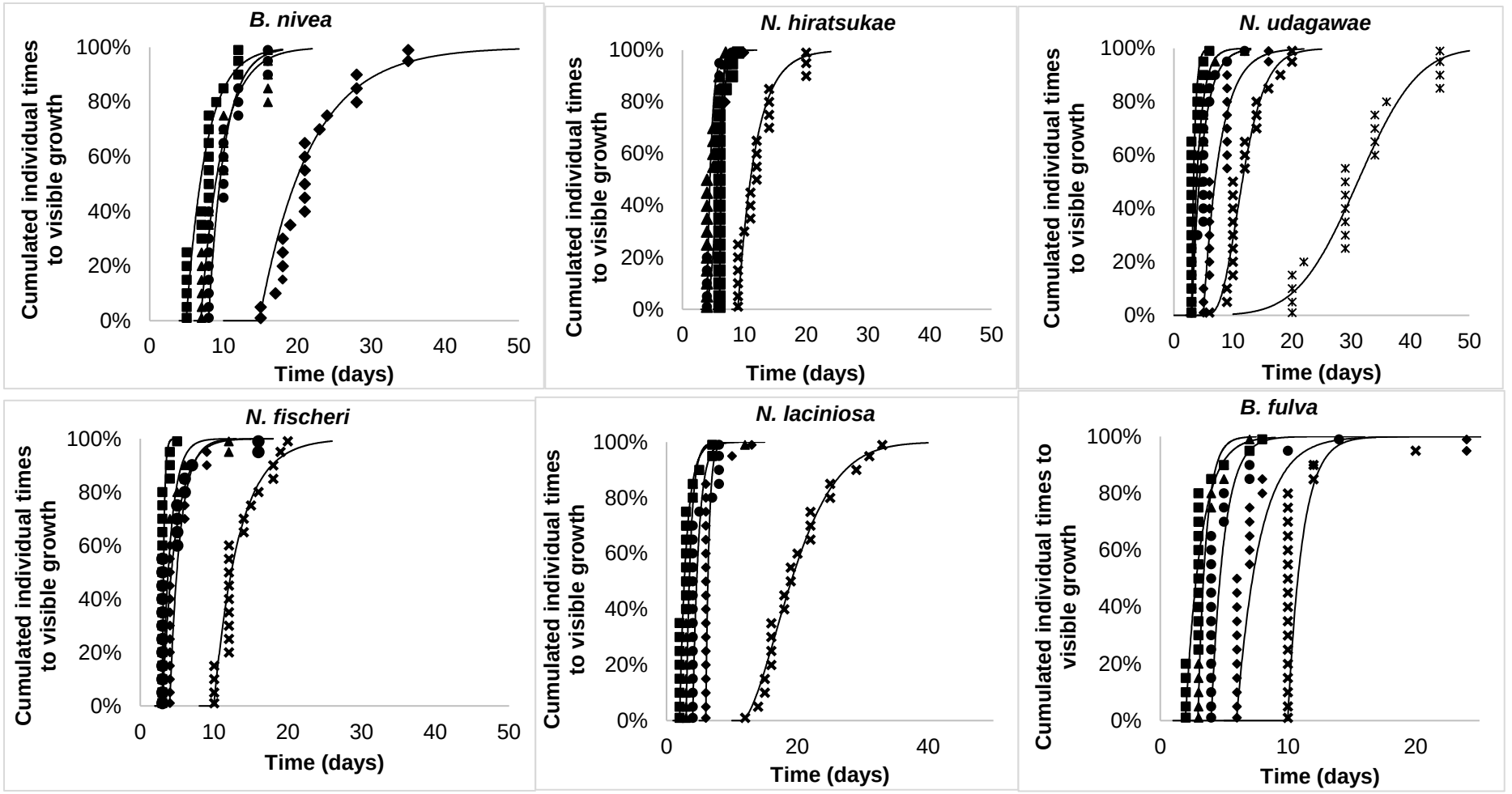

Figure 4.1. Cumulated individual times to visible growth of spores of *Byssochlamys* and *Neosartorya* isolates on acidified PDA (pH= 3.6) adjusted to 44°Brix (■), 47°Brix (▲), 50°Brix (●), 53°Brix (♦), 56°Brix (✕) and 59°Brix. (✳) at 30°C.

Table 4.3. Estimated parameters of cumulative distributions fitted to the individual times to growth (t_v , days) data obtained on aPDA (pH=3.5) plates stored at temperatures ranging from 7°C to 14°C. No growth means that no visible mycelium was observed in any of the replicates.

HRM	Temperature (°C)	Distribution	t_v (days) ± SD	5th percentile	95th percentile
N. hiratsukae	7	RiskExpon(4,4902;RiskShift(26,9707))	32 ± 4.05	27	40
	8	RiskExpon(4,2432;RiskShift(21,9771))	25 ± 3.88	22	33
	10	RiskExpon(4,1735;RiskShift(9,9787))	14 ± 3.91	10	22
	12	RiskPareto(4,5953;9)	12 ± 6.13	9	27
	14	RiskPareto(4,8743;6)	8 ± 2.46	6	10
N. udagawae	7	No Growth	-	-	-
	8	No Growth	-	-	-
	10	RiskExpon(2,654;RiskShift(18,9908))	22 ± 3.34	19	30
	12	RiskExpon(2,1294;RiskShift(10,9875))	13 ± 3.28	11	19
	14	RiskExpon(0,93469;RiskShift(8,99618))	10 ± 3.24	6	10
N. laciniosa	7	No Growth	-	-	-
	8	No Growth	-	-	-
	10	RiskExpon(5,0704;RiskShift(21,9745))	27 ± 3.88	22	35
	12	RiskExpon(4,6838;RiskShift(11,9828))	17 ± 3.68	13	25
	14	RiskExpon(0,76891;RiskShift(8,99677))	10 ± 3.39	9	13
N. fischeri	7	No Growth	-	-	-
	8	No Growth	-	-	-
	10	RiskExpon(2,75;RiskShift(19,9596))	23 ± 3.08	20	29
	12	RiskExpon(4,1546;RiskShift(10,9572))	15 ± 4.43	11	24
	14	RiskExpon(0,90909;RiskShift(7,97934))	9 ± 0.86	8	11
B. nivea	7	No Growth	-	-	-
	8	No Growth	-	-	-
	10	No Growth			
	12	RiskLogistic(40,9177;3,7612)	40 ± 7.28	24	46
	14	RiskNormal(27,5886;5,7337)	28 ± 5.73	18	35
B. fulva	7	No Growth	-	-	-
	8	No Growth	-	-	-
	10	RiskLognorm(6,7016;3,0758;RiskShift(23,1762))	30 ± 3.40	27	38
	12	RiskNormal(22,6957;2,5287)	23 ± 2.53	16	27
	14	RiskExpon(4,1075;RiskShift(7,9558))	12 ± 5.14	8	23

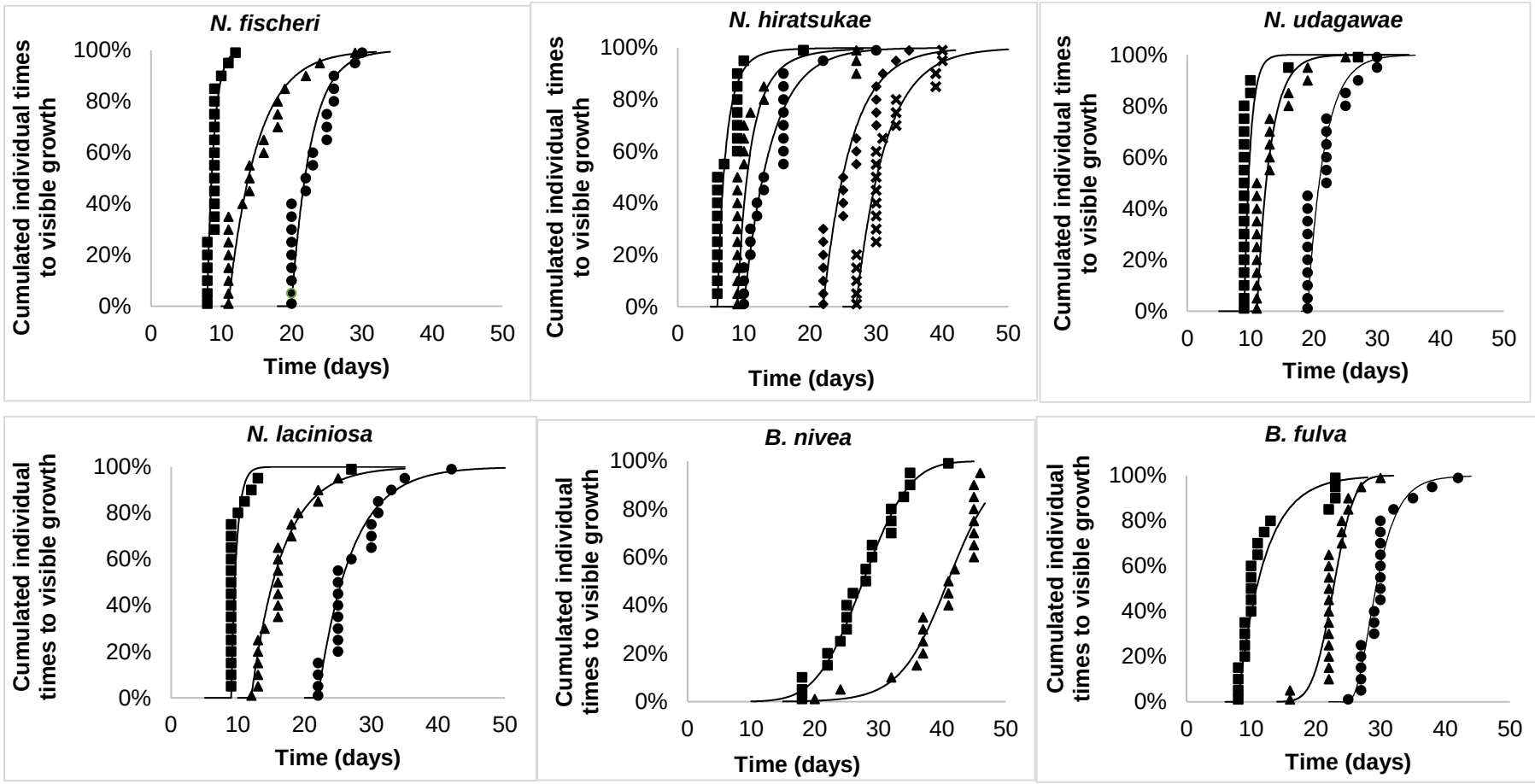

Figure 4.2. Cumulated individual times to visible growth of spores of *Byssochlamys* and *Neosartorya* spp. strains in acidified PDA (pH= 3.6) stored at 14°C (■), 12°C (▲), 10°C (●), 8°C (♦) and 7°C (✕)

Fig. 4.2 shows the cumulative t_v's of of *Byssochlamys* and *Neosartorya* spp. arising from ascospores inoculated on aPDA and stored at temperatures ranging from 7 to 14°C. Reduction of temperature (14°C - 4°C) appears to have a more pronounced effect on the distribution and spread of individual t_v's compared to the effect of sugar content (°Brix) in the evaluated range (44-59°Brix). This can be also deduced from the large distances between the cumulative t_v curves at different temperatures (Fig. 4.2). Most of the curves were determined to be exponential right-skewed, most t_v-values concentrated in the lower boundary (=shorter times). Besides, *N. hiratsukae*, which were the most low-temperature tolerant HRM strain evaluated in this study, had individual ascospores t_v's expressed by right-skewed Pareto distributions at 12 and 14°C. These are characterized by a shape and scale parameter (α, β): (4.59, 9) and (4.87, 6), respectively. These curves are similar to an exponential distribution, with its density decreasing from its mode in α at a rate β. In difference, the curves obtained for the *Byssochlamys* isolates were represented by logistic, normal and lognormal distributions (Table 4.3) and characterized by larger t_v ranges (Fig. 4.2). Whilst logistic curves are similar to normal curves, they distinguished by generating most likely values at extreme conditions (tails).

4.4 Discussion

Fungi have, in general, relatively simple nutritional requirements for growth. Dormancy is usually broken after the ascospores are exposed to an external trigger such as heat, high pressure or chemicals (Dijksterhuis, 2007; Wyatt et al., 2013). The transition from a dormant to an active metabolic state is characterized by the degradation of compatible solutes, decrease in the viscosity of the cytoplasm and disruption of the thick cell wall, allowing nutrient uptake by the activated ascospores and initiation of the germination process. This comprises formation and elongation of the tube, followed by hyphal extension and branching (Burgain et al., 2013, Gougouli et al, 2013, Wyatt et al., 2013). The t_v, comprises the time just after lag time preceding the visible mycelial linear growth. Therefore, there is a trend to replace the lag time for t_v in studies aiming to predict fungal spoilage, as the last is of much more applicability for this type of approach (Burgain et al., 2013, Dantigny, 2016, Berni et al., 2017, Santos et al., 2017, Gougouli and Koutsoumanis, 2017).

As previously mentioned in Ch. 1 (see § 1.*3.5*), a_w is the most important factor influencing fungal germination and outgrowth. As a result, this parameter has been assessed by the majority of available studies focused on HRMs growth (Panagou et al., 2010, Roland and Beuchat, 1984, Berni et al., 2017, Samson et al., 2010). However, while a_w is an useful parameter among food microbiologists, the effect of sugar concentrations (°Brix) is universally applied in the fruit processing industry. Despite this, scarce data are currently available

regarding the effect of °Brix on the HRMs growth (Berni et al., 2017, Beuchat and Toledo, 1977).

Berni et al. (2017) recently determined the sugar concentration limiting conditions (ºBrix) of *Neosartorya* strains (*N. hiratsukae, N. pseudofischeri*, *N. glabra*) on fruit-based media. The authors observed that *Neosartorya* sp. could tolerate sugar concentrations from 49-56°Brix, dependent on the species. These results were slightly below those observed in our study (53-59°Brix). In contrast to our findings, *N. hiratsukae* was only able to grow at Brix values ≤50°Brix in fruit-based media. This could be a result of differences in the media (aPDA vs. fruit based media) and strains used. Beuchat and Toledo (1977) investigated the behavior of *B. nivea* ascospores in fruit products supplemented with sucrose (20-60% soluble solids). The authors reported that the majority of fruit products containing 60% soluble solids did not support the growth of *B. nivea*, whilst visible mycelia were observed on fruit products with 40% sucrose after 3-13 days (at 30°C) and 4-34 days (at 21°C) (Beuchat and Toledo, 1977). In contrast to our results, Panagou et al. (2010) did not observe growth when *B. nivea* and *B. fulva* were inoculated on non-acidified Malt Extract Agar whose a_w had been adjusted with glycerol to values ≤ 0.88, regardless of the storage temperature (10-45ºC).

HRMs can be classified as thermo-tolerant microorganisms, i.e., which are able to tolerate (=to growth) temperatures as high as 45-50°C and lower than 20°C (Mouchacca, 2007, Samson et al., 2010, Pitt and Hocking, 2009, Panagou et al., 2010). In agreement with our results, *B. nivea* was inhibited in diverse fruit products incubated at 7°C (Beuchat and Roland, 1977). Whilst the majority of HRMs have not been reported to growth at chilled conditions, one of the evaluated strains, *N. hiratsukae*, was able to grow out at 7°C after almost one month. Although much less attention is given to *N. hiratsukae* in literature compared to other HRMs, it has been isolated from soil, fruit, fruit juice, spoiled tea-based beverage and indoor environments (Berni et al., 2017, Samson et al., 2007, Santos et al., 2018a).

It is known that t_v may be highly dependent on some factors, including media composition, temperature and inoculum size (Burgain et al., 2013, Zimmerman et a., 2011, Zimmerman et al., 2013, Valík and Picková, 2001, Dagnas et al., 2017, Walker and White, 2005). Berni et al. (2017) observed shorter times for detection of visible mycelium of *Neosartorya* strains at lower °Brix values. As an example, while the visible growth of *N. glabra* took place after 6-8 days at 47-51°Brix, visible colonies were only detected after 20-38 days at 56°Brix. It has also been demonstrated in some studies that similar growth parameters are obtained when HRMs were inoculated on distinct (different types of) fruit matrices with the same a_w- values (Zimmerman et al., 2011, Zimmerman et al., 2013, Berni et al., 2017). This is in contradiction with the findings

of Beuchat and Toledo (1977) who observed discrepancies on the time required for growth when *B. nivea* ascospores were inoculated in different fruit juices and nectars with similar a_w-values. As an example, when *B. nivea* was inoculated in apple juice (a_w= 0.92 and 40% sucrose) it was able to form visible mycelium after 17 days at 21°C, while in grape juice it took only four days for the appearance of the first mycelium. In contrast, cranberry juice stored at 21°C did not support the growth of HRMs when the °Brix was as low as 48.5 (a_w=0.94) (Beuchat and Toledo, 1977). The lower values for maximum °Brix for HRMs growth observed in fruit-based medium compared to our results may be a result of compositional differences. Fruits contain phenolic compounds and organic acids that may have a significant effect on the germination and outgrowth of HRMs (Amaeze, 2013, Beuchat, 1977, Panagou et al., 2010, Valík & Piecková, 2001, Zimmermann et al., 2013).

The interval (spread) of individual t_v's corresponds to their natural biological variability and therefore cannot be reduced by increasing the number of performed experiments. Larger spreads of the t_v's of individual ascospores were observed as the conditions became more sub-optimal, suggesting that greater variability occurs when fungal growth is stressed. While no data are currently available regarding the biological variability of ascospore, such variability has been reported for individual heat sensitive spores (conidia) (Garcia et al., 2010, Gougouli and Koutsoumanis, 2013, Samapundo et al., 2007, Dagnas et al., 2015, Dagnas et al., 2017). Garcia et al. (2010) studied the effect of a_w and temperature on the growth of heat sensitive fungi at various inoculum size and observed that low inoculum levels and suboptimal conditions lead to high variability of estimated lag times. Dagnas et al. (2017) quantified the inhibitory effect of a_w and storage temperature on lag times of single spore of moulds isolated from spoiled bakery products and concluded that stressful conditions result in discrepancy between individual and population lag time. Likewise, Samapundo et al. (2007) assessed the effect of a_w and temperature on the individual lag times of moulds associated with the spoilage of maize and observed larger variabilities (spread) on growth parameters at suboptimum conditions. Nevertheless, uncertainties referring to possible variations within replicates may also have contributed to the spread of the t_v's.

The t_v was defined in this study as the time required for visual detection of mycelium, i.e., the time taken for the colonies to grow to a diameter ≥2 mm. This threshold has also been used by other authors (Zimmerman et al., 2013), while a threshold of 3mm has been applied in other studies (Dantigny, 2016, Gibson et al., 1994, Gougouli et al., 2011, Valík and Piecková, 2001). It is worth mentioning that the t_v's observed in this study may underestimate the real time required before rejection time of fruit products. Despite the aPDA mimic low pH found in most of fruit products, it does not take into account the presence of other fruit compounds, such as

organic acids and preservatives, reduced oxygen content and other stress factors which may potentially contribute to longer t_v's. Moreover, the t_v-values observed are much shorter than the typical shelf-lives of fruit products stored at ambient temperature of a few to several months. At the same time, it is important to emphasize that the ascospores inoculated in this study were not submitted to high intensities pasteurization (as done for pasteurized fruit products), which can sub-lethally injure the ascospores resulting in longer times to visible growth. Ultimately, the t_v's of large inoculums as used in this study (*ca.* 100 ascospores per plate), is mostly represented by ascospores with relatively short germination and lag times and may highly differ than contamination by one or a few spores (Burgain et al., 2013, Gougouli et al., 2011). Hence, it is important to validate such data in real fruit products. Nevertheless, our data represents worst case scenarios, which may be very useful for developing fail safe predictive (shelf-life) models.

4.5 Conclusions

The study of environmental factors (intrinsic and extrinsic) preventing the growth of spoilage microorganisms is crucial to maintain and/or increase the microbial stability of food products. In this study growth/no growth limits for temperature and water activity were established for 6 different HRM species isolated from fruit and fruit based products. Moreover, the variability inherent of individual ascospores regarding their individual time to form visible growth was quantified and described as parametric statistics distributions. Large ranges of individual times to form visible mycelia were mainly observed under conditions at the growth/no growth regions. Ultimately the generated data will be used in predictive and microbial risk assessment studies.

Chapter 5

Development of an experimental set-up to assess the effect of low oxygen levels on the microbial growth in solid synthetic media

Redrafted after

Santos, J. L. P., Pimentel, G.C. Samapundo, S., Van Impe, J., Sant´Ana, A. S. & Devlieghere, F. Development of an experimental set-up to assess the effect of low oxygen levels on the microbial growth in synthetic media (manuscript under preparation).

Abstract

This study aimed to develop an experimental set-up (method) to attain and maintain low-oxygen-levels (<1%) in a synthetic solid medium for up to two months. This set-up would enable the effect of low oxygen (O_2) levels on the growth of moulds to be assessed. The set-up was developed into two parts. Firstly, the evolution of dissolved O_2 as function of depth (0-12 cm) in a solid medium was determined. Thereafter, a method based on the use of an O_2 scavenger was developed to establish and maintain low desired O_2 levels. From the five depths assessed, the O_2 level (%) was significantly different ($p<0.05$) at only three depths (0, 3 and 6cm). It was also deduced relying on the O_2 gradient created as function of depth in solid medium may be unreliable as any change in the gas concentration in one layer (at one depth) would influence the overall gradient and the concentrations of gases in the other layers. Nevertheless, 3-cm-deep glass jars were selected as it was possible to maintain the set (desired) level of dissolved O_2 in these jars for up to two months. The method developed in brief consists of obtaining desired levels of dissolved O_2 by adjusting the headspace O_2 levels in the jars *via* O_2 scavengers. The suitability of this method was not only due to the possibility of setting one level per jar with better gas control, but also due to its feasibility. Short times (1-5 days) were required to obtain the desired dissolved O_2 level within the synthetic medium after equilibration with the headspace. The proposed method can be ultimately used to assess the growth limiting conditions of microorganisms and to develop predictive models aiming to increase the shelf-lives (microbial stability of food products).

5.1 Introduction

The control of microorganism´s growth associated with food spoilage and food poisoning is crucial to maintain and/or increase the microbial quality and safety of food products. This control is mainly obtained through robust formulations, suitable processing, packaging and storage conditions. Numerous studies are currently available regarding the quantitative effect of intrinsic and extrinsic factors such as water activity (a_w), pH and temperature on the growth of microorganisms (Dagnas et al., 2014, Nguyen Van Long et al., 2017a, Ross et al., 2003, Singh et al., 2016, Syamaladevi et al., 2016). Conversely, only a few studies have included the effect of gases on microbial growth/inhibition (Couvert et al., 2017, Couvert et al., 2019, Devlieghere et al., 1998, Guillard et al., 2016, Nguyen Van Long et al., 2017b). Besides, the majority of these studies have focused on the use of modified atmospheric packaging (MAP) (Farber et al., 1994, Devlieghere et al., 1998, Nguyen Van Long et al., 2017b, Pin et al., 2000, Samapundo et al., 2011, Taniwaki et al., 2001, Taniwaki et al., 2010).

Modifying the composition of the atmosphere by adding carbon dioxide (CO_2) and reducing O_2 is an effective alternative to control microbial growth and degradation reactions, such as oxidation and enzyme reactions in order to extend the shelf-life of food products. However, the use of MAP does not allow for the total removal of O_2 and, at the same time, it is not applicable (feasible) to all food products i.e. fruit juices. In addition, while many microorganisms have an absolute requirement for O_2, others, such as anaerobes and facultative anaerobes, are very tolerant of reduced O_2 and/or elevated CO_2 (Couvert et al., 2019, Hillmann et al., 2015, King et al., 1969, Nielsen et al., 1989, Taniwaki et al., 2009). Besides being directly associated with microbial stability of food products, the available O_2 may compromise their sensorial characteristics by decreasing the overall nutritional value and freshness during storage (Choe and Min, 2006).

The total amount of available O_2 determines microbial growth rather than only the O_2 tension (Pitt & Hocking, 2009). As a result, two important phenomena need to be taken into account to assess the effect of gases during storage of processed food: gas solubility and diffusivity (Chaix et al., 2014). Atmospheric O_2 permeates through packaging materials to the headspace followed by dissolution at food surface and ultimately diffusion through the food (Chaix et al., 2014, Bhunia et al., 2016). Thus, it is necessary to know not only the O_2 concentration in the headspace, but also the amount (mg/L, ppm) that has dissolved and diffused into the water phase in order to predict the growth of microorganisms.

Recently, the use of non-destructive methods for O_2 measurements have been adopted through the use of light-sensitive sensor chips (Bhunia et al., 2016, Kinouchi et al., 2014, Muñoz et al., 2017). These O_2 sensors are usually placed inside packages, allowing for measurement of the gas concentration in both the headspace, and within the food during storage. They have been used to assess the diffusion of O_2 in food/model packaging systems (Muñoz et al., 2017, Samapundo et al., 2011) and to assess low levels of dissolved O_2 levels (0-3 mg/L) in airtight glass bottles (Kinouchi et al., 2014). Nonetheless, O_2 assessments using such available methods are usually performed for shor period of time. Moreover, no efficient method to reach and accurately maintain very low levels of dissolved O_2 is currently available. Unlike, these are limited to the use of gas flushing and/or temperature to reach and maintain such levels (Kinouchi et al., 2014, Nguyen Van Long et al., 2017b, Taniwaki et al., 2009), which may restrict the type of experiments to be performed.

Predictive models are valuable tools to assess growth limiting conditions of microorganisms and increase shelf-life and microbial stability of food products. These quantitative studies have been performed, in some cases, during an extended period in order to obtain more realistic

predictions, according to the shelf-life of a specific food product (Deschuyffeleer et al., 2015, Gougouli and Koutsoumanis, 2017). Likewise, it is crucial to have a perfectly closed system to maintain the gas concentration over time once O_2 and/or CO_2 is included as a factor in the development of these models. Thus, a method needs to be developed to establish desired levels of dissolved O_2 in solid medium. This would enable the effect of dissolved O_2 level on the growth of microorganisms to be studied more systematically.

Therefore, this study aimed to develop a suitable set-up which would: (i) provide a fast equilibrium between O_2 concentrations in headspace and solid medium; (ii) allow assessment of O_2 levels in the headspace and solid medium (dissolved O_2) during time; (iii) be suitable to assess and maintain low O_2 levels (<1%) for up to two months (60 days).

5.2 Material and Methods

5.2.1 Oxygen measurement

The O_2 concentrations (%) in the headspace and dissolved in synthetic media were measured by a non-destructive optical method. The system consists of two parts: (i) oxygen sensitive films Oxydot´s and (ii) a reader pen with an infrared detector which is connected to an OxySense® 200T (OxySense, Inc.,Dallas, TX) running on Oxysense's Gen-III software. In brief, the Oxydot´s contain an immobilized metal organic fluorescent dye which is excited by a blue light emitted from the reader pen. The principle of the measurement relies on the fact that the fluorescent light from the dye changes in intensity and lifetime proportionally to the oxygen partial pressure in the container/package. Each measurement was repeated five times through O_2-sensitive films (Oxydot®) attached to the jars. A schematic representation of the oxygen analyser method is shown in Figure 5.1.

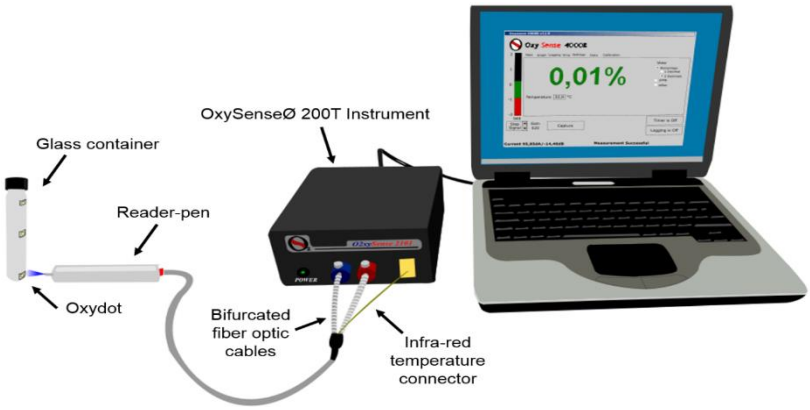

Figure 5.1. Schematic representation of a non-destructive optical method for oxygen measurement.

5.2.2 Set-up development

In order to establish constant low levels of dissolved O_2 (<1%), the study was divided into two parts. Firstly the profile of dissolved oxygen as function of depth was assessed. Next, a set-up based on the use of an oxygen scavenger was developed and monitored.

5.2.2.1 Oxygen profile as a function of depth

This part of the study was developed based on the hypothesis that an O_2 gradient is obtained in synthetic agar based medium depending on the distance from the agar-headspace interface. Three factors were evaluated: i) size of glass container, ii) volume of headspace and iii) temperature. Three different containers were evaluated; these are depicted in Figure 5.2. In order to non-invasively assess the O_2 profile as a function of depth in solid media, O_2-sensitive films (Oxydot®, European Tech Serv NV, Belgium) were attached to different depths of the container using translucent silicone sealant (RTV 108 - Momentive, United States). After the molten medium (agar 1.5%. w/v) had being poured and allowed to solidified in the jars, a target headspace oxygen concentration of 0.9% was achieved in the headspace by means of a Multivac packaging machine (Multivac Sepp. Haggenmüller, 127 Wolfertschwenden, Germany). Thereafter the jars were sealed and incubated at 22°C for two months. During this period, the O_2 levels at different depths in these jars was assessed periodically.

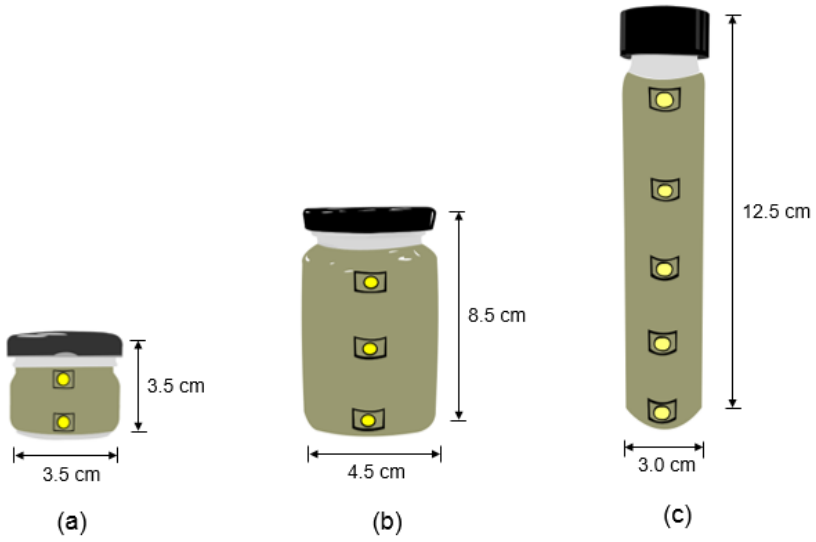

Figure 5.2. Illustration of the glass jars (a), (b), (c) with Oxydot®'s attached at different depths in the solid agar-based culture medium.

5.2.2.2 Set-up development: Oxygen profile using oxygen scavenger

The development of the set-up was based on the use of an O_2 scavenger to obtain specific low levels of dissolved O_2 in synthetic solid medium contained in glass jars. A schematic representation of the set-up is shown in Figure 5.3. One Oxydot® each were attached at the side walls at bottom and top of the glass jars which were subsequently sterilized at 121°C for 15 min. A 1-cm-thick layer of acidified Potato Dextrose Agar (Sigma-Aldrich, Germany) (aPDA, pH = 3.5) was then poured into the recipient after which the lid was closed. The glass jars were disinfected externally with sodium hypochlorite solution 10% and subsequently placed inside high O_2 barrier plastic bags with O_2 and CO_2 permeability's of 2.3 and 6.5 $cm^3.m^{-2}.d^{-1}.bar^{-1}$ at 23°C and 0% relative humidity, respectively. For each bag (= one O_2 level), three jars (=three replicates) were prepared. In order to reach and assess O_2 levels (0.1 - 0.9%), O_2 scavenger sachets (AnaeroGen Compact, Oxoid Ltd, Basingstoke, UK) were added to each bag. Subsequently, the lid of each jar was opened to allow the O_2 from the media and headspace to be consumed by the scavenger. Thus, the rate of O_2 uptake was measured through the Oxydot® attached at the top (headspace) of the glass jar. Different sizes of plastic bags and O_2 scavengers were tested (data not shown), in order to maximize the rate of O_2 uptake. When the desired O_2 concentration was achieved in the headspace, the bag was sealed as shown in Figure 5.3 (steps 3 and 4), which allowed the O_2 scavenger to be separated from the glass jars. The equilibration time, which is the time it takes for the O_2 in the culture medium to reach the same level as in the headspace, was assessed at: 8 and 22°C. Once equilibrium was reached, the lids were closed, after which the jars were withdrawn from the bag and stored at 22°C.

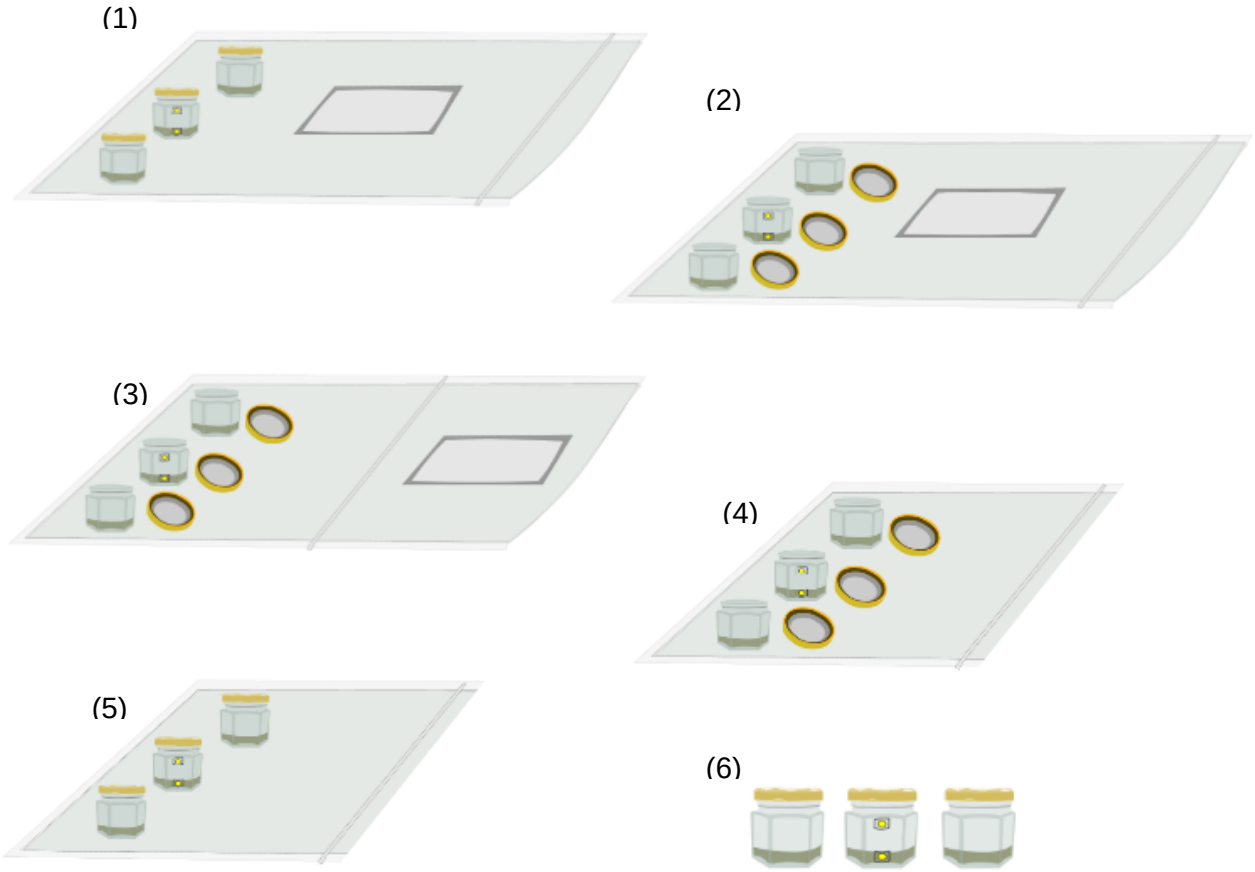

Figure 5.3. Representation of the set-up using an oxygen scavenger: (1) Oxygen scavenger and disinfected jars are added to the plastic bag and sealed; (2) All lids are opened and O_2 uptake is measured through the Oxydot®; (3) O_2 scavenger is sealed off the bag when the desired O_2 level in the headspace is achieved; (4) Bag is stored at 8°C for 1-3 days until the O_2 level in the media is in equilibrium with the O_2 in the headspace; (5) After equilibrium is reached, lids are closed while jars are still inside the bag; (6) the glass jars are stored at pre-determined temperatures.

5.2.3 Statistical analysis

Statistical analysis were performed in R software version *3.3.1*. The normality of the distributions were evaluated by means of boxplot and QQ-plot, and the equality of variances checked by Levene's test. Thereafter, one-way ANOVA with a Tukey's post hoc test was performed to evaluate where significant differences occurred. All tests were performed at 95% confidence level ($\alpha = 0.05$).

5.3 Results

5.3.1 Dissolved O_2 level as a function of depth in a solid agar-based medium

This part of the study aimed to assess: i) whether the level of dissolved O_2 in the media varied as a function of depth in a solid agar-based medium, ii) which O_2 levels could be reached at each depth after setting a specific level of O_2 at the headspace and iii) if these levels would remain constant for 1-2 months. To this end, three factors were also taken into consideration in this study - size of jars, volume of headspace, and temperature. The effects of these factors are described below in detail.

5.3.1.1 Effect of size of jar

The O_2 profile in three types of glass jars evaluated as a function of depth in the medium are shown in the Figure 5.4 (A-C). As can be seen, the O_2 levels in jar A decreased from 7.4% to 1.4% and from 7.2% to 1.4% at the top (depth = 0 cm) and bottom (depth = 3 cm), respectively, during the first five days of incubation. After the equilibration time (= 5 days), the O_2 levels remained relatively constant for the following 53 days at average values of 1.41 ± 0.04 %O_2 and 1.36 ± 0.04 % O_2 at the top and 3cm depth, respectively. O_2 levels at the top were significantly higher than at 3cm depth ($p<0.05$) after equilibration (see Table 5.1).

Table 5.1. Average oxygen concentration of oxygen after equilibrium was reached, at different depths in the 3 jars evaluated.

Glass jar	Average O_2 ± Standard Deviation (%)					Days*
	0 cm	3 cm	6 cm	9 cm	12 cm	
	1.41 ± 0.04^{a}	1.36 ± 0.04^{b}	-	-	-	53
	0.58 ± 0.05^{c}	0.30 ± 0.07^{d}	0.15 ± 0.09^{e}	-	-	53
	1.19 ± 0.11^{f}	0.30 ± 0.07^{d}	0.05 ± 0.03^{g}	0.01 ± 0.01^{g}	0.00 ± 0.00^{g}	22

Different superscript letters indicate where significant differences occurred between the oxygen levels as a function of the depth ($p<0.05$). (*) Days of incubation after equilibrium was reached.

For jar type B (6cm long) the O_2 levels were measured at the top (0cm depth), center (3cm depth) and bottom (6 cm depth). The O_2 levels at these three depths decreased to 0.58, 0.18 and 0.02% at depths 0, 3 and 6cm, respectively during the first five days of incubation, during which equilibration occurred. Thereafter, there were slight fluctuations in the O_2 level over time followed by slow increase after 40-50 days incubation. The average concentrations at the top,

center and bottom during the following 53 days of experiment were 0.58 ± 0.05 %, 0.30 ± 0.07 % and 0.15 ± 0.09 %, respectively. O_2 concentrations at the three depths were significantly different ($p<0.05$), with the levels decreasing as a function a depth as can be seen in Fig. 5.4b. Figure 5.4c illustrates the O_2 profile from tallest glass jars (type C). Five O_2 measurements were done at five depths - 0, 3, 6, 9 and 12 cm. The O_2 concentration decreased from 3.24% to 0.91 and from 0.75% to 0.19% after two days incubation at depths of 0 and 3 cm, respectively. Afterwards, the levels at the gas-medium interface and at 3cm depth slightly increased to 1.12% and 0.27% O_2, respectively after 15 days of incubation. Overall, it was observed that as the depth increased, i.e., as far as the layer is from the gas/medium interface, the gas level tend to converge to zero, with very barely noticeable fluctuations. Significant difference ($p<0.05$) was observed between the O_2 concentrations at these two uppermost layers. In the three deepest layers (at 6, 9 and 12 cm), the initial O_2 concentration (of 2.32%, 4.47% and 3.24%, respectively) decreased gradually to 0% after 10 days at all three depths. Whilst no significant differences ($p>0.05$) were observed between the O_2 concentrations at these three layers, they were significantly ($p<0.05$) lower than the O_2 levels measured in the upper two layers.

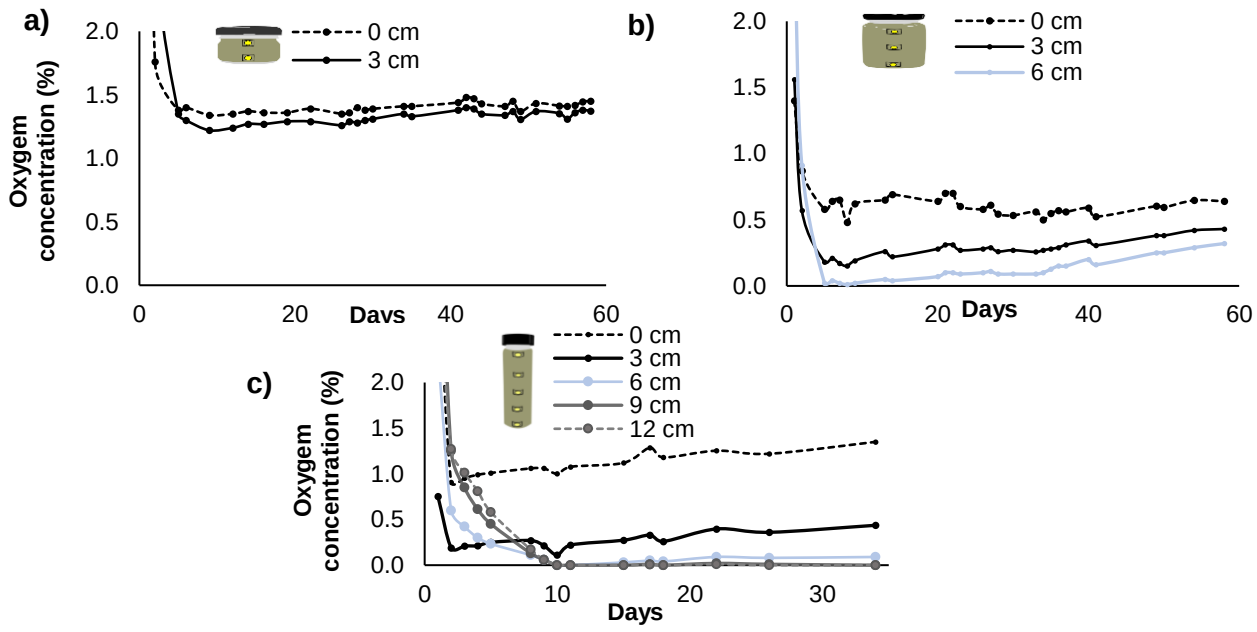

Figure 5.4. Evolution of O_2 concentration as a function of depth in the solid medium: (a) gas/product ratio: 1.7:1; (b) gas/product ratio: 1.2:1; (c) gas/product ratio: 1.6:1. The depths at which Oxydot®s were attached are shown in the Figure. The three samples were filled with agar, with 0.9% O_2 in the headspace and incubated at 22 °C.

5.3.1.2 Effect of headspace volume

Another factor that could influence the distribution of oxygen within the culture medium is the volume of the headspace. Figure 5.5 shows the evolution of the O_2 levels in jars (types A and B) with headspace: agar ratios of (a) 1:2 and (b) 1:12 and gas/product ratio of (a) 1.9 : 1 and 1.1 : 1, (the last defined by the volume of the jar and the volume of medium added to the jar). As shown in Figure 5.5a, an Oxydot® was attached to the glass in the headspace, whilst the Oxydots® at the gas-agar interface and 3 cm into the agar where in contact with the agar. After two days, the agar at the gas-medium interface had the same O_2 level as the headspace. During the following 32 days, the O_2 concentration in the headspace and at the gas-medium interface did not significantly differ ($p>0.05$). At a depth of 3 cm into the medium, the O_2 level decreased during the first 15 days to 0.20% (± 0.03) after which it remained constant for the following 35 days. The O_2 profile for the glass jar illustrated in Figure 5.5b was previously discussed in §5.3.1.1. No significant differences were observed ($p>0.05$) between layers of culture media at gas-medium interface (0 cm) in both jars, which were set with similar oxygen level in the headspace (0.9%). Both interfaces reached 0.89% O_2 after one day of incubation at 22°C. Likewise, similar levels were observed at a depth of 3cm, despite the equilibrium time being longer in the jar type A (ratio 1:2) compared to the jar with smaller headspace (ratio 1:12). Therefore, changing the headspace: medium volume from 1:12 to 1:2 did not influence the O_2 levels after equilibrium when the depth in the media was limited to 3 cm. Of note, it was determined that the gas-medium interface had similar O_2 levels to those set in the headspace regardless of the headspace volume.

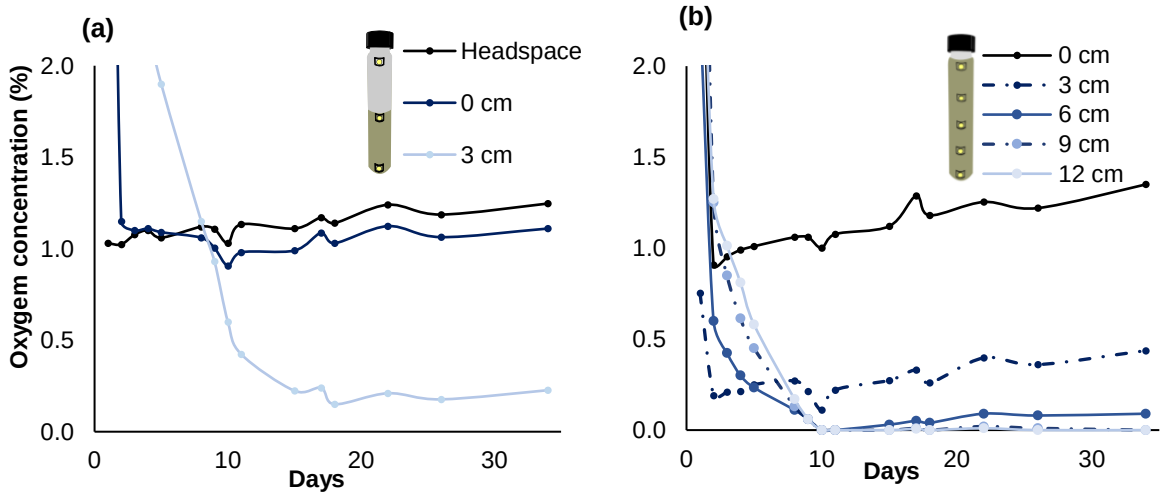

Figure 5.5. Oxygen profile developed in glass jar filled with agar 1.5% and with headspace: agar ratios of (a) 1:2 and (b) 1:12. Both (a) and (b) were filled with 0.9 % O_2 at the headspace.

5.3.1.3 Temperature

Glass tubes filled with 1.5% agar and 0.9% O_2 in the headspace were stored at 8 and 22 °C (see Figure 5.6a and b, respectively). It was observed that temperature has a large effect on the solubility of O_2 in the agar. There was a gradual decrease in the O_2 concentrations from 7.6% to 3.29% and from 5.8% to 4.4%, in the central and bottom layers, respectively, of the samples incubated at 8°C for an 18 day period. At 22°C the O_2 levels decreased rapidly until an equilibrium value of 1.19 % O_2 was attained at the gas-medium interface and 0% O_2 at depths 6 and 12cm, respectively, after 10 days. At the interface agar-headspace (0 cm depth), the level of oxygen was slightly higher compared to the O_2 level set in the headspace (0.9%): 1.19 ± 0.11 % (after equilibrium) and 1.86 ± 0.00 % O_2 (during 18 days of measurements) at jars stored at 22°C and 8°C, respectively.

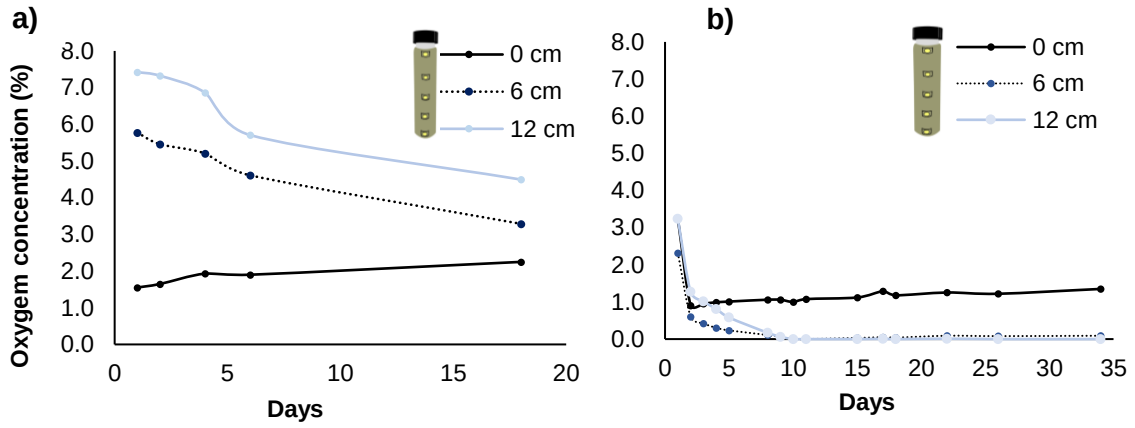

Figure 5.6. O_2 profile developed in glass jar filled with agar and stored at (a) 8 °C and (b) 22 °C. Both (a) and (b) initially had a headspace O_2 level of 0.9 %.

5.3.2 Set-up development: Oxygen profile using oxygen scavenger

Because it was observed that both, the gas-medium interface and the headspace reached similar O_2 concentrations after equilibration, the proposed set-up was further elaborated in glass jars with a 1 cm thick layer of medium (acidified PDA) (Figure 5.7). In this way one could control the level of dissolved O_2 in the culture media by simply setting the desired O_2 level in the headspace by means of O_2 scavengers (AnaeroGen™). In order to minimize the time of O_2 removal (scavenging) from the headspace, the impact of two types of O_2 scavengers (AnaeroGen™ Compact and Anaerogen™ 2.5L) was evaluated. Ideally, the time should be sufficiently short so that the experiments remain feasible and sufficiently long so that the scavenger can be sealed-off (isolated from the glass jars) in the bag at the correct moment (=

when the desired O_2 level is achieved). While it took more than 22 hours to attain low O_2 levels (0.1%) when an AnaeroGen™ Compact was used, a 2.5 L-sachet enabled similar levels to be attained in about half an hour in jars placed inside high O_2 barrier 30 x 39 cm plastic bags. Therefore, the 2.5L sachets were selected for this set-up.

Figure 5.7. Glass jar used in the set-up based on the use of oxygen scavenger.

After sealing off the scavenger, the equilibrium time, which is the time it takes for the culture medium to achieve the same O_2 concentration as in the headspace, was assessed at 8 and 22°C and determined to range from 1 to 3 days at both temperatures.

As previously described in §5.2.3.2, three glass jars were placed and opened inside a plastic bag together with a O_2 scavenger sachet in order to obtain the desired O_2 concentration in the headspace. Some experiments were performed to verify whether the level of O_2 in the headspace would result in the same level of dissolved O_2 in the medium in the three replicates (= 3 jars) placed in the same bag. For this purpose, Oxydot®s were attached to all three jars and the set-up performed. Afterwards, the dissolved O_2 levels were measured as a function of time for a period of 20-30 days. These results are shown in the Table 5.2. Four headspace O_2 levels were tested: 0.4, 0.3, 0.2, and 0.1%. When the O_2 level in the headspace was set at the desired value of 0.4%, the three replicates had actual levels of 0.37 ± 0.03, 0.39 ± 0.05 and 0.35 ± 0.03 % O_2. No significant differences ($p>0.05$) were found between the replicates. The same was observed at the other headspace O_2 levels tested, which implied that it would be sufficient in this set-up to measure the O_2 levels in one of the three replicates from the same bag.

Table 5.2. Average oxygen concentration (%) in the culture media of replicates from the same bag.

O_2 (%) set at the headspace	**Days of measurement**	**Average O_2 (%) in the culture media**		
		Replicate 1	Replicate 2	Replicate 3
0.4	30	0.37 ± 0.03^a	0.39 ± 0.05^a	0.35 ± 0.03^a
0.3	30	0.25 ± 0.07^b	0.23 ± 0.04^b	0.21 ± 0.06^b
0.2	20	0.16 ± 0.03^c	0.17 ± 0.03^c	0.15 ± 0.03^c
0.1	30	0.04 ± 0.03^d	0.03 ± 0.03^d	0.04 ± 0.03^d

Equal letters in the superscript represents no significant difference ($p>0.05$).

5.4 Discussion

The microbial safety and quality of packaged foods are highly dependent on intrinsic and extrinsic factors, such as the amount of O_2 in the headspace and that dissolved in the food itself. Reduced O_2 levels results in metabolic modification and reduction of microbial growth rates (Farber et al., 1994, Devlieghere et al., 1998, Taniwaki et al., 2001). The reduction and/or depletion of O_2 is achieved by modifying the composition of the atmospher by adding CO_2 and N_2 to the headspace to partially or fully replace O_2 (Chaix et al., 2015, Devlieghere et al., 1998, Nguyen Van Long et al., 2017b) or by means of active packaging incorporating O_2 scavengers to remove residual O_2 (García-Torres et al., 2009, Zahra et al., 2016). During the storage, a fraction of the O_2 present at the headspace is dissolved in the food, followed by diffusion within the matrix. It is known that these two phenomena are highly affected by the composition of the food, temperature and chemical reactions in the food (Pénicaud et al., 2010, Chaix et al., 2014, Bhunia et al., 2016). Therefore, the total amount of available O_2 (dissolved and un the headspace) determine microbial growth rather than only the O_2 tension (Pitt & Hocking, 2009). Therefore, it is essential to have a suitable set-up to assess dissolved and headspace O_2 levels in different matrices.

Muñoz et al. (2017) studied O_2 solubility and diffusivity in a model food system comprising of tryptic soy broth supplemented with various agar concentrations. The model food systems were placed in high O_2 barrier pouches at 0, 3 and 6 cm depth. Similar to our findings, Muñoz et al. (2017) observed that the diffusivity of O_2 decreased as the depth into the model food system increased. In addition, very low O_2 levels were also observed at the bottom (at a depth of 6 cm), reaching 0% after 2-5 days of storage, depending on the storage temperature. As expected, higher amount of dissolved O_2 was detected at 8°C, as the solubility of most gases strongly increases with reduction of temperature (Kinouchi et al., 2014, Muñoz et al., 2017). Similar findings have been observed with regards O_2 solubility in water and various food matrices, where temperature plays an important role in both, gas solubility and diffusivity (Chaix et al., 2014). Unlike solubility, decrease in temperature results in reduced diffusivity. This can be attributed to less energy among the gas molecules at lower temperatures, which impairs the diffusion process (Bhunia et al., 2016, Muñoz et al., 2017). This explains why in our study the levels of dissolved O_2 in the samples stored at 8 °C decreased at a much slower rate in comparison to the samples stored at 22°C.

In the first part of the study, the levels of dissolved O_2 set at different depths in the media remained nearly constant for up to two months. However, long times, from 5 to 10 days, were

required for the O_2 levels to equilibrate in the agar at 22 °C. It is worth mentioning that this temperature is favorable for the microbial growth. As an example it is known that the germination of fungal spores can occur within 3-7 days at 20-25 °C even at O_2 levels lower than 0.27% (King et al., 1969, Taniwaki et al., 2009). Moreover, despite being possible to reach low dissolved O_2 levels in different depths in a solid agar-based medium, the number of layers with significantly different levels that developed in the glass jars were limited to a maximum of three depths (0cm, 3cm and 6cm, see Fig. 5.5). The presence of agar in the medium used in our study resulted in higher viscosity, which reduce the mobility of O_2 through the medium (Muñoz et al., 2017). Moreover, increasing in dry matter levels has been reported to decrease O_2 solubility (Pénicaud et al., 2012). Therefore, these factors may have contributed to the very low levels of O_2 measured at the greatest depths. The equilibration (1-3 days) at 22°C might not be suitable as there is a risk of microbial germination/growth during equilibration, particularly if only one stress factor (low level of O_2) is taken into account, with all other conditions favoring their growth. Thus, in order to avoid germination of spores during equilibration, the storage temperature during this period was set at 8 °C. After equilibrium, the glass jars can be incubated at the desired temperatures. It is important to mention that the equilibration time is an estimate, i.e. O_2 measurements should always be performed during an experiment to verify the dissolved O_2 levels over time. Lastly, it should be taken into account that the use of this method to assess microbial growth would be impaired by their O_2 consumption when they grow at one depth, which would affect the O_2 concentration in the other layers, as well as the overall gradient changes.

Some protocols have been developed to assess low oxygen levels and its effect on the growth/inhibition of target microorganisms. They include the use of desiccators, hypoxic chamber, O_2 scavengers, flushing of gas mixtures, gas-flow manometer control, analyzer infrared sensor and gas analysis chromatograph (Couvert et al., 2019, King et al., 1969, Nguyen Van Long et al., 2017b, Nielsen et al., 1989, Taniwaki et al., 2001). However, the available methods have some limitations such as the need for constant gas-flow of gases with the desired composition followed by gas sampling and analytical gas quantification; the use of barrier plastic bags that may not be suitable to perform experiments for long period due to small amounts of oxygen that may permeate through packaging. Moreover, as mentioned previously, many of them have assessed the depletion of O_2 by adding CO_2 instead of evaluating the single effect of O_2. The feasibility of the developed set-up is a result of its fast equilibration time (at 8 and 22°C), the possibility of assessing low levels dissolved O_2 in one single layer of culture media and its easier control as it is defined by the headspace. Moreover, this set-up allows low O_2–levels to be attained and thereafter maintained in solid synthetic media for long periods. In this regard, it may be appropriate for application in studies focused

on evaluating the effect of headspace and/or dissolved O_2 concentration on the growth of spoilage and/or pathogenic microorganisms.

5.5 Conclusions

In this study a set-up was developed which can be used in studies intending to assess the effect of low O_2 levels (<1%) in solid synthetic medium on microbial growth. Firstly, different tests were performed to investigate the hypothesis that an O_2 gradient occurs in solid agar-based media as a function of depth or distance from the gas-medium interface. However, from the five depths assessed, only three of them were statistically different ($p<0.05$). Nevertheless, a 3cm-deep glass jar was selected as it set (desired) O_2 levels which could be maintained for up to two months. Subsequently, a set-up based on the use of an O_2 scavenger was developed in which one O_2 level was set and thereafter monitored per jar. The suitability of this protocol was based on short times required to reach the desired dissolved O_2 levels and its ability of maintaining the gas concentration constant for long incubation periods (up to 60 days). Nevertheless, the set-up needs to be further validated in different matrices as the composition of the medium may affect the amount of dissolved O_2. Ultimately, the proposed set-up can be used in quantitative studies to assess the effect of the level of O_2 on microbial growth in solid medium and potentially in real food products. This would generate data which can be used to develop strategies to increase the microbial stability and/or safety of food products.

Chapter 6

Assessment of minimum oxygen concentrations for the growth of heat-resistant moulds

Redrafted after

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Abstract

This study evaluated the effect of both gaseous and dissolved oxygen (O_2) concentration (0 - 21%) on the growth of six heat-resistant moulds (HRMs) (*Neosartorya* and *Byssochlamys* spp.) previously isolated from high-acid fruit products. The study was performed in acidified potato dextrose agar (aPDA) with all six HRMs and with *B. fulva* and *N. fischeri* in strawberry, apple and orange juice-based media. At ≥ 0.15% O_2, visible growth of the HRMs occurred within 3-6 days. Complete inhibition on aPDA did not occur even at very low levels of dissolved O_2 (*ca*. 0.01% O_2). With the exception of *B. fulva*, decrease of the O_2 concentration to ≤0.03% resulted in significantly ($p<0.05$) longer times to visible growth. The growth of *N. laciniosa, N. fischeri , B. nivea* and *B. fulva* was inhibited for 30 days when they were incubated under strict anaerobic conditions. As in aPDA, *B. fulva* and *N. fischeri* grew in the three fruit-based media at O_2 concentrations ≥ 0.15%. Significantly slower ($p<0.05$) growth was observed for *N. fischeri* in orange juice medium. Strategies to inhibit the growth of HRMs should therefore not be based entirely on establishing low headspace O_2 levels. With this in mind, the effect of low O_2 concentrations (<1%) should be studied in combination with other factors (hurdles) such as antioxidants, organic acids, sugars (a_w), storage temperature and pasteurization intensity, in order to predict the growth inhibition of the HRMs.

6.1 Introduction

The ability of heat-resistant moulds (HRMs) to withstand thermal and non-thermal processes commonly applied by the food industry and to grow in a broad range of conditions, makes them a threat for the stability of high-acid processed fruit products (Berni et al., 2017, Evelyn and Silva, 2015, Evelyn and Silva, 2017, Houbraken et al., 2006, Panagou et al., 2010, Tournas, 1994). To tackle this issue, it is crucial to determine the conditions that may prevent and/or inhibit the germination of ascospores and fungal growth. To date, the effects of sugar composition and concentration, water activity (a_w) and storage temperature on the growth of HRMs have been addressed (Berni et al., 2017, Panagou et al., 2010, Tremarim et al., 2015). Conversely, studies focusing on the effect of the atmosphere on the growth of these microorganisms are still very scarce. Moreover, the few available studies have mostly focused on the use of modified atmosphere packaging (MAP) i.e., the combination of oxygen (O_2) reduction by adding carbon dioxide (CO_2) to inhibit microbial growth (Taniwaki et al., 2001, Taniwaki et al., 2009, Taniwaki et al., 2010, Yates et al., 1967).

HRMs belonging to *Byssochlamys* sp. and *Aspergillus* sp. with *Neosartorya*-type ascospores are well known for their economical relevance and their high incidence of occurrence in fruits and fruit products (Pitt and Hocking, 2009, Samson et al.,2010, Tranquillini et al., 2017). Although many fungal species associated with food spoilage require O_2 for growth, HRMs have been reported to be able to grow under nearly anaerobic conditions; at levels as low as 0.1% O_2 (King et al., 1969, Kotzekidou, 2014, Pitt and Hocking, 2009, Taniwaki et al., 2009). Moreover, some *Byssochlamys* and *Neosartorya* strains have been reported to produce mycotoxins in atmospheres with very low O_2 concentrations and/or 80% CO_2, such as in packaged fruit juices (Kotzekidou et al., 2014, Nielsen et al., 1989, Sant'Ana et al., 2010, Taniwaki et al., 2010).

Food spoilage by HRMs is caused by their mycelial growth followed by the production of CO_2 and pectic enzymes (Kotzekidou et al., 2014). Thus, available O_2 is not only associated with the microbial stability of food products, but also with sensorial and nutritional changes which can compromise their quality (Choe and Min, 2005). According to Pitt and Hocking (2009), it is necessary to study the available O_2 in both gaseous and water phases as they both determine microbial growth, rather than the isolated study of O_2 tension. The few studies that addressed the effect of O_2 depletion on the growth of HRMs, were performed by controlling the gas phase alone (King et al., 1969, Nielsen et al., 1989). The dissolved O_2 level and its evolution over time were not evaluated in these studies. Moreover, the data available on the minimum amount of O_2 required for growth inhibition is very limited, existing for only a few HRM species (King et al., 1969, Nielsen et al., 1989).

Therefore, this study aimed to assess the O_2 concentration necessary for the growth of different HRMs isolated from fruit products by (i) determining their times to visible growth in a general growth medium at 22°C as a function of low O_2 concentrations; (ii) assessing the effect of O_2 concentration on the growth of two HRMs in three fruit-based media and, (iii) by assessing the evolution of gaseous and dissolved O_2 profiles over time at all conditions studied.

6.2 Material and Methods

6.2.1 Strains

Six HRMs, previously isolated from raw and processed fruit products (Ch.2), were investigated in this study. These were *Byssochlamys nivea* (Bysso nivea 76-1) and *Neosartorya laciniosa* (Neosart laciniosa 67-2) - isolated from pasteurized strawberry puree, *Byssochlmays fulva*

(Bysso fulva 56-2) and *Neosartorya hiratsukae* (Neosar hiratsukae 77-5)- isolated from strawberries, *Neosartorya udagawae* (Neosar udagawae 54-3) - isolated from sieved strawberry puree, and *Neosartorya fischeri* (Neosar fisheri 95-1)- isolated from extracted orange juice. The isolates were maintained in the culture collection of the Laboratory of Applied Mycology (MYCOLAB, Department of Food Technology, Safety and Health, Ghent University, Belgium).

6.2.2 Fruit medium preparation

Three fruit-based media were prepared by adding bacteriological agar (Oxoid™, Hampshire, UK) to diluted strawberry puree, apple puree and concentrated orange juice at the rates shown in table 6.1. The a_w values were set at 0.960 by adding appropriate amount of water to simulate fruit purees aimed for the retail. Firstly, fruit puree or concentrate was added to water and heated in a microwave (1 min at 750 W) to facilitate dissolution. Subsequently, bacteriological agar was added after which the media were heated for 1-2 min. at 750 W in a microwave to facilitate mixing. The fruit media were ultimately sterilized at 115˚C for 10 min. and characterized by measuring the a_w, degree Brix (°Brix) and pH after sterilization. These values and the formulations used are summarized in Table 6.1.

Table 6.1 Ratio of fruit concentrate, water, and agar used to prepare fruit-based medium.

Fruit	Concentrate (g)	Water (g)	Agar (g)	a_w	°Brix	pH
Apple	70	30	5	0.96	22	3.90
Orange	30	70	13	0.96	24	3.83
Strawberry	70	30	10	0.96	26	3.90

6.2.3 Experimental design

In order to assess the effect of low O_2 on the growth of the six HRMs, four O_2 levels were evaluated in acidified Potato Dextrose Agar (aPDA, pH=3.5, HCl 6M, a_w=0.995, 4°Brix): 0% (strict anaerobic), 0.03, 0.15, and 0.9%. Every condition was examined in triplicate and the experiments were independently performed twice (n=6). In the second part of the study, two of the six HRMs, *B. fulva* and *N. fischeri* were inoculated in the three fruit-based media whose O_2 concentrations were set at 0.15% and 21% (atmospheric air). Every condition in this part of the study was examined in triplicate (n=3).

6.2.4 Preparation of ascospore suspensions

HRMs ascospores suspensions were performed as described in Ch. 3 (see § *3.2.2*). The final (non-activated) ascospore suspensions were kept at 2°C for up to one month.

6.2.5 Data collection

The final suspensions were then standardized in sterile acidified phosphate buffer (pH=3.5) to 10^3 ascospores/ml. The diluted spore suspensions were activated before use by heat shocking them for 10 minutes at 80°C. Glass jars filled with molten agar (1cm layer) were inoculated with 100µL of standardized suspension (*ca*.100 ascospores) and thoroughly mixed. In this way, it was possible to obtain a homogeneous distribution of the ascospores within the culture medium. The method based on the use of O_2 scavengers to obtain and maintain the desired low O_2 levels previously described in Ch. 5 (see § *5.2.2.2*) was used. The rate of O_2 uptake was measured *via* the Oxydot attached in the headspace of the jar. The bags containing the open jars were then stored at 8°C, a temperature at which no germination could occur (data not shown), until the O_2 levels in the headspace and medium had equilibrated. After equilibrium, the lids were tightly closed before the jars were withdrawn from the bag and stored at 22°C. The incubation temperature was selected in order to simulate the storage condition of shelf-stable pasteurized fruit products, such as fruit purees and concentrated juices. For the strict anaerobic condition, the aPDA was supplemented with sodium thioglycolate (1.0g.L^{-1}, Sigma-Aldrich, USA) and Resazurin salt (0.001g.L^{-1}, Sigma-Aldrich, USA). Additionally, the O_2 scavengers were kept inside bags with the inoculated closed jars during the whole experiment. Measurements of composition (O_2 and CO_2 levels) of the air inside the bags were performed by means of the CheckMate 3 headspace gas analyzer (Dansensor A/S, Denmark) just after equilibration. The jars were checked every two days for visible growth (at colony diameters of *ca*. 2 mm) at the agar surface and along the walls, for up to 30 days or until growth was observed. In addition, the O_2 concentrations during the course of the study were determined by means of an OxySense® 200T (OxySense, Inc., Dallas, TX).

6.2.6 Statistical analysis

All statistical tests were performed in R version 3.3.1 (R Foundation for Statistical Computing, Austria). Differences between average times to visible growth were analyzed using one-way ANOVA and post-hoc analysis by means of Tukey's test when the normality and equality of variances were confirmed. When equality of variances was not verified, one-way ANOVA followed by the Games-Howell post-hoc test was used. All tests were performed at $\alpha = 0.05$.

6.3 Results and Discussion

6.3.1 Effect of O_2 on the growth of HRMs in acidified PDA

In order to assess the effect of low O_2 levels on the time to visible growth of HRMs, four conditions were assessed in PDA: strict anaerobic, 0.03%, 0.15%, and 0.90% O_2. The growth of six HRMs (*B. fulva*, *B. nivea*, *N. laciniosa*, *N. hiratsukae*, *N. udagawae* and *N. fischeri*) and the O_2 concentrations in the headspace and in the water phase (medium) were monitored for up to 30 days. Fig. 6.1 shows the O_2 profile for each HRM at each condition evaluated. At the highest O_2 level assessed (0.9%), no growth inhibition was observed. The O_2 concentration generally decreased rapidly in both the headspace and the water phase followed by visible growth of *B. nivea* and the *Neosartorya* isolates within 3-5 days (see Fig. 6.2). Under this condition, no significant differences ($p>0.05$) were observed between the times to visible growth of the six HRMs (see Fig. 6.2). The highest level to be evaluated in the study was set at 0.9% as filamentous fungi are not likely to be inhibited at levels ≥1% O_2 when other growth determining factors are set at optimal levels (Nguyen Van Long and Dantigny, 2017a, Nielsen et al., 1989). The decrease in the O_2 concentration was attributed to consumption by the HRMs. Growth was observed promptly after the O_2 level in the water phase of the medium had reached very low levels (≤0.05%). With regards to *B. fulva*, there was no indication of appreciable O_2 consumption before visible growth was observed (see Fig. 6.1).

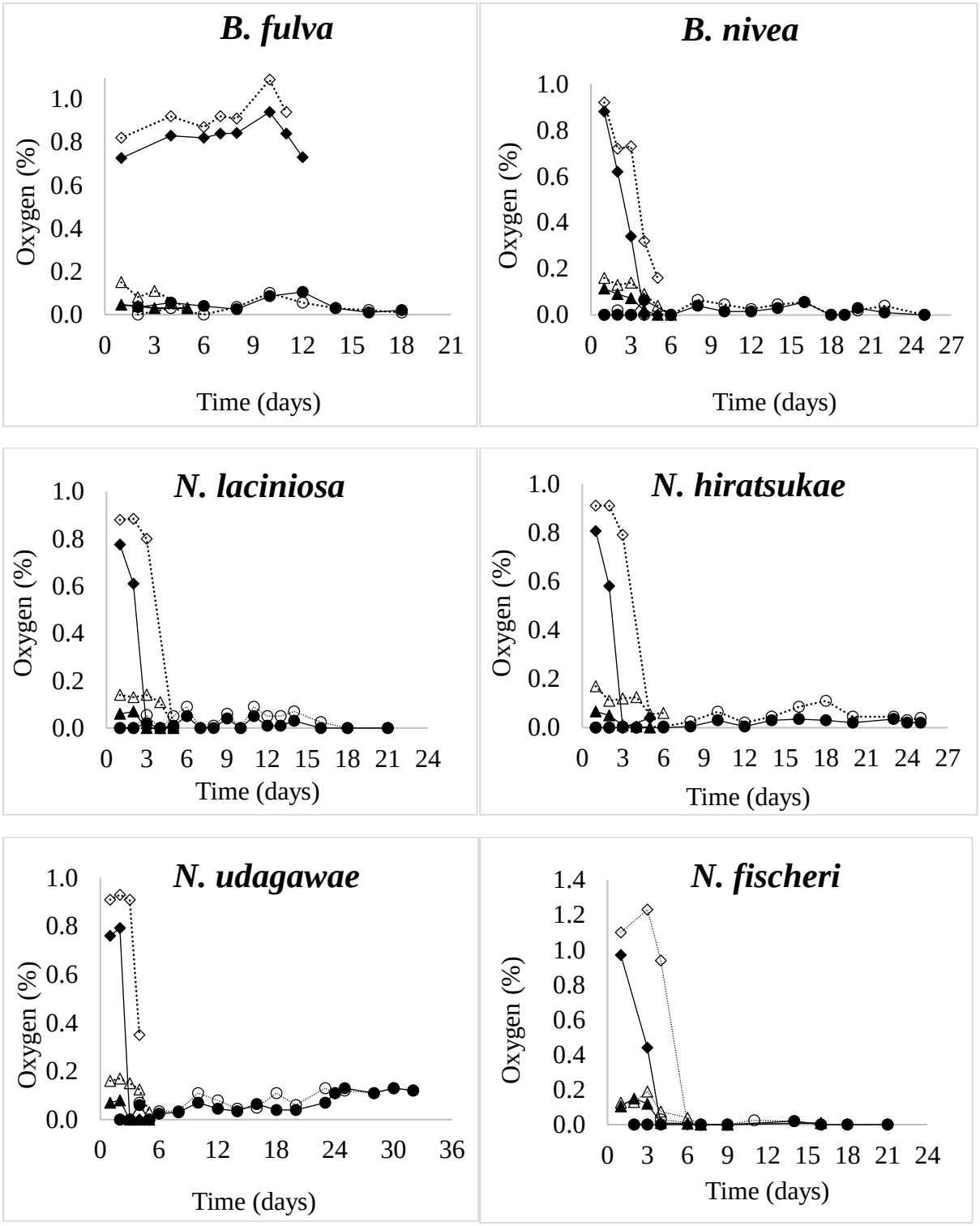

Fig. 6.1. O_2 profile (%) at headspace (dotted line) and dissolved in acidified PDA (solid lines) in jars inoculated with *B. fulva*, *B. nivea*, *N. laciniosa*, *N. hiratsukae*, *N. udagawae* and *N. fischeri* and stored at 22°C. The O_2 concentrations were initially set at 0.03% (●), 0.15% (▲) and 0.9% (♦). Each symbol represents the average O_2 level of two repetitions.

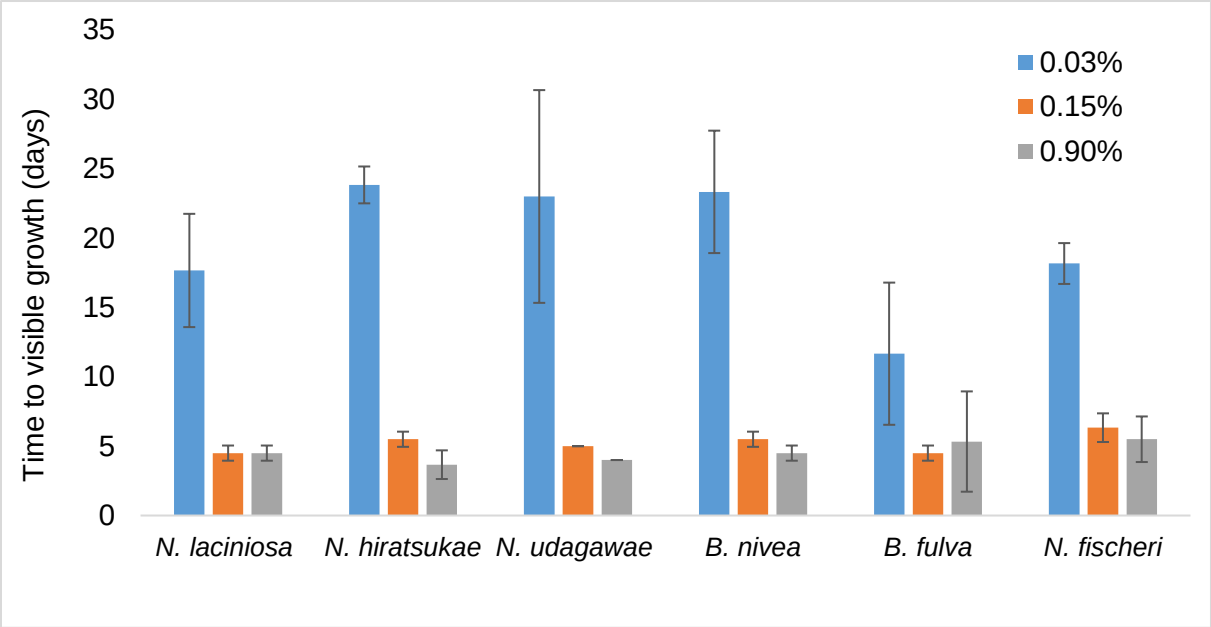

Figure 6.2. Time to visible growth in aPDA for *N. laciniosa*, *N. hiratsukae*, *N. udagawae*, *B. nivea*, *B. fulva* and *N. fischeri* at three initial headspace O_2 levels (0.03, 0.15 and 0.9%).

Subsequently, growth of the HRMs was assessed in a headspace set at 0.15% O_2. In this case, a slight decrease in the O_2 concentrations was observed before visible growth of the HRMs occurred after 5-7 days of incubation (see Fig. 6.1). With the exception of *N. hiratsukae*, the times to visible growth at these two O_2 levels were non-significantly different ($p>0.05$) for all isolates evaluated (see Fig. 6.2). The O_2 was rapidly consumed and growth was visible after the O_2 level had reached nearly 0% in both the water phase and headspace.

As the growth of all six HRMs was not inhibited at 0.15% O_2, a very low level of 0.03% O_2 was also assessed. Despite the slight fluctuations in the O_2 levels observed over time under this initial O_2 level, the O_2 level in all replicates remained under 0.1% O_2 (see Fig. 6.1). The mean O_2 values and respective standard deviations are depicted in the Table 6.2. Despite the delay observed in the growth of the HRMs when the O_2 level was reduced to 0.03%, all six HRMs were still able to grow out and form visible colonies within 4-30 days of incubation (see Fig.6. 3). *B. fulva* had in general the shortest and the most variable times to visible growth between the replicates, which ranged from 4-18 days. *B. fulva* was followed by *N. laciniosa* which was able to form visible colonies after 11-21 days of incubation. *N. fischeri* and *N. hiratsukae* required 17-21 days and 22-25 days to form visible colonies, respectively. Spores from the most O_2-sensitive HRMs evaluated in this study, *B. nivea* and *N. udagawae*, may require up to one month to form visible colonies under this condition. One-way ANOVA analysis showed that, with the exception of *B. fulva*, the HRMs exhibited significantly higher ($p<0.05$) times to visible growth under extremely low O_2 levels (0.03% O_2) (see Fig. 6. 2).

Table 6.2. Mean oxygen values and the respective standard deviations assessed in the headspace and culture medium (aPDA) in jars inoculated with HRMs and incubated at 22°C for 30 days when the initial concentration was set at 0.03 % O_2.

Strain	O_2 (%) headspace	O_2 (%) culture medium
B. fulva	0.03 ± 0.04	0.04 ± 0.05
B. nivea	0.03 ± 0.03	0.02 ± 0.03
N. laciniosa	0.03 ± 0.03	0.01 ± 0.02
N. hiratsukae	0.03 ± 0.04	0.01 ± 0.02
N. fischeri	0.00 ± 0.01	0.00 ± 0.00
N. udagawae	0.07 ± 0.05	0.06 ± 0.04

The temporal reduction of O_2 observed in the headspace and in the culture medium was attributed to metabolism of the HRMs, which use O_2 as a final electron acceptor in respiration (Deacon, 2006, Hull, 1939). Decrease in headspace O_2 levels due to fungal growth has been previously reported by several authors (Ellis et al., 1994, Hull, 1939, Rice, 1980, Tournas, 1994, Weng and Hotchkiss, 1991). Hull (1939) inoculated canned plums with *B. fulva* and stored them at 30°C. The O_2 concentrations in the artificially contaminated cans decreased from 10.5% on the second day of storage to 0% on the sixth day, whereas, the CO_2 concentration increased up to as much as 52%. Rice (1980) inoculated canned grape juice with *B. nivea* and observed a reduction in the O_2 level from 10% to 0.5% at 25°C within 10 days, accompanied by patulin production. Ellis (1994) inoculated peanuts with *Aspergillus flavus* and observed a decrease in the headspace O_2 within 1-3 days of incubation.

B. fulva and *B. nivea* did not form any visible colonies under strict anaerobic conditions over a thirty day incubation period. The mean O_2 levels during this period were 0.00 ± 0.01% in both the headspace and medium of the jars inoculated with *B. fulva* and 0.00 ± 0.00% in both the headspace and medium of the jars inoculated with *B. nivea*. Amongst the *Neosartorya* isolates, the jars inoculated with *N. fischeri* had mean O_2 values of 0.01 ± 0.01% in the headspace and 0.00 ± 0.00% in the medium whereas the jars inoculated with *N. laciniosa* had mean O_2 levels of 0.04 ± 0.06% in the headspace and 0.00 ± 0.01% in the medium. No visible growth was observed for these isolates. In difference, visible growth was observed in one of the three replicates (jars) inoculated with *N. udagawae* and *N. hiratsukae* after 24 days, in jars which had O_2 concentrations of 0.02 ± 0.02% and 0.00 ± 0.00% in the headspace and 0.04 ± 0.07% and 0.00 ± 0.01% in the medium, respectively. It is important to highlight that the O_2 measurements were only performed in one of the three replicate jars. Therefore, the visible growth observed in jars without Oxydot® may be due to either possible (albeit most likely small) differences in the initial O_2 concentrations, gas leakage and/or the presence of residual O_2 on the medium at the start of incubation. Nevertheless, this assessment reinforces the fact that

despite the tolerance of the HRMs evaluated in this study to very low O_2 levels, they are unlikely to grow in conditions without available O_2.

The ability of *Neosartorya* and *Byssochlamys* sp. to grow under low O_2 tensions was previously reported (King et al., 1969; Nielsen et al., 1989; Taniwaki et al., 2001, 2009). As an example, *N. fischeri* was reported to exhibit growth under 1% O_2 on CYA at 25 °C (Nielsen et al., 1989), whilst *B. fulva* was reported to grow under 0.27% O_2 on PDA after three days of incubation at room temperature (King et al., 1969). The growth of *Byssochlamys* sp. at extremely low O_2 levels (<0.1%) was also observed by King et al. (1969) and Taniwaki et al. (2009). In agreement with our results, King et al. (1969) observed no growth after three weeks when *B. fulva* was inoculated on PDA and incubated under strict anaerobic conditions. Nielsen et al. (1989) reported absence of growth of *N. fischeri* under 0.0095% O_2 after a 38-day incubation period at 25 °C. Based on these finding, it might be appropriate to classify these fungi as facultative anaerobes as already proposed by some authors including Hesseltine et al. (1985) and Taniwaki et al. (2010).

Overall, the results showed that HRMs respond differently to O_2 depletion. Moreover, potential variability in response of single spores within a population to O_2 level cannot be neglected. These variabilities appeared to increase as the O_2 level was reduced to very low levels. The high variability on the time to visible growth between replicates incubated under 0.03% O_2 may be due to the higher degree of stress that the ascospores were submitted to as a result of the near absence of O_2. It is known that stress may result in increased variability in fungal growth response (Amaeze, 2013, Dagnas et al., 2015, 2017). In addition, variability may be influenced by experimental uncertainties due to potential differences in inoculum as the repetitions were performed independently. Nevertheless, the biological variability of germination times of single spores from the same population could also have influenced the results (Gougouli and Koutsoumanis, 2012, Judet et al., 2008). Regarding the inter-species variability, *B. fulva* was significantly ($p<0.05$) more tolerant than *B. nivea* to low levels of O_2. Whilst the ability to form visible colonies was not significantly different ($p>0.05$) among the three *Neosartorya* isolates, the time to visible growth of *N. hiratsukae* was significantly longer ($p<0.05$) than those of *N. laciniosa* and *N. fischeri*. Variability between fungal species regarding their O_2 tolerance, were also reported by Gibb and Walsh (1980).

Although the time to visible growth was the main variable investigated in the present study, it is worth mentioning that the size of the colonies when the headspace was set at 0.03 % O_2 was markedly smaller compared to those that developed under 0.9 and 0.15% O_2. The influence of the composition of the headspace on the mycelium weight has been studied by

several authors (Hillman et al., 2015, Hull, 1939, Taniwaki et al., 2009 and 2010, Yates et al., 1967). Therefore, the outgrowth of fungi growing at lower O_2 concentrations is expected to not only be delayed, but also to present less dense colonies compared to growth under higher O_2 concentrations.

It is worth noting that the O_2 scavenger used in the set-up generates CO_2 whilst absorbing atmospheric O_2 from the headspace (Oxoid, 2018). Therefore, the headspace CO_2 concentrations were determined after the lids of the jars were closed (= just after equilibration). The mean CO_2 concentrations in the samples set to initial O_2 levels of 0.9%, 0.15%, 0.03% were 9.00 ± 0.78%, 9.52 ± 0.61% and 1.08 ± 0.58%, respectively. 16.9 ± 1.7% CO_2 was obtained in the strictly anaerobic condition. It has been already reported that CO_2 at high partial pressures has a fungistatic effect, even though the threshold for inhibition may vary considerably between species (Nguyen Van Long and Dantigny, 2017, Pitt & Hocking, 2009, Taniwaki et al., 2010). Studies indicate that concentrations higher than 40% noticeably inhibit the growth of most spoilage fungi (Taniwaki et al.,2009; Zardetto, 2005). Although limited information is available on the effect of intermediate partial CO_2 pressures (0.03-20%) on the growth of fungi (Nguyen Van Long and Dantigny, 2017), there is some indication that the intermediate concentrations inadvertently attained in this study (1-9.5%) might have a stimulatory effect on the fungal growth. This effect has also been observed in other studies including Gibb and Walsh (1980), Nguyen Van Long and Dantigny (2017), Taniwaki et al. (2010), and Wells and Uota (1970). As an example, Gibb and Walsh (1980) reported a general stimulatory effect of CO_2 at levels up to 4% in combination with 0.1% O_2 for *Fusarium moniliforme.* Furthermore, Wells and Uota (1970) observed growth stimulation of several fungi when CO_2 in the headspace was 10% and O_2 was as low as 2%. This occurs possibly due to the heterotrophic CO_2 fixation capacity observed in most fungi. CO_2 is fixed into acids of the citric acid cycle which afterwards are used for energy and growth (Walker and White, 2005, Wells and Uota, 1970). To the extent of our knowledge, no data is available on the effect of intermediate CO_2 levels on the growth of the HRMs investigated in the present study. Thus, we strongly recommend that further studies are performed focused on assessing the combined effects of CO_2 and O_2 or evaluating the effect of O_2 alone (using methods which do not generate CO_2 or which scavenge produced CO_2).

The HRMs investigated have been recently isolated from various raw materials of the fruit processing industry (Ch.2, Tranquillini et al., 2017). Moreover some of them have been associated with the spoilage of pasteurized and canned fruit products (Chapman et al., 2007, Kotzekidou, 1997, Tournas, 1994). The time to visible growth of the six HRMs evaluated ranged from 3-30 days, depending on the species and initial O_2 concentration. However, this

period is much shorter than the typical shelf-life of fruit products stored at room temperature, which may vary from a few to several months. Therefore, O_2 concentration cannot be used alone as a hurdle to inhibit the growth of HRMs and ensure stability of food products during shelf-life.

It is also worthwhile to acknowledge that the experiments were performed in acidified PDA, which leaves the possibility that the response of these HRMs could differ in real fruit products. Composition of the growth medium has been determined to have an effect on the solubility of O_2 in fruit products (Renard and Maingonnat, 2012). Other factors that may also affect O_2 solubility include the °Brix, temperature (Schumpe et al.,1982) and antioxidants (García-Torres et al., 2009). The effect of some of these factors was taken into account in the second part of the study whereby the growth potential of the HRMs was assessed in fruit-based media.

6.3.2 Effect of O_2 in fruit-based media

In addition to the assessment of the effect of O_2 level in aPDA on the growth of HRMs, the effect of O_2 level on the growth of *N. fischeri* and *B. fulva* was investigated in three types of media based on concentrated orange juice, strawberry puree and apple puree. Only two O_2 levels, 0.15% and 21% O_2 (atmospheric air), were assessed in this study. The two isolates were selected due to their potential to spoil fruit and fruit-based products and for exhibiting the most tolerance to very low O_2 levels (Pitt and Hocking, 2009, Samson et al., 2010, Tournas, 1994). The composition of the three fruit-based media is shown in Table 6.1.

Table 6.2. Ratio of fruit concentrate, water, and agar used to prepare fruit-based medium.

Fruit	Concentrate (g)	Water (g)	Agar (g)	a_w	°Brix	pH
Apple	70	30	5	0.96	22	3.90
Orange	30	70	13	0.96	24	3.83
Strawberry	70	30	10	0.96	26	3.90

The evolution of the O_2 concentrations in the headspace and water phase of the fruit-based medium is shown in Fig. 6.3. Overall, the headspace O_2 concentrations were higher than those of dissolved O_2 in the medium. Initially, growth of the HRMs was assessed in atmospheric air (21% O_2). Under this condition, the mean O_2 concentration in the fruit medium inoculated with *B. fulva* were 9.7 ± 2.1%, 13.8 ± 4.8%, and 12.8 ± 6.4% in orange, strawberry and apple medium, respectively. A slight increase in the O_2 concentrations of orange and strawberry medium inoculated with *B. fulva* was observed during the first four and two days, of incubation respectively. Two important phenomena occur during gas transfer on food as well as in

synthetic media: solubility and diffusivity (Chaix et al., 2014). The gas present in the headspace first needs to dissolve at the foods surface followed by diffusion through the matrix. The initial increase in the O_2 levels may be therefore a result of O_2 diffusing into the medium. After four days, the O_2 levels rapidly decreased followed thereafter by visible grow in all three medium inoculated with *B. fulva*. The initial O_2 concentration in the fruit medium inoculated with *N. fischeri* were respectively 13% in orange medium; 5% in strawberry medium and 7% in apple medium. As observed for *B. fulva*, there was a slight increase in the O_2 concentrations during the first 4 days in strawberry and apple puree medium at 21% O_2. Thereafter, there was a gradual decrease followed by a sharp fall in the O_2 levels in apple puree medium until the O_2 was depleted and visible growth was observed within 5-8 days. Similar trends were observed in strawberry puree medium inoculated with *N. fischeri* albeit three days later than observed in apple puree medium. Interestingly, the evolution of the O_2 concentration in orange juice medium inoculated with *N. fischeri* was marked by a sharp decline during the first two days of incubation, followed by a slight increase and then reduction until the O_2 was depleted and visible growth was observed within 9-13 days (see Fig. 6.3).

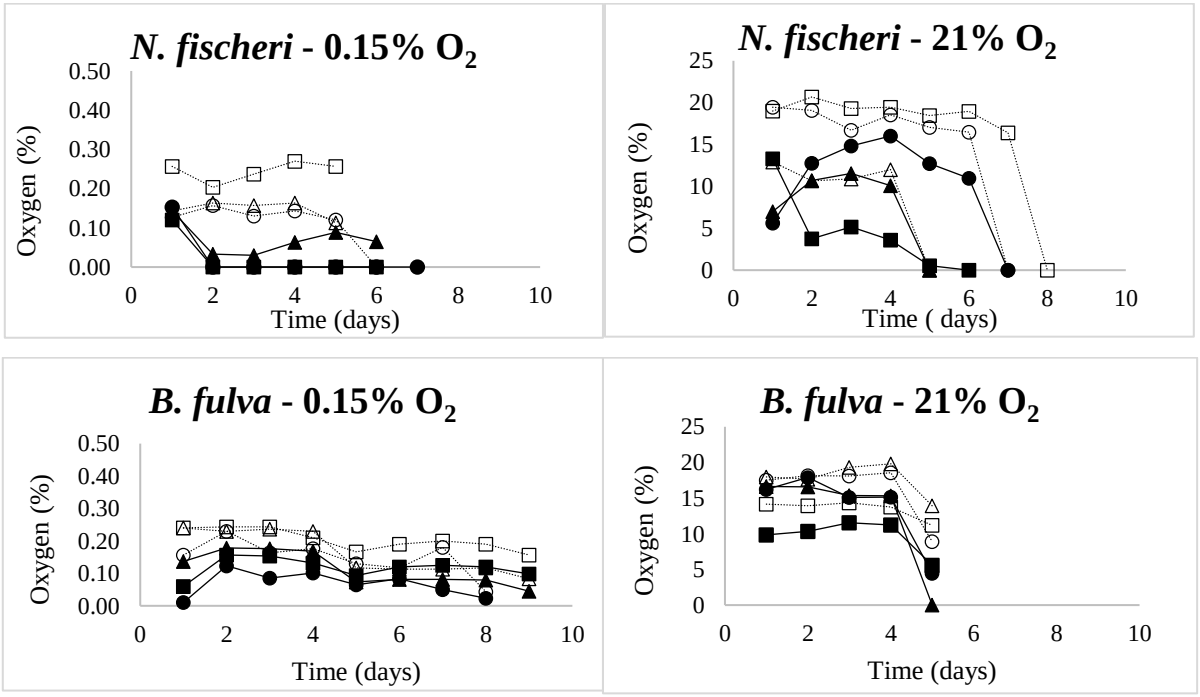

Fig.6.3. Profile of O_2 (%) in the headspace (dotted line) and dissolved in fruit-based medium (solid lines) in apple puree (▲), strawberry puree (●) and orange juice (■). The medium were inoculated with *N. fischeri* and *B. fulva* and stored at 22°C. The O_2 concentrations were set at 0.03% (●), 0.15% (▲) and 0.9% (♦). Each symbol represents the average O_2 level of the 2 repetitions.

As previously observed in aPDA, *B. fulva* seems to consume O_2 at a slower rate than the other HRMs. This could be deducted from the presence of dissolved O_2 in the medium after visible

growth had occurred (Fig. 6. 4). Moreover, this HRM seems to exhibit a high hypoxic tolerance compared to the other species, as it was able to germinate and growth after just 4 days when the O_2 concentration was lowered to 0.03%. It is known that under O_2-limited conditions, the energy metabolism of filamentous fungi will not be entirely depends on O_2, but also on the available carbon source, such as glucose (Hillman et al., 2015, Zhou et al. 2010). Therefore, these findings imply that more studies are required to better understand the physiological characteristics of *B. fulva* regarding its alternative respiration processes. On the other hand, the results show that *N. fischeri* consumes O_2 at very early stages of outgrowth, most probably during the germination process. It was also noticed that, despite the experiments being performed without O_2 control (in atmospheric air), the O_2 level found in fruit medium differed according to the isolates (species) inoculated in the medium. The O_2 levels in fruit media inoculated with *N. fischeri* were markedly lower than those found in the media inoculated with *B. fulva*. For instance, the initial O_2 decreased from 13 to 0% and from 9 to 6 % when *N. fischeri* and *B. fulva* were inoculated, respectively, in orange juice.

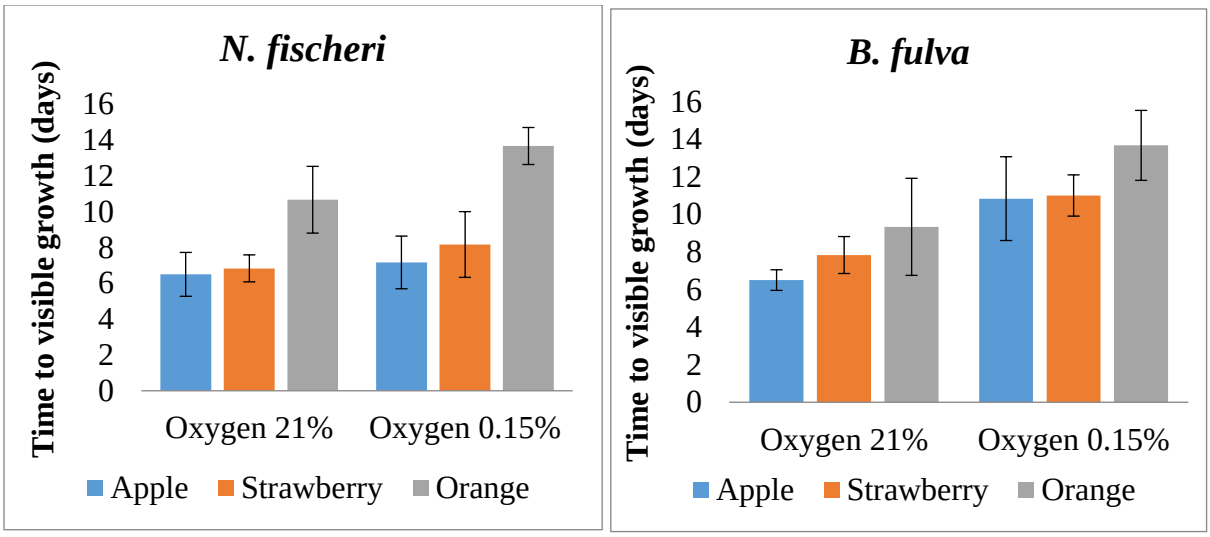

Fig. 6 4. Time to visible growth (D≥2mm) of *N. fischeri* and *B. fulva* inoculated in fruit-based medium (aw=0.960) at 0.15% O2 and 21% O2 (atmospheric air) and 22°C. The bars represent the average time (days) with the respective standard deviations.

The evolution of O_2 concentrations when the initial value was set at 0.15% is depicted in Fig. 6.3. Under this condition, the initial and final O_2 values were respectively 0.07 and 0.04% in orange juice medium; 0.02 and 0% in strawberry puree medium and, 0.13 and 0.05% in apple puree medium. Overall, the O_2 profile in fruit media inoculated with *B. fulva* were characterized by an initial slight increase, fluctuations and a slight reduction which was accompanied by appearance of visible colonies within 9-13 days in apple medium, 10-12 days in strawberry medium and, 10-15 days in orange juice medium (see Fig.6.4). At the same condition, the

initial and final O_2 concentration in fruit medium inoculated with *N. fischeri* were, respectively, 0.12 and 0% in orange juice medium, 0.15 and 0% in strawberry puree medium and, 0.14 and 0.06% in apple puree medium (see Fig.6.4). These levels decreased rapidly, reaching 0.00% after two days in orange juice and strawberry puree medium. Thereafter the O_2 levels remained constant before visible growth was ultimately observed after 6-10 days in strawberry medium and 12-15 days in orange juice medium. For apple puree medium, the evolution of the O_2 concentration was characterized by an initial rapid decrease followed by slight fluctuations and visible growth within 6-9 days (see Fig. 6.3).

Reduction of the O_2 level resulted in a significant increase ($p<0.05$) in the time to visible growth of *N. fischeri* in orange juice medium and that of *B. fulva* in all three fruit medium (see Fig. 6.4). Both *N. fischeri* and *B. fulva* had longer times to visible growth in orange juice medium than they had in apple and strawberry puree medium. The time to growth of *N. fischeri* was nearly two times longer and significantly higher ($p<0.05$) in orange juice medium (10.7 ± 1.9 days) than it was in strawberry and apple puree medium (5-6 days) under 21% O_2. Similar results were observed when the initial O_2 concentration was decreased to 0.15% for *N. fischeri*, Likewise, the time to growth of *B. fulva* was longer in orange juice medium at both O_2 levels investigated. However, the times did not differ significantly ($p>0.05$) between the three fruit medium for these HRMs (see Fig. 6.4). The lower O_2 concentration found in orange juice medium when exposed to atmospheric air may be due to the fact that orange juice is rich source of antioxidants, such as ascorbic acid, which would potentially consume dissolved O_2 (García-Torres et al., 2009, Wang et al., 1996). This implies that HRMs have to compete for available O_2 with chemical reactions such as ascorbic acid oxidation and oxidation of flavor and color compounds in the food matrix (Kefford et al., 1959), which therefore resulted in longer times to visible growth in orange juice medium.

In general, shorter times to visible growth were observed in aPDA than in fruit medium. For instance, the times to visible growth of the HRMs at 0.15% O_2, were nearly twice as long in fruit medium (7-14 days) than they were in aPDA (4-7 days). It should be noted that the a_w values of aPDA and the fruit-based medium used in this study differed, with the latter having higher values. The lower a_w values of the fruit medium (a_w = 0.96) together with the fruits composition may have contributed to the longer times to visible growth observed in these medium. The effect of sugars, such as glucose and fructose, commonly present in fruit and fruit-products, may strongly influence fungal germination and growth (Amaeze, 2013, Panagou et al., 2010, Valík & Piecková, 2001, Zimmermann et al., 2013). Moreover, it is known that at sugar concentrations above 20% (20° Brix), such as those of the fruit-based medium used in this study, the germination of heat shocked ascospores may be delayed or inhibited (Amaeze,

2013). In addition, oranges and strawberries are rich sources of citric acid, which is known as an important food preservative and has been reported to strongly retard and/or inhibit the growth of ascospores (Ackermann et al., 1992, Amaeze, 2013, Campo and Santos, 2006, Sturm et al., 2003). This may in part explain the faster growth of the HRMs in apple puree medium compared to orange juice and strawberry puree medium. Ultimately, it cannot be neglected that O_2 in the headspace may also be consumed by the food while it diffuses through the matrix (Chaix et al., 2014). As a result, lower O_2 levels were expected in the fruit medium than in aPDA. In this way, the O_2 that was able to diffuse into the fruit medium might not have been entirely available for the HRMs.

6.4 Conclusions

This study aimed to assess the minimum inhibitory concentration of O_2 towards six HRMs (*N. laciniosa, N. fischeri, N udagawae, N. hiratsukae, B. nivea* and *B. fulva*) previously isolated from fruit products. All six HRMs were able to grow in aPDA under atmospheres with $\geq$ 0.03% O_2 within 30 days at 22°C. Decrease in the initial O_2 concentration resulted in markedly smaller colonies and longer times to visible growth. With the exception of *B. fulva*, all the HRMs exhibited significantly ($p<0.05$) longer times to visible growth when exposed to 0.03% O_2. No growth was observed for up to 30 days when *N. laciniosa, N. fischer, B. nivea* and *B. fulva* were incubated under strict anaerobic conditions at 22°C. Conversely, *N udagawae, N. hiratsukae,* exhibited visible growth in at least one of three replicates after 24 days. The effect of O_2 level (0.15 and 21% O_2) on the growth of *B. fulva* and *N. fischeri* was also evaluated in three fruit-based media. The time to visible growth in the fruit-based medium was in general almost twice as long as those observed in aPDA at the same O_2 concentrations. Both isolates grew faster in apple puree medium, followed by strawberry puree and then orange juice medium. However, it should be noted that the delay observed in orange juice medium was only significant ($p<0.05$) for *N. fischeri*. Furthermore, the wide range of times to visible growth observed for some isolates when O_2 was nearly depleted highlights the large biological variability between single ascospores. This issue needs to be taken into account in future studies i.e. by assessing more replicates. The times to visible growth observed under all conditions where O_2 was present were much shorter than the actual shelf-life of fruit-based products. Therefore, the complete inhibition of the growth of HRMs in acid fruit-based should not be based alone on establishing low headspace O_2 levels. Instead, a more effective approach would encompass the combined effect of low O_2 concentration (<1%) (in the headspace and food matrix) with intrinsic factors specific for the food e.g. antioxidants, organic acids, sugars (a_w), optimized heat treatments, high O_2 barrier packaging, and storage temperature.

Chapter 7

Effect of storage temperature, water activity, oxygen headspace concentration and pasteurization intensity on the time to growth of *Neosartorya fischeri*

Redrafted after

Santos, J.L.P, Samapundo, S., Djunaidi, S., Vermeulen, A., Sant'Ana, A.S., Van Impe, J., Devlieghere, F. Effect of storage temperature, water activity, oxygen headspace concentration and pasteurization intensity on the time to growth of *Neosartorya fischeri* (manuscript under preparation).

Abstract

In addition to their high thermal tolerance, the ascospores of heat resistant moulds (HRMs) are known to have very high tolerance to adverse environmental conditions. As a result, HRMs can compromise the microbial stability of processed fruit products. Understanding their tolerance towards suboptimal growth conditions and multiple sources of stress may provide valuable information for preventing their germination and subsequent outgrowth in processed fruit products. This study aimed to assess, by means of a full factorial design, the combined effect of storage temperature (10-30°C), water activity (a_w, 0.87-0.89), headspace oxygen (O_2) level (0.15-0.80%) and pasteurization intensity (95°C, 100°C or 105°C/15sec) on the time to visible growth (t_v, days) of *Neosartorya fischeri* on acidified Potato Dextrose Agar (aPDA, pH 3.6) for up to 90 days. Moreover, to quantify the effect of a fruit matrix, 13 conditions were selected and assessed in strawberry-puree based medium. Ultimately, the effect of O_2 (0.05 and 1%) and pasteurization intensity (95°C and 105°C/15sec) were evaluated on totally 22 real fruit purees (single strength and concentrates) over a 60 day storage period. Overall, storage temperature had the greatest effect on the t_v of *N. fischeri*. At 10°C, no visible growth was observed over the 90 day incubation period, whilst visible mycelia (diameter ≥ 2mm) were present in 37% and 89% of the conditions at 22°C and 30°C, respectively. Pasteurization intensity had only a minor effect on the outgrowth of *N. fischeri*. Growth inhibition was observed when a_w was reduced to 0.870 ± 0.005 in combination with very low headspace O_2 levels (0.15%± 0.10), regardless of the incubation temperature and heat pasteurization intensity. Overall, longer t_v's were required when incubation was done at 22°C compared to 30°C. The combination of low a_w (0.87) and low O_2 (0.15%) resulted in inhibition of the growth of *N. fischeri* in both, aPDA and strawberry media at 22°C and 30°C. Growth inhibition was only observed at O_2 levels ≤ 0.05% on single strength fruit purees (a_w≥0.980) stored at 22°C. Combination of multiple stress factors effectively inhibited growth of *N. fischeri*. In general, storage of fruit purees at low temperatures (<10°C) or distribution in the form of concentrates can be considered as important strategies to prevent the growth of spoilage associated heat-resistant moulds. Nevertheless, the stability of high a_w fruit purees will be entirely based on depletion of headspace O_2 and/or fruit composition, when mild pasteurization intensities are applied and storage is done at room temperature.

7.1 Introduction

Different attempts have been made to deal with the persistence of HRMs in fruit processing and heat-treated products during storage (Sant'Ana et al., 2009, Rico-Munoz, 2017, Berni et al., 2017). However, this is a great challenge as HRMs are well known for their high tolerance

to heat, low pH, low oxygen (O_2) and high carbon dioxide (CO_2) atmospheres (King et al., 1969, Samson et al., 2010, Pitt and Hocking, 2009, Evelyn and Silva, 2017).

Neosartorya fischeri is known for its economic importance as a spoilage fungi, being prevalent in soil, raw materials and pasteurized food products (Jesenská et al., 1992, Pitt and Hocking, 2009, Salomão et al., 2008, Samson et al., 2010). Moreover, *N. fischeri* is able to grow in a broad range of environmental conditions (Amaeze, 2012, Evelyn et al., 2016, Salomão et al., 2007, Evelyn and Silva, 2017, Souza et al., 2017, Samson et al., 2010, Baglioni et al., 1999, Zimmerman et al., 2011, Berni et al., 2017). Besides, some *N. fischeri* strains have been reported to produce mycotoxins such as terrein, fumitremorgins and verruculogen (Nielsen et al., 1988, Samson et al., 2007, Samson et al., 2010).

Although the inactivation (thermal and non-thermal) of *N. fischeri* has already been extensively studied (Beuchat, 1986, Slongo and Aragão, 2008, Evelyn and Silva, 2017, Delgado et al., 2012, Souza et al., 2017, Menezes et al., 2019), data regarding its potential growth in synthetic medium and fruit products are very scarce (Amaeze, 2013, Baglioni et al., 1999, Zimmerman et al., 2011, Zimmerman et al., 2013, Valík and Piecková, 2001). Moreover, only two studies focusing on the effect of heat treatment intensity on subsequent growth of HRMs are available (Ch.3, Samapundo et al., 2018). To the best of our knowledge, no data are currently available regarding the combined effect of heat pasteurization and multiple sources of stress such as O_2 and a_w (reduction) on the subsequent growth or inhibition of HRMs during storage.

Therefore, this study aimed to assess: (i) the combined effect of storage temperature, a_w, O_2 and pasteurization intensity on the time to growth of *N. fischeri* on acidified potato dextrose agar (aPDA), (ii) to validate some of the conditions on strawberry puree based medium and, (ii) to assess the effect of O_2 and pasteurization intensity on the growth of *N. fischeri* in fruit purees.

7.2 Material and Methods

7.2.1 Isolate

Neosartorya fischeri strain (Neosar fisheri 95-1) previously identified and isolated from processed fruit products (Ch. 2) was used in this study. This HRM strain was selected based on its high level of occurrence in high-acid fruit products, including pasteurized products, its high heat resistance (see Appendix) and its capability to growth out on several suboptimum conditions (Ch.4 and Ch.6). The isolate was maintained in the culture collection of the

Laboratory of Applied Mycology (MYCOLAB; Department of Food Technology, Safety and Health, Ghent University, Belgium).

7.2.2 Preparation of ascospores suspensions

Suspensions of ascospores of *N. fischeri* strains were prepared as described in Ch. 3 (see *§3.2.2*). Final suspensions were then standardized to 10^4–10^5 ascospores/ml in 9ml-tubes of sterile acidified buffer glucose solutions (pH=3.6, 10% tartaric acid, KH_2PO_4/ Na_2HPO_4, Sigma Aldrich, Germany) in order to obtain ~ 100 ascospores (initial inoculum) after the ascospores suspensions being submitted to the pasteurization treatments in later experiments (see *§7.2.6)*. The ascospores suspensions were prepared weekly and kept at 2 °C for at most 7 days before use.

7.2.3 Growth media

The experiments were carried out on two culture media: sugar-based and fruit-based medium, (aPDA, pH=3.6, HCl 6N) and concentrated strawberry puree were used as bases for the sugar-based and fruit-based media, respectively. Both were adjusted to a_w values of 0.87, 0.88 and 0.89 by addition of fructose (F-0127, Sigma) and glucose (G-8270, Sigma) (1:1) (Table. 7.1). Each medium was sterilized at lower temperatures than usual, more specific 115 °C for 10 min, to avoid undesirable reactions reducing the sugar levels. The a_w values and °Brix were determined by means of a a_w meter (Sprint TH500, Novasina Thermoconstanter, Pfapfikkon, Switzerland) and a pocket refractometer HR25/800 (Kruess, Germany), respectively.

Table 7.1 Sugar-based and fruit-based media composition.

Media	Fruit (g)	PDA (g)	Fructose (g)	Glucose (g)	Agar (g)	a_w	°Brix	pH
PDA[1]		19.8	125	125		0.870	56	3.6
PDA		19.8	120	120		0.880	53	3.6
PDA		19.8	115	115		0.890	50	3.6
Strawberry	100		46	46	5	0.870	57	3.89
Strawberry	100		43	43	5	0.880	54	3.98
Strawberry	100		40	40	5	0.890	52	3.87

[1]Potato Dextrose Agar.

7.2.4 Setting of O_2 concentrations at headspace

To set and assess low O_2 levels (<1%) inside 40 ml gas-tight-glass jars with metallic lids, a previously developed method based on the use of O_2 scavenger was used (Ch. 5). The O_2 concentrations in the headspace (%) were determined non-invasively by means of the OxySense® 200T (OxySense, Inc.,Dallas, TX) through a reader-pen device connected to Oxysense's Gen-III software.

7.2.5 Evaluation of N. fischeri time to visible growth (t_v)

In the first part of this study, a full factorial design experiment was used to assess the combined effect of a_w (sugar content), pasteurization intensity, O_2 and storage temperature on the time to visible growth (t_v, days) of *N. fischeri* on adjusted aPDA (see *§7.2.3*). Three levels were set for each factor: Temperature (10, 22 and 30°C), a_w (0.870, 0.880 and 0.890), headspace O_2 level (0.15, 0.40 and 0.80%) and pasteurization intensity (95°C, 100°C or 105°C/15sec), with three replicates (= three jars) at each level and the experiments were repeated twice (N=486). A validation on strawberry-medium was subsequently performed using conditions identified in the growth/no growth regions by inoculating the same isolate on strawberry-medium. Each part of this experiment is described in more details below.

7.2.6 Pasteurization treatments

Thermal pasteurization was performed in sterile heat-sealed capillary tubes, filled with 500µl of the adjusted spore suspensions (see *§7.2.2*). In order to standardize the initial temperature and reduce the come-up time, the inoculated tubes were immersed in a pre-heated water bath at 80°C for a few seconds. Subsequently, the tubes were fully immersed in a pre-heated oil bath for 15sec at 105 ± 1°C, 100 ± 1°C and 95 ± 1°C. After the heat treatment, the tubes were promptly cooled down in an ice-water bath. A reference tube containing a thermocouple placed at the approximate center of the heating medium was heated simultaneously with sample tubes to monitor the temperature profile in each experiment. After cooling, the content of each tube were aseptically transferred to sterile Eppendorf's tubes for further use as inoculum. In order to control the number of ascospores in the inoculum, 100µl of heat-treated ascospores were spread plated in duplicate on aPDA plates. Enumeration was performed after incubation for 3-5 days at 30 °C. Treatments carried out at 105±1°C and 100±1°C for 15sec resulted in ca. 2 log reduction in the concentration of ascospores suspensions, while treatments at 95±1°C resulted in ca. 1 log reduction (data not shown). Therefore, the initial concentration of ascospores suspensions prior pasteurization were set as previously described (see *§7.2.2*).

7.2.7 Inoculation and incubation conditions

It was observed in a preliminary experiment that dissolved O_2 levels (0.05-1.0%) in non-inoculated sugar-based and fruit-based media did not remain stable during storage at 10-30°C (unreported data). Based on these findings, the level of O_2 was only set and assessed in the headspace phase in this study. Therefore O_2 sensitive dots (Oxydot®, European Tech Serv NV, Belgium) were previously attached to the inner walls in the headspace area of one of the

three glass jars (replicates) to enable non-invasive measurement of the O_2 levels. The sterile jars were then filled with ten ml of adjusted aPDA until it solidifies. Subsequently, 10µL of heat treated ascospores (10-10^2 ascospores) was centrally inoculated on the media surface. The jars with inoculated media were immediately transferred to a temperature-controlled room (4 ± 1°C) to reach equilibrium concentrations and to avoid early germination of the ascospores. The O_2 levels were then set at 0.15, 0.40 and 0.80% in the headspace as described in §*7.2.4*. As soon as the desired O_2 level had been reached in the headspace (at 4°C), the jars were transferred to incubators adjusted to 10, 22 and 30°C.

7.2.8 Data collection

Fungal visible growth, i.e., formation of visible colonies with diameter $\geq$ *2 mm* at the surface of the agar was periodically determined for a maximum period of 90 days. Additionally, the a_w of media from non-inoculated jars was determined weekly to ensure a_w stability.

7.2.9 Validation in strawberry-based medium

In order to validate the results obtained on aPDA, 13 combinations of a_w and O_2 concentrations were selected and evaluated in triplicate on strawberry media (see *§7.2.3*), using the method described above. The growth/no growth conditions and the time before the first colony became visible on strawberry medium were then compared to those observed on aPDA.

7.2.10 Evaluation of N. fischeri time to visible growth in actual fruit purees

In the last part of the study, the effect of pasteurization (95°C and 105°C for 15sec) and O_2 concentration (0.05% and 1.0%) on the growth/no growth conditions and t_v of *N. fischeri* were evaluated in triplicate in actual fruit purees. Two types of purees were acquired from Belgium fruit processing companies: concentrated purees and single strength purees (= produced without water removal). The single strength fruit purees were evaluated at 22°C and comprised single fruits of strawberry, raspberry, sour cherry, mango, blueberry, kiwi and blackberry with a_w values which ranged from 0.98-0.99. The concentrated purees were evaluated at 30°C and comprised concentrates of single fruits of passiflora, rosheship, lemon, sour cherry, raspberry, blueberry, pineapple, orange, mango, aronia, black current, strawberry, lychee, apple and plum with a_w values which ranged from 0.85-0.86. The experiments were performed as previously described (see §*7.2.6* and §*7.2.7*) over an eight week (60 days) incubation period.

7.2.11 Statistical analysis

The data of t_v (days) (n=6) were described by a number of values including minimum, maximum, median, mean and standard deviation. Significant differences (at α=0.05) between t_v were determined by comparing their 95% CI's for overlap or lack thereof.

7.3 Results and Discussion

7.3.1 Effect of a_w, pasteurization intensity, headspace O_2 level and storage temperature on the time to visible growth of N. fischeri on aPDA

The effect of a_w (adjusted with 1:1 glucose/ fructose), pasteurization intensity, headspace O_2 level and storage temperature were evaluated on the time to visible growth of *N. fischeri* (*Nf*1) on aPDA over a 90 day incubation period. Three levels were evaluated for each factor based on previous studies (Ch. 4 and Ch. 6), which assessed the effects of a_w, O_2 and temperature and their proximity to the growth/no growth regions. The pasteurization treatments were chosen to mimic treatments commonly applied in the fruit processing industry.

The results regarding the growth/no growth conditions of *N. fischeri* at 22°C and 30°C are displayed in Fig. 7.1. The minimum, maximum, median and mean time to visible growth for the conditions showing growth as well as the number of replicate(s) showing growth are summarized in Tables 7.2. and 7.3. Overall, incubation temperature had a large effect on the ability of *N. fischeri* ascospores to germinate and grow out into visible colonies. At 10°C, no visible growth was observed in any of the conditions evaluated (results not shown). At an incubation temperature of 30°C, 8 of the 9 combinations of O_2 level and a_w evaluated showed growth, irrespective of the pasteurization intensity. At 22°C, on the other hand, visible growth was observed only in 2 to 4 of the 9 combinations of O_2 level and a_w evaluated, depending on the pasteurization intensity. Interestingly, the pasteurization temperature (all for 15 seconds) showed to have only a minor impact on the subsequent outgrowth. At 30°C this effect was negligible ($p>0.05$), whereby growth after all pasteurization treatments was only inhibited on media with a_w = 0.87, stored under 0.15% headspace O_2. When incubation was done at 22°C, outgrowth was much more limited in comparison to incubation at 30°C. Growth was not observed at 22°C after the most severe heat treatment was applied (105°C/15sec) in combination with a a_w reduction ($a_w \leq 0.880$), irrespective of the headspace O_2 level. It is known that milder pasteurization, i.e., at 75-85°C, will potentially result in activation of dormant ascospores of HRMs, instead of their inactivation (Zimmerman et al., 2011, Paula et al., 2006, Samapundo et al., 2018, Tournas, 1994). Nonetheless it should be noted that the few studies

that have evaluated the growth of *N. fischeri* only took into account the ascospore activation, whereby milder pasteurization intensities of 75°C-80°C/10-30min were commonly applied (Berni et al., 2017, Zimmerman et al., 2011). Information on the effects of high temperature and short time thermal processes, which are often applied in fruit processing industry, and are investigated here is not available in literature.

The evaluated a_w-values were close to the minimum a_w required for the growth of *N. fischeri* and correspond to high sugar contents (48-53°Brix). The a_w range evaluated was narrow, making it difficult to see a clear effect of this parameter. Hence careful analysis of the data was performed and the a_w values were determined to be 0.870±0.005, 0.880±0.005 and 0.890±0.005. At 30°C, the growth inhibition of *N. fischeri* was only observed when the a_w was reduced to 0.870 in combination with a very low headspace O_2 levels (0.15%). Conversely, this effect was more pronounced at 22°C, where mycelial growth was inhibited not only at a_w 0.87, but also at a_w 0.88 and 0.89 in combination with low headspace O_2 levels and/or high pasteurization intensities (Fig. 7.1). Regarding the effect of the headspace O_2 level, *N. fischeri* was able to grow at extremely low levels, even when this was combined with the other stress factors (heating and a_w). The exception was at the lowest level (0.15%), where no growth was observed when incubation was done at 22°C irrespective of the a_w or pasteurization intensity and at 30°C on media with a_w 0.870.

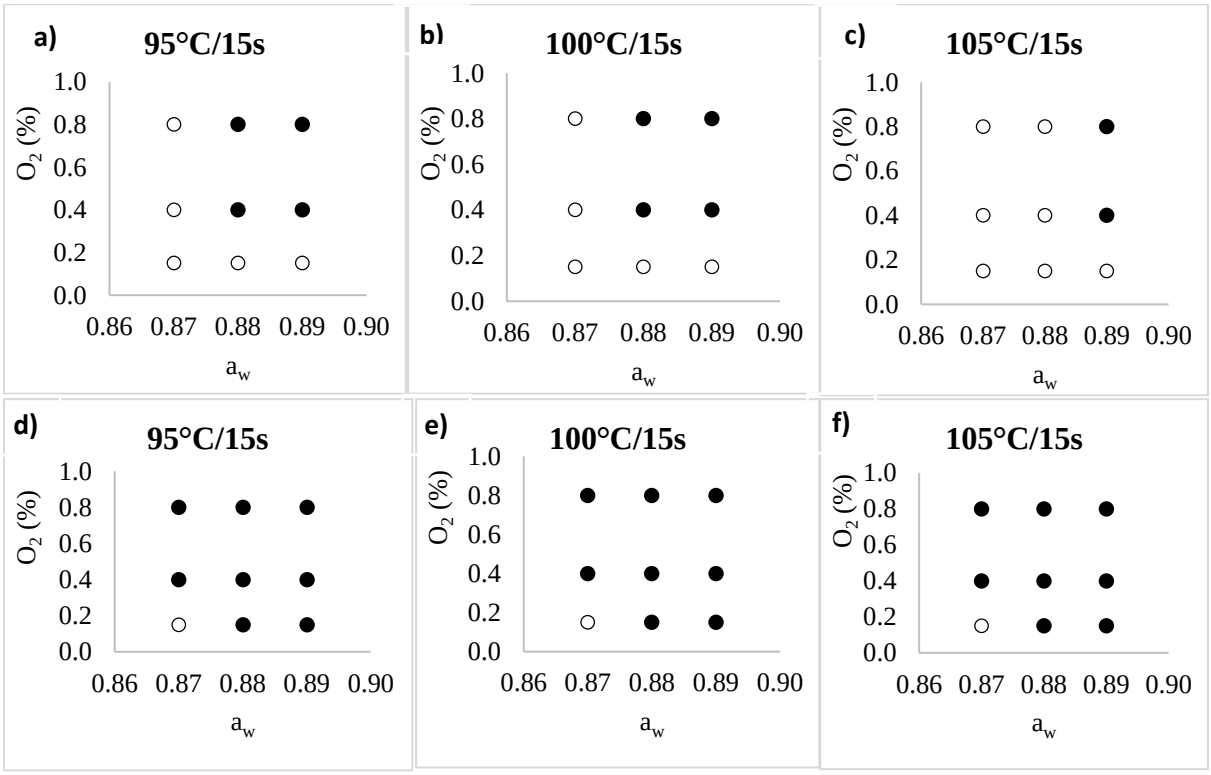

Figure 7.1. Growth/no growth regions of *N. fischeri* inoculated on aPDA as a function of a_w, headspace O_2 level (%), pasteurization intensity when stored at 22°C (a-c) or 30°C (d-f) for 90 days.

Each time to visible growth (t_v, days) (Tables 7.2. and 7.3) corresponds to the formation of at least one single colony in one replicate (=one jar). Each colony was assumed to have originated from one ascospore. Thus differences in t_v's are considered to arise from biological variability inherent to ascospores coming from the same population (= same inoculum) and/or experimental uncertainties. Data at 10°C are not shown as no growth was observed over the 90 day incubation period. As can be seen in Table 7.2, longer t_v's were observed at 22°C compared to 30°C ranging from 27 to 85 days respectively. At this storage temperatures, the shortest t_v was associated with the less stressful growth condition (a_w 0.890 and 0.80% headspace O_2), after the least intense pasteurization intensity evaluated (95°C/15sec). Under these conditions all the ascospores germinated and grew out into visible colonies. Although some of the ascospores germinated and grew after just 27 days at 22°C, the maximum and median t_v's for this condition were 67 and 54 days, respectively (Table 7.2) indicating a large variability probably due to presence of some sub lethally injured ascospores needing long times to recover before they germinate and grow out. On the other hand, the longest t_v observed at 22°C was associated with the most intense pasteurization applied (105°C/15sec), a_w=0.880, and a headspace O_2 level of 0.80% (Table 7.2). At a_w 0.890 no significant difference ($p>0.05$) was found between the t_v's as the pasteurization intensity and headspace O_2 concentration were increased (Table 7.2) while those effects were significant ($p<0.05$) when the a_w was reduced to 0.880. In addition, differences ($p<0.05$) were found between these two a_w-values as the conditions became less conducive for growth. These results confirm that a_w is an important parameter to be considered in preventing the growth of HRMs as small differences result in significant delay or inhibition of growth.

Table 7.2. Time to visible growth (t_v) of *N. fischeri* inoculated on aPDA as a function of a_w, headspace O_2 level (%) and heat pasteurization intensity when stored for up to 90 days at 22°C.

Pasteurization	O_2 (%)	a_w	N[1]	Time to visible growth (days)				
				min	max	median	mean	SD
95°C/15s	0.40	0.88	4/8	62	70	69	67[a]	3.3
		0.89	9/12	48	84	57	61[ab]	11.1
95°C/15s	0.80	0.88	6/10	51	84	61	64[ab]	10.1
		0.89	9/9	27	67	54	51[b]	11.9
100°C/15s	0.40	0.88	3/3	69	74	74	72[a]	2.4
		0.89	6/12	44	69	59	57[bc]	10.3
100°C/15s	0.80	0.88	3/6	61	61	61	61[b]	0.0
		0.89	3/6	36	60	60	52[ab]	11.3
105°C/15s	0.80	0.88	2/5	85	85	85	85[c]	0.0
		0.89	3/6	58	65	58	60[ab]	3.3

[1]Number of replicates where visible growth was observed out of total number of replicates. Once visible growth was observed the minimum, maximum, median, mean and standard deviation were determined. [a] Different superscript letter indicates where significant differences ($p < 0.05$) occur between time to visible growth from different conditions.

As previously mentioned, incubation at 30°C was more favorable than 22°C for the growth of *N. fischeri*. Overall, at that a_w the t_v's ranged from 10 to 86 days (Table 7.3). At the lowest a_w (0.870), no growth was observed after all the three pasteurization treatments when the headspace O_2 level was low (0.15) during incubation. The appearance of mycelia was delayed for more than one month when the headspace O_2 level was reduced to this level after pasteurization at 95°C/15sec and inoculation on media with a_w 0.880 and after 105°C/15sec and inoculation on media with a_w 0.880. Nevertheless, no statistical differences ($p>0.05$) were found between the t_v's as a function of the headspace O_2 concentrations evaluated in this study. A marked delay ($p>0.05$) in the t_v's (63-84 days) was also observed at 30°C after 100°C/15sec, a_w 0.870 and 0.40% ± 0.10 O_2 (Table 7.3). Shorter t_v's were mostly observed at the highest a_w-value evaluated (a_w 0.890), regardless the headspace O_2 level and pasteurization intensity. However, the t_v's were only significantly shorter ($p<0.05$) between a_w 0.890 and 0.870 after treatment of 95°C/15sec followed by incubation under a headspace O_2 level of 0.15% (Table 7.3). The highest variability was observed after a heat treatment of 105°C/15sec inoculation on media with a_w 0.870 incubation under a headspace with 0.8% O_2, which presented a very stressful condition. At 30°C, the effect of a_w was less pronounced and the t_v's were in general not significantly different ($p>0.05$) between the three a_w values evaluated (Table 7.3).

Temperature is one of the most important factors determining fungal growth and therefore the potential spoilage risk posed by ascospores. According to our results, the appearance of colonies of *N. fischeri* is not likely to occur before at least one month of incubation at 22°C within the a_w and headspace O_2 ranges evaluated. On the other hand, increasing temperature to 30°C will potentially increase the risk of germination and outgrowth of *N. fischeri*. Despite no visible growth being observed in this study at 10°C (after at most 90 days), previous investigation showed that *N. fischeri* is able to grow at refrigerated temperatures (≥10°C) when the other growth determining factors are optimal (Ch. 4). Accordingly, Tremarim et al. (2015) assessed the growth kinetics of *N. fischeri* in diluted apple juice (a_w 0.99) from 10°C to 30°C and observed lag times ranging from 19 days, at the lowest temperature, to less than one day, when the temperature was optimum for growth (30°C). The same authors observed that reduction of the temperature from 25-37°C to 15°C drastically reduced the growth rates.

Table 7.3.Time to visible growth (t_v) of *N. fischeri* inoculated on aPDA as a function of a_w, headspace O_2 level (%) and pasteurization intensity when stored for up to 90 days at 30°C.

				Time to visible growth (days)				
Pasteurization	**O_2 (%)**	**a_w**	**N[1]**	**min**	**max**	**median**	**mean**	**SD**
95°C/15s	0.15	0.88	6/6	44	62	54	53[a2]	7.4
		0.89	6/6	16	40	17	19[b]	3.3
95°C/15s	0.40	0.88	3/6	19	37	28	28[ab]	6.6
		0.89	9/9	14	48	26	30[ab]	11.7
95°C/15s	0.80	0.87	3/3	23	31	25	26[b]	3.1
		0.88	3/6	23	54	23	33[ab]	14.6
		0.89	9/9	16	55	30	32[ab]	16.4
100°C/15s	0.15	0.88	8/9	14	42	25	28[ab]	8.4
		0.89	2/6	22	45	34	34[ab]	11.5
100°C/15s	0.40	0.87	4/6	63	82	73	73[ab]	9.5
		0.89	6/9	15	42	34	32[ab]	10.2
100°C/15s	0.80	0.87	4/4	25	57	35	39[ab]	10.7
		0.88	3/3	14	23	18	18[b]	3.7
		0.89	6/6	10	36	15	19[b]	9.2
105°C/15s	0.15	0.88	5/9	37	86	52	57[ab]	18
		0.89	6/9	16	44	35	33[ab]	11.7
105°C/15s	0.40	0.87	4/9	26	55	37	39[ab]	10.5
		0.88	5/6	32	49	39	39[a]	6.3
		0.89	2/6	22	40	24	29[ab]	8.1
105°C/15s	0.80	0.87	6/6	16	83	23	40[ab]	27.6
		0.88	3/3	33	43	39	38[ab]	4.1
		0.89	3/3	13	16	16	15[b]	1.4

[1]Number of replicates where visible growth was observed out of total number of replicates. Once visible growth was observed the minimum, maximum, median, mean and standard deviation were determined. [2] Different superscript letter indicates where significant differences ($p < 0.05$) occur between time to visible growth from different conditions.

Even though *N. fischeri* growth may occur under headspace O_2 levels as low as 0.1% O_2, when combined with other stress sources, such as high sugar concentrations and storage temperature, it may potentially inhibit outgrowth. Despite the lack of information regarding the actual dissolved O_2 concentrations in heat treated fruit products, concentrations under 3% are relevant in controlled-atmosphere storage (Morales et al., 2007). These levels are commonly reached by vacuum packaging aiming to prolong the shelf-life. However, it is not assured that no residual O_2 remains in the food product during storage. This is even more important as the total amount of available O_2 comprises both the O_2 in the headspace and that dissolved in the matrix, which during storage may change due to food chemical reactions, biological activity and nature of the storage conditions (Pénicaud et al., 2010, Chaix et al., 2014, Bhunia et al., 2016). Moreover, when a limited amount of O_2 is available, slow growth rates are expected in addition to changes in the mycelial size and morphological characteristics of colony (Hull, 1939, Taniwaki et al., 2010, Yates et al., 1967). For example, Nielsen et al. (1989) observed that when *N. fischeri* was inoculated on Czapek Yeast Autolysate agar (CYA) and incubated under

0.1% O_2, very slow growth rates were obtained (1mm/day) compared to 1.01% O_2 (4mm/day) at 25°. Additionally, same authors observed that *N. fischeri* is able to produce mycotoxins such as fumitremorgins A and C and verruculogen at O_2 levels as low as 0.1%.

7.3.2 Validation in strawberry-based medium

Thirteen combinations of a_w, O_2, pasteurization and storage temperature were selected for the validation study performed on a strawberry-based medium with *N. fischeri*. The conditions, the growth/no growth outcomes and t_v's are shown in Table 7.4. The conditions which inhibited the growth of *N. fischeri* on aPDA also resulted in inhibition on strawberry medium. These conditions included, for example, combinations of single stressor such as low temperature (10°C) with less growth limiting conditions such as the highest a_w (0.890) and headspace O_2 (0.8%) levels and the lowest pasteurization intensity (95°C/15sec). Combinations of the lowest a_w (0.87) with low O_2 (0.15%) and/or high pasteurization intensities (105°C/15sec) effectively inhibited the growth *N. fischeri* in both aPDA and strawberry media for up to 90 days when incubated at 22°C. In difference to the results observed on aPDA, no growth was observed on strawberry medium in six of the conditions in which *N. fischeri* grew on aPDA. These were associated with reduced headspace O_2 levels (0.40-0.15%, see Table 7.4). Increasing temperature, a_w and/or headspace O_2 level resulted in shorter times (14- 44 days) to the visible growth of *N. fischeri* on aPDA, while these effects were less pronounced on strawberry medium (Table 7.4). From all the conditions were growth was observed on aPDA, only 33.3% resulted in visible growth on strawberry medium (after 50 - 64 days). Two of them were associated with the highest headspace O_2 level evaluated (0.8%) and the other one with the highest a_w (0.89) and the lowest pasteurization intensity (95°C/15sec). It is worthwhile mentioning that whilst visible growth occurred on both media under this condition, t_v of *N. fischeri* was much longer on strawberry medium (50 days) than it was on aPDA (14 days) (Table 7.4). The difference in inhibition of outgrowth of *N. fischeri* on strawberry medium can be attributed to its composition, such as the presence of organic acids and natural antioxidants (Campo and Santos, 2006, Huang et al., 2012, Wang et al., 1996). Strawberries are rich source of citric acid which is known to be antifungal (Amaeze, 2013, Campo and Santos, 2006). On the other hand, the presence of powerful antioxidants such as vitamin C and anthocyanin, may also have strongly contributed to the inhibition of *N. fischeri* growth by reducing or depleting available O_2, especially when the initial headspace O_2 was already low.

Table 7.4. Growth/no growth conditions of *N. fischeri* in sugar-based medium (aPDA) and fruit-based medium during 90 days.

Condition				Time to visible growth (days)[1]	
T (°C)	**a_w**	**O_2 (%)**	**Pasteurization**	aPDA	Strawberry media
10	0.89	0.80	95°C/15s	NG	NG
22	0.87	0.15	95°C/15s	NG	NG
22	0.87	0.80	105°C/15s	NG	NG
22	0.88	0.40	95°C/15s	62	NG
22	0.88	0.80	105°C/15s	85	60
22	0.89	0.15	105°C/15s	NG	NG
30	0.87	0.15	105°C/15s	NG	NG
30	0.87	0.40	95°C/15s	NG	NG
30	0.87	0.80	105°C/15s	16	64
30	0.88	0.15	95°C/15s	44	NG
30	0.88	0.40	105°C/15s	32	NG
30	0.89	0.15	105°C/15s	16	NG
30	0.89	0.15	95°C/15s	14	50

[1] Shortest time in which *N. fischeri* mycelia (D=2mm) was observed in at least one of the three replicates.

7.3.3 Evaluation of N. fischeri times to visible growth in actual fruit purees

This part of the study evaluated the effect of pasteurization intensity (95°C and 105°C/15sec) and headspace O_2 level (0.05% and 1.0%) on the growth/no growth and t_v of *N. fischeri* in actual fruit purees which are used as ingredients in the food industry. The a_w of the fruit purees were maintained at their original values i.e. single strength purees (a_w 0.98-0.99) and concentrated purees (a_w 0.85-0.86). Storage was performed at 22°C to simulate ambient temperatures. On the other hand, concentrated purees were also stored at 30°C, the optimum temperature for the growth of *N. fischeri*. It is important to mention that the a_w-values of these purees differed from the ones used to determine the growth/no growth conditions in the first part of this study. Nevertheless, both sets of data provide very valuable information regarding inhibition of the outgrowth of *N. fischeri*. With regards to the concentrated purees, no visible growth was observed during the first 30 days of incubation in any of the evaluated conditions on all fruit purees. As expected, the very low a_w-values (0.85-0.86) of these purees in combination with pasteurization and low headspace O_2 levels were sufficient to completely inhibit outgrowth. On the other hand, inoculation of *N. fischeri* in single strength purees (produced without water removal) resulted in outgrowth in almost all of the conditions evaluated (Fig. 7.2). Interestingly, the effect of the headspace O_2 level was very pronounced. Under 1.0% O_2, growth of *N. fischeri* was observed in all fruit purees within 1-5 weeks of incubation, regardless of the pasteurization intensity. Decreasing the headspace O_2 level to 0.05% significantly increased ($p>0.05$) the time required by *N. fischeri* to form visible colonies in almost all of the purees. The only exception was on blackberry puree which in general had the

shortest t_v's observed (1-2 weeks) after pasteurization at 95°C/15s, at both headspace O_2 concentrations. When pasteurization at 95°C for 15sec was combined with low headspace O_2 levels (0.05%), outgrowth of *N. fischeri* in strawberry, mango and kiwi purees occurred within 4-5 weeks. On the other hand, decrease in the headspace O_2 level from 1.0 to 0.05% resulted in inhibition of the outgrowth of *N. fischeri* in raspberry, sour cherry and blueberry for both pasteurization treatments. At the same time, increase in pasteurization intensity from 95 to 105°C/15sec in combination with a headspace O_2 level of 0.05% strongly limited the ability of *N. fischeri* to grow out. Under this condition, visible mycelia was only observed in strawberry puree after seven weeks of incubation at 22°C (Fig. 7.2).

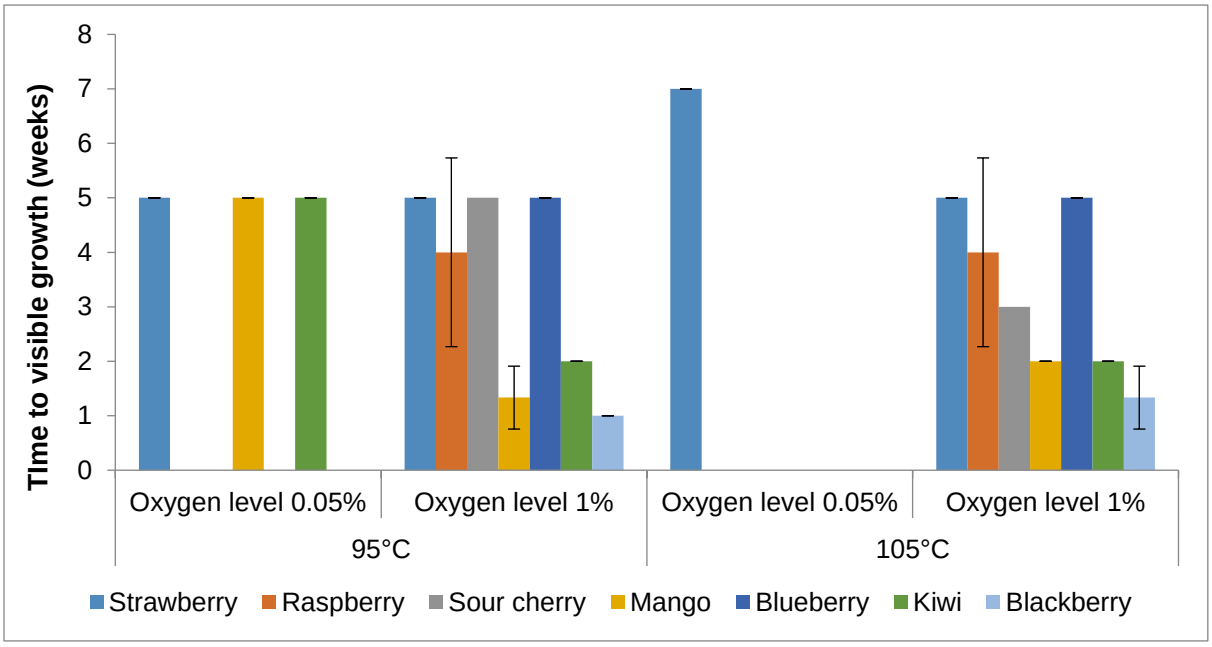

Figure 7.2. Effect of headspace O_2 level (%) and pasteurization intensity on the time to visible growth of *N. fischeri* in diverse fruit purees after storage at 22°C during 8 weeks.

Berries are known as good source of natural antioxidants such as vitamin A, C and E and anthocyanin's. When present, these compounds will potentially consume the available O_2, reducing the amount available for fungi in the food product. The presence of anthocyanin's in berries and their high antioxidant capacity may have contributed to the growth inhibition of *N. fischeri* in comparison to mango and kiwi purees. Moreover, the total antioxidant capacity (flavonoid and anthocyanidin) found in blueberries, blackberries and raspberries have been reported to be significantly higher (about 3-fold) than that of strawberries which may have accounted to the greater inhibition of *N. fischeri* on these purees (Skrovankova et al., 2015, He and Giusti, 2009, Lee et al., 2015, Olivas-Aguirre et al., 2016, Leong et al.,2002, Huang et al., 2012). Nevertheless, more studies are needed to assess the effect of different fruit products

(= fruit composition) on the dissolved levels of oxygen during time and on the HRMs growth inhibition.

As observed in our results, *N. fischeri* are reported to grow at extremely low O_2 levels when the other growth determining conditions (such as temperature, a_w) are optimal (Nielsen et al., 1989). Therefore, attempts to prevent the spoilage of high a_w fruit purees should not be entirely based on establishing low headspace O_2 levels. Rather, these attempts should be combined with other hurdles, such as storage at low temperature and application of high intensity pasteurization processes i.e. 105°C/ for at least 15 sec.

The data generated in this study (in both aPDA and fruit purees) are of practical importance for the fruit processing industry as the estimation of the time to visible growth (t_v) is strongly associated with rejection time by consumer (spoilage). Thus, this parameter provides very useful information about shelf-life prediction and extension of the t_v directly equates to longer mould-free shelf-lives. According to the results obtained in this study, two alternatives to use the data of this chapter may be suggested. Firstly, the t_v data can be included as stochastic data (range of values) in the prediction of HRM-free shelf-lives (as explained in chapter 4 and performed in chapter 8). Secondly, the t_v data can be taken into account as worst case scenario, which in this case would be the shortest t_v at each condition (combination of factors) evaluated. The last approach may result in an underestimation of the shelf-life because food products are commonly colonized by low number of spores. As our data allow including the variability of t_v, it is therefore suggested to incorporate them as stochastic values leading to information about the actual variability of shelf life of individual packages which has been reported to be highly variable (Dijksterhuis, 2017, Dagnas et al., 2017).

Lastly, it is worth noting that due to the high incidence of ascospores of HRMs in fruits and processed fruit products and their ability to withstand and form visible colonies after pasteurization, it would be expected that more spoilage incidents by the HRMs would be reported while this is to our knowledge not really the case. This may be explained by several reasons, such as the low level of ascospores contamination in real products (Ch.2) in comparison with the higher inoculum evaluated in this study, the non-uniform contamination distribution over the packages (units), the presence of natural preservatives i.e. organic acids and antioxidants, the heterogeneous distribution of ascospores inside fruit matrix and perhaps due to the low chance of observation by consumers of small colonies formed within the packed product.

7.4 Conclusions

Growth inhibition of HRMs in thermally treated fruit products is crucial to prevent their spoilage. The findings from this study demonstrated that the application of multiple stress factors (hurdles) is effective (necessary) in inhibiting the growth of *N. fischeri* in synthetic media, strawberry-based media and actual fruit purees. The results showed that the microbial stability of concentrated fruit purees is mainly due to their high sugar content (i.e. low a_w). However, when these purees are formulated in the growth/no growth region (a_w = 0.86-0.88), their stability for long periods will be highly dependent on the headspace O_2 level and storage temperature. Decreasing the storage temperature from 30°C to 22°C resulted in an extension of the t_v and pronounced increase of the no-growth region stressing the importance of temperature control even during ambient storage. The ability of *N. fischeri* to form visible colonies was impaired or delayed in a strawberry-based medium in comparison to aPDA with the same a_w value. The stability of high-a_w fruit purees (a_w≥0.980) is mainly based on their storage under chilled conditions (≤ 8°C) or by reaching extremely low O_2 concentrations in the headspace (≤0.15%) in combination with high pasteurization intensities, if the products are stored at ambient temperatures i.e., 22°C. Furthermore, the high content of antioxidants such as flavonoids and anthocyanins in some berries, may contribute to the delay or inhibition of the outgrowth of *N. fischeri*. Our findings showed that blueberry, raspberry and sour cherry purees did not support the growth of *N. fischeri* when the headspace O_2 level was reduced to 0.05%, regardless of the pasteurization intensity. With exception of strawberry puree, no visible growth was observed on all the high-a_w fruit purees inoculated with ascospores that had being heated at 105°C for 15sec and incubated with 0.05% O_2 at the headspace. The results provide very useful information that could be applied in establishing (predicting) mould-free shelf-lives of fruit based products such as fruit purees. Nevertheless, more studies taking into account the effect of multiple (sub-optimum) conditions on real fruit products are needed.

Chapter 8

Towards Practical Applications: a Spoilage Risk Assessment Modelling Approach

Redrafted after

Santos, J. L.P.; Membré, J-M, Samapundo, S.; Jacxsens, L.; Van Impe, J.; Sant'Ana, A. S.; Devlieghere, F., 2019. Risk assessment modelling of pasteurized fruit purees spoilage by heat resistant moulds (HRMs) (manuscript under preparation).

This chapter aims at transferring the obtained results of previous chapters into practical implications. Therefore, it introduces a "proof-of-concept" of a spoilage risk assessment approach for heat-resistant moulds (HRMs). The development of a risk spoilage model of pasteurized fruit purees by HRMs was obtained by aggregating results from the various previous chapters and by making an attempt of generating and interpreting scenarios. Firstly, an overview of the risk model and an explanation of each step are provided (*see 8.1.*). These are followed by a description of scenarios and the software used for run simulations (*see 8.2.*) Next, the outcomes (=spoilage risk) obtained from the scenarios simulations are elaborated (*see 8.3*). Lastly, a general discussion of this PhD is provided (*see 8.4.*).

8.1 Development of the risk spoilage model

8.1.1 Overview of the development of the risk spoilage model

This study aimed to quantify the spoilage risk inherent of Heat-Resistant-Moulds (HRMs) in packaged fruit purees under various scenarios regarding formulation, processing and storage. The risk model was focused on three types of fruit purees: strawberry puree concentrate (SP_C, i=1) and single strength strawberry puree (SP_{SS}, i=2) both contaminated with *Neosartorya fischeri* strains and aimed for use as a food ingredient, and apple puree (AP, i=3) contaminated with *Byssochlamys nivea* strains intended for direct consumer use. The general model comprises three main steps: (1) Initial contamination level of raw material (fruits) by ascospores, (2) thermal inactivation of ascospores and (3) germination and formation of visible mycelia by the ascospores during storage. The model inputs comprise original data collected in fruit puree processing industries as well as data obtained in the previous chapters. Additionally, the following assumptions were considered: (i) there is no post contamination after pasteurization, (ii) the raw material (apples and strawberries) are not treated with sanitizers (e.g. chlorine dioxide) during washing prior processing. The estimated spoilage risk of the fruit purees was associated with the ability of HRMs ascospores form visible mycelia ($D = 2\,mm$) during storage inside "Packs" which refer to 100g-portions for fruit puree used as an ingredient, and 100g-packs for fruit puree intended to direct consumer use. The general framework for the risk model is depicted in Figure 8.1.

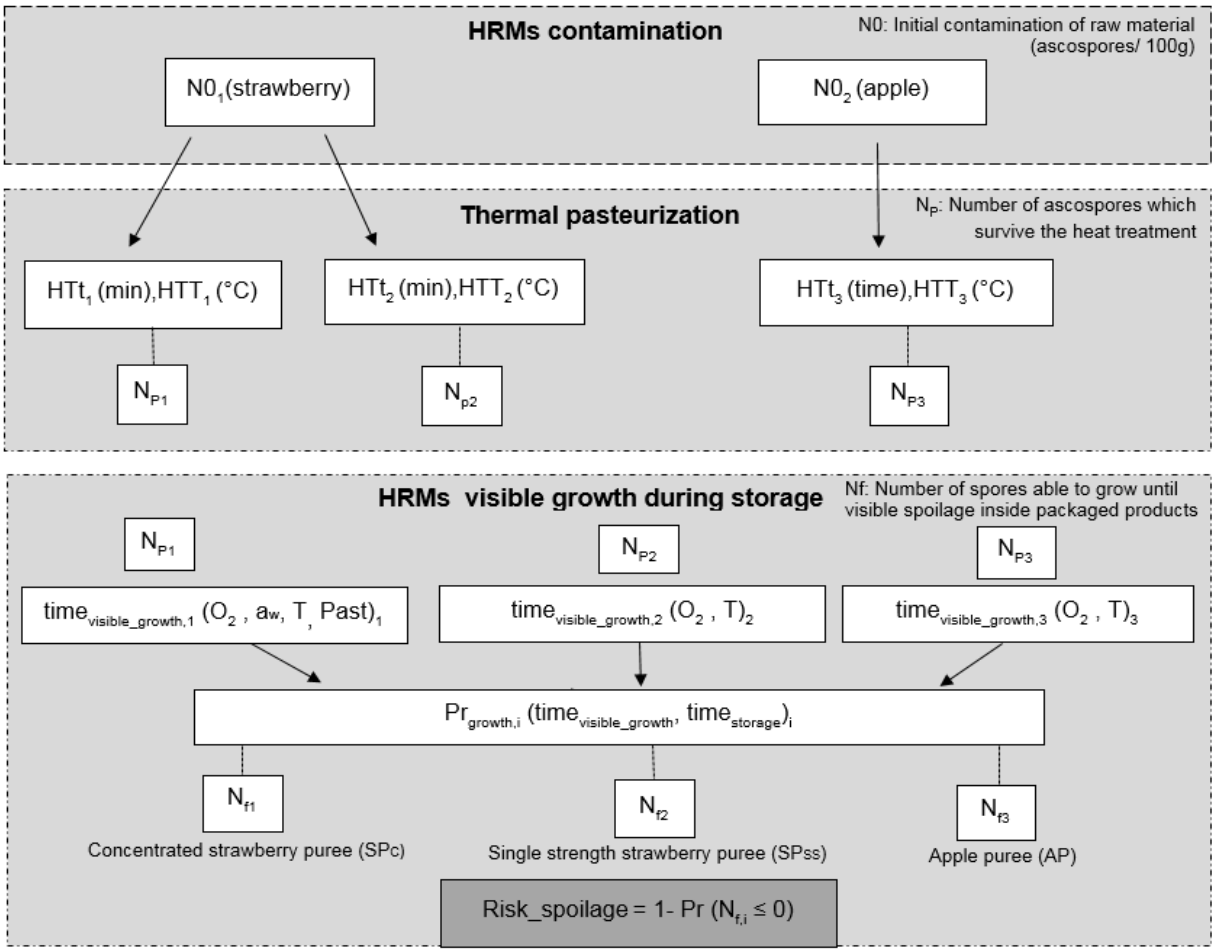

Figure 8.1 Flowchart of the process by which the model for predicting the risk of spoilage of 100g-packs of fruit purees was developed. Bracketed squares delineate the model three steps: (i) the initial ascospore contamination level ($N0_i$, ascospores/100g) in the raw materials (fruits); (ii) determination of number of ascospores which survive thermal pasteurization (N_{pi}) and (iii) determination of the number of ascospores which are able to survive the heat treatment and subsequently form visible mycelia ($D \geq 2$mm) during storage (N_{fi}).

8.1.2 Initial contamination levels of raw material ($N0_i$)

The data of initial contamination of *N. fischeri* in strawberries (n=29) and *B. nivea* in apples (n=52) previously described in Ch. 2 were used to represent the initial levels of ascospores in these products before processing. Statistical distributions (Gamma, Lognormal, Weibull and Exponential) were fitted to the non-censored (≥1/100g) and censored contamination data (0< ascospores/100g <1) in R *3.5.2* by means of the package fitdistrplus version 1.0-14 through the functions descdist, fitdist, bootdist, fitdistcens and bootdistcens. Bootstrapping was used to quantify the uncertainty on the cumulative distributions functions (CDFs). The goodness-of-fit was evaluated visually and by means of Loglikelihood obtained as follows: −2lnL with L the likelihood in its optimum and, by Akaike's information criterion AIC. As lower the AIC better the approximation of the model to the experimental dataset (AIC=−2 ln L+2 k, with k the number

of parameters in the fitted model). The (best fitting) distributions that were eventually used in the first step of developing the risk models are shown in Table 8.1.

8.1.3 Inactivation of the ascospores during thermal processing and determination of number of ascospores which survive pasteurization (Np_i)

After the fruits (raw materials) are sieved, grinded and milled into fruit purees, they are subjected to a thermal treatment (pasteurization). The intensity of the pasteurization applied depends on the type of fruit and nature of the desired puree (single strength or concentrated). In this study two groups of purees are represented regarding their heating sensitivity. The pasteurization treatments commonly applied to strawberry purees range from 85°C to 95°C from 15sec to 1min. While more intense heat treatments may be applied to apple purees ranging from 95 °C to 105 °C for few seconds. The differences between these matrices are mainly due to sensorial qualities (e.g. color) that need to be preserved after heating. Data of inactivation kinetics from *B. nivea* (Bysso nivea 76-1) and *N. fischeri* (Neosar fisheri 95-1) previously obtained in Ch. 3 and Appendix, respectively, were used in this study. The heat resistance parameters, $p_{iT°C}$, δ_i, were estimated from the Weibull model (Mafart et al., 2001) (Eq. 8.1) from data obtained in acidified buffer glucose solution (pH = 3.6, 13°Brix) at 85°C, 90°C and 93°C (Table 8.1), while $log\delta_{ref,i}$ and $Z_{T,i}$ were obtained from Eq. 8.2. All the parameters were estimated by means of the package nlstools version *1.0-2* available in R. The parameter $p_{iT°C}$, describes the survival curve shape, i.e., n<1= curves which concave upwards (tails), n>1 = curves which concave downwards (shoulders) and n=1 corresponds to log-linear curves at temperature $T°C$; $logN_i$ corresponds to the log of ascospores population (cfu/ml) at time t_i (min); $logN0_i$ is the log of the initial population of ascospores (cfu/ml); $\varepsilon\ (\mu, \sigma)_i$ stands for normal distribution of Root Mean Square Error (RMSE) as follow: Normal (Pack, 0, RMSE); δ_i is the time (min) for the first decimal reduction following a non-linear equation; HTT_i is the temperature of the thermal treatment; $Tmean,i$ is the mean temperature (°C) calculated from kinetic curves at 85°C (n=3), 90°C (n=3) and 93°C (n=3); $log\delta_{ref,i}$ stands for the time for the first log reduction at $Tmean,i$ and $Z_{T,i}$ is the temperature (°C) required to obtain a tenfold reduction of δ-value. The index i:1→3 stands for concentrated strawberry puree (SP_c), single strength strawberry puree (SP_{SS}) and apple puree (AP), respectively.

$$logN_i = logN0_i - \left(\frac{t_i}{\delta_i}\right)^{p_{iT°C}} + \varepsilon\ (\mu, \sigma)_i \qquad \text{(Eq. 8.1)}$$

$$log\delta_i = log\delta_{ref,i} - \frac{(HTT_i - Tmean,i)}{Z_{T,i}} \qquad \text{(Eq. 8.2)}$$

The number of ascospores (Np_i) surviving the heat treatment at ($HTT_i, °C$) was described by a Poisson distribution (Eq. 8.3), where $N0_i$ stands for the number of ascospores initially present in the raw material and $Pr_{ascospore,i}$ the probability of one ascospore to survive the heat treatment as proposed by Nauta, 2001 (Eq. 8.4). The Poisson distribution was used to simplify a binomial distribution suggested by Nauta (2001) and Membré and Valdramidis (2016).

$$N_{p_i} = Poisson\ (N0_i \cdot Pr_{ascospore,i}) \quad \text{(Eq. 8.3)}$$

$$Pr_{ascospore,i} = 10^{(-\frac{t_i}{\delta_i})^{p_i}} \times 10^{\varepsilon\,(\mu,\sigma)_i} \quad \text{(Eq. 8.4)}$$

8.1.4 Determination of number of ascospores which are able to survive heat treatment and form visible mycelia during storage (N_{fi})

The population of ascospores (N_{fi}) which survive the heat treatment at (HTT_i) and are able to form visible mycelia during storage time ($time_{storage,i}$) was determined by a Poisson distribution ($N_{p_i} \cdot Pr_{growth,i}$) (Eq. 8.6), where N_{p_i} stands for the number of ascospores which survive to pasteurization (t_i, HTT_i) and $Pr_{growth,i}$, the probability of one ascospore to germinate and present visible growth (Eq. 8.5). The input $time_{visible\ growth,i}$ came from the data previously obtained in Ch. 4, 6 and 7. This variable was described as parametric or non-parametric distributions according to the sample size and study performed. For instance, the data obtained in Ch. 6 and 7 (n=6) has been expressed as uniform or discrete distribution, while the data from Ch. 4 (n=100) has been used in the form of lognormal and normal distributions. In all cases the parameter time (days) to visible growth stands for the time when HRMs visible mycelia was observed by the naked eye ($D = 2\text{mm}$) (Zimmerman et al., 2013).

$$Pr_{growth,i} = time_{visible\ growth,i} < time_{storage,i} \quad \text{(Eq. 8.5)}$$

$$\begin{matrix} 0 & if\ time_{visible\ growth,i} > time_{storage,i} \\ 1 & if\ time_{visible\ growth,i} < time_{storage,i} \end{matrix}$$

$$N_{fi} = Binomial\ (Np_i, Pr_{growth,i}) \quad \text{(Eq. 8.6)}$$

8.1.5 Storage of fruit purees

After pasteurization, strawberries purees are cooled and packed in bags-in-boxes, bags-in-drums, drums or tins and stored frozen, chilled or at ambient temperatures for 2 to 24 weeks, depending on the storage conditions. Pasteurized apple purees are usually hot filled in 100g-plastic cups and/or in laminated polyester bags (pouches) followed by cooling (to 25-40°C)

and storage at chilled or ambient temperatures. Apple purees have shelf-lives ranging from 6-18 months.

8.1.6 Spoilage risk estimation during storage

According to the probability theory, the cumulative distribution function F(x) gives the probability that the variable X is less than or equal to x (Vose, 2008): F(x)=P(X ≤ x). Consequently, the percentage (%) of spoilage packs (or 100 g portions) considering to have at least one ascospore capable of growing out during storage was calculated using Eq. 8.7, with N_{fi} being the quantity of visible mycelium in a 100 g pack calculated in Eq. 8.6. Ultimately the number of risky packs of the fruit purees was estimated by multiplying the $\mathrm{Risk\ spoilage}_i$ by the total packs produced (Eq. 8.8).

$$\mathrm{Risk\ spoilage}_i = 1 - \mathrm{F}(0) = 1 - \Pr(N_{fi} \leq 0) \quad \text{(Eq. 8.7)}$$

$$\mathrm{Risky\ packs}_i = \mathrm{Risk\ spoilage}_i \; x \; Packs \quad \text{(Eq. 8.8)}$$

8.1.7 Model software and simulations

The model was developed in R software version *3.5.2*. Uncertainties regarding occurrence (distributions), inactivation (RMSE from predictive models) and growth (parametric and non-parametric distributions) were taken into account in the risk prediction. Each scenario was simulated three times with 10^4 iterations (= number of 100g packs). After each simulation the number of 'risky' packs were estimated. Thus, the outcomes comprise the number of 100g-packs/portions of fruit purees on a batch of 10^4 units contaminated with one ascospore capable of forming visible colonies during storage (= number of packs that will be spoiled). After each simulation the number of 'risky' packs were estimated.

Table 8.1 Parameters and distributions used in the spoilage risk assessment of fruit purees.

Parameters	Description	Unit	Value/Distribution/Model	Reference
Packs	Number of 100g-pack of fruit puree	100g	10^4 (= n iterations)	
$N0_1$	Incidence of *N. fischeri* ascospores on strawberries	ascospores/100g	Lognormal(0.66,1.623)	Chapter 2
$N0_2$	Incidence of *B. nivea* ascospores on apples	ascospores/100g	Lognormal(-2.05,2.68)	Chapter 2
HTT_i	Temperature of the thermal treatment	°C	[85, 90, 95, 100, 105]	Chapter 3, Company info
t_i	Duration of the thermal treatment	min	0.25-1.0	Chapter 3, Company info
$T_{mean(Nf)}$	Mean temperature (calculated from kinetic curves)	°C	89.3	Chapter 3, Appendix
$T_{mean(Bn)}$	Mean temperature (calculated from kinetic curves)		90.1	
$\delta_{ref(Nf)}$	*N. fischeri heat resistance* at $T_{mean(Nf)}$	min	2.48	Equation 8.1
$\delta_{ref(Bn)}$	*B. nivea* heat resistance at $T_{mean(Bn)}$		0.05	
Z_{T1}	Thermal death of *N. fischeri*	°C	4.5	Equation 8.2
Z_{T2}	Thermal death of *B. nivea*		5.8	
$p_{iT°C}$	Shape parameter from inactivation curves at HTTi		$p_{iT°C}$	Equation 8.1, Appendix
ε_i	Root Mean Square Error (RMSE) from inactivation models		Normal(Pack, 0, $rmse_i$)	Equation 8.1
$Pr_{ascospore,i}$	Probability for one ascospore to survive the pasteurization	%		Equation 8.4
N_{pi}	Ascospores which survived pasteurization	ascospores/Pack	Binomial ($N0_i$,$Pr_{ascospore,i}$)	
N_{fi}	Ascospores which survived pasteurization and form visible growth	ascospores/Packs	Binomial (N_{p1},$Pr_{growth,i}$)	
T_i	Storage temperature	°C	8-30	Chapter 4 and 7
$time_{storage,i}$	Storage time before use (SP_c ,SP_{SS}) or consumption (AP)	days	≤90	Company info
$A_{w,\,i}$	a_w of the fruit puree		0.850-0.980	Company info
$O_{2,\,i}$	Oxygen level in the headspace of fruit puree pack	%	0.00-21.0%	Chapters 6, 7
$time_{visible\ growth,i}$	Time required for one ascospore form visible colony	days		Chapters 4, 6, 7
$Pr_{growth,i}$	Probability of one ascospore to germinate and present visible growth	%		Equation 8.5
$Risk\_spoilage_{,\,i}$	Spoilage risk of n packs during storage	%	(Nf_i /Packs)	

8.2 Scenario analysis

Three scenarios regarding processing, formulation and/or storage condition were simulated for each type of fruit puree (n=9 scenarios). These where selected according to the available data, industrial interest and outcomes obtained. The scenarios, parameters, distributions and media in which growth data were collected are depicted in Table 8.2. A brief description of each scenario is described below.

Concentrated strawberry puree (SPc)

Scenario 1: Increasing pasteurization intensity

The effect of pasteurization temperature (85°C, 90°C, 95°C, 100°C) and time (15sec, 30sec, 45 sec, 60sec) were evaluated on the production of SP_C with a_w=0.880, 0.8% O_2 in the headspace, stored for 90 days at 22°C.

Scenario 2: Storage time and temperature

The effect of storage time (30, 60 and 90 days) and temperature (10°C, 22°C and 30°C) were evaluated on the spoilage risk of SP_C with a_w=0.880, 0.8% O_2 at the headspace after pasteurization at 95°C/15sec.

Scenario 3: Decreasing Aw and O_2 in the headspace

The effect of a_w (0.86,0.87,0.88) and headspace O_2 level (0.00, 0.15, 0.40 and 1.00%) were evaluated on the spoilage risk of SP_C after pasteurization at 95°C/15sec and storage at 30°C for 60 days.

Single strength strawberry puree (SPss)

Scenario 4: Increasing pasteurization intensity

The effect of pasteurization temperature (85°C, 90°C, 95°C, 100°C) and time (15sec, 30sec, 45 sec, 60sec) were evaluated on the spoilage risk of SP_{SS} with a_w=0.985, 21% O_2 in the headspace (atmospheric air), stored for 30 days at 22°C.

Scenario 5: Storage time and temperature

The effect of storage time (10, 20 and 30 days) and temperature (8°C, 10°C, 12°C, 14°C) were evaluated on the spoilage risk of single strength strawberry purees (SP_{SS}) with a_w=0.985, 21% O_2 in the headspace (atmospheric air) after pasteurization at 95°C/15sec.

Scenario 6: Decreasing oxygen in the headspace

The effect of headspace O_2 level (0.00, 0.05 and 1.00%) as function of storage time (28 days, 42 days, 56 days) were evaluated on the spoilage risk of single strength strawberry purees (SP_{SS}) with a_w=0.985, after pasteurization at 95°C/15sec and storage at 22°C for 60 days.

Apple puree (AP)

Scenario 7: Increasing pasteurization intensity

The effect of pasteurization temperature (85°C, 90°C, 95°C, 100°C) and time (15sec, 30sec, 45 sec, 60sec) were evaluated on the spoilage risk of AP with a_w=0.985, 1.0% O_2 in the headspace, stored for 30 days at 22°C.

Scenario 8: Storage time and storage temperature

The effect of storage time (30, 60 and 90 days) and storage temperature (10°C, 12°C, 14°C, 22°C) were evaluated on the spoilage risk of AP with a_w=0.985, 21% O_2 in the headspace (atmospheric air) after pasteurization at 95°C/15sec.

Scenario 9: Decreasing headspace O_2 level

The effect of the headspace O_2 level (0.00, 0.03, 0.15 and 1.00%) as function of storage time (14, 28, 42 days) were evaluated on the spoilage risk of AP with a_w=0.985, after pasteurization at 95°C/15sec and storage at 22°C.

Table 8.2 Description of scenarios (processing, formulation, storage) parameters and distributions for estimation of spoilage risk (% number of spoilage packs) of fruit purees by heat-resistant moulds.

nr	Scenario Description	Parameters									Reference
		HTT_i	HTt_i	$p_{iT°C}$	$a_{w,i}$	Headspace $O_{2,i}$	T_i	$time_{visible\_growth,}$ (days)	$time_{storage,i}$ (days)	Media	
Concentrated strawberry puree (SP_C)											
1	Increasing pasteurization intensity	85 90 95 100	0.25-1.00	1.60 0.97 0.58 0.58	0.88	0.8	22	Discrete({51,60,61,67,84},{1,1,2,1,1})	≤90	aPDA	
2	Storage time and temperature	95	0.25	0.58	0.88	0.8	10 22 30	No Growth Discrete({51,60,61,67,84},{1,1,2,1,1}) Discrete({23,54},{2,1})	30,60,90	aPDA	
3	Decreasing a_w and headspace O_2	95	0.25	0.58	0.86	0 0.15 0.4 0.8	30	No Growth	≤60	aPDA	Chapters 2, 7 and Appendix
					0.87	0 0.15 0.4 0.8	30	No Growth No Growth Discrete({19, 30, 37},{1,1,1}) Discrete({23,25,28,31},{2,1,1,1})			
					0.88	0 0.15 0.4 0.8	30	No Growth Discrete({44,54,62},{1,1,1}) Discrete({19,25,30,37},{1,1,1,1}) Discrete({23,54},{2,1})			
Single strength strawberry puree (SP_{SS})											
4	Increasing pasteurization intensity	85 90 95 100	0.25-1.00	1.60 0.97 0.58	0.985	21	22	Discrete({5,6,8)},{1,3,2})	≤30	Strawberry	Chapters 2, 6 and Appendix
5	Storage time and temperature	95	0.25	0.58	0.985	21	8 10 12 14	No Growth Lognormal(3.12,0.13) Lognormal(2.68,0.27) Lognormal(2.18,0.09)	10,20,30	aPDA	Chapters 2, 4 and Appendix
6	Decreasing headspace O_2	95	0.25	0.58	0.985	0 0.05	22	No Growth Uniform(36,52)	28,42,56	aPDA Strawberry	Chapters 2, 6 and 7

						1		Uniform(10,36)			
Apple puree (AP)											
7	Increasing pasteurization intensity	85 90 95 100	0.25-1.00	0.35 0.26	0.985	1	22	Uniform(4,5)	≤30	aPDA	Chapters 2, 3 and 6
8	Storage time and temperature	95	0.25	0.26	0.985	21	10 12 14 22	No Growth Normal(40.02, 7.19) Normal(27.58,5,71)	30,60,90	aPDA	Chapters 2, 3 and 4
9	Decreasing headspace O_2	95	0.25	0.26	0.985	0 0.03 0.15 1	22	No Growth Discrete({18,19,23,25,30},{1,1,1,2,1}) Uniform(5,6) Uniform(4,5)	14,28,42	aPDA	Chapters 2, 3 and 6

8.3. Model outcomes: Spoilage risk prediction of fruit purees

Fig. 8.2 – 8.4 show the estimated risks (number of risky packs per 10^4 units of produced packs) for each for concentrate strawberry puree (SP_C) being potentially spoiled by *N. fischeri* in three different scenarios (scenarios 1, 2 and 3). In the first scenario, the effect of various pasteurization intensities was determined. A significant percentage of risky packs, roughly 67% of SP_C (a_w =0.88) after receiving a mild pasteurization of 85°C for 15sec to 1min and being stored for three months at 22°C was estimated. When the pasteurization temperature was increased to 90°C and applied for 1 min, the risk was decreased by only 40% (3998 ± 55 risky packs). A practically significant reduction of the risk was only predicted to occur when the pasteurization intensity was increased to 95°C for 45 sec or more (59 ± 4 risky packs). The application of very intensive heating, such as 100°C for 15 sec, seems to reduce the risk to 0% (Fig. 8.2). Taking these results into account, the simulations for the other two scenarios evaluated for SP_C were run considering pasteurization at 95°C for 15sec. This treatment was selected because it represents what is commonly applied by fruit processors and because it enabled the estimation of the potential risk reductions looking as function of other factors and variables. The second scenario was intended to assess the effect of storage conditions (time and temperature) on the spoilage risk of SP_C (Fig. 8.3). The storage temperature played an important role on the estimated spoilage risk level. When the simulation was performed at 30°C, which is the optimum temperature for growth of the HRMs, the number of risky packs were 787 ± 27 (7.8%), 1176 ± 34 (11.7%) and 1198 ± 30 (11.9%) after 30, 60 and 90 days of storage, respectively. Decreasing the storage temperature to 22°C drastically reduced the number of risky packs stored after 30 days to 0% (0±0) and to 1.99% after 60 days (199±10). However, for SP_C stored for long periods, i.e., 90 days or longer, no significant reduction was observed between these two storage temperatures (Fig. 8.3). Conversely, storage of SP_C at temperatures ≤10°C resulted in complete inhibition of the outgrowth of the ascospores of *N. fischeri* (spoilage risk=0 %) (Fig. 8.3). The last scenario run for SP_C aimed to evaluate the effect of reducing two important parameters, a_w and headspace O_2 level (%) (Fig. 8.4). The three a_w values assessed belong to the growth/no growth region for *N. fischeri* as determined in Ch. 7. By reducing the a_w to ≤ 0.86, complete growth inhibition (no risk of spoilage) was observed, regardless of the initial headspace O_2 level within the pack. Nevertheless, small differences of a_w within this range may drastically change the risk. When, for instance, the a_w was increased to 0.880, for instance, the number of risky packs highly increased to 1177 ± 33 (11.7%), 1152 ± 13 (11.5%), 786 ± 12 (7.9%) and 0 ± 0 (0%) at headspace O_2 levels of 0.80%, 0.40%, 0.15% and 0.0%, respectively. For SP_C with a_w = 0.870, decreasing the oxygen from 0.40% to 0.15% resulted in complete risk reduction (Fig. 8.4).

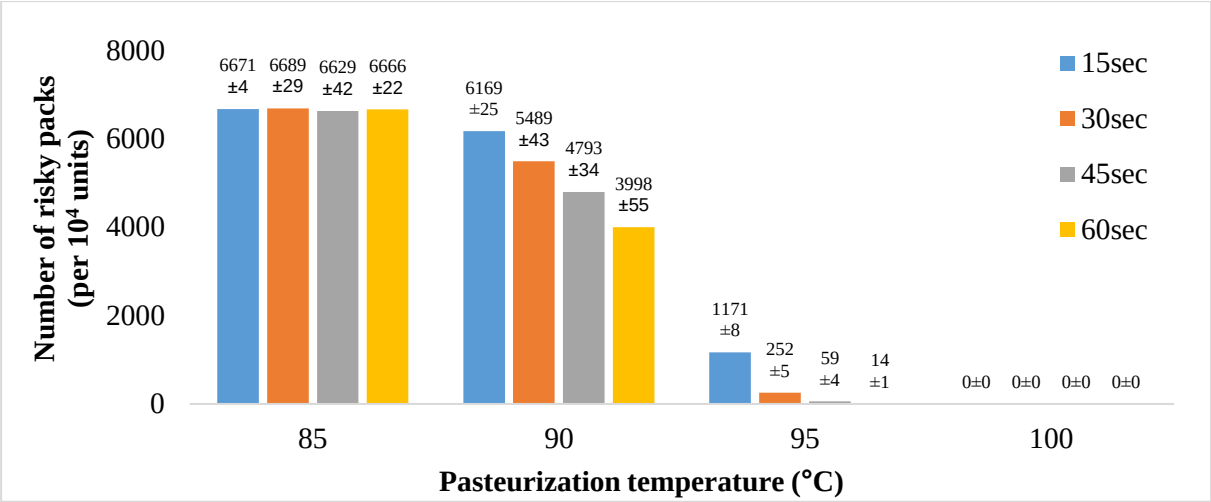

Figure 8.2. **Scenario 1** – Simulation of the effect of increasing pasteurization intensity (time and temperature) on the spoilage risk of concentrated strawberry purees with a_w=0.880 (53°Brix), 0.8% O_2 in the headspace, stored for 90 days at 22°C.

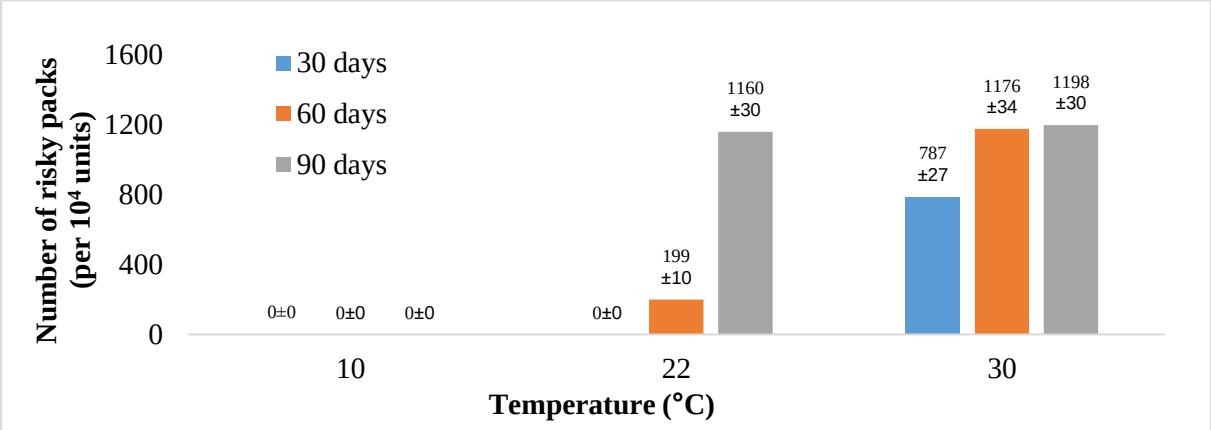

Figure 8.3. **Scenario 2** – Simulation of the effect of increasing storage time (30, 60 and 90 days) and temperature (10°C, 22°C and 30°C) on spoilage risk of concentrated strawberry purees with a_w=0.880, 0.8% O_2 in the headspace after pasteurization at 95°C/15sec.

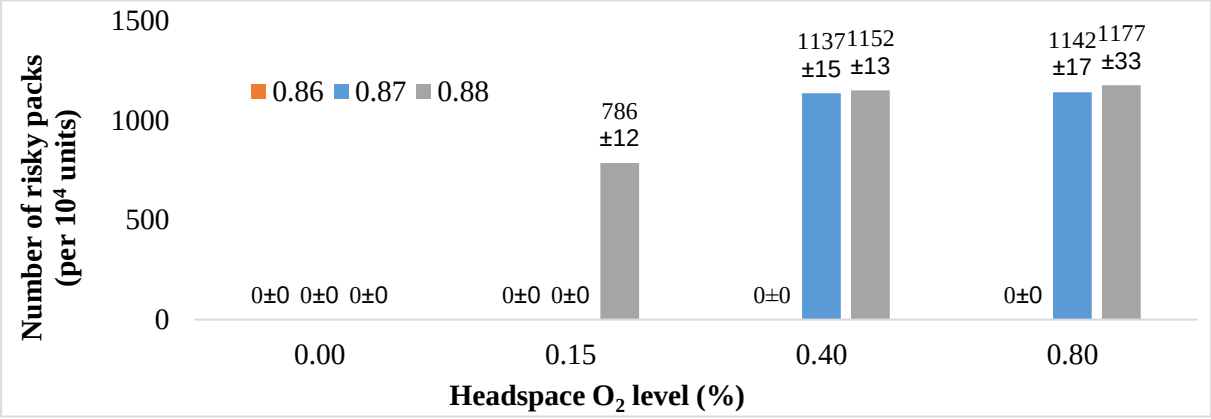

Figure 8.4. **Scenario 3** – Simulation of the effect of a_w (0.86,0.87,0.88) and headspace O_2 level (0.00, 0.15, 0.40 and 0.80%) on the spoilage risk of concentrated strawberry purees, after pasteurization at 95°C/15sec and storage at 30°C for 60 days.

The scenarios run for estimating the spoilage risk of SP_{SS} are displayed in Fig. 8.5 – 8.7 (scenarios 4, 5 and 6). In scenario 4, the effect of pasteurization intensity was assessed in the same manner of concentrate purees (Fig. 8.5). Likewise SP_C, high probabilities of risky packs (≥40%) were obtained after milder pasteurization treatments (85 and 90°C /15sec-60sec) when SP_{SS} was stored at 22°C for up to 30 days. The spoilage risk was only effectively reduced by applying pasteurization temperatures ≥ 95°C for 45sec or more (Fig. 8.5). The risk was eliminated (0%) when pasteurization was done at temperatures ≥ 100°C. The effect of storage conditions (time and temperature) on the spoilage risk of SP_{SS} is shown in Fig. 8.6 (scenario 5). In contrast to SP_C, lowering the storage temperature to 10°C did not result for SP_{SS} in a spoilage risk=0%. Rather, at this temperature, the number of risky packs increase as longer is the storage time. For instance, when SP_{SS} were stored for 10 days, the number of estimated risky packs was 0, while this number increased to 206 ± 16 and 1154 ± 45 after being stored for 20 and 30 days, respectively (Fig. 8.6). No spoilage risk was observed only when SP_{SS} was stored at ≤ 8°C for up to 30 days. Storing the purees at 12°C and 14°C for more than 20 days will result in a high number of risky packs (≥ 10%). However, when they were stored at 12°C for a short period (10 days), the risk was reduced from 10% to 0.9% (Fig. 8.6). In scenario 6 (Fig. 8.7), the effect of reducing the headspace O_2 level and storage time on the spoilage risk of SP_{SS} stored at 22°C for up to 60 days was evaluated. As previously reported for SP_C, the total depletion of oxygen (0.0%) at the headspace resulted in no risk of spoilage for SP_{SS}. On the other hand, the presence of a small residual oxygen content (0.05%) in the headspace seems to strongly affect the risk spoilage of such purees. However, this risk depends a lot on the storage time. 0±0, 442± 18 and 1176 ± 20 risky packs were estimated when SP_{SS} with 0.05% oxygen in the headspace were stored at 22°C for 28, 42 and 56 days respectively (Fig. 8.7). Increasing the oxygen to levels ≥ 1.0% results in high probability of spoilage (> 8%) when SP_{SS} are stored for periods of ≥28 days.

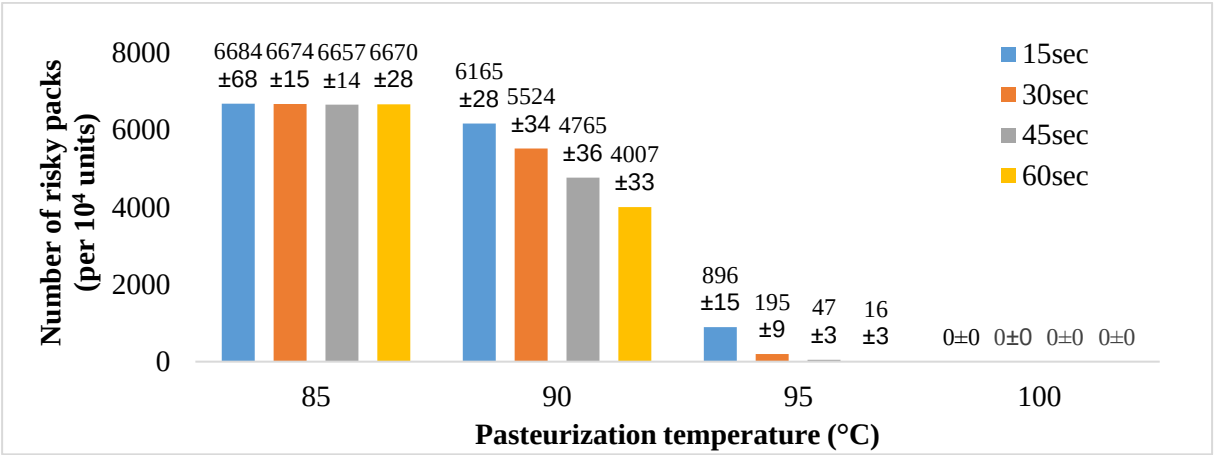

Figure 8.5 **Scenario 4** – Simulation of the effect of pasteurization temperature (85, 90, 95, and 100°C) and time (15, 30, 45, and 60sec) on the spoilage risk production of SP_{SS} with a_w=0.985, 21% O_2 in the headspace (atmospheric air), stored for 30 days at 22°C.

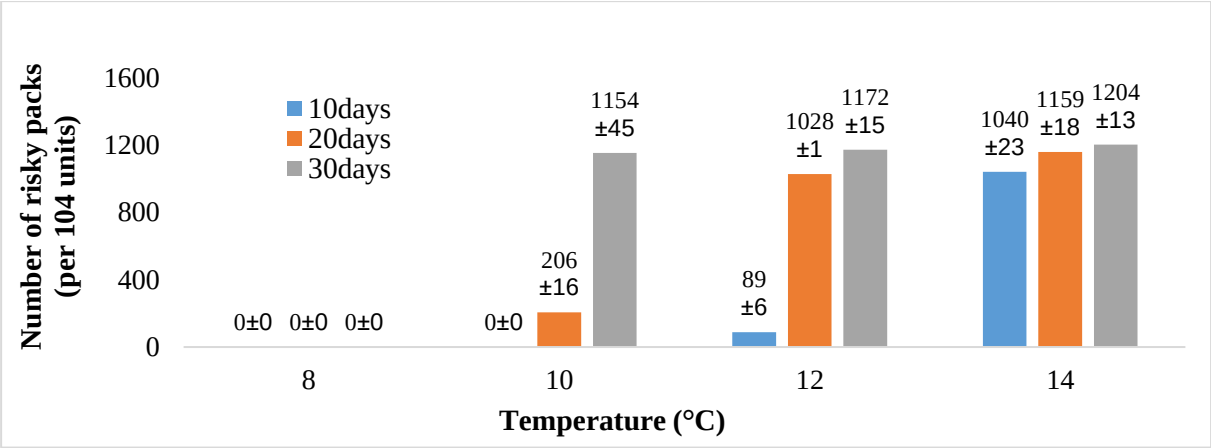

Figure 8.6. **Scenario 5** – Simulation of the effect of increasing storage time (10, 20 and 30 days) and temperature (8, 10, 12, and 14°C) on the spoilage risk of SP_{SS} with a_w=0.985, 21% O_2 in the headspace (atmospheric air) after pasteurization at 95°C/15sec.

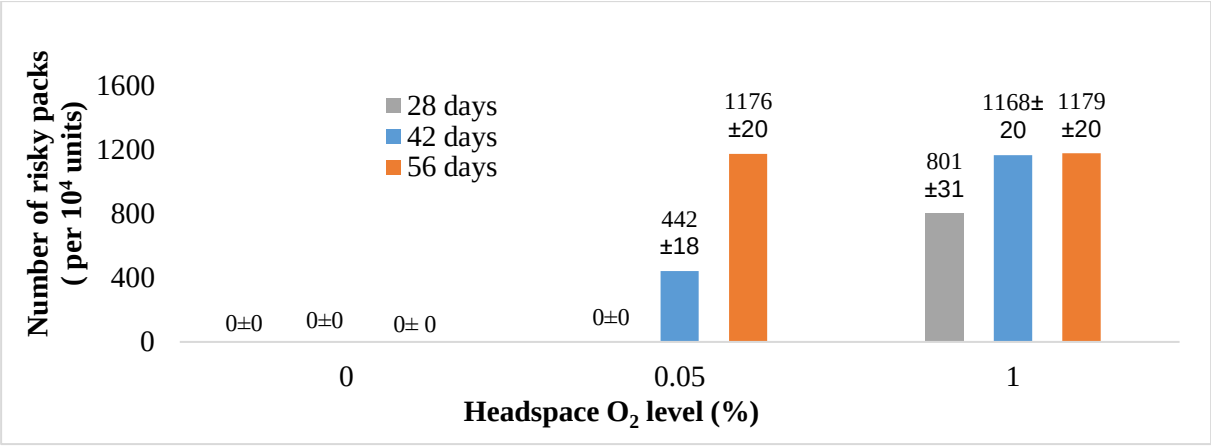

Figure 8.7. **Scenario 6** – Simulation of the effect of headspace O_2 level (0.00, 0.15 and 1.00%) and storage time (28, 42, and 56 days) on the spoilage risk of SP_{SS} with a_w=0.985, after pasteurization at 95°C/15sec and storage at 22°C for 60 days.

The spoilage risk of apple purees (AP) by *B. nivea* was estimated by running simulations for the scenarios 7-9 (Fig. 8.8 – 8.10). In scenario 7 the effect of the pasteurization intensity was assessed (see Fig. 8.8). By increasing the holding time during a mild treatment (85°C) from 15 to 60 sec, the spoilage risk of AP was reduced two-fold from 1338±46 (13.4%) risky packs to 689±43 (6.9%) packs. The same trend was observed when the holding times were increased (from 15 to 60 sec) at 90°C and 95°C. A drastic risk reduction was also observed when the temperature was increased. For instance, when the treatments were applied for 60 sec, the number of risky packs decreased from 689±43 (6.9%) at 85°C to 237±4 (2.4%) and 17±1 (0.17%) at 90 and 95°C, respectively. No spoilage risk was predicted only when the pasteurization temperature was increased to 100°C (see Fig. 8.8). The effect of the storage conditions (time and temperature) on the spoilage risk of AP was evaluated by means of scenario 8 (Fig. 8.9). A zero spoilage risk was estimated in such purees for temperatures ≤

10°C while. At 12°C, the number of risky AP packs increased ten-fold when the storage time was extended from 30 to 60 days. On the other hand, by storing AP at temperatures ≥14°C, *ca*. 1% of packs are expected to be spoiled, regardless the storage time. Lastly, the scenario 9 was intended to evaluate the effect of decreasing the headspace O_2 level on the spoilage of AP stored at 22°C for 30 days (Fig. 8.10). Anaerobic conditions (0.0% O_2) resulted in no spoilage risk of AP by *B. nivea* ascospores. Conversely the presence of only 0.03% O_2 in the headspace already resulted in 106±5 and 12±12 risky AP packs after 28 and 42 days storage, respectively (Fig. 8.10). Higher levels of oxygen (0.15% or higher) did not result in a lot more risky packs when they were stored for 28 days or longer.

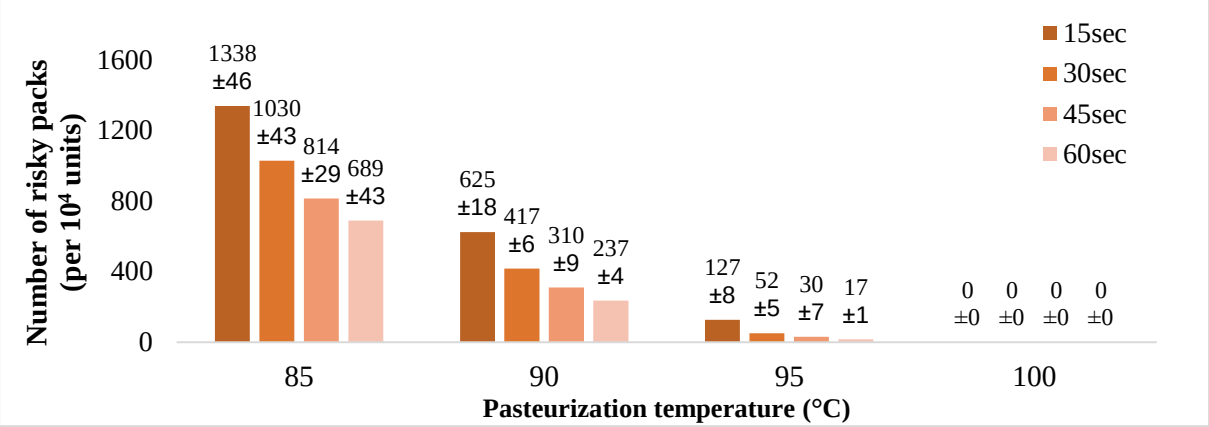

Figure 8.8 **Scenario 7** – Simulation of the effect of pasteurization temperature (85, 90, 95, and 100°C) and time (15, 30, 45, and 60sec) on the spoilage risk of AP with a_w=0.985, 1.0% O_2 in the headspace (atmospheric air), stored for 30 days at 22°C.

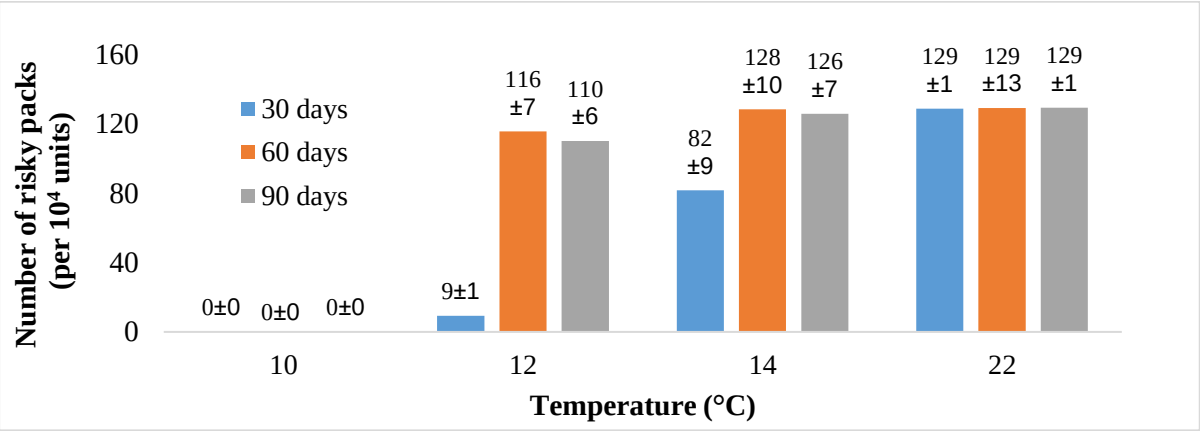

Figure 8.9 **Scenario 8** – Simulation of the effect of storage time (30, 60 and 90 days) and temperature (10, 12, 14, and 22°C) on the production of AP with a_w=0.985, 21% O_2 in the headspace (atmospheric air) after pasteurization at 95°C/15sec.

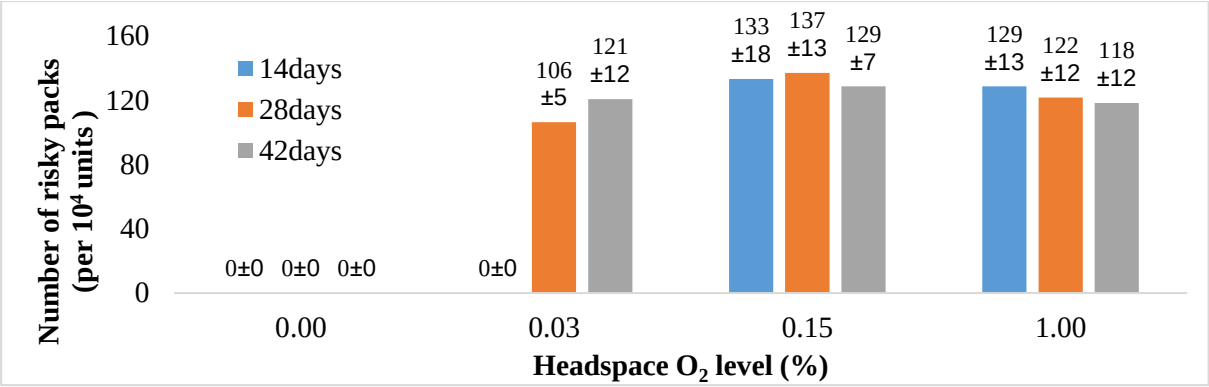

Figure 8.10**. Scenario 9** - Simulation of the effect of the headspace O_2 level (0.00, 0.03, 0.15 and 1.00%) over time (14, 28, 42 days) on the spoilage risk of AP with a_w=0.985, after pasteurization at 95°C/15sec and storage at 22°C.

8.4. General discussion

The spoilage of pasteurized fruit-based products is a topic of great concern among fruit processors worldwide, influencing both import and export markets. The main objective of this PhD study was to quantify the distribution levels of HRMs contamination and to assess various effects regarding process, product and storage on the spoilage risk of pasteurized high-acid fruit based products to build a probabilistic model to prevent and predict the spoilage of pasteurized high-acid fruit products by heat-resistant moulds (HRMs). To do so, several studies were performed in order to collect original quantitative data as well as to explore relevant topics which have not yet been well investigated. These include, for instance, the investigation of inter- and intra-species variability among HRMs strains (Ch. 3), the effect of heat treatment intensity on the subsequent outgrowth of ascospores (Ch. 3 and 7) and the effect of extremely low levels of oxygen on the time to visible growth of HRMs (Ch. 6 and 7). For the latter a suitable experimental set-up needed to be developed first (Ch.5).

8.4.1 Effect of HRMs contamination levels

Although HRMs are known as potential spoilage microorganisms in heat-treated fruit products, to the date there is little quantitative data available regarding their prevalence in pasteurized product as in the raw material. Moreover, the effect of processing steps other than heat pasteurization (such as peeling, sieving etc.) on the contamination level of ascospores has not been reported yet. Therefore, the first experimental part of this thesis (Ch. 2) was intended to quantify and identify heat-resistant fungal ascospores in samples collected throughout the processing of pasteurized high-acid fruit products (strawberry puree, apple puree and orange concentrate juice). Our results suggest that despite the high occurrence of ascospores across all stages in fruit processing (59.3%), HRMs are not likely to occur at levels higher than 1

ascospore/g as the counts were predominantly low (76.8%, <0.1 ascospores/g) (Ch. 2). This reinforces the importance of the quality and control of primary raw, not only because of the high occurrence of ascospores in unprocessed fruits (apples, strawberries and oranges), but also because the most encountered species prior to processing are also predominant in the resulting pasteurized products (Ch. 2). Even though Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP) and sanitation programs are widely used to control HRMs, 69% of fruit manufacturers agree that these techniques are not sufficient to prevent spoilage by the HRMs (Snyder and Worobo, 2018, Rico-Munoz, 2017).

The ascospores of HRMs are known as the most resilient eukaryotic cells, able to persist in hostile environments for long periods (Snyder et al., 2018, Conner and Beuchat, 1987a; Goddard et al., 2005). Besides being carried to processing facilities in the raw materials themselves, other sources of HRMs have been reported within processing environment, including empty packages (PET and laminated paperboard), wooden pallets, palletizers, cap boxes, airveyors, conveyors for bottles, rinsers, fillers, cappers, cooler, forklifts, ingredient coolers, pallet jacks, among others (Sant'Ana et al. 2010, Delgado et al., 2012, Rico-Munoz, 2017). Ideally, high-quality fruits previously selected, washed, and disinfected should be used in further fruit processing. However, in reality, fruits which are not considered suitable for retail, are destined to processing of fruit juices and fruit purees. These may include damaged fruits and fruit with (potential) fungal contamination. Visual or optical sorting of damaged and/or moulded fruits is therefore essential to avoid spoilage of processed fruits, especially when mild pasteurization regimes are applied.

A recent study has shown that treatments with chlorine dioxide (ClO_2) at 100 ppm for at least 10 min or Iodine (iodophor) at levels of 75 ppm, for 16h are required to inactivate ascospores of HRMs (Dijksterhuis et al., 2018). However, many fruit processors do not use chemical sanitizers during washing of fruits prior processing due to safety issues, strict regulations, and the need of high intensity sanitation programs, such as previously mentioned for reaching HRMs inactivation. When no sanitation is used, the reduction of ascospores during the first stages of processing will only occur during sieving and peeling, as was observed during apple puree processing (Ch. 2, Fig. 2.3). This will mainly be the case for fruits from which the peel is removed before further processing.

N. fischeri strains were predominant among the isolates recovered from the strawberry puree processing line, being present in 55.7% and 86.2% of the samples and batches, respectively (Ch. 2, Table 2.1), whilst *B. nivea* isolates were largely recovered from the apple puree processing line, being present in 14.3% and 42.3% of the samples and batches, respectively.

These two HRMs comprised the top three species isolated from pasteurized fruit product, together with *N. fumigata* (see Fig. 2.4). Even though *N. fumigata* was frequently isolated from samples collected on the three fruit processing lines evaluated, we decided to not include this HRMs in the proceedings experiments based on some experimental limitations: (i) difficulties in obtaining the sexual stage (*A. fumigatus*), which may require very long incubation times (>60 days) on specific growth media, such as Oatmeal agar, (ii) their spores can easily be spread through the air, which could potentially contaminate the other HRMs species. Therefore, based on the results and the economic importance of *B. nivea* and *N. fischeri* in apple puree and strawberry puree processing, respectively, they were selected and their data included during the development of the spoilage risk models. The two lognormal distributions used as input for the spoilage risk models were obtained from the counts observed on the raw materials, i.e., occurrence of *N. fischeri* in crushed strawberries and *B. nivea* in crushed apples. These distributions are different from the ones obtained in Ch. 2, where the data of all process steps and HRM species have been taken into account. Nevertheless, both uncertainties and variabilities inherent of contamination by HRMs were considered in the final spoilage risk predictions of fruit purees. The variability on the contamination levels in apples or strawberries may be the result of the differences in geographical sources of fruits and/or application of efficient sanitations/sorting programs and agricultural practices i.e. preventing the fruits from contacting the soil, which is the main source of ascospores contamination (Frąc et al., 2015). A large part of the uncertainties are associated with the censored contamination data, i.e., data below the detection limit (0< ascospores/100g <1).

The high spoilage risk of strawberry purees is also related to the high incidence of *N. fischeri* ascospores on the raw material, in comparison to the occurrence of *B nivea* ascospores in apple puree (Ch.2). Our data imply that the spoilage prevention in strawberry purees will be based on the application of 95°C heat treatments in combination with other hurdles, which will be further discussed in the other sections of this discussion. On the other hand, during the thermal processing of apple puree, higher temperatures are feasible to be applied in comparison to strawberry puree. Besides the advantage of the use of a more intensive pasteurization, the level of contamination of *B.nivea*, considered here as the specific spoilage organism (SSO) of apples purees, is likely to be very low. This might have affected the much lower number of risky packs in comparison to the other two fruit purees (SP_C and SP_{SS}) spoiled by *N. fischeri*.

As discussed in Ch. 2, no criteria have yet been set regarding the acceptable level of ascospores in food products. However, it has been shown that even when present in low levels, HRMs with the potential to survive pasteurization and grow out will represent a threat

to the microbial stability of processed fruit products. Moreover, the presence of one fungal spore is sufficient to cause spoilage under favorable conditions (Sant'Ana et al., 2010, Gougouli and Koutsoumanis, 2017). Accordingly, the risk of spoilage of fruit purees due to HRMs was defined in this chapter as the probability of one ascospore to germinate and present visible growth ($D = 2\text{mm}$) during storage. Nevertheless, some specification and recommendation are further proposed in the last chapter of this manuscript.

8.4.2 Effect of pasteurization intensity

The pasteurization intensities assessed in the scenarios: 85-100°C/15-60sec mimic real treatments applied by the fruit processing industry. However, to the data, no data are available on the HRMs inactivation at high temperatures (>95°C). To tackle this issue, some assumptions were made in this study. Firstly, a secondary model (Eq.8.2) was fitted to the inactivation (survivor) curves of *N. fischeri* and *B. nivea* to estimate their z- and δ_{ref}- values. These parameters together with $p_{iT°C}$ were included as input in the spoilage risk models. Secondly, we assessed whether the pasteurization temperature had an effect on the shape parameter, $p_{iT°C}$. For *B. nivea* it was observed that $p_{iT°C}$ attained a plateau ($p_{iT°C}$ =0.26) from 90°C onwards. So, this parameter was fixed to this value for the pasteurization treatments ranging from 90 to 100°C. For *N. fischeri*, different trends were observed. The parameter $p_{iT°C}$ changed as a function of the pasteurization temperature. Therefore, attempts were made to obtain $p_{iT°C}$ values for the highest pasteurization temperatures. However, the values obtained by extrapolation indicated no biological consistency. Nonetheless, the $p_{iT°C}$ estimated at 93°C was fixed (0.58) and used when pasteurization was done at 95 and 100°C.

The effect of pasteurization intensities on the number of risky packs assessed in the scenarios 1 and 4 for concentrated strawberry puree and single strength strawberry puree, respectively, showed similar outputs. Based on the results, the use of milder heat treatments, in this case 85°C-90°C for up to 1 min, will potentially result in a high percentage of risky packs (≥40 % units). Increasing the holding time within the range that is feasible for the fruit industry (up to 1 min) seems to not affect the risk of spoilage at these temperatures. It is worth mentioning that even when differences occur i.e. in the a_w and O_2 content of both types of purees, SP_C and SP_{SS} are prone to subsequent spoilage after milder treatments and storage at ambient temperatures, i.e., 22°C. Nevertheless, as the strawberry purees are intended to be used as ingredients in a range of food products, they may also present a risk as a contaminated ingredient in these food products. This risk, however, will be highly dependent on the subsequent processing the products are subjected to as well as to the proportion of the purees in the finished products and their storage conditions. Increasing the pasteurization intensity to

95°C/30sec seems feasible to maintain the sensorial and quality characteristics of strawberries, as reported by fruit processors. However, this pasteurization alone is not enough to drastically reduce the risk, even for SP_C (a_w =0.880). While heat treatments applied at very high temperatures, such as 100°C, have shown to be effective in maintaining the microbial stability of such purees during storage, they may result in sensory and quality alterations.

It is noteworthy mention that the effect of soluble solids on the heat resistance was not integrated in this study as the inactivation data from Ch. 3 were obtained in buffer glucose (pH = 3.6, 13°Brix) and not in real fruit purees. While our data may well represent the inactivation in high a_w fruit purees (SP_{SS} and AP) it may affect the ascospores resistance in concentrated purees (SP_{CC}). Indeed, the thermal resistance of HRMs is not only influenced by the temperature but also by the soluble solids content (Souza et al., 2017, Beuchat, 1986, Tournas, 1994). Thus, it might be expected that a higher number of ascopores would survive heating (N_P) due to protection by the sugars and other compounds present in fruit purees (Baglioni, 1998). Therefore our data may underestimate the heat resistance of the HRMs in some real fruit products.

The use of high temperatures, i.e., 100°C/15sec, as reported by fruit manufactures, is one of the main strategies for decreasing the spoilage risk of such purees. Moreover, the effect on reducing the spoilage risk by increasing pasteurization holding times may be associated with the lower thermal resistance of *B. nivea* compared to *N. fischeri* (Ch. 3, Souza et al., 2017). While the growth data (= time to visible growth) on SP_{SS} and AP were collected after heat activation, i.e., 80°C/10 min (Ch. 4, 6 and 7), SP_C data were obtained in Ch. 7 after being submitted to the same pasteurization treatments used in the second step of our risk models (§8.1.3).

Spoilage zero risk was estimated after simulating high intensity pasteurization (100°C/15 sec). This is in agreement with literature, as even though *N. fischeri* is extremely resistant to thermal processes, where it is expected that the application of pasteurization at temperatures ≥100°C, which corresponds to commercial sterility, would strongly inhibit the ascospores (Dijksterhuis, 2019, Snyder, 2018). Instead, cleistothecia, which are spores fruiting bodies containing ascospores, were reported to withstand treatments as severe as 100 °C/30min (Katan, 1985, Van der Spuy et al ., 1975, Snyder et al., 2018). In Ch. 7, it was shown that after long times of incubation, *N. fischeri* ascospores may germinate and subsequently grow out after treatments at 100 and 105°C for 15sec (Ch. 7, Fig. 7.2 and 7.3). Perhaps, the heating method used in the latter may have influenced the results. Despite the attempt to control pasteurization temperatures by placing a thermocouple in the pre-heated oil bath, some temperature

variations may be expected in the inoculated tubes (Ch.7), which might have influenced the results.

8.4.3 Effect of storage temperature and shelf-life duration

Both, *N. fischeri* and *B. nivea* are known to be able to germinate and grow in a wide range of temperatures (11 an 43 °C for *B. nivea*; and 10 and 52 °C for *N. fischeri*) (Nielsen et al., 1989, Panagou et al., 2010, Pitt and Hocking, 1997, Zimmermann et al., 2011). By assessing the growth limits of HRMs in Ch. 4, it was observed that the ability of *N. fischeri* and *B. nivea* to form visible mycelia in high-a_w Potato Dextrose Agar (a_w = 0.985, PDA) was impaired by reducing the temperature to 8°C and 10°C, respectively. The growth data (Ch. 4, Table 4.3) were then included as input in the risk model for SP_{SS} in order to predict the spoilage risk. As previously reported, storing the SP_{SS} at chilled temperatures ≤ 8°C will drastically reduce the risk of spoilage of these purees to null during 30 days of storage. Our results showed that when the strawberry puree is produced as a concentrate, i.e., $a_w \leq 0.880$, it is not likely to present spoilage risk at temperatures ≤ 10°C for 90 days. However, no growth data were collected in our study between 10 and 22°C. Perhaps the growth inhibition of *N. fischeri* at high sugar concentrations is reached even at higher temperatures than 10°C. *B. nivea* ascospores, on the other hand, showed less ability to germinate and form visible colonies as temperature decreased (Ch. 4). Therefore, no spoilage risk was estimated for AP stored at temperatures ≤ 10°C for 3 months, which represents chilled and abuse chilled temperatures. Additionally, it will take at least one month for the appearance of *B. nivea* visible mycelia at 12°C, associated with a very low spoilage risk, 0.09% or 9 in 10^4 packs.

Slight temperature abuse, especially during summer, may compromise the microbial stability of those purees and increase the spoilage risk over time. It has been reported (Ch. 3 and Ch. 7) that some injured ascospores may require long times to recover after a heating treatment or other sources of stress. This may result in a considerable delay of their time to visible growth and in an increase of spoilage risk as the storage time increases. Despite the temperature plays a big role on the microbial stability of fruit purees regarding the HRMs ascospores, it is difficult to have it under control in the entire food chain. Complexity of temperature traceability and maintenance of the cold chain in both import and export markets are a major challenge for fruit processors worldwide. For this reason, many of the fruit-based products used as ingredients intended to be exported or imported are preferentially traded as concentrates or stored at chilled or freezing conditions.

Nonetheless, it is important to mention that the cooling step after hot filling/pasteurization of purees intended to be stored under refrigeration or frozen conditions may in practice last many hours or even days. This period may pose a threat for the quality of processed purees. Once the conditions of packed purees are favorable, activated ascospores may start to germinate during cooling step, followed by outgrowth. As discussed throughout this thesis, the time for appearance of visible mycelia (=spoilage) is highly associated with spoilage and rejection by consumers. This time can be long or very short, dependent on the product formulation, storage conditions and level of HRMs contamination (Ch. 4, Ch. 6 and Ch.7). For instance, visible growth ($D = 2mm$) of *B. nivea* was observed after ≥ 8 days in papaya juice (a_w=0.93) at 30°C (Zimmerman et al., 2013). The same authors observed that by increasing the a_w to 0.99**,** it took only 17 hours for observing mycelial colonies in papaya juice. Roland and Beuchat (1984) reported that 8 days or more are required for growth of *B. nivea* in apple syrups at 21°C and above 2 days if they are stored at 30°C. While Sant'Ana et al. (2010) observed that the appearance of visible mycelia of *B. fulva* in clarified apple juice may occur just after 1 to 4 days after heat treatment. It is therefore essential to monitor the time/temperature profile during the cooling step end to insure conditions which will not allow proliferation of HRMs.

8.4.4 Effect of a_w and reduction of oxygen in the headspace

Because HRMs will grow very fast at a_w-values ≥ 0.900, the water activity effect was only assessed for different formulations of concentrates purees (SP_{CC}) which was demonstrated in preliminary research. For this type of purees, it has been discussed that small changes in a_w value or in the soluble solids concentrations (°Brix) may strongly affect the ability of *N. fischeri* to form visible mycelia, with consequent spoilage (Ch. 7). In this study, the experimental range of a_w of 0.860-0.880 was chosen in order to ensure an effect of changing a_w's as it belongs to the growth/ no growth region of this HRMs (Ch. 7, Fig. 7.3). However, it is worth mentioning that these values may represent a worst case scenario, as most of the concentrates purees, including strawberry have a_w values ≤ 0.870, which will strongly decrease the risk of spoilage. Furthermore, when this parameter is reduced to values ≤ 0.860, no spoilage is expected, regardless other factors such as high temperature storage or long storage periods. However, slight increases of the a_w to 0.870 will result in a potential increase of the spoilage risk, depending on the level of oxygen at the headspace. Therefore, our data imply that by choosing the right a_w, more robust formulations of purees can be obtained.

The role of oxygen on the growth of HRMs such as *N. fischeri* and *B. nivea* has extensively been study in this PhD (Ch. 6 and 7). As discussed in Ch. 7, reducing the oxygen to extremely low levels, such as 0.05%, will not assure the microbial stability of single strength fruit purees

if they are stored at moderate and ambient temperatures. In order to maintain the microbial stability of fruit products, strict anaerobic in-pack conditions are required. During processing of fruit purees, an attempt to deplete the headspace oxygen takes place by applying vacuum or steam during packaging. However, controlling and quantifying the residual level of oxygen in the headspace is a challenge for the processors. Moreover, it is known that many variables may influence the total package oxygen content such as the fill temperature, storage temperature, headspace volume and the type of package (Snyder et al., 2018). In addition, the presence of natural antioxidants (flavonoids, vitamins and anthocyanin) may also contribute to reducing the levels of oxygen during storage, depending on the initial oxygen levels set at the packaging headspace (Ch. 7, Fig.7.4). Therefore, single strength purees obtaining after mild pasteurization treatment and containing very low residual level of oxygen and stored at ambient temperature, such as ready-to-eat strawberry purees, need to be consumed within a short time (ca. one month).

8.4.5 Quantitative Microbial Spoilage Risk Assessment (QMSRA) model to assess spoilage risk of heat-resistant moulds (HRMs)

Quantitative microbial exposure risk assessment (QMRA) has been extensively applied within the context of food safety to estimate and characterize the risk associated with a foodborne illness (Codex Alimentarius Commission, 1999, FAO et al., 2001, Cassin et al., 1998; Delignette-Muller et al., 2008, Ross et al., 2009). However, according to the European Commission, the term “unsafe food” may not only be associated with consumption of foodborne pathogens/microbial toxins but also with foods which are unfit for human consumption, i.e., i.e. foods with a certain degree of deterioration (European Commission, 2002). Also the food industry makes a lot of efforts to avoid having spoiled products in the market and to obtained logistically long enough shelf-lives.

In contrast to the vast number of QMRA studies in food safety, there is a paucity of using this approach to food spoilage issues (QMSRA). There are indeed only a limited number of studies available using this approach. Examples are estimations of the spoilage risk of yogurt by *Aspergillus niger* (Gougouli and Koutsoumanis, 2017) and canned green beans by *Geobacillus stearothermophilus* (Rigaux et al., 2014). To develop such risk models some adjustments in comparison with the classical QMRA approach need to be taken into account. The terminology for the two approaches are depicted in Table 8.3. Whilst QMSRA studies are developed to predict the probability (risk) of having spoiled packs of food, QMRA approach is mainly intended to estimate the probability (risk) of developing foodborne illness upon consumption of pathogen and its potential adverse health effects (Membré and Boué, 2018).

Table 8.3 Comparison of terminology used in QMRA and QMSRA approaches.

	QMRA	QMSRA
Target microorganism	Pathogen	Spoilage
Hazard	Foodborne illness	Spoilage defects
Exposure assessment	Intake of a pathogen	Microbial concentration/ spoilage defect*
Risk characterization	Adverse health effects	Food quality changes and spoilage risk

*e.g. visible fungal mycelia, sensorial and quality changes (off-odors, discoloration, etc.)

As discussed throughout this PhD, the microbiological variability inherent to HRMs at spore, strain and species levels may strongly influence spoilage predictions and fungal response (growth or inactivation). Each one of them was approached to some extent during this PhD. Variability at spore level was explored in Ch. 4, by assessing the time to growth of individual ascospores within the same population although with the same historic treatment and physiological state (Gougouli and Koutsoumanis, 2012; Judet et al., 2008; Nanguy et al., 2010). The differences in times to visible growth at the spore level may be the result of heterogeneity in germination time and growth rate of the mycelium (Chitarra and Dijksterhuis, 2007, Chitarra et al., 2004, Dijkterhuis, 2017). Additionally, the effect of microbiological variability at the spore level was also observed in Chapters 6 and 7. In these studies, albeit, the same inoculum of ascospores was used, distinct times to growth among replicates were observed. An exploration of intra-strain variability was carried out in Ch.3. The results obtained for different HRMs strains within *Byssochlamys* sp. showed the importance of including strain variability in model predictions (model development). Estimating kinetic parameters from individual strains allows for different behaviors and marginal cases to be taken into account which may imply more accurate predictions of the risk of spoilage. The assessment of inter-species variability, on the other hand, was performed in Ch.3, 4 and 6, where it was demonstrated that different species respond differently to environmental and physicochemical stresses.

Moreover, for QMSRA studies focusing on fungal spoilage, the variability inherent to consumers perception of visible mycelia may also be considered. As this corresponds to the rejection time of the food, shelf-life estimation may be more accurately predicted by considering this parameter as a distribution, which can be incorporated in QMSRA models (Gougouli and Koutsoumanis, 2017).

Final conclusions, limitations and future perspectives

The vast **quantitative data generated** from this PhD comprise very useful information for both researchers and the fruit processing industry. As discussed throughout the thesis, the different types of data can be effectively incorporated in QMSRA models intended to prevent the spoilage of processed fruit products. Moreover, the outcomes obtained during this PhD emphasize the importance of preventing the spoilage of fruit based products due to HRMs, as well as the need for more research in this area. The main conclusions, limitations, recommendations and perspectives for future research are presented in more detail below.

Additionally to the prediction of the spoilage of fruit purees, the presented "proof-of-concept" **microbial spoilage risk assessment model** focusing on HRMs can be potentially applied for other heat treated fruit matrices, such as juices, concentrates, etc., as long as data regarding occurrence, inactivation and growth are available. For example, the quantitative data regarding the contamination of concentrate orange juice by HRMs (Ch.2) can be used to assess the spoilage risk of orange juices prepared from concentrates. Additionally, the risk model can be used to assess the effect of **sorting** and **decontamination** (after treatment with sanitizers) on the initial contamination reduction of HRMs and final spoilage risk of packaged fruit purees. Yet, to do so, more **quantitative data** still need to be generated regarding potential HRMs inactivation of such procedures. Furthermore, the developed risk models still need to be completed by introducing data of heat resistance variability at the strain level (Ch.3) finalized by increasing the number of simulations to 10^5-10^6 iterations using Latin Hypercube sampling. This will increase the chances of having inputs selected from the tails of the distributions, i.e., the marginal conditions, leading to more accurate predictions at the growth regions.

The use of universal primers targeting internal transcribed spacer (ITS) regions of rDNA, ITS4 and ITS5, used in Ch.2 for molecular identification of the HRMs isolates may not result in enough resolution to discriminate between closely related species, such as *Aspergillus* section *Fumigati* (*Neosartorya* morph). Therefore, it is strongly recommended to use primers targeting β-tubulin or calmodulin gene to find full resolution (identification) of HRMs at species level.

Future investigations may be performed to **track** the sources and establish the routes via which spoilage associated **HRMs strains** contaminate fruit based products. This could be performed by using molecular techniques. The outcomes could be useful to trace the real cause of contamination and to set control measures.

There is a paucity in data (on the heat resistance and growth limiting conditions) of ***N. fumigata*** while this was one of the most occurring HRMs species in the fruit processing lines evaluated

in this PhD; so more studies (inactivation and growth) should be performed which are focused on this HRM.

The developed **set-up** (Ch. 5) intended to assess the effect of low levels of oxygen (headspace and dissolved) by using oxygen scavengers and gas-tight-glass jars can be used to evaluate the individual **effect of oxygen** or combined with multiple factors (Ch.7) on the growth of all kind of relevant oxygen dependent microbial species. However, it is important to notice that when the range of this variable is very narrow, as in our studies (Ch. 6 and 7), the uncertainties regarding to small oxygen fluctuations over time may impair the results and many replicates will be needed. Moreover, attention should be paid to select oxygen scavengers that preferentially do not generate carbon dioxide (Ch. 6).

Various factors (intrinsic and extrinsic)/hurdles may influence the microbial stability of fruit purees during storage. Our data imply that $\mathbf{a_w}$ (adjusted by sugar) plays a major role on the this stability and it is often used to classify purees (as concentrates or single strength). The a_w **growth/no growth** region determined in this work provides useful information for the development of robust formulations which in combination with other key variables, such as storage temperature and, less pronounced, oxygen content (%) may contribute to long shelf-lives. Despite of the similar conditions (a_w, O_2, pasteurization and storage temperature) assessed for both matrices, synthetic medium (aPDA) and strawberry medium, the ability of HRM to growth up to visible mycelia was, in general, impaired in fruit-based media (Table 7.4). Therefore, it is recommended that future studies are performed directly in fruit products or at least in fruit-based media (as presented in Table 6.3 and Table 7.1).

Follow-up research should be carried out to determine the effect of different **fruit products** (= fruit composition) on the dissolved levels of **oxygen** during time (=product storage), i.e. in non-inoculated fruit products, as well as on the time to visible growth of HRMs in such matrices. Therefore, additional studies with real fruit products focusing on evaluating the growth kinetics of HRMs as function of low levels of oxygen (as the ones assessed in this PhD) should be performed together with evaluating the oxygen consumption of the products as a function of time. Ultimately these data could also be incorporated in risk assessment models.

The effect of **thermal pasteurization** on **subsequent germination and outgrowth** of ascospores which was initially explored in this PhD still need to be further elaborated. Future experiments should mainly cover high intensities treatments (> 95°C/ for short times), as already applied by some fruit industries. To do so, accurate and suitable experimental set-ups need to be firstly obtained. The method applied for heating of ascospores at 95°C-105°C in oil

bath still need to be improved (Ch.7). It is important to assess if such treatments may result in ascospore activation, besides potential inactivation. In order to simulate real contamination on fruit products, it is suggested that further studies are performed with low level of inoculum (such as the contamination ranges from Ch. 2). In addition, more investigations should be carried out on the **thermal inactivation** of less occurring HRMs species, such as the ones isolates in Ch.2. These studies may be preferentially performed or validated in real fruit products, as the presence of sugar and other fruit compounds may strongly affect the inactivation kinetics of the ascospores.

Once some injured HRMs ascospores may require very long periods to recover, germinate and grow out (up to 85 days) (Ch. 7, Table 7.2), it is very important that **experiments** aiming to determine HRMs-free shelf-lives are performed for **long periods**, such as performed in this PhD (2-3 months). By doing so, more realistic and accurate results can be obtained (e.g., shelf-life determination and HRMs growth inhibition during realistic shelf-lives).

Even though the **inter** and **intra- species variability** of HRMs was partly investigated in this PhD, this topic should be further explored in greater detail: by (i) increasing the number of strains (>20) and (ii) by assessing more data intensive marginal/suboptimal conditions. Due to the potential biological variability of HRMs, it is suggested that growth or inactivation parameters are incorporated in quantitative studies (predictive model and risk assessment model) as stochastics parameters, i.e., statistical distributions.

Despite of not being part of the scopus of this PhD, it is known that potential spoilage HRMs may produce (myco)toxins which are very often claimed to be a hazard to human health. However, more studies need to be carried out to: (i) detect and quantify mycotoxins in pasteurized fruit products, (ii) to investigate the health risk of such mycotoxins through dietary exposure assessments and (iii) to investigate whether HRMs visible growth will result or not in mycotoxin production in stress conditions such as those studied in this thesis.

From the industrial point of view, the generated data can be applicable in defining **process settings**, establishing **control measures**, defining robust **formulations** and setting **shelf-lives**. In general, it is highly recommended to sort, wash and disinfect the raw material prior to processing in order to reduce the initial load of ascospores. Additionally, the application of strict cleaning and disinfection programs in critical areas of processing such as the raw material storage and packaging areas are strongly advised. Ultimately, a set of **specifications for HRMs** in raw material and pasteurized product have been proposed (Table 9.1). The threshold levels (cfu/100g) were defined based on a target of 10^{-4} spoilage risk, i.e., one spoilage pack

out of 10^4 units produced. Moreover, the proposed specifications were set taking into account the potential risk of spoilage regarding formulation, process and storage conditions (Fig. 9.1), the real HRMs levels of contamination found in the raw materials (Fig. 9.2) and in pasteurized purees (see §*2.3.1*, §*2.3.3* and Fig. 8.13).

Therefore, in order to reduce the risk of spoilage to 0.01% or 10^{-4}, target and tolerance levels of contamination for HRMs in raw material and pasteurized product have been proposed. High contamination levels of HRMs have been reported to occur in strawberries (89.7% of samples, 1-215 cfu/100g) and apples (84.6%, 1-84 cfu/100g) (Fig. 9.2, modified from Fig. 2.2, Ch.2). However, as presented in Fig. 9.2 and already discussed in this thesis, such levels are most likely to be low, i.e., ≤ 20 cfu/100g and ≤ 10 cfu/100g in strawberries and apples, respectively. Therefore, a target value of 20 cfu/100g and a maximum tolerance value of 100cfu/100g are suggested for **raw material** used to produce: (i) stable fruit purees (concentrates), (ii) purees intended to be stored in the cold chain, i.e., frozen or at chilled conditions (≤ 7°C) and (iii) heat sensitive purees (HSP) (e.g. berries purees) (Table 9.1). For raw material used to produce heat resistant purees (HRP) which can withstand high intensity pasteurization (such as 100°C/15sec) a target value of 10cfu/100g and a maximum tolerance of 50 cfu/100g have been suggested. These threshold were established based on the most likely level (=target value) and on the highest contamination level at 95% CI (=tolerance value) found in the raw materials (apples) (Fig. 9.2).

With regards to **specifications for pasteurized purees** produced as concentrates or stored in the cold chain, a target value of 10cfu/100g and a maximum tolerance of 35 cfu/ 100g are recommended. These comprise the most likely contaminated value and the maximum value within 90% CI, respectively, for pasteurized HSP (Fig. 9.3). On the other hand, absence of HRMs in 100g of pasteurized product is suggested for both HRP and HSP (single strength purees) intended to be stored at ambient temperatures, as the HRMs growth is highly favorable on such formulations and temperatures. Ultimately, in order to drastically reduce the contamination levels and the spoilage risk, it is suggested the use of high quality raw material and pasteurization at 95°C for at least 45-60sec for such types of fruit purees (Fig.9.1).

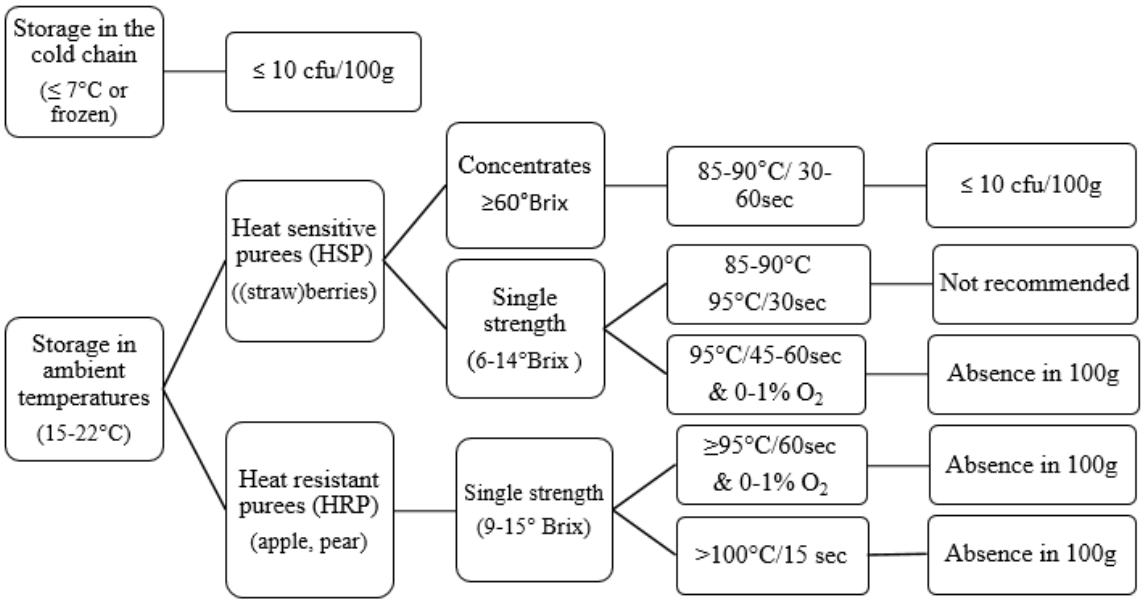

Figure 9.1. Flow chart for HRMs target specifications in pasteurized fruit purees according their formulation, process and storage conditions.

Table 9.1. Specifications for HRMs maximum levels in raw materials and in pasteurized fruit purees.

				Contamination in raw material		Contamination in pasteurized puree	
Type of puree	**Formulation**	**Pasteurization**	**Storage temperature**	**HRMs target**	**HRMs tolerance**	**HRMs target**	**HRMs tolerance**
All types of fruit purees		85-90°C/ 30-60sec	Frozen or chilled[3]	≤ 20 cfu/100g	100 cfu/100g	≤ 10 cfu/100g	35 cfu/100g
HSP[1]	≥60°Brix	85-90°C/ 30-60sec	Ambient[4]	≤ 20 cfu/100g	100 cfu/100g	≤ 10 cfu/100g	35 cfu/100g
HSP	6-14°Brix, 0-1% O_2	95°C/ 45-60sec	Ambient	≤ 20 cfu/100g	100 cfu/100g	Absence in 100g	Absence in 100g
HRP[2]	9-15°Brix, 0-1% O_2	≥ 95°C/60sec	Ambient	≤ 10 cfu/100g	50 cfu/100g	Absence in 100g	Absence in 100g
HRP	9-15°Brix	>100°C/15sec	Ambient	≤ 10 cfu/100g	50 cfu/100g	Absence in 100g	Absence in 100g

[1] Heat sensitive fruit purees, [2]Heat resistant fruit purees [3] T ≤ 7°C, [4] T:15-22°C.

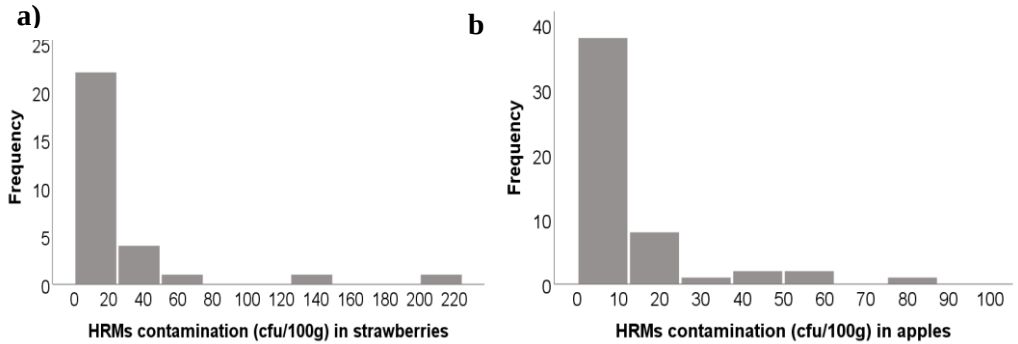

Figure 9.2. Distribution of HRMs contamination levels (cfu/100g) in raw material: (a) strawberries (b) apples (data obtained from Ch.2).

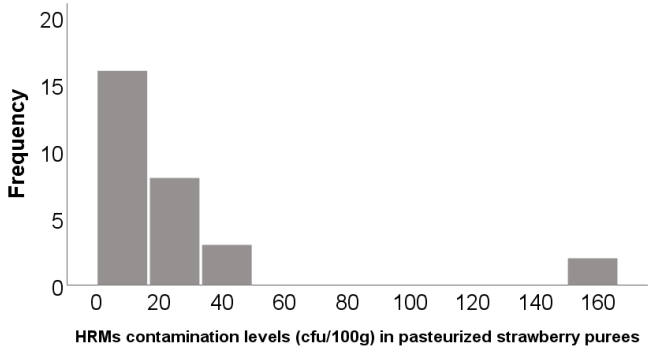

Figure 9.3. Distribution of HRMs contamination levels (cfu/100g) in pasteurized strawberry purees (data obtained from Ch.2).

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Appendix

Appendix - Data of *Neosartorya fischeri* heat resistance

This appendix provides the data of heat resistance of *Neosartorya fischeri* (Neosar fisheri 95-1) used in the risk assessment model developed in Ch.8 (*see* §*8.1*). Inactivation kinetic data were collected as described in Ch. 3 (*see* §*3.2*) and the heat resistance parameters, p, δ, $log\delta_{ref,i}$ and $Z_{T,i}$ estimated by means of package "nlstools" version *1.0-2* available in R software version *3.5.2* (Eq. 8.1 and Eq. 8.2, *see* §8.1.3).

Table A.1. Inactivation kinetics (survival data) of *Neosartorya fischeri* collected in buffer glucose solution (adjusted to pH 3.6 and 13°Brix) at 85 °C, 90 °C and 93 °C.

85°C				90°C				93°C			
	R1*	R2	R3		R1	R2	R3		R1	R2	R3
Time[1]	logN[2]	logN	logN	Time	logN	logN	logN	Time	logN	logN	logN
0	6.15	6.20	6.90	0	6.73	6.69	6.48	0.0	7.09	7.02	7.01
5	5.89	6.24	6.77	1	6.59	6.52	6.33	0.2	5.89	5.74	5.82
10	5.54	5.90	6.77	2	6.20	6.04	5.95	0.5	5.13	4.81	4.50
15	5.86	5.58	6.29	3	5.46	5.53	5.51	0.8	4.51	4.24	4.27
20	5.18	5.64	6.03	4	4.42	4.43	4.47	1.2	4.75	4.16	3.95
30	3.93	4.83	5.04	5	3.64	3.55	4.25	1.5	4.02	4.17	3.91
40	3.58	3.65	4.13	7	2.88	2.88	2.99	1.8	4.03	3.83	3.32
50	2.12	3.07	3.06	10	<2.00	<2.00	<2.00	2.2	3.77	2.98	3.20

[1] Time in minutes [2] log of surviving ascospores (cfu/ml) *Replicates.

Table A.2. Weibull model parameters for the survival of *Neosartorya fischeri* after heat treatment at 85 °C, 90 °C and 93 °C in acidified buffer glucose solution (pH=3.6, 13°Brix).

	R1	R2	R3		R1	R2	R3	
T (°C)	p[1]			Mean (sd)*	δ (min)[2]			Mean (sd)
85	1.66	1.47	1.66	1.6 (0.09)	20.60	22.80	20.30	21.23 (1.11)
90	0.99	0.92	1.01	0.97 (0.04)	2.00	2.40	2.00	2.13 (0.19)
93	0.56	0.61	0.57	0.58 (0.02)	0.08	0.07	0.07	0.08 (0.01)

[1]survival curve shape [2] time for first log reduction * mean, standard deviation.

Curriculum Vitae

Juliana Lane Paixão dos Santos was born in Campinas-São Paulo, Brazil on the 10th October 1986. In 2012 she obtained a Bachelor degree in Food Engineering from Federal University of Viçosa. During her years at university she worked as a Project Manager in a Food Engineering Junior Enterprise (2008) and as an undergraduate researcher at the Cereal Chemistry Research Laboratory-UFV (2010) with scholarship received from FAPEMIG, Brazil. In 2013 she was granted with CAPES scholarship to pursue master degree in Food Science at Quantitative Food Microbiology Laboratory at University of Campinas. Her master thesis was focused on the prevention of multigrain whole meal bread spoilage by fungi. After receiving her master diploma in Food Science (2015), she joined Ghent University as a PhD student at the Food Microbiology and Food Preservation Research Unit with financial support from National Council for Scientific and Technological Development (CNPq), Brazil. During her PhD she cooperated with fruit product companies, presented in several international conferences, published in international journals, supervised seven Bachelor's and Master thesis' students and assisted in the practical sessions of the course 'food microbiology and analysis'. In addition, part of her research was performed at the Unité de Recherché Sécurité des Aliments et Microbiologie (Oniris, Nantes, France).

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International Commission on Food Mycology – ICFM 2016 Workshop: Current and Future Trends in Food Mycology - Methods, Taxonomy, and Emerging Problems, 13th -15th June 2016 – Freising, Germany.

5th International Symposium on Mycotoxins and Toxigenic Moulds: Challenges and Perspectives, 11th May 2016 – Ghent, Belgium.

XII Congresso Latino Americano de Microbiologia e Higiene de Alimentos, IAFP's Latin American Symposium on Food Safety, 13º Simpósio Internacional ABRAPA de Segurança de Alimentos, Symposium of the International Commission on Food Mycology, 12th-15th October 2014 – Foz do Iguaçu, Brazil.

10th Latin American Symposium on Food Science (SLACA 2013), 3th-6th November 2013– Campinas, Brazil.