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

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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.

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, 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; Tremarin 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, 2009, 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; Santos et al., 2018a; 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

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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.

2. Material and methods

2.1. Strains

Six HRMs, previously isolated from raw and processed fruit products (Santos et al., 2018a), 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, Ghent University, Belgium).

2.2. Fruit medium preparation

Three fruit-based medium 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 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 medium were heated for 1-2 min at 750 W in a microwave to facilitate mixing. The fruit medium 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 1.

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).

Table 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

2.4. Preparation of ascospore suspensions

The HRMs were grown for 30 days at 30 °C on ten Malt Extract Agar plates (MEA, Oxoid, Hampshire, UK) to ensure adequate ascospore production. Subsequently, ascosporous suspensions were prepared by flooding each Petri plate with 8 ml of sterile 0.1% Tween 80 (Sigma-Aldrich, USA), followed by filtration through sterile cotton and centrifugation for three times at 8.000 g for 15 min at 4 °C. The final suspensions were obtained by adding 10 ml of sterile distilled water to the pellet obtained after centrifugation. When asci were present in high numbers, sonication (amp: 45%, 30s-interval) was applied for up to 4 min to separate the ascosporous clusters (Vibra ≈ Cell™ 75,186, United States). The counts of ascospores in the final suspensions were determined by spread plating serial decimal dilutions of the suspensions on MEA. The serial decimal dilutions were prepared in test tubes with 9 ml of sterile distilled water. Spread plating of the decimal dilutions was preceded by heat shocking the ascospores at 80 °C for 10 min. Enumeration was performed after 5-7 days of incubation at 30 °C. The final (non-activated) ascospore suspensions were kept at 2 °C for up to one month.

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 min at 80 °C. A method based on the use of O_2 scavengers to obtain and maintain the desired low O_2 levels was used. The method relies on initially setting the desired O_2 level in the headspace of gas-tight-40 ml glass jars closed with metal lids. Ten ml of molten aPDA (*ca.* 48 °C) were poured into each of three glass jars (= three replicates) resulting in a 1 cm thick layer of medium. One of the three jars contained two Oxydot's® (European Tech Serv NV, Belgium) which were attached to the inner walls prior to the addition of the molten aPDA. One of the Oxydots was attached in the region of the headspace whereas the other one was attached at the bottom of the jar. Thereafter, the molten agar was inoculated with 100 µL 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 glass jars were externally disinfected and placed inside a high O_2 barrier plastic bag with an O_2 scavenger sachet (AnaeroGen Compact, Oxoid Ltd, Basingstoke, UK). Subsequently, the bag was sealed with a heat-sealing machine after which the lids of the jars were opened to facilitate the consumption of the O_2 in the medium and headspace by the scavenger. The rate of O_2 uptake was measured *via* the Oxydot attached in the headspace of the jar. When the desired gas concentration was achieved in the headspace, the bag was divided into two compartments with a heat-sealing machine in such a way that the O_2 scavenger was separated from the jars. 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).

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 $\alpha = 0.05$.

3. Results and discussion

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. 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. 2). Under this condition, no significant differences ($p > 0.05$) were observed between the times to visible growth of the six HRMs (see Fig. 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 $\geq$1% O_2 when other growth determining factors are set at optimal levels (Nguyen Van Long and Dantigny, 2017; Nielsen et al., 1989). The decrease in the O_2 concentration was attributed to consumption by the HRMs. Growth was observed promptly after the O_2 level in the water phase of the medium had reached very low levels ($\leq$0.05%). With regards to *B. fulva*, there was no indication of appreciable O_2 consumption before visible growth was observed (see Fig. 1).

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. 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. 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. 1). The mean O_2 values and respective standard deviations are depicted in Table 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. 3). *B. fulva* had in general the shortest and the most variable times to visible growth between the replicates, which ranged from 4 to 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. 2).

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; Nguyen Van Long and Dantigny, 2016). 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 three 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 ascosporous 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

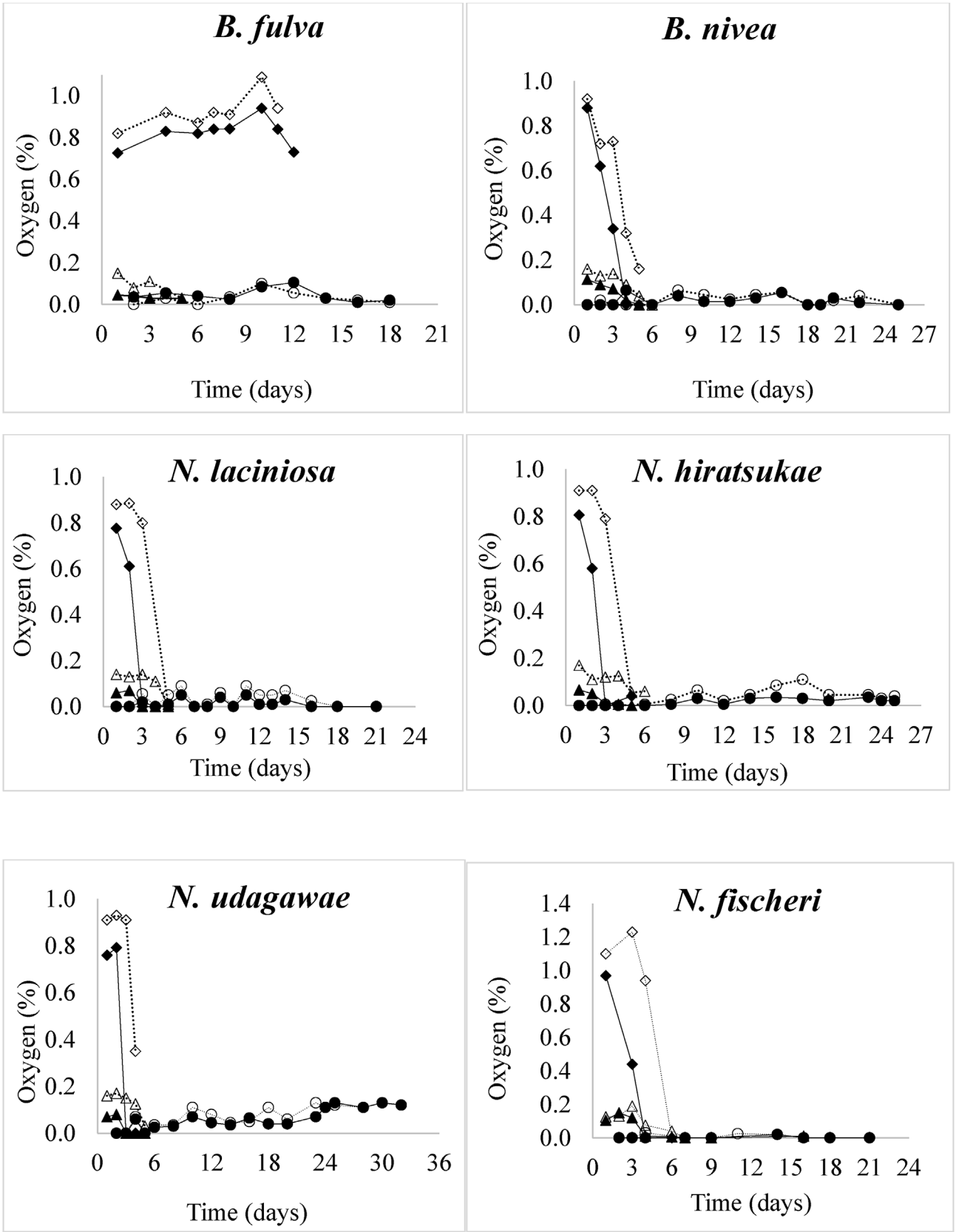

Fig. 1. O_2 profile (%) at headspace (dotted line) and dissolved in acidified PDA (solid lines) in jars inoculated with *B. fulva*, *B. nivea*, *N. laciniosa*, *N. hiratsukae*, *N. udagawae* and *N. fischeri* and stored at 22 °C. The O_2 concentrations were initially set at 0.03% (●), 0.15% (▲) and 0.9% (♦). Each symbol represents the average O_2 level of two repetitions.

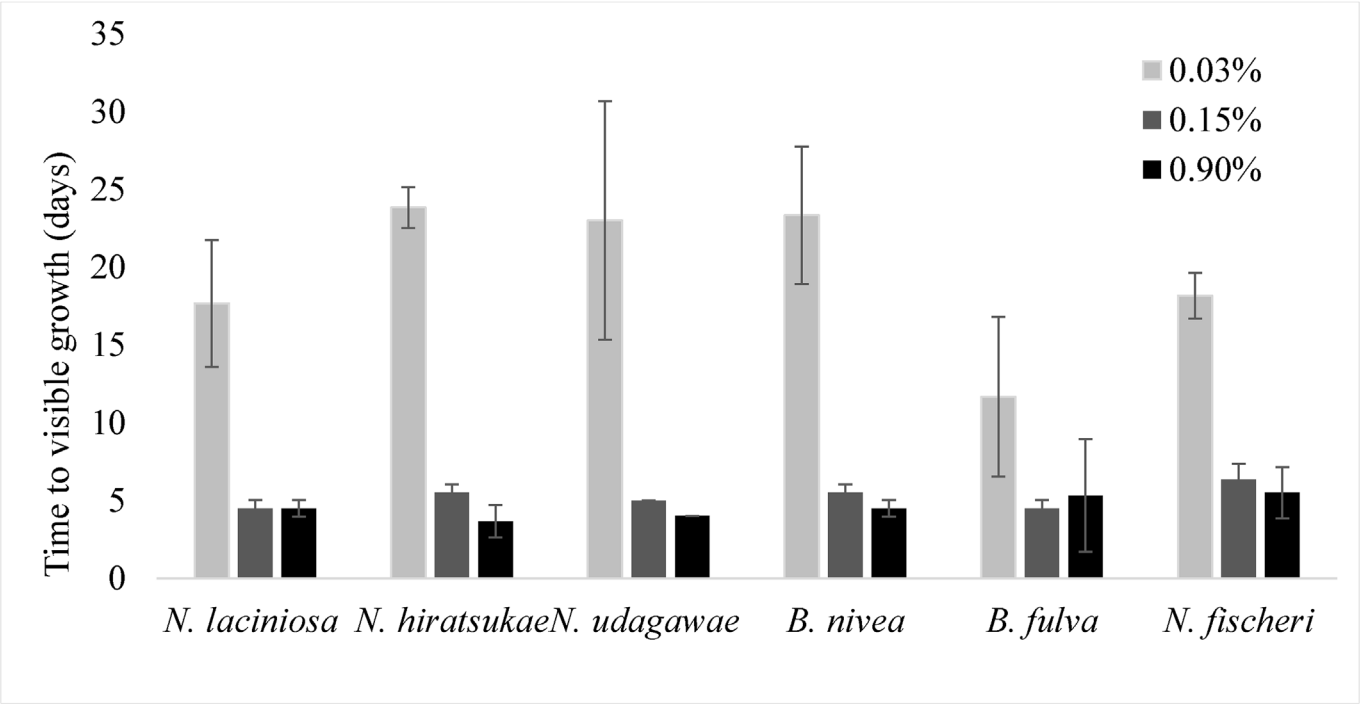

Fig. 2. Time to visible growth in aPDA for *N. laciniosa*, *N. hiratsukae*, *N. udagawae*, *B. nivea*, *B. fulva* and *N. fischeri* at three initial headspace O_2 levels (0.03, 0.15 and 0.9%).

Table 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

growth response (Amaeze, 2013; Dagnas et al., 2015, 2017; Santos et al., 2018b). 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, 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 and Hocking, 2009; Taniwaki et al., 2010). Studies indicate that concentrations higher than 40% noticeably inhibit the growth of most spoilage fungi (Taniwaki et al.,2010; 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 (Santos et al., 2018a; Tranquillini et al., 2017). Moreover some of them have been associated

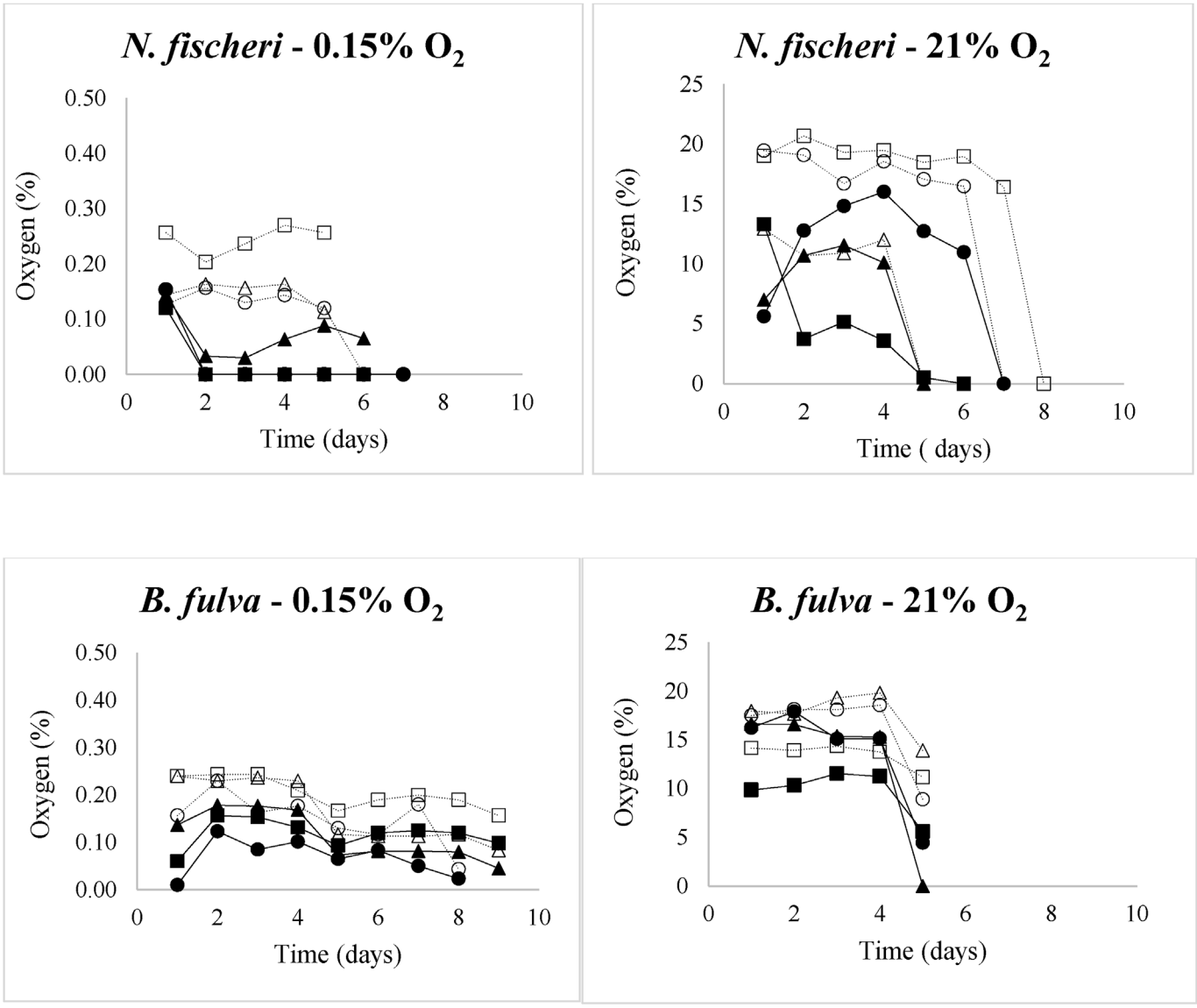

Fig. 3. Profile of O_2 (%) in the headspace (dotted line) and dissolved in fruit-based medium (solid lines) in apple puree (▲), strawberry puree (●) and orange juice (■). The medium were inoculated with *N. fischeri* and *B. fulva* and stored at 22 °C. The O_2 concentrations were set at 0.03% (●), 0.15% (▲) and 0.9% (♦). Each symbol represents the average O_2 level of the 2 repetitions.

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 to 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.

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; Santos et al., 2018a; Tournas, 1994). The composition of the three fruit-based media is shown in Table 1.

The evolution of the O_2 concentrations in the headspace and water phase of the fruit-based medium is shown in Fig. 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. 3).

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. 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.

The evolution of O_2 concentrations when the initial value was set at 0.15% is depicted in Fig. 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. 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. 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. 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. 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. 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

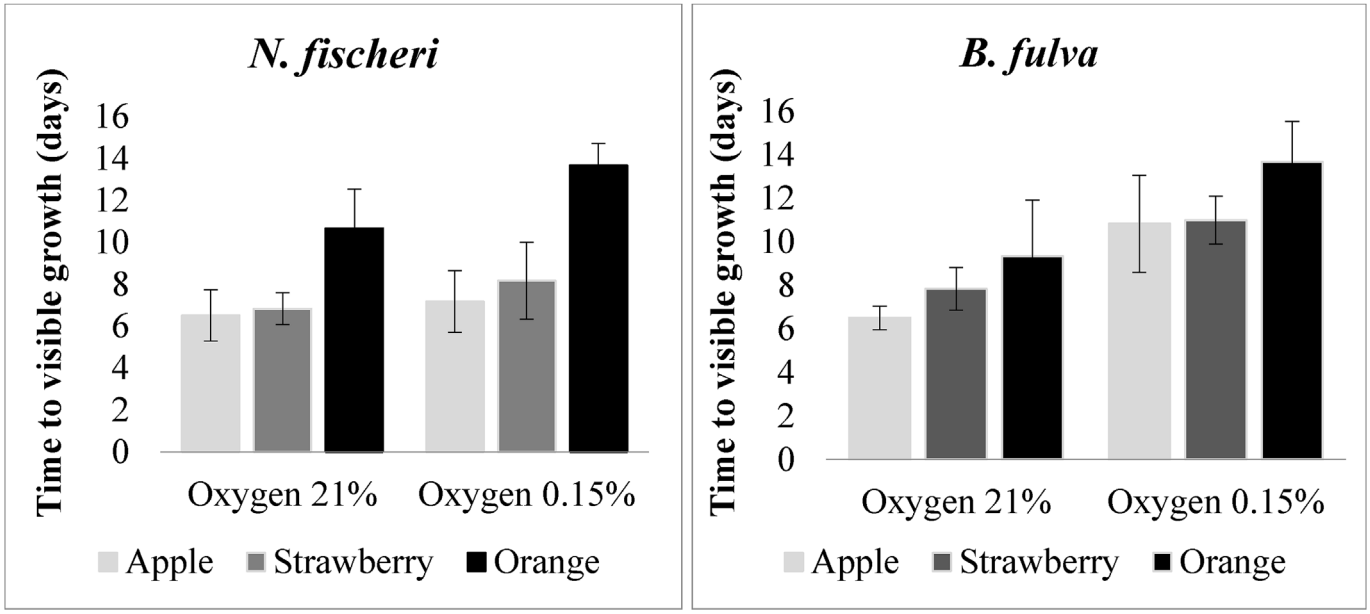

Fig. 4. Time to visible growth ($D \geq 2$ mm) of *N. fischeri* and *B. fulva* inoculated in fruit-based medium ($a_w = 0.960$) at 0.15% O_2 and 21% O_2 (atmospheric air) and 22 °C. The bars represent the average time (days) with the respective standard deviations.

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 and 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.

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.

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