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Evaluation of the inhibitory effect of alginate oligosaccharide on yeast and mould in yoghurt

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ABSTRACT

The use of alginate oligosaccharides (AOS) in fermented dairy products and tested against common spoilage organisms was investigated. In a broth model, yeast growth was measured by optical density, while hyphae propagation of mould was assessed with a live-cell imaging system. Reduction of the growth rate of *Candida parapsilosis*, *Debaryomyces hansenii* and *Meyerozyma guilliermodii* was observed in broth supplemented with 2% (w/v) AOS. Colony forming units (cfu) were enumerated to quantify yeast growth in fresh yoghurt prepared with AOS. Direct observation and pictures were taken to assess mould growth. The time to reach the spoilage limit (10^6 cfu g^{-1}) with 2% AOS was significantly increased for the yeasts *C. parapsilosis* and *M. guilliermodii*, by 11.8% and 22.5% respectively, while no effect of AOS was observed for all mould species. AOS could potentially be used as a food preservative against some yeasts, although a relatively high concentration is required.

1. Introduction

Throughout the past few decades, an increasing concern about the use of food additives has emerged (Wu, Zhang, Shan, & Chen, 2013). Diverse types of chemical compounds such as benzoates, nitrites, sulphites and sorbates are commonly used to supress the growth of microorganisms in food (Abdulmumeen, Risikat, & Sururah, 2012). Recent studies showed consumers are more aware of the use of food additives in foods and they are increasingly concerned about their impact on human health (Shim et al., 2011; Siegrist & Sütterlin, 2017). Despite the generally accepted safety of food additives by the scientific and regulatory communities, a study in Australia reported 30% of the participants considered food additives as the greatest potential hazard to safety and quality of a food product, and more than half perceived those compounds as having a negative impact on human health (Williams, Stirling, & Keynes, 2004). The use of food additives by industry is in conflict with the emerging consumer desire to seek naturalness in food products (Aoki, Shen, & Saijo, 2010; Bearth, Cousin, & Siegrist, 2014).

Alginate is a structural linear biopolymer found in the cell wall of various species of brown algae and has a natural high degree of polymerisation ranging from 50 to 3000 corresponding to a molecular mass ranging from 10 to 600 kDa (Pawar & Edgar, 2012). Alginate is mainly composed of building blocks of β-D-mannuronic acid called M-block and its C-5 epimer, α-L-guluronic acid, G-block, linked by a (1,4)-glycosidic bond (Grasdalen, 1983; Johnson, Craig, & Mercer, 2011). In food, alginates are frequently used in the form of sodium alginate for a wide range of applications, mainly based on the gelling properties of the polymer (Qin, Jiang, Zhao, Zhang, & Wang, 2018). Recently, studies investigated alginate oligosaccharides (AOS) obtained by either acid hydrolysis or enzymatic degradation with alginate lyase (An et al., 2009; Lu et al., 2015; Wong, Preston, & Schiller, 2000) and their potential application (Akiyama et al., 1992; Ramnani et al., 2012; Wang, Han, Hu, Li, & Yu, 2006).

The antifungal activity of AOS towards diverse species of *Candida* and *Aspergillus* has recently been described (Tøndervik et al., 2014). Moreover, AOS was also described to potentiate the activity of certain antibiotics (Khan et al., 2012) as well as antifungal drugs. A study also reported AOS to present a strong scavenging activity against free radicals (Falkeborg et al., 2014). The antioxidant properties as well as the calcium chelating capacity of poly-G-blocks make AOS a strong candidate for

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preserving the biochemical integrity of fermented dairy products by avoiding fat oxidation and protein aggregation. Thus, this leads to the hypothesis that AOS could be of interest in food applications as a potential preservative agent against yeast and mould spoilage.

Dairy products represent a major food type in many diets and are as many other product subjects to contamination and spoilage, most frequently by opportunistic fungi (Lu & Wang, 2017). However, the negative attitude of consumers to the use of chemicals in dairy products (Collins, 1967) is a key factor that drives innovation towards cleaner labels and more natural solutions.

In this work, we investigated the potential antifungal effect of AOS toward dairy spoilage, focussing mainly on yoghurt. To assess the activity of AOS, a diverse list of spoilage fungi from dairy sources was initially selected. Different concentrations of AOS were tested in a broth model, and subsequently tested in a yoghurt matrix using single culture experiments. This work aimed to answer the question whether AOS could be used as a potential novel food additive for dairy applications. This study is believed to be the first in which the antifungal activity of AOS in food has been investigated.

2. Material and methods

2.1. Fungal strains and cultivation methods

The strains used in this study (Table 1) were selected to be representative of dairy spoilage fungi and were obtained from the Leibniz Institute DSMZ-German collection of microorganisms and cell cultures (Braunschweig, Germany). Each species was reactivated following the protocol provided by DSMZ. The dry pellet was resuspended into 5 mL of YM broth (BD Difco, Heidelberg, Germany). Yeast strains were incubated aerobically for 48 h at 30 °C, and the purity of the suspension was checked by streaking on agar plates. Subsequently, frozen stocks were prepared from the broth by adding glycerol to a final concentration of 20% (v/v) and stored at −60 °C. Mould strains were spread on YM-agar plates and incubated aerobically at 25 °C for 5 days (±2 days). Frozen stocks were prepared by harvesting the spores from the surface of the agar plate with a saline solution (per litre: 8.5 g NaCl, 1 g casein peptone) supplemented with 0.25% (v/v) Tween 80 and mixed with glycerol for a final concentration of 20% (v/v) and stored at −60 °C. The purity of the suspension was checked by streaking on agar plates.

Table 1
List of microorganisms.

Species	DSM number	Source
Yeasts		
Candida parapsilosis	DSM-70125	Sausage
Debaryomyces hansenii	DSM-70590	Harzer cheese
Meyerozyma guilliermondii	DSM-70052	Butter
Yarrowia lipolytica	DSM-70562	Marzipan
Moulds		
Mucor racemosus	DSM-5266	Unknown
Penicillium commune	DSM-2211	Cheese
Penicillium roqueforti	DSM-1079	Gorgonzola cheese

2.2. Preparation of alginate oligosaccharides

Alginate oligosaccharides (AOS) were supplied by DuPont Nutrition and Health (Sandvika, Norway). The alginate was extracted from the stem of brown seaweed *Laminaria hyperborea*, subsequently subjected to high temperature acid hydrolysis, followed by neutralisation with Na_2CO_3 before spray drying.

2.3. High-performance anion-exchange chromatography-pulsed amperometry detection analysis

AOS size distribution was assessed by high-performance anion-exchange chromatography with pulsed amperometry detection (HPAEC-PAD). An aqueous solution of 1% (w/v) AOS was simultaneously injected with 15% 1 M sodium hydroxide solution onto a Dionex CarboPac PA100 column (4 × 50 nm) (ThermoScientific, Roskilde, Denmark). Elution was performed using a linear gradient of 0–85% 1 M sodium acetate/25 mM sodium hydroxide over 60 min using a flow of 0.4 mL min^{-1}.

2.4. NMR analysis

Ten milligrams of AOS was dissolved in 2 mL 150 mM sodium phosphate D2O buffer with 0.1% (w/v) 3-trimethylsilyl-[2,2,3,3-D4]-propionate (TSP) as an internal standard and used for NMR characterisation according to the ASTM standard protocol F2259-10-(2012)e1 for analysis of sodium alginate (ASTM International, 2012). NMR analysis was performed on a 600 MHz Bruker Avance III spectrometer equipped with a 5-mm BBO smart probe (Bruker Biospins, Rheinstetten, Germany). A standard ^1H pulse-sequence was used to acquire ^1H NMR spectra at 353 K. A total of 64 scans collected into 64K data points were acquired with a spectral width of 20.55 ppm, a recycle delay of 20 s and an acquisition time of 2.66 s. Each spectrum was automatically phased and referenced to the TSP signal at 0 ppm using TopSpin 3.2 (Bruker BioSpin, Rheinstetten, Germany). Automated determination of the M/G ratio and block structure from the NMR spectra was performed using Python 2.

2.5. Inhibition assay

The potential antifungal effect of AOS was tested against each fungal species in a single culture experiment in a 96-well microtiter plate (ThermoScientific, Roskilde, Denmark). Yeasts were pre-cultured for 24 h into YM broth and inoculated at an initial level of 10^4 cfu mL^{-1} into a YM-broth. The final volume inside the microtitre plate was 200 μL. AOS were added to the media at 0, 0.1, 0.5 and 2% (w/v) final concentration. Each concentration was tested in triplicate against each strain, and every experiment was performed in duplicate. Potassium sorbate was used as an antifungal reference at a concentration of 0.03% (w/v). The microtiter plate was incubated for 72 h at 25 °C. Every 3 h the plate was shaken at medium speed for 10 s and optical density (OD_{600}) was measured by an Elx808 microplate reader (BioTek Instruments Inc, Hørsholm, Denmark). Mould strains were inoculated from frozen stocks at 10^3 spores mL^{-1} following the same experimental design as described for the yeasts. Mould growth was monitored with an oCelloscope (Biosense solutions, Copenhagen, Denmark). The live-cell imaging

system of oCelloscope provided a better insight of hyphae formation compared with OD measurement.

2.6. Challenge study in yoghurt

Reconstituted milk was prepared by mixing 300 g of medium heat skimmed milk powder (Arla Foods, Aarhus, Denmark) with 2700 mL of water. AOS was added to the reconstituted milk at a final concentration of 0, 0.1, 0.5 and 2% (w/v). Heat treatment was performed at 85 °C for 20 min. The milk was then cooled to 45 °C and inoculated with 0.02% (w/w) of the starter culture F-DVS-L901: blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* species (Chr. Hansen, Hørsholm, Denmark). Fermentation was carried out at 45 °C. When the pH reached the value of 4.5, the yoghurt was cooled to 10 °C. A yoghurt supplemented with 0.03% (w/v) potassium sorbate was used as an antifungal reference. A portion (75 g) of yoghurt was poured into a plastic beaker and inoculated with a single culture of yeast or mould. Yeasts were pre-cultured for 24 h in YM broth and inoculated into the yoghurt to give a concentration of 10 cfu g^{-1} of yoghurt. Mould strains were directly diluted from frozen stock and added into the yoghurt to give a concentration of 10 spores g^{-1}. All yoghurts were incubated at 10 °C for 15 days. Yeasts were enumerated by plate counting on YPD-agar (BD Difco, Heidelberg, Germany) supplemented with 0.1 g L^{-1} of chloramphenicol and oxytetracycline (Sigma, Søborg, Denmark). Mould growth was visualised photographically using a EOS 400D Canon camera. For each experiment, two biological replicates with three technical replicates were performed. According to literature, a spoilage limit of 10^6 cfu g^{-1} was fixed as the limit of detection for which off-odours and change of aspect of the yoghurt due to microbial spoilage can be perceived by consumers (Liptáková, Valík, & Bajúsová, 2006; Sperber & Doyle, 2009).

2.7. Statistical analysis

Statistical analysis was performed with the open-access "R" software. Using the package "Growthcurver" (Sprouffske & Wagner, 2016) a logistic model was fitted, and a growth rate was determined for every replicate of the broth model, then compared using a one-way ANOVA to calculate significant difference between growth condition. A model based on the same logistic equation was fitted using "drc" package (Ritz, Baty, Streibig, & Gerhard, 2015) for every growth kinetic of the yoghurt trial. Employing the ED () function, the time to reach spoilage limit of 10^6 cfu g^{-1} was estimated and similarly compared by one-way ANOVA.

3. Results

3.1. Alginate oligosaccharides characterisation

The chromatogram obtained from the HPAEC analysis is presented in Fig. 1. The AOS was composed of a mixture of oligomers with a degree of polymerisation ranging from approximately 1 to 40. A large portion of the solution was characterised as mono-, di- and trisaccharide. Following the standardised protocol of ASTM International, NMR analysis presented in Table 2 revealed the alginate source contained 35% mannuronic acid and 65% guluronic acid, as well as a calculated number average degree of polymerisation of the AOS solution of approximately 20.

3.2. Broth model experiment

Growth curves obtained from OD measurement are presented Fig. 2. Direct observation of the curves indicated a dose-dependent effect of AOS against *Candida parapsilosis*, *Debaryomyces hansenii* and *Meyerozyma guilliermodii*. After calculation of growth capacities, *C. parapsilosis* and *D. hansenii* showed a significant reduction of their growth rates in media supplemented with either 2% (w/v) AOS or potassium sorbate (Fig. 3), decreasing from approximately 0.21 h^{-1} to 0.16 h^{-1} and 0.14 h^{-1} to 0.107 h^{-1} respectively. No other differences were observed at any concentration lower than 2% AOS. Interestingly, the growth rate of *M. guilliermodii* was statistically reduced at every condition compared with control from approximately 0.145 h^{-1} to 0.120–0.125 h^{-1}. Finally, no satisfactory growth measurement using optical density was possible for *Yarrowia lipolytica* probably due to its partial hyphal-like growth characteristics. The *p*-value above 0.05 obtained from the one-way ANOVA of the growth rate confirmed that difference within the values were not due to the experimental design but from another source. Therefore, it was also investigated with oCelloscope. No reduction of the growth of *Y. lipolytica* was observed in media supplemented with 0.1, 0.5 and 2% AOS compared with control. Furthermore, no effect of the addition of potassium sorbate in the media was observed. These results, in agreement with previous literature, showed the capacity of *Y. lipolytica* to grow at low concentration of common preservatives (Praphailong & Fleet, 1997).

The mould growth was measured by oCelloscope. A typical example of the growth of the mould is presented Fig. 4. Using the algorithm "Background corrected absorption", the light intensity for each pixel detected as "background pixel" and "object pixel" was computed and growth curves (Fig. 5) were generated based on the evolution of the object compared with the background. No significant difference of growth for all the moulds was observed at all concentration of AOS tested. Growth reduction was only observed with potassium sorbate for all the mould species.

3.3. Yoghurt assay

Fresh yoghurts were prepared by fermentation of the reconstituted milk supplemented with the various concentrations of AOS. No changes in pH were observed after addition of the AOS to the milk, and no significant difference of the pH profile during fermentation was seen. Yeast cell concentration was measured by plate counting (Fig. 6). The growth curves of both *C. parapsilosis* and *M. guilliermodii* inoculated into yoghurt with 2% (w/v) AOS were noticeably delayed when meeting the spoilage limit of 10^6 cfu g^{-1}. No growth was observed throughout the experiment when adding potassium sorbate into yoghurt for *C. parapsilosis*, *D. hansenii* and *M. guilliermodii*. *Y. lipolytica* showed an increasing growth after 8 days, without reaching 10^6 cfu g^{-1}.

Using a logistic equation to model the growth of each yeast, an estimated time to reach the spoilage limit was calculated. At the highest concentration of AOS (2%) the growth of *C. parapsilosis* and

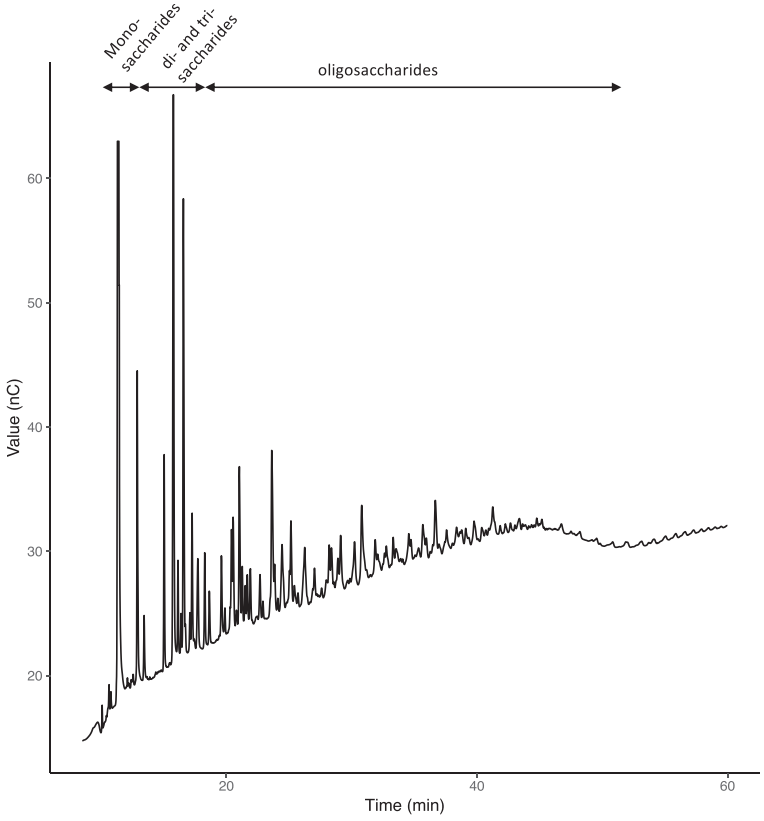

Fig. 1. Characterisation of a 1% (w/v) solution of alginate oligosaccharides (AOS) with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Table 2
Fractions of monades, diades and triades of alginate oligosaccharides (AOS) obtained by NMR analysis using a standard ^{1}H pulse-sequence.[a]

M/G	GG	MG	MM	GGM	MGM	GGG	DP_N
0.3504 (±0.015)	0.6353 (±0.003)	0.1053 (±0.006)	0.1542 (±0.014)	0.0377 (±0.003)	0.0675 (±0.003)	0.5976 (±0.001)	20.135 (±4.16)

[a] Abbreviations are: M, mannuronic acid; G, guluronic acid; DP_N, number average degree of polymerisation.

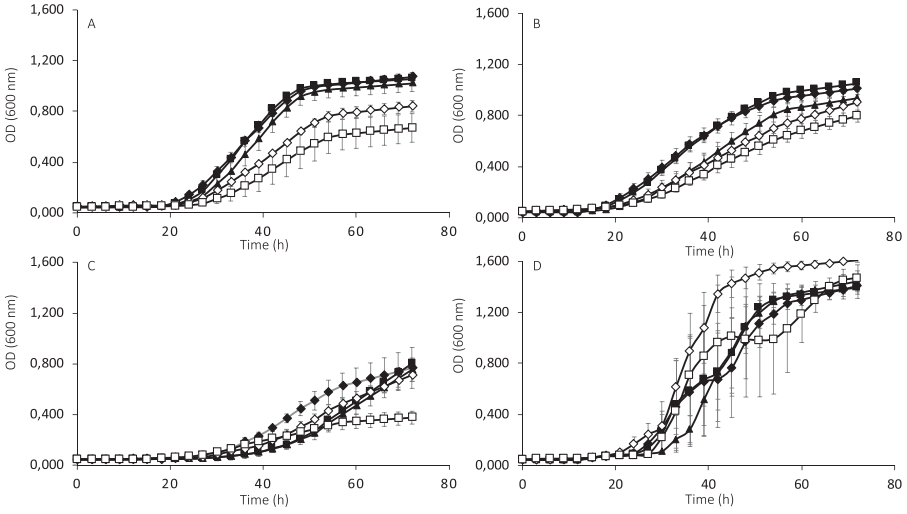

Fig. 2. Growth curves of *Candida parapsilosis* (A), *Debaryomyces hansenii* (B), *Meyerozyma guillermondii* (C) and *Yarrowia lipolytica* (D) incubated at 25 °C for 72 h. Each species was inoculated into YM media supplemented with 0.0 (◆), 0.1 (■), 0.5 (▲), 2% (w/v) AOS (◇) or 0.03% (w/v) potassium sorbate (□).

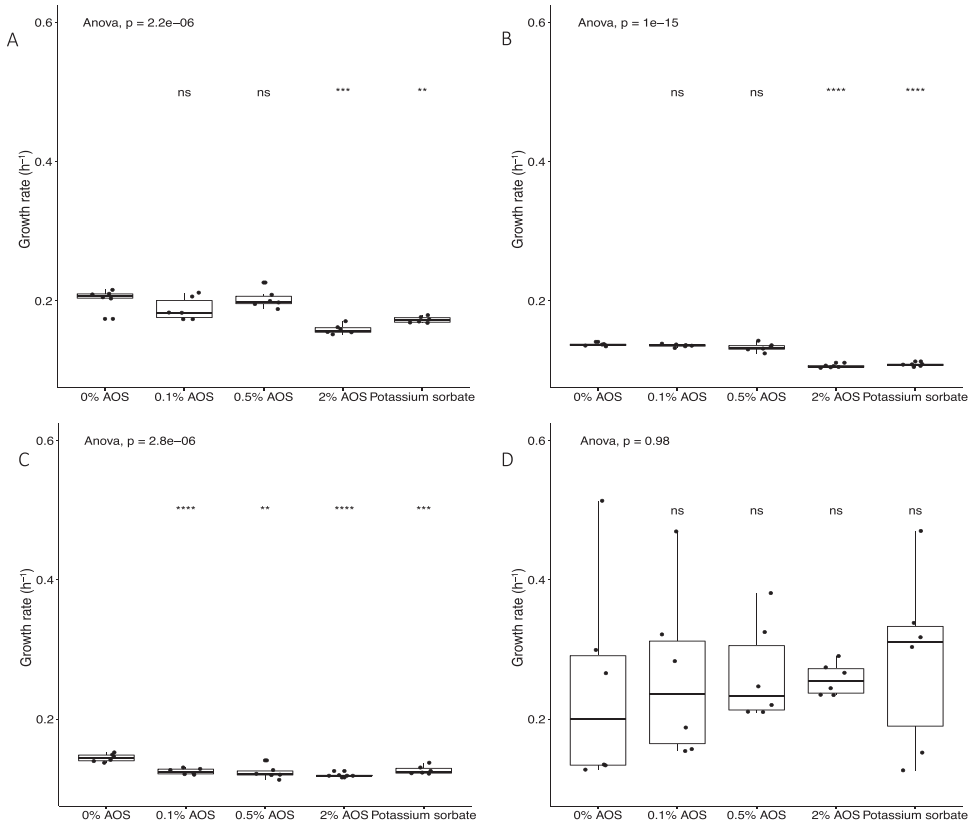

Fig. 3. Growth rates of *Candida parapsilosis* (A), *Debaryomyces hansenii* (B), *Meyerozyma guillermondii* (C) and *Yarrowia lipolytica* (D) incubated at 25 °C for 72 h (n = 6). Each species was inoculated into YM media supplemented with either 0.0, 0.1, 0.5, 2% (w/v) AOS or 0.03% (w/v) potassium sorbate. The optical density was measured at regular intervals and growth rates were calculated using the Growthcurver "R" package.

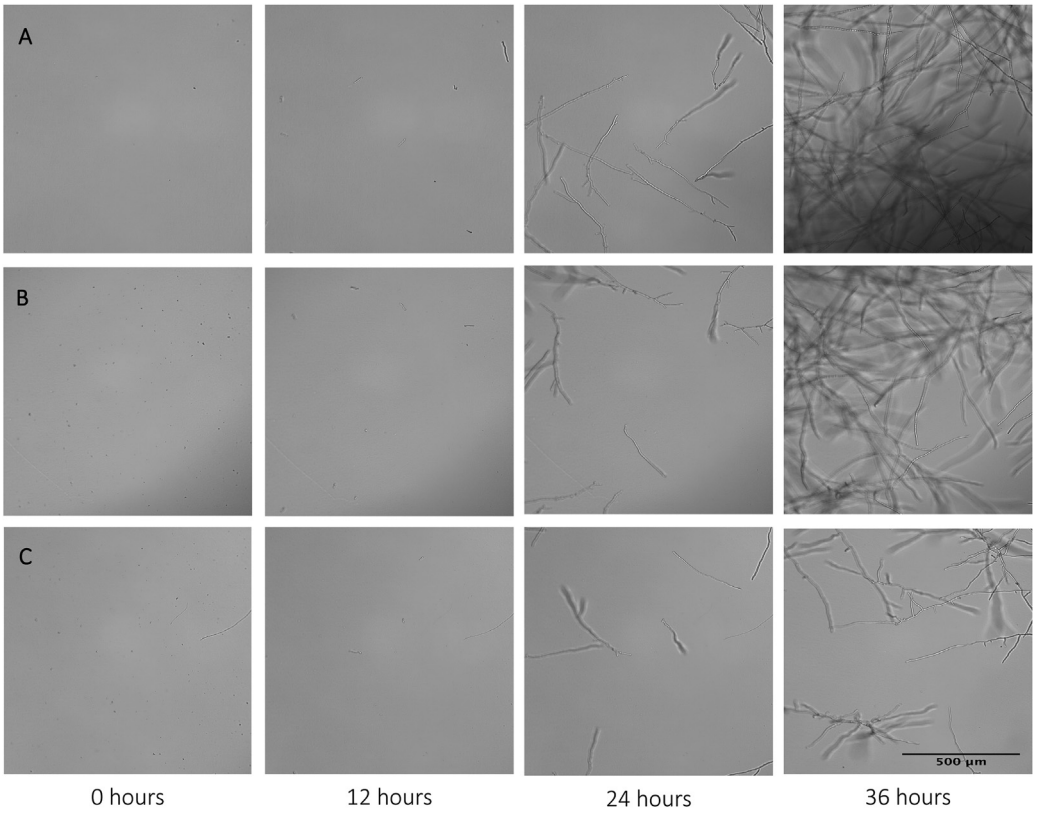

Fig. 4. Pictures of *Penicillium commune* DSM 2211 grown under different conditions. Each picture was taken with an oCelloscope at 12 h interval. A) control medium YM media; B) YM broth supplemented with 2% (w/v) AOS, C) YM media supplemented with 0.03% (w/v) potassium sorbate.

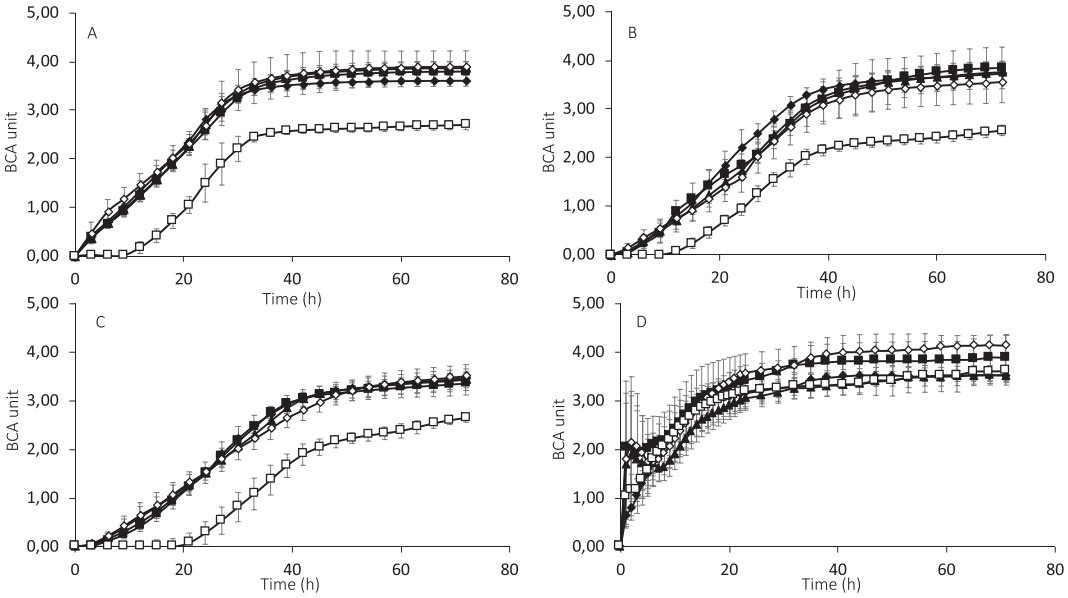

Fig. 5. Computed growth curves of *Mucor racemosus* (A), *Penicillium commune* (B), *Penicillium roqueforti* (C) and *Yarrowia lipolytica* (D) incubated in YM broth at 25 °C for 72 h. Each species was incubated into YM media supplemented with 0.0 (◆), 0.1 (■), 0.5 (▲), 2% (w/v) AOS (◇) or 0.03% (w/v) potassium sorbate (□).

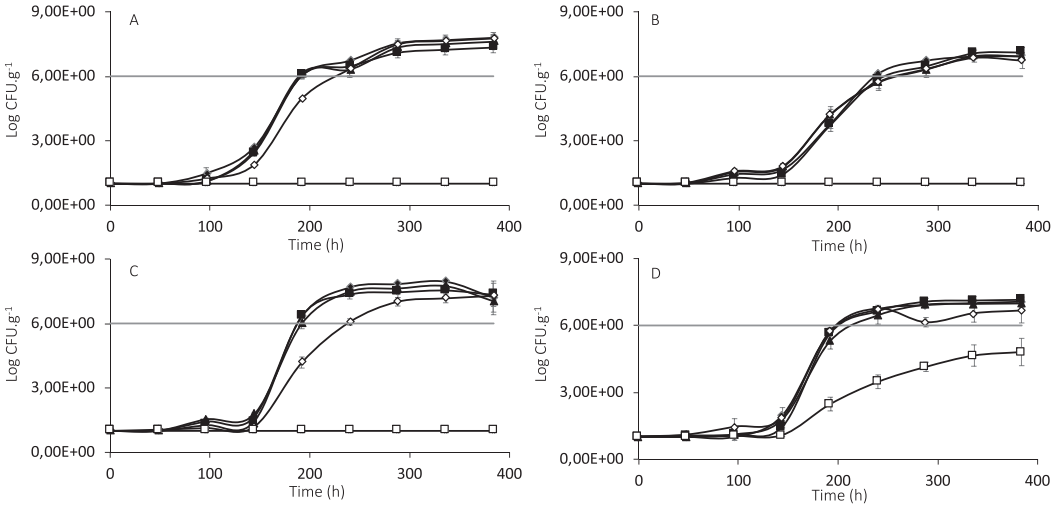

Fig. 6. Growth curves of *Candida parapsilosis* (A), *Debaryomyces hansenii* (B), *Meyerozyma guillermondii* (C) and *Yarrowia lipolytica* (D) incubated at 10 °C for 15 days. Each species was incubated into freshly produced yoghurt supplemented with 0.0 (◆), 0.1 (■), 0.5 (▲), 2% (w/v) AOS (◇) or 0.03% (w/v) potassium sorbate (□); spoilage limit of 10^6 cfu g^{-1} (▬ ▬).

M. guilliermodii was significantly delayed. The time to reach the spoilage limit was increased from 196.48 ± 3.70 h to 219.61 ± 4.20 h for *C. parapsilosis* and 190.26 ± 1.13 h to 233.14 ± 4.73 h for *M. guilliermodii* corresponding to a delay of approximately 23 h and 43 h respectively (Table 3).

Finally, the moulds species showed no significant reduction of their growth in yoghurt when supplemented with the AOS at all concentrations (Fig. 7). Clear reduction of the growth was noted in presence of potassium sorbate. Texture analysis was performed on yoghurts after fermentation (data not shown). A slight increase in

Table 3
Estimated time in hours to reach spoilage limit (10^6 cfu g^{-1}) in yoghurt for each yeast species.[a]

Species	AOS (%)			
	0	0.1	0.5	2
Candida parapsilosis	196.48 ± 3.70	196.21 ± 4.0	197.47 ± 4.15	219.61 ± 4.20***
Debaryomyces hansenii	236.21 ± 3.8	249.47 ± 7.9	253.52 ± 7.56*	252.45 ± 12.14
Meyerozyma guilliermondii	190.26 ± 1.13	186.80 ± 2.84	192.03 ± 0.77	233.14 ± 4.73***
Yarrowia lipolytica	199.58 ± 3.19	200.93 ± 2.65	211.38 ± 3.63*	193.58 ± 1.46

[a] With potassium sorbate (0.03%), no growth above 10^6 cfu g^{-1} was observed during the time of experiment. Asterisks indicate statistical differences between values in a row (n = 6): *$p < 0.5$; ***$p < 0.001$.

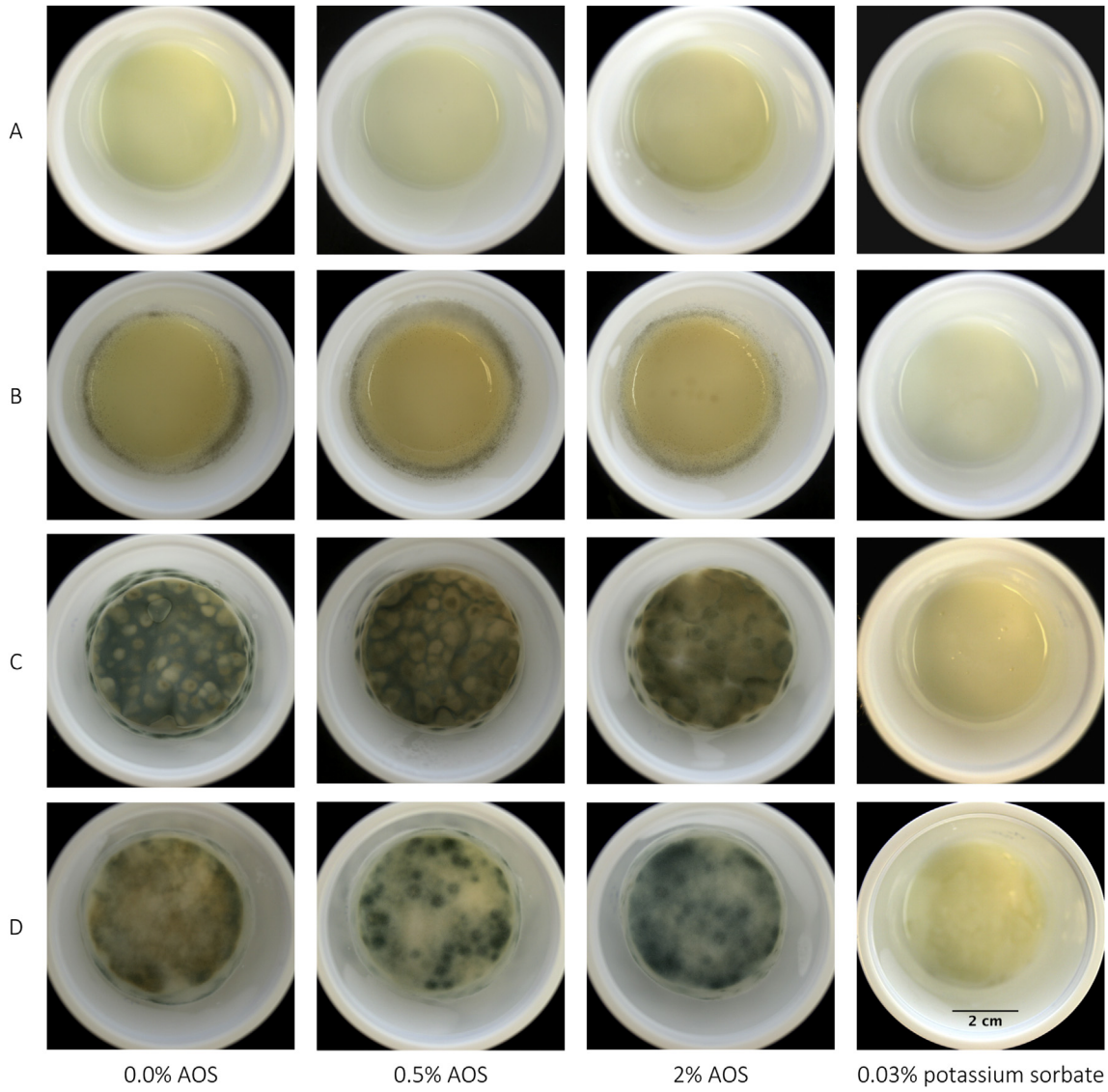

Fig. 7. Yoghurt after 15 days of storage at 10 °C supplemented with different concentration of AOS or potassium sorbate. Yoghurts were without inoculation (A) or inoculated with 10 spores g^{-1} of *Mucor racemosus* (B), *Penicillium commune* (C) or *Penicillium roqueforti* (D).

the firmness and the cohesiveness of yoghurts was observed in a dose-dependent manner for AOS addition.

4. Discussion

Alginate is a common food additive used for diverse applications mainly based on its gel-forming properties (Grasdalen, 1983; Johnson et al., 2011). Recent studies demonstrated that AOS exhibit direct antifungal properties as well as potentiating the performance of antifungal drugs (Tøndervik et al., 2014) and selected antibiotics (Khan et al., 2012). These properties may therefore present an opportunity for the food industry for the inhibition of opportunistic spoilage fungi. This type of spoilage has led the scientific community into an intensive research for food preservatives to extend the shelf-life of food products (Barreteau, Delattre, & Michaud, 2006; Brul & Coote, 1999). In this study, structured in a two-step approach, the potential antifungal activity of AOS against dairy spoilage fungi was investigated.

AOS obtained were characterised by HPAEC and NMR. The molecular mass distribution after acid hydrolysis observed by chromatography was similar to AOS previously reported (Khan et al., 2012). The NMR profile correlated with previous analyses using alginate from *L. hyperborea* (Grasdalen, 1983; Stephen, Phillips, & Williams, 2006) while also indicating a number average degree of polymerisation of the oligomers of approximately 20, which is in good agreement for alginate-derived oligosaccharides (Onsoyen, Myrvold, Onsøyen, & Myrvold, 2014). As such, the AOS in this study were well defined and presented a pattern of depolymerisation expected with respect to the current literature.

Results obtained with the broth model indicated similar reduction of the growth rate of *C. parapsilosis*, *D. hansenii* and *M. guilliermodii* when incubated into YM-broth supplemented with 2% (w/v) AOS or 0.03% (w/v) potassium sorbate (maximal concentration allowed by the EC 1333/2008 of the European commission in flavoured fermented dairy product). In agreement with these results, the estimated time to reach a concentration of 10^6 cfu g^{-1} in yoghurt with 2% AOS was significantly increased for both *C. parapsilosis* and *M. guilliermodii* by 11.8% and 22.5% respectively. Interestingly, no growth reduction effect was observed within the

yoghurt for any concentration tested of AOS against *D. hansenii*. Furthermore, while the reduction of the growth rate in the broth model was equivalent for both 2% AOS and 0.03% potassium sorbate, the effect of potassium sorbate was greatly superior in the yoghurt. The results demonstrated the need for the yoghurt challenge study to correctly assess the antifungal property of AOS in a food matrix.

No change of pH due to the addition of the AOS in the yoghurt was seen whereas an increase in the firmness and cohesiveness were observed. While this study did not focus on the impact of AOS onto the yoghurt texture, it is hypothesised the poly-G fragment of high polymerisation degree may conserve a high binding affinity for calcium ions, giving a texturing capacity of the AOS without forming a gel.

Sorbic acid and its salts have been widely described and used as preservatives in food (Lück, 1990; Sofos & Busta, 1981). The main mechanisms of inhibition of the spoilage organisms relies on the interaction of the sorbate with the cellular membrane, the inhibition of transport systems and/or key enzymes, the deregulation of proton flux or a combination of these cited actions. Unlike the sorbates, the mechanism of action of AOS remains unclear. AOS are essentially composed of weak acids (mannuronic acid and guluronic acid) mainly in form of monomers, dimers and trimers. Although the mode of action of the sorbates is still a source of discussion (Stratford & Anslow, 1998), it is speculated that these oligosaccharides may have acted like a weak acid preservative against the fungal species and having a similar effect as the sorbates.

Tøndervik et al. (2014) demonstrated the potential of AOS to directly interact with biofilms as well as the cell membrane of bacteria (Khan et al., 2012). As such, their results described a clear loss of motility of the cell membrane and morphological changes. Nevertheless, they later refuted the possible binding of AOS with the fungal cell wall while still observing morphological changes. Thus, the direct antifungal effect of AOS remains unknown.

Although this study described an activity against certain species of yeast, many more species still remained to be investigated to assess the full potential of AOS. Besides, the AOS used in this study were obtained by acid hydrolysis, a recent review emphasised the importance of enzymatic degradation for the yield and recovery of bioactive compounds (Charoensiddhi, Conlon, Franco, & Zhang, 2017). Consequently, the use of enzymatic depolymerisation method remains to be investigated. Synergic effects with other preservatives could be an adequate way to improve the efficiency of the AOS while reducing the final quantity of preservatives in the food product. However, further investigations are needed to assess the full potential of these oligosaccharides as well as to define a clear mechanism of action.

5. Conclusion

AOS has a potential antifungal effect against certain dairy spoilage yeasts although relatively a high concentration is required. No clear effect was observed against mould species. These oligomers present therefore an opportunity for the food industry to change consumer perception of preservatives, while ensuring safety of the food product for which yeast is the main source of spoilage.

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