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Quantitative antifungal activity of reuterin against food isolates of yeasts and moulds and its potential application in yogurt

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ABSTRACT

Reuterin is an antimicrobial agent produced by conversion of glycerol and excreted by several bacterial species including the food grade lactic acid bacterium *Lactobacillus reuteri*. Several inhibitory activities have been reported to reuterin against a broad range of Gram-positive and Gram-negative bacteria, bacterial spores, moulds, yeasts and protozoa. However, the antifungal and anti-yeast activity of reuterin is poorly documented.

The aim of the current work was:1) To quantify the minimum inhibitory activity (MIC) and the minimum fungicidal activity (MFC) of reuterin against a representative panel of the most abundant fungi and yeast species associated with food contamination; 2) To investigate the application of reuterin as antifungal agent for bio-preservation of yogurt.

Reuterin was produced by *L. reuteri* ATCC 53608 in MRS and glycerol solution then purified before using. Our data showed that purified reuterin inhibited the growth of tested microorganisms at a concentration of 11 mM or less. Moreover, reuterin showed a fungicidal activity (killed 99.9% of all tested microorganisms) at concentrations equal or below 15.6 mM as indicated by MFC. Values of MFC were comprised between 1.0 and 4.8 of the MIC values, suggesting a potent fungicidal mechanism on both yeasts and filamentous moulds with one exception only. In yogurt, reuterin showed a fungistatic effect at a concentration of 1.38 mM while a fungicidal effect was obtained at 6.9 mM. Therefore, reuterin has a high potential as a food preservative, particularly owing to its biochemical properties and antibacterial and antifungal activities.

1. Introduction

Yeasts and filamentous moulds are commonly identified as spoilage microorganisms of food products, stored crops and feed (Pitt and Hocking, 2009). Yeasts such as *Candida*, *Kluyveromyces* and *Rhodotorula* are common spoilage of dairy and meat products while *Saccharomyces*, *Schyzosaccharomyces*, *Torulospora* and *Zygosaccharomyces* are frequently found in beverages.

Fungi are recognized as food spoilage microorganisms. Therefore, they render the human consumption of food which represents huge economic losses for the food industry.

Aspergillus, *Aureobasidium*, *Eurotium* and *Penicillium* are reported as spoilage filamentous moulds for a wide range of food products including meat, marine, dairy products and cereals (Ledenbach and Marshall, 2009; Pitt and Hocking, 2009). Fungal spoilage threatens both food quality and public health due to the possible production of mycotoxins. (Pitt and Hocking, 2009). Furthermore, mould growth on food products such as bakery products is a serious economic concern as it is the major factor limiting shelf life of these products (Saranraj, 2012).

To control these microorganisms, preservatives are commonly added. The most commonly used antifungal compounds for the preservation of foods are the weak acids or natamycin (also called pimaricin) (Chen et al., 2008; Pitt and Hocking, 2009). Weak acids, such as benzoic or sorbic acids, cause the microbial death by inducing a stress response that restores homeostasis, resulting in the reduction of available energy for growth and other essential metabolic functions (Brul and Coote, 1999). Natamycin is a member of the polyene antibiotic family which binds specifically to ergosterol, preventing it from performing its functional effects (Welscher et al., 2008).

Food spoilage yeasts and moulds are becoming resistant to antibiotics and also to preservatives (Brul and Coote, 1999). In this regard, some fungal species possess mechanisms of resistance to the preservatives. For instance, a number of *Penicillium* species have acquired

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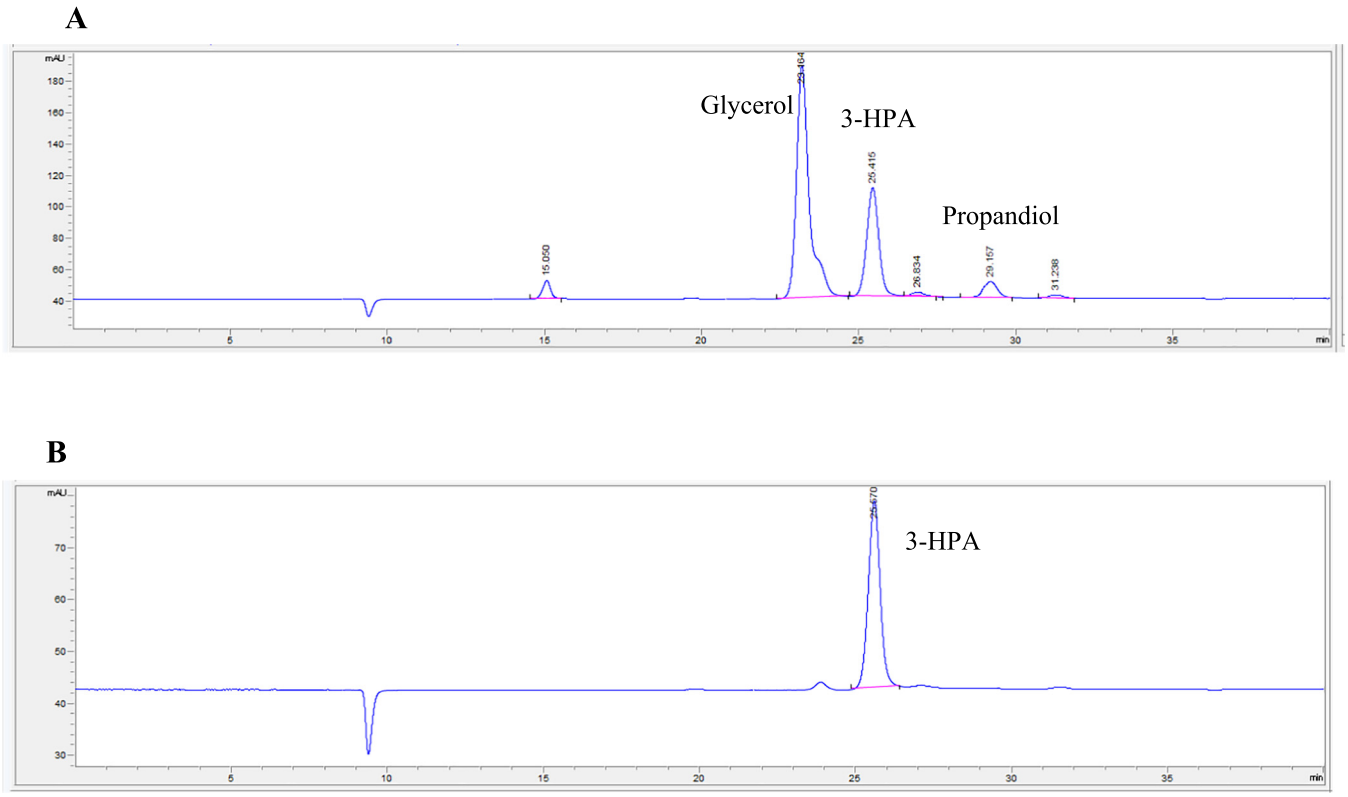

Fig. 1. HPLC chromatograms of reuterin: A) non-purified supernatant; B) purified reuterin.
Ion exclusion HPLC was performed using Coregel ION-300 column (7.8 × 300 mm, sulfonated polystyrene/divinylbenzene copolymers) at 40 °C. Elution was made with isocratic 10 mM H_2SO_4 with a flow rate of 0.4 mL/min and a refractive index detector use polystyrene/divinylbenzene copolymers. The detected peaks are: Glycerol, RT: 23.5; Reuterin (3-HPA), RT: 25.5; and Propandiol, RT:29.2.

the ability to degrade sorbate by its decarboxylation into trans-1,3-pentadiene, causing an off-odor and a "kerosene-like" flavor (Stopforth et al., 2005). The growing consumer concerns about food safety issues have raised the interest in producing bio-preservatives such as protective culture and their metabolites, including reuterin.

Reuterin is an antimicrobial agent produced by *Lactobacillus reuteri*, a hetero-fermentative lactic acid bacterium found in a variety of ecological niches like food fermentations or intestinal gut. Reurerin could also be produced by other genera of bacteria including *Bacillus*, *Citrobacter*, *Clostridium*, *Enterobacter* and *Klebsiella.* Reuterin is produced by converting the glycerol into 3-hydroxypropionaldehyde (3-HPA) through a coenzyme B12-dependent enzymatic reaction catalysed by the glycerol dehydratase (Vollenweider and Lacroix, 2004). Until 2016, reuterin was defined as a dynamic system, containing 3-HPA, its hydrate 1,1,3-propanetriol and the dimer 2-(2-hydroxyethyl) – 4-hydroxy-1,3-dioxane (Vollenweider and Lacroix, 2004). Recently, Engels et al. (2016) demonstrated that 3-HPA spontaneously dehydrates in aqueous solution to form acrolein. They therefore proposed to include acrolein in the definition of reuterin. By the means of 3-HPA and acrolein, reuterin has been proposed to induce oxidative stress in cells, most likely by modifying thiol groups in proteins and small molecules, causing the depletion of glutathione and modification of functional enzymes in particular (Engels et al., 2016).

Regarding its antimicrobial activities, reuterin has been reported to inhibit a broad range of Gram-positive and Gram-negative bacteria, bacterial spores, fungi and protozoa, including various food spoilers and pathogens (Ávila et al., 2014). Moreover, reuterin has been reported as a high potential food preservative (Vollenweider and Lacroix, 2004). However, its antifungal properties are poorly documented, in particular because the inhibiting molecules inside reuterin were not yet chemically characterized. To our knowledge, the only available minimal inhibitory concentrations (MIC) for reuterin against moulds were established from a supernatant of *L. reuteri* 1063 grown in the presence of glycerol (Chung et al., 1989). However, the authors reported the MIC as activity units rather than providing exact effective concentrations. The results showed that MIC comprised between 2 and 14 unit/mL for four yeast strains, and between 8 and 36 unit/mL for two mould strains. These concentration were later on recalculated based on the concentration of the aldehydic monomer and reported as molar concentrations (Stevens et al., 2011). Nakanishi et al., (Nakanishi et al., 2002) also reported a complete growth inhibition of four yeast strains and 11 moulds after 24 h of contact with the supernatant containing reuterin.

Therefore, this study aimed to characterize the antifungal properties of pure reuterin by providing exact effective MIC and minimal fungicidal concentrations (MFC) against 10 moulds and 14 yeasts that represent the most abundant fungi and yeast species associated with food contamination.

2. Materials and methods

2.1. Fungal strains and media

Fungal strains used in this study were isolated from the environment, food or dairy products and were referenced in four different strain collections (Table 1). They represent some of the most abundant fungal species found as food contaminant (Pitt and Hocking, 2009). Moulds were grown at 25 °C for at least 7 days on Potato Dextrose Agar (PDA, BD-Difco, Sparks, MD, USA) while yeasts were grown at 30 °C for 48 h in YPD broth composed of 10 g/L of yeast extract (BD-Difco), 10 g/L of peptone (BD-Difco) and 20 g/L of dextrose (Thermo Fisher Scientific Inc., Ontario, Canada). All fungal strains were reactivated from a

Table 1
In vitro MIC ranges of reuterin and natamycin against filamentous moulds and yeasts as determined by broth microdilution followed by reading to eye, OD at 595 nm or XTT. No significant difference was observed between the three methods used (Tukey's HSD test, $P < 0.05$, $n = 3$).

Fungal species	Strain no.	Origin[a]	Reuterin (mM)			Natamycin (μM)		
			CLSI	OD_{595nm}	XTT	CLSI	OD_{595nm}	XTT
Moulds								
Aspergillus niger	3071-13	D[1]	0.4	0.1–0.2	0.5–1.0	1.9	0.9–1.9	1.9
Aspergillus versicolor	LMA-370	D[2]	0.1–0.4	0.1	0.5	3.8–5.3	1.9–3.8	3.8
Aureobasidium pullulans	27,164	D[3]	2.0	1.0–2.0	2.0	1.9	0.9–1.9	1.9
Eurotium rubrum/amstelodami	3071-14a	D[1]	1.0	1.0	1.0	0.9–1.9	0.9–1.9	0.9
Paecilomyces spp.	5332-9a	D[1]	7.8	3.9–7.8	7.8–11.0	1.9	1.9	1.9
Penicillium chrysogenum	LMA-212	D[2]	0.5–1.0	0.2–0.5	1.0	1.3–2.7	0.5–1.9	0.9
Penicillium citrinum	27,165	D[3]	0.5	0.5	1.0–2.0	3.8	3.8	7.5
Penicillium commune	27,163	D[3]	0.5–2.0	0.2–1.0	0.5–2.0	1.9–5.3	0.9–1.9	1.9
Penicillium crustosum	27,159	D[3]	0.2–1.0	0.2–1.0	0.5–2.0	1.9–2.7	0.9–1.9	0.9
Penicillium roqueforti	27,161	D[3]	0.4–0.5	0.2–1.0	0.5–1.0	1.3–7.5	0.5–1.9	1.9–2.7
All moulds			**0.1–7.8**	**0.1–7.8**	**0.5–11.0**	**0.9–7.5**	**0.5–3.8**	**0.9–7.5**
Yeasts								
Candida guilliermondii	27,168	D[3]	0.7–1.0	0.5–1.0	1.0	7.5	7.5	7.5
Candida intermedia	27,171	D[3]	0.5	0.5	ND	3.8	3.8	ND
Candida lusitaniae	27,170	D[3]	0.5	0.5	0.5	7.5	7.5	7.5
Candida parapsilosis	27,167	D[3]	0.5	0.5	0.5	7.5	7.5–15.0	7.5
Kluyveromyces lactis	LL12_098	T[4]	0.1–0.2	0.1–0.2	0.2	1.9	1.9	1.9
Kluyveromyces marxianus	27,175	D[3]	1.0	0.5–1.0	1.0	3.8	3.8–7.5	3.8
Lachancea thermotolerance	LL12_031	T[4]	0.2	0.1–0.2	0.2	3.8	1.9–3.8	3.8
Rhodotorula mucilaginosa	27,173	D[3]	0.5	0.2–0.5	0.5	7.5	3.8–7.5	7.5
Saccharomyces boulardii	27,169	D[3]	1.0	0.5–1.0	1.0	3.8	3.8	3.8
Saccharomyces uvarum	LL14_180	T[4]	0.1–0.2	0.1	0.1–0.2	3.8	1.9–3.8	3.8
Schyzosaccharomyces japonicus	LL14_020	T[4]	0.2	0.2	0.2	3.8	3.8	3.8
Torulospora delbrueckii	27,172	D[3]	0.2–0.5	0.2–0.5	0.2–0.5	2.7–5.3	3.8–7.5	3.8
Wickerhamomyces anomalus	LL11_104	A[4]	1.0–2.0	1.0–2.0	1.0–2.0	3.8	3.8	7.5
Zygosaccharomyces rouxii	LL12_088	B[4]	0.1–0.4	0.1–0.2	0.1–0.2	7.5	7.5	3.8–7.5
All yeasts			**0.1–2.0**	**0.1–2.0**	**0.1–2.0**	**1.9–7.5**	**1.9–15.0**	**1.9–7.5**
All organisms			**0.1–7.8**	**0.1–7.8**	**0.1–11.00**	**0.9–7.5**	**0.5–15.0**	**0.9–7.5**

The bold numbers are summarizing the results for All mould, all yeasts or all organisms.
[a] A: Apple, B: Bumblebee, D: Dairy products, T: Tree bark, 1: strain collection of Denis Roy (Laval University), 2: strain collection of LMA, Laboratoire de Microbiologie Alimentaire (Laval University), 3: strain collection of General Mills Yoplait (France), 4: strain collection of Christian Landry laboratory (Laval University) (Charron et al., 2014).

50% glycerol stock stored at −80 °C and cultured three times before using.

2.2. Preparation of inocula

The preparation of inocula was different for moulds and yeasts. After two weeks of growth at 25 °C on PDA agar, mould conidia were harvested and dispersed in RPMI 1640 medium (with glutamine and without sodium bicarbonate, MultiCell media, Wisent Inc., Montreal, Québec, Canada) supplemented with 0.164 M of 3-(N-morpholino) propane-sulfonic acid (MOPS, Oakville, ON, Canada) to prepare a conidia suspension. Yeast were transplanted from YPD broth in the same RPMI 1640 medium and incubated at 30 °C for 48 h. Finally, conidia and yeast suspensions were diluted 100-fold in sterile saline (9 g/L NaCl), enumerated with a hematometer. Dilutions were made to adjust conidia and yeast suspensions within 2×10^4 and 1×10^5 conidia or yeast per mL in RPMI 1640 medium. Conidia or yeast suspensions were used within a maximum of 1 h after preparation.

2.3. Production, purification and quantification of reuterin

Reuterin was produced, purified and quantified following the protocol described previously with some minor modifications (Cleusix et al., 2007).

Briefly, reuterin (3-hydroxypropionaldehyde) was produced by the strain *Lactobacillus reuteri* ATCC 53608 (STELA collection, University Laval) according to the Doleyres protocol (Pitt and Hocking, 2009). The strain was subcultured in MRS broth and incubated under an anaerobic atmosphere (10% H_2, 10% CO_2, and 80% N_2) at 37 °C for 24 h in a Forma anaerobic chamber (Thermo Scientific, Waltham, MA, USA). The following day, 1 L of sterile MRS medium supplemented with 20 mM glycerol was inoculated at 1% with the *L. reuteri* strain and was incubated at 37 °C for 16 h under anaerobic conditions. After incubation, the bacterial suspension was centrifuged at 1500*g* at 20 °C for 10 min, the supernatant was removed, and the pellet was washed with 500 mL of 0.1 M potassium phosphate buffer pH 7. After a further centrifugation at 1500*g* for 10 min, the pellet was suspended in 90 mL of a sterile glycerol solution at 300 mM and incubated for 45 min at room temperature under anaerobic conditions. The suspension was then centrifuged at 15,000*g* at 4 °C for 10 min to remove the majority of the microorganisms and supernatant was collected and filter-sterilized using a 0.22 μm syringe filter.

Reuterin was purified on a silica gel 60 chromatography column (Merck, Darmstadt, Germany), with 200 mL acetone:ethyl acetate (2:1) as eluent. Forty fractioned of 5 mL each were collected. After purification, reuterin was detected and quantified according to the colorimetric method of (Circle et al., 1945) that is based on the dehydration of reuterin into acrolein followed by the formation of a tryptophan-acrolein colored complex. Acrolein was used as a standard. The reuterin was detected in the fractions from 18 to 23. The fractions with reuterin were combined. The reuterin purity was verified by an HPLC system (Waters, Milford, MA) equipped with an ICSep-ion300 column (Transgenomics, San Jose, CA) using an isocratic mobile phase of 10 mM H_2SO_4 solution at 40 °C with a flow rate of 0.4 mL/min and a refractive index detector (Hitachi, L-7490 model, Tokyo, Japan). After purification, reuterin was quantified by HPLC by integrating the area under the peak corresponding to reuterin at the retention time of 25.5 min. External calibration was performed using dilutions of a reference standard of pure reuterin that previously purified and quantified. The percentage yield was calculated from the ratio of produced

Table 2
In vitro MFC ranges of reuterin and natamycin against filamentous moulds and yeasts as determined by plating. Ratio between the means of MFC and MIC are presented. For each molecule, different letters indicate significant difference between strains (Tukey's HSD test, $P < 0.05$, n = 3).

		Reuterin (mM)				Natamycin (μM)			
Fungal species	Strain no.	MFC	MFC / MIC CLSI	MFC / MIC OD595nm	MFC / MIC XTT	MFC	MFC / MIC CLSI	MFC / MIC OD595nm	MFC / MIC XTT
Moulds									
Aspergillus niger	3071–13	1.0	2.8	4.8	1.2	15.0–30.0	13.3	20.0	13.3
Aspergillus versicolor	LMA-370	0.5	2.3	4.0	1.0	15.0–120.0	15.2	20.8	17.3
Aureobasidium pullulans	27,164	2.0	1.0	1.2	1.0	7.5–15.0	6.7	8.0	6.7
Eurotium rubrum/amstelodami	3071-14a	1.0–2.0	1.3	1.3	1.3	60.0–120.0	72.5	80.0	106.7
Paecilomyces spp.	5332-9a	7.8–15.6	1.7	2.5	1.5	15.0–30.0	13.3	13.3	13.3
Penicillium chrysogenum	LMA-212	1.0–2.0	2.0	3.2	1.3	15.0–30.0	12.8	22.9	26.7
Penicillium citrinum	27,165	1.0–2.0	2.7	2.7	1.0	120.0–240.0	42.7	42.7	21.3
Penicillium commune	27,163	1.0–2.0	1.3	2.3	1.1	15.0–240.0	50.4	105.6	88.0
Penicillium crustosum	27,159	0.5–3.9	2.9	2.9	1.4	60.0–240.0	75.2	115.2	192.0
Penicillium roqueforti	27,161	1.0–2.0	3.3	2.3	1.6	60.0–240.0	47.0	128.0	84.4
All moulds		**0.5–15.6**	**1.0–3.3**	**1.2–4.8**	**1.0–1.6**	**7.5–240.0**	**6.7–75.2**	**8.0–128.0**	**6.7–192.0**
Yeasts									
Candida guilliermondii	27,168	1.0	1.1	1.2	1.0	7.5	1.0	1.0	1.0
Candida intermedia	27,171	0.5	1.0	1.0	ND	3.8	1.0	1.0	ND
Candida lusitaniae	27,170	0.5	1.0	1.0	1.0	7.5–15.0	1.3	1.3	1.3
Candida parapsilosis	27,167	0.5	1.0	1.0	1.0	7.5–15.0	1.7	1.3	1.7
Kluyveromyces lactis	LL12_098	0.2	1.2	1.5	1.0	1.9	1.0	1.0	1.0
Kluyveromyces marxianus	27,175	1.0	1.0	1.2	1.0	3.8–7.5	1.3	1.0	1.3
Lachancea thermotolerance	LL12_031	0.2	1.0	1.2	1.0	3.8	1.0	1.2	1.0
Rhodotorula mucilaginosa	27,173	1.0	2.0	2.4	2.0	7.5	1.0	1.2	1.0
Saccharomyces boulardii	27,169	1.0	1.0	1.5	1.0	3.8–7.5	1.3	1.3	1.3
Saccharomyces uvarum	LL14_180	0.2	1.5	2.0	1.5	3.8	1.0	1.2	1.0
Schyzosaccharomyces japonicus	LL14_020	0.2	1.0	1.0	1.0	3.8	1.0	1.0	1.0
Torulospora delbrueckii	27,172	0.5	1.2	1.5	1.2	7.5–15.0	2.6	2.0	2.7
Wickerhamomyces anomalus	LL11_104	1.0–2.0	1.0	1.0	1.0	7.5	2.0	2.0	1.7
Zygosaccharomyces rouxii	LL12_088	0.2	1.0	1.2	1.2	15.0–30.0	3.3	3.3	4.0
All yeasts		**0.1–2.0**	**1.0–2.0**	**1.0–2.4**	**1.0–2.0**	**1.9–30.0**	**1.0–3.3**	**1.0–3.3**	**1.0–4.0**
All organisms		**0.1–15.6**	**1.0–3.3**	**1.0–4.8**	**1.0–2.0**	**1.9–240.0**	**1.0–75.2**	**1.0–128.0**	**1.0–192.0**

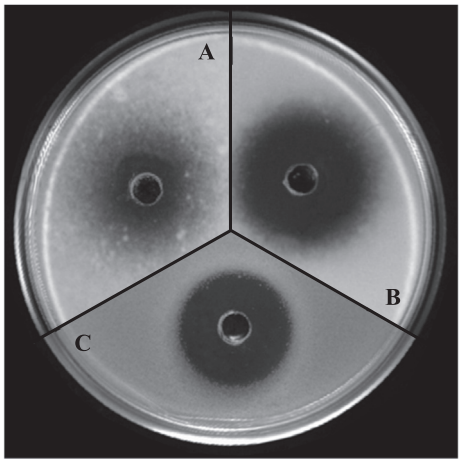

Fig. 2. Inhibition zones induced by non-purified reuterin (120 mM) against: (A) *Aspergillus versicolor* LMA-370, (B) *Penicillium chrysogenum* LMA-212 and (C) *Saccharomyces boulardii* 27,169 grown in YEG soft agar as shown by agar diffusion assay.

reuterin to the initial glycerol concentration. The biotransformation yield was calculated from the ratio of reuterin to the initial concentration of glycerol. Purified reuterin was concentrated by lyophilization and stored at −20 °C until further use.

Natamycin (Sigma) was used as a positive control for fungal inhibition. Before experiment, reuterin and natamycin were diluted in RPMI 1640 medium following the dilution procedure of the broth microdilution method from the National Committee for Clinical Laboratory Standards, currently known as the Clinical and Laboratory Standards Institute (CLSI) guidelines M38-P for moulds and M27A2 for yeasts (Espinel-Ingroff and Canton, 2007a, 2007b).

2.4. Susceptibility testing

Susceptibility test of all the fungal strains presented in Table 1 was performed following the CLSI guidelines M38-P or M27A2 (Espinel-Ingroff and Canton, 2007a, 2007b) using the broth microdilution method in 96 well microtiter plate. Reuterin and natamycin were tested at maximum concentrations of 31.25 mM and 240 μM, respectively. Minimal inhibitory concentrations (MIC) were determined after 48 h of incubation at 35 °C using three different methods including the visual reading by four trained persons as proposed by the CLSI, the reading of the OD at 595 nm with a microplate reader (Infinite® F200 PRO, Tecan Inc., Durham, NC, USA) and using the XTT assay for cell viability (Roehm et al., 1991). The OD of the well must be the same as the negative control (uninoculated well) for being considered inhibited. Minimal fungicidal concentrations (MFC) were determined by spotting a 10 μL of each well showing absence of growth onto Dichloran Rose-Bengal Chloramphenicol agar (DRBC, BD-Difco) plates. After incubation at 35 °C for 48 h, the lowest dilution with no growth indicated the MFC. By extrapolation from the conventional definition used for bacterial testing, a compound was considered fungicidal when the MFC/MIC ratio was ≤4 and fungistatic when the MFC/MIC ratio was > 4 (Pfaller et al., 2004).

2.5. Antifungal activity in yogurt

The antifungal activity of reuterin in yogurt was evaluated against *P. chrysogenum* LMA-212 and *Mucor racemosus* LMA-722 in commercial 1% F.M. plain yogurt (Astro, QC, Canada) as described before (Fernandez et al., 2017). Yogurt was placed in 6 well flat bottom plates (3.5 cm in diameter, Falcon, Corning, NY, USA) supplemented with 0.138; 0.69; 1.38; 6.9; 13.8; 69 and 138.1 mM of purified reuterin. A positive control was prepared with the fungal strain to the yogurt with no reuterin added and a negative control prepared with 50 mL sterile

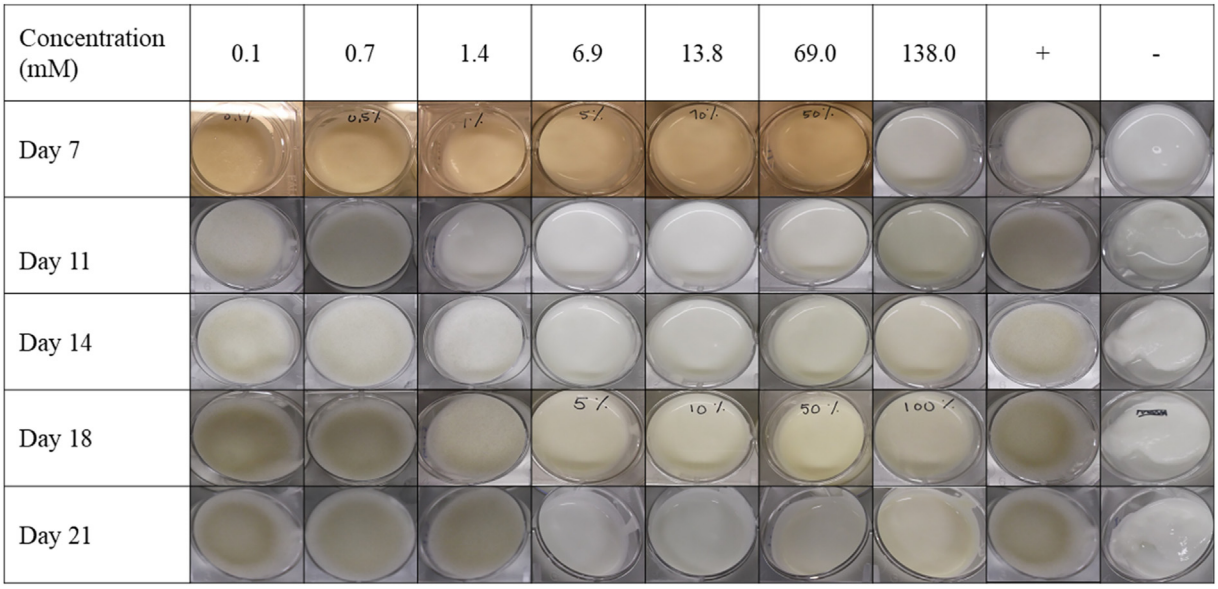

Fig. 3. Effect of reuterin on the growth of *P. chrysogenum* LMA-212 mould in yogurt under different storage times up to 21 days. The positive and the negative controls are represented by + and –, respectively.

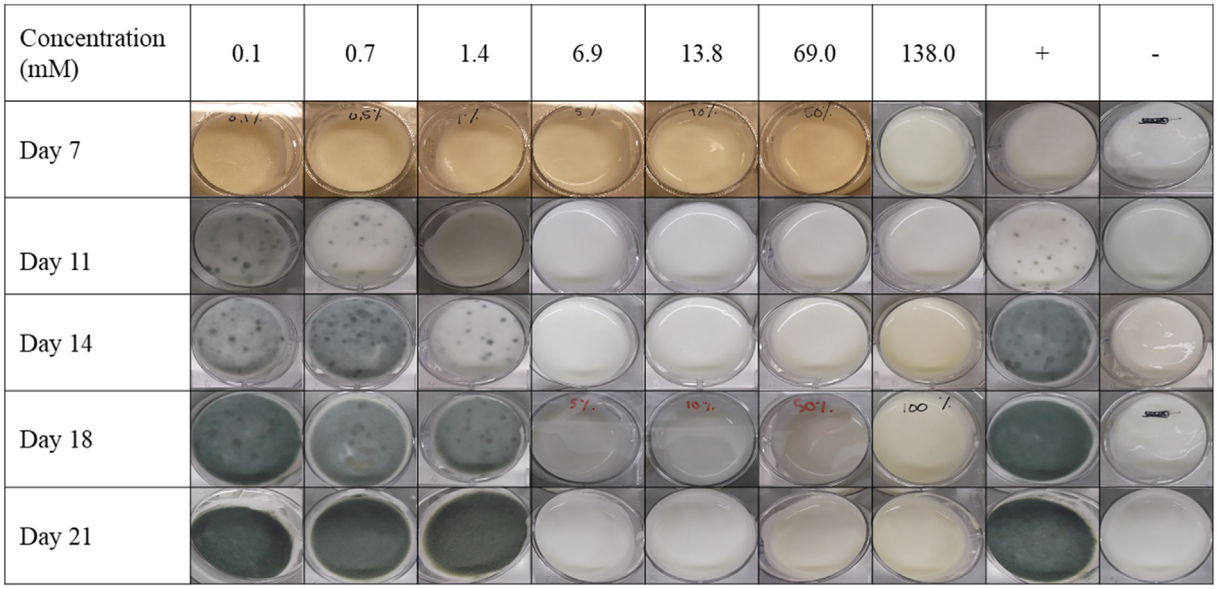

Fig. 4. Effect of reuterin on the growth of Mucor racemosus LMA-722 mould in yogurt under different storage times up to 21 days. The positive and the negative controls are represented by + and –, respectively.

water was included in the test. The samples and the positive control were inoculated with 50 µl of *P. chrysogenum* or *M. racemosus* spores prepared at 10^6 spores/samples. All samples were mixed for 1 min with a sterile probe. Plates were stored in the darks at 4 °C for 21 days and observed every 3 to 4 days by two different skilled technicians. Results were recorded using the same scale as stated by Fernandez et al., 2017.

2.6. Data analysis

All statistical analyses were performed using JMP® software version 10.0 (SAS Institute Inc., Cary, NC). All experiments were done in biological triplicate. For each method, MIC and MFC comparison was performed using the one-way analysis of variance (ANOVA) general linear model followed by Tukey's HSD test. For all analysis, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Production and purification of reuterin

Reuterin was produced from biotransformation of 80 mL of 300 mM glycerol solution by *Lactobacillus reuteri* ATCC 53608. The biotransformation resulted in a solution containing 187 mM reuterin with a bioconversion yield of 62%. The HPLC chromatogram of the reuterin solution after the bioconversion shows three main peaks identified as glycerol, reuterin (3-HBA) and propanediol (Fig. 1, Chromatogram A). The subsequent purification of reuterin with silica gel was performed on the supernatant that contains reuterin. Reuterin was detected by colorimetric method. Fractions with reuterin were collected, combined and analyzed by HPLC. The HPLC chromatogram of purified reuterin showed a single peak confirming the purity of reuterin (Fig. 1, Chromatogram B). Finally, the produced reuterin was 93.2% pure (12.75 M).

3.2. MIC and MFC of reuterin

MIC of reuterin and natamycin against filamentous moulds and yeasts were determined by broth microdilution followed by visual reading, OD measurement at 595 nm and XTT assay (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)) as presented in Table 1. For both molecules, no significant difference was observed between the three methods used to determine the MIC ($P > 0.05$). Purified reuterin exhibited a broad spectrum of activity by inhibiting all tested strains, with MIC ranging from 0.1 to 2.0 mM for all yeast and mould strains except for *Paecilomyces* spp. 5332-9a that required MIC of 11.0 mM. Natamycin showed also a broad spectrum of activity with MIC ranging from 0.5 to 15.0 μM. The MIC of natamycin against yeasts and moulds are in agreement with what has been previously reported (Stern, 1978).

MFC of reuterin ranged between 0.1 and 3.9 mM for all fungal strains except *Paecilomyces* spp. 5332-9a that required MFC of 15.6 mM (Table 2). By extrapolation from the conventional definition used for bacterial testing (Pfaller et al., 2004), reuterin acted with a fungicidal mechanism towards filamentous moulds and yeasts since MFC/MIC ratio was < 4 with only one exception in case of *Aspergillus niger* that showed MFC/MIC ratio of 4.8 when measured by OD_{595}. Moreover, no significant difference between MIC and MFC was observed ($P > 0.05$). Regarding natamycin, MFC were comprised between 7.5 and 240.0 μM, and 1.9 and 30.0 μM against filamentous moulds and yeasts respectively. MFC were significantly higher than MIC with ratio comprised between 6.7 and 192 for filamentous moulds and 1.0 and 4.0 for yeasts ($P < 0.05$). Therefore, natamycin acted with a fungicidal mechanism on yeasts and fungistatic mechanism on moulds, while reuterin was fungicidal for all microorganisms tested".

3.3. Reuterin application in yogurt

Increasing concentration from 0.138 to 138 mM of reuterin was tested at 4 °C for 21 days in plain yogurt against two fungal strains *P. chrysogenum* LMA-212 and *Mucor racemosus* LMA-722. Reuterin showed fungal inhibition of the two strains at concentrations equal to or above 1.38 mM. The growth of the fungi was visually delayed 3 days compared to the positive control (Fig. 2–4). The growth was detected at the 7th day of incubation for the positive control and at the 11th day for the sample with 1.38 mM of reuterin for the two strains. Reuterin also completely inhibited the visual growth of the two fungal stains at concentration of 5 mM and more during all the 21 days of incubation. However, reuterin affected the color where a yellowish color was detected in the samples with high reuterin concentrations: 69 mM after 18 days and 138.1 mM after 14 day.

4. Discussion

In the context of increasing consumer demand for naturally preserved foods, reuterin could be a promising alternative to control these fungal spoilage microorganisms. It has previously been recognized as a promising food preservative owing its activity against both prokaryotic and eukaryotic organisms (including pathogen and spoilage microorganisms), its chemical characteristics (water soluble, active at wide pH-range, salt concentration, and temperatures that are relevant for foods, and not sensitive to lipases and proteases) and the possibility to large scale production to produce high amounts of reuterin in a relatively cheap biotechnological process (Vollenweider and Lacroix, 2004; Stevens et al., 2011). In addition, no natural resistance mechanism has been described and consequently no resistance genes have been identified.

Reuterin is currently defined as a multi-compounds system containing 3-HPA, its hydrate, the dimer and acrolein (Engels et al., 2016). Our results showed that out of these components, only reuterin existed in the supernatant. The reuterin was confirmed by three different ways: 1. the colorimetric method; 2. The HPLC method; and 3. Microbiologically. These results were in agreement with the Talarico and Dobrogosy (1989) that confirmed the production of reuterin under similar condition with LC-MS. In addition, produced by several bacterial species or through traditional chemistry, reuterin exhibits inhibitory activity against a broad range of Gram-positive and Gram-negative bacteria, bacterial spores, moulds, yeasts and protozoa (Ávila et al., 2014; Cleusix et al., 2007). Contrary to its antibacterial activity, its inhibitory activity against moulds and yeasts is poorly documented (Chung et al., 1989; Nakanishi et al., 2002). A direct comparison with published data is difficult due to the differences between the experimental protocols and the expression of the results that were either expressed as poorly defined arbitrary units or arbitrary scale of inhibition. In this study, the MIC and MFC of pure reuterin for moulds and yeasts isolated from dairy and maple syrup environments were quantified using three different methods including Clinical & Laboratories Standard Institute (CLSI) method.

Our data showed that reuterin is an effective fungicide for all tested moulds and yeasts that were inhibited by concentrations equal or below 11 mM and killed by concentrations equal or below 15.6 mM. Our results are in agreement with those that were reported for reuterin inactivation of yeasts and moulds (Chung et al., 1989; Stevens et al., 2011), and also against intestinal bacteria with MIC comprised between 1.9 and 50 mM, and MBC between 1.9 and 120 mM (Cleusix et al., 2007). It has been previously observed that the sensitivity of yeasts and moulds to reuterin was comparable to that of bacteria (Chung et al., 1989). Moreover, it is worth noting that the sensitivity of yeast and moulds to reuterin appeared to be similar with a fungicidal effect of reuterin against both yeasts and moulds, unlike natamycin which acted with a fungicidal mechanism on yeasts and fungistatic mechanism on moulds.

Few studies have explored the effectiveness of reuterin in food matrix. It has been reported that reuterin that was added at concentrations of 150 arbitrary units per gram in cottage cheese reduced the viability of *Listeria monocytogenes* by 5.0 log cycles after storage at 7 °C for 7 days, while the viability of the cells was increased by 0.4 log cycles in the reuterin-free control. In UHT skim milk, the inhibition of listerial cells was higher than that in cottage cheese (El-Ziney and Debevere, 1998). Moreover, the fat content in the milk up to 3% did not affect the reuterin activity. In addition, reuterin was proposed as the best option to control *Clostridium* spp. spores and vegetative cell growth in dairy products. Concentrations up to 32.5 mM inhibited and/or killed vegetative cells and spores of twelve *Clostridium* species in milk (Ávila et al., 2014). Reuterin could also be used to successfully decontaminate food contact surfaces and to prevent the development of microorganism resistance, as proposed by (El-Ziney and Jakobsen, 2009). On stainless steel surface, a reduction higher than 6 log cycles of *Escherichia coli* and *Listeria innocua* was observed after a treatment with 200 au/mL during 5 min at 30 °C. Since most of the studies established the effectiveness of reuterin under laboratory conditions in standard growth media, which do not necessarily reflect conditions in food or environment, additional studies on its activity and its stability in different food matrices over storage time are still necessary to ensure activity against spoilage and pathogen microorganisms at sufficient rates.

Despite the classification of *L. reuteri* as safe by qualified presumption of safety approach by the European Food Safety Authority (EFSA Panel on Biological Hazards, 2017) and the acceptance of its use with glycerol (food additive E-422), data on the toxicity of reuterin are limited. Additional toxicology research is needed before granting the use of reuterin, including the long-term exposure, its toxicokinetics and the mechanisms of toxic action, in particular due to the limitations to extrapolate *in vitro* results to *in vivo* situations.

Our results show that the fungal grow was reduced at reuterin concentration of 1.38 mM and the fungi were killed at 6.9 mM. Those concentrations were similar to those found with the official method. It was previously reported that the reuterin has a killing effect on

Penicillium spp. in fermented milk at 10 mM (Ortiz-Rivera et al., 2017).

In conclusion, this study reports for the first-time numerical MIC and MFC of reuterin against various filamentous moulds and yeasts of food interest. Reuterin acted with a fungicidal mechanism on all microorganisms which were inhibited and/or killed at concentrations equal or below 3.9 mM except *Paecilomyces* spp. that required up to 15.6 mM. Reuterin has a high potential as an antifungal preservative in dairy products, and more broadly antimicrobial food preservative, due to both its chemical characteristics and its inhibitory activity against fungi and bacteria. Further studies on its toxicity and stability in other complex alimentary matrices are still needed before its use as food preservative becomes possible.

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