

MISS MARÍA DEL ROSARIO AGUSTÍN (Orcid ID : 0000-0002-2908-4928)

DR. MARIA SOLEDAD VELA GUROVIC (Orcid ID : 0000-0003-3583-1958)

Article type : Original Article

Effect of quorum sensing molecules and natamycin on biofilms of *Candida tropicalis* and other yeasts
isolated from industrial juice filtration membranes

Maria del Rosario Agustín^a, Fatima R. Viceconte^b, Maria S. Vela Gurovic^{a,b,*}, Andrea Costantino^c,
Lorena I. Brugnoni^{a,d}

^a Department of Biology, Biochemistry and Pharmacy, Universidad Nacional del Sur (UNS), San Juan
670, 8000 Bahía Blanca, Argentina

^b CERZOS UNS-CONICET, Camino La Carrindanga Km7, B8000FWB Bahía Blanca, Argentina

^c INQUISUR, Departamento de Química, Universidad Nacional del Sur (UNS)-CONICET, Av. Alem 1253,
8000 Bahía Blanca, Argentina

^d Institute of Biological and Biomedical Sciences of the South (INBIOSUR), Universidad Nacional del
Sur (UNS), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), 12 de Octubre 991,
8000 Bahía Blanca, Argentina

Abbreviated running headline “Yeast biofilm inhibition in juice”

This article has been accepted for publication and undergone full peer review but has not
been through the copyediting, typesetting, pagination and proofreading process, which may
lead to differences between this version and the Version of Record. Please cite this article as
doi: 10.1111/jam.14248

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Corresponding author

Vela Gurovic María Soledad, svela@uns.edu.ar

Tel: 54 (0291) 4861666 Ext. 180, Fax: 54 (0291) 4862882

ORCID 0000-0003-3583-1958

Abstract

Aims

Cells limit the cell number of dense biofilms by releasing self-inhibitory molecules. Here, we aim to assess the effectiveness of yeast quorum sensing (QS) molecules and the antifungal agent natamycin against yeast biofilms of strains commonly isolated from fruit juice ultrafiltration membranes.

Methods and results

Yeast QS molecules such as tyrosol, 2-phenylethanol and farnesol were detected by solvent extraction and HS SPME GC-MS in *C. tropicalis* cultures. The effect of quorum sensing (QS) molecules on mono and multi species biofilms formed by *Rhodotorula mucilaginosa*, *Candida tropicalis*, *Candida krusei* and *Candida kefyr* was evaluated by plate count and epifluorescence microscopy. Farnesol caused a decrease in cell number and disrupted mono and multispecies yeast biofilms during adhesion (0.6 mmol l^{-1}). 2-phenyl ethanol 1.2 mmol l^{-1} stimulated biofilm density and increased cell number in both mono and multispecies biofilms, while tyrosol did not show effects when tested against *C. tropicalis* biofilms ($0.05\text{-}1.2 \text{ mmol l}^{-1}$). Natamycin caused a strong decrease on cell number and disruption of biofilm structure in *C. tropicalis* biofilms at high concentrations (0.3 to 1.2 mmol l^{-1}). The combination of farnesol 0.6 mmol l^{-1} and natamycin at 0.01 mmol l^{-1} , the maximum concentration of natamycin accepted for direct addition into fruit juices, effectively reduced cell counts and disrupted the structure of *C. tropicalis* biofilms.

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Conclusion

Farnesol 0.6 mmol l^{-1} significantly increased the inhibition exerted by natamycin 0.01 mmol l^{-1} (~5 ppm) reducing biofilm development from juice on stainless steel surfaces.

Significance and impact of the study

These results support the use of QS molecules as biofilm inhibitors in beverages and would certainly inspire the design of novel preservative and cleaning products for the food industry based on combinatory approaches.

Keywords: quorum sensing, biofilm, juice, farnesol, *Candida* spp., natamycin

Introduction

Yeasts predominate among microbial communities as spoilage microorganisms, particularly in acidic food products like apple juice, due to their ability to grow at low pH, high sugar concentration and low water activity conditions and to resist inactivation by heat processing (Stratford *et al.* 2000). Yeasts attach and develop biofilms on ultrafiltration (UF) membranes used in beverage clarification (Tarifa *et al.* 2013) and in pre and post-UF processing equipment (Brugnoni *et al.* 2007). *Candida* spp. are among the most common yeast species associated to UF membranes in fruit juice processing plants (Tarifa *et al.* 2018). *C. tropicalis* L5, a strain previously isolated from UF membranes, strongly adheres to the surface of stainless steel, forming resistant biofilms which may confer a competitive advantage over other yeasts (Brugnoni *et al.* 2014; Tarifa *et al.* 2013; Tarifa *et al.* 2015). As cleaning procedures cannot easily remove yeast in such sites (Brugnoni *et al.* 2012; Tarifa *et al.* 2013), it was assumed that this community is able to persist over time on food production surfaces.

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Among new approaches to eradicate biofilms, targeting the quorum sensing (QS) mechanism is being evaluated as a promising alternative against bacterial biofilms (Coughlan *et al.* 2016). The ability of these molecules to disrupt yeast biofilms is currently being studied in the clinical field (Wongsuk *et al.* 2016). Additionally, the combination of antifungals with QS molecules has been studied especially against *Candida albicans* biofilms (Cordeiro *et al.* 2015; Katragkou *et al.* 2015; Bozó *et al.* 2016; Kóvacs *et al.* 2016; Shanmughapriya *et al.* 2014). To the best of our knowledge, there is no information available about the effectivity of combinatory treatments against yeast biofilms inhabiting food and food processing devices.

Currently, there is no antifungal in use in the food industry with a demonstrated effectiveness against biofilms. Among antimicrobials for the food industry, natamycin has been widely used to control fungal development in dairy-based food products and is considered safe for human use when applied on food surfaces (Reps, *et al.* 2002; Gallo and Jagus 2006; El-Diasty *et al.* 2008). Although natamycin effectively reduces the viability of *Saccharomyces* in whey cheese (Gallo and Jagus 2006; Ollé Resa *et al.* 2014), there are no reports testing this antifungal against *Candida* spp. in beverages.

With the aim of finding effective treatments against biofilms developed in the fruit industries, we identified *Candida* spp. quorum sensing molecules in *C. tropicalis* cultures and evaluated the antibiofilm properties of three selected alcohols. We also compare for the first time the effects of natamycin against biofilms developed from juice, and the effects of the combination of natamycin with one of the identified alcohols showing antibiofilm activity. Cell number and biofilm morphology were evaluated at different concentrations, in mono and multispecies biofilms growing over stainless steel surfaces at different stages of biofilm development.

Materials and methods

Materials

Alcohols were dissolved in sterile water immediately before testing excepting farnesol, which was dissolved in dimethyl sulfoxide (DMSO). The toxicity of DMSO was previously assessed. Neither cell number nor morphology were affected by DMSO at the highest concentration used 0.08 % v/v. Tyrosol, farnesol and 2-phenylethanol were from Sigma Aldrich (St. Luis, Mo, USA) and commercial natamycin was kindly provided by Ing. Jesica Ostapchuk (Biotec S.A., Argentina).

Microorganisms and culture conditions

Candida tropicalis L5, *Candida krusei* L9, *Candida kefyr* L11, and *Rhodotorula mucilaginosa* L1 were previously isolated from the surfaces of polyvinylidene–fluoride ultrafiltration (UF) membranes, obtained from a large-scale apple juice processing industry located in Villa Regina, Río Negro, Argentina (Tarifa *et al.*, 2013). Stock cultures of yeasts were stored at -70°C in yeast extract glucose-chloramphenicol (YGC, Biokar) broth supplemented with 20% v/v glycerol. A loop of frozen cells of yeasts was suspended in YGC broth at 25± 1°C until reaching the stationary phase (48h). Cultures were then harvested by centrifugation at 1200 g for 5 min (Labofuge 200, Kendro, Germany) and washed twice with Phosphate-Buffered Saline (PBS). Pellets were subsequently resuspended in sterile clarified 12 °Brix apple juice to achieve a population of ca. 6.0 log Colony Forming Units (CFU) mL⁻¹. The 12 °Brix clarified apple juice used in the successive assays was prepared from 72 °Brix concentrated apple juice obtained from a large-scale apple juice processing industry located in Argentina and sterilized by microfiltration (pore size 0.45 µm). The major components of apple juice are carbohydrates, acids, nitrogen compounds, polyphenols, minerals and vitamins; mean composition: fructose: 70 g l⁻¹, glucose: 35 g l⁻¹, sucrose: 16 g l⁻¹, malic acid: 0.4 - 3.4 g l⁻¹, citric acid: <

1 g l⁻¹, ascorbic acid: < 40 mg l⁻¹, potassium: 1 g l⁻¹, calcium: 0.05 - 0.4 g l⁻¹, phosphorus 70 - 100 mg l⁻¹, sodium: 20 mg l⁻¹, free aminoacids: 1 - 5 g l⁻¹ (Lozano 2006), pH: 4.0 ± 0.2, ionic strength 0.023 mol l⁻¹.

Culture conditions for identification of volatiles

The system used to obtain the supernatant consisted of beakers in which stainless steel (SS) disks (50 mm diameter, 0.5 mm thickness; AISI 304 2B, food grade) were placed. Previously, the disks and the beakers were soaked for 15 min with 2% v/v of a detergent solution (Extran MA 02 neutral, Merck KGaA, Darmstadt, Germany) at 50°C, and rinsed with hot tap water, followed by autoclaving for 15 min at 120°C.

To allow attachment, the beakers were filled with the *C. tropicalis* culture suspension, with a working volume of 25 ml per beaker. Initially, the suspension was deposited on the SS disk surfaces for 24 h at 25 ± 1°C. Afterwards, the food matrix was replaced at 24 h with sterile juice to provide fresh nutrients for the cells. At the selected times, cultures were centrifuged at 1200 g for five min and the cell-free supernatants were filter sterilized (0.22-µm pore size).

Identification of volatiles from cultures of *C. tropicalis*

Identification by solvent extraction and Gas Chromatography – Mass Spectrometry (GC-MS)

Production of volatiles was followed in cultures of *C. tropicalis* for four weeks. An aliquot of 15 ml of fresh supernatant was extracted three times with an equal volume of ethyl acetate. The extracts were concentrated under reduced pressure at 45°C and stored at -20°C until use. Before injection, the final volume of each extract was adjusted to one ml with ethyl acetate.

GC-MS analyses were performed with a GC MS 7890B chromatograph equipped with a 5977A mass spectrometer (Agilent Technologies). The ionization was performed by electron impact with an ionization energy of 70 eV. Samples (one μL , ethyl acetate) were injected in the splitless mode into a HP-5MS capillary column (30 m \times 0.25 mm i.d. \times 0.25 μm film thickness). Temperatures were programmed from 50°C to 250°C at a rate of 5°C min^{-1} , then increased to 280°C at a rate of 120°C min^{-1} and held at the final temperature for 5 min. The injector and detector temperature were held at 250°C, and the carrier gas was helium at a flow rate of 1 mL min^{-1} . The solvent delay was 4.5 min. The MSD transfer line was maintained at 250°C; the ion source temperature was 230°C; the quadrupole temperature was 150°C. Based on the mass scan range of 50–550 atomic mass units (a.m.u.) with SCAN mode, retention times of compounds were determined by comparing the MS fragmentation pattern of the standards and the National Institute of Standards and Technology (NIST) 2.0 GC-MS library.

Identification of volatiles by Head Space Solid Phase Microextraction (HS-SPME) and GC-MS

Cultures of *C. tropicalis* were incubated for five days and supernatants were immediately subjected to extraction. Volatiles were extracted from the supernatant using headspace-solid-phase microextraction (HS-SPME) and analysed by gas chromatography-mass spectrometry (GC-MS). The fiber was purchased from Supelco (Aldrich, Bellefonte, PA, code 57328-U). This SPME fiber assembly consists of a 50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) with L=1 cm coating bonded to a flexible fused silica core, with a needle size of 24 ga. This coating is recommended for detection of volatiles and semi-volatiles with number of carbons between 3 and 20 and molecular weights in the range of 40-275. The SPME fiber was conditioned at 250°C for 15 min in the GC injector port. For headspace sampling, 16 ml of each supernatant fraction was introduced into a 30-ml glass vial. The vial was capped with a headspace septum and an aluminium cap after addition of 3.2 g of NaCl and agitation at 250 rev min^{-1} in a thermostatically controlled bath

adjusted to 40°C for 30 min. Following this step, the SPME fiber was manually inserted into the sample vial headspace for 30 min and then introduced into the GC injection port at 250°C and kept for 20 min for alcohol thermal desorption. The oven temperature was programmed from 35°C to 150°C at 2°C/min, raised again to 220°C at 10°C/min and held at this final temperature for 15 min. Masses were scanned in the range of 50 to 300 m/z. The identification of *C. tropicalis* metabolites was achieved by comparing the mass spectra with those of the data system library and 2-phenylethanol and farnesol retention times were compared with those of reference substances. A comparable analysis was done with juice supernatants (growth medium). Otherwise stated, the gas chromatograph was operated as described above.

Microbiological assays. Testing of selected alcohols on biofilms

Effect of chemicals on adherent cells and subsequent biofilm formation

Assays were carried out on stainless steel coupons (SSC) (25 x 15 x 1 mm) type AISI-304. The coupons were degreased and sterilized according the protocol described in Brugnoli *et al.* (2007). A coupon was placed into each well of a sterile 24 well plate. Adjusted yeast suspensions ($OD_{550}=0.125 \sim 10^6$ CFU mL⁻¹) with the corresponding amount of alcohols were poured into each well to achieve yeast attachment to the SSCs at 25°C. The effect of the tested molecules was assessed both in mono and multi species biofilms. The same suspension containing mono or multispecies without the testing substances was poured into the well as a control for each assay, and, in the successive, treated in the same manner as the cultures containing the tested substances. After the first two hours, the SSC were cleaned once with sterile water, and juice with tested molecules and without tested molecules for controls was renewed. Effects were evaluated at 2, 6, 24 and 48h. Coupons were carefully removed after the incubation period using sterile forceps and rinsed by immersing in five ml sterile

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water for one min to remove the loosely attached cells. The colonized coupons were used for viable counts and epifluorescence microscopy. All experiments were run at least per duplicate.

Quantification of single and mixed cultures

To determine the composition of the resulting surface attached cultures, the cell number was estimated by placing the SSC into a test tube with glass beads. Each suspension was sonicated once for two min at 20°C (Digital Ultrasonic Cleaner, PS-10A). To remove the adherent microorganisms, each tube with the coupon was sonicated for two min at 20°C (Digital Ultrasonic Cleaner, PS-10A) and vortexed at full speed for two min (Lindsay and von Holy 1997). Then, samples were serially diluted with sterile deionized water and counts determined by spread plate technique on CHROMAgar *Candida* and YGC agar, by triplicate. Samples were incubated for 24-48 h. The results were expressed as CFU cm⁻².

Epifluorescence microscopy

Biofilms formed on stainless steel were observed by regular epifluorescence microscopy (Carl Zeiss, Primo Star). After incubation of the sample, the coupons were rinsed two times with sterile water to remove planktonic cells and stained with Live/Dead BaLight (Molecular Probes, USA). The stain package consisted of a mixture of two nucleic acid-binding stains: SYTO 9 and propidium iodide. These stains differed both in their spectral characteristics and in their ability to penetrate viable bacterial cells. SYTO 9 stains cells green, while propidium iodide penetrates cells whose cell membrane has been damaged, staining them red. Stains were mixed together in equal amounts (1.5 µL + 1.5 µL), diluted in distilled water (1000 µL) and vortexed in the dark. 10 µL of the mixture were used for covering the coupon surface and incubated in the dark at room temperature for 20 min. Two washes were carried out with distilled water to remove residues of the dyes. Biofilms on

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stainless steel coupons were observed using an epifluorescence microscope equipped with a Carl Zeiss camera (AxioCam ERc5s) and filters to detect Syto 9 and PI, and software for microscopy imaging (ZEN core, Carl Zeiss).

Statistical analysis

Cell counts were converted to decimal logarithmic values ($\log \text{CFU cm}^{-2}$) to nearly match the assumption of a normal distribution. In all analyses, triplicate tests were performed under identical conditions in two independent trials and the results expressed as means and standard deviations (mean \pm SD). When appropriate, Student's t-test was used for comparison of means. Confidence level equal or higher than 95% was considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Identification of volatiles from cultures of *C. tropicalis*

As shown in Table 1, differences were found between the two methods employed. Regarding solvent extraction, the most abundant compound from day 2 to day 21 was 2-phenylethanol. After incubation for 28 days, the relative percentage of 2-phenylethanol considerably decreased, whereas tyrosol, which was among the minority during the first three weeks, increased. Other molecules detected at almost all times in supernatants but not present in juice were N-acetyl tyramine and prenol. Volatiles of juice detected by HS-SPME were a mixture of 94 % furfural and 6% methoxy phenyl oxyme. Volatiles of *C. tropicalis* cultures detected by this technique were mainly a mixture of amyl alcohols. Although at very low percentages, 2-phenylethanol and farnesol were also detected by HS-SPME. Since 2-phenylethanol, tyrosol and farnesol have been previously identified as QS

molecules in other strains of *Candida* spp., we selected these molecules to further assess their potential as biofilm inhibitors.

Effects of low molecular weight alcohols on *C. tropicalis* biofilms and monospecies biofilms of other yeast isolated from ultrafiltration membranes

We first tested the effects of increasing concentrations of farnesol, 2-phenylethanol and tyrosol at different times during *C. tropicalis* biofilm development (Fig. 1). Regarding cell number, farnesol (0.05-1.2 mmol l⁻¹) caused a significant decrease at 2h, 24h and 48h. This effect was evidenced specially at adhesion (2h, 0.6 mmol l⁻¹) and at 24h (0.05 mmol l⁻¹), when reductions surpassed the logarithmic unit ($p < 0.001$). Effects of 2-phenylethanol on *C. tropicalis* biofilms were not dose dependent (0.05-1.2 mmol l⁻¹), while cells treated with tyrosol at the same concentrations did not differentiate from controls for which this molecule was not further tested. We further explored the effects on the morphology of monospecies biofilms of *C. tropicalis* and monospecies biofilms of three other yeasts isolated from the same source (Fig. 2). Effects were only evident at 2h (adhesion). Here, 0.6 mmol l⁻¹ farnesol and 1.2 mmol l⁻¹ 2-phenylethanol showed contrary effects regarding morphology and pseudohyphae production, being farnesol inhibitory and 2-phenylethanol stimulatory for all *Candida* species. Under the microscope, *R. mucilaginosa* showed no differences between treatments.

Effects of farnesol and 2-phenylethanol on multispecies biofilms

To simulate processing conditions in the industry, we further evaluated the effects of 0.6 mmol l⁻¹ farnesol and 1.2 mmol l⁻¹ 2-phenylethanol on multispecies biofilms (Fig. 3). During adhesion (2h), 2-phenylethanol significantly increased the cell number of all yeast strains. The effect of farnesol became evident in mature biofilms (48h), significantly reducing the number of viable cells of all

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strains when compared with respective controls. Regarding morphology (Fig. 4), the stimulatory activity of 2-phenylethanol on multispecies biofilms became evident at 6h and persisted over the time, while the inhibition exerted by farnesol was clearly observed during adhesion with persistence of disruption zones in mature biofilms (24h and 48h).

Effects of natamycin and combinations with farnesol

Facing the lack of a commonly accepted positive control of biofilm inhibition and aiming to compare the effect of the alcohols produced by *C. tropicalis* against juice biofilms simulated in the laboratory, we assessed the effect of natamycin, an antifungal traditionally used as food preservative. As expected, a strong reduction in cell number (Fig. 5) and disruption of biofilm structure (not shown) were observed at all concentrations tested (0.3-1.2 mmol l⁻¹) during all the stages of biofilm development. At 24h and concentrations of 0.6 and 1.2 mmol l⁻¹, natamycin was able to reduce the cell number of *C. tropicalis* biofilms by more than five logarithmic units (Fig. 5). The combination with farnesol 0.6 mmol l⁻¹ did not cause mayor inhibition at these high concentrations of natamycin.

We further tested the combination of farnesol 0.6 mmol l⁻¹ and natamycin 0.01 mmol l⁻¹, a concentration of natamycin that is near the maximum accepted for direct addition into juices according to the FDA ([https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=578&sort=GRN_No&order=DESC&startrow=1&type=basic&search=578,\[2019.02.19\]](https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=578&sort=GRN_No&order=DESC&startrow=1&type=basic&search=578,[2019.02.19])). Here, farnesol 0.6 mmol l⁻¹ clearly contributed to a better reduction on biofilm cell density from six hours on in co-treatment with natamycin 0.01 mmol l⁻¹ (Fig. 6). Regarding biofilm cell number, farnesol significantly enhanced the effects exerted by natamycin at all incubation times (Fig. 7). If the log number of CFU in controls is considered as 100% growth at each incubation time, natamycin alone caused a reduction of 12%, 20%, 35% and 60% at 2h, 6h, 24h and 48h, respectively. The addition of farnesol caused an

additional reduction of 9%, 30%, 23% and 12% respectively. While the effects of natamycin increased with time, the additional effects attributed to the combination with farnesol reached a maximum at 6h. However, only the combination caused a log reduction of 5 log units (48h).

Discussion

Highly polar compounds such as free acids were only detected by solvent extraction, while all molecules detected by HS-SPME were alcohols, esters and less polar molecules (Table 1). It is expected that extraction with ethyl acetate would lead to more polar compounds with higher molecular weights than HS-SPME. In the latter method, molecules must be in the vapor phase to be extracted, which depends on molecular properties such as molecular weight and polarity (Vas and Vékey 2004). Furfural derivatives were observed in the juice where they are known to be produced under certain processing and storage conditions (Gökmen and Acar 1999), for which these compounds may not be biosynthesized by *C. tropicalis*. Among detected molecules, farnesol, 2-phenylethanol and tyrosol function as quorum sensing molecules in *C. albicans*, as reported by some previous studies. In 1969, Lingappa *et al.* identified molecules such as tyrosol and 2-phenylethanol in cultures of *C. albicans* that functioned as “auto-inhibitors”. Tyrosol was later identified as a quorum sensing molecule of *C. albicans* by Chen *et al.* (2004). Farnesol is another QS alcohol released by different *Candida* species with inhibitory effects on yeast (Alem *et al.* 2006; Weber *et al.* 2008; Martins *et al.* 2010, Monteiro *et al.* 2017). *C. albicans* sessile cells also produce farnesol and 2-phenylethanol, both molecules being associated to modulation of biofilm development (Martins *et al.* 2007, 2010; Johansen and Jespersen 2017).

Among the three molecules tested only farnesol showed a clear inhibitory effect on *C. tropicalis* biofilms (Fig. 1 and 2) and biofilms of other yeast isolated from the juice filters, in both mono (Fig. 2) and multispecies systems (Fig. 3 and 4). The inhibitory effects on biofilm morphology were more

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evident at adhesion (2h). This has been previously observed on studies on *C. albicans*, showing that the effect of farnesol is evident during adhesion, when pseudohyphae inhibition is clearly observed (Ramage *et al.* 2002). The reduction in cell number in multispecies biofilms was not evident at adhesion but in mature biofilms instead (Fig. 3). The presence of other yeast may have protected the biofilm against farnesol at early stages of biofilm development. However, in mature biofilms farnesol 0.6 mmol l⁻¹ caused a decrease in cell number on *C. tropicalis* both monospecies (Fig. 1) and multispecies biofilms (Fig. 3). Although statistically different from controls, the decrease in cell number was not greater than one log unit. In accordance with previous studies (Weber *et al.* 2010; Han *et al.* 2012), farnesol clearly affected cell morphology more than cell number. Monteiro *et al.* (2017) also reported a decrease of around one log unit in dual biofilms of *C. albicans* and *C. glabrata*, but at 60 mmol l⁻¹ farnesol, a concentration 100 times higher than the one used in this study. A higher potency of farnesol in the present study may be explained by differences in methodology or different susceptibility to farnesol between species. Fleishmann *et al.* (2017) found that *C. krusei* exhibits communal interactions forming mycelia towards itself and towards *C. albicans* and *C. glabrata*, but the opposite did not occur although all species produced farnesol. This supports that susceptibility or intensity of response to farnesol and other alcohols may be different between species.

The effect of natamycin on *C. tropicalis* biofilms depended on exposure time and concentration. The dependence of such variables is in accordance with one study on inactivation of *S. cerevisiae* cultures (Gallo and Jagus 2006). The addition of farnesol to cells co-treated with natamycin slightly stimulated the cell number during the first six hours of biofilm formation (Fig. 5). However, this was only evident for biofilms treated with high concentrations of natamycin, while in mature biofilms no significant differences were found. Natamycin is an antifungal polyene which binds specifically to ergosterol (te Welscher *et al.* 2010). Contrary to other antifungal agents like nystatin, it does not cause membrane permeabilization but rather inhibits glucose and aminoacid protein transporters in the membrane by a sterol dependent mechanism (te Welscher *et al.* 2012). Farnesol inhibits germ

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tube formation and upregulates many metabolic pathways in *C. albicans* (Han *et al.* 2012, Polke *et al.* 2017). Cells that were submitted to the pressure of both inhibitors, would hypothetically have experienced a blockade of protein transporters and inhibition of germ tube formation, together with an upregulation of metabolism. When surviving cells were released from the inhibitors, probably the upregulation exerted by farnesol would have prevailed, causing the increase in viable counts at 2H and 6H (Fig. 5). These experiments showed that the potency of farnesol was clearly inferior to that shown by natamycin. However, the highest concentration of natamycin in beverages allowed by the FDA is 5 ppm ($\sim 0.01 \text{ mmol l}^{-1}$), which is lower than the concentrations used for comparison with farnesol ($0.3\text{-}1.2 \text{ mmol l}^{-1}$, Fig. 5). When tested in combination with natamycin 0.01 mmol l^{-1} , the inhibitory effects of this alcohol improved natamycin performance against biofilms (Fig. 6 and 7). The combination of natamycin 0.01 mmol l^{-1} and farnesol 0.6 mmol l^{-1} caused a clear disruption of the biofilm structure in comparison with cells treated with natamycin 0.01 mmol l^{-1} alone from early stages (6h) of biofilm formation on. The combination of farnesol and natamycin at these concentrations significantly enhanced the inhibitory effects exerted by natamycin at all incubation times, causing an additional reduction of 0.5, 2.0, 1.7 and 0.9 log numbers at 2h, 6h, 24h and 48h respectively. Similar effects are observed when comparing the results of natamycin at 0.6 and 1.2 mmol l^{-1} 24h and 48h (Fig. 5) with those of natamycin 0.01 mmol l^{-1} plus farnesol 0.6 mmol l^{-1} at 48h (Fig. 7). At these points the maximal effect is shown, corresponding to a reduction of 5 log units. This suggests that the concentration of natamycin could be reduced when combined with farnesol without affecting the performance of natamycin against yeast.

Farnesol, tyrosol and 2-phenylethanol were detected in *C. tropicalis* cultures. From these, only farnesol exerted inhibition against *C. tropicalis* and other yeast isolated from fruit juice filtration membranes in mono and multispecies biofilms. Natamycin efficiently inhibited *C. tropicalis* biofilms in concentrations between 0.6 mmol l^{-1} to 1.2 mmol l^{-1} . By adding farnesol, the dose of natamycin can be reduced 100 times with no detrimental effects on biofilm inhibition. If intended to be added to a 12 °Brix apple juice to prevent biofilm formation and to avoid exceeding approved limits,

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natamycin could be used in combination with farnesol, a food additive permitted for direct addition to food for human consumption (<https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.515&SearchTerm=farnesol>, [2019.02.19]). This combination could also be used to design preservatives and cleaning products to prevent the formation of biofilms in juice processing plants.

Acknowledgements

This work was supported by the National Agency of Scientific and Technical Research ANPCyT [Brugnoni LI, PICT 2015-0156]. M. R. Agustín acknowledges ANPCyT for a doctoral fellowship and F. R. Viceconte acknowledges CONICET for a doctoral fellowship. L. I. Brugnoni and M. S. Vela Gurovic are researchers of the National Scientific and Technical Research Council (CONICET).

Conflict of Interest

None declared.

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Accepted Article

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Figure captions

Fig. 1 Cell counts of *C. tropicalis* biofilms treated with farnesol ●, 2-phenylethanol □ and tyrosol ◇ (0.05-1.2 mmol l⁻¹, logarithmic scale) at different stages of biofilm development (2, 6, 24 and 48h). Cell numbers of controls are those values at 0 mmol l⁻¹ for each incubation time. Farnesol caused a significant reduction in cell number at 2h, 24h and 48h at all concentrations, exceeding one log unit reduction at 2h (0.6 mmol l⁻¹) and 24h (0.05 mmol l⁻¹).

Fig. 2 Monospecies biofilm structure at adhesion (2h) in the presence of farnesol 0.6 mmol l⁻¹ (left) and 2-phenylethanol 1.2 mmol l⁻¹ (right). For all *Candida* species, farnesol exerted a disruption on biofilm structure, while 2-phenylethanol increased biofilm density when compared with controls.

Fig. 3 Effects of farnesol 0.6 mmol l⁻¹ (**black**) and 2-phenylethanol 1.2 mmol l⁻¹ (**grey**) on cell counts of multispecies biofilms at different stages of biofilm development (2-48h). Cell numbers of controls at each incubation time are highlighted in **white**. Farnesol affected significantly the cell number of mature biofilms (48h). The stimulating effect of 2-phenylethanol was predominantly observed at adhesion (2h). (**p* < 0.05, ***p* < 0.01 ****p* < 0.001).

Fig. 4 Multispecies biofilms at different stages of development (2-48h) in the presence of farnesol 0.6 mmol l⁻¹ (left) and 2-phenylethanol 1.2 mmol l⁻¹ (right). The inhibitory effect of farnesol was clearly observed at adhesion (2h).

Fig. 5 Cell number reduction on *C. tropicalis* biofilms treated with natamycin (0.3-1.2 mmol l⁻¹) alone ● and in combination with farnesol 0.6 mmol l⁻¹ □ at different stages of development. Cell numbers of controls are those values at 0 mmol l⁻¹ for each incubation time. Natamycin deeply altered biofilm cell number at all stages. The addition of farnesol did not cause a major inhibition but rather increased cell number at early stages of biofilm development. (**p* < 0.05, ***p* < 0.01 ****p* < 0.001).

Fig. 6 Epifluorescence microscopy of *C. tropicalis* biofilms after treatment with natamycin 0.01 mmol l⁻¹ (left) and combination with farnesol 0.6 mmol l⁻¹ (right) at each stage of biofilm development (2-48h). Both treatments strongly disrupted the biofilm architecture. Differences in biofilm cell density between treatments were evident after addition (2h), showing that farnesol clearly enhanced biofilm inhibition exerted by natamycin at 48h.

Fig. 7 Number of *C. tropicalis* cells attached to stainless steel after treatment with natamycin 0.01 mmol l⁻¹ (— grey narrow line) and co-treatment with farnesol 0.6 mmol l⁻¹ (— black wide line) at different stages of biofilm development (2-48h) in comparison with controls (..... dashed line). Significant differences were found between treatments at all time points ($p < 0.001$). Natamycin caused a decrease of more than four log numbers at 48h. Farnesol enhanced the inhibitory effect of natamycin by around one log number.

Table 1. Identification of volatiles from cultures of *C. tropicalis* in apple juice 12 °Bx *

	By solvent extraction							By HS-SPME		
	Rt (min)	juice	Incubation [d]					Rt (min)	5	
			2	7	14	21	28			
5-methylfurfural	6.5	-	18.5	17.3	8.0	8.1	13.7	isoamyl alcohol	4.1	54.2
isobutyl formate	7.7	-	-	-	-	-	6.9	amyl alcohol	4.2	16.0
succinic anhydride	7.9	-	-	9.8	-	5.2	-	furfuryl alcohol	8.4	5.2
sorbic acid	9.1	-	-	3.7	5.5	2.2	3.6	isoamyl acetate	8.9	1.6
2-furoic acid methyl ester	9.5	4.3	-	-	-	0.6	-	styrene	9.3	1.7
2-phenyl ethanol	10.3	-	34.0	43.3	50.8	39.7	3.6	methoxy phenyl oxyme [‡]	11.5	2.4
2-furoic acid	10.5	20.5	16.2	-	8.6	-	-	2-ethyl hexanol	17.9	0.8
pyranone	11.3	1.4	-	-	-	1.6	1.5	dimethyl octenol	20.7	0.3
p-vinyl anisole	11.4	-	0.4	-	-	-	-	2-phenyl ethanol	22.5	0.5
prenol	12.3	-	-	-	2.9	1.4	1.9	camphor	25.1	0.8
butenylmethylether	12.4	-	5.1	-	-	-	-	cytronellal dimethyl acetal	27.4	4.2
5-hydroxymaltol	12.5	1.4	-	0.9	-	1.3	-	farnesol	45.6	0.1
5-methyl-2-furanmethanol	13.5	-	1.4	-	-	-	-	farnesene	45.9	0.6
5-hydroxymethylfurfural	13.7	65.3	-	-	-	-	-	squalene [‡]	46.3	0.1
2'-furanil-1,2-ethanediol	13.9	-	-	-	-	20.1	33.1	nerolidol	52.1	3.1
10-dodecen-1-ol propionate	14.1	-	1.2	-	-	-	-	trimethyl pentanediol	54.0	1.2
methyl 2-phenylethanoate	14.2	-	-	-	3.0	-	-	isopropyl myristate	60.9	0.8
chavicol	14.3	-	0.7	-	-	-	-			
1,3-octanediol	14.4	5.9	5.1	2.6	3.4	4.9	6.3			
butanedioic acid	17.2	-	2.7	0.9	0.5	-	-			
tyrosol	18.9	-	4.8	14.2	5.8	6.4	14.1			

nerolidol acetate	19.3	-	0.4	-	-	-	-	
N-acetyl tyramine	20.9	-	5.3	5.2	11.4	8.2	7.8	
betulin	21.7	-	0.9	-	-	-	-	
Total		98.8	96.9	98.0	99.8	99.7	92.6	93.6
NI [†]		1.2	3.1	2.0	0.2	0.3	7.4	6.4

* Values are expressed as relative percentages and compounds are listed by elution order according to GC-MS analyses. [†] Not identified. [‡] This compound was also detected in juice by the same method.













