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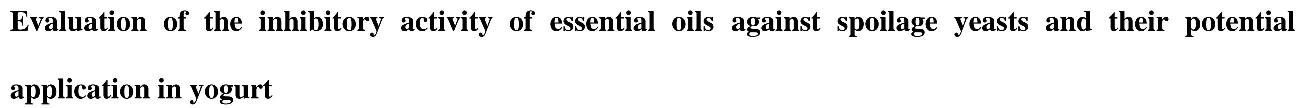

Evaluation of the inhibitory activity of essential oils against spoilage yeasts and their potential application in yogurt

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Abstract

Yeasts are the leading cause of spoilage in yogurt. Considering the high demand from consumers to use natural products as an alternative to additives, essential oils (EOs) could be a promising solution to guarantee high microbiological standards. The present study highlighted the *in vitro* antifungal potential of cinnamon, ginger, lemongrass, mandarin, orange, lemon and lime EOs against spoilage yeasts isolated from yogurts prepared with pasteurized buffalo milk. A total of 74 isolates represented by 14 different species of *Candida*, *Rhodotorula*, *Debaryomyces*, *Kluyveromyces* and *Yarrowia* genera were subjected to a disk diffusion assay, showing lemongrass EO to have the highest antifungal activity (40.97 ± 9.86 mm), followed by cinnamon (38.46 ± 6.59 mm) and orange (12.00 ± 4.52 mm) EOs. *Yarrowia lipolytica* was less susceptible to lemongrass EO than *Candida sake* and *Yarrowia deformans* isolates. Ginger EO exhibited the lowest efficacy. A minimum inhibitory concentration (MIC) assay showed the ability of lemongrass and cinnamon EOs to inhibit the growth of all selected isolates at concentrations between ≤ 0.31 and 1.25 µL/mL. Therefore, for the first time, the two best-performing EOs (lemongrass and cinnamon) based on *in vitro* assays were assessed for their potential roles as preservatives in an *in vivo* yogurt model prepared at the laboratory scale. Since some limitations, such as the inhibition of lactic acid bacteria by cinnamon EO, consequently leading to fermentation failure as well as species-specific antifungal activity of lemongrass EO, were observed, further studies are needed to explore the possibility of using a slightly higher concentration of lemongrass EO and/or combinations of different EOs and/or their components. Finally, since yogurt spoilage could also be prevented by correct sanitation procedures, the sanitizers commonly used in the food industry were tested against all isolates, showing the high efficiency of alcohol-based sanitizers and the ineffectiveness of chlorine-based sanitizers.

Keywords: essential oils, yogurt spoilage yeasts, antifungal activity, sanitizers, *in vivo* yogurt model

1. Introduction

Yeasts are well-known microorganisms with a positive impact on the quality of several fermented foods and beverages, such as dairy products, leavened baked goods and alcoholic beverages (Mayoral et al., 2005). However, they can cause spoilage of foods and beverages, resulting in product deterioration (Vimont et al., 2019). Dairy products are susceptible to yeast spoilage mainly due to yeast species ascribed to the genera *Debaryomyces*, *Kluyveromyces*, *Yarrowia*, *Rhodotorula*, *Candida*, *Pichia*, and *Saccharomyces*. In particular, yogurt is susceptible to yeast spoilage due to the ability of some species to grow at refrigeration temperatures, to ferment sugars such as lactose and sucrose with gas production and to hydrolyze milk proteins and fat (Buehler et al., 2018; Mayoral et al., 2005). The effects on yogurt of such yeast metabolic activities include the swelling of the yogurt bowl, the presence of yeasty off-flavors coupled with reduced quality of the texture and sometimes the appearance of visible yeast colonies on the surface of the yogurt. Overall, the shelf life and commercial value of yogurt are strongly affected by yeast spoilage, thus causing economic losses for the dairy industry (Bernardi et al., 2019; Mayoral et al., 2005; Vimont et al., 2019). The source of yeast contamination during food production is mainly linked to the production environment (such as equipment, surfaces, indoor air), food handlers and/or the additional ingredients used (Bernardi et al., 2019; Buehler et al., 2018; Osimani et al., 2016; 2017). Therefore, a correct sanitation plan and the selection of adequate sanitizers and hygiene procedures applied during food processing are crucial to prevent and control yeast spoilage of yogurt. To the author's knowledge, only a few studies have been published on testing the activity of commercial sanitizers against food spoilage yeasts (Bernardi et al., 2019; Osimani et al., 2017; Salo and Wirtanen, 2005).

Furthermore, the addition of chemical antifungals such as sorbic or benzoic acid or their salts to the product is an additional strategy that may be applied to avoid yeast spoilage of yogurt. However, some yeasts possess genetic or acquired resistance mechanisms to weak organic acids, including the ability to degrade them or to pump out dissociated anions (Dawidowicz and Rado, 2010; Monu et al., 2016). Recently, a growing consumer demand for products without traditional preservatives has been observed, with consequent difficulties for producers to ensure the shelf life of the foods. To this end, the use of natural compounds from vegetable origin as preservatives in the food industry, including essential oils (EOs), may be promising. EOs,

also called volatile or ethereal oils, are aromatic volatile compounds that are obtained from different parts of the plant (i.e., flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) (Tariq et al., 2019). EOs are well known for their pleasant flavoring properties and have a generally recognized as safe (GRAS) status (Çoşkun et al., 2016; Tariq et al., 2019). EOs are characterized by the presence of phenols, aldehydes, ketones, alcohols, esters or hydrocarbons responsible for well-documented antimicrobial properties (Burt, 2004; Tariq et al., 2019). Nevertheless, to the author's knowledge, scarce information is available regarding the antifungal capabilities of EOs in food matrices (Brr and Mahmoud, 2005; Çoşkun et al., 2016; Monu et al., 2016; Sacchetti et al., 2005; Souza et al., 2007).

In this context, the aim of the present study was to screen the *in vitro* activities of cinnamon, ginger, lemongrass, mandarin, orange, lemon and lime EOs against 74 spoilage yeasts isolated from buffalo milk yogurts. These EOs were selected for their possible positive attributions to yogurt flavor and odor. Indeed, the potential role of best-performing EOs as preservatives in an *in vivo* model of yogurt prepared at the laboratory scale was also assessed. Moreover, the efficacies of three sanitizing agents commonly used in the food industry were evaluated against yogurt spoilage yeast isolates.

2. Material and methods

2.1 Essential Oils

Lime (*Citrus aurantifolia)*, ginger (*Zingiber officinale*), lemongrass (*Cymbopogon citratus*), mandarin (*Citrus reticulata*), cinnamon (*Cinnamonum zeylanicum*), orange (*Citrus sinensis*) and lemon (*Citrus limon*) EOs were obtained from Sigma Aldrich (Saint Louis, USA) and their quality parameters (appearance, color, purity, odor, density, refraction index) were described in an accompanying technical report.

2.2 Sampling

Three batches of spoiled buffalo milk yogurt, characterized by yeasty flavor but without visible structure alterations, were purchased from a local producer located in Marche Region (Italy). The yogurts from each batch were sampled in duplicate immediately after production and transported to the laboratory under refrigerated conditions. After arrival, the samples were stored at two different storage temperatures (4°C and

25°C) for 25 days. The analyses were performed immediately after arrival (T0), after 12 days (T1) and after 25 days (T3).

2.3 pH measurements

The pH measurements of buffalo yogurt samples were carried out using a model 300 pH meter equipped with an HI2031 solid electrode (Hanna Instruments, Padova, Italy). Two independent measurements were performed for each sample and the results were expressed as the mean value ± standard deviation.

2.4 Microbial viable counts

Ten grams of each yogurt sample were added with 90 mL of sterile peptone H_2O (peptone, 1 g/L, w/v), homogenized for 2 minutes at 260 rpm in a Stomacher 400 Circulator apparatus (VWR International PBI, Milan, Italy) and subsequently ten-fold serially diluted. For the enumeration of the yeasts, an aliquot (100 μL) of each dilution was inoculated by spread plate method on differential WL agar plates (Merck KGaA, Darmstadt, Germany) added with chloramphenicol (100 mg/L) and incubated at 25°C for 72 h. The pour plate method was used for the enumeration of lactic acid bacteria (LAB) on MRS agar (VWR International, Milan, Italy) added with cycloheximide (250 mg/L) and incubated at 37°C for 48 h. The analyses were performed in duplicate and the results were expressed as mean values of the log of colony forming units (CFU) per g of sample ± standard deviation.

2.5 Isolation and molecular identification of yeasts

A total of 74 isolates were obtained from buffalo yogurt samples, including different batches, sampling times and storage temperatures. In detail, the yeast colonies grown on differential WL agar plates used for the viable counts were selected on the basis of different colony morphologies and/or colors, successively isolated and purified by streaking on the same growth substrate. The purified isolates were stored at -80°C in a mixture of YPD broth (yeast extract 10 g/L, peptone 20 g/L, D-glucose 20 g/L) and glycerol at a ratio of 3:2. The DNA was extracted from each isolate following the procedure described by Makimura et al. (1999). The quantity and the purity of the extracted DNAs were determined by Nanodrop ND 1000 (Thermo Fisher Scientific) and further standardized to 25 ng/μL. Molecular identification of the isolates at the species level

was performed by PCR amplification of the ITS1-5.8S-ITS2 rDNA region of extracted DNAs using the ITS1-ITS4 primer pair as described by Osimani et al. (2015) followed by sequencing at Genewiz (Takeley, UK) and Basic Local Alignment Search Tool (BLAST) analysis (Altschul et al.,1990) as previously detailed by Osimani et al. (2019).

2.6 Susceptibility of yeast isolates to sanitizers

The pool of 74 spoilage yeasts isolated from buffalo milk yogurts was tested for susceptibility to 3 commercial sanitizers (S1, S2, S3) commonly used in the food industry, whose compositions and in-use concentrations are reported in Table 1. To avoid loss of efficiency, the sanitizers were diluted to their in-use concentration right before the beginning of each assay. The susceptibility test was performed in duplicate for all isolates by their exposure to each sanitizer for 5 minutes at 20°C, as detailed by Osimani et al. (2017). The results were expressed as the reduction of the number of viable yeast cells (log CFU/mL) after exposure to sanitizing agents with respect to the appropriate controls exposed to sterile physiological solution (NaCl, 0.85%, w/v).

2.7 Disc diffusion assay

A disc diffusion assay was employed to determine the antifungal activities of the 7 EOs against 74 yeast isolates obtained from spoiled buffalo milk yogurts. The isolates were preliminarily grown on YPD agar plates at 25°C for 48 h. Two loopfuls (10 µL capacity) of each pure culture were collected from the YPD plates and resuspended in 10 mL of sterile physiological solution (0.85% NaCl). The concentration of yeast cells in each suspension was determined using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) by measuring the optical density (OD) at 600 nm. Each suspension was diluted with sterile physiological solution to reach a final concentration of 6 log cells/mL before spreading 100-µL aliquots on two YPD agar plates contemporarily. The plates were left to dry in the laminar flow hood with the lid slightly off for 30 minutes. Seven sterile filter paper discs (6 mm diameter) (Schleicher & Schuell, Germany), each soaked with 10 µL of an EO, were distributed among two YPD agar plates for each isolate. The plates were incubated at 25°C for 48 h, and the diameters of the inhibition zones were measured in millimeters. The disk diffusion assay, including 7 EOs for each isolate, was performed in duplicate, and the

results were expressed as the mean value (mm) ± standard deviation. The best-performing EOs were selected for further analyses.

2.8 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of lemongrass and cinnamon EOs against 20 selected yeast isolates was determined by the broth microdilution method performed in Sabouraud broth (peptone 10 g/L, D-glucose 20 g/L). A loopful (10 µL capacity) of each isolate, preliminarily grown on YPD agar plates at 25°C for 48 h, was inoculated in 10 mL of Sabouraud broth and incubated overnight at 25°C. The concentration of overnight cultures was determined spectrophotometrically as described above and adjusted to approximately 6 log cells/mL. Hence, 100-µL aliquots were distributed in 96-well microdilution plates. EO solutions were prepared as previously described by Souza et al. (2005) and serially diluted. For the susceptibility testing, performed over twofold serial dilutions ranging from 10 to 0.31 µL/mL for both EOs, an aliquot (100 µL) of each dilution was added to microplate wells containing yeast cultures. A positive (inoculated Sabouraud broth without EO) and negative control (noninoculated Sabouraud broth) were included for each tested isolate to demonstrate adequate microbial growth during the incubation period and media sterility, respectively. The microplates were aseptically sealed and incubated at 25°C for 48 h (Sahin et al., 2004; Viljoen et al., 2003). The MIC was defined as the lowest concentration of the EO able to inhibit visible yeast growth at the end of the incubation period. All tests were performed in duplicate.

2.9 Lab-scale yogurt production

The potential role of the selected EOs as preservatives was tested directly in yogurts intentionally contaminated by a selected pool of yeast spoilage isolates. To produce yogurts at a laboratory scale, whole bovine U.H.T. (Ultra-High Temperature) processed milk (12.14% dry matter; 3.74% fat; 3.35% protein; 4.65% lactose; and 0.74% ash) was fermented with two commercial yogurt starter cultures, namely, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* at a concentration of 6 log cells/mL. Three yeast isolates, namely, *Debaryomyces hansenii* (isolate code 35), *Candida pararugosa* (isolate code 59) and *Yarrowia deformans* (isolate code 86), were used for the contamination of yogurts, each added at low and high concentrations of 2 and 4 log cells/mL, respectively. To test the effectiveness of

EOs against spoilage yeasts, yogurts were supplemented with 1.25 µl/mL EO (cinnamon or lemongrass) at the beginning of the fermentation process. Contemporarily, 3 types of control yogurts (negative and positive) were prepared: i) negative control [Ctrl (N)] without the addition of yeasts and EOs to test the efficiency of LAB starter cultures, ii) negative controls [Ctrl (NL) and Ctrl (NC)] supplemented with lemongrass and cinnamon EO, respectively, to test the effect of EOs on LAB starter cultures, and iii) positive control yogurts [Ctrl (P2) and Ctrl (P4)] added with a mixture of three selected yeast isolates at both the tested concentrations (2 or 4 log cells/mL, respectively) but without EOs, to test the growth of spoilage yeasts in experimental conditions. All experiments were performed in duplicate. The samples were incubated at 45°C until a pH value of 4.6 was reached, followed by 28 days of storage at 4°C. pH measurements and viable counts were performed at the beginning and after 8 h, 4 days, 8 days and 28 days of fermentation. The serial dilutions of yogurt samples were prepared as described in section 2.4. Lactic acid bacteria (LAB) were enumerated on M17 agar medium (Merck, Darmstadt, Germany) supplemented with 400 mg/L cycloheximide after incubation at 45°C for 48 h under anaerobic conditions (Camu et al., 2008). The enumeration of the yeasts was performed on YPD agar medium supplemented with 200 mg/L chloramphenicol to inhibit bacterial growth after incubation at 25°C for 96 h aerobically. All analyses were performed in duplicate.

2.10 Statistical analysis

The results of each performed analysis were subjected to one-way analysis of variance (ANOVA). The differences were considered significant at $P < 0.05$. All the statistical analyses were performed using JMP statistical software v. 11.0 (SAS Institute, Cary, NC, USA).

3. Results and Discussion

3.1 pH measurement and viable counts

The pH values of the buffalo milk yogurts (3 different batches) measured for 25 days of storage at 2 different temperatures (4°C and 25°C) are reported in Table 2. As expected, the initial pH was between 4.24 ± 0.00 (batch 2) and 4.67 ± 0.01 (batch 3), which are the values commonly reported for different types of yogurt

(Aryana & Olson, 2017). A slight, statistically insignificant decrease in pH was observed during storage. This trend was more evident for the samples stored at 25°C, with values ranging from 3.18 ± 0.00 (batch 3) to 3.54 ± 0.01 (batch 2), registered at the end of the shelf life (25 days, T2). Even if the samples stored at 25°C were characterized by pH values lower than those measured for the refrigerated samples, the differences were not significant.

The yeast spoilage of yogurt is usually recognized by gas production, which may result in swelling of the yogurt's package or even by visible yeast colonies on the surface of the product in the case of a high contamination level. Other spoilage characteristics, such as structural loss or yeasty odor and flavor, have been reported (Snyder et al., 2016). The hypothesis that the yeasty flavor of buffalo milk yogurts in the present study is due to yeast spoilage was checked by viable counts. Tirloni et al. (2015) reported that yogurt should not contain more than 1 yeast cell/g at the time of production and, if stored in refrigerated conditions, should not undergo yeast spoilage, which is perceived when loads reach 5 to 6 log CFU/g (Fleet, 1990). The results of the viable counts (Table 3) confirmed our hypothesis, showing that the initial yeast load was between 0.33±0.46 and 2.40 ± 0.01 log CFU/g. The number of yeasts increased significantly (approximately 3-4 logs) after 12 days (T1) of storage under refrigerated conditions and remained stable until the end of shelf life (25 days, T2). The same trend, except for batch 3, was observed for the samples stored at room temperature during the first 12 days of storage, followed by a decrease in the number of yeasts enumerated on the 25th day (T2), with values between 0.85 ± 1.20 log CFU/g (batch 1) and 1.82 ± 0.83 log CFU/g (batch 2). Conversely, the number of yeasts counted at T0 for batch 3 did not increase significantly during storage. Considering the storage conditions, the proliferation of the yeasts was evidently supported by the low storage temperature (4°C) during the whole shelf life.

Since LAB play a crucial role in the fermentation of milk and milk products, the enumeration of this group of microorganisms was performed for all the samples during storage, with the results reported in Table 3. According to Italian Standard Uni 10358 (Uni Ente Italiano di Normazione, 1993), the minimum viable count of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in yogurt should be ≥ 6 log CFU/g during the entire shelf life, while their sum must be at least 7 log CFU/g. In the present study, the initial LAB load attested at approximately 6.5 log CFU/g for batches 1 and 2 decreased progressively during storage, showing a quite low load (~3.4 log CFU/g) at the end of the shelf life (T2). In contrast, batch 3 was characterized by a

low initial load of LAB (4.54 ± 0.03 log CFU/g), which remained stable during the entire storage period. The samples stored at 25°C showed a different trend with the maximum LAB load (> 7 log CFU/g) after 12 days (T1) of storage. Finally, except for batch 3, a significantly higher LAB concentration was observed in samples stored at 25°C when compared to refrigerated samples.

Generally, the results of the present study showed that refrigerated conditions favor the proliferation of yeasts, while higher storage temperatures act selectively on LAB. The competitive advantage of yeasts at low storage temperatures with respect to starter LAB cultures has already been reported by Fleet (2011) and Snyder et al. (2016).

3.2 Yeast identification

The molecular identification of 74 spoilage yeasts isolated from 3 different batches of buffalo milk yogurt during their shelf life highlighted remarkable biodiversity (Supplementary Table 1), represented by 14 different species (*Candida aaseri*, *Candida intermedia*, *Candida parapsilosis*, *Candida pararugosa*, *Candida sake*, *Clavispora lusitaniae* (anamorph: *Candida lusitaniae*), *D. hansenii, Debaryomyces subglobosus, Kluyveromyces lactis*, *Rhodotorula babjevae*, *Rhodotorula diobovata*, *Rhodotorula glutinis*, *Y. deformans* and *Yarrowia lipolytica*) from 5 genera, distributed as shown in Figure 1. *Y. lipolytica* and *D. hansenii* largely prevailed between isolates. These two species, together with *C. parapsilosis*, *Candida diffluens*, *Kluyveromyces marxianus*, *Rhodotorula mucilaginosa* and *Zygosaccharomyces bailii*, are reported to be among the most frequently detected yeast contaminants in fermented milks and yogurts, especially in those with high sugar or fruit contents (Mayoral et al., 2005).

Y. lipolytica and *D. hansenii* are commonly isolated from lipid- or protein-rich foods such as cheese, yogurt, butter, meat and olive oil due to their high potential of producing enzymes such as lipases and proteases. The frequent spoilage of yogurt by these species might be related to their capacity to grow well at low storage temperatures and in highly acidic foods as well as to their resistance to weak acid preservatives, such as benzoic and sorbic acids, when compared to other spoilage yeasts (Praphailong and Fleet, 1997). Although some *Y. lipolytica* strains can cause food spoilage evidenced by off-flavors or brown pigments, no cases of negative effects on human health have been reported (Liu et al., 2015). Moreover, *Y. lipolytica* strain B9014

has been reported to be able to improve the viability of *Lactobacillus bulgaricus* after coinoculation in yogurt (Liu and Tsao, 2009).

Regarding *Candida*, six different species were sporadically identified among isolates, with *C. parapsilosis* and *C. sake* isolated from batches 1 and 3 and *C. intermedia*, *C. pararugosa* and *C. aaseri* isolated exclusively from batches 1, 2 and 3, respectively (Supplementary Table 1). The species from this genus may cause yeasty and bitter off-flavors as well as swelling of containers due to gas produced when the cell concentration reaches above 5 log CFU/g (Hommel, 2014). Twenty-four different *Candida* species have already been reported as responsible for dairy product spoilage, with *C. parapsilosis* and *C. intermedia* most frequently isolated from spoiled yogurt (Mayoral et al., 2005; Tilbury et al., 1974), while *C. pararugosa* and *C. sake* were previously isolated only from traditional Tanzanian fermented milk, "mtindi" (Mlimbila et al., 2013), and from yogurts produced using traditional methods in the North East Anatolian Region of Turkey (Biberoğlu and Ceylan, 2013), respectively. To the best of the authors' knowledge, this is the first report of the lipolytic yeast *C. aaseri* detected in yogurt. *Cl. lusitaniae*, isolated exclusively from batch 1, is reported to cause disseminated candidiasis. This species was first isolated from the digestive tracts of warm-blooded animals and some environmental sources, such as cornmeal, citrus peel, fruit juices, milk from cows with mastitis and spoiled yogurt. In contrast to our study, Green and Ibe (1987) reported this species as the most abundant among 100 analyzed yogurt samples.

Considering *Rhodotorula*, only a few species were sporadically identified among freshly produced yogurt samples (T0), with *R. babjevae* isolated from batch 2 and *R. diobovata* and *R. glutinis* isolated from batch 3. The environment, working surfaces, worker hands and equipment are reported to be the main sources of contamination by *Rhodotorula* species in the dairy industry (Yeeh, 1999). Some representatives of the genus *Rhodotorula* can cause staining and a bitter taste to the products (Savova and Nikolova, 2002). Moreover, yeast species, mainly from the genera *Candida* and *Rhodotorula,* have been reported to be able to decrease the quality of dairy products by lactose assimilation (Wood, 1985).

3.3 Susceptibility of yeast isolates to sanitizers

Although the yeasts causing the spoilage of the yogurt may derive from the raw milk, this possibility is significantly reduced by its pasteurization. Eumycetes have been frequently isolated from dairy

environments, so inadequately sanitized production equipment or working surfaces represent the most common sources of contamination, especially evident after the pasteurization process (Buehler et al., 2018). The failure of the sanitizing procedure is usually due to the selection of inappropriate sanitizers or their incorrect use, including inaccurate in-use concentrations and exposure times. The sanitizing agents used in the food industry are typically based on alcohol, chlorine, hydrogen peroxide, persulphate, peracetic acid, quaternary ammonium and iodophor compounds (Salo and Wirtanen, 2005). Not only the selection of appropriate sanitizers but also the characteristics of spoilage microorganisms present in the environment need to be considered for effective fungal control. Information on the efficiency of the main sanitizers allowed in the food industry against principal food spoilage yeasts can help in selecting the best sanitizing agent for the control of hygiene, which could significantly reduce the economic losses caused by food spoilage (Bernardi et al., 2019). In the present study, the efficiency of 3 commercial sanitizers (S1, S2, S3) was tested against spoilage yeasts isolated from buffalo milk yogurts. The results of the susceptibility test (Table 4, Supplementary Table 2) showed alcohol-based sanitizers (S1 and S2) to be able to reduce the number of viable yeast cells by more than 4 log units. According to European standard EN 13697 (2001), the basic requirement for each liquid sanitizer to be recognized as effective is at least a 3-log-unit reduction of fungal viable cells when compared with the corresponding positive control. The S2 sanitizer (30–50% isopropyl alcohol) was the most efficient, causing an average reduction of 5.62 ± 0.43 log CFU/mL of the yeast cells after 5 minutes of exposure. The mean reduction of the spoilage yeasts by sanitizer S1 (20–30% isopropyl alcohol; 0.005% didecyl dimethyl ammonium chloride) was 4.35 ± 1.67 log CFU/mL, except for *C. pararugosa* isolates characterized by lower susceptibility (< 3 log CFU/mL reduction). Sanitizer S3 (3% active chlorine, 1% in-use concentration) was not efficient against the tested yeasts except for *Cl. lusitaniae* isolates, which were susceptible (4.56 ± 0.27 log CFU/mL reduction). The obtained results confirm the high efficacy of alcohol-based sanitizers as well as the ineffectiveness or reduced efficacy of chlorine-based sanitizers against viable yeast cells, as previously reported by Salo and Wirtanen (2005) and Osimani et al. (2017), respectively.

3.4 Disc diffusion assay

A disc diffusion assay was carried out to test the antifungal activities of the 7 EOs (lime, ginger, lemongrass, mandarin, cinnamon, orange and lemon) against the 74 spoilage yeast isolates. The average results, expressed in millimeters of growth inhibition zone for each species represented by at least 2 isolates, are reported in Table 5, while Supplementary Table 3 contains the results for each of the 74 isolates separately. Considering all the tested isolates, statistical analysis showed lemongrass EO to have the highest antifungal activity (40.97 ± 9.86 mm), followed by cinnamon (38.46 ± 6.59 mm) and orange EOs (12.00 ± 4.52 mm). Since the thickness of the growth medium and the type of solvent used for the assay can influence the diffusion rate of the EOs, the comparison of the results obtained in different studies is not feasible (Burt, 2004). Based on the inhibition zone generated by the diffusion of the EOs into plates containing growth media inoculated by yeast strains, Conner and Beuchat (1984) proposed EOs to be classified as i) strongly active with an inhibition zone > 12 mm, ii) moderately active with an inhibition zone between 6 and 12 mm, and iii) inactive with an inhibition zone < 6 mm. Accordingly, the lemongrass, cinnamon, and orange EOs assayed in the present study may be classified as strongly active EOs. The strong antimicrobial activity of lemongrass EO is mainly due to its principal component, citral, which is a mixture of two chiral isomers, geranial and neral. Additional active ingredients, such as limonene, citronellol, myrcene, geraniol, geranyl acetate and nerol, may be found in lemongrass EO (Shah et al., 2011). The literature indicates that citral may inhibit the growth of yeast cells by oxidative stress (Khan et al., 2011). Among the different species represented by at least two isolates, *C. sake* and *Y. deformans* isolates showed higher susceptibility to lemongrass EO than *Y. lipolytica* isolates (Table 5). The strong *in vitro* activity of lemongrass EO and citral against *Candida* spp. yeasts was previously described by da Silva et al. (2008). Although the antifungal activity of lemongrass EO has been reported frequently, mostly against phytopathogens and dermatophytes, its activity against food-spoilage yeasts has scarcely been studied (Sacchetti, 2005).

As shown in Table 5, no significant differences were seen among species when cinnamon EO was used as a growth-inhibiting agent. The antimicrobial activity of cinnamon EO is mainly due to its major constituent, cinnamaldehyde, and other active components, such as eugenol and cinnamic acid. The strong activity of cinnamon EO and cinnamaldehyde against pathogenic bacteria from foods has been reported frequently; thus, this EO and its components have been widely used as antimicrobials and antioxidants in the food industry (Chu et al., 2020). Cinnamon EO inhibits the growth of yeast cells by altering cell membrane

permeability and fluidity, as in the case of *C. albicans* and *S. cerevisiae*, or even by preventing cell division (Shanina et el., 2018). Brr and Mahmoud (2005) tested the activity of cinnamon EO against *Candida*, *Cryptococcus*, *Debaryomyces*, *Issatchenkia*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Trichosporon* and *Yarrowia* species isolated from Egyptian fresh chicken meat products, showing the strong activity of cinnamon EO at 20% concentration against *C. albicans*, *D. hansenii* and *S. cerevisiae* isolates.

Among *citrus* EOs (orange, mandarin, lemon and lime) assayed in the present study, only orange EO was strongly active against the tested isolates. The most frequently isolated species, such as *D. hansenii*, *Y. lipolytica* and *Y. deformans,* were less susceptible to orange EO than the other tested yeasts. On average, lime EO was moderately active (6.35 ± 4.36 mm) against 74 isolates with reduced activity against *Y. lipolytica* isolates, while mandarin and lemon EOs showed low antifungal activity, with an inhibition zone < 6 mm. Several studies reported EOs extracted from the *Citrus* genus to have good antibacterial and antifungal activity (Chanthaphon et al., 2008; Dosoky and Setzer, 2018), attributed principally to components such as L-linalool, D-limonene, or citral, which are present in different portions (Viuda-Martos et al., 2008).

Finally, ginger EOs exhibited the lowest efficacy against the tested isolates, with all but *C. pararugosa* isolates being resistant. This finding is in accordance with Whiley et al. (2018), where ginger EO is listed among EOs showing only moderate or limited antifungal activity.

3.5 The minimum inhibitory concentration (MIC) of lemongrass and cinnamon EOs

Since the lemongrass and cinnamon EOs showed the highest inhibitory activity against spoilage yeasts in the study, their MIC values were determined against 20 yeast isolates (Table 6), chosen to represent each of 14 detected species in relation to their abundance. Accordingly, one or two isolates were selected from less abundant species, while the most abundant species, such as *Y. lipolytica* and *D. hansenii,* were represented by three isolates each, selected among those showing different sensitivities to lemongrass and cinnamon EOs. The MIC values obtained by using a broth microdilution method are reported in Table 6. Both EOs were able to inhibit the growth of the selected yeast isolates at concentrations between ≤ 0.31 and 1.25 µL/mL. Two isolates from the *Rhodotorula* genus, namely, *R. diobovata* and *R. glutinis,* showed the highest sensitivity to both essential oils, as they were inhibited by the lowest concentration (≤ 0.31 ± 0.00 µL/mL) applied in the present study. Additionally, the same MIC value was registered when cinnamon EO was used

as an inhibiting agent against *C. intermedia* and *C. sake* isolates. Moreover, the growth of the latter two *Candida* species, together with *C. pararugosa*, *Cl. lusitaniae*, *D. hansenii*, *D. subglobosus*, *K. lactis*, *Y. deformans* (isolate 86) and *Y. lipolytica* (isolate 76), was inhibited by the highest lemongrass EO concentration (1.25 ± 0.00 µL/mL). The same result was observed for cinnamon EO when used to inhibit the growth of *C. parapsilosis*, *C. pararugosa*, and *Cl. lusitanie*, *D. hansenii* (isolate 35), *K. lactis* and *Y. deformans* (isolate 86). Analogous to what was reported for the disk diffusion method, the comparison of MIC values from different studies could be difficult due to the use of nonstandardized quantitative or semiquantitative methods (Thielmann et al., 2019). However, the results of the study performed by Çoşkun et al. (2016) aimed to investigate the antifungal activity of nine EOs (cinnamon, clove, laurel, oregano, marigold, mint, mustard, coriander, and tea tree oil) against some food spoilage yeasts, such as *Candida lambica*, *Candida keyfr*, *Candida zeyland*, *C. sake*, and *S. cerevisiae,* which showed cinnamon EO to have the lowest MIC value (7.8 µL/mL) among the tested EOs. Regarding the lemongrass EO, the MIC analysis performed by Sacchetti et al. (2005) resulted in its high efficiency against food spoilage yeasts such as *C. albicans*, *R. glutinis*, *Schizosaccharomyces pombe*, *S. cerevisiae* and *Y. lypolitica*. Similarly, Pattnaik et al. (1996) reported lemongrass EO to be able to inhibit the growth of 3 yeast-like and 9 filamentous fungi, showing MIC values between 0.25 µL/mL and 10 µL/mL.

3.6 Efficacy of lemongrass and cinnamon EOs as preservatives in yogurts produced at the laboratory scale

According to the disk diffusion and MIC assay results, lemongrass and cinnamon EOs showed the highest efficacy against different yogurt spoilage yeasts; thus, their potential role as preservatives was tested directly in yogurts produced at the laboratory scale and intentionally contaminated by a selected pool of spoilage yeasts. The yeasts were selected among those showing the highest MIC values (1.25 µL/mL) for both EOs, with *D. hansenii* (isolate 35), *C. pararugosa* (isolate 59) and *Y. deformans* (isolate 86) each added in two different concentrations (2 and 4 log cells/mL, respectively). The U.H.T. whole bovine milk, used instead of buffalo milk for the convenience and easier reproducibility of the experiment, was supplemented with 1.25 µL/mL EOs before fermentation with two commercial yogurt starter cultures followed by 28 days of storage of the obtained yogurts in refrigerated conditions. The pH dynamics of yogurt samples monitored during fermentation and storage are reported in Figure 2.

When lemongrass EO was used as an antifungal agent (Figure 2, panel A), the initial pH value of 6.7 decreased significantly after 8 h of incubation at 45°C, reaching values ≤ 4.5 in all the samples, thus indicating an effective fermentation process. A further slight decrease in the pH was observed during storage at 4°C, attesting to the values of approximately 4.2 for all the samples at the end of the experiment (28 days). The viable counts of LAB and spoilage yeasts were evaluated throughout the entire experimental period. Based on the pH results as well as the presence of typical yogurt appearance (consistency and color), showing successful yogurt production after the addition of lemongrass EO, it was hypothesized that this EO does not inhibit the growth of LAB starter cultures under experimental conditions. Indeed, the hypothesis was confirmed by viable counts where the initial LAB concentration of approximately 6-7 log CFU/g (inoculation level) increased significantly after 8 h of fermentation (from 8.86 to 9.01 log CFU/g) and remained stable during the 28 days of storage at 4°C, attesting to values between 8.14 and 8.95 log CFU/g in all samples including negative control Ctrl (NL) (Table 7). Regarding the antifungal activity of the lemongrass EO, it was species-specific, showing the complete growth inhibition (<1 log CFU/g) (data not shown) of *D. hansenii* and *Y. deformans* in all sampling time points and reduced growth of *C. pararugosa* isolates. As reported in Supplementary Figure 1, the initial concentration (2 and 4 log CFU/g, respectively) of *C. pararugosa* (isolate 59) remained stable during the first 8 days of storage, followed by a 2 log-unit increase in the number of viable yeast cells at the end of the trial (28 days). To the authors' knowledge, data regarding the use of lemongrass EO in yogurt with inhibitory activity against spoilage yeasts is lacking in the scientific literature, thereby preventing further comparison.

A different pH trend, demonstrated in Figure 2 (panel B), was observed when cinnamon EO was used to inhibit the growth of the spoilage yeasts. After 8 h incubation at 45°C, the pH values of all but control samples [negative Ctrl (N) and positive Ctrl (P2) and Ctrl (P4) controls] not supplemented with cinnamon EO remained higher than 6.0, thus indicating fermentation failure. Therefore, these samples were not transferred at 4°C for storage but were further incubated at 45°C. Although the pH of all the samples, including the Ctrl (NC) (negative control supplemented with cinnamon EO), reached values ≤ 4.8 after 4 days of incubation, typical milk and no yogurt-like texture was observed. The samples were further stored at 4°C, showing pH values between 4.15 (yogurt contaminated with 4 log cells/mL of *C. pararugosa*) and 4.48 (yogurt contaminated with 2 log cells/mL of *D. hansenii*) at the end of the 28-day storage period.

Fermentation failure was hypothesized to be the consequence of the strong antimicrobial activity of cinnamon EO, as confirmed by the results of LAB viable counts. After 8 h of incubation at 45°C, the initial 6-7 log CFU/g concentration of commercial yogurt LAB starter cultures added to milk strongly decreased in all samples supplemented with cinnamon EO, including negative control Ctrl (NC), showing values below 2 log CFU/g throughout the whole storage period (Table 7). The strong growth inhibition of yogurt starter cultures by cinnamon, cardamom and clove EOs at a 0.05-5 µL/g concentration has been previously reported by Bayoumi (1992). In contrast, Suliman et al. (2019) demonstrated that the addition of cinnamon powder to cow milk yogurt enhanced the LAB load and simultaneously reduced the total bacterial, coliform and *Escherichia coli* loads. Similarly, Illupapalayam et al. (2014) reported that the presence of cardamom, cinnamon and nutmeg spice oleoresins in yogurts did not negatively affect the probiotic strains during 4 weeks of refrigerated storage. Since the previous studies reported contradictory results regarding the effect of cinnamon EO on LAB, in the present study EOs were tested directly in an *in vivo* system, thus allowing us to predict the effectiveness of selected EOs in a real food system and fermentation conditions. As reported by Calo et al. (2015), intrinsic factors, such as pH, water activity, protein and fat content, enzymes and salt concentration, as well as extrinsic factors, including temperature, the presence of microorganisms and packaging differences, can potentially reduce or even enhance the antimicrobial activity of EOs in different food matrices.

Although the addition of cinnamon EO triggered the failure of yogurt production, its strong antifungal activity was confirmed by the viable counts of the yeasts showing < 1 log CFU/g in all samples and sampling time points (data not shown).

4. Conclusions

Among the seven tested EOs, cinnamon and lemongrass EOs were the most effective against spoilage yeasts isolated from buffalo milk yogurt. Both EOs inhibited the growth of the selected yeasts at relatively low concentrations, between ≤ 0.31 and 1.25 µL/mL, which represented a good basis for directly evaluating their antifungal activity in intentionally contaminated yogurts. Later, the strong antifungal activity of cinnamon EO was confirmed in the yogurt model, but unfortunately, its strong antimicrobial activity against LAB

starter strains caused incomplete fermentation of milk. By contrast, lemongrass EO was found to be the most promising since it did not have a negative influence on milk fermentation, and its antifungal activity resulted in the complete growth inhibition of *D. hansenii* and *Y. deformans* and reduced the growth of *C. pararugosa* isolates. Although the antifungal activity of lemongrass EO seems to be species-specific, a slightly higher concentration of this EO could inhibit the growth of all spoilage yeasts in yogurt. Furthermore, the combinations of different EOs and/or their components could represent a promising alternative to avoid this problem. However, since the addition of EOs in yogurts could affect the sensory properties of final product, which is very critical from both scientific and marketing points of view, further investigations including sensory analysis of the resulting products are strongly needed.

Finally, since correct sanitation and hygiene procedures applied during yogurt production are crucial to prevent spoilage, the results of the present study also showed the high efficiency of alcohol-based sanitizers against all tested isolates.

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Author contributions

Vesna Milanović: Investigation, Data curation, Writing-original draft, Writing-review & editing; **Riccardo Sabbatini**: Investigation, Data curation; **Cristiana Garofalo**: Conceptualization, Supervision, Writing-original draft, Writing-review & editing; **Federica Cardinali**: Data curation; **Marina Pasquini**: Resources; **Lucia Aquilanti**: Supervision; **Andrea Osimani**: Investigation, Resources, Supervision, Writing-review & editing.

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FIGURE CAPTIONS

Figure 1. Species distribution of the 74 spoilage yeasts isolated from 3 batches of buffalo milk yogurt.

The number of isolates for each species is reported.

Figure 2. pH dynamics of yogurt samples supplemented with lemongrass EO (panel A) and cinnamon EO (panel B) during the 28 days storage.

Ctrl (N), negative control (yogurt without the addition of EOs and yeasts); Ctrl (P2) and Ctrl (P4), positive controls (yogurt intentionally contaminated with a mixture of the three selected yeast isolates at concentration of 2 and 4 log cells/mL, respectively, not supplemented with EOs); Ctrl (NL) and Ctrl (NC), negative controls (yogurt supplemented with lemongrass and cinnamon EO, respectively, not contaminated by yeasts); *D. h.* (2), *D. h.* (4), *C. p.* (2), *C. p.* (4), *Y. d.* (2), *Y. d.* (4), yogurt contaminated with 2 and 4 log cells/g of *Debaryomyces hansenii* (isolate 35), *Candida pararugosa* (isolate 59) and *Yarrowia deformans* (isolate 86), respectively, supplemented with lemongrass (panel A) or cinnamon (panel B) EO. Error bars indicate standard deviations.

Table 1. The commercial sanitizers tested against spoilage yeasts isolated from buffalo milk yoghurts.

Sanitizer	Composition*	In-use concentration*
S1	20–30% isopropyl alcohol; 0.005% didecyl dimethyl ammonium chloride	100%
S2	30–50% isopropyl alcohol	100%
S3	3% active chlorine	1%

*based on information from the manufacturers.

Table 2. pH values of buffalo milk yoghurt samples stored at 4°C and 25°C during 25 days shelf-life.

Batch	**Storage temperature**	**Sampling time**		
		T0	**T1**	**T2**
1	4°C	4.34±0.03	4.16±0.02	4.19±0.00
	25°C	4.34±0.03	4.02±0.00	3.48±0.01
2	4°C	4.24±0.00	4.23±0.00	4.22±0.01
	25°C	4.24±0.00	3.86±0.00	3.54±0.01
3	4°C	4.67±0.01	4.52±0.01	4.36±0.01
	25°C	4.67±0.01	3.40±0.00	3.18±0.00

Mean values ± standard deviations of double biological and technical experiments are shown; TO, 0 days; T1, 12 days; T2, 25 days. No significant differences among samples were observed at $P < 0.05$.

Table 3. Microbial counts (log CFU/g) of buffalo milk yoghurt samples stored at 4°C and 25°C for 25 days shelf-life.

Batch	Storage temperature	Yeasts			Lactic acid bacteria		
		T0	T1	T2	T0	T1	T2
1	4°C	$1.69 \pm 1.34^{b*}$	5.12 ± 0.28^{aA}	4.78 ± 0.11^{abA}	$6.67 \pm 0.09^{a*}$	4.48 ± 0.01^{bB}	3.73 ± 0.12^{cB}
	25°C	$1.69 \pm 1.34^{a*}$	3.17 ± 0.05^{aB}	0.85 ± 1.20^{aB}	$6.67 \pm 0.09^{b*}$	7.20 ± 0.09^{aA}	5.86 ± 0.12^{cA}
2	4°C	$0.33 \pm 0.46^{b*}$	5.08 ± 0.06^{aA}	4.07 ± 0.21^{aA}	$6.48 \pm 0.05^{a*}$	4.47 ± 0.03^{bB}	3.14 ± 0.42^{cB}
	25°C	$0.33 \pm 0.46^{b*}$	4.19 ± 0.91^{aA}	1.82 ± 0.83^{abA}	$6.48 \pm 0.05^{b*}$	7.56 ± 0.05^{aA}	7.60 ± 0.01^{aA}
3	4°C	$2.40 \pm 0.01^{c*}$	5.44 ± 0.03^{bA}	5.87 ± 0.03^{aA}	$4.54 \pm 0.03^{a*}$	4.58 ± 0.12^{aA}	4.62 ± 0.08^{aA}
	25°C	$2.40 \pm 0.01^{a*}$	0.81 ± 1.15^{aB}	1.30 ± 0.79^{aB}	$4.54 \pm 0.03^{b*}$	7.42 ± 0.02^{aA}	4.75 ± 0.09^{bA}

Mean values ± standard deviations of double biological and technical experiments are shown; TO, 0 days; T1, 12 days; T2, 25 days. Means followed by different letters (a, b, c) within each row indicate significant differences ($P<0.05$) over time; means followed by different letters (A, B) indicate significant differences ($P<0.05$) among storage temperatures (4°C and 25°C) for each batch and sampling time; *, statistical analysis non applicable.

Table 4. Susceptibility of spoilage yeasts isolated from buffalo milk yoghurts to 3 commercial sanitizers (S1, S2, S3). The results are expressed as the reduction (log CFU/mL) of viable yeast cells after exposure to sanitizers with respect to the appropriate controls exposed to sterile physiological solution.

Species	**Sanitizer**		
	S1	**S2**	**S3**
Candida parapsilosis	- 3.90 ± 0.16 $^{b\ AB}$	- 5.71 ± 0.54 $^{a\ A}$	- 0.32 ± 0.14 $^{c\ BCD}$
Candida pararugosa	- 2.77 ± 0.57 $^{b\ B}$	- 5.79 ± 0.19 $^{a\ A}$	- 0.36 ± 0.42 $^{c\ CD}$
Candida sake	- 5.63 ± 0.30 $^{a\ A}$	- 5.63 ± 0.30 $^{a\ A}$	- 1.37 ± 0.43 $^{b\ B}$
Clavispora lusitaniae	- 5.97 ± 0.21 $^{a\ A}$	- 5.97 ± 0.21 $^{a\ A}$	- 4.56 ± 0.27 $^{b\ A}$
Debaryomyces hansenii	- 5.09 ± 0.97 $^{b\ A}$	- 5.61 ± 0.30 $^{a\ A}$	- 0.83 ± 0.74 $^{c\ BC}$
Rhodotorula babjevae	- 5.94 ± 0.16 $^{a\ A}$	- 5.94 ± 0.16 $^{a\ A}$	- 0.59 ± 0.60 $^{b\ BCD}$
Yarrowia deformans	- 4.86 ± 1.17 $^{a\ A}$	- 5.37 ± 0.65 $^{a\ A}$	- 0.44 ± 0.39 $^{b\ CD}$
Yarrowia lipolytica	- 3.21 ± 1.79 $^{b\ B}$	- 5.53 ± 0.48 $^{a\ A}$	- 0.46 ± 0.50 $^{c\ D}$
Total (74 isolates)	- 4.35 ± 1.67 ^{b}	- 5.62 ± 0.43 ^{a}	- 0.81 ± 0.92 ^{c}

Mean values ± standard deviations of double experiments are shown. S1, 20–30% isopropyl alcohol with 0.005% dodecyl dimethyl ammonium chloride; S2, 30–50% isopropyl alcohol; S3, 3% active chlorine. Means followed by different letters within each row (a, b, c) and within each column (A, B, C…) indicate significant differences ($P<0.05$) among sanitizers and yeast species, respectively. To underline significant differences among species, only those represented with at least two isolates were subjected to ANOVA.

Table 5. The activity of 7 essential oils (EO) against different spoilage yeasts isolated from buffalo milk yoghurts, as detected by agar disc-diffusion assay.

Species	Essential oil*						
	Lime	Ginger	Lemongrass	Mandarin	Cinnamon	Orange	Lemon
Candida parapsilosis	6.50 ± 3.84 AB bc	0.00 ± 0.00 B c	44.00 ± 6.48 AB a	4.00 ± 4.00 ABC bc	41.00 ± 3.61 A a	12.50 ± 1.66 BC b	5.50 ± 5.55 AB bc
Candida pararugosa	11.33 ± 4.50 A bc	2.67 ± 4.13 A c	39.00 ± 13.43 AB a	1.33 ± 3.27 BC c	43.33 ± 3.27 A a	23.33 ± 9.00 A b	2.67 ± 4.13 AB c
Candida sake	8.00 ± 2.62 A bc	0.00 ± 0.00 B c	51.75 ± 4.83 A a	8.50 ± 0.93 A bc	43.75 ± 12.30 A a	15.75 ± 6.27 B b	7.00 ± 4.54 A bc
Clavispora lusitaniae	11.00 ± 2.00 A c	0.00 ± 0.00 B d	48.50 ± 2.52 AB a	4.00 ± 4.62 ABC cd	34.00 ± 9.38 A b	12.50 ± 2.52 BC c	4.00 ± 4.62 AB cd
Debaryomyces hansenii	8.43 ± 1.04 A cd	0.00 ± 0.00 B e	42.14 ± 10.33 AB a	2.90 ± 3.96 B e	36.43 ± 5.87 A b	11.38 ± 2.19 C c	6.29 ± 4.16 A d
Rhodotorula babjevae	10.00 ± 2.31 A bc	0.00 ± 0.00 B c	39.50 ± 6.40 AB a	8.50 ± 1.00 A bc	38.00 ± 8.49 A a	18.00 ± 6.93 AB b	6.00 ± 4.00 AB c
Yarrowia deformans	6.33 ± 4.81 AB cd	0.00 ± 0.00 B e	46.17 ± 6.85 A a	0.00 ± 0.00 BC e	35.83 ± 5.75 A b	8.67 ± 3.23 C c	2.67 ± 4.16 AB de
Yarrowia lipolytica	3.07 ± 4.19 B c	0.00 ± 0.00 B d	37.05 ± 9.23 B a	0.86 ± 2.50 C cd	38.36 ± 5.65 A a	10.39 ± 2.39 C b	3.04 ± 3.77 B cd
Total (74 isolates)	6.35 ± 4.36 d	0.22 ± 1.30 f	40.97 ± 9.86 a	2.49 ± 3.79 e	38.46 ± 6.59 b	12.00 ± 4.52 c	3.97 ± 4.52 e

*Diameters (mm) of yeast growth inhibition zones expressed as the mean value of the isolates from the same species ± standard deviation. Means followed by different letters within each row (a, b, c…) and within each column (A, B, C…) indicate significant differences (P<0.05) between essential oils and yeast species, respectively. To underline significant differences among species, only those represented with at least two isolates were subjected to ANOVA.

Table 6. Minimum Inhibitory Concentration (MIC) values of lemongrass and cinnamon essential oils against 20 selected spoilage yeasts isolated from 3 different batches of buffalo milk yoghurts.

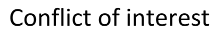
Isolate code	**Species**	**MIC (µL/mL)**	
		Lemongrass	**Cinnamon**
74	*Candida aaseri*	0.62±0.00	0.62±0.00
38	*Candida intermedia*	1.25±0.00	≤ 0.31±0.00
19	*Candida parapsilosis*	0.62±0.00	1.25±0.00
59	*Candida pararugosa*	1.25±0.00	1.25±0.00
33	*Candida sake*	1.25±0.00	≤ 0.31±0.00
24	*Clavispora lusitaniae*	1.25±0.00	1.25±0.00
42	*Clavispora lusitaniae*	1.25±0.00	1.25±0.00
35	*Debaryomyces hansenii*	1.25±0.00	1.25±0.00
64	*Debaryomyces hansenii*	1.25±0.00	0.62±0.00
65	*Debaryomyces hansenii*	1.25±0.00	0.62±0.00
93	*Debaryomyces subglobosus*	1.25±0.00	0.62±0.00
61	*Kluyveromyces lactis*	1.25±0.00	1.25±0.00
51	*Rhodotorula babjevae*	0.62±0.00	0.62±0.00
73	*Rhodotorula diobovata*	≤ 0.31±0.00	≤ 0.31±0.00
72	*Rhodotorula glutinis*	≤ 0.31±0.00	≤ 0.31±0.00
63	*Yarowia deformans*	0.62±0.00	0.62±0.00
86	*Yarrowia deformans*	1.25±0.00	1.25±0.00
46	*Yarrowia lipolytica*	0.62±0.00	0.62±0.00
76	*Yarrowia lipolytica*	1.25±0.00	0.62±0.00
89	*Yarrowia lipolytica*	0.62±0.00	0.62±0.00

The results are expressed as means ± standard deviation of two independent measurements.

Table 7. Lactic acid bacteria counts (log CFU/g±standard deviation) of model yoghurt samples supplemented with lemongrass (L) or cinnamon (C) essential oil with their respective controls.

Yoghurt samples	**Sampling time points**				
	0 hours	**8 hours**	**4 days**	**8 days**	**28 days**
Ctrl (N)	7.04±0.04 [b]	8.94±0.02 [a]	8.79±0.02 [a]	8.85±0.02 [a]	8.80±0.00 [a]
Ctrl (P2)	7.09±0.01 [b]	9.00±0.01 [a]	8.60±0.08 [a]	8.64±0.01 [a]	8.68±0.09 [a]
Ctrl (P4)	7.18±0.19 [b]	9.01±0.06 [a]	8.80±0.01 [a]	8.79±0.01 [a]	8.80±0.01 [a]
Ctrl (NL)	7.21±0.14 [b]	8.99±0.11 [a]	8.91±0.05 [a]	8.24±0.01 [a]	8.14±0.01 [a]
D.h. L (2)	7.07±0.01[b]	8.89±0.03 [a]	8.89±0.04 [a]	8.95±0.00 [a]	8.33±0.03 [a]
C.p. L (2)	7.08±0.03 [b]	8.93±0.01 [a]	8.89±0.07 [a]	8.45±0.12 [a]	8.31±0.05 [a]
Y.d. L (2)	6.94±0.00 [b]	8.86±0.08 [a]	8.74±0.08 [a]	8.38±0.08 [a]	8.31±0.05 [a]
D.h. L (4)	7.11±0.02 [b]	8.91±0.17 [a]	8.89±0.01 [a]	8.46±0.09 [a]	8.43±0.04 [a]
C.p. L (4)	7.15±0.09 [b]	8.97±0.02 [a]	8.84±0.04 [a]	8.44±0.02 [a]	8.36±0.01 [a]
Y.d. L (4)	7.03±0.15 [b]	8.93±0.07 [a]	8.81±0.02 [a]	8.45±0.02 [a]	8.36±0.03[a]
Ctrl (NC)	7.15±0.20 [a]	<1 [b]	<1 [b]	<1 [b]	<1 [b]
D.h. C (2)	7.06±0.07 [a]	<1 [b]	1.19±0.83 [b]	<1 [b]	<1 [b]
C.p. C (2)	7.04±0.00 [a]	<1 [b]	1.27±0.10 [b]	<1 [b]	<1 [b]
Y.d. C (2)	7.08±0.00 [a]	<1 [b]	<1 [b]	<1 [b]	<1 [b]
D.h. C (4)	7.13±0.01 [a]	<1 [b]	1.61±0.44 [b]	<1 [b]	<1 [b]
C.p. C (4)	7.14±0.00 [a]	<1 [b]	1.30±0.06 [b]	<1 [b]	<1 [b]
Y.d. C (4)	7.04±0.08 [a]	<1 [b]	1.29±0.20 [b]	<1 [b]	<1 [b]

Ctrl (N), negative control (yoghurt without the addition of EOs and yeasts); Ctrl (P2) and Ctrl (P4), positive controls (yogurt intentionally contaminated with a mixture of the three selected yeast isolates at concentration of 2 and 4 log cells/mL, respectively, not supplemented with EOs); Ctrl (NL) and Ctrl (NC), negative controls (yogurt supplemented with lemongrass and cinnamon EO, respectively, not contaminated by yeasts); *D. h.* (2), *D. h.* (4), *C. p.* (2), *C. p.* (4), *Y. d.* (2), *Y. d.* (4), yoghurt contaminated with 2 and 4 log cells/g of *Debaryomyces hansenii* (isolate 35), *Candida pararugosa* (isolate 59) and *Yarrowia deformans* (isolate 86), respectively, supplemented with lemongrass (L) or cinnamon (C) EO. Means followed by different letters within each row (a, b, c) indicate significant differences ($P<0.05$).



Conflict of interest

I declare that **there is no conflict of interest**.

Highlights

- Seven essential oils (EOs) were tested *in vitro* against 74 yogurt spoilage yeasts.
- Lemongrass and cinnamon EOs showed the highest *in vitro* antifungal activity.
- Cinnamon and lemongrass EO inhibited spoilage yeasts in an *in vivo* yogurt model.
- Inhibition of lactic acid bacteria by cinnamon EO in *in vivo* system was observed.
- High efficiency of alcohol-based sanitizers on yogurt spoilage yeasts was seen.

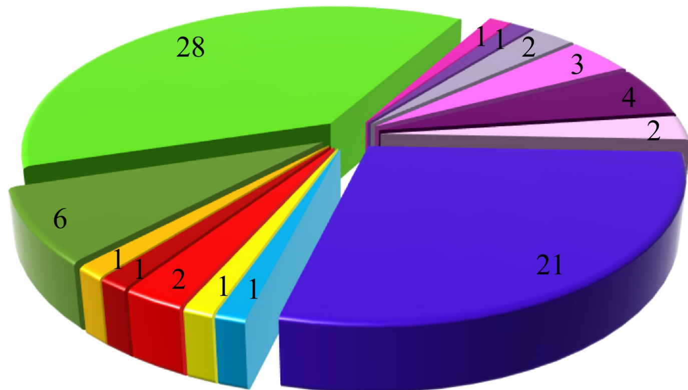


Figure 1

A)

B)

Figure 2