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A three-step approach to assess efficacy of alternative chemical treatments to preserve fresh fruit juices: Application to pineapple (*Ananas comosus* 'Queen Victoria')

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ABSTRACT

Fresh pineapple juice exhibits a shelflife of a few days limited by the development of yeasts and molds. An approach was implemented to assess the efficacy of treatments of juice, based on three successive steps. The first step was to validate the use of a microbial cocktail which leads to juice spoilage. In a second step, the efficacy of treatments was assessed on the growth of fungi in two conditions. The treatment chosen was the addition of *Thymus leptobotrys* (Tl) and *Thymus maroccanus* (Tm) essential oils (EO). These extracts were mainly composed of the monoterpene carvacrol, and inhibited fungal growth in culture medium. The addition of EO to pasteurized juice inoculated with a fungal cocktail limited fungal population below 4.6 log CFU/mL during 14 days. The low pH of pineapple juice probably strengthened the effect of antifungal compounds acting on proton gradient across plasma membrane. Lastly, the efficacy of the treatments was assessed in fresh pineapple juice harboring its natural microbial population. The extracts TlEO at 0.25‰ or TmEO at 0.05‰ limited fungal growth below 5 log CFU/mL during 14 days. Thyme scent was detected after EO addition but the characteristic descriptors of pineapple and sugary were maintained.

1. Introduction

Consumption of fresh pure fruit juice has increased in recent years, particularly to meet the growing demand for healthier products (Priyadarshini & Priyadarshini, 2018). Compared to carbonated and flavored drinks, fruit juices benefit of a better nutritional status. In spite of their content of sugars, pure fruit juices contain high levels of vitamins and minerals. "Queen Victoria" pineapple is particularly appreciated for its sweet flavor (Sun, Zhang, Soler, & Marie-Alphonsine, 2015).

Inappropriate processing or storage of fresh products can lead to their rapid deterioration due to the presence of micro-organisms. In particular, contrarily to foodborne pathogens, yeasts and molds are well adapted to fruit juice conditions, with low pH and high sugar content. Their role in fresh fruit based food spoilage is largely described (Pitt & Hocking, 2009; Tournas, Heeres, & Burgess, 2006), including in pineapple (Leneveu-Jenvrin, Quentin, Assemat, Hoarau, et al., 2020). The implication of *Penicilllium citrinum, Talaromyces amestolkiae, Rhodotorula mucilaginosa, Saccharomyces cerevisiae,* and *Meyerozyma caribbica* in the spoilage of minimally-processed pineapple cuts was demonstrated (Leneveu-Jenvrin, Quentin, Assemat, Hoarau, et al., 2020).

The most used method to limit the presence of microorganisms that can cause damage is heat treatment. It inactivates microorganisms and enzymes, but deteriorates sensory properties (Rattanathanalerk, Chiewchan, & Srichumpoung, 2005; Wurlitzer et al., 2019). Recently, to extend the shelf life of the pineapple juice, the impact of a mild pasteurization treatment has been described (Leneveu-Jenvrin, Quentin, Assemat, & Remize, 2020). A treatment at 60 °C ensures a good microbiological quality for 30 days and sensory tests revealed the absence of significant differences between freshly prepared and

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pasteurized juice. However, many alternatives to thermal treatment to increase fruit juice shelf-life are under investigation, either physical, chemical or biological methods (Bevilacqua et al., 2018; Leneveu-Jenvrin, Charles, Barba, & Remize, 2019).

Studies aiming to assess alternative treatments to preserve fruit juice are either based on assays on product batches which harbor their natural microflora (Gouma, Álvarez, Condón, & Gayán, 2020; Riganakos, Karabagias, Gertzou, & Stahl, 2017; Yang, Wu, Huang, & Miao, 2017), or on inoculated pasteurized products (Nierop Groot, Abee, & van Bokhorst-van de Veen, 2019; Timmermans et al., 2019), hence targeting different microbial groups, and different indicators of juice quality. The main drawback of using freshly prepared products is that the microbial diversity they contain is considerably dependent on the selected batch. The advantages of using a well-defined microbial target are related to its relevance for development in the juice and ability to lead to spoilage. The side-effects of the treatment are usually evaluated in a second phase through physicochemical, sensory or nutritional indicators of quality (Gabrić et al., 2018). Hence, we propose in this study an approach based on the use of a defined fungal cocktail able to induce spoilage of juice, prior to assays in fresh juice, with an alternative treatment.

The alternative treatment chosen was addition of thyme essential oils, whose plant-derived volatiles with a hydrophobic character are known to inhibit the growth of foodborne pathogens or spoilage microflora (Helal, Sarhan, Shahla, & EI-Khair, 2006; Ju et al., 2019; Sadekuzzaman, Mizan, Kim, Yang, & Ha, 2018; Trinetta, Morgan, Coupland, & Yucel, 2017; Wang & Sun, 2020). Recently, the use of some oil constituents (e.g. thymol and carvacrol) has been proved to decrease postharvest decays resulting from the growth of fungal pathogens on fruit (Bill, Korsten, Remize, Glowacz, & Sivakumar, 2017; Chillet, Minier, Hoarau, & Meile, 2019; Elshafie, Mancini, Camele, Martino, & De Feo, 2015; Elshafie, Mancini, Sakr, et al., 2015). The inhibitory effect of thymol, carvacrol or thyme essential oils on the growth of yeasts and molds in fruit juice has been demonstrated (Patel & Ghosh, 2020; Wang & Sun, 2020). However, the balance between microbial control and sensory acceptability of juices containing essential oils still requires investigations (Barcelos Leite et al., 2016). In this study, two Moroccan thyme essential oils were assayed for their application to increase the shelf-life of fresh pineapple juice, with a special attention to the consequences of their addition on microbiological and sensory qualities.

2. Materials and methods

2.1. Fruit juice preparation

Queen Victoria pineapples at a similar stage of ripeness were collected from local markets in Reunion island (France). They were transferred to the laboratory and stored at 10 °C until processing within 24 h of collection.

2.1.1. Fresh juice processing and sampling

For juice processing, 18 fruit were manually peeled and cut. Juice was recovered with an extractor (Wismer EW-01, CAPAVENIR, France). The juice was then dispensed into sterile bottles (1 L). A total of seven bottles (for each repetition) were prepared for sensory, physico-chemical and microbiological analyses and stored at 4 °C.

2.1.2. Pasteurized juice processing and sampling

At least 21 fruit were peeled, cut and processed into juice (Extractor Wismer EW-01, CAPAVENIR, France) so as to obtain at least 8 L of juice. The pineapple juice was pasteurized in a tubular heat exchanger in Simaco (Bouzonville, France) at 60 °C for 90 ± 5 s (average residence time). The hot juice was then dispensed into pre-sterilized 1 L glass bottles and closed with screw caps. These bottles were cooled in a cold-water bath (5 °C), labelled and then stored in a cold room (4 °C).

2.2. Juice quality determination

2.2.1. Microbial analysis

To enumerate yeasts and molds, 20 mL of fruit juice was sampled from a bottle, and serial decimal dilutions were made in saline peptone water (SPW, Condalab, Torrejón de Ardoz, Madrid, Spain). Yeast and mold colony forming units (CFU) were counted on Sabouraud glucose agar with 100 mg/L chloramphenicol (SGA, Biokar diagnostic, Solabia, Allonne, France) after incubation at 30 °C for 5 days.

2.2.2. Analysis of pH, total soluble sugar and color

The pH was determined by a pH meter (5231 and GLP22, Crison Instruments S.A. Barcelona, Spain), and the titratable acidity (TA) was determined by titration with 0.05 M NaOH (TitroLine easy, Schott, Mainz, Germany). TA was expressed as g citric acid equivalents per 100 mL.

The total soluble sugars (TSS) of pineapple, expressed in °Brix, were determined using a hand refractometer (Atago, Tokyo, Japan) at room temperature.

The color of samples (12 mL) was assessed from the determination of the three parameters L*, a* and b* with a spectrophotometer CM 3500d (Minolta®, Carrières-sur-Seine, France). Color difference, $\Delta E = \Delta E = \sqrt{(L_e^* - L_c^*)^2 + (a_e^* - a_c^*)^2 + (b_e^* - b_c^*)^2}$, in which L^*_e, a^*_e and b^*_e refer to the assay condition and L^*_c, a^*_c and b^*_c to the control (Day 0 samples) condition, was calculated.

2.2.3. Sensory quality

The sensory quality of the pineapple juice was assessed by a panel of thirteen trained judges. The pineapple juice was placed at room temperature 1 h before the sensory analysis. Each batch was encoded differently, with a three-digit code.

In order to determine if there was a detectable difference between lab-pasteurized juice stored and lab-pasteurized juice inoculated with the fungal cocktail, stored 7 day at 4 °C, a triangle test was used according to ISO 4120–2004 (Sensory analysis - Methodology - Triangle test). Descriptors were given by the judges to qualify the perceived odor of each juice.

To classify fresh juices (with or without thyme extracts at different concentrations) and lab-pasteurized juice (without fungal cocktail; with or without thyme extracts at different concentrations) after seven days of storage at 4 °C, a ranking test was used by the judges. The ISO 8587 method (Perceptible difference between several products according to a given characteristic) was used. Juices were classified by the panel in descending order of preference and judges assigned descriptors. To compare the frequency of occurrence of each descriptor between juices, the geometric mean GM = $\sqrt{(F \times I)}$ (Dravnieks, Bock, Powers, Tibbetts, & Ford, 1978) was calculated for each juice, with F, the frequency of citation of the descriptor, and I, the relative cumulative intensity of the descriptor. Geometric mean was expressed as a percentage of the sum of the geometric means of all juices.

Table 1
Fungal isolates.

Reference	Identification	Days of 4 °C storage of pineapple cuts before isolation
R	*Penicillium citrinum*	*14*
20	*Talaromyces amestolkiae*	*3*
A	*Rhodotorula mucilaginosa*	*7*
C	*Saccharomyces cerevisiae*	*3*
F	*Meyerozyma caribbica*	*3*

2.3. Fungal isolates

Fungal isolates (Table 1) were collected from pineapple cuts stored at 4 °C for several days and their ability to spoil pineapple cuts was confirmed (Leneveu-Jenvrin et al., 2020a). For isolate reactivation, a loop of −80 °C glycerol stock was used to inoculate Sabouraud glucose agar and was incubated for 3 days at 30 °C prior to further inoculation.

2.4. Ability of the fungal isolates to induce spoilage of commercial juice

Ten milliliters of shelf stable commercial juice (LawLam®, Réunion, France) were inoculated with *ca.* 4.6 log CFU/mL of each isolate, previously grown independently. Fungal enumeration, visual observation of modification and gas production, and pH value determination were performed after 7 days of storage of inoculated juice at 4 °C. The obtained data were compared to control, non-inoculated juice stored under the same conditions. This test was performed three times with independent fungal cultures.

2.5. Thymol, thyme essential oils and antifungal activity

2.5.1. Thymol and essential oils

Crystals of pure thymol (number CAS 89–83-8) from Xeda International SA (Saint Andiol, France) were solubilized in Sabouraud dextrose agar or pineapple juice at the desired final concentration, 0.25‰ or 0.05‰.

The aerial parts of two Moroccan endemic thyme species, namely *Thymus leptobotrys* (TlEO) and *Thymus maroccanus* (TmEO) were harvested during the flowering stage of the year 2018 from their natural habitats located, respectively, in Tafraout (N 29°72'/W 09°74′) and Ait Ourir (N 31°33'/W 07°40') regions. The identification of the species was confirmed by the botanist Prof. A. Abbad from the Faculty of Science Semlalia, Marrakesh, Morocco, and Voucher specimens (TL089 for *T. leptobotrys* and TM0739 for *T. maroccanus*) were deposited at Laboratoire Bioressources et Sécurité Sanitaire des Aliments, Faculté des Sciences et Techniques, Université Cadi Ayyad, Marrakech, Morocco. The freshly harvested plant aerial parts were dried in the shade (≈25 °C), then were subjected to hydrodistillation for 3h using a Clevenger-type apparatus. Obtained essential oils were dried with anhydrous sodium sulphate, weighed and stored at 4 °C until use. The yields of essential oils calculated on dry plant materials basis (v/w) were 1.5 ± 0.3% and 1.7 ± 0.2% for *T. maroccanus* and *T. leptobotrys*, respectively.

Qualitative and quantitative analysis of the EO chemical profiles were performed using gas chromatography/mass spectrometry (GC/MS) method. An Agilent GC–MSD system (Agilent Technologies) was used with Agilent DB5 MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, model number 122–5532) programmed from 60 °C to 246 °C at 3 °C/min. The transfer, source and quadrupole temperatures were 280 °C, 230 °C and 150 °C, respectively, operating at 70 eV ionization energy and scanning the m/z range 41–450. The carrier gas was helium (high purity) at a constant linear velocity of 37 cm/s. Essential oil samples (60 μL) were diluted with acetone (2 mL). The injection volume was 1.0 μL, the split ratio was 1:50 and the injector temperature was 260 °C. Identification of the individual components was accomplished by comparison of their mass spectra with the mass spectra of authentic reference compounds where possible and by reference to WILEY275, NBS75K, and Adams terpene library (Adams, 2007) and by comparison of their retention times with those of authentic compounds or literature data. The normalized peak area of each compound was used without any correction factors to establish abundances.

Thymol and essential oils (TlEO and TmEO) were used at two concentrations: 0.25‰ or 0.05‰.

2.5.2. Determination of antifungal activity in SGA

Thymol or EOs were added to molten SGA prior to plate pouring. A loop of each fungal culture was deposed on the center of SGA (with or without thymol or EO) plate. The growth diameter of each fungal isolate was measured after 7 days at 30 °C. Relative growth diameter was calculated as percent of control (without thymol and EO) colony diameter.

2.5.3. Determination of antifungal activity in lab-pasteurized juice

Thymol, TlEO or TmEO were added to 1 L of lab-pasteurized juice. If indicated, the juice was then inoculated with a suspension containing *ca.* 4.6 log CFU/mL of each of the five fungal isolates. Juice was stored for 14 days at 4 °C. Negative control corresponded to juice inoculated with the fungal cocktail but without thymol or EO.

2.6. Statistical analysis

The statistical analysis of the data was performed with XLSTAT software (Addinsoft, Paris, France). One-way analysis of variance (ANOVA) or ANOVA with repeats were used to compare the effects of conditions or conditions according to time, respectively. The REGWQ test (Ryan-Einot-Gabriel-Welsh F) was used for pairwise comparisons. Comparison to a control condition was performed with Dunnet's test.

3. Results and discussion

3.1. Validation of the spoilage ability of a defined fungal cocktail in pineapple juice

The efficacy of a fungal cocktail, composed of two mold and three yeast isolates, was recently demonstrated for the spoilage of minimally processed pineapple cuts (Leneveu-Jenvrin, Quentin, Assemat, Hoarau, et al., 2020). Involvement of each isolate of yeasts or molds in the deterioration of commercial pineapple juice at cold temperature was first investigated. For each isolate, visual change, gas production, pH, and fungal isolate population in commercial pineapple juice, were determined after seven days of storage at 4 °C (Table 2). All the isolates exhibited a population increase within 7 days at 4 °C in pineapple juice. No visual change was observed but the three yeast isolates led to gas production. An increase of pH value, higher than 0.15 unit, compared to the control juice (not inoculated) was noted for *Penicillium citrinum* (R), *Talaromyces amestolkiae* (20) and *Meyerozyma caribbica* (F). On the opposite, *Rhodotorula mucilaginosa* (A) and *Saccharomyces cerevisiae* (C) led to a decrease of pH value, above 0.15 unit. Hence, each fungal isolate was able to grow in these conditions and to modify commercial juice characteristics.

The validation of the ability of the fungal cocktail containing all five isolates to spoil pineapple juice was assessed with lab-pasteurized pineapple juice. Sensory triangle tests (olfactory and aspect) were performed on lab-pasteurized pineapple juice inoculated or not with the fungal cocktail and stored for 7 days at 4 °C (Fig. 1). The presence of the five-isolate cocktail in the juice led to changes of the sensory properties compared to the control juice, with a 99.99% confidence. The control juice was preferred by 67% of the panel (Fig. 1). The panel proposed descriptors for the inoculated juice: the most frequent descriptor was

Table 2
Isolate reference, identification, gas production (from 0 no production to 1 gas production detected), pH change (pH difference comparatively to the non-inoculated juice exhibiting a pH value of 3.55) and population (initial inoculation *ca.* 4.6 log CFU/mL) in commercial pineapple juice stored 7 days at 4 °C.

Isolate	Identification	Population (log_{10} CFU/mL)	Gas production	pH change
R	*P. citrinum*	6.60 ± 0.00	0	>0.15
20	*T. amestolkiae*	6.75 ± 0.00	0	>0.15
A	*R. mucilaginosa*	6.88 ± 0.03	1	<0.15
C	*S. cerevisiae*	7.09 ± 0.05	1	<0.15
F	*M. caribbica*	6.78 ± 0.00	1	>0.15

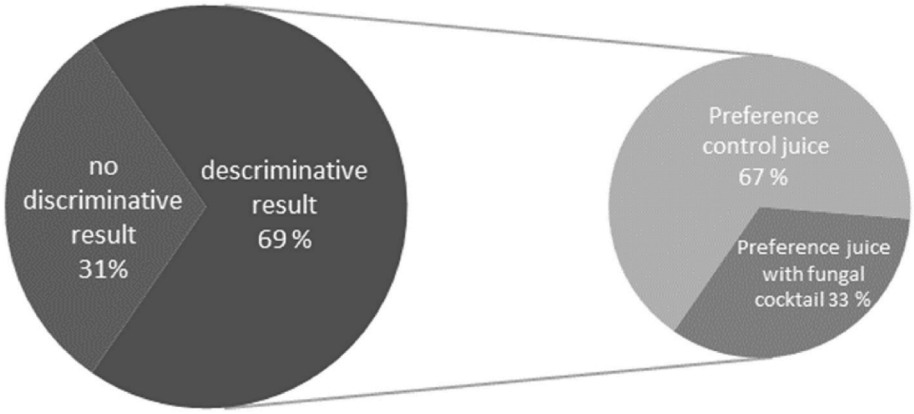

Fig. 1. Proportion of panelist answers for triangle test comparison of pasteurized juice vs. pasteurized juice with fungal cocktail after 7 days of storage at 4 °C.

"fermented". The ability of the fungal cocktail to spoil pineapple juice was thus confirmed.

3.2. Assay of thyme essential oils in vitro and on inoculated pasteurized juice

3.2.1. In vitro effect

The growth of yeast or mold isolates was determined in SGA medium. Thymol or thyme extracts (TlEO or TmEO) were added at two concentrations (0.25‰ or 0.05‰) (Fig. 2). Thymol was used as a positive control.

In SGA medium, thymol at 0.25‰ completely inhibited the growth of the five isolates. By reducing fivefold (0.05‰) thymol concentration in SGA medium, the growth of molds *P. citrinum* (R) and *T. amestolkiae* (20) was respectively reduced to 16% and 17% to that of the negative control (100% of growth), whereas for yeasts, growth was 53%, 52% and 66% for isolates *R. mucilaginosa* (A), *S. cerevisiae* (C) and *M. caribbica* (F) respectively. Thymol antifungal activity has been previously demonstrated and levels of 0.75‰ thymol completely stopped *Colletotrichum gloeosporioides* anthracnose necrosis on mango (Chillet et al., 2019; Wang & Sun, 2020).

T. amestolkiae (20) was totally inhibited by TlEO and TmEO at 0.25‰, and partially inhibited to 51% (TlEO) or 52% (TmEO) of the negative control for the lowest EO concentration. The inhibition of *Talaromyces purpureogenus* growth by *Thymus vulgaris* essential oil vapor was shown in previous work (Ambindei et al., 2017).

TlEO 0.25‰ completely inhibited growth of yeast *R. mucilaginosa* (A), but the inhibition effect was relieved for a concentration of 0.05‰.

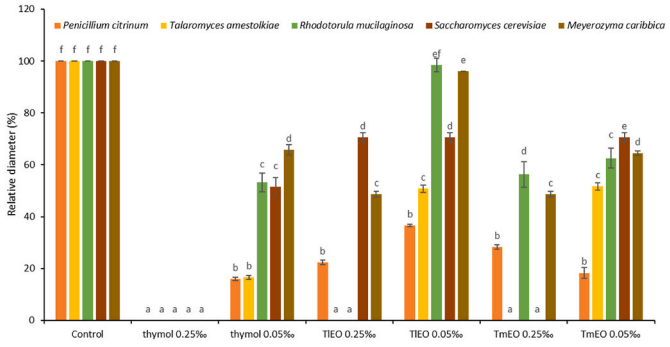

Fig. 2. Relative growth diameter (% of control condition) of fungal isolates (*Penicillium citrinum* (R), *Talaromyces amestolkiae* (20), *Rhodotorula mucilaginosa* (A), *Saccharomyces cerevisiae* (C) and *Meyerozyma caribbica* (F), on SGA medium supplemented or not with thymol solution or thyme EO (different letters indicate significant differences). Negative control: SGA; positive control was thymol 0.25‰.

With TmEO 0.25‰ and 0.05‰, growth of *R. mucilaginosa* (A) was partially inhibited, to 43.7% and 37.5% of the control, respectively. *S. cerevisiae* (C) growth was totally inhibited at TmEO 0.25‰ and partially inhibited (29.4%) in the presence of TlEO 0.25‰ and TlEO or TmEO at 0.05‰.

Growth of *P. citrinum* (R) mold or *M. caribbica* (F) yeast was partially inhibited regardless of EO and concentration, but the inhibition was more pronounced for the mold than for the yeast. Antifungal properties of *Thymus moroderi* or *Thymus piperella* essential oils (0.25‰) against *Penicillium chrysogenum*, *R. mucilaginosa*, and *S. cerevisiae* growth have been shown previously (Chavan & Tupe, 2014; Ruiz-Navajas, Viuda-Martos, Sendra, Perez-Alvarez, & Fernández-López, 2013).

3.2.2. Effect of thyme extracts on fungal spoilage of pasteurized pineapple juice

Changes in physicochemical parameters were determined over the shelf life of lab-made pasteurized pineapple juice, inoculated with the fungal cocktail, and in which EO were added. Table 3 shows mean values and data dispersion among the initial condition (day of preparation) and the six conditions (thymol 0.25‰ or 0.05‰, TlEO 0.25‰ or 0.05‰, TmEO 0.25‰ or 0.05‰) after 10 days of refrigerated storage. Initial pasteurized juice values were pH: 3.5 ± 0.1, TA: 0.83% ± 0.14%, TSS: 17.0°Brix ±0.1°Brix, L*: 69.2 ± 4.7, a*: 1.3 ± 1.1 and b*: 43.8 ± 8.2. After 10 days at refrigerated storage, whatever the added extract, pH, TA, TSS or color components (L*, a* and b*) did not significantly change compared to initial condition.

Yeast and mold's (Y&M) population during storage is shown in Fig. 3. In lab-made pasteurized juice, fungal population increased from 7.8 log CFU/mL to 11.5 log CFU/mL, during refrigerated storage, confirming the previous observations of spoilage could be linked to fungal population increase. The addition of thymol or essential oils to pasteurized juice had a negative impact on Y&M 's population whatever the condition. From the third day of storage, population inactivation was observed, down to 3 log CFU/mL (detection limit). The most inhibiting condition was thymol 0.25‰ for which Y&M population remained below the detection limit after 14 days of storage. The condition TmEO 0.25‰ resulted in a Y&M population of 3.9 log CFU/mL after 14 days of juice storage.

In pineapple juice inoculated with the fungal cocktail, the addition of TlEO or TmEO was an efficient way to limit the development of Y&M during refrigerated storage. The inactivation of pathogenic bacteria (*Listeria monocytogenes, Escherichia coli* and *Salmonella Enteritidis*) added to pineapple juice by Cymbopogon citratus *EO* was reported, hypothesized to result from the antibacterial activity of citral and geraniol (Barcelos Leite et al., 2016). The mechanisms of antifungal activity of thyme EO are mainly attributed to thymol and carvacrol (Chavan & Tupe, 2014; Kordali et al., 2008; Sakkas & Papadopoulou, 2017). On *Aspergillus flavus*, thyme EO triggered nuclear condensation, plasma

Table 3
Visual changes and physicochemical parameters of pasteurized pineapple juice inoculated with fungal cocktail (with or without thyme extract) after 10 days at 4 °C. Different letters in the same column indicates significant differences between same days or compared to initial condition (day 0) (p-value<0.05).

Condition	pH	TA (%)	TSS	L*	a*	b*	Change of aspect	Gas production
Initial condition (day 0)	3.5 ± 0.1 a	0.83 ± 0.14 a	17.0 ± 0.1 a	69.2 ± 4.7 a	1.3 ± 1.1 a	43.8 ± 8.2 a	–	–
Inoculated juice	3.5 ± 0.1 a	0.77 ± 0.07 a	16.7 ± 1.5 a	67.7 ± 3.6 a	0.2 ± 1.3 a	42.2 ± 7.1 a	yes	yes
Thymol 0.25‰	3.4 ± 0.1 a	0.85 ± 0.17 a	17.0 ± 0.2 a	68.6 ± 4.1 a	1.2 ± 1.0 a	42.6 ± 6.6 a	no	no
Thymol 0.05‰	3.5 ± 0.1 a	0.81 ± 0.22 a	16.7 ± 0.2 a	65.4 ± 5.0 a	0.8 ± 1.8 a	41.8 ± 7.3 a	no	no
TlEO 0.25‰	3.5 ± 0.1 a	0.88 ± 0.16 a	16.8 ± 0.3 a	65.4 ± 6.4 a	1.2 ± 0.3 a	40.9 ± 4.2 a	no	no
TlEO 0.05‰	3.4 ± 0.1 a	0.90 ± 0.14 a	16.9 ± 0.2 a	71.2 ± 2.9 a	0.9 ± 1.2 a	45.8 ± 4.8 a	yes	yes
TmEO 0.25‰	3.4 ± 0.1 a	0.85 ± 0.15 a	17.3 ± 0.1 a	68.6 ± 4.3 a	0.4 ± 0.6 a	35.2 ± 1.2 a	no	no
TmEO 0.05‰	3.4 ± 0.2 a	0.89 ± 0.12 a	17.6 ± 0.1 a	60.6 ± 2.2 a	0.1 ± 1.0 a	33.7 ± 5.1 a	yes	yes

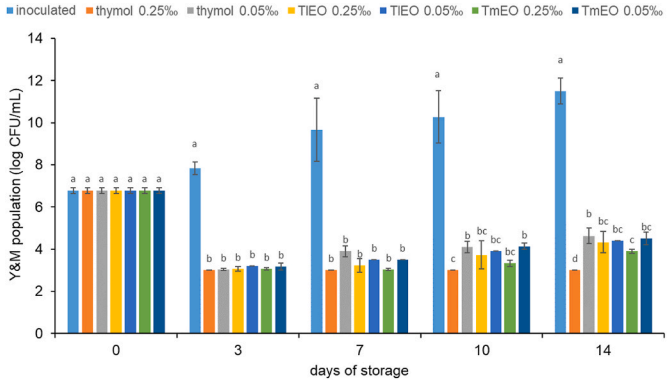

Fig. 3. Yeasts and molds population (log CFU/mL) during refrigerated storage of lab-made pasteurized juice inoculated with fungal cocktail, supplemented or not with thymol or thyme extracts. Each bar represents mean and standard deviation. The threshold 3 log CFU/mL corresponded to the detection limit. Different letters indicate significant differences for a same duration of storage ($p < 0.001$).

membrane damages and transcriptional changes on secondary metabolite pathway regulation, leading to apoptotic-like cell death (Oliveira, Carvajal-Moreno, Correa, & Rojo-Callejas, 2020). The low pH of pineapple juice probably enhances the antifungal effect of bioactive compounds of EO which act by dissipating the proton gradient across the plasma membrane.

In our study, the main physicochemical parameters did not vary, except for a slight color change, significant after 10 days for TlEO and TmEO at the highest level, *i. e.* 0.05‰. The color modification could reflect changes in the oxidation level of coloring compounds. Slight color parameter changes in lamb's lettuce were observed during refrigerated storage and was modulated in the presence of thyme/oregano EO and oregano/carvacrol (Siroli et al., 2015). The color of fresh figs was maintained during storage by using a coating of chitosan loaded with thymol (Saki, ValizadehKaji, Abbasifar, & Shahrjerdi, 2019). More recently, it has been shown that majoram (*Origanum majorana* L.) EO modulated the activity of superoxide dismutase, catalase and peroxidase, hereby modifying the hydrogen peroxide concentration and lipid peroxidation level, in shredded carrots during cold storage (Xylia, Clark, Chrysargyris, Romanazzi, & Tzortzakis, 2019). The effect of EO on meat color, in relation to the protein oxidative status is also documented (Estévez, Ventanas, & Cava, 2005; Kirkpinar, Ünlü, Serdaroğlu, & Turp, 2014).

3.2.3. Chemical composition of the two thyme essential oils

The GC-MS analysis of two essential oils from *T. leptobotrys* (TlEO) and *T. maroccanus* (TmEO) allowed the identification of twenty-three compounds representing more than 96% of the total compounds in the oils (Table 4). The compounds were grouped based on their chemical structures into four classes: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpenes hydrocarbons and others. The most

Table 4
Chemical composition of *Thymus maroccanus* and *Thymus leptobotrys* essential oils.

Compound	RI[a]	*T. maroccanus*	*T. leptobotrys*
α-Thujene	928	0.7	0.5
α-Pinene	936	3.7	1.6
Camphene	951	-[b]	0.3
β-Pinene	980	–	0.2
Myrcene	990	1.3	0.8
3-Octanol	993	–	0.3
α-Phellandrene	1007	–	0.3
α-Terpinene	1018	1.6	0.6
p-Cymene	1025	6.5	6.7
Limonene	1030	1.2	0.4
γ-Terpinene	1059	8.8	3.2
Sabinene hydrate	1067	–	0.2
Linalool	1099	–	0.5
Borneol	1169	–	0.4
Terpinen-4-ol	1179	–	0.3
Carvacrol methyl ether	1244	–	0.2
Thymol	1290	0.5	0.4
Carvacrol	1302	70.1	75.1
(*E*)-caryophyllene	1426	1.4	1.9
Aromadendrene	1445	0.9	1.1
Viridiflorene	1501	1.3	1.0
β-Bisabolene	1511	1.1	–
δ-Cadinene	1528	–	0.2
Monoterpenes hydrocarbons		23.8	14.6
Oxygenated monoterpenes		70.6	77.1
Sesquiterpenes hydrocarbons		4.7	4.2
Others		–	0.3
Total identified (%)		99.1	96.2

[a] RI: retention index relative to n-alkanes on the non-polar BD-5 column.
[b] -: compounds not detected in the oil.

prevalent group for both essential oils was oxygenated monoterpenes (70.6%–77.1%), followed by monoterpenes hydrocarbons (14.6%–23.8%). The two oils were shown to be mainly composed of the phenolic monoterpene carvacrol (70.1–75.1%) (Table 4). These results are in accordance with previous report by Jamali et al. (2012). Comparing the two thyme EOs, GC/MS analysis indicated that carvacrol was more abundant in EO obtained from *T. leptobotrys* (75.1%) than that extracted from *T. maroccanus* (70.1%). In contrast, the content of its precursor γ-terpinen was greater in EO extracted from *T. maroccanus* (8.8%) compared to the oil from *T. leptobotrys* (3.2%) (Table 4). In agreement to what has been reported previously (Jamali et al., 2012), thymol was detected as a minor component (0.4%–0.5%) in both essential oils.

The inhibition of fungal isolates is impacted by the composition of thyme essential oils. Thus, their antifungal activity could be due to their high content of carvacrol. This phenolic compound is known to have a significant antimicrobial activity because of its potency to disturb membrane permeability and proton gradient across the membrane (Chavan & Tupe, 2014; Ultee, Bennik, & Moezelaar, 2002; Xu, Zhou, Ji, Pei, & Xu, 2008). Hence, this effect was shown to be pH-dependent, being more efficient at low pH. The presence of thymol as a minor compound could also contribute to the observed antimicrobial activity.

It has been reported that a combination of carvacrol and thymol may exhibit additive or synergistic antimicrobial activity (Lee, Kim, Beuchat, Kim, & Ryu, 2020). Thyme essential oil from Thymus zygis L. (0.1563 μL/mL) slightly inhibited *Leuconostoc citreum* in tomato juice (Lee et al., 2020). In addition, synergy between carvacrol and its precursor p-cymene has been also noted (Kordali et al., 2008; Ultee et al., 2002). Furthermore, γ-terpinene was reported to contribute, in addition to carvacrol, to antifungal activity against *Sporothrix schenckii* (Couto et al., 2015). Finally, interactions of minor compounds of essential oils could contribute to synergistic antimicrobial effects (Lee et al., 2020).

3.3. Effect of alternative treatments against fungal spoilage and quality descriptors in fresh pineapple juice

Thyme extract's impact was investigated in fresh pineapple juice harboring its natural flora. Physicochemical parameters and Y&M population were determined during storage. Table 5 shows mean values and data dispersion for the initial condition (fresh juice, day of preparation), negative control (fresh juice stored for 14 days) and the six conditions with thymol or EO added (thymol 0.25‰ or 0.05‰, TlEO 0.25‰ or 0.05‰, TmEO 0.25‰ or 0.05‰) after 14 days at 4 °C. pH, TA, TSS and a* and b* parameters did not significantly change. Only L* parameter presented a slight change (p-value 0.037) between the juice which contained TlEO 0.25‰ and the juice with thymol 0.05‰. Table 6 shows the color difference during refrigerated storage. After three days, color of juices with thymol 0.25‰, TlEO 0.25‰ and TlEO 0.05‰ changed significantly compared to the initial juice (day 0). This change further increased when storage duration increased for juice with TlEO 0.25‰. The data presented in Fig. 4 show that the initial natural fungal population of fresh juice was 4.2 log CFU/mL and increased during refrigerated storage to 6.9 log CFU/mL. The behavior of the fungal cocktail used in pasteurized juice mimicked this tendency though with a faster and larger population increase. Thymol 0.05‰ was poorly inhibiting as the corresponding Y&M population was similar for each storage duration to that of the control juice. Addition of thymol or EO at a level of 0.25‰ resulted in a transient decrease of fungal population, reaching initial levels after 7–14 days depending on the condition.

Industrial standards generally require less than 6 log CFU/mL of yeasts and molds (Y&M) population for fruit juices at consumption level. Here, the addition of essential oils (other than thymol at 0.05‰) allows lower levels up to 14 days of storage. At high concentration (0.25‰) both TlEO and TmEO exert similar effect on Y&M, but a color change compared to the negative control is noticed for TlEO. At lower concentration (0.05‰), TmEO is more efficient than TlEO in inhibiting fungal growth and no change in color quality was noticed compared to the negative control.

Table 5
Physicochemical parameters (mean and standard deviation) of fresh pineapple juice (with or without thyme extract) after 14 days at 4 °C. Different letters in the same column indicate significant differences (p-value<0.05).

Condition	pH	TA (%)	TSS	L*	a*	b*
Initial condition (day 0)	3.5 ± 0.1 a	1.13 ± 0.02 a	17.3 ± 1.1 a	67.3 ± 2.7 ab	0.4 ± 0.8 a	37.6 ± 3.4 a
Negative control	3.5 ± 0.1 a	1.08 ± 0.05 a	16.6 ± 0.7 a	62.5 ± 3.0 ab	−0.3 ± 0.5 a	37.6 ± 3.9 a
Thymol 0.25‰	3.4 ± 0.1 a	1.03 ± 0.08 a	16.5 ± 0.5 a	62.1 ± 1.4 ab	0.5 ± 1.0 a	38.2 ± 4.6 a
Thymol 0.05‰	3.4 ± 0.0 a	1.06 ± 0.04 a	17.8 ± 1.0 a	67.8 ± 7.4 b	0.8 ± 0.8 a	34.7 ± 7.0 a
TlEO 0.25‰	3.4 ± 0.1 a	1.07 ± 0.01 a	16.6 ± 0.9 a	58.3 ± 2.1 a	−0.7 ± 0.3 a	32.3 ± 5.8 a
TlEO 0.05‰	3.4 ± 0.1 a	1.10 ± 0.05 a	16.7 ± 0.1 a	63.0 ± 1.3 ab	0.1 ± 0.6 a	39.7 ± 5.1 a
TmEO 0.25‰	3.4 ± 0.1 a	1.11 ± 0.03 a	17.3 ± 0.9 a	66.3 ± 1.5 ab	0.2 ± 0.3 a	36.7 ± 3.6 a
TmEO 0.05‰	3.4 ± 0.1 a	1.11 ± 0.05 a	16.9 ± 0.8 a	65.8 ± 2.5 ab	0.3 ± 0.9 a	38.8 ± 2.0 a

Table 6
Color difference of fresh juice (supplemented or not with thyme extracts) during refrigerated storage compared to initial juice (day 0). Data are means and standard deviations. Different letters in the same column indicate significant differences compared to day 0 juice.

Condition	Color difference				
	Day 0	Day 3	Day 7	Day 10	Day 14
Negative control	0.0	2.3 ± 1.1 a	4.0 ± 1.6 a	5.2 ± 0.5 a	5.1 ± 0.5 a
Thymol 0.25‰	0.0	7.9 ± 5.9 b	6.3 ± 1.7 b	7.7 ± 1.3 b	5.6 ± 1.0 b
Thymol 0.05‰	0.0	2.2 ± 1.0 a	3.4 ± 3.3 a	4.8 ± 3.3 a	4.9 ± 3.9 a
TlEO 0.25‰	0.0	7.0 ± 2.8 b	8.7 ± 3.1 b	9.0 ± 2.6 b	10.6 ± 2.4 c
TlEO 0.05‰	0.0	7.7 ± 4.5 b	7.2 ± 5.1 b	7.2 ± 5.3 b	7.1 ± 5.0 a
TmEO 0.25‰	0.0	1.4 ± 0.4 a	1.9 ± 0.7 a	1.5 ± 0.6 a	1.6 ± 0.9 a
TmEO 0.05‰	0.0	0.5 ± 0.3 a	1.5 ± 0.8 a	3.2 ± 1.9 a	3.1 ± 2.2a
p-value	–	*0.010*	*0.009*	*0.006*	*0.004*

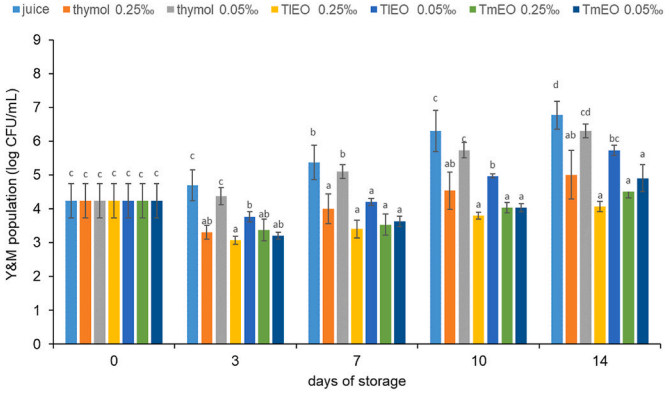

Fig. 4. Yeasts and molds population (log CFU/mL) during refrigerated storage of fresh pineapple juice, supplemented or not with thymol or thyme extracts. Each bar represents mean and standard deviation. The threshold 3 log CFU/mL corresponded to the detection limit. Different letters indicate significant differences for a same duration of storage ($p < 0.001$).

A sensory analysis was performed to describe fresh or lab-made pasteurized (without fungal cocktail) pineapple juice supplemented or not with thymol or thyme extract, and stored for 7 days at 4 °C. After 7 days, the fresh juice was still acceptable. Olfactive descriptors and preference ranks are presented in Table 7. Fresh or pasteurized juices without extracts are classified first, with the descriptors: "pineapple" or "sugared".

Pasteurized pineapple juice without EO or thymol was preferred by the panel. The juices TlEO or TmEO at 0.05‰ were ranked just after, and before the other juices. The "pineapple" or "sugared" scent was reported for the juices containing thymol or TlEO or TmEO at the lowest level, contrarily to juices supplemented at the highest level. The "thyme" scent was reported for all the juices with thyme extracts or thymol, with maximal scores, up to 7 (% geometric mean) for juice with thymol 0.25‰ and TlEO 0.25‰.

Similar observations were performed with fresh pineapple juices. The control juice was preferred, followed by the juices containing thymol 0.05‰ and TlEO 0.05‰. The "thyme" scent was reported for all juices except control, with the highest score (7%) for thymol 0.25‰, and the lowest for juice with TlEO 0.05‰ (3%). The "pineapple" or "sugared" scent was reported four out of the six juices, but other descriptors such as "chemical", "alcohol" or "fermented" were also reported.

Table 7
Ranking test and main olfactive descriptors of fresh or pasteurized juice, supplemented or not with thyme extracts, and stored 7 days at 4 °C. Descriptors (Desc.) proposed by the panel are presented with their geometric mean (GM, %)., if above the value of 2% for pasteurized juice and above 3% for fresh juice.

Condition	Control		Thymol 0.25‰		Thymol 0.05‰		TlEO 0.25‰		TlEO 0.05‰		TmEO 0.25‰		TmEO 0.05‰	
	Desc.	GM	Desc.	GM	Desc.	GM	Desc.	GM	Desc.	GM	Desc.	GM	Desc.	GM
Pasteurized	pineapple	2	thyme	7	thyme	2	thyme	7	thyme	2	thyme	6	thyme	2
	sugared	2	Lemon	2	sugared	2	carbonated	4	pineapple	2			sugared	2
Rank	1		6		4		7		2		5		3	
Fresh	pineapple	5	Thyme	7	thyme	5	thyme	5	pineapple	5	thyme	5	thyme	4
	sugared	4	Pineapple	3	pineapple	3	lemon	3	sugared	4	sugared	3	alcohol	3
	Sweet	4			fermented	3	chemical	3	thyme	3	chemical	3		
Rank	1		7		2		5		3		4		6	

Eventually, the condition with TlEO 0.05‰ was the closest to the control juices but perception of thyme was present though at a low score. Though the results obtained were promising, more extensive examination of sensory acceptability has to be performed after lowering the EO addition and through a consumer's test. As addition of TlEO or TmEO at a level of 0.05‰ were very efficient to limit fungal growth, further assays with different fresh juices, to represent the variability of initial fungal contaminants of pineapple juice, and lower levels of EO, would be required before a commercial use. Lastly, another approach would be to incorporate thyme EO in packaging of juice, so that a gradual release would allow the anti-fungal effects together with a lower content in food (Wu, Wang, Hu, & Nerín, 2018).

4. Conclusion

In this study, we have validated the use of a defined fungal cocktail to mimic spoilage flora of pineapple juice and used this cocktail to determine whether the addition of thyme EO would preserve pineapple juice. The efficacy of thyme EO at low level to preserve microbiological quality of fresh juice opens new perspective for industrial application.

The developed approach based on three successive steps, *i. e.* validation of the spoilage ability of the selected microflora, assay of alternative treatment efficacy in vitro and in inoculated pasteurized juice against the fungal cocktail, examination of side-effects on fresh juice with natural microflora, ensures to assess the efficacy of the treatments on the relevant microbial group independently of the batch of juice and to encompass side-effects of treatments on quality indicators. This approach can be easily adapted to other treatments, including physical treatments such as ultrasounds, pulsed-electric fields or UV-C, and can be used to compare treatments efficacy to increase fruit juice shelf-life.

CRediT authorship contribution statement

Charlène Leneveu-Jenvrin: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. **Aouatif Aboudia:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Sophie Assemat:** Methodology, Investigation. **Fabienne Remize:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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