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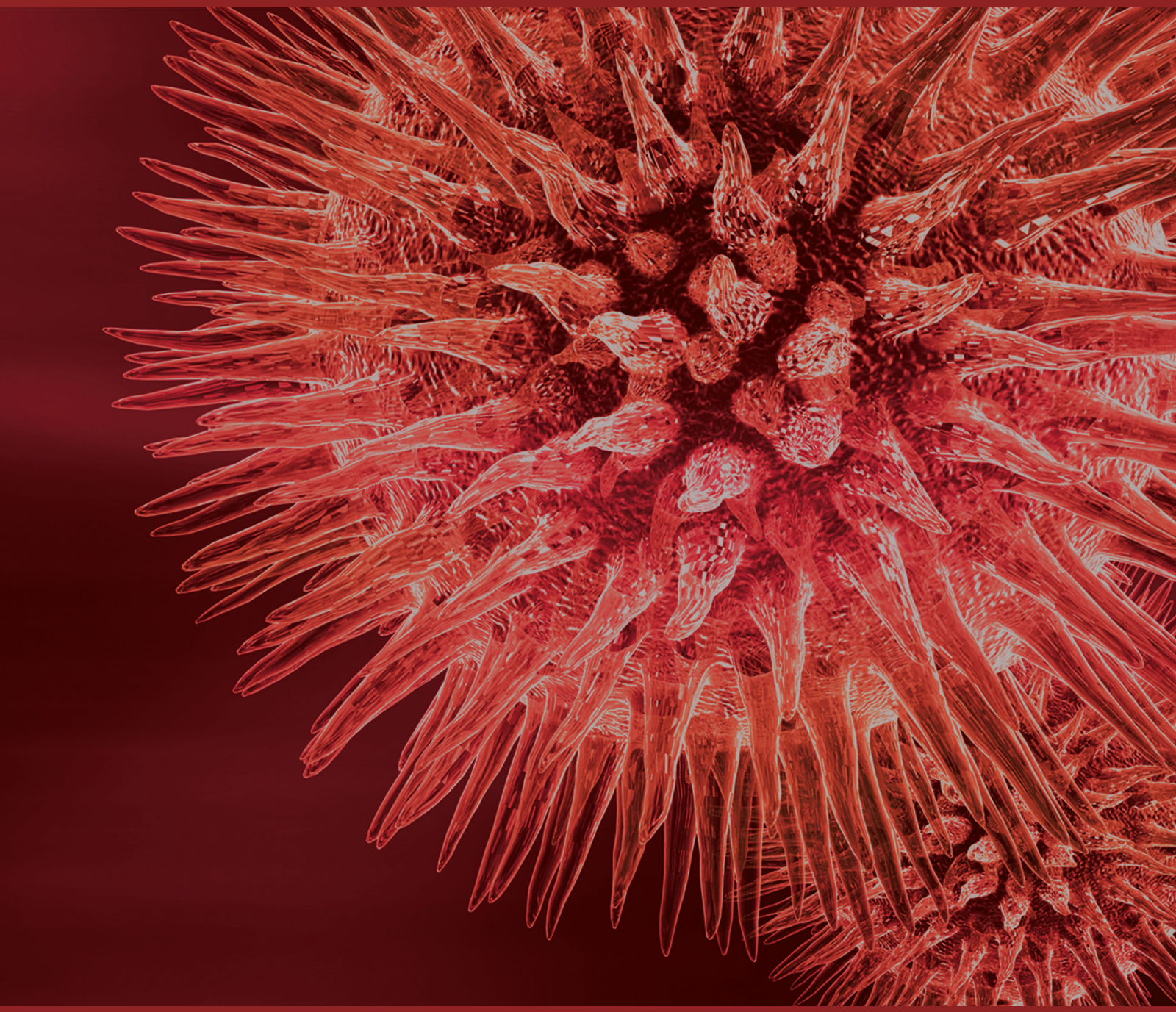

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Editorial

Bioactive Natural Products: Facts, Applications, and Challenges

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Today, there are a strong debate and interest regarding the safety aspects of chemical preservatives added widely in many food products to prevent mainly growth of spoilage and pathogenic microbes. Synthetic compounds are considered responsible for carcinogenic and teratogenic attributes and residual toxicity. To avoid the aforementioned problems, consumers and authorities have increased pressure on food manufacturers to substitute the harmful artificial additives with alternative, more effective, nontoxic, and natural substances. In this context, the use of natural compounds with antimicrobial action presents an intriguing case. Natural antioxidants also demonstrate a wide range of biological and pharmacological activities and are considered to have beneficial effects in nutrition and health [1, 2]. Natural products are currently used in several product preparations mainly as flavouring agents, fragrances, and functional additives by the cosmetic and pharmaceutical industries [3], while their individual components are also used as flavourings [4]. These natural substances have been suggested for use in foodstuffs [5], as they are known to display significant antimicrobial properties [6–8].

In order to extend our knowledge on the effectiveness of natural bioactive products and explore their application as antimicrobial systems and in functional foods production, research must be focused on the following issues: the elucidation of the molecular cell mechanisms through which microorganisms respond against natural bioactive products; the definition of matrix effects on the antimicrobial efficiency of a natural bioactive product in combination with other hurdles; the use of emerging technologies in combination with natural products, which may act synergistically for microbial growth prevention; the determination of other biological activities of natural products, for example, those relative to antioxidant and anticancer potential, and the identification of possible mechanism(s) of action; the understanding of consumer attitudes and quality perception.

Additionally, more emphasis should be given on prevalence assays of pathogenic microorganisms in connection with the use of natural antimicrobials during various production stages in industry. The inclusion of several factors, such as matrix and physiological stage of microorganisms, into mathematical models describing microbial growth and death, would represent a significant advancement in quantitative studies when compared with the empirical, descriptive models of microbial growth of limited predictive capability, currently used by the industries [9–11].

The main objective of this special issue is to provide a number of documents focused on the facts, applications, and challenges of bioactive natural products and present the methodologies in use for their effectiveness evaluation. Moreover, the challenges that industry faces with respect to the use of bioactive natural products as antimicrobial agents in terms

of safety and microbial growth prevention are discussed. A better understanding of the proposed mechanisms of action for some natural compounds and relevant key molecular factors in bacterial biofilm formation and their regulation, such as the chemical signalization machinery involved in bacteria-environment interaction, are also referred to. Furthermore, the application of high hydrostatic pressure treatment as a reliable nonthermal pasteurization method to extend the microbiological shelf life of various foodstuffs is thoroughly discussed. Finally, the potential of various plant-derived compounds to control pathogenic bacteria and especially the diverse effects exerted by plant compounds on virulence factors that are critical for pathogenicity is highlighted and assessed.

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Research Article

Genetic and Technological Characterisation of Vineyard- and Winery-Associated Lactic Acid Bacteria

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Vineyard- and winery-associated lactic acid bacteria (LAB) from two major PDO regions in Greece, Peza and Nemea, were surveyed. LAB were isolated from grapes, fermenting musts, and winery tanks performing spontaneous malolactic fermentations (MLF). Higher population density and species richness were detected in Nemea than in Peza vineyards and on grapes than in fermenting musts. *Pediococcus pentosaceus* and *Lactobacillus graminis* were the most abundant LAB on grapes, while *Lactobacillus plantarum* dominated in fermenting musts from both regions. No particular structure of *Lactobacillus plantarum* populations according to the region of origin was observed, and strain distribution seems random. LAB species diversity in winery tanks differed significantly from that in vineyard samples, consisting principally of *Oenococcus oeni*. Different strains were analysed as per their enological characteristics and the ability to produce biogenic amines (BAs). Winery-associated species showed higher resistance to low pH, ethanol, SO_2, and $CuSO_4$ than vineyard-associated isolates. The frequency of BA-producing strains was relatively low but not negligible, considering that certain winery-associated *Lactobacillus hilgardii* strains were able to produce BAs. Present results show the necessity of controlling the MLF by selected starters in order to avoid BA accumulation in wine.

1. Introduction

In winemaking, a secondary fermentation known as malolactic fermentation (MLF) often takes place following the cease of yeast activity. During MLF, L-malate is converted into L-lactate by the lactic acid bacteria (LAB) of wine. This bioconversion is a desirable process in red winemaking and also in the production of certain white wines of high acidity, due to the organoleptic advantages that LAB activity confers. These include a decline in the total acidity and an increase of soft mouth feel, flavour, and microbiological stability of the wine [1]. However, MLF often entails certain risks, that is, the production of off-flavours, reduction in colour, and most importantly the formation of biogenic amines (BAs) [2, 3].

Currently, there is a growing concern regarding the limits of BAs in wines because of their potential health implications [4]. Although not regulated uniformly worldwide, BAs are generally confronted under similar regulations as for allergens. As a matter of fact, wines containing elevated amounts of histamine are rejected from certain markets due to recommended or suggested existing limits [4], while recently the Panel on Biological Hazards of the European Food Safety Authority (EFSA) released a scientific opinion on risk based control of BA formation in fermented foods [5]. Therefore, MLF in wine needs to be regulated to avoid the accumulation of BAs by LAB. This may be accomplished by the use of selected LAB strains tested for low production of BAs [6, 7] or able to degrade BA in wine [8].

Selected strains of *Oenococcus oeni*, the principal malolactic bacterium, have been launched in the market over the last decades. Nevertheless, wineries often face difficulties when conducting MLF by current commercial starters, as the induction of the process is not always successful [9]. Still several wineries prefer to conduct spontaneous malolactic

fermentations by the native microbiota [10]. In these cases, the indigenous bacteria actualize MLF more effectively than commercial *O. oeni*, since native strains can deal with microbial incompatibilities and are better acclimatized to the local wine and practices [11, 12]. In addition, spontaneous MLF typically involves a composite bacterial community that may confer a more complex flavour to wine [1].

To this end, the wine industry seeks for novel MLF starters bearing positive technological and flavouring attributes [12]. The use of LAB species other than *O. oeni* is also being considered [13]. Grape resident microbial diversity forms an untapped reservoir of indigenous bacteria strains and may be primarily considered in an MLF starter selection scheme. Here we explored the local vineyard- and winery-associated LAB culturable populations in two key viticultural regions in Greece, Nemea and Peza. By using different molecular techniques various species and strains of enological importance were identified and characterised.

2. Materials and Methods

2.1. Sampling and LAB Isolation. Grape samples belonging to the Greek grapevine (*Vitis vinifera*) varieties "Vilana" (white), "Mandilaria" (red), and "Kotsifali" (red) were collected from 16 vineyards (1VP–16VP) within the Peza PDO region in Crete. Grapes of the "Agiorgitiko" cultivar (red variety) were collected from 11 vineyards (1VN–11VN) in the Nemea PDO region, Peloponnese. Samples consisting of healthy grape bunches were collected from at least 3 distant sampling points (sites) within each vineyard, placed into sterile plastic bags and transferred at 4°C to the laboratory. Grapes were crushed with a stomacher and let to ferment spontaneously in sterile bottles. Fermentation progress was daily followed by weight determinations. LAB were isolated from grapes or fermenting grape juice at the middle stage (MF) when about 50% of sugars were consumed, the final stage (EF) when sugars were depleted, and after the end of alcoholic fermentation. LAB were also isolated from wine samples collected from 9 tanks (T1–T9) of a winery in Nemea during spontaneous MLF. No spontaneous MLF was conducted in Peza winery. For bacteria enumeration, appropriate dilutions were spread onto MRS agar medium (pH 5.5) supplemented with 100 mg/L cycloheximide and incubated in anaerobic jars at 28°C for 3–8 days. Colonies were randomly selected from plates and examined microscopically. Bacterial colonies were further examined for Gram stain and catalase reaction. Isolates were maintained in liquid cultures in MRS broth with 30% glycerol at −80°C until further analysis.

2.2. Species Identification. DNA was extracted as previously described [14]. The 16S rDNA region of bacteria isolates was PCR-amplified using primers pA and pH [15]. For restriction analysis of the amplified 16S rDNA region (16S-ARDRA), approximately 500 ng of PCR product was digested with the restriction endonuclease *Mse*I [15] and fragments were analyzed by agarose gel electrophoresis. For the differentiation of *Lactobacillus plantarum*, *Lactobacillus pentosus*, and *Lactobacillus paraplantarum*, a multiplex PCR assay was performed with the *recA* gene-based primers paraF, pentF, planF, and pREV, according to Torriani et al. [16]. For sequence analysis, the V1–V3 region of 16S rDNA was amplified using the primers P1V1 and P4V3 as previously described [17]. PCR products of representative isolates per distinct PCR-ARDRA pattern were sequenced (Macrogen; http://www.macrogen.com/). BLAST searches of sequences were performed at the NCBI/GenBank database.

2.3. Strain Typing and Genetic Analysis. Repetitive element sequence-based PCR (rep-PCR) using the single primer (GTG)5 or the primer pair REP1R-Dt and REP2R-Dt [18, 19] and RAPD analysis using the single primer RAPD1 or RAPD2 [20], 5′-ACGCGCCCT-3′ [21], and 1283 [22] were initially evaluated. The banding patterns corresponding to isolates from the same vineyard were considered as a vineyard population. UPGMA clustering of vineyard populations was conducted by using the PopGene 1.32 software [23].

2.4. Detection of BA-Producing Genes. For simultaneous detection of four genes involved in the production of major BAs in wine by LAB, that is, histamine (*hdc*), tyramine (*tyrdc*), and putrescine (*odc* and *agdi*), a multiplex PCR assay was applied as described elsewhere [24]. Briefly, the *hdc* and *tyrdc* genes were targeted with the primer pairs HDC3/HDC4 and TD2/TD5, respectively, while the primers ODC1/ODC2 and AGD1/AGD2 were used for the detection of *agdi* and *odc* genes, respectively. The 16S rRNA gene was concomitantly targeted with the universal primers BSF8/BSR1541 [25].

2.5. Technological Characterization of LAB. Tests were performed on MRS agar (pH 4) containing 7% ethanol unless otherwise stated. Ethanol tolerance was determined at ethanol contents of 10, 12, or 14%. SO_2 resistance was evaluated at 5, 15, or 30 mg/L. Tolerance to low pH was determined at pH values of 3.0, 3.5, 4.0, or 5.5 adjusted by the addition of HCl. $CuSO_4$ tolerance was evaluated at concentrations of 5 or 20 mg/L. Biogenic amines formation was determined on modified decarboxylating agar (MDA) plates (per litre: 5.0 g tryptone, 8.0 g meat extract, 4.0 g yeast extract, 0.5 g Tween 80, 0.2 g $MgSO_4$, 0.05 g $MnSO_4$, 0.04 g $FeSO_4$, 0.1 g $CaCO_3$, 0.06 g bromocresol purple, and 20.0 g agar) supplemented with 2% of either tyrosine, histidine, or arginine. The formation of biogenic amines was indicated by a purple halo around the bacterial colony as a result of amino acid decarboxylation [26]. Isolates were spot inoculated (ca. 10^6 cells/mL) on the surface of agar medium. Growth was evaluated after anaerobic incubation for up to 8 days at 28°C.

3. Results and Discussion

3.1. Bacterial Abundance. Grapes and wine fermentations constitute complex microbial ecosystems consisting of highly dynamic yeast and bacteria communities. Despite the importance of LAB populations in shaping the wine quality, our current knowledge on the spatiotemporal distribution of LAB populations in grapes and musts during the alcoholic or malolactic fermentation is still limited. Here we analyzed the LAB culturable communities in two distant viticultural zones

Table 1: Species identification of bacteria isolates based on 16S-ARDRA profiles and sequence analysis.

Profile	Approximate Sizes of Restriction Fragments (bp)	Species
I	610 + 280 + 260 + 190 + 130 + 90	*Lactobacillus graminis*
II	420 + 270 + 200 + 130 + 110 + 90	*Lactobacillus hilgardii*
III	480 + 290 + 270 + 160 + 140 + 110 + 90	*Lactobacillus plantarum*[*]
IV	400 + 380 + 270 + 180 + 160 + 140	*Lactococcus lactis*
V	610 + 250 + 200 + 130	*Oenococcus oeni*
VI	400 + 270 + 230 + 150 + 130 + 80	*Pediococcus parvulus*
VII	290 + 260 + 250 + 130 + 120 + 110 + 90	*Pediococcus pentosaceus*
VIII	610 + 410 + 290 + 140 + 80	*Staphylococcus epidermidis*
IX	400 + 270 + 240 + 200 + 140 + 80	*Weissella* sp.

[*] *Lactobacillus plantarum* was differentiated from *L. pentosus* and *L. paraplantarum* with a multiplex PCR assay using *rec*A gene-derived primers.

in Greece, Peza in Crete and Nemea in Peloponnese. Samples included grapes and the respective fermenting musts. Sampling was also conducted after the end of the alcoholic fermentation (AF) and *in situ* in winery tanks during spontaneous MLF.

LAB were detected at relatively low frequencies on grapes. About 28% of grape samples from the Nemea region harbored bacteria at populations ranging from 1.4 to 3.8 log CFU/mL. In grapes from Peza, the bacterial populations were below the detection limit. The low incidence of LAB populations on wine grapes, as detected here, is in accordance with previous studies that suggest limited LAB population density (<3 log CFU/g) in vineyards, due to their nutritional requirements [10, 27–31].

Musts from grape samples were allowed to ferment spontaneously and at the middle stage of the AF (MF stage) bacteria could be recovered from 16% of the samples from either region. In the case of Nemea, population densities were relatively low (1.4–3.7 log CFU/mL), except for a single population that reached 8.7 log CFU/mL. Similarly, in Peza samples, populations at stage MF ranged from 0.9 to 3.3 log CFU/mL, except for one sample (ca. 7.2 log CFU/mL). At the end of the AF (EF stage), the number of Nemea samples with detectable populations decreased to 9%, while counts ranged from 1.3 to 7.0 log CFU/mL. As opposed, the respective percentage of Peza samples increased (24%), with populations ranging from 1.9 to 4.3 log CFU/mL. No bacterial populations were detected in samples from Nemea or Peza regions after the completion of AF. Present results show that, with a few exceptions, the bacterial growth is limited during the AF. Similarly low bacterial densities during the AF, ranging from 2 to 4 log CFU/mL, have been recorded previously [32]. These populations may further decline at the end of AF, with the exception of *O. oeni* [28, 32–36]. It is most likely that bacterial growth is prevented by the accumulating ethanol, the lack of nutrients, or the competition with indigenous yeast biota [28, 36]. Contracting this general observation, tumultuous bacterial growth during AF, as reported here, has been occasionally associated with musts infected with certain *Lactobacillus* spp. [28]. As opposed to vineyard-associated samples, relatively high bacterial densities (ca. 7 log CFU/mL) were recovered from winery tanks T1–T6. Populations of ca. 4 log CFU/mL were detected in tanks T7 and T8. Bacteria were below the detection limit in tank T9.

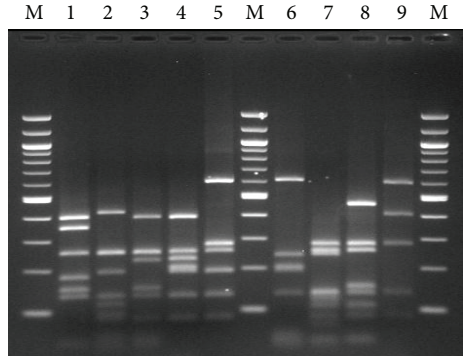

Figure 1: 16S-ARDRA patterns obtained after digestion with *Mse*I. Lanes: 1, *Lactococcus lactis*; 2, *Lactobacillus hilgardii*; 3, *Pediococcus parvulus*; 4, *Weissella* sp.; 5, *Lactobacillus graminis*; 6, *Oenococcus oeni*; 7, *Pediococcus pentosaceus*; 8, *Lactobacillus plantarum*; 9, *Staphylococcus epidermidis*; M, 100 bp molecular marker.

3.2. Species Identification. 16S-ARDRA grouped 626 isolates according to their banding profiles (profiles I to IX) (Table 1; Figure 1). Phylogenetic analysis of the V1–V3 region of 16S rDNA of representative isolates from each group assigned them to the species *Lactobacillus graminis*, *Lactobacillus hilgardii*, *Lactobacillus pentosus/plantarum*, *Lactococcus lactis*, *Oenococcus oeni*, *Pediococcus parvulus*, *P. pentosaceus*, *Staphylococcus epidermidis*, and *Weissella* sp. According to the above analysis, isolates within group III showed 100% sequence similarity to both *Lactobacillus pentosus* JCM 1558^T (D79211) and *Lactobacillus plantarum* NRRL B-14768^T (AJ965482) followed by 99.8% to *Lactobacillus paraplantarum* DSM 10667^T. Since 16S rDNA sequence is identical or highly similar among these species, a multiplex PCR assay with *recA* gene-based primers was applied for the identification of isolates within group III, as previously suggested [16], revealing that all isolates belong to the species *Lactobacillus plantarum*.

3.3. LAB Species Diversity and Succession. *Pediococcus pentosaceus* and *Lactobacillus graminis* were the most abundant LAB species in grape samples from Nemea (12.5 and 9.4%, resp.), followed by *Weissella* sp. and *Lactococcus lactis* at percentages lower than 7%. Typically, LAB species diversity associated with grape surfaces is rather limited mainly due

Table 2: Distinct genotypes according to RAPD2-PCR patterns of vineyard-associated LAB populations.

LAB species	Region of origin	No. of isolates	No. of distinct patterns	Percentage of biodiversity*	Common patterns among vineyards	Common patterns between regions
Lactobacillus plantarum	Nemea	64	3	4.7	1	2
	Peza	319	13	4.1	3	
Pediococcus pentosaceus	Nemea	61	5	8.2	4	1
	Peza	16	1	6.3	—	
Lactobacillus graminis	Nemea	37	5	13.5	—	—
	Peza	nd**	—	—	—	
Lactococcus lactis	Nemea	21	3	14.3	—	—
	Peza	nd	—	—	—	
Weissella sp.	Nemea	11	2	18.2	—	—
	Peza	nd	—	—	—	

*Ratio between the number of patterns and the number of isolates [45].
**Not detected.

to their nutritional requirements [28]. Species that have been reported to occur on grapes belong to the genera *Lactobacillus* (*Lactobacillus casei*, *Lactobacillus hilgardii*, *Lactobacillus kunkeei*, *Lactobacillus lindneri*, *Lactobacillus mali*, and *Lactobacillus plantarum*), *Pediococcus*, and *Leuconostoc* [29, 37, 38]. By applying a culture independent approach Renouf et al. [39] revealed a broader LAB diversity than previously described, including species within the genera *Enterococcus* and *Weissella*. Here we also detected *Lactococcus lactis*, a species that is quite scarce on grapes and a potentially novel *Weissella* species.

At the MF stage in Nemea samples, *Pediococcus pentosaceus* showed a higher level of persistence compared to the other species encountered on grapes. All other grape-associated populations were undetectable except for *Lactobacillus graminis*, which replaced *S. epidermidis* in one case. *Lactobacillus plantarum* emerged for the first time in two out of five samples, in which initial LAB populations on grapes were below the detection limit. At the EF stage, LAB were detected in three samples and all isolates were identified as *Lactobacillus plantarum*. Although in Peza grape samples bacteria were below the detection limit, LAB populations then emerged during the AF. At stage MF, *Lactobacillus plantarum* was the only species detected in all samples. At the EF stage, all samples were exclusively dominated by *Lactobacillus plantarum*, except for one sample in which *P. pentosaceus* thrived.

Previous studies have also shown that *Lactobacillus plantarum* is scarce on grapes [29, 30], but frequent in fermenting musts [10]. *Oenococcus oeni*, the principal malolactic bacterium often isolated from wines, was not detected on grapes or fermenting musts, collaborating previous suggestions about the absence or low population of this species in Greek vineyards [10].

The dominant population in winery-associated samples was *O. oeni* that could be recovered from all tanks performing spontaneous MLF. In 75% of the samples, *Pediococcus parvulus* was also isolated, albeit at significant lower populations than *O. oeni*. In one case, *Lactobacillus hilgardii* was also isolated along with *P. parvulus*, again at much lower population density than *O. oeni* (ca. 3 versus 7 log CFU/mL, resp.). The high occurrence of *P. parvulus* in the present samples needs further consideration since it is often associated with ropiness and oiliness of wine [40]. Furthermore, *P. parvulus* and *Lactobacillus hilgardii* were identified as the main spoilage, high histamine producing bacteria [41]; therefore their presence during MLF needs to be controlled.

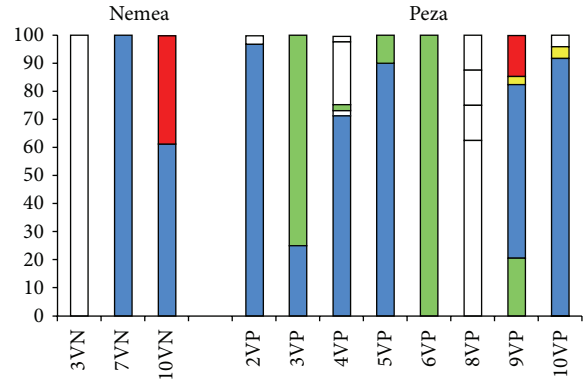

Figure 2: Distribution of *Lactobacillus plantarum* genotypes (%) in different vineyards of Nemea and Peza regions. Common genotypes are represented with the same colour. Unique genotypes are shown in white colour.

3.4. Genotypic Diversity. For the discrimination of different LAB genotypes, various PCR-based fingerprinting methods were initially evaluated, including rep-PCR using the primer (GTG)5 or the primer set REP1R-Dt/REP2R-Dt and RAPD analysis with various primers. Among them, PCR using the primer RAPD2 (RAPD2-PCR) generated clear and reproducible banding patterns and also showed the highest discriminatory capacity in our tests (data not shown). Therefore, it was retained as the fingerprinting method of choice in the present genotyping analysis. The primer RAPD2 has been successfully applied previously in RAPD-PCR assays to differentiate strains within various LAB species [20, 42].

In the case of *Lactobacillus plantarum* isolates, RAPD2-PCR generated a total of 45 polymorphic bands and 14 distinct banding patterns (hereafter referred to as genotypes) were identified (Table 2). The number of different genotypes detected within a vineyard (all sampling points included) ranged from 1 to 5 (Figure 2). Recent metagenomic studies by using next generation sequencing technology suggest

Table 3: Distinct genotypes according to RAPD2-PCR patterns of winery-associated LAB populations.

LAB species	Tank (T1–T9)	No. of isolates	No. of distinct patterns	Percentage of biodiversity*
Lactobacillus hilgardii	T6	4	3	75.0
Oenococcus oeni	T1–T8	46	12	26.1
Pediococcus parvulus	T1–T6	38	23	60.5

* Ratio between the number of patterns and the number of isolates [45].

Table 4: Technological characteristics and biogenic amines production of vineyard- and winery-associated LAB species. The total number of strains analysed per species and the number of strains that produced positive reactions are indicated.

LAB species	No of strains	Biogenic amines			pH			SO_2 (mg/L)			Ethanol (%)			$CuSO_4$ (mg/L)	
		Putrescine	Tyramine	Histamine	3.0	3.5	4	5	15	30	10	12	14	5	20
Lactobacillus graminis	5	1	0	1	0	0	2	2	2	2	1	0	0	2	1
Lactobacillus hilgardii	3	1	3	0	0	0	3	3	3	3	3	3	2	3	3
Lactobacillus plantarum	14	0	0	0	0	0	11	11	11	8	8	6	0	11	4
Lactococcus lactis	3	1	0	0	0	0	3	3	3	3	1	0	0	3	3
Pediococcus parvulus	23	0	0	0	17	21	22	21	21	21	21	21	21	21	21
Pediococcus pentosaceus	5	5	0	0	0	0	5	5	5	2	4	0	0	5	0
Oenococcus oeni	12	0	0	0	3	9	11	10	10	6	12	10	10	10	9
Weissella sp.	2	0	0	0	0	0	1	2	1	0	0	0	0	1	1

that different wine-growing regions may maintain different microbial communities [43, 44]. As far as regional variation in wine characteristics may be influenced by the local grape microflora, the so-called microbial "*terroir*" concept, it is very important to examine in more detail the spatiotemporal distribution of various strains. In this study, population genetic analysis was conducted in isolates of different vineyards (populations) and the existence of genetic structure between populations of the two geographical zones of origin (groups of Peza and Nemea) was evaluated. Results from UPGMA cluster analysis showed that the spatial distribution of genotypes within a vineyard is rather random (data not shown). Measures of genetic identity (Nei's coefficient) showed that most vineyard populations shared a relatively high degree of genetic similarity (>0.7). The UPGMA tree of vineyard populations showed no clustering according to the zone of origin (Figure 3).

The isolates from four more vineyard-associated LAB populations belonging to the species *Lactobacillus graminis*, *Lactococcus lactis*, *P. pentosaceus*, and *Weissella* sp. were analysed by RAPD2-PCR. Five distinct genotypes of *P. pentosaceus* were identified in samples originating from the Nemea region. Peza samples harbored a single *P. pentosaceus* genotype, which was also found in Nemea suggesting that it may be a cosmopolitan genotype. The species *Lactobacillus graminis*, *Lactococcus lactis*, and *Weissella* sp. were only detected in the Nemea region. The number of isolates analysed, the distinct banding patterns per population, and the percentage of biodiversity are summarized in Table 2.

Three different bacterial populations were associated with spontaneously fermenting wines in winery tanks. These included 12, 23, and 3 distinct genotypes for *O. oeni*, *P. parvulus*, and *Lactobacillus hilgardii*, respectively. The number of genotypes identified in different tanks is presented in Table 3. One up to five *O. oeni* distinct genotypes were isolated from the same tank. The respective range for *P. parvulus* was 2 to 7.

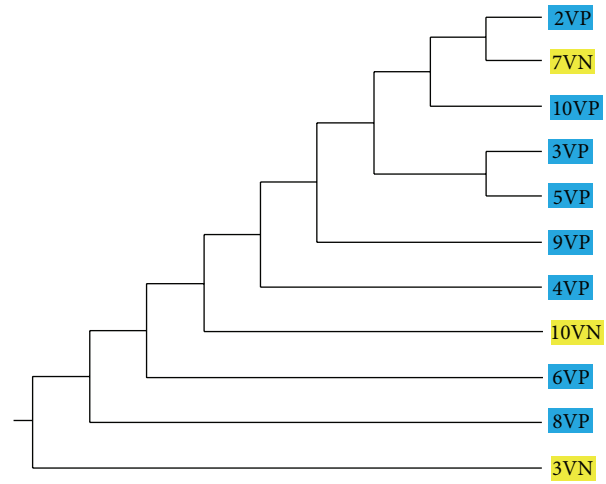

Figure 3: UPGMA dendrogram based on Nei's genetic distances among *Lactobacillus plantarum* vineyard populations. Populations from Nemea and Peza are yellow- and blue-highlighted, respectively.

In the case of *Lactobacillus hilgardii* all different genotypes were isolated from the same tank. Present results suggest that the genetic biodiversity of LAB species within a winery may be quite high (Table 3). Most importantly, different strains of the same LAB species may coexist in the same tank during MLF.

3.5. Technological Characterization. Distinct genotypes within each species were evaluated as per their technological and enological characteristics (Table 4). Among LAB species, only *O. oeni* and *P. parvulus* isolates were able to grow at low pH, that is, at 3 or 3.5 in the presence of 7% ethanol. Growth at pH 4 was supported by all other species, albeit at different percentages. Winery-associated species showed

higher resistance to SO_2 than vineyard-associated isolates. Among the latter, several isolates of *Lactobacillus plantarum*, *Lactococcus lactis*, and *Pediococcus pentosaceus* tolerated up to 30 mg/L SO_2. *Lactobacillus graminis* exhibited a moderate resistance, while *Weissella* sp. could grow only up to 30 mg/L SO_2. Differences between winery- and vineyard-associated species were more profoundly reflected in ethanol tolerance. All vineyard-associated isolates could grow only up to 10% ethanol, except *Weissella* sp. A percentage of 43% of *Lactobacillus plantarum* strains could withstand 12% ethanol. Yet, winery-associated isolates could be considered as highly ethanol tolerant, resisting up to 14% ethanol. Again, the winery-associated isolates showed higher resistance to $CuSO_4$ than vineyard-associated isolates did. *P. pentosaceus* was the most sensitive species to $CuSO_4$, as none of the strains could tolerate a concentration of 20 mg/L.

3.6. BA-Producing LAB. LAB are the main producers of biogenic amines (BAs) in wine. Therefore, LAB should be evaluated for their ability to produce BAs, before being used as malolactic starters. By using appropriate culture media [26], we analysed the different strains identified in this study for their ability to produce the three major BAs in wine, that is, putrescine, tyramine, and histamine. As it is shown in Table 4, except for *Lactobacillus plantarum*, *P. parvulus*, and *Weissella* sp., certain strains from the other species were able to produce putrescine. The percentage of putrescine-producing strains was rather low, except for *Pediococcus pentosaceus*. Tyramine was found to be produced only by *Lactobacillus hilgardii* strains.

Recently, a PCR method was developed for the simultaneous detection of four genes involved in the production of the above BAs [24]. We applied this multiplex PCR to screen the above LAB strains. The PCR results were in good agreement with those obtained by the culture method. There was only one mismatch regarding a *Lactobacillus hilgardii* strain that produced tyramine but the corresponding gene (*tyrdc*) was not amplified. Thus the percentage of mismatching was rather low (1.5% of the strains), being slightly lower than the one detected by Coton et al. [24] (2.5%). It is likely that this discrepancy may be attributed to the existence of novel BA-producing genes not amplifiable by the present degenerate primers [24]. The relatively low frequency of BA-producing strains identified in this study is in accordance to previous results for wine-associated LAB, particularly as regards the low percentage of histamine-producing strains [24].

All three *Lactobacillus hilgardii* strains isolated from one winery tank performing spontaneous MLF produced tyramine and/or putrescine. Present results show the necessity of controlling the MLF by selected starters in order to avoid BA accumulation in the final product, since spontaneous fermentation may allow the occurrence of BA-producing strains.

4. Conclusions

The present study shows that the LAB species richness and population densities on grapes may differ considerably between regions or vineyards. Yet, *Lactobacillus plantarum* was the most abundant species in both regions and dominated the alcoholic fermentations. However, there was not any genetic structure in the *Lactobacillus plantarum* populations examined. As expected, *O. oeni* was quantitatively the principal LAB in the winery tanks during the MLF. Present results point to relatively high genotypic and phenotypic diversity within most LAB species identified, including *O. oeni*. Most importantly, various strains of the same species may coexist in the same tank during the MLF. Winery-associated species showed higher resistance to low pH, ethanol, SO_2, and $CuSO_4$ than vineyard-associated isolates. Most LAB strains did not produce BAs in our tests. Further PCR analysis targeting BA-producing genes verified that the frequency of BA-producing LAB was low. However, a few LAB strains isolated from a winery tank conducting MLF did produce major BAs, strengthening the need for novel superior LAB starters to control the MLF.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Formation of Biofilms by *Pseudomonas aeruginosa*: A Review of the Natural and Synthetic Compounds Interfering with Control Mechanisms

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P. aeruginosa is an opportunistic pathogenic bacterium responsible for both acute and chronic infections. Beyond its natural resistance to many drugs, its ability to form biofilm, a complex biological system, renders ineffective the clearance by immune defense systems and antibiotherapy. The objective of this report is to provide an overview (i) on *P. aeruginosa* biofilm lifestyle cycle, (ii) on the main key actors relevant in the regulation of biofilm formation by *P. aeruginosa* including QS systems, *GacS*/*GacA* and *RetS*/*LadS* two-component systems and C-di-GMP-dependent polysaccharides biosynthesis, and (iii) finally on reported natural and synthetic products that interfere with control mechanisms of biofilm formation by *P. aeruginosa* without affecting directly bacterial viability. Concluding remarks focus on perspectives to consider biofilm lifestyle as a target for eradication of resistant infections caused by *P. aeruginosa*.

1. Introduction

The misuse and abuse of antibiotics are recognized to create selective pressure, resulting in the widespread development of resistant bacterial strains [1, 2]. Antibiotics are also known to kill "good/beneficial" indigenous bacteria, which may have protective role against pathogenic bacteria [3, 4]. Another important point to consider is that antibiotics have been found to be less effective in biofilm-growing bacteria [5].

Facing these limitations of antibiotics, there is an increasing need for the discovery and the development of antimicrobial agents that present novel or unexplored properties to efficiently control and manage bacterial infectious diseases [6]. Inhibition of bacterial virulence and/or biofilm formation by targeting nonmicrobicidal mechanisms are examples of increasingly explored antipathogenic approaches [7–9]. Among opportunistic pathogenic bacteria, *P. aeruginosa*, which produces several virulence factors, is known to be an important human and plant pathogen, responsible for various infections, particularly in immunocompromised persons [10]. Besides this, the remarkable ability of *P. aeruginosa* to form biofilms in many environments renders antibiotic treatments inefficient and therefore promotes chronic infectious diseases [5, 11].

Three global nonmicrobicidal strategies have been proposed to struggle against pathogenic bacteria with biofilm formation ability by (i) avoiding microbial attachment to a surface; (ii) disrupting biofilm development and/or affecting biofilm architecture in order to enhance the penetration of antimicrobials; and (iii) affecting biofilm maturation and/or inducing its dispersion and degradation [8, 12, 13].

The present review covers the scope of natural compounds from both prokaryote and eukaryote organisms that have been identified to disrupt biofilm lifestyle cycle in *P. aeruginosa* without affecting directly bacterial viability. As a prerequisite and for a better understanding of the proposed mechanisms of action of some of the identified compounds, relevant key molecular actors in *P. aeruginosa* biofilm formation and its regulation, such as the chemical signalization machinery involved in bacteria-environment

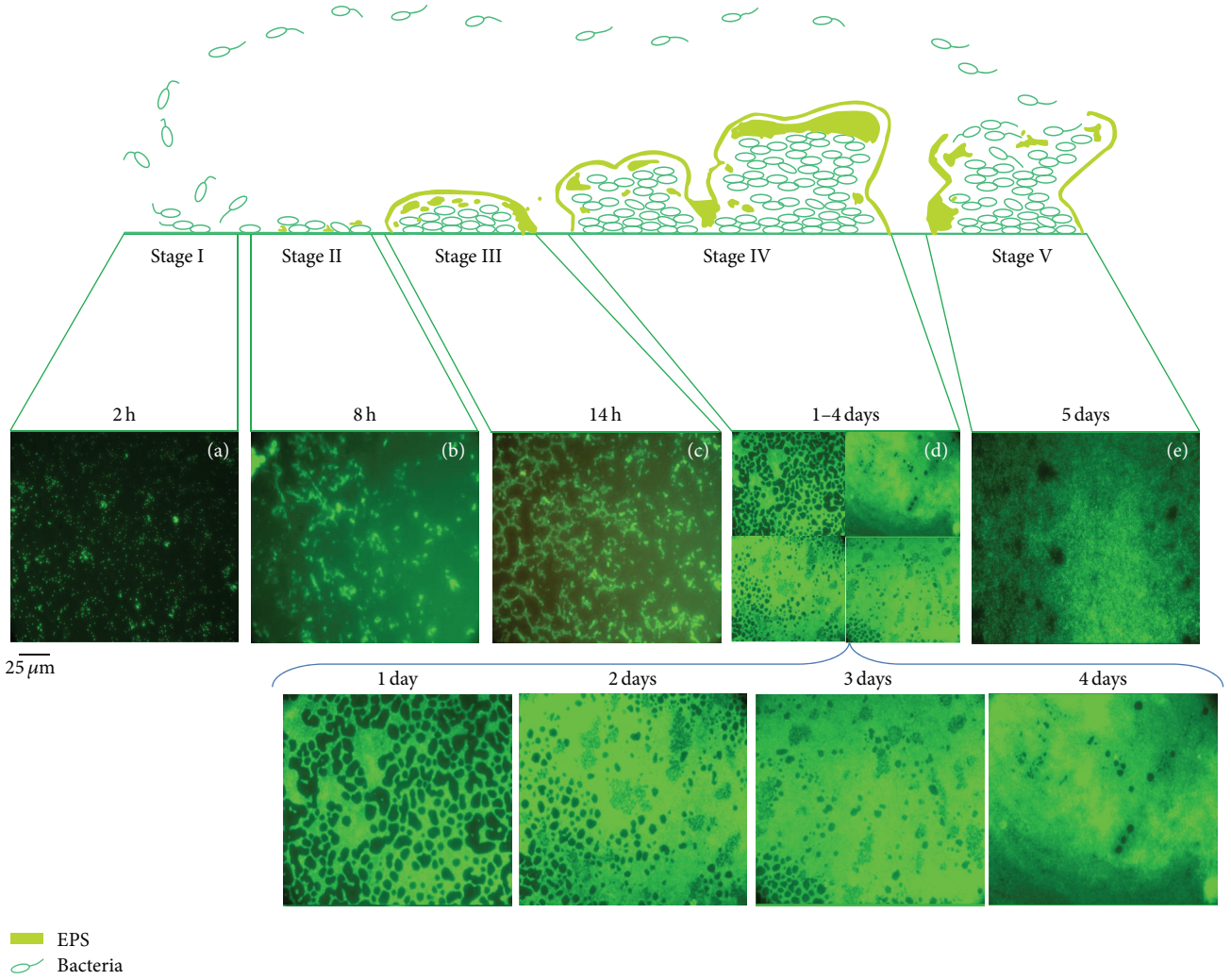


FIGURE 1: Biofilm lifestyle cycle of *P. aeruginosa* PAO1 grown in glucose minimal media. In stage I, planktonic bacteria initiate attachment to an abiotic surface, which becomes irreversible in stage II. Stage III corresponds to microcolony formation. Stage IV corresponds to biofilm maturation and growth of the three-dimensional community. Dispersion occurs in stage V and planktonic bacteria that are released from the biofilm to colonize other sites. The biofilm formation by *P. aeruginosa* PAO1 was revealed with Syto9 and visualized in Leica DM IRE2 inverted fluorescence microscope with 400x magnification at 2 h (Stage I), 8 h (Stage II), 14 h (Stage III), 1 to 4 days (Stage IV), and 5 days (Stage V). Images represent a 250 × 250-μm field.

interaction, including quorum sensing (QS) pathways, will be summarized.

2. Biofilm Lifestyle Cycle of *P. aeruginosa*

Biofilm formation is an endless cycle, in which organized communities of bacteria are encased in a matrix of extracellular polymeric substances (EPS) that hold microbial cells together to a surface [14, 15]; these are thought to be determinant in 65–80% of all microbial infections [16–18]. In this microscopic world, biofilms are metaphorically called a "city of microbes" [19, 20] with EPS, which represents 85% of total biofilm biomass, as "house of the biofilm cells" [21]. EPS is composed mainly of biomolecules, exopolysaccharides, extracellular DNA (eDNA), and polypeptides that form a highly hydrated polar mixture that contributes to the overall structural scaffold and architecture of the biofilm [22–24].

Depending on *P. aeruginosa* strains and/or nutritional conditions, different biofilm phenotypes can be developed [25]. For instance, in glucose minimal media, biofilm lifestyle cycle of *P. aeruginosa* PAO1 can be subdivided into five major phenotypic steps (Figure 1). The process begins by the reversible adhesion of planktonic bacteria onto a surface suitable for growth (Figure 1(a), Stage I), followed by irreversible attachment of bacteria, which thereafter form microcolonies in EPS matrix (Figure 1(b), Stage II). Progressively, bacterial microcolonies expand and their confluences lead to a more structured phenotype with noncolonized space (Figure 1(c), Stage III). Then, noncolonized spaces are filled with bacteria, which finally cover the entire surface (Figure 1(d), Stage IV).

Meanwhile, the growth of three-dimensional communities is observed (Figure 1, Stages III and IV). Finally, bacteria disperse from the sessile structure and reenter in planktonic state to spread and colonize other surfaces [15, 26] (Figure 1(e), Stage V).

P. aeruginosa produces at least three polysaccharides (alginate, Pel, and Psl) that are determinant for the stability of the biofilm structure [27, 28]. Mucoid and nonmucoid *P. aeruginosa* strains differ by the qualitative composition of their polysaccharides in the biofilm matrix, predominantly alginate or Psl/Pel, respectively [29–31]. Alginate, a linear unbranched polymer composed of D-mannuronic acid and L-guluronic acid [32], contributes to the structural stability and protection of biofilms as well as to the retention of water and nutrients [33]. The Pel polysaccharide is mainly a glucose-rich matrix material, with still unclarified composition [34, 35], while Psl comprises a repeating pentasaccharide consisting of D-mannose, L-rhamnose, and D-glucose [36]. Pel and Psl can serve as a primary structure scaffold for biofilm development and are involved at early stages of biofilm formation [30, 37, 38].

eDNA constitutes an important functional component of *P. aeruginosa* biofilm matrix; indeed (i) *P. aeruginosa* biofilm formation is prevented by exposition to DNase I [39]; (ii) biofilms that are deficient in eDNA have been shown to be more sensitive to the detergent sodium dodecyl sulfate [40]; (iii) eDNA facilitates the twitching motility-mediated biofilm expansion by maintaining coherent cell alignments [41]; (iv) eDNA has been proposed to play an important role in the initial and early development of *P. aeruginosa* biofilms as a cell-to-cell interconnecting compound [24, 42, 43]; and (v) finally, eDNA constitutes a nutrient source for bacteria during starvation [44, 45].

Beyond their role in bacterial motilities [46–48], *P. aeruginosa* extracellular appendages flagella, type IV pili and cup fimbriae, are also considered to be matrix components that play adhesive roles in the cell-to-surface interactions (irreversible attachment) as well as in microcolony formation in biofilms. Mutants defective in flagellar-mediated motility and mutants defective in biogenesis of the polar-localized type IV pili do not develop microcolonies compared to the wild type strains [49–51].

3. Overview of Global Regulating Systems Involved in *P. aeruginosa* Biofilm Formation

The complex regulation of biofilm formation involves multiple bacterial machineries, including the QS systems and the two-component regulatory systems that both interact mainly with EPS production [52]. Deficiency in the network regulation required for biofilm matrix formation effectively results in the alteration of the biofilm structure and architecture and, therefore, of its protective role. The main key actors relevant in the regulation of biofilm formation by *P. aeruginosa* are summarized in Figure 2.

3.1. QS Mechanisms and Biofilm Formation. QS is a cell-to-cell communication used by many bacteria to detect their population density by producing and perceiving diffusible signal molecules that coordinate virulence factors production, motility, and biofilm formation [53, 54]. *P. aeruginosa* possesses two main QS systems (*las* and *rhl*) which drive the production (throughout synthases LasI and RhlI) and the perception (by the transcription factors LasR and RhlR) of the autoinducer signaling molecules *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL) (Figure 3(a)), respectively [54]. A third QS system, based on quinolone signals (PQS system), interacts with the acyl homoserine lactones (AHLs) systems in an intricate way [54].

Davies et al. [55] have evidenced the role of the *las* system for biofilm formation and maturation; compared to wild type biofilm, the biofilm of *lasI* mutant appears flat, undifferentiated, and quickly dispersed from the surface upon exposure to sodium dodecyl sulfate. The precise implication of *las* system in biofilm formation is not yet clear. However, Gilbert et al. [56] reported that the QS regulator LasR can bind to the promoter region of the *psl* operon, suggesting that QS can regulate *psl* expression. The *rhl* system has been reported to intervene in *P. aeruginosa* biofilm formation [57] by enhancing Pel polysaccharide biosynthesis; transcription of the *pel* operon is actually reduced in *rhlI* mutant. The PQS system, for its part, is linked to eDNA release during biofilm development; biofilm formed by *pqsA* mutant contains less eDNA than biofilm formed by the wild type [40, 42]. All together these data indicate that the three QS systems known in *P. aeruginosa* play roles in biofilm lifestyle cycle.

Importantly, an indirect link between biofilm formation and QS has been reported, through the control of swarming and twitching motilities, as well as rhamnolipids and lectins production. The swarming motility, a form of organized surface translocation, depends on extensive flagellation and cell-to-cell contact [58, 59]; regulated by the *rhl* system [60], swarming motility is implicated in early stages of *P. aeruginosa* biofilm establishment. Strains grown under conditions that promote swarming motility (growth medium with glutamate or succinate as carbon source) form flat and uniform biofilm while strains with limited swarming motility result in biofilm containing nonconfluent cell aggregates [25]. Twitching motility, a flagella-independent form of bacterial translocation, occurs by successive extension and retraction of polar type IV pili [47]. Known to be regulated by the *rhl* system on Fe-limited minimal medium [61], twitching motilities are necessary for the assembly of a monolayer of *P. aeruginosa* cells into microcolonies [49].

Beyond their biosurfactant and virulence factor roles [62], rhamnolipids, whose production is under the *rhl* system control [63], present multiple roles in biofilm formation by *P. aeruginosa*. Indeed, they are believed to be involved in (i) forming microcolonies [64]; (ii) maintaining open channel structures that prevent bacterial colonization by disrupting both cell-to-cell and cell-to-surface interactions [26]; (iii) facilitating three-dimensional mushroom-shaped structures formation in *P. aeruginosa* biofilms [64]; and (iv) facilitating the cell dispersion from the biofilm as *P. aeruginosa*

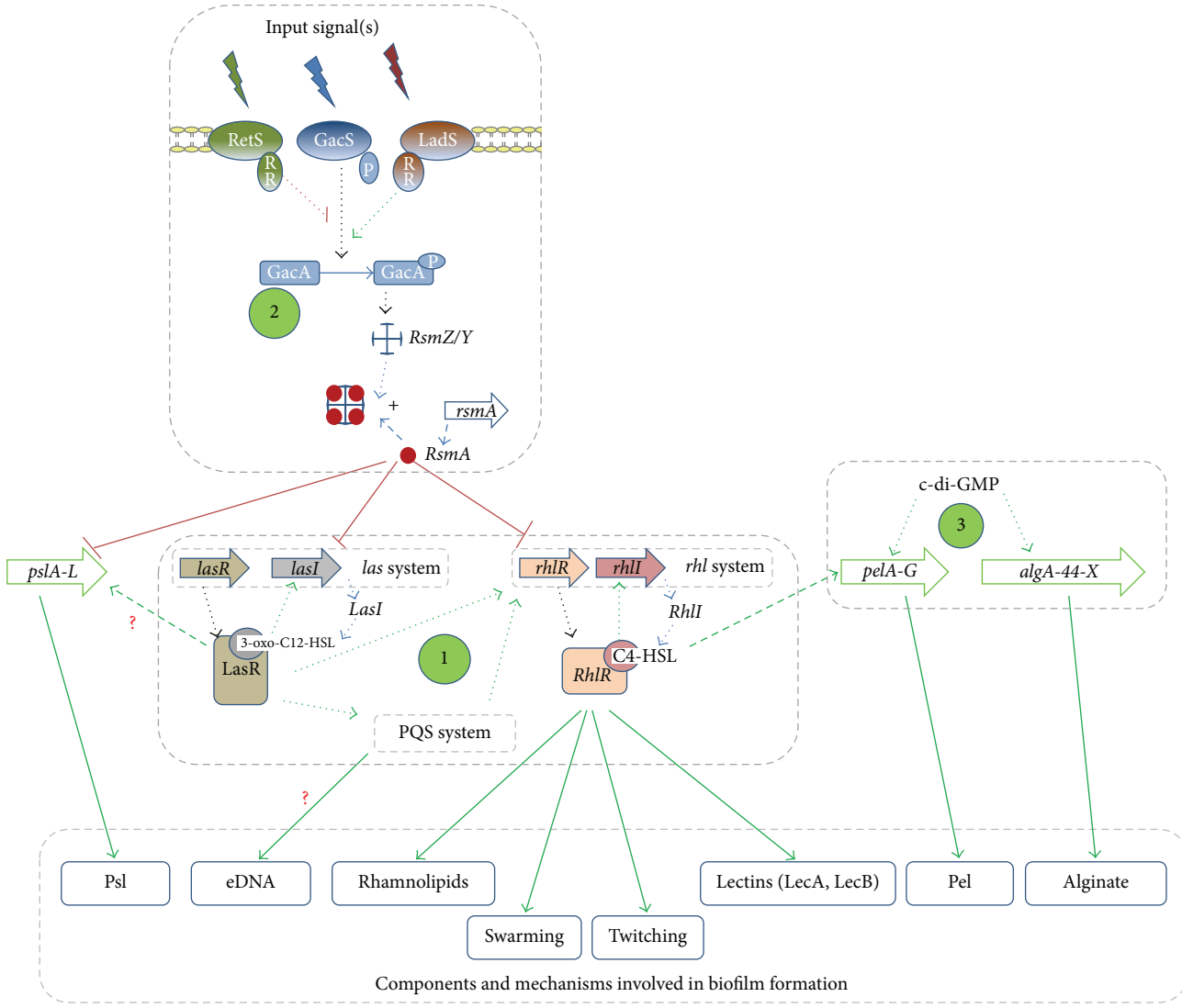


FIGURE 2: Relevant bacterial systems and factors implicated in the regulation of *P. aeruginosa* biofilm formation. (1) Quorum sensing system; (2) Two-component regulatory system *GacS*/*GacA* and *RetS*/*LadS* (RR: response regulator domain receiver; P: phosphorylation) pathway; (3) Exopolysaccharides production and c-di-GMP pool regulation. See text for explanation.

variants which produce more rhamnolipids than wild-type *P. aeruginosa* exhibit hyper-detaching properties [65, 66]. Finally, the cytotoxic virulence factor, galactophilic lectins LecA and LecB, has been proposed to contribute to biofilm development in *P. aeruginosa*, since LecA and LecB mutants form thin biofilms as compared to the wild type bacteria [67, 68]. Both LecA and LecB expressions are regulated by the *rhl* QS system [69].

3.2. Biofilm Regulation by GacS/GacA and RetS/LadS Two-Component Systems. Among the 60 two-components systems found in the genome of *P. aeruginosa* [70], the *GacS*/*GacA* system acts as a super-regulator of the QS system and is involved in the production of multiple virulence factors as well as in biofilm formation [71]. The Gac system consists of a transmembrane sensor kinase (GacS) that, upon autophosphorylation, transfers a phosphate group to its cognate regulator (GacA) which in turn upregulates the expression of the small regulatory RNAs (*RsmZ* and *RsmY*). *RsmZ* and *RsmY* capture the small RNA-binding regulatory protein RsmA (encoded by *rsmA* gene), a repressor that posttranscriptionally regulates the *psl* locus (*pslA-L*) [72–74]. The *GacS*/*GacA* system also has a control on the AHL system as it inactivates free RsmA which negatively controls the synthesis of C4-HSL and 3-oxo-C12-HSL and therefore the extracellular virulence factors controlled by the *las* and *rhl* systems [75–77].

The hybrid sensor histidine kinase RetS is known to repress biofilm formation [78, 79] whereas the histidine kinase LadS antagonizes the effect of RetS [80]. Indeed, Δ*retS* mutant form more structured biofilms as compared to wild type *P. aeruginosa* PAO1 [78]; the PA14 strain (naturally deficient in *ladS* gene) displays attenuated biofilm formation compared to PA14 *LadS*$^+$ strain [81]. It is reported that RetS

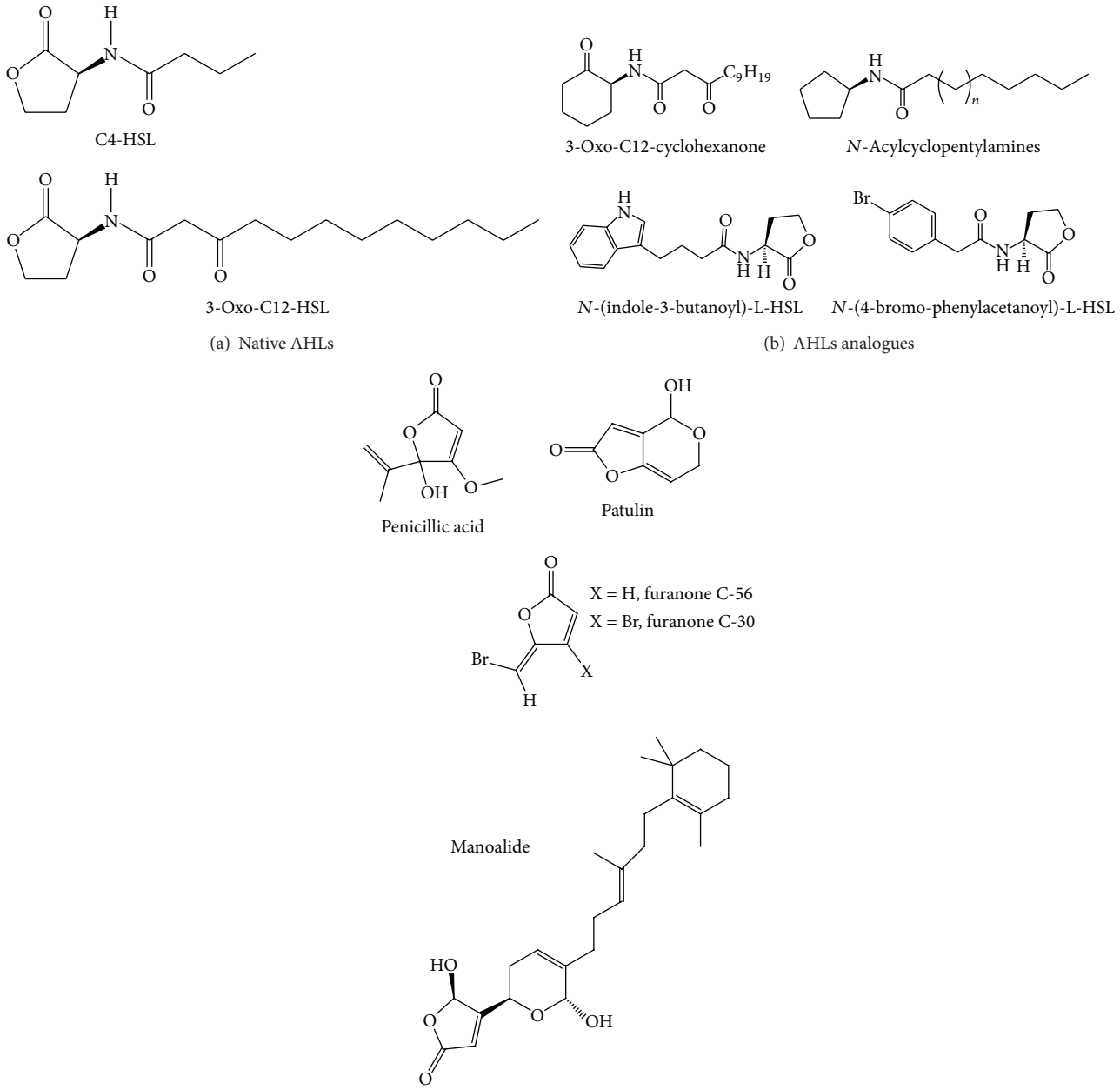


Figure 3: Structure of natural and synthetic AHL-based compounds which inhibit biofilm formation by *P. aeruginosa*. (a) Native *N*-acyl-l-homoserine lactone, signal molecules of *P. aeruginosa* (C4-HSL and 3-oxo-C12-HSL), (b) synthetic analogue of AHLs with side aromatics and synthetic analogues of AHLs with modified lactone rings, and (c) natural (manoalide, penicillic acid, and patulin), and synthetic (furanones) compounds with lactone ring analogues.

and LadS interact with the *GacS*/*GacA* system by modulating the phosphorylation state of GacS, which consequently inhibits and promotes, respectively, the phosphorylation of GacA [82, 83].

It is interesting to note that *GacS*/*GacA* and *RetS*/*LadS* systems are proposed to be involved in mediating the transition of the *P. aeruginosa* phenotype from an acute to chronic phase infection [78].

3.3. C-di-GMP-Dependent Polysaccharides Biosynthesis and Biofilm Formation. Polysaccharides production is dependent on the intracellular pool of bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) [84, 85], a ubiquitous intracellular second messenger widely distributed in bacteria [86]. In bacterial cells, c-di-GMP is generated from two molecules of guanosine triphosphate by diguanylate cyclases and broken down into 2-GMP by specific phosphodiesterases [86].

High levels of c-di-GMP promote the biosynthesis of polysaccharides (alginate and Pel). Indeed, a binding process of c-di-GMP to PelD and Alg44 proteins is required for Pel and alginate polymer formation, respectively [85, 87]. However, the exact molecular mechanism by which this interaction regulates the polymerization of sugar precursors is not known.

Conversely, low levels of c-di-GMP promote bacterial motilities by enhancing flagellar formation and bacterial dispersion [85].

4. Natural and Synthetic Products That Affect *P. aeruginosa* Biofilm Formation

Plants and animals are naturally exposed to bacterial infections and they respond to bacterial components and signal molecules in different manners, including the activation of defense mechanisms and/or the expression of stress management genes [88–93]. Therefore, it is obvious to expect that eukaryotes have developed chemical mechanisms to combat pathogens by killing them or silencing virulence mechanisms such as QS system and/or biofilm formation. Tables 1 and 2 summarize the reported natural and synthetic products that affect *P. aeruginosa* biofilm formation.

4.1. Antibiofilm Compounds with Anti-QS Activity. Several classes of molecules have been reported to present both antibiofilm formation and anti-QS properties in *P. aeruginosa* [94–96].

Some AHL analogues (Figure 3(b)) have been shown to exhibit this double inhibitory activity. Geske et al. [97] have reported that synthetic analogues of AHLs with additional aromatic moieties [*N*-(indole-3-butanoyl)-L-HSL and *N*-(4-bromo-phenylacetanoyl)-L-HSL] display inhibitory activity on LasR-based QS system as well as biofilm formation in *P. aeruginosa* PAO1. Synthetic AHLs analogues, where the homoserine lactone ring is replaced by a cyclohexanone ring, downregulate expression of the *LasI* AHL synthase, resulting in a reduced expression of the virulence factors pyocyanin and elastase and in an alteration of biofilm morphology/phenotype [98]. Nonhydrolysable cyclopentyl analogues of AHLs (*N*-acyle cyclopentylamides) inhibit the *lasI* and *rhlA* expression, the production of virulence factors, including elastase, pyocyanin, and rhamnolipids, and the biofilm formation, without affecting bacterial growth [99].

Halogenated furanones (particularly furanones C-30 and C-56), inspired from natural compounds produced by the marine macroalga *Delisea pulchra*, exhibit biofilm reduction and target the *las* and *rhl* systems in *P. aeruginosa* [55, 100, 101]. Besides, in mouse lungs infected with *P. aeruginosa*, they were found to inhibit bacterial colonization to improve the clearance of bacteria from the host and to reduce the tissue damage [102].

Among the macrolide antibiotics, azithromycin, derived from *Saccharopolyspora erythraea*, has been the most investigated anti-QS antibiotic that presents a strong QS and biofilm inhibitory effect in *P. aeruginosa* [103–105]. Indeed, at subinhibitory azithromycin concentration (2 μg/mL), *P. aeruginosa* produces lower AHL signal molecules and virulence factors [106, 107] suggesting that the observed biofilm inhibition is at least partially due to the reduction of both C4-HSL and 3-oxo-C12-HSL production [108]. Interestingly, azithromycin has been reported to diminish the expression of *GacA* but also *RsmA* at translational level [109], to inhibit the synthesis of alginate [103] and to reduce the three types of motility (swimming, swarming, and twitching) [110].

Penicillic acid and patulin, two secondary fungal metabolites from *Penicillium* species, were shown to effect QS-controlled gene expression in *P. aeruginosa*, most likely by affecting the RhlR and LasR regulatory proteins at posttranscriptional level. *In vitro* studies showed that *P. aeruginosa* PAO1 biofilms treated with patulin and tobramycin were considerably more susceptible to the antibiotic as compared to control biofilms exposed to either tobramycin or patulin alone [111]. However, treatment with patulin alone did not affect development of the biofilm and no hypothesis of mechanisms of action was proposed by authors. The genotoxicity of patulin certainly limits its potential usefulness [112].

Manoalide, a sesterterpenoid from the marine organism *Luffariella variabilis*, exhibits antibiofilm and anti-QS activities (*las* system) in *P. aeruginosa* without bactericidal effects [113], although presenting antibiotic activity against gram-positive bacteria [114].

Solenopsin A alkaloid, isolated from the ant *Solenopsis invicta*, inhibits *P. aeruginosa* pyocyanin production, probably through disruption of the *rhl* signaling system and reduces biofilm production in a dose-dependent manner [115].

Mammalian cells release enzymes called paraoxonases 1 (extracted from human and murine sera) that have lactonase activity; degrading *P. aeruginosa* AHLs, they prevent, in an indirect way, QS and biofilm formation [116, 117].

The phenolic compound curcumin, a major constituent of turmeric roots (*Curcuma longa* L.), downregulates virulence factors (pyocyanin, elastase, and protease) in *P. aeruginosa* PAO1 and inhibits adherence of the bacteria to polypropylene surfaces. This was correlated with a decrease in 3-oxo-C12-HSL production [118]. Rosmarinic acid, a natural phenolic compound produced by the root of *Ocimum basilicum* L. upon *P. aeruginosa* infection, prevents biofilm formation but fails to penetrate mature biofilm under *in vivo* and *in vitro* conditions [89]. Structure-based virtual screenings against LasR and RhlR receptor proteins effectively indicate that rosmarinic acid is a potential QS inhibitor [119]. Ellagic acid derivatives, from *Terminalia chebula* Retz., have been shown to downregulate *lasIR* and *rhlIR* genes expression with a concomitant AHLs decrease, resulting in the attenuation of virulence factor production and in an enhanced sensitivity of biofilm towards tobramycin [120]. Girennavar et al. [121] demonstrated that the furocoumarins from grapefruit juice, bergamottin and dihydroxybergamottin, inhibit the activities of the autoinducers AI-1 (*N*-3 hydroxybutanoyl-homoserine lactone) and AI-2 (furanosyl borate diester) in a *V. harveyi* bioassay. Besides, these authors showed that AI-1 and AI-2 inhibit biofilm formation in *E. coli* O157:H7, *Salmonella typhimurium*, and *P. aeruginosa* without affecting bacterial growth. However, the mechanisms of action remain unclear.

Table 1: Natural inhibitory compounds for *P. aeruginosa* biofilm formation.

Natural products compounds	Origin	Class	Activities				References
			QS inhibition	Biofilm inhibition[(a)]	Dispersion promotion	Synergy with antibiotic	
Alginate lyase	*P. aeruginosa*	Enzyme	−	+	+	+[(1)]	[134]
Ursolic acid	*Diospyros dendo* Welw.	Triterpenoid	−	+ (24 h) motility	NC	NC	[141]
p-Coumaroyl-hydroxy-ursolic acid	*Diospyros dendo* Welw.	Coumarate ester of triterpene	NC	+ (24 h)	NC	NC	[142]
Zingerone	*Zingiber officinale* Rosc.	Phenolic compound	NC	+ (168 h) c-di-GMP	NC	+[(2)]	[138, 139]
Casbane diterpene	*Croton nepetaefolius* Baill.	Diterpenoid	NC	+ (24 h) adherence	NC	NC	[140]
DNase I	Bovine pancreas	Enzyme	NC	+ (18–24 h)	+	+[(3)]	[137]
DNase-1L2	Human *stratum corneum*						[136]
Paraoxonases 1	Human and murine sera	Enzyme (lactonase)	+	+ (24 h)	NC	NC	[116, 117]
Manoalide	*Luffariella variabilis* (Polejaeff, 1884) (marine organism)	Sesterterpenoid	+ (*las* system)	+ (24 h)	NC	NC	[113, 114]
Solenopsin A	*Solenopsis invicta* (insect; ant)	Alkaloid	+ (*rhl system*)	+ (24 h)	NC	NC	[115]
Catechin	*Combretum albiflorum* (Tul.) Jongkind	Flavonoid	+ (*las rhl systems*)	+ (24 h)	NC	NC	[124]
Naringenin	Commercial	Flavonoid	+ (*las rhl systems*)	+ (48 h)	NC	NC	[125]
Coumarate ester	*D. trichocarpa* Baker.	Phenolic compound	+ (*las rhl systems*)	+ (48 h)	+	+[(1)]	[126]
Ajoene	*Allium sativum* L.	Organosulfur	+ (*las rhl systems*)	+ (96 h)	NC	+[(1)]	[130]
Ellagic acid derivatives	*Terminalia chebula* Retz.	Phenolic compound	+ (*las rhl systems*)	+ (72 h)	NC	+[(1)]	[120]
Rosmarinic acid	*Ocimum basilicum* L.	Phenolic compound	+ (*las rhl systems*)	+ (18 h)	−	NC	[89, 119]
Eugenol	*Syzygium aromaticum* (L.) Merr. Et Perry	Phenylpropanoid	+ (*las pqs* systems)	+ (24 h)	NC	NC	[128]
Curcumin	*Curcuma longa* L.	Phenolic compound	+ (AHLs)	+ (48 h)	NC	NC	[118]
Bergamottin and dihydroxybergamottin	*Citrus paradisi* Macfad. (Rio Red and Marsh White grapefruits)	Furocoumarins	+ (AI-1 and AI-2)	+ (24 h)	NC	NC	[121]
Penicillic acid	*Penicillium* species	Furanone	+ (LasR, RhlR)	NC	NC	NC	[111]
Patulin		Furopyranone	+ (LasR RhlR)	—[‡]	NC	+[(1)]	
Emodin	*Rheum palmatum* L.	Anthraquinone	+ (docking traR)	+ (72 h)	NC	+[(3)]	[123]
Baicalein	*Scutellaria baicalensis* Georgi.	Flavonoid	+ (docking traR)	+ (72 h)	NC	+[(3)]	[122]

+: yes; −: no; NC: not communicated.

[‡]Patulin does not affect the development of biofilm.

[(a)]Experiment duration.

[(1)]Aminoglycosides, [(2)]ciprofloxacin, [(3)]ampicillin.

Table 2: Synthetic inhibitory compounds in *P. aeruginosa* biofilm formation.

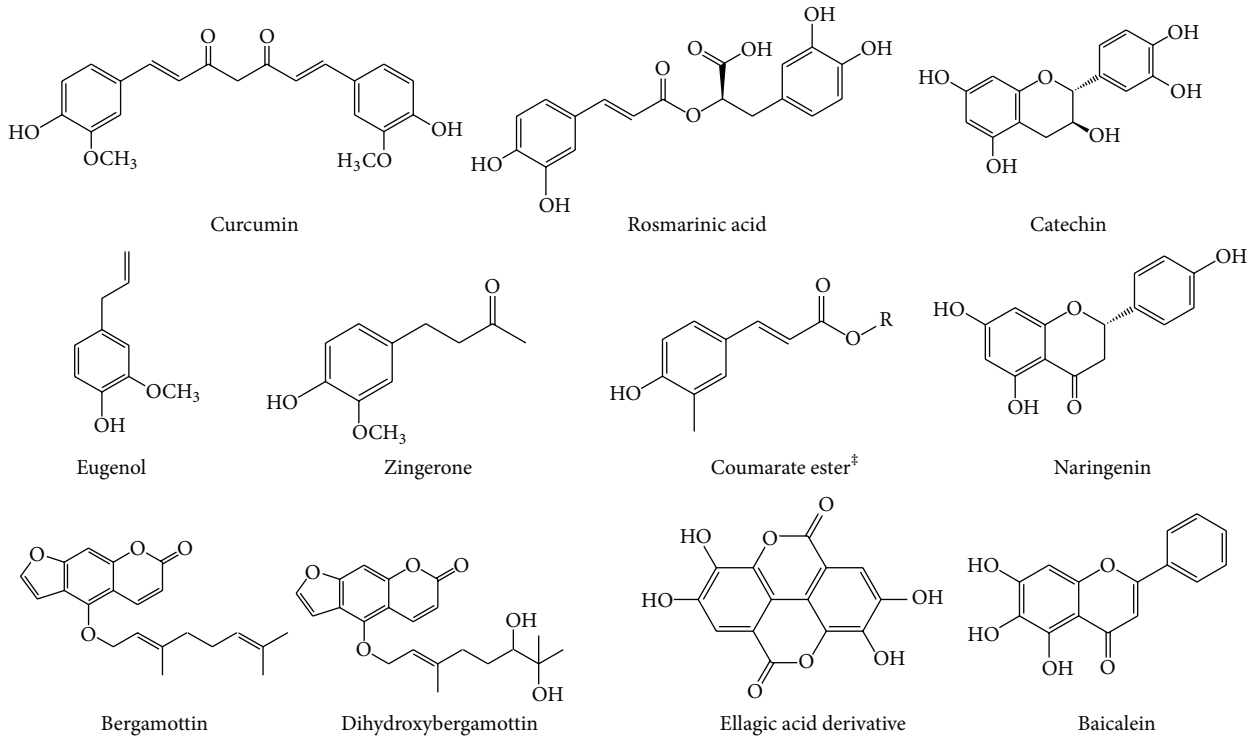
Synthetic compounds (Natural compound origin)	QS inhibition	Activities			References
		Biofilm inhibition[(a)]	Dispersion promotion	Synergistic antibiotic and/or immune defense effect	
TAGE and CAGE (Bromoageliferin)	NC	+ (24 h)	+	NC	[132]
Dihydrosventrin (Sventrin)	NC	+ (24 h)	+	NC	[133]
N-(4-bromo-phenylacetanoyl)-l-HSL; *N*-(indole-3-butanoyl)-L-HSL (AHLs)	+ AHLs antagonist (*las* system)	+ (24 h)	NC	NC	[97]
3-oxo-C12-cyclohexanone (AHLs)	+ AHLs antagonist (*las* system)	+ (24 h)	NC	NC	[98]
C10-cyclopentylamide (AHLs)	+ (*lasI* and *rhlA*)	+ (24 h)	NC	NC	[99]
Furanone C-30 and C-56 (Furanone)	+ (*las, rhl* systems)	+ (24 h)			[55, 100, 101]
S-phenyl-L-cysteine sulfoxide (Cysteine sulfoxide alliin)	+ (*las, rhl systems)*	+ (24 h)	NC	NC	
Diphenyl disulfide (Disulfide derivatives of the alliinase mediated reactions)	+ *las system*	+ (24 h)	NC	NC	[148]
Azythromycin[‡] (Erythromycin)	+ (*gacA, las and rhl systems*)	+ (72 h)	+	+[(1)]	[103, 108, 109]

+: yes; −: no; NC: not communicated.

[‡]At subinhibitory concentration.

[(a)]Experiment duration.

[(1)]Aminoglycosides.



‡Radical under elucidation

Figure 4: Phenolic compounds and derivatives with antibiofilm and anti-QS proprieties.

Docking screening for QS inhibitors predicted that the flavone baicalein, obtained from the roots of *Scutellaria baicalensis* Georgi, could interact with *A. tumefaciens* QS transcription activator protein TraR. Effectively, at 20 μM, baicalein promotes the proteolysis of the signal receptor TraR protein in *Escherichia coli* biosensor, significantly inhibiting the biofilm formation by *P. aeruginosa* [122]. Similarly, the screening of traditional Chinese medicinal plants identified the anthraquinone emodin, extracted from rhubarb (*Rheum palmatum* L.); emodin actually inhibits the *P. aeruginosa* biofilm formation at 20 μM, increasing the activity of ampicillin [123].

The flavan-3-ol catechin, isolated from the bark of *Combretum albiflorum* (Tul.) Jongkind, as well as the flavanone naringenin, both at 4 mM final concentration, do interfere with QS mechanism in *P. aeruginosa* PAO1 by affecting autoinducers perception and biofilm formation [124–126]. A coumarate ester isolated from the bark extract of Malagasy endemic *Dalbergia trichocarpa* Baker interferes with *P. aeruginosa* QS systems (*las* and *rhl*), inhibits the biofilm formation and increases the effectiveness of the antibiotic tobramycin in killing biofilm-encapsulated *P. aeruginosa* [126] (Figures 4 and 5).

Recently, *Meliaceae*, *Melastomataceae*, *Lepidobotryaceae*, and *Sapindaceae*, collected from neotropical rainforests in Costa Rica, presented significant anti-QS activities in a *Chromobacterium violaceum* bioassay and/or inhibition of biofilm formation by *P. aeruginosa* PA14 [127]. Although the exact natures of the active constituents are not yet elucidated, the authors suggest that they could belong to polar polyphenols similar to tannic acid.

A recent screening of various herbal extracts revealed that clove extract (*Syzygium aromaticum* (L.) Merr. Et Perry) inhibits QS-controlled gene expression (*las* and *pqs* systems) in *P. aeruginosa* with eugenol as major active constituent [128]. Eugenol, at subinhibitory concentrations (400 μM) inhibited virulence factors production including elastase, pyocyanin and biofilm formation. In agreement with this finding, subinhibitory concentrations of the clove essential oil significantly reduces *las*- and *rhl*-regulated virulence factors, exopolysaccharide production, and biofilm formation by *P. aeruginosa* PAO1 [129].

Ajoene, an allyl sulfide isolated from garlic (*Allium sativum* L.), has been reported to affect QS-regulated genes in *P. aeruginosa*, including the production of rhamnolipids. Additionally, ajoene synergizes with the antibiotic tobramycin in killing biofilm-encapsulated *P. aeruginosa*, improving the clearance of *P. aeruginosa* from lungs in a mouse model of pulmonary infection [130]. A naturally-inspired organosulfur compound (*S*-phenyl-L-cysteine sulfoxide) and its derivative (diphenyl disulfide) have been reported to significantly reduce the amount of biofilm formation by *P. aeruginosa* [131]. The *S*-phenyl-L-cysteine sulfoxide antagonizes both the *las* and *rhl* QS systems whereas the diphenyl disulfide only interferes with the *las* system.

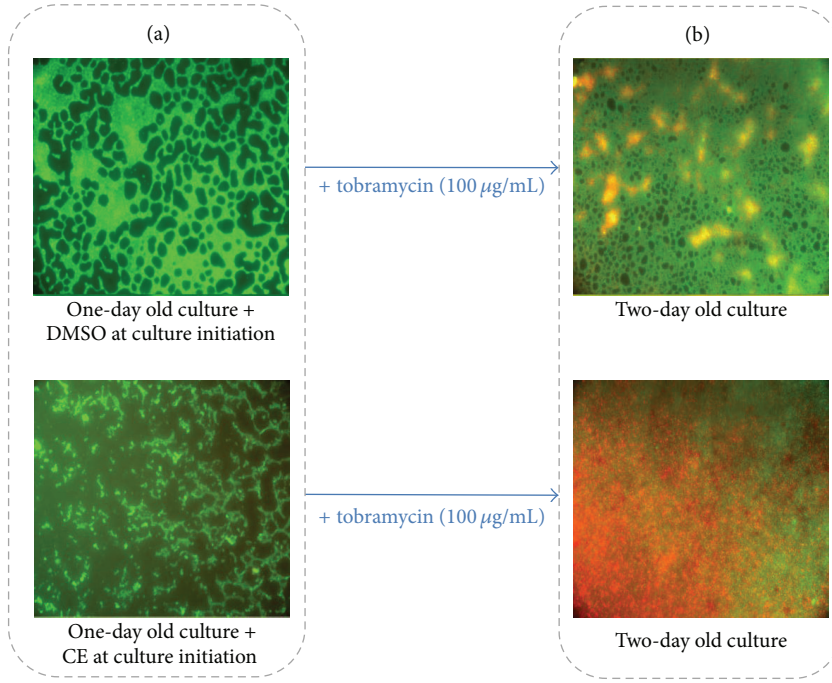


Figure 5: *P. aeruginosa* biofilm phenotypes and effectiveness of tobramycin treatment in presence of DMSO 1% or coumarate ester (CE) at 300 μg/mL. (a) After 1 day of incubation, *P. aeruginosa* fails to form structured confluent aggregate in presence of CE as compared to DMSO treatment. (b) CE considerably increases the susceptibility of *P. aeruginosa* to tobramycin (100 μg/mL), as shown by the increased proportion of dead cells compared with DMSO. The bacterial viability was assessed by staining the cells with SYTO-9 (green areas—live bacteria) and propidium iodide (red areas—dead bacteria) furnished in the LIVE/DEAD *Bac*Light kit. Cells were visualized using a Leica DM IRE2 inverted fluorescence microscope using a 40x objective lens and colored images were assembled using Adobe Photoshop.

4.2. Antibiofilm Compounds without or with Unspecified Anti-QS Activity. Various organisms, including prokaryotes and eukaryotes (marine organisms, animals, and plants) have been reported to produce secondary metabolites which exert antibiofilm activity. Some of those natural compounds have been used as models to build synthetic antibiofilm compounds against *P. aeruginosa*.

Bromoageliferin, pyrrole-imidazole alkaloids from marine sponges (*Agelas conifer*, *Agelaceae*), has been the scaffolding for the development of two derivatives, *trans*-bromoageliferin analogue 1 (TAGE) and *cis*-bromoageliferin analogue 2 (CAGE). Both synthetic derivatives inhibit biofilm formation and furthermore are able to disperse preexisting *P. aeruginosa* PAO1 biofilms without demonstrating a bactericidal or growth-inhibiting effect [132]. Analogues based upon the oroidin template, parent molecules of bromoageliferin, have been synthesized and screened in *P. aeruginosa* for their antibiofilm ability [133]. The authors found that the most potent analogue turned out to be dihydrosventrin, a variant of the pyrrole-imidazole alkaloids sventrin (from *Agelas sventres*) which exhibits biofilm inhibition and biofilm dispersion for different strains of *P. aeruginosa* without any microbicidal activity.

Alginate lyase, produced by *P. aeruginosa* itself, promotes biofilm dispersion and acts synergically with antibiotics for successful elimination of mucoid strains of *P. aeruginosa* established in the respiratory tracts of cystic fibrosis patients [134]. However, a recent study demonstrated that this effect cannot be attributed to the catalytic activity of the enzyme. Indeed, bovine serum albumin or simple amino acids lead to the same results. The authors postulate that alginate lyase acts simply as a nutrient source, modulating cellular metabolism and thus inducing cellular detachment and enhancing tobramycin efficacy [135].

Bovine pancreatic Dnase I and Dnase-1L2, extracted from human *stratum corneum*, exhibited strong antibiofilm activity in *P. aeruginosa* [136]. Indeed, the degradation of extracellular DNA leads to an altered biofilm that permits increased antibiotics penetration [137].

Extracts of Ginger (*Zingiber officinale* Rosc.), long used by Indians, Asians, and Arabs to treat numerous ailments [137], inhibit *P. aeruginosa* PA14 biofilm formation through the reduction of c-di-GMP production and consequent reduction of total polysaccharides production [138]. The ginger extract revealed no AHL-based QS inhibition in the *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NT1 reporter biosensor systems. The major component of dry ginger root, zingerone (vanillyl acetone), has been shown to inhibit biofilm formation, to increase the susceptibility of *P. aeruginosa* PAO1 to ciprofloxacin [139] and to inhibit swimming, swarming, and twitching motilities. However, authors did not propose any mechanism of action.

The casbane diterpene, isolated from the ethanolic extract of *Croton nepetaefolius* Baill., a plant native from northeastern Brazil, inhibits biofilm formation in several clinical relevant species, including *P. aeruginosa* (at 250 μg/mL) without

affecting the planktonic growth. Authors suggest that this inhibition of biofilm formation may be related to an interaction between casbane diterpene and lipopolysaccharides present on the cell surface, which might affect their adherence properties [140].

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) from *Diospyros dendo* Welw. is identified to inhibit biofilm formation without interfering with QS systems in *E. coli*, *P. aeruginosa,* and *V. harveyi*; ursolic acid, at 10 μg/mL, has been found to reduce 72% of *E. coli* JM109 biofilm. Transcriptomic analyses led to the conclusion that ursolic acid inhibits biofilm formation by inducing motility [141]. The *3β-O-cis-p*-coumaroyl-20β-hydroxy-12-ursen-28-oic acid, isolated from the same plant, strongly inhibits biofilm formation by *P. aeruginosa* PAO1 [142]. However, the mechanism of activity was not investigated.

5. Concluding Remarks and Perspectives

There is increasing evidence that biofilm-mediated infection facilitates the development of chronic infectious diseases and recurrent infections [143–145]. Relevance in using antibiofilm compounds is based on the restoration of antibiotic effectiveness by facilitating their penetration through compromised biofilm structure. Moreover, a degradation of the biofilm matrix could render infectious bacteria reachable to immune defenses (e.g., polymorphonuclear leukocytes, innate, and specific antibodies) [146, 147]. Thus, antibiofilm compounds could be interesting antibiotic adjuvants to prevent or treat chronic infections. Similarly, relevance in using anti-QS compounds is based on the concomitant drastic reduction of virulence factors expression, which gives the necessary time for immune defense systems to elaborate appropriate responses by the recruitment of immune cells and production of specific antibodies. Unlike antibiofilm compounds, anti-QS compounds are interesting to prevent or jugulate acute infection. However, it should also be noted that (i) anti-QS and antibiofilm compounds may lose their appeal in immune compromised patients who often harbor bacteria that are still alive but present in a disorganized and less virulent stage; (ii) QS systems do not control the totality of virulence factors expression; and (iii) the development of anti-QS bacterial resistance cannot be excluded [148]. These facts partly explain why the discovery of QS modulators has not yet led to major therapeutic breakthroughs. In our opinion, such bioactive compounds will probably not substitute antibiotics but rather optimize the effectiveness of infectious diseases treatment, notably through biofilm disruption and antibiotic dose reduction; their use is also appealing to optimize the use of microbicidal products by reducing biofilm encroachment on biomaterials and medical devices.

In the perspective of therapeutic application, very few studies have been progressed to clinical trial. To the best of our knowledge, garlic is the only extract with anti-QS and antibiofilm to have been tested in a clinical trial with non-significant results, contrary to its drastic *in vitro* bioactivity effect [149]. One reason of this fact is that behavior of clinical isolates may be different when grown in laboratory condition and in human body which could lead to unexpected biofilm development. Thus, before progressing in clinical trial of relevant bioactive compounds, effort on the improvement of experimental *in vitro* and *in vivo* conditions should be addressed and clinical trial protocols should be discussed.

Potent antibiofilm agents are considered interesting if they exert a sustainable bioactivity; this can be indicated by an activity that resists accumulating bacterial toxins, enzymes, and metabolites for more than 48 h in culture media. As less than half of bioactive products have been tested up to 48 hours, further investigations are warranted to select those compounds with sustained activities, which would have more chances to be active in clinical conditions. Halogenated furanones have been widely studied for their powerful anti-QS and antibiofilm activities (<10 μM) [100]. However, their toxic and carcinogenic properties relegate them so far to the role of positive QS inhibitory controls in laboratory experiments [150, 151]. In this regard, herbal phenolic compounds and their derivatives, frequent in food components, and more particularly those already present in popular and approved herbal drugs (i.e., rosmarinic acid in *Melissa officinalis* L.), are promising candidates to develop antibiofilm agents; however, structure-activity studies are still required to better assign essential structural features responsible for antibiofilm activity. In the same perspective, searching for compounds active at nanomolar levels should be privileged as these could presumably present lower toxicity risks. The QS system is an obvious target for biofilm-associated infections as QS interacts, directly and/or indirectly, in different steps of biofilm formation. Intriguingly, even if QS inhibition is the most extensively studied approach against *P. aeruginosa*, several anti-QS natural compounds have not been yet investigated for their antibiofilm activity (e.g., human sexual hormones and some antibiotics at subinhibitory concentration, notably ceftazidime and ciprofloxacin) [103, 152]. Attractive therapeutic agents are those which modulate QS system(s) with an extending or particular impact on biofilm lifestyle; they could then be helpful as a preventive or curative approach and at every step of infectious diseases (acute and chronic). However, finding universal antibiofilm compounds represents a challenge as biofilm lifestyle, composition, and phenotype strongly depend on several parameters, such as nutritional conditions. In this regard, we support the hypothesis that compounds which target *GacS*/*GacA* pathway are worthy of interest with respect to the pathway hierarchically upstream position that controls positively both QS system and exopolysaccharides biosynthesis (Psl) (Figure 2). Such compounds could possibly impair almost all the biofilm lifestyle cycle of *P. aeruginosa*, from irreversible attachment to dispersion stages (Table 3) and could be powerful allies for conventional antibiotics in the struggle against bacterial biofilm-mediated infections [8, 12, 95].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Table 3: Factors affecting biofilm lifestyle regulation.

Biofilm lifestyle cycle	Stage I Reversible attachment	Stage II Irreversible attachment	Stage III Maturation-1 (microcolony development)	Stage IV Maturation-2 (maintenance)	Stage V Dispersion
Implicated factors	c-di-GMP	(i) Flagella and type IV pili (ii) Adhesins (iii) Psl, Pel	(i) Cell-to-cell communication *lasI* (ii) Type IV fimbriae (iii) Matrix components: Psl, Pel, eDNA (iv) Lectin A, B (v) *rhlA*	(i) Cell-to-cell communication (ii) Matrix components: Psl, Pel, eDNA (iii) *rhlA*	Rhamnolipids
General target	(i) Promoting planktonic lifestyle (ii) Blocking switch from planktonic to biofilm lifestyle	Reducing initial adhesion and interaction	Interfering with QS communication	(i) Reactivating metabolic activity for antibiotic efficiency. (ii) Interfering with QS communication	Promoting dispersion and degradation
Examples of extracts or compounds	(i) Ginger extract (ii) Ursolic acid	(i) Casbane diterpene (ii) Coumarate ester	Coumarate ester	(i) Solenopsin A (ii) Naringenin (iii) Furanone C-30 and -56 (iv) Flavan-3-ol Catechin (v) Ajoene (vi) Coumarate ester	DNAse I, 1L2

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Research Article

The Influence of Tea Tree Oil (*Melaleuca alternifolia*) on Fluconazole Activity against Fluconazole-Resistant *Candida albicans* Strains

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The aim of this study was to evaluate the activity of fluconazole against 32 clinical strains of fluconazole-resistant *Candida albicans*, and *C. albicans* ATCC 10231 reference strain, after their exposure to sublethal concentrations of tea tree oil (TTO) or its main bioactive component terpinen-4-ol. For all tested fluconazole-resistant *C. albicans* strains TTO and terpinen-4-ol minimal inhibitory concentrations (MICs) were low, ranging from 0.06% to 0.5%. The 24-hour exposure of fluconazole-resistant *C. albicans* strains to fluconazole with sublethal dose of TTO enhanced fluconazole activity against these strains. Overall, 62.5% of isolates were classified as susceptible, 25.0% exhibited intermediate susceptibility, and 12.5% were resistant. For all of the tested clinical strains the fluconazole MIC decreased from an average of 244.0 μg/mL to an average of 38.46 μg/mL, and the fluconazole minimal fungicidal concentrations (MFC) decreased from an average of 254.67 μg/mL to an average of 66.62 μg/mL. Terpinen-4-ol was found to be more active than TTO, and strongly enhanced fluconazole activity against fluconazole-resistant *C. albicans* strains. The results of this study demonstrate that combining natural substances such as TTO and conventional drug such as fluconazole, may help treat difficult yeast infections.

1. Introduction

Essential oils are antiseptic substances produced by plants. Tea tree oil (TTO) is the essential oil obtained by steam distillation from the Australian native plant *Melaleuca alternifolia* and is used medicinally as a topical antiseptic. It has a broad spectrum of antimicrobial activity against a wide range of bacteria, viruses, and fungi, including yeasts and dermatophytes. TTO is a mixture of more than 100 different compounds, primarily terpenes (mainly monoterpenes and sesquiterpenes). The physical properties and chemical composition of TTO are variable, and it is, therefore, important to determine international standards. The Australian Standard for tea tree oil (AS 2782-1985) includes directives relating to the levels of two components: the minimum content of terpinen-4-ol should be at least 30% and the maximum content of 1,8-cineole should be less than 15% of the oil volume [1]. The international standard for tea tree oil (ISO 4730:2004) includes maximum and minimum percentage values for the 15 most important TTO components. TTO obtained by steam distillation of the leaves and terminal branches of *Melaleuca alternifolia* Cheel, *Melaleuca linariifolia* Smith, *Melaleuca dissitiflora* F. Mueller, and other species of *Melaleuca* should conform to this standard [2].

TTO has been used for centuries in Australian folk medicine, predominantly for wound treatment [3, 4]. In the 1920s, Penfold described for the first time the properties and chemical composition of TTO, and he later confirmed the antiseptic properties of TTO and its components [5–8]. In the 1930s, consecutive publications appeared which demonstrated the powerful antimicrobial activity of TTO when used in inhalation therapy, aseptic surgery, dental surgery, wound disinfection, and oral cavity rinsing [9–11].

Currently, TTO is used as a local agent for treating various diseases, predominantly dermatoses (e.g., recurrent herpes labialis, acne, pustules, dandruff, and rash). TTO is also used

to treat *Staphylococcus aureus* infections of the oral cavity and the pharynx, vaginitis, and respiratory tract diseases. Numerous studies have confirmed the broad antimicrobial activity of TTO against bacteria, fungi, and viruses, as well as microorganisms that are resistant to conventional drugs [12–16]. This is important due to the increase in infections that are difficult to treat, as TTO can be used as an alternative to or in combination with conventional drugs (including antibiotics and chemotherapeutic agents).

Treatment of infections can be based on monotherapy (using one antimicrobial drug) or combined therapy (two or more drugs). The primary aim of combined therapy is to enhance the action of the drugs while decreasing the dosages, through synergism. When monotherapy or combined therapy based on conventional drugs is unsuccessful, then combined treatment including a natural agent may be more effective. Several recent studies have reported the increased antimicrobial activity of natural substances combined with conventional drugs as compared to conventional drug treatment alone [17–20].

The aim of this study was to evaluate the activity of fluconazole against clinical strains of fluconazole-resistant *Candida albicans* and reference strain *C. albicans* ATCC 10231, after their exposure to sublethal concentrations of TTO or its main bioactive component terpinen-4-ol.

2. Materials and Methods

2.1. Candida albicans Strains. This study included 32 clinical *Candida albicans* strains, which were isolated from the following materials: swabs of the pharynx and oral cavity ($n = 5$), vagina ($n = 15$), sputum ($n = 8$), or faeces ($n = 4$). These strains were isolated from culture on Sabouraud agar (bioMèrieux, Marcy l'Etoile, France), and species identification was performed using the biochemical test ID 32C (bioMèrieux, Marcy l'Etoile, France). We also used the reference strain *C. albicans* ATCC 10231, which was purchased from Oxoid Ltd. (Basingstoke, Great Britain). We previously determined the sensitivity of *C. albicans* strains to fluconazole by the Kirby-Bauer disk diffusion susceptibility test [21] using 6 mm filter paper disks impregnated with 10 μg of fluconazole obtained from DHN (Cracow, Poland) and YNB agar (Yeast Nitrogen Base-Difco 0.5%, glucose 3%, agar 1.8%, pH = 7) also obtained from DHN (Cracow, Poland). The *C. albicans* strains were classified as exhibiting susceptibility (diameter of growth inhibition zone ≥18 mm), intermediate susceptibility (diameter of growth inhibition zone from 14 mm to 17 mm), or resistance (diameter of growth inhibition zone <14 mm) to fluconazole (the data were described in chapter 3). The fluconazole MIC (minimal inhibitory concentration) and MFC (minimal fungicidal concentration) values were determined by the broth dilution method according to the Clinical and Laboratory Standards Institute (CLSI document M27-A3-2008) [22]. Using this standard, the *C. albicans* strains were classified as exhibiting susceptibility (MIC ≤ 8 μg/mL), intermediate susceptibility (MIC from 9 μg/mL to 63 μg/mL), or resistance (MIC ≥ 64 μg/mL) to fluconazole (the data were presented in chapter 3).

Table 1: Composition of the TTO used in this study compared to ISO standard 4730:2004 [2].

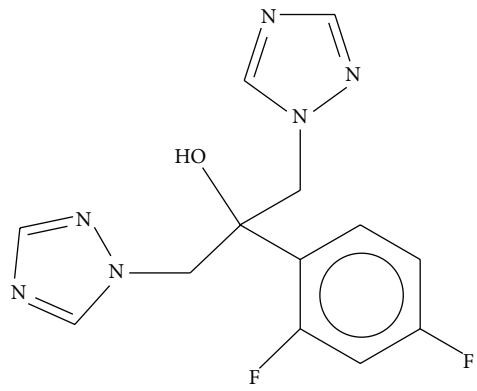
Components	Content (%) according to ISO standard 4730	Content (%) of TTO sample
α-Pinene	1–6	2.5
Sabinene	Trace-3.5	0.1
α-Terpinene	5–13	8.1
Limonene	0.5–1.5	1.0
p-Cymene	0.5–8	4.4
1,8-Cineole	Trace-15	2.8
γ-Terpinene	10–28	19.6
Terpinolene	1.5–5	3.2
Terpinen-4-ol	30–48	41.0
α-Terpineol	1.5-8	3.0
Aromadendrene	Trace-3	1.3
Ledene (syn. viridiflorene)	Trace-3	No data available
δ-Cadinene	Trace-3	No data available
Globulol	Trace-1	No data available
Viridiflorol	Trace-1	No data available

2.2. Tea Tree Oil (TTO). In this study, we used Australian tea tree oil (*Melaleuca alternifolia*) from Thursday Plantation (Integria Healthcare, Eight Mile Plains, QLD, Australia) series 270930 that conforms to the ISO standard 4730:2004 [2] (Table 1). TTO was distilled from specially selected *Melaleuca alternifolia* leaves, a plant native to the coastal regions of northern New South Wales and south eastern Queensland in Australia. The analysis of TTO composition was carried out by gas chromatography according to the international standard ISO 4730 [2]. It was performed in the following conditions: fused-silica column (50 m × 0,20 mm i.d., film thickness 0,25 μm) and flame ionisation type of detector were used, the carrier gas was hydrogen (flow rate of 1 mL/min), the oven temperature programme was from 70°C to 220°C at a rate of 2°C/min, the injector temperature was 230°C, the detector temperature was 250°C, the volume of injected TTO was 0,2 μL, and the split ratio was 1 : 100.

In our study, we also used terpinen-4-ol, which was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Fluconazole. In this study, we used the antifungal drug fluconazole (Polfarmex, Kutno, Poland). The structure of the fluconazole molecule is shown in Figure 1.

2.4. Preparation of the Initial Candida albicans Suspension. *C. albicans* cells cultured for 24 h on Sabouraud agar were suspended in a saline solution (0.85% NaCl) and adjusted to a 0,5 McFarland density standard ($1{,}5 \times 10^8$ CFU/mL). This suspension was later diluted to a density of 6×10^4 CFU/mL. The suspension was then used to estimate the MIC and MFC values for TTO, terpinen-4-ol, and fluconazole.



$C_{13}H_{12}F_2N_6O$

2-(2,4-difluorophenyl)-1,3-bis(1*H*-1,2,4-triazol-1-ilo)propan-2-ol

Figure 1: Chemical structure of fluconazole [23].

2.5. Determination of MIC and MFC Values for TTO and Terpinen-4-ol. The TTO activity against the *C. albicans* strains tested was determined by broth macrodilution using the general dilution standards as described by PN-EN ISO 20776-1:2007 [24]. TTO was serially diluted in liquid Sabouraud medium with 10% Tween 80 to final TTO concentrations of 1% to 0.0075%. The Tween 80 detergent helps dissolve the TTO. The same volume of the *C. albicans* suspension was added to each tube to obtain a final density of 3×10^3 CFU/mL. After 24 h of incubation at 35°C, the cell growth was assessed visually in the tubes with TTO and the positive control tube (without TTO). The MIC was defined as the lowest concentration of TTO that led to no visible growth of the cell strains tested. The MFC value was defined as the lowest concentration of TTO that showed no growth of *C. albicans* colonies. The experiment was performed triply. The terpinen-4-ol MIC and MFC values were determined identically as described above. The TTO and terpinen-4-ol MICs were used to calculate the sublethal doses of TTO and terpinen-4-ol used in the following experiments.

2.6. Brief Pretreatment of Candida albicans with 1/4 MIC TTO. For each sample, a tube was prepared containing saline solution with 10% Tween 80 and TTO to a final concentration of 1/4 MIC TTO. A control tube with no TTO was also prepared. Next, the *C. albicans* suspension was added to tubes to obtain a final density of 3×10^3 CFU/mL. The suspensions were then incubated at 35°C for 30 minutes. The samples were then rinsed twice and centrifuged between rinses (3000 ×g, 15 minutes), and the cells were resuspended to a density of 6×10^4 CFU/mL. The suspension was then used to determine the fluconazole MIC and the minimal fungicidal concentration (MFC) of fluconazole. The study was performed in triplicate.

2.7. Determination of the Fluconazole MIC and MFC Values after Brief Pretreatment of Candida albicans with 1/4 MIC TTO. The fluconazole activity against the *C. albicans* strains tested was determined by broth macrodilution using the general dilution standards as described by PN-EN ISO 20776-1:2007 [24]. Serial, parallel dilutions of fluconazole ranging from 256.0 μg/mL to 0.125 μg/mL were prepared in liquid Sabouraud medium, and a control tube without the drug was included. For each of the tubes, the same volume of *C. albicans* cells suspension pretreated with 1/4 MIC TTO was added, and the inoculum was adjusted to a final density of 3×10^3 CFU/mL. After 24 h of incubation at 35°C, the cell growth in each tube was assessed visually. The MIC value was defined as the lowest concentration of fluconazole that resulted in no visible growth of the strains tested. The cells from the tube identified as the MIC, as well as several of the surrounding dilutions, were plated to Sabouraud agar. After 24 h of incubation at 35°C, the *C. albicans* colonies were counted. The MFC value was defined as the lowest concentration of fluconazole that showed no growth of *C. albicans* colonies. The experiment was performed in triplicate. The *C. albicans* strains were classified as exhibiting susceptibility, intermediate susceptibility, or resistance to fluconazole according to CLSI document M27-A3-2008 [22], as described in Section 2.1.

2.8. Prolonged Pretreatment of Candida albicans with Fluconazole and Sublethal Dose of TTO or Terpinen-4-ol. Serial, parallel dilutions of fluconazole ranging from 256.0 μg/mL to 0.125 μg/mL were prepared in liquid Sabouraud culture medium. Two positive controls were included. All tubes contained 10% Tween 80, and TTO was added to each dilution and one of the control tubes to achieve a final concentration of 1/4 MIC TTO. The second control tube contained only the liquid medium. Next, an equal volume of *C. albicans* suspension was added to each tube to a final density of 3×10^3 CFU/mL. All the tubes were incubated at 35°C for 24 h. After incubation, the cell growth in each tube was evaluated visually, and the fluconazole MIC and MFC values were defined, as described previously. The cells from the tube identified as the MIC, as well as several of the surrounding dilutions, were plated to Sabouraud agar. After 24 h of incubation at 35°C, the *C. albicans* colonies were counted, and the fluconazole MFC value was defined. The experiment was performed in triplicate. The prolonged pretreatment of *C. albicans* with fluconazole and terpinen-4-ol was performed identically as described above.

2.9. Statistical Methods. The results are presented as the arithmetic mean and the median. The statistical differences between the mean values were determined by Student's t-test and the Mann-Whitney U test, depending on how well the results correlated with a normal distribution. Values of $P \leq 0.05$ were considered statistically significant. The programme STATISTICA version 10 (StatSoft, Cracow, Poland) was used to perform the statistical analyses.

3. Results

The *Candida albicans* strains tested were resistant to fluconazole and susceptible to low concentrations of TTO. The clinical *C. albicans* strains and *C. albicans* ATCC 10231

Table 2: Susceptibility of fluconazole-resistant clinical *Candida albicans* strains to fluconazole after exposure to 1/4 MIC TTO.

Candida albicans strains ($n = 32$)	Number (%) of *Candida albicans* strains with the indicated susceptibility to fluconazole		
	Resistant	Intermediate susceptibility	Susceptible
Strains not exposed to TTO (control)	32 (100%)	0	0
Strains exposed to 1/4 MIC TTO for 30 minutes	32 (100%)	0	0
Strains exposed to 1/4 MIC TTO and fluconazole for 24 hours	4 (12.5%)	8 (25.0%)	20 (62.5%)

reference strain, tested by the Kirby-Bauer disk diffusion susceptibility test, did not exhibit the zone of inhibition of growth. All the studied *C. albicans* strains were classified as exhibiting resistance to fluconazole. The fluconazole MIC values for the 32 clinical *C. albicans* strains ranged from 64.0 μg/mL to 256.0 μg/mL (average = 244.0 ± 47.22 μg/mL). The most common values were 256.0 μg/mL (30 strains) and 64.0 μg/mL (2 strains). For *C. albicans* ATCC 10231 reference strain the fluconazole MIC was 256.0 μg/mL.

The TTO MICs for the 32 clinical *C. albicans* strains ranged from 0.06% to 0.5% (average = 0.19 ± 0.09%). The most common values were 0.125% (15 strains) and 0.25% (15 strains). The TTO MICs of the two remaining strains were 0.06% and 0.5%. For *C. albicans* ATCC 10231 reference strain, the TTO MIC was 0.125%. These results indicate that the *C. albicans* strains tested did not exhibit any cross-resistance to TTO and fluconazole. The TTO MIC values were used to calculate the sublethal doses (1/4 MIC TTO) used in the rest of the study.

The brief pretreatment of 32 clinical *C. albicans* strains and of *C. albicans* ATCC 10231 reference strain with 1/4 MIC TTO did not change the fluconazole MIC and MFC values. Exposing *C. albicans* strains to 1/4 MIC TTO and fluconazole for 24 hours (prolonged pretreatment) significantly increased susceptibility yeast strains to fluconazole. Out of 32 fluconazole-resistant *C. albicans* clinical strains, 28 strains (87.5%) exhibited then high or intermediate susceptibility to fluconazole (Table 2).

Exposure of fluconazole-resistant *C. albicans* strains for 24 h to 1/4 MIC TTO and fluconazole enhanced fluconazole activity against these strains. Overall, 62.5% of isolates were classified as susceptible, 25.0% exhibited intermediate susceptibility, and 12.5% were resistant. For all of the tested clinical strains, the average fluconazole MIC decreased from 244.0 μg/mL to 38.46 μg/mL after this prolonged pretreatment, and the average fluconazole MFC decreased from 254.67 μg/mL to 66.62 μg/mL (Table 3). The MIC and MFC values for the susceptible strains ($n = 20$) and strains with intermediate susceptibility ($n = 8$) were statistically low compared to the analogous values obtained for the control sample and for the samples that were only briefly pretreated with TTO. For the group of susceptible isolates, the fluconazole MIC decreased to an average of 0.52 μg/mL, and the fluconazole MFC decreased to an average of 4.25 μg/mL. Prolonged pretreatment of *Candida albicans* ATCC 10231 standard strain with 1/4 MIC TTO and fluconazole did not increase the susceptibility of this strain to fluconazole, like the four fluconazole-resistant clinical *C. albicans* strains studied.

Terpinen-4-ol, the main bioactive component present in TTO, strongly enhanced fluconazole activity against fluconazole-resistant *C. albicans* strains. The terpinen-4-ol MICs for clinical *C. albicans* strains ranged from 0.06% to 0.25% (average = 0.11 ± 0.09%). For *C. albicans* ATCC 10231 standard strain, the terpinen-4-ol MIC was 0.06%. The *C. albicans* strains tested did not exhibit any cross-resistance to terpinen-4-ol and fluconazole. Exposure of fluconazole-resistant clinical and standard *C. albicans* strains for 24 h to fluconazole and sublethal doses (1/4 MIC) of terpinen-4-ol strongly enhanced fluconazole activity against these strains, and all of *C. albicans* isolates were classified as susceptible (fluconazole MIC decreased to 0.125 μg/mL). We summed up the results of this study, and the most important data are presented in a table form (Table 4).

4. Discussion

TTO is the most commonly used essential oil for its antibacterial and antifungal properties [3, 25]. In this study, we evaluated the change in fluconazole activity *in vitro* against fluconazole-resistant clinical *Candida albicans* strains exposed to the sublethal concentrations of TTO or terpinen-4-ol, the main bioactive component of TTO. The earlier *in vitro* studies of the sensitivity of *Candida* spp. to TTO have shown that TTO is highly active against these microbes, as well as azole-resistant strains, for which the TTO MICs ranged from 0.25% to 0.5% [14, 26]. For the *C. albicans* strains that were resistant to both fluconazole and itraconazole, the TTO MICs ranged from 0.25 to 1.0%, the TTO MIC_{50} was 0.5%, and the TTO MIC_{90} was 1% [27]. Another study showed that three fluconazole-resistant clinical *C. albicans* strains had very low TTO MICs (0.15% for two strains and 0.07% for the third strain) [15].

The experiments performed in this study confirm the results from previously published studies in that all of the tested fluconazole-resistant *C. albicans* strains were sensitive to TTO [14, 15, 28, 29]. The determined TTO MICs were low, ranging from 0.06% to 0.5%. The TTO antimicrobial activity is attributed mainly to terpinen-4-ol, the main bioactive component present in TTO [3, 14]. The determined MIC values for terpinen-4-ol were very low, ranging from 0.06% to 0.25%. Our study and other studies show that *C. albicans* does not exhibit cross-resistance to TTO and azole agents [14, 15, 26, 27]. Clinical resistance to TTO has not been reported. Multicomponent nature of TTO may reduce the potential for resistance to occur spontaneously, and multiple simultaneous mutations may be required to overcome all of

Table 3: Fluconazole MIC (a) and MFC (b) values (μg/mL) for fluconazole-resistant *Candida albicans* clinical strains after their exposure to 1/4 MIC TTO.

(a) Fluconazole MIC values (μg/mL)

	C. albicans ($n = 32$)[a]			*C. albicans* ($n = 20$)[b]			*C. albicans* ($n = 8$)[c]		
	Control	Brief pretreatment with TTO	Prolonged pretreatment with TTO and fluconazole	Control	Brief pretreatment with TTO	Prolonged pretreatment with TTO and fluconazole	Control	Brief pretreatment with TTO	Prolonged pretreatment with TTO and fluconazole
Range of MICs	64.0–256.0	64.0–256.0	0.125–256.0	256.0-256.0	256.0-256.0	0.125–2.67	64.0–256.0	64.0–256.0	12.0–42.67
Average MIC	244.0 ± 47.22	244.0 ± 47.22	38.46 ± 84.35	256.0 ± 0.0	256.0 ± 0.0	0.52 ± 0.56	208.0 ± 88.88	208.0 ± 88.88	24.54 ± 11.54
P[d]			$P < 0.0001$[e] $P < 0.0001$[f]			$P < 0.0001$[e] $P < 0.0001$[f]			$P < 0.0002$[e] $P < 0.0002$[f]

(b) Fluconazole MFC values (μg/mL)

	C. albicans ($n = 32$)[a]			*C. albicans* ($n = 20$)[b]			*C. albicans* ($n = 8$)[c]		
	Control	Brief pretreatment with TTO	Prolonged pretreatment with TTO and fluconazole	Control	Brief pretreatment with TTO	Prolonged pretreatment with TTO and fluconazole	Control	Brief pretreatment with TTO	Prolonged pretreatment with TTO and fluconazole
Range of MFCs	213.33–256.0	256.0-256.0	0.17–256.0	256.0-256.0	256.0-256.0	0.17–23.33	213.33–256.0	256.0-256.0	14.67–213.33
Average MFC	254.48 ± 7.54	256.0 ± 0.0	66.62 ± 96.16	256.0 ± 0.0	256.0 ± 0.0	4.25 ± 6.19	250.67 ± 15.08	256.0 ± 0.0	127.83 ± 70.42
P[d]			$P < 0.0001$[e] $P < 0.0001$[f]			$P < 0.0001$[e] $P < 0.0001$[f]			$P < 0.0003$[e] $P < 0.0002$[f]

[a]All 32 tested fluconazole-resistant clinical *Candida albicans* strains.
[b]Fluconazole-resistant clinical *Candida albicans* strains ($n = 20$) that exhibited susceptibility to fluconazole after prolonged pretreatment with TTO.
[c]Fluconazole-resistant clinical *Candida albicans* strains ($n = 8$) that exhibited intermediate susceptibility to fluconazole after prolonged pretreatment with TTO.
P[d]: the level of statistical significance for the average MIC/MFC values.
P[e]: statistical significance compared to the control.
P[f]: statistical significance compared to the group that was pretreated briefly.

Table 4: MIC and MFC values of fluconazole, TTO, terpinen-4-ol, and fluconazole with TTO or terpinen-4-ol, for fluconazole-resistant *Candida albicans* strains.

Reagents	*C. albicans* ATCC 10231		*C. albicans* clinical strains ($n = 32$)			
	MIC	MFC	MIC		MFC	
			Range	Average	Range	Average
Fluconazole (μg/mL)	256.0	256.0	64.0–256.0	244.0 ± 47.22	213.33–256.0	254.48 ± 7.44
TTO (% v/v)	0.125	0.25	0.06–0.5	0.19 ± 0.09	0.125–0.5	0.37 ± 0.13
Fluconazole (μg/mL) with sublethal dose of TTO	256.0	256.0	0.125–256.0	38.46 ± 84.35	0.17–256.0	66.62 ± 96.16
Terpinen-4-ol (% v/v)	0.06	0.125	0.06–0.25	0.11 ± 0.09	0.125–0.5	0.22 ± 0.19
Fluconazole (μg/mL) with sublethal dose of terpinen-4-ol	0.125	0.125	0.125-0.125	0.125 ± 0.0	0.125–1.0	0.38 ± 0.42

the antimicrobial actions of each of the components [3]. Thus, TTO can be used as a topical antiseptic to effectively treat superficial mycoses caused by fluconazole-resistant *Candida* spp. and other azole-resistant yeast. Unfortunately, TTO can be potentially toxic when it is ingested in high doses, and, therefore, TTO should not be administrated orally. The acute oral toxicity of TTO is similar to the oral toxicity of other common essential oils, for example, such as eucalyptus oil [30, 31]. The lipophilic nature of TTO, which enables it to penetrate the outer layers of skin, potentiates not only the antiseptic actions but also the possibility of TTO toxicity due to dermal absorption. TTO can cause skin irritation at higher concentrations and may cause allergic reactions in predisposed individuals [3, 31, 32]. Zhang and Robertson observed ototoxic effect of 100% TTO [33]. The toxicity of TTO is dose-dependent, and the majority of adverse events can be avoided through the use of TTO in a diluted form [31]. TTO is not mutagenic or genotoxic [34, 35].

There is increasing interest not only in the activity of natural substances against resistant microbes but also in the synergistic interactions between these substances and conventional drugs [19, 20, 36–38]. Fluconazole is one of the azole antifungal agents widely used for both prophylaxis and therapy of *Candida* infections [39–41]. In this study, we explored changes in the activity of fluconazole against fluconazole-resistant *C. albicans* strains after exposure to sublethal concentrations of TTO or terpinen-4-ol. We used exclusively fluconazole-resistant strains because identifying synergistic treatments for these strains would be especially important. We tested sublethal concentrations of TTO and terpinen-4-ol because we expected concentrations lower than the MIC to weaken the cell structure without killing the cells, facilitating the activity of fluconazole and consequently inhibiting *C. albicans* resistance to fluconazole. Our results show that brief (0.5 h) exposure of fluconazole-resistant *C. albicans* strains to sublethal concentration of TTO (1/4 MIC TTO) had no influence on the antifungal activity of fluconazole. However, exposing *C. albicans* cells to sublethal concentration of TTO and then treating them with fluconazole inhibited the resistance to fluconazole in 87.5% of the tested strains. These results suggest that there is a synergistic interaction between fluconazole and TTO against fluconazole-resistant *C. albicans*. TTO was used to permeabilise the yeast cell membranes, markedly increasing the susceptibility to fluconazole. The TTO becomes embedded in the lipid bilayer membrane, which disrupts its structure, resulting in increased permeability and impaired physiological function. TTO also inhibits the formation of germ tubes or mycelial conversion in *C. albicans* and inhibits respiration in *C. albicans* in dose-dependent manner [3]. Fungal cells exposed to TTO will eventually rupture. Sublethal concentrations of TTO also weaken *Candida* spp. cells vitality [41, 42]. The mechanism of fluconazole antifungal activity is different. It was demonstrated that fluconazole interferes with the cytochrome P-450-dependent enzyme C-14α-demethylase, which is responsible for production of ergosterol. The disruption of ergosterol synthesis causes structural and functional changes in the fungal cell membrane, which predispose the fungus cells to damage. Inhibition of cytochrome *c* oxidative and peroxidative enzymes is an additional antifungal activity of fluconazole [39]. Several mechanisms have been described for fluconazole resistance in *C. albicans* isolates: increased production of lanosterol 14α-demethylase encoded by *ERG11* gene and decreases in the affinity of lanosterol 14α-demethylase for fluconazole because of mutations in *ERG11* gene and a defect in Δ5-6 desaturase encoded by *ERG3* gene causing loss of function in the ergosterol pathway. The other mechanism of fluconazole resistance in *C. albicans* is the active transport of drugs across the plasma membrane by "efflux pumps," which requires the expression of the *CDR1/2* and *MDR1* genes [39, 43–47]. TTO-induced cell membrane damage can disrupt the function of "efflux pumps," thus making the fungal cell more susceptible to fluconazole [48, 49].

Our data show that there is a synergistic effect *in vitro* of sublethal concentrations of TTO and fluconazole against fluconazole-resistant *C. albicans* strains. However, the fluconazole-resistant *C. albicans* ATCC 10231 standard strain and four clinical *C. albicans* strains did not increase the susceptibility to fluconazole. The differences in mechanisms of resistance of these strains to fluconazole were probable cause of this effect. In our *in vitro* study the TTO main component terpinen-4-ol was more active than TTO and strongly enhanced fluconazole activity against all studied fluconazole-resistant *C. albicans* strains. Mondello et al. [14] as well as

Ninomiya et al. [50] observed that *in vivo* TTO and terpinen-4-ol were similarly effective against candidiasis caused by azole-resistant *C. albicans*. The mechanisms underlying the synergy between fluconazole and TTO did not elucidate. Yu et al. [51] confirmed the synergism between fluconazole and triclosan against clinical isolates of fluconazole-resistant *C. albicans*. Liu et al. [52] observed synergistic effect between fluconazole and glabridin against *C. albicans* related to the effect of glabridin on cell envelope. Ahmad et al. [53] described synergistic activity of thymol and carvacrol with fluconazole against *Candida* isolates. Both monoterpenes inhibited efflux by 70–90% showing their high potency to block drug transporter pumps.

Previous studies also have evaluated the activity of TTO against various microorganisms in combination with other antimicrobial substances. A synergistic effect was observed for itraconazole and TTO in a thermosensitive gel used to treat vaginal candidiasis [26]. Synergistic effects have also been observed between essential oils and ciprofloxacin, gentamicin, cefixime, and pristinamycin [20]. In a disc diffusion test using *C. albicans, C. glabrata, C. tropicalis, C. krusei, C. guilliermondii*, and *C. parapsilosis*, larger growth inhibition zones occurred around discs impregnated with TTO and amphotericin B than around discs containing only TTO [17]. In a study of *Staphylococcus aureus*, larger zones of growth inhibition occurred around discs impregnated with TTO and other essential oils compared to discs impregnated with TTO only [54].

The synergistic action of antimicrobial substances has also been shown using *time-kill* curves. The short pretreatment of *Pseudomonas aeruginosa* with a substance that disrupts the cytoplasmic membrane (carbonyl cyanide m-chlorophenylhydrazone, polymyxin B nonapeptide, or ethylenediaminetetraacetic acid) enhanced the bactericidal activity of TTO, as demonstrated by the increased speed of microbe killing in the *time-kill* curves [55, 56]. However, in a study using the E-test method, *Escherichia coli, Salmonella enteritidis, Salmonella typhimurium, Staphylococcus aureus*, and coagulase-negative staphylococci (CoNS) exposed to sublethal concentrations of TTO for 72 hours exhibited increased resistance to gentamicin, streptomycin, chloramphenicol, tetracycline, erythromycin, trimethoprim, ampicillin, fusidic acid, mupirocin, linezolid, and vancomycin [57, 58]. Increased antimicrobial activity was observed when essential oils were combined with their isolated components (e.g., terpinen-4-ol from *Melaleuca alternifolia*) [59] and when TTO was combined with silver ions [60, 61].

The fractional inhibition concentration (FIC) index, also referred to as the FICI, is used to determine whether two substances are synergistic or antagonistic. FIC values can be interpreted differently, however, in general, an FIC index lower than 0.5 indicates synergism and an FIC index higher than 4 indicates antagonism [18, 19, 38, 59]. The FIC index value for TTO and tobramycin was 0.37 for *Escherichia coli* and 0.62 for *Staphylococcus aureus*, indicating that these two substances are synergistic [19]. A minor synergistic effect was observed when treating *Candida albicans* with TTO and amphotericin B and *Klebsiella pneumoniae* with TTO and ciprofloxacin. TTO and ciprofloxacin exhibit antagonistic effects against *Staphylococcus aureus* [18]. There is no synergistic effect between TTO and lysostaphin, mupirocin, gentamicin, or vancomycin against methicillin-resistant *Staphylococcus aureus* strains. In fact, the FIC index indicated that TTO and vancomycin are antagonistic [38].

The results of this study and other previous studies demonstrate that combining natural substances such as TTO and conventional drugs such as fluconazole may help treat difficult yeast infections. However, additional *in vitro* studies are needed to identify the antimicrobial activity of natural medicinal substances and detect synergistic interactions with commonly used antimicrobial agents.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Combating Pathogenic Microorganisms Using Plant-Derived Antimicrobials: A Minireview of the Mechanistic Basis

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The emergence of antibiotic resistance in pathogenic bacteria has led to renewed interest in exploring the potential of plant-derived antimicrobials (PDAs) as an alternative therapeutic strategy to combat microbial infections. Historically, plant extracts have been used as a safe, effective, and natural remedy for ailments and diseases in traditional medicine. Extensive research in the last two decades has identified a plethora of PDAs with a wide spectrum of activity against a variety of fungal and bacterial pathogens causing infections in humans and animals. Active components of many plant extracts have been characterized and are commercially available; however, research delineating the mechanistic basis of their antimicrobial action is scanty. This review highlights the potential of various plant-derived compounds to control pathogenic bacteria, especially the diverse effects exerted by plant compounds on various virulence factors that are critical for pathogenicity inside the host. In addition, the potential effect of PDAs on gut microbiota is discussed.

1. Introduction

Human population growth with its global effects on the environment over the past million years has resulted in the emergence of infectious diseases [1, 2]. Development of agriculture further contributed to this, since these infections could only be sustained in large and dense human populations [3]. The discovery of antibiotics during the twentieth century coupled with significant advances in antimicrobial drug development improved human health through improved treatment of infections [4, 5]. However, prolonged use of antibiotics led to bacterial adaptation, resulting in the development of multidrug resistance in bacteria [2, 5–8]. This has significantly limited the efficacy of antibiotics, warranting alternative strategies to combat microbial infections.

The persistence of bacteria in the environment and their interaction with humans is central to most infections and illnesses. Bacterial illnesses are orchestrated by means of an array of virulence factors that facilitate various aspects of their pathophysiology critical for disease in the host [9]. These include adhesins and membrane proteins that mediate bacterial attachment, colonization, and invasion of host cells. In addition, microbial toxins cause host tissue damage, and bacterial cell wall components such as capsular polysaccharide confer resistance against host immune system [10, 11]. Biofilm formation and spore forming capacity are additional virulence factors that help in the persistence of pathogens in harsh environmental conditions.

Since ancient times, plants have played a critical role in the development and well-being of human civilization. A plethora of plant products have been used as food preservatives, flavor enhancers, and dietary supplements to prevent food spoilage and maintain human health. In addition, plant extracts have been widely used in herbal medicine, both prophylactically and therapeutically for controlling diseases. The antimicrobial activity of several plant-derived compounds has been previously reported [12–23], and a wide array of active components have been identified [24]. A majority of these compounds are secondary metabolites and are produced as a result of reciprocal interactions between plants, microbes, and animals [25]. These compounds do not appear to play a direct role in plant physiology [26]; however they are critical for enhancing plant fitness and defense against predation [27]. The production of secondary

metabolites is often restricted to a limited set of species within a phylogenetic group as compared to primary metabolites (amino acids, polysaccharides, proteins, and lipids), which are widespread in the plant kingdom [28]. Also, they are generated only during a specific developmental period of plant growth at micro- to submicromolar concentration [28, 29].

The primary advantage of using plant-derived antimicrobials (PDAs) for therapeutic purposes is that they do not exhibit the side effects often associated with use of synthetic chemicals [30]. In addition, to the best of our knowledge, no reports of antimicrobial resistance to these phytochemicals have been documented, probably due to their multiple mechanisms of action which potentially prevent the selection of resistant strains of bacteria. The marked antimicrobial effect, nontoxic nature, and affordability of these compounds have formed the basis for their wide use as growth promoters in the livestock and poultry industry, effective antimicrobials and disinfectants in the food industry, components of herbal therapy in veterinary medicine, and source for development of novel antibiotics in pharmaceutics.

The antimicrobial properties of various plant compounds that target cellular viability of bacteria have been adequately discussed previously [12, 31–33], but very few reviews have highlighted the effects of these compounds in modulating various aspects of bacterial virulence, critical for pathogenesis in the host. In this review, we have focused on a wide array of PDAs, with special emphasis on the diverse biological effects exerted by these compounds on bacterial virulence. The important classes of plant compounds and selected antimicrobial mechanisms have been discussed.

2. Plant-Derived Antimicrobials

Most plant-derived compounds are produced as secondary metabolites and can be classified based on their chemical structure, which also influences their antimicrobial property (Table 1). The major groups of phytochemicals are presented here.

2.1. Phenolics and Polyphenols. These are a diverse group of aromatic secondary metabolites involved in plant defense. They consist of flavonoids, quinones, tannins, and coumarins [33–35].

2.1.1. Flavonoids. Flavonoids are pigmented compounds found in fruits and flowers of plants and mainly consist of flavone, flavanones, flavanols, and anthocyanidins [34, 35]. They facilitate pollination by acting as chemoattractants for insects, modulate plant physiology by signaling to beneficial microbiota in rhizosphere, and protect plants against predation due to their antimicrobial nature [36]. The marked antimicrobial property of flavonoids against a variety of bacterial [37–39] and fungal pathogens [40] is mediated by their action on the microbial cell membranes [41]. They interact with membrane proteins present on bacterial cell wall leading to increased membrane permeability and disruption. Catechins belonging to this group exhibit inhibitory activity against both Gram-positive and Gram-negative organisms [42].

2.1.2. Quinones. Quinones are organic compounds consisting of aromatic rings with two ketone substitutions. Quinones are known to complex irreversibly with nucleophilic amino acids in protein, often leading to their inactivation and loss of function [43]. The major targets in the microbial cell include surface-exposed adhesin proteins, cell wall polypeptides, and membrane-bound enzymes [44]. Quinone such as anthraquinone from *Cassia italica* was found to be bacteriostatic against pathogenic bacteria such as *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal against *Burkholderia pseudomallei* [45].

2.1.3. Tannins. Tannins are a group of water-soluble oligomeric and polymeric polyphenolic compounds, with significant astringent properties. They are present in the majority of plant parts, including bark, leave, fruits, and roots [46]. They are widely used in leather industry, in food industry, and, as antimicrobials, in healthcare industry [47]. The mode of antimicrobial action of tannins is potentially due to inactivation of microbial adhesins and cell envelope transport proteins [47–49]. Besides their efficacy against bacteria, tannins have been reported to be inhibitory on fungi and yeasts [46, 50].

2.1.4. Coumarins. Coumarins are a group of aromatic benzopyrones consisting of fused benzene and alpha pyrone rings [51]. Approximately, 1300 coumarins have been identified since 1996 [44] and are used as antithrombotic and anti-inflammatory compounds [52]. Recently, coumarins such as scopoletin and chalcones have been isolated as antitubercular constituents of the plant *Fatoua pilosa* [53]. In addition, phytoalexins, which are hydroxylated derivatives of coumarins, which are produced in plants in response to microbial infections, have been found to exert marked antifungal activity.

2.2. Alkaloids. Alkaloids are a group of heterocyclic nitrogenous compounds with broad antimicrobial activity. Morphine and codeine are the oldest known compounds in this group [54]. Diterpenoid alkaloids, commonly isolated from Ranunculaceae or buttercup family of plants, are found to possess antimicrobial properties [55]. The mechanism of action of aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA thereby resulting in impaired cell division and cell death [33].

2.3. Terpenoids. Terpenes represent one of the largest and most diverse groups of secondary metabolites consisting of five carbon isoprene structural units linked in various configurations [43]. The action of terpene cyclase enzymes along with subsequent oxidation and structural rearrangement imparts a rich diversity to the group with over 55,000 members isolated so far [56]. The major groups

Table 1: Chemical structure, examples, and antimicrobial spectrum of major groups of plant-derived antimicrobials.

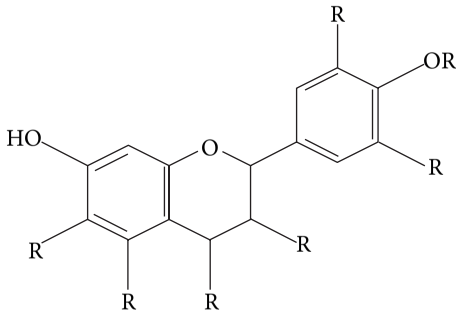
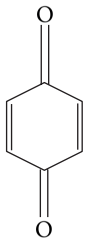
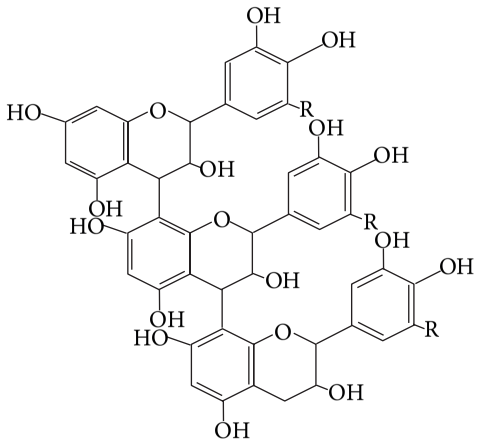
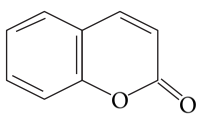
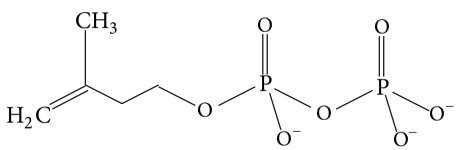
Plant-derived antimicrobials	Chemical structure	Examples*	Selected antimicrobial spectrum	References
		Phenolics and polyphenols		
Flavonoids	 (Beecher, 2003) [263]	Flavones(apigenin, chrysin, rutin) Flavanones(naringenin, fisetin) Catechins(catechin, epicatechin) Anthocyanins(cyanidin, petunidin)	*Listeria monocytogenes* *Staphylococcus aureus* *Escherichia coli* O157:H7 *Salmonella enterica* *Vibrio cholera* *Pseudomonas aeruginosa* *Acinetobacter baumannii* *Klebsiella pneumonia* *Aspergillus flavus* *Penicillium* sp. *Cladosporium* sp.	Beecher, 2003 [263]; Chye and Hoh, 2007 [264]; Orhan et al., 2010 [265]; Rattanachaikunsopon and Phumkhachorn, 2010 [266]; Ozçelik et al., 2008 [267]; Cushnie and Lamb, 2005 [268]
Quinones	 (Cowan, 1999) [34]	Anthraquinone Benzoquinone Naphthoquinone Plastoquinone Pyrroloquinoline quinone	*Staphylococcus aureus* *Pseudomonas aeruginosa* *Bacillus subtilis* *Cryptococcus neoformans*	Ignacimuthu et al., 2009 [269]; Singh et al., 2006 [270]; Cowan, 1999 [34]
Tannins	 (Scalbert, 1991) [46]	Tannic acid Gallic acid Proanthocyanidins	*Staphylococcus aureus* *Bacillus cereus* *Listeria monocytogenes* *Salmonella enterica* *Campylobacter jejuni*	Engels et al., 2009 [271]; Scalbert, 1991 [46]

Table 1: Continued.

Plant-derived antimicrobials	Chemical structure	Examples*	Selected antimicrobial spectrum	References
Coumarins	 (Cowan, 1999) [34]	Ammoresinol Ostruthin Anthogenol Agasyllin	*Staphylococcus aureus* *Listeria monocytogenes* *Escherichia coli* O157:H7 *Salmonella* Typhimurium *Salmonella* Enteritidis *Vibrio parahaemolyticus*	Basile et al., 2009 [272]; Ulate-Rodríguez et al., 1997 [273]; Venugopala et al., 2013 [274]; Saleem et al., 2010 [275]; Cowan, 1999 [34]
		Alkaloids		
Terpenoids	 (Ulubelen, 2003) [276]	Carotenoids Terpinene Isopentenyl pyrophosphate	*Staphylococcus aureus* *Pseudomonas aeruginosa* *Vibrio cholera* *Salmonella typhi*	Ulubelen, 2003 [276]; Bach et al., 2011 [277]; Batista et al., 1994 [278]; Mathabe et al., 2008 [279]
Lectins and polypeptides	 Concanavalin A (Hardman and Ainsworth, 1972) [280]	Concanavalin A Wheat germ agglutinin (WGA) *Aleuria aurantia* lectin (AAL)	*Staphylococcus aureus* *Bacillus subtilis* *Escherichia coli* *Pseudomonas aeruginosa* *Candida albicans*	Hardman and Ainsworth, 1972 [280]; Petnual et al., 2010 [281]; Kheeree et al., 2010 [282]; Peumans and Van Damme, 1995 [283]

*The examples discussed in the table are only representative for the group. For an extended list of examples of each group, the readers are requested to peruse review articles in the References section and other sources.

consist of diterpenes, triterpenes, tetraterpenes as well as hemiterpenes, and sesquiterpenes [44]. When the compounds contain additional elements, frequently oxygen, they are termed terpenoids. Compounds such as menthol and camphor (monoterpenes), farnesol and artemisinin (sesquiterpenoids) are terpenoids synthesized from acetate units and share their origins and chemical properties with fatty acids [34]. Sesquiterpenoids are known to exhibit bactericidal activity against Gram-positive bacteria, including *M. tuberculosis* [35, 53]. The mechanism of antimicrobial action of terpenoids is not clearly defined, but it is attributed to membrane disruption in microorganisms [57].

2.4. Lectins and Polypeptides. In 1942, it was first reported that peptides could be inhibitory on microorganisms [58]. Although recent interest has chiefly focused on studying anti-HIV peptides and lectins, the inhibition of bacteria and fungi by these molecules has long been known [59]. The mechanism of action of peptides and lectins is presumed to be due to the formation of ion channels in the microbial membrane [60] or due to competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors [61]. Lectin molecules are larger and include mannose-specific molecules obtained from an array of plants [62]. Lectins such as MAP30 from bitter melon [63], GAP31 from *Gelonium multiflorum* [64], and jacalin [65] are inhibitory on viral proliferation, including HIV and cytomegalovirus by potentially inhibiting viral interaction with critical host cell components. Due to the versatile antifungal, antibacterial, and antiviral functions delivered by these compounds, it is advantageous to investigate in depth their exact mechanism of action.

3. Critical Antimicrobial Properties of PDAs

3.1. Membrane Disruption and Impaired Cellular Metabolism. Although the exact mechanisms by which PDAs exert their antimicrobial action are not well defined, several potential methods have been reported. These include disruption of bacterial cell membrane leading to loss of membrane potential, impaired ATP production, and leakage of intracellular contents [66, 67]. Furthermore, chelation of metal ions, inhibition of membrane-bound ATPase, and altered membrane permeability brought about by PDAs affect normal physiology of bacteria and cause cell death [12, 32, 34, 68–71]. Plant-derived antimicrobials such as carvacrol, thymol, eugenol, and catechins act by disruption of cell membrane, followed by the release of cell contents and loss of ATP [12, 70, 72, 73]. However, cinnamaldehyde has been reported to result in the depletion of intracellular ATP by inhibiting ATPase dependent energy metabolism along with the inhibition of glucose uptake and utilization [32, 69, 70, 74]. Lysis of cell wall has also been documented in bacteria exposed to phenolic compounds [32, 75].

3.2. Antibiofilm Activity. Bacterial biofilms are surface-associated microbial communities enclosed in a self-generated exopolysaccharide matrix [76, 77]. They are a cause of major concern, especially in the food industry and hospital environments due to their recalcitrance to commonly used antimicrobials and disinfectants [78–82], thereby resulting in human illnesses, including endocarditis, cystic fibrosis, and indwelling device-mediated infections [83].

Extensive research exploring the potential of alternative strategies for microbial biofilm control has highlighted the efficacy of several PDAs in controlling biofilm formation in major pathogens, including *Listeria monocytogenes* [84], *Staphylococcus aureus* [85–89], *Pseudomonas aeruginosa* [90, 91], *Escherichia coli* [92, 93], and *Klebsiella pneumoniae* [94]. *Trans*-cinnamaldehyde, an aromatic aldehyde obtained from bark of cinnamon trees, was found to inhibit biofilm formation and inactivate mature biofilm of *Cronobacter sakazakii* on feeding bottle coupons, stainless steel surfaces, and uropathogenic *E. coli* on urinary catheters [95, 96]. Similarly, terpenes such as carvacrol, thymol, and geraniol and essential oils of *Cymbopogon citratus* and *Syzygium aromaticum* were found to exhibit marked antibiofilm activity against both fungal [97–99] and bacterial biofilms [86, 87, 100] encountered in food processing environments and biomedical settings.

As observed in antibiotics [101–103], PDAs at subinhibitory concentrations (SICs, concentrations not inhibiting the growth of microbes) are reported to modulate bacterial gene transcription [84, 96, 104–106], which could be a contributing factor to their antibiofilm property. In a study by Amalaradjou and Venkitanarayanan [96], *trans*-cinnamaldehyde was found to modulate the transcription of genes critical for biofilm formation, motility, attachment, and quorum sensing in *C. sakazakii*. Similarly, Brackman and coworkers [107] observed the inhibitory effects of *trans*-cinnamaldehyde on biofilms of *Vibrio* spp. These authors found that *trans*-cinnamaldehyde was able to mitigate autoinducer 2 based quorum sensing and biofilm formation without inhibiting bacterial growth, probably due to its effect on gene transcription. Similar transcription modulatory effects have been observed in other major pathogens such as *Salmonella* [108] and *P. aeruginosa* [109] following exposure to PDAs. Since quorum sensing is one of the key processes involved in cell-to-cell communication and social behavior in microbes, the aforementioned reports could provide new insights into the development of novel therapeutics targeting key physiological processes in microbes.

Despite exhibiting effective antibiofilm properties, the use of PDAs has been thwarted by various confounding factors such as the requirement for more contact time, difficulty in administration, and organoleptic considerations when used on food contact surfaces. Therefore several researchers have investigated the efficacy of new delivery methods such as biodegradable polymers, micellar encapsulation, and polymeric films to potentiate the antibiofilm action of plant compounds. For example, micellar encapsulated eugenol and carvacrol were found to inhibit and inactivate *L. monocytogenes* and *E. coli* O157:H7 colony biofilms [110]. Similarly, reduced biofilm formation was observed on polymeric films containing carvacrol and cinnamaldehyde [88]. Nanoparticle-based drug delivery systems have been

more frequently investigated for potentiating the antimicrobial efficacies of drugs [111]. The major advantages of nanoparticle-based drug delivery include sustained release, higher stability, and enhanced interaction of active ingredients with pathogens at their molecular level [112], thereby potentiating their antimicrobial action. The antimicrobial potential of nanoparticles containing plant-derived compounds such as *trans*-cinnamaldehyde, eugenol [113], and resveratrol [114] or essential oil of *Nigella sativa* [115] and garlic [116] has been recently investigated. These researchers found that nanoparticle formulations were more stable and highly effective in inhibiting the growth of major bacterial pathogens, including *Salmonella* and *Listeria* spp. Currently research is underway to investigate the potential of various nanoparticle-based delivery systems containing PDAs [117] for eradicating biofilms from hospital devices [118] and food processing environments [119]. In a recent study, Iannitelli and coworkers [117] prepared carvacrol encapsulated poly (DL-lactide-co-glycolide) (PLGA) nanoparticles and found that they were significantly effective in inactivating microbial biofilms of *Staphylococcus epidermidis*. In another study, PLGA containing cinnamaldehyde and carvacrol coatings were found to inhibit biofilms of *E. coli*, *S. aureus*, and *P. aeruginosa* [120].

3.3. Inhibiting Bacterial Capsule Production. Polysaccharide capsule is an important virulence determinant [121, 122] in many pathogenic bacteria, including *Streptococcus pneumonia* [123–125], *S. aureus* [126], *K. pneumoniae* [127], and *Bacillus anthracis* [128]. It protects bacteria from phagocytosis [123], thereby enhancing bacterial survival inside the host [126]. In addition, the presence of a capsule enhances bacterial adhesion and biofilm formation [129] in the environment [10, 130]. Bacterial capsule has also been observed to cause pathology in plants. For example, capsular polysaccharide of *Pseudomonas solanacearum* was found to occlude xylem vessels resulting in plant death [131]. Since salicylic acid is a signal molecule involved in plant defense [132], several researchers have investigated the effect of salicylic acid [133] or its derivatives such as sodium salicylate [134], bismuth subsalicylate [135], and bismuth dimercaprol [136] on modulating bacterial capsule production. These researchers found that salicylic acid or its derivatives were effective in significantly reducing capsule production by modulating the expression of global regulators controlling capsular synthesis in *S. aureus*. Similar inhibitory effects have been observed with sub-MICs and MICs of various antibiotics [137–140]. Thus, plant-derived compounds represent a valuable resource for the development of therapeutics targeting bacterial capsule production.

3.4. Increasing Antibiotic Susceptibility in Drug Resistant Bacteria. As the understanding of antimicrobial resistance mechanisms in pathogens is increasing, multifold strategies to combat infections and reverse bacterial antibiotic resistance are being explored. Many researchers have reported PDAs as potential resistance modulating compounds, in addition to their inherent antimicrobial nature. In a study by Brehm-Stecher and Johnson [141], low concentrations of sesquiterpene such as nerolidol, bisabolol, and apritone increased bacterial sensitivity to multiple antibiotics, including ciprofloxacin, clindamycin, tetracycline, and vancomycin. Similarly, Dickson et al. [142] reported that plant extracts from *Mezoneuron benthamianum*, *Securinega virosa*, and *Microglossa pyrifolia* increased the susceptibility of major drug resistant fungi such as *Trichophyton* spp. and *Microsporum gypseum* and bacteria such as *Salmonella* spp., *Klebsiella* spp., *P. aeruginosa*, and *S. aureus* to norfloxacin. In addition, geraniol (present in essential oil of *Helichrysum italicum*) was found to restore the efficacy of quinolones, chloramphenicol, and β-lactams against multidrug resistant pathogens, including *Acinetobacter baumannii* [143]. Similar synergism was observed between antibiotics and various other medicinal plant extracts, including those of *Camellia sinensis* [144], *Caesalpinia spinosa* [145], oil of *Croton zehntneri* [146], carvacrol [147], and baicalein, the active component derived from *Scutellaria baicalensis* [148]. This modulatory effect of plant compounds is potentially due to the attenuation of three main resistance strategies employed by drug resistant pathogens to survive the action of antibiotics, namely, enzymatic degradation of antibiotics [149], alteration of antibiotic target site [150], and efflux pumps [151]. In addition, recent reports suggest that the combination therapy of antibiotics with PDAs acts through inhibition of multiple targets in various pathways critical for the normal functioning or virulence of the bacterial cell.

Generation of β-lactamase enzymes is an example of microbial strategy that is responsible for resistance to β-lactam antibiotics [152]. Several plant compounds have been identified with inhibitory activity towards β-lactamases [153]. Gangoué-Piéboji and coworkers [154] screened medicinal plants from Cameroon and found that extracts from *Garcinia lucida* and *Bridelia micrantha* exhibited significant inhibitory activity towards β-lactamases. Similarly, epigallocatechin gallate was found to inhibit penicillinase activity, thus increasing the sensitivity of *S. aureus* to penicillin [155] and augmenting the antimicrobial properties of ampicillin and sulbactam against Methicillin resistant *S. aureus* (MRSA).

Numerous studies in the past two decades have shown the efficacy of PDAs as potent efflux pump inhibitors against Gram-positive microbes [156–158]. Gram-negative bacteria pose an even greater challenge owing to the presence of potent efflux pumps, especially, AcrAB-TolC pumps [159]. In a recent investigation, five PDAs, namely, *trans*-cinnamaldehyde, β-resorcylic acid, carvacrol, thymol, and eugenol, or their combinations were found to increase the sensitivity of *Salmonella enterica* serotype Typhimurium phage type DT104 to five antibiotics [160]. Since the mechanism of antimicrobial resistance in *Salmonella* Typhimurium DT104 is mainly mediated by interaction between specific transporters of antibiotics and AcrAB-TolC efflux pump, the aforementioned plant compounds could be acting through modulation of these efflux pumps to increase the antibiotic sensitivity of the pathogen [161].

3.5. Attenuating Bacterial Virulence. The pathophysiology of microbial infection in a host is mediated by multiple virulence factors, which are expressed at different stages of

infection to cause the disease. Reducing production of these virulence factors could control infections in humans. With major advancement in the fields of comparative genomics, transcriptomics, and proteomics, a better understanding of the key virulence mechanisms of pathogenic bacteria has been achieved. Thus, virulence factors are the prime targets for therapeutic interventions and vaccine development [11]. Quorum sensing controls the expression of genes encoding various virulence factors in many microorganisms [162, 163]. A growing body of evidence suggests that plants produce antiquorum sensing compounds that interfere with cell-to-cell communication, thereby downregulating the expression of virulence genes in microbes [164–166]. We previously reported that *trans*-cinnamaldehyde reduced the expression of *luxR*, which codes for transcriptional regulator of quorum sensing in *C. sakazakii* [96]. Similarly, Bodini and coworkers found that garlic extract and p-coumaric acid inhibited quorum sensing in quorum sensing reporter strains, indicating that plant compounds potentially modulate virulence by affecting quorum sensing in microbes.

For the majority of enteric pathogens, adhesion to and invasion of intestinal epithelium are critical for virulence and infection in a host. Specific proteins contribute to adhesion and invasion in various microbes. For example, Inl A and Inl B are surface proteins that facilitate receptor-mediated entry of *L. monocytogenes* in intestinal cells [167]. Several PDAs have been shown to reduce these virulence attributes in major food-borne pathogens such as *L. monocytogenes* [105], uropathogenic *E. coli* [168], and *Salmonella enterica* serovar Enteritidis [104] by downregulating the expression of virulence genes. In addition, reduction in capsule production has been documented in *K. pneumoniae* on exposure to PDAs [169], which affects its virulence and survival inside the host. These results highlight the ability of plant compounds to successfully target virulence factors critical for pathogenicity and pave the way for the development of compounds that target bacterial virulence.

3.6. Reducing Toxin Production. Microbial toxins are chemical compounds critical for virulence and pathogenesis in the host and therefore are prime targets for therapeutic interventions. Microbial toxins include exotoxins (secreted by the bacteria) and endotoxins (released after bacterial lysis), whereas mycotoxins are toxic secondary metabolites produced by fungi with diverse chemical structures and biological activities causing a variety of illnesses in humans. The drugs of choice for treating bacterial infections have been antibiotics; however the use of antibiotics to kill toxigenic microorganisms has several disadvantages such as resistance development [170], disruption of normal microbiota [171], and enhanced pathogenesis due to increased toxin production and cell lysis as observed in *E. coli* O157:H7 [172, 173]. Moreover, toxin-mediated pathogenesis can continue in the host even after bacterial clearance [174]. Therefore, antibiotics in general are contraindicated to treat toxigenic organisms and it is beneficial to employ an alternative approach to counteract the toxin-mediated virulence of pathogens.

In the past, plant extracts and their active molecules have proven effective against bacterial toxins produced by *Vibrio* spp., *S. aureus*, *E. coli*, and fungal toxins from *Aspergillus* spp. For example, a natural plant-derived dihydroisosteviol has been observed to prevent cholera toxin-mediated intestinal fluid secretion [175]. Plant polyphenols such as RG-tannin and apple phenols have been reported to inhibit ADP-ribosyltransferase activity critical for cholera toxin action [176, 177]. These researchers also observed a reduction in the toxin induced fluid accumulation in mouse ileal loops. In a recent study by Yamasaki et al. [178], extracts from spices such as red chilli, sweet fennel, and white pepper were found to substantially inhibit the production of cholera toxin. These researchers found that capsaicin was an important component among the tested fractions and significantly reduced the expression of major virulence genes of *V. cholerae*, including *ctxA*, *tcpA*, and *toxT*. Similarly, eugenol, an essential oil from clove, was observed to significantly reduce the production of *S. aureus* α-hemolysin, enterotoxins (SEA, SEB), and toxic shock syndrome toxin 1 [106]. Transcriptional analysis conducted by these researchers revealed a reduction in the expression of critical virulence genes (*sea, seb, tst, and hla*) involved in various aspects of *S. aureus* toxin production. Similarly, a compound from olive, 4-hydroxytyrosol, was found to successfully inactivate *S. aureus* endotoxin production *in vitro* [179].

Enterohemorrhagic *E. coli* (EHEC) is responsible for causing severe human infections, characterized by hemorrhagic colitis and hemorrhagic uremic syndrome [180]. In a recent study by Doughari and coworkers [181], extracts of *Curtisia dentata* were found to inhibit expression of *vtx1* and *vtx2* genes in EHEC. The extracts from this plant have been traditionally used as an antidiarrheal agent [182]. Similar verotoxin inhibitory activity was observed in other plant extracts such as *Haematoxylon brasiletto* [183], *Limonium californicum* (Boiss.), *Cupressus lusitanica*, *Salvia urica* Epling, and *Jussiaea peruviana* L. [184]. Inactivation of Shiga toxins by antitoxin antibodies [185] and by certain synthetic carbohydrate and peptide compounds designed to compete with the active site of the toxin for receptor sites on cell membranes has also been investigated [186–189]. Quiñones and coworkers [190] found that grape seed and grape pomace extracts exhibited strong anti-Shiga toxin-2 activity and conferred cellular protection against Shiga toxin-2. Likewise, Daio (*Rhei rhizoma*), apple, hop bract, and green tea extracts have been shown previously to inhibit the release of Shiga toxin from *E. coli* O157:H7 [176, 191].

Aflatoxins, produced by *Aspergillus flavus, A. parasiticus, A. nomius, A. tamari, A. bombycis,* and *A. pseudotamarii*, cause both acute and chronic toxicity in humans and animals [192–195]. Common food products associated with mycotoxicosis include peanuts, corn grain, cottonseed [196, 197], chicken meat [198] cheese [199], canned mushrooms [200], raw milk [201, 202], and pork [203, 204]. Several studies have highlighted the efficacy of essential oils in reducing mycotoxin production. Crude aqueous extracts of garlic, carrot, and clove have been shown to exert a significant inhibitory effect on aflatoxin production in rice [205]. Capsanthin and capsaicin, the coloring and pungent ingredients of red chilli (*Capsicum annum*), completely inhibited both the growth and toxin production in *A. flavus* [206]. Mahmoud [207]

studied the effect of several plant essential oils on growth and toxin production of *A. flavus* and found that five essential oils, namely, geraniol, nerol, citronellol, cinnamaldehyde, and thymol, completely suppressed the growth of *A. flavus* and prevented aflatoxin synthesis in a liquid medium. Similarly, curcumin and essential oil from *Curcuma longa* have also been reported to inhibit *A. flavus* toxin production [208]. In another study, cumin and clove oils have been found to exert inhibitory effects on toxin production in *A. parasiticus* [209], wherein aflatoxin production was decreased by 99%. Similar findings have been observed with ochratoxin-producing aspergilli, where essential oil from wild thyme reduced ochratoxin production by more than 60% [210]. In addition, essential oils have been found to inhibit spore germination in toxin producing *Aspergillus* species [211]. In a recent study, Kumar and coworkers [212] demonstrated that amaryllin, a 15-kDa antifungal protein from *Amaryllis belladonna* bulbs, exerts significant inhibitory effect against toxin producing *A. flavus* and *Fusarium oxysporum*. The aforementioned studies collectively suggest that plant polyphenols and other plant compounds are potential agents that can be used to protect humans against toxin-mediated food-borne diseases.

3.7. Beneficial Effects on Host Immune System. Pioneering research has demonstrated the existence of intriguing parallels between plant and animal immune responses against microbial infections. These include recognition of invariant pathogen-associated molecular patterns (PAMPs) [213], apoptosis of infected cells [214, 215], and production of antimicrobial peptides [216, 217]. However, unlike microbe-specific immune response in animals, plants depend on innate immunity of individual cells coupled with signals emanating from the site of infection [28, 218–220] to combat infections. This is mediated by the production of a wide variety of low molecular weight secondary metabolites [26, 221]. A mounting body of evidence suggests that plants extracts, in addition to their role in plant defense, exert immune-modulatory effects in animals [222, 223] and are increasingly being used for treating inflammatory diseases, allergy, and arthritis [224]. For example, tea tree [225, 226] and lavender oils [227] were found to ameliorate allergy symptoms by reducing histamine release [228, 229] and cytokine production [230]. The immune-modulatory effects of many PDAs have been demonstrated in mouse, chicken, and human cell lines [231–233]. Since the majority of the enteric pathogens colonize and invade the gut epithelium, followed by systemic spread via macrophages resulting in infection, the intestinal mucosal immune response (IMIS) is critical for conferring protection against such bacterial infections. A growing body of evidence suggests that PDAs in addition to attenuating bacterial virulence modulate IMIS [224, 234] through both nonspecific inflammatory response and antigen specific adaptive interactions in the intestine, thereby affecting pathogen survival. Plant preparations such as *Eucalyptus* oil [224], babassu mesocarp extract [235], and oil from seeds of *Chenopodium ambrosioides* L. [236] were found to activate the phagocytic activity of macrophages, whereas essential oils from *Petroselinum crispum* [234], *Artemisia iwayomogi* [237], and Jeju plant extract [116] were found to suppress activity of splenocytes and macrophages, indicating that the two oils may act through different mechanisms.

3.8. Beneficial Effects on Gastrointestinal Microflora. The human intestinal tract hosts a vast population of diverse bacterial communities that amount to as many as 10^{12} cells per 1 g of fecal mass in an average human being [238, 239]. The gut microbiota interacts with the host and influences various biological processes [240], including microbial defense [241]. With advances in high throughput sequencing and metagenomics and development of gnotobiotic animals, the ability to explore the variations in gut microbiota composition and their effect on human health has significantly improved [242, 243]. Modulations in dietary components have been associated with fluctuations in the composition of gut microbial population and diversity [244, 245], which in turn affects host's metabolic functions [246] and susceptibility to gastrointestinal bacterial infections [247]. David and coworkers [248] observed that short-term macronutrient variation leads to a change in the gut microbial community structure, with animal protein-based diet increasing the abundance of bile-tolerant microorganisms (*Alistipes*, *Bilophila*, and *Bacteroides*) and reducing the levels of Firmicutes that metabolize dietary plant polysaccharides (*Roseburia*, *Eubacterium rectale*, and *Ruminococcus bromii*). Bailey and group [249] demonstrated that stress exposure disrupted commensal microbial populations in the intestine of mice and led to increased colonization of *Citrobacter rodentium*. These researchers in their subsequent study observed that *Lactobacillus reuteri* attenuated the stress-enhanced severity of *C. rodentium* infection in mice [250]. Interestingly, recent studies have shown that PDAs that are highly bactericidal towards enteric pathogens exert low antimicrobial effect against commensal gut microbiota [251, 252]. Thapa and coworkers [253] found that nerolidol, thymol, eugenol, and geraniol inhibited growth of enteric pathogens such as *E. coli* O157:H7, *Clostridium difficile*, and *S. Enteritidis*. Moreover, the degree of inhibition was more on the pathogens than the commensal bacteria. Since PDAs and probiotics exert their antimicrobial effects by different mechanisms [254], a combinatorial approach using both could be more effective in controlling pathogens as compared to using them separately. However, research investigating their synergistic interactions is scanty. Further research is necessary to comprehensively elucidate the mechanism of action of such dietary interventions and their effect on gut microbiota for designing effective therapies that promote health by targeting diverse microbial communities.

4. Challenges Associated with Using PDAs for Pathogen Control

The efficacy of PDAs in controlling pathogens in the environment, high-risk foods, or their virulence in the host depends on various intrinsic and extrinsic factors. Physiochemical properties of PDAs such as solubility in aqueous solutions, hydrophobicity, biodegradability, and stabilities are major

challenges that thwart their usage as natural biocontrol agents in the environment [32, 255]. In addition, factors such as environmental temperature and atmospheric composition also modulate their antimicrobial efficacy [256]. In food products, the presence of fat [257], carbohydrates [258], and proteins [259] affects the efficacy of PDAs. Moreover, chemical variability in PDAs, originating from differences in extraction protocols [260, 261], affects the antimicrobial efficacy [12]. Another concern for PDAs is their strong aroma, which may modulate the organoleptic property and taste profile of food products. Therefore, careful selection of PDAs based on their chemical composition and effect on sensory attributes of food product is warranted before recommending their usage as food preservatives or direct oral supplements for human consumption [262].

5. Future Directions

With an increasing body of supporting literature, PDAs are now recognized to play a critical role in the development of effective therapeutics, either alone or in combination with conventional antibiotics. However, the major challenges to this include finding compounds with sufficiently lower MICs, low toxicity, and high bioavailability for effective and safe use in humans and animals.

Based on their modes of action, PDAs are classified into three categories, including conventional antimicrobials, multidrug resistance inhibitors, and compounds that target specific or multiple virulence factors in microbes [221]. As new approaches that target specific regulatory pathways and bacterial virulence are becoming the paradigm of antibacterial therapeutics in recent years, characterization of the mechanism of action of these compounds would pave the way for the development of novel drugs that can circumvent antimicrobial resistance and control infectious diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effect of High Hydrostatic Pressure Processing on Microbiological Shelf-Life and Quality of Fruits Pretreated with Ascorbic Acid or $SnCl_2$

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In the current study, the processing conditions required for the inactivation of *Paenibacillus polymyxa* and relevant spoilage microorganisms by high hydrostatic pressure (HHP) treatment on apricot, peach, and pear pieces in sucrose (22°Brix) solution were assessed. Accordingly, the shelf-life was determined by evaluating both the microbiological quality and the sensory characteristics (taste, odor, color, and texture) during refrigerated storage after HHP treatment. The microbiological shelf-life of apricots, peaches, and pears was prolonged in the HHP-treated products in comparison with the untreated ones. In all HHP-treated packages for apricots, peaches, and pears, all populations were below the detection limit of the method (1 log CFU/g) and no growth of microorganisms was observed until the end of storage. Overall, no differences of the L^*, a^*, or b^* value among the untreated and the HHP-treated fruit products were observed up to the time at which the unpressurized product was characterized as spoiled. HHP treatment had no remarkable effect on the firmness of the apricots, peaches, and pears. With regard to the sensory assessment, the panelists marked better scores to HHP-treated products compared to their respective controls, according to taste and total evaluation during storage of fruit products.

1. Introduction

High hydrostatic pressure (HHP) processing is a novel nonthermal method which has revealed great potential in producing microbiologically safer products while maintaining the natural characteristics of the food products [1]. The use of HHP in food preservation has been acknowledged as an alternative method to thermal processes [2]. HHP practical application in the food industry has taken place in the past twenty years [3–5], usually for a range of pressures between 100 and 800 MPa.

One of the principal advantages of the HHP method is the substantial increase in shelf-life and improvement of food safety due to the inactivation affected in the microbial population [1, 5]. HHP destroys vegetative cells and inactivates certain enzymes [6] with an insignificant change in the sensory characteristics [7, 8]. The resistance of microorganisms is variable, depending on the type of organism and the food matrix involved [1]. Spores show higher resistance to inactivation by HHP. *Bacillus* spp. form high-pressure resistant spores and they have been suggested to be used as the target organism in the development of standards for HHP treatments [9, 10]. There are few reports on the behavior of HHP-treated *Bacillus* spp. in foods [11, 12], whereas in all cases a combination of HHP and mild temperature had to be used to accomplish a noteworthy loss of viability.

During the last years, processed fruit products have become well known due to the increased demand of consumers for healthy diets. Fresh-cut fruits have occurred as common snacks in food services [13]. Overall, they have a short shelf-life because of enzymatic browning, tissue softening, and microbial growth [14]. The use of the HHP treatment in such products is an effective tool to enhance the microbiological safety and shelf-life of various types of fruit products and juices [15, 16].

A number of various methods may be used to control the browning reactions in fruit products and extend their shelf-life. In this respect, the most effective process is the heat treatment, but it leads to changes in quality characteristics of the fruit products that are not friendly to the consumer. Browning can also be delayed by the elimination of oxygen and by the use of oxygen-impermeable packaging [14]. An alternative approach to control browning is through the use of additives such as ascorbic acid or $SnCl_2$, which may act by reducing the formed o-quinones back to o-diphenolic compounds, avoiding thus the existence of the secondary pigment formation [14]. Undesirably, when ascorbic acid is oxidized, the o-quinones can amass with browning reactions [17].

In the present study, HHP treatment was applied to preserve fresh-cut apricot, peach, and pear pieces in sucrose solution. To our knowledge, there is limited, if any, information relevant to the potential use of HHP treatment in fresh-cut apricot, peach, or pear pieces preservation. Consequently, the process conditions toward inactivation of *Paenibacillus polymyxa* (a microorganism relevant to the spoilage of fresh-cut fruit pieces) by HHP were optimized for the first time. A shelf-life study of apricot, peach, and pear pieces in sucrose solution HHP treated was assessed, where color, texture, and sensory evaluation were monitored in parallel with the microbiological control during refrigerated storage at 5°C.

2. Materials and Methods

2.1. Preparation of the Apricot, Peach, and Pear Pieces. For the series of experiments, apricots (cultivar Bebekou), peaches (cultivar Andross), and pears (cultivar Williams) were bought in a local market and thoroughly washed with water and 70% ethanol. Consequently, apricots and peaches were dipped for 30 seconds in boiling water containing 2% NaOH to loosen skins, dipped quickly in cold water, and peeled. Then the fruits were cut in half with a sterile knife and the pits were removed. The pear fruits were peeled, cut in half with a sterile knife and the core was removed. Finally, all fruits were cut with a sterile knife into approximately 0.5 cm thick cube pieces.

2.2. Production of Paenibacillus polymyxa Spores. The strain used in this study was the *Paenibacillus polymyxa* DSM 36 (DSMZ, Germany). The strain was revived from a stock culture stored at −80°C, by subculturing twice in 10 mL nutrient broth (LAB014, LAB M), incubated for 48 h at 30°C. The fresh cultures were heat shocked (80°C for 10 min) prior to inoculation on agar plates to allow uniform sporulation. Subsequently, the above suspension was spread on nutrient agar (LAB008, LAB M) plates containing 0,06 g/L $MgSO_4$ *και* 0,25 g/L K_2HPO_4 (pH adjusted at 7.0) (NA-MK) and incubated for approximately 7 days at 30°C to allow time for the cells to sporulate. When at least 90% cells have sporulated (evaluated by contrast phase microscopy), the spores were harvested by depositing 1 mL of sterile water onto each NA-MK plate and transferred into a sterile centrifugation tube. The spore suspension was centrifuged at 4000 ×g for 20 min at 4°C and the pellet was rinsed with ice-cold distilled water. The latter procedure was repeated four times. The final pellet was resuspended in a small volume of ice-cold distilled water and stored at 4°C until use.

2.3. Inoculation of the Samples. The prepared apricot, peach, and pear cuts were inoculated with spores of *P. polymyxa* to reach a final concentration of 400 CFU/g on the fruits. A part of the fruit samples was not inoculated with *P. polymyxa* to serve as samples for the sensory analyses. The inoculated or not samples were subsequently treated with the following preprocessing handlings.

2.4. Preprocessing of the Samples Prior to the HHP Treatment. The fruit preparation as well as the following 1st and 2nd handling was performed according to different processing procedures followed in canned food industry for each fruit type. The 3rd handling was followed to evaluate the effect of HHP processing on the cut fruits without additional thermal treatment.

1st Handling. 100 g of cut-fruit pieces was dipped in hot water ($T > 95$°C) for 2 min, subsequently transferred to sterile polyethylene bags (180 mm × 300 mm, film thickness 90 μm, FlexoPack) and 100 mL of boiling-hot sucrose solution (22.7% sucrose/22°Brix) was added. The average final temperature of the product was 81°C.

2nd Handling. 100 g of cut-fruit pieces was washed in cold water, subsequently transferred to sterile polyethylene bags (180 mm × 300 mm, film thickness 90 μm, FlexoPack) and 100 mL of boiling-hot sucrose solution (22.7% sucrose/22°Brix) was added. The average final temperature of the product was 61°C.

3rd Handling. 100 g of cut-fruit pieces was washed in cold water, subsequently transferred to sterile polyethylene bags (180 mm × 300 mm, film thickness 90 μm, FlexoPack) and 100 mL of sucrose solution ($T = 30$°C) (22.7% sucrose/22°Brix) was added. The final temperature of the product was 25°C.

The handlings 1, 2, and 3 were applied to apricots and peaches, whereas handlings 1 and 3 were applied to pears. In half packages with apricots and peaches, $SnCl_2$ (30 ppm) was added, while in half packages with pears ascorbic acid (0.1%) was added. Then, the pouches were heat-sealed after careful removal of air, moved directly to the HHP unit, and treated in a single run at 600 MPa for 5 min at 10°C,

as described below. Additionally, samples treated according to 1st handling without any additive and HHP treatment served as control samples. The HHP-treated and control samples were subsequently stored at 5°C. Each experiment was replicated three times (three different batches of each fruit).

2.5. High Hydrostatic Pressure (HHP) Treatment. HHP inactivation experiments were conducted in triplicate at pressure of 600 MPa and temperature 10°C for processing time of 5 minutes. The high pressure unit (Food Pressure Unit FPU 1.01, Resato International BV, Roden, Holland) comprised of a pressure intensifier and a multivessel system consisting of a central vessel of 250 mL capacity, with a maximum operating pressure and temperature of 1000 MPa and 90°C. The pressure transmitting fluid used was polyglycol ISO viscosity class VG 15 (Resato International BV, Roden, Holland). Process temperature in the vessel was achieved by liquid circulation in the outer jacket controlled by a heating cooling system [18, 19]. The desired value of pressure was set and after pressure build up (20 MPa/s) the pressure vessels were isolated. The pressure of the vessel was released after a preset time interval (according to the experimental design) by opening the corresponding pressure valve [18, 19]. The initial adiabatic temperature increase during pressure build up was taken into consideration in order to achieve the desired operating temperature during pressurization. Pressure and temperature were constantly monitored and recorded (in 1 s intervals) during the process [18, 19]. The come-up rate was approximately 100 MPa per 7 sec and the pressure release time was 3 sec. Pressurization time reported in this work does not include the pressure come-up and release times.

2.6. Microbiological Analysis. Samples were analyzed throughout storage at regular time intervals during storage of apricot (0, 103, 166, 231, and 287 days), peach (0, 48, 104, 170, and 226 days), and pear (0, 67, 122, and 185 days). Fruit pieces were weighed aseptically, added to sterile 1/4 strength Ringer's solution, and homogenized in a stomacher (Lab Blender 400, Seward Medical, London, UK) for 60 s at room temperature. Decimal dilutions in 1/4 strength Ringer's solution were prepared and duplicate 1 or 0.1 mL aliquots of appropriate dilutions was pour- or spread-plated on the following media: (i) plate count agar (CM0325, Oxoid) for total viable counts, incubated at 30°C for 48–72 h; (ii) de Man-Rogosa-Sharp (MRS) medium (CM 0361, Oxoid) for lactic acid bacteria (LAB), adjusted in pH 5.7, overlaid with the same medium, and incubated at 30°C for 48–72 h; (iii) tryptone dextrose extract agar (CM0075, Oxoid) containing 0.1% activated carbon; (iv) rose Bengal chloramphenicol agar base (LAB 36 supplemented with selective supplement X009, LAB M), for yeasts/molds incubated at 25°C for 72 h. In all growth media incubation time was extended by 1-2 days to allow recovery of lethally/sublethally injured or stressed by HHP treatment cells.

2.7. Firmness Measurement. The firmness of the fruit pieces was determined using a TA.HD *plus* texture analyser equipped with a Kramer shear cell (Stable Microsystems, Surrey, UK) with the following test parameters: load cell = 500 kg, test speed = 5 mm/s, Krammer shear cell (Stable Microsystems, Surrey, UK). The fruit cuts of a total weight of ca. 20 g were placed on the Kramer cell. The procedure was followed at least 4 times for each case. The speed setting was 200 mm/sec, whereas the penetration force was measured in N. The values were recorded and firmness was calculated dividing the maximum shear compression force by the total weight of fruits and expressed as N/g of product.

2.8. Color Measurement. Color change was measured using a Minolta Chroma Meter fitted with a CR-300 measuring head (Minolta, Osaka, Japan). The apparatus was calibrated with a standard white tile ($X = 78.66$, $Y = 83.31$, and $Z = 88.40$). The recorded values X, Y, and Z were converted to CIE L^*, a^*, and b^* color values. The L^* value indicates the visual lightness or the luminance on a scale of 0 to 100 (0 = perfect black, 100 = perfect white). Positive a^* values indicate red direction; negative a^* value is the green direction. Positive b^* values are the yellow direction, and negative b^* values are the blue direction. At each sampling time, 5 random measurements of the fruits of each different treatment were performed from duplicate samples, from 3 different batches of each fruit.

2.9. Sensory Analysis. For the sensory evaluation, taste and total evaluation (color, texture, taste, and odor) of the noninoculated products were assessed from a panel of eight members (staff from the laboratory). The same trained persons were used in each evaluation, and all were blinded to which sample was being tested. The sensory evaluation was carried out in artificial light and the temperature of packaged product was similar to ambient temperature. Assessment was designed to identify spoilage conditions exclusively. A persistent dull appearance, or unusual color or appearance, was considered unacceptable. Taste was scored on a three-point hedonic scale where 0 = good, 1 = acceptable, and 2 = totally unacceptable. Scores above 1 rendered the product spoiled and indicated the end of the product's shelf-life. Total evaluation was scored on a ten-point hedonic scale where 10 = very good, 5 = acceptable, and 1 = totally unacceptable. Scores below 5 rendered the product spoiled and indicated the end of the product's shelf-life.

3. Results

3.1. HHP Inactivation of Microorganisms in Apricot, Peach, and Pear Pieces in Syrup. The growth of spoilage microorganisms and *P. polymyxa* was monitored during refrigerated storage of the apricot, peach, and pear pieces in sucrose solution after HHP treatment at 600 MPa for 10 min. In all HHP-treated packages for apricots (Table 1), peaches (Table 2), and pears (Table 3), no growth of microorganisms was observed, whereas all populations were below the detection limit of the method (1 log CFU/g). In the respective control cases for each fruit (Tables 1, 2, and 3), growth was observed for total viable counts, lactic acid bacteria, and yeasts, and all the control samples were spoiled until the end of storage. The

Table 1: Evolution of microbiological counts (log CFU/g), firmness, L^*, a^*, b^* values, and sensory evaluation in HHP-treated at 600 MPa for 5 min at 10°C and untreated (control case) apricot pieces in sucrose solution with or without $SnCl_2$ during storage (5°C). Each data point is an average ± standard deviation from three individual batches.

	Time (days)						Time (days)				
	0	103	166	231	287		0	103	166	231	287
P. polymyxa						L^* value					
Control	1.3 ± 0.1	2.1 ± 0.4	4.2 ± 0.7	ND	ND	Control	29.3 ± 0.5	32.5 ± 1.2	37.7 ± 1.4	37.8 ± 2.3	38.5 ± 1.1
1st handling without $SnCl_2$	No growth detected (<dl during 287 days)					1st handling without $SnCl_2$	29.5 ± 1.7	31.9 ± 2.5	32.4 ± 2.2	32.9 ± 1.1	35.9 ± 1.2
1st handling with $SnCl_2$	No growth detected (<dl during 287 days)					1st handling with $SnCl_2$	30.4 ± 1.4	33.5 ± 0.8	31.0 ± 1.5	33.9 ± 3.4	34.7 ± 2.1
2nd handling without $SnCl_2$	No growth detected (<dl during 287 days)					2nd handling without $SnCl_2$	31.2 ± 0.9	33.1 ± 2.6	31.9 ± 0.7	31.8 ± 1.9	34.8 ± 1.4
2nd handling with $SnCl_2$	No growth detected (<dl during 287 days)					2nd handling with $SnCl_2$	30.5 ± 1.6	31.7 ± 1.2	32.0 ± 2.3	35.0 ± 1.7	35.2 ± 2.0
3rd handling without $SnCl_2$	No growth detected (<dl during 287 days)					3rd handling without $SnCl_2$	29.9 ± 1.5	31.8 ± 2.3	29.4 ± 0.6	34.6 ± 2.2	31.8 ± 0.8
3rd handling with $SnCl_2$	No growth detected (<dl during 287 days)					3rd handling with $SnCl_2$	30.1 ± 1.6	30.2 ± 2.2	31.9 ± 1.0	35.8 ± 0.5	35.3 ± 1.9
Total viable counts						a^* value					
Control	1.7 ± 0.2	5.2 ± 1.3	6.8 ± 0.5	ND	ND	Control	0.2 ± 0.0	0.4 ± 0.1	−0.1 ± 0.0	1.4 ± 0.1	1.3 ± 0.1
1st handling without $SnCl_2$	No growth detected (<dl during 287 days)					1st handling without $SnCl_2$	0.1 ± 0.0	0.2 ± 0.0	−0.5 ± 0.0	−0.1 ± 0.0	−0.1 ± 0.0
1st handling with $SnCl_2$	No growth detected (<dl during 287 days)					1st handling with $SnCl_2$	0.4 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.1 ± 0.1	0.2 ± 0.0
2nd handling without $SnCl_2$	No growth detected (<dl during 287 days)					2nd handling without $SnCl_2$	0.3 ± 0.0	0.6 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	−0.1 ± 0.0
2nd handling with $SnCl_2$	No growth detected (<dl during 287 days)					2nd handling with $SnCl_2$	0.7 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	0.6 ± 0.1	0.1 ± 0.0
3rd handling without $SnCl_2$	No growth detected (<dl during 287 days)					3rd handling without $SnCl_2$	1.4 ± 0.2	1.5 ± 0.2	1.1 ± 0.0	1.6 ± 0.2	1.2 ± 0.1
3rd handling with $SnCl_2$	No growth detected (<dl during 287 days)					3rd handling with $SnCl_2$	1.9 ± 0.1	1.8 ± 0.1	2.2 ± 0.2	1.8 ± 0.2	1.2 ± 0.1
Lactic acid bacteria						b^* value					
Control	<dl	1.4 ± 0.1	2.2 ± 0.3	ND	ND	Control	10.5 ± 0.3	12.6 ± 0.2	14.6 ± 0.3	16.9 ± 0.3	16.5 ± 0.8
1st handling without $SnCl_2$	No growth detected (<dl during 287 days)					1st handling without $SnCl_2$	10.2 ± 0.2	11.4 ± 0.2	10.2 ± 1.0	10.4 ± 0.2	14.5 ± 0.3
1st handling with $SnCl_2$	No growth detected (<dl during 287 days)					1st handling with $SnCl_2$	12.1 ± 0.3	13.3 ± 0.4	11.5 ± 0.3	13.1 ± 0.5	13.8 ± 0.6
2nd handling without $SnCl_2$	No growth detected (<dl during 287 days)					2nd handling without $SnCl_2$	12.0 ± 0.2	13.0 ± 0.4	12.0 ± 0.8	11.4 ± 0.7	13.5 ± 0.5
2nd handling with $SnCl_2$	No growth detected (<dl during 287 days)					2nd handling with $SnCl_2$	11.9 ± 0.1	12.3 ± 0.2	11.9 ± 0.9	14.6 ± 0.8	13.8 ± 0.2
3rd handling without $SnCl_2$	No growth detected (<dl during 287 days)					3rd handling without $SnCl_2$	12.4 ± 0.2	13.2 ± 0.1	10.7 ± 0.7	14.7 ± 0.5	10.7 ± 0.3
3rd handling with $SnCl_2$	No growth detected (<dl during 287 days)					3rd handling with $SnCl_2$	11.2 ± 0.1	11.9 ± 0.4	14.0 ± 0.2	16.4 ± 1.4	14.2 ± 0.6
Yeasts						Taste[a]					
Control	<dl	4.9 ± 0.7	6.7 ± 0.4	ND	ND	Control	0.0	1.4	2.0	ND	ND
1st handling without $SnCl_2$	No growth detected (<dl during 287 days)					1st handling without $SnCl_2$	0.0	0.3	0.8	0.5	0.4
1st handling with $SnCl_2$	No growth detected (<dl during 287 days)					1st handling with $SnCl_2$	0.0	0.1	0.9	0.3	0.7
2nd handling without $SnCl_2$	No growth detected (<dl during 287 days)					2nd handling without $SnCl_2$	0.0	0.4	0.9	1.3	0.5
2nd handling with $SnCl_2$	No growth detected (<dl during 287 days)					2nd handling with $SnCl_2$	0.0	0.8	1.0	0.9	0.5
3rd handling without $SnCl_2$	No growth detected (<dl during 287 days)					3rd handling without $SnCl_2$	0.0	1.0	1.4	1.1	1.3
3rd handling with $SnCl_2$	No growth detected (<dl during 287 days)					3rd handling with $SnCl_2$	0.1	0.9	1.4	0.9	0.9
Firmness						Total evaluation[b]					
Control	34.0 ± 2.4	20.5 ± 1.2	15.8 ± 2.2	24.5 ± 3.5	15.0 ± 2.3	Control	10.0	3.8	2.8	ND	ND
1st handling without $SnCl_2$	34.1 ± 1.7	29.6 ± 3.3	14.9 ± 1.1	18.0 ± 2.2	13.5 ± 2.5	1st handling without $SnCl_2$	10.0	6.3	6.4	6.6	4.9
1st handling with $SnCl_2$	34.1 ± 1.7	33.6 ± 2.8	25.4 ± 2.5	22.5 ± 1.4	18.0 ± 3.1	1st handling with $SnCl_2$	10.0	7.8	6.4	8.4	4.6
2nd handling without $SnCl_2$	32.4 ± 3.0	49.9 ± 2.5	38.5 ± 3.0	40.5 ± 3.2	43.0 ± 2.6	2nd handling without $SnCl_2$	10.0	7.4	3.9	3.1	6.7
2nd handling with $SnCl_2$	32.4 ± 3.0	42.2 ± 3.7	37.8 ± 2.3	34.8 ± 2.0	36.5 ± 2.9	2nd handling with $SnCl_2$	10.0	5.3	4.9	6.0	7.8
3rd handling without $SnCl_2$	35.0 ± 2.3	33.3 ± 3.2	35.9 ± 1.7	30.0 ± 2.6	31.0 ± 1.7	3rd handling without $SnCl_2$	10.0	4.0	4.0	3.8	2.4
3rd handling with $SnCl_2$	35.0 ± 2.3	32.1 ± 2.4	24.9 ± 2.1	29.0 ± 4.4	30.0 ± 2.8	3rd handling with $SnCl_2$	10.0	6.0	4.0	5.5	6.2

ND: not determined-spoiled product, dl: detection limit (1 log CFU/g); [a]the taste is the mean value of scores given by 8 panelists-0: good, 1: acceptable, 2: spoiled; [b]the total evaluation is the mean value of odor, color, texture, and taste of 8 panelists. -10: very good, 5: acceptable, and 1: totally unacceptable.

Table 2: Evolution of microbiological counts (log CFU/g), firmness, L^*, a^*, b^* values, and sensory evaluation in HHP-treated at 600 MPa for 5 min at 10°C and untreated (control case) peach pieces in sucrose solution with or without $SnCl_2$ during storage (5°C). Each data point is an average ± standard deviation from three individual batches.

	Time (days)						Time (days)				
	0	48	104	170	226		0	48	104	170	226
P. polymyxa						L^* value					
Control	1.5 ± 0.2	3.1 ± 0.5	5.2 ± 0.6	ND	ND	Control	39.8 ± 2.3	40.4 ± 1.1	40.9 ± 3.4	41.8 ± 1.9	40.0 ± 1.7
1st handling without $SnCl_2$	No growth detected (<dl during 226 days)					1st handling without $SnCl_2$	39.5 ± 1.6	36.9 ± 2.5	40.0 ± 2.2	42.2 ± 1.2	39.7 ± 2.5
1st handling with $SnCl_2$	No growth detected (<dl during 226 days)					1st handling with $SnCl_2$	38.4 ± 1.4	39.5 ± 0.8	39.7 ± 1.5	38.5 ± 0.5	38.2 ± 1.7
2nd handling without $SnCl_2$	No growth detected (<dl during 226 days)					2nd handling without $SnCl_2$	37.2 ± 0.9	35.7 ± 2.0	39.9 ± 0.8	40.7 ± 1.1	41.4 ± 1.4
2nd handling with $SnCl_2$	No growth detected (<dl during 226 days)					2nd handling with $SnCl_2$	40.5 ± 1.6	42.3 ± 1.2	43.3 ± 2.3	40.3 ± 1.5	39.8 ± 2.0
3rd handling without $SnCl_2$	No growth detected (<dl during 226 days)					3rd handling without $SnCl_2$	39.9 ± 1.5	40.8 ± 2.3	40.4 ± 0.6	41.1 ± 2.2	37.0 ± 1.9
3rd handling with $SnCl_2$	No growth detected (<dl during 226 days)					3rd handling with $SnCl_2$	40.1 ± 1.6	40.2 ± 2.2	42.0 ± 1.6	40.3 ± 2.3	40.2 ± 1.6
Total viable counts						a^* value					
Control	1.8 ± 0.1	5.4 ± 0.8	6.5 ± 0.4	ND	ND	Control	−3.2 ± 0.0	−3.0 ± 0.1	−2.3 ± 0.0	−2.7 ± 0.1	−2.4 ± 0.2
1st handling without $SnCl_2$	No growth detected (<dl during 226 days)					1st handling without $SnCl_2$	−2.7 ± 0.0	−1.9 ± 0.0	−2.7 ± 0.0	−3.0 ± 0.0	−1.6 ± 0.1
1st handling with $SnCl_2$	No growth detected (<dl during 226 days)					1st handling with $SnCl_2$	−3.0 ± 0.1	−2.8 ± 0.1	−2.3 ± 0.1	−2.5 ± 0.0	−1.1 ± 0.1
2nd handling without $SnCl_2$	No growth detected (<dl during 226 days)					2nd handling without $SnCl_2$	−2.9 ± 0.0	−2.2 ± 0.2	−2.8 ± 0.0	−2.8 ± 0.0	−1.1 ± 0.1
2nd handling with $SnCl_2$	No growth detected (<dl during 226 days)					2nd handling with $SnCl_2$	−3.7 ± 0.1	−3.4 ± 0.2	−3.8 ± 0.1	−2.4 ± 0.0	−1.6 ± 0.0
3rd handling without $SnCl_2$	No growth detected (<dl during 226 days)					3rd handling without $SnCl_2$	−3.4 ± 0.2	−3.0 ± 0.2	−1.9 ± 0.0	−1.5 ± 0.5	−1.9 ± 0.1
3rd handling with $SnCl_2$	No growth detected (<dl during 226 days)					3rd handling with $SnCl_2$	−3.9 ± 0.1	−3.2 ± 0.1	−2.2 ± 0.2	−2.9 ± 0.1	−1.3 ± 0.1
Lactic Acid Bacteria						b^* value					
Control	<dl	1.1 ± 0.1	2.2 ± 0.2	ND	ND	Control	17.8 ± 0.3	17.9 ± 0.3	18.0 ± 0.2	18.1 ± 0.2	17.9 ± 0.1
1st handling without $SnCl_2$	No growth detected (<dl during 226 days)					1st handling without $SnCl_2$	17.2 ± 0.2	18.0 ± 0.2	16.3 ± 1.0	18.1 ± 0.2	15.9 ± 0.4
1st handling with $SnCl_2$	No growth detected (<dl during 226 days)					1st handling with $SnCl_2$	17.1 ± 0.3	16.9 ± 0.4	17.4 ± 0.3	16.9 ± 0.3	13.2 ± 0.3
2nd handling without $SnCl_2$	No growth detected (<dl during 226 days)					2nd handling without $SnCl_2$	18.0 ± 0.2	17.6 ± 0.4	18.0 ± 0.8	18.7 ± 0.3	16.6 ± 0.5
2nd handling with $SnCl_2$	No growth detected (<dl during 226 days)					2nd handling with $SnCl_2$	19.9 ± 0.1	19.3 ± 0.2	19.9 ± 0.9	17.8 ± 0.8	17.1 ± 0.6
3rd handling without $SnCl_2$	No growth detected (<dl during 226 days)					3rd handling without $SnCl_2$	18.4 ± 0.3	18.8 ± 0.1	18.8 ± 0.7	18.6 ± 0.1	16.0 ± 0.4
3rd handling with $SnCl_2$	No growth detected (<dl during 226 days)					3rd handling with $SnCl_2$	18.2 ± 0.1	18.3 ± 0.4	19.3 ± 0.2	18.1 ± 0.4	17.0 ± 0.2
Yeasts						Taste[a]					
Control	<dl	3.8 ± 0.8	6.3 ± 0.7	ND	ND	Control	0.0	1.3	2.0	ND	ND
1st handling without $SnCl_2$	No growth detected (<dl during 226 days)					1st handling without $SnCl_2$	0.0	0.4	0.9	1.1	1.2
1st handling with $SnCl_2$	No growth detected (<dl during 226 days)					1st handling with $SnCl_2$	0.0	0.2	0.7	0.9	0.8
2nd handling without $SnCl_2$	No growth detected (<dl during 226 days)					2nd handling without $SnCl_2$	0.1	0.5	0.9	1.2	1.5
2nd handling with $SnCl_2$	No growth detected (<dl during 226 days)					2nd handling with $SnCl_2$	0.0	0.6	1.0	1.1	0.9
3rd handling without $SnCl_2$	No growth detected (<dl during 226 days)					3rd handling without $SnCl_2$	0.0	1.1	1.3	1.2	1.6
3rd handling with $SnCl_2$	No growth detected (<dl during 226 days)					3rd handling with $SnCl_2$	0.1	0.8	1.2	1.1	1.3
Firmness						Total evaluation[b]					
Control	104.1 ± 1.0	94.1 ± 1.7	92.6 ± 2.4	82.4 ± 3.2	85.0 ± 2.3	Control	10.0	3.7	2.9	ND	ND
1st handling without $SnCl_2$	73.0 ± 2.3	53.6 ± 2.8	52.6 ± 2.2	48.0 ± 3.7	35.5 ± 3.2	1st handling without $SnCl_2$	10.0	6.5	6.0	6.3	4.6
1st handling with $SnCl_2$	73.0 ± 2.3	55.4 ± 2.5	49.0 ± 3.5	54.5 ± 2.3	31.9 ± 1.7	1st handling with $SnCl_2$	10.0	7.9	6.8	6.4	4.9
2nd handling without $SnCl_2$	91.0 ± 2.2	72.5 ± 1.4	68.2 ± 3.2	81.5 ± 2.0	69.0 ± 2.6	2nd handling without $SnCl_2$	10.0	7.1	3.8	3.3	4.7
2nd handling with $SnCl_2$	91.0 ± 2.2	68.0 ± 3.0	49.0 ± 2.6	73.0 ± 2.9	69.0 ± 1.7	2nd handling with $SnCl_2$	10.0	6.3	4.7	5.0	5.8
3rd handling without $SnCl_2$	103.0 ± 1.9	70.5 ± 1.2	63.8 ± 2.8	77.5 ± 3.5	84.5 ± 2.1	3rd handling without $SnCl_2$	10.0	4.2	3.6	3.3	2.8
3rd handling with $SnCl_2$	103.1 ± 1.9	85.8 ± 2.2	80.2 ± 2.4	84.5 ± 2.3	84.0 ± 4.4	3rd handling with $SnCl_2$	10.0	7.0	5.0	5.9	5.2

ND: not determined-spoiled product, dl: detection limit (1 log CFU/g); [a]the taste is the mean value of scores given by 8 panelists-0: good, 1: acceptable, 2: spoiled, [b]the total evaluation is the mean value of odor, color, texture, and taste of 8 panelists. -10: very good, 5: acceptable, and 1: totally unacceptable.

Table 3: Evolution of microbiological counts (log CFU/g), firmness, L^* values, and sensory evaluation in HHP-treated at 600 MPa for 5 min at 10°C and untreated (control case) pear pieces in sucrose solution with or without ascorbic acid during storage (5°C). Each data point is an average ± standard deviation from three individual batches.

	Time (days)					Time (days)			
	0	67	122	185		0	67	122	185
P. polymyxa					*Firmness*				
Control	1.2 ± 0.2	2.4 ± 0.2	4.7 ± 0.5	ND	Control	141.0 ± 12.5	70.5 ± 5.0	67.0 ± 8.0	57.0 ± 6.5
1st handling without ascorbic acid	No growth detected (<dl during 185 days)				1st handling without ascorbic acid	75.5 ± 7.5	101.0 ± 8.0	96.0 ± 6.5	92.5 ± 9.5
1st handling with ascorbic acid	No growth detected (<dl during 185 days)				1st handling with ascorbic acid	75.5 ± 7.5	73.5 ± 5.5	78.5 ± 7.5	79.5 ± 7.0
3rd handling without ascorbic acid	No growth detected (<dl during 185 days)				3rd handling without ascorbic acid	100.0 ± 10.5	116.5 ± 6.0	122.5 ± 10.5	116.5 ± 11.5
3rd handling with ascorbic acid	No growth detected (<dl during 185 days)				3rd handling with ascorbic acid	100.0 ± 10.5	107.0 ± 9.5	116.5 ± 11.0	133.0 ± 12.5
Total Viable Counts					L^* value				
Control	1.4 ± 0.2	4.3 ± 0.4	6.9 ± 0.7	ND	Control	39.5 ± 3.3	40.1 ± 4.0	38.6 ± 3.0	39.6 ± 2.4
1st handling without ascorbic acid	No growth detected (<dl during 185 days)				1st handling without ascorbic acid	38.8 ± 2.4	38.9 ± 2.6	40.3 ± 2.5	38.4 ± 1.0
1st handling with ascorbic acid	No growth detected (<dl during 185 days)				1st handling with ascorbic acid	37.4 ± 2.5	37.1 ± 3.1	40.0 ± 4.2	34.8 ± 3.7
3rd handling without ascorbic acid	No growth detected (<dl during 185 days)				3rd handling without ascorbic acid	36.9 ± 3.1	36.1 ± 2.4	37.8 ± 1.7	37.4 ± 2.2
3rd handling with ascorbic acid	No growth detected (<dl during 185 days)				3rd handling with ascorbic acid	37.1 ± 1.6	36.9 ± 2.0	34.7 ± 3.2	31.4 ± 2.8
Lactic acid bacteria					Taste[a]				
Control	<dl	1.2 ± 0.1	2.0 ± 0.4	ND	Control	0.0	1.2	2.0	ND
1st handling without ascorbic acid	No growth detected (<dl during 185 days)				1st handling without ascorbic acid	0.0	0.4	0.5	0.5
1st handling with ascorbic acid	No growth detected (<dl during 185 days)				1st handling with ascorbic acid	0.1	0.3	0.3	0.8
3rd handling without ascorbic acid	No growth detected (<dl during 185 days)				3rd handling without ascorbic acid	0.1	1.4	1.4	1.4
3rd handling with ascorbic acid	No growth detected (<dl during 185 days)				3rd handling with ascorbic acid	0.0	0.8	0.6	0.8
Yeasts					Total Evaluation[b]				
Control	<dl	4.1 ± 0.5	6.3 ± 0.6	ND	Control	10.0	6.4	3.1	ND
1st handling without ascorbic acid	No growth detected (<dl during 185 days)				1st handling without ascorbic acid	10.0	8.0	4.6	5.6
1st handling with ascorbic acid	No growth detected (<dl during 185 days)				1st handling with ascorbic acid	10.0	8.5	8.3	7.9
3rd handling without ascorbic acid	No growth detected (<dl during 185 days)				3rd handling without ascorbic acid	10.0	3.0	4.0	3.5
3rd handling with ascorbic acid	No growth detected (<dl during 185 days)				3rd handling with ascorbic acid	10.0	5.5	5.9	5.8

ND: not determined-spoiled product, dl: detection limit (1 log CFU/g); [a]the taste is the mean value of scores given by 8 panelists-0: good, 1: acceptable, 2: spoiled; [b]the total evaluation is the mean value of odor, color, texture, and taste of 8 panelists. -10: very good, 5: acceptable, and 1: totally unacceptable.

same results were monitored for the inoculated *P. polymyxa*, which was found to be below the detection limit in HHP-treated packages for apricots (Table 1), peaches (Table 2), and pears (Table 3).

3.2. Effect of HHP Treatment on the Quality Parameters of Apricot, Peach, and Pear Pieces in Syrup during Storage. An increase was observed in lightness (L^*) of apricot samples during storage in all treatments, with changes found to be more intense in control samples (Table 1). a^* values were found to increase during storage of control samples, indicating a gradual browning of the samples. Browning was also observed in samples treated according to the 3rd handling with or without $SnCl_2$ after the HHP application in comparison with control samples, but no further browning was observed during storage. No browning was observed during storage of samples treated according to 1st or 2nd handling with or without $SnCl_2$ (Table 1). Finally, b^* values were found to increase especially during storage of control and 3rd handling samples, meaning that there is an increase in samples yellowness (Table 1). A decrease was observed in firmness of apricot cuts of all treatments during storage, except from the 2nd handling cases, where a hardening of apricot cuts was noticed (Table 1). Moreover, the firmness values of samples treated according to the 1st handling decreased earlier in comparison with the rest of handlings. However, the addition of $SnCl_2$ showed a positive effect in the firmness of apricot samples treated according to 1st handling. Finally, no changes were observed between the samples within the same treatment of 1st or 3rd handling, with or without the addition of $SnCl_2$.

No specific trend was observed in L^* or b^* values of the peach samples of all treatments during storage (Table 2). However, an increase was monitored in a^* values of samples for all cases (browning) with changes being observed earlier in 3rd handling samples without the addition of SnCl2 (Table 2). A decrease was observed in firmness of samples for all treatments during storage. The application of HHP had no effect in the firmness of the samples, since the samples treated with the 3rd handling had similar firmness values with control. However, an additional effect of the thermal processing on reducing the firmness of samples treated according to the 1st and 2nd handling was observed, which was more intense in the case of 1st handling (stronger thermal treatment). Finally, no particular changes were observed between the samples of the same treatment after the addition of $SnCl_2$.

In pear samples, no remarkable changes were observed in the color parameters. Indicatively, L^* values are given in Table 3. It was observed that L^* values decreased slightly during storage of samples treated with the 3rd handling with ascorbic acid. Similarly, a^* values showed an increase in the latter case only, while b^* values showed no particular changes (data not shown). It was observed that, in control samples, the firmness decreased during storage. On the other hand, the application of HHP had as a result a slight decrease in firmness values (3rd handling), with no further reduction observed during storage (Table 3). This effect increased with the previous thermal treatment of the pear cuts (1st handling), with no further reduction during storage being observed in this case as well.

3.3. Sensory Analysis. For the sensory evaluation, taste and total evaluation (color, texture, taste, and odor) of the products were assessed from a panel of eight members. The shelf-life of the products treated with the different handlings in comparison with the control is presented in Tables 1, 2, and 3. The panelists marked with better scores the HHP-treated products compared to their respective controls, according to taste and total evaluation during storage of apricots, peaches, and pears (Tables 1, 2, and 3). The addition of $SnCl_2$ or ascorbic acid gave similar or higher scoring for total evaluation and taste of the products within the same treatment. More specifically, the scores of the total evaluation were higher during storage of HHP-treated samples of all fruits with the $SnCl_2$ or ascorbic acid, while the corresponding taste scores were found to be similar (Tables 1, 2, and 3). In addition, the samples treated with the 1st handling gave better scores in comparison with the rest of handlings (especially the 3rd one) that scores were found to be below the acceptable boundaries. It has to be noted that for all cases of HHP treated fruit samples (irrespective of the fruit type), a more transparent appearance of the fruit cuts was observed by the panelists.

4. Discussion

In the current study, the potential of HHP treatment to preserve fresh-cut apricot, peach, and pear pieces in sucrose solution was assessed. Therefore, the processing conditions toward inactivation of *P. polymyxa*—a microorganism relevant to the spoilage of fresh-cut fruit pieces—were studied. In addition, a shelf-life study of HHP-treated apricot, peach, and pear pieces in sucrose solution was evaluated, in which color, texture, and sensory evaluation were assessed in parallel to the microbiological quality during refrigerated storage at 5°C.

In the present work, the syrup in which the apricot, peach, or pear pieces were dipped into did not only provide an acidic environment, but also a high concentration of sucrose. Several authors have observed the protective effect of such solutions on the inactivation of bacteria and yeasts, despite the low a_w [14, 20–22]. Although sucrose solution provided a protected environment on the bacteria and yeast cells, a total reduction of microorganisms (below the detection limit of the method) was achieved with the application of HHP, irrespective of the previous thermal treatment of the fruit cuts. These results are in accordance with other studies relevant to the reduction of microorganisms caused by HHP [14, 23, 24]. In addition, several authors have reported that HHP sensitizes bacteria cells to low pH [25–28], while others have reported that, even after a pressure level of 600 MPa, cells are able to grow in such acidic fruit products during refrigerated storage [29, 30]. Furthermore, the inactivation of the *P. polymyxa* spores after the application of HHP treatment is in accordance with previous reports that a minimum pressure level of 600 MPa is needed for the inactivation of bacteria and molds spores [31–33].

Generally, the presence of $SnCl_2$ or ascorbic acid in the HHP-treated samples had no effect on the color parameters within the same treatment. This indicates that either the HHP application gives the maximum effect that can be given or the applied $SnCl_2$ or ascorbic acid concentration as indicated from the industry should be elevated to meet the needs of the new processing method. However, the different handlings seemed to have an effect on the color, especially in the browning of apricot (Table 1). It was shown that the application of thermal treatments—a strong one (1st handling) or a milder (2nd handling)—had a positive effect in preventing the fruit browning, most probably due to the thermal inactivation of the enzymes. In contrast, fruit samples treated with the 3rd handling exhibited higher a^* values (similar or higher to the control samples), indicating the nessecary thermal treament of fruits cuts, irrespective of the HHP application. It has been reported that, for natural peach puree and peach puree containing ascorbic acid or cysteine and treated with HHP (517 MPa/5 min), an increased color maintenance of HHP-processed purees was observed [34]. In another report [35], the authors managed to prevent browning on HHP-treated apples during storage by using pineapple juice.

The changes in the firmness values of fruit cuts were different according to the fruit type. The application of the HHP alone (3rd handling) had no effect in the firmness of peach and apricot cuts but reduced the firmness of pears. Moreover, the previous heat treatments of the samples (1st and 2nd handling) did not initially affect the firmness of apricots but reduced the firmness of pears and peaches. However, in most of the cases, the HHP application resulted in higher firmness values of the samples during storage, in comparison with the control samples. It has been reported that when the fruits are stored in pouches with syrup, as in the current study, texture degradation may occur due to solubilization and depolymerization of water-soluble pectin sodium carbonate-soluble pectin [36]. The pectin degradation and softening of flesh previously treated with thermal and HHP processing (600 MPa for 5, 10, or 30 min) can be delayed with a storage temperature of $4 \pm 1^{\circ}C$ [36]. A firming effect of HHP at 600 MPa for 5 min on apple pieces was reported previously [35], while in another study, no significant decrease of hardness was observed during storage of HHP-treated (600 MPa for 10 min) apple pieces, in comparison with the untreated ones that showed a significant decrease [14]. This could be due to the damage of the fruit tissue caused by the high population of the yeasts/molds in the products [14].

The scores of the total sensory evaluation were higher during storage of HHP-treated samples of all fruits with the $SnCl_2$ or ascorbic acid, while the corresponding taste scores were found to be similar (Tables 1, 2, and 3). In addition, the samples treated with the 1st handling gave better scores in comparison with the rest of handlings, especially the 3rd one that scores were found to be below the acceptable boundaries. Moreover, for all cases of HHP treated fruit samples (irrespective of the fruit type), the fruit cuts had a more transparent appearance, which is in agreement with other reports [14, 22]. The changes in the nature and overall appearance of the products may be attributed to the damage of the fruit tissues caused by HHP [37].

5. Conclusion

The HHP treatment was applied to enhance the microbiological quality and extend the shelf-life of apricot, peach, and pear pieces in sucrose solution. Treatment at 600 MPa for 5 min with the combination of thermal processing of the fruits can remarkably extend the shelf-life of these fresh products, with minor changes on their color and firmness. However, additional research is needed in order to optimize the handling prior to HHP, aiming to better quality and sensory attributes of such products.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Nonthermal Pasteurization of Fermented Green Table Olives by means of High Hydrostatic Pressure Processing

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Green fermented olives cv. Halkidiki were subjected to different treatments of high hydrostatic pressure (HHP) processing (400, 450, and 500 MPa for 15 or 30 min). Total viable counts, lactic acid bacteria and yeasts/moulds, and the physicochemical characteristics of the product (pH, colour, and firmness) were monitored right after the treatment and after 7 days of storage at 20°C to allow for recovery of injured cells. The treatments at 400 MPa for 15 and 30 min, 450 MPa for 15 and 30 min, and 500 MPa for 15 min were found insufficient as a recovery of the microbiota was observed. The treatment at 500 MPa for 30 min was effective in reducing the olive microbiota below the detection limit of the enumeration method after the treatment and after 1 week of storage and was chosen as being more appropriate for storing olives for an extended time period (5 months). After 5 months of storage at 20°C, no microbiota was detected in treated samples, while significant changes for both HHP treated and untreated olives were observed for colour parameters only (minor degradation). In conclusion, HHP treatment may introduce a reliable nonthermal pasteurization method to extend the microbiological shelf-life of fermented table olives.

1. Introduction

High hydrostatic pressure (HHP) processing has a great potential in producing high quality foods that are microbiologically safer and with an extended shelf-life [1]. Recently, different HHP treatments have been applied in the food industry on various food products such as meat, fisheries, fruits, and vegetables [2–5]. Due to technological improvement in HPP equipment, industrial application is widespread for a range of pressures between 100 and 800 MPa depending on the desired objective [2]. High pressure is transmitted immediately and uniformly throughout the pressure vessel (Pascal principle) and the process is adiabatic; therefore the food is prevented from being deformed or heated which would modify its quality properties [2, 6, 7].

Table olive processing relies on the microbiota naturally present on fruit surface, processing water and equipment, and in fermentation vessels [8]. Most fermented olives are distributed throughout the market "in bulk" (available in open containers), stored at ambient temperature, and thus exposed to high risk of contamination from the environment [8]. The final product may also be marketed to local markets or exported abroad in glass and plastic containers, as well as in tins, or in other packaging materials such as polyethylene or multilaminated pouches, filled with brine or gases (modified atmospheres) [9, 10]. The latter packages are more convenient to be distributed through the market and at the same time provide added value to the product [10]. However, irrespective of the packaging material, industry usually applies a subsequent thermal pasteurization step to stabilize microbiologically the product [9]. Thermal processing of olives is often associated with quality deterioration, especially if the process is not optimized, resulting in softening of olive

tissue, loss of green colour, and development of cooking taste that affects the sensory attributes of the final product [11, 12].

Very limited information is available in the literature about the use of HHP on table olives as an alternative to conventional pasteurization treatment. Pradas et al. [13] studied the effect of HHP on Cornezuelo dressed olives, measuring several physicochemical parameters of HHP treated or untreated olives during storage. The authors reported that no hazardous microorganisms could be enumerated on olives with the exception of yeasts and moulds that were found to be less than 10^6 CFU/g which is in agreement with the IOC trade standard for table olives [14]. On the other hand, a recent study of Abriouel et al. [11] presented the effect of different levels of HHP and antimicrobials on total viable counts (TVC) and yeasts of Manzanilla Alorena cracked table olives. It was reported that a pressure of 300 MPa for 5 min was effective in reducing yeast population below the detection limit of the enumeration method, but even a pressure of 700 MPa for 5 min was not efficient to suppress the growth of TVC.

According to the above, there is a knowledge gap about the possible HHP treatments that are effective in reducing the population of olive microbiota below the detection limit of the enumeration method, thus guaranteeing an extended shelf life of the product. In this context, the aim of this study was to evaluate the effect of different HHP treatments (combinations of different levels of HHP and processing times) on the indigenous microbiota and the physicochemical parameters of fermented green table olives cv. Halkidiki in an attempt to investigate the efficacy of HHP as an alternative treatment to thermal pasteurization.

2. Materials and Methods

2.1. Olive Packaging. Green olives cv. Halkidiki from three different batches were obtained from Konstantopoulos S.A. table olive processing industry located in Northern Greece. After the end of the fermentation process, olives were withdrawn from the fermentation vessels and selected by hand to remove defective drupes. Samples (100 g) of fermented green olives were placed in polyethylene pouches, covered with freshly prepared 6% brine (w/v, NaCl) containing 0.2% citric and 0.15% ascorbic acid, and heat sealed. The packages were finally subjected to different HHP treatments as explained below.

2.2. Experimental Design

2.2.1. Effect of Different High Pressure Treatments. To investigate the effectiveness of different HHP treatments, inactivation tests were conducted in triplicate at pressures of 400, 450, and 500 MPa for 15 and 30 minutes, respectively. The pressurized packages (HHP samples) were stored at 20°C for 7 days in high precision (0.5°C) incubation chambers (VELP Scientifica, Italy), to allow for recovery of injured cells on the olive fruits and cover brine and thus select the optimum HHP treatment for further storage experiments. Packages without any treatment served as control samples and followed storage at the same conditions as the HHP samples. Microbiological and physicochemical analysis was conducted for both HHP and control samples, at day 0 (right after the HHP treatment) and after 7 days of storage.

2.2.2. Selection of the Most Appropriate HHP Treatment and Storage for 5 Months. Further on, based on the selection of the most effective combination of HHP level and pressurization time, additional packages of green olives were prepared as described above, subjected to HHP processing, and stored for a period of 5 months at 20°C to mimic storage conditions in retail outlets (supermarkets, hypermarkets). Duplicate packages of three different batches of olives were randomly removed and analyzed at preselected time intervals of 0, 7, and 15 days and 1, 2, 3, 4, and 5 months.

2.3. High Hydrostatic Pressure (HHP) Treatment. HHP inactivation experiments were conducted in triplicate at pressures of 400, 450, and 500 MPa for 15 and 30 minutes, respectively. Pressurization was carried out at room temperature (18–20°C). The high pressure unit (Food Pressure Unit FPU 1.01, Resato International BV, Roden, Holland) comprised a pressure intensifier and a multivessel system consisting of a central vessel of 250 mL capacity, with a maximum operating pressure and temperature of 1000 MPa and 90°C. The pressure transmitting fluid was polyglycol ISO viscosity class VG 15 (Resato International BV, Roden, Holland). The desired value of pressure was set and, after pressure buildup (20 MPa/s), the pressure vessels were isolated. The pressure of the vessel was released after a preset time interval by opening the corresponding pressure valve. Pressure and temperature were constantly monitored and recorded (in 1 s intervals) during the process [15, 16]. The come-up rate was approximately 100 MPa per 7 sec and the pressure release time was 3 sec. Pressurization time reported in this work does not include the pressure come-up and release times. Further details of the high pressure system and operating conditions can be found elsewhere [17, 18].

2.4. Microbiological Analyses. Immediately after the HHP treatment, the enumeration of microorganisms was performed on both olive and brine samples. Specifically, brine samples (1 mL) were aseptically transferred to 9 mL sterile 1/4 Ringer's solution (BR0052G, Oxoid). In the case of olive samples, 10 g of olive flesh was aseptically added to 90 mL sterile 1/4 Ringer's solution and homogenized in a stomacher (Stomacher 400 Circulator, Seward) for 60 s at room temperature. The resulting suspensions were serially diluted in the same diluent and 1 or 0.1 mL samples of the appropriate dilutions were poured or spread on nonselective and selective agar plates. To reduce the detection limit of the enumeration method (for spread plating) to 1 log CFU/g for olive samples and 0 log CFU/mL for brine samples, 1 mL from olive homogenate or 1 mL of brine, respectively, was spread equally on 3 agar plates of each substrate. The selected agar media were the following: Plate Count Agar (CM0325, Oxoid) for total viable counts, incubated at 30°C for 48–72 h; de Man-Rogosa-Sharp (MRS) medium (CM

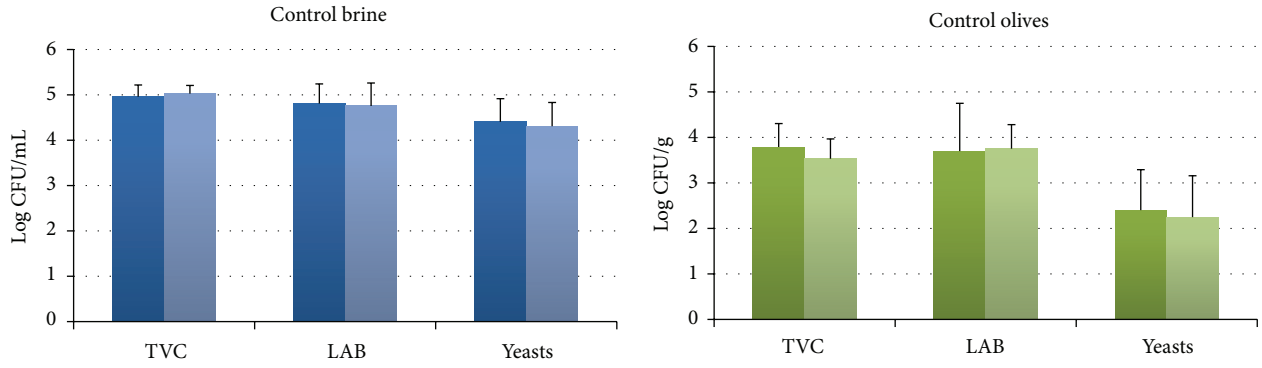

Figure 1: Changes in the population of the indigenous microbiota in brines at day 0 (dark blue) and day 7 (light blue) and in olives at day 0 (dark green) and day 7 (light green) without any HHP treatment. The detection limit of the enumeration method was 0 log CFU/mL for brines and 1 log CFU/g for olives. Data are mean values ± standard deviation of duplicate pouches analyzed from three different batches of olives.

0361, Oxoid) for LAB, adjusted to pH 5.7 and supplemented with 0.05% (w/v) cycloheximide (Sigma), overlaid with the same medium, and incubated at 30°C for 48–72 h; Rose Bengal Chloramphenicol Agar Base (LAB036 supplemented with selective supplement X009, LAB M) for yeasts/moulds incubated at 25°C for 48–72 h; Violet Red Bile Glucose Agar (CM 0485, Oxoid) for Enterobacteriaceae overlaid with the same medium and incubated at 37°C for 24 h; Pseudomonas agar base (CM559 supplemented with selective supplement CFC SR0103, OXOID), for *Pseudomonas* spp. incubated at 25°C for 48 hours. In all growth media incubation time was extended by 1-2 days to allow recovery of lethally injured or stressed cells.

2.5. pH Measurement. The pH value of olives and brine was measured with a digital pH meter (HI 2211 pH-ORP Meter, HANNA Instruments, USA). The pH of brine was recorded by immersing the electrode directly in the brine of the package, whereas the pH of olive fruits was measured in the olive homogenate (stomacher homogenate) after the end of the microbiological analysis.

2.6. Colour Measurement. The olive colour was assessed by taking at least 10 random measurements from the surface of different olives using a Minolta Chroma Meter fitted with a CR-300 measuring head (Minolta, Osaka, Japan). The CIE (Commission Internationale de l' Eclairage) L^*, a^*, b^* colorimetry system was used for colour determination. L^* indicates lightness, and its values range from 0 (an ideal black object) to 100 (an ideal white object). Positive a^* values indicate red direction, negative a^* value is the green direction, positive b^* values are the yellow direction, and negative b^* values are the blue direction. The instrument was calibrated with a standard white tile ($L^* = 96.10$, $a^* = +0.98$, and $b^* = +7.27$). At each sampling time, 10 olives from each sample (package) of each different treatment were analyzed in duplicate (2 measurements at random locations on each olive). Thus for each time point and treatment a total of 120 measurements were recorded (3 batches × 2 samples × 20 measurements). Chroma (C^*) and hue angle (h^*) values were also calculated based on the following equations:

$$C^* = \sqrt{a^{*2} + b^{*2}},$$
$$h^* = \tan^{-1}\left(\frac{b^*}{a^*}\right). \quad (1)$$

2.7. Firmness Measurement. Firmness of olives was determined using a TA.HD *plus* Texture Analyser equipped with a needle probe and a 50 Kg load cell (Stable Microsystems, Surrey, UK). The speed setting was 30 mm/min, whereas the penetration force was measured in N. At each sampling time, 10 olives from each sample (package) of each different treatment were analyzed. Thus for each time point and each treatment a total of 60 measurements were recorded (3 batches × 2 samples × 10 measurements).

2.8. Data Analysis. Each experiment was repeated three times (three different batches of olives) with duplicate samples (packages) opened at each time point. Counts of the different microbial groups were transformed to log CFU/g or log CFU/mL values before computing means and standard deviations. The effects of different treatments on the physicochemical parameters of HHP treated or untreated olives were analyzed using the t-test of Excel. Initially, an F-test was performed on the dataset to determine if the variances of the tested populations were equal or unequal and in continuance a t-test was performed assuming equal or unequal variances, respectively, at 95% confidence interval ($P < 0.05$).

3. Results and Discussion

3.1. Effect of Different High Pressure Treatments

3.1.1. Effect on the Microbial Community. Results showed that the indigenous microbiota of olives prior to treatment comprised LAB followed by yeasts (Figure 1). The initial mean population of LAB was 4.81 ± 0.43 log CFU/mL and 3.70 ± 1.05 log CFU/g in the brines and olives, respectively,

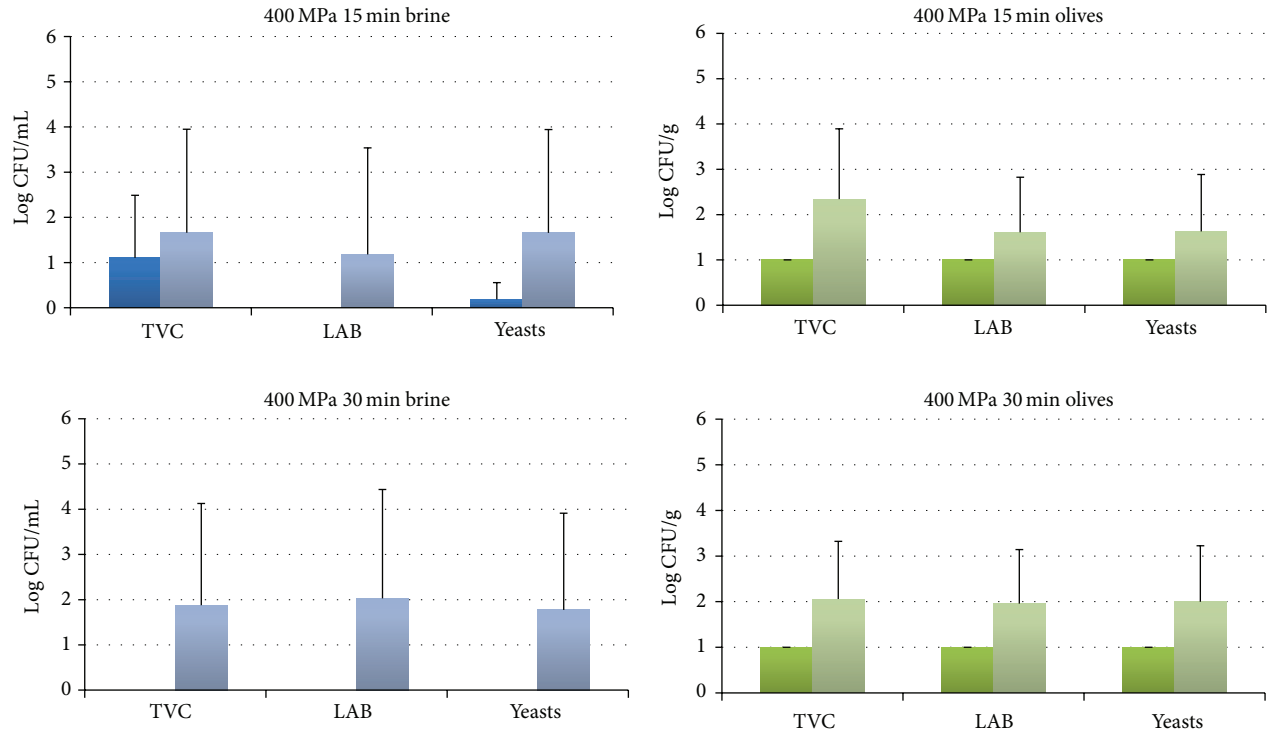

Figure 2: Changes in the population of the indigenous microbiota in brines at day 0 (dark blue) and day 7 (light blue) and in olives at day 0 (dark green) and day 7 (light green) treated at 400 MPa for 15 or 30 min. The detection limit of the enumeration method was 0 log CFU/mL for brines and 1 log CFU/g for olives. Data are mean values ± standard deviation of duplicate pouches analyzed from three different batches of olives.

whereas the corresponding population of yeasts was 4.04 ± 1.31 log CFU/mL in the brines and 2.40 ± 0.89 log CFU/g in olives (Figure 1). These results are within the expected range of LAB and yeasts reported previously for fermented black or green olives with or without covering brines [12, 19–21]. The microbial population showed no changes during storage at 20°C for 7 days for the control olive samples (Figure 1). *Pseudomonas* spp. and enterobacteria were not detected at any stage of the above storage period for both HHP treated and control samples.

The HHP treatment resulted in the reduction of the microbial populations in both brines and olives below the detection limit of the enumeration method in all cases right after the treatment (Figures 2–4), with the exception of 400 MPa for 15 min where a recovery of yeasts was observed in the brines (Figure 2). The subsequent storage of the HHP treated samples for 7 days at 20°C resulted in the recovery of LAB and yeasts in all studied treatments except from 500 MPa for 30 min where no growth of LAB and yeasts was observed (Figures 2–4). More specifically, LAB were recovered in both brines and olives in treatments of 400 MPa for 15 and 30 min (Figure 2) and 450 MPa for 15 min (Figure 3) as well as in olives at 450 MPa for 15 min (Figure 3). Yeasts were found to be more resistant than LAB and were recovered in all cases except 500 MPa for 30 min (Figures 2–4). These findings are in contrast with a previous study of Abriouel et al. [11] who reported no viable yeast counts in Manzanilla Aloreña cracked olives at pressures of 300 MPa or higher for 5 min. On the other hand, the same authors have shown that even a pressure of 700 MPa for 5 min was not capable of reducing the bacteria below the detection limit that were found to be more HHP resistant than yeasts. Sánchez et al. [22] reported that a pressure of 450 MPa for 10 min was not sufficient to reduce bacteria and yeasts/moulds in olive paste below the detection limit, whereas a treatment of 600 MPa for 5 or 10 min was effective against yeasts/moulds but not bacteria.

Thus, according to the findings of this work, the treatment at 500 MPa for 30 min was chosen as the most suitable condition of pressure/time to study the effect of the specific HHP treatment on the storage of green table olives.

3.1.2. Effect on the Physicochemical Parameters. The initial pH values in olive samples prior to pressurization were found to be 3.97 ± 0.07 and 4.12 ± 0.08 in brine and olives, respectively, and did not change significantly after 1 week. Similar values were observed for the HHP treated samples (Table 1).

The HHP processing was found to reduce significantly the L^* value of the samples right after treatment at 500 MPa for 30 min in comparison with the control samples, indicating slightly darker olive products due to HHP processing. Moreover, the L^* values decreased and a^* values increased after 1-week storage for HHP samples in all treatments except from the case of 400 MPa for 15 min (Table 1). Increasing a^* values during 7-day storage indicate an increase in the red component of the colour of olives. The HHP effect on the b^* values was a reduction ($P < 0.05$) in samples treated

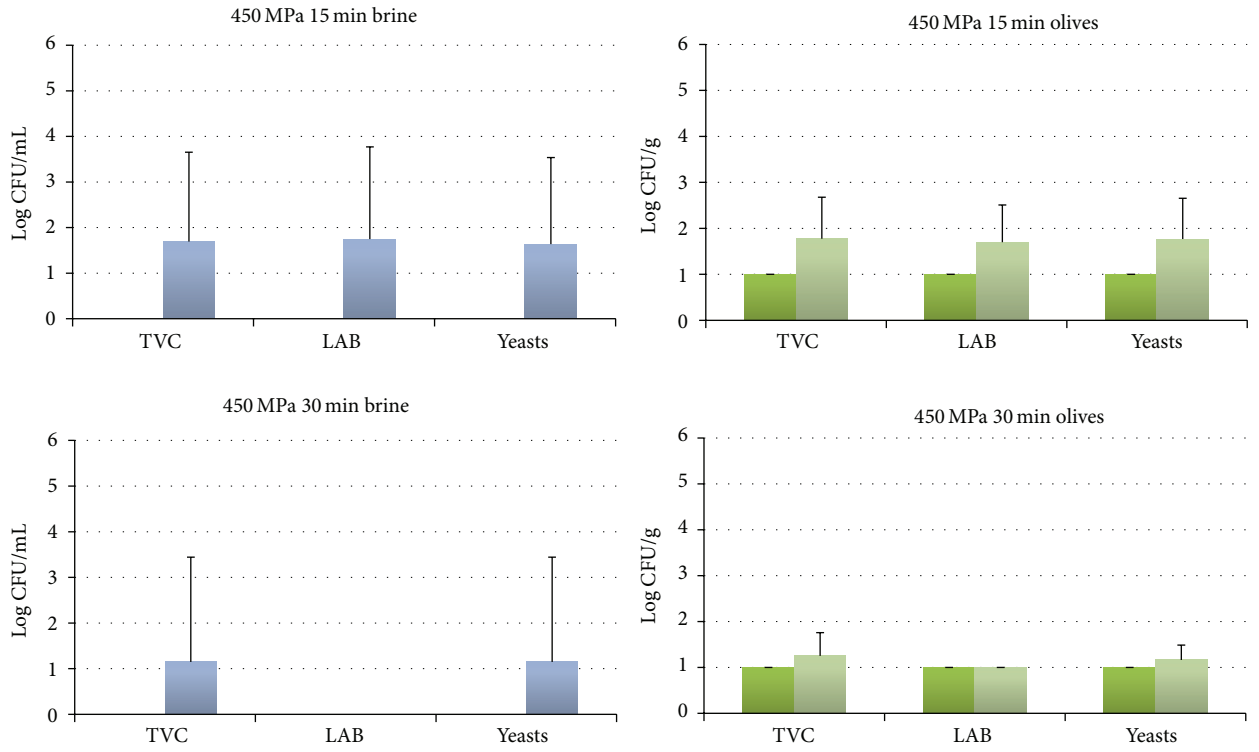

Figure 3: Changes in the population of the indigenous microbiota in brines at day 0 (dark blue) and day 7 (light blue) and in olives at day 0 (dark green) and day 7 (light green) treated at 450 MPa for 15 or 30 min. The detection limit of the enumeration method was 0 log CFU/mL for brines and 1 log CFU/g for olives. Data are mean values ± standard deviation of duplicate pouches analyzed from three different batches of olives.

for 30 min irrespective of the pressure applied. However, no further reduction was observed after 1 week of storage. On the contrary, all the samples treated for 15 min at all pressure levels did not show reduced b^* values right after the treatment, but a slight reduction was observed after 7 days indicating a minor loss in the yellow tonalities (Table 1).

Finally, no significant effect ($P < 0.05$) on the firmness of the HHP samples was observed neither after the treatment, nor after 7 days of storage.

3.2. Effect of 500 MPa for 30 min on the Storage of Olives

3.2.1. Effect on the Microbial Evolution. HHP treatment at 500 MPa for 30 min resulted in the reduction of all the indigenous microbiota below the detection limit of the enumeration method. Moreover, no growth was observed during storage for 5 months at 20°C. Regarding the control (unpressurized) samples, only minor changes in the population of LAB and yeasts were observed after 5 months of storage (data not shown). The initial mean population of LAB was 4.98 ± 0.28 log CFU/mL and 4.53 ± 0.34 log CFU/g in the brines and olives, respectively, whereas the corresponding population of yeasts was 4.06 ± 0.77 log CFU/mL in the brines and 2.68 ± 0.73 log CFU/g in olives. The final population of LAB was 5.10 ± 0.11 log CFU/mL and 4.48 ± 0.42 log CFU/g in brines and olives, respectively, whereas the corresponding population of yeasts was 4.01 ± 0.65 log CFU/mL in brines and 3.22 ± 0.24 log CFU/g in olives. No *Pseudomonas* spp. or enterobacteria were detected at any stage of the storage period in control or HHP treated samples.

3.2.2. Effect on the Physicochemical Parameters. Statistically significant changes during storage of both HHP treated and control samples were observed only for colour parameters. The remainder of the studied parameters (firmness and pH) did not show any significant changes (Table 2). These results are in contrast with a previous study of Pradas et al. [13] where softening of olives and decrease in pH were reported during storage for both HHP treated and untreated Cornezuelo dressed olives. This could be attributed to the fact that these olives were not previously fermented when subjected to HHP treatment but they were packed in brine and dressed with sodium chloride, vinegar, and various herbs (thyme, garlic, and fennel).

Colour has a key contribution in the marketability of green table olives as a vivid green colour is an essential characteristic of the product, especially in Spanish-style processing [23]. The lightness (L^*) was fairly high at the beginning of storage and showed a slight decrease at all cases. The a^* values were initially negative indicating green tonalities. During storage however, a gradual decrease was observed in both control and HHP samples indicating a slight decrease in the green olive colour. Finally, the values of b^*

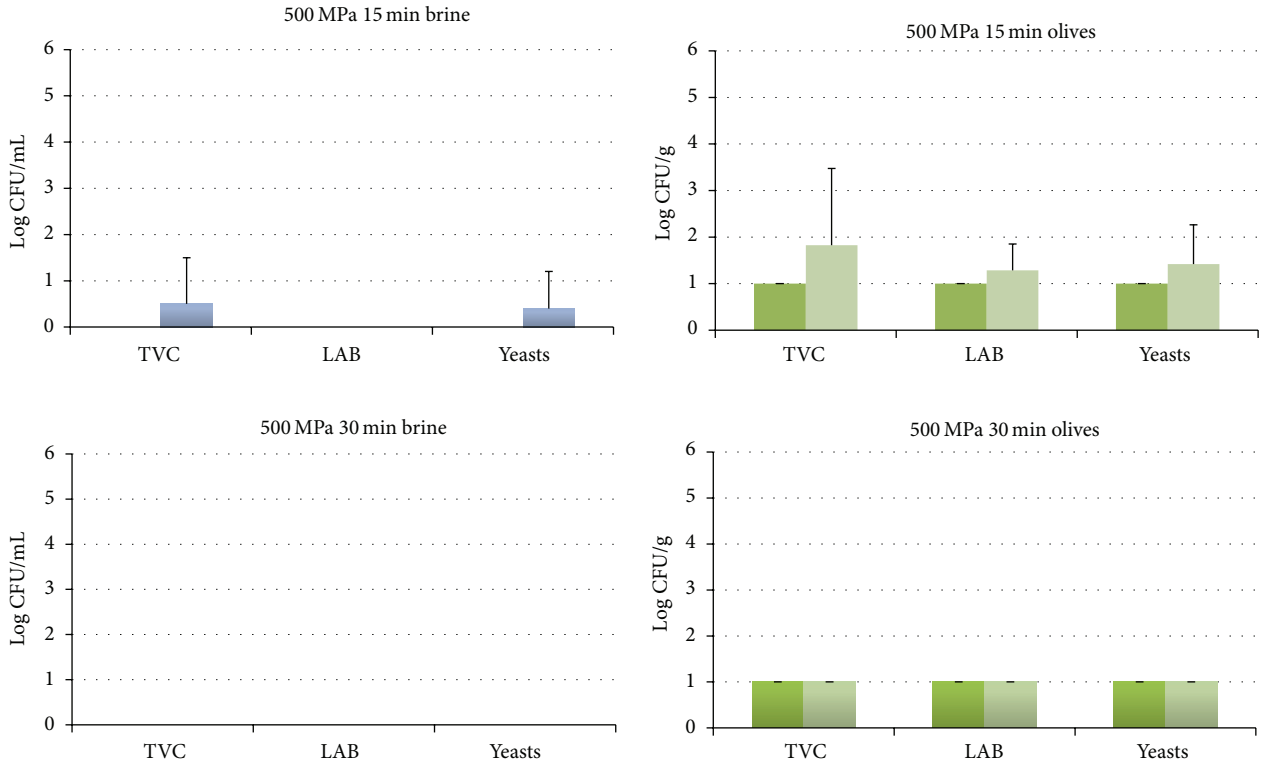

Figure 4: Changes in the population of the indigenous microbiota in brines at day 0 (dark blue) and day 7 (light blue) and in olives at day 0 (dark green) and day 7 (light green) treated at 500 MPa for 15 or 30 min. The detection limit of the enumeration method was 0 log CFU/mL for brines and 1 log CFU/g for olives. Data are mean values ± standard deviation of duplicate pouches analyzed from three different batches of olives.

were also positive (yellow) at day 0 and showed a decrease, indicating a decrease in olive yellowness (Table 2).

Regarding the control samples, the L^* and b^* values were found to decrease during storage, with statistically significant reduction being observed after the 1st month. The a^* values were found to increase during storage, with statistically significant reduction being observed after the 2nd month of storage (Table 2). Concerning the HHP treated samples, the changes for all colour parameters were observed earlier in comparison with the control (i.e., at the 15th day of storage) and were more intense (Table 2). Moreover, at the last month of storage of HHP treated samples, the a^* value was found to be positive indicating a light browning of olives. A gradual decrease in chroma (C^*) values was observed in both HHP treated and control olive samples throughout storage (Figure 5) that was higher in pressure treated olives (*ca.* 10 units) compared to control samples (*ca.* 5 units), indicating slightly higher colour intensity in untreated samples. Concerning hue angle values (h^*), a slight increase of 4-5° towards yellow colour (hue angle 90°) was recorded with no significant difference between the applied treatments (data not shown). Similar results have been reported by Pradas et al. [13] for a moderate degradation of the colour in both HHP treated and untreated samples, with no further details given about the differences between the treatments.

A possible solution for further improving the colour in HHP treated olives is the addition of ascorbic acid, which was shown to enhance colour of Manzanilla Aloreña cracked table olives [24]. In the latter study, the highest concentration of ascorbic acid that was also shown to have the best effect on colour was 15 g/L. Since in this study the initial concentration of ascorbic acid added in the brines was 1.5 g/L, a future increase in the concentration of the acid could improve the colour maintenance of olives during storage.

4. Conclusions

In conclusion, the HHP treatment was applied to extend the microbiological shelf-life and the quality of green table olives. The treatment at 500 MPa for 30 min can significantly extend the shelf-life of these products, since it was found efficient at reducing the indigenous microbiota below the detection limit. Additionally, no microbial growth was observed after storage of the HHP treated olives at 20°C for 5 months. Thus, HHP processing may introduce a reliable nonthermal method to extend the shelf-life of fermented green table olives. However, additional research is needed in order to establish HHP processing as a useful tool for the table olive industry.

Table 1: Evolution of the physicochemical parameters of olives pressurized or not (control) at 400, 450, or 500 MPa for 15 or 30 min and stored at 20°C for 7 days.

Pressure (MPa)	Processing time (min)	Colour					
		L^* value		a^* value		b^* value	
		0 days	7 days	0 days	7 days	0 days	7 days
Control (unpressurized)	—	$53.95 \pm 2.44^{a,x}$	$54.71 \pm 1.64^{a,x}$	$-4.17 \pm 0.70^{a,x}$	$-4.43 \pm 1.01^{a,x}$	$31.53 \pm 2.67^{a,x}$	$32.54 \pm 2.29^{a,x}$
400	15	$53.84 \pm 2.50^{a,x}$	$53.02 \pm 1.81^{b,x}$	$-4.66 \pm 0.66^{a,x}$	$-4.17 \pm 0.74^{a,b,x}$	$31.50 \pm 2.86^{a,x}$	$29.89 \pm 2.02^{b,y}$
	30	$53.97 \pm 1.45^{a,x}$	$51.87 \pm 1.70^{b,c,y}$	$-4.58 \pm 0.66^{a,x}$	$-3.86 \pm 0.76^{b,c,y}$	$30.55 \pm 2.22^{b,x}$	$29.25 \pm 1.93^{b,x}$
450	15	$54.28 \pm 3.09^{a,x}$	$51.95 \pm 1.89^{b,c,y}$	$-3.96 \pm 0.71^{a,x}$	$-3.28 \pm 0.87^{c,y}$	$31.59 \pm 3.76^{a,x}$	$29.44 \pm 1.83^{b,y}$
	30	$53.85 \pm 3.46^{a,x}$	$52.75 \pm 1.97^{b,c,x,y}$	$-3.90 \pm 0.68^{a,x}$	$-3.23 \pm 0.79^{c,y}$	$30.89 \pm 3.25^{b,x}$	$29.66 \pm 1.75^{b,y}$
500	15	$53.12 \pm 2.58^{a,x}$	$51.91 \pm 2.04^{b,c,y}$	$-4.34 \pm 0.65^{a,x}$	$-3.39 \pm 0.61^{c,y}$	$31.23 \pm 2.36^{a,x}$	$30.08 \pm 1.72^{b,y}$
	30	$52.01 \pm 3.07^{b,x}$	$50.71 \pm 2.77^{b,c,y}$	$-4.22 \pm 1.26^{a,x}$	$-3.13 \pm 0.44^{c,y}$	$30.79 \pm 2.17^{b,x}$	$30.22 \pm 1.30^{b,x}$
		Firmness (Nt)		pH			
				Brine		Olives	
		0 days	7 days	0 days	7 days	0 days	7 days
Control (unpressurized)	—	$4.24 \pm 0.94^{a,x}$	$4.42 \pm 1.08^{a,x}$	$3.97 \pm 0.07^{a,x}$	$3.99 \pm 0.08^{a,x}$	$4.12 \pm 0.08^{a,x}$	$4.10 \pm 0.08^{a,x}$
400	15	$4.23 \pm 0.91^{a,x}$	$4.15 \pm 1.48^{a,x}$	$3.99 \pm 0.04^{a,x}$	$4.02 \pm 0.06^{a,x}$	$4.14 \pm 0.06^{a,x}$	$4.11 \pm 0.04^{a,x}$
	30	$4.89 \pm 1.03^{a,x}$	$4.14 \pm 1.42^{a,x}$	$3.88 \pm 0.16^{a,x}$	$4.09 \pm 0.06^{a,x}$	$4.18 \pm 0.01^{a,x}$	$4.16 \pm 0.01^{a,x}$
450	15	$4.50 \pm 0.85^{a,x}$	$4.61 \pm 1.08^{a,x}$	$3.90 \pm 0.01^{a,x}$	$3.87 \pm 0.04^{a,x}$	$4.01 \pm 0.15^{a,x}$	$4.04 \pm 0.03^{a,x}$
	30	$4.92 \pm 0.91^{a,x}$	$4.94 \pm 0.97^{a,x}$	$3.93 \pm 0.01^{a,x}$	$3.90 \pm 0.01^{a,x}$	$4.11 \pm 0.09^{a,x}$	$4.04 \pm 0.00^{a,x}$
500	15	$4.25 \pm 1.05^{a,x}$	$4.07 \pm 1.12^{a,x}$	$4.04 \pm 0.09^{a,x}$	$4.04 \pm 0.07^{a,x}$	$4.12 \pm 0.06^{a,x}$	$4.10 \pm 0.09^{a,x}$
	30	$4.75 \pm 1.29^{a,x}$	$4.77 \pm 1.17^{a,x}$	$4.07 \pm 0.04^{a,x}$	$4.02 \pm 0.02^{a,x}$	$4.15 \pm 0.02^{a,x}$	$4.12 \pm 0.04^{a,x}$

^{a,b,c}Different letters within the same column indicate significant differences between different treatments at the specific storage time ($P < 0.05$).
^{x,y}Different letters within the same row indicate significant differences between 0th and 7th day at the same treatment ($P < 0.05$).

Table 2: Evolution of the physicochemical parameters of olives pressurized or not (control) at 500 MPa for 30 min and stored at 20°C for 5 months.

	0 days	7 days	15 days	1 month	2 months	3 months	4 months	5 months
500 MPa								
L	$52.05 \pm 1.31^{a*}$	51.89 ± 2.69^{a}	48.17 ± 1.57^{b}	48.32 ± 2.11^{b}	47.27 ± 2.04^{c}	47.18 ± 2.16^{c}	45.98 ± 1.17^{d}	44.92 ± 1.49^{e}
a	-3.85 ± 0.62^{a}	-3.82 ± 0.79^{a}	-1.75 ± 0.94^{b}	-2.07 ± 0.82^{b}	$-1.77 \pm 0.60^{b,c}$	-1.50 ± 0.77^{c}	-0.61 ± 0.76^{d}	0.67 ± 0.52^{d}
b	30.95 ± 2.17^{a}	30.29 ± 2.43^{a}	26.12 ± 2.13^{b}	26.18 ± 2.32^{b}	$25.12 \pm 1.88^{b,c}$	$24.62 \pm 2.50^{c,d}$	23.61 ± 2.51^{d}	21.65 ± 2.25^{e}
Firmness	4.27 ± 1.10^{a}	3.88 ± 1.23^{a}	3.45 ± 0.96^{a}	3.93 ± 1.37^{a}	3.65 ± 1.06^{a}	3.66 ± 1.05^{a}	3.72 ± 1.22^{a}	3.77 ± 0.94^{a}
pH brine	3.94 ± 0.17^{a}	3.94 ± 0.07^{a}	4.03 ± 0.16^{a}	4.14 ± 0.01^{a}	4.07 ± 0.04^{a}	4.10 ± 0.03^{a}	3.86 ± 0.02^{a}	3.86 ± 0.01^{a}
pH olives	4.18 ± 0.11^{a}	4.11 ± 0.08^{a}	4.17 ± 0.04^{a}	4.18 ± 0.01^{a}	4.17 ± 0.01^{a}	4.18 ± 0.06^{a}	4.13 ± 0.06^{a}	4.19 ± 0.02^{a}
Control								
L	52.94 ± 2.24^{x}	52.69 ± 1.68^{x}	53.14 ± 2.52^{x}	51.94 ± 2.24^{y}	51.01 ± 2.65^{y}	48.47 ± 2.17^{z}	47.63 ± 1.89^{z}	47.79 ± 1.34^{z}
a	-3.78 ± 0.97^{x}	-3.72 ± 0.72^{x}	-3.78 ± 1.23^{x}	$-3.77 \pm 0.59^{x,y}$	-2.84 ± 1.29^{y}	-2.27 ± 1.05^{y}	-2.26 ± 0.53^{y}	-1.22 ± 0.21^{z}
b	31.57 ± 3.06^{x}	31.49 ± 1.89^{x}	32.37 ± 2.05^{x}	30.36 ± 1.39^{y}	29.29 ± 1.33^{y}	29.44 ± 1.83^{y}	27.28 ± 1.64^{z}	26.42 ± 0.72^{z}
Firmness	4.24 ± 0.91^{x}	4.46 ± 1.07^{x}	4.34 ± 1.09^{x}	4.39 ± 1.09^{x}	4.42 ± 1.08^{x}	4.29 ± 0.93^{x}	4.24 ± 0.94^{x}	4.20 ± 1.03^{x}
pH brine	3.90 ± 0.20^{x}	3.99 ± 0.06^{x}	3.98 ± 0.11^{x}	4.03 ± 0.07^{x}	3.89 ± 0.09^{x}	4.03 ± 0.01^{x}	4.08 ± 0.03^{x}	3.93 ± 0.09^{x}
pH olives	4.18 ± 0.01^{x}	4.10 ± 0.03^{x}	4.12 ± 0.08^{x}	4.18 ± 0.01^{x}	4.07 ± 0.07^{x}	4.16 ± 0.02^{x}	4.06 ± 0.03^{x}	4.13 ± 0.06^{x}

*Different letters within the same row indicate significant differences between each storage time ($P < 0.05$).

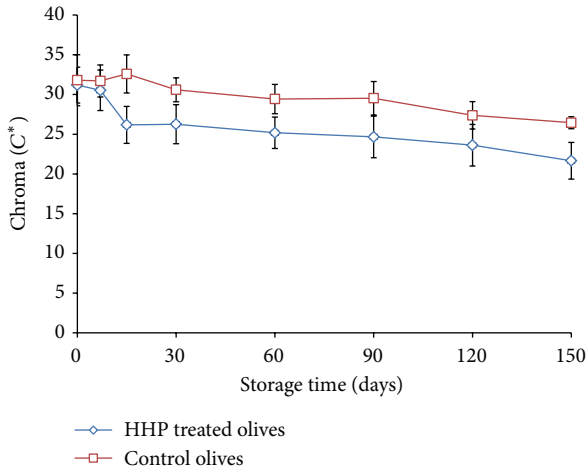

FIGURE 5: Changes in chroma (C^*) values of HHP treated and control olives during storage for 5 months at 20°C. Data are mean values ± standard deviation of duplicate pouches analyzed from three different batches of olives.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Eucalyptus Essential Oil as a Natural Food Preservative: *In Vivo* and *In Vitro* Antiyeast Potential

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In this study, the application of eucalyptus essential oil/vapour as beverages preservative is reported. The chemical composition of eucalyptus oil was determined by gas chromatography-mass spectrometry (GC-MS) and solid phase microextraction GC-MS (SPME/GC-MS) analyses. GC-MS revealed that the major constituents were 1,8-cineole (80.5%), limonene (6.5%), α-pinene (5%), and γ-terpinene (2.9%) while SPME/GC-MS showed a relative reduction of 1,8-cineole (63.9%) and an increase of limonene (13.8%), α-pinene (8.87%), and γ-terpinene (3.98%). Antimicrobial potential of essential oil was initially determined in vitro against 8 different food spoilage yeasts by disc diffusion, disc volatilization, and microdilution method. The activity of eucalyptus vapours was significantly higher than the eucalyptus oil. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) varied from 0.56 to 4.50 mg/mL and from 1.13 to 9 mg/mL, respectively. Subsequently, the combined efficacy of essential oil and thermal treatment were used to evaluate the preservation of a mixed fruit juice in a time-dependent manner. These results suggest eucalyptus oil as a potent inhibitor of food spoilage yeasts not only in vitro but also in a real food system. Currently, this is the first report that uses eucalyptus essential oil for fruit juice preservation against food spoiling yeast.

1. Introduction

Eucalyptus is an evergreen, tall tree, or shrub, belonging to Myrtaceae family. Although it is native to Australia and Tasmania, nowadays it has extensively spread to other countries [1]. The genus *Eucalyptus* contains about 700 species; among them, more than 300 contain volatile oils in their leaves. Essential oils of various eucalyptus species are used in the pharmaceutical, toiletries, cosmetics, and food industries [1]. These broad applications are due to the antiseptic, antihyperglycemic, anti-inflammatory, flavouring, and antioxidant properties of the molecules present in the oil [2]. The antimicrobial activity of eucalyptus oils has been found to vary significantly within microbial species and within microbial strains. The strong antimicrobial activity may be directly associated with their major compounds in the oil (such as 1,8-cineole and α-pinene) or with the synergy between the major and minor constituents [3]. Previous results reported that Gram positive bacteria are more susceptible than Gram negative bacteria; moreover, activity against fungi and yeasts (*Candida albicans* and *Saccharomyces cerevisiae*) has also been detected [4]. According to one of the reports, *Eucalyptus odorata* had the strongest activity against bacteria and yeasts while *E. bicostata* had the best antiviral activity [3]. Although several studies about eucalyptus oils have been published [5–7], only few of them evaluated their activity

against pathogenic and food spoilage microorganisms [4, 8]. Despite the well-reported antimicrobial activity *in vitro*, the food industry has applied eucalyptus essential oils mainly as flavouring agents. Therefore, the use of essential oils as preservatives in food has been limited. Because the required concentration against microorganisms is affected by the interactions of the oil compounds with the food matrix components, higher concentrations are needed to achieve sufficient activity. This negatively impacts the organoleptic properties of the final product [9]. To overcome this problem, a promising alternative is the use of a combination of mild temperature treatment with essential oils [10]. A mild thermal treatment, in fact, enhances the antimicrobial efficacy of the essential oil influencing the vapour pressure of the molecules [11].

In the present study, after a chemical characterization by GC-MS, *in vitro* effect of eucalyptus oil against 8 different food spoilage yeast species was studied through the disc diffusion method, the disc volatilisation method, and MIC/MFC. Moreover, to evaluate the antiyeast activity *in vivo*, we employed a real food system based on the preservation of a mixed fruit juice inoculated with *S. cerevisiae* and stored at room temperature for 8 days. Further, in order to improve the efficiency of the essential oil, the combined effect of oil and thermal treatment was also evaluated in the same real system.

2. Materials and Methods

2.1. Chemicals and Strains. The essential oil was procured from Erbamea, "*olio essenziale naturale,*" Italy, and stored in an airtight sealed glass bottle at 4°C till further use. Growth media and Tween 80 were purchased from Oxoid Ltd., Basingstoke, Hampshire, UK, and Merck Schuchardt, Germany, respectively. Different yeast strains (*Saccharomyces cerevisiae* SPA, *Zygosaccharomyces bailii* 45, *Aureobasidium pullulans* L6F, *Candida diversa* T6D, *Pichia fermentans* T2A1, *Pichia kluyveri* T1A, *Pichia anomala*, and *Hansenula polymorpha* CBS 4732) were obtained from the strain collection of the Dipartimento di Scienze degli Alimenti, University of Bologna, Italy, and used to evaluate the effect of essential oil. The yeast strains were grown in yeast peptone dextrose (YPD) medium at 28°C for 24 h in an orbital shaking incubator (Universal Table Shaker 709, Milan, Italy) at 120 rpm.

2.2. Gas Chromatography-Mass Spectrometry (GC-MS) and Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry (SPME/GC-MS) Analyses of Eucalyptus Essential Oil. GC-MS analyses were carried out on an Agilent 7890 gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5975 mass selective detector operating in electron impact mode (ionization voltage, 70 eV). A CP-Wax 52 CB capillary column (50 m length, 0.32 mm inner diameter, and 1.2 μm film diameter) was used. The temperature program started from 50°C and then was programmed at 3°C/min to 240°C which was maintained for 1 min. Injector, interface, and ion source temperatures were 250°C, 250°C, and 230°C, respectively. Injections were performed in split mode and helium (1 mL/min) was used as carrier gas. The mass selective detector was operated in the scan mode between 20 and 400 m/z. Data acquisition started 4 min after injection. Five millilitres of 10 ppm solution of the eucalyptus oil was placed in 10 mL vials and the vials were sealed by PTFE/silicone septa. 1 μL of the samples was injected directly into the column with a split ratio of 1 : 100. Component separation was achieved following the method described above.

For the SPME analysis, a divinylbenzene-poly(dimethylsiloxane) coated stable flex fiber (65 μm) and a manual SPME holder (Supelco Inc., Bellefonte, PA, USA) were used in this study after preconditioning according to the manufacturer's instruction manual. Samples were put into sealed vials for 10 min at room temperature. The SPME fiber was exposed to each sample for 10 min by manually penetrating the septum, and, finally, the fiber was inserted into the injection port of the GC for 10 min sample desorption.

The identification of the molecules was based on comparison of mass spectra of compounds both with those contained in available databases (NIST version 2005) and with those of pure standards (Sigma-Aldrich, Milan, Italy) analyzed under the same conditions.

2.3. Antiyeast Activity of Eucalyptus Oil and Vapour

2.3.1. Disc Diffusion Method. The agar disc diffusion method was employed for the determination of antimicrobial activities of the essential oils [12] (NCCLS, 1997). Briefly, a suspension of the tested microorganism (100 μL of 1×10^6 CFU/mL) was confirmed by viable counts and spread on the YPD agar media plates. These plates were allowed to dry. Filter paper discs, 6 mm in diameter (Schleicher & Schuell, Germany), were soaked with 10 μL of the oil and placed on the inoculated plates and, after storing at 4°C for 2 h, were incubated at 28°C for 48 h. Volume of essential oils tested was varied (10, 20, or 30 μL) by using appropriate number of sterile discs. The diameters of the inhibition zones were measured in millimetres.

2.3.2. Disc Volatilisation Method. Standard experimental setup as described by López et al. [13] was used. Briefly, a 100 μL portion of each suspension containing approximately 10^6 CFU/mL was spread over the surface of YPD agar plate and allowed to dry. A paper disc (diameter 6 mm; Schleicher & Schuell, Germany) was laid on the inside surface of the upper lid and 10 μL eucalyptus oil was soaked on each disc. The plates inoculated with microorganisms were immediately inverted on top of the lid and sealed with parafilm to prevent leakage of eucalyptus oil vapour. Plates were incubated at 28°C for 48 h and the diameter of the resulting inhibition zone in the yeast lawn was measured.

2.3.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC). Broth microdilution assays were performed as recommended by NCCLS [12]. All tests were performed in YPD agar supplemented with Tween 80 (final concentration of 0.5% v/v). Yeast strains were cultured overnight at 28°C in YPD broth. Test strains were suspended in YPD to give a final density of 1×10^6 CFU/mL. Geometric dilutions ranging from 0.036 mg/mL to 72 mg/mL of the eucalyptus oil were

prepared in a 96-well microtiter plate, including one growth control (YPD broth + Tween 80) and one sterility control (YPD broth + Tween 80 + test oil). Plates were incubated at 30°C for 48 h. The yeast cell growth was indicated by the presence of a white pellet on the well bottom. The MIC values were determined as the lowest concentration of oil preventing visible growth of microorganisms. MFC was defined as the lowest concentration at which no growth was observed after subculturing into fresh media.

2.4. Mixed Fruit Juice Preservation by Eucalyptus Oil and Thermal Treatment

2.4.1. Preparation of Fruit Juice Mixture Inoculated with S. cerevisiae SPA. Apples (Golden Delicious) and oranges at commercial maturity were purchased from a local market (Ipercoop, Cesena). After being washed, apples were cut into about 35 × 25 × 5 mm slices and then immersed in 0.2% ascorbic acid solution (to avoid undesirable enzymatic browning during the processing), drained quickly, and made into juices using a blender. After being washed, oranges were peeled off and made into juices. Both juices were mixed in 1 : 1 ratio. The suspension of yeast strain (*S. cerevisiae* SPA) was mixed with fruit juice mixture to result in final concentration of 10^3 CFU/mL and the inoculated juice mixtures were transferred into 10 mL sterilized glass vials.

2.4.2. Effect of Thermal Treatment. The effect of thermal treatment was studied by exposing the mixed juice samples at 70°C for 30, 60, and 90 s. Subsequently, the treated vials were stored at room temperature up to 8 days and samples were drawn on 0, 2nd, 4th, and 8th day.

2.4.3. Effect of Eucalyptus Oil. 1.0% ethyl alcohol solution of eucalyptus oil was mixed in the inoculated fruit juice mixture at MIC level (4.5 mg/mL), half of MIC level (2.25 mg/mL), and one-fourth of MIC level (1.125 mg/mL). Fruit juice sample inoculated with *S. cerevisiae* alone was considered as positive control. Subsequently, the treated vials were stored at room temperature up to 8 days and samples were drawn on 0, 2nd, 4th, and 8th day.

2.4.4. Effect of Eucalyptus Oil and Thermal Treatment: Combined Effect. A set of inoculated fruit juice mixtures vials added with three different concentrations of eucalyptus oil were exposed to thermal treatment (70°C) for 30, 60, and 90 s. Each condition was treated in triplicate. Subsequently, the treated vials were stored at room temperature up to 8 days and samples were drawn on 0, 2nd, 4th, and 8th day. All treated samples were serially diluted and plated on PDA. The plates were incubated for 72 h at 28°C and CFU counts were made. The efficacy of the thermal treatment alone and the combination with different doses of eucalyptus oil were quantified in time-dependent manner by the variation in log CFU/mL of the inoculated yeast strains.

2.5. Statistical Analyses. All the experiments were done in triplicate and repeatability was established. Significance of differences among treatments ($P \leq 0.05$) was analysed using one-way ANOVA (SPSS, 10.0 version). For all experiments, three replicates were used and the data presented here represents the mean of these replicates with standard error or deviation. Moreover, as regards yeast load counts during juice storage, a principal component analysis (PCA) was carried out with Statistica 6.1 (StatSoft Italy srl, Vigonza, Italy), using the different concentrations of eucalyptus essential oil and duration of the thermal treatments as variables.

Table 1: Chemical constituents of eucalyptus essential oil obtained through GC-MS.

RT (min)	Compound	Percentage
15.776	α-Pinene	5.02
17.843	β-Pinene	0.54
18.267	β-Myrcene	0.77
19.092	α-Phellandrene	0.53
19.685	Terpinolene	0.10
20.128	Limonene	6.45
20.646	1,8-Cineole	80.44
21.75	γ-Terpinene	2.90
23.237	4-Carene	0.34
23.616	Linalool	0.16
25.889	Pinocarveol	0.17
27.69	4-Terpineol	0.55
28.296	α-Terpineol	1.72
	Monoterpene hydrocarbons	16.67
	Oxygenated monoterpenes	83.04
	Total of identified compound	99.71

RT: retention time (min), relative area percentage. Results are based on GC-MS. MS acquisition started after 4 min.

3. Results and Discussion

3.1. Chemical Composition of Eucalyptus Oil. Thirteen compounds were identified by GC-MS for the total of 99.7% (Table 1). The major constituents of the oil were 1,8-cineole (80.4%), followed by limonene (6.5%), α-pinene (5%), and γ-terpinene (2.9%). On the contrary, seventeen molecules were detected by SPME/GC-MS for the total of 99.9% (Table 2). The major constituents were the same as the reported ones in Table 1 but with different relative composition: 1,8-cineole (63.9%), limonene (13.8%), α-pinene (8.9%), and γ-terpinene (3.9%). The differences between the chemical contents of oil and vapour and their reasons were also evaluated in our previous reports [4]. As reported in the literature, essential oil of eucalyptus was characterized by very high concentration of 1,8-cineole. Damjanović-Vratnica et al. [14] determined an 85.8% 1,8-cineole in eucalyptus essential oil from Montenegro and reported its significant activity against different bacteria and yeasts. Moreover, Elaissi et al. [3] showed strong antibacterial, antifungal, and antiviral effect of eight eucalyptus oils from Tunisia.

3.2. Antiyeast Activity of Eucalyptus Oil. In this work, the antiyeast activity of eucalyptus oil was evaluated with 8

Table 2: Chemical constituents of eucalyptus essential oil obtained through SPME/GC-MS.

RT (min)	Compound	Percentage
8.069	α-Pinene	8.87
11.315	β-Pinene	0.82
13.422	β-Myrcene	1.07
13.677	α-Phellandrene	1.05
13.908	3-Carene	0.19
14.323	Terpinolene	0.19
15.221	Limonene	13.84
15.877	1,8-Cineole	63.96
17.255	γ-Terpinen	3.98
18.423	o-Cymene	4.13
18.948	4-Carene	0.15
22.443	*trans*-5-Methyl-2-isopropyl-2-hexen-1-al	0.05
25.958	p-Cymene	0.14
30.132	Linalool	0.10
32.868	4-Terpineol	0.26
35.08	Pinocarveol	0.08
36.609	α-Terpineol	0.43
	Monoterpene hydrocarbons	34.46
	Oxygenated monoterpenes	64.83
	Total of identified compound	99.29

RT: retention time (min), relative area percentage. Results are based on GC-MS.

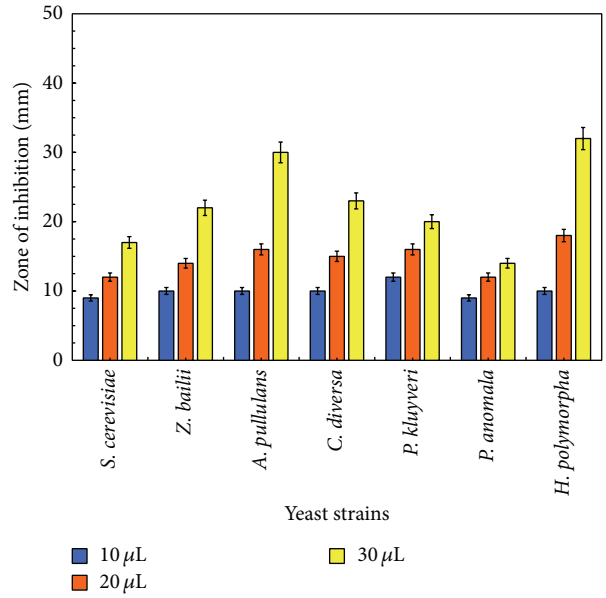

Figure 1: Antiyeast potential of eucalyptus oil evaluated with disc diffusion method. Zone of inhibition due to the different concentrations (10, 20, and 30 μL) of eucalyptus oil against *S. cerevisiae* SPA, *Z. bailii* 45, *A. pullulans* L6F, *C. diversa* T6D, *P. kluyveri* T1A, *P. anomala*, and *H. polymorpha* CBS 4732 was measured. Column height represents the mean of triplicate results and error bar represents the standard deviation.

different food spoilage yeasts: *S. cerevisiae*, *Z. bailii*, *A. pullulans*, *C. diversa*, *P. fermentans*, *P. kluyveri*, *P. anomala*, and *H. polymorpha*.

3.2.1. Disc Diffusion Method. The antiyeast activity of eucalyptus essential oil was assessed by the presence or the absence of inhibition zones. Three different concentrations of the oil (10, 20, and 30 μL) were tested. The highest susceptible yeast was *H. polymorpha* (10, 18, and 32 mm), followed by *A. pullulans* (10, 16, and 30 mm), *C. diversa* (10, 15, and 23 mm), *Z. bailii* (10, 14, and 22 mm), *P. kluyveri* (12, 16, and 20 mm), and *S. cerevisiae* (9, 12, and 17 mm) (Figure 1). *P. anomala* presented the lowest inhibition zone (9, 12, and 14 mm). The results demonstrated that eucalyptus oil was effective against all the considered strains. Previous data already reported that eucalyptus oil possesses antimicrobial activity against different microorganisms [14–18]. For example, *Eucalyptus staigeriana* oil showed strong activity (with 14.3–18.2 mm zones of inhibition) against several microorganisms (*Escherichia coli*, *Staphylococcus aureus*, and *Alcaligenes faecalis*) and no activity against yeast *C. albicans* [19]. *Eucalyptus cinerea* oil exhibited significant activity against *S. pyogenes* (26 mm) and *P. aeruginosa* (17 mm). The zone of inhibition for *S. aureus* was 13 mm. Only isolated and purified 1,8-cineole (eucalyptol) presented no antimicrobial activity against *S. aureus* and *C. albicans* [20]. Also, Vilela et al. [21] tested the antimicrobial activity of both the eucalyptus essential oil and 1,8-cineole against two *Aspergillus* species. They reported a complete fungal growth inhibition when using the essential oil, while a reduced activity was detected when using 1,8-cineole alone. This shows that possible synergistic effect of minor and major components determines the final antimicrobial activity of the essential oils [22]. Based on the chemical composition, it can be concluded that the antimicrobial activity of the oil is apparently attributed to its high content of oxygenated monoterpenes.

3.2.2. Disc Volatilisation Method. The zone of inhibition in yeast strains due to eucalyptus essential oil vapours is shown in Figure 2. Zone of inhibition due to essential oil vapour increased in a dose-dependent manner similar to disc diffusion method. The inhibition zones observed using 30 μL of eucalyptus oil vapours were *P. kluyveri* (22 mm) < *S. cerevisiae* (36 mm) < *Z. bailii* (38 mm) = *P. anomala* (38 mm) < *C. diversa* (39 mm) < *A. pullulans* (42 mm) < *H. polymorpha* (44 mm). *H. polymorpha* was the most susceptible yeast to eucalyptus oil vapours since 14, 26, and 44 mm inhibition zones were generated using 10, 20, and 30 μL eucalyptus oil vapours, respectively (Figure 2). As compared to the oil, the eucalyptus vapours resulted in a significantly larger zone of inhibition ($P \leq 0.05$) in all the strains tested. This better result could be attributed to the variation in relative composition of the oil and vapours, as already shown in Table 2.

3.2.3. MIC and MFC of Eucalyptus Oil. MIC and MFC of eucalyptus essential oil were determined against food spoiling yeasts (Table 3). The MIC values varied from 0.56 mg/mL to 4.5 mg/mL. MIC value for *S. cerevisiae* and *A. pullulans* was higher (i.e., 4.5 mg/mL) than that for *Z. bailii*, *C. diversa*,

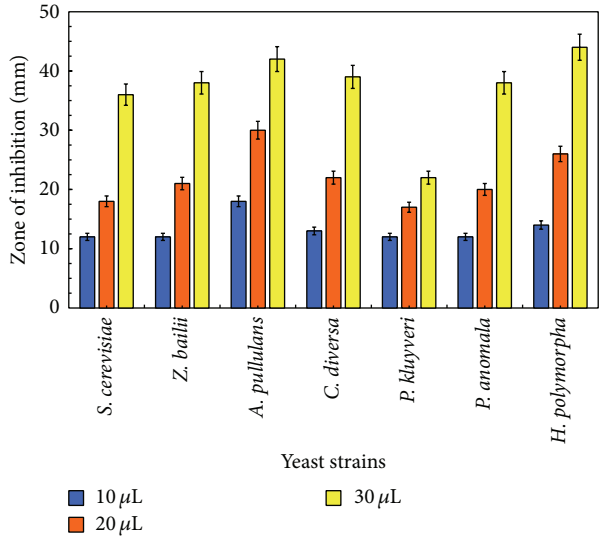

Figure 2: Antiyeast potential of eucalyptus oil vapour evaluated with disc volatilisation method. Zone of inhibition due to the different concentrations (10, 20, and 30 μL) of eucalyptus oil against *S. cerevisiae* SPA, *Z. bailii* 45, *A. pullulans* L6F, *C. diversa* T6D, *P. kluyveri* T1A, *P. anomala*, and *H. polymorpha* CBS 4732 was measured. Column height represents the mean of triplicate results and error bar represents the standard deviation.

Table 3: MIC/MFC of eucalyptus oil for different yeast strains.

S. number	Name of the strain	MIC (mg/mL)	MFC (mg/mL)
1	*Saccharomyces cerevisiae* SPA	4.5	9
2	*Zygosaccharomyces bailii* 45	2.25	4.5
3	*Aureobasidium pullulans* L6F	4.5	9
4	*Candida diversa* T6D	2.25	4.5
5	*Pichia fermentans* T2A1	2.25	4.5
6	*Pichia kluyveri* T1A	0.56	2.25
7	*Pichia anomala*	1.13	2.25
8	*Hansenula polymorpha* CBS 4732	2.25	4.5

MIC: minimum inhibitory concentration by microbroth dilution method; MFC: minimum fungicidal concentration by streak plate method ($n = 3$; $P \leq 0.05$).

P. fermentans, and *H. polymorpha* (i.e., 2.5 mg/mL). *P. anomala* and *P. kluyveri* showed the lowest MIC values, 1.13 mg/mL and 0.56 mg/mL, respectively. MFC varied from 1.13 mg/mL to 9 mg/mL and showed a similar pattern to MIC; that is, *S. cerevisiae* and *A. pullulans* (9 mg/mL) had higher values than *Z. bailii*, *C. diversa*, *P. fermentans*, *H. polymorpha* (4.5 mg/mL), *P. anomala.* (2.25 mg/mL), and *P. kluyveri* (1.13 mg/mL). Silva et al. [20] reported the minimal inhibitory concentrations of the eucalyptus essential against different bacteria: *Streptococcus pyogenes* (MIC: 0.39 mg/mL), *Staphylococcus aureus* (MIC: 0.78 mg/mL), *Pseudomonas aeruginosa* (MIC: 1.56 mg/mL), and *Candida albicans* (MIC: 0.78 mg/mL). Damjanović-Vratnica et al. [14] reported that the MICs of eucalyptus oil from Montenegro against 17 microorganisms, including food poisoning and spoilage bacteria and human pathogens, varied between 0.3 and 3.13 mg/mL, which can be attributed to the different amount of active molecules observed in the tested eucalyptus oils. In fact, according to Soković et al. [23] and Inouye et al. [24], not only the major compounds (1,8-cineole) but also the minor ones (such as γ-terpinene, α-pinene, β-pinene, myrcene, and linalool) play a significant role in the antimicrobial activity.

3.3. Mixed Fruit Juice Preservation by Eucalyptus Oil and Thermal Treatment

3.3.1. Effect of Thermal Treatment. A thermal treatment for 30 and 60 s at 70°C did not have effect on the growth of *S. cerevisiae* in the mixed fruit juice samples (Figure 3(a)). Indeed, only a 0.49 log CFU/mL reduction was observed in samples, subjected to a thermal treatment for 90 sec, after eight days at room temperature. Hence, this kind of treatment was almost ineffective for preserving the juice. A similar pattern was also observed in our previous studies [25, 26].

3.3.2. Effect of Varying Concentrations of Eucalyptus Oil. Since eucalyptus oil was able to kill several food spoilage yeasts in *in vitro* tests, its activity in a real food system (mixed fruit juice) has also been studied. The reduction inviability of *S. cerevisiae* due to eucalyptus oil treatment in dose-dependent manner (MIC, 1/2 MIC, and 1/4 MIC level) and time-dependent manner (i.e., 0, 2, 4, and 8 days) was evaluated. As shown in Figure 3(b), a complete growth inhibition was observed in the mixed fruit juice when MIC levels of essential oil were used. However, 1/2 MIC and 1/4 MIC level samples showed a significant reduction in the final number of cells (3.2 log CFU/mL and 6.2 log CFU/mL, resp.) compared to untreated sample (7.2 log CFU/mL). These data represented that the yeast growth has been inhibited in a dose-dependent manner even in food matrix. As previously reported [27], different essential oils showed an excellent activity against food spoilage yeasts (*Saccharomyces cerevisiae*, *Candida rugosa*, *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Rhodotorula glutinis*, *Rhodotorula minuta*, *Trichosporon cutaneum*, *Yarrowia lipolytica,* and *Zygosaccharomyces rouxii*). For example, cardamom oil acted as a good antimicrobial agent in real system such as pine apple fruit juice [28], sweet orange juice [29], and apple juice [30]. In the present study, it is the first attempt to evaluate the antiyeast potential of eucalyptus oil in fruit juice mixture. In some cases, the natural compounds performed even better than the chemical preservatives [27].

3.3.3. Combined Effect of Eucalyptus Oil and Thermal Treatment. The combined effect of eucalyptus oil (at MIC, 1/2 MIC, and 1/4 MIC level) with thermal treatment (at 70°C for 30, 60, and 90 sec) on *S. cerevisiae* growth was determined in a time-dependent manner (i.e., 0, 2, 4, and 8 days) at room temperature (Figure 4). MIC and 1/2 MIC levels of eucalyptus oil combined with thermal treatment showed complete growth inhibition of *S. cerevisiae* after two days. In fact, the same growth recovery was also found in samples treated with only eucalyptus oil at 1/2 MIC dose up to

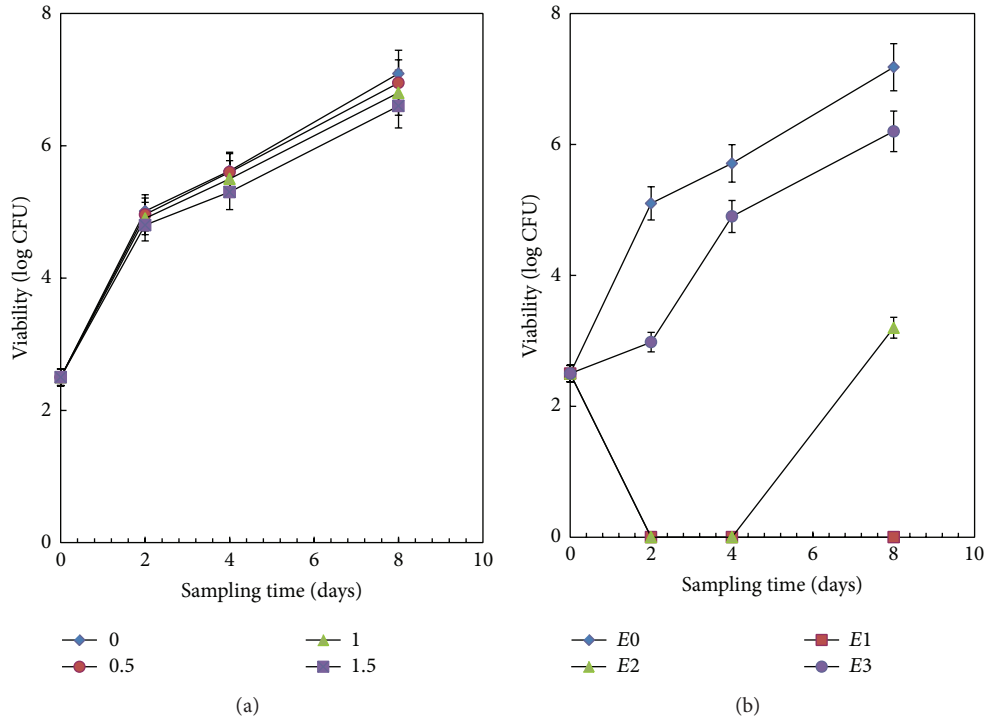

FIGURE 3: Variation in viability of *S. cerevisiae* SPA in fruit juice mixtures during storage after (a) thermal treatment at 70°C for 0.5, 1, and 1.5 min; (b) eucalyptus oil treatment at different concentrations (*E*1 = MIC, *E*2 = 1/2 MIC, *E*3 = 1/4 MIC level, and *E*0 = control). The data represents the mean of triplicate results and error bar represents standard deviation.

eight days of storage. However, the 30 and 60 sec thermal treatments combined with the oil produced a final reduction of 4.5 and 5.16 log CFU/mL, respectively (Figure 4), when compared with 3.98 log CFU/mL measured in those treated with essential oil alone (Figure 3(b)). Finally, the samples with 1/4 MIC level of the oil were not affected by 30 sec thermal treatment, if compared with Figure 3(b). Nevertheless, the 60 and 90 sec of thermal treatments reduced the growth by 0.89 to 1.90 log CFU/mL, respectively, as compared to the control.

It has been reported that the use of thermal treatment affects the volatile compounds by increasing their vapour pressure, which in turn improves the possibility to solubilize the yeast cell membrane. Though, the use of only one treatment cannot guarantee the microbial stability of the beverages without affecting the final organoleptic properties of the product [31]. The combination of thermal treatment with essential oils offers a very useful synergy whereby increase in temperatures during storage could enhance the vapour phase concentration of volatiles, thereby enhancing the antimicrobial activity for better food preservation [25]. In some of our previous reports [32–34], it was also observed that antimicrobial activity of essential oils was higher in vapour phase than in liquid phase, which was observed by different microscopic techniques: scanning electron microscope, transmission electron microscope, and atomic force microscope. Basically, the differences in inhibition of yeast strain obtained from essential oil (liquid phase, direct contact with the culture media) and the vapour can be attributed to the differences in diffusion coefficients of the antimicrobial compounds present in the eucalyptus oil when they have to diffuse in the agar compared to the diffusion in vapour phase [35]. In our study, the oil dose requirement was significantly reduced with the combination of the two treatments. This combination can be used as a better preservative with minimal impact on the organoleptic properties of the beverage. Even our previous studies using a combination of oils (mentha and lemongrass) and thermal treatment have been published [25, 26]; this is the first report that uses eucalyptus essential oil for fruit juice preservation against *S. cerevisiae*.

3.3.4. Principal Component Analysis. In order to confirm the interactive effects between the different variables (concentrations of eucalyptus essential oil and thermal treatments) on the yeast growth, a principal component analysis (PCA) was carried out. Figure 5 reports the PCA loadings plot on the first two factors of the samples. As expected, factor 1 (essential oil concentration) accounted for the great part of variability (about 94%) while factor 2 (thermal treatment) had a limited effect. In particular, four clusters can be identified. In the first, the control juice and the heat treated juices (without eucalyptus essential oil) were grouped. In the second, the juices added with 1/4 MIC thermal treated or not were grouped together. This cluster was characterized by a lower difference compared with cluster 1 in relation to factor 1. This means that the addition of this amount of oil had scarce activity on the effectiveness of heat treatment on yeast viability during storage. At last, clusters 3 and 4 were characterized by pronounced differences with respect to the

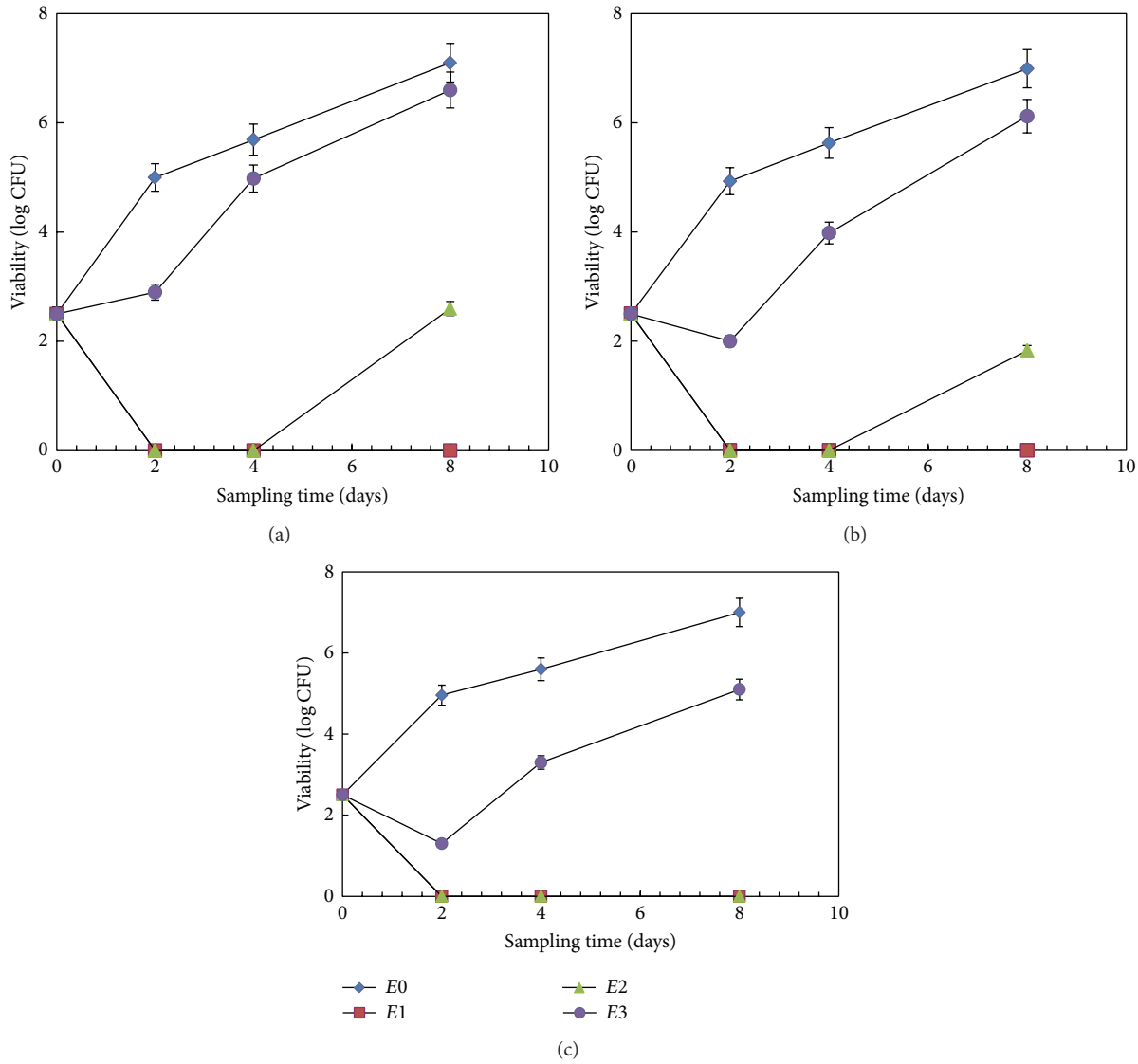

Figure 4: Combined effect of essential oil and thermal treatment. Variation in viability of *S. cerevisiae* SPA in fruit juice mixtures was estimated. Different concentrations of eucalyptus oil ($E1$ = MIC, $E2$ = 1/2 MIC, $E3$ = 1/4 MIC level, and $E0$ = control) combined with different thermal treatments at 70°C for (a) 30, (b) 60, and (c) 90 sec were tested. The growth of the yeast was followed up to 8 days after the treatment. The data represents the mean of triplicate results and error bar represents standard deviation.

sample without essential oil. Cluster 3 was formed by juices with 1/2 MIC, not treated and thermal treated for 30 and 60 seconds. The last cluster grouped all the samples having better antiyeast results (juices with MIC level and the sample with 1/2 MIC thermal treated for 90 seconds). The composition of the latter cluster highlights the equivalence of the antiyeast effectiveness of this last sample with the juices containing a double concentration of oil. This fact can allow for obtaining the same antiyeast effect using a concentration of essential oil with a lower impact on organoleptic profile of juices.

4. Conclusion

The results of this work demonstrated that eucalyptus essential oil could be used as a potential antimicrobial compound against food spoilage yeasts (*in vitro* and in a real food system). The chemical identification of the different molecules characterizing the eucalyptus oil evidenced the presence of oxygenated monoterpenes responsible for the antimicrobial activity. The use of the combination of eucalyptus essential oil with thermal treatment successfully inhibited the development of yeast (*S. cerevisiae* SPA) in fresh fruit juices. The results provide an excellent record of eucalyptus oil as antimicrobial agent and suggest its potential application for beverages preservation. Additional studies should be conducted to confirm the potentiality of eucalyptus essential oil in order to use it as a preservative in other food models.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

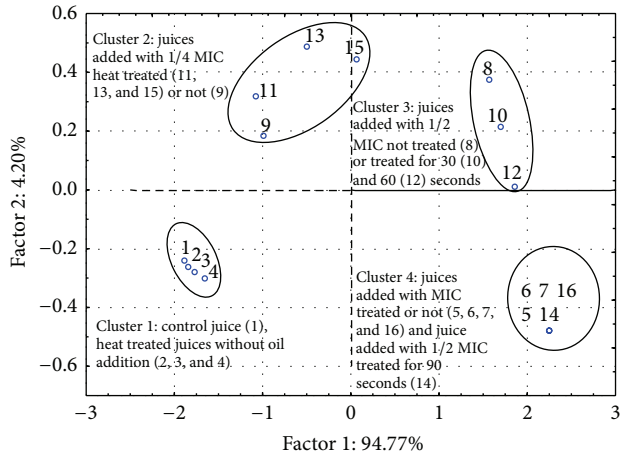

Figure 5: PCA loadings plot on the two first factors of control and treated juice. The clusters generated were as follows. Cluster 1: control juice (1), heat treated juices without essential oil (2, 3, and 4). Cluster 2: juices added with 1/4 MIC heat treated (11, 13, and 15) or not (9). Cluster 3: juices added with 1/2 MIC not treated (8) or treated for 30 (10) and 60 (12) seconds. Cluster 4: juices added with MIC treated or not (5, 6, 7, and 16) and juice added with 1/2 MIC treated for 90 seconds (14).

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Research Article

High-Level Antimicrobial Efficacy of Representative Mediterranean Natural Plant Extracts against Oral Microorganisms

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Nature is an unexplored reservoir of novel phytopharmaceuticals. Since biofilm-related oral diseases often correlate with antibiotic resistance, plant-derived antimicrobial agents could enhance existing treatment options. Therefore, the rationale of the present report was to examine the antimicrobial impact of Mediterranean natural extracts on oral microorganisms. Five different extracts from *Olea europaea*, mastic gum, and *Inula viscosa* were tested against ten bacteria and one *Candida albicans* strain. The extraction protocols were conducted according to established experimental procedures. Two antimicrobial assays—the minimum inhibitory concentration (MIC) assay and the minimum bactericidal concentration (MBC) assay—were applied. The screened extracts were found to be active against each of the tested microorganisms. *O. europaea* presented MIC and MBC ranges of 0.07–10.00 mg mL^{-1} and 0.60–10.00 mg mL^{-1}, respectively. The mean MBC values for mastic gum and *I. viscosa* were 0.07–10.00 mg mL^{-1} and 0.15–10.00 mg mL^{-1}, respectively. Extracts were less effective against *C. albicans* and exerted bactericidal effects at a concentration range of 0.07–5.00 mg mL^{-1} on strict anaerobic bacteria (*Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, and Parvimonas micra*). Ethyl acetate *I. viscosa* extract and total mastic extract showed considerable antimicrobial activity against oral microorganisms and could therefore be considered as alternative natural anti-infectious agents.

1. Introduction

The Hippocratic statement "Nature, without instruction or knowledge, does what is necessary" wisely acknowledges the healing contribution of the vegetable kingdom to the treatment of various diseases. The potential of a small fraction of natural plant extracts to cure serious infections of the human body has been well established to date [1–4]. In particular, various Mediterranean natural herb products have been screened profitably for their antioxidant and antimicrobial effects [5–8]. *Origanum dictamnus*, *Olea europaea*, Chios mastic gum, *Inula viscosa*, *Petroselinum crispum*, and *Sideritis* spp. are some of the most well-known examples [7, 9, 10]. Nevertheless, the biological behavior of over 300.000 existing plant species needs to be further studied [11].

The remarkable immunostimulatory activity of several plant species is also responsible for the low occurrence of infectious processes in their wild counterparts. The most known broad-spectrum defense mechanisms of plant-originated antimicrobial agents are related to the presence of "phytoalexins." The latter are small-molecule antibiotics (molecular weight: MW < 500) and according to their

classification include polyphenols, flavonoids, terpenoids, and glycosteroids [12]. In order to conquer the pathogenic attackers, the aforementioned mild antimicrobials usually act synergistically. Moreover, the production of callose, a sugar polymer with (1–3)-β-D-glucan subunits, at the microbial invasion sites on the plant cell wall constitutes a typical pathogen-specific response of vegetable organisms [13]. The secretion of resistance (*R*) proteins triggered by avirulence (Avr) genes in the presence of attacking microorganisms is also another microbe-specific defense of plants [14].

Bacteria, viruses, and fungi are the cause of numerous infectious diseases. They are official residents of the human body and are capable of forming biofilms, dynamic microbial networks, on human substrata [15]. In the oral cavity in particular, more than 700 microbial species have been recovered, either being in a planktonic form or being embedded in a polysaccharide-affluent extracellular layer [16]. Despite the unfriendly conditions on tooth surfaces and gingival tissues, oral microorganisms are skilled at surviving through complicated physicochemical intercommunication patterns with the oral substrata [17]. The harmful microbial impact on these surfaces results in the genesis of caries, gingivitis, or periodontitis.

Recent years have seen a focus in oral microbiology research on the elucidation and elimination of biofilm-related dental diseases [18, 19]. Microbial biofilm communities have been found to be up to 1000 times more resistant to antibiotics against their equivalent planktonic cultures, while multidrug-resistant oral microorganisms have been identified [19]. The presence of vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) underlines the limits of antibiotics in facing this primary health hazard with regard to the oral cavity [20]. Furthermore, an endocarditis-associated *Enterococcus faecalis* virulence gene named efaA has been recovered from therapy-resistant strains in root canals [21].

The need to discover new efficient treatment strategies against oral microbial species has raised interest in natural vegetable sources. Novel antimicrobial agents of plant origin aim to reintroduce traditional treatment paradigms to modern medicine. Indeed, plants exhibit a remarkable pharmaceutical range due to their secondary metabolism-mediated chemical responses to various microorganisms and the synergistic paradigms they develop. The latter prevent microbes from resisting antibiotic monotherapy and, therefore, becoming untreatable.

The aim of the present report was to examine the antimicrobial activity of natural plant and fruit extracts of Mediterranean origin against various microbial species. More specifically, five different extracts from olive leaves, table olives, mastic gum, and *Inula viscosa* (Syn. *Dittrichia viscosa*) were screened against a panel of nine relevant pathogenic microorganisms, which constitute typical residents of the oral microflora, including one strain of *Candida albicans*. Additionally, *Staphylococcus aureus* and *Escherichia coli*, normally a part of skin and intestinal flora, served as reference bacterial strains. The null hypothesis of this report was that the aforementioned extracts have no antimicrobial impact on the tested microorganisms. For this purpose, two antimicrobial assays—the minimum inhibitory concentration (MIC) assay and the minimum bactericidal concentration (MBC) assay—were utilized.

2. Materials and Methods

2.1. Extraction Process

2.1.1. Olive Leaves. *Olea europaea* leaves were dried in a well-ventilated shady place and subsequently stored in a dark room. Before their extraction, the leaves were ground using an Allen West type SCIS grinder with a sieve of 3 mm. The leaves (3.5 Kg) were extracted by mechanical stirring for 12 h with acetone (2 × 2.5 L). The extract was evaporated completely and washed with a mixture of CH_2Cl_2/MeOH 98 : 2 (3 L). The insoluble material was separated and dried under reduced pressure, producing a yellow powder (360 g) containing 60% oleuropein [22].

2.1.2. Table Olive Processing Wastewater. Olive fruits were cured for a period of 4 months, using an aqueous solution of 8% NaCl (w/v). The water extract (400 mL) was applied to Amberlite XAD-4 resin and the column eluted with 96% ethanol. After desorption, ethanol was evaporated by drying at 40°C and the phenolic fraction (1.34 g) was recovered [22, 23].

2.1.3. Conventional Extraction of Mastic Gum. A quantity of mastic gum (500 g) was diluted in ethyl acetate (500 mL). 1,500 mL of methanol was then added. After a period of 2 days, a layer of poly-β-myrcene (150 g) was decanted. Filtration was applied in order to obtain a clear supernatant solution and the solvent mixture was evaporated in a rotary evaporator at 45°C with an 80 kPa vacuum (extraction A). The resulting semisolid residue was dried in a desiccator at 70°C and 1,000-mbar vacuum and resulted in a white powder (350 g) [24].

2.1.4. Inula viscosa. *Inula viscosa* (Asteraceae) was extracted with pressurized liquid extraction. For that purpose, a Dionex accelerated solvent extraction (ASE) 300 System (Dionex, Sunnyvale, CA) with 100 mL stainless steel vessels was used. Specifically, 20 g of ground *I*. viscosa aerial parts was placed into tubular extraction cells. These were then placed into the carousel and the samples were extracted under the specified conditions: temperature: 70°C, pressure: 120 bar (preset by the instrument), preheat time: 1 min, heat time: 5 min, 2 extraction cycles of 5 min static time each, flush volume: 100%, and purge: 120 sec. Separate preparations of ethyl acetate extracts and methanol extracts were made. Analytical grade ethyl acetate and methanol were used and were evaporated to dryness under reduced pressure using a rotary evaporator (Buchi Rotavapor R-200) at 40°C. The obtained yields were 2.08 gr for the ethyl acetate extraction and 3.51 gr for the methanol.

2.2. Chemical Analysis of Extracts. The qualitative and quantitative determination of the tested extracts (olive leaves,

table olive processing wastewater, and *Inula viscosa*) was performed in a HPLC-DAD system: Thermo Finnigan HPLC system (Thermo Finnigan, San Jose, CA) coupled with a Spectral System UV6000LP PDA detector. A two-solvent gradient method was used: (A) H_2O and (B) acetonitrile. The flow rate was set at 1 mL/min and the following elution program was applied: 0–60 min linear gradient from 0% A to 50% B; 60–65 min linear gradient to 100% B; 65–70 min 100% B isocratic; 70–75 min linear gradient to 0% B; 75–85 min 0% B isocratic. The analysis was performed at 25°C and the injection volume was 20 μL. The detection was done at 280 nm and the column used was Supelco Analytical Discovery HS C18 (25 cm × 4.6 mm i.d., 5.0 μm). Standard solutions of oleuropein and hydroxytyrosol were prepared and run under the same conditions in the case of olive leaves and table olive processing wastewater extracts [22, 23].

Concerning mastic gum the tested extract was divided into two fractions, an acidic and a neutral one. The acidic fraction after several chromatographic separations afforded the major triterpenic acids: oleanonic acid, moronic acid, 24Z-masticadienonic acid, 24Z-isomasticadienonic acid, 24Z-masticadienolic acid, and 24Z-isomasticadienolic acid. The neutral fraction afforded five neutral triterpenic compounds: tirucallol, dammaradienone, 28-norolean-12-en-3-one, oleanonic aldehyde, and oleanolic aldehyde. All the above constituents were identified by NMR (1H, 13C, COSY, HMQC, and NOESY) and MS and by comparison with data in the literature. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker DRX 400 and Bruker AC 200 (50.3 MHz for 13C NMR) instruments at 295 K. Gas chromatography mass spectroscopy (MS) analysis was performed on a Finnigan GCQ Plus mass spectrometer [22].

2.3. Bacterial and Fungal Strains. A total of ten bacterial strains from eight different species and one *Candida albicans* strain were investigated. Eight of the tested bacterial strains and *C. albicans* occur in the oral flora, whereas *Staphylococcus aureus* and *Escherichia coli* are mainly recovered from the skin and intestinal flora, respectively. The latter two were used as reference microorganisms to specifically compare the oral inhibitory effect of natural extracts to their general antimicrobial activity. In particular, *Streptococcus mutans* DSM 20523, *Streptococcus sobrinus* DSM 20381, *Streptococcus oralis* ATCC 35037, *Enterococcus faecalis* ATCC 29212, and *S. aureus* ATCC 25923 are facultative anaerobic Gram-positive species, whereas *E. coli* ATCC 25922 is also facultative anaerobic but has a Gram-negative cell wall. *Porphyromonas gingivalis* W381, *Prevotella intermedia* ATCC 25611, *Fusobacterium nucleatum* ATCC 25586, and *Parvimonas micra* ATCC 23195 are obligate anaerobes. All bacterial and fungal strains were kindly supplied by the Division of Infectious Diseases and the Institute of Medical Microbiology and Hygiene of the Albert-Ludwigs University, Freiburg. The microorganisms were deposited at −80°C in basic growth medium containing 15% (v/v) glycerol until their use.

2.4. Determination of the Minimum Inhibitory Concentration (MIC). First, an overnight culture of each bacterial and fungal strain was prepared according to the CLSI guidelines [25, 26]. Each dilution was plated on Columbia blood agar (CBA) plates or yeast-cysteine blood agar (HCB) plates. Facultative anaerobic bacteria and *C. albicans* were incubated on CBA agar plates at 37°C and 5%–10% CO_2 atmosphere for 24 h. Anaerobic bacteria were placed on HCB agar plates at 37°C for 48 h (anaerobic chamber, Genbox BioMérieux SA, Marcy/Etoile, France). For the microdilution assay, all facultative anaerobic strains were inoculated in Mueller-Hinton broth (MHB), anaerobic bacteria in Wilkins-Chalgren broth (WCB), and *C. albicans* in Sabouraud dextrose broth (SDB), each to be tested at 10^6 colony forming units (CFU) mL^{-1}. Afterwards, appropriate volumes of the MHB/WCB/SDB microbial cultures were transferred into a 96-well microtiter-plate using a multichannel pipette. The prepared natural extracts were then dissolved in dimethyl sulfoxide (DMSO, Sigma, Steinheim, Germany) and diluted in aqua destillata. All extract solutions in DMSO were screened in a concentration series ranging from 10 mg mL^{-1} to 0.02 mg mL^{-1} at dilution levels starting from 2-fold to 512-fold. The experiments were performed in duplicate. A 0.5/1 A McFarland standard suspension was prepared in normal saline for bacteria and fungi, respectively. Each well of the 96-well microtiter-plate had a total volume of 200 μL. In order to exclude potential antimicrobial effects of the DMSO residuals, a dilution series of DMSO was examined in parallel. Wells containing solely MHB/WCB/SDB or 0.2% chlorhexidine (CHX) served as positive and negative controls for bacterial growth, respectively. The possibility of contamination was minimized by using sterile MHB/WCB/SDB. Thereafter, facultative anaerobic bacteria and *C. albicans* were incubated at 37°C and 5%–10% CO_2 atmosphere for 24 h, while anaerobic bacteria were kept at 37°C for 48 h (anaerobic chamber, Genbox BioMérieux SA, Marcy/Etoile, France). All assays for each bacterial and fungal strain were performed at least in duplicate. The highest minimum inhibitory concentration (MIC) values were taken into consideration in case the MIC values of a specific strain were not identical. MIC was defined as the lowest concentration of each natural extract at which visible inhibition of bacterial growth was induced and was thus expressed by the percentage of bacterial growth at that particular concentration. The inhibitory impact of DMSO was taken into consideration if bacterial growth was observed in the cotested DMSO dilution series.

2.5. Determination of the Minimum Bactericidal Concentration (MBC). The minimum bactericidal concentration (MBC) was also assessed according to the CLSI guidelines [25, 26]. After completion of the MIC assay, the aforementioned 96-well microtiter-plates were further incubated for MBC testing. In brief, 10 μL from each well containing the tested natural extract concentration series was plated on Columbia blood agar (CBA) or yeast-cysteine blood agar (HCB) plates. In particular, the facultative anaerobic bacteria and *C. albicans* were incubated on CBA plates at 37°C and 5%–10% CO_2 atmosphere for 2 days. Strictly anaerobic bacteria were cultivated on HCB plates at 37°C for 5 days (anaerobic chamber, Genbox BioMérieux SA, Marcy/Etoile,

France). The colony forming units (CFU) were determined visually. The MBC was defined as the concentration at which a three-log decrease in bacterial growth (=99.9%) was detected compared to the positive control.

3. Results

3.1. Olea europaea. Two extracts produced from olive processing byproducts were tested. Extraction of olive leaves with acetone afforded a polar fraction which was defatted with a mixture of CH_2Cl_2/MeOH 98 : 2. The obtained extract contained 60% oleuropein. The extract coming from table olive processing wastewater contained as its main compound, the degradation product of oleuropein, hydroxytyrosol, in a percentage around 15%.

The mean MIC and MBC values for each of the *O. europaea* extracts as well as the tested bacterial/fungal strains are presented in Table 1.

In general, table olive extract was more active than olive leaf extract. It was effective against almost all of the tested microorganisms, with a mean concentration range of 0.15 mg mL^{-1} (*Porphyromonas gingivalis, Parvimonas micra*) to 10.00 mg mL^{-1} (*Candida albicans*). For the facultative anaerobic bacteria MIC values varied between 1.25 mg mL^{-1} (*Streptococcus mutans*, *Streptococcus oralis*) and 5.00 mg mL^{-1} (*Enterococcus faecalis*). The MBC values of the table olive extract ranged from 0.60 to 10.00 mg mL^{-1}. Obligate anaerobes (*Prevotella intermedia*, *P. micra*) were more easily eradicated (0.60 mg mL^{-1}) when compared to *E. faecalis* and *C. albicans* (10.00 mg mL^{-1}).

Olive leaf extracts showed a milder inhibitory effect against oral pathogens. The MIC values of the eradicated microbial strains were between 0.07 mg mL^{-1} (*S. oralis*) and 10.00 mg mL^{-1} (*C. albicans*, *Escherichia coli*). The MBC values demonstrated the persistence of facultative anaerobes in the presence of *O. europaea* leaf extracts (10.00 mg mL^{-1}). However, *P. gingivalis* was inhibited by 0.60 mg mL^{-1} of the extract, while 1.25 mg mL^{-1} killed 99.9% of *Fusobacterium nucleatum* and *P. micra*.

3.2. Mastic Gum. Table 2 summarizes the MIC and MBC values of the tested mastic gum extract for all screened microbial strains. A total mastic extract without polymer was prepared after removal of the contained insoluble polymer. The extensive characterization of the extract by NMR and MS revealed that it contained triterpenic acids, triterpenic alcohols, and aldehydes.

Total mastic extract was effective against all of the microorganisms with MIC values ranging from 0.02 mg mL^{-1} (*P. gingivalis*) to 10 mg mL^{-1}. The mean MBC values were between 0.07 mg mL^{-1} (*P. gingivalis*, *P. micra*) and 10.00 mg mL^{-1} (*S. mutans*, *S. sobrinus*, *E. faecalis*, *C. albicans*, and *E. coli*). Extract concentrations between 0.07 and 2.50 mg mL^{-1} exerted bactericidal effect mainly on strict anaerobic, Gram-negative bacteria (*P. gingivalis*, *P. intermedia*, and *F. nucleatum*).

3.3. Inula viscosa. Table 3 summarizes the range of MIC and MBC values of the two tested *I. viscosa* extracts against the selected oral bacterial/fungal strains. Aerial parts of *I. viscosa* were extracted with two solvents of different polarity, following a separate extraction. Ethyl acetate extract afforded medium polarity compounds, mainly flavonoids such as 3-*O*-acetylpadmatin, 7-*O*-methylaromadendrin, hispidulin, apigenin, luteolin, sesquiterpenic compounds, and triterpenoids. While the methanol extract contained some common compounds with the ethyl acetate extract, phenolic acids and flavonoids were also detected.

Among the two *I. viscosa* extracts, ethyl acetate extract presented a more robust antimicrobial effect against the screened microorganisms compared to the methanol extract. Under its influence, a mean inhibitory concentration range of 0.07 mg mL^{-1} (*P. gingivalis*) to 2.50 mg mL^{-1} (*S. sobrinus*, *E. faecalis*, and *E. coli*) was observed. The MBC values of the ethyl acetate extract varied from 0.15 to 5.00 mg mL^{-1}. Obligate anaerobes such as *P. gingivalis* (0.15 mg mL^{-1}) were efficiently eliminated by reduced extract concentrations as compared to the more persistent *E. faecalis* and *E. coli* strains (5.00 mg mL^{-1}).

The *I. viscosa* methanol extract also had a considerable inhibitory impact on oral bacteria and fungi. The MIC values of the eradicated microorganisms ranged between 0.15 mg mL^{-1} (*P. gingivalis*) and 10.00 mg mL^{-1} (*E. coli*). The MBC values ranged from 0.30 to 10.00 mg mL^{-1}. Hence, *P. gingivalis* was eliminated by 0.30 mg mL^{-1} of the extract, while 0.60 mg mL^{-1} induced a three-log reduction in bacterial growth for *P. intermedia* and *S. oralis*.

4. Discussion

Taking the antibiotic resistance of oral biofilms into consideration, the present study aimed to introduce novel plant-derived antimicrobial agents in the treatment of therapy-persistent dental diseases. Hence, the antimicrobial impact of five different Mediterranean natural plant and fruit extracts was investigated on representative oral bacterial strains and *C. albicans*. To the best of our knowledge, this is the first report on the antimicrobial efficacy of *Olea europaea*, mastic gum, and *Inula viscosa* against microorganisms which belong to the commensal flora of the oral cavity.

In this study, the tested *O. europaea* extracts were found to be especially effective against Gram-negative, anaerobic periodontal pathogens such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum*. Indeed, there are several reports on the excellent antibacterial activity of *O. europaea* extracts and pure compounds using diverse microbial species [7, 27–29]. Sudjana et al. substantiated the narrow-spectrum antibacterial action of commercial olive leaf extract against *Campylobacter jejuni*, *Helicobacter pylori*, and *Staphylococcus aureus,* as well as against methicillin-resistant *S. aureus* (MRSA) [30]. This highlights the regulatory interference of *O. europaea* extracts with gastric Gram-negative microorganisms such as *C. jejuni* and *H. pylori*. In another report olive leaf extracts succeeded in eliminating the Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*,

Table 1: Antimicrobial activity in mg mL^{-1} of olive leaf and table olives extracts (*O. europaea*).

	Olea europaea					
Sample	Olive leaf extract		Table olives extract		DMSO (%)	
c/mg mL^{-1}	MIC*	MBC**	MIC	MBC	MIC	MBC
Streptococcus mutans DSM 20523	1.25	NA	1.25	5.00	5.00	40.00
Streptococcus sobrinus DSM 20381	2.50	NA	2.50	5.00	20.00	20.00
Streptococcus oralis ATCC 35037	0.07	NA	1.25	2.50	10.00	20.00
Enterococcus faecalis ATCC 29212	0.60	NA	5.00	NA	20.00	80.00
Candida albicans DSM 1386	NA	NA	NA	NA	10.00	10.00
Escherichia coli ATCC 25922	NA	NA	5.00	NA	20.00	40.00
Staphylococcus aureus ATCC 25923	2.50	5.00	2.50	2.50	20.00	40.00
Porphyromonas gingivalis W381	0.30	0.60	0.15	1.25	5.00	20.00
Prevotella intermedia ATCC 25611	2.50	5.00	0.30	0.60	2.50	5.00
Fusobacterium nucleatum ATCC 25586	0.60	1.25	0.60	2.50	10.00	10.00
Parvimonas micra ATCC 23195	0.30	1.25	0.15	0.60	10.00	20.00

NA: no activity observed; MIC or MBC was measured at 10.00 mg mL^{-1}.
*MIC: extract concentration at which the optical density (OD) measurement revealed minimal bacterial growth.
**MBC: extract concentration at which a three-log reduction (99.9%) of the bacterial growth was induced.

Table 2: Antimicrobial activity in mg mL^{-1} of four mastic gum extracts.

	Mastic gum			
Sample	Total mastic extract		DMSO (%)	
c/mg mL^{-1}	MIC*	MBC**	MIC	MBC
Streptococcus mutans DSM 20523	NA	NA	5.00	40.00
Streptococcus sobrinus DSM 20381	NA	NA	10.00	20.00
Streptococcus oralis ATCC 35037	2.50	5.00	10.00	20.00
Enterococcus faecalis ATCC 29212	NA	NA	10.00	80.00
Candida albicans DSM 1386	NA	NA	10.00	10.00
Escherichia coli ATCC 25922	NA	NA	10.00	10.00
Staphylococcus aureus ATCC 25923	2.50	5.00	20.00	80.00
Porphyromonas gingivalis W381	0.02	0.07	10.00	20.00
Prevotella intermedia ATCC 25611	0.07	0.30	2.50	5.00
Fusobacterium nucleatum ATCC 25586	2.50	2.50	10.00	20.00
Parvimonas micra ATCC 23195	0.60	0.60	2.50	10.00

NA: no activity observed; MIC or MBC was measured at 10.00 mg mL^{-1}.
*MIC: extract concentration at which a three-log reduction (99.9%) of the bacterial growth was induced.
**MBC: extract concentration at which a three-log reduction (99.99%) of the bacterial growth was induced.

and *Klebsiella pneumoniae* [31]. However, Gram-negative microorganisms are often resistant to conventional antimicrobial drugs because of their expression of active efflux pumps [32]. Their tenacious nature is also assisted by the release of degrading enzymes and molecular metamorphosis of antibiotic targets [33]. Moreover, the overdelicate structure of Gram-negative bacteria, which involves structural discrepancies between the cell wall and exterior membrane, influences their susceptibility to various antimicrobial agents [34].

Furthermore, table olive extract presented milder antistaphylococcal activity, suggesting an additional impact on Gram-positive bacteria. A previous report described the antimicrobial efficacy of *O. europaea* leaves towards the Gram-positive *Bacillus cereus* [27]. The exact infiltration and destruction mechanism of the Gram-positive cell wall by *O. europaea* extracts probably implies the presence of antiquorum sensing (QS) compounds [35]. Since the pathogenicity of Gram-positive microorganisms such as *S. aureus* is based on small peptides named "quormones" triggered by the *agr* operon or analogous QS communication patterns, *O. europaea* could assumingly interfere with their action [36]. Contrary to the results of an earlier study, *Candida albicans* was not found susceptible to table olive extract [27]. The conflicting outcomes can be attributed to the differing extraction methods which were used, including boiling. However, which of the numerous phenolic or other compounds was responsible for this favorable effect remains unknown. In the present study, the two main compounds of the extracts were oleuropein in olive leaves and hydroxytyrosol in table olive processing wastewater. A lot of studies claim the strong antimicrobial activity of the two compounds [22, 37].

Table 3: Antimicrobial activity in mg mL^{-1} of *I. viscosa* extracts.

	Inula viscosa					
Sample	Ethyl acetate extract		Methanol extract		DMSO (%)	
c/mg mL^{-1}	MIC*	MBC**	MIC	MBC	MIC	MBC
Streptococcus mutans DSM 20523	0.15	1.25	1.25	5.00	5.00	40.00
Streptococcus sobrinus DSM 20381	2.50	2.50	2.50	5.00	20.00	20.00
Streptococcus oralis ATCC 35037	0.30	0.30	0.60	0.60	10.00	20.00
Enterococcus faecalis ATCC 29212	2.50	5.00	5.00	5.00	20.00	80.00
Candida albicans DSM 1386	1.25	2.50	5.00	5.00	10.00	10.00
Escherichia coli ATCC 25922	2.50	5.00	NA	NA	20.00	40.00
Staphylococcus aureus ATCC 25923	0.60	1.25	1.25	2.50	20.00	40.00
Porphyromonas gingivalis W381	0.07	0.15	0.15	0.30	5.00	20.00
Prevotella intermedia ATCC 25611	0.15	0.30	0.30	0.60	2.50	5.00
Fusobacterium nucleatum ATCC 25586	0.30	0.60	0.60	1.25	10.00	10.00
Parvimonas micra ATCC 23195	0.15	0.60	0.60	1.25	10.00	20.00

NA: no activity observed; MIC or MBC was measured at 10.00 mg mL^{-1}.
*MIC: extract concentration at which the optical density (OD) measurement revealed minimal bacterial growth.
**MBC: extract concentration at which a three-log reduction (99.9%) of the bacterial growth was induced.

Mastic gum constitutes a stem-derived resinous exudate of the Mediterranean tree *Pistacia lentiscus* var. *chia. P. gingivalis*, *P. intermedia*, *F. nucleatum*, and *P. micra* demonstrated high susceptibility toall of the mastic gum extracts studied. The numerous reports on the favorable antimicrobial properties of mastic gum also underline its effectiveness against various pathogens [6, 24, 38–40]. More specifically, a commercial product containing mastic in the liquid form (2%) showed a narrow antibacterial spectrum against Gram-negative *P. gingivalis* and *Prevotella melaninogenica* [40]. Gram-positive bacteria (*Streptococcus mutans*, *S. aureus*) as well as fungi (*C. albicans*) were not influenced. Takahashi et al. confirmed a decrease in the total number of bacterial salivary colonies after 4 hours of chewing mastic gum [41]. Since side effects such as tooth discoloration and enhanced cell toxicity accompany the application of conventional oral antibacterial chemicals such as chlorhexidine (CHX), the introduction of mastic-derived mouth rinses could promote antiplaque activity with minimal drawbacks.

In general, mastic gum is composed of various volatile ingredients, the most important of which are α-pinene, β-myrcene, β-caryophyllene, β-pinene, and limonene [6]. The herein tested extract, though, contained mostly triterpenic acids, which could be the active principles. It was previously found that total mastic extract without polymer containing mostly major triterpenic acids can efficiently eliminate other persistent microorganisms such as *H. pylori*. Reduced activity was observed for the triterpenic alcohols and aldehydes [24]. The additionally contained triterpenic alcohols and aldehydes have not showed a respective antimicrobial action. Koutsoudaki et al. detected moderate to high susceptibility of *B. subtilis* and *S. aureus* to β-myrcene, while *E. coli* exhibited no sensitivity to the substance [39]. Furthermore, β-pinene proved to be ineffective against *E. coli* and *S. aureus,* whereas *B. subtilis* showed only a mild response. These varying outcomes suggest that a synergistic activity of the different components of mastic gum extracts may be responsible for its antimicrobial action. Thus, the results of the present study indicate that the interdependent antimicrobial activity of the particular mastic ingredients focuses on eradicating therapy-resistant Gram-negative oral microorganisms.

In this study, *I. viscosa* was highly effective against not only Gram-negative anaerobic pathogens, but also streptococci (*S. mutans*, *S. oralis*). Although its advantageous effects in the oral cavity were demonstrated here for the first time, there are many reports appraising its general antibacterial, antifungal, and antiviral properties [42–45]. The difference between the activities of the two extracts could be attributed to the different chemical profile. Generally the two extracts contain a lot of common metabolites; however, the most polar ones are found at the methanol extract. In a previous report, methanol extracts of the plant demonstrated effective antimicrobial behavior against Gram-positive bacteria such as *S. aureus,* and *E. faecalis*, *C. albicans*, and *Candida tropicalis* were also found to be sensitive to *I. viscosa*, whereas Gram-negative bacteria were less susceptible [45]. The antimicrobial impact of *I. viscosa* was also observed on *Bacillus cereus* and *Salmonella typhimurium*. In addition, it was revealed that a compound of the plant named 3,3′-di-O-methylquercetin can deteriorate the cytoplasmic membranes after penetrating various bacterial cell walls [44]. Moreover, Wang et al. reported that *I. viscosa* presented fungicidal properties against pathogens of the families Oomycetes, Ascomycetes, and Basidiomycetes [46]. This is in agreement with the findings of this study, in which *I. viscosa* exerted bactericidal effects at a concentration range of 2.50–5.00 mg mL^{-1} towards *C. albicans*.

Medicinal plants have proven to be a bountiful reservoir of numerous biologically active components with advanced antibacterial properties. It is well known that synthetic chemicals, which are commonly used in dental products such as toothpastes and oral mouthwashes, can induce tooth discoloration, cell toxicity, regurgitation, or diarrhea [47, 48]. Thus, plant extracts are considered to be potent alternative

compounds for the new generation of oral pharmaceuticals. In fact, novel plant-derived constituents of dental chemotherapeutics (mouth rinses, irrigation solutions, and intracanal medicaments) are crucial future sources of the dental industry specializing in plaque-control strategies, since they allow for efficient treatment regarding antibiotic-resistant pathogens, immunocompromised individuals, and financially weak developing countries.

5. Conclusion

In conclusion, the results of this report promote interest in the discovery of alternative natural compounds with antimicrobial activity against therapy-resistant oral microorganisms. Overall, extracts from *O. europaea*, mastic gum, and *I. viscosa* were active against the tested oral pathogens, especially Gram-negative anaerobic bacteria, and could therefore be considered as alternative natural anti-infectious agents which could be used against periodontitis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Use of Antimicrobial Films and Edible Coatings Incorporating Chemical and Biological Preservatives to Control Growth of *Listeria monocytogenes* on Cold Smoked Salmon

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The relatively high incidence of *Listeria monocytogenes* in cold smoked salmon (CSS) is of concern as it is a refrigerated processed food of extended durability (REPFED). The objectives of this study were to compare and optimize the antimicrobial effectiveness of films and coatings incorporating nisin (Nis) and sodium lactate (SL), sodium diacetate (SD), potassium sorbate (PS), and/or sodium benzoate (SB) in binary or ternary combinations on CSS. Surface treatments incorporating Nis (25000 IU/mL) in combination with PS (0.3%) and SB (0.1%) had the highest inhibitory activity, reducing the population of *L. monocytogenes* by a maximum of 3.3 log CFU/cm^2 (films) and 2.9 log CFU/cm^2 (coatings) relative to control samples after 10 days of storage at 21°C. During refrigerated storage, coatings were more effective in inhibiting growth of *L. monocytogenes* than their film counterparts. Cellulose-based coatings incorporating Nis, PS, and SB reduced the population of *L. monocytogenes*, and anaerobic and aerobic spoilage flora by a maximum of 4.2, 4.8, and 4.9 log CFU/cm^2, respectively, after 4 weeks of refrigerated storage. This study highlights the effectiveness of cellulose-based edible coatings incorporating generally regarded as safe (GRAS) natural and chemical antimicrobials to inhibit the development of *L. monocytogenes* and spoilage microflora thus enhancing the safety and quality of CSS.

1. Introduction

Listeria monocytogenes has long been established as an important food-borne pathogen with a fatality rate of 25–30% [1]. However, the incidence of food-related listeriosis has increased dramatically in the last few years, where *L. monocytogenes* has been listed in the top five highest-ranking pathogens with respect to the total cost of foodborne illness in the United States in terms of loss of income by the affected individual, cost of health care, loss of productivity due to absenteeism, costs of investigations of an outbreak, loss of income due to closure of businesses, consumer litigations, or losses of product sales when consumers avoid particular products [2].

L. monocytogenes infection has been associated with consuming a variety of meat, poultry, fish, and dairy products [3]. The prevalence of this organism in cold smoked fish in particular is relatively high and typically between 10 to 40% [4, 5]. This high prevalence is likely due to the low temperature inherent with cold smoking process. Indeed, this condition would be congenial for the proliferation of *L. monocytogenes* if the raw salmon harbored the pathogen or acquired it from the processing environment [6]. Hence, processing of cold smoked salmon (CSS) includes no recognizable critical control point for *L. monocytogenes* and therefore this product cannot be completely free of this pathogen [7]. For this reason, smoked seafood, including CSS, has been categorized as having a high risk of listeriosis [7].

Since postprocess contamination of smoked fish with *L. monocytogenes* is highly problematic, antimicrobial additives are sought after to prevent growth of this bacterium

in food products and to ensure safety. Several studies have reported the effectiveness of nisin (a polycyclic antibacterial peptide) in delaying and reducing growth of *Listeria* spp. in model systems [8, 9] and in RTE products [10] including CSS [11, 12]. Nisin is a heat-stable bacteriocin that kills sensitive pathogens by disrupting their cell membranes, leading to leakage of cellular material and ultimately cell death [13]. In the United States, nisin has a generally regarded as safe (GRAS) status for use in pasteurized processed cheese and petitions have been filed for its use in other products as well [14, 15]. The efficacy of nisin as an antimicrobial agent in raw or minimally processed seafood is limited by several factors [16]. These include the potential for proteolytic activity in raw food that would cause rapid degradation of nisin [17], as well as the rapid decrease in the antimicrobial activity of nisin due to increased resistance of the pathogens [18].

Thus, a multiple-hurdle approach relying on the combination of nisin with other antimicrobials, such as chemical preservatives, is desirable. Sodium lactate (SL) is a GRAS additive that is widely used to enhance flavor, control microbial growth, and increase shelf life of meat, poultry, and fish products [19–22]. The use of lactates as antimicrobial agents is primarily due to their ability to reduce pH and water activity. Currently, the addition of SL is allowed at 4.8% for the decontamination of seafood products [16]. Sodium diacetate (SD), a derivative of acetic acid, is used to achieve an antimicrobial effect in baked goods, fats and oils, gravies and sauces, snack foods, meat products, and soups and soup mixes, as well as to flavor these foods [16]. It is also a GRAS substance recommended for use at levels not exceeding 0.25% [16]. Potassium sorbate (PS) is a salt of sorbic acid and common usage levels of PS in various food products have ranged from 0.5 to 1.0% [23]. Depending on the processing conditions, PS is usually applied to whole or eviscerated fish or fillets, prior to or immediately after smoking [24]. Sorbates may be used at a level not exceeding 0.3%, and at this concentration sorbates do not contribute to flavor [23]. Sodium benzoate (SB) is also used as an antimicrobial agent and is currently allowed at 0.1% [16]. These chemical preservatives have been shown to inhibit growth of gram-positive bacterial pathogens such as *L. monocytogenes* in media, meat [25–30], and seafood [31, 32].

Antimicrobial coatings and films allow the controlled diffusion and gradual release of embedded antimicrobials onto the food surface [33]. Significant inroads have been made in antimicrobial packaging to control the proliferation of *L. monocytogenes* on CSS. Neetoo et al. [34] found that alginate coatings supplemented with 2.4% SL and 0.25% SD significantly delayed the growth of *L. monocytogenes* in CSS during a 30-day storage at 4°C. Moreover, Ye et al. [35] showed that prior frozen storage enhanced the effect of alginate-based coatings and chitosan-based films incorporated with SL (1.2 or 2.4% w/w) or SD (0.125 or 0.25% w/w) against *L. monocytogenes* on CSS during subsequent refrigerated storage. Reductions ranging from 0.5 to 4.5 log CFU/cm^2 compared to uncoated samples were reported. Growth of *L. monocytogenes* on the surface of CSS was inhibited by whey-protein films incorporating a lactoperoxidase system [36]. Seydim and Sarikus [37] showed that strong antimicrobial activity of oregano essential oil impregnated in whey-protein isolate-based edible films against *L. monocytogenes*. Similarly, Tammineni et al. [38] developed an edible antimicrobial film using potato peel waste incorporating oregano essential oil against *L. monocytogenes* on CSS. The films reduced the inoculum by greater than 2 log CFU/g during storage at 4°C for 28 days. However, all aforementioned studies have tested the antilisterial efficacy of films or coatings in isolation and lack a systematic comparison of the relative efficacy of each approach.

The objective of this study was to compare cellulose-coated plastic films and edible cellulose-based coatings incorporating binary or ternary combinations of food-approved antimicrobials to inhibit growth of *L. monocytogenes* and spoilage microflora on vacuum-packaged CSS during refrigerated storage (4°C).

2. Materials and Methods

2.1. Listeria monocytogenes Inoculum Preparation. Five *L. monocytogenes* strains, PSU1 (serotype 1/2a), PSU21 (serotype 4b), PSU9 (serotype 1/2b), F5069 (serotype 4b), and Scott A (serotype 4b) (Courtesy of Rolf Joerger, University of Delaware), were used. The strains were maintained on tryptic soy agar plus 0.6% yeast extract (TSAYE) plates and stored at 4°C. Each strain was grown independently in tryptic soy broth plus 0.6% yeast extract (TSBYE) for 24 h at 37°C and a loopful of each overnight culture was transferred to 10 mL of fresh TSBYE and incubated at 37°C for 24 h. On the day of the experiment, 1 mL volume of each culture was combined to provide a five-strain mixture and then readjusted with 0.1% peptone water to a final cell density of ca. 10^8 CFU/mL, which served as the inoculum. Serial dilutions were plated onto TSAYE plates and incubated at 37°C for 24 h to determine cell numbers.

2.2. Inoculation of CSS Samples. Freshly processed CSS (*Salmo salar*) was obtained from a producer. It was kept frozen at −20°C and thawed at 2 ± 2°C (<4°C) for 1 day immediately before use. Slices of CSS were punched aseptically into 5.7 cm diameter round pieces weighing 10 ± 1 g. The samples were surface-inoculated with a 10^8 CFU/mL dilution of the five-strain cocktail of *L. monocytogenes* to achieve final concentrations of 10^5 CFU/cm^2 (or 5×10^5 CFU/g) of salmon surface. After inoculation, salmon samples were kept at room temperature for 30 min to allow bacterial attachment. *L. monocytogenes* populations in CSS are generally low (1–10^3 CFU/g) with 90–99% of cases below 10^2 CFU/g and less than 1% between 10^3 and 10^4 CFU/g as reported by Jørgensen and Huss [39] and Farber and Peterkin [40]. However, pathogen levels as high as 10^5–10^7 CFU/g have also been reported previously [1]. In this experiment, we used an initial load of ~5 log CFU/g to provide a worst-case scenario. From

a risk assessment point of view, using an inoculum size of 5 log CFU/g to test the antilisterial efficacy of antimicrobial films and coatings provides greater confidence or assurance.

2.3. Antilisterial Effectiveness of Films and Coatings Incorporating Binary Combinations of Antimicrobials

2.3.1. Preparation of Coating Solution. Methylcellulose (MC; 7.0 g) and hydroxypropylmethylcellulose (HPMC; 3.0 g) were mixed with 200 mL of 95% ethanol and 200 mL of sterile distilled water and stirred to which 6 mL of polyethylene glycol 400 was subsequently added. This coating stock solution was then supplemented with the various antimicrobial preparations. Briefly, 1.3 g of nisin was dissolved in 60 mL of 0.02 M acetic acid, and 12 mL of the prepared nisin solution was then supplemented with either 1.29 g SD, 1.54 g PS, 0.51 g SB, or 2.57 g of a-60% SL syrup. The antimicrobial solution was then made up to 50 mL with the MC/HPMC carrier solution. The solution was sufficient to coat 10 salmon discs.

2.3.2. Preparation of Films. The MC and HPMC were mixed with ethanol and water to prepare a coating solution as described above. The coating solution was then supplemented with the various antimicrobials. Briefly, 3.0 g nisin was dissolved in 60 mL 0.02 M acetic acid, and 12 mL of the prepared nisin solution was then supplemented with 3.0 g SD, 3.6 g PS, 1.2 g SB, or 6 g of 60% of SL syrup. The antimicrobial solution was then made up to 42 mL with the MC/HPMC carrier solution. The solution was then used to coat 3 glass plates, lined with LDPE films covering a surface area of 400 cm^2 each, using a thin layer chromatography (TLC) plate coater.

2.3.3. Treatment of Inoculated CSS Samples with Antimicrobial Coatings and Films. The inoculated CSS discs were coated with a 500 μL aliquot of a HPMC/MC coating solution containing nisin (25,000 IU/mL) alone or in combination with SL (0.3%), SD (0.25%), PS (0.3%), or SB (0.1%) as summarized in Table 1. The samples were then air-dried by leaving them in a laminar-flow hood under ventilation for 20 min. Samples were then flipped and similarly coated with an equal volume of the antimicrobial coating solution followed by drying. Alternatively, inoculated CSS discs were wrapped in different antimicrobial-coated films as shown in Table 1.

Inoculated samples without films or coatings were also prepared as untreated controls. Controls and treated samples (film-wrapped or coated) were then inserted into 3 mm thick high barrier pouches and subsequently sealed using a vacuum-packaging machine. The samples were stored at room temperature (21°C) for 10 days and analyzed microbiologically every other day.

2.3.4. Enumeration of L. monocytogenes from Samples. For microbial analysis, the package was aseptically cut and the sample transferred to a sterile stomacher bag that contained 40 mL of 0.1% sterile peptone water and stomached for 2 min. Serial dilutions were made in 0.1% peptone water and counts of *L. monocytogenes* were determined by an overlay method [41]. Briefly, the serial dilutions were spread-plated on solidified TSAYE and the plates were incubated at 35°C for 3 h. where Modified Oxford Medium (~7 mL) tempered at 45°C was overlaid on the plates. These plates were then incubated at 35°C for 48 h and small black colonies with black haloes were counted. Occasionally, suspect colonies were confirmed using a BAX for Screening/*Listeria monocytogenes* PCR assay. The numbers of *L. monocytogenes* per cm^2 of salmon were calculated by dividing the total count of *L. monocytogenes* per salmon disc by the total surface area (51.4 cm^2). The absence of the pathogen in the CSS samples was confirmed by a primary enrichment in UVM broth (Difco Laboratories) and a secondary enrichment in Fraser broth (Difco Laboratories) according to the USDA Microbiology Laboratory Guidebook [42].

2.4. Antilisterial Effectiveness of Films and Coatings Incorporating Ternary Combinations of Antimicrobials. The CSS samples were inoculated to a final concentration of 10^5 CFU/cm^2 of salmon surface. Antimicrobials with the highest antilisterial activity (PS and SB) were selected for further testing. Inoculated salmon discs were coated with a 500 μL aliquot of HPMC/MC coating solution on each side containing Nis (25000 IU/mL) with PS (0, 0.15 or 0.3%) or Nis (25000 IU/mL) with PS (0, 0.15, or 0.3%) and SB (0, 0.05, or 0.1%). The samples were air-dried and packaged. In addition, inoculated CSS samples were wrapped with LDPE films coated with a solution containing Nis (25000 IU/mL) with PS (0, 0.15, or 0.3%) or Nis (25000 IU/mL) with PS (0, 0.15, or 0.3%) and SB (0, 0.05, or 0.1%). Samples were then packaged and stored at 21°C for 10 days. The formulations for the various binary or ternary combinations of antimicrobials incorporated in films or coatings are summarized in Table 2. Samples were then microbiologically analyzed for *L. monocytogenes* as described earlier.

2.5. Effectiveness of Films and Coatings Containing Selected Antimicrobial Combinations against L. monocytogenes and Spoilage Microflora. The CSS samples were surface-inoculated to a final concentration of approximately 10^3 CFU/cm^2 (or 5×10^3 CFU/g). Uninoculated samples were also prepared. Inoculated and uninoculated samples were wrapped with film or coated with an antimicrobial coating solution, packaged, and stored at 4°C for 4 weeks. The combinations of antimicrobials incorporated in films and coatings for the refrigerated storage study are summarized in Table 3.

Inoculated samples were analyzed weekly for *L. monocytogenes* as described previously. Uninoculated samples were also analyzed weekly for spoilage aerobic and anaerobic bacteria. Spoilage anaerobic bacterial counts were determined by plating on Liver Veal Agar and plates incubated in anaerobic jars with anaerobic GasPak (BBL) for two days at 35°C. Aerobic bacterial counts were determined by plating onto TSAYE and plates incubated aerobically for two days at 35°C.

2.6. Statistical Analysis. Three independent trials were conducted for all experiments. Colony counts were converted to $\log_{10}$ CFU/cm^2 and means and standard deviations were

Table 1: Binary combinations of antimicrobials incorporated in films and edible coatings.

Antimicrobial films	Antimicrobial coatings
Control (plain LDPE films)	Plain MC/HPMC
Nis (25000 IU/mL)	Nis (25000 IU/mL)
Nis (25000 IU/mL) + SD (0.25%)	Nis (25000 IU/mL) + SD (0.25%)
Nis (25000 IU/mL) + SB (0.1%)	Nis (25000 IU/mL) + SB (0.1%)
Nis (25000 IU/mL) + SL (0.3%)	Nis (25000 IU/mL) + SL (0.3%)
Nis (25000 IU/mL) + PS (0.3%)	Nis (25000 IU/mL) + PS (0.3%)

Table 2: Binary and ternary combinations of antimicrobials incorporated in films and edible coatings.

Nisin (IU/mL)	PS (%)	SB (%)
0	0.0	0.0
25000	0.0	0.0
25000	0.0	0.05
25000	0.0	0.1
25000	0.15	0.0
25000	0.15	0.05
25000	0.15	0.1
25000	0.3	0.0
25000	0.3	0.05
25000	0.3	0.1

Table 3: Combinations of antimicrobials incorporated in films and coatings for the refrigerated storage study.

Nisin (IU/mL)	PS (%)	SB (%)
0	0	0
25000	0.3	0
25000	0.3	0.1

calculated using Microsoft Excel. A Tukey-Kramer test was used to determine differences in the populations of *L. monocytogenes*, aerobes, and anaerobes on CSS samples. Significant differences were considered at the 95% confidence level ($P < 0.05$).

3. Results

3.1. Antilisterial Effectiveness of Films and Coatings Incorporating Binary Combinations of Antimicrobials. The fate of *L. monocytogenes* on CSS slices treated with different binary combinations of antimicrobials in films and coatings is represented in Figures 1(a) and 1(b), respectively. The initial load of the inoculum was about 6 log CFU/cm^2. After 2 days, the control (untreated) samples had higher counts than all other treatments with the Nis + SB (5.2 log CFU/cm^2) and Nis + PS (4.8 log CFU/cm^2) coatings showing significantly ($P < 0.05$) lower counts than the control groups. After 10 days, *L. monocytogenes* population on samples treated with Nis + SB (4.9–6.3 log CFU/cm^2) and Nis + PS (4.3–4.7 log CFU/cm^2) was appreciably lower compared to the control untreated samples (7.7 log CFU/cm^2) although the results were not statistically significant ($P > 0.05$).

3.2. Antilisterial Effectiveness of Films and Coatings Incorporating Ternary Combinations of Antimicrobials. Figures 2(a) and 2(b) show the effect of film and coating treatments incorporating ternary combinations of antimicrobials on the growth of *L. monocytogenes* on CSS. The initial counts of *L. monocytogenes* on CSS were about 5.6 log CFU/cm^2 and increased steadily over the 10-day period reaching a maximum count of 7.6 log CFU/cm^2. Over the storage period, the counts for all treatments were consistently lower than the control for either method of antimicrobial application. Ternary combinations of Nis (25000 IU/mL), PS (0.15 or 0.3%), and SB (0.05 or 0.1%) in films and coatings significantly ($P < 0.05$) inhibited the growth of *L. monocytogenes* after 10 days, lowering the population by 2.0–3.3 log CFU/cm^2 and 2.2–2.9 log CFU/cm^2, respectively.

3.3. Effectiveness of Films and Coatings Containing Selected Antimicrobial Combinations against L. monocytogenes and Spoilage Microflora. The two combined treatments chosen for this study were Nis + PS (0.3%) and Nis + PS (0.3%) + SB (0.1%). *L. monocytogenes* counts on inoculated CSS treated with antimicrobial films and coatings incorporating Nis + PS or Nis + PS + SB are shown in Figures 3(a) and 3(b), respectively. The mean population of *L. monocytogenes* on CSS recovered just after inoculation was 2.7 log CFU/cm^2. The pathogen grew unhindered in untreated samples stored at 4°C, reaching approximately 4.8 log CFU/cm^2 after 4 weeks. All the antimicrobial treatments (films or coatings) brought about a significant reduction ($P < 0.05$) in the population achieving reductions of 2.0–2.7 log CFU/cm^2 by the end of the storage period. Ternary combinations of Nis (25000 IU/mL) with PS (0.3%) and SB (0.1%) were more effective than binary combinations of Nis (25000 IU/mL) with PS (0.3%) for either method of application although the difference was not statistically significant ($P > 0.05$).

The total anaerobic and aerobic counts of the uninoculated samples are shown in Figures 4(a) and 4(b). Throughout

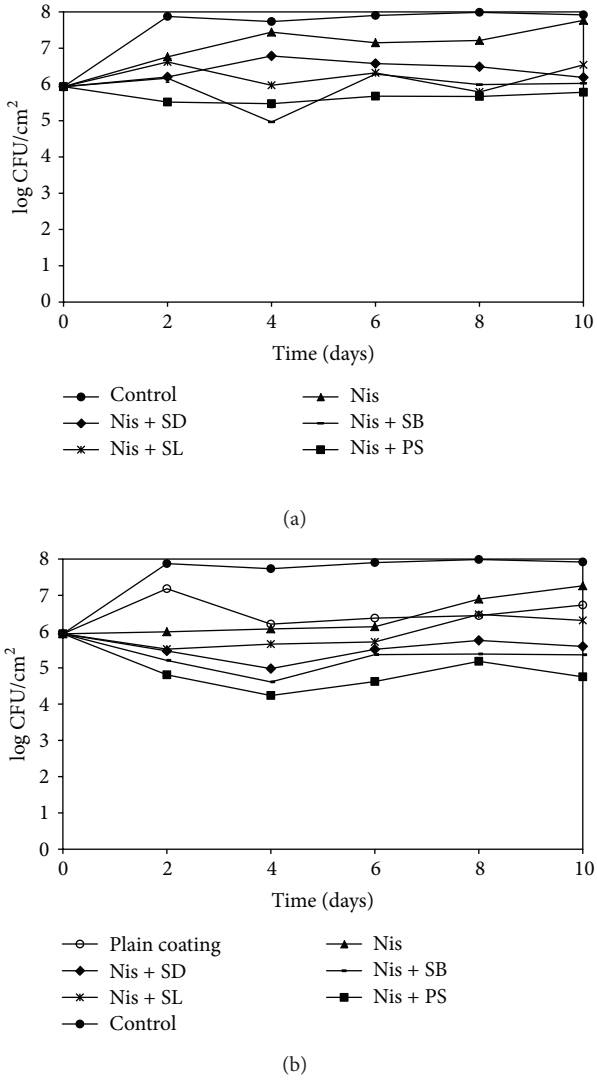

Figure 1: Fate of *L. monocytogenes* on CSS slices packaged with antimicrobial films (a) and coatings (b) incorporating nisin (25000 IU/mL), SL (0.3%), SD (0.25%), SB (0.1%), and PS (0.3%) and stored at ambient temperature. Error bars are omitted from the chart for the sake of clarity.

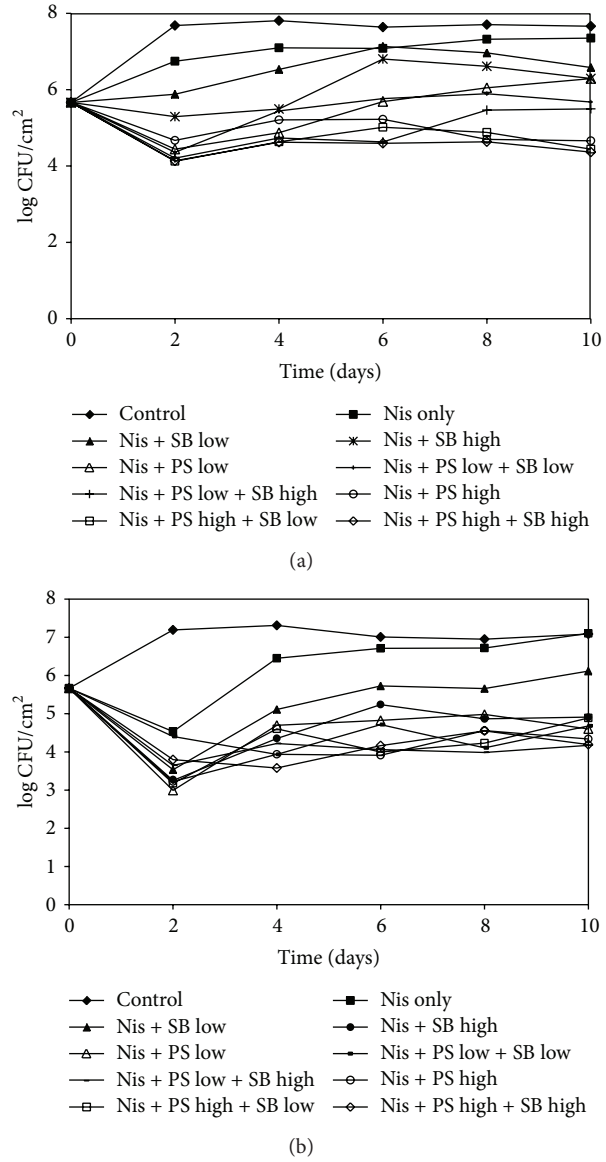

Figure 2: Populations of *L. monocytogenes* on CSS slices packaged with antimicrobial films (a) and coatings (b) incorporating nisin (25000 IU/mL) with PS at low (0.15%) or high (0.3%) concentrations or SB at low (0.05%) or high concentrations (0.1%) and stored at ambient temperature. Error bars are omitted from the chart for the sake of clarity.

the storage study, the population of mesophilic aerobic and anaerobic bacteria in treated samples was consistently lower than their untreated counterpart with a maximum reduction of 4.9–5.6 log CFU/cm^2 by the end of the storage period. Although both films and coatings were effective in delaying the development of background flora, coatings incorporating Nis + PS and Nis + PS + SB resulted in greater ($P > 0.05$) population reduction than their film counterparts.

4. Discussion

Cold smoked salmon (CSS) is considered a high-risk food because the temperature used during the cold smoking operation is not lethal to *L. monocytogenes*. In addition, CSS is a type of minimally processed food, also called Refrigerated Processed Foods of Extended Durability (REPFED) [43], and concern has been expressed about the survival and growth of this pathogen during the product's prolonged shelf life. During the past decade, there have been several recalls of smoked fish because of *L. monocytogenes* contamination [44] and it has been generally assumed that the presence of the pathogen on fish products is the result of postprocess contamination on the surface of the product [44]. Moreover, several studies [45, 46] have reported that bacterial population in

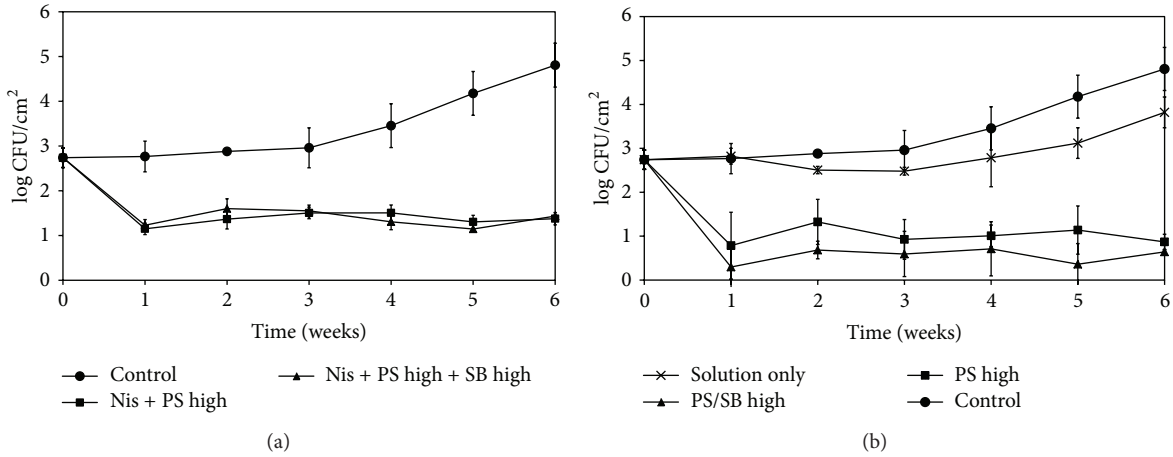

Figure 3: Development of *Listeria monocytogenes* on CSS slices treated with antimicrobial films (a) and coatings (b) incorporating Nis (25000 IU/mL) with high concentrations of PS (0.3%) with or without SB (0.1%) during storage at 4°C.

cold smoked salmon can increase by 3-4 log CFU/g in a few weeks during refrigerated storage.

Several forms of interventions have thus been recommended to reduce the risks from *L. monocytogenes* in these products: (1) elimination or reduction of *L. monocytogenes* on the outside surface of frozen or fresh fish before filleting, (2) prevention of recontamination and growth of *L. monocytogenes* during all stages of processing, and (3) the inhibition of any possible survivors or recontamination during processing and distribution [44]. Numerous papers have been published on the inhibition of *L. monocytogenes* in cold smoked fish using physical interventions including gamma irradiation [47], X-ray irradiation [48], E-beam [49], high-pressure processing [50], chemical preservatives [51–53], natural antimicrobials [54, 55], and protective cultures [56, 57]. Extensive research has also been performed in the last decade on the application of antimicrobial packaging to specifically enhance the safety and extend the shelf life of fish and fish products. However, despite considerable efforts, this area of research remains challenging. This is primarily due to the intrinsic characteristics of fishery products themselves, namely, their almost neutral pH and presence of endogenous proteolytic enzymes [58], which can decrease the efficacy of acid antimicrobials and bacteriocins, respectively. Indeed, the effectiveness of salts of organic acids as antimicrobials is known to differ widely depending on the pH of the food matrix [16]. Moreover, in cold processed foods such as CSS, proteases can affect nisin stability [59].

Because of the aforementioned reasons, films and coatings have garnered more interest by virtue of their ability to not only provide a barrier against gases and moisture [60], but also act as carriers of antimicrobials. Alishahi and Aïder [61] pointed to the promising application of chitosan as an excellent antimicrobial, used stand alone or in combination, in herring, cod, cold smoked salmon, and trout. Reductions of the order of 1–3 log CFU/g of *L. monocytogenes*, following chitosan application on CSS, have been reported previously [32, 62]. Moreover, Gómez-Estaca et al. [63] coated cold smoked sardines with gelatin-based films (4%) enriched with oregano (1.25%) and rosemary extracts (20%) and showed that growth of TVC was retarded by 2 log and 2.5 log (respectively) compared to uncoated samples after 16 days of storage. Lu et al. [64] tested, in snakehead fish fillets, alginate coatings (20 mg/mL) with cinnamon EO (10 μL/mL), EDTA (150 μg/mL), and nisin (2000 IU/mL) alone and with their mixes against *Pseudomonas* spp., TVC, and psychrotrophic bacteria during storage at 4°C. The inhibitory effect of those antimicrobials on TVC followed the order: cinnamon + EDTA + nisin or cinnamon (5.5 log CFU/g) > nisin + EDTA (1.5 log CFU/g) compared to controls. Song et al. [65] reported that composite films of barley bran protein and gelatin containing grapefruit seed extract brought about a reduction of 0.5 log CFU/g of *E. coli* O157:H7 and *L. monocytogenes* on salmon after 15 days of storage at 4°C. The antilisterial effect of a calcium alginate coating incorporating oyster lysozyme in the presence or absence of nisin on the surface of smoked salmon was also investigated previously [66]. Although the coatings supplemented with nisin and lysozyme were able to delay or slow down the growth of *L. monocytogenes*, the treatment was not highly inhibitory. Taken together, these findings point to the highly variable antimicrobial efficacy of edible films and coatings (reductions of 0.5–5.5 log CFU/g), which is dependent on the type, concentration, and combination of antimicrobials as well as the test product of interest.

In our current study, the antimicrobial efficacy of cellulose-coated films and cellulose-based coatings incorporating Nis, SL, SD, PS, and SB in different concentrations and combinations was compared. Findings revealed that coatings incorporating nisin (25000 IU/mL), PS (0.3%), and SB (0.1%) were most effective and reduced the population of *L. monocytogenes* and anaerobic and aerobic spoilage flora by a maximum of 4.2, 4.8, and 4.9 log CFU/cm^2, respectively, after 4 weeks of refrigerated storage. The listeriostatic activity

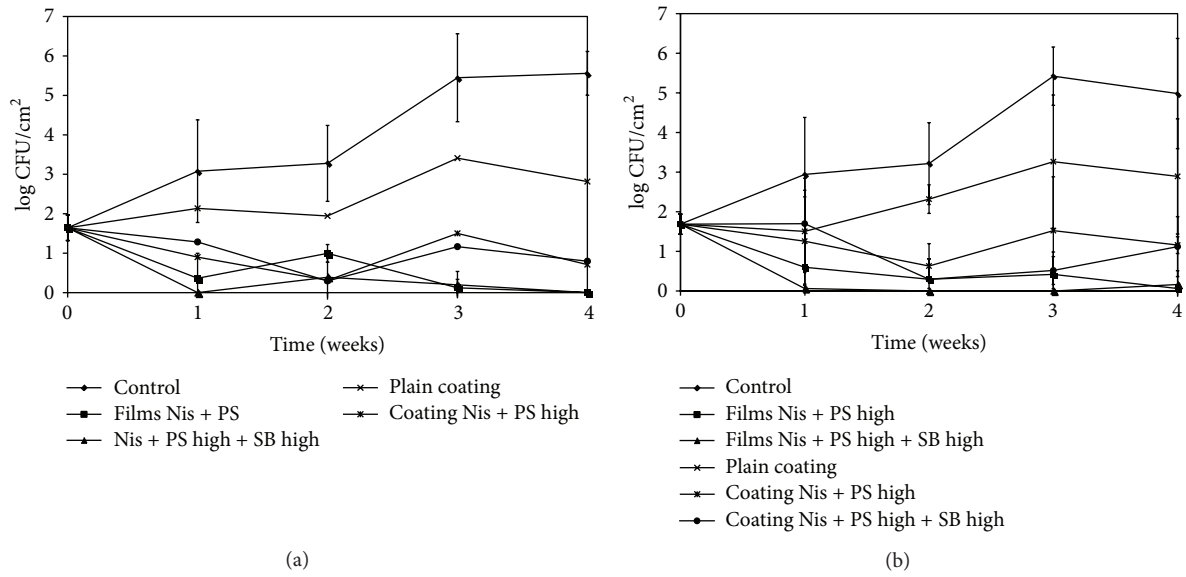

Figure 4: Development of mesophilic anaerobes (a) and aerobes (b)on CSS slices treated with antimicrobial films and coatings incorporating Nis (25000 IU/mL) with high concentrations of PS (0.3%) with or without SB (0.1%) during storage at 4°C.

of nisin on cold smoked fish, at levels ranging from 100 IU/mL to 2000 IU/mL, alone or in combination with LAB protective cultures [67], or natural antimicrobials such as lyzozyme and polylysine [68], or chemical preservatives such as lactate or diacetate [69] has been demonstrated previously. Unlike our findings, Tang et al. [69] reported that a binary combination of Nisin and lactate had greatest antilisterial effectiveness. On the other hand, Wan Norhana et al. [70] showed that ternary combinations of nisin-PS-EDTA reduced the population of *L. monocytogenes* as well as psychrotrophic bacteria on vacuum-packaged shrimps by 1.3 and 4.0 log CFU/g, respectively. Neetoo et al. [31] also indicated that PS had considerable antilisterial activity when combined with nisin on CSS pâté and fillets. Sorbates have also been shown to kill or inhibit *L. monocytogenes* previously [25, 30, 71] and its activity can be further enhanced by the addition of nisin [72]. Other authors [72, 73] have similarly demonstrated the listeriostatic and listericidal ability of Nis + PS *in vitro* as well as on packaged beef kept at refrigeration temperature for up to 4 weeks.

Moreover, our data indicate that edible coatings containing nisin (25000 IU/mL) + PS (0.3%) or nisin (25000 IU/mL) + PS (0.3%) + SB (0.1%) reduced the population of *L. monocytogenes* and spoilage bacteria to a greater extent than their film counterparts. Recent studies have also highlighted the application of organic acids or their salts alone [74] or in combination with other hurdles such as CO_2 [75] to delay the development of spoilage flora on salmon. Other overriding advantages of edible coatings are that they reduce packaging waste, they are environment-friendly, and they are low-priced [76, 77] when compared to films, which are synthetic packaging materials. Hence, edible coatings constitute an alternative, environmentally sustainable, and cost-effective technology [78] for the salmon industry. In the current work, the carrier of choice was cellulose, a widely available, low-cost, versatile polysaccharide biopolymer [77] and a compatible matrix for the embedded antimicrobials. Polysaccharide-based edible coatings are more popular than other hydrocolloids because they are generally transparent, cohesive, and homogeneous with adequate mechanical properties [60, 79].

Antimicrobials tested in this study are regarded as direct food additives and their application is thus limited by governmental legislation [16]. Sorbates may be directly added to food or incorporated into the packaging, at a level not exceeding 0.3% [23] while sodium benzoate is currently allowed at 0.1% [16]. Levels of PS (0.15 and 0.3%) and SB (0.05 and 0.1%) investigated in the current experiments were within legal limits of 0.3 and 0.1%, respectively. Presently, nisin is commercially added to smoked salmon in the United States (US) to control the growth of *L. monocytogenes*, although the maximum allowable level of this additive in smoked fish has not been stipulated [80]. The US Food and Drug Administration (FDA) has set a maximum limit of 10,000 IU/g for use of nisin in processed cheese although no such upper limit exists in Australia, France, or Great Britain [80, 81]. In the current study, the level of nisin incorporated into the films and coatings was 25000 IU/mL, translating to a maximum concentration of 2500 IU/g assuming complete leaching into the food product.

In recognition of the fact that CSS is (i) susceptible to postprocess surface contamination by *L. monocytogenes*, (ii) a refrigerated processed food of extended durability, and (iii) consumed without any heat-killing step, it is regarded as a high-risk product. This study reiterates the usefulness of antimicrobial packaging for cold smoked salmon,

and it also underscores the effectiveness of cellulose-based coatings incorporating GRAS antimicrobials to control the development of pathogens and spoilage microbiota, thereby enhancing the microbiological safety and quality of this product.

Conflict of Interests

The authors declare no conflict of interests. Neither author has any commercial associations that might create a conflict of interests in connection with this paper.

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Research Article

Antifungal and Antiproliferative Protein from *Cicer arietinum*: A Bioactive Compound against Emerging Pathogens

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The emergence of epidemic fungal pathogenic resistance to current antifungal drugs has increased the interest in developing alternative antibiotics from natural sources. *Cicer arietinum* is well known for its medicinal properties. The aim of this work was to isolate antimicrobial proteins from *Cicer arietinum*. An antifungal protein, C-25, was isolated from *Cicer arietinum* and purified by gel filtration. C-25 protein was tested using agar diffusion method against human pathogenic fungi of ATCC strains and against clinical isolates of *Candida krusei*, *Candida tropicalis*, and *Candida parapsilosis*, and MIC values determined were varied from 1.56 to 12.5 μg/mL. The SEM study demonstrated that C-25 induces the bleb-like surface changes, irregular cell surface, and cell wall disruption of the fungi at different time intervals. Cytotoxic activity was studied on oral cancer cells and normal cells. It also inhibits the growth of fungal strains which are resistant to fluconazole. It reduced the cell proliferation of human oral carcinoma cells at the concentration of 37.5 μg/mL (IC_{50}) and no toxic effect was found on normal human peripheral blood mononuclear cells even at higher concentration of 600 μg/mL. It can be concluded that C-25 can be considered as an effective antimycotic as well as *antiproliferative* agent against human oral cancer cells.

1. Introduction

The major healthcare problem is the antibiotic resistance which arises the lack of effective therapeutics for microbial infection. During the past few years a wide spectrum of plant antimicrobial proteins has been identified and has enhanced the activity in low duration to prevent the development of resistant by microbes.

There are several classes of proteins having antimicrobial properties which include thionins, lipid transfer proteins, plant defensins, chitinases, glucanases, 2S albumins, ribosome inactivating proteins, and lectin [1, 2]. Lectins are proteins or glycoproteins of a ubiquitous distribution in nature, which have at least one carbohydrate or derivative binding site without catalytic function or immunological characteristics. They have the unique ability to recognize and bind reversibly to specific carbohydrate ligands without any chemical modification which distinguishes lectins from other carbohydrate binding proteins and enzymes and makes them invaluable tools in biomedical and glycoconjugate research. In plant, lectin plays an important role in the defence against harmful fungi, insects, and bacteria. Several lectins have been found to possess anticancer properties in human case studies, where they are used as therapeutic agents binding to the cancer cell membrane or their receptors causing cytotoxicity, apoptosis, and inhibition of tumor growth [3, 4].

Cicer arietinum (chickpea) is a legume and belongs to the Fabaceae family. It contains 75% fibres and low fat protein. It has been reported that the use of *Cicer arietinum* helps in diabetes and cardiovascular diseases and in some cancers. Some lectins having hemagglutination activity were isolated earlier from *Cicer arietinum* [5]. This study focused on isolation and characterization of a lectin protein possessing medicinal properties from the seeds of *Cicer arietinum*.

2. Methods

2.1. Ethics. The Ethics Committee of All India Institute of Medical Sciences (AIIMS), New Delhi, India, approved the study protocol (IEC/NP-374/2013) and informed consent was obtained.

2.2. Isolation and Purification of Protein from Cicer arietinum. *Cicer arietinum* seeds were soaked, homogenized in 10 mM Tris-Cl buffer (pH7.2), and centrifuged at 13,000 ×g for 30 min. at 4°C. The resulting crude extract was treated with ammonium sulphate with 30% saturation under cold condition and the precipitant was centrifuged at 13,000 ×g for 30 min. at 4°C. The salt was removed from the resultant supernatant by dialysis membrane (10 kDa) in the same buffer.

The dialysed sample was loaded onto Sephadex G-100 gel filtration column preequilibrated with 10 mM Tris-HCl (pH7.2) and 150 mM NaCl. The proteins were eluted using the same buffer and simultaneously monitored at 280 nm. Each fraction was tested for antimicrobial activity. One fraction showed inhibition activity against fungi and it was characterized further.

2.3. Characterization of the Purified Protein

2.3.1. Molecular Mass Determination. The concentration of proteins was estimated by BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) using Bovine serum albumin as a standard. The 12% SDS-PAGE of the protein was carried out using Laemmli system of buffers [6] in the presence and absence of 2-mercaptoethanol. The electrophoretic mobility of the protein and protein marker were compared to determine the molecular weight of the protein.

2.3.2. N-Terminal Amino Acid Sequence Analysis. The N-terminal sequence analysis of the C-25 protein was done by Edman degradation on a Procise Protein Sequencer (Applied Biosystems). The database was searched for other antifungal proteins with similar sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

2.3.3. Hemagglutination Activity and Sugar Inhibition Assays. Hemagglutination studies of the purified protein were carried out using human erythrocytes in a 96-well microtiter plate. 50 μL of purified protein solution (0.8 mg/mL) was placed in the first well and twofold serially diluted into the successive wells with phosphate buffered saline, pH 7.4. Then, 50 μL of 4% human erythrocyte suspension was added to all the wells. Hemagglutination was visualized in the plate after 1 h of incubation at 37°C.

Hemagglutination inhibition assays [7] with the purified protein were performed by placing 50 μL of different sugar solutions (40 mM) including inulin, D-mannose, D-glucose, D-ribose, N-acetyl-D-galactosamine, and melibiose in respective wells of the plate and serially twofold diluted. Then, 50 μL of the purified protein (0.8 mg/mL) was added to each well and incubated for 30 min. at 37°C. Later, 50 μL of 4% erythrocyte suspension was added and the plate was incubated for 1 h at 37°C. Hemagglutination inhibition titre was scored visually.

2.3.4. Detection of Antifungal Activity. Antifungal activity of the purified protein was tested using agar diffusion method against human pathogenic fungi such as *Candida parapsilosis* ATCC22019, *Candida krusei* ATCC6258, and *Candida tropicalis* ATCC13803. All *Candida* species were grown overnight on Sabouraud's dextrose agar plates. Each colony was inoculated in 5 mL of 0.9% (w/v) normal saline to make inoculum suspension adjusted with 0.5 Mc Farland standard solutions and the cell suspension was spread by sterile cotton swab over the Mueller Hinton agar (MHA) plates under aseptic conditions. The wells were bored with a borer and 0.1 mL of purified protein (200 μg/mL) was added to respective wells. Fluconazole disc (25 mcg) was used as positive control. The plates were incubated at 35°C for 24 h and the zone of inhibition was observed.

2.3.5. Determination of Minimum Inhibitory Concentration (MIC). The MIC was performed on *Candida parapsilosis* ATCC22019, *Candida krusei* ATCC6258, *Candida tropicalis* ATCC13803, and clinical isolates of the same strains from 45 patients with *Candida* infection. The patient samples were taken from Department of Microbiology, AIIMS. The MIC was determined according to the CLSI (Clinical and Laboratory Standards Institute) guidelines [8]. Pure colonies of *Candida* species were suspended in 5 mL of sterilized saline (0.9% w/v) to a concentration of 5×10^6 CFU/mL as matched with 0.5 McFarland Standard solutions. 100 μL of purified protein (200 μg/mL) solution was added and twofold serially diluted using RPMI-1640 media. 100 μL of each final inoculum suspension (2.5×10^3 CFU/mL) was added to the respective wells and the plates were incubated at 35°C for 24 h. The fungal strains *C. krusei* and *C. parapsilosis* without treatment and uninoculated RPMI-1640 media were used as growth and media control, respectively. The MIC was calculated as the lowest concentration at which cell growth was inhibited. Fluconazole drug was used as a positive control. The experiment was performed in triplicate.

2.3.6. Scanning Electron Microscopic (SEM) Studies. The inoculated *Candida krusei* ATCC6258 was incubated in MHB (Mueller Hilton broth) media at 35°C overnight, which was further incubated for another 2 h at 35°C in fresh media for exponential growth phase. After washing with PBS, cells were suspended in 10 mM phosphate buffer (pH 7.4) at a final concentration of 1×10^6 CFU/mL. The protein at concentrations of 12.5 μg/mL (2xMIC) was added to the cell suspension and was incubated at different time intervals at 35°C. High concentration of test sample is chosen in order to achieve killing of a high number of yeast cells [9]. The cells were prepared for SEM study by treatment with 1% osmium tetroxide for 1 h at 4°C [10]. The cells were visualized under Electron Microscope (LEO, Cambridge, UK). Images were digitally acquired by using a CCD camera attached to the microscope.

2.3.7. Determination of Cytotoxicity. Cytotoxic effect of protein was analyzed using oral carcinoma cell line (KB cells) using MTT dye reduction assay [11]. Briefly, 5×10^3 cells/100 μL media (EMEM) were seeded in 96-well plates 24 h before the experiment. The cells were then incubated with different concentrations (9–600 μg/mL in EMEM) of protein for 48 h. 10 μL of MTT solution (10 mg/mL in PBS) was then added to each well and plates were further incubated for 3 h at 37°C. The formazan crystals formed were dissolved by adding 100 μL of DMSO. The cells were treated with 1% Tris-HCl (same concentration as used for the solvent of C-25) which was then subtracted from all the cytotoxic values. Absorbance was measured by a microplate reader at 570 nm and the reference filter 650 nm was used. The data obtained were presented as percentage of cell survival in the best-fit (linear) dose response curves. The IC_{50} value at 95% confidence interval was calculated. Each concentration was used in triplicate.

To examine the cytotoxicity effect of this protein on PBMCs, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, USA) as per the standard protocol [12] from healthy human blood and collected in heparinized tubes and diluted 1 : 2 with fresh sterile phosphate-buffered saline (PBS). The proliferation analysis of C-25 was performed by MTT assay as described above.

2.3.8. Kinetic Analysis with p38α MAP Kinase. The kinetic analysis of protein was done with p38α MAP kinase as this signaling molecule was found to be overexpressed in oral cancer [13]. Hence, binding study of protein was performed with recombinant p38α MAP kinase (mitogen activated protein kinase) using both ELISA and BIAcore to ensure the anticancer activity.

(1) By ELISA. The assay was performed in 96-well microtitre plate coated with ATF-2 protein at 37°C. 12 μg of p38α protein was incubated with six different concentrations of protein (1, 2.5, 5, 10, 15, 20, and 25 μM) for 1.5 h. The kinase mixture (purified p38α incubated with C-25 protein, 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 100 μg/mL BSA, 1 mM DTT, 0.1 mM Na_3VO_4, and 100 μM ATP) was added and incubated for 1 h at 37°C. After washing, the plates were incubated with anti-phospho ATF-2 antibody (1 : 400) (Biovision) for 1 h at 37°C and subsequently with alkaline phosphates conjugated goat anti-rabbit IgG (1 : 4000) (Chemicon) for 1 h at 37°C. Finally, the chromogenic substrate solution 4-nitrophenyl phosphate (4-NPP) in 0.1 M Tris-HCl, pH 8.1, and 0.01% $MgCl_2$ (Cayman Chemical Company, USA) was added for 1.5 h at 37°C and the formation of nitrophenolate was measured at 405 nm which analyzed the extent of phosphorylation of ATF-2. The assay was performed in triplicate for each concentration and mean ± SD values were used to calculate the IC_{50} value.

(2) By Surface Plasmon Resonance (SPR). The His-tagged recombinant p38α protein was immobilized on the NTA sensor chip via Ni^{2+}/NTA chelation at 25°C in BIAcore-2000 (GE Healthcare, Sweden). The surface was first activated with Ni^{2+} forming a chelating complex with NTA which further binds with His-tag of recombinant protein. 2 mM $NiCl_2$ solution was passed at a flow rate of 5 μL/min. One flow cell was used as a reference cell and, on the other, 20 μL of His-tagged p38α (9 mg/mL) was injected at a flow rate of 5 μL/min. for immobilization. The binding parameters of the C-25 were measured by injecting three different concentrations (4.6×10^{-6} M, 9.2×10^{-6} M, and 13.8×10^{-6} M) over the immobilized protein. BIAevaluation 3.0 software was used to determine the dissociation constant (KD) of the inhibitory protein.

3. Results

3.1. Purification and Molecular Characterization. Crude protein extract from *Cicer arietinum* was subjected to ammonium sulphate precipitation to remove unwanted proteins. Three peaks were obtained after gel filtration with Sephadex G-100 column (Figure 1(a)). In SDS-PAGE of these fractions, the third peak showed a single band corresponding to molecular mass of 25 kDa named as C-25. The antifungal activity was found in peak 3 fractions (Figure 1(b)). Both Lane 2 and Lane 3 showed a single band of C-25 in the presence and absence of mercaptoethanol, respectively, which revealed the protein to be a monomer (Figure 1(c)).

3.2. N-Terminal Amino Acid Sequence Analysis. N-terminal amino acid sequence of the purified C-25 from *Cicer arietinum* is shown in Table 1 and it was compared with other antifungal proteins using Blast from NCBI website. This protein exhibited 100% sequence similarity of 10 amino acid residues with sequence of lectin from other plant sources.

3.3. Hemagglutinating Activity and Inhibition Assay. C-25 protein from *Cicer arietinum* readily agglutinated human erythrocytes showing the hemagglutination activity. Hemagglutination-inhibition assay was performed with C-25 to investigate its sugar specificity. The results showed that agglutination activity of C-25 was inhibited strongly by N-acetyl-D-galactosamine and not by any other sugar moieties, indicating that the acetamido moiety of this sugar might have interacted with C-25. Agglutination activity of C-25 was inhibited by 20 mM of N-acetyl-D-galactosamine (S1) indicating that C-25 specifically binds with N-acetyl-D-galactosamine.

3.4. Assay of Antifungal Activity. The pure C-25 obtained from gel filtration was tested for antifungal activity against *C. parapsilosis*, *C. krusei*, and *C. tropicalis* by agar well diffusion method using fluconazole drug as a positive control. The zone of inhibition around the test sample was found in all the above mentioned *Candida* spp. (Figures 2(a), 2(b), and 2(c)). The MIC values of a C-25 against the above mentioned fungi and clinical isolates of *Candida* species from 45 patients were found to be varied from 1.56 to 12.5 μg/mL after 24 h incubation period. Among 45 clinical isolates strains, the MIC of ≤8 μg/mL are susceptible, 16 to 32 μg/mL are susceptible-dose dependent (SDD), and ≥64 μg/mL are resistant to fluconazole

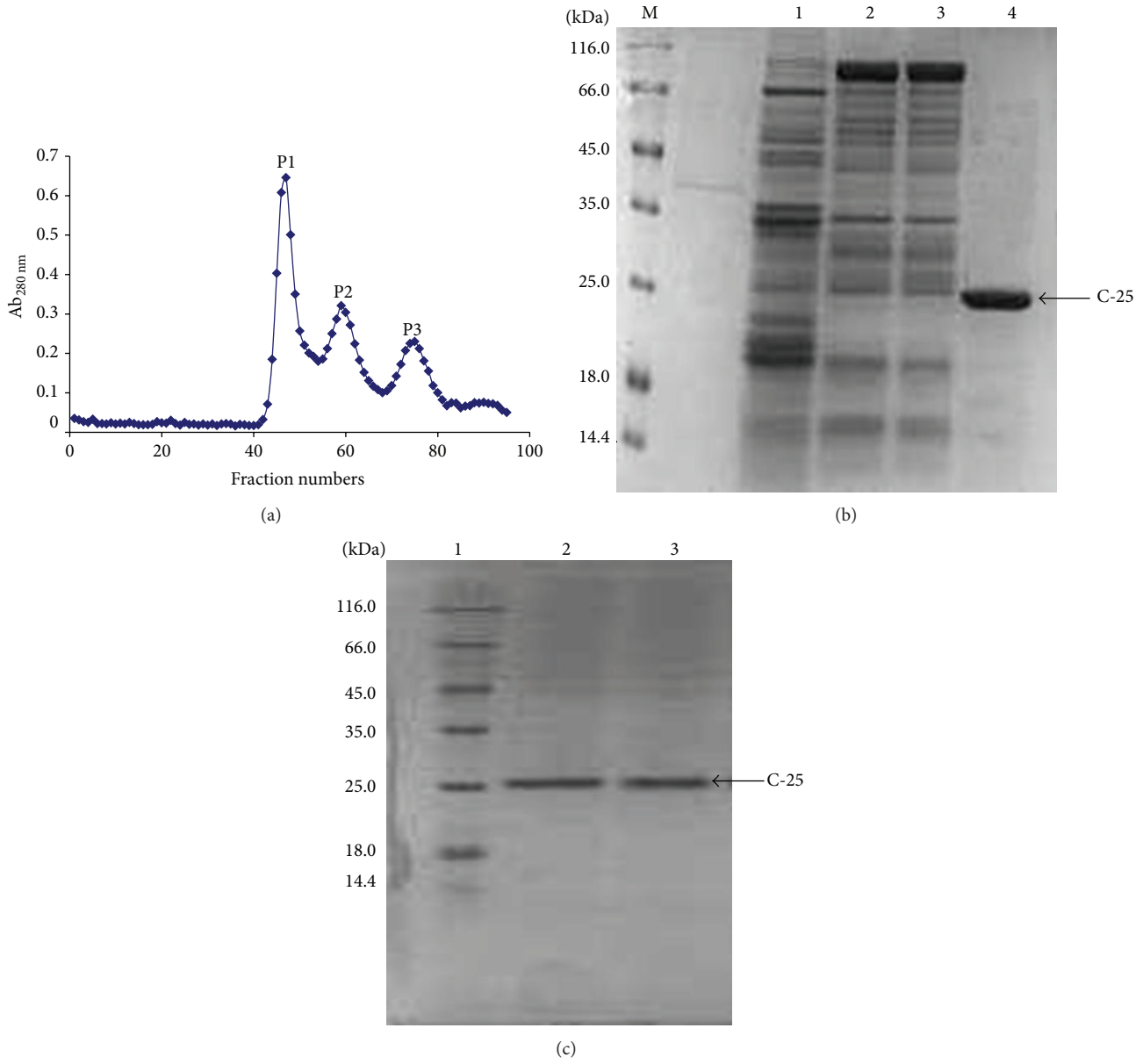
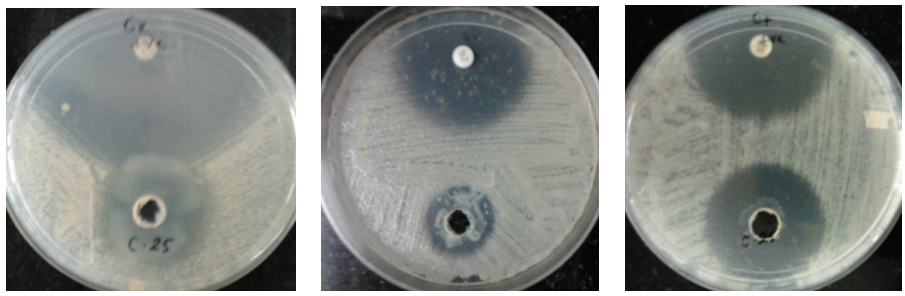


Figure 1: (a) Elution profile of *C. arietinum* protein crude extract from gel filtration on Sephadex G-100 column showing three peaks P1, P2, and P3. SDS-PAGE of protein fractions from gel filtration: (b) from left to right: Lane M is molecular mass marker, Lane 1 is eluent of P1, Lanes 2 and 3 are P2, and Lane 4 is P3 (C-25). (c) From left to right: Lane 1 is molecular mass marker, Lane 2 is P3 in the absence of mercaptoethanol under nonreducing conditions, Lane 3 is P3 in the presence of mercaptoethanol under reducing conditions.

Table 1: A comparison between N-terminal amino acids sequence of C-25 (TKTGYINAAF) and sequences of other proteins.

Protein	Sequence	Accession number	% identity
C-25 protein	TKTGYINAAF	AGN33419	100
Seed albumin 2 *(Pisum sativum)*	TKTGYINAAF	CAH55839.1	100
Albumin 2 *(Pisum sativum)*	TKTGYINAAF	P08688.1	100
Crystal structure of Ls24 (*Lathyrus sativus)*	TKPGYINAAF	Pdb:3LP9A	90
Crystal structure of albumin *(Cicer arietinum)*	TKTGYINAAF	3S18	100



(a) (b) (c)

Figure 2: Antifungal assay of the C-25 protein showing zone of inhibition against (a) *Candida krusei*, (b) *C. parapsilosis*, and (c) *C. tropicalis*. Fluconazole disc was taken as positive control.

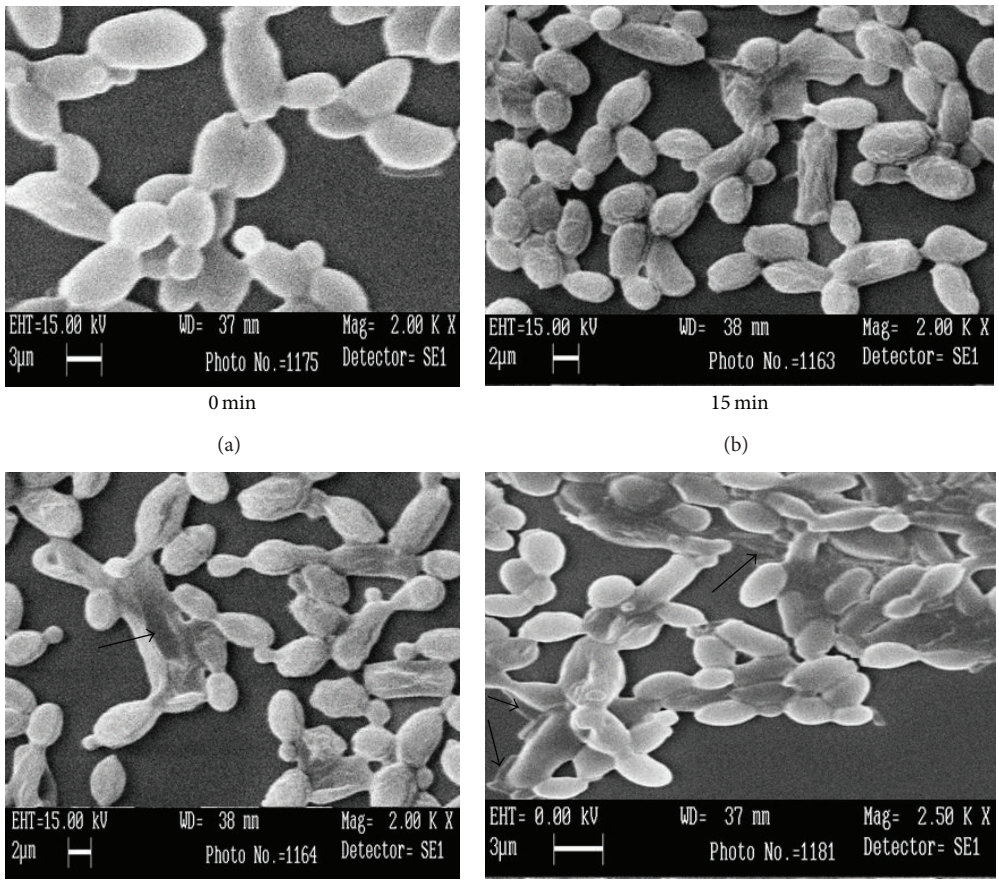


Figure 3: SEM study showing the cell wall disruption of *Candida krusei* treated with (a) 10 mM PBS buffer (control) and (b), (c), and (d) 2xMIC value (12.5 μg/mL) of C-25 protein at different time scales. The arrows indicate the cell wall disruption and cytoplasmic leakage.

as per CLSI document M27-A3, although C-25 was showing fungicidal activity on these strains (Table 2).

3.5. SEM Studies. To understand the mechanism of action of C-25 on the cell wall of fungi, SEM studies were performed with cells of *C. krusei* at different times of incubation with 12.5 μg/mL of C-25 and the changes in the morphology of cell wall of the *C. krusei* were examined. Figure 3(a) showed the morphology of the untreated cells (control). The effect of 12.5 μg/mL concentrations of C-25 showed different consequences on the cell wall. Figure 3(b) showed the bleb-like surface changes and cell shrinkage at 15 min, and

Table 2: MIC assay: ATCC and clinical isolates of *Candida* species from 45 patients treated with C-25 protein and fluconazole drug (control).

ATCC number/ patient ID	Organism	Source	MIC (μg/mL) of fluconazole	MIC (μg/mL) of C-25
22019 (QC)	*C. parapsilosis*	ATCC	1	6.25
6258 (QC)	*C. krusei*	ATCC	16	6.25
13803 (reference)	*C. tropicalis*	ATCC	1	6.25
AID 19	*C. tropicalis*	Blood	2	6.25
AID 20	*C. tropicalis*	Blood	2	6.25
AID 21	*C. tropicalis*	Blood	2	6.25
AID 37	*C. parapsilosis*	Blood	1	6.25
AID 45	*C. tropicalis*	Blood	1	6.25
AID 47	*C. tropicalis*	Blood	1	6.25
2549	*C. tropicalis*	Blood	0.5	1.56
4347	*C. tropicalis*	Blood	1	3.125
7004	*C. tropicalis*	Blood	0.5	1.56
9097	*C. parapsilosis*	Urine	1	3.125
9409	*C. parapsilosis*	Urine	1	3.125
8995	*C. parapsilosis*	Urine	4	6.25
8399	*C. tropicalis*	Urine	4	6.25
8509	*C. tropicalis*	Urine	2	6.25
8110	*C. tropicalis*	Urine	2	6.25
9853	*C. tropicalis*	Urine	1	6.25
9697	*C. tropicalis*	Urine	1	3.125
9183	*C. tropicalis*	Urine	1	3.125
9814	*C. tropicalis*	Urine	1	3.125
9239	*C. tropicalis*	Urine	2	6.25
9762	*C. tropicalis*	Urine	1	3.125

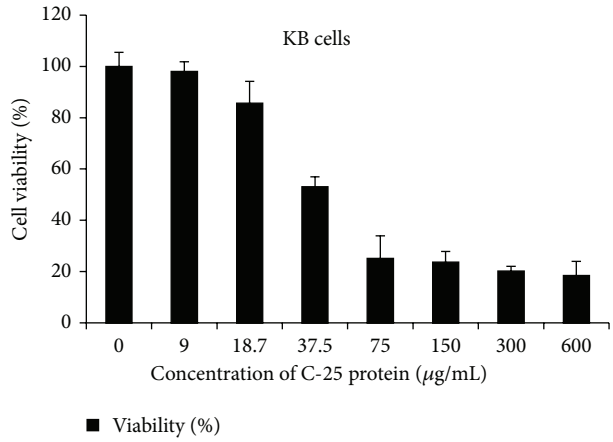


Figure 4: Cell viability of cancer cells (KB cell line) treated with different concentrations of C-25 protein.

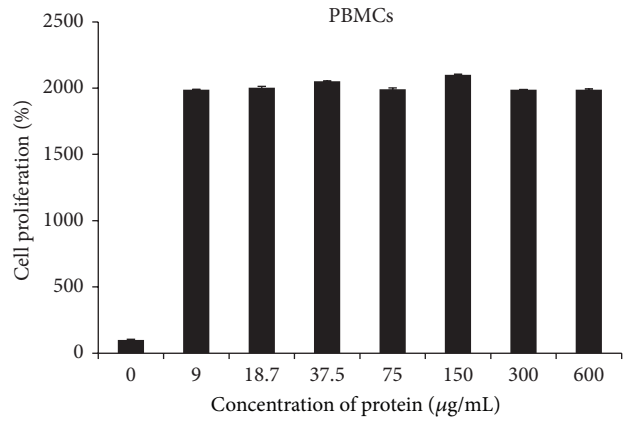


Figure 5: Cell proliferation of normal PBMCs at different concentrations of C-25 protein.

Figures 3(c) and 3(d) showed irregular cell surface, cell wall disruption, and cytoplasmic leakage at different times, 30 and 60 min., respectively.

3.6. Cytotoxicity. The cytotoxicity of the C-25 against KB cell line was investigated using MTT assay. 50% of KB cell survival was reduced by treating with 37.5 μg/mL (IC_{50}) of C-25. At 75 μg/mL, it significantly inhibited the survival of KB cells in 48 h incubation period (Figure 4).

In the case of normal mammalian cells (PBMCs), no toxic effect of C-25 lectin was found even at higher concentration of 600 μg/mL but it enhanced the normal cell proliferation

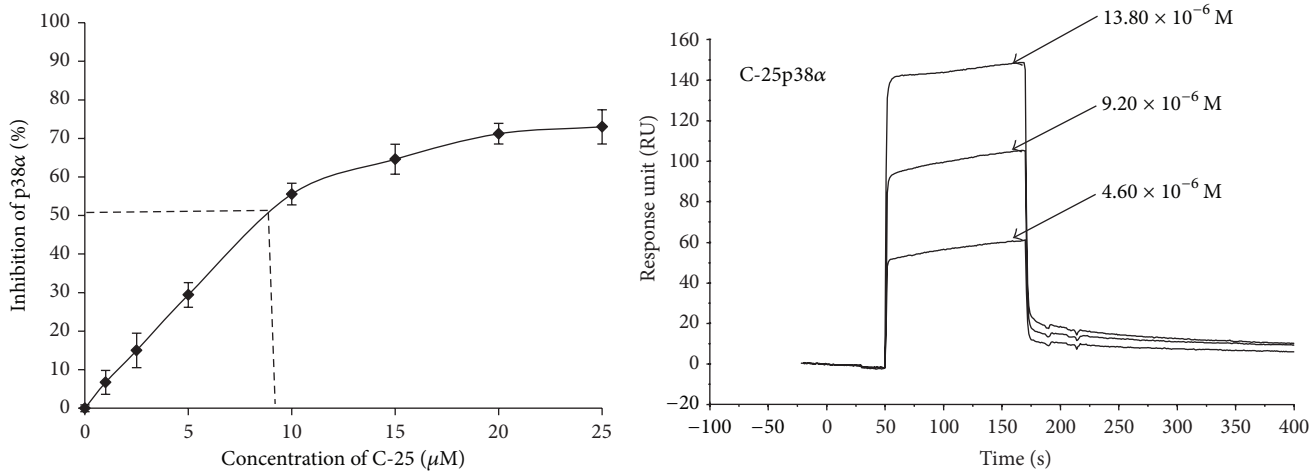


Figure 6: Inhibition assay: (a) % inhibition of p38α with increasing concentration of C-25; (b) Sensorgram showing the binding interaction of p38α with increasing concentrations of C-25.

(Figure 5). Hence, it indicates that the C-25 inhibits the proliferation of cancer cells selectively.

3.7. Kinetic Analysis of C-25 with p38α MAP Kinase. p38α is a cell signaling molecule and is reported to be overexpressed in oral cancer [13]. Hence, binding study of C-25 was performed with recombinant p38α MAP kinase (mitogen activated protein kinase) using both ELISA and BIAcore to ensure the antiproliferative activity.

3.7.1. By ELISA. The pure p38α was incubated with C-25 and the phosphorylation activity of p38α was tested in the presence of ATP. It inhibited p38α by competing with ATP. Thus, it prevented the phosphorylation of the activated transcription factor-2 (ATF-2). The IC_{50} value of C-25 was found to be 7.9 μM against the pure p38α protein (Figure 6(a)).

3.7.2. By SPR Technology. The specific bindings of C-25 were determined in the form of binding capacity on to immobilized p38α protein. The change in RU (resonance unit) with different concentrations denoted the change in bound mass on the sensor chip with time giving the KD value of C-25, 2.69×10^{-7} M. The sensorgram in Figure 6(b) shows the binding of varying concentrations of C-25 over p38α.

Hence, by ELISA and SPR it can be revealed that C-25 can inhibit the activity of p38α.

4. Discussion

Cicer arietinum has been used in many traditional medical purposes. C-25 protein isolated from *Cicer arietinum* exhibited strong antifungal activities against human pathogens: *Candida krusei*, *Candida tropicalis*, and *Candida parapsilosis* of MIC values 1.56–12.5 μg/L. It also inhibits the growth of fungal strains which are resistant and susceptible-dose dependent to fluconazole. The MIC of C-25 on fungal growth was comparable to the antifungal lectins of other leguminous plants. Though the exact mode of action of lectin on fungal growth is not clearly known it was previously observed by SEM that lectin disrupted the cell wall and resulted in leakage of cytoplasm [14]. In the present investigation, C-25 also acts primarily on the cell wall of *Candida* species, by disrupting the cell wall and distorting the cellular morphologies.

Lectins are widely used in agriculture as antimicrobials and pesticides. Some lectins have been isolated from plants having antifungal properties in plant pathogens [15–22]. The present study reveals the isolation of lectin (C-25) of molecular weight 25 kDa from *Cicer arietinum*. The C-25 was found to be monomer as the molecular mass obtained by SDS-PAGE analysis was the same in both reducing and nonreducing conditions. N-terminal sequence of the C-25 protein had some amino acids sequence similarity with the previously isolated lectin from other plant sources having a different molecular weight. The database search using BLAST indicated that the sequence showed 100% homology with lectins of *Pisum sativum*, *Lathyrus sativus*, and *Cicer arietinum*. The characteristic properties of lectin isolated previously from *Cicer arietinum* (PDB 3S18) are not reported. The present study isolated lectin C-25 from chickpea (*Cicer arietinum*) and reported the biological properties. Many sugar binding lectins from seeds of leguminous plants are well characterised and offer many biological functions. The hemagglutination activity of C-25 was inhibited by N-acetyl-D-galactosamine and showed to be N-acetyl-D-galactosamine-specific protein.

It is well recognized that lectins exhibit an anticancer activity. The intensive cancer research is going on the basis of different cell surface sugar moieties of cancerous cells [23]. The different mode of cytotoxic effect was observed by different lectin. Lectin isolated from different sources differentially inhibited the type of cancer cell proliferation like leukemia L1210 cells [24], HeLa and FemX cells [25], breast cancer MCF7 cells and hepatoma HepG2 cells [26], hepatoma (HepG2) cells [27, 28], and KB cell line. Earlier

studies have reported the inhibitory effect of ethanol/acetone extract from *Cicer arietinum* on the proliferation of Caco-2 cells [29] as well as the antiproliferative effect of *Cicer arietinum* PIC on breast and prostate cancer cell lines [30]. In the present study, MTT assay demonstrated a significant cell death of oral cancer cell line (KB cell line) treated with C-25. The inhibition of KB cell line viability with C-25 was concentration dependent. But even at high doses it is nontoxic to normal mammalian PBMCs; rather it induces proliferation of normal cells which is the characteristic of many plant lectins [31].

This lectin also inhibits the p38α MAP kinase in presence of substrate (ATP) and showed binding affinity with p38α. The p38α plays a central role in the production of inflammatory cytokines IL-1β, TNF-α, and IL-6. The overproduction of these cytokines causes tumor growth. There is an evidence of overexpression of p38α in oral cancer patients and its declination after treatment [13]. Hence, it may be assumed that C-25 inhibits the oral cancer cell lines (KB cells) growth by targeting p38α MAP kinase.

It can be concluded that a lectin C-25 isolated from *Cicer arietinum* possessed carbohydrate specificity and antifungal and antiproliferative activity. Hence, C-25 only after *in vivo* studies can be considered to be an effective bioactive compound.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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