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Antimicrobial Properties of Basil (*Ocimum basilicum* L.), Sage (*Salvia officinalis* L.), Lavender (*Lavandula officinalis* L.), Immortelle (*Helichrysum italicum* (Roth) G. Don), and Savory (*Satureja montana* L.) and Their Application in Hard Cheese Production

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Abstract: The aim of the study was to evaluate the antimicrobial activity of the extracted plants basil (*Ocimum basilicum* L.), sage (*Salvia officinalis* L.), lavender (*Lavandula officinalis* L.), immortelle (*Helichrysum italicum* (Roth) G. Don), savory (*Satureja montana* L.), and rosemary (*Salvia rosmarinus* Spenn.) against foodborne and clinical pathogens. Dried plants were used in the production of Dalmatian cow's milk hard cheese at concentrations of 0.5, 1.0, 1.5, and 2% to evaluate the microbiological safety and sensory properties of novel cheeses. The broadest antimicrobial activity was found in rosemary and sage, inhibiting ten indicator pathogens, and the strongest antimicrobial activity was found in immortelle and sage, which showed the widest zones of inhibition. The most sensitive indicators were *Staphylococcus* species and *Yersinia enterocolitica*. The supplemented cheeses met the official microbiological criteria and were mycotoxin negative. The surface mycobiota of control and experimental cheeses consisted mainly of *Penicillium sollitum*, based on the sequence analysis of the beta-tubulin and calmodulin genes. The antifungal effect of the added plants was clearly demonstrated in cheeses with added basil and sage ($p < 0.05$).

Keywords: hard cheese; plant extracts; dried plants; safety; quality

Citation: Zdolec, N.; Franičević, M.; Klanac, L.; Kavain, I.; Batinić, J.; Zadravec, M.; Pleadin, J.; Čobanov, D.; Kiš, M. Antimicrobial Properties of Basil (*Ocimum basilicum* L.), Sage (*Salvia officinalis* L.), Lavender (*Lavandula officinalis* L.), Immortelle (*Helichrysum italicum* (Roth) G. Don), and Savory (*Satureja montana* L.) and Their Application in Hard Cheese Production. *Hygiene* **2024**, *4*, 135–145. https://doi.org/10.3390/hygiene4020010

Academic Editor: Günter Kampf

Received: 21 February 2024
Revised: 17 March 2024
Accepted: 26 March 2024
Published: 29 March 2024

1. Introduction

Aromatic plants and medicinal plants have been used in folk medicine and as food preservatives since ancient times. They contain several active ingredients that have antibacterial, antifungal, antiparasitic, antioxidant, and anti-inflammatory properties, among others, and the best known of these plants originate from the Mediterranean region [1–3]. Antimicrobial properties are most commonly associated with essential oils and may help to prevent and slow down food spoilage, extend shelf life, and be a potential substitute for chemical preservatives [4].

Before looking at their practical applications and effects in food production, the antimicrobial properties of plants are first tested in vitro on their extracts. In this respect, several species from the broad spectrum of Mediterranean wild plants can be highlighted. For example, basil extract shows significant antibacterial and antifungal properties [5–8], and linalool, one of the dominant substances in basil (*Ocimum basilicum* L.), is responsible for its general antimicrobial activity [9]. On the other hand, sage (*Salvia officinalis* L.) has

significant clinical importance in medicine [10,11], but its antimicrobial and antioxidant activity has also been noted in food (soft cheeses) preservation [12,13]. The antibacterial and antifungal effects of lavender (*Lavandula officinalis* L.) have also been reported in many recent studies [14]. Rosemary (*Salvia rosmarinus* Spenn.) has long been known for its strong antioxidant activity, and its antimicrobial effect is also confirmed in food by inhibiting several foodborne pathogens, such as *L. monocytogenes* [12,15]. Still, Nieto et al. [16] emphasize that the antioxidant and antimicrobial properties of rosemary depend on many intrinsic and extrinsic factors. Immortelle (*Helichrysum italicum* (Roth) G. Don) is primarily thought to have an anti-inflammatory effect [17], and research also confirms a good antimicrobial activity of its ethanol extracts against Gram-positive bacteria [18,19], but its use in food matrices is rarely reported. Finally, savory (*Satureja montana* L.) compounds may affect the mycobiota commonly found during cheese ripening [20].

Therefore, the use of plants and their natural preservatives and antioxidants can be significantly promoted as part of the agenda for sustainable food production systems with a focus on biodiversity conservation, e.g., in cheese production [21]. Several studies have shown the positive impact of phytochemicals on the protection and improvement of the safety and quality of different cheeses [22] by demonstrating their antibacterial (sage, lemon, thyme, basil; [23,24]), antioxidant (rosemary, oregano; [25–28]), or antifungal activity (basil and savory; [20,29]). In this context, the aim of this work was to investigate the antimicrobial activity of ethanol extracts from wild Mediterranean plants from the region of Dalmatia, Croatia—rosemary, basil, sage, immortelle, savory, and lavender—against foodborne and clinical (animal) bacterial isolates. Dried forms of the plants were also used in the production of hard cheese from cow's milk to develop new products and evaluate their microbiological safety.

2. Materials and Methods

2.1. Plants and Extracts

Five dried aromatic plants from the Dalmatian region of Croatia were used for testing antimicrobial properties and experimental cheese production: basil (*Ocimum basilicum* L.), sage (*Salvia officinalis* L.), lavender (*Lavandula officinalis* L.), immortelle (*Helichrysum italicum* (Roth) G. Don), and savory (*Satureja montana* L.). In addition, rosemary (*Rosemarinus officinalis* L.) was tested in some laboratory trials.

To obtain plant extracts intended for testing antimicrobial properties in vitro, a Soxhlet apparatus was used. All plants were first crushed and dried. After drying, 30 g were taken from each plant and divided into cones. Each flask was placed in its own chamber of the Soxhlet apparatus. Flasks containing 150 mL of 70% ethanol heated to 80 °C were placed under the chambers. Above the chambers containing the cones with the dried plants are coolers in which the vaporized ethanol condenses. After condensation, the ethanol drips onto the funnel with the dried plant and collects in the chamber. When the amount of ethanol in the chamber reaches a certain level, it is poured back into the flask and evaporates again. The extraction continued until the ethanol collected in the chamber became completely transparent. The flasks with the accumulated ethanol extract were then removed from the Soxhlet apparatus and filtered through filter paper. The resulting liquid was evaporated in a rotary evaporator at a bath temperature of 27 °C. The pressure in the evaporator was set to 58 mbar, and the rotation was carried out at a speed of 40 revolutions per minute. After evaporation, the extracts (25% w/v) were filtered again, placed back on a rotary evaporator and evaporated to a volume of 120 mL. A further filtration was then carried out, and the extracts were stored in a cool place in dark bottles.

2.2. Antimicrobial Activity of Plant Extracts Tested by Agar Disk Diffusion

The antimicrobial potential of ethanol extracts was evaluated via a disk diffusion test [30] against 17 bacterial strains of species: *Salmonella enterica* ser. Infantis, *erm*A+ *Staphylococcus haemolyticus*, *erm*C+ *Staphylococcus haemolyticus*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Listeria innocua*, *Listeria welshimeri*, *Listeria ivanovii*, *Listeria monocytogenes*, vanB+ *Enterococcus faecalis*, vanA+ *Enterococcus faecium*, and vancomycin-resistant *Enterococcus faecium*. Bacterial

cultures were grown in appropriate broth and inoculated on selective agars for the final preparation of a cell suspension of 0.5 McFarland to be used in the disk diffusion assay. Suspensions were streaked on Brain Hearth Infusion agar (BHI, Biolife, Milano, Italy) and disks (6 mm in diameter) soaked with filtered herbal extracts were placed onto agar plates and incubated for 24 h at 37 °C. The widths of inhibition zones were measured by an automatic device (Scan 1200, Interscience, Saint-Nom-la-Bretèche, France). Inhibition below 10 mm was considered as weak (+), 10–20 mm as medium (++), and >20 mm as a strong effect (+++). Two extract preparations and two measures were performed.

2.3. Antimicrobial Activity of Plants and Extracts on Dairy Starter Culture

The effects of cold-sterilized sage, immortelle, rosemary, and their extracts against the commercial cheese starter culture DI-PROX LH1 (Bioprox, Levallois-Perret, France) in pasteurized milk were evaluated. To avoid microbiological interactions in the milk, the dried herbs were irradiated at the Co-60 panoramic irradiation source (8 kGy, P = 5.23 Gy/s) at the Laboratory of Radiation Chemistry and Dosimetry of the Ruđer Bošković Institute Zagreb, Croatia. The herbal extracts were prepared according to the Soxhlet protocol and then filtered and evaporated under a rotational vacuum. Different concentrations of the sterilized herbs and extracts (0.25, 0.5 and 1% *v*/*w*) were added to pasteurized milk inoculated with a starter (3 $\log_{10}$ CFU/mL) and incubated at 30 °C for 24 h.

2.4. Cheese Production and Analyses

The production of hard cheese Dalmatinski sir (Puđa Ltd., Trilj, Croatia) supplemented with dried basil (*Ocimum basilicum* L., 'Genovese'), sage (*Salvia officinalis* L.), lavender (*Lavandula officinalis* L.), immortelle (*Helichrysum italicum* (Roth) G. Don), and savory (*Satureja montana* L.) was started in June/July 2022. Each herb species was used in 4 different concentrations: 0.5%, 1.0%, 1.5%, and 2.0%. The exact weight of added dried herbs was calculated in relation to the weight of single mature cheese rounds (2.7 kg on average) and were, therefore, as follows: 13.5 g for 0.5%; 27.0 g for 1.0%; 40.5 g for 1.5%; and 54.0 g for 2.0%. Immortelle was prepared in two concentrations, 0.5% and 1.5%, due to it being very voluminous even after milling. The herbs were weighed and submerged for 24 h in previously boiled and cooled water. The water and herb mixtures were added to the stirring vessel before rennet. Cheeses were ripened to maturity for two months and vacuum sealed until analyses.

2.4.1. Microbiological Analyses of Cheese

For the microbiological analysis, 25 g of cheese was taken after the surface layers of the product had been sterilely separated. The sample was diluted in 225 mL buffered peptone water (Biolife, Milano, Italy) and homogenized at 200 rpm for 2 min (Stomacher 400, Seward, Worthing, UK). Serial decimal dilutions were then prepared to determine the total viable count (TVC), lactic acid bacteria (LAB), enterococci, yeasts and molds, enterobacteria, *Escherichia coli*, sulfite-reducing clostridia, coagulase-positive staphylococci, *Listeria monocytogenes*, and the presence of *Salmonella* spp.

TVC was determined according to ISO 4833-1 [31] using plate count agar (PCA, bioMerieux, Craponne, France) through incubation at 30 °C for 72 h. The number of LAB was determined based on ISO 15214 [32] using de Man–Ragosa–Sharpe agar (MRS, Merck, Darmstadt, Germany) through incubation at 30 °C for 24–48 h, and the amount of enterococci was measured through growth on chromogenic Compass Enterococcus agar medium (BIOKAR, Beauvais, France) with incubation at 44 °C for 24 h. The number of yeasts and molds was determined on oxytetracycline–glucose–yeast agar (OGY, Merck, Darmstadt, Germany) through incubation at 25 °C for 48–72 h (ISO 21527-1) [33]. The number of *E. coli* was counted on rapid *E. coli* chromogenic medium (BIOKAR, Beauvais, France) incubated at 37 °C for 24 h and enterobacteria on violet red bile agar with glucose (VRBG, Merck, Darmstadt, Germany) through incubation at 37 °C for 24 h (ISO 21528-1) [34]. The number of coagulase-positive staphylococci was determined on

Baird-Parker agar (Merck, Darmstadt, Germany) incubated at 37 °C for 24–48 h (ISO 6888-1) [35], and the number of clostridia was determined according to ISO 15213 [36] on iron sulfite agar (bioMerieux, Marcy l'Etoile, France) incubated anaerobically at 37 °C for 72 h (Anaerocult, Merck, Darmstadt, Germany). The number of *L. monocytogenes* was assessed according to the ISO 11290-2 [37] method and the presence of *Salmonella* according to ISO 6579-1 [38]. Catalase, oxidase, and coagulase tests for LAB, enterobacteria, and staphylococci, respectively, were performed prior to counting. The results are expressed as logarithmic values for the number of colonies per gram of cheese ($\log_{10}$ CFU/g) as an average of three measurements ± SD.

2.4.2. Molecular Detection of Molds

The methods for mold identification were provided by Lešić et al. [39]. Briefly, the visible mold colonies and cheese surface swabs were subsequently transferred onto a DG-18 agar (dichloran 18%–glycerol, Merck, Darmstadt, Germany). After a seven-day incubation in darkness at 25 ± 1 °C, individual mold cultures were sub-cultivated on DG-18 agar, malt extract agar (MEA, BD Difco, Franklin Lakes, NY, USA), and Czapek yeast extract agar (CYA, BD Difco, Franklin Lakes, NY, USA) and incubated for seven days at 25 ± 1 °C in darkness to the end of identification using a traditional method based on macro- and micro-morphology and growth characteristics according to the literature [40,41]. DNA was extracted from isolated mold colonies using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Primers specific to beta-tubulin (benA)—the Bt2a forward primer (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and the Bt2b reverse primer (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′)—and calmodulin (CaM) loci—the Cmd5 forward primer (5′-CCGAGTACAAGGARGCCTTC- 3′) and the Cmd6 reverse primer (5′-CCGATRGAGGTCATRACGTGG-3′)—were selected for polymerase chain reaction (PCR) amplification (Macrogen, Amsterdam, The Netherlands). After purification using the ExoSAP-IT PCR clean-up reagent (Affymetrix, Santa Clara, CA, USA), amplicons were sent to a commercial facility for sequencing (Macrogen, Amsterdam, The Netherlands). Sequences were aligned and edited using the DNASTAR Software 16 (Lasergene, Madison, WI, USA) and then compared to those available from the GenBank database using the BLAST algorithm.

2.4.3. Determination of Mycotoxins

For the determination of aflatoxin B1 (AFB1), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), and citrinin (CIT), cheese samples were homogenized using a Grindomix GM 200 (Retsch, Haan, Germany) and stored at +4 °C prior to analyses. Different sample preparation procedures were implemented through the use of chemicals of an analytical grade, as follows:

AFB1: An amount of 20 mL of acetonitrile (50%) was added to 3 g of the sample and shaken for 90 min. After centrifugation (15 min, 5000 rpm, 10 °C), 3 mL of supernatant were diluted in 12 mL of deionized water and cleaned using ISOLUTE Myco columns (60 mg/3 mL, Biotage, Sweden) and conditioned using 2 mL of acetonitrile and water. A total of 3 mL of sample was applied on the column and then washed with 6 mL of water and 10% acetonitrile. After drying, columns were eluted with 2 mL of 0.1% formic acid in acetonitrile and 2 mL of methanol. The obtained eluate was evaporated and dissolved in 500 µL of methanol/water solution (35/65).

OTA: An amount of 0.5 mL of 1 M H_3PO_4 and 6 mL of ethyl acetate were added to 1 g of the sample, and the components were mixed and centrifuged (1 min, 3000 rpm). A total of 6 mL of ethyl acetate was added to the supernatant, the layers were combined, and 3 mL of 0.26 M $NaHCO_3$ was added, mixed, and centrifuged (5 min, 3000 rpm). A total of 800 µL of the lower aqueous phase was transferred, heated in a water bath for 5 min at 100 °C, shaken, and supplemented with 200 µL of 0.225 M HCl and 1 mL of 0.13 M $NaHCO_3$.

DON and ZEN: Glucoronidase/arylsulphatase Helix pomatia (Art No. 4114, Merck) (20 µL) were added to a sample (1 g), incubated for 3 h at 37 °C, and centrifuged (3 min,

3000 rpm). A total of 100 µL of methanol (for ZEN) or water (for DON) was added to the upper layer of the sample. The resulting solution (50 µL) was used for further analyses.

CIT: A quantity of 20 mL of 80% methanol was added to 5 g of the sample and then the components were mixed and centrifuged (10 min, 4000 rpm). A total of 12 mL of the supernatant was diluted with 60 mL of 0.1% Tween 20 PBS buffer and gently mixed. A total of 48 mL of the sample solution was applied to the immunoaffinity columns Easi-extract Citrinin® (R-Biopharm, Darmstadt, Germany). The mixture was washed with 20 mL of PBS buffer without Tween, dried for 3 min, eluted with 1 mL of 100% methanol, and washed with 1 mL of ultrapure water. The supernatants were diluted with deionized water (1 + 1).

After sample preparation, the determination of mycotoxin concentration was performed using competitive ELISA kits, completely according to the test procedures declared by the kits' manufacturers for each mycotoxin (R-Biopharm, Darmstadt, Germany) and using a ChemWell autoanalyzer (Awareness Technology Inc., Palm City, FL, USA).

2.5. Statistical Analysis

The results were processed using the methods of descriptive statistics (Statistica 13.5) and presented as mean values of three measurements with standard deviation (x ± SD). To determine statistically significant differences in the number of microorganisms between different types of cheese, a one-way analysis of variance (one-way ANOVA) was used for indicators that followed a normal distribution, and a Kruskall–Wallis analysis of variance was used for indicators that did not follow a normal distribution. The differences between the individual bacteria groups were determined using a post hoc analysis at a probability level of 0.05.

3. Results and Discussion

3.1. Antibacterial Capacity of Plant Extracts

The ethanol extracts showed an inhibitory effect on 12 of 17 strains tested (Table 1). Staphylococci and *Yersinia enterocolitica* were the most sensitive, being inhibited by all extracts. Rosemary extract inhibited the broadest spectrum, i.e., ten indicator bacteria, and lavender inhibited the fewest, i.e., three of them. The immortelle extract showed the strongest inhibitory activity in the form of the broadest inhibition zones against *erm*A+ *Staphylococcus haemolyticus*, *erm*C+ *Staphylococcus haemolyticus*, and *Staphylococcus aureus*. After three months of storage, the antimicrobial activity of sage, rosemary, and immortelle extracts was maintained, although attenuated. The susceptibility of vancomycin-resistant enterococci including *vanB*+ *Enterococcus faecalis* and *vanA*+ *Enterococcus faecium* strains was recorded in response to sage, rosemary, and immortelle extracts. *Listeria* species and *S.* Infantis strains were the most resistant indicator bacteria to applied plant extracts.

In general, it is assumed that Gram-negative bacteria are more resistant to the antimicrobial effect of ethanol extracts from plants and other antimicrobial agents compared to Gram-positive bacteria due to cell protection by the outer membrane [42]. This is evident in our results in the case of *Salmonella* Infantis strains, which were resistant to almost all extracts, while the other Gram-negative pathogenic bacteria, *Y. enterocolitica* 4:O3, showed moderate sensitivity to basil, sage, rosemary, and immortelle. These observed differences between species of Gram-negative bacteria in sensitivity to plant extracts have been reported previously [43]. *Staphylococcus aureus* has been shown to be an important pathogen in dairy production, from farm (mastitis) to cheese safety (enterotoxin poisoning), and any new antimicrobial solution against this pathogen could be relevant. Our study shows that *S. aureus* and *S. haemolyticus* strains, which are carriers of transferable resistance genes (*erm*), are very sensitive to all plant extracts tested, with the exception of savory. These data are consistent with those of Piras et al. [44] and Oppedisano et al. [45], who even listed 39 different plants growing in Italy that produce extracts effective against *S. aureus*, implying that this is generally very sensitive to plants extracts. Unfortunately, the second most relevant foodborne pathogen in cheese production, *L. monocytogenes*, was not affected by the extracts used in our study, as was the case for other *Listeria* species tested. Weak inhibition was

found only for rosemary against *L. innocua* and *L. welshimeri*, which is consistent with previous studies [46]. These authors emphasized that the ability of rosemary to combat *Listeria* is species- and strain-dependent and depends on the types and concentrations of the extracts. Ceruso et al. [47] reported the anti-listeria activity of only 12 extracted plants out of 800 tested from a worldwide selection. The favorable results in our study were found in the inhibition of vancomycin-resistant enterococci (VRE), which could pose a significant public health problem. Sage extracts have previously been described as good inhibitors of VRE even in the soft cheese matrix [48], as well as rosemary in vitro [49]. A more recent study by Duraisamy et al. [50] showed an inhibitory effect of basil ethanol extracts against VRE, but this was not the case with our three strains. To our knowledge, immortelle extracts have never been reported to be active against VRE. Therefore, further research is needed to confirm our preliminary results and to identify active compounds that can potentially be used to combat antimicrobial resistance.

Table 1. Inhibitory activity of plant extracts against selected indicator bacteria.

	Basil	**Sage**	**Lavender**	**Rosemary**	**Immortelle**	**Savory**
Salmonella Infantis 78	-	-	-	+	-	+
Salmonella Infantis 186	-	-	-	-	-	+
Salmonella Infantis 81	-	-	-	-	-	+
Salmonella Infantis 94	-	+	-	-	-	+
*erm*A+ *Staphylococcus haemolyticus* 422	+	++	-	++	+++	-
*erm*C+ *Staphylococcus haemolyticus* 231	++	+++	+	+++	+++	+
Staphylococcus aureus ATCC 25923	+++	+++	++	+++	+++	-
Yersinia enterocolitica 4/O:3	++	++	-	++	++	+
Listeria innocua ATCC 33090	-	-	-	+	-	-
Listeria welshimeri	-	-	-	+	-	-
Listeria ivanovii ATCC 19111	-	-	-	-	-	-
Listeria innocua	-	-	-	-	-	-
Listeria monocytogenes NCTC 10527	-	-	+	-	-	-
Listeria monocytogenes ATCC 7644	-	-	-	-	-	-
vanB+ Enterococcus faecalis	-	++	-	++	++	-
vanA+ Enterococcus faecium	-	+	-	++	++	-
VR *Enterococcus faecium*	-	+	-	++	++	-

- no effect, + weak effect, ++ medium effect, +++ strong effect.

In the subsequent experiments, the selected extracts with the strongest antibacterial effect and dried herbs (sage, rosemary, and immortelle) were used in pasteurized milk inoculated with commercial starter cultures. To avoid microbiological interactions in the raw material (milk), the dried herbs were irradiated with the Co-60 panoramic irradiation source (8 kGy, P = 5.23 Gy/s). The irradiated herbs were free of sporogenic bacteria and molds. The starter culture *Lactobacillus helveticus*/*Lactobacillus lactis* reached 7.4 log CFU/mL in pasteurized milk at 30 °C/24 h. When dried herbs were used, the growth of the milk cultures was not affected regardless of the concentrations used. The higher the concentration of sage and immortelle extracts, the lower the number of starter cultures, which was, however, within the growth rate of the control sample. These preliminary results

showed that dried sage, immortelle, and rosemary and their extracts do not negatively affect the target starter culture *Lactobacillus helveticus*/*Lactobacillus lactis*, which is important for their use in pilot cheese production. The effect of plant extracts on milk starter cultures and the quality of dairy products have been the focus of many studies and were reviewed by Granato et al. [51]. In general, studies showed the positive effect of plant extracts used in a wide range of dairy products, including cheese, and the growth of starter cultures was not affected by their presence, such as oregano essential oil [52]. In fact, there are few studies focusing on the plants used in our study in terms of their effects on cheese starter cultures under laboratory conditions. However, plant extracts have recently been proposed as functional food ingredients that can improve the viability of probiotic starter cultures in fermented milk beverages [53].

3.2. Microbiological Properties of Cheeses Produced with Dried Plants

In the final stage of our study, the grounded plants (sage, immortelle, savory, basil, and lavender) were used in cheese production. Maturated cheeses supplemented with dried herbs were microbiologically safe at the end point of the process. *Salmonella* spp. was absent in all samples, and *L. monocytogenes*, coagulase-positive staphylococci, *E. coli*, sulfite-reducing clostridia, and enterobacteria were found to be below the detection limits of the methods (<1 $\log_{10}$ CFU/g). These results are in line with previous studies and are a confirmation of the high-level standards in the production process. These results also prove the effectiveness of this technology [54]. The addition of grounded plants had no significant influence on the microbiota studied ($p < 0.05$) compared to control cheese (Table 2). Although the differences are small, the addition of lavender showed the best antibacterial activity ($p < 0.05$) when considering the total viable count and lactic acid bacteria. On the other hand, the use of immortelle showed the opposite effect on these populations but significantly reduced the count of enterococci compared to other plants.

Table 2. Microbial populations of cheeses supplemented with dried aromatic herbs.

Parameter ($\log_{10}$ CFU/g)	Basil	Sage	Lavender	Immortelle	Savory	Control Cheese
Total viable count	7.06 ± 0.71	7.23 ± 0.19	6.85 ± 0.76 a	7.38 ± 0.08 a	6.96 ± 0.28	7.30 ± 0.15
Lactic acid bacteria	6.98 ± 0.16	7.28 ± 0.39	6.50± 0.57 b	7.87 ± 0.03 b	7.45 ± 0.20	7.69 ± 0.21
Enterococci	1.63 ± 0.22	3.63 ± 0.13 c	2.97 ± 0.45	1.62 ± 0.21 c	2.72 ± 0.76	2.43 ± 0.10
Yeasts and molds	<2.00 d	1.74 ± 1.19	2.21 ± 1.47	3.00 ± 0.00	3.09 ± 0.58	3.30 ± 0.10 d

values marked with the same letters in the same line differ statistically at the level of 0.05.

Most importantly, it was evident that the addition of basil showed significant antifungal capacity ($p < 0.05$), contributing to improvement in the hygienic quality of the final product. The antifungal capacity of basil is well documented [55] and reported in different cheese types as a promising defense against contaminating molds [56,57].

Since fungal contamination in cheese production can pose a hygienic threat during the ripening process, the mycobiota was collected from the cheese surfaces and molecularly identified in this study. In addition, the presence of mycotoxins was assessed. Regardless of the plant species used in cheese production, the majority of cheese samples were contaminated with *Penicillium solitum*. In addition, other species were also identified—*Penicillium nalgoviense*, *Penicillium salamii*, *Penicillium discolor*, *Penicillium citrinum*, and *Aspergillus proliferans*—noticeably so when using savory, probably as a result of the highest fungal contamination compared to other added herbs (Table 2). Of the identified species, only *P. citrinum* was found to be relevant to food safety due to the possible production of the mycotoxin citrinin. As a mesophilic mold, *P. citrinum* is often isolated from various foods such as cereals, nuts, fermented cured meats, and

cheese [58]. The mold contamination of cheese during the ripening process is a common hygienic problem that affects both quality and safety [59]. The composition of spoilage mycobiota depends on many factors, such as climate, season, microclimate in the dairies, general hygiene, and others. However, it is generally accepted that *Penicillium* and *Aspergillus* species are the most common cheese-contaminating molds. The most frequently isolated species in cheese production worldwide is *Penicillium commune* [59]. *Penicillium solitum*, as the most commonly identified species in our cheese production, can also be found as part of the contaminating mycobiota in other cheeses such as Italian Grana cheese [60]. In any case, our study is the first report on the molecular identification of molds isolated from plant-supplemented hard cheeses in Croatia. Based on the molecular profiles, only the cheese produced with savory was additionally contaminated with *Penicillium salamii*, a new species recently discovered in the production of Italian salami [61]. Its presence in cheese mycobiota has never been reported before, which prompted us to further investigate the contamination pathways in cheese production with grounded plants.

4. Conclusions

The use of local wild medicinal and aromatic plants offers great potential for the development of new autochthonous cheese varieties. The antimicrobial properties of the plants used in this study can also contribute to the hygienic quality of the product. The antifungal effect of basil during cheese ripening seems to be the most promising finding from a hygienic point of view. Considering the environmental contamination of cheese surfaces, the antifungal properties of basil extracts can be considered as a potential alternative to natamycin, a common antifungal coating for cheese surfaces.

Author Contributions: Conceptualization, N.Z. and M.K.; methodology, N.Z., L.K., M.F., M.Z. and J.P.; software, M.K. and M.Z.; validation, J.P., formal analysis, L.K., M.K., I.K., J.B., M.Z., J.P. and N.Z.; investigation, N.Z.; resources, D.Č.; data curation, M.K.; writing—original draft preparation, N.Z.; writing—review and editing, J.P., M.Z. and M.K.; supervision, N.Z.; project administration, M.K. and N.Z.; funding acquisition, N.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by European Commission—European Regional Development Fund, project Center of competence 3LJ, grant number KK.01.2.2.03.0017.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are presented within the paper.

Conflicts of Interest: The authors declare no conflicts of interest. Mr. Darko Čobanov is employed at Pudja Ltd. (cheese making company) and provided available materials and equipment to conduct research in pilot production of cheese; The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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