






## Article

# Effect of Dough-Related Parameters on the Antimold Activity of *Wickerhamomyces anomalus* Strains and Mold-Free Shelf Life of Bread

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**Abstract:** The aim of the present study was to assess the antimold capacity of three *Wickerhamomyces anomalus* strains, both in vitro and in situ, and to identify the responsible volatile organic compounds. For that purpose, two substrates were applied; the former included brain heart infusion broth, adjusted to six initial pH values (3.5, 4.0, 4.5, 5.0, 5.5, 6.0) and supplemented with six different NaCl concentrations (0.0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%), while the latter was a liquid dough, fortified with the six aforementioned NaCl concentrations. After a 24 h incubation at 30 °C, the maximum antimold activity was quantified for all strains at 5120 AU/mL, obtained under different combinations of initial pH value and NaCl concentration. A total of twelve volatile compounds were detected; ethanol, ethyl acetate, isoamyl alcohol and isoamyl acetate were produced by all strains. On the contrary, butanoic acid-ethyl ester, acetic acid-butyl ester, ethyl caprylate, 3-methyl-butanoic acid, 2,4-di-tert-butyl-phenol, benzaldehyde, nonanal and octanal were occasionally produced. All compounds exhibited antimold activity; the lower MIC was observed for 2,4-di-tert-butyl-phenol and benzaldehyde (0.04 and 0.06 µL/mL of headspace, respectively), while the higher MIC was observed for butanoic acid-ethyl ester and ethyl caprylate (5.14 and 6.24 µL/mL of headspace, respectively). The experimental breads made with *W. anomalus* strains LQC 10353, 10346 and 10360 gained an additional period of 9, 10 and 30 days of mold-free shelf life, compared to the control made by commercially available baker's yeast. Co-culture of the *W. anomalus* strains with baker's yeast did not alter the shelf-life extension, indicating the suitability of these strains as adjunct cultures.

**Keywords:** yeasts; adjunct culture; inhibitory capacity; *P. chrysogenum*; volatile organic compounds

## 1. Introduction

The application of antagonist yeasts, as biological control agents (BCAs) in the production of clean label products, has been at the epicenter of intensive study over the past few years, as a promising alternative to chemical preservatives. Several possible mechanisms have been reported for the biocontrol activity exerted by yeasts, among which are competition for space and nutrients, biofilm formation and secretion of killer toxins, volatile organic compounds (VOCs) and hydrolytic enzymes [1]. The use of *Wickerhamomyces anomalus* strains as antimold agents has received considerable attention due to the antagonistic features of the yeast [2].

*Wickerhamomyces anomalus* is an ascomycetous yeast, formerly known as *Hansenula anomala*, *Pichia anomala* and *Saccharomyces anomalus*, the anamorph name of which is *Candida pelliculosa* [3]. Its frequent isolation from a wide variety of ecological niches, ranging from soil, plants and water to dairy and baked products, indicates the highly competitive nature of this yeast species [4,5]. In addition, the ability of *W. anomalus* strains to ferment many carbon and nitrogen sources, combined with their capacity to survive in harsh conditions, in terms of temperature, initial pH value, water activity and osmotic pressure, suggest a robust character [6]. Due to the ability of *W. anomalus* strains to grow in a broad range of temperatures, its implication as an opportunistic pathogen has been claimed and its occasional identification in clinical settings has raised some consideration [7]. However, the European Food Safety Authority (EFSA) addressed these safety concerns, by granting the Qualified Presumption of Safety (QPS) status to *W. anomalus* for use in food industries [8]. Regarding the antimold activity assessment of *W. anomalus* strains, several studies have associated their biocontrol activity with VOCs production [9,10], with ethyl acetate and 2-phenylethanol being reported as the major ones [11,12].

Despite the fact that the application of *W. anomalus* strains for postharvest decay control in fruits and vegetables has extensively been reported [13,14], only scarce is the literature concerning their effect on the shelf life of foodstuff in general, and particularly bread. Bread spoilage represents a serious concern for food industries, leading to significant economic losses and posing a major threat for human health. The most common molds causing bread spoilage belong to *Penicillium*, *Aspergillus* and *Cladosporium* genera [15]. The applicability of *W. anomalus* strains to control their proliferation has only been marginally assessed [16].

In our previous study [17], the inhibitory activity of three *W. anomalus* strains, namely LQC 10346, 10353 and 10360, against the growth of *P. chrysogenum*, was reported and attributed to the production of non-proteinaceous compounds. Thus, the aim of the present study was to assess the effect of substrate, initial pH value and NaCl content on the growth and antimold activity of the three *W. anomalus* strains, identify the responsible VOCs and to evaluate the effect on the shelf life of experimental breads.

## 2. Materials and Methods

### 2.1. Microbial Strains and Culture Conditions

Three sourdough-derived yeast strains, namely *W. anomalus* LQC 10346, 10353 and 10360 [18], and one mold strain belonging to the species *P. chrysogenum* (moldy bread isolate) were used throughout this study. The yeast strains were able to produce non-proteinaceous compounds with inhibitory activity against the mold strain [17].

The yeast strains were stored at  $-20\text{ }^{\circ}\text{C}$  in Nutrient Broth (LAB M, Lancashire, UK), supplemented with 50% glycerol (Applichem, Darmstadt, Germany). The mold strain was stored in Brain Heart Infusion (BHI) agar (LAB M) slants, at  $4\text{ }^{\circ}\text{C}$ . Before experimental use, *W. anomalus* strains were grown twice in BHI broth at  $30\text{ }^{\circ}\text{C}$  for 48–72 h, while mold was grown on BHI agar, at  $25\text{ }^{\circ}\text{C}$  for 5 days.

### 2.2. Effect of pH and NaCl on Growth and Antimold Activity of *W. anomalus* Strains

Overnight cultures of the three *W. anomalus* strains were washed twice with sterile saline, resuspended in the same medium and used to inoculate BHI broth adjusted to six initial pH values (3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) and supplemented with NaCl at concentrations (0.0%, 0.5%, 1.0%, 1.5%, 2.0% and 2.5%). The inoculum level was  $6\text{ log CFU/mL}$ . Incubation took place at  $30\text{ }^{\circ}\text{C}$  for 24 h. Yeast enumeration was performed by plating serial dilutions on BHI agar. The well diffusion assay (WDA), as previously described by Syrokou et al. [17], with minor modifications, was applied to assess the antimold activity. More accurately, wells were aseptically punched, with the aid of a Pasteur pipette, in freshly prepared lawns of BHI agar previously surface inoculated with the *P. chrysogenum* strain. Twenty-four-hour yeast cultures were centrifuged ( $12,000\times g$ ; 15 min;  $4\text{ }^{\circ}\text{C}$ ) to obtain cell-free supernatants (CFS), which were further neutralized and treated with catalase (Sigma-Aldrich, St. Louis, MO, USA). An amount of  $25\text{ }\mu\text{L}$  of each CFS was added to each well. Incubation took

place at 25 °C for 5 days. The antimold activity was quantified by applying the two-fold serial dilution approach on the CFS and expressed in AU/mL. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution, exhibiting a clear inhibition zone, multiplied by 40 to obtain AU/mL. All analyses were performed in triplicate and the average values are presented.

Liquid wheat dough was prepared by mixing sterilized wheat flour (10 g) and tap water (30 mL). The liquid wheat dough at pH 6.0 was supplemented with NaCl at six concentrations (0.0%, 0.5%, 1.0%, 1.5%, 2.0% and 2.5%). Overnight cultures of the three *W. anomalus* strains were washed twice with sterile saline, resuspended in the same medium and used to inoculate liquid wheat dough at 6 log CFU/mL. Incubation took place at 30 °C for 24 h. Yeast enumeration and WDA were performed as previously described.

### 2.3. Identification of Yeast Volatile Compounds by SPME-GC-MS

*W. anomalus* VOCs obtained after growth of the strains in conditions, which previously revealed the maximum antimold activity, were determined by SPME-GC-MS, according to Xagoraris et al. [19], with some minor modifications. Regarding the isolation of VOCs, SPME was performed using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber 50/30 µm (Supelco, Bellefonte, PA, USA) with 1 cm length. For fiber activation, heating to 260 °C was applied. At the same time, two mL of *W. anomalus* CFSs were introduced into 15 mL screw top vials with PTFE/silicone septa and water bath followed at 35 °C for 15 min. Then, the needle of the SPME fiber was inserted into the vial and exposed to the sample headspace. The sampling time lasted 15 min. Then, the fiber was removed from the vial and inserted into the gas chromatograph injector for analysis of the isolated VOCs. VOCs were analyzed applying a Trace Ultra gas chromatograph (GC) (Thermo Scientific Inc., Waltham, MA, USA), coupled to a mass spectrometer (MS) (DSQII, Thermo Scientific Inc., Waltham, MA, USA). The desorption conditions were as follows: GC inlet temperature 260 °C in the splitless mode for 3 min, with a 0.8 mm injector liner (SGE International Pty Ltd., Ringwood, Australia). The column used was a Restek Rtx-5MS (30 m × 0.25 mm i.d., 0.25 µm film thickness) and the flow rate of carrier gas (helium) was 1 mL/min. The column was adjusted to 40 °C for 6 min, then increased to 120 °C at a rate of 5 °C/min, followed by another increase to 160 °C at a rate of 3 °C/min, then heated to 250 °C with a step of 15 °C/min and kept at 250 °C for 1 min. The transfer line and injector temperatures were maintained at 290 and 220 °C, respectively. Electron impact was 70 eV, and mass spectra were recorded at the 35–650 mass range. The peak identification was carried out with the Wiley 275 mass spectra library, its mass spectral data and arithmetic index provided by Adams. VOCs were tentatively identified by comparing their elution order and mass spectra with data from the NIST 98, Wiley 275 mass spectra libraries.

### 2.4. Antimold Activity Assessment of Pure Volatile Compounds against *P. chrysogenum*

Pure standards of 12 previously identified VOCs, namely ethanol, ethyl acetate, acetic acid-butyl ester, isoamyl alcohol, isoamyl acetate, 3-methyl-butanoic-acid, butanoic acid-ethyl ester, ethyl caprylate, benzaldehyde, octanal, nonanal and 2,4-ditert-butyl-phenol, purchased from Sigma Aldrich, were used for inhibitory activity assessment against *P. chrysogenum*, according to Oro et al. [12], with some modifications. In brief, an agar plug (6-mm diameter), obtained from actively growing culture of *P. chrysogenum*, was placed in the center of freshly prepared lawn of BHI agar. Then, different concentrations of the 12 VOCs, ranging from 0.02 to 6.45 µL/mL of headspace, were placed on paper filter (Whatman No. 1, 90 mm diameter), which was placed in the center of the Petri dish lid. Distilled water was used as control. The dishes were immediately closed and sealed with parafilm. Incubation took place at 25 °C for 5 days. The concentration of each VOC that inhibited growth of the mold (i.e., growth less than 5 mm around the agar plug) was considered as the minimum inhibitory concentration (MIC) of each compound. The experiment was performed in triplicate.

### 2.5. Bread Preparation and Shelf-Life Assessment

Liquid wheat dough supplemented with 1.0% NaCl concentration, in which the maximum antimold activity of the yeast strains was recorded, was prepared by mixing 20 g wheat flour and 60 mL tap water. Overnight cultures of the three *W. anomalus* strains were treated as previously described and used to inoculate the liquid wheat dough at 6 log CFU/mL. Each yeast strain was inoculated in monoculture as well as in coculture with commercial baker's yeast. Liquid wheat dough inoculated only with commercial baker's yeast was used as control. Incubation took place at 30 °C for 24 h. Then, 50 g of each incubated liquid dough was added to a mixture of 250 g wheat flour, 150 mL water and 4.5 g NaCl (final NaCl concentration 1%). After mixing, the doughs were placed in separate containers and proofing took place (30 °C for 2.5 h). Baking of doughs took place at 200 °C for 40 min. After baking, the bread loaves were cooled at room temperature for 3 h. Then, slices of ca. 20 g were cut from each loaf and placed in polyethylene bags. The shelf life of bread slices was monitored on a daily basis, at room temperature, until visible signs of mold presence were detected. The shelf life was monitored until 30 d after control bread exhibited visible mold growth. The experiment was performed in triplicate.

### 2.6. Statistical Analysis

The contribution of the initial pH value and NaCl on the antimold activity and population of the three *W. anomalus* strains was evaluated through Multifactor Analysis of Variance. The method of 95% LSD was applied as post-hoc comparison test to discriminate among the means, at  $p < 0.05$ . All calculations were performed in Statgraphics Centurion XVII (Statgraphics Technologies, Inc., The Plains, VA, USA).

## 3. Results

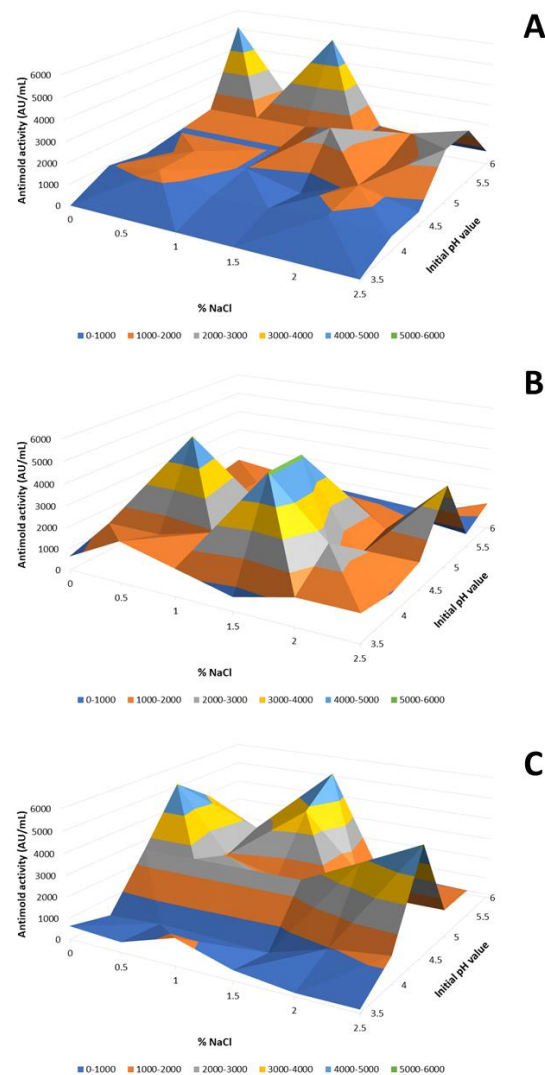
### 3.1. Effect of pH and NaCl on Growth and Antimold Activity of *W. anomalus* Strains

Antimold activity in the three *W. anomalus* strains, namely LQC 10346, 10353 and 10360, after incubation in BHI broth, adjusted to six initial pH values (3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) and six NaCl concentrations (0.0%, 0.5%, 1.0%, 1.5%, 2.0% and 2.5%), is presented in Figure 1. After 24 h incubation at 30 °C, the population of yeast strains LQC 10346, 10353 and 10360 ranged between 7.11–7.45, 7.03–7.39 and 7.03–7.46 log CFU/mL, respectively.

The maximum antimold activity, by all three yeast strains, was quantified at 5120 AU/mL. However, the aforementioned activity was achieved at different combinations of initial pH value and NaCl concentrations. More specifically, strain LQC 10346 reached maximum antimold activity in BHI broth adjusted to initial pH 6.0, supplemented with 0.0 and 1.0% NaCl. Regarding strain LQC 10353, maximum antimold activity was achieved in BHI broth adjusted to initial pH 4 supplemented with 1.5% NaCl and in BHI broth adjusted to initial pH 4.5 and supplemented with 0.5 and 1.5% NaCl. Finally, the maximum antimold activity of strain LQC 10360 was obtained in BHI broth adjusted to initial pH 5, without NaCl addition, and in BHI broth adjusted to initial pH 4.5, supplemented with 2.5% NaCl.

Multifactor Analysis of Variance was applied to assess the effect of initial pH value and NaCl on the antimold activity of each yeast strain. Initial pH value, NaCl concentration and their in-pair interactions were found to significantly affect the antimold activity exhibited by all three strains ( $p < 0.05$ ). The method of 95% LSD was applied as a post-hoc comparison test, so as to elucidate how the antimold activity of the three yeast strains was differentiated among the six levels of both pH values and NaCl concentrations (Table S1). In the case of strain LQC 10346, the mean antimold activities obtained after growth in BHI broth at pH 3.5, 4.0 and 4.5 were similar in most treatments and that was the case for the activities recorded at pH 5.0 and 5.5. Their differentiation from the mean activity, corresponding to initial pH 6.0, was evident only in a few cases, namely at 0.0 and 1.0% NaCl. Regarding the contribution of the different NaCl concentrations to the mean antimold activity, supplementation of BHI broth adjusted to pH 3.5, 5.0 and 6.0 with 0.0 and 1.0% NaCl revealed overlapping activities, which were differentiated from those at 1.5% NaCl. In the case of strain LQC 10353, the effect of pH values on the antimold activity

revealed no statistically significant differences in the majority of cases, with some minor exceptions. More accurately, in BHI broth at 1.5% NaCl, the mean activities corresponding to pH 3.5, 5.0 and 6.0 were similar and were further differentiated from those at pH 4.0 and 4.5. In addition, overlapping mean activities were recorded in the same medium at pH 3.5, 4.5, 5.0 and 5.5, at 2.0% NaCl, which were significantly different from those at pH 4.0 and 6.0, with the latter two being differentiated in a pH-dependent manner, as well. In terms of the contribution of the different NaCl levels, the mean activities recorded upon growth at pH 5.5 were similar among the different NaCl concentrations. On the other hand, the adjustment of BHI broth to pH 4.0 resulted in a NaCl-dependent antimold capacity. In brief, similar activities were recorded at 0.0, 0.5 and 2.5% NaCl, which were differentiated from those at 1.5% and 2.0%, between which significant differences were displayed as well. Regarding strain LQC 10360, overlapping mean antimold activities were recorded upon growth in BHI broth at pH 3.5 and 4.0, corresponding to 0.0, 0.5, 1.0 and 2.0% NaCl, respectively. Similar was the case for the mean activities at pH 5.0 and 5.5, at 0.5, 1.5, 2.0 and 2.5% NaCl, respectively. Concerning the effect of different NaCl levels on the antimold activity, overlapping means were observed after growth in BHI broth supplemented with 0.0, 0.5 and 1.0% NaCl, at pH 4.0, 4.5 and 5.5, respectively, which were further differentiated from those corresponding to 2.5% NaCl.



**Figure 1.** Antimold activity (AU/mL), exhibited by the three *W. anomalus* strains, LQC 10346 (A), 10353 (B) and 10360 (C), after growth in modified BHI broth adjusted to 6 initial pH values (3.5, 4.0, 4.5, 5.0, 5.5, 6.0) and 6 NaCl concentrations (0.0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%), at 30 °C for 24 h.

Regarding the effect of initial pH value and NaCl on the growth of yeast strains, Multifactor ANOVA revealed statistically significant differences among the mean populations obtained from the different levels of NaCl and initial pH values, either as single factors or as a combination ( $p < 0.05$ ). However, these differences lack biological significance, since yeast populations ranged within less than half a logarithm.

As far as the antimold activity of the three *W. anomalous* strains, namely LQC 10346, 10353 and 10360, was concerned, the 24 h incubation at 30 °C in liquid dough supplemented with six NaCl concentrations (0.0%, 0.5%, 1.0%, 1.5%, 2.0% and 2.5%) resulted in populations ranging between 7.28–7.45, 7.21–7.32 and 7.26–7.37 log CFU/mL, respectively. The maximum antimold activity of all strains was quantified at 5120 AU/mL and was recorded in liquid dough supplemented with 1.0% NaCl (Table S2). The statistically significant contribution of the NaCl concentration on the antimold activity was also exhibited ( $p < 0.05$ ).

### 3.2. Identification of Yeast Volatile Compounds by SPME-GC-MS

Cell-free supernatants of the three *W. anomalous* strains, grown under conditions in which the highest inhibitory activity against *P. chrysogenum* was observed, were subjected to SPME-GC-MS analysis. The qualitative analysis of VOCs is summarized in Table 1. Twelve compounds were detected, among which three alcohols (ethanol, isoamyl alcohol, 2,4-di-tert-butyl-phenol) three aldehydes (benzaldehyde, nonanal, octanal), one acid (3-methyl-butanoic acid), and five esters (isoamyl acetate, ethyl acetate, butanoic acid-ethyl ester, acetic acid-butyl ester, ethyl caprylate) were identified. Ethanol, ethyl acetate, isoamyl alcohol and isoamyl acetate were detected in all samples. Ethyl acetate was the dominant compound in all samples, with ethanol and isoamyl alcohol being in relative abundance as well; the % area of these three compounds ranged between 77.6% and 89.2% of the total peak area of the samples. The rest of the volatile compounds were not produced in all conditions tested. Acetic acid-butyl ester and 2,4-di-tetr-butyl-phenol were produced by all yeast strains, butanoic acid-ethyl ester and ethyl caprylate were only produced by strain LQC 10346, 3-methyl-butanoic acid and octanal by strain LQC 10353, while nonanal by both LQC 10346 and 10353. Finally, benzaldehyde was produced by strains LQC 10353 and 10360.

**Table 1.** VOCs produced by three *W. anomalous* strains, after a 24 h incubation at 30 °C, in modified BHI broth and liquid wheat dough at different pH value and NaCl combinations. Identification was performed by SPME-GC-MS.

Strain/Substrate	pH Value	NaCl (%)	Compound	Kovats Index	Area (%)
LQC 10346/BHI broth	6.0	0.0	Ethanol	<800	9.11
			Ethyl acetate	<800	63.43
			Isoamyl alcohol	<800	15.10
			Butanoic acid-ethyl ester	<800	0.24
			Acetic acid-butyl ester	814	1.81
			Isoamyl acetate	879	0.62
			Nonanal	1105	1.25
			Ethyl caprylate	1197	0.33
	6.0	1.0	Ethanol	<800	6.28
			Ethyl acetate	<800	73.74
			Isoamyl alcohol	<800	9.14
			Butanoic acid-ethyl ester	<800	0.16
			Acetic acid-butyl ester	814	1.48
			Isoamyl acetate	879	1.57
Ethyl caprylate	1197	0.20			

Table 1. Cont.

Strain/Substrate	pH Value	NaCl (%)	Compound	Kovats Index	Area (%)
LQC 10353/BHI broth	4.0	1.5	Ethanol	<800	11.17
			Ethyl acetate	<800	56.58
			Isoamyl alcohol	<800	15.01
			3-methyl-butanoic acid	854	0.33
			Isoamyl acetate	879	0.61
			Benzaldehyde	965	1.72
	2,4-di-tert-butyl-phenol	1505	0.44		
	4.5	0.5	Ethanol	<800	5.23
			Ethyl acetate	<800	74.31
			Isoamyl alcohol	<800	9.68
			Acetic acid-butyl ester	814	1.75
			Isoamyl acetate	879	2.69
	4.5	1.5	Ethanol	<800	6.87
			Ethyl acetate	<800	70.48
			Isoamyl alcohol	<800	11.79
			Isoamyl acetate	879	1.24
			Benzaldehyde	965	0.66
			Octanal	1002	0.38
Nonanal	1105	1.05			
LQC 10360/BHI broth	4.5	2.5	Ethanol	<800	17.76
			Ethyl acetate	<800	43.50
			Isoamyl alcohol	<800	16.39
			Isoamyl acetate	879	0.40
			Benzaldehyde	965	2.98
			2,4-di-tert-butyl-phenol	1505	0.19
	5.0	0.0	Ethanol	<800	10.82
			Ethyl acetate	<800	63.40
			Isoamyl alcohol	<800	12.40
			Acetic acid-butyl ester	814	1.69
			Isoamyl acetate	879	0.62
			2,4-di-tert-butyl-phenol	1505	0.59
	6.0	1.0	Ethanol	<800	15.93
			Ethyl acetate	<800	51.64
			Isoamyl alcohol	<800	17.67
			Acetic acid-butyl ester	814	1.81
			Isoamyl acetate	879	0.36
	LQC 10346/Liquid wheat dough	6.0	1.0	Ethanol	<800
Ethyl acetate				<800	51.60
Isoamyl alcohol				<800	11.42
Isoamyl acetate				879	0.66
2,4-di-tert-butyl-phenol				1505	0.35
LQC 10353/Liquid wheat dough	6.0	1.0	Ethanol	<800	24.81
			Ethyl acetate	<800	44.05
			Isoamyl alcohol	<800	14.26
			Isoamyl acetate	879	0.61
			2,4-di-tert-butyl-phenol	1505	0.31
LQC 10360/Liquid wheat dough	6.0	1.0	Ethanol	<800	20.36
			Ethyl acetate	<800	57.27
			Isoamyl alcohol	<800	10.83
			Isoamyl acetate	879	0.57

The incubation of yeast strains in liquid wheat dough resulted in the production of fewer volatile compounds, compared to those identified after growth in BHI broth. More accurately, the stable presence of ethanol, ethyl acetate, isoamyl alcohol and isoamyl acetate was detected in all samples assessed, in a strain- and substrate-independent manner. Additionally, 2,4-di-tert-butyl-phenol was identified in fewer samples; however, it was retrieved from incubation in both substrates. Regarding the VOCs identified in the *W. anomalus* CFS after growth in BHI broth, the presence of acetic acid-butyl ester, butanoic acid-ethyl ester, ethyl caprylate, benzaldehyde, nonanal, octanal, and 3-methyl-butanoic acid was detected as well, in a strain, substrate, initial pH value and NaCl-dependent manner.

Generally, the most abundant compound in every sample assessed, generated by % area, was ethyl acetate (ranging from 43.50 to 74.31%). In addition, isoamyl alcohol and ethanol were constantly detected in significant amounts as well (ranging from 9.14 to 17.67% and from 5.23 to 24.81% of total area, respectively). Acetic acid-butyl ester and nonanal exceeded 1.0% of total area in samples detected. Similar was the case for benzaldehyde, the abundance of which was recorded at 1.72% and 2.98% of total area in two assessed samples,

but in another one, was found less than 1.0%. Regarding the abundance of isoamyl acetate, only in some cases, it exceeded 1.0% of the total area.

### 3.3. Antimold Activity Assessment of Pure Volatile Compounds against *P. chrysogenum*

In Table 2, the MIC of pure VOCs against *P. chrysogenum* is presented. All compounds but ethanol exhibited antimold activity. Benzaldehyde and 2,4-di-tert-butyl-phenol were characterized by the lowest MICs, with only 0.06 and 0.04  $\mu\text{L}/\text{mL}$  of headspace, respectively. The rest of the aldehydes, namely nonanal and octanal, could also effectively suppress the growth of *P. chrysogenum*, with a MIC of 0.34 and 0.42  $\mu\text{L}/\text{mL}$  of headspace, respectively. As far as esters were concerned, higher MICs were recorded, with butanoic acid-ethyl ester and ethyl caprylate needing 5.14 and 6.24  $\mu\text{L}/\text{mL}$  of headspace, respectively, to inhibit mold growth.

**Table 2.** Antimold activity of pure VOCs against *P. chrysogenum*.

Volatile Organic Compounds	MIC ( $\mu\text{L}/\text{mL}$ of Headspace)
Alcohols	
Ethanol	-
Isoamyl alcohol	0.97 (0.00) <sup>e</sup>
2,4-di-tert-butyl-phenol	0.04 (0.00) <sup>a</sup>
Aldehydes	
Octanal	0.42 (0.02) <sup>c</sup>
Nonanal	0.34 (0.00) <sup>b</sup>
Benzaldehyde	0.06 (0.00) <sup>a</sup>
Acids	
3-methyl-butanoic acid	0.67 (0.03) <sup>d</sup>
Esters	
Isoamyl acetate	2.15 (0.00) <sup>g</sup>
Ethyl acetate	2.01 (0.01) <sup>f</sup>
Butanoic acid-ethyl ester	5.14 (0.03) <sup>i</sup>
Acetic acid-butyl ester	4.93 (0.03) <sup>h</sup>
Ethyl caprylate	6.24 (0.00) <sup>j</sup>

Statistically significant differences are indicated with different letters <sup>a-j</sup> ( $p < 0.05$ ), according to the method of 95% LSD applied as post-hoc comparison test.

### 3.4. Bread Preparation and Shelf-Life Assessment

Bread produced with 20% addition of liquid dough, inoculated with the monocultures of the three *W. anomalus* strains or their cocultures with baker's yeast, gained an additional period of mold-free shelf life, ranging from 9 to 30 days, compared to the control (bread made only with commercial yeast). More accurately, the greatest prolongation of bread shelf life was recorded with the addition of strain LQC 10360, which led to a shelf-life extension of 30 days. On the other hand, bread made with the incorporation of strain LQC 10353 presented an increased shelf life of 9 days. A similar shelf-life extension (10 days) was recorded with the addition of strain LQC 10346. Co-culture with baker's yeast did not alter the shelf-life extension.

## 4. Discussion

Mold spoilage in bread represents an ongoing safety and quality concern for various food industries. The use of lactobacilli as biocontrol agents in the sourdough-bread-making process has been extensively assessed and reported to confer shelf-life prolongation and a delay in the staling of baked products, in a strain-specific manner [20]. However, the



application of yeasts as antimold agents for the production of bakery products has been only marginally studied [16,21].

The ability of *W. anomalus* strains to thrive in NaCl-rich mediums and at a wide range of initial pH values has already been reported [22,23]. The NaCl and pH-tolerant profile of this species was verified in our study as well. The antimold activity was attributed to compounds of a non-protein nature [17]; thus, the production of killer toxins was excluded and the production of VOCs was further considered. Many studies have correlated the inhibitory activity of *W. anomalus* strains with the production of VOCs [9,13]. However, only a few of them reported VOCs identification. In our study, the most abundant compounds produced by yeasts were ethyl acetate, ethanol and isoamyl alcohol, while the stable presence of isoamyl acetate was detected as well. In agreement with our observations, a recent study by Khunnamwong et al. [10] reported that the biocontrol activity of *W. anomalus* strains against five phytopathogenic fungi was mainly attributed to the synthesis of VOCs, with isoamyl alcohol and isoamyl acetate being the most abundant. In addition, Oro et al. [12] demonstrated that VOCs synthesized by *W. anomalus* exhibited the highest inhibitory activity against *Botrytis cinerea* (87% total inhibition), while *P. digitatum* was suppressed only at 1.5%. Consistent with our results, the main VOC produced by *W. anomalus* was ethyl acetate. In another study, by Hua et al. [11], the biocontrol activity of a *W. anomalus* strain, WRL-076, was attributed to 2-phenylethanol, which further suppressed the spore germination and aflatoxin production of *Aspergillus flavus*. In an earlier study by Masoud et al. [24], *W. anomalus* strains, grown both on malt yeast glucose peptone and coffee agar, were found to inhibit the growth of *A. ochraceus* and concomitant ochratoxin A production. Regarding VOCs production during coffee processing, the main esters identified with GC-MS were ethyl acetate and isobutyl acetate, while the abundance of isoamyl alcohol was also reported [24].

It has been proposed that the biocontrol activity of VOCs against mold species decreases, according to the following order: organic acid > aldehyde > alcohol > ether > ketone > ester > lactone [25]. However, this is not always the rule, since there are cases opposing the aforementioned statement [26,27]. This was similar to our study, where the MIC of 3-methyl-butanoic acid was higher than the respective of 2,4-di-tert-butyl-phenol and benzaldehyde, which required only 0.04 and 0.06  $\mu\text{L}/\text{mL}$ , respectively. The increased antimold effect of 2,4-di-tert-butyl-phenol has probably been attributed to the free functional group (hydroxyl), coupled to the presence of hydrophobic alkyl groups [26]. Alkyl groups contribute to the antimold activity of phenol, since their hydrophobicity enhances the affinity for cell membranes, thus, impairing the integrity and leading to disruption. Varsha et al. [28], reported that at 400  $\mu\text{g}/\text{disc}$ , an inhibition zone of 1.5 cm against *P. chrysogenum*, was evident, while Padmavathi et al. [29] demonstrated that a complete suppression of fungal growth was exhibited, with an MIC of 100  $\mu\text{g}/\text{mL}$ . In the case of ethanol, its mold-inhibiting effects have already been reported [30]. According to Dao and Dantigny [31], the concentration of ethanol applied, combined with the microorganism assessed, exert distinct effects on mold inhibition. Results obtained in our study revealed that 98% ethanol could not prevent the growth of *P. chrysogenum*. Similarly, Rogawansamy et al. [32] reported that 70% ethanol was ineffective to suppress the growth of *P. chrysogenum* and *A. fumigatus*, while in another study, by Druvefors and Schnürer [33], ethanol was found to exert a minor but synergic effect on the antimold activity of *W. anomalus* J121. In general, ethanol is a short-chain alcohol, the inhibitory activity of which is lower against mold species, compared to longer-chain alcohols, namely isoamyl alcohol, due to the increased affinity of the latter for the cell membrane [34].

Among the aldehydes assessed, benzaldehyde had the most pronounced antimold effect in our study, while nonanal and octanal needed a higher MIC in order to inhibit the growth of *P. chrysogenum*. In accordance with our results, Calvo et al. [27] demonstrated that only 0.063 mL/L of benzaldehyde was necessary to suppress the growth of *B. cinerea* and *P. expansum*. In addition, Zhang et al. [35] reported that the MIC of nonanal for the complete suppression of *P. cyclopium* was 0.35  $\mu\text{L}/\text{mL}$ , while octanal needed 0.50  $\mu\text{L}/\text{mL}$

for total mycelial growth inhibition of both *P. italicum* and *P. digitatum* [36]. However, in another study by Pishawikar and More [37], the opposite results were demonstrated. More accurately, analogs with aromatic aldehydes exhibited decreased inhibitory activity compared to those with aliphatic aldehydes against *Candida albicans*. The reason for that was the presence of structural characteristics, which interacted via hydrophobic or Van der Waals forces with the target molecules.

Regarding the antimold activity of the other VOCs, higher MICs were needed to suppress the growth of *P. chrysogenum*. In brief, the MICs corresponding to isoamyl alcohol and isoamyl acetate for mold inhibition were 0.97 and 2.15  $\mu\text{L}/\text{mL}$ , respectively. These results were in agreement with the previous findings by Ando et al. [38], which demonstrated that the amount of isoamyl acetate used for the complete inhibition of *P. chrysogenum* was two-times higher than the respective isoamyl alcohol. The reason for this could be related to the presence of reactive hydroxyl groups in the case of isoamyl alcohol, thus, being less volatile, in contrast to esters, which are characterized by increased volatility due to the absence of free functional groups. Among esters, ethyl acetate has been reported to suppress the growth of *B. cinerea* at 8.97  $\text{mg}/\text{cm}^3$  and gray mold on strawberry fruit at 0.718  $\text{mg}/\text{cm}^3$  [12], while according to Masoud et al. [24], at 48  $\mu\text{g}/\text{L}$  of headspace, it was able to reduce the growth of *A. ochraceus* only at 15%. The association of butanoic acid-ethyl ester, acetic acid-butyl ester and ethyl caprylate with the volatile metabolome of microbial strains with antimold activity has been documented, as well [39–41].

As far as the use of *W. anomalus* strains on sourdough bread making was concerned, only a few studies have assessed their application as starter cultures in sourdough fermentations. More accurately, Coda et al. [16] shed some light by reporting that dough produced with a co-culture of *Lactiplantibacillus plantarum* 1A7 and *W. anomalus* LCF1695 exhibited good organoleptic features and further contributed to the mold-free shelf-life extension of bread slices, up to 28 days of storage at room temperature. Similar were the findings obtained in our study, according to which, bread made with the *W. anomalus* strain LQC 10360 gained one additional month of mold-free shelf life, compared to the control. Finally, the suitability of all *W. anomalus* strains to be used as an adjunct culture in breadmaking was exhibited, since the co-presence of *W. anomalus* and baker's yeast displayed the same antimold effect compared to monoculture trials.

## 5. Conclusions

In this work, the antimold capacity of three sourdough-derived *W. anomalus* strains, as well as their suitability as adjunct cultures in bread making, were exhibited. The former was attributed to a set of VOCs produced, some of which were common among all strains, and growth conditions were examined. Regarding their applicability as starter cultures in dough preparation, with further use in bread making, the antimold capacity of the yeast strains was retained, even in the presence of commercial baker's yeast, providing an additional mold-free shelf life of up to 30 days. Thus, the findings of the present study included, among others, the production of yeasted breads with extended shelf life; however, the combination of the antimold *W. anomalus* strains with selected LAB, for further application in sourdough fermentation, is yet to be conducted. From a technological perspective, the collaboration among non-conventional yeasts and acidifying LAB will lead to the production of leavened sourdough products, characterized by microbiological stability and desired flavor attributes. Therefore, moving towards the production of green label products, the assessment of antimold starter cultures, both in vitro and in situ, is mandatory.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app12094506/s1>, Table S1: Antimold activity (AU/mL) of three *W. anomalus* strains, recorded after a 24 h growth at 30 °C, in modified BHI broth at six different pH values (3.5, 4.0, 4.5, 5.0, 5.5, 6.0) and six NaCl concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5%). Table S2: Antimold activity (AU/mL) of three *W. anomalus* strains, recorded after a 24 h growth at 30 °C, in liquid dough at six NaCl concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5%).

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