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New insights into the antifungal activity of lactic acid bacteria isolated from different food matrices

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SUMMARY: The anti-mold activity of 397 strains of lactic acid bacteria was evaluated using both the spot method in Petri plates and co-culture in liquid medium. The study led to the selection of 34 strains isolated from table olives or olive brines, 15 strains from dairy products, and 10 strains from sourdoughs, all able to inhibit a strain of *Penicillium crustosum* and/or a strain of *Aspergillus* section *Nidulantes*, prevailing in two Calabrian olive brines. Seven representative strains were identified as *Lactobacillus pentosus* (four strains) and *Lactobacillus sanfranciscensis* (three strains) and are currently under testing for their antifungal activity during table olive fermentation. This research constitutes an initial contribution to the control of fungal growth and mycotoxin accumulation during table olive fermentation. The selected strains could be used as adjunct cultures in table olive fermentation, allowing for the biological control of table olive safety.

KEYWORDS: *Antifungal activity;* Aspergillus *section* Nidulantes*; Lactic acid bacteria;* Penicillium*; Table olive fermentation*

RESUMEN: ***Nuevos conocimientos sobre la actividad antifúngica de las bacterias del ácido láctico aisladas de diferentes matrices alimentarias.*** La actividad antimoho de 397 bacterias del ácido láctico se evaluó utilizando tanto el método puntual en placas de Petri como el co-cultivo en medio líquido. El estudio condujo a la selección de 34 cepas aisladas de aceitunas de mesa o salmueras de oliva, 15 cepas de productos lácteos y 10 cepas de masa madre, todas capaces de inhibir una cepa de *Penicillium crustosum* y/o una cepa de *Aspergillus* sección *Nidulantes*, que prevalecen en dos salmueras de aceituna de Calabria. Se identificaron siete cepas representativas como *Lactobacillus pentosus* (cuatro cepas) y *Lactobacillus sanfranciscensis* (tres cepas) y actualmente se están probando su actividad antifúngica durante la fermentación de aceituna de mesa. Esta investigación constituye una primera contribución para controlar el crecimiento de hongos y la acumulación de micotoxinas durante la fermentación de aceitunas de mesa. Las cepas seleccionadas podrían usarse como cultivos adjuntos en la fermentación de aceitunas de mesa.

PALABRAS CLAVE: *Actividad antifúngica;* Aspergillus *sección* Nidulantes*; Bacterias de ácido láctico; Fermentación de aceituna de mesa;* Penicillium

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1. INTRODUCTION

In Mediterranean countries, table olives are among the most commonly consumed fermented food. They are considered a functional food due to their nutritional value, content in bioactive compounds and dietary fiber, fatty acid composition and presence of several antioxidants (Campus *et al.,* 2018).

In table olive fermentation, mold growth can cause huge economic losses and reduce the product safety (El Adlouni *et al.,* 2006; Ghitakou *et al.,* 2006). Molds of the genera *Aspergillus* and *Penicillium* have been described in different olive fermentation processes (Heperkan *et al.,* 2006) and both are considered undesirable microorganisms. They can produce mycotoxins and cause the softening of fruits because of their cellulase and xylanase activities (Arroyo-López *et al.,* 2016). *Penicillium crustosum* is one of the most prevalent molds in fermented black table olives and is able to produce toxic metabolites such as dehydrocyclopeptin, andrastin A, cyclopenol, penitrem A, roquefortine C, viridicatol (Bavaro *et al.,* 2017) and thomitrem A and E (Rundberget and Wilkins, 2002). *Aspergillus* section *Nidulantes* includes several species able to produce mycotoxins, like aflatoxins, sterigmatocystin, emestrin, fumitremorgins, asteltoxins, and paxillin; four species (*A. astellatus*, *A. miraensis*, *A. olivicola*, *A. venezuelensis*) produce aflatoxin B1 (Chen *et al.,* 2016).

Different chemical, physical and biological methods have been proposed to prevent mold growth or to eliminate or reduce aflatoxins during table olive fermentation. For example, the spoilage of table olive by molds can be reduced using chemical preservatives, such as potassium sorbate and sodium benzoate (Turantaş *et al.,* 1999) or natamycin (Hondrodimou *et al.,* 2011). Değirmencioğlu *et al.* (2014) studied the effect of washing solutions -acetic acid, lactic acid, chlorine dioxide- on dry-salted olives during 7 months of storage at 4 °C. The chlorine dioxide solution (10 ppm) combined with vacuum packaging was the best approach for controlling mold and yeast growth. In addition, the effectiveness of high hydrostatic pressures and citrinin against mold growth in table olives has been demonstrated (Tokuşoğlu *et al.,* 2010; Argyri *et al.,* 2014).

In view of growing consumer concern for food safety issues, including additive content (Bearth *et al.,* 2014), the food industry is seeking biological alternatives in order to inhibit undesirable molds. Among the novel approaches, biopreservation and, more specifically, the selection of protective cultures has been identified as one of the more promising alternatives.

It is well known that lactic acid bacteria (LAB) can produce several antifungal metabolites (Schnürer and Magnusson, 2005). LAB are able to inhibit molds related to bread spoilage (Corsetti *et al.,* 1998); consequently, the use of antifungal sourdough in the bakery industry is now a common practice to ensure the microbiological safety of bread (Gerez *et al.,* 2009). Antifungal lactobacilli can also be found in raw milk (Delavenne *et al.,* 2012); therefore, specifically selected LAB strains can also be used as biopreservatives in fresh cheeses (Fernandez *et al.,* 2017). The antifungal activity of LAB, specifically *Lactobacillus plantarum*, and their ability to reduce aflatoxin B1 during olive storage has also been demonstrated (Kachouri *et al.,* 2014).

Considering the state of the art, the aim of the present study was to find LAB able to inhibit molds commonly associated with table olive fermentation and spoilage.

2. MATERIALS AND METHODS

2.1. Microorganisms

Three hundred ninety-seven strains of LAB from the Collection of the Laboratory of Microbiology (Department of Agraria, *Mediterranea* University of Reggio Calabria, Reggio Calabria, Italy) were used. All the strains were previously classified to genus level, according to Cogan *et al.,* (1997). Each strain was tested for its Gram reaction, catalase using 3 g H_2O_2/L, shape by observation of overnight cultures using a phase contrast microscope Standard 20 (Carl Zeiss, Göttingen, Germany), and for the heterofermentation or homofermentation of sugars (Abd-el-Malek and Gibson, 1948). The growth of coccal-shaped bacteria was examined in M17 broth after incubation at 10 °C for 7 days, at 45 °C for 2 days, and in M17 broth containing 20, 40, and 65 g NaCl/L after incubation at 30 °C for 4 days. The LAB was tested against two mold strains -one *Penicillium* and one *Aspergillus*- prevailing in two different Calabrian olive brines. *Penicillium* was identified as *Penicillium crustosum* according to

Visagie *et al.,* (2014); *Aspergillus* was included in the section *Nidulantes* according to Chen *et al.,* (2016). Out the 397 strains of LAB, 198 strains were isolated from table olives or olive brines, 115 strains from dairy products, and 84 strains from sourdough.

Finally, the seven more representative LAB were identified as *Lactobacillus pentosus* (four strains) and *Lactobacillus sanfranciscensis* (three strains) by molecular methods. DNA was extracted by the InstaGene matrix (Bio-Rad) from LAB and subjected to amplification using specific primers (Young *et al.,* 1991). LAB identification was performed with PCR-ARDRA, comparing the profile isolates to those previously described in the literature and to International Collection reference strains (Aquilanti *et al.,* 2007; Torriani *et al.,* 2001).

2.2. Preliminary screening

The LAB were screened for their antagonistic activity using the agar spot method (Spelhaug and Harlander, 1989) with some modifications. All the strains were stored at −80 °C using a cryopreservative bead storage system Microbank TM (Pro-Lab Diagnostics, Canada). The LAB were cultured in a deMan Rogosa Sharpe (MRS) broth at 30 °C for 48 hours. Subsequently, 0.1 ml of each culture were inoculated in MRS agar plates (60 mm), incubated at 30 °C for 48 hours. For each strain, the biomass was collected using a sterile loop and spotted in triplicate in MRS agar plates (90 mm). Then, the plates were overlaid with 10 ml of Yeast Extract Peptone Dextrose agar (agar 0.7%) containing 0.1 ml of an abundant spore suspension of each mold. After 72 h at 30 °C, the plates were checked for the presence of inhibition zones around the spots of each LAB.

2.3. Test for antifungal activity of LAB by co-culture in a liquid medium

The LAB that exhibited antagonistic activity with the agar spot method were tested by co-culture in a liquid medium against three serial dilutions of spore suspensions of the two molds. *Penicillium crustosum* and *Aspergillus* section *Nidulantes* were inoculated in MRS agar plates and incubated at 30 °C for 48 h in order to test their ability to grow in this medium and adapt them to the subsequent conditions. Spore suspensions were prepared from the same plates as described above and diluted at 1:10, 1:100 and 1:1000 in sterile physiological solution. Co-cultures were prepared by inoculating 0.1 ml of pre-culture of each LAB in tubes containing 10 ml of MRS broth; 0.1 ml of each dilution of the spore suspensions was added. The tubes were incubated at room temperature for sixteen days and the antagonistic activity was evaluated based on the absence of mold growth at the top of the MRS broth.

3. RESULTS

3.1. Preliminary screening

One hundred and fifteen strains (28.97% of the total strains tested) exhibited antagonistic activity in the agar spot method: 60 strains isolated from table olives or olive brines, 20 dairy strains and 35 sourdough strains. The strains which were isolated from the same sample, exhibited the same cellular morphology, and showed an identical antagonistic activity were considered duplicates. Consequently, the 115 strains were reduced to 59:34 strains isolated from table olives or olive brines (listed in Table 1), 15 dairy strains (listed in Table 2), and 10 sourdough strains (listed in Table 3).

3.2. Test for antifungal activity of LAB by co-culture in a liquid medium

The antagonistic activity of the 59 pre-selected strains of LAB against the two molds was studied by co-culture in a liquid medium at three different concentrations of the two molds; all strains exhibited good antagonistic activity against one or both tested molds (Figure 1). When the LAB were inoculated together with the less diluted spore suspensions (1:10), 58 strains (98.3% of all 59 LAB) exhibited antagonistic activity against *Penicillium crustosum* and 57 (96.6% of all 59 LAB) against *Aspergillus* section *Nidulantes* after sixteen days. All the LAB strains were able to inhibit the growth of *Penicillium crustosum* at the spore suspension 1:100; 58 strains (98.3% of all 59 LAB) inhibited the growth of *Aspergillus* section *Nidulantes* at the same concentration. All the LAB tested inhibited the growth of both molds at the most diluted (1:1000) spore suspensions after sixteen days.

TABLE 1. List of the 34 lactic acid bacteria isolated from olives and exhibiting antagonistic activity

Lactic acid bacteria			Activity against olive moulds	
Strain	**Sample**	**First identification**	***Penicillium spp.***	***Aspergillus spp.***
B 200	Olive_1	*Lactobacillus spp.*	+	-
B 212	Olive brine_18	*Lactobacillus spp.*	+	-
B 221	Olive_2	*Lactobacillus spp.*	+	-
B 229	Olive_16	*Lactobacillus spp.*	+	-
B 248	Olive brine_5	*Lactobacillus spp.*	+	-
B 283	Olive brine_13	*Lactobacillus spp.*	-	+
B 284	Olive_14	*Lactobacillus spp.*	-	+
B 318	Olive brine_12	*Lactobacillus spp.*	+	-
B 337	Olive brine_11	*Lactobacillus spp.*	+	-
B 348	Olive_2015_C_15gg	*Lactobacillus spp.*	+	-
B 350	Olive_2015_D_15gg	*Lactobacillus spp.*	+	-
B 354	Olive_2015_E_15gg	*Lactobacillus spp.*	+	-
B 358	Olive_2015_G_15gg	*Lactobacillus spp.*	+	-
B 364	Olive_2015_L_15gg	*Lactobacillus spp.*	+	-
B 366	Olive_2015_N_15gg	*Lactobacillus spp.*	+	+
B 384	Olive_2015_B_30gg	*Lactobacillus spp.*	+	-
B 386	Olive_2015_C_30gg	*Lactobacillus spp.*	-	+
B 389	Olive_2015_D_30gg	*Lactobacillus spp.*	+	+
B 391	Olive brine_MF3_67gg	*Lactobacillus spp.*	+	+
B 524	Olive_2015_G1_240gg	*Lactobacillus spp.*	+	-
B 525	Olive_2015_G2_240gg	*Lactobacillus spp.*	+	-
B 526	Olive_2015_H1_240gg	*Lactobacillus spp.*	+	-
B 527	Olive_2015_H2_240gg	*Lactobacillus spp.*	+	-
B 529	Olive_2015_I1_240gg	*Lactobacillus spp.*	+	-
B 531	Olive_2015_L1_240gg	*Lactobacillus spp.*	+	-
B 534	Olive_2015_M1_240gg	*Lactobacillus spp.*	+	-
B 536	Olive_2015_N1_240gg	*Lactobacillus spp.*	+	-
B 539	Olive_2015_O1_240gg	*Lactobacillus spp.*	+	-
B 540	Olive_2015_O2_240gg	*Lactobacillus spp.*	+	-
B 542	Olive_2015_E1_240gg	*Lactobacillus spp.*	+	-
B 543	Olive_2015_M1_240gg	*Lactobacillus spp.*	+	-
B 545	Olive_2015_D1_240gg	*Lactobacillus spp.*	+	-
B 546	Olive_2015_B1_240gg	*Lactobacillus spp.*	+	-
B 560	Olive brine_2016	*Lactobacillus spp.*	+	-

3.3. LAB identification

Among the 34 LAB listed in Table 1, 91.2% of them exhibited antagonistic activity against *Penicillium crustosum* but only 17.6% exhibited antagonistic activity against *Aspergillus* section *Nidulantes*. The following three strains are representative of the three typologies of behavior against the two molds:

- strain B221, identified as *Lactobacillus pentosus*, which exhibited antagonistic activity against *Penicillium crustosum*.
- strain B283, identified as *Lactobacillus pentosus*, which exhibited antagonistic activity against *Aspergillus* section *Nidulantes*.
- strain B391, identified as *Lactobacillus pentosus*, which exhibited antagonistic activity against both strains of mold.

All 15 LAB listed in Table 2 exhibited antagonistic activity against *Penicillium crustosum* but none of them exhibited antagonistic activity against *Aspergillus* section *Nidulantes*. The strain B167, identified as *Lactobacillus pentosus*, is representative of this typology of behavior against the two molds.

Among the 10 LAB listed in Table 3, 90% exhibited antagonistic activity against *Penicillium crustosum* but only 10% exhibited antagonistic activity against *Aspergillus* section *Nidulantes*. The following three strains are representative of the three typologies of behavior against the two molds:

1. Strain B426, identified as *Lactobacillus sanfranciscensis*, which exhibited antagonistic activity against *Penicillium crustosum*.
2. Strain B551, identified as *Lactobacillus sanfranciscensis*, which exhibited antagonistic activity against the strain of *Aspergillus* section *Nidulantes*.
3. Strain B511, identified as *Lactobacillus sanfranciscensis*, which exhibited antagonistic activity against both strains of mold.

The seven representative strains of LAB are currently under testing for their antifungal activity during table olive fermentation (unpublished data).

4. DISCUSSION

In the present study, 59 LAB exhibited antifungal activity. Both in agar and in co-culture, the LAB exhibited greater inhibitory activity against *Penicillium crustosum* compared to *Aspergillus* section *Nidulantes*. The antifungal

TABLE 2. List of the 15 dairy lactic acid bacteria exhibiting antagonistic activity

Lactic acid bacteria			Activity against olive moulds	
Strain	**Sample**	**First identification**	***Penicillium spp.***	***Aspergillus spp.***
B 3	PP_A03_1	*Lactobacillus spp.*	+	-
B 15	PP_M3	*Lactobacillus spp.*	+	-
B 17	PP_P4	*Lactobacillus spp.*	+	-
B 27	PP_A03_2	*Lactobacillus spp.*	+	-
B 28	PP_A03_3	*Lactobacillus spp.*	+	-
B 54	UK_TH1	*Lactobacillus spp.*	+	-
B 59	UK_SR1	*Lactobacillus spp.*	+	-
B 62	UK_WB1	*Lactobacillus spp.*	+	-
B 76	RAF_M1	*Lactobacillus spp.*	+	-
B 79	PM_M2	*Lactobacillus spp.*	+	-
B 147	M_DN1	*Lactobacillus spp.*	+	-
B 164	M_FP1	*Lactobacillus spp.*	+	-
B 167	P_M3	*Lactobacillus spp.*	+	-
B 172	CC_4	*Lactobacillus spp.*	+	-
B 179	MC_5	*Lactobacillus spp.*	+	-

TABLE 3. List of the 10 lactic acid bacteria isolated from sourdough and exhibiting antagonistic activity

Lactic acid bacteria			Activity against olive moulds	
Strain	**Sample**	**First identification**	***Penicillium spp.***	***Aspergillus spp.***
B 426	Sourdough_SC_SA1	*Lactobacillus spp.*	+	-
B 435	Sourdough_CL_IF1	*Leuconostoc spp.*	+	-
B 455	Sourdough_CL_IF2	*Lactobacillus spp.*	+	-
B 470	Sourdough_CZ_IFAL1	*Pediococcus spp.*	+	-
B 481	Sourdough_CZ_IFAL2	*Lactobacillus spp.*	+	-
B 489	Sourdough_RC_LSDMA1	*Lactobacillus spp.*	+	-
B 503	Sourdough_VV_CF1	*Lactobacillus spp.*	+	-
B 511	Sourdough_GI_C1	*Lactobacillus spp.*	+	+
B 551	Sourdough_VV_SF1	*Lactobacillus spp.*	-	+
B 553	Sourdough_VV_LS1	*Lactobacillus spp.*	+	-

LAB strains so identified belong to two species (*Lactobacillus pentosus* and *Lactobacillus sanfranciscensis*), whose ability to inhibit or reduce mold growth is well known (Corsetti *et al.,* 1998; Schnürer and Magnusson, 2005).

The antifungal activity of LAB is related to their ability to produce antifungal metabolites, e.g. organic acids, proteinaceous compounds, reuterin, and 3-hydroxylated fatty acids (Schnürer and Magnusson, 2005). For example, the inhibitory properties of phenyllactic acid (PLA) produced by LAB against several fungal species isolated from food have been demonstrated (Valerio *et al.,* 2004). Regarding proteinaceous compounds, bacteriocin-producing LAB were already isolated from fermented olives and it was observed that their ability to produce bacteriocins was affected by NaCl, pH and temperature (Hurtado *et al.,* 2011). Moreover, the bacteriocin production by LAB seemed to be affected by the presence of other bacteria during olive fermentation (Ruiz-Barba *et al.,* 2010).

The antifungal activity of LAB could also be due to a synergic effect between the sodium acetate present in the MRS medium and lactic acid and other compounds produced by LAB (Cabo *et al*., 2002). Schillinger and Villareal (2010) demonstrated how sodium acetate in a culture medium can influence the inhibitory activity of LAB; in their experiment, LAB which exhibited antifungal activity in MRS agar with sodium acetate did not have the capacity to inhibit molds in MRS agar without sodium acetate. Also, Cheong *et al*., (2014) reported that LAB which exhibited antifungal activity in MRS agar with sodium acetate did not exhibit the same behavior in MRS agar without sodium acetate; however, the same strains were able to inhibit *Penicillium commune* in cottage cheese.

Lind *et al*., (2005) demonstrated how the absence of sodium acetate in MRS agar did not influence the antifungal activity of *Propionibacterium* against *Penicillium roqueforti* and *Aspergillus fumigatus*. Magnusson *et al*., (2003) performed an HPLC analysis of the supernatants of the LAB exhibiting antifungal activity, previously tested in MRS broth, and the concentration of lactic acid was equal to or even higher than concentrations in strains devoid of inhibitory activity; in addition, the concentration of acetic acid was similar to that found in MRS broth. This demonstrated that the activity was probably due to the production of other antifungal compounds.

In the present study we decided to carry out the tests using MRS with sodium acetate. *Penicillium crustosum* and *Aspergillus* section *Nidulantes* grew well in all the control samples, so the inhibition was probably due to the production of antifungal substances by LAB.

5. CONCLUSIONS

In our opinion, this study makes a useful contribution to solving the problem of fungal growth and potential mycotoxin accumulation during table olive fermentation, thereby improving its safety.

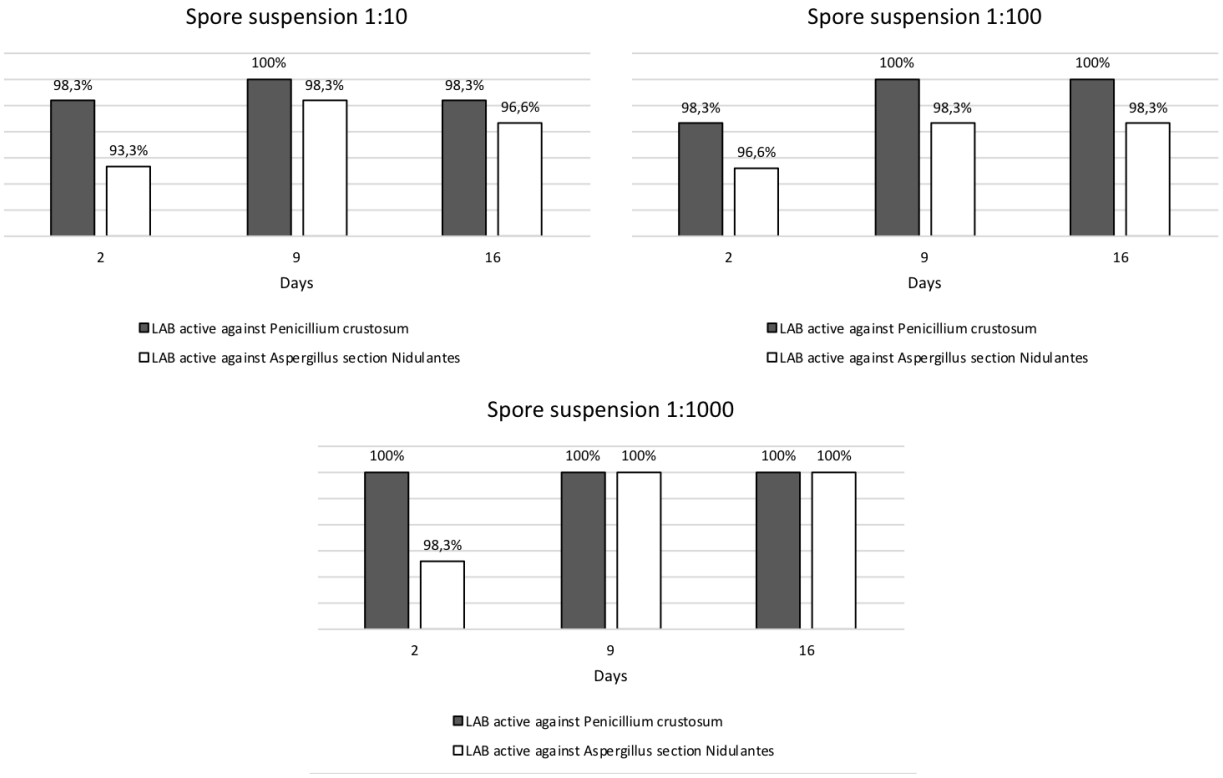

FIGURE 1. Percentage of the selected 59 strains of lactic acid bacteria that confirmed their activity against *Penicillium crustosum* in liquid medium co-culture (■) and *Aspergillus* section *Nidulantes* (□) at the three concentrations of spore suspension.

Summarizing:

Almost 400 strains of LAB were screened for anti-mold activity.

One strain of *Penicillium crustosum* and one strain of *Aspergillus* section *Nidulantes* were used;

Almost 60 strains of LAB resulted in the ability to inhibit one or both molds.

The antagonistic activity was evaluated both using the spot method and by co-culture in liquid medium.

Starting from the present results, a consortium of the best anti-mold LAB could be tested in table olive fermentation.

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