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Antimicrobial activity of *Lactobacillus plantarum* strains isolated from different environments: a preliminary study

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Abstract

The aim of this study was the investigation of the antimicrobial activity expressed by *Lactobacillus plantarum* strains isolated from different fermented matrices (wines, cheese, fermented sausages, and sourdoughs). A total of 106 strains of *Lb. plantarum* (producers) were tested against 33 undesirable microorganisms (indicators), including both moulds and bacteria. The antimicrobial activity exerted by growing cells (GC) was evaluated by the spot-on-the-lawn, while the activity of cell free supernatants (CFS), neutralised CFS (nCFS) and CFS treated with proteases (pCFS) was assessed by the agar well diffusion assay. The antagonistic effect produced by GC of *Lb. plantarum* isolated from wines was higher than that exibited by cells isolated from other fermented matrices. Moreover, 5 CFS - all from wine strains - as well as the corresponding nCFS and pCFS were able to inhibit different bacteria and moulds. The results suggested a relationship between the origin of *Lb. plantarum* strains and their antimicrobial properties, while no relation was found between the intensity of inhibition and the origin of indicator strains. This fact highlights that the knowledge of conditions characterising different ecosystems can be helpful in the detection and isolation of *Lb. plantarum* strains to be used as protective agents.

Introduction

Lactobacillus plantarum is a versatile and widespread microorganism found in different environments, ranging from food to human gastrointestinal tract (Kleerebezem *et al*., 2003; Basso *et al*., 2004; Ricciardi *et al*., 2014; Papadimitriou *et al*., 2015). As evidenced by several Authors (Zotta *et al*., 2012; Ferrando *et al*., 2015), the great adaptability of *Lb. plantarum* to various environments is due to its ability to cope with different stress conditions. Moreover, some strains of *Lb. plantarum* are known for their ability to produce several natural antimicrobial substances, such as bacteriocins, BLIS, phenyllactic acid, organic acids (mainly lactic and acetic acid) and hydrogen peroxide (Prema *et al*., 2010; Todorov *et al*., 2011; Reis *et al*., 2012; Rumjuankiat *et al*., 2015), thus inhibiting competitors that share the same niche. Lowe *et al*. (1993) evidenced that the growth of most chemoorganotrophic anaerobes is naturally associated with the generation of toxic end products, which requires a sort of dynamic adaptation mechanism or tolerance to their catabolic end products. Moreover, in some *Lactobacillus* species specific bacteriocin production seems to be enhanced under unfavourable growth conditions, such as low temperatures or presence of potentially toxic compounds, like ethanol (De Vuyst *et al*., 1996).

In the case of *Lb. plantarum*, the natural genomic architecture is the basis of its versatility (Siezen *et al*., 2011) and of its success in industrial applications, not only as starter culture but also as bio-protective agent. In this last field, the *in vitro* screening of bacterial protective properties represents a challenge for researchers, due to the substantial amount of screening procedures required to test numerous strains isolated from different food matrices. Taking into account that several studies highlighted the impact of food stress conditions on the occurrence of specific microbial strains (Ricciardi *et al*., 2012; Filannino *et al*., 2014; Heunis *et al*., 2014; Olguin *et al*., 2015), it could be as much important to define the influence of different environments on the ability of strains to exert antimicrobial activities. To our knowledge, a relationship between the strain resistance to food stress conditions and the ability to produce antimicrobial effects was little explored, and only few studies on specific food matrices are available in the literature (Neysens *et al*., 2003; Lee *et al*., 2010; Butler *et al*., 2013; Arena *et al*., 2016). In the light

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Table 1. Main features of fermented food and beverages used as isolation source of 106 *Lactobacillus plantarum* strains tested for their antimicrobial activity against several bacteria and moulds (see Table 2).

Number of strains	Short IS	Strains	Isolation source	Features of isolation source				References
				pH	aw	alcohol (% vol)	NaCl %	
17	C_	C_11; C_12; C_21; C_25; C_29-30; C_35-36; C_43; C_54; C_56; C_63; C_66; C_68; C_71; C_74; C_78	cheese (Caciocavallo)	5.55 - 5.75	0.96 - 0.97	nd	1.9 - 2.2	Coppola *et al.* (2003)
13	FS_	FS_8; FS_14; FS_22; FS_24; FS_28; FS_32; FS_36; FS_39; FS_41; FS_52; FS_54; FS_58; FS_63	fermented sausage (Soppressata)	5.75 - 5.80	0.94 - 0.97	n.d.	2.8 - 3.1	Coppola *et al.* (1998)
9	FS_	FS_CV11; FS_ CV21; FS_CV25; FS_CV28; FS_CV30; FS_IV2. FS_IV29; FS_IV38; FS_IV87	fermented sausage (Ventricina)	5.15 -5.18	0.93 - 0.94	n.d.	3.0 - 3.5	Tremonte *et al.* (2005); Pannella (2013)
5	W_	W_A1-A5	red wine (Aglianico)	3.71 - 3.88	n.d	13.6	n.d.	Testa *et al.* (2014)
12	W_	W_M2; W_M5; W_M11-12; W_M14; W_M16-20; W_M23; W_M26	red wine (Montepulciano)	3.60 - 3.80	n.d	11.8 - 13.5	n.d.	Testa *et al.* (2014)
2	W_	W_P2; W_P5	red wine (Piedirosso)	3.62 - 3.65	n.d	12.4 - 12.8	n.d.	Testa *et al.* (2014)
3	W_	W_PI6; W_PI8; W_PI9	red wine (Pentro d'Isernia)	3.66 - 3.77	n.d	11.3 - 11.6	n.d.	Testa *et al.* (2014)
3	W_	W_R1; W_R2; W_R4	red wine (Rosso Molise)	3.62	n.d	12.5	n.d.	Testa *et al.* (2014)
5	W_	W_T1; W_T4; W_T13-14; W_T17	red wine (Tintilia)	3.66	n.d	14	n.d.	Testa *et al.* (2014)
6	W_	W_TA1; WTA4-8	red wine (Taurasi)	3.68	n.d.	14.2	n.d.	Testa *et al.* (2014)
6	S_	S_9-10; S_20; S_24; S_29; S_33	sourdough from Campania Region	3.7 - 4.0	0.98	n.d	0.8 -1.2	Pannella (2013)
18	S_	S_B1; S_D2; S_D3; S_L4; S_M1; S_M2; S_M3; S_M4; S_N1-N2; S_Q1-Q4; S_R1-R4	sourdough from Molise Region	3.6 - 4.1	0.97 - 0.98	n.d	0.7 - 1.1	Reale *et al.* (2011)
7	S_	S_J14; S_J22; S_J35; S_SEP11; S_SEP16; S_W1-W2	sourdough from Molise Region	3.6 - 4.2	0.98	n.d	0.9 -1.0	Reale *et al.* (2005)

of previous findings, this research was addressed to the investigation of possible relationships between the antimicrobial activities exerted by *Lb. plantarum* strains and the source of isolation.

Materials and Methods

Matrices, producer and indicator strains

One hundred and six *Lactobacillus plantarum* strains (producers), belonging to the Department of Agricultural Environmental and Food Sciences (DIAAA), were isolated from different fermented foods (sourdoughs, wines, cheese and fermented sausages). The main features of food samples and the number of *Lb. plantarum* strains isolated are reported in Table 1. All the strains were tested for their antimicrobial activity against 33 undesirable microbial strains (indicators), listed in Table 2. Prior their use, indicator strains were propagated twice for 16 h at 28°C in proper culture media (Table 2), while producer strains were revitalised in MRS broth (Oxoid, Milan, Italy) in the same incubation conditions.

Detection of the antimicrobial activity exerted by growing cells

The spot-on-the-lawn technique was performed against each indicator to detect growing cells (GC) of producers having inhibitory properties. The method used was that described by Tremonte *et al.* (2007). Briefly, overnight cultures in MRS broth (Oxoid) of each *Lb. plantarum* strain were spotted (75 µL) onto the surface of MRS agar plates and incubated for 24 h at 28°C. A maximum of four strains (spaced approximately 3 cm apart) was spotted per plate. Each indicator strain was inoculated (2% v/v) into 7 mL of the proper soft medium (containing 0.7% agar) at a final concentration of about 10^7 CFU/mL. The content of the tubes was gently mixed and poured over the plates on which *Lb. plantarum* strains were grown. After incubation at 28°C for 24–48 h, plates were checked for inhibition zones, and the presence of a distinguishable halo around the spots was considered as positive antagonistic effect. A calibrated-densitometer (GS-800, Bio-Rad, Hermles CA, USA) was used for imaging acquisition and Adobe Photoshop CS4 Extended software was used for the measurement of clearing zones. On the basis of acquired images, the degree of inhibition was defined as low (5 mm < Ø < 15 mm), moderate (15 mm ≤ Ø < 25 mm), strong (25 mm ≤ Ø < 35 mm), or very strong (35 mm ≤ Ø < 45 mm). Each experiment was carried out in triplicate.

Detection of the antimicrobial activity exerted by cell free supernatants

The antimicrobial activity of cell free supernatants (CFS) was detected by the agar well diffusion assay described by Moraes *et al*. (2010), following the modifications of Tremonte *et al.* (2010; 2016). Cell free supernatants were obtained by overnight cultures in MRS broth of each producer strain. After centrifugation (12000 rpm for 10 min at 4°C, Centrifuge 5415 R, Eppendorf, Hamburg, Germany), each supernatant was filter-sterilised (0.22 µm pore size, Schleider & Schuell, Dassel, Germany). Then

Table 2. Microbial strains used as indicators, their source of isolation and conditions adopted for cultivation.

Species	Strains	Origin	Collection	Cultivation	References
Lactobacillus brevis	A4, B2	sourdough	DIAAA	MRS broth, 28 °C	Reale *et al*. (2011)
Lactobacillus casei	SERB108, SERB69	wine	DIAAA	MRS broth, 28 °C	Sorrentino *et al.* (2010)
Listeria innocua	DSM 20649^T	bovine brain	DSMZ	BHI, 28 °C	
Brochotrix thermosphacta	DSM 20171^T	fresh pork sausage	DSMZ	Corin broth, 28 °C	
Clostridium sporogenes	DSM 795^T	soil	DSMZ	RCM, 28 °C	
Pseudomonas fluorescens	RMFL3	raw milk	DIAAA	Nutrient broth, 28 °C	Tremonte *et. al.* (2014)
Pseudomonas fragi	RMFR5	raw milk	DIAAA	Nutrient broth, 28 °C	Tremonte *et. al.* (2014)
Pseudomonas putida	RMPU12	raw milk	DIAAA	Nutrient broth, 28 °C	Tremonte *et. al.* (2014)
Acetobacter aceti	DSM 3508^T	vinegar	DSMZ	MYP broth, 28 °C	
A. aceti	111, 111E, ASRT, ASC	vinegar	DIAAA	MYP broth, 28 °C	Pannella (2013)
Acetobacter pasteurianus	DSM 3509^T	beer	DSMZ	MYP broth, 28 °C	
Acetobacter tropicalis	DSM 15551^T	coconut juice	DSMZ	MYP broth, 28 °C	
Gluconacetobacter hansenii	DSM 5602^T	vinegar	DSMZ	MYP broth, 28 °C	
Ga. hansenii	194BV, ASAC4, ASR, ARLA, AC1, 141A	wine	DIAAA	MYP broth, 28 °C	Pannella (2013)
Ga. hansenii	203B1	fruit	DIAAA	MYP broth, 28 °C	Pannella (2013)
Ga. liquefaciens	DSM 5603^T	dried fruit	DSMZ	MYP broth, 28 °C	
Gluconobacter oxydans	146B, AC6	wine	DIAAA	MYP broth, 28 °C	Pannella (2013)
Penicillium spp.	T1, T2, T3, T4, T5	black truffle	DIAAA	MYP broth, 28 °C	Sorrentino *et al.* (2013)

agar media, specific for the different indicators (Table 2), were poured in Petri dishes and overlaid with 7 mL of the same soft medium (0.7% agar) inoculated with an overnight culture of each indicator strain (final concentration of about 10^7 CFU/mL). Wells of 3.0 mm in diameter were bored into plates and 75 µL of each *Lb. plantarum* CFS were placed into each well. After 24-48 h of incubation at 28°C, dishes were observed for zones of inhibition, and inhibition halos were normalised using the following formula:

Inhibition Score (IS) = Ø inhibition halo (mm) / Ø well (mm)

On this basis, the antimicrobial effect was considered as low ($1 < IS < 3$), moderate ($3 \leq IS < 5$), strong ($5 \leq IS < 7$), or very strong ($7 \leq IS < 9$).

Dishes inoculated with each indicator strain and without CFS were used as control. To detect the presence of acids or proteins with inhibitory effect produced by *Lb. plantarum*, the agar-well diffusion assay was also performed including two additional tests:

1) nCFS: filter-sterilised CFS of each *Lb. plantarum* strain, neutralised with 1N NaOH (Sigma-Aldrich, St. Louis, MO) up to pH 7;

2) pCFS: filter-sterilised CFS of each *Lb. plantarum* strain added with α-chymotrypsin, proteinase K, and trypsin (Moraes *et al.*, 2010) to a final concentration of 1 mg/mL each. All proteases were supplied by Sigma-Aldrich.

Each experiment was carried out in triplicate.

Statistical analysis

Mean values and standard deviations were determined with the OriginPro 7.5 software (OriginLab Corporation, Northampton, MA, USA). Calculation of similarities in the antimicrobial profiles, in terms of activity and susceptibility of producers and indicators, respectively, was obtained with the software Genesis through a hierarchical cluster analysis based on the Euclidean distance metric and the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm. Data were shown in a pseudo-heat map with producer strains reported in rows and indicator strains in columns.

Results and Discussion

A total of 106 *Lactobacillus plantarum* strains, previously isolated from different fermented matrices (Coppola *et al*., 1998; 2003; Reale *et al*., 2005; 2011; Pannella *et al.*, 2013; Testa *et al*., 2014), were analysed in this study. The spot-on-the-lawn test evidenced different effects of *Lb. plantarum* growing cells (GC) against undesirable microorganisms (Figure 1). As general consideration, the antimicrobial activity expressed by producers was strain-dependent, confirming what reported by other Authors (Engelhardt *et al.*, 2015). Moreover, Gram positives seemed to be more sensitive than Gram negatives to the effect of *Lb. plantarum* GC, as also described by Arena *et al.* (2016).

In fact, out of 106 GC tested, 38 (36 from wines and 2 from sourdoughs) produced a very

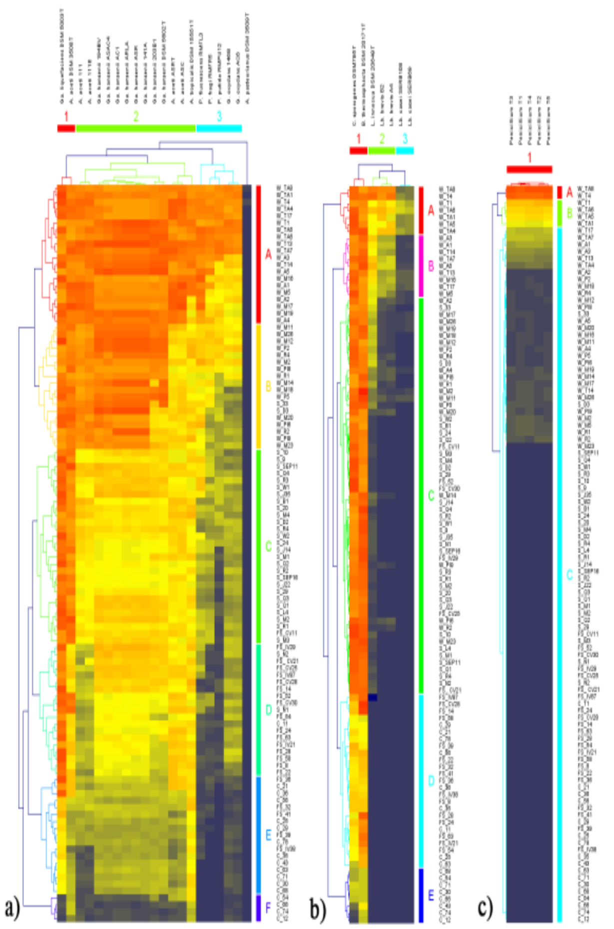

Figure 1. Pseudo-heat map showing the similarity in the antimicrobial profiles of GC from 106 *Lactobacillus plantarum* against Gram negative bacteria (a), Gram positive bacteria (b) and moulds (c). Coloured numbers and letters indicate different clusters of indicators and producers, respectively, individuated on the basis of the inhibition level. Cromatic scale of inhibition level: , very strong; , strong; , moderate; , low.

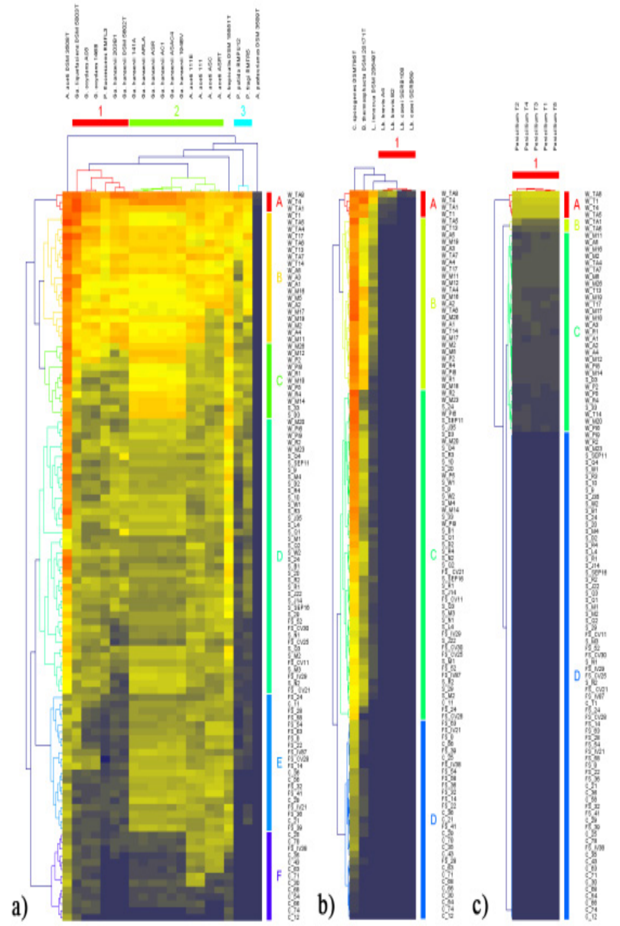

Figure 2. Pseudo-heat map showing the similarity in the antimicrobial profiles of CFS from 106 *Lactobacillus plantarum* against Gram negative bacteria (a), Gram positive bacteria (b) and moulds (c). Coloured numbers and letters indicate different clusters of indicators and producers, respectively, individuated on the basis of the inhibition level. Cromatic scale of inhibition level: , very strong; , strong; , moderate; , low.

strong to a moderate inhibitory activity against all the assayed Gram negative bacteria (clusters A and B), except for *Acetobacter pasteurianus* type strain (Figure 1a). Clusters C and D grouped 47 GC (29 from sourdoughs and 18 from fermented sausages), whose inhibitory activity was principally strong or moderate. The remaining 21 GC (4 from fermented sausages and 17 from cheese) had moderate, low or no detectable antimicrobial activity (clusters E and F). Among Gram negative bacteria, acetic acid bacteria (except *A. pasteurianus* type strain) showed the highest sensitivity to the action of *Lb. plantarum* GC.

The assay against Gram positive bacteria (Figure 1b) showed 16 GC (all from wine strains) having a very strong to a moderate inhibitory action (clusters A and B). Other 57 GC (31 from sourdough, 21 from wine and 5 from fermented sausage) produced a very strong/strong inhibition against *Clostridium sporogenes* and *Brochothrix thermosphacta* type strains and a moderate or low activity against the remaining Gram positive indicators (cluster C). The remaining 33 GC (16 from fermented sausages and 17 from cheese) had low or no detectable inhibition (clusters D and E). Generally, *B. thermosphacta* and *C. sporogenes* showed the highest sensitivity, while a moderate inhibition against *Listeria innocua* type strain was observed.

Results obtained against moulds (Figure 1c) evidenced that 91 GC (about 86% of the assayed *Lb. plantarum* strains) were unable to inhibit *Penicillium* spp. Only 5 GC (all from wine strains) showed a very strong inhibitory activity against tested moulds (cluster A), while 10 GC (9 from wine and 1 from sourdough) caused a moderate or a low inhibition (cluster B). These results are in agreement with those obtained by Coloretti *et al.* (2007), which showed a high inhibition against moulds of only one *Lb.*

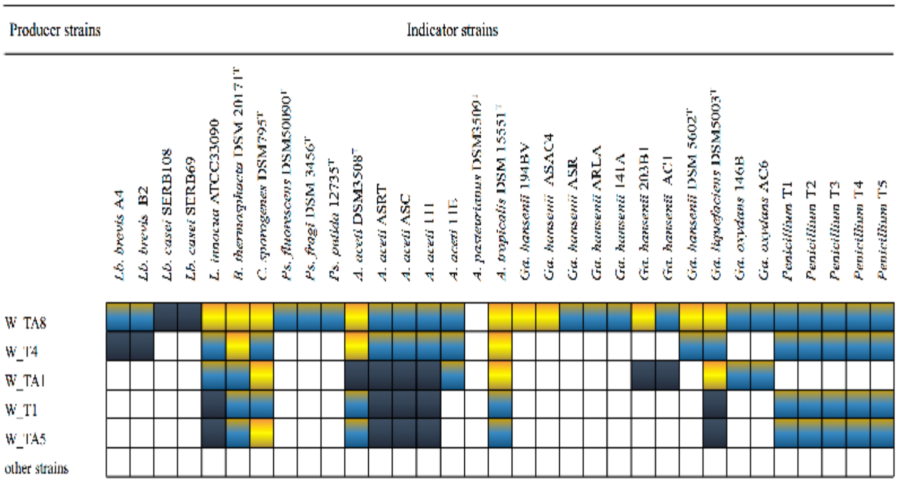

Figure 3. Antimicrobial activity expressed by neutralised Cell Free Supernatants (nCFS) from 106 *Lactobacillus plantarum* strains isolated from different food and beverages. Cromatic scale of inhibition level: , very strong; , strong; , moderate; , low; , no inhibition.

plantarum strain isolated from salami. Conversely, in our study the strains isolated from fermented sausages showed a low inhibitory activity against *Penicillium* spp., confirming that the antimicrobial activity is a strain-dependent character.

The agar well diffusion assay was applied to evaluate the activity of *Lb. plantarum* cell free supernatants (CFS), neutralised CFS (nCFS) and CFS treated with proteases (pCFS). Specifically, nCFS and pCFS were used to assess the involvement of acid and/or proteinaceous compounds in the inhibitory process. Overall, the antimicrobial activity exerted by CFS on both bacteria and moulds was lower than that exhibited by GC (Figure 2), but results also highlighted a considerable number of CFS with a remarkable antimicrobial activity against several indicator strains. In detail, the analysis of the inhibitory action expressed by CFS against Gram negative indicators produced 6 different clusters (Figure 2a). Cluster A grouped 3 CFS, all from wine strains, having a strong inhibitory activity against all the assayed Gram negative bacteria, with the exception of *A. pasteurianus* type strain. Cluster B grouped 19 CFS, all from wine strains, showing a strong or a moderate antimicrobial activity against numerous indicator strains and no detectable antimicrobial action against *A. pasteurianus.* Eleven CFS (9 from wine and 2 from sourdough strains), grouped in cluster C, strongly inhibited all the *Gluconoacetobacter hansenii* strains and the type strains of *Acetobacter aceti* and *Acetobacter tropicalis*, while a moderate or a low action against the other assayed indicators was detected. Forty CFS, grouped in cluster D (5 from wine, 6 from fermented sausage and 29 from sourdough strains), showed a strong or a moderate inhibition against *A. aceti* and *A. tropicalis* type strains, and a moderate or a low inhibition against all the other bacteria. The remaining 33 CFS (from cheese and fermented sausage strains) were grouped in clusters E and F and were characterised by a moderate, a low, or no detectable inhibitory action against all the assayed Gram negative bacteria.

The results of the CFS activity against Gram positive bacteria are reported in Figure 2b. Producer strains were grouped into 4 clusters. Twenty-nine CFS clustering in A and B (all from wine strains) showed a very strong, a strong and a moderate inhibition against *C. sporogenes, B. thermosphacta* and *L. innocua*. The remaining indicator strains were inhibited to a lesser extent. Interestingly, the wine strains clustering in A and B showed a high intra-specific biodiversity, as highlighted in a previous study (Testa *et al.*, 2014).

A lower spectrum of antagonism was detected for 48 CFS (from all the investigated matrices) clustered in C, while the lowest antimicrobial activity was detected for 29 CFS (from fermented sausages and cheese) clustered in D. The activities of CFS against moulds are reported in Figure 2c. As general consideration, tested moulds showed the lowest sensitivity to the CFS. Only 4 CFS, all from wine strains, produced a strong inhibitory activity against *Penicillium* spp. The CFS of the remaining 102 strains exhibited a moderate, a low or no detectable inhibition.

Previous data indicated a good correspondence between the antimicrobial activity of most CFS and GC. This fact suggest that the inhibitory effect was only in part influenced by antagonistic mechanisms involving a direct interaction between producer and indicator strains. A more detailed information was obtained using neutralised CFS (data not shown). In that case, no inhibitory activity was recorded for 101 producer strains. This evidence implies that the antimicrobial activity of almost all the assayed *Lb. plantarum* strains was mainly due to the production of organic acids, primarily lactic acid (De Keersmaecker *et al*., 2006).

However, 5 nCFS, all from wine strains (Figure 3), showed the ability to inhibit all (producer strain WTA8) or several (producer strains WT4, WTA1, WT1 and WTA5) indicators. Remarkably, the same antimicrobial activity persisted also when the CFS was exposed to proteinases (pCFS). The analysis of data reported previously suggests the existence of an association between the antimicrobial properties expressed by assayed strains and their isolation source, characterised by different physico-chemical parameters (see Table 1). In fact, *Lb. plantarum* strains isolated from wines (both GC and CFS) evidenced the largest spectrum of antimicrobial activity, and this fact is possibly the result of selective pressures more accentuated in wines than in the other investigated food matrices. Several studies were addressed to the response of *Lb. plantarum* strains to different stresses characterising food products (De Angelis and Gobbetti, 2004; Guidone *et al*., 2013). However, possible relationships between the antimicrobial ability of lactobacilli and their isolation environments were not revealed or investigated. Other Authors (Guidone *et al*., 2014) found an association between probiotic properties and isolation sources of bacteria, but no correlation between the antimicrobial activity and the isolation environment was highlighted. To our knowledge, only some studies revealed a certain link between the matrix and the presence of bacteriogenic strains, as the case of Corsetti *et al*. (2008).

The research proposed here confirmed that the inhibition of undesirable microorganisms by *Lactobacillus plantarum* was strain-dependent, as also reported by Arena *et al.* (2016). Moreover, the cluster analysis evidenced a high correlation between the antimicrobial ability exerted by *Lactobacillus plantarum* strains and their isolation source, characterised by different stress conditions. To date, only few studies focused the attention on this topic (Bergonzelli *et al*., 2006; Butler *et al*., 2013) assuming that proteins involved in the stress response, such as chaperones, could play a crucial role in the antimicrobial activity.

Moreover, it is important to underline that environmental conditions that are normally present in wine, in particular acid pH and ethanol concentration, are normally lethal for lactic acid bacteria (Eva *et al.*, 2004), thus allowing the proliferation of tolerant bacteria. Our results also showed that the antimicrobial activity of the 95% assayed strains was mainly due to the production of organic acids, as highlighted by the absence of inhibition when neutralised CFS was used.

However, the nCFS of five producer strains involved the permanence of the antagonistic effect, and they maintained their antimicrobial activity when treated with proteinases (pCFS). Such evidence supports the hypothesis that the inhibition was due to the production of extracellular compounds having neither acid nor proteinaceous nature. This datum will be further investigated, since only few studies ascertained the presence of non acid or non proteinaceus antimicrobial compounds produced by *Lb. plantarum* (Niku-Paavola *et al*., 1999).

Noteworthy, a relationship between the antimicrobial activity expressed by strains of *Lb. plantarum* and their isolation environment was found. In fact, those environments characterised by hard conditions (high ethanol levels and low pH), such as wines, harboured higher numbers of antagonistic *Lb. plantarum* strains than other fermented matrices (i.e. cheese or fermented sausages). The relation between environmental conditions and antagonistic properties of *Lb. plantarum* is further strengthened by examining the results of the antimicrobial activity expressed by strains isolated by the same matrix, still having different physico-chemical features. In fact, strains from wines with higher ethanol content (i.e. Taurasi and Tintilia, Table 1) evidenced a stronger antimicrobial activity than those isolated from wines characterised by lower ethanol content (i.e. Pentro d'Isernia and Montepulciano, Table 1).

Data reported in this study indicate that specific food conditions can influence the occurrence of certain strains of *Lb. plantarum* able not only to respond to specific adverse conditions, but also to compete with other bacterial populations. Cao *et al.* (2013), who found an association between the antibacterial activity of *Bacillus amyloliquefaciens* and the adaptation to environmental stress conditions, made a similar remark. In our opinion, the most important conclusion we draw from our research is that the choice of the source of isolation could be an important preliminary tool for the selection of antagonistic strains of *Lb. plantarum.*

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