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SHORT COMMUNICATION

Ecology and technological capability of lactic acid bacteria isolated during Grillo grape vinification in the Marsala production area

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Abstract Grapes of the "Grillo" variety, used to produce Marsala wine, were harvested from five vineyards with different climatic and agronomic parameters, in order to obtain a first mapping of lactic acid bacteria (LAB) inhabiting the production area. Marsala base wine production was followed at a large-scale, and also two experimental vinifications, with different lysozyme and SO_2 concentrations and in combination, were carried out at pilot-plant scale. LAB communities and conventional chemical parameters were periodically analysed. LAB were found on grapes at an average concentration of about 10^2 CFU g^{-1} which decreased during the transformation process. A total of 146 colonies were collected, but only 35 were recognized as presumptive LAB. On the basis of phenotypic differences and isolation source, 16 isolates were then subjected to genotypic identification and assembled into the following species: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Enterococcus faecium*, *Leuconostoc fallax* and *Sporolactobacillus nakayamae* subsp. *nakayamae*. *Lactococcus lactis* subsp. *lactis* strains were the most frequently isolated during winemaking which showed the highest resistance to SO_2 and lysozyme.

Keywords Lactic acid bacteria · Grillo grapes · Marsala wine · Lysozyme · SO_2

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Introduction

Lactic acid bacteria (LAB) occur naturally on grapes; their ability to grow in grape juice and wine is well documented (Davis et al. 1985; Bartowsky et al. 2004; Neeley et al. 2005). LAB have a defining role in wine production since their activities can be beneficial or detrimental for the quality of wine, depending on the species and/or strain and also on the stage of the vinification process at which they develop (Lonvaud-Funel 1999). The growth of LAB in wine is influenced by many factors such as temperature, alcohol concentration, pH, nutrient availability and sulphur dioxide (SO_2) (Fugelsang 1997).

Lysozyme (EC. 3.2.1.17, muramidase) has been proposed as an alternative to SO_2 for controlling the proliferation of LAB in red and white wine, or as a means for delaying malolactic fermentation (Gerbaux et al. 1997). However, this chemical compound does not exhibit the antioxidant properties of SO_2. The efficacy of lysozyme in inhibiting LAB growth in wine is generally dependent on several factors, including the susceptibility of the bacteria, its dosage, pH, polyphenolic compound and SO_2 concentration of wine (Bartowsky et al. 2004).

The objectives of this study were: (1) to isolate and identify LAB from Grillo grapes and wine samples collected during the whole transformation process; (2) to evaluate the influence of lysozyme and SO_2 on LAB

communities during winemaking; and (3) to determine the sensitivity of LAB to lysozyme and SO_2.

Materials and methods

Sample collection

Grapes from cv Grillo were collected from five wineyards located within the Marsala (Sicily, Italy) wine production area, but characterized by different climatic and agronomic parameters. Must and wine samples were collected during the different stages of three different wine productions (Table 1).

Winemaking process A was performed at the winery "Cantina Sociale Birgi" located in Marsala, following the factory protocol using must (230.50 g l^{-1} reducing sugars, pH 3.40) inoculated with commercial dry yeasts (CDY) Premium Blanc 12V *Saccharomyces cerevisiae* (Enologica Vason, Verona, Italy). The fermentation was carried out at a temperature ranging from 16 to 18°C. Two experimental vinifications were performed with different concentrations and combinations of SO_2 and lysozyme: B, in the presence of 25 mg l^{-1} free SO_2; C in the presence of 20 mg l^{-1} free SO_2 and 200 mg l^{-1} lysozyme. Both processes were carried out following the same protocol of the industrial process (vinification A) employing *Saccharomyces cerevisiae* strain GRA21, belonging to the culture collection of SENFIMIZO Department (University of Palermo), as starter culture in a total volume of 100 l.

Chemical analysis

Chemical measurements (Table 2) were performed as reported by Francesca et al. (2010). Glycerol, malic and lactic acid contents were determined by means of enzymatic kits (R-Biofarm, Darmstadt, Germany).

LAB counts and isolation

Grape samples were prepared as follows: 20 bunches were harvested from several plants and 500 g of berries were randomly collected, crushed in sterile bags and blended in a stomacher (Laboratory Blender Stomacher 400; Seward Medical, UK) for 5 min at high speed. Grape, must and wine samples were serially diluted in physiological (0.9% NaCl, w/v) solution and aliquots of 1 ml were pour plated onto MRS (Oxoid, Basinkstoke, UK). At the end of fermentation, LAB were counted onto MRS with added tomato juice (15% v/v) (MRSt) and adjusted to pH 4.8. Plates were anaerobically incubated at 28°C for 5 days.

Colonies were randomly picked up from Petri dishes. Presumptive LAB, Gram-positive (determined by Gregersen's KOH method; Gregersen 1978), catalase-negative (determined by transferring fresh colonies from agar medium to a glass slide and adding 5% H_2O_2) isolates were maintained in glycerol stocks at −80°C. The cultures were purified by successive subculturing.

Grouping and identification of LAB

LAB grouping was performed on the basis of gas formation from glucose (Pilone et al. 1991).

Genotypic identification was carried out by means of 16S rRNA gene sequencing. DNA from LAB was extracted following the method reported by Lopez et al. (2003). PCR reactions were performed as previously described by Weisburg et al. 1991. DNA fragments were visualized after staining with ethidium bromide (0.5 μl ml^{-1}) by a UV transilluminator. The amplicons of about 1,600 bp were purified by the QIAquick purification kit (Quiagen, Milan, Italy) and sequenced using the same primers employed for PCR amplification. DNA sequences were determined by the dideoxy chain termination method with the DNA sequencing kit (Perkin-Elmer Cetus, Emeryville, CA, USA) according to the manufacturer's instructions. The

Table 1 Scheme of sampling during the winemaking processes

Winemaking processes	Must		Alcoholic fermentation				Steel tank			Bottling
	Just pressed	Clarified	3days	6days	14days	21days	17days	32days	78days	
Vinification A[a]	A0	n.c.	n.c.	FA2	n.c.	FA4	A1	A2	A3	A4
Microvinification B[b]	M0	M1	F1	F2	F3	F4	B1	B2	B3	B4
Microvinification C[c]							C1	C2	C3	C4

[a] Industrial process carried out in the presence of 20 mg l^{-1} free SO_2

[b] Microvinification carried out in the presence of 25 mg l^{-1} free SO_2

[c] Microvinification carried out in the presence of 20 mg l^{-1} free SO_2 and 200 mg l^{-1} lysozyme

n.c., not collected

Table 2 Chemical composition of musts and wines obtained during the industrial winemaking process (A) and the micro-vinifications (B and C)

Samples	pH	Reducing sugars (g l^{-1})	TTA[a]	VA[b]	Malic acid (g l^{-1})	Lactic acid (g l^{-1})	Alcohol (% v/v)	Glycerol (mg l^{-1})	Total SO_2 (mg l^{-1})	Free SO_2 (mg l^{-1})	Dry extract (g l^{-1})	TP[c]	Tannins (ppm catechin)
A0	3.40±0.01	230.50±0.04	5.00±0.01	n.d.	1.08±0.01	n.d.	n.d.	1.33±0.04	48.00±0.18	16.00±0.01	20.30±0.33	382.00±0.87	15.40±0.80
A1	3.32±0.01	1.50±0.04	5.00±0.01	0.23±0.01	0.85±0.01	0.45±0.01	13.56±0.03	5.56±0.04	51.00±0.09	15.00±	20.60±0.12	340.00±0.87	15.40±0.61
A2	3.39±0.01	1.60±0.03	5.20±0.01	0.25±0.01	0.91±0.01	0.41±0.02	13.56±0.07	5.92±0.02	58.00±0.12	15.00±0.01	21.90±0.09	382.00±1.25	16.20±0.42
A3	3.39±0.00	1.60±0.05	5.20±0.03	0.28±0.01	0.91±0.03	0.43±0.01	13.56±0.03	5.85±0.02	66.00±0.10	17.00±0.01	21.60±0.12	398.00±0.98	16.40±0.60
A4	3.34±0.01	1.60±0.01	5.40±0.02	0.24±0.01	0.95±0.012	0.43±0.01	13.61±0.05	5.89±0.01	99.00±0.18	35.00±0.01	22.90±0.05	384.00±1.45	16.20±0.41
M0	3.48±0.00	261.40±0.04	6.50±0.01	n.d.	0.97±0.02	n.d.	n.d.	2.84±0.01	46.00±0.22	17.00±0.05	28.60±0.25	303.00±1.02	32.70±0.18
B1	3.50±0.01	1.10±0.05	6.40±0.05	0.20±0.01	1.30±0.02	n.d.	15.80±0.08	9.00±0.01	81.00±0.31	23.00±0.07	25.30±0.08	334.00±0.89	15.70±0.21
B4	3.53±0.01	1.10±0.01	6.00±0.03	0.18±0.01	1.17±0.02	n.d.	15.80±0.01	9.00±0.02	92.00±017	28.00±0.02	22.90±0.09	379.00±1.22	17.60±0.20
C1	3.51±0.01	1.40±0.03	5.90±0.02	0.26±0.01	1.20±0.01	n.d.	15.80±0.02	9.50±0.04	98.00±0.12	24.00±0.02	26.80±0.02	341.00±1.09	15.00±0.20
C4	3.55±0.00	1.40±0.03	5.60±0.02	0.28±0.01	1.17±0.02	n.d.	15.80±0.02	9.50±0.04	102.00±0.10	25.00±0.02	28.90±0.02	338.00±0.96	15.20±0.21

[a] TTA total titratable acidity (g l^{-1} tartaric acid)

[b] VA volatile acidity (g l^{-1} acetic acid)

[c] TP total polyphenols (ppm gallic acid)

n.d. Not detected (value < detection limit of method)

sequences were compared by a BLAST search in GenBank/EMBL/DDBJ database (Altschul et al. 1997).

Sensitivity to lysozyme and SO_2

The resistance of strains to lysozyme and SO_2 was assessed using the well diffusion assay as follows: MRS agar plates were overlaid with 3.0 ml MRS soft agar (0.7% w/v) containing 0.1 ml of culture of each strain developed overnight. Wells (7 mm in diameter) were cut into agar plates and filled with 50 μl of the different chemical compounds (lysozyme and SO_2) at varying concentrations (100–1,600 mg l^{-1} of lysozyme; 100–1,600 mg l^{-1} of SO_2; lysozyme and SO_2 together with the latter at constant 100 mg l^{-1} and lysozyme from 100 to 1,600 mg l^{-1}). The plates were kept at 4°C for 6 h to allow the radial diffusion of the inhibitors into the agar medium. The Petri dishes were then incubated in anaerobic condition at 30°C for 24 h and were examined for the inhibition zone diameter (mm).

Results

Chemical analysis

Chemical analysis were carried out on musts used in both experimental and industrial vinifications, as well as on wines during refining and bottling (Table 2). The musts showed a high concentration of reducing sugars which strongly reduced at the refining stage. Final pH of wines was 3.53 and 3.55 for experimental vinification B and C, respectively, while a lower pH (3.34) was shown by the industrial process. All three wines showed a low volatile acidity. Lactic acid was only detected in wine obtained from the industrial process. Malic acid did not greatly vary from must to wines for all three wine productions. Alcohol percentage was higher for experimental winemaking than industrial process. A similar trend was observed for glycerol. The three wines showed comparable concentrations of total SO_2 at the bottling stage, whereas wine A showed a higher concentration of free SO_2. Total polyphenols increased during vinifications A and B.

LAB enumeration, isolation and identification

Results of presumptive LAB concentration are reported in Table 3. The majority of grapes hosted a similar LAB concentration of about 10^2 CFU g^{-1}. In the case of sample GR1, LAB were found at three orders of magnitude higher than the average level. A cell concentration of 1.1×10^2 CFU ml^{-1} was registered for the must used for experimental vinifications, which decreased to $1.2\times$ 10 CFU ml^{-1} after clarification. The must employed for

Table 3 Presumptive LAB populations associated with grape, must and wine samples

Samples[a]	UFC $g^{-1}(ml^{-1})$[b]	Isolates
GR1	$1.0 (\pm 0.3)\times10^{5}$	1
GR2	$6.7 (\pm 0.4)\times10^{2}$	0
GR5	$1.0 (\pm 0.4)\times10^{2}$	2
GR7	$1.1 (\pm 0.2)\times10^{2}$	0
GR9	$1.2 (\pm 0.4)\times10^{2}$	0
GR10	$4.2 (\pm 0.3)\times10^{2}$	0
GR12	$2.7 (\pm 0.2)\times10^{2}$	0
GR15	$4.0 (\pm 0.1)\times10^{2}$	0
GR26	$2.3 (\pm 0.1)\times10$	0
A0	$1.0 (\pm 0.4)\times10^{3}$	1
FA2	$1.6 (\pm 0.1)\times10$	0
FA4	$1.8 (\pm 0.1)\times10$	0
A1	$1.3 (\pm 0.2)\times10$	3
A2	5.0 (± 0.1)	4
A3	$2.0 (\pm 0.1)\times10$	2
A4	5.0 (± 0.1)	3
M0	$1.1 (\pm 0.2)\times10^{2}$	6
M1	$1.2 (\pm 0.1)\times10$	4
F1	6.3 (± 0.2)	1
F2	4.0 (± 0.1)	1
F3	6.0 (± 0.1)	0
F4	3.1 (± 0.1)	1
B1	1.0 (± 0.1)	0
B2	1.0 (± 0.1)	1
B3	1.0 (± 0.1)	0
B4	4.0 (± 0.2)	1
C1	1.0 (± 0.1)	1
C2	2.0 (± 0.1)	1
C3	3.1 (± 0.2)	1
C4	3.0 (± 0.1)	1

[a] *GR* Grapes, *A0* must just pressed in industrial vinification, *FA2, FA4* alcoholic fermentation in industrial vinification, *A1–A3* industrial wine refining, *A4* industrial wine bottling, *M0* must just pressed in micro-vinifications, *M1* must clarified in microvinifications, *F1–F4* alcoholic fermentation in microvinifications, *B1–B3* wine refining in microvinification B, *B4* bottling in microvinification B, *C1–C3* wine refining in microvinification C, *C4* bottling in microvinification C

[b] Results are expressed per grams of grapes and per millilitres of all other samples

industrial winemaking process contained 10^3 CFU ml^{-1} of LAB. During the 21 days of fermentations, LAB were in the range 3.1–6.3 CFU ml^{-1} for the experimental processes and 1.8×10 CFU ml^{-1} for the industrial process. Very low LAB levels were found in experimental wines at the refining stages in steel (samples B1 to B3 and C1 to C3, Table 3) or in bottle (samples A4, B4, C4, Table 3), whereas a concentration of one log higher was found for the industrial wine.

One hundred and forty-six colonies were isolated as follows: five colonies were picked up from each plate count and, in the case of the lower concentration (<5 colonies per plate), all colonies were isolated from the Petri dishes. All isolates were subjected to a set of preliminary tests (Gram, catalase and spore formation) in order to verify their belonging to the LAB group: only 31 isolates were found to be Gram+, catalase negative and non-spore-forming, while four isolates were Gram+, catalase-negative and spore-forming.

According to the gas production from glucose, 31 isolates were found to be homofermentative, while the remaining four were heterofermentative. On the basis of CO_2 production (Table 4) and isolation source, 16 representative strains were genotypically identified as *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Enterococcus faecium*, *Leuconostoc fallax* and *Sporolactobacillus nakayamae* subsp. *nakayamae* (Table 4).

Development in presence of lysozyme and SO_2

The 16 strains were evaluated for their growth in the presence of different concentrations of inhibitory substances (Table 4). The majority of *Lc. lactic* subsp. *lactis* strains (5 out of 8) showed a resistance to 200 mg l^{-1} of lysozyme, even though a strain (Vm231) was found to develop colonies up to 800 mg l^{-1}. *Leuconostoc fallax* strains were those characterized by the strongest capacity to grow in the presence of lysozyme, since neither strain was inhibited by a concentration of 1,600 mg l^{-1}, while the species that showed the lower resistance to the chemical compound was *S. nakayamae* subsp. *nakayamae* which was controlled in growth by concentrations higher than 100 mg l^{-1}. Regarding SO_2, all lactococci, enterococci and one *Ln. fallax* strain were resistant to 1,600 mg l^{-1}, with a lower resistance found for *S. nakayamae* subsp. *nakayamae* and one *Ln. fallax*. The resistance to lysozyme increased in presence of 100 mg l^{-1} of SO_2: almost all strains, except *Lc. lactis* subsp. *cremoris*, were able to develop at a concentration of 1,600 mg l^{-1} of lysozyme.

Discussion

This study was mainly aimed at characterizing the composition of LAB associated with grapes and wine produced within the Marsala area and to test their resistance to inhibitory compounds. Three different vinifications were followed: one carried out at industrial level and two additional wine-making processes performed at pilot-plant scale in order to test the influence of lysozyme and SO_2 on the LAB flora. The samples collected before and during fermentation, as well as at the bottling stage, were analyzed by a culture-dependent approach.

Table 4 Bacterial species and resistance to lysozyme and SO_2

Samples	Strains	CO_2 production from glucose	Species	Accession no.	Identity (%)	Lysozyme (mg l^{-1})[a]	SO_2 (mg l^{-1})[a]	Lysozyme and SO_2 (mg l^{-1})[a]
F1	Vm199	–	*Lactococcus lactis* subsp. *lactis*	HM638430	97	200	1600	1600
C1	Vm209	–	*Lactococcus lactis* subsp. *lactis*	HM638422	98	200	1600	1600
C2	Vm141	–	*Lactococcus lactis* subsp. *lactis*	HM638416	99	200	1600	1600
C3	Vm115	–	*Lactococcus lactis* subsp. *lactis*	HM638420	97	200	1600	1600
C4	Vm118	–	*Lactococcus lactis* subsp. *lactis*	HM638419	99	100	1600	1600
B4	Vm231	–	*Lactococcus lactis* subsp. *lactis*	HM638431	98	800	1600	1600
A4	Vm214	–	*Lactococcus lactis* subsp. *lactis*	HM638423	99	200	1600	800
GR1	Vm79	–	*Lactococcus lactis* subsp. *lactis*	HM638418	99	400	1600	400
M0	Vm72	–	*Lactococcus lactis* subsp. *cremoris*	HM638424	99	200	1600	800
F4	Vm180	–	*Lactococcus lactis* subsp. *cremoris*	HM638425	99	400	1600	400
M0	Vm3	–	*Enterococcus faecium*	HM638426	99	800	1600	1600
B2	Vm137	–	*Enterococcus faecium*	HM638421	98	800	1600	1600
A0	Vm162	+	*Leuconostoc fallax*	HM638428	98	1600	1600	1600
GR5	Vm181	+	*Leuconostoc fallax*	HM638429	98	1600	200	1600
A1	Vm28A	–	*Sporolactobacillus nakayamae* subsp. *nakayamae*	HM638417	99	100	800	1600
A1	Vm28B	–	*Sporolactobacillus nakayamae* subsp. *nakayamae*	HM638427	98	100	800	1600

[a] The results of resistance to lysozyme and SO_2 refer to the highest concentration allowing growth

LAB were found on grapes at an average concentration of about 10^2 CFU g^{-1}. Similar LAB values have been reported for freshly extracted grape juice (Fleet 1993; Fugelsang 1997). LAB concentration decreased during alcoholic fermentation, refining and bottling until reaching levels of a few (<10) colonies per millilitre of wine.

Despite the large number of colonies isolated from the media generally used for LAB count and isolation, only 35 were recognized as presumptive LAB. This result highlights the difficulties encountered in the isolation of LAB from the wine environment, and, for this reason, a pre-enrichment procedure may be necessary when no LAB are detected by both culture-dependent and -independent methodologies (Bae et al. 2006). On the basis of phenotypic differences and source of isolation, 16 strains were subjected to genotypic identification. From the present work, lactococci (*Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) were the LAB most frequently isolated, since they were identified from almost all the vinification steps of the three processes followed. LAB community also included *E. faecium* and *Ln. fallax*. Although *Lactococcus* spp. have been identified from wine environments (Yanagida et al. 2008; Bae et al. 2006), so far they have been found associated only to grapes and wineyard, while no publication has reported their presence during vinification. In the present work, the species *E. faecium* and *Ln. fallax* are generally revealed during winemaking, but not beyond the beginning of fermentation.

The spore-forming *S. nakayamae* subsp. *nakayamae* was also isolated. *Sporolactobacillus* species were isolated from grapes cultivated in Australia (Bae et al. 2006) and winery soils in Korea (Chang et al. 2008); hence, this is the first report on the presence of the species *S. nakayamae* subsp. *nakayamae* at the refining stage. This species was only found during the industrial vinification, which included the addition of CDY. Thus, the ultimate source of contamination of wine with the species *S. nakayamae* subsp. *nakayamae* could be the CDY itself. In fact, contaminations of yeast starter preparations by LAB have been reported at concentrations of about 10^4 CFU g^{-1} (Scartezzini et al. 2009). However, the isolation of two strains of *S. nakayamae* subsp. *nakayamae* is technologically interesting. *Sporolactobacillus* genus includes spore-forming bacteria that produce lactic acid homofermentatively (Yanagida et al. 1997), but which are not included in the group of LAB (Bae et al. 2006). The capacity to produce spores determines an important ecological advantage of the species allotted to this group, since they may survive the adverse conditions generated by lethal concentrations of chemical compounds, as well as extreme temperatures and deficiency of nutrients. For these reasons, the species *S. nakayamae* subsp. *nakayamae*, being associated with a food matrix, deserves a deeper characterization for its future use in food fermentation.

The data resulted from the chemical analysis of wines showed that the three vinification processes did not greatly

vary from one another and that they were in agreement with those reported for other commercial productions.

All 16 strains identified were tested for their resistance to different concentrations and combinations of lysozyme and SO_2. Due to their possible biotechnological applications, *S. nakayamae* subsp. *nakayamae* strains were also included in the assays. Lysozyme showed a higher inhibitory capacity than SO_2, especially against *S. nakayamae* subsp. *nakayamae*. *Enterococcus faecium* and *Lc. lactis* did not seem to be negatively influenced by the concentrations tested in this study. As a matter of fact, *Lc. lactis* persisted during the entire vinification processes until bottling of all wines. The metabolic traits of our *Lc. lactis* strains need to be investigated, because wine represents an unusual environment for these species. In fact, lactococci, but also enterococci, are thought not to be able to grow at the high ethanol concentrations of wine (Stiles and Holzapfel 1997). They have been found in the winery ecosystem only associated with grapes (Bae et al. 2006). In our study, lactococci were isolated from wine samples at very low concentrations (<10 CFU ml^{-1}) which are not dangerous, but the high pH and the low TTA of wines obtained in hot climates, such as Sicily, and the resistance of certain strains to lysozyme and SO_2, as well as their high alcohol tolerance, could allow their proliferation until spoilage.

In general, the persistence of LAB at high concentrations may be detrimental for the quality of wine, because they may cause the degradation of citric acid, tartaric acid, glycerol and determine several alterations (Comi et al. 2005).

Strains belonging to the species *Oenococcus oeni*, mainly responsible for malo-lactic fermentation (Van Vuuren and Dicks 1993), were not isolated in the present work. This finding was not surprising since malic acid concentration (Table 2) did not greatly vary during wine transformation and lactic acid was detected at low levels in the sole industrial vinification. Furthermore, except for two strains of *Ln. fallax*, no heterofermentative LAB were isolated.

The two experimental vinifications did not produce negative effects in terms of LAB composition and concentrations. Moreover, neither process differed from the industrial production.

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