International Journal of Food Microbiology 159 (2012) 25–29

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Lysozyme resistance of the ropy strain *Pediococcus parvulus* IOEB 8801 is correlated with beta-glucan accumulation around the cell

Joana Coulon [a], Anne Houlès [a], Maria Dimopoulou [b], Julie Maupeu [a], Marguerite Dols-Lafargue [b,*]

[a] Microflora, ISVV, 210 chemin de Leysotte, CS 50008-33882 Villenave d'Ornon cedex, France
[b] Université de Bordeaux, ISVV, EA 4577, Unité de recherche OENOLOGIE, INRA USC 1219, 210 chemin de Leysotte, 33882 Villenave d'Ornon cedex, France

ARTICLE INFO

Article history:
Received 1 May 2012
Received in revised form 13 July 2012
Accepted 26 July 2012
Available online 31 July 2012

Keywords:
Pediococcus parvulus
Ropiness
Wine
Lysozyme
Glucanase
Capsule

ABSTRACT

Lactic acid bacteria (LAB) are often exploited to carry out malolactic fermentation in wine. However, a few specific LAB strains and, more precisely, some *Pediococcus parvulus* strains synthesize a β-glucan, which can be deleterious to wine quality as it confers a ropy texture to the wine that can no longer be commercialized. Although molecular methods exist to detect these unwanted microorganisms, ropy *Pediococcus* still remain difficult to remove from wine, because of their natural resistance to traditional wine stabilizing treatments. In this work, we show that ropy *P. parvulus* are resistant to lysozyme. We clearly demonstrate that this resistance may be ascribed to the presence of the β-glucan that forms around the cell a protective barrier against anti-bacteria agents. Moreover, this resistance increases during bacterial growth. We show that using lysozyme with β-glucanase can strongly improve the treatment against ropy strains, in model media as well as red and white wine based media. This work not only brings potential solutions to the wine industry, but also opens interesting perspectives for studying β-glucan producing bacteria which are widespread in the food industry.

1. Introduction

"Ropy" wines are characterized by an increased viscosity and a slimy texture ("graisse"). Even though the aromatic profile of the wine is not affected, the dramatic visual appearance and texture on the palate render its marketing impossible. This problem can occur in tanks, barrels, or even after bottling, mainly in wines displaying high pH (>3.8). At the onset of ropiness, these wines contain high populations of lactic acid bacteria, in most cases belonging to the species *Pediococcus parvulus* which is the only recognized agent of ropiness in Bordeaux wines (Dols-Lafargue and Lonvaud-Funel, 2009). The spoilage also occurs in beer and in cider (Dols-Lafargue and Lonvaud-Funel, 2009; Duenas et al., 1995). In each case, the pediococci strain synthesizes a high molecular weight β-1,3-glucan with β-1,2-linked branched units consisting of a single glucose. Synthesis is performed via a membrane spanning glucosyltransferase, called Gtf, catalyzing both the polymerization of UDP-glucose and the export of the neosynthesized β-glucan (Werning et al., 2006). The Gtf protein is encoded by the *gtf* gene, located on a 5.5 kb plasmid for red wine pediococci and on a 35 kb plasmid for cider pediococci (Dols-Lafargue and Lonvaud-Funel, 2009). Other lactic acid bacteria species also carry this gene: *Lactobacillus diolivorans* and *Lactobacillus suebicus* have it on a 5.5 kb plasmid whereas in some *Oenococcus oeni* strains it is located on the chromosome (Dols-Lafargue et al., 2008; Garai-Ibabe et al., 2010; Walling et al., 2005; Werning et al., 2006).

Based on the *gtf* gene sequence (at least 97% identical in all the species studied), rapid and highly sensitive real-time PCR reactions were developed to detect ropy bacteria (Delaherche et al., 2004; Ibarburu et al., 2010). The main advantage of these techniques is that they allow an early detection of the spoiling agent (detection limit 40 CFU.mL^{-1}). In this case, the winemaker needs to immediately prevent pediococci development, because β-glucan production is largely associated with bacterial growth. Only 12 mg/L is sufficient to spoil the wine (Dols-Lafargue and Lonvaud-Funel, 2009). Sulfur dioxide is commonly used in winemaking to protect the wine from undesirable bacteria development (Ribéreau-Gayon et al., 2000). However, the antimicrobial effect of SO_2 decreases with increasing pH, and ropy pediococci generally display high levels of resistance to SO_2 (Dols-Lafargue et al., 2008; Lonvaud-Funel and Joyeux, 1988). Furthermore, SO_2 is subjected to maximum authorized levels and the European Commission has recognized the health issues raised by its use in the wine industry (Commission Regulation (EC) N° 606/2009; OIV, 2011). Enterocin AS-48 has been proposed to inactivate ropy lactic acid bacteria from cider, but today, its use in wine is still not permitted (Martínez-Viedma et al., 2010). Lysozyme is an alternative to prevent bacterial spoilage in wine, especially those which harbor a high pH. Isolated from egg white, it displays a muramidase activity and hydrolyzes the bacterial cell wall. Its use in wine is authorized in EU since October 2001 (OIV, 2011). It is recommended for the control of spontaneous malolactic fermentation which often leads to increased volatile acidity or biogenic amine formation, due to undesirable lactic bacteria species or strain development (Bartowsky, 2009; Delfini et al., 2004; Gerbaux et al., 1997; Lasanta et al., 2010; Lopez et al., 2009). Besides the fact

* Corresponding author. Tel.: +33 5 57 57 58 32; fax: +33 5 57 57 58 13.
E-mail address: dols@enscbp.fr (M. Dols-Lafargue).

0168-1605/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2012.07.024

that, like SO_2, it can cause allergic reactions in susceptible individuals, its effectiveness is reduced in acid wines, wines with high polyphenol or metal cation concentrations or wines treated with bentonite (Blättel et al., 2009; Guzzo et al., 2011; Lin and Georgiou, 2005; Weber et al., 2009). In addition, some wine lactic acid bacteria are resistant to lysozyme even though the mechanisms underlying this characteristic are not totally understood (Blättel et al., 2009; Delfini et al., 2004). Thus, it is not uncommon to find ropy pediococci in wines despite lysozyme treatment. These ropy pediococci strains are indeed known to show a high tolerance to various growth inhibitors in wine (Dols-Lafargue et al., 2008; Lonvaud-Funel and Joyeux, 1988). Compared to other lactic acid bacteria, they are able to grow in what is considered to be harsh wine conditions (low pH, high ethanol or SO_2 concentrations). The ropy β-glucan produced by these bacteria can be partially associated with the cells, hence forming a protective layer and allowing the adhesion of the bacteria to different biotic and abiotic surfaces (Blättel et al., 2011; Dols-Lafargue et al., 2008; Fernandez de Palencia et al., 2009).

The aims of this work are to demonstrate the increased resistance to lysozyme of ropy pediococci, to identify the origin of this resistance and to suggest ways to improve ropy pediococci remediation in red and white wines.

2. Materials and methods

2.1. Strains and growth conditions

The ropy *P. parvulus* IOEB 8801 belongs to our collection (Enology Institute of Bordeaux). The strain *P. parvulus* IOEB 8801 was chosen as a model strain for this study because (i) among the pediococci in the IOEB collection, it is the one harboring the most ropy phenotype and (ii) we obtained a non-ropy isogenic mutant of this strain (*P. parvulus* IOEB 0206) by plasmid curing experiments (Walling et al., 2005).

Bacteria were propagated in liquid MRS containing ($g.L^{-1}$): glucose 20; yeast extract, 4; beef extract, 8; bactopeptone, 10; sodium acetate, 5; trisodium citrate, 2; K_2HPO_4, 2; $MgSO_4$, $7H_2O$, 0.2; $MnSO_4$, H_2O, 0.1; Tween 80, 1 mL and adjusted to pH 5.0. Ethanol (10%) was added prior to inoculation. For solid media, agar (20 $g.L^{-1}$) was added prior to sterilization.

The red wine used was a blend of different varieties of red grapes (vintage 2008), with a pH of 3.7. It was treated with H_2O_2 to eliminate the total SO_2. The white wine used was a Chardonnay (vintage 2010), which did not initially contain any free SO_2. Its pH was adjusted to 3.6 with KOH 5 N. Both wines were sterilized by filtration (0.2 μm cut-off membrane). The ethanol content was 12% vol for both. The ropy pediococci were progressively transferred (4 intermediate cultures with growing proportions of wine) from pure MRS medium to a medium containing 75% of wine and 25% of MRS-ethanol. The inoculation rate was of 10% vol/vol (pre-culture duration 3 to 6 days) and the cultures were incubated at 25 °C without agitation.

2.2. Analytical methods

Growth was followed by measuring the absorbance at 600 nm. Precise determinations were carried out by colony counts on solid MRS media using several dilutions for plating. Results were expressed as $CFU.mL^{-1}$ based on weighted mean calculations (AFNOR, 1998). Survival rates were calculated as the ratio of colony forming units between treated samples and untreated samples. EPS concentrations were determined after ethanol precipitation and phenol-sulfuric assay and glucose concentration was assayed by HPLC as previously described (Dols-Lafargue et al., 2008).

2.3. Enzyme treatment

The enzyme preparations used were the oenology quality lysozyme “Lacticid” (Lamothe Abiet, France), a β-1,3-β-1,6 glucanase (EC: 3.2.1.58) from *Trichoderma harzianum* in a laboratory grade powder form named Glucanex® (from Novozyme and distributed by Sigma-Aldrich, l"Isle d'Abeau, France) and in an oenology quality liquid form (Vinoflow® Max A, VMA, from Novozyme and distributed by Lamothe Abiet). A 100× concentrated enzyme stock solution was prepared in sterile water and immediately added (concentration indicated in the text) to the cell culture. Sterile water was added to the control assay. Reactions were performed at 25 °C in the culture broth.

After the indicated contact time, serial dilutions were performed in NaCl 9 $g.L^{-1}$ and plated onto MRS agar. Determinations were done in triplicate. The ropy character of the colonies and microscope observations were used to confirm that the colonies corresponded to pediococci.

2.4. Viability assays

The absence of viable but not cultivable (VBNC) cells was checked by epifluorescence microscopy (Olympus Bx 41 microscope), using the ChemChrome V6 Kit, as recommended by the supplier (Chemunex, Ivry-sur-Seine, France). The quantification limit of the method is of $1.5 \cdot 10^3$ $cells.mL^{-1}$ and the detection limit can be estimated at $1.5 \cdot 10^2$ $cells.mL^{-1}$.

2.5. Microscopy

To visualize the glucan capsule before and after glucanase treatment, MRS grown cells of *P. parvulus* IOEB 8801 were treated (or not) for 4 h with 0.5 $mg.mL^{-1}$ Glucanex®. For transmission electron microscopy (TEM), bacteria were fixed for 2 h in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde, at room temperature. Fixed bacteria were stored at 4 °C in the fixative solution. They were rinsed in cacodylate buffer, included in 1% gelatin and postfixed (i) with 1% osmium tetroxide containing 1.5% potassium cyanoferrate and (ii) with 3% uranyl acetate at 4 °C. They were gradually dehydrated in ethanol (30% to 100%) and embedded in Epon. Thin sections (70 nm) were collected on 150-mesh cooper grids before examination with a HITACHI H7650 TEM.

2.6. Repeatability

All the experiments described in the paper were performed twice using independent cultures.

3. Results and discussion

The growth of *P. parvulus* IOEB 8801 on MRS medium was accompanied by the production of soluble exopolysaccharides. Production occurred mainly during growth phase after a short delay and was thus partially connected with growth (Fig. 1A). The ropy character appeared after 1 day of culture. All media used in this study yielded similar growth profiles.

At different growth phases, samples of *P. parvulus* IOEB 8801 cultures were collected and subjected to lysozyme treatment: the first sample was taken at the exponential growth phase (after 1 day of growth, Fig. 1B), the second on entry into stationary phase (after 3 days of growth, Fig. 1C) and the last one, during late stationary phase (after 6 days, Fig. 1D). The survival rates after lysozyme treatment were estimated using these 3 samples. It appeared clearly that bacteria treated during the exponential growth or during the stationary phase were totally eliminated by treatment with 0.2 $mg.mL^{-1}$ (20 g/hL) of lysozyme (survival rate $<10^{-8}$, no colony detected on the agar plates, Fig. 1B and D). On the contrary, when lysozyme treatment was performed on cells entering into stationary growth phase, the pediococci were more resistant. Fig. 1C shows that, after 2 days of treatment with 0.2 $mg.mL^{-1}$ of lysozyme, the survival rate was of 10^{-4} and a slight growth was observed between 2 and 3 days of

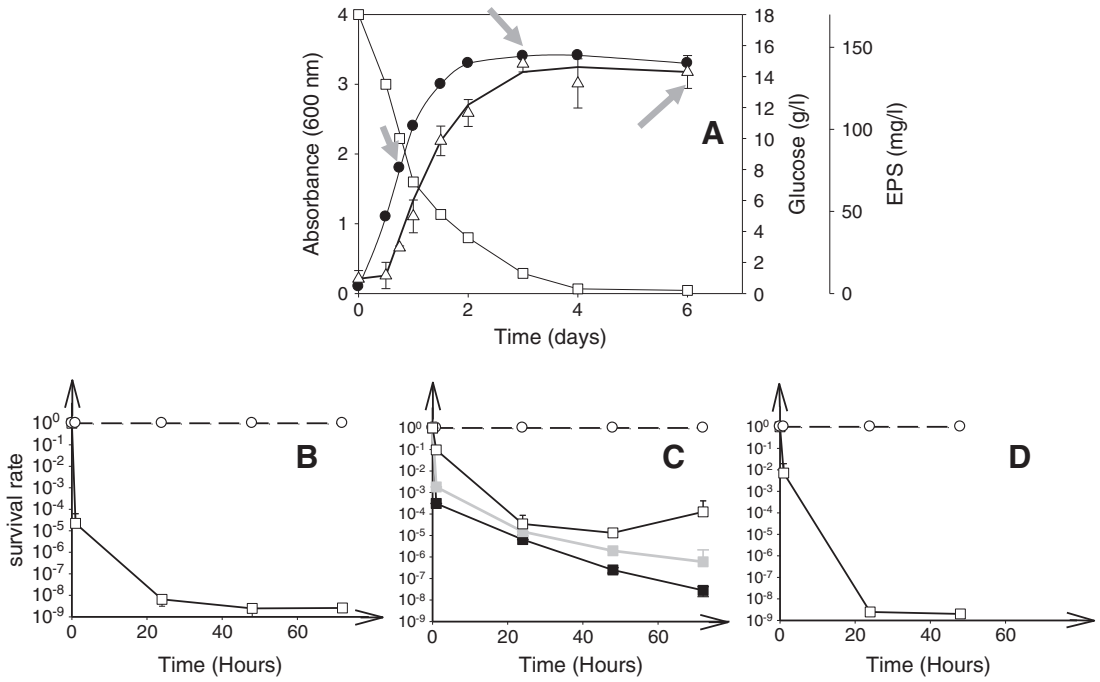

Fig. 1. A. *P. parvulus* IOEB 8801 growth and exopolysaccharide production in MRS + ethanol (10% vol). ● Absorbance; □ glucose; △ exopolysaccharides; the gray arrows indicate time of growth before sampling in order to perform lysozyme treatment (see panels B to D). Survival rate of *P. parvulus* cells sampled after B. 1 day of culture, C. 3 days of culture and D. 6 days of culture, and immediately treated with the indicated lysozyme concentration. ○ Non-treated control; □ lysozyme 0.2 mg.mL^{-1}; ■ lysozyme 0.4 mg.mL^{-1}; ■ lysozyme 0.5 mg.mL^{-1}.

treatment. Treatments of wine pediococci collected at the entry into stationary growth phase were repeated using different doses of lysozyme: the survival rate remained high (10^{-3}–10^{-4}) even in the presence of 0.4 mg.mL^{-1} of lysozyme. Furthermore, the maximum dose of lysozyme recommended by the supplier (50 g/hL, i.e. 0.5 mg.mL^{-1}) was not sufficient to completely eliminate the ropy pediococci in this physiological state (Fig. 1C).

P. parvulus IOEB 8801 cells isolated during exponential growth or at the entry of the stationary phase of growth were examined by electron microscopy. As shown in Fig. 2A, the cells were surrounded by a loose coat of β-glucan. The coat became thicker at the end of the exponential growth (Fig. 2B). We thus hypothesized that the coat of β-glucan prevented the lysozyme from reaching the cell wall, thus protecting it.

To verify this hypothesis, we performed two types of experiments. First, the ropy *Pediococcus* (IOEB 8801) and its non-ropy mutant (IOEB 0206), both sampled at the entry of stationary growth phase were treated with lysozyme. The non-ropy mutant IOEB 0206 does not display the *gtf* gene and does not produce β-glucan in neither soluble nor capsular form (Walling et al., 2005). The results presented in

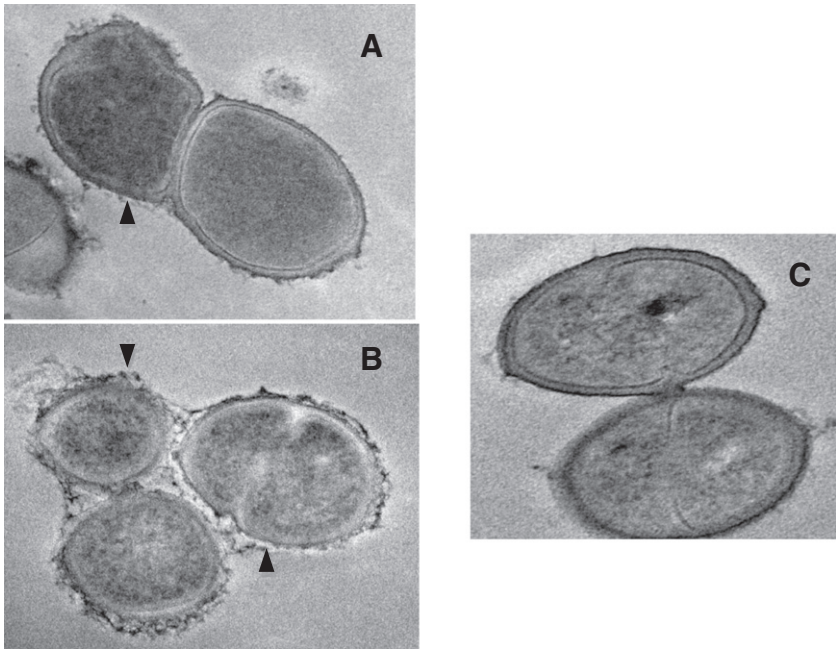

Fig. 2. Transmission electron microscopy of *P. parvulus* IOEB 8801 (A) isolated during early exponential growth — 1 day growth, (B) isolated at the entry of the stationary phase — 3 day growth, and (C) isolated at the entry of the stationary phase and subsequently treated for 4 h with 0.5 mg/mL of Glucanex. The black arrows indicate the β-glucan layer.

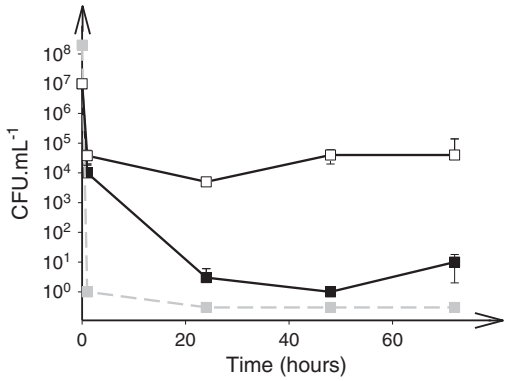

Fig. 3. Population dynamics of *P. parvulus* IOEB 8801 and its non-ropy mutant IOEB 0206, after lysozyme treatment. Prior to enzyme treatment, the cells were grown for 3 days in MRS + ethanol 10% vol. □ *P. parvulus* IOEB 8801 + lysozyme 0.2 mg.mL^{-1}; ■ *P. parvulus* IOEB 8801 + lysozyme 0.5 mg.mL^{-1}. ▩ *P. parvulus* IOEB 0206 + lysozyme 0.2 mg.mL^{-1}.

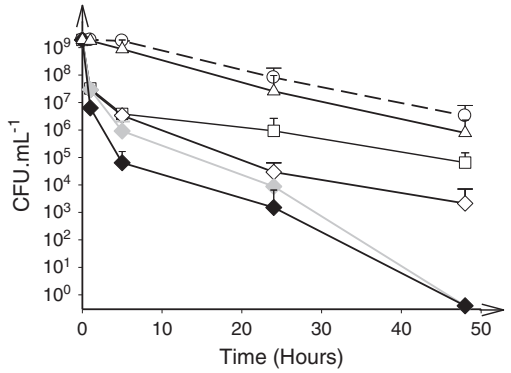

Fig. 4. Population dynamics of *P. parvulus* IOEB 8801 after lysozyme and glucanase treatment. Prior to enzyme addition, the cells were grown for 3 days in MRS + ethanol 10% vol. ○: non-treated control; △ Glucanex® 0.5 mg.mL^{-1}; □ lyzozyme 0.2 mg.mL^{-1}; ◊ lyzozyme 0.2 mg.mL^{-1} + Glucanex® 0.1 mg.mL^{-1}; ◆ lysozyme 0.2 mg.mL^{-1} + Glucanex® 0.25 mg.mL^{-1}; ♦ lysozyme 0.2 mg.mL^{-1} + Glucanex® 0.5 mg.mL^{-1}.

Fig. 3 indicate that the non-ropy mutant was completely eliminated by treatment with 0.2 mg.mL^{-1} lysozyme in the very first hours of treatment, whereas, in the same conditions, the ropy pediococci remained at a population level higher than 10^4 CFU.mL^{-1}.

The second test combined lysozyme with Glucanex®, a β-1,3, β-1,6 fungal glucanase, known to hydrolyze the β-glucan from *Botrytis cinerea* but also from *P. parvulus* (Dubourdieu et al., 1985; Llaubères et al., 1990). The results are shown in Fig. 4. First, the survival curves show that the untreated control and culture treated only with Glucanex® were very similar. Hence, β-glucanase did not display any proper significant bactericidal activity. However, electron microscopy scans clearly showed that the glucanase treated cells had lost their glucan coat (Fig. 2C). Furthermore, β-glucanase improved the bactericidal action of lysozyme used at 0.2 mg.mL^{-1} in a dose-dependent way (Fig. 4). As a result, ropy pediococci were totally eliminated after 48 h by the joint action of Glucanex® (0.25 mg.mL^{-1} or more) and lysozyme (0.2 mg.mL^{-1}). No viable but non-cultivable cells were detected in the assays (data not shown).

All the tests carried out in model media were repeated in the presence of red (Fig. 5) or white (not shown) wine based media, using either the laboratory grade (Glucanex®) or an oenological preparation (VMA) of glucanase. It appeared that, in the presence of red wine, lysozyme alone was not sufficient to completely eliminate the ropy pediococci. Furthermore, the effective glucanase concentrations needed to be higher than in the model media: in the presence of 0.2 mg.mL^{-1} of lysozyme, 0.3 μL.mL^{-1} of VMA or 0.5 mg.mL^{-1} of Glucanex® were necessary to completely eliminate the ropy pediococci. In the presence of higher doses of lysozyme (0.5 mg.mL^{-1}), it was possible to lower the effective doses of glucanase (0.25 mg.mL^{-1} of Glucanex® or 0.1 μL.mL^{-1} of VMA). In the presence of white wine, even though pediococci populations and their associated ropy character were at levels similar than in red wine media, the bacteria were less resistant to lysozyme treatment and the effective enzyme concentrations were similar to those described for model media (not shown). Several studies had reported that lysozyme is less active in red than in white wine, due to the inhibitory effect of polyphenolic compounds and particularly tannic acid (Delfini et al., 2004; Guzzo et al., 2011). Nevertheless, even in red wine based media, pediococci were totally eliminated by the combined action of lysozyme and glucanase, used at their maximal authorized/recommended level. Beta-1,3 glucanases are generally used by winemakers to improve wine filterability or clarification (Villetaz et al., 1984; Dubourdieu et al., 1985). They were also proved to be efficient to eliminate spoilage yeasts (Blättel et al., 2011; Enrique et al., 2010). Our results indicate that they are useful as well to eliminate ropy pediococci from wine, in association with lysozyme, even in the toughest conditions: (i) cell concentration higher than what is generally described for ropy wines (10^7 cells.mL^{-1}), (ii) strong ropy character, (iii) cells in a physiological state enabling the highest resistance to lysozyme, and (iv) presence of lysozyme wine inhibitors such as ethanol or polyphenols. Assays in naturally occurring ropy wines will allow to determine the exact efficient doses of lysozyme and glucanase in pure wine in the future.

These results may be generalized to other wine or cider lactic acid bacteria producing beta glucans, since these polymers are highly identical (Dols-Lafargue and Lonvaud-Funel, 2009). For example, the association of β-glucanase and lysozyme was proven to kill all

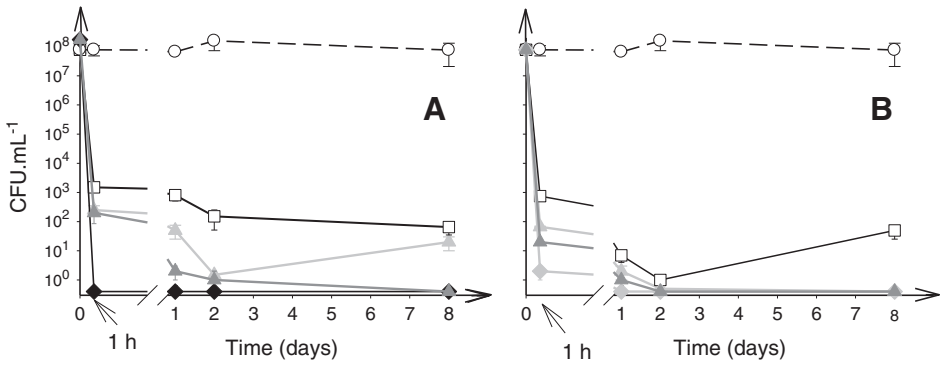

Fig. 5. Influence of lysozyme and oenological glucanase treatment on *P. parvulus* IOEB 8801 survival. Prior to enzyme addition, pediococci were grown for 3 days in red wine base medium (25% MRS-ethanol + 75% red wine). A. Lysozyme 0.2 mg.mL^{-1}; B. lysozyme 0.5 mg.mL^{-1}. ○: non-treated control; □ lysozyme alone; △ lyzozyme + VMA 0.1 μL.mL^{-1}; ▲ lyzozyme + VMA 0.3 μL.mL^{-1}; ◆ lyzozyme + Glucanex® 0.25 mg.mL^{-1}; ♦ lyzozyme + Glucanex® 0.5 mg.mL^{-1}.

the ropy *O. oeni* in the IOEB collection, while lysozyme alone did not (not shown). In addition, most of the lactic acid bacteria produce polysaccharide structures which accumulate to form very thin or large capsules around the cell. As with ropy beta glucan, these polysaccharidic structures may protect the peptidoglycan wall and partially prevent the action of lysozyme, hence explaining the ineffectiveness of lysozyme treatments in some wines (Delfini et al., 2004). Furthermore, repeated lysozyme treatments in wineries may favor the persistence and prevalence of lysozyme resistant encapsulated species or strains. Indeed, the ropy *O. oeni* were all isolated from white wines, where lysozyme treatment is more frequent than in red wines (Dols-Lafargue et al., 2008).

This work also opens interesting perspectives for studying β-glucan producing bacteria from other ecological niches (cider, cheese …). The presence of this specific glucan at the surface of bacteria considerably modifies their adhesion capacities or their resistance to stress and thus their probiotic status (Deutsch et al., 2012; Fernandez de Palencia et al., 2009; Stack et al., 2010; Werning et al., 2008). As for lysozyme resistance, differences in β-glucan capsule thickness depending on the growth phase may also explain some of the contradictory results reported in the literature regarding the stress resistance or the adhesion capacity conferred by the production of β-glucan.

4. Conclusion

The present paper clearly shows that the glucan capsule thickness varies during *P. parvulus* growth and modulates the bacteria resistance towards muramidase activity. As a result, the association of β-glucanase with lysozyme is essential for ropy pediococci remediation, in model media as well as red and white wine based media.

Acknowledgments

M. Dimopoulou is the recipient of a grant from the Agence Nationale de la Recherche (ANR).

The microscopy was done in the Bordeaux Imaging Center of the University of Bordeaux Segalen. The authors would like to thank Melina Petrel and Etienne Gontier for their assistance in electronic microscopy and Gaëlle Renou (Lamothe-Abiet company) for providing the Vinoflow Max A enzyme preparation.

References

AFNOR, 1998. Règles pour le comptage des colonies et pour l'expression des résultats. Cas des dénombrements en milieu solide. XP V 08–102.

Bartowsky, E.J., 2009. Bacterial spoilage of wine and approaches to minimize it. Letters in Applied Microbiology 48, 149–156.

Blättel, V., Wirth, K., Claus, H., Schlott, B., Pfeiffer, P., König, H., 2009. A lytic enzyme cocktail from *Streptomyces* sp. B578 for the control of lactic and acetic acid bacteria in wine. Applied Microbiology and Biotechnology 83, 839–848.

Blättel, V., Larisika, M., Pfeiffer, P., Nowak, C., Eich, A., Eckelt, J., König, H., 2011. Beta-1,3-glucanase from *Delftia tsuruhatensis* strain MV01 and its potential application in vinification. Applied and Environmental Microbiology 77, 983–990.

Commission Regulation (EC) N° 606/2009 of 10 July 2010 laying down certain details rules for implementing Council regulation (EC) N°479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions.

Delaherche, A., Claisse, O., Lonvaud-Funel, A., 2004. Detection and quantification of *Brettanomyces bruxellensis* and "ropy" *Pediococcus damnosus* strains in wine by real time polymerase chain reaction. Journal of Applied Microbiology 97, 910–915.

Delfini, C., Cersosimo, M., Del Prete, V., Strano, M., Gaetano, G., Pagliara, A., Ambrò, S., 2004. Resistance screening essay of wine lactic acid bacteria on lysozyme: efficacy of lysozyme in unclarified grape musts. Journal of Agricultural and Food Chemistry 52, 1861–1866.

Deutsch, S.M., Parayre, S., Bouchoux, A., Guyomarc'h, F., Dewulf, J., Dols-Lafargue, M., Baglinière, F., Cousin, F., Falentin, H., Jan, G., Foligné, B., 2012. Surface β-glucan polysaccharide affects the physicochemical and immunomodulatory properties of *Propionibacterium freudenreichii*. Applied and Environmental Microbiology 78, 1765–1775.

Dols-Lafargue, M., Lonvaud-Funel, A., 2009. Polysaccharide production by grapes, must and wine microorganisms. In: König, H., Unden, G., Fröhlich, J. (Eds.), Biology of Microorganisms on Grapes, in Must and Wine. Springer-Verlag, Berlin Heidelberg, pp. 243–260.

Dols-Lafargue, M., Lee, H.Y., Le Marrec, C., Heyraud, A., Chambat, G., Lonvaud-Funel, A., 2008. Studies on *gtf*, a glucosyltransferase gene shared by *Pediococcus parvulus* and *Oenococcus oeni*. Applied and Environmental Microbiology 74, 4079–4090.

Dubourdieu, D., Desplanques, C., Villettaz, J.C., Ribereau-Gayon, P., 1985. Investigations of an industrial β-D-glucanase from *Trichoderma harzianum*. Carbohydrate Research 144, 277–287.

Duenas, M., Irastorza, A., Fernandez, K., Bilbao, A., 1995. Heterofermentative *Lactobacilli* causing ropiness in Basque country ciders. Journal of Food Protection 58, 76–80.

Enrique, M., Ibáñez, A., Marcos, J.F., Yuste, M., Martínez, M., Vallés, S., Manzanares, P., 2010. Beta-glucanases as tools for the control of wine spoilage yeasts. Journal of Food Science 75, M41–M45.

Fernandez de Palencia, P., Werning, M.L., Sierra-Filardi, E., Dueñas, M.T., Irastorza, A., Corbí, A.L., López, P., 2009. Probiotic properties of the 2-substituted (1,3)-beta-D-glucan-producing bacterium *Pediococcus parvulus* 2.6. Applied and Environmental Microbiology 75, 4887–4891.

Garai-Ibabe, G., Areizaga, J., Aznar, R., Elizaquivel, P., Prieto, A., Irastorza, A., Dueñas, M.T., 2010. Screening and selection of 2-branched (1,3)-beta-D-glucan producing lactic acid bacteria and exopolysaccharide characterization. Journal of Agricultural and Food Chemistry 58, 6149–6156.

Gerbaux, V., Villa, A., Monamy, C., Bertrand, A., 1997. Use of lysozyme to inhibit malolactic fermentation and to stabilise wine after malolactic fermentation. American Journal of Enology and Viticulture 48, 49–54.

Guzzo, F., Cappello, M.S., Azzolini, M., Tosi, E., Zapparoli, G., 2011. The inhibitory effects of wine phenolics on lysozyme activity against lactic acid bacteria. International Journal of Food Microbiology 148, 184–190.

Ibarburu, I., Aznar, R., Elizaquível, P., García-Quintáns, N., López, P., Munduate, A., Irastorza, A., Dueñas, M.T., 2010. A real-time PCR assay for detection and quantification of 2-branched (1,3)-beta-D-glucan producing lactic acid bacteria in cider. Journal of Food Microbiology 143, 26–31.

Lasanta, C., Roldan, A., Caro, I., Pérez, L., Palacios, V., 2010. Use of lysozyme for the prevention and treatment of heterolactic fermentation in the biological aging of sherry wines. Food Control 21, 1442–1447.

Lin, S.C., Georgiou, G., 2005. A biocatalyst for the removal of sulphite from alcoholic beverages. Biotechnology and Bioengineering 89, 123–127.

Llaubères, R.M., Richard, B., Lonvaud, A., Dubourdieu, D., Fournet, B., 1990. Structure of an exocellular β-D-glucan from *Pediococcus* sp, a wine lactic bacteria. Carbohydrate Research 203, 103–107.

Lonvaud-Funel, A., Joyeux, A., 1988. Une altération bactérienne des vins: la «maladie» des vins filants. Sciences des Aliments 8, 33–49.

Lopez, I., Santamaria, P., Tenorio, C., Garijo, T., Gutierrez, A.R., Lopez, R., 2009. Evaluation of lysozyme to control vinification process and histamine production in Rioja wines. Journal of Microbiology and Biotechnology 19, 1005–1012.

Martínez-Viedma, P., Sobrino-López, A., Ben-Omar, N., Abriouel, H., Lucas-López, R., Martín-Belloso, O., Gálvez, A., 2010. Increased inactivation of exopolysaccharide-producing *Pediococcus parvulus* in apple juice by combined treatment with enterocin AS-48 and high-intensity pulsed electric field. Journal of Food Protection 73, 39–43.

O. I. V., 2011. International Oenological Codex.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A., 2000. Handbook of Enology. Volume 1. The Microbiology of Wine and Vinifications. John Wiley & Sons, LTD., Chichester-New York-Weinheim-Brisbane-Singapore-Toronto.

Stack, H.M., Kearney, N., Stanton, C., Fitzgerald, G.F., Ross, R.P., 2010. Association of beta-glucan endogenous production with increased stress tolerance of intestinal lactobacilli. Applied and Environmental Microbiology 76, 500–507.

Villetaz, J.C., Amado, R., Neukom, H., 1984. The use of a beta-glucanase as an enzyme in wine clarification and filtration. American Journal of Enology and Viticulture 35, 253–256.

Walling, E., Gindreau, E., Lonvaud-Funel, A., 2005. A putative glucan synthase gene dps detected in exopolysaccharide-producing *Pediococcus damnosus* and *Oenococcus oeni* strains isolated from wine and cider. International Journal of Food Microbiology 98, 53–62.

Weber, P., Kratzin, H., Brockow, K., Ring, J., Steinhart, H., Paschke, A., 2009. Lysozyme in wine: a risk evaluation for consumers allergic to hen's egg. Molecular Nutrition & Food Research 53, 1469–1477.

Werning, M.L., Ibarburu, I., Duenas, M.T., Irastorza, A., Navas, J., Lopes, P., 2006. *Pediococcus parvulus gtf* gene encoding the GTF glucosyltransferase and its application for specific PCR detection of β-D-glucan producing bacteria in food and beverages. Journal of Food Protection 69, 161–169.

Werning, M.L., Corrales, M.A., Prieto, A., Fernandez de Palencia, P.F., Navas, J., López, P., 2008. Heterologous expression of a position 2-substituted (1→3)-beta-D-glucan in *Lactococcus lactis*. Applied and Environmental Microbiology 74, 5259–5262.