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Combined cold, acid, ethanol shocks in *Oenococcus oeni*: Effects on membrane fluidity and cell viability

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Abstract

The effects of combined cold, acid and ethanol on the membrane physical state and on the survival of *Oenococcus oeni* were investigated. Membrane fluidity was monitored on intact whole *O. oeni* cells subjected to single and combined cold, acid and ethanol shocks by using fluorescence anisotropy with 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe. Results showed that cold shocks (14 and 8 °C) strongly rigidified plasma membrane but did not affect cell survival. In contrast, ethanol shocks (10–14% v/v) induced instantaneous membrane fluidisation followed by rigidification and resulted in low viability. Acid shocks (pH 4.0 and pH 3.0) exerted a rigidifying effect on membrane without affecting cell viability. Whatever the shock orders, combined cold (14 °C) and ethanol (14% v/v) shocks resulted in strong membrane rigidification. Interestingly, *O. oeni* survived combined cold and ethanol shocks more efficiently than single ethanol shock. Membrane rigidification was induced by ethanol-and-acid (10% v/v - pH 3.5) shock and correlated with total cell death. In contrast, *O. oeni* recovered its viability when subjected to cold (8 °C)-then-ethanol-and-acid shock which strongly rigidified the membrane. Our results suggested a positive short-term effect of combined cold, acid and ethanol shocks on membrane fluidity and viability of *O. oeni*.

Keywords: Combined shock; Membrane fluidity; Cell viability; *Oenococcus oeni*

1. Introduction

The lactic acid bacterium *Oenococcus oeni* is responsible for the malolactic fermentation (MLF), a so-called secondary fermentation which plays a major role in winemaking processes [1,2] because it decreases acidity, enhances organoleptic properties and increases the microbiological stability of wine [2]. This step naturally follows alcoholic fermentation carried out by yeasts. However, the harsh conditions characteristic of wine (low pH, high content of ethanol, variations in cellar temperatures) can hinder bacterial growth in wine. Any delay in the beginning of the MLF can lead to deterioration in wine quality [1]. To overcome this problem, the use of malolactic starters is widely recommended. Therefore, improved knowledge of stress tolerance and adaptation mechanisms of the malolactic bacterium *O. oeni* is essential to improve the efficiency of the malolactic starter in wine.

Performance of microorganisms under stress conditions requires the maintenance of the main function of the cell membrane to control ionic permeability and to regulate the exchange of solutes between the cell and the external environment [3–5]. The barrier properties of the cytoplasmic membrane are of special importance for the energy transduction of the cell and they are known to depend critically upon the physical state of lipid bilayers [6] which is susceptible to changes in external temperature. Indeed, it is widely acknowledged that normal cell function requires the membrane lipid bilayers to be in a liquid-crystalline state at physiological temperatures. At lower temperatures, lipid bilayers undergo a reversible change of state from a disordered to an ordered array of fatty acid chains [6].

The effect of ethanol on the cell membrane is well documented [7–10]. Ethanol affects the physical state and

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biological functions of cell membranes. It interacts with membranes at the lipid–water interface, weakening the hydrophobic barrier to the free exchange of polar molecules, thereby perturbing membrane structure and function [3,9]. In comparison, little work has been carried out to elucidate the physical effect of acidity on biomembranes. A correlation between the physical state of the membrane and proton permeability has been established [11,12], i.e., proton permeability increases with increasing membrane fluidity.

The effects of various combined shocks on the cell membrane have been studied mainly for food safety applications. The combinations of high acidity, high ethanol content, high or low temperatures are applied in order to inhibit or prevent growth of pathogenic microorganisms in food products. For instance, combinations of ethanol and organic acids at low pH have been used as a bactericidal treatment [13,14]. These treatments are more effective at killing pathogenic bacteria because they damage membranes and disrupt the capacity for pH homeostasis [13,14]. In desirable bacteria, very few studies have focused on such combined shocks at the membrane level probably because of the complexity in performing such shocks and in explaining results. The combined effects of osmotic pressure and temperature on the viability of *Saccharomyces cerevisiae* were investigated [15]. Yeast resistance to high osmotic stress can be enhanced when coupled with cold shocks [15].

In this study, we focused on combined shocks applied to the lactic acid bacterium *O. oeni* by changing the application orders of three main shocks involved in winemaking processes namely cold, acid and ethanol shocks. The variations in membrane fluidity monitored by using fluorescence anisotropy with a hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) with regard to cell viability were investigated to gain an insight into the effects of combined shocks on the physical state of cell membranes.

2. Materials and methods

2.1. Strain, medium and growth conditions

Oenococcus oeni ATCC BAA-1163 (formerly *Oenococcus oeni* IOB 8413) was cultured at 30 °C in modified FT80 (mFT80) medium [16] at pH 5.3. Stock cultures (kept frozen at −70 °C) were grown until the stationary phase then diluted 20-fold in the mFT80 medium. After a 24-h incubation period, these precultures were used to inoculate the mFT80 medium at an initial optical density 600 nm (OD_{600}) of 0.1. *O. oeni* cells were harvested in the middle of the exponential growth phase (OD_{600}=0.6–0.8).

2.2. Cell energisation and fluorescence anisotropy measurements

The method for membrane fluidity assays was previously validated [17]. Cell energisation with glucose was necessary to prevent a decrease in membrane fluidity caused by bacterial death independent of the nature of the stress [17]. This step was performed at room temperature. Exponential phase cells (20 ml culture at an OD_{600} of 0.7) were harvested by centrifugation (6300×g, 10 min) and washed once in 20 ml of 50 mM MES (2-[*N*-morpholino]ethanesulfonic acid)-KOH buffer (pH 5.5) containing 10 mM glucose. The cell pellet was resuspended in the same buffer. The OD_{600} of the cell suspension was adjusted to 0.6 in every measurement. The cell suspension was immediately used for fluorescence anisotropy measurements.

Membrane fluidity was determined continuously by measuring fluorescence anisotropy in intact whole cells by using hydrophobic 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes) as a probe. The determinations of fluorescence anisotropy were optimised as follows: a total of 5 μl 1.5 mM DPH solution was added to 2.5 ml of cell suspension (final probe concentration 3.0 μM). Samples were then placed in the stirred and thermostated cuvette holder of the spectrofluorometer (FLUOROLOG-3, Jobin Yvon, Inc. USA) in a T format. The temperature of the samples was measured with a thermometer immersed in the analysed suspension. To ensure probe stabilisation for optimal anisotropy determinations, cells were incubated with the DPH probe in 50 mM MES-KOH buffer, 10 mM glucose, pH 5.5, 30 °C for 15 min before shock and labelled cells were then directly stressed during the measurement.

Excitation and emission wavelengths were 352 and 402 nm, respectively. Anisotropy values (r) were automatically calculated by the spectrofluorometer according to Shinitzky [18]:

$$r = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2.G.I_{VH}} \quad \text{with } G = \frac{I_{HV}}{I_{HH}}$$

$_V$ and $_H$ stand for polarisation direction (vertical and horizontal directions) I: corrected fluorescence intensity de fluorescence obtained by:

$$I_{HH} = I_{(L)HH} - I_{(BUFFER+CELL)HH} - I_{(BUFFER+PROBE)HH} + I_{(BUFFER)HH}$$

$I_{(L)}$: fluorescence intensity of probe-labelled cell suspension; $I_{(BUFFER+CELL)}$: fluorescence intensity of non-labelled cell suspension; $I_{(BUFFER+PROBE)}$: fluorescence intensity of the buffer incubated with the DPH probe; $I_{(BUFFER)}$: fluorescence intensity of the sole buffer. This calculation was repeated for the other three signals (I_{HV}, I_{VV}, I_{VH}).

Fluorescence anisotropy (inversely proportional to membrane fluidity) was measured for 45 min with determinations made every 7 s. Each experiment was performed at least three times from independent cultures.

2.3. Single shocks on intact whole O. oeni cells

The conditions and the amplitudes of shocks tested (various temperatures, pH and ethanol content) were chosen depending on real conditions in winemaking processes [1,2]. For cold shocks, the temperature of cell suspensions was automatically reduced from 30 °C to 20, 17, 14 and 8 °C by the Peltier system. The cooling rate of the cell suspension was set at 3.8±0.4 °C min^{-1}. For ethanol shocks, ethanol (purity 99.8%) was added to the samples under continuous stirring to final concentrations of 10, 12 and 14% (v/v). During cold and ethanol shocks, the pH of the cell suspension was unchanged (pH 5.5). For acid shocks, 2 M HCl solution was added to the samples under continuous stirring to reach final pH values of 3.0, 3.5 and 4.0. During acid shocks, the temperature of the cell suspension was unchanged (30 °C).

2.4. Combined shocks on intact whole O. oeni cells

Ethanol-then-cold shock was performed by combining firstly the single ethanol shock (14% v/v) then 14 °C-cold shock was applied as described above. Cold-then-ethanol shock was carried out by reversing the shock-application order: as soon as the temperature of the cell suspension reached 14 °C, ethanol was added to obtain a final concentration of 14% (v/v). We verified that cell suspensions retained their pH (5.5) during combined cold and ethanol shocks. Pure ethanol and 2 M HCl solution were simultaneously added to the cell suspension to perform ethanol-and-acid shock (10% v/v ethanol, pH 3.5, 30 °C). Cold-then-acid-and-ethanol shock was performed by combining first the single cold shock at 14 °C or 8 °C and then ethanol-and-acid shock.

2.5. Cell viability determinations

Cell viability was determined by using the CFU method, by which cells were plated in mFT80 medium supplemented with 20 g of agar per litre. Cell suspensions were taken at 5 and 30 min during the shock and diluted to appropriate dilutions which were automatically spread on Petri dishes by the

EDDY JET system (IUL SA, Spain). These Petri dishes were then incubated at 30 °C for 6 days. Viability was determined by comparing the numbers of shocked and control *O. oeni* colonies. Each experiment was performed in triplicate.

2.6. Statistical analysis

The significance of the difference among cell viabilities or among fluorescence anisotropy values was determined by a two-tailed Student *t*-test. The confidence interval for a difference in the means was set at 95% ($P \leq 0.05$) for all comparisons.

3. Results

3.1. Effects of single shocks on membrane fluidity and viability of O. oeni

Instantaneous variations in fluorescence anisotropy values of DPH during cold (8, 14, 17, 20 °C); ethanol (10, 12, 14% v/v) and acid (pH 3.0, 4.0) shocks on intact *O. oeni* cells were continuously monitored for a 30-min period. The evolutions of membrane fluidity expressed in anisotropy percentage compared to its initial anisotropy value are shown in Fig. 1.

3.1.1. Effect of cold shock

Cold shock strongly rigidified the membrane of *O. oeni*. Anisotropy levels of *O. oeni* cells increased by 20, 28, 34, and 70% compared to their initial values for cold shock at 20, 17, 14 and 8 °C, respectively (Fig. 1A). The lower the temperature of shock, the more rigid the membrane of *O. oeni* became. The rigidifying effect attributed to cold shock was observed as long as the temperature of the cell suspension remained low. After 30 min of cold shock, when the temperature was raised to the initial one (30 °C), the fluidity membrane of *O. oeni* cells returned to its initial level (data not shown). The reversible effect of cold shock was confirmed. In addition, we verified that changes in membrane fatty acid composition and proteins did not occur after 30 min of cold shocks by fatty acid analysis of *O. oeni* membranes and chloramphenicol treatment of *O. oeni* cells (S Chu-Ky, personal communication). Thus, cold shock only induced physical effects on the membrane.

Cell viability was determined at 5 min and 30 min during the shock in order to assess the impact of cold shock on the physiological state of *O. oeni* (Table 1). We found that all cold shock tested did not affect the viability of *O. oeni* because the bacterium retained almost total viability after 5 and 30 min of any cold shock tested.

3.1.2. Effect of acid shock

Acid shock at pH 3.0 induced a progressive increase in anisotropy values in *O. oeni* cells (Fig. 1B). After 30 min of acid shock, the anisotropy level increased by 30% compared to its initial level. This increase indicated that the membrane was more rigid than its initial state. However, acid shock at pH 4.0 did not induce any significant changes in anisotropy values. After a 30-min period, the pH of the cell suspension was raised to the initial value (pH 5.5). However, anisotropy values did not return to the initial values (data not shown). Acid shock induced an irreversible effect on the membrane of *O. oeni*.

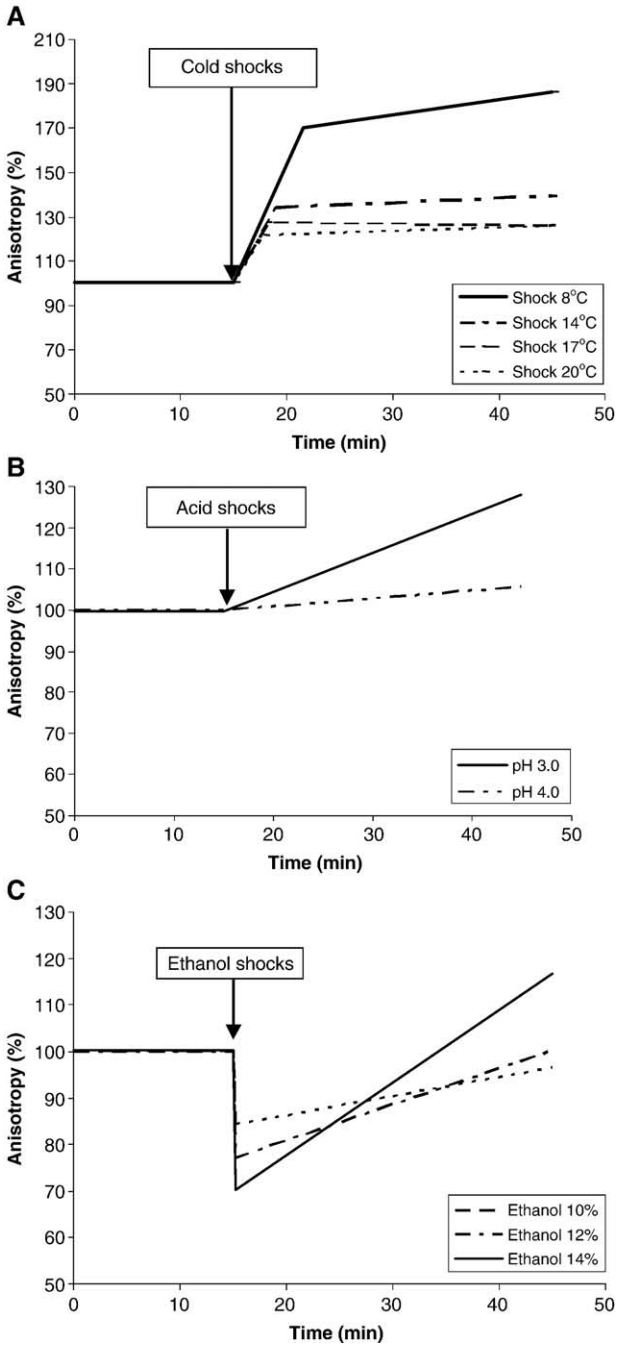

Fig. 1. Evolution of membrane fluidity (anisotropy percentage) in *O. oeni* cells during cold shocks (8, 14, 17, and 20 °C), pH 5.5 (A); acid shocks at pH 3.0 and pH 4.0, 30 °C (B); ethanol shocks 10, 12, and 14% (v/v), 30 °C, pH 5.5 (C). The initial fluorescence anisotropy value (100%) of *O. oeni* cells was equal to 0.115 ± 0.016.

Cell viability was also determined to evaluate the impact of acid shock on the physiological state of *O. oeni* cells (Table 1). Viability was maintained near 100% for the acid shock at pH 4.0. At least 75% cells remained viable after 30 min for the acid shock at pH 3.0.

Table 1
Viability of *O. oeni* cells after 5 min and 30 min of different simple and combined shocks

Shock type	Shock amplitude	Cell viability[a] (%)	
		5 min	30 min
Cold shocks	8 °C	99±21	105±3
	14 °C	94±16	86±10
	17 °C	93±12	91±9
	20 °C	93±13	95±10
Acid shocks	pH 4.0	86±7	98±5
	pH 3.0	87±8	74±9
Ethanol shocks	10% (v/v)	65±12	73±8
	12% (v/v)	77±7	45±6
	14% (v/v)	75±7	43±13
Ethanol-then-cold shock	14% (v/v) ethanol–14 °C	83±13	76±12
Cold-then-ethanol shock	14 °C–14% (v/v) ethanol	69±7	69±9
Acid-and-ethanol shock	pH 3.5–10% (v/v) ethanol	55±12	3±1
Cold-then-acid-and-ethanol shock	14 °C–pH 3.5–10% (v/v) ethanol	87±11	92±7

[a] Percentages of viability of *O. oeni* cells after 5 min and 30 min of different simple and combined shocks±standard deviations.

3.1.3. Effect of ethanol shock

Ethanol shock (10, 12 and 14% [v/v]) resulted in an abrupt decrease, by 16, 23 and 30%, of the initial anisotropy value, indicating an instantaneous increase in membrane fluidity (Fig. 1C). The higher the concentrations of ethanol, the more fluid the membrane became. The fluidising effect induced by ethanol was transitory as the membrane state returned to its initial state after 30-min shocks of 10 and 12% ethanol. However, for 14% ethanol shock, the anisotropy level increased by 20% compared to its initial value after a 30-min shock, indicating membrane rigidification.

The impact of different ethanol shocks on the physiological state of *O. oeni* was also assessed by determining cell viability at 5 min and 30 min during the shock (Table 1), *O. oeni* retained high cell viability (at least 70%) after 5 min of all ethanol shocks tested. However, after a 30-min period, ethanol shocks of 12 and 14% resulted in low viability (45%) whereas higher cell viability (more than 70%) was observed for 10% ethanol shock.

3.2. Effects of combined shocks on membrane fluidity and viability of O. oeni

3.2.1. Effect of cold and ethanol shock

As we had previously observed antagonistic effects induced by cold and ethanol shocks on the membrane and the viability of *O. oeni*, we tested the combination of these two shocks at membrane fluidity level with regard to cell viability (Fig. 2). We performed two combinations. In the first case, ethanol-then-cold shock firstly induced a slight but sharp increase in membrane fluidity (a 9% decrease in anisotropy value) which could be attributed to the fluidising effect of ethanol. Then, we observed an increase in rigidity as if the membrane of *O. oeni* was rigidified by the 14 °C cold shock (Fig. 2A). When we proceeded with cold-then-ethanol shock, the anisotropy values behaved in a way similar to that of ethanol-then-cold shock. After a 30-min period of shock, the temperature was raised to the initial one (30 °C), the anisotropy value decreased to that comparable to its initial level whatever the combination of shocks.

After 30 min of combined shocks, cell viability was significantly higher than that following ethanol shock alone (Table 1). The viability of cells subjected to combined cold and ethanol shocks was at least 70% compared to 40% obtained with cells subjected to the 14% (v/v) ethanol shock alone.

3.2.2. Effect of acid-and-ethanol shock

Ethanol and HCl solution were simultaneously added to the cell suspension to perform acid-and-ethanol shock. Because of instantaneous and high mortality of the combined acid (pH 3.0) and ethanol (14%) shock (data not shown), we reduced the ethanol concentration to 10% (v/v) and raised the pH to 3.5. Acid-and-ethanol shock firstly brought about an abrupt drop (20%) in the anisotropy level, corresponding to instantaneous membrane fluidisation (Fig. 3A). The rigidifying effect of acid did not diminish the fluidising effect of ethanol as the level of anisotropy fell to that induced by the 10% ethanol shock alone. After this transitory fluidisation, the anisotropy value significantly increased to reach nearly 150% of its initial level. This increase indicated an increase in membrane rigidity. Cell

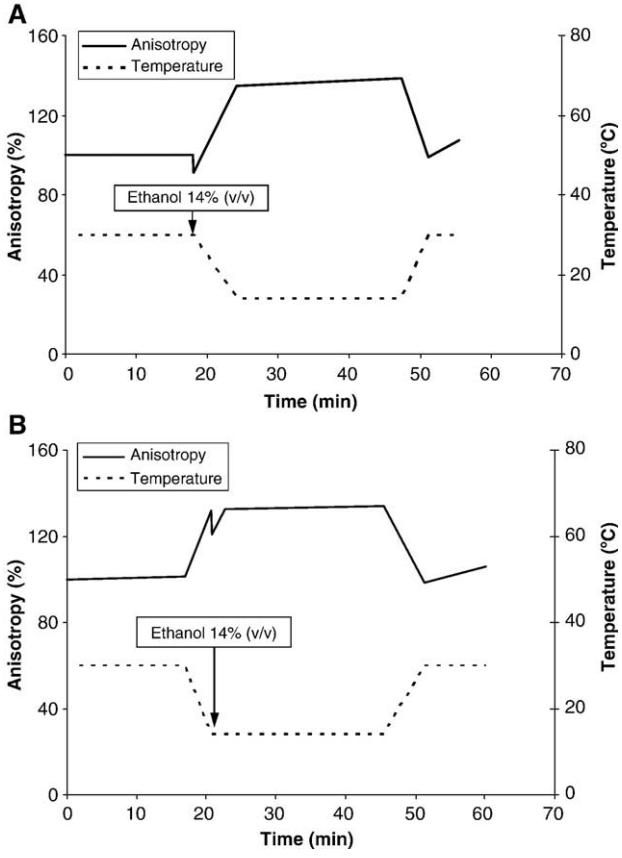

Fig. 2. Evolution of membrane fluidity (anisotropy percentage) in *O. oeni* cells during ethanol-then-cold shock (14% ethanol [v/v] then 14 °C), pH 5.5 (A) and cold-then-ethanol shock (14 °C then 14% ethanol [v/v]), pH 5.5 (B).

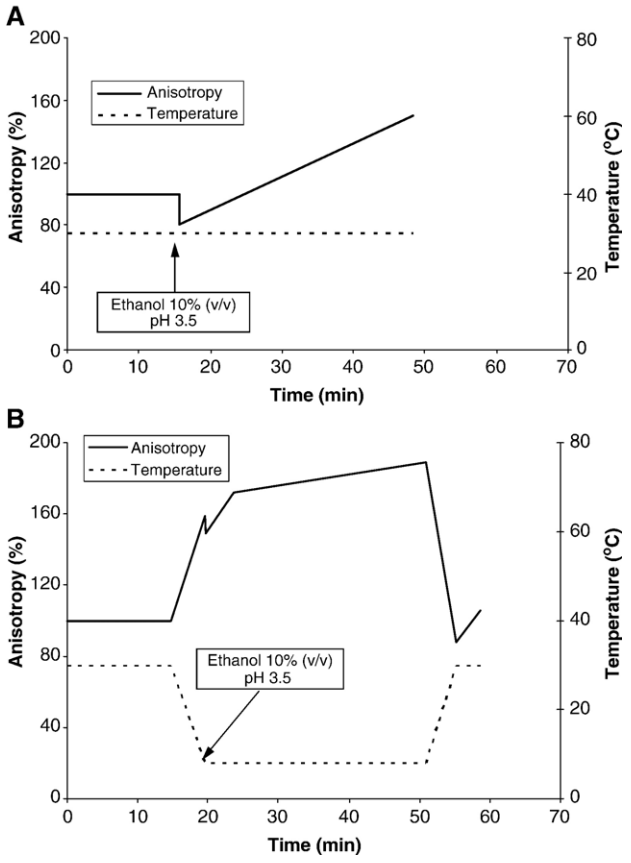

Fig. 3. Evolution of membrane fluidity (anisotropy percentage) in *O. oeni* cells during acid-and-ethanol shock (30 °C, pH 3.5 and 10% ethanol [v/v]) (A); and during cold then-acid-and-ethanol shock (8 °C, pH 3.5 and 10% ethanol [v/v]) (B).

viability fell to 55% after only 5 min of shock and total cell death was observed after 30 min of shock (Table 1).

3.2.3. *Effect of cold shock over acid-and-ethanol shock*

As observed above, cold shock was shown to cancel out the negative effects of ethanol shock. We tested whether cold shock was capable of counteracting the deleterious effect of acid-and-ethanol shock. When 14 °C-cold shock was applied just before acid-and-ethanol, we did not obtain any improvement in cell viability compared to that after 30 min of acid-and-ethanol shock (data not shown). In this case, we suggest that the amplitude of 14 °C cold-shock was not high enough to counterbalance the deleterious effect induced by acid-and-ethanol shock. Therefore, we tested cold shock at a higher amplitude (8 °C), while the acid-and-ethanol shock remained unchanged. As expected, cell viability was restored not only after 5 min but also after 30 min of cold-then-acid-and-ethanol shock (Table 1). With respect to membrane fluidity, anisotropy values evolved in a similar way to those for cold-then-ethanol but with higher membrane rigidification (Fig. 3B). Cold shock at 8 °C also cancelled out acid-and-ethanol shock. After 30 min of shock, when the temperature of the cell suspension was raised to 30 °C, the anisotropy level of the *O. oeni* membrane returned to its initial value.

4. Discussion

Various techniques using fluorescence probes have been used to study the physical state of membranes in term of fluidity [17,19–21]. The DPH probe is widely used in such studies because it partitions very favourably to the membrane interior, it has intense fluorescence, does not appear to bind to proteins and it is sensitive to the physical state of the membrane [17,22]. We first focused our investigations on the kinetics of variations in membrane fluidity by instantaneously monitoring the impact of shock induced by three main physical parameters involved in vinification: cold, acid and ethanol.

The physical effects of ethanol on the membrane have been well documented in the literature [3,7,9,23]. The fluidity of the cytoplasmic membrane in *O. oeni* cells instantaneously increased when ethanol was added. This result is in agreement with other studies reporting that ethanol fluidises biomembranes [7,9,24]. The ethanol-induced fluidisation of membrane lipids observed could be due to the interfacial binding of ethanol that is capable of disordering the entire length of the acyl chains [9]. Exposure to ethanol also results in increased permeability of the cytoplasmic membrane, therefore, enhancing passive proton influx and concomitant loss of intracellular material [10]. This may partially account for very low cell viability observed in cells shocked at 14% (v/v) ethanol. Nonetheless, this fluidising effect was transitory and was followed by membrane rigidification which may be explained

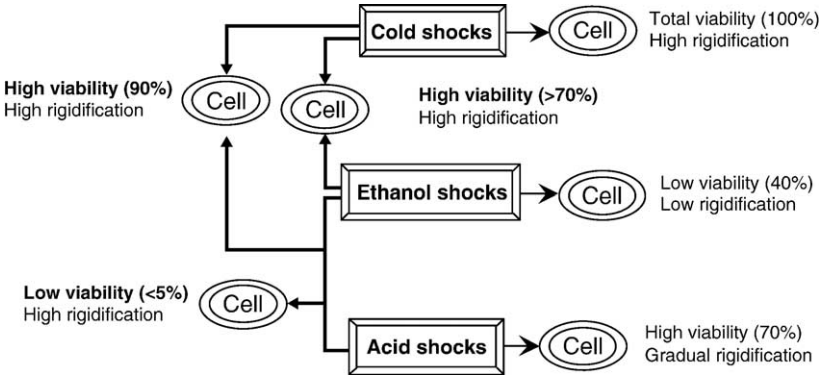

Fig. 4. Schematic representation of effects of single and combined cold, ethanol and acid shocks on the physical state of the membrane and on the cell viability of *O. oeni*.

by the *cis*-to-*trans* isomerisation of monounsaturated fatty acids in bacterial lipid bilayers [25]. This short-term modification allows cells to increase membrane rigidity in response to the fluidising effect of ethanol. For ethanol shock at 10 and 12% (v/v), cells retained the anisotropy level after 30 min of shock which correlated with high viability. On the contrary, for 14% (v/v)-ethanol shock, the final membrane state was much more rigid than its initial state. This rigidity may result from strong disorganisation of the membrane and also explain the very high cell mortality observed.

We showed in this study that cold shock can counterbalance the deleterious effect of ethanol by combining cold and ethanol shocks. *O. oeni* cells responded to cold shock by significantly increasing the rigidity of the membrane. At physiological temperatures, rotational isomerisation about the C– C bonds of the fatty acyl chains results in the formation of cis isomers producing kinks in the chains. When thermal agitation is weak at a sufficiently low temperature, the fatty acyl chains are fully extended in an all-trans configuration, lipid chains are thus in a parallel position and in a narrow contact with each other [26], resulting in increased membrane rigidity. Membrane lipid bilayers are therefore transformed from disordered to ordered state [27]. Thus, the cold-induced rigid membrane prevented an increase in permeability induced by ethanol, therefore diminishing passive proton influx and loss of intracellular materials. In this case, bacterial cells remained viable. A combination of ethanol and cold shock has been shown to provide *Escherichia coli* with high viability [28]. In addition, the rigidifying effect of cold shock was shown to be reversible in combined cold and ethanol shocks. When the effect of cold shock was removed by returning the temperature to the initial one, the anisotropy level also restored to the initial level, suggesting in this case a cancel of the fluidising effect of ethanol.

More interestingly, cold shock was shown to be capable of counteracting the combined acid-and-ethanol shock which was very deleterious to the bacterial cell. Combined ethanol and acid dramatically slow down growth of *O. oeni* [29] but these two shocks seem to have a slight influence in malic acid metabolism [30]. The increased membrane fluidity induced by ethanol can result in increased membrane permeability which in turn promotes stronger proton influx and loss of essential metabolites. These changes lead to a dissipation of the proton motive force resulting in less effective energy transduction [23]. The non-specific increase in plasma membrane permeability also affects its function as a selective barrier, which could account for very low viability observed in this study even after 5 min of shock. Combinations of low pH and ethanol have proved to be particularly effective bactericidal treatments because of cytoplasmic membrane damage and disruption of the capacity for pH homeostasis [13,14]. In terms of fluidity, the membrane was fluidised because of the instantaneous effect of ethanol. The strong perturbation caused by combined ethanol and acid shock induced strong membrane rigidification which indicates the highly disorganised state of the cell membrane. We suggested that protein denaturation could lead to this highly disorganised state, resulting in the high cell mortality observed. We showed in this study that the membrane of *O. oeni* cells was pre-adapted by changing from a disordered to a much more ordered state when subjected to cold shock at 8 °C. This rigid state of the membrane could reduce the fluidising effect of ethanol so that the synergistically deleterious effect of acidity and ethanol may be minimised.

Fluorescence anisotropy is a useful tool to investigate the behaviour of *O. oeni* cells subjected to multiple stresses. This study points out the effects of three main shocks involved in the winemaking process. Cold shocks strongly rigidified the cell membrane without affecting bacterial survival. In contrast, ethanol shock induced instantaneous and transitory membrane fluidisation and resulted in very low viability. Acid shock exerted a gradual rigidifying effect on cell membranes without affecting viability. Moreover, our approach gives insights into the synergetic and antagonistic effects of combined cold, ethanol and acid shocks on the physical state of the membrane and on the cell viability of the wine bacterium *O. oeni* (the results are summarised in Fig. 4). We have shown that cold shock played a major role in counterbalancing the deleterious effects induced by both ethanol shock and combined acid and ethanol shocks whatever the application order of shocks in *O. oeni*. Cold shock reserved viability in *O. oeni* in the hostile environments characteristic of wine. We propose here an original approach to performing combined shocks in order to minimise the effects of deleterious shocks on the viability of *O. oeni* malolactic starters.

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