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Physiological functions at single-cell level of *Lactobacillus* spp. isolated from traditionally fermented cabbage in response to different pH conditions

Magdalena A. Olszewska*, Aleksandra M. Kocot, Łucja Łaniewska-Trokenheim

Chair of Industrial and Food Microbiology, Faculty of Food Science, University of Warmia and Mazury in Olsztyn, Plac Cieszyński 1, 10-726 Olsztyn, Poland

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ABSTRACT

Changes in pH are significant environmental stresses that may be encountered by lactobacilli during fermentation processes or passage through the gastrointestinal tract. Here, we report the cell response of *Lactobacillus* spp. isolated from traditionally fermented cabbage subjected to acid/alkaline treatments at pH 2.5, 7.4 and 8.1, which represented pH conditions of the gastrointestinal tract. Among six isolates, four species of *Lactobacillus plantarum* and two of *Lactobacillus brevis* were identified by fluorescence *in situ* hybridization (FISH). The fluorescence-based strategy of combining carboxyfluorescein diacetate (CFDA) and propidium iodine (PI) into a dual-staining assay was used together with epifluorescence microscopy (EFM) and flow cytometry (FCM) for viability assessment. The results showed that the cells maintained esterase activity and membrane integrity at pH 8.1 and 7.4. There was also no loss of culturability as shown by plate counts. In contrast, the majority of 2.5 pH-treated cells had a low extent of esterase activity, and experienced membrane perturbation. For these samples, an extensive loss of culturability was demonstrated. Comparison of the results of an *in situ* assessment with that of the conventional culturing method has revealed that although part of the stressed population was unable to grow on the growth media, it was deemed viable using a CFDA/PI assay. However, there was no significant change in the cell morphology among pH-treated lactobacilli populations. These analyses are expected to be useful in understanding the cell response of *Lactobacillus* strains to pH stress and may facilitate future investigation into functional and industrial aspects of this response.

1. Introduction

Lactobacilli are considered to be probiotic potential microorganisms. In fact, some *Lactobacillus* spp. have a confirmed probiotic status (Gbassi et al., 2011). The species, such as *Lactobacillus plantarum* and *Lactobacillus brevis*, are a small part of the autochthonous microbiota of raw vegetables and have been found to occur in the spontaneous fermentation of olives, cabbages, cucumbers, eggplants, and caper berries (Rodríguez et al., 2009). The production of such fermented foods aims at improving these products' nutritional, therapeutic and prophylactic properties, only if certain criteria will be fulfilled (Bunthof et al., 2001; Rodríguez et al., 2009). One of the requirements is resistance of bacteria to the food processing system in which they are employed, and the other is their persistence to stress in the gastrointestinal tract in order to perform health-promoting effects (Kristo et al., 2003). Evaluation of lactobacilli viability in response to pH- and bile salts-induced stress along their passage through gastrointestinal tract is therefore of great importance.

Acid-alkaline stresses are found to be significant environmental conditions that affect lactic acid bacteria (LAB) growth and survival (Bunthof et al., 2001). It is recognized that LAB are able to regulate the pH_i and overcome acid stress by the action of F_1F_0-ATPase proton pumps, the ADI (arginine deiminase) pathway, NH_3 production, the GAD (glutamate decarboxylase) system, and a mechanism known as the acid-tolerance response (ATR), as well as production of acid shock proteins (ASPs) (Cotter and Hill, 2003; De Angelis and Gobbetti, 2004). On the other hand, it was found that LAB may counteract alkaline stress by pumping protons into the cells *via* ATP synthase and Na^+/H^+ antiporters. It was identified that a variety of proteins are up- and down-regulated as a result of alkaline stress, *e.g.*, ATP-dependent protease (ClpB) (Lee et al., 2011). Bile salts are, in turn, inhibiting factors that strongly affect cell metabolic processes, alter the lipid profiles of cell membranes and also influence

* Corresponding author. Tel.: +48 89 523 37 29; fax: +48 89 523 46 15.
E-mail address: magdalena.olszewska@uwm.edu.pl (M.A. Olszewska).

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the shape of cells (Perrin et al., 2000; Zavaglia Gómez et al., 2002). In response to adverse effects induced by bile salts, bacteria have developed a protective mechanism known as bile salt hydrolase (BSH) (Ferrari et al., 1980). BSH, upon deconjugation of bile salts, releases bile acids, probably resulting in a less detrimental effect on cells as compared to bile salts themselves (Gopal-Srivastava and Hylemon, 1988; Gunn, 2000; Klaver and van der Meer, 1993).

Apart from these studies, we still know too little about lactobacilli viability with respect to cell functionality upon stress. Fluorescent labelling approaches for viability evaluation at the single cell level are thus desired where certain physiological functions can be measured (Bunthof et al., 2001). Various fluorescent probes in combination with fluorescence microscopy (EFM) and flow cytometry (FCM) are used. The different probes are employed to assess different physiological functions of bacteria, *e.g.*, membrane potential probes such as Rh123, $DiOC_6(3)$, $DiBAC_4(3)$, membrane integrity probes such as SYTO-9, SYTO-13, propidium iodide (PI), TOTO-1, Sytox Green, dehydrogenase activity and esterase activity labels: CTC and FDA, CFDA, CFDA-AM, BCECF-AM, Calcein-AM, ChemChrome, respectively (Joux and Lebaron, 2000). Fluorescent dyes, CFDA and PI were applied as viability indicators of *Lactobacillus rhamnosus* by Ananta et al. (2004), who studied the response of lactobacilli to high pressure-induced stress. Moreover, Sunny-Roberts et al. (2007) and Sunny-Roberts and Knorr (2008), using a CFDA and PI fluorescence approach, studied the same species under trehalose and sucrose-induced stress, respectively. They revealed that the high hydrostatic pressure and sugars exerted different effects on the physiological and metabolic status of *L. rhamnosus*. It was found that the pressure deactivated cells were still in possession of enzyme activity and not completely membrane compromised, but scored as nonculturable according to the results of the cultivation method. On the other hand, none of the sugars caused a significant culturability loss, but differences in the metabolic and physiological behaviour of cells subjected to osmotic stress were observed. Preservation of membrane integrity and enzymatic activity was recorded in response to sucrose-induced stress and a low degree of membrane permeabilization was shown for trehalose-treated cells, pointing out their importance in sugar-rich foods, in which exposure to high osmotic pressure is expected. Bunthof et al. (2001) analysed populations of *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Pediococcus* species under pH and bile salt stress using CFDA, PI and TOTO-1 probes. They demonstrated that the combination of CFDA and TOTO-1 makes the best live/dead assay with lactic acid bacteria and found that at pH 2 and in the presence of 0.25% of deconjugated bile salts there were hardly any surviving cells, which was in contrast to the high extent of membrane-compromised cells. Papadimitriou et al. (2006) assessed the physiological status of *Streptococcus macedonicus* and its response to acid stress with $DiBAC_4(3)$, PI and CFDA probes. The physiological studies of the stressed cells revealed the existence of three subpopulations in accordance with their fluorescence profile and culturability, representing intact/culturable, permeabilized/dead and injured cells that exhibited both metabolic activity and membrane permeabilization as well as decreased culturability. All of these studies have confirmed the usefulness of fluorescent probes in assessing the cell functionality of lactic acid bacteria under certain stress conditions, and a developed EFM and FCM live/dead assay is also applied in this study.

The aim of our investigation was to evaluate the viability of *Lactobacillus* spp. isolated from traditionally fermented cabbage under different pH conditions. For this purpose, fluorescence *in situ* hybridization (FISH) was applied for lactobacilli identification. The pH levels of 2.5, 7.4 and 8.1 represented the possible pH conditions of the gastrointestinal tract. For in-depth studies of physiological state under stress conditions, CFDA and PI (carboxyfluorescein diacetate/propidium iodide) fluorescent staining combined with epifluorescence microscopy and flow cytometry was introduced. Plate counting was applied for cell culturability assessment and comparison.

2. Materials and methods

2.1. Isolation and pH treatments of bacterial strains

Strains of *Lactobacillus* spp. used in this study were isolated from sour cabbage obtained from domestic producers from different regions in Poland. The isolation procedure was followed by streaking sour cabbage juice on MRS-agar (Merck, Darmstadt, Germany) and anaerobic incubation (Anaerocult C; Merck, Darmstadt, Germany) at 30 °C for 48 h. The representative colonies of lactic acid bacteria were further used for lactobacilli identification. The identified *Lactobacillus* strains were stored as Microbank™ (Biocorp, Warsaw, Poland) bead cultures at −20 °C. These cultures were later used to propagate bacterial strains by transferring beads into MRS broth (Merck, Darmstadt, Germany) and overnight incubation at 30 °C. The MRS broth was further used to inoculate final broths of different pH (pH of 8.1, 7.4 adjusted with NaOH and 2.5 with hydrochloric acid, Avantor, Gliwice, Poland) with a bacterial culture of optical density [OD_{600}] = 0.1. The pH levels of 2.5, 7.4 and 8.1 represent the pH conditions of the stomach, saliva and intestine, respectively. Treatments were carried out at 30 °C over a period of 1 h, spun down (13,000 × *g* for 3 min, MPW 65R, Med. Instruments, Warsaw, Poland) and re-suspended in phosphate-buffered saline (PBS) at pH 7.4 (130 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4; Sigma–Aldrich, Poznan, Poland), and put on ice until analysis. The results shown are representatives of three replicate trials.

2.2. Lactobacilli identification with FISH

Identification of the *Lactobacillus* spp. strains was carried out with fluorescence *in situ* hybridization (FISH) according to Blasco et al. (2003) with own modifications. The hybridization procedure was performed using rRNA oligonucleotide probes fluorescently labelled at –5′ end with 6-FAM, Cy3 or Cy5 and listed in Table 1, purchased from Bionovo (Legnica, Poland). Prior to hybridization, 500 μl of bacterial cultures were centrifuged at 13,000 × *g* for 3 min (MPW 65R, Med. Instruments, Warsaw, Poland), and pellets were fixed by adding a 1:1 mixture of phosphate-buffered saline (PBS) at pH 7.4 (130 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4; Sigma–Aldrich, Poznan, Poland) and 96% (v/v) ice-cold ethanol (≥99.8%, Merck, Darmstadt, Germany), and stored at −20 °C for 30 min. After fixation, the cells were centrifuged at 13,000 × *g* for 3 min (MPW 65R, Med. Instruments, Warsaw, Poland) and suspended in 200 μl of a 10 mg/ml lysozyme solution (Merck, Darmstadt, Germany) in a 100 mM Tris–HCl, 50 mM EDTA (pH 8.0) buffer. The lysozyme permeabilization was carried out for 15 min at 37 °C. After that, the cells were washed with PBS solution and spotted on polycarbonate filters Ø 13 mm, 0.22 μm (Isopore, Millipore, Darmstadt, Germany). Air-dried filters were placed on glass slides and covered with 20 μl of the hybridization solution, composed of 18 μl of hybridization buffer (20 mM Tris–HCl pH 7.2, Sigma–Aldrich, Poznan, Poland; 0.9 M NaCl, Stanlab, Lublin, Poland; 0.01% sodium dodecyl sulphate, SDS, Sigma–Aldrich, Poznan, Poland) and 2 μl of a 50 ng/μl fluorescent oligonucleotide probe solution. Then, they were transferred into a hybridization chamber, equilibrated with the hybridization buffer as described above and incubated at 43 °C for 4 h (GFL Incubator, Burgwedel, Germany). After the hybridization step, the filters were transferred to Falcon tubes containing a pre-warmed (to 48 °C) washing solution (20 mM Tris–HCl pH 7.2, 0.9 M NaCl, 0.01% SDS) and freely incubated floating at 48 °C for 20 min (GFL Incubator). After the washing step, the air-dried filters were stained