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Cryobiology 50 (2005) 294–307
CRYOBIOLOGY
www.elsevier.com/locate/ycryo

Influence of cooling temperature and duration on cold adaptation of *Lactobacillus acidophilus* RD758 ☆

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Received 10 November 2004; accepted 1 March 2005
Available online 18 April 2005

Abstract

The effect of different cooling temperatures and durations on resistance to freezing and to frozen storage at −20 °C in *Lactobacillus acidophilus* RD758 was studied, by using a central composite rotatable design. A cold adaptation was observed when the cells were maintained at moderate temperature (26 °C) for a long time (8 h) before being cooled to the final temperature of 15 °C. These conditions led to a low rate of loss in acidification activity during frozen storage (0.64 min day^{-1}) and a high residual acidification activity after 180 days of frozen storage (1011 min). The experimental design allowed us to determine optimal cooling conditions, which were established at 28 °C during 8 h. Adaptation to cold temperatures was related to an increase in the unsaturated to saturated fatty acid ratio and in the relative cycC19:0 fatty acid concentration. Moreover, an increased synthesis of four specific proteins was observed as an adaptive response to the optimal cooling conditions. They included the stress protein ATP-dependent ClpP and two cold induced proteins: pyruvate kinase and a putative glycoprotein endopeptidase.

Keywords: Lactic acid bacteria; Probiotic bacteria; Cooling; Temperature; Duration; Cryotolerance; Fatty acid composition; Proteome

Lactic acid and probiotic bacteria are often subjected to stress conditions during industrial processing and preservation. These conditions are detrimental to their physiological state, and hence to the quality of starters [19]. Among them, cold stresses appear during the cooling and freezing steps, and during frozen storage. They lead to a loss in the viability and activity of starters [12,25].

Following these stresses, adaptive physiological responses have been observed in probiotic and lactic acid bacteria. The first adaptive response consists of changes in the fatty acid composition of

☆ This work was funded by institutional sources.
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doi:10.1016/j.cryobiol.2005.03.001

cellular membranes, which modulate the membrane permeability. An increase in the unsaturated fatty acid content decreases the solid-to-fluid transition temperature and then, maintains membrane fluidity during cold stress [31]. A higher content in unsaturated fatty acids was related to a better cryotolerance in *Streptococcus thermophilus* [3]. In addition, the synthesis of some specific fatty acids plays a major role in stress response. The C16:0 fatty acid concentration was found to increase in response to low temperature in *Lactobacillus acidophilus* [10,40]. The C18:1 fatty acid content increases during adaptation to low temperatures in *Lactobacillus plantarum* [32]. A high cycC19:0 concentration favours the cryotolerance of *Lactobacillus bulgaricus* [35], *Lactobacillus helveticus*, and *Lb. acidophilus* [15]. This higher synthesis of the cycC19:0 fatty acid allowed the membrane fluidity to be maintained [15,37].

Synthesis of cold shock proteins is the second adaptive response to cold stress. These proteins are able to bind to RNA in a cooperative manner and, for some of them, to function as RNA chaperones, thus facilitating the translation process under low positive temperatures [16]. They also act to desaturate membrane fatty acids. In *Lactococcus lactis* subsp. *lactis*, 12 proteins were oversynthesised up to threefold at 8 °C, as compared to 30 °C [24]. In *S. thermophilus*, four non-constitutive proteins were induced at 15 °C [28], and six proteins were maximally synthesised after a temperature shift to 20 °C [43]. A family of cold shock proteins (CspA–CspE), characterised by a molecular weight of ~7 kDa, has been identified and described. Among them, the overproduction of CspB and CspE resulted in an increased survival of *Lc. lactis* after freezing [42], and the overproduction of CspP in *Lb. plantarum* led to an enhanced survival after freezing [9]. Finally, 10 cold acclimation proteins were produced in *Enterococcus faecalis* during growth at 8 °C [23]. Five of them were distinct from already known cold shock proteins.

These two kinds of physiological adaptations, which appear after cold stress, may be induced during frozen starter production, to improve the cellular cryotolerance. For example, growing the bacteria at a low temperature or a low pH improved their resistance to freezing and frozen storage [3,10,40]. Nevertheless, these methods led to low cell concentrations at the end of the fermentation step, which limits their interest for starter production.

Other adaptations may take place after the fermentation step. Incubating *Lc. lactis* at 8 °C for 48 h after culture led to an enhanced survival to freezing (95%) [27]. In *Lb. bulgaricus*, cooling the cells at 30 °C for 1 h led to 80% survival after freezing [8]. *Lc. lactis* cells were more resistant to freezing, after a treatment at a sublethal temperature of 10 °C for 4 h [21] or for 2 h [5]. A cold shock at 20 °C during 4 h, applied to *S. thermophilus*, increased 10^3 times the survival rate after freezing [43]. Finally, low temperatures (4 °C) during the cryoprotection step led to a better recovery of acidification activity in *Lb. bulgaricus* and *S. thermophilus* after freezing [12]. However, an adaptation temperature below the minimum growth temperature of 15 °C did not result in an effective cold adaptation in *S. thermophilus* [43].

These observations suggest that an effective cold adaptation taking place after the culture step may be of interest to reduce the loss in viability and activity during starter preservation. Nevertheless, results vary according to the authors and the strains studied, from 4 to 30 °C and during 1–48 h. Moreover, most of the studies have been conducted so far with cells recovered at acidic pH, which leads to confusion between the effects of cold stress and acid stress. As a consequence, determining the optimal cold adaptation conditions that have to be used to obtain a good cryotolerance of lactic acid bacteria is of great interest. This study was made to adapt *Lb. acidophilus* RD758 to freezing and frozen storage, by applying different temperature and duration conditions during the cooling step that follows fermentation. Our aim was to analyse the membrane fatty acid composition and the proteome of adapted cells to gain a better insight into the cellular adaptive responses.

Materials and methods

Bacterial strain and media

Frozen aliquots of *Lb. acidophilus* RD758 (Danisco, Dangé-Saint-Romain, France) were