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Viability of *Lactobacillus acidophilus* NCFM Howaru Dophilus during storage at refrigeration temperatures

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Abstract: A strain *Lactobacillus acidophilus* NCFM Howaru Dophilus is a probiotic bacterium available in dairy products and dietary supplements since 1970s. Its positive health effects have been proved by many studies. This work deals with the examination of NCFM strain's viability during its storage at stable and unstable temperature in MRS broth and ultra-pasteurized milk. In nutritionally rich environment, *Lb. acidophilus* NCFM was able to survive and to metabolize the media. The relevant decrease of viable cells was observed in MRS broth about 30 days after inoculation, and in milk after 21 to 45 days at both stable and unstable temperatures, respectively. The average rate of decrease of viable cells was approximately two to three times higher in experiments at unstable temperature ($Gr_{MRS,unst}$ = –0.149 log CFU.ml^{-1}.d^{-1} in MRS broth, $Gr_{milk,unst}$ = 0.030 log CFU.ml^{-1}.d^{-1} in milk) compared with that at stable temperature ($Gr_{MRS,st}$ = –0.079 log CFU.ml^{-1}.d^{-1} in MRS broth, $Gr_{milk,st}$ = 0.009 log CFU.ml^{-1}.d^{-1} in milk). In the MRS broth exhausted by overnight cultivation of NCFM strain the decrease of viable cells started practically immediately ($Gr_{overnigh,unst}$ = –0.137 log CFU.ml^{-1}.d^{-1}). Maintenance of the culture in milk at stable temperature was proved to be the most appropriate form of its storage.

Keywords: *Lactobacillus acidophilus* NCFM, survival, predictive microbiology

Introduction

Many intrinsic and extrinsic factors including temperature and media composition affect the growth and metabolism of microorganisms. However, "the growth of bacterial cultures generally obeys relatively simple laws" (Ross and McMeekin, 1994) and the reactions of bacterial populations to environmental factors are reproducible and they are basis of predictive microbiology. The predictive microbiology would not only enable to focus on foodborne pathogens but also to predict the behavior of lactic acid bacteria during fermentation and storage of dairy products. Since the temperature is one of the most important factors in the microorganism growth, in control of bioprocesses and safe handling in food industry, many predictive models describe the effect of temperature on the microbial growth parameters. From them, the models introduced by Baranyi et al. (1993), Rosso et al. (1993), Gibson et al. (1994) and Daughtry et al. (1997) were used in this work and we will come to them later.

A bacterial culture *Lactobacillus acidophilus* NCFM Howaru Dophilus has been used as a common component of conventional dairy products and dietary supplements since the seventies of the 20th century (Sanders and Klaenhammer, 2001). The probiotic strain NCFM (Howaru Dophilus) was isolated from a human gastrointestinal tract. Its taxonomic status has been confirmed by using phenotypic and genotypic techniques, including carbohydrate fermentation tests, whole genome sequencing, 16S rRNA gene sequence analysis and hybridization to a species-specific probe as a type A1 *Lb. acidophilus* (Sanders and Klaenhammer, 2001). The ratio of G+C content of the A1 lactobacilli group is generally about 35 %, in the case of NCFM Howaru the ratio of G+C content is 38.4 %. Similarly to related species of the genus *Lactobacillus*, it lacks biosynthetic capacity for most amino acids, vitamins and cofactors. On the other hand, it is able to encode a wide range of transport and fermentation systems (Altermann et al., 2005), including a unique transport system — L-aspartate-L-alanine exchanger forming a proton-motive force across the bacterial membrane, and a formyl coenzyme A transferase gene (*frc*) with an oxalyl coenzyme A decarboxylase gene (*oxc*) whose expression specifically improves its survival in the presence of oxalic acid (Azcarate-Peril et al., 2006, Altermann et al., 2011).

The EFSA Panel on Dietetic Products, Nutrition and Allergies in its recent Scientific Opinion (EFSA, 2011) confirmed that *Lb. acidophilus* NCFM Howaru Dophilus helps to strengthen the natural defenses of a human body and contributes to enhance body's resistance against infectious agents. The strain is able to prevent a bacterial infection through the inhibition of pathogens and competing for nutrients and/or binding sites on epithelial cells and by production of specific and/or nonspecific metabolites. The studies using co-cultures of *Lb. acidophilus*

NCFM with various foodborne disease agents have indicated an antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Salmonella* Typhimurium and *Clostridium perfringens* (Dobson et al., 2007, Collado et al., 2007, Kim and Mylonakis, 2012).

Several studies also proved an ability of NCFM strain to survive in a human gastrointestinal tract (Mäkeläinen et al., 2009; Lahtinen et al., 2012). For enhancement the gut health of patients suffering from the functional bowel disorders, diarrhoea and food intolerance, dietary supplements containing the studied strain are used. It was endorsed that the supplement of diet with the combination of *Lb. acidophilus* NCFM and *Bifidobacterium lactis* Bi-07 significantly reduced volunteers' symptoms of bloating, distension and nausea (Ringel-Kulka et al., 2011). During supplementary diet with Howaru combined with lactitol it was determined that the fecal *Lb. acidophilus* NCFM levels were positively correlated with the prostaglandin E2 (PGE2) concentrations. PGE2s increase has been suggested to be associated with an improvement of physiological gastrointestinal functions including motility and cytoprotection against NSAID-induced injury in elderly patients (Ouwehand et al., 2009, Björklund et al., 2012). An immunomodulatory capacity of the strain was not considered as negligible. The cytotoxicity of NK cells was significantly increased by consumption of the probiotic cheese containing *Lb. rhamnosus* HN001 and *Lb. acidophilus* NCFM. Daily intake of probiotics enhanced parameters of innate immunity in elderly volunteers (Ibrahim et al., 2010). Gad et al. (2011) determined a correlation between the consumption of NCFM strain and the levels of IL-10 and IL-12. This effect was related to the interaction of the bacteria with the host dendritic cells and the consequent regulation of their immune functions (Konstantinov et al., 2008). It is necessary to note that stimulation of cytokines production is not a beneficial physiological effect per se and it needs to be linked to a corresponding clinical outcome (EFSA, 2011). Moreover, consumption of probiotic *Lb. acidophilus* NCFM resulted in the retardation of the growth of tumor volume and the enhancement of the apoptosis of tumor cells (Chen et al., 2012). The cells of this strain are reported to adhere to the HT-29 and Caco-2 cells derived from colon carcinoma (Goh and Klaenhammer, 2010).

Despite the fact, the culture has been characterized by a large number of in vitro and in vivo studies, on the field of scientific interest, data describing its ability to survive in commonly available dietary product are not generally available. That is why this work deals with the quantification of temperature and media composition effect on the survival and metabolism of *Lb. acidophilus* NCFM Howaru Dophilus in a real and an artificial growth medium.

Experimental

Materials and Methods

Microorganism

The strain *Lactobacillus acidophilus* NCFM (Howaru Dophilus) of Danisco A/S (Copenhagen, Denmark) was provided for our study by Rajo, a.s. (Bratislava, Slovak Republic). Identification and the monoculture composition of the culture was confirmed by the Gram staining and microscopic examination, by the API 50CHL test (BioMeriuéx, Marcy-l'Étoile, France) and by the PCR analysis of the 16S rRNA gene presence.

Inoculation and cultivation conditions

The strain of *Lb. acidophilus* NCFM was kept in MRS broth (Biokar Diagnostics, Beauvais, France) at 5 ±1 °C. The standard suspension of the microorganism was prepared from a 24 h old culture of *Lb. acidophilus* NCFM grown in the MRS broth at 37 °C and 5 % CO_2. This culture was inoculated into 300 ml of pre-tempered ultra-pasteurized (UHT) milk (Rajo, Bratislava, Slovak Republic) or into the MRS broth (Biokar Diagnostics, Beauvais, France) in concentration of approximately 10^8 CFU. ml^{-1} (colony forming unit per milliliter). Three parallel samples of model media were incubated aerobically without shaking at stable (t_{st} = 6 ± 0.5 °C) or at unstable temperature (t_{unst} ~ 6—9 °C) in a refrigerator. For the examination of strain's survival after overnight incubation (24 h, 37 °C, 5 % CO_2), the strain was kept in 10 ml of the original medium and incubated in the refrigerator.

Determination of Lb. acidophilus NCFM in milk and in MRS broth

In relevant time intervals required amounts of *Lb. acidophilus* NCFM were taken to determine the actual microorganism density on the MRS agar (Biokar Diagnostics, Beauvais, France) according to ISO 20128:2006. Typical colonies were white, round to oblate, convex and morphology of bacterial cells was sporadically confirmed by microscopic examination.

Determination of active acidity

At the same time as the microbiological determinations were determined, the sample pH values were done using the WTW Inolab 720 pH meter (Weilheim, Germany).

Fitting the growth curves and calculating the growth parameters

The growth data (lag-phase, the rate of decrease and others), curves and parameters of the strain under study were analyzed, fitted and calculated, respectively, using the mechanistic modelling technique of Baranyi et al. (1994) which is incorporated in the DMFit tools kindly provided by Dr. J. Baranyi (IFR Norwich, UK).

Results and Discussion

To describe the effect of the incubation temperature and media composition on the survival of *Lb. acidophilus* NCFM Howaru Dophilus, the experiments in ultra-pasteurized milk and in MRS broth were carried out. The average values of growth parameters, including the rate of decrease Gr (log CFU.ml^{-1}.d^{-1}), lag-phase duration λ (d), initial counts of NCFM strain N_0 (log CFU.ml^{-1}) and rate of pH value decrease pH_r (d^{-1}) obtained from three parallel growth curves in every experiment are summarized in Table 1.

Although metabolism, genetics and clinical effects of *Lb. acidophilus* NCFM have been characterized comprehensively, there is still a lack of information about its viability in the wider temperature range. Therefore, this work is specialized on the examination of NCFM strain's viability during its storage at stable temperature 6 °C and unstable temperature in a refrigerator. Considering types of products containing *Lb. acidophilus* NCFM Howaru Dophilus available for Slovak consumers, experiments were realized in an artificial medium of MRS broth and in a real medium of ultra heat treated milk.

It is recommended to keep dairy products and dietary supplements cooled to prevent probiotic cultures' decrease. Similarly, culture storage in an appropriate liquid medium at the temperatures allowing its survival is customary in laboratory practice. Storage in an ordinary refrigerator maintaining constant temperature is impossible due to frequent door opening and a natural temperature fluctuation occurs. Therefore the experiments at stable and unstable temperature were done.

The compliant starting state with initial counts N_0 = 7.98 ± 0.02 log CFU.ml^{-1} (CV = 0.28 %) were achieved, except for the experiment of strain's survival after overnight incubation, where initial count $N_{0,overnight}$ = 8.82 log CFU.ml^{-1} was reached.

In nutritionally rich environment *Lb. acidophilus* NCFM was able to survive, even to ferment available carbohydrates forming organic acids and lowering the pH value. Relevant decrease of viable cells in MRS broth was observed 30 or 29 days after inoculation at stable and unstable temperature, respectively. As Figure 1 shows, duration of lag-phase during cultivation in milk media was approximately 21 or 45 days at stable and unstable temperature, respectively).

Although the influence of the constancy of storage temperature on duration of the lag-phase was not clearly proven, experiments showed that the rate of decrease was two times slower during cultivation in MRS broth at stable temperature ($Gr_{MRS,st}$ = –0,079 log CFU.ml^{-1}d^{-1}) than in the refrigerator ($Gr_{MRS,unst}$ = –0,149 log CFU.ml^{-1}d^{-1}). Similar results were observed in experiment realized in milk. The rate of decrease of viable cells in milk was due to temperature fluctuations in the range from 6 to 9 °C approximately three times higher ($Gr_{milk,unst}$ = 0.030 log CFU.ml^{-1}d^{-1}) than at 6 ± 0.5 °C ($Gr_{milk,st}$ = 0.009 log CFU.ml^{-1}d^{-1}). Figure 1 also shows that while at stable temperature (dashed line in Figure 1a background) the rate of decrease was relatively stable too. On the other hand fluctuating temperature (dashed line in Figure 1b background) during cultivation caused fluctuations in actual rate of decrease.

A bacterial cell is able to adapt to the unfavorable external conditions by lowering its metabolic processes. During temporary temperature increase the intermediate acceleration of metabolism occurs. The cell in stage of replication is less protected against environmental changes than in stage of rest, therefore the re-decrease of temperature can cause its death (Pommerville, 2010). Although the milk medium had been shown to be less suitable for the growth of NCFM strain (Mančušková et al., 2013), it was able to survive for longer time, compared to MRS broth thanks to slower metabolism. These results are consistent with results published by Nosrati et al. (2014) who discovered that viability of *Lactobacillus* sp. is higher in media, where its growth rate is lower.

Storage of lactobacilli cultures in MRS broth at the refrigeration temperatures is customary in laboratory practice. As shown in Figure 1b, the decrease of viable cells of *Lb. acidophilus* NCFM from initial counts $N_{0,overnight}$ = 8.82 log CFU.ml^{-1} started practically immediately after its transfer into the refrigerator with rate of decrease ($Gr_{overnight,unst}$ = –0,137 log CFU.ml^{-1}d^{-1}) comparable with the experiment where inoculation of culture into nutritionally rich MRS broth was achieved.

The intensive growth and metabolism at the pre-incubation phase of the culture was probably the reason of the depletion of available nutrients and of the practically zero lag-phase. Due to this, the density of NCFM strain in this experiment reached the initial numbers of lactobacilli inoculated into the fresh MRS broth already in about 5 days.

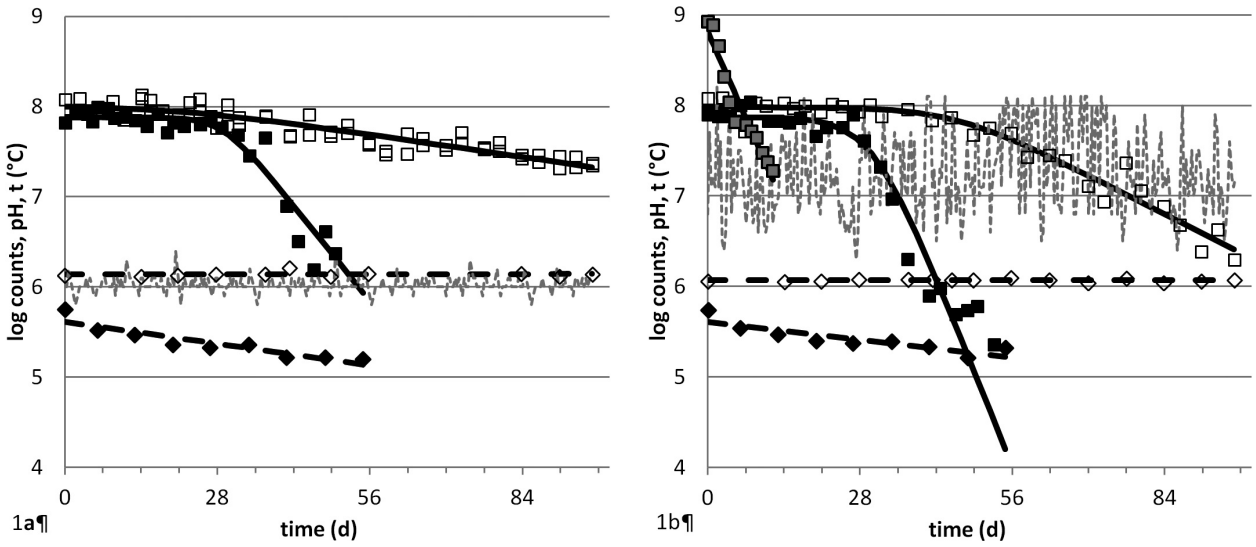

Fig. 1. Viability of *Lactobacillus acidophilus* NCFM Howaru Dophilus during storage at stable (**Fig. 1a**, t_{st} = 6 ± 0.5 °C) and unstable (**Fig. 1b**, t_{unst} ~ 6—9 °C) temperature (■ log counts in MRS broth, □ log counts in milk, ◆ pH in MRS broth, ◇ pH in milk, ■ log counts in MRS broth after overnight cultivation, --- temperature).

Tab. 1. Growth parameters of *Lactobacillus acidophilus* NCFM Howaru Dophilus during storage under different conditions.

Storage conditions	Gr (log CFU.ml^{-1}.d^{-1})	λ (d)	N_0 (log CFU.ml^{-1})	pH_r (d^{-1})
MRS, t_{st}	–0.079	29.2	7.87	–0.009
MRS, t_{unst}	–0.149	30.2	7.87	–0.007
overnight, t_{unst}	–0.137	0.00	8.82	–
milk, t_{st}	–0.030	21.2	8.08	< ±0.001
milk, t_{unst}	–0.009	45.4	8.08	< ±0.001

Gr is rate of decrease, λ is lag-phase duration, N_0 is initial counts, pH_r is rate of pH, t_{st} is storage at stable temperature, t_{unst} is storage at unstable temperature.

Conclusions

The survival of the probiotic strain *Lactobacillus acidophilus* NCFM Howaru Dophilus during storage at refrigeration temperatures as affected by the composition of cultivation media and stability of storage temperature was studied. Considering different types of products containing *Lb. acidophilus* NCFM available for Slovak consumers, experiments were realized in an artificial medium of MRS broth and in a real medium of ultra-pasteurized milk. The results obtained by the models of predictive microbiology showed that maintenance of the culture in milk at stable temperature is probably the most appropriate form of its storage. Whereas retail chains and consumers exert the pressure on producers of dairy products and nutrient supplements to extend the shelf life of their products, a knowledge of the probiotic strain survival in artificial and real media is essential. Enhancement of its ability to survive could be performed by usage of an appropriate storage media and by storage at stable temperature. Examination of suitable media composition and storage temperature will be the subject of our next work.

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