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Factors influencing the stability of freeze-dried stress-resilient and stress-sensitive strains of bifidobacteria

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ABSTRACT

Freeze-drying is a common method for preservation of probiotics, including bifidobacteria, for further industrial applications. However, the stability of freeze-dried bifidobacteria varies depending on the freeze-drying method and subsequent storage conditions. The primary goals of this study were to develop an optimized freeze-drying procedure and to determine the effects of temperature, water activity, and atmosphere on survival of freeze-dried bifidobacteria. To address these goals, a commercially used bifidobacteria strain that is resilient to stress, *Bifidobacterium animalis* ssp. *lactis* Bb-12, and a characterized intestinal strain that is more sensitive to stress conditions, *Bifidobacterium longum* DJO10A, were used. A freeze-drying protocol was developed using trehalose as the cryoprotectant, which resulted in almost no loss of viability during freeze-drying. Resuscitation medium, temperature, and time did not significantly influence recovery rates when this cryoprotectant was used. The effects of temperature (−80 to 45°C), water activity (0.02 to 0.92), and atmosphere (air, vacuum, and nitrogen) were evaluated for the stability of the freeze-dried powders during storage. Freeze-dried *B. animalis* ssp. *lactis* Bb-12 was found to survive under all conditions tested, with optimum survival at temperatures up to 21°C, water activities up to 0.44, and all 3 atmospheres tested. The intestinal-adapted strain *B. longum* DJO10A was much more sensitive to the different storage conditions, but could be adequately maintained using optimum conditions. These optimum storage conditions included frozen storage, replacement of oxygen with nitrogen, and water activities between 0.11 and 0.22. These results indicated that an optimized storage environment is required to maintain viability of stress-sensitive bifidobacteria strains, whereas stress-resilient bifidobacteria strains can maintain viability over a wide range of storage conditions, which is practical in countries where controlled cold storage conditions may not be readily available.

Key words: *Bifidobacterium longum*, *Bifidobacterium animalis* ssp. *lactis*, probiotic, trehalose

INTRODUCTION

The observations of Metchnikoff and Mitchell (1907) on the health benefits of ingesting lactic acid–producing bacteria laid the groundwork for the current probiotic era, which is a rapidly growing field worldwide. Dairy foods are particularly good vehicles for the delivery of probiotics to humans, as lactic acid–producing cultures are suited to this environment. The first commercial probiotic drink Yakult (Yakult Honsha Co. Ltd., Tokyo, Japan), containing *Lactobacillus casei* Shirota, was introduced in 1935 in Japan and is still sold today throughout the world (Fukushima and Hurt, 2011). Bifidobacteria were subsequently introduced because of their association with healthy intestines in breast-fed infants and their reduced numbers in the elderly, with a concomitant reduction in gut health. In the last 20 yr, the global probiotic market has been growing due to considerable progress in probiotic research, and sales are estimated to reach $19.6 billion in 2013 (Granato et al., 2010). Currently, *Lactobacillus* and *Bifidobacterium* are the most common probiotic genera used in food products.

Many challenges exist in the development of probiotic-containing food products, such as selection of effective strains and their survival during processing and storage. An important component of current selection practices for bifidobacteria for use as probiotics is their ability to survive food processing and storage. Their viability has been a technological issue for food manufacturers, particularly in fermented foods such as yogurt. As bifidobacteria are largely obligate anaerobic bacteria and not very acid tolerant, they are less stable in yogurts compared with lactobacilli (El-Dieb et al., 2012). Hence, the viability of bifidobacteria becomes an important issue and until technological advancements are made in protecting their viability in foods, strains are selected primarily for this feature rather than the

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myriad other characteristics that pertain to their probiotic functioning in the gut.

Drying cultures can enable their long-term storage and transportation without the need for refrigeration, if conditions are optimized. However, conditions are not the same for all cultures, with some, particularly many bifidobacteria cultures, being particularly sensitive (Meng et al., 2008). Although dried cultures have advantages during shipping and storage, critical parameters affect viability following their incorporation into foods, such as the nature of the food matrix, rehydration temperature, rehydration time, and powder-to-liquid ratios (Champagne, 2009). Freeze-drying and spray-drying are the 2 methods currently used to dry probiotics, with spray-drying being the preferred choice because of its cost effectiveness. In terms of viability, freeze-drying is the better process but its cost has hindered its use in large-scale processes. Spray-drying can be used for some cultures, but the conditions require optimization because probiotics are sensitive to heat (Chávez and Ledeboer, 2007). Studies have confirmed that bifidobacteria in general are very sensitive to spray-drying and showed superior survival rates following freeze-drying in different media (Wang et al., 2004; Chávez and Ledeboer, 2007; Wong et al., 2010). Based on these findings, freeze-drying became a popular method of stabilizing bifidobacteria before incorporation into food products.

Maintaining viability not only during the freeze-drying process but also during storage is a critical challenge for commercial production of bifidobacteria for probiotic applications (Saarela et al., 2005). Cryoprotectants such as polymers and sugars have been involved in the freeze-drying process to improve the survival rate of bifidobacteria (Kiviharju et al., 2005; Saarela et al., 2005). However, the abilities to survive during freeze-drying and subsequent storage are not linked. Therefore, factors that affect survival during storage need to be defined (Champagne et al., 2005). Storage temperature, exposure to oxygen, and water activity are several critical factors that affect viability of dried probiotics (Chávez and Ledeboer, 2007). Only a few studies show the detrimental effects of storage temperature and water activity on freeze-dried bifidobacteria (Champagne et al., 1996; Bruno and Shah, 2003; Abe et al., 2009a).

Although bifidobacteria with high stress tolerance are commonly used in food products, there is interest in using other less-tolerant strains that may be more suited to competing in the gut, provided that suitable conditions are developed to maintain their viability in foods. The objectives of this study were to develop a freeze-drying protocol for bifidobacteria, including stress-sensitive strains, and to evaluate the effects of temperature, water activity, and atmosphere during subsequent storage of the freeze-dried powders. Two potential probiotic bifidobacteria with different stress tolerances were used to achieve these objectives. One strain represented the most stress-adapted group of bifidobacteria, *Bifidobacterium animalis* ssp. *lactis* (Simpson et al., 2005), and the other strain, *Bifidobacterium longum* DJO10A, a characterized intestinal strain with minimum pure culture adaptation, represented a highly stress-sensitive group of bifidobacteria.

MATERIALS AND METHODS

Bacterial Strains, Growth Media, and Culture Growth Conditions

Two strains of bifidobacteria were used for this study: *B. animalis* ssp. *lactis* Bb-12 and *B. longum* ssp. *longum* DJO10A. Strain Bb-12, a common commercial probiotic used in many food products, was obtained from Chr. Hansen Inc. (Milwaukee, WI). Strain DJO10A is an intestinal strain that was isolated and characterized in our laboratory (Islam, 2006). Cultures were used from frozen stocks stored at −80°C in de Man, Rogosa, and Sharpe medium (**MRS**, BD Biosciences, San Jose, CA) containing 15% glycerol. Cultures were cultivated in MRS and incubated at 37°C anaerobically. A selective medium for bifidobacteria, bifidobacteria iodoacetate selective medium (**BIM-25**; Muñoa and Pares, 1988), was used, consisting of 3.8% reinforced clostridial agar (Oxoid, Basingstoke, UK), 0.005% L-cysteine·HCl, 0.002% nalidixic acid, 0.00085% polymyxin B sulfate, 0.005% kanamycin sulfate, 0.0035% iodoacetic acid, 0.0025% 2,3,5 triphenyl tetrazolium chloride, and 1.8% agar.

Preparation of Cultures for Freeze-Drying

Strains of Bb-12 and DJO10A were inoculated from stock cultures into tubes of MRS containing 0.05% L-cysteine·HCl and incubated anaerobically at 37°C for 1 d. Each culture was subinoculated at 2% into 4 L of MRS + 0.05% L-cysteine·HCl and incubated anaerobically at 37°C. Optical density at 600 nm (**OD_{600}**) was checked for determination of growth kinetics; when an OD_{600} of 1.0 was reached, the freeze-drying procedure was applied.

Preparation of Freeze-Drying Buffers

Sodium phosphate buffer (pH = 6.8) was used as the base buffer after autoclaving at 121°C for 15 min. The base buffer was supplemented with dried skim milk (**DSM**; Difco), trehalose, sucrose, or combinations

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