

Comparison of Temperature and Additives Affecting the Stability of the Probiotic *Weissella cibaria*

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Daily use of probiotic chewing gum might have a beneficial effect on oral health, and it is important that the viability of the probiotics be maintained in this food product. In this study, we examined the stability of probiotic chewing gum containing *Weissella cibaria*. We evaluated the effects of various factors, including temperature and additives, on the survival of freeze-dried probiotic *W. cibaria* powder. No changes in viability were detected during storage at 4°C for 5 months, whereas the viability of bacteria stored at 20°C decreased. The stability of probiotic chewing gum decreased steadily during storage at 20°C for 4 weeks. The viability of the freeze-dried *W. cibaria* mixed with various additives, such as xylitol, sorbitol, menthol, sugar ester, magnesium stearate, and vitamin C, was determined over a 4-week storage period at 20°C. Most of the freeze-dried bacteria except for those mixed with menthol and vitamin C were generally stable during a 3-week storage period. Overall, our study showed that *W. cibaria* was more stable at 4°C than that at 20°C. In addition, menthol and vitamin C had a detrimental effect on the storage stability of *W. cibaria*. This is the first study to examine the effects of various chewing gum additives on the stability of *W. cibaria*. Further studies will be needed to improve the stability of probiotic bacteria for developing a novel probiotic *W. cibaria* gum.

Key Words: Temperature; Chewing gum; Probiotics

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INTRODUCTION

The food industry often refers to foods supplemented with probiotics as functional foods. Several health benefits are associated with the consumption of live probiotic bacteria.¹ Because of the therapeutic benefits of live probiotics, there has been an increase in the incorporation of probiotic bacteria in a wider variety of food products, including yogurts, cheese, drinks, chewing gum, and dietary supplements.^{2,3} Probiotics are live microorganisms that can confer a health benefit to the host when consumed in adequate amounts.⁴ Interest in the use of foods that contain probiotics is increasing, and various functional foods that contain these microorganisms have been developed. The viability of the probiotics must be maintained in the food product until the time of consumption, and the probiotics

should be present in significant numbers, at least 10⁷ viable cells per gram or milliliter of a product.⁴ The survival of probiotics is genus-, species-, and strain-dependent, and the composition of the other ingredients in the preparation will also affect viability.⁵ For this reason, changes in the number of viable probiotic bacteria during storage of functional foods should be studied more extensively.

Weissella species are lactic acid bacteria that were formerly included in the genus *Lactobacillus*.⁶ *Weissella cibaria* is a Gram-positive, non-spore-forming, nonmotile, heterofermentative, catalase-negative bacillus that is normally isolated from fermented foods. Recently, our research group showed that *W. cibaria* isolates from human saliva can be used as a probiotic owing to an inhibitory effect on biofilm formation and volatile sulfur compound formation.^{7,8} Previous studies have demonstrated that daily use

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of probiotic chewing gum has a beneficial effect on oral health.^{9,10} Hence, the goal of this study was to investigate the stability of probiotic chewing gum containing *W. cibaria*.

For the development of functional foods, including probiotic chewing gum, probiotics should have the ability to remain viable and to retain their probiotic properties during production and storage of the probiotic food product.⁴ Commercially available chewing gums are often supplemented with various ingredients, such as sugars, vitamins, and various flavors in various amounts. All these ingredients will have an effect on the growth and activity of probiotic bacteria. To avoid such variability, it is important to examine the stability of probiotic bacteria in a complex mixture. Therefore, the objective of this study was to examine the effect of temperature on the storage stability of freeze-dried probiotic *W. cibaria* as well as the influence of various additives on the viability of *W. cibaria* in a mixture. This study aimed to gain a better understanding of the role of these ingredients on probiotic survival in order to develop a novel probiotic gum.

MATERIALS AND METHODS

1. Bacterial strain

The strain *W. cibaria* CMU, which has been shown to possess probiotic properties,⁸ was used as a model microorganism.

2. Viability of freeze-dried *W. cibaria* during storage

Freeze-dried *W. cibaria* powders were produced and provided by CTC BIO (Seoul, South Korea). The powders were stored in sterile plastic tubes at 4°C or 20°C for 5 months. The viable counts of the stored powders were determined after 0, 1, 2, 3, 4, and 5 months of storage. To count the viable cells of *W. cibaria*, 0.1 g of lyophilized powder was homogenized in 1 ml of phosphate-buffered saline. Serial dilutions were spread onto De Man, Rogosa, Sharpe agar plates (MRS agar; Difco, Detroit, MI, USA), which were then incubated aerobically at 37°C for 24 to 48 hours.

3. Stability of probiotic chewing gum during storage

The probiotic chewing gums were manufactured and provided by Xybita (Seoul, South Korea). The gums contained 2% freeze-dried *W. cibaria* powder, which corresponded to 1.7 g per tablet. The concentration of *W. cibaria* in the gum was 8.8×10^9 colony-forming units (CFU) per gum. The gum also contained xylitol, sorbitol, menthol, sugar ester, magnesium stearate, vitamin C, glycerin, and flavor. The pieces of gum were stored in sterile plastic tubes at 20°C for 4 weeks. One tablet of chewing gum was aseptically taken for microbial analysis at weekly intervals. To investigate the viability of *W. cibaria*, the probiotic chewing gum tablet was ground and homogenized in 10 ml of phosphate-buffered saline for 20 min. Serial dilutions were spread onto MRS plates, which were incubated aerobically at 37°C for 24 to 48 hours.

4. Effect of additives in chewing gum on the stability of *W. cibaria*

To evaluate the effects of the various additives used in chewing gum on the viability of *W. cibaria*, equal amounts of xylitol, sorbitol, menthol, sugar ester, magnesium stearate, and vitamin C, respectively, were mixed with *W. cibaria* powder. *W. cibaria* powder was used as a control. Control and mixed samples were stored at 20°C for 4 weeks. Samples of approximately 0.1 g were aseptically taken for microbial analysis at weekly intervals. The viability was assessed by bacterial counts, which were performed on MRS plates as described above.

5. Statistics

Results are expressed as means \pm standard deviations of three independent experiments. Statistical analysis was carried out by using SPSS (Statistical Packages for Social Science version 17.0; SPSS Inc., Chicago, IL, USA). A Mann-Whitney U test was used to identify any statistically significant differences in the experiments.

RESULTS

1. Storage stability of freeze-dried *W. cibaria*

The viability of freeze-dried *W. cibaria* stored at 4°C or 20°C for 5 months is shown in Fig. 1. Both conditions showed an average initial viable count of 1.08×10^{11} CFU/ml. After 2 months, the survival rates for *W. cibaria* at 20°C did not differ significantly from those at 4°C. However, a steady loss in viability was detected, and the viability of *W. cibaria* stored at 20°C was approximately $50.0 \pm 8.3\%$ of the viability after a storage period of 3 months. After a 4-month storage period, a significantly higher viable population was observed in the dried samples incubated at 4°C than in those stored at 20°C ($p < 0.05$). The viable count of bacteria at 20°C decreased to $2.64 \times 10^8 \pm 1.64 \times 10^8$ CFU/ml at the end of the storage period. However,

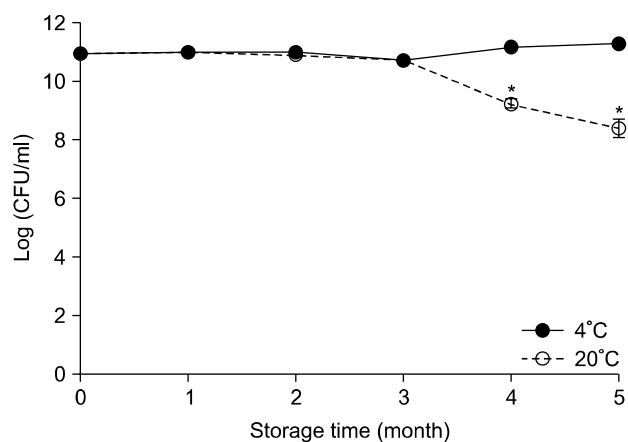


FIG. 1. The viable cells of freeze-dried *W. cibaria* as a function of storage time at a storage temperature of 4°C and 20°C. * $p < 0.05$ for 20°C vs. 4°C. The values are reported as the mean \pm SD of three independent experiments.

no changes in viability were detected during storage at 4°C.

2. Storage stability of probiotic chewing gum

The stability of probiotic chewing gum was examined during storage at 20°C for 4 weeks (Fig. 2). The viability of *W. cibaria* rapidly decreased with storage period. The cell number of *W. cibaria* was initially higher ($8.80 \times 10^9 \pm 1.20 \times 10^9$ CFU/ml) and decreased by 7-log cycles ($4.50 \times 10^7 \pm 2.70 \times 10^7$ CFU/ml) at the end of the storage period. The viability was approximately $0.5 \pm 0.2\%$ after storage for 4 weeks.

3. Effect of additives in chewing gum on the storage stability of *W. cibaria*

The viable cell counts of the freeze-dried *W. cibaria* mixed with various additives were determined over a 4-week storage period at 20°C (Table 1). In addition, viabilities of the freeze-dried *W. cibaria* mixed with xylitol, sorbitol, menthol, sugar ester, magnesium stearate, and vitamin C were determined compared with control (Fig. 3). In

general, the viability of *W. cibaria* stored with various additives decreased over the 4-week storage period when compared with control. After a 2-week storage period, the viability of bacteria stored with menthol, vitamin C, or sorbitol was approximately $12.0 \pm 1.1\%$, $48.1 \pm 3.1\%$, and $78.8 \pm 12.4\%$, respectively. Freeze-dried bacteria mixed with xylitol or sugar ester were stable during a 3-week storage period. However, a significantly lower viable population was observed in the mixed samples compared with the control at the end of the storage period ($p < 0.05$). No significant changes in the viability of bacteria stored with magnesium stearate were detected during a 4-week storage period.

DISCUSSION

The product in which probiotics are ingested or delivered in the oral cavity can affect the oral colonization of the probiotic. Hence, because the daily consumption of probiotics might lead to a transient or permanent colonization of these bacteria, consumption of probiotics might have a

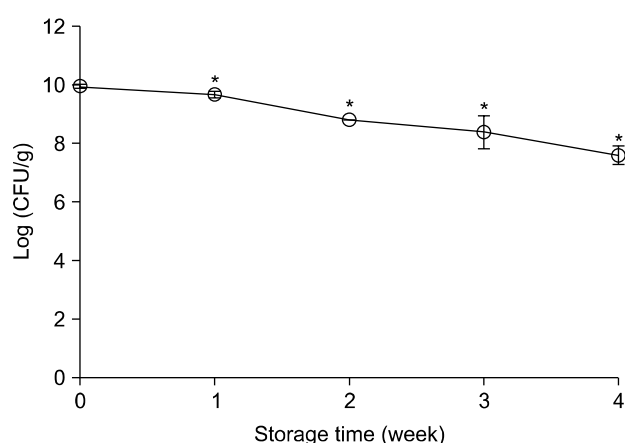


FIG. 2. Storage stability of probiotic *W. cibaria* chewing gum as a function of storage time at a storage temperature of 20°C. Viable cell numbers of *W. cibaria* in chewing gum containing *W. cibaria* were examined. * $p < 0.05$, compared with the initial storage time. The values are reported as the mean \pm SD of three independent experiments.

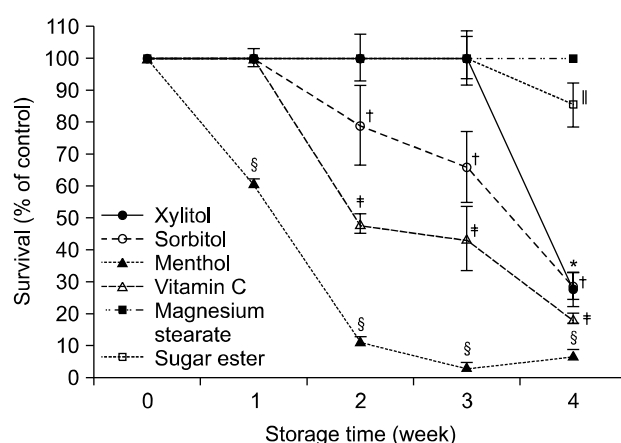


FIG. 3. Comparative effect of additives on the survival rate of freeze-dried *W. cibaria* as a function of storage time at 20°C. *, †, ‡, §, || $p < 0.05$, compared with control. The values are reported as the mean \pm SD of three independent experiments.

TABLE 1. Effect of additives on the viable cell numbers of freeze-dried *W. cibaria* as a function of storage time (week) at 20°C

Group	Viable cell count (Log CFU/ml)				
	0 w	1 w	2 w	3 w	4 w
Control	10.98 \pm 0.10	11.10 \pm 0.10	11.32 \pm 0.02	11.47 \pm 0.15	11.07 \pm 0.10
Xylitol	11.21 \pm 0.07	11.10 \pm 0.08	11.32 \pm 0.02	11.47 \pm 0.11	10.51 \pm 0.18
Sorbitol	10.94 \pm 0.02	11.10 \pm 0.10	11.21 \pm 0.06	11.28 \pm 0.07	10.53 \pm 0.05
Menthol	11.02 \pm 0.05	10.88 \pm 0.10	10.40 \pm 0.03	9.96 \pm 0.03	9.91 \pm 0.03
Vitamin C	11.13 \pm 0.10	11.10 \pm 0.10	11.00 \pm 0.04	11.10 \pm 0.05	10.34 \pm 0.07
Magnesium stearate	11.11 \pm 0.08	11.10 \pm 0.08	11.32 \pm 0.02	11.47 \pm 0.12	11.07 \pm 0.10
Sugar ester	11.10 \pm 0.10	11.10 \pm 0.10	11.32 \pm 0.02	11.47 \pm 0.14	11.00 \pm 0.07

An equal amount of xylitol, sorbitol, menthol, sugar ester, magnesium stearate, and vitamin C was mixed with *W. cibaria* powder, respectively. *W. cibaria* powder was used as control. The values are reported as the mean \pm SD of three independent experiments.

beneficial effect on the oral cavity. Specifically, the daily use of high levels of probiotic bacteria is required to confer health benefits. For this reason, the viability of probiotics must be maintained in the food product until the time of consumption.⁴ Therefore, changes in the number of viable probiotic bacteria during the storage of probiotic-containing products should be studied more extensively.¹¹

Freeze-drying has traditionally been used for preserving the starter culture and probiotic bacteria.^{12,13} This is because the low temperatures used during freeze-drying are expected to be less injurious to labile biological organisms than drying at ambient or higher temperatures.¹⁴ Many factors contribute to the stability of freeze-dried probiotic preparations, including the type of probiotic, method of drying, the composition of the matrix, and storage condition.^{13,15} The stability of freeze-dried cultures is generally higher at low-temperature storage. Storage under nonrefrigerated conditions at high relative humidity and exposure of powders to an oxygen-containing atmosphere can contribute to a decrease in viability of probiotics over time. The current study showed that the survival of *W. cibaria* at 20°C decreased as the storage time increased. *W. cibaria* survival during storage was higher at 4°C than at 20°C. The results of this study agree with the findings of other similar studies; for example, the survival of freeze-dried lactic acid bacteria was shown to decrease at higher storage temperatures.¹⁶

Several authors have suggested that chewing gums containing probiotic *Lactobacillus reuteri* could have a beneficial effect on oral health.^{9,10} The results of these studies have shown that chewing gum containing probiotic bacteria reduces the levels of salivary mutans streptococci as well as the levels of pro-inflammatory cytokines in gingival crevicular fluid. In addition, our previous studies on *W. cibaria* were the first to use a more scientifically based step-by-step approach to identify a probiotic for the treatment or prevention of oral disease.^{7,8} It can be anticipated that the repeated daily use of probiotic *W. cibaria*-containing foods, such as chewing gum, over a long period of time will increase the level of probiotic bacteria in the oral cavity.

Commercially available chewing gums contain various sugars, vitamins, and flavors at different concentrations. All of these ingredients can affect the growth and activity of probiotic bacteria. To avoid such variability, it is important to examine the stability of probiotic bacteria in a complex mixture. Menthol is a monocyclic monoterpene that is the main compound in the essential oil of *Mentha piperita*.¹⁷ It is an organic compound that is naturally occurring in mint plants. It is routinely used as a cooling, anesthetic, antipruritic, and antiseptic agent in many pharmaceutical formulations, toothpastes, and gums.¹⁸ Tampieri et al.¹⁹ suggested that the essential oil of *Mentha piperita* can inhibit the growth of *Staphylococcus species*, *Escherichia coli*, and *C. albicans*. Similarly, the present study showed that menthol had a detrimental effect on the storage stability of probiotic *W. cibaria*. Therefore, a possi-

ble mechanism for the decrease in storage stability is that menthol inhibits the viability of *W. cibaria*.

Previous studies have demonstrated that damage to the cell membrane occurs during long-term storage, and some antioxidants have been reported to protect membrane lipids against such damage.²⁰ The addition of antioxidants may increase the storage stability of the dried probiotic bacteria.^{15,21} Vitamin C or ascorbic acid is a weak sugar acid that is structurally related to glucose. It usually acts as an antioxidant. It typically reacts with oxidants of the reactive oxygen species, such as the hydroxyl radical formed from hydrogen peroxide.²² Several studies have shown that vitamin C can be beneficial to probiotic survival during storage, presumably because it is an oxygen scavenger, thus promoting a more favorable anaerobic environment.^{20,23} On the other hand, vitamin C has long been known for its antibacterial properties.²⁴ In the present study, vitamin C did not improve the survival of probiotics. We suggest that the viability of *W. cibaria* might be decreased by the antibacterial properties of vitamin C.

The polyols most frequently used in chewing gum are sorbitol, a hexitol derived from glucose, and xylitol, a pentitol that occurs widely in nature. Xylitol is a natural sugar alcohol that is commonly used as a sweetener in chewing gums and candy.^{25,26} Sucrose fatty acid esters, which are generally called sugar esters, are nonionic surfactants consisting of sucrose as a hydrophilic group and a fatty acid as the lipophilic group.²⁷ This compound increases chewing gum plasticity, softness, and chewiness and prevents adherence to the teeth during chewing. Magnesium stearate is insoluble in water and has been used as a filling agent in food supplements. In the present study, the viabilities of *W. cibaria* mixed with polyols, sugar ester, and magnesium stearate were generally stable during the storage period. Among the additives tested, magnesium stearate resulted in the highest survival when mixed with the bacteria.

In this study, the stability of freeze-dried *W. cibaria* powders at different temperatures were determined. Our findings showed that *W. cibaria* did not survive well at 20°C. In addition, this is the first study to examine the effects of various gum additives on the stability of *W. cibaria*. The data indicate that menthol and vitamin C had detrimental effects on the storage stability of *W. cibaria*. Further studies will be needed to improve the stability of probiotic bacteria for developing a novel probiotic *W. cibaria* gum.

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