JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2014. Vol. **8**(3), p. 2151-2154

Survival of Probiotic Bacteria in a Chocolate

Wei Ye[1]*, Guicheng Huo[2], Jie Han[3], Qingbo Ding[3] and Ballevre Oliver[3]

[1]Shanghai Medical Instrumentation College, University of Shanghai for Science and Technology, Shanghai, China.
[2]Key Lab of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin, China.
[3]Nestlé Research R&D Center Shanghai Ltd, Shanghai, China.

(Received: 18 November 2013; accepted: 09 January 2014)

The object of this study was to evaluate the effect of chocolate as a delivery vehicle on the survival of probiotics including *L. acidophilus* NCFM, *L. acidophilus* R5002 and *S. boulardii* at different temperature 4℃, 20℃ and 30℃ with different water activity. In this work, chocolate could be used as an effective carrier for probiotic bacteria to storage at 4! when inoculated at 8 log g^{-1} or more. All the strain could maintained above required level for health benefit over the storage period of 12 weeks. Even at 20! the losses of probiotics were no more than 2 logs except *S. boulardii* in 72.28% chocolate samples. The losses of *L. acidophilus* R5002 in 86.94% chocolate sample were 0.88 log and the losses of *S. boulardii* in 89.22% chocolate sample were 0.49 log. Both were less than 1 log in 12 weeks. That chocolate samples could be the effective carrier for these two probiotics to storage at standard temperature.

Key words: Probiotics, *L. acidophilus* NCFM, *L. acidophilus* R5002, *S. Boulardii,* stability.

Probiotics have been used to improve the health of humans and animals on various aspects. Their benefits include improving the intestinal microflora balance, enhancing immune system, preventing cancer, treatment of diarrhea[1], allergic diseases[2], antagonistic activity against pathogens. Some of them have clinical trials such as Irritable bowel syndrome[3] and cholesterol [4].

The functional food with probiotics has to face some questions, such as strain selection, addition level steps, adaptation of procession steps, the effect of different ingredient, survival of probiotics during storage, oxygen-sensitive, packing material and identification and numeration of the viable populations. In this experiment three probiotics were choosed, including *Lactobacillus acidophilus* NCFM, *Lactobacillus acidophilus*-R5002 and *Saccharomyces boulardii*. *L.acidophilus* NCFM™ strain has been thoroughly researched. From the manufacturer's point of view, other technological qualities are demanded. It is believed that the cells between 10^6-10^8CFU/g of intestinal contents can have a significantly affect on the GI tract[5].

Food matrix formulation is a major technological factor that influences the functionality of probiotics[6]. Most reports are the application of probiotics on milk, cheese[7,8]. There are some reports on the ice cream[9], milk- and water-based cereal puddings[10], cheese-based dips[11] and sausage[12,13]. It's a great challenge that applied the probiotics in non-dairy products such as cereal products, oat and confectionary. In non-dairy products it's important to maintain viability of probiotics of formulation in shelf life since the probiotics don't usually multiply. So the survival

* To whom all correspondence should be addressed.
Tel.: 086-021-65502675-3353;
E-mail: beatificy@gmail.com

of probiotics is critical. In these products the water activity, oxygen tension and temperature become increasingly important.

This experiment evaluated the effect of chocolate samples with different water activity on the survival of three probiotics *L. acidophilus NCFM, L. acidophilus* R5002 and *S. boulardii* at 4!, 20! and 30!.

MATERIAL AND METHODS

Strain and samples

The strain used in the study were *Lactobacillus acidophilus* NCFM, *Lactobacillus acidophilus* R5002 and *Saccharomyces boulardii*. chocolate and glycerol with different content was mixed to test its water activity. The a_w of different recipes were recorded. The recipes of a_w between 0.15-0.30 was choosed to estimate the scale of the content of component which was suitable to be used for the food system. According to this scale designed the experiment to test the survival of probiotics at different water activity (Table 1).

Preparation of food sample

Melted and mixed the chocolate samples to make the ingredient equally inside and outside. Weighted the components that as designed in Table 2. Each sample was divided into 15 parts. Added the probiotics into the chocolate until the temperature below 40!, after mixing well added the glycerol. First put some samples into the small cup for water activity test. Then packed the bags and sealed them. Sticked the label and separated each sample as out date and storage temperature. Put samples of the same out date and storage temperature together in the big bag to do the storage test at 4!, 20! and 30! .

Microbiological determinations of food system

Weighted about 10g of the food sample and put it into the stomacher bag. Added the TS water into the blender jar until the weight was 100g. Homogenize the sample by stomaching for 2min. The content of the stomacher bag was 1/10 dilution of the original sample. Prepared the dilutions and poured the MRS agar into the plate. Incubated sample plates with *L.acidophilus* NCFM and *L. acidophilus* R5002 at 30! for 72h. Spreaded the dilutions of *S. boulardii* samples on the GM17 plate and incubated at 25!for 5 days.

Colony Counting and analysis

The colony counts were recorded in the table using plates that fit the criteria of the counting rules. The plate which counts between 20 and 300 was choosed. The curve was drawed according to the test weeks and microbial counting (shown in Log). If the difference of log (counting) was more than 1, then a significant difference was taken.

RESULT AND DISCUSSION

The survival of probiotics at different temperature

All the three probiotics kept the best survival at 4°C. This result was the same with the report of Hughes and Hoover[14] that *Bifidobacterium* and *Lb. acidophilus* didn't lost much after 15 days at 4°C. The viable probiotics lost most quickly at 30°C. At 4°C storage the survival of the probiotics is *S. boulardii*> *L. acidophilus* R5002> *L. acidophilus* NCFM and the losses of *S. boulardii* and *L. acidophilus* R5002 wasn't more than 0.5 logs at 12th week. In cheese-based dips the losses of *L. acidophilus* was more than 1 log at 10 weeks at 4 °C[11]. It suggested that chocolate was more suitable to be the vehicle of probiotics at 4 °C. At 20°C, Losses of *L. acidophilus R5002* in 86.94% chocolate sample and *S. boulardii* in 89.22% chocolate sample were still less than the losses in cheese-based dips. The losses of probiotics were no more than 2 logs except *S. boulardii* in 72.28% chocolate samples. The survival population still maintained above 10^6 CFU/g that the required level for health benefit. 30°C wasn't suitable to storage that the viable strain lost too quickly.

The viable probiotics lost very quickly at 30°C especially the first 4 weeks and nearly no viable probiotics at 12th week. So we analyzed the losses of the probiotics in the first 4 weeks. The probiotics *L. acidophilus* NCFM and *L. acidophilus* R5002 were more stable in 70% chocolate sample than in 86.94% chocolate sample, and the losses of probiotics in later samples was more than 0.5 log than in 70% sample at 30°C.This result was different from the results at 4°C and 20°C in 12 weeks. Maybe the higher temperature the higher a_w wasn't suitable for *L. acidophilus* NCFM and *L. acidophilus* R5002 storage, and the strains were more stable at lower a_w. But *S. boulard* got the same result at 30°C with storage at 4°C and

20°C, that its losses the least in 89.22% chocolate samples and losses the most in 72.28%.

The effect of chocolate with different water activity on the survival of probiotics

L. acidophilus NCFM was more stable when a_w was 0.303 than 0.256 in chocolate samples, *L. acidophilus* R5002 was more stable when a_w was 0.316 than 0.185 in chocolate samples and *S. boulardii* was more stable when a_w was 0.322 than 0.199 in chocolate samples at 4°C and 20°C. These results were different from what we thought before the experiment. We thought the lower a_w the more stable the probiotics would be. That seems it didn't always work in all the situations that in this experiment the probiotics were more stable when a_w was 0.3 than 0.2. But at 30°C, the probiotics were more stable when a_w was 0.2 than 0.3. Therefore both temperature and a_w were important factors that influence the survival of the probiotics.

There were some reports about the effect of food ingredient on the probiotics. The fat content of yogurt mix don't seem to influence the growth of the probiotics[15]. It was evaluated the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. The results presented in their study indicated that malt, wheat and barley extracts exhibit a significant protective effect on the viability of *L.acidophilus* under acidic conditions, which could be mainly attributed to the amount of sugar present in the cereal extracts [16]. Maybe it was also the same reason that samples which a_w was 0.3 contained more chocolate than 0.2, that meant more sugar in the samples. So the survival of probiotics in 0.3 a_w samples were more stable than in 0.2 a_w samples. That needs more works to identify.

CONCLUSION

The results obtained in this study suggested that chocolate could be used as an effective carrier for probiotic bacteria to storage at 4! when inoculate at 8 log g^{-1} or more. All the strain can maintained above required level for health benefit during the storage period of 12 weeks. Even at 20! chocolate samples could also be the effective carrier for *L. acidophilus* R5002 and *S. boulardii*. These three probiotics were more stability when a_w around 0.3 than 0.2 at 4°C and 20°C, Maybe the sugar in chocolate samples have the effect on the stability of probiotics. 30°C isn't suitable to storage that the viable strain lost too quickly. But it maybe looked as the accelerating tests that with the storage prolongation the tendency of survival probiotics at 4 and 20°C. Some investigation about influence factors sill need to do in the further.

ACKNOWLEDGEMENTS

Thanks Nestle R&D centre Shanghai for the financial support. Thanks A. Bousbaine for designing the recipe of experiment. The paper was supported partially by Shanghai Medical Instrumentation college, Project Number: A2500130104, and University of Shanghai for Science and Technology, Project Number: slg14065.

REFERENCES

1. Johnston BC, Ma SS, Goldenberg JZ, Thorlund K, Vandvik PO, Loeb M, Guyatt GH.Probiotics for the prevention of Clostridium difficile-associated diarrhea: a systematic review and meta-analysis. *Ann Intern Med*. 2012; **157**(12): 878-88.
2. West CE, Hammarström ML, Hernell O. Probiotics in primary prevention of allergic disease - follow-up at 8-9 years of age.Allergy. 2013; **68**(8):1015-20.
3. Korpela R, Niittynen L. Probiotics and irritable bowel syndrome.*Microb Ecol Health Dis.* 2012; 23.
4. Pavlovi N, Stankov K, Mikov M. Probiotics—interactions with bile acids and impact on cholesterol metabolism. *Appl Biochem Biotechnol.* 2012; **168**(7):1880-95.
5. Richardson, D. Probiotics and product innovation. *Nutrition and food Science*. 1996; **4**: 27-35.
6. Sanders ME, Marco ML. Food formats for effective delivery of probiotics.*Annu Rev Food Sci Technol*. 2010; **1**:65-85.
7. Escobar MC, Van Tassell ML, Martínez-Bustos F, Singh M, Castaño-Tostado E, Amaya-Llano SL, Miller MJ. Characterization of a Panela cheese with added probiotics and fava bean starch. *J Dairy Sci*. 2012; **95**(6): 2779-87.
8. Santillo A, Albenzio M, Bevilacqua A, Corbo MR, Sevi A.Encapsulation of probiotic bacteria in lamb rennet paste: effects on the quality of Pecorino cheese. *J Dairy Sci*. 2012; **95**(7): 3489-

500.
9. Di Criscio T, Fratianni A, Mignogna R, Cinquanta L, Coppola R, Sorrentino E, Panfili G. Production of functional probiotic, prebiotic, and synbiotic ice creams. *J Dairy Sci*. 2010; **93**(10): 4555-64.
10. Merete H. Helland, Trude Wicklund and Judith A. Narvhus. Growth and metabolishm of selected strain of probiotic bacteria in milk-and water-based cereal puddings. *International Dairy Journal*. 2004; **14**: 957-965.
11. Nalayini Tharmaraj, Nagendra P. Shah. Survival of Lactobacillus acidophilus, Lactobacillus paracasei subsp. Paracasei, Lactobacillus rhamnosus, Bifidobacterium animalis and Propionibacterium in cheese-based dips and the suitability of dips as effective carriers of probiotic bacteria. *International Dairy Journal*. 2004; **14**: 1055-1066
12. Rouhi M,Probiotic fermented sausage: viability of probiotic microorganisms and sensory characteristics. Crit Rev Food Sci Nutr. 2013; **53**(4): 331-48.
13. Klingberg TD, Budde BB. The survival and persistence in the human gastrointestinal tract of five potential probiotic lactobacilli consumed as freeze-dried cultures or as probiotic sausage. *Int J Food Microbiol*. 2006; **109**(1-2): 157-9.
14. Hughes D.B. and Hoover D.G..Viability and enzymatic activity of bifidobacteria in milk. *Journal of Dairy Science*. *J Dairy Sci*. 1995; **78**(2): 268-276.
15. Micanel N., Haynes I.N., and Playne, M.J., Viability of probiotic cultures in commercial Australian yoghurts. *Australian Journal of Dairy Technology*. 1997; **52**: 24-27.
16. Charalampopoulos D, Pandiella SS, Webb C., Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. *International Journal of Food Microbiology.* 2003; **82**: 133-141.