The DNA-based gels for oral delivery of probiotic bacteria

Benjamas Jonganurakkun[1], Yoshinobu Nodasaka[2], Nobuo Sakairi[1], Norio Nishi*[3]

[1]Graduate School of Environmental Science, Hokkaido University, Sapporo 060-0810, Japan

[2]Graduate School of Dentistry, Hokkaido University, Sapporo 060-8586, Japan

*[3]To whom the correspondence should be addressed

Graduate School of Engineering, Hokkaido University, Kita 13, Nishi 8, Sapporo 060-8628, Japan

Fax: +81-11-706-7584

E-mail: nishin@ees.hokudai.ac.jp

Keywords: biomaterials; DNA; hydrogels; probiotic strains, drug delivery system

Summary

A single-stranded DNA readily extracted from industrial discarded salmon milt, was used to prepare hydrogels and complex gels by cross-linking with gelatin and *κ*-carrageenan, for the oral delivery of probiotic bacteria. The complex gels showed a higher protective capability over the hydrogels for approximately one log scale. However, the hydrogels were more stable during 4°C storage. The *Lactobacillus* and *Lactococcus* due to protection of the hydrogels could better tolerate to acid than the *Bifidobacterium*. Furthermore, food-graded hydrogels were prepared and optimized to a similar protective capability for future applications.

Introduction

DNA can be used as a remarkable natural biomaterial due to its particular structure and properties. The phosphate groups of DNA provide the poly-anionic character which allows bonding with the cationic polymers by ionic strength. The DNA extracted from industrial discarded salmon milts and shellfish gonads were studied and applied in many aspects by our groups. The DNA could be used for environmental purposes [1-3], biological and genetic applications [4] and medical therapeutic aims [5, 6]. DNA was acknowledged as digestible by deoxyribonuclease I (DNase I). [7] The human DNase I is excreted from the pancreas and sensitive to acidic conditions. [8] Thus, DNA might be usable as a protective biomaterial to carry functional materials which are sensitive to the gastric acidity in the human intestine and colon, for food and drug delivery applications.

Lactic acid-producing bacteria, *Lactobacillus*, *Bifidobacterium*, *Lactococcus* etc., are being promoted as probiotics. Probiotic bacteria are the bacteria that survive passage through the gastrointestinal tract and have beneficial effects on the host. [9] However, the low pH of the stomach environment is detrimental to their survival. [10]

In a previous study [11], the DNA-based gels were prepared to protect the lactic acid bacteria present in commercial drinking yogurts from extreme acidic conditions. The DNA could form complex with gelling agents, gelatin and *κ*-carrageenan at low pH

values. Two kinds of gel were prepared and called hydrogels and complex gels. The hydrogels were easily prepared by cooling the hot mixture solution of DNA, gelatin and κ-carrageenan. The bacteria were directly fixed in the gel when applying the yogurt containing bacteria into the pre-cooled hot solution. On the other hand, the complex gels were produced by previously preparing the emulsion of yogurt and cacao oil before being mixed in the gel solution containing the DNA, gelatin and κ-carrageenan. Therefore, the bacteria were collected in the oil particles dispersed in the complex gel, which were formed after blending the solidified emulsion. Only the complex gels were intensively studied and optimized since they could highly protect the bacteria, while the bacteria protected in the hydrogels were almost undetectable after simulated gastric incubations similar to the results of the free cells. Nevertheless, the nourishing components, such as proteins, fats, carbohydrates, flavors and odors in the yogurt [12] might be the factors that interfere with the results of the protective capability of the hydrogels.

In the present study, 6 strains of probiotic bacteria were used instead of the commercial yogurts containing the bacteria. The survival of the bacteria by the protection of the hydrogels and the complex gels were measured and reported. Scanning electron microscopy was used to observe the morphology of the gels. In addition, the

food-graded gels were intentionally produced and optimized to satisfy one of the study's purposes to develop a healthy food product from remarkable natural biomaterials.

Experimental Part

Materials

Single-stranded DNA (sodium salt from salmon milt, M_w approx. = 100000, Lot. No. 02037) was purchased from Nippon Chemical Feed (Hakodate, Japan), and used without further purification. Gelatin, κ-carrageenan, lecithin (from soy bean) and potato starch were purchased from Wako (Osaka, Japan). Cacao oil was purchased from Merck Hoei (Osaka, Japan).

For preparing the food-graded hydrogels, single-stranded DNA (sodium salt from salmon milt, M_w approx. = 100000, Lot. No. 050303) was purchased from Nippon Chemical Feed (Hakodate, Japan) repeated. Gelatin (#200) and κ-carrageenan (KK-9) were kindly donated from Nitta Gelatin (Osaka, Japan) and Marine Science (Tokyo, Japan), respectively.

Bacterial strains, culture conditions and bacterial suspension preparations

The probiotic bacteria used in this study are *Lactobacillus* strains, *Lactococcus* strains

and *Bifidobacterium* strains as shown in Table 1. Cultivation was carried out by inoculation of the bacteria in the GAM broth (Gifu anaerobic medium broth for general culture and susceptibility test; Nissui Pharmaceutical, Tokyo, Japan) then anaerobically incubated overnight at 37°C. The anaerobic conditions were achieved using the Culture System for Anaerobic Gas Jet Method AG-2 (Sanshin Industrial, Yokohama, Japan) and Anaero Pack® (Mitsubishi Gas Chemical Company, Tokyo, Japan). Cells were harvested after 12 h incubation by centrifugation at 3000 x g for 10 min, 4°C, washed and then re-suspended in phosphate-buffered saline (PBS; pH 7.2). The cells were adjusted to 10^9-10^{10} CFU/ ml for gel preparation by PBS buffer after estimated cell concentration by measuring the OD_{600}. The cell suspensions were subsequently used either directly (free cells) in the assays or subjected to gel preparation as described in a latter section.

Gel preparations

Initially, the gel solution was prepared by dissolving 4 g DNA in 100 g sterile distilled water, stirred at room temperature. Thirteen g gelatin and 0.9 g *κ*-carrageenan were added to the prepared solution, boiled at 70°C for 30 min, cooled and incubated at 35°C. The hydrogels were obtained by aseptically adding 3.3 ml of cell suspensions to the pre-cooled gel solution, then stirring for 5-10 min and kept refrigerated.

The complex gels were obtained by preparing a water-in-oil emulsion by the Hatschek transpiration method.[13] Cells suspensions were gradually dropped together by jet air by passing through a thin nozzle into the oil phase, which contained 49 wt.-% cacao oil and 2 wt.-% lecithin, at 35°C. Upon cooling, the aqueous emulsion solidified at 4°C, and 2 g of the solidified emulsion was ground with 4 g potato starch. The mixture was then dispersed into the prepared gel solution composed of DNA, gelatin and *κ*-carrageenan as described above. The gel was formed at 10°C, and kept refrigerated.

Determination of acid tolerance

Essentially, either 1 ml of the free cells or a gram gels, hydrogels and complex gels, were incubated in 9 ml of simulated gastric juices (0.7% HCl, 0.2% NaCl, and 0.32% Pepsin, pH 1.2) at 37°C. Bacterial viability was measured at 0, 2 and 4h of incubation. The incubated free cells and gels were harvested by centrifugation (3000 × g at 4°C for 10 min), washed and re-suspended with PBS. Homogenization (Stomacher® 80 Biomaster, Organo, Tokyo, Japan) was performed to release the bacteria from the gels. Viable cells were determined by a ten-fold serial dilution followed by an anaerobic spread plate on MRS agar (Oxoid, Hampshire, UK) after incubation for 48 h at 37°C.

The viability was reported as colony forming unit (CFU) per milliliter cell suspension and per gram gels. Survival of the bacteria was calculated using the following equation:

Survival = Log (viable cell after 2h or 4h treated) / Log (viable cell before treated)

where each value was done in triplicate.

Gel morphology

Gel specimens were fixed using 2 vol.-% glutaraldehyde and 1 vol.-% osmium tetroxide (OSO_4), dried at the critical point using a Hitachi Critical Point Dryer HCP-1 and Pt/Pd-sputtered using a Hitachi E-1030 ion sputter. Scanning electron microscopy (SEM) was performed using a Hitachi S-2380N electron microscope operating at an acceleration voltage of 10 kV.

Food-graded hydrogel preparations

For the oral delivery applications, food-graded components were used to prepare the hydrogel and *B. longum* JCM1217 was used as the representative probiotic strain. The effect of food-graded components was measured by preparing the 3 kinds of hydrogel by altering a new commercial lot DNA, a food-graded gelatin and a food-graded κ-carrageenan, respectively. These prepared hydrogels were incubated in simulated

gastric juice at 37°C for 2h. The survival of the bacteria in each gel was measured and compared to the original hydrogels.

Results and Discussion

The morphology of the prepared hydrogels and complex gels were observed by SEM. The scanning electron micrographs of the gels are shown in Figure 1. The *L. gasseri* JCM1131 was used as a representative of the probiotic strain. The bacteria were fastened with the fiber-like structure of the hydrogel which occurred from the cross-linking of DNA, gelatin and κ-carrageenan as shown in Figure 1a. On the other hand, the bacteria were gathered inside the oil particles fixed in the complex gel. The oil particles were formed after blending the solidified emulsion of cacao oil, lecithin and the bacterial suspension. Figure 1b shows the various sizes, 10-50 μm in diameter, of spherical oil particles fixed in the complex gel. The bacteria in the oil particle were exposed from the melted oil particles, as shown in Figure 1c, by incubating the gel overnight at 37°C.

The protective capability of the gels was measured from the survival of bacteria after incubation in the simulated gastric juice (pH 1.2) at 37°C. When compared to the hydrogel and complex gel, the free cells without protection were almost undetectable

after the 2h and 4h incubations in the simulated gastric juice (data not shown). On the contrary, both hydrogels and complex gels could highly protect the bacteria from the acidic conditions as shown in Figure 2. The survival of all 6 probiotic strains secured in the complex gels after the 2h and 4h incubations were both higher than those protected in the hydrogels for about the 1-2 log scale. Moreover, within the 2 h incubation of the hydrogels, *Lactobacillus* species, JCM1131 and JCM1132, and *Lactococcus* species, R-704, could better tolerate the acid than the *Bifidobacterium* species, Bb-12, JCM1217 and JCM1255. *L. gasseri* JCM1131 showed the highest survival due to protection by the hydrogel, and showed an undistinguished survival in both the hydrogels and the complex gels, within the 2 h incubation.

The gel stability during storage at 4°C was observed by measuring the viability of bacteria in the gels at 0, 1, 2, 3, 4, 6, 7, 10 and 12 weeks of storage. *L. gasseri* JCM1131 was used as the representative probiotic strain. The results are shown in Figure 3. The viability of the bacteria in the complex gel gradually decreased on a log scale within 3 weeks, and continually dropped until undetectable in the 10^{th} week. On the other hand, the viability of the bacteria in the hydrogel could be detected in the 12^{th} week with a decreasing log scale. This might because the oil particles could not be used for releasing the waste or acid that was produced by the bacteria fixed inside. Since the

probiotic bacteria are acid producible, especially the *Lactobacillus* species, the *Lactococcus* species and the *Bifidobacterium* species can produce the lactic acid by their metabolic pathway [14, 15], consequently, the bacteria could not survive in an adverse acidic environment that was produced by itself. These results suggested that the hydrogels were more suitable for the bacteria to survive in refrigerated storage. These effective results correspond to the product shelf life [16, 17] that should be considered for future applications.

In this study, the hydrogel was considered for use in future applications of the oral delivery of probiotic bacteria since the hydrogel was easy to prepare, more stable than the complex gel during long refrigerated storage, and showed a high protective ability for the survival of bacteria in the simulated gastric juice incubations. The edible hydrogels were prepared using food-graded gelatin and *κ*-carrageenan. Figure 4a shows the effect of the food-graded components on the survival of bacteria. The hydrogels prepared from a new commercial lot of DNA and those prepared from the food-graded *κ*-carrageenan showed insignificant differences from the control, the original hydrogels. However, the distinct result was measured from the hydrogels prepared from the food-graded gelatin. Therefore, food-graded gelatin was the main effective component that should be optimized for increasing the protective capability of the food-grade

hydrogels. The hydrogels were prepared using a new commercial lot of DNA and food-graded κ-carrageenan in varying amounts of food-graded gelatin. The optimal quantity of the food-graded gelatin was measured and the results are shown in Figure 4b. As a result, a 12.3 % consistency of food-graded gelatin showed the most protective ability towards the bacteria in the simulated gastric juice, in contrast to the other values. These results indicated that a commercially food-graded DNA, gelatin and *κ*-carrageenan could be substituted for the original lab-graded components with a similar protective capability for the survival of bacteria under the simulated gastric conditions.

Conclusion

According to the attempt to produce innovative healthy food products from new kinds of biomaterials, DNA-based hydrogels and complex gels, which could significantly protect the probiotic bacteria from adverse simulated gastric conditions, were prepared. The complex gels showed a higher protective capability than the hydrogels. However, the easier prepared hydrogels were more stable during 4°C storage which corresponds to the long shelf life of the product that might be applied in the future. The optimized food-graded hydrogels also showed effective results for the survival of the probiotic

bacteria. Therefore, the DNA-based hydrogels should be able to be used for the oral administrative of probiotic bacteria in the future.

Acknowledgements

We would like to thank the Ministry of Education, Science, Sports and Culture of Japan for Grants-in-Aid for Science Research (No. 11694114, No. 11450359, No. 10555327, No. 13132201 and No. 14350491) and also the Hokkaido Foundation for the Promotion of Scientific and Industrial Technology (Hokscitec) for supporting this work.

References

[1] X. D. Liu, Y. Murayama, M. Yamada, M. Nimizu, M. Matsunaga, N. Nishi, *Int. J. Biol. Macromol.* **2003**, *32*, 121.

[2] S. Satoh, B. Fugetsu, M. Nomizu, N. Nishi, *Polym. J.* **2005**, *37*, 94.

[3] M. Yamada, M. Yokota, M Kaya, S. Satoh, B. Jonganurakkun, M. Nomizu, N. Nishi, *Polymer* **2005**, *46*, 10102.

[4] M. Kaya, Y. Toyama, K. Kubota, Y. Nodasaka, M. Ochiai, M. Nomizu, N. Nishi, *Int. J. Biol. Macromol.* **2005**, *35*, 39.

[5] S. Rikimaru, Y. Wakabayashi, N. Nomizu, N. Nishi, *Polym. J.* **2003**, *35*, 255.

[6] C. Zhao, X. D. Liu, M. Nomizu, N. Nishi, *Biomaterials* **2003**, *24*, 3747.

[7] S. A. Lacks, *J. Biol. Chem.* **1981**, *256*, 2644.

[8] T. Yasuda, H. Takeshita, T. Nakajima, K. Mogi, Y. Kaneko, M. Ueki, R. Iida, K. Kishi, *FEBS Lett.* **2002**, *510*, 22.

[9] R. Fuller, *J. Appl. Bacteriol.* **1989**, *66*, 365.

[10] R. A. Gianella, S. A. Broitman, N. Zamcheck, *Gut* **1972**, *13*, 251.

[11] B. Jonganurakkun, X. D. Liu, Y. Nodasaka, M. Nomizu, N. Nishi, *J. Biomater. Sci. Polymer Edn.* **2003**, *14*, 1269.

[12] R. Ranganathan, T. A. Nicklas, S. J. Yang, G. S. Berenson, *J Am Diet Assoc.* **2005**,

105, 1391.

[13] E. G. Richardson, *J. Colloid Sci.* **1950**, *5*, 404.

[14] L. Axelsson, "Lactic Acid Bacteria: Clssification and Physiology", in: *Lactic Acid Bacteria*, 2nd Edition, S. Salminen, A. V. Wright, Eds., Marcel Dekker, New York 1998, p. 1.

[15] A. Samona, R. K. Robinson, S. Marakis, *Food Microbiol*. **1996**, *13*, 275

[16] R. P. Ross, S. Morgan, C. Hill, *Int J Food Microbiol.* **2002**, *79*, 3.

[17] E. Al-Kadamany, I. Toufeili, M. Khattar, Y. Abou-Jawdeh, S. Harakeh, T. Haddad, *J. Dairy Sci.* **2002**, *85*, 1023

Figure Captions

Table 1. Bacterial strains used in present study.

[a]) Chr. Hansen Lab, Christian Hansen Laboratory.

[b]) JCM, Japan Collection of Microorganisms.

Figure 1. Scanning electron micrographs of the DNA-based gels showed (a) bacteria were fixed in the hydrogels, (b) oil particles embedded in the complex gel, (c) exposing of bacteria gathered in the oil particle after incubating the complex gel at 37°C overnight.

Figure 2. Survival of the 6 probiotic strains in 2h and 4h simulated gastric juice incubations, in compare between the protection capability of the hydrogels and the complex gels.

Figure 3. Gels stability during storage at 4°C. The hydrogels and the complex gels were kept refrigerated and the survival of bacteria in each gel was measured during 12 weeks of storage.

Figure 4. Food-graded hydrogels (a) effect of food-graded components on the survival of bacteria; each gel prepared by altering with a new commercial lot DNA, food-graded *κ*-carrageenan and food-graded gelatin; control was the hydrogel prepared by lab-graded components; the gels were incubated in simulated

gastric juice and measured for the survival of bacteria at 2h and 4h of incubation, (b) optimization of the food-graded gelatin; *the basis is the quantity of water in the gel composition; survival of bacteria by the protection of the gels in varying of gelatin quantities was measured after 2h incubation in simulated gastric juice.

Bacteria	Source
Commercial strains	
B. lactis Bb-12	Chr. Hansen Lab, Denmark
Lc. lactis subsp. *cremoris* and	Chr. Hansen Lab, Denmark
Lc. lactis subsp. *lactis* R-704	
Type strains	
B. longum 1217	JCM, Japan
B. bifidum 1255	JCM, Japan
L. gasseri 1131	JCM, Japan
L. acidophilus 1132	JCM, Japan

Table 1

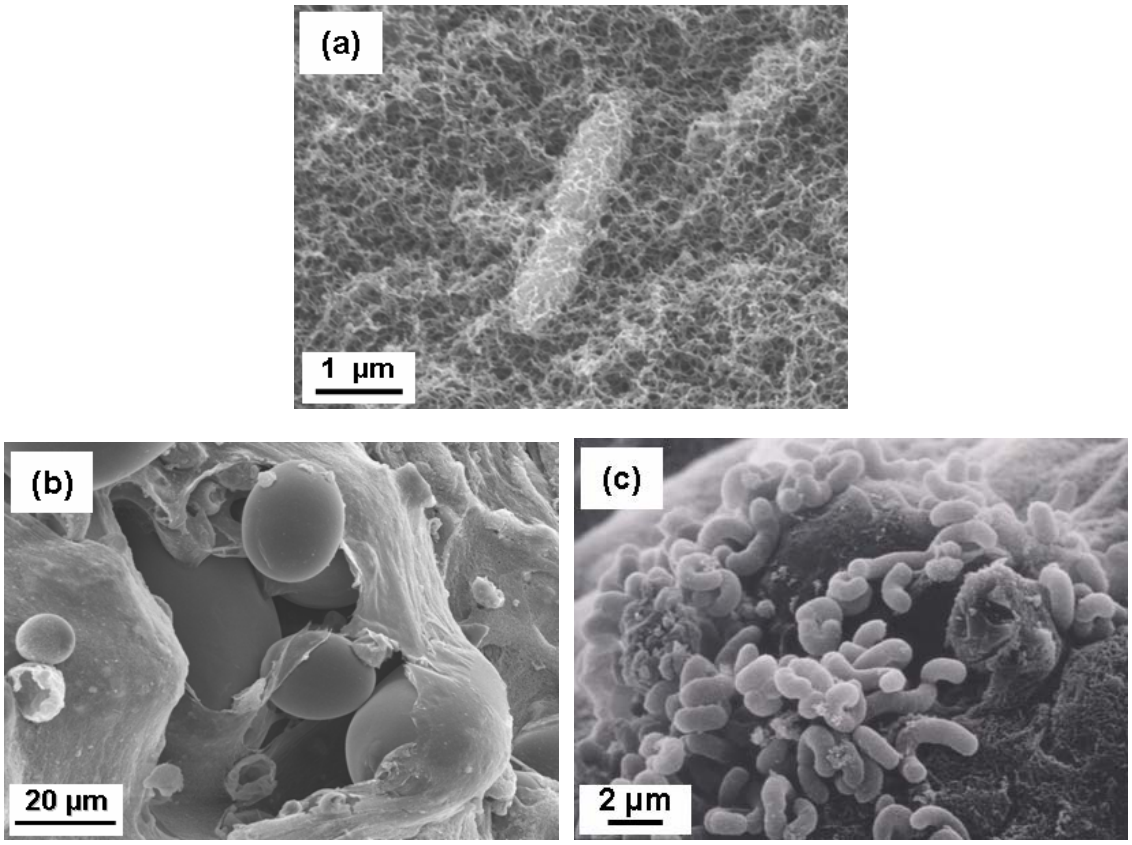

Figure 1

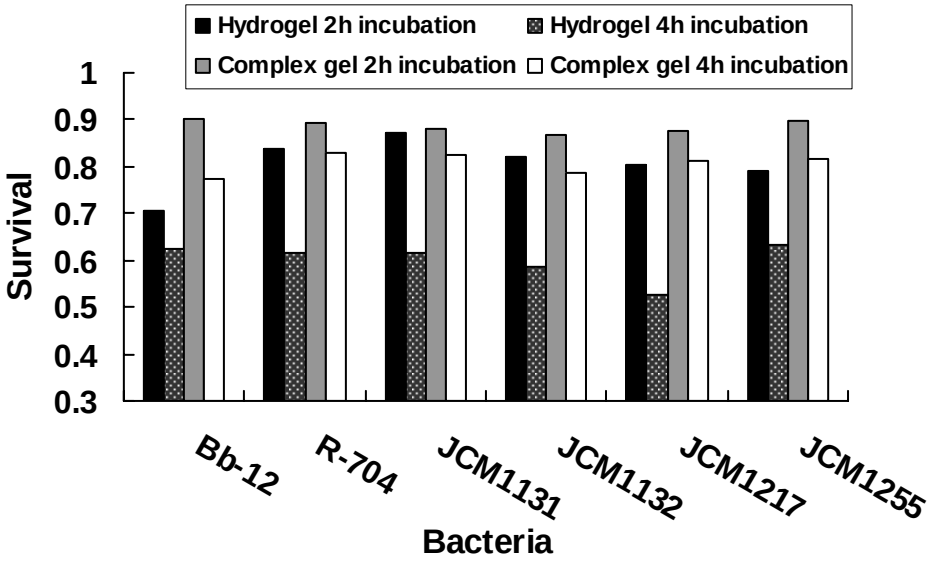
Hydrogel 2h incubation
Hydrogel 4h incubation
Complex gel 2h incubation
Complex gel 4h incubation
Survival
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
Bb-12
R-704
JCM1131
JCM1132
JCM1217
JCM1255
Bacteria

Figure 2

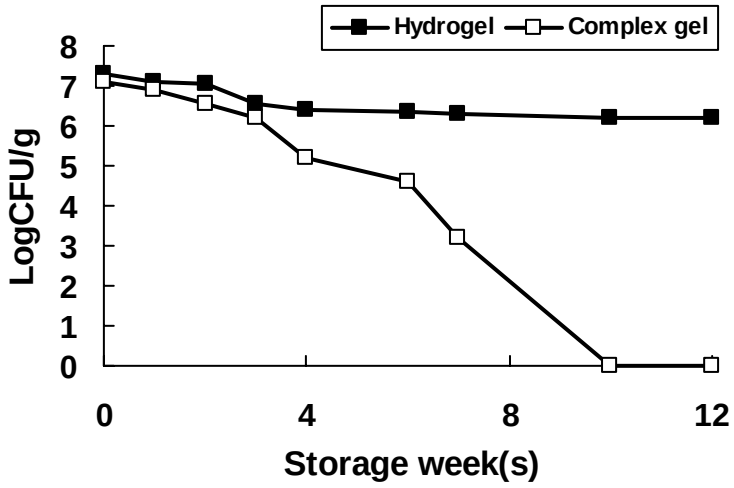
Hydrogel
Complex gel
LogCFU/g
8
7
6
5
4
3
2
1
0
0
4
8
12
Storage week(s)

Figure 3

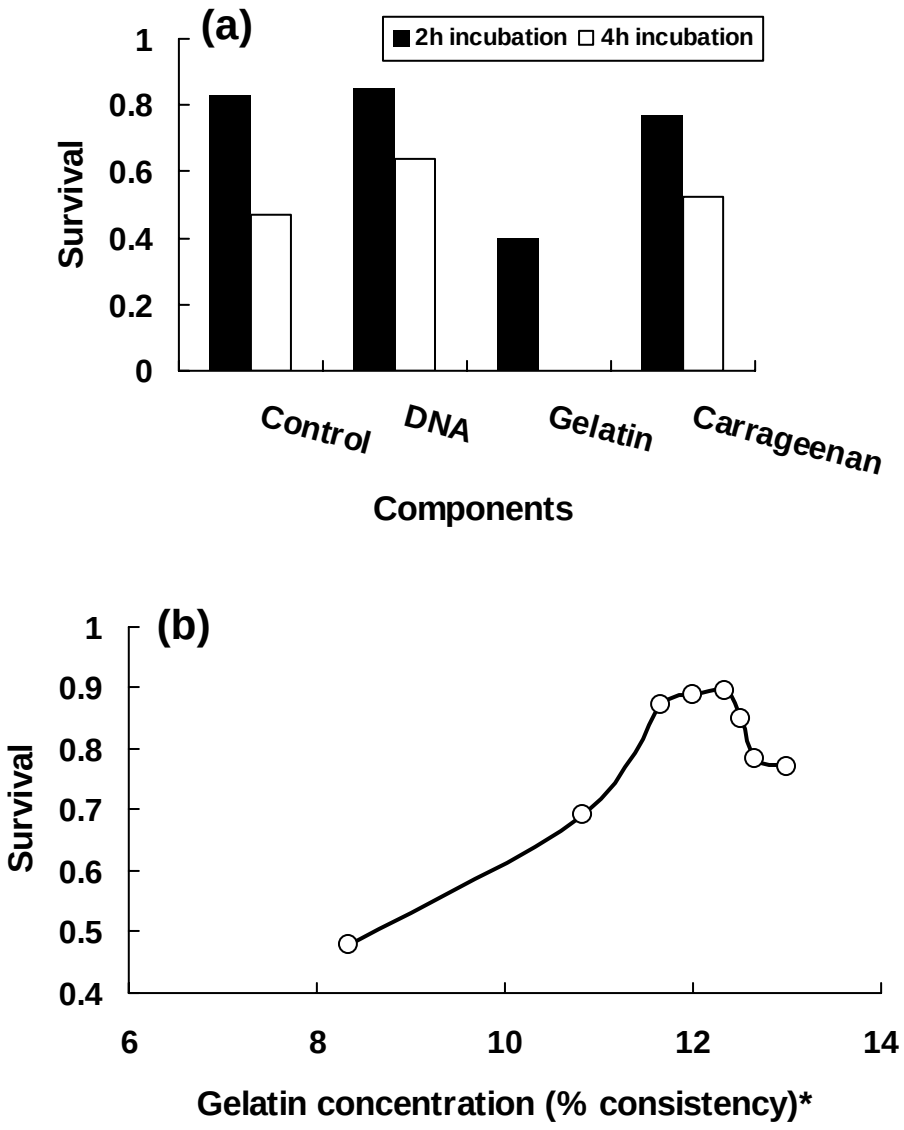
(a)
2h incubation
4h incubation
Survival
1
0.8
0.6
0.4
0.2
0
Control
DNA
Gelatin
Carrageenan
Components
(b)
Survival
1
0.9
0.8
0.7
0.6
0.5
0.4
6
8
10
12
14
Gelatin concentration (% consistency)*

Figure 4

Table of Contents

DNA-based gels were prepared from single-stranded salmon milt DNA, gelatin and κ-carrageenan. These components could form a complex in low acidity (pH 1.2) medium. The gels were used as the protective vehicle for the probiotic bacteria from simulated gastric acidity. Food-graded gels were produced and optimized to provide a highly protective capability for human oral delivery.