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β-Lactoglobulin tablets as a suitable vehicle for protection and intestinal delivery of probiotic bacteria

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ABSTRACT

The use of succinylated β-lactoglobulin as a novel functional tablet excipient for the protection of probiotic bacteria against the adverse gastric conditions and their delivery in the intestine was studied. Tablets were produced by direct compression of a dry mixture of *Bifidobacterium longum* HA-135 and the tested excipient. The results showed that tablets made of native β-lg did not ensure cell survival while grafting carboxylic acid groups on the protein revealed to be an innovative method to create a gastroresistant matrix that could allow the survival of up to 10^8 CFU and 10^7 CFU after 1 h and 2 h gastric incubation, respectively. When compared to other polymers, succinylated β-lg promoted the best survival both upon compression and after simulated gastric passage. The proportion of succinylated β-lg in the formulation could be lowered to 60% without modifying the protective ability of the matrix. Additionally, the tablets proved to be stable over a period of 3 months when refrigerated. Succinylated β-lg tablets are an interesting vehicle for the protection of acid-sensitive bacteria during transit in the upper gastro-intestinal tract.

1. Introduction

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host, as defined by the Food and Agriculture Organization and the World Health Organization (FAO/WHO, 2006). Since Metchnikoff (2004) first exposed his thoughts on the benefits of ingesting specific bacterial strains a hundred years ago, probiotics have made their way through the nutraceutical sector to become a multibillion dollars a year market (BCC Research, 2008). Global interest is linked to the numerous studies that have attributed, and continue to attribute, several health benefits to probiotic-containing products. These beneficial actions range between the alleviation of lactose intolerance, the reduction of symptoms caused by viral and antibiotic associated diarrhea, the modulation of the immune system, the prevention of inflammatory bowel disease and the reduction of risks associated with mutagenicity and carcinogenicity (Fooks et al., 1999; Saxelin et al., 2005; Vasiljevic and Shah, 2008).

However, to confer such health benefits to the host, probiotic microorganisms must be viable when they reach their site of action, meaning that they must survive the adverse conditions encountered in the upper gastro-intestinal tract (Mattila-Sandholm et al., 2002; Del Piano et al., 2006; Ding and Shah, 2007). One of the main issues in probiotic research is therefore to develop strategies to ensure survival during gastric transit and during storage of the product containing beneficial microorganisms. The design of tablets made of functional polymers to improve the stability and survival of probiotics is currently gaining attention, as they allow accurate dosage, ease of administration, good patient acceptance, stability upon storage and large-scale production (Klayraung et al., 2009). Different polymers have been studied to form the protective matrix, namely sodium alginate in combination with hydroxypropylcellulose (Chan and Zhang, 2002, 2005), carboxymethyl high amylose starch (Calinescu et al., 2005), carboxymethyl high amylose starch in combination with chitosan (Calinescu and Mateescu, 2008), hydroxypropylmethylcellulose acetate succinate (Stadler and Viernstein, 2003) and hydroxypropylmethylcellulose phthalate (Klayraung et al., 2009). In all cases, the tablets had the capacity to maintain their physical integrity in gastric fluid, minimize the penetration of solvent at acidic pH values and permitted some survival of the encapsulated bacteria.

Our research group recently demonstrated the effective use of succinylated food proteins, including β-lactoglobulin, as natural tablet excipients to protect acid-labile compounds from the gastric environment and to delay the release of active molecules (Caillard et al., 2009, 2011). β-Lactoglobulin is the main component of whey proteins. Thus, β-lactoglobulin constitutes a natural and largely available material. Moreover, β-lactoglobulin is a co-product from dairy industry that makes it a low cost biopolymer. Finally, because

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of its dairy origin, β-lactoglobulin seems well adapted to the protection of dairy bacteria such as probiotics.

The aim of the present work was therefore to extend the use of these novel excipients to the protection of probiotic microorganisms during the gastrointestinal transit. To achieve this, matrix-type tablets made of succinylated β-lactoglobulin and freeze-dried *Bifidobacterium longum* were characterized regarding their processability, their ability to maintain bacterial viability during gastric incubation and their stability in time. *B. longum* was chosen because of its sensibility to gastric pH (Boylston et al., 2004). Moreover, *B. longum* is a well recognized probiotic and is one of the 16 species eligible for probiotic claims in Canadian regulation (Health Canada, 2009).

2. Materials and methods

2.1. Materials

Freeze-dried *B. longum* HA-135, the model probiotic bacterium used in the present work, was kindly provided by Harmonium International (Mirabel, QC, Canada). BioPURE β-lactoglobulin (β-lg) isolate was donated by Davisco Foods International (Le Sueur, MN, USA). MRS agar, according to de Man, Rogosa and Sharpe, and cysteine hydrochloride used to prepare the culture media were purchased from EMD Chemicals (Gibbstown, NJ, USA) and Sigma–Aldrich (St. Louis, MO, USA) respectively. Pepsin from porcine gastric mucosa and other chemicals used for dissolution experiments, peptone water, sodium alginate and succinic anhydride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pectin DE35 was obtained from CP Kelco (Lille Skensved, Denmark) and hydroxypropylmethylcellulose acetate succinate (AQOAT) was given by Shin-Etsu Chemical Co. (Tokyo, Japan). Alginate (Alginic acid sodium salts) from brown algae was purchased from Sigma Aldrich (St. Louis, MO, USA). Gelatin gelcaps were bought from a local pharmacist.

2.2. Succinylation of β-lactoglobulin

β-Lactoglobulin was succinylated at levels of 50% and 100% using succinic anhydride according to the method of Caillard et al. (2009, 2011) for soy proteins with slight modifications. Briefly, succinic anhydride was added gradually to a 10% β-lg solution maintaining the pH value between 8.0 and 8.5 using 2 M NaOH. Once all the succinic anhydride was added and the pH stabilized, the solutions were dialyzed overnight at 4 °C using 1 kDa membranes. The solutions were finally freeze-dried and ground to a fine powder.

2.3. Tablet preparation

Tablets with a constant weight of 400 mg were prepared by direct compression of a homogeneous mixture of excipient and freeze-dried *B. longum* using a Carver press equipped with a 13 mm diameter flat-faced punch (Autopellet Laboratory press, Carver Incorporation, Wabash, IN, USA). All the punch pieces were disinfected with 70% ethanol before each tablet was produced. Unless otherwise specified, all tablets were formed at an applied pressure of 67 MPa.

2.4. Dissolution media

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the US pharmacopoeia (The United States Pharmacopoeial Convention, 2004). SGF consisted of 2 g NaCl dissolved in 993 mL HPLC grade water and 7 mL 37% hydrochloric acid (final pH of 1.2). 3.2 g (924 units/mg of protein) pepsin were added to 1000 mL of SGF. SIF was prepared by dissolving 6.8 g monobasic potassium phosphate in 250 mL HPLC-grade water to which were added 190 mL 0.2 M sodium hydroxide and 400 mL HPLC grade water. The pH was adjusted to 7.5 ± 0.1 with 0.2 M NaOH and the final volume was brought to 1000 mL with HPLC grade water. Dissolution steps in SIF were conducted without the addition of pancreatin as it did not influence cell viability. Preliminary experiments showed that incubation of *B. longum* in SIF with ($10\,g\,L^{-1}$) or without pancreatin resulted in a difference of 0.1 log in the number of viable cells, a difference that proved to be statistically non-significant.

2.5. Bacterial mortality during tabletting

To determine the relationship existing between the applied pressure and bacterial mortality, 400 mg tablets composed of 10% freeze-dried *B. longum* and 90% native β-lg were formed at pressure values ranging from 30 to 300 MPa. After compression, the tablets were immediately dissolved in 100 mL simulated intestinal fluid. The resulting suspension was serially diluted in 0.1% sterile peptone water and 1 mL of each dilution was plated in duplicate in MRS agar supplemented with 0.05% cysteine hydrochloride (MRS-C). The Petri dishes were incubated for 48 h at 37 °C in a 2.5 L AnaeroJar (Oxoid Ltd, Hampshire, UK) using an AnaeroGen sachet (Oxoid Ltd, Hampshire, UK) to create an anaerobic environment. The colony forming units (CFU) were finally enumerated.

The initial number of CFU in 40 mg lyophilised *B. longum* was determined by following the same procedure without compressing the powder mixture.

2.6. Survival to gastric conditions

Tablets made of 360 mg native β-lg, 50% succinylated β-lg or 100% succinylated β-lg and 40 mg lyophilized *B. longum* were incubated in 400 mL simulated gastric fluid with pepsin at 37 °C for 30, 60 or 120 min. An incubator shaker (Lab Line Instruments Inc., Melrose Park, IL, USA) was used to promote a constant agitation of 160 rpm. Immediately after gastric incubation, the tablets were transferred to 25 mL simulated intestinal fluid and dissolved. To determine the number of viable bacteria remaining after gastric passage, culture and enumeration were conducted as described previously. If the tablet was completely dissolved during gastric incubation, no transfer to SIF was possible and the SGF was used as the mother suspension for the following steps.

As a control, 40 mg non-compressed freeze-dried *B. longum* was treated following the same procedure.

2.7. Comparison to other excipients

Other polymeric excipients were studied regarding their ability to prevent bacterial mortality during gastric incubation. The studied dosage forms were tablets composed of 360 mg of sodium alginate, pectin, or AQOAT and 40 mg bacterial lyophilizate, tablets combining 216 mg AQOAT mixed with 144 mg sodium alginate and 40 mg freeze-dried *B. longum* as well as gelatin gelcaps filled with 40 mg freeze-dried *B. longum* and 360 mg methylcellulose. 24 h after tablets preparation, tablets specific volumes were calculated as an approximation of tablets initial porosity. The weight of each tablet was determined and the height of tablets was measured using a Mitutuyo Indicator (Mitutuyo, Japan). Tablet specific volume (V_{sp}) was calculated as follows:

$$V_{sp} = \frac{\pi \times r^2}{w}$$

where w is the tablet weight, h is the tablet height and r is the tablet radius (6.5 mm). Each measurement was performed in triplicate.

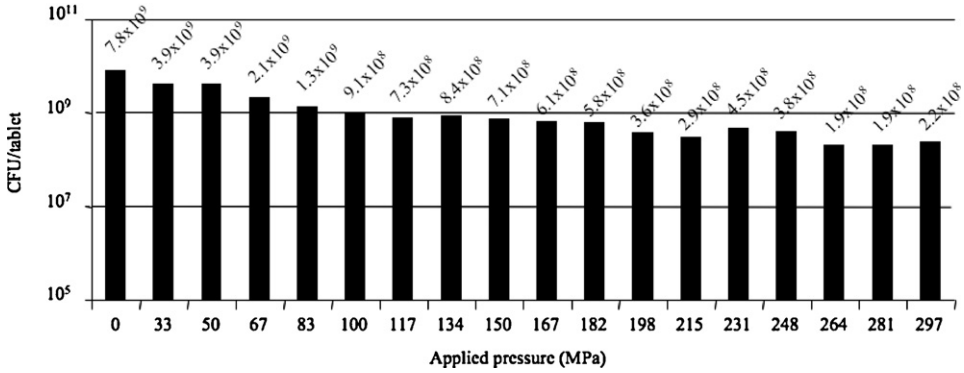

Fig. 1. Effect of the applied pressure to form the tablet on cell viability.

Tablets and gelcaps were incubated in 400 mL SGF with pepsin at 37 °C for 60 min under a constant agitation of 160 rpm and then transferred in 25 mL SIF for complete dissolution. Serial dilutions were plated in MRS-C, the Petri dishes were incubated as described before and viable bacteria were enumerated. Again, if the dosage form completely dissolved during the gastric step, SGF was used as the initial bacterial suspension.

2.8. Tablet loading rate

The impact of the β-lg/*B. longum* ratio on bacterial survival to gastric conditions was studied for tablets (400 mg) with loading rates of 10%, 20% and 40%. Native β-lg, 50% succinylated β-lg and 100% succinylated β-lg were used as excipients. The initial content in bacteria was determined by immediately dissolving the tablet upon formation and following the culture and enumeration protocols already described. To evaluate the degree of mortality in acidic environments, tablets were incubated in SGF with pepsin for 60 or 120 min at 37 °C under a constant agitation of 160 rpm. They were then transferred to 25 mL SIF, totally dissolved and the culture and enumeration procedures were done as previously described.

2.9. Storage stability

400 mg tablets containing 10% freeze-dried *B. longum* were stored in amber bottles at room temperature and 4 °C for a period of 3 months. Sample tablets were taken out periodically (1, 2, 3, 4, 5, 6, 8 and 12 weeks), dissolved in 100 mL SIF and bacterial viability was assessed following the aforementioned protocol.

2.10. Statistical analysis

Statistical analyses were conducted using JMP software (version 7.0.1, SAS Institute, Cary, NC, USA). Data were compared using the Student *t*-test ($P < 0.05$).

3. Results and discussion

3.1. Bacterial mortality during tabletting

Fig. 1 presents the evolution of cell viability inside the tablets as a function of the applied pressure to form the tablets. As previously reported by other authors (Plumpton et al., 1986a,b; Blair et al., 1991; Chan and Zhang, 2002), increasing the compression force resulted in increasing the mortality level. In fact, compared to the non-compressed sample, 50% of the cells died when applying a pressure of 33 MPa while the mortality rate went up to 97% at the highest tested pressure of 297 MPa; the overall reduction of viable bacteria was therefore of 1.6 log. The relationship existing between bacterial survival and pressure was however not linear: 90% of the viable cells reduction was observed from 0 to 117 MPa and the remaining 7% mortality was observed between 117 and 300 MPa. This goes hand in hand with the results obtained by Caillard and Subirade (submitted for publication), which showed that the specific volume of β-lg tablets diminished predominantly up to a pressure of 130 MPa. Plumpton et al. (1986b) indicated that the lethal effect of compression is attributable to the shearing forces caused by interparticulate movement and the pore size reduction inside the matrix, leading to mechanical damage and death of the cells. Thus, the highest extent of mortality was observed in the pres-

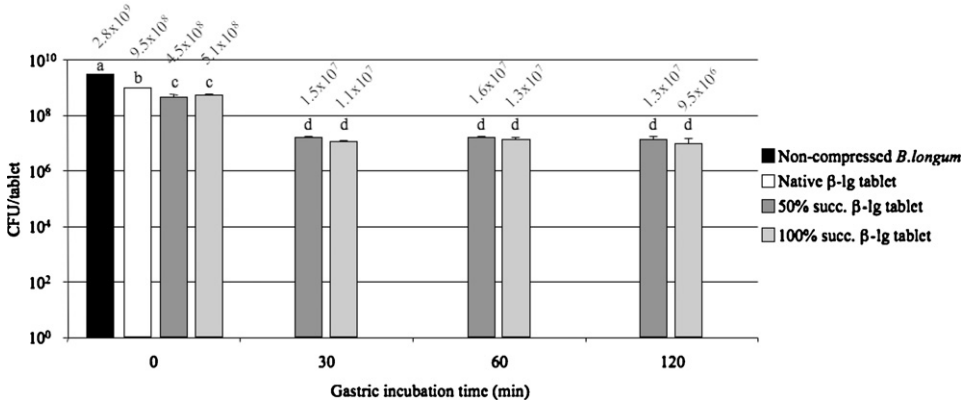

Fig. 2. Bacterial survival after gastric incubation (30, 60 or 120 min) of tablets made of native β-lg, 50% succinylated β-lg or 100% succinylated β-lg. Columns with different letters are significantly different ($P < 0.05$).

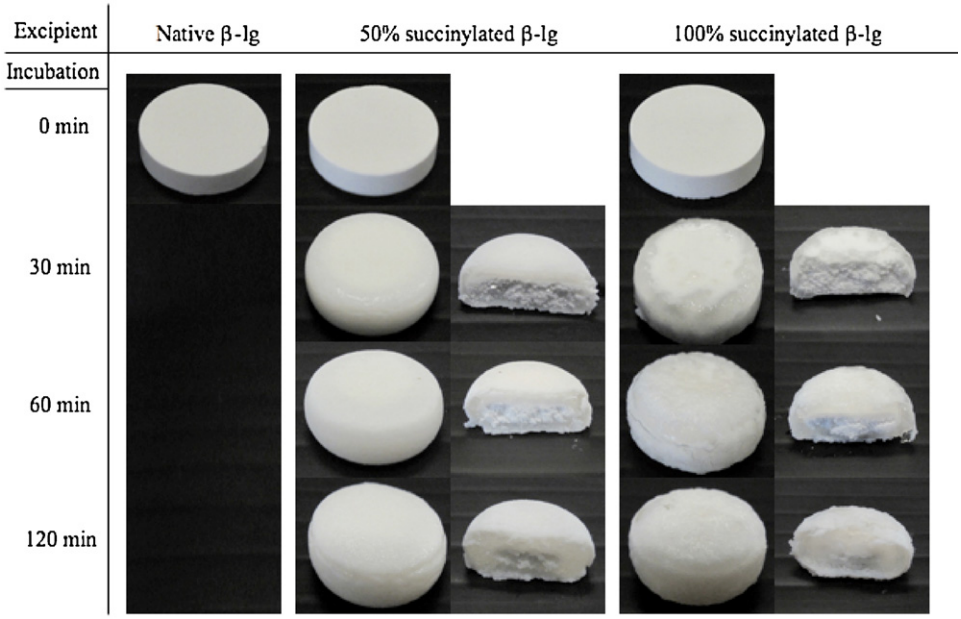

Fig. 3. Pictures of tablets made of native, 50% succinylated or 100% succinylated β-lg before and after incubation in simulated gastric fluid with pepsin for 30, 60 or 120 min.

sure range corresponding to the most drastic decrease in specific volume, i.e., reduction of the pore size inside the tablet. At higher pressure values, when maximal compression was reached, particle rearrangement was hindered and shear forces were weakened, explaining the relatively small degree of bacterial kill (Blair et al., 1991).

For the following experiments, the working pressure was chosen to be 67 MPa as rigid and easy to manipulate tablets were obtained. Moreover, these tablets contained 10^9 viable bacteria, a viability level that meets the general requirement of 10^8–10^9 living cells per serving for effective probiotic products (Lahtinen et al., 2006).

3.2. Survival to gastric conditions

The *B. longum* content of β-lactoglobulin tablets before and after gastric incubation is presented in Fig. 2. When the freeze-dried bacteria was not compressed, the number of viable cells decreased radically, passing from 2.8×10^9 to an undetectable level after only 30 min in SGF with pepsin. This result was expected, as bifidobacteria are known to be sensitive to acidic environments (Takahashi et al., 2004; Anal and Singh, 2007). Compacting the lyophilizate as a tablet in a native β-lg matrix did not improve bacterial survival during gastric incubation. After only 30 min in simulated gastric fluid, the tablet was completely dissolved as shown in Fig. 3 and no viable cells could be enumerated. In its native form, β-lg thus failed to demonstrate any protective ability against the harsh gastric conditions. Fig. 2 however indicates that β-lg succinylation had a marked effect on *B. longum* survival. In fact, using succinylated protein at a level of 50% or 100% as excipient resulted in the survival of 10^7 CFU/tablet after gastric incubation, corresponding to a 1.5 log reduction of the number of viable cells. Moreover, Fig. 2 shows that this extent of viability was measured independently of the duration of the gastric passage. Chan and Zhang (2005) also observed an exponential decrease in cell viability at the beginning of the exposure to SGF and a subsequent stabilization. The difference in behavior between the native and modified proteins is a consequence of the grafted carboxylic groups. Replacing positively charged lysine by negatively charged carboxylic groups lowered both the protein's isoelectric point (pI) and it's solubility at pH values under the pI, as reported by Caillard et al. (2009, 2011). In acidic environments, the carboxylate groups were protonated and lead to a compact structure that ensured local buffering properties (Calinescu et al., 2005; Caillard et al., 2011). A gelled layer formed at the surface of the tablets shortly after they were put

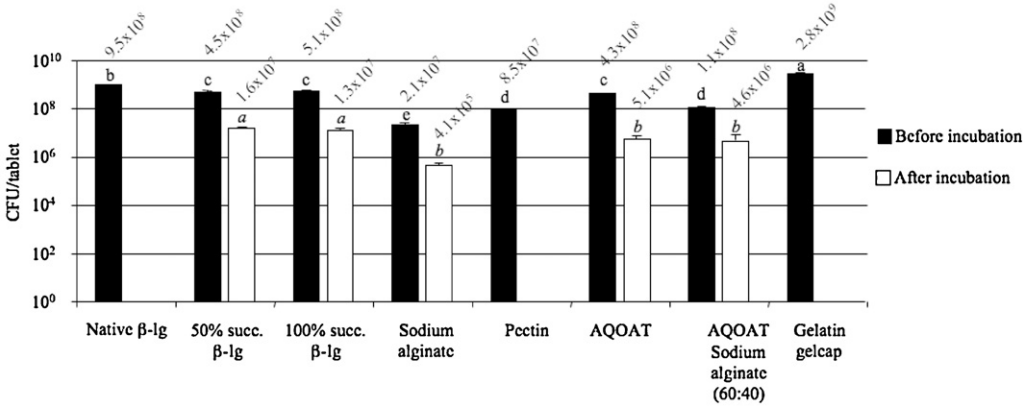

Fig. 4. Comparison of different excipients regarding their ability to maintain bacterial viability after 60 min gastric incubation. Columns with different letters are significantly different ($P < 0.05$); values were compared before and after incubation.

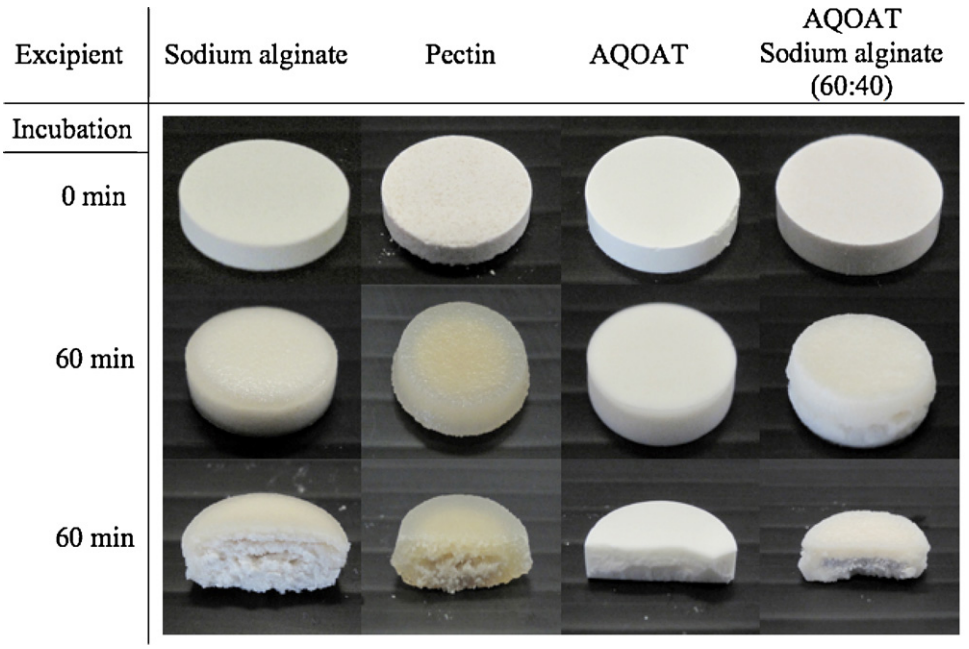

Fig. 5. Pictures of tablets made of sodium alginate, pectin or AQOAT before and after 60 min incubation in simulated gastric fluid with pepsin.

in SGF. This layer, which slowed down the solvent uptake and the tablet swelling, combined to the buffering capacity of the matrix are the factors explaining the protection and high survival of *B. longum* even after 120 min incubation. The formation of a hydrogel around the cell pellet was also given as the explanation for bacterial protection in the works of Chan and Zhang (2005). The observed mortality is thought to be that of the cells situated on the very surface of the tablet and therefore not taking advantage of the protective ability of the matrix. The external and internal appearances of the tablets at different stages of incubation are pictured in Fig. 3. The gelled layer formed on succinylated β-lg tablets thickened with time at pH 1.2, but even after 2 h in SGF the core was still dry and powdery.

The survival rates observed for succinylated β-lg tablets were found to be in the same range of those of previous studies regarding tablets reported in the literature, namely between 10^4 and 10^8 CFU (Chan and Zhang, 2002, 2005; Stadler and Viernstein, 2003; Calinescu et al., 2005; Calinescu and Mateescu, 2008; Klayraung et al., 2009). Works regarding probiotics protection are however difficult to compare as none of them study the same bacterial strain, which impacts strongly on the behavior in acidic environments. Additionally, the survival of the unprotected bacterium when put in gastric media is not systematically stated, which makes it difficult to fully assess the protective capacity of the compressed matrix.

3.3. Comparison to other excipients

In order to position succinylated β-lg tablets regarding their protective capacity against gastric conditions, tablets containing *B. longum* HA-135 were made with other polymers. The bacterial content upon tablet formation and after 60 min incubation in SGF of the different dosage forms is presented in Fig. 4. As shown, the nature of the polymeric excipient used to produce the tablet modified the bacterial content after compression at 67 MPa. Compared to non-compressed freeze-dried bacteria, native β-lg induced the lowest mortality upon compression with a reduction of 0.5 log while sodium alginate caused a viable cells reduction of 2.1 log. Succinylated β-lg and AQOAT showed a similar mortality of 0.8 log. Blair et al. (1991) previously reported that in addition to the applied compression force and the bacterial strain, cell mortality during tabletting is related to the excipient used to form the tablet matrix. In fact, the size of the powder particles and their physical behavior under high pressure, namely brittle fracturing and/or plastic deformation, have an important influence on the extent of bacterial killing (Plumpton et al., 1986b; Blair et al., 1991). In addition to a cell mortality excipient related phenomenon, it is thought that initial mortality would be connected to tablet initial porosity. While porosity is diminished, cell mortality would be logically increased (Plumpton et al., 1986b). Tablet initial porosity is proportional to the ratio between tablet density ($1/V_{sp}$) and polymer true density (that is not known here) (Zhao et al., 2006). When the powder bed densifies to a greater extent, it eliminates more of the air in the powder bed and voids in individual particles. The increased densification process (the decrease in tablet specific volume) results in a tablet with lower porosity (Crowley et al., 2004). Consequently, for each tablet, specific volume was measured as an approximation of tablet initial porosity. These results are reported in Table 1. It appeared that globally, these data would fit our initial mortality measurements: the higher the tablet specific volume, the lower the initial mortality (see Table 1 *versus* Fig. 4).

The nature of the polymer used to protect the bacteria also had an impact on the survival rate observed after incubation in simulated gastric media. Fig. 5 presents pictures of the tablets before and after incubation in SGF. The encapsulation of *B. longum* HA-135 in a gelatin gelcap, a widely used oral dosage form, or in a tablet made of pectin did not allow survival of the bacteria. The gelcap quickly hydrated and dissolved after immersion in SGF after what no cultivable cells could be grown. In the case of the pectin tablet, it is interesting to note that even if it did not dissolve in SGF, it did not prove to have any protective capacity. This therefore indicates that

Table 1
Tablets specific volume (V_{sp}) as a function of the polymer used to form the tablet.

	V_{sp} (cm^3 g^{-1})	*t*-test results
Native β-lg	0.932 ± 0.007	a
50% succ. β-lg	0.893 ± 0.003	b
100% succ. β-lg	0.894 ± 0.003	b
Sodium alginate	0.860 ± 0.008	c
Pectin	0.845 ± 0.019	c
AQOAT	0.897 ± 0.007	b
AQOAT-Sodium alginate (60:40)	0.841 ± 0.027	c

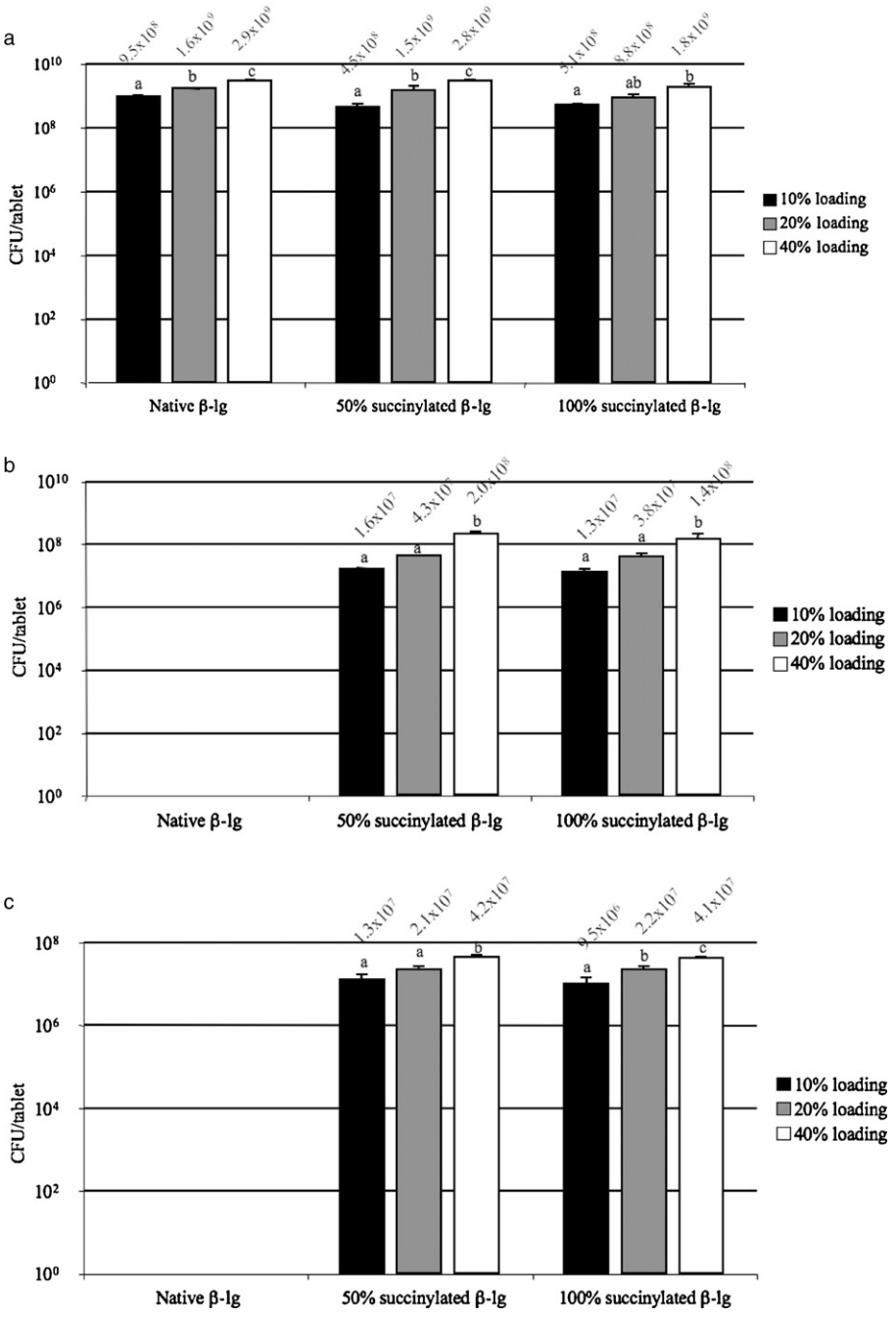

Fig. 6. Influence of the loading rate on the bacterial content of tablets made of native β-lg, 50% succinylated β-lg or 100% succinylated β-lg (a) after compression, (b) after 60 min gastric incubation and (c) after 120 min gastric incubation. Columns identified with different letters are significantly different ($P < 0.05$); values were compared for each excipient.

the nature of the polymer and its buffering capacity are decisive factors to ensure cell viability after gastric transit. Tablets made of succinylated β-lg at levels of 50 or 100%, sodium alginate, AQOAT or a mix of both all showed a similar protective ability. In each case, the reduction in the number of viable cells was of approximately 1.5 log. This similitude is thought to be linked to the homology of the functional groups bore by the polymers. In fact, these polymers have in common their numerous carboxylate groups responsible for their low solubility in acidic media and their buffering capacity, hence their ability to preserve bacterial viability in SGF. However, given that the tablets initial content in live bacteria was different for each excipient, there was an important difference in the number of cultivable cells after gastric incubation. Survival levels of 10^5, 10^6 and 10^7 were measured for a matrix made of sodium alginate, AQOAT and succinylated β-lg, respectively. Thus, succinylated β-lg proved to be the excipient that permitted to preserve the highest number of viable bacteria both after tabletting and incubation in simulated gastric fluid for 60 min.

3.4. Tablet loading rate

As a way to deliver an increased number of viable probiotic bacteria to the intestine but also to evaluate the critical excipient content of tablets required to still observe gastric protection, different *B. longum* loading rates were studied. Fig. 6a, which presents the results of the initial content in bacteria right after tabletting,

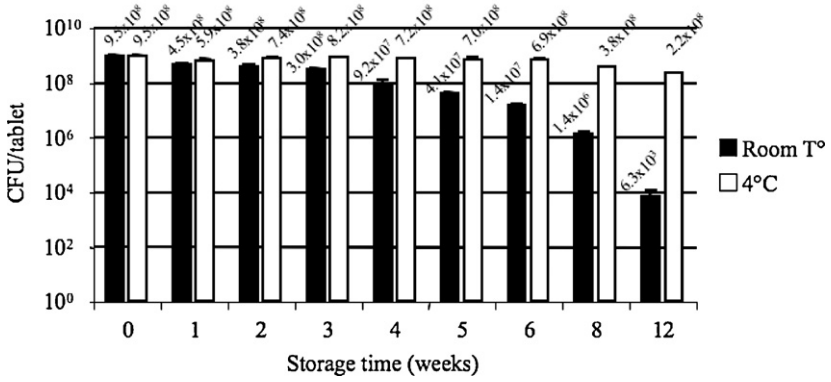

Fig. 7. Effect of storage time and temperature on bacterial viability inside β-lg tablets.

reveals that increasing the proportion of freeze-dried bacteria in the formulation resulted in a proportional increase of the viable cells content of tablets made of either native or succinylated β-lg. In fact, the number of cultivable bacteria doubled when raising the lyophilizate quantity from 10 to 20% and doubled again when passing from a loading rate of 20% to 40%. The same phenomenon was outlined by Klayraung et al. (2009) in their work on mixed hydroxypropylmethylcellulose phtalate/*Lactobacillus fermentum* tablets with loading rates varying between 10 and 44%. Fig. 6b presents the survival of bacteria after 60 min in simulated gastric fluid for the different loading rates and excipients studied. When native β-lg was used to form the matrix, no bacterial survival could be measured no matter what the proportion of lyophilizate was. On the contrary, high levels of survival were noted when the succinylated protein was used. Tablets containing 20% freeze-dried *B. longum* did not lead to a survival level that was significantly higher than tablets containing 10% bacteria. However, at a loading rate of 40%, the increase was significantly more important: the number of viable cells was 11 times higher compared to a loading rate of 10%. This is in accordance with the works of Klayraung et al. (2009) who noted that survival was increased when the proportion of freeze-dried bacteria in the formulation was augmented. Mention was also made that this increase in survival was not proportional to the actual increase in the lyophilizate content as passing from 10 to 20% and from 10 to 40% lyophilizate in the tablet resulted in survival levels 10× and 25× higher, respectively. The authors explained these results by the cell–cell interactions that could improve the protection against external stresses and the decrease of the adverse effects that the polymeric excipient could have on the bacterial cell surface. In our case, the phenomenon is thought to be associated to the low solubility of the lyophilizate. Combined with the low solubility of succinylated β-lg at low pH values, it limited even more the penetration of SGF inside the matrix and therefore improved the protective capacity of the tablet and the survival level of *B. longum*. After 120 min incubation, higher loading rates also lead to higher survival levels for succinylated β-lg tablets, as shown in Fig. 6c. Contrary to the results obtained after 60 min, the survival increase factor corresponded to the increase in the loading rate. Survival was therefore two times higher at 20% loading and four times higher at 40% loading. These results indicate that it is possible to decrease the proportion of the functional excipient down to 60% while keeping the protective capacity of the matrix. It also points out that there is place for minor ingredients that could be needed to improve the processability of the formulation for large-scale production of tablets.

3.5. Storage stability

Fig. 7, which presents the evolution of the number of viable bacteria inside β-lg tablets stored for 3 months, indicates that the storage temperature had a major impact on bacterial survival as established before for freeze-dried bacteria by Champagne et al. (1996). When stored at 4 °C, the survival of *B. longum* inside the tablets was high: the global reduction in the number of viable cells over 12 weeks was of 0.6 log. On the opposite, increasing the storage temperature to room temperature resulted in a high level of mortality, specifically a reduction of 5.2 log. In their work on *Lactobacillus acidophilus* tablets, Chan and Zhang (2002) reported a mortality of 2 log after 5 weeks storage at 25 °C which is in agreement with our results after the same period at room temperature.

This effect would be connected to the impact of redox potential on probiotic storage. During our experiments, there is no reason to think that the atmosphere composition was different between our refrigerator and the laboratory. But it is well known that the presence of oxygen (positive redox potential) in probiotic-containing products can have a detrimental effect on the viability of probiotics and more specifically of *Bifidobacterium* spp., that are anaerobic (Vasiljevic and Shah, 2008). As every chemical reaction, oxidation is temperature-dependent (Arrhenius law). Consequently, the higher mortality at room temperature than at 4 °C would be simply due to the increase of oxidation phenomena at 25 °C.

These results indicate that the formulated tablets are stable when refrigerated, a common practice for products containing probiotic microorganisms. However, oxygen impermeable packaging or "modified" atmosphere storage conditions would be also interesting in order to improve product stability.

4. Conclusion

To sum it up, succinylated β-lactoglobulin revealed to be a suitable natural excipient to form tablets containing probiotic bacteria and promote their survival against gastric conditions. In fact, it was possible to produce rigid tablets by direct compression with a bacterial content as high as 10^9 CFU. While native β-lg did not protect *B. longum* in gastric fluid, grafting carboxylic acids via succinylation modified the protein's physico-chemical properties and consequently its behavior in acidic medium resulting in survival of up to 10^8 CFU after 1 h incubation and 10^7 CFU after 2 h. The level of succinylation (50% or 100%) had no significant impact on the properties of the matrix. It was also demonstrated that the functional excipient proportion could be lowered to 60% and the same protective effect was measured. Moreover, among all the polymers tested to form the tablets' matrix, succinylated β-lg was the one showing the better properties in terms of survival. Finally, such tablets proved to be very stable over a period of 3 months when stored at 4 °C. This work highlights the great potential of succinylated food proteins as novel excipient-carriers for probiotic microorganisms to be delivered alive and in high numbers in the lower gastrointestinal tract.

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Appendix A. Supplementary data

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