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Research paper

Carboxymethyl high amylose starch (CM-HAS) as excipient for *Escherichia coli* oral formulations

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Abstract

Carboxymethyl high amylose starch (CM-HAS) is proposed as a novel excipient for oral tablet formulation of bioactive agents ensuring their protection in the stomach and delivery in the intestine. Three variants of CM-HAS, with different degrees of substitution, were synthesized by starch treatment with various amounts of monochloroacetic acid. The products were dried in powder form and tablets were obtained by direct compression of mixed powders of polymeric excipient and lyophilized *Escherichia coli* (*E. coli*) bacteria. Dosage forms of CM-HAS are unswollen and compact in acidic medium, ensuring protection of active agents against acidity. Release of bacteria from CM-HAS tablets is based on the fast swelling of the tablets during the passage from gastric acidity to alkaline intestinal medium, enzymatic hydrolysis triggering their rapid, almost total dissolution. The bacteria thus formulated displayed higher survival rates in acidic gastric conditions and for longer periods than the free bacteria or than the bacteria formulated with the non-derivatized starch. The CM-HAS selected matrix also assured a good viability of bacteria after 6 months under refrigeration.

Keywords: Carboxymethyl high amylose starch; Oral administration; Drug delivery; Gastric resistance; Intestinal matrix dissolution; *Escherichia coli*; Probiotics; Vaccin

1. Introduction

The interest for polymers as pharmaceutical excipients and carriers is continuously growing. In this context, vaccine delivery systems formulated with biodegradable synthetic polymers, mostly microspheres, have received considerable attention [1–4]. Their role is to protect the active agent from the acidic medium of the stomach and to deliver it to the mucosal intestinal site [5].

A wide range of polymeric matrices, many of them based on polysaccharides (i.e. starch, cellulose) are of interest in drug delivery. For instance, High Amylose Starch (HAS) with more than 70% amylose (non-ramified 1,4-α-polysaccharide) and less than 30% amylopectin (branched with multiple side chains) is largely used in pharmaceutics as filler, binder or disintegrant [6]. The hydroxyl groups play an important role in the organization of the matrix network, which is an important factor in the control of the drug release [7,8]. Many chemical modifications may be achieved by partial substitution of hydroxylic groups of HAS with various agents, such as monochloroacetic acid, leading to carboxymethyl groups (Fig. 1) [9,10]. We are now proposing gastroresistant formulations based on the hypothesis that carboxylic functions as salts (carboxylates)

Abbreviations: CM-HAS, non-cross-linked Carboxymethyl High Amylose Starch; CM, carboxymethyl; HAS, High Amylose Starch; ETEC, Enterotoxigenic *Escherichia coli*; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; DNS, 35-dinitrosalicylic acid; DS, degree of substitution; CF, compression force; CFU, colony forming units.

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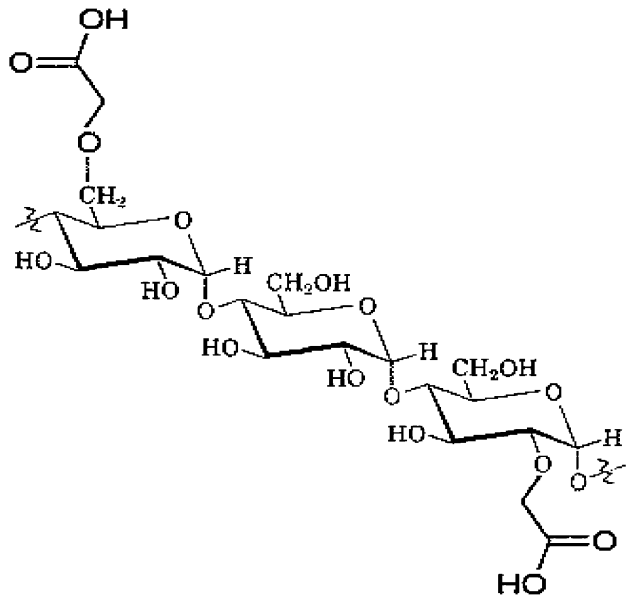

Fig. 1. Schematic representation of Carboxymethyl High Amylose Starch (CM-HAS).

would exchange the cation for a proton in acidic (gastric) media, resulting in a compact structure of the tablets. This ensures local (limited to surface surroundings) buffering properties, protecting thus the carried active agent against acidic denaturation. When in neutral or weak alkaline media, the protonated (carboxyl) form will change the proton for cation. This will facilitate the hydration, swelling and polymeric material dissolution, allowing the release of the bioactive agent. Protonation, ionization, solubilisation or enzymatic degradation of the polymers may be part of the chemical erosion mechanisms [11].

Enterotoxigenic *E. coli* (ETEC) are associated with neonatal and post-weaning diarrhea in pigs [12]. The bacteria adhere to the brush border receptors on porcine intestinal epithelial cells by means of fimbriae, an essential step in intestinal colonization during the infection process [12–14]. Parenteral immunization of pregnant swine with fimbrial preparations leads to the production of specific antibodies in the colostrum and in the milk, protecting the newborn pigs against infections with ETEC strains [15]. As this protection ceases at weaning, the weaned pigs become susceptible again to ETEC. Since parenteral immunization does not induce protective immunity at mucosal surfaces [16], it has been suggested that oral immunization may be a more effective means of inducing a protective mucosal immune response against ETEC infection, leading to subsequent prevention of diarrhea in weaned pigs [17].

In this in vitro study, *E. coli* bacteria, a potential candidate as an oral vaccine for post-weaning diarrhea due to ETEC, were directly incorporated in tablets based on carboxylated polymeric excipients such as CM-HAS. The tablet susceptibility to alpha-amylase (from pancreatin) was determined in the simulated intestinal fluid (SIF), a medium that was also used to evaluate the release of the bacteria from CM-HAS tablets. For many bioactive agents, it is necessary to modulate the velocity of disintegration in function of the desired location of bioactive agent delivery. It was therefore of interest to evaluate the influence of carboxymethyl (CM) groups of the non-cross-linked HAS excipient on the protection of bacteria within tablets at low pH and on their release in SIF. The ability of these matrices to modulate bacterial delivery in SIF as a function of the degree of substitution was also investigated.

Hence, the aims of this study were first to evaluate in vitro the swelling and erosion of CM-starch derivatives and to see whether formulations with CM-HAS at different degrees of substitution (DS) can ensure the protection of *E. coli* bacteria against acidic denaturation and its liberation in SIF and then, to determine the most appropriate formulation for the *E. coli* active agent.

2. Materials and methods

2.1. Materials

High amylose corn starch (Hylon VII; National Starch, USA), pancreatin (porcine pancreas) eight times strength with α-amylase, lipase and proteolytic activities (American Chemicals, USA), agar powder USP (Anachemia Chemicals Ltd., Canada), yeast extract (Difco Laboratories, USA), 3,5-dinitrosalicylic acid, monochloroacetic acid and other chemicals (reagent grade) were used without further purification.

Non-pathogenic *E. coli* bacteria (serotype O8:K87:H7) were lyophilized with a medium containing 5% dextran T-40, 7% saccharose and 1% monosodium glutamate, at the *Escherichia coli* Laboratory (Faculté de médécine vétérinaire, Université de Montréal, Saint-Hyacinthe, Canada).

2.2. Synthesis of polymeric derivatives (CM-HAS)

Derivatives were synthesized as described previously by Schell et al. [9] and Mulhbacher et al. [10] with slight modifications. Three variants of CM-HAS with different degrees of substitution (CM-HAS1, CM-HAS2 and CM-HAS3) were obtained. Essentially, 70 g of HAS were suspended in 170 ml of distilled water and warmed at 50 °C under continuous stirring in a Hobart planetary mixer. Then, 235 mL of 1.5 M NaOH were added slowly and the reaction medium was homogenized for 20 min at 50 °C. The different substitution degrees of the polymeric derivatives were obtained by adding to the alkaline reaction medium various amounts of monochloroacetic acid dissolved in minimal volumes of distilled water. Thus, 5 g of monochloroacetic acid were added for CM-HAS1, 45.5 g for CM-HAS2 and 70 g for CM-HAS3 synthesis. The pH was maintained between 9 and 10 by adding small volumes, if necessary, of 10 M NaOH

solution to the alkaline suspension (about 55 ml of 10 M NaOH for CM-HAS2 and 100 ml of 10 M NaOH for CM-HAS3 synthesis; none for CM-HAS1 synthesis). To attain substitution, the reaction media were maintained under continuous stirring for 1 h at 50 °C. When the reactions were ended, 130 mL of distilled water at 50 °C were added to each gel slurry. Each synthesis was then neutralized by slowly adding, while stirring, 250–350 mL of acetic acid solution (20 mL of glacial acetic acid in 380 mL of distilled water preheated at 50 °C). The final pH of each suspension was 6.8–7.0. The reaction media were cooled at room temperature.

2.2.1. Washing and drying of polymeric derivatives (CM-HAS)

A volume of 600 mL of pure acetone was added slowly to each of the neutralized suspensions under continuous stirring for 30 min at room temperature. The suspensions were subsequently filtered and gels remaining on the filters were recovered. Each gel was resuspended in 600 mL of acetone/water solution (60:40, v/v) and maintained under stirring for 30 min and then filtered again. The last procedures (resuspension and filtration) were repeated. For the final drying, each recovered gel was resuspended in 600 mL of pure acetone in the same conditions and then filtered. Washing with acetone was continued until a white wet powder was obtained. The powders were expanded, kept overnight at room temperature for drying and then sieved, retaining fractions of 75–300 μm.

The degree of substitution was determined by potentiometric titration of CM groups with 0.2 N NaOH. Each CM-HAS derivative was kept 20 min in a 0.2 N HCl solution under agitation (for activation by protonation), and then filtered. In the case of CM-HAS2 and CM-HAS3 polymers, due to their higher solubility, acetone was added for precipitation, after protonation and before the filtration step. The powders were washed with a distilled water: acetone solution (50: 50, v/v) until a neutral pH was reached and then dried with acetone (100%). One gram of each CM-HAS powder was completely dissolved in distilled water for the potentiometric titration with 0.2 N NaOH. We determined the number of mmoles of carboxylic groups / g of polymeric powder, and the degree of substitution was expressed in number of carboxylic functional groups per 100 glucose units.

2.3. Preparation of a non-derivatized high amylose starch (HAS-0)

A non-derivatized starch treated with NaOH (1 h, 50 °C) for gelatinization but not with monochloroacetic acid, neutralized and dried as for CM-HAS, was used as control in all experiments (hereto called HAS-0).

2.4. Stability of the various starch types (CM-HAS and HAS-0) in tablet forms at gastric pH

Tablets (200 mg) based on HAS-0, CM-HAS1, CM-HAS2 and CM-HAS3 polymers were obtained by direct compression of mixed powder of polymer and 4-nitrophenol as pH indicator (10 mg/tablet), at 2.5 T/cm^2 using a manual hydraulic press Carver (USA) and 9.0 mm cylinder outfits. Tablets were incubated in 50 mL of Simulated Gastric Fluid (SGF, pH 1.2), with or without pepsin, prepared following the US Pharmacopeia [18], for 2 h at 37 °C, agitated at 50 rpm using a series 25D incubator shaker (New Brunswick Scientific Co., New Jersey, USA). The behavior of these tablets (integrity and color modifications) was visually monitored; they were then cross-sectioned and the modification of the pH indicator color was evaluated.

2.5. Stability of the various starch types (CM-HAS and HAS-0) in tablet forms in the presence of pancreatin

Stability and kinetics of pancreatin-catalyzed hydrolysis of CM-HAS tablets were studied. The tablets of HAS-0, CM-HAS1, CM-HAS2 and CM-HAS3 (200 mg each, excipients only) were obtained by the same procedure as above. They were incubated in 50 mL of SIF containing 1 USP pancreatin unit [18] at 37 °C under agitation at 50 rpm using the same incubator shaker. The tablet shape was examined visually every hour for 5 h. Aliquots were sampled every hour for determination of the equivalents of maltose liberated (due to the amylolytic action of pancreatin), by titration with 1% 3,5-dinitrosalicylic acid (DNS) as described by Noelting and Bernfeld [19]. Essentially, 2 mL of each aliquot were treated with 1 mL of DNS reagent and the samples were heated in a boiling bath for 5 min and then rapidly placed in an ice-cold water bath to stop the reaction and diluted with 15 mL of distilled water. The absorbency was read at 535 nm and the liberated reducing sugars calculated versus a calibration curve with maltose. Each experiment was performed in triplicate.

2.6. Viability of E. coli bacteria in the SGF acidic medium

Tablets (200 mg) based on HAS-0, CM-HAS1, CM-HAS2 or CM-HAS3 and containing 10 mg of lyophilized *E. coli* (approximately 10^9 bacteria), were formulated by direct compression at 2.5 T/cm^2. The initial amount of *E. coli* in the lyophilized preparation (number of bacterial colony forming units, CFU/10 mg lyophilized *E. coli*) was determined in sterile pancreatin-free SIF (pH 6.8) at room temperature.

Tablets were placed individually in 50 mL of sterile SGF (pH 1.2) for different times at 37 °C (simulating the gastric passage), under agitation at 50 rpm, using the same incubator shaker as above. Their shape was examined visually. The viability of bacteria was evaluated after 30, 60, 90 and 120 min in SGF. After the appropriate period of incubation in SGF, the tablets were transferred into 50 mL

of sterile pancreatin-free SIF (pH 6.8), crushed and aliquots of 1 mL were ten-fold serially diluted. A volume of 100 μL of each dilution was plated on a nutrient 2% agar plate in order to determine the number of CFU. The tablets based on HAS-0 were used as control for polymers. As control for the active agent, 10 mg of free (non-formulated) lyophilized bacteria was used.

2.7. E. coli delivery in the SIF medium

The same formulations as above were incubated in 50 mL of sterile SGF (pH 1.2) for 1 h at 37 °C, under agitation (50 rpm) and then transferred into 50 mL of SIF containing pancreatin [18], and incubated for 5 h at 37 °C (50 rpm). The tablet shapes were examined visually during the entire incubation period. Samples of 1 mL were taken after 1 h in SGF and every hour in the SIF and they were serially diluted in order to evaluate the viability of the bacteria liberated from the swollen tablets. The number of CFU was evaluated as previously described (Section 2.6). As a control for the stability of the non-protected *E. coli*, 10 mg of free, non-formulated bacteria were incubated in SIF. Samples of 1 mL were taken at hourly intervals to determine the number of bacterial CFU.

All the tests were performed in triplicate and the colonies were counted after aerobic incubation at 37 °C for 24 h.

2.8. Influence of compression force (CF) on E. coli viability

Tablets (200 mg) based on CM-HAS2 and containing 10 mg of lyophilized *E. coli* were formulated by direct compression at 1.0, 2.5 and 5.0 T/cm^2. The formulations were added to 50 mL of sterile pancreatin-free SIF (pH 6.8) and immediately crushed at room temperature in order to determine the number of CFU, as described in Section 2.6.

2.9. Stability of E. coli formulated with CM-HAS2 after 6 months storage under refrigeration

CM-HAS2 tablets (200 mg) containing 10 mg of lyophilized *E. coli* were formulated by direct compression at 2.5 T/cm^2 and stored under refrigeration (4 °C) for 0 (zero), 3 and 6 months. As control for the CM-HAS2 polymer, the same number of tablets was formulated using HAS-0 and lyophilized *E. coli*. As control for the free bacteria, 10 mg of lyophilized *E. coli* were stored at 4 °C. After each storage period, the CM-HAS2 and HAS-0 tablet formulations were transferred into 50 mL of sterile pancreatin-free SIF (pH 6.8) and rapidly crushed at the room temperature. Aliquots of 1 mL were serially diluted and the number of CFU was determined as described in Section 2.6. For the free *E. coli*, the number of CFU was determined in pancreatin-free SIF (pH 6.8).

2.10. Statistical analysis

Statistical analyses were performed using the one-way ANOVA test. Pair-wise comparisons were performed via Tukey HSD (Honestly Significantly Different) test and statistical significance was assessed for $P \leq 0.05$. Asterisks represent significant differences observed within the same group of samples. To evaluate the influence of compression force on bacterial viability in CM-HAS2 tablets, statistical analyses were performed between the three groups of samples ($n = 3$ for each group).

3. Results and discussion

3.1. Synthesis of non-cross-linked polymeric derivatives (CM-HAS)

The DS were determined as 2 for CM-HAS1, 11 for CM-HAS2 and 22 (carboxylic functional groups per 100 glucosidic units) for CM-HAS3.

3.2. Stability of various High Amylose Starch (CM-HAS and HAS-0) tablets at gastric pH

The tablets based on CM-starch maintained their shape almost entirely in SGF after 2 h at 37 °C under 50 rpm, whereas the tablets based on the non-derivatized starch (HAS-0) disintegrated. This different behavior seems due to the presence or the absence of the carboxylic groups. Protonated carboxyl groups from neighboring chains can be dimerized by hydrogen bonding, stabilizing the network. Furthermore, the hydroxylic groups of the polymeric backbone (HAS) can also be involved in formation of new hydrogen bonds with the carboxylic groups, thus enhancing the stability of CM-HAS tablets. For CM-HAS2, and especially for CM-HAS3, a gel layer was formed at the surface of the tablets. The gel front seemed to progress toward the center of the tablet and after 2 h in SGF, the CM-HAS3 tablet had gelified entirely but maintained its shape in solution. Thus, a kind of supramolecular rearrangement may occur after hydration of CM-HAS tablets, allowing the formation of a gel. A good stability of CM-HAS tablets was also found in SGF containing pepsin.

In the pH stability studies, 4-nitrophenol contained in the tablets is colorless at a pH of less than 5.4 and yellow at a pH greater than 7.5. For HAS-0, the disintegrated parts of the tablets were colorless indicating that HAS-0 did not stabilize the pH indicator in the tablet. The CM-HAS3 tablets afforded the greatest protection against high acidity (yellow inside the tablet) followed by CM-HAS2 and CM-HAS1, suggesting that the higher substitution degree (CM-HAS3) afforded a better pH stability within the tablets (Fig. 2).

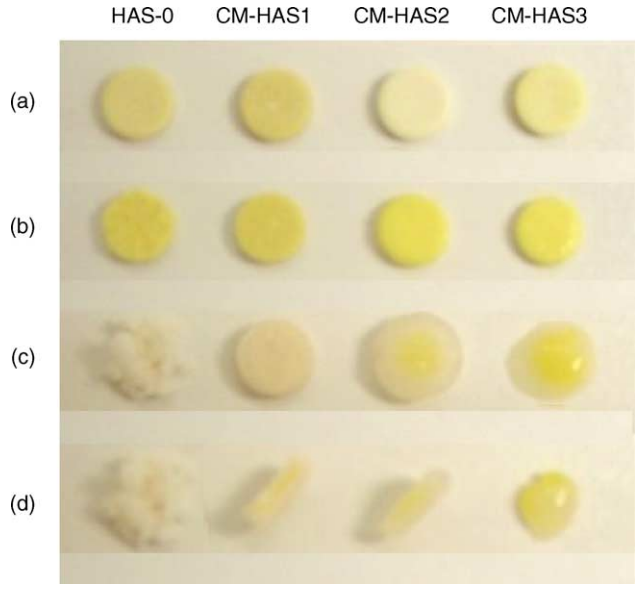

Fig. 2. Evaluation of pH stability of tablets incubated in SGF. Tablets based on non-derivatized (HAS-0) and functionalized (CM-HAS1, CM-HAS2 and CM-HAS3) polymers containing 4-nitrophenol as pH indicator were obtained by direct compression (2.5 T/cm^2) and incubated in SGF. Presence of the yellow color within the tablets indicates pH stability against gastric acidity. Treatments: (a) untreated tablets (tablets in air), (b) 5 min in distilled water, (c) 2 h in SGF - complete tablets and (d) 2 h in SGF—cross-sections of the tablets.

3.3. Stability of derivatized (CM-HAS) and non-derivatized (HAS-0) tablets in SIF in the presence of pancreatin

The HAS-0 tablets were disintegrated within 30 minutes in SIF whereas the tablets based on substituted starch maintained their shape for more than 2 h. The CM-HAS1 tablets exhibited a capping phenomenon after 3 h in SIF whereas the CM-HAS2 and CM-HAS3 tablets were partially solubilized after 4 and 3 h, respectively (Table 1). During the compaction, an elastic deformation of starch is supposed [20] and the relaxation of the tablet at hydration can differ on various directions and induce capping. The swelling volumes of tablets were found, as expected, to increase with the DS of the polymer. At higher DS, following the exchange protons/cations (Na^+), the CM-HAS chains allow the penetration of higher volumes of water (enhanced by Na^+) into the tablet and a more gelatinous structure of the matrix is obtained.

The CM-HAS tablets were found to be susceptible to erosion, hydrolysis and final dissolution under intestinal α-amylase action, even though the starch in these tablets was chemically modified. Different amounts of maltose were liberated as amylolysis products. CM-HAS1 tablets showed the highest stability to the α-amylase (Fig. 3). A slightly higher, but not significant, susceptibility to amylolysis was observed for CM-HAS2 tablets when compared to CM-HAS3 tablets. All CM-HAS tablets were more resistant to amylolysis than the HAS-0 tablets, in agreement with previous data on cross-linked CM-HAS [21]. The CM groups afforded a higher stability to amylolysis, probably via a steric and/or an ionic interaction of CM groups with the α-amylase active site involved in amylolysis. On the other hand, a higher substitution degree makes the matrix more hydrophilic, facilitating enzyme access to the site of degradation. The ability of CM-HAS1 tablets to swell was lower than that of CM-HAS2 and CM-HAS3 tablets, explaining the highest stability of CM-HAS1 to amylolysis. For CM-HAS2 tablets, there appeared to be a balance between the substitution and swelling degrees of the matrix, allowing a higher access of the enzyme to the hydrolysis sites than for CM-HAS1 tablets.

3.4. Viability of bacteria in the acidic medium (SGF)

In contrast to the HAS-0 tablets, that were completely disintegrated during the first 30 minutes of incubation in SGF, CM-HAS1 tablets presented a very low swelling, whereas CM-HAS2 and CM-HAS3 tablets showed a low and moderately-low swelling, respectively, after 2 h in SGF.

The *E. coli* viability tests showed that the CM-HAS tablets were able to protect the bacteria against 2 h of acidic denaturation. On the other hand, tablets based on HAS-0, which disintegrated, did not protect the bacteria, no viable bacteria being observed even after 30 min (Fig. 4). After 30 min of acidic treatment, the number of viable bacteria formulated with CM-HAS was higher for all substituted polymers than for the non-formulated free *E. coli*. The bacterial viability was not significantly different at time 0 (zero) and after 120 min of incubation in SGF for

Table 1
Stability of tablets based on polymeric CM-HAS derivatives, in SIF containing pancreatin (USP), at 37 °C and 50 rpm

Polymer	1 h SIF	2 h SIF	3 h SIF	4 h SIF	5 h SIF
HAS-0	Disintegrated	Disintegrated	Disintegrated	Disintegrated	Disintegrated
CM-HAS1	One piece shape low swelling	One piece shape moderate swelling	Capping moderate swelling	Capping moderate swelling	Capping moderate swelling
CM-HAS2	One piece shape moderate swelling	One piece shape moderate-high swelling	One piece shape high swelling	Partially dissolved	Dissolved
CM-HAS3	One piece shape moderate-high swelling	One piece shape high swelling	Partially dissolved	Dissolved	Dissolved

Tablets were compressed at 2.5 T/cm^2 ($n = 3$ tablets for each polymeric derivative).

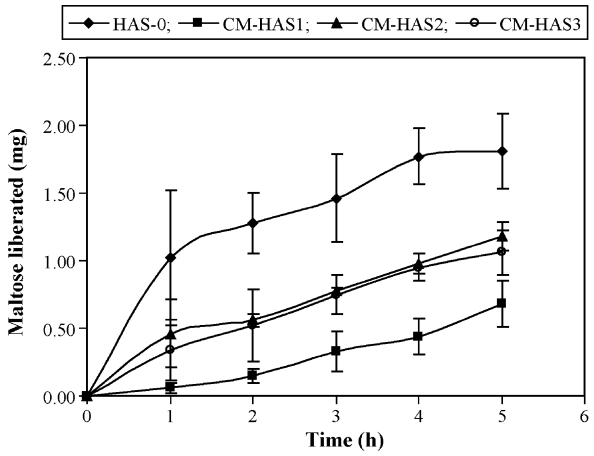

Fig. 3. Kinetics of pancreatin (α-amylase)-catalyzed hydrolysis of HAS-0 and CM-derivatives tablets. The kinetics of enzymatic hydrolysis and maltose release were followed in 50 mL of SIF medium, at 37 °C and 50 rpm (Mean ± SD, $n = 3$).

the CM-HAS1 and CM-HAS2 tablets, whereas for the CM-HAS3 tablets, a statistically significant reduction of viability was observed at 120 min (Fig. 4). The highest protection was always afforded by CM-HAS1 followed, in order, by CM-HAS2 and CM-HAS3.

For the free *E. coli* suspension, viable bacteria were still observed after 30 min of incubation in acidic medium (pH 1.2). This was surprising, considering that HAS-0 did not protect the bacteria against the acidic medium, even after only 30 minutes. It is possible that a proportion of the bacteria are liberated in the acidic conditions, but not being resistant, are rapidly killed. Another proportion of the bacteria possibly adhered to HAS-0 particles and when the tablet disintegrated, these bacteria were not liberated in the acidic medium. A similar finding has been already reported by Bundy and Fenselau [22], suggesting that carbohydrates captured bacteria via microbial lectins expressed on their surfaces. Alternately, HAS-0 may bind and weaken the barrier function of the outer membrane of *E. coli*, rendering bacteria more sensitive to the action of the acidic medium. A similar explanation was reported for chitosan [23].

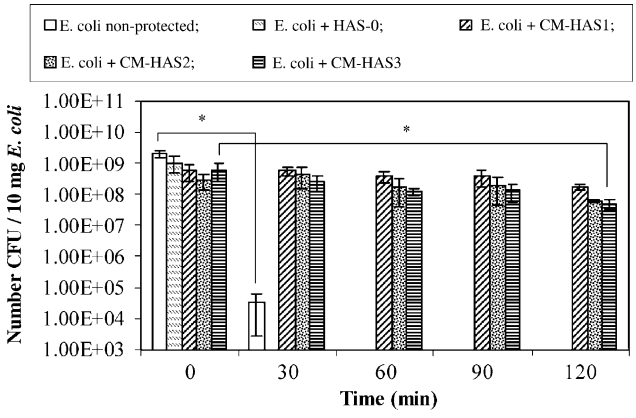

Fig. 4. Stability of bacteria formulated in tablets with HAS-0 and CM-derivatives following incubation in an acidic medium. The tablets were incubated in 50 mL of SGF (37 °C and 50 rpm) and colony-forming units (CFU) were measured (Mean ± SD, $n = 3$). Asterisks stand for significant differences within the same group of samples ($P \leq 0.05$).

3.5. Bacterial delivery in the SIF

The release of the bacteria is related to tablet swelling and dependent on the substitution degree. Erosion, some turbidity and dissolution of the partially swollen polymer occurred during the incubation in SIF, especially for CM-HAS2 and CM-HAS3. The swelling volume of the HAS derivatives increased at higher pH values. The CM-HAS2 and CM-HAS3 tablets were partially dissolved after 2–3 h in SIF and liberated the bacteria. The CM-HAS1 tablets presented a capping phenomenon after 1–3 h and released a higher amount of viable bacteria than the CM-HAS2 and CM-HAS3 tablets (Fig. 5). However, due to the capping, this liberation could not be controlled.

Initially, after 1 h in SGF, no viable liberated bacteria were found in the gastric medium from the tablets of CM-substituted polymers and control (HAS-0). During the period of 5 h in SIF, no CFU were found for the control (HAS-0) (data not shown). The CM-HAS1 tablets partially liberated bacteria during the first hour in SIF, in contrast to the CM-HAS2 and CM-HAS3 tablets, which liberated no bacteria during this interval. The gel forming around the tablet, which may provide a mechanism for delayed liberation, could explain this lack of bacterial release for CM-HAS2 and CM-HAS3. The gel would prevent access of water and α-amylase into the deeper layers of the tablet. After 2 h in SIF, the bacterial liberation from CM-HAS2 and CM-HAS3 tablets appears to be controlled by erosion of

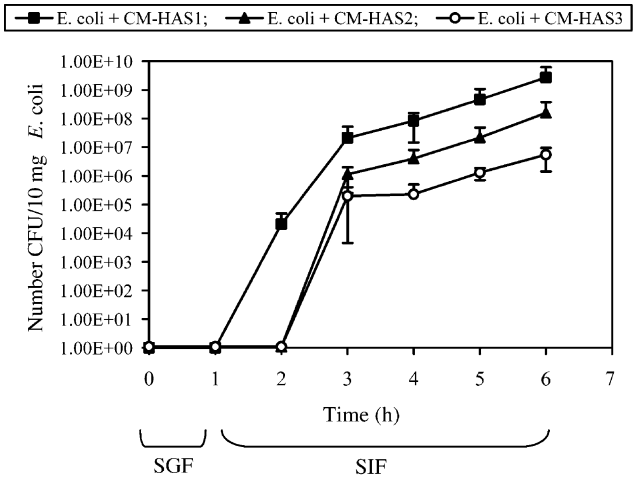

Fig. 5. Release of live bacteria formulated in tablets with HAS-0 and CM-derivatives following incubation in stimulated gastric and intestinal medium. The tablets were incubated in SGF for 1 h followed by 5 h in SIF at 37 °C and 50 rpm and colony forming units (CFU) were measured (Mean ± SD, $n = 3$).

the swollen polymer. In fact, the enzyme contact with the external surface of the swollen tablets is only partial. This external hydrated gel layer limits the deeper penetration of the α-amylase enzyme into the tablet. Due to the polymer swelling, the gel layer thickness first increases by hydration and then decreases because of erosion (due to the α-amylase activity) [24] and tablet dissolution. In the case of CM-HAS1, the polymer swelling and dissolution are negligible and the bacteria are released from the matrix predominantly by diffusion. The capping phenomenon (particular case of CM-HAS1) will accelerate bacterial liberation by increasing the release area. In addition, this will increase the accessibility of the α-amylase to the polymeric substrate and consequently, the rate of hydrolysis increases. Thus, diffusion could contribute to bacterial liberation, particularly in case of low substituted CM-HAS1.

The solubility of CM-HAS derivatives is a function of their hydrophilic properties and a higher carboxylation increases the water uptake in the tablet, its dissolution and, consequently, is expected to increase bacterial release. However, a decrease rather an increase in liberation of live bacteria was observed for a higher degree of substitution. This could be explained by differences in the resistance afforded by various starch derivatives against the effects of acid pH. Although CM-HAS3 provided the best buffering properties (Fig. 2), it was also the most hydrophilic derivative, resulting in highly swollen tablets. Thus, it was the most susceptible to the effects of acid pH and liberated only a small number of live bacteria in SIF.

It was found that the conditions of the intestinal medium allow the multiplication of free bacteria (data not shown). Based on these data, it is supposed that bacteria liberated from a tablet with a CM-derivative can multiply during incubation in the SIF medium. This behavior seems important for the pharmaceutical formulation of the *E. coli* with the CM-HAS excipients, suggesting a good efficiency of this oral dosage form.

3.6. Influence of compression force (CF) on viability of bacteria

It was found that a CF of 1.0, 2.5 and 5.0 T/cm^2 exerted no significant effects on the viability of the bacteria formulated with the CM-HAS2 polymer, at time zero only (data not shown).

It has been reported that an increase in CF results in a decrease in the porosity of the certain substituted HAS matrix [25]. In our case, it is probable that the void space within the tablet will have been reduced but this does not appear to have affected significantly the bacterial cell viability. Furthermore, water taken up by the tablets could generate interchain hydrogen bonding and stabilize the matrix homogeneously at distances much lower than those within the dry matrix, irrespective of the compression force and initial porosity.

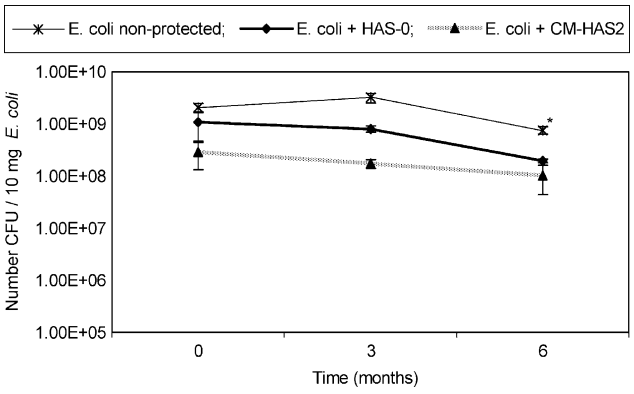

Fig. 6. Stability of formulated *E. coli* based on CM-HAS2 at 4 °C compared with free-bacteria and formulated-bacteria based on HAS-0. The stability tests were performed in 50 mL of pancreatic-free SIF (pH 6.8) at room temperature (Mean ± SD, $n = 3$). *$P \leq 0.05$.

3.7. Stability of E. coli formulated with CM-HAS2

The count of viable bacteria formulated as tablets with CM-HAS2 and HAS-0 decreased slightly when stored for 6 months under refrigeration at 4 °C. For the unprotected, free *E. coli*, a slight decrease of the bacterial viability was also observed after six months of storage in similar conditions (Fig. 6).

4. Conclusion

Carboxymethyl high amylose starch appears to be an interesting excipient-carrier for microorganism transportation through the stomach and delivery in the gut. The low substituted CM-HAS1 liberated the highest amount of bacteria in simulated intestinal conditions, but, due to the capping, this bacterial liberation is difficult to control. The CM-HAS2 derivative, with a moderate degree of substitution, appears to be the most interesting excipient for bacterial transportation through the gastrointestinal tract. The compression force showed a non-significant effect on the viability of the bacteria formulated as tablets with CM-HAS2 and viability remained stable after six months of storage under refrigeration for bacteria formulated as tablets with this matrix, although similar results were also found for free bacteria.

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