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Research paper

Carboxymethyl high amylose starch: Chitosan self-stabilized matrix for probiotic colon delivery

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ABSTRACT

A new hydrophilic tablet dosage system based on an ionic self-stabilization of a carboxylated (carboxymethyl high amylose starch, CM-HAS) and an amino (Chitosan) excipient was proposed for probiotic colon delivery. CM-HAS (protonated and compacted in acidic medium) ensures gastro-protection and Chitosan (low soluble in intestinal media) prevents early release of *Lactobacillus rhamnosus* bacteria. Thus, in CM-HAS:Chitosan monolithic tablets, increasing percentage and molecular weight (MW) of Chitosan generated a decrease of bacteria release rate, bacteria being the most effectively retarded by the highest MW of Chitosan (2.2×10^6 g/mol). The monolithic formulations containing high percentages of CM-HAS (80%) delivered bacteria after 2 h of incubation in gastrointestinal conditions for all the Chitosan MWs used. A combined mechanism of bacteria release is proposed for CM-HAS:Chitosan monolithic tablets, involving the swelling of the tablets (due to the Chitosan), followed by the erosion and dissolution of CM-HAS. In addition, a gel-forming barrier of Chitosan in acidic conditions also contributed to the delay of the bacteria delivery. The CM-HAS dry-coated monolithic tablets changed the effect of Chitosan molecular weight on bacteria liberation and improved the percentage of delivered bacteria in simulated intestinal conditions.

1. Introduction

The interest of probiotics as remedies for a broad number of gastrointestinal diseases is continuously growing. Probiotics are live microorganisms administered as food supplements in order to improve the host intestinal microbial balance and to confer major health benefits such as modulation of the immune system [1,2], enhanced healing of damaged gastrointestinal mucosa [3], relapsing of *Clostridium difficile* diarrhea [4] and antagonism against pathogens (i.e. *Lactobacilli* able to compete with, exclude and displace pathogenic gastrointestinal bacteria when incubated together [5]). Widespread prescription of antibiotics is often associated with the disruption of the protective flora and can lead to predisposition to infections. The control of infections without using antibiotics is a large advantage and probiotic therapy represents a promising alternative. Among probiotic bacteria, *Lactobacilli* and *Bifidobacteria* strains are largely present in humans. It has been shown that fecal levels of these strains were reduced in patients with inflammatory bowel disease (IBD), such as Crohn's disease and ulcerative colitis [6,7], suggesting an alteration of the colonic flora as one of the factors responsible for these diseases. Lactic acid bacteria impart further nutritional and therapeutic benefits such as: improved digestion of lactose [8], control of some types of cancer [9] and control of cholesterol levels [10]. To be effective, orally administered probiotics should be efficiently implanted in the intestine and adhesion to the intestinal mucosa is considered one of the beneficial health effects of probiotics. This requires that the cells survive during the preparation of dosage forms and passage through the acidic environment. Reaching the intestine, these microorganisms should be able to establish themselves, remain viable and perform their beneficial actions. In this context, oral formulations have to protect the bioactive agent from the gastric acidity and to deliver it to the intestinal site. Colon target is the main objective of drug formulation for the prevention and the topical treatment of IBD or other infectious disorders and for the therapy of colorectal cancer. Oral formulations for the colonic delivery should afford gastric protection of bioactive agents in the stomach and delay their release through the small intestine in order to allow their complete release in the colon.

We have recently proposed CM-HAS as an excipient for bioactive agent transport in simulated gastrointestinal conditions [11,12]. Thus, in gastric acidity, CM-HAS (Na^+) changes the cation for a proton, resulting in a compact structure of the tablet and conferring gastro-stability of the active agent against acidic and enzymatic denaturation. In intestinal fluid, the protonated form of CM-HAS (H^+) will change the proton for cations, enhancing the water uptake and generating the polymeric swelling and matrix

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dissolution and the release of the bioactive agents. Thus, due to its relatively fast and total dissolution, which can be enhanced by enzymatic hydrolysis of alpha-amylase, CM-HAS alone may not be suitable in formulations of drugs aimed at colon delivery. Recently, we have proposed an association CM-HAS:Chitosan [13] in which both macromolecular excipients contribute to a physical and chemical stabilization of the matrix to formulate small molecules for intestinal delivery. We are now reporting a hydrophilic matrix based on binary mixtures of CM-HAS:Chitosan for colon delivery of lactic acid bacteria. It is well known that Chitosan, a linear polysaccharide consisting of β-(1-4)-linked 2-amino-2-deoxy-D-glucose still presenting a certain number of 2-acetamido-2-deoxy-D-glucose units, is a good matrix for the delayed liberation of active agents in colon. Chitosan can be obtained by the partial alkaline [14] or enzymatical [15] *N*-deacetylation of naturally occurring chitin. An important advantage of Chitosan for its successful use in colon-targeting consists in its stability against the enzymes in duodenum and the lower intestinal tract, but susceptible to a certain degradation by colonic bacterial enzymes [16]. However, an important limitation can be its dissolution in the gastric medium due to the protonation of amino groups. To prevent this, the association of Chitosan with another excipient carrying carboxylic functions such as alginate [17] or xanthan [18] has been suggested. The association of CM-HAS with Chitosan in monolithic tablet formulations is expected to assure a good stability of the tablet and a good viability of bacteria in gastric conditions (due to the presence of CM-HAS) and, at the same time, to delay the bacteria liberation in intestinal media (due to the presence of Chitosan). To overcome certain aspects related to the high solubility of Chitosan in the simulated gastric media, we have also tested a novel double-layer system based on CM-HAS:Chitosan tablets coated with CM-HAS polymer.

The aim of this study was to investigate the effect of CM-HAS:Chitosan excipient association on the stability of monolithic and of CM-HAS dry-coated tablets and the effect of the Chitosan molecular weight on the delaying *Lactobacillus rhamnosus* (*L. rhamnosus*) probiotic delivery.

2. Materials and methods

2.1. Materials

High amylose starch (Hylon VII) was from National Starch (Bridgewater, NJ, USA). Chitosan 1 (98% degree of deacetylation, DDA) and Chitosan 2–4 (84% DDA) from crab shells were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA), where the numbers were ascribed to represent different molecular weights used in our formulations. Pepsin (porcine gastric mucosa) was from Sigma–Aldrich Chemical Company and pancreatin (porcine pancreas extract with alpha-amylase, lipase and proteolytic activities) from A&C American Chemicals Ltd. (Montréal, Que., Canada).

The *Lactobacillus rhamnosus* probiotic bacteria (HA-111 strain) were generously supplied by Harmonium International Inc. (Mirabel, Que., Canada). Difco™ *Lactobacilli* MRS (DeMan, Rogosa and Sharpe) agar, for the culture media, was from Fisher Scientific Company (Ottawa, Ont., Canada).

The other chemicals were of reagent grade and used without further purification.

2.2. Synthesis of polymeric derivative CM-HAS

The starch derivative was synthesized as previously described [12]. The CM-HAS powder, held overnight to air at room temperature, was ground in a blender, and then sieved to obtain a powder (particles granulometry between 75 and 300 μm), used to prepare the tablets.

The CM-HAS structure was analyzed by Fourier transform infrared spectroscopy (FTIR) and the degree of substitution (DS) was determined by nuclear magnetic resonance (^{1}H NMR) – 300 MHz (Varian Gemini) in deuterated dimethyl-d_6 sulfoxide (99.9 atom%D, CDN Isotopes, Que., Canada) as previously described [12].

2.3. Chitosan preparation

All the purchased Chitosans were dissolved in 2% acetic acid solution and every viscous solution was passed through a filter paper to remove the undissolved residues. A solution of 1.0 M NaOH was added to the homogeneous filtrate until a pH of 6.5 and then, Chitosan was precipitated with acetone. The precipitated polymer was thoroughly washed with distilled water, dried with 100% acetone and then dried at room temperature. The powders were sieved retaining fractions smaller than 300 μm for tablet formulations.

2.4. Molecular weight determination of Chitosan polymers

The molecular weight (MW) of the Chitosan samples was determined viscometrically, using a Cannon-Ubbelohde semi-micro viscometer (State College, PA, USA) for increasing Chitosan concentrations (0.01–0.15%) in 0.3 M acetic acid/0.2 M sodium acetate buffer at 25 °C ± 0.05 for Chitosan MW1 [19] or in 0.2 M acetic acid/0.1 M sodium acetate buffer at 30 °C ± 0.05 for Chitosan MW2–4 [20]. The flow time data were used to calculate the intrinsic viscosity ($[\eta]$), as the intercept of the linear regression of reduced viscosity versus concentration extrapolated to zero concentration [20]. The average MW (MW_v) for each Chitosan type was determined from the intrinsic viscosity, using the Mark–Houwink–Sakurada Eq. (1) and the parameters K and a, obtained following Rinaudo et al. [19] and Wang et al. [20].

$$[\eta] = K \times MW_v^a \quad (1)$$

2.5. Monolithic and CM-HAS dry-coated tablet formulation

2.5.1. Preparation of monolithic tablets

Monolithic tablets (200 mg) based on CM-HAS:Chitosan excipients (different ratios) were obtained by direct compression of a homogenous mixture of dry powders containing 190 mg of CM-HAS and Chitosan excipient polymers and 10 mg of probiotic *L. rhamnosus* bacteria (representing 10^9 colony-forming units, CFU). The mixture was compressed at 2.5 T/cm^2 using a manual hydraulic Carver press (Wabash, IN, USA) and 9.0 mm cylinder outfits with flat-faced punches.

2.5.2. Preparation of CM-HAS dry-coated tablets

Monolithic tablets (200 mg) based on CM-HAS:Chitosan and containing 10 mg of bacteria were formulated as previously described. Then, they were double-faced coated with 300 mg CM-HAS polymer only: 130 mg CM-HAS on the inferior side and 170 mg CM-HAS on the lateral and upper sides of the monolithic tablet in the die. The compression was done at 2.5 T/cm^2, using 13.0 mm cylinder outfits with flat-faced punches (obtaining dry-coated tablets of 500 mg).

2.6. Bacteria delivery in the simulated gastric and intestinal media

Monolithic (200 mg) and CM-HAS dry-coated (500 mg) tablets were incubated for 1 h in 50 mL of sterile simulated gastric fluid (SGF, pH 1.2) [21] and then transferred into 50 mL of sterile simu-

lated intestinal fluid (SIF, pH 6.8) [21] and incubated for 24 h at 37 °C and 50 rpm, using an incubator shaker (Series 25 D, New Brunswick Scientific Co., NJ, USA). Samples of 100 μL were taken after 1 h in SGF and at every hour in the SIF over the first 12 h and after 24 h of SIF to evaluate the viability of the liberated bacteria from the tablets. After 1 h in SGF and 24 h in SIF, the tablets were crushed in the SIF medium to determine the number of bacteria that still remained inside the tablet.

The number of CFU was counted on the aliquots removed and serially diluted in 0.1% sterile bacteriological peptone water and then cultured on MRS nutrient agar plates at 37 °C for 48 h, to calculate the number of living bacteria liberated in function of time.

The initial number of bacterial CFU in 10 mg of lyophilized bacteria was determined in sterile phosphate-buffered saline (PBS, pH 7.4) at room temperature. As control, 10 mg of free bacteria was incubated in the same conditions as in the case of formulated bacteria: 1 h in 50 mL sterile SGF medium and, respectively, 24 h in 50 mL sterile SIF at 37 °C and 50 rpm. All the colonies were counted after aerobic incubation at 37 °C for 48 h.

2.7. Water uptake and erosion studies

Tablets based on 100% Chitosan (MW1–4), on 50% CM-HAS:50% Chitosan (MW1–4) and on 100% CM-HAS, each containing 10 mg of lyophilized *L. rhamnosus*, were kept in the same conditions as for the dissolution tests (at 50 rpm and 37 °C). For the water uptake study, the hydrated tablets of 100% Chitosan (MW1–4) and of 50% CM-HAS:50% Chitosan (MW1–4) were removed from the dissolution medium after 1 h SGF and after every 2 h in SIF (during an overall duration of 12 h in SIF), blotted with tissue paper to eliminate the excess surface water and then weighed. The remaining dry weight of tablets was determined in an oven at 50 °C after 1 h in SGF and after 2, 12 and 24 h of SIF incubation for matrices based on 100% Chitosan (MW1–4) and on 50% CM-HAS:50% Chitosan (MW1–4). Concerning the 100% CM-HAS matrix, the tests were performed after 1 h in SGF and after 2, 3 and 4 h only of SIF incubation due to rapid tablet dissolution. The percentage of water uptake and that of erosion were determined gravimetrically as previously described [12].

2.8. Scanning electron microscopy

2.8.1. Sample preparation

Monolithic and CM-HAS dry-coated tablets based on 100% Chitosan (MW1–4), on 50% CM-HAS:50% Chitosan (MW1–4) and on 100% CM-HAS, prepared as previously described (Section 2.5), were incubated for 1 h in SGF and then transferred for 6 h in SIF (50 rpm, 37 °C). Due to their faster dissolution in simulated intestinal media, the 100% CM-HAS monolithic tablets were incubated for 1 h in SGF and only 2 h in SIF (50 rpm, 37 °C). Then, the tablets were frozen and lyophilized for a week. The dry tablets were mounted on metal supports and their surface and intern (section)

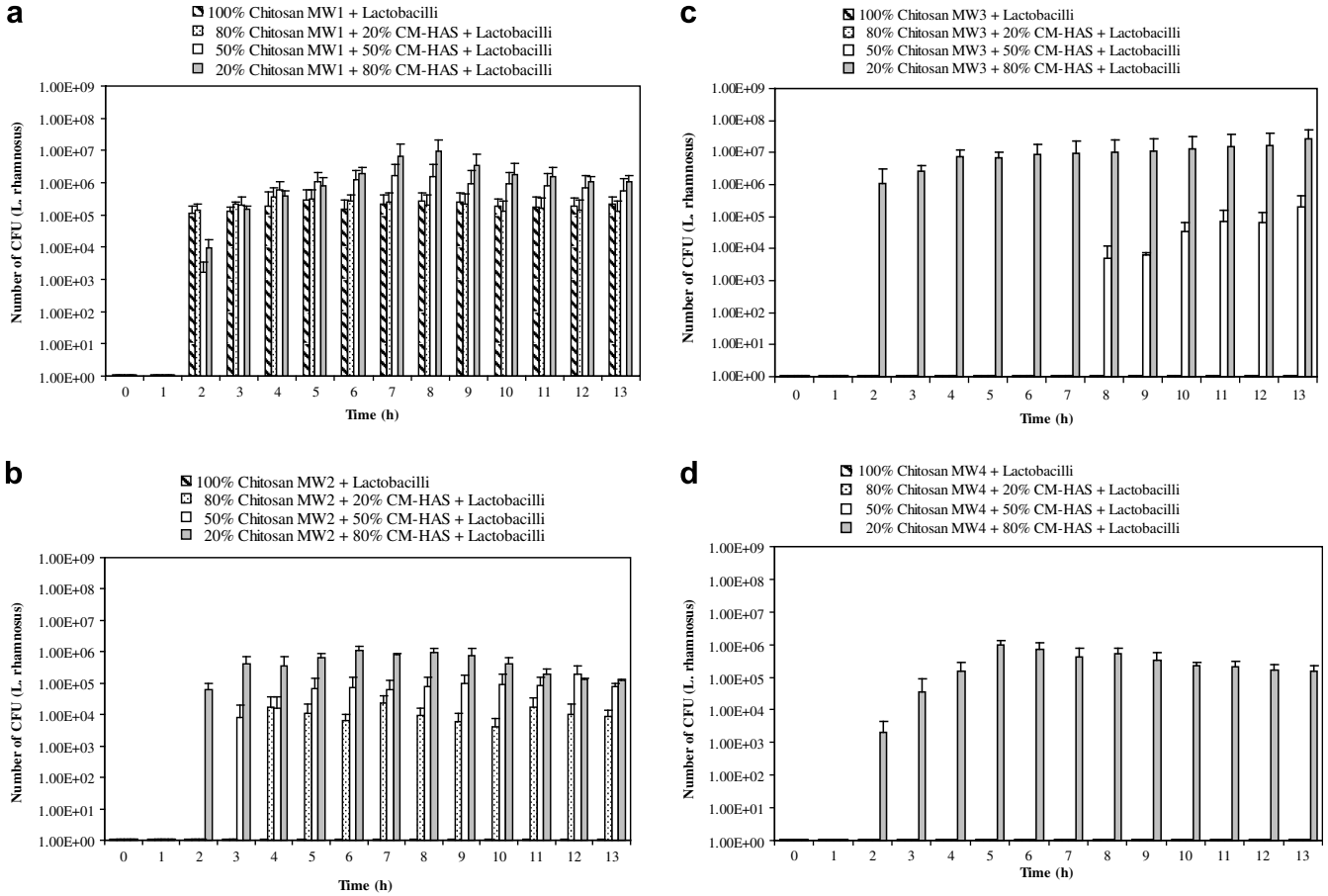

Fig. 1. Liberation of living *Lactobacillus rhamnosus* formulated in CM-HAS:Chitosan (MW1–4) monolithic tablets following incubation in gastric and intestinal medium. The tablets were incubated for 1 h in simulated gastric fluid and then transferred into simulated intestinal fluid for 12 h at 37 °C and 50 rpm. CM-HAS:Chitosan formulations based on (a) Chitosan MW1: 1.2×10^5 g/mol, (b) Chitosan MW2: 6.3×10^5 g/mol, (c) Chitosan MW3: 9×10^5 g/mol and (d) Chitosan MW4: 2.2×10^6 g/mol ($n = 3$). The bacterial number at the undetectable level was considered to be zero. CFU, colony-forming units.

Table 1
Modulation of bacteria liberation from CM-HAS:Chitosan formulations by different molecular weights of Chitosan

Tablets	Monolithic (h)				CM-HAS dry-coated (h)			
Chitosan Type	100%	80%	50%	20%	100%	80%	50%	20%
MW1	2	2	2	2	5	4	4	4
MW2	–	4	3	2	3	3	5	5
MW3	–	–	8	2	4	3	3	5
MW4	–	–	–	2	2	3	3	4

Tablets based on different ratios of CM-HAS:Chitosan (where the degree of substitution of CM-HAS was 0.25 and Chitosan was of different MW1–4) have been incubated for 1 h in simulated gastric fluid (SGF, pH 1.2) and 12 h in simulated intestinal fluid (SIF, pH 6.8), at 37 °C and 50 rpm. (–) means no detectable level of liberated bacteria after 1 h in SGF and 12 h in SIF. Chitosan MW1: 1.2×10^5 g/mol, Chitosan MW2: 6.3×10^5 g/mol, Chitosan MW3: 9×10^5 g/mol and Chitosan MW4: 2.2×10^6 g/mol.

morphology were examined by an Hitachi S-4300SE/N variable pressure-scanning electron microscope (Hitachi High Technologies America, Pleasanton, CA, USA), using an environmental-scanning electron detector.

3. Results and discussion

The initial count of *L. rhamnosus* free cells dropped from 10^9 CFU to an undetectable level after exposure to a pH 1.2 (SGF) for 1 h, as expected, since *L. rhamnosus* is known to be sensitive in an acidic environment. When incubated in SIF (pH 6.8) for 24 h, the free bacteria population was maintained (data not shown).

Chitosan is a polycationic polymer in acidic environments and the basic nature of Chitosan depends on its degree of deacetylation. Before solubilization, Chitosan rapidly forms gels in acidic environments and this makes Chitosan interesting in relation to the development of slow-release dosage forms for oral administration. The MW of Chitosan excipients was determined by viscosimetry for Chitosan 1 (MW1: 1.2×10^5 g/mol), Chitosan 2 (MW2: 6.3×10^5 g/mol), Chitosan 3 (MW3: 9×10^5 g/mol) and Chitosan 4 (MW4: 2.2×10^6 g/mol).

The substitution of high amylose starch polymer was confirmed by FTIR (data not shown) and its degree of substitution (DS 0.25) was determined by NMR spectroscopy of a CM-HAS hydrolytically depolymerized sample.

The monolithic tablet formulations based on high percentages of Chitosan MW1 presented a capping phenomenon after 1 h in

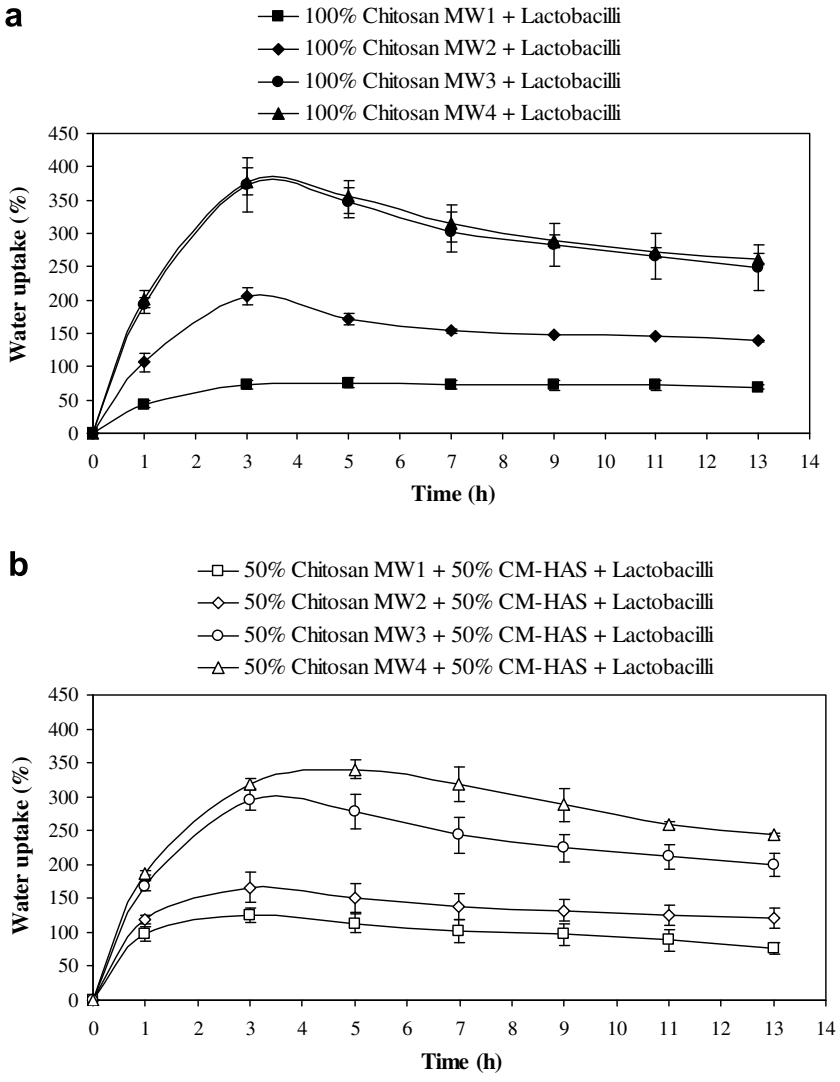

Fig. 2. Water uptake of monolithic matrices based on CM-HAS:Chitosan (MW1–4). The tablets were incubated for 1 h in simulated gastric fluid and 12 h in simulated intestinal fluid, at 37 °C and 50 rpm. (a) 100% Chitosan MW1–4 monolithic tablets and (b) 50% CM-HAS:50% Chitosan (MW1–4) monolithic tablets ($n = 3$). Chitosan MW1: 1.2×10^5 g/mol, Chitosan MW2: 6.3×10^5 g/mol and Chitosan MW3: 9×10^5 g/mol, Chitosan MW4: 2.2×10^6 g/mol.

SGF and 1 h in SIF (100% Chitosan) and, respectively, after 2 h in SIF (80% Chitosan:20% CM-HAS), and the bacteria were rapidly liberated (Fig. 1a). An increase in CM-HAS percentage afforded a good stabilization of the CM-HAS:Chitosan MW1 monolithic tablet in intestinal medium. Thus, formulations based on 50% CM-HAS:50% Chitosan MW1 and on 80% CM-HAS:20% Chitosan MW1 preserved the shape of the tablet after 1 h in SGF and 12 h in SIF at 37 °C and 50 rpm, without any capping. This stabilization could be partially due to an *in situ* interaction between the carboxylic function of CM-HAS and amino groups of Chitosan which could appear at the external layer and, in a certain extension, within the tablets, particularly with the advancement of the aqueous front.

From monolithic tablets based on 100% Chitosan MW2–4, there was no detectable level of bacteria liberation during 12 h of incubation in SIF, at 37 °C and 50 rpm (Fig. 1b–d). However, after 24 h of SIF incubation, low levels of released bacteria were found in the case of 100% Chitosan MW2–3 formulations and, at crushing of these tablets, a considerable amount of viable bacteria retained inside the tablets was found. The situation was different for the monolithic tablets based on 100% Chitosan MW4, which did not liberate the bacteria at all after 12/24 h of SIF incubation, but a marked amount of living *L. rhamnosus* was still found inside the tablet (data not shown).

The Chitosan in solution is known to present some antibacterial activity [22]. As the pK_a of Chitosan is about 6.5, below this pH the amino groups are positively charged and can interact with anionic groups of the microbial cell surface. Thus, Chitosan may bind and weaken the barrier function of the outer membrane of bacteria, altering the membrane permeability and producing metabolic disturbance and eventual death of bacteria [23]. In the case of bacteria formulated in monolithic tablet form, the gastric acidity in contact with the Chitosan is limited only to surface surroundings of the monolithic tablets. Furthermore, due to the protection afforded by the protonated carboxylic groups of CM-HAS [11] and because of possible hydrogen-bonding between the amino groups of Chitosan and hydroxyl and carboxyl groups of CM-HAS and, at the same time, of possible ionic stabilization between the amino groups of Chitosan and carboxylic functions of CM-HAS, its antibacterial effect seems greatly reduced. Increasing the ratio of the CM-HAS in the matrix, a larger number of CM-HAS macromolecules came in contact with SIF dissolution medium and, hence, the matrix erosion is enhanced and a higher amount of living bacteria is delivered (Fig. 1a–d). The increasing MW of Chitosan generates a delay in delivery times of the active principle [24]. Therefore, bioactive agent release *in vitro* can be controlled by adequate choice of the amount and of the MW of Chitosan in the tablet (Table 1, Fig. 1a–d), bacteria being the most effectively retarded by the highest MW of Chitosan. The monolithic formulations containing high percentages of CM-HAS (80%) delivered bacteria after 2 h for all the Chitosan MWs (Table 1).

The water uptake of CM-HAS:Chitosan monolithic tablets increased with the increase of Chitosan MW for both 100% Chitosan and 50% Chitosan:50% CM-HAS formulations (Fig. 2a and b). The Chitosan tablets presented a high water retention which could be advantageous for the development of slow-release formulations, because it might facilitate the formation of gels that would better control drug release.

The matrices based on 100% Chitosan (MW1–4), on 50% CM-HAS:50% Chitosan (MW1–4) and on 100% CM-HAS presented almost the same percentage of erosion after 1 h of SGF incubation (Fig. 3). At increased percentage of CM-HAS in CM-HAS:Chitosan monolithic formulations, the tablet erosion in SIF conditions was accelerated. Thus, tablets based on 100% Chitosan (MW1–4), with no CM-HAS at all, showed an erosion of only 16–27%, while those based on 50% CM-HAS:50% Chitosan (MW1–4) presented an erosion between 55% and 65% after 12/24 h of incubation in SIF (Fig. 3). The 100% CM-HAS matrix (particles granulometry between 75 and 300 μm) was rapidly eroded: 100% of matrix erosion was registered only after 1 h in SGF and 4 h in SIF (Fig. 3). A combined mechanism of bacteria release is proposed for CM-HAS:Chitosan monolithic tablets, involving the swelling of the tablets due to the Chitosan, followed by the erosion and dissolution of CM-HAS. In addition, a gel-forming barrier of Chitosan in acidic conditions also contributed to the delay of the bacteria delivery and depended on the amount (%) and on the MW of the Chitosan used in the formulation.

In the case of CM-HAS dry-coated tablets, the external double-layer of CM-HAS protected the CM-HAS:Chitosan tablet core against the acidity of SGF and, then, at a SIF pH of 6.8, it was deprotonated and eroded, affording a greater protection of bacteria. Since the gastric acidity did not enter into direct contact with Chitosan within the tablet, the amino groups of Chitosan may not be positively charged and thus, the antibacterial effect of Chitosan could be lowered. The CM-HAS dry-coating of monolithic tablets generated an unexpected change in the effect of Chitosan MW on bacterial liberation (Table 1), markedly improving the percentage of delivered bacteria in simulated intestinal conditions (Fig. 4a–d) in comparison with the monolithic formulations (Fig. 1a–d). The observed enhanced release of bacteria was attributable first to the erosion and dissolution of the CM-HAS external layer, favoring a substantial water uptake of Chitosan and, then, to the erosion and dissolution of the CM-HAS from the monolithic remaining core. Due to the presence of CM-HAS at the external sur-

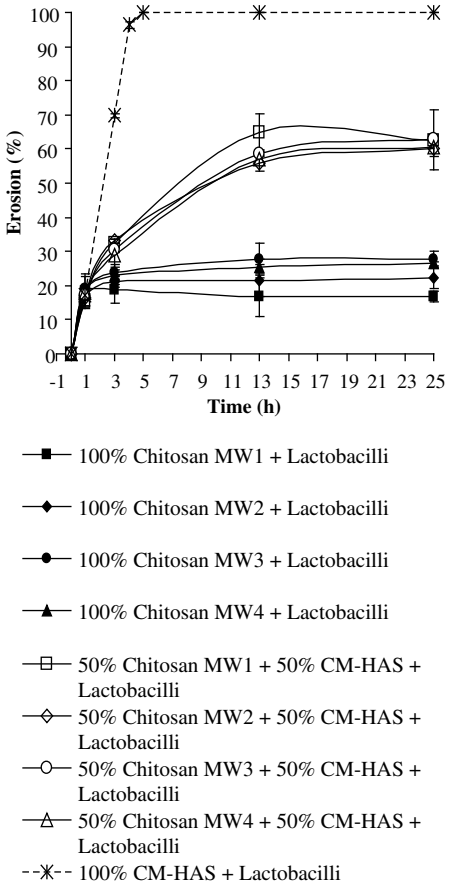

Fig. 3. Erosion studies of CM-HAS:Chitosan (MW1–4) monolithic tablets. The tablets were incubated for 1 h in simulated gastric fluid and 24 h in simulated intestinal fluid, at 50 rpm and 37 °C ($n = 3$). Chitosan MW1: 1.2×10^5 g/mol, Chitosan MW2: 6.3×10^5 g/mol, Chitosan MW3: 9×10^5 g/mol and Chitosan MW4: 2.2×10^6 g/mol.

face of the tablet, the acidity did not enter until the core of the tablet and no Chitosan gel can be formed in SGF. Thus, in the absence of the Chitosan gel-controlling barrier, the bacteria were rapidly liberated. Even the CM-HAS dry-coated formulations which contain 100% Chitosan core (MW2–4) liberated a large quantity of *L. rhamnosus* during the 12 h of SIF incubation (Fig. 4a–d).

In the case of CM-HAS:Chitosan (MW2–4) monolithic formulations (84% DDA), with the increasing MW of Chitosan, the bacteria release was delayed. This effect was not present in CM-HAS dry-coated formulations. Concerning the effect of the DDA on bacteria liberation, no conclusion could be reached from this study because the Chitosans used had different MWs (Chitosan MW1: 98% DDA and Chitosan MW2–4: 84% DDA). Since only the free (non-acetylated) amino groups bind protons, the charge density of the Chitosan depends on the ratio of the two monomers in a chain. It is known that for the same Chitosan MW, at increasing DDA, the viscosity of Chitosan increases because the polyelectrolytic characteristics of Chitosan become more marked. Thus, at a high DDA (98% for Chitosan MW1), Chitosan is highly charged in acidic solution and less charged for smaller DDA (as 84 % for Chitosan MW2–4). Sakkinen et al. (2002) found that the drug release rate was controlled in a lesser extent by the DDA of high MW of Chitosan [25]. In contrast, Sabnis et al. (1997) reported that the changing the DDA of Chitosan could control the drug release [26] and that higher DDA results in increasing formation of a rate-limiting Chitosan gel-barrier and in decreasing drug release rates from tablets (pH 1.2). However, the Chitosan of low MW used in the later case may explain the different findings from the two studies.

The surface morphology of CM-HAS and/or Chitosan tablets was analyzed by scanning electron microscopy (SEM) for monolithic and CM-HAS dry-coated tablets. Generally, the surface of the 100% Chitosan monolithic tablets was dense (Fig. 5a). The external surface of 50% CM-HAS:50% Chitosan monolithic matrices showed large voids within a dense matrix (Fig. 5b), similar results being obtained for Chitosan MW2–4. For the matrix based on 50% CM-HAS:50% Chitosan MW1, the surface structure was different (Fig. 5c). The 100% CM-HAS matrix had a porous structure (Fig. 5d), drastically differing from that of 100% Chitosan matrix (Fig. 5a).

After 1 h in SGF and 6 h in SIF, there were some morphology differences between the monolithic and the CM-HAS dry-coated formulations based on 100% Chitosan and on 50% CM-HAS:50% Chitosan, with the loss of dense surface for the formulations coated with CM-HAS (Fig. 5a′–b′), similar results being observed for MW2–4 of Chitosan. This aspect confirmed the fact that, in the presence of CM-HAS external layer, the Chitosan within the tablet did not enter into direct contact with the gastric acidity and, thus, did not form the dense, gel-barrier that controls the bacteria liberation in SIF medium. Concerning the internal structure of CM-HAS dry-coated 50% CM-HAS:50% Chitosan formulations, there were no morphology differences between the tablets made with the four MWs of Chitosan (data not shown).

In vivo, Chitosan is known to be degraded by enzymes produced by bacteria present in the colon. Zhang and Neau [27] reported that both MW and DDA affect the Chitosan degradation by colonic enzymes. Lower MW and DDA of Chitosan favored a faster degrada-

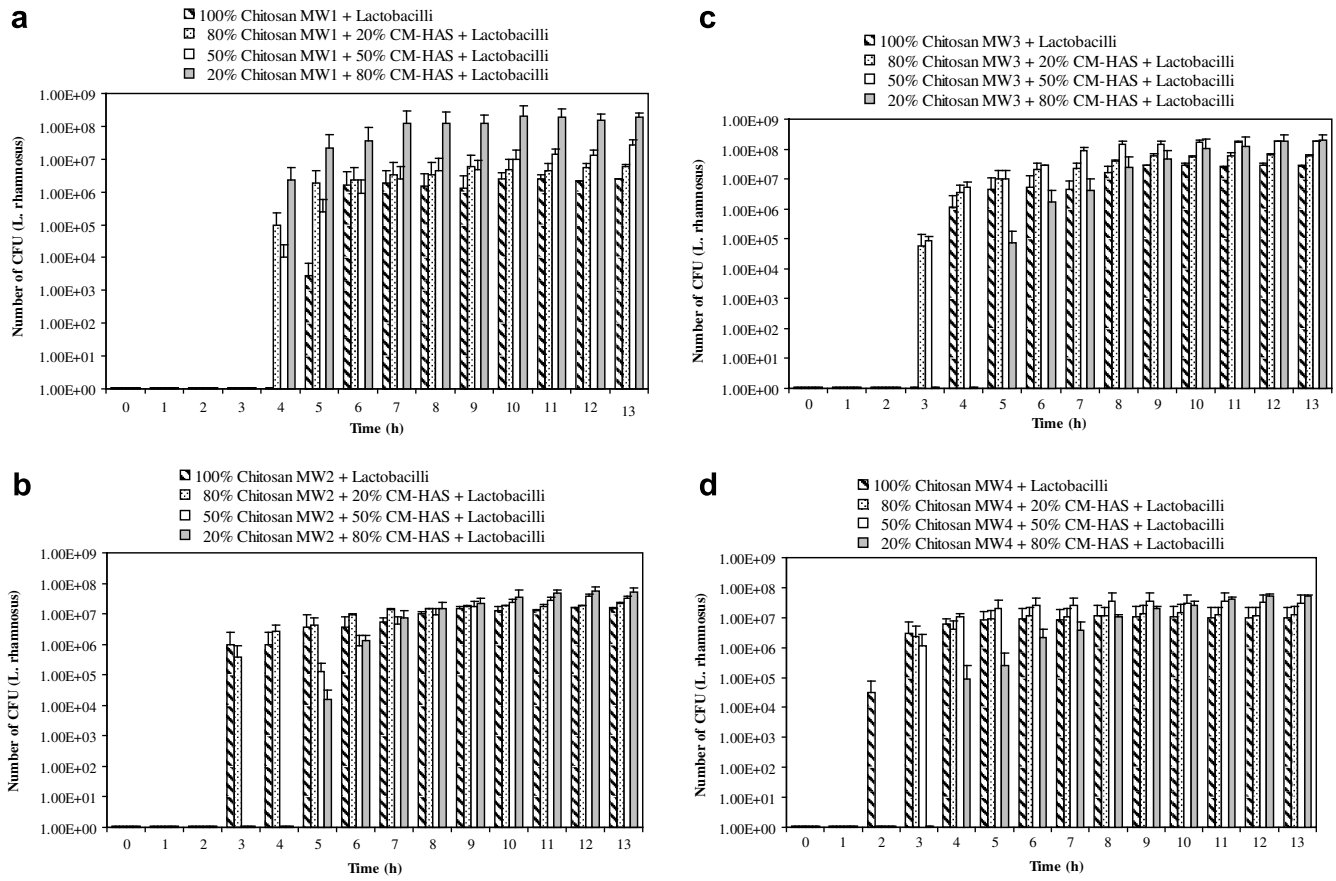

Fig. 4. Liberation of living *Lactobacillus rhamnosus* formulated in CM-HAS dry-coated tablets containing CM-HAS:Chitosan (MW1–4) core following incubation in gastric and intestinal medium. The tablets were incubated for 1 h in simulated gastric fluid and then transferred into simulated intestinal fluid for 12 h, at 37 °C and 50 rpm. CM-HAS:Chitosan formulations based on (a) Chitosan MW1: 1.2×10^5 g/mol, (b) Chitosan MW2: 6.3×10^5 g/mol, (c) Chitosan MW3: 9×10^5 g/mol and (d) Chitosan MW4: 2.2×10^6 g/mol ($n = 3$). The bacterial number at the undetectable level was considered to be zero. CFU, colony-forming units.

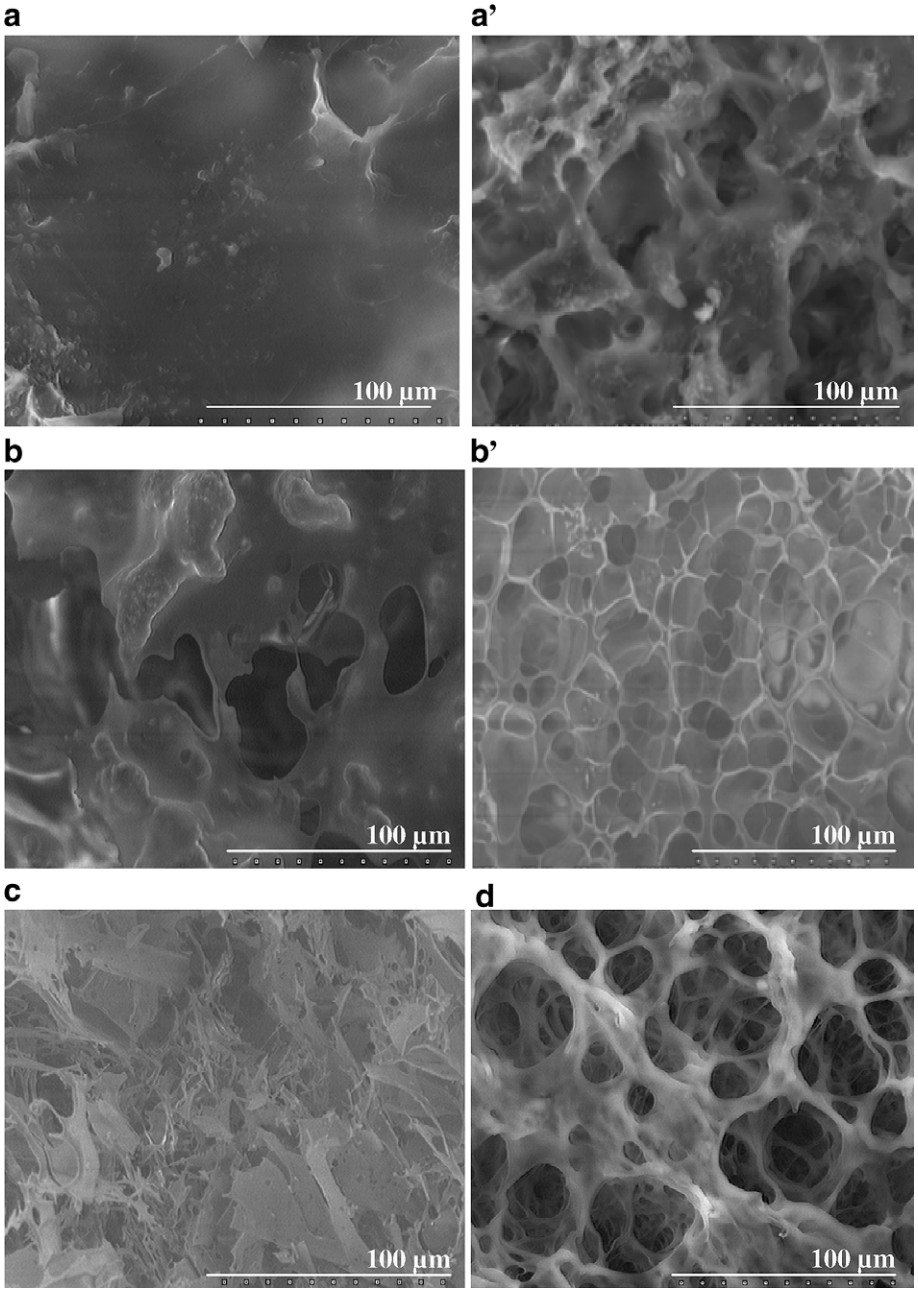

Fig. 5. Scanning electron microscopy images from surface structures of CM-HAS:Chitosan matrices. (a) 100% Chitosan MW4 monolithic and (a′) dry-coated tablet, (b) 50% CM-HAS:50% Chitosan MW4 monolithic and (b′) dry-coated tablet, (c) 50% CM-HAS:50% Chitosan MW1 monolithic tablet, (d) 100% CM-HAS monolithic tablet. Only with the exception of 100% CM-HAS monolithic tablet (which was kept for 1 h in simulated gastric fluid/SGF and 2 h in simulated intestinal fluid/SIF), all the other tablets were kept for 1 h in SGF and 6 h in SIF, at 37 °C and 50 rpm. The tablets were frozen and lyophilized for a week and then, they were analyzed by scanning electron microscopy ($n = 3$). Chitosan MW1: 1.2×10^5 g/mol and Chitosan MW4: 2.2×10^6 g/mol.

tion rate. It was also reported that the pH may decrease in the colon as compared with the pH of the small intestine [28] due to the acidification of the colonic contents by the products of bacterial fermentation. This lower pH could contribute to enhanced Chitosan degradation and to a better colonic liberation of bioactive agent.

The novelty of our formulation is the ionic self-stabilization of the two carboxylated (CM-HAS) and amino (Chitosan) polymers which ensure a two-step pH sensitive protection of bioactive agents. Thus, CM-HAS (protonated and compacted in acidic medium) ensures gastro-protection and it is susceptible to alpha-amylase attack, modulating the bacteria release, whereas Chitosan hydrogel (deprotonated in intestinal fluid) ensures stabilization against pancreatic enzymes. Furthermore, Chitosan, low soluble in neutral media, prevents early release of bacteria, which can be thus delivered to the colon. The advantage of the use of CM-HAS versus other carboxylic matrices (i.e. alginate, xanthan) associated with Chitosan is that our CM-HAS, with less carboxyl equivalents/monomer, is supposed to be less adhesive in the early gastrointestinal segments, enhancing thus the colon delivery.

In conclusion, CM-HAS association with Chitosan (monolithic tablets) can modulate the Chitosan excipient properties as probiotic agent carrier, increasing the number of liberated living bacteria, with the bacteria release modulated by the molecular weight of Chitosan. Concerning the dry-coated tablets, the CM-HAS external

layer afforded a better protection of bacteria, with an enhanced number of CFU of *L. rhamnosus* delivered in simulated intestinal conditions. In this context, the CM-HAS could be an efficient excipient for dry-coated formulations for colon delivery.

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