According to section 2.3.4.2, chitosan coating has a considerable positive effect on the protection of encapsulated probiotics, but a high number of coatings and materials combinations do not bring any main advantage on probiotic protection

The effect of alginate in the protection of probiotics from the harsh conditions of digestion

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Introduction

Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit to the host. However, to accomplish this positive influence on Human health, probiotics should survive to the passage through the upper digestive tract in large numbers to unsure a desired beneficial effects in the host. Several encapsulation methods have been used to protect probiotics. Alginate is the most used biopolymer in the production of these systems, although its performance is totally dependent of its characteristics. In this work, alginates with different molecular weights and different M/G ratio were used in the encapsulation of *Lactococcus lactis* spp. *cremoris* (LLC) aiming the protection of this probiotic bacteria against the harsh conditions of digestion. In the first set of experiments, variables such as flow rate, needle-CaCl2 solution distance and the stirring speed, CaCl2 concentration, alginate type, alginate concentration and needle diameter were studied in order to understand how they affect the formation of alginate beads.

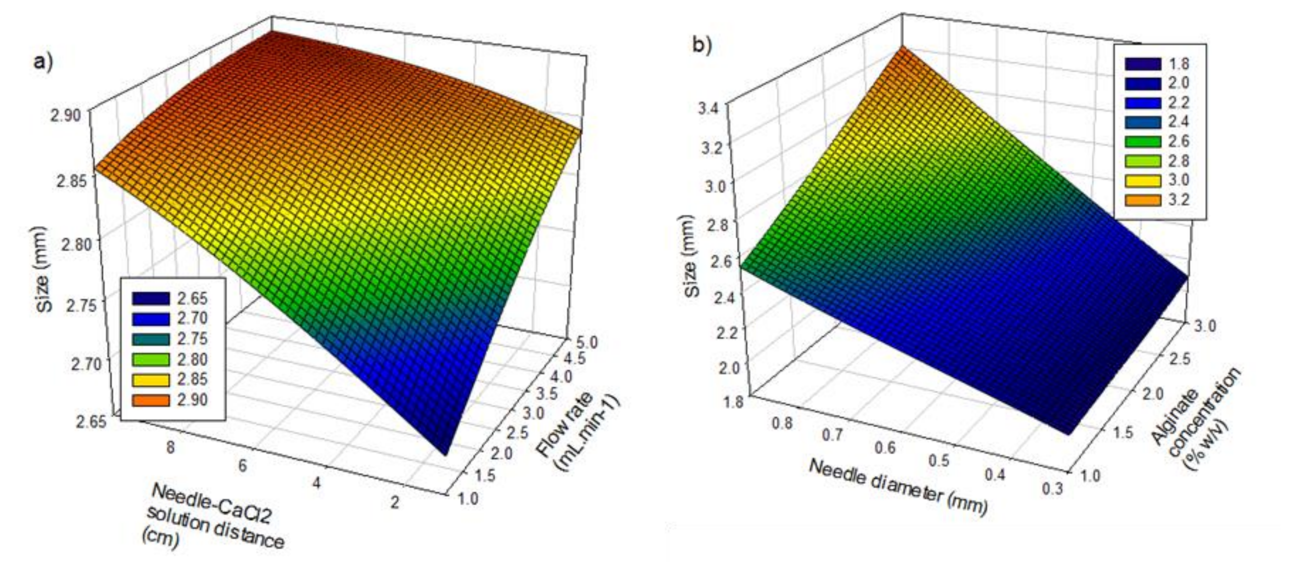
Methods

Alginate beads production were studied based on two experimental designs where external and internal parameters were evaluated separately Briefly, a volume of 10 mL of alginate solution (with the concentration tested in each experiment), was dropped in 90 mL of a solution of CaCl2, with different concentrations. After that the alginate solution was transferred to a syringe, and dropwise, with the help of a syringe pump, at different flow rates, through needles with different diameters, into the CaCl2 solution that was placed at variable distances, and magnetically stirred (with different stirring speeds). Afterwards, the alginate beads were freeze-dried. LLC encapsulation was performed by using a volume of 9 mL of sterile alginate solution (1 % w/v), combined with 1 mL of cells + PBS, previously prepared, and 90 mL of a sterile 0.25 mol.L^{-1} solution of CaCl2, 1 mL.min^{-1} of flow rate; 300 rpm for the stirring speed; 1 cm for the needle-CaCl2 solution distance; 0.4 mm of needle diameter; and a stirring/hardening time of 20 min.

The in vitro digestion was conducted using a gastrointestinal simulation test based on the protocol presented by Minekus et al. (2014)[1] with some modifications. The solutions used were Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF). The first step was the introduction of a 5 g sample in a 50 mL Falcon with 4 mL of SSF. This mixture was incubated for 2 min at 37 ºC (all the incubation steps are performed at this temperature and shaking conditions). The next step was to add 8 mL of SGF and a volume of porcine pepsin in water (an exact volume to achieve a 2000 U.mL-1 in the final mixture), adjusting the pH to 3 (using an HCl 1 mol.L-1 solution). The mixture was incubated during 2 h. After this, an exact volume of pancreatin suspension to achieve a 200 U.mL-1 in the final mixture, an exact volume of bile solution to achieve a final concentration of 10 mmol.L-1 and 8 mL of SIF were added. Falcons were incubated during 2 h and sampling was performed each 30 min during the gastric and intestinal simulation. All the experiments were performed in triplicate.

Results

The smaller capsules were formed with lower flow rates and smaller needle-CaCl2 solution distance, leading to the formation of beads with a size of 2.670 ± 0.186 mm. Higher flow rates produced bigger beads, that is explained by the flow rate and the rate at which the droplets fall down[2]. Higher needle-CaCl2 distances will influence the diving of the drop into the calcium solution and thus will highly influence the alginate drop structure. This factor will lead to a less organized bead, allowing a bigger penetration of calcium ions and consequently beads with higher sizes. The needle diameter variable was the most influencing variable on the size of the beads, being the smallest beads obtained with a needle diameter of 0.3 mm (1 % (w/v) alginate solution, 1 mol.L-1 of CaCl2), with a size of 1.940 ± 0.094 mm. These results were expected considering that the size of the drops created, that corresponds approximately to the beads' final size, are influenced directly by the needle diameter. Higher alginate concentrations will also create bigger beads, due to the higher viscosity of the polymer solution that will originate bigger drops. Figure 1 c) shows that the beads' size increase when alginates with high molecular weight are used, such as CR8223. This fact is justified by the creation of less organized structures when high molecular weight alginates are used. In general, bigger molecules will create bigger beads. The lower size molecules (low molecular weight) of alginate allow an easy construction and fitting of a higher mass of molecules, thus creating beads with smaller sizes. Fourier transform infrared (FTIR) spectroscopy showed relevant differences between beads produced proving the impact of different M/G ratios in the beads' chemical structure. In general, low molecular weight and low M/G ratio alginate (LFR5/60) proved to produce the most well organized (according to SEM analyses), less permeable (pore diameter of 2.52 mm) and stronger alginate beads, moreover molecular weight and M/G ratio proved to be an important variable on the protection of probiotics against the harsh conditions of digestion. Figure 2 shows a constant decrease on LLC viability during time, in all tested systems. The beads produced with alginate CR8133 and LFR5/60 showed to be the best protection of the LCC bacteria, being the values of cell viability with beads produced with these two alginates statistically equivalent ($p<0.05$). For the beads produced with alginate CR8223 a substantial loss of LLC viability is observed during the experiment, up to 120 min (end of stomach simulation). This difference might be related to the high molecular weight of this type of alginate (250-350 kDa comparing with LFR5/60 - 20-60 kDa and CR8133 - 90-180 kDa) that benefited the formation of a less organized and more porous structure. The molecular weight influences the porosity of the microcapsules created, being responsible for a higher permeability that leads to a higher contact of LLC with the outside medium. This phenomenon will be responsible for the decrease of LLC viability in that experiment, once it facilitates the diffusion of the simulated stomach phase into the beads and therefore the contact of probiotics with harsh conditions.



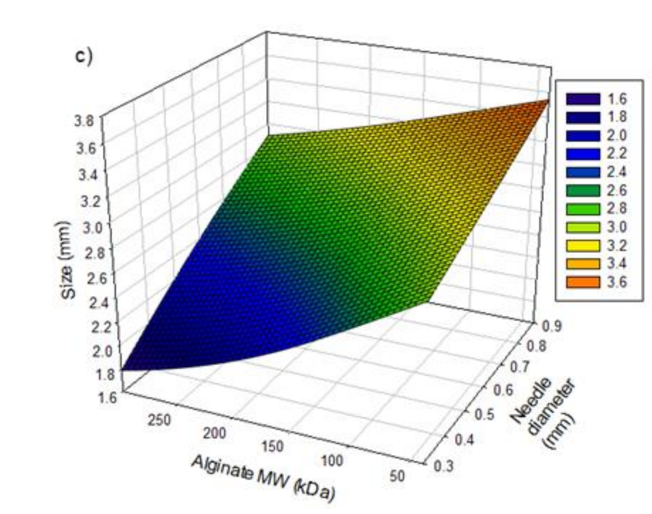


Figure 1 - LLC viability during the gastrointestinal digestion simulation (first 120 min - simulation of the stomacal digestion; subsequent 120 min (until 240 min) - simulation of the intestinal digestion. In black is the encapsulated LLC viability and in grey is the free LLC viability.

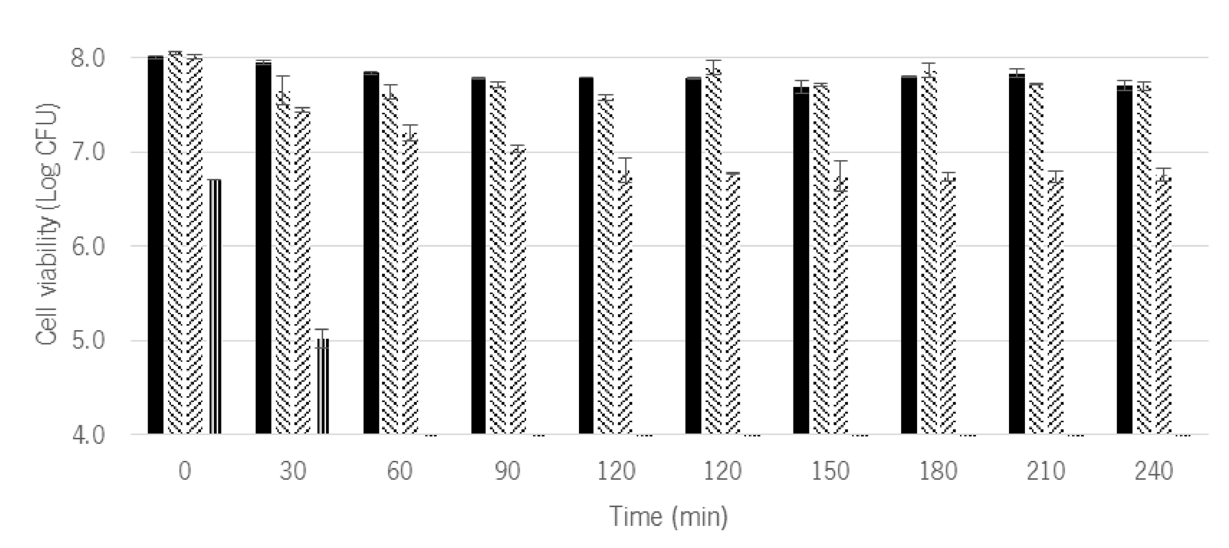


Figure 2 - Viability of free and microencapsulated LLC under a gastrointestinal simulation.

Conclusions

The variables that most influenced the beads' size in the extrusion technique were the alginate concentration, alginate type (molecular weight and M/G ratio) and the nozzle diameter. These variables are directly correlated with the size of the produced beads. Alginate's characteristics such as the molecular weight and M/G ratio, demonstrated to have a positive impact in order to accomplish a system able to protect probiotics from the harsh conditions of digestion. It is important to refer that strong differences were achieved in the protection of probiotics in tests where the only difference was the molecular weight (and/or M/G ratio) of the alginates used. Therefore, alginate LFR5/60, a low molecular weight and high G content alginate, showed to produce the biggest, strongest and best suitable beads for probiotics protection in a digestive system.

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Acknowledgements

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684) and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte.

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