Probiotics and Antimicrobial Proteins (2018) 10:157–167
https://doi.org/10.1007/s12602-017-9355-x

Study and Understanding Behavior of Alginate-Inulin Synbiotics Beads for Protection and Delivery of Antimicrobial-Producing Probiotics in Colonic Simulated Conditions

Abdelbasset Atia[1,2,3] · Ahmed Gomaa[1,2,4] · Benoit Fernandez[1,2] · Muriel Subirade[1,2] · Ismail Fliss[1,2]

Published online: 27 November 2017

Abstract
According to the World Health Organization (WHO), using antibiotics as growth promoters for livestock—particularly swine—is the principal cause of antibiotic resistance. It is therefore clear that finding an alternative to antibiotics becomes an emergency. Hundreds of recent studies have appointed probiotics as potential candidates to replace or to be used in combination with antibiotics. However, bringing probiotics alive to the colon—their site of action—remains a big challenge because of different physiological barriers encountered in proximal gastrointestinal tract (GIT) such as acidic pH and bile salts that may affect the viability of probiotic cultures. To overcome this problem, in previous studies, we developed and characterize a synbiotic formula consisting of beads of a mixture of alginate and inulin. Three potential probiotics strains namely *Pediococcus acidilactici* UL5 (UL5), *Lactobacillus reuteri* (LR), and *Lactobacillus salivarius* (LS) were encapsulated to study their release and the behavior of this synbiotic formula throughout the GIT using in vitro models. The survival and the release of bacteria from beads were studied by specific PMA-qPCR counting. The microscopic aspects of the beads were studied using scanning electron microscopy (SEM). Moreover, the microbial dynamics inside beads were studied by fluorescence microscopy using the live/dead test. Our results have shown that the beads containing 5% inulin were the most stable in the stomach and throughout the small intestine. However, beads were completely degraded in approximately 3 h of incubation in the fermented medium that mimic the colon. These results were confirmed by SEM and fluorescence microscopy images. Therefore, it can be stated that the AI5 formulation well protected the bacteria in the upper part of the digestive tract and allowed their controlled release in the colon.

Keywords Colonic delivery · Colonic behavior · Inulin-alginate beads · prebiotics · probiotics

Introduction

In modern pharmaceutical field, drugs are defined as delivery system of an active ingredient, which follows the conventional steps of the LADMER system (Liberation, Absorption, Distribution, Metabolism, Excretion and Response). The first letter of this acronym "L" refers to the step of liberation (release) which is a crucial step [1, 2]. For biopharmacists, a rapid release of the active ingredient is generally sought to induce a therapeutic activity in a short time as in the case of pain killers which are intended to relieve the patient quickly [3, 4]. However, in some cases, a slowed or delayed release of active ingredient may be desired to prolong the duration of therapeutic action or to reach relatively distant target sites in the digestive tract as in the case of probiotics which act mainly in the colon [5–7].

The colonic microbiota contains a very complex and diverse ecosystem [8]. The cell population of the gut is estimated to be ten times higher than the total cells constituting the host [9, 10]. This cell population is composed of over than 1100 different species and contains 100 times more genes than the host [11]. The colon is of such importance in farmed

✉ Ismail Fliss
Ismail.Fliss@fsaa.ulaval.ca

[1] Department of Food Science, Université Laval, Québec G1V 0A6, Canada

[2] Institute of Nutrition and Functional Foods (INAF), Université Laval, Québec G1V 0A6, Canada

[3] Biena | Probiotics and Lactic Cultures, 2955 Rue Cartier, Saint-Hyacinthe, QC J2S 1L4, Canada

[4] Food Science and Nutrition Department, National Research Center, Dokki, Cairo, Egypt

animals that some researchers proclaim its integration as a parameter of animal phenotyping [9]. The main role of the colonic microbiota is to facilitate digestion and absorption of non-digestible sugars and/or complex lipids. The colonic microbiota may influence systemically energy metabolism by acting on the metabolism of host cells and can also influence immune system [11]. Thus, the modulation of the colonic bacterial population is an effective way to influence the state of health and overweight condition of livestock.

There are different strategies to modulate the activity of the colonic bacterial microbiota. Administration of probiotics, prebiotics, or an association of both named synbiotic can modulate the activity of the colonic bacterial microbiota.

Probiotics are defined as "live microorganisms, which when consumed in adequate amounts, confer health benefits to the host" [12]. The health benefits of probiotics for livestock especially in swine were frequently investigated in literature. Prebiotics are defined as non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of probiotics, and thus improves host health [13].

The idea of combining prebiotics and probiotics led to the possibility of encapsulating probiotic in a prebiotic matrix. Thus far, there are a wide variety of encapsulation methods. That makes the choice of the appropriate technique complicated. The various approaches are often based on the use of a single or a mix of polymer. They could be in a particular structure that provides chemical and physical resistance enough to transport the probiotics through the digestive tract and control their release in the colon [14].

Encapsulation of probiotics requires the use of soft methods, which do not require high temperatures and physicochemical conditions that impact bacterial survival [15, 16]. Among methods of probiotics encapsulation, spray drying, extrusion, and emulsification technologies are the most used [14, 17]. The efficiency of these methods must be evaluated using several techniques exploring physico-chemical properties of the matrix [18, 19], microbiological characteristics of the probiotic [18–20], and simulating the physiological conditions under which these products ending up once ingested [21, 22].

In a previous study (Atia et al. [23]), we developed synbiotic formulations based on a mixture of three potential probiotic strains (*Pediococcus acidilactici* UL5, *Lactobacillus reuteri* ATCC 53608, and *Lactobacillus salivarius*) encapsulated by an extrusion/ionotropic gelation method in an alginate/inulin prebiotic matrix. We demonstrate that the matrix containing 2% alginate and 5% inulin (named AI5) was the most effective formulation in terms of gastrointestinal protection and probiotic delivery in the colon. To our knowledge, works that have succeeded to deliver live and functional probiotics to the colon are very rare. Since the target of AI5 formulation is the colon which is considered as an integrated metabolic space [24], and described by some authors as a superorganism [8, 25], the behavior of the formulation needs to be studied precisely in colonic conditions.

Thus, the aim of the current work is to study the following: (i) the behavior of AI5 formulation in the fermented (FM) or unfermented (UFM) simulated colonic media and (ii) the bacterial dynamics of encapsulated strains inside beads during the passage through the digestive tract.

Materials and Methods

Materials

Alginic acid sodium salt from brown algae (4–12 cps for 1% *w*/*v* aqueous solution at 25 °C, mannuronic/guluronic acid ratio of 0.65), calcium chloride ($CaCl_2$), sodium citrate, isopropanol, agarose, and ammonium acetate were purchased from Sigma Chemical Company, St Louis, MO, USA. Inulin Frutafit® was kindly provided by Sensus America (Lawrenceville, NJ, USA); sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from fisher scientific (Ottawa, ON, Canada). Dimethylsulfoxide (DMSO) was purchased from Serva (Heidelberg, Germany). Sodium chloride (NaCl), potassium chloride (KCl), and potassium dihydrogen phosphate (KH_2PO_4) were purchased from EMD (Darmstadt, Germany).

Methods

Bacterial Strains and Growth Conditions

Lactobacillus reuteri ATCC 53608 (LR), a reuterin producer [26] (American Type Culture Collection, Rockville, MD, USA); *Pediococcus acidilactici* UL5 (UL5), a pediocin PA-1 producer [27]; and *Lactobacillus salivarius* (LS) [28] (Dairy Research Centre, Laval University culture collection, Quebec, Canada) were used as probiotic strains. *Pediococcus acidilactici* UL5 and *Lactobacillus salivarius* were grown in MRS broth incubated aerobically for 24 h at 37 °C [27], whereas *Lactobacillus reuteri* was incubated anaerobically for 24 h at 37 °C [26]. Bacterial strains were subcultured three times (1%, *v*/*v*) in MRS prior to experiments. Experiments were carried out aseptically in a laminar flow cabinet.

Preparation of Beads

For this work, all the three tested strains were encapsulated simultaneously in the beads. One hundred milliliters of bacterial suspension grown as previously described was centrifuged for 10 min at 10,000 rpm, and pellets ($\approx 10^{11}$ cfu) were washed two times with 10 mL of phosphate-buffered saline (PBS). Collected pellets were suspended in 10 mL of the alginate-inulin solutions (2–5%*v*/*v*). The three strains were

encapsulated simultaneously by thoroughly mixing; then, beads were prepared by the extrusion/ionotropic gelation method as described by Atia et al. [23]. Briefly, 10 mL of bacterial suspension was poured through a drop-by-drop system with a constant flow (2 mL/min) into 90 mL of 0.1 M calcium chloride solution at low magnetic stirring (40 rpm) [29]. The formed beads were then separated, using a sieve, from the calcium chloride solution for characterization and simulation of digestion further analysis.

Gastrointestinal Simulation

Gastrointestinal simulation experiments were performed according to the following diagram (Fig. 1). Experiments were designed in two main steps; the first step simulated the upper parts of the digestive tract, while the second simulated lowers parts (colon).

Behavior of Synbiotic Beads in the Upper Parts of the Gastrointestinal Tract

Simulation of upper part of the gastrointestinal tract was done with flow-through method as described by Gao [30]. A two 12-mm flow cells USP 4 assembly (Leap Technologies, Carrboro, N.C., USA) were used during this study. USP4 cells were prepared using a check valve ruby bead (5 mm) in the apex of each cell with a glass-bead bed of 1 mm in the cone area of each cell; then, 5 g of synbiotic bead sample was positioned on the glass bead bed. The system was then set in closed loop configurations and placed in a water bath with controlled temperature at 37.0 ± 0.5 °C. Beads underwent exposure to simulated gastric fluid (SGF) supplemented with pepsin gastric lipase at pH 1.2 for half an hour followed by exposure for 1.5 h to pH 4.5, and finally to simulated intestinal fluid (SIF) supplemented with pancreatin and bile salts at pH 6.8 during 4 h. Samples of 10 beads (0.100 g ± 0.003) and 1 mL of media were taken at the beginning of the experiment and at the end of each phase. The bacterial count of the samples was measured to monitor the survival and release of bacteria during simulated gastrointestinal conditions.

Behavior of Synbiotic Beads in the Colon

Colonic environment was simulated using the medium described by Macfarlane et al. [31] and modified to match swine colonic conditions as described by tanner et al. [32]. The medium was used before and after a fermentation with colonic microbiota as described by Le lay et al. [33]. Fermented medium was taken at the end of the stabilization period, centrifuged at 10,000*g* for 10 min then sterilized by filtration with a 0.2 μm filter. Simulation of colonic part was performed in a sequential form using 12-well plates. Each well was filled with 20 digested beads (0.2 g ± 0.005) from USP4 step, in 2 mL of fermented or unfermented Macfarlane media. The percentage of released bacteria was calculated in relations to the initial number of encapsulated bacteria.

Monitoring of the Survival and Release of Bacteria During the Digestion

During the experiment, sampling was done at different time points as shown in Fig. 1. Beads were washed with PBS buffer and solubilized in sodium citrate buffer at pH 6.0 (1 g of beads in 9 mL of 55 mM sodium citrate)

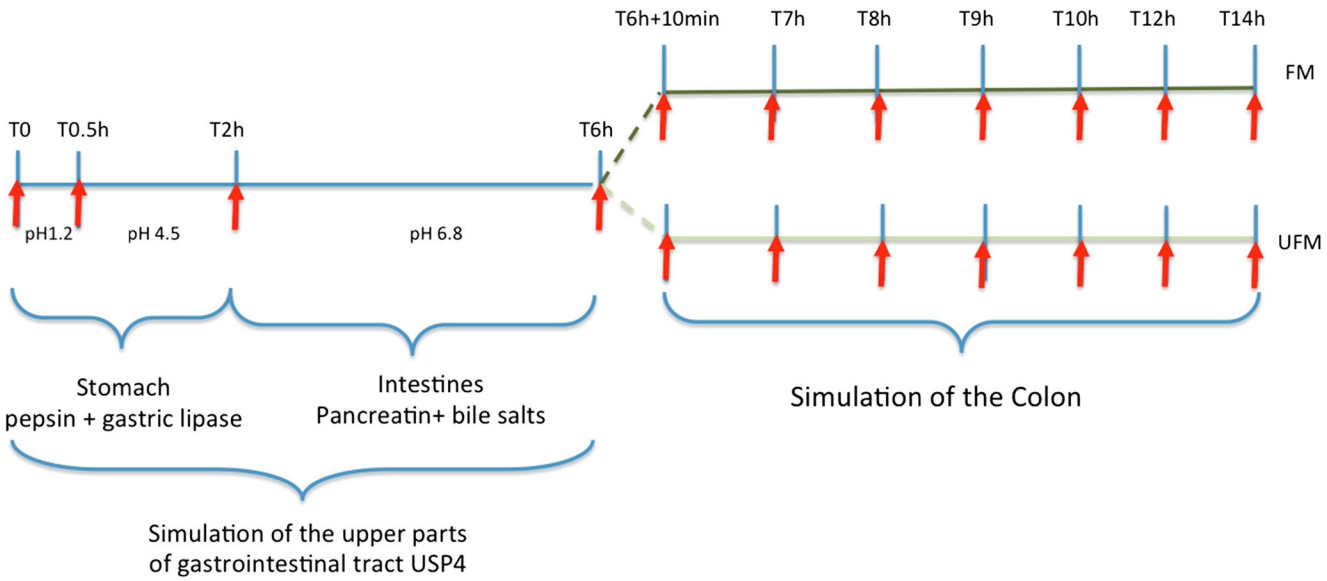

Fig. 1 Schedule of the gastrointestinal simulation steps. FM: fermented medium, UFM: unfermented medium, arrows: sampling times

[34]. Bead samples were used to track the survival of strains while media samples were used to track the release of the strains from beads. Enumeration of bacterial strains was performed by quantitative polymerase chain reaction combined with propidium monoazide treatment (PMA-qPCR) as described below.

Propidium Monoazide Treatment Propidium monoazide treatment was performed according to the protocol described by Fernandez et al. [35]. Briefly, a stock solution of propidium monoazide (Biotium, Hayward, CA, USA) in DMSO 20% was prepared and stored in the dark at − 20 °C. An aliquot of 2.5 μL was added to 1 mL of fresh samples. Samples were incubated for 5 min in clear Eppendorf tubes in the dark with periodic mixing during the incubation. Following the incubation, the Eppendorf tubes were placed on ice and exposed to a 500-W halogen light source at a distance of 20 cm for 5 min [36]. The Eppendorf tubes were turned over manually every minute of illumination. Finally, samples were immediately frozen by immersion in liquid nitrogen followed by storage at − 80 °C until DNA extraction.

DNA Extraction The employed DNA extraction protocol was based on the protocol developed by by Fernandez et al. [35, 37]. Samples were washed three times in Tris–EDTA buffer (20 mM Tris–HCl, 2 mM EDTA), and the centrifugal pellet was resuspended in 200-μL Tris–EDTA buffer containing 40 mg mL^{-1} of lysozyme, 200 U mL^{-1} of mutanolysin, and 4 μg mL^{-1} of proteinase K followed by incubation for 1 h at 37 °C. Subsequent steps were performed following the manufacturer's instructions of the Wizard® genomic DNA Purification Kit handbook (Promega, Madison, WI, USA). Purity of the DNA sample was checked by measuring the 260/280 nm ratio using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Bacterial Enumeration by qPCR Quantitative polymerase chain reaction (qPCR) was carried out using Fast SYBR Green qPCR Master Mix (Applied Biosystemd, Carlsbad, CA, USA). Experiments were run in 96-well plates. Each qPCR reaction mixture was prepared as shown in Table 1. Negative control was introduced for each assay.

Table 1 composition of each PCR reaction

Reagents	Volume
forward primer 5 μM	1 μL
reverse primer 5 μM	1 μL
10× diluted purified DNA	5 μL
2× SYBR Green master mix	12.5 μL
DNase-free water	5.5 μL
Total	25 μL

The primer pairs developed by Mora et al. (2006), forward −5′-GGACTTGATAACGTACCCGC-3′; reverse 5′-GTTC CGTCTTGCATTTGACC-3′ targeting the *ldhD* gene was used to quantify *Pediococcus acidilactici* UL5. This primer generated an amplicon of 449 base pairs (bp) [38]. *Lactobacillus salivarius* was quantified using primers developed by Harrow et al. (2007): forward −5′-GTCG TAACAAGGTAGCCGTAGGA-3′ and reverse 5′-TAAA CAAAGTATTCGATAAATGTACAGGTT-3′. They give an amplicon of 97 bp [39]. Finally, *Lactobacillus reuteri* primers were as follows: forward −5′- TTGGAAATGTTCCA CAAGAC-3′ and reverse 5′-TTGTGAGTTTGGAT TGAACC-3′ [40]. qPCRs were performed on an ABI 7500 real-time PCR system (Applied Biosystems, Streetsville, ON, Canada). Standard curves were generated from plots of threshold cycle (Ct) versus bacterial count (cfu/mL). The bacterial count (cfu/mL) was interpolated from the averaged standard curves. A detection limit near 5×10^3 cfu/mL was calculated for all strains.

Study of the Macrostructure and Microstructure of the Beads During Gastrointestinal Digestion

Bead macrostructure was analyzed at different sampling times, using a Bio-Rad ChemiDoc Imager (Bio-Rad Laboratoires, ON, Canada). Bead samples were also examined under scanning electron microscope (SEM) (JSM-5310LV Scanning Microscope, Tokyo, Japan). They were mounted on metal grids using double-sided adhesive tape and gold coated under vacuum. Observations were performed at low (× 50), high (× 2700), and very high (× 9000) magnification power.

Monitoring the Distribution of Bacteria Inside the Beads During Gastrointestinal Digestion

Microbial distribution inside beads during gastrointestinal digestion was studied using L-7012 LIVE/DEAD ® BacLight ™ Bacterial Viability kit, according to manufacturer's instructions [41]. The kit composed of two fluorochromes: SYTO 9, green-fluorescent nucleic acid dye and propidium iodide, red-fluorescent nucleic acid dye. The maximum excitation/emission was 480 nm/500 nm for SYTO 9 and 490 nm/635 nm for propidium iodide. Thus, with an appropriate SYTO 9/propidium iodide mixture, bacteria with intact cell membranes emit green fluorescence, whereas bacteria with damaged membranes emit red fluorescence. Bead samples at different times of digestion were collected, split into two halves, colored, and examined under Olympus BX51 fluorescence microscope (Tokyo, Japan). Obtained images were analyzed with the GNU Image Manipulation Program (GIMP) software, for semi-quantitative determination of live and dead bacteria inside the beads during the digestion.

Statistical Analysis

All measurements were performed at least in triplicate. Data was statistically analyzed by analysis of variance (ANOVA) using SPSS software. Mean comparisons were performed using Tukey's honest significant difference (HSD) test with a significance level of $p < 0.05$.

Results and Discussion

Monitoring the survival and release of bacteria during the digestion

Bead samples were used to track survival of the used strains during digestion. Indeed, the probiotics strains used in this work were selected because of their potential antimicrobial properties [42, 43] and also for their compatibility; indeed, no effect was observed between the three strains when they were encapsulated together as demonstrated in our previous works [23]. Furthermore, our previous studies demonstrated the resistance of AI5 formulation in the upper parts of the gastrointestinal tract [44]. Thus, the current work was a study to complement and provide missing information about the behavior of this formulation in the fermented and unfermented colonic media.

The count of each bacterial strain was performed using PMA treatment followed by qPCR. The PMA treatment stops the amplification of DNA from dead bacteria and therefore only quantifies living one [45, 46]. Specific primers were used to detect each strain in a very precise way. Figure 2 shows the survival profiles of the three bacterial strains. Profiles obtained were similar regardless of the used strain. During the first 6 h of simulated digestion that corresponds to the upper parts of gastrointestinal tract, no mortality was observed and the survival of the bacteria was constant for the three strains. This ascertainment confirms the results of dissolution tests reported previously in reference [47].

The digestion of the beads in media simulating the upper parts of gastrointestinal tract has been performed using USP4, which is one of the systems listed in the pharmacopeia and recommended by FDA [48, 49]. USP4 system offers several advantages compared to other dissolution systems USP1, USP2, and USP3 [50]. Due to the continuous circulation of dissolution medium in the USP4 cell system that easily maintains of the "sink" conditions [51, 52], this system presents an easy change in the composition and pH of the medium over the course of the test [30, 49, 53]. It also reduces the handling of the experience and limits the risk of contamination.

During this stage, pH 1.2 and pH 4.5 were used to mimic acid conditions of the fasting and filled stomach, respectively [54]. After acidic conditions, pH 6.8 was used to mimic neutral intestinal conditions. Media were supplemented with gastric and intestinal enzymes and bile salts to closely simulate in vivo conditions [55, 56]. The purpose of this step was to obtain a bead digests after their passage through the gastro intestinal upper parts namely the stomach and the small intestine.

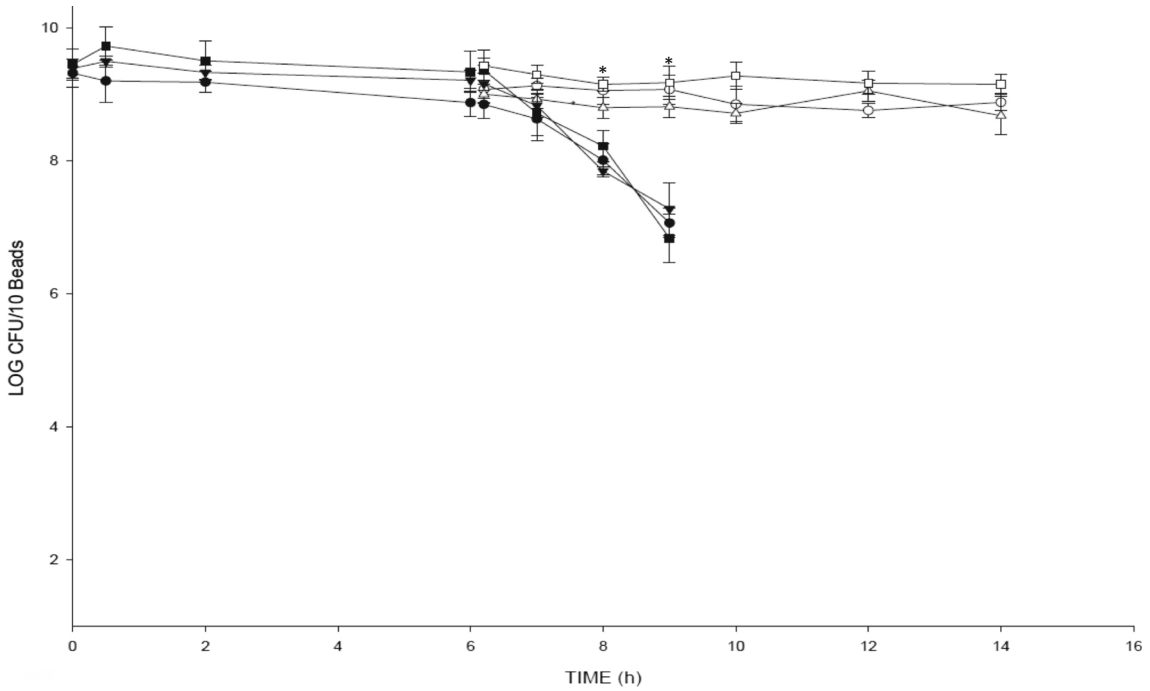

Fig. 2 Survival of probiotic strains encapsulated together in the upper parts of the gastrointestinal tract at pH 1.2 (30 min), pH 4.5 (1 h and 30 min), and in pH 6.8 (4 h), followed by simulation of fermented (FM) (black) and unfermented (UFM) (white) colonic media. *Pediococcus acidilactici UL5* (triangle) *Lactobacillus salivarius*(circle) *Lactobacillus reuteri* (square). *Significant difference between FM and UFM

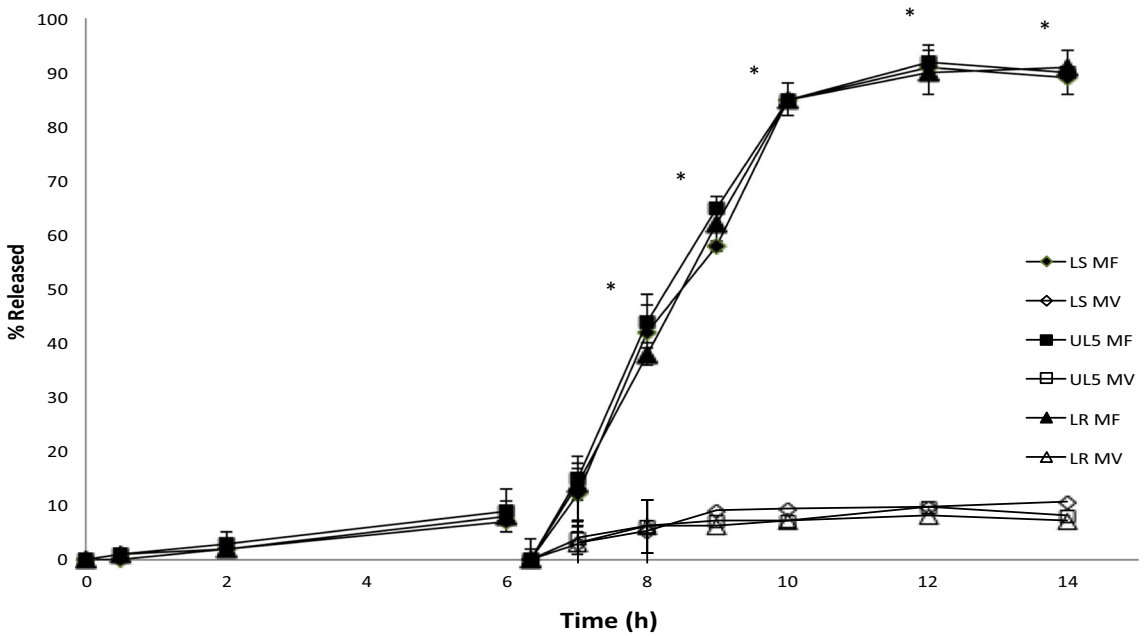

Fig. 3 Bacterial release from beads in the upper parts of the gastrointestinal tract at pH 1.2 for 30 min, in pH 4.5 for 1 h and 30 min and pH 6.8 for 4 h, followed by simulation of fermented (black) and unfermented (white) colonic media . *Pediococcus acidilactici UL5* (square) *Lactobacillus salivarius* (diamond) *Lactobacillus reuteri* (triangle). *Significant difference between FM and UFM

In the lower parts of gastrointestinal tract, no differences were observed between the three strains incubated in the same medium. In unfermented medium, bacterial survival continued to be constant. However, survival of strains in fermented medium started to decline after 1 h of incubation. Significant differences were observed between the survival of strains between fermented and unfermented medium starting after 2 h of incubation.

To monitor the release profiles of the tested strains, enumeration was done in dissolution media. Figure 3 shows the

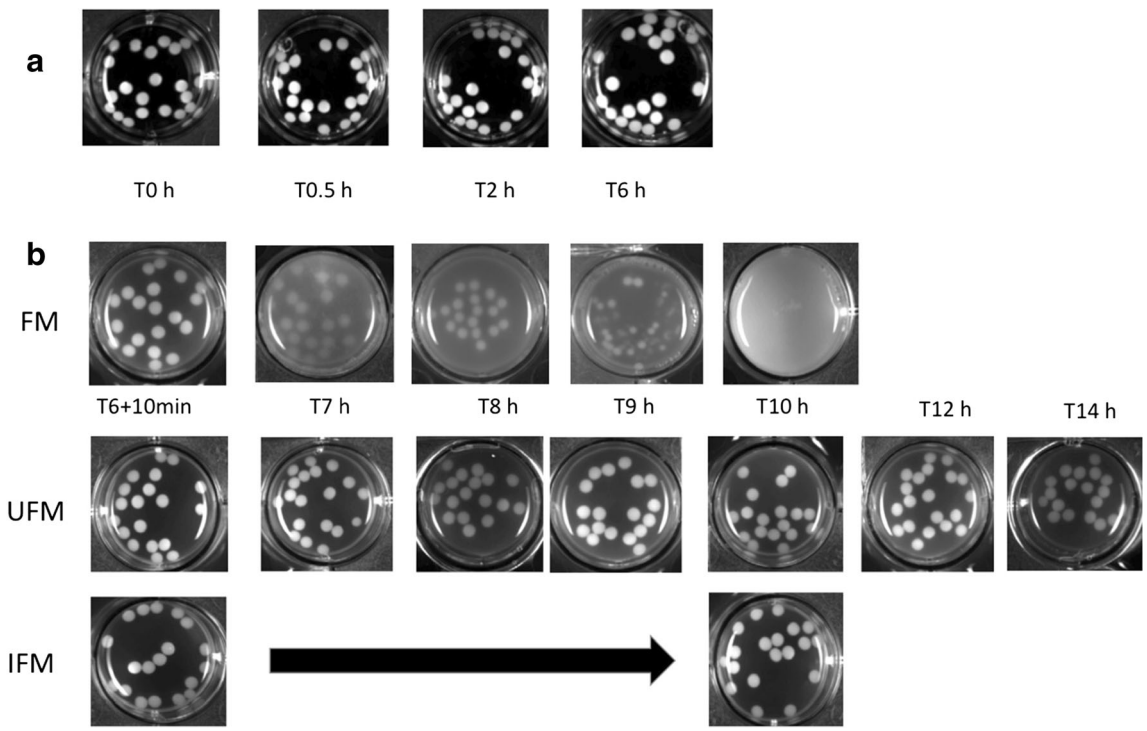

Fig. 4 Evolution of the macroscopic appearance of beads during gastrointestinal simulation. **a** In the upper parts of the gastrointestinal tract. **b** In colon. FM: fermented medium, UFM: unfermented medium, IFM: inactivated fermented medium

release profiles of the tested strains. As stated in the survival profiles, the release profile of bacterial strains had similar patterns in the same medium regardless of the bacterial strain. In the upper parts of gastrointestinal tract, the release was very slow that reached only 7% after 6 h of digestion. This tendency is maintained during the simulated digestion in the unfermented medium until the end of the colonic phase where release reached 10% at the end of the digestion process. Conversely, release in fermented medium was very fast; it reaches the almost total release and was more than 85% after 4 h. A significant difference was observed between the survival of strains in fermented and unfermented medium after 2 h of incubation; at this point, the release in fermented medium was more than the 45% while still less than 10% for unfermented one.

During the second stage of the study, colon conditions using fermented and unfermented medium were simulated. Comparison of the bead digest behaviors in these two media was indispensable to recognize any possible differences between these media. The fermented medium was obtained from a fermentation of colonic media with a specific amount of colonic flora; hence, it is very rich in bacterial enzymes that are able to degrade prebiotics which were indigestible in upper parts of gastrointestinal tract. On the other hand, the unfermented medium was very poor of enzyme from colonic flora [57, 58].

To understand the behavior of beads in each media, macroscopic aspects of beads during the gastrointestinal simulation were studied. The images showed that the macroscopic appearance of beads during gastrointestinal and colonic simulation is shown in Fig. 4. These images showed that beads remained intact throughout the duration of gastrointestinal upper part simulation. The beads also remained intact for 8 h in unfermented medium; however, in fermented medium, beads degraded gradually and disappear after 4 h of incubation. To elucidate the cause of the degradation of the beads in the fermented medium, a control experiment was carried out by incubating the beads in inactivated fermented medium (IFM) which is merely fermented medium after having undergone a thermic treatment of 100 °C for 10 min. This control shows that the beads remain intact in IFM even after 10 h of incubation. This suggests that the degradation of beads in fermented

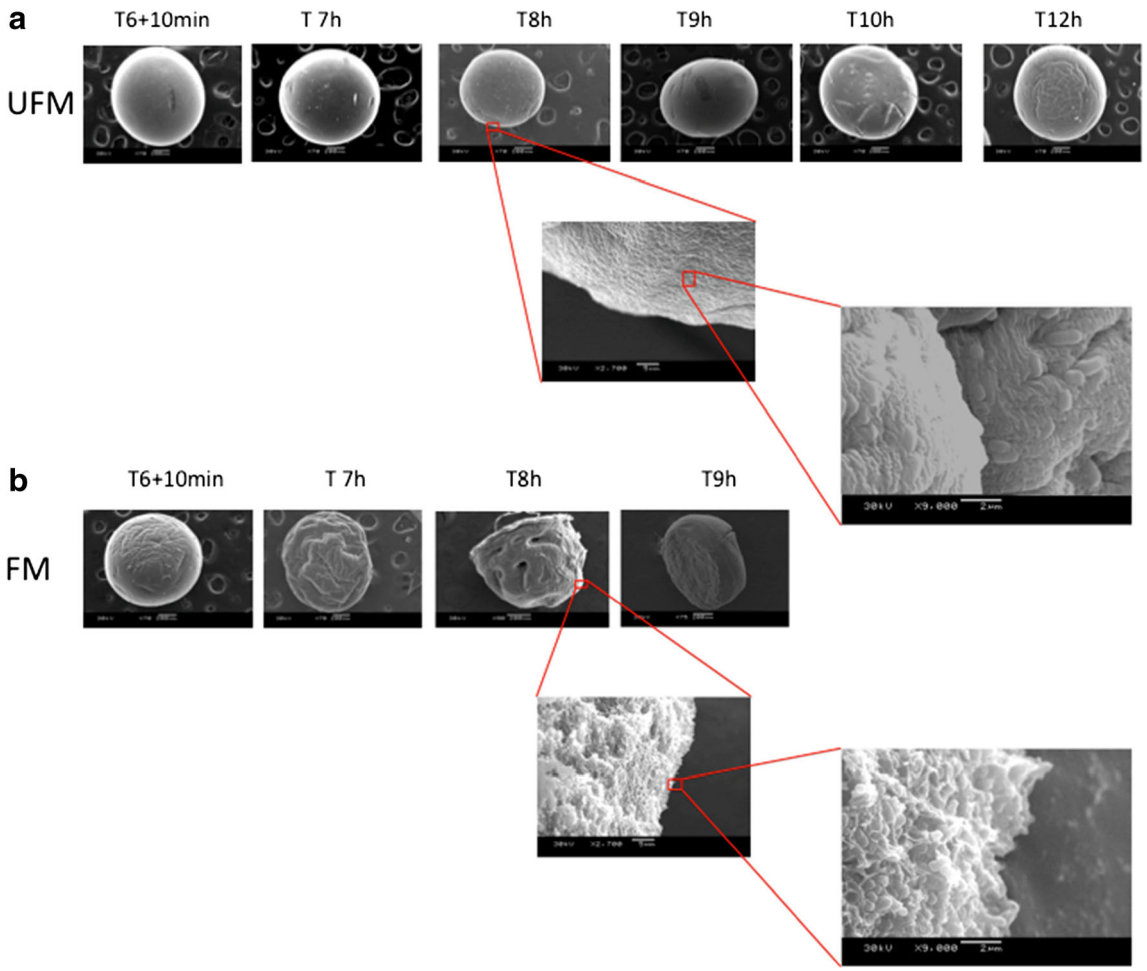

Fig. 5 Scanning electron micrograph (SEM) photographs of beads during colonic digestion at low (× 50), high (× 2700), and very high magnification (× 9000); **a** unfermented medium and **b** fermented medium

medium is mainly caused by enzymes which are abundantly present in this media.

Monitoring the Microscopic Appearance of the Beads During the Digestion

The microstructure of the beads was then investigated using SEM. Figure 5 shows the microstructure of beads during incubation in fermented or unfermented medium. Low magnification SEM showed that the spherical shape of the beads and their smooth aspect was preserved during incubation in the unfermented medium. However, in the fermented medium, beads begin to deform and become rough after the first hour of incubation and continue to deteriorate gradually until they disappear. By zooming on the bead surface at high and very high magnification, obvious differences were distinguished between beads of unfermented and fermented medium. In unfermented medium, beads remained smooth and cottony with some apparent bacteria on the surface, whereas in fermented medium, beads were very rigorous that reflect the degradation of the beads.

Since there is no pH difference between UFM and FM, the degradation in FM is definitely due to the effect of enzymes that are abundantly present in FM media in contrast to UFM media. In this work, the bacterial dynamics inside beads during digestion was also investigated.

Monitoring the Distribution of Bacteria to the Inside the Beads During the Digestion

Figure 6 shows images of bead sections captured at different steps of digestion. Images were captured after staining bead sections by fluorescence microscopy with the dead/live kit. Figure 6a represents the image of the bead sections after digestion in the upper parts of gastrointestinal tract. The images presented in Fig. 6a shows colored spots scattered uniformly in the matrix. More than 80% of the spots were green while the red spots covered less than 20% of the beads. In this part, green and red spots had a regular circular shape reflecting aspect of bacteria colony inside matrix; moreover, their distribution in the matrix was very homogeneous.

This result supports the findings observed in Atia et al. [23] regarding the distribution of bacteria inside the matrix. In colon part (Fig. 6b), the circular aspect of colored spotes has been replaced by stain diffuse aspect occupying large and continuous surfaces through the matrix (data not shown).

This change in the aspect of bacterial colony may demonstrate the growth of this latter in the unfermented colonic media in contrast to GSF and SIF where appearance of colony aspect remained static. Another possible cause of this change is the presence of Hemin (protoporphyrin IX) which is a compound that emits fluorescence in the same wave lengths that the components of the live/dead test cause interference and change the appearance of images obtained.

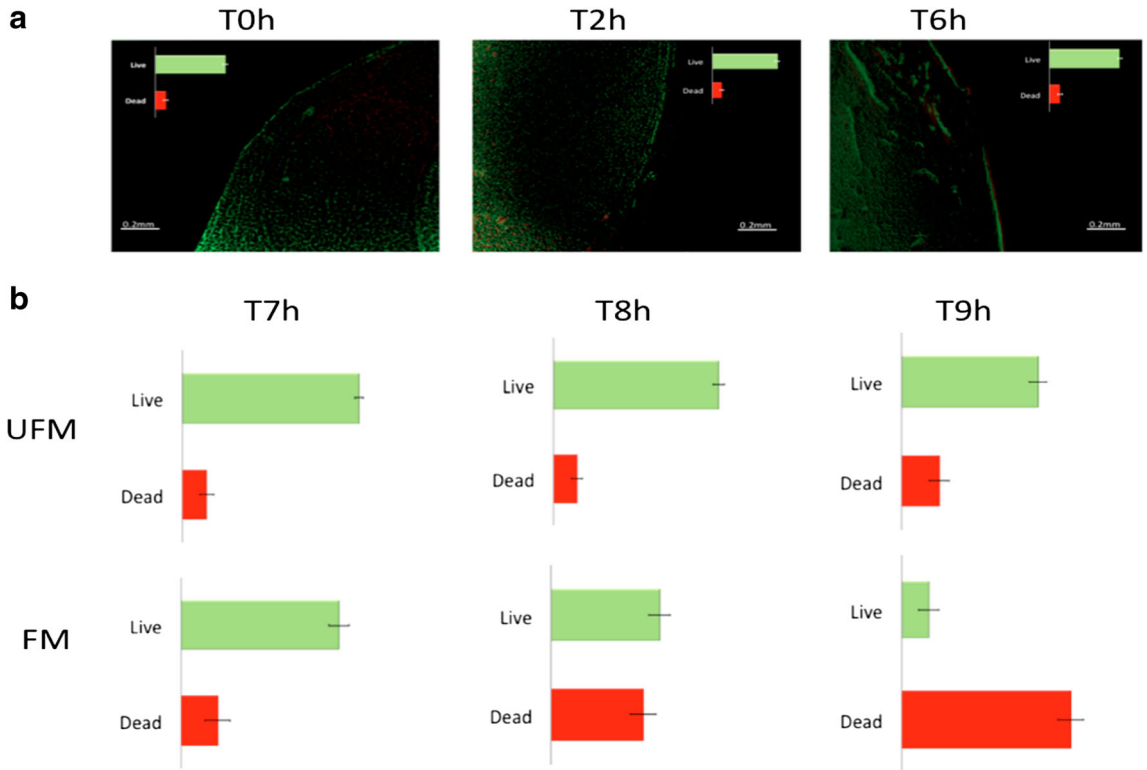

Fig. 6 Monitoring the dynamic bacterial inside the beads during the gastro intestinal simulation by live/dead staining. **a** In the upper parts of the gastrointestinal tract. **b** In colon. FM: fermented Medium, UFM: unfermented medium

In unfermented medium, the green/red proportions were between 10/90 and 20/80 and they did not fluctuate significantly during the incubation; dead cells (red) usually occupy deep part of the beads while the living cells (green) set location on surface as well as bead core. Such positioning can also be explained by the growth of living bacteria inside beads incubated in unfermented medium. In fermented medium green/red colors, proportions fluctuate and move from 80/20 in beginning of incubation to 10/ 90 at the end, respectively. This change is mainly due to degradation of beads by the enzymes abundantly presented in the media and which attacks the surface of the beads causing the deformation of these latter losing their spherical and regular character.

These findings give an idea about bead behavior; the colonic media where bacterial strains were released under the effect of the enzyme which plays a decisive role in the degradation of AI5 matrix.

Conclusion

The conclusions drawn from the current study are as follows:

- The digestion results of AI5 formulation in media simulating the upper part of the gastrointestinal tract showed that this formulation provided protection to bacterial strains against acidity and enzymes of the stomach and also against bile salts at the intestinal level, which supported the results from the previous work [23, 47].
- In unfermented colonic media, beads remained stable for 8 h, while in the fermented medium, they completely degraded in less than 4 h. This rapid degradation was due to enzymes generally present abundantly in fermented medium. These enzymes have the ability to metabolize inulin that deteriorates the bead surface as shown by SEM fluorescence microscopy images, and thus causes a rapid release of bacteria into the fermented colonic media.
- The results of bacterial dynamics inside the bead studies are consistent with of survival and liberation profiles obtained in this work.
- Although colonic conditions (fermented medium) favor the release of bacteria, their survival rate drastically decreases.

Acknowledgements The authors would like to thank Ms. Diane Gagnon (Institut de recherche sur la nutrition et les aliments fonctionnels, Université Laval, Québec, QC, Canada) and Richard Janvier (Plateforme de microscopie, l'Université Laval).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Perumal OP, Haywood A, Glass B, Ho PC-L (2011) Pharmacokinetics and biopharmaceutics. In: Pharmaceutical Science and Technology, Garg, Sanj. Pharmaceutical Press, London, pp 41–86
2. Panchagnula R, Thomas NS (2000) Biopharmaceutics and pharmacokinetics in drug research. Int J Pharm 201(2):131–150. https://doi.org/10.1016/S0378-5173(00)00344-6
3. Sheftell FD, Dahlöf CGH, Brandes JL, Agosti R, Jones MW, Barrett PS (2005) Two replicate randomized, double-blind, placebo-controlled trials of the time to onset of pain relief in the acute treatment of migraine with a fast-disintegrating/rapid-release formulation of sumatriptan tablets. Clin Ther 27(4):407–417. https://doi.org/10.1016/j.clinthera.2005.04.003
4. Tahara K, Yamamoto K, Nishihata T (1995) Overall mechanism behind matrix sustained release (SR) tablets prepared with hydroxypropyl methylcellulose 2910. J Control Release 35(1):59–66. https://doi.org/10.1016/0168-3659(95)00021-Y
5. Philip AK, Philip B (2010) Colon targeted drug delivery systems: a review on primary and novel approaches. Oman Med J 25(2):79
6. Stella VJ, Rao VM, Zannou EA, Zia V (1999) Mechanisms of drug release from cyclodextrin complexes. Adv Drug Deliv Rev 36(1):3–16. https://doi.org/10.1016/S0169-409X(98)00052-0
7. Metz DC, Vakily M, Dixit T, Mulford D (2009) Review article: dual delayed release formulation of dexlansoprazole MR, a novel approach to overcome the limitations of conventional single release proton pump inhibitor therapy. Aliment Pharmacol Ther 29(9):928–937. https://doi.org/10.1111/j.1365-2036.2009.03984.x
8. BOURLIOUX P, Corthier G, Gobert J-G, Butel M-J (2014) Pourquoi la flore intestinale a-t-elle vocation à devenir médicament ? Ann Pharm Fr 72(5):325–329. https://doi.org/10.1016/j.pharma.2014.03.005
9. Calenge F, Martin C, Floch NLE, Phocas F, Morgavi D, Sup V, Ouest A (2014) Intégrer la caractérisation du microbiote digestif dans le phénotypage de l ' animal de rente : vers un nouvel outil de maîtrise de la santé en élevage ? INRA Prod Anim 27(3):209–222
10. Castillo M, Martín-Orúe SM, Nofrarías M, Manzanilla EG, Gasa J (2007) Changes in caecal microbiota and mucosal morphology of weaned pigs. Vet Microbiol 124(3-4):239–247. https://doi.org/10.1016/j.vetmic.2007.04.026
11. Le Lay C (2015) UL719 et la nisine : une nouvelle approche dans le traitement des infections à Clostridium difficile Lactococcus lactis ssp . lactis biovar . diacetylactis UL719 et la nisine : une nouvelle approche dans le traitement des infections à Clostridium difficil. Laval University
12. WHO and FAO(2001) Probiotics in food FOOD AND NUTRITION. Food and Nutrition Paper.
13. Gobinath D, Prapulla SG (2014) Permeabilized probiotic lactobacillus plantarum as a source of β-galactosidase for the synthesis of prebiotic galactooligosaccharides. Biotechnol Lett 36(1):153–157. https://doi.org/10.1007/s10529-013-1345-9
14. Cook MT, Tzortzis G, Charalampopoulos D, Khutoryanskiy VV (Aug. 2012) Microencapsulation of probiotics for gastrointestinal delivery. J Control Release 162(1):56–67. https://doi.org/10.1016/j.jconrel.2012.06.003
15. Gbassi GK, Atheba P, Yolou FS, Vandamme T (2013) Macrobeads based-polysaccharides: development and morphological analysis. World Appl Sci J 22(5):732–737
16. Gbassi GK (2010) Aspects physicochimiques de l'encapsulation et de la désencapsulation des probiotiques.
17. Sathyabama S, Ranjith Kumar M, Bruntha Devi P, Vijayabharathi R, Brindha Priyadharisini V (2014) Co-encapsulation of probiotics with prebiotics on alginate matrix and its effect on viability in

simulated gastric environment. LWT Food Sci Technol 57(1):419–425. https://doi.org/10.1016/j.lwt.2013.12.024

18. Pimentel TC, Madrona GS, Garcia S, Prudencio SH (2015) Probiotic viability, physicochemical characteristics and acceptability during refrigerated storage of clarified apple juice supplemented with lactobacillus paracasei ssp. paracasei and oligofructose in different package type. LWT Food Sci Technol 63(1):415–422. https://doi.org/10.1016/j.lwt.2015.03.009
19. Arief II, Wulandari Z, Aditia EL, Baihaqi M (2014) Physicochemical and microbiological properties of fermented lamb sausages using probiotic lactobacillus plantarum IIA-2C12 as starter culture. Procedia Environ Sci 20:352–356. https://doi.org/10.1016/j.proenv.2014.03.044
20. Alegre I, Viñas I, Usall J, Anguera M, Abadias M (Feb. 2011) Microbiological and physicochemical quality of fresh-cut apple enriched with the probiotic strain lactobacillus rhamnosus GG. Food Microbiol 28(1):59–66. https://doi.org/10.1016/j.fm.2010.08.006
21. Havenaar R, Anneveld B, Hanff LM, de Wildt SN, de Koning BAE, Mooij MG, Lelieveld JPA, Minekus M (2013) In vitro gastrointestinal model (TIM) with predictive power, even for infants and children? Int J Pharm 457(1):327–332
22. Havenaar R, Bellmann S, Zeijdner E (2014) Dynamic gastrointestinal in vitro model (TIM) for reliable prediction of stability, availability for absorption, and luminal efficacy of clinical foods and ingredients. Pharm Nutr 2(3):85–86. https://doi.org/10.1016/j.phanu.2013.11.032
23. Atia A, Gomaa A, Fliss I, Beyssac E, Garrait G, Subirade M (2016) A prebiotic matrix for encapsulation of probiotics: physicochemical and microbiological study. J Microencapsul:1–13
24. Blaser M, Bork P, Fraser C, Knight R, Wang J (2013) The microbiome explored: recent insights and future challenges. Nat Rev Microbiol 11(3):213–217. https://doi.org/10.1038/nrmicro2973
25. Wilson DS, Sober E (1989) Reviving the superorganism. J Theor Biol 136(3):337–356. https://doi.org/10.1016/S0022-5193(89)80169-9
26. Heavens D, Tailford LE, Crossman L, Jeffers F, MacKenzie DA, Caccamo M, Juge N (2011) Genome sequence of the vertebrate gut symbiont lactobacillus reuteri ATCC 53608. J Bacteriol 193(15):4015–4016. https://doi.org/10.1128/JB.05282-11
27. Daba H, Lacroix C, Huang J, Simard RE, Lemieux L (1994) Simple method of purification and sequencing of a bacteriocin produced by Pediococcus acidilactici UL5. J Appl Bacteriol 77(6):682–688. https://doi.org/10.1111/j.1365-2672.1994.tb02819.x
28. Lo Verso L, Lessard M, Talbot G, Subirade M, and Fliss I (2013) Isolation and antibacterial activity of potential probiotic bacteria from pig gastrointestinal tract. In 6th Symposium of Swine and Poultry Infectious Diseases Research Center. Congrès de l'ACFAS Colloque 227 –le microbiote animal : une question d'équilibre. p 45
29. Smrdel P, Bogataj M, Mrhar A (2008) The influence of selected parameters on the size and shape of alginate beads prepared by ionotropic gelation. Scientia Pharmaceutica 76(1):77
30. Gao Z (2009) In vitro dissolution testing with flow-through method: a technical note. AAPS Pharm Sci Tech 10(4):1401–1405
31. Macfarlane GT, Macfarlane S, Gibson GR (1998) Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. Microb Ecol 35(2):180–187. https://doi.org/10.1007/s002489900072
32. Tanner SA, Zihler Berner A, Rigozzi E, Grattepanche F, Chassard C, Lacroix C (2014) In vitro continuous fermentation model (PolyFermS) of the swine proximal colon for simultaneous testing on the same gut microbiota. PloS One 9(4):e94123
33. Le Lay C, Fernandez B, Hammami R, Ouellette M, Fliss I (2015) On Lactococcus lactis UL719 competitivity and nisin (Nisaplin(®)) capacity to inhibit Clostridium Difficile in a model of human colon. Front Microbiol 6:1020. https://doi.org/10.3389/fmicb.2015.01020
34. Häuselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block, Aydelotte MB, Kuettner KE, Thonar EJ (Jan. 1994) Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J Cell Sci 107:17–27
35. Fernandez B (2014) Activité biologique et impact sur le microbiote intestinal des bactéries lactiques bactériocinogènes. Collection des thèses et mémoires électroniques de l'Université Laval
36. Josefsen MH, Löfström C, Hansen TB, Christensen LS, Olsen JE, Hoorfar J (2010) Rapid quantification of viable campylobacter bacteria on chicken carcasses, using real-time PCR and propidium monoazide treatment, as a tool for quantitative risk assessment. Appl Environ Microbiol 76(15):5097–5104. https://doi.org/10.1128/AEM.00411-10
37. Fernandez B, Savard P, Fliss I (2015) Survival and metabolic activity of Pediocin producer Pediococcus acidilactici UL5: its impact on intestinal microbiota and listeria monocytogenes in a model of the human terminal ileum. Microb Ecol 72(4):931–942. https://doi.org/10.1007/s00248-015-0645-0
38. Mora D, Fortina MG, Parini C, Manachini PL (2006) Identification of Pediococcus acidilactici and Pediococcus pentosaceus based on 16S rRNA and ldhD gene-targeted multiplex PCR analysis. FEMS Microbiol Lett 151(2):231–236. https://doi.org/10.1111/j.1574-6968.1997.tb12575.x
39. Harrow SA, Ravindran V, Butler RC, Marshall JW, Tannock GW (2007) Real-time quantitative PCR measurement of ileal lactobacillus salivarius populations from broiler chickens to determine the influence of farming practices. Appl Environ Microbiol 73(22):7123–7127. https://doi.org/10.1128/AEM.01289-07
40. Million M, Maraninchi M, Henry M, Armougom F, Richet H, Carrieri P, Valero R, Raccah D, Vialettes B, Raoult D (2012) Obesity-associated gut microbiota is enriched in lactobacillus reuteri and depleted in Bifidobacterium animalis and Methanobrevibacter smithii. Int JJ Obes (2005) 36(6):817–825. https://doi.org/10.1038/ijo.2011.153
41. Molecular Probes (2001) LIVE/DEAD BacLight bacterial viability kits. Manuals and Product Inserts. [Online]. Available: http://www.mobitec.de/probes/docs/media/pis/mp07007.pdf. Accessed 21 Sep 2015
42. Dabour N, Zihler A, Kheadr E, Lacroix C, Fliss I (2009) Vivo study on the effectiveness of pediocin PA-1 and Pediococcus acidilactici UL5 at inhibiting listeria monocytogenes. Int J Food Microbiol 133(3):225–233. https://doi.org/10.1016/j.ijfoodmicro.2009.05.005
43. Fernandez B, Hammami R, Savard P, Jean J, Fliss I (2014) Pediococcus acidilactici UL5 and Lactococcus lactis ATCC 11454 are able to survive and express their bacteriocin genes under simulated gastrointestinal conditions. J Appl Microbiol 116(3):677–688. https://doi.org/10.1111/jam.12391
44. Atia A, Gomma AI, Fliss I, Beyssac E, Garrait G, and Subirade M (2017) Molecular and biopharmaceutical investigation of alginate–inulin synbiotic coencapsulation of probiotic to target the colon. J Microencapsul 1–13
45. Kim M, Wuertz S (2015) Survival and persistence of host-associated Bacteroidales cells and DNA in comparison with Escherichia Coli and enterococcus in freshwater sediments as quantified by PMA-qPCR and qPCR. Water Res 87:182–192. https://doi.org/10.1016/j.watres.2015.09.014
46. Truchado P, Gil MI, Kostic T, Allende A (2016) Optimization and validation of a PMA qPCR method for Escherichia Coli quantification in primary production. Food Control 62:150–156. https://doi.org/10.1016/j.foodcont.2015.10.014

47. Atia A, Gomaa A, I Fliss, Beyssac E, Garrait G, and Subirade M (2016) Prebiotic matrix for encapsulation of probiotics: biopharmaceutical study of a controlled release formulation targeting colon. Eur J Pharm Biopharm
48. Kauffman JS (2005) Qualification and validation of USP apparatus 4. Dissolut Technol 12:41–43
49. Beyssac E, Lavigne J (2005) Dissolution study of active pharmaceutical ingredients using the flow through apparatus USP 4. Dissolut Technol 12(2):23–25. https://doi.org/10.14227/DT120205P23
50. Marles RJ, Barrett ML, Barnes J, Chavez ML, Gardiner P, Ko R, Mahady GB, Low Dog T, Sarma ND, Giancaspro GI, Sharaf M, Griffiths J (2011) United States pharmacopeia safety evaluation of spirulina. Crit Rev Food Sci Nutr 51(7):593–604. https://doi.org/10.1080/10408391003721719
51. Damitz R, Chauhan A (2015) Rapid dissolution of propofol emulsions under sink conditions. Int J Pharm 481(1–2):47–55. https://doi.org/10.1016/j.ijpharm.2015.01.045
52. Seidlitz A, Nagel S, Semmling B, Grabow N, Martin H, Senz V, Harder C, Sternberg K, Schmitz K-P, Kroemer HK, Weitschies W (2011) Examination of drug release and distribution from drug-eluting stents with a vessel-simulating flow-through cell. Eur J Pharm Biopharm 78(1):36–48. https://doi.org/10.1016/j.ejpb.2010.12.021
53. Scheubel E (2010) Predictive in vitro dissolution tools : application during formulation development. Université d'Auvergne - Clermont-Ferrand I
54. Xie X, Cardot J-M, Garrait G, Thery V, El-Hajji M, Beyssac E (2014) Micelle dynamic simulation and physicochemical characterization of biorelevant media to reflect gastrointestinal environment in fasted and fed states. Eur J Pharm Biopharm 88(2):565–573. https://doi.org/10.1016/j.ejpb.2014.05.020
55. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, Carrière F, Boutrou R, Corredig M, Dupont D, Dufour C, Egger L, Golding M, Karakaya S, Kirkhus B, Le Feunteun S, Lesmes U, Macierzanka A, Mackie A, Marze S, McClements DJ, Ménard O, Recio I, Santos CN, Singh RP, Vegarud GE, Wickham MSJ, Weitschies W, Brodkorb A (2014) A standardised static in vitro digestion method suitable for food - an international consensus. Food Funct 5(6):1113–1124. https://doi.org/10.1039/c3fo60702j
56. Stefanelli M, Vichi S, Stipa G, Funari E, Testai E, Scardala S, Manganelli M (2014) Survival, growth and toxicity of Microcystis Aeruginosa PCC 7806 in experimental conditions mimicking some features of the human gastro-intestinal environment. Chem Biol Interact 215:54–61. https://doi.org/10.1016/j.cbi.2014.03.006
57. Naeem M, Kim W, Cao J, Jung Y, Yoo J-W (2014) Enzyme/pH dual sensitive polymeric nanoparticles for targeted drug delivery to the inflamed colon. Colloids Surf B: Biointerfaces 123:271–278. https://doi.org/10.1016/j.colsurfb.2014.09.026
58. Campos-Vega R, Vázquez-Sánchez K, López-Barrera D, Loarca-Piña G, Mendoza-Díaz S, Oomah BD (2015) Simulated gastrointestinal digestion and in vitro colonic fermentation of spent coffee (Coffea Arabica L.): bioaccessibility and intestinal permeability. Food Res Int 77:156–161. https://doi.org/10.1016/j.foodres.2015.07.024