DESIGN OF A MATRIX ABLE TO CONTROL RELEASE OF BACTERIA DURING IN VITRO DIGESTION

Burgain, J., Guerin, J., Borges, F., Scher, J., Gaiani, C., – Lorraine University, LIBio, France

INTRODUCTION & OBJECTIVES

Milk matrix are increasingly used to encapsulate probiotic bacteria and the matrix composition plays a crucial role for the controlled release of viable and functional bacteria in the intestine. The aim of this work was to develop instrumental tools of microscopy to correlate the survival of probiotic bacteria and the microstructure of microparticles during gastrointestinal simulated digestion. For this purpose, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were used to understand the protection efficiency of the milk proteins matrices. *L. rhamnosus* GG (LGG) was encapsulated by an emulsification method. The influence of different dairy matrix formulation on matrix integrity, bacterial location, matrix delivery capacity and probiotic survival during gastrointestinal simulated digestion were investigated. The objective was to find the best matrix permitting to protect bacteria during simulated gastric digestion and to release bacteria in viable state in the intestine.

MATERIAL & METHODS

Material

Micellar casein (Promilk 872 B) and whey protein (Promilk 752 FB) powders were provided by Ingredia IDI (Arras, France). Micellar caseins and whey proteins solutions were prepared by rehydrating powders separately in distilled water at a concentration of 12.5 % (w/w). The rehydration occurred during 2h at room temperature and then overnight at 4 °C.

Chymosin was purchased from CHR Hansen (Chymax Plus, CHR Hansen, Hørsholm, Denmark). The initial solution (200 IMCU/mL) was diluted ten times to obtain a concentration of 2 IMCU/mL before the encapsulation process.

The strain LGG (ATCC 53103) was used in this study. LGG growth was performed in a laboratory-scale reactor. Culture was stopped at the beginning of the stationary phase and bacterial cells were harvested from the broth by gentle centrifugation (3000 *g*, 10 min). The pellet was then lyophilized for 72 h and stored at 4 °C before use.

Microencapsulation process

Different matrix formulation were used to encapsulate LGG. Micellar caseins and whey proteins solutions were mixed at different ratios (solid basis): 100/0, 80/20 and 60/40, respectively. To this end, 0.5 g (between 10^8 and 10^9 UFC/g) of freeze-dried bacteria was added to 200 mL of protein mixture. Then, 18 mL of the freshly prepared chymosin solution was added to the 200 mL protein and bacterial mixture. The microencapsulation was performed by an emulsification method described in a recent patent (Patent EP2807932A1) (Burgain *et al.,* 2014).

Microparticles digestion

Microparticles digestion was performed using an *in vitro* digestion method as described by Minekus *et al.,* (2014). For the gastric digestion, microparticles containing LGG were dispersed in simulated gastric fluid (6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$) containing porcine pepsin (2000 U/mL) and 0.075mM of $CaCl_2$. The pH was checked to reach a final pH of 3.0 at the beginning of the gastric phase (but not controlled during the phase). The gastric digestion occurred during 2 hours at 37 °C. After 2 hours, the gastric chyme was mixed with simulated intestinal fluid (6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$) containing pancreatine (800 U/mL), fresh bile (160 mM) and CaCl2 (0.3 mM). The pH was neutralized to obtain pH 7.0 at the beginning of the intestinal digestion. The intestinal digestion occurred during 2h at 37 °C.

Microparticles were analyzed with imaging techniques at various stages of the digestion: at the beginning of the gastric phase, at the end of the gastric phase and at the end of the intestinal phase

Scanning electron microscopy

The microparticles at various stages of the digestion were observed with a high-resolution field-emission scanning electron microscope (SEM) type JEOL JSM-7100F supplied with a hot (Schottky) electron gun (JEOL Ltd., Tokyo, Japan). The resolution of the equipment was around 1 nm at 30 kV.

Confocal laser scanning microscopy

The viability of *L. rhamnosus* GG during digestion was performed by CLSM using an LSM700 confocal scanning laser microscope (Carl Zeiss Ltd., New South Wales 2113, Australia). For this purpose, microparticles during digestion sampling were neutralized with a phosphate buffer and gently centrifuged to remove the buffer. To visualize the bacterial viability, microparticles were stained with a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular probes, Thermofisher). For this microparticles were stained with a mixture of SYTO9 and propidium iodide (60 mM and 300 mM, respectively) during 30 min. Observations were done with a 63x oil-immersion objective. The excitation/emission used for SYTO 9 stain was 488/500 nm (green fluorescence signal) and 488/635 nm for propidium iodide (red fluorescence signal).

RESULTS & DISCUSSION

Effect of matrix composition on bacterial location

To study the influence of matrix formulation on bacterial spatial distribution in microparticles, the core and the surface of the microparticles were observed by SEM (**Figure 1**). For the formulation 100/0, solely composed of caseins, only few bacteria were visualized inside the microparticle but they were located at the surface of the microparticle. By contrast, most of bacteria were located inside the microparticles for the two formulations containing whey proteins. The matrix composition also influenced the matrix density: an increase in whey proteins ratio in the formulation led to a higher porosity. Bacteria spatial distribution in the microparticles seems to be clearly governed by the matrix composition. Recent works demonstrated that LGG presents the ability to interact specifically with whey proteins and more particularly with β-lactoglobulin (Guerin *et al.*, 2016). This interaction may be responsible of the preferential bacterial localization in the microparticles.

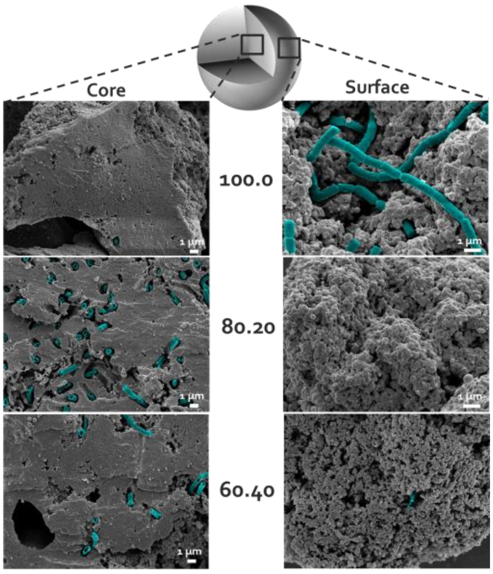

Figure 1: LGG location in cut and surface of microparticles produced with the three different matrices (100.0, 80.20 and 60.40).

Effect of matrix composition on bacteria viability and bacteria delivery

The effect of matrix composition on bacteria viability and bacteria delivery was assessed by microscopic technology. During simulated digestion, SEM was used to follow the matrix integrity and porosity and CLSM was used to study the bacterial survival.

SEM study demonstrated that the microparticles formulations 100/0 and 80/20 were stable during the gastric digestion. On the contrary, the microparticle formulation 60/40 was partially digested at the beginning of the stomach digestion. At the end of the intestinal digestion, the microgel structure of the formulation 100/0 was poorly affected and microparticles remained stables. On the contrary, majority of microparticles of the formulation 80/20 were destroyed and bacteria were released in the media. For the formulation 60/40, only residual fragments were observed at the end of the gastric digestion. When only caseins were present in the matrix, the matrix appeared very dense and the structural network was preserved along the digestion tract. On the contrary, the addition of whey proteins resulted in a more porous, spongy and swollen matrix after digestion.

In addition, CLSM was employed to check bacterial viability directly in the microparticles. For the formulations 100/0 and 80/20, a lot of live bacteria were observed after the gastric phase and a majority of live bacteria were still observed after the intestinal phase. But differences in matrix integrity were observed: live bacteria were still embedded in the formulation 100/0 whereas free live bacteria were observed for the formulation 80/20. On the opposite, presence of dead bacteria was noticed for the 60/40 formulation from the beginning of the gastric phase until the end of the intestinal digestion.

Only the formulation 80/20 allowed a good bacterial protection in the stomach and release of viable bacteria during intestinal digestion (**Figure 2**).

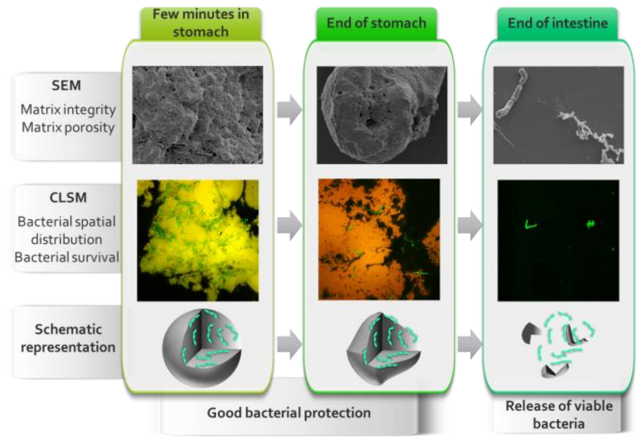

Figure 2: Example of microscopy results obtain for the best matrix 80/20.

CONCLUSIONS & PERSPECTIVES

For the first time, SEM and CLSM were used as complementary microscopy techniques to highlight the importance of matrix formulation on probiotic delivery system efficiency. An ideal matrix composed of caseins (80 %) and whey proteins (20 %) was identified to preserve the bacterial viability during the gastric phase and to release bacteria in viable state in the intestine.

REFERENCES

Burgain, J., Gaiani, C., Jeandel, C., Ghoul, M., & Scher, J. (2014). Method for encapsulating bioactive molecules in dairy matrices and microparticles obtained. Patent.

Guerin, J., Bacharouche, J., Burgain, J. et al. (2016). Pili of Lactobacillus rhamnosus GG mediate interaction with β-lactoglobulin. Food Hydrocolloid, 58, 35–41.

Minekus, M., Alminger, M., Alvito, P. et al. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. Food & Function, 5(6), 1113–1124.

Acknowledgement and Full address

The authors would like to thank the European grant (Milk PEPPER n°621727, International Outgoing Fellowship grant) for their financial support and acknowledge the scientific and technical assistance provided by the School of Agriculture and Food Sciences (SAFS) and the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy and Microanalysis (CMM) at The University of Queensland. Sarah Lebeer is thanked for providing the strain *L. rhamnosus* GG (ATCC 53103).