P-045 **Quantification of probiotics entrapped in Ca-alginate beads during storage**

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INTRODUCTION AND OBJECTIVES

Probiotics are live bacteria that can contribute to the well-being of the host by maintaining or improving microbial equilibrium in the gastrointestinal tract. Viability of probiotic microorganisms is a prerequisite for their efficiency, but microbiological analyses often show poor stability of these bacteria in different products, especially those containing lyophilised bacteria, during storage. One of the possibilities to improve stability of probiotics against negative environmental effects is microencapsulation. In this study we encapsulated three *Lactobacillus* probiotic strains in Ca-alginate beads. Besides the most widespread plate-count technique, we wanted to examine the possibility, use an alternative cultivation-independent technique, quantitative polymerase chain reaction (»real-time PCR«) combined with PMA treatment of the samples, for quantification of encapsulated probiotic lactobacilli.

MATERIALS AND METHODS

Microorganisms

Lactobacillus gasseri K7 (IM 105)
Lactobacillus bulgaricus L89 (IM 396, Selur 19)
Lactobacillus bulgaricus BF (IM 411, Selur 6)

Electrostatic extrusion

For extrusion we used modified procedure described by Nedovic et al. (2001). Each tested strain was seeded (1 % inoculum) into 100 ml of MRS broth in Erlenmeyer flasks. After , incubation at 37 °C for 20 h, we aseptically transferred cultures into 50 ml centrifuge tubes and centrifuged 10 min at 10 °C and 5000 min^{-1}. We washed two-times the pellets thoroughly with 40 ml of 0,1 % sterile peptone buffered solution(1 g peptone /l and 8,5 g NaCl /l). Afterwards the bacterial cell pellets were resuspended in 10 ml of peptone buffered solution and transferred into 50 ml of 2 % Na-alginate solution.

A plastic syringe was filled with Na-alginate mixture and placed onto the screw press of the encapsulation apparatus. Mixture was extruded through an 700 µm orifice of the needle into 300 ml of 40 mM $CaCl_2$ solution for alginate beads hardening. Working conditions of extrusion apparatus were: fluid flow rate: 1ml/min; distance between needle orifice and hardening solution: 2 cm; voltage: 5,0 kV (this can be lowered in time to 4,5 kV, when the alginate heats up causing its viscosity to drop). We left the beads for 20 min in the $CaCl_2$ solution, than we filtered them onto sterille filter paper and transfered them into 200 ml of 10 % reconstituted milk. They were stored at 4°C.

For plate counts of samples of Na-alginate encapsulated bacteria, we dissolved 1 g of beads in 9 g of 2 % Na-citrate and homogenised well the samples. The appropriate decimal dilutions were prepared in ¼ Ringer solution and plated on MRS-agar plates. The incubation was carried out at 37 °C for 48 h. The initial bacterial suspension were directly diluted in ¼ Ringer solution and plated on MRS agar.

Treatment with PMA

We transferred two 1 ml aliquotes of homogenised samples in two centrifuge tubes and added 2,5 µl of 50 µM PMA. After 5 min incubation in the dark the tubes were placed on ice and exposed for 2 minutes to a halogen lamp (650 W, 120 V). After fotoinduced crosslinking of PMA to DNA (free DNA or DNA within the cells with damaged cell membranes) we centrifuged the samples for 5 minutes at 5000 g. The pellets were used for DNA isolation (Kramer 2009) and (Bogovic Matijasic 2010).

DNA isolation

We added 400 µl of TE buffer and 100 µl of enzyme mixture (25 mg/ml lysozyme and 10 U/ml mutanolysin) to each of the samples of pelleted bacteria. Pellets were resuspended by thorough mixing with vortex and incubated 2 h at 37 °C until the cell lysis was completed. We transferred entire content of centrifuge tube into the cartridge of »Maxwell - DNA tissue kit« for DNA isolation with Maxwell system (Promega) following the instructions of the producer. DNA was eluted by 300 µl of elution buffer and stored at –20 °C.

Amplification

PCR amplifications were performed in a 25-µl reaction volume, containing Maxima SYBR Green qPCR Master Mix (2x, Fermentas), 0.2 µM of each primer, and 5 µl of genomic DNA extract. The sequences of *Lactobacillus* specific primers Lacto R'F and LbFR were previously described by Songjinda et al. (2007). The PCR amplification was performed with the MX3000P (Stratagene, La Jolla,CA, USA) instrument. All samples were subjected also to melting-curve analysis in order to establish the specificity of the amplification. Standard curves were prepared from the pure cultures of three test strains. The ratio (%) of the cells with entire cell membrane was calculated from the results obtained by real-time PCR analysis of PMA-treated and non treated samples.

RESULTS AND DISCUSSION

Microcapsules produced were small caviar like translucent spheres that we suspended in reconstituted milk. We measured the diameter of spheres and besides the beads containing bacteria throughout the volume, control spheres without microorganisms were made. The average diameters were as following: d (control beads) = (1529 ± 50) μm,
d (beads with IM 105 strain) = (1351 ± 36) μm,
d (beads with IM 396 strain) = (1460 ± 24) μm,
d (beads with IM 411 strain) = (1566 ± 32) μm

Table 1: Number of bacteria from alginate beads from colonies that grew on MRS-agar plates, number of bacteria from real time-PCR count and percentage of intact cells for each strain. Units used are CFU/g where not stated otherwise.

		Plate count (cfu/g) / % intact cells (PMA-qPCR)			
		Before encapsulation (cfu/ml)	**t0 - after encapsulation (cfu/g)**	**t1 - 1m storage (cfu/g)**	**t3 - 3m storage (cfu/g)**
IM105	**Plate count**	$7,9\times10^{10}$	$9,6\times10^{10}$	$3,1\times10^{9}$ (t1/t0: 3,2 %)*	$2,3\times10^{9}$ (t3/t0: 2,4 %)**
	% intact cells	n.d.	n.d.	13,04	n.d.
IM396	**Plate count**	$3,05\times10^{9}$ CFU/ml	$8,2\times10^{9}$	n.d.	$5,5\times10^{8}$ (t3/t0: 6,7 %)
	% intact cells	n.d.	n.d.	15,71	n.d.
IM411	**Plate count**	$5,3\times10^{9}$	$1,3\times10^{10}$	$2,5\times10^{8}$ (t1/t0: 1,9%)*	$3,0\times10^{5}$ (t3/t0: 0,1%)**
	% intact cells	n.d.	n.d.	1,34	n.d.

n.d. no data
The results present the average of two batches.
*The ratio (%) of cfu/g determined after 1 month of storage, compared to the initial cfu/g determined after encapsulation
**The ratio (%) of cfu/g determined after 3 month of storage, compared to the initial cfu/g determined after encapsulation

Optimization of real-time PCR showed that 1:100 dilution of DNA isolated from microcapsules samples was the most appropriate. The melting curve analysis confirmed good specificity of PCR amplification using *Lactobacillus* genus specific primers. We were able to reach efficiency up to 89, 4 %. As shown in Table 1, plate count analysis showed that during the first month of storage the number of cultivable K7 cells decreased for about 1,5 log, and the number of IM411 for about 1,7 log (results not available for IM396). The survival of IM396 during entire 3 months was the best compared to the other two strains. The survival of both *L. bulgaricus* strains was much different showing the strain specificity in this regard. Also the ratio (%) of the undamaged cells (with intact membrane) calculated from the results of real-time PCR of PMA-treated and PMA-untreated samples showed the highest % for IM396 strain, followed by *L. gasseri* K7 strain.

CONCLUSIONS

Real time PCR in combination with PMA treatment was found successful for the quantification of three strains of *Lactobacillus* in the samples of Ca-alginate microencapsulated bacteria, using *Lactobacillus* specific primers. PMA treatment of the samples with homogenized Na-alginate encapsulated lactobacilli enabled selective PCR-amplification of DNA derived from undamaged cells. The correlation between the plate counting (CFU/g) and the results of PMA-real-time PCR quantification were however not always consistent which is not surprising since the plate counting is based on the cultivability, while the PMA-real-time PCR on the integrity of the cell membrane. The PMA-real-time PCR method is promising for future use in monitoring the survival of microencapsulated probiotic bacteria, especially in combination with plate counting.

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