Arch. Biol. Sci., Belgrade, 64 (4), 1465-1472, 2012 DOI:10.2298/ABS1204465R

QUANTIFICATION OF VIABLE SPRAY-DRIED POTENTIAL PROBIOTIC LACTOBACILLI USING REAL-TIME PCR

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Abstract - The basic requirement for probiotic bacteria to be able to perform expected positive effects is to be alive. Therefore, appropriate quantification methods are crucial. Bacterial quantification based on nucleic acid detection is increasingly used. Spray-drying (SD) is one of the possibilities to improve the survival of probiotic bacteria against negative environmental effects. The aim of this study was to investigate the survival of spray-dried *Lactobacillus plantarum* 564 and *Lactobacillus paracasei* Z-8, and to investigate the impact on some probiotic properties caused by SD of both tested strains. Besides the plate count technique, the aim was to examine the possibility of using propidium monoazide (PMA) in combination with real-time polymerase chain reaction (PCR) for determining spray-dried tested strains. The number of intact cells, *Lb. plantarum* 564 and *Lb. paracasei* Z-8, was determined by real-time PCR with PMA, and it was similar to the number of investigated strains obtained by the plate count method. Spray-dried *Lb. plantarum* 564 and *Lb. paracasei* Z-8 demonstrated very good probiotic ability. It may be concluded that the PMA real-time PCR determination of the viability of probiotic bacteria could complement the plate count method and SD may be a cost-effective way to produce large quantities of some probiotic cultures.

Key words: Spray-drying, potential probiotic, real-time PCR, propidium monoazide

INTRODUCTION

The majority of autochthonous microflora of traditional dairy products are lactic acid bacteria (LAB) and their variety is a result of the great number by different strains. Beside selection for their application as starter or adjunct cultures (Radulović et al., 2011), their selection as a potential probiotic is relatively new. Probiotics have recently been defined as "live microbes which transit the gastrointestinal tract and in doing so benefit the health of the consumer" (Tannock et al., 2000). They are capable of surviving the low pH in the stomach. Most probiotic bacteria belong to the *Lactobacillus* genera and *Bifidobacterium* strains (Prasad et al., 1998).

Several approaches have been adopted in endeavoring to improve probiotic survival in traditional probiotic foods. Freeze-drying and spray drying are the most commonly used microencapsulation methods. The process of spray drying is economical, it can be operated on a continuous basis, it is easily scaled-up and uses equipment readily available in the food industry (Gibbs et al., 1999). Researchers have found that there is no difference in microbial viability between spray drying and freeze-drying (Teixeira et al.,

1994). The disadvantage is that the high temperature used in the process may not be suitable for encapsulating probiotic bacterial cultures. However, proper adjustment of the processing conditions, such as the inlet temperature, can achieve viable encapsulated cultures.

Plate counting is one of the most widespread techniques for determining the viability of spray-dried bacteria, though there are obvious disadvantages, such as the limiting of microbial recovery and relatively long times needed for the growth of colonies. The use of molecular techniques has largely resolved the problem of the lack sensitivity and specificity of plating on selective media. Real-time PCR is the most widely applied technique for direct quantification of bacteria in mixed samples (Justé et al., 2008). However, its main drawback is the inability to discriminate between live and dead bacteria because the DNA can be amplifiable although the cells are dead (Justé et al., 2008). EMA and PMA have been shown to be useful for differentiating between live and dead bacteria (Nocker et al., 2006). PMA is a DNA-intercalating dye with the azide group, which enables the covalent binding to DNA under bright visible light and, consequently strongly inhibits PCR amplification.

The aim of this work was to study the effect of spray drying on the viability of two previously isolated autochthonous strains, *Lb. plantarum* 564 and *Lb. paracasei* Z-8, in simulated gastrointestinal conditions. Furthermore, we also examined the possibility of using PMA in combination with real-time PCR with SYBR Green I chemistry for selective quantification of live tested strain cells after SD.

MATERIALS AND METHODS

Strains and culture conditions

Two autochthonous strains, *Lb. plantarum* 564 and *Lb. paracasei* Z-8, isolated from traditionally made white brined cheese (Radulović et al., 2010) were used for examination. These strains belong to the strain collection of the Department for Industrial Microbiology, Faculty of Agriculture, University of Belgrade, Serbia. The strains were cultured in MRS broth (Merck, Darmstadt, Germany) at 37˚C. For both strains, cell enumerations were carried out on MRS agar (Merck, Darmstadt, Germany) for 48 h at 37˚C under anaerobic conditions (Gas Pak, BBL, Germany).

Investigation of the probiotic properties of the tested strains before spray drying

Tolerance to simulated gastric juice

To determine the transit tolerance of the examined strains through simulated gastric juice, the method of Doleyeres et al. (2004) was used with a slight modification. The simulated gastric juice was a solution of pepsin (0.3% w/v) and NaCl (0.5% w/v) adjusted to pH 2.5. Overnight cultures (1 ml) were centrifuged (5000 x g, 10 min, 10˚C), washed twice in 0.1% peptone water, resuspended in 0.1% peptone and stored on ice until use. Thirty µl of washed cell suspension and 270 µl of simulated gastric juice were mixed in a microtiter plate and stored at 37˚C under anaerobic conditions for 30 min. Dilution was carried out in PBS buffer pH 7.2, and the total viable counts were determined using the spot method. The plates were incubated anaerobically at 37˚C for 48 h (Gas Pak, BBL, Germany).

Tolerance to simulated duodenal conditions

The ability of strains to grow in simulated duodenal conditions was determined according to the method of Doleyeres et al. (2004), with some modifications. The simulated duodenal environment was a solution of bile salts (Sigma, 0.4% w/v) and pancreatin (Sigma, 0.2% w/v). Bacterial cells from overnight cultures were harvested (5000 x g, 10 min, 10˚C). The pellet was washed twice with 1 x PBS buffer, pH 7.2, resuspended in the same buffer and stored on ice until use. 270 µl of the simulated duodenal juice and 30 µl of the cell suspension were mixed in a microtiter plate and stored at 37˚C under anaerobic conditions for 60 min. Total viable counts were performed, as detailed above.

Spray drying

Examination of heat resistance of tested strains

This method was described by Teixeira et al. (1997). Experiments in this work involved determining heat resistance in a reconstituted skim milk (RSM, 20% w/v) medium subsequently used for spray drying. Two 50 ml portions of the RSM (20% w/v) were stirred with magnetic stir bars and placed in a water bath at the following test temperatures: 55, 58, 59, 60 and 61˚C. One bottle was used for monitoring the temperature, and after temperature equilibration, a 1% inoculum of an overnight culture was added to the second bottle. In the next 4 min, at every 1 min, 1 ml samples were removed from the bottles, serially diluted and pour-plated onto MRS agar. The plates were incubated anaerobically at 37˚C for 48 h (Gas Pak, BBL, Germany). The decimal reduction time (*D values*) were determined as follows: D = τ /logNo-logN (Stumbo, 1965).

Spray-drying conditions

Spray-drying tests were performed using the method of Petrović (2011). Overnight cultures (300 ml) were centrifuged (4500 x g, 15 min, 15˚C). The pellet was washed twice in 50 mM K_2HPO_4 (pH 6.5) and resuspended in 300 ml of sterile RSM (20% w/v). Both tested strains were spray-dried with a laboratory scale spray-dryer (model B-290 Buchi mini spray dryer, Switzerland) by using a constant inlet air temperature of 170˚C and outlet temperature of 80˚C.

Determination of viability of tested strains in spray-dried powders

Plate count method

The colony forming units (CFU) of cultures were assessed by plate count using MRS agar. 9 ml of Na-citrate (2% w/v) was added to 1 g spray-dried powder and the preparation allowed to rehydrate before further dilutions were performed and appropriate dilutions plated (1ml) onto MRS agar. The plates were placed in anaerobic conditions and incubated at 37˚C for 48 h (Gas Pak, BBL, Germany). The number of bacteria before drying was determined and compared with the number of bacteria per spray-dried powder; CFU/g dry weight was determined from CFU/ml after drying a known volume of the original sample used for plating. Survival rates were calculated as follows: % survival = N/No x 100, where No represented the number of bacteria before drying and N was the number of the bacteria after drying.

Real-time PCR for selective quantification

Sample preparation

Both spray-dried tested strains were well resuspended in a Na-citrate (2%) solution to prepare 1% (w/w) solution. One milliliter of 1% spray-dried suspension was centrifuged (5000 x g, 10 min), and the DNA was extracted from the pellet by the method described below. Samples were serially diluted ten-fold prior to PCR analysis.

PMA treatment

The spray-dried tested strains were treated with PMA as described by Nocker et al. (2006). PMA (Biotium, Inc, CA, USA) was dissolved in dH_2O to create a 20 mM PMA stock solution and stored at -20˚C in the dark. Powders of each tested strain were resuspended in the Na-citrate solution to prepare a 1% (w/v) solution. An adequate volume of PMA stock solution was added to 500 µl of the aliquots of the spray-dried cultures to make a final concentration of 50 µM. This was followed by an incubation period of 5 min in the dark with occasional mixing to allow the PMA to penetrate the dead cells and to bind to the DNA. Samples were light-exposed to a halogen light source (650W, 150V) for 5 min; the samples were laid horizontally on ice to avoid excessive heating and placed about 20 cm from the light source. After photo-induced cross-linking, the cells were pelleted at 5000 x g for 10 min prior to DNA isolation by the protocol described below.

DNA isolation

Total genomic DNA from the samples was extracted using a MaxwellTM 16 cell tissue DNA purification kit applied with a MaxwellTM 16 instrument (Kramer et al., 2009). Four hundred µl of TE buffer and 100 µl of lysozyme (25 mg/ml) with mutanolysin (10 U/ml) were added to the pellet which was further resuspended and incubated for 2 h at 37˚C for bacterial cell lyses, before isolation by the Maxwell system. The whole volume of the prepared samples was transferred into the first well of the cartridge and further treated according to the manufacturer's instructions. Finally, the DNA was resuspended in 300 µl of elution buffer with added 1.5 µl RNAse (4 mg/ml).

Real-time PCR

PCR amplifications were performed with an MX3000P (Strategene, LA Jolla, CA, USA) instrument, as previously described by Kramer et al. (2009). The reaction mixture (25 µl) contained Platinum SYBR Green qPCR Super Mix UDG (11733; Invitrogene, Carlsbad, CA, USA), 0.2 µM of each primer and 5 µl of genomic DNA diluted -10 fold. The following primers were used LactoR`F (5`-CACAATGGACG(A/C)AAGTCTGATG-3`) and LBFR (5`-CGCCACTGGTGTTCTTCCAT-3`) (Songjinda et al., 2007). The amplification program was 50˚C for 2 min and 95˚C for 2 min, 35 cycles of 95˚C for 30 s, 60˚C for 15 s, 72˚C for 20 s, and then 95˚C for 1 min and 55˚ for 30 s.

Standard curves relating cell numbers to real-time PCR Ct values for tested strains

Standard curves were prepared with the DNA isolated from the pure cultures of the two tested strains. The tested strains were grown for 18 h in MRS broth. Two-fold dilution series from target species genomic DNA preparations were amplified by real-time PCR. The viable counts of each strain were determined by plate counting on MRS agar. The correlation between Ct values and CFU/ml was determined by the Strategene System's program.

Investigation for probiotic properties of spray-dried tested strains

Gastric juice and bile salts tests were performed using a modified version of the method of Dolereyes et al. (2004), described above. The only differences were in the way the samples were prepared; 1 g of spray-dried tested strains were dissolved in 9 ml Na-citrate (2% w/v).

Statistical analysis

The Ct values were automatically generated by Invitrogene software. Experiments were replicated at least three times. Means and standard deviations were calculated using one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Tolerance to simulated gastric juice and duodenal conditions

The viability of the tested strains in simulated gastrointestinal conditions is shown in Table 1. Both tested strains demonstrated a very good ability to survive the simulated gastric and duodenal conditions. About 2.5 l of gastric juice at pH 2–2.5 is eradiated each day in the stomach (Charteris et al., 1998), which causes the death of most microorganisms. In this work, *Lb. plantarum* 564 and *Lb. paracasei* Z-8 strains showed very good survival characteristics when exposed to a low pH solution, 91.20% and 85.00%, respectively.

Resistance to bile salts is also necessary to evaluate the ability of potential probiotic bacteria. The relevant concentrations of human bile salts are from 0.3% to 0.5% (Dunne et al., 1999). Both tested strains demonstrated a very high survival percentage in duodenal conditions, 91.20% for *Lb. plantarum* 564 and 84.00% for *Lb. paracasei* Z-8. The presence of bile salts was equally inhibiting for both tested strains, like gastric juice. This study demonstrates the good ability of both tested strains to survive simulated gastric juice and duodenal conditions. However, further

Table 1. Effect of simulated gastric juice and duodenal conditions on the viability of the tested strains.

Strains	Start number[a]	Resistance to gastric juice[a]	Resistance to duodenal conditions[a]
Lb. plantarum 564	9.44 ± 0.09	8.61 ± 0.01	8.61 ± 0.00
Lb. paracasei Z-8	10.32 ± 0.02	8.75 ± 0.01	8.67 ± 0.01

[a] Mean values (log CFU/ml) and standard deviation were calculated from three parallel plate count analyses.

Table 2. The number of cells of spray-dried tested strains performed by the plate count method, real-time polymerase chain reaction (PCR) and propidium monoazide (PMA) real time PCR.

Strains	Plate count[a]	Real-time PCR without PMA[b]	Real-time PCR with PMA[b]
Lb. plantarum 564	9.45 ± 0.05	9.31 ± 1.11	9.20 ± 0.98
Lb. paracasei Z-8	10.52 ± 0.04	10.42 ± 0.4	10.35 ± 0.50

[a] Mean values (log CFU/g) and standard deviation were calculated from three parallel plate count analyses.
[b] Mean values (log CFU/g) and standard deviation calculated from Ct values; based on two parallel DNA extracts from which two real-time cycles were run.

Table 3. The effect of simulated gastric juice and duodenal conditions on the viability of tested strains after spray-drying.

Strains	Start number[a]	Resistance to gastric juice[a]	Resistance to duodenal conditions[a]
Lb. plantarum 564	9,45 ± 0,05	8,65 ± 0,05	6,92± 0,03
Lb. paracasei Z-8	10,52 ± 0,04	8,96 ± 0,03	6,98 ± 0,01

[a] Mean values (log CFU/g) and standard deviation were calculated from three parallel plate count analyses.

research is necessary to provide more information about the probiotic potential of these species.

Heat resistance of the tested strains

Both tested strains possessed a high ability to survive at 55˚C. At 58, 59, 60, 61˚C the viability of *Lb. plantarum* 564 decreased dramatically, by 5 log. The heat resistance of microorganisms can also be defined by the *D* value. The *D* values obtained in this work for the tested strains were lower than the *D* values previously reported for the strain of *Lb. rhamnosus* E 800 and *Lb. salivarius* UCC 500 (Corcoran et al., 2004), and they were higher than the values previously reported for *Lb. salivarius* UCC 118 at 61˚C (Gardnier et al., 2000). It should be noted that both tested strains were in stationary phases, which may have resulted in increased heat resistance as it has been previously demonstrated that stationary-phase cultures are more resistant to heat stress than cells in the exponential growth phase (Texeira et al., 1994).

Viability of potential probiotic lactobacilli in spray-dried powders

Following the PCR reaction optimization, standard curves were constructed from the known concentrations (CFU/ml) of viable bacterial cells of *Lb. plantarum* 564 and *Lb. paracasei* Z-8. Suspensions of the standard samples prepared for DNA extraction were plate counted. The first two dilutions in each dilution series exhibited a PCR inhibitory effect, probably because of the too high DNA concentration or the presence of some other interfering substances (Kramer et al., 2009). The standard curve parameters for *Lb. plantarum* 564, obtained by a two-fold dilution series, were R^2=0.997, amplification efficiency 98.5%, and slope -3.225, and for the *Lb. paracasei* Z-8

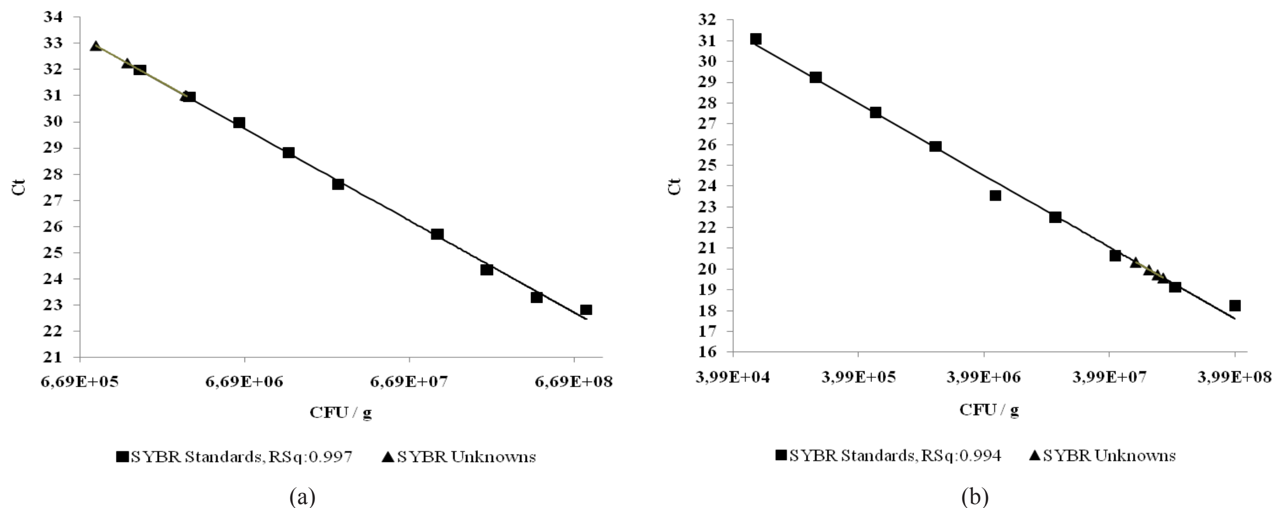

Fig. 1. Standard curves between CFU/g of standard samples or diluted product samples and the Ct values detected by real-time PCR for (a) *Lactobacillus plantarum* 564, and (b) *Lactobacillus paracasei* Z-8.

three-fold dilution series, they were R^2=0.994, amplification efficiency 94.7%, and slope -3.456 (Fig. 1).

Real-time PCR methods have been used for the quantification of probiotic bacteria in several studies; however, most often in different foods, such as fermented milk products (Grattepanche et al., 2005). One of the aims of this study was to evaluate the possibility of using PMA in combination with real-time PCR using SYBR Green I chemistry for the selection of viable lactobacilli in spray-dried powders. Fujimoto et al. (2010) attempted the EMA treatment to enumerate viable cells using real-time PCR, but the number of viable cells with EMA treatment exhibited a clear declining trend, about ten times lower than the number obtained by the PMA treatment. Therefore, in this work, PMA treatment with real-time PCR was chosen to enumerate accurately the viable cells of the tested strains. Bacterial counts derived from real-time PCR determination of PMA-treated and non-treated samples of spray-dried tested strains were compared to the plate count method (Table 2). The survival rates of the tested *Lb. plantarum* 564 and *Lb. paracasei* Z-8 strains after spray drying, determined by plate counting, were very high, 93% and 95.3%, respectively, calculated according to the formula described in materials and methods.

Quantitative molecular methods are in general more sensitive than traditional methods. The DNA of dead cells could be intact and degraded in the product. The treatment of bacterial cells with PMA before DNA isolation resulted in selective suppression of the amplification of DNA from dead cells (Lee and Levin, 2006). The concentrations log (CFU/g) of the tested strains obtained by the plate count method were not significantly ($p < 0.05$) different from the values obtained by real-time PCR analysis.

Investigation for probiotic properties of spray-dried tested strains

The application of these methodologies for improving probiotic survival during gastrointestinal transit is relatively new. Encapsulation in calcium alginate improved the survival of *Lactobacillus* in ice milk (Sheu et al., 1993). A potential disadvantage of spray drying as a way to protect cultures is the damage caused to bacterial cells during the process. Both spray-dried tested strains were investigated in terms of probiotic

properties *in vitro* and the results are shown in Table 3. The spray-dried tested strains showed a reduction in cell counts less than 1 log when maintained at pH 2.5 for 30 min. There was no difference compared with the results obtained before spray drying for both tested strains maintained at the same conditions. Bile salts with pancreatin showed a negative influence on the cell viability of both spray-dried tested strains.

Free and encapsulated cells exhibited different rates of decline in simulated duodenal conditions. Spray drying did not promote survival; in fact, the levels of free cells were less than 2 log higher in the simulated duodenal conditions. From the results obtained in this study, further work is required to establish successful encapsulation processes for *Lactobacillus* strains.

CONCLUSION

The tested strains were found to possess some potential probiotic properties. These strains are good candidates for further research in *in vivo* studies to elucidate their potential health benefits. The viability of the spray-dried tested strains was not affected by the simulated gastric juice; however, the simulated duodenal conditions drastically reduced the viability of both tested strains after spray drying. The results of this study indicate that the spray-drying process of microencapsulation did not offer any protection to the tested strains in simulated gastric juice and duodenal conditions. Treatment of the spray-dried tested strains with PMA, followed by real-time PCR analysis, as presented in this study, appears to be a promising approach for the routine determination of viable cells in spray-dried powders.

Acknowledgments - The authors acknowledge the financial support from the Ministry of Education and Science of the Republic of Serbia (National Project No. 046010 (2011-2014) and the Bilateral Project of the Republic of Serbia and the Republic of Slovenia (2010-2011), BI-SL/10-11-035).

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