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# Application of propidium monoazide coupled with quantitative PCR to evaluate cell viability of *Bifidobacterium animalis* subsp. *lactis* in a non-dairy probiotic beverage

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## Abstract

**Purpose:** In this study, a PMA-qPCR assay was developed for the enumeration of *Bifidobacterium animalis* subsp. *lactis* BB-12 viable cells in a non-dairy probiotic beverage.

**Methods:** Probiotic viability was monitored in three formulations of probiotic passion fruit juice microencapsulated by spray drying, during 30 days of storage at 4 °C. Viable cells were quantified using qPCR and PMA-qPCR assays targeting *tuf* gene and by plate counting method.

**Results:** The limit of detection for all samples was $10^3$ genome copies, corresponding to 21.3 pg of DNA. Higher CFU values were obtained for *B. lactis* BB-12 enumeration by qPCR, when compared to those obtained by PMA-qPCR and plate count, for all probiotic juice microcapsules. Similar quantification values were obtained by PMA-qPCR and plate counting for all samples and remained above 8 log CFU/g during the storage period.

**Conclusion:** These results demonstrated that the PMA-qPCR technique is a promising approach for *B. lactis* BB-12 viable cell enumeration in complex matrices such as passion fruit juice microcapsules. This PMA-qPCR assay allowed the achievement of reliable results faster than with the traditional plate counting method.

**Keywords:** Functional beverage, Bifidobacteria, Microencapsulation, qPCR, PMA-qPCR

## Introduction

Probiotics are widely known to provide several benefits to the human health, since their administration in adequate amounts improves the balance and composition of the gut microbiota (Hill et al. 2014). The genus *Bifidobacterium* is considered one of the most important groups of probiotic bacteria, which have been widely used (Prasanna et al. 2014).

Fermented dairy products still are the most representative category of probiotic products on the food market (Granato et al. 2019). However, there is a growing interest in the development of non-dairy probiotics, as an alternative for consumers with restrictions on the consumption of dairy products such as those with lactose intolerance (Panghal et al. 2018). In this sense, seeking the diversification of the functional food market, fruit juices have been studied as potential matrices for the incorporation of probiotic microorganisms

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(Chaikham 2015; Pimentel et al. 2015). Yellow passion fruit juice was investigated to be a carrier matrix of probiotic microorganisms and presented promising results for this purpose (Dias et al. 2018; Santos et al. 2017).

The recommendation to achieve beneficial health effects is a minimum concentration of 6 log CFU per gram of probiotic product throughout its shelf life, ensuring adequate amounts of viable cells at the time of product consumption (Tripathi and Giri 2014). This requirement represents one of the main technological challenges related to probiotic incorporation in fruit juices, since these products present some characteristics considered unfavorable to the maintenance of bacterial viability, such as the high concentration of organic acids and low pH (Shori 2016). Therefore, microencapsulation methods have been proposed aiming to protect probiotic microorganisms against adverse conditions. The application of the spray-drying technique for microencapsulation of probiotic fruit juices is a promising alternative, which allows their conversion into powder resulting in more stable products (Shishir and Chen 2017). The protective effect conferred on probiotics through the microencapsulation process depends on the physical characteristics of the matrix that constitutes the microcapsules. Therefore, different encapsulating agents may result in different levels of probiotic survival (Anekella and Orsat 2013).

The main challenge in the spray-drying microencapsulation process is the exposure of bacterial cells to high temperatures and the rapid dehydration. These conditions are stressful and may result in a decrease of bacterial count after the process and during the storage (Huang et al. 2017). Therefore, monitoring the survival of these cultures is necessary, since bacterial viability may influence the desired probiotic effects (Corona-Hernandez et al. 2013).

Classical microbiological methods are commonly used to identify and quantify probiotic species in foods. However, these methods present some inconvenience such as the time required to obtain results and the possibility to underestimate the viable cell counts. Culture-independent DNA-based methods have been proposed as an alternative to classical microbiology methods. The qPCR-based methods have been used for rapid and specific quantification of microorganisms in diverse food matrices (Achilleos and Berthier 2013; Ilha et al. 2016; Lawley et al. 2018). However, this method is unable to differentiate viable and non-viable cells, since DNA can persist in the environment even after cell death (Fittipaldi et al. 2012). In this sense, qPCR assays associated with nucleic acid intercalating dyes, such as propidium monoazide (PMA), have been developed as a rapid tool for the detection and quantification of viable cells of different microorganisms in several food matrices (Laidlaw et al. 2019; Liu et al. 2018). The PMA-qPCR technique is based on cell membrane integrity, since PMA dye penetrates only cells with damaged membranes and it intercalates DNA (Elizaquível et al. 2013; Nocker et al. 2006).

A PMA-qPCR assay was developed to enumerate viable cells of three probiotic strains (*Lactobacillus rhamnosus* R0011, *Lactobacillus helveticus* R0052, and *Bifidobacterium animalis* subsp. *lactis* BB-12) in maple sap beverages during refrigerated storage (Lupien-Meilleur et al. 2016). PMA-qPCR was used to estimate the lactic acid bacteria population in wine (Rizzotti et al. 2015). However, the use of qPCR combined with the PMA treatment for the quantification of viable *Bifidobacterium* BB-12 cells microencapsulated by spray drying in a non-dairy matrix has not been described to date.

The present study aimed to develop a PMA-qPCR assay for enumeration of *Bifidobacterium animalis* subsp. *lactis* BB-12 viable cells in probiotic passion fruit juice microencapsulated by spray drying. The viability of probiotic culture was assessed during refrigerated storage of the microcapsules by plate counting in selective medium and *tuf* gene-based qPCR assay, combined or not with PMA treatment.

## Material and methods

### Bacterial strains and culture conditions

Freeze-dried culture of *Bifidobacterium animalis* subsp. *lactis* (Nu-trish® BB-12®, Chr. Hansen, Hønsholm, Denmark) was prepared as described by Fritzen-Freire et al. (2012) and stored as a stock solution at − 20 °C into sterile glass bottles. The bacterial strain used as the negative control (*Lactobacillus paracasei* ATCC 10746) was grown as described previously by Scariot et al. (2018).

### Microencapsulation by spray drying

#### *Preparation of the bacterial suspension*

The preparation of the probiotic bacterial suspension was performed prior to the microencapsulation process as described by Dias et al. (2018). The stock solution of *B. lactis* BB-12 was inoculated in MRS broth (Difco, Sparks, USA) modified with the addition of 0.2% (w/w) lithium chloride (Vetec, Rio de Janeiro, Brazil) and 0.3% (w/w) sodium propionate (Sigma Chemical Co., St. Louis, USA) incubated under anaerobic condition (AnaeroGen®, Oxoid, Hampshire, UK) at 37 °C for 48 h. Afterwards, the cells were harvested by centrifugation (1000 × *g*, 15 min, 4 °C) and washed three times with sterile saline solution 0.9% (w/v). After the successive washes, cell pellets were suspended in sterile saline solution.

#### *Preparation of the feed solutions*

Commercial frozen yellow passion fruit pulp, without added sugars and preservatives (DeMarchi, Jundiaí, Brazil), was thawed and mixed with distilled water (1:1 w/w). The sample was filtered twice to reduce the

content of suspended solids and seed fractions. The filtrate was called passion fruit juice.

The feed solutions were prepared by the addition of the encapsulating agents to the passion fruit juice. The encapsulating agents used were maltodextrin DE 16.5–19.5 (Sigma Chemical Co., St. Louis, USA) and inulin (Orafti HPX, Orafti, Tienen, Belgium—degree of polymerization ≥ 23) added in different proportions (Table 1). All the feed solutions were homogenized until complete dissolution, submitted to heating at 80 °C for 1 min and cooled to 25 °C. Before spray-drying process, the feed solutions were divided into two parts: inoculated (with the addition of probiotic bacterial suspension) and control (without the addition of probiotic bacteria suspension). The inoculated feed solutions were added with 10% (v/v) of the probiotic bacterial suspension containing 9.61 log CFU/mL of *B. lactis* BB-12, in order to obtain final concentrations of about 8 log CFU/g in the microcapsules after the spray-drying process.

### *Spray-drying and sampling*

The microencapsulation process was performed in a laboratory scale spray dryer (B-290 Mini Spray Dryer, Büchi, Flawil, Switzerland) using the operating parameters described by Dias et al. (2018). The drying process was conducted at a constant air inlet temperature of 130 °C and an outlet temperature of 43 ± 3 °C. At the end of the procedure, the microcapsules were collected from the base of the cyclone and stored in sterile vials. The microcapsules were kept at 4 °C, and the sampling was performed at 1 and 30 days after the microencapsulation process. Immediately, all samples were used for plate counting in MRS-LP agar (Vinderola and Reinheimer 2000).

### Propidium monoazide treatment

Aliquots of 100 mg of each probiotic microcapsule were submitted to centrifugation (6000 × *g*, 2 min), and pellets were washed twice with 0.9% NaCl. The pellets were suspended in 500 μL ultra-pure water, and then, PMA treatment was performed. PMA (Biotium Inc., Hayward, CA, USA) treatment was carried following the procedures described by Scariot et al. (2018). After the treatment, the samples were stored at − 80 °C until DNA extraction.

**Table 1** Composition of the feed solutions for each formulation used in the spray drying process

| Formulation | Encapsulating agents (% m/v) | | Concentration of probiotic culture (% v/v) |
|---|---|---|---|
| | Maltodextrin | Inulin | |
| M1 | 20 | – | 10 |
| M2 | 10 | 10 | 10 |
| M3 | – | 20 | 10 |
| Control M1 | 20 | – | – |
| Control M2 | 10 | 10 | – |
| Control M3 | – | 20 | – |

### DNA extraction

Bacterial DNA was extracted from control and probiotic samples by DNAzol® method (Achilleos and Berthier 2013) with modifications, as described by Scariot et al. (2018). DNA quality and concentration were estimated on a Thermo Scientific Nanodrop 2000 spectrophotometer (Wilmington, DE, USA) with measurements at 260 and 280 nm.

### Quantitative PCR

In order to quantify the presence of *Bifidobacterium animalis* subsp. *lactis* in microcapsule samples, primer pair TUF (Solano-Aguilar et al. 2008), which amplifies a fragment of 117 bp, was used by qPCR. Quantitative real-time PCR was performed in the ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA).

Amplification reactions were carried out in a final volume of 25 μL containing 12.5 μL of 2× SYBR Green Master Mix (Applied Biosystems), 200 nmol/L TUF Forward, 150 nmol/L TUF Reverse, water, and 10 ng of template DNA. The following cycling conditions were applied for all reactions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The fluorescence signal was measured at the end of each 60 °C step. Melting curve analysis was performed automatically after amplification reactions by continuous heating from 65 to 95 °C. All real-time PCR runs were analyzed using automatic software settings.

### Construction of standard curves

Standard curves (Cq versus log DNA copy number and Cq versus log CFU) were prepared with serial dilutions of genomic DNA isolated from *B. animalis* subsp. *lactis* BB-12 pure culture and each of the three formulations of microencapsulated probiotic passion fruit juice (1 day after microencapsulation process). Genomic DNA was 10-fold serially diluted in ultra-pure water to final copy number ranging from $10^7$ to $10^0$ genome copies per reaction, equivalent to 21.3 to 2.13 × $10^{-6}$ ng. The number of genome copies was calculated on the basis of the size of the *Bifidobacterium animalis* subsp. *lactis* BB-12 (GenBank NC_017214.1) genome (1.9422 Mbp). The corresponding CFU was calculated based on plate counting of the same sample. Each standard curve was performed in three different qPCR runs in triplicate. Amplification efficiencies were determined as described previously (Ilha et al. 2016).

### Enumeration of *Bifidobacterium animalis* subsp. *lactis* BB-12 by qPCR and plate counting

Enumeration of BB-12 by plate counting (CFU/g) was performed using a selective medium (MRS-LP agar) under anaerobic conditions (Dias et al. 2018). Bacterial counts estimated by qPCR and PMA-qPCR were calculated as previously described (Scariot et al. 2018).

### Statistical analysis

The results were expressed as the mean ± standard deviation (SD). The quantification methods (qPCR, PMA-qPCR, and plate count) were compared by linear regression analysis with 95% of confidence interval (95% CI), using STATISTICA 7.0 software (StatSoft Inc. Tulsa, OK, USA)

## Results and discussion

### qPCR parameters for *Bifidobacterium animalis* subsp. *lactis* BB-12 quantification

In order to detect and quantify *B. animalis* subsp. *lactis* BB-12, TUF primers were used. The specificity was previously reported by Solano-Aguilar et al. (2008). Standard curves obtained from 10-fold dilutions of *B. animalis* subsp. *lactis* BB-12 DNA isolated from pure culture, and three formulations of microencapsulated probiotic passion fruit juice were used to determine the qPCR parameters (efficiency, slope, and correlation coefficient). The standard curves presented linear correlation coefficients with $R^2$ value higher than 0.99, and efficiency values in the range 81–92% for 6 standard curves using DNA extracted from pure culture (Table 2) and 82%, 88%, and 93% using DNA extracted from each formulation of microencapsulated probiotic passion fruit juice (Table 3). The limit of detection (LOD) for both samples was $10^3$ genome copies, corresponding to 21.3 pg of DNA.

The presence of residual interfering substances from the microcapsules co-extracted with DNA, mainly the polysaccharides used as encapsulating agents, i.e., maltodextrin and inulin, could interfere in qPCR efficiency values (Schrader et al. 2012). Efficiency values around 90% were observed by Odooli et al. (2018) for a *tuf* gene-based qPCR assay targeting *B. animalis* subsp. *lactis* BB-12 quantification in commercial probiotic yogurts. So considering the matrix effect of interfering substances in qPCR inhibition (Schrader et al. 2012), acceptable efficiency values were obtained here. To our knowledge, the use of TUF primers to quantify *B. animalis* subsp. *lactis* BB-12 viable cells has not been reported yet.

**Table 2** qPCR parameters of standard curves for *Bifidobacterium animalis* subsp. *lactis* (Nu-trish®BB-12®) extracted from *B. lactis* grown in culture medium

| qPCR run | Efficiency (%) | Slope | $R^2$ |
|---|---|---|---|
| 1 | 81 | − 3.890 | 0.9965 |
| 2 | 87 | − 3.685 | 0.9997 |
| 3 | 87 | − 3.689 | 0.9998 |
| 4 | 90 | − 3.588 | 0.9994 |
| 5 | 88 | − 3.638 | 0.9993 |
| 6 | 92 | − 3.526 | 0.9991 |

**Table 3** Parameters of standard curves for *Bifidobacterium animalis* subsp. *lactis* (Nu-trish®BB-12®) quantification using *tuf* primers and bacterial DNA extracted from three formulations of microencapsulated probiotic passion fruit juice 1 day after microencapsulation process

| qPCR run | Efficiency (%) | Slope | $R^2$ |
|---|---|---|---|
| **M1 (20% maltodextrin)*** | | | |
| 1 | 94 | − 3.464 | 0.9988 |
| 2 | 98 | − 3.378 | 0.9983 |
| 3 | 88 | − 3.642 | 0.9993 |
| **M2 (10% maltodextrin 10% inulin)*** | | | |
| 1 | 85 | − 3.755 | 0.9997 |
| 2 | 92 | − 3.520 | 0.9995 |
| 3 | 86 | − 3.690 | 0.9983 |
| **M3 (20% inulin)*** | | | |
| 1 | 78 | − 3.987 | 0.9957 |
| 2 | 80 | − 3.920 | 0.9817 |
| 3 | 87 | − 3.681 | 0.9978 |

*Encapsulating agents (% m/v)

### Quantification of *Bifidobacterium* BB-12 viable cells in microencapsulated probiotic passion fruit juice samples by PMA-qPCR

The quantification of viable cells in probiotic food products is important since to exert health benefits on the host, most probiotic cells must be viable and not just present (Davis 2014). Culture-independent methods associated with intercalating DNA agents, such as PMA-qPCR, have been proposed for monitoring and quantification of viable cells in several different food matrices, also for probiotic dairy products (Padilha et al. 2016; Scariot et al. 2018). However, only few studies have reported the development of PMA-qPCR assays intended for the determination of probiotic cultures viability in non-dairy products (Kramer et al. 2009; Lupien-Meilleur et al. 2016).

In our study, we developed a PMA-qPCR assay to quantify *B. animalis* subsp. *lactis* BB-12 viable cells in probiotic passion fruit juice microcapsules and control microcapsules. All samples were quantified by plate count and qPCR assay simultaneously. Standard curves Cq versus log CFU were plotted using mean CFU counts

obtained by plate counting of *B. animalis* subsp. *lactis* BB-12 from probiotic microcapsules (1 day after microencapsulation process). Afterwards, the *Bifidobacterium* BB-12 counts (log CFU/g) were calculated using the Cq values obtained (Additional file 1: Table S1) and the equation described by Ilha et al. (2016). *Bifidobacterium* BB-12 counts of microencapsulated probiotic passion fruit juice samples were obtained by three quantification techniques during storage at 4 °C (Table 4).

*Bifidobacterium* BB-12 was not detected in control microcapsules, since there was no amplification signal in qPCR and PMA-qPCR assays using DNA extracted from these samples. Additionally, control samples showed no growth when submitted to plating in a selective medium. These results demonstrated that there was no contamination between control and probiotic feed solutions during the microencapsulation process, which could cause false-positive signals and inaccuracy in the quantification of probiotic bacteria in the microcapsules.

The enumeration log CFU/g of *B. animalis* subsp. *lactis* BB-12 by qPCR showed higher values than those obtained by PMA-qPCR and plate count, for all the microcapsules samples. The difference observed between the bacterial counts by qPCR and PMA-qPCR methods is due to the inability of qPCR assay to differentiate viable and non-viable cells, since total DNA is amplified, including the DNA from non-viable cells, leading to an overestimated number of viable cells in samples (Postollec et al. 2011). Similar results were previously reported by other studies that evaluated the efficiency of PMA-qPCR methods for the quantification of viable cells in probiotic products (Matias et al. 2016; Scariot et al. 2018).

In this study, the presence of non-viable cells in the microcapsule samples can be attributed to the spray-drying microencapsulation process, since the bacterial cultures were submitted to stressful conditions and may present reduction or loss of viability (Tripathi and Giri 2014).

Similar counts were obtained by PMA-qPCR and plate count assays for all microcapsule samples (Additional file 1: Table S2). These results showed that the treatment with PMA dye was efficient to discriminate viable and non-viable cells. Similar results were observed in other studies. García-Cayuela et al. (2009) evaluated the viability of different species of lactic acid bacteria in a commercial symbiotic product by qPCR combined with PMA and by plating in selective media and observed similar results between these two quantification methods. Santos et al. (2018) observed a correlation between the traditional plating method and the PMA-qPCR method for the evaluation of *Bifidobacterium* BB-12 viability in probiotic food emulsions.

During the storage period, the counts of *B. animalis* subsp. *lactis* BB-12 (obtained by PMA-qPCR assay and plate count) remained similar in all of the microcapsule formulations (Table 4), and values obtained were above 8 log CFU/g, which represent effective dosages, according to the recommended probiotic criterion (> 6 log CFU/g or mL), necessary to achieve health benefits (Bakr 2015). These results are in accordance with those observed in a previous study (Dias et al. 2018) carried out with the same formulations of microencapsulated probiotic passion fruit juice. In the former study, the refrigerated storage was favorable and necessary to ensure the stability of the probiotic culture in samples. However, different from the former study, a significant effect of cell recovery during storage at 4 °C was not observed in the present study, since the differences observed between cell counts performed on days 1 and 30 were considered of little microbiological significance. Similarly, Kramer et al. (2009) reported high bacterial counts of *B. lactis* in a lyophilized product, obtained by PMA-qPCR—as well as by plate count, which remained practically unchanged during 90 days of storage.

According to the results obtained by both PMA-qPCR and traditional plate count methods, all microencapsulated probiotic passion fruit juice formulations supported the probiotic survival during the storage period and could be a good alternative as a non-dairy food matrix to deliver live probiotic microorganisms.

## Conclusion

This study showed the suitability of the PMA-qPCR method to assess the viability of *Bifidobacterium animalis* subsp. *lactis* BB-12 in a complex non-dairy food matrix such as probiotic passion fruit juice powders. PMA-qPCR assay is a specific and sensitive method to

**Table 4** Comparison of *B. animalis* subsp. *lactis* BB-12 count (log CFU/g) obtained by qPCR, PMA-qPCR, and plate count of the three formulations of microencapsulated probiotic passion fruit juice samples collected at 1 and 30 days of storage

| Sample | qPCR count (log CFU/g)[a] | | PMA-qPCR count (log CFU/g)[a] | | Plate count (log CFU/g)[b] | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 30 | Day 1 | Day 30 | Day 1 | Day 30 |
| M1* | 10.49 ± 0.17 | 10.43 ± 0.17 | 7.95 ± 0.08 | 8.78 ± 0.30 | 8.74 ± 0.08 | 8.78 ± 0.04 |
| M2* | 10.76 ± 0.12 | 10.22 ± 0.20 | 8.69 ± 0.09 | 8.89 ± 0.14 | 8.69 ± 0.05 | 8.64 ± 0.05 |
| M3* | 10.36 ± 0.28 | 9.95 ± 0.24 | 8.92 ± 0.51 | 8.50 ± 0.35 | 8.51 ± 0.04 | 8.49 ± 0.03 |

[a]Values are mean ± SD ($n$ = 12), qPCR duplicate of two independent DNA extracts in three independent runs
[b]Values are mean ± SD ($n$ = 4), quadruplicate plate count
*Encapsulating agents (% m/v): M1 20% maltodextrin, M2 10% maltodextrin 10% inulin, M3 20% inulin

quantify viable cells in food samples when compared with the culture-dependent methods. Furthermore, the PMA-qPCR method allows a faster quantification when compared to the time needed to obtain the results with the plate count method and lower waste production since that anaerobic atmosphere generation systems are necessary for plate count techniques.

This method appears to be a promising approach for quick assessment of the viability of probiotic *Bifidobacterium* BB-12 in a food product for routine monitoring in industries. To our knowledge, the application of qPCR coupled with PMA for the quantification of microencapsulated *Bifidobacterium animalis* subsp. *lactis* BB-12 viable cells in a non-dairy matrix has not been described to date.

## Supplementary information

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s13213-020-01566-9.

**Additional file 1: Table S1.** Cq and Tm obtained by qPCR assay using DNA extracted from samples three formulations of microencapsulated probiotic passion fruit juice. **Table S2.** Parameters of linear regression analysis with 95 % of confidence interval (95 % CI).

### Authors' contributions

All the authors designed the study; C.O.D. prepared the samples under the guidance of R.D.M.C.A.; C.O.D. and M.C.S. performed the qPCR analysis under the guidance of A.C.M.A., C.O.D. and M.C.S. performed the data analysis. All the authors wrote, read and approved the final manuscript.

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### Ethics approval and consent to participate

N/A

### Competing interests

The authors declare that they have no competing interests.

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