Food Microbiology 27 (2010) 236–242

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Development of a real-time RT-PCR method for enumeration of viable *Bifidobacterium longum* cells in different morphologies

Sebastian Reimann [a], Franck Grattepanche [a], Enea Rezzonico [b], Christophe Lacroix [a,*]

[a] Laboratory of Food Biotechnology, Institute of Food Science and Nutrition, Swiss Federal Institute of Technology, Schmelzbergstrasse. 7 8092 Zurich, Switzerland
[b] Food and Health Microbiology, Nestlé Research Center, P.O. Box 44, CH-1000 Lausanne 26, Switzerland

ARTICLE INFO

Article history:
Received 9 July 2009
Received in revised form
8 October 2009
Accepted 14 October 2009
Available online 21 October 2009

Keywords:
Bifidobacterium
qRT-PCR
mRNA-stability
Cell aggregation

ABSTRACT

Viability of probiotic bacteria is traditionally assessed by plate counting which has several limitations, including underestimation of cells in aggregates or chains morphology. We describe a quantitative PCR (qPCR)-based method for an accurate enumeration of viable cells of *Bifidobacterium longum* NCC2705 exhibiting different morphologies by measuring the mRNA levels of *cysB* and *purB*, two constitutively expressed housekeeping genes. Three primer-sets targeting short fragments of 57-bp of *cysS* and *purB* and one 400-bp fragment of *purB* were used. Cell quantification of serially diluted samples showed a good correlation coefficient of R^2 0.984 ± 0.003 between plate counts and qRT-PCR for all tested primer sets. Loss of viable cells exposed to a lethal heat stress (56 °C, 10, 20 and 30 min) was estimated by qRT-PCR and plate counts. No significant difference was observed using qRT-PCR targeting the 400-bp fragment of *purB* compared to plate counts indicating that this fragment is a suitable marker of cell viability. In contrast, the use of the 57-bp fragments led to a significant overestimation of viable cell counts (18 ± 3 and 7 ± 2 fold for *cysB* and *purB*, respectively). Decay of the mRNA fragments was studied by treatment of growing cells with rifampicin prior qRT-PCR. The 400-bp fragment of *purB* was faster degraded than the 57-bp fragments of *cysB* and *purB*. The 400-bp fragment of *purB* was further used to enumerate viable cells in aggregate state. Cell counts were more than 2 $\log_{10}$ higher using the qRT-PCR method compared to plate counts.

Growing interest in probiotic characteristics of aggregating bacteria cells make this technique a valuable tool to accurately quantify viable probiotic bacteria exhibiting heterogeneous morphology.

1. Introduction

Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). It is generally accepted that probiotic food products should contain a minimal level of viable cells of 10^6 per gram or milliliter of product (Ouwehand and Salminen, 1998), although this value is relative since beneficial effect depends on the strain and targeted health benefit (Lacroix and Yildirim, 2007). Viability of probiotic bacteria is traditionally assessed by plate counting which has several limitations, including underestimation of cells in aggregates or chains morphology. Auto-aggregation trait of *Bifidobacterium longum* strains isolated from human gastric juice and intestine has been associated with their ability to adhere to epithelial cells (Del Re et al., 2000) which is considered as an important functionality of probiotic bacteria for some beneficial health effects such as to prevent attachment of pathogens to gut mucosa (Resta-Lenert and Barrett, 2003; Cesena et al., 2001; Castagliuolo et al., 2005).

However, bacterial cell aggregates lead to underestimation of cell counts with traditional, culture-dependent methods (Daley, 1977; Auty et al., 2001). Enumeration techniques with fluorescent stains targeting bacterial membrane potentials or enzymatic activities were proposed as alternative methods to plate counts (Breeuwer and Abee, 2000; Hewitt and Nebe-Von-Caron, 2001; Alakomi et al., 2005). The accuracy of these methods using fluorescence probes, generally combined with flow cytometry can be affected by clustering and chaining of cells (Wallner et al., 1995; Nebe-von-Caron et al., 2000; Bibiloni et al., 2001).

The use of real-time PCR techniques to quantify bifidobacteria and lactic acid bacteria by specific DNA or rRNA targeting showed good correlation with plate counts (Masco et al., 2007; Friedrich and Lenke, 2006; Matsuda et al., 2007). However, their use as accurate viability markers is limited since rRNA and DNA can be detected hours or even days after cell death (TolkerNielsen et al., 1997; Sheridan et al., 1998; Hellyer et al., 1999; Aellen et al., 2006; Keer and Birch, 2003; Lahtinen

* Corresponding author. Fax: +41 44 632 14 03.
E-mail address: christophe.lacroix@ilw.agrl.ethz.ch (C. Lacroix).

0740-0020/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2009.10.010

et al., 2008). Molecules of mRNA in contrast have short half-life times ranging between 0.5 and 50 min (Belasco et al., 1986; Hellyer et al., 1999; Takayama and Kjelleberg, 2000) and are therefore a more suitable viability marker for bacterial cells. Yeasts and pathogenic bacteria were quantified using mRNA in combination with quantitative reverse transcription PCR (qRT-PCR) (Bleve et al., 2003; Jacobsen and Holben, 2007; Coutard et al., 2007; Gonzalez-Escalona et al., 2009). The use of instable mRNA as a target molecule however requires special attention to minimize losses during RNA extraction and purification (Bustin, 2002; Johnson et al., 2005).

In this study, we describe the development of a qRT-PCR-based method for the quantification of viable *B. longum* NCC2705 cells. Serial dilutions of bacterial culture aliquots were used to generate qRT-PCR calibration curves targeting three mRNA fragments (two short amplicons of 57-bp of *cysS* or *purB* and one of 400-bp of *purB*) and plotted against plate counts. Spiked internal reference mRNA was used to monitor mRNA loss during sample processing and thus to provide accurate mRNA comparison across different experimental conditions (Johnson et al., 2005). Stability of the three mRNA fragments was tested by comparing cell counts obtained by qRT-PCR and plate counts of culture exposed to lethal heat stress. Instability of the different mRNA fragments was also assessed by measuring mRNA decay after rifampicin treatment. The mRNA fragment selected was then used to estimate viable cell numbers in culture samples containing macroscopic cell aggregates.

2. Materials and methods

2.1. Bacterial strain

Bifidobacterium longum NCC2705 (Nestlé Culture Collection, Nestlé Research Center, Lausanne, Switzerland) was grown at 37 °C in rubber-lid flasks containing 100 ml MRS broth (de Man et al., 1960) (BioLife, Milan, Italy) supplemented with 0.5 g/l L-cysteine (Sigma–Aldrich, Buchs, Switzerland) (MRSC) and inoculated at 2% (v/v) with an overnight MRSC pre-culture. The headspace of flasks was flushed with CO_2 to ensure anaerobic conditions. Colony forming units (CFU) were determined by plating serial dilutions of culture aliquots in duplicate on MRSC agar plates 1.5% (w/v) (Becton Dickinson, Basel, Switzerland) and incubating at 37 °C for 48 h in anaerobic jars containing atmosphere generation system packs (AnaeroGen, Oxoid, Pratteln, Switzerland).

2.2. Preparation of bacterial samples for calibration curves

Cultures were grown at 37 °C in a 2000 ml reactor (Bioengineering, Wald, Switzerland) containing 1800 ml MRSC inoculated at 0.5% (v/v) with pre-culture. pH was maintained at 6.0 by addition of NaOH (5 M), agitation rate was set at 200 rpm using a flat blade impeller and anaerobic conditions were maintained by flushing CO_2 in the headspace of the reactor. Cells were harvested in end-exponential growth phase and centrifuged at 6000 × g for 2 min. Pellets were suspended in an equal volume of a cryoprotectant solution composed of MRSC supplemented with glycerol 10% (w/v) (Fluka, Buchs, Switzerland), shock frozen in liquid nitrogen and stored at −80 °C before RNA extraction and plate counts. Standards with cell concentrations ranging from about 3.2×10^4 to 4.7×10^8 CFU/ml determined by plate counts were prepared by serial dilution in MRSC before RNA extraction.

2.3. Production of aggregated cells

B. longum NCC2705 cells aggregates were produced during continuous culture with immobilized cells grown in a two-stage reactor system as described by Doleyres et al. (2004), with some modifications. Briefly, the first reactor (R1) was a 500 ml glass reactor (Sixfors, Infors-HT, Bottmingen, Switzerland) with a working volume of 240 ml, containing 80 ml of gel beads pre-colonized with *B. longum* NCC2705 as described by Cinquin et al. (2004) and stirred between 150 and 250 rpm. The second 2000 ml reactor (Bioengineering) was run in series at a volume of 1800 ml, agitated at 250 rpm and inoculated with cells produced in R1. CO_2 was injected in the headspace of the two reactors to maintain anaerobic conditions during culture. The continuous system was fed with MRSC medium supplemented with 1.5 g/l $CaCl_2$ (Sigma–Aldrich) and 40 g/l glucose at a flow rate of 360 ml/h. Continuous fermentation was run for 20 days at 37 °C and pH was maintained at pH 6.0 by addition of NaOH (5 M) in both reactors. Culture samples from the effluent of both reactors were periodically collected and processed as described above.

2.4. Heat stress

A 100-ml volume of MRSC was inoculated at 1% (v/v) with *B. longum* NCC2705 and incubated in a rubber-lid flask at 37 °C for 16 h. Cells were collected by centrifugation at 6000 × g for 2 min. Supernatant was discarded and pellet suspended in the same volume of fresh MRSC. Cell suspensions were split in aliquots of 1 ml and immediately added to 9 ml MRSC pre-warmed at 56 °C and kept for 10, 20 and 30 min at this temperature or to 9 ml MRSC at room temperature as control experiment. After heating, cells were immediately cooled to room temperature in a waterbath for 3 min, collected by centrifugation, washed and suspended in the initial volume of MRSC for plate counting. 0.5 ml of the cell suspension was added to prepared screw-cap tubes as described below and immediately frozen in liquid nitrogen for RNA extraction. Experiments were performed in triplicate.

2.5. Rifampicin test

MRSC (100 ml) in a rubber-lid flask was inoculated at 5% (v/v) with pre-culture and incubated at 37 °C until the culture reached mid-exponential phase (OD_{600} 0.5–0.8). Rifampicin (Sigma–Aldrich) was then added to a final concentration of 400 μg/ml. Samples were taken 2.5, 7.5, 14, 20, 25 and 45 min after rifampicin-addition. Cells were collected by centrifugation, washed in fresh MRSC and suspended in the initial volume of MRSC for RNA extraction. The half-life time ($T_{½}$) of mRNA was determined from the degradation rate constant (k) corresponding to the slope of a semi-logarithmic plot of mRNA amount as a function of time with the relation: $T_{½} = \ln 2/k$. Experiments were performed in duplicate.

2.6. RNA isolation and reverse transcription reaction

RNA extraction was performed according to Stevens et al. (2008). Briefly, 0.5 ml of fresh or stored cell samples was centrifuged and the pellet was washed once in MRSC, transferred to 2-ml screw-cap tubes containing a mix of 500 mg glass beads of 100 μm diameter (Sartorius, Goettingen, Germany), 500 μl of a 1:1 solution of phenol/chloroform (Sigma–Aldrich), 30 μl of 10% (w/v) sodium dodecyl sulfate (Sigma–Aldrich) and 30 μl of 3 M sodium acetate (pH 5.2) and immediately frozen in liquid nitrogen. Frozen cells were then disrupted by bead-beating in a homogenizer (FastPrep FP 120, Q-Biogene, Carlsbad CA, USA), four times for 40 s at speed 4.0 intercalated with cooling periods on ice. Afterwards, the mixtures were centrifuged (21,000 × g, 3 min at 4 °C) to remove glass beads and cell debris. The aqueous phase was transferred to a new tube and traces of phenol were removed by extraction with pre-chilled chloroform. The resulting aqueous phase was mixed with an equal volume of 70%-ethanol and applied to RNA purification column using RNeasy Mini Kit (Qiagen, Basel, Switzerland). Further purification was performed