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Suppression subtractive hybridisation and real-time PCR for strain-specific quantification of the probiotic *Bifidobacterium animalis* BAN in broiler feed

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ABSTRACT

To ensure quality management during the production processes of probiotics and for efficacy testing *in vivo*, accurate tools are needed for the identification and quantification of probiotic strains. In this study, a strain-specific qPCR assay based on Suppression Subtractive Hybridisation (SSH) for identifying unique sequences, was developed to quantify the strain *Bifidobacterium animalis* BAN in broiler feed. Seventy potential BAN specific sequences were obtained after SSH of the BAN genome, with a pool of closely related strain genomes and subsequent differential screening by dot blot hybridisation. Primers were designed for 30 sequences which showed no match with any sequence database entry, using BLAST and FASTA. Primer specificity was assessed by qPCR using 45 non-target strains and species in a stepwise approach. Primer T39_S2 was the only primer pair without any unspecific binding properties and it showed a PCR efficiency of 80% with a C_q value of 17.32 for 20 ng BAN DNA. Optimised feed-matrix dependent calibration curve for the quantification of BAN was generated, ranging from 6.28×10^3 cfu g^{-1} to 1.61×10^6 cfu g^{-1}. Limit of detection of the qPCR assay was 2×10^1 cfu g^{-1} BAN. Applicability of the strain-specific qPCR assay was confirmed in a spiking experiment which added BAN to the feed in two concentrations, 2×10^6 cfu g^{-1} and 2×10^4 cfu g^{-1}. Results showed BAN mean recovery rates in feed of $1.44 \times 10^6 \pm 4.39 \times 10^5$ cfu g^{-1} and $1.59 \times 10^4 \pm 1.69 \times 10^4$ cfu g^{-1}, respectively. The presented BAN-specific qPCR assay can be applied in animal feeding trials, in order to control the correct inclusion rates of the probiotic to the feed, and it could further be adapted, to monitor the uptake of the probiotic into the gastrointestinal tract of broiler chickens.

1. Introduction

Bifidobacteria are beneficial bacteria that inhabit the gastrointestinal tract of mammals and birds (Apajalahti et al., 2004). Different species of this genus are widely used as probiotics in food-producing animals (Gaggia et al., 2010). Formerly, a *B. animalis* subsp. *animalis* strain (DSM 16284), which is mentioned hereafter, by the code BAN, has been isolated and identified as a probiotic strain for use in a multi-species feed additive in chicken (Klose et al., 2006). In order to evaluate the efficacy of a probiotic strain *in vivo*, appropriate methods for the identification and quantification of the strain are important. It is also important to assure the required inclusion rate of the probiotics in feed, because adequate amounts of probiotics are required to confer health benefits to the host (Fuller, 1989). The method of choice should be strain-specific, quantitative, and applicable for the analysis of feed. Culture-dependent methods for the enumeration of *Bifidobacteria* are available (Leuschner et al., 2003), but these techniques are time consuming (2–5 days) and do not allow for differentiation between different species or even strains (Davis, 2014). Several molecular typing methods are available to differentiate between strains of a certain species, such as Pulsed-field Gel Electrophoresis (PFGE) (Yeung et al., 2004) or Random Amplified Polymorphic DNA (RAPD)-PCR (Welsh and McClelland, 1990). Similar to culture-dependent methods, these techniques are time consuming and are of limited use for complex samples derived from broiler feed, faeces, or content of the gastrointestinal-tract (GIT). Real-time PCR (qPCR) is a very common technique for detecting and quantifying various bacterial species from different sources, like food (De Martinis et al., 2007), rumen (Tajima et al., 2001) or faeces (Rinttila et al., 2004). This method is highly sensitive and allows for quantification within a wide dynamic range (10–10^9 copy numbers). A calibration curve is required for absolute quantification by qPCR and should be generated from reference samples which are similar to test samples, in order to ensure comparable PCR efficiencies and thus, avoid over- or underrepresentation of the amount of target DNA. A commonly used approach is by adding a defined number of target cells to the environmental sample of interest, which is free of target (Bustin et al., 2009). The amplification efficiency of the target

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DNA is influenced by the background DNA and other possible PCR inhibitory substances, originating from the sample matrix (for example, proteins, polysaccharides, or lipids). Thus, a major obstacle to DNA extraction from feed samples is obtaining good quality DNA without PCR-inhibitory substances, and at the same time, extracting the total genomic DNA from the bacterial community (Zoetendal et al., 2001). Highly specific primers are crucial for the correct quantification of target sequences, despite the presence of closely related bacterial DNA. Genes commonly used for taxonomic identification (for example, 16S rRNA, *rpoB*) are not sufficient for strain differentiation, due to sequence homology (Yeung et al., 2002). Alternatively, several reports describe the development of strain-specific primers, using discriminatory RAPD-derived DNA fragments (Ahlroos and Tynkkynen, 2009; Fujimoto et al., 2008, 2011; Maruo et al., 2006; Tilsala-Timisjarvi and Alatossava, 1998) or by identifying phage-related sequences (Brandt and Alatossava, 2003). Suppression Subtractive Hybridisation (SSH) is applied to filter and identify genomic DNA fragments present in one, but not another closely related genome (Agron et al., 2002; Huang et al., 2007; Saxena et al., 2005). This method is especially useful, when the genome sequence information of the strain of interest is not available and thus, *in silico* genome comparison is not possible, in the search for specific sequences. The strategy to develop a strain-specific qPCR for environmental samples, based on comparative genomics was only reported in a few studies of qPCR (Konstantinov et al., 2005; Peng et al., 2011). The aim of this study was to develop a strain-specific qPCR assay for detection of the strain *B. animalis* BAN (DSM 16284) in broiler chicken feed. To increase the probability of SSH to detect BAN specific DNA fragments, a pool of different *B. animalis* subspecies was employed as SSH driver, for the whole genome subtraction. To the best of our knowledge, this is the first time the use of SSH in combination with qPCR is reported, for specific quantification of a *Bifidobacterium* strain in feed.

2. Materials and methods

2.1. Bacterial culturing conditions

Bifidobacterium strains were grown in specific growth medium containing 10 g L^{-1} peptone from casein, 5 g L^{-1} meat extract, 2.5 g L^{-1} yeast extract, 10 g L^{-1} glucose, 1 g L^{-1} tween 80, 0.5 g L^{-1} cystein-HCl, 2 g L^{-1} K_2HPO_4, 0.6 g L^{-1} $MgSO_4x7H_2O$, 0.25 g L^{-1} $ZnSO_4x7H_2O$, 0.15 g L^{-1} $CaCl_2$, 0.08 g L^{-1} $FeCl_3x6H_2O$ and 10 g L^{-1} inulin. Cells were grown under anaerobic conditions for 24–48 h at 37 °C. *Enterococcus*, *Lactobacillus*, and *Pediococcus* strains were grown in Man Rogosa Sharpe medium (MRS; Oxoid, Hampshire, UK) under anaerobic conditions at a temperature of 37 °C for 24 h.

2.2. DNA extraction from culture broth and broiler feed

DNA extraction from bacterial cultures was done, following a protocol for Gram-positive bacteria (Chan et al., 2003), with additional lysis steps, using lysozyme (2.5 mg mL^{-1}) and Proteinase K (125 μg mL^{-1}) as earlier described (Sattler et al., 2014).

Microbial DNA from broiler feed was extracted from a sample volume of 40 g. Feed was mixed with 150 mL peptone water, containing 0.01% Triton X-100 and shaken for 30 min. The mixture was then, smashed and filtered through a stomacher bag. Bacterial cells of the filtrate were pelleted and further used for DNA extraction. Briefly, the cells were lysed with lysozyme (100 mg mL^{-1}) for 45 min at 37 °C. Thereafter, ASL buffer (4 mL), from the DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) was added and heated for 5 min at 95 °C. The Stool kit protocol or procedure for pathogen detection was followed from there. Isolated DNA was visualised by agarose gel electrophoresis and concentration was determined by NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnology GmbH, Erlangen, Germany).

2.3. SSH and mirror orientation selection (MOS) clone library

SSH was performed using the PCR-select bacterial genome subtraction Kit (Clonetech Laboratories, Mountain View, CA, USA) following the manufacturer's instructions. BAN was used as tester sample to subtract unique genomic DNA from a driver sample; a mixture of genomic DNA from four strains (*B. animalis* subsp. *animalis* (DSM 20104), *B. animalis* subsp. *animalis* (LMG 18900) *B. animalis* subsp. *lactis* HN019 (Danisco, Madison, USA) and *B. animalis* subsp. *lactis* (DSM 10140). Advantage 2 Polymerase Kit (Clonetech Laboratories, Mountain View, CA, USA) was used for all PCR mixtures according to the SSH protocol with minor modifications. A higher volume of the secondary PCR product (48 μL instead of 24 μL) was required to continue with MOS procedure, to remove non-differential background PCR products. Secondary PCR products were purified with phenol–chloroform–isoamylalcohol (25:24:1), precipitated with 4 M ammonium acetate solution and ethanol and then, digested with Xma I enzyme at 37 °C for 2 h, to remove adaptor 1. PCR products were denatured at 98 °C for 1.5 min and reannealed at 68 °C for 3 h. Samples were then mixed with 200 μL dilution buffer (included in the SSH kit) and incubated for 7 min at 70 °C. Reaction mix for MOS-PCR was prepared according to SSH primary PCR with 10 μM MOS primer NP2Rs (Rebrikov et al., 2000). PCR was performed with an initial step at 72 °C for 2 min, followed by 26 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and elongation at 72 °C for 1.5 min. During this MOS PCR step, only target fragments containing the SSH, with nested Primer 2R at both ends were amplified exponentially. MOS PCR products were cloned into pJET 1.2/blunt cloning vectors, using the CloneJet PCR Cloning Kit (Fermentas, Burlington, CA, USA) transformed into ElectroMAX DH10B cells by electroporation as earlier described (Sattler et al., 2014). Rapid plasmid preparation and insert amplification were performed from clones as previously described (Dong et al., 2007). Size and purity of the amplified inserts were analysed by agarose gel electrophoresis and DNA was purified directly from the PCR reaction mix or the gel (when more than one band appeared), using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).

2.4. Differential screening using dot blot hybridisation

Differential screening using dot blot hybridisation assay was performed for BAN (SSH tester) specific fragments, obtained from the clone library. PCR fragments were spotted on a nylon membrane and hybridised with biotin labeled and RsaI digested pool of genomic driver DNA, as previously described (Sattler et al., 2014). DNA dots were visualised using the Biotin Chromogenic Detection Kit (Fermentas, Burlington, CA, USA), following the manufacturer's instructions. Fragments showing no hybridisation reaction with the driver DNA were sequenced and further used for primer design.

2.5. Primer design and specificity testing

Potential BAN-specific sequences were searched for sequence similarity using BLAST (Altschul et al., 1990) and FASTA (Pearson and Lipman, 1988) program. Sequences with no match found in the databases were selected for primer design in the PrimerQuest program. Primers were checked for secondary structures and sequence similarities with BLAST. Specificity testing of primers was conducted in two rounds, applying several non-template strains (Tables 1 and 2). PCR reactions with a total volume of 15 μL contained 1 × KAPA SYBR Mix (VWR international GmbH, Erlangen, Germany), 100 nM of each primer, and 10 ng template DNA. The program was run for a 2 min initial denaturation period, at a temperature of 95 °C, followed by 40 cycles at 95 °C for 15 s, annealing at 56 °C or 60 °C for 20 s, and an elongation at 72 °C for 20 s. To ensure that the correct PCR product was amplified, a melting curve analysis was added at the end of the PCR program, using the default settings of the realplex2 Mastercycler ep-gradient S instrument (Eppendorf, Hamburg, Germany). For primers that were not