



## Optimisation of a droplet digital PCR for strain specific quantification of a probiotic *Bifidobacterium animalis* strain in poultry feed

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### ABSTRACT

The use of probiotics in animal nutrition to provide health benefits is widely accepted. *Bifidobacterium animalis* (BAN) is an example of a commonly used beneficial strain. BAN is applied in a multi-strain feed additive for poultry. As part of the increased demand for tracking and tracing of feed additives within modern quality management, it is crucial to determine the quantity of the active strain after mixing the probiotic product into feed. A real-time PCR protocol, already developed some years ago, was replaced with a Droplet Digital PCR (ddPCR) assay, as this third generation PCR method is known for higher precision, sensitivity and does not require standard curves. Each sample is partitioned into thousands of small subsamples that are measured individually and an absolute result value for each sample is extrapolated via Poisson distribution. The following parameters were evaluated for the ddPCR assay: optimal annealing temperature (59 °C), concentration of primers (500 nM) and probe (400 nM), and PCR cycle number (50 cycles). The linearity of the optimised ddPCR assay was tested with BAN DNA extracted from pure culture. The obtained standard curve was linear ( $R^2 = 0.9982$ ) and the efficiency (E) of the method was 99.98%. To finalise the development, the Limit of Blank (LoB =  $9.17 \times 10^2$  copies  $g^{-1}$ ), Limit of Detection (LoD =  $1.15 \times 10^3$  copies  $g^{-1}$ ) and Limit of Quantification (LoQ =  $1.57 \times 10^3$  copies  $g^{-1}$ ) for the assay were determined using poultry feed free of BAN and feed spiked with different concentrations of the strain. A BAN strain-specific, probe-based ddPCR assay for the quantification in poultry feed was developed.

### 1. Introduction

The genus *Bifidobacterium* consists currently of more than 50 different known species. These beneficial bacteria constitute part of the intestinal microbiota of mammals, birds and insects. The health benefits of this genus, even though not completely unveiled, are associated with (i) the complex dynamic interplay among bifidobacteria, (ii) interaction with other members of the microbiota and (iii) interaction with the host (Hidalgo-Cantabrana et al., 2017). The positive effect of increased bifidobacteria levels and in general probiotics on the overall composition and metabolism in the gut was repeatedly described (Enomoto et al., 2014; Sugahara et al., 2015). Bifidobacteria are commonly used as probiotics in productive livestock (Gaggia et al., 2010). The *Bifidobacterium animalis* subsp. *animalis* strain (DSM 16284), which is mentioned hereafter by the abbreviation BAN, has been isolated and identified as a probiotic strain for use in a multi-species feed additive in poultry (Klose et al., 2006; Mountzouris et al., 2010). The identification, quantification and inclusion rate of the probiotic strain in feed is very important to confer health benefits to the host (Fuller, 1989) and

to evaluate the efficacy of the probiotic strain in vivo. Application of Droplet Digital PCR (ddPCR) for the strain-specific quantification of BAN in feed could provide a superior method compared to the available real-time PCR assay.

Digital PCR (dPCR) is a third generation PCR method for target quantification. One of the commercially available approaches is ddPCR from Bio-Rad (QX200™ Droplet Digital™ PCR system, Bio-Rad, Munich, Germany). For ddPCR, each PCR sample is partitioned into thousands of droplets, with each droplet containing either 0 or 1 (or more) copies of the target sequence (Hindson et al., 2011). After partitioning in about 20,000 droplets with a volume of 0.85 nL, PCR is performed and the droplets are read out. Based on the proportion of positively and negatively read droplets, the absolute number of targets can be extrapolated from the positive fraction via Poisson distribution. Compared to real-time PCR, ddPCR provides several significant advantages such as higher precision, sensitivity and tolerance towards PCR inhibitors (Hugget et al., 2013; Pinheiro et al., 2012). Additionally, absolute quantification can be performed without the requirement of a standard curve. This results in shortened laboratory work and guarantees higher intra- and

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**Table 1**  
Primers and Probe used in this study. Purification quality: desalt.

Primer/probe name	Sequence	Supplier	Origin
BAN_T39_S2_fw	5'-CCATCTTCTGGTATCTGCAACAA-3'	Sigma-Aldrich	Fibi et al., 2016
BAN_T39_S2_rv	5'-GTAAGCGCAAATGTCACAAAGA-3'	Sigma-Aldrich	Fibi et al., 2016
BAN_T39_S2_probe	[6FAM]ACGTAGCGCCCTCAGAAGTGCAACAAA[BHQ1]	Sigma-Aldrich	this study

inter-laboratory reproducibility. DdPCR is regularly applied in the field of diagnostic analyses (Lin et al., 2016; Memon et al., 2017) and has also been reported for bacterial quantification (Witte et al., 2016a, 2016b). Thus, ddPCR was the method of choice to improve a previously established strain-specific real-time PCR method for quantification of BAN in poultry feed (Fibi et al., 2016). A probe for BAN-specific ddPCR was designed, evaluated and included in the method. The DNA-extraction procedure was adapted to the specific needs of ddPCR. Real-time PCR is still a valid method, but its limitations compared to ddPCR became obvious. The labour and resource intensive preparation of standard curves for each type and set of samples and the detrimental effect of sample autofluorescence on the results make real-time PCR inferior to ddPCR. The latter method does not require standard curves and tolerates inhibitors much better. Taken together, a new ddPCR method starting with DNA extraction from complex feed samples to the PCR run was developed to overcome real-time PCR limitations.

The aim of this study was to develop a strain-specific, probe based ddPCR assay for the detection of BAN (DSM 16284) in poultry feed. To the best of our knowledge, this is the first time the use of ddPCR to quantify a probiotic *Bifidobacterium* strain in feed has been reported.

## 2. Materials and methods

### 2.1. Bacterial culturing conditions

*Bifidobacterium animalis* (DSM 16284, BAN) was used in this study. The strain was grown in a growth medium containing 10 g L<sup>-1</sup> peptone from casein, 5 g L<sup>-1</sup> meat extract, 2.5 g L<sup>-1</sup> yeast extract, 10 g L<sup>-1</sup> glucose, 1 g L<sup>-1</sup> Tween 80, 0.5 g L<sup>-1</sup> cysteine-HCl, 2 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 0.6 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.25 g L<sup>-1</sup> ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.15 g L<sup>-1</sup> CaCl<sub>2</sub>, 0.08 g L<sup>-1</sup> FeCl<sub>3</sub>·6H<sub>2</sub>O and 10 g L<sup>-1</sup> inulin. Cells were grown under anaerobic conditions for 24–48 h at 37 °C.

### 2.2. DNA-extraction and quantification from culture broth and poultry feed

DNA extraction from bacterial cultures was performed following a protocol for Gram-positive bacteria (Chan et al., 2003), with additional lysis steps, using lysozyme (2.5 mg mL<sup>-1</sup>) [Sigma-Aldrich L6876] and Proteinase K (125 µg mL<sup>-1</sup>) [Qiagen DNA Stool Mini Kit, Hilden, Germany] as described earlier (Sattler et al., 2014).

Microbial DNA from different poultry feed was extracted from a 40 g sample. Briefly, feed was mixed with 150 mL peptone water (0.1% peptone [Sigma-Aldrich 70169, MO, USA] and 0.01% Triton X-100 [Fluka, Sigma-Aldrich 93426, MO, USA]) and shaken for 30 min. The mixture was smashed and filtered through a stomacher bag (Smasher, AES Chemunex, Bruz, France). An aliquot of 250 µL of the filtered peptone water extract was used for further steps. The pelleted cells were lysed with lysozyme (100 mg mL<sup>-1</sup>) for 45 min at 37 °C. Thereafter, the DNA Stool Mini Kit protocol for pathogen detection (Qiagen GmbH, Hilden, Germany) was followed. The concentration of the DNA samples and their purity was determined by NanoDrop™ One spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, U.S.A) and Qubit 3.0 Fluorometer with the Qubit dsDNA HS Assay Kit (Invitrogen, California, USA). The resulting data are summarised in Supplementary Table 1. DNA was stored at 4 °C until ddPCR was performed and for long term storage at -20 °C.

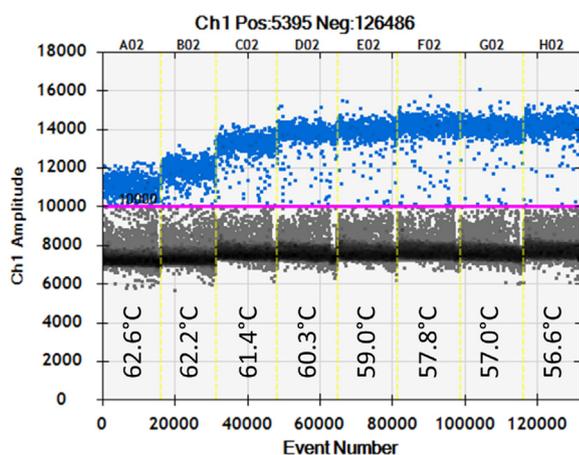
### 2.3. ddPCR-setup

The probe for the ddPCR assay was designed by Oligo Techservice of Sigma-Aldrich (Sigma-Aldrich, MO, USA) based on the BAN specific sequence and the already established primers, amplicon size with these primers was 119 bp (Fibi et al., 2016). Optimisation of the ddPCR assay was performed considering different chemical and physical parameters: annealing temperature (thermal gradient from 59 °C to 62.2 °C), primers and probe concentration (from 100 nM to 1000 nM) and number of cycles (40 and 50 cycles) with different BAN DNA dilutions. Mastermix and PCR program were initially setup according to Bio-Rad (Bio-Rad, Munich, Germany) recommendations in the manual. Final conditions for one ddPCR reaction are 20 µL final volume: 1 × ddPCR™ Supermix for Probes (No dUTP) [Bio-Rad, Munich, Germany], 500 nM of each primer and 400 nM of the probe (Table 1) and 3 µL of DNA. Reactions were prepared in duplicate or triplicate with 10% excess volume. According to the manufacturer's instructions, 20 µL of each reaction were loaded into a sample well of an 8-well cartridge for the QX200™ Droplet Generator (Bio-Rad, Munich, Germany) followed by 70 µL of droplet generation oil for probes (Bio-Rad, Munich, Germany) into the oil wells. QX200™ Droplet Generator (Bio-Rad, Munich, Germany) was used to generate the droplets (~20,000 droplets are possible). For each ddPCR run a non-template control (NTC), with nuclease-free water instead of DNA, was included. The emulsion (~40 µL) was transferred to a 96-well plate. The plate was heat sealed using pierceable PCR Plate Heat Seal foil (Bio-Rad, Munich, Germany) and the PX1™ PCR Plate Sealer (Bio-Rad, Munich, Germany). The final two-step PCR protocol applied was: initial polymerase activation period of 10 min at 95 °C, 50 cycles of denaturation at 95 °C for 30 s and annealing temperature of 59 °C for 1 min and at the end final enzyme deactivation at 98 °C for 10 min. For all steps a ramp rate of 2 °C s<sup>-1</sup> was used with the C1000 Touch™ Thermal Cycler (Bio-Rad, Munich, Germany). After amplification, the plate was placed in the QX200™ Droplet Reader (Bio-Rad, Munich, Germany) for analysis. Data evaluation was performed using QuantaSoft™ Software 1.7 (Bio-Rad, Munich, Germany).

The result is displayed as copies per reaction. As feed samples were subjected to dilutions during the sample preparation process, copies per gram of feed were calculated accordingly. One reaction contained 3 µL DNA from the 50 µL DNA extract, thus, the number was first multiplied by 50 × 3<sup>-1</sup>. Additionally, only 0.25 mL of the initial 150 mL feed extract were used, leading to a multiplication by 150 × 0.25<sup>-1</sup>. To calculate 1 g of feed instead of the 40 g used for the feed extract, the value was finally divided by 40 in order to calculate copies per gram of feed.

### 2.4. ddPCR efficiency

As PCR efficiency is crucial for a ddPCR assay, BAN DNA was diluted serially (1:10, 1:50, 1:100, 1:500, 1:1000, 1:5000 and 1:10000) to guarantee linearity of the reaction results. Dilutions were prepared in triplicate, then measured independently in duplicates on the QX200™ ddPCR System (Bio-Rad, Munich, Germany) with the parameters described above. Results were subjected to regression analysis and efficiency was calculated according to the formula:



**Fig. 1.** Amplitude plot showing the clusters of negative (grey) and positive (blue) droplets for reactions with different separation annealing temperatures. High annealing temperatures led to a decreased separation of the clusters, whereas too low temperatures might lead to non-specificity. 59 °C was chosen as annealing temperature for BAN-specific ddPCR. The corresponding events plot is displayed in Supplementary Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$$E = \frac{\text{slope}_{\text{measured}}}{\text{slope}_{\text{expected}}} \times 100$$

### 2.5. Limit of blank (LoB) in poultry feed

LoB was defined as the highest BAN concentration that was expected to be found when testing replicates of samples not containing BAN (Armbruster and Pry, 2008). Feed from a local supplier (broiler starter feed from Lagerhaus, Tulln, Austria) was used to determine the LoB. DNA was extracted eight times from the feed sample, resulting in a total of 8 extracts. For each extract, ddPCR samples were prepared in technical triplicates.

For the LoB calculation, the following formula was used (Armbruster and Pry, 2008):

$$\text{LoB} = \text{mean}_{\text{blank}} + 1.645 \times (\text{SD}_{\text{blank}})$$

Where  $\text{mean}_{\text{blank}}$  stands for the mean of the 8 extracts (mean of the replicates of a blank sample) and  $\text{SD}_{\text{blank}}$  stands for the standard deviation of the calculated mean of the 8 extracts (standard deviation of mean result of replicates of a blank sample).

### 2.6. Limit of detection (LoD) in poultry feed

Based on the definition: LoD is the lowest BAN concentration which can be detected and reliably distinguished from LoB (Armbruster and Pry, 2008). The LoD was determined by using the measured LoB and test replicates of samples known to contain different concentrations of BAN. Broiler starter feed, used for LoB determination, was spiked with different concentrations of BAN to get the following final concentrations:  $2.00 \times 10^6 \text{ cfu g}^{-1}$ ;  $5.00 \times 10^5 \text{ cfu g}^{-1}$ ;  $1.25 \times 10^5 \text{ cfu g}^{-1}$ ;  $3.13 \times 10^4 \text{ cfu g}^{-1}$ ;  $7.81 \times 10^3 \text{ cfu g}^{-1}$ ;  $1.95 \times 10^3 \text{ cfu g}^{-1}$ . Therefore, the highest concentration of BAN was mixed into 1 kg of feed, resulting in  $2.00 \times 10^6 \text{ cfu g}^{-1}$ . This feed was then diluted in 1:4 steps with blank feed to get approximately 1 kg of each feed mixture at the end. From each of the six spiked feed mixtures four times 40 g samples were extracted, resulting in a final number of 24 DNA extracts. Each extract was subjected to ddPCR in technical triplicates using the conditions described above.

The LoD was calculated using the following equation (Armbruster and Pry, 2008):

$$\text{LoD} = \text{LoB} + 1.645 \times (\text{SD}_{\text{low concentration sample}})$$

Whereby  $\text{SD}_{\text{low concentration sample}}$  is the standard deviation of the mean result of the samples known to contain the lowest concentration of analyte, in this case  $1.95 \times 10^3 \text{ cfu g}^{-1}$  of BAN.

### 2.7. Limit of quantification (LoQ) in poultry feed

LoQ is the lowest concentration at which BAN can be reliably quantified (Armbruster and Pry, 2008). To determine the LoQ, more than one type of feed was applied in order to check for the robustness of the assay in different feed matrices. Three different feed types (1: broiler starter feed, 2: layer concentrate and coarsely ground corn in ratio 40:60 and 3: poultry grower feed, all from the local supplier: Lagerhaus, Tulln, Austria) were spiked with a final concentration of  $2.00 \times 10^6 \text{ cfu g}^{-1}$  of BAN. From each spiked feed type a sample was removed for DNA extraction. DNA was extracted twice from each sample resulting in 6 extracts. Each extract was serially diluted in 1:4 steps in nuclease free water to reach a dilution of 1:4096. For ddPCR analysis, reactions were done in technical triplicates and results were subjected to regression analysis.

## 3. Results

### 3.1. ddPCR conditions

Important for ddPCR are well-separated clusters of positive and negative droplets. Samples with less than 10,000 droplets were not included in the analysis because too low droplet numbers decrease the precision of the applied Poisson distribution. By the use of BAN DNA extracted from pure culture, the following optimised parameters were defined. As displayed in Fig. 1, positive and negative droplets were well separated at 59 °C and lower temperature. Because annealing temperatures below 59 °C did not show better separation, but might increase unspecific binding, and because annealing temperatures above 59 °C lowered separation quality, 59 °C was determined as the optimal annealing temperature.

The Bio-Rad recommendation according to their manual was a primer concentration of 900 nM and a probe concentration of 250 nM for ddPCR. To provide enough probe for each reaction of the primer concentration optimisation process, the probe concentration was set at 900 nM and primer concentration varied from 100 nM to 1000 nM. With increasing primer concentration, a better separation between positive and negative droplets was seen (Supplementary Figs. 2 and 3). However, cluster separation was similar when primer concentrations from 500 nM to 1000 nM were applied. Thus, optimisation of the probe concentration was carried out with two fixed primer levels: (i) 500 nM and (ii) as recommended by the supplier, 900 nM.

As shown in Supplementary Figs. 4 and 5, the fluorescence levels for 500 nM and 900 nM primers with different probe concentrations were similar. Thus, 500 nM of primers were used in subsequent experiments. Increased probe concentration led to increased fluorescence levels of the positive droplets and negative droplets. A good separation between the positive and the negative droplets was observed starting from 400 nM of probe. Therefore, 400 nM was chosen as the probe concentration for subsequent experiments.

A comparison of 40 versus 50 cycles during PCR was performed (Fig. 2). The run with 50 cycles displayed less droplets with fluorescence levels between the negative and positive droplets, commonly referred to as “rain”, compared to the run with 40 cycles. Therefore, subsequent experiments were performed with a PCR including 50 cycles.

### 3.2. ddPCR efficiency

To evaluate the linearity of the ddPCR assay, seven dilutions from

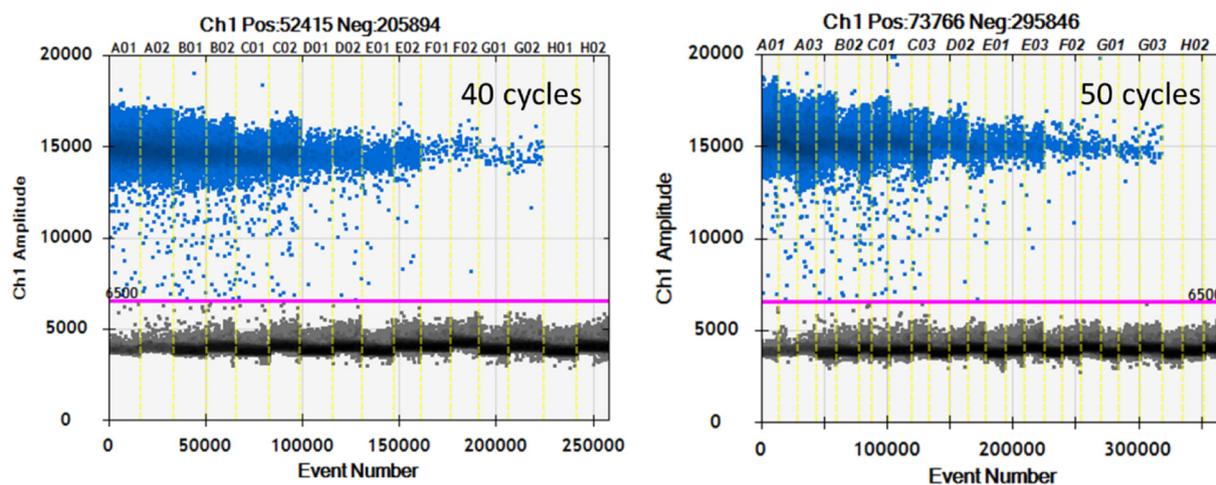


Fig. 2. Amplitude plot for ddPCR based on 40 and 50 cycles of PCR amplification. Less “rain” was obtained with 50 amplification cycles. The corresponding events plot is displayed in Supplementary Fig. 6.

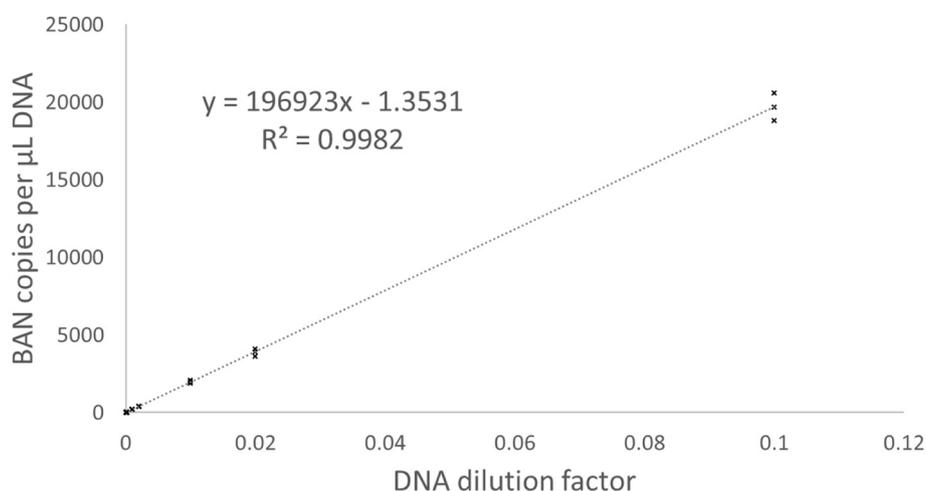


Fig. 3. Regression curve for seven pure BAN DNA dilutions to test for linearity of the ddPCR assay.

1:10 to 1:10000 of BAN DNA extracted from pure culture were analysed in three independent runs. Results fit well to the dilution steps as shown in Fig. 3. The efficiency was 99.98% (it was calculated by dividing the slope of the trendline from Fig. 3 [196923] by the slope of the trendline from the expected values [196958] [data not shown] and followed by a multiplication with factor 100). In order to evaluate the applicability of ddPCR quantification in the more complex feed matrix, BAN was quantified in poultry feed samples spiked with the strain.

### 3.3. Limit of blank (LoB) in poultry feed

DNA (8 replicates) was extracted from broiler starter feed and measured via ddPCR, in order to determine the LoB. Out of the 8 samples, one did not give any positive droplet, whilst some positive droplets were detected in the remaining samples, from which a LoB of  $9.17 \times 10^2$  copies  $g^{-1}$  feed was calculated. Every value measured below this limit was defined as negative for BAN. Results are summarised in Supplementary Table 2.

### 3.4. Limit of detection (LoD) in poultry feed

The LoD was determined together with the LoB for poultry feed, to define a limit at which the presence of BAN could reliably be detected, by the measurement of 24 extracts in four independent ddPCR assays. A LoD of  $1.15 \times 10^3$  copies  $g^{-1}$  feed was determined and values below

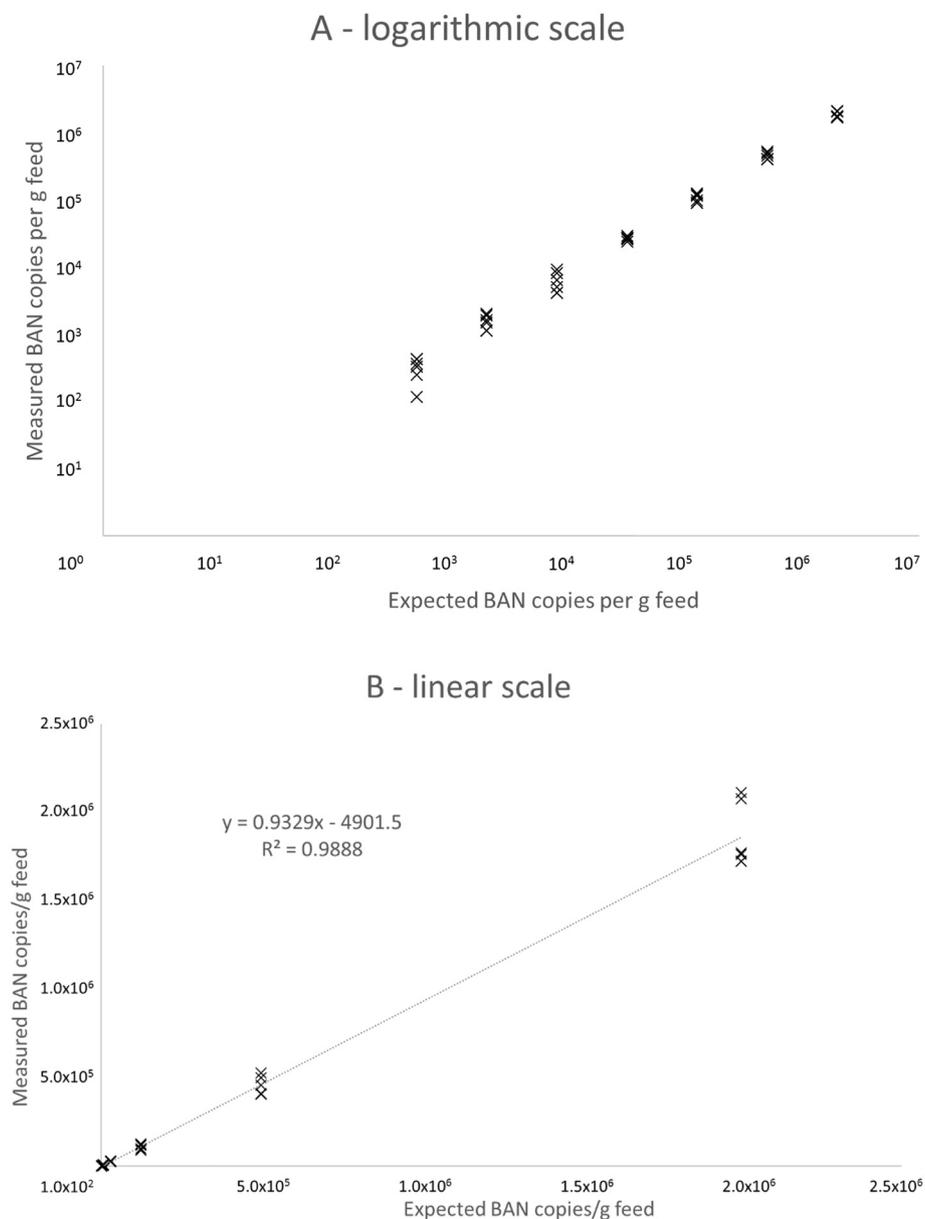
the LoD were not considered for subsequent experiments. Results are summarised in Supplementary Table 3.

### 3.5. Limit of quantification (LoQ) in poultry feed

To define the LoQ, three different feeds were spiked with  $2.00 \times 10^6$  cfu  $g^{-1}$  of BAN. The ddPCR results from 6 extracts fit well together up to a dilution of 1:1024 (Fig. 4). Linearity of the ddPCR for BAN from feed could be shown until a LoQ of  $1.57 \times 10^3$  copies  $g^{-1}$ . Every result above this value could be quantified. If results between LoD and LoQ appeared, only a qualitative assumption on the BAN content could be made. Results are summarised in Supplementary Table 4.

## 4. Discussion and conclusion

The beneficial effect of *Bifidobacterium animalis* as a probiotic has been described in many reports (Ezendam et al., 2008; Jungersen et al., 2014; Mountzouris et al., 2010; Paveljšek et al., 2018). To see these beneficial effects in animals a certain dose of probiotic strains is necessary (Yan et al., 2018). Previously, the microbial quantification procedure for *Bifidobacterium animalis*, strain BAN, could be shortened by a specific real-time PCR assay (Fibi et al., 2016) that replaced the microbiological technologies (Norin et al., 1991). The advantages of ddPCR, such as less sensitivity to PCR inhibitors in the feed and no



**Fig. 4.** A: Plot for seven dilutions of each of the 6 extracts to test for linearity of the ddPCR assay with feed samples. The first 6 dilutions up to 1:1024 show linearity, dilution number 7 shows already a slightly different trend and was not included in regression analysis. B: Regression plot for the first 6 dilutions showing linearity and a good correlation for expected and measured values. Thus, quantification could be performed in the range of  $1.57 \times 10^3$  copies  $g^{-1}$  to  $1.86 \times 10^6$  copies  $g^{-1}$  of BAN in feed.

requirement for a standard curve, was expected to allow the development of a shortened and more accurate strain-specific quantification method of BAN.

The higher sensitivity of ddPCR compared to real-time PCR (Hindson et al., 2011) encouraged us to completely rework the DNA extraction procedure to make it easier and faster. In the final procedure, 45 mL of feed extract for DNA extraction (Fibi et al., 2016) were replaced by 0.25 mL. Thereby, it was possible to carry out the extractions in easy-to-handle small volume reaction tubes with snap caps.

To increase specificity a probe was designed and used for ddPCR (Selvaraj et al., 2018; Srisutham et al., 2017; Witte et al., 2016a). The changes in the DNA extraction method and the application of a probe made it impossible to compare samples between the current real-time PCR and the newly developed ddPCR methodology. As ddPCR provides absolute values calculated with Poisson distribution, a comparison of the results with those from real-time PCR was not necessary. Finally, feed sample analysis could be performed without the need of the

laborious processing of standard curves.

We were able to determine the best conditions for high amplification and good cluster separation of the positive and negative droplets by varying annealing temperatures, primer and probe concentrations and PCR cycle number. Optimisation of the first three parameters is common for real-time PCR and ddPCR assays (Dalmira et al., 2016; Weerakoon et al., 2016), whereas the optimisation of PCR cycle number is not as common. The number of PCR cycles for ddPCR could have a severe impact on the droplet “rain”, the fraction of droplets appearing at fluorescence levels between the negative and the positive fraction (Witte et al., 2016b). We could observe an influence of PCR cycle number on the droplet “rain”, but in contrast to Witte (2016b), the effect was less pronounced. However, we could still observe a reduction in droplet “rain”, when 50 cycles were used, instead of 40 cycles. Based on our experience during the general optimisation of real-time PCR processes, small changes of annealing temperature or primer/probe concentration directly influenced the  $c_q$  value and thus the outcome. An

end-point method such as ddPCR was less sensitive to such changes. Changes of 1 °C or variations of 100 nM in primer/probe concentration were tolerated according to the optimization results.

Since in blank feed very small numbers of droplets (maximum  $n = 2$ ) were detected, the LoB was included as suggested by Armbruster and Pry (2008). The authors described statistical methods, not specific for ddPCR, but based on our experience with real-time PCR, the methods are well suited and applicable. Real-time PCR LoD ( $2 \times 10^1$  cfu  $g^{-1}$ ) was lower compared to  $1.15 \times 10^3$  copies  $g^{-1}$  with the current ddPCR method. This fact is likely due to the change in the DNA extraction procedure, as 0.25 mL of feed extract contained a lower total number of BAN cells compared to 45 mL of the same extract when using previous extraction method. On the contrary, LoQ could be reduced from  $6.28 \times 10^3$  cfu  $g^{-1}$  with real-time PCR to  $1.57 \times 10^3$  cfu  $g^{-1}$  with ddPCR. This shows the strength of ddPCR method and its superiority in regards to sensitivity.

Experiments were performed according to “Minimum Information for Publication of Quantitative Digital PCR Experiments (MIQE)” guidelines (Hugget et al., 2013).

In conclusion, a BAN specific ddPCR method was developed to quantify the probiotic strain in poultry feed samples without the necessity of a standard curve.

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## Declarations of interest

none.

## Declaration of potential conflict of interest

We declare that this work was performed in cooperation with the company BIOMIN Holding GmbH, who provided the probiotic strain. However, authors confirm that this does not alter the adherence to all policies on sharing data and materials.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mimet.2019.105646>.

## References

Armbruster, D.A., Pry, T., 2008. Limit of blank, limit of detection and limit of quantitation. *Clin. Biochem. Rev.* 29, S49–S52 (doi:citeulike-article-id:3416410).

Chan, R.K., Wortman, C.R., Smiley, B.K., Hendrick, C.A., 2003. Construction and use of a computerized DNA fingerprint database for lactic acid bacteria from silage. *J. Microbiol. Methods* 55, 565–574. [https://doi.org/10.1016/S0167-7012\(03\)00186-6](https://doi.org/10.1016/S0167-7012(03)00186-6).

Dalmira, F.U., Melina, P.U., José-Benigno, V.T., Josefina, L.F., Raymundo, G.E., Abraham, A.S., 2016. Development, optimization, and evaluation of a duplex droplet digital PCR assay to quantify the T-nos/hmg copy number ratio in genetically modified maize. *Anal. Chem.* 88, 812–819. <https://doi.org/10.1021/acs.analchem.5b03238>.

Enomoto, T., Sowa, M., Nishimori, K., Shimazu, S., Yoshida, A., Yamada, K., Furukawa, F., Nakagawa, T., Yanagisawa, N., Iwabuchi, N., Odamaki, T., Abe, F., Nakayama, J., Xiao, J., 2014. Effects of bifidobacterial supplementation to pregnant women and infants in the prevention of allergy development in infants and on fecal microbiota. *Allergol. Int.* 63, 575–585. <https://doi.org/10.2332/allergolint.13-OA-0683>.

Ezendam, J., De Klerk, A., Gremmer, E.R., Van Loveren, H., 2008. Effects of *Bifidobacterium animalis* administered during lactation on allergic and autoimmune responses in rodents. *Clin. Exp. Immunol.* 154, 424–431. <https://doi.org/10.1111/j.1365-2249.2008.03788.x>.

Fibi, S., Klose, V., Mohnl, M., Weber, B., Haslberger, A.G., Sattler, V.A., 2016. Suppression subtractive hybridisation and real-time PCR for strain-specific quantification of the probiotic *Bifidobacterium animalis* BAN in broiler feed. *J. Microbiol. Methods* 123, 94–100.

Fuller, R., 1989. Probiotics in man and animals. *J. Appl. Bacteriol.* 66, 365–378.

Gaggia, F., Mattarelli, P., Biavati, B., 2010. Probiotics and prebiotics in animal feeding for safe food production. *Int. J. Food Microbiol.* 141, S15–S28. <https://doi.org/10.1016/j.ijfoodmicro.2010.02.031>.

Hidalgo-Cantabrana, C., Delgado, S., Ruiz, L., Ruas-Madiedo, P., Sàncnes, B., Margolles, A., 2017. Bifidobacteria and their health-promoting effects. *Microbiol. Spectr.* 5 (BAD-0010-2016. doi:10.1128/microbiolspec.BAD-0010-2016.Correspondence).

Hindson, B., Ness, K., Masquelier, D., Belgrader, P., Heredia, N., Makarewicz, A., 2011. High-throughput droplet digital PCR system for absolute quantification of DNA copy number. *Anal. Chem.* 83, 8604–8610. <https://doi.org/10.1021/ac202028g>.

Hugget, J.F., Foy, C.A., Benes, V., Emslie, K., Garson, J.A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R.D., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., Bustin, S.A., 2013. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. *Clin. Chem.* 59, 892–902. <https://doi.org/10.1373/clinchem.2013.206375>.

Jungersen, M., Wind, A., Johansen, E., Christensen, J., Stuer-Lauridsen, B., Eskesen, D., 2014. The science behind the probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12<sup>®</sup>. *Microorganisms* 2, 92–110. <https://doi.org/10.3390/microorganisms2020092>.

Klose, V., Mohnl, M., Plail, R., Schatzmayr, G., Loibner, A.P., 2006. Development of a competitive exclusion product for poultry meeting the regulatory requirements for registration in the European Union. *Mol. Nutr. Food Res.* 50, 563–571. <https://doi.org/10.1002/mnfr.200500166>.

Lin, C.T.M., Leibovitch, E.C., Almira-Suarez, M.I., Jacobson, S., 2016. Human herpesvirus multiplex ddPCR detection in brain tissue from low- and high-grade astrocytoma cases and controls. *Infect. Agent. Cancer* 11, 1–10. <https://doi.org/10.1186/s13027-016-0081-x>.

Memon, A.A., Zöllner, B., Hedelius, A., Wang, X., Stenman, E., Sundquist, J., Sundquist, K., 2017. Quantification of mitochondrial DNA copy number in suspected cancer patients by a well optimized ddPCR method. *Biomol. Detect. Quantif.* 13, 32–39. <https://doi.org/10.1016/j.bdq.2017.08.001>.

Mountzouris, K.C., Tsiroskos, P., Palamidi, I., Arvaniti, A., Mohnl, M., Schatzmayr, G., Fegeros, K., 2010. Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. *Poult. Sci.* 89, 58–67. <https://doi.org/10.3382/ps.2009-00308>.

Norin, K.E., Persson, A.K., Saxerholt, H., Midtvedt, T., 1991. Establishment of *Lactobacillus* and *Bifidobacterium* species in germ-free mice and their influence on some microflora-associated characteristics. *Appl. Environ. Microbiol.* 57, 1850–1852. <https://doi.org/10.1001/jama.2014.17841>.

Paveljšek, D., Juvan, P., Košir, R., Rozman, D., Hacin, B., Ivicač-Kocjan, K., Rogelj, I., 2018. *Lactobacillus fermentum* L930BB and *Bifidobacterium animalis* subsp. *Animalis* IM386 initiate signalling pathways involved in intestinal epithelial barrier protection. *Benef. Microbes* 9, 515–525. <https://doi.org/10.3920/BM2017.0107>.

Pinheiro, L.B., Coleman, V.A., Hindson, C.M., Herrmann, J., Hindson, B.J., Bhat, S., Emslie, K.R., 2012. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Anal. Chem.* 84, 1003–1011. <https://doi.org/10.1021/ac202578x>.

Sattler, V.A., Mohnl, M., Klose, V., 2014. Development of a strain-specific real-time PCR assay for enumeration of a probiotic *Lactobacillus reuteri* in chicken feed and intestine. *PLoS One* 9. <https://doi.org/10.1371/journal.pone.0090208>.

Selvaraj, V., Maheshwari, Y., Hajeri, S., Chen, J., McCollum, T.G., Yokomi, R., 2018. Development of a duplex droplet digital PCR assay for absolute quantitative detection of “*candidatus liberibacter asiaticus*”. *PLoS One* 13, 1–16. <https://doi.org/10.1371/journal.pone.0197184>.

Srisutham, S., Saralamba, N., Malleret, B., Réna, L., Dondorp, A.M., Imwong, M., 2017. Four human *Plasmodium* species quantification using droplet digital PCR. *PLoS One* 12, 1–16. <https://doi.org/10.1371/journal.pone.0175771>.

Sugahara, H., Odamaki, T., Fukuda, S., Kato, T., Xiao, J.Z., Abe, F., Kikuchi, J., Ohno, H., 2015. Probiotic *Bifidobacterium longum* alters gut luminal metabolism through modification of the gut microbial community. *Sci. Rep.* 5, 1–11. <https://doi.org/10.1038/srep13548>.

Weerakoon, K.G., Gordon, C.A., Gobert, G.N., Cai, P., McManus, D.P., 2016. Optimisation of a droplet digital PCR assay for the diagnosis of *Schistosoma japonicum* infection: a duplex approach with DNA binding dye chemistry. *J. Microbiol. Methods* 125, 19–27. <https://doi.org/10.1016/j.mimet.2016.03.012>.

Witte, A.K., Fister, S., Mester, P., Schoder, D., Rossmanith, P., 2016a. Evaluation of the performance of quantitative detection of the *Listeria monocytogenes* prfA locus with droplet digital PCR. *Anal. Bioanal. Chem.* 408, 7583–7593. <https://doi.org/10.1007/s00216-016-9861-9>.

Witte, A.K., Mester, P., Fister, S., Witte, M., Schoder, D., Rossmanith, P., 2016b. A systematic investigation of parameters influencing droplet rain in the *Listeria monocytogenes* prfA assay - reduction of ambiguous results in ddPCR. *PLoS One* 11, 1–13. <https://doi.org/10.1371/journal.pone.0168179>.

Yan, F.F., Murugesan, G.R., Cheng, H.W., 2018. Effects of probiotic supplementation on performance traits, bone mineralization, cecal microbial composition, cytokines and corticosterone in laying hens. *Animal* 1 (9). <https://doi.org/10.1017/S175717311800109X>.