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Underrecognized niche of spore-forming bacilli as a nitrite-producer isolated from the processing lines and end-products of powdered infant formula

Tae Jin Cho, Min Suk Rhee*

Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul, 02841, South Korea

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ABSTRACT

Although nitrite in powdered milk formula (PIF) is a recognized health risk for infants, the presence of nitrite in PIF has only been investigated as a chemical contaminant during the inspection of end-products. The risk posed by microbial sources of nitrite during the PIF manufacturing process has not been considered. This is the first study to report the taxonomy and physiological characteristics of nitrite-producing bacteria isolated from PIF processing environments. All isolates identified as nitrite-producers (133 out of 501 strains collected over four years) from work-in-process and end-products of PIF were spore-forming bacilli. Nitrite-producing metabolism under PIF processing conditions was found in not only thermophilic isolates (3 *Bacillus*, 60 *Geobacillus* from 63 strains; 100%) but also in mesophilic isolates (65 *Bacillus*, 1 *Anoxybacillus* from 70 strains; 65.7%). *Geobacillus* was the only highly heat-resistant sporeformer and vigorous nitrite-producer exhibiting dramatic increases in nitrite over short periods of incubation (a maximum value within 3 h). High conversions of nitrate to nitrite (up to 88.8%) was also observed, highlighting bacteria as a key source of nitrite in PIF processing lines. Further research into the diversity of metabolic activity observed in this study can facilitate specialized management of nitrite-producers in PIF processing lines.

1. Introduction

Nitrite has been regarded as a cause of the fatal infant disease methemoglobinemia, the so called 'blue baby syndrome', in which symptoms of cyanosis and convulsions are apparent (Gapper et al., 2004; Singh et al., 2017). Nitrite either directly ingested or produced by the reduction of nitrate in the human body can convert fetal hemoglobin to methemoglobin, which is incapable of oxygen delivery and associated with infant enteritis (ATSDR, 2013; Pun et al., 2016). Infant death and/or hospitalization incidents linked to excessive intake of nitrate/nitrite have raised concerns about the detrimental effects of contaminated foods (Montague-Jones, 2011; Vuong et al., 2016). The Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) and the European Commission's Scientific Committee on Food (EC-SCF) have set an acceptable daily intake (ADI) of nitrate and nitrite as 0–3.7 and 0–0.07 mg kg^{-1} bw day^{-1}, respectively (Speijers and Van den Brandt, 2003).

Besides breast-feeding, most infants depend on powdered infant formula (PIF) for food (Kim et al., 2011). This potentially exposes infants to deleterious contaminants in PIF (e.g., pathogenic microorganisms, heavy metals, veterinary drug residues, etc.) (Choi et al., 2013; Ha and Kang, 2014; Sorbo et al., 2014; Zhan et al., 2013). Milk powder products can be naturally contaminated with nitrate and/or nitrite from raw materials, manufacturing environments, and nitric acid used as cleaning agent in processing lines (Yeh et al., 2013). In China, the regulatory upper limits for nitrate and nitrite in PIF are set at 100 and 2 ppm (mg kg^{-1} PIF), respectively (MOH, 2010), and recalls resulting from excessive levels of nitrate/nitrite have been reported (Aras, 2015; CFDA, 2015). However, the current management system for nitrite in PIF is limited to the testing of raw materials and/or end-products samples without any preventative measures (Erkekoglu and Baydar, 2009; Odhiambo, 2014). Since nitrite, a natural contaminant of the external environment, is perceived as an uncontrollable chemical risk, the impact of nitrite formation by bacteria within PIF processing lines has been disregarded.

From the viewpoint of nitrite as a metabolite, we focused on examining for the presence and activity of nitrite-producing bacteria in manufacturing environments and re-evaluated the current paradigm of nitrite management to consider microbiological inputs during the various processing steps of PIF production. Nitrate in nature is an electron acceptor that can result in the nitrite production (Vavilin and Rytov, 2015) during bacterial respiration. Since this reaction [i.e., reduction of nitrate

* Corresponding author. Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, 145, Anam-ro, Seongbuk-gu, Seoul, 02841, South Korea.
E-mail address: rheems@korea.ac.kr (M.S. Rhee).

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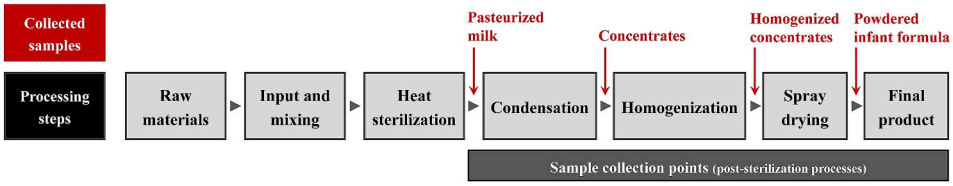

Fig. 1. Flow chart of powdered infant formula processing steps and sample collection points.

(NO_3^-) to nitrite (NO_2^-)] is a common step in the metabolic pathways of the nitrogen cycle (e.g., denitrification and dissimilatory reduction to ammonium), previous research on nitrate-reducing microorganisms has focused on the biosphere (e.g. soil and marine environments) (Gao et al., 2017; Kraft et al., 2011; Morse et al., 2015). In the case of the dairy industry, there has been speculation that raw materials may represent a source of bacteria with nitrate-reducing activity (Lima et al., 2006; Woollard and Indyk, 2014). Bacterial contamination of raw materials and manufacturing environments is regarded as a major factor affecting PIF quality (André et al., 2017; Kim et al., 2010), but the nitrate-reducing activity of microorganisms in processing lines has only rarely been considered, although it would provide information on the origin and fate of nitrite during the manufacture of PIF.

We previously reported the microbiological compositions of materials along PIF processing lines and the growth temperature optima (mesophilic, thermophilic) and spore-forming ability (total bacterial and spore counts) of these microorganisms (Cho et al., 2018). Heat-sterilization was found not to completely inactivate these bacteria and thermophiles were not affected by any process. A predominance of endospores indicated that heat-resistant spore supported the bacterial survival which could result in the growth of survived spore-formers in post-sterilization steps (condensation, homogenization) (Flint et al., 2001; Hill and Smythe, 2012; Yuan et al., 2012). Other research reported germination and vegetative growth of spore-formers during the manufacturing process of powdered milk. Thermophilic bacteria grow under temperatures that are typically used during post-sterilization steps (55–65 °C) and can result in a high concentration of microbiota (up to 10^6 CFU/g) in the end-product (Burgess et al., 2010; Karaman and Alvarez, 2014; Scott et al., 2007). In this regard, we hypothesized that spore-formers capable of nitrate-respiration during post-sterilization steps would result in nitrite in PIF products. However, previous research on the physiological characterization of bacteria capable of deteriorating the quality and safety of dairy products only focused on spoilage and infectious diseases (Chen et al., 2003; Dancer et al., 2009; Lücking et al., 2013; Sadiq et al., 2016). Little attention has been paid to changes in nitrite concentration during PIF processing. The extent of nitrite production from bacterial metabolic activity during PIF processing has never been estimated because no investigations of nitrite-producing bacteria in manufacturing environments have been performed.

Here we examined the major microbial contaminants of PIF (mesophilic and thermophilic bacteria) for nitrate/nitrite respiration. Mesophilic and thermophilic bacteria were isolated from work-in-process (WIP) products at post-sterilization steps, as well as in end-product samples to identify and characterize each isolate in axenic cultures with respect to nitrite production and heat resistance. Based on key findings regarding the microbial sources of nitrite production within the manufacturing process, we address key factors for the establishment of measures to control nitrite formation during PIF manufacture and decrease reliance on end product testing.

2. Materials and methods

2.1. Source of isolates

A total of 501 bacterial isolates from WIP (three processing plants) and PIF end-products (53 products) collected over four years (2013–2017) were included in this study. Isolates were kindly provided from a biobank managed by Maeil Dairies, Co., Ltd (Pyeongtaek, Korea). To differentiate the potential for bacterial growth during PIF processing steps and to exclude heat-susceptible bacteria that are easily inactivated by heat treatments, raw materials and WIP products at steps preceding heat-treatment were not used for isolation purposes. Samples were therefore those taken from WIP intermediate processing steps (pasteurized products, concentrates, and homogenized concentrates) and end-products (Fig. 1).

Mesophilic and thermophilic isolates were obtained as follows. Samples (25 g and 25 ml for solid-phase and liquid-phase samples, respectively) were each placed in a stomacher bag (Circulator 400 standard bags, Seward, Worthing, UK) with 225 ml sterile 0.85% (w/v) saline and homogenized using a stomacher (Circulator 400, Seward) operating at 230 rpm for 2 min. One milliliter of homogenized sample was then transferred to 9 ml sterile 0.85% saline for serial 10-fold dilution. One hundred microliters of dilutions were then spread-plated on plate count agar (PCA, Difco, Sparks, MD, USA) plates and incubated at 37 °C and 55 °C for 48 h to differentiate mesophilic and thermophilic isolates, respectively. Single colonies with distinct morphologies on PCA were randomly transferred to tryptic soy agar (TSA, Difco) plates and incubated under the conditions described above. Then single colonies from sub-streaked TSA plates were propagated in 5 ml tryptic soy broth (TSB, Difco) (37 °C, 48 h for mesophilic isolates and 55 °C, 48 h for thermophilic isolates) and mixed with 50% glycerol solution for storage at −80 °C until further characterization.

2.2. Isolation and identification of nitrate-reducing bacteria

2.2.1. Initial screen of isolates metabolizing nitrate during bacterial growth

Stock suspensions (50 μl) of mesophilic and thermophilic isolates were incubated in 4.95 ml TSB at 37 °C for 24 h and at 55 °C for 48 h, respectively. Cultures were centrifuged at 15,814 × *g* for 3 min using a micro-centrifuge (Smart R17 plus; Hanil Scientific Inc., Korea). The pellets were washed twice and re-suspended in sterile 0.85% saline. Then 50 μl suspensions of each isolate were inoculated in nitrate broth (TSB supplemented with 100 ppm KNO_3) and incubated under the conditions described above. After incubation, 1 ml culture was centrifuged (15,814 × *g*, 3 min) to separate particulate matters in the sample. The supernatant (50 μl) was tested for the formation of nitrite using the Griess reagent system (Promega, Madison, WI, USA) according to the manufacturer's instructions (Saleh-Lakha et al., 2008). Reaction of nitrite with sulfanilamide and N-1-napthylethylenediamine dihydrochloride solution results in the production of a purple/magenta color. To avoid false-negative results, zinc powder, which immediately reduces nitrate to nitrite, was added to all negative samples and when no color change occurred, the nitrite produced from nitrate was considered to have been reduced further to nitrogen oxides. In both cases (color change or complete exhaustion of nitrate and nitrite in the sample), nitrate-reducing organisms were considered to be responsible.

2.2.2. Identification of nitrate-reducing bacteria

Genetic (16S rRNA sequencing) and biochemical-based (VITEK2 system) methodologies were used for identification. All isolates were cultivated in TSB under appropriate conditions (48 h at 37 °C for mesophilic isolates and 48 h at 55 °C for thermophilic isolates) and then streaked onto TSA. After incubation, representative colonies were collected by suspension in 0.85% sterile saline.

For genetic identification, the 16S rRNA gene in DNA extracted from a colony suspension was amplified with universal primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), 1492R (5′-GGTTACCTTGTTACGA CTT-3′), 518F (5′-GTATTACCGCGGCTGCTGG-3′), 805R (5′-GACTACC AGGGTATCTAATC-3′), and 785F (5′-GGATTAGATACCCTGGTA-3′). The sequencing reaction was performed using BigDye Terminator v3.1 cycle sequencing Kits (Applied Biosystems, CA, USA), and the data obtained were analyzed using the ABI 3730XL DNA Analyzer (Applied Biosystems).

Depending on the results of the genetic analysis, the VITEK2 system (bioMérieux, Marcy l'Étoile, France) was used for bacterial identification according to the manufacturer's instructions using appropriate conditions for the genus of each isolate.

2.2.3. Phylogenetic analysis of nitrate-reducing bacteria

16S rRNA coding sequences were aligned using the CLUSTAL W program and analyzed using BLASTn to find homologous sequences in the NCBI database. Reference strains used for phylogenetic analysis and their GenBank accession numbers are listed in Supplementary Table 1. Phylogenetic trees were constructed using the Maximum Likelihood method based on the Kimura 2-parameter model using MEGA 7.0 software (Kumar et al., 2016). *Alicyclobacillus acidocaldarius* subsp. rittmannii DSM 11297 (GenBank accession: NR_040874.1) was used as the outgroup. Evaluation of the statistical strengths of the branches in the trees was conducted by 1000 replicated bootstrap analysis. The percentage of trees in which the associated taxa clustered together is presented next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and selecting the topology with the superior log likelihood value. The tree was drawn to scale, with branch lengths measured by the number of substitutions per site. Positions containing gaps and missing data were eliminated.

2.3. Characterization of nitrate-reducing bacteria

To estimate the ability of nitrate-reducing bacteria obtained in Section 2.2 to produce nitrite during PIF manufacture, the nitrite profile of each isolate cultivated at typical operating temperatures of post-sterilization processing steps (e.g. 55–65 °C during condensation, homogenization, and storage in buffer tank) (Burgess et al., 2010; Karaman and Alvarez, 2014; Scott et al., 2007) and spore heat-resistance were evaluated. Results were obtained from at least six replicates for each strain.

2.3.1. Analysis of nitrite production and consumption patterns

Bacterial suspensions of nitrate-reducing isolates were prepared using the methods described in Section 2.2.1 [i.e., incubation in TSB followed by centrifugation and re-suspension]. Suspensions (50 μl) were then inoculated into nitrate broth and incubated at 55 and 65 °C. After 6, 12 and 24 h, Griess tests were conducted to detect the presence of nitrate and/or nitrite. The pattern of production and consumption of nitrite was recorded according to the results from the Griess test, i.e. case 1 was defined as the occurrence of complete consumption of nitrate and nitrite, case 2 was defined as the production of nitrite during growth.

2.3.2. Spore-forming ability and spore heat-resistance

Bacterial suspensions (200 μl) prepared according to Section 2.3.1 were spread onto sporulation agar (nutrient agar supplemented with 1 μg/ml of Mn^{2+}) (Difco) followed by incubation at 37 °C for 5 days (mesophilic isolates) or 55 °C for 7 days (thermophilic isolates) (Furukawa et al., 2009; Watanabe et al., 2003). Following incubation, cultures were observed by microscopy (Eclipse E200, Nikon, Tokyo, Japan) to ensure that more than 90% of the cells had sporulated. Spores were harvested into 5 ml of cold sterile distilled water (DW) by using a sterile loop to scrape growth from the surface of agar plates. One milliliter of harvested spores was centrifuged (15,814 × *g*, 3 min) and the spore pellet was successively washed twice, followed by resuspension in DW (Kim et al., 2014). To inactivate vegetative cells, the spore suspension were subjected to heat treatment at 80 °C for 15 min and spore survival was examined by the spread-plate method (Reich et al., 2017).

Highly heat-resistant spores were defined as those surviving a temperature of 100 °C for 20 min (excluding spores from isolates producing less than five colonies on a plate) (Lücking et al., 2013; Witthuhn et al., 2011). Spore suspensions were transferred to tubes and diluted in DW to adjust their concentration to 2–3 $\log_{10}$ CFU/ml. After heat treatment in an autoclave (100 °C, 20 min), the suspension was immediately cooled in an ice bath and spread-plated on TSA. Mesophilic and thermophilic isolates were incubated at 37 °C and 55 °C for 24–48 h, respectively.

2.4. Quantitative assay of the nitrite profile

According to the nitrite production profile, as described in Section 2.3.1, vigorously metabolizing strains exhibiting the distinct phenotypic trait of nitrite production and consumption (case 1: detection of nitrite produced during the growth, case 2: complete consumption of nitrate and nitrite) were used in the assay. These strains showed either the complete consumption of nitrate/nitrite within 6 h, or the presence of nitrite at all sampling points (Tables 2 and 3).

Suspensions of isolates were prepared according to the methods described in Section 2.2.1 and adjusted to 4–5 $\log_{10}$ CFU/ml to reflect the high bacterial load of processing lines reported in previous researches (Cho et al., 2018; Scott et al., 2007; Watterson et al., 2014). Then 50 μl suspensions were inoculated into nitrate broth followed by cultivation at 55 °C and 65 °C. Samples were collected at appropriate time intervals (time-points for the measurement were set according to the results of preliminary tests), immediately transferred to an ice bath for cooling followed by the centrifugation at 15,814 × *g* for 3 min. The resulting supernatants were stored at −80 °C until analyzed (Behrendt et al., 2010). For the quantitative assay, 50 μl of thawed supernatants were sequentially mixed with an equal volume of each Griess reagent, and the concentration of nitrite was quantified by reading the absorbance at 530 nm of the magenta and/or purple colored reaction products. A calibration curve produced using the same media with standard nitrite solution (Promega) was prepared for each assay to account for any background caused by components of the growth medium (i.e. nitrate broth). All experiments were performed in triplicate.

3. Results

3.1. Isolation and identification of nitrate-reducing bacteria

3.1.1. Initial screen for isolates that use nitrate as an electron acceptor

Of the 501 isolates from processing lines and commercial PIF products tested, 133 (70 of 193 mesophilic isolates, and 63 of 308 thermophilic isolates) were identified as being capable of reducing nitrate during growth. Nitrate reduction was demonstrated either by accumulation of nitrite or complete consumption of both nitrate and nitrite. These isolates were adopted as targets for bacterial identification, and characterization for nitrite production/consumption and the formation of heat-resistant endospores.

3.1.2. Identification of nitrate-reducing bacteria

Table 1 shows the overall results of isolate identification categorized according to isolation source. On the basis of biochemical (VITEK2) and genetic-based (16s rRNA sequencing) identification, all of the nitrate-reducing bacteria were grouped into families *Bacillaceae* (*Anoxybacillus*, *Bacillus*, *Geobacillus*) and *Paenibacillaceae* (*Brevibacillus*, *Paenibacillus*). Mesophilic isolates belonged to the genera *Bacillus* (n = 65, 92.9%), *Paenibacillus* (n = 3, 4.3%), *Anoxybacillus* (n = 1, 1.4%) and

Table 1
Identification of nitrate-reducing bacteria categorized by the source of isolation.

Source of isolation		No. of strains analyzed from bacterial identification					
		Mesophilic isolates				Thermophilic isolates	
		Bacillus	*Paenibacillus*	*Brevibacillus*	*Anoxybacillus*	*Geobacillus*	*Bacillus*
Intermediate products (WIP products [a])	Pasteurized products	16	1	1	–	20	–
	Concentrates	4	–	–	–	12	–
	Homogenized concentrates	3	–	–	–	6	–
End-products	PIF [b]	42	2	–	1	22	3
Total		**65**	**3**	**1**	**1**	**60**	**3**

[a] WIP products: work-in-process products.
[b] PIF: powdered infant formula.

Brevibacillus (n = 1, 1.4%) whereas thermophilic isolates were *Geobacillus* (n = 60, 95.2%) and *Bacillus* (n = 3, 4.8%). *Bacillus* and *Geobacillus* were the dominant genera of the mesophilic and thermophilic bacteria, respectively, and were detected in all isolation sources investigated in this study (pasteurized products, concentrates, homogenized concentrates, and PIF end-products).

Fig. 2 shows the phylogenetic trees constructed from the 16S rRNA gene sequences of the mesophilic and thermophilic nitrate-reducing isolates. There were 1268 and 1356 positions in the final dataset for mesophiles and thermophiles, respectively. The phylogenetic tree for the mesophilic isolates delineated four clusters with high bootstrap values, and strains in each cluster showed a high similarity within the same genus; *Paenibacillus* (bootstrap value: 97%), *Brevibacillus* (99%), *Anoxybacillus* (99%), and *Bacillus* (99%). Clusters with *Bacillus* strains were classified into two monophyletic clades with 99% bootstrap support as the *Bacillus cereus* group (i.e., *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis, B. cytotoxicus,* and *B. toyonensis*) (Guinebretière et al., 2013; Jiménez et al., 2013; McIntyre et al., 2008) and *Bacillus subtilis* group (i.e., *Bacillus subtilis* subsp. subtilis, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus mojavensis*, *Bacillus vallismortis*, *Bacillus subtilis* subsp. spizizenii and *Bacillus sonorensis*) (Wang et al., 2007).

In the case of thermophilic isolates, the phylogenetic tree clearly yielded two clusters with 100% bootstrap support. All isolates in the large cluster (n = 60) formed a monophyletic clade with the type strain of *G. stearothermophilus* (NCBI GenBank accession number: NR_115284.2), while the isolates in the small cluster (n = 3) were similar to the type strain of *B. coagulans* (NR_041523.1).

3.2. Characterization of nitrite-producing bacteria: nitrate/nitrite production and consumption, formation of highly heat-resistant spore

Overall results are shown in Tables 2 and 3 for mesophilic and thermophilic isolates, respectively. Nitrite was produced as a transiently accumulated metabolite (resulting from the depletion of all nitrite from the medium) or as a residual product within 24 h of incubation. Thermal resistance of the spores was categorized as either heat-resistant or highly heat-resistant.

3.2.1. Mesophilic isolates

Majority of mesophilic isolates (45 out of total 70 strains, 64.3%; Table 2) belonged to the genus *Bacillus* and showed complete consumption of both nitrate and nitrite at 55 °C, whereas nitrate/nitrite metabolism was not observed at 65 °C. For 82.2% (37 out of 45 strains) of these strains, nitrite was detected within 6 h of incubation. The other 20 *Bacillus* isolates were incapable of performing nitrate-nitrite conversion at either temperature. One *Anoxybacillus* isolate (strain ID: FHS-PPAM212) was the only one in which nitrate to nitrite reduction occurred at 65 °C, and it showed different patterns of nitrate reduction at each temperature (i.e. Complete consumption of nitrate and nitrite occurred within 6 h incubation at 55 °C, while at 65 °C residual nitrite could be detected at any time-point). None of the isolates identified as *Brevibacillus* and *Paenibacillus* produced nitrite at either 55 or 65 °C.

All isolates (*Anoxybacillus*, *Bacillus*, *Brevibacillus*, *Paenibacillus*) produced heat-resistant endospores. Whereas highly heat-resistant spores were detected from only *Bacillus* (n = 5), but nitrite-production by those isolates was unavailable.

3.2.2. Thermophilic isolates

Three *Bacillus* isolates out of 63 (4.8%, Table 3) converted nitrate to nitrite at 55 °C only, whereas all *Geobacillus* strains (n = 60; 95.2%) were capable of nitrate reduction at 55 and 65 °C, with the effect of temperature on metabolic activity being strain-dependent. The majority of *Geobacillus* strains (52 out of total 60 strains; 86.7%) completely consumed nitrate and nitrite during incubation at 55 and/or 65 °C. Whereas the remaining eight *Geobacillus* strains could produce nitrite from nitrate, but residual nitrite was detected from all trials within 24 h of incubation.

Five isolates of *Geobacillus* showed evidence of rapid nitrate reduction within 6 h incubation at 55 and 65 °C. Complete consumption of nitrate and nitrite occurred with *Geobacillus* sp. FHS-PHGT51 and FHS-PCGT429 isolated from homogenized concentrates, while nitrite was produced but not metabolized further by *Geobacillus* sp. strains FHS-PPGT111 and PCGT134 isolated from pasteurized products, or by FHS-PPGT130 isolated from concentrates. These vigorously metabolizing bacteria were used in the quantitative assay as described in Section 3.3.

All isolates of *Geobacillus* produced highly heat-resistant endospores, whereas endospores from all isolates of *Bacillus* were heat-resistant but could not survive the conditions meeting the definition of highly heat-resistant spores (i.e. inactivated after exposure to 100 °C for 20 min).

3.3. Nitrite profile of vigorously metabolizing bacteria

The nitrite metabolizing profile of *Geobacillus* strains with vigorous nitrate/nitrite-metabolizing activity is shown for those exhibiting complete consumption of nitrate/nitrite (Fig. 3a and b) and those where residual nitrite could be detected (Fig. 3c and d).

Isolates exhibiting the case 1 phenotype rapidly produced and then consumed nitrite. *Geobacillus* sp. FHS-PCGT429 initiated nitrite production after 225 min (37 μM) at 55 °C and the level increased to a peak value of 881 μM after 285 min, which was followed by a decrease to an undetectable concentration at 360 min. The initiation of nitrite production and its subsequent metabolism proceeded faster at 65 °C than at 55 °C, with the peak nitrite concentration occurring at 165 min and then decreasing to an undetectable concentration at 210 min.

A similar pattern was observed for *Geobacillus* sp. FHS-PHGT51. Nitrite production was first detected at 270 min (46 μM), and then sharply increased from 299 to 655 μM between 285 and 315 min. Nitrite was detected up until 375 min. In the case of incubation at 65 °C, nitrite production was first detected after 135 min at 19 μM with a

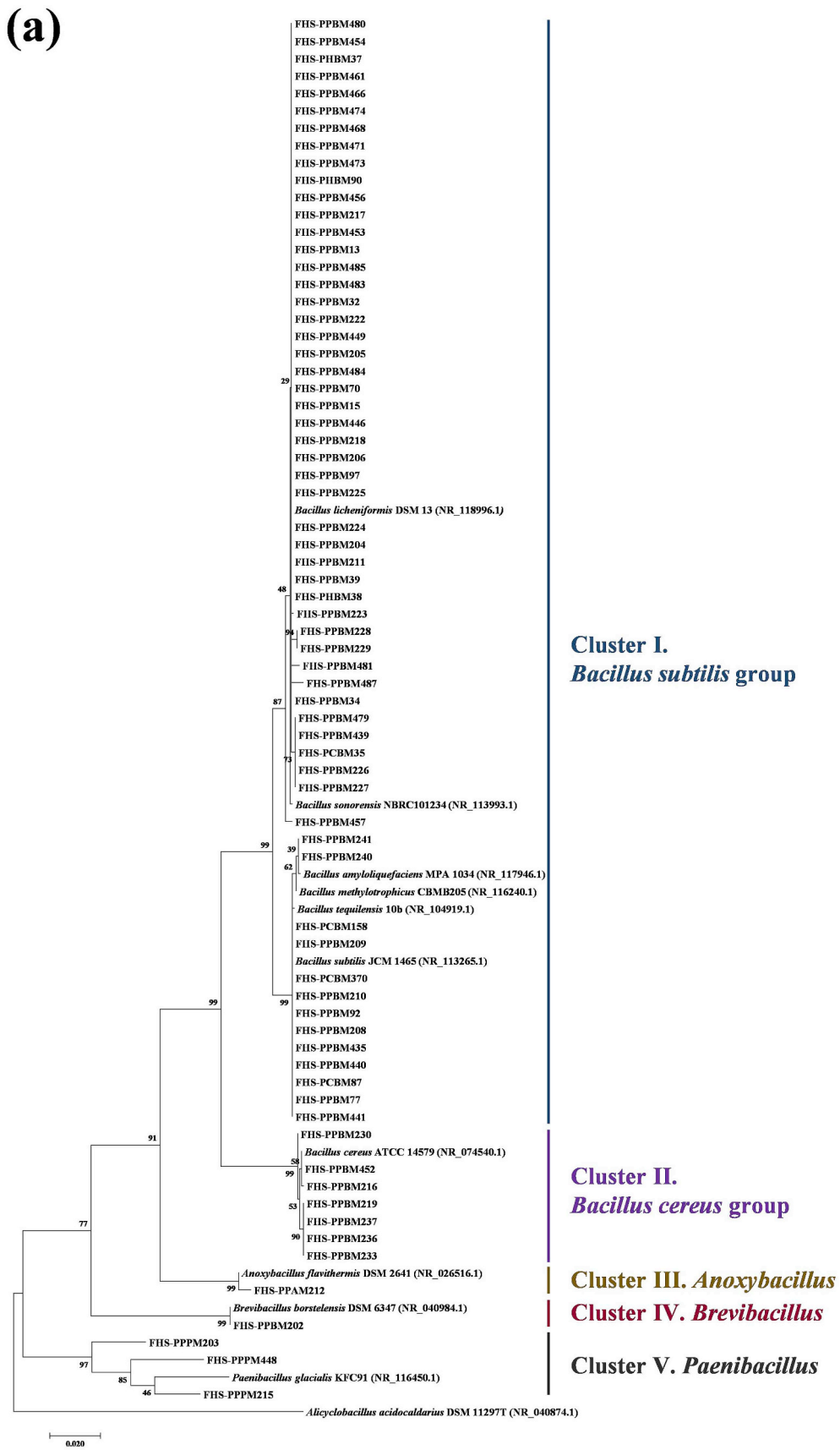

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Fig. 2. Phylogenetic trees of nitrate-reducing bacteria based on the 16S rRNA gene sequences: (a) mesophilic isolates (n = 70), (b) thermophilic isolates (n = 63). The tree was constructed by using the Maximum Likelihood method based on the Kimura 2-parameter model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The numbers at the branches are bootstrap confidence percentages (based on 1000 replications). *Alicyclobacillus acidocaldarius* subsp. rittmannii DSM 11297 (GenBank accession: NR_040874.1) was used as the outgroup. Bar, % estimated substitutions per nucleotide position. GenBank accession numbers are indicated in parentheses. Sequences of the strain from the species which showed the highest sequence similarity with bacterial isolates analyzed in this study were obtained from NCBI GenBank and were also indicated in each phylogenetic tree: *Anoxybacillus flavithermus* DSM 2641 (NCBI GenBank accession number: NR_026516.1), *Bacillus amyloliquefaciens* MPA 1034 (NR_117946.1), *Bacillus cereus* ATCC 14579 (NR_074540.1), *Bacillus licheniformis* DSM 13 (NR_118996.1), *Bacillus methylotrophicus* CBMB205 (NR_116240.1), *Bacillus sonorensis* NBRC 101234 (NR_113993.1), *Bacillus subtilis* JCM 1465 (NR_113265.1), *Bacillus tequilensis* ATCC BAA-819 (NR_104919.1), *Brevibacillus borstelensis* DSM 6347 (NR_040984.1), and *Paenibacillus glacialis* DSM 22343 (NR_116450.1).

further increase to 697 μM at 240 min. The concentration then sharply decreased to 245, 139, and 22 μM at 255, 270, 285 min, respectively, before becoming completely undetectable at 300 min.

In terms of isolates with the case 2 phenotype, identical values for the rate of nitrite production were observed, but the consumption of nitrite by *Geobacillus* sp. strains FHS-PHGT51 and FHS-PCGT429 was relatively slow. The effect of temperature on metabolic activity was strain-dependent (FHS-PPGT1111: 65 °C > 55 °C; FHS-PPGT130: 65 °C > 55 °C; FHS-PPGT134: 55 °C > 65 °C). *Geobacillus* sp. FHS-PPGT1111 accumulated nitrite in the medium to 33–852 μM after 270–330 min incubation with a decrease in nitrite concentration to 275 μM between 360 and 450 min. Concentrations varied between 116 and 256 μM from 480 min onwards. Incubation at 65 °C showed a similar pattern with an immediate increase to 878 μM occurring after 300 min followed by a decrease to 450 μM after 360 min. From 390 to 720 min, the nitrite concentration gradually decreased from 408 to 126 μM. In the case of *Geobacillus* sp. FHS-PPGT130 incubated at 55 °C, the nitrite concentration increased over the period of 210–330 min and then gradually decreased to 176 μM after 720 min. Incubation at 65 °C also produced a rapid increase to 837 μM after 270 min and then a decrease to 347 μM after 360 min, resulting in a sustained concentration of nitrite of 223–301 μM from 390 to 720 min. *Geobacillus* sp. FHS-PPGT134 had a peak value as 838 μM at 300 min with the first appearance of nitrite (23 μM) occurring at 210 min. The nitrite concentration then decreased to 169 μM after 720 min. A delay in the initiation of nitrate production at 65 °C was observed, with relatively low nitrite concentrations of 17–77 μM being measured between 300 and 390 min. The nitrite concentration then increased to reach 658 μM and 839 μM at 420 and 450 min respectively, and then gradually decreased to 386 μM after 720 min.

4. Discussion

Beyond the generally recognized microbiological issues associated with bacterial growth during the manufacture of PIF (e.g., off-flavor, discoloration, curdling, etc.) (André et al., 2017; Machado et al., 2017), this study reveals the potential niche of spore-forming bacteria as nitrite-producer in PIF processing lines. Our previous research revealed that spore-formers dominate the bacterial community of PIF processing lines (Cho et al., 2018), and the present study identified them as being capable of nitrite production under PIF processing conditions. Since bacterial metabolism during post-sterilization steps prior to the drying process can result in the accumulation of residual nitrite in the end-product, interventions designed for processing lines are required to prevent this occurring. We obtained a set of bacterial nitrite-producers and found that each genus had distinctive characteristics (i.e. nitrate/nitrite metabolism and spore heat-resistance). Although metabolic activity varied among isolates within the same genus, phenotypic groups were identified with different patterns of nitrite production and consumption.

The characteristics of major genera of nitrite-producing mesophilic and thermophilic isolates with the ability to grow at the temperatures of general post-sterilization processes (55–65 °C) are shown in Tables 2 and 3, respectively. While *Bacillus* strains could be identified among thermophilic isolates (3 out of 63 strains) (Table 3), the majority were mesophilic isolates (65 out of 70 strains) (Table 2). However, most of them (48 out of 68 strains) only displayed nitrate-reducing metabolism at temperatures under 55 °C with no metabolic activity at 65 °C, confirming that 55 and 65 °C is their maximal temperature growth range. This suggests that, although previous taxonomic studies differentiated and re-classified thermophilic isolates of the genus *Bacillus* into new taxa (e.g. *Anoxybacillus flavithermis*, *Geobacillus stearothermophilus*, and *Geobacillus thermoleovorans* previously assigned to *Bacillus*) (Nazina et al., 2001; Pikuta et al., 2000), moderately thermophilic *Bacillus* spp. should be regarded as nitrite-producers in post-sterilization processes operating within their growth temperatures (Burgess et al., 2010). *Anoxybacillus*, a thermophilic genus with a broad range of growth temperatures (30–72 °C) (Pikuta et al., 2000), was the only strain identified among the mesophilic isolates that exhibited nitrate/nitrite metabolism at both 55 °C and 65 °C (Table 2). In the case of *Paenibacillus* and *Brevibacillus*, the inability to produce nitrite at elevated temperatures (55–65 °C) is consistent with previous studies reporting their temperature growth ranges (Aw et al., 2016; Li et al., 2014; Logan et al., 2004; Shida et al., 1995), and thus nitrate-nitrite conversion by members of this genus is expected to be inhibited by processes (Karaman and Alvarez, 2014; Kim et al., 2017). Members of the genus *Geobacillus* comprised most thermophilic isolates (60 out of 63 strains) and all could reduce nitrate at 55 °C and 65 °C (Table 3).

Highly heat-resistant spores were detected in only five mesophilic *Bacillus* isolates (7.1%) but they could not reduce nitrate between 55 and 65 °C (Table 2). All *Geobacillus* strains (95.2% of thermophilic isolates) formed highly heat-resistant spores (Table 3), demonstrating the potential for a high prevalence of spore-formers in PIF processing steps (Gopal et al., 2015; Seale et al., 2012; Stoeckel et al., 2016; Watterson et al., 2014). Moreover, the growth of *Geobacillus* in post-sterilization stages of milk powder production (e.g., plate heat exchangers, evaporator preheaters, and buffer tanks) has been recognized as a major concern (Flint et al., 2001; Hill and Smythe, 2012). We also found that *Geobacillus* displayed nitrite production and high thermal resistance, however, the prevalence of *Geobacillus* in PIF processing environments might have been underestimated due to its thermophilic nature. Since *Geobacillus* is an obligate thermophile and identified only in thermophilic isolates (Table 1), its population cannot be estimated by general microbiological inspection methods (i.e., aerobic plate counts). To identify and quantify major nitrite-producers, additional inspections for thermophilic bacteria should be adopted.

Analysis of the nitrite profiles along with bacterial growth showed distinct differences with respect to the fate of nitrate (i.e., depletion of both nitrate and nitrite, or residual nitrite), demonstrating variability in the extent of nitrite production and consumption across strains. Vigorously metabolizing *Geobacillus* strains clearly belonged to the two phenotypic groups (Table 3); *Geobacillus* sp. strain FHS-PHGT51 and PCGT429 completely consumed both nitrate and nitrite, whereas FHS-PPGT111, PPGT130, and PCGT134 showed only nitrite production and persistence within the analysis period (i.e., 6–24 h). The results of the quantitative assay (Fig. 3) reflect the differences in nitrite consumption between groups. Since complex growth medium (TSB supplemented with nitrate) was used to promote metabolic activity (Verbaendert

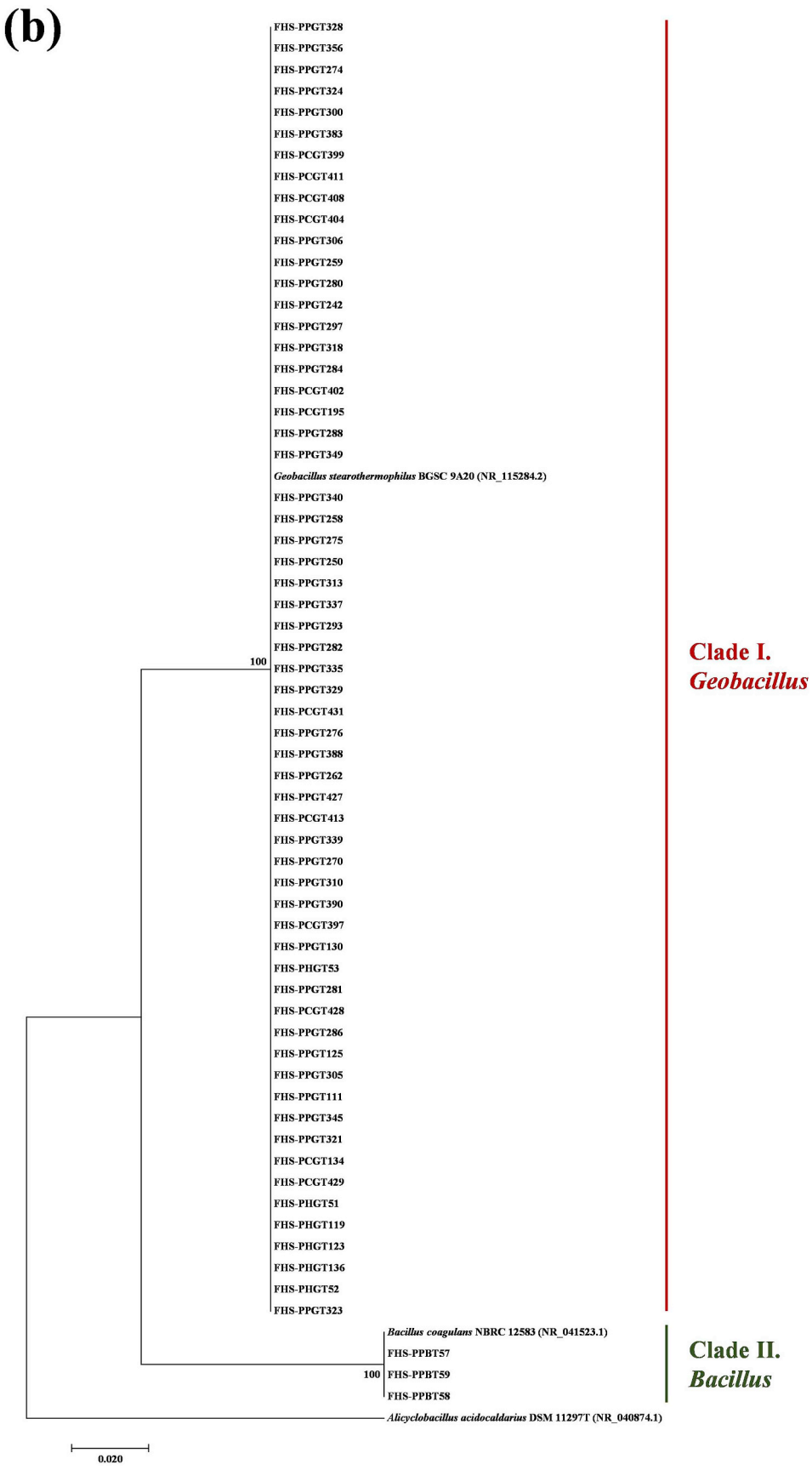

Fig. 2. (*continued*)

Table 2
Characterization of nitrate/nitrite metabolism and spore heat-resistance of mesophilic isolates.

Bacterial species	Characterization of isolates							Number of strains
	Production of nitrite from nitrate [a]						Heat resistance of spore [b]	
	55 °C			65 °C				
	6 h	12 h	24 h	6 h	12 h	24 h		
Bacillus sp.	+	CC	CC	–	–	–	HRS	37
	–	CC	CC	–	–	–	HRS	1
	–	–	CC	–	–	–	HRS	7
	–	–	–	–	–	–	HRS	15
	–	–	–	–	–	–	HHRS	5
Anoxybacillus sp.	CC	CC	CC	+	+	+	HRS	1
Brevibacillus sp.	–	–	–	–	–	–	HRS	1
Paenibacillus sp.	–	–	–	–	–	–	HRS	3

[a] +: detection of nitrite, -: no nitrite detection, CC: Complete consumption of nitrate and nitrite during the incubation.
[b] HRS: heat-resistant spore (resistant to heat treatment at 80 °C for 15 min); HHRS: highly heat-resistant spore (resistant to heat treatment at 100 °C for 20 min).

et al., 2011) and rapid production of nitrite was observed in both groups, differences in the extent and rate of nitrite consumption is likely due to metabolic diversity (e.g., denitrification, the dissimilatory reduction of nitrate to ammonia, etc.) between phenotypic groups (Shapleigh, 2013; Vavilin and Rytov, 2015). Comparative analysis of the metabolic pathways between isolates mediating different nitrite reduction patterns should be performed to reveal any variations in metabolism and to discover key functional genes for the gene-based management of nitrite-producers (Liu et al., 2013). Different regulatory modules in a metabolic pathway have been predicted to affect nitrite metabolism profile (Philippot, 2002).

Vigorously metabolizing strains showed a rapid onset of nitrate-nitrite conversion with a high level of nitrite production. In the case of *Geobacillus* sp. strain FHS-PCGT429, nitrite production started at 105 min and nitrite concentration reached 801 μM within 165 min (Fig. 3d). Moreover, up to 878 μM of nitrite was produced at a conversion ratio of 88.8% by *Geobacillus* sp. FHS-PPGT1111. Given that the regulatory upper limit of the ADI for nitrite ($< 0.07\ mg\ kg^{-1}$ bw day^{-1}) is set lower than corresponding limits for nitrate ($< 3.7\ mg\ kg^{-1}$ bw day^{-1}) according to the health risk considerations (MOH, 2010; Speijers and Van den Brandt, 2003), the high conversion ratios observed in this study suggest that nitrite-producing bacteria are a source of nitrite in PIF.

The results of quantitative assays of bacterial nitrite-production have been reported previously. However, the metabolic activities reported in the present study are difficult to compare with those of previous reports because these reports mainly focused on biospheres dominated by mesophiles (e.g., soil, marine, etc.) (Gao et al., 2017; Morse et al., 2015). Whereas studies on thermophiles have been limited to their presence in biological nitrogen removal systems for wastewater (Lopez-Vazquez et al., 2014). The *Geobacillus* strains isolated in this study showed more rapid nitrite production than those of thermophiles reported in previous studies (Gokce et al., 1989; Mishima et al., 2009; Nara et al., 2009; Natcheva and Beschkov, 2003; Yang et al., 2013).

Table 3
Characterization of nitrate/nitrite metabolism and spore heat-resistance of thermophilic isolates.

Bacterial species	Characterization of isolates							Number of strains
	Production of nitrite from nitrate [a]						Heat resistance of spore [b]	
	55 °C			65 °C				
	6 h	12 h	24 h	6 h	12 h	24 h		
Bacillus sp.	+	+	+	–	–	–	HRS	3
Geobacillus sp.	CC	CC	CC	CC	CC	CC	HHRS	2 [c]
	+	CC	CC	CC	CC	CC	HHRS	1
	+	CC	CC	+	CC	CC	HHRS	9
	–	CC	CC	+	CC	CC	HHRS	26
	–	CC	CC	–	CC	CC	HHRS	4
	–	+	CC	+	CC	CC	HHRS	4
	–	+	CC	–	CC	CC	HHRS	1
	+	CC	CC	–	+	CC	HHRS	1
	+	CC	CC	+	+	+	HHRS	1
	–	CC	CC	+	+	+	HHRS	2
	–	+	CC	+	+	+	HHRS	1
	–	+	+	+	+	+	HHRS	1
	–	+	+	–	+	+	HHRS	2
	–	+	+	–	–	+	HHRS	2
	+	+	+	+	+	+	HHRS	3 [d]

[a] +: detection of nitrite, -: no nitrite detection, CC: Complete consumption of nitrate and nitrite during the incubation.
[b] HRS: heat-resistant spore (resistant to heat treatment at 80 °C for 15 min); HHRS: highly heat-resistant spore (resistant to heat treatment at 100 °C for 20 min).
[c] *Geobacillus* sp. strain FHS-PHGT51, FHS-PCGT429.
[d] *Geobacillus* sp. strain FHS-PPGT111, FHS-PPGT130, FHS-PCGT134.

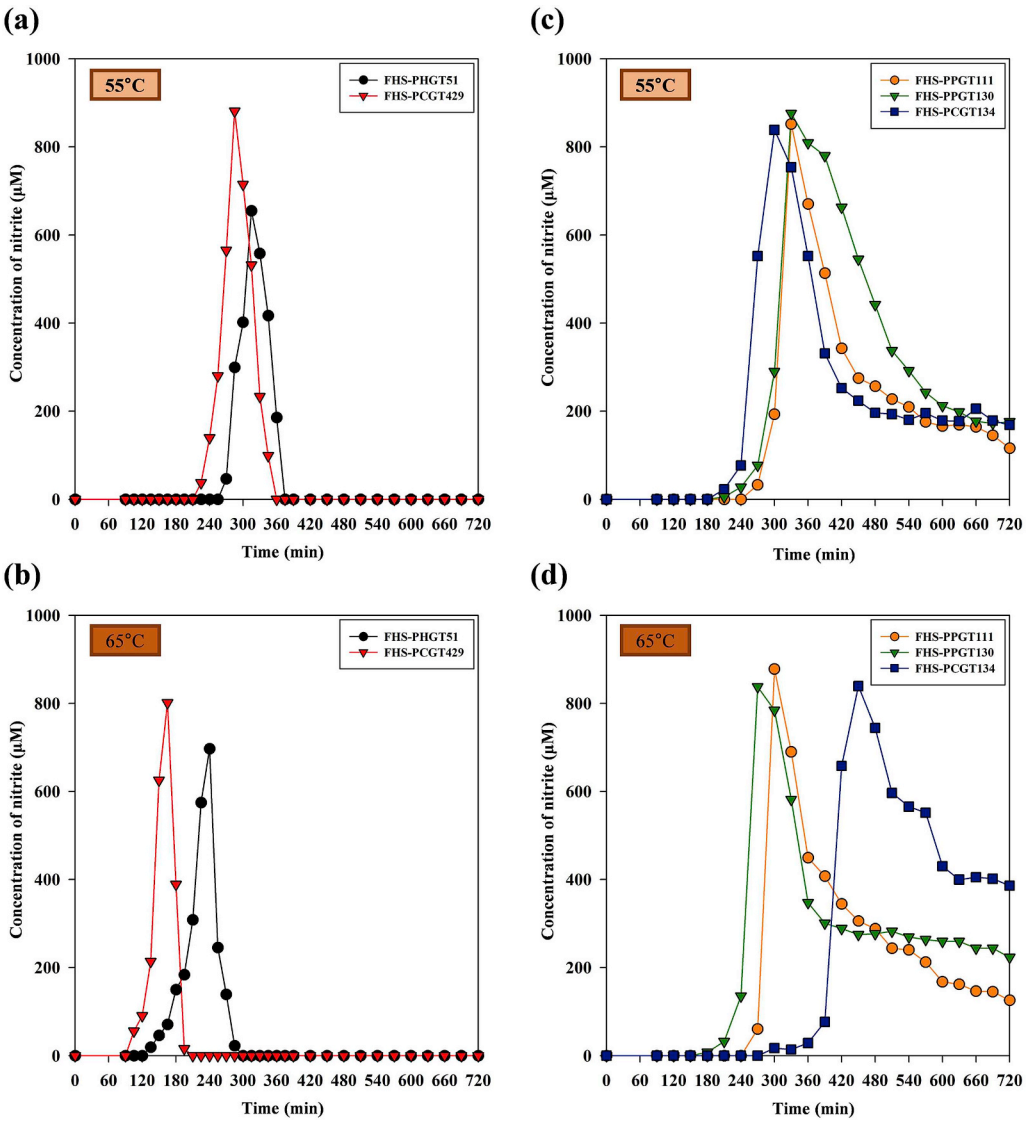

Fig. 3. Nitrite profile representing the nitrite concentration along the incubation of vigorously nitrate/nitrite-metabolizing strains: *Geobacillus* sp. strain FHS-PHGT51 (●), FHS-PCGT429 (▼) under **(a)** 55 °C and **(b)** 65 °C; *Geobacillus* sp. strain FHS-PPGT111 (●), FHS-PPGT130 (▼), and FHS-PCGT134 (■) under **(c)** 55 °C and **(d)** 65 °C. Data shown are average values from triplicate replications of incubations.

Metabolic activities observed in this study were even analogous to those of *Geobacillus* strain artificially mutated to optimize for nitrate-reducing metabolism (Downey et al., 1969). Since rapid nitrite production occurring without any perceptible changes in the product due to bacterial spoilage (color, odor, and viscosity) is likely to be underestimated (Kakagianni et al., 2016), microbes producing nitrite in PIF processing lines should receive at least as much consideration as those causing spoilage. Moreover, complementary research using physiological and genetic analyses is expected to provide clues to which factors are involved in vigorous nitrite-producing activity (Bergaust et al., 2011; Liu et al., 2013).

Since bacterial metabolism occurring at different WIP stages has not previously been considered a source of nitrite, the management of nitrate/nitrite in PIF is generally limited to the inspection of raw materials and/or end-products (Erkekoglu and Baydar, 2009; Gapper et al., 2004; Pistó and Mollo, 2011). Our findings heighten the need for improvements in the current management system of PIF with respect to nitrite. This should involve 1) inspection of raw materials by quantitative tests specific for nitrite-producing bacteria, 2) risk assessment through the simulation of the major processing conditions allowing the growth of nitrite-producers.

Firstly, the inspection of raw materials would help to prevent the introduction of active nitrite-producers into processing lines and keep the population level low to inhibit the onset of nitrite production during PIF processing. Reduction of the population level and/or the substrate for the nitrite production (i.e., nitrate) in raw materials is expected to delay the initiation of nitrate metabolism and to decrease metabolic rates (Bergaust et al., 2011; Cornwell et al., 1999). Our results showed that nitrite production is strain-dependent (i.e., lag phase for the initiation of nitrate-nitrite conversion, and extent and rate of nitrite production/consumption). Thus, further characterization of nitrite-producers obtained in this study should be performed to identify the optimal conditions for metabolic activity, which could then be used to inform the establishment of the permissible limits for the populations of nitrite-producers.

Secondly, simulation analysis of the processing conditions allowing the development of a predictive model for the activity of nitrite-producers is needed for accurate risk assessment (Membré and Boué, 2018). Major determining factors contributing to the increase in nitrite formation (e.g., time, initial concentration of nitrate and nitrite, oxygen supply, nutritional composition) should be identified to allow manipulation of optimal processing conditions with the aim of preventing nitrite production (Shapleigh, 2013). As shown in this study, temperature was the key factor determining the activity of nitrate/nitrite metabolism (Wallenstein et al., 2006). In this regard, dominant strains in the bacterial community may vary according to processing

conditions and thus dominant nitrite-producers at each processing step should be identified. Since growth and spoilage models for thermophilic spore-former in dairy food have reported (Kakagianni et al., 2016; Remenant et al., 2015), the nitrite profiles of the bacteria reported in this study (e.g., initiation time of nitrite production, peak value of nitrite, rate of nitrite production and consumption, etc.) will improve these models and permit a more accurate determination of nitrite metabolism.

These management strategies require the availability of analytical tools to evaluate the prevalence and identities of nitrite producers in the bacterial communities of raw materials and/or WIP stages. Investigations into the abundance of functional genes has been used to quantify nitrite-producers (Wallenstein et al., 2006), and thus genetic analysis of the isolates obtained in this study may facilitate the identification of specific marker genes in nitrite-producers in PIF processing lines. Due to the high diversity in the sequences of functional genes governing nitrogen metabolism (Green et al., 2010), whole genome sequencing (WGS) is needed for gene identification and construction of metabolic pathways (Kraft et al., 2011). However, genotypic research into nitrate/nitrite metabolism of Gram-positive bacteria suffers from several major drawbacks, such as limited background information because previous relevant studies have been biased toward Gram-negative bacteria (Verbaendert et al., 2011). Although WGS of spore-forming thermophilic bacilli has been reported (Hussein et al., 2015; Studholme, 2015), the application of bioinformatics in the context of nitrite production and consumption is rare (Heylen and Keltjens, 2012; Sun et al., 2016). Such work calls for the analysis of genome sequences of isolates mainly in representative phenotypic groups and comparative genomics with previously-reported nitrite-producers to find common and/or unique functional genes (Throbäck et al., 2004).

5. Conclusions

This study pioneers the identification and characterization of nitrite-producers from the perspective of their potential risk of producing nitrite in PIF. Major findings, especially those pertaining to the metabolic activity of thermophiles, provide novel insights into nitrite as a microbiologically-derived risk. Inherent bacterial characteristics involved in nitrite production and the formation of highly heat-resistant spore classified active and dominant genera responsible for the nitrite level in PIF. Implications of this study can be summarized as follows: 1) all nitrite-producers obtained from processing lines and end-products of PIF were spore-forming bacilli, 2) bacterial characteristics of nitrite-production and spore heat-resistance suggest novel niches of obligate or moderately thermophilic *Bacillus*, *Anoxybacillus*, and *Geobacillus* that may be determinants of the nitrite concentration in PIF, 3) *Geobacillus* was the only highly heat-resistant spore-forming nitrite-producer and was the predominant organism in processing lines, 4) vigorously metabolizing *Geobacillus* isolates demonstrated characteristic nitrite-production profiles and further analysis of the functional genes from metabolic pathways governing those phenotypes may facilitate the understanding of nitrite metabolism in PIF processing lines, and 5) both the inspection of end-products to detect the presence of nitrate/nitrite and testing for nitrite-producers in raw materials should be adopted as the primary management strategy against nitrite in PIF, 6) risk assessment based on the simulation of processing environments should be implemented to establish a risk management system for PIF.

Declarations of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.12.012.

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