



Short communication

Nitrite production by thermophilic aerobic bacteria isolated during the manufacture of milk powder

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ABSTRACT

Nitrite contamination in milk powder is hypothesised to be caused by thermophilic bacteria that form biofilms in milk powder manufacturing plants. Regulatory limits in some countries have made nitrite contamination in milk powder an issue influencing product sales. In this study, thermophilic bacteria were isolated from milk samples obtained from a milk processing plant to investigate the potential of different isolates to convert nitrate to nitrite. Eight bacteria species were identified through 16s rDNA gene sequencing (*Bacillus licheniformis*, *Bacillus aerius*, *Bacillus subtilis*, *Bacillus sonorensis*, *Bacillus firmus*, *Enterococcus faecium*, *Geobacillus stearothermophilus* and *Macrococcus caseolyticus*). Most bacteria isolated were able to convert nitrate to nitrite, and surprisingly in aerobic conditions. The most interesting species was *G. stearothermophilus* as its nitrate reducing capability was highly variable between different isolates. This could explain why there was no relationship reported between nitrite level in milk powder and high thermophile count.

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1. Introduction

Nitrate (NO₃) and nitrite (NO₂) are commonly encountered in food. The presence of nitrate and nitrite in food may be associated with microbiological contamination, residual chemical cleaners used in the manufacturing plant or the addition of nitrates as preservatives in some foods. Consumer concerns of adverse health effects of nitrate and nitrite are controversial (Bedale, Sindelar, & Milkowski, 2016).

The contamination of milk powder with nitrite may result in monetary losses for the dairy industry due to rejection of product by customers. Many countries have regulatory limits for nitrate and nitrite in foods. For example, through the European Commission, the EU has a maximum limit for baby foods of 200 ppm nitrate (EC, 2011). In China the permissible limit of nitrate in infant formula is 100 ppm (National Standard of the People's Republic of China, 2012). There is a need to understand what influences the nitrate/nitrite levels in milk powder to meet regulatory requirements.

We hypothesised that the conversion of nitrate to nitrite is caused by the main microbial contaminants in milk powder such as *Geobacillus stearothermophilus* or *Bacillus licheniformis*. Nitrite contamination of dairy products may result from the activity of

these thermophilic bacteria; however, this has not been proven. The conditions that result in the reduction of nitrate to nitrite have not been established. For these reasons, this study screened thermophilic bacteria isolated from milk from a dairy processing plant for the ability to convert nitrate to nitrite and to study the effect of oxygen and presence of milk fat on the potential for thermophilic bacteria to convert nitrate to nitrite.

2. Materials and methods

2.1. Source and cultivation of bacteria

Twenty seven isolates of bacteria, from samples taken at different stages of milk powder manufacture in a milk powder manufacturing plant, were selected on the basis of colony morphology.

This study was limited to aerobic bacteria that were able to be cultured on Milk Plate Count Agar (Merck, Germany) based on the International Standards Organisation method for aerobic plate counts (ISO 4833-1:2013; ISO, 2013) using incubation temperatures of 55 °C or 37 °C and examination and 18 h incubation. This aimed to isolate the true thermophilic bacteria and those that grow at high temperatures (37–50 °C) but struggle to grow at 55 °C.

The reduction of nitrate to nitrite was determined in Nitrate Broth (BD, United States) and in skim and whole UHT milk to which

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1 g potassium nitrate was added from a sterile solution of 10% potassium nitrate.

Tests for nitrate reduction followed 18 h of growth of bacteria in both aerobic and anaerobic conditions. Nitrate reduction was tested using the Fluka nitrate reduction test (Fluka Analytical & Sigma-Aldrich Co, 2012).

Tests were done in duplicate.

3. Results and discussion

Twenty-seven isolates were obtained from samples from a milk powder manufacturing plant. Table 1 contains the identities of the isolates from 16s rDNA sequencing.

The results for the nitrate reducing capability of the isolates are summarised in Table 2. All eleven isolates of *Bacillus licheniformis* reduced nitrate to nitrite both in nitrate broth and in both skim and whole milk supplemented with potassium nitrate, irrespective of aerobic conditions and temperature of incubation (37 °C and 55 °C). There is a high possibility that all 11 *B. licheniformis* isolated were the same, due to the uniform response to nitrate reduction.

With regard to other *Bacillus* species, a single isolate of *Bacillus aerius*, first described as a close relative of *B. licheniformis* by Shivaji et al. (2006), was able to reduce nitrate in all conditions tested. Four isolates of *Bacillus sonorensis* reduced nitrate to nitrite in all conditions tested. Two isolates of *Bacillus subtilis* also reduced nitrate in all conditions tested. A single isolate of *Bacillus firmus*, reduced nitrate in broth media and in whole milk when grown aerobically but not anaerobically. There was no nitrate reduction in trim milk. *B. firmus* is known to have nitrate reducing capabilities that can vary between different strains (Berger, 2015), but the negative results for trim milk were not expected.

Four isolates of *G. stearothermophilus* showed varied in the ability to reduce nitrate (Table 2). These results are in line with the information from existing literature, and highlighted the fact that *G. stearothermophilus* is highly variable between different isolates (Vos et al., 2011), although this is the first time this has been demonstrated in dairy isolates. This variability may explain why there is no reported correlation between the nitrite level and high thermophile counts, e.g., growth of *G. stearothermophilus* hF in milk would result in no reduction of nitrate.

While both aerobic and anaerobic environments allow nitrate reduction to occur, there were two exceptions in our isolates. *G. stearothermophilus* h7 completely reduced nitrate into either nitrogen or ammonia gas in trim milk supplemented with potassium nitrate under an anaerobic conditions and *B. firmus* reduced nitrate into nitrite in whole milk supplemented with potassium nitrate under anaerobic conditions. The results of these two exceptions could be due to their variability in nitrate reduction capability across different strains.

With regard to other isolates, one isolate of *Micrococcus caseolyticus* reduced nitrate in all conditions tested, while three enterococcus isolates failed to reduce nitrate under all conditions tested.

Considering the effect of whole and trim milk on nitrate reducing activity, *G. stearothermophilus* h7 completely reduced nitrate into either nitrogen or ammonia gas in trim milk, but not whole milk, supplemented with potassium nitrate under anaerobic conditions and *B. firmus* reduced nitrate into nitrite in whole milk, but not skim milk, supplemented with potassium nitrate under anaerobic conditions. These results suggest that milk fat may have an influence on nitrate reduction of these isolates; however, overall, the presence or absence of milk fat does not appear to have any consistent effect on bacteria nitrate reducing capability.

Table 1
Identities of isolates.

Isolate	Source	Number of isolates
<i>Bacillus aerius</i>	Evaporator	1
<i>Bacillus firmus</i>	Evaporator	1
<i>Bacillus licheniformis</i>	Evaporator	4
<i>Bacillus licheniformis</i>	Milk powder	5
<i>Bacillus licheniformis</i>	Concentrate buffer tank	2
<i>Bacillus sonorensis</i>	Evaporator	4
<i>Bacillus subtilis</i>	Evaporator	1
<i>Bacillus subtilis</i>	Concentrate buffer tank	1
<i>Enterococcus faecium</i>	Evaporator	3
<i>Geobacillus stearothermophilus</i>	Evaporator	3
<i>Geobacillus stearothermophilus</i>	Milk powder	1
<i>Micrococcus caseolyticus</i>	Evaporator	1

Table 2
Nitrate to nitrite conversion for bacterial isolates from milk powder.^a

Isolate	Nitrate broth		Whole milk		Trim milk	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
<i>B. licheniformis</i> (11)	+	+	+	+	+	+
<i>B. sonorensis</i> (4)	+	+	+	+	+	+
<i>B. aerius</i> (1)	+	+	+	+	+	+
<i>B. firmus</i> (1)	+	+	+	–	–	–
<i>B. subtilis</i> (2)	+	+	+	+	+	+
<i>G. stearothermophilus</i> h3	+	+	+	+	+	+
<i>G. stearothermophilus</i> hf	+	+	–	–	–	–
<i>G. stearothermophilus</i> h7	–	–	–	–	–	–
<i>G. stearothermophilus</i> h14	+	+	+	+	+	+
<i>M. caseolyticus</i> (1)	+	+	+	+	+	+

^a Number of isolates given in parentheses.

4. Conclusions

B. licheniformis, *B. aerius*, *B. subtilis*, *B. sonorensis* and *M. caseolyticus* are able to reduce nitrate to nitrite in milk, while *B. firmus* and *G. stearothermophilus* vary in their ability to reduce nitrate. The variability in nitrate reducing capability between different *G. stearothermophilus* isolates explains why there is no observed link between high numbers of thermophilic bacteria and nitrite levels in milk powder. Nitrate reduction by thermophilic bacteria under aerobic conditions is a potential source of nitrite contamination in milk.

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