Screening for nitrite producing *Bacillus licheniformis* from various origins.

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Abstract

Nitrite, which has been considered a chemical danger, can cause infant methemoglobinemia. *Bacillus licheniformis* is one of the spores forming nitrite-producing bacteria that serves as a potential risk in the dairy industry based on its roles in foodborne illness and dairy spoilage. This study was carried out to assess the nitrite production by 10 strains of *B. licheniformis* under different environmental conditions. *B. licheniformis* isolated in this research comes from various sources, including a pasteurisation system, tuber feed, and milk samples obtained from the Massey University Microbiology Lab. This research focused on five different temperatures (25°C, 30°C, 37°C, 55°C and 60°C) approximate processing conditions found in the dairy industry checking the *B. licheniformis* growth and measuring optical density at 570nm at all five temperatures. This study also examined the production of nitrite in aerobic and anaerobic conditions. All isolated strains were able to convert nitrate into nitrite at all 5 temperatures at both aerobic and anaerobic conditions except some isolates from spring (15, 17) and winter (36, 43, 50,55) convert nitrite into some other nitrogenous compounds under 60°C anaerobic conditions, suggesting that there is a potential variability in metabolic pathways. However, oxygen availability does not affect nitrate reduction. It was observed that optimal growth occurred between 30°C and 55°C. These findings have shown the likely health hazards associated with *B. licheniformis*, as the organism can convert nitrate into nitrite. The finding suggests a need for further research on the metabolic pathways of these isolates to understand their behaviour and mitigate associated risks.

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1. Introduction

Nitrate and nitrite are commonly found in milk, fruit and vegetables. Microbiological contamination can be one of the sources of food contamination. The food industry is trying to eliminate or stop the addition of nitrate and foodborn pathogens due to consumer concerns about the harmful health impacts of these substances (Bedale et al., 2016). Microbial contamination in milk product manufacture has been mentioned as an important risk factor leading to the development of disease in infants. The presence of nitrite in dairy products, especially in powdered infant formula, is reported to cause methemoglobinemia which affects oxygen transportation among infants.(Chan, 2011). Additionally, nitrosamines, which cause cancer and mutation in humans, can be ingested directly or produced endogenously from nitrate and nitrite as a result of nitrites and secondary amines interacting in food. In various animal experiments, it has been shown that specific N-nitroso compounds can induce cancer (Erkekoglu & Baydar, 2009). Infants may be at risk when consuming water or formula that contains high concentrations of nitrite.

The dairy business may suffer monetary losses if milk powder is contaminated with nitrite because consumers may reject the product. According to Tajitsu and Rajagopalan's (2013) report, New Zealand considers 5 mg of nitrite per kilogram of milk powder to be safe for human consumption. While, in China, the acceptable limit of nitrate and nitrite in the product is 100 and 2 ppm, respectively (Bugang & Woolsey, 2010). To satisfy legal standards, it is necessary to recognize the factors that affect the amount of nitrite in milk powder.

Manufacturers of powdered dairy products are particularly concerned about spore-formers, which fall into one of three categories: thermophilic, mesophilic, or psychrotolerant, with thermophilic spore-formers being more prevalent in the end product(Gopal et al., 2015). According to a global examination of milk powders, 39.2% of milk products include *B. licheniformis*, a common thermophilic spore former. According to studies, it is the most widespread thermophilic bacillus in Chinese milk powders (36.8%), and it is present in both the summer and the winter. Its high frequency in milk products can be attributed to its extensive presence in dairy habitats, such as feed, feces, and raw milk(Gopal et al., 2015). For these reasons, this review examines the nitrite production capabilities of *B. licheniformis* sourced from various stages of dairy processing, focusing on its ability to convert nitrate to nitrite and the effects of oxygen and temperature on this process.

1.1 Research aim and objectives

The objective of the study is to establish how different *B. licheniformis* strains reduce nitrate at different temperatures and in different environmental conditions, and whether all strains produce nitrite, with

particular emphasis on the evaluation of the possible exposure risk of nitrites in powdered infant formula.

1.2 Research questions

1. Is every strain of *B. licheniformis* capable of reducing nitrate into nitrite at every tested temperature (25°C, 30°C, 37°C, 55°C, and 60°C)?
2. Does temperature affect bacterial growth and nitrate reduction?
3. Do aerobic and anaerobic conditions affect the growth and nitrate reduction?

2. Literature review

2.1 Bacterial contamination in powdered infant formula and its health impact

A report of 2015 from the World Health Organization (WHO) stated that 33 million years of healthy lives are lost annually due to foodborne illness, with one in ten people worldwide falling ill as a result of eating contaminated food and 420,000 deaths(Lang & Sant'Ana, 2021). Of all the etiological agents, bacteria are responsible for two-thirds of outbreaks and nearly one-third of deaths among children under five(Kirk et al., 2015). As a result, food safety is a concern for ecology, public health, food security, and the economy (Guillier et al., 2016). Milk is used as a raw material as an ingredient in the formulation of foods, or as a final product. Milk is a highly nutritive food product therefore it is an ideal medium for the growth of some microorganisms. Milk is initially sterile but can become contaminated with bacteria from several sources: soil and pastures, sick animals, unhygienic methods of milking, unhygienic storage of milk and growth during transportation of milk and dairy products and if the raw milk is stored for many days under refrigeration many genera of bacteria such as *Enterococcus, Lactococcus, Streptococcus, Leuconostoc, Lactobacillus, Microbacterium, Propionobacterium, Micrococcus, Proteus, Pseudomonas, Bacillus* and *Listeria* can grow (Moreira, 2019). Some of these microorganisms (e.g., *Micrococcus*) can survive heat treatment including pasteurization and can be present in the final product (Martin et al., 2018). It is essential to ensure the safety of powdered infant formula because infants are sensitive to microorganisms due to their developing immune systems (Lang & Sant'Ana, 2021). Research conducted by Wong and Flint found 27 isolates from samples taken at different stages of milk powder manufacture in a milk powder manufacturing plant and hypothesised that *Geobacillus stearothermophilus* or *Bacillus licheniformis* are the main microbial contaminants responsible for conversation of nitrate to nitrite (Wong & Flint, 2019). Acceptable daily intakes (ADIs) for nitrate and

nitrite were set at 0-3.70 and 0-0.07 mg/kg of body weight, respectively, by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) and the Joint Expert Committee on Food Additives (JECFA) of the Food and the Scientific Committee on Food (EC-SCF) of the European Commission(Park et al., 2022; Singh et al., 2017; Speijers & Van den Brandt, 2003). While, in China, the acceptable limit of nitrate and nitrite in the product is 100 and 2 ppm, respectively (Bugang & Woolsey, 2010). Nitrite is acknowledged as a potential threat to babies and toddlers and as a significant contributor to methemoglobinemia, which is characterised by cyanosis, breathing difficulties and convulsions(Chan, 2011). Infants under three months old are vulnerable to methemoglobinemia (Chan, 2011; Greer et al., 2005; Savino et al., 2006). After ingestion, nitrites are thought to be responsible for most toxic effects, especially since nitrites can oxidise ferrohemoglobin to form methemoglobin. Methemoglobin does not bind oxygen and can cause hypoxemia and methemoglobinemia. As a greater proportion of the hemoglobin in newborns is fetal hemoglobin with a high likelihood of oxidation, and their methemoglobin reductase is immature before 5–6 months old, they are more susceptible to developing this condition. The fact that infants are substantially more vulnerable than other age groups, such as adults, to ingested nitrite makes nitrite contamination of their food particularly concerning (Erkekoglu & Baydar, 2009)(Agency for Toxic Substances and Disease Registry 1991; Greer et al., 2005).

2.2 Possible sources of nitrite in powder infant formula

Products made from milk powder may naturally contain nitrate or nitrite from the production environment, raw materials, and nitric acid used as a cleaning agent in processing lines(Yeh et al., 2013). Nitrates and nitrites can enter the bodies of dairy cows through their food and water (Genualdi et al., 2020). Over the course of five days, Croitoru et al. examined the endogenous nitrate and nitrite concentrations in milk and fodder (hay, grass, maize, bran, alfalfa, and molasses). The median milk concentrations throughout this period were less than 1% of the feed values, and they corresponded to around 2–3.5 mg/kg of nitrate and 0.035–0.8 mg/kg of nitrite in powdered milk. Both the application of fertilizers on crops and pastures and the presence of nitrates in feed and water cause endogenous concentrations (Croitoru et al., 2015).

Direct or indirect hot spray drying techniques can be used to dry milk. The combustion products get into contact with the milk during the drying process when using the direct heating method. A heat exchanger is used to transport heat to the milk during indirect heating, removing any chance of contamination from combustion products. Nitrogen oxide gases may be produced during direct heated spray drying if combustion occurs with excess air present. In the milk powder, these gasses further dissolve to generate acids, which then take the form of nitrates and nitrites (Kelly et al., 1989)

2.3 Nitrate reduction pathways

Denitrification is one of the processes in the nitrogen cycle which is performed by different types of microorganisms and is important for the change of such environmental and industrial processes. The initial step of the pathway is to take nitrate (NO_3^-) from the environment and convert it into nitrite with the help of nitrate reductase, which needs a cofactor such as NADH and NADPH(Cabello et al., 2004). In this process, there are two main types of nitrate reductase, which are assimilatory nitrate reductase (ANR) and dissimilatory nitrate reductase (DNR)(Cabello et al., 2004). The nitrite produced is further reduced by the nitrite reductase enzymes These enzymes have two subdivisions which are Copper-containing enzymes which can reduce nitrite to nitric oxide (NO) and heme-containing enzymes which also produce nitric oxide (NO) or nitrous oxide (N_2O)(Cabello et al., 2004). *B. licheniformis* has nitrate reductase, which produces nitrite from nitrate(He et al., 2023). Some factors may affect their efficiency such as temperature and environmental conditions.

2.4 *Bacillus licheniformis* overview

B. licheniformis is a gram-positive, spore-forming, thermophilic/mesophilic microorganism normally found in soil, feed, and dairy environments. The use of this species as a probiotic is a concern as a threat to the dairy industry based on potential foodborne diseases and dairy product spoilage (Hongchao et al., 2024). Moreover, the presence of nitrification and denitrification-enabling enzyme systems in *B. licheniformis* enables the organisms to reduce nitrate to nitrite and ammonia, to nitrogen.(He et al., 2023).

It has been demonstrated that *B. licheniformis* and *B. cereus* are among the most common *Bacillus* species found in raw milk and throughout the dairy processing spectrum(Gopal et al., 2015; Kalogridou-Vassiliadou, 1992; Scheldeman et al., 2006). Although not classified as a human pathogen, *B. licheniformis* spores are known to adversely affect milk's organoleptic and functional qualities, cause milk and dairy products to deteriorate, and cause issues with specification compliance(Crielly et al., 1994; Dhakal et al., 2014; Gopal et al., 2015). In an examination of 28 milk powder samples from 18 different nations, *B. licheniformis* was the second most prevalent thermophilic spore-former found, accounting for 39.2% of the overall occurrence(Gopal et al., 2015; Rückert et al., 2004). According to the same study, a particular strain of *B. licheniformis* was found in 27 of the 28 milk powder samples, indicating how widespread this soil isolate is (Gopal et al., 2015; Rückert et al., 2004). According to previous research, *B. licheniformis* was shown to be the most common thermophilic bacillus in Chinese

milk powders, occurring in 36.8% of 801 isolates from infant milk formula (IMF) and whole milk powders examined from various Chinese manufacturers(Gopal et al., 2015; Yuan et al., 2012).

Despite its disadvantages in the dairy industry, such as potential issues with spoilage or contamination, *B. licheniformis* offers numerous advantages. Its distinct genetic origin sets it apart as an important component of the industrial microbiota, resulting in its remarkable uniqueness and specific uses in the food industry(He et al., 2023). *B. licheniformis* is widely utilized in fermentation, the biosynthesis of edible compounds, raw material processing, and the biological treatment of food industrial waste(He et al., 2023). Due to its absence of endogeneous toxins, *B. licheniformis* is recognized as a food safety strain and is a beneficial microorganism with significant uses in the food business(He et al., 2023). Because *B. licheniformis* can withstand relatively high ambient temperatures during food processing and offers a diverse range of enzymes(e.g., thermostable α-amylases), it is the perfect source microorganism for functional enzymes in the food industry(He et al., 2023). These highly effective functional enzymes have found useful uses in the food production industry, such as lowering dough viscosity and delaying bread aging in the bread industry or transforming low-sweetness, low-solubility lactose into sweeter, more soluble glucose in the dairy industry(He et al., 2023). From upstream food processing to downstream waste treatment, *B. licheniformis* is involved in many facets of the food business and can be characterized as an efficient microbial facilitator for the industry's quick growth(He et al., 2023).

2.5 Sources of *Bacillus licheniformis* and nitrite production

2.5.1 Tuber feed

Farm environment (tuber feed) used in dairy farming can be a potential source of *B. licheniformis (Crielly et al., 1994)*. The bacterium can enter the dairy production chain through contaminated feed and may cause nitrite contamination in milk products.

2.5.2 Pasteurization processes

Pasteurization is intended to kill pathogenic microorganisms that may be present in a food product. However, bacillus licheniformis is a thermophilic/mesophilic and spore-forming bacterium, the spores of which can survive pasteurization and UHT temperatures (Janštová & Lukášová, 2001).

2.5.3 Milk

Milk is one of the important sources of bacterial contamination for human health. Among the *Bacillus* species, *B. licheniformis* is reported to be the most common species in raw milk and at all stages of milk

processing (Kalogridou-Vassiliadou, 1992). They can survive all the heat treatment of milk, and they can be in the final product, and this could be a reason for nitrite production.

2.6 Nitrate reduction test

Nitrate reduction by microorganisms involves nitrate reductase enzyme(Cabello et al., 2004).Nitrite may be reduced to several nitrogen compounds such as NO, N2O, N2, and NH3, depending on the enzyme system of the microorganism and the environment in which the microorganism is growing(Buxton, 2011). To measure nitrite formation from nitrate there are two reagents called sulfanilic acid(Reagent A) and N, N-dimethyl-a-naphthylamine(Reagent B) which react with nitrite and form a red azo dye indicating the presence of NO_2^-(Khattak et al., 2004). Acetic acid in reagents A and B together acidifies NO2 to form HNO2 in the presence of NO2, which initiates the colour reaction.(Buxton, 2011). There could be several reasons why the colour doesn't change in two minutes. The organism could either (i) not be able to reduce NO3- at all, (ii) be able to reduce NO2-, or (iii) reduce NO3 directly to molecular nitrogen. However, zinc powder is also used to force nitrate reduction, if there is no colour change in the test tube then we can add zinc to check the presence of nitrate. If there is a presence of nitrate then zinc reacts with nitrate and immediately produces nitrite and forms a red colour. (Buxton, 2011).

3. Materials and methods

3.1 Source of *Bacillus licheniformis* strains

The 10 isolated *B.licheniformis* strains used in this study were collected from the microbial culture collection lab of Massey University which is collected directly from cattle feed, details are listed in Table 1. The isolated colony was streaked onto TSA slant and maintained as a working culture was stored at 4˚C, and the sub-culture was prepared by inoculating *B. licheniformis* into Tryptic Soy Broth and incubated at 37˚C.

Table 1. Identities of isolates

No	ID	Sample type	Season
15	*B. licheniformis*	Pasture	Spring
17	*B. licheniformis*	Pasture	Spring
27	*B. licheniformis*	Tuber feed	Autumn

36	*B. licheniformis*	Tuber feed	Winter
37	*B. licheniformis*	Tuber feed	Autumn
42	*B. licheniformis*	Milk	Autumn
43	*B. licheniformis*	Tuber feed	Winter
44	*B. licheniformis*	Milk	Autumn
50	*B. licheniformis*	Pasture	Winter
55	*B. licheniformis*	Milk	Winter

3.2 Growth medium

To cultivate and maintain various isolates before inoculating them into the test medium, Tryptic Soy Broth (BD, TSB) and Tryptic Soy Agar (BD, TSA) were prepared according to the manufacturer's instruction. TSA was formed into slants to preserve pure isolates. Sterilization of the prepared media was done for 15 minutes at 121 ˚C.

3.3 Test medium

Nitrate Broth (BD) was used for testing the nitrate-reducing properties of different isolates and prepared according to the directions on the box label. It was then dispensed into bottles of 10mL each. Nitrate Agar (BD) Slants were prepared according to the instructions on the packaging label, for *B. aerius* isolates (used as a positive control) require a solid surface to grow. Sterilization of the prepared media was done for 15 minutes at 121 ˚C.

3.4 Nitrate reduction test reagent

Sulfanilic acid solution (reagent A) and N, N-Dimethyl-1-naphthylamine (reagent B) were used for the nitrate reduction test. Eight grams of sulfanilic acid was dissolved in 1 litre of 5N acetic acid to create Reagent A, which was then kept in an opaque bottle. Reagent B was directly used as manufacturer instruction (Sigma-Aldrich. co), which does not need any modifications and purification.

3.5 Growth experiments in broth

The *B. licheniformis* working culture was inoculated into TSB and incubated for 24 hrs at different temperatures (25°C,30°C,37°C,55°C, and 60°C) to measure growth at all these 5 temperatures and in

aerobic and anaerobic conditions. This experiment was performed in three replicates. The optical density at 570nm (Md Zain et al., 2017) was measured to determine growth.

3.6 Screening of *Bacillus licheniformis* for aerobic nitrate reducing activity

All 10 strains of *B. licheniformis* were inoculated into 10 ml of Tryptic soy broth and then incubated for 24 hours at different temperatures (25°C,30°C,37°C,55°C, and 60°C). One loopful of the culture was then inoculated into nitrate broth for the nitrate reduction test. Inoculated nitrate broth was then incubated for 24 hours at all five temperatures. After 24 hours of incubation of nitrate broth, a few drops of reagents A and B were added to the broth. The broth would turn pink or reddish if there was a nitrate-to-nitrite reduction. Zinc powder was added to nitrate broth if it was colourless. After the addition of zinc if the broth turns into a red or pink colour that means nitrate is present in the broth and the result is negative. If broth remains colourless after adding zinc the result is positive. Positive controls were prepared by adding *B.aerius* as it can reduce nitrate and convert it into nitrite. By adding zinc into freshly sterilised nitrate broth negative controls were prepared to see the negative reaction.

3.7 Screening of *B. licheniformis* for anaerobic nitrate-reducing activity

The procedure followed was identical to screening isolates for aerobic nitrate reduction. However, all bottles were incubated in anaerobic jars rather than under aerobic conditions. To create the anaerobic environment The Becton Dickinson Company's AnaeroPack was used which absorbs oxygen and releases carbon dioxide.

Screening tests were conducted to determine the nitrate reduction capacity of the microorganisms. There was a total of 10 screening tests:

Test No.	Condition	Temperature
1	Aerobic, Nitrate broth	25°C
2	Anaerobic, Nitrate broth	25°C
3	Aerobic, Nitrate broth	30°C
4	Anaerobic, Nitrate broth	30°C
5	Aerobic, Nitrate broth	37°C
6	Anaerobic, Nitrate broth	37°C
7	Aerobic, Nitrate broth	55°C
8	Anaerobic, Nitrate broth	55°C
9	Aerobic, Nitrate broth	60°C

10	Anaerobic, Nitrate broth	60°C

3.8 Experimental conditions

3.8.1 Temperature effect on growth and nitrite production

To determine the temperature effect on growth and nitrite production, 10 strains of *B. licheniformis* were incubated and tested at 5 temperatures (25°C, 30°C, 37°C, 55°C, and 60°C) for 24 hours and the optical density was measured at 570 nm to record bacterial growth (Md Zain et al., 2017). Seasonal variation may affect the microbiological quality of milk.(Kmiha et al., 2017). Experiments conducted by (Zhou et al., 2023) On the effect of carbon source and temperature on the growth of *Bacillus licheniformis* and *Bacillus cereus* and the formation of biofilms, concluded that the organism grew well under 30 to 55 ˚C.

3.8.2 Aerobic vs Anaerobic conditions

The effect of oxygen on nitrite formation was also demonstrated by comparing the aerobic and anaerobic conditions. Wong and Flint(Wong & Flint, 2019) isolated 27 microorganisms from a milk powder manufacturing plant and studied nitrite production in both aerobic and anaerobic conditions and found that *Bacillus aerius,* four isolates of *Bacillus sonorensis,* two isolates of *Bacillus subtilis,* one isolate of *Micrococcus caseolyticus,* and eleven isolates of *B. licheniformis* produces nitrite in both aerobic and anaerobic conditions and found variability into the ability to reduce nitrate in *B. firmus, G. stearothermophilus(Wong & Flint, 2019)*. In aerobic conditions, bacteria use oxygen for their energy production known as aerobic respiration and convert nitrate into nitrite. On the other hand, In the absence of oxygen, most of these bacteria use nitrate as an electron acceptor (*B. megaterim* and *B. pumilus* are notable exceptions), and a small number, including *B. cereus, B. licheniformis and B. thuringiensis*, ferment sugars in the absence of external electron acceptors(Shariat et al., 1995). It was stated (Schulp & Stouthamer, 1970) that bacteria behave differently in varying oxygen levels. They have stated that in the presence of oxygen, there was no activity of the enzyme nitrate reductase which is responsible for reducing nitrate into nitrite, and in weak aeration or anaerobic conditions activity of nitrate reductase was observed. They explained that oxygen availability significantly influences nitrite production and bacterial response to environmental changes.

On the other hand,(Roco et al., 2017) Performed experiments by isolating organisms from well-aerated soil and stated that organisms had distinct genotypes and phenotypes for nitrate reduction and that many bacteria are capable of aerobic nitrate reduction.

4. Results

From various sources, ten *B. licheniformis* isolates were collected. The name of the organism and its source are listed in Table 1. The main goal was to determine nitrate reduction under different conditions and temperatures.

4.1 Effect of temperature on nitrate reduction capabilities

The nitrate reduction capability data are presented in the Table 2. All ten isolates of Bacillus licheniformis were capable of converting nitrate to nitrite at all five temperatures in nitrate broth. Overall, temperature (Range: - 25°C,30°C,37°C,55°C, and 60°C) did not have any effect on nitrate reducing capability of *B. licheniformis*.

The nitrate reduction results, shown in Figure 1, confirm the ability of *B. licheniformis* to reduce nitrate to nitrite, as indicated by the formation of a reddish-pink-coloured complex.

4.2 Effect of oxygen on nitrate-reducing capacity

It was stated by (Schulp & Stouthamer, 1970)) when bacteria were grown under the presence of oxygen there was no activity of the enzyme Nitrate reductase and cell density was not too high, while bacteria grown under weak aeration or in anaerobic conditions nitrate reductase was active. The aerobic environment did not influence nitrate reduction in any of the trials that were carried out. In both aerobic and anaerobic conditions, a uniform response was observed by every isolate that reduced nitrate. After isolating bacteria from well-aerated soils,(Roco et al., 2017) concluded that many bacteria are capable of aerobic nitrate reduction and that the isolates "had distinct genotypes and phenotypes for nitrate reduction." In this study, all ten isolates showed no difference between aerobic and anaerobic conditions, which may be explained by the occurrence of aerobic nitrate reduction.

However, the isolates from spring (15,17) and winter (36,43,50,55), as well as one from autumn (27), seem to be able to change nitrite into other forms under anaerobic conditions at 60°C. For these species, nitrate reduction occurred in one or two of the tubes during three replica tests as shown in Figure 2 and Figure 3. After adding both reagent and zinc to check for the presence of nitrate and nitrite, no red colour developed, which indicates that neither nitrate nor nitrite was present. Notably, nitrite was not found in these instances, indicating that the bacteria may be further metabolizing nitrite or changing it into other molecules in these circumstances. The results could be due to variability across different strains in

nitrate reduction as stated by (Roco et al., 2017). Further investigation is needed to understand this phenomenon.

4.3 Growth measurement

The growth of all 10 *B. licheniformis* strains was measured by OD at 570 nm in aerobic and anaerobic conditions(Md Zain et al., 2017). The effect of temperature ranges from 25 to 60°C on *B. licheniformis* growth is presented in the graph below. The amount of growth of all ten isolates in both aerobic and anaerobic conditions peaked at 30°C,37°C, and 55°C, lower at 60°C and moderate at 25 °C. Out of the two conditions tested, there was increased growth of *B. licheniformis* in the anaerobic condition than in the aerobic one.

The growth of *B. licheniformis* under aerobic and anaerobic conditions is illustrated in Figure 4 and Figure 5, respectively demonstrating a higher growth in anaerobic conditions.

Table 2. Nitrate reduction for all 10 *B. licheniformis* isolates for both Aerobic and anaerobic conditions.

Name of organism	25 °C	30°C	37 °C	55 °C	60°C
B. licheniformis 15	+	+	+	+	+
B. licheniformis 17	+	+	+	+	+
B. licheniformis 27	+	+	+	+	+
B. licheniformis 36	+	+	+	+	+
B. licheniformis 37	+	+	+	+	+
B. licheniformis 42	+	+	+	+	+
B. licheniformis 43	+	+	+	+	+
B. licheniformis 44	+	+	+	+	+
B. licheniformis 50	+	+	+	+	+
B. licheniformis 55	+	+	+	+	+

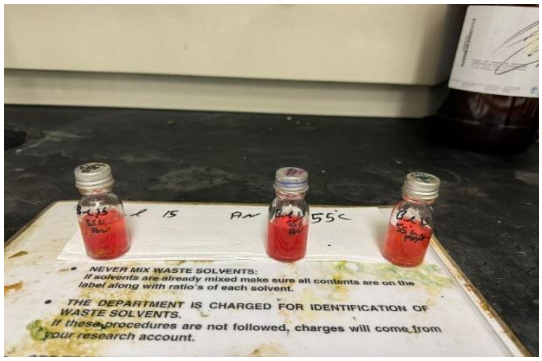

Figure 1Nitrate reduction by *B.licheniformis* (reddish-pink complex formation)

Figure 2 Nitrate and nitrite absence in isolates under anaerobic conditions at 60°C (no red colour formation after reagent and zinc addition).

Figure 3 Nitrate and nitrite absent in isolates under anaerobic conditions at 60°C (no red colour formation after reagent and zinc addition).

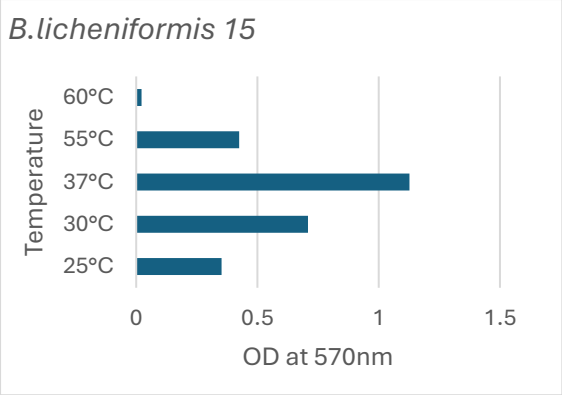

Figure 4 Growth under Aerobic Condition

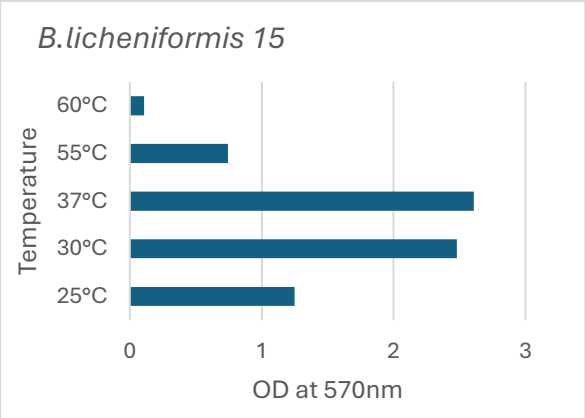

Figure 5 Growth under Anaerobic Condition

5. Discussion

This study highlights the nitrate reduction capability of ten *B. licheniformis* isolates and their growth characteristics under 5 different temperatures and aerobic and anaerobic conditions. All ten isolates were able to reduce nitrate under all conditions tested. These results are comparable with the results reported by (Wong & Flint, 2019), who tested 11 isolates of *B. licheniformis* under different conditions and found that all *B. licheniformis* isolates were able to reduce nitrate under all conditions tested. Due to their capacity to produce toxins(Logan, 2012; Mezian et al., 2022; Pukall et al., 2008; Rowan et al., 2001), and their ability to reduce nitrate, *B. licheniformis* detection in PIF poses serious health risks(Di Pinto et al., 2013).

Moreover, the growth of the organism was measured at different temperatures, which shows all 10 strains of *B. licheniformis* grew well under 30°C to 55°C, this result is comparable with the findings of (Zhou et al., 2023), who also studied the effect of temperature on the growth of *B. licheniformis*. In addition, *B. licheniformis* grew maximum under anaerobic conditions, and there is a higher possibility

that maximum nitrite production occurs in anaerobic conditions than in aerobic conditions, because (Shariat et al., 1995) stated that nitrate reduction increases 2.5-fold when grown anaerobically in the presence of glucose and nitrate.

Interestingly, all isolates reduce nitrate, however, isolates from spring (15, 17) and winter (36, 43, 50,55) were capable of reducing nitrite into some other form under anaerobic conditions at 60°C. This was observed while there was nitrate reduction in nitrate broth but, there was no nitrite in one or two tubes during three replica tests. These results suggest that the organism may possess different metabolic pathways or enzymes, yet to be discovered, as stated by (Roco et al., 2017). Under these experimental conditions (60°C, anaerobic), the lack of nitrite may indicate that nitrite-producing bacteria are not active in considerable amounts. This does not mean that the product is risk-free. Even if there is no nitrite present, the bacteria may still be generating toxic acids (Logan, 2012; Mezian et al., 2022; Pukall et al., 2008; Rowan et al., 2001) or other dangerous compounds that could be dangerous.

The results of the nitrate reduction and growth studies should be useful for further understanding of organism behaviour under different conditions. Additionally, further investigation is needed to measure nitrite levels produced in all tested conditions, including a milk environment, as this study did not examine those conditions. This information is crucial for addressing potential risks associated with *B. licheniformis* in the dairy industry, particularly in powdered infant formula.

6. Conclusion

All ten isolates of *B. licheniformis* can reduce nitrate to nitrite in nitrate broth under all five temperatures and both aerobic and anaerobic conditions. Optimal growth was observed at 37°C, notably, enhanced growth was observed under anaerobic conditions compared to aerobic conditions.

Importantly, some isolates of spring and winter can reduce nitrate into some other form rather than nitrite under 60°C anaerobically. It highlights that there may be differences in metabolic pathways and enzymes across isolates, which need further investigation as it was indicated by(Roco et al., 2017). There is variability across different strains in nitrate reduction. This study suggests that there is a lack of knowledge about the metabolic pathway and enzymes of this organism, and their behaviour in the milk environment, which should be important to understand its behaviour in different conditions and to control its risk in the dairy industry especially in milk powder manufacture plant and in powder infant formula.

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Appendices

Materials

- sterile petri dish

- 10 ml bottles
- Micropipette
- Tips
- Wire loop
- Weighing aid
- Spatula
- Hand gloves
- Marker pen
- Dissolving purify water

Instruments

- Wigh balance
- Autoclave
- Spectrophotometer
- Hot air oven
- Laminar air flow
- Magnetic stirrer
- Incubator (25°C,30°C,37°C,55°C, and 60°C)
- Chemicals: -
- Sulfanilic acid solution
- N, N-Dimethyl-1-naphthylamine

Media

- Tryptic soy broth
- Tryptic soy agar
- Nitrate broth

Media composition

Tryptic soy broth

Ingredients	g/l
Pancreatic digest of casein	17.0 g

Papaic digest of soybean	3.0 g
Dextrose	2.5 g
Sodium chloride	5.0 g
Dipotassium Phosphate	2.5 g
PH after sterilization	7.3±0.2

Tryptic soy agar

Ingredients	g/l
Pancreatic digest of casein	15.0 g
Papaic digest of soybean	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
PH after sterilization	7.2±0.2

Nitrate Broth

Ingredients	g/l
Beef Extract	3.0 g
Peptone	4.0 g
Proteose Peptone No. 3	1.0 g
Potassium Nitrate	1.0 g
PH after sterilization	7.0±0.2

Preparation of media

- Placed a weighing aid on the weighing pan of the calibrated balance, large enough to accommodate the quantity to be weighed, and pressed tare to zero the weight.
- Prepared the medium in a sufficiently sized container to allow proper mixing and heating.
- Opened the bottle containing the dehydrated media. With the help of a clean and dried spatula/spoon, add the dehydrated media powder to the weighing aid.

- For the preparation of the media, accurately weigh the quantity of media as mentioned on the media container.

- After adding the media, make up the volume of water as required.
- Sterilized the medium at 121°C for 15 minutes after properly dispensing the media into the bottle. Labelled the media indicating name and date.

Storage of media

After the preparation of media, all media were stored in at 4°C cold room to maintain stability.