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Assessing spore-forming bacteria in milk powder: A study of bacterial spoilage in dairy products from selected developing countries

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KEY CONTRIBUTION

The dairy sector can create more efficient methods to lessen the negative impact that aerobic spore-forming bacteria have on the safety and quality of its products. The identification and counting of these bacterial pollutants can help with the implementation of corrective and preventive actions. Greater comprehension of the characteristics of mesophilic and thermophilic biofilms is also necessary for more effective control tactics.

ABSTRACT

The global increase in milk consumption has led to a rise in milk production, with a particular emphasis on dried milk products to extend shelf life. However, a significant challenge faced by the dairy industry, especially in Algeria, is the contamination of milk powders by resilient aerobic spore-forming bacteria. These microorganisms can withstand high processing temperatures and adhere to stainless steel surfaces, forming persistent biofilms. These mature biofilms become a source of contamination, releasing spores and vegetative cells into the liquid products during processing, resulting in poor product quality and a limited shelf life. This study examines the occurrence of aerobic spore-forming bacteria in the dairy sector, focusing specifically on their contamination of milk powders. A comprehensive analysis of their distinctive characteristics, growth conditions, mechanisms of inactivation, and biofilm development highlights their potential to cause both pathogenic and spoilage problems in dairy products. A deeper understanding of these factors can help the dairy industry develop more effective strategies to mitigate the adverse effects of aerobic spore-forming bacteria on product quality and safety.

Introduction

Milk and dairy products play a vital role in human nutrition. They are concentrated sources of macro and micronutrients (FAO, 2013). The increasing consumption of milk and dairy products has driven growth in their production, with particular focus on dried milk products to improve shelf life (McHugh et al., 2017). However, milk powder and other dried milk products are often contaminated with aerobic spore-forming bacteria, including *Bacillus licheniformis, Geobacillus stearothermophilus, Anoxybacillus flavithermus, Bacillus cereus*, and *Bacillus subtilis* (Ronimus et al., 2006; Reginensi et al., 2011; Yuan et al., 2012; Buehner et al.,2014; Sadek et al., 2015). The presence and persistence of microorganisms in milk and dairy products depend on the initial contamination level of the raw milk and on their resistance to the treatments applied.

Due to their adhesion ability, spores can form biofilms, attach to surfaces where they may germinate under favorable conditions, and withstand high processing temperatures. The liquid product that flows over the biofilm is contaminated by spores and vegetative cells. Therefore, due to their pathogenic potential, toxins, and extracellular enzymes, Bacillus species can cause food poisoning and spoilage (Tirloni et al., 2022). As a result, large quantities of milk and dairy products are wasted each year. Although Algeria is the largest consumer of dairy products in North Africa, its milk production is insufficient to meet industry needs, which remain largely disconnected from the agricultural sector. Only a small portion of national milk demand is supplied by local dairy farms (Kacimi-El-Hassani, 2013). Consequently, Algeria's dairy industry relies heavily on imported raw materials, milk powder, and anhydrous milk fat, making it the world's second-largest importer of milk powder. Algeria has significant needs for milk and dairy products. Milk consumption through dairy products increased rapidly from 54 liters per person per year in 1970 to 112 liters per person per year in 1990, reaching 120 liters in 2013 (Kacimi-El-Hassani, 2013). According to the Algerian Ministry of Agriculture and Rural Development statistics, domestic consumption reached 5 billion liters of milk per year, and domestic production is estimated at 2.5 billion liters (Algeria Press Service, 2015). Thus, billions of liters of milk are imported yearly, mainly in milk powder. European countries, including Poland, France, and Belgium, were Algeria's leading milk powder suppliers until 2003. However, a significant increase in dairy product prices in these countries reduced their exports to Algeria. So, Algerian milk importation turned to New Zealand, Ukraine, and the United States, increasing the diversity of milk powder origins. Pasteurized milk, sterilized concentrated milk, UHT milk, sweetened condensed milk, cream, ice cream, fresh and processed cheese, yogurt, and dairy desserts are among the reconstituted and recombined products that contain milk powder. Additionally, a lot of manufactured food products contain milk powder as an ingredient. The components of milk products, such as lactose, protein, fat, and milk salts, all help the food product achieve its intended qualities. Milk powders contribute to the final food product's texture and sensory appeal in addition to their nutritional value as a source of nutrients (Baxter et al., 2011).

However, the increasing production and use of dairy powders can pose significant safety and economic challenges, particularly regarding the control of microbial loads. The obvious result is Algeria's pasteurized milk's fragile quality: After two days or even on the same day, this substance coagulates (Malek, 2023).

Impact of milk powder use and associated spoilage bacterial spores on dairy products

Losses in the dairy industry appear to be primarily caused by microbial spoilage (Remenant et al., 2015). Losses for whole and non-fat dry milk were estimated by the USDA Economic Research Service

(USDA, 2012) to be above 42%, while losses for all dairy products were over 31%. Dairy foods have a wide range of different types of spoilage microorganisms due to the selective effects of production, formulation, processing, packing, storage, distribution, and handling procedures.

In Algeria, insufficient attention is given to contamination of milk powder by mesophilic or thermophilic bacilli; unlike many other countries, there is no legislation limiting the presence of *Bacillus* species in dairy powders. The presence of aerobic spore-forming bacteria in imported milk powder presents significant safety and quality concerns.

Bacillus spp*. cause structural, chemical, and sensory defects that reduce the shelf life of milk powder and dairy products.* In Algeria, the shelf life of pasteurized milk often does not exceed 24 hours, and microbiological spoilage is frequently reported in yogurt (Moussa-Boudjemaa et al., 2004; Malek, 2013). In yogurt, slow acidification has been shown to promote increased *B. cereus* growth during fermentation. Under such conditions, *B. cereus* counts increased by 4–5 log units instead of the expected 3 log units (Brisabois et al., 1997).

Pasteurized milk and UHT milk

Pasteurized milk is one of the most highly consumed dairy products with, for example, a percentage of 89% of consumption milk, an average production of 1.6 x 10^9 L, and consumption of more than 130 L per capita in Algeria in 2016 (Data given by National Inter-professional Milk Board. Algeria).

Despite all the exigencies of heat-treatment and other conditions of production, many consumers complain about the quality of pasteurized milk packaged in bags, especially for its shelf life, which sometimes does not exceed 24 hours, and for its abnormal odour and flavour. Studies conducted in Algerian dairies have reported high levels of contamination in pasteurized milk and in manufacturing equipment by aerobic spore-forming bacteria (Moussa-Boudjemaa et al., 2004; Chérif Antar, 2015). This spoilage is largely attributed to the activation of bacterial spores present in milk powder during heat treatments such as pasteurization. These bacterial spores germinate and proliferate rapidly if the cold chain is compromised, as is often observed in the Algerian retail market. Moreover, bacterial spores in dairy equipment can form biofilms, and inadequate cleaning can cause milk contamination (Moussa-Boudjemaa et al., 2004; Chérif Antar, 2015).

Fermented milk

Fermented milk such as yogurt and Leben are perishable cultured dairy products with a shelf life of up to 60 days under refrigeration. The storage duration depends on the degree of sanitation during the processing and packaging of these products (Karagül-Yüceer et al., 2001). While molds and yeasts are the primary contaminants in yogurt, bacterial contamination by heat-resistant spore-forming bacteria can also cause a gradual decline in product quality. Common flaws in yogurts include high acidity that results in a strong acetaldehyde flavour, flaws in texture and appearance, and—most importantly—bitter flavours in the finished product brought on by the proteinase activity of spore-forming bacteria like *B. cereus* or *B. subtilis* (Mistry, 2001).

Despite the high consumption of yogurt in Algeria, no study reports the effect of aerobic spores on this dairy product due to the addition of milk powder. The evolution of spore-forming bacteria populations in yogurt has been studied. *B. cereus* can grow during the acidification of yogurt milk but not in the final product, which is too acidic. *B. licheniformis* failed to grow under any conditions applied in the study on yogurt (Karagül-Yüceeret al., 2001). *B. licheniformis* can actively grow between pH 5.5 and pH 8.5. However, it is unable to grow at 4°C (Gibson and Gordon, 1974). The naturally acidic environment of yogurt, combined with the further decrease in pH during storage,

likely accounts for the loss of viability of most aerobic spore-forming bacteria (Karagül-Yüceer et al., 2001).

Cheeses

In order to produce cheese, dairy ingredients (cheese, butter, and milk powder) and non-dairy products (agents, emulsifiers, and salts) must be combined, heated, and texturized. The cheese is heated for four to fifteen minutes at temperatures ranging from 70 to 95 °C (Richonnet, 2016). A variety of raw ingredients, including cheddar cheese, milk powder, lipids, milk proteins, modified starch, chemical products (melting salts), and pH regulators, are mixed in Algeria. For 10 minutes, the mixture is heated to 72–86 °C or 92–94 °C for pasteurized cheese, and for 2–4 seconds, it is heated to around 140 °C for UHT-sterilized cheese. In Algeria, studies by Benamara et al. (2016) have reported the presence of *Bacillus* species and/or their toxins in Algerian processed cheese. Sixty samples of four brands of Algerian melted cheese were analyzed. For four isolates identified, the spores of *B. cereus* belonging to group III, according to the classification of Guinebretière et al. (2010), were found. In Egypt, Sadek et al. (2015) reported the presence of *B. cereus* in all the 150 tested samples of locally made processed cheese and some ingredients used to manufacture processed cheese, such as skim milk and powdered cheddar, with a varying percentage. Other studies have demonstrated the presence and persistence of this bacterium during cheddar cheese production (Oliveira et al., 2016), which is the basic cheese for the manufacture of processed cheese, milk powder (McHugh et al., 2017; Sadek et al., 2015; Lücking et al., 2013), which represents one of the major raw materials in the manufacture of processed cheeses, and modified starch (Gonzalez et al., 1997).

According to Glass and Doyle (2005), the quality of the ingredients, the degree of heat treatment, the type of packaging and its technology, and the storage temperature all affect the processed cheese's microbiological stability and safety. Sterilization is necessary to provide microbiologically stable products with a long shelf life since processed cheese has a pH value higher than 4. However, after manufacture, processed cheese that has undergone light heat treatment (pasteurization) needs to be kept chilled. In general, the applied heat treatments are sufficient to destroy vegetative cells but are often insufficient to eliminate bacterial spores. The vegetative cells of foodborne pathogens and spoilage organisms are rendered inactive by mild heat treatment. Spore-forming bacteria, on the other hand, can endure (Glass and Doyle, 2005). Various microorganisms are responsible for the spoilage of powdered milk and dairy products, including sporulated bacteria. *Bacillaceae* is one of the two major spore-forming bacterial families in the processed cheese business, according to Sadek et al. (2016). These bacteria can cause foodborne illness or product spoilage. Bacilli species include *B. licheniformis, B. cereus, B. subtilis*, and *G. stearothermophilus* can proliferate during cheese production and cause a variety of spoiling kinds (Fernández-No et al., 2011). Examples include the "flat sour" spoiling caused by *G. stearothermophilus* because of its ability to produce acid but not gas, and the coagulation of casein by the protease produced by some *Bacillus* or *Geobacillus* species (Lücking et al., 2013). Ensuring the microbiological safety and stability of processed cheese represents a significant challenge for the cheese-making industry to meet the growing market demand for this product (Cusato et al., 2013).

Aerobic spore-forming bacteria in milk powder

Spore-forming bacteria are present in many food-processing environments and may threaten food quality and safety. They have been frequently isolated and identified in the raw, powdered, and milk

processing continuum (Postollec et al., 2012). It is well established that aerobic mesophilic and thermophilic bacilli are significant contaminants of dairy products throughout the world; they have been extensively studied (Ronimus et al., 2006; Reginensi et al., 2011; Yuan et al., 2012; Buehner et al., 2014; Sadek et al., 2015).

Ronimus et al. (2003) analyzed milk powders produced in New Zealand to identify the types of thermophilic contaminants. Over 98% of the 1470 isolates examined (grown at 55 °C) were assigned to the species *G. stearothermophilus, A. flavithermus*, *B. licheniformis,* and *B. subtilis.* According to a survey by Rückert et al. (2004) using random amplified polymorphic DNA (RAPD), *G. stearothermophilus* was responsible for 11% of all isolates (742) examined in 28 milk powders from Australia, Chile, France, Germany, Great Britain, Mexico, the Netherlands, New Zealand, Poland, South Africa, and the United States. The highest values of 5 104, 2.7 x 10^4, and 3 x 10^4 CFU.g-1 were identified in milk powders made in the USA, France, and Great Britain, respectively, while the lowest value of 8 CFU.g-1 was observed in milk powders made in Germany and Mexico.

Ronimus et al. (2006) analyzed milk powders from New Zealand. Spore counts were all low, with a maximum of 330 cells. g^{-1}, the two dominant species in this old milk powder were *B. licheniformis* and *B. subtilis.* Reginensi et al. (2011) analyzed 22 powdered milk samples from Uruguay representative of spring and summer production. The mean thermophilic cell counts per gram of sample incubated at 55 °C and 37 °C were 2.2*10^3 and 1.0*10^3, respectively. These contamination levels were close to the limits established by New Zealand regulations (Rückert et al., 2004) and remained below the higher counts reported by Ronimus et al. (2003).

Buehner et al. (2014) showed levels of spores in milk powders processed in the Midwestern USA of about 3.6 Log CFU.g-1. The predominant organisms identified in their study were *B. licheniformis*, *B. pumilus, B. sonorensis,* and *G. stearothermophilus*. In China, *B. licheniformis, A. flavithermus*, and *G. stearothermophilus* were also prevalent microorganisms in Chinese milk powders (Yuan et al., 2012). In another Chinese study, three milk powders were analyzed for thermophilic and mesophilic spore-formers. *B. licheniformis* was the most prevalent bacterium with incredible diversity, followed by *G. stearothermophilus* and *A. flavithermus* (Sadek et al., 2015). From these studies, *A. flavithermus*, *G. stearothermophilus*, and *B. licheniformis* appear as the major species of the family *Bacillaceae* isolated from different dairy powders. The other species, *B. cereus, B. subtilis*, *B. megaterium,* and *B. pumilus,* were also isolated from many milk power samples (Ronimus et al., 2003; Rückert et al., 2004; Reginensi et al., 2011; Sadek et al., 2015).

Evolution of aerobic spore-forming bacteria populations during milk powder processing

The bacterial spore concentration in milk powders is higher than in raw milk. Spores are concentrated during milk powder production. High concentrations of these spore-formers reduce milk powder quality. It is an indicator of poor manufacturing practices and low levels of hygiene in the plant (Sadek et al., 2015). Raw milk in the farm environment may contain spore counts of up to 10^4 CFU/mL and is regarded as the primary source of milk contamination (Coorevits et al., 2008). Bacterial spores can survive the elevated temperatures used in industrial processing. Therefore, heat treatments during processing cannot completely inactivate spore-forming bacteria (Hornstra et al., 2009). The milk concentration in the evaporator is a critical hazard during milk drying. Water is eliminated by boiling at low pressure. The evaporation temperatures are below 66-68 °C, not to decrease dry milk quality. This temperature range does not inactivate bacterial spores. However, these temperatures are favorable to the development of thermophilic bacteria. Bacterial spores can adhere to steel surfaces and form biofilms. Then, bacterial spores can recontaminate milk powder during processing (Flint et

al., 2001). These conditions select thermophilic bacilli, which can influence the stability of dairy powders and reconstituted dairy powders. Murphy et al. (1999) isolated *G. stearothermophilus* and *B. licheniformis* from the tubular preheater and the evaporator before milk drying. In 2007, Scott et *al.* isolated *A. flavithermus* and *Geobacillus* spp. from the preheater (plate heat exchanger) and evaporator in a milk powder processing plant. According to Murphy et al. (1999), evaporator contamination increased rapidly within 4 hours and became significant after 8 hours. Scott et al. (2007) discovered that during whole milk powder processing cycles, spore-former counts rose in the preheater and evaporator phases from 9 to 18 hours. The product becoming contaminated due to biofilm accumulation and fouling organisms shedding their skins has been blamed for the rise in spore-forming bacteria.

Spray drying does not get rid of bacterial spores after evaporation. The air in the spray chamber has an intake temperature of nearly 200 °C and an output temperature of nearly 90 °C. However, the temperatures into and on the surface of the concentrated milk droplet tare between the wet bulb temperature close to 50 °C and the outlet air temperature. Moreover, the passage of milk particles in the spray chamber is rapid, within a few seconds. So, their thermal damages are limited (Schuck et al., 2005).

Conditions influencing the growth, survival, and biofilm formation by aerobic spore formers in the dairy environment

Environmental conditions for spore-forming bacteria growth

The main environmental factors that affect spore-forming bacteria's growth are temperature, pH, and water activity (McClure, 2006). Spore-formers are categorized as mesophilic, psychrotolerant, and thermophilic. *B. cereus, B. licheniformis, B. pumilus, and B. subtilis species* represent mesophilic spore-forming species and are detected in various dairy products, including milk powders*. B. cereus* strains can survive industrial pasteurization processes due to the production of spores while psychrotolerant strains can survive refrigeration temperatures, and can affect the shelf-life of pasteurized milk and cream (Griffiths, 1992). The most common species in summertime raw milk is *B. cereus* (Scheldeman et al., 2006). The cardinal values could be used to describe these bacteria's growth (X_{min}, X_{opt}, X_{max}). Table 1 summarizes the values for the main species of spore-forming bacteria isolated from milk powder. *B. licheniformis* is one of the most prevalent *Bacillus* species found in raw milk and along the dairy processing continuum (Scheldeman et al., 2006). *B. licheniformis* spores are often detected in milk powders. They are known to be among spore-formers capable of producing spoilage enzymes (Reginensi et al., 2011). The thermophilic bacilli *Geobacillus* spp. and *A. flavithermus* are the predominant bacteria able to grow in warm conditions, from 50 °C to 70 °C, in milk powder processing plants. *Geobacillus* spores often survive the pasteurization of raw milk and subsequently adhere to surfaces and germinate to form biofilm and contaminate process vessels (Murphy et al., 1999). Milk powder processing selects thermophilic species such as *Geobacillus* spp. and *A. flavithermus.* Thermophilic bacilli spores appear to be the predominant spoilage organisms in the final milk powder product (Burgess et al., 2010).

Heat resistance of spore-forming bacteria isolated from milk powder

Bacillus spp. endospores are considered the most challenging microbial forms to be inactivated in the dairy industry. However, different heat treatments can be used during the manufacturing of milk powder or dairy products such as UHT and pasteurized milk, cheese, or yogurt. During these

processes, the populations of bacterial spores will evolve, decreasing or increasing according to their thermoresistance and growth abilities. Heat treatments could negatively affect dairy product quality depending on the required intensity. Hence, bacterial spore heat resistance knowledge is needed to optimize heat treatment processes (Hornstra et al., 2009). Table 2 summarizes typical D-values (decimal reduction times) and z-values reported for spore thermal inactivation.

Table 1. Lower, optimum, and higher conditions limit the growth of spore-forming bacteria species in milk powder

Organism	Growth cardinal values			
	pH	Temp (°C)	a_w	NaCl (%)
Bacillus cereus	Min 4.3 [b] Opt6-7 [b] Max 9.3 [b]	Min3 [a] Opt30-37 [b] Max55 [b]	Min 0.92 [b] Opt 0.99 [b]	Max growth at 5% [b]
Bacillus subtilis	Min 4.5 [c] Max 8.5[l]	Min 10 [d] Max 45-55[l]	Min 0.90[e]	Growth at 7% [l]
Bacillus licheniformis	Min 4.5 [c] Max 8.5[l]	Min 5-10 [f] Max 50-55[l]	Min 0.89-0.91 [g]	Growth at 7% [l]
Bacillus pumilus	Min 5.2 [c] Max 8.5[l]	Min 4-7 [h] Max 50-55[l]	Min 0.95 [e]	Growth at 7% [l]
Geobacillus stearothermophilus	Min 5 [i] Max7[j]	Min 40 [k] Opt 55 [k] Max 65-68[l]	Min 0.93 [e-k]	No growth at 7% [l]
Anoxybacillus flavithermus	Min 8 [j] Max 10.5 [j]	Min 30-45 [j] Opt 55 [j] Max 75 [j]	-	No growth at 7% [l]
Bacillus sporothermodurans	-	Min 20 Max 45-55 [l]	-	No growth at 7% [l]

[a](Drobniewski, 1993); [b](ANSES, 2011); [c](Lindsay et al., 2000a); [d](Shapton and Shapton, 1993); [e](Troller and Christian, 1978); [f](Gibson and Gordon, 1974); [g](Alzamora et al., 1995); [h](Meer et al., 1991); [i](Hill and Fields, 1967); [j](McClure, 2006); [k](Kotzekidou, 2013); [l](Burgess et al., 2010).

According to Berendsen et al. (2015), heat tolerance of spores varies not only between species but also among strains within the same species. *Bacillus cereus, Bacillus subtilis, G. stearothermophilus,* and *Bacillus sporothermodurans* are among the species that can produce extremely heat-resistant spores that can withstand the heating conditions frequently employed in food preservation (Scheldeman et al., 2006). According to Table 2, the $D_{100\,°C}$ value for *B. cereus* varied between 1.45 and 5.5 with a z-value between 9.4 °C and 40.7 °C (Ingram, 1969; Janstova and Lukasova, 2001). For different groups of *B. cereus* spores, the $D_{90\,°C}$ values are as follows: group VI: 1.7 minutes, group II, V, IV: 20 to 30 minutes, group III, VII: 40 to 90 minutes (ANSES, 2011).

Reported z-values for B. subtilis range from 7.4 to 15.7 °C, and D_{100}°C values from 2.13 to 13.3 minutes. However, Berendsen et al. (2015) shown that there can be significant variation in the heat tolerance of spores from several strains within the *B. subtilis* group. When strains are divided into two groups, one group shows an average D_{120}°C of 0.33 s, whereas the other shows an average D_{120}°C of 45.7 s, a markedly higher value. Jagannath and Tsuchido (2003) reported D-values for *B. subtilis* in whole, skimmed, and half-fat UHT milk of D_{95}°C = 5.76 min, D_{95}°C = 5.13 min, and D_{100}°C = 1.18 min, respectively.

Another example is *B. sporothermodurans*, which produces spores that are extremely heat-resistant (Huemer et al., 1998; Esteban et al., 2013). For this bacterium, distinct variations in decimal reduction times (D-value) for spores of strains from different isolation sources at 100 °C were noted (Scheldeman et al., 2006). Further complicating the optimization of heating treatment during milk operations are the observed differences in spore heat resistance for various strains within a species.

Thus, a deeper understanding of spore heat resistance is needed, especially how strain heterogeneity affects it. Further complicating the translation of the results to practical flow inactivation procedures is the fact that the majority of inactivation kinetics are established in batch heating trials (Witthuhn et al., 2011).

Table 2. The heat resistance of spore-forming bacteria is often isolated from milk or milk powder

Organism	D (minutes)			Z°C
	100°C	121°C	140°C	
Bacillus cereus	5 [a]			10 [a]
	5.5 [b]			9.4 [e]
	2.0- 5.4 [f]			9.7 [b]
	2.06 [i]			12.8 [i]
	1.45 *[j]			40.7* [j]
Bacillus subtilis	11 [a]	0.1 [d]		7 [a]
	13.3 [b]			7.4 [b]
	2.13 [i]			15.7 [i]
Bacillus licheniformis	13 [a]	0.135 [b]		6 [a-b]
	13.5 [b]			16.8 [i]
	4-8 [c]			8 *[k] 7.88**[k]
	5.6 [i]			
Bacillus pumilus	0.83[i]			9.7 [g-i]
Geobacillus stearothermophilus	3000 [b]	4-5[a]	0.9 [g]	7 [a-b]
				7.54*[k]
				7.89**[j]
				9.1[g]
Bacillus sporothermodurans		0.32-0.57[h]	3.4-7.9 [g]	13.1-14.2 [g]

[a](Ingram, 1969); (Briggs, 1966) ; [c] (Brown, 2000); [d] (Jacobs et al., 1973); [e] (Shehata and Collin, 1972); [f] (Wong et al., 1988); [g] (Huemer et al., 1998) ; [h] (Meier et al., 1996); [i](Janstova and Lukasova, 2001); [j](Evelyn and Silva, 2016); [k](Behringer and Kessler, 1992).(*) in skim milk, (**) skim concentrated milk

Several key steps are involved in producing dairy powders, including pasteurization, separation, evaporation, and spray drying. Scott et al. (2007) isolated *A. flavithermus* and *Geobacillus* spp. from multiple sampling points in a whole-milk powder processing plant. The authors noted that *A. flavithermus* was the predominant organism in the preheating processing steps, but both *Geobacillus* spp. and *A. flavithermus* were isolated at subsequent processing steps.

By legislation, pasteurized milk, pasteurized cheese, and yogurt produced based on milk powder are all treated at temperatures below 95 °C. As noted above, spores are not inactivated by the pasteurization treatment during dry milk processing due to their extreme resistance to heat (Cregenzán-Alberti et al., 2016). Therefore, the heat treatments applied in the Algerian dairy industry cannot inactivate Bacillus spores; instead, they may trigger germination under favorable conditions, leading to contamination of final products. Despite treatment of Algerian UHT milk at a temperature of 140 °C for a few seconds, spore-forming bacteria can also contaminate this product. The studies of Huemer et al. (1998) and Esteban et al. (2013) showed that *B. sporothermodurans* and *G. stearothermophilus* can survive UHT treatments.

Spore-forming bacteria biofilms and their control in the dairy industry

Biofilm formation

Spore-former bacteria, which might be vegetative cells, spores, or unattached biofilm clumps that stick to the surface components, contaminate milk processing lines and dairy products from the milk powder used in the dairy sector (Gopal et al., 2015). Stainless steel is used for equipment in dairy

manufacturing plants due to its mechanical strength, resistance to corrosion, and longevity. The *Bacillus* genus is frequently detected in common biofilms in the dairy industry. Process biofilms are formed from these organisms (Flint et al., 1997). The *Bacillus* and *Geobacillus* genera include the aerobic thermophilic/thermoduric spore-formers found in dairy manufacturing facilities and dairy products: *B. subtilis, B. cereus, B. licheniformis*, and *Geobacillus* sp. (mostly *G. stearothermophilus*) have been commonly found in dairy biofilms (Flint et al., 1997; Murphy et al., 1999; Scott et al., 2007; Burgess et al., 2009; Shaheen et al., 2010) For the thermophilic spore-former *A. flavithermus*, previous publications have demonstrated its capabilities of biofilm formation and have focused on the physiology of mature (18 h) biofilms related to the effect of different cleaning regimes (Parkar et al*.,* 2003).

The hydrophobic exosporium of *B. cereus*, which differs among species, along with surface appendages on spores, contributes to their strong adhesive properties (Faille et al., 2001). According to Sharma and Anand (2002), *B. cereus* accounted for about 12% of the microbiota present in the biofilms formed by dairy plants. At the air-liquid interface, a thicker biofilm of *B. cereus* forms than in a submerged system. This implies that *B. cereus* biofilm may form, especially in industrial piping and storage systems, where residual liquid has remained after a production cycle or if the system is partially filled during operation (Wijman et al., 2007). Biofilm formation by *B. cereus* is considered more as a survival strategy than a virulence factor (Ehling-Schulz et al., 2004). Several studies have investigated *B. cereus* biofilm formation on various substrates, including stainless steel, polypropylene, plastic, and glass wool (Houry et al., 2010). Spores have the most potential to form biofilms among the various physiological stages of *B. cereus*. In biofilms, spores can account for up to 90% of the total viable cell population (Wijman et al., 2007). In addition, the adhesion of *B. cereus* to stainless steel surfaces increases with a rise in temperature, and the pH increases over time (Peña et al., 2014). Biofilm formation can even be observed at low temperatures. Kumari and Sarkar (2014) reported that, among 144 isolates from milk, cheese, and ice cream, 71–90% were capable of forming biofilms at 4 °C, while all isolates from butter (100%) were positive. It has been demonstrated by Shaheen et al. (2010) that some strains of *B. cereus* can form a biofilm in whole milk but not in water-diluted milk, likely because whole milk contains surface-active compounds such as phospholipids, which is a surface-active compound found in the fat globules of whole milk. This indicates that *B. cereus* requires additional milk components, such as natural surfactants and phospholipids, to colonize stainless steel surfaces.

B. subtilis, mesophilic bacilli, can develop different types of biofilms according to the culture conditions, including colony biofilms at the air–solid interface, floating pellicles at the air-liquid interface, and submerged biofilms at the liquid-solid interface. *B. subtilis* is a model spore-forming bacterium to study biofilm formation and structure. By contrast, *B. licheniformis* has rarely been studied for its ability to form robust biofilms (Randrianjatovo et al., 2015). Randrianjatovo-Gbalou et al. (2017) conducted an in-situ analysis of *B. licheniformis* biofilms, this study has given new insight into the complex interconnection of the leading EPS components that regulate the adherence and aggregation of the matrix in *B. licheniformis*.

Zhao et al. (2013) studied biofilms formed by thermophilic spore-formers in the dairy industry. The most potent biofilm-forming *Geobacillus* strains isolated belong to G. *stearothermophilus* and *G. thermoglucosidans* species. *Geobacillus* species have a more remarkable ability to form biofilms at air-liquid interfaces rather than submerged surfaces. Thermostable spore-producing *G. thermoglucosidans* requires proteolytic compounds from other spoilage organisms to form biofilms (Zhao et al., 2013). Flint et al. (2001) examined the development of *G. stearothermophilus* biofilms

on stainless steel surfaces in the presence of milk. *G. stearothermophilus* formed biofilms after approximately a 6-hour incubation period using a laboratory continuous flow reactor. After 12 hours, the biofilm contained up to 10^6 cells/cm^{-2}. Doyle et al. (2015) stated that *G. stearothermophilus* causes long-term persistent contamination of dairy processing lines as they form biofilm on the stainless-steel surfaces of processing equipment. It is challenging to eliminate thermophilic bacilli due to their wide temperature growth range, their fast growth rate, the resistance of their spores to heat and biocides, and their ability to form biofilms (Flint et al., 2001; Parkar et al., 2003). One of the most prevalent contaminants of milk powders is *Geobacillus* sp., which can withstand industrial pasteurization of raw milk. Its spores then stick to surfaces and sprout to create biofilms, which causes milk products to degrade (Murphy et al., 1999). It has been found that *G. stearothermophilus* has a high capacity for adhesion and biofilm formation (Jindal et al., 2016).

Biofilm formation by *A. flavithermus* was observed by Burgess et al. (2009), who demonstrated that biofilm formation and sporulation can occur very rapidly, within 6–8 hours, with spore concentrations in the biofilm ranging from 10% to 50% on total viable counts. This important observation shows that spores within the biofilm could greatly increase its resistance to cleaning and high temperatures in a dairy processing environment.

Bacterial cells in biofilms are more resistant to biocides and cleaning processes than planktonic bacteria. So, their elimination from the dairy industry is challenging. Aerobic spore-forming bacteria such as *B. cereus* and *Geobacillus* sp. are involved in biofilm formation in different dairy processing surfaces, which increases their resistance to cleaning and disinfection procedures (Kumari and Sarkar, 2016). Moreover, these biofilms detach from the surface and disperse, colonizing the processing environments and leading to product contamination. Three distinct mechanisms can cause biofilm detachment: (i) erosion brought on by fluid shear forces; (ii) abrasion, which happens when particles collide; and (iii) sloughing, which is the immediate loss of significant portions of the entire biofilm (Garny et al., 2008). Biofilms have the potential to provide a number of risks to the dairy industry. Businesses must uphold strict biofilm monitoring and control procedures and appropriate hygiene standards (Flint et al., 1997; Shaheen et al., 2010).

Biofilm control

Several approaches can be used to control biofilms. The most common approaches include altering surface chemistry to prevent cell adhesion, applying antimicrobial agents to surfaces, optimizing equipment and process design, and using rigorous cleaning procedures such as Cleaning-in-Place (CIP). For improved performance, these entail cleaning and sanitizing milk processing lines with extra antimicrobial materials (Gopal et al., 2015). Generally, because of their capacity to eliminate both organic (protein and fat) and inorganic (calcium and other minerals) fouling layers, CIP systems employ a series of caustic (sodium hydroxide) and acidic (nitric acid) wash phases (Flint et al., 1997). The existence of various bacterial species that can withstand the industrial pasteurization process can limit the effectiveness of heat treatments and the tactics used by industry to control the biofilm of pathogens and spoilage-causing microbes. Due to the fact that aerobic spore-forming bacteria can withstand hostile environments and produce resistant biofilms, dairy manufacturing facilities experience financial losses and equipment run durations that are shortened (Gopal et al., 2015). Fouling of milk processing equipment and subsequent contamination of final products are major problems for the dairy industry. Aerobic spore-formers, especially *B. cereus* biofilms, are present after cleaning and disinfection procedures. The assessment of the microflora of biofilms formed on the surfaces of dairy equipment shows high contamination levels. Malek (2023) found that *B. cereus*

group members are predominant in biofilms attached to dairy equipment surfaces. The study by Chérif Antar (2015) showed that *B. cereus* spores remained attached to equipment surfaces after the pasteurization of milk and after Clean–in–place procedures. However, CIP has proven to be a valuable strategy since its introduction. So, biofilms can become resistant to CIP chemical treatments. In this case, the use of enzymatic polysaccharides and proteolytic therapies, in addition to conventional CIP cleanings,

effectively eliminates biofilms (Lequette et al., 2010). Efforts have been made to develop new strategies based on advances in bacterial genetics, systems biology, materials science, mechanical engineering, and chemical biology.

There have also been suggestions for additional methods to manage biofilm in the dairy sector. Among these are the so-called green strategy for biofilm control, which includes the use of enzyme-based detergents, bacteriophages, and metabolite molecules or microbial interactions (Simões et al., 2010); disinfection and removal of biofilm layers using ozone (O_3) water and hydrogen peroxide solution (Tachikawa and Yamanaka, 2014); ultrasound treatments combined with heat and/or pressure; high-intensity focused ultrasound; the use of biotherapeutic agents like lactoferrin (Ammons and Copié, 2014); and a range of antimicrobials like nisin, lauricidin, or reuterin (El-Ziney and Jakobsen, 2009). However, these approaches are rarely used in the dairy sector.

Incidence of aerobic spore-forming bacteria on milk powders

Aerobic spore-forming bacteria affect food safety due to the production of toxins and food quality due to the production of spoilage enzymes.

Production of toxins and foodborne illnesses

Some *Bacillus* spore-formers are known to cause food poisoning by producing toxins. The most common species responsible for such outbreaks are some strains of *B. cereus,* which demonstrate widely different phenotypes and pathological effects (Logan and De Vos, 2009). *B. cereus* may cause two types of food poisoning syndromes: an emetic type and a diarrheal type. The emetic symptom is caused by cereulide, a heat-stable cyclic dodecadepsipeptide encoded by the CES genes (Ehling-Schulz et al., 2004; Ehling-Schulz et al., 2005). The diarrheal syndrome is caused by three heat-labile enterotoxins: hemolysin BL, non-hemolytic enterotoxin, and cytotoxin K. Hemolysin BL is encoded by the genes hblA (encoding B), hblC (encoding L2), and hblD (encoding L1), which are organized in a single operon (Bhunia, 2008). The non-hemolytic enterotoxin (NHE) complex is encoded by nheA, nheB, and nheC and organized in one operon (Granum et al., 1999). The cytotoxin K (cytK) protein is highly cytotoxic. CytK is implicated as the primary virulence factor in *B. cereus* diarrhea and may also be necrotic and hemolytic (Lund et al., 2000). Several Bacillus species carry genes encoding enterotoxins, including *B. subtilis, B. pumilus,* and *B. licheniformis* (Beattie and Williams, 1999) (Table 3).According to Taylor et al. (2005), *B. megaterium, B. firmus*, and *B. simplex* strains produced thermostable toxins, which showed similar physical characteristics to cereulide, the emetic toxin of B*. cereus* (Taylor et al., 2005). However, Lücking et al. (2013) reported that cytotoxicity was detected only in *B. cereus,* suggesting that the risk of food poisoning by aerobic, thermoresistant spore-formers outside the *B. cereus* group is relatively low. Around the world, *B. cereus* has been implicated in various foodborne outbreaks (Bennett et al., 2013). However, food poisoning associated with *Bacillus cereus* or *Bacillus* in general may be vastly underreported and can confused with

Staphylococcus aureus and *Clostridium perfringens* food poisoning due to similar symptoms. This leads to underestimating the number of foodborne outbreaks caused by *Bacillus*.

Table 3. Bacillus species present in milk powder and production of toxins

Strains	Production of toxins	References
Bacillus subtilis	Production and functionality of heat-labile toxins	(Beattie and Williams, 1999; Lindsay et al., 2000b)
Bacillus licheniformis		(Beattie and Williams, 1999; Lindsay et al., 2000b)
Bacillus licheniformis	Production of novel heat-stable toxins resembling the physicochemical characteristics of cereulide	(Beattie and Williams, 1999; From et al., 2005); Taylor et al., 2005)
Bacillus pumilus		(From et al., 2005)
Bacillus subtilis		(Beattie and Williams, 1999; From et al., 2005)

Production of spoilage enzymes

Bacteria from *B. cereus* groups, *B. subtilis* groups, and *B. licheniformis* are the most important spoilage microorganisms (Lücking et al., 2013). *Bacillus* spp. produces a variety of extracellular enzymes, including amylases, hemolysins, lecithinases, phospholipases, proteases, beta-lactamases, and sphingomyelinases (Chen et al., 2004; Logan and De Vos, 2009). The presence and growth of these vegetative cells and their associated enzymes primarily affect the quality of milk and milk products.

Milk powder with low water activity is less affected. The water activity of whole milk powder ranges from 0.25 to 0.35 (Baechler et al., 2005), while that of skim milk powder ranges from 0.32 to 0.43 (Shrestha et al., 2008). These low water activity levels greatly limit microbial growth and enzymatic activity. Due to their relatively fragile nature, the stability of enzymes is significantly influenced by water activity (Acker, 1969). Most enzymes and proteins must maintain conformation to remain active. Maintaining critical a_w levels to prevent or entice enzyme conformational changes is important for food quality.

However, data suggest that the changes in milk powders during storage are not only caused by chemical reactions (Stapelfeldt et al., 1997) but are also caused by a low enzymatic activity (Chen, 2000). The low water activity in milk powder explains the absence of recoverable vegetative bacterial cells, but bacterial enzymes can still remain active (Chen et al., 2003). Most enzymatic reactions are slowed down at water activities below 0.80. Muir (1996) suggests that even if bacterial extracellular enzyme activity in a milk processing procedure is high, degradation of milk powders produced from this process would be slow because of the low water content. However, some reactions occur even at low a_w values of 0.30 (Labuza, 1970). The research conducted by Chen et al. (2004) has shown that lipolysis can occur in whole milk powders with a moisture content (aw) of 0.3. After storage for two weeks at 37 °C, the levels of short-chain FFAs in the powders exceeded the sensory threshold in reconstituted whole milk powder at 125 g L^{-1}.

When water activity increases to approximately 0.80 in milk products made from recombined milk powder, enzymatic activity increases, and bacterial spores can germinate and grow. In addition, the vegetative cells of spore-forming bacteria produce extracellular enzymes such as protease, lipase, and amylase. For *Bacillus* spore-forming species present in dairy products, such as *B. cereus*, *B. licheniformis*, *B. subtilis*, *B. pumilus* (From and Boor, 2004), *A. flavithermus* and *Geobacillus* (Burgess et al., 2009), the associated enzymes produced are presented in Table 4.

Table 4. Various characteristics of enzymes obtained from *Bacillus* species

Enzymes	Origin	T_{min}	T_{opt}	T_{max}	pH_{min}	pH_{opt}	pH_{max}	References
Amylase	*Bacillus sp.* SMIA-2 (Thermophilic)	40	90	100	6	8.5	12	(Simões et al., 2010)
α-amylase	Bacillus sp. TS-23	_	70	_	_	9	_	(Lin et al., 1998)
α-amylase	*Bacillus sp.* BKL20	_	60-70	_	4	7	12	(Kubrak et al., 2010)
α-amylase	*Bacillus sp.* PN5	30	90	110	4	10	12	(Saxena et al., 2007)
α-amylase	*Bacillus sp.* YX-1	30	45	70	4	5	11	(Liu and Xu, 2008)
α-amylase	*Bacillus licheniform* TSI-14	50	70	90	5	7	9	(Shukla and Singh, 2015)
α-amylase	*Bacillus subtilis* MTCC 121	30	40	55	6.6	7.1	8.6	(Raul et al., 2014)
α-amylase	*Geobacillus stearothermophilus* DSM 5934	35	70	90	4	7	11	(Fincan and Enez, 2014)
α-amylase	*Anoxybacillus sp* YIM 342	30	80	90	3	9	11	(Zhang and al., 2016)
lipase	*Bacillus sp. FH5*	10	60	80	4	9	11	(Ghori et al., 2011)
lipase	*Bacillus sp.*	30	60	70	4	5.5-7.2	9.5	(Sugihara et al., 1991)
lipase	*Bacillus sp. Thermophilic*	_	60-65	_	_	8.5	_	(Nawani et al., 2006)
lipase	*Bacillus cereus*	30	40	60	NM	7.5	NM	(El-Shafei and Rezkallah, 1997)
lipase	*Bacillus coagulans* BTS-3	_	55	_	6,5	8.5	9.5	(Kumar et al., 2005)
lipase	*Bacillus coagulans ZJU318*	40	45	60	7	9	10	(Lianghua and Liming, 2005)
lipase	*Bacillus licheniformis*	30	60	80	6	9	14	(Kaur et al., 2016)
lipase	*Bacillus licheniformis* VSG1	20	55	70	8	9	12	(Sangeetha et al., 2010)
lipase	*Bacillus subtilis A.S.1.1700*	37	43	47	7	8.5	9.5	(Ma et al., 2006)
lipase	*Bacillus subtilis* 168	15	35	55	6	10	12	(Lesuisse et al., 1993)
lipase	*Bacillus thermoleovorans* ID-1	_	70-75	_	_	7.5	_	(Lee et al., 1999)
Protease	*Bacillus sp. NCDC 180*	_	50-55	_	6	11.12	12	(Kumar et al., 1999)
Protease	*Bacillus cereus* HUTBS62	25	80	100	4.8	6.8	10.8	(Aqel et al., 2012)
Protease	*Bacillus cereus* MCM B-326	25	55	65	6	9	12	(Nilegaonkar et al., 2007)
Protease	*Bacillus licheniformis*	_	55	65	_	9	_	(Al Shehri and Mostafa, 2004)
Protease	*Bacillus licheniformis* VSG1	20	45	70	5	9	12	(Sangeetha et al., 2010)
Protease	*Bacillus subtilis*	20	40	80	_	14	_	(Badhe et al., 2016)
Protease	*Geobacillus stearothermophilus* B-1172	_	90	100	5	9	11	(Iqbal et al., 2014)

Flavour quality and textural problems in milk products have been attributed to the enzymatic activity of heat-stable protease. The most important spoilage organism in the dairy industry is undoubtedly *B. cereus*, causing 'bitty cream' and 'sweet curdling' defects (Heyndrickx and Scheldeman, 2002). The former is caused by lecithinase activity, and the latter by proteolytic activity. In addition, the lipolytic spoilage of milk due to enzymes can produce flavour defects associated with fat degradation. Free fatty acids are liberated and give rise to off flavours, such as rancid, butyric, unclean, soapy, and astringent flavors (Bhunia, 2008). Several factors, such as temperature, pH, and a_w, influence the enzymatic activities. Table 4 presents the temperature and pH limits of enzymatic activities for *Bacillus* species.

The optimum temperature for protease activity produced by *B. licheniformis* (Al Shehri and Mostafa, 2004) and *B. cereus* (Nilegaonkar et al., 2007) has been reported to be 55 °C, although Sangeetha, Geetha, and Arulpandi (2010) reported an optimal temperature of 45 °C for a protease produced by the *B. licheniformis* VSG1 strain. A lower optimum temperature of 40 °C has been reported for *B. subtilis* (Badhe et al., 2016). For *Geobacillus*, Iqbal et al. (2014) report 90 °C as an optimum temperature for protease produced by *Geobacillus stearothermophilus* B-1172. A temperature ranging from 55 °C to 60 °C has been reported as the optimal temperature for lipase activity produced by several *Bacillus* species, such as *B. licheniformis* (Sangeetha et al., 2010; Kaur et al., 2016). High temperatures of 70 °C to 90°C have been reported to be best for the activity of α-amylase by *B. licheniformis* TSI-14 (Shukla and Singh, 2015) and *Anoxybacillus sp. YIM 342* (Zhang et al., 2016). In contrast, an optimum temperature of 40 °C has been reported by Raul et al. (2014) for *B. subtilis* MTCC 121. A medium pH affects all enzymatic processes and the transportation of various components across the cell membrane. Since the proton motive force in chemiosmosis is affected by a medium pH value, it is possible that the relative metabolic efficiency is high under an optimum pH range. Hence, it is essential to consider this parameter (Sharma et al., 2017).

Conclusion

Aerobic spore-forming bacteria, especially mesophilic and thermophilic bacilli, are significant bacterial contaminants in the dairy industry, particularly in milk powder production. Several studies have demonstrated the contamination of milk powder and dried milk products with aerobic, spore-forming bacilli. These bacteria can survive heat treatments and persist during processing. Bacillus spp. may cause food poisoning and/or food spoilage due to their toxigenic and pathogenic nature and extracellular enzymes. Therefore, it is essential that imported milk powder be selected based on microbiological quality, especially the concentration of bacterial spores. Implementing remedial and preventive measures can be aided by the identification and enumeration of these bacterial contaminants, especially in the manufacturing process steps prior to heat treatment. Dairy production operations must also adequately heat-treat the milk before use in order to eliminate the spores. Deeper control strategies also require a deeper understanding of the nature of mesophilic and thermophilic biofilms in the milk processing environment and how they relate to spore development.

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